

Iron and the Pathogenicity of Bacteria
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I. Gram-negative bacterial iron uptake. We are studying the ability of pathogenic bacteria to obtain the element iron (Fe) in human and animal hosts. This research spans several decades, which may be briefly summarized with a few statements. First, not just microorganisms, but essentially all organisms, require iron for a variety of metabolic processes, including energy generation by cytochrome-containing proteins, DNA synthesis, as a cofactor in metabolic enzymes, and for detoxification of reactive oxygen species. Secondly, humans and animals sequester iron within the body, in forms like transferrin, lactoferrin and ferritin, as a means of defense against prokaryotic infection, but microorganisms synthesize and secrete small organic molecules called siderophores that actively chelate iron and remove it from eukaryotic iron-binding proteins (Fig. 1). Furthermore, some bacterial pathogens may directly utilize the iron in transferrin or lactoferrin. Thus on the molecular level, iron is a valuable commodity that is a key

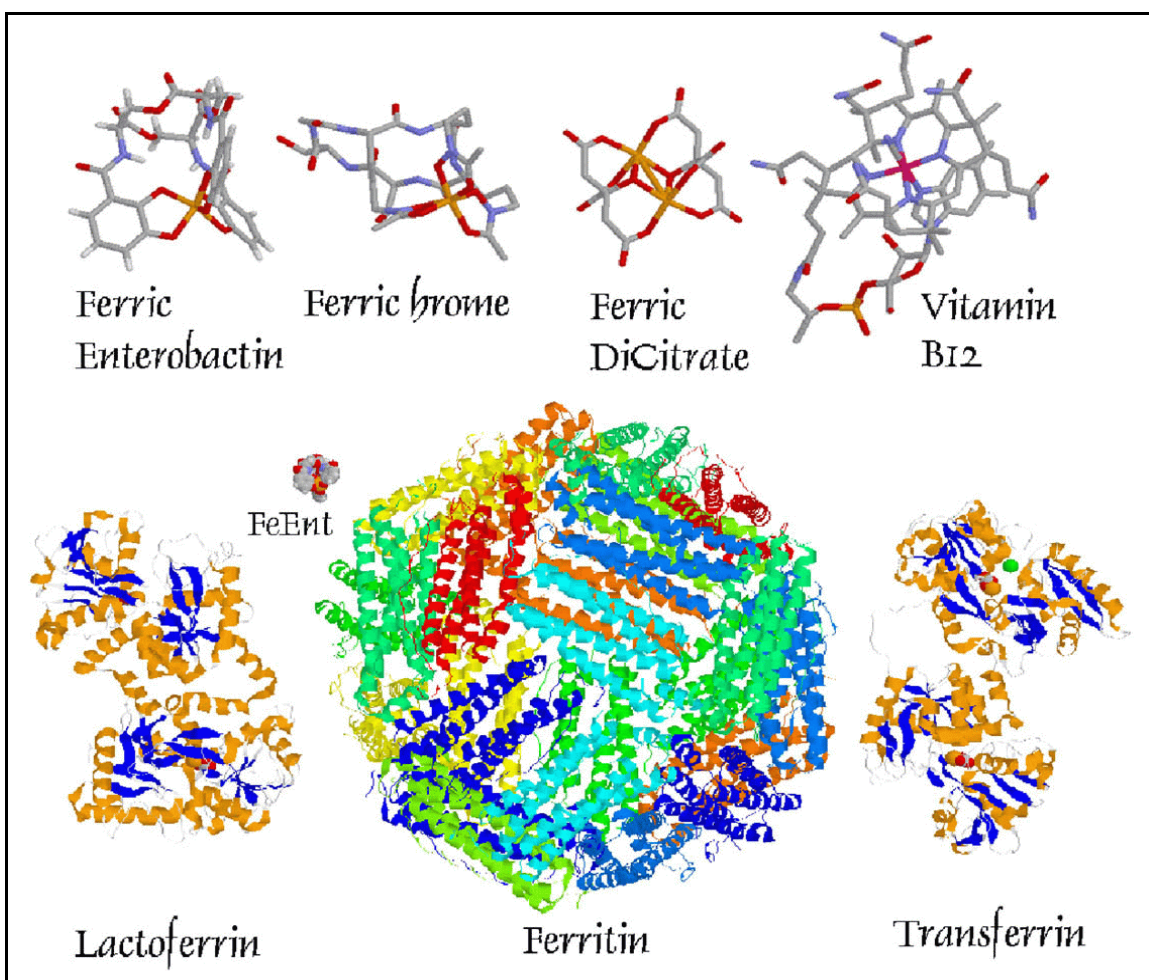


Figure 1. Structure of iron-binding microbial siderophores (top; molecular mass 700 - 1000 D) and iron-binding eukaryotic proteins (bottom: molecular mass 80, 000 - 500,000 D). For comparison, the actual size and structure of ferric enterobactin is shown next to that of ferritin.

element of bacterial pathogenesis. But unfortunately, the process of iron acquisition is not well understood in either Gram-positive or Gram-negative bacteria.

Regarding Gram-negative organisms, our experiments focus on the uptake of iron through the outer membrane (OM) of *Escherichia coli* (Fig. 2), a prototypic bacterium that is like many other pathogens, including *Salmonella typhi* (typhoid fever), *Vibrio cholerae* (cholera), *Shigella dysenteriae* (dysentery), *Neisseria meningitidis* (meningitis) and *Yersinia pestis* (plague). The use of *E. coli*, which is itself not pathogenic, simplifies the experiments, but its iron transport systems are exactly comparable to those of the more severe pathogens. *E. coli* primarily obtains iron by the synthesis of the siderophore enterobactin, that binds iron with very high affinity in the extracellular environment. The bacteria then transport ferric enterobactin, in a process that involves several stages. First, the iron complex binds to a receptor protein in the OM, called FepA, and through a series of incompletely understood reactions, FepA internalizes ferric enterobactin (FeEnt) into the cell (1-3, 5-9, 11-13). This process requires energy and the action of another cell envelope protein, TonB, but the biochemical mechanism of transport is not known.

The OM of gram-negative bacteria is an unusual, asymmetric bilayer that creates selective permeability: it permits nutrients and vitamins to enter the cell, but it also excludes

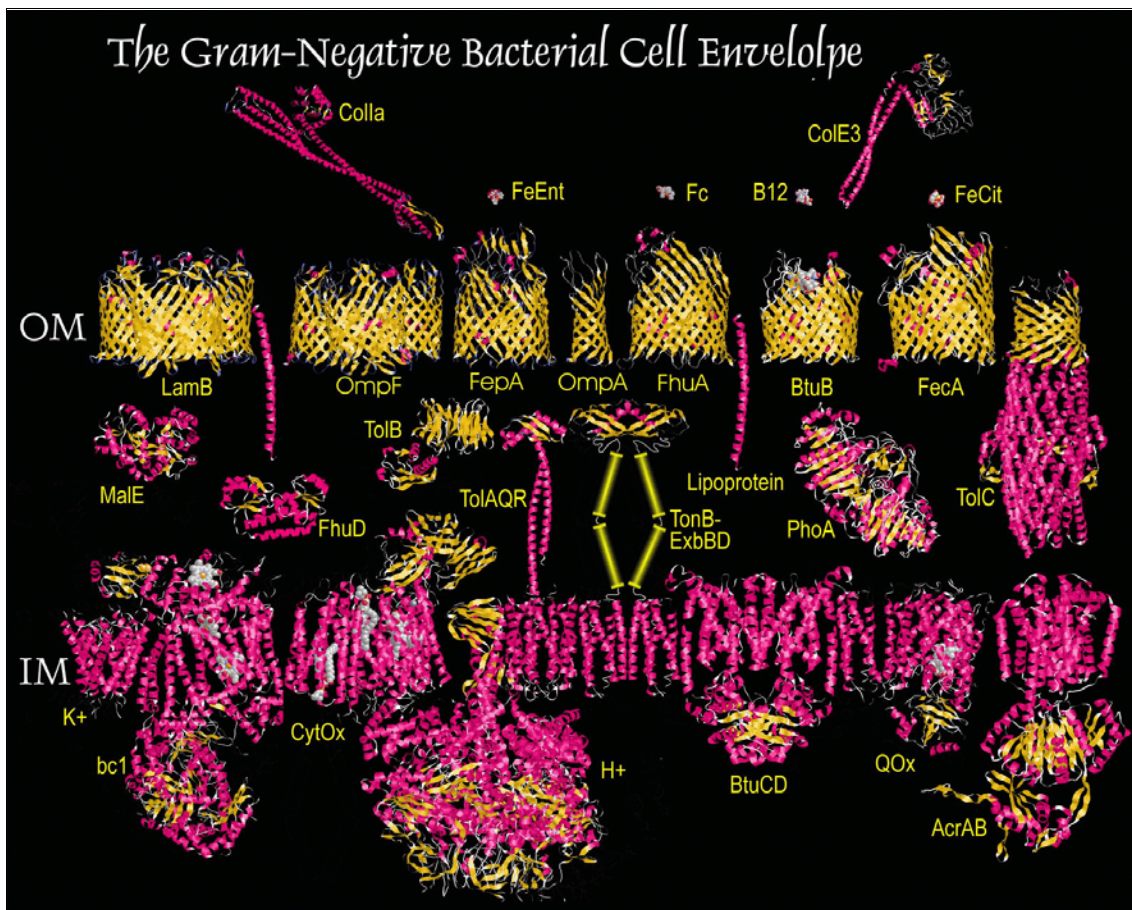


Figure 2. Transport through the Gram-negative cell envelope. Many experiments in our laboratory consider the transport mechanisms of small molecules through proteins of the outer and inner membranes of bacteria, depicted here from their crystallographic coordinates.

many toxic molecules, like detergents and antibiotics. These selective permeability properties are critical to the survival of bacteria, including all of the pathogenic gram-negative bacteria that cause diseases in animals and man. One of the ultimate goals of our research program is to learn enough about the fundamental biochemistry of outer membrane transport processes as to design strategies to disrupt them, and thereby combat disease. Without a known exception, OM proteins transport molecules into the bacterial cell, and one of the projects in our laboratory centers around the characterization of their structure and function. In the past few years a lot of progress has occurred in this area, primarily from the X-ray crystallographic solution of the structure of a class of outer membrane proteins, called porins. Porins are proteins that form **po**res through which materials come **in**to the cell. Unlike most other membrane proteins, porins are not anchored in the membrane bilayer by hydrophobic alpha helices. Rather, they contain a series of membrane-spanning, amphipathic beta strands that wrap around to form a barrel. The outside of the barrel is hydrophobic, and the interior is hydrophilic. Porin beta-barrels sit in the outer membrane, creating a relatively rigid, hydrophilic channel across the bilayer.

The recent crystal structure of FepA, which is a special type of porin, has greatly facilitated our experiments (Fig. 3). Research on this project include a variety of approaches to understand the mechanism of iron transport. Our main methodology is molecular biological: we genetically engineer mutant proteins to attempt to understand how the FepA receptor protein passes FeEnt through the OM bilayer. Another technique that we've utilized is called site-

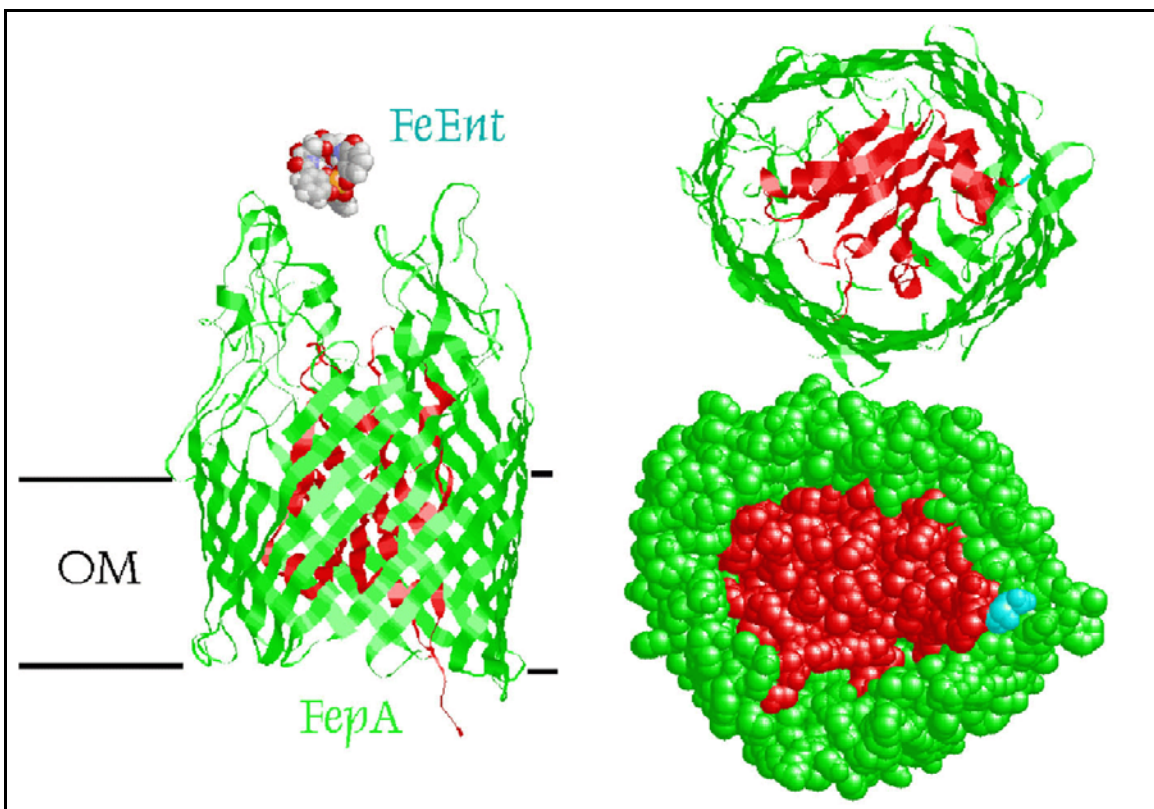


Figure 3. Structure of FepA and ferric enterobactin. The N-terminal 150 amino acids of the receptor protein, which form a globular domain (N-domain), are colored red; the C-terminal 575 residues (C-domain), which form a transmembrane β -barrel are colored green. Note that the N-domain inserts itself within the C-domain, effectively closing the channel.

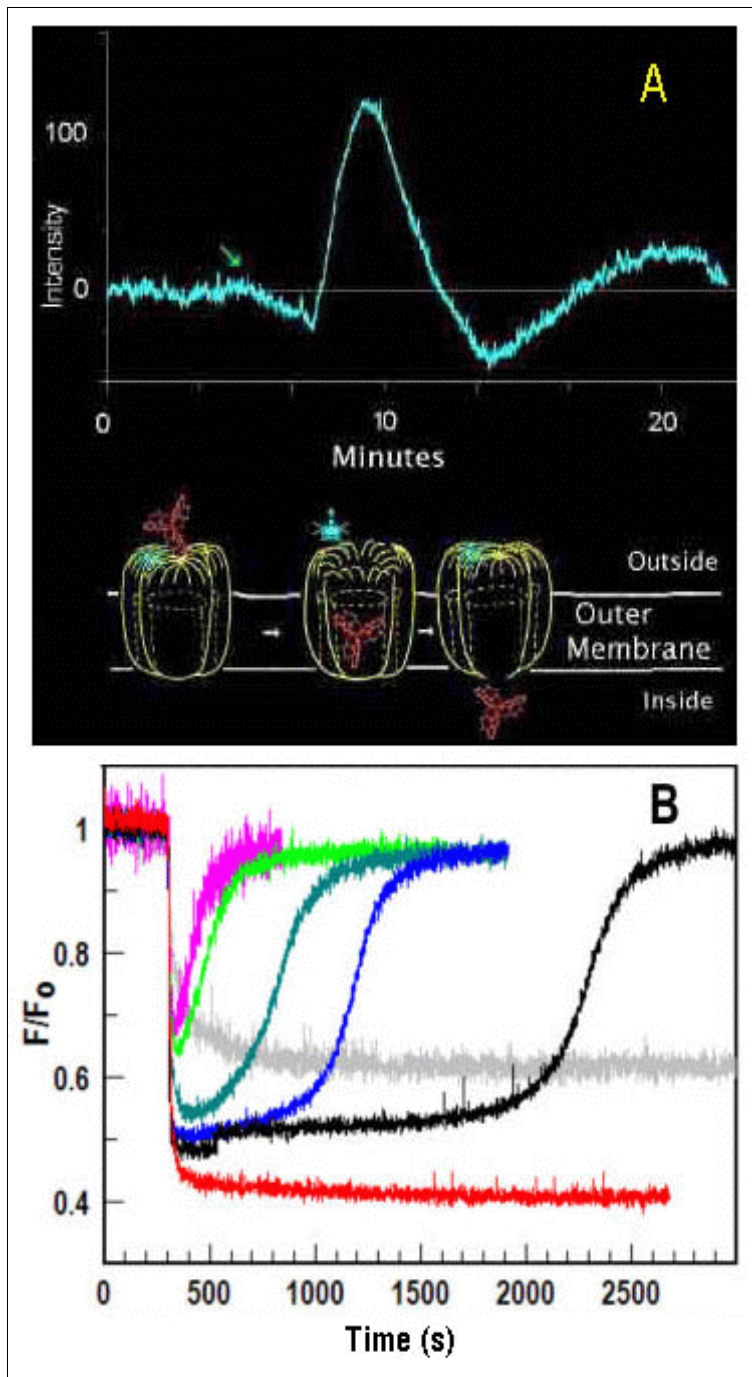


Figure 4. Spectroscopic measurements of transport.
For an explanation, see references 3 and 4.

dislodging from the existing channel so that it becomes open for transport. Lastly, in order for transport of the siderophore to occur, FepA must act in concert with another cell-envelope protein, called TonB. It is known that this interaction between FepA and TonB requires metabolic energy, in the form of proton-motive force, but essentially all of the other details of the

directed spectroscopic labeling, in which we introduce spectroscopic labels (either fluorescent or paramagnetic) into the protein structure by covalently attaching them to genetically engineered cysteine residues at positions of interest. Once labeled in this way, we then indirectly monitor the transport of FeEnt through the receptor by spectroscopic observations. Microbiological and biochemical assays of iron binding and transport are also important to the understanding of the transport process.

FepA contains the same basic characteristics as general porins: an amphipathic beta-barrel that forms a hydrophilic channel across the outer membrane bilayer. However, FepA is different in that its channel is closed by an unusual N-terminal domain that resides within the channel itself. At present, our laboratory is focused on three questions about the transport of iron-containing siderophores by ligand-gated porins like FepA (Fig. 5). First, how does the receptor properly recognize its correct ligand, FeEnt, in the environment. Secondly, how does the metal complex pass through the channel, given that the pore is completely blocked by the N-terminal domain? The N-terminus must change in some significant way during transport, either by forming an opening through which the ferric siderophore passes, or by

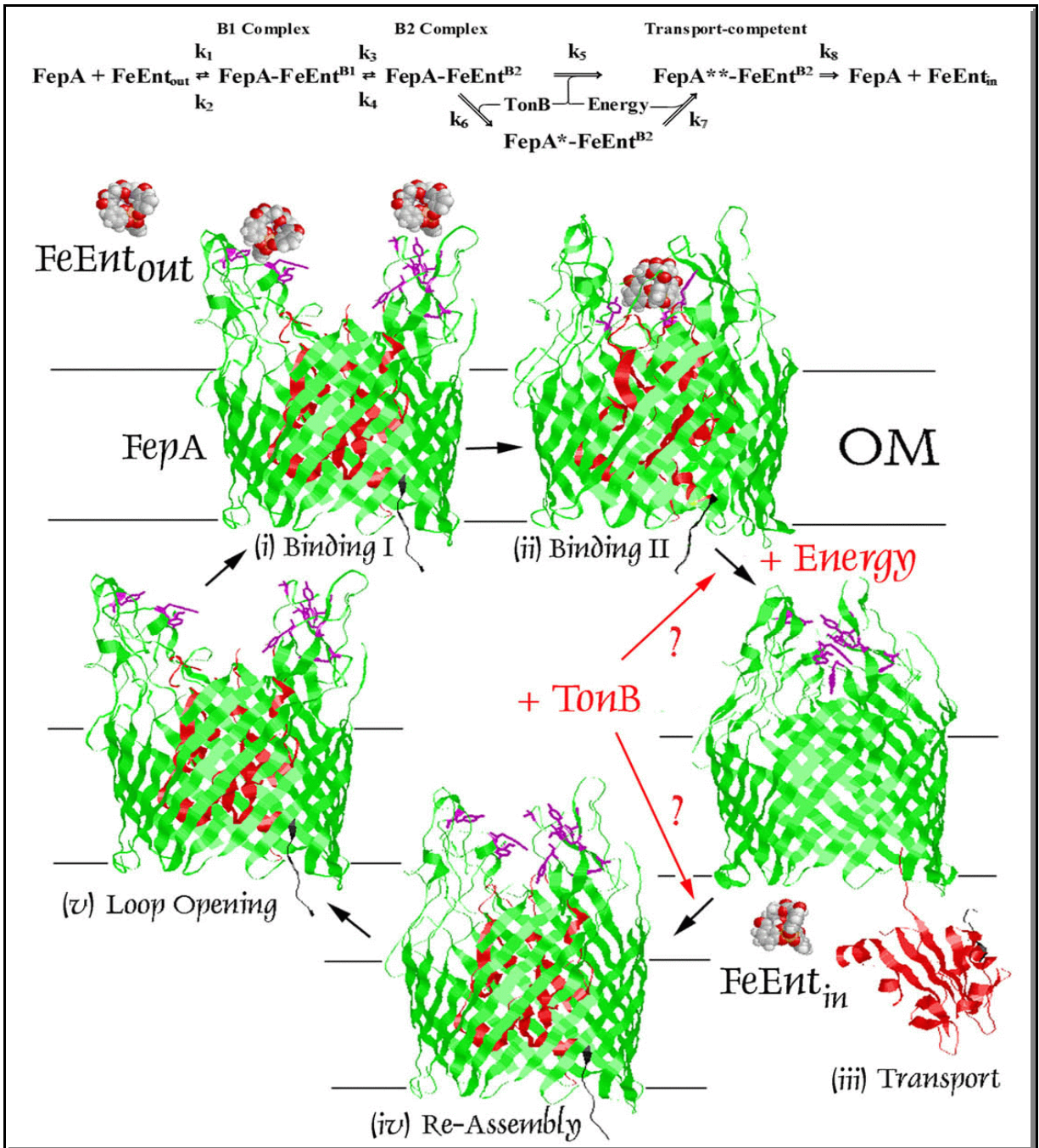


Figure 5. Proposed mechanism of FeEnt uptake by FepA. We know that initially, FeEnt binds to aromatic and basic residues in the surface loops of FepA. Recent experiments showed that the loops assume an **open conformation** that is receptive to the ferric siderophore, and that during the binding reaction the loops contract into a **closed conformation** that holds the iron complex above the N-domain, ready for transport. We hypothesize that the next stage of transport involves dislodgement of the N-domain from the pore, which pulls FeEnt through the membrane bilayer into the cell. This reaction probably involves the input of energy and the actions of TonB, although these assumptions are not yet proven.

FepA transport mechanism are unknown. The final major objective of our research is to determine what is the function of TonB in the siderophore uptake process.

Ligand-gated porins like FepA are dynamic receptor proteins that undergo conformational changes during their transport reactions. Thus besides their inherent interest and importance as the basis of the connection between iron and bacterial virulence, they are prototypic receptors that illustrate some of the most interesting phenomena of membrane proteins. In particular, we desire to understand how they are energized, and how TonB acts to facilitate their transport activities. Our approaches to these problems involve genetic engineering of FepA and TonB, and then analysis of the mutant proteins that we create by a variety of biochemical and biophysical methodologies. The results of these experiments usually provide insight into the mechanism of the iron transport process. In our laboratory you will acquire a working knowledge of site-directed mutagenesis, DNA sequencing, ferric siderophore and membrane protein purification, immunology, and several other techniques related to membrane protein biochemistry. Some of our most interesting recent experiments involve the combination of molecular biology and biophysics, using genetically engineered Cys residues to introduce paramagnetic or fluorescent labels into the structure of bacterial membrane proteins. With these constructions we were able to monitor the transport of ferric enterobactin into living bacterial cells (Fig. 4; (3, 4)). These data ultimately led to our working model of FepA transport (Fig. 5).

II. Gram-positive bacterial iron uptake. For all bacteria, the need for iron is problematical. Iron is unavailable in the aerobic microbial world, either because it is insoluble or because it is sequestered. In wild aqueous environments ferric iron rapidly precipitates as hydroxide polymers, and within animal hosts iron binding proteins like transferrin, lactoferrin and ferritin bind the metal with high affinity. Iron also circulates in the body as hemoglobin, which is normally ensconced within red blood cells. Indeed, iron is essential to the vast majority of organisms, but its sequestration by transferrin in serum and lymph, by lactoferrin in mucosal secretions, and by ferritin and heme compounds in cells, normally renders these fluids and tissues void of prokaryotic life. However, efficient pathogens overcome this barrier, by either producing siderophores or by utilizing iron-containing eukaryotic proteins.

The Gram-positive cell envelope is much different from, and much less characterized than that of Gram-negative bacteria. Gram positive organisms, like *Staphylococcus aureus* (skin infections), *Streptococcus pyogenes* (Scarlett fever), *Bacillus anthracis* (anthrax) and *Listeria monocytogenes* (meningitis) do not contain an outer membrane. Instead their inner membrane (IM) is covered by a thick layer of peptidoglycan (PG), in which proteins and lipids are anchored and extend to the cell surface. Gram-positive bacteria require iron in comparable amounts to Gram-negative organisms, but their iron transport systems are comparatively obscure, in that none of them are biochemically defined. Among all the Gram-positive bacteria not even a single cell envelope protein is unambiguously known to transport iron. In the past few years, nevertheless, many Gram-positive bacterial genomes were completely sequenced, including those of all the organisms listed above. All of the genomes contain loci with homology to iron transporters of Gram-negative bacteria (Fig. 6). These sequence data present a unique opportunity, the discovery of previously unknown and uncharacterized membrane transport proteins, with potentially different mechanisms than those of *E. coli* and its relatives.

***Listeria monocytogenes*, an ubiquitous pathogen.** *Listeria monocytogenes* is a prototypic Gram-positive bacterium that is widespread in nature. It does not normally belong to the human commensal flora, but its ability to grow at 4 °C allows it to contaminate food and cause digestive infections that may become systemic in immunocompromised individuals and/or pregnant women. The severity of listeriosis (an overall mortality of 25-30% in spite of antibiotic therapy) mainly results from a high frequency of neurological damage associated with its systemic infections. *L. monocytogenes* is an intracellular pathogen: its ability to survive and multiply in a variety of cell types and tissues, including macrophages and epithelial cells like hepatocytes, is a key element of its pathogenicity. Although the iron uptake processes of Gram-positive bacteria are not well known, the fact that *Listeria* actively proliferates within cells and spreads from cell to cell suggests that it adeptly obtains iron in the intracellular environment. Nevertheless, at present the relationship between iron and listerial virulence remains unclear. *Listeria* is not known to secrete siderophores, but uses exogenously available ferric siderophores made by other organisms.

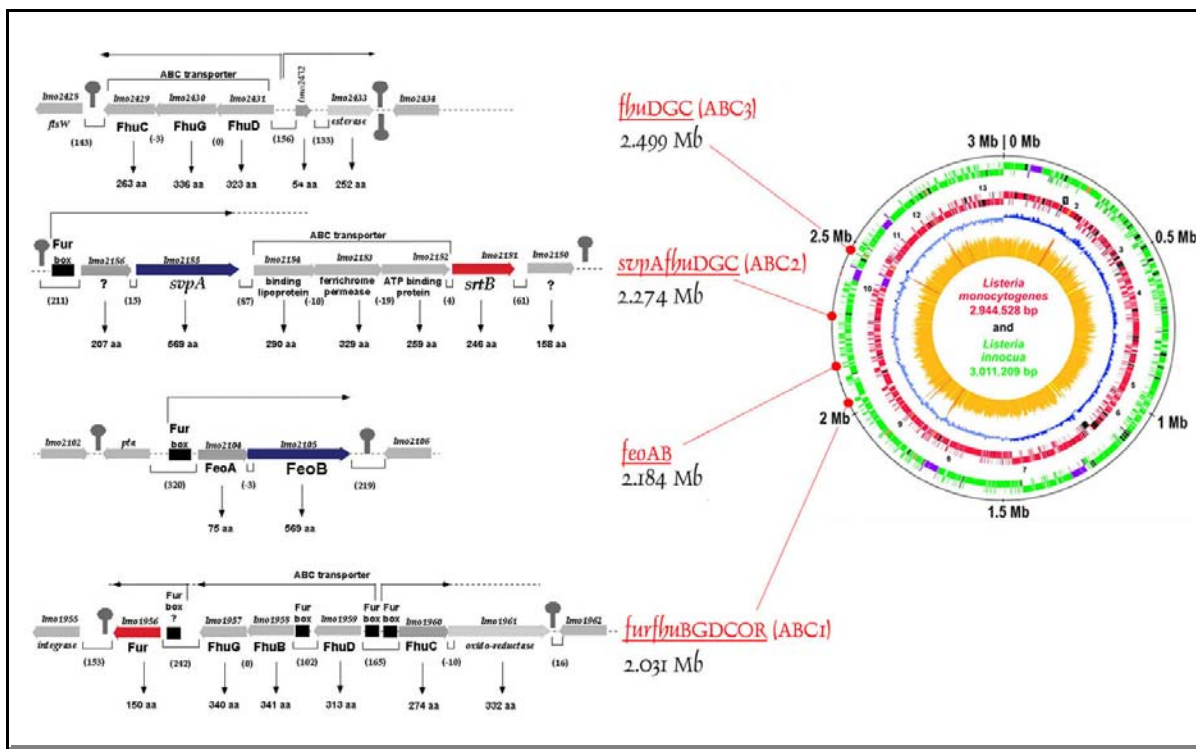


Figure 6. Putative iron transport loci in the genome of *L. monocytogenes*. The indicated genes are *fur* regulated and show homology to ferric siderophore transporters of the *E. coli* inner membrane.

In our laboratory, we are working on the mechanisms of iron acquisition by *Listeria*, and the relationship between those systems and virulence in a mouse model system. One of the approaches we use involves genomic and proteomics: we search for genes that are under the control of promoters potentially iron-regulated, and construct *Listeria* strains in which these genes are deleted from the chromosome. This enables us to study the effect of those genes on the growth of the strain under iron-restricted conditions. The basis of this approach is to use a

technique called allelic replacement to create deletions of target genes (Fig. 6), and then to analyze the mutant *L. monocytogenes* strains for their ability to transport iron, and their ability to cause disease in mice.

Another approach is to analyze the profile of proteins produced by the strain under iron-deficient conditions. When bacteria are deprived of iron, they turn on their cell envelope iron acquisition systems. These iron-regulated membrane proteins appear in SDS-PAGE analyses of the listerial cells. After visualization in this manner, we purify the proteins that are under iron control, micro-sequence them to determine their primary structure, and then identify the genes that encode them from genomic sequence data. In this different way we find a potentially different group of cell envelope (or secreted) proteins that may participate in iron uptake. At present we are studying four different regions of the *Listeria* chromosome, that were identified by this method, and generating deletions in them by site-directed deletion mutagenesis.

Several iron acquisition systems were described in *L. monocytogenes*, including an inducible **ferric citrate transport system** and a **cell-surface localized reductase** that recognizes naturally occurring iron-containing catecholamines and siderophores. *L. monocytogenes* was proposed to use catecholamines as siderophore-like compounds to bind iron in the blood, and then reduce the ferric-catecholamine complexes, releasing ferrous iron intracellularly. However, no specific surface receptors for iron or iron-containing siderophores are known to exist in *L. monocytogenes*. Rather, the cell surface reductase was proposed to broadly recognize the iron-centers of different ferric complexes and reduce them. A third

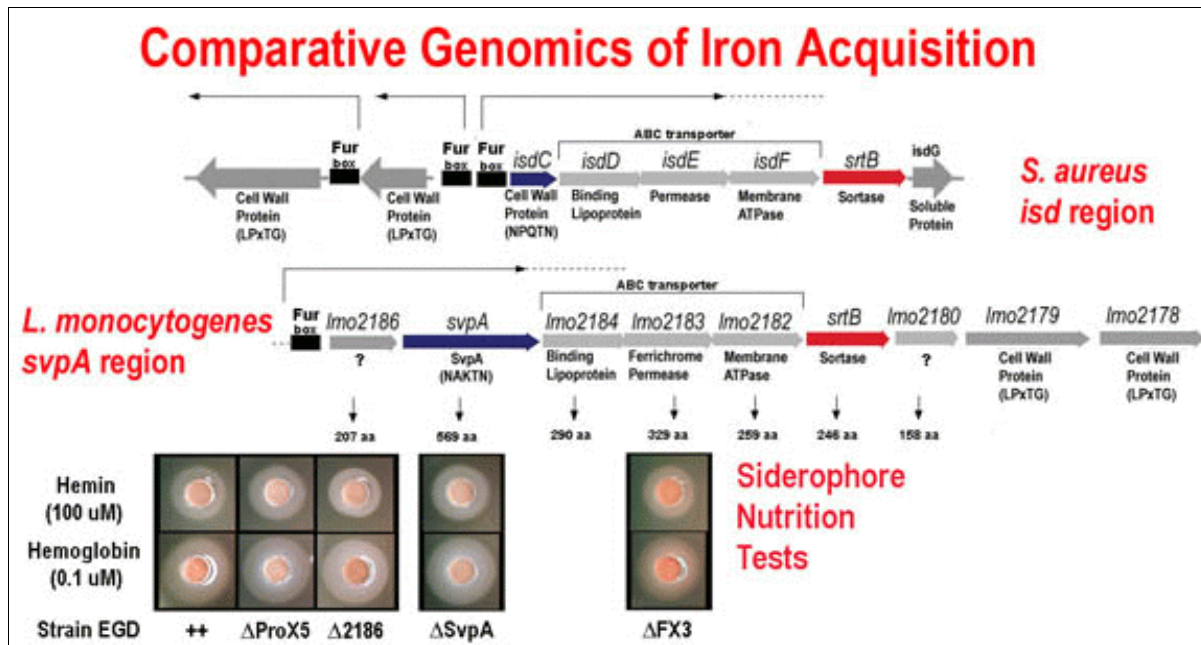


Figure 7. Comparative genomics of iron uptake. Our recent experiments characterized and compared, using genomic data, the chromosomal systems for iron uptake from hemin/hemoglobin by the proteins encoded by the *isd* operon of *Staphylococcus aureus* and the *svpA-srtB* locus of *L. monocytogenes*. Although they are genetically very similar, *L. monocytogenes* apparently does not acquire iron from hemin/hemoglobin with the *svpA-srtB* system (10).

mechanism of iron acquisition may involve a bacterial cell surface-located **transferrin-binding protein**, but the existence of this system has not yet been experimentally demonstrated. *Listeria* does acquire iron from transferrin by an as yet undetermined process, but no loci with homology to transferrin receptors were found in the *Listeria* genome, perhaps because of structural nuances in the cell envelope proteins of Gram-positive bacteria. In infected cells *L. monocytogenes* may use eukaryotic iron-containing proteins as a source of the metal, but the genes for these proposed iron transporters are not known. We devised a test, called the “*siderophore nutrition assay*,” that shows the ability of *L. monocytogenes* to use various different sources of iron (Fig. 7). The results of such assays show that the bacterium can acquire iron from a variety of ferric siderophores and eukaryotic iron binding proteins, and we are currently working to determine the connection between these abilities and the pathogenesis of *Listeria* in humans and animals.

Regulation. We are also interested in the regulation of iron acquisition in Gram positive bacteria.

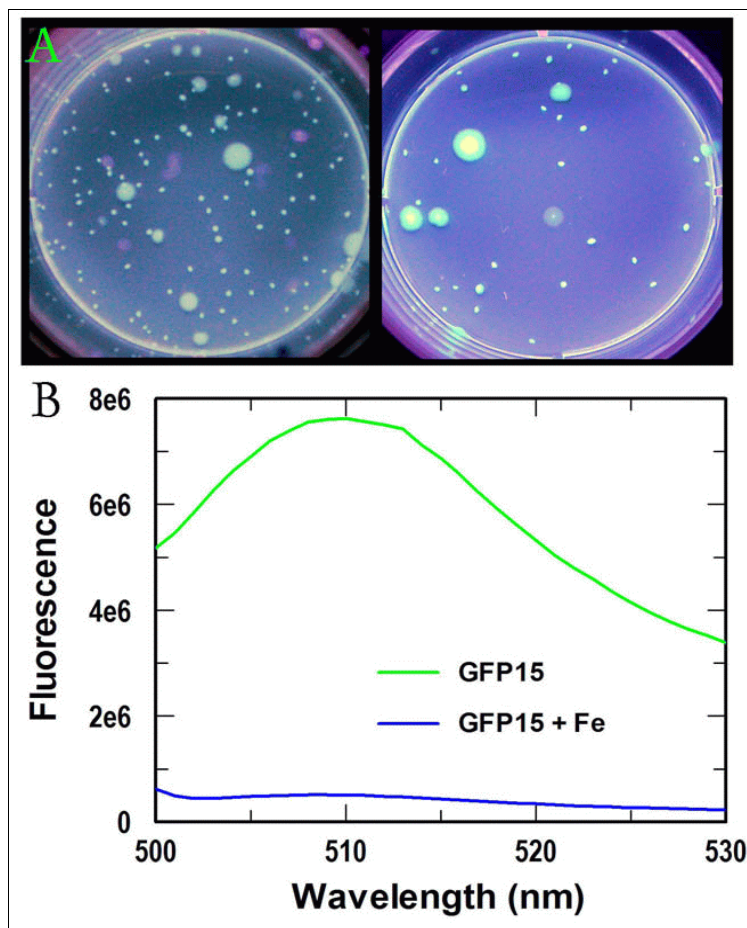


Figure 8. Expression of GFP under control of the *svpA-srtB* promoter. When grown on iron deficient plates (A) or liquid media (B), cells harboring pGFP15 became highly fluorescent, demonstrating the iron-regulation of this locus.

We are also interested in the regulation of iron acquisition in Gram-positive bacteria. Fur (Ferric uptake regulator) is the cytoplasmic regulator of many iron transport reactions in Gram-negative bacteria, and a homolog exists in Gram-positive bacteria. However, our experiments indicate that other regulatory systems also operate in *Listeria*, and we are attempting to define them. In recent work, we fused the structural gene for the green fluorescent protein (GFP) to potential iron transport genes in the *L. monocytogenes* chromosome to demonstrate their regulation by iron availability (Fig. 8; (10)).

Significance. This research project will systematically identify and characterize the iron uptake pathways of one Gram-positive organism, *Listeria*. The experiments will involve extensive genomic and proteomic studies of this pathogenic bacterium, followed by cloning of target genes of interest, site-directed mutagenesis to generate deletions *in vitro*, allelic replacement by double recombinations *in vivo* (Fig. 9). After these genetic constructions, we use analyses of

membrane transport by *Listeria* to identify its iron uptake pathways, and determinations of the

relationship between iron acquisition and virulence, using the mouse model system.
 Its intracellular route of infectivity and ability to cross barriers within the body

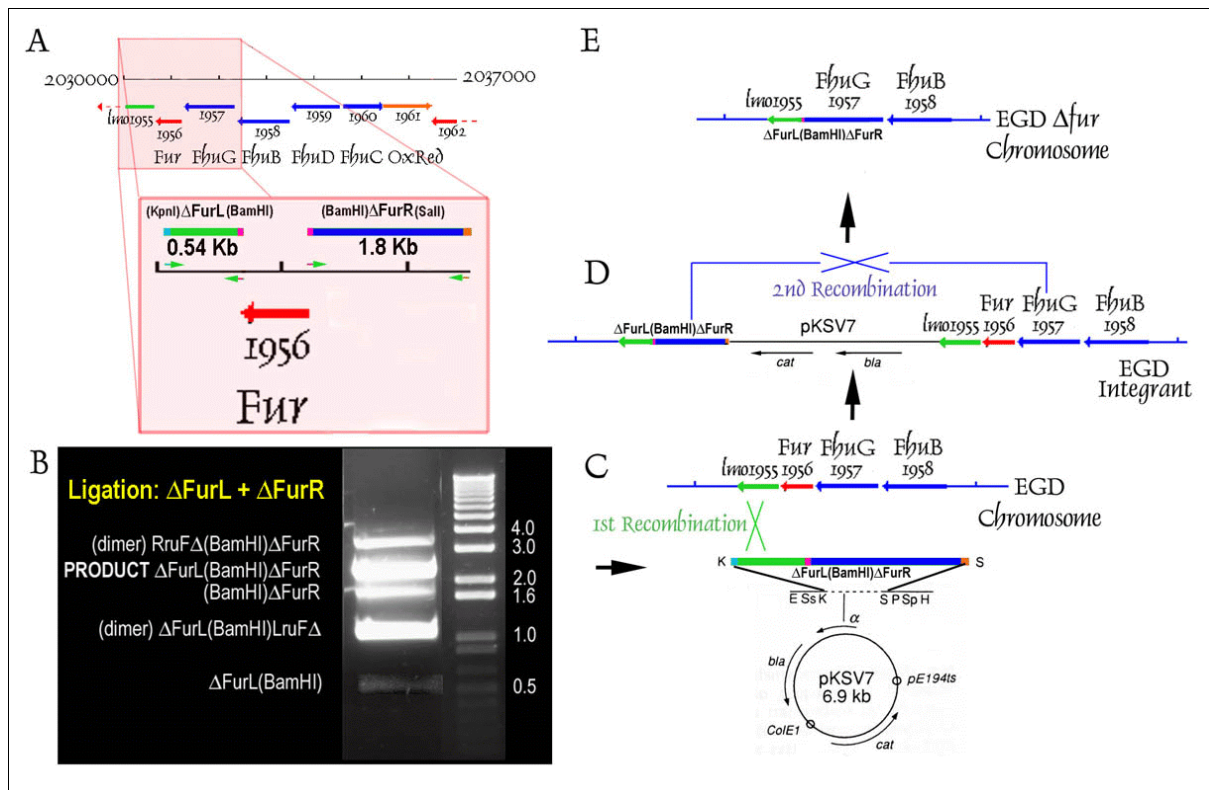


Figure 9. Allelic replacement in *L. monocytogenes*. The figure depicts the method of allelic exchange that we use for construction of site-directed deletions (10).

distinguish *L. monocytogenes* as a pathogen; the mechanisms of iron acquisition in these microenvironments are obscure. In various European nations human outbreaks of listeriosis strongly link to raw and unpasteurized cheeses, milks, ice creams, and raw meats or fish. 2% of raw milk worldwide and 16% of dairy cows are infected. In the United States, *Listeria* contaminates poultry, and in particular, processed meats. Besides its often tragic consequences in human infections (the second major bacterial cause of food-borne death after *Salmonella*), *Listeria* has superseded *E. coli* and *Salmonella* as the most common cause of food recalls in the US.

Although the understanding of Gram-negative membrane transport systems dramatically progressed in the past decade, knowledge of Gram-positive uptake mechanisms is much less advanced. The general pathways of solute uptake through the Gram-positive cell envelope are largely undefined, and even less is known about the mechanism by which iron-containing molecules, including both small ferric siderophore complexes and larger iron-binding eukaryotic proteins, pass through the multilamellar peptidoglycan layer. Most importantly, the biochemical characterization of cell envelope iron transport systems in a prototypic Gram-positive organism will provide a basis to compare and contrast their efficiencies, specificities and mechanisms with the equivalent systems of Gram-negative bacteria.

References

1. **Annamalai, R., B. Jin, Z. Cao, S. M. Newton, and P. E. Klebba** 2004. Recognition of ferric catecholates by FepA J Bacteriol. **186**:3578-89.
2. **Cao, Z., Z. Qi, C. Sprencel, S. M. Newton, and P. E. Klebba** 2000. Aromatic components of two ferric enterobactin binding sites in escherichia coli fepA Mol Microbiol. **37**:1306-17.
3. **Cao, Z., P. Warfel, S. M. Newton, and P. E. Klebba** 2003. Spectroscopic observations of ferric enterobactin transport J Biol Chem. **278**:1022-8.
4. **Jiang, X., M. A. Payne, Z. Cao, S. B. Foster, J. B. Feix, S. M. Newton, and P. E. Klebba** 1997. Ligand-specific opening of a gated-porin channel in the outer membrane of living bacteria Science. **276**:1261-4.
5. **Jin, B., S.M. C. Newton, A. Charbit and P.E. Klebba**. In preparation. Iron acquisition systems of Listeria.
6. **Klebba, P. E.** 2003. Three paradoxes of ferric enterobactin uptake Frontiers in Bioscience. **8**:1422-1436.
7. **Klebba, P. E.** 2004. Transport Biochemistry of FepA. ASM Press.
8. **Newton, S. M., J. S. Allen, Z. Cao, Z. Qi, X. Jiang, C. Sprencel, J. D. Igo, S. B. Foster, M. A. Payne, and P. E. Klebba** 1997. Double mutagenesis of a positive charge cluster in the ligand-binding site of the ferric enterobactin receptor, FepA Proc Natl Acad Sci U S A. **94**:4560-5.
9. **Newton, S. M., J. D. Igo, D. C. Scott, and P. E. Klebba** 1999. Effect of loop deletions on the binding and transport of ferric enterobactin by FepA Mol Microbiol. **32**:1153-1165.
10. **Newton, S. M., P. E. Klebba, C. Raynaud, Y. Shao, X. Jiang, I. Dubail, C. Archer, C. Frehel, and A. Charbit** 2005. The svpA-srtB locus of Listeria monocytogenes: Fur-mediated iron regulation and effect on virulence Mol Microbiol. **55**:927-940.
11. **Payne, M. A., J. D. Igo, Z. Cao, S. B. Foster, S. M. Newton, and P. E. Klebba** 1997. Biphasic binding kinetics between FepA and its ligands J Biol Chem. **272**:21950-5.
12. **Scott, D. C., Z. Cao, Z. Qi, M. Bauler, J. D. Igo, S. M. Newton, and P. E. Klebba** 2001. Exchangeability of N termini in the ligand-gated porins of Escherichia coli J Biol Chem. **276**:13025-33.
13. **Scott, D. C., S. M. Newton, and P. E. Klebba** 2002. Surface loop motion in FepA J Bacteriol. **184**:4906-11.