Salmochelin, a new siderophore of *E. coli*: biosynthesis, transport and utilization

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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 (Di Russo 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 B₁₂ 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 surrounding. 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 Fe³⁺ and is easily 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 10⁵ 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.,
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 categories 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 hydroxamate 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 microorganisms. 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). EntF 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 siderophile, 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 Siderophile receptors in the outer membrane and transport systems of *E. coli* and *S. enterica*

Iron siderophile are recognized by specific receptors located in the outer membrane in bacteria. In previous studies, many siderophile 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 siderophile receptor in *E. coli* which utilizes the siderophile 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 siderophile 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-siderophile 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
dihydroxybenzyolserine 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 receptors 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; Oelschlaege, 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).

Table 1: Catecholate siderophore receptors of S.enterica were summarized by Rabsch et al., 2003

<table>
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<tr>
<th>Receptor</th>
<th>Catecholate siderophore</th>
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<tr>
<td>FepA</td>
<td>Enterobactin</td>
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<td></td>
<td>Dihydroxybenserin (DHBS)</td>
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<tr>
<td></td>
<td>Salmochelin</td>
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<tr>
<td>IroN</td>
<td>Enterobactin</td>
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<td></td>
<td>DHBS</td>
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<td></td>
<td>Corynebactin</td>
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<tr>
<td></td>
<td>Salmochelin</td>
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<tr>
<td>Cir</td>
<td>DHBS</td>
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<td></td>
<td>Amonabactins P2 and T2</td>
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<tr>
<td></td>
<td>Salmochelin</td>
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</tbody>
</table>

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 FepB C D G ABC transport system (Pierce et al., 1983; M o eck & 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

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:
The basic structure of salmochelin is 2,3-dihydroxybenzoylserine 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 pH 4-7, 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-trypotine

**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
Material and Methods

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:
  Miniplasmid isolation kits, Taq DNA-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 R 250 and G 250

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.