UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

GENETIC AND BIOCHEMICAL CHARACTERIZATION OF HYDROXAMATE TRANSPORTERS IN LISTERIA MONOCYTOGENES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF

DOCTOR OF PHILOSOPHY

BY

YI SHAO
Norman, Oklahoma
2007

UMI Number: 3291243

UMI Microform 3291243

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

GENETIC AND BIOCHEMICAL CHARACTERIZATION OF HYDROXAMATE TRANSPORTERS IN LISTERIA MONOCYTOGENES

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

BY

Phillip E. Klebba

Ann West

Helen Zgurskaya

Chuanbin Mao

Roger Harrison

This work is dedicated to my late parents.

May they be proud of me in heaven!

Acknowledgements

Firstly, I would like to thank Dr. Klebba, my major professor. It has been my privilege to work with him. His guidance, knowledge, support, and ideas have helped to finish this work. His passion on bacterial iron transport biochemistry gave me great inspiration to build my career goal. His strictness on research trained me how to be an independent thinker and a professional. I would also like to take this opportunity to thank Dr. Salete Newton, who has been giving me tremendous source of suggestions, technique supports, training and discussions. Her kindness has made us feel the lab is our 2nd home.

I appreciate the direction and guidance given me by the members of my committee throughout my graduate studies.

I also would like to extend my thanks to all the previous and current members in Dr. Klebba's lab, who helped me a lot and whom I have always had great fun to work with and always enjoyed keen debates on research or any other science issues. They are, Daniel Schuerch, Bo Jin, Rajasekran Annamalai, Wallace Kaserer, Xiaoxu Jiang, Li Ma, Qiaobin Xiao and Marj Montague.

Lastly, my gratitude also goes out to Jason and Luke, my best friends, for taking a walk with me through the hardest time of my graduate study. Thanks you for making my life worthwhile everyday.

TABLE OF CONTENT

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Listeria monocytogenes is a Gram-positive bacterial pathogen that can cause severe opportunistic infection in humans and animals, and iron is a virulence determinant in this pathogen. By sequence homologies, the genome of *Listeria monocytogenes* shows at least 4 potential cell envelope iron uptake systems: at 2.031 Mb (the *fur* region), 2.184 Mb (the *feo* region), 2.27 Mb (the *srtB* region) and 2.499 MB (which we later designated as the *hupDGC* region). Herein we biochemically determined the relationship of those systems to the mechanisms of listerial iron acquisition. We created systematic chromosomal deletions of genes in each of those loci. Mutations in the *fur* and *hupDGC* regions showed defects in the uptake of ferric hydroxamates and hemin/hemoglobin, respectively. The other locus *srtB*, which showed greatest homology to the *isd* locus in *S. aureus*, didn't show any phenotype in terms of iron uptake. In the *fur* locus, deletion of *fhuD(lmo1959)* or *fhuC(lmo1960),* which encodes a putative iron-binding lipoprotein and a membrane ATPase of an ABC transporter, severely impaired the uptake of ferrichrome, ferrichrome A and ferrioxamine B, but they didn't show any attenuation of virulence in mouse model. On the other hand, elimination of *hupC (lmo2429)* created defects in hemin/hemoglobin uptake and reduced infectious virulence in the mouse model system. I further characterized the function of fhuD by cloning the gene in an expression vector and purifying the expressed protein. The *fhuD* gene encoded a lipoprotein that was important for the utilization of iron (III)-hydroxamates by *Listeria monocytogenes*.

Full length FhuD (no signal peptide cleavage) showed low expression in *E. coli* but when the signal peptide was deleted by genetic engineering, the resultant mature FhuD was overexpressed in the cytoplasm. I purified the protein and studied the function of the FhuD protein in greater detail, demonstrating that it binds several different hydroxamates siderophores (with or without iron chelated) with different specificity and affinities, but it did not bind to non-hydroxamate siderophores. Those intrinsic fluorescence measurements reveals the K_Ds for iron(III)-ferrichrome, iron(III)- desferrioxamine B, iron(III)-ferrichrome A and iron(III)-aerobactin as 306nM, 123 nM, 451 nM and 231 nM, respectively. My data demonstrated that listerial FhuD is specific for ferrioxamine B, but it can recognize and bind other hydroxamate siderophores with less affinity. It absorbed apo-ferrichrome but not to apo-ferrichrome A. To our further surprise, FhuD absorbed to apo-ferrioxamine B with greater affinity than for any other compound tested. The possibility of redundancy in S. aureus and b. subtilis regarding hydroxamate siderophore transport systems but in *L. monocytogenes* the FhuGBCD permease (lmo1957/1958/1959/1960) that FhuD (lmo1959) is the primary transporter of hydroxamate siderophores. Another, secondary hydroxamate siderophore transport system may exist in *L. monocytogenes* or another binding lipoprotein may share the same traffic ABC transporter, creating slightly different selectivity, specificity, affinity and transport velocity.

Chapter 1

INTRODUCTION

1. 1 Cell Envelopes of Bacteria

1.2 Pathogenicity of *Listeria monocytogenes*

1.2.1 *Listeria monocytogenes,* a ubiquitous pathogen.
1.2.2 Life cycle of listerial intra-cellular parasitism
1.2.3 Surface virulence factors and sorting systems of *Listeria*

1.3 The Role of Iron in Bacterial Infection

1.4 General Mechanisms of Bacterial Iron Uptake

1.5 Fhu vs. Hn/Hb uptake system

1.5.1 Overall description
1.5.2 FhuD Iron-uptake System
1.5.3 Isd Iron Uptake System

1.6 Bacterial Lipoprotein and Importance of Binding Lipoprotein

1.6.1 Lipid Modification of Protein
1.6.2 Structure, Formation and Localization of Bacterial Lipoprotein
1.6.3 Binding lipoprotein

1.7 Siderophores

1.8 Fur----Iron-mediated Regulation

1.9 Genomic Analysis of *Listeria*

1.10 Goals and significance of research on iron acquisition in *L. monocytogenes*

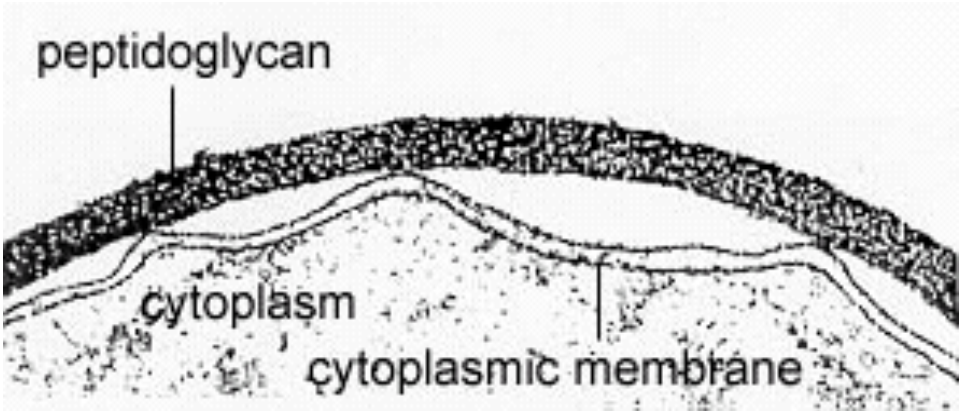
1. 1 Cell Envelopes of Bacteria

Bacteria can be categorized into two classes: Gram-positive bacteria and Gram-negative bacteria. This is because they differ in cell envelopes and therefore show different colors in Gram staining. Most Gram-negative bacteria have a trilaminar cell envelope, composed of an inner membrane (IM), an outer membrane (OM) and in between an aqueous space called periplasm (PP). In the periplasmic space, a cell wall, which is an assembly of a few layers of peptidoglycan (PG), is closely attached to the outer membrane. Gram-positive bacteria, on the other hand, only have a cytoplasmic membrane (CM), surrounded by a much thicker cell wall, up to 50-100 angstroms, which is also composed of PG (Dmitriev, Ehlers et al. 1999). (**Fig. 1.1**)

In Gram-negative bacteria, the outer membrane usually functions as a permeability barrier to toxic molecules but meanwhile it may also block the entrance of nutrients and the exit of wastes. To solve those problems, bacteria develop sophisticated transport systems that are associated on the cell envelopes to help import what they need or export virulence factors during their invasion of infection if a bacterium is a pathogen. In Gram-positive bacteria, the extremely thick cell wall protects the cytoplasmic membrane and is the first contact point between the microbes and their host environment. So the cell envelope proteins, either peptidoglycan-associated, membrane-associated or trans-membrane, play a critical role in transporting necessary substances from host or aqueous environment to inside of the

cells, as well as in the infectious process between the pathogenic organism and the host (Navarre and Schneewind 1999).

Gram-positive



Gram-negative

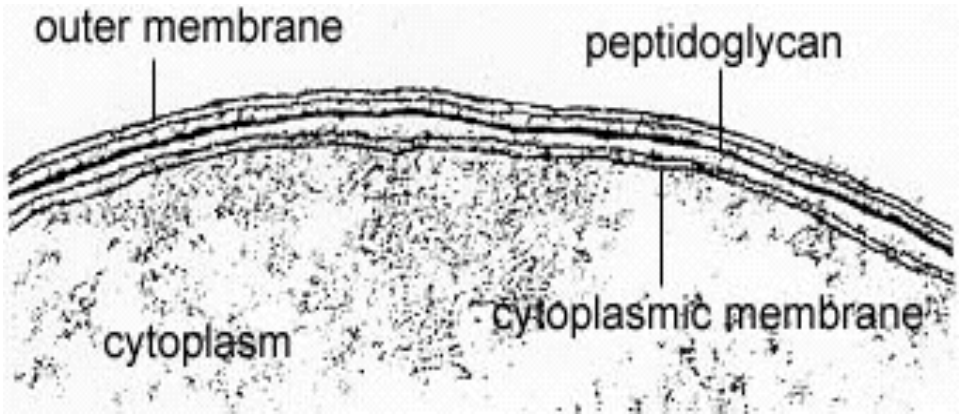


Fig. 1.1 Cell envelopes of Gram-positive and Gram-negative bacteria

Taken from http://www.cat.cc.md.us/courses/bio141/lecguide/unit1/prostruct/gncw.html

1.2 Pathogenicity of *Listeria monocytogenes*

1.2.1 *Listeria monocytogenes*, a ubiquitous pathogen.

Listeria monocytogenes is a ubiquitous, rapidly growing, Gram-positive bacterium of very low G+C content (39%), closely related to *Bacillus, Staphyloccus*, and *Streptoccocus* species. Because *Listeria* has a very broad ecological niche and host range, it is widely distributed throughout the environment, inhabiting soil, decaying vegetable matter, sewage, water, animal feed, fresh and frozen poultry, processed meats, raw milk, cheese, and humans. But primary habitats are considered to be soil and decaying vegetable matter where it lives saprophytically (Watkins and Sleath 1981). *Listeria* also can survive in many extreme conditions such as high salt concentrations, high pH, and high temperature. Different from most human pathogens, it can multiply at refrigeration temperatures (Lammerding and Doyle 1989). Since its discovery in the early 20's, *Listeria* was not thought to be an important human pathogen until in the mid 80's when there was a sharp increase in human outbreak in both Europe and North America, which made *L. monocytogenes* one of most important food-borne human pathogens (Seeliger 1988). The general mild infection of *L. monocytogenes* causes symptoms which are more like flu-type illness and digestive infections such as nausea, vomiting and diarrhea. The severe infection of *L. monocytogenes*, named listeriosis, is one of the most deadly bacterial infections, with a mean mortality rate of 20% to 39% in humans despite antibiotic treatment. This may be due to the fact that the organism is able to survive in macrophages, to invade and

replicate in non phagocytic cells and to breach the intestinal- , the blood-brain- and the placental barriers (Lecuit, Vandormael-Pournin et al. 2001). The groups at high risk of Listeriosis are immunocomprised individuals, pregnant women, newborns, and elders (McLauchlin 1990; McLauchlin 1990). But even with all of the problems caused by *L. monocytogenes* to humans, it is now being researched as a cancer vaccine because of its ability to induce potent innate and adaptive immunity (Brockstedt, Giedlin et al. 2004).

1.2.2 Life cycle of listerial intra-cellular parasitism.

Many bacterial pathogens have a striking characteristic ability to attach to many types of mammalian cells, including non-professional phagocytes during infection. The life cycle of pathogenic *Listeria,* consists of three stages: adherence and entrance into the cell, escape from a vacuole, and cell to cell spread (Vazquez-Boland, Kuhn et al. 2001). (**See Fig. 1.2**) After the bacterium is taken in either by phagocytosis or by active invasion with the aid InlA and InlB, two of major surface virulence factors produced by *L. monocytogenes* (Gaillard, Jaubert et al. 1996), there are two fates awaiting internalized bacterium: it is either killed or escapes from a host vacuole into the cytosol and begins to grow rapidly. The stage of listerial escape from the vacuole largely depends on the virulence factors listeriolysin O (LLO) and phospholipases C (PLCs)(Cossart and Lecuit 1998). LLO inserted into the phagosomal membrane forms a pore, which acts as a channel for the passage of

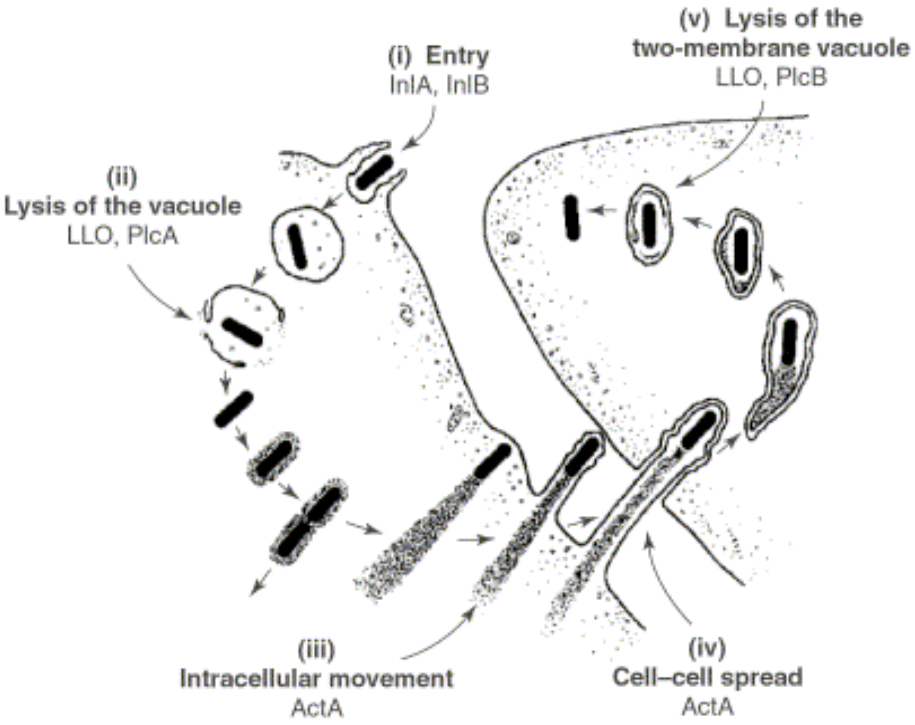


Fig. 1.2 Infectious Life Cycle of *L. monocytogenes*. The cartoon was adapted from (Tilney and Portnoy 1989)

(i) *L. monocytogenes* induces its entry into a non-professional phagocyte. A bacterium is internalized into a vacuole (also known as a phagosome) by InlA and InlB. (ii) The membrane of the vacuole is disrupted by the secretion phospholipase, PlcA and the pore-forming toxin listeriolysin O. (iii) Bacterium is released into the cytoplasm, where it replicates rapidly and starts to polymerize actin with the help by ActA, as observed by the presence of the characteristic actin tails. (iv) Actin polymerization allows bacteria to pass into a neighbouring cell by forming protrusions in the plasma membrane. (v) On entry into the neighbouring cell, bacteria are present in a double-membraned vacuole, from which a new cycle of infection can proceed.

proteins from the vacuole. Then the bacterial PLCs pass through this channel and act on the vacuole, leading to its dissolution. Shortly after entry into the mammalian cytosol, *L. monocytogenes* induces the polymerization of host actin filaments with the aid of another important surface virulence factor, ActA, and uses the force generated by actin polymerization to move to the surface of the cell, where a bacterium containing protrusion forms and is taken up by a secondary cell. The bacterium then escapes from the double membrane vacuole in the secondary cell and enters the cytosol again (Cameron, Giardini et al. 2000; Portnoy, Auerbuch et al. 2002).

1.2.3 Surface virulence factors and sorting system of *Listeria*

The cell wall of *Listeria monocytogenes* is formed by a multilaminar peptidoglycan that confers rigidity and acts as the first point of contact between the microorganism and the environment (Navarre and Schneewind 1999). Therefore, surface proteins of many Gram-positive pathogens play various key roles in pathogenicity and they are of great interest in terms of understanding the infection process and have potential as targets for therapy (**Fig. 1.4**). During the life cycle of *L. monocytogenes*, the first stage involves invasion and internalization of *L. monocytogenes* into the host cell. This is often mediated by one or more bacterial surface proteins, collectively named internalins, among which internalin A, B and C are best characterized (Braun and Cossart 2000). Internalin A promotes binding and internalization by E-cadherin (Mengaud, Ohayon et al. 1996) whereas Internalin B binds to the Met receptor tyrosine kinase (Cossart 2001)and mediates synergistically

with Internalin A to internalize *L. monocytogenes* into the host cells (Dramsi, Dehoux et al. 1997). Internalin A is covalently anchored to the cell wall by a sorting system that is widely found in Gram-positive bacteria (Bierne, Mazmanian et al. 2002). The mechanism of the sorting system (**Fig. 1.3**) lies in that the transaminase Sortase A can recognize a specific motif (LPxTG) on the C-terminal region of InlA and form an amide bond between C-terminal threonine and the side chain amino group of m-diaminopmelic acid within cell wall peptides. This mechanism, first found in *Staphylococcus aureus* (Schneewind, Fowler et al. 1995), is widespread in Gram-positive bacteria (Navarre and Schneewind 1999). The relevance of the sorting mechanism to bacterial virulence is evident from the attenuation of virulence as found among many *srtA* mutants in *S. aureus* and *Streptococcus pyogenes* (Mazmanian, Liu et al. 2000; Lee and Boran 2003). In *L. monocytogenes*, virulence of srtA mutant was also attenuated, confirming the importance of surface proteins in the ability to cause disease (Bierne, Mazmanian et al. 2002). Internalin B, however, does not have a hydrophobic C-terminal region but has a third region of repeats. It associates with the cell wall non-covalently by a different mechanism involving tandem repeats of 80 amino acids. A large amount of InlB still can be found in the supernatant of *Listeria* cell cultures. InlC, which also has an LPxTG sorting motif, is believed to be anchored to the cell wall by sortaseA, but it may play a role in a late stage of infection rather than in the initial uptake of *L. monocytogenes* by the mammalian cells (Dramsi, Dehoux et al. 1997).

A second sorting mechanism exists in *S. aureus, S. pyogenes* and *L. monocytogenes* that requires sortase B (Barnett and Scott 2002; Mazmanian, Ton-That et al. 2002; Bierne, Garandeau et al. 2004). In *S. aureus*, Sortase B anchors a surface protein involved in iron uptake, IsdC, through an anchoring motif NPQTN. In *L. monocytogenes,* gene srtB is found in svpA-srtB locus, in which SvpA was initially named by its function as a surface virulence protein A. In this locus, genes svpA (lmo2185) and lmo2186 share a conserved motif NxxTN that might be recognized by SrtB and cleaved from its C-terminal threonine and anchored to the cell wall. Only about 10% of the total SvpA is attached to PG, whereas 90% is released to the supernatant (Newton, Klebba et al. 2005). The svpA-srtB locus will be discussed in detail in a later chapter.

ActA is another important surface virulence factor during the pathogenic life cycle of *L. monocytogenes*. It is a transmembrane protein whose C-terminus is anchored to the membrane (Cossart and Jonquieres 2000). ActA functions in actin polymerization and propels one end of a listerial cell to move inside the host cell to the other. Its hydrophobic stretch of about 20 amino acids followed by positively charged amino acids at C terminus acts as a stop-transfer signal and helps to anchor on the cytoplasmic membrane (Kocks, Gouin et al. 1992).

Furthermore, membrane proteins can be lipoproteins and they hang around the cell envelop surface by the lipid moiety. Cleavage of a characteristic signal peptide generates an N-terminal cysteinyl residue that becomes lipidated. The lipid moiety

helps the protein tether to the bacterial membrane (Navarre, Daefler et al. 1996). FhuD (lmo1959) and HupD (lmo2430) are good examples of those lipoproteins whose preproteins have a conserved "LTAC" motif in the signal peptide region that can be recognized by signal peptidase II and this cysteine becomes glyceride and serves as the attachment site for two ester-linked fatty acids and one amide-linked fatty acid (von Heijne 1989). Those two surface lipoproteins also may serve as binding proteins to bind their specific iron sources and transport them through their specific ABC-type transport systems. Their iron acquisition system may also be involved in listerial virulence pathways because in *Hup* locus, the deletion of gene *hupC* in this locus attenuated the virulence of listerial EGD-e wild type by an increase of 50 fold in LD_{50} (Jin, Newton et al. 2006).

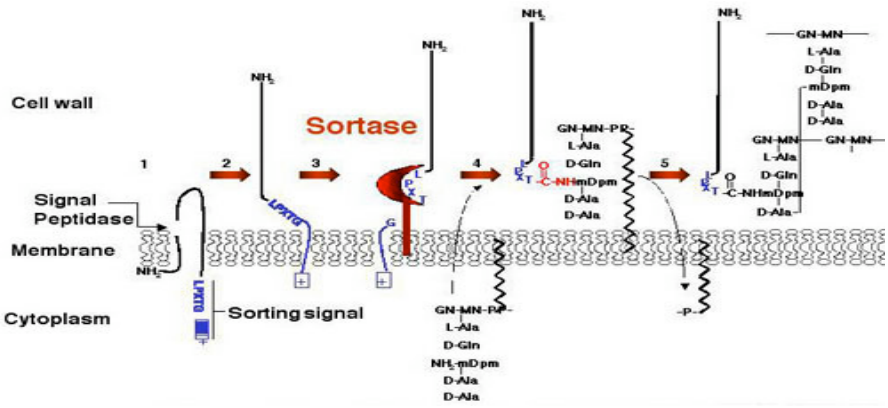


Fig. 1.3 Sorting Mechanisms of *L. monocytogenes.* Adapted from (Mazmanian, Ton-That et al. 2001). Surface proteins harboring a C-terminal sorting signal with an LPxTG motif are covalently linked to the cell wall peptidoglycan by a transamidase named sortase. Two genes encoding putative sortases, termed srtA and srtB, were identified in the listerial genome.

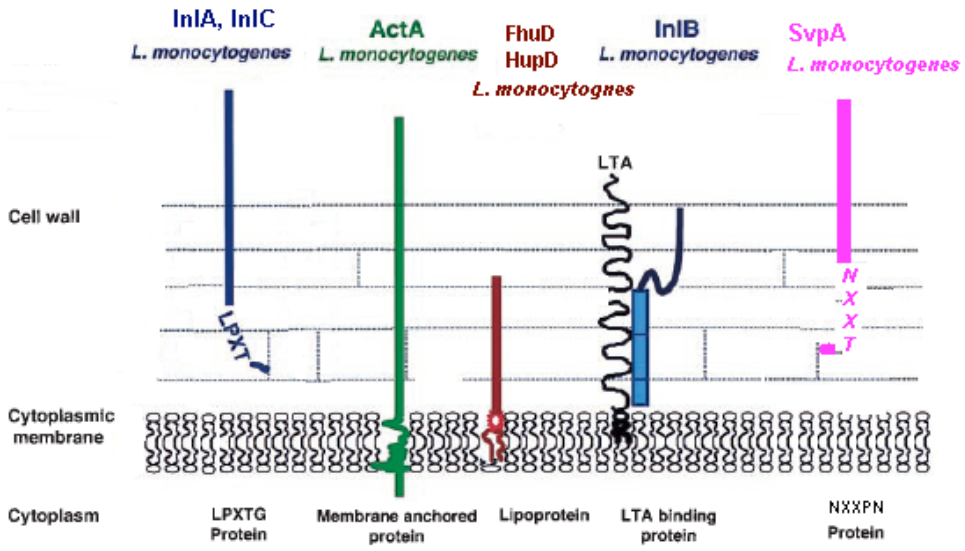


Fig. 1.4 Major Surface Virulence Proteins in *L. monocytogenes.* Adapted from (Cossart and Jonquieres 2000).

L. monocytogenes expresses surface proteins that have critical roles in host-bacterium interactions. Surface virulence proteins InlA, InlC are anchored to cell wall covalently through LPxTG motif by Sortase A (Braun and Cossart 2000); InlB associates to cell wall non-covalently via LTA (Bierne and Cossart 2002); ActA is transmembrane protein whose C-terminus is anchored to the membrane (Cossart and Jonquieres 2000); SvpA, which was initially named by its function as a surface virulence protein A (Borezee, Pellegrini et al. 2001), is anchored to the cell wall through NAKTN motif by Sortase B and later found not involved in virulence (Bierne, Garandeau et al. 2004; Newton, Klebba et al. 2005); FhuD and HupD are lipoproteins whose N-termini are anchored to the membrane after cleavage of signal peptide and HupD is found involved in virulence by attenuation of LD_{50} in mouse model (Jin, Newton et al. 2006).

1.3 The Role of Iron in Bacterial Infection---a nutrient barrier for most bacteria and a virulence determinant for most bacterial pathogens

The redox potential of Fe^{3+}/Fe^{2+} spans from +300 mV to -500 mV in nature. This renders iron an exceptional ability to participate in a relatively wide range of electron-transfer reactions in a biological environment composed of ligands and proteins (Guerinot 1994). For example, many proteins including cytochromes, respiratory proteins and tricarboxylic acid metalloenzymes, use iron as a cofactor. Therefore, iron plays a central role in many redox enzymes that function in electron-transport chains of intermediary metabolism. Also most microorganisms that have been studied are known to utilize iron, only with a few exceptions such as that *lactobacilli* utilizes manganese and cobalt as biocatalysts in place of iron and pathogenic *Borrelia burgdorferi* avoids the need for the iron by eliminating genes encoding most iron-dependent proteins from the genome and by using cations other than iron as cofactors for the remaining metalloproteins, (Imbert and Blondeau 1998; Brown and Holden 2002). In summary, iron is an essential element for the growth of most bacteria. It is estimated that 10^5 free irons are required per bacterial cell and it is tested that at least a Fe^{3+} concentration of 0.4 ~ 4 uM is needed to support bacterial growth (Braun 2001). Although iron is abundant in nature, in most environments, iron uptake is limited not by its presence but by the fact that it is insoluble and inaccessible. In aerobic environments, iron exists primarily in the oxidized ferric form Fe(III) and its concentration at pH7 is extremely low (10^{-18} M), due to the fact that free ferric

irons in aqueous environments rapidly precipitate as hydroxide polymers (Braun and Killmann 1999; Clarke, Tari et al. 2001). Even though in anaerobic conditions, ferrous Fe(II) is soluble and can be diffused through the membranes and used directly by bacteria, its concentration also has to be up to micromolar level and the Fe(II) often activates the Fenton reaction [$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH$], leading to the partial reduction of oxygen into hydroxyl radicals that are harmful for most macromolecules (Klebba, McIntosh et al. 1982; Wandersman and Delepelaire 2004). Also in physiological conditions, in order to combat against microbial infection or fight against generation of harmful free radicals caused by iron, animals strictly limit the availability of free iron in their blood or tissue by carrying high-affinity iron-binding proteins, such as transferrin in the blood, lactoferrin in secretory fluids and ferritin within host cells, to sequester the free iron from above (Braun and Killmann 1999). The iron homeostasis is so strictly regulated that there is barely any free iron in living organisms (Weinberg 2000). Therefore, on one hand, such limited availability of iron in the hosts provides one form of non-specific immune defense that bacterial pathogens need to overcome before they can grow and cause further infection (Rouault 2004). On the other hand, the ability to acquire iron is key determinant in establishing bacterial virulence in vivo (Raymond, Dertz et al. 2003)

1.4 General Mechanisms of Bacterial Iron Uptake

Faced with iron shortage no matter whether in environment or in the infected host, most bacteria have developed different mechanisms to acquire iron from the various sources they may encounter in their diverse habitats. Basically there are three distinctive mechanisms found among all the bacteria that have been studied;

Receptor-mediated mechanism: In this mechanism, bacteria usually have surface receptors to bind ferrated siderophophilins, which are those iron-containing eukaryotic proteins such as transferrin(Tf), lactoferrin(Lf), Ferritin(Tn) and hemoglobin(Hb), and extract heme or iron at the cell surface and transport into the cell through a sophisticated but not yet well characterized multi-component ABC transporters (Wandersman and Delepelaire 2004).

Siderophore-mediated mechanism: This mechanism is dependent on the use of either exogenous or endogenous siderophores. The siderophores are less than 1000 Da molecular weight, iron-chelating compounds with extremely high affinities to iron so that they can either scavenge free iron at very low concentrations or directly capture iron from siderophilins. Bacteria and fungi are induced to secret siderophores into extracellular media to scavenge iron from a variety of iron sources when suffering from stress of low iron concentration. More than 100 siderophores have been reported so far (Neilands 1984; Neilands 1991). In general, most of them can be classified into two major types: Catecholate and Hydroxamate, in which Ferric enterobactin porin system (FepA) and Ferric hydroxamate uptake System (FhuA) represent good

examples of those two types of siderophore iron-uptake. We will discuss Fhu uptake system in detail in the later section of this chapter.

Feo uptake mechanism: The Feo system, which has a surface ferric reductase in *Listeria monocytogenes* and *Salmonella enterica,* is essential for iron acquisition of ferrous iron (Boyer, Bergevin et al. 2002). It involves bacterial utilization of insoluble ferric iron (Fe^{3+}) by reducing it to ferrous iron (Fe^{2+}) on the cell envelope surface and diffusing ferrous iron into the cell (Deneer, Healey et al. 1995).

Among those three mechanisms, the first one is a quite common attribute among pathogenic bacteria. But microbial strains that use siderophilin binding often have a very narrow host range (Weinberg 1999).However, not every pathogen that uses siderophilin binding has a narrow host range. *Staphylococcus aureus* is a good example that can use different source of transferrin and be virulent for a variety of mammalian species. This might be explained by the fact that *S. aureus* were found to be able to produce 3 kinds of siderophores, Staphyloferrin A, Staphyloferrin B and aureochelin (Courcol, Trivier et al. 1997). Each of these small molecules helps bacteria withdraw iron from transferrins synthesized by a variety of host species. Besides above, erythrocyte lysis, digestion of hemoglobin, and heme assimilation are also available to strains of *S. aureus* and Schneewind's group from University of Chicago has already found an operon called Iron surface determinants (Isd) involved in binding hemoglobin and then transport heme across cell wall and membrane (Mazmanian, Skaar et al. 2003). We will discuss Isd locus in detail in the later part.

The general mechanism of active transport of free irons or iron-containing complexes through cell envelopes of both gram-positive and gram-negative bacteria is a system of multi-components. Those transport systems rely on proteins at the cell surface that bind iron or iron-containing molecules. In gram-positive bacteria, those iron sources are recognized by specific binding proteins either anchored to the inner-membrane or to the cell wall by a covalently linked lipid, and then are transported by ABC permeases. In gram-negative bacteria, when free iron or iron-containing complexes traverse against their own concentrations, they first bind to the specific outer membrane receptors with much higher affinity and specificity. Once in the periplasm, those irons or iron containing molecules are much more concentrated and they bind to the binding lipoproteins of ABC transporter similar to those of Gram-positive bacteria, but with less affinity compared to the OM receptors. Finally, they are delivered to the IM ABC type permeases. Those ABC transporters usually consist of a transmembrane permease and an ATP-binding lipoprotein. Even though these proteins share consensus sequences which identify them as belonging to a particular family, they cannot replace each other in different iron-uptake systems.

1.5 Fhu vs. Hn/Hb uptake system

1.5.1 Overall description

Fhu system, which stands for ferric hydroxamate uptake, is a typical and also well characterized siderophore-mediated iron uptake system found in both Gram-

positive and Gram-negative bacteria. Hn/Hb uptake system, on the other hand, represents a good example for the receptor-mediated mechanism as described in the earlier section. Both heme utilization and hydroxamate biosynthesis appear to contribute to *S. aureus*'s infectivity (Dale, Doherty-Kirby et al. 2004);(Skaar, Humayun et al. 2004). As for most human bacterial pathogens, heme or hemoglobin can be a good iron source. Heme itself is a Fe(III) protoporphyrin IX molecule. Heme-iron is penta-coordinated to four nitrogens in the porphyrin ring and to the imidazole of one histidine residue. The sixth coordination is either free (in methemoglobin) or bound to oxygen in oxyhemoglobin. Heme is also a hydrophobic molecule and can be complexed with many proteins, such as cytochromes, hemoglobin and haptoglobin. Hemoglobin and haptoglobin exist in the blood. The former is found within the blood cell whereas the latter is found in the serum. Heme can be released from hemoglobin after hemoglobin released from red blood cells by hemolysis. Haptoglobin also can be a iron source in a sort of similar way as hemoglobin because its function in serum is to bind hemoglobin and prevent it from releasing heme. Since many bacterial pathogens are able to cause hemolysis in blood or tissues, abundance of heme or hemoglobin would provide those hemolytic pathogens good opportunities to overcome the iron-deficient barrier and survive in the tissue or body fluid and cause further severe infections. Those hemolytic pathogens can achieve those above goals via Hn/Hb transport system which has a surface receptor to bind hemoglobin or haptoglobin on the bacterial cell envelope, pirates the

heme from hemoglobin, and internalizes heme through membrane. Those Hn/Hb transport systems have been found in many bacteria. Hn/Hb transport system was first reported in a Gram-negative bacterium, *Vibrio cholerae* (Stoebner and Payne 1988; Henderson and Payne 1993). Then more Gram-negative bacterial pathogens were found to contain this transport system, such as *Escherichia coli* O157 (Torres and Payne 1997), *Plesiomonas shigelloides* (Daskaleros, Stoebner et al. 1991), *Neisseria meningitidis* (Khun, Kirby et al. 1998), and *Yersinia pestis* (Thompson, Jones et al. 1999). While a detailed elucidation of mechamism of Hn/Hb transport system in Gram-negative bacteria has not been completed yet, discovery of such transport system in Gram-positive bacterial is booming and many progresses have been made in the last few years. A cell surface hemin receptor was first found in *S. pneumoniae* (Tai, Wang et al. 1997). But whether this receptor was able to bind hemoproteins, such as hemoglobin or haptoglobin was not elucidated and genes related to the membrane-based ABC transporter were not identified. Later, HmoTUV, which encodes Hn/Hb ABC transporter, and HmuO that encodes heme oxygenase were found in *C. diphtheriae*. This is the first report in Gram-positive bacteria that described not only how heme is internalized through this ABC type transporter but also how heme is degraded after being transported. Similar transport system was also found in *S. aureus*. This iron-regulated surface determinants (isd) system has many cell-wall-based components that facilitate iron acquisition besides a membrane-base ABC transporter. Components homologous to IsdG and IsdH were also later found in another Gram-

positive pathogen, *B. anthraci* (Skaar, Gaspar et al. 2004). But no abc type transporters have been reported in that species.

Unlike Hn/Hb uptake system, Fhu system can be set as one of prototypes to elucidate the mechanism of ferric-siderophore iron acquisition. The system was first found in *E. coli* and it consists of several components on the cell envelope. Hydroxamate siderophores are internalized across the cell envelope of *E. coli* with the aid of an outer membrane receptor (FhuA), periplasmic binding lipoprotein (FhuD), and associated ABC type transporter (FhuBC). FhuA has a single specificity to ferrichrome. However, the periplasmic binding protein FhuD is less specific. FhuD can bind several hydroxamate siderophores internalized from the other OM receptors, eg. rhodoturulate and coprogen from FhuE, ferrioxamine B from FhuF and aerobactin from LutA. Then it transports all the hydroxamate siderophores that it is able to bind through the same FhuBC membrane complex (Guerinot 1994). Therefore, FhuD catalyzes the uptake of the different hydroxamate siderophores from periplasm, through the cytoplasmic membrane, and to the cytosol. Similar mechanism and cellular organization were also found in Gram-positive bacteria. However, Gram-positive bacteria do not have outer membrane. So instead of having outer membrane receptor, FhuD, which is hydroxamate binding lipoprotein, can act as surface receptor. So instead of calling it FhuA system in Gram-negative bacteria, we called it FhuD uptake system in Gram-positive bacteria. The first FhuD hydroxamate uptake system was characterized in *B. subtilis* (Schneider and Hantke 1993). Two hydroxamate

uptake systems, FhuD and FoxD, exist in this non-pathogenic bacterial species. FhuD is a hydroxamate-binding lipoprotein that can recognize a broad range of hydroxamate substrates, including ferrichrome, ferrichrysin, ferricrocin, and coprogen while FoxD is another surface binding protein only can bind ferrioxamine B and E. But both FhuD and FoxD share the same traffic FhuCB transmembrane components to internalize all the hydroxamates into cytosol. Similar Fhu systems were also found in Group B *streptococcus and S. aureus* (Sebulsky and Heinrichs 2001; Clancy, Loar et al. 2006). The Fhu system in *S. aureus* is composed of five proteins: FhuD1, Fhu2, and FhuBCG. FhuB and FhuG are two integral membrane proteins and FhuC is an ATPase. Unlike fhu system in *E. coli*, genes of FhuD1, FhuD2 and FhuBCG are not from the same operon. FhuD1 and FhuD2 both encode hydroxamate binding lipoproteins and they can bind the same substrates tested. However, the affinity of FhuD2 for hydroxamate siderophores is much higher than that of FhuD1 and even greater than *E. coli* FhuD.

A summary of Fhu and Hn/Hb uptake systems in Gram-positive bacteria is made through Table 1.1. FhuD system and Isd Hn/Hb uptake system in *S. aureus,* as representative models for Gram-positive bacteria, will be discussed in detail in the next two sections.

Table1.1 Fhu and Hn/Hb uptake systems in Gram-positive bacteria

organism	transporter	substrate	Note	Reference
B. subtilis	FhuD	hydroxamate	Binding lipoprotein specific for hydroxamate other than ferrioxamines; iron is internalized via a common traffic fhuBCG membrane components	(Schneider and Hantke 1993)
	FoxD	ferrioxamine	Binding lipoprotein only specific for ferrioxamines; iron is internalized via the same traffic fhuBCG membrane components	(Schneider and Hantke 1993)
S. aureus	FhuD1	hydroxamate	A secondary binding lipoprotein for hydroxamates with less specificity	(Sebulsky, Speziali et al. 2004)
	FhuD2	hydroxamate	Primary hydroxamate transport	(Sebulsky, Shilton et al. 2003)
	Isd	Hn/Hb	Well characterized Hn/Hb transport system	(Mazmanian, Skaar et al. 2003)
	Hst	Hn	Putative hemin transport system by homology alignment preliminary characterization	(Skaar, Humayun et al. 2004)
Group B *streptococcus*	FhuD	hydroxamate	Hydroxamate transport system similar to FhuD2 of S. aureus	(Clancy, Loar et al. 2006)
B. anthracis	Isd	Hn/Hb	Hn/Hb transport system similar to isd found in S. aureus	(Skaar, Gaspar et al. 2004)
S. pyogenes	ShuA-C	Hn	A hemoprotein-binding iron transport system	(Bates, Montanez et al. 2003)
S. pneumoniae	Haemin receptor	Hn	A cell surface hemin binding protein, not sure if other hemoproteins can be bound and no other components have been found	(Tai, Wang et al. 1997)
C. diphtheriae	HmuOTUV	Hn/Hb	Hn/Hb transport system	(Drazek, Hammack et al. 2000)
L. monocytogenes	hydroxamate-like furfhuBGCD ABC transporter	?	Later was identified as hydroxamate transport system in our study	In this study
	svpA-srtB ABC transporter	?	Iron-regulated but its function is still unknown	In this study
	hydroxamate-like ABC transporter	?	Later was identified to be involved in Hn/Hb transport in our study	In this study

1.5.2 FhuD system

FhuD refers to a hydroxamate-binding lipoprotein to bind hydroxamate siderophores. It was first found in *E. coli* Gram positive bacteria. For Gram-positive bacteria, it was first found in *B. subtilis* (Schneider and Hantke 1993). Later, more FhuD systems were characterized in many other gram-positive bacteria such as *S. aureus, Group B streptococcus* (GBS) (Schneider and Hantke 1993; Sebulsky and Heinrichs 2001; Clancy, Loar et al. 2006). One fundamental difference between the iron uptake systems in Gram-negative bacteria versus Gram-positive bacteria is the lack of an outer membrane in the latter. In general, the OM is the home of high affinity ferric siderophores receptors whereas the periplasmic binding proteins do not necessarily posses high affinity as most OM receptors do for the substrates because substrates are relatively "concentrated" in the periplasm. However, the FhuD in Gram-positive bacteria can serve as a surface receptor. Thus, the affinity of FhuD as a cell surface receptor may be higher than the FhuD in gram-negative bacteria, close to or slightly less than the affinity of FhuA in *E. coli.* In *S. aureus*, two FhuDs were found, in which FhuD2 plays a major role in binding and transporting hydroxamate siderophores (Sebulsky and Heinrichs 2001). So far there are no good explanations for the redundancy of two hydroxamate siderophores uptake systems existing in the same strain. However, FhuD2 is not observed to undergo any significant conformational changes upon binding hydroxamate siderophores in vitro, which indirectly suggests that FhuD2 might have closer structural homology to *E. coli* FhuD (Sebulsky, Shilton

et al. 2003). Because the shallow binding pocket of crystal structure of *E. coli* FhuD is predominantly hydrophobic, suggesting binding and release of neutral charge siderophores does not need large scale opening and closing of the binding site (Clarke, Ku et al. 2000). However, there is one clear difference between FhuD2 and *E. coli* FhuD in that FhuD2 does not have an arginine residue that can be found present within the predicted binding pocket whereas Arg-84 of *E. coli* FhuD plays a key role in the interactions with hydroxamate siderophores. Furthermore, in Gram-positive bacteria, the following ABC type cytoplasmic membrane components are slightly different from those in gram-negative bacteria. Instead of having transmembrane homodimer of FhuB as permease, the cytoplasm membrane permease of FhuD in Gram-positive bacteria is composed of hetrodimer, FhuB and FhuG. (**Fig. 1.5**)

1.5.3 Isd Iron Uptake System

Isd is an iron regulated "Iron surface determinants" genomic locus found in *S. aureus* (Mazmanian, Skaar et al. 2003). It is also relatively enlightened model for receptor-mediated iron uptake system in Gram-positive bacterial since little is well-known about iron transport systems in Gram-positive bacteria. (**Fig. 1.6**) This whole operon is composed of 10 genes and their gene products are directly into different locations in the cell: IsdD, IsdE, IsdF and SrtB (membrane), IsdA, IsdB, IsdC, IsdH (cell wall), IsdG and IsdI (cytoplasm).All the cell wall proteins are anchored by SrtA except that IsdC is anchored by SrtB from the same locus(Maresso and Schneewind 2006). *S. aureus* is a major virulent pathogen causing infection within red blood cells.

Once entry into the blood circulation system, *S. aureus* starts to secrete virulence factor called "hemolysin" to disrupt the red blood cells. On one hand, bacteria suffer iron-deplete stress in the host's body fluid; on the other hand, bacteria are also faced with abundant iron source from hemoglobins (Hb) and heptoglobin (Hpt) disrupted from red blood cells. Therefore under iron-deficient stress, Fur, the global iron regulator of *S. aureus*, is removed from binding site in the promoter region of isd locus. The genes of isd locus start to express. *isd* locus is a very elegant system in utilizing Heme from Hb/Hpt. At first, cell wall anchored proteins IsdB/H act as receptor to bind either Hb or Hpt and pirate heme from those heme-containing proteins. Then heme passes through the thick cell wall by the aid of two other cell wall proteins, IsdA and IsdC. Finally Heme is internalized into the cytoplasm by a Ferrichrome-like ABC transporter IsdDEF. Once entry into the cytosol, heme is degraded and Fe(II) is released by two heme monooxygenases, IsdG/I. However, even in this model, how the heme is extracted from Hb or Hpt by IsdB/H is not understood and the binding affinity and transport parameters are still lacking.

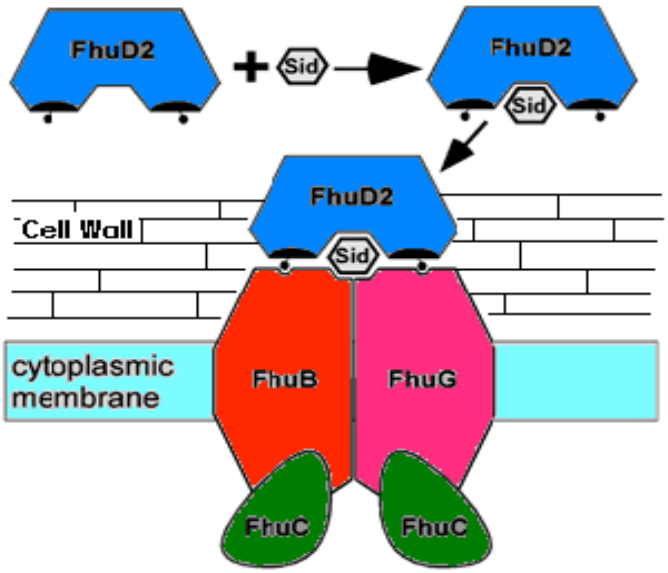


Fig. 1.5 Model for FhuD transport system in *S. aureus* and other gram-positive bacteria. Adapted from (Sebulsky, Shilton et al. 2003)

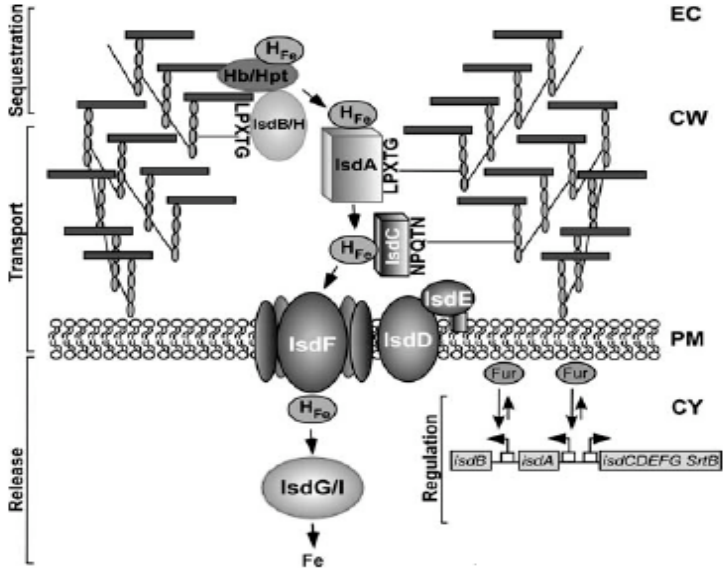


Fig..1.6 Model for Heme/hemoglobin acquisition in *S. aureus.* EC=extracellular, CW=cell wall, PM=plasma membrane, CY=cytoplasm. Adapted from (Maresso and Schneewind 2006)

1.6 Bacterial Lipoprotein and Importance of Binding Lipoprotein

1.6.1 Lipid Modification of Protein

Many intercellular and cell-surface proteins are covalently or non-covalently linked to one or more lipidic groups. For many such proteins these lipid modifications are important and sometimes critical to the protein's cellular functions. One of the more common biological roles of protein lipidation is to influence the subcellular distributions of proteins, for example, by associating and targeting a protein to a particular membrane compartment or submembrane domain (Epand 1997). Proteins with lipid modifications can be divided into two classes:

Proteolipids are a specific set of polypeptides that bind **nonconvalently** to arrays of lipid and form water-soluble complexes.

Lipoproteins are a functionally diverse class of proteins that **covalently** bind to lipid moiety at their N-termini.

1.6.2 Structure, Formation and Localization of Bacterial Lipoprotein

Covalently modification of proteins with lipids appears to ubiquitous in all living cells. It was first identified in 1969 in the outer membrane protein of *E. coli* (Braun and Rehn 1969). Then the structure (N-acyl-S-diacylglyceryl Cysteine at the N-terminal) of the lipid was elucidated by Braun in 1973 and therefore it is called Braun's lipoprotein (Hantke and Braun 1973). Subsequently more than 700 proteins with the same modification have been reported in all known bacteria (Madan Babu and Sankaran 2002)**.** However, some bacterial toxins from species such as *Bordetella*

pertussis and pathogenic *Esherichia coli* have been shown to be N-acylated on lysine residues (Stanley, Packman et al. 1994), not on the usual cysteine. For all the lipoproteins identified so far, N-Acyl Diacyl Glyceryl lipid moiety (derived from phospholipids) serves to anchor these proteins to the membrane-aqueous cell surface. Lipoproteins are synthesized as pre-prolipoproteins and mature by post-translational modifications (Chattopadhyay and Wu 1977). The post-translational modifications are directed by a consensus sequence of lipoprotein modification/processing located within the C-region of signal peptide of prolipoprotein. L-X-X-C at -3 to +1 position represents the conserved cleavage region in three fourths of all lipoprotein signal peptides in bacteria (Madan Babu and Sankaran 2002). The common pathway for the biosynthesis of bacterial lipoproteins (**See Fig.1.7**) involves the following three steps: diacylglyceryl modification, cleavage of signal peptide only by signal peptidase II, and N-acylation. Each step needs a unique enzyme (Sankaran and Wu 1994)

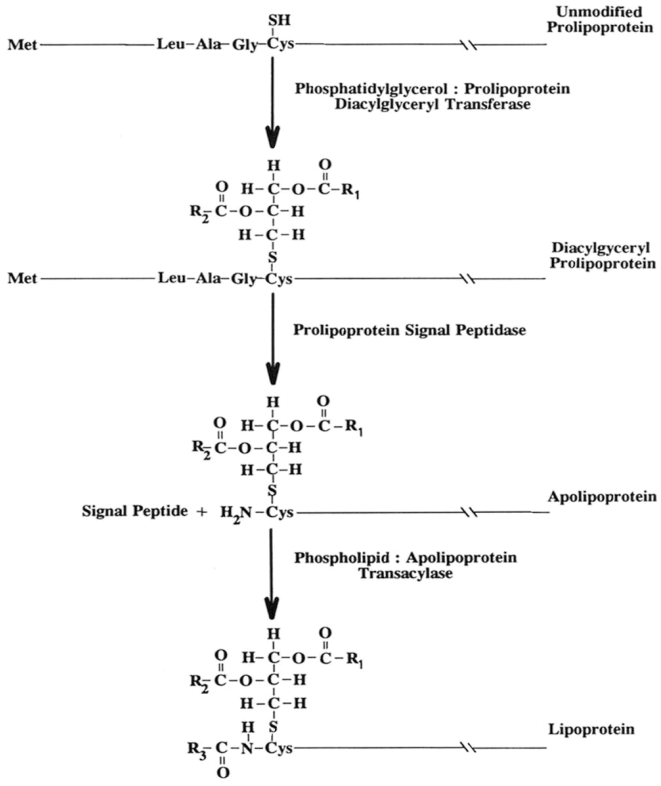


Fig. 1.7 Biosynthesis pathway of lipoprotein, adapted from (Sankaran and Wu 1994).

The lipid adaptor on the N-terminal of lipoprotein anchors the protein to the outer membrane in Gram-negative bacteria (Braun's lipoprotein) or to the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria. However, its C-terminus also can serve to anchor the protein to the cell wall of bacteria and this kind of lipoproteins is called murein lipoprotein (Hayashi and Wu 1990). To verify if it is a lipoprotein, a test of inhibition by globomycin can be applied, and the results can be obtained from the fluorograph of [^{35}S]-methoinine-labelled protein of interest. This is because globomycin inhibits signal peptidase II during its processing of prolipoprotein (Inukai, Takeuchi et al. 1978) and stops maturation of lipoprotein. Bands of both

prolipoprotein (higher MW) and lipoprotein (bit lower MW) can be observed from sample with no treatment of globomycin and only band of prolipoprotein (higher MW) can be observed from the sample treated with globomycin (Wu, Hou et al. 1977; Schneider and Hantke 1993). This phenomenon is found to be characteristic among all the bacterial lipoproteins. Other methods such as membrane localization, electrophoretic mobility shift and MS analysis have been used to identify the lipoprotein converted from a non-lipoprotein from a non-lipoprotein (with or without signal sequences) by in vitro lipid modification with a hydrophobic anchor at the N-terminus (Gan, Gupta et al. 1993; Kamalakkannan, Murugan et al. 2004).

1.6.3 Binding lipoprotein

Since Braun's lipoprotein was found in 1969 in the outer membrane of *E. coli*, more than 700 lipoproteins have been found and more than 400 of them have been identified with their functions (Madan Babu and Sankaran 2002). These lipoproteins are structural proteins, antigens, toxins, enzymes, binding proteins or transporters that perform essential functions at the membrane-aqueous interface. Also all the lipoproteins that bind ferric siderophores, {categorized by a database of bacterial lipoprotein (DOLOP) (Babu, Priya et al. 2006)}, are about 315 to 322 amino acid long, located either in the periplasmic space of Gram-negative bacteria or in the cell envelope of Gram positive bacteria. Among those, FhuD was identified as the binding lipoproteins for ferrichrome. It has not been reported that any of those lipoproteins are anchored to the peptidoglycan (PG). However, one heme-binding lipoprotein reported

from DOLOP shows that the protein is 547 AA long. The siderophore or heme-binding lipoprotein may have to follow two rules: 1) In the signal peptide region of the prolipoprotein, a signature motif of "LXXC" exists that defines the cleavage of signal peptide. Lipid modification occurs at the cysteine. 2) In the coding sequence of the mature protein, some conserved regions characteristic for heme or siderophore binding also exists. With the rapid expansion of the bacterial genomic database and reports on the roles of lipoproteins in bacterial homeostasis and pathogenesis, we will be able to update and highlight the various features, especially the functional assignments to predicted lipoproteins.

1.7 Siderophores

As previously stated in the earlier chapter, most bacteria employ a prominent strategy of expressing iron chelators called siderophores, from the Greek: "iron carriers". Siderophores are generally less than 1000 Da, iron chelating molecules secreted by bacteria and fungi under iron-deficient conditions (Neilands 1984). More than 100 different siderophores have been found since its first discovery in the early 50's. In general, most siderophores possess either phenolate or carboxylate oxygen that can tightly bind to Fe(III), not Fe(II). Due to the extremely low environmental concentration of soluble Fe(III) (less than 10^{-18} M), microbes have to synthesize and export potent and specific chelators to selectively solubilize Fe(III) from a large pool of cations. Therefore, siderophores have very high affinity to Fe(III), greater than $10^{30}M^{-1}$. Structurally most siderophores have a peptide backbone with several non-protein amino acid analogs including both modified and D-amino acids. The microbial bioavailability

of iron is largely determined by the coordination chemistry of siderophores (Neilands 1995). Based on their chemistry, siderophores are classified into three major types: catecholates, hydroxamates and the mixed of those first two. We will mainly discuss the first two types of siderophores used in my study **(see table 1.2).**

Catecholates:

FeEnt

Ferric enterobactin (FeEnt), enterobactin binding with Ferric iron, is a prototypical catechol-type siderophores. Enterobactin (Ent) itself is the native siderophores of *E. coli*. It is synthesized by 7 genes, entA-G (Crosa and Walsh 2002). It consists of three dihydroxybenzoyl serine groups (DHBS) that are esterified to form a trilactone backbone. However, in FeEnt, Fe(III) ion is wrapped by the three catechol rings. The three catechol rings render a hexa-coordination, which with Fe(III) around gives a net charge of -3 (Raymond, Isied et al. 1976). This hexa-coordination is essentially important because it renders the ability to bind iron with greatest affinity, about $10^{52} M^{-1}$ among all kinds (Cooper, McArdle et al. 1978). FeEnt has a chirality of Δ and also has very high structure specificity because its mirror image, ferric enantioenterobactin, does not stimulate the bacterial growth (Neilands 1981).

FeCrn

Corynebactin (also called bacillibactin) is a catecholate siderophore produced by the gram-positive bacilli, Corynebacterium and Bacillus (May, Wendrich et al. 2001; Bluhm, Hay et al. 2002). Similar to enterobactin, corynebactin also incorporates a trilactone ring and three catecholate binding groups. However, Corynebactin has three features different from enterobactin: i) corynebactin arms contain a glycine

spacer between the catecholamide and the trilactone backbone. ii) The ring is methylated. iii) The substitution of threonyl glycines for the smaller serines renders the conformation of ferric corynebactin to be opposite of FeEnt, that is, the chirality of ferric corynebactin is Λ, not like Δ for ferric enterobactin (Bluhm, Kim et al. 2002).

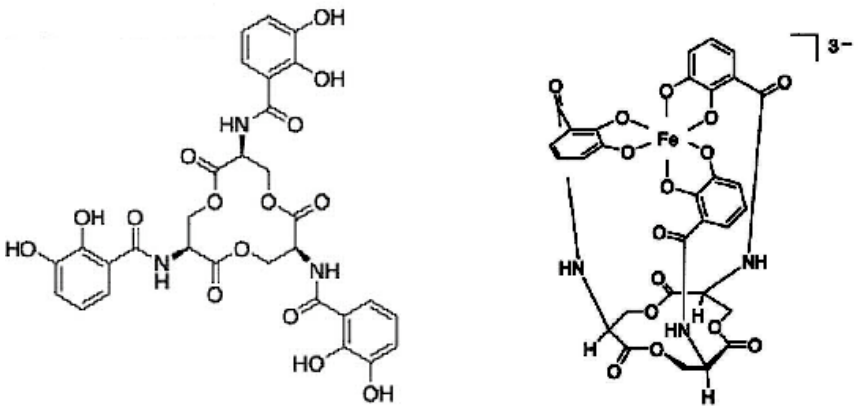


Fig. 1.8 Structure Diagram of Enterobactin (left) and Fe-Enterobactin (right)

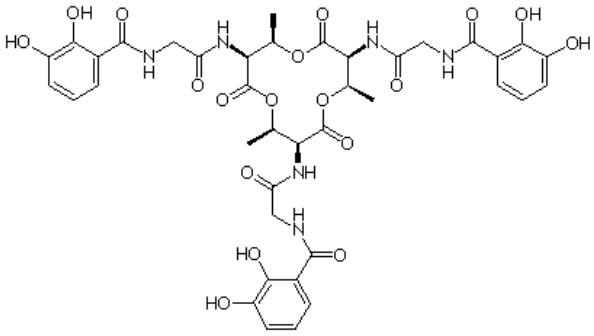


Fig. 1.9 Structure Diagram of Corynebactin

Hydroxamate:

Ferrichrome

Ferrichrome (Fc) is a hydroxamate type siderophore synthesized by smut fungus, *Ustilago sphaerogena* (Neilands 1983). Actually most fungal siderophores are of the hydroxamate group. Although ferrichrome is a fungal siderophore, it is also rapidly taken up by enteric bacteria, such as, *E. coli* and *Samonella*. Apoferrichrome (apo-Fc), without Fe(III) chelated, is a cyclic hexapeptide backbone of triglycyl-tri (N5 acetyl- N5-hydroxy- L- ornithine). Apoferrichrome binds Fe^{3+} forming neutral ferrichrome (Fc). In Fc, the central iron is coordinated octahedrally by 3 deprotonated hydroxyl groups and 3 carbonyl oxygens of the hydroxamic acid moieties. Ferrichrome itself is crystallized in the geometry of Λ–cis coordination (Neilands 1995). But much different from FeEnt, the mirror image of Ferrichrome, enantio-ferrichrome, can also be recognized by the ferrichrome uptake system (Winkelmann and Braun 1981).

Ferrichrome A

Ferrichrome A (FcA) was also extracted from fungus, *Ustilago sphaerogena (Warren and Neilands 1965)*. It also shares basic structural unit N5-acetyl- N5-hydroxyornithine. But Ferrichrome A's hexapeptide ring is made of one glycine, two serine, and three N5-hydroxyornithine amino acid residues, the latter acylated by trans-(α-methyl)-glutaconic acid residues. The membrane receptors of fungi can recognize both Fc and Fc A but transport FcA with less efficiency. Most bacteria cannot utilize FcA at all, except *L. monocytogenes*, even though ferrichrome and ferrichrome A are quite similar in conformation. There are only some minor differences existing in the hexapeptide ring and acyl substitution of the ornithine residues might be responsible for the observed differences in transport activity of Fc and FcA.

Ferrioxamine B

The ferrioxamines is a group of trihydroxamate siderophores produced by actinomycetes (Dhungana, White et al. 2001). Desferrioxamine B, a simple linear ferrichrome-mimicking compound, has been developed clinically to treat toxicity in iron-overload patients. It is also a hexadentate ligand with three asymmetrical bidentate functional unites that can theoretically result in 16 geometrical and optical isomers. That's why the crystal structure of ferrioxamine B contains a racemic mixture of Λ and Δ isomers. In ferrioxamine B, the Fe(III) is coordinated with the six hydroxamate oxygen atoms to form a distorted octahedral geometry around metal center. The structure features at the octahedrally coordinated Fe(III) center in ferrioxamine B are similar to iron complexes of other ferrioxamines and ferrichrome and ferrichrome A. However the iron transport activity of Ferrioxamine B is comparable to ferrichrome system, not those other ferrioxamines whose structures are more closely related. This is because Ferrioxamine B has a unique carbonyl face like those ferrichrome-type which helps recognition during iron uptake process.

Aerobactin

Aerobactin was first isolated from cultures of *Aerobacterium aerogenes* (Gibson and Magrath 1969). It is a conjugate of 6-(N-acetyl-N-hydroxyamino)-2-aminohexanoic acid with a central citric acid moiety. This central citrate moiety also renders aerobactin the ability to be photonreative. The structure of Fe(III)-aerobactin indicates retention of the Λ chirality around the iron (Kupper, Carrano et al. 2006). Aerobactin production is one of several virulence factors of invasive strains of *E. coli*,

enabling bacterial proliferation in the iron-deficient intercellular environment of mammalian tissues (Valvano, Silver et al. 1986). This is because *E. coli* cells can use the siderophore enterobactin to steal iron from human proteins such as transferrin. However, the immune system protein, siderocalin, can seize enterobactin upon human immune response to interrupting bacterial infection but it can't recognize "stealth siderophores" such as aerobactin and salmochelin. Therefore, study of structural nuance of siderophores will help us understand the specificity during binding of siderophore to the target proteins or receptors.

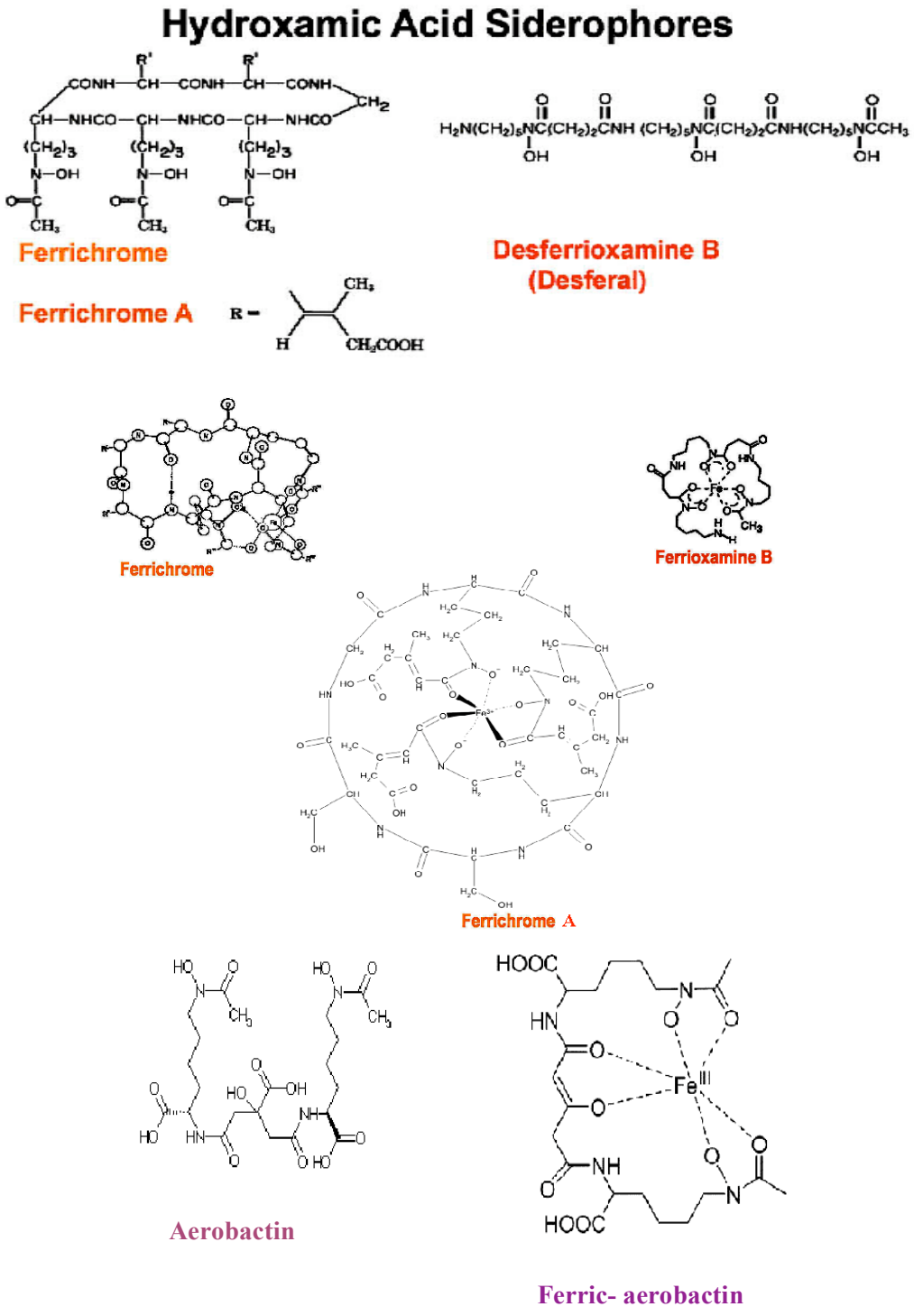


Fig. 1.10 Structures of Hydroxamate Siderophores. Top left: Linear structures of apo-ferrichrom, apo-ferrichrome A and desferrioxamine B. Bottom left: 3-D structures of ferrichrome, ferrichrome A and ferrioxamine B. Bottom Right: structures of aerobactin and coordinated with ferric iron.

Siderophore	Type	Native source	Structure	Iron complex	Chirality	Net charge of iron complex
Enterobactin	Catecholate	*Escherichia. coli*	Tricatecholate; dihydroxybenzoyl serine groups (DHBS) moiety; Backbone:cyclic triserine lactone	hexa-coordination; Ka=10^{52}	Δ	-3
Corynebactin	Catecholate	*Corynebacterium diphtheriae & Bacillus subtilis*	Triscatecholate;Glycine spacer;backbone: Trithreonine lactone; methylated ring	hexa-coordination	Λ	-3
Ferrichrome	Hydroxamate	*Ustilago sphaerogena*	Tri-hydroxamate moiety; backbone:cyclic hexapeptide of triglycyl-tri (N5 acetyl- N5-hydroxy- L-ornithine).	Octahedral coordination; Ka=10^{29}	Λ	0
Ferrichrome A	Hydroxamate	*Ustilago sphaerogena*	Tri-hydroxamate moiety; backbone:cyclic hexapeptide of one glycine, two serine, and three N5-acetyl- N5-hydroxyornithine	Octahedral coordination; Ka=10^{29}	Λ	-3
Ferrioxamine B	Hydroxamate	*actinomycetes*	Tri-hydroxamate moiety;	Distorted Octahedral coordination; Ka=10^{31}	a racemic mixture of Λ and Δ	0
aerobactin	Hydroxamate	*Aerobacterium aerogenes*	Dihydroxamate moiety with additional central citric acid moiety;	Ka=10^{26}	Λ	0

Table 1.2 Property of siderophores. (Raymond, Isied et al. 1976) (Neilands 1981) (Bluhm, Kim et al. 2002) (Cooper, McArdle et al. 1978) (Winkelmann and Braun 1981) *(Warren and Neilands 1965)* (Dhungana, White et al. 2001) (Kupper, Carrano et al. 2006).

1.8 Fur ─ Iron-mediated Regulation

Although iron is indispensable to the bacterial growth, its excess in the cytoplasm can be toxic for the cell, as it catalyzes the Fenton reaction, leading to formation of hydroxyl radicals (OH), which is the most active radicals in the oxidative destruction of DNA, lipids, and proteins. Thus, the iron homeostasis is strictly controlled and iron-uptake genes are tightly negatively regulated, primarily at transcriptional level through metalloproteins using Fe(II) as corepressor.

The most common global iron regulator of this kind, called "Fur" (Ferric uptake regulator), was found in *E. coli* and *Salmonella typhimurium* 30 years ago (Ernst, Bennett et al. 1978; Hantke 1981). It is a 17-kDa polypeptide with high histidine content. Under iron-rich growth condition, Fur, which regulates the iron uptake genes and biosynthesis of siderophores, is complexed with ferrous iron, bind to a classic consensus binding sequence, GATAATGATTATCATTATC, known as the "Fur-box", and shut down the transcription of genes involved in iron transport systems or biosynthesis of siderophores (Bagg and Neilands 1987). **(Fig. 1.11)** Similar highly conserved "Fur-boxes" are also found in the promoter of many iron-regulated genes in other bacteria (Lavrrar and McIntosh 2003). However systematic studies on deviation from the *E. coli* Fur box are relatively lacking. It is only reported in *B. subtilis* that a 7-1-7 inverted repeat (TGAtAATnATTaTCA, lower case stands for the less highly conserved) is more accurate to stand for the consensus sequence of Fur box than the classic 19 bp site. And the classical 19-bp sequence can be thought as two overlapping

7-1-7 repeats (Baichoo and Helmann 2002). In vitro, Fur can not only bind to Fe(II), it also can bind to some other divalent cations such as CoII, ZnII and MnII, probably because Fur in some strain, like *B. subtilis*, is regulated by PerR, which is a MnII-binding Fur homolog (Fuangthong, Herbig et al. 2002).

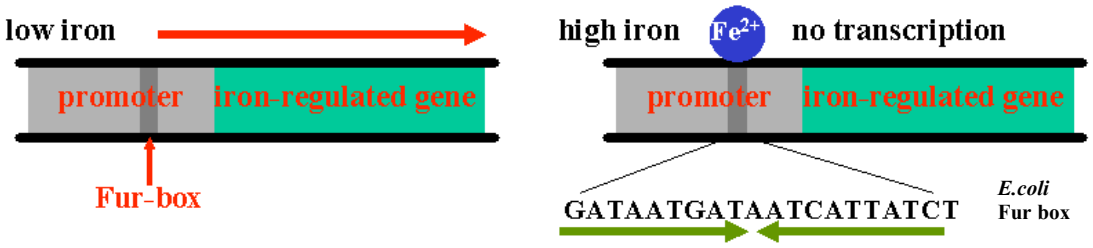


Fig. 1.11 Mechanism of iron-uptake genes regulated by Fur

Under low iron conditions, not enough irons can bind to the protein Fur, and Fur thus cannot bind to the Fur-box in the promoter region and block the RNA polymerase from transcribing iron-regulated genes. Therefore, under iron starving stress, or in the *fur* mutant, the iron-mediated genes regulated by Fur are overexpressed. Fur has an iron binding domain as well as a DNA binding domain. Under high Fe(II) concentration inside the cell, Fur first binds to Fe(II) and then binds to Fur-box in the promoter and represses the iron – regulated genes from transcription. The 19 bp sequence of GATAATGATAATCATTATCT is a canonical Fur box that has been found highly conserved in many bacteria.

A second global iron regulator, called DtxR (Diphtheria toxin Regulator), was the first gram-positive iron-dependent repressor found in *Corynebacterium diphtheriae* as controlling iron-uptake genes and also virulence genes (Schiering, Tao et al. 1995). DxtR is now believed to be a major iron regulator in gram-positive bacteria with high G+C content and regulates a set of genes similar to those regulated by Fur in many gram-negative bacteria.

1.9 Genomic Analysis of Listeria – 4 putative iron-regulated loci

The genomes of both *Listeria monocytogenes EGD-e* and *Listeria innocua* have been sequenced by a European collaboration (Glaser, Frangeul et al. 2001). *L. monocytogenes* has a single circular chromosome. The genome of *Listeria monocytogenes EGD-e* is 2,944,528 bp long with 2853 open reading frames and a G+C content of 39%. Surprisingly, many encoded proteins are very similar to those of non-pathogenic bacterium *Bacillus subtilis* as well as to those of pathogenic *Staphylococcus aureus*. The ability of *Listeria* to inhabit a wide range of environments coincides with the presence of 331 genes encoding different transport proteins, consisting of 11.6% of the total genome in *L. monocytogenes*. The availability of those data allowed us to find open reading frames in the *L. monocytogenes* genome that are homologous to the known iron transport genes of other bacteria. By comparing *L. monocytogenes* genomic sequence to current nucleic acid and protein database, we found four putative loci for iron utilization. All of them have Fur-box.

1. **The furfhuBGDCOR locus** (2.031 Mb;orfs 1956-1961)

The presence of Fur, a global iron regulator, together with Fur-box adjacent to a typical ferrichrome-like ABC transporter makes this locus very likely to be involved in iron transport, particularly in hydroxamate siderophores iron uptake.

2. **The svpA-srtB locus** (2.274 Mb; orfs 2180-2186)

SvpA is a 64 kd Surface virulence-associated protein required for intercellular survival of *L. monocytogenes.* As a newly-found surface virulence factor, it is both

secreted in culture supernatant and anchored to the cell wall by the SrtB in the same locus (Newton, Klebba et al. 2005).Even though physiological function of SvpA in *Listeria* is not yet known, its structural gene is part of a 7-gene operon (the svpA locus) that contains another putative ferrichrome-like ABC transporter. Furthermore, its promoter region contains a well-conserved "Fur-box" (18/19 conserved bases homology to *B. subtilis* Fur-box), a DNA sequence to which the ferric uptake regulator binds when iron concentrations are high. This suggests that this virulence factor might be iron-regulated during bacterial pathogenesis.

3. **The fhuDGC locus** (2.499 Mb; orfs 2429-2431)

This region contains a third ferrichrome-like ABC type transporter, with an esterase homology on one of the ends. Even though it does not contain a canonical Fur-box, before lmo2431, a homology of Fur-box was also found.

4. **The feoAB locus** (2.184 Mb; orfs 2104-2105)

feoAB encodes a ferrous uptake system that is also found in E. coli and many other species. Again a typical Fur-box precedes the two structural genes. Also there was a report experimentally showing the existence of ferrous uptake system in *L. monocytogenes* before its genomic sequence was found. Therefore, the feoAB locus is not the focus of my PHD research here.

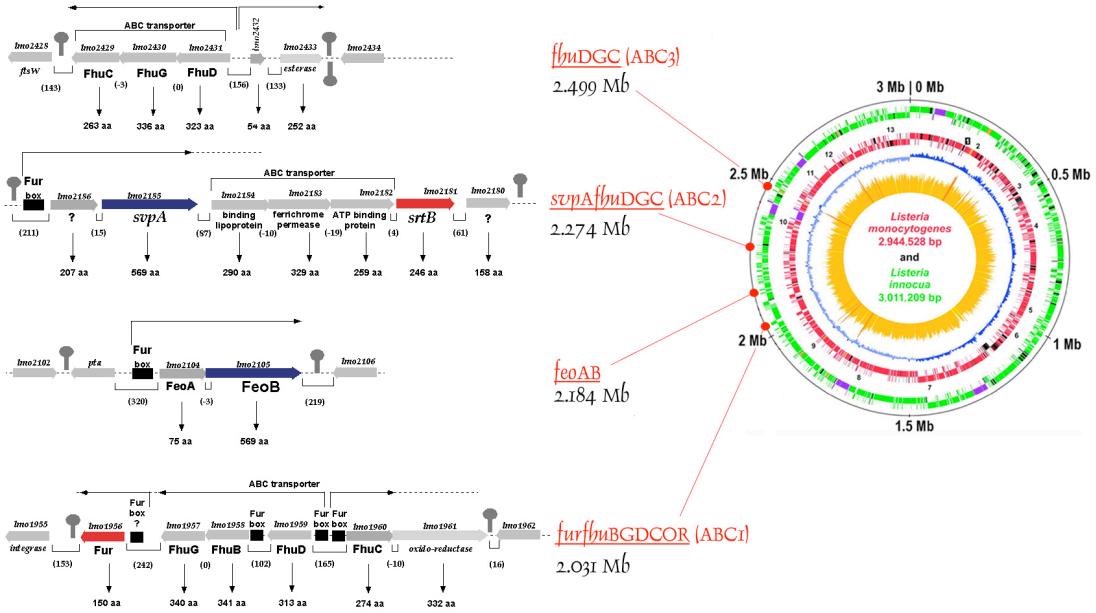


Fig. 1.12 Four putative iron-regulated membrane transport operons in the genome of *L. monocytogenes,* adapted from http://cheminfo.chem.ou.edu/faculty/pek.html

1.10 Goals and significance of our research on iron acquisition in *Listeria monocytogenes*

A variety of specialized iron uptake systems encoded by their genomic operons had been well-characterized experimentally in Gram-negative bacteria whereas iron transport systems in Gram-positive bacteria were comparatively obscure when my PHD research project started. Barely any papers describing the molecular basis of iron uptake by Gram-positive bacteria were published during that time. However, completion of genome sequences for important Gram-positive pathogens during that period allowed us to identify genes encoding potential iron transporters by genomic sequence alignment with sequences of known iron transporters from Gram-positive bacteria and also has stimulated studies on the mechanisms of iron uptake in Gram-positive pathogens. When this searches project started, little was known about listerial iron-uptake mechanism or which genes encode for its iron transporters. What we did know during that time was that the bacterium is not known to synthesize any siderophores but it can utilize many of the siderophores made by other bacteria (Simon, Coulanges et al. 1995; Coulanges, Andre et al. 1997). Only four different mechanisms from preliminary studies were described for listerial iron acquisition: (i) inducible ferric citrate uptake (Adams, Vartivarian et al. 1990); (ii) a surface-associated reductase described by Deneer et al (Deneer, Healey et al. 1995) and/or an excellular reductase described by Barchini et al. (Barchini and Cowart 1996); (iii) a cell-surface-associated transferrin binding protein (Hartford, O'Brien et al. 1993); and

(iv) utilization of exogenous siderophores (Simon, Coulanges et al. 1995; Coulanges, Andre et al. 1997). However, none of them elucidated corresponding genes of their functions and kinetic parameters of binding affinity and specificity of each transporter are lacking. Furthermore, even though we had some genomic predictions from preliminary computer-generated data showing the existence of iron transporters in *L. monocytogenes*, we cannot elucidate mechanism of iron uptake in *L monocytogenes* or Gram-positive bacteria until we verify it by the traditional biochemical methods.

So the first goal of my PHD study was set to make biochemical identification of Fur. It encodes a global regulator to control the expression of those iron-regulated transport systems and it is located within one of genomic regions of putative ABC type iron transporters ─ the furfhuBGDCOR locus. My second goal was to determine the function of svpA-srtB locus. We wanted to find the answers for the questions: Is SvpA iron-regulated? Is it a cell surface iron-binding protein? What source of iron it can utilize if it is involved in iron acquisition? Is SvpA involved in virulence? Thirdly, I wanted to characterize the specificity of FhuD, which we later found is involved in uptake of hydroxamate siderophores. Finally, we wanted to find out which operon or protein within those loci is actually involved in the listerial virulence by the study of bacterial infection in mouse. This is because a study showed *L. monocytogenes* could increase virulence in mice loaded with iron and the effect was reversed by removing excess iron using iron-chelators (Cowart and Foster 1985). This suggested that iron acquisition may contribute to the pathogen's virulence.

Due to the essential role of iron in cellular metabolism, toxicity and pathogenesis, our research on iron acquisition systems in *L. monocytogenes* will help to design novel therapeutic agents. It is reported that conjugation of antibiotics to siderophores, a very common iron-chelator used by the most bacteria to sequester iron from their environment, has shown promise for therapeutic control of bacterial infections (Roosenberg, Lin et al. 2000). Also even though the mechanisms of iron-uptake systems in Gram-negative bacteria have been dramatically enlightened in the last 20 years, knowledge of Gram-positive iron-uptake systems, particularly in pathogenic *L. monocytogenes*, still remain relatively obscure. Furthermore, as in the post-genomic era, a combination of genomic and proteomic, biochemical approaches will help us to identify more and more iron uptake systems and thus gain deeper insights into the molecular mechanisms of pathogenic iron uptake.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

2.2 Growth media and condition

2.3 Preparation of siderophores

2.4 Preparation of competent Cells

2.5 Preparation of chromosomal DNA from Listeria monocytogenes EGD

2.6 Quantification of secreted proteins in Listerial cells

2.7 Site-directed chromosomal deletion in *L. monocytogenes*

2.8 FhuD overexpression and purification

2.9 Intrinsic fluorescence.

2.10 Antibody generation

2.11 Western immunoblots

2.12 Complementation of Fc uptake deficiency in *ΔfhuD*

2.13 Virulence study in the mouse model system

2.14 Nutrition tests

2.15 Molecular analyses of genes and proteins

2.1 Bacterial strains and plasmids

All *E. coli* strain used in these studies are derivatives of *E. coli* K-12, listed in Table 2.1 with their characteristics and reference. *B. subtilis* 168 was used for purification of corynebactin**.**

Table 2.1 Bacterial strains and plasmids used in this study

Stain,plamids	Usage	Reference
E. coli		
DH5α	*E. coli* lab strain for in vivo or in vitro cloning	(Hanahan 1983)
BN1071	*E. coli* with FepA function	(Klebba, McIntosh et al. 1982)
KDF541	*E. coli* strain *fepA, fhuA*	(Rutz, Liu et al. 1992)
AN102	*E. coli* strain for purification of enterobactin	(Yeowell and White 1982)
BL21	*E. coli* strain for overexpression protein of T7 promoter constructs	(Studier, Rosenberg et al. 1990)
XL-1-Blue	*E. coli* strain for midi prep of pPL2	(Bullock et al. 1987)
DP-E-4189	*E. coli* host strain carrying pPL2	(Lauer, Chow et al. 2002)
SM10	*E. coli* donor strain for conjugation	(Lauer, Chow et al. 2002)
B. subtilis		
168	Wild type	Dr. JD Ballard at OU
L. monocytogenes		
EGD-e	wild type	(Trost, Wehmhoner et al. 2005)
lmo*fur*	Listerial EGD strain of fur deletion	(Newton, Klebba et al. 2005)
lmo*fhuD*	Listerial EGD strain of fhuD deletion	(Jin, Newton et al. 2006)
lmo*fhuC*	Listerial EGD strain of fhuC deletion	(Jin, Newton et al. 2006)
lmo*hupC*	Listerial EGD strain of hupC deletion	(Jin, Newton et al. 2006)
lmo*hupC/fhuC*	Listerial EGD strain of hupC/fhuC double deletion	In this lab
Plasmid		
pUC18	Cloning of a target gene and its expression using lac promoter	(Yanisch-Perron, Vieira et al. 1985)
pET28a	Histag fusion expression vector	Novagen
pKSV7	E. coli-gram positive shuttle vector	(Smith and Youngman 1992)
pMAD	*E. coli-L. monocytogenes* shuttle vector	(Arnaud, Chastanet et al. 2004)
pPL2	*E. coli-L. monocytogenes* shuttle vector	(Lauer, Chow et al. 2002)

2.2 Growth media and Condition

Luria-Bertani (LB) broth (Difco) and Brain Heart Infusion (BHI) broth (Difco) were used as rich media to routinely grow *E. coli* and *L. monocytogenes* respectively. T media (Klebba, McIntosh et al. 1982) and TE/SMM media (May, Wendrich et al. 2001) were used as iron-deficient media for purification of enterobactin and corynebactin. Solid media were obtained by the addition of 0.7% (w/v) Bacto-agar (Difco).

α,α-bipyridyl (BP) was added to BHI if iron deprivation was needed. For bacterial growth in KRM medium, which is an iron-deficient synthetic medium based RPMI 1640, we subcultured (1%) EGD-e strains first from BHI broth, and grew the culture until cells reached stationary phase (OD600~1.2), and then subcultured again into KRM (1%) and let them grow to mid-log phase. Ferrichrome (50 μM) was added to KRM if required. Growth of bacterial cultures was performed at 37 °C. Iron-free water was used for all experiments and was obtained by double distillation of di-ionized water (reverse osmosis).

Table 2.2 Media used in this study

Media	**References**
Luria-Bertani	Miller et al., 1972
T-media	Klebba et al., 1982
MOPs media	Neidhart et al, 1974
RPMI1640	Sigma
KRM	Newton et al, 2005
KRMT	Bo Jin et al, 2006
SMM	JJ May et al, 2001
TE/SMM	JJ May et al, 2001
Brain Heart Infusion	Difco

2.3 Preparation of Siderophores

Apo-ferrichrome and apo-ferrichrome A were purified from cultures of *Ustilago sphaerogena*. Enterobactin and corynebactin, the siderophores of Gram-negative and Gram-positive bacteria respectively, were purified from *E. coli* and *B. subtilis*. The ferric iron complexes of those siderophores were purified by passage over Sephadex LH20. Ferrioxamine B (FxB) was a gift from J. B. Neilands. We purchased purified hemin (Hn), bovine hemoglobin (Hb), bovine holotransferrin (Htf), equine ferritin (Ftn), ferric citrate (Fe-Cit) and ferrous sulfate (FeSO4) from Sigma-Aldrich (St. Louis, Mo)

2.3.1 Preparation of ferric-enterobactin

Enterobactin was purified from the supernatant of AN102 cultures grown to late exponential phase in 15 L of T-Media. After centrifugation (4,000 rpm for 40 min), the cultured broth was extracted three times with 0.1 L ethyl acetate per liter of supernatant. Subsequently, the volume of the pooled organic extracts was reduced to 100 ml in a rotary evaporator at no higher than 30 °C, and the concentrated ethyl acetate extract was washed 1X with 0.1 M sodium citrate buffer (pH 5.5) and water, respectively. The organic layers were dried overnight in anhydrous $MgSO_4$, the $MgSO_4$ was removed by filtration, and the filtrate was concentrated in a roto vapor to a total volume of 10 mL. Hexanes were slowly added until crystals formed and then the solution was centrifuged for 5 minutes to pellet the enterobactin.

Ferric enterobactin was prepared by dissolving 1mg of enterobactin in 0.5 ml of methanol, and 0.5 ml of 4 mM $FeSO_4$ in dilute HCl was added, and incubated at room temperature for 1 hour to allow for complex formation. Then NaH_2PO_4, pH 6.9, was added to make the final buffer concentration of 2.5 mM. The mixture was loaded onto a Sephadex LH20 column equilibrated with 2.5 mM NaH_2PO_4, pH 6.9 and eluted with the same buffer. The concentration of ferric enterobactin was determined by measuring the absorbance at 495 nm on a DU Beckman 640 spectrophotometer. The purity of ferric enterobactin was determined by ratio of absorbance between 393 nm and 495 nm (optimum; 0.666). Ferric enterobactin was stored on ice and when necessary repurified by chromatography the Sephadex LH20 column.

2.3.2 Preparation of ferric bacillibactin

2.3.2.1 Growth Conditions for Siderophore Extraction

To test various *B. subtilis* strains under iron deprivation, cells were grown in Spitzien's minimal medium supplemented with 0.2 (w/v) casamino acids and 0.5% (w/v) glucose. Iron was added at various concentrations (0.1–1000 mM) from a freshly prepared solution of $FeCl_3$, and 10 ml of cells were incubated at 250 rpm for 48 h at 37 °C in 50-ml polyethylene tubes. To avoid cross contamination with iron, all glassware was rinsed with concentrated HCl, and solutions were stored in bottles made of polycarbonate or polyethylene.

2.3.2.2 Siderophore Extraction from

For siderophore extraction, 1 liter of cells from *B. subtilis* strains 168 was cultured for 48 h at 37 °C in Spitzien's minimal medium supplemented with 50 mM $FeCl_3$. After centrifugation (10,000 rpm for 20 min), the cultured broth was extracted three times with equal volumes of ethyl acetate. Subsequently, the volume of the pooled organic extracts was reduced to 100 ml in a rotary evaporator at 37 °C, and the remainder was washed two times with 0.1 M sodium citrate buffer (pH 5.5) and water, respectively. The organic layer was evaporated to dryness, and the residue was resuspended in a small volume (200 ml) of methanol. The resulting suspension was cleared by centrifugation (13,000 rpm for 5 min), and the supernatant was stored at -20 °C, or evaporated to dryness.

2.3.2.3 Detection and Analysis of the Siderophore by Ferric Hexadecyltrimethylammonium Bromide-Chrom-Azurol-S (CAS) Assay

For the detection of siderophore-producing *B. subtilis* strains, organic extracts of their cultured broth were applied to a CAS assay as described by Schwyn and Neilands (Schwyn and Neilands 1987). Additionally, the *B. subtilis* strains were streaked out on CAS plates and tested for growth and the ability to breakdown the CAS complex.

2.4 Preparation of Competent Cells

2.4.1 *E. coli*

A 5 mL LB culture of an E. coli strain was grown for overnight before it was subcultured (1:100) into a 500 mL of LB broth with the appropriate antibiotics. When the OD_{600} reached 0.5, the culture was chilled on ice for 15 minutes. Bacteria were spun at 8000g for 15 minutes. The bacterial pellet was washed once with 500 ml, and twice with 250ml of ice cold distilled water, and then with 50ml of ice cold distilled water contains 10% glycerol respectively. Finally, the pellets were resuspended in 1 ml of distilled water with 10% glycerol, aliquoted into microtubes (40µl) and stored at -80 °C.

2.4.2 *Listeria monocytogenes*

EGD-e was grown in 25ml of BHI overnight and subcultured into 500 ml BHI (1:50 dilution). Penicillin G was added to 0.12 µg/mL when the OD_{600} reached to 0.3. The cell culture was harvested at 8000 rpm for 20 minutes immediately after the OD_{600} reached to 0.8~0.9. The pellet was washed with 100ml, 50ml and 3X25 ml of 1mM Hepes/500mMsucrose. Finally, the pellet was resuspended in 500 µl Hepes/Sucrose with 15% glycerol and aliquoted into 40µl/microtube and stored at -80 °C.

2.5 Preparation of chromosomal DNA from *Listeria monocytogenes* EGD

25 mL of *L. monocytogenes* strain EGD-e was grown in BHI broth overnight. The cells were harvested at 8000 rpm for 15 minutes and then kept on ice. The pellets were resuspended in 1 ml ice cold distilled water and the cells were broken by the Fast Prep Bead-beater at an intensity of 6.5 for 30 seconds with 3 cycles. In each cycle, the cell suspension was immediately chilled on ice for 30 seconds before the next cycle. The bead-beater tubes were centrifuged for 2 minutes and the supernatants were transferred to an eppendorf tube. NaCl was added to a final concentration of 100 mM, and the supernatant was extracted with buffered-phenol twice, at a portion of 1:1. The supernatant was removed each time to a fresh tube. Next, the supernatant was extracted twice with chloroform/isoamyl-alchol. Ultimately the chromosomal DNA was precipitated with 2 volumes of EtOH and pelleted in a refrigerated microcentrifuge by centrifugation for 30 minutes. The pellet was washed with 70% ethanol. DNA was resuspended in 100 µl TE+ 2 µl 0.5mg/ml RNAse.

2.6 Quantification of secreted proteins in Listeria cells

An overnight culture of EGD-e cells was subcultured to 25 ml BHI (1:100). After the OD_{600} reached between 0.1 and 0.2, culture was divided into 2 flasks, and one was added with bypiridyl to1mM and the other was added with 1mM bypiridyl and 10 µM $FeSO_4$. The flasks were put back to 37 °C shaker and the OD was monitored every two hours until the culture reached the stationary stage. The cells

were harvested and centrifuged at 8000 rpm for 15 minutes, and the supernatant was transferred to a Corex tube and TCA was added to 1N. The tubes were covered with parafilm and left on the ice in the cold room overnight. The next day, the tubes were centrifuged at 8000 rpm for 30 minutes, and the supernatant was immediately discarded. The pellet was resuspended in 0.5 ml 80% acetone and centrifuged again in a refrigerated micro-centrifuge tube for 20 minutes. The supernatant was discarded and the pellet was resuspended in appropriate volume of distilled water to normalize the amount of cells by cell density at the end of the growth (for Listeria; 1 OD_{600}= $2X10^8$ cells/ml and all samples were adjusted to 10^7 cells/ µl). Aliquots (20 µl, 10 µl, 5 µl) were subjected to SDS-PAGE or western blot.

2.7 Site-directed chromosomal deletion in *L. monocytogenes*

Site-directed chromosomal deletion in wild-type *Listeria monocytogenes* EGD-e or in the mutant derivatives was done by in vivo recombination. Two chromosomal sequences, upstream and downstream of the target gene of deletion interest, were amplified by PCR with appropriate restriction digestion sites designed to flank on both ends of each PCR segment. After digestion with restriction enzymes, the segments were joined together by ligase. The ligated fragment that eliminated the target gene was cloned into a thermosensitive shuttle vector, pKSV7 (Smith and Youngman 1992). pKSV7 carrying the deletion construct was first electroporated into DH5α. White colonies were picked and analyzed to confirm with the right size of the plasmid by PCR. The purified vector was then transformed by electroporation again into wild

type EGD-e, or the EGD-e competent mutant derivative strain. The transformants (Cm^R) were picked at permissive temperature of 30 °C with 5μg/mL of chloramphenicol, and then subcultured and grown at 37 °C with 5μg/mL of chloramphenicol. At 37 °C, the new construct integrants into the chromosome by homologous recombination with DNA flanking the target gene. The integrant was subcultured again at 37 °C, but without chloramphenicol, and passaged at least 6 generations at 37 °C without chloramphenicol. After several passages, integrated plasmid excises from the chromosome by a second homologous recombination event, with the result of deletion of target gene on the chromosome. Such mutants were screened by chloramphenicol sensitivity test, and were verified by colony PCR with appropriate primers designed to show the size of the deletion.

2.8 FhuD overexpression and purification

The *fhuD* gene and *fhuD* without the predicted signal sequence (21 amino acid long), were PCR-amplified as an 1.0-kb DNA fragment, digested with HindIII and EcoRI (Sites were incorporated into the oligonucleotides and all the restriction endonucleases were purchased from New England Biolabs;) and then cloned in-frame into HindIII- and EcoRI-digested pET28A(+). Those constructs were named as pET28FhuD and pET28FhuDΔ21. They have 6 histidine tags in N termini. The recombinant pET28A (+) vectors were introduced into *E. coli* BL21 for protein overexpression. Cells of BL21 with pET28FhuD or with pET28FhuDΔ21 were grown to a mid-log phase before isopropyl-1-thio-β-D-galactopyranoside (1.0 mM) was added

and growth continued for another 4.5 h before the cells were harvested. For purification, cells were suspended in lysis buffer (50 mM NaH_2PO_4, pH 7.5, 300 mM NaCl, 10 mM imidazole, 1mM PMSF), with 10 ug/ml RNase and DNase on ice for 15' and then lysed by French Press at 14,000 psi. The lysate was centrifuged at 8,000 x *g* for 20 min; the resulting supernatant was spun in the ultracentrifuge at 35,000 rpm for 45 minutes to pellet membrane fraction, which were resuspended in PBS for 30 min at 25 °C and extracted with 0.2% Triton 100. The sample was centrifuged again at 35,000 rpm for 45 min, and the solubilized membranes were saved. Both cytoplasmic and membrane fractions (up to 40ml= extract from 1 L of cell culture) were passed through a 7-ml pQE-9™ histag nickel column (Qiagen) equilibrated with lysis buffer. Columns with adsorbed His_6-FhuD or His_6-FhuDΔ21 were washed with 10 column volumes of Lysis buffer and another 10 column volumes of wash buffer (lysis buffer plus 20 mM imidazole) at a flow rate of 1ml/min, and eluted with a linear gradient of imidazole (80–250 mM) at a low flow rate of 1.5 ml/hr. Protein purity was assessed by SDS-PAGE. All purification procedures were performed at 4°C. FhuD or FhuDΔ21-containing fractions were pooled and dialyzed against TBS buffer, and protein concentrations were determined by the MicroBCA assay (Pierce, Rockford, Ill.), using bovine serum albumin as a standard. The mature FhuD protein has a calculated molecular mass of 37.8 kDa with the His tag in the N-termini. The mature FhuD Δ21 protein has a calculated molecular mass of 35.9 kDa with the His-tag also in the N-termini. The collected fractions were concentrated with a PES membrane

centrifuge filter unit (Millipore Co Ltd: M_f cut–off is 10kDa). For purification from membrane fractions, the samples were pretreated with lysis buffer (PBS, pH8.0, 0.02% TritionX100, 0.05% Tween20) and washed twice with the same lysis buffer. Alternatively, buffer exchange was performed by dialysis. Impure fractions were further subjected to gel filtration (Sephadex G75). After removal of imidazole and high salts by dialysis, the concentrated or collected pure fractions were mixed with glycerol, aliquoted into 1 ml /microtubes, and stored at -20 °C.

2.9 Intrinsic fluorescence.

All fluorescence spectra and titrations were measured in an SLM Instruments ratio recording spectrofluorimeter. Buffers were filtered to eliminate precipitates. Using an SLM 8000C fluorimeter, upgraded to 8100 capability with automated shutters and polarizers (SLM Instruments, Rochester, N.Y.), the excitation and emission slits were set at 1 and 10 nm, respectively. The excitation band pass was 8 nm, and the sample cell was maintained at 20 °C with a circulating water bath. The excitation and emission maxima for FhuD were 283 and 327 nm, respectively. These settings, which are optimized for excitation/emission of tryptophan, were used for fluorescence measurements of siderophore binding by FhuD. At 20 °C, using purified FhuD, different siderophore-binding reaction mixtures reached equilibrium from a few seconds to a few minutes (data not shown). With an integration time of 5 min, we recorded fluorescence intensities after the addition of various amounts of siderophores to FhuD (62.5 nM) in TBS (pH 7.4). After subtraction of the emission spectrum of the

siderophore itself (in TBS [pH 7.4]), the data were corrected for dilution effects and contaminating fluorescence from impurities in the sodium phosphate buffer. Finally, as a negative control of Ferrichrome binding, the fluorescence of bovine serum albumin in TBS (pH 7.4), was recorded in the presence and absence of Fc. No changes in bovine serum albumin fluorescence occurred, demonstrating the specificity of the binding of hydroxamate siderophores to FhuD.

The K_D was then calculated using the 'Bound-versus-Total' equation from Grafit 5.09 (Erithacus, Middlesex, UK), the non-linear fit of equation performed by the computer.

$$K_D = \frac{(\text{Cap-Bound})*(\text{Total-Bound})}{\text{Bound}}$$

Where $B=K_D+Total+Cap$; total is the amount of ligand added to the assay, and K_D and Cap(capacity) are the two parameters determined by the least squared fitting using the bound-vs-total equation program from Grafit 5.09.

2.10 Antibody generation

Purified FhuDΔ21, denatured by boiling in 1% SDS for 10 min, was added to the purified native FhuDΔ21 in a 1:1 molar ratio. For polyclonal antisera, the mixture was emulsified with complete Freund's adjuvant and 100 μg of protein was injected into mice. The animals were boosted with the same amount, emulsified in incomplete Freund's adjuvant, weekly for a month, and serum was collected.

2.11 Western immunoblots

Whole-cell lysates (5×10^8 cells/lane) were solubilized in SDS-PAGE sample buffer by boiling for 5 min, resolved on 12% polyacrylamide gels, electro-transferred to nitrocellulose paper, and suspended in 25 ml of blocking buffer (TBSBA: 10 mM Tris-Cl, pH 7.4, 0.9% NaCl, 1% BSA, 0.2% NaN_3) for overnight in the cold room. The nitrocellulose paper was incubated with primary antibody (1:2000 in TBS+1% gelatin) in a shaker for 1 hour and washed with TBST (TBS with 0.05% Tween-20) 5 times. The paper was next incubated with anti-mouse alkaline phosphatase (1:1000 TBS+1% gelatin) with gentle shaking for another hour, and washed again with TBST 5 times. The substrate was prepared during the last wash. 17 mg of bromochloroindoyl phosphate (BCIP) was dissolved in 0.5 ml distilled water and 33 mg nitroblue tetrazolium (NBT) was dissolved in 0.5 ml 70% dimethyl formamide. Those solutions were added to 50 ml of substrate buffer (per liter, 98 g of diethanolamine and 1 g of $MgCl_2$-$6H_2O$, pH9.8). The nitrocellulose paper was incubated in substrate for 5 minutes or until color developed appropriately. For quantification of FhuD expression, the nitrocellulose was incubated overnight with mouse polyclonal anti-FhuD sera, incubated with ^{125}I-protein A, and subjected to autoradiography.

2.12 Complementation of Fc uptake deficiency in lmofhuD

Plasmid pPL2, which is a shuttle vector between *E. coli* and *L. monocytogenes* and also a thermo-sensitive integration vector in *L. monocytogenes,* was purified from DP-E-4189 (Lauer, Chow et al. 2002). The *fhuD* gene, together with it is promoter sequence was PCR-amplified as a about 1.2-kb DNA fragment, (Comp-fhuD-BamHI 5' CCCCCC**GGATCC**CGCTCCAATTTAAAGTTAAG3' and Comp-fhuD-Pst1 5'CCCCCC**CTGCAG**TTAGTTGGACGCAAG3') were cloned into BamHI- and PstI-digested pPL2 and the plasmid was transformed into *E. coli* XL-blue for enormous replication. pPL2 carrying fhuD and its promoter was then transformed into competent *E. coli* SM10 conjugative donor strain and was grown in LB broth containing 20ug/ml chloramphenicol at 30 °C. The *lmofhuD* streptomycin-resistant recipient was grown in BHI at the same temperature. A Millipore 0.45 um filter was washed with 5 ml of LB or BHI. 2.5 ml of donor culture were mixed with 1.5 ml of conjugative recipient culture. The mixture was filtered, and the filter was washed with 10 ml BHI. The filter was placed on a fresh BHI plate at 30 C for two hours. The cells were gently resuspended for five minutes in 2.5 ml BHI, and portions (25 ul, 5l ul and 100 ul) were plated in LB soft agar on BHI plates containing 100ug/ml streptomycin and 7.5 ug/ml chloramphenicol. The plates were incubated at 30 °C overnight and then shifted to 37 °C for pPL2fhuD integration to the listerial chromosomal DNA. The integration of fhuD onto chromosomal DNA is site-specific but different from its original fhuD locus. Chloramphenicol-resistant, streptomycin-resistant colonies appeared at frequency of about 10^{-4} per donor.

2.13 Virulence study in the mouse model system

Animal virulence studies were performed in the mouse model to evaluate the effects of the site-directed chromosomal mutations on bacterial virulence by measuring lethal dose 50 (LD_{50}) values. Four groups of Balb/c mice were inoculated by intravenous injection with different doses, in ten-fold dilutions (10^5 10^6, 10^7, 10^8 cells/ ml). Mortality was scored for the following 7 to 10 days and LD50 was determined by the Probit statistical method.

2.14 Nutrition tests

Bacteria were grown in BHI and exposed to BP at 0.1 mM between OD600=0.1~0.2, and then grown until the OD reached to mid log. 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 with appropriate concentration were applied to the discs, and the plates were incubated overnight at 37°C. The diameters of the growth halos were measured and pictures were taken.

2.15 Molecular analyses of genes and proteins

We obtained listerial genes from listerial genome sever (http://genolist.pasteur.fr/ListiList/) and subjected the translated sequences to blastP analysis (http://www.ncbi.nlm.nih.gov/blast/). For homology alignment of genes or proteins of interest to those in the other strains, we subjected them to ClustalW (http://www.ebi.ac.uk/clustalw/). The signal peptide sequence was predicted by signalP (http://www.cbs.dtu.dk/services/SignalP/).

Chapter 3

Systematic mutagenesis to create site-directed chromosomal deletions

3.1 Candidate genes

Because four potential Fur-regulated iron transport systems were found based on their homology (**Fig. 1.13**), creation of systematic mutant strains devoid of certain secreted or cell envelope proteins involved in iron uptake pathways will allow us to evaluate the phenotypic properties from the resulting mutant bacteria. The most important phenotypic tests were the effects on the iron acquisition process and the effects on the virulence of the bacteria in the mouse model system.

Among all the structural genes in the four loci, I was involved in chloramphenicol sensitivity screening of *Δlmo2105 (ΔfeoB)*, and I generated de novo chromosomal deletion mutants of *Δlmo1956 (Δfur), Δlmo2429 (ΔhupC), Δlmo1957/1958 (Δ fhuBG),* and one double mutants of *Δlmo2429/1960 (ΔhupC/fhuC)*. Other mutants were also made in our lab, including *Δlmo1959 (ΔfhuD), Δlmo1960 (ΔfhuC), Δlmo1961 (Δor), Δlmo943 (Δfri), Δlmo929 (ΔsrtA), Δlmo2181 (ΔsrtB), lmoΔ2185 (ΔsvpA), Δlmo2183, Δlmo2186, ΔsrtAB, ΔInlAB, Δlmo2431 (ΔhupD), Δlmo2430 (ΔhupG), and ΔprfA*. The single deletion mutations of the remaining genes

in those four operons or multi-deletion mutations within one operon or between operons constitute future research in our laboratory.

3.2 Computer-based Comparative Analysis

Understanding nature of genes or proteins of interests from their sequence encryptions helped narrow down the scope of the candidate genes that I could start with and also helped for later experimental trouble shooting. I made four major homology alignments throughout the projects I have been working on.

3.2.1 Searching for the candidate Fur-box

Since Fur is a global regulator controlling metal ion homeostasis in different microorganisms, eg. iron-uptake in particular, the search for both conserved target genes and for conserved Fur-binding sites were useful for the identification of genes belonging to the Fur regulon. From the conserved 19-bp sequence of classical *E. coli* Fur-box, GATAATGATTATCATTATC, I first used the 6-nucleotide array, "GATTAAT", to search for the Fur-box in listerial genome by sequence alignment. However, I found more than 700 candidates that were strictly homologous to this six-nucleotide sequence upstream the ORFs. Considering the fact that Fur in *B. subtilis* can regulate about 50 operons, this 6-nucleotide array certainly was too small to target the genes of interest that might function as iron transporters. If I used at least 18 out of 19 conserved nucleotides from the classical 10-bp Fur-box as probe to align the whole genome, it only gave one or two candidates genes that seemed to be iron-

regulated, in which svpA (lmo2185) was identified to be one of the candidates(Newton, Klebba et al. 2005). It seemed that selecting a good length of sequence from the classic Fur-box for the alignment was the first step to narrow down the scope and target good candidates of putative iron-regulated transporters. This is because too long a sequence with high fidelity would end up with no candidate genes after alignment but too short would end up with too many. Therefore, I introduced te following rules to further reduce the redundancy of false positives in the initial pool of candidate Fur boxes: (1) candidates had to be located <200 nt from the proposed initiation of translation of the potential target gene; (2) increase the length of array sequence by incorporating exhibited conservation of key nucleotides known to be protected by Fur binding in *E. coli*; (3) change those less conserved nucleotides in the array sequence to "n", which stands for any of four nucleotides. I found it much easier and also more accurate and effective to use "TGAtAATnATTaTCA", a 7-1-7 inverted repeat conserved among all the Fur-boxes in *B. subtilis* (Baichoo and Helmann 2002), as sequence query to search for the Fur boxes in *L. monocytogenes*. Also by checking data from DNA sequence logos of *B. subtilis* Fur-box (Panina, Mironov et al. 2001) (**Fig. 3.1**), I chose to use "TGAnAATnATTnTCA" for alignment and identified about 20 genes to have this canonical Fur-box in the promoter region upstream their genes (less than 200 nucleotides)(**Table 3.1**). All the Fur-boxes within the four putative iron-regulated loci we previously predicted were included by this array of alignment, and in addition, I found two more candidates. lmo0541 is homologous to a binding

lipoprotein in an ABC transporter, and lmo1131 encodes for a putative ABC permease with ATPase domain.. Furthermore, lmo2431 and lmo2432 shared the same Fur-box but they are transcribed in the opposite direction. It is of interest that downstream lmo2432, lmo2433 has homology to an esterase, because uptake of ferric enterobactin by *E. coli* requires the function of inner membrane esterase. Considering the fact that the Fur-box exists in the promoter region of lmo2432-2433, this operon might be involved in the degradation of enterobactin since *L. monocytogenes* also can utilize this exogenous enterobactin for its own growth. Also by this alignment, lmo0484, a homology to isdG that was a heme-degrading monooxygenase in *S. aureus* and *B. anthracis* was found (Skaar, Gaspar et al. 2004; Skaar, Gaspar et al. 2006). Furthermore, it was surprising to find three Fur boxes within the locus of furfhuBGDCOR. Two distinctive Fur boxes were located between fhuD (lmo1959) and fhuC (lmo1960); these genes are transcribed in opposite directions. Within the poly-cistronic fhuBGD region, there was another Fur-box existing in the intergenic of region fhuD and fhuB. I noticed that fur itself was not included if using canonical sequence "TGAnAATnATTnTCA" for the sequence alignment. After I rechecked the conserved sequences of all the Fur-boxes in Gram-positive bacteria, I found this 15-bp sequence was less conserved in 11th and 14th nucleotides. After changing those less conserved nucleotides into "n" and using "TGAnAATnATnnTnA" again for alignment in search for putative structural genes with Fur-box, I found almost all the genes that were previously predicted to be iron-regulated, were included by this

alignment, including fur and fri, which encodes bactoferritin (Dussurget, Dumas et al. 2005). Therefore, even though systematic studies on deviations from the *E. coli* Fur box are relatively lacking, "TGAnAATnATnnTnA" that I found can be used as an effective sequence of query or even as Fur-box with good length and deviations to probe the putative iron-regulated genes during genomic alignment.

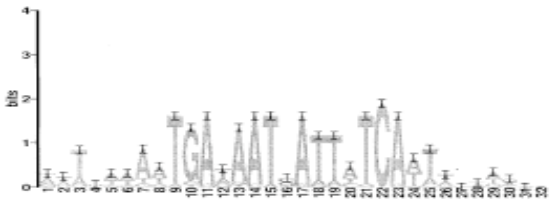


Fig 3.1 A Sequence-Logo representation of the most highly conserved bases in the aligned Fur box elements of *B. subtilis*. Adapted from (Panina, Mironov et al. 2001). This also reiterates the sequence of TGAtAATnATTaTCA, a 7-1-7 inverted repeat conserved among all the Fur-boxes in *B. subtilis*.

bp from start codon of gene		Gene translated pattern sequence	Fur-box
-	-44	lmo0362: similar to conserved hypothetical protein	ggtttcgatttagaattaac**tgataatgattatca**ttttcatttaaagaatggag
+	-39	lmo0365: similar to conserved hypothetical protein	gaacttggagaatatgataa**tgataatcattttca**attagaaaggaggatgaatg
+	-76	lmo0484: putative isdG	ccattccctaaaattgacat**tgagaatcattatca**atataatggaaggaactagc
-	-34	lmo0541: similar to ABC transporter (binding protein)	aattacttttgtaacgataa**tgaaaatcattttca**attagggaggaaatacacaa
+	-55	lmo1007:	ctataagatggttttaataa**tgataatcattttca**gttagaaatgattacttcaa
-	-179	lmo1006: similar to aminotransferases (to *B. subtilis* PatA protein)	ttgaagtaatcatttctaac**tgaaaatgattatca**ttattaaaaccatcttatag
+	-172	lmo1131: similar to ABC transporters, ATP-binding proteins	aaataaaaaataaatgacaa**tgagaatcattatca**aatgatgatttttgtgatat
-	-91	lmo1130: similar to transcription regulators	atatcacaaaaatcatcatt**tgataatgattctca**ttgtcatttatttttattt
-	-57	fhuB: similar to ferrichrome ABC transporter (permease)	gatataattttctttgcgat**tgataattattatca**cttaaaacgagcggataatt
+	-146	fhuC: similar to ferrichrome ABC transporter (ATP-binding protein)	aattgaacccctcctgtaac**tgataataattctca**gttagtatagcaactttatt
-	-33	lmo1959: fhuD similar to ferrichrome binding protein	aataaagttgctatactaac**tgagaattattatca**gttacaggaggggttcaatt
+	-42	fhuC: similar to ferrichrome ABC transporter (ATP-binding protein)	aaaactctatacttaaccat**tgagaatgattatca**ccttaactttaaattggagc
-	-137	lmo1959: fhuD similar to ferrichrome binding protein	gctccaatttaaagttaagg**tgataatcattctca**atggttaagtatagagtttt
+	-52	lmo2104: feoA	cgtgataaaatgaacatagt**tgataatgattatca**tgttcattacataacataaa
-	-121	lmo2186: svpA	aaataatctgttgttgacaa**tgataatcattatca**attaaaatgataattaacgt
-	-64	lmo2261: similar to unknown proteins	tttttgagaaattcttataa**tgaaaatcattctca**tatatgatacaataaatgta
+	-81	lmo2432: unknown protein	caaaaagacgaacccctaat**tgagaattattttca**tctatgtttaaatagtcgat
-	-89	lmo2431: hupD similar to *B. subtilis ferrichrome* ABC transporter fhuD precursor (ferrichrome-binding protein)	atcgactatttaaacataga**tgaaaataattctca**attaggggttcgtctttttg
-	-108	lmo2801: similar to a putative N-acetylmannosamine-6-phosphate epimerase	caactatccacgctaaaaca**tgaaaatcattttca**tttaattgatattgataaaa

Table 3.1 Fur boxes found by Sequence Alignment using an array of "TGAnAATnATTnTCA". Underlines in green are newly found iron regulated ABC transporters; highlight in red are putative iron-regulated iron transporters in this study.

3.2.2 Homologous Alignment of lmo1956 (fur)

The first systematic mutant that I made was Δlmo1956 (Δfur). Fur encodes a global iron regulator that regulates the genes of iron acquisition or siderophore synthesis. Deletion of this iron regulator was expected to have a great effect on the iron-regulated transport genes because their operons were preceded by Fur boxes. Also we wanted to observe any changes of expression for the genes of interest between iron-rich and iron-deficient conditions. After studying the listerial genome, we found lmo1956 seemed a good candidate to function as Fur because of its high homology to the known Fur of other species.

From **Table 3.2** and **Fig. 3.2**, lmo1956 has the highest similarity scores of 71% 74% to the FURs in those low G+C content Gram-positive bacteria, *S. aureus* and *B. subtilis,* respectively. In Gram-negative bacteria, Fur of *E. coli* and *S. typhi* seemed to be identical (similarity >=99%), but both of them showed only about 31% identity and 70% similarity to lmo1956. Also from detailed amino acid sequence alignment, four highly-conserved regions were found in Fur among all the strains. The C terminus indicates the presence of a conserved metal binding domain (HTHHHH) and two motifs (CXXCG and CXXXXC) involved in coordination with the binding metal ions and formation of the dimer. The N terminus contains a Helix-Turn-Helix (HTH) domain conserved among most of the metalloregulators, such as Fur and DxtR (Cook, Kar et al. 1998; Pohl, Holmes et al. 1999; Pohl, Holmes et al. 1999; Xiong, Singh et

al. 2000). From those alignment data, we believed that lmo1956 encodes listerial Fur, the global iron regulator in *L. monocytogenes*. Therefore, we deleted the gene.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	*B. subtilis*	149	2	*L. innocua*	152	74
1	*B. subtilis*	149	3	*L. monocytogenes*	150	74
1	*B. subtilis*	149	4	*S. aureus*	149	74
1	*B. subtilis*	149	5	*E. coli*	148	31
1	*B. subtilis*	149	6	*S. typhi*	150	30
2	*L. innocua*	152	3	*L. monocytogenes*	150	98
2	*L. innocua*	152	4	*S. aureus*	149	71
2	*L. innocua*	152	5	*E. coli*	148	31
2	*L. innocua*	152	6	*S. typhi*	150	31
3	*L. monocytogenes*	150	4	*S. aureus*	149	71
3	*L. monocytogenes*	150	5	*E. coli*	148	31
3	*L. monocytogenes*	150	6	*S. typhi*	150	31
4	*S. aureus*	149	5	*E. coli*	148	29
4	*S. aureus*	149	6	*S. typhi*	150	29
5	*E. coli*	148	6	*S. typhi*	150	97

Table 3.2 Homology scores of Fur by sequence alignment.

The score of the alignment is obtained and the expect value E for that score is calculated using statistical parameters previously found for gapped alignment using scoring matrix and gap penalty combination used in the similarity search. In ClustalW program, when generating the multiple sequence alignment, an identity matrix which gives a score of 10 to two identical amino acids and a score of zero otherwise, are supplied. The higher the score, the more similarity it is. But the score itself cannot be used as percentage of identity or similarity.

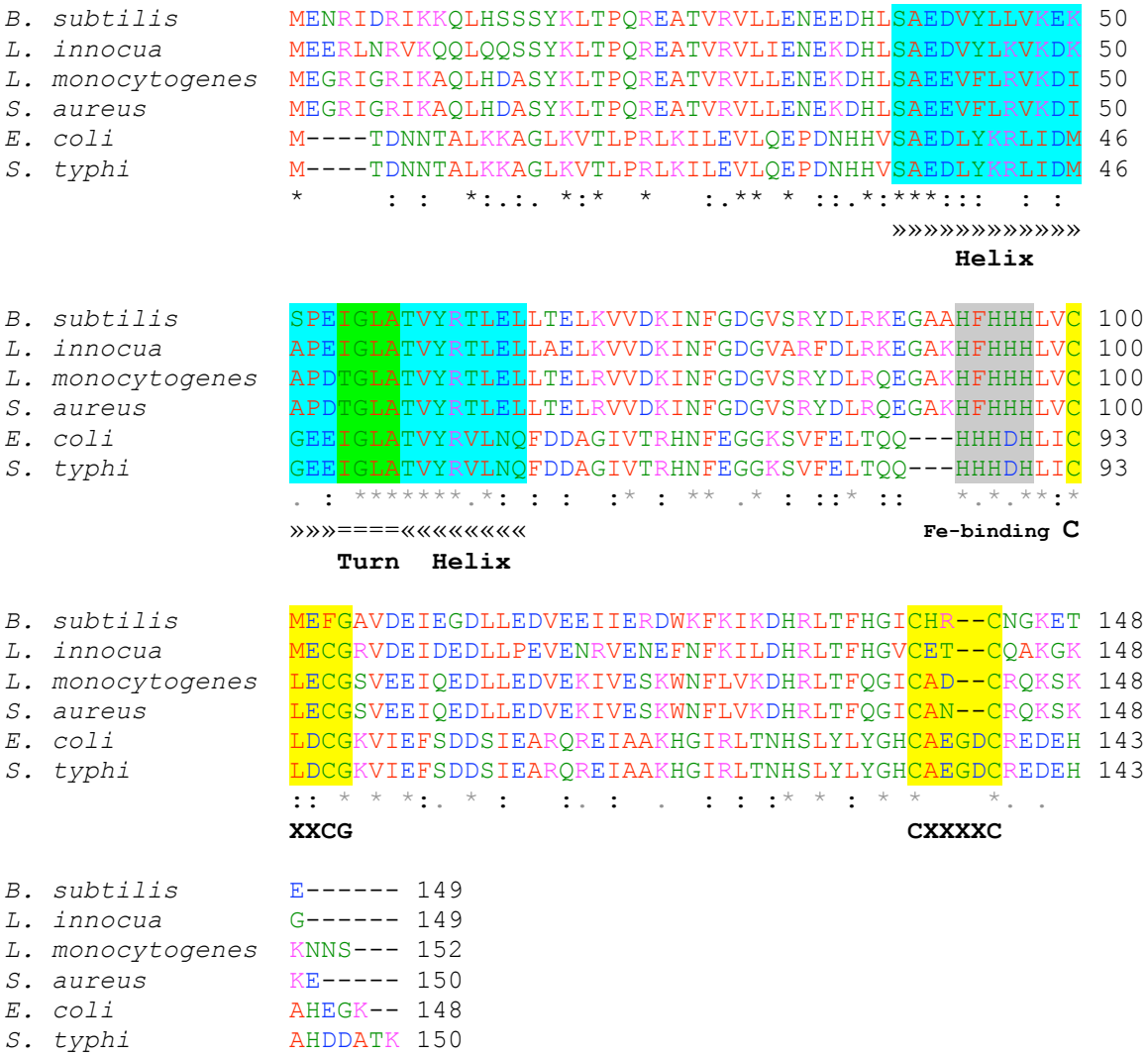


Fig. 3.2 Alignment of amino acid sequence of Fur (lmo1956) of *L. monocytogenes* with that of *B. subtilis, L. innocua, S. aureus, E. coli, and S. typhi* by the ClustalW program described by Thompson et al (1994). Highlights in blue and green are those consensus sequences of HTH (Helix-Turn-Helix); highlight in grey is conserved metal-binding motif (HXHHH); highlights in yellow are two (CXXC) motifs that coordinate metal binding and dimerization of the protein.

3.2.3 Homologous Alignment of svpA-srtB locus

The reason why we had special interests in svpA locus is that besides the facts that it has "Fur-box" and SvpA itself is a surface-associated virulence protein, svpA locus of *Listeria* is very similar to the isdC locus of *Staphylococcus aureus*, which has already been identified to participate in the uptake of heme. Therefore, we were eager to find out if this locus showed any phenotypes related with its ability to acquire heme or any other iron sources. Here is the comparison between svpA locus of *L. monocytogenes* and isd locus of *S. aureus*. (**Fig. 3.3**)

Similarities:

1) IsdC and lmo2186 (unknown function) share 33% identity. They are encoded at similar position of it own operon and have similar molecular weights.

2) Both loci contain a Fur-box in their promoter region.

3) Immediately downstream svpA and IsdC, there are three genes which resemble ABC-transporters in the CM possibly involved in iron uptake

4) In both loci, there are two genes encoding SrtA-dependent cell wall proteins with typical LPxTG motifs.

5) Both loci have genes encoding SrtB downstream the whole locus and they share 35% identity. Both SvpA and IsdC are anchored to the cell wall by downstream SrtB from the same locus.

Differences:

1) In front of svpA, there is a gene (lmo2186) encoding for unknown function, regulated by "Fur-box".

2) Cell wall proteins which seem to be SrtA-dependent in S. aureus have their own "Fur-box" and orient in the opposite direction of the isd operon.

3) The putative ABC-transporter clusters of lmo2184, lmo2183 and lmo2182 are more homologous to a typical ferrichrome transporter in IM of Gram-negative bacteria. (see blast *Listeria* genome at http://genolist.pasteur.fr/ListiList/).

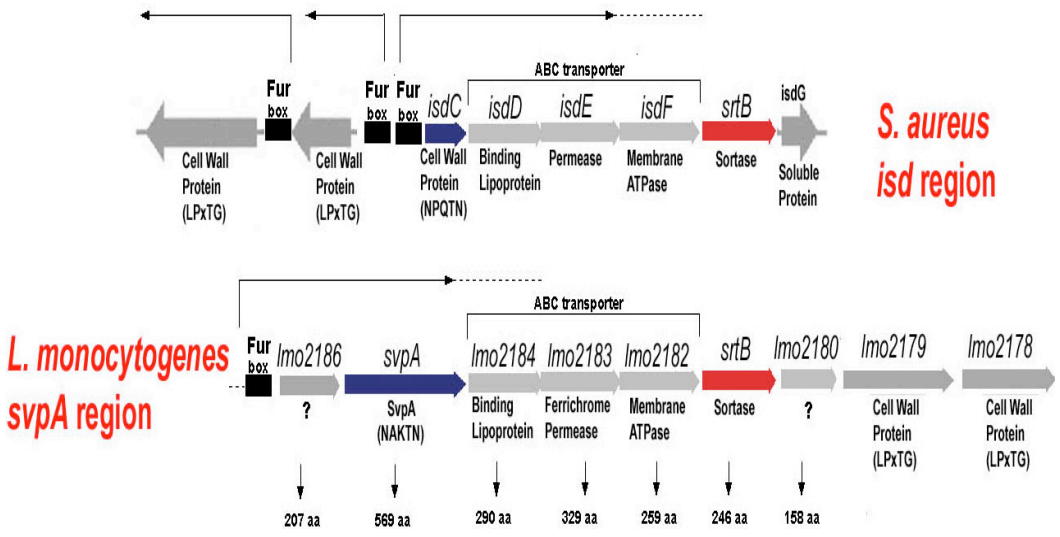


Fig. 3.3 Comparison of *L. monocytogenes* svpA region with *S. aureus* isd region

3.2.4 Homologous alignment of lmo1959

By homology alignment with the FhuD in the other Gram positive bacteria, lmo1959 seemed to encode FhuD in *L. monocytogenes.* First it had a typical signature motif of LXXC in the signal peptide region, with possible signal peptidase II cleavage. Also using the SignalP prediction program, it was further verified that the cleavage site of signal peptide should be between positions 19 and 20: VLT-AC, leaving the uncleaved cysteine on the N terminus to be lipidated and anchored to the membrane. (**Fig.3.4**) Even the overall sequence of lmo1959 shared only about 18% identity to the FhuDs in *S. aureus* and *B. subtilis*, it had up to 68% similarities to FhuDs in the above strains. The detailed alignment, (**Fig 3.5**) showed three conserved motifs, I, II, and III, which are present in a variety of Fe(III)-siderophore binding proteins.

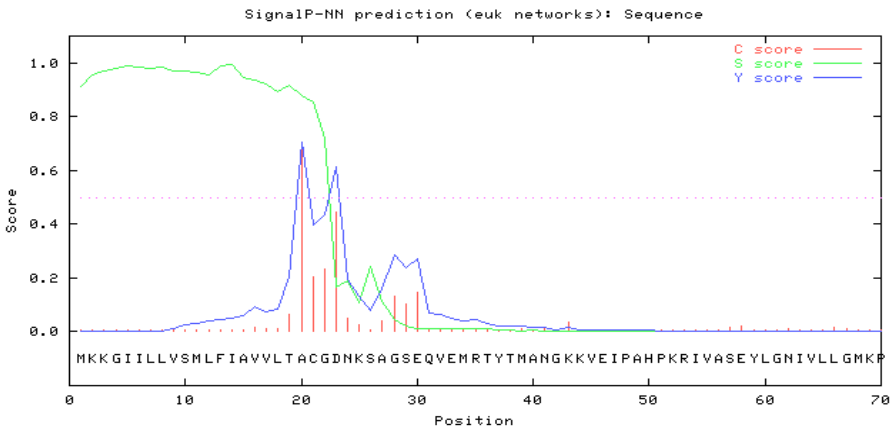


Fig. 3.4 Predictions of signal peptide sequence and cleavage site using SignalP

Likely cleavage site is between pos. 19 and 20: VLT-AC, with a cut-off probability of 0.48.

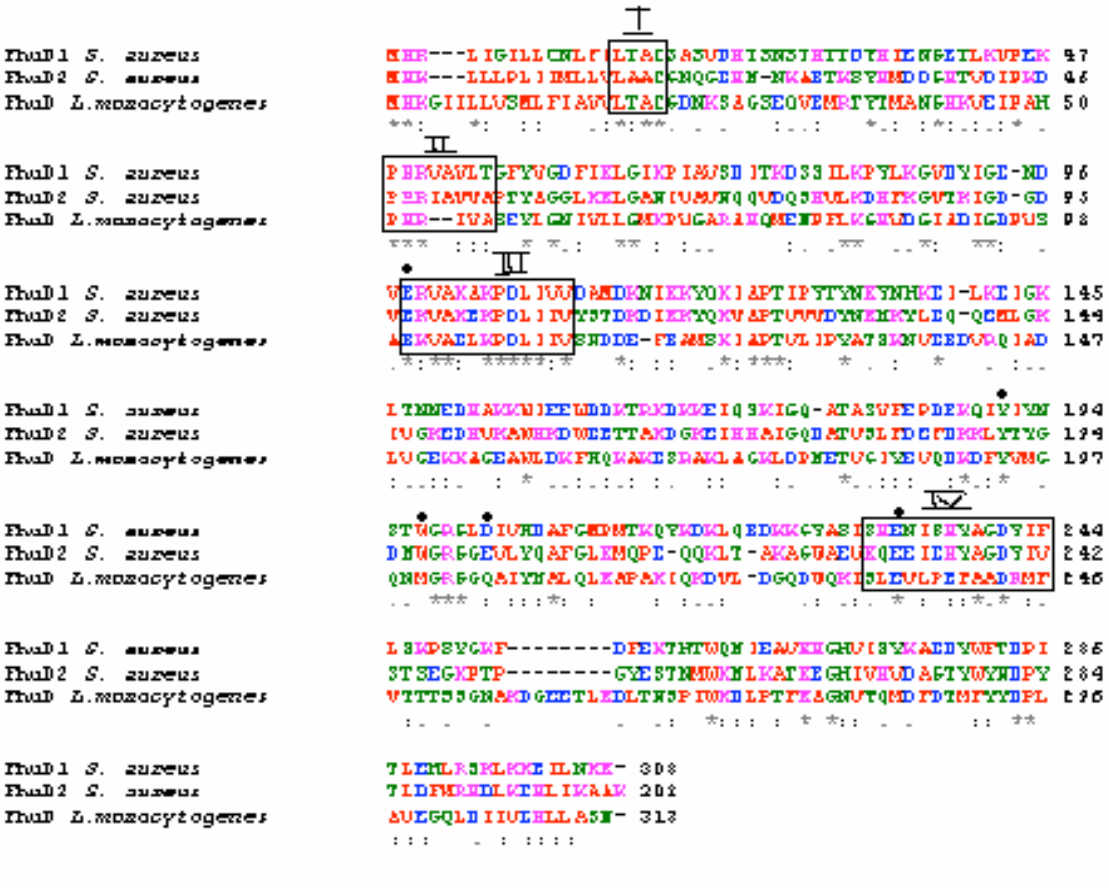


Fig. 3.5 Sequence alignment of FhuD (lmo1959) in *L. monocytogenes* with two FhuDs in *S. aureus*. The amino acid sequences were aligned using the ClustalW Program. Asterisk and light dots below the residues represent identical and similar amino acid residues respectively. Box I stands for the conserved signature motif (LxxC) within the signal peptide. Boxes II, III and IV stand for three conserved regions in heme or siderophore binding proteins (Braun, Kantke, et al. 1998). Five black dots above the residues are shown to contribute to the specificity of siderophore binding and transport by the FhuD2 in *S. aureus*. E97 and E231 are conserved residues that might be involved in interaction between FhuD2 and its cognate membrane counterpart. Y191, W197 and E202 are conserved residues involved in hydroxamate siderophore binding by FhuD (Sebulsky, Shilton et al. 2003).

3.3 Experimental Strategies

3.3.1 Transposon insertion of antibiotic-cassette VS. In-frame, full and clean deletion

To make chromosomal deletion mutant of bacterial strain, there are basically two different strategies that can be applied: one is called transposon mutagenesis and the other is called allelic replacement mutagenesis.

A transposon is a piece of DNA which can hop around within a genome (**Fig. 3.6**). If a transposon hops into the middle of a gene it will disrupt the gene. Many bacterial transposons themselves carry drug resistant genes. This offers an alternative, simpler strategy to screen the transformed bacterial cells for the acquisition of drug resistance by including the relevant antibiotic in the media. The advantages of this strategy is easy to handle and less time-consuming. Thus, transposon mutagenesis is quite efficient in making bacterial chromosomal deletion. However, sometimes it does cause some problems. The genes sometimes are not fully disrupted by insertion of transposons. Dr. Newton and her colleagues detected a small truncated form of SvpA that still could be recognized by the polyclonal SvpA antibody in the *svpA* mutated by insertion of transposon carrying kanamycin Tn7 cassette (EGD*ΔsvpA K7*). (**See Fig. 3.7**). The molecular weight of this truncated peptide was about 1/3 of the size of SvpA. The expression of truncated or misfolded OM proteins was already shown to cause toxicity in many gram-negative bacteria and show different physiology from the changes in the growth rate or virulence. Both changes of growth rate and attenuation

in virulence were observed from the *EGDΔsvpAK7*. However, the other chromosomal deletion mutant made by allelic replacement, *EGDΔsvpA,* was not shown to have such changes. In order to avoid such problems caused by transposon antibiotic cassette insertion, we chose to make a full and clean chromosomal deletion for *L. monocytogenes* mutants by applying allelic replacement in vivo.

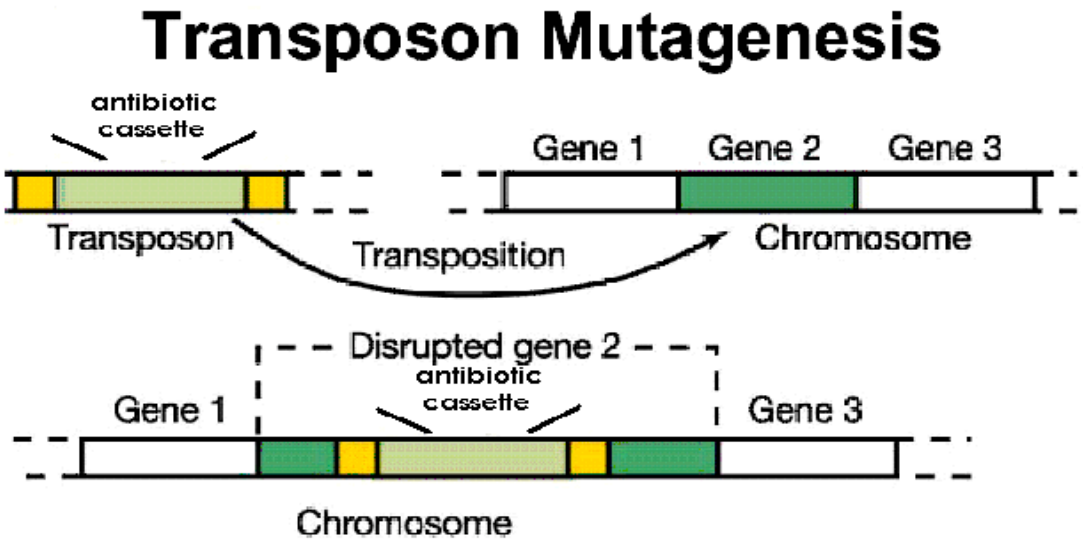


Fig. 3.6 Diagram of Transposon mutagenesis with insertion of antibiotic cassette

A typical engineered transposon: Two Insertion Sequences (in Yellow) + antibiotic resistance gene(s) (in Light Green). Transposon can hop around chromosome. Once it hops into the genes of interest on the chromosome by random, the gene is disrupted and its function might be completed lost.

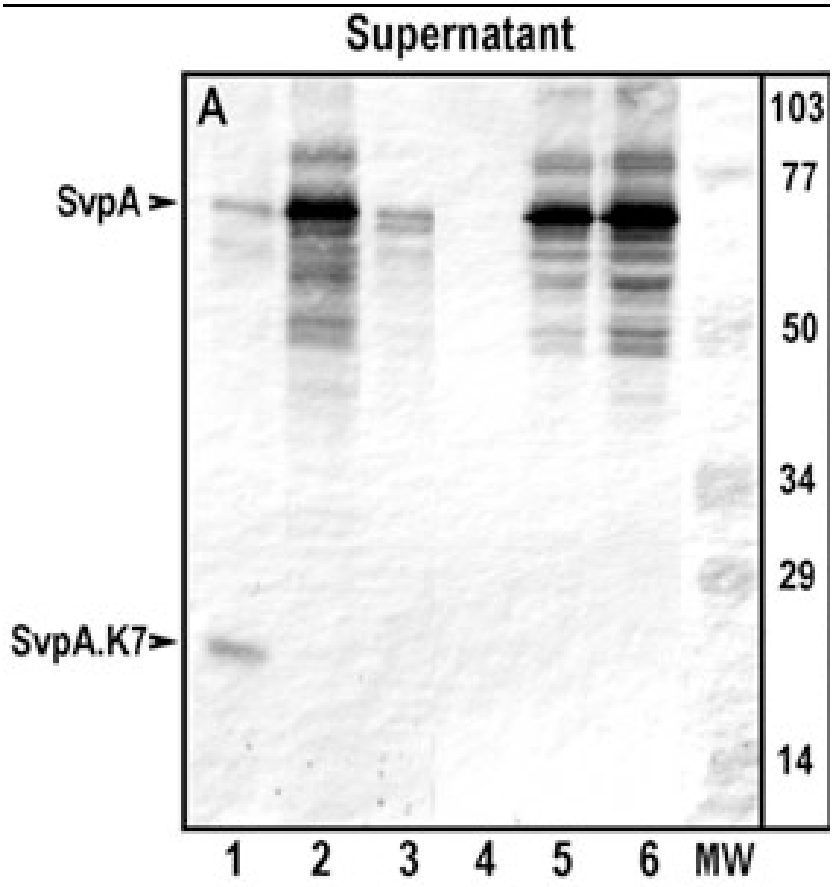


Fig. 3.7 A Western blot of BHI culture supernatant proteins from *L. monocytogenes* strains ΔSvpA.k7, EGD-e, ΔPROX5, ΔSvpA, Δ2186 and ΔFX3 (lanes 1–6 respectively) with anti-SvpA antibodies, showing the 20 kD truncated SvpA fragment in the ΔSvpA.k7. However, by allelic exchange mutagenesis of ΔSvpA, the SvpA is fully not detectable **(Newton, Klebba et al. 2005)**

3.3.2 Site-directed, in-frame, full and clean chromosomal deletion

fur (lmo1956)

To construct *fur* mutant, I first amplified two fragments with proper restriction digestion sites incorporated upstream and downstream of the gene lmo1956. After I digested the two PCR fragments with proper restriction enzymes, I ligated those two PCR fragments together and recovered from the agarose gel. (**See Fig. 3.8 A and B**) Then I tried to follow the general procedure as shown in Fig 3.7, to ligate this joint PCR fragment onto pKSV7 also digested with the same "extremity" restriction enzymes but no success. However, hardly any colonies were grown on the plates after electroporating ligation product into DH5α. So I religated the joint PCR fragment to another vector pUC18, which has the similar characteristics as pKSV7 but with much smaller size. After success in ligating joint PCR fragment onto pUC18, the vector with new construct was purified by midi-prep and digested with BamHI and PstI. The PCR1-PCR2 ligated fragment was cut and recovered from agarose gel and ligated onto pKSV7 again, with greater amount. The clone was transformed into *E. coli* strain DH5α and verified by PCR. Then this pKSV7 with new construct was purified and transformed in competent cells of *L. monocytogenes*. Bacteria were incubated first at 30 C and the transformants were verified by PCR colony test. Then the transformants were grown at 37 C with 5µgl/ml of chloramphenicol in BHI. pKSV7 only replicates as plasmid at 30C because its thermosensitive replication origin can only stand temperature no higher than 30C. At 37 C or above, the plasmid integrates into the

chromosome of *L. monocytogenes*. That is when the first recombination event happens. Then I kept passaging the integrants for at least 6 generations at the same temperature but without any chloramphanicol. Finally, I plated out the culture on BHI plates with a dilution of 10^6 or 10^7. Then I picked at least 200 colonies for chloramphenicol sensitive test. The mutants were checked by two extremity primers with appropriate size. (**See Fig. 3.8 C~E**)

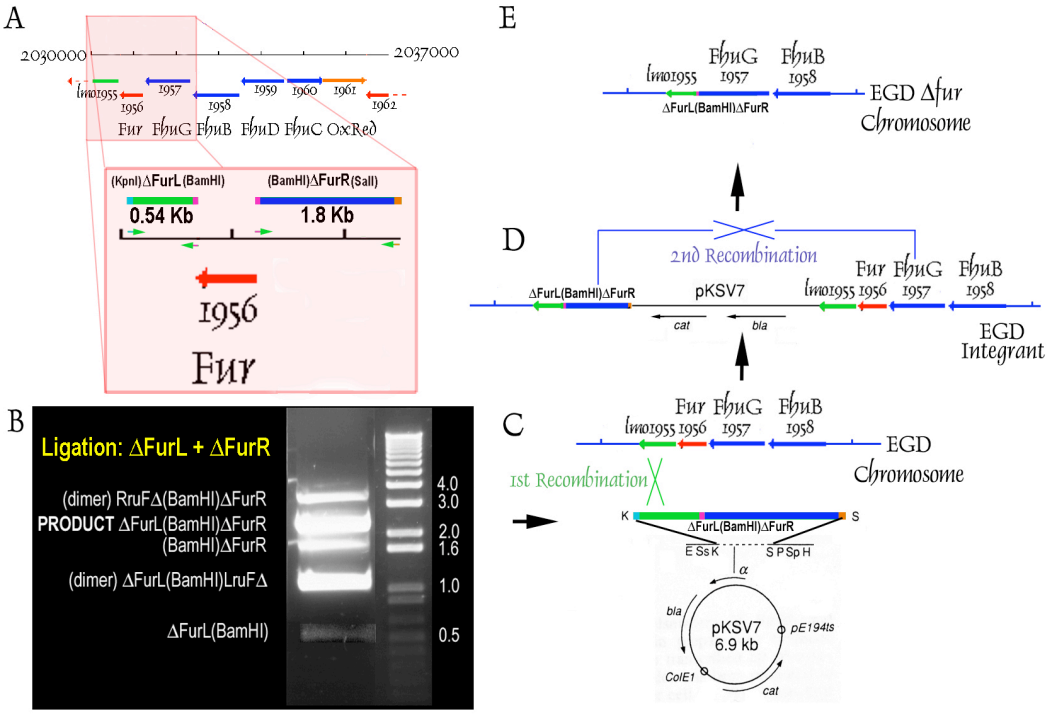


Fig. 3.8 Diagram of fur whole procedure. The figure depicts the method of allelic exchange that we used for construction of site-directed deletions (Newton, Klebba et al. 2005). A. Two chromosomal sequences, upstream and down stream of the gene of deletion interest, were amplified by PCR with appropriate restriction digestion sites designed to flank on both

ends of each PCR segment. B. After digested with restriction enzymes, they were joined together by ligase. C.The ligated fragment, with the elimination of my target gene, was cloned to a thermosensitive shuttle vector pKSV7. The pKSV7 carrying the deletion construct was transformed into *L. monocytogenes* and the new constructed vector was integrated into the chromosome by homologous recombination with DNA flanking the target gene at non-permissive temperature of 37°C with 5μg/mL of chloramphenicol. D. The integrant was passaged for at least 6 generations at 37°C without chloramphenicol and the second homology recombination occurred with the result of deletion of target gene on the chromosome. E. The mutant was screened by chloramphenicol sensitivity test. Mutants were verified by colony PCR with appropriate primers designed to show the size of the deletion.

hupC(lmo2429)

To make deletion mutant of lmo2429, I employed strategy slightly different from *fur* mutagenesis in the step of ligation. Instead of ligating those two PCR fragments first, I ligated those two PCR fragments, each digested with restriction enzymes designed, directly with pKSV7 that was digested and recovered from agarose gel. This strategy was called "Triple Ligation". (**Fig 3.9~10**) Unexpectedly, I immediately got the transformants containing the pKSV7 with two PCR fragments ligated together. The next steps followed the same procedure described above for *fur* mutagenesis.

fhuBG(lmo1957/1958)

To make deletion of lmo1957, which spans a large DNA length, I employed the same strategy used for creation of *hupC(lmo2429)*. There is only one difference in that I ligated the digested pKSV7 directly to the two digested PCR fragments with no recovery from the agarose gel. The general purpose of vector recovery from agarose

gel after digestion with restriction enzymes was to get rid of undigested supercoiled vector which might overpopulate the transformants of interest after electroporation and thus made it harder to screen for the transformants. However, the vector recovered from agarose after digestion sometimes did cause problems. It made following ligation hard to work. If with the gel recovery, there were only one or two white colonies per plate able to grow after transformation. However, if without gel recovery, there could be more than 200 white colonies per plate growing. 9 out of 20 colonies I tested by the latter method were verified to be the transformants with right size. However, the genes I wanted to delete seemed quite large and it caused much more difficult to get integrants and the final mutant might occur in a very low rate after the second homology recombination event. I am working on the screening of final mutant following the same procedure of in vivo allelic exchange in *L. monocytogenes*. We might still need to make deletion mutants of *lmo1957 (fhuG*) and *lmo1958 (fhuB)* individually if the deletion of *fhuBG (lmo1957/1958)* is too big to make.

fhuC/hupC(lmo1960/2429)

fhuC/hupC (lmo1960/2429) was the first double chromosomal deletion mutant that I made. The two genes were on the different loci. Both of them encode an ATPase of a typical ABC transporter respectively. I first transformed the vector pKSV7 containing PCR fragment generated from the previous *hupC(lmo2429)* cloning into competent *fhuC(lmo1960)* mutant. The next steps followed the same in vivo

integration and recombination procedures. The final double mutant was confirmed by fhuC and hupC check primer with the right sizes.

feoB(Δlmo2105)

I started to make *feoB* mutant after the plasmid pKSV7 containing feoB deletion PCR fragment was transformed into *L. monocytogenes* by Dr. Klebba in France. I followed the same procedures for the creation of other deletion mutants described above. I screened more than 300 colonies and eventually identified 2 out of 7 Cm sensitive colonies to be the *feoB* deletion mutant.

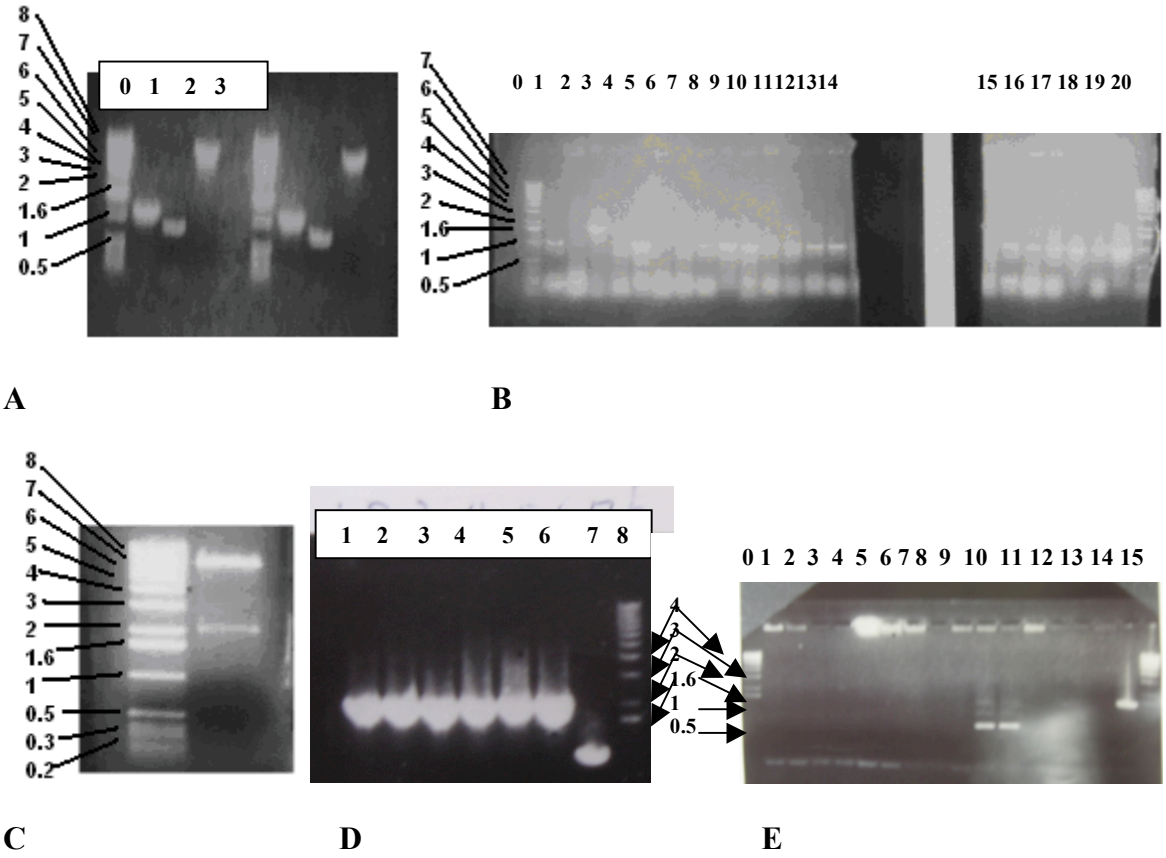


Fig. 3.9 1% agarose gel pictures showing the complete cloning process of *Δ2429* using triple-ligation strategy (numbers in each DNA marker are in the units of kb)

A. Double digestion of PCR1 (EcoRI/XbaI), PCR2 (XbaI/PstI), upstream and downstream gene 2429 respectively, and pKSV7 (EcoRI/PstI). Lane 0, 1kb ladder from invitrogen; lane 1, PCR1 ≈ 1.1 kb; lane 2, PCR2≈ 0.8 kb; lane 3 pKSV7≈7 kb.
B. Confirmation by M13 primers of transformants in *E. coli* DH5α after triple ligation. Lane 1: Ladder, Lane 1~20 Clones 1~20. Clone #3 showed a band of 1.9 kb, which correlated with the size of two PCR products after they are ligated. Thus Clone #3 was the transformant.
C. Double digestion of new constructed vector (EcoRI/PstI) again to confirm the two fragments ligated onto pKSV7. Lower band: Ligation product of PCR1+PCR2 ≈ 1.9 Kb; Upper band: pKSV7 ≈ 7 Kb.
D. Confirmation of listerial transformants by M13 primers. Lane 1~6, 6 listerial clones picked; Lane 7, as negative control EGD-e wild type clone amplified by LLO check primers; Lane 8 Ladder.
E. Confirmation of the final chromosomal deletion mutant with newly designed check primers. Lane 0, Ladder; Lane 1~14, Cm sensitive clones in which clones 10 and 11 showed to be the mutant with right deletion size; Lane 15, negative control EGD-e amplified by LLO primers.

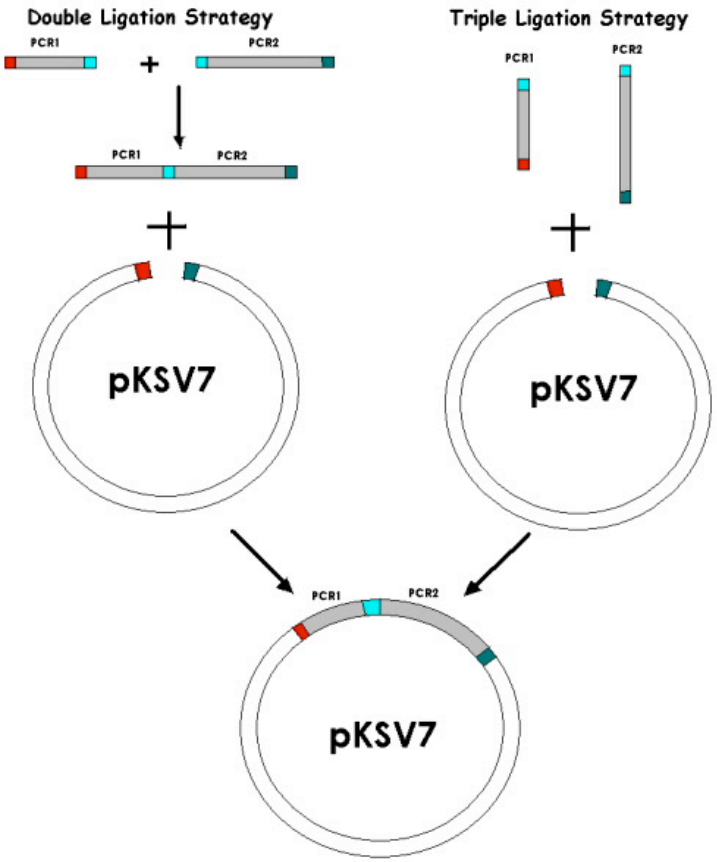


Fig. 3.10 Diagram of Double-fragment ligation and triple-fragment ligation.

Left: double-fragment ligation strategy. Two PCR fragments, PCR1 and PCR2, each digested with two restriction enzymes engineered on two ends, were first ligated together. Then the joint PCR fragment was ligated onto the vector pKSV7, which was digested with the same two extremity enzymes.

Right: triple-fragment ligation strategy. Three fragments, PCR1, PCR2 and pKSV7, each digested with two restriction enzymes, were ligated all together with proper ratio.

3.3.3 Confirmation of chromosomal deletion mutants

To check all the important mutants of EGD-e we constructed in our lab, I designed the two primers (called "check" primers for certain deletion), each of them was complimentary to the sequence within 0.5 kb upstream and downstream deleted gene, to show the size of the deletion. The control was always the EGD-e wt strain. The difference in sizes of PCR colony test between mutant and wild type showed the size of gene of interest we deleted. Mutants were also sequenced.

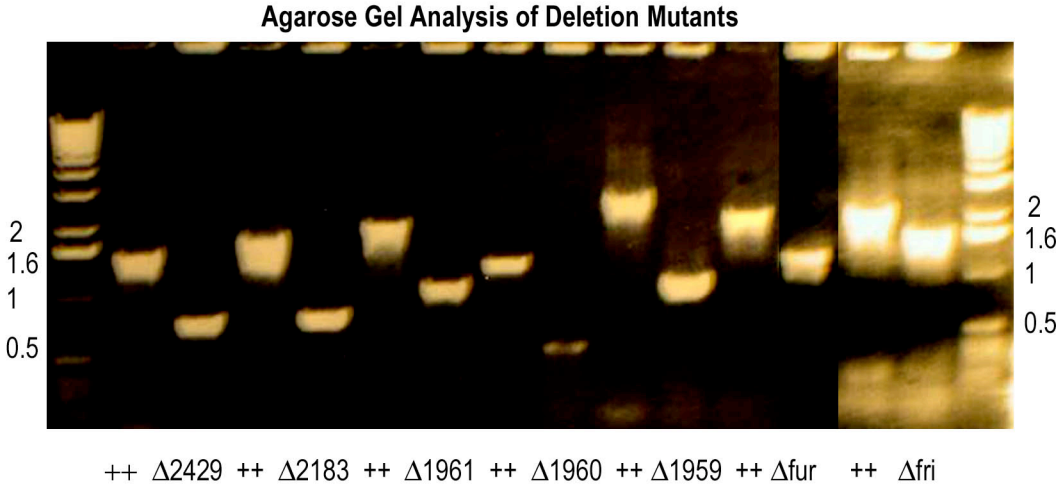


Fig. 3.11 Agarose Gel Analysis of Deletion mutants.

Mutants were confirmed by check primers designed. Every two lanes showed PCR products with the same check primers designed to show the size of the deletion. " ++ " stands for the EGD-e wild type. Numbers shows the size of ladder in Kb.

3.4 Discussion

Since no specific genes encoding iron-regulator transporter were identified *L. monocytogenes* before this project started, a genomic approach by combining denotation of open reading frames flanked by Fur regulatory sites with sequence analysis and structural predictions seemed to be an effective way to identify a fraction of candidate genes that encode cell envelope proteins negatively iron-regulated by Fur. Even though the overall length and the complete sequence of Fur-box still remains in debate, using "tganaatnatnntna" for a quick survey of listerial genome empirically revealed more than 30 open reading frames adjacent to Fur-box. However, from P.E. Klebba's personal data showed that some proteins whose synthesis was enhanced by iron repletion but obviously Fur-independent. Furthermore, enough evidence also showed that not all the Fur-dependent genes were as iron-regulated. They can be involved in some other metabolic stress pathways. Therefore, DNA Sequence alignment or protein homology comparative analysis can be quite useful but not adequate tools to determine a gene's function until we eliminated it.

Given the large number of genes we were interested to delete, the insert ional mutagenesis was first thought to be able to disrupt genes quickly and allow a rapid analysis of the roles of those genes. However, this approach can be problematic in three ways: (1) a truncated protein still can be expressed and hard to be detected if the gene to be disrupted is quite large and thus this non-specifically expressed protein might interfere with the normal growth of the strain and affect the virulence of the

strain in mouse model; (2) gene disruption by insertion of a transposon cassette can be unstable and phenotype of mutant can be reversible to its wild type. (3) Even a creation of mutants with a stable gene disruption sometimes appears to have polar effect, which is, to affect the expression of downstream genes. However, even though creation of in-frame chromosomal deletion by in vivo allelic exchange seemed to be laborious and time-consuming, it was full and clean deletion and could avoid the above problems caused by insertional mutagenesis. While learning the methodologies with Drs. Klebba and Newton, we improved the whole methodology by incorporating strategy of triple-fragment ligation. (**See Fig. 3.10**) Among all the steps, ligation seemed to be the most critical step to the success of creating in-frame chromosomal deletion by in vivo allelic exchange. Ligating the two PCR fragments first could significantly reduce the amount of joint PCR fragment for the further successful ligation with the vector, especially after recovery of the fragment from the agarose gel. Therefore, ligation is the rate limiting step of the whole process. In addition, gel recovery is also another important step to determine the success of mutagenesis. Normally the agarose gel recovery for vectors after restriction enzyme digestion was to get rid of undigested supercoiled vector in case they might overpopulate the transformants of interest after electroporation and thus made it harder to screen for the transformants. However, this step also could cause problems. Usually few colonies were observed to grow after gel recovery step. It could be possible that gel recovery somehow caused to lose some of the sticky ends of DNA product after restriction

enzyme digestion or some unremoved agarose gel was still left in the vector ligation product and could be toxic to the transformed cells. Furthermore, since all shuttle vectors (pKSV7, pAUL-A and pMAD) we used for listerial in vivo allelic exchange were very large (about 7 to 10 kb), it was extremely difficult to make a good estimate of relative ratio between vector and segment that were to be ligated. To minimize the loss of product we wanted to ligate and decrease chance of introducing anything toxic to the final product, triple-fragment ligation without any agarose gel recovery seemed to be very effective and reduced the whole amount of input time for chrosomal deletion mutagenesis from 3-6 months to less than 1 month.

Chapter 4

Phenotypic characterization of mutants

4.1 Quantitative analysis of SvpA

4.1.1 Deletion of srtB on over-expression of secreted SvpA

SvpA (lmo2186) was the first gene in the svpA-srtB locus whose promoter region contains a highly conserved Fur box. Even though whether SvpA functioned as a surface virulence factor in *L. monocytogenes* (Borezee, Pellegrini et al. 2001; Newton, Klebba et al. 2005) was still in debate, its promoter region was found tightly controlled by iron concentration and the regulator Fur (Newton, Klebba et al. 2005). The expression of SvpA was strictly regulated by the concentration of iron that at iron-deficient condition, (iron was chelated by BP), a second, lower molecular weight band appeared when using Anti-SvpA antibody to immunoblot against SvpA. Also SvpA was verified to be anchored to the cell wall through its NAKTN motif. However, only less than 10% of SvpA is anchored to the cell wall while 90% of it is secreted. As we precisely eliminated the complete srtB gene and the resulting strain was analyzed for expression of secreted SvpA in BHI, BHI + BP, and BHI +BP+Fe. In the iron-depleted condition (BHI+BP), The SvpA was observed to be overexpressed with a second and lower MW band showing up. However, even though the culture was first grown in iron-depleted condition, when rendered with Fe (BHI+BP+Fe), the expression of SvpA returned to its original level, the same as iron-rich condition

(BHI). (**Fig. 4.1**) However, the Δ*srtB* increased production of secreted SvpA in both iron-rich and iron-deficient media, compared with that in EGD-e wild type, and a second lower MW band showed up again. But the addition of BP did not increase it more. (**Fig. 4.1 and 4.2**)**.** It is possibly because in Δ*srtB* mutant, no more SrtB exists to help SvpA anchor to the cell wall and thus all the SvpA expressed is secreted, as in Δ*srtA* mutant, more SvpA was expressed in iron depleted condition. And deletion of promoter region (Fur-box included) of svpA-srtB operon prevent SvpA from expression. And there was not any SvpA found in *ΔPROX5* deletion.

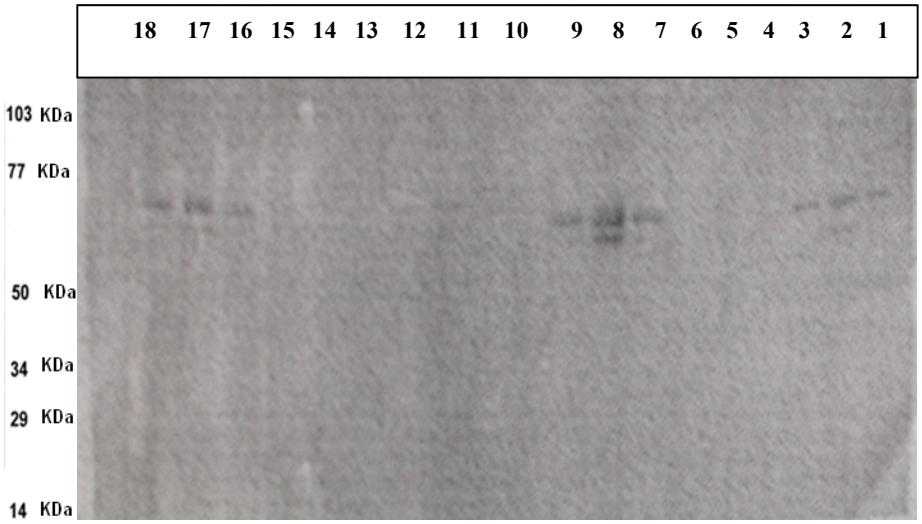


Fig. 4.1 Immunoblot of secreted SvpA developed by ^{125}I-Protein A using Fe-citrate as iron source.

Expression of SvpA in different media. Culture supernatants from EGD-e and its mutant derivatives were concentrated by TCA precipitation and subjected to SDS-PAGE and Western immunoblot with rabbit anti-SvpA

Lane 1~9 sample loaded in 15 µl, lane 10~18 the same sample loaded in 5 µl.

1. EGD + BHI
2. EGD + BHI + BP
3. EGD + BHI + BP + Fe-Citrate
4. *ΔProX5* + BHI
5. *ΔProX5* + BHI + BP
6. *ΔProX5* + BHI + BP + Fe-Citrate
7. *ΔSrtB* + BHI
8. *ΔSrtB* + BHI + BP
9. *ΔSrtB* + BHI + BP + Fe-Citrate

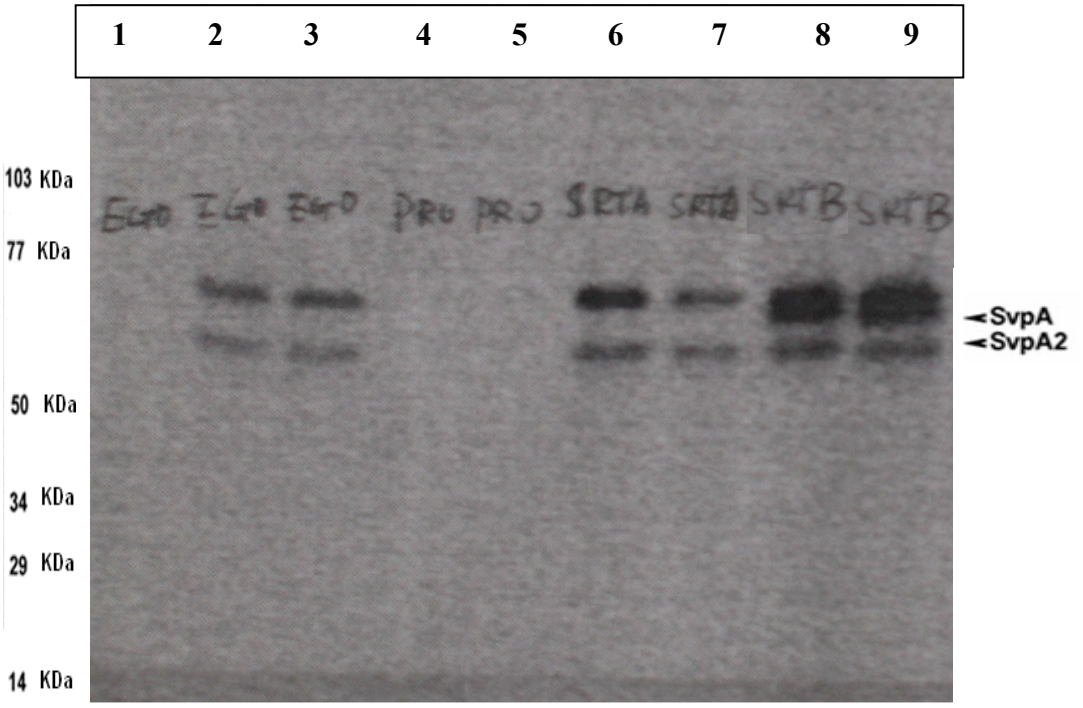


Fig. 4.2 Immunoblot of secreted SvpA developed by ^{125}I-Protein A using $FeSO_4$ as iron source.

Expression of SvpA in different media. Culture supernatants from EGD-e and its mutant derivatives were concentrated by TCA precipitation and subjected to SDS-PAGE and Western immunoblot with rabbit anti-SvpA.

Lane #:

1. EGD + BHI
2. EGD + BHI + BP
3. EGD + BHI + $FeSO_4$
4. *ΔProX5* + BHI + BP
5. *ΔProX5* + BHI + $FeSO_4$
6. *ΔSrtA* + BHI + BP
7. *ΔSrtA* + BHI + BP + $FeSO_4$
8. *ΔSrtB* + BHI + BP
9. *ΔSrtB* + BHI + BP + $FeSO_4$

4.1.2 Deletion of fur on over-expression of SvpA

We deleted the putative fur gene of *L. monocytogenes*, *lmo1956* (*ΔFur*). Fur negatively regulates the transcription of bacterial proteins involved in iron acquisition (De Lorenzo, Herrero et al. 1988). However, the identity of fur in *L. monocytogenes* was unknown when the sequential chromosomal deletion experiments began. But from our computer-based genomic analyses in chapter 3 (**Fig.3.2 and table 3.2**), we identified that lmo1956 had a very high homology (around 75% of similarity) to fur of *B. subtilis* and *S. aureus*. We precisely eliminated the complete fur gene and analyzed the resulting strain for SvpA expression in BHI and BHI + BP. The Δ*fur* increased production of SvpA in both iron-rich and iron-deficient media, compared with that in EGD-e wild type, but the addition of BP did not increase it more. And deletion of promoter region (Fur-box included) of svpA-srtB operon prevent SvpA from expression. SvpA reached maximum levels in culture supernatants when iron was sequestered by BP, or when Fur was deleted. This was again when a second, lower molecular weight form of the protein (SvpA2), appeared in the immunoblots. SvpA2 seemed to be associated with overexpression in response to either iron-depleted or the absence of Fur **(see Fig. 4.3**).

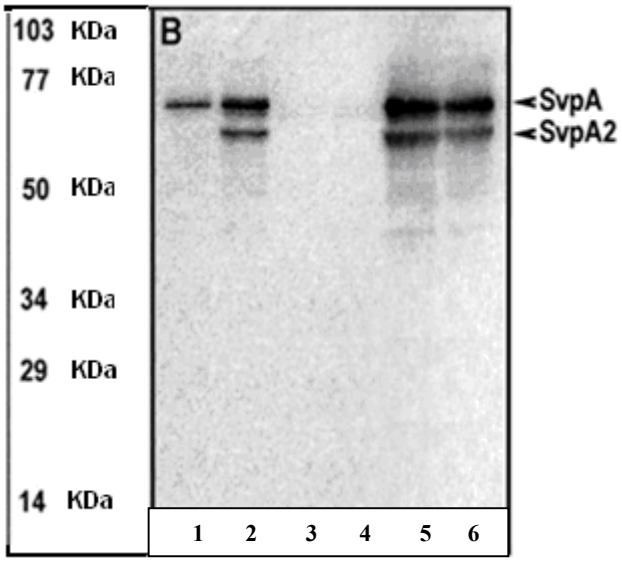


Fig. 4.3 An immunoblot of supernatant SvpA developed by ^{125}I-ProteinA

Expression of SvpA in different media. Culture supernatants from EGD-e and its mutant derivatives were concentrated by TCA precipitation and subjected to SDS-PAGE and Western immunoblot with rabbit anti-SvpA.

In EGD-e wild type (Lanes 1 and 2), *ΔPROX5* (Lanes 3 and 4), and *Δfur* (Lanes 5 and 6). Samples in BHI were in odd lanes and samples in BHI with 1mM BP were in even lanes.

4.2 Siderophore nutrition test of mutants

Siderophore nutrition test is a qualitative assay to determine the ability of *L. monocytogenes* wild type strain and its derivative mutants to utilize different iron sources for their growth. We used BP as iron chelator in both liquid and solid media. The bacteria was first grown overnight and subcultured in 1:100 in BHI media until OD600 reached between 0.1~0.2. Then BP was added to the culture to a final concentration of 1 mM. The culture was grown for a few hours until it reached to mid

log. Then 200 ul of each strain was put into 8 ml of softened BHI top agar +1mM BP, mixed well and poured out into a plate. After agar became solid, a paper disc with certain amount of iron source was put into the plate. After overnight incubation at 37 C, halos should be found for those strains of bacteria that are able to utilize certain iron sources.

Results of nutrition tests (**Fig. 4.4 and Table 4.1**) showed EGD-e wild type could utilize all the iron-sources we tested here. Even though we could not show halos of bacterial growing by utilization of FeEnt and FeCrn by nutrition test, we knew EGD-e wild type was able to use those two iron source from the later ^{59}Fe-siderophore uptake assay testified by Bo Jin. All the constructed Listerial mutants could utilize all the compounds of iron sources more or less except that *ΔfhuD (Δ1959)* and *ΔfhuC (Δ1960)* could not utilize ferrichrome, ferrichrome A and ferrioxamine B (the three hydroxamate siderophores we tested here) and *ΔhupC (Δ2429)* could not utilize Hemin and hemoglobin. Furthermore, relatively smaller but brighter halos were found all in Hn and Hb groups. This was probably because Hn or Hb was very poorly diffused around paper disc, which made the local concentration of Hn or Hb relatively higher and colonies growing on it appeared to be denser. However, the deletion of genes in svpA-srtB locus didn't show any defects in utilization any iron-sources.

Furthermore, the double deletion mutant that I made, *ΔhupC/fhuC (Δ2429/1960)*, was also observed unable to utilize both Hn/Hb and Ferrichrome. (**Fig. 4.5**)

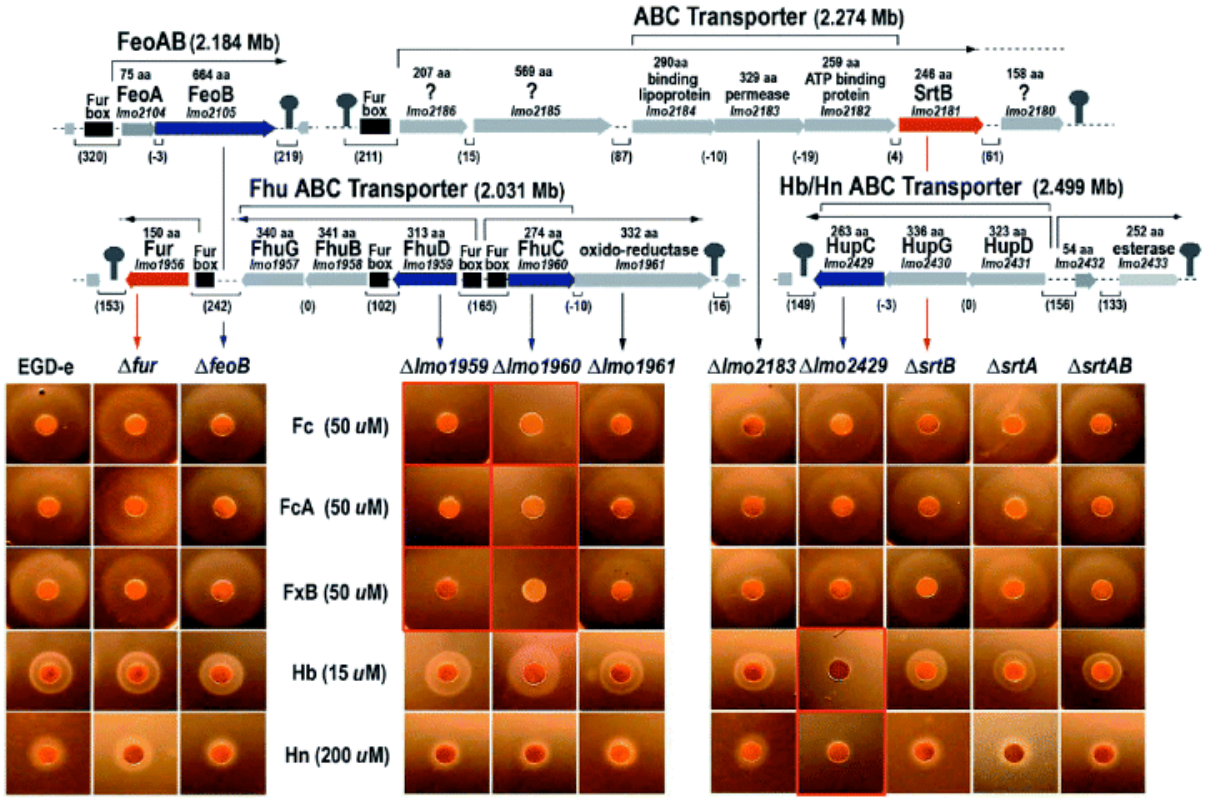


Fig. 4.4 Chromosomal loci of interest and nutrition tests with ferric hydroxamates, Hn and Hb. (Jin, Newton et al. 2006)

Chromosomal loci of interest (above) and nutrition tests (below) with ferric hydroxamates, Hn and Hb. Four loci that contain Fur binding sites and encode potential transport systems were studied for their participation in iron uptake. Siderophore nutrition tests revealed that Δ*fhuD* and Δ*fhuC* strains lost the ability to transport Fc; the *hupDGC* locus (2.499 Mb) encodes a third ferrichrome-like ABC transporter from listerial genome database; deletion of the gene *hupC*, which encodes a putative membrane ATP binding protein, prevented *L. monocytogenes* from acquiring iron from Hb and Hn. The nutrition tests shown below were performed in BHI top agar containing 0.1 mm BP.

Table 4.1 Siderophore nutrition tests and mouse infection experiments with EGD-e and its mutant derivatives.

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

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. The concentration of each siderophore in the unit is of micromolar. The results of 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 triplicate tests, which had minimal variation. NA, not applicable; ND, no data. (Jin, Newton et al. 2006)

EGD-e W.T ***ΔfhuC(Δlmo1960)***

ΔhupC(Δlmo2429) ***ΔfhuC/hupC(Δlmo1960/2429)***

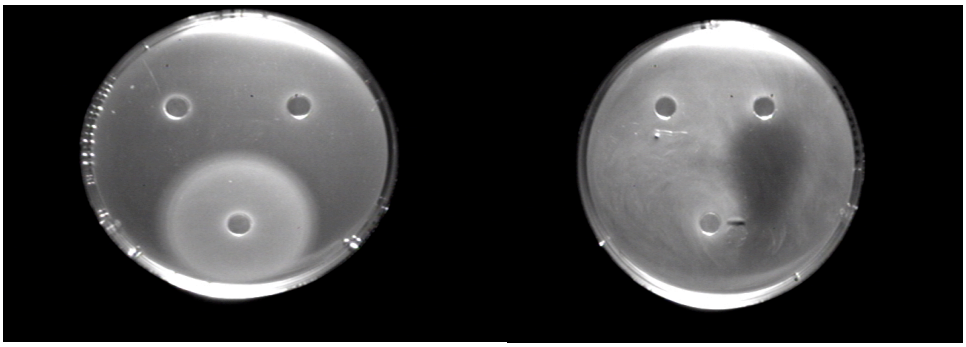


Fig. 4.5 Nutrition test of double mutant *ΔfhuC/hupC(Δlmo1960/2429)*

In the paper discs in each plate, the top left was rendered with 15 uM Hemoglobin; top right was rendered with 200uM Hemin; bottom was rendered with 50 uM Ferrichrome. EGD-e showed to utilize Hn, Hb and Fc; *ΔfhuC* displayed to be unable to use Fc; *ΔhupC* showed defects in using Hn/Hb; *ΔfhuC/hupC* showed to be unable to use all of them.

4.3 Growth curves of mutants

After all the derivative mutants were constructed, their growth was studied in either BHI or KRM media. Only *fur, fhuC(lmo1960), fri, fhuC/hupC* were found to have serious defects in growth (**Fig 4.6**). *feoB* showed slightly retard in growth. All the other mutants showed to grow normal, compared with EGD-e wild type. Even though *Δlmo1959 (ΔfhuD), Δlmo1960 (ΔfhuC), Δlmo1961 (Δor)* were from the same locus, only *Δlmo1960 (ΔfhuC)* showed retard in growth, suggesting that this locus that was predicted to be involved in hydroxamate siderophore uptake, might have the same traffic ABC membrane transporter but may have different hydroxamate-binding lipoproteins. The deletion of one of lipoproteins may not impair the function of the other lipoprotein if they share the same membrane ABC transporter. However, deletion of traffic membrane components severely may disrupt the function of the whole transport system.

On the other hand, Fur is defined by its function to regulate iron uptake as well as control some other oxidative stresses upon gene expression. For this reason, a *fur* mutant may have a higher influx of iron or higher gene expression of many genes than a wild type strain and thus grow more slowly than a w.t. strain. This phenotype has been reported in many other strains (Rea, Gahan et al. 2004).

fri encodes for bactoferritin, the iron storage protein. It was reported the elimination of bactoferritin retarded the bacterial growth in *L. monocytogenes*.

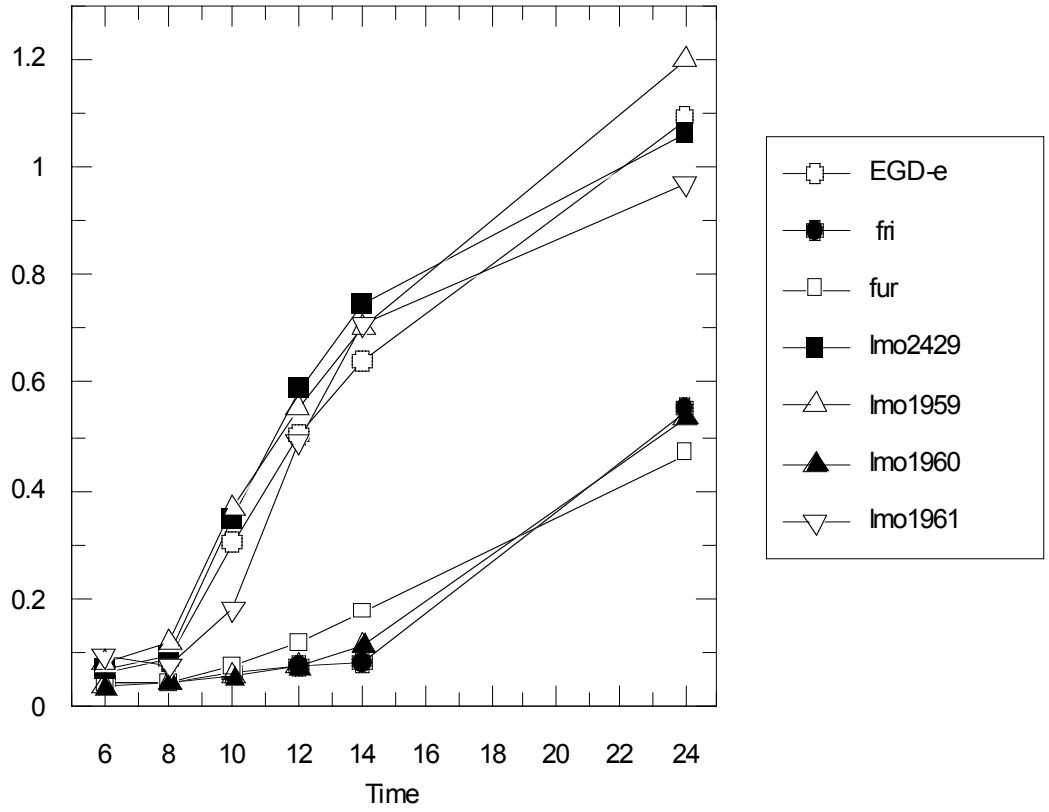


Fig. 4.6 The study of derivative mutants' growth curves in KRM.

Only *lmo1960 (hupC), fur, fri* showed retard in growth while all the other mutants was not observed to have any impair in growth.

4.4 Virulence study of mutants

I made virulence studies of three different mutants (*ΔfeoB, Δfur* and *Δ1959*) in the mouse model (**also see table 4.1**). *Δfur* showed a 3 log decrease in LD_{50}. *ΔfeoB and Δ1959* showed no attenuation of virulence. However the mutant *hupC* that I constructed was tested t by Dr. Newton and showed a 2 log decrease in LD_{50}.

4.5 Discussion

Transport of iron from the environment milieu into the bacterial cytosol involves translocation of molecules across the 50-100 nm diameter of the cell wall envelope and cytoplasmic membrane of *L. monocytogenes*. At least three iron-regulated ABC type transporter systems were described by listerial genome database in both pathogenic *Listeria monocytogenes* and non-pathogenic *Listeria innocua* (**http://genolist.pasteur.fr/ListiList/**) after the genomic sequencing was completed in 2001 (Glaser, Frangeul et al. 2001). Those ABC transporters are hypothesized to utilize ferric hydroxamate siderophores via a sequential process of a surface receptor to bind siderophores, membrane permease to translocate siderophores that are still complexed with iron across hydrophobic membrane bilayer, and an ATPase to provide energy by hydrolysis of ATP. Besides interests in determining the mechanism of iron transport system, we were also interested in finding new virulence factors involved in iron transport. This is because many virulence factors of bacterial pathogens are surface associated or secreted proteins. In Gram positive bacteria, sortaseA, the first sortase of this kind, anchors proteins to the cell wall at C- termini through the sorting motif of LPxTG consensus in many cell-wall-based proteins. Sortase A is believed to function as a major enzyme to anchor many cell-wall virulence factors because the deletion of the srtA attenuated the whole cell virulence. A second class of sortase, sortase B, was found in both *S. aureus* and *L. monocytogenes*. Contrary to sortase A, which recognizes wide range of proteins, sortase B only works on a few proteins,

among those SvpA was found SrtB dependent in *L. monocytogenes*. However, SvpA, even though was initially named as a surface virulence protein, was found to have nothing to do with virulence. But its expression was hypothesized to be iron-regulated because of the Fur-box upstream the gene. In order to test that hypothesis, we first made make a *fur* mutant. Fur encodes the global iron regulator protein that not only regulates the genes within its own operon but all the operons or genetic loci that may be iron-regulated. Using fur mutant, we first characterized the regulation of svpA-srtB in iron-sufficient and iron-deficient conditions and we also determined the effects of *fur* and *srtB* deletions on SvpA synthesis in the above conditions. Those experiments demonstrated that iron-availability regulated the svpA-srtB locus, mediated by Fur. However, we didn't find any phenotypes of specific iron sources that this ABC transporter was able to utilize even after each gene of this locus was deleted. The function of svpA-srtB locus are still under study, Whether this locus is involved in iron is still in debate even though in vitro the SvpA was able to bind with hemin in solution and SvpA's expression is in response to the iron concentration,. Among four loci that we expected to be putative iron-regulated membrane transporters by genomic analysis, only furfhuBGDCOR locus were shown to confirm with what we expected, a typical hydroxamate transporter; svpA-srtB locus showed no phenotype in terms of iron transport even though the locus itself displayed to be very homologous to isd locus in *S. aureus*; to our surprise, the other locus fhuDGC, which was predicted to be ferrichrome-like ABC transporter, was tested to be involved in hemin/hemoglobin

uptake. This result again showed that computer-based genomic comparative analysis is useful but not adequate an approach to determine the complete function of a gene or an operon. Even though no obvious phenotypes were found in svpA-srtB locus, we still could not conclude that it was not involved in iron transport because much evidence showed that this operon is strictly iron-regulated. It could be because of limited sorts of siderophores we tested here. Or maybe this locus is involved in transporting some metabolites that may level up oxidative stress which is also regulated by Fur and iron concentration. It was reported that Fur also regulates a variety of iron-dependent cellular processes, such as the acid-shock response and oxidative-stress response. *fur* mutant was also found to grow slowly than the wild type, which showed the same result from Hill et al (Rea, Gahan et al. 2004). This is possibly because deletion of Fur, a global iron regulator, changes many genes' expression. The attenuation of *fur* again supported the idea that iron acquisition is an important determinant for a bacterial pathogen to survive in a host.

L. monocytogenes, the intracellular pathogen, has shown to have increased virulence in mice loaded with iron, an effect which is reversed by removing iron in the host using iron-chelator. Iron availability may therefore be of great importance for this pathogen. *L. monocytogenes* is widely distributed in the environment and does not produce siderophores. So even though there are lots of similarities between those ABC type transporters and known siderophore-uptake transporters in both Gram-positive and Gram-negative bacteria, it does not aid the identification of their substrates. As

shown in our data that three loci, only two showed impairs in iron uptake while the third one, which had homology to isd locus in S. aureus, didn't show any impair of iron uptake.

In aerobic and neutral pH conditions, iron exists predominantly as Fe(III). However, Fe(III) is insoluble and cannot be directly assimilated. Under anaerobic conditions, iron exists in the Fe(II) oxidation state. Fe(II) iron is highly soluble and can be diffused freely through the outer membrane porins of gram-negatively bacteria or the thick cell wall of gram-positive bacteria, and thus becomes bio-available to the anaerobes. Then this ferrous iron usually is transported through the cytoplasmic membrane by a feo transport system conserved in many species.

Chapter 5

Characterization of the binding specificity of FhuD (lmo1959) in *Listeria monocytogenes*

5.1 Purification of FhuD.

I used pET28(a), which is a very common expression vector, to purify listerial FhuD. (**Fig 5.1**) 1.1 kb of *fhuD* gene was PCR-amplified and cloned into the HindIII/EcoRI sites of pET28(a). This construct encoded *L. monocytogenes* FhuD tagged at the N terminus with His_6. The recombinant pET28A(+) vector was introduced into *E. coli* BL21(λ DE3) for protein overexpression. Strains expressing fusion proteins were grown to an approximate A_{600} of 0.7. Following the addition of isopropyl-1-thio-β-D-galactopyranoside (1mM), growth was allowed to continue another 3 h before the cells were lysed. The resulting supernatants were centrifuged at 35,000 rpm to remove insolubles and then passed across a 7-ml pQE-9^{TM} histag nickel column (Qiagen) for purification. Protein purity was assessed by SDS-PAGE.

After I successfully transformed pET28fhuD into *E. coli* BL21, This 6his-tagged listerial FhuD in *E. coli* BL21 was not over-expressed as we expected (**Fig. 5.2**). Without cutting its signal peptide sequence, only a small amount of FhuD was observed to express from BL21 after adding IPTG. (**Fig 5.2 Lane 6**) We observed

FhuD were expressed in both cytoplasmic and membrane fractions. Even from 15 Liter of cell lysate, and the purification was not good enough and also we did not have enough amount of purified protein to prepare for further binding test or crystallization. (**Fig. 5.3**)

However, from the genomic alignment of FhuD in *L. monocytogenes, S. aureus and B. subtilis* (**See Fig. 3.5**) it showed that they had similar signal peptide sequences. First, they all contain a LXXC motif in the first 18 to 21 that was characteristic of a lipoprotein secretion signal and could be recognized by signal peptidase II. Also from signalP 3.0 sever http://www.cbs.dtu.dk/services/SignalP/, it was predicted to be about 90% of the most likely cleavage site between pos. 19 and 20: VLT-AC. (**see Fig. 3.4**) In *L. monocytogenes*, an unpaired Cys residue (C21) from its motif maybe be lipidated to form a membrane anchor, which explained $_6$His-FhuD found in both cytoplasmic and membrane fraction. (**Fig. 5.3**) We were not sure if this signal peptide, which led the premature FhuD out of cytoplasmic membrane, could cause FhuD to be expressed in a low expression level in *E. coli* system. FhuD of *S. aureus,* also cleaved of its signal peptide, was found to be expressed in a high level in *E. coli*. It suggested that we could cleave the signal peptide to get high level expression of listerial FhuD of in *E. coli*.

After I redesigned FhuD, with deletion of signal peptide onto pET28 expression vector, the new construct is called pET28FhuDΔ21, devoid of 21 amino

acids for signal peptide sequence. FhuDΔ21 showed great expression than FhuD in BL21. (**See Fig. 5.4**) Then $_6$HisFhuDΔ21 was purified from Ni-NTA column and purification of FhuDΔ21 was a lot better than that of FhuD. (**See Fig. 5.5 and 5.6**) The new FhuD started to elute when the imidazole concentration only reached to 40mM, suggesting interaction between 6 histidines of the protein and the nickel was not a strong binding. Since the FhuDΔ21 was so overexpressed that even added with phenylmethanesulphonylfluoride (PMSF), some degradation products of the protein appeared from SDS-PAGE gel of purification. This may be because the protein concentration from lysates was too high because of large overexpression or it may indicate that the protein itself is relatively unstable in *E. coli.*

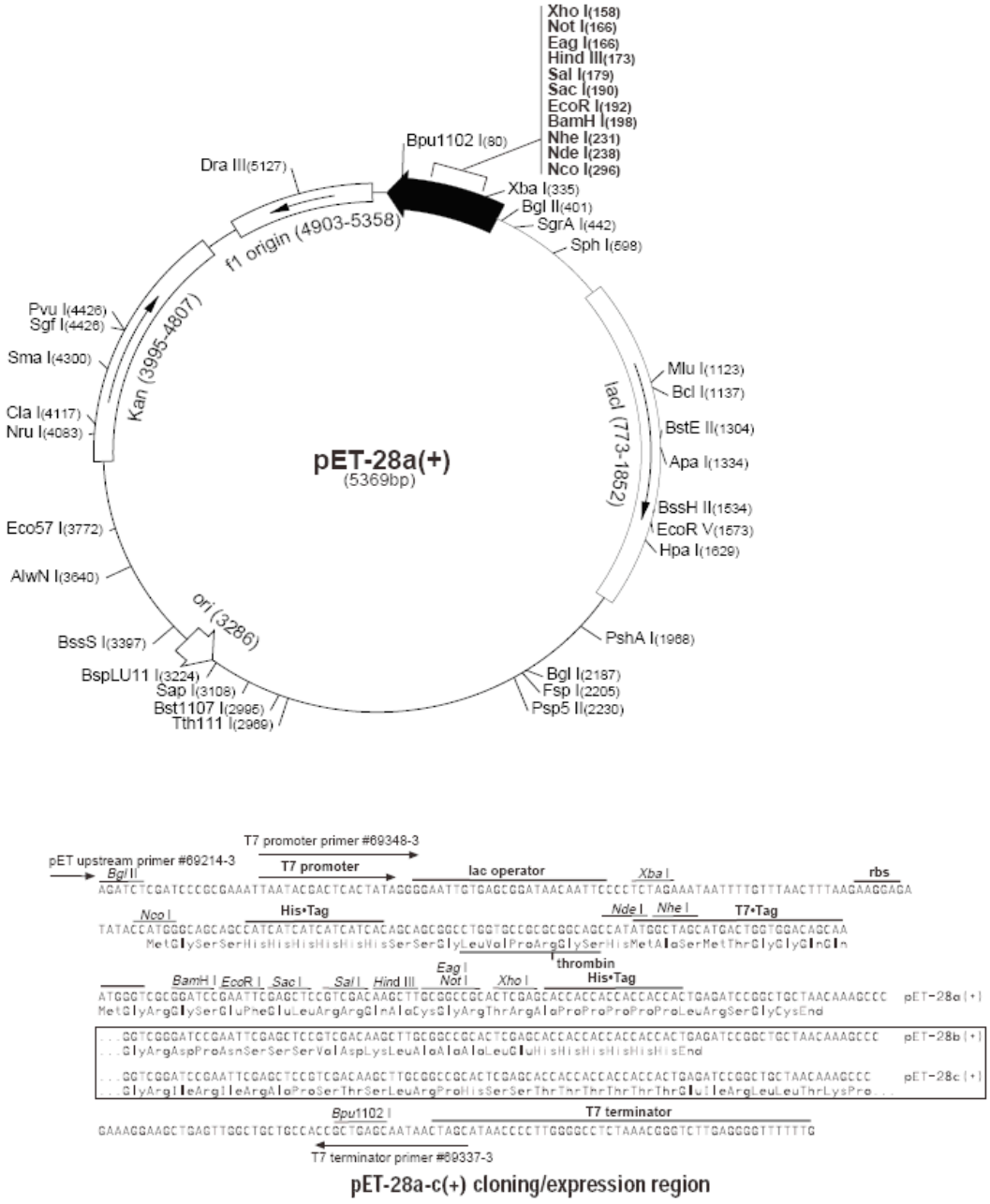


Fig. 5.1 pET28(a) expression vector.

http://www.emdbiosciences.com/docs/docs/PROT/TB074.pdf

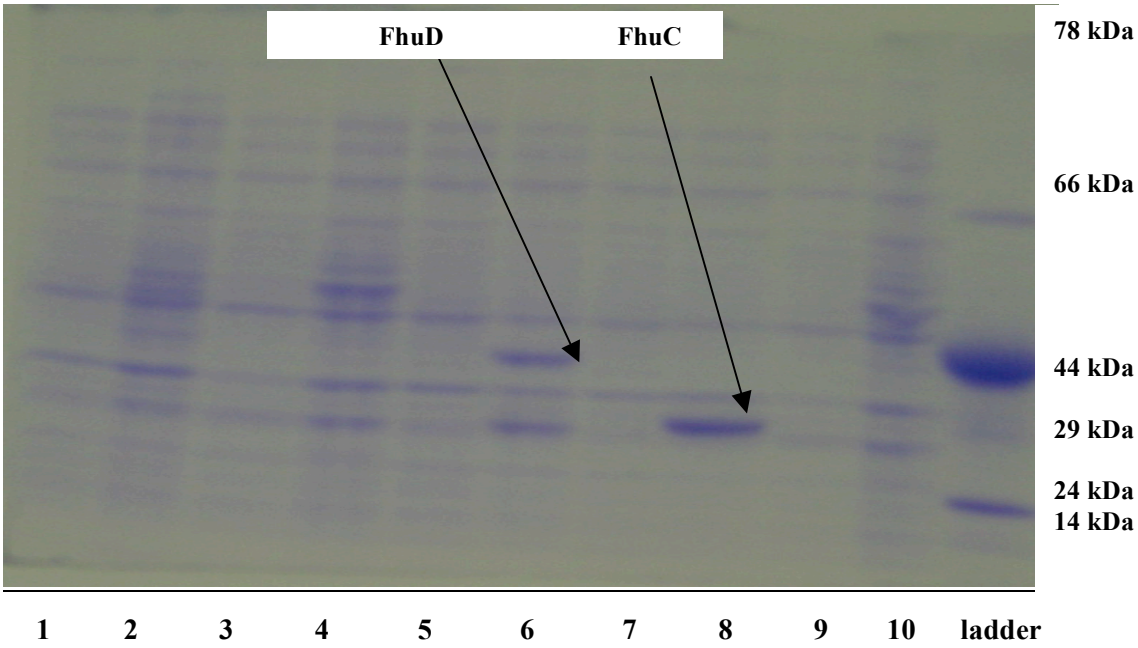


Fig. 5.2 SDS-PAGE of expression check of $_6$His FhuD.

Lane 1~10 Cell lysate of different strains, each fully grown strain was normalizd to the same cell density before culture was lysed; Lane11-Ladder

Lane 1, 2- BL21 w/o and w IPTG; Lane 3, 4- DH5α/pET6HisFhuD;
Lane 5, 6- BL21/ pET6HisFhuD; Lane 7, 8- BL21/ pET6HisFhuC;
Lane 9, 10- BL21/ pET28
Molecular Mass Marker consisted of Phosphorylase b(94kDa), Conal Bomin(78kDa), BSA(66kDa), Egg Albomin(44kDa), carbonic anhydrase (29 kDa), Trypsin (24kDa) and lysozyme (14 kDa); $_6$His FhuD is expected to be 37.8kDa.

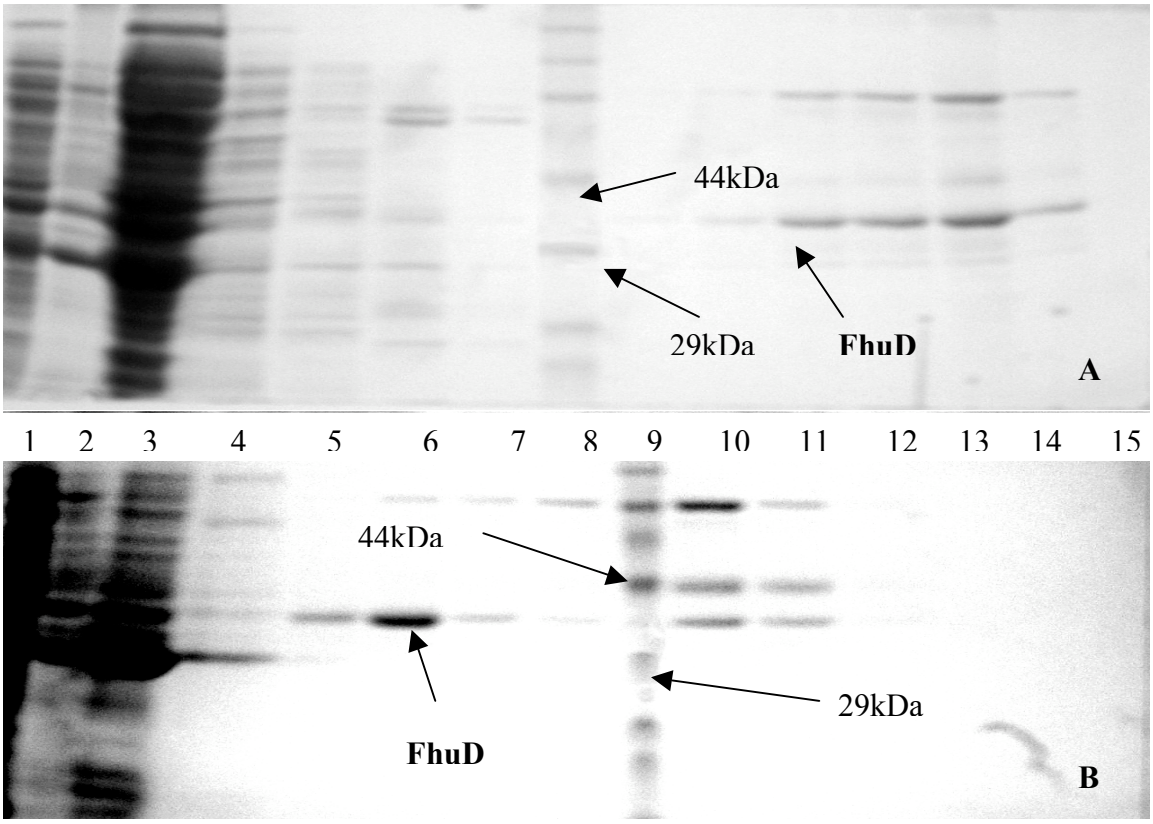


Fig. 5.3 SDS-PAGE showed purification of FhuD by nickel column from 15 Liter LB broth.

A. Cytoplasm fraction. Lanes 1, 2 Flow through; Lane 3, 4, 5, 5 x volume of wash by 10 to 30 mM imidazole, respectively; Lanes 6, 7, 9, 10, 11, 12, 13, 14, 15, eluted at concentrations of 40mM to 250 mM imidazole; Lane 8, Ladder.

B. Membrane fraction. All the buffers were added with 0.2% triton 100. Lanes 1, 2 Flow through;. Lane 3, 4. 5. 5 x volume of wash by 10 to 30 mM imidazole, respectively; Lanes 6, 7, 8, 10, 11, 12, 13, 14, 15, eluted at concentrations of 40mM to 250 mM imidazole; Lane 9, Ladder. Molecular Weight Marker consisting of Phosphorylase (94kDa) Conal Bomin(78kDa), BSA(66kDa), Nadase (55kDa), Egg Albomin(44kDa), carbonic anhydrase (29 kDa), Trypsin (24kDa) and lysozyme (14 kDa);. $_6$His FhuD is expected to be 37.8kDa.

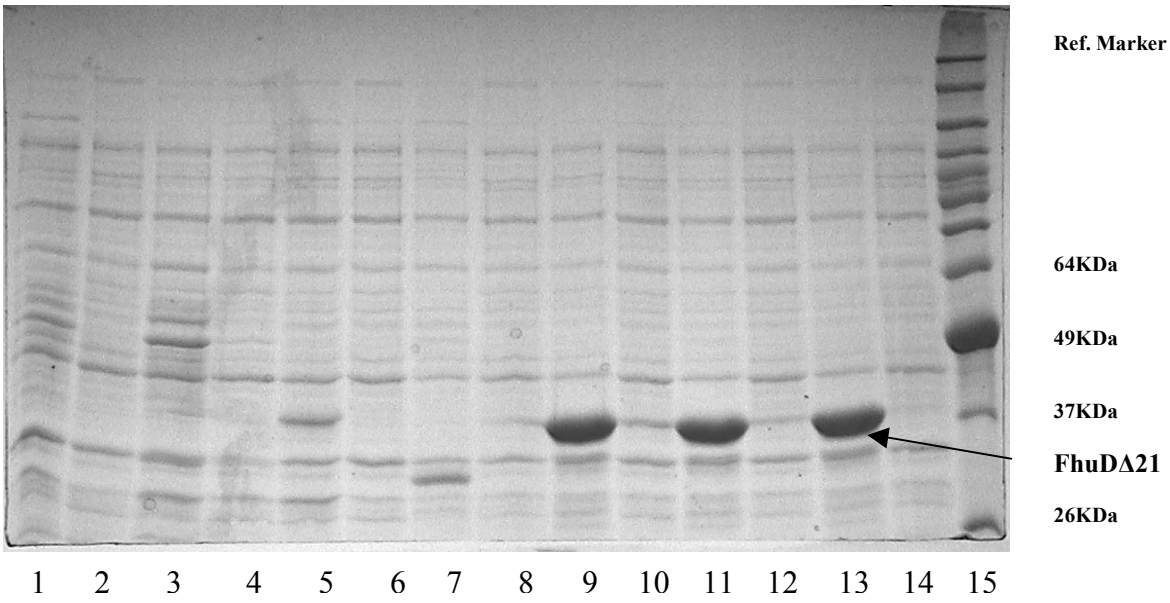


Fig. 5.4 High Level Expression of FhuDΔ21 in *L. monocytogenes*.

Lanes 1, 2– BL21 w/wo IPTG

Lanes 3, 4 – BL21/pET28 w/wo IPTG

Lanes 5, 6 – BL21/pET28 FhuD

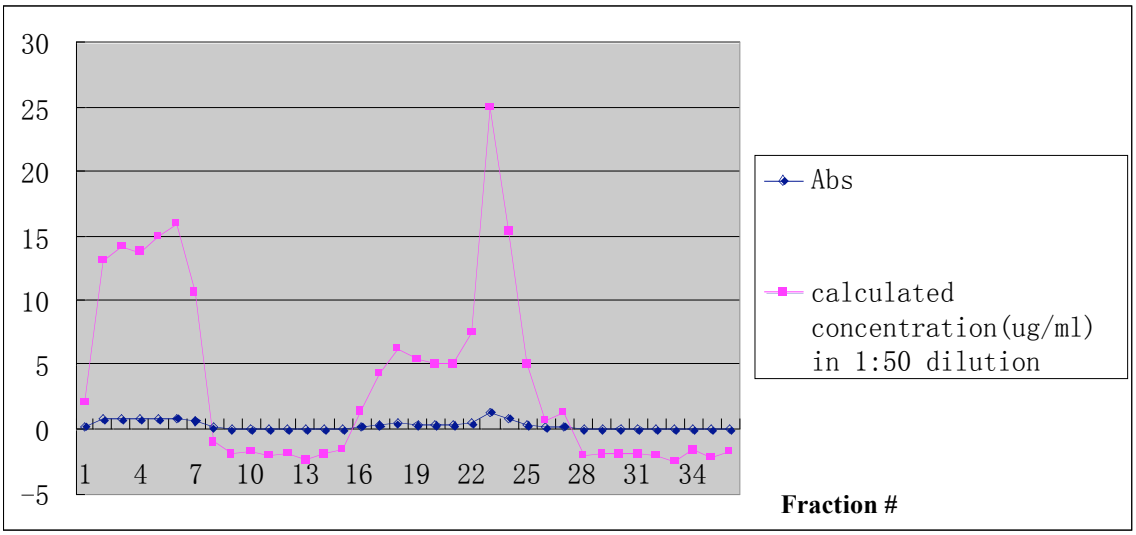
Lanes 7, 8 – BL21/pET28 FhuC

Lanes 9, 10–BL21/pET28 FhuDΔ21 clone 1

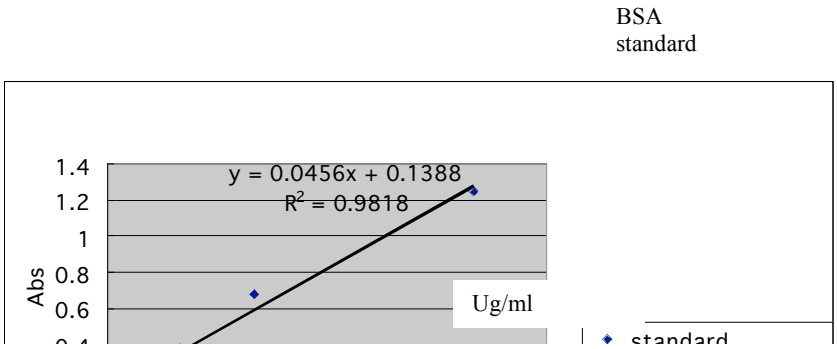
Lanes 11, 12– BL21/pET28 FhuDΔ21 clone 2

Lanes 13, 14–BL21/pET28 FhuDΔ21 clone 3

All the 3 new constructs of pET28FhuD Δ21 were sequenced and shown to be correct.



A



B

Fig. 5.5 Protein concentration was determined by Bradford Assay.

A. OD reading at 495nm and converted concentration (Ug/ml) for $_6$His FhuDΔ21 purification fractions.

Fraction #:1~6 flow through; Fraction #: 7~16: wash; Fraction #:17~27 eluant.

B. Standard of BSA

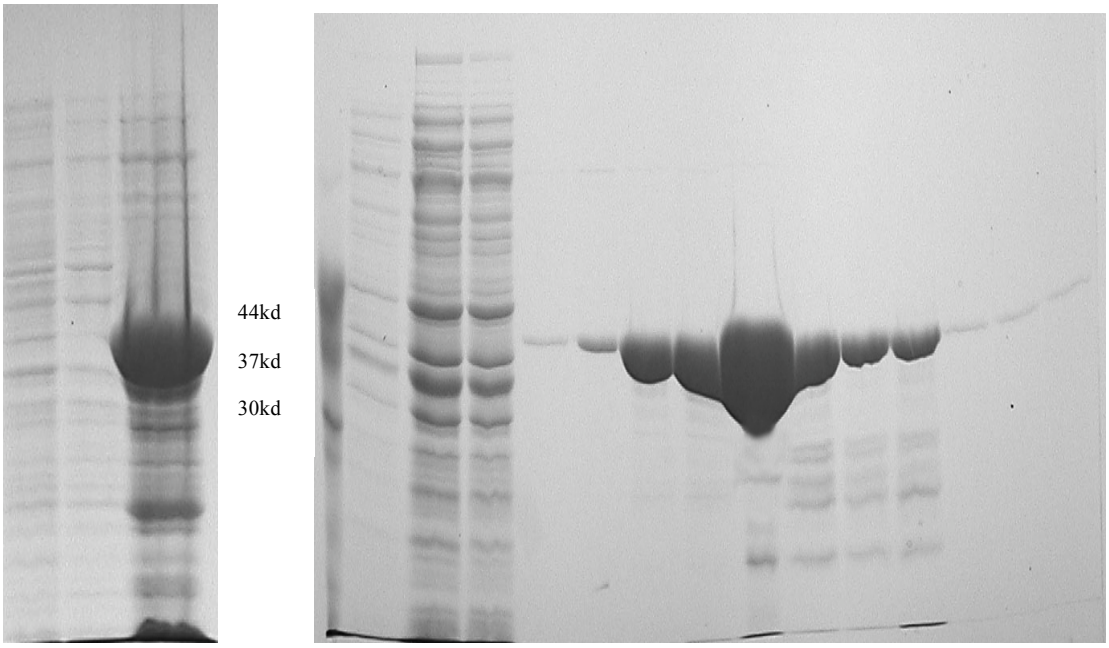


Fig. 5.6 SDS-PAGE of nickel column purification of $_6$His FhuDΔ21 from 1 Liter broth. (without signal peptide)

Left- huge expression of $_6$His FhuDΔ21 before nickel column purification

Lanes 1,2 – BL21/pET28 FhuD w/wo IPTG

Lanes 3, – BL21/pET28FhuDΔ21 w IPTG

Right-Gradient imidazole elute of $_6$His FhuDΔ21 after nickel column purification

Lane 0 Ladder

Lanes 1, 2 flow through

Lanes 3~5 10 to 30 mM imidazole wash

Lanes 6~12 40 to 100 mM imidazole eluates

Lane 13~14 250 mM imidazole eluates

5.2 Intrinsic fluorescence

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 (Schneider and Hantke 1993). Listerial FhuD was assumed to be homology to *E. coli* FhuD. (See discussion part I). Four tryptophans were found in the binding pocket from the crystal structure of *E. coli* FhuD. And those four tryptophans can be used to track change of fluorescence intensity upon FhuD's binding of siderophores. Four tryptophans also exist in mature listerial FhuD, which provided another measure of the affinity of the interaction with ferric siderophores. Binding of the ferric siderophore did not shift the excitation (290nm) or emission (327nm) maxima of purified FhuD (Fig. 5.4, pooled fractions 6 to 9; >90% 35.9-kDa band), suggesting that the tryptophans did not experience any significant change in environment. However, saturation with Ferrichrome, one of major hydroxamate siderophores greatly reduced the fluorescence intensity of FhuD by approximately 45%. (**Fig. 5.7**). The concentration dependence of this decrease showed a midpoint (K_D) at 306 nM, roughly three fold lower than FhuD in *E. coli* but four fold higher than FhuA in *E. coli*.

Among all the hydroxamate siderophores, which all can be utilized by *L. monocytogenes* wild type by the nutrition tests and radioactive Fe transport assay, FhuD binds them with different affinities as well as specificities (See **Table 5.1.**).

Among four hydroxamate siderophores, FhuD displayed highest affinity for iron(III)-desferroxamine, with a K_D (nM) = 123, two fold less affinity as described for FhuD2 from *Staphylococcus aureus* but 350 fold higher affinity of FhuD found in *E. coli*. Also since *Listeria monocytogenes* is the only strain which can utilize Ferrichrome A, a hydroxamate siderophore that hardly can be utilized by most bacteria, its binding affinity of K_D is about 451 nM. FhuD shows less binding of Apo-ferrichrome as well as Apo-ferrichrome A, except for desferrioxamine B. FhuD shows no binding to non-hydroxamate siderophore, eg, Fe-Enterobactin. (**See Fig. 5.7 b and Table 5.1.**)

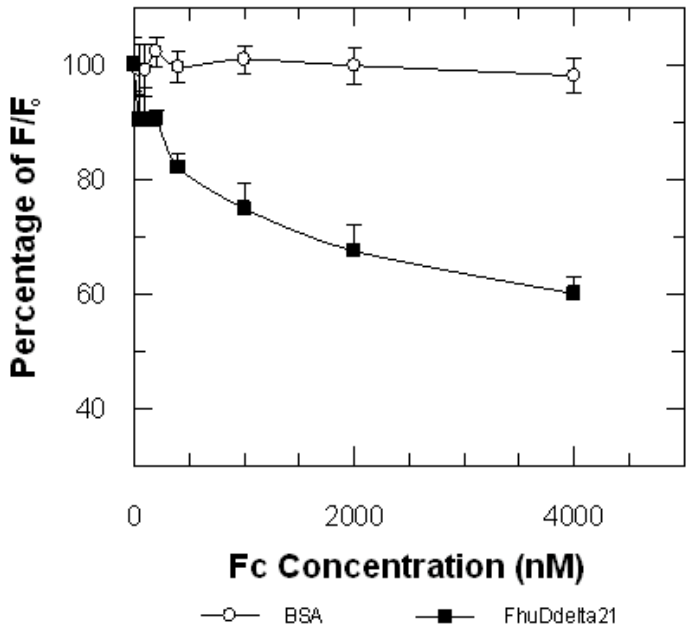


Fig. 5.7 Comparison of Fc Binding by FhuD and BSA via Fluorescence spectroscopy

FhuD fluorescence emissions were quenched when the protein bound the Fe(III)ferrichrome, and control of BSA shows no binding. For FhuD, F/F_0 is Fc concentration dependent. $(1 - F/F_0)$ was used to estimate the affinity (K_D) of the interactions.

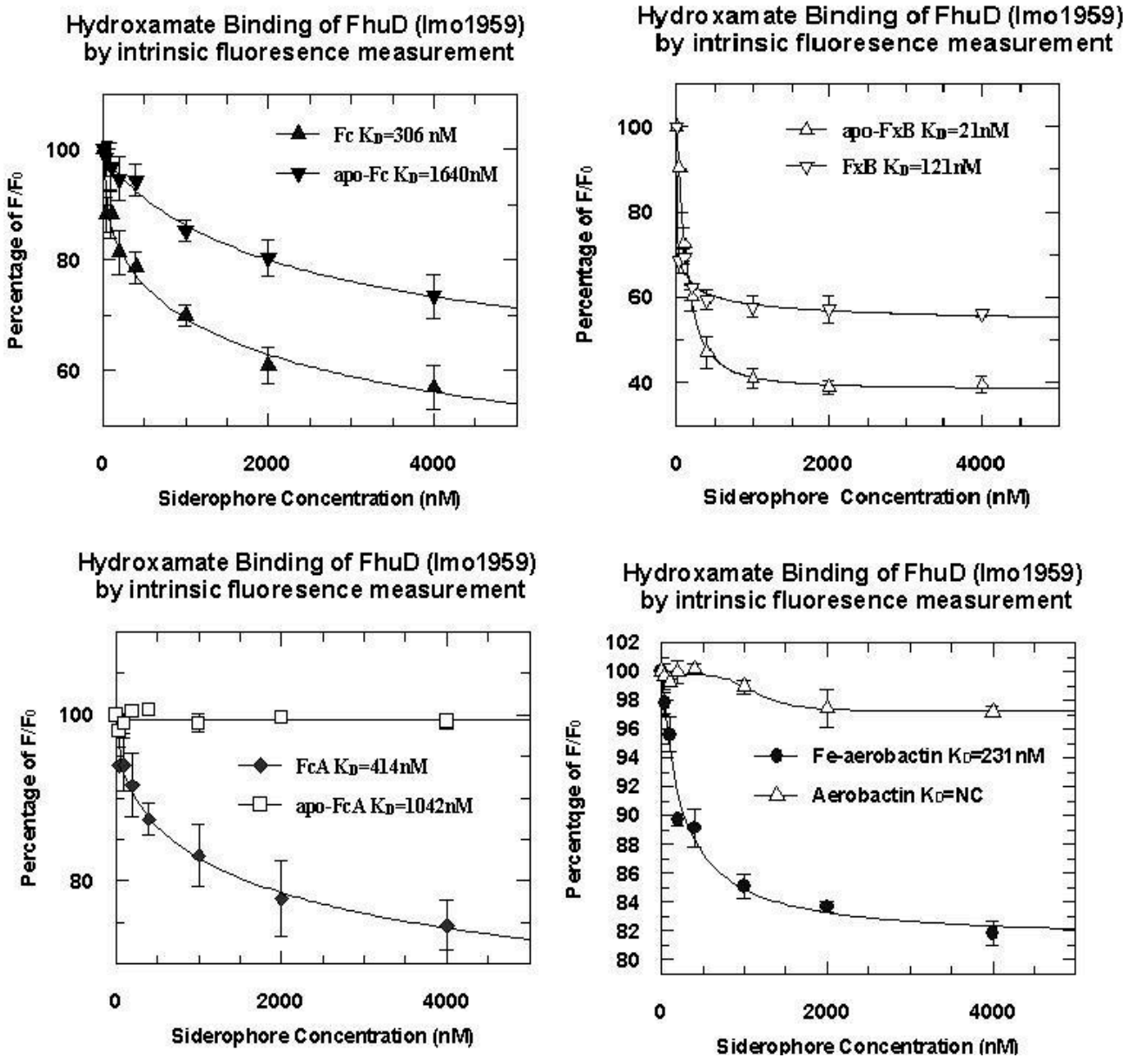


Fig. 5.8 Hydroxamates binding of FhuD(1959) by intrinsic fluorescence measurement. Top left: both Fc and apo-Fc showed quenching of fluorescence upon ligand binding with FhuD; Top right:FxB and apo-FxB showed quenching of fluorescence upon binding, however the Calculated Kd was higher in apo-FxB than in FxB; Bottom left: FcA showed quenching but FcA showed slightly any quenching; bottom right: Ferric-aerobactin showed quenching while Ferric but aerobactin didn't.

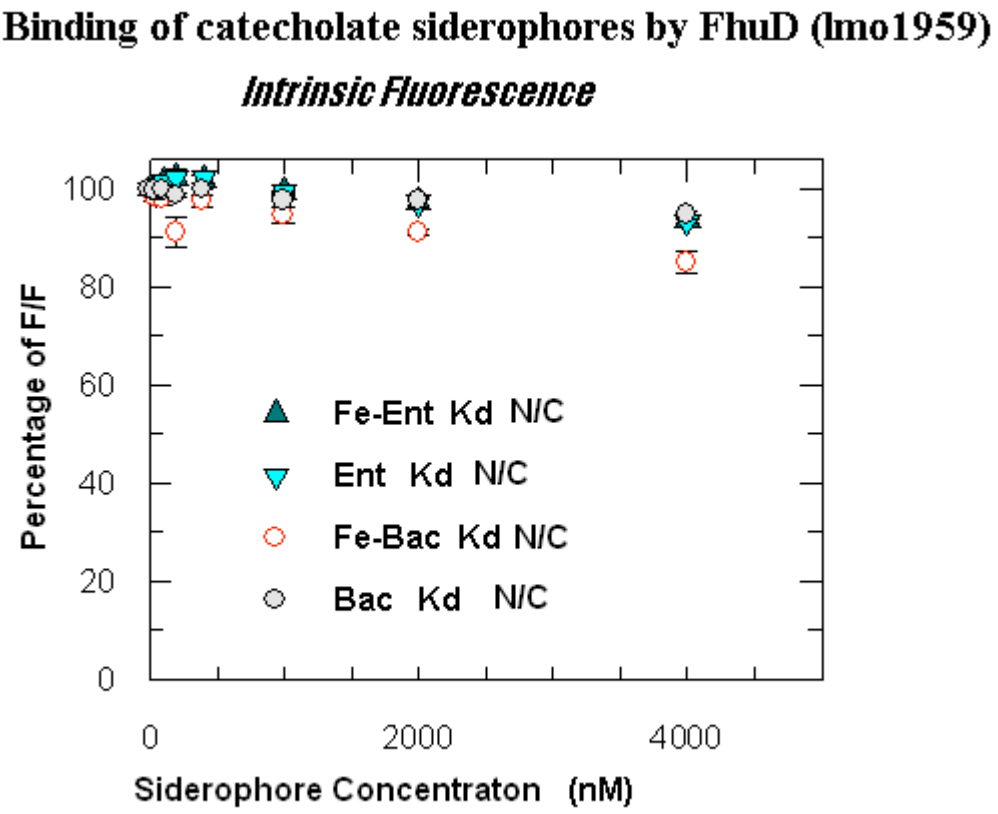


Fig. 5.9 Catecholate siderophore binding of FhuD(1959) by intrinsic fluorescence measurement. All the siderophores tested here showed no quenching and the K_Ds are not calculable.

Hydroxamate		Catecholate	
Siderophore	**Kd (nM)**	**Siderophore**	**Kd**
FxB	**121**	**Fe-Ent**	**N/C**
Apo-FxB	**21**	**Ent**	**N/C**
Fc	**306**	**Fe-Bac**	**N/C**
Apo-Fc	**1640**	**Bac**	**N/C**
FcA	**414**		
Apo-FcA	**1024**		
Aerobactin	**N/C**		
Fe-aerobactin	**231**		

Table 5.1 Kd of siderophore binding measure by intrinsic fluorescence spectroscopy. N/C means the K_D is not calculable because of the numbers has too large standard deviations.

5.4 Discussion

5.4.1 Comparison of *E. coli* and *L. monocytogenes* FhuD

The crystal structure of E. coli FhuD was first solved in complexed with gallichrome, a homolog of ferrichrome, in 2000 (Clarke, Ku et al. 2000). The siderophore-binding site is located in a shallow cleft between the two lobes. Because the interior of this shallow cleft is predominantly composed of aromatic residues, the binding site is hydrophobic. Siderophore binds to FhuD through both hydrophobic and hydrophilic interactions. The shallow cleft and the hydrophobicity of the siderophore-binding site suggest that large conformational change does not occur upon siderophore binding. From its crystal structure, I found 4 tryptophans (W43, W68, W217, W273) in proximity to the ligand in the binding pocket. (**Fig. 5.10**). Because of those tryptophans within the binding site, I used intrinsic fluorescence quenching experiments to characterize FhuD and determine its binding affinity and specificity for hydroxamate siderophores. The substrate specificity and affinity of *E. coli* FhuD for iron or siderophore uptake have been determined. The K_D for hydroxamate binding to FhuD ranged from 300 to 400 nM for coprogen and aerobactin, to 1 μM for ferrichrome, and to around 40 μM for ferrioxamines (Rohrbach, Braun et al. 1995). In the previous chapter, I demonstrated that the listerial FhuD had high sequence homology to *S. aureus* FhuD. (**Fig. 3.5**) Since the crystal structure of any Gram-positive FhuD has not yet been solved, listerial FhuD was compared to the *E. coli* homolog in terms of both structure and function.

Although listerial FhuD has astonishingly low identity (13.7%) with *E. coli* FhuD, the mature proteins still have very significant similarity (53.4% by sequence alignment using ClustalW). However, the four *E. coli* tryptophans that were found to be involved in binding from its crystal structure were not fully conserved in listerial FhuD. Only *L. monocytogenes* W229 was found close to *E. coli* W217 in sequence alignment and this tryptophan of listerial FhuD may reside within the binding pocket. Tryptophan fluorescence was quenched upon ferric siderophore binding to FhuD, verifying this assumption. (**Fig. 5.11**)

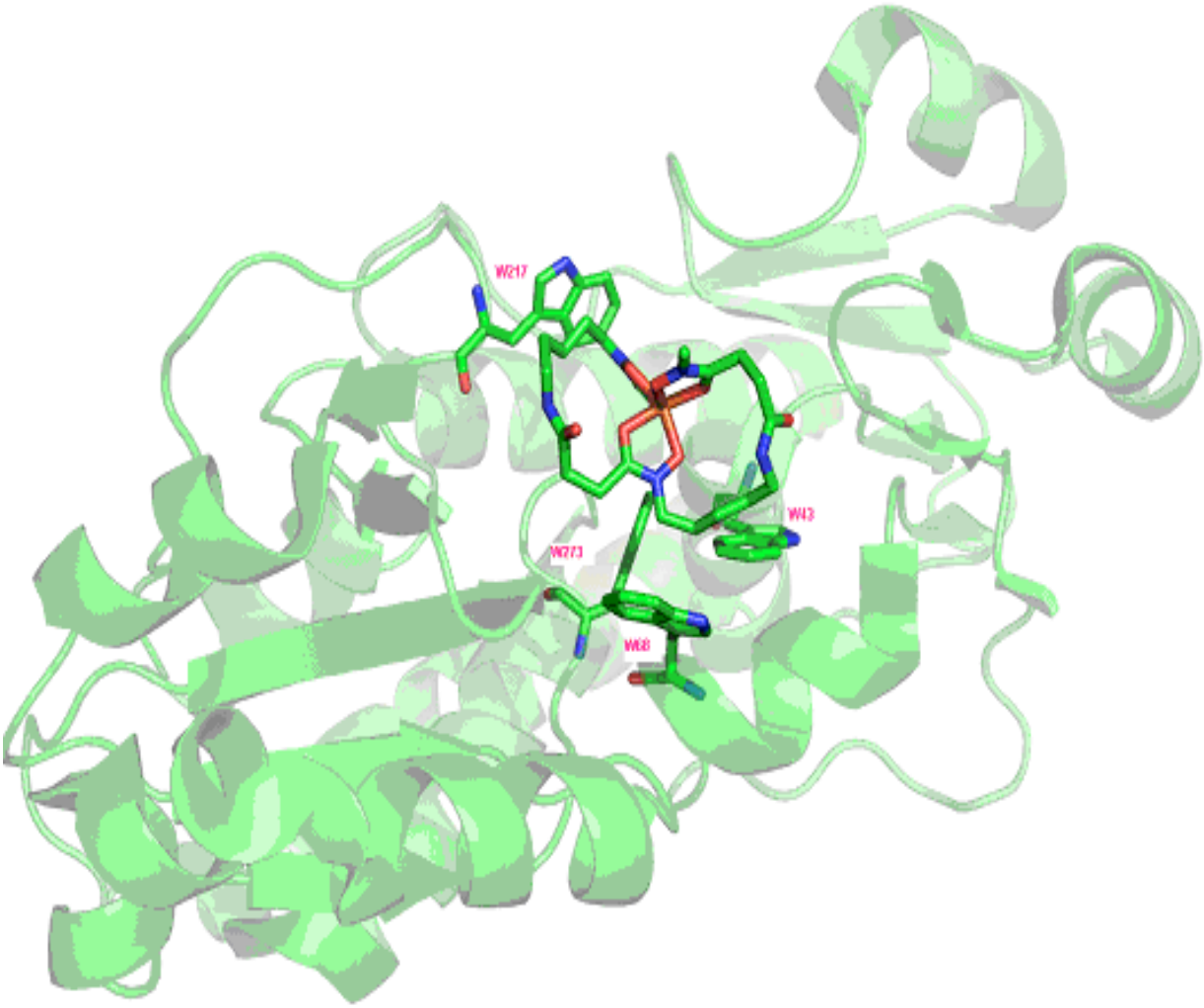


Fig. 5.10 Crystal structure of *E. coli* FhuD in complex with desferal.

The crystal structure showed 4 tryptophans were in the ligand binding domain. Those are W43, W68, W217, and W273.

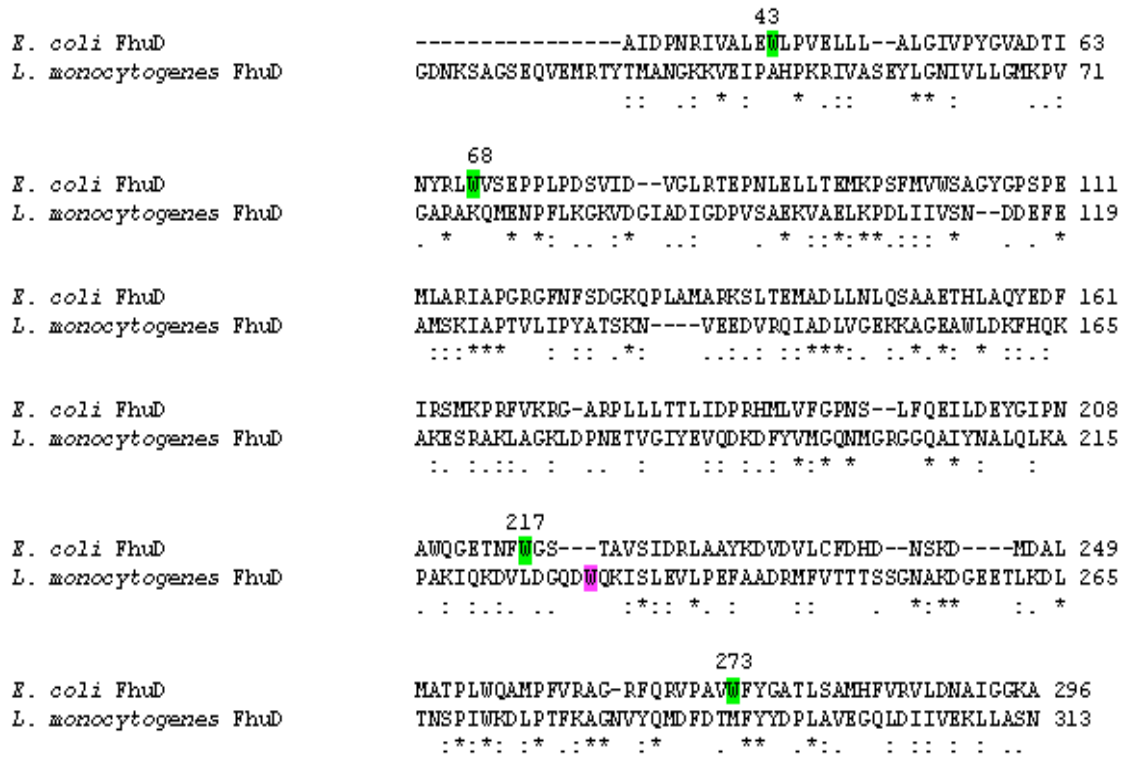


Fig. 5.11 Amino acid sequence alignment between mature E. coli FhuD and listerial FhuD

Even though listerial FhuD has percentage of identity (13.7%) that is even lower than the cut-off of sequence homolog, the overall similarity is still very high (53.4%), suggesting the two proteins may have the similar structures. 7 tryptophans were found in mature *E. Coli* FhuD and 3 tryptophans were found in listerial FhuD. From this alignment, none of the 4 tryptophans within the binding pocket are conserved in listerial FhuD, except W255 that is located far below the binding pocket. However, *L. monocytogenes* W229 (Purple) and *E. coli* W217 (Green) are close to each other in the sequence and W229 may be located in the binding pocket of *L. monocytogenes*. Even though it is not known which tryptophans account for the fluorescence quenching, it is still feasible to determine dissociation constant from changes in the intrinsic fluorescence of listerial FhuD as a result of substrate binding.

5.4.2 Affinity of FhuD

Previous studies in our laboratory showed that the *fhuD(lmo1959)* was unable to utilize three hydroxamate siderophores, Fc, FcA and FxB (Jin, Newton et al. 2006). The gene was from locus furfhuBGCD and itself resembled a typical lipoprotein that was one of three components of a typical ABC type transporter which was predicted to be ferrichrome-like transporter by homology alignment with that in *S. aureus* (see **Fig. 3.5**). Ferrichome-like iron transporter system belongs to siderophore-mediated iron transport systems in the two major bacterial iron uptake mechanisms.

My fluorescence quenching experiments determined that purified listerial FhuD bound hydroxamate-type siderophores with different affinities. Using this technique, dissociation constants were determined from changes in intrinsic fluorescence of FhuD as a result of ligand binding. Similar experiments were also performed to characterize specificity and affinity of substrate binding for FhuD in *Escherichia coli, Staphylococcus aureus, and group B streptococcus.* The results are summarized in Table 5.2.

Fe(III)-siderophores	K_D (uM)			
	Listerial FhuD (this study)	*S. aureus* FhuD2 (Sebulsky, Shilton et al. 2003)	GBS FhuD (Clancy, Loar et al. 2006)	*E. coli* FhuD (Rohrbach, Braun et al. 1995)
FerrioxamineB	0.12	0.05	0.05	40
Ferrichrome	0.3	0.02	0.1	1
Aerobactin	0.23	0.3	3	0.4
Rhodotorulic acid	NA	3	3	NA
Coprogen	NA	1.7	9.3	0.3
Ferrichrome A	0.41	NA	NA	NB
Enterobactin	NB	NB	3	NB

Table 5.2 Comparison of dissociation constants of iron(III)-siderophores bound to FhuD in L. monocytogenes, *S. aureus*, Group B *streptococcus* (GBS) and *E. coli* by fluorescence quenching experiments. NA-not available; NB-no binding.

Listerial FhuD has the highest affinity for ferrioxamine B with a K_D of 0.12 uM, twice as much as those in *S. aureus* and GBS and more than 300 fold less than *E. coli* FhuD. The affinities of listerial FhuD for ferrichrome and aerobactin are in the close range to each other. Affinity of aerobactin for listerial FhuD is close to that in *S. aureus* and *E. coli* but higher than in GBS. Ferrichrome A, which most bacteria can hardly utilize but *L. monocytogenes* can from our nutrition test, has about the same affinity compared to all the other ferric hydroxamate we tested. Ferrichrome A is unique because most of the bacteria are unable to utilize it, neither as hydroxamate siderophores nor catecholate siderophore. Ferrichrome A is structurally related to those hydroxamates, but it is -3-charged while most hydroxamate siderophores are of neutral charge (**see Table. 1.2**) On the other hand, even though ferrichrome A is -3 charged, the same as found in many catecholate siderophores, they are not structurally related. (**Table. 1.2**)

For ferrichrome, a fungal siderophore that can used in many bacteria, listerial FhuD has an affinity that is 10-15 fold lower than that in GBS and in *S. aureus* but still 3 fold higher than that of *E. coli*. K_D of ferrichrome for FhuD in all the three Gram-positive stains was lower than that of FhuD in *E. coli*. However, compared to the K_D (=0.1 nM) of FhuA (Scott, Cao et al. 2001), which is the outer membrane receptor with a strict specificity for ferrichrome in *E. coli*, K_Ds of FhuD of those Gram-positively bacteria including *Listeria* we tested are considerably lower than the K_D of FhuA. This is because many of the outer membrane binding receptors in Gram-negative bacteria are usually ligand-gated porins to transport those molecules that are larger than 600 dalton and unable to passively diffuse through general porins across these membranes (Nikaido and Wu 1984). Ligand-gated porins are very specific to their substrates and have very high affinities, in which the K_Ds range from 0.1 nM~100 uM (Stintzi, Barnes et al. 2000). For example, FepA has a very high affinity for ferric enterobactin (K_D=0.1 nM). However, the binding constants of their binding lipoprotein in the periplasm are larger than those in the outer membrane. The affinity of E. coli FepB for its substrate is estimated at approximately 30 nM by fluorescence quenching experiments and 145 nM by chromatographic measurement of ^{59}FeEnt-FepB binding (Sprencel, Cao et al. 2000). *E. coli* FhuD, which is also a binding lipoprotein, binds ferrichrome with less affinity, only with a K_D=1 uM (Koster and Braun 1990). In general, the outer membrane of Gram-negative bacteria is home to high affinity iron(III)-siderophore receptors, whereas periplasmic components, eg

FhuD in *E. coli*, need not possess as high an affinity for substrates because substrates transported across the outer membrane are "concentrated" in the periplasm, whereas the cognate outer membrane receptors of gram-negative bacteria are the first contact of iron source and have to pirate any iron from very low iron concentration in the surroundings.

5.4.3 Specificity of FhuD

Comparing the affinities of FhuD in the above species, as expected, listerial FhuD bound hydroxamate siderophores similar to those counterparts but showed highest affinity for ferrioxamine B like GBS. If the substrate which shows the highest binding affinity defines the protein of interest, then listerial FhuD is specific for ferrioxamine B, not ferrichrome as we expected because its operon was originally mis-named as ferrichrome-like ABC transporter by homolog alignment to *E. coli* FhuD transporter and *B. subtilis* FhuD system. Even for *E. coli* fhuD, the best substrate is not ferrichrome but coprogen that shows the highest affinity. The biological significance of different affinities for different hydroxamate siderophores is not clear for *L. monocytogenes*. When living saprophytically in woods, soil and decaying vegetables as primary habitats, *Listeria monocytogenes* may manage to use ferrioxamine B, ferrichrome, or ferrichrome A as major iron sources in those habitats because hydroxamates are produced by fungi and molds and are relatively abundant. It is also possible that *Listeria monocytogenes* causes intestinal infections via the raw milk

cheese, which contains hydroxamate siderophores from fungi used to produce the cheese. This may help the bacteria to survive in refrigeration temperature (Goulet, Jacquet et al. 1995).

FhuD is a prototype for a large and growing subfamily of iron(III)-siderophore-binding lipoproteins from Gram-positive bacteria. This subfamily of iron(III)-siderophore-binding proteins function as "high affinity" receptors at the external face of the cytoplasmic membrane in Gram-positive bacteria, as compared with the periplasmic location of FhuD homologs in Gram-negative bacteria. Our experiments and the measurements of FhuDs in the other Gram-positive bacteria by other groups showed that FhuD has broad specificity and but still can be regarded as high affinity receptors. Our experiments also confirmed that listerial FhuD does not bind catecholate siderophore such as ferric-bacillibactin or ferric enterobactin, which is contrary to the GBS FhuD that was found to be able to bind enterobactin still with a decent affinity (K_D=3 uM). To our surprise, listerial FhuD binds to the apo-ferrioxamine B with greater affinity than to ferrioxamine B. It is reported that the binding site of FhuA, the outer membrane receptor for ferrichrome, possesses a higher affinity for ferrichrome-iron than for apo ferrichrome (Boulanger, le Maire et al. 1996). In the ligand binding structure, Tyr^{244} of FhuA comes in close contact with the iron atom of the ferrichrome-iron molecule. This observation may explain the decreased affinity for apo ferrichrome. However, FhuD does not show any homology to FhuA in primary sequence and tertiary structure, because the former is a

transmembrane protein while the latter is a membrane-anchored peripheral lipoprotein. Therefore, difference in binding to the apo-siderophore may exist, which makes a unique feature of listerial FhuD. Furthermore, broad specificity of FhuD in *B. subtilis* was also observed. Deletion of fhuD impairs the transport of hydroxamates of ferrichrome, coprogen Rhodotorulic acid but not ferrioxamines. Another lipoprotein in *B. subtilis*, FoxD, was identified to be involved in uptake of ferrioxamines (Schneider and Hantke 1993). Analogously two binding proteins with different specificities to hydroxamate siderophores may exist in *Listeria monocytogenes* as well.

Although the affinities displayed by listerial FhuD for particular siderophores were very comparable to that in the other Gram-positive bacteria, differences exist as described above. To address such issue of relationship between structure and function in FhuD in terms of substrate binding, some amino acid sequence alignments were made between listerial FhuD and two other FhuDs in *S. aureus*. Despite three regions that were conserved among all siderophore-binding and heme-binding proteins of most bacteria were found in listerial FhuD, from amino acid homology alignment with *S. aureus* FhuD, four amino acids (E97, E231, W197 and E202) of listerial FhuD may appear to be critical for binding with ligand and interaction with cognate membrane permease. The importance of those conserved residues for siderophores binding by listerial FhuD are currently unknown in the absence of its crystal structure. Crystallization of listerial FhuD is still underway.

5.4.4 A secondary hydroxamate iron transporter exists?

From our genomic analysis, the FhuD(lmo1959) has only about 25% identity and 54% similarity to FhuD in *B. subtilis*, which is the first FhuD found Gram-positive bacteria, and even lower identity and similarity to FhuD in *E. coli*. Another binding lipoprotein, lmo0541 was found to have a typical Fur-box in its promoter region (table 3.1). From homology alignment by ClustalW and BlastP, this binding lipoprotein shares 27% identify and 45% similarity with FhuD in *B. subtilis*. They also shared the same typical three regions of siderophore binding within all the FhuD reported so far. Such transport redundancy is known in many other iron regulated transport systems. In *B. subtilis*, both FoxD and FhuD are the hydroxamate-binding lipoproteins using the same traffic ABC type transporter, FhuCB to internalize the hydroxamate siderophores through cytoplasmic membrane. The only difference between those two lipoproteins is that FoxD appears to strictly specific to ferrioxamines while FhuD can use wider range of hydroxamate siderophores. But both of them cannot utilize aerobactin. In our research, *L. monocytogenes* can use almost all kinds of siderophores we have tested. It is possible that a second hydroxamate uptake system or a second hydroxamate binding protein with different substrate specificity may exist in *L. monocytogenes*. It is notable from our previous growth test that the only the deletion of *fhuC* showed great retardation in growth while *fhuD* didn't. It is possible that this FhuC is a traffic ATPase within in a membrane transporter but they have different receptors on the cell surface.

Taken together, these results support the conclusion that FhuD(lmo1959) is an effective receptor for ferric hydroxamate transport in *L. monocytogenes* and the ability to acquire iron from the surrounding environment is critical to the growth of virtually all bacteria. *L. monocytogenes* imports ferric iron associated with hydroxamate-type siderophores through the Fhu (ferric hydroxamate uptake) system. The Fhu system in many *L. monocytogenes* strains is composed of five proteins: FhuD, and FhuCBG. The FhuCBG proteins represent components of a traffic ATPase (FhuB and FhuG are integral membrane proteins and FhuC has signature *L. monocytogenes*). In our research, we have expanded our understanding of the Fhu system in *L. monocytogenes* by characterizing the FhuD protein and establishing its role in the transport process. From our genetic alignment studies, FhuD showed homolog to lipoproteins and from our biochemical characterization FhuD acts as a receptor for ferric hydroxamate complexes with quite high affinity in *L. monocytogenes*. Although our data suggest that FhuD is the more functionally relevant binding protein, our evidence is based solely on data derived from experiments performed in the laboratory with a small subset of hydroxamate siderophores. However, because redundancy of multiple hydroxamate siderophore was found in *S. aureus* and other Gram-negative bacteria, more genomic analysis should be studied to find genes that may be homologous to FhuD.

Appendix

1. Simple Theory of Fluorescence

Absorbance spectrum is often used to study the properties of macromolecules or their interaction with other molecules. This is based on the principle that when light in the ultraviolet/visible part of the electromagnetic spectrum is passed through a sample in solution, some light energy may be absorbed. Molecules that are capable of absorbing light are called chromophores. The wavelengths at which the light is absorbed are affected by both the structure and the environment of the chromophore and λ_{max} is characteristic for a chromophore under standard conditions.

However, for some chromophores, absorption of light is followed by the emission of light of a longer wavelength. This phenomenon is called fluorescence and such chromophores are called fluors or fluorophores. Not all chromophores are able to fluoresce and the rigidity of a chromophore determines whether such chromophore turns into a fluor. This can be explained by an energy-level diagram, shown in **Fig. 6.1**. When an electron of a molecule absorbs light energy, it moves from a lower (ground state) to a higher energy level (excited state), or we say the electron is excited. Upon excitation to higher electronic and vibrational levels, the excess energy is quickly dissipated and the electron returns its original ground state. If all the excess energy absorbed from light is dissipated as heat, the chromophore just has an experience of absorption, no emission of light and no fluorescence. However, if the

excess energy is not totally dissipated as heat but some of it is used to emit a photon, the chromophore experiences both absorption and emission. From an energy diagram of fluorescence, some of the excess energy is lost as heat when the electron passes through various vibrational energy of excited state, leaving the fluorophore in the lowest vibrational level of excited state. It is from this position that the photon will be emitted. Vibrational energy only can be lost as heat in collision with solvent molecules. If the vibrational energy levels of the excited state overlap with those of the ground state, the electron simply can return all the way down to ground state by passing through all the small vibrational energy levels (small arrows) in both the excited and ground states. All the excess energy is lost as heat. If the vibrational energy levels of the excited state do not overlap with those of ground state, however, some excess energy turns into emission of light. Rigid molecules usually have a limited range of vibration energy levels and the vibrational energy levels of the excited state and the ground state often do not overlap with each other. In such molecules, fluorescence may occur. Since at least some of the light energy initially absorbed is lost in transitions between vibrational energy levels, the fluorescent light emitted always has lower energy and longer wavelength (i.e. lower energy) than that absorbed. Such phenomenon is also called “the Stokes shift”. This is one of the important characteristics of fluorescence emission. Another important property of a fluor is that even though it has a characteristic fluorescence or emission spectrum as well as a

characteristic absorption spectrum, emission spectra are typically independent of the excitation wavelength (Sheehan, D., 2000).

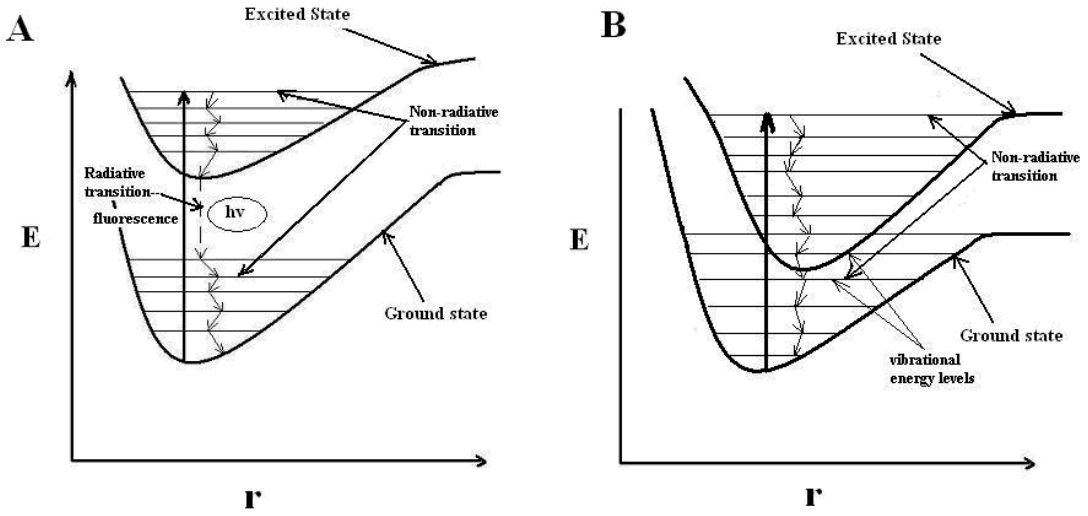


Fig. 6.1 Physical basis of fluorescence

An Energy level diagram of fluorescence and absorption: Heavy lines stand for the ground and first excited states, respectively. The vibrational levels are the thin lines. The chromophore in A is able to fluoresce because the vibrational energy levels of the ground and excited states do not overlap. Upon excitation to higher electronic and vibrational levels, the excess energy is quickly dissipated, leaving the fluorophore in the lowest vibrational level of excited state. It is from this position that the photon will be emitted. However, besides the radiative transition, there is also non-radiative transition from the vibrational losses (small wavy arrows) in both excited and ground states. B. This chromophore can only experience absorption but fails to fluoresce because the vibrational energy levels of ground and excited states overlap. All the excess energy from absorption is lost as heat when the electron passes all the way down through non-radiative vibrational energy levels.

We can quantify fluorescence by the quantum yield, Q (D. M. Freifelder 1982):

$$Q = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}} \qquad \text{[Eq.6.2]}$$

Under given conditions, Q usually has a fixed value for a fluor, with a maximum value of 1. However, it is experimentally difficult to determine Q. We often use relative intensities of fluorescence in two different situations (eg. in the presence and absence of an agent) in practice. More sensitive to environmental changes than absorption spectroscopy, fluorescence spectroscopy often can provide information about conformation, binding sites, solvent interactions, degree of flexibility, and intermolecular distances as well as the rotational diffusion coefficient of macromolecules of interest.

2. Mechanism of Fluorescence Quenching (Eftink, M.R., 1991).

Fluorescence quenching is a process which decreases the intensity of the fluorescence emission of a sample. A molecule in solution that causes the decrease fluorescence intensity of the flour is called quencher. There are a wide variety of quenching processes such as molecular rearrangements, excited state reactions, ground state complex formation, and energy transfer. Since fluorescence spectroscopy is more sensitive to environmental changes than absorbance spectroscopy, fluorescence

quenching experiments can be used to determine the accessibility of quencher to a fluorophore and monitor conformational changes or association reactions of the fluorescence of a protein as a result of substrate binding.

Quenching by small molecules either in the solvent or bound to the protein in close proximity to the fluorophore can greatly decrease the quantum yield of a protein. Quenching may occur by several mechanisms:

- **Collisional or dynamic quenching:** This occurs when the quencher collides with the excited fluor leading to the loss of some energy from the excited state as kinetic energy.
- **Static quenching:** This happens when the quencher and the excited fluor form a stable complex and this complex is non-fluorescent. Some energy from the excited state is lost during the process.
- **Quenching by fluorescence resonance energy transfer (FRET):** This happens only when two fluors (intrinsic or extrinsic) are very close to each other (less than 80 Å) and emission λ_{max} of one fluor(A) overlaps with the absorbance λ_{max} of a second fluor (B). It is possible for some or all of the emission light energy from fluor A to be absorbed by fluor B and be emitted as part of B's emission spectrum (Cheung, H.C., 1995).

Both collisional quenching and static quenching need an interaction between the fluorophore and quencher whereas FRET does not. When quenching occurs by a

collisional mechanism, the quencher must diffuse to the fluorophore during the lifetime of the excited state and upon collision, the fluorophore itself returns to the ground state without emission of a photon. Such quenching involves collision between the two molecules with the fluor losing kinetic energy. This quenching is an additional process besides radiative emission that deactivates the excited state. The decrease in fluorescence intensity equates to the decrease in fluorescence lifetime. The dependence of the emission intensity, *F,* on quencher concentration *[Q]* is given by the Stern-Volmer equation (Joseph R. R. Lakowicz*,* 2006):

$$F_o/F = T_o/T = 1 + k_q T_o[Q] \qquad \text{[Eq.6.3]}$$

where T and T_o is the lifetime in the presence and absence of quencher, respectively; and k_q is the bimolecular rate constant for the dynamic reaction of the quencher with the fluorophore. The product of $k_q T_o$ is referred to as the dynamic *Stern-Volmer quenching constant* or $K_{SV.}$ This constant indicates the sensitivity of the fluor to a quencher.

However, when quenching only results from a collision process and a stable bimolecular complex is formed then:

$$F + q \rightarrow Fq \qquad \text{[Eq.6.4]}$$

The ratio between F_o and F is also given by:

Fo/F=1+Ka[Q] [Eq.6.5]

Where *Ka* is the association constant determining the complex formed. This is how K_D (dissociation constant and the reciprocal of the association constant) is determined using fluorescence quenching measurements by addition of a quencher. Therefore, it is useful to note that $1/K_{SV}$ *or* K_D, is the quencher concentration at which $F_0 / F = 2$, or 50% of the intensity is quenched. Mathematical determination of K_D will be discussed in part III.

A major difference between dynamic and static quenching is that temperature affects the two processes in opposite ways. Dynamic quenching is a diffusion-controlled process which increases with temperature. Static quenching, on the other hand, does not affect temperature, and efficiency is decreased at higher temperatures since the fluor-quencher complex is less stable under those conditions. Therefore, in practice, it is necessary to measure F_o/F under a controlled temperature.

As described above, FRET is another way to cause fluorescence quenching. This process is strongly dependent on the distance, *R*, between two fluors. And it may be used to measure distances in proteins, membranes and macromolecules when the distances fall within the range of 10-80 Å. One way to calculate *R* is by the following equation (Sheehan, D., 2000):

$$E = \frac{R_0^6}{R_0^6 + R^6} \qquad \text{[Eq.6.6]}$$

Where R is the distance between the donor and acceptor fluors and R_0 is a constant related to the donor-acceptor pair which can be calculated from their absorption and emission spectra. E can be determined either from the fluorescence intensity (F) in the presence (p) or absence (a) of the acceptor as follows:

$$E = 1 - \frac{F_p}{F_a} \qquad \text{[Eq.6.7]}$$

After E is determined, R can be calculated if R_0 is known.

In overall speaking, even though fluorescence quenching can be used as a sensitive probe to monitor environmental changes and tell us the information about both the properties of macromolecules and their interactions with other molecules, lots of factors can contribute to fluorescence quenching and thus we should be cautious when interpreting quenching data. Many factors, such as properties of the fluorophore, spectrum shifts to shorter wavelengths or the change of polarity of the solvent, affect the intensity of fluorescence of a fluor (Freifelder, D. M., 1982). So whether quenching occurs is a combination of all effects. Also even if fluorescence quenching is observed from experiments determining protein binding affinity, it is hard to say whether it results from conformational changes or from substrate binding. It is unwise to always correlate ligand binding with a result from conformational changes. Therefore, alternative binding tests sometimes should be applied besides fluorescence quenching when we need to determine a protein's binding affinity.

3. Use of Intrinsic Fluorescence Measurements for Determination of K_D in Binding Proteins.

Two types of fluors are used in fluorescence analysis of macromolecules of biochemistry − intrinsic fluors and extrinsic fluors. Tryptophan, tyrosine and phenylanine are the only three intrinsic fluors in proteins. Among those three intrinsic fluors, tryptophan has the highest value of Q, which makes it more commonly used in fluorescence studies. This is because phenylalanine has a very low Q and tyrosine has a very weak fluorescence signal when quenched. The fluorescence of tyrosine is almost totally quenched when it is ionized or near a protonated acidic group, or even a tryptophan. The main reason to study intrinsic fluorescence of proteins is to obtain information about their conformation. Also, binding of ligands to proteins often causes conformational changes in their structure. If this structural change has an effect on the micro-environment of the intrinsic fluors (tryptophans in particular) within the binding pocket or domain of a protein, this will result in measurable changes in the fluorescence spectrum. Therefore, changes of intensity of fluorescence at a particular wavelength can be used to determine the dissociation constant (K_D) of the protein for the ligand where K_D is a measure of the binding affinity of the protein for the ligand. K_D is derived as below:

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL \qquad \text{[Eq.6.8]}$$

$$K_D = \frac{[P][L]}{[PL]} = 1/Ka = \frac{k_{off}}{k_{on}} \qquad \text{[Eq.6.9]}$$

Where P stands for the protein and L represents the ligand; k_{on} is the association rate constant or on rate while k_{off} is the dissociation rate constant or off rate. *Ka* and K_D seem to be simply the ratio of the two rates. However, they have different meanings. *Ka*, known as the association or equilibrium constant, has a unit of the reciprocal of a concentration (M^{-1}). However, K_D, dissociation constant, has a physical unit of a concentration (M). In biochemistry, K_D is usually determined in preference to *Ka* because determination of *Ka* requires the reaction to proceed to equilibrium whereas K_D can be derived from reactions in which half of the concentration of ligand is complexed with the protein.

If it is simple binding with no cooperativity, the simplest form of binding equation is used:

$$y = \frac{[L]*Cap}{K_d + [L]} \qquad \text{[Eq.6.10]}$$

In this expression, *y* is either the amount bound or is some factor proportional to it (e.g. radioactivity, absorbance, fluorescence intensity etc.). The capacity for binding ligand, *Cap*, is either a saturated amount bound in terms of moles of ligand, or a stoichiometric quantity in terms of moles of ligand per mole protein depending upon the definition of *y*. K_D is the concentration of free ligand added to the sample when the

bound reaches half of the Cap. The concentration of free ligand, [L], is the *x* data axis. **Also see Fig. 6.2.**

As demonstrated before, if we plot fluorescence intensity or the factor F_o/F against concentration of a quencher, which F_o stands for starting fluorescence intensity of the sample and F is the fluorescence intensity after addition of a quencher, we will observe similar curve as described in Fig 6.2, but with a tendency of decrease in F_o/F as the concentration of the quencher increases. K_D can be calculated using bound-versus-total equation of Grafit 5.09 (Erithacus, Ltd., Meddlesex, United Kingdom), the non-linear fit of equation performed by the computer (Grafit 2002).

$$K_D = \frac{(\text{Cap-Bound})*(\text{Total-Bound})}{\text{Bound}} \qquad \text{[Eq.6.11]}$$

$$\text{Bound}^2-(\text{Cap}+\text{Kd})*\text{Bound}+\text{Cap}*\text{Total}=0 \qquad \text{[Eq.6.12]}$$

$$\text{Bound} = \frac{b- \sqrt{b^2- 4*\text{Total}*\text{Cap}}}{2} \qquad \text{[Eq.6.13]}$$

Where B=Kd+Total+Cap; total is the amount of ligand added to the assay, and K_D and Cap(capacity) are the two parameters determined by the least squared fitting using the bound-vs-total equation program from Grafit 5.09.

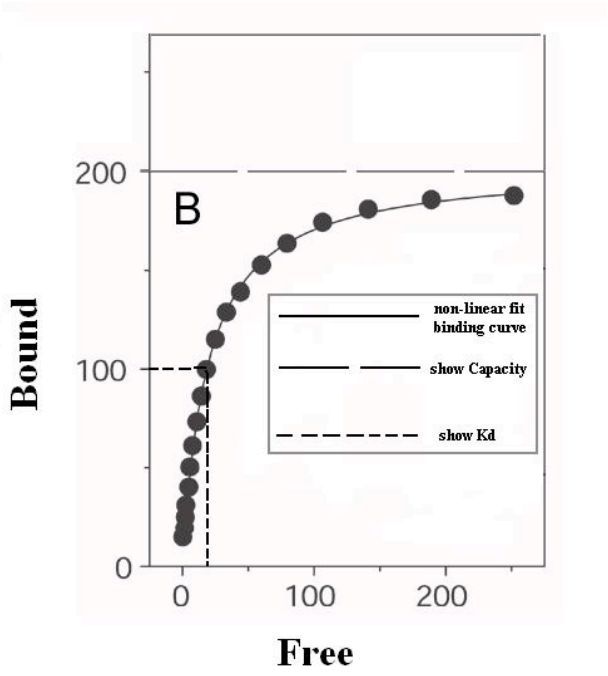


Fig. 6.2 Binding Graph

From this typical binding graph, the capacity and K_d are shown. The curved line is a non-linear fit of the equation performed by computer and the fit is good because it indicates that binding follows the simple 1:1 model.
K_d can be calculated from the non-linear fit equation.
.

In summary, fluorescence spectroscopy is a sensitive tool to study the conformation, binding sites, and solvent interactions of proteins of interest. The intrinsic fluorescence especially can give us a quick and convenient survey of how the ligand binds to the protein. However, the use of measurements of intrinsic fluorescence in proteins is based on empirical principles from studies with model compounds whose structure and conformation are well known. Therefore, some caution must be taken when data are interpreted. For example, if a ligand binds to a protein and tryptophan fluorescence is quenched, either there is a gross conformational change as a result of binding or some tryptophan is in or very near the binding site. Also if the λ_{max} of the tryptophans fluorescence spectrum does not shift to shorter

wavelength, quenching occurs as the polarity of the solvent increase or as tryptophans is exposed to some neighbouring charged groups.

REFERENCE

Adams, T. J., S. Vartivarian, et al. (1990). "Iron acquisition systems of Listeria monocytogenes." Infect Immun **58**(8): 2715-8.

Arnaud, M., A. Chastanet, et al. (2004). "New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria." Appl Environ Microbiol **70**(11): 6887-91.

Babu, M. M., M. L. Priya, et al. (2006). "A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins." J Bacteriol **188**(8): 2761-73.

Bagg, A. and J. B. Neilands (1987). "Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli." Biochemistry **26**(17): 5471-7.

Baichoo, N. and J. D. Helmann (2002). "Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence." J Bacteriol **184**(21): 5826-32.

Barchini, E. and R. E. Cowart (1996). "Extracellular iron reductase activity produced by Listeria monocytogenes." Arch Microbiol **166**(1): 51-7.

Barnett, T. C. and J. R. Scott (2002). "Differential recognition of surface proteins in Streptococcus pyogenes by two sortase gene homologs." J Bacteriol **184**(8): 2181-91.

Bierne, H. and P. Cossart (2002). "InlB, a surface protein of Listeria monocytogenes that behaves as an invasin and a growth factor." J Cell Sci **115**(Pt 17): 3357-67.

Bierne, H., C. Garandeau, et al. (2004). "Sortase B, a new class of sortase in Listeria monocytogenes." J Bacteriol **186**(7): 1972-82.

Bierne, H., S. K. Mazmanian, et al. (2002). "Inactivation of the srtA gene in Listeria monocytogenes inhibits anchoring of surface proteins and affects virulence." Mol Microbiol **43**(4): 869-81.

Bluhm, M. E., B. P. Hay, et al. (2002). "Corynebactin and a serine trilactone based analogue: chirality and molecular modeling of ferric complexes." Inorg Chem **41**(21): 5475-8.

Bluhm, M. E., S. S. Kim, et al. (2002). "Corynebactin and enterobactin: related siderophores of opposite chirality." J Am Chem Soc **124**(11): 2436-7.

Borezee, E., E. Pellegrini, et al. (2001). "SvpA, a novel surface virulence-associated protein required for intracellular survival of Listeria monocytogenes." Microbiology **147**(Pt 11): 2913-23.

Boyer, E., I. Bergevin, et al. (2002). "Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium." Infect Immun **70**(11): 6032-42.

Braun, L. and P. Cossart (2000). "Interactions between Listeria monocytogenes and host mammalian cells." Microbes Infect **2**(7): 803-11.
Braun, V. (2001). "Iron uptake mechanisms and their regulation in pathogenic bacteria." Int J Med Microbiol **291**(2): 67-79.
Braun, V. and H. Killmann (1999). "Bacterial solutions to the iron-supply problem." Trends Biochem Sci **24**(3): 104-9.
Braun, V. and K. Rehn (1969). "Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the E. coli cell wall. The specific effect of trypsin on the membrane structure." Eur J Biochem **10**(3): 426-38.
Brockstedt, D. G., M. A. Giedlin, et al. (2004). "Listeria-based cancer vaccines that segregate immunogenicity from toxicity." Proc Natl Acad Sci U S A **101**(38): 13832-7.
Brown, J. S. and D. W. Holden (2002). "Iron acquisition by Gram-positive bacterial pathogens." Microbes Infect **4**(11): 1149-56.
Cameron, L. A., P. A. Giardini, et al. (2000). "Secrets of actin-based motility revealed by a bacterial pathogen." Nat Rev Mol Cell Biol **1**(2): 110-9.
Chattopadhyay, P. K. and H. C. Wu (1977). "Biosynthesis of the covalently linked diglyceride in murein lipoprotein of Escherichia coli." Proc Natl Acad Sci U S A **74**(12): 5318-22.
Clancy, A., J. W. Loar, et al. (2006). "Evidence for siderophore-dependent iron acquisition in group B streptococcus." Mol Microbiol **59**(2): 707-21.
Clarke, T. E., S. Y. Ku, et al. (2000). "The structure of the ferric siderophore binding protein FhuD complexed with gallichrome." Nat Struct Biol **7**(4): 287-91.
Clarke, T. E., L. W. Tari, et al. (2001). "Structural biology of bacterial iron uptake systems." Curr Top Med Chem **1**(1): 7-30.
Cook, W. J., S. R. Kar, et al. (1998). "Crystal structure of the cyanobacterial metallothionein repressor SmtB: a model for metalloregulatory proteins." J Mol Biol **275**(2): 337-46.
Cooper, S. R., J. V. McArdle, et al. (1978). "Siderophore electrochemistry: relation to intracellular iron release mechanism." Proc Natl Acad Sci U S A **75**(8): 3551-4.
Cossart, P. (2001). "Met, the HGF-SF receptor: another receptor for Listeria monocytogenes." Trends Microbiol **9**(3): 105-7.
Cossart, P. and R. Jonquieres (2000). "Sortase, a universal target for therapeutic agents against gram-positive bacteria?" Proc Natl Acad Sci U S A **97**(10): 5013-5.
Cossart, P. and M. Lecuit (1998). "Interactions of Listeria monocytogenes with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling." Embo J **17**(14): 3797-806.
Coulanges, V., P. Andre, et al. (1997). "Utilization of iron-catecholamine complexes involving ferric reductase activity in Listeria monocytogenes." Infect Immun **65**(7): 2778-85.

Courcol, R. J., D. Trivier, et al. (1997). "Siderophore production by Staphylococcus aureus and identification of iron-regulated proteins." Infect Immun **65**(5): 1944-8.

Cowart, R. E. and B. G. Foster (1985). "Differential effects of iron on the growth of Listeria monocytogenes: minimum requirements and mechanism of acquisition." J Infect Dis **151**(4): 721-30.

Crosa, J. H. and C. T. Walsh (2002). "Genetics and assembly line enzymology of siderophore biosynthesis in bacteria." Microbiol Mol Biol Rev **66**(2): 223-49.

De Lorenzo, V., M. Herrero, et al. (1988). "Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of fur gene in Escherichia coli." Eur J Biochem **173**(3): 537-46.

Deneer, H. G., V. Healey, et al. (1995). "Reduction of exogenous ferric iron by a surface-associated ferric reductase of Listeria spp." Microbiology **141 (Pt 8)**: 1985-92.

Dhungana, S., P. S. White, et al. (2001). "Crystal structure of ferrioxamine B: a comparative analysis and implications for molecular recognition." J Biol Inorg Chem **6**(8): 810-8.

Dmitriev, B. A., S. Ehlers, et al. (1999). "Layered murein revisited: a fundamentally new concept of bacterial cell wall structure, biogenesis and function." Med Microbiol Immunol **187**(3): 173-81.

Dramsi, S., P. Dehoux, et al. (1997). "Identification of four new members of the internalin multigene family of Listeria monocytogenes EGD." Infect Immun **65**(5): 1615-25.

Dussurget, O., E. Dumas, et al. (2005). "Listeria monocytogenes ferritin protects against multiple stresses and is required for virulence." FEMS Microbiol Lett **250**(2): 253-61.

Epand, R. M. (1997). "Studies of membrane physical properties and their role in biological function." Biochem Soc Trans **25**(3): 1073-9.

Ernst, J. F., R. L. Bennett, et al. (1978). "Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of Salmonella typhimurium." J Bacteriol **135**(3): 928-34.

Ferguson, A. D. and J. Deisenhofer (2002). "TonB-dependent receptors-structural perspectives." Biochim Biophys Acta **1565**(2): 318-32.

Ferguson, A. D., E. Hofmann, et al. (1998). "Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide." Science **282**(5397): 2215-20.

Gaillard, J. L., F. Jaubert, et al. (1996). "The inlAB locus mediates the entry of Listeria monocytogenes into hepatocytes in vivo." J Exp Med **183**(2): 359-69.

Gan, K., S. D. Gupta, et al. (1993). "Isolation and characterization of a temperature-sensitive mutant of Salmonella typhimurium defective in prolipoprotein modification." J Biol Chem **268**(22): 16544-50.

Gibson, F. and D. I. Magrath (1969). "The isolation and characterization of a hydroxamic acid (aerobactin) formed by Aerobacter aerogenes 62-I." Biochim Biophys Acta **192**(2): 175-84.

Guerinot, M. L. (1994). "Microbial iron transport." Annu Rev Microbiol **48**: 743-72.

Hanahan, D. (1983). "Studies on transformation of Escherichia coli with plasmids." J Mol Biol **166**(4): 557-80.

Hantke, K. (1981). "Regulation of ferric iron transport in Escherichia coli K12: isolation of a constitutive mutant." Mol Gen Genet **182**(2): 288-92.

Hantke, K. (2001). "Iron and metal regulation in bacteria." Curr Opin Microbiol **4**(2): 172-7.

Hantke, K. and V. Braun (1973). "[The structure of covalent binding of lipid to protein in the murein-lipoprotein of the outer membrane of Escherichia coli (author's transl)]." Hoppe Seylers Z Physiol Chem **354**(7): 813-5.

Hartford, T., S. O'Brien, et al. (1993). "Utilization of transferrin-bound iron by Listeria monocytogenes." FEMS Microbiol Lett **108**(3): 311-8.

Hayashi, S. and H. C. Wu (1990). "Lipoproteins in bacteria." J Bioenerg Biomembr **22**(3): 451-71.

Imbert, M. and R. Blondeau (1998). "On the iron requirement of lactobacilli grown in chemically defined medium." Curr Microbiol **37**(1): 64-6.

Inukai, M., M. Takeuchi, et al. (1978). "Mechanism of action of globomycin." J Antibiot (Tokyo) **31**(11): 1203-5.

Jin, B., S. M. Newton, et al. (2006). "Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in Listeria monocytogenes." Mol Microbiol **59**(4): 1185-98.

Kamalakkannan, S., V. Murugan, et al. (2004). "Bacterial lipid modification of proteins for novel protein engineering applications." Protein Eng Des Sel **17**(10): 721-9.

Klebba, P. E., M. A. McIntosh, et al. (1982). "Kinetics of biosynthesis of iron-regulated membrane proteins in Escherichia coli." J Bacteriol **149**(3): 880-8.

Kocks, C., E. Gouin, et al. (1992). "L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein." Cell **68**(3): 521-31.

Kupper, F. C., C. J. Carrano, et al. (2006). "Photoreactivity of iron(III)-aerobactin: photoproduct structure and iron(III) coordination." Inorg Chem **45**(15): 6028-33.

Lammerding, A. M. and M. P. Doyle (1989). "Evaluation of enrichment procedures for recovering Listeria monocytogenes from dairy products." Int J Food Microbiol **9**(3): 249-68.

Larson, J. A., H. L. Howie, et al. (2004). "Neisseria meningitidis accelerates ferritin degradation in host epithelial cells to yield an essential iron source." Mol Microbiol **53**(3): 807-20.

Lauer, P., M. Y. Chow, et al. (2002). "Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors." J Bacteriol **184**(15): 4177-86.

Lavrrar, J. L. and M. A. McIntosh (2003). "Architecture of a fur binding site: a comparative analysis." J Bacteriol **185**(7): 2194-202.

Lecuit, M., S. Vandormael-Pournin, et al. (2001). "A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier." Science **292**(5522): 1722-5.

Lee, S. F. and T. L. Boran (2003). "Roles of sortase in surface expression of the major protein adhesin P1, saliva-induced aggregation and adherence, and cariogenicity of Streptococcus mutans." Infect Immun **71**(2): 676-81.

Lewis, L. A., M. H. Sung, et al. (1998). "Transport of intact porphyrin by HpuAB, the hemoglobin-haptoglobin utilization system of Neisseria meningitidis." J Bacteriol **180**(22): 6043-7.

Madan Babu, M. and K. Sankaran (2002). "DOLOP--database of bacterial lipoproteins." Bioinformatics **18**(4): 641-3.

Maresso, A. W. and O. Schneewind (2006). "Iron acquisition and transport in Staphylococcus aureus." Biometals **19**(2): 193-203.

May, J. J., T. M. Wendrich, et al. (2001). "The dhb operon of Bacillus subtilis encodes the biosynthetic template for the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin." J Biol Chem **276**(10): 7209-17.

Mazmanian, S. K., G. Liu, et al. (2000). "Staphylococcus aureus sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections." Proc Natl Acad Sci U S A **97**(10): 5510-5.

Mazmanian, S. K., E. P. Skaar, et al. (2003). "Passage of heme-iron across the envelope of Staphylococcus aureus." Science **299**(5608): 906-9.

Mazmanian, S. K., H. Ton-That, et al. (2001). "Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus." Mol Microbiol **40**(5): 1049-57.

Mazmanian, S. K., H. Ton-That, et al. (2002). "An iron-regulated sortase anchors a class of surface protein during Staphylococcus aureus pathogenesis." Proc Natl Acad Sci U S A **99**(4): 2293-8.

McLauchlin, J. a. (1990). "Human listeriosis in Britain, 1967-85, a summary of 722 cases. 1. Listeriosis during pregnancy and in the newborn." Epidemiol Infect **104**(2): 181-9.

McLauchlin, J. b. (1990). "Human listeriosis in Britain, 1967-85, a summary of 722 cases. 2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence." Epidemiol Infect **104**(2): 191-201.

Mengaud, J., H. Ohayon, et al. (1996). "E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells." Cell **84**(6): 923-32.

Navarre, W. W., S. Daefler, et al. (1996). "Cell wall sorting of lipoproteins in Staphylococcus aureus." J Bacteriol **178**(2): 441-6.
Navarre, W. W. and O. Schneewind (1999). "Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope." Microbiol Mol Biol Rev **63**(1): 174-229.
Neidhardt FC, B.P., Smith DF (1974). "Culture medium for enterobacteria." J Bacteriol. 119(3):736-47.
Neilands, J. B. (1981). "Microbial iron compounds." Annu Rev Biochem **50**: 715-31.
Neilands, J. B. (1983). "Siderophores." Adv Inorg Biochem **5**: 137-66.
Neilands, J. B. (1984). "Siderophores of bacteria and fungi." Microbiol Sci **1**(1): 9-14.
Neilands, J. B. (1991). "A brief history of iron metabolism." Biol Met **4**(1): 1-6.
Neilands, J. B. (1995). "Siderophores: structure and function of microbial iron transport compounds." J Biol Chem **270**(45): 26723-6.
Newton, S. M., P. E. Klebba, et al. (2005). "The svpA-srtB locus of Listeria monocytogenes: fur-mediated iron regulation and effect on virulence." Mol Microbiol **55**(3): 927-40.
Panina, E. M., A. A. Mironov, et al. (2001). "Comparative analysis of FUR regulons in gamma-proteobacteria." Nucleic Acids Res **29**(24): 5195-206.
Pohl, E., J. C. Haller, et al. (2003). "Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator." Mol Microbiol **47**(4): 903-15.
Pohl, E., R. K. Holmes, et al. (1999). "Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain." J Mol Biol **292**(3): 653-67.
Pohl, E., R. K. Holmes, et al. (1999). "Crystal structure of the iron-dependent regulator (IdeR) from Mycobacterium tuberculosis shows both metal binding sites fully occupied." J Mol Biol **285**(3): 1145-56.
Portnoy, D. A., V. Auerbuch, et al. (2002). "The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity." J Cell Biol **158**(3): 409-14.
Raymond, K. N., E. A. Dertz, et al. (2003). "Enterobactin: an archetype for microbial iron transport." Proc Natl Acad Sci U S A **100**(7): 3584-8.
Raymond, K. N., S. S. Isied, et al. (1976). "Coordination isomers of biological iron transport compounds. VI. Models of the enterobactin coordination site. A crystal field effect in the structure of potassium tris(catecholato)chromate(III) and -ferrate(III) sesquihydrates, K3(M(O2C6H4)3)-1.5H2O, M = Cr, Fe1." J Am Chem Soc **98**(7): 1767-74.
Rea, R. B., C. G. Gahan, et al. (2004). "Disruption of putative regulatory loci in Listeria monocytogenes demonstrates a significant role for Fur and PerR in virulence." Infect Immun **72**(2): 717-27.
Rohrbach, M. R., V. Braun, et al. (1995). "Ferrichrome transport in Escherichia coli K-12: altered substrate specificity of mutated periplasmic FhuD and interaction

of FhuD with the integral membrane protein FhuB." J Bacteriol **177**(24): 7186-93.
Roosenberg, J. M., 2nd, Y. M. Lin, et al. (2000). "Studies and syntheses of siderophores, microbial iron chelators, and analogs as potential drug delivery agents." Curr Med Chem **7**(2): 159-97.
Rouault, T. A. (2004). "Microbiology. Pathogenic bacteria prefer heme." Science **305**(5690): 1577-8.
Rutz, J. M., J. Liu, et al. (1992). "Formation of a gated channel by a ligand-specific transport protein in the bacterial outer membrane." Science **258**(5081): 471-5.
Sankaran, K. and H. C. Wu (1994). "Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol." J Biol Chem **269**(31): 19701-6.
Schiering, N., X. Tao, et al. (1994). "Crystallization and preliminary X-ray studies of the diphtheria Tox repressor from Corynebacterium diphtheriae." J Mol Biol **244**(5): 654-6.
Schneewind, O., A. Fowler, et al. (1995). "Structure of the cell wall anchor of surface proteins in Staphylococcus aureus." Science **268**(5207): 103-6.
Schneider, R. and K. Hantke (1993). "Iron-hydroxamate uptake systems in Bacillus subtilis: identification of a lipoprotein as part of a binding protein-dependent transport system." Mol Microbiol **8**(1): 111-21.
Schwyn, B. and J. B. Neilands (1987). "Universal chemical assay for the detection and determination of siderophores." Anal Biochem **160**(1): 47-56.
Sebulsky, M. T. and D. E. Heinrichs (2001). "Identification and characterization of fhuD1 and fhuD2, two genes involved in iron-hydroxamate uptake in Staphylococcus aureus." J Bacteriol **183**(17): 4994-5000.
Sebulsky, M. T., B. H. Shilton, et al. (2003). "The role of FhuD2 in iron(III)-hydroxamate transport in Staphylococcus aureus. Demonstration that FhuD2 binds iron(III)-hydroxamates but with minimal conformational change and implication of mutations on transport." J Biol Chem **278**(50): 49890-900.
Seeliger, H. P. (1988). "Listeriosis--history and actual developments." Infection **16 Suppl 2**: S80-4.
Simon, N., V. Coulanges, et al. (1995). "Utilization of exogenous siderophores and natural catechols by Listeria monocytogenes." Appl Environ Microbiol **61**(4): 1643-5.
Skaar, E. P., A. H. Gaspar, et al. (2004). "IsdG and IsdI, heme-degrading enzymes in the cytoplasm of Staphylococcus aureus." J Biol Chem **279**(1): 436-43.
Skaar, E. P., A. H. Gaspar, et al. (2006). "Bacillus anthracis IsdG, a heme-degrading monooxygenase." J Bacteriol **188**(3): 1071-80.
Smith, K. and P. Youngman (1992). "Use of a new integrational vector to investigate compartment-specific expression of the Bacillus subtilis spoIIM gene." Biochimie **74**(7-8): 705-11.

Sprencel, C., Z. Cao, et al. (2000). "Binding of ferric enterobactin by the Escherichia coli periplasmic protein FepB." J Bacteriol **182**(19): 5359-64.
Stanley, P., L. C. Packman, et al. (1994). "Fatty acylation of two internal lysine residues required for the toxic activity of Escherichia coli hemolysin." Science **266**(5193): 1992-6.
Studier, F. W., A. H. Rosenberg, et al. (1990). "Use of T7 RNA polymerase to direct expression of cloned genes." Methods Enzymol **185**: 60-89.
Tilney, L. G. and D. A. Portnoy (1989). "Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes." J Cell Biol **109**(4 Pt 1): 1597-608.
Trost, M., D. Wehmhoner, et al. (2005). "Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic Listeria species." Proteomics **5**(6): 1544-57.
Valvano, M. A., R. P. Silver, et al. (1986). "Occurrence of chromosome- or plasmid-mediated aerobactin iron transport systems and hemolysin production among clonal groups of human invasive strains of Escherichia coli K1." Infect Immun **52**(1): 192-9.
Vazquez-Boland, J. A., M. Kuhn, et al. (2001). "Listeria pathogenesis and molecular virulence determinants." Clin Microbiol Rev **14**(3): 584-640.
von Heijne, G. (1989). "The structure of signal peptides from bacterial lipoproteins." Protein Eng **2**(7): 531-4.
Wandersman, C. and P. Delepelaire (2004). "Bacterial iron sources: from siderophores to hemophores." Annu Rev Microbiol **58**: 611-47.
Warren, R. A. and J. B. Neilands (1965). "Mechanism of Microbial Catabolism of Ferrichrome A." J Biol Chem **240**: 2055-8.
Watkins, J. and K. P. Sleath (1981). "Isolation and enumeration of Listeria monocytogenes from Sewage, Sewage Sludge and River Water." J Appl Bacteriol **50**(1): 1-9.
Weinberg, E. D. (1999). "Iron loading and disease surveillance." Emerg Infect Dis **5**(3): 346-52.
Weinberg, E. D. (2000). "Microbial pathogens with impaired ability to acquire host iron." Biometals **13**(1): 85-9.
Wu, H. C., C. Hou, et al. (1977). "Biochemical characterization of a mutant lipoprotein of Escherichia coli." Proc Natl Acad Sci U S A **74**(4): 1388-92.
Xiong, A., V. K. Singh, et al. (2000). "Molecular characterization of the ferric-uptake regulator, fur, from Staphylococcus aureus." Microbiology **146 (Pt 3)**: 659-68.
Yanisch-Perron, C., J. Vieira, et al. (1985). "Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors." Gene **33**(1): 103-19.
Yeowell, H. N. and J. R. White (1982). "Iron requirement in the bactericidal mechanism of streptonigrin." Antimicrob Agents Chemother **22**(6): 961-8.

Adams, T. J., S. Vartivarian, et al. (1990). "Iron acquisition systems of Listeria monocytogenes." Infect Immun **58**(8): 2715-8.

Arnaud, M., A. Chastanet, et al. (2004). "New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria." Appl Environ Microbiol **70**(11): 6887-91.

Babu, M. M., M. L. Priya, et al. (2006). "A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins." J Bacteriol **188**(8): 2761-73.

Bagg, A. and J. B. Neilands (1987). "Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli." Biochemistry **26**(17): 5471-7.

Baichoo, N. and J. D. Helmann (2002). "Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence." J Bacteriol **184**(21): 5826-32.

Barchini, E. and R. E. Cowart (1996). "Extracellular iron reductase activity produced by Listeria monocytogenes." Arch Microbiol **166**(1): 51-7.

Barnett, T. C. and J. R. Scott (2002). "Differential recognition of surface proteins in Streptococcus pyogenes by two sortase gene homologs." J Bacteriol **184**(8): 2181-91.

Bates, C. S., G. E. Montanez, et al. (2003). "Identification and characterization of a Streptococcus pyogenes operon involved in binding of hemoproteins and acquisition of iron." Infect Immun **71**(3): 1042-55.

Bierne, H. and P. Cossart (2002). "InlB, a surface protein of Listeria monocytogenes that behaves as an invasin and a growth factor." J Cell Sci **115**(Pt 17): 3357-67.

Bierne, H., C. Garandeau, et al. (2004). "Sortase B, a new class of sortase in Listeria monocytogenes." J Bacteriol **186**(7): 1972-82.

Bierne, H., S. K. Mazmanian, et al. (2002). "Inactivation of the srtA gene in Listeria monocytogenes inhibits anchoring of surface proteins and affects virulence." Mol Microbiol **43**(4): 869-81.

Bluhm, M. E., B. P. Hay, et al. (2002). "Corynebactin and a serine trilactone based analogue: chirality and molecular modeling of ferric complexes." Inorg Chem **41**(21): 5475-8.

Bluhm, M. E., S. S. Kim, et al. (2002). "Corynebactin and enterobactin: related siderophores of opposite chirality." J Am Chem Soc **124**(11): 2436-7.

Borezee, E., E. Pellegrini, et al. (2001). "SvpA, a novel surface virulence-associated protein required for intracellular survival of Listeria monocytogenes." Microbiology **147**(Pt 11): 2913-23.

Boulanger, P., M. le Maire, et al. (1996). "Purification and structural and functional characterization of FhuA, a transporter of the Escherichia coli outer membrane." Biochemistry **35**(45): 14216-24.

Boyer, E., I. Bergevin, et al. (2002). "Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium." Infect Immun **70**(11): 6032-42.

Braun, L. and P. Cossart (2000). "Interactions between Listeria monocytogenes and host mammalian cells." Microbes Infect **2**(7): 803-11.

Braun, V. (2001). "Iron uptake mechanisms and their regulation in pathogenic bacteria." Int J Med Microbiol **291**(2): 67-79.

Braun, V. and H. Killmann (1999). "Bacterial solutions to the iron-supply problem." Trends Biochem Sci **24**(3): 104-9.

Braun, V. and K. Rehn (1969). "Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the E. coli cell wall. The specific effect of trypsin on the membrane structure." Eur J Biochem **10**(3): 426-38.

Brockstedt, D. G., M. A. Giedlin, et al. (2004). "Listeria-based cancer vaccines that segregate immunogenicity from toxicity." Proc Natl Acad Sci U S A **101**(38): 13832-7.

Brown, J. S. and D. W. Holden (2002). "Iron acquisition by Gram-positive bacterial pathogens." Microbes Infect **4**(11): 1149-56.

Cameron, L. A., P. A. Giardini, et al. (2000). "Secrets of actin-based motility revealed by a bacterial pathogen." Nat Rev Mol Cell Biol **1**(2): 110-9.

Chattopadhyay, P. K. and H. C. Wu (1977). "Biosynthesis of the covalently linked diglyceride in murein lipoprotein of Escherichia coli." Proc Natl Acad Sci U S A **74**(12): 5318-22.

Clancy, A., J. W. Loar, et al. (2006). "Evidence for siderophore-dependent iron acquisition in group B streptococcus." Mol Microbiol **59**(2): 707-21.

Clarke, T. E., S. Y. Ku, et al. (2000). "The structure of the ferric siderophore binding protein FhuD complexed with gallichrome." Nat Struct Biol **7**(4): 287-91.

Clarke, T. E., L. W. Tari, et al. (2001). "Structural biology of bacterial iron uptake systems." Curr Top Med Chem **1**(1): 7-30.

Cook, W. J., S. R. Kar, et al. (1998). "Crystal structure of the cyanobacterial metallothionein repressor SmtB: a model for metalloregulatory proteins." J Mol Biol **275**(2): 337-46.

Cooper, S. R., J. V. McArdle, et al. (1978). "Siderophore electrochemistry: relation to intracellular iron release mechanism." Proc Natl Acad Sci U S A **75**(8): 3551-4.

Cossart, P. (2001). "Met, the HGF-SF receptor: another receptor for Listeria monocytogenes." Trends Microbiol **9**(3): 105-7.

Cossart, P. and R. Jonquieres (2000). "Sortase, a universal target for therapeutic agents against gram-positive bacteria?" Proc Natl Acad Sci U S A **97**(10): 5013-5.

Cossart, P. and M. Lecuit (1998). "Interactions of Listeria monocytogenes with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling." Embo J **17**(14): 3797-806.

Coulanges, V., P. Andre, et al. (1997). "Utilization of iron-catecholamine complexes involving ferric reductase activity in Listeria monocytogenes." Infect Immun **65**(7): 2778-85.

Courcol, R. J., D. Trivier, et al. (1997). "Siderophore production by Staphylococcus aureus and identification of iron-regulated proteins." Infect Immun **65**(5): 1944-8.

Cowart, R. E. and B. G. Foster (1985). "Differential effects of iron on the growth of Listeria monocytogenes: minimum requirements and mechanism of acquisition." J Infect Dis **151**(4): 721-30.

Crosa, J. H. and C. T. Walsh (2002). "Genetics and assembly line enzymology of siderophore biosynthesis in bacteria." Microbiol Mol Biol Rev **66**(2): 223-49.

Dale, S. E., A. Doherty-Kirby, et al. (2004). "Role of siderophore biosynthesis in virulence of Staphylococcus aureus: identification and characterization of genes involved in production of a siderophore." Infect Immun **72**(1): 29-37.

Daskaleros, P. A., J. A. Stoebner, et al. (1991). "Iron uptake in Plesiomonas shigelloides: cloning of the genes for the heme-iron uptake system." Infect Immun **59**(8): 2706-11.

De Lorenzo, V., M. Herrero, et al. (1988). "Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of fur gene in Escherichia coli." Eur J Biochem **173**(3): 537-46.

Deneer, H. G., V. Healey, et al. (1995). "Reduction of exogenous ferric iron by a surface-associated ferric reductase of Listeria spp." Microbiology **141 (Pt 8)**: 1985-92.

Dhungana, S., P. S. White, et al. (2001). "Crystal structure of ferrioxamine B: a comparative analysis and implications for molecular recognition." J Biol Inorg Chem **6**(8): 810-8.

Dmitriev, B. A., S. Ehlers, et al. (1999). "Layered murein revisited: a fundamentally new concept of bacterial cell wall structure, biogenesis and function." Med Microbiol Immunol **187**(3): 173-81.

Dramsi, S., P. Dehoux, et al. (1997). "Identification of four new members of the internalin multigene family of Listeria monocytogenes EGD." Infect Immun **65**(5): 1615-25.

Drazek, E. S., C. A. Hammack, et al. (2000). "Corynebacterium diphtheriae genes required for acquisition of iron from haemin and haemoglobin are homologous to ABC haemin transporters." Mol Microbiol **36**(1): 68-84.

Dussurget, O., E. Dumas, et al. (2005). "Listeria monocytogenes ferritin protects against multiple stresses and is required for virulence." FEMS Microbiol Lett **250**(2): 253-61.

Epand, R. M. (1997). "Studies of membrane physical properties and their role in biological function." Biochem Soc Trans **25**(3): 1073-9.

Ernst, J. F., R. L. Bennett, et al. (1978). "Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of Salmonella typhimurium." J Bacteriol **135**(3): 928-34.
Gaillard, J. L., F. Jaubert, et al. (1996). "The inlAB locus mediates the entry of Listeria monocytogenes into hepatocytes in vivo." J Exp Med **183**(2): 359-69.
Gan, K., S. D. Gupta, et al. (1993). "Isolation and characterization of a temperature-sensitive mutant of Salmonella typhimurium defective in prolipoprotein modification." J Biol Chem **268**(22): 16544-50.
Gibson, F. and D. I. Magrath (1969). "The isolation and characterization of a hydroxamic acid (aerobactin) formed by Aerobacter aerogenes 62-I." Biochim Biophys Acta **192**(2): 175-84.
Glaser, P., L. Frangeul, et al. (2001). "Comparative genomics of Listeria species." Science **294**(5543): 849-52.
Goulet, V., C. Jacquet, et al. (1995). "Listeriosis from consumption of raw-milk cheese." Lancet **345**(8964): 1581-2.
Guerinot, M. L. (1994). "Microbial iron transport." Annu Rev Microbiol **48**: 743-72.
Hanahan, D. (1983). "Studies on transformation of Escherichia coli with plasmids." J Mol Biol **166**(4): 557-80.
Hantke, K. (1981). "Regulation of ferric iron transport in Escherichia coli K12: isolation of a constitutive mutant." Mol Gen Genet **182**(2): 288-92.
Hantke, K. and V. Braun (1973). "[The structure of covalent binding of lipid to protein in the murein-lipoprotein of the outer membrane of Escherichia coli (author's transl)]." Hoppe Seylers Z Physiol Chem **354**(7): 813-5.
Hartford, T., S. O'Brien, et al. (1993). "Utilization of transferrin-bound iron by Listeria monocytogenes." FEMS Microbiol Lett **108**(3): 311-8.
Hayashi, S. and H. C. Wu (1990). "Lipoproteins in bacteria." J Bioenerg Biomembr **22**(3): 451-71.
Henderson, D. P. and S. M. Payne (1993). "Cloning and characterization of the Vibrio cholerae genes encoding the utilization of iron from haemin and haemoglobin." Mol Microbiol **7**(3): 461-9.
Imbert, M. and R. Blondeau (1998). "On the iron requirement of lactobacilli grown in chemically defined medium." Curr Microbiol **37**(1): 64-6.
Inukai, M., M. Takeuchi, et al. (1978). "Mechanism of action of globomycin." J Antibiot (Tokyo) **31**(11): 1203-5.
Jin, B., S. M. Newton, et al. (2006). "Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in Listeria monocytogenes." Mol Microbiol **59**(4): 1185-98.
Kamalakkannan, S., V. Murugan, et al. (2004). "Bacterial lipid modification of proteins for novel protein engineering applications." Protein Eng Des Sel **17**(10): 721-9.
Khun, H. H., S. D. Kirby, et al. (1998). "A Neisseria meningitidis fbpABC mutant is incapable of using nonheme iron for growth." Infect Immun **66**(5): 2330-6.

Klebba, P. E., M. A. McIntosh, et al. (1982). "Kinetics of biosynthesis of iron-regulated membrane proteins in Escherichia coli." J Bacteriol **149**(3): 880-8.

Kocks, C., E. Gouin, et al. (1992). "L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein." Cell **68**(3): 521-31.

Koster, W. and V. Braun (1990). "Iron (III) hydroxamate transport into Escherichia coli. Substrate binding to the periplasmic FhuD protein." J Biol Chem **265**(35): 21407-10.

Kupper, F. C., C. J. Carrano, et al. (2006). "Photoreactivity of iron(III)-aerobactin: photoproduct structure and iron(III) coordination." Inorg Chem **45**(15): 6028-33.

Lammerding, A. M. and M. P. Doyle (1989). "Evaluation of enrichment procedures for recovering Listeria monocytogenes from dairy products." Int J Food Microbiol **9**(3): 249-68.

Lauer, P., M. Y. Chow, et al. (2002). "Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors." J Bacteriol **184**(15): 4177-86.

Lavrrar, J. L. and M. A. McIntosh (2003). "Architecture of a fur binding site: a comparative analysis." J Bacteriol **185**(7): 2194-202.

Lecuit, M., S. Vandormael-Pournin, et al. (2001). "A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier." Science **292**(5522): 1722-5.

Lee, S. F. and T. L. Boran (2003). "Roles of sortase in surface expression of the major protein adhesin P1, saliva-induced aggregation and adherence, and cariogenicity of Streptococcus mutans." Infect Immun **71**(2): 676-81.

Madan Babu, M. and K. Sankaran (2002). "DOLOP--database of bacterial lipoproteins." Bioinformatics **18**(4): 641-3.

Maresso, A. W. and O. Schneewind (2006). "Iron acquisition and transport in Staphylococcus aureus." Biometals **19**(2): 193-203.

May, J. J., T. M. Wendrich, et al. (2001). "The dhb operon of Bacillus subtilis encodes the biosynthetic template for the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin." J Biol Chem **276**(10): 7209-17.

Mazmanian, S. K., G. Liu, et al. (2000). "Staphylococcus aureus sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections." Proc Natl Acad Sci U S A **97**(10): 5510-5.

Mazmanian, S. K., E. P. Skaar, et al. (2003). "Passage of heme-iron across the envelope of Staphylococcus aureus." Science **299**(5608): 906-9.

Mazmanian, S. K., H. Ton-That, et al. (2001). "Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus." Mol Microbiol **40**(5): 1049-57.

Mazmanian, S. K., H. Ton-That, et al. (2002). "An iron-regulated sortase anchors a class of surface protein during Staphylococcus aureus pathogenesis." Proc Natl Acad Sci U S A **99**(4): 2293-8.

McLauchlin, J. a. (1990). "Human listeriosis in Britain, 1967-85, a summary of 722 cases. 1. Listeriosis during pregnancy and in the newborn." Epidemiol Infect **104**(2): 181-9.

McLauchlin, J. b. (1990). "Human listeriosis in Britain, 1967-85, a summary of 722 cases. 2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence." Epidemiol Infect **104**(2): 191-201.

Mengaud, J., H. Ohayon, et al. (1996). "E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells." Cell **84**(6): 923-32.

Navarre, W. W., S. Daefler, et al. (1996). "Cell wall sorting of lipoproteins in Staphylococcus aureus." J Bacteriol **178**(2): 441-6.

Navarre, W. W. and O. Schneewind (1999). "Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope." Microbiol Mol Biol Rev **63**(1): 174-229.

Neilands, J. B. (1981). "Microbial iron compounds." Annu Rev Biochem **50**: 715-31.

Neilands, J. B. (1983). "Siderophores." Adv Inorg Biochem **5**: 137-66.

Neilands, J. B. (1984). "Siderophores of bacteria and fungi." Microbiol Sci **1**(1): 9-14.

Neilands, J. B. (1991). "A brief history of iron metabolism." Biol Met **4**(1): 1-6.

Neilands, J. B. (1995). "Siderophores: structure and function of microbial iron transport compounds." J Biol Chem **270**(45): 26723-6.

Newton, S. M., P. E. Klebba, et al. (2005). "The svpA-srtB locus of Listeria monocytogenes: fur-mediated iron regulation and effect on virulence." Mol Microbiol **55**(3): 927-40.

Nikaido, H. and H. C. Wu (1984). "Amino acid sequence homology among the major outer membrane proteins of Escherichia coli." Proc Natl Acad Sci U S A **81**(4): 1048-52.

Panina, E. M., A. A. Mironov, et al. (2001). "Comparative analysis of FUR regulons in gamma-proteobacteria." Nucleic Acids Res **29**(24): 5195-206.

Pohl, E., R. K. Holmes, et al. (1999). "Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain." J Mol Biol **292**(3): 653-67.

Pohl, E., R. K. Holmes, et al. (1999). "Crystal structure of the iron-dependent regulator (IdeR) from Mycobacterium tuberculosis shows both metal binding sites fully occupied." J Mol Biol **285**(3): 1145-56.

Portnoy, D. A., V. Auerbuch, et al. (2002). "The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity." J Cell Biol **158**(3): 409-14.

Raymond, K. N., E. A. Dertz, et al. (2003). "Enterobactin: an archetype for microbial iron transport." Proc Natl Acad Sci U S A **100**(7): 3584-8.

Raymond, K. N., S. S. Isied, et al. (1976). "Coordination isomers of biological iron transport compounds. VI. Models of the enterobactin coordination site. A crystal field effect in the structure of potassium tris(catecholato)chromate(III)

and -ferrate(III) sesquihydrates, K3(M(O2C6H4)3)-1.5H2O, M = Cr, Fe1." J Am Chem Soc **98**(7): 1767-74.

Rea, R. B., C. G. Gahan, et al. (2004). "Disruption of putative regulatory loci in Listeria monocytogenes demonstrates a significant role for Fur and PerR in virulence." Infect Immun **72**(2): 717-27.

Rohrbach, M. R., V. Braun, et al. (1995). "Ferrichrome transport in Escherichia coli K-12: altered substrate specificity of mutated periplasmic FhuD and interaction of FhuD with the integral membrane protein FhuB." J Bacteriol **177**(24): 7186-93.

Roosenberg, J. M., 2nd, Y. M. Lin, et al. (2000). "Studies and syntheses of siderophores, microbial iron chelators, and analogs as potential drug delivery agents." Curr Med Chem **7**(2): 159-97.

Rouault, T. A. (2004). "Microbiology. Pathogenic bacteria prefer heme." Science **305**(5690): 1577-8.

Rutz, J. M., J. Liu, et al. (1992). "Formation of a gated channel by a ligand-specific transport protein in the bacterial outer membrane." Science **258**(5081): 471-5.

Sankaran, K. and H. C. Wu (1994). "Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol." J Biol Chem **269**(31): 19701-6.

Schneewind, O., A. Fowler, et al. (1995). "Structure of the cell wall anchor of surface proteins in Staphylococcus aureus." Science **268**(5207): 103-6.

Schneider, R. and K. Hantke (1993). "Iron-hydroxamate uptake systems in Bacillus subtilis: identification of a lipoprotein as part of a binding protein-dependent transport system." Mol Microbiol **8**(1): 111-21.

Schwyn, B. and J. B. Neilands (1987). "Universal chemical assay for the detection and determination of siderophores." Anal Biochem **160**(1): 47-56.

Scott, D. C., Z. Cao, et al. (2001). "Exchangeability of N termini in the ligand-gated porins of Escherichia coli." J Biol Chem **276**(16): 13025-33.

Sebulsky, M. T. and D. E. Heinrichs (2001). "Identification and characterization of fhuD1 and fhuD2, two genes involved in iron-hydroxamate uptake in Staphylococcus aureus." J Bacteriol **183**(17): 4994-5000.

Sebulsky, M. T., B. H. Shilton, et al. (2003). "The role of FhuD2 in iron(III)-hydroxamate transport in Staphylococcus aureus. Demonstration that FhuD2 binds iron(III)-hydroxamates but with minimal conformational change and implication of mutations on transport." J Biol Chem **278**(50): 49890-900.

Sebulsky, M. T., C. D. Speziali, et al. (2004). "FhuD1, a ferric hydroxamate-binding lipoprotein in Staphylococcus aureus: a case of gene duplication and lateral transfer." J Biol Chem **279**(51): 53152-9.

Seeliger, H. P. (1988). "Listeriosis--history and actual developments." Infection **16 Suppl 2**: S80-4.

Simon, N., V. Coulanges, et al. (1995). "Utilization of exogenous siderophores and natural catechols by Listeria monocytogenes." Appl Environ Microbiol **61**(4): 1643-5.
Skaar, E. P., A. H. Gaspar, et al. (2004). "IsdG and IsdI, heme-degrading enzymes in the cytoplasm of Staphylococcus aureus." J Biol Chem **279**(1): 436-43.
Skaar, E. P., A. H. Gaspar, et al. (2006). "Bacillus anthracis IsdG, a heme-degrading monooxygenase." J Bacteriol **188**(3): 1071-80.
Skaar, E. P., M. Humayun, et al. (2004). "Iron-source preference of Staphylococcus aureus infections." Science **305**(5690): 1626-8.
Smith, K. and P. Youngman (1992). "Use of a new integrational vector to investigate compartment-specific expression of the Bacillus subtilis spoIIM gene." Biochimie **74**(7-8): 705-11.
Sprencel, C., Z. Cao, et al. (2000). "Binding of ferric enterobactin by the Escherichia coli periplasmic protein FepB." J Bacteriol **182**(19): 5359-64.
Stanley, P., L. C. Packman, et al. (1994). "Fatty acylation of two internal lysine residues required for the toxic activity of Escherichia coli hemolysin." Science **266**(5193): 1992-6.
Stintzi, A., C. Barnes, et al. (2000). "Microbial iron transport via a siderophore shuttle: a membrane ion transport paradigm." Proc Natl Acad Sci U S A **97**(20): 10691-6.
Stoebner, J. A. and S. M. Payne (1988). "Iron-regulated hemolysin production and utilization of heme and hemoglobin by Vibrio cholerae." Infect Immun **56**(11): 2891-5.
Studier, F. W., A. H. Rosenberg, et al. (1990). "Use of T7 RNA polymerase to direct expression of cloned genes." Methods Enzymol **185**: 60-89.
Tai, S. S., T. R. Wang, et al. (1997). "Characterization of hemin binding activity of Streptococcus pneumoniae." Infect Immun **65**(3): 1083-7.
Thompson, J. M., H. A. Jones, et al. (1999). "Molecular characterization of the hemin uptake locus (hmu) from Yersinia pestis and analysis of hmu mutants for hemin and hemoprotein utilization." Infect Immun **67**(8): 3879-92.
Tilney, L. G. and D. A. Portnoy (1989). "Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes." J Cell Biol **109**(4 Pt 1): 1597-608.
Torres, A. G. and S. M. Payne (1997). "Haem iron-transport system in enterohaemorrhagic Escherichia coli O157:H7." Mol Microbiol **23**(4): 825-33.
Trost, M., D. Wehmhoner, et al. (2005). "Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic Listeria species." Proteomics **5**(6): 1544-57.
Valvano, M. A., R. P. Silver, et al. (1986). "Occurrence of chromosome- or plasmid-mediated aerobactin iron transport systems and hemolysin production among clonal groups of human invasive strains of Escherichia coli K1." Infect Immun **52**(1): 192-9.

Vazquez-Boland, J. A., M. Kuhn, et al. (2001). "Listeria pathogenesis and molecular virulence determinants." Clin Microbiol Rev **14**(3): 584-640.
von Heijne, G. (1989). "The structure of signal peptides from bacterial lipoproteins." Protein Eng **2**(7): 531-4.
Wandersman, C. and P. Delepelaire (2004). "Bacterial iron sources: from siderophores to hemophores." Annu Rev Microbiol **58**: 611-47.
Warren, R. A. and J. B. Neilands (1965). "Mechanism of Microbial Catabolism of Ferrichrome A." J Biol Chem **240**: 2055-8.
Watkins, J. and K. P. Sleath (1981). "Isolation and enumeration of Listeria monocytogenes from Sewage, Sewage Sludge and River Water." J Appl Bacteriol **50**(1): 1-9.
Weinberg, E. D. (1999). "Iron loading and disease surveillance." Emerg Infect Dis **5**(3): 346-52.
Weinberg, E. D. (2000). "Microbial pathogens with impaired ability to acquire host iron." Biometals **13**(1): 85-9.
Wu, H. C., C. Hou, et al. (1977). "Biochemical characterization of a mutant lipoprotein of Escherichia coli." Proc Natl Acad Sci U S A **74**(4): 1388-92.
Xiong, A., V. K. Singh, et al. (2000). "Molecular characterization of the ferric-uptake regulator, fur, from Staphylococcus aureus." Microbiology **146 (Pt 3)**: 659-68.
Yanisch-Perron, C., J. Vieira, et al. (1985). "Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors." Gene **33**(1): 103-19.
Yeowell, H. N. and J. R. White (1982). "Iron requirement in the bactericidal mechanism of streptonigrin." Antimicrob Agents Chemother **22**(6): 961-8.

Book reference for appendix:

Cheung, H.C. (1995). Resonance energy transfer. In Lokowicz., J.R. (ed.), Topics in fluorescence Spectroscopy Vol. 2; Principles. Plenum Press, New York, 127-176.
Eftink, M.R. (1991). Fluorescence Quenching: Theory and Applications. In Lokowicz., J.R. (ed.), Topics in fluorescence Spectroscopy Vol. 2; Principles. Plenum Press, New York, 53-126.
Sheehan, D (2000). Chapter in Fluorescence Spectroscopy. (ed), John Wiley Press, England, 74-86.
David M Freifelder (1982). Physical Biochemistry: Applications to Biochemistry and Molecular biology, W H Freeman Press.
Joseph R. R. Lakowicz, Chris D. Geddes, (2006) (3rd ed), Principles of Fluorescence Spectroscopy, Springer-Verlag Press, New York.
Grafit 5.09 User guide (2002). Erithacus, Ltd., Meddlesex, United Kingdom, 230-235.