

# Sortase independent and dependent systems for acquisition of haem and haemoglobin in *Listeria monocytogenes*

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## Summary

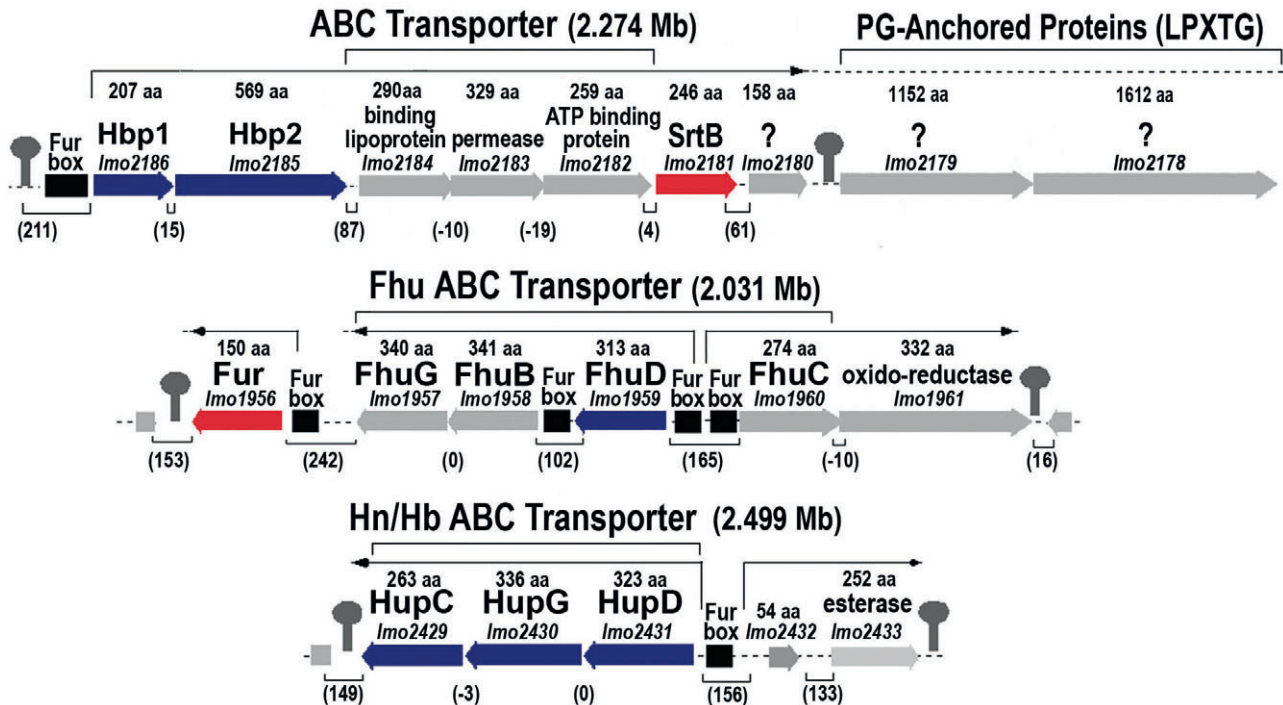
We studied three Fur-regulated systems of *Listeria monocytogenes*: the *srtB* region, that encodes sortase-anchored proteins and a putative ABC transporter, and the *fhu* and *hup* operons, that produce putative ABC transporters for ferric hydroxamates and haemin (Hn)/haemoglobin (Hb) respectively. Deletion of *lmo2185* in the *srtB* region reduced listerial [<sup>59</sup>Fe]-Hn transport, and purified Lmo2185 bound [<sup>59</sup>Fe]-Hn ( $K_D = 12$  nM), leading to its designation as a Hn/Hb binding protein (*hbp2*). Purified Hbp2 also acted as a haemophore, capturing and supplying Hn from the environment. Nevertheless, Hbp2 only functioned in [<sup>59</sup>Fe]-Hn transport at external concentrations less than 50 nM: at higher Hn levels its uptake occurred with equivalent affinity and rate without Hbp2. Similarly, deletion of sortase A had no effect on ferric siderophore or Hn/Hb transport at any concentration, and the *srtA*-independence of listerial Hn/Hb uptake distinguished it from comparable systems of *Staphylococcus aureus*. In the cytoplasmic membrane, the Hup transporter was specific for Hn: its lipoprotein (HupD) only showed high affinity for the iron porphyrin ( $K_D = 26$  nM). Conversely, the FhuD lipoprotein encoded by the *fhu* operon had broad specificity: it bound both ferric siderophores and Hn, with the highest affinity for ferrioxamine B ( $K_D = 123$  nM). Deletions of Hup permease components *hupD*, *hupG* or

*hupDGC* reduced Hn/Hb uptake, and complementation of  $\Delta$ *hupC* and  $\Delta$ *hupG* by chromosomal integration of *hupC*<sup>+</sup> and *hupG*<sup>+</sup> alleles on pPL2 restored growth promotion by Hn/Hb. However,  $\Delta$ *hupDGC* did not completely eliminate [<sup>59</sup>Fe]-Hn transport, implying the existence of another cytoplasmic membrane Hn transporter. The overall  $K_M$  of Hn uptake by wild-type strain EGD-e was 1 nM, and it occurred at similar rates ( $V_{max} = 23$  pmol  $10^9$  cells<sup>-1</sup> min<sup>-1</sup>) to those of ferric siderophore transporters. In the  $\Delta$ *hupDGC* strain uptake occurred at a threefold lower rate ( $V_{max} = 7$  pmol  $10^9$  cells<sup>-1</sup> min<sup>-1</sup>). The results show that at low (< 50 nM) levels of Hn, SrtB-dependent peptidoglycan-anchored proteins (e.g. Hbp2) bind the porphyrin, and HupDGC or another transporter completes its uptake into the cytoplasm. However, at higher concentrations Hn uptake is SrtB-independent: peptidoglycan-anchored binding proteins are dispensable because HupDGC directly absorbs and internalizes Hn. Finally,  $\Delta$ *hupDGC* increased the LD<sub>50</sub> of *L. monocytogenes* 100-fold in the mouse infection model, reiterating the importance of this system in listerial virulence.

## Introduction

Iron, the second-most abundant metal in the Earth's crust, precipitates in water as an oxyhydroxide polymer, resulting in poor bio-availability (Neilands, 1974). Despite the paucity of soluble iron in solution, it is usually essential to pro- and eukaryotes from its activities as an oxygen carrier and metabolic or redox cofactor. Animals, plants and microbes compete for iron, and successful pathogens obtain it in the host environment. The majority of mammalian iron exists as haem (Hn) in myoglobin and haemoglobin (Hb), but in aerobic organisms iron functions in energy generation as an electron transport chain cofactor, in lipid, steroid, xenobiotic and drug oxidation by cytochrome P450s, and in antioxidant defence mechanisms involving superoxide dismutases. Free Hn is toxic at excessive concentrations (Robinson *et al.*, 2009; Immenschuh *et al.*, 2010; Reeder, 2010) and bacteria regulate its accumulation (Stauff and Skaar, 2009). The upshot is that the multiple iron-containing systems of animals and bacteria lead to delicately balanced adversarial mechanisms for iron sequestration by the former (iron-binding proteins

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**Fig. 1.** Chromosomal loci. A schematic representation of the *srtB*, *fhu* and *hup* regions of the *L. monocytogenes* chromosome shows loci involved in iron acquisition. The *srtB* region (2.274 Mb) contains genes for secreted, peptidoglycan-associated proteins (*Imo2185*, 6), the sortase (*srtB*) that anchors them to the cell wall, a putative ABC-transporter (*Imo2182-2184*) and two SrtA-dependent cell wall proteins (*Imo2178*, *2179*) (Bierne *et al.*, 2004; Newton *et al.*, 2005). The *fhu* region (2.031 Mb) encodes a substrate binding lipoprotein (*FhuD*) and a putative ferric hydroxamate ABC-transporter (*FhuBCG*) (Jin *et al.*, 2005). The *hupDGC* locus (2.499 Mb) encodes a substrate binding lipoprotein (*HupD*) and a putative ABC transporter for Hn/Hb (Jin *et al.*, 2005). Parenthetical values refer to the distance (bp) between adjacent genes. This figure is available in colour online on [wileyonlinelibrary.com](http://wileyonlinelibrary.com).

like ferritin, transferrin, lactoferrin, lipocalin), and iron acquisition by the latter organisms (siderophores and membrane iron/Hn transporters; Konopka *et al.*, 1981; 1982; Tidmarsh *et al.*, 1983; Braun, 2005; Arslan *et al.*, 2009; Singh *et al.*, 2009).

Bacteria produce membrane transport systems for Hn utilization during infections of animal hosts. In Gram-negative cells TonB-dependent outer membrane receptors, periplasmic binding proteins and ABC transporters capture Hn and concentrate it intracellularly (Perry *et al.*, 2003; Wyckoff *et al.*, 2004; Schneider and Paoli, 2005; Zhao *et al.*, 2010). ATP hydrolysis energizes Hn transport through the inner membrane (IM) (Stojiljkovic and Hantke, 1994; Thompson *et al.*, 1999; Tong and Guo, 2007; Burkhard and Wilks, 2008). In Gram-positive cells ABC transporters for Hn (Drazek *et al.*, 2000; Skaar *et al.*, 2004; Jin *et al.*, 2005) underlie an extensive, loosely cross-linked peptidoglycan (PG) layer, that permits diffusion of small solutes through its 20- 70 Å diameter pores (Demchick and Koch, 1996; Touhami *et al.*, 2004; Meroueh *et al.*, 2006). Ferric siderophores like ferrioxamine B (FxB) and ferrichrome (Fc) need not interact with PG or proteins anchored to it: CM-resident ABC transporters are their only membrane uptake component (Jin *et al.*,

2005). The different architecture of the Gram-positive bacterial cell wall makes the pathway of Hn uptake less certain, but sortase-anchored proteins in the PG matrix extract the porphyrin from iron-containing proteins like Hb by a cascade of binding reactions that ultimately deliver it to a CM transporter (Lei *et al.*, 2002; 2003; Nygaard *et al.*, 2006; Zhu *et al.*, 2008a; Ran *et al.*, 2010).

Hn/Hb transporters exist in *Staphylococcus aureus* (Grigg *et al.*, 2010), *Streptococcus pyogenes* (Lei *et al.*, 2002), *Bacillus anthracis* (Tarlovsky *et al.*, 2010) and *Listeria monocytogenes* (Jin *et al.*, 2005), which contribute to bacterial virulence (Carniel, 2001; Stork *et al.*, 2004; Braun, 2005; Jin *et al.*, 2005). In *S. aureus* the *isd* gene cluster encodes sortase B (*SrtB*), an *SrtB*-anchored Hn binding protein (*IsdC*), an ABC transporter and other sortase A-dependent PG-anchored proteins (Mazmanian *et al.*, 2003). *L. monocytogenes* has a comparable *srtB*-containing genetic system, but deletion of its components, including the *IsdC* homologue *Lmo2185*, did not influence Hn/Hb uptake (Jin *et al.*, 2005). Nor did  $\Delta$ *lmo2185* or  $\Delta$ *lmo2186* (Fig. 1) reduce the virulence of *L. monocytogenes* in a mouse model (Jin *et al.*, 2005). Rather, iron acquisition from both the porphyrin and the protein required the activity of the putative ABC-transporter

encoded by the *hup* chromosomal region (2498.3–2501.1 kb);  $\Delta hupC$  impaired Hn and Hb uptake and decreased virulence (Jin *et al.*, 2005).

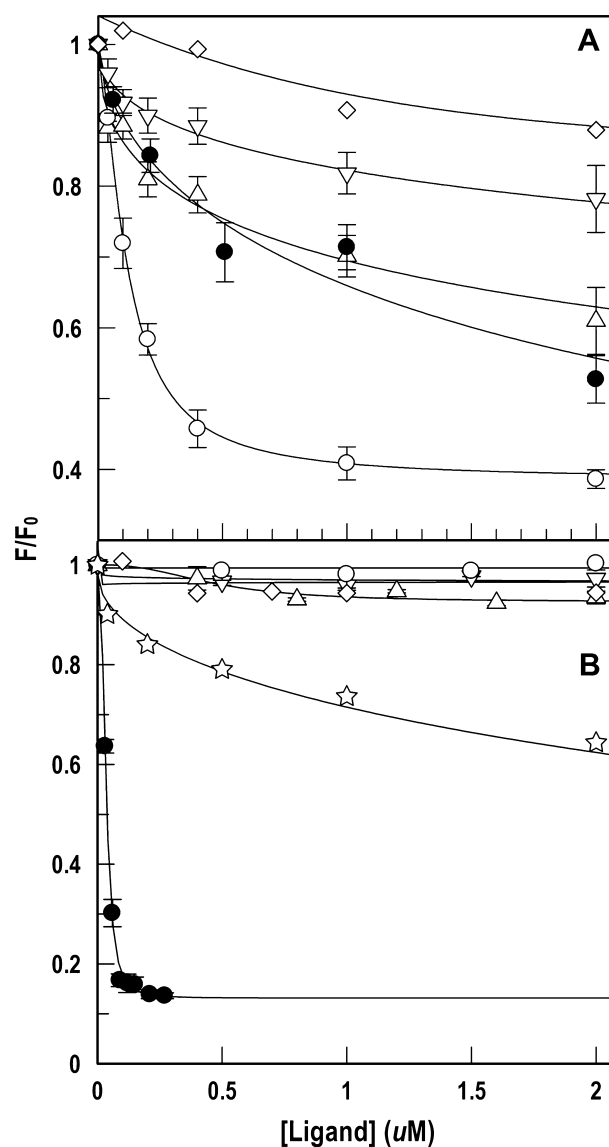
Because of uncertainties in the Gram-positive bacterial Hn transport pathway we further studied its uptake by *L. monocytogenes*. The putative Hn binding protein, HupD, displayed selectivity for the iron porphyrin, which contrasted the behaviour of the ferric hydroxamate binding protein, FhuD, that showed broad recognition of numerous ferric siderophores. These data, and the impairment of Hn/Hb uptake by deletion of *hupG* (*Imo2430*), *hupD* (*Imo2431*) or the whole operon ( $\Delta hup$ ) reiterated the specificity of the Hup membrane permease. High sensitivity [ $^{59}\text{Fe}$ ]-Hn uptake assays defined the kinetic properties of the HupDGC transporter, identified a second Hn/Hb uptake system in *L. monocytogenes*, and showed a role for SrtB-anchored Lmo2185 in capture of the iron porphyrin. Sortase A-deficient bacteria, on the other hand, showed no defects in Hn/Hb acquisition.

## Results

### Specificities of the FhuD and HupD binding lipoproteins

In *L. monocytogenes* the Fur-regulated (Baichoo *et al.*, 2002) *fhu* and *hup* operons (Fig. 1) contain genes for binding proteins (*fhuD*, *hupD*), membrane permeases (*fhuB*, *fhuG*, *hupG*) and ATPase components (*fhuC*, *hupC*) of putative ABC transporters that function in the acquisition of ferric hydroxamate siderophores and Hn/Hb respectively (Jin *et al.*, 2005). To define the selectivities of the two systems we cloned, overexpressed and purified their binding proteins, and then assayed their adsorption of ferric siderophores and porphyrins. Although neither listerial protein is structurally solved, both are related to *Escherichia coli* FhuD [EcoFhuD; PDB 1ESZ (Clarke *et al.*, 2002)]: LmoFhuD is 19% identical and 59% similar; LmoHupD is 17% identical and 57% similar. All three binding proteins contain Trp: LmoFhuD at residues 158, 229 and 271, LmoHupD at residue 280, and EcoFhuD at residues 43, 68, 102, 210, 217, 255 and 273. EcoFhuD W273, situated in the solute binding site, aligns with W271 in LmoFhuD and W280 in LmoHupD.

Trp in proximity to the binding cavity permitted fluorescence spectroscopic measurements of ligand adsorption to purified *L. monocytogenes* 6H-FhuD and 6H-HupD. These data (Fig. 2; Table 1) revealed a hierarchy of multispecificity in the former protein. It had the highest affinity for FxB ( $K_D = 0.1 \mu\text{M}$ ), with lower affinities for other ferric hydroxamate siderophores [FcA ( $K_D = 0.5 \mu\text{M}$ ), Fc ( $K_D = 0.5 \mu\text{M}$ )]. But, 6H-FhuD also bound porphyrins [PPIX ( $K_D = 0.3 \mu\text{M}$ ), Hn ( $K_D = 0.6 \mu\text{M}$ )], and even FeEnt ( $K_D = 2.3 \mu\text{M}$ ), which is structurally distinct from the hydroxamates or porphyrins. The ferric catecholate



**Fig. 2.** Fluorescence spectroscopic comparison of FhuD and HupD binding specificities. *L. monocytogenes* 6H-FhuD and 6H-HupD were purified by affinity chromatography on  $\text{Ni}^{2+}$ -NTA agarose and tested for their ability to bind ferric siderophores and Hn, by monitoring quenching of intrinsic Trp fluorescence. (Top panel) FhuD, the ferric hydroxamate binding protein, recognized a variety of iron complexes, including Hn, whereas (bottom panel) HupD was specific for its only ligand, Hn [(○) ferrioxamine B (FxB); (△) ferrichrome (Fc); (▽) ferrichrome A (FcA); (◇) ferric enterobactin (FeEnt); (●) Hn; (☆) protoporphyrin IX (PPIX)]. Removal of their 6H tags (by cleavage with thrombin) had no effect on the specificities or affinities of the binding proteins (Table 1).

caused less quenching, suggesting its incomplete occupancy of the binding site. We conducted the initial measurements of these binding reactions with purified proteins that retained an N-terminal 6-His tag (Fig. 2), but because hexahistidine may coordinate  $\text{Fe}^{2+}$  (e.g. at the iron centre of Hn), we also assayed purified FhuD from

**Table 1.** Affinities of listerial proteins FhuD (Lmo1959) and HupD (Lmo2431) for iron chelates.

Iron complex	Binding $K_D^a$			
	FhuD-6H	FhuD	HupD-6H	HupD
Fc	523 ± 243	243 ± 13	NB	ND
FcA	497 ± 130	ND	NB	ND
FxB	123 ± 9.6	44 ± 3	NB	ND
FeEnt	2330 ± 678	ND	NB	ND
Hn	588 ± 79	711 ± 183	36 ± 11	26 ± 5
PPIX	276 ± 32	ND	1148	ND

a. The data derive from repeated measurements (3–5 trials) of intrinsic fluorescence quenching (Fig. 2), from which we used Grafit 7.0.1 to calculate the binding  $K_D$  (nM) for each iron complex. The data are expressed as  $K_D \pm$  the standard deviation of the mean values (the mean SD of all determinations was 20.2%).

NB, no binding; ND, not determined.

which the 6-His sequence was proteolytically removed. The hexahistidine tag had little or no effect on measured affinities for ferric siderophores and Hn (Table 1).

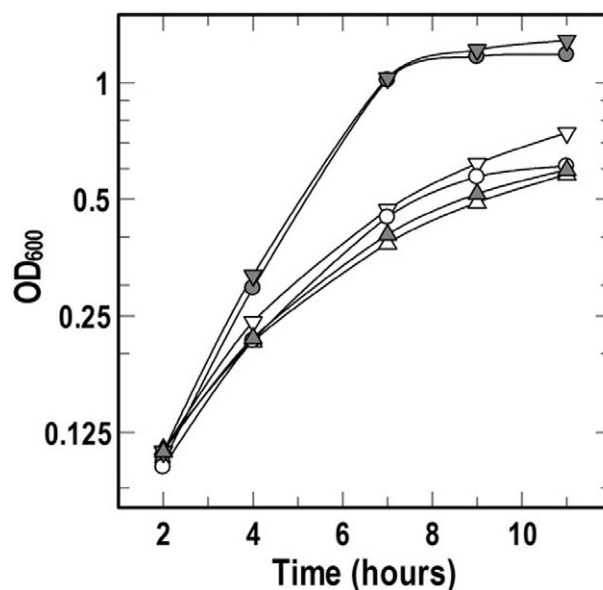
In contrast to LmoFhuD, the high affinity and specificity of 6H-HupD for Hn was apparent even during its purification. When expressed in *E. coli* BL21 and purified by Ni<sup>2+</sup>-NTA chromatography, solutions of 6H-HupD were yellow, red or brown, and their visible spectra showed Soret peaks characteristic of Hn. Removal of the bound iron porphyrin from 6H-HupD required either anion exchange chromatography or SDS-denaturation of the protein. Unlike FhuD, purified 6H-HupD was virtually monospecific: it bound Hn with fivefold higher affinity ( $K_D = 36$  nM) than the most avid LmoFhuD binding interaction, and did not measurably adsorb the other iron complexes that we tested. Only PPIX also bound to LmoHupD, but with 40-fold lower affinity, indicating the importance of iron in the recognition reaction. Again, proteolytic removal of the 6H tag did not significantly alter the affinity of HupD for Hn (Table 1). In summary, the two binding proteins of the ABC transporter systems were different: the former had lower affinity and broad specificity for ferric siderophores, whereas the latter manifested high affinity and monospecificity for the iron porphyrin.

#### Effects of *hup* operon deletions on growth of *L. monocytogenes* in iron-deficient media

*Listeria monocytogenes* does not utilize the complex of Fe<sup>3+</sup> and 2,2'-bipyridyl (BP) as an iron source (Jin *et al.*, 2005; Newton *et al.*, 2005), so we employed BP to render the bacteria iron-deficient in microbiological media. In BHI media inoculated with the wild-type strain (EGD-e) or its mutant derivatives the addition of 1 mM BP inhibited growth, but when 0.2 mM Hn was added EGD-e returned to its original growth rate and ultimately reached a higher density. However, otherwise isogenic strains with  $\Delta hupC$ ,  $\Delta hupG$  or a complete, precise deletion of the entire *hup* operon ( $\Delta hupDGC$ ) were not stimulated by Hn, unless we

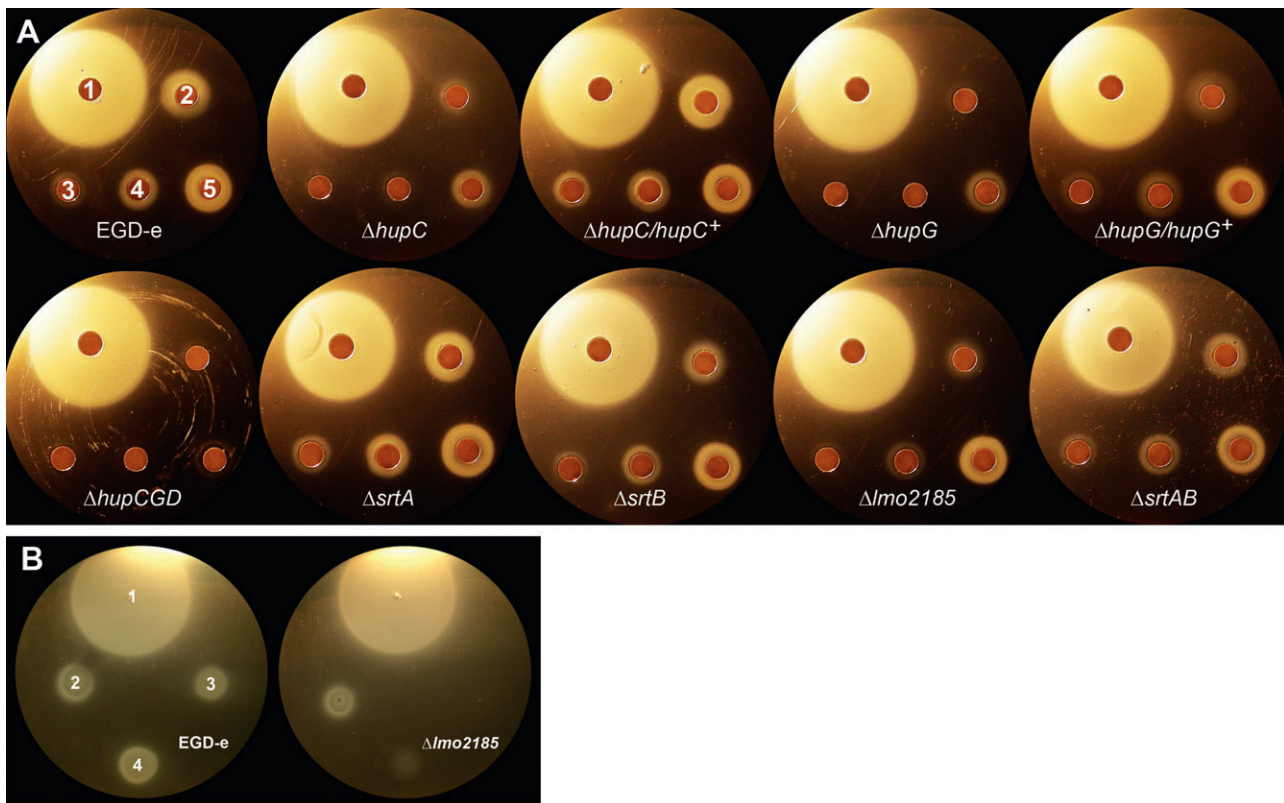
complemented the chromosomal deletions by introduction of the corresponding wild-type gene ( $\Delta hupC/hupC^+$  and  $\Delta hupG/hupG^+$ ; Fig. 3). These findings reiterated the specificity of the *hup* operon:  $\Delta hupC$ ,  $\Delta hupG$  and  $\Delta hupDGC$  impaired Hn capture, while chromosomal reinstatement of those genes restored it.

We also assessed the ability of mutant strains to utilize Hn by microbiological nutrition tests (Wayne *et al.*, 1976; Newton *et al.*, 2005). In a previous study only relatively high concentrations of Hn (200  $\mu$ M; Jin *et al.*, 2005) stimu-



**Fig. 3.** Growth of mutants in BHI with 2,2'-bipyridyl. Cells were grown in BHI overnight, and  $2.5 \times 10^7$  cells were subcultured into BHI containing 1 mM 2,2'-bipyridyl. After 2 h, Hn (2 mM in 40% DMSO) was diluted into the media to 0.2  $\mu$ M. Growth curves defined by open symbols derive from parent cultures to which no Hn was added; curves from filled symbols represent data derived from cultures to which 0.2  $\mu$ M Hn was added: (○) EGD-e, (△)  $\Delta hupG$ , (▽)  $\Delta hupG\_pPL2hupG^+$ . For clarity, equivalent results with EGD-e derivatives carrying  $\Delta hupC$  or  $\Delta hupDGC$  are not shown. The plotted data represent a single experiment, but the experiment was repeated at least 3 times with the same results.





**Fig. 4.** Iron nutrition tests of *L. monocytogenes* strains. EGD-e and its mutants were grown in BHI, subjected to iron deprivation at mid-log by addition of 1 mM bipyridyl, and plated on BHI agar containing 0.25 mM bipyridyl (Newton *et al.*, 2005). A. Hn/Hb utilization. Paper discs were placed on the agar, and 10  $\mu$ l aliquots of different iron compounds were applied to the discs: (1) 50  $\mu$ M Fc; (2) 15  $\mu$ M Hb; (3–5) 0.5  $\mu$ M, 5  $\mu$ M and 50  $\mu$ M Hn respectively. Mutants in the *hup* operon had reduced iron supply from Hn and Hb, whereas sortase mutants and  $\Delta$ *lmo2185* showed little reduction in this assay. All the strains normally utilized the hydroxamate siderophore Fc. B. Utilization of iron from purified Lmo2185. Ten-microlitre aliquots of compounds were applied with a micropipette tip directly to the agar: (1) 50  $\mu$ M Fc; (2) 0.5  $\mu$ M Hn; (3) 10.5  $\mu$ M apo-Lmo2185; (4) partially saturated Lmo2185 (1.6  $\mu$ M Lmo2185 containing 0.7  $\mu$ M Hn). Both apo- and holo-Lmo2185 supplied iron to EGD-e, but not to its  $\Delta$ *lmo2185* derivative.

lated the growth of EGD-e, and we found that the low sensitivity derived from poor solubility of Hn in aqueous buffers. Hn may dimerize and precipitate in aqueous solution (Collier *et al.*, 1979, de Villiers *et al.*, 2007, Asher *et al.* 2009), but a solvent/buffer system containing DMSO (*Experimental procedures*) minimized formation of the  $\pi$ - $\pi$  and/or  $\mu$ -oxo-dimers, improving its solubility. When stored in DMSO stocks and diluted into media, Hn stimulated growth at concentrations as low as 0.5  $\mu$ M. Under these conditions, deletions in the *hup* operon ( $\Delta$ *hupC*,  $\Delta$ *hupD*,  $\Delta$ *hupG* or  $\Delta$ *hupDGC*) decreased or eliminated Hn (and Hb) uptake (Fig. 4). The iron nutrition tests showed greater impact from loss of the membrane permease (HupG) than loss of the ATPase component (HupC), in that the  $\Delta$ *hupG* strain did not respond to Hn or Hb at 5  $\mu$ M, but the  $\Delta$ *hupC* mutant showed a faint halo around that concentration. This result may derive from the ability of ATPase components of different ABC transporters to substitute for each other. Complementation of  $\Delta$ *hupC* and  $\Delta$ *hupG* by integration of pPL2 vectors (Lauer *et al.*, 2002)

carrying their corresponding wild-type genes restored Hn/Hb uptake. However, our attempted complementation of  $\Delta$ *hupD* was unsuccessful, even though pPL2*hupD*<sup>+</sup> integrated into the chromosome of the *hupD* deletion mutant. We also attempted complementation when pPL2*hupD*<sup>+</sup> contained the stronger *clpB* promoter (Chastanet *et al.*, 2004), but again without success. *hupD* is the first gene in the operon, and its deletion may cause a polar effect on downstream genes.

Among listerial sortase mutants, nutrition tests did not show an effect of  $\Delta$ *srtA* on Hn or Hb uptake. These and other data (see following) indicated that SrtA-anchored proteins have no obvious role in ferric siderophore or Hn/Hb acquisition. On the other hand, SrtB anchors the product of *lmo2185* (formerly known as SvpA) to PG. The improved-sensitivity Hn nutrition assay showed that  $\Delta$ *lmo2185* reduced uptake of Hn at 0.5 and 5  $\mu$ M; at 50  $\mu$ M Hn the strain behaved like EGD-e (Fig. 4A). Furthermore, Lmo2185 bound Hn (Jin *et al.*, 2005; see below), and it promoted growth of *L. monocytogenes*

**Table 2.** Binding and transport properties of selected strains.

Strain	Binding <sup>a</sup>		Transport <sup>a</sup>	
	$K_D$	Capacity	$K_M$	$V_{max}$
EGD-e	2.4 ± 0.47	129.5 ± 5.6	1.2 ± 0.2	22.2 ± 0.9
Δ <i>hup</i>	2.5 ± 0.45	105 ± 4.2	1.1 ± 0.2	7.5 ± 0.3
Δ <i>srtA</i>	2.3 ± 0.16	129 ± 1.9	1.4 ± 0.2	23.1 ± 0.6
Δ <i>srtB</i>	9.9 ± 1.3	18.7 ± 0.9	7.1 ± 7.5	3.5 ± 1.6
Δ <i>srtAB</i>	5.1 ± 3	9 ± 1.5	15.1 ± 6.4	5.6 ± 1.3
Δ <i>lmo2185</i>	11.4 ± 2.6	30.1 ± 2.9	NS	NS

**a.** The data derive from repeated measurements (3–5 trials) of [<sup>59</sup>Fe]-Hn binding or transport (Fig. 5), from which we used Grafit 7.0.1 to calculate its binding  $K_D$  (nM) and capacity (pmol 10<sup>9</sup> cells<sup>-1</sup>), and its transport  $K_M$  (nM) and  $V_{max}$  (pmol 10<sup>9</sup> cells<sup>-1</sup> min<sup>-1</sup>) in the different genetic backgrounds. The data are expressed as the measured parameter ± the standard deviation of the mean values (the mean SD for  $K_D$ , capacity,  $K_M$  and  $V_{max}$  were 23%, 7%, 39% and 16% respectively). NS, non-saturation transport process.

EGD-e in iron nutrition tests (Fig. 4B), demonstrating its activity as a haemophore (Arnoux *et al.*, 1999). However, both apo- and holo-Lmo2185 were inactive or barely stimulatory to a Δ*lmo2185* strain, suggesting that Hn uptake by this route also requires the SrtB-anchored form of the cell envelope protein.

#### Hn binding and transport

We synthesized [<sup>59</sup>Fe]-Hn (*Experimental procedures*) and measured its binding and uptake by EGD-e and derivatives with deletions in the *hup* operon (Table 2, Fig. 5), again using conditions to avoid rapid formation of Hn dimers. When grown in MOPS-L (*Experimental procedures*) the binding capacity of EGD-e for [<sup>59</sup>Fe]-Hn was 130 pmol 10<sup>9</sup> cells<sup>-1</sup> and the  $K_D$  of the binding interaction was 2.4 nM. Complete deletion of the *hup* operon did not eliminate Hn adsorption, but decreased binding capacity by ~20%, to 105 pmol 10<sup>9</sup> cells<sup>-1</sup>. Further experiments ascribed the residual Hn binding to two systems: an additional cytoplasmic membrane transporter, and to sortase B-anchored proteins in the cell wall (see below).

Again, the Δ*srtA* strain lacked a phenotype in Hn binding studies. It adsorbed [<sup>59</sup>Fe]-Hn with roughly the same affinity and precisely the same capacity as EGD-e. We saw a slight (twofold) difference in [<sup>59</sup>Fe]-Hn binding capacity between the Δ*srtB* (19 pmol 10<sup>9</sup> cells<sup>-1</sup>) and Δ*srtAB* (9 pmol 10<sup>9</sup> cells<sup>-1</sup>) strains. But, when compared with the ~10-fold higher capacity of both EGD-e and the Δ*srtA* mutant (130 pmol 10<sup>9</sup> cells<sup>-1</sup>) the significance of this variation was questionable, and complicated by the error inherent in measurements of weak residual binding by the Δ*srtB* strain. Thus, sortase A had no impact on Hn binding; it was the Δ*srtB* mutation that decreased the Hn binding capacity in the double mutant.

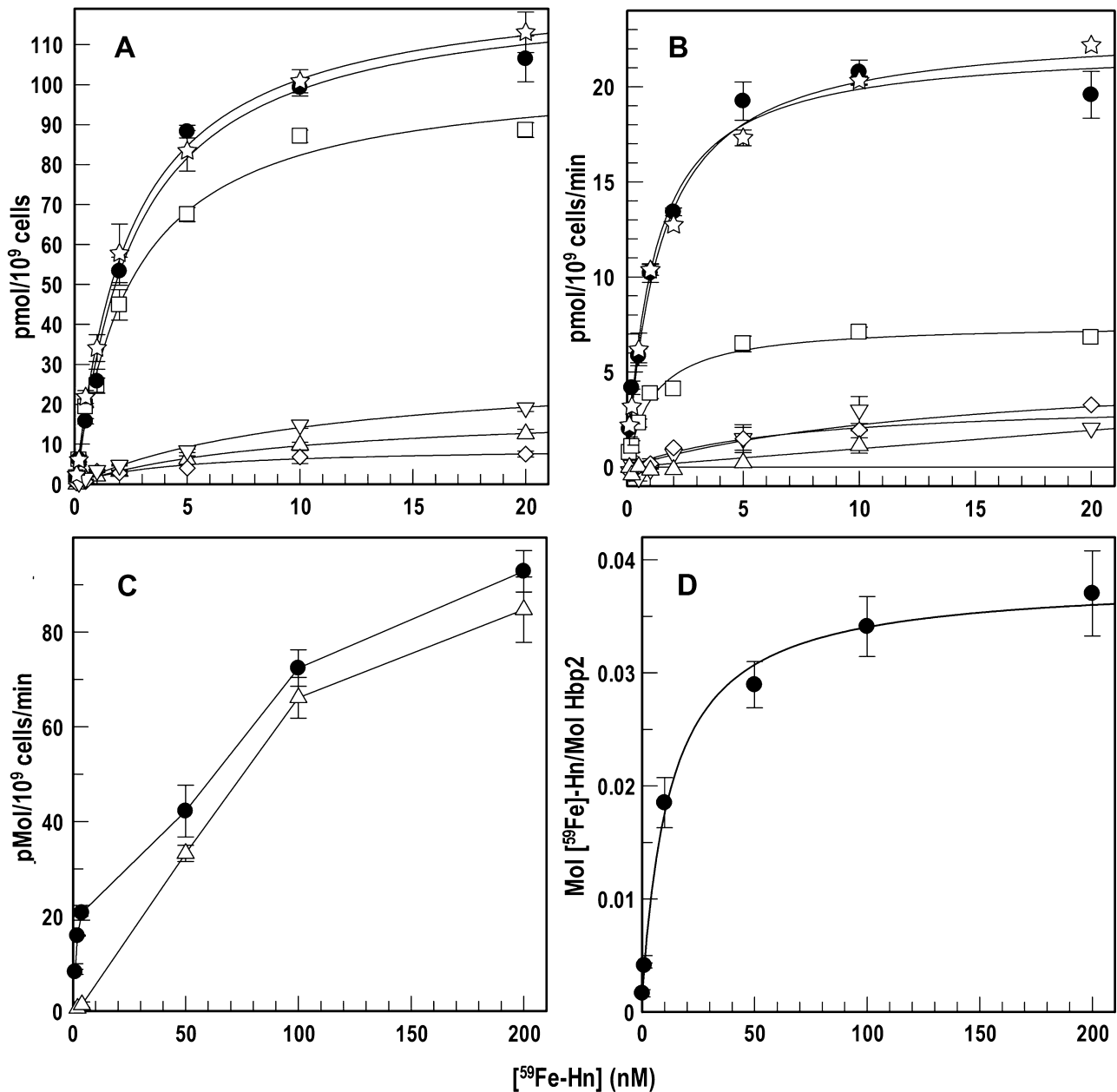
[<sup>59</sup>Fe]-Hn uptake studies showed the presence of a second Hn transporter. For EGD-e the  $V_{max}$  of [<sup>59</sup>Fe]-Hn uptake was 23 pmol 10<sup>9</sup> cells<sup>-1</sup> and its overall uptake

$K_M$  was ~1 nM. In a Δ*hupDGC* strain [<sup>59</sup>Fe]-Hn uptake persisted, but  $V_{max}$  decreased to 7.5 pmol 10<sup>9</sup> cells<sup>-1</sup> min<sup>-1</sup>, 27% of the wild-type rate (Fig. 5). Thus, Hup is not the only CM transporter for Hn in *L. monocytogenes*, and the kinetic and thermodynamic properties of the residual system are similar to those of Hup system:  $V_{max}$  was lower, but  $K_M$  was the same (~1 nM). Subtraction of the residual accumulation in Δ*hup* from that of the wild-type gave an estimate of the Hup-dependent transport rate:  $V_{max}$  was 14 pmol 10<sup>9</sup> cells<sup>-1</sup> min<sup>-1</sup>.

#### Sortase B and Lmo2185 in Hn binding and uptake

The availability of [<sup>59</sup>Fe]-Hn also allowed evaluation of sortase-dependent, PG-anchored proteins in Hn transport. Lmo2185 is partly secreted into the extracellular environment, but a portion remains anchored to PG by sortase B (Newton *et al.*, 2005). The primary structures of Lmo2185 and Lmo2186 (from the same transcriptional unit) show considerable identity with the Hn binding protein SaulsdC (Fig. 6; *Discussion*). Hn associates with Lmo2185 (Newton *et al.*, 2005), so we determined the effect of Δ*lmo2185* and Δ*srtB* on binding and transport of the iron porphyrin. The improvements in Hn solubility enhanced its activity in nutrition tests with EGD-e, but these assays still did not reveal effects of Δ*lmo2185* or Δ*srtB* on Hn uptake (Fig. 4). We only observed their impact in quantitative binding and transport experiments at lower concentrations (Fig. 5; Table 2), where Δ*lmo2185* and Δ*srtB* reduced [<sup>59</sup>Fe]-Hn binding capacity and transport rate to about 20% of what was seen in EGD-e. In the same tests Δ*srtA* did not affect [<sup>59</sup>Fe]-Hn binding  $K_D$  nor capacity, nor its transport  $K_M$  nor  $V_{max}$ . The double mutant Δ*srtAB* was indistinguishable from Δ*srtB* alone (Table 2). Hence, in *L. monocytogenes* Hn/Hb acquisition was always *srtA*-independent, but *srtB*-dependent at low concentrations.

These results agreed with the high affinity of purified Lmo2185 for Hn: in equilibrium determinations of



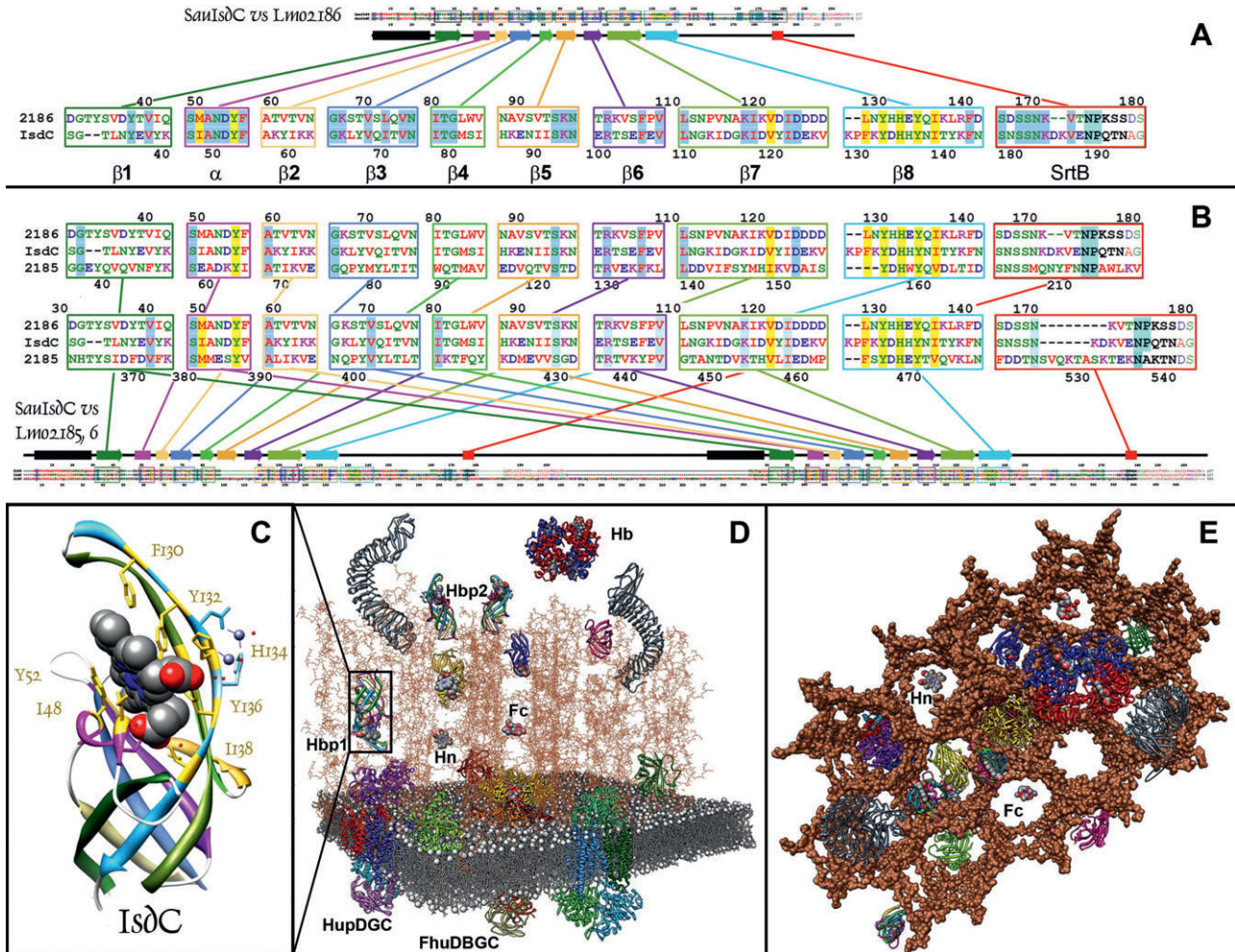
**Fig. 5.** Hn binding and transport by EGD-e and its derivatives. In panels A-C, wild-type strain EGD-e (●), or its derivatives carrying the individual mutations  $\Delta hup$  (□),  $\Delta srtA$  (☆),  $\Delta srtB$  (▽),  $\Delta srtAB$  (◇) and  $\Delta lmo2185$  (△) were grown in BHI, subcultured twice in MOPS-L, and tested for their ability to bind or transport [ $^{59}\text{Fe}$ ]-Hn (Jin *et al.*, 2005). Binding (A) and transport (B) assays revealed impaired Hn acquisition by  $\Delta hup$ ,  $\Delta srtB$  and  $\Delta lmo2185$  at low nanomolar concentrations of the iron porphyrin, but no effect of  $\Delta lmo2185$  at Hn concentrations  $\geq 50$  nM (C). In panel D, purified Lmo2185 was mixed with increasing concentrations of [ $^{59}\text{Fe}$ ]-Hn and the protein was collected on filters, which were washed and counted to determine the amount of Hn bound (*Experimental procedures*). The experiments were repeated 3 or 4 times; bars on the data points represent standard deviations of the mean values. In (A), from the 6 fitted curves (bound versus total) the mean standard deviation was 6.7% for  $K_D$  values and 22.3% for capacity; in (B), from the 5 fitted curves (enzyme kinetics) the mean standard deviation was 8.5% for  $K_M$  values and 20.7% for  $V_{max}$ ; in (D), from the fitted curve (bound versus total) the mean standard deviation was 4.1% for the  $K_D$  value and 18.7% for capacity.

[ $^{59}\text{Fe}$ ]-Hn binding to Lmo2185 the  $K_D$  was 12 nM (Fig. 5). Furthermore, like HupD above, purified Lmo2185 contained bound Hn (at  $\sim 50\%$  saturation), as evidenced by a Soret band (at 405 nm) in its visible spectrum and a positive pyridine haemochrome test (data not shown).

#### *Role of the hup locus in virulence and intracellular multiplication of L. monocytogenes*

We determined the infectivity of the triple deletion mutant EGD-e $\Delta hupDGC$  by intravenous inoculation of Swiss





**Fig. 6.** Sequence, structural and potential spatial relationships of haem binding proteins in the Gram-positive cell envelope.

A. Lmo2186 (Hbp1) and SaulsdC. The sequences of LmoHbp1 and SaulsdC are 30% identical overall (69 of 227 residues), confirming their structural relatedness. The foundation of their conservation is in  $\alpha$  and  $\beta$  secondary structure [the coloured boxes in (A) and (B) correlate with the depiction of SaulsdC crystal structure (2O6P) in (C)]: in the  $\alpha$  and  $\beta$  elements the extent of sequence identity is 78%.

B. Lmo2185 (Hbp2), LmoHbp1 and SaulsdC. LmoHbp2 comprises a duplication of LmoHbp1, separated by ~200 amino acids (residues 163–351) in the middle of the protein. Its N-terminal half (a.a. 1–219) has 21% (43 of 207 residues) and 23% (51 of 227 residues) overall identity to LmoHbp1 and SaulsdC respectively. The main sequence diversity occurs in loops and turns; in the  $\alpha$  and  $\beta$  elements the extent of identity is 48% and 59% respectively. Its C-terminal half (a.a. 352–569) has 25% (52 of 207 residues) and 22% (50 of 227 residues) overall identity to LmoHbp1 and SaulsdC, with 58% and 57% identity in  $\alpha$  and  $\beta$  structure respectively.

C. SaulsdC. Eight residues its single  $\alpha$ -helix and  $\beta$ -strands 7 and 8 complex haem in SaulsdC (2O6P). These amino acids in IsdC (yellow) are identical or conservatively substituted in Hbp1 and Hbp2 (both the N- and C- domains), including Y52 and Y132, which coordinate iron in the porphyrin ring. The only exception is I48 in IsdC, substituted by E59 in Hbp2.

D and E. Sortase-dependent and independent haem transport through the Gram-positive bacterial cell envelope. The cytoplasmic membrane (phospholipids in stick format; phosphate atoms in space filling form) contains transporters for iron compounds, such as the HupDGC and FhuDBGC putative ABC-transporters (shown in ribbon format). The PG polymer in the vertical scaffold model (Meroueh *et al.*, 2006) [shown in wire format in (D), and as space filling surfaces in (E)] surrounds the cell as a series of conjoined hexagonal pores with approximately 50 Å diameter (coordinates through the courtesy of S. Mobashery; Meroueh *et al.*, 2006). This architecture creates pathways for diffusion of small solutes, like ferric siderophores (Fc) or porphyrins (Hn), through the PG matrix [best viewed in (E)]. At concentrations above 50 nM, Hn permeates the PG layer and directly adsorbs to lipoproteins of the ABC-transporters. On the other hand, sortases A and B attach haem binding proteins (for example, listerial Hbp1 and Hbp2) and other proteins to the cell wall matrix by peptide bonds to Lys or DAP in PG. The anchored proteins create a sortase-dependent pathway of Hn/Hb (Hb: PDB file 3HF4) uptake. At very low Hn concentrations Hbp1 and Hbp2 initiate transport by capturing the iron porphyrin and subsequently transferring it to the underlying ABC transporters. In this cartoon Hbp1 and Hbp2 were modelled from their relationship to IsdC (2O6P); HupDGC and FhuDBGC were modelled from their relationship to the iron ABC transporter of *Haemophilus influenzae* (2NQ2) and the maltose ABC transporter of *E. coli* (3FH6) respectively. The molecules were rendered and assembled with Chimera (UCSF); high resolution images with more detailed explanations are provided as Fig. S4).



**Table 3.** LD<sub>50</sub> of *L. monocytogenes* strains.

Strain	LD <sub>50</sub>
EGD-e	10 <sup>4.5</sup>
$\Delta hupC$	10 <sup>5.5</sup>
$\Delta hupG$	10 <sup>5.7</sup>
$\Delta hupD$	10 <sup>5.5</sup>
$\Delta hupDGC$	10 <sup>6.4</sup>

Wild-type strain EGD-e and site-directed deletion mutants were evaluated in the mouse infection model, and LD<sub>50</sub> values were determined by the probit graphic method (Roth, 1961).

mice (Table 4). Its LD<sub>50</sub> was 10<sup>6.4</sup>, as compared with 10<sup>4.5</sup> for the parental strain EGD-e. These data were consistent with previous estimation of LD<sub>50</sub> for the single mutant EGD-e $\Delta hupC$  (10<sup>6.2</sup>; Jin *et al.* 2005). The 100-fold attenuation of virulence relative to the wild-type strain reiterated that the *hup* locus plays a role in *L. monocytogenes* pathogenesis in the mouse model.

We also monitored the intracellular multiplication of the single deletion mutants  $\Delta hupC$ ,  $\Delta hupG$ ,  $\Delta hupD$  and  $\Delta hupDGC$  in bone marrow-derived macrophages (Fig. S3). None of them showed growth defects relative to EGD-e, indicating that the *hup* loci do not affect intracellular survival of *L. monocytogenes*.

## Discussion

The comparative biochemistry of iron uptake through the Gram-positive and Gram-negative cell envelopes is a question of interest. Towards that end we quantitatively characterized, for the first time, the membrane Hn/Hb acquisition systems of *L. monocytogenes*. In the wild-type strain the uptake process was similar to ferric siderophore transport. The steady-state Hn uptake rate was nearly identical to that of the listerial CM Fc transporter (FhuDBG:  $V_{max} = 22$  pmol 10<sup>9</sup> cells<sup>-1</sup> min<sup>-1</sup>; Jin *et al.*, 2005), which is approximately half the rate of enteric bacterial Fc transport (*E. coli* FhuA:  $V_{max} = 45$  pmol 10<sup>9</sup>

cells<sup>-1</sup> min<sup>-1</sup>; Newton *et al.*, 2010). The main distinction of listerial iron acquisition was lower overall affinity. For Hn or Fc transport,  $K_D \approx K_M \approx 1$  nM, 10-fold lower affinity than that of ferric siderophore transport systems of *E. coli* [for ferric enterobactin or Fc,  $K_D \approx K_M \approx 0.1$  nM (Newton *et al.*, 1999; Scott *et al.*, 2001; Annamalai *et al.*, 2004)].

Gram-negative bacteria initiate iron uptake with tight binding of metal complexes to the external surfaces of the OM proteins that transport them. Despite their lack of an OM, Gram-positive cells also produce high-affinity cell surface binding sites for adsorption of eukaryotic haemoproteins (Lei *et al.*, 2002; 2003; Mazmanian *et al.*, 2002; 2003). The physiological role of Lmo2185 in iron transport was in doubt, but several of our findings showed that the sortase B-anchored protein binds Hn with high affinity. Sequence homologies in the context of the staphylococcal IsdC crystal structure revealed its relatedness to Lmo2185 and Lmo2186 (Fig. 6). The three proteins contain sufficient sequence identity to assure their structural similarity (Kryshchavych *et al.*, 2005; Ginalski, 2006), and the conservation of IsdC Hn-binding residues in Lmo2185/6 agrees with the high affinity of purified Lmo2185 for [<sup>59</sup>Fe]-Hn. Therefore, we propose to rename Lmo2186 as Hbp1 (Hn/Hb binding protein), and Lmo2185, which duplicates Lmo2186 (Fig. 6), as Hbp2.

In the *srtB* locus of *L. monocytogenes*, *hbp1* and *hbp2* encode IsdC homologues with NPKSS and NAKTN sorting motifs respectively (Bierne *et al.*, 2004). Synthesis of both proteins is Fur-regulated and they are over-produced in iron-deficient environments. The post-secretion fate of the former is unknown, whereas 10–50% of Hbp2 is anchored to PG, depending on growth conditions (Newton *et al.*, 2005). In the nanomolar concentration range of [<sup>59</sup>Fe]-Hn,  $\Delta srtB$  and  $\Delta hbp2$  decreased its binding capacity 80% (from 130 to 30 pmol 10<sup>9</sup> cells<sup>-1</sup>), while  $\Delta hup$  only dropped it 20% (to 105 pmol 10<sup>9</sup> cells<sup>-1</sup>). Therefore, at low external concentrations Hbp2 is the primary Hn adsorption site, and Hup accounts for the remainder. Hup is fourfold less abundant than Hbp2, as estimated by [<sup>59</sup>Fe]-Hn binding capaci-

**Table 4.** Gram-positive bacterial Hn/Hb and ferric siderophore binding and transport proteins.

Organism	Iron complex	Secreted or PG-binding protein	CM permease	References
<i>L. monocytogenes</i>	Hn/Hb	Hbp1, Hbp2	HupDGC	This report
	FxB, Fc, FcA	None	FhuDBG	Jin <i>et al.</i> (2005)
<i>B. subtilis/anthracis</i>	Hn/Hb	IsdX1, IsdX2	ND	Fabian <i>et al.</i> (2009)
	Fc	None	FhuDBG	Schneider and Hantke (1993)
<i>S. aureus</i>	Hn/Hb	IsdA, B, C, H	IsdDEF, HtsABC	Skaar <i>et al.</i> (2004); Zhu <i>et al.</i> (2008a)
	Staphylobactin	None	SirABC	Dale <i>et al.</i> (2004)
<i>S. pyogenes</i>	Fc, FxB,	None	FhuCBG	Sebulsky <i>et al.</i> (2000); Cabrera <i>et al.</i> (2001)
	Hn/Hb	Shr, Shp	HtsABC	Lei <i>et al.</i> (2002); Liu and Lei (2005) (Nygaard <i>et al.</i> (2006); Zhu <i>et al.</i> (2008b)
	Fc	None	FtsABCD	Hanks <i>et al.</i> (2005)

See Discussion for an explanations of the various transport systems.

ties, immunoblots (data not shown) and proteomics data (Ledala *et al.*, 2010). Second, besides being the predominant Hn binding constituent, Hbp2 has the highest affinity for it. Measurements of whole cell affinity for [<sup>59</sup>Fe]-Hn ( $K_D = 2$  nM) were comparable to those of purified Hbp2 ( $K_D = 12$  nM), and  $\Delta hbp2$  caused a fivefold reduction in Hn binding affinity ( $K_D$  to  $\sim 10$  nM), while  $\Delta hup$  caused no observable change. [<sup>59</sup>Fe]-Hn uptake results agreed with the binding data:  $\Delta srtB$  and  $\Delta hbp2$  decreased the uptake rate 80% or more, and reduced overall uptake affinity 5- to 10-fold ( $\Delta hbp2$  resulted in a non-saturable uptake process). These data show that at concentrations  $< 50$  nM Hn acquisition is SrtB-dependent in *L. monocytogenes*, as proposed in *S. aureus* (Mazmanian *et al.*, 2003; Maresso *et al.*, 2006). Still, in radioisotopic tests neither Hbp nor SrtB affected uptake above this 50 nM threshold, so in environments containing more Hn the iron complex directly interacts with its CM transporter(s). *L. monocytogenes* is  $\beta$ -haemolytic, and proteolytic degradation of haemoglobin at sites of infection or abscess will result in release of free haemin.

In Gram-positive bacteria sortase-anchored proteins bind Hb in the PG framework (Mazmanian *et al.*, 2003; Maresso *et al.*, 2006), presumably facilitating the subsequent extraction of Hn for delivery to CM transporters. The family of known and putative Gram-positive bacterial haem binding proteins (Table 4) has considerable sequence and structural variability. Among the secreted or PG-(sortase)anchored binding proteins, Hbp1 and Hbp2 of *L. monocytogenes*, IsdC, IsdX1 and IsdX2 of *B. anthracis*, and IsdC of *S. aureus* form a subfamily. Hbp1, IsdC and IsdX all contain the same 8-stranded  $\beta$ -barrel nucleus (Fig. 6); Hbp2 and IsdX2 duplicate this domain, linked by an intervening polypeptide of  $\sim 200$  and 370 a.a. respectively. With the exception of IsdX1, all these proteins contain potential SrtB cleavage sites (NPXZN). However, IsdX1, IsdX2 (Maresso *et al.*, 2008; Fabian *et al.*, 2009) and a large fraction of Hbp2 (Newton *et al.*, 2005) are secreted to the environment, where the three proteins act as haemophores (Fabian *et al.*, 2009; this report). The streptococcal proteins Shp (Lei *et al.*, 2002) and Shr (Zhu *et al.*, 2008b) are unique among the group: the former has a different structural format than the other haem-binders, and unexpectedly, the latter shows the most relatedness to InIA (20% identity). From their sequence relatedness and SrtA-mediated attachment to PG, the staphylococcal proteins IsdA, IsdB and IsdH form another subfamily of haem binding proteins. IsdB, IsdH and IsdA are proposed to bind Hb, extract Hn and transfer it to SrtB-dependent IsdC, which passes the porphyrin to the CM ABC transporters IsdDEF or HtsABC (Mazmanian *et al.*, 2002; 2003; Reniere *et al.*, 2007). According to this scheme the staphylococcal Hn/Hb uptake system

requires sortase A-anchored proteins. Nevertheless, for *L. monocytogenes* the  $\Delta srtA$  mutation did not influence listerial Hn/Hb uptake in any assay, at any concentration, indicating that sortase A-anchored proteins play no role in listerial Hn acquisition.

Deletion of the *hup* operon or its individual membrane permease components decreased but did not eliminate Hn uptake, implying the existence of additional CM Hn transport systems in *L. monocytogenes*. The Hup permease is the primary CM Hn transporter, because its steady-state uptake rate is twice that of the residual system. The overall affinity of the secondary CM Hn transporter is similar to that of HupDGC. Other Gram-positive bacterial CM transporters of iron and haem are known (Table 3). Listerial FhuDBG (Jin *et al.*, 2005), staphylococcal FhuCBG (Sebulsky *et al.*, 2000) and streptococcal FtsABCD (Hanks *et al.* 2005) all transport ferric hydroxamates. Their micromolar affinities for the hydroxamate iron complexes leave no doubt about their specificity. However, LmoFhuD was also promiscuous for other iron complexes, including Hn, raising the possibility that FhuDBG provides an auxiliary pathway for Hn uptake. In *S. aureus* both IsdDEF and HtsABC ABC-transporters are thought to transport Hn (Skaar *et al.*, 2004); the HtsABC complex also functions in *Streptococcus pyogenes* (Liu and Lei, 2005; Nygaard *et al.*, 2006). LmoHupDGC is most closely related to SauHtsABC (28%, 26% and 46% identity, respectively, between the individual components), even though the staphylococcal *srtB* region encodes the proposed ABC-transporter IsdDEF.

The pathways of haem and haemoglobin passage through the Gram-positive cell wall, and the interactions among potential uptake components are not obvious: how do binding proteins attached within the PG matrix facilitate Hn transport through ABC-transporters as much as 50–100 Å away, in the CM? The answer is that at concentrations greater than 50 nM sortase-anchored proteins are not needed for Hn uptake by *L. monocytogenes*. The cell wall architecture (Fig. 6) contains pores that allow diffusion of the porphyrin to CM permeases. Thus, besides the dispensability of SrtA-anchored proteins, even SrtB-anchored proteins have a limited role in Hn uptake. They are only advantageous at external Hn concentrations  $< 50$  nM and at higher levels SrtB and Hbp are inconsequential. Corroborating this point, the cytoplasmic HupDGC membrane transporter, not the sortase-anchored proteins, was most crucial to listerial virulence. We note that LD<sub>50</sub> determinations of strains containing complemented *hup* operon deletions are still needed to complete the analysis of its contributions to pathogenesis. Furthermore, the exact role of Hup-mediated Hn/Hb uptake in bacterial infection remains mysterious. RNome analysis (which studies the structure and function of non-

**Table 5.** Bacterial strains and plasmids.

Strain/plasmid	Markers/characteristics	Reference/source
<b>Strains</b>		
<i>E. coli</i>		
DH5 $\alpha$	<i>supE44 <math>\Delta</math>lacU169(<math>\Phi</math>80lacZ <math>\Delta</math>M15) hsdR17 recA1 endA1 gyra96 thi-1 relA1</i>	Hanahan (1983)
SM10	<i>F<sup>-</sup> thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44 (Mu<sub>C</sub>) <math>\lambda^-</math> Km<sup>r</sup> Tra<sup>r</sup></i>	Lauer <i>et al.</i> (2002)
XL-1 blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i>	Stratagene
<i>L. monocytogenes</i>		
EGD-e	Wild-type strain	Glaser <i>et al.</i> (2001)
$\Delta$ HupC	<i><math>\Delta</math>hupC</i>	Jin <i>et al.</i> (2005)
$\Delta$ HupG	<i><math>\Delta</math>hupG</i>	This study
$\Delta$ HupD	<i><math>\Delta</math>hupD</i>	This study
$\Delta$ Hup	<i><math>\Delta</math>hupC, <math>\Delta</math>hupG, <math>\Delta</math>hupD</i>	This study
$\Delta$ HupC.comp	<i><math>\Delta</math>hupC, complemented by pPL2_ <i>hupC</i><sup>+</sup></i>	This study
$\Delta$ hupG.comp	<i><math>\Delta</math>hupG, complemented by pPL2_ <i>hupG</i><sup>+</sup></i>	This study
$\Delta$ SrtA	<i><math>\Delta</math>srtA</i>	Bierne <i>et al.</i> (2002)
$\Delta$ SrtB	<i><math>\Delta</math>srtB</i>	Bierne <i>et al.</i> (2004)
$\Delta$ SrtAB	<i><math>\Delta</math>srtA, <math>\Delta</math>srtB</i>	Pascal Cossart
$\Delta$ Imo2185	<i><math>\Delta</math>Imo2185</i>	Newton <i>et al.</i> (2005)
<b>Plasmids</b>		
pKSV7	<i>E. coli</i> – <i>L. monocytogenes</i> shuttle vector	Smith and Youngman (1992)
pPL2	Site-specific shuttle integration vectors	Lauer <i>et al.</i> (2002)
pPro	pPL2 harbouring <i>hup</i> promoter	This study
pCHupC	pPL2 harbouring <i>hup</i> promoter and <i>hupC</i>	This study
pCHupG	pPL2 harbouring <i>hup</i> promoter and <i>hupG</i>	This study

coding RNAs) revealed that incubation of *L. monocytogenes* in human blood upregulates several virulence genes (Toledo-Arana *et al.*, 2009), implicating them in blood stage dissemination. HupC expression was unchanged in these conditions. *In vivo* transcriptome profiling showed increased Fur expression *in vivo* in mice (7.67-fold at 48 h; Camejo *et al.*, 2009), but this upregulation did not correlate with increased repression of Fur-regulated iron ABC transporters. Instead, the Fur-controlled *srtB* locus was upregulated *in vivo*, whereas expression of the *FhuDGBC* and *hupDGC* loci did not vary. Thus, the relationship between external iron availability and Fur-regulated gene expression encompasses complex relationships and/or regulatory cascades that affect pathogenesis, but by still unknown mechanisms

## Experimental procedures

### Bacterial strains, plasmids and media (Table 5)

*Escherichia coli* strains DH5 $\alpha$  (Dower *et al.*, 1988) or XL-1 blue (Li *et al.*, 1990) hosted the thermosensitive shuttle vector pKSV7 (Glaser *et al.*, 2001). Using this plasmid we deleted chromosomal genes of *L. monocytogenes* strain EGD-e (Jin *et al.*, 2005). Using the site-specific phage integration vector pPL2 (Lauer *et al.*, 2002) we re-introduced wild-type alleles of the deleted genes into the chromosome, to verify their ability to complement the original mutations (*hupC* and *hupG*).

We selected spontaneous streptomycin-resistant clones of all the EGD-e mutant derivatives, which facilitated their growth in minimal media without contamination. Bacteria

were routinely grown on plates and in broth [*E. coli*: Luria–Bertani broth (LB; Difco); *L. monocytogenes*: brain heart infusion (BHI; Difco)]. For cultivation of *L. monocytogenes* in iron-deficient conditions we use medium MOPS-L, a minimal medium that we adapted from the original MOPS formulation for Enterobacteriaceae (Neidhardt *et al.*, 1974). Because *L. monocytogenes* is more fastidious, we increased the concentration of glucose from 0.4% to 1%, and added additional micronutrients, vitamins, cysteine and glutamine (Premaratne *et al.*, 1991; Tsai and Hodgson, 2003; Table 6) To prepare the modified medium we added the following components at the indicated final concentrations to MOPS medium: Cys (0.1 mg ml<sup>-1</sup>), Glu (0.6 mg ml<sup>-1</sup>), casamino acids (0.1%), tryptophan (50  $\mu$ g ml<sup>-1</sup>), riboflavin (0.5  $\mu$ g ml<sup>-1</sup>), biotin (1  $\mu$ g ml<sup>-1</sup>), thiamine (1  $\mu$ g ml<sup>-1</sup>), lipoic acid (0.005  $\mu$ g ml<sup>-1</sup>) (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O (3  $\times$  10<sup>-7</sup> M), CoCl<sub>2</sub>·6H<sub>2</sub>O (3  $\times$  10<sup>-6</sup> M), HBO<sub>3</sub> (4  $\times$  10<sup>-5</sup> M), CuSO<sub>4</sub>·5H<sub>2</sub>O (10<sup>-6</sup> M), MnCl<sub>2</sub> (8  $\times$  10<sup>-6</sup> M), ZnCl<sub>2</sub> (10<sup>-6</sup> M), and glucose (1%).

### Site-directed deletions

We used homologous recombination to delete chromosomal genes of strain EGD-e (Bierne *et al.*, 2004; Jin *et al.*, 2005; Newton *et al.*, 2005), and verified the correct structure of the resulting mutants by colony-PCR and DNA sequencing. For PCR reactions, we utilized Taq 2X mix (New England Biolabs) and oligonucleotide primers synthesized by Invitrogen. Primer sequences are listed in Table S1.

### Complementation of deletions

By PCR, we amplified the 155-bp DNA fragment containing the promoter region of the *hup* operon (upstream of *Imo2432*)



**Table 6.** Components of iron-deficient media.

Component	Concentration in medium				
	KRM <sup>a</sup>	MWB <sup>b</sup>	HTM <sup>c</sup>	MOPS <sup>d</sup>	MOPS-L
MOPS			100 mM	40 mM	40 mM
K <sub>2</sub> HPO <sub>4</sub>		48.2 mM	4.82 mM	1.32 mM	1.32 mM
NH <sub>4</sub> Cl				9.52 mM	9.52 mM
MgCl <sub>2</sub>				0.523 mM	0.523 mM
K <sub>2</sub> SO <sub>4</sub>				0.276 mM	0.276 mM
CaCl <sub>2</sub>				5 × 10 <sup>-4</sup> mM	5 × 10 <sup>-4</sup> mM
NaCl				50 mM	50 mM
Na <sub>2</sub> HPO <sub>4</sub>		115.5 mM	11.55 mM		
MgSO <sub>4</sub>		1.70 mM	1.70 mM		
KH <sub>2</sub> PO <sub>4</sub>					
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>			0.6 mg ml <sup>-1</sup>		
NaHCO <sub>3</sub>	0.2%				
Ferric citrate		360 μM			
Glucose	0.4%	0.99%	0.99%	0.4%	1.0%
RPMI1640	1.04%				
Thiamine	1.0 μg ml <sup>-1</sup>	2.96 μM	2.96 μM		1.0 μg ml <sup>-1</sup>
Riboflavin	0.5 μg ml <sup>-1</sup>	1.33 μM	1.33 μM		0.5 μg ml <sup>-1</sup>
Biotin	1.0 μg ml <sup>-1</sup>	2.05 μM	2.05 μM		1.0 μg ml <sup>-1</sup>
Lipoic acid	5 × 10 <sup>-3</sup> μg ml <sup>-1</sup>	24.0 pM	24.0 pM		5 × 10 <sup>-3</sup> μg ml <sup>-1</sup>
Histidine		0.1 mg ml <sup>-1</sup>			
Tryptophan	0.05 mg ml <sup>-1</sup>	0.1 mg ml <sup>-1</sup>			0.05 mg ml <sup>-1</sup>
Leucine		0.1 mg ml <sup>-1</sup>			
Isoleucine		0.1 mg ml <sup>-1</sup>			
Valine		0.1 mg ml <sup>-1</sup>			
Arginine		0.1 mg ml <sup>-1</sup>			
Cysteine		0.1 mg ml <sup>-1</sup>	0.1 mg ml <sup>-1</sup>		0.1 mg ml <sup>-1</sup>
Methionine		0.1 mg ml <sup>-1</sup>	0.1 mg ml <sup>-1</sup>		
Glutamine		0.6 mg ml <sup>-1</sup>			0.6 mg ml <sup>-1</sup>
Casamino acids	0.1%				0.1%
Micronutrients	+			+	+

a. Newton *et al.* (2005).

b. Premaratne *et al.* (1991).

c. Tsai and Hodgson (2003).

d. Neidhardt *et al.* (1974).

e. Micronutrients contain: (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O (3 × 10<sup>-7</sup> M), CoCl<sub>2</sub>·6H<sub>2</sub>O (3 × 10<sup>-6</sup> M), HBO<sub>3</sub> (4 × 10<sup>-5</sup> M), CuSO<sub>4</sub>·5H<sub>2</sub>O (10<sup>-6</sup> M), MnCl<sub>2</sub> (8 × 10<sup>-6</sup> M), ZnCl<sub>2</sub> (10<sup>-6</sup> M).

on the EGD-e chromosome, flanked by BamHI and PstI restriction sites. We transferred this PCR fragment into pPL2 [Cm<sup>r</sup>; (Lauer *et al.*, 2002)] to generate pPro, and then separately cloned wild-type *hupC* and *hupG* into it using the PstI site upstream and a KpnI (in the multiple cloning site) downstream. We individually transformed the resulting constructs (pHupC, pHupG) into *E. coli* SM10, and used the resulting *E. coli* strains as donors for plasmid transfer to *L. monocytogenes* Δ*hupC* or Δ*hupG* (Sm<sup>r</sup>). After mixing the strains in BHI broth and incubating for 2 h at 37°C, we spread the mixture on BHI plates containing both antibiotics and incubated overnight. From double resistant colonies we isolated the complementation constructs and verified them by DNA sequencing.

#### Growth assays

EGD-e and its mutant derivatives were grown in BHI at 37°C overnight, and 2.5 × 10<sup>7</sup> cells were subcultured (1%) into 10 ml BHI containing 1 mM 2,2'-bipyridyl (BP), with or without Hn. We spectrophotometrically monitored the growth of the cultures at 600 nm until stationary phase.

#### Nutrition tests

Fc was purified from cultures of *Ustilago sphaerogena* (Emery, 1971). Hn and bovine Hb were from Sigma-Aldrich (St. Louis). *L. monocytogenes* was grown in BHI until OD<sub>600</sub> = 0.2, 1 mM BP was added to the culture and the cells were grown for another 3.5 h, when 200 μl aliquots were mixed with 8 ml BHI top agar containing 0.25 mM BP and poured onto a BHI plate. Paper discs were applied to the agar surface, aliquots of solutions of Fc, Hn or bovine Hb were applied to the discs, and the plates were incubated overnight at 37°C (Jin *et al.*, 2005; Newton *et al.*, 2005).

#### [<sup>59</sup>Fe]-Hn

We chemically synthesized [<sup>59</sup>Fe]-Hn by inserting <sup>59</sup>Fe into protoporphyrin IX (PPIX) (Babusiak *et al.*, 2005). Fifty microlitres of a solution of PPIX (Sigma-Aldrich; 6 mg ml<sup>-1</sup> in pyridine) was added to 450 μl of glacial acetic acid, and maintained at 60°C under N<sub>2</sub> in a sealed flask. Approximately 0.25 μl of thioglycolic acid (Sigma-Aldrich) was added to a 30 μg of <sup>59</sup>FeCl<sub>3</sub> (Perkin-Elmer) in 50 μl of 0.5 M HCl, and the

mixture was immediately injected into the PPIX solution. After 30 min at 60°C, the solution was stirred for 1.5 h at room temperature and then transferred to 20 ml of ethyl ether, and washed five times with 30 ml of 1 M HCl to remove unreacted iron and porphyrin. The organic phase was dried overnight under a mild flow of nitrogen, and the dried [<sup>59</sup>Fe]-Hn was stored desiccated at 4°C. Its purity was evaluated by thin layer chromatography: [<sup>59</sup>Fe]-Hn in dimethylsulphoxide (DMSO) was spotted on silica gel plate (60 F<sub>254</sub>, Merck) and developed with a mixture of 2, 6-lutidine and water (5:3.5, v/v) (Falk, 1964; Fig. S1). The concentration of the [<sup>59</sup>Fe]-Hn stock solution was determined by UV-VIS spectrophotometry in 40% DMSO, using a millimolar extinction coefficient of 180 mM<sup>-1</sup>cm<sup>-1</sup> at 400 nm (Sinclair, *et al.*, 1999). We synthesized [<sup>59</sup>Fe]-haem under reducing conditions, but upon its addition to culture media it oxidized to [<sup>59</sup>Fe]-haemin. Hence, in our experiments haemin was the primary porphyrin under investigation, but these solutions contained some haem. It was not possible to analytically monitor the concentrations of the two porphyrins, or to functionally discriminate between them.

#### [<sup>59</sup>Fe]-Hn binding experiments

We prepared and stored solutions of [<sup>59</sup>Fe]-Hn in DMSO, prior to dilution into transport media and/or buffers (Collier *et al.*, 1979). We also evaluated 50 mM NaOH to maintain Hn in soluble, monomeric form. The porphyrin had good initial solubility and gave the same results in fresh NaOH solutions as in DMSO, but it tended to dimerize over time, which we did not observe in DMSO, making it the solvent of choice. Additionally, the hydrophobicity of its porphyrin ring system promotes the adsorption of Hn to many filtration materials, and to reduce its non-specific binding to the solid phase we added 0.05% Tween-20 to the binding and transport washing buffers. EGD-e and mutant derivatives were grown in 20 ml BHI at 37°C overnight, subcultured (1%) in MOPS-L and grown to OD<sub>600</sub> = 0.8, then subcultured (1%) again into 50 ml MOPS-L, shaken at 37°C until the OD<sub>600</sub> reached 0.6–0.8, and finally assayed for binding and uptake of [<sup>59</sup>Fe]-Hn. Binding experiments were performed on ice. Appropriate amounts of [<sup>59</sup>Fe]-Hn were added to 25 ml of ice cold MOPS-L and the mixtures were immediately poured onto 100 µl aliquots of bacterial cells in 50 ml test tubes, and incubated for 1 min before collection by filtration. The 0.45 micron Durapore filters (Millipore) were washed with 25 ml of 50 mM Tris-HCl, pH 9.0, containing 0.05% Tween-20, and counted in a Packard Cobra gamma counter. CPM from adsorption of [<sup>59</sup>Fe]-Hn alone to the filters at each concentration were measured as background and subtracted from the CPM of the cell binding reactions. Data were collected in triplicate at each concentration, averaged, and K<sub>D</sub> and binding capacity were calculated using the 'Ligand Binding (1 Site + Background)' equation of Grafit 6.0 (Erithacus, Middlesex, UK).

Purified Hbp2 (see below) was assayed by initially diluting it to 30.6 pmol ml<sup>-1</sup> in TBS, pH 8.0, and incubating at room temperature for 1 h to allow refolding of the protein. [<sup>59</sup>Fe]-Hn was prepared at various concentrations in DMSO, and 100 µl aliquots were mixed with 4.9 ml of TBS pH 8.0, or TBS containing purified Hbp at pH 8.0. The reactions were transferred

to ice and after 30 min each sample was collected on cellulose acetate filters and washed with 5 ml of TBS pH 8.0 plus 0.05% Tween-20. The radioactivity on each filter was measured in a Packard Cobra gamma counter. Each concentration of [<sup>59</sup>Fe]-Hn was performed in triplicate and averaged. The radiation accumulated on the filters without protein present was subtracted as a background control, and the differential counts were used to determine the K<sub>D</sub> of Hbp2 for Hn.

#### [<sup>59</sup>Fe]-Hn uptake

*Listeria monocytogenes* strain EGD-e and its mutant derivatives were inoculated in 10 ml BHI broth at 37°C overnight. In the next morning, the stationary phase BHI culture was subcultured at 1% into 20 ml MOPS-L and grown to stationary phase. The full-grown MOPS-L culture was subcultured again at 1% into 20 ml of MOPS-L, and grown to an OD<sub>600</sub> of 0.8–1. The bacteria were then subjected to [<sup>59</sup>Fe]-Hn uptake assays at 37°C: 100 µl aliquots of the cell cultures were mixed with 10 ml MOPS media pre-warmed to 37°C, containing varying concentrations of [<sup>59</sup>Fe]-Hn. After incubation at 37°C for 5 s or 1 min, the reaction was filtered through a 0.45 micron Durapore filter and washed with 10 ml ice cold wash buffer (50 mM Tris, 0.05% Tween-20, pH 9). The radiation on the filters was measured in a Packard Cobra gamma counter. At each concentration, data were collected in triplicate and averaged. The radiation accumulated during the 5 s time point was subtracted from that of the 1 min time point to determine the uptake rate. Transport K<sub>M</sub> and V<sub>max</sub> were calculated using the 'Enzyme Kinetics' equation of Grafit 6.0 (Erithacus).

#### Purification of FhuD and HupD

For expression and purification of FhuD and HupD of *L. monocytogenes*, we cloned their structural genes into pET28a, devoid of their signal sequences (FhuD: residues 1–23; HupD: residues 1–20), and transformed the clones (p6HΔssFhuD, p6HΔssHupD) into *E. coli* BL21. The elimination of their signal sequences resulted in high-level overexpression of the two binding proteins as soluble, rather than periplasmic proteins. This strategy dramatically enhanced expression, which facilitated their Ni<sup>2+</sup>-NTA chromatographic purification. We grew the strains to mid-log phase (OD<sub>600</sub> ≈ 0.5) in LB broth plus kanamycin, added IPTG (0.5 mM), and continued shaking the cultures at 37°C for another 3–4 h. The cells were pelleted by centrifugation (3000 g, 20 min), resuspended in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing RNase and DNase (both at 10 µg ml<sup>-1</sup>), and lysed by passage through a French pressure cell at 14 000 p.s.i. The lysate was clarified by centrifugation at 4000 g for 20 min, and the supernatant was centrifuged at 100 000 g for 1 h. The supernatant (cytoplasm) was passed through a Ni<sup>2+</sup>-NTA column (Qiagen) equilibrated with lysis buffer. After loading, the column was washed with 10 volumes of lysis buffer, followed by 10 volumes of 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0, and eluted with a linear gradient of imidazole (40–250 mM) in lysis buffer. Fractions were collected and subjected to SDS-

PAGE (Fig. S2). Fractions containing purified 6H-FhuD and 6H-HupD were pooled, dialysed against Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris, pH 7.4), and stored frozen at  $-20^{\circ}\text{C}$ . Protein concentrations were determined by Bradford assay.

In the case of 6H-HupD, pooled fractions of the purified protein were coloured, varying from light yellow to brown: the more concentrated the protein fraction, the darker its colour. UV-visible spectroscopy showed absorption around 400 nm, and Soret peaks that are diagnostic of an iron porphyrin. 6H-HupD was isolated with bound Hn, and the chromophore was not removed by dialysis. To test whether the bound Hn derived from the growth medium or was synthesized by the bacteria, we purified 6H-HupD from bacteria grown in MOPS minimal medium (which does not contain any Hn), but the purified binding protein was still loaded with Hn. Thus, the porphyrin bound by 6H-HupD was synthesized by BL21 cells. To separate the Hn from the binding protein, we mixed 6H-HupD with 1% SDS, boiled the sample for 5 min and chromatographed it over Sephacryl S100 HR equilibrated in 10 mM Tris-Cl, 1% SDS, pH 8.0. 1 ml fractions were collected and spectroscopically analysed at 280 nm and 405 nm. This method separated Hn from 6H-HupD, but left the protein in a denatured state. Nevertheless, ion exchange chromatography of the native complex also yielded the apo-protein. 6H-HupD was loaded onto DEAE Sepharose ( $1.5 \times 50$  cm) equilibrated with 10 mM, pH 8.0 Tris-Cl. After loading the column was washed with 5 volumes of buffer and eluted with a linear gradient of 0–1 M NaCl in the same buffer. Purified apo-6H-HupD was dialysed against Tris-HCl, and spectrophotometric analyses showed that it was 98–99% free of Hn. A pyridine haemochrome test (Zhu *et al.*, 2008b) confirmed the absence of Hn in the purified apo-6H-HupD (data not shown).

The pET28a expression vector inserts thrombin cleavage site between the N-terminal 6H tag and the initial residue of cloned proteins. We also removed the hexahistidine tags from both 6H-FhuD and 6H-HupD by incubation with biotinylated thrombin (EMD Biosciences), followed by chromatography of the reaction mixture over avidin-sepharose (Fig. S2). The eluted FhuD and HupD proteins were subsequently chromatographed over another  $\text{Ni}^{++}$ -NTA column to remove the cleaved hexahistidine tags.

#### Purification of Hbp2 (*Lmo2185*)

*Escherichia coli* BL21 containing pET28a encoding *lmo2185* was grown in LB broth at  $37^{\circ}\text{C}$  until  $\text{OD}_{600} = 0.5$ . IPTG was added to 1 mM and the culture was harvested after 3 h. The cells were harvested by centrifugation at 10 000 *g* for 15 min; the pellets were weighed and resuspended in a volume of lysis buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 2 mM DTT, 3 mg DNase, 3 mg RNase, and a cocktail of protease inhibitors) that was 5 times their wet weight (g). The cell suspension was passed through the French Press twice at 14000 p.s.i., and centrifuged at 10 000 *g* for 15 min to remove debris and unbroken cells. The supernatant was collected and centrifuged at 100 000 *g* for 1 h to remove the cell envelope fraction. The supernatant was dialysed twice against 4 l of column buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl and 5 mM imidazole, pH 7.4), and loaded onto a column of cobalt-NTA sepharose (Talon Superflow; Pierce)

equilibrated in the same buffer. The resin was washed with column buffer until absorbance at 280 nm reached background levels, and then further washed with steps of 20 and 40 mM imidazole in column buffer until the absorbance at 280 nm was not above background. His-tagged Hbp2 was eluted with 150 mM imidazole in column buffer. The protein was further purified by chromatography of its peak fractions (slightly orange in colour) over a Sephacryl S-100 size exclusion column (Pharmacia). Fractions containing purified, holo-6H-Hbp2 were combined and assayed to determine their protein concentration. The purified protein was boiled for 3 min in the presence of 0.5% SDS to remove bound Hn. The SDS and liberated Hn were removed by acetone precipitation: 9 volumes of ice cold acetone were added to the sample, which was incubated at  $-20^{\circ}\text{C}$  overnight, and pelleted by centrifugation at 16 000 *g* for 30 min at  $0^{\circ}\text{C}$ . The supernatant was immediately removed and the pellet was resuspended and renatured in TBS, pH 8.0, by incubation at room temperature for an hour or more. Purified, renatured 6H-Hbp2 was assayed for its ability to bind [ $^{59}\text{Fe}$ ]-Hn. We attempted to remove its hexahistidine residues by thrombin cleavage, but we did not observe an alteration of its mobility in SDS-PAGE gels, suggesting that the 6H tag was resistant to cleavage. Nevertheless, the hexahistidine residues did not affect the affinity of either FhuD or HupD for Hn (*vide infra*).

#### Measurements of ferric siderophore and Hn binding

The affinities of 6H-FhuD, FhuD, 6H-HupD and HupD for ferric siderophores and Hn were determined by intrinsic fluorescence quenching, using an SLM-AMINCO 8000 fluorimeter (Rochester, NY, USA) upgraded to 8100 functionality. 20 nM FhuD or 33 nM apo-HupD in 3 ml TBS was added to a quartz cuvette and the fluorescence was recorded from 320 nm to 340 nm. Using constant agitation from a small stir bar, various concentrations of ferric siderophores or Hn (in DMSO) were added to the solutions of binding proteins and fluorescence was recorded after each addition. The background fluorescence (various concentrations of ferric siderophores or Hn in TBS) and volume changes were accounted for and the data were analysed with the bound versus total function of GraFit 6.0 (Erithacus Software Ltd) to determine the affinities ( $K_D$  values) of the binding proteins for the various iron complexes.

#### Determination of virulence in the mouse model system

Bacteria were grown, prepared and intravenously inoculated into pathogen-free, female Swiss mice (Janvier, Le Genest St. Isle, France), as previously described (Jin *et al.*, 2005). Bacteria were grown for 18 h in BHI broth, centrifuged, appropriately diluted in 0.15 M NaCl, and inoculated (0.5 ml) intravenously (i.v.) into mice via the lateral tail veins. Groups of five mice were challenged i.v. with various doses of bacteria, and mortality was monitored for 10 days. The virulence of the strains was estimated by the  $\text{LD}_{50}$  using the graphic probit method (Roth, 1961).

#### Intracellular multiplication

Bone marrow-derived macrophages from a BALB/c mouse were obtained and cultured (de Chastellier and Berche,



1994), and invasion assays were carried out essentially as previously described (Bigot *et al.*, 2005). Cell monolayers were incubated for 30 min at 37°C with the bacterial suspensions in Dulbecco modified Eagle medium (multiplicities of infection = 0.1) to allow penetration of the bacteria. After washing ( $t = 0$  in the kinetic analysis), the cells were incubated for several hours in fresh culture medium containing gentamicin (50 µg ml<sup>-1</sup>) to kill extracellular bacteria. At several points cells were washed three times in RPMI and processed for counting of infecting bacteria. The cells were lysed with distilled water, and the titre of viable bacteria released from the infected cells was determined by spreading dilutions onto BHI medium plates. The assays were performed in triplicate for each strain and time point.

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