Molecular characterization of the Fur protein of *Listeria monocytogenes*

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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.

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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 haemin for 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 (Escobar *et al.*, 1999; Dorman, 1994). In *Bacillus subtilis*, Fur functions as a regulator of iron uptake (Bsai *et al.*, 1998). In corynebacteria and mycobacteria, the iron-uptake process is regulated by a Fur functional

Abbreviations: EMSA, electrophoretic mobility shift assay; ICP-MS, inductively coupled plasma MS.

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 δ -aminolaevulinic 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 *fhu* 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 (*fhu*) 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 *fhuD* and *fhuC*. 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'-CGGGATCCATGGAAGGTCGTATTGGACG-3' and downstream 5'-GGAATTCTTATCCAAACTCTCGCATT-3') were designed for amplification of the *fur* ORF by PCR. A *Bam*HI restriction site was incorporated into the upstream primer and an *Eco*RI 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 *Bam*HI and *Eco*RI, and the fragment was gel-purified and subcloned into pRSETa (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

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
<i>L. monocytogenes</i>		
10403S	Serotype 1/2a	Bishop & Hinrichs (1987)
<i>E. coli</i>		
JM109	<i>recA1 supE44 hsdR17 gyRA96 relA1 thi (lac-proAB) F'(traD36 proAB⁺ lacI^qZ M15)</i>	Yanish-Perron <i>et al.</i> (1985)
BLR(DE3)(pLysS)	<i>F⁻ ompT hsdS_B(r_B⁻ m_B⁻) gal dcm (srl-recA) 306::Tn10(DE3) pLysS Cam^r Tet^r</i>	Novagen
BLR(DE3)(pLysS)- <i>fur</i>	BLR(DE3)(pLysS) containing pLmo- <i>fur</i>	This work
Plasmids		
PCR2.1	PCR product cloning vector; Amp ^r Kan ^r	Invitrogen
PCR2.1- <i>fur</i>	PCR2.1 containing PCR product of <i>fur</i> ORF from <i>L. monocytogenes</i>	This work
pRSETa	Overexpression vector	Invitrogen
pLmo- <i>fur</i>	pRSETa containing <i>fur</i> cloned at <i>Bam</i> HI and <i>Eco</i> RI; Amp ^r	This work

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 *fhvDC* (165 bp) and *fur* (247 bp) promoters, using forward primers incorporated with *Xba*I and reverse primers with *Kpn*I sites. Oligonucleotide primers for the *fhvDC* promoter were: forward primer 5'-CGCGTCTAGATTGAACCCCTCCTGTAAC-3' and reverse primer 5'-CGTCAGGTACCTTTTCATCGCTCCAATTTA-3'. For amplification of the *fur* promoter, forward primer 5'-CGCGTCTAGATAATAATAATTATTGATAACGAGAA-3' and reverse primer 5'-CGTCAGGTACCTATAGTTCCCTCCCTTGGTT-3' were used. PCR-amplified promoter sequences were digested with *Xba*I, 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 equilibrated by incubating in 20 μ l binding buffer (20 mM Tris-acetate/HCl, pH 8, 1 mM MgCl₂, 50 mM KCl, 5%, v/v, glycerol, 0.5 mM DTT) with 1 μ g BSA and 1 μ g sheared calf thymus DNA for 15 min at room temperature. To these buffer samples containing different concentrations of Fur protein, 5 nM end-labelled *fhvDC* or *fur* promoter was added, and samples were incubated for an additional 15 min at room temperature. For each sample, 3 μ l loading buffer (1 $\times$ binding buffer containing 50% glycerol (v/v) and 0.002% bromophenol blue) was added, and the sample was loaded onto a 7.5% non-denaturing PAGE gel. Gel electrophoresis was performed in buffer containing 20 mM Tris-acetate (pH 8) and 0.1 mM DTT for 2.5 h at 90 V and 4 °C. The gels were dried and visualized by exposure to X-ray film. Binding-assay experiments were also performed for the *fhvDC* promoter in the above buffer with 1 and 2 mM EDTA. A similar binding assay was also 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'-TGTTCTAGACAGTGTGAGGTGCAATATCT-3' and 5'-CGGGATCCATGGAAGGTCGTATTGGACG-3'.

DNase I protection assay. An experiment 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 μ g plasmid was digested with *Eco*RI followed by *Xba*I. The fragment was gel-purified and end-labelled by the Klenow filling reaction. A DNase I protection assay was performed in a 50 μ l total volume of 1 $\times$ binding buffer, as described by Leblanc & Moss (1994). This buffer contained 20 nM end-labelled DNA, 1 μ g BSA and different concentrations of Fur protein. After initial incubation for 25 min at room temperature, 50 μ l cofactor solution composed of 10 mM MgCl₂ and 5 mM CaCl₂ was added. Later, an enzymic 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 μ l 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).

Fur transcription analysis in response to iron availability. For *fur* gene transcription analysis, *L. monocytogenes* cultures were grown overnight and diluted 1:100 in 5 ml TSB containing 20 μ M tropolone, supplemented with various concentrations of ferric citrate, and grown at 37 °C with constant shaking, as described by Andre *et al.* (2003). Ferric citrate (20 μ M) was added to achieve a low level of iron in the medium, and for high levels of iron, 100 and 500 μ M ferric citrate were added. When the cultures reached OD₆₀₀ 0.7, the cells were harvested by centrifugation and total RNA was isolated using an RNeasy Mini kit (Qiagen). Equal amounts of RNA (30 μ g) for each condition were electrophoresed in a 1% agarose/0.66 M formaldehyde gel. The RNA was transferred to a nylon membrane (Millipore), and the blot was probed with radiolabelled *fur* probe under aqueous-phase conditions at 65 °C. The probe was prepared using the Prime-a-Gene labelling system (Promega) in the presence of [α -³²P]dCTP (specific activity > 3000 Ci mmol⁻¹; > 111 TBq mmol⁻¹).

Other biochemical and analytical methods. The concentration of total protein in different preparations was determined using the Bradford reagent (Pierce). Metal content of the purified Fur was

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 column 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

Fig. 1. (a) Schematic organization of *fur* and *fhu* loci in *L. monocytogenes*. The identity and position of *fur* and *fhu* genes are shown. Arrows indicate orientation of the ORFs, and empty boxes represent promoter regions containing consensus Fur box sequences. (b) Nucleotide sequence containing a putative Fur box (enclosed) sequence upstream of *fur* (*lmo1956*). A Shine–Dalgarno sequence is underlined and the nucleotide sequence ATG, encoding the first amino acid methionine, is shown in bold type. (c) Nucleotide sequence between *fhuD* (*lmo1959*) and *fhuC* (*lmo1960*). Putative Fur box sequences are enclosed in boxes, the Shine–Dalgarno sequences are underlined, and the nucleotide sequences TTG and ATG, encoding the first amino acid methionine for FhuD and FhuC, respectively, are shown in bold type.

Fig. 2. SDS-PAGE of Fur. His-tagged Fur was overexpressed in *E. coli* and purified as described in Methods. Lane 1, standard protein markers; lane 2, uninduced cell lysate of *E. coli* with pLMO-*fur*; lane 3, IPTG-induced cell lysate of *E. coli* carrying pLMO-*fur*; lane 4, 5 µg pure protein fraction of His-tagged Fur (indicated by arrow).

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 *fhuDC* promoter, binding was readily observed in the presence of 0.02 µM 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 µM Fur protein (Fig. 3b, lanes 6–8).

Interaction between Fur and its promoter

EMSA was performed with 5 nM end-labelled *fur* promoter. Similar to the *fhuDC* promoter, the binding of the Fur protein to its promoter did not require addition of Mn^{2+} or Fe^{2+} , nor was it inhibited in the presence of EDTA (data not shown). In the EMSA using 5 nM *fur* promoter, a decrease in mobility was observed on addition of ≥ 0.9 µM Fur protein (Fig. 4). The binding was competitively inhibited when ninefold excess cold *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 *fur* promoter compared to that of the *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^{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; Stojiljkovic & Hantke, 1995; Gonzalez de Peredo *et al.*, 1999, 2001). It is also believed that Fe^{2+} present in the metal-binding site can be exchanged with Mn^{2+} . Our experiments indicate that the Fur protein does not carry any intrinsic

Fig. 3. (a) EMSA of Fur binding to 5 nM end-labelled *fhuDC* promoter (lanes 1–5) or non-specific DNA sequence (lanes 6 and 7). Lane 1, *fhuDC* promoter without Fur protein; lane 2, end-labelled *fhuDC* promoter with 0.21 µM Fur in the presence of 100 µM MnCl_2 ; lane 3, end-labelled *fhuDC* promoter with 0.21 µM Fur in the absence of 100 µM MnCl_2 ; lane 4, end-labelled *fhuDC* promoter with 0.21 µM Fur in the presence of 1 mM EDTA; lane 5, end-labelled *fhuDC* promoter with 0.21 µM Fur in the presence of 2 mM EDTA; lane 6, end-labelled non-specific DNA without Fur; lane 7, end-labelled non-specific DNA with 0.21 µM Fur. C and F indicate promoter DNA bound or unbound to the Fur protein, respectively. (b) EMSA of Fur binding to the *fhuDC* promoter. Lane 1, 5 nM end-labelled *fhuDC* promoter without Fur protein; lanes 2–5, 5 nM end-labelled *fhuDC* promoter with 0.02, 0.085, 0.14 or 0.21 µM Fur; lanes 6–8, EMSA competition assay using 5 nM end-labelled *fhuDC* promoter with 0.21 µM Fur and 20, 40 or 80 nM cold *fhuDC* promoter. C and F indicate promoter DNA bound or unbound to the Fur protein, respectively.

Fe^{2+} or Mn^{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 *B. subtilis* have shown that binding of Fur to the promoter of the *dhb* 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^{2+} and Fe^{2+} ions (Jacquemet *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^{2+} or Mn^{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

Fig. 4. EMSA of Fur binding to the *fur* promoter. Lane 1, 5 nM end-labelled *fur* promoter without Fur protein; lanes 2–5, 5 nM end-labelled *fur* promoter with 0.6, 0.75, 0.9 or 1.1 μ M Fur; lanes 6–8, 5 nM end-labelled *fur* promoter with 1.1 μ M Fur protein and 5, 15 or 45 nM cold *fur* promoter. C and F indicate promoter DNA bound or unbound to the Fur protein, respectively.

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 μ M Fur protein, in the absence of Fe^{2+} and Mn^{2+} . 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'-GATAATGATGATAATTTAG-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 Fe^{2+} and Mn^{2+} 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 protection assay further confirms the iron-independent binding of Fur to Fur box sequences.

Fig. 5. Mapping of the binding site of Fur on the *fur* promoter region. Lanes 1 and 2, AC and AG reactions, respectively, of 20 nM end-labelled *fur* promoter digested with 0.15 U DNase I; lanes 3–5, DNase I-digested *fur* promoter with 5.6, 7.6 or 11.6 μ M Fur protein; lane 6, DNase I-digested *fur* promoter in the absence of Fur protein. 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; Escobar *et al.*, 1999). The transcription of *fur* was repressed in the presence of 100 and 500 μ M 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 μ M ferric citrate. We observed that the bacteria grew slowly in medium supplemented with 20 μ M ferric citrate when compared to 100 or 500 μ M 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 μ M tropolone, followed by addition of 20, 100 or 500 μ M 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 μ M ferric citrate, respectively. The probe hybridized with a single transcript of $\sim$ 0.45 kb, as indicated by the arrow. (b) Thirty micrograms total RNA isolated from cultures grown in TSB treated with 20 μ M tropolone, followed by addition of 20, 100 or 500 μ M 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 *pfr* 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 *pfr* 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.

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