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Comparative Transcriptome Analysis of *Listeria monocytogenes* Strains of the Two Major Lineages Reveals Differences in Virulence, Cell Wall, and Stress Response▽†

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***Listeria monocytogenes* is a food-borne, opportunistic, bacterial pathogen causing a wide spectrum of diseases, including meningitis, septicemia, abortion, and gastroenteritis, in humans and animals. Among the 13 *L. monocytogenes* serovars described, human listeriosis is mostly associated with strains of serovars 4b, 1/2b, and 1/2a. Within the species *L. monocytogenes*, three phylogenetic lineages are described. Serovar 1/2a belongs to phylogenetic lineage I, while serovars 4b and 1/2b group in phylogenetic lineage II. To explore the role of gene expression in the adaptation of *L. monocytogenes* strains of these two major lineages to different environments, as well as in virulence, we performed whole-genome expression profiling of six *L. monocytogenes* isolates of serovars 4b, 1/2b, and 1/2a of distinct origins, using a newly constructed *Listeria* multigenome DNA array. Comparison of the global gene expression profiles revealed differences among strains. The expression profiles of two strains having distinct 50% lethal doses, as assessed in the mouse model, were further analyzed. Gene ontology term enrichment analysis of the differentially expressed genes identified differences in protein-, nucleic acid-, carbon metabolism-, and virulence-related gene expression. Comparison of the expression profiles of the core genomes of all strains revealed differences between the two lineages with respect to cell wall synthesis, the stress-related sigma B regulon and virulence-related genes. These findings suggest different patterns of interaction with host cells and the environment, key factors for host colonization and survival in the environment.**

Listeria monocytogenes is a gram-positive, facultative, intracellular bacterium that causes severe food-borne infections, such as gastroenteritis, septicemia, abortion, and meningitis, in humans and animals (60). *L. monocytogenes* is able to cross the intestinal barrier, the blood-brain barrier, and the fetoplacental barrier and to invade and replicate inside epithelial and professional phagocytic cells. *L. monocytogenes* is widely present in nature, and it has also been isolated from numerous animals, including cattle, sheep, and goats (21). Furthermore, *L. monocytogenes* has the important capacity to adapt to and survive in extreme environments, such as high salt concentration (10% NaCl), a broad pH range (from 4.5 to 9.0), and a wide temperature range. Its ability to grow at temperatures between −1°C and 45°C increases the risk of contamination in dairy products, meats, seafood, and other processed food products via selective enrichment during refrigeration. *Listeria* can also survive long periods of drying and freezing with subsequent thawing (38, 54). *L. monocytogenes* is an environmental bacterium living, for example, on decomposing plants. However, the presence of virulence factors, which have most probably been acquired by a common ancestor through horizontal gene transfer (for reviews see references 7 and 56), allows *L. monocytogenes* to infect humans and other mammalian hosts. Most susceptible to listeriosis are immunocompromised individuals, elderly people, pregnant women, fetuses, and neonates. Listeriosis is characterized by a low infection rate but a high mortality rate, and thus, *L. monocytogenes* is a concern for public health and for the food industry.

The infectious process is dependent on the production of several virulence factors. Proteins necessary for adhesion and the invasion of eukaryotic cells (the internalins InlA and InlB, the autolysin Ami, the cell wall hydrolase p60 [Iap], and the pore-forming listeriolysin O [LLO]); proteins involved in intracellular life, such as those responsible for escaping from

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TABLE 1. Characteristics of *Listeria monocytognes* strains used for expression profiling

Strain[a]	Origin	Serovar	Country	Yr	Designation	Chick embryo mortality (%)	Reference(s)
CLIP80459	Listeriosis epidemic in humans	4b	France	1999	Clinical	100	11
CLIP90602	Listeriosis epidemic in humans	1/2b	France	2002	Listeriosis	100	Unpublished
CLIP93667	Healthy human	4b	France	1992	Human	100	46, 47
CLIP93666	Healthy human	1/2a	France	1991	Carriage	20	
CLIP93665	Environment	4b	France	2000	Environmental	100	Unpublished
CLIP93649	Environment	1/2a	France	2000	Industrial plant	100	Unpublished

[a] CLIP denotes strain designations of the *Listeria* collection at the Institut Pasteur.

phagosomes (LLO and the phospholipases PlcA and PlcB), actin-based motility, and cell-to-cell spread (the surface protein ActA); and a protein involved in the bacterium's intracytoplasmic replication (the hexose phosphate transporter Hpt) are among the best studied to date (19). Virulence gene expression is tightly regulated. The major *L. monocytogenes* virulence genes are regulated by the transcriptional activator PrfA, whose regulon is under complex environmental control. It has been demonstrated that both temperature sensing and chemical components of the extracellular environment play important roles in regulating the expression of the PrfA regulon (4, 31, 40). Additionally, the transcription of a subset of *L. monocytogenes* virulence genes seems to be regulated by a network that can include activation by both PrfA and sigma B (33, 41, 58) and interplay between PrfA and sigma B has been suggested (40).

The species *L. monocytogenes* comprises 13 serovars; among those, serovars 1/2a, 1/2b, and 4b account for over 98% of all human listeriosis cases (29, 57). Even though serovar 1/2a is the most frequently isolated from food and environmental sources, most major food-borne outbreaks and a majority of sporadic cases of listeriosis are caused by serovar 4b strains (1, 28, 30, 43, 66). Moreover, the heterogeneous virulence of *L. monocytogenes* clinical strains and strains of different serovars has been demonstrated in a mouse model of infection and in the Caco-2 cell line (26, 49, 50). Comparative genomic analysis of the complete genome sequences of *L. monocytogenes* EGDe, of *Listeria innocua* CLIP11262 (25), and of three additional *L. monocytogenes* strains (42), as well as the comparison of over 100 *L. monocytogenes* strains of different serovars and origins, by DNA/DNA hybridization using DNA arrays (7, 8, 15, 68) revealed that differences in gene content exist between strains of different serovars and origins. Some of these differences may be implicated in the various disease potentials of *L. monocytogenes* strains. However, differences among strains may also be due to different gene expression/regulation of the core genes of *L. monocytogenes* as already shown for other bacterial species, such as *Escherichia coli* (16, 35) and *Pseudomonas aeruginosa* (55).

In order to better understand the differences occurring between strains, as well as between the two major phylogenetic lineages of the species *L. monocytogenes*, in niche adaptation and probably also in disease potential, we compared the transcriptional signatures of six *L. monocytogenes* strains under in vitro growth conditions in defined, rich medium and late exponential phase—conditions designed to maximize the number of genes expressed. The virulence of these six strains was assessed in the mouse model of infection and in the chicken embryo model. Variations in the expression of virulence-, cell wall-, carbohydrate metabolism-, and sigma B-related genes correlated with lineage designations, suggesting differences in the expression of genes for pathways related to interactions with the host.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and RNA extraction. The *Listeria monocytogenes* strains used in this study are described in Table 1. The strains were grown overnight in BHI (brain heart infusion) medium (Difco), inoculated at an optical density at 600 nm (OD_{600}) of 0.1 into 10 ml of the defined medium MCDB 202 (CryoBioSystem) supplemented with 1% glucose, and grown at 37°C until late exponential phase (OD_{600}, 0.9).

For each strain, two independent cultures were used. Cells were harvested by centrifugation at 4°C and flash frozen in a dry ice-alcohol mixture. RNA extraction was done as previously described (40). Briefly, pellets were resuspended in 400 μl of buffer (10% glucose, 12.5 mM Tris [pH 7.6], 5 mM EDTA) and 60 μl of 0.5 MEDTA. Then, cells were mechanically disrupted in a FastPrep apparatus in an acid phenol (pH 4.5) glass bead (Sigma) mixture. For extraction, Trizol (Invitrogen) was used, and RNA was precipitated with 2-propanol. After centrifugation for 30 min at 4°C, the pellets were washed with 70% ethanol and dried at room temperature prior to being dissolved in 50 μl of DNase- and RNase-free deionized water treated with 0.001% diethylpyrocarbonate (MP Biomedicals). The RNA concentration was estimated by using a spectrophotometer, and the RNA quality was checked on an agarose gel containing ethidium bromide.

Macroarray construction and hybridization. For this study, we designed a *Listeria* multigenome DNA macroarray. It contains PCR products representing 2,816 genes predicted in the completely sequenced *L. monocytogenes* EGDe genome (40) and 153 additional PCR products specific for genes predicted in *L. monocytogenes* strain CLIP80459 that are not present in strain EGDe (15; unpublished data). This *Listeria* multigenome DNA array contains probes representing *L. monocytogenes* serovar 1/2a (EGDe) and *L. monocytogenes* serovar 4b (CLIP80459) genes, thus allowing comparisons of strains belonging to the two serovars.

The construction of the macroarray and the hybridization of RNA samples were done as previously described (40), with slight modifications. Briefly, for cDNA synthesis and labeling, 1 μg of total RNA was mixed with 6 μl of 5× AMV reverse transcriptase buffer (Roche), 3 μl of a mixture of dATP, dGTP, and dTTP (10 mM), and 5 μg of random hexamers [Primer Random $p(dN)_6$, Roche], and diethylpyrocarbonate-treated water was added to a final volume of 21 μl. After heating at 90°C for 2 min and temperature adjustment to 42°C, 3 μl of [α-^{33}P]dCTP (2,000 to 3,000 Ci mmol^{-1}; Amersham) and 2 μl (50 U/μl) of AMV reverse transcriptase (Roche) were added. After a 2-h incubation at 42°C, the labeled cDNA was purified using a QIAquick nucleotide removal column (QIAquick nucleotide removal kit; QIAGEN). The cDNA obtained was denatured by heating at 99°C for 5 min just before hybridization. Macroarray hybridization was carried out as previously described (40). The macroarrays were then exposed to a phosphorimager screen and scanned with a Typhoon phosphorimager (Pharmacia-Amersham). Two hybridizations were performed for each independent RNA extraction. In total, four hybridizations were performed for each strain. The array design is available at the GEO database under the accession number GPL2811 (http://www.ncbi.nlm.nih.gov/geo/).

Data acquisition, preprocessing, and statistical analysis. After the signal intensity of each spot was scanned, it was quantified using ArrayVision software (Imaging Research). Raw and normalized data are available at the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The intensity values were normalized

arbitrarily by setting the mean of each array to 1. To study the similarities and variability of the expression signals among the strains, cluster analysis and *p*rincipal *c*omponent *a*nalysis (PCA), respectively, were used (51). The analysis was performed using the Euclidean distance and the complete linkage algorithm. In order to identify differentially expressed genes, the probability (Pr) that the normalized expression level G of a given gene g is greater in one strain than in the other, $\Pr(G_A > G_B)$, was calculated, where A and B represent all pairwise combinations of the strains studied: $\Pr(G_{\text{CLIP80459}} > G_{\text{CLIP90602}}), \ldots, \Pr(G_{\text{CLIP93665}} > G_{\text{CLIP93649}})$. The expression levels were assumed to be normal variables. The same approach was used to search for genes with expression levels associated with the results of the virulence profile calculating the probabilities $\Pr(G_C > G_D > G_E)$ and $\Pr(G_C < G_D < G_E)$, where C and E are the strains with extreme 50% lethal dose (LD_{50}) results and D represents the set of all other strains: $\Pr(G_{\text{CLIP80459}} > G_{\text{others}} > G_{\text{CLIP93649}})$ and $\Pr(G_{\text{CLIP80459}} < G_{\text{others}} < G_{\text{CLIP93649}})$. A gene is called significantly differentially expressed when the Pr of being interrogated is greater than 80% (combinatorial pair-wise analysis or profile-matching analysis). In spite of the analyses differing in their appropriate context, for the sake of clarity we called the output of both analyses differential expression of genes. Along with the statistical significances, the magnitudes (M) of differences were calculated as usual with the log fold changes with the appropriate groupings of C and D: $M = \log_2(G_A/G_B)$. With the list of differentially expressed genes, we performed a *g*ene *o*ntology (GO; http://www.geneontology.org) enrichment analysis using the BayGO method (62). The GO annotations were obtained at the EBI GOA-Proteomes web page (http://www.ebi.ac.uk/GOA/proteomes.html) (downloaded in February 2007). BayGO results showing GO categories with enrichment significance values of P smaller than 0.05 were further considered. The complete data set is available (see the supplemental material).

In vivo infection models. Assessment of the virulence of *L. monocytogenes* in chick embryos was done as described previously (46). Briefly, *L. monocytogenes* strains were grown in BHI medium at 37°C to mid-log phase (OD_{600}, 1.0). After being washed in phosphate-buffered saline (PBS), cells were suspended in PBS to an initial cell density of 3×10^7 CFU ml^{-1} to 3×10^8 CFU ml^{-1} and serially diluted. Fourteen-day-old embryos were inoculated with 100 ml of the 10^{-5} dilution via the chorioallantoic membrane. At least five embryos were used per strain tested. The embryos' vitality was monitored daily for 6 days using transillumination. The mean time to death was determined for each strain tested.

The LD_{50} of the six selected *L. monocytogenes* strains was determined after growth in BHI medium at 37°C to mid-log phase (OD_{600}, 0.8). After being washed in PBS, pellets were resuspended in sterile 0.9% NaCl. LD_{50} experiments were carried out by injecting 300-μl serial dilutions of inoculum intravenously into the tail veins of 8-week-old female BALB/c mice (Charles River). The LD_{50} values were determined by the probit method (3) after the infection of groups of four mice.

RESULTS

***Listeria monocytogenes* strains of different origins and epidemiological characteristics show different in vivo virulences.** For this study, six *L. monocytogenes* strains isolated from different sites, showing different epidemiological characteristics, and belonging to the two major lineages present within this species were selected. The two clinical strains were *L. monocytogenes* CLIP80459 (serovar 4b), which was responsible for 32 cases of illness during an epidemic of listeriosis in France (11), and *L. monocytogenes* CLIP90602 (serovar 1/2b), which was responsible for 7 clustered cases of listeriosis in France in 2002 (personal communication from C. Jacquet). Strains *L. monocytogenes* CLIP93667 (serovar 4b) and CLIP9366 (serovar 1/2a) were isolated from healthy humans, and strains *L. monocytogenes* CLIP93665 (serovar 4b) and CLIP93649 (serovar 1/2a) were isolated from a cheese plant (Table 1). The growth curves of these six strains (MCDB202 medium) were compared and did not show any significant differences (data not shown). Furthermore, the gene contents of the six selected strains were tested on the multiple-genome DNA array, as well as on a focused "*Listeria* biodiversity" array carrying genes specific to three *L. monocytogenes* and one *L. innocua* strain (27, 63). The results showed that the six selected strains contained the core genome of 2,695 genes (see Table S1 in the supplemental material), including all known virulence genes (data not shown).

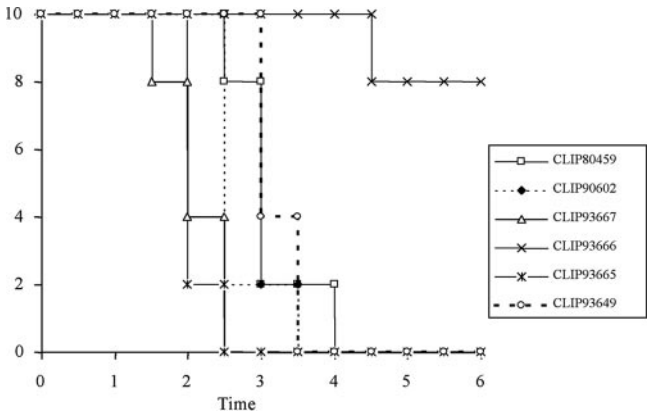

FIG. 1. Assessment of the virulence of the six studied *L. monocytogenes* strains in the chicken embryo model. The survival curve for the chick embryos is shown. *L. monocytogenes* cells were inoculated into 14-day-old chick embryos (0.3×10^2 to 1×10^2 CFU per egg) via the chorioallantoic membrane. The survival of the embryos was monitored daily for 6 days.

The virulence of the six *L. monocytogenes* isolates used in this study was determined in two models, the chicken embryo and the mouse model of infection. Infection of chick embryos resulted in 100% mortality within 3 days for all strains except for strain CLIP93666 (H1), which induced only 20% mortality 6 days after infection (Fig. 1). This strain harbors a point mutation at position 1474 of *inlA* generating a nonsense codon in the coding sequence which results in the translation of a truncated protein of 47 kDa. The presence of a truncated internalin may explain its reduced virulence in the chicken embryo model (46). However, the reduced virulence might also be due to other, not-yet-identified differences present in this strain, as restoration of internalin A functionality did not result in full virulence in chicken embryo assays (45). The LD_{50} of these six strains was also determined after intravenous injection into BALB/c mice. Two strains presented virulence phenotypes that were distinct from those of the rest of the group. The epidemic strain CLIP80459 showed a high virulence, with an LD_{50} of 1.7×10^2 CFU, and the environmental strain CLIP93649 showed a reduced virulence, with an LD_{50} of 8.7×10^4 CFU (Fig. 2). This result suggests that the chicken embryo test may allow the definition of strains expressing a truncated InlA. Furthermore, the mouse model identified one of the two epidemic strains as having a higher virulence than the environmental or carrier strains. However, the second clinical strain, CLIP90602 of serovar 1/2b, showed an LD_{50} of 2.2×10^3 CFU, which, like those of the four remaining strains, was intermediate compared to those of CLIP80459 and CLIP93649, suggesting that the mouse model of infection does not always reflect epidemiological characteristics (Fig. 2).

Global gene expression profiles of *L. monocytogenes* strains show differences and correlate with virulence. The *L. monocytogenes* multigenome array used in this study contains all the genes predicted in *L. monocytogenes* strain EGDe (serovar 1/2a) and all the genes specific for *L. monocytogenes*

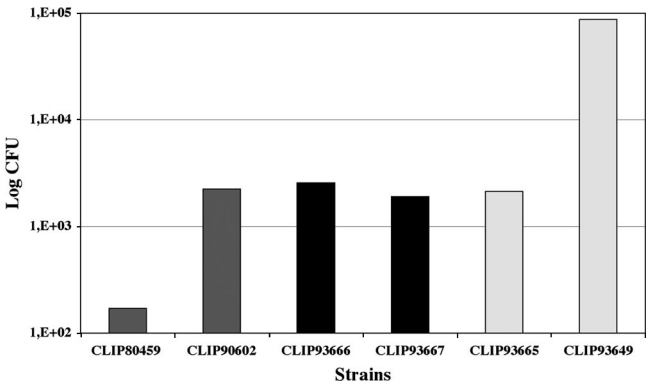

FIG. 2. Virulence of six *L. monocytogenes* strains in BALB/c mice. LD_{50}s of two epidemic strains (left bars), two carriage strains (middle bars), and two environmental strains (right bars) were determined after intravenous injection of increasing inocula of each strain in BALB/c mice.

CLIP80459 (serovar 4b) with respect to EGDe. *L. monocytogenes* strain EGDe is a representative of lineage I and strain *L. monocytogenes* CLIP80459 of lineage II of the three divergent evolutionary lineages found within the species *L. monocytogenes* (6, 15, 48, 64, 66). These two strains thus belong to the most common serovars found in foods and in human disease. The DNA array newly constructed here allows the comparison of isolates belonging to the two major lineages, as it contains genes of serovar 1/2a and of serovar 4b strains. To analyze differences and similarities in gene expression among the six *L. monocytogenes* strains chosen, the expression profile of cells cultured to an OD_{600} of 0.9 in MCDB broth was determined for each strain and then compared using different methods.

Unsupervised clustering methods offer efficient ways of finding overall patterns and tendencies in gene expression data. Thus, hierarchical clustering may disclose gene expression-based patterns that classify/split the tested strains, by their virulence potential, serovar, or lineage characteristics, for instance. However, analysis of the clusters obtained showed that, by searching for similarities in global gene expression profiles, no such obvious patterns were identified (Fig. 3). This method defined two clusters. Cluster 1 contained the two epidemic strains of serovar 4b and 1/2b and an environmental strain of serovar 4b (CLIP80459, CLIP93665, and CLIP90602). Cluster 2 contained the two carriage strains of serovar 1/2a and 4b and the environmental serovar 1/2a strain (CLIP93666, CLIP936667, and CLIP93649) (Table 1). PCA showed that only about 12% of the variance among the transcriptomes of the strains is significantly linked to the LD_{50} results, suggesting that there may be a specific set of genes associated with different levels of virulence as assessed in the mouse model. Our primary analysis focused on finding genes preferentially expressed in the strain defined as most virulent in the mouse model of infection, showing high $\Pr(G_{\text{CLIP80459}} > G_{\text{others}} > G_{\text{CLIP93649}})$, and those preferentially expressed in the least virulent strain in the same model of infection, showing high $\Pr(G_{\text{CLIP80459}} < G_{\text{others}} < G_{\text{CLIP93649}})$.

In order to investigate whether the clustering was dependent on the strain-specific genes, we excluded those genes from the analysis and reran the PCA. The relative disposition of the strains did not change when strain-specific genes were excluded, suggesting that the source of variation in the data relates to the differential expression of common *L. monocytogenes* genes (data not shown). Thus, we subsequently compared the strains with respect to the expression of their core genomes to better understand the possible impact on lineage-related differences in disease potential and niche adaptation. This analysis focused on a total of 2,695 genes common among the strains (see Table S1 in the supplemental material).

Differences in proteins, nucleic acids, carbon metabolism, and virulence-related gene expression characterize the epidemic strain CLIP80459. CLIP80459 is a clinical isolate responsible for a listeriosis outbreak and presents high virulence as assessed in the mouse model. Its expression profile was compared to those of the other five strains selected for this

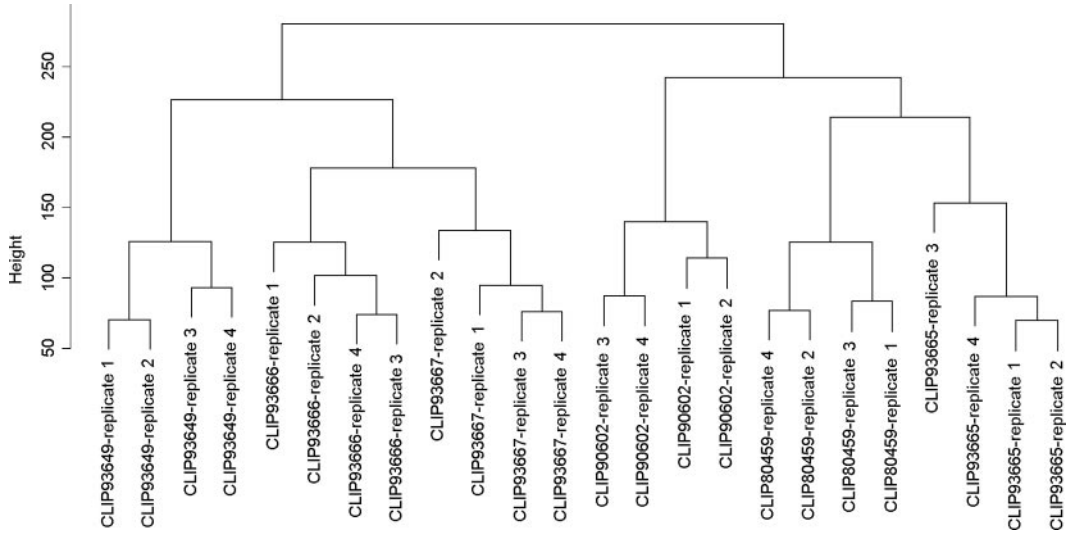

FIG. 3. Hierarchical clustering. The analysis was performed using the Euclidean distance and the complete linkage algorithm. Numbers 1 to 4 refer to the experimental replicates.

TABLE 2. Functional annotation of genes highly expressed in strain CLIP80459[a]

Locus tag(s)	GO term identification no.	Description	*P*
lmo0284 lmo0448 lmo2362	GO:0006865	Amino acid transport	0
lmo2193	GO:0015833	Peptide transport	0.04
lmo2545 lmo2546	GO:0009088	Threonine biosynthetic process	0
lmo1522 lmo1619	GO:0019478	D-Amino acid catabolic process	0
lmo1221 lmo1520 lmo1660	GO:0006418	tRNA aminoacylation for protein translation	0
lmo1096	GO:0004808	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase activity	0.03
lmo1359	GO:0003715	Transcription termination factor activity	0.03
lmo1235	GO:0004072	Aspartate kinase activity	0.05
lmo1517	GO:0006808	Regulation of nitrogen utilization	0.04
lmo1516	GO:0008519	Ammonium transporter activity	0.04
lmo0001	GO:0006275	Regulation of DNA replication	0.01
lmo1885	GO:0043101	Purine salvage	0.02
lmo1891 lmo1942	GO:0006310	DNA recombination	0.05
lmo1096 lmo1238	GO:0008033	tRNA processing	0.05
lmo1482	GO:0030420	Establishment of competence for transformation	0.03
lmo1600	GO:0016832	Aldehyde-lyase activity	0.04
lmo1600	GO:0004106	Chorismate mutase activity	0.02
lmo2524	GO:0016836	Hydro-lyase activity	0.05
lmo1952 lmo2363	GO:0016831	Carboxy-lyase activity	0
lmo1072	GO:0004736	Pyruvate carboxylase activity	0.02
lmo1917	GO:0008861	Formate *C*-acetyltransferase activity	0.03
lmo2363	GO:0019752	Carboxylic acid metabolic process	0.03
lmo0482 lmo1407 lmo1661	GO:0051539	4 Iron, 4 sulfur cluster binding	0

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. GO terms are accessible at http://www.geneontology.org. *P* indicates values of significance for GO term enrichment.

study. We explored whether there exist genes presenting an expression profile distinguishing this strain as did the virulence profile, with expression levels in CLIP80459 greater than in those strains presenting moderate virulence potentials and greater even than the levels in CLIP93649. A total of 78 core genome genes corresponded to these characteristics (Pr greater than 80%; see Table S2 in the supplemental material). GO term enrichment analysis was used for the functional analysis of these differentially expressed genes (9, 67). The major GO categories of the genes expressed differentially between CLIP80459 and the remaining five strains are genes coding for proteins associated with transport and protein and nucleic acid metabolism, as well as carbon utilization (Table 2). Major virulence genes also showed significant, *n*-fold variations. Surprisingly, *prfA*, encoding the master regulator of *Listeria* virulence gene expression (34); the two phospholipase-encoding genes *plcA* and *plcB* (24, 36, 39, 59); and the major invasion protein-encoding genes *inlA* and *inlB* (17), as well as the more recently characterized *bsh* gene, coding for a bile salt hydrolase and regulated by PrfA (18), showed lower expression levels in the epidemic strain CLIP80459 (Table 3).

In contrast to the epidemic strain CLIP80459, which had a very low LD_{50}, *L. monocytogenes* CLIP93649 (serovar 1/2a), which was isolated in a French cheese plant in 2000, showed the highest LD_{50} in the mouse model. In order to investigate whether the differences in virulence observed in the mouse model are reflected in differences in gene expression patterns, the expression profile of this strain was analyzed in detail. Eighty-two genes were statistically differentially regulated with respect to their regulation in the remaining strains (see Table S3 in the Supplemental Material). The ontology term analyses pointed to several GO terms related to environmental monitoring and stress responses like heat shock and oxidative stress as the major groups of genes characterizing this strain (Table 4).

Virulence-, sigma B-, and cell wall-related gene expression differ between *L. monocytogenes* lineages I and II. Differential gene expression analysis between strains belonging to lineage I (CLIP93666 and CLIP93649) and lineage II (CLIP80459, CLIP90602, CLIP93667, and CLIP93665) was carried out in an attempt to reveal core genome features that may distinguish the two groups. This analysis identified a total of 1,034 genes, including core genome and strain-specific genes, as being differentially expressed between the two groups (see Table S4 in the supplemental material). Several of those genes that showed overexpression in lineage I compared to their level of expression in lineage II were cell wall-associated core genome genes common to serotype 1/2 and 4 strains (Table 5). Differences in cell wall-associated genes are somewhat expected as there are structural differences between the cell walls of *L. monocyto-*

TABLE 3. Virulence-related genes differentially expressed in CLIP80459 and CLIP93649[a]

Locus tag	Gene; function	*M*	*P*
lmo0200	*prfA*; listeriolysin-positive regulatory protein	−25.1	0.89
lmo0201	*plcA*; phosphatidylinositol-specific phospholipase C	−29.7	0.79
lmo0205	*plcB*; phospholipase C	−38.6	0.91
lmo0433	*inlA*; internalin A	−26.4	0.86
lmo0434	*inlB*; internalin B	−3.85	0.89
lmo2067	*bsh*; bile salt hydrolase	−4.89	0.81
lmo2785	*kat*; catalase	−17.7	0.91

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. $M = \log_2(G_{CLIP80459}/G_{CLIP93649})$. Negative *M* value indicates gene repression in CLIP80459. $P = \Pr(G_{CLIP93649} > G_{CLIP80459})$.

TABLE 4. Functional annotation of genes highly expressed in strain CLIP93649[a]

Locus tag(s)	GO term identification no.	Description	*P*
lmo0641	GO:0005261	Cation channel activity	0
lmo2064	GO:0005216	Ion channel activity	0.01
lmo2680	GO:0006813	Potassium ion transport	0.04
lmo2174	GO:0000155	Two-component sensor activity	0.05
lmo2673 lmo2679	GO:0006950	Response to stress	0.03
lmo1472	GO:0031072	Heat shock protein binding	0.02
lmo2785	GO:0006979	Response to oxidative stress	0.03
lmo0205	GO:0004629	Phospholipase C activity	0.05
lmo0905 lmo0938 lmo2230	GO:0006470	Protein amino acid dephosphorylation	0
lmo1014 lmo1421	GO:0015171	Amino acid transporter activity	0.01
lmo1579	GO:0000286	Alanine dehydrogenase activity	0.01
lmo2551	GO:0003715	Transcription termination factor	0.02
lmo0211	GO:0008097	5S rRNA binding	0.04
lmo1471	GO:0006479	Protein amino acid methylation	0.04
lmo1538 lmo2695	GO:0006071	Glycerol metabolic process	0.01
lmo2205	GO:0004619	Phosphoglycerate mutase activity	0.01
lmo0014 lmo0970 lmo1372 lmo1439 lmo1688 lmo1830 lmo2573	GO:0016491	Oxidoreductase activity	0.02
lmo0014 lmo2389 lmo2390	GO:0006118	Electron transport	0.04
lmo1931	GO:0009060	Aerobic respiration	0.05
lmo1227 lmo1639	GO:0006284	Base excision repair	0.02
lmo1929	GO:0009209	Pyrimidine ribonucleoside triphosphate biosynthetic process	0.02

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. GO terms are accessible at http://www.geneontology.org. *P* indicates values of significance for GO term enrichment.

genes strains belonging to serogroups 1/2 and 4 (22, 23). The somatic component of the serotype designation of *Listeria* is related to the teichoic acid (TA; polyribitol phosphate covalently linked to peptidoglycan) present in the cell wall. Glycosidic substitutions of the ribitol phosphate units render this component variable, structurally and antigenically (32). Such differences could account for distinct patterns of interaction with host cells, with a direct impact on the virulence of the different serovars. Serotype 1/2 and 4 *L. monocytogenes* strains show structural differences in the cell wall due to distinct genome content (22, 23). However, here we show that the gene expression of common cell wall-regulated genes is consistently different between the two lineages, suggesting also common features of the cell walls of 1/2b and 4b strains. Interestingly, differences are also seen in *prfA* and PrfA-regulated genes. As depicted in Table 5, *prfA*, *plcB*, *plcA*, *hly*, *actA*, and *inlAB* are highly expressed in lineage I strains compared to their levels of expression in lineage II strains.

Basal levels of the *sigB* operon genes (*rsbR-rsbS-rsbT-rsbU-rsbV-rsbW-sigB-rsbX*) are provided by transcription initiating at a promoter positioned upstream of the *rsbR* gene. A second SigB-dependent promoter is located between *rsbU* and *rsbV* and is induced by all SigB-activating environmental and metabolic stimuli described thus far. Several virulence-related genes, such as *inlA* and *inlB*, are also under the influence of sigma B. Like the virulence genes mentioned above, *sigB* was overexpressed in lineage I, as were *rsbV*, *rsbW*, and *rsbX*. We thus specifically analyzed the expression of the genes reported to be under the control of sigma B as described by Kazmierczak and colleagues (33). This analysis additionally showed that 20 genes described as belonging to the sigma B regulon were up-regulated in lineage I (Table 6).

TABLE 5. Selected core genome features differentially expressed between *L. monocytogenes* strains belonging to lineages I and II[a]

Locus tag	Gene; function	*M*	*P*
lmo0200	*prfA*; listeriolysin-positive regulatory protein	13.5	0.87
lmo0201	*plcA*; phosphatidylinositol-specific phospholipase C	21.3	0.98
lmo0202	*hly*; listeriolysin O precursor	8.8	0.9
lmo0204	*actA*; actin-assembly inducing protein precursor	0.54	0.96
lmo0205	*plcB*; phospholipase C	19.4	0.84
lmo0433	*inlA*; internalin A	15.3	0.93
lmo0434	*inlB*; internalin B	2.35	0.95
lmo1074	Similar to TA translocation permease protein TagG	0.67	0.88
lmo1075	Similar to TA translocation ATP-binding protein TagH	1.55	0.96
lmo0842	Putative peptidoglycan-bound protein (LPXTG motif)	0.24	0.98
lmo0441	Similar to penicillin-binding protein (D-alanyl-D-alanine carboxypeptidase)	−1.12	0.94
lmo0540	Similar to penicillin-binding protein	−2.54	0.97
lmo1892	Similar to penicillin-binding protein 2A	−3.64	0.96
lmo2229	Similar to penicillin-binding protein	−1.98	0.99

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. $M = \log_2(G_{\text{linI}}/G_{\text{linII}})$, where linI and linII are lineages I and II. $P = \Pr(\text{LinI} > \text{LinII})$ when M is a positive number, and $P = \Pr(\text{LinII} > \text{LinI})$ when M is a negative number.

TABLE 6. Sigma B-related genes overexpressed in *L. monocytogenes* strains belonging to lineage I[a]

Locus tag	Gene; function	*M*	*P*
lmo1421	Similar to glycine betaine/carnitine/choline ABC	1.95	0.94
lmo1426	*opuCC*; transporter (membrane protein)	3.64	0.83
lmo1427	*opuCB*; similar to glycine betaine/carnitine/choline ABC	0.908	0.67
lmo1428	*opuCA*; similar to glycine betaine/carnitine/choline ABC	1.94	0.81
lmo0893	Anti-anti-sigma factor (antagonist of RsbW)	2.67	0.84
lmo0894	RNA polymerase sigma-37 factor (sigma B)	2.29	0.79
lmo0895	Sigma B activity negative regulator RsbW	2.34	0.78
lmo0896	Indirect negative regulation of sigma B-dependent gene	1.54	0.81
lmo2230	Expression (serine phosphatase) similar to arsenate reductase	5.33	0.88
lmo0783	Similar to mannose-specific phosphotransferase system component IIB	19.3	0.94
lmo0784	Similar to mannose-specific phosphotransferase system component IIA	9.94	0.96
lmo2398	Low-temperature-requirement C protein	3.21	0.94
lmo2602	Phosphotransferase system component IIA cation-transporting ATPase	2	0.99
lmo1539	Similar to glycerol uptake facilitator	0.748	0.92
lmo0669	Transporter (ATP-binding protein) similar to oxidoreductase	2.93	1
lmo1694	Similar to CDP-abequose synthase	3.09	0.99
lmo2695	Similar to dihydroxyacetone kinase	2.3	0.86
lmo2205	Similar to phosphoglyceromutase 1	9.5	0.82
lmo2085	Putative peptidoglycan-bound protein (LPXTG motif)	2.81	0.94
lmo0880	Similar to cell wall-associated protein precursor (LPXTG motif)	2.17	0.83

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. $M = \log_2(G_{\text{linI}}/G_{\text{linII}})$, where linI and linII are lineages I and II. $P = \Pr(\text{LinI} > \text{LinII})$.

Penicillin binding- and antibiotic response-related genes were also identified as markers that differentiate the two groups. Four of the genes overexpressed in this category in lineage II (lmo0441, lmo0540, lmo1892, and lmo222) (Table 5) present the characteristics of penicillin-binding proteins. Penicillin-binding proteins are transpeptidases involved in different aspects of cell wall synthesis in bacteria, and their overexpression in lineage II corroborates distinct cell wall activities in the two groups under the studied conditions.

Motility- and chemotaxis-related genes were overexpressed in lineage II (Table 7), suggesting that the signals for the expression of these genes may be perceived in a different way by the two groups. Distinct patterns of regulation may act upon the pathogen's efficiency in cell invasion and its interaction with the host—in particular, the immune system.

Strain-specific genes expressed are mainly cell wall related. As reported recently, strain-specific genes are differentially expressed during intracellular growth of *L. monocytogenes*

TABLE 7. Motility- and chemotaxis-related genes overexpressed in *L. monocytogenes* strains belonging to lineage II[a]

Locus tag	Gene; function	*M*	*P*
lmo0678	Similar to flagellar biosynthesis protein FliR	−0.613	0.72
lmo0679	Similar to flagellar biosynthesis protein FlhB	−0.964	0.78
lmo0680	Similar to flagellum-associated protein FlhA	−0.129	0.63
lmo0681	Similar to flagellar biosynthesis protein FlhF	−0.417	0.78
lmo0682	Similar to flagellar hook-basal body protein FlgG	−0.478	0.8
lmo0683	Similar to chemotactic methyltransferase CheR	−0.986	0.83
lmo0685	Similar to motility protein (flagellar motor rotation) MotA	−0.323	0.88
lmo0686	Similar to motility protein (flagellar motor rotation) MotB	−1.49	0.88
lom0689	Similar to CheA activity-modulating chemotaxis protein CheV	−0.933	0.89
lmo0690	Flagellin protein	−7.61	0.74
lmo0691	Chemotaxis response regulator CheY	−0.868	0.81
lmo0692	Two-component sensor histidine kinase CheA	−2.57	0.78
lmo0693	Similar to flagellar motor switch protein FliY C-terminal part	−0.248	0.77
lmo0697	Similar to flagellar hook protein FlgE	−2.44	0.8
lmo0698	Weakly similar to flagellar switch protein	−0.353	0.82
lmo0699	Similar to flagellar switch protein FliM	−0.55	0.83
lmo0700	Similar to flagellar motor switch protein fliY	−0.676	0.8
lmo0705	Similar to flagellar hook-associated protein FlgK	−0.817	0.9
lmo0706	Similar to flagellar hook-associated protein 3 FlgL	−0.689	0.913
lmo0707	Similar to flagellar hook-associated protein 2 FliD	−1.44	0.88
lmo0710	Similar to flagellar basal-body rod protein FlgB	−1.19	0.919
lmo0711	Similar to flagellar basal-body rod protein FlgC	−0.514	0.88
lmo0712	Similar to flagellar hook-basal body complex protein FliE	−0.239	0.9
lmo0713	Similar to flagellar basal-body M-ring protein FliF	−0.636	0.87
lmo0714	Similar to flagellar motor switch protein FliG	−1.02	0.902

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. $M = \log_2(G_{\text{linI}}/G_{\text{linII}})$, where linI and linII are lineages I and II. $P = \Pr(\text{LinII} > \text{LinI})$.

TABLE 8. Strain-specific genes expressed in *L. monocytogenes* strains belonging to lineage II[a]

Locus tag	Gene function; description or name	*M*	*P*
lm4b0014	Weakly similar to similar to autolysin (amidase)	0.62	0.81
lm4b0015	Weakly similar to similar to autolysin (amidase)	0.39	0.86
lm4b0290	Similar to internalin D	0.32	0.97
lm4b0349	Similar to internalin, putative peptidoglycan-bound protein (LPXTG motif)	0.34	0.97
lm4b0372	Similar to internalin, peptidoglycan-bound protein (LPxTG motif)	0.99	0.94
lm4b0484	Similar to transcription regulator (VirR from *Streptococcus pyogenes*)	0.49	0.96
lm4b0687	Similar to internalin proteins, putative peptidoglycan-bound protein (LPXTG motif)	0.48	0.93
lm4b0943	Similar to ABC transporter, ATP-binding protein	0.60	0.99
lm4b1092	Similar to autolysin (*N*-acetylmuramoyl-L-alanine amidase)	15.3	0.98
lm4b1101	Similar to galactosamine-containing minor TA biosynthesis protein GgaA	0.64	0.88
lm4b1102	Similar to *Bacillus subtilis* TagF protein (probable CDP-glycerol glycerophosphotransferase)	1.75	0.92
lm4b1103	Similar to TA biosynthesis protein B precursor	0.16	0.82
lm4b1887	Similar to hypothetical proteins containing ChW repeats	0.77	0.91
lm4b2360	Similar to putative transcription regulator	0.91	0.99
lm4b2526	Autolysin, amidase	5.82	0.91
lm4b2631	Similar to internalin; putative peptidoglycan-bound protein (LPXTG motif)	1.22	0.96
lm4b2727	Similar to glycosyltransferase; *gltA*	2.15	0.94
lm4b2728	Similar to glycosyltransferase; *gltB*	2.29	0.94

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. $M = \log_2(G_{\text{linI}}/G_{\text{linII}})$, where linI and linII are lineages I and II. $P = \Pr(\text{LinII} > \text{LinI})$.

(10). Thus, we investigated whether strain-specific genes were also expressed under our study conditions. Genes considered to be strain specific were those defined as present only in the *L. monocytogenes* EGDe or in the *L. monocytogenes* CLIP80459 genome sequence according to the best BLASTP hits and an amino acid sequence similarity lower than 70% over two-thirds of the length of the protein. Table 8 shows 18 genes (out of a total of 151 *L. monocytogenes* serovar 4b strain-specific genes present on the array) that were significantly expressed in lineage II strains under the study conditions. Fourteen of these genes code for proteins related to cell wall biosynthesis or cell wall-associated proteins.

In lineage I, 13 specific genes (out of 139 *L. monocytogenes* serovar 1/2a strain-specific genes present on the array) were significantly expressed. Among those, genes coding for cell wall-associated proteins, like the clusters lmo1076 to 1088 and lmo1090 to 1091 involved in cell wall biosynthesis, again were the most prominent ones (Table 9). These results are in agreement with the fact that both groups differ in cell wall-related activities with a possible impact on host-pathogen interaction. The carbohydrate metabolism-related gene *bvrA* (lmo2788), encoding an antiterminator of the BglG family (4), implicated in the virulence of *L. monocytogenes* and absent in lineage II strains (14) was also expressed in lineage I (Table 9).

DISCUSSION

All *L. monocytogenes* strains found in foods are considered to be pathogenic; however, the relative virulence of individual *L. monocytogenes* isolates can vary substantially in selected animal models (5, 53, 66). The genetic basis underlying these virulence differences is not yet understood. Comparative genomics by the hybridization of over 100 *L. monocytogenes* strains revealed important differences in gene content among different isolates (8, 15, 68), giving some clues as to how virulence differences might have evolved. However, complete understanding of the variations in virulence was not obtained. Global gene expression profiling has been shown to be a powerful tool for comparing differences in gene expression/regulation that might be related to virulence. Transcriptional pro-

TABLE 9. Strain-specific genes expressed in *L. monocytogenes* strains belonging to lineage I[a]

Locus tag	Gene function; description or name	*M*	*P*
lmo1077	Similar to TA biosynthesis protein B	0.27	0.87
lmo1080	Glycosyl transferase	0.63	0.93
lmo1081	Glucose-1-phosphate thymidylyltransferase	1.59	0.96
lmo1082	dTDP-4-dehydrorhamnose 3,5-epimerase	2.09	0.91
lmo1083	dTDP-glucose 4,6-dehydratase	1.89	0.88
lmo1084	dTDP-4-dehydrorhamnose reductase	3.87	0.92
lmo1085	Similar to TA biosynthesis protein B	0.47	0.98
lmo1086	Similar to CDP-ribitol pyrophosphorylase	4.66	0.88
lmo1087	Similar to glucitol dehydrogenase	1.64	0.89
lmo1088	Similar to TA biosynthesis protein B precursor; *tagB*	0.67	0.93
lmo1090	Glycosyl transferase	0.54	0.92
lmo1091	Glycosyl transferase domain protein; putative	0.47	0.85
lmo2788	Transcription antiterminator; BglG family	0.82	1

[a] lmo indicates locus tags of *L. monocytogenes* strain EGDe accessible at http://genolist.pasteur.fr/ListiList/. $M = \log_2(G_{\text{linI}}/G_{\text{linII}})$, where linI and linII are lineages I and II. $P = \Pr(\text{LinI} > \text{LinII})$.

filing and comparison of two clinical isolates of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, identified differences in the transcriptomes that provided clues to their pathogenesis (44). Comparison of the transcriptomes of two cystic fibrosis-causing epidemic strains of *Pseudomonas aeruginosa* that displayed enhanced virulence and antimicrobial resistance and of a laboratory strain revealed differences in the transcriptomes that were related to the phenotypes (55). A similar approach applied to *Escherichia coli* (16, 35) led to the discovery of selection-driven transcriptional profile differences and distinct differences in the expression of virulence and stress-related genes.

We used the comparison of global gene expression profiles and two different in vivo infection models to learn more about strain- and lineage-specific differences, some of which might also be related to virulence differences in *L. monocytogenes* strains. Six *L. monocytogenes* strains with different epidemiological backgrounds (epidemic, carrier, and environmental isolates) belonging to the two major disease-related lineages I and II were chosen for this first multiple-strain transcriptome comparison *of L. monocytogenes*. The virulence of these strains as assessed in the mouse infection model and in the chicken embryo varied according to the model used. The infection of chick embryos identified as having very low virulence one strain (CLIP93666) that was previously shown to be impaired in its ability to invade target cells via the interaction of InlA and its receptor E-cadherin, due to a truncated *inlA* gene (47), whereas the other five strains did not show significant virulence differences in this model. The murine model distinguished the epidemic serovar 4b strain (CLIP80459) from one environmental strain (CLIP93649) as having very high virulence and very low virulence, respectively, and these were clearly distinct from the four remaining strains for which virulence was not distinguished in this model. Interestingly, comparison of global gene expression and virulence as assessed in the mouse model showed a good correlation, indicating that differences in virulence may indeed be reflected in gene expression. Due to the many differences identified in genes encoding functions of the basic metabolism, such as carbohydrate metabolism and amino acid and cell wall biosynthesis, and those encoding functions expressed under specific circumstances, such as stress-related and virulence genes expected to be important for adaptation to the environment and the infected host, we specifically focused the analysis on the transcriptional data for these two groups of genes.

Like many other pathogens that can live saprophytically in the environment, *L. monocytogenes* must have tightly regulated virulence gene expression. In this study, we observed a clear repression of the *prfA*- and PrfA-dependent genes (*plcA*, *plcB*, *hly*, *inlA*, and *inlB*) in CLIP80459 in comparison to their levels of expression in the other group of strains. Interestingly, strain CLIP80459 was the most virulent one in the murine model of infection and was responsible for an epidemic of listeriosis in France from the end of 1999 to the beginning of 2000 (11). This observation is in agreement with our previous results showing that the virulence gene expression of the epidemic *L. monocytogenes* serovar 4b strain PAM 14 was lower than that of the *L. monocytogenes* EGDe strain (40). Furthermore, only low levels of haemolysin and lecithinase have been reported in clinical isolates of *L. monocytogenes* when grown in rich medium (53, 61), which might indicate that it is an advantage not to express these genes under in vitro or "environmental" conditions. However, another recent study comparing the invasion capacity of and the expression of the *inlA* and *inlB* genes in 27 clinical and 37 nonclinical *L. monocytogenes* strains in Caco-2 and HepG2 cells also identified significant differences in their invasion capacities that were correlated with gene expression. Clinical strains showed a lower invasion capacity and lower expression levels of *inlA* and *inlB* and induced lower interleukin-8 levels in HepG2 cells than nonclinical strains (65). Taken together, these findings show that in vitro gene expression and invasion into human cell lines correlate and suggest that the lower capacity of clinical strains to invade HepG2 cells, which is correlated with lower *inlAB* expression and the lower induction of interleukin-8, is possibly a mechanism of immune evasion used by specific *L. monocytogenes* strains.

Interestingly, overexpression of motility genes was observed in lineage II *L. monocytogenes* strains. Flagella are of great importance for the virulence of several pathogens (12, 20, 37, 52). Even though they mediate or facilitate the adhesion and invasion of eukaryotic host cells by *L. monocytogenes* (2, 13), the role of flagella in vivo is still not clear. The repression of virulence-related genes and overexpression of motility- and chemotaxis-related genes possibly characterize free-living *L. monocytogenes*.

The gene expression of lineage I strains compared to that of lineage II strains revealed many differentially expressed genes, highlighting important metabolic differences between the two serotypes. TA biosynthesis genes common to serotype 1/2 and 4 were clearly differentially expressed, pointing not only to structural differences in the TA composition but also to differential regulation of the biosynthesis of TA. Glycosylated TA components are important antigenic determinants in *L. monocytogenes*, even though their specific role in infection has not been elucidated (32). Due to their surface exposure and immunogenicity, their importance in interactions between bacteria and their host cells, with other free-living organisms, and with the extracellular matrix is thus likely. In agreement with these observations, a number of genes encoding cell wall-associated proteins were also detected as being differentially expressed between the two groups. We also detected the differential expression of *sigB* and SigB-regulated genes. In *L. monocytogenes*, SigB confers stress resistance and contributes to pathogenesis since it affects *prfA* transcription, also differentially expressed between the two groups (33). Links between environmental stress responses and virulence suggest that SigB might have a central role in the survival and pathogenesis of *L. monocytogenes*. The diversity of genes affected by this global regulator could reflect the way strains respond to environmental stress. Thus, one of the major themes that emerges from the analysis of the differential gene expression between lineages I and II is that they seem to interact differently with the milieu or their host. This is in agreement with their dissimilar patterns of distribution in foods, the environment, and the infected hosts. However, gene expression profiling in in vitro conditions in rich medium does not necessarily reflect the conditions encountered in the environment or the host. Thus, our results represent a first basis of gene expression profiles, allowing it to be shown that differences among strains exist in vitro and suggesting that differences may exist also in vivo. The exciting

experiment for the future will be the comparison of a greater number of strains in vivo and in conditions encountered in the food environment.

In conclusion, this study presents the first comparison of the gene expression profiles of distinct strains of *L. monocytogenes*, providing new insights into strain-specific differences in gene expression and new results for the better understanding of the response of *L. monocytogenes* to the different environments it encounters. Our results suggest that differential gene regulation of core genes of the *L. monocytogenes* genome may have an impact on its clinical behavior, suggesting niche adaptation. Differences in the gene expression profiles of representative strains of the two major lineages within the species *L. monocytogenes* that are responsible for human disease reflect diverse possibilities of interaction with their hosts.

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