The Dps-like protein Fri of *Listeria monocytogenes* promotes stress tolerance and intracellular multiplication in macrophage-like cells

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INTRODUCTION

*Listeria monocytogenes* is a Gram-positive food-borne pathogen capable of causing listeriosis in humans and animals. The natural route of infection is through the gastro-intestinal passage, following ingestion of contaminated food (Farber & Peterkin, 2000; Portnoy *et al*., 2002; Vázquez-Boland *et al*., 2001). Following infection, the mammalian host has several strategies to defend itself against invading microbes. One of these is to decrease the amount of available iron by binding free extracellular iron to iron-binding proteins such as transferrin and lactoferrin (Hartford *et al*., 1993). Iron is essential for most bacterial species, as it functions as a co-factor in different proteins, is involved in various cellular functions, and is a major environmental stimulus capable of regulating virulence gene expression in many bacterial pathogens (Adams *et al*., 1990; Coulanges *et al*., 1996). In addition to the low concentration of free iron, the bacteria are faced with severe oxidative stress due to the release of reactive oxygen intermediates, such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), from the phagolysosome. These compounds are only mildly reactive, but they can be transformed into the highly reactive hydroxyl radical (OH·) in the presence of iron, and cause severe damage to proteins, DNA and lipids (Henle & Linn, 1997; Luo *et al*., 1994).

In order to avoid the production of OH·, bacteria store iron in a manner that prevents the participation of iron in these reactions. Two major families of proteins are involved in sequestering iron: the haem-containing bacterioferritins and the non-haem ferritins (Andrews, 1998). In addition, members of the Dps protein family resemble the ferritins as they form spherical protein complexes with the ability to bind iron, and have been implicated in tolerance to oxidative stress. In this study of the food-borne pathogen *Listeria monocytogenes*, the *fri* gene encoding a Dps homologue was deleted, and, compared to wild-type cells, it was found that the resulting mutant was less resistant to hydrogen peroxide, and demonstrated reduced survival following long-term (7–11 days) incubation in laboratory media. In view of this, it is shown that *fri* gene expression is controlled by the hydrogen peroxide regulator PerR, as well as the general stress sigma factor σB. When *fri* mutant cells were transferred to iron-limiting conditions, growth was retarded relative to wild-type cells, indicating that Fri may be required for iron storage. This notion is supported by the observation that the *L. monocytogenes* genome appears not to encode other ferritin-like proteins. Given the role of Fri in resistance to oxidative stress, and growth under iron-limiting conditions, the ability of the *fri* mutant to infect mice was examined. When injected by the intraperitoneal route, the *fri* mutant demonstrated a reduced capacity to proliferate in the organs of infected mice relative to the wild-type, whereas when the bacteria were supplied intravenously this effect was mitigated. In addition, the mutant was impaired in its ability to survive and grow in J774.A1 mouse macrophage cells. Thus, the data suggest that Fri contributes to the ability of *L. monocytogenes* to survive in environments where oxidative stress and low iron availability may impede bacterial proliferation.
bind iron (Bozzi et al., 1997; Grant et al., 1998; Ilari et al., 2000; Tonello et al., 1999; Yamamoto et al., 2002; Zhao et al., 2002). While the ferritins consist of 24 subunits binding more than 4000 iron atoms (Andrews, 1998), the Dps homologues form dodecamers that can bind approximately 500 iron molecules (Bozzi et al., 1997; Ishikawa et al., 2003; Tonello et al., 1999; Yamamoto et al., 2002; Zhao et al., 2002). Dps was originally identified in *Escherichia coli* as one of the major proteins induced in late stationary growth phase (Altuvia et al., 1994). During exponential growth, it is induced by carbon and nitrogen starvation, osmotic stress or the presence of H$_2$O$_2$. The key regulatory elements include RpoS, IHF and OxyR (Altuvia et al., 1994; Lomovskaya et al., 1994; Michán et al., 1999). Mutants lacking *dps* are sensitive to H$_2$O$_2$ in stationary growth phase, and have an increased rate of some base substitutions caused by oxidative agents (Martinez & Kolter, 1997). *In vitro* and *in situ* studies have revealed that Dps binds DNA in a non-sequence-specific manner, and it has been proposed that Dps has a dual role in protecting cells against oxidative stress, either by binding directly to DNA (Altuvia et al., 1994; Martinez & Kolter, 1997; Wolf et al., 1999), or by sequestering iron, thus avoiding the oxidative damage mediated by Fenton chemistry (Zhao et al., 2002).

Dps homologues are found in several Gram-negative and Gram-positive bacteria. While most of these are able to bind iron, some members appear unable to bind DNA, including the *Helicobacter pylori* NapA, the *Listeria innocua* ferritin-like protein, the *Streptococcus mutans* Dpr, and Dlp-1/Dlp-2 from *Bacillus anthracis* (Bozzi et al., 1997; Papinutto et al., 2002; Tonello et al., 1999; Yamamoto et al., 2002). In addition to the *E. coli* Dps, DNA binding has been demonstrated for the *Bacillus subtilis* MrgA, the *Synechococcus* DpsA and the *Mycobacterium smegmatis* Dps (Chen & Helmann, 1995; Gupta & Chatterji, 2003; Gupta et al., 2002; Peña & Bullerjahn, 1995). In a recent report, it was suggested that DNA binding requires either an extended C-terminus, or positively charged residues in the N-terminus of Dps homologues, which allow a disordered and flexible structure (Ceci et al., 2003). *L. monocytogenes* also contains a homologue of Dps named Flp (Hébraud & Guzzo, 2000) or Fri (Glaser et al., 2001; Polidoro et al., 2002). We have chosen to use the latter designation to avoid confusion with the Flp recombinate system (Sadowski, 1986). Previous studies have revealed that *fri* expression is induced by a number of stress conditions, including heat and cold shock, and to a lesser extent SDS, ethanol and deoxycholate (Hébraud & Guzzo, 2000; Liu et al., 2002; Phan-Thanh & Gorman, 1995). Northern blot analysis showed that induction by heat and cold occurred at the transcriptional level, and that the Fri protein is expressed from a monocistronic mRNA (Hébraud & Guzzo, 2000). Furthermore, the Fri amino acid sequence is 98% identical to the *L. innocua* Dps homologue that binds iron (Bozzi et al., 1997), and is induced by iron-limiting conditions (Polidoro et al., 2002). Although the mechanisms by which *L. monocytogenes* acquires iron are obscure (Brown & Holden, 2002; Cowart & Foster, 1985), several studies have shown that iron availability plays an important role in virulence (Bockmann et al., 1996; Conte et al., 1996, 2000; Coulanges et al., 1996; Sword, 1966). To determine if Fri contributes to virulence, we have deleted the corresponding gene in *L. monocytogenes* EGD, and analysed the resulting mutant.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *L. monocytogenes* EGD was obtained from Werner Goebel (Biozentrum). The sigB mutant derivative was obtained from Lone Brøndsted (Brondsted et al., 2003), and the *perR* mutant was constructed as described by Rea et al. (2004). Strains were grown in rich growth medium (brain heart infusion, BHI; Oxoid) or improved minimal medium (Phan-Thanh & Gorman, 1997), with continuous shaking at 37°C unless otherwise indicated. When required, erythromycin was added to a final concentration of 5 μg ml$^{-1}$. Investigations were carried out with strains grown to stationary (OD$_{600}$ ~2) or exponential phase (OD$_{600}$ ~0.5). *E. coli* TOP 10 (Invitrogen) was grown routinely at 37°C in Luria–Bertani broth (LB; Oxoid), with continuous shaking at 37°C. When required, erythromycin was added to a final concentration of 150 μg ml$^{-1}$.

**Stress tolerance assays.** For investigation of the ability to form c.f.u. when subjected to iron deprivation, *L. monocytogenes* strains were grown in BHI until stationary phase, and transferred to BHI agar treated with 50 mg ml$^{-1}$ Chelex 100 (Sigma-Aldrich). The ability to grow in iron-deprived broth was investigated by growing the strains to exponential phase in BHI, and shifting the cells to improved minimal medium containing 0 or 335 mM ferric citrate.

Survival during oxidative stress was examined by growing cultures to either stationary or exponential phase, washing once in saline (5000 g, 5 min), and resuspending in saline containing H$_2$O$_2$ at concentrations of 50 mM (stationary phase) or 20 mM (exponential phase). The cells were incubated at 37°C, and the samples were collected at different time points, serially diluted, and plated onto BHI agar. The numbers of c.f.u. were counted after 24 h incubation at 37°C.

Survival during long-term growth was examined by daily withdrawing samples for determination of c.f.u. from cultures grown to stationary growth phase in BHI, and then left with shaking at 37°C for a period of 11 days.

**DNA manipulations.** Extraction of plasmid DNA from *E. coli* was performed using the QiAprep Spin Miniprep Kit (Qiagen), as recommended by the manufacturer. *E. coli* was transformed as described by Cohen et al. (1972), and electrottransformation of *L. monocytogenes* was achieved as described elsewhere (Dramsi et al., 1995). PCR reagents (*Taq* polymerase and deoxynucleoside triphosphates) were purchased from Roche, and used according to the manufacturer’s instructions.

**Primer extension analysis.** Total RNA was extracted using the Nucleospin RNA extraction kit (Macherey-Nagel) according to the manufacturer’s directions. The bacterial cells were lysed by incubation with 10 mg lysozyme ml$^{-1}$ and 200 μl glass beads (106 μm and finer) (Sigma-Aldrich). Three times during the 10 min incubation period, the cell suspension was mixed by vortexing for 30 s. Primer extension analysis was performed as described by Michán et al. (1999) using 3 mg total RNA per reaction. A 5′ 32P-labelled primer, dps-B (see below), was used for detection of *fri* transcription start sites.

**Construction of the *fri* deletion mutant.** A *L. monocytogenes* mutant with an internal 381 bp deletion in the *fri* gene was...
constructed by the splicing by overlap extension PCR procedure (Horton et al., 1990). Primers were designed to amplify two fragments (~600 and ~300 bp, respectively), one comprising the 5’ end of fri, which was amplified by primers dps-a (5’-TGC TCT AGA CCC AAA ACC ACC AAT AAA GTC ATT GG-3’) and dps-b (5’-CCG TAC TTG GTG ATT C-3’), and the other comprising the 3’ end of the gene, which was amplified by primers dps-c (5’-GAA TCA CCA AGT AGC GGA CAA ACA TAT CTG GAT G-3’) and dps-d (5’-TAT CCC AAG CTG CAT GAA ACC ATT TAT TTG CTT GGT CCG-3’).

The resulting products were gel extracted, mixed in a 1:1 ratio, and reamplified using the dps-a and dps-d primers. The amplified 948 bp product was digested with XbaI and HindIII, and inserted into the temperature-sensitive shuttle vector PAUL-A (Schaferkordt & Chakraborty, 1995). The resultant plasmid was electroporated into L. monocytogenes, and forced chromosomal integration of the plasmid was achieved by growing transformed strains at 42 °C in the presence of erythromycin. In order to allow allelic exchange between the intact gene and the truncated gene to take place, as well as subsequent loss of erythromycin. In order to allow allelic exchange between the intact gene and the truncated gene to take place, as well as subsequent loss of erythromycin. In order to allow allelic exchange between the intact gene and the truncated gene to take place, as well as subsequent loss of erythromycin. In order to allow allelic exchange between the intact gene and the truncated gene to take place, as well as subsequent loss of erythromycin.

Macrophage assay. The murine-macrophage-like cell line J774.A1 was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing Glutamax (Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco). Cells were cultured in 5 % CO₂ at 37 °C, and were seeded at a density of approximately 5 × 10⁵ cells ml⁻¹ per well in 24-well tissue culture plates for invasion assays. Monolayers produced after 24 h incubation in 5 % CO₂ at 37 °C were used for infection studies. Bacteria grown in BHI broth to stationary phase or exponential phase were pelleted by centrifugation, washed once with PBS (0.15 M sodium chloride, pH 7.4), and adjusted to a concentration of 5 × 10⁶ c.f.u. ml⁻¹. The bacteria were then sononized with 10 % heat-inactivated fetal bovine serum for 30 min at 37 °C, and subsequently washed with PBS. Macrophages were infected at an m.o.i. of 10 bacteria per cell in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (time zero of the assay). Contact between bacteria and cells was facilitated by centrifugation (150 g, 5 min). After a 1 h incubation, the cell monolayer was washed with Hanks’ balanced salt solution (HBSS) containing 10 mM HEPES (Gibco), and then overlaid with fresh cell culture medium containing 100 μg gentamicin ml⁻¹ (Gibco) to kill extracellular bacteria. After 1 h, the cell monolayer was washed with HBSS containing 10 mM HEPES, and 1 ml fresh medium containing 25 μg gentamicin ml⁻¹ was added to each well. Thereafter, at defined time points, the cell monolayers were washed with HBSS, and subsequently lysed with 1 ml 0.1 % Triton X-100 (Sigma). Lysates were serially diluted 10-fold, plated on BHI agar, and c.f.u. were determined after 24 h incubation at 37 °C. The experiments were performed twice, with duplicate determinations per time point.

**Mouse virulence assay (intraperitoneal).** Five 6-week-old female BALB/c mice were inoculated intraperitoneally with either 100 μl L. monocytogenes grown in 50 ml BHI for 18 h at 37 °C, or 100 μl L. monocytogenes grown to the exponential phase; both cultures were diluted in PBS, pH 7.2, to approximately 2 × 10⁶ ml⁻¹. Numbers of c.f.u. in the inocula were determined by plate-spreading on BHI agar. After 3 days, mice were killed by cervical dislocation, and livers and spleens were removed and homogenized in 0.15 M NaCl. Tenfold serial dilutions were plated in duplicate on BHI agar to determine the numbers of c.f.u. in the organs. The experiments were performed twice. All mice were treated in accordance with institutional guidelines for treatment of animals. The data were analysed statistically by analysis of variance, examining the effects of the strain and the run (experiment). In order to achieve a uniform variance, the bacterial counts were log transformed before the data were analysed. Measurement of a strain consisted of five mice per run and two runs. Exponential- and stationary-phase cultures were analysed separately. The variance analysis was carried out by the GLM procedure in SAS (SAS Institute). There were no significant interactions between the strain and the run.

**Mouse virulence assay (intravenous).** Five 8-week-old female BALB/c mice were inoculated intravenously with 50 μl L. monocytogenes, which had been grown in 50 ml BHI for 18 h at 37 °C, and then diluted 25-fold in PBS, pH 7.2. Numbers of c.f.u. in the inocula were determined by plate-spreading on BHI agar. After 1, 6 and 48 h, mice were killed by cervical dislocation, and livers were removed and homogenized in 0-15 M NaCl. Tenfold serial dilutions were plated in duplicate on BHI agar. All mice were treated in accordance with institutional guidelines for treatment of animals.

**Lecithinase and haemolysin activity.** Using charcoal-supplemented egg-yolk agar plates. PlcB activity was estimated as the size of a halo of precipitation around the bacterial colony, essentially as described by Ermolaeva et al. (2003). The concentration of activated charcoal was 0.2 %. Ten per cent of an egg-yolk suspension, prepared by adding one fresh egg yolk to 25 ml sterile saline, was added to molten BHI agar. Inoculated egg-yolk medium was incubated at 37 °C for 2 days. Listeriolysin O activity in culture supernatants of exponential and stationary cultures grown in BHI, or BHI supplemented with 0.2 % activated charcoal (Merck), was assayed by quantification of cell lysis activity in serial dilutions of supernatants using calf blood, essentially as described by Leimeister-Wachter & Chakraborty (1989).

**RESULTS**

**Expression of L. monocytogenes fri is controlled by PerR and σB**

A homologue of Dps was recently identified in the human and animal pathogen L. monocytogenes (Hébraud & Guzzo, 2000). Characterization of the corresponding gene, fri (lm943, GenBank accession number NC003210; Fig. 1A), revealed that it is expressed from a monocistronic transcript of 800 nt (Fuangthong et al., 2002). In a study of fri in L. innocua, primer extension analysis showed that transcription is initiated from either of two promoters located 36 nt (σA dependent) or 85 nt (σB dependent) upstream of the fri start codon (Polidoro et al., 2002). Similar cis regulatory elements are also present in the L. monocytogenes fri promoter region (Fig. 1B), as is a putative binding site for the peroxides stress regulator PerR, with only two mismatches relative to the 15 bp consensus sequence (Fuangthong & Helmann, 2003; Fuangthong et al., 2002).

To determine if the regulatory elements identified in the L. monocytogenes fri promoter region are involved in controlling fri expression, we performed a primer extension analysis of wild-type, sigB (Brondsted et al., 2003) and perR (Rea et al., 2004) mutant cells grown to exponential phase in BHI (Fig. 1C). These analyses showed that in L. monocytogenes EGD, fri was expressed primarily from a transcript initiated 36 nt upstream of the translational start site corresponding to a σB-dependent promoter (SigA1, Fig. 1C, lane 1). In the absence of PerR (Fig. 1C, lanes 3
and 4), the amount of this transcript was dramatically increased, demonstrating that PerR is a repressor of fri expression. While PerR repression is often relieved by peroxide stress, this is not the case for the fri promoter, since the addition of H$_2$O$_2$ failed to induce SigA1 promoter activity (Fig. 1C, lane 7).

In the wild-type strain, we also observed a faint band corresponding to a transcript initiated from the $\sigma^B$ promoter element (SigB, Fig. 1C). This transcript was absent in the sigB mutant strain (Fig. 1C, lane 2), and the amount was increased by heat, which is known to induce the general stress response controlled by $\sigma^B$ (Fig. 1C, lane 5). Interestingly the amount of $\sigma^B$-dependent transcript was also increased in the absence of PerR (Fig. 1C, lanes 3 and 4), indicating that PerR blocks transcription initiated from the $\sigma^B$ promoter. Upstream of the $\sigma^B$ promoter, we identified a third transcript initiated 149 bp upstream of the translation start site, which corresponded to a $\sigma^A$-type promoter element indicated as SigA2 in Fig. 1(B). Thus, fri expression in L. monocytogenes is initiated from three promoters, and it is controlled by PerR and $\sigma^B$.

**Fri improves survival of cells exposed to H$_2$O$_2$**

With the aim of determining if Fri plays a protective role against peroxide stress in L. monocytogenes, we constructed an in-frame deletion of fri by removing a DNA fragment encompassing an internal 381 bp of the gene using the temperature-sensitive plasmid pAUL-A. Since Fri

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**Fig. 1.** The L. monocytogenes fri gene location and promoter region. (A) The location of the fri gene is indicated together with the transcriptional orientation of the indicated ORFs (arrows) and transcriptional terminators (T). The positions of the primers used for construction of the fri deletion mutant are indicated (a, b, c and d). (B) Nucleotides marked in upper-case letters are not identical between L. monocytogenes and L. innocua. Promoter elements corresponding to SigA1- (double underlined), SigA2- (bold) and SigB- (single underlined) dependent promoter elements are marked according to the transcriptional start sites determined in (C). The grey box identifies a putative PerR box. The start codons of fri and htpG are in bold. (C) Primer extension analysis of the fri promoter region using exponentially growing cells of L. monocytogenes wild-type at 37 °C (lane 1), sigB mutant (lane 2), perR mutant, small colony variant as described by Rea et al., 2004 (lane 3), perR mutant, large colony variant as described by Rea et al., 2004 (lane 4), wild-type shifted to 49 °C (lane 5), sigB mutant shifted to 49 °C (lane 6), and wild-type exposed to H$_2$O$_2$ (lane 7).
is expressed from a monocistronic transcript, it is highly unlikely that the in-frame fri deletion mutation will have polar effects on expression of other genes. Despite this notion, we still attempted to complement the mutant strain by introducing the fri gene in trans; however, numerous attempts proved unsuccessful in cloning the intact fri gene and promoter region in a plasmid vector when using either E. coli or L. monocytogenes. While the reason for the lack of success remains obscure, a previous study also reported difficulties when attempting to clone the fri gene (Polidoro et al., 2002). However, when we examined growth of the fri deletion mutant, we found that it was identical to the wild-type strain in both rich growth medium and improved minimal medium (data not shown). In contrast, when cells grown to either exponential or stationary growth phase were exposed to H₂O₂ at 20 mM (Fig. 2A) or 50 mM (Fig. 2B), respectively, the survival of fri mutant cells was greatly reduced when compared with wild-type cells. Notably, the fri mutant cells appeared to be more sensitive to oxidative stress during the exponential growth phase compared with cells present in stationary growth phase.

Fri improves survival in stationary growth phase

In order to determine if Fri protects cells against the stress experienced by prolonged incubation in stationary growth phase, we followed the ability of mutant and wild-type bacteria to form colonies after extended incubation in rich growth medium. Over a period of 11 days, samples were enumerated daily, and the results demonstrate that the fri mutant has a reduced ability to form colonies compared with the wild-type, reaching a plateau fivefold lower than the wild-type after 9 days in stationary growth phase (Fig. 3).

Fri promotes growth of L. monocytogenes at low iron concentrations

Since the Fri protein of L. innocua is able to bind iron, we speculated that Fri might facilitate iron storage in L. monocytogenes when transferred to low-iron conditions. To address this question, we followed growth of the wild-type and the fri mutant when exponentially growing cells were shifted from rich broth containing iron to improved minimal medium with or without ferric citrate. In the presence of 335 μM ferric citrate, the fri mutant grew slightly slower than the wild-type (Fig. 4), whereas in the absence of iron, the wild-type strain initiated growth with a generation time of approximately 2 h, while the fri mutant doubled every 3–4 h without reaching the same level as the wild-type. Also, when transferred to solid media treated with the iron chelator Chelex 100, wild-type stationary cells grown in rich media were able to form normal colonies, whereas the fri mutant formed micro-colonies only (not shown). Thus, our results indicate that Fri promotes growth under iron-limiting conditions.

Fri affects virulence of L. monocytogenes in mice

During infection, L. monocytogenes meets both iron-limiting conditions and bactericidal reactive oxygen species. Since Fri stimulates growth under iron-limiting conditions, as well as protecting against oxidative stress, we speculated that Fri might contribute to virulence. To investigate this, we infected mice intraperitoneally with the fri mutant or
wild-type cells grown to either exponential or stationary phase. After 3 days, the number of bacteria established in the spleen and liver was determined. When the infection was performed with bacteria in stationary growth phase, significantly fewer bacteria were recovered from mice infected with fri mutant cells compared with wild-type cells (Table 1). In contrast, no difference was observed when the infections were performed using exponentially growing bacterial cells (Table 1), or when the bacteria were injected intravenously (data not shown). To determine if the reduced virulence was caused by altered virulence gene expression, we determined lecithinase and haemolysin activities of both mutant and wild-type cells; however, repeated experiments failed to reveal significant differences (data not shown).

**Impaired intracellular proliferation of fri mutant cells in macrophages**

Following *L. monocytogenes* infection, inflammatory phagocytes constitute the first phase of host defence. To determine if the fri mutant is impaired at this stage of infection, we infected the murine-macrophage-like J774.A1 cell line with fri mutant or wild-type cells (m.o.i. of 10) to determine potential differences in intracellular survival. When we used bacterial cells either in exponential (Fig. 5A) or stationary phase (Fig. 5B), we found that intracellular survival was substantially reduced for the mutant compared with the wild-type. During the 6 h infection assay, the number of intracellular wild-type bacteria increased almost 10-fold, while levels of the mutant were reduced. In order to ensure that this difference in survival did not result from different sensitivities of the two strains to gentamicin, survival tests were carried out in the presence of various concentrations of gentamicin (1–100 μg ml⁻¹), and these tests showed that the two strains were equally able to tolerate gentamicin (data not shown).

### Table 1. Recovery of *L. monocytogenes* wild-type and fri mutant cells from tissues of mice after intraperitoneal infection with bacteria in exponential or stationary growth phase

Values are LS (least-squares) means (SD) from 10 inoculated mice.

<table>
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<tr>
<th>Strain</th>
<th>Growth phase</th>
<th>Postinfection log₁₀ c.f.u. per organ</th>
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<td>Spleen</td>
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<td>Wild-type*</td>
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<td>Stationary</td>
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<td>fri mutant†</td>
<td>Exponential</td>
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<td>Stationary</td>
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*Inoculum, 2·1×10⁴ c.f.u.
†Inoculum, 3·1×10⁴ c.f.u.
‡P=0·7.
§P=0·6.
‖P=0·007.
¶P=0·03.
DISCUSSION

During the infection process, the acquisition of iron is essential for bacterial pathogens (Brown & Holden, 2002). After uptake, the low solubility and toxicity of free iron necessitates its storage, and the major iron storage proteins, the ferritins, generally provide this function. When we searched the L. monocytogenes genome (Glaser et al., 2001), we were unable to detect homologues of the ferritin protein family. To determine if the Dps-like protein Fri may contribute to iron storage, we examined the response to low-iron conditions, and found that growth of the fri mutant was reduced compared with the wild-type when shifted from rich broth to a medium depleted of iron. Also, the ability of the fri mutant to form colonies in the presence of an iron-chelating compound was severely inhibited compared with the wild-type. Although the absence of Fri did not completely inhibit growth, the difference between mutant and wild-type cells indicates that Fri is important as an iron reservoir under these conditions. While similar findings have not been obtained for other Dps-like proteins, studies have shown that the ferritins from E. coli and Campylobacter jejuni, encoded by ftnA and cft, respectively, are also required for growth when shifted from iron-rich to iron-depleted conditions (Abdul-Tehrani et al., 1999; Wai et al., 1996).

Members of the Dps protein family have been shown to protect cells entering stationary phase (Martinez & Kolter, 1997). When we followed viability during prolonged incubation in rich growth medium, we observed a greater loss of viability of fri mutant cells compared with the wild-type after 6 days of incubation. Generally, we observed a dramatic decrease in viability for both cell types during the first 7–9 days of incubation, which subsequently stabilized during the rest of the experiment. This profile is in accordance with similar studies regarding long-term survival of L. monocytogenes (Herbert & Foster, 2001) and Staphylococcus aureus (Watson et al., 1998). The apparent difficulty of the fri mutant in maintaining viability compared to the wild-type may be the result of the inability to cope with oxidative damage inflicted by reactive oxygen species produced during stationary phase (Dukan & Nystrom, 1998; Kolter et al., 1993), as fri mutant cells also proved more sensitive to oxidative stress than wild-type cells.

The expression of fri has been shown to be only marginally induced by entry into stationary phase (Polidoro et al., 2002). In general, expression of members of the Dps family of proteins appears to fall into two classes: those induced by entry into stationary phase, such as Dps of E. coli and B. subtilis, and those that are relatively unaffected by growth phase, namely Dps of C. jejuni (Ishikawa et al., 2003), Dpr of Streptococcus suis (Pulliainen et al., 2003) and Fri. However, despite the lack of stationary-phase induction of fri expression, the promoter region carries a consensus sequence of the general stress σ factor SigB, which in B. subtilis is responsible for the stationary-phase induction of Dps (Antelmann et al., 1997). Primer extension analysis revealed that fri is expressed from three promoters, namely two SigA-type and one SigB-type promoter elements. In addition, the fri promoter region carries a consensus binding site for the peroxide regulator PerR, and in the absence of PerR, expression from the fri proximal promoter was strongly increased. Interestingly, the amount of σB-dependent transcript was also increased in the absence of PerR, indicating that PerR is controlling expression initiated from both the SigA1 and the SigB promoter. Despite the involvement of PerR in controlling fri expression, we failed to see an induction of expression by H2O2, suggesting that fri belongs to the subclass of PerR-controlled genes that is not part of the peroxide regulon (Fuangthong et al., 2002).

To determine if Fri is of importance to virulence, we infected BALB/c mice intraperitoneally with wild-type and fri mutant cells, and found that the lack of Fri decreased the ability of L. monocytogenes to multiply in the organs. A major defence of the human body against invading pathogens is the bactericidal activity of the macrophages. When examining uptake and viability in the macrophage-like cell line J774.A1, we found that the absence of Fri reduced the ability of L. monocytogenes to multiply intracellularly, resulting in a 10-fold reduction in recovery of fri mutant cells compared with wild-type cells 6 h after infection. The antimicrobial activities of macrophages include the production of reactive oxygen metabolites (Forbes & Gros, 2001), and these compounds may be responsible for the accelerated killing of fri mutant cells inside macrophages. Indeed, L. monocytogenes cells lacking Fri were significantly more sensitive to H2O2 than wild-type cells, as shown for other members of the Dps protein family (Chen & Helmann, 1995; Ishikawa et al., 2003; Martinez & Kolter, 1997; Nair & Finkel, 2004). Since the amino acid sequence of Fri from L. monocytogenes only differs at two positions from the iron-binding Fri of L. innocua (Bozzi et al., 1997), the mechanism by which this protection occurs is likely to involve sequestration of iron away from processes generating toxic hydroxyl radicals in combination with H2O2 (Almirón et al., 1992; Ishikawa et al., 2003; Martinez & Kolter, 1997; Zhao et al., 2002). Curiously, when using the mouse model, we only observed a significant difference in virulence between mutant and wild-type if the bacterial cells were in stationary growth phase, and injected in the peritoneum, whereas in the macrophage assay, the virulence of fri mutant cells was reduced independently of the growth phase of the bacteria. Although we are currently unable to explain the reason for this difference, it may reflect that Fri is only required for proliferation of L. monocytogenes in specific host cells, or compartments that are only reached during some types of infections. In a recent study of the Gram-negative dental pathogen Porphyromonas gingivalis, the inactivation of a Dps-like protein reduced survival in human umbilical vein endothelial cells (Ueshima et al., 2003), and in Salmonella enterica serovar Typhimurium, Dps promoted survival in...
murine macrophages as well as in organs of infected mice (Halsey et al., 2004). These reports, together with our findings, provide evidence that Dps-like proteins are important for virulence of distantly related bacterial pathogens.

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