Transcriptomic Response of *Listeria monocytogenes* to Iron Limitation and *fur* Mutation^{∇†}

Nagender Ledala, Mrittika Sengupta, Arunachalam Muthaiyan,
Brian J. Wilkinson, and R. K. Jayaswal*

School of Biological Sciences, Illinois State University, Normal, Illinois 61790-4120

Received 14 June 2009/Accepted 5 November 2009

Iron is required by almost all bacteria, but concentrations above physiological levels are toxic. In bacteria, intracellular iron is regulated mostly by the ferric uptake regulator, Fur, or a similar functional protein. Iron limitation results in the regulation of a number of genes, especially those involved in iron uptake. A subset of these genes is the Fur regulon under the control of Fur. In the present study, we have identified Fur- and iron-regulated genes in *Listeria monocytogenes* by DNA microarray analysis using a *fur* mutant and its isogenic parent. To identify genes regulated exclusively in response to iron limitation, the whole-genome transcriptional responses to the iron limitation of a *fur* mutant and its isogenic parent were compared. Fur-regulated genes were identified by comparing the transcriptional profile of the parent with the transcriptional profile of the isogenic *fur* mutant. Our studies have identified genes regulated exclusively in response to iron and those that are negatively regulated by Fur. We have identified at least 14 genes that were negatively regulated directly by Fur. Under iron-limited conditions, these genes were upregulated, while the expression of *fur* was found to be downregulated. To further investigate the regulation of *fur* in response to iron, an ectopic *fur* promoter-*lacZ* transcriptional fusion strain was constructed, and its isogenic *fur* and *perR* mutant derivatives were generated in *L. monocytogenes* 10403S. Analysis of the iron limitation of the *perR* mutant indicated that the regulation of genes under the negative control of Fur was significantly inhibited. Our results indicate that Fur and PerR proteins negatively regulate *fur* and that under iron-limited conditions, PerR is required for the negative regulation of genes controlled by Fur.

Iron is an important nutrient required by almost all bacteria and is an abundant element in nature. However, it is not readily available for bacteria to use, especially under aerobic conditions because iron is present in the insoluble Fe³⁺ form. In the human host, free iron is present in almost negligible concentrations, as it is sequestered by high-affinity proteins such as albumin, transferrin, and lactoferrin, which are also potential sources of iron for many pathogenic bacteria (5, 25). It has been repeatedly demonstrated that the presence of Fur as well as the iron acquisition ability of bacteria are important requirements for survival in the host (23, 48). In order to acquire iron from diverse sources, bacteria have evolved various mechanisms (26). However, iron uptake needs to be tightly controlled since excess levels of iron can generate toxic hydroxyl radicals by the Fenton reaction.

In most bacteria, iron uptake regulation is under the control of Fur or a similar protein of equivalent function (1). When iron is available to the bacteria in adequate levels, Fur complexes with iron and represses several genes by binding to their promoters at a consensus nucleotide sequence, the Fur box (6, 21). Some studies have shown that Fur can function as a repressor even in the absence of iron (20, 33). Moreover, for some bacteria, the Fur protein was identified as being a positive regulator (14, 16).

In order to identify genes under the control of Fur, methods such as systematic evolution of ligands by exponential enrichment (SELEX) and Fur titration assay (FURTA) have been developed. The recent use of DNA microarrays has greatly improved our understanding of the role of Fur and iron in bacteria (2, 12, 17). However, little is known about iron- and Fur-regulated genes in *Listeria monocytogenes*.

L. monocytogenes is a Gram-positive, rod-shaped, facultative, intracellular pathogen that can cause serious food-borne illness in humans (10, 13, 29, 46). In the host environment, the availability and acquisition of iron are important for the survival of this bacterium (11, 52). In *L. monocytogenes*, Fur is understood to be the regulator of iron acquisition systems that help to utilize diverse iron sources, such as transferrin, ferritin, lactoferrin, hemin, catecholamine, and siderophores, produced by other bacteria (28). We have recently cloned, purified, and characterized the Fur protein from *L. monocytogenes*. Our studies showed that, *in vitro*, the purified Fur protein binds to Fur box-containing promoters (P_{fur} and P_{fnuc}) without iron, and *fur* is expressed in the presence of iron (33). Studies from other laboratories have shown that *fur* regulates virulence genes and almost all iron acquisition genes in this bacterium, while the expression of *fur* seems to be negatively regulated by PerR (41, 47). Although few roles for Fur and iron in *L. monocytogenes* have been described, the independent functions of these two factors are not completely known.

In this study, *L. monocytogenes* EGDe and its isogenic *fur* deletion mutant were compared for genome-wide expression changes in response to iron limitation. The implementation of this approach helped to identify genes that were regulated

* Corresponding author. Mailing address: School of Biological Sciences, Illinois State University, Normal, IL 61790-4120. Phone: (309) 438-5128. Fax: (309) 438-3722. E-mail: drjay@ilstu.edu.

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

[∇] Published ahead of print on 20 November 2009.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or primer sequence (5'→3') ^a	Source or reference
Strains		
<i>E. coli</i> JM109	<i>recA1 supE44 hsdR17 gyrA96 relA1 thi (lac-proAB) F'</i> (<i>traD36 proAB⁺ lacI^qZ M15</i>)	This study
<i>L. monocytogenes</i>		
10403S	Serotype 1/2a	This study
Pfl	10403S P _{<i>fur</i>} :: <i>lacZ fur⁺</i>	This study
Pf-1	PFL <i>fur</i> :: <i>pAUL-A fur</i> mutant	This study
Pf-2	PFL <i>perR</i> :: <i>pAUL-A perR</i> mutant	This study
EGDe	Serotype 1/2a	47
EGDeΔ <i>fur</i>	<i>fur</i> deletion mutant of EGDe	47
Plasmids		
pHK77	Integratable <i>lacZ</i> fusion vector	31
pHK77-F	720-bp upstream region of <i>fur</i> cloned into pHK77	This study
pIntFM	pAUL-A containing 275-bp <i>fur</i> internal PCR fragment	This study
pIntPM	pAUL-A containing 275-bp <i>perR</i> internal PCR fragment	This study
Primers		
514F	TATGCGCAAACTCCATGAA	
514R	CTTTGAGTCCTGCAGCATTTT	
FB1F	CGCTTGTTTTACCATACGGAG	
FB1R	CCATAAAGTTTTAAGCGATGGC	
HK091	GTTTCCCCTCGAATTC	
HKlacZ	TTCTGGTGCCGAAACCAG	
HKFF	GCTAGCGTTGTGCTTTAATGCGTCCA	
HKFR	GGTACCGGATGGCAGGAAATATTTGG	
Paf-F	GCAAATGGTACCTGCACCTGACACTGGCCT	
Paf-R	TGAATCGGAGCTCCCACTTCGATTTCGACGATTT	
PaP-F	CCTGAAAGCTTTTAACTCACATACACACCCAACC	
pap-R	TGAATGGATCCATATCCGGTCATATGTGCTGC	
Svpa1F	AGATGCAATTAGTTATGATCACTGGT	
Svpa1R	AGATGCAATTAGTTATGATCACTGGT	
qRT-PCR primers		
16S rRNART-F	CCCTTATGACCTGGGCTACA	
16S rRNART-R	CCTACCGACTTCGGGTGTTA	
lmo0485RT-F	CGTATTGCACACACTTGCTG	
lmo0485RT-R	CCTGCTTTTAAATCCGCAAT	
lmo1956RT-F	TGGCCTTGCAACCGTTTATAG	
lmo1956RT-R	TTATCAACCACGCGCAATTC	
lmo1960RT-F	CCAACTAGAGACGTTGCCAAA	
lmo1960RT-R	ACTTTGATGCGGAGAACGTC	
lmo2185RT-F	GATGCAATTAGTTATGATCACTGGT	
lmo2185RT-R	CCATCCGAAAGAGTCACAGG	

^a Underlining indicates restriction endonuclease sites.

exclusively by either iron or Fur. We found 7 new genes with Fur boxes in their upstream putative promoter regions. Among the Fur-regulated genes, iron limitation resulted in a significant upregulation of many genes except *fur*. To confirm the microarray data, Northern blotting and quantitative reverse transcription-PCR (qRT-PCR) analysis were performed with cells grown in iron-limiting KRM medium (41) and iron-chelated (2,2'-dipyridyl [DPD]) brain heart infusion (BHI) medium, and the expressions of *fur*, *feoB*, *lmo0514*, and *lmo2185* (previously named *svpA*) were analyzed. Furthermore, the regulation of *fur* gene expression and the role of PerR were investigated by using a *fur*::*lacZ* fusion strain of *L. monocytogenes* 10403S and its isogenic *fur* and *perR* mutant derivatives.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *Escherichia coli* and *L. monocytogenes* used in this study are listed in Table 1. *E. coli* was grown at 37°C on LB agar or in LB broth (Difco, Sparks, MD). *L. monocytogenes* EGDe and its

isogenic *fur* deletion mutant were grown at 30°C or 37°C in BHI broth, BHI agar (Difco, Sparks, MD), or KRM medium. The composition and preparation of KRM medium was previously described by Newton et al. (41). To prepare KRM medium, all ingredients except metal ions were dissolved in water and treated with 10 g of Chelex per liter of medium for 5 h at room temperature, and Chelex was removed by using a 0.22-μm filter (Nalgene, Rochester, NY). A total of 10.4 g liter⁻¹ of RPMI 1640 medium (Sigma, St. Louis, MO) and all the required metals except iron were added to this solution, and filter sterilization was performed by using 0.22-μm filter units. Iron-replete conditions were achieved by adding 20 μM sterile FeSO₄ to KRM minimal medium. To reduce the iron in the cultures, the bacteria were grown overnight in BHI broth, diluted 1:100 in KRM medium, and grown to stationary phase. This culture was diluted once again (1:100) in fresh KRM medium and grown to the exponential phase. Iron contamination was minimized by washing and then rinsing all the glassware with concentrated HCl. In addition, all the required metals were dissolved in Chelex-treated water.

For the preparation of iron-depleted BHI medium, cultures grown overnight in BHI medium were diluted 1:100 in BHI broth and grown to an optical density at 600 nm (OD₆₀₀) of ~0.1, 0.3 mM 2,2'-dipyridyl (DPD) (Sigma, St. Louis, MO) was then added, and the cultures were grown to an OD₆₀₀ of ~0.7 as described previously by Jin et al. (28).

When antibiotic selection pressure was required, ampicillin (50 μg ml⁻¹),

erythromycin (300 $\mu\text{g ml}^{-1}$), or kanamycin (50 $\mu\text{g ml}^{-1}$) for *E. coli* or chloramphenicol (10 $\mu\text{g ml}^{-1}$), erythromycin (3 $\mu\text{g ml}^{-1}$), or neomycin (10 $\mu\text{g ml}^{-1}$) for *L. monocytogenes* was added to the medium.

Construction of a *fur::lacZ* fusion in *L. monocytogenes*. To construct the transcriptional fusion consisting of the *fur* promoter and *lacZ*, two primers, hkf-F and hkf-R, with NheI and KpnI restriction sites, respectively, were used to amplify a 720-bp region upstream of the *fur* coding region from *L. monocytogenes* chromosomal DNA. The PCR product was digested with the enzymes KpnI and NheI (Promega, Madison, WI), ligated into NheI- and KpnI-digested plasmid pHK77, and transformed into *E. coli* JM109 cells. The resulting clone was designated pHK77-F.

To create a *fur::lacZ* fusion in an ectopic region of the chromosome, pHK77-F was transformed by electroporation into *L. monocytogenes* competent cells. The resulting transformants were selected at 30°C on BHI agar plates containing chloramphenicol. The resultant colonies were transferred into BHI broth without antibiotics and grown overnight. This culture was then diluted 1:1,000 in BHI broth with neomycin (10 $\mu\text{g ml}^{-1}$) and grown overnight at 41°C without shaking to facilitate the integration of the plasmid. This process was sequentially repeated 5 times. Later, the culture was diluted 1:1,000 with fresh BHI medium, and 50 μl was plated onto BHI agar containing neomycin. Several colonies were inoculated into BHI broth containing neomycin and grown at 30°C overnight to facilitate plasmid excision. Diluted cultures were plated onto BHI agar containing neomycin, and the resultant colonies were tested for chloramphenicol sensitivity to confirm the double-crossover recombination and integration of *P_{fur}::lacZ* as described previously (31). The recombinants, which were neomycin resistant and chloramphenicol sensitive, were tested for β -galactosidase activity on BHI agar plates containing 50 $\mu\text{g ml}^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) (Invitrogen, Carlsbad, CA).

Construction of *fur* and *perR* mutants of the *L. monocytogenes* 10403S *fur::lacZ* strain. The procedure for the construction of the mutants by using plasmid pAUL-A was previously described by Chakraborty et al. (9). To construct mutations in the *fur* and *perR* regions of the *fur::lacZ* fusion strain, an internal fragment from each respective coding region was generated from the chromosomal DNA of *L. monocytogenes* 10403S by PCR amplification. To create a mutation in the *fur* region, primers paf-F and paf-R with KpnI and SacI restriction sites, respectively, were used to amplify a 275-bp fragment internal to the *fur* coding region. Similarly for the construction of the *perR* mutation, primers pap-F and pap-R with HindIII and BamHI restriction sites, respectively, were used to generate a 275-bp PCR product internal to the *perR* coding region. The PCR fragment corresponding to the regions of *fur* or *perR* were cloned separately into pAUL-A, a temperature-sensitive integratable plasmid, by appropriate restriction enzyme digestions, ligated using T4 DNA ligase (Promega, Madison, WI), and transformed into *E. coli* JM109 cells. The resultant clones were designated pintFM, for pAUL-A:*fur* internal fragment, and pintPM, for pAUL-A:*perR* internal fragment. These plasmids were purified and transformed separately into the *fur::lacZ* strain of *L. monocytogenes* 10403S and selected by incubation at 30°C on BHI agar plate containing erythromycin. For the integration of the plasmids into either *fur* or *perR* coding regions, colonies were transferred twice by streaking onto BHI agar containing erythromycin and incubated at 42°C. Mutants of *fur* (Pfl-1) or *perR* (Pfl-2) were confirmed by PCR, sequencing, and transcript analysis.

Construction of an *L. monocytogenes* EGDe *perR* mutant. Plasmid pAUL-A, containing the *perR* internal fragment constructed as described above, was transformed into competent EGDe cells. Integration of the plasmid and mutant confirmation were performed as described above for *L. monocytogenes* 10403S.

RNA purification and analysis. For the extraction of total RNA, 3 ml of culture was mixed with 6 ml of bacterial RNA Protect solution (Qiagen, Valencia, CA), and the mixture was centrifuged for 20 min at $3,000 \times g$ in a swinging-bucket rotor centrifuge to pellet the cell. These pellets were resuspended in 1 ml of Trizol (Invitrogen, Carlsbad, CA), and the cells were broken using the Fast-Prep system (Qiagen, Irvine, CA) at a speed of 6.0 for 40 s. RNA was extracted from the broken-cell lysate according to the manufacturer's instructions. Extracted RNA was treated with RNase-free DNase I (Qiagen, Valencia, CA) and purified by using the RNeasy minikit (Qiagen, Valencia, CA). The concentration of RNA was measured by using a spectrophotometer, and its quality was assessed by agarose gel electrophoresis before being used for microarray, Northern blot, or qRT-PCR analysis. Three independent bacterial cultures for each test and controls were prepared as biological replicates for RNA isolation.

Probe synthesis and microarray hybridization. cDNA was generated by reverse transcription using random hexamers (Invitrogen, Carlsbad, CA) as primers. The primers were annealed (70°C for 10 min, followed by snap-freezing in ice for 1 min) to 5 μg of total RNA, extended with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a buffer containing 0.1 M dithiothreitol–12.5

mM deoxynucleoside triphosphate (dNTP)/5-(3-aminoallyl)-dUTP (Ambion, Austin, TX), and incubated overnight at 42°C. Residual RNA was removed from these samples by alkaline treatment and neutralized, and cDNA was purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Purified amino-allyl-modified cDNA was labeled with Cy3 or Cy5 monofunctional *N*-hydroxy-succinimide (NHS) ester cyanogen dyes (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Labeled cDNA was purified by using a QIAquick PCR purification kit.

The full genome of the *L. monocytogenes* array consists of single-stranded (70-mer) epoxy-coated oligonucleotides representing 6,347 open reading frames (ORFs) from *L. monocytogenes* strains EGDe, 4b F2365, 1/2a F6854, and 4b H7858 (<http://pfgrc.tigr.org>). Purified labeled cDNA was hybridized with *L. monocytogenes* genome microarrays as previously described (37, 40). Briefly, the slides were prehybridized in a buffer containing $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS, and 0.1% bovine serum albumin (BSA) and then washed and blown to dryness. The two different labeled cDNA samples were dried and resuspended in hybridization solution (40% deionized formamide, $5\times$ SSC, 0.1% SDS, 0.6 $\mu\text{g ml}^{-1}$ sheared salmon sperm DNA). The labeled cDNA samples were hybridized with slides at 42°C for 18 to 20 h. After hybridization, the slides were washed in low-stringency buffer ($2\times$ SSC, 0.1% SDS) for 2 min, in medium-stringency buffer ($0.1\times$ SSC, 0.1% SDS) for 5 min, and, finally, in high-stringency buffer ($0.1\times$ SSC) for 10 min. The slides were dried and scanned by use of a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA).

Data analysis. Scanned tagged image file format (TIFF) images obtained from hybridized microarray slides were analyzed by using TIGR-Spotfinder (<http://www.tm4.org/spotfinder.html>). Spots were analyzed by adaptive quantification, and the local background was subsequently subtracted. Spots with background-corrected signal intensities (median) in both channels with less-than-twofold background intensity (median) were rejected from further analyses. Data normalization was performed on the remaining spots by use of the LOWESS algorithm (block mode; smooth parameter, 0.33) using TIGR-MIDAS software (<http://www.tm4.org/midas.html>), and the normalized $\log_2$ ratio of test/reference signal for each spot was recorded. Genes with less than three data points were considered unreliable and were excluded from further analysis. For the remaining genes, the averaged $\log_2$ ratio was calculated, and the change of significance in expression for a gene was determined with Significance Analysis of Microarrays (SAM) software (www.stat.stanford.edu/~tibbs/SAM/index.html) (56) using one class mode ($\Delta = 1.0$) [the measurement is the $\log(\text{test/reference})_2$ ratio from two labeled samples hybridized to an array]. In this analysis, SAM assigns a score to each gene on the basis of the change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR). A “*q* value” is assigned to each gene and corresponds to the lowest false discovery rate at which the gene is considered significant. The differentially expressed genes identified by SAM were further filtered on the basis of previously defined criteria (29, 35, 38). Several controls were employed to minimize the technical and biological variations. To ensure the quality of the data, the following steps were performed: (i) each ORF was present as a $4\times$ replicate on each array; (ii) array slides were prepared in duplicate for each experiment, and fluorophore dyes were swapped between replicates to account for dye bias; and (iii) the experiment was repeated twice.

To examine changes in the levels of transcripts among different functional categories, we classified these genes using in-house Gene Sorter software according to the categories described by the comprehensive microbial resource of TIGR (<http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi>).

Fur box analysis. To identify putative Fur boxes, intergenic regions of the *L. monocytogenes* EGDe genome were analyzed by using a custom position weight matrix (CPWM) sequence generated from a previously identified Fur box nucleotide sequence (31, 36). The generation of CPWM and whole-genome analysis were done using an online bioinformatics software tool, Virtual Footprint Analysis (<http://www.prodoric.de/vfp>) (39).

Northern blot analysis. Ten micrograms of purified total RNA samples from wild-type *L. monocytogenes* EGDe and its isogenic *fur* mutant, obtained from cultures grown in KRM medium with or without added iron, was separated on a 1.0% agarose–0.66 M formaldehyde gel and transferred onto a nylon membrane. RNA isolation, hybridization, and detection were performed as described previously (36). To confirm the microarray data, regulations of *lmo0514*, *fur* (*lmo1956*), *lmo2185*, and *feoB* (*lmo2105*) transcripts were analyzed by Northern blotting. Total RNA was also isolated from *L. monocytogenes* 10403S cultures grown in BHI medium or BHI medium containing 0.3 mM DPD and analyzed

for changes of *fur* and *lmo2185* gene expression. The PCR primers used for the preparation of radioactive probes are shown in Table 1.

Quantitative real-time PCR analysis. To analyze the effects of iron limitation on the regulation of various Fur repression control (FRC) genes, 0.3 mM 2,2'-dipyridyl (DPD) was added to cultures growing in BHI medium. In order to titrate out the DPD, 0.1 mM ferrous sulfate was added to the samples. Cells were grown in biological triplicates as follows: cultures in BHI medium grown overnight were diluted 1:100 in 10 ml BHI broth and grown to an OD₆₀₀ of 0.1, 0.3 mM DPD was added to the first set, 0.3 mM DPD and 0.1 mM FeSO₄ were added to the second set, and the third set had no DPD and FeSO₄ and thus served as a control. In addition, the effect of excess iron on gene expression was quantified for samples treated with 0.1 mM, 0.5 mM, or no ferric citrate and grown in biological triplicates. Cultures grown overnight were diluted 1:100 in 10 ml BHI medium supplemented with (i) no ferric citrate (control), (ii) 0.1 mM ferric citrate, and (iii) 0.5 mM ferric citrate. All the cultures were grown until they reached an OD₆₀₀ of 0.7 before total RNA was isolated.

To investigate gene expression, 0.5 µg of total RNA was reverse transcribed to cDNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed by using Power SYBR green PCR master mix (Applied Biosystems) in a 7300 Fast system instrument (Applied Biosystems) according to the manufacturer's instructions. The levels of gene expression in the treated cells and the nontreated controls were calculated by relative quantification using the 16S rRNA gene as the endogenous reference gene (30). All the samples were amplified in triplicates, and data analysis was carried out using 7300 Fast system software (Applied Biosystems). Primers were designed specifically to amplify 100-nucleotide stretches from the genes being analyzed (Table 1).

β-Galactosidase assay for *fur* expression analysis. Cultures of the *fur::lacZ* fusion strain and its isogenic *fur* or *perR* mutants of *L. monocytogenes* were grown overnight in BHI medium. These cultures were diluted 1:100 and grown to an OD₆₀₀ of 0.1. At this culture density, 0.3 mM DPD was added, and the culture was grown until it reached an OD₆₀₀ of 0.7. For β-galactosidase assays, cells were permeabilized with 0.5% toluene and 4.5% ethanol, and the activity was measured as previously described (40). The activity for each sample was calculated as follows: $1,000 \times (\text{OD}_{420} - 1.75 \times \text{OD}_{550}) / (\text{OD}_{600} \times \text{reaction time} \times \text{volume})$.

Microarray data accession number. The data set discussed in this publication is shown in Table S1 in the supplemental material and was deposited in the Gene Expression Omnibus (GEO) (19), accessible through GEO series accession number GSE12735 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12735>).

RESULTS AND DISCUSSION

Bacterial growth in KRM medium. KRM medium, which was used to grow the bacteria, was previously described to be very low in iron (46). In this medium, EGDe and the *fur* mutant reached an approximate final-density OD₆₀₀ of 0.9 (see Fig. S1 in the supplemental material). Although a growth defect for the *fur* mutant in iron-limited tropolone-containing medium was reported previously (48), we did not observe any defect in growth for the mutant in KRM medium. Reasons for the difference in the growth of the mutant in different media reported by the two studies are not completely clear. Experiments described previously by Jin et al. (28) showed that *L. monocytogenes* can efficiently grow in RPMI-based medium and that this medium has about 1 µM iron. From our experience with various media, we have observed that the minimum concentration of iron required to support growth varies from one medium to another. For example, the minimum iron concentration required for medium containing or treated with tropolone would not be the same as that required for RPMI-based medium. Hence, the minimum amount of iron is dependent on the medium, growth conditions, and, specifically, the presence of the iron-chelating agent added or used. Furthermore, the iron toxicity for a bacterium is an intrinsic physiological phenomenon and is determined by its state of growth, oxygen requirement, and efficiency in regulating iron transport. Researchers have reported that the concentration of iron in

BHI medium is about 25 µM, and in our experiments, we used up to 1,000 µM iron, but this did not result in any growth defect. However, we did not test whether 1,000 µM or any other concentration of iron was toxic to the *fur* mutant.

To perform our experiments, total RNA was isolated from strain EGDe and its *fur* mutant grown to exponential phase (OD₆₀₀ = 0.55). We chose this growth phase because it is considered to be the metabolically active phase of growth for bacteria. Recent studies have shown that the transcriptomic response of exponential-phase-grown cultures is closely related to the response elicited by *Listeria* cells growing *in vivo* (7).

Microarray experiments. Microarray experiments were aimed at determining significant transcriptional changes of the *fur* mutant compared to EGDe when iron is available for both strains (experiment a), the *fur* mutant compared to EGDe when iron is limited for both strains (experiment b), when iron is limited for the *fur* mutant only (experiment c), and when iron is limited for the parent only (experiment d). A similar experimental design was used to study iron acquisition and regulation in *Campylobacter jejuni* (45) and Fur- and iron-regulated gene expression in *Helicobacter pylori* (20).

In our study, genes that were significantly regulated were identified by estimating the false discovery rate (FDR) using SAM software ($\Delta = 1.01$; FDR of 5%; $q < 0.05$). The false discovery rate has been well described to be a suitable method for microarray analysis and has become one of the most important standards by which genes showing significant changes in transcription are listed and sorted (40, 42, 51). In a recent proteomic study, researchers compared *t* statistics and SAM methods to analyze the liquid chromatography-mass spectrometry (LC/MS) proteomic data, and the results indicated that SAM identified a large number of different expressed proteins while overcoming the false-positive results that one would encounter with a *t* test (50).

In our experiments, iron deficiency resulted in transcriptional changes of a variety of genes in the parent and the *fur* mutant (see Table S1 in the supplemental material). In the parent, these changes are likely mediated through Fur, which is known as an iron-responsive transcriptional regulator for almost all bacteria. Thus, the genes that are repressed by Fur are commonly observed to be upregulated when iron is limited or when *fur* is mutated, as was observed in experiments a, b, and d. However, bacteria have several genes that are regulated directly by iron and not by Fur. In our study, genes that were exclusively iron dependent were identified by comparing data from experiment c to data from experiment d.

***L. monocytogenes* response to iron limitation.** Iron limitation resulted in significant changes in the expression of several genes in *L. monocytogenes* EGDe and the *fur* mutant. From the microarray experiments, we obtained genes that responded especially to iron and those that were negatively regulated by Fur.

Genes regulated exclusively in response to iron limitation. Comparison of gene lists from experiments c and d showed that iron limitation resulted in the upregulation of 10 genes (Table 2) and the downregulation of 15 genes (Table 3).

(i) Genes induced in response to iron limitation. Iron limitation resulted in the induction of genes involved in arginine biosynthesis, *argH* and *argG*. Bioinformatic analysis of these two genes revealed that they encode argininosuccinate synthase

TABLE 2. Genes upregulated exclusively in response to iron limitation

Locus tag	Predicted function (gene)	Fold value ^a			
		Δfur +Fe/ WT+Fe	Δfur -Fe/ WT-Fe	Δfur -Fe/ Δfur +Fe	WT-Fe/ WT+Fe
Amino acid biosynthesis					
<i>lmo2090</i>	Argininosuccinate synthase (<i>argG</i>)	—	—	2.43	7.47
<i>lmo2091</i>	Argininosuccinate lyase (<i>argH</i>)	—	-1.42	3.41	3.2
<i>lmo2252</i>	Aspartate aminotransferase (<i>aspB</i>)	-1.38	-1.31	2.27	2.47
Energy metabolism					
<i>lmo0014</i>	Similar to quinol oxidase AA3, subunit I (<i>qoxB</i>)	—	-1.46	2.02	2.54
<i>lmo0016</i>	Similar to quinol oxidase AA3-600 chain IV (<i>qoxD</i>)	1.37	—	2.77	2.81
Protein fate					
<i>lmo2068</i>	Chaperone protein, GroEL	1.6	—	2.31	2.28
Protein synthesis					
<i>lmo0569</i>	Histidyl-tRNA synthetase (<i>hisZ</i>)	—	—	2.07	2.1
Transport and binding proteins					
<i>lmo1738</i>	Similar to amino acid ABC transporter (binding protein)	—	-1.52	3.88	4.68
<i>lmo1739</i>	Similar to amino acid (glutamine) ABC transporter (ATP-binding protein)	—	-1.34	2.58	8.31
<i>lmo2251</i>	Similar to amino acid ABC transporter (ATP-binding protein) (GlnQ)	—	—	2.45	6.01

^a Numbers in the fold value column are ratios of expression levels in the *fur* mutant (Δfur) and in the *L. monocytogenes* wild type (WT) in the presence of iron (+Fe) or in iron-limiting conditions (-Fe). —, no change or data not available.

and argininosuccinate lyase, respectively, from a single operon and are involved in a two-step process to enzymatically convert citrulline to L-arginine. In our experiments, various other genes, *lmo0569* (*hisZ*), *lmo2252* (aspartate aminotransferase), and amino acid transport genes of the ABC type, *lmo1738*, *lmo1739*, and *lmo2251*, were induced under low-iron conditions. *lmo2251* belongs to an operon consisting of two additional structural genes, *arpJ* and arginine ABC transporter. Researchers reported previously that *arpJ* is highly induced in *L. monocytogenes*-infected mammalian cells and that the mutant for this gene was defective in intracellular growth (32). The induction of these two genes was also observed for a *Vibrio cholerae* mouse infection study (8).

In our study, the induction of two genes, *lmo0014* and *lmo0016*, known to encode subunits for the enzyme quinol oxidase, indicates that oxygen is still a preferred electron acceptor under iron-deprived growth conditions. This condition seems to be causing damage to some proteins, as shown by the upregulation of *lmo2068* (chaperone protein GroEL) in our experiments (Table 2). In bacteria, GroEL is an important heat shock chaperone that works together with GroES to help cells cope with damaged or improperly folded proteins. In *L. monocytogenes*, these genes are present in a single operon and are known to be induced in response to various environmental stress stimuli (36).

(ii) **Genes repressed in response to iron limitation.** In the amino acid biosynthesis group, the most downregulated genes were found to be involved in leucine (*lmo1988* to *lmo1990*) and isoleucine (*lmo1986*) biosynthesis. It appears that in the absence of iron, bacteria try to carefully regulate branched-amino-acid biosynthesis to ensure that iron is properly utilized since many enzymes involved in amino acid biosynthesis are iron dependent: the adaptive “iron-sparing” response (24). We also found

that the *lmo0515*, *lmo0576*, *lmo0585*, *lmo0587*, and *lmo0850* genes, encoding hypothetical proteins, were downregulated. Bioinformatic analysis of the corresponding representative proteins, Lmo0850 and Lmo0515, showed that they are similar to a transcriptional regulator and universal stress protein (UspA), respectively. In *E. coli*, UspA is known to protect the bacteria from different stress conditions generated during growth, such as hydrogen peroxide stress. Hence, the repression of this gene in our experiments may suggest lower oxidative stress generated under low-iron growth conditions (43). Other genes encoding several putative cell wall- or membrane protein-related genes, such as *lmo0160*, *lmo0576*, *lmo0584*, *lmo0732*, and *lmo2522*, were downregulated in the absence of iron. Protein sequence analysis for Lmo0160 and Lmo0732 showed that they have LPXTG wall-anchoring motifs and could serve as substrates for sortase A (SrtA), whose function is to recognize the motif and display the protein on the bacterial cell surface (3). Analysis of the other sequences showed that Lmo0584 is similar to YubA, a membrane protein of unknown function, whereas Lmo0576 is similar to mucin binding protein (MucBP). In *Lactobacillus reuteri* 1063, *mucBP* was shown to be involved in adhesion to mucin (49). Downstream of the *lmo0584* gene, there is a Csc-like cluster present, comprised of the genes *lmo585* to *lmo587*. Among the proteins encoded in this cluster, we found that Lmo0585 (CscB-like) and Lmo0587 (CscC-like) have a WxL domain on their C termini, which may help in associating with the bacterial surface (4). The function of Lmo2522 is not completely known; experiments have shown that this protein has two LysM domains and is released into the supernatant of a bacterial culture (55). We found that *lmo2254* (xanthine/uracil transport system permease protein) and *lmo0733*, a transcriptional fac-

TABLE 3. Genes downregulated exclusively in response to iron limitation

Locus tag	Predicted function (gene)	Fold value ^a			
		Δfur +Fe/WT+Fe	Δfur −Fe/WT−Fe	Δfur −Fe/ Δfur +Fe	WT−Fe/WT+Fe
Amino acid biosynthesis					
<i>lmo1986</i>	Similar to ketol-acid reductoisomerase (<i>ilvC</i>)	1.45	1.32	−2.38	−4.38
<i>lmo1988</i>	3-Isopropylmalate dehydrogenase (<i>leuB</i>)	1.4	—	−2.63	−3.36
<i>lmo1989</i>	3-Isopropylmalate dehydratase (large subunit) (<i>leuC</i>)	—	—	−2.86	−2.57
<i>lmo1990</i>	3-Isopropylmalate dehydratase (small subunit) (<i>leuD</i>)	1.47	—	−3.49	−2.05
Cellular processes					
<i>lmo0515</i>	Universal stress protein, UspA-like	—	—	−2.32	−3.53
Hypothetical proteins					
<i>lmo0584</i>	Similar to membrane protein YubA	−1.6	−1.8	−3.03	−4.03
<i>lmo0585</i>	Unknown, CscB-like	—	1.27	−2.68	−4.96
<i>lmo0587</i>	Unknown, CscC-like	−1.37	−1.84	−2.79	−3.68
<i>lmo0576</i>	Cell wall-associated MucBP	—	—	−2.33	−2.97
<i>lmo0850</i>	Transcriptional regulator	—	—	−2.45	−4.17
Regulatory functions					
<i>lmo0733</i>	Putative transcriptional regulator	—	−1.28	−2.01	−3.36
Transport and binding proteins					
<i>lmo2254</i>	Xanthine/uracil permease family protein	—	—	−2.08	−2.19
Unclassified					
<i>lmo0160</i>	Putative peptidoglycan-bound protein (LPXTG motif)	−1.49	−1.68	−2.53	−3.86
<i>lmo0732</i>	Putative peptidoglycan bound protein (LPXTG motif)	−1.47	−1.28	−2.25	−7
<i>lmo2522</i>	Cell wall-binding protein, unknown	—	−1.74	−2.28	−3.94

^a Numbers in the fold value column are ratios of expression levels in the *fur* mutant (Δfur) and in the *L. monocytogenes* wild type (WT) in the presence of iron (+Fe) or in iron-limiting conditions (-Fe). —, no change or data not available.

tor, were downregulated (Table 3). However, we do not understand the significance of the regulation of these two genes.

Genes regulated by Fur. To identify the genes under the direct control of Fur, the gene expression profiles from experiments a, b, and d were compared. In this approach, the genes that were observed to be simultaneously regulated either as a consequence of *fur* mutation (experiments a and b) or as a response to low-iron conditions in the medium (experiment d) can be considered to be regulated by Fur. The difference in the responses of the Fur-regulated genes in experiments a and b is due to the different regulation of *fur* in the parent; *fur* is significantly repressed in the absence of iron. Our analysis showed several genes to be negatively regulated by Fur, and only one gene, *lmo0514* (contains the LPXTG sorting signal) appeared to be positively regulated in the presence of iron. However, we did not experimentally determine whether *lmo0514* is directly or indirectly regulated by Fur. Also, we did not find any consensus Fur box sequence in the upstream putative promoter region of this gene.

Genes negatively regulated by Fur under iron limitation conditions. Analysis of the iron limitation response showed that several genes under FRC were upregulated (Table 4). Northern blot analysis showed that the regulation of *fur*, which is also under Fur regulation, was significantly repressed. Based

on these preliminary results, the response of the FRC genes to iron limitation seems to mimic the response observed when *fur* is mutated in this bacterium.

Comparative analysis of the FRC genes in the *fur* mutant showed that their response (fold value) was much higher in medium containing iron (Δfur +Fe/WT+Fe) than when this iron was limited (Δfur -Fe/WT-Fe). For example, the fold value for the expression of *fur* changed from -12.75-fold (Δfur +Fe/WT+Fe) to -3.25-fold (Δfur -Fe/WT-Fe), and that of *lmo2185* changed from 8.91-fold (Δfur +Fe/WT+Fe) to 1.58-fold. Based on these results, we hypothesized that (i) the presence of an optimal concentration of iron is required for *fur* to be maximally induced, and any significant limitation of iron would result in the repression of *fur*, and (ii) it is the repression of *fur* rather than the availability of iron that is important for the regulation of genes under Fur repression control (FRC).

Some of the FRC genes identified by microarray analysis were previously studied for *L. monocytogenes* EGDe (18, 22, 28). It was previously shown that that deletion of *fhuD*, *fhuC*, or *hupC* reduced the uptake of ferric hydroxamate and hemin/hemoglobin, respectively (28). Moreover, the authors of that study also observed that iron transport was impaired when *fri* was mutated, but the deletion of *fur*, *feoB*, *lmo2183*, *lmo2184*, or *lmo2185* did not result in the same impairment (28). Anal-

TABLE 4. Genes regulated by Fur under iron limitation conditions

Locus tag	Predicted function (gene)	Fold value ^a			
		Δfur +Fe/WT+Fe	Δfur -Fe/WT-Fe	Δfur -Fe/ Δfur +Fe	WT-Fe/WT+Fe
Cell envelope					
<i>lmo0366</i>	Similar to putative lipoprotein involved in iron transport	27.61	2.1	—	5.47
Energy metabolism					
<i>lmo0485</i>	Nitroreductase family protein	3.83	1.65	1.61	2.17
<i>lmo0943</i>	Nonheme iron-binding ferritin (<i>fri</i>)	2.4	1.89	1.18	2.35
<i>lmo1131</i>	Unknown, similar to ABC transporter, ATP-binding protein	9.93	1.39	—	5.97
Hypothetical proteins					
<i>lmo0365</i>	Similar to high-affinity iron transporter	25.03	2.43	—	5.58
<i>lmo1007</i>	Unknown	5.61	—	—	2.48
<i>lmo2132</i>	Unknown	2.04	1.4	1.26	2
<i>lmo2181</i>	Sortase B (<i>srtB</i>)	12.83	1.34	-1.21	4.6
<i>lmo2104</i>	Ferrous iron transport protein (A)	15.17	—	1.22	7
<i>lmo2105</i>	Ferrous iron transport protein (B)	7.76	1.27	—	4.34
Regulatory functions					
<i>lmo1956</i>	Ferric uptake regulator	-12.75	-3.25	—	1.44 ^b
Transport and binding proteins					
<i>lmo0541</i>	Similar to iron compound binding lipoprotein, ABC Transporter	7.01	—	—	2.62
<i>lmo1957</i>	Ferrichrome ABC transporter (permease)	5.86	1.79	-1.27	2.22
<i>lmo1958</i>	Iron compound ABC transporter, permease protein (<i>fhuB</i>)	4.7	1.44	—	2.01
<i>lmo1960</i>	Iron compound ABC transporter (ATP binding) (<i>fhuC</i>)	2.44	—	—	2.02
<i>lmo2182</i>	Ferrichrome ABC transporter (ATP binding)	13.3	1.34	—	3.97
<i>lmo2183</i>	Ferrichrome ABC Transporter (permease)	15.76	1.41	—	4.48
Unclassified					
<i>lmo0361</i>	Twin-arginine-targeting protein translocase (<i>tatC</i>)	22.75	2.85	-1.17	3.61
<i>lmo0362</i>	Twin-arginine-targeting protein translocase (<i>tatA</i>)	19.75	3.21	-1.16	4.29
<i>lmo2180</i>	Unknown, similar to GP157 family of protein	4.09	—	—	4.71
<i>lmo2185</i>	(<i>svpA</i>)	8.91	1.58	—	2.99
<i>lmo2186</i>	Unknown	9.48	—	—	4.24
<i>lmo0367</i>	Putative iron-dependent peroxidase	25.83	1.57	—	4.07
Unknown function					
<i>lmo1961</i>	Similar to oxidoreductase	3.58	—	—	2.4

^a Numbers in the fold value column are ratios of expression levels in the *fur* mutant (Δfur) and in the *L. monocytogenes* wild type (WT) in the presence of iron (+Fe) or in iron-limiting conditions (-Fe). —, no change or data not available.

^b Downregulated.

ysis of FhuD from *L. monocytogenes* showed that it is similar to FhuD from *E. coli* and FhuD2 from *Staphylococcus aureus*, but unlike some *S. aureus* strains, no gene duplication was observed. Studies of other organisms have shown that Fur regulates *fhuA* in *E. coli* and *fhuD* in *Bacillus subtilis* (6, 25). As expected, other iron uptake genes, *feoAB* (*lmo2104* and *lmo2105*), *fhuGB* operon genes (*lmo1957* and *lmo1958*), *fhuD* (*lmo1959*), *fhuC* (*lmo1960*), *lmo2183*, and *lmo2431*, were found to be significantly upregulated in response to iron limitation.

Studies have shown that in bacteria, the Feo transport system, Fe²⁺ type, is used for the uptake of the Fe²⁺ form of iron and is under the negative control of Fur (25). In *E. coli*, the proteins involved in this system are encoded from the *feoABC* operon. Among the Gram-positive *Bacillus* species, the *feoAB*

operon is present in *Bacillus cereus* but not in *Bacillus subtilis*. In *L. monocytogenes*, both the *feoA* and *feoB* genes are present.

In our study, we have found that the *tatC* (*lmo0361*), *tatA* (*lmo0362*), *lmo0365* to *lmo0367*, *lmo0485*, *lmo0541*, and *lmo1007* genes are under the regulation of Fur. Extensive studies of the Tat system of *E. coli* showed that this machinery translocates proteins that have twin-arginine motifs on their N termini and also possess hydrophobic H domains. In *E. coli*, this machinery is made of several Tat proteins, encoded by *tatABCD*, but in *L. monocytogenes*, only *tatA* and *tatC* are present. It seems that one of the substrates translocated by this system is an iron-dependent peroxidase, encoded by *lmo0367* (15), and we found a significant induction of this gene in our experiments. Nucleotide sequence analysis of this gene showed that it is part of the operon consisting of *lmo0365* to *lmo0367*.

FIG. 1. (a) Effect of iron concentration on β -galactosidase expression of *L. monocytogenes fur::lacZ* fusion strain Pfl (10403S). Error bars represent standard deviations for triplicate experiments. (b) Effect of iron limitation caused by 2,2'-dipyridyl on β -galactosidase expression of *L. monocytogenes fur::lacZ* fusion strains, Pfl (10403S), Pfl-1 (*fur* mutant), and Pfl-2 (*perR* mutant). Errors bar represent standard deviations for triplicate experiments.

Analysis of the protein products encoded by this operon and Lmo0541 indicated that they are all probably involved in iron transport. We found that Lmo0365 was 40% identical to YwbL, Lmo0366 was 43% identical to YwbM, and Lmo0367 was 51% identical to YwbN of *B. subtilis*. It is known that all these proteins in *B. subtilis* are involved in iron transport and are regulated by Fur in response to iron.

In our microarray analysis, we also identified *lmo0485*, encoding a nitroreductase family protein, to be upregulated. Protein sequence alignment showed that it is 28% identical to *B. subtilis* YfhC. Experiments done with *B. subtilis* showed that *yfhC* is regulated by peroxide stress and iron limitation (2, 38). Furthermore, analysis of Lmo0541 showed that it is about 30% identical to FhuD in *B. subtilis*, and when Lmo1131 was analyzed, it was found to be 26% identical to *B. subtilis* CydC (44). However, more experiments are needed to fully understand and confirm their function in *L. monocytogenes*.

Identification of Fur boxes. For the identification of Fur boxes, all the intergenic regions within the whole-genome sequence of *L. monocytogenes* EGDe were analyzed using a custom position weight matrix generated from Fur box sequences. Fur boxes in the upstream putative promoter sequences were identified in 14 genes, and the locations of these consensus sequences in the genome are listed in Table S2 in the supplemental material.

While several genes were observed to be regulated by *fur* mutation or due to iron limitation, we found Fur boxes in only a few of them, such as the ones involved in iron acquisition and virulence. We performed an electrophoretic mobility shift assay (EMSA) for these putative Fur box sequence-containing promoter regions and confirmed that the purified Fur protein can efficiently shift the mobility of Fur box-containing DNA upon binding (see Fig. S2 in the supplemental material). Comparison of all the identified Fur box sequences showed that they have a small number of nucleotide differences from one another; such differences were also observed previously for *B. subtilis* (2). In our present study, we have identified Fur boxes in 7 new genes. Among the 14 Fur-regulated genes, *fur* was downregulated only in KRM medium or when DPD was added. We hypothesized that this mode of regulation may be a way to effectively reduce Fur protein synthesis to relax the genes under its direct control. This mechanism seems plausi-

ble, as there are only a few genes under the direct control of Fur. Moreover, no genes except *lmo0514*, which would require the presence of this protein for their expression, were identified. To understand whether iron availability or *fur* regulation is important for controlling FRC genes, we performed experiments to observe the regulation of *fur* in the presence of excess and limited concentrations of iron.

Regulation of *fur* in *L. monocytogenes* in response to iron limitation. In most bacteria, *fur* is regulated positively or negatively in response to iron in an autoregulatory manner. In autoregulatory control, *fur* has been shown to be iron dependent where Fur complexes with metals and binds to a putative Fur box sequence present in its promoter region. In addition to this mode of regulation, *fur* is also understood to be regulated by other proteins such as PerR. In *L. monocytogenes*, *fur* has a Fur box and PerR box in the upstream promoter region. We have previously shown that the purified Fur protein can bind a putative Fur box in the absence of iron and proposed an autoregulatory mode of regulation for this gene (33). In our microarray analysis experiment, *fur* was observed to be downregulated in response to iron limitation, and in our previous report, we showed that the addition of excess iron also results in the repression of *fur*. PerR was previously shown to be a negative regulator of *fur* in *L. monocytogenes* (47). From all these studies, *fur* appears to be under the dual control of the Fur and PerR proteins, and knowledge of the regulatory mechanism is important for an understanding of its global regulatory control.

In order to understand the regulation of *fur*, we constructed a *fur::lacZ* promoter fusion in the ectopic chromosomal region of the parent and generated its derivatives with mutations in *fur* and *perR* regions. Analysis of β -galactosidase expression showed that the addition of either iron or DPD in increasing concentrations for the parent growing in BHI medium resulted in a decrease of β -galactosidase activity (Fig. 1a and b). To understand the mechanism behind the repression of *fur* under iron-limiting conditions, DPD was added to *fur* or *perR* mutant derivatives. In this assay, we found high levels of β -galactosidase activity for *fur* or *perR* mutants growing in BHI medium (Fig. 1b). The addition of DPD at 0.3 mM or 0.5 mM concentrations resulted in a significant repression of the activity in the *fur* mutant and its parent fusion strain, but no significant

change in the activity was observed for the fusion strain with the *perR* mutation. For these studies, we could not include complementation studies for either the *fur* or *perR* mutant. Several attempts to complement the *fur* mutant or make a *perR* and *fur* double mutant were unsuccessful, and we believe that it was due partly to the significant downregulation of *comK* in the *fur* mutant, as observed for microarray analyses (experiment a). We encountered a different problem in the case of complementing the *perR* mutant, where we were unable to transform pCU1 containing the *perR* structural gene. We concluded that it was not a transformation problem since we were able to successfully transform plasmid pCU1 alone into the *perR* mutant.

However, we have taken several steps as a quality control measure to assess the stability of plasmid pAUL-A integration. We checked the mutants by growing them in medium with or without antibiotics for 24 h and 48 h. Cultures from these two time points were then serially diluted and plated onto appropriate antibiotic-containing BHI agar medium. Simultaneously, genomic DNA was also isolated from several colonies growing on agar medium and checked by Southern blotting. For both of these analyses, we found that the plasmid integration was stable. For β -galactosidase assays or Northern blot analysis (pAUL-A insertion mutants), we grew the bacteria overnight in the presence of antibiotics, but antibiotics were eliminated from the diluted cultures. We did not make any attempt to understand the response of our strains or the regulation of the FRC genes when iron was limited at the stationary phase of growth. Although an understanding of the regulation in this phase is important, we believe that this study should be carried out separately because the stationary phase would add significant metabolic and other nutrient limitation stresses to the already subsisting iron limitation stress.

Our results suggest that the regulation of *fur* is autoregulatory and negatively regulated by PerR. When iron is limited, *fur* is repressed, and this is mediated through the change in activity of PerR. Previously, PerR from *B. subtilis* was biochemically characterized, and it was proposed that this protein associates with Fe(II) as a cofactor that is sensitive to peroxide (27). When intracellular iron levels are low, PerR binds Mn(II) and represses several genes under its regulon, which include *fur* (27, 34, 54). Based on our data and data from previous studies of PerR, we hypothesize that in the absence of iron, PerR changes from a less active (complexed to Fe) to a more active (uses Mn as cofactor) form, which can cause the repression of *fur*, resulting in the upregulation of the Fur repressor control genes. However, it is not known if PerR mediates any factors that may target *fur* mRNA for degradation or reduce the repression caused by Fur.

Northern analysis. To confirm microarray results, expression levels of four genes, *lmo0514*, *fur*, *feoB*, and *lmo2185*, were analyzed with the *fur* deletion mutant and its isogenic parent grown in minimal medium (KRM medium) with or without added iron (see Fig. S3 and S4 in the supplemental material). Under low-iron conditions (KRM medium) or because *fur* was deleted, both *fur* and *lmo0514* were found to be repressed, while *feoB* and *lmo2185* were induced. It should be noted that the absence of *fur* mRNA in the mutant is due to the complete deletion of the structural gene.

In order to confirm that the repression of *fur* in KRM medium was due to iron limitation and that this regulation requires PerR,

FIG. 2. Northern blot analysis to determine the expression of *fur* and *lmo2185* from *L. monocytogenes* in response to 2,2'-dipyridyl. (a) Northern blot analysis for *fur* in the *perR* mutant in response to iron limitation by DPD. Ten micrograms of total RNA was isolated from parent cells grown in BHI medium (lane 1) or 0.3 mM DPD (lane 2) or the *perR* mutant grown in BHI medium (lane 3) or 0.3 mM DPD (lane 4). (b) Northern blot analysis of *lmo2185* expression. The amounts of RNA and lanes are similar to those described above (a).

total RNA isolated from the cultures grown in BHI medium with DPD was analyzed by Northern blotting (see Fig. S5 in the supplemental material). These samples were also analyzed to confirm the induction of *lmo2185* in the presence of DPD. We probed for *lmo2185* transcripts for control purposes in these experiments, as the regulation of this gene is a good indication of iron limitation in the environment (41). In the case of the *perR* mutant, *fur* expression was upregulated. However, when the same mutant was grown in the presence of DPD, no significant change in *lmo2185* expression was observed (Fig. 2).

qRT-PCR analysis. To demonstrate that *fur* is regulated in the presence of excess iron and under conditions of iron limitation, we performed qRT-PCR using *fur* (*lmo1956*), *lmo0485*, *lmo1960*, and *lmo2185* in wild-type *L. monocytogenes* EGDe (see Tables S3 and S4 in the supplemental material). The data generated by the qRT-PCR analysis supported data from the Northern blot analysis. The presence of excess iron, in the form of ferric citrate, showed a direct proportional downregulation of the *fur* transcript. However, the level of repression was less than that observed when iron was limited. Among other genes, *lmo0485* showed an upregulation as in the microarray analysis. *lmo0485* is a member of the nitroreductase family and is similar to *yfhC* of *B. subtilis*. In *B. subtilis*, *yfhC* is induced upon exposure to hydrogen peroxide and paraquat (38). The iron compound ABC transporter gene *fluC* (*lmo1960*) is also upregulated initially in the presence of iron, but increasing concentrations of iron resulted in a decreased level of expression. *lmo1960* is responsible for the uptake of iron, but excess iron is detrimental to the cell, hence the reduced levels of expression. In this analysis, the downregulation of *fur* and *lmo2185* could also be a result of the sensing of excess iron by some unknown factor. Work is under way in our laboratory to understand the mechanism of regulation of *fur* and FRC genes under excess-iron conditions.

qRT-PCR data obtained from the experiments under iron-depleted conditions, i.e., in the presence of DPD, also support

FIG. 3. Proposed model of PerR-mediated iron acquisition in *L. monocytogenes*. PerR is constitutively expressed, and it is in a less active state (associated with Fe). When iron is limited, PerR binds to manganese (more active state; complexed with Mn) and represses *fur*, which consequently results in a reduction of Fur expression and up-regulation of FRC genes. Broken lines indicate a change in control over FRC genes by Fur due to a decrease in expression. Alternatively, PerR in the active form may be interacting with unknown factors to cause the same effect on *fur* and FRC gene regulation.

the microarray and Northern blot results. A downregulation of *fur* and *lmo1956* in the absence of iron was observed. However, the level of repression was reduced in the presence of 0.1 mM FeSO_4 , thus demonstrating that the DPD added was effectively chelating iron in our experiments. On the other hand, *lmo0485*, *lmo1960*, and *lmo2185* showed markedly decreased levels of expression in the presence of 0.1 mM FeSO_4 compared to those under iron-depleted conditions.

The results observed from *fur::lacZ* fusions, Northern blot analysis, and qRT-PCR clearly indicated that *fur* is repressed when iron availability is limited. Several studies demonstrated that the regulation of genes under the control of Fur changes as a consequence, resulting from the deliberate deletion of *fur*, repression of *fur*, degradation of *fur* mRNA, or intracellular inactivation of Fur repression activity. Although any of the above-mentioned processes could be operating in *Listeria*, our studies support the hypothesis that the repression of *fur* is the probable way of regulating FRC genes when iron is limited. In this bacterium, *fur* is under the control of its own protein and PerR. Based on all our studies, we believe that *fur* repression is essential for regulating FRC genes because a characterization of the Fur protein in *Listeria* indicated that this protein can actively bind in the absence of metal ions (33). *fur* is repressed under iron limitation conditions, but repression was significantly inhibited when *perR* was mutated. Using microarray analysis, we have also observed that the addition of DPD to the *perR* mutant does not result in significant changes in *fur* or FRC genes (data not shown). We hypothesize that iron limitation and the regulation of iron acquisition genes depend on the activity of PerR but not on the availability of iron as a cofactor for the Fur protein. Fur complexing with iron is a generally observed mechanism of regulation seen for many bacteria. In *B. subtilis*, PerR functions as a transcriptional repressor of *fur*, but no role in the degradation of *fur* mRNA or inactivation of Fur has been demonstrated. However, we do not have sufficient evidence to rule out the involvement of

PerR-mediated molecules in the regulation of Fur or iron acquisition genes. Based on our current understanding of *fur* and FRC gene expression in *L. monocytogenes*, we have proposed a model for regulation, as shown in Fig. 3.

ACKNOWLEDGMENTS

We thank Colin Hill for providing the *fur* deletion mutant of *L. monocytogenes* EGDe and Abraham L. Sonenshein for generously providing plasmid pHK77. The *L. monocytogenes* microarrays were obtained through the NIAID Pathogen Functional Genomics Resource Center, managed and funded by the Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS, and operated by The Institute for Genomic Research (TIGR). We appreciate Anthony J. Otsuka for critical reading of the manuscript.

This work was partially supported by the Illinois State University Research Program, a grant from the NIH to R.K.J., and award 2006-35201-17356 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture.

REFERENCES

- Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones. 2003. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27:215–237.
- Baichoo, N., T. Wang, R. Ye, and J. D. Helmann. 2002. Global analysis of the *Bacillus subtilis* Fur regulon and the iron starvation stimulon. *Mol. Microbiol.* 45:1613–1629.
- Bierne, H., and P. Cossart. 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. *Microbiol. Mol. Biol. Rev.* 71:377–397.
- Brinster, S., S. Furlan, and P. Serror. 2007. C-terminal WxL domain mediates cell wall binding in *Enterococcus faecalis* and other gram-positive bacteria. *J. Bacteriol.* 189:1244–1253.
- Brown, J. S., and D. W. Holden. 2002. Iron acquisition by Gram-positive bacterial pathogens. *Microbes Infect.* 4:1149–1156.
- Bsat, N., A. Herbig, L. Casillas-Martinez, P. Setlow, and J. D. Helmann. 1998. *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Mol. Microbiol.* 29:189–198.
- Camejo, A., C. Buchrieser, E. Couve, F. Carvalho, O. Reis, P. Ferreira, S. Sousa, P. Cossart, and D. Cabanes. 2009. *In vivo* transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. *PLoS Pathog.* 5:e1000449.
- Camilli, A., and J. J. Mekalanos. 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol. Microbiol.* 18:671–683.
- Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans. 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J. Bacteriol.* 174:568–574.
- Chakraborty, T. 1999. Molecular and cell biological aspects of infection by *Listeria monocytogenes*. *Immunobiology* 201:155–163.
- Conte, M. P., C. Longhi, M. Polidoro, G. Petrone, V. Buonfiglio, S. Di Santo, E. Papi, L. Seganti, P. Visca, and P. Valenti. 1996. Iron availability affects entry of *Listeria monocytogenes* into the enterocyte like cell line Caco-2. *Infect. Immun.* 64:3925–3929.
- Conway, T., and G. K. Schoolnik. 2003. Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. *Mol. Microbiol.* 47:879–889.
- Cossart, P. 2002. Molecular and cellular basis of the infection by *Listeria monocytogenes*: an overview. *Int. J. Med. Microbiol.* 291:401–409.
- Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato. 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. *Mol. Microbiol.* 42:1297–1309.
- Dilks, K., R. W. Rose, E. Hartmann, and M. Pohlshroder. 2003. Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. *J. Bacteriol.* 185:1478–1483.
- Dubrac, S., and D. Touati. 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. *J. Bacteriol.* 182:3802–3808.
- Ducey, T. F., M. B. Carson, J. Orvis, A. P. Stintzi, and D. W. Dyer. 2005. Identification of the iron-responsive genes of *Neisseria gonorrhoeae* by microarray analysis in defined medium. *J. Bacteriol.* 187:4865–4874.
- Dussurget, O., E. Dumas, C. Archambaud, I. Chafsey, C. Chambon, M. Hebraud, and P. Cossart. 2005. *Listeria monocytogenes* ferritin protects against multiple stresses and is required for virulence. *FEMS Microbiol. Lett.* 250:253–261.
- Edgar, R., and T. Barrett. 2006. NCBI GEO standards and services for microarray data. *Nat. Biotechnol.* 24:1471–1472.
- Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J.

- Kuipers, M. Kist, A. H. van Vliet, and G. Homuth. 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. *Microbiology* **151**:533–546.
21. Escobar, L., J. Perez-Martin, and V. de Lorenzo. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. *J. Bacteriol.* **181**:6223–6229.
22. Fiorini, F., S. Stefanini, P. Valenti, E. Chiancone, and D. De Biase. 2008. Transcription of the *Listeria monocytogenes* *fri* gene is growth-phase dependent and is repressed directly by Fur, the ferric uptake regulator. *Gene* **410**:113–121.
23. Friedman, D. B., D. L. Stauff, G. Pishchany, C. W. Whitwell, V. J. Torres, and E. P. Skaar. 2006. *Staphylococcus aureus* redirects central metabolism to increase iron availability. *PLoS Pathog.* **2**:e87.
24. Gaballa, A., H. Antelmann, C. Aguilar, S. K. Khakh, K. B. Song, G. T. Smaldone, and J. D. Helmann. 2008. The *Bacillus subtilis* iron-sparing response is mediated by a Fur-regulated small RNA and three small, basic proteins. *Proc. Natl. Acad. Sci. U. S. A.* **105**:11927–11932.
25. Hantke, K. 2001. Iron and metal regulation in bacteria. *Curr. Opin. Microbiol.* **4**:172–177.
26. Heinrichs, D. V., A. Rahn, E. Dale, and M. T. Sebuly. 2004. *Staphylococcus*, *Streptococcus*, and *Bacillus*, p. 387–401. In J. H. Crosa, A. R. Mey, and S. M. Payne (ed.), *Iron transport in bacteria*. ASM Press, Washington, DC.
27. Herbig, A. F., and J. D. Helmann. 2001. Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Mol. Microbiol.* **41**:849–859.
28. Jin, B., S. M. Newton, Y. Shao, X. Jiang, A. Charbit, and P. E. Klebba. 2006. Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in *Listeria monocytogenes*. *Mol. Microbiol.* **59**:1185–1198.
29. Kathariou, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.* **65**:1811–1829.
30. Kenny, J. G., D. Ward, E. Josefsson, I. M. Jonsson, J. Hinds, H. H. Rees, J. A. Lindsay, A. Tarkowski, and M. J. Horsburgh. 2009. The *Staphylococcus aureus* response to unsaturated long chain free fatty acids: survival mechanisms and virulence implications. *PLoS One* **4**:e4344.
31. Kim, H. J., M. Mittal, and A. L. Sonenshein. 2006. CcpC-dependent regulation of *citB* and *lmo0847* in *Listeria monocytogenes*. *J. Bacteriol.* **188**:179–190.
32. Klarsfeld, A. D., P. L. Goossens, and P. Cossart. 1994. Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA*, *purH*, *purD*, *pyrE* and an arginine ABC transporter gene, *arpJ*. *Mol. Microbiol.* **13**:585–597.
33. Ledala, N., S. L. Pearson, B. J. Wilkinson, and R. K. Jayaswal. 2007. Molecular characterization of the Fur protein of *Listeria monocytogenes*. *Microbiology* **153**:1103–1111.
34. Lee, J. W., and J. D. Helmann. 2007. Functional specialization within the Fur family of metalloregulators. *Biometals* **20**:485–499.
35. Lin, J. T., M. B. Connelly, C. Amolo, S. Otani, and D. S. Yaver. 2005. Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. *Antimicrob. Agents Chemother.* **49**:1915–1926.
36. Liu, S., J. E. Graham, L. Bigelow, P. D. Morse, and B. J. Wilkinson. 2002. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. *Appl. Environ. Microbiol.* **68**:1697–1705.
37. Mongodin, E., J. Finan, M. W. Climo, A. Rosato, S. Gill, and G. L. Archer. 2003. Microarray transcription analysis of clinical *Staphylococcus aureus* isolates resistant to vancomycin. *J. Bacteriol.* **185**:4638–4643.
38. Mostertz, J., C. Scharf, M. Hecker, and G. Homuth. 2004. Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. *Microbiology* **150**:497–512.
39. Munch, R., K. Hiller, A. Grote, M. Scheer, J. Klein, M. Schobert, and D. Jahn. 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* **21**:4187–4189.
40. Muthaiyan, A., J. A. Silverman, R. K. Jayaswal, and B. J. Wilkinson. 2008. Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. *Antimicrob. Agents Chemother.* **52**:980–990.
41. Newton, S. M., P. E. Klebba, C. Raynaud, Y. Shao, X. Jiang, I. Dubail, C. Archer, C. Frehel, and A. Charbit. 2005. The *svpA-srtB* locus of *Listeria monocytogenes*: Fur-mediated iron regulation and effect on virulence. *Mol. Microbiol.* **55**:927–940.
42. Norris, A. W., and C. R. Kahn. 2006. Analysis of gene expression in pathophysiological states: balancing false discovery and false negative rates. *Proc. Natl. Acad. Sci. U. S. A.* **103**:649–653.
43. Nystrom, T., and F. C. Neidhardt. 1994. Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. *Mol. Microbiol.* **11**:537–544.
44. Ollinger, J., K. B. Song, H. Antelmann, M. Hecker, and J. D. Helmann. 2006. Role of the Fur regulon in iron transport in *Bacillus subtilis*. *J. Bacteriol.* **188**:3664–3673.
45. Palyada, K., D. Threadgill, and A. Stintzi. 2004. Iron acquisition and regulation in *Campylobacter jejuni*. *J. Bacteriol.* **186**:4714–4729.
46. Portnoy, D. A., V. Auerbuch, and I. J. Glomski. 2002. The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *J. Cell Biol.* **158**:409–414.
47. Rea, R., C. Hill, and C. G. Gahan. 2005. *Listeria monocytogenes* PerR mutants display a small-colony phenotype, increased sensitivity to hydrogen peroxide, and significantly reduced murine virulence. *Appl. Environ. Microbiol.* **71**:8314–8322.
48. Rea, R. B., C. G. Gahan, and C. Hill. 2004. Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. *Infect. Immun.* **72**:717–727.
49. Roos, S., and H. Jonsson. 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology* **148**:433–442.
50. Roxas, B. A., and Q. Li. 2008. Significance analysis of microarray for relative quantitation of LC/MS data in proteomics. *BMC Bioinformatics* **9**:187.
51. Schafer, E. R., M. J. Oneal, M. L. Madsen, and F. C. Minion. 2007. Global transcriptional analysis of *Mycoplasma hyopneumoniae* following exposure to hydrogen peroxide. *Microbiology* **153**:3785–3790.
52. Sword, C. P. 1966. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. *J. Bacteriol.* **92**:536–542.
53. Reference deleted.
54. Traore, D. A., A. El Ghazouani, L. Jacquamet, F. Borel, J. L. Ferrer, D. Lascoux, J. L. Ravanat, M. Jaquinod, G. Blondin, C. Caux-Thang, V. Duarte, and J. M. Latour. 2009. Structural and functional characterization of 2-oxohistidine in oxidized PerR protein. *Nat. Chem. Biol.* **5**:53–59.
55. Trost, M., D. Wehmhoner, U. Karst, G. Dieterich, J. Wehland, and L. Jansch. 2005. Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic *Listeria* species. *Proteomics* **5**:1544–1557.
56. Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U. S. A.* **98**:5116–5121.