

## Microarray-Based Characterization of the *Listeria monocytogenes* Cold Regulon in Log- and Stationary-Phase Cells<sup>∇†</sup>

Yvonne C. Chan, Sarita Raengpradub, Kathryn J. Boor, and Martin Wiedmann\*

Department of Food Science, Cornell University, Ithaca, New York 14853

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Whole-genome microarray experiments were performed to define the *Listeria monocytogenes* cold growth regulon and to identify genes differentially expressed during growth at 4 and 37°C. Microarray analysis using a stringent cutoff (adjusted  $P < 0.001$ ;  $\geq 2.0$ -fold change) revealed 105 and 170 genes that showed higher transcript levels in logarithmic- and stationary-phase cells, respectively, at 4°C than in cells grown at 37°C. A total of 74 and 102 genes showed lower transcript levels in logarithmic- and stationary-phase cells, respectively, grown at 4°C. Genes with higher transcript levels at 4°C in both stationary- and log-phase cells included genes encoding a two-component response regulator (Imo0287), a cold shock protein (*cspL*), and two RNA helicases (Imo0866 and Imo1722), whereas a number of genes encoding virulence factors and heat shock proteins showed lower transcript levels at 4°C. Selected genes that showed higher transcript levels at 4°C during both stationary and log phases were confirmed by quantitative reverse transcriptase PCR. Our data show that (i) a large number of *L. monocytogenes* genes are differentially expressed at 4 and 37°C, with more genes showing higher transcript levels than lower transcript levels at 4°C, (ii) *L. monocytogenes* genes with higher transcript levels at 4°C include a number of genes and operons with previously reported or plausible roles in cold adaptation, and (iii) *L. monocytogenes* genes with lower transcript levels at 4°C include a number of virulence and virulence-associated genes as well as some heat shock genes.

*Listeria monocytogenes* is a gram-positive, psychrotolerant, food-borne pathogen that has the ability to grow at temperatures as low as  $-0.4^{\circ}\text{C}$  (48, 93). *L. monocytogenes*' ability to grow at low temperatures is a concern for refrigerated ready-to-eat food products in which *L. monocytogenes* can grow to high levels that may cause human disease, particularly if products are stored at refrigeration temperatures for a prolonged time (43). Previous studies have identified some *L. monocytogenes* genes and proteins that are involved in cold shock and cold adaptation or that are upregulated at low temperatures (2, 5, 13, 27, 55, 61, 62, 101), including ATP-binding cassette (ABC) transporters, such as the carnitine transport system (encoded by *opuCABCD*) and the glycine betaine porter II system (encoded by *gbuABC*), which allow for the uptake of compatible solutes that appear to facilitate *L. monocytogenes* growth at low temperatures (2, 27, 55). In addition, an oligopeptide binding protein (encoded by *oppA*), which is part of an ATP-dependent oligopeptide permease, has been shown to be required for *L. monocytogenes* growth at 5°C in brain heart infusion (BHI) broth (13). Other genes with potential contributions to the growth of *L. monocytogenes* at low temperatures include the cold-inducible gene *fri*, which encodes ferritin (39), and *ltrC*, which encodes a low-temperature-requirement C protein (101).

Thermoregulation of several *L. monocytogenes* virulence genes has been well documented (45, 58, 64), including temperature-dependent translation of *prfA* (45), which encodes

the positive regulatory factor PrfA. PrfA, which is preferentially translated at 37°C over 30°C (45), regulates the transcription of a number of virulence and virulence-associated genes (12, 59, 78), including genes located in the main *L. monocytogenes* pathogenicity island (e.g., *plcA*, *hly*, *mpl*, *actA*, and *plcB*) as well as genes located in other areas of the genome (e.g., *inlA*, *inlB*, and *bsh*) (21, 56).

A number of studies have used genomic tools, including microarrays, to study microbial gene expression during cold shock (i.e., cold exposure for  $<1$  h) as well as during cold growth (31, 47, 49), including in *Bacillus subtilis* (7, 16, 31, 49), a close relative of *L. monocytogenes*. For example, Budde et al. (16) recently used genome-wide microarray-based transcriptome analyses to identify 279 *B. subtilis* genes that showed higher transcript levels during cold growth at 15°C than during growth at 37°C. While these data provided initial insights into cold adaptation in some gram-positive bacteria, no microarray studies of *L. monocytogenes* gene expression during cold growth or cold shock have been published to date. Studies using other techniques for the identification of differentially expressed genes or proteomic approaches have been performed with *L. monocytogenes* but have revealed only small numbers of cold-induced genes or proteins (5, 62, 74). A comprehensive, genome-wide understanding of *L. monocytogenes* gene expression during cold growth is critical to provide a better understanding of cold adaptation in this food-borne pathogen and to allow for the rational development of novel strategies to control the growth of this pathogen during refrigerated storage of foods. We thus used a whole-genome microarray strategy to identify *L. monocytogenes* genes differentially expressed at 4 and 37°C during log- or stationary-phase growth, with confirmation of selected cold-induced genes by quantitative reverse transcriptase PCR (qRT-PCR).

\* Corresponding author. Mailing address: 412 Stocking Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 254-2838. Fax: (607) 254-4868. E-mail: mw16@cornell.edu.

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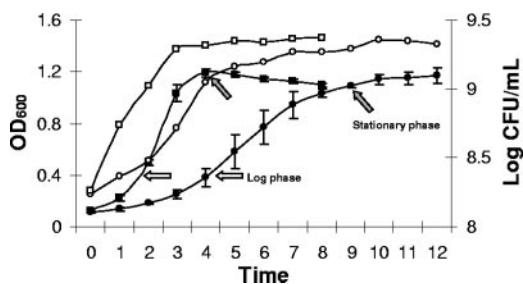


FIG. 1. Growth of *L. monocytogenes* strain 10403S at 4 and 37°C in BHI broth. The OD<sub>600</sub> of *L. monocytogenes* 10403S was measured once a day (for 12 days) for cells grown at 4°C (●) and once an hour (for 8 h) for cells grown at 37°C (■). The data shown represent the average OD<sub>600</sub> values for three independent experiments, and error bars indicate standard deviations. Cell counts were performed once a day (for 12 days) for cells grown at 4°C (○) and once an hour (for 5 h) for cells grown at 37°C (□); the cell count data shown represent the log CFU/ml determined for one growth experiment (average from duplicate platings). Numbers on the x axis represent days (for growth at 4°C) and hours (for growth at 37°C). White arrows indicate the time points used for the collection of log-phase cells (OD<sub>600</sub> of 0.4 ± 0.05 for both temperatures) for microarray experiments; gray arrows indicate the time points used for the collection of stationary-phase cells (OD<sub>600</sub> of 1.0 ± 0.25; reached 9 days and 4 h after inoculation for cells grown at 4 and 37°C, respectively).

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *L. monocytogenes* serotype 1/2a strain 10403S (10) was used throughout this study. For each experiment, *L. monocytogenes* 10403S was streaked onto BHI (Difco, Sparks, MD) agar from glycerol stock cultures (stored at -80°C), followed by incubation at 37°C for 24 h. A single colony was subsequently inoculated into BHI broth and grown at 37°C overnight with shaking (250 rpm), and a 1-ml aliquot of this overnight culture was used to inoculate 99 ml of BHI broth. After growth to log phase (an optical density at 600 nm [OD<sub>600</sub>] of 0.4), the *L. monocytogenes* suspension was used to inoculate 75 ml of prechilled (4°C) or prewarmed (37°C) BHI broth (in a 300-ml Erlenmeyer flask) to a starting OD<sub>600</sub> between 0.1 and 0.2. Cells were then incubated either at 4°C for up to 12 days or at 37°C for up to 8 h (without shaking). OD<sub>600</sub> values were measured every day for cells grown at 4°C and every hour for cells grown at 37°C; in addition, bacterial cell numbers were determined during one experiment by spread plating on BHI agar plates at each time point.

Bacterial cells were collected for RNA isolation at (i) log phase (defined as an OD<sub>600</sub> of 0.4 ± 0.05 for both temperatures) and (ii) stationary phase (defined as an OD<sub>600</sub> of 1.0 ± 0.25; reached at day 9 and 4 h after inoculation for cells grown at 4 and 37°C, respectively) (Fig. 1); the *L. monocytogenes* cell densities at each of the two growth stages (log and stationary phases) were similar for both temperatures, indicating that observed differences in transcript levels are not likely due to different cell densities in *L. monocytogenes* cells grown at 4 and 37°C (Fig. 1), a consideration raised previously (73). Bacterial cultures were treated with RNeasy Protect bacterial reagent (QIAGEN, Valencia, CA), according to the manufacturer's protocol, to stabilize RNA prior to RNA isolation. Three biological replicates were performed for each growth experiment.

**Total RNA isolation.** Total RNA from *L. monocytogenes* cells grown to log or stationary phase at 4°C or 37°C was isolated using an RNeasy midi kit (QIAGEN) as described by Kazmierczak et al. (53) with a minor modification. Briefly, QIAGEN's protocol for "enzymatic lysis with mechanical disruption" was used, with the exception that cells were lysed by sonication on ice three times with 30-s bursts at 18 to 21 W by using a Sonicator 3000 (Misonix, Farmingdale, NY). To remove contaminating DNA, total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI) in the presence of RNase inhibitor (RNasin; Promega) as previously described (53). RNA concentration and purity were evaluated by gel electrophoresis and absorbance readings at 260 and 280 nm, using a Nanodrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE).

**Microarray construction.** A whole-genome microarray was constructed to include 70-mer oligonucleotides representing 2,857 open reading frames (ORFs), identified based on the annotated genome for *L. monocytogenes* EGD-e (28); in addition, an *inlD* probe was designed, using ArrayOligoSelector (<http://arrayoligoselector.sourceforge.net/>), based on the *inlD* sequence for *L. monocytogenes* 10403S (as *inlD* is not present in strain EGD-e [28]). Probes targeting five *Saccharomyces cerevisiae* genes were used as nonhybridizing controls as previously described (64, 97). Salmon sperm DNA and serial dilutions of chromosomal *L. monocytogenes* 10403S DNA were also spotted on the glass array for quality control and signal normalization purposes, respectively. The Array-Ready Oligo sets for 2,857 ORFs from *L. monocytogenes* EGD-e and all other 70-mer oligonucleotides were purchased from Operon Technologies (Huntsville, AL). *L. monocytogenes* strains EGD-e and 10403S represent the same *L. monocytogenes* lineage (II), serotype (1/2a), and ribotype (DUP-1039C) (94), and probes designed based on the EGD-e genome were thus expected to hybridize well with 10403S genes. As an unfinished genome sequence for strain 10403S has recently (and after initiation of this project) become available (15), we were also able to verify cross-hybridization identities (CHI) between the EGD-e probes and the target genes in strain 10403S; 2,107, 2,578, and 2,695 of the probes targeting ORFs in the EGD-e genome showed CHI values of 100, ≥95, and ≥90, respectively; only 45 probes showed CHI values of <90. A total of 117 of the EGD-e-based microarray probes were not detected in the 10403S genome, likely reflecting sequence divergence, deletion of these genes in 10403S, or genes located in the gaps of the unfinished genome sequence for 10403S. We thus were confident that the array used here would allow for the 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). Mismatches to the selected target genes are unlikely to yield false positives (i.e., genes identified as differentially regulated even if they are not) since the same mismatches will occur with both RNAs (i.e., the RNA from cells grown at 4 and 37°C).

The 70-mer oligonucleotides and controls were spotted in duplicate on Corning UltraGAPS slides (Corning, NY), using a custom-built XYZ arrayer at the Microarray Core Facility at Cornell University (Ithaca, NY). After spotting of the oligonucleotides, the slides were UV cross-linked (300 mJ for 1 min) to immobilize the oligonucleotides onto the slide. Slides were stored in a desiccator at room temperature until use.

**cDNA labeling and competitive microarray hybridization.** Total RNA (representing about 10 µg RNA for each RNA sample) was reverse transcribed into cDNA and labeled with Alexa Fluor dyes (either Alexa Fluor 555 or Alexa Fluor 647) by using the SuperScript Plus indirect cDNA labeling system (Invitrogen Inc., Carlsbad, CA). Fluorescently labeled *L. monocytogenes* cDNA from cells grown to the same growth phase (log or stationary) at 4 or 37°C was used in the competitive microarray hybridization. Immediately prior to hybridization, microarray slides were blocked and washed essentially as previously described (64). For each array, the two fluorescently labeled cDNAs (from cells grown at 4 and 37°C) were combined into one tube, dried, and resuspended in 50 µl of 1× hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, 0.1 mM dithiothreitol, 0.5× formamide, 600 µg/ml salmon sperm DNA), denatured at 95°C for 5 min (two times), and applied to the microarray slide by using mSeries LifterSlips (Erie Scientific, Portsmouth, NH). The competitive hybridization was then performed at 42°C overnight. After the hybridization, the microarray slide was washed to remove unbound fluorescently labeled cDNA in a series of low-, medium-, and high-stringency washes as previously described (64). Slides were then centrifuged briefly to dry and scanned using a GenePix 4000B scanner at the Microarray Core Facility at Cornell University (Ithaca, NY). Microarray images were analyzed using GenePix Pro 6.0 (Molecular Devices Corp., Sunnyvale, CA). Microarray experiments were performed using RNA from three biological replicates.

**Microarray data analyses.** Microarray data were analyzed using LIMMA (linear models for microarray data) software (83) in R/Bioconductor (26), including the performance of background correction and the normalization and assessment of differential expression (DE). Background corrections were performed for each microarray slide by using the "normexp" method (83), resulting in strictly positive values and reducing variability in the log ratios for genes with low transcript levels. For within-array normalization, the "print-tip loess" method (84) was used to correct for spatial variation and intensity-dependent bias. Between-array normalization was performed to scale the normalization of log ratios to the same median absolute deviation across arrays in a given data set. Correlation between duplicate spots on each array was calculated, and a linear model was fitted to the normalized log ratios for each gene; moderated *t* statistics and *P* values were generated to identify genes with DE. Statistical significance of DE results was assessed based on adjusted *P* values (*P* values were adjusted for multiple comparisons by controlling for the false discovery rate).

**GSEA.** Gene Set Enrichment Analysis (GSEA) (87) was conducted using GSEA software v2.0.1 (<http://broad.mit.edu/GSEA>) and the *M* values (log<sub>2</sub> changes [*n* = fold]) obtained from the fitted normalized data in LIMMA. Data

TABLE 1. Sequences of primers and TaqMan probes used in this study<sup>a</sup>

Gene	Primer sequence <sup>b</sup>	TaqMan probe sequence <sup>c</sup>
lmo0287	5'-GCTCGTGTGAAAGCCAACCTTG-3' 5'-TTCCTCGGCTGTGCTTGAG-3'	FAM-5'-CCGTCACAGCCAAGT-3'-MGB NFO
lmo0866	5'-GCGGTTTACGGTGGTAGTGATAT-3' 5'-GGCGTACCAACTACGATTTGTG-3'	FAM-5'-CGTCAAATCCGTTCACT-3'-MGB NFO
lmo1364 ( <i>cspL</i> )	5'-CCAAGGCGACGGATTCAA-3' 5'-CGCGTTGGCCTTCTTCAA-3'	FAM-5'-CAAGCAGTAACTTTCG-3'-MGB NFO
lmo1722	5'-CAAAGACGGTGCAGATGTACTTG-3' 5'-GGCAGTGCATAAGCCACTGTT-3'	FAM-5'-AGTATCACCAACTGGAAC-3'-MGB NFO

<sup>a</sup> The primers and TaqMan probes for *rpoB* and *gap* have previously been described (82, 88).

<sup>b</sup> The first sequence listed is that of the forward primer, and the second sequence listed is that of the reverse primer.

<sup>c</sup> FAM, 6-carboxyfluorescein; MGB, minor groove binder; NFO, nonfluorescent quencher.

for log- and stationary-phase cultures were analyzed separately. For analysis of biological function, genes were classified into "main role category: subrole category" (e.g., "cellular processes: chemotaxis and motility") based on The Institute for Genomic Research Comprehensive Microbial Resource (<http://cmr.tigr.org>) role categories for *L. monocytogenes* EGD-e. For analysis of enrichment of genes in the  $\sigma^B$ , CodY, and PrfA regulons among genes with high or low transcript levels at 4°C, genes were classified as follows: all 168 genes found by microarray analyses to be positively regulated by  $\sigma^B$  in *L. monocytogenes* cells grown under at least one of two  $\sigma^B$ -activating conditions (adjusted  $P < 0.05$ ;  $>2.0$ -fold change) (S. Raengpradub, M. Wiedmann, and K. J. Boor, submitted for publication) were considered members of the  $\sigma^B$  regulon, all 85 CodY-dependent genes identified by Bennett et al. (8) were considered members of the CodY regulon, and all 12 positively regulated PrfA-dependent genes with upstream PrfA boxes (i.e., group I genes), as reported by Milohanic et al. (65), were considered members of the PrfA regulon. GSEA parameters were set to calculate 1,000 permutations, and the gene set size parameters were adjusted to include gene sets of all sizes, except gene sets with fewer than five members. For each data set (log and stationary phases), we report those gene sets found to be significant at a false discovery rate of  $<5\%$  (i.e.,  $q < 0.05$  [ $q$  represents the estimated probability of a false positive]).

**qRT-PCR.** Real-time qRT-PCR was used to confirm selected genes that showed higher transcript levels in cells grown at 4°C than in cells grown at 37°C in the microarray analyses (see Results). Primers and TaqMan minor groove binder probes were designed using Primer Express 1.0 (Applied Biosystems, Foster City, CA) (Table 1); primers and probes for the housekeeping genes *rpoB* and *gap* have previously been reported (82, 88).

qRT-PCR was performed as previously described (54). All qRT-PCR experiments were performed using the three different RNA preparations (representing the three independent growth experiments) that were also used for the microarray experiments. qRT-PCR data were  $\log_{10}$  transformed and normalized to the geometric means for the housekeeping genes *rpoB* and *gap* as also previously described (54). Statistical analyses were performed in SAS v 9.1 (SAS Institute, Inc., Cary, NC) using a general linear model (GLM), with multiple comparisons performed using Tukey's studentized range test. An  $\alpha$  value of  $<0.05$  was considered significant for qRT-PCR data.

**Microarray data accession number.** Raw data and microarray files (MIAME format) are available at the Gene Expression Omnibus (GEO) database (4) under accession number GSE7465.

## RESULTS

**Microarray-based definition of *L. monocytogenes* cold stress genes.** Competitive whole-genome microarray hybridizations were performed to experimentally define the *L. monocytogenes* cold stress regulon, using (i) cells grown to log phase at 4°C and 37°C and (ii) cells grown to stationary phase at 4°C and 37°C (Fig. 1). Statistical analysis was performed separately for each microarray data set. Initial analyses that used an adjusted  $P$  value of  $<0.05$  and at least twofold DE to identify differentially expressed genes revealed (i) 110 and 237 genes, respec-

tively, with significantly higher transcript levels in log- and stationary-phase cells grown at 4°C and (ii) 74 and 187 genes, respectively, with significantly lower transcript levels in log- and stationary-phase cells grown at 4°C (see Fig. S1 in the supplemental material); all genes identified as differentially transcribed with an adjusted  $P$  value of  $<0.05$  are listed in Tables S1 through S4 in the supplemental material. Based on the large numbers of differentially expressed genes identified when an adjusted  $P$  value of  $<0.05$  was used as the cutoff, and in order to include only genes identified with high confidence as differentially expressed in our analyses, we modified the  $P$  value cutoff to an adjusted  $P$  value of  $<0.001$ , consistent with other studies that have used  $P$  value cutoffs lower than 0.05 to define genes differentially expressed at low temperatures (16). Thus, the two criteria used to identify genes that showed either higher or lower transcript levels at 4°C (compared to 37°C) were (i) a conservatively adjusted  $P$  value of  $<0.001$  and (ii) at

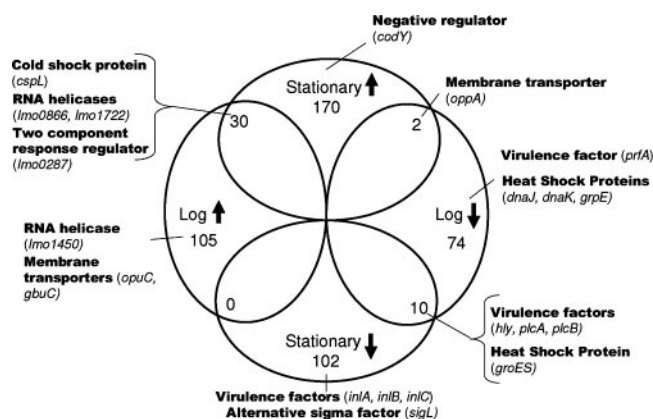


FIG. 2. Venn diagram of *L. monocytogenes* 10403S genes differentially transcribed at 4 and 37°C in log- and stationary-phase cells. Only genes that are differentially transcribed based on stringent cutoff criteria ( $\geq 2$ -fold change; adjusted  $P < 0.001$ ) are shown (Fig. S1 in the supplemental material represents a similar Venn diagram but shows all genes that showed evidence of differential transcription based on less stringent criteria [ $\geq 2$ -fold change; adjusted  $P < 0.05$ ]). In each circle, the total number of genes upregulated ( $\uparrow$ ) at 4°C or downregulated ( $\downarrow$ ) at 4°C under a given condition (log or stationary phase) is shown. Selected genes with relevant known or putative functions are also indicated.

TABLE 2. Genes identified by microarray analysis to be upregulated at 4°C in both log- and stationary-phase cells<sup>a</sup>

Protein category and gene	Protein function(s) <sup>b</sup>	Change ( <i>n</i> -fold) in cells grown to <sup>c</sup> :	
		Log phase	Stationary phase
<b>Cell membrane function</b>			
lmo0540	Similar to penicillin binding protein	2.3	3.1
lmo0581 ( <i>met</i> )	Putative <i>S</i> -adenosylmethionine-dependent methyltransferase	4.7	2.2
lmo1713	Similar to cell-shape-determining proteins	2.5	5.0
lmo1864	Similar to hemolysin III proteins, putative integral membrane protein	4.1	8.3
lmo2522	Similar to hypothetical cell wall binding protein from <i>B. subtilis</i>	10.6	4.5
<b>Lipid and/or carbohydrate metabolism</b>			
lmo0624	Acetyltransferase, Gcn5-related <i>N</i> -acetyltransferase family	3.6	2.1
lmo0625	Putative lipase/acylhydrolase	2.7	3.1
lmo1936 ( <i>gpsA</i> )	Similar to NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	2.2	4.2
lmo2480	Similar to acetyltransferase	2.2	2.4
<b>Motility</b>			
lmo0677	Similar to flagellar biosynthesis protein FliQ	3.2	2.8
<b>Signal transduction</b>			
lmo0287	Similar to two-component response regulator	2.5	3.5
<b>Transcription or translation</b>			
lmo0866	Similar to ATP-dependent RNA helicase	2.4	3.0
lmo1067	Similar to GTP-binding elongation factor	3.1	3.3
lmo1364 ( <i>cspL</i> ) <sup>d</sup>	Similar to cold shock protein	7.1	7.0
lmo1722	Similar to ATP-dependent RNA helicases	2.4	3.9
<b>Amino acid metabolism</b>			
lmo0055 ( <i>purA</i> )	Highly similar to adenylosuccinate synthetase	2.2	3.0
<b>Other or hypothetical proteins</b>			
lmo0047	Unknown	2.8	5.6
lmo0189	Highly similar to <i>B. subtilis</i> Veg protein	12.2	5.2
lmo0391	Unknown	3.8	4.1
lmo0392	Highly similar to <i>B. subtilis</i> YqfA protein	3.1	2.8
lmo0393	Unknown	3.7	3.8
lmo0592	Unknown	3.4	2.4
lmo0604	Similar to <i>B. subtilis</i> YvIA protein	2.8	3.5
lmo0905	Unknown	2.5	3.0
lmo0954	Unknown	2.8	5.0
lmo1245	Unknown	4.7	4.6
lmo1487	Similar to unknown proteins	2.3	3.0
lmo1670	Similar to conserved hypothetical proteins	2.5	4.0
lmo1937 ( <i>engA</i> )	GTP-binding protein	2.6	5.4
lmo2713	Secreted protein with one GW repeat	2.5	3.4

<sup>a</sup> Only the 30 genes that met the stringent criteria for being upregulated at 4°C in both log- and stationary-phase cells (i.e., a >2-fold change and an adjusted *P* value of <0.001) are listed here; all genes upregulated at 4°C in either log- or stationary-phase cells are listed in Tables S1 and S2 in the supplemental material.

<sup>b</sup> Protein functions are based on annotations provided by ListiList (<http://genolist.pasteur.fr/ListiList/>), TIGR (<http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi>), the NCBI (<http://www.ncbi.nlm.nih.gov/>), and the KEGG Sequence Similarity Database (<http://www.genome.jp/kegg/ssdb/>).

<sup>c</sup> Change (*n*-fold) indicates the transcript level ratio between *L. monocytogenes* 10403S cells grown at 4°C and those grown at 37°C (as determined by microarray analysis); positive values indicate that transcript levels are higher at 4°C than at 37°C (e.g., a value of 2.3 indicates a 2.3-fold-higher transcript level at 4°C than at 37°C).

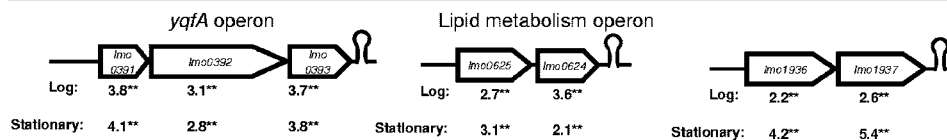
<sup>d</sup> *cspL* has also been designated *cspLA*.

least twofold DE in mRNA transcript levels between *L. monocytogenes* cells grown at 4°C and those grown at 37°C. Using these criteria, we identified a total of 245 genes that showed higher transcript levels in either log- or stationary-phase cells at 4°C, including (i) 105 genes that showed higher transcript levels in log-phase cells and (ii) 170 genes that showed higher transcript levels in stationary-phase cells; 30 of these genes showed higher transcript levels in both log- and stationary-phase cells grown at 4°C (Fig. 2; Table 2). In addition, 166 genes showed lower transcript levels in either log- or stationary-phase cells at 4°C (compared to 37°C), including 74 genes

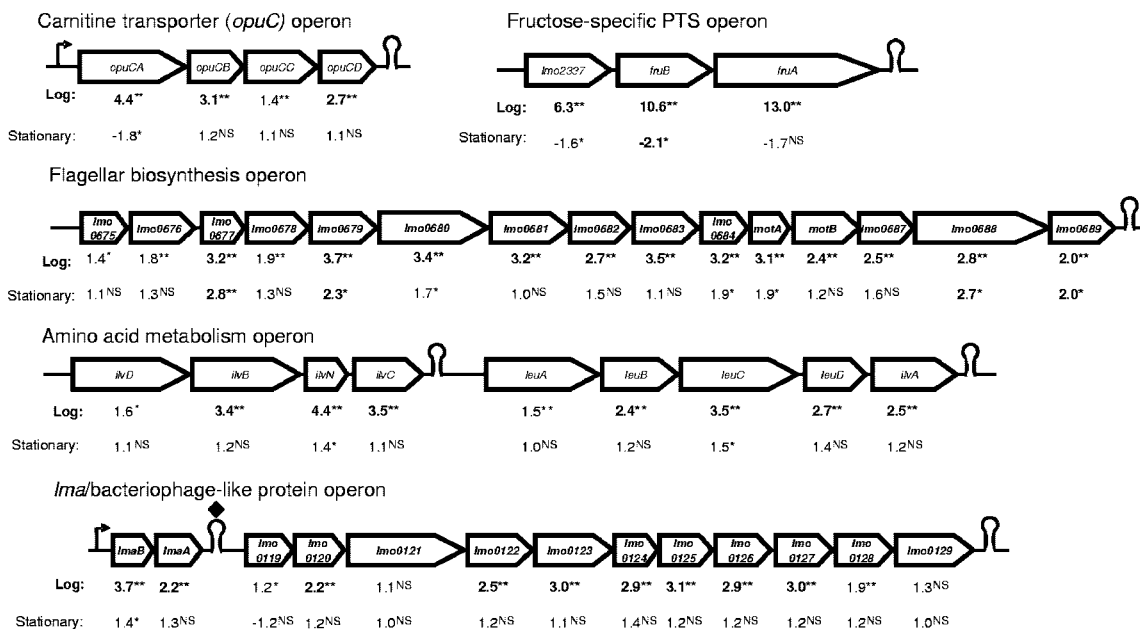
and 102 genes that showed lower transcript levels in log- and stationary-phase cells, respectively (Fig. 2).

***L. monocytogenes* genes that show higher transcript levels at 4°C. (i) Genes with higher transcript levels at 4°C in both log- and stationary-phase cells.** Genes with higher transcript levels at 4°C in both log- and stationary-phase (Table 2) could be considered part of the “core” cold growth regulon. The 30 *L. monocytogenes* genes that were grouped into this category included three operons (Fig. 3) as well as genes encoding a cold shock protein (*cspL*), two RNA helicases, a two-component response regulator (lmo0287), a flagellar biosynthesis protein

**(A) Operons with higher transcript levels in log- and stationary-phase cells grown at 4°C**



**(B) Operons with higher transcript levels in log-phase cells grown at 4°C**



**(C) Operons with higher transcript levels in stationary-phase cells grown at 4°C**

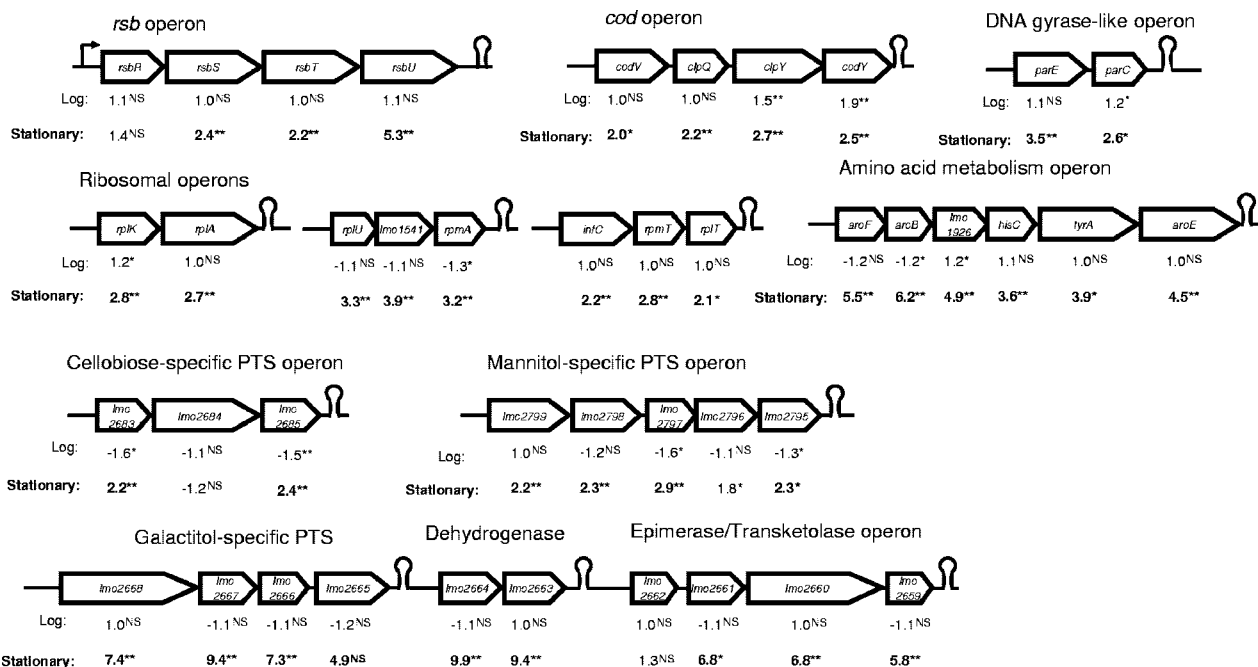


FIG. 3. Illustration of selected putative *L. monocytogenes* operons identified by microarray analysis to be upregulated at 4°C in log and stationary phases (A), log phase only (B), and stationary phase only (C). Operons were included even if some genes did not meet the stringent cutoff criteria (i.e., a  $\geq 2$ -fold difference in transcript levels and an adjusted  $P$  value of  $< 0.001$ ). Transcript level ratios are given under each gene. Positive values indicate higher transcript levels at 4°C than at 37°C; while most genes have positive values, some genes have negative values for

(lmo0679), and a number of genes with unknown functions (see Table 2 for a complete listing). A number of these genes encode proteins with confirmed or likely roles in cold growth and cold adaptation. For example, the putative ATP-dependent DEAD-box RNA helicases (encoded by lmo0866 and lmo1722; Table 2) likely act as chaperones facilitating translation at low temperatures, as supported by evidence from studies of *Escherichia coli* and *B. subtilis* (42, 91). The GTP-binding elongation factor homolog (encoded by lmo1067), which was also identified here as part of the *L. monocytogenes* "core" cold growth regulon, may assist in translation elongation at low temperatures; the lmo1067 homolog in *B. subtilis* was found to be upregulated at 15°C (7), and *E. coli* *bipA* (53.2% amino acid [aa] sequence identity to lmo1067) (28) was shown to be required for growth at low temperatures (72). While only one flagellar biosynthesis protein (encoded by lmo0679) showed higher transcript levels in both log- and stationary-phase cells at 4°C with an adjusted *P* value of <0.001, three more genes in the flagellar biosynthesis operon (consisting of lmo0675 to lmo0689) also showed higher transcript levels in both log- and stationary-phase cells but with an adjusted *P* value of <0.05 for DE in stationary phase (Fig. 3).

Interestingly, seven genes and one operon (consisting of lmo0624 and lmo0625) with higher transcript levels at 4°C in both log- and stationary-phase cells had putative or confirmed functions in lipid metabolism or represented cell membrane proteins (Table 2), consistent with the importance of membrane modifications for cold adaptation (3, 22). One of these genes, lmo0581 (*met*), encodes a putative *S*-adenosylmethionine-dependent methyltransferase; an *L. monocytogenes* deletion in this gene resulted in impaired growth under alkaline conditions and under ethanol stress (77). Another one of these genes, lmo0540, encodes a putative penicillin binding protein; this class of proteins is critical for bacterial cell wall peptidoglycan assembly (35, 100); peptidoglycan polymerization and modification appear to contribute to stress resistance, including osmotic stress, in some bacteria (75).

**(ii) Genes with higher transcript levels only in log-phase cells at 4°C.** The 75 genes that were found to have higher transcript levels only in log-phase cells grown at 4°C included at least 10 operons (representing 45 of these genes) (Fig. 3; see also Table S1 in the supplemental material) as well as 30 individual genes. A number of these operons and genes encode proteins and/or pathways with confirmed or likely roles in cold growth and cold adaptation, such as compatible solute transporters, an RNA helicase, and amino acid synthesis pathways providing precursors for low-melting-point, branched-chain fatty acids. For example, both the *opuCABCD* operon (Fig. 3), which encodes a carnitine transporter, and *gbuC*, which encodes the binding protein of glycine betaine transporter II (see Table S1 in the supplemental material), had higher transcript

levels in log-phase cells at 4°C. These transporters are known to facilitate the uptake of compatible solutes important for *L. monocytogenes* growth at low temperatures (2). lmo1450 (see Table S1 in the supplemental material) encodes a DEAD-box family RNA helicase that may act as an RNA chaperone at low temperatures (42, 91). Interestingly, the protein encoded by *lmaA* was previously found to be secreted in cultures growing at 4 and 20°C but not at 37°C, and it has been hypothesized that *lmaA* may encode a protein transport system or surface structures that may contribute to growth at low temperatures (81). Most genes in a large operon directly downstream of *lmaA* (consisting of lmo0117 to lmo0129) also showed higher transcript levels in log-phase cells at 4°C (Fig. 3); the genes in this operon encode a number of proteins with similarity to A118 phage proteins.

A number of operons with higher transcript levels in log-phase cells at 4°C have previously been shown to be downregulated by the pleiotropic transcriptional repressor CodY in *L. monocytogenes* (8); these CodY-repressed operons include the operon consisting of lmo0675 to lmo0689 (encoding flagellar biosynthesis proteins), the operon consisting of lmo0984 to lmo0988 (encoding proteins with unknown functions), and the *ilvD-ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD-ilvA* operon. Interestingly, the proteins encoded by *ilvD-ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD-ilvA* are important for the biosynthesis of amino acids, including isoleucine, which is an important precursor for the synthesis of anteiso-branched-chain fatty acids essential for ensuring membrane fluidity in *L. monocytogenes* cells grown at low temperatures (3, 102). In addition, the CodY-repressed genes *flaA*, which encodes a flagellin protein, and lmo2824, which encodes a D-3-phosphoglycerate dehydrogenase, were also found to have higher transcript levels in log-phase cells at 4°C.

**(iii) Genes with higher transcript levels only in stationary-phase cells at 4°C.** The 140 genes that showed higher transcript levels only in stationary-phase cells at 4°C included at least 24 operons (representing 58 of these genes) (Fig. 3; see also Table S2 in the supplemental material). A number of these operons and genes encode proteins and/or pathways with confirmed or likely roles in cold growth and cold adaptation, including a putative DNA gyrase protein, transcriptional regulators, ribosomal proteins, phosphotransferase systems (PTS), a peptide transporter, a two-component sensor histidine kinase, and a protein-folding enzyme. Specifically, the *parE-parC* operon, which showed higher transcript levels in stationary-phase cells at 4°C (with an adjusted *P* value of <0.05 for *parC*; Fig. 3), encodes DNA gyrase-like proteins that may have a role in relieving negative supercoiling at low temperatures (30, 46). Ribosomal operons with higher transcript levels (Fig. 3) include *rplK-rplA* (encoding ribosomal proteins L11 and L1), *rplU-lmo1541-rpmA* (encoding the ribosomal proteins L21 and

transcript level ratios, indicating higher transcript levels at 37°C (e.g., a value of -2.1 indicates a 2.1-fold-higher transcript level at 37°C). Transcript level ratios of  $\geq 2$  (indicating  $\geq 2$ -fold differences in transcript levels) are in bold; adjusted *P* values for significance of differential expression are indicated by \*\* (*P* < 0.001), \* (*P* < 0.05), and NS (not significant) (*P* > 0.05). Known promoters (GenBank accession no. AF0322444 and Y09161) (16a, 81) are marked with small arrows. Rho-independent terminators were predicted for *L. monocytogenes* as described by de Hoon et al. (19) and are shown as hairpins; the previously determined Rho-dependent terminator downstream of *lmaA* (81) is shown as a hairpin with a diamond. Genes are not drawn to scale.

L27 and a protein with unknown function), and *infC-rpmT-rplT* (encoding translation initiation factor IF-3, ribosomal protein L35, and ribosomal protein L20). In addition, six individual genes encoding ribosomal proteins were also found to be upregulated at 4°C, including lmo1480, lmo1787, lmo2548, lmo2620, lmo2627, and lmo2856 (see Table S2 in the supplemental material).

Operons encoding PTS-associated proteins (Fig. 3) that showed higher transcript levels at 4°C include a cellobiose-specific PTS operon (consisting of lmo2683 to lmo2685), a mannitol-specific PTS operon (consisting of lmo2799 to lmo2795), and a galactitol-specific PTS operon (consisting of lmo2668 to lmo2665), indicating that the expression of these particular PTS may be important for carbon assimilation during stationary-phase growth at low temperatures. In addition, two operons located immediately adjacent to the galactitol-specific PTS operon (consisting of lmo2668 to lmo2665), including the operon consisting of lmo2664 and lmo2663 (encoding sorbitol dehydrogenase and polyol dehydrogenase, respectively) and that consisting of lmo2661, lmo2660, and lmo2659 (encoding ribulose-5-phosphate-3-epimerase, transketolase, and ribulose-phosphate-3-epimerase, respectively), also showed higher transcript levels at 4°C (Fig. 3). Ribulose-5-phosphate-3-epimerase, transketolase, and ribulose-phosphate-3-epimerase are part of the phosphogluconate pathway, which is involved in the production of NADPH for fatty acid synthesis as well as in the conversion of pentoses and hexoses for nucleotide synthesis and glycolysis (86). The *aroF-aroB-lmo1926-hisC-tyrA-aroE* amino acid biosynthesis operon (Fig. 3) also showed higher transcript levels in stationary-phase cells at 4°C, consistent with two-dimensional protein gel electrophoresis data that showed the induction of *B. subtilis* AroF after 3 h of exposure to low temperatures (33).

Individual genes with higher transcript levels in stationary-phase cells at 4°C also included lmo2196 (*oppA*), which encodes an oligopeptide binding protein previously shown to be required for *L. monocytogenes* growth at 5°C (13), as well as *lisK*, which encodes a two-component regulatory system histidine kinase that is part of a response system that affects membrane composition in *L. monocytogenes* (see Table S2 in the supplemental material) (18). Two genes encoding prolyl isomerases also showed higher transcript levels at 4°C, including *tig*, which has been reported to be important for protein folding at low temperatures in *E. coli* (52), and lmo2376. While lmo2376 showed only 1.99-fold-higher transcript levels at 4°C in *L. monocytogenes* (adjusted  $P < 0.05$ ) (see Table S2 in the supplemental material), *ppiB*, the *B. subtilis* homolog of lmo2376, has been found to encode a protein that shows increased synthesis at low temperatures (33) and that is involved in protein folding (29), supporting that lmo2376 plays a role in cold growth.

Finally, some genes encoding regulatory proteins were also found to show higher transcript levels in stationary-phase cells grown at 4°C. Specifically, the four genes (i.e., *rsbRSTU*) representing the  $\sigma^A$ -dependent 5' portion of the eight-gene *L. monocytogenes sigB* operon show higher transcript levels at 4°C than at 37°C (Fig. 3). While the *codV-clpQ-clpY-codY* operon, which includes the gene encoding the transcriptional repressor CodY, showed higher transcript levels in stationary-phase cells at 4°C (Fig. 3), two previously identified CodY-repressed oper-

ons (i.e., those consisting of lmo2664 and lmo2663 and lmo2661 to lmo2659; see above) also showed higher transcript levels in stationary-phase cells at 4°C than in cells at 37°C.

***L. monocytogenes* genes that show lower transcript levels at 4°C. (i) Genes with lower transcript levels at 4°C in both log- and stationary-phase cells.** A total of 10 genes, including virulence genes and genes encoding heat shock proteins, showed  $\geq 2.0$ -fold-lower transcript levels (adjusted  $P < 0.001$ ) at 4°C in both log- and stationary-phase cells (Table 3). Interestingly, three of the six virulence genes in the major *L. monocytogenes* virulence gene island (i.e., *hly*, *plcA*, and *plcB*) showed lower transcript levels in log- and stationary-phase cells grown at 4°C (Table 3; Fig. 4), consistent with the fact that the transcription of these virulence genes is activated by PrfA (12, 59), which is preferentially translated at 37°C (45). In addition to *groES*, which showed significantly lower transcript levels, with an adjusted  $P$  value of  $< 0.001$ , in both log- and stationary-phase cells grown at 4°C, *groEL* also showed lower transcript levels under both conditions (even though only at an adjusted  $P$  value of  $< 0.05$  in stationary-phase cells) (Table 3), indicating that the *groES-groEL* operon, which encodes class I heat shock proteins, is downregulated at 4°C independent of growth phase.

**(ii) Genes with lower transcript levels only in log-phase cells grown at 4°C.** The 64 genes that were found to have lower transcript levels only in log-phase cells at 4°C included a total of seven operons (representing 20 of these genes) (Fig. 3; see also Table S1 in the supplemental material) as well as 44 individual genes, including *prfA* and an operon encoding a mannose-specific PTS (consisting of lmo0096 to lmo0098) (see Table S3 in the supplemental material). Interestingly, the *hrcA-grpE-dnaK* operon showed 3.4- to 4.3-fold-lower transcript levels in log-phase cells grown at 4°C (see Table S3 in the supplemental material). This finding was surprising as the HrcA-repressed *groES-groEL* operon also showed lower transcript levels, but this finding may be a consequence of post-transcriptional regulation of HrcA activity (89). *cspB*, which encodes a putative cold shock protein, shows 36.1-fold-lower transcript levels at 4°C than at 37°C (see Table S3 in the supplemental material), consistent with observations that the *cspB* homologs in *E. coli* and *B. subtilis* do not seem to be critical for growth at low temperatures (32, 99).

**(iii) Genes with lower transcript levels only in stationary-phase cells at 4°C.** The 92 genes found to have lower transcript levels only in stationary-phase cells at 4°C included at least 16 operons (representing 29 of these genes) (see Table S4 in the supplemental material). While approximately 30 of these genes encode proteins with unknown or hypothetical functions (see Table S4 in the supplemental material), three genes encoding internalins also showed lower transcript levels at 4°C, including the *inLAB* operon (Fig. 4) and *inlC*. Interestingly, *relA*, which encodes a (p)ppGpp synthetase involved in the stringent response, was also found to have lower transcript levels in stationary-phase cells grown at 4°C. Furthermore, *cspD*, which encodes a putative cold shock protein similar to *E. coli* CspC (59.0% aa identity) (11) and *B. subtilis* CspD (81.5% aa identity) (57), showed lower transcript levels at 4°C than at 37°C (adjusted  $P < 0.05$ ). *clpB*, which encodes an ATP-binding endoprotease, also showed considerably lower transcript levels in stationary-phase cells at 4°C (15.7-fold-lower transcript lev-

TABLE 3. Genes identified by microarray analysis to be downregulated at 4°C in both log- and stationary-phase cells<sup>a</sup>

Protein category and gene	Protein function <sup>b</sup>	Change ( <i>n</i> -fold) in cells grown to <sup>c</sup> :	
		Log phase	Stationary phase
<b>Virulence factors</b>			
lmo0201 ( <i>plcA</i> )	Phosphatidylinositol-specific phospholipase C	-8.3	-8.8
lmo0202 ( <i>hly</i> )	Listeriolysin O precursor	-20.7	-26.1
lmo0205 ( <i>plcB</i> )	Phospholipase C	-5.3	-3.3
<b>Heat shock proteins</b>			
lmo2069 ( <i>groES</i> )	Class I heat shock protein (chaperonin) GroES	-5.8	-4.4
lmo2068 ( <i>groEL</i> )	Class I heat shock protein (chaperonin) GroEL	-5.7	-3.1 <sup>d</sup>
<b>Metabolism</b>			
lmo0210 ( <i>ldh</i> )	Similar to l-lactate dehydrogenase	-4.0	-3.8
<b>Transcription or translation</b>			
lmo0822	Similar to transcriptional regulators	-2.2	-2.4
<b>Other or hypothetical proteins</b>			
lmo0104	Unknown	-2.6	-3.7
lmo0355	Similar to flavocytochrome <i>c</i> fumarate reductase chain A	-9.2	-3.9
lmo1219	Unknown	-2.5	-4.8
lmo2453	Similar to lipolytic enzyme	-2.1	-5.5

<sup>a</sup> While only 10 genes met the stringent criteria for being downregulated at 4°C in both log- and stationary-phase cells (i.e., a ≥2-fold change and an adjusted *P* value of <0.001), 11 genes are listed here. *groEL* was included (even though *groEL* differential expression in stationary-phase cells did not meet the criterion of an adjusted *P* value of <0.001) as this gene is in the same operon as *groES* (which met the criteria of a ≥2-fold change and an adjusted *P* value of <0.001); all genes downregulated at 4°C in either log- or stationary-phase cells are listed in Tables S3 and S4 in the supplemental material.

<sup>b</sup> Protein functions are based on annotations provided by ListiList (<http://genolist.pasteur.fr/ListiList/>), TIGR (<http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi>), the NCBI (<http://www.ncbi.nlm.nih.gov/>), and the KEGG Sequence Similarity Database (<http://www.genome.jp/kegg/ssdb/>).

<sup>c</sup> Change (*n*-fold) indicates the transcript ratio between *L. monocytogenes* 10403S cells grown at 4°C and those grown at 37°C (as determined by microarray analysis); negative values indicate transcript levels that are lower at 4°C than at 37°C (e.g., a value of -8.6 indicates an 8.6-fold-lower transcript level at 4°C than at 37°C).

<sup>d</sup> For lmo2068 (*groEL*), differential transcription in stationary-phase cells was significant at an adjusted *P* value of <0.05 but not at an adjusted *P* value of <0.001.

els at 4°C, representing one of the genes with the largest differences in transcript levels between 4 and 37°C in stationary phase). Interestingly, *ltrC*, encoding the low-temperature-requirement C protein (101), and *sigL*, encoding the alternative sigma factor σ<sup>L</sup>, which appears to be important for cold growth in *B. subtilis* (95), also showed lower transcript levels at 4°C (6.9- and 2.6-fold-lower transcript levels, respectively, during

stationary-phase growth at 4°C than during growth at 37°C) (see Table S4 in the supplemental material).

**GSEA of genes in different role categories.** GSEA identified three role categories that are overrepresented among the genes that showed higher transcript levels in log-phase cells grown at 4°C, including (i) “cellular processes: chemotaxis and motility” (*q* = 0.001), (ii) “amino acid biosynthesis: pyruvate

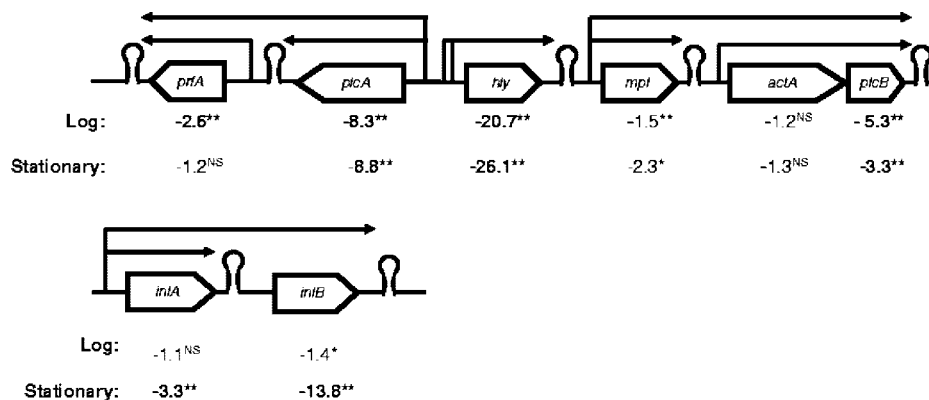


FIG. 4. Illustration of selected *L. monocytogenes* virulence genes identified by microarray analysis to be downregulated at 4°C. Transcript level ratios are given under each gene. Negative values indicate lower transcript levels at 4°C than at 37°C (e.g., a value of -3.3 indicates a 3.3-fold-lower transcript level at 4°C). Transcript level ratios of ≥2 (indicating ≥2-fold differences in transcript levels) are in bold; adjusted *P* values for significance of differential expression are indicated by \*\* (*P* < 0.001), \* (*P* < 0.05), and NS (not significant) (*P* > 0.05). Previously reported promoters and terminators (79) are indicated by small arrows and hairpins, respectively. Genes are not drawn to scale.



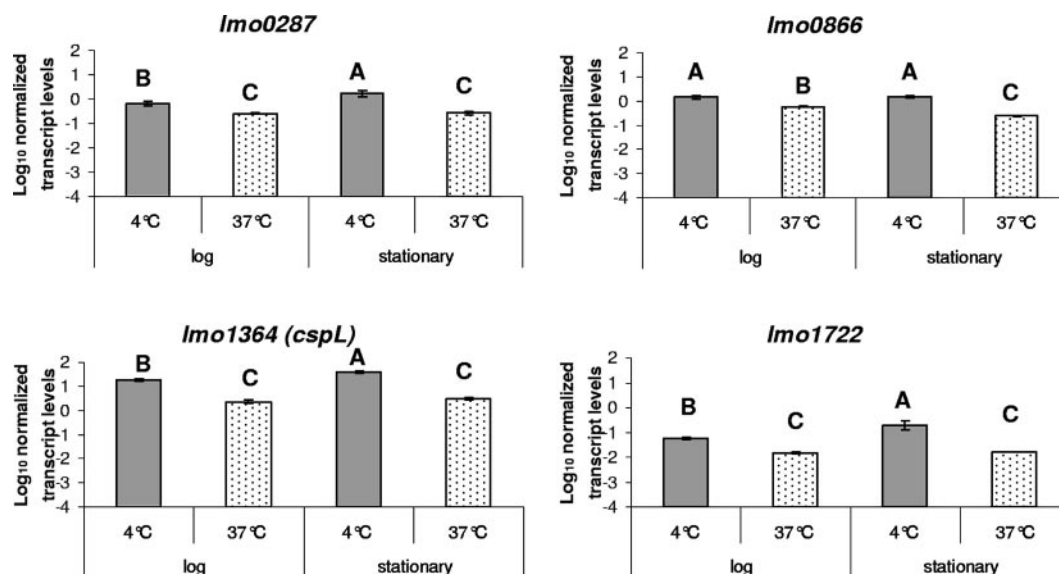


FIG. 5. Transcript levels for putative cold stress genes as determined by qRT-PCR. Transcript levels for the housekeeping genes *rpoB* and *gap* and for four putative cold stress genes were determined by qRT-PCR for *L. monocytogenes* 10403S cells grown in BHI broth to log or stationary phase at 4°C or 37°C. Transcript levels for the putative cold stress genes lmo0287, lmo0866, *cspL*, and lmo1722 were normalized to the geometric means for *rpoB* and *gap*. While *rpoB* and *gap* transcript levels were significantly affected ( $P < 0.0001$  and  $P < 0.01$ , respectively) by growth phase, with lower absolute transcript levels (per 10  $\mu\text{g}$  RNA) observed in stationary-phase cells, consistent with previous qRT-PCR data (16a), normalization of the transcript levels of the four target genes to the geometric means for *rpoB* and *gap* transcript levels was performed to adjust transcript levels to account for physiological differences between bacterial cells in different growth phases. The fact that temperature did not significantly ( $P > 0.1$ ) affect *rpoB* and *gap* transcript levels supports that analyses of target gene transcript levels normalized to *rpoB* and *gap* transcript levels provide robust data for evaluating the effect of temperature on target gene transcript levels. Data represent the averages for three biological replicates; error bars indicate standard deviations. For a given gene, different letters above the bars indicate significantly different transcript levels.

family” ( $q = 0.008$ ), and (iii) “purines, pyrimidines, nucleosides, and nucleotides: pyrimidine ribonucleotide biosynthesis” ( $q = 0.025$ ). Chemotaxis and motility-related genes include the operon consisting of lmo0675 to lmo0689, which encodes flagellar biosynthesis proteins, while genes involved in pyruvate family amino acid biosynthesis include the *ilvD-ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD-ilvA* operon. Two role categories (i.e., “protein synthesis: tRNA aminoacylation” [ $q = 0.002$ ] and “protein fate: protein folding and stabilization” [ $q = 0.019$ ]) were overrepresented among the genes with lower transcript levels in log-phase cells grown at 4°C. Genes in the “tRNA aminoacylation” subrole category that showed significantly lower transcript levels in log-phase cells at 4°C encode a number of tRNA synthetases (phenylalanyl-, glycyl-, alanyl-, and asparaginyl-tRNA synthetases), while the “protein folding and stabilization” category includes several genes encoding class I heat shock proteins (i.e., *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL*).

GSEA of stationary-phase microarray data identified four and two gene sets, respectively, that were enriched among genes with higher and lower transcript levels at 4°C. The role categories overrepresented among genes with higher transcript levels at 4°C include (i) “protein synthesis: ribosomal proteins—synthesis and modification” ( $q = 0.002$ ), (ii) “cell envelope: biosynthesis of murein sacculus and peptidoglycan” ( $q = 0.001$ ), (iii) “cellular processes: chemotaxis and motility” ( $q = 0.025$ ), and (iv) energy metabolism: ATP-proton motive force interconversion ( $q = 0.041$ ). Nine of the 21 genes in the ATP-proton motive force gene set are organized into two operons (one consisting of *atpD-atpC* and the other consisting of *atpI-atpB-atpE-atpF-atpH-atpA-atpG*),

which encode various chains of the  $\text{H}^+$ -transporting ATP synthase; 8 of these 9 genes were found to have significantly higher transcript levels at 4°C (adjusted  $P$  value of  $< 0.05$ ; 1.6- to 2.4-fold change). The role categories overrepresented among genes with lower transcript levels in stationary phase at 4°C include (i) “energy metabolism: amino acids and amines” ( $q = 0.002$ ) and (ii) cellular processes: pathogenesis” ( $q = 0.006$ ). The latter subrole category contains many of the internalin genes (i.e., *inlA*, *inlB*, and *inlC*) but none of the genes in the *prfA* virulence gene island, as many of these genes were categorized into separate subrole categories.

**GSEA of  $\sigma^B$ , CodY-, and PrfA-dependent genes.** GSEA found that genes in the CodY regulon were significantly enriched among genes with higher transcript levels in log-phase cells ( $q = 0.011$ ) and stationary-phase cells ( $q < 0.001$ ) at 4°C, suggesting the derepression of CodY at 4°C. Genes in the PrfA regulon were also significantly enriched among genes with lower transcript levels in log-phase cells ( $q = 0.006$ ) and stationary-phase cells ( $q = 0.007$ ) at 4°C, consistent with the enhanced expression of PrfA at 37°C (45, 58). Genes in the  $\sigma^B$  regulon were significantly enriched among genes with higher transcript levels in log-phase cells at 4°C ( $q = 0.02$ ) but were also significantly enriched among genes with lower transcript levels in stationary-phase cells at 4°C ( $q < 0.001$ ), suggesting that growth phase may be more important than temperature in regulating transcription of the  $\sigma^B$  regulon.

**qRT-PCR confirmation of selected *L. monocytogenes* genes that have higher transcript levels at 4°C.** For four genes that were found by microarray analysis to show higher transcript

levels at 4°C than at 37°C in both log- and stationary-phase cells, differential transcription was also evaluated by qRT-PCR. Genes selected for qRT-PCR confirmation included lmo0287 (encoding a two-component response regulator) and lmo1364 (*cspL*, encoding cold shock protein L) as well as lmo0866 and lmo1722, both of which encode ATP-dependent DEAD-box RNA helicases; all four of these genes encode proteins with plausible roles in cold growth and cold adaptation (42, 44, 66, 91). The transcript levels of these genes were normalized to the geometric means for the housekeeping genes *rpoB* and *gap*; the factor “temperature” (4°C or 37°C) did not have a significant effect on either *rpoB* or *gap* transcript levels ( $P > 0.1$ ; GLM). The analysis of normalized transcript levels for the four target genes (lmo0287, lmo0866, *cspL*, and lmo1722) showed that normalized transcript levels for all four genes were significantly ( $P < 0.0005$ ; GLM) affected by temperature. For all four target genes tested, transcript levels were higher at 4°C than at 37°C in both log- and stationary-phase cells (Fig. 5), confirming the microarray data, which also showed higher transcript levels for these four genes at 4°C in both log- and stationary-phase cells (Table 2).

## DISCUSSION

Overall, our data show that (i) a large number of *L. monocytogenes* genes are differentially expressed at 4 and 37°C, (ii) *L. monocytogenes* genes with higher transcript levels at 4°C include a number of genes and operons with previously reported or plausible roles in cold adaptation, and (iii) *L. monocytogenes* genes with lower transcript levels at 4°C include a number of virulence and virulence-associated genes as well as some heat shock genes.

**(i) A large number of *L. monocytogenes* genes are differentially expressed at 4 and 37°C.** Among the 2,857 *L. monocytogenes* ORFs included in our microarray, a total of 245 genes (8.6% of all ORFs in the array) showed higher transcript levels ( $\geq 2.0$ -fold change; adjusted  $P$  value  $< 0.001$ ) in stationary- or log-phase cells grown at 4°C than in cells grown at 37°C. A previous study using selective capture of transcribed sequences identified 24 genes to be more highly transcribed in *L. monocytogenes* 10403S cells grown at 10°C than in cells grown at 37°C (62). While some genes (i.e., *flaA* and *aroA*) identified in this previous study (62) as upregulated at 10°C also showed higher transcript levels at 4°C in our study, other genes identified in this previous study (62) as upregulated at 10°C were not identified as cold induced here, most likely due to differences in experimental conditions (e.g., use of different growth temperatures) and/or methods used to measure differential transcription. A previous study by Bayles et al. (5), which used two-dimensional gel electrophoresis to identify cold shock proteins in *L. monocytogenes* 10403S cells exposed to 5°C for up to 120 min and cold acclimation proteins in *L. monocytogenes* cells exposed to 5°C for up to 5 h, found 12 cold shock proteins and 4 cold acclimation proteins, which were not further identified. Two-dimensional protein gel electrophoresis studies of other psychrotolerant microorganisms (e.g., *Pseudomonas fragi* and *Arthrobacter globiformis*) found 20 *P. fragi* proteins that were present at higher levels in cells grown at 4°C for 270 min than in cells grown at 30°C (38) and 9 *A. globiformis* proteins that were induced at 4°C after cold shock for 240 min (9).

While fairly small numbers of cold-induced proteins are usually identified using traditional two-dimensional gel electrophoresis approaches, including proteins in *L. monocytogenes* (5, 9, 38, 47), whole-genome microarray analyses can clearly provide more-comprehensive analysis of the cold shock regulon. Similar to our studies, microarray-based transcriptome analyses of other bacteria revealed large numbers of genes that were upregulated during cold shock and cold growth (16, 25). For example, transcriptome analysis of *B. subtilis* (16) identified 279 of 4,107 ORFs to be upregulated ( $\geq 2$ -fold change; adjusted  $P$  value  $\leq 0.01$ ) at 15°C compared to those at 37°C. In our study, a total of 166 *L. monocytogenes* genes (i.e., 5.8% of all ORFs in the array) showed lower transcript levels at 4°C (in either stationary-phase or log-phase cells). By comparison, microarray analyses of *B. subtilis* found 301 genes (7.3% of ORFs) to be downregulated in cells grown at 15°C (compared to those in cells grown at 37°C) (16).

Transcriptome analysis of bacteria exposed to other stress conditions, e.g., heat shock (1, 41), also revealed stress response regulons of considerable size. For example, in *B. subtilis*, 124 of approximately 4,100 genes present in a microarray (i.e., 3.0% of genes) were induced during heat shock (i.e., at 3, 10, and 20 min after exposure of cells to 48°C) (41). In *Staphylococcus aureus*, 98 of approximately 3,300 ORFs included in the Affymetrix *S. aureus* GeneChip (i.e., 3.0% of genes) were upregulated after 30 min of heat shock at 42°C (1).

**(ii) *L. monocytogenes* genes upregulated at 4°C include a number of genes and operons with previously reported or plausible roles in cold adaptation.** Bacteria that transition to low temperatures have to overcome a number of well-recognized problems, including decreased membrane fluidity, which reduces nutrient uptake capabilities, increased superhelical coiling of DNA, which may negatively affect a bacterium's ability to replicate or transcribe DNA, secondary structures in RNA, which affect translation, reduced enzyme activities, inefficient or slow protein folding, and reduced ability of ribosomes to function properly at low temperatures (33). A considerable number of the *L. monocytogenes* genes and operons we identified to be upregulated in cells grown at 4°C appear to facilitate bacterial responses to these challenges, as discussed in more detail below.

*L. monocytogenes* genes that are upregulated at 4°C and encode proteins likely to facilitate replication at low temperatures include *parE* and *parC*, which encode subunits of DNA gyrase. DNA gyrases likely play a role in relieving negative supercoiling at low temperatures, supported by observations that DNA gyrases are important for cellular processes in *B. subtilis* at low temperatures (30) and that gyrase-encoding genes are cold inducible in *E. coli* (46). Three genes encoding RNA helicases were also found to be upregulated at 4°C in *L. monocytogenes*, consistent with the observation that some DEAD-box RNA helicases can act as RNA chaperones that resolve secondary structures in mRNA that are formed at low temperatures (42, 91). Similar to our findings, the transcription of genes encoding RNA helicases has also been found to be induced in other bacteria exposed to low temperatures, including *E. coli*, where *rhIE* (*csdA*), encoding a putative ATP-dependent RNA helicase, showed higher transcript levels in cells exposed to 16°C (76), and *Yersinia pestis*, where four genes (*rhIE*, *srmB1*, *dbpA*, and *deaD*), encoding RNA helicases, showed higher transcript levels at 10°C (36). The importance

of helicases for cold growth is also supported by the observation that an *E. coli* strain with a site-directed mutation in the DEAD motif of the helicase CsdA (91) showed reduced growth at low temperatures. In addition to RNA helicases, some cold-inducible cold shock proteins also facilitate translation by binding to single-stranded mRNA (presumably preventing mRNA that has been unwound by RNA helicases from refolding) until the ribosome initiates translation at low temperatures (33, 42). For example, in *B. subtilis*, RNA helicases such as CshA and CshB have been found to work together with cold shock proteins (e.g., CspB) to help keep mRNA single stranded so that the ribosome can initiate translation (42). Interestingly, we also found that a gene encoding an *L. monocytogenes* cold shock protein (*cspL*) is induced in cells grown at 4°C, consistent with the fact that *B. subtilis* *cspC*, which shares 90% aa sequence identity with *cspL* (57), has also been found to have higher transcript levels at 15°C (32), which may at least be partially due to increased *cspC* mRNA stability in cells grown at low temperatures (50). In addition to the upregulation of genes facilitating translation at 4°C, we also found that *tig*, which encodes a putative prolyl isomerase important for protein folding (33), shows higher transcript levels at 4°C, consistent with the observation that an *E. coli* *tig* mutant is cold sensitive (52). Thus, genes upregulated in *L. monocytogenes* cells grown at 4°C include genes encoding proteins important for replication as well as both translation and proper folding of translated proteins.

We also found that a number of genes encoding translation initiation factors and ribosomal proteins showed higher transcript levels in stationary-phase *L. monocytogenes* cells grown at 4°C. Previous two-dimensional protein gel electrophoresis studies have also found that some ribosomal proteins are induced at low temperatures in *B. subtilis* (i.e., ribosomal proteins S6 and L7/L12) (31) and in *E. coli* (i.e., initiation factor IF-2) (47). Translation initiation factors may aid tRNA binding to the ribosomal subunit in *E. coli* (47), and ribosomal proteins may be important for the correct assembly of rRNA at low temperatures (31). Genome-wide transcriptional profiling of *B. subtilis* also found that genes encoding ribosomal proteins and translation initiation factors were upregulated in bacteria exposed to cold shock at 15 and 18°C (7, 49), further supporting the importance of ribosomal proteins and translation initiation factors in bacterial cold adaptation.

*L. monocytogenes* has two major compatible solute transport systems, i.e., carnitine and glycine betaine transporters, that are known to be critical for adaptation at low temperatures (2). In our study, *gbuC*, encoding a glycine betaine binding protein, and the *opuC* operon, encoding the carnitine transport system in *L. monocytogenes*, were induced at 4°C during log-phase growth, consistent with previous evidence supporting a critical role for these osmolyte transporter systems in growth at low temperatures (2). Similarly, transcription of *oppA*, which encodes an oligopeptide binding protein transporter that is required for *L. monocytogenes* growth at low temperatures (13), was also upregulated at 4°C in stationary-phase cells. The upregulation of similar oligopeptide and osmolyte transporter genes (e.g., the *opuCABCD* operon, *oppC*) has previously been observed in *B. subtilis* exposed to low temperatures (16, 49), indicating a conserved role for these transporters in cold growth across different gram-positive genera.

Genes with higher transcript levels in *L. monocytogenes* ex-

posed to low temperatures also included a number of genes with apparent involvement in modifications of membrane fatty acid composition, which may facilitate adjustments of membrane fluidity to allow for growth at low temperatures. Specifically, upregulation of the amino acid biosynthesis operon *ilvD-ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD-ilvA* may be important at low temperatures as the amino acid isoleucine is a critical precursor for the synthesis of anteiso-branched-chain fatty acids (102), which have a lower melting temperature than iso-branched-chain fatty acids and thus allow *L. monocytogenes* to maintain membrane fluidity at low temperatures (3, 22, 69, 80, 102). Genes in this operon were also induced in *B. subtilis* grown at 15°C (33, 49). A critical role for this pathway in adaptation to growth at low temperatures is further supported by findings that anteiso-C<sub>15:0</sub> fatty acids are more abundant in the cell membrane of *L. monocytogenes* cells grown at low temperatures than in that of *L. monocytogenes* cells grown at 37°C (3). Interestingly, *L. monocytogenes* lmo0287, which was upregulated at 4°C, encodes a two-component response regulator with 83.5% aa sequence identity to *B. subtilis* *yccF* (57). While this two-component response regulator appears to be essential in *L. monocytogenes* (51, 96) and in *B. subtilis* (23), as supported by the inability to generate null mutants in the respective gene in either of these two species, a null mutation in the nonessential *yccF* ortholog in *Streptococcus pneumoniae* was found to affect the transcription of fatty acid biosynthesis genes and transport-associated genes that alter membrane composition (66), suggesting that this regulator contributes to alterations of cell membrane composition that may facilitate cold growth.

Additional noteworthy genes with higher transcript levels in *L. monocytogenes* at 4°C include a flagellum-associated operon (consisting of lmo0675 to lmo0689), which showed higher transcript levels in log phase at 4°C, as well as other flagellum-associated genes that showed higher transcript levels in stationary-phase cells at 4°C. This is consistent with previous studies that found the transcription of *flaA* and the operon consisting of lmo0675 to lmo0689 to be temperature regulated (20, 70). The observation that chemotaxis and motility genes were found to be enriched among genes with higher transcript levels at 4°C likely represents MogR-mediated repression of flagellar gene transcription at elevated temperatures (34) and is consistent with the observation that *L. monocytogenes* motility is generally observed only in cells grown at temperatures below 37°C (20, 34). Interestingly, while transcriptional analysis of *E. coli* also revealed that flagellum-associated genes show higher transcript levels in cells exposed to 15°C (73), in *B. subtilis*, chemotaxis and motility genes appeared to be repressed at low temperatures (16).

Our microarray analyses revealed that *codY* showed higher transcript levels in stationary-phase cells grown at 4°C than in stationary-phase cells grown at 37°C. *codY* encodes a pleiotropic transcriptional regulator, which appears to actively repress transcription during log phase at 37°C and appears to have reduced repressing activity as *L. monocytogenes* enters stationary phase at 37°C (8). CodY represses genes involved in amino acid metabolism, nitrogen assimilation, and sugar uptake, and derepression of the CodY regulon has been found to be important for virulence in *L. monocytogenes* (8, 67). Interestingly, we found that a number of CodY-repressed genes (8, 67) showed higher transcript levels in log-phase cells grown at 4°C

than in log-phase cells grown at 37°C, including a flagellar biosynthesis operon and an amino acid metabolism-associated operon (*ilvD-ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD-ilvA*), which was found to be CodY dependent in *B. subtilis* (67). In stationary-phase cells, most of these CodY-repressed genes and operons were no longer differentially expressed at 4°C and 37°C, even though two other operons that have been shown to be repressed by CodY at 37°C (8) showed higher transcript levels in stationary-phase cells at 4°C than in stationary-phase cells at 37°C, despite the fact that *codY* transcription itself was upregulated. This may be explained by the fact that CodY needs corepressors to repress transcription (85); therefore, CodY-dependent genes may not be repressed even if *codY* transcription is enhanced. Overall, our data indicate that *codY* transcription as well as transcription of a number of genes in the CodY regulon may be affected by growth temperature, consistent with the proposed role for CodY in virulence (8), which would suggest temperature-dependent regulation of CodY to appropriately express virulence genes at the host body temperature. Our data indicating that *L. monocytogenes* CodY appears to contribute to the regulation of genes important for growth at low temperatures are consistent with the observation that *B. subtilis* CodY regulates a large number of genes that encode proteins important for adaptation to poor growth conditions (67). *L. monocytogenes* CodY thus appears to contribute to both virulence and stress response (e.g., cold stress), similar to  $\sigma^B$  (53). While both the CodY and PrfA regulons showed consistent differential expression patterns in log- and stationary-phase cells (with genes in the CodY regulon more likely to show higher transcript levels at 4°C and genes in the PrfA regulon more likely to show lower transcript levels at 4°C), differential transcription of the  $\sigma^B$  regulon appeared to be affected more by growth phase than by temperature. Genes in the  $\sigma^B$  regulon were enriched among genes with higher transcript levels in log-phase cells at 4°C and among genes with lower transcript levels in stationary-phase cells at 4°C, suggesting induction of the  $\sigma^B$  regulon in log-phase cells at 4°C (compared to that in log-phase cells at 37°C), consistent with observations by Becker et al. (6). While genes in the  $\sigma^B$  regulon seemed to show lower transcript levels in stationary-phase cells at 4°C, the 5' portion of the *sigB* operon, which encodes four regulators of  $\sigma^B$  (RsbS), showed higher transcript levels in stationary-phase cells grown at 4°C, indicating that higher transcript levels for *rsb* genes does not necessarily result in higher  $\sigma^B$  activity, consistent with extensive posttranslational regulation of Rsb and  $\sigma^B$  activities (17). While *L. monocytogenes sigB* null mutants did not show a cold growth defect in rich media (16a), *sigB* null mutants showed reduced growth at refrigeration temperatures in minimal media and in meats (6, 68). In *B. subtilis*, a *sigB* null mutant showed reduced growth at 15°C (14). A number of *B. subtilis* genes induced during growth at low temperatures appear to be part of the  $\sigma^B$  regulon (16, 71, 92). While  $\sigma^B$  thus appears to contribute to the regulation of gene expression during growth at low temperatures, further studies are needed to more clearly define specific roles for  $\sigma^B$  and its regulon in *L. monocytogenes* cold adaptation, including their roles in different growth phases and under different growth conditions.

(iii) *L. monocytogenes* genes downregulated at 4°C include a number of virulence and virulence-associated genes as well as

some heat shock genes. Stress response genes with lower transcript levels in *L. monocytogenes* cells grown at 4°C included the *groES-groEL* operon, consistent with the fact that these genes encode well-documented class I heat shock proteins (24, 37, 40). Interestingly, some *L. monocytogenes* genes annotated as genes encoding cold shock proteins were also found to be downregulated in cells grown at 4°C, including *cspD* as well as *cspB*, a homolog of *E. coli cspC* (11) and *B. subtilis cspD* (57). These findings are consistent with observations that *E. coli cspC* is not cold inducible and is expressed at 37°C (98) and with data suggesting that *B. subtilis cspD* has a more important role at 37°C (32). Some of the conserved features of cold shock proteins thus may be important for functions required under conditions other than cold growth.

Consistent with the well-documented, temperature-dependent transcription of PrfA-dependent *L. monocytogenes* virulence genes (45, 58), we also found that many of the PrfA-dependent genes in the *prfA* virulence gene island, as well as PrfA-dependent genes in other locations (e.g., *bsh*), showed higher transcript levels at 37°C than at 4°C, generally regardless of growth phase. On the other hand, *inlA*, *inlB*, and *inlC*, which are all coregulated by  $\sigma^B$  and PrfA (60, 63, 64), showed higher transcript levels at 37°C than at 4°C in stationary-phase cells but not in log-phase cells. Overall, these findings suggest different patterns of temperature-dependent regulation for  $\sigma^B$ - and PrfA-coregulated internalins, compared to genes regulated only by PrfA, consistent with previous observations of temperature-dependent regulation of PrfA- and  $\sigma^B$ -dependent genes (64). Finally, *relA*, which encodes a (p)ppGpp synthetase reported to be involved in the regulation of *L. monocytogenes* virulence (90), also showed higher transcript levels at 37°C than at 4°C in stationary-phase cells, thus identifying another protein with regulatory function, in addition to PrfA,  $\sigma^B$ , and CodY, that may be involved in temperature-dependent regulation of *L. monocytogenes* virulence genes.

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