1.Introduction

1.1 The cell wall of bacteria

To withstand the outside and inside pressure, bacteria contain cell walls. They determine the bacterial shape and rigidity. Based on the gram staining, bacteria can be divided into two major groups: gram-positive ones (such as *Bacillus subtilis*) and gram-negative ones (such as *Escherichia coli*). The cell wall of gram positive bacteria and gram negative bacteria differ markedly. The gram-positive bacteria have a rigid, thick layer of peptidoglycan (or murein), while gram- negative bacteria have an outer membrane superimposed on a thin peptidoglycan layer. A characteristic component of the outer membrane is the lipopolysaccharide. The space between the outer surface of the cytoplasmic membrane and the inner surface of the outer membrane is the periplasm where in addition to the peptidoglycan enzymes and many binding proteins are located.

Protein-porins are present in the outer membrane as substrate transport channels, they make the outer membrane of gram negative bacteria permeable to small substrates. The cytoplasmic membrane is not only a barrier separating the cell content from outside but it is also functioning as a permeability barrier. The cytoplasmic membrane is made of a double layer of phospholipid with various types of proteins anchored in the membrane. These membrane proteins are very important for the stability of the cytoplasmic membrane and also critically responsible for substrate uptake and export of proteins and small molecules (Koebnik *et. al*., 2000; Schulz, 2000).

1.2 The outer membrane of Gram-negative bacteria

In gram-negative bacteria, the outer membrane is a lipid bilayer superimposed on the peptidoglycan. The outer layer of the bilayer is made of lipopolysaccharide (LPS) while the inner layer is composed of phospholipids. Besides the porins there are several proteins which allow uptake of only certain substrates (Lugtenberg &Van Alphen, 1983).

There are three types of mechanism for the transport across the outer membrane: diffusion, facilitated diffusion and active transport (Nikaido & Saier, 1992).

1.2.1 Diffusion

General diffusion is an energy independent passive substance transport along the concentration gradient of the substance. Several factors influence the permeation of a substance through the outer membrane: size, pH and polycations which may influence the activity of the porins (Delcour, 2003). The porins work as channels for the transport of substances . Only substances with a molecular weight less than 600 Dalton pass through the porins of *E. coli* (Nikaido, 1993). *E.coli* K-12 has three porins, OmpF, OmpC and PhoE for phosphates (details in 1.2.2) which have a low specificity for their substrates which has been studied on the example of the beta-lactam antibiotic transport. (Nikaido & Saier, 1992; Ceccarelli *et al*., 2004). These three porins show a high sequence homology and always form trimers in vivo (Mizuno *et al*., 1983; Gehring & Nikaido, 1989).

1.2.2 Facilitated diffusion

Facilitated diffusion is similar to the general diffusion as discussed above. With specific porins in the outer membrane, some substrates, for example, maltose and maltodextrins are transported into *E. coli*. In this case, maltodextrins are recognized and transported via the specific porin LamB. LamB is a trimer and located in the outer membrane and it facilitates the diffusion of maltose and maltooligosaccharides through the outer membrane. Each monomer of LamB consists of a β-barrel formed by 8 β-strands and 9 surface loops (Charbit, 2003).

The phage T6-receptor Tsx is another specific porin in the outer membrane of *E. coli*; it is specific for nucleosides (Hantke, 1976). Tsx forms a monomeric, twelve stranded β-barrel with a long and narrow channel spanning the membrane. The channel is like a keyhole, and contains several distinct nucleotide specific binding sites. (Ye &Van Den Berg, 2004**)**. Other specific porins for facilitated diffusion include: ScrY (sucrose porin) for sucrose specific transport (Kim *et al.*, 2002), PhoE for phosphates and FadL responsible for the transport of long-chain fatty acid (DiRusso *et al.*, 1999; Lee *et al.*, 2004; Van den Berg *et al.*, 2004).

1.2.3 Active transport

An active transport system against the concentration gradient of the substrate is also found in the outer membrane. It consists of a specific receptor in the outer membrane which recognizes specific substrates and binds them with high affinity in the nanomolar range. In *E.coli* the TonB protein delivers the energy from the cytoplasmic membrane and allows release of the substrate from the receptor into the periplasm (Postle, 1993; Braun *et al*., 1994). In *E.coli* K-12, both iron uptake systems and vitamin B_{12} uptake system use this type of active transport. (Braun, 2003; Ludrigan *et al*., 1991). The uptake system studied in this work is an active transport system for ferric iron from an iron poor environment.

1.3 Iron and bacterial growth

1.3.1 Bacterial growth is iron dependent

Iron is one of the most important trace elements of bacteria; it plays an essential role in electron transport and enzyme function. Only a few bacterial species, such as *Lactobacillus plantarum* and *Borrelia burgdorferi* seem to need no or very little iron (Archibald & Duong, 1983; Posey & Gherardini, 2000). *Lactobacilli* replace iron with other cofactors and they obtain heme for a few heme containing proteins from their sourrounding. *Borrelia burgdorferi* does not contain iron regulated genes usually found in other bacteria. Vertebrates developed a defence strategy to lower the iron level in their serum.

Although iron is the fourth abundant trace element in the earth crust, it is difficult for bacteria to obtain the iron from the environment because usually iron is insoluble at physiological pH. At pH 7.0 and under aerobic conditions, most irons exist as Fe^{3+} and is easyly hydrolyzed and forms $(FeOOH)_n$. Under this condition, the free ferric iron concentration is only 10^{-12} μM. The concentration of iron needed for bacterial growth is about 10^{-1} μM (Braun & Hantke, 2001). Bacteria contain about 10^5 iron ions per cell. Most of the iron is bound to iron sulfur centers of redox proteins and less than 10% of the iron is bound to heme (Matzanke *et al*.,

1991). In the human body, iron is complexed to transferrin in the serum and lactoferrin in the secretory fluids, and intracellularly it is stored in ferritin.

Most of the iron (about 78%) in vertebrates is bound to hemoglobin and myoglobin. Both heme and bound iron have to be mobilized by the bacteria to satisfy their iron requirement for growth in the host (Bullen *et.al*, 1999; Ratledge & Dover, 2000). In order to obtain iron from their host, most bacteria secrete substances, named siderophores to complex the iron and utilize the iron containing siderophores.

1.3.2 Iron supply systems contribute to the virulence of bacteria

Because bacteria need iron for growth they secrete high-affinity siderophores to complex iron. This leads to a competition between the host iron stock system and the iron transport system in bacteria. The virulence of bacteria is related to their iron transport systems (Kunkle & Schmitt, 2005).

For instance *Pseudomonas aeruginosa* produces two high-affinity siderophores, pyoverdine and pyochelin. Both siderophores are necessary for the efficient growth of *P. aeruginosa* in the lung of the infected host. In *Helicobacter pylori*, *pfr* and *feo* are relevant for colonization and infection of the host. Pfr is a ferritin like protein for storage of iron, and *feo* encodes a ferrous iron transporter (Waidner *et al*., 2002**;** Velayudhan *et al.,* 2000).

A long known example of an iron regulated virulence factor is the diphtheria toxin. Its regulator, the diphtheria toxin repressor (DtxR) regulates iron transport. In iron poor conditions iron transport systems and the toxin are derepressed in *Corynebacterium diphtheriae* (Schmitt & Holmes, 1991; De Zoysa *et al.*, 2005).

1.3.3 The catecholate siderophores of *Escherichia coli* and *Salmonella enterica*.

Generally, siderophores are divided into different catagories depending on the functional groups: catecholate siderophores form an octahedral complex; examples are enterobactin, salmochelin, vibriobactin, chrysobactin; hydroxamate siderophores, such as ferrichrome and aerobactin, have hexadentate forms (Matzanke *et al.*, 1991; Van der Helm *et al.*, 1987; Griffiths *et al.*, 1984) and hydroxamate carboxylate siderophores such as aerobactin.

Nearly all *E. coli* and *S. typhimurium* strains produce the catecholate siderophore enterobactin. Although some enterobacteriaceae strains also may produce the hydroxmate siderophore aerobactin, enterobactin is still the major siderophore in enterobacteriaceae (Payne, 1988; Crosa, 1984**)**.

In *E. coli* K-12, enterobactin (cyclic trimer of 2,3-dihydroxybenzoylserine) is the only siderophore produced albeit the 7 receptors recognize and accept many siderophores produced by other miroorganisms. Hence, catecholate siderophore-enterobactin (also enterochelin) and its linear breakdown products, trimer-$(DHBS)_3$, dimer-$(DHBS)_2$ and monomer-DHBS are the major siderophores not only in *E.coli* but also in many other enterobacteriaceae.

In *E. coli* and *S. typhimurium* the *ent* gene cluster is responsible for the biosynthesis of enterobactin (Elkins *et al*., 1988; Liu *et al.*, 1990) which is synthesised from chorismic acid, the branch point for aromatic amino acids (see appendix Fig.49). Enterobactin biosynthesis includes 2 parts, the conversion of chorismate to DHB, and synthesis of enterobactin from DHB and serine (Young *et al.*, 1969). Ent F activates and binds serine as peptide synthetase, EntE activates DHB as adenylate-forming acyl-activating enzyme, and it also transamidates to the EntF-serine (Bryce & Brot, 1971,1972; Reichert *et.al*., 1992; Earhart, 1996). Then EntB/G and EntD linke DBS by esterification (Earhart, 1996; Hantash & Earhart, 2000).

The *fep* gene cluster encodes the transport system of iron-enterobactin in the cytoplasm (Elkins & Earhart, 1988). In the *fep* gene cluster, FepA is the receptor for recognizing enterobactin in the outer membrane, FepB is the periplasmic binding protein, FepD and FepC are the permease proteins spanning the cytoplasmic membrane, FepC is the ATPase that provides the energy. FepBCDG system forms a typical ABC transport system (Chenault & Earhart, 1991; Chenault & Earhart, 1992; Stephens *et al.*, 1995).

Recently a new catecholate siderophore, salmochelin, was found in *S. enterica* serovar Typhimurium (Hantke *et al.*, 2003). This iron transport system will be discussed in details in this dissertation.

1.3.4 Siderophore receptors in the outer membrane and transport systems of *E. coli* and *S. enterica*

Iron siderophore are recognized by specific receptors located in the outer membrane in bacteria. In previous studies, many siderophore receptors have been described and the crystal structures of some siderophores have been determined.(Buchanan *et al.* 1999; Ferguson *et al*., 1998; Locher *et al.*, 1998). All these receptors are regulated by the iron concentration in the environment. The receptors in the outer membrane are usually not very specific and could also recognize other substrates besides siderophores, for example, bacteriophages, colicins, antibiotics etc. (Pilsl *et al.*, 1993; Salomon & Farias, 1993; Killmann *et al.*, 1995**)**.

The receptors may be divided into two categories: receptors which utilize siderophore produced by other microorganisms and receptors which utilize the siderophores produced by the organisms itself. An example for the first type is FhuA, a siderophore receptor in *E.coli* which utilizes the siderophore ferrichrome which is produced by fungi. FepA is an example of the second type of receptors in *E.coli* which recognizes ferric enterobactin. Enterobactin is the catecholate siderophore produced by *E.coli* itself. There are 6 ferric transport systems and one ferrous-dependent transport system in *E.coli* (Hantke, 1990; Braun *et al.*, 1991). Different receptors with the molecular weight between 74 and 83 kDa are located in the outer membrane for recognizing different iron-siderophore complexes and transport the complex into the periplasm. These receptors in the outer membrane recognize colicin, phages and antibiotics. They include FhuA for ferrichrome (Fecker & Braun, 1983; Koster 1991), albomycin, rifamycin, microcin J25 and certain phages. FhuE recognizes ferric-coprogen (Sauer *et al*., 1990; Wachi *et al.*, 1999), FepA recognizes the enterobactin complex and salmochelin iron complexes, (Lundrigan & Kadner, 1986; Hantke *et al*., 2003); FecA binds diferric-dicitrate (Frost & Rosenberg, 1973; Hussein *et al.*, 1981; Sauter & Braun, 2004), Iut binds ferric-aerobactin (Krone *et al*., 1983**;** Smajs *et al.*, 2003); Fiu and Cir bind 2,3

dihydroxybenzoylserine or dihydroxybenzoate (Hantke, 1984; Hantke, 1990). The Feo system is responsible for the uptake and transport of ferrous ions in *E.coli* (Kammler *et al.*, 1993; Stojiljkovic *et al.*, 1993).

S.enterica expresses 3 types of outer membrane recetptors for the catecholate siderophores, IroN, Cir and FepA (Fernandez-Beros *et al.*, 1989; Tsolis *et al.*,1995; Baumler *et al.*, 1998; Rabsch *et al.*, 1999). Their molecular weight varies between 79 kDa and 83 kDa. All three receptors could recognize and bind many natural and artificial siderophores. FepA is a receptor for ferric enterobactin and the largest receptor in the outer membrane.(Neilands, 1982; Annamalai *et al.*, 2004). IroN is another ferric enterobactin complex receptor in *S. enterica*.(Rabsch *et al.*, 1999; Sorsa *et al.*, 2003; Oelschlaeger *et al.*, 2003). It differs from FepA in that it binds besides enterobactin, IroN transports corynebactin which is produced by corynebacteria, while FepA transports one of the myxobacterial siderophores, myxochelin C (Rabsch *et al.*, 1999). Cir is a receptor for colicin I, and also a receptor for catecholate siderophore broke down products, but its character is still not clear (Curtis *et al.*, 1988; Williams *et al.*, 1990; Critchley *et al.*, 1991).

Catecholate siderophore receptors of *S.enterica* were summarized by Rabsch *et al.*, 2003

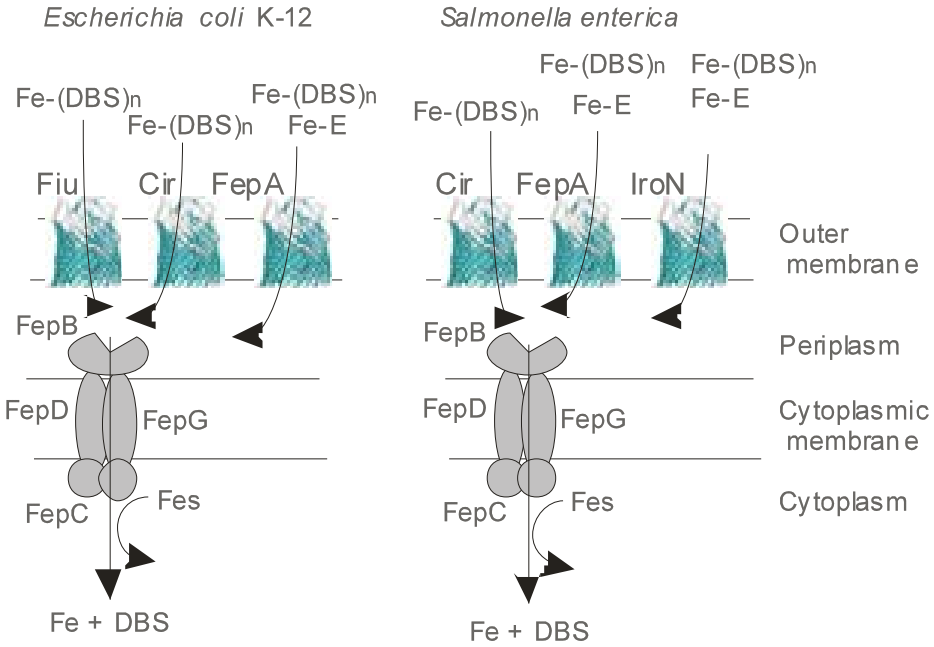
Table 1: Catecholate siderophore receptors of *S.enterica*

Receptor	Catecholate siderophore
FepA	Enterobactin
	Dihydroxybenserin (DHBS)
	Salmochelin
IroN	Enterobactin
	DHBS
	Corynebactin
	Salmochelin
Cir	DHBS
	Amonabactins P2 and T2
	Salmochelin

In *Salmonella enterica*, it also a number of hydroxamate siderophores, for example, aerobactin, shizokinen, rhodotorulic acid can be utilized. In this dissertation, it will not be discussed in detail.

It is an active process for iron to be transported into the cytoplasm. It is dependent on energy and needs the assistance of the proteins TonB, ExbB, ExbD (Postle & Good, 1983; Eick-Helmerich & Braun, 1987, 1989; Postle, 1993; Ferguson & Deisenhofer, 2002). When catecholate iron-complexes are taken into the periplasm, the complexes will further be transported into the cytoplasm by the FepBCDG ABC transport system (Pierce *et al.*, 1983; Moeck & Letellier, 2001). The transport system in the cytoplasmic membrane is less specific for the substrates compared with the receptors in the outer membrane.

1.3.5 The uptake pathway of catecholate siderophores in *E. coli* and *S. enterica*



Firgure 1: Comparison of enterobactin transport and its degraded products transport in *E. coli* K-12 and *S. enterica*

In both *E. coli* and *S. enterica*, ferric enterobactin and ferric salmochelin complexes are transported mainly through FepA across the outer membrane, the linear degradation products from enterobactin are taken up mainly by Fiu and FepA in *E.coli*. In *S. enterica*, the uptake and transport system for salmochelin must be verified and has been studied in this work.

1.3.6 The molecular structure of salmochelin S4, S2, S1, Sx, DHBS and the pathway of biosynthesis and degradation.

The salmochelins were concentrated from the bacterial culture supernatant by binding to DE52-cellulose. S4, S2, S1 were isolated by HPLC. From mass spectroscopy, the molecular weight of the salmochelin, the structures of salmochelin Sx, S1, S2 and S4 and the pathway of biosynthesis were hypothesized as follows:

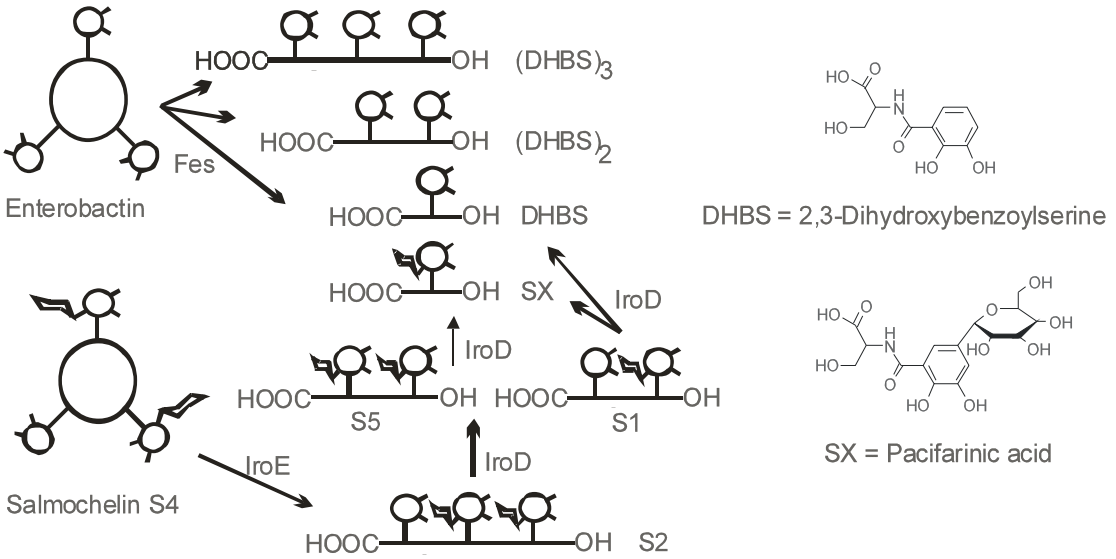


Figure 2: Structure of enterobactin and salmochelin S4 and their degradation products detected in culture supernatants by HPLC analysis. Arrows indicate the reactions catalyzed by the indicated enzyme, as shown previously for Fes (Bryce & Brot, 1971, 1972; Brickman & Mcintosh,1992) and in the current study for IroE and IroD.

The basic structure of salmochelin is 2,3-dihydroybenzoylserine with one glucose residue C-glycosidic bound to C5 of the catechole (Fig 2) (Bister *et al.*, 2004). In previous work, this molecule was named pacifarinic acid (Wawszkiewicz *et al*., 1975). Here it is often called as salmochelin SX; The dimer is called S1, the linear trimer S2 and the cyclic, diglucosylated trimer is called S4.

1.4 The aims of this dissertation

In previous studies, a new siderophore salmochelin was found which may play a critical role in the pathogenicity of *S.enterica*. In addition, the structures of cyclic salmochelin S4, linear salmochelin S2, S1 and SX were elucidated in a recent publication (Hantke *et al.*, 2003; Bister *et al.*, 2004).

The aims of this work were: to construct a plasmid with the *iroBCDEN* gene cluster and to study its expression in *E.coli* K-12. There are two types of hydrolases predicted in the *iroBCDEN* gene cluster, IroD and IroE. What is the function of these two hydrolases? To solve this question both genes were cloned and the proteins were purified to check their enzyme activities in vitro.

2.Material and Methods

2.1 Material

2.1.1 Chemicals and instruments

Amersham Bioscience, Freiburg:
Immobiline DryStrip pH4-7, 18cm, Auto Read Sequencing Kit

Amicon, Beverly MA, USA:
Zentrifugatinsfilterröhrchen, Centriprep YM-30

Applichem, Darmstadt:
DTT, Urea, SDS

Biolab, Frankfurt am Main:
Vectors, restriction endonucleases

Biometra, Göttingen:
Gelelectrophoresekammer for polyacrylamidegel electrophoresis

Bio-Rad, München:
Trans-Blot-System

Carl Roth GmbH & Co, Karlsruhe:
BCIP, NBT, Acrylamid Rotiphorese Gel A , Bisacrylamid Rotiphorese Gel B

Difco Laboratories, Detroit MI, USA:
Macconkey-Agar (BASIS), Bacto-agar, Bacto-tryptone

Eppendorf AG, Köln:
Photometer 1101M, Tischzentrifuge

Gentra systems, Minnesota, USA:
Genomic DNA Purification Kit

Heraeus Instruments GmbH, Hanau:
Biofuge fresco centrifugation instrument

Invitrogen, Groningen, Netherland:
DNA-standard (1kb marker), pBAD/His vectors, pBAD/Myc-his

Kurt Hillerkurs, Krefeld:
Gerät-pH-Meter 26

Life Technology Inc., Rockville, MD., USA:
Flatbed-electrophoreseapparatur Modell Horizon 58

Macherey-Nagel GmbH &Co.KG, Düren:
Nucleobond AX-100 Kit, NucleoSpin Extract Kit

MBI Fermentas GmbH, St. Leon-Rot:
Protein ladder

MWG-Biotech, Ebersberg:
Synthetic oligonucleotide

New England Biolabs GmbH, Schwalbach:
Restriction endonucleases, Klenow fragment

Novagen, Darmstadt:
Clone vectors

Pall Life Science, Dreieich:
Metricel Membrane Filter

Peqlab Biotechnologie GmbH, Erlangen:
Miniplasmidisolation kits, Taq DANN-polymerase and buffer

Pierce, Rockford, Illinois, USA:
BCA Protein Assay

Roche Diagnostics GmbH, Mannheim :
Restriction endonucleases, alkaline phosphatase, Complete-EDTA free, T4 DNA-ligase, Long template PCR kit, Rapid ligation kit

Qiagen GmbH, Hilden:
Ni-NTA-spin-column, Anti-6xHis antibody

Satorius AG, Göttingen:
Nitrocellulosefilter 0,45µm

Serva Feinbiochemika GmbH, Heidelberg:
APS, Ampicillin, Temed, Servablau R250 and G250

Schleicher & Schuell GmbH, Dassel
Nitrocellulose membrane

Sigma-Aldrich Chemie GmbH, Taufkirchen:
Trizma base, minimum 99.9% titration, BSA, Anti-mouse IgG (Fab specific), Polypropylene chromatography columns, L-arabinose, Chloramphenicol, 2’2-dipyridyl, IPTG

Sorvall GmbH, Bad Homburg:
Sorvall centrifuge RC 5B Plus

Stratagene:
RoboCycler Gradient 96, Temperature Cycler with the Hot Top Assembly

E.Merck AG, Darmstadt
All other chemicals not mentioned above were from Merck AG.