The svpA-srtB locus of *Listeria monocytogenes*: Fur-mediated iron regulation and effect on virulence

Satele M. C. Newton,1,2 Phillip E. Klebba,1,2 Catherine Raynaud,1 Yi Shao,2 Xiaoxu Jiang,2 Iharilalao Dubail,1 Crystal Archer,2 Claude Frehel1 and Alain Charbit1*

1INSERM U-570, CHU Necker, Enfants Malades, 156, rue de Vaugirard, 75730 Paris Cedex 15, France.
2Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK, 73019, USA.

Summary

In *Listeria monocytogenes* the promoter region of the svpA-srtB locus contains a well-conserved Fur box. We characterized the iron-regulation of this locus: real-time polymerase chain reaction analyses and anti-SvpA immunoblots showed that, in response to iron deprivation svpA transcription and SvpA production markedly increased (80-fold and 10-fold respectively), when initiated by either the addition of the iron chelator 2,2′-bipyridyl to BHI media, or by growth in iron-restricted minimal media. Green fluorescent protein (GFP) reporter constructs also showed increased activity of the svpA-srtB promoter in *Escherichia coli* (37-fold) and in *L. monocytogenes* (two- to threefold) when the bacteria were grown in iron-deficient conditions. A Δfur mutant of *L. monocytogenes* constitutively synthesized SvpA, as well as GFP fused to the svpA-srtB promoter. Cellular fractionation data revealed that in iron-rich media wild-type SvpA was exclusively secreted to the culture supernatant. However, both the Δfur derivative and wild-type *L. monocytogenes* grown in iron-deficient media anchored a fraction of the SvpA proteins (~5%) to peptidoglycan, and produced a lower-molecular weight, wholly secreted form of SvpA. Together these data establish that iron availability controls transcription of the svpA-srtB locus (through Fur-mediated regulation), and attachment of SvpA to the cell wall (through SrtB-mediated covalent linkage). SvpA bears homology to IsdC, a haemin-binding protein of *Staphylococcus aureus*, and haemin bound to SvpA in solution. However, site-directed deletions of four structural genes and the promoter of the svpA-srtB locus did not impair haemin, haemoglobin or ferrichrome utilization in nutrition tests. We did not find strong evidence to support the notion that the svpA-srtB locus participates in haemin acquisition, as was reported for the homologous isd operon of *S. aureus*. Furthermore, the svpA-srtB mutant strains showed no significant attenuation of virulence in an intravenous mouse model system, but we found that the mutations reduced the persistence of *L. monocytogenes* in murine liver, spleen and intestines after oral administration.

Introduction

*Listeria monocytogenes* is a Gram-positive bacterium of very low G+C content, closely related to *Bacillus*, *Clostridium* and *Staphylococcus* species. *L. monocytogenes* can be isolated from soil, water, food sources and animal feces. Several factors contribute to the success of *L. monocytogenes* as a food contaminant: it tolerates high salt, low pH and contrary to most human pathogens; it multiplies at refrigeration temperatures (Lammerding and Doyle, 1989). Ingestion of *Listeria*-contaminated food is probably common, but its high LD₅₀ (around 10⁸) leads to low incidence of listeriosis under normal circumstances (2–8 cases per million per year; Rocourt and Browch, 1992). Nevertheless, listeriosis is one of the most deadly bacterial infections, with a mean mortality rate of 20–30% in humans, even with antibiotic treatment. (McLauchlin, 1990a,b; Schuchat *et al.*, 1991) The severity of the disease is often associated with the ability of the strain to cross the blood–brain barrier and infect the central nervous system.

Both the interaction of *L. monocytogenes* with mammalian cells and its ability to multiply in the host involve surface-associated proteins (for review see Cabanes *et al.*, 2002); multiple systems anchor proteins to the cell wall of Gram-positive bacteria. For example, the enzyme sortase A (SrtA) (Ton-That *et al.*, 1999; 2000) recognizes the motif LPxTG on secreted proteins, hydrolyses the peptide bond between T and G, and synthesizes an amide bond between the resulting carboxyl group of threonine and the amino group of m-diaminopimelic acid of cell wall peptidoglycan (PG). The importance of sorting mechanisms to bacterial virulence is evident from the attenuation of *Staphylococcus aureus* and...
Another Gram-positive sorting mechanism involves the enzyme sortase B (SrtB), which acts on the motif NxyTN. The srtB region is similar in *S. aureus* and *L. monocytogenes*. In *S. aureus* the gene cluster contains an upstream Fur box, suggesting its involvement in iron uptake, and it produces both SrtB and a protein (IsdC) that SrtB attaches to PG (Mazmanian et al., 2003). IsdC, which contains the motif NPQTN, was reported to participate in iron (haemin) acquisition (Mazmanian et al., 2003). In *L. monocytogenes* the svpA-srtB locus also contains a promoter with a Fur box that precedes seven open reading frames; strong transcriptional terminators flank the region. The genetic organization suggests that the seven genes encode a single, iron-regulated operon. The first gene of the svpA-srtB cluster, *lmo2186*, encodes a 207 residue protein with 33% identity to IsdC. *Lmo2186* contains a potential SrtB-dependent sorting motif (NKVTN), but it is not known whether SrtB attaches it to PG. The second gene in the region encodes SvpA, a 569 residue protein that *L. monocytogenes* may either secrete to the culture supernatant or covalently attach to PG, by the actions of SrtB on its motif NAKTN (Bierne et al., 2004). The central portion of SvpA contains homology to both IsdC and Lmo2186. Neither is it known if Lmo2186 and/or SvpA are involved in iron utilization, but downstream from svpA are three genes with homology to ferric hydroxamate ABC transporters: *lmo2184* specifies a potential lipoprotein, *lmo2183* a potential permease component, and *lmo2182* a potential adenosine triphosphate (ATP)-binding protein. A comparable ABC-type transporter system occurs in the *S. aureus* isdC-srtB cluster. It was reported that a mutant containing a partial deletion of SvpA was attenuated 100-fold in mice (Boreeze et al., 2001), but a ΔsrtB mutant was not attenuated (Bierne et al., 2004).

In this study we characterized the regulation of svpA-srtB in iron-sufficient and iron-deficient conditions, including analysis of svpA transcription by real-time polymerase chain reaction (PCR), quantification of SvpA production by 125I-protein A immunoblots, and expression of an svpA-srtB promoter–green fluorescent protein (GFP) fusion. We also determined the effects of a fur deletion on SvpA synthesis. These experiments demonstrated that iron availability regulates the svpA-srtB locus, mediated by Fur. Regarding the involvement of the svpA-srtB locus in haemin uptake, although haemin associated with SvpA in solution, independent deletions in the svpA-srtB locus did not impair haemin, haemoglobin or ferrichrome utilization in nutrition tests, and these mutations had only limited impact on the virulence of *L. monocytogenes* in the mouse model system.

**Results**

**Fur-mediated iron-regulation of the svpA-srtB region**

The Fur box in its promoter region (Fig. 1) suggests that iron regulates the svpA-srtB locus. To evaluate this prediction and to consider the impact of different genes on iron utilization and virulence, we genetically engineered deletions in the svpA-srtB region (Fig. 1) that individually eliminated the promoter region (∆PROX5), *lmo2186* (∆l2186), svpA (∆SvpA) and *lmo2182-lmo2183* (∆FX3). The latter three mutations eliminated the two proteins that are homologous to *S. aureus* isdC, and portions of the putative membrane permease and ATPase of the locus. For each mutation, we generated PCR products flanking the region of interest, joined them by restriction and ligation, electroporated the constructs into *L. monocytogenes*, and selected for replacement of the target locus by the genetically engineered deletion (*Experimental procedures*). We confirmed the deletions (all in

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**Fig. 1.** A. Schematic representation of the svpA-srtB locus of *L. monocytogenes*. Arrows indicate the orientation and approximate sizes of the open reading frames; the number of amino acids in each putative polypeptide is listed above, and parenthetic numbers give the sizes (in bp) of the intergenic regions. The locations of genetically engineered deletions are shown as dashed boxes below the open reading frames. Terminators flank the region. The positions and identities of svpA and srtB in the locus are also shown. B. Nucleotide sequence of the region upstream of *lmo2186*, containing the Fur box. The Shine-Delgarno sequence is underlined, and the N-terminal Met (M1) encoded by the ATG start codon is shown in bold type.
strain EGD-e) by PCR from colonies carrying them. For Δ2186 and ΔSvpA, the deletions removed almost all, or all of the structural genes: in the former case three N-terminal codons remained, and in the latter case the deletion was complete. We designed both of these mutations in-frame to avoid polarity effects. For svpA the nature of the deletion was important, because we compared its phenotype to that of a partial deletion construct (ΔSvpA.K7; Borezee et al., 2001) which contained a kanamycin cassette in the mutated gene.

Because of its purported relationship to virulence, SvpA was a focal point of the experiments. We initially used anti-SvpA serum (Borezee et al., 2001) to monitor expression from the svpA-srtB region (Fig. 1). When L. monocytogenes was grown in BHI broth, these sera revealed SvpA in the culture supernatants (Fig. 2, top), as previously reported (Borezee et al., 2001). Immunoblots also showed that ΔSvpA.K7 produced a previously undetected fragment of SvpA, consistent in size with the position of the kanamycin insertion into its structural gene. Cell lysates and supernatants (Fig. 2) of ΔSvpA, on the other hand, were devoid of reactivity with the anti-SvpA sera, as anticipated. Δ2186 and ΔFX3, which contained deletions upstream and downstream of svpA respectively, had normal expression of the full-length SvpA protein. Enzymatic (alkaline phosphatase) immunoblots showed that a promoter deletion (strain ΔPROX5) reduced SvpA expression, and experiments with 125I-protein A immunoblots, quantified by phospho-imagery, determined that SvpA levels in ΔPROX5 were <1% of wild type levels.

BHI broth is iron-replete ([Fe] = 8 μM, see Experimental procedures), but α,α-Dipyridyl (BP) chelates iron in solution, making it unavailable to many bacteria (Sebulsky et al., 2000; Sebulsky and Heinrichs, 2001; Mazmanian et al., 2003). We rendered BHI broth iron-deficient by addition of BP (1 mM), and supernatants from these cells showed increased production of SvpA (Fig. 2, top), suggesting that extracellular iron availability regulates expression from svpA-srtB.

We also deleted the putative fur gene of L. monocytogenes, lmo1956 (ΔFur). Fur negatively regulates the transcription of bacterial proteins involved in iron acquisition (de Lorenzo et al., 1988). The identity of fur in L. monocytogenes was unknown when these experiments began, but in silico analyses identified lmo1956 as highly similar to fur of B. subtilis (Bsat and Helmann, 1999). We

![Fig. 2. Anti-SvpA immunoblots of EGD-e and its mutants. Top. Expression of SvpA in different media. Culture supernatants from EGD-e and its mutant derivatives were concentrated by TCA precipitation and subjected to SDS-PAGE and Western immunoblot with rabbit anti-SvpA. A. An immunoblot of BHI culture supernatant proteins from strains ΔSvpA.K7, EGD-e, ΔPROX5, ΔSvpA, Δ2186 and ΔFX3 (lanes 1–6 respectively) was developed with goat-anti-rabbit IgG-alkaline phosphatase. B. An immunoblot containing supernatant proteins from strains EGD-e wild type (lanes 7 and 8), ΔPROX5, ΔSvpA, Δ2186 and ΔFX3 (lanes 11 and 12) was developed with 125I-protein A; the samples derived from cells grown in either BHI broth (odd lanes) or KRM broth containing BP (1 mM; even lanes). Prestained SDS-PAGE standards (Bio-Rad; low range) were included as a molecular mass reference (MW). Bottom. Localization of SvpA in different cell fractions by Western immunoblot. Cytoplasmic (A), cytoplasmic membrane (B), PG-associated (C) and culture supernatant proteins (D) were prepared from strains EGD-e wild type and ΔFur, grown in either BHI broth or KRM defined media. Equivalent quantities from each fraction (see Experimental procedures) were subjected to SDS-PAGE and transferred to nitrocellulose, which was developed with rabbit anti-SvpA and 125I-Protein A, and quantified by phosphoimagery.](image-url)
precisely eliminated the complete fur gene and analysed the resulting strain for SvpA production in BHI and BHI + BP. The fur deletion increased production of SvpA (Fig. 2, top), and the addition of BP did not increase it more. SvpA reached maximum levels in culture supernatants when iron was sequestered by BP, or when Fur was deleted. In addition, immunoblots showed a second, lower molecular weight form of the protein (SvpA2) associated with overexpression. SvpA2 appeared in response to either iron-deprivation or the absence of Fur (Fig. 2).

Cellular localization of SvpA

To study the response of L. monocytogenes to iron-deficient conditions we developed an RPMI-based, iron-restricted, defined medium (KRM) that supported growth to substantially higher cell densities than other minimal media. The concentration of iron in KRM, determined by atomic adsorption spectrometry, was <1 μM. After growing the wild-type strain and ΔFur in BHI broth or in KRM media, we collected their culture supernatants, prepared cytoplasmic, cytoplasmic membrane and PG-associated fractions (Experimental procedures), and compared their levels of SvpA using 125I-protein A immunoblots. The experiment showed that, as suggested by its NAKTN motif, SvpA may attach to PG, but when EGD-e was grown in BHI essentially all of the SvpA appeared in the culture supernatant (Fig. 2, bottom: C and D). Secondly, the attachment of SvpA to PG was itself iron-regulated: SvpA was not linked to PG when wild-type L. monocytogenes was grown in (iron-rich) BHI media; the anchoring reaction only occurred when the cells were grown in (iron-deficient) KRM, or if fur was deleted. These data concurred with the fact that SrtB, which attaches SvpA to PG (Bierne, 2004), originates from the same iron-regulated locus. Under maximum derepression conditions (ΔFur in BHI), 5% of the total SvpA was anchored to PG, and the majority of the remainder (except a small fraction in the cytoplasm), was secreted. In KRM media, again about 5% of the total SvpA associated with PG, but in this case we detected significant quantities of the protein in the cytoplasmic and cytoplasmic membrane fractions. We did not observe a protein with the molecular weight of Lmo2186 (22 kDa), another potential target for SrtB, in either culture supernatants or PG fractions of EGD-e or ΔFur, but antibodies do not yet exist to this protein, and without them such localization experiments are inconclusive.

Regulation of SvpA in bacteria grown in culture and in infected cells

We fused the svpA-srtB promoter to the GFP structural gene and used the construct to study the extent of iron regulation in L. monocytogenes and Escherichia coli. We amplified 175 bp preceding the first gene of the operon (lmo2186), and inserted the product upstream from gfp in pUC18. In the resulting vector, pUCgfp15, the Fur box is situated at position –110 from the initial ATG codon of GFP. Colonies of E. coli strain DH5α/pUCgfp15 were not initially fluorescent on Luria–Bertani (LB) plates, but they became yellow after longer incubation (24 h) at 37°C, and they fluoresced green under UV light. We used two methods to restrict iron availability in liquid E. coli cultures. First, we grew DH5α/pUCgfp15 in (iron-deficient) MOPS minimal media (Neidhardt et al., 1974; Newton et al., 1999) which induced a high level of fluorescence in the cells. Conversely, the addition of 20 μM FeSO₄ to the medium repressed fluorescence (Fig. 3). Next, we plated single colonies on LB agar containing the iron-sequestering agent apoferrichrome A (E. coli cannot acquire iron

Fig. 3. A. Fluorescence of (svpA-srtB promoter–GFP in response to iron availability. DH5α/pUCgfp15 (which carries svpA-srtB promoter–gfp) was grown in MOPS minimal medium either without added iron, or with 20 μM FeSO₄ added. The fluorescence of the cells was measured in an SLM-Aminco fluorimeter.

B. Quantitative RT-TaqMan PCR. Induction ratio of svpA transcription in different conditions of growth; samples were normalized to RNA from transcription of gyrA. (i) Growth in BHI + BP versus BHI; (ii) Growth in Caco-2 cells versus in KRM; (iii) Growth in HepG-2 cells versus in KRM.
from ferrichrome A; Wayne et al., 1976; Newton et al., 1999). In these iron-restricted conditions DH5α/pUCgfp15 clones synthesized GFP, resulting in highly fluorescent colonies (data not shown). As in minimal media, addition of iron reduced GFP expression, producing non-fluorescent colonies (data not shown).

For expression in L. monocytogenes, we transferred the promoter–GFP fusion construct to pAT28 by restriction fragment exchange, creating pATgfp7. The L. monocytogenes strain was not visibly fluorescent on BHI plates, but addition of bipyridyl (1 mM) turned on fluorescence, as did growth in KRM media, albeit to lower levels than observed for the E. coli strain grown in MOPS media (Fig. 3). When grown in BHI plus BP or KRM liquid media, EGD-e/pATgfp7 was fluorescent, but much less so than DH5α/pUCgfp15 (Table 1). The addition of iron to KRM repressed fluorescence, whereas deletion of fur maximally enhanced it, in either medium.

To assess svpA transcription levels in infected cells, we synthesized oligonucleotide primers to sites within the gene and used real-time PCR (TaqMan, Stratagene) to compare the svpA transcription levels of bacteria either proliferating in animals or growing in iron-rich and iron-poor media. The addition of BP to BHI increased the svpA transcription rate 80-fold, and in bacteria growing in Caco-2 enterocytes or HepG-2 hepatocytes it was 10-fold higher than in cells cultured in KRM (Fig. 3). The fold-increase in svpA transcription in vivo was less than that caused by addition of BP to BHI broth, because we compared the transcription rates of bacteria in animal cells to those of bacteria growing in KRM, which itself contains about 10-fold less iron than BHI (<1 versus 8 μM). Thus, in BHI iron represses the transcription of svpA-srtB, in KRM partial derepression occurs, and in the host cells more severe iron deprivation takes place, increasing svpA transcription to a higher level. These data indicate that iron is less available in mammalian cells than in the synthetic medium (which contained <1 μM Fe).

**Influence of the svpA-srtB locus on growth, virulence and intracellular multiplication**

We determined the growth rates of strains EGD-e, ΔPROX5, Δ2186, ΔSvpA, ΔFX3 and ΔSvpA.K7 in BHI broth (Fig. 4). As previously described, strain ΔSvpA.K7 showed a marked lag and a reduction in growth rate at 37°C. However, ΔSvpA, which carried a precise chromosomal deletion of svpA, and all the other mutant strains, grew like wild-type strain EGD-e. These data suggested that the fragment of SvpA synthesized by ΔSvpA.K7 (Fig. 2) was responsible for the reduced growth rate of the strain.

Table 1. Fluorescence of bacteria harbouring svpA-srtB promoter–gfp constructs.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Medium</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>LB broth</td>
<td>0.2</td>
</tr>
<tr>
<td>DH5α/pUCgfp15</td>
<td>MOPS</td>
<td>7.3</td>
</tr>
<tr>
<td>DH5α/pUCgfp15</td>
<td>MOPS + FeSO4 (20 μM)</td>
<td>2.9</td>
</tr>
<tr>
<td>EGD-e/pATgfp7</td>
<td>BHI broth</td>
<td>0.2</td>
</tr>
<tr>
<td>EGD-e/pATgfp7</td>
<td>BHI broth + BP (1 mM)</td>
<td>1.6</td>
</tr>
<tr>
<td>EGD-fur/pATgfp7</td>
<td>BHI broth</td>
<td>2.0</td>
</tr>
<tr>
<td>EGD-fur/pATgfp7</td>
<td>BHI broth + BP (1 mM)</td>
<td>3.8</td>
</tr>
<tr>
<td>EGD-e/pATgfp7</td>
<td>KRM</td>
<td>4.1</td>
</tr>
<tr>
<td>EGD-e/pATgfp7</td>
<td>KRM + Haemin (20 μM)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The mean fluorescence intensities of E. coli DH5α and L. monocytogenes EGD-e were determined by cytofluorimetry in broth and defined minimal media. When iron was rendered unavailable by addition of BP, the cells were grown to mid-log, the chelator was added to 1 mM, and growth was continued with shaking for 30 min prior to flow cytometry.
Previous studies concluded that SvpA is a virulence factor: in *L. monocytogenes* strain LO28 the ΔsvpA.k7 mutation created a two-log increase in LD₅₀ (Borezee *et al.*, 2001). We observed an identical, 100-fold decrease in virulence when ΔsvpA.k7 was constructed in EGD-e (Table 2). But conversely, ΔSvpA (in EGD-e) was only slightly attenuated when injected into mice by the intravenous route (twofold increase in LD₅₀), and the other deletions in svpA-srtB produced similarly small, or no effects on virulence. The kinetics of infection by ΔPROX5 in livers and spleen (at a dose of 10⁶ bacteria per mouse) were identical to those of the wild-type strain (data not shown). These findings were unexpected for ΔSvpA and ΔPROX5, which in one case completely eliminated the putative virulence protein, and in the other reduced its expression to minimal levels.

We infected Caco-2 cells with EGD-e, ΔPROX5, Δ2186, ΔSvpA and ΔFX3 at a ratio of 10 bacteria per cell, and followed intracellular multiplication for 4 h. All strains invaded and multiplied at the same rate as the wild type (data not shown). Bone marrow-derived macrophages were used to evaluate the ability of the mutants to escape the phagosomal vacuole and polymerize actin. Two hours after infection with 20 bacteria per cell, macrophages were stained with phalloidin to reveal actin polymerization. The percentage of cells surrounded by actin indicates their escape from the phagosome and the process of migration to neighbour cells. No impairment of multiplication or phagosomal escape was observed for any of the mutants (data not shown).

Finally, we followed the survival of EGD-e and ΔPROX5 in *vivo* after intragastric inoculation of mice (4 × 10⁷ bacteria per mouse). Viable counts were determined in liver, spleen and the small intestinal tissue of BALB/c mice, 3 days after inoculation. The results (Fig. 4) indicated a 10-fold reduction in survival for ΔPROX5 in the three tissues. These data intimate that the srtB-svpA locus may function in the crossing of the digestive barrier by *L. monocytogenes*.

**The svpA-srtB locus and haem utilization by *L. monocytogenes***

In *S. aureus* the *isd* region encodes a haemin–haemoglobin uptake system. Sequence homologies raise the possibility that the *L. monocytogenes* svpA-srtB locus also facilitates iron acquisition from haemmin or haemoglobin. We first addressed this question with nutrition tests, which show the ability of bacteria to utilize specific iron sources (Fig. 5). However, EGD-e strains carrying individual deletions in the region (ΔPROX5, Δ2186, ΔSvpA and ΔFX3) were not impaired in ability to use haemmin or haemoglobin. Neither was growth on ferrichrome impaired in any of these strains. We observed these same results in liquid KRM medium: addition of haemmin or haemoglobin equally stimulated all the strains (data not shown). The nutrition tests (Fig. 5, Table 3) showed characteristic, concentration-dependent halos for haemmin (diameter: 13 mm at 15 mM), haemoglobin (17 mm at 15 mM), and ferrichrome (25 mm at 50 mM), that did not appreciably change in any of the mutants. These data implied that the svpA-srtB region of *L. monocytogenes* does not significantly affect the uptake of the three compounds.

SvpA and Lmo2186 are homologous to IsdC and contain SrtB-dependent sorting motifs. But despite its NAKTN motif, SvpA is largely secreted rather than anchored to PG (Fig. 2). Therefore, we tested the secreted proteins of

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**Table 2. LD₅₀ values of *L. monocytogenes* strains in mice.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD₅₀</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGD-e</td>
<td>10⁴±⁵</td>
<td>Borezee <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>EGD-e</td>
<td>10⁴±⁶</td>
<td>This work</td>
</tr>
<tr>
<td>ΔsvpA.k7</td>
<td>10⁷±²</td>
<td>This work</td>
</tr>
<tr>
<td>ΔPROX5</td>
<td>10⁴±⁶</td>
<td>This work</td>
</tr>
<tr>
<td>Δ2186</td>
<td>10⁴±⁶</td>
<td>This work</td>
</tr>
<tr>
<td>ΔSvpA</td>
<td>10⁴±⁶</td>
<td>This work</td>
</tr>
<tr>
<td>ΔFX3</td>
<td>10⁴±⁶</td>
<td>This work</td>
</tr>
</tbody>
</table>

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**Table 3. Nutrition tests with *L. monocytogenes***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Haemoglobin</th>
<th>Haemin</th>
<th>Ferrichrome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15.5 5 1</td>
<td>200 100 10</td>
<td>100 50 10</td>
</tr>
<tr>
<td>EGD</td>
<td>17 16 14</td>
<td>13.2 (0.7) 10 –</td>
<td>25 25 20</td>
</tr>
<tr>
<td>ΔPROX5</td>
<td>17 16 14</td>
<td>13.4 (0.8) 10 –</td>
<td>26 25 22</td>
</tr>
<tr>
<td>Δ2186</td>
<td>17 16 14</td>
<td>13.2 (0.7) 10 –</td>
<td>25 25 20</td>
</tr>
<tr>
<td>ΔSvpA</td>
<td>17 15 14</td>
<td>13.2 (1.2) 10 –</td>
<td>26 28 22</td>
</tr>
<tr>
<td>DFX3</td>
<td>17 16 14</td>
<td>12.4 (0.5) 9 –</td>
<td>26 25 20</td>
</tr>
<tr>
<td>DStrB</td>
<td>17 16 14</td>
<td>12.6 (0.5) 9 –</td>
<td>25 25 20</td>
</tr>
</tbody>
</table>

Bacterial strains were grown in BHI broth, plated in BHI top agar containing 1 mM BP, and iron compounds were applied to paper discs at the indicated concentrations (micromolar). The plates were incubated 15 h at 37°C and the diameter of the growth haloes (mm) was measured with a ruler. The tabulated data represent the results of a single experiment, except in the case of 200 μM haemmin, where five tests were made and averaged. The parenthetic values are the standard deviations of the means.
L. monocytogenes for the ability to bind haemin. We first chromatographed concentrated culture supernatant proteins from the Δfur strain over haemin agarose (Fig. 5). SvpA and another secreted protein, P60 (identified by micro-Edman degradation), adsorbed to the resin, whereas two other proteins, of masses 63 kDa and 55 kDa did not bind. A solution of haemin (200 μM) did not release any proteins; only 1% SDS eluted SvpA and P60 from the haemin agarose (Fig. 5). Because these data were inconclusive about the ability of SvpA to bind haemin, we tested the secreted proteins of L. monocytogenes for haemin binding in solution. After growth of the wild-type and the ΔPROX5 strains in KRM minimal medium, we collected and concentrated the culture supernatants, incubated them with haemin and fractionated the mixtures by gel-permeation chromatography on Sephacryl S100HR (Fig. 6). Haemin cochromatographed with the supernatant proteins, that included SvpA (Frs. 26–31), and less haemin cochromatographed with the secreted proteins from ΔPROX5. Haemin did not adsorb to P60 (Frs. 22–25), which was excluded from the resin and eluted in the void volume. These data indicated that haemin adsorbs to SvpA, and that other secreted proteins also bind the porphyrin, because it also cochromatographed with supernatant proteins from ΔPROX5, which are essentially devoid of SvpA. However, we also tested albumin and in the same assay: haemin adsorbed to it and cochromatographed with it over S100HR (Fig. 6).

Discussion

Many virulence factors of pathogenic bacteria are surface-associated and secreted proteins. In Gram-positive organisms, sortases anchor proteins to the PG layer. Sortase A, the first such enzyme described, functions in Listeria, Staphylococcus and Streptococcus, which explains the attenuation of srtA mutants in all three species. (Schneewind et al., 1993; Mazmanian et al., 2000; Bierne et al., 2002; Jonsson et al., 2002; Kharat and Tomasz, 2003) A second enzyme, sortase B, exists in S. aureus and L. monocytogenes. Contrary to the widespread activity of sortase A (20 genes encode the LPxTG domain in
sortase B apparently only acts on a few proteins. In both Staphylococcus and Listeria, only a single cell surface protein is known as SrtB-dependent for attachment to PG (IsdC and SvpA respectively).

SrtB (Imo2181) is the penultimate gene in the Fur-regulated, seven-gene, svpA-srtB locus of L. monocytogenes. The second gene in the region encodes SvpA, a 569 amino acid protein that is secreted except during growth in iron-deficient conditions, when SrtB covalently links a fraction of it to PG (this report). Whereas the sorting motif NPQTN occurs in IsdC, the sequence NAKTN exists in SvpA (Bierne et al., 2004). Our experiments characterized, for the first time, the manner in which iron availability controls the expression and localization of products of the svpA-srtB locus. Real-time PCR data showed that iron deprivation increased transcription of svpA-srtB, whether the bacteria were growing in culture (BHI + BP or KRM), or in animals (Caco-2 or HepG-2 cells), confirming that the locus is active in vivo during intracellular growth of L. monocytogenes (Bierne et al., 2004). Because BP is a chelator with broad specificity for metals, we performed additional experiments to substantiate iron regulation. The Fur box upstream of the

Fig. 6. Co-chromatography of haemin with secreted proteins from L. monocytogenes. Absorbance profiles at 280 nm (open symbols) and 393 nm (filled symbols) revealed the elution of protein and haemin respectively, when supernatant proteins from strains EGD-e (A, circles) and ΔPROX5 (B, triangles) were incubated with 20 μM haemin and chromatographed on a column of Sephacryl S100hr in 50 mM Tris, pH 8. An anti-SvpA immunoblot (C), developed with 125I protein A, and an SDS-PAGE gel (D) of the EGD-e secreted proteins, showed the coelution of haemin and SvpA in fractions 27–30. Uncomplexed haemin chromatographed in fractions 40–50, with the media components. The small amount of SvpA in culture supernatants can be seen as the top band in the doublet above P60; in C, D and E the ‘BenchMark’ prestained protein ladder (Invitrogen) was included as a molecular mass reference (MW). In the absence of SvpA (the ΔPROX supernatant in B and the SDS-PAGE gel in E), haemin bound to other proteins and cochromatographed with them. Furthermore, when haemin was mixed with bovine serum albumin, it bound to it and cochromatographed through S100HR (A, diamonds).
svpA-srtB promoter is almost identical (18/19 residues, Fig. 1) to the consensus Fur box of *B. subtilis* (Baichoo and Helmann, 2002). However, in *silico* analyses alone may misrepresent potential Fur-mediated iron regulation of bacterial operons (Baichoo et al., 2002). Some genes with conserved Fur boxes are not Fur-regulated (e.g. the *Bacillus subtilis* *ywcC* gene, which contains a sequence with identity at 15/19 positions), and Fur may recognize operator sites in genes that do not contain the canonical Fur box (e.g. the *B. subtilis* *yusV* and *feuABC* genes). Therefore, we experimentally defined the regulation of the *svpA-srtB* locus. The *svpA-srtB* promoter (–GFP fusion) was repressed by iron: the fluorescence of both *Listeria* and *E. coli* cells harbouring this construct depended iron availability in the medium. In EGD-e Δfur SvpA synthesis dramatically increased, confirming that Fur regulates its production. RT-PCR experiments suggested that the seven-gene *svpA-srtB* cluster constitutes a single transcriptional unit: the first two genes, *lmo2186* and *svpA*, and the first gene of the ABC transporter (*lmo2184*) are co-transcribed (Bierne et al., 2004). Our data substantiate this conclusion: both *svpA* upstream in the locus and *srtB* downstream, were derepressed by iron deprivation and/or deletion of fur; the intervening region (including the 87 bp between *svpA* and *lmo2184*) does not contain a Fur recognition sequence. Thus, the locus is an operon, and Fur-regulated at the upstream promoter.

Although previous data indicated that in BHI broth SrtB links a significant percentage of SvpA to the cell wall (Bierne et al., 2004), 125I-protein A immunoblots more accurately showed that in BHI the vast majority of SvpA is not linked to PG, but secreted into the medium. This discrepancy originated in the different immunochromatographic methodologies: enzymatic and radiochemical methods gave different characterizations of SvpA expression and localization (compare SvpA expression in ΔPROX5: Fig. 2, top: A and B). The former procedure inaccurately portrays relative antigen concentrations, because of substrate depletion in regions of the solid phase that contain high amounts of antigen (i.e. high enzyme concentrations). 125I-protein A immunoblots, on the other hand, are not subject to this caveat because they involve a stoichiometric complex of the antigen, antibody and protein A, that accurately quantifies relative antigen concentrations (Nakamura et al., 1986). The radiochemical method showed that iron deprivation had three effects on SvpA: it increased the rates of its production; it caused the formation of a lower-molecular weight form of SvpA; and it activated the linkage of only the higher-molecular weight form to PG. The latter conclusion concurs with the fact that the anchoring enzyme, SrtB, originates from the same, iron-regulated transcriptional unit (Bierne et al., 2004). The predicted molecular mass of SvpA, without cleavage of a signal sequence, is 63,380, slightly less than the observed mass of the SvpA band in SDS-PAGE gels and immunoblots. The mobility of the SvpA2 band, on the other hand, correlates with a mass of 60 kDa (Fig. 5). This decrease in mass is almost exactly commensurate with cleavage of the polypeptide by SrtB at the NAKTN motif, which removes the C-terminal 29 amino acids (3043 D). Hence it appears that the SvpA2 band, which was not linked to PG, may consist of SvpA proteins that are digested by the sortase but not bound to the cell wall.

Other prior experiments with strain LO28 concluded that SvpA is a virulence factor, because a mutant harbouring an inactivated version of the gene had a two-log increase in *LD*50 by the intravenous route. (Borezee et al., 2001). We obtained the same results with the more virulent strain EGD-e, carrying the same deletion and kanamycin cassette (ΔsvpA.K7). SvpA is an acronym for surface virulence protein, and the protein was thought to function in the early multiplication of *Listeria* inside host cells. However, the srtB deletion mutant was not attenuated (Bierne et al., 2004), and our strains carrying deletions in the *svpA-srtB* locus, which eliminated or drastically reduced *svpA*, were not severely attenuated. Although ΔPROX5 reduced SvpA expression to 1% of its normal level, it did not impair growth of *L. monocytogenes* in vitro and did not decrease its virulence in mice (*LD*50 of 101.5 versus 101.8, by the intravenous route; Table 2). The precise deletion that did not introduce an antibiotic cassette (ΔsvpA) gave the same results. Strains with two other deletions in the *svpA-srtB* locus (Δ2186 and ΔFX3) produced SvpA at normal levels, grew like the wild-type EGD-e and were only slightly attenuated. The small increases in *LD*50 that we observed were not explained by defects in multiplication in Caco-2 cells or macrophages, nor by reduced actin polymerization (data not shown). The only significant phenotype that the mutations created was a 10-fold reduction of persistence of ΔPROX5 in spleen, liver and intestine after oral immunizations. When ingested, the vast majority of *L. monocytogenes* are destroyed, so very high inoculums (=1010 bacteria per mouse) are required to produce a lethal infection by the oral route. The *svpA-srtB* locus may enhance multiplication and/or persistence after oral infection, facilitating survival and increasing virulence. Other such systems are known to exist. For example, in acidic conditions that mimic the stomach the bacterium mounts a tolerance response that increases survival (Saklani-Jusforgues et al., 2000). Nevertheless, a primary conclusion of our study is that the advantages conferred to pathogenesis by *svpA-srtB* are slight: EGD-e carrying ΔsvpA was not attenuated 100-fold in mice, but only twofold, and when carrying ΔsrtB strains it was not attenuated (Bierne et al., 2004). The more deleterious mutation ΔsvpA.K7 contains an internal 79 bp deletion and a kanamycin cassette. It
still encodes and secretes the amino terminal part of SvpA, and the reduction in virulence that it caused probably derives from synthesis of this truncated polypeptide. Aberrant proteins are often toxic to bacteria, retarding multiplication in vivo and allowing more efficient clearance from the host. These results underscore the limitations of transposon insertion or inactivations in the study of bacterial physiology and pathogenesis: precise deletions that completely remove the target gene are preferable.

IsdC, a protein linked to the S. aureus cell wall by sortase B, was reported to bind haemin (Mazmanian et al., 2003), and two neighbouring genes (isdG and isdI) encode cytoplasmic enzymes involved in haemin degradation (Skaar et al., 2004a). Lmo2186 and IsdC are homologous throughout their mature sequences, and 33% identical. SvpA contains three sequential, contiguous domains of homology to Lmo2186 and IsdC. These regions within SvpA show 30%, 29% and 30% identity to Lmo2186 (by blast analysis), and 23%, 26% and 28% identity to IsdC respectively. Overall, SvpA has 25% identity to IsdC. Therefore, we tested the L. monocytogenes svpa-srtB locus for a role in haemin/haemoglobin utilization, and also considered its involvement in ferrichrome uptake, because lmo2183-lmo2181 share homology with hydroxamate ABC-transporters. In nutrition tests Δsvpa, Δlmo2186, ΔFX3 and ΔPROXS did not produce discernible effects on haem, haemoglobin or ferrichrome uptake. The assay gave reproducible, concentration-dependent stimulation of L. monocytogenes growth for a wide range of siderophores, as previously reported (Coulanges et al., 1998). The siderophore nutrition test is sensitive to even slight defects in iron transport (Wayne and Neilands, 1975), reflecting, for example, as small as three- to fivefold differences in transport $K_a$ or $K_m$ (Newton et al., 1999; Scott et al., 2001; Annamalai et al., 2004). The normal behaviour of the svpa-srtB mutants in this test suggests that the locus does not play a central role in iron acquisition from haemin, haemoglobin or ferrichrome.

Similar to what was reported for S. aureus, certain of our data relate the svpa-srtB locus to haemin/haemoglobin uptake. Although we could not analyse the properties of Lmo2186, because we did not observe it either in the membranes, attached to PG or in culture supernatants, we did study SvpA. It absorbed to haemin agarose, but haemin did not elute it, and the adsorption of P60, a putative glycolase without an apparent relationship to iron acquisition (Pilgrim et al., 2003), raised questions about the specificity of the affinity chromatography. The exclusive elution of SvpA from the haemin column by SDS intimated that in culture supernatants it was denatured, and non-specifically retained by the resin. Co-chromatography of haemin with SvpA and other secreted proteins on Sephacryl S100HR, an approach that was more interpretable, suggested a binding interaction with the iron porphyrin. But we also observed haemin adsorption to serum albumin in the same assay. Albumin contains non-polar patches on its surface that non-specifically absorb hydrophobic molecules, like lipids, in serum. The adsorption of haemin to serum albumin illustrates the uncertainties of conclusions about membrane transport from the observation of haemin binding to SvpA. Haemin that is uncomplexed by protein is only marginally soluble, and readily adsorbs to proteins. Furthermore, the minor effect that deletions had on virulence was consistent with their lack of influence on haemin/haemoglobin uptake. Thus the physiological function of haemin binding to SvpA and to other secreted proteins of L. monocytogenes remains for further study. A more recent report on staphylococcal iron acquisition (Skaar et al., 2004b) indicates that the isd operon is not the primary transport system for haem or haemoglobin; our results on the homologous svpa-srtB locus of L. monocytogenes are consistent with this conclusion.

**Experimental procedures**

**Bacterial strains, plasmids and media**

*Listeria monocytogenes* strain EGD-e (Bierne, 2004) was used for all mutant constructions. We reproduced in EGD-e a mutation of lmo2185 (svpa∆aphA3, herein called ΔsvpaK7), which was formerly studied in strain LO28 (Boreeze et al., 2001). Plasmid pUC19 was used to clone PCR-amplified sequences from EGD-e and to construct deletions, which were then transferred to the thermo-sensitive shuttle vector pAUL-A for recombination into the chromosome. *E. coli* strain DH5α was the host for plasmid constructions prior to their introduction in EGD-e, and for studies with a svpa-srtB promoter–gfp gene fusion.

*Escherichia coli* was grown in LB (Miller, 1972) or tryptose soy broth (Difco); *L. monocytogenes* was grown in BHI broth or on BHI plates (Difco). In some experiments we subcultured *L. monocytogenes* into an iron-deficient minimal media (KRM), that was based on RPMI 1640 (Sigma), with the following supplements (Sigma): casamino acids (0.1%), adenine (10 μg ml⁻¹), thiamine (1 μg ml⁻¹), riboflavin (0.5 μg ml⁻¹), biotin (1 μg ml⁻¹), lipoic acid (0.005 μg ml⁻¹), (NH₄)₂MoO₄·4H₂O (3 × 10⁻⁶ M), CoCl₂·6H₂O (3 × 10⁻⁴ M), HBO₃ (4 × 10⁻⁵ M), CuSO₄·5H₂O (10⁻⁶ M), MnCl₂ (8 × 10⁻⁶ M), ZnCl₂ (10⁻⁴ M).

We determined the concentration of iron in media by atomic adsorption spectrometry, using an iron hollow cathode lamp in a Varian 50B spectrometer. The results were compared to a set of standards, prepared from elemental iron ignited in moist hydrogen (reference grade; 100% Fe by assay); the assay was linear over a concentration range from 1 to 100 μM Fe. The iron concentrations were: RPMI minimal media, <1 μM; KRM minimal media, <1 μM; LB broth, 6 μM; BHI broth, 8 μM; Trypticase Soy broth, 29 μM.

**Construction of deletion mutants**

We performed two kinds of inactivations: deletions combined with insertion of a kanamycin cassette (ΔsvpaK7, ΔFX3), or...
complete, in-frame deletions without cassette insertions (all other constructs; Fig. 1). We created the latter mutations by joining segments of 600–1200 bp upstream and downstream of each gene, forming a 1.2–2 kb sequence of DNA lacking the coding sequence of a target gene. In the svpA-srtB locus, the promoter deletion (ΔPROX5) removed 175 nucleotides, from –186 to –11, which included the Fur box; it did not intrude into the downstream genes, which remain unaltered from start codon to termination codon. Almo2186 removed 189 of 207 codons (codons 3–192), in-frame. The new deletion in Imo2185 (ΔsvpA) precisely removed the complete structural gene, from start codon to termination codon. For the putative Fhu membrane permease complex [lmo2184 (binding protein), Imo2183 (permease) and Imo2182 (ATPase)], we used natural restriction sites to create a deletion. Primers FerrEco685 and FerrBam3542 were used to amplify a 2.8 kb region, and two natural ClaI sites were used to make a deletion and simultaneously insert a kanamycin-resistant cassette (855 bp). The deletion removed the C-terminal 90 residues encoded by Imo2183 and the N-terminal 197 residues encoded by Imo2182 (Fig. 1), leaving a kanamycin resistance cassette between the remnants of the two genes. The Δfur mutation eliminated the complete structural gene (lmo1956).

We used Taq polymerase (New England BioLabs) or AmpliTaqGold (Applied Biosystems) for PCR, according to manufacturer’s recommendations. Deletions were introduced in the EGDe chromosome by electroporating PAULA carrying the constructs into competent strains of L. monocytogenes (Poyart and Trieu-Cuot, 1997), and performing allelic replacement (Poyart and Trieu-Cuot, 1997). Primers for all the constructs (Table 4) were purchased from Eurogentec, France or IDT Biotechnologies, USA. We tested colonies of L. monocytogenes for the presence of desired plasmids, or their integration into or excision from the chromosome, by resuspension of a small portion of a bacterial colony into a 50 μl PCR reaction, containing appropriate primers. The annealing temperature was 50°C, and the extension time depended on the size of the expected fragments (1 min kb–1).

**Growth of L. monocytogenes strains in iron-limiting media**

Preliminary experiments showed that L. monocytogenes did not utilize iron complexed by BP, so we used the chelator to sequester iron in BHI cultures. Bacteria were grown in BHI to OD600 = 0.1, BP was added to 1 mM, and the cells were shaken at 37°C until the cultures reached an OD600 of 0.9. For experiments in iron-restricted KRM media, L. monocytogenes strains were cultured overnight in BHI, subcultured at 1% into KRM, grown to stationary phase (OD600 of approximately 0.9), and then subcultured again into KRM (1%) and grown to mid-log phase.

**Cell fractionation**

Listeria monocytogenes EGD-e was grown in 200 ml of liquid media, pelleted by centrifugation at 5000 g for 20 min, and the cell supernatant, which contained the secreted proteins, was concentrated using centrifugal filters (Amicon; 10 000 D cutoff; 4000 g for 20 min) and adjusted to a final volume that was commensurate with cell density (0.25 ml/1010 cells). We

**Table 4. Oligonucleotide primer sequences.**

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resuspended the cells in 1.5 ml of phosphate-buffered saline (PBS) and lysed them either with a Fast Prep bead-beater (Savant; three 30-s bursts at setting 6), or by two passages through a French pressure cell (SLM-Amino) at 20 000 pounds per square inch. After removing debris and unlysed cells by a 20 min centrifugation at 5000 g, we separated the cytoplasm from the cell envelope by centrifugation at 100 000 g for 45 min. We collected the supernatant (cytoplasm), resuspended the pellet in a volume of 0.5% sodium sarkosinate in PBS that was commensurate with the original cell density (0.25 ml 10^{-10} cells), and incubated the mixture at 25°C for 30 min with reciprocal shaking. After centrifugation at 100 000 g for 45 min, we collected the supernatant, containing the cytoplasmic membrane proteins, and resuspended the pellet, which contained the PG layer of the cell wall, in a volume of PBS that was commensurate with the original cell density (0.25 ml 10^{-10} cells). PG-associated proteins were released by treatment with bacteriophage amylase [1 μg ml^{-1}, 25°C for 15 min (Loessner et al., 1995)], followed by centrifugation at 100 000 g for 45 min. The phage amylase cleaves PG and release covalently anchored proteins. We purified the overexpressed enzyme from E. coli HPLL511 (a gift from Dr Martin Loessner, Technical University of Munich, Germany) by His-tag chromatography over Ni-NTA agarose (Qiagen). In summary, the procedure yielded the cell supernatants, cytoplasmic membranes, and PG-associated proteins in equivalent volumes that were commensurate with cell density.

**SDS-PAGE and Western immunoblots**

SDS-PAGE (Ames, 1975) was performed on 0.8 mm slabs. For quantitative comparisons of protein concentrations in different cell fractions, equal volumes of concentrated culture supernatants, cytoplasmic membranes and PG-associated proteins were solubilized with sample buffer and loaded into gels; equivalent volumes of cytoplasmic proteins were also calculated, solubilized and electrophoresed. SvpA production was visualized and analysed by Western immunoblots with rabbit anti-SvpA (Boreeze et al., 2001), developed with either goat-anti-rabbit-IgG alkaline phosphatase and bromochloroindolyl phosphate/nitroblue tetrazolium (Newton et al., 1999) or with 125I-protein A. In the latter case the immunoblots were quantified by phosphoimagery (Stormscan, Molecular Dynamics).

**Quantitative real-time (TaqMan) PCR**

Total RNA isolated from cultures of L. monocytogenes grown in two different conditions was converted into DNA with reverse transcriptase, and quantitative PCR using sets of primers from different genes was performed to evaluate their relative level of expression. We compared cells grown in BHI and BHI + BP, and in a separate experiment, compared cells growing inside Caco-2 or HepG-2 cells with cells growing free in KRM media. For RNA preparation cells were broken with a bead-beater (Savant) in a solution of 1 ml Trizol (Invitrogen). Total RNA was extracted with 300 μl of chloroform/isoamyl alcohol, isopropanol-precipitated and resuspended in diethylpyrocarbonate 5DEPC-treated water. Contaminating DNA was removed by digestion with DNase I. Reverse transcription (RT)-PCR experiments were made from 1 μg of RNA with 2.5 pmol of specific primers for the genes gyrA (gyrase, constitutively expressed) and svpA, in a volume of 8 μl. After denaturation at 65°C for 10 min, 12 μl of the mixture containing 2 μl of dNTP (25 mM), 4 μl of 4× buffer, 2 μl of DTT, 1 μl of Rnasin (Promega) and 1.5 μl of Superscript II (Invitrogen) were added. Samples were incubated for 60 min at 42°C, heated at 75°C for 15 min and then chilled on ice. PCR conditions were identical for all reactions. The 25 μl reactions, performed in sealed tubes, consisted of 12.5 μl of PCR master mix (PE Applied Biosystems) containing Sybr Green, 4 μl of template, and 5 pmol of each primer. For real-time quantitative PCR, we used the ABI Prism 7700 sequence detection system with TaqMan Universal PCR master mix (Applied Biosystems). Results were normalized to the amount of gyrA mRNA, which was constant under our growth conditions; measurements were made in triplicate. In experiments with Caco-2 or HepG-2 cells, the induction was measured in comparison to Listeria cells grown in KRM media.

**Cloning of GFP under svpA-srtB promoter control**

We used the Pro5’Eco and Pro3’Kpn primers, which are flanked by EcoRI and KpnI restriction sites (Table 4), to amplify the 175 bp promoter region of the svpA-srtB locus. Primers GFP5’Kpn and GFP3’Sai were used to amplify the 723 bp GFP gene encoded in plasmid pAMGFP (a gift from Rebecca L. Wilson; Wilson et al., 2001), flanked by KpnI and Sall restriction sites. The resulting amplified fragments were cloned as EcoRI/KpnI/Sall insert in vector pUC18 (Invitrogen). E. coli cells (DH5α) were transformed with the resulting vector, pGFP15. We transferred the svpA-srtB promoter–gfp insert from pGFP15 to the listerial vector pAT28 (Trieu-Cuot et al., 1990) by restriction fragment exchange, using EcoRI and Sall, creating pATgfp7.

**Nutrition tests**

Overnight BHI cultures of L. monocytogenes were subcultured at 1% in BHI broth and shaken at 37°C for 2.5 h. BP was added to 1 mM, and growth was continued for 3.5 h. A total of 200 μl of cells were mixed with 8 ml of BHI top agar, and BP was added to 0.1 mM. The mixture was poured into Petri dishes, allowed to solidify, and sterile paper discs were placed on the surface of the agar. Then 10 μl aliquots of solutions of ferrichrome, haemin or haemoglobin were added to the discs and the assays were scored after 18 h at 37°C.

**Affinity chromatography on haemin agarose**

We 10-fold concentrated (200 ml to 20 ml) the supernatant of a BHI EGD-Δfur culture, that overexpresses SvpA, by centrifugal filtration (Amicon; 10 000 D cutoff), and chromatographed the supernatant proteins over a column (4 ml packed volume) of haemin agarose (SIGMA) in PBS, pH 7.4. The column was washed with 36 ml of PBS (pH 7.4), then 36 ml 100 μM haemin in PBS (pH 10), and 36 ml of 1% SDS. Three millilitre fractions were collected and analysed by Coomassie

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blue-stained SDS-PAGE gels, and by anti-SvpA immunoblots, developed with $^{125}$I-protein A.

**Gel permeation chromatography on Sephacryl S100hr**

Culture supernatants from EGD-e and aPROX5, grown in KRM media, were concentrated 100-fold (200 ml to 2 ml) by centrifugal filtration (Amicon; 10 000 D cutoff) and haemin was added to 20 μM. The mixtures were incubated on ice for 10 min, and then chromatographed on a column of Sephacryl S100HR (Pharmacia; 0.5 × 55 cm) in 50 mM Tris buffer, pH 8. As a control, bovine serum albumin (0.25 mg ml$^{-1}$) was mixed with 20 μM haemin and chromatographed on the same resin. The void and included volumes of the column were determined by chromatography of a group of protein standards that spanned the range of molecular weights 10 000–150 000.

**Multiplication in Caco-2 cells and bone marrow-derived macrophages**

We used the human colon carcinoma cell lines Caco-2 and HepG-2 from the American Type Culture Collection (Manassas, VA, USA). Primary bone marrow-derived macrophages from BALB/c mice were cultured and infected for growth curves at a cell/bacterium ratio of 10:1. Cells were exposed to $L$. monocytogenes cells for 15 min at 37°C and non-invasive bacteria were removed by several washings. Fresh media was added to infected cells and multiplication of intracellular bacteria was followed by plating appropriate dilutions after lysis of the cell monolayer (Autret et al., 2001).

**Persistence in organs after oral immunization**

For oral infection, groups of five mice (5–6 week old female BALB/c) were starved for 24 h and inoculated orally with $4 \times 10^{10}$ bacteria in 0.2 ml aliquots. After 3 days, the spleen, liver, and the small intestines were recovered. Small intestines were rinsed in Dulbecco’s modified Eagle’s medium (Invitrogen) to remove the intestinal content. Intestines were homogenized in 0.15 M NaCl and plated on BHI agar. Livers and spleens were homogenized and also plated in BHI agar at appropriate dilutions.

**Determination of virulence in the mouse model**

LD$_{50}$ was calculated by the Probit method, after intravenous inoculation of groups of five mice (6–8 week old female Swiss) with various doses of $L$. monocytogenes suspensions in sterile (Autret et al., 2001).

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**References**


