Bacterial iron acquisition in the host environment.

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Iron acquisition by both pathogens and their hosts relates to bacterial disease. Prokaryotes and eukaryotes alike require iron for metabolic biochemistry in the TCA cycle, for energy generation by the electron transport chain, for DNA synthesis (via ribotide reductase), and for defense against toxic reactive oxygen. Iron deprivation inhibits bacterial growth, reducing or eliminating virulence. Research on this subject confirmed what originally seemed intuitive: bacteria need iron for metabolism, they produce biosynthetic and transport systems to obtain the metal, and impairment or abrogation of these processes reduces bacterial virulence. The central role of iron in aerobic biochemistry makes it a focal point of pathogenesis and invasiveness: eukaryotic hosts sequester iron as a defense against infection, but successful pathogens overcome this strategy and capture the metal. Gram-negative bacteria, like *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, *Yersinia pestis*, and *Neisseris meningitidis* contain redundant, TonB-dependent iron uptake pathways. that either internalize ferric siderophores or strip the metal from eukaryotic iron proteins. Gram-positive bacteria, like *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus anthracis* produce extracellular heme (Hn)/hemoglobin (Hb) binding proteins that anchor in the peptidoglycan (PG) matrix surrounding the cell. However, none of the prokaryotic uptake systems are fully defined, which complicates the use of iron deprivation as a strategy against pathogenesis. The iron acquisition process often begins when cell surface receptors recognize Fe^{+++} complexes, and ultimately ends when cytoplasmic membrane (CM) transporters internalize, and in some cases reduce the metal to Fe^{++}, which then enters cytoplasmic metabolic pools. Despite many advances the fundamental question perseveres in both Gram-negative and Gram-positive bacteria: how does iron (III) traverse the bacterial cell envelope?

I. The hemin /hemoglobin uptake (Hup) transporter of *Listeria monocytogenes*. The Gram-positive bacterial cell envelope has a different architecture than that of Gram-negative cells. Organisms like *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus anthracis* and *Listeria monocytogenes* lack an outer membrane, and instead they

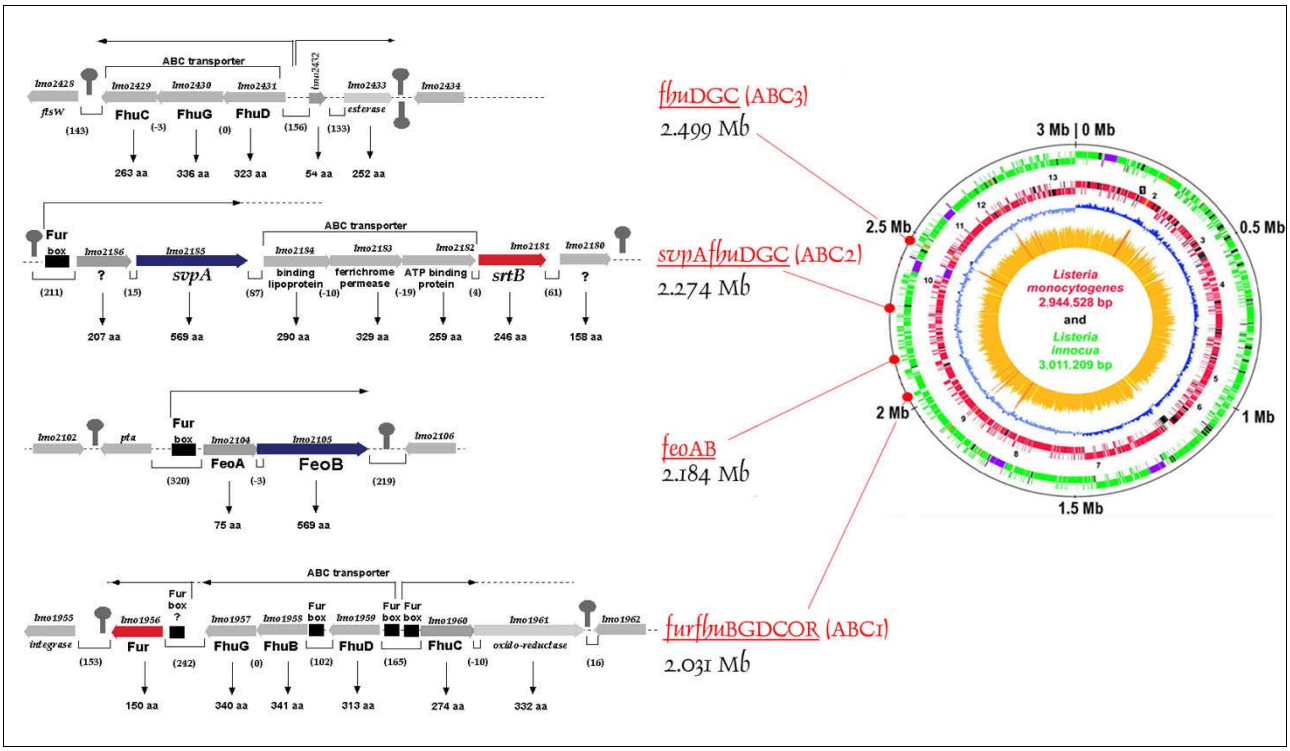

Figure 1. **The ferric hydroxamate and hemin/hemoglobin transporters of L. monocytogenes.** Genomic analyses allowed site-directed deletions by allelic exchange, that eliminated the transport ferric hydroxamates (Fc, FxB) and hemin/ hemoglobin (Hn/Hb) in *L. monocytogenes*. These and other data identified loci underlying two membrane transporters

produce a thick layer of PG that polymerizes outside their CM. Proteins, polysaccharides and lipids associate with or anchor within the multilamellar coating of PG and extend to the cell surface. Gram-positive bacteria require iron in comparable amounts to Gram-negative organisms, but their transport systems are comparatively obscure, in that only a

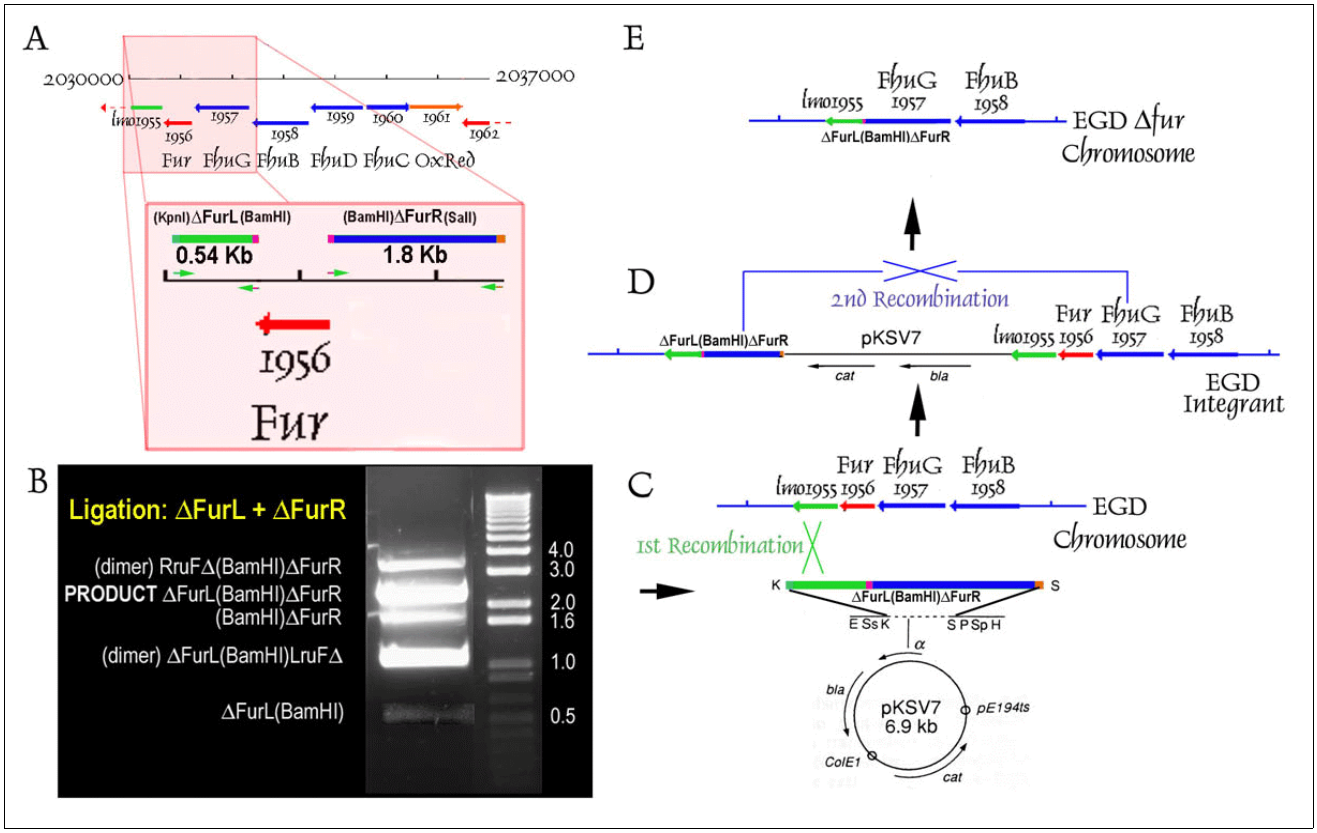

Figure 2. **The molecular biological strategy of allelic exchange to create chromosomal deletions.** For further details see Newton et al. (5), in which we deleted the the *fur* gene of *L. monocytogenes*.

few are known and those are not yet fully characterized. Nevertheless, the rapidly expanding library of Gram-positive bacterial genomic information created a different path to the discovery and definition of their systems, which are of interest because they may function by dissimilar mechanisms to those of *E. coli* and its relatives. Gram (+) bacteria contain many homologous loci to the iron transporters of Gram (-) bacteria: their CM (Gram-positive) and IM (Gram-negative) systems are functionally and structurally equivalent with regard to transport of many solutes. Therefore, using genomic and proteomic analyses as starting points, we investigated the iron acquisition mechanisms of *L. monocytogenes* by systematic chromosomal deletions and phenotypic characterizations. These experiments (1-3) identified two ABC-transporter systems for iron, one that functions to acquire ferric hydroxamates (Fhu), and another with specificity for hemin/hemoglobin (Hup).

The genomic approach supercedes many of the genetic, and to some extent biochemical experiments that delineated Gram-negative bacterial membrane transport systems. Once identified by deletion mutagenesis, Gram-positive bacterial iron acquisition systems often closely correlated with those of *E. coli*. Furthermore, although IM transporters of Gram-negative cells are shielded by the OM, CM transporters of Gram-positive cells are more accessible to chemical modification from without (by the same biophysical techniques we've developed for *E. coli* FepA; see the link on Gram-negative bacterial iron uptake), because of the absence of an OM. We are genetically introducing modifiable Cys residues in the two ABC-permease transporters of *L. monocytogenes* noted above, and applying fluorescence spectroscopic techniques to understand their mechanisms. One target is the Hup transporter (2, 6), because of its multiple ligands, ability to extract hemin or iron from hemoglobin, and its relationship to the virulence of *L. monocytogenes*, an intracellular pathogen that crosses the blood-brain barrier (see also below). Experiments on the Hup transporter complement studies on *E. coli* FepA in several ways. Both systems actively transport iron, in one case through the OM, in the other through the CM. The Gram-negative bacterial OM transporter utilizes proton-motive force for energization, whereas the listerial CM ABC-transporter hydrolizes ATP. Hence, both membrane proteins undergo energized conformational motion, but by two different biochemical mechanisms that involve proton-motive force and ATP-hydrolysis, respectively.

A. Sortase-independent and -dependent systems for Hn/Hb uptake by Gram-positive bacteria. While they lack the OM transport systems of Gram-negative bacteria, *L. monocytogenes* and its Gram-positive relatives contain other novel approaches to both the interaction with eukaryotic cells, and iron acquisition in animal tissues. Various proteins encoded by Fur-regulated transport operons of Gram-positive bacteria are secreted to the cell exterior, or covalently

attached to PG by a class of cell envelope enzymes called sortases (3). Some of these PG-anchored protein function in bacterial adherence to animal cells, and others may act in the recognition and transport of heme by providing an initial binding site for the porphyrin and for heme-containing proteins like hemoglobin or haptoglobin. We completed thermodynamic and kinetic analyses of hemin binding and transport that revealed the role of sortase-anchored cell envelope proteins in iron uptake by *L. monocytogenes* (2,5,6). These data indicate that a protein anchored to PG by sortase B, the product of *lmo2185*, indeed functions to bind heme. In addition, the proteins encoded by *lmo2186* and *lmo2185*, which we designated Hbp1 and Hbp2 (heme/hemoglobin binding protein), respectively, bear a striking sequence relatedness to the IsdC protein of *Staphylococcus aureus*, which was already shown to bind hemin in a crystal structure (7). HbpA, HpbB and IsdC are secreted proteins that are apparently anchored to PG by sortase B. However, the mechanism by which they transfer heme to CM hemin ABC-transporters is unknown, and we are studying this phenomenon.

B. Host colonization. Since the early 70's biochemists and microbiologists suspected that iron acquisition by both pathogens and their hosts, relates to bacterial disease. Research on this subject confirmed what originally seemed intuitive: bacteria need iron for metabolism, they produce biosynthetic and transport systems to obtain the metal, and impairment or abrogation of these processes reduces bacterial virulence. The central role of iron in aerobic biochemistry makes it a focal point of pathogenesis and invasiveness: eukaryotic hosts sequester iron as a defense against invasion, but successful pathogens overcome this strategy and capture the metal (8). Consequently, the characterization of systems that pathogenic bacteria employ for iron uptake *in vivo*, and the evaluation of their importance to bacterial infection and colonization, are central goals of our research efforts. We are studying the function of siderophore uptake systems in colonization by Gram-negative (*E. coli*, *S. typhimurium*) bacteria, and the participation of hemin/hemoglobin uptake systems in infection by Gram-positive (*L. monocytogenes*, *S. aureus*) species.

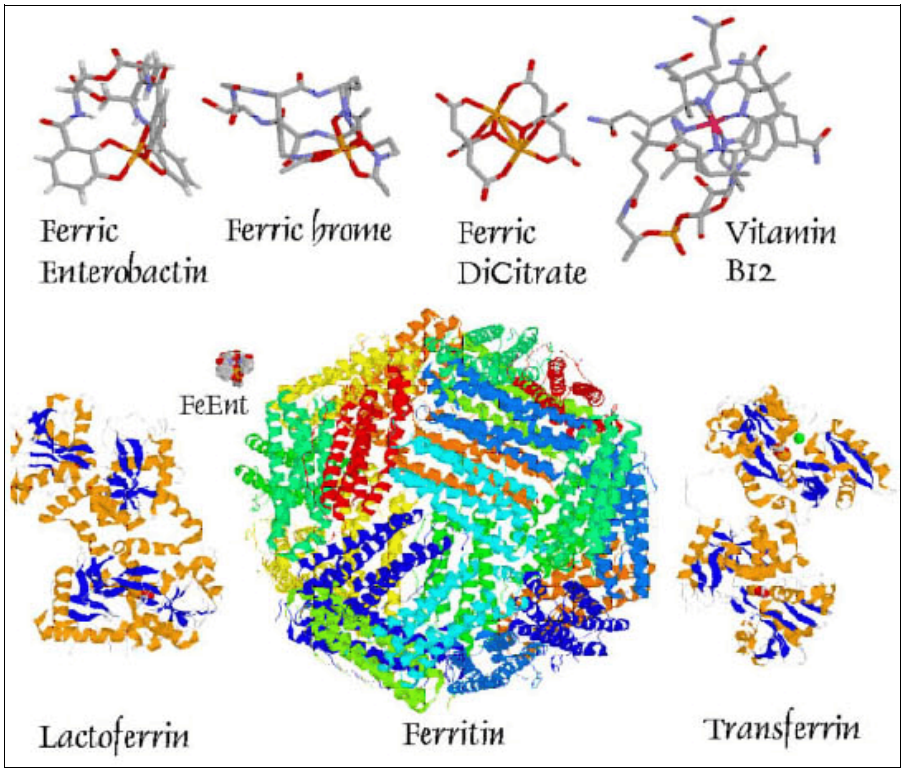

Figure 3. **Prokaryotic siderophores and eukaryotic iron binding proteins.** Although much smaller in size (top, 700 - 1000 Da, the affinity of microbial siderophores for iron exceeds that of eukaryotic binding proteins (bottom: 80, 000 - 500,000 Da; the relative size of ferric enterobactin (FeEnt) is also shown). Although not an iron complex, cobalt-containing vitamin B_{12} is recognized and transported by bacteria in a similar manner to that of ferric siderophores.

Primarily because of its utility in redox processes, iron is an essential metal in the metabolic processes of most terrestrial organisms. This iron requirement is problematical for bacteria, because Fe^{+++} precipitates as un-transportable hydroxide polymers in aqueous environments, and it is sequestered by binding proteins in eukaryotic fluids and tissues. Within mammals iron circulates as hemin/ hemoglobin (normally ensconced within red blood cells); in serum, lymph and intracellularly, transferrin, lactoferrin and ferritin bind it with high affinity. However, efficient microbial pathogens overcome this barrier by either secreting siderophores that chelate Fe^{+++} with even higher affinity, or by producing membrane transport systems that directly acquire iron from the eukaryotic proteins. The organisms under study in our laboratory exhibit both modes of pathogenesis. *L. monocytogenes*, a saprophytic Gran-positive organism that is widespread in nature, is also a lethal intracellular pathogen that does not elaborate siderophores, but uses exogenous ferric siderophores from other organisms or directly extracts iron from hemoglobin, holotransferrin or ferritin (Fig. 3). We are studying the relationship between iron acquisition and virulence in *L. monocytogenes* (in our laboratory and that of Dr. Alain Charbit at Institut Necker in

Paris), with animal infection experiments that evaluate the effects of site-directed chromosomal mutations on bacterial virulence (LD_{50} values). We also measure bacterial multiplication in target organs (spleen, liver, brain) by oral infection of mice with wild *L. monocytogenes* or its genetically engineered mutants. Finally, its intracellular mode of pathogenesis and ability to directly utilize eukaryotic iron sources makes *L. monocytogenes* prototypic for studies of iron and virulence. We are determining the pathogenicity of iron uptake mutants in murine macrophages and cell lines, including intracellular multiplication in primary macrophages prepared from murine bone marrow, and in human enterocytes (CACO-2 cells).

II. Systems Biology: iron acquisition and microbial pathogenesis. The experiments in our lab center on membrane transport biochemistry, with particular interest in the acquisition of iron by bacteria. We are characterizing outer membrane transport reactions in Gram-negative bacteria, and cytoplasmic membrane uptake systems in Gram-positive bacteria. Both systems involve the uptake of iron (III), and as a result they relate to microbial pathogenesis. Iron availability is a key global regulator of bacterial metabolism, which makes iron acquisition a focal point of prokaryotic systems biology. In the host environment, the success or failure of iron uptake processes impacts the outcome of pathogenesis. Hence this global regulatory and transport network intimately connects to human and animal disease. We are presently studying the mechanistic biochemistry of metal transport by prokaryotes as our primary research goal, but our program is connected to microbial systems biology, and our current experiments focus on a multi-disciplinary, systems biology approach to the discovery of new pharmaceutical agents to thwart bacterial pathogenesis. These research programs endeavor to block prokaryotic iron acquisition, and thereby prevent microbial growth in human and animal tissues.

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