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Identification of *Listeria monocytogenes* Genes Contributing to Intracellular Replication by Expression Profiling and Mutant Screening†

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A successful transition of *Listeria monocytogenes* from the extracellular to the intracellular environment requires a precise adaptation response to conditions encountered in the host milieu. Although many key steps in the intracellular lifestyle of this gram-positive pathogen are well characterized, our knowledge about the factors required for cytosolic proliferation is still rather limited. We used DNA microarray and real-time reverse transcriptase PCR analyses to investigate the transcriptional profile of intracellular *L. monocytogenes* following epithelial cell infection. Approximately 19% of the genes were differentially expressed by at least 1.6-fold relative to their level of transcription when grown in brain heart infusion medium, including genes encoding transporter proteins essential for the uptake of carbon and nitrogen sources, factors involved in anabolic pathways, stress proteins, transcriptional regulators, and proteins of unknown function. To validate the biological relevance of the intracellular gene expression profile, a random mutant library of *L. monocytogenes* was constructed by insertion-duplication mutagenesis and screened for intracellular-growth-deficient strains. By interfacing the results of both approaches, we provide evidence that *L. monocytogenes* can use alternative carbon sources like phosphorylated glucose and glycerol and nitrogen sources like ethanolamine during replication in epithelial cells and that the pentose phosphate cycle, but not glycolysis, is the predominant pathway of sugar metabolism in the host environment. Additionally, we show that the synthesis of arginine, isoleucine, leucine, and valine, as well as a species-specific phosphoenolpyruvate-dependent phosphotransferase system, play a major role in the intracellular growth of *L. monocytogenes*.

Listeria monocytogenes is a gram-positive, food-borne bacterium that is widely distributed in nature and can cause serious infection in susceptible individuals (9). The infection pathogenesis of this facultative intracellular pathogen includes phases where the bacteria successfully proliferate in the challenging environment in a variety of mammalian cell types like epithelial cells, endothelial cells, hepatocytes, dendritic cells, and macrophages. While invading the host cells, *L. monocytogenes* is confronted with suboptimal growth conditions, such as a rapid pH drop within the phagosome (1). Following early escape from the primary phagosome by membrane lysis, the pathogen is released into the cytosol where it encounters conditions of nutrient and iron starvation. The genes essential for the internalization of *L. monocytogenes* by the mammalian host cells (*inlA* and *inlB*), phagosomal escape (*hly*, *plcA*, and *plcB*), and intra- and intercellular motility (*actA*) have been extensively studied in the past years (45). All these virulence genes are controlled by PrfA, a central transcription regulator, which activates gene transcription by binding to a specific site termed the PrfA box and recruiting RNA polymerase to the PrfA-dependent promoters (6). Using a microinjection technique, we observed a *prfA* mutant of *L. monocytogenes* to replicate much less efficiently than the wild-type strain. However, none of the known virulence genes was shown to be responsible for this reduced intracellular replication (17). Likewise, a *Listeria innocua* strain equipped with the *prfA* and *hly* genes encoding listeriolysin for phagosomal escape into the host cell's cytosol also showed a substantially lower cytosolic replication efficiency than that of *L. monocytogenes* (39).

While it is evident that the products of the virulence gene cluster and their regulator, PrfA, are indispensable for *L. monocytogenes* to reach the cytoplasm of mammalian cells, the specific set of genes necessary for cytosolic proliferation still lacks detailed description despite several approaches to characterize the genes and proteins involved in intracellular replication: the selective induction of 32 proteins was shown by two-dimensional electrophoresis when *L. monocytogenes* EGD was grown in J774 macrophages, but the nature of the proteins is unknown (19). By screening a library of Tn*917-lac* insertion mutants, the specific induction of genes for purine and pyrimidine biosynthesis as well as for arginine uptake in J774 cells was observed (21). Furthermore, the results of analysis of some *L. monocytogenes* mutants auxotrophic for amino acids and nucleobases indicated that synthesis of all three aromatic amino acids and adenine was essential for efficient cytosolic

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TABLE 1. Bacterial strains used in this study

Strain	Characterization	Source or reference
E. coli		
XL2-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r) Amy Cam^r]	Stratagene
DH5α	*deoR endA1 gyrA96 hsdR17*(r_K^- m_K^+) *recA1 relA1 supE44 λthi-1* Δ(*lacZYA-argF*)*U169*	18
L. monocytogenes		
EGD	Sv1/2a, wild-type	S. H. E. Kaufmann
EGD-e	Derivative of EGD	G. B. Mackaness
Pkp1	Sv1/2a Δ*plcA-hly-mpl-actA-plcB*, Kan^r	10
EGDΔ*glpD* (lmo1293)	In-frame deletion of *glpD* (lmo1293)	This study
EGDΔ1538	In-frame deletion of lmo1538	This study
EGDΔ1538Δ*glpD* (lmo1293)	Double mutant of lmo1538 and *glpD* (lmo1293)	This study
EGD-eΔ1968-1974	In-frame deletion of the gene cluster lmo1968-1974 specific for *L. monocytogenes*	This study

replication in J774 cells (27). Reduced proliferation in the cytosol of these mammalian host cells was also observed for a mutant defective in *oppA* encoding an oligopeptide-binding protein (2), a result that suggests that efficient uptake of oligopeptides may favor intracellular growth of *L. monocytogenes* in macrophages.

Recently, a transporter for phosphorylated hexoses, encoded by *hpt*, was identified in *L. monocytogenes* with high homology to the UhpT transporter of *Escherichia coli*. Expression of *hpt* proved to be strictly PrfA dependent, and an *hpt* mutant exhibited significantly reduced efficiency in cytosolic replication (8). However, complementation of *L. innocua* with the *L. monocytogenes hpt* gene did not substantially improve the replication capacity of this strain in the host cell's cytosol, suggesting further requirements of *L. monocytogenes* for efficient growth in this cellular compartment. Interestingly, an *L. monocytogenes* mutant lacking the lipoate protein ligase LplA1 was also found to be defective for growth specifically in the host cell cytosol. Further analysis suggested that abortive growth was due to loss of pyruvate dehydrogenase function whose E2 subunit is a major target for LplA1 (32). Specific metabolic requirements for replication in macrophages and epithelial cells have also been reported for *Salmonella enterica* serovar Typhimurium (22), *Mycobacterium tuberculosis* (43), and *Shigella flexneri* (26). These intracellular bacteria, however, replicate in specialized phagosomal compartments, and the growth requirements in these compartments are likely to be different from the requirements in the host cell's cytosol where *L. monocytogenes* multiplies.

To obtain a more comprehensive overview of genes deployed by *L. monocytogenes* to efficiently proliferate in mammalian cells, we determined the complete expression profile of *L. monocytogenes* replicating in the cytosol of mammalian cells. For that purpose, we compared the overall transcriptional activity of *L. monocytogenes* grown in the cytosol of epithelial cells to that of the same strain cultured extracellularly in rich medium by using whole-genome microarrays. This approach allowed the identification of 279 genes exhibiting increased mRNA levels and 272 genes with reduced mRNA levels under in vivo conditions. In parallel, we established a listerial mutant library by insertion duplication mutagenesis that represents approximately 13% of the genes of *L. monocytogenes* and screened it for strains with impaired replication capacity in Caco-2 cells, but not in brain heart infusion (BHI) medium. Seventeen percent of the listerial genome is devoted to carbon and nitrogen metabolism and transport proteins involved therein (16); of these genes, in this study we found an upregulation of 65 genes intracellularly, and one of these genes, encoding a putative phosphoenolpyruvate-dependent phosphotransferase system (PTS) enzyme, is present in *L. monocytogenes*, but not in the nonpathogenic species *L. innocua*. This study shows for the first time a genome-wide analysis of gene regulation in intracellularly replicating *L. monocytogenes*. By combining two different approaches, microarray analysis and mutant screening, we partially expose the biological relevance of the observed adaptations of the listerial transcriptome to the intracellular environment.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and cell lines. Strains used in this study are listed in Table 1. *Escherichia coli* strains XL2-Blue (Stratagene, La Jolla, CA) and DH5α were cultivated in Luria-Bertani (LB) medium at 37°C. *L. monocytogenes* EGD (serovar 1/2a) was grown in brain heart infusion (BHI) at 37°C or at 30°C and 42°C. When necessary, media were supplemented with erythromycin (Sigma, St. Louis, MO) to final concentrations of 300 μg/ml for *E. coli* or 5 μg/ml for *L. monocytogenes*. For solid media, 1.5% agar (wt/vol) was added. Human colon epithelial cells (Caco-2 cells) were received from the American Type Culture Collection (ATCC HTB-37) and were cultured at 37°C and 5% CO_2 in RPMI 1640 (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). In order to monitor the in vitro growth of *L. monocytogenes* cells, 100 μl of an overnight culture were diluted into 10 ml prewarmed BHI medium and shaken at 190 rpm. The optical density of the cultures was measured every hour in a Klett-Summerson colorimeter (Klett Manufacturing Co., Inc., New York, NY). For storage at −80°C, strains were grown to an optical density at 550 nm of 0.6, and glycerol (final concentration, 15%) was added to yield 1-ml samples.

General techniques. PCR amplifications, cloning procedures, isolation of chromosomal DNA, and DNA manipulations were carried out according to standard procedures (37). Cycle sequencing was performed using the CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter, Fullerton, CA), and sequencing reactions were run on a XL2000 Beckman Coulter sequencer. The *Listeria* home page of the Institut Pasteur (http://www.genolist.pasteur.fr/ListiList/) was used for sequence analysis and gene annotation. Oligonucleotides used for PCRs are listed in Table S1 in the supplemental material.

Construction of an insertion mutant library of *L. monocytogenes*. A random, mutagenic fragment library of *L. monocytogenes* strain Pkp1 was constructed in the temperature-sensitive shuttle vector pLSV101 that is a shortened version of pLSV1 (46). The methylase gene from pLSV1 was amplified with primer pair MSLmethylase1 and MSLmethylase2, generating a 1,322-bp HindIII-HindIII DNA fragment which was cloned into a 4,170-bp fragment derived from HindIII treatment of pLSV1, resulting in plasmid pLSV101 (5,491 bp). This vector replicates in *Listeria* at 30°C, while it is lost after several cell divisions at 42°C due to two transition mutations in the *repF* gene. DNA fragments of *L. monocytogenes* EGD were randomly generated by sonication by the protocol of Lee et al.

(24) with the following modifications. Chromosomal DNA (100 μg) was prepared as described previously (10). DNA was suspended in 1 ml of sonication buffer and sonicated for 120 s with repeated interruptions for cooling on ice. After ethanol precipitation, the DNA fragments were redissolved, and an aliquot of 1 μg was analyzed by agarose gel electrophoresis (2% agarose) to confirm the generation of short DNA fragments. The fractionated DNA was digested with 1 U MboI (= Sau3A) per μg DNA and separated as described above. Gel slices containing unirradiated DNA fragments from 200 to 400 bp were isolated from the gel using dialysis bags, purified, and redissolved by standard procedures (37). In parallel, the pLSV101 vector was restricted with the MboI-compatible restriction endonuclease BamHI. Aliquots of 100 ng vector DNA were treated with shrimp alkaline phosphatase (Boehringer, Mannheim, Germany) as recommended by the supplier and ligated with 250 ng fragment DNA. Ligation samples were transformed into *E. coli* strain XL2-Blue, and *E. coli* transformants were selected at 37°C on LB agar containing erythromycin. Colonies were pooled in sets of 60 to 380 clones, and 300 ng DNA isolated from each pool was transformed into *L. monocytogenes* EGD. The *L. monocytogenes* EGD fragment library clones were selected at 30°C in the presence of erythromycin. The clones were streaked out at 30°C, and single colonies were suspended in 200 μl BHI in a 96-well microtiter plate. Twenty microliters of each suspension was dropped with a multichannel pipette into wells on prewarmed BHI agar plates containing erythromycin and incubated at 42°C for 2 days. Selected insertion mutants of *L. monocytogenes* EGD were isolated in 96-well microtiter plates in BHI and regrown overnight at 42°C in the presence of erythromycin. To determine the site of insertion-duplication mutagenesis, the respective strain of the mutagenic library was used as a template to amplify the cloned fragment with the primer pair LSV3 and LSV-4380rev. To recover wild-type strains that had lost the plasmid, mutants were streaked out on BHI agar plates without erythromycin and grown overnight at a permissive temperature. This step was reiterated at least four times, and complete curing of integrated plasmid was confirmed by growing single colonies on BHI agar plates with erythromycin at 30°C.

Construction of deletion mutants. In-frame deletions of *glpD* (lmo1293), lmo1538, and a species-specific region spanning from lmo1968 to lmo1974 (lmo1968-1974) were performed in parental strains Sv1/2a, EGD, and EGD-e, respectively, as described previously (42). To construct EGDΔ*glpD*, two fragments of 518 bp and 529 bp were amplified using the oligonucleotide pairs glpDa/glpDb2 and glpDc2/glpDd and ligated via the introduced BglII sites. Following nested PCR using the oligonucleotides glpD-nested1 and glpD-nested3 and the ligation mixture as a template, the resulting fragment was cloned into pLSV1 via BamHI and EcoRI, giving rise to pLSVΔ*glpD*. pLSVΔ*glpD* was transformed into *L. monocytogenes* EGD by electroporation, and erythromycin-resistant bacteria growing at 43°C harboring the chromosomally integrated plasmid were selected. Cointegrates were resolved as described above, and erythromycin-sensitive clones were screened by PCR to identify a mutant in which the second recombination step resulted in a deletion of *glpD* (lmo1293). For the deletion of the lmo1968-1974 gene cluster, a 906-bp downstream fragment of lmo1968 was amplified by using the oligonucleotides CAH1 and CAH2, and a second, 861-bp fragment, which is localized upstream of lmo1974, was amplified with oligonucleotides CAH3 and CAH4 from chromosomal DNA derived from strain EGD-e. The fragments were cut with BamHI-XhoI and XhoI-EcoRI, respectively, and cloned in one ligation step into pLSV1 via BamHI and EcoRI. The deletion mutant EGD-eΔ1968-1974 was then generated as described above. A similar strategy was chosen to delete lmo1538 using the oligonucleotides gkA to gkD. The resulting fragments were cut with BamHI and SalI, respectively, and cloned into pLSV101 in two steps. The correct orientation of the fragments was confirmed by PCR. *glpD* (lmo1293) was also deleted in mutant EGDΔ1538 using pLSVΔ*glpD* as described above, resulting in mutant EGDΔ1538Δ*glpD*. The gene deletions in all four mutants were confirmed by PCR analysis and sequencing.

Epithelial cell infection assays. A total of 2.5×10^5 Caco-2 cells per well were seeded in a 24-well culture plate and cultivated until infection. Cells were washed twice with phosphate-buffered saline (PBS) supplemented with Mg^{2+} Ca^{2+} (PBS-$Mg^{2+}Ca^{2+}$) and covered for 1 hour with 500 μl RPMI 1640 containing 1.5 μl of a bacterial culture grown overnight. The average multiplicity of infection (MOI) was calculated to range from 6 to 14. To test deletion mutants, glycerol stocks were thawed, and the bacteria were sedimented and washed twice with PBS and resuspended in 1 ml PBS. After the Caco-2 cells were washed once with PBS-$Mg^{2+}Ca^{2+}$, extracellular bacteria were removed by adding 0.5 ml RPMI 1640 containing 10 μg/ml gentamicin. After 7 hours of incubation, the infected Caco-2 cells were washed again with PBS-$Mg^{2+}Ca^{2+}$ and then lysed in 1 ml cold Triton X-100 (0.1%). Intracellular replication behavior of the mutants and the wild type was quantified by plating dilutions of the lysed cells on BHI agar plates that were incubated at 42°C for 1 day. If appropriate, the plates contained 5 μg/ml erythromycin. To examine adhesion properties of bacterial strains, the infection time was reduced to 35 min, and before lysis, cells were washed four times with PBS-$Mg^{2+}Ca^{2+}$. The capability of bacterial cells to invade Caco-2 cells was investigated as described above, but lysis of the epithelial cells was performed after 1 hour, and a higher gentamicin concentration of 50 μg/ml was used.

RNA isolation. Since the two samples to be compared in this study were not homogeneous, they were treated as follows to make them comparable. (i) Eight 250-ml tissue culture flasks with confluent Caco-2 cells were infected with *L. monocytogenes* at an MOI of 20. Caco-2 cell infection assays were performed as described above. Cells were lysed 6 h postinfection with cold distilled water. Mammalian cell debris was removed by centrifugation at 1,000 × *g* for 10 min at 4°C, leaving only the bacteria in the supernatant. Bacterial numbers were determined by plating dilutions on BHI agar plates. Bacteria were pelleted at 6,000 × *g* for 10 min at 4°C, shock frozen in liquid nitrogen, and stored at −70°C for RNA isolation. (ii) *L. monocytogenes* bacteria were grown in BHI to an optical density at 600 nm of 1.0 corresponding to the late logarithmic phase. To normalize the background resulting from Caco-2 cell debris, eight 250-ml flasks of confluent Caco-2 cells were lysed with cold distilled water. After the addition of an appropriate number of cells corresponding to that of the infected pellets, the suspension was treated in the same way as described above. RNA from these two samples was extracted using the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol with some modifications to lyse the bacteria. Cell pellets were suspended in lysis buffer and placed in a 2-ml BLUE tube, filled with silica-sand (Bio 101 Inc., La Jolla, CA). The tube was shaken three times for 45 s each time with a 1-minute interval on ice between each shaking at a speed setting of 6.5 in a bead beater FP120 FastPrep cell disrupter (Savant Instruments, Inc., Farmingdale, NY). Residual DNA was removed on a column with QIAGEN RNase-free DNase (QIAGEN, Hilden, Germany).

Microarray hybridization and data analysis. Transcriptome analyses were performed using whole-genome DNA microarrays that contained synthetic 70-mer oligodeoxyribonucleotides covering all open reading frames of the *L. monocytogenes* genome. The oligonucleotides (Operon Co.) were spotted on epoxy-coated glass slides from Quantifoil according to the manufacturer's instructions by T. Chakraborty (Institut für Medizinische Mikrobiologie, Giessen, Germany). Each oligonucleotide was spotted twice on a slide to generate two replicates for each oligonucleotide on a slide. A total of six RNA samples were prepared for cDNA labeling and hybridization. Briefly, equal amounts (40 μg) of the RNAs were used to synthesize cDNA differentially labeled with Cy3-dCTP and Cy5-dCTP (Amersham Pharmacia, Freiburg, Germany) during a first-strand reverse transcription reaction with Superscript II RNase H^- reverse transcriptase and 9 μg random primers (Life Technologies, Karlsruhe, Germany). Dye swap was performed as follows. Three intracellular listerial cDNA samples were generated using Cy3-dCTP, and the other three were generated using Cy5-dCTP. The two cDNA samples were combined, diluted with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) sodium dodecyl sulfate, hybridized to the microarray, and incubated at 50°C for 16 h. After the slides were washed, they were scanned using ScanArray HT and analyzed using the ScanArray express software (Perkin-Elmer, Boston, MA). Spots were flagged and eliminated from analysis when the signal-to-noise ratio was less than three or in obvious instances of high background or stray fluorescent signals. The LOWESS method of normalization (47) was performed on the background-corrected median intensity of the spots. The normalized ratios were analyzed further with Microsoft Excel (Microsoft, Redmond, WA) and SAM (significance analysis of microarrays) software for statistical significance (44) (see Table S2 in the supplemental material). The Δ value was adjusted to 2.1, which results in false significant median of 0.29 (see Table S3 in the supplemental material). To determine the significance of differential expression, RNA was isolated from *L. monocytogenes* grown in BHI, and 30 to 40 μg of this RNA was labeled either with Cy3-dCTP or with Cy5-dCTP. The two cDNA probes generated were hybridized onto the same slide, and the data were analyzed as mentioned above. Since the values spread from 0.6 to 1.6, we considered values >1.6 and <0.6 to be significant in our experiments. To exclude the possibility of cross hybridization to host cell RNA, one slide was hybridized with Cy3-dCTP-labeled cDNA derived from Caco-2 cell RNA and with Cy5-dCTP-labeled cDNA obtained from *L. monocytogenes* grown in BHI, and the other slide was hybridized with the same cDNA but with the dyes swapped. No significant signal intensity was detected using the cDNA from Caco-2 cells (data not shown).

Real-time RT-PCR. Real-time reverse transcriptase PCR (RT-PCR) was conducted on total RNA isolated independently from that used for transcriptome analysis experiments. Before real-time RT-PCR was performed, the absence of DNA from RNA samples was verified by PCR amplification of the genes to be assayed with 1 μg RNA as the template. cDNA synthesis was performed as described above from 5 μg total RNA. Instead of the labeled nucleotides, equal amounts (20 mM) of dATP, dCTP, dGTP, and dTTP were used. Real-time

RT-PCR in a final volume of 20 μl was carried out in a MJ Research PTC-200 cycler according to the manufacturer's protocol of the qPCRCore kit for SYBR green-I (Eurogentec, Liege, Belgium). The primers used for real-time RT-PCR are listed in Table S1 in the supplemental material.

Statistical analysis for overrepresentation of genes identified by microarray analysis and insertion-duplication mutagenesis. The average knockout fraction (F) of the mutant library was determined to be 16% using the general features of the *L. monocytogenes* genome (16) and the formulas of Lee and coworkers (24) [$q = (G - I)/L$ and $F\ (\%) = 1 - (1 - q)^n$, where G is the average size of a gene (918 bp), I is the insert size (244 bp), L is the genome size of *L. monocytogenes* (2,944,528 bp), F is given as a percentage, and n is the size of the library (760 mutants)]. We extrapolated the 16 overlapping genes to a library saturation of 100%, resulting in an estimated number of 100 attenuating mutant genes in the group of up-regulated genes. Seventy-two mutant genes out of 456 genes (16% saturation) extrapolated to 450 mutants out of 2,853 genes (100% saturation), and 279 up-regulated genes from 2,853 genes gives a probability of 0.0153 [(450/2,853) × (279/2,853) = 0.158 × 0.097] of genes which overlap between the two techniques by chance (2,853 × 0.0153 = 43.6). To the expected number of 43.6 genes and the observed number of 100 overlapping genes (extrapolated), we applied the test for binominal distribution and the chi-square test, and both tests rejected the null hypothesis ($P < 0.01$), i.e., the overrepresentation is significant.

RESULTS

Global transcriptional profile of *L. monocytogenes* in epithelial cells. We compared the expression profile of *L. monocytogenes* grown in BHI (RNA_{BHI}) with that of *L. monocytogenes* grown in the cytosol of Caco-2 cells (RNA_{Caco}). RNA was isolated and purified from *L. monocytogenes* EGD either grown in vitro or intracellularly, and the differentially labeled RNAs (RNA_{Caco}/RNA_{BHI}) were then hybridized against whole-genome microarrays containing oligonucleotides of all genes identified in the genome sequence of *L. monocytogenes* EGD-e (16). We chose to isolate RNA 6 hours after infection, a time point that reflects active intracellular replication as shown previously (14). In total, the statistical analysis using SAM software (44) identified 541 genes with transcriptional alterations during intracellular growth of *L. monocytogenes*. We observed 259 listerial genes whose expression is down-regulated in intracellular *L. monocytogenes* compared to those cultivated in BHI. Most noticeable is the strong repression of genes involved in glycolysis, namely, *eno* (3.3-fold), lmo2460 (3.3-fold), *pfk* (2.5-fold), *pgk* (2.5-fold), *pgm* (2-fold), *pykA* (2.5-fold), and *tpi* (2.5-fold), while the other repressed genes mainly encode functions involved in DNA replication, recombination, transcription, protein biosynthesis, cell wall synthesis, and energy metabolism. The down-regulation of these listerial genes most likely reflects the slower growth of *L. monocytogenes* in Caco-2 cells (generation time about 40 min) compared to that in BHI (25 min) (34). The differentially up-regulated genes were grouped into several categories according to their cellular functions, such as virulence, carbon and nitrogen metabolism, stress response, regulation, and those with unknown functions (Table 2 and Fig. 1).

To verify the microarray transcription profiling data, we conducted real-time RT-PCR analysis of a sample of 15 genes, including genes encoding virulence factors and genes encoding components of specific metabolic pathways. Real-time RT-PCR identified induced transcription of *plcA*, *mpl*, *actA*, and *plcB*, and the values obtained correlated well with the respective microarray data (Fig. 2). Qualitative correlation between hybridization experiments and real-time RT-PCR was shown for two genes of a species-specific gene cluster (lmo1971 and lmo1974), for *glnA* encoding glutamine synthetase (GS), for a regulatory gene (lmo1517) encoding a potential activator for GS, for lmo1734 that codes for a glutamate synthase subunit, and for a gene putatively involved in sugar uptake (lmo0784). The real-time RT-PCR analysis led to a confirmation of the microarray-derived expression data of the investigated genes with a high level of concordance ($r = 0.80$), despite quantitative variations between the results of the two applied techniques as reported earlier (40).

Identification of mutants with impaired cytosolic replication efficiency. To further validate the biological relevance of the derived pattern of listerial genes with induced expression in Caco-2 cells, a random mutant library of *L. monocytogenes* was constructed by insertion-duplication mutagenesis (23) and screened for intracellular-growth-deficient strains. The stability of plasmid pLSV101 inserted into the chromosome in these mutants during infection of Caco-2 cells was assured by plating aliquots of lysed cells from five independent infection assays on BHI agar plates with and without erythromycin, but the numbers of viable listeriae did not differ significantly (data not shown). Approximately 760 nonredundant insertion mutants that represent approximately 13% of all listerial genes (25) were tested in an epithelial cell infection assay. Mutants that exhibited attenuated intracellular replication behavior in at least three independent experiments were further characterized. To distinguish between gene knockouts that affect cell entry and gene knockouts that affect intracellular proliferation, the abilities of mutants to adhere to and to invade epithelial cells were investigated. All mutants but one, a *inlA*(*1724*) mutant carrying pLSV101, were not significantly affected in their invasion capability. To genetically characterize the attenuated mutants, the respective clones of the mutant library were used as a template for PCR amplification, and the fragment was subjected to sequence analysis. By comparison with the *L. monocytogenes* EGD-e genome sequence, the obtained nucleotide sequences revealed the chromosomal sites that had been targeted by homologous recombination in the respective mutants. We identified 72 insertions that did not affect the growth of the mutants in BHI medium at 37°C (data not shown). To confirm that no mutation independent from the site-specific insertion is responsible for the observed attenuated phenotype, 15 insertion mutants were reverted to the wild-type genotype upon growth at 30°C for 10 generations and without antibiotic. All vector-cured strains were shown to behave as the wild-type strain does in the infection assay. We then interfaced the 72 gene loci that led to impaired listerial growth in the cytosol of Caco-2 cells with the genes found to be up-regulated in mammalian host cells by transcriptome analysis and real-time RT-PCR. Interestingly, 16 of these genes were also shown to be intracellularly up-regulated (Table 2). This overrepresentation of genes with increased transcription among the group of attenuated genes proved to be significant (Materials and Methods). The replication rates of 15 of the respective insertional mutants within Caco-2 cells in comparison to the wild-type strain are shown in Fig. 3.

Investigation of polar effects resulting from insertional gene knockout. Insertional mutagenesis has the potential for undesired side effects, such as polar termination-induced reduction of downstream gene expression. Four of the 16 genes identified by both approaches (lmo1244, lmo1538, lmo2434, and *gidA*)

TABLE 2. Interfacing transcriptome analysis with attenuated insertion-duplication mutagenesis mutants

Category	O[a]	Gene	Function	RTL[b] (fold)	IR[c] (%)
Virulence genes		lmo0200 (*prfA*[d])	Listeriolysin-positive regulatory protein	3.1	
		lmo0201 (*plcA*[d])	Phosphatidylinositol-specific phospholipase C	3.1	
		lmo0202 (*hly*[d])	Listeriolysin O precursor	9.4	
		lmo0203 (*mpl*[d])	Zinc metalloproteinase precursor	5.2	
		lmo0204 (*actA*[d])	Actin assembly-inducing protein precursor	18.2	
		lmo0205 (*plcB*[d])	Phospholipase C	9.8	
		lmo0433 (*inlA*[d])	**Internalin A**	**5.6**	**0.0035**
		lmo0433 (*inlB*[d])	Internalin B	3.9	
		lmo1786 (*inlC*[d])	Internalin C	5.7	
Transporters					
PTS systems		lmo0097	Similar to mannose-specific enzyme IIC	1.8	
		lmo0299	Similar to β-glucoside-specific enzyme IIB	2.0	
	↑	lmo0782	Similar to mannose-specific enzyme IIC	*2.4*	
	↑	lmo0783[f]	Similar to mannose-specific enzyme IIB	2.7	
	↑	lmo0784	Similar to mannose-specific enzyme IIA	*1.7*	
	↑	lmo1969[d]	Similar to 2-keto-3-deoxygluconate-6-phosphate aldolase	2.7	
	↑	**lmo1971[d]**	**Similar to pentitol-specific enzyme IIC**	**4.3**	**25.1**
	↑	lmo1972[d]	Similar to pentitol-specific enzyme IIB	2.2	
	↑	lmo1973[d]	Similar to PTS system enzyme IIA	2.0	
	↑	lmo1974[d]	Similar to transcription regulators (GntR family)	2.3	
		lmo2000	Similar to PTS mannose-specific enzyme IID	2.0	
		lmo2335 (*fruA*)	Highly similar to fructose-specific enzyme IIABC	2.3	
		lmo2665	Similar to PTS system galactitol-specific enzyme IIC	2.5	
		lmo2762[f]	Similar to PTS cellobiose-specific enzyme IIB	2.7	
ABC transporters		lmo0155	Similar to high-affinity zinc ABC transporter (membrane protein)	2.2	
		lmo0541	Similar to ABC transporter (binding protein)	1.7	
		lmo1422	Similar to glycine betaine/carnitine/choline ABC transporter (membrane protein)	2.8	
	↓	lmo1427 (*opuCA*)	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	2.6	
	↓	lmo1428 (*opuCB*)	Similar to glycine betaine/carnitine/choline ABC transporter (membrane protein)	2.3	
		lmo1652	**Similar to ABC transporter (ATP-binding protein)**	**1.9**	**22.8**
		lmo1960 (*fhuC*)	**Similar to ferrichrome ABC transporter (ATP-binding protein)**	**2.5**	**20.1**
		lmo2182	Similar to ferrichrome ABC transporter (ATP-binding protein)	2.2	
		lmo2196[f]	Similar to pheromone ABC transporter (binding protein)	*1.9*	
		lmo2251	Similar to amino acid ABC transporter (ATP-binding protein)	2.1	
		lmo2751	Similar to ABC transporter, ATP-binding protein	2.0	
Other transporters		lmo0838 (*hpt*[d,f])	Highly similar to hexose phosphate transport protein	4.5	
		lmo1516[f]	Similar to ammonium transporter NrgA	2.3	
		lmo1539	Similar to glycerol uptake facilitator	*1.9*	
		lmo2469	Similar to amino acid transporter	2.4	
		lmo2818	**Similar to transmembrane efflux protein**	**1.7**	**38**
C metabolism					
PP cycle[g]		lmo0499[f]	Similar to ribulose-5-phosphate-3-epimerase	*2.1*	
		lmo2660	Similar to transketolase	4.9	
		lmo2674	Similar to ribose-5-phosphate epimerase	2.3	
Gluconeogenesis		lmo2094	Similar to L-fuculose-phosphate aldolase	2.0	
		lmo2336 (*fruB*[f])	Fructose-1-phosphate kinase	2.1	
Glycolysis		lmo1570 (*pykA*)	Highly similar to pyruvate kinases	0.4	
		lmo1571 (*pfk*)	Highly similar to 6-phosphofructokinase	0.4	
		lmo2455 (*eno*)	Highly similar to enolase	0.3	
		lmo2456 (*pgm*)	Highly similar to phosphoglycerate mutase	*0.5*	
		lmo2457 (*tpi*)	Highly similar to triose phosphate isomerase	0.4	
		lmo2458 (*pgk*)	Highly similar to phosphoglycerate kinase	0.4	
		lmo2460	Similar to *B. subtilis* CggR hypothetical transcriptional regulator	0.3	
Other		lmo1034	Similar to glycerol kinase	2.1	
	↓	lmo1174 (*eutA*)	Similar to ethanolamine utilization protein EutA (putative chaperonin)	1.8	
	↓	**lmo1175 (*eutB*[e,f])**	**Similar to ethanolamine ammonia-lyase, heavy chain**	**1.8**	**11.2**
	↓	lmo1176 (*eutC*[e])	Similar to ethanolamine ammonia-lyase, light chain	2.3	
		lmo1538	**Similar to glycerol kinase**	**2.2**	**23.1**
		lmo1969	Similar to 2-keto-3-deoxygluconate-6-phosphate aldolase	2.7	
		lmo2205	Similar to phosphoglyceromutase 1	3.8	

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TABLE 2—*Continued*

Category	O[a]	Gene	Function	RTL[b] (fold)	IR[c] (%)
Anabolic pathways					
Amino acids		lmo1299 (*glnA*[f])	Highly similar to glutamine synthetases	2.7	
		lmo1591 (*argC*)	Similar to *N*-acetylglutamate γ-semialdehyde dehydrogenases	3.7	
	↓	lmo1628 (*trpB*)	Highly similar to tryptophan synthase, β subunit	1.9	
	↓	lmo1630 (*trpC*)	Highly similar to indol-3-glycerol phosphate synthases	2.1	
	↓	lmo1632 (*trpG*)	Highly similar to anthranilate synthase β subunit	2.0	
	↓	**lmo1983 (*ilvD*[f])**	**Similar to dihydroxy-acid dehydratase**	**1.7**	**18.2**
	↓	lmo1984 (*ilvB*)	Similar to acetolactate synthase (acetohydroxy-acid synthase), large subunit	1.9	
	↓	lmo1985 (*ilvN*)	Similar to acetolactate synthase (acetohydroxy-acid synthase), small subunit	3.0	
	↓	lmo1986 (*ilvC*)	Similar to ketol-acid reductoisomerase (acetohydroxy-acid isomeroreductase)	2.0	
		lmo1991 (*ilvA*)	Similar to threonine dehydratase	2.0	
		lmo2825 (*serC*)	Highly similar to phosphoserine aminotransferase	*2.6*	
		lmo2434	**Similar to glutamate decarboxylases**	**2.0**	**49.6**
Co enzymes	↓	lmo1202 (*cbiK*[e,f])	Similar to anaerobic cobalt chelatase in cobalamin biosynthesis	*2.7*	
	↓	lmo1203 (*cbiL*[e])	Similar to *S*-adenosyl-methionine:precorrin-2 methyltransferase	2.3	
	↓	lmo1204	Similar to cobalamin biosynthesis protein M	2.4	
	↓	lmo1147	Similar to bifunctional cobalamin biosynthesis protein CopB (cobinamide kinase, cobinamide phosphatase guanylyltransferase)	3.0	
		lmo2022	**Unknown, similar to a NifS-like protein required for NAD biosynthesis**	**1.8**	**33.9**
Nitrogen metabolism		lmo1299 (*glnA*)	Highly similar to glutamine synthetases	2.7	
		lmo1517	Similar to nitrogen regulatory PII protein	1.6	
	↑	lmo1733	Similar to glutamate synthase, small subunit	2.8	
	↑	lmo1734	Similar to glutamate synthase, large subunit	3.3	
	↑	lmo1735 (*gltC*)	Transcription activator of glutamate synthase operon GltC	2.5	
	↓	lmo1591 (*argC*)	Similar to *N*-acetylglutamate γ-semialdehyde dehydrogenases	3.7	
	↓	**lmo1588 (*argD*)**	**Similar to *N*-acetylornithine aminotransferase**	**2.0**	**31.5**
Stress response		lmo1473 (*dnaK*)	Class I heat shock protein (molecular chaperone) DnaK	2.1	
		lmo2068 (*groEL*)	Class I heat shock protein (chaperonin) GroEL	2.4	
		lmo1474 (*grpE*)	Heat shock protein GrpE	2.5	
		lmo1138	Similar to ATP-dependent Clp protease proteolytic component	*2.5*	
Regulators		lmo1475 (*hrcA*)	Transcription repressor of class I heat shock gene HrcA	2.4	
		lmo0178[f]	Similar to xylose repressor	0.5	
		lmo0229	Highly similar to transcription repressor of class III stress genes (CtsR)	3.7	
		lmo0294	Similar to transcription regulator LysR-GltR family	2.0	
		lmo0459[f]	Similar to transcription regulator (VirR from *Streptococcus pyogenes*)	2.2	
		lmo0480[f]	Similar to putative transcriptional regulator	0.6	
		lmo0649	Similar to transcription regulators	0.5	
		lmo0806	Similar to transcription regulators	0.5	
		lmo0822[f]	Similar to transcription regulators	1.7	
		lmo0926	Similar to transcription regulators (TetR/AcrR family)	3.2	
		lmo0948	Similar to transcription regulators	2.4	
		lmo1150[e,f]	Regulatory protein similar to *Salmonella enterica* serovar Typhimurium PocR protein	*2.4*	
		lmo1172[e]	Similar to two-component response regulator	2.5	
		lmo1478	Similar to transcription regulators (MerR family)	2.0	
		lmo1788	Similar to transcription regulators	1.8	
		lmo1850	Similar to transcriptional regulators (MarR family)	2.3	
		lmo2086	Unknown, weakly similar to transcription regulators	1.9	
		lmo2088	Similar to transcription regulators	2.9	
		lmo2099	Similar to transcription antiterminator	0.5	
		lmo2328	Similar to transcription regulators	2.1	
		lmo2329	Similar to a putative repressor protein (bacteriophage A118)	2.3	
		lmo2337	Similar to regulatory protein (DeoR family)	3.8	
		lmo2441[f]	Similar to transcription regulators	2.4	
		lmo2460	Similar to *B. subtilis* CggR hypothetical transcriptional regulator	0.3	
		lmo2731	Similar to transcription regulator (RpiR family)	0.6	

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TABLE 2—*Continued*

Category	O[a]	Gene	Function	RTL[b] (fold)	IR[c] (%)
		lmo2739	Similar to regulatory proteins of the SIR2 family	0.6	
		lmo2814	Similar to transcriptional regulator	2.4	
		lmo2820	Unknown, amino-terminal domain similar to transcription regulators	3.3	
		lmo2827	Unknown, similar to transcriptional regulator (MarR family)	3.3	
		lmo2842[f]	Similar to transcriptional regulator	2.3	
		lmo2500 (*phoR*)	Two-component sensor histidine kinase	0.5	
		lmo2501 (*phoP*[f])	Two-component response phosphate regulator	0.4	
Miscellaneous		**lmo0090**	**Similar to ATP synthase α chain**	**2.1**	**36.3**
		lmo0705	**Similar to flagellar hook-associated protein FlgK**	***2.0***	**35.4**
		lmo2810 (*gidA*)	**Similar to GidA protein**	**2.1**	**23.3**
	↓	lmo1159[e]	Similar to carboxysome structural protein	2.1	
		lmo1160[e]	Similar to *Salmonella enterica* PduL protein	2.3	
		lmo1169 (*cobD*[e])	Similar to *S. enterica* serovar Typhimurium CobD protein and to histidinol-phosphate aminotransferase	*2.8*	
		lmo2206 (*clpB*)	Similar to endopeptidase Clp ATP-binding chain B (ClpB)	4.1	
		lmo1244	**Unknown, weakly similar to phosphoglycerate mutase 1**	**1.9**	**19.3**
		lmo2734	**Hypothetical protein**	**2.1**	**52.0**
Unknown		lmo0119	Unknown	2.3	
		lmo0207	Hypothetical lipoprotein	2.3	
		lmo0227	Unknown, conserved hypothetical protein	9.3	
		lmo0704	Unknown	3.2	
	↓	lmo0748[d]	Unknown	6.0	
		lmo0749[d]	Unknown	3.4	
		lmo0750[d]	Unknown	2.7	
		lmo0751[d]	Unknown	5.5	
	↓	lmo0752[d]	Unknown	3.6	
		lmo0759	**Unknown**	**1.7**	**35.5**
		lmo0819	Unknown	1.9	
		lmo0991	Unknown, conserved hypothetical protein	2.0	
		lmo0998[f]	Unknown	5.5	
		lmo1137	Unknown	2.4	
		lmo1140	Unknown	2.6	
		lmo1183[e]	Unknown	2.2	
		lmo1697	Similar to putative transmembrane proteins	2.9	
		lmo2066	Unknown	2.4	
		lmo2361	Unknown, conserved hypothetical protein	2.3	

[a] Arrows mark regulated genes probably organized in an operon, and the direction indicates the direction of transcription.
[b] RTL, relative transcript level. Numbers in italics indicate ratios that were determined by SAM not to be significant but showed a consistent up-regulation of ≥1.6.
[c] Mean percentage of intracellular replication (IR) and whose mutation led to attenuation describes the degree of attenuation of the respective mutant with respect to a control group of 107 insertion mutants (cf. Fig. 3). The genes that are intracellularly up-regulated, which are shown in bold type.
[d] Genes specific for *L. monocytogenes*.
[e] Genes clustered in a 55-kb fragment involved in vitamin B_{12} synthesis and the coenzyme B_{12}-dependent degradation of 1,2-propanediol and ethanolamine.
[f] Genes that contain a *cre* box in their promoter region with up to two mismatches to the consensus sequence reported recently (29).
[g] PP cycle, pentose phosphate cycle.

are monocistronically transcribed according to the listerial genome annotation. The remaining 13 genes are organized in known or putative operon structures, and exploration of the microarray data revealed at least one intracellularly up-regulated gene located downstream of six of the disrupted genes (lmo1652, lmo2022, *ilvD*, lmo1971, *eutB*, and *inlA*). Five other mutated genes encode subunits of a transporter (lmo2818), of a putative ATP synthase (lmo0090), or of the flagellar apparatus (lmo0705) or are followed by a gene that is probably involved in the same cellular pathway (lmo2734 and *argD*). Putative polar effects resulting from pLSV101 insertions into the chromosome were also investigated by real-time RT-PCR. First, we confirmed by RT-PCR that the lmo1968 to lmo1974 genes are polycistronically transcribed (data not shown). cDNA obtained from *L. monocytogenes* grown in BHI was used as a template for RT-PCR with five oligonucleotide pairs, and each pair amplified a DNA fragment that overlapped two or three genes of the operon (data not shown). RNAs from strain EGD-lmo1971::pLSV101 and the wild type were isolated and transcribed into cDNA using random hexamers, and the cDNA was used for real-time RT-PCR with oligonucleotides rt and rtRev specific for lmo1968. The results are shown in Fig. 4, indicating that an insertion of pLSV101 into lmo1971 does not significantly affect the transcription of the most distantly located gene of the putative species-specific operon lmo1974-1968 at least in vitro, possibly due to promoter elements present on pLSV101 (15).

Intracellular growth deficiencies of deletion mutants. Nonpolar and in-frame deletions of lmo1538 encoding a glycerol kinase and of the putative operon lmo1974-1968 were constructed as described above. To further investigate the contribution of glycerol metabolism to intracellular growth, the monocistronic gene *glpD* (lmo1293) encoding glycerol-3-phosphate-dehydogenase was deleted both in the wild-type strain EGD and in the mutant EGDΔ1538. The first six codons at the 5′ ends of *glpD* and lmo1538 (11 codons in lmo1974) and the

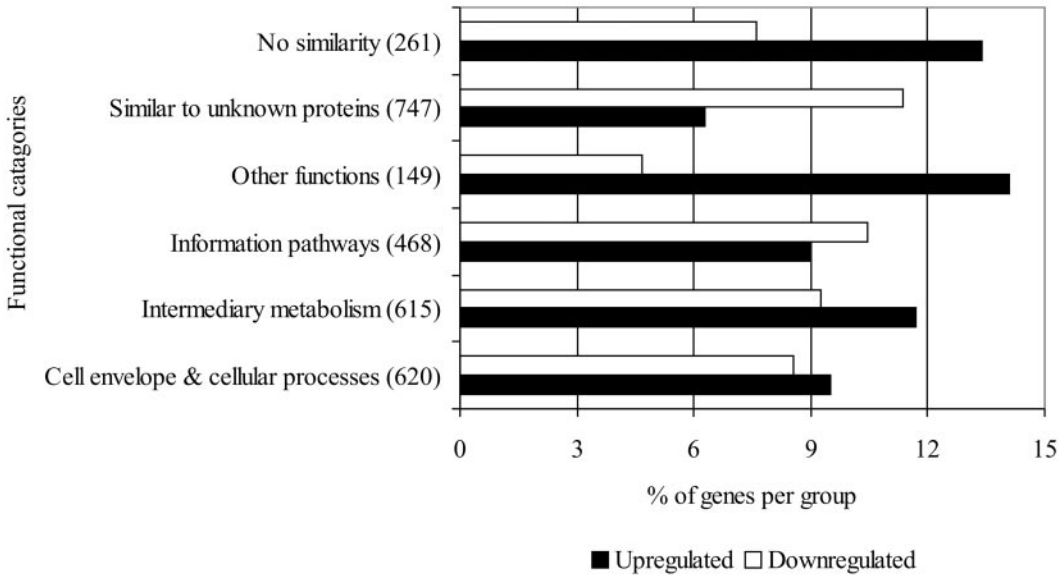

FIG. 1. Changes in expression of genes belonging to functional groups. The bars show the percentages of listerial genes in each group that exhibit altered expression inside epithelial cells. The white bars indicate the proportion of genes that are down-regulated, and the black bars represent the proportion of up-regulated genes for each functional group. The total number of genes per functional category is shown in parentheses and is equivalent to 100%.

last 12 codons at the 3′ ends of these genes (9 codons in lmo1968) were retained on the chromosome to exclude any effects of the deletions on the regulation of neighboring genes. When cultivated in liquid culture, mutants EGDΔ*glpD*, EGDΔ1538, EGDΔ1538Δ*glpD*, and EGD-eΔ1968-1974 grew at a rate indistinguishable from that of the wild-type strain (data not shown). All strains were then tested for their ability to grow in cultured Caco-2 cells. Significant intracellular growth deficiencies of the four deletion mutants were observed after 7 hours of infection. In comparison to the wild-type strain (100%), the two single mutants were less attenuated than the double mutant, which showed a survival rate (52.7%) similar to that of mutant EGD-eΔ1968-1974 (Fig. 5). These data confirmed the results of the infection assays and provide strong evidence that *glpD* (lmo1293), lmo1538, and the species-specific gene cluster lmo1968-1974 are required for the wild-type-like intracellular growth of *L. monocytogenes*.

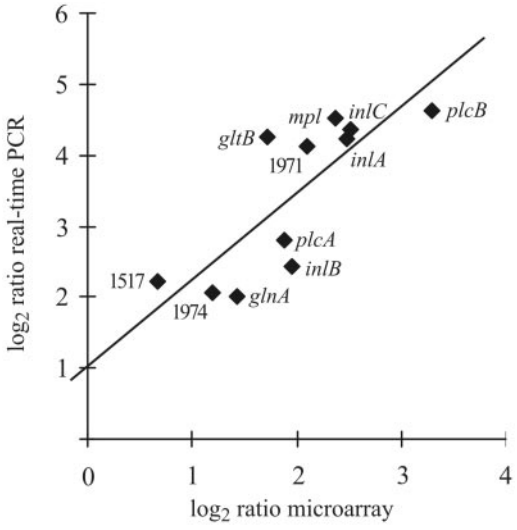

FIG. 2. Correlation of microarray and real-time RT-PCR analyses of selected genes. The changes in intracellular gene expression compared to that in BHI (RNA_{Caco}/RNA_{BHI}) were log transformed. The relative expression of the genes studied was normalized to the expression of the housekeeping gene *rpoB* as described elsewhere (28, 41). The genes studied included lmo1517 (1517), lmo1974 (1974), and lmo1971 (1971). Six independent real-time RT-PCR experiments were performed.

DISCUSSION

PrfA-dependent virulence genes. PrfA-regulated virulence genes are absolutely required for intracellular listerial replication, and they were also found to be highly up-regulated in *L. monocytogenes* growing in Caco-2 cells (Table 2). The observed alterations in the expression profiles of the PrfA-induced genes are in accordance with previous studies which analyzed the expression of virulence genes of *L. monocytogenes* grown inside mammalian cells by real-time RT-PCR or by using reporter gene fusions which also showed the transcriptional induction of several PrfA-controlled virulence genes, including *plcA*, *hly*, and in particular *actA* and *plcB* (3, 12). It is, however, interesting to note that in the cytosol of Caco-2 cells, transcription of *prfA* is up-regulated only 3.2-fold, while PrfA-dependent genes, such as *mpl*, *actA*, *plcB*, and *inlC*, showed much higher levels of transcriptional induction (Table 2 and Fig. 2). This finding could be explained by one or more of the following possibilities. (i) The *prfA* transcript(s) is rather unstable inside the host cells. (ii) Activation of PrfA by the cytosolic milieu of the mammalian cells is more important than increased PrfA production. (iii) PrfA functions as a catalyst, and thus, a small increase in PrfA protein level could still result in a large increase in PrfA-dependent transcripts. The latter assump-

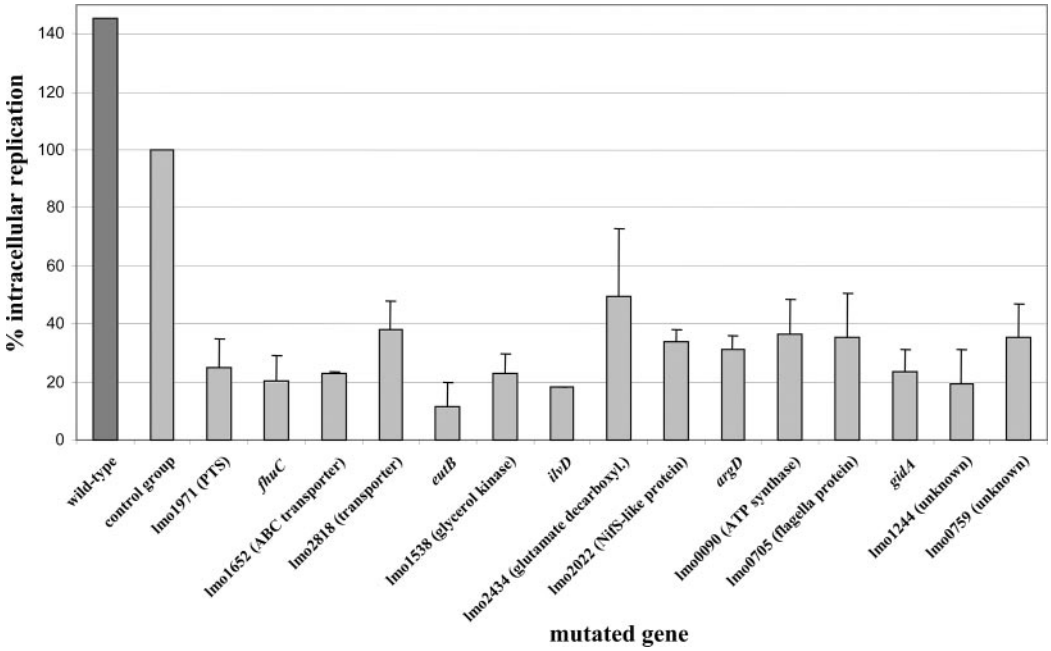

FIG. 3. Effects of insertional knockouts on the intracellular replication of *L. monocytogenes*. Caco-2 cells were infected with either the wild-type strain or insertional mutants as described in Materials and Methods. Mutated genes are in the same order as in Table 2, and putative functions are indicated in parentheses. The number of CFU recovered after 7 hours was determined, and reduced survival of the mutants was calculated as a percentage compared to the control group. The control group comprised 107 insertional mutants whose intracellular growth was not significantly affected. Error bars represent the standard deviations from the mean ($n \geq 3$ for insertional mutants; $n = 13$ for the wild type). The significance level was ≤ 0.02 according to Student's t test. lmo2434 encodes a glutamate decarboxylase (glutamate decarb.).

tion is supported by the previous demonstration that up-regulation of *prfA* transcripts is essential for *L. monocytogenes* in the cytosol (13).

Previous in vitro studies (28) have shown that PrfA especially in its constitutively active PrfA* form (33) can induce, in addition to the PrfA-dependent virulence genes, transcription of a large number of other (mainly SigB-dependent) genes. It is interesting to note that induction of many of these genes was not observed in the intracellular milieu, indicating that the bacterium adapts to this environment in a different manner.

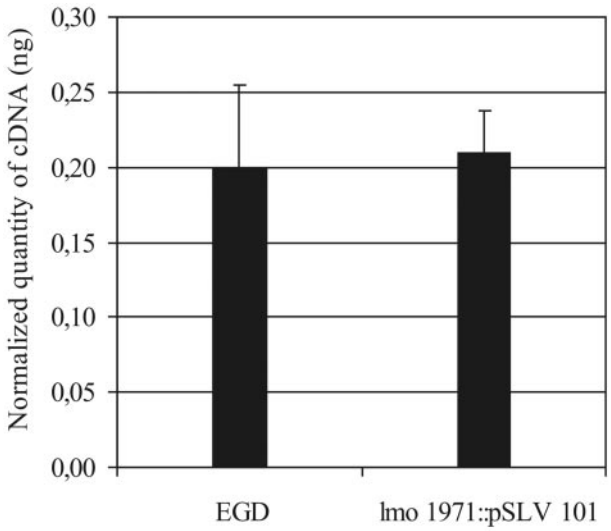

FIG. 4. Real-time RT-PCR to study the effects of pLSV101 insertion on the transcription of genes located downstream. cDNAs obtained from wild-type strain EGD (Wt Egd) and mutant EGD-lmo1971::pLSV101 (lmo 1971 :: pLSV 101) were used as a template for real-time RT-PCR with oligonucleotides specific for lmo1968. The quantity of lmo1968-specific cDNA was normalized to the quantity of *rpoB* cDNA, indicating that the concentration of lmo1968-specific cDNA and thus mRNA is not reduced in the mutant strain.

Genes responsible for uptake and utilization of carbon sources. We identified several up-regulated genes coding for ABC transporters and enzymes of the PTS and other transport systems that are required for the uptake and utilization of carbon sources (Table 2). The importance of these genes for intracellular replication of *L. monocytogenes* inside the host cell's cytosol is further shown by the fact that mutations in some of these genes reduce the efficiency of listerial growth in epithelial cells. Some of these genes seem to be under carbon catabolite repression (CCR) control, since their up-regulation is also observed in *ccpA* and *hprK* mutants (S. Mertins, unpublished data). The presence of catabolite-responsive elements (*cre*) in the promoter regions of two genes identified in this study, namely, lmo0783 and lmo2762, encoding mannose-specific and cellobiose-specific PTS enzymes, indicates that part of the listerial metabolism is released of CCR control when the bacteria replicate in the host cell cytosol (Table 2). Together with the down-regulation of the glycolysis genes (Fig. 1) and the up-regulation of *hpt*, this observation strengthens the assumption that the level of free glucose is low and glucose is not a predominant carbon source for listeria inside host cells. This is in line with our observation that the use of glucose, in addition to weakening the host cell's own carbon supply, would lead to the counterproductive inactivation of PrfA (Mertins,

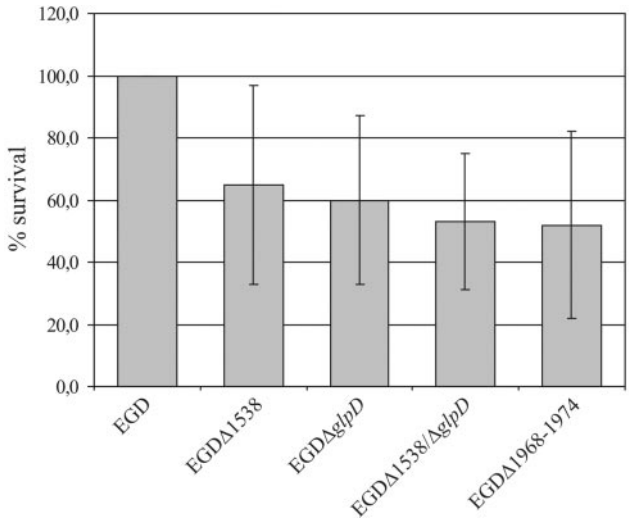

FIG. 5. Effects of nonpolar deletions of *glpD* (lmo1293), lmo1538, and lmo1968-1974 on the intracellular replication of *L. monocytogenes*. Caco-2 cells were infected with either the wild-type strain (EGD) or the mutants to an MOI of 6 to 14, and the numbers of bacteria recovered after 7 hours of infection were determined. Mutant strains with deletions of lmo1538 (Δ1538), *glpD* (Δ*glpD*), lmo1538 and *glpD* (Δ1538/Δ*glpD*), and lmo1968-1974 (Δ1968-1974) were studied. Eleven independent infections were performed for each strain. Error bars represent the standard deviations from the means. The application of the Student *t* test and the use of pair differences revealed significant differences in the replication efficiencies of the tested mutants from that of the wild-type strain.

unpublished), and it is also possible that listeria uses this low concentration of glucose as a signal to induce the PrfA regulon. Among the differentially regulated genes involved in C metabolism are remarkably many that are involved in the pentose phosphate cycle (Table 2). Their up-regulation provides evidence that the pentose phosphate cycle is the major catabolic pathway for the generation of necessary intermediates and for gluconeogenesis when carbon sources other than glucose or phosphorylated glucose are utilized. The induced transcription of the genes encoding glycerol kinases (lmo1034 and lmo1538) suggests that glycerol may also play a role as a carbon source for listerial growth in the cytosol of the host cells. The reduced growth of strains with insertion and deletion mutations of lmo1538 and *glpD* (lmo1293) seems to confirm this hypothesis (Fig. 5). This assumption is further strengthened by the strong up-regulation of *plcB* (9.8-fold) encoding a broad-spectrum phospholipase which could provide glycerol from host-derived phospholipids (Fig. 6A). The gene encoding a putative α-keto-deoxygluconate aldolase (lmo1969) known to be involved in the Entner-Doudoroff pathway is part of a gene operon specific for *L. monocytogenes* to which the genes lmo1973-1971 encoding a putative pentitol-specific PTS also belong. Their intracellular up-regulation, together with the finding that the EGD-lmo1971:pLSV101 and EGD-eΔ1968-1974 mutants showed significantly attenuated growth in Caco-2 cells (Table 2 and Fig. 2 to 4), demonstrate that the species-specific gene cluster lmo1968-1974 is required for wild-type-like intracellular replication of *L. monocytogenes*.

Another striking result of this study is the induction of 10 genes within the 55-kb gene cluster ranging from lmo1142 (*pduS*) to lmo1208 (*cbiP*), which encode the factors for coenzyme B_{12} synthesis and for the vitamin B_{12}-dependent degradation of 1,2-propanediol and ethanolamine (Table 2; also see Table S1 in the supplemental material). Vitamin B_{12} has been shown to be essential, together with ethanolamine, for the induction of the *eut* operon in *S. enterica* serovar Typhimurium (34, 35) under anaerobic conditions. The similar composition of the listerial *eut* operon suggests the same regulatory mechanism in *L. monocytogenes*. The induced transcription of the genes involved in vitamin B_{12} biosynthesis thus supports the idea that the use of ethanolamine as a C or N source might be critical for intracellularly growing *Listeria* bacteria (4). Furthermore, propanediol utilization via the *pdu* genes has been reported to play a role in the virulence of *S. enterica* serovar Typhimurium (4, 20). In our approach, we show the up-regulation of *eutABC* and the reduced replication efficiency of an insertional knockout of *eutB* in the cytosol of Caco-2 cells (Table 2), indicating that utilization of ethanolamine, probably deriving from phosphatidylethanolamine by the activity of PlcB (Fig. 6A), is indeed a requirement for replication of *L. monocytogenes* growing in mammalian cells.

Genes involved in anabolic pathways. Among the differentially regulated genes involved in anabolic pathways, we primarily observed up-regulation of genes involved in the synthesis of amino acids that *L. monocytogenes* bacteria, but not the host cells, are able to biosynthesize, notably tryptophan, isoleucine, leucine, valine, and arginine. The importance of isoleucine and valine biosyntheses for intracellular growth was confirmed by a threefold attenuation of an insertional *ilvD* mutant (Fig. 3). Recently, it was shown that CcpA regulates the expression of branched-chain amino acids in *Bacillus subtilis* (38, 42). The presence of a *cre* box in the promoter region of *ilvD* leads to the assumption that the synthesis of branched-chain amino acids might be regulated by CcpA. Although serine and glutamine as nonessential amino acids could be provided by the host cell, the up-regulation of key genes such as *glnA* for their biosynthesis in cytosolically grown *L. monocytogenes* suggests that the intracellular level of these amino acids is low and that cytosolically replicating listeriae depend on their biosynthesis for efficient intracellular growth (see below). Synthesis of arginine revealed another significant adaptation of listeriae to the host cell cytosol. The gene cluster *argCJBDF* of which two genes, *argC* and *argD*, were found to be intracellularly up-regulated by a factor of 3 and 1.9, respectively, is involved in the conversion of glutamate to ornithine, and an insertional mutation of *argD* led to a reduced replication rate in Caco-2 cells (Fig. 3).

Up-regulated genes required for nitrogen metabolism. *L. monocytogenes* lacks the genes for nitrate reduction (16) and is therefore entirely dependent on reduced nitrogen sources, such as amino acids or ammonia, for its nitrogen supply. Within the host cell cytosol, the amount of ammonia is low, as excess ammonia is lethal to mammalian cells. Therefore, it was not surprising to see the up-regulation of genes involved in the glutamine-glutamate synthesis pathway, such as *glnA* (lmo1299) and *gltAB* (lmo1733-lmo1734). The amino group of glutamate is the nitrogen donor for the biosynthesis of approximately 85% of the cell's nitrogenous compounds in the low-GC-containing bacterium *B. subtilis* (11), a fact that strengthens the assumption that high-level expression of GS is essential

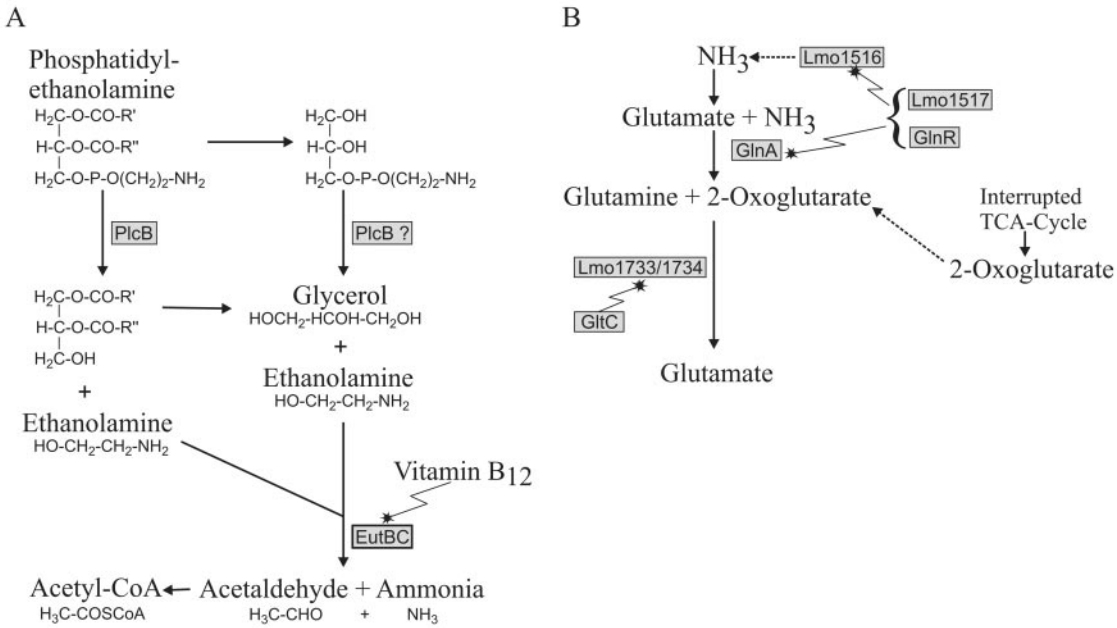

FIG. 6. (A) Proposed model for the interaction of phospholipases with *eut* genes to provide alternative C and N sources to intracellular *L. monocytogenes*. Phosphatidylethanolamine derived from phospholipids of host cell membranes could be broken down by the listerial phospholipase PlcB (which is highly active intracellularly) into glycerol and ethanolamine which then provides additional C or N sources by the action of the EutBC (ethanolamine ammonia-lyase). Acetyl-CoA, acetyl coenzyme A. (B) Proposed model for the regulation and fine tuning of nitrogen metabolism by *L. monocytogenes* for intracellular proliferation. TCA-cycle, tricarboxylic acid cycle.

for *L. monocytogenes* to proliferate in the intracellular environment. The importance of GS in the pathogenicity of *M. tuberculosis* (43) and *S. enterica* serovar Typhimurium (22) has been previously studied, and it was shown that deletion of GS resulted in reduced replication of these bacteria in human macrophages and guinea pigs. In this context, it is interesting to note that *L. monocytogenes* has an interrupted citrate cycle due to the absence of the gene for 2-oxoglutarate dehydrogenase (W. Eisenreich, personal communication), and accumulated 2-oxoglutarate could be channelled into the glutamate metabolic pathway (Fig. 6B).

Little is known about the regulation of nitrogen metabolism in *L. monocytogenes*. Nitrogen metabolism in gram-positive bacteria is regulated by the availability of rapidly metabolizable nitrogen sources, but not by any mechanism analogous to the two-component system NtrBC found in enteric bacteria (11). Of the three *B. subtilis* regulators involved in nitrogen metabolism, namely, TnrA, GlnR, and NrgB, homologues of only the latter two are present in *L. monocytogenes* and are encoded by *glnR* and lmo1517. The regulatory function of the homologue of lmo1517 (GlnK) in nitrogen metabolism of *Corynebacterium glutamicum* has been recently established (5). We propose that the product of lmo1517 has a similar function in regulation of nitrogen metabolism in *L. monocytogenes* along with GlnR and GltC (Fig. 6B). Thus, increased glutamine-glutamate synthesis regulated by GlnR, GltC, and the product of lmo1517 seems to be an adaptation by intracellular *L. monocytogenes* to proliferate in the cytosolic milieu.

Genes involved in stress response and gene regulation. The transcription of a number of listerial genes, such as *dnaK*, *groEL*, lmo1138, and *grpE*, genes that are known to be essential for overcoming bacterial stress, was found to be induced during intracellular growth like (Table 2). Their up-regulation suggests that their products help the bacteria to adapt to the harsh intracellular conditions. Of particular interest is the fourfold up-regulation of *clpB* encoding a subunit of the ATP-dependent Clp protease. This proteolytic enzyme in bacterial cells has been shown previously to be an important factor for efficient intracellular growth of *L. monocytogenes* (7, 30, 31, 36). By degradation of unnecessary proteins, Clp may provide amino acids, which could also serve as C and/or N sources for intracellularly growing *L. monocytogenes*. As expected, a large number of listerial genes encoding transcriptional regulators were found to be up- or down-regulated inside the host cells, reflecting the altered gene regulation during adaptation of *L. monocytogenes* to the intracellular environment. The induction of five of these genes is probably due to the release of CCR control. It is also worth mentioning that a substantial number of listerial genes of entirely unknown function were highly up-regulated in the intracellularly growing listeriae. The insertional knockout of one of these genes, lmo0759, resulted in a threefold-reduced intracellular proliferation with respect to the control group (Fig. 3). Four up-regulated genes, lmo0748-lmo0751, are part of a gene cluster specific for *L. monocytogenes*. The contribution of these genes to intracellular listerial replication deserves further investigation.

Concluding remarks. Despite extensive studies concerning the key steps in the life cycles of facultative intracellular pathogens, knowledge of the specific set of genes required for replication within the cytosolic environment is still rather limited. The up-regulation of all genes of the major virulence gene cluster, including *hly*, *plcA*, *mpl*, *actA*, *plcB* as well as the other

known PrfA-regulated genes, *inlA*, *inlB*, *inlC*, and *hpt*, is obviously the most prominent response of *L. monocytogenes* to the growth conditions of the host cell's cytosol. While most of these genes determine special functions essential for reaching the final destination in the infected cells (9, 45), the latter gene encodes a hexose phosphate transporter and was the first indication that specific physiological adaptation mechanisms may be essential for the successful colonization of the host cells by this intracellular pathogen (8).

Apart from the previously reported metabolic requirements for the growth of *S. enterica* serovar Typhimurium and *M. tuberculosis* in macrophages, this study identified differentially regulated genes in intracellular *L. monocytogenes* that belong to other functional categories. This is not too surprising, considering the entirely different compartments of the host cells in which these microorganisms multiply, namely, the cytosol versus specifically modified phagosomes. The present study provides the first, still rather incomplete view of the metabolism of an intracellularly growing bacterial pathogen. On the basis of these results, we would like to conclude that intracellular bacteria that are able to efficiently replicate inside host cells avoid competing with the host cell for its major carbon and nitrogen sources but rather take advantage of alternative carbon and nitrogen products of the host cells, such as phosphorylated glucose deriving from the host cell's glycogen (M. Beck, unpublished data) and ethanolamine derived from phospholipids. This strategy probably allows a longer survival of the host cell, in which the invading bacterium could survive and replicate for a longer period. In addition to the discussed metabolic adaptations of the bacteria by the host cell's cytosolic milieu, it should be considered that these bacteria will probably also induce functions during cytosolic growth which may modify host cell activities to favor their multiplication.

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