INFECTION AND IMMUNITY, Feb. 2006, p. 1323–1338 Vol. 74, No. 2
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.2.1323–1338.2006

Intracellular Gene Expression Profile of *Listeria monocytogenes*†

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Received 23 May 2005/Returned for modification 10 July 2005/Accepted 26 October 2005

Listeria monocytogenes **is a gram-positive, food-borne microorganism responsible for invasive infections with a high overall mortality.** ***L. monocytogenes*** **is among the very few microorganisms that can induce uptake into the host cell and subsequently enter the host cell cytosol by breaching the vacuolar membrane. We infected the murine macrophage cell line P388D1 with** ***L. monocytogenes*** **strain EGD-e and examined the gene expression profile of** ***L. monocytogenes*** **inside the vacuolar and cytosolic environments of the host cell by using whole-genome microarray and mutant analyses. We found that ~17% of the total genome was mobilized to enable adaptation for intracellular growth. Intracellularly expressed genes showed responses typical of glucose limitation within bacteria, with a decrease in the amount of mRNA encoding enzymes in the central metabolism and a temporal induction of genes involved in alternative-carbon-source utilization pathways and their regulation. Adaptive intracellular gene expression involved genes that are associated with virulence, the general stress response, cell division, and changes in cell wall structure and included many genes with unknown functions. A total of 41 genes were species specific, being absent from the genome of the nonpathogenic** ***Listeria innocua*** **CLIP 11262 strain. We also detected 25 genes that were strain specific, i.e., absent from the genome of the previously sequenced** ***L. monocytogenes*** **F2365 serotype 4b strain, suggesting heterogeneity in the gene pool required for intracellular survival of** ***L. monocytogenes*** **in host cells. Overall, our study provides crucial insights into the strategy of intracellular survival and measures taken by** ***L. monocytogenes*** **to escape the host cell responses.**

Listeriosis is a food-borne disease with high mortality rates. *Listeria monocytogenes*, the causative agent, can survive in diverse habitats, including extracellular abiotic and intracellular environments, and infect a large number of vertebrate and invertebrate hosts. All of the identified virulence genes in *L. monocytogenes* are known to be under the direct or partial control of positive regulatory factor A (PrfA) (9, 34). Entry into nonprofessional phagocytes is mediated by surface-associated gene products internalin A and B (12, 31). Early after internalization, the bacteria disrupt the phagosomal membrane of the host by expressing a pore-forming toxin, listeriolysin (Hly), and a phospholipase (PlcA) to access the cytoplasm of the host cell. Intracellular movement of the bacteria inside the host cell is mediated by ActA, which polymerizes the host actin molecules and propels itself inside the cytosol of the host cell. Spreading from one cell to another is dependent on hemolysin (Hly) and another phospholipase (PlcB) (39). Mutations have been introduced into virulence genes of *L. monocytogenes* that lead to debilitating phenotypes in the mouse model of infection. In addition, a hexose phosphate transporter, UhpT, that is required for efficacious intracellular growth has recently been described (11). Recently, a global view of the PrfA regulon has been obtained through whole-genome expression profiling (35).

At present there is a lack of knowledge about intracompartmentally expressed genes and their products in *L. monocytogenes*. To obtain information towards this end, we explored and quantified gene expression through whole-genome transcriptome profiling of *L. monocytogenes* EGD-e isolated directly from the cytosol of infected cells. We also exploited current information on the roles of virulence gene factors to examine bacterial gene expression in the vacuolar compartment following uptake. Our data provide a comprehensive view of changes in gene expression as the bacterium transits from the extracellular milieu and adapts to growth in the cytoplasm of the infected host.

In this work we characterized lmo0206, lmo0207, and lmo2219 as new members of the PrfA regulon and further investigated the involvement of lmo0206 (*orfX*), lmo0207 (*orfZ*), lmo0229 (*ctsR*), *sigB*, lmo1298 (*tnrA*), lmo2200 (*ohrR*), and lmo2219 (*prsA*) genes in intracellular survival by producing deletion mutants. Our data show that the deletion of lmo0206, lmo0207, lmo0229, lmo2200, and lmo2219 affected the intracellular survival of *L. monocytogenes* inside murine macrophage (P388D1) cells.

MATERIALS AND METHODS

Strains and growth conditions. *L. monocytogenes* EGD-e (20) and its several isogenic mutants were used in this study. Bacteria were grown in brain heart infusion (BHI) broth (Difco) at 37°C with shaking. *Escherichia coli* InvαF′ cells (Invitrogen) were grown in Luria-Bertani broth containing 300 μg of erythromycin/ml to select for the plasmid pAUL-A (9). For *L. monocytogenes* EGD-e, pAUL-A was selected with 5 μg of erythromycin/ml. pMV158 (36) was selected on BHI containing 5 μg of tetracycline/ml. A list of strains and plasmids used in this study is presented in Table 1. All experiments were done with bacterial cultures at an optical density at 600 nm of 1.0.

General molecular biological techniques. Standard molecular techniques were used for DNA manipulation (45). Plasmid DNA was transferred into *E. coli* InvαF′ (Invitrogen) by using the method of Hanahan (24). The electroporation protocol of Park and Stewart (37) was used for the transformation of *L. monocytogenes* strains.

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

TABLE 1. Strains and plasmids used in this study

Organism or plasmid	Genotype or strain	Reference
L. monocytogenes	EGD-e	20
	Δ*hly* Δ*plcA*	38
	Δ*prfA*	This study
	Δlmo0206 (Δ*orfX*)	This study
	Δlmo0207 (Δ*orfZ*)	This study
	Δlmo0229 (Δ*ctsR*)	This study
	Δ*sigB*	This study
	Δlmo1298 (Δ*tnrA*)	This study
	Δlmo2200 (Δ*ohrR*)	This study
	Δlmo2219 (Δ*prsA*)	This study
E. coli	InvαF′	Invitrogen
pAUL-A		46
pMV158		36

Construction of deletion mutants. Mutants with chromosomal in-frame deletions were constructed by generating the 5′ (with primers P1 and P2) and the 3′ (with primers P3 and P4) flanking regions of the genes concerned. Primers used to generate the flanking regions are shown in Table S1 in the supplemental material (restriction sites are underlined). The purified PCR fragments were digested with either NotI or BamHI (as stated in Table S1 in the supplemental material) and ligated (for lmo1298, ligation was carried out as described in reference 41). Following ligation, the fragments were amplified using the 5′ upstream and 3′ downstream primers (P1 and P4) of the 5′ and 3′ flanking regions, respectively, of the genes concerned, digested with appropriate restriction enzymes (see Table S1 in the supplemental material), and ligated into the temperature-sensitive suicide vector pAUL-A, which was digested with the same enzymes and used to transform *E. coli* InvαF′ electrocompetent cells. Plasmid DNA of pAUL-A bearing the fragments was isolated from the recombinants and used to transform *L. monocytogenes* EGD-e to generate the chromosomal deletion mutants by using previously described methods (46). The deletions in the genes concerned were identified by PCR and confirmed by sequencing of the PCR fragments by using primers P1 and P4.

Cell culture and infection model. P388D1 murine macrophage cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories) in 6-well or 24-well tissue culture plates for intracellular growth assays and in 85-mm-diameter tissue culture plates for RNA isolation. Intracellular growth assays were performed as previously described in reference 31 at 1, 4, and 8 h postinfection. For extraction of listerial RNA, P388D1 cells were grown to about 80% confluence. Bacteria were added to the cell monolayer at a multiplicity of infection (MOI) of 10 per eukaryotic cell for EGD-e and 500 per eukaryotic cell for the Δ*hly* Δ*plcA* mutant. Infection was carried out for 45 min, followed by the addition of fresh medium containing 20 μg of gentamicin/ml. The medium on the plates (containing 20 μg of gentamicin/ml) with EGD-e was replaced 2 h postinfection with fresh medium containing 50 μg of gentamicin/ml. At each step, the plates were washed extensively with 1× phosphate-buffered saline. Incubations were carried out in a humidified incubator for up to 8 h.

Plaque formation assays. L929 cells were grown to monolayers in 6-well tissue culture plates in RPMI 1640 supplemented with 5% FCS. The cells were infected with 8 μl of a 10^{-4} dilution of a washed bacterial culture with an optical density at 600 nm of 1.0 for 2 h at 37°C in a humidified incubator and subsequently incubated for 30 min in the presence of 50 μg of gentamicin/ml. The agarose overlay contained 1% agarose in minimal essential medium, 5% FCS, glutamine, nonessential amino acids, and 10 μg of gentamicin/ml. Experiments were carried out in duplicate. The plates were examined for plaques after an additional incubation at 37°C for 3 days postinfection.

Confocal and immunofluoresence microscopy. P388D1 cells were grown on coverslips and infected with either *L. monocytogenes* EGD-e or the Δ*hly* Δ*plcA* mutant carrying pMV158 (Δ*hly* Δ*plcA*::pMV158) for constitutive green fluorescent protein expression. Infection was carried out as described above. After 1 h (for the Δ*hly* Δ*plcA* mutant) and 8 h (for EGD-e), cells were fixed with 3.7% formaldehyde in 1× phosphate-buffered saline for 30 min at room temperature. Coverslips infected with the Δ*hly* Δ*plcA*::pMV158 mutant were stained with 100 nM LysoTracker red (Molecular Probes) to stain acidic vacuoles, mounted with 50 mg of moviol/ml, and analyzed under a confocal microscope (Leica DM IRBE).

Coverslips infected with *L. monocytogenes* EGD-e were stained with fluorescein isothiocyanate-phalloidin for actin tail staining, and *L. monocytogenes* was detected by using α-ActA monoclonal antibody N81 (35a) followed by a secondary Cy3-conjugated goat anti-mouse polyclonal antibody (Dianova). Coverslips were mounted with 50 mg of moviol/ml and analyzed under an immunofluoresence microscope (Axiophot Zeiss).

Generation of a microarray for *L. monocytogenes* EGD-e. To create a whole-genome microarray for *L. monocytogenes* EGD-e, 2,840 unique primer pairs were selected using two independent software tools: GenomePRIDE (23) and the CAAT-Box (16), a modified version of Primer3 (44). Fifty-four and 46% of the open reading frame-specific primers were generated using the GenomePRIDE software and the CAAT-Box tool, respectively, with amplicon sizes ranging from 100 to 600 bp in length.

PCR amplifications were performed with HotStar *Taq* DNA polymerase (QIAGEN), with chromosomal DNA as the template. After purification of PCR products and inspection by agarose gel analysis to ensure that the products were free of extraneous material, the DNA concentrations were determined with SYBR green I (Molecular Probes) by using a SpectraMAX GEMINI XS spectrofluorometer (Molecular Devices). DNA was prepared for spotting by diluting the purified PCR products in dimethyl sulfoxide at a final concentration of 25 to 50 ng/μl.

Whole-genome microarray slides for *L. monocytogenes* EGD-e were produced by using a Generation III microarray spotter (GE Health Care). Each microarray contained 2,840 PCR products spotted in duplicate onto Type 7*(star) slides (GE Health Care) and UV cross-linked with 50 mJ in a Stratalinker UV cross-linker 1800 (Stratagene).

RNA isolation and purification, labeling with Cy dyes, and hybridization. Bacteria were isolated from infected host cells as described previously (14). Macrophages were lysed using 0.1% (wt/vol) sodium dodecyl sulfate, 0.1% (vol/vol) acidic phenol, and 19% (vol/vol) ethanol in water for 30 min on ice. Bacteria from 85-mm-diameter tissue culture plates were pooled from plates containing the Δ*hly* Δ*plcA* mutant and EGD-e. Total bacterial RNA was extracted using the RNeasy mini kit (QIAGEN) after a wash in SET buffer (50 mM NaCl, 5 mM EDTA, and 30 mM Tris-HCl [pH 7.0]) containing 10% sodium dodecyl sulfate and a pretreatment at 37°C for 30 min with shaking at 350 rpm (Eppendorf Thermomixer Comfort) with 0.1 ml of Tris-HCl (pH 6.5) containing 50 mg of lysozyme/ml, 25 U of mutanolysin, 40 U of SUPERase, and 0.2 mg of proteinase K (Ambion) and finally treated with 4 U of DNase I (RNase free; Ambion). The total RNA thus isolated was ethanol precipitated and quantified by absorbance at 260 and 280 nm, and quality was analyzed by using the Agilent 2100 bioanalyzer. cDNA was generated and labeled with Cy dyes (both Cy3 and Cy5 for each probe) by using 3 μg of total RNA with the CyScribe postlabeling kit (GE Health Care). The cDNA thus generated was quantified by absorbance at 550 and 650 nm for Cy3 and Cy5, respectively, by using an ND-1000 spectrophotometer (NanoDrop Technologies).

Hybridization was performed with an ASP base hybridizer (GE Health Care) for 12 h at 42°C [per the instructions of the Type 7*(star) slide manufacturer] by using 10 pmol of Cy dye-labeled cDNA of the wild-type and mutant strains, 50 μl of hybridization buffer (GE Health Care), and 100 μl of deionized formamide (Ambion) per slide. Hybridizations were conducted with cDNA of (i) the Δ*hly* Δ*plcA* mutant in BHI versus that of the Δ*hly* Δ*plcA* mutant 1 h postinfection, (ii) EGD-e in BHI versus that of EGD-e 4 h postinfection, and (iii) EGD-e in BHI versus that of EGD-e 8 h postinfection.

Imaging and microarray analysis. Hybridized Type 7*(star) slides were imaged with a Generation III array scanner (GE Health Care). The fluorescent signal intensities from each spot on the microarray were quantified using Spotfinder software (GE Health Care).

Data from image analysis were averaged over the duplicate spots present in one array and then normalized in a two-step process; first, an intrachip normalization was carried out in order to standardize signals from the Cy5 or the Cy3 channel. In a second step, we normalized over the entire series to bring the means to a common value. The intrachip normalization was carried out using a robust local regression function (56) as implemented in the BIOCONDUCTOR package marrayNorm (http://www.bioconductor.org/). The parameters were as follows: global local regression by the LOWESS function, with consideration of the inner 40% of data, i.e., those values where the log ratio was between the 30th and the 70th percentiles of the distribution. For the second interchip normalization, the signals from the Cy3 channel of the first chip were taken as a reference, and for all other signals the median of the difference of the logarithmic values was taken as a correcting factor. In a prior check of the correlation coefficient, low-signal values were excluded from the calculation when they deviated from linearity. For details, see reference 6. The performance of the normalization procedure was controlled by inspecting scatter plots for all possible combinations of signals. Missing data from the cDNA microarray analyses were replaced by the corresponding mean gene expression values calculated from the biological replicates or eliminated when this information was not available. Correlation matrices were obtained using Pearson's correlation coef-

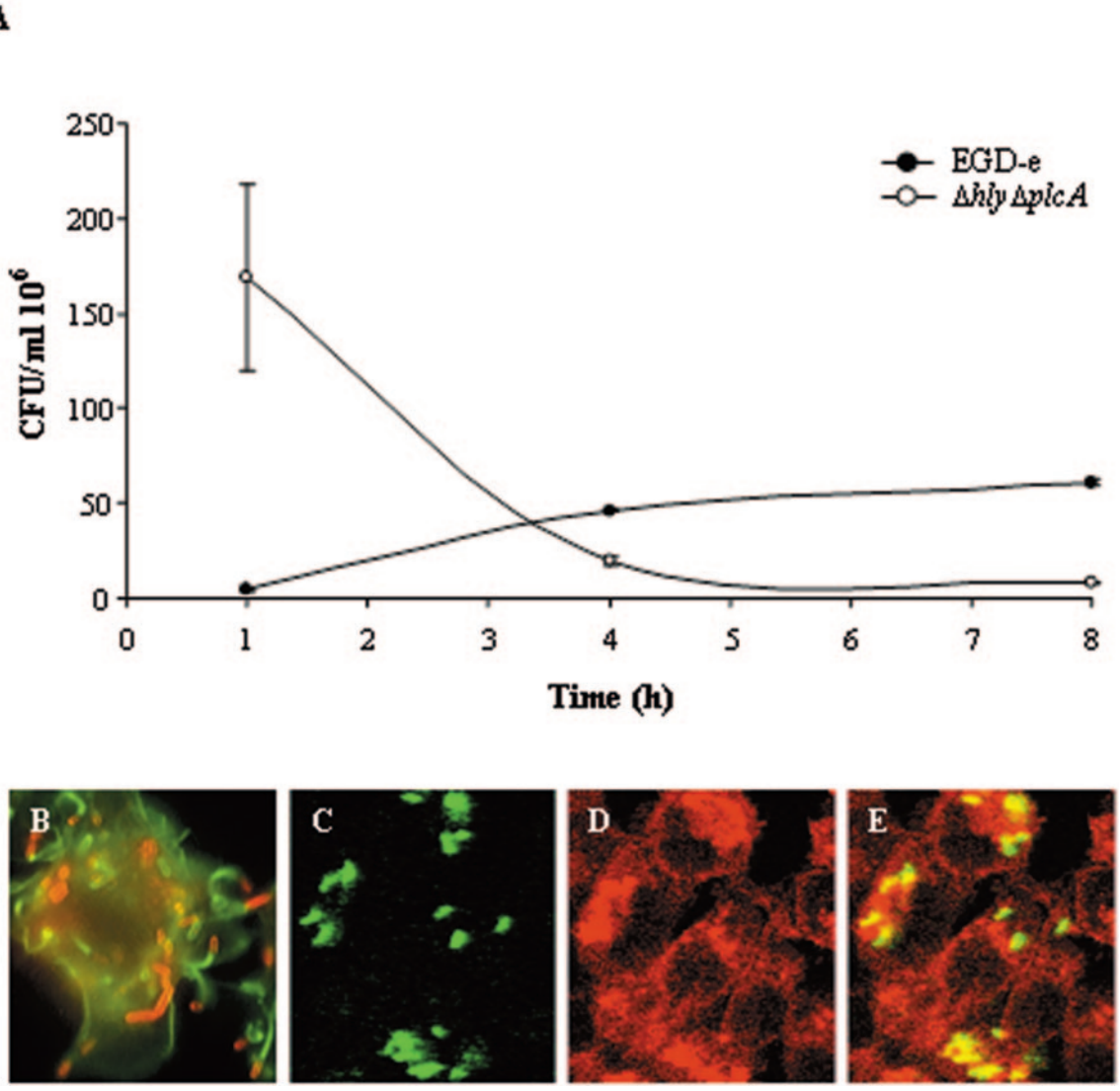

FIG. 1. Survival of *L. monocytogenes* EGD-e and the isogenic double-deletion Δ*hly* Δ*plcA* mutant intacellularly. (A) P388D1 murine macrophage cells were infected with the wild-type strain EGD-e and the isogenic double-deletion Δ*hly* Δ*plcA* mutant at MOIs of 10 and 500, respectively, in a 6-well tissue culture plate. One, 4, and 8 h postinfection, eukaryotic cells were lysed with cold water and plated onto agar plates for CFU counts. The data shown here are representative of results from three independent experiments. (B) Immunofluorescence microscopic image of P388D1 cells infected with wild-type EGD-e 8 h postinfection. (C to E) Confocal microscopic images of P388D1 cells infected with the Δ*hly* Δ*plcA* mutant 1 h postinfection. (C) Δ*hly* Δ*plcA* mutant bacteria expressing a constitutive green fluorescent protein. (D) P388D1 cells stained with LysoTracker red. (E) Merge of the diagrams in panels C and D.

ficient (*r*) for both biological replicates as well as across experiments and revealed *r* values of 0.972 and 0.753. The significance analysis of microarrays (SAM) program was used to analyze the data after downloading of SAM into Excel (49a). Briefly, for each gene *i*, SAM computes a statistic, *di*, measuring the strength of the relationship between gene expression and the response variable. It uses repeated permutations to determine if the expression of any gene is significantly related to the response. The program creates a profile of observed versus expected values, and values which lie outside a user-defined region of this profile are considered to indicate a significant relationship to the response or, in this case, significantly regulated genes. In this study, one-class response analyses were chosen to test if the mean level of gene expression for each of the 2,831 genes differed from a hypothesized mean. Therefore, SAM was conducted with $\log_2$ ratios of gene expression values from three experiments, those with (i) the Δ*hly* Δ*plcA* mutant in BHI versus the Δ*hly* Δ*plcA* mutant 1 h postinfection, (ii) EGD-e in BHI versus EGD-e 4 h postinfection, and (iii) EGD-e in BHI versus EGD-e 8 h postinfection. Seven hundred and twenty permutations were made, and the delta value giving the lowest false discovery rate was chosen to set the cutoff for significantly altered genes. The resulting three gene lists were then filtered for genes with a false discovery rate of ≤0.22 and a fold change of ≥2. By using these criteria, a final set of 484 significantly regulated genes was collated from all three experiments (Table S2 in the supplemental material).

Quantitative PCR analysis. Quantitative real-time PCR was performed on the ABI Prism 7700 sequence detection system. Forward and reverse primers (Table S1 in the supplemental material) were purchased from Sigma Genosys and were designed using Primer Express 1.0 software to produce an amplicon length of about 150 bp. Total RNA was isolated as described above with an additional treatment with the DNase-free kit (Ambion). Quantitative PCR was performed for 40 cycles with 5 μl of cDNA, 25 μl of 2× SYBR green PCR master mix (Applied Biosystems), and 10 pM (each) forward and reverse primers in a final volume of 50 μl. A standard curve was generated for each primer pair by using different copy numbers of genomic DNA from *L. monocytogenes* EGD-e. For each primer pair, a negative control (water), an RNA sample without reverse transcriptase (to determine genomic DNA contamination), and a sample with a known amount of template copies (to determine the efficiency of the reaction) were included as controls during

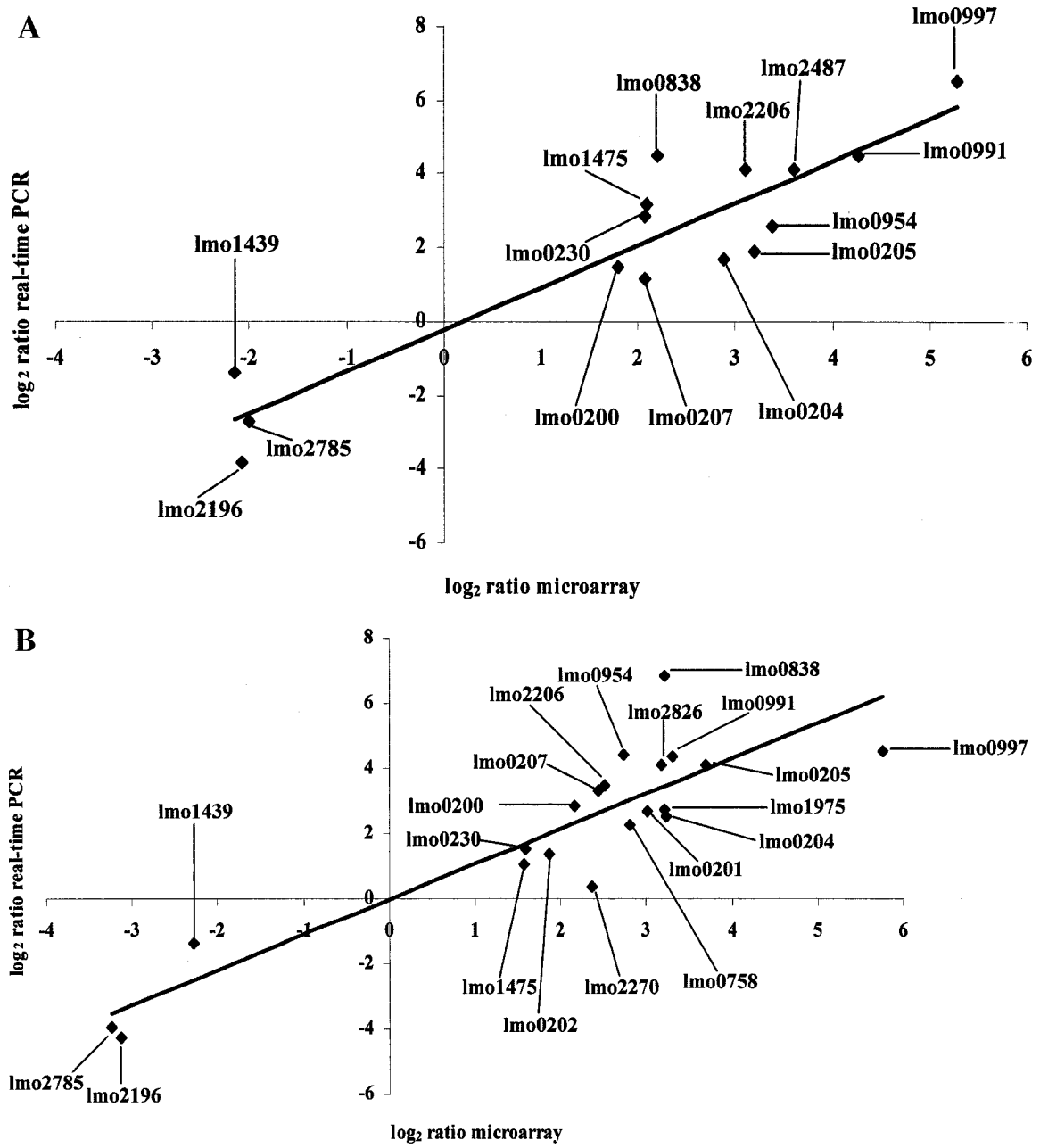

FIG. 2. Validation of microarray data with quantitative real-time PCR analysis. (A) Validation of the intravacuolarly expressed genes. (B) Validation of intracytosolically expressed genes at 4 h postinfection. (C) Validation of intracytosolically expressed genes at 8 h postinfection.

cDNA quantification. All samples after real-time PCR were run on a 1.5% agarose gel to verify that only a single band was produced.

RESULTS

Intracellular growth properties of *L. monocytogenes* EGD-e and the Δ*hly* Δ*plcA* mutant in P388D1 cells. *L. monocytogenes* EGD-e was used to infect two different murine macrophage cell lines, J774 and P388D1, and intracellular replication of bacteria was monitored as described in Materials and Methods. In the study reported here, we used the P388D1 cell line, rather than the widely used cell line J774, because preliminary experiments revealed that the latter is more sensitive to killing by *L. monocytogenes* EGD-e infection (data not shown). By using the P388D1 cell line, growth of wild-type intracellular bacteria could be monitored even at 8 h postinfection (Fig. 1A). In addition, the isogenic Δ*hly* Δ*plcA* double-deletion mutant that lacks both phosphatidylinositol phospholipase C and listeriolysin, whose activities are required for exit of bacteria to the intracytoplasmic compartment, is rapidly killed in this cell line (Fig. 1A). As shown in Fig. 1C, D, and E, Δ*hly* Δ*plcA* mutant bacteria were entrapped within the acidic host cell vacuoles and were rapidly eliminated. Thus, to model adaptation and growth of listeriae within different intracellular compartments, we used the wild-type strain and its isogenic Δ*hly*

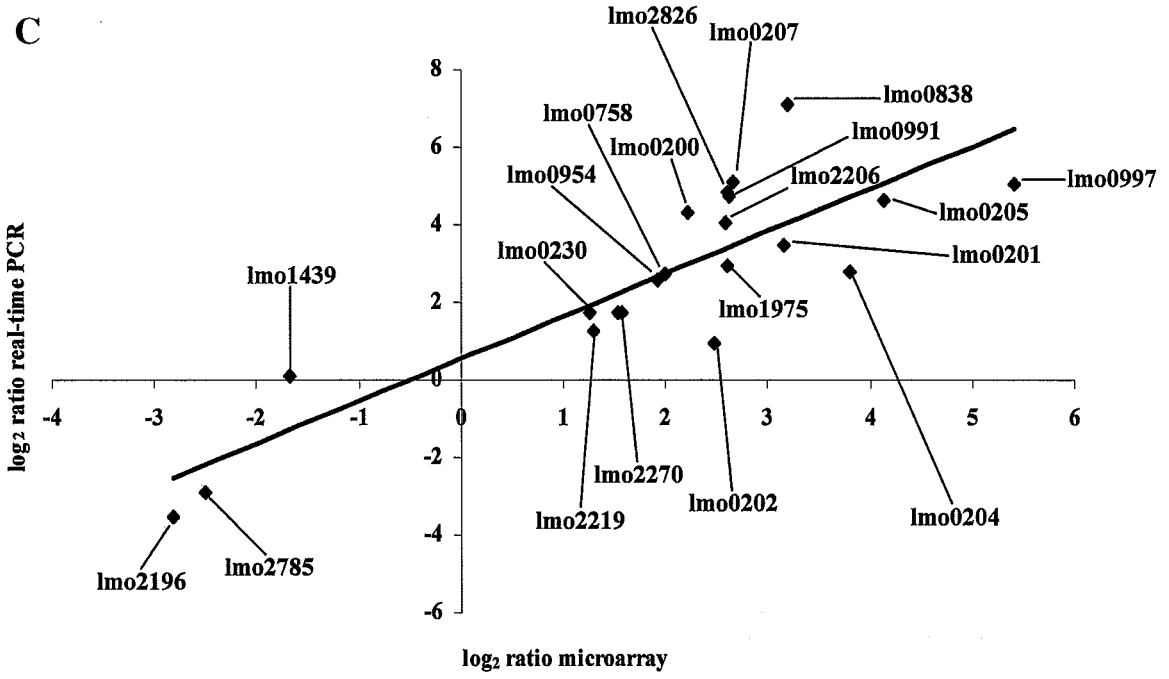

FIG. 2—*Continued.*

Δ*plcA* double-deletion mutant. Bacterial cells were collected from infected P388D1 host cells at 1 h (for the Δ*hly* Δ*plcA* mutant) and at 4 and 8 h (for EGD-e) postinfection.

Collection of bacterial RNA from infected cells has been limited by the inherent difficulties of separating large quantities of host cell RNA. We performed RNA isolation using the cold phenol-ethanol protocol described in reference 14, which allows the disruption of the eukaryotic cell and stabilization of the bacterial RNA at the same time. At each time point, excellent-quality bacterial total RNA was isolated which was almost free from eukaryotic RNA contamination (data not shown). We were able to isolate about 1.5 to 3 μg of prokaryotic total RNA per 85-mm-diameter tissue culture plate. RNA was reverse transcribed and labeled and hybridized to the cDNA microarray as described in Materials and Methods.

Alterations in the transcriptome of *L. monocytogenes* EGD-e during growth in cellular compartments. To understand the mechanisms *L. monocytogenes* EGD-e employs for intracellular survival, we used the whole-genome microarray to search for genes that showed changes in expression during the transition of bacteria from the vacuoles into the cytoplasm of P388D1 cells. To study the expression within the phagosomal compartments of the host cells, we used cDNA of the *L. monocytogenes* Δ*hly* Δ*plcA* mutant obtained 1 h after infection of P388D1 cells, when bacteria were found entirely in acidic vacuolar compartments (Fig. 1C, D, and E). The whole-genome transcription profile of the wild-type EGD-e bacteria growing in the cytosol was obtained by preparing cDNA transcripts from bacteria harvested at 4 and 8 h postinfection, when bacteria were found primarily in the host cytoplasm (Fig. 1B). In both cases, the intracellular bacterial expression profile was compared to that of the bacteria growing in BHI medium at exponential phase. Each data set in our study represents the average of results from three independent biological experiments, each including two technical replicates. Additionally, we compared the expression profiles of the Δ*hly* Δ*plcA* mutant and EGD-e growing in BHI medium and found them to be highly correlated, with an *r* of 0.956. Similar comparisons between extracytosolic and intracytosolic EGD-e revealed an *r* of between 0.679 and 0.771. This allowed us to directly compare the intraphagosomal and intracytosolic expression profiles of the bacterial strains used in this study.

Overall, a total of 484 genes representing ~17% of the total genome were mobilized to enable adaptation for intracellular growth (Table S2 in the supplemental material). Of these 484 genes, 301 were up regulated and 182 were down regulated during intracellular growth. There were 66 genes specifically up regulated for survival in the phagolysosome and 115 genes up regulated for growth in the intracytosolic compartment. In addition, 42 and 76 genes were specifically down regulated in vacuolar and cytosolic compartments, respectively. Among genes commonly regulated in either compartment, 120 were up regulated and 64 were down regulated. A single gene (lmo1254) was down regulated in the vacuolar compartment and up regulated in the cytosolic compartment of the host cell.

To identify candidate virulence genes specific to *L. monocytogenes* EGD-e, all of the genes regulated during intracellular growth were evaluated for the presence or absence of a homolog in the nonpathogenic *Listeria innocua* CLIP11262 strain (Table 2). This analysis revealed that 41 genes were uniquely present in *L. monocytogenes* EGD-e. Of the 41 putative *L. monocytogenes* species-specific genes, 10 have been previously implicated in the intracellular growth of this bacterium. We also examined whether any of the genes regulated during intracellular growth were strain specific by searching for the presence of regulated genes in the previously sequenced *L. monocytogenes*

F2365 serotype 4b genome. A total of 25 genes were detected (Table 2), suggesting heterogeneity in the gene pool required for intracellular survival of *L. monocytogenes* EGD-e.

Expression of virulence and virulence-associated genes. The virulence gene cluster of *L. monocytogenes* comprising the genes *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB* (lmo0200 to lmo0205) was up regulated and belongs to the class of genes that are strongly regulated during intracellular growth. Transcription of the virulence gene cluster and other virulence genes is dependent on the PrfA transcriptional activator protein. We also detected the up-regulated transcription of lmo206 (*orfX*) and lmo0207 (*orfZ*), encoding gene products of unknown functions. Neither lmo0206 nor lmo0207 has to date been implicated in the virulence process of *L. monocytogenes*, but the expression patterns of these genes identify them as potential virulence factors. Among the genes encoding the internalins, *inlA* (lmo0433), *inlB* (lmo0434), and *inlC* (lmo1786) were observed to be up regulated in this study. Another PrfA-regulated gene, *uhpT* (lmo0838), encoding a hexose phosphate transporter, was uniformly up regulated during cytosolic growth. The gene *prsA* (lmo2219) that encodes a product homologous to the posttranslocation molecular chaperone protein PrsA of *Bacillus subtilis* was also up regulated, albeit at the late cytosolic phase of growth. We detected the up regulation of *sepA* (lmo2157) in the cytosol. SepA is reported to be under the control of both PrfA and SigB in *L. monocytogenes* (28, 35).

The listerial *agr* locus comprising lmo0048 to lmo0051 was down regulated in our experiment. lmo0051, coding for AgrA, was previously described to be virulence associated in *L. monocytogenes* in an in vivo mouse model but not in tissue culture experiments (4).

Metabolic gene expression. The observation that nonpathogenic listeriae such as *L. innocua* are unable to grow efficiently in the host cytoplasm (47) has led to the suggestion that adaptation is required to enable growth of *L. monocytogenes* in the intracellular milieu. We examined the expression profiles of genes involved in sugar catabolism and de novo synthesis of macromolecular precursors and their transport to gain insight into substrates that were available intracellularly. Intermediary metabolism and energy production systems were down regulated following entry of bacteria into the phagososomal compartment, and components of bacterial nucleotide sugar metabolism were also down regulated throughout the infection cycle.

In particular, transcript levels of genes encoding enzymes involved in the second part of glycolysis (lmo1570, lmo2455, lmo2456, lmo2458, and lmo2459), i.e., the five steps corresponding to the conversion of glyceraldehyde-3-phosphate into pyruvate, were reduced during infection. In addition, the enzyme encoded by *pgi* (lmo2367), responsible for the conversion of glucose-6-phosphate into fructose-6-phosphate, and that encoded by *ldh* (lmo0210), responsible for the conversion of pyruvate into lactate, were down regulated. Genes encoding pyruvate dehydrogenase, the *pdhABC* operon (lmo1052 to lmo1054), were down regulated, suggesting interruption of the citric acid cycle and gluconeogenesis during intracellular growth.

A PrfA-regulated gene, *uhpT*, is highly up regulated during intracellular growth, promoting the uptake of phosphorylated hexoses (glucose-1-phosphate and glucose-6-phosphate). In addition, we found up regulation of the operon encoding the glycerol kinase and the glycerol uptake facilitator (lmo1538 to lmo1539) and glycerol-3-phosphate dehydrogenase (lmo1293), indicating that glycerol, probably deriving from the activity of the phospholipases A and B on cellular lipids, is an additional carbon source for intracellular growth. Components of the fatty acid biosynthesis pathway (lmo0970, lmo1808, lmo2201, and lmo2202) were down regulated throughout the infection cycle.

Other carbon sources that may be used intracellularly include carbohydrates and polyols such as mannose, fructose, cellobiose, mannitol, and pentitol transported by the phosphotransferase (PTS), entering the cell as phosphorylated substrates, and degraded by sugar hydrolases or β-glucosidases. Significantly, lmo1002, encoding a PTS phosphocarrier protein, Hpr, was down regulated inside the cytosol. The amount of Hpr is known to decrease in the absence of glucose (3). This suggests that the other substrates are preferred over glucose. Glucose inhibits *prfA* expression and hence the expression of other virulence genes in this pathogen (27).

During intravacuolar survival, we found that particularly the expression of genes for enzymes involved in the synthesis of branched-chain amino acids (encoded by *ilv* and *leu*) was highly enhanced (lmo1983 to lmo1991). The TnrA protein, a negative regulator of branched-chain-amino-acid expression in *B. subtilis*, has strong homology to the product of lmo1298, which was down regulated (49). The major source of nitrogen inside the host cell is preferably the amino acids and oligopeptides accessible in the host cell cytosol, as suggested by the up regulation of the oligopeptide transporters (lmo0135, lmo0136, and lmo0152). Also, down regulation of aminoacyl tRNA synthase genes *glyS*, *serS*, *cysS*, *alaS*, *hisS*, *valS*, *thrS*, *ileS*, *leuS*, *tyrS*, and *trpS* suggests availability of the respective amino acids within the cytosol. An ammonium transporter gene, *nrgA* (lmo1516), was strongly down regulated following initiation of the infection cycle.

We found that only the nonoxidative stage of the pentose phosphate pathway was up regulated, leading to the production of xylulose- and ribose-5-phosphate, both of which are precursors of nucleotide metabolism in the cell.

Stress response genes. Responses to environmental insults are followed by the induction of specific patterns of stress response genes. Genes responsible for the class I stress response include *dnaJ*, *dnaK*, *grpE*, and *hrcA* (lmo1472 to lmo1475) and *groEL* and *groES* (lmo2068 to lmo2069), all of which encode the classical chaperone proteins. These were clearly up regulated during survival in the vacuolar and cytosolic compartments (except for *groEL* and *groES* genes, which were specifically up regulated in the vacuolar compartment) (Table 3). Members of the CtsR-regulated class III stress proteins, which are ATP-dependent proteases required for degradation of abnormal and short-lived proteins, were strongly induced in both the vacuolar and intracytoplasmic compartments. In particular, expression of the protease genes *clpP*, *clpC*, *clpE*, and *clpB* (lmo2468, lmo0232, lmo0997, and lmo2206) and lmo0230 and lmo0231 (similar to *mcsA* and *mcsB*, the modulators of the CtsR regulon) was induced in the phagolysosome and was maintained throughout intracellular growth. In fact, *clpE* is the most highly regulated gene in our data set. These changes were accompanied by moderate up regulation of the *ctsR* gene (lmo0229), albeit only in the vacuolar compartment. Similarly, the *htrA* gene (lmo0292), encoding an envelope-associated

TABLE 2. Species- and strain-specific genes of *L. monocytogenes* EGD-e regulated intracellularly[a]

IV gene	FC	IC gene (4 h)	FC	IC gene (8 h)	FC	Gene designation	Description of product
Species-specific genes							
lmo0200	3.45	lmo0200	4.48	lmo0200	4.66	*prfA*	Listeriolysin-positive regulatory factor A
		lmo0201	8.08	lmo0201	8.97	*plcA*	Phosphatidylinositol-specific phospholipase C
		lmo0202	3.66	lmo0202	5.57	*hly*	Listeriolysin O
lmo0203	10.82	lmo0203	18.76	lmo0203	19.24	*mpl*	Zinc metalloproteinase precursor
lmo0204	7.33	lmo0204	9.40	lmo0204	13.96	*actA*	Actin assembly-inducing protein precursor
lmo0205	9.12	lmo0205	12.97	lmo0205	17.63	*plcB*	Phospholipase C
		lmo0206	2.84	lmo0206	3.12		lmo0206 protein
lmo0257	3.06	lmo0257	2.66	lmo0257	2.15		Similar to unknown protein
lmo0419	−3.44	lmo0419	4.16				Similar to unknown protein
				lmo0433	2.84	*inlA*	Internalin A
		lmo0434	2.05	lmo0434	2.06	*inlB*	Internalin B
lmo0445	2.04	lmo0445	3.30	lmo0445	2.62		Similar to transcription regulator
		lmo0748	12.44	lmo0748	7.25		lmo0748 protein
lmo0750	3.31	lmo0750	30.42	lmo0750	23.29		lmo0750 protein
lmo0751	2.55	lmo0751	25.84	lmo0751	16.02		lmo0751 protein
lmo0752	2.79	lmo0752	32.47	lmo0752	21.24		Weakly similar to a putative haloacetate dehalogenase
		lmo0753	3.56	lmo0753	2.58		Similar to transcription regulator, Crp/Fnr family
		lmo0754	4.48	lmo0754	3.63		Weakly similar to a bile acid 7-alpha dehydratase
lmo0838	4.58	lmo0838	9.30	lmo0838	9.22	*uhpT*	Highly similar to hexose phosphate transport protein
		lmo1081	2.11				Similar to glucose-1-phosphate thymidyl transferase
		lmo1082	−3.70	lmo1082	−2.49		Similar to dTDP-sugar epimerase
lmo1083	−2.48	lmo1083	−2.59				Similar to dTDP-D-glucose 4,6-dehydratase
lmo1084	−2.09	lmo1084	−2.10				Similar to dTDP-L-rhamnose synthetase
lmo1100	7.57					*cadA*	Cadmium resistance protein
lmo1786	2.88	lmo1786	7.20	lmo1786	9.55	*inlC*	Internalin C
		lmo1876	−2.32				
		lmo1877	−2.35	lmo1877	−2.03		Similar to formyl-tetrahydrofolate synthetase, N-terminal part
		lmo1968	4.55	lmo1968	3.68		Similar to creatinine amidohydrolases
		lmo1969	4.33	lmo1969	3.74		Similar to 2-keto-3-deoxygluconate-6-phosphate aldolase
		lmo1970	3.55	lmo1970	2.74		Similar to putative phosphotriesterase-related proteins
		lmo1971	3.12	lmo1971	3.01		Similar to pentitol PTS system enzyme IIC component
		lmo1973	6.16	lmo1973	6.32		Similar to PTS system enzyme IIA component
		lmo1974	10.53	lmo1974	8.78		Similar to transcription regulators, GntR family
lmo2085	2.97	lmo2085	6.16	lmo2085	4.41		Putative peptidoglycan-bound protein with LPXTG motif
		lmo2157	2.73	lmo2157	2.37	*sepA*	SepA
		lmo2226	2.86				Similar to unknown proteins
lmo2257	2.13	lmo2257	2.25	lmo2257	2.43		Hypothetical protein
		lmo2270	5.15	lmo2270	2.91	*comK*	Similar to competence transcription factor ComK, N terminal part
		lmo2672	2.80				Weakly similar to transcription regulator
		lmo2773	2.13				Similar to transcription antiterminator
		lmo2826	9.06	lmo2826	6.08		Similar to efflux proteins
Strain-specific genes							
lmo0398	2.55						Similar to PTS system enzyme IIA
lmo0399	2.84	lmo0399	2.46				Similar to fructose-specific PTS enzyme IIB
lmo0400	2.42						Similar to fructose-specific PTS enzyme IIC
		lmo0434	2.05	lmo0434	2.06	*inlB*	Internalin B
lmo0445	2.04	lmo0445	3.30	lmo0445	2.62		Similar to transcription regulator
		lmo0748	12.44	lmo0748	7.25		lmo0748 protein
lmo0750	3.31	lmo0750	30.42	lmo0750	23.29		lmo0750 protein
lmo0751	2.55	lmo0751	25.84	lmo0751	16.02		lmo0751 protein
		lmo0783	2.96	lmo0783	2.67		Similar to mannose-specific PTS system enzyme IIB
		lmo0888	−2.13	lmo0888	−2.27		Similar to *B. subtilis* YdcE protein
		lmo1081	−2.11				Similar to glucose-1-phosphate thymidyl transferase
		lmo1082	−3.70	lmo1082	−2.49		Similar to dTDP-sugar epimerase
lmo1083	−2.48	lmo1083	−2.59				Similar to dTDP-D-glucose 4,6-dehydratase
lmo1084	−2.09	lmo1084	−2.10				Similar to dTDP-L-rhamnose synthetase
lmo1100	7.57					*cadA*	Cadmium resistance protein
		lmo1876	−2.32				
		lmo1877	−2.35	lmo1877	−2.03		Similar to formyl-tetrahydrofolate synthetase, N-terminal part
		lmo1968	4.55	lmo1968	3.68		Similar to creatinine amidohydrolases
		lmo1969	4.33	lmo1969	3.74		Similar to 2-keto-3-deoxygluconate-6-phosphate aldolase
		lmo1970	3.55	lmo1970	2.74		Similar to putative phosphotriesterase-related proteins
		lmo1971	3.12	lmo1971	3.01		Similar to pentitol PTS system enzyme IIC component
		lmo1973	6.16	lmo1973	6.32		Similar to PTS system enzyme IIA component
		lmo1974	10.53	lmo1974	8.78		Similar to transcription regulators, GntR family
lmo2269	8.39	lmo2269	7.48	lmo2269	5.59		lmo2269 protein
		lmo2270	5.15	lmo2270	2.91	*comK*	Similar to competence transcription factor ComK, N terminal part

[a] Species- and strain-specific genes of EGD-e were identified by comparing the genome sequences of *L. monocytogenes* EGD-e, *L. innocua*, and *L. monocytogenes* F2365 serotype 4b by using an identity of >50% and >75% coverage of the protein sequences. Fold change (FC) indicates the ratio of expression intensities of the intravacuolar (IV) and intracytosolic (IC) *L. monocytogenes* genes to those of genes of in vitro-growing *L. monocytogenes*.

TABLE 3. Intracellularly expressed genes of *L. monocytogenes* EGD-e controlled by different regulators[a]

IV gene	FC	IC gene (4 h)	FC	IC gene (8 h)	FC	Gene designation	Description of product
RpoN-regulated genes							
lmo0048	−3.05	lmo0048	−7.02	lmo0048	−4.18		Similar to *Staphylococcus* two-component sensor histidine kinase AgrB
		lmo0049	−3.18	lmo0049	−3.01		lmo0049 protein
				lmo0122	2.00		Similar to phage proteins
lmo0136	2.20	lmo0136	2.53				Similar to oligopeptide ABC transporter; permease protein
lmo0152	5.56	lmo0152	2.67				Similar to oligopeptide ABC transporter-binding protein
lmo0210	−2.14					*ldh*	Similar to L-lactate dehydrogenase
		lmo0520	3.09	lmo0520	2.75		Similar to transcription regulator
		lmo0646	2.73	lmo0646	3.29		Similar to unknown proteins
lmo0972	2.66					*dltC*	D-Alanyl carrier protein
lmo0974	4.95					*dltA*	D-Alanine-D-alanyl carrier protein ligase
lmo1053	−4.03					*pdhB*	Highly similar to pyruvate dehydrogenase, E1 beta subunit
lmo1055	−2.74					*pdhD*	Highly similar to E3 subunit of pyruvate dehydrogenase complex
lmo1293	2.25	lmo1293	3.88	lmo1293	3.29	*glpD*	Similar to glycerol-3-phosphate dehydrogenase
	−2.93	lmo1388	−4.13	lmo1388	−3.17	*tcsA*	T-cell-stimulating antigen; lipoprotein
lmo1407	−3.25					*pflC*	Pyruvate-formate lyase-activating enzyme
lmo1538	2.20	lmo1538	3.11	lmo1538	2.46	*glpK*	Similar to glycerol kinase
lmo1934	−6.46	lmo1934	−9.33	lmo1934	−5.84	*hup*	Similar to nonspecific DNA binding protein Hup
lmo2032	−2.53	lmo2032	−3.99	lmo2032	−2.60	*ftsZ*	Highly similar to cell division initiation protein FtsZ
lmo2114	5.45	lmo2114	3.94	lmo2114	4.13		Similar to ABC transporter-ATP binding protein
lmo2115	5.87	lmo2115	2.99	lmo2115	3.33		Similar to ABC transporter; permease
lmo2468	3.34	lmo2468	2.55	lmo2468	2.26	*clpP*	ATP-dependent Clp protease, proteolytic subunit
		lmo2478	−2.63			*trxB*	Thioredoxin reductase
lmo2617	2.51	lmo2617	5.22	lmo2617	5.22	*rplF*	Ribosomal protein L6
		lmo2829	2.82	lmo2829	2.62		Similar to yeast protein Frm2p involved in fatty acid signaling
SigB-regulated genes							
		lmo0013	−2.12			*qoxA*	AA3-600 quinol oxidase subunit II
		lmo0405	2.17				Similar to phosphate transport proteins
lmo0593	2.21	lmo0593	3.17	lmo0593	2.72		Similar to transport proteins
lmo0880	2.39	lmo0880	2.52	lmo0880	2.39		Similar to wall-associated protein precursor; has LPXTG motif
lmo0911	2.35	lmo0911	5.11	lmo0911	4.60		lmo0911 protein
		lmo0956	2.84	lmo0956	2.84		Similar to *N*-acetylglucosamine-6*P*-phosphate deacetylase
		lmo1421	2.70	lmo1421	2.35	*opuBA*	Similar to glycine betaine-carnitine-choline ABC transporter; ATP-binding protein
lmo1433	2.00	lmo1433	2.49	lmo1433	2.21		Similar to glutathione reductase
lmo1539	4.20	lmo1539	7.09	lmo1539	5.45		Similar to glycerol uptake facilitator
lmo1883	4.80	lmo1883	3.82	lmo1883	2.99		Similar to chitinases
lmo2085	2.97	lmo2085	6.16	lmo2085	4.41		Putative peptidoglycan-bound protein with LPXTG motif
lmo2205	4.38	lmo2205	3.79	lmo2205	4.41	*gpmA*	Similar to phosphoglyceromutase 1
lmo2269	8.39	lmo2269	7.48	lmo2269	5.59		lmo2269 protein
lmo2485	2.41			lmo2485	2.06		Similar to *B. subtilis* YvlC protein
PrfA-regulated genes							
		lmo0019	2.83	lmo0019	2.59		lmo0019 protein
		lmo0169	2.53	lmo0169	2.05		Putative sugar uptake protein
lmo0200	3.45	lmo0200	4.48	lmo0200	4.66	*prfA*	Listeriolysin positive regulatory factor A
		lmo0201	8.08	lmo0201	8.97	*plcA*	Phosphatidylinositol-specific phospholipase C
		lmo0202	3.66	lmo0202	5.57	*hly*	Listeriolysin O
lmo0203	10.82	lmo0203	18.76	lmo0203	19.24	*mpl*	Zinc metalloproteinase precursor
lmo0204	7.33	lmo0204	9.40	lmo0204	13.96	*actA*	Actin assembly-inducing protein
lmo0205	9.12	lmo0205	12.97	lmo0205	17.63	*plcB*	Phospholipase C
lmo0278	2.41	lmo0278	3.30	lmo0278	2.52		Similar to sugar ABC transporter; ATP-binding protein
lmo0439	2.36	lmo0439	2.63	lmo0439	2.74		Weakly similar to a module of peptide synthetase
lmo0602	2.24	lmo0602	3.01	lmo0602	2.29		Weakly similar to transcription regulator
lmo0641	−3.22						Zinc uptake P-type ATPase
lmo0654	2.72	lmo0654	3.29	lmo0654	2.68		lmo0654 protein
		lmo0676	2.61	lmo0676	2.06		Similar to flagellar biosynthesic protein FliL
		lmo0781	2.29	lmo0781	2.25		Similar to mannose-specific PTS system component IID

Continued on following page

TABLE 3—*Continued*

IV gene	FC	IC gene (4 h)	FC	IC gene (8 h)	FC	Gene designation	Description of product
		lmo0782	2.42	lmo0782	2.24		Similar to mannose-specific PTS system component IIC
		lmo0783	2.96	lmo0783	2.67		Similar to mannose-specific PTS system component IIB
				lmo0796	2.93		Conserved hypothetical protein
lmo0838	4.58	lmo0838	9.30	lmo0838	9.22	*uhpT*	Highly similar to hexose phosphate transport protein
		lmo0913	2.52	lmo0913	3.29		Similar to succinate semialdehyde dehydrogenase
lmo0953	2.13	lmo0953	2.59	lmo0953	2.27		lmo0953 protein
		lmo2213	2.69	lmo2213	2.28		Similar to unknown protein
				lmo2219	2.45	*prsA*	Similar to posttranslocation molecular chaperone
lmo2391	2.87						Conserved hypothetical protein similar to *B. subtilis* YhfK protein
		lmo2477	−2.22			*galE*	UDP-glucose 4-epimerase
lmo2571	2.46						Similar to nicotinamidase
lmo2572	2.61						Similar to chain A, dihydrofolate reductase
lmo2573	2.71						Similar to zinc binding dehydrogenase
		lmo2696	2.70				Similar to hypothetical dihydroxyacetone kinase
		lmo2697	2.44				lmo2697 protein
		lmo2748	2.16				Similar to *B. subtilis* stress protein YdaG
PrfA- and SigB-regulated genes							
				lmo0433	2.84	*inlA*	Internalin A
		lmo0434	2.05	lmo0434	2.06	*inlB*	Internalin B
lmo0794	2.68	lmo0794	3.64	lmo0794	3.37		Similar to *B. subtilis* YwnB protein
		lmo0994	3.44	lmo0994	2.94		lmo0994 protein
lmo1694	2.40	lmo1694	2.79	lmo1694	2.58		Similar to CDP-abequose synthase
lmo1786	2.88	lmo1786	7.20	lmo1786	9.55	*inlC*	Internalin C
		lmo2157	2.73	lmo2157	2.37	*sepA*	SepA
		lmo2230	3.65	lmo2230	3.68		Similar to arsenate reductase
lmo2570	2.47						lmo2570 protein
lmo2673	2.24						Conserved hypothetical protein
		lmo2695	2.58				Similar to dihydroxyacetone kinase
PrfA-, SigB-, and RpoN-regulated genes							
		lmo1602	2.34	lmo1602	2.24		Similar to unknown proteins
CtsR-regulated genes							
lmo0229	2.60					*ctsR*	Highly similar to transcription repressor of class III stress genes
lmo0230	4.21	lmo0230	3.03	lmo0230	2.40		Similar to *B. subtilis* YacH protein
lmo0231	4.55	lmo0231	3.76	lmo0231	3.10		Similar to arginine kinase
lmo0232	2.83	lmo0232	2.61	lmo0232	2.62	*clpC*	Endopeptidase Clp ATP-binding chain C
lmo0997	38.80	lmo0997	54.22	lmo0997	42.40	*clpE*	ATP-dependent protease
lmo2206	8.57	lmo2206	5.74	lmo2206	6.04	*clpB*	Similar to endopeptidase Clp, ATP binding chain B
lmo2468	3.34	lmo2468	2.55	lmo2468	2.26	*clpP*	ATP-dependent Clp protease, proteolytic subunit
HrcA-regulated genes							
lmo1472	3.34	lmo1472	2.62	lmo1472	2.75	*dnaJ*	Heat shock protein DnaJ
lmo1473	3.23					*dnaK*	Class I heat shock protein; molecular chaperone DnaK
lmo1474	3.84	lmo1474	2.77	lmo1474	2.77	*grpE*	Heat shock protein GrpE
lmo1475	4.23	lmo1475	2.98	lmo1475	2.96	*hrcA*	Transcription repressor of class I heat shock gene *hrcA*
lmo2068	3.01					*groEL*	Class I heat shock protein; chaperone GroEL
lmo2069	2.82					*groES*	Class I heat shock protein; chaperone GroES
OhrR-regulated genes							
lmo2199	4.93	lmo2199	4.75	lmo2199	3.25	*ohrA*	Similar to OhrA
lmo2200	3.81	lmo2200	3.77	lmo2200	2.25	*ohrR*	Similar to transcription regulator OhrR

[a] IV, intravacuolar; IC, intracytosolic; FC, fold change.

quality control protease that degrades misfolded proteins and prevents their accumulation on the cell surface, was also up regulated intracellularly (54).

The class II stress response is mediated by sigma factor B (SigB). Although in our study *sigB* itself was not regulated, a total of 26 genes in our data set have previously been classified as being SigB regulated (28). We detected the up regulation of the *opuBA* gene (lmo1421 and lmo0903, encoding a glycine betaine uptake system) and a gene similar to the *osmC* gene during the intracellular growth. Both the genes are also known to be induced under osmotic stress situations (22, 51).

Three genes of the universal stress protein (Usp) family (lmo0515, lmo2673, and lmo2748) and genes for cholate, sodium, and pH homeostasis (lmo2378, lmo2381, and lmo2382) were up regulated intracellularly. Additionally, we detected the up regulation of lmo0754 (*btlB*), needed to combat bile stress (5).

DNA repair genes. An intriguing observation in this study is the increased expression of the truncated *comK* gene (lmo2270) during bacterial growth in the cytoplasm. The ComK locus is the site of integration of the A118 bacteriophage in listerial species. In *L. monocytogenes*, the prophage inserted at this position of the chromosome has been rendered inactive by gene deletions. Nevertheless, strong expression of bacteriophage genes lmo2270 to lmo2274 and lmo2276 was observed during intracellular growth. In *B. subtilis*, expression of *comK* is associated with DNA repair (7); in our study, transcription of DNA repair genes was also observed to be up regulated. Thus, we observed induction of the *lexA* (lmo1302), *recA* (lmo1398), and *polIV* (lmo1975) genes in the cytoplasm. A gene encoding a DNA repair protein, *radA* (lmo0233), and lmo2676, coding for a UV-damage repair protein, were also up regulated in the cytosol of the host cell.

Cell division and replication genes. To counteract the effects of virulence factors, the host cell may develop defense mechanisms to limit intracellular pathogen multiplication. We found 10 genes, *divIVA* (lmo2020), *ftsZA* (lmo2032 to lmo2033), *ftsXE* (lmo2506 to lmo2507), *ftsL* (lmo2040), *ftsH* (lmo0220), *minC* (lmo1545), *minD* (lmo1544), and *smc* (lmo1804), to be down regulated during transit in the phagolysosome and during intracellular growth. Thus, down regulation of *ftsZ* and *ftsA*, the major bacterial cell division determinant, and the *ftsXEL* complex, whose product localizes to the septal ring and is required for the recruitment of FtsK and other subsequent division proteins, suggests lowered cell division activity. We also observed down regulation of *minC* and *minD* genes, which mediate spatial regulation of cytokinesis in bacteria (42), and the *smc* gene, which is essential for chromosomal condensation, segregation, and cell cycle progression (21). In addition, repression of *divIVA* (lmo2020), encoding a protein involved in chromosomal segregation and control of the site specificity of cell division, lends further credence to the suggestion that cell division processes were affected during intracellular growth (15).

We observed the down regulation of listerial chromosomal replication initiation gene *dnaA* (lmo0001) and DNA primase gene *dnaG* (lmo1455) intracellularly. This indicates that along with the cell division, replication of intracytosolic bacteria was also negatively regulated.

Peptidoglycan and cell wall gene expression. Expression levels of the major autolysin genes *iap* (lmo0582), *murA* (lmo2691), and *spl* (lmo2505) of *L. monocytogenes* were significantly decreased during intracellular growth. A putative regulator gene, lmo2690, located upstream of the *murA* autolysin gene was reciprocally regulated and probably acts to repress production of the *murA* gene product.

We observed up regulation of the *dlt* operon (lmo0971 to lmo0974) that encodes enzymes that incorporate D-alanine residues into the cell wall-associated lipotechoic acids. These enzymes were up regulated in the vacuole. The increased positive charge conferred by these molecules on the cell surface may mediate resistance to cationic peptides present in the phagolysosome of the host cell. In this context, *murC* (lmo1605), responsible for the replacement of alanine with glycine in the cell wall (33), was down regulated.

We also detected intracellular up regulation of eight putative cell wall-associated genes (*inlA*, *inlB*, lmo0514, lmo0610, lmo0880, lmo2085, lmo2713, and lmo2714), all of which encode products containing the LPXTG motif (except *inlB* and lmo2713, which encode products containing the GW motif). Of these, lmo0514 and lmo0610 are predicted to encode internalin-like proteins (8).

Transport of ions. Metal ions are essential cofactors for functional expression of many proteins in bacterial systems. Thus, any expressional alteration of bacterial ion transport genes intracellularly reflects the accessibility of those ions in the altered environment. Among the genes involved in bacterial ion uptake systems, the zinc transporter genes *zurA* (lmo1447) and lmo1671 (encoding a high-affinity zinc uptake system protein) were up regulated during growth in the cytosol but lmo0641 (encoding zinc uptake P-type ATPase) was down regulated in the vacuolar compartment and the potassium uptake protein gene (lmo1023) and *kdpA* (lmo2681) were up regulated in the vacuoles of the host cells. All three manganese ABC transporter genes (lmo1847 to lmo1849) were down regulated inside the cytosol, and another gene (lmo1424) with homology to the eukaryotic *Nramp* genes was down regulated throughout the experiment. Phosphate uptake is regulated by a two-component system, PhoRP, in *B. subtilis*. *L. monocytogenes* also harbors a homolog of the *phoRP* two-component system. In our experiment, the *phoRP* operon (lmo2500 to lmo2501) was down regulated in the cytosolic phase of growth. The ferric uptake protein genes *fri* (lmo0943) and *fur* (lmo1956) were down regulated in both the vacuolar and cytosolic compartments. Collectively, we conclude that potassium and zinc in the vacuoles of the host cells were lacking and that the cytosol is a rich source of ions for the bacteria as suggested by the down regulation of magnesium, iron, and phosphate transport systems and the absence of regulation of other metal transporter systems.

Free radical production. Both the genes encoding superoxide dismutase (lmo1439) and catalase (lmo2785) were down regulated during bacterial growth inside the cell. We validated this finding independently through quantitative real-time PCR. An operon whose open reading frames have homology to *ohrAR* (lmo2199 and lmo2200) of *B. subtilis* encoding organic hydroxyperoxide resistance and its regulator protein (17) was up regulated during intracellular growth. In addition, the glutathione reductase gene (lmo1433), lmo0646 (encoding a protein similar to lactoyl glutathione lyase), and a sulfur limitation-regulated protein gene (lmo2350) were up regulated. We

also detected lmo2191, a gene similar to the *B. subtilis spx* gene, to be up regulated during growth of bacteria inside the host cell. The Spx protein is required for transcriptional induction of genes that function in thiol homeostasis (57).

Oxygen level. We detected an increase in the menaquinone biosynthesis gene *menF* (lmo1676) intracellularly, indicating that components of the respiratory chain are utilized during intracellular growth. A decrease in the expression of cytochrome *d* genes (lmo2715 to lmo2718) inside the host cell indicates that the bacterial cells are not oxygen starved intracellularly, but the absence of cytochrome *b* or cytochrome *o* (the preferential path of electron flow for cells growing aerobically) suggests a more complex situation. In this respect, up regulation of the nitrite transporter gene (lmo0593) suggests that nitrite may constitute an alternative terminal electron acceptor allowing *L. monocytogenes* to carry out respiration under oxygen-restricted conditions. The down regulation of the cytochrome *d* complex genes and components of the oxidative phosphorylation system, *qoxA* (lmo0013), *qoxB* (lmo0014), *qoxD* (lmo0016), and lmo0355, coding for flavocytochrome *c*, suggest that electron acceptors other than oxygen are used under intracellular growth conditions.

Validation of microarray gene expression results. We validated our expression profile data by performing quantitative real-time PCR analysis. A subset of 22 genes was selected to encompass a range of expression values from among the data set obtained. As shown in Fig. 2, the real-time PCR data correlated strongly with the expression profile data (*r* of greater than 0.86 in all cases).

We used the wild-type strain and its isogenic Δ*prfA* mutant to examine whether the genes lmo0206, lmo0207, and *prsA* (lmo2219) are indeed PrfA regulated. The expression levels of all three genes were higher ($P < 0.01$) in the wild-type bacteria than in the Δ*prfA* strain by at least threefold (data not shown). We therefore conclude that lmo0206, lmo0207, and *prsA* are novel PrfA-regulated genes.

Phenotypic characterization of mutants with intracellularly active gene products. As a first step to analyze the roles of genes described herein for intracellular survival, we used information from the literature and performed a DNA binding motif search analysis to classify genes that are controlled by known regulatory proteins, i.e., PrfA (35), CtsR (10), SigB (28), OhrR (17), TnrA (49), RpoN (2), and HrcA (52) (Table 3). For the bioinformatic analysis, we used a nucleic acid pattern search program called fuzznuc (part of the EMBOSS package) to search for the respective DNA binding motifs in the 500-bp upstream regions of the 484 genes identified above, with one mismatch. A list of the genes and their respective putative regulators is given in Table 3.

We used this information to generate specific isogenic deletion mutants of the following regulators: the lmo1298 product (TnrA), the lmo2200 product (OhrR), SigB, and the lmo0229 product (CtsR). We also created deletions in the PrfA-regulated genes lmo0206, lmo0207, and *prsA* (lmo2219) that were discovered in this study and examined their relative contributions to intracellular growth and plaque formation. As shown in Fig. 3A and B, intracellular growth of the Δlmo0206 (Δ*orfX*), Δlmo0207 (Δ*orfZ*), Δlmo0229 (Δ*ctsR*), Δlmo2200 (Δ*ohrR*), and Δlmo2219 (Δ*prsA*) mutant strains was impaired compared to that of the parental EGD-e strain. On the other hand, neither the Δ*sigB* nor the Δlmo1298 (Δ*tnrA*) mutant was compromised for intracellular growth. As in other cell lines used in previous studies, the intracellular growth of the Δ*hly* Δ*plcA* and Δ*prfA* mutants was completely abolished in the P388D1 cell line.

We also looked for plaque formation ability, a measure of intracellular growth and cell-to-cell spread of these mutants. As shown in Fig. 3C, the Δ*prsA*, Δ*orfX*, and Δ*ctsR* mutants formed visibly smaller plaques than the wild type and the Δ*prfA* and Δ*hly* Δ*plcA* mutants were unable to form plaques inside the host cells. The plaque formation ability of the Δlmo0207 (Δ*orfZ*), Δ*sigB*, Δlmo1298 (Δ*tnrA*), and Δlmo2200 (Δ*ohrR*) mutants was similar to that of the wild type.

DISCUSSION

Adaptive gene expression permits intracellular pathogens to successfully persist and disseminate during encounters with the host defenses in the diverse intracellular microenvironments within the cell. Since *L. monocytogenes* has a facultative intracellular lifestyle, identification of genes uniquely expressed intracellularly is important for understanding infection processes and for identifying new approaches to limit listerial infections. Currently, there are no experimental approaches that accurately reconstruct the intracellular microenvironments faced by the bacterium, and inclusion of all variables influencing bacterial transcription is not currently feasible. As a first step to analyze bacterial responses throughout the course of infection, we have used listerial infection of mouse macrophages to examine the complex host-bacterium interaction. We aimed at identifying *L. monocytogenes* transcripts that correlate with ex vivo infection of mouse macrophages, a cell type central to the control of infections mediated by this bacterium.

Shortly following entry into the vacuolar compartment, strong reduction in the expression of genes involved in glycolysis and the citric acid cycle was observed, suggesting a depletion of bacterial intracellular glucose levels. We also observed massive transcriptional changes that reflected temporal adjustment of the listerial cellular metabolism, firstly to augment the transport and degradation of alternate metabolizable carbohydrates and the transport and metabolism of oligopeptides, aminosugars, fatty acids, and branched-chain amino acids. Branched-chain amino acids are the most abundant amino acids in proteins and form the hydrophobic core of most proteins. They are also precursors for the biosynthesis of iso- and anteiso-branched-chain fatty acids in bacterial cells. In *B. subtilis*, the production of branched-chain amino acids is complex and subject to many levels of regulation (48). The product of the *tnrA* gene represses expression of these operons involved in regulation, and attenuation is mediated by uncharged tRNA. Here we observed that the gene product of lmo1298 that is homologous to TnrA of *B. subtilis* was down regulated intravacuolarly. Similarly, expression of genes encoding tRNA synthetases, i.e., *alaS* (lmo1504), *hisS* (lmo1520), *ileS* (lmo2019), *glyS* (lmo1458), *serS* (lmo2747), *cysS* (lmo0239), *valS* (lmo1552), *thrS* (lmo1559), *leuS* (lmo1660), *tyrS* (lmo1598), and *trpS* (lmo2198), was decreased, facilitating production of branched-chain amino acids during intracellular growth and contributing to a metabolic transition from carbohydrate to amino acid fermentation. Previously, it has been demonstrated for *B. subtilis* (32) that a link exists between the expression of carbon and nitrogen pathways mediated via expression of the *ilv-leu* operon. It will be important to

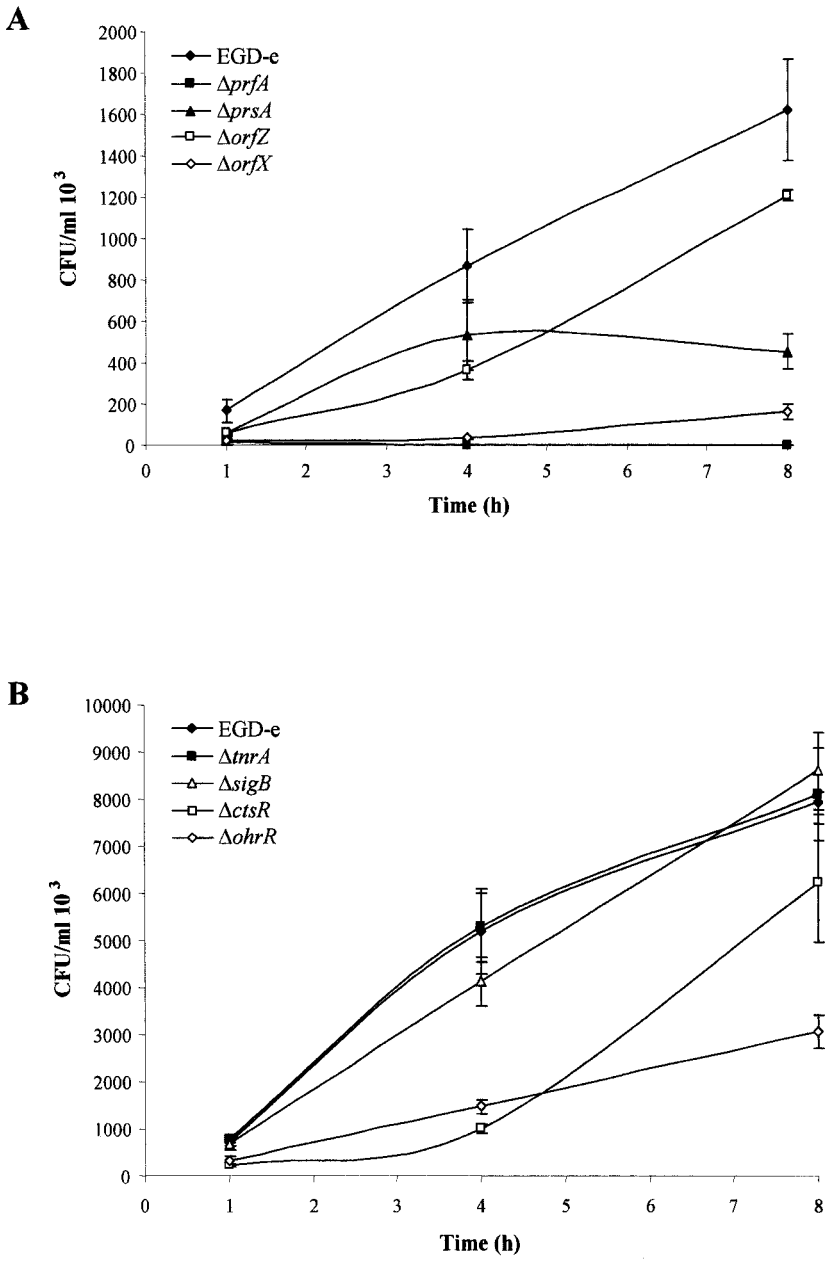

FIG. 3. Characterization of bacterial deletion mutants. (A and B) Intracellular survival of *L. monocytogenes* EGD-e compared to that of the Δ*prfA*, Δ*orfX* (lmo0206), Δ*orfZ* (lmo0207), Δ*ctsR* (lmo0229), Δ*sigB*, Δ*tnrA* (lmo1298), Δ*ohrR* (lmo2200), and Δ*prsA* (lmo2219) isogenic deletion mutants. P388D1 murine macrophage cells were infected with the wild-type strain EGD-e and its isogenic deletion mutants at MOIs of 10 in 24-well plates, and bacterial CFU were counted on agar plated following lysis of the P388D1 cells at 1, 4, and 8 h postinfection. (C) A bacterial plaque formation assay was performed with L929 murine fibroblast cells. The cells were infected with *L. monocytogenes* EGD-e and its isogenic deletion mutants for 2 h at 37°C in a humidified incubator and subsequently incubated for 30 min in the presence of 10 μg of gentamicin/ml. The plates were then agarose overlaid and were observed for plaques after an additional incubation at 37°C 3 days postinfection. The diameters of the different plaques obtained were determined by processing the images for Adobe Photoshop version 5.5 and are derived from measuring 25 plaques for each strain tested. The mean plaque size of the wild-type strain was defined as 100%. The mean size (%) with standard deviation is given for each strain in the experiment. n.d. (not detected) indicates that strains produced no visible plaques under the conditions used. The data shown here are representative of results of three independent experiments.

determine whether the differential regulation seen with genes involved in glycolysis and the branched-chain amino acids is a *conditio sine qua non* for intracellular growth.

Transcript levels of genes encoding several ribosomal subunits (S5, S11, S17, L5, L6, L14, L17, L18, L24, L30, and L35) and the translational initiation factor (IF3), the molecular chaperones such as GroEL, DnaJ, and DnaK, and the class II ATP-dependent proteases ClpP and ClpC, etc., were the most

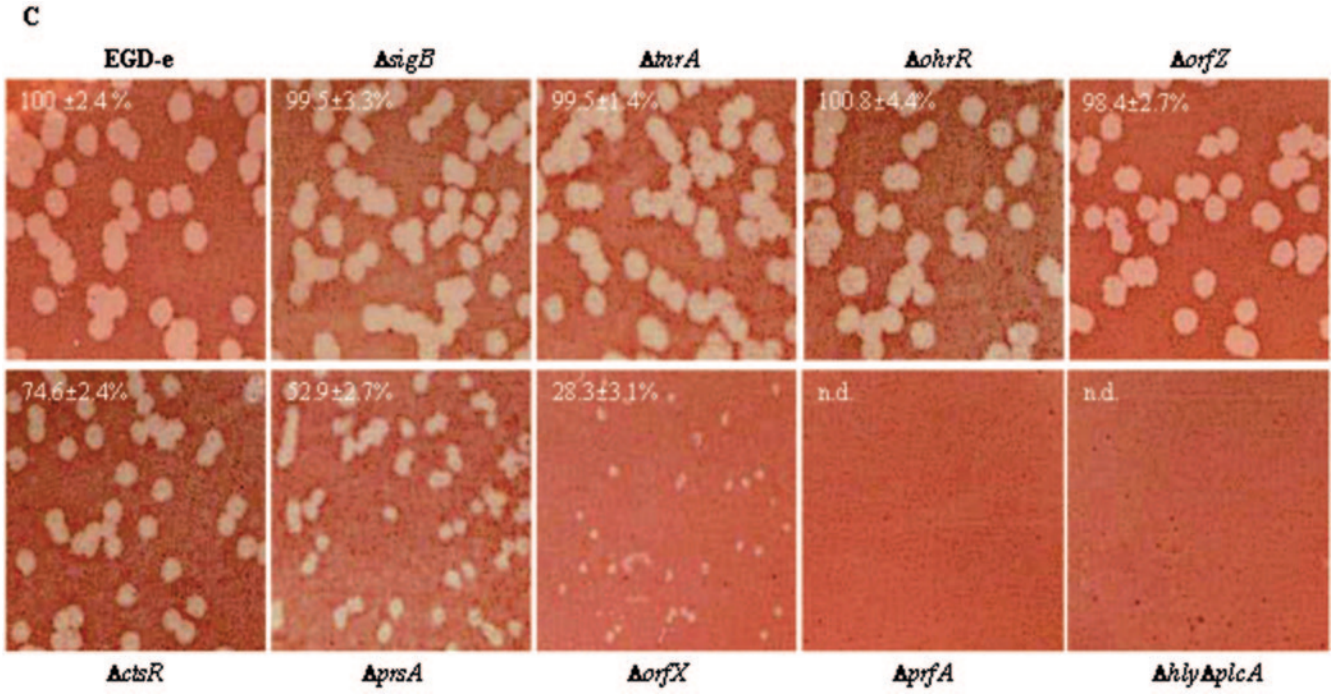

FIG. 3—*Continued.*

highly regulated genes during intravacuolar survival. The data are in agreement with those in previous publications documenting intracellular expression of the *groES-groEL* operon (19), the requirement of DnaK for enhanced survival following phagocytosis (25), and the major role of the Clp stress proteins in promoting early bacterial escape from the vacuoles of infected macrophages and achieving virulence in the mouse model of infection (43). Accumulating evidence for a number of bacterial pathogens indicates that heat shock proteins and chaperones play an important role in virulence. Thus, in *Staphylococcus aureus*, the two major chaperones DnaK and GroES-GroEL are induced during infection of human epithelial cells (40). Activation of *clpP* has been shown to be required for growth and survival of *Salmonella entrica* serovar Typhimurium within macrophages (55).

An important component of the stress response to bacteria is SigB, the master regulator of stress and stationary phase. We found 26 genes that were previously classified as members of the SigB regulon (28) to be up regulated during intracellular bacterial growth, although *sigB* expression itself was not significantly regulated throughout the infection period. When a Δ*sigB* mutant was examined for its ability to grow intracellularly, its properties were indistinguishable from those of wild-type bacteria, suggesting that the gene expression pattern for intracellular bacteria is not equivalent to that for in vitro stationary phase bacterial growth. Thus, other overlapping mechanisms of regulatory control may be dominant for the 26 genes described in this study.

Nevertheless, the data presented here suggest that intracellularly growing bacteria may be experiencing some level of stress as indicated by the expression of the *htrA* gene, encoding a protease required for quality control of proteins presented to the bacterial cell surface, and the *prsA* gene, encoding a chaperone component that is required for regulated secretion of surface components and secreted proteins. Similarly, we observed induction of genes involved in the translesional repair of DNA (*recA*, *lexA*, *radA*, lmo2627, and *polIV*) that is characteristic of an SOS response in bacteria. The operon *ohrAR* (lmo2199 and lmo2200) encoding an organic hydroxyperoxidase and its regulator protein is up regulated during intracellular growth. The regulatory OhrR protein in *B. subtilis* has been implicated in sensing of the overall thiol-disulfide redox balance of the cell deriving from oxidation of its single conserved cysteine residue. Organic hydroperoxidases have been shown to be active in the detoxification of bioactive lipids such as lysophosphatidic acid of the eukaryotic cell. Finally, a listerial homolog of the *B. subtilis* Spx regulator gene (lmo2191), required for the transcriptional induction of genes that function in thiol homeostasis, is highly up regulated during intracellular growth of the bacteria. The regulation of thiol oxidation appears to be an important process within bacteria growing in the intracellular environment.

In *L. monocytogenes*, the largest family of surface proteins are the internalins, comprising 25 members. The requirement for internalins A and B for attachment to and invasion of target host cells, the involvement of InlC and InlH (13, 30) in virulence, and the presence of only eight members of this family in apathogenic *L. innocua* have led to the notion that many of the internalin genes are part of the virulence gene repertoire of pathogenic *L. monocytogenes*. We discovered that apart from *inlA*, *inlB*, and *inlC*, the expression of six other internalin-like protein genes, lmo0514, lmo0610, lmo0880, lmo2085, lmo2713, and lmo2714, was increased within 1 h of infection. Although the ex vivo growth technique used in this study successfully mimics some of the conditions existing within the cell, it does not replicate the highly complex envi-

ronment that pathogens encounter during natural infections. Thus, the role of additional internalin-like proteins may be revealed only in whole-animal infections. Alternatively, since *L. monocytogenes* is known to have a wide host range, these proteins may be required for infection of various hosts.

Overall, up regulation of genes corresponding to a further seven cell wall-associated proteins (excluding internalins) was observed. Nevertheless, there was evidence for extensive remodeling of the cell surface of intracellular listeriae. We found up regulation of the gene encoding the succinyl-aminopimelate desuccinylase (*dapE*; lmo0265) that produces the amino acids of mesodiaminopimelate as a key intermediate of peptidoglycan synthesis as well as the essential amino acid lysine. Enhanced production of diaminopimelate could be required for the anchoring of cell surface proteins to the peptidoglycan. Similarly, up regulation of the genes of the *dlt* operon, required for the incorporation of D-alanine residues into cell wall lipoteichoic acids, modifies cell surface charge, thus mediating resistance to cationic peptides. Indeed, it was previously shown that a *dltA* deletion mutant exhibited increased susceptibility to the cationic peptides colistin, nisin, and polymyxin B (1).

Expression of the major autolysin genes *iap* (lmo0582), *spl* (lmo2505), and *murA* (lmo2691) of *L. monocytogenes* was down regulated during growth in the vacuole and the host cytoplasm. The combined activities encoded by these autolysin genes on the listerial cell wall would result in the production of muramyl dipeptide intracytoplasmically, activating an inflammatory response mediated by the Nod proteins in the eukaryotic cytosol (19a).

We also detected induction of expression of *mogR* (lmo0674) (21a), encoding a repressor of flagellin gene (*flaA*) expression. Despite this, expression of flagellin and the membrane-associated *fliP* (lmo0676) was also induced at 4 h postinfection, suggesting that additional levels of control apart from transcriptional repression by MogR exist for these genes. Flagellin from *L. monocytogenes* has been shown to possess potent proinflammatory activity by activation of host inflammatory processes through the Toll-like receptor 5 (26).

An important finding of this study was the demonstration that all of the PrfA-regulated genes previously implicated in virulence were represented in the data set as highly up-regulated genes. Induction of the *prfA* regulator and virulence genes was already visible in the vacuolar phase; only *inlA*, *inlB*, and *prsA* were found to be up regulated within the cytosol. In this study, we detected three newly identified PrfA-regulated genes that demonstrate increased expression during intracellular growth. Isogenic deletion mutants generated for the *orfX*, *orfZ*, and *prsA* genes revealed clearly that these genes are required for intracellular growth of the bacterium. In a previous study, insertional mutagenesis of the *orfX* gene resulted in no visible defect when the mutant was examined in a plaque assay (50). Cumulatively, the data point to a definite role for *orfX* and *orfZ* in intracellular survival and warrant further study. The listerial PrsA homolog encoding a chaperone-like protein had previously been described as a novel member of the PrfA regulon (35). We provide here the first evidence for its role in intracellular survival; the substrate(s) of this protein remains unknown and will require further analysis.

An interesting observation in this study is the expression of strain-specific intracellularly expressed genes defined as those genes absent from a sequenced *L. monocytogenes* serotype 4b isolate. For four of the loci involved, i.e., lmo0398-lmo0399-lmo0400, lmo0434, lmo0783-lmo0784, and lmo0888, the corresponding genes appear to have been either truncated or fused as a result of mutation(s). All of the other genes are part of a larger set of genes that have been partially lost from either the *L. monocytogenes* 4b strain or *L. innocua* or that are absent in both the *L. monocytogenes* 4b and *L. innocua* strains. These include lmo0748, lmo0750, lmo0751, lmo1081 to lmo1084, and lm01968 to lmo1974. The contribution of these genes to intracellular survival is presently unclear. Recently, it has been shown that the lmo0754 gene is a novel member of the bile salt regulon of *L. monocytogenes* (5). This particular gene has also been retained in the *L. monocytogenes* 4b strain but is absent from the genome of the nonpathogenic *L. innocua* strain.

Previously, different studies employing a strategy analogous to in vivo expression technology used a library of listerial subgenomic fragments fused to a promoterless copy of *hly* in a listeriolysin-negative in vivo expression technology host strain to screen for recombinants with increased survival potential in the mouse model (18). Comparison of the loci obtained in those studies to the data set reported here revealed only one common gene, lmo2684, which is part of a cellobiose uptake PTS system. A comparison with transcriptional fusions to *lacZ* that had been identified with higher expression inside a macrophage-like cell line than in a rich broth medium in another study also showed an overlap of a single gene encoding PlcA (29). Comparison of our data with those from an additional study that identified in vivo-induced transcription fusions to *gfp* following infection of J774 macrophages by using fluorescence-activated cell sorter analysis showed an overlap of two genes: one encoding ActA and a second encoding a mannose-specific IIAB PTS system (lmo0783) (53).

Our study provides a first comprehensive analysis of how *L. monocytogenes* adapts to growth in the vacuolar and cytosolic compartments of infected macrophages. Following entry, *L. monocytogenes* immediately remodels transcription through the coordinated expression of regulatory, metabolic, stress, and virulence genes. The study presented here indicates that listerial virulence is dependent on a large network of genes, many of whose functions are not understood. Nevertheless, the data provide a rich resource to describe and understand how adaptation to growth in intracellular environments evolved in a pathogen that has a large variety of ecological habitats.

ACKNOWLEDGMENTS

We acknowledge Martina Hudel, Stanislav Lechnow, Alexandra Amend, Nelli Schklarenko, and Maria Hass for technical assistance. We also thank Manvi Porwal for helping us with confocal microscopic images and Rohit Ghai for helpful discussions. T.C. thanks Anindita Chakraborty for help in analyzing the gene lists.

This work was supported by grants from the Bundesministerium fuer Bildung and Forschung, Berlin, Germany (NGFN 01GS0111), and the Competence Network PathoGenomik (PTJ-BIO//03u213B) to T.C. and T.H.

REFERENCES

1. **Abachin, E., C. Poyart, E. Pellegrini, E. Milohanic, F. Fiedler, P. Berche, and P. Trieu-Cuot.** 2002. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Mol. Microbiol. **43:**1–14.
2. **Arous, S., C. Buchrieser, P. Folio, P. Glaser, A. Namane, M. Hebraud, and**

Y. Hechard. 2004. Global analysis of gene expression in an *rpoN* mutant of *Listeria monocytogenes*. Microbiology **150:**1581–1590.
3. **Asanuma, N., and T. Hino.** 2003. Molecular characterization of HPr and related enzymes, and regulation of HPr phosphorylation in the ruminal bacterium *Streptococcus bovis*. Arch. Microbiol. **179:**205–213.
4. **Autret, N., C. Raynaud, I. Dubail, P. Berche, and A. Charbit.** 2003. Identification of the *agr* locus of *Listeria monocytogenes*: role in bacterial virulence. Infect. Immun. **71:**4463–4471.
5. **Begley, M., R. D. Sleator, C. G. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. Infect. Immun. **73:**894–904.
6. **Beissbarth, T., K. Fellenberg, B. Brors, R. Arribas-Prat, J. Boer, N. C. Hauser, M. Scheideler, J. D. Hoheisel, G. Schutz, A. Poustka, and M. Vingron.** 2000. Processing and quality control of DNA array hybridization data. Bioinformatics **16:**1014–1022.
7. **Berka, R. M., J. Hahn, M. Albano, I. Draskovic, M. Persuh, X. Cui, A. Sloma, W. Widner, and D. Dubnau.** 2002. Microarray analysis of the *Bacillus subtilis* K-state: genome-wide expression changes dependent on ComK. Mol. Microbiol. **43:**1331–1345.
8. **Cabanes, D., P. Dehoux, O. Dussurget, L. Frangeul, and P. Cossart.** 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol. **10:**238–245.
9. **Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.
10. **Chastanet, A., I. Derre, S. Nair, and T. Msadek.** 2004. *clpB*, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. J. Bacteriol. **186:**1165–1174.
11. **Chico-Calero, I., M. Suarez, B. Gonzalez-Zorn, M. Scortti, J. Slaghuis, W. Goebel, and J. A. Vazquez-Boland.** 2002. Hpt, a bacterial homolog of the microsomal glucose-6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. Proc. Natl. Acad. Sci. USA **99:**431–436.
12. **Dramsi, S., I. Biswas, E. Maguin, L. Braun, P. Mastroeni, and P. Cossart.** 1995. Entry of *Listeria monocytogenes* into hepatocytes requires expression of *inIB*, a surface protein of the internalin multigene family. Mol. Microbiol. **16:**251–261.
13. **Dramsi, S., P. Dehoux, M. Lebrun, P. L. Goossens, and P. Cossart.** 1997. Identification of four new members of the internalin multigene family of *Listeria monocytogenes* EGD. Infect. Immun. **65:**1615–1625.
14. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47:**103–118.
15. **Errington, J., R. A. Daniel, and D. J. Scheffers.** 2003. Cytokinesis in bacteria. Microbiol. Mol. Biol. Rev. **67:**52–65.
16. **Frangeul, L., P. Glaser, C. Rusniok, C. Buchrieser, E. Duchaud, P. Dehoux, and F. Kunst.** 2004. CAAT-Box, Contigs-Assembly and Annotation Tool-Box for genome sequencing projects. Bioinformatics **20:**790–797.
17. **Fuangthong, M., S. Atichartpongkul, S. Mongkolsuk, and J. D. Helmann.** 2001. OhrR is a repressor of *ohrA*, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. J. Bacteriol. **183:**4134–4141.
18. **Gahan, C. G., and C. Hill.** 2000. The use of listeriolysin to identify in vivo induced genes in the gram-positive intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. **36:**498–507.
19. **Gahan, C. G., J. O'Mahony, and C. Hill.** 2001. Characterization of the *groESL* operon in *Listeria monocytogenes*: utilization of two reporter systems (*gfp* and *hly*) for evaluating in vivo expression. Infect. Immun. **69:**3924–3932.
19a. **Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti.** 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem. **278:**8869–8872.
20. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849–852.
21. **Graumann, P. L.** 2001. SMC proteins in bacteria: condensation motors for chromosome segregation? Biochimie **83:**53–59.
21a. **Grundling A., L. S. Burrack, H. G. Bouwer, and D. E. Higgins.** 2004. *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. Proc. Natl. Acad. Sci. USA **101:**12318–12323.
22. **Gutierrez, C., and J. C. Devedjian.** 1991. Osmotic induction of gene *osmC* expression in *Escherichia coli* K12. J. Mol. Biol. **220:**959–973.
23. **Haas, S. A., M. Hild, A. P. Wright, T. Hain, D. Talibi, and M. Vingron.** 2003. Genome-scale design of PCR primers and long oligomers for DNA microarrays. Nucleic Acids Res. **31:**5576–5581.
24. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
25. **Hanawa, T., M. Fukuda, H. Kawakami, H. Hirano, S. Kamiya, and T. Yamamoto.** 1999. The *Listeria monocytogenes* DnaK chaperone is required for stress tolerance and efficient phagocytosis with macrophages. Cell Stress Chaperones **4:**118–128.
26. **Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem.** 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **410:**1099–1103.
27. **Huillet, E., S. Larpin, P. Pardon, and P. Berche.** 1999. Identification of a new locus in *Listeria monocytogenes* involved in cellobiose-dependent repression of *hly* expression. FEMS Microbiol. Lett. **174:**265–272.
28. **Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann.** 2003. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. J. Bacteriol. **185:**5722–5734.
29. **Klarsfeld, A. D., P. L. Goossens, and P. Cossart.** 1994. Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA*, *purH*, *purD*, *pyrE* and an arginine ABC transporter gene, *arpJ*. Mol. Microbiol. **13:**585–597.
30. **Lingnau, A., T. Chakraborty, K. Niebuhr, E. Domann, and J. Wehland.** 1996. Identification and purification of novel internalin-related proteins in *Listeria monocytogenes* and *Listeria ivanovii*. Infect. Immun. **64:**1002–1006.
31. **Lingnau, A., E. Domann, M. Hudel, M. Bock, T. Nichterlein, J. Wehland, and T. Chakraborty.** 1995. Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. Infect. Immun. **63:**3896–3903.
32. **Ludwig, H., C. Meinken, A. Matin, and J. Stulke.** 2002. Insufficient expression of the *ilv-leu* operon encoding enzymes of branched-chain amino acid biosynthesis limits growth of a *Bacillus subtilis ccpA* mutant. J. Bacteriol. **184:**5174–5178.
33. **Mahapatra, S., D. C. Crick, and P. J. Brennan.** 2000. Comparison of the UDP-*N*-acetylmuramate:L-alanine ligase enzymes from *Mycobacterium tuberculosis* and *Mycobacterium leprae*. J. Bacteriol. **182:**6827–6830.
34. **Mengaud, J., S. Dramsi, E. Gouin, J. A. Vazquez-Boland, G. Milon, and P. Cossart.** 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol. Microbiol. **5:**2273–2283.
35. **Milohanic, E., P. Glaser, J. Y. Coppee, L. Frangeul, Y. Vega, J. A. Vazquez-Boland, F. Kunst, P. Cossart, and C. Buchrieser.** 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol. Microbiol. **47:**1613–1625.
35a. **Niebuhr, K., T. Chakraborty, M. Rohde, T. Gazlig, B. Jansen, P. Köllner, and J. Wehland.** 1993. Localization of the ActA polypeptide of *Listeria monocytogenes* in infected tissue culture cell lines: ActA is not associated with actin "comets." Infect. Immun. **61:**2793–2802.
36. **Nieto, C., and M. Espinosa.** 2003. Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid **49:**281–285.
37. **Park, S. F., and G. S. Stewart.** 1990. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. Gene **94:**129–132.
38. **Paschen, A., K. E. Dittmar, R. Grenningloh, M. Rohde, D. Schadendorf, E. Domann, T. Chakraborty, and S. Weiss.** 2000. Human dendritic cells infected by *Listeria monocytogenes*: induction of maturation, requirements for phagolysosomal escape and antigen presentation capacity. Eur. J. Immunol. **30:**3447–3456.
39. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
40. **Qoronfleh, M. W., C. A. Bortner, P. Schwartzberg, and B. J. Wilkinson.** 1998. Enhanced levels of *Staphylococcus aureus* stress protein GroEL and DnaK homologs early in infection of human epithelial cells. Infect. Immun. **66:**3024–3027.
41. **Rea, R. B., C. G. Gahan, and C. Hill.** 2004. Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. Infect. Immun. **72:**717–727.
42. **Rothfield, L. I., Y. L. Shih, and G. King.** 2001. Polar explorers: membrane proteins that determine division site placement. Cell **106:**13–16.
43. **Rouquette, C., C. de Chastellier, S. Nair, and P. Berche.** 1998. The ClpC ATPase of *Listeria monocytogenes* is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. Mol. Microbiol. **27:**1235–1245.
44. **Rozen, S., and H. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. **132:**365–386.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Schaferkordt, S., and T. Chakraborty.** 1995. Vector plasmid for insertional

mutagenesis and directional cloning in *Listeria* spp. BioTechniques **19:**720–725.

47. **Slaghuis, J., M. Goetz, F. Engelbrecht, and W. Goebel.** 2004. Inefficient replication of *Listeria innocua* in the cytosol of mammalian cells. J. Infect. Dis. **189:**393–401.
48. **Tojo, S., T. Satomura, K. Morisaki, J. Deutscher, K. Hirooka, and Y. Fujita.** 2005. Elaborate transcription regulation of the *Bacillus subtilis ilv-leu* operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. Mol. Microbiol. **56:**1560–1573. [Online.] doi:10.1111/j.1365-2958.2005.04635.x.
49. **Tojo, S., T. Satomura, K. Morisaki, K. Yoshida, K. Hirooka, and Y. Fujita.** 2004. Negative transcriptional regulation of the *ilv-leu* operon for biosynthesis of branched-chain amino acids through the *Bacillus subtilis* global regulator TnrA. J. Bacteriol. **186:**7971–7979.

49a. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:**5116–5121.

50. **Vazquez-Boland, J. A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60:**219–230.
51. **Wemekamp-Kamphuis, H. H., R. D. Sleator, J. A. Wouters, C. Hill, and T. Abee.** 2004. Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of *Listeria monocytogenes* at low temperatures. Appl. Environ. Microbiol. **70:**2912–2918.
52. **Wiegert, T., and W. Schumann.** 2003. Analysis of a DNA-binding motif of the *Bacillus subtilis* HrcA repressor protein. FEMS Microbiol. Lett. **223:**101–106.
53. **Wilson, R. L., A. R. Tvinnereim, B. D. Jones, and J. T. Harty.** 2001. Identification of *Listeria monocytogenes* in vivo-induced genes by fluorescence-activated cell sorting. Infect. Immun. **69:**5016–5024.
54. **Wonderling, L. D., B. J. Wilkinson, and D. O. Bayles.** 2004. The *htrA* (*degP*) gene of *Listeria monocytogenes* 10403S is essential for optimal growth under stress conditions. Appl. Environ. Microbiol. **70:**1935–1943.
55. **Yamamoto, T., H. Sashinami, A. Takaya, T. Tomoyasu, H. Matsui, Y. Kikuchi, T. Hanawa, S. Kamiya, and A. Nakane.** 2001. Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar Typhimurium results in persistent infection in mice, and development of persistence requires endogenous gamma interferon and tumor necrosis factor alpha. Infect. Immun. **69:**3164–3174.
56. **Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed.** 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.
57. **Zuber, P.** 2004. Spx-RNA polymerase interaction and global transcriptional control during oxidative stress. J. Bacteriol. **186:**1911–1918.

Editor: J. L. Flynn