Sortase independent and dependent systems for acquisition of haem and haemoglobin in

$_D$ = 12 nM), leading to its designation as a Hn/Hb binding protein (*Hbp 2*). Purified Hbp2 also acted as a haemophore, capturing and supplying Hn from the

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

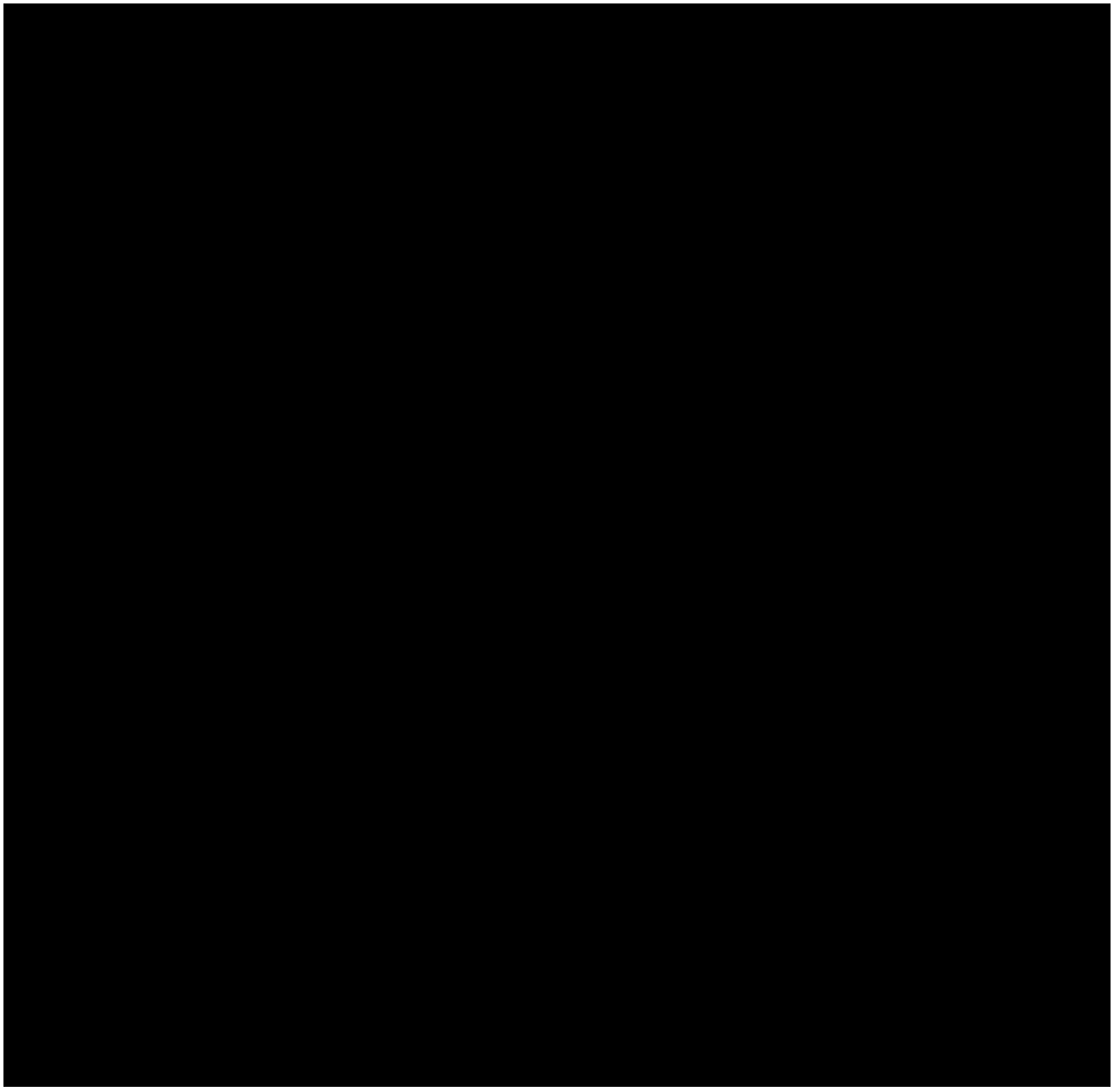
encoded by the *hup* chromosomal region (2498.3–2501.1 kb); D*hupC* impaired Hn and Hb uptake and decreased virulence (Jin *et al*., 2005).

Because of uncertainties in the Gram-positive bacterial Hn transport pathway we further studied its uptake by

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 D*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 [^{59}Fe]-Hn (*Experimental procedures*) and measured its binding and uptake by EGD-e and derivatives with deletions in the(*hup* opeasTet254.9(,)]TJ8182.0046 -1.3333 .9((Fig.)-25.8(4B46t)-27ga70.746tbind46t(deleti46t)-294



Furthermore, like HupD above, purified Lmo2185 contained bound Hn (at ~ 50% saturation), as evidenced by a Soret band (at 405 nm) in its visible spectrum and a positive pyridine haemochrome test (data not shown).

multiplication of L. monocytogenes

We determined the infectivity of the triple deletion mutant EGD-e Δ*hupDGC* by intravenous inoculation of Swiss

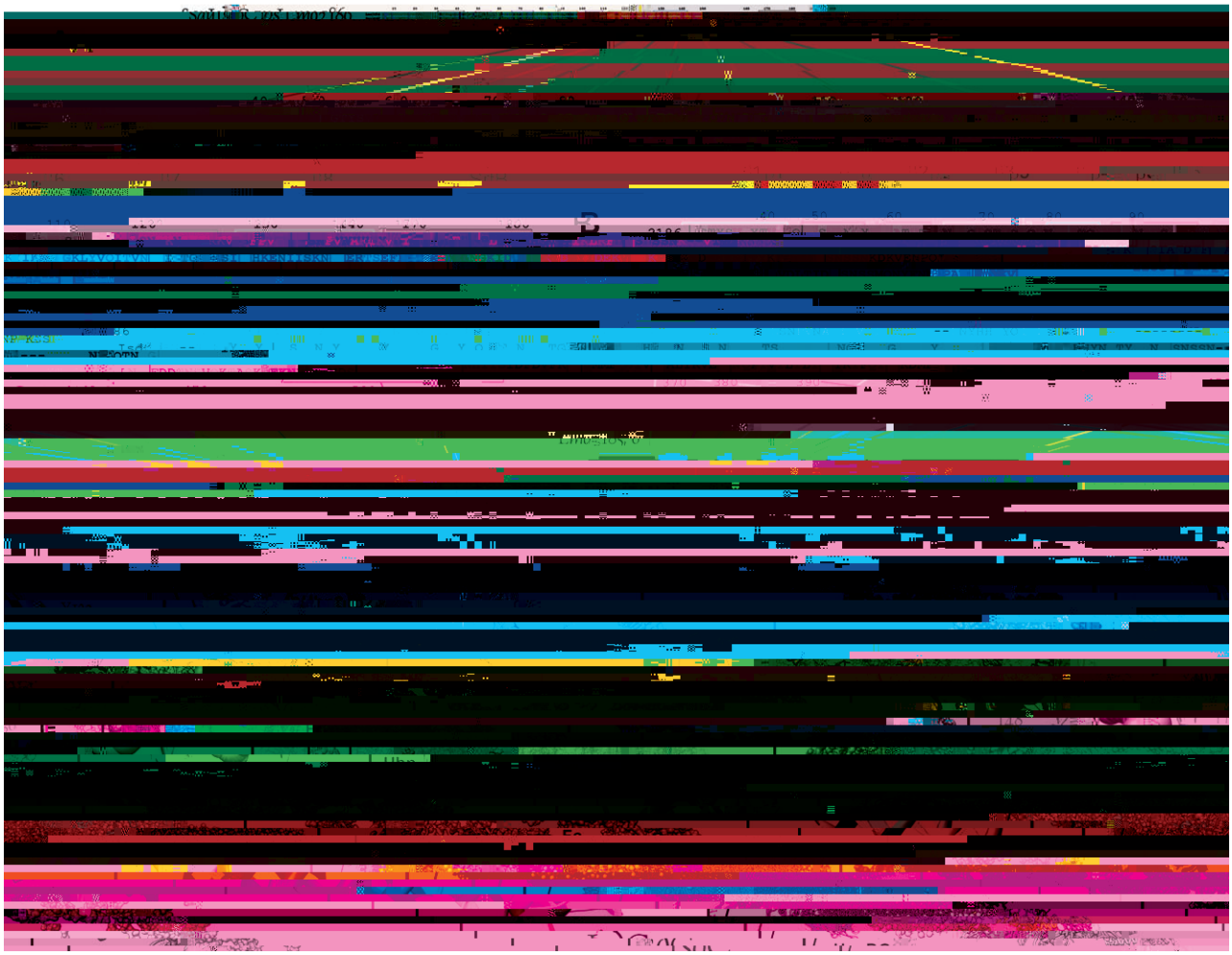


Fig. 6. Sequence, structural and potential spatial relationships of haem binding proteins in the Gram-positive cell envelope.
A. Lmo2186 (Hbp1) and SauIsdC. The sequences of LmoHbp1 and SauIsdC are 30% identical overall (69 of 227 residues), confirming their structural relatedness. The foundation of their conservation is in α and β secondary structure [the coloured boxes in (A) and (B) correlate with the depiction of SauIsdC crystal structure (2O6P) in (C)]: in the α and β elements the extent of sequence identity is 78%.
B. Lmo2185 (Hbp2), LmoHbp1 and SauIsdC. 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 SauIsdC respectively. The main sequence diversity occurs in loops and turns; in the α and β 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 SauIsdC, with 58% and 57% identity in α and β structure respectively.

mice (Table 4). Its LD_{50} was $10^{6.4}$, as compared with $10^{4.5}$ for the parental strain EGD-e. These data were consistent with previous estimation of LD_{50} for the single mutant EGD-eΔ*hupC* ($10^{6.2}$; Jin *et*

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 [^{59}Fe]-Hn (K_D = 2 nM) were comparable to those of purified Hbp2 (K_D = 12 nM), and D*hbp2* caused a fivefold reduction in Hn binding affinity (K_D to ~ 10 nM), while D*hup* caused no observable change. [^{59}Fe]-Hn uptake results agreed with the binding data:

coding RNAs) revealed that incubation of *L. monocytoge-*
nes

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

PAGE (Fig. S2). Fractions containing purified 6H-FhuD and 6H-HupD were pooled, dialysed against Tris-buffered saline

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* =

Sinclair, P.R., Gorman, N., and Jacobs, J.M. (1999) *Current Protocols in Toxicology*. San Francisco, CA: John Wiley and Sons.

Singh, A., Isaac, A.O., Luo, X., Mohan, M.L., Cohen, M.L., Chen, F., *et al*. (200 8.xnadsl2((200 brals)-(200 iros200 homeostasis200 insmostasis23k(200 insmostasis)-(23a