Molecular characterization of the Fur protein of Listeria monocytogenes

Nagender Ledala, Stacy L. Pearson, Brian J. Wilkinson and R. K. Jayaswal

Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, IL 61790-4120, USA

Iron is essential for the survival of almost all organisms, although excess iron can result in the generation of free radicals which are toxic to cells. To avoid the toxic effects of free radicals, the concentration of intracellular iron is generally regulated by the ferric uptake regulator Fur in bacteria. The 150 aa fur ORF from Listeria monocytogenes was cloned into pRSETa, and the His-tagged fusion protein was purified by nickel affinity column chromatography. DNA binding activity of this protein was studied by an electrophoretic mobility shift assay using the end-labelled promoters P_{fhuDC} and P_{fur}. The results showed a decrease in migration for both promoter DNAs in the presence of the Fur protein, and the change in migration was competitively inhibited with an excess of the same unlabelled promoters. No shift in migration was observed when a similar assay was performed using non-specific end-labelled DNA. The assay showed that binding of Fur to P_{fur} or P_{fhuDC} was independent of iron or manganese ions, and was not inhibited in the presence of 2 mM EDTA. Inductively coupled plasma MS of the Fur protein showed no iron or manganese, but 0.48 mole zinc per mole protein was detected. A DNase I protection assay revealed that Fur specifically bound to and protected a 19 bp consensus Fur box sequence located in the promoters of fur and fhuDC. There was no requirement for iron or manganese in this assay also. However, Northern blot analysis showed an increase in fur transcription under iron-restricted compared to high-level conditions. Thus, the study suggests that under in vitro conditions, the affinity of the Fur protein for the 19 bp Fur box sequence does not require iron, but iron availability regulates fur transcription in vivo. Thus, the regulation by Fur in this intracellular pathogen may be dependent on either the structure of the DNA binding domain or other intracellular factors yet to be identified.

INTRODUCTION

Iron is an essential element for most bacteria, as many enzymes involved in cellular metabolism require iron as a co-factor (Griffiths, 1999). In bacteria, the level of iron determines the expression of several virulence factors (Braun, 2005; Litwin & Calderwood, 1993). Although iron is considered an abundant element in nature, under aerobic conditions, most iron exists in the insoluble Fe^{3+} form. Reduction of Fe^{3+} to Fe^{2+} is toxic to cells because Fe^{2+} has the ability to generate hydroxyl radicals by catalysing the Fenton reaction (Gutteridge et al., 1982; Imlay et al., 1988). Several proteins, such as albumin, ferritin, lactoferrin and transferrin, present in humans, reduce this toxicity by sequestering free Fe^{2+} and oxidizing it to insoluble Fe^{3+}, which is not readily available to support bacterial growth (Weinberg, 1978). Hence, the survival and growth of bacteria during infection depends on their ability to interact with and acquire iron from the host. Bacteria have evolved several mechanisms to help utilization of host iron-bound compounds directly, or to separate iron from other host sources. Under iron-limiting conditions, most bacteria produce siderophores, which can solubilize iron in the environment and present it to specific receptors for transport into the bacteria. Several reports have indicated that Gram-positive pathogenic bacteria can utilize transferrin, haemoglobin and haem in the provision of iron (Brown & Holden, 2002).

For bacteria, the uptake of iron or iron sources requires tight regulation, in order to reduce the generation of toxic radicals in the cell resulting from the accumulation of high cytoplasmic iron levels (Braun, 1997; Miller & Britigan, 1997; Storz & Imlay, 1999). In almost all bacteria, this regulation process is performed by Fur and Fur homologues (de Lorenzo et al., 1988). Generally, Fur proteins sense intracellular iron levels, and negatively regulate siderophore biosynthesis and iron-uptake genes by binding a consensus nucleotide sequence, known as the Fur box, in the promoter of these genes (Escolar et al., 1999; Dorman, 1994). In Bacillus subtilis, Fur functions as a regulator of iron uptake (Bsat et al., 1998). In corynebacteria and mycobacteria, the iron-uptake process is regulated by a Fur functional
homologue known as DtxR (Pohl et al., 2003; Rodriguez et al., 2002; Schiering et al., 1995). Earlier studies have shown that Fur also regulates a diverse range of metabolic functions in bacteria, such as respiration, the tricarboxylic acid cycle, glycolysis, amino acid biosynthesis, DNA synthesis, purine metabolism, and redox stress conditions (Andrews et al., 2003; McHugh et al., 2003). In a few bacteria, Fur is understood to have a positive regulatory function. For example, in Bradyrhizobium japonicum, the β-aminolaevulinc acid synthase-coding gene hemA is positively regulated by Fur (Hamza et al., 2000).

Listeria monocytogenes is a rod-shaped, Gram-positive, facultative intracellular pathogen that can cause serious infections in humans (Chakraborty, 1999; Cossart, 2002; Kathariou, 2002; Portnoy et al., 2002; Vazquez-Boland et al., 2001). It has been reported that during listerial infection, the availability of iron determines the survival and invasiveness of the bacterium (Conte et al., 1996; Fisher & Martin, 1999; Polidoro et al., 2002; Rea et al., 2004; Sword, 1966). When present in a low-iron environment, such as in a phagosome, activation of PrfA-regulated virulence genes such as hly, plcA, actA and plcB occurs to induce lysis of the phagocytic vacuole, and to enhance the spread of the bacteria to neighbouring cells (Cowart & Foster, 1985; Geoffroy et al., 1987). Iron has been shown to modify the surface hydrophobicity and protein profile of L. monocytogenes, resulting in a greater invasion of host cells (Conte et al., 1996). Although growth of this bacterium is completely inhibited in the absence of iron, addition of ferric citrate reverses the growth inhibition (Andre et al., 2003). Similar to Streptococcus pneumoniae, no siderophore synthesis has been detected in L. monocytogenes, and whole-genome sequence data of L. monocytogenes indicate that such genes are absent in this bacterium (Cowart & Foster, 1985; Tai et al., 1993; Glaser et al., 2001). It has been reported that L. monocytogenes can utilize transferrin, ferritin, lactoferrin, haemin, catecholamine, and siderophores produced by other bacteria in the environment, as sources of iron (Hartford et al., 1993; Jin et al., 2006; Mikael et al., 2002; Newton et al., 2005; Simon et al., 1995). Additionally, extracellular iron reductase activity has also been reported in this bacterium (Barchini & Cowart, 1996; Coulanges et al., 1997, 1998; Cowart & Foster, 1985; Deneer et al., 1995). Recent studies of this bacterium have shown that flu genes help in the uptake of ferric hydroxamate, but no change in iron transport is observed when fur alone is mutated (Jin et al., 2006).

Recently it has been shown that the disruption of fur in L. monocytogenes results in severe attenuation of virulence in a mouse model of infection (Rea et al., 2004; Newton et al., 2005). In our laboratory, we have been investigating the mechanisms of iron homeostasis in L. monocytogenes. Inspection of the L. monocytogenes genome sequences has shown the presence of genes for the ferric uptake regulator fur, and for the ferrichrome hydroxamate (flu) iron-acquisition system. To study the function of Fur, we have cloned, overexpressed and purified Fur from L. monocytogenes. Our electrophoretic mobility shift assay (EMSA) indicated that Fur protein can interact with its own promoter and a common promoter for fluD and fluC. These experiments showed that the interaction is specific, does not require iron, and that binding is not inhibited in the presence of EDTA. Furthermore, DNase I footprinting analysis of the fur promoter showed that addition of the Fur protein protects the consensus Fur box sequence independently of iron. However, Northern blot analysis showed that low levels of iron cause an increase in fur transcription; therefore, in vivo, fur is autoregulated.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used for this study are listed in Table 1. Escherichia coli strains were grown on Luria–Bertani (LB) agar or in LB broth, and L. monocytogenes was grown in Tryptic soy broth (TSB), or brain heart infusion (BHI, Difco) broth or agar media at 37°C. When selective pressure was required, the bacterial cultures were grown in the presence of ampicillin (50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹) or tetracycline (10 μg ml⁻¹).

**DNA and plasmid isolation.** DNA from L. monocytogenes was isolated using the DNA isolation kit (Promega) as described by Zhu et al. (2005). The plasmid DNA was purified according to the manufacturer’s protocol using a QIAprep plasmid isolation kit (Qiagen).

**fur gene cloning and overexpression.** Based on the DNA sequence of the L. monocytogenes fur gene, two oligonucleotide primers (upstream 5′-CGGGATCCATGGAAGGTCTGATGAC3′ and downstream 5′-GGAATTCTTGCTAATGGCAG-3′) were designed for amplification of the fur ORF by PCR. A BamHI restriction site was incorporated into the upstream primer and an EcoRI site into the downstream primer. PCR was performed using pfx DNA polymerase (Invitrogen), and the amplified fragment was cloned into PCR2.1 (Invitrogen), according to the manufacturer’s instructions. PCR2.1 containing fur was digested with BamHI and EcoRI, and the fragment was gel-purified and subcloned into pSETA (Invitrogen) to generate pLMo-fur. pLMo-fur was transformed into E. coli BLR(DE3)(pLysS) for protein expression. Overexpression and purification of Fur was done as described by Xiong et al. (2000). Overnight cultures of E. coli BLR(DE3)(pLysS) transformants were inoculated into 200 ml fresh LB broth and grown in a 1000 ml Erlenmeyer flask. When the culture reached OD₆₀₀ 0.4, the cells were induced for fur expression by adding 1 mM IPTG for 2 h. For purification of the Fur protein, the culture was centrifuged at 16,000 g at 4°C for 10 min. The cell pellet was resuspended in 5 ml binding buffer (1 mM PMSF, 500 mM NaCl, 20 mM Tris/HCl, 5 mM imidazole, pH 7.9). The cell suspension was subjected to sonication [10 pulses of 15 s, spaced 40 s apart, using a sonicator (Branson Ultrasonics) set at control 5 and 50% duty cycle]. The sonicated culture was centrifuged at 30,000 g for 15 min at 4°C, and the supernatant was applied to a nickel-charged histidine-binding resin column (Novagen) and eluted with 1 M imidazole. Eluted fractions were analysed by 15% SDS-PAGE, and fractions containing the overexpressed His-tagged Fur were dialysed against buffer (25 mM Tris/HCl, 10 mM MgCl₂, 5% v/v, glycerol, pH 7.8). The His-tag was then removed by digestion with Enterokinase (Invitrogen), according to the manufacturer’s protocol. The Fur protein and the His-tag were separated by passing them through a nickel affinity column. Finally, the Fur protein obtained in the flow-through was dialysed against buffer containing 25 mM
Tris/HCl, pH 7.8, 50 mM NaCl, 10 mM MgCl₂, 0.1 mM DTT and 5% (v/v) glycerol, and stored in the same buffer at −20°C. This protein was used for all the subsequent experiments.

**EMSA.** This assay was performed with amplified fluDC (165 bp) and fur (247 bp) promoters, using forward primers incorporated with XbaI and reverse primers with KpnI sites. Oligonucleotide primers for the fluDC promoter were: forward primer 5’-CGGGTCTAGATTGAAACCCTCTCTGTAAAC-3’ and reverse primer 5’-CTAGATTGAAACCCTCTCTGTAAAC-3’. For amplification of the fur promoter, forward primer 5’-CGGGTCTAGATTGAAACCCTCTCTGTAAAC-3’ and reverse primer 5’-CTAGATTGAAACCCTCTCTGTAAAC-3’ were used. PCR-amplified promoter sequences were digested with XbaI, and then end-labelled with [α-32P]dCTP (ICN Biochemicals) by a filling-in reaction using the Klenow fragment of DNA polymerase I, as described by Sambrook et al. (1989). For the binding assay, various concentrations of Fur protein were diluted in buffer (20 μl), and then incubated with [α-32P]dCTP (ICN Biochemicals) by a filling-in reaction using the Klenow fragment of DNA polymerase I, as described by Sambrook et al. (1989). For the binding assay, various concentrations of Fur protein were diluted in buffer (20 μl). A similar binding assay was performed for the fur promoter. To determine the specificity of the Fur protein for the promoters, a binding assay was performed with an amplified non-specific region between nt 2031032 and 2031196 of the L. monocytogenes genome using the oligonucleotide primers 5’-CTAGATTGAAACCCTCTCTGTAAAC-3’ and reverse primer 5’-CTAGATTGAAACCCTCTCTGTAAAC-3’.

**DNase I protection assay.** An assay was performed using a 247 bp amplified fur promoter DNA fragment cloned into PCR2.1. E. coli JM109 containing the PCR2.1 fur promoter was grown in 100 ml LB medium. Plasmid was isolated using Wizard Plus Maxiprep columns (Promega). Approximately 50 μg plasmid was digested with EcoRI followed by XbaI. The fragment was gel-purified and end-labelled by the Klenow filling reaction. A DNase I protection assay was performed in a 50 μl total volume of 1x binding buffer, as described by Leblanc & Moss (1994). This buffer contained 20 mM end-labelled DNA, 1 μg BSA and different concentrations of Fur protein. After initial incubation for 25 min at room temperature, 50 μl cofactor solution composed of 10 mM MgCl₂ and 5 mM CaCl₂ was added. Later, an enzyme DNase reaction was performed by adding 0.15 U DNase I, and after 1 min, the reaction was stopped by adding an equal volume of stop solution (100 μl 1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0). All of the samples were purified by extraction with phenol/chloroform followed by ethanol precipitation, before running on an 8% polyacrylamide sequencing gel. The DNase I protected region was identified by simultaneously performing AC and AG digestion reactions of Maxam–Gilbert nucleotide sequencing on the fur promoter, as described by Sambrook et al. (1989).

### Table 1. Strains and plasmids used in this study

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<td>E. coli JM109</td>
<td>recA1 supE44 hisdR17 gyrA96 relA1 thi (lac-proAB) F’(traD36 proAB + lacP’ M15)</td>
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<td>BLR(DE3)(pLysS)</td>
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**Iron homeostasis in L. monocytogenes**

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IP: 54.70.40.11
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analysed using inductively coupled plasma MS (ICP-MS) at the University of Illinois at Urbana-Champaign. Other molecular procedures were performed as described by Sambrook et al. (1989).

RESULTS AND DISCUSSION

Analysis of fur in the L. monocytogenes genome

Genome analysis of L. monocytogenes between nt 2 029 698 and 2 036 536 showed the presence of a 450 bp ORF (fur) and putative ferric hydroxamate-uptake genes fhuG, fhuB, fhuD and fhuC (Fig. 1a) (Glaser et al., 2001). Hydrophathy index analysis by the Kyte & Doolittle (1982) method has suggested that the Fhu proteins belong to the ATP-binding cassette transporter family. Additionally, molecular analyses by Jin et al. (2006) have indicated that FhuD of L. monocytogenes is similar to E. coli FhuD protein. Further analysis has shown that a conserved Fur box sequence is present in upstream promoter sequences of fur (lmo1956), fhuB (lmo1958), fhuD (lmo1959) and fhuC (lmo1960) (Fig. 1b, c), also reported earlier by Jin et al. (2006). Moreover, analysis of these Fur box-like sequences present upstream of fur, fhuD and fhuC has shown about five, six and seven mismatches, respectively, when compared to E. coli consensus Fur box sequence 5'-GATAATGATAATCATTATC-3'.

In the present analysis, an upstream region of L. monocytogenes fhuB also showed six mismatches with the consensus Fur box sequence (putative fhuB promoter sequence not shown).

Analysis of the fur gene showed that it encoded a putative 150 aa polypeptide with a pI of 5.9 and the apparent molecular mass of 17.3 kDa. Amino acid sequence analysis showed that this protein had 76 % identity to the B. subtilis Fur protein. In addition, the sequence at the C terminus indicated the presence of a conserved metal-binding domain (HxHHH), and four cysteine residues forming two motifs (CxxCG and CxxC) known to coordinate the binding of metal ions. In E. coli, Cys92 and Cys95 are present in the Fur protein, but cysteine residues were not observed at these positions in L. monocytogenes. The N-terminal sequence of the protein showed a helix–turn–helix (HTH) DNA binding motif commonly present in most metalloregulatory proteins. Hence, these features associated with the secondary structure of the Fur strongly suggest that it is a metallo-regulatory protein also.

Overexpression and purification of Fur

To determine the biochemical properties of Fur, the L. monocytogenes fur gene was cloned into pRSETa for overexpression and purification. The Fur protein was expressed by IPTG induction of the lac promoter and purified as a His-tagged protein using nickel affinity chromatography. Analysis on SDS-PAGE of the dialysed pure protein showed migration at ~21 kDa (Fig. 2). The activity of the purified protein was determined by performing a gel-shift analysis using fur and fhuDC promoters.

Fur protein and EMSA studies

To determine the activity of the purified His-tag-free Fur protein, EMSA was performed in the presence of lmo1959–fhuC (fhuDC) and fur promoters. On a 7.5 % non-denaturing polyacrylamide gel, 5 nM fhuDC promoter showed retarded migration upon addition of 0.21 μM Fur protein in either the presence or absence of Mn²⁺ (Fig. 3a, lanes 2 and 3). To avoid contamination by Fe²⁺, Mn²⁺ or other divalent cations, binding assays for the fhuDC promoter were performed in the presence of 1 and 2 mM EDTA (Fig. 3a, lanes 4 and 5). The results indicated that
the presence of EDTA does not inhibit the binding of Fur to the promoters. No binding was observed when 5 nM labelled non-specific DNA was used (Fig. 3a, lanes 6 and 7). In experiments with the \textit{fhuDC} promoter, binding was readily observed in the presence of 0.02 mM Fur (Fig. 3b, lanes 1–5). Further, this binding was competitively inhibited in the presence of 16-fold excess concentration of unlabelled promoter and 0.21 mM Fur protein (Fig. 3b, lanes 6–8).

### Interaction between Fur and its promoter

EMSA was performed with 5 nM end-labelled \textit{fur} promoter. Similar to the \textit{fhuDC} promoter, the binding of the Fur protein to its promoter did not require addition of Mn\textsuperscript{2+} or Fe\textsuperscript{2+}, nor was it inhibited in the presence of EDTA (data not shown). In the EMSA using 5 nM \textit{fur} promoter, a decrease in mobility was observed on addition of \( \geq 0.9 \) mM Fur protein (Fig. 4). The binding was competitively inhibited when ninefold excess cold \textit{fur} promoter was added (Fig. 4, lanes 4 and 5). Based on the EMSA, it seems that a greater amount of Fur protein is required to cause a shift of the \textit{fur} promoter compared to that of the \textit{fhuDC} promoter.

In several bacteria, Fur has been described as an iron-responsive repressor protein. Additionally, Fur is known to be active in a dimeric form in which each monomer is believed to bind one Fe\textsuperscript{2+} ion. Previous studies have shown that the N-terminal region of Fur is required for interaction with DNA, whereas the C-terminal region is necessary for dimerization and metal binding (Coy & Neilands, 1991; Stojilkovic & Hantke, 1995; Gonzalez de Peredo et al., 1999, 2001). It is also believed that Fe\textsuperscript{2+} present in the metal-binding site can be exchanged with Mn\textsuperscript{2+}. Our experiments indicate that the Fur protein does not carry any intrinsic Fe\textsuperscript{2+} or Mn\textsuperscript{2+}, and the activity does not require addition of divalent metal ions. Although Fur is generally referred to as an iron-dependent repressor protein, studies on \textit{B. subtilis} have shown that binding of Fur to the promoter of the \textit{dbh} operon, which is involved in dihydroxybenzoate siderophore biosynthesis, does not require iron (Bsat & Helmann, 1999). Generally, it is believed that the C-terminus of the Fur protein monomer has metal-binding sites occupied by Zn\textsuperscript{2+} and Fe\textsuperscript{2+} ions (Jacquamet et al., 1998). Such intrinsic divalent metal ions in the protein are difficult to chelate by EDTA.

ICP-MS analysis showed that there was no Fe\textsuperscript{2+} or Mn\textsuperscript{2+} present, but 0.48 ± 0.09 mole zinc per mol protein was observed. Based on metal-analysis data, it seems that only 50% of the protein molecules have zinc bound to them. A
low ratio of zinc ions to Fur protein has been reported in E. coli (Jacquamet et al., 1998; Smith et al., 1996). Additionally, in E. coli and Pseudomonas aeruginosa, it has been shown that zinc alone may have a role in the activity of the Fur protein (Althaus et al., 1999; Ochsner et al., 1995). Lewin et al. (2002) have demonstrated that Fur with zinc alone at the metal-sensing site can actively bind to the pvds promoter in P. aeruginosa. Hence, in L. monocytogenes, we believe that Fur containing zinc, but not metal-free Fur, is responsible for binding in vitro. Experiments are under way in our laboratory to validate this hypothesis.

**DNase I protection assay**

The assay was performed to show that the Fur protein interacts at specific nucleotides on the fur promoter. Fur proteins are generally known to protect about 27−30 bp of promoter DNA (Ochsner et al., 1995). A 247 bp EcoRI/XbaI-digested fur promoter fragment was isolated from PCR2.1-fur and labelled by the Klenow filling reaction at the XbaI site, for DNase I footprint analysis. As described in Methods, 20 nM end-labelled fur promoter fragment was incubated for 25 min with various amounts of Fur protein, and then digested with 0.15 U DNase I. As predicted, protection of ~29 nt on the fur promoter DNA was clearly observed in the presence of 11.6 μM Fur protein, in the absence of Fe2+ and Mn2+. The protected area was compared to standard AC and AG sequencing reactions of the fur promoter generated by the Maxam–Gilbert method (Sambrook et al., 1989). Comparative analysis revealed that the protected region comprised 19 bp with a sequence of 5′-GATAATGATGATAATTAG-3′, which is similar to the consensus Fur box found in most bacterial promoters under the control of the fur regulon (Fig. 5). Experiments using the 165 bp fhuDC promoter in the absence of Fe2+ and Mn2+ also showed protection of both the Fur box sequences shown in Fig. 1(c) (DNase protection assay data for the fhuDC promoter are not shown). Thus, the DNase I-protected nucleotide sequence is shown on the right.

**Effect of iron on fur transcription**

RNA was isolated from L. monocytogenes grown in TSB containing either a low or a high level of iron. Northern blot analysis showed a change in fur mRNA transcription due to differences in the amount of iron available in the medium (Fig. 6a, b). Based on the data, fur appeared to be auto-regulated, as seen in E. coli (de Lorenzo et al., 1988; Escolar et al., 1999). The transcription of fur was repressed in the presence of 100 and 500 μM ferric citrate. These data indicate that iron strongly regulates fur gene expression. From our in vitro studies, however, the binding of Fur to its own promoter appears to be independent of the level of iron. In our preliminary experiments, we observed expression of fur when L. monocytogenes was grown in TSB or BHI medium without any iron-chelating agent (data not shown). However, to mimic iron-depleted conditions, medium was chelated with tropolone and supplemented with 20 μM ferric citrate. We observed that the bacteria grew slowly in medium supplemented with 20 μM ferric citrate when compared to 100 or 500 μM ferric citrate. Based on the Northern blot data, we suggest that L. monocytogenes generally expresses a basal level of Fur protein but, when iron
Fig. 6. (a) Northern blot analysis of fur in L. monocytogenes. Total RNA was isolated from cultures grown in TSB treated with 20 μM tropolone, followed by addition of 20, 100 or 500 μM ferric citrate. Thirty micrograms total RNA from each sample was electrophoresed on a 1.4 % formaldehyde gel under denaturing conditions, transferred to nitrocellulose, and probed with radiolabelled fur. Lanes 1, 2 and 3, cells grown in the presence of 20, 100 and 500 μM ferric citrate, respectively. The probe hybridized with a single transcript of ~0.45 kb, as indicated by the arrow. (b) Thirty micrograms total RNA isolated from cultures grown in TSB treated with 20 μM tropolone, followed by addition of 20, 100 or 500 μM ferric citrate, were electrophoresed on a 1.4 % formaldehyde gel under denaturing conditions and stained with ethidium bromide.

levels are in excess, the protein represses transcription of its own expression and that of iron-uptake genes under its control, to maintain homeostasis.

In B. subtilis, in vitro experiments have shown that purified Fur can actively bind to the dhb promoter without addition of any divalent metal ions (Bsat & Helmann, 1999). Previous experiments have shown that Fur does not bind to the regulatory region of fur, and this gene is under the control of PerR (Fuangthong et al., 2002). These authors have proposed that the absence of intracellular inhibitory factors is responsible for the in vitro iron-independent binding activity of the Fur protein. Further, in vivo genetic analysis has indicated that direct interaction of iron with Fur is required for repression activity. Thus, it is possible that the absence of inhibitory factors, or the high sensitivity and level of activation of this protein under the assay conditions we have used, is responsible for the in vitro iron-independent activity of the L. monocytogenes Fur protein. The in vitro binding ability of Fur to the Fur box sequence present in its own promoter, and the change in fur expression in response to iron availability, indicate that Fur may autoregulate its own expression.

In contrast to the classical iron-dependent regulation of fur, regulation of fur in Anabaena and Vibrio cholerae is not sensitive to iron. In Anabaena, Fur binds to its own promoter, and the gene regulation is insensitive to the concentration of iron (Hernandez et al., 2002). In V. cholerae, Brad. japonicum, Bartonella and Staphylococcus aureus, no significant iron-box sequences have been identified in the upstream region of fur, indicating non-autoregulation of fur (Hamza et al., 1999; Litwin et al., 1992; Park et al., 2001; Xiong et al., 2000). Similarly, studies in Brad. japonicum indicate that regulation of many iron-responsive genes is not Fur-mediated, and that fur expression is constitutive and insensitive to iron (Hamza et al., 2000; Nienaber et al., 2001). In this bacterium, irr regulation is iron-dependent and mediated by Fur, but the binding to the promoter is at a sequence dissimilar to the Fur box (Hamza et al., 2000). Furthermore, in some bacteria, Fur also plays a dual role. For example, in Helicobacter pylori, Fur seems to actively bind to promoters in the absence and presence of iron. In this study, Fur was shown to bind sodB and pf promoters and repress transcription in the absence of iron, although the regulation of frpB by Fur has been shown to be iron-dependent (Delany et al., 2001). However, the interactions between the Fur-binding sequences in the sodB promoter are not similar to those generally found for Fur boxes, and in the pf promoter, the binding appears to involve Fur boxes along with additional sequences. In this bacterium, Fur has been shown to autoregulate its own expression by binding to three different operators containing Fur-box-like sequences with about six and eight mismatches in operators I and II, respectively, and 10 mismatches in operator III, when compared with E. coli consensus Fur box sequences (Delany et al., 2003). Here, binding of protein to one of the operators was iron-independent. Delany et al. (2003) have proposed that the iron-independent binding activity of the Fur protein to this operator exists to protect complete repression of fur. From the above-mentioned studies, Fur appears to differ among bacteria. Thus, we suggest that this protein in L. monocytogenes is another divergent form of Fur, whose regulation and expression are influenced by the concentration of iron.

In conclusion, in vitro binding to the Fur box in the promoters is not dependent on iron or inhibited in the absence of divalent ions, but, in vivo, L. monocytogenes fur is autoregulated in response to iron. L. monocytogenes can proliferate in a remarkably diverse range of environments, ranging from soil and decaying vegetation, in which it has been described as expressing its benign environmental ‘Dr Jekyll’ personality, to another life as an intracellular pathogen (‘Mr Hyde’) (Gray et al., 2006). Profound changes in gene expression occur in L. monocytogenes in different environments. Chatterjee et al. (2006) have recently reported the transcriptomes of the organism in the vacuolar compartment and cytosol of infected cells. The environments in which L. monocytogenes can grow are expected to vary greatly in their iron availability. Undoubtedly, Fur is an important player in the iron homeostasis of Listeria, and it is tempting to speculate that the apparent independence of Fur from iron plays a role in the ability of the organism to grow in these diverse environments.
ACKNOWLEDGEMENTS

We would like to thank Anthony Otsuka for critical reading of the manuscript. This work was partially supported by grant R15-GM071363-01 from the National Institutes of Health to R.K.J.

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