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Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in *Listeria monocytogenes*

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Summary

Listeria monocytogenes **is a Gram-positive bacterium that causes severe opportunistic infections in humans and animals. We biochemically characterized, for the first time, the iron uptake processes of this facultative intracellular pathogen, and identified the genetic loci encoding two of its membrane iron transporters. Strain EGD-e used iron complexes of hydroxamates (ferrichrome and ferrichrome A, ferrioxamine B), catecholates (ferric enterobactin, ferric corynebactin) and eukaryotic binding proteins (transferrin, lactoferrin, ferritin, haemoglobin). Quantitative determinations showed 10–100-fold lower affinity for ferric siderophores ($K_m \approx 1$–10 nM) than Gram-negative bacteria, and generally lower uptake rates. V_{max} for [^{59}Fe]-enterobactin (0.15 pMol per 10^9 cells per minute) was 400-fold lower than that of *Escherichia coli*. For [^{59}Fe]-corynebactin, V_{max} was also low (1.2 pMol per 10^9 cells per minute), but EGD-e transported [^{59}Fe]-apoferrichrome similarly to *E. coli* ($V_{max} = 24$ pMol per 10^9 cells per minute). *L. monocytogenes* encodes potential Fur-regulated iron transporters at 2.031 Mb (the *fur-fhu* region), 2.184 Mb (the *feo* region), 2.27 Mb (the *srtB* region) and 2.499 Mb (designated *hupDGC* region). Chromosomal deletions in the *fur-fhu* and *hupDGC* regions diminished iron uptake from ferric hydroxamates and haemin/haemoglobin respectively. In the former locus, deletion of *fhuD* (*lmo1959*) or *fhuC* (*lmo1960*) strongly reduced [^{59}Fe]-apoferrichrome uptake. Deletion of *hupC* (*lmo2429*) eliminated the uptake of haemin and haemoglobin, and decreased the virulence of *L. monocytogenes* 50-fold in mice. Elimination of *srtB* region genes (Δ*lmo2185*, Δ*lmo2186*, Δ*lmo2183*), both sortase structural genes (Δ*srtA*, Δ*srtAB*), *fur* and *feoB* did not impair iron transport. However, deletion of bacterioferritin (Δ*fri*, *lmo943*; 0.97 Mb) decreased growth and altered iron uptake: V_{max} of [^{59}Fe]-corynebactin transport tripled in this strain, whereas that of [^{59}Fe]-apoferrichrome decreased 20-fold.**

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Introduction

Iron acquisition was proposed as a determinant of bacterial virulence in the early 1970s (Bullen, 1974), and although arguments were made to the contrary (Benjamin *et al*., 1986), the correlation between iron uptake and virulence was repeatedly verified (Cornelissen and Sparling, 1994; Furman *et al*., 1994; Bearden *et al*., 1998; Stork *et al*., 2004; Braun, 2005), and recently reiterated by proteomic analyses (Frisk *et al*., 2004; Snyder *et al*., 2004). With few exceptions, bacteria require iron as a cofactor in indispensable metabolic systems, including glycolysis, energy generation, DNA synthesis, photosynthesis and detoxification of oxygen radicals (Neilands, 1995). This iron requirement is problematical, because in aqueous solutions Fe^{+++} precipitates as hydroxide polymers, and in animal fluids, cells and tissues it associates with transferrin, lactoferrin, haem/haemoglobin (Hb) and ferritin (Ftn). The elaboration of siderophores (Neilands, 1976), which generally chelate iron with higher affinity than eukaryotic proteins, is one way that microorganisms overcome iron sequestration. In Gram-negative bacteria, trilaminar cell envelope uptake systems bind and internalize ferric siderophore complexes (Klebba, 2004; Braun, 2005), and in some cases, haem and iron binding proteins (Cornelissen and Sparling, 1994; Wandersman and Delepelaire, 2004). Similar transport systems exist in Gram-positive cells (Sebulsky and Heinrichs, 2001), which also produce siderophores, and studies of *Bacillus anthracis* (Cendrowski *et al*., 2004) showed their importance to pathogenesis. Despite research on *B. subtilis* (Schneider and Hantke, 1993) and *Staphylococcus aureus* (Sebulsky *et al*., 2000; 2003; Sebulsky and Heinrichs, 2001), the biochemical properties of Gram-positive iron transport systems are not yet fully understood.

Listeria monocytogenes is a ubiquitous Gram-positive bacterium that lives saprophytically in nature. This species is not a usual constituent of the human flora, but its ability to grow at 4°C allows it to contaminate foodstuffs, some-

times resulting in digestive infections that may become systemic in immunocompromised individuals. The severity of listeriosis (an overall mortality of 25–30% in spite of antibiotic therapy) mainly results from a high frequency of neurological damage in such infections. *L. monocytogenes* survives and multiplies in many cell types and tissues, including macrophages and hepatocytes (Portnoy *et al*., 2002). This intracellular survival is fundamental to its pathogenicity, and each step of its intracellular parasitism depends on the production of virulence factors (Cossart, 2002; Kreft *et al*., 2002). The major virulence genes so far identified cluster in two loci on the chromosome, and are controlled by a single pleiotropic regulatory activator, PrfA, which is also required for virulence (Sheehan *et al*., 1995; Wong and Freitag, 2004). One locus contains the *hly* gene that encodes the major virulence factor listeriolysin O (LLO). This protein is a pore-forming cytolysin that promotes bacterial escape from the phagosomal compartment into the cytoplasm (Portnoy *et al*., 2002). Inactivation of *hly* prevents access to the cytosol (where the bacterium usually multiplies), resulting in complete avirulence in the mouse infection model. LLO also lyses erythrocytes (Parrisius *et al*., 1986), and thus can provide an abundant source of iron-loaded Hb.

The proliferation of *L. monocytogenes* within cells, and its spread from cell to cell, suggest that the organism readily obtains iron in the intracellular environment. But, *L. monocytogenes* does not secrete iron chelators. Rather, it utilizes the ferric siderophores of other organisms or other iron-containing compounds, including catecholamines (Simon *et al*., 1995; Coulanges *et al*., 1996), citrate (Adams *et al*., 1990) and transferrin (Hartford *et al*., 1993). The genetic systems, membrane permeases, or surface receptors for such ferric complexes are not yet known. In the related organism *S. aureus,* a ferrichrome (Fc) uptake system exists with a cytoplasmic membrane-anchored binding proteins (Sebulsky and Heinrichs, 2001) and an ABC-type membrane permease (Sebulsky *et al*., 2000). A particularly informative report on the binding protein of this uptake system, FhuD2 (Sebulsky *et al*., 2003), showed that it has higher affinity for ferric hydroxamates than the homologous periplasmic binding protein of *Escherichia coli*, FhuD.

Like *Staphylococcus* (Mazmanian *et al*., 2003)*, Streptococcus* (Brown *et al*., 2001) and *Corynebacteria* (Drazek *et al*., 2000), *L. monocytogenes* utilizes haemin (Hn) and Hb (Newton *et al*., 2005). *S. aureus* contains *Isd*-encoded, sortase-anchored surface proteins that were proposed to function in Hn/Hb uptake (Mazmanian *et al*., 2002; 2003; Skaar *et al*., 2004). *L. monocytogenes* contains homologous, sortase-dependent proteins in its Fur-regulated *srtB* locus, but these do not transport Hn/Hb (Newton *et al*., 2005). A cell surface-localized reductase (Deneer *et al*., 1995; Cowart, 2002) was postulated to transport iron in *L. monocytogenes,* by broadly recognizing the iron-centres of different ferric complexes, reducing the metal, and releasing Fe^{++} into the cell (Coulanges *et al*., 1997; 1998). The experiments that we report defined the biochemical parameters of ferric siderophore uptake by *L. monocytogenes* strain EGD-e (Glaser *et al*., 2001), demonstrated its ability to acquire iron from several eukaryotic iron binding proteins, identified the structural genes for transporters of ferric hydroxamates and Hn/Hb, and evaluated the virulence of EGD-e mutants with deletions in the potential iron uptake loci. Only the elimination of Hn/Hb transport (Δ*hupC*) increased the 50% lethal dose (LD_{50}) of *L. monocytogenes* (50-fold in mice).

Results

Iron uptake by L. monocytogenes*: siderophore nutrition tests*

To initially assess the ability of strain EGD-e to utilize different iron sources, we adapted the siderophore nutrition test (Wayne *et al*., 1976; Newton *et al*., 1999) to the Gram-positive organism. ApoFcA (Neilands, 1976) deprives Gram-negative bacteria of iron (Wayne *et al*., 1976), but it was ineffective for *L. monocytogenes*, which utilizes it (Table 1). α,α-bipyridyl (BP), on the other hand, restricted growth of *L. monocytogenes* in brain–heart infusion (BHI), and nutrition assays on BHI agar containing BP (0.1 mM) showed the ability of *L. monocytogenes* to obtain iron from siderophores and mammalian proteins (Table 1). Contrary to previous reports (Coulanges *et al*., 1996; 1997), EGD-e utilized the hydroxamates Fc and ferrichrome A (FcA). No bacteria of which we are aware are able to transport FcA. EGD-e also utilized Hn and Hb in nutrition assays, but the test did not reveal uptake of elemental iron, ferric catecholates, or from holotransferrin (HTf) or Ftn (data not shown). Regarding the eukaryotic iron binding proteins, BP has sufficient affinity to remove their iron, thereby preventing them from supplying it to *L. monocytogenes.* Therefore, we also conducted nutrition tests with KRMT agar, where iron is limited by alkaline pH (see *Experimental procedures*). These assays confirmed the stimulation of EGD-e growth by HTf, Ftn, ferric citrate (FeCit) and $FeSO_4$ (Table 1). Subsequent experiments found transport of ferric enterobactin (FeEnt) and ferric corynebactin (FeCrn), but at very low rates that the nutrition test did not detect (see following).

Iron acquisition and growth in iron-restricted minimal media: KRM

In our hands, *L. monocytogenes* grew poorly in existing minimal media (Pine *et al*., 1989; Premaratne *et al*., 1991;

Table 1. Siderophore nutrition tests and mouse infection experiments with EGD-e and its mutant derivatives.

		Growth											
		Fc/A		FxB		Hb	Hn	HTf	Ftn	FeCit	$FeSO_4$		
Strain	Region	50	0.5	50	0.5	15	200	13	8.5	20	20	LD_{50}	Origin
EGD-e	NA	25	16	23	14	15	10	20	14	15	14	$10^{4.5}$	Glaser *et al.* (2001)
Δ*fri* (*lmo943*)	*fri*	28	17	28	18	15	10	20	12	14	14	ND	This study
Δ*fur* (*lmo1956*)	*fur-fhu*	31	22	28	18	16	10	14	11	12	12	$10^{7.5}$	Newton *et al.* (2005)
Δ*fhuD* (*lmo1959*)	*fur-fhu*	0	0	0	0	15	10	19	14	15	15	$10^{4.5}$	This study
Δ*fhuC* (*lmo1960*)	*fur-fhu*	0	0	0	0	16	10	18	14	14	14	ND	This study
Δ*lmo1961*	*fur-fhu*	23	15	22	10	15	9	18	14	14	14	$10^{4.5}$	This study
Δ*feoB* (*lmo2105*)	*feo*	25	15	25	13	14	9	19	15	15	15	$10^{4.5}$	This study
Δ*lmo2183*	*srtB*	24	15	24	14	14	10	20	12	14	14	ND	This study
Δ*srtB* (*lmo2181*)	*srtB*	25	15	25	13	14	9	20	15	14	13	$10^{4.5}$	Bierne *et al.* (2004)
Δ*hupC (lmo2429)*	*hupDGC*	25	14	22	12	0	0	18	15	15	15	$10^{6.2}$	This study
Δ*srtA* (*lmo929)*	*srtA*	23	14	22	12	14	9	18	11	15	15	$10^{6.4}$	Garandeau *et al.* (2002)
Δ*srtAB*	NA	25	15	25	13	14	9	17	11	14	14	ND	Bierne *et al.* (2004)

For nutrition tests, the tabulated values represent the diameter (in mm) of the halo of growth surrounding a paper disc embedded with 10 μl aliquots of the test compound. Fc and FcA, FxB, Hb and Hn were tested on BHI agar containing 0.1 mM BP; HTf, Ftn, FeCit and $FeSO_4$ were tested on KRMT agar plates. Fc and FcA were evaluated at 50 and 0.5 μM; the two hydroxamate siderophores always gave similar growth halos with all the strains, and the results of three or four experiments with each compound were averaged and tabulated. The concentrations of all other iron compounds are also micromolar; each tabulated value represents the mean of three or four tests, which had minimal variation. NA, not applicable; ND, no data.

Phan-Thanh and Gormon, 1997), and we devised Klebba's RPMI modification (KRM) to study its iron acquisition processes. RPMI contains no extrinsic iron, and its components are highly purified for cell culture applications. We added other supplements (vitamins, trace metals, casamino acids, glucose and adenine; Newton *et al.*, 2005) to increase growth rate and maximum cell density. The concentration of adventitious iron in KRM was $<10^{-6}$ M (Newton *et al.*, 2005), and growth in this medium rendered *L. monocytogenes* iron-deficient, as seen by increased doubling time and decreased final cell density. These effects were reversed by addition of elemental iron or ferric siderophores.

Tests of iron acquisition in KRM recapitulated the nutrition test results, and further showed that elemental iron ($FeSO_4$, $FeCl_3$), catecholate ferric siderophores (FeEnt, FeCrn) and mammalian proteins (Hb, HTf, Ftn) were effective iron sources (Fig. 1). When any of these compounds was added to KRM, EGD-e had a doubling time of about 1 h at 37°C, and reached a final density of 4–5×10^8 cells ml^{-1}.

Binding and transport of ^{59}Fe metal complexes

Using ^{59}Fe (Newton *et al.*, 1999), we determined the affinity and velocity with which EGD-e transports ferric siderophores. The adsorption of [^{59}Fe]-complexes of apoFc, corynebactin and enterobactin to listerial cells was a saturable process with roughly the same affinity: $K_d \approx 10$ nM (Fig. 2). The affinity of these binding reactions was about 100-fold lower than that of *E. coli* FepA and FhuA for FeEnt and Fc respectively; *E. coli* does not adsorb FeCrn (Annamalai *et al.*, 2004). The capacities of EGD-e for both FeCrn and Fc was 50–60 pMol per 10^9 cells; the capacity for FeEnt was lower, 20 pMol per 10^9 cells (Fig. 2). Above 200 nM, the amounts of all three ^{59}Fe-complexes bound to the cells began to linearly increase with concentration (data not shown), indicating a non-specific binding process in this higher range. However, with K_m values of about 10 nM, this non-specific binding did not significantly distort the transport affinity and rate parameters that we observed.

Despite their negative siderophore nutrition tests, EGD-e bound and transported ^{59}FeEnt and ^{59}FeCrn (Table 2; Fig. 2). The listerial uptake reactions for ferric catecholates were significantly less efficient than those of Gram-negative bacteria. The overall uptake affinity of EGD-e for FeEnt ($K_m = 2$–4 nM; Fig. 2) was 20-fold lower than the affinity of *E. coli* FepA for the same compound ($K_m = 0.1$–0.2 nM; Annamalai *et al.*, 2004; Klebba, 2004), and its uptake affinity for FeCrn was another log lower ($K_m = 10$ nM). Second, the rate of ferric catecholate transport by *L. monocytogenes* was 100-fold (FeCrn; $V_{max} = 0.4$ pMol per 10^9 cells per minute) to 400-fold (FeEnt; $V_{max} = 0.1$ pMol per 10^9 cells per minute) slower than that of FeEnt uptake by *E. coli* FepA [$V_{max} = 40$ pMol per minute per 10^9 cells, for the chromosomal system (Newton *et al.*, 1999; Klebba, 2004)]. Next, the presence of FeEnt in the growth media enhanced the rate of ^{59}FeEnt uptake (Fig. 2), suggesting that the Gram-negative ferric catecholate positively regulates the synthesis of its own transport system. This apparent positive regulation of FeEnt uptake differentiates *L. monocytogenes* from most Gram-negative species, which (with some exceptions, e.g.

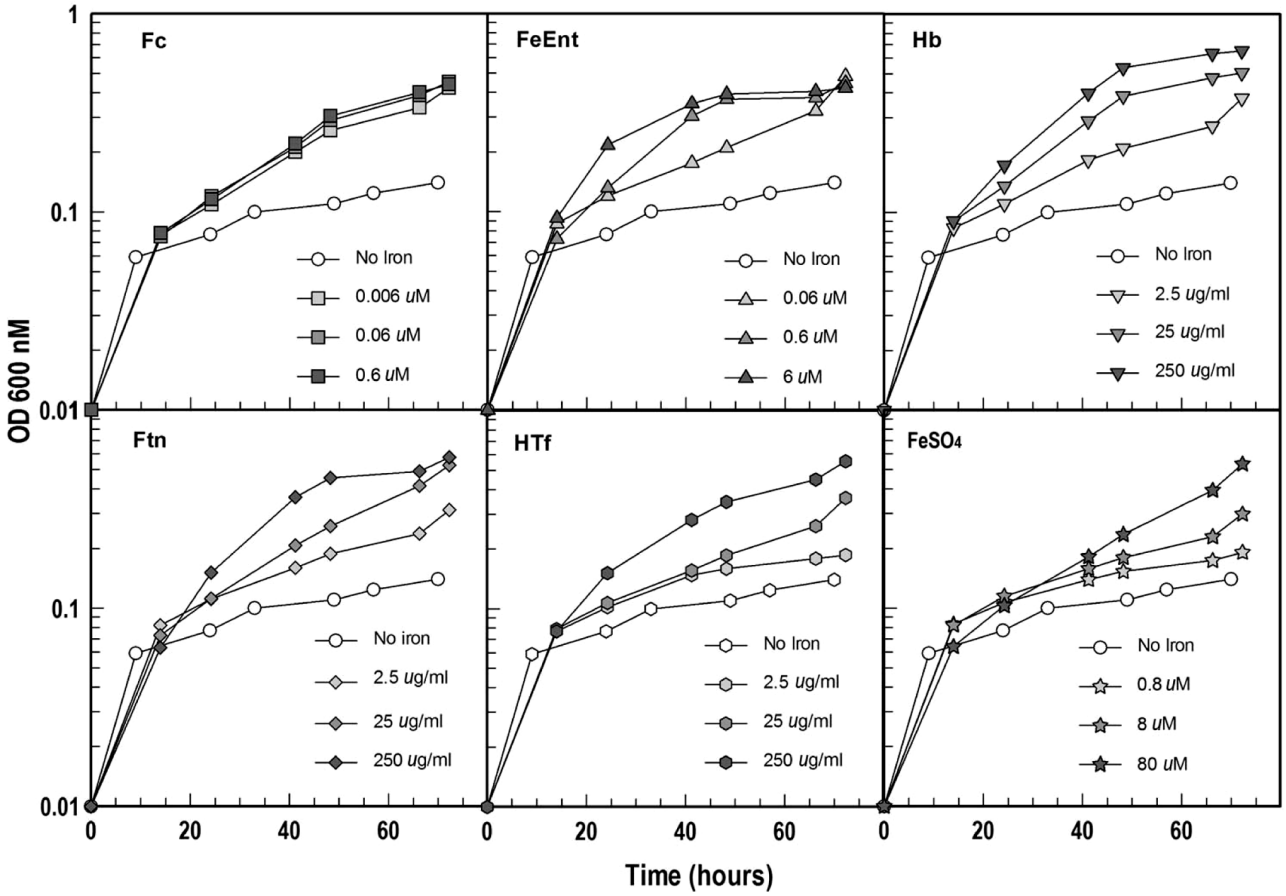

Fig. 1. Growth of EGD-e in KRM medium, containing different iron sources. EGD-e was grown in BHI broth overnight, subcultured (1%) into KRM and grown overnight at 37°C with shaking at 150 rpm. The bacteria were subcultured (1%) into KRM at 37°C and shaken at 150 rpm in the presence of ferric siderophores or iron binding proteins at the indicated concentrations. Growth was monitored by optical density at 600 nm.

Table 2. Transport properties of EGD-e and its mutants.

	^{59}Fe transport			
	FeCrn		Fc	
Strain	K_m	V	K_m	V
EGD ++	10	1.2	10	24
Δ*fri* (*lmo943*)	1.2	2.9	0.4	1.5
Δ*fhuD* (*lmo1959*)	26	1.8	0.2	2.1
Δ*fhuC* (*lmo1960*)	ND	ND	0.1	1.1
Δ*lmo1961*	8	1	7	24
Δ*feoB* (*lmo2105)*	8	1.2	ND	ND
Δ*lmo2183*	ND	ND	10.4	25
Δ*hbuC3*(*lmo2429*)	ND	ND	13.5	25

Bacteria were cultured in BHI broth overnight, subcultured in KRM media to stationary phase, and subcultured a second time in KRM to mid-log. Uptake of ^{59}Fe complexes of corynebactin and apoFc was determined and plotted by Grafit 5.09 (Erithacus, London). K_m (nanomolar) and V_{max} (pMol per 10^9 cells per minute) were calculated using the 'Enzyme Kinetics' algorithm. The mean standard deviations of K_m and V_{max} determinations for FeCrn and Fc were 42% and 14%, and 41% and 8.5% respectively.

Pseudomonas) negatively regulate the FeEnt uptake (Klebba *et al*., 1982). Conversely, the addition of FeCrn to KRM did not stimulate ^{59}FeCrn uptake (data not shown), intimating that FeEnt and FeCrn are acquired by separate transport systems.

The overall uptake affinity of *L. monocytogenes* for Fc was also 100-fold lower (K_m = 10 nM) than that of Gram-negative transport systems for this compound [K_m of *E. coli* FhuA for Fc is 0.2 nM (Scott *et al*., 2001)]. On the other hand, the rate of Fc uptake by *L. monocytogenes* was about the same (V_{max} = 24 pMol per 10^9 cells per minute) as that of *E. coli* (Scott *et al*., 2001). So, although the Fc uptake process had lower affinity in *L. monocytogenes* than in *E. coli*, the maximal rates of ^{59}Fc transport by the two bacteria were equivalent. EGD-e transported the ferric hydroxamate roughly 20- and 200-fold faster than FeCrn and FeEnt respectively. Finally, unlike FeEnt, the presence of Fc in culture media decreased the rate of ^{59}Fc uptake by the same cells (Fig. 2). Thus, synthesis of the Fc transport system was negatively regulated by iron availability, just as it is in

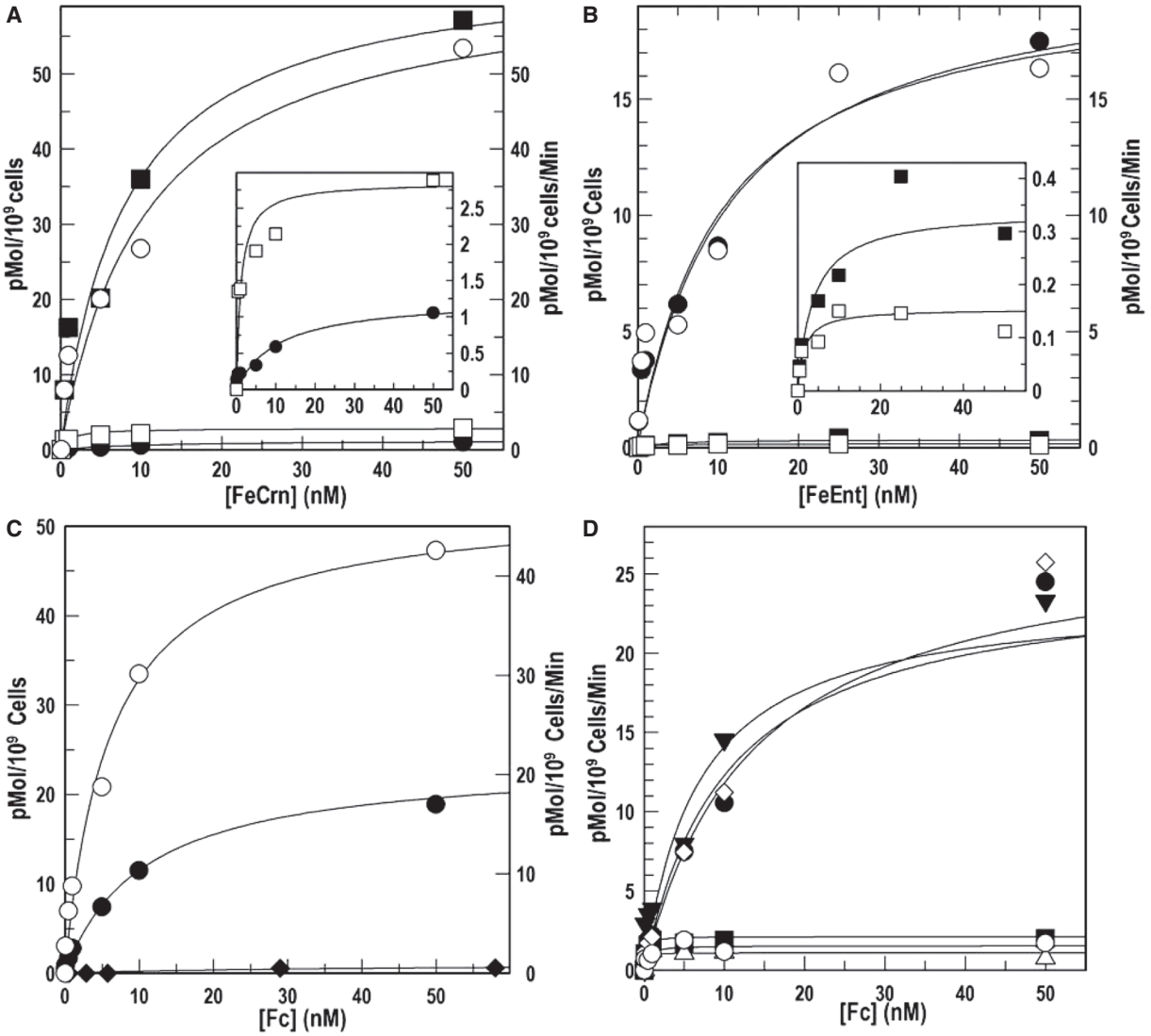

Fig. 2. Binding and uptake of [^{59}Fe]-siderophores by EGD-e and its mutant derivatives. Bacteria were grown in BHI broth, subcultured (1%) in KRM and grown to stationary phase, then subcultured (1%) into KRM again and grown to mid-log phase.
A. FeCrn (see also Table 2). Binding of FeCrn to EGD-e (○) and Δ*fri* (■) was measured by a 15 s incubation with 2×10^7 cells; transport of FeCrn by EGD-e (●) and Δ*fri* (□) was monitored for 1 h, and the uptake rate was calculated on a per-minute basis. The inset shows the saturation processes for transport by both strains, and the higher rate of uptake by Δ*fri*.
B. FeEnt. Binding (circles) and transport (squares) of FeEnt by EGD-e grown in KRM (open symbols) or KRM containing 2 μM FeEnt (filled symbols) was measured as described for FeCrn. The inset shows the saturation processes for transport in both conditions, and the higher rate of uptake by EGD-e grown in the presence of FeEnt. The mean standard deviations of K_m and V_{max} valued calculated from these data were 44% and 8% respectively.
C. Fc (See also Table 2). Binding (open symbols) and transport (filled symbols) of Fc was measured as described for FeCrn and FeEnt. Bacteria were grown in KRM (circles) or KRM containing 2 μM Fc (diamonds), which repressed its own uptake reaction.
D. Fc transport by mutants of EGD-e. Derivatives of EGD-e carrying chromosomal deletions of *lmo1961* (▼), *lmo2183* (◇) and *hupC* (●) transported Fc like wild type, whereas deletions of *fhuC* (△), *fhuD* (■) and *fri* (○) were strongly impaired in Fc uptake.

Gram-negative bacteria, and these data concur with its Fur-mediated regulatory system (see following).

Iron transport by chromosomal deletion mutant derivatives of EGD-e

On the basis of their sequence similarities to iron metabolism or acquisition systems of other organisms, we chose target genes in five regions (Fig. 3) of the *L. monocytogenes* genome (Glaser *et al.*, 2001), and engineered individual, precise, in frame chromosomal deletions. Some of the site-directed mutants were impaired in iron uptake, others had deficiencies in iron metabolism, and some had no phenotypes.

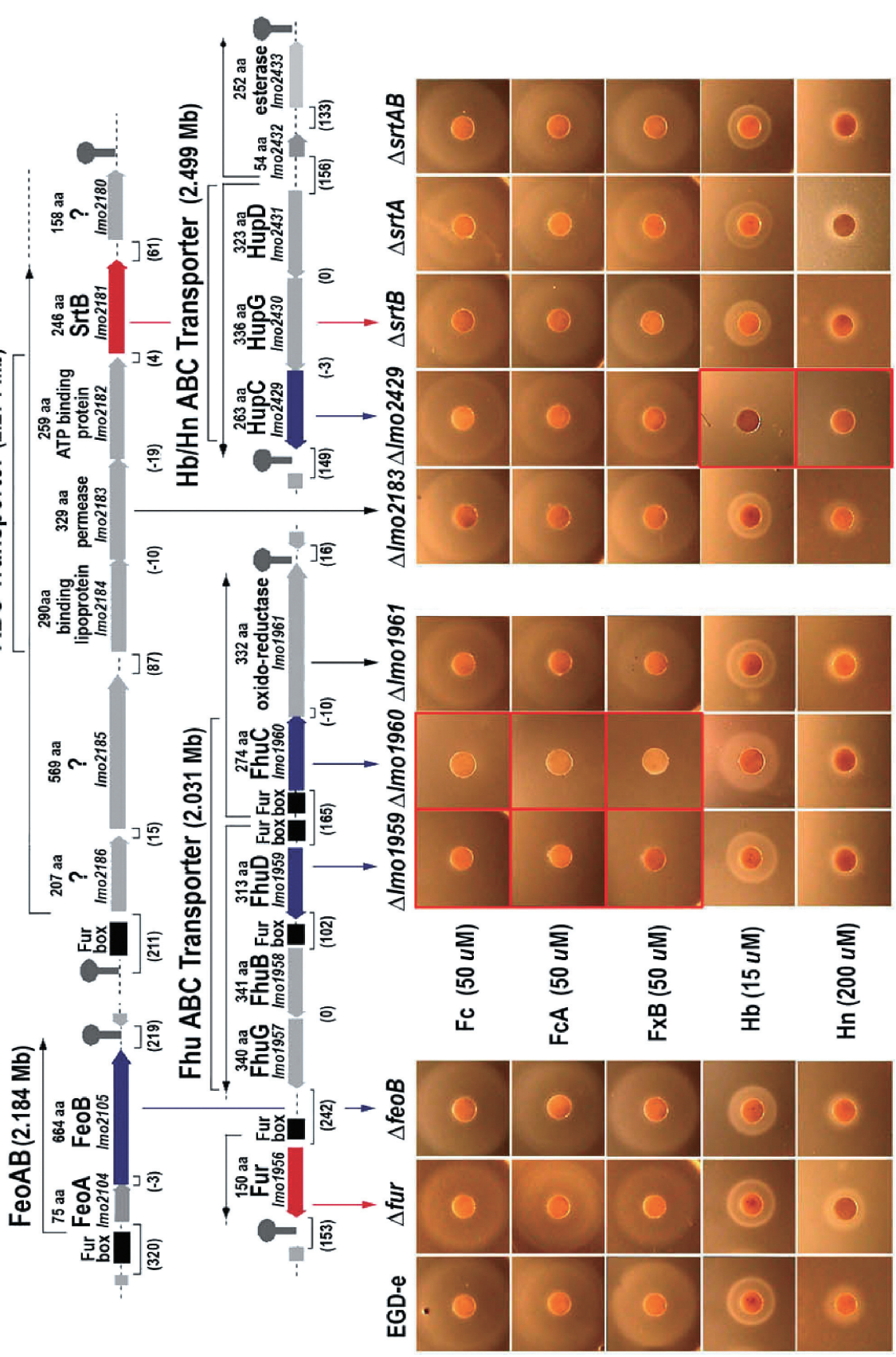

Fig. 3. Chromosomal loci of interest and nutrition tests with ferric hydroxamates, Hn and Hb. We studied four loci that contain Fur binding sites and encode potential transport systems, and the *fri* locus (not shown), for their participation in iron uptake. Siderophore nutrition tests revealed, and [^{59}Fe]-apoFc uptake experiments confirmed, that Δ*fhuD* and Δ*fhuC* strains lost the ability to transport Fc. Similarly, The *hupDGC* locus (2.499 Mb) encodes a third ABC transporter, and a deletion of the gene encoding its putative membrane ATP binding protein (Δ*hupC*) prevented *L. monocytogenes* from acquiring iron from Hb and Hn. The nutrition tests shown below were performed in BHI agar containing 0.1 mm BP.

The *fur-fhu* region (2.031 Mb) encodes *lmo1956*, a 150-residue protein with 76% identity to *fur* of *B. subtilis*. *fur* precedes *lmo1957–lmo1960*, a potential ABC-transporter for ferric hydroxamate siderophores, and *lmo1961*, a putative oxidoreductase. Although we ultimately generated deletions in three different putative ferric hydroxamate ABC-transporters, only deletions in the *fur-fhu* region affected ferric hydroxamate transport. Δ*lmo1959* and Δ*lmo1960*, which eliminated a putative binding protein and membrane ATP binding permease component respectively, both prevented the uptake of Fc, FcA and ferrioxamine B (FxB) in siderophore nutrition tests (Table 1), without affecting the uptake of Hn, Hb, transferrin or Ftn. These data demonstrate the specificity of the transport system encoded by the *fur-fhu* locus for ferric hydroxamates. Both Δ*lmo1959* and Δ*lmo1960* severely reduced ^{59}Fe uptake from the iron hydroxamates. (Fig. 2, Table 2). Although Δ*lmo1959* and Δ*lmo1960* grew like wild type in BHI broth and KRM, the growth of Δ*lmo1960* became restricted when it was repeatedly subcultured in KRM (doubling time >6 h; final density of 2×10^8 cells ml^{-1}). Deletion of the putative oxidoreductase (Δ*lmo1961*) did not impair either growth or iron uptake.

The *srtB* region (2.274 Mb) contains genes for a secreted, peptidoglycan-associated surface protein (*lmo2185*), the sortase (*srtB*) that anchors it to the cell wall (Bierne *et al*., 2004; Newton *et al*., 2005), and a putative ABC-transporter, *lmo2182–2184.* A precise deletion of *lmo2183*, the putative cytoplasmic permease of the *srtB* region, did not affect the uptake of any of the iron-containing compounds we tested. Besides siderophore nutrition tests on Fc, FcA, FxB, Hn, Hb, HTf and Ftn, we performed ^{59}FeEnt and ^{59}FeCrn uptake studies on the Δ*lmo2183* derivative of EGD-e, but we did not observe a phenotype in any of these assays. Similarly, deletions of the upstream genes *lmo2186* and *lmo2185* [formerly called *svpA* (Newton *et al*., 2005)], and the downstream gene, *srtB,* did not affect iron uptake from Fc, Hb or Hn (Fig. 3; Table 2). We also tested a deletion of the unlinked sortase A structural gene, *srtA* (*lmo0929*; Bierne and Cossart, 2002; Garandeau *et al*., 2002) and the double mutant Δ*srtAB*: neither mutation, nor the double mutation, impaired iron uptake from the compounds we surveyed.

The *hup* region (2.499 Mb) encodes a third ABC transporter containing an ATP binding component (*lmo2429*) that was annotated for potential participation in ferric hydroxamate uptake. Yet, the deletion of *lmo2429* did not impair the transport of iron hydroxamates. Instead, Δ*lmo2429* eliminated the uptake of Hn and Hb (Fig. 3; Table 1). Because of its poor solubility, the halos around Hn in nutrition tests are smaller than those seen for Hb, but the effect of Δ*lmo2429* was distinct: neither Hb nor Hn was utilized by this strain. Conversely, the deletion did not affect uptake of HTf or Ftn, demonstrating the selectivity of the permease system for Hn/Hb. Thus we named the locus *hupDGC* for Hn/Hb uptake, and designated *lmo2429* as *hupC.*

The *feoAB* region (2.184 Mb) contains a Fur box followed by contiguous *feoA (lmo2104*) and *feoB (lmo2105*) genes that encode 75 and 664 residue proteins respectively, identical in length to those of *E. coli*. Listerial FeoB is 34% identical and 51% similar to *E. coli* FeoB (Kammler *et al*., 1993). In aerobic (Table 1) and anerobic (data not shown) nutrition tests, and in aerobic ^{59}FeCrn uptake experiments (Table 2), EGD-e Δ*feoB* behaved like wild-type strain EGD-e.

Δ*fri* (0.979 Mb). In our hands also, the elimination of bacterioferritin retarded bacterial growth in defined media (Olsen *et al*., 2005). In early log phase in KRM, EGD-e Δ*fri* had a doubling time of about 1.25 h at 37°C, and only reached a density of 3.5×10^8 cells ml^{-1} in stationary phase. Δ*fri* also decreased cytoplasmic iron availability (see following), and doubled the V_{max} of FeCrn uptake. This increased efficiency of FeCrn uptake likely derived from the depletion of intracellular iron stores, resulting in overexpression of the FeCrn transporter. Unexpectedly, Δ*fri* impaired the uptake of ferric hydroxamates in nutrition tests, and [^{59}Fe]-apoFc in transport studies (Fig. 3).

To better understand these data, we determined the effect of Δ*fri* on intracellular iron availability, relative to wild-type EGD-e and Δ*fur*, by transforming the three strains with pATgfp7(Newton *et al*., 2005). The plasmid carries the green fluorescent protein under the control of the Fur-regulated *srtB* locus promoter (Fig. 4). In BHI broth at stationary phase, EGD-e/pATgfp7 was predominantly non-fluorescent (mean fluorescence <1), indicating that intracellular iron was sufficient to repress the *srtB promoter-gfp* construct. A fraction of the cells (about 5%) was significantly fluorescent (mean fluorescence between 10 and 50). At the other extreme, most of the Δ*fur* cells were fluorescent (mean fluorescence of 3), and a fraction of these bacteria (10%) was highly fluorescent (mean fluorescence between 20 and 100). The main Δ*fri* population was intermediate between EGD-e and Δ*fur* (mean fluorescence of 1.6), and it also contained a fraction (20%) that was highly fluorescent (mean fluorescence between 20 and 100). These data show that the Δ*fri* mutation impaired the ability of EGD to regulate intracellular iron availability, which in some cells resulted in complete derepression of Fur-regulated promoters.

Pathogenicity of mutants in the mouse model system

We performed animal pathogenesis studies in the mouse infection model to evaluate the effects of the site-directed chromosomal mutations on bacterial virulence, by measuring LD_{50} values. Groups of outbred Swiss mice were inoculated by intravenous injection with different doses, in

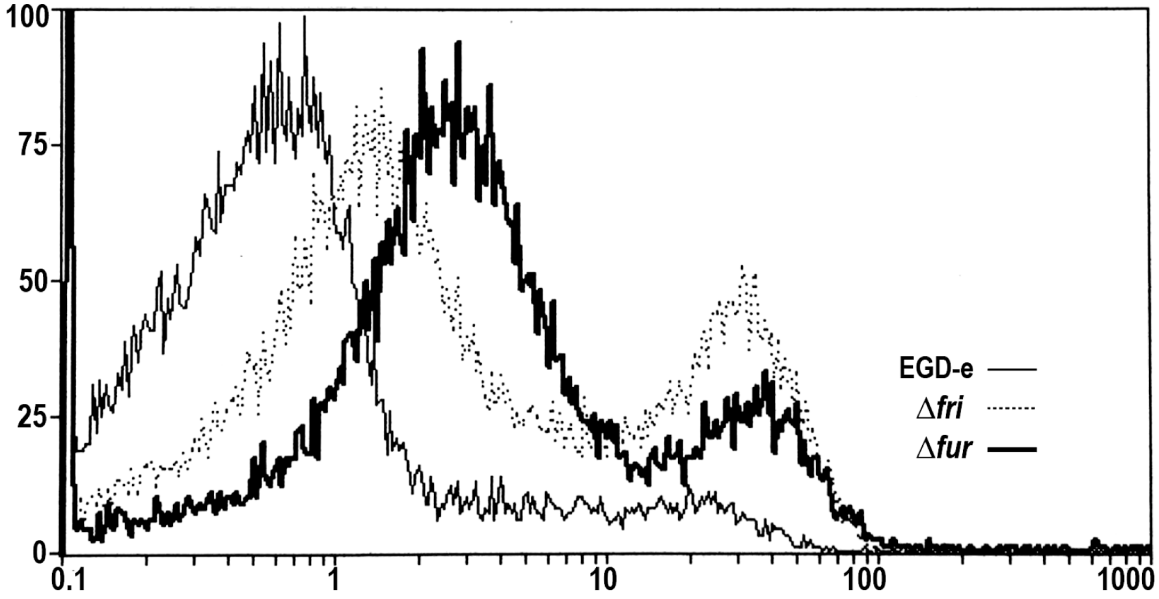

Fig. 4. Cytofluorimetry of EGD-e and its Δ*fur* and Δ*fri* derivatives harbouring pATgfp7. pATgfp7 contains a *gfp* structural gene under control of the Fur-regulated *srtB* promoter from *L. monocytogenes* (Newton *et al.*, 2005). Bacteria were inoculated in BHI broth, grown to stationary phase, and their fluorescence at 520 nm was measured in a Beckman-Coulter Epics Elite flow cytometer.

10-fold dilutions. Mortality was scored for the following 7–10 days, and LD_{50} determined by the graphic probit method (Roth, 1961). Three mutations reduced the pathogenesis of *L. monocytogenes* in mice: Δ*fur*, Δ*hupC* (Table 1) and Δ*fri* (Dussurget *et al.*, 2005; Olsen *et al.*, 2005). Among loci involved in ferric hydroxamate uptake, Δ*fhuD* and Δ*lmo1961* had no effect on LD_{50}; the decrease in growth rate conferred by Δ*fhuC* prevented us from fairly evaluating its impact on virulence.

Molecular analyses of L. monocytogenes *FhuD, FhuC and HupC*

BLAST analyses indicated similarity of *L. monocytogenes* FhuD to the *E. coli* periplasmic binding proteins FhuD, BtuF and MalE. The listerial protein was most similar to FhuD2 of *S. aureus*, a membrane anchored binding protein for ferric hydroxamates (Sebulsky *et al.*, 2003). Like FhuD2, the listerial binding protein contains an unpaired Cys residue (C21) that may be lipidated to form a membrane anchor. Sequence analyses indicated that FhuC and HupC of *L. monocytogenes*, which are themselves very closely related in primary structure, are similar to know ATP binding subunits of ABC-type membrane permeases, including the crystallized proteins BtuD, HisP and MsbA of *E. coli*. Using their most obvious identities and similarities as a basis, we correlated the sequences of the *L. monocytogenes* proteins to the crystal structures of FhuD and BtuD (Fig. 5). In the case of the FhuD family, the most similar regions of primary structure distribute throughout both lobes of the binding protein, in sites that give rise to ligand-contact residues. In the ATP binding membrane permease component family, we found a higher degree of homology, which was more uniformly distributed throughout tertiary structure.

Discussion

Like many infective bacteria, *L. monocytogenes* acquired iron from ferric siderophores and eukaryotic proteins. The experiments reported herein provide the first thermodynamic and kinetic descriptions of Gram-positive bacterial iron transport *in vivo*, which are of additional interest because *L. monocytogenes* is an intracellular pathogen. We furthermore identified, also for the first time, listerial cytoplasmic membrane transporters for particular iron-containing compounds. In light of other data (Sebulsky *et al.*, 2003), the results suggest that these ABC-type permeases are the principal components of Gram-positive bacterial iron uptake systems.

Listeria monocytogenes utilized FcA, a hydroxamate iron complex that most other bacteria do not transport. The unsuitability of apoFcA for sequestration of iron from *L. monocytogenes* led us to seek another iron-deprivation agent, and BP effectively rendered the cells iron deficient. Unlike apoFcA, which is triply negatively charged, BP may permeate through membrane bilayers. We found, however, that addition of iron or iron complexes reversed growth inhibition by BP (Fig. 3, data not shown), indicating that the chelator is not generally cytotoxic to

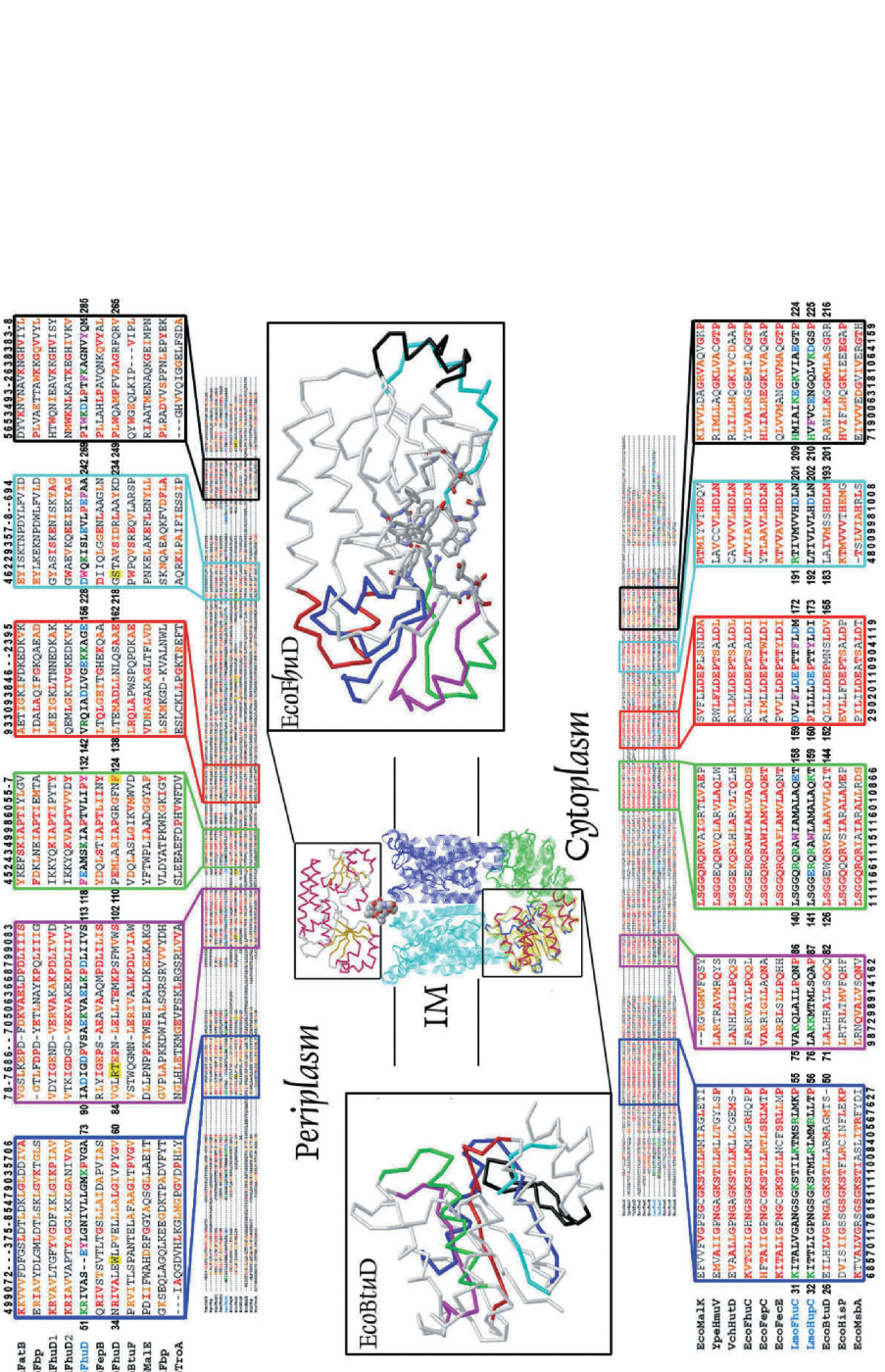

Fig. 5. BLASTP and CLUSTALW analyses *L. monocytogenes* FhuD, FhuC and HupC. The central cartoon shows the overall relationships of these proteins in a typical ATP-dependent, ABC-type membrane permease system, which contains a binding protein, membrane-spanning permease, and ATP binding components. FhuD is a binding protein that is equivalent to *E. coli* FhuD; FhuC and HupC are ATP binding permease components equivalent to *E. coli* BtuD.
Top. Close homologues of FhuD (LmoFhuD) were identified in *Vibrio anguillarum* (VanFatB), *Neisseria gonorrhoeae* (NgoFbp), *S. aureus* (SauFhuD1, SauFhuD2), *E. coli* (EcoFepB, EcoFhuD, EcoBtuF, EcoMalE), *Haemophilus influenzae* (HinFbp) and *Treponema pallidum* (TreTroA). The crystal structures of the latter five proteins are solved. The alignment illustrates six regions of similarity between FhuD and its closest homologues, which are all binding proteins associated with ABC transporters. Residues in LmoFhuD are colour coded: acidic, blue; basic, green; aromatic, magenta; Pro, red; Cys, cyan; all others, black. Identical residues in other proteins are coloured red; similar residues (BLOSUM 62 matrix) are coloured orange; the numerical listings above or below the alignments tabulate the similarity (on a scale from 2 to 11). Ligand contact residues of *E. coli* FhuD are highlighted yellow; in the structural depiction, ligand-contact residues are coloured in CPK format and shown in stick representation. I and S denote the per cent identity and similarity, respectively, of the protein of interest to its homologues.
Bottom. Close homologues of FhuC (including LmoHupC) were identified in *E. coli* (EcoMalK, EcoFhuC, EcoFepC, EcoFecE, EcoBtuD, EcoHisP, EcoMsbA) *Yersinia pestis* (YpeHmuV) and *Vibrio cholerae* (VchHutD). The crystal structures of EcoBtuD, EcoHisP and EcoMsbA are solved. The alignment shows six regions of similarity that HupC and FhuC show to each other, and to their closest homologues, other ATP binding proteins of inner membrane permeases. The colour-coding of the residues, tabulation of similarity, and the denotation of per cent identity/similarity are the same as in the top panel. It is noteworthy that despite their strong sequence conservation (44% identity), HupC and FhuC participate in distinctly different transport systems. In both the top and bottom sequence alignments, stretches of similarity are boxed in different colours that map onto regions of the *E. coli* FhuD or BtuD crystal structures, coloured with the same scheme.

L. monocytogenes and that its effects are related to iron availability. The consistency of BP-based nutrition tests and [^{59}Fe]-apoFc uptake studies supports this conclusion. In addition, BP was previously used to study iron metabolism or transport in both Gram (+) [*Bacillus* (Baichoo *et al*., 2002), *Staphylococcus* (Lim *et al*., 1998)] and Gram (–) [*Salmomella* (Reissbrodt *et al*., 1997; Zaharik *et al*., 2002; Ho *et al*., 2004), *Vibrio* (Holmstrom and Gram, 2003)] bacteria, often at 10-fold higher concentrations (1 mM) than we used (0.1 mM). The single discrepancy we observed with BP, the inability of FeEnt and FeCrn in nutrition assays, was not likely attributable to use of BP as an iron sequestration agent, because in plate tests with *E. coli*, BP acted just like apoFcA (data not shown). We found instead that *L. monocytogenes* transports both catecholate ^{59}Fe-complexes extremely slowly, at low rates that may preclude the formation of growth halos on plates.

Site-directed chromosomal deletions identified the structural loci for two iron transport systems and elucidated other aspects of listerial iron acquisition. (i) Elimination of *lmo1960* or *lmo1959* reduced Fc transport more than 90%. These genes, in the *fur-fhu* region, encode the primary ferric hydroxamate transporter of *L. monocytogenes.* Nevertheless, both the Δ*fhuC* and Δ*fhuD* strains had low-level, residual ^{59}Fc uptake (V_{max} = 2 pMol per 10^9 cells per minute; K_m = 0.1–0.2 nM), suggesting that EGD-e contains a second, slower Fc uptake system, which becomes visible when the *fhuCDBG* transporter is inactivated by deletion. Such transport redundancy is known in other systems. A single membrane protein may recognize multiple substrates [the Fhu permease system of *E. coli* transports Fc, FxB, ferric aerobactin, and ferric rhodotorulate (Rohrbach *et al*., 1995)], or, multiple membrane transporters may exist for a single compound [in *E. coli*, FepA and FecA both transport FeEnt (Annamalai *et al*., 2004; Zhou *et al*., 1995)]. In *L. monocytogenes*, a second Fc uptake system exists with higher affinity but lower rate, whose activity is most relevant when ferric hydroxamates are present at low concentrations. (ii) Among the 11 deletion mutants in this study, only the elimination of *lmo2429* prevented the uptake of iron from Hn/Hb. This gene encodes a putative ATP binding membrane permease component with homology to other known Hn/Hb transporters, as for example those that were genetically identified in *Corynebacterium* [*hmuV* (Drazek *et al*., 2000)] and *Streptococcus* [*shp* (Liu and Lei, 2005)] HupC has identity/similarity to these proteins at levels of 37%/57% and 40%/61% respectively. The acquisition of iron from mammalian proteins is a common attribute of pathogens (Cornelissen and Sparling, 1994), and EGD-e Δ*hupC* was attenuated in mice, consistent with other findings on strains that are defective in Hn/Hb uptake (Tai *et al*., 1993; Stojiljkovic *et al*., 1995; Stevens *et al*., 1996; Torres and Payne, 1997). In *Yersinia pestis*, though, Hn/Hb utilization is not connected to virulence (Simpson *et al*., 2000). (iii) The elimination of bacterioferritin decreased intracellular iron availability, as shown by enhanced expression of a Fur-regulated GFP reporter construct, and by an increase in the rate of FeCrn uptake. Fc uptake in the Δ*fri* mutant was paradoxical. The *fhu* locus is negatively regulated by Fur, and Δ*fri* decreased intracellular iron availability. Therefore, we expected Δ*fri* to enhance FhuBCDG biosynthesis, and consequently, the Fc uptake rate. Instead, the precipitous drop in Fc uptake in the Δ*fri* strain intimates a functional relationship between bacterioferritin and the Fc transport system. These data suggest a previously unrecognized link between the iron storage system and ferric hydroxamate transport. (iv) The *feoAB* locus encodes a membrane uptake system for ferrous iron (Kammler *et al*., 1993). FeoAB homologues contribute to the virulence of *Helicobacter pylori* (Velayudhan *et al*., 2000) and *Legionella pneumoniae* (Robey and Cianciotto, 2002); conflicting findings exist on the relationship of FeoAB to the pathogenesis of *Salmonella typhimurium* (Tsolis *et al*., 1996; Boyer *et al*., 2002). EGD-e Δ*feoB* was neither defective in iron transport, nor attenuated in mice.

Data that we report address a fundamental question about Gram-positive transport mechanisms: the participation of sortase-anchored proteins in iron acquisition from mammalian iron binding proteins. Whereas experiments on *S. aureus* indicated that proteins bound to peptidoglycan by *srtA* or *srtB* act in the uptake of iron from Hb (Mazmanian *et al*., 2002; 2003; Skaar *et al*., 2004), our analysis of Δ*srtA,* Δ*srtB* and Δ*srtAB* mutants found that sortase-anchored proteins play no discernible role in iron (haem/Hb or ferric siderophore) utilization by *L. monocytogenes*. The elimination of sortase genes (Δ*srtA,* Δ*srtB* or Δ*srtAB*) did not impair listerial iron acquisition from any of the ferric siderophores or iron binding proteins we tested. These results conflict with the idea (Mazmanian *et al*., 2003; Skaar *et al*., 2004) that sortase-anchored proteins play a major role in iron acquisition. It is conceivable, although perhaps unlikely, that these two closely related organisms acquire iron from Hb by different mechanisms. On the other hand, even if sortase-anchored iron binding proteins exist in *L. monocytogenes* and/or *S. aureus*, it remains unclear how adsorption to the PG layer may facilitate iron uptake into the cell. Once bound, no obvious driving force exists to transfer iron, ferric siderophores Hn, Hb or Fe-transferrin from the peptidoglycan polymer to CM permeases.

Listeria monocytogenes EGD-e used all of the iron sources we tested, including several (FcA, FeCrn, FxB, Hb, Hn HTf, Ftn) that are not utilized by *E. coli*. Relevant to this broad specificity, others postulated a surface-localized membrane reductase that catalyses iron uptake

(Adams *et al*., 1990; Deneer *et al*., 1995; Barchini and Cowart, 1996; Cowart, 2002). Yet, the identification of two ABC-transporters with differential selectivity for ferric hydroxamates and Hn/Hb argues against this idea. Reduction of iron from these compounds on the cell surface will eliminate the specificity of their cytoplasmic membrane transport reactions. Furthermore, thermodynamic and kinetic barriers exist to the removal of iron from haem by biological reduction. Rather, Hn/Hb and ferric hydroxamates enter through two different ABC-transport systems. Uptake of Fc has moderately high affinity ($K_m = 10$ nM) and a maximum velocity (24 pMol per 10^9 cells per minute) comparable with that of the equivalent *E. coli* transporter. The listerial permease components also equate with those of hydroxamate transporters in *S. aureus* (Sebulsky *et al*., 2000; 2003) and *B. subtilis* (Schneider and Hantke, 1993).

Experimental procedures

Bacterial strains, plasmids and media

We used *L. monocytogenes* strain EGD-e (Glaser *et al*., 2001) for mutant constructions, and the thermosensitive shuttle vector pKSV7 (Smith and Youngman, 1992) for construction of deletions and their recombination into the chromosome. *E. coli* DH5α was the host for plasmids prior to their introduction in EGD-e. *E. coli* was grown in Luria–Bertani (LB) (Miller, 1972) or trypticase soy broth (Difco); *L. monocytogenes* was grown in BHI broth or on BHI plates (Difco). We subcultured (1%) EGD-e strains from BHI broth into KRM (Newton *et al*., 2005), an iron-deficient synthetic medium based on RPMI 1640. Because insoluble $Fe(OH)_n$ forms at alkaline pH, growth at pH 7.4 renders Gram-negative bacteria iron-deficient (McIntosh and Earhart, 1976). So, in some experiments we buffered KRM with 0.1 M Tris-Cl, pH 7.4 (KRMT), to study listerial iron uptake.

Siderophores and iron binding proteins

Ferrichrome and FcA were purified from cultures of *Ustilago sphaerogena* (Emery, 1971). Enterobactin and corynebactin, the native siderophores of Gram-negative and Gram-positive bacteria, were purified from *E. coli* and *B. subtilis* respectively, and their iron complexes (FeEnt and FeCrn) were purified by passage over Sephadex LH20 (Wayne and Neilands, 1975; Annamalai *et al*., 2004). FxB was a gift from J. B. Neilands. We purchased purified Hn, bovine Hb, bovine HTf (approximately 80% saturated with Fe^{+++}) equine Ftn, FeCit and ferrous sulphate ($FeSO_4$) from Sigma-Aldrich (St. Louis).

Construction of deletion mutants

We constructed precise, in frame deletions of structural genes in the four genetic regions of interest, and also of *fri*, by allelic replacement (Domann *et al*., 1992; Poyart and Trieu-Cuot, 1997; Bergmann *et al*., 2002; Bierne *et al*., 2004; Newton *et al*., 2005). Each mutant of EGD-e was verified by PCR reactions from chromosomal DNA (PCR-colony tests) with appropriate primers designed to show the size of the deletion. The constructions were confirmed by PCR sequence analysis of chromosomal DNA from the mutants.

For PCR we used Taq polymerase (New England BioLabs) or AmpliTaqGold (Applied Biosystems) according to the manufacturer's instructions, and oligonucleotide primers from Eurogentec, France, IDT Biotechnologies, or Invitrogen. We performed PCR-colony tests of *L. monocytogenes* for the presence of plasmids, or their integration into or excision from the chromosome, by resuspension of a small portion of a colony into a 50 µl PCR reaction containing appropriate primers. The annealing temperature was 50°C; the extension time depended on the size of the expected fragments (1 min per kb).

Growth in iron-limiting media

Previous experiments showed that *L. monocytogenes* did not utilize iron complexed by BP, so we used the chelator to sequester iron in BHI (Newton *et al*., 2005). After growth in BHI to $OD_{600} = 0.1$, BP was added to 0.1 mM, and the cells were shaken at 37°C until the cultures reached an OD_{600} of 0.9. For experiments in defined media, EGD-e and its mutants were grown overnight in BHI, subcultured into KRM, grown to stationary phase (OD_{600} ~1.2), and then subcultured again into KRM (1%) and grown to mid-log phase.

Nutrition tests

Bacteria were grown in BHI and exposed to BP as described above, and 2×10^7 cells were plated in BHI agar containing 0.1 mM BP. Paper discs were applied to the agar, 10 µl aliquots of sterile ferric siderophores or iron binding proteins were applied to the discs, and the plates were incubated overnight at 37°C. The diameters of the growth halos were measured.

^{59}Fe binding and uptake experiments

For binding and transport studies we prepared and chromatographically purified ^{59}Fe complexes of corynebactin, enterobactin and apoFc (specific activity 150–1000 cpm $pMol^{-1}$). We measured their binding and transport (Newton *et al*., 1999) over a range of concentrations, by adding appropriate amounts of ^{59}Fe-complexes to two aliquots of 2×10^7 cells of EGD-e or its mutants, and incubating them for 15 s, or 1 h and 15 s respectively, before collecting and washing the cells on 0.2 micron filters. The 15 s aliquot measured the amount initially bound to the cells, which we subtracted from the second time-point to obtain the amount transported during a 1 h period. The long duration of the transport reactions was necessary to measure the low uptake rates we observed for some of the ferric siderophores. At each concentration, data were collected in triplicate and averaged. The K_d and capacity of ^{59}Fe-siderophore binding were determined by using the 'Bound-vs.-Total' equation of Grafit 5.09 (Erithacus, Middlesex, UK), and K_m and V_{max} of transport were calculated using the 'Enzyme Kinetics' equation.

In uptake experiments on bacteria that were cultured in the presence of ferric siderophores, when the cells reached mid-log phase they were pelleted by centrifugation at 5000 *g* for 10 min at 4°C, washed three times with fresh KRM media to remove adsorbed iron complexes, and resuspended in fresh media before measurement of the uptake of [^{59}Fe]-siderophore complexes.

*Assessment of intracellular iron availability in EGD-e Δ*fri

We transformed EGD-e and its Δ*fur* and Δ*fri* derivatives with the Fur-regulated-*srtB* promoter–*gfp* construct pATgfp7 (Newton *et al*., 2005), and cytofluorimetrically measured the extent of expression of the reporter green fluorescent protein, with a Beckman Coulter Epics Elite flow cytometer.

Determination of virulence in the mouse model

Bacteria were grown in BHI medium overnight at 37°C with agitation. Pathogen free Swiss female mice (Janvier), 6–8 weeks old, were intravenously inoculated (0.5 ml per mouse) in the lateral tail vein with bacterial suspensions diluted in 0.15 M NaCl. Groups of five mice were challenged with various doses of bacteria, and the mortality was followed for 10 days. The virulence of the strains was estimated by the LD_{50} using the graphic probit method (Roth, 1961).

Molecular analyses of genes and proteins

We obtained chromosomal DNA sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) or from Institut Pasteur [http://www.pasteur.fr/externe], and subjected the translated sequences to BLASTP analysis (http://www.ncbi.nlm.nih.gov/blast/). Among homologues of the *L. monocytogenes* proteins of interest (FhuD, FhuC and HupC), we selected related binding proteins and membrane permease ATP binding subunits respectively, including several in each class whose structures are known from crystallography. We subjected these families of proteins to CLUSTALW (http://www.ebi.ac.uk/clustalw/) and protein domain search analyses. We ultimately mapped the sequences of *L. monocytogenes* binding protein FhuD, and ATP binding permease components FhuC and HupC, according to the known structures of *E. coli* FhuD and BtuD respectively. X-ray structure co-ordinates were obtained from the Protein Data Bank (http://pdbbeta.rcsb.org/pdb).

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