1 Introduction

Escherichia coli is an inhabitant of the human intestinal tract constituting part of the normal intestinal flora (comensal). In the host, this bacterium can find enough iron present, although the amount available can be extremely limited, because most iron is held within cells as haem or ferritin and outside the cells iron is attached to the iron-binding glycoproteins transferrin and lactoferrin. Many of these *E. coli* strains are not pathogenic. In fact, the presence of these strains may contribute to a normal, healthy intestinal flora and may prevent colonization by pathogenic bacteria. Various studies have demonstrated that some *E. coli* strains are able to produce virulence factors such as hemolysins, certain toxins and adhesins. In addition, antibiotically active metabolites like microcin, colicin, and short-chain fatty acids are important factors produced during microbial competition (Kuhar and Žgur-Bertok, 1999; Patzer *et al*., 2003). Furthermore, it is known that these bacteria synthesize highly efficient iron acquisition systems to scavenge iron from the environment under iron-restricted conditions. These low-molecular-mass organic ferric chelators are termed siderophores (Lankford, 1973; Thulasiraman *et al*., 1998; Winkelmann, 2002). Indeed, bacteria which synthesize a particular siderophore possess the cognate transport systems, but many enteric bacteria also express transport systems recognizing siderophores produced by other microorganisms. This ability is highly advantageous because they can reduce iron availability to other bacteria and further obtain iron with minimum expenditure of energy.

1.1 Biochemical significance of iron in Enterobacteriaceae

Iron is the fourth-most common element in the earth crust, and the second-most common metal after aluminum. The most important oxidation states of iron are +2 (ferrous) and +3 (ferric).

Iron is an essential micronutrient for almost all microorganisms except for Lactobacilli that lack haem and use a cobalt type of ribonucleotide reductase (Archibald, 1983; Briat, 1992).

The physiological importance of Fe^{+3} is its function as a cofactor in a large number of enzymes indispensable to the oxido-reduction processes of the respiratory chain

including cytochromes and hydroperoxidases. It is a constituent of some ribonucleotide reductases and is essential for the activity of nitrogenases (Neilands, 1981a; Escolar *et al*., 1999). In the electron transport chain a series of redox reactions leads to the production of energy which is used to generate ATP, the high energy phosphate that all organisms require to enable metabolism.

Although iron is amongst the most plentiful of metals, it is difficult for biological systems to acquire. So, to maintain an internal iron concentration of 10^{-6} M, growing bacteria of 10^{-9} cm^3 require 10^5 to 10^6 ferric ions per generation (Braun and Killmann, 1999; Raymond *et al*., 2003). Iron is not readily available due to its low solubility in the presence of oxygen and in humans it is tightly associated to iron carrier proteins or to heme in hemoproteins. The insolubility of ferric hydroxides at neutral pH limits the concentration of [Fe^{3+}] to ~10^{-18} M, whereas *Escherichia coli* requires approximately a minimum of 10^{-8} M for growth and 10^{-6} M for iron sufficiency (Raymond *et al*., 2003).

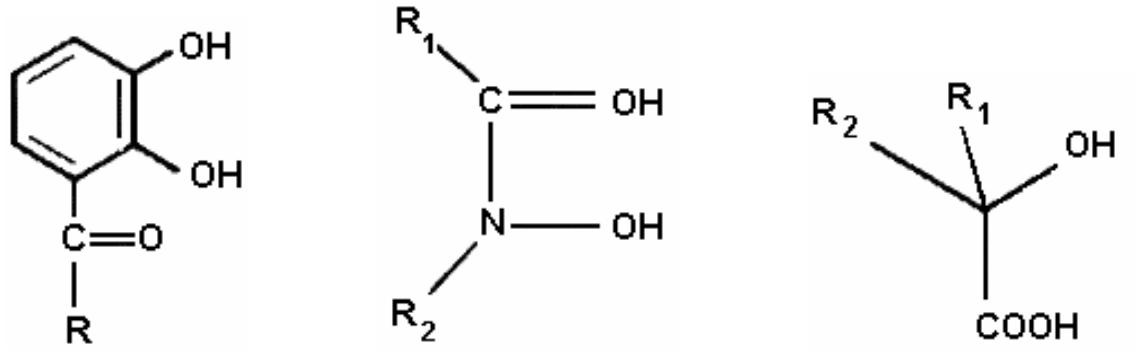
While in anaerobic conditions ferrous iron is soluble at physiological pH and can be directly imported by the G protein-like transporter, FeoB, the exposure to oxygen converts this ion to ferric iron and forms insoluble hydroxides, making ferrous iron very scarce. Some pathogens have the ability to utilize, host–iron complexes (transferrin, lactoferrin, haem, haemoglobin) directly as iron sources. Furthermore, in enteric bacteria the bacterial iron storage proteins (ferritin, bacterioferritin) provide intracellular iron reserves for use when external supplies are restricted, and iron detoxification proteins are employed to protect the chromosome from iron-induced free radical damage.

1.2 Siderophores secreted by enteric bacteria. Adventages for growth

Highly efficient iron acquisition systems are used to scavenge iron from the environment or from iron-protein complexes such as transferrin and lactoferrin under iron-restricted conditions (Weinberg, 2000; Winkelmann, 2002). These iron acquisition systems are designated siderophores. They are low-molecular-mass organic compounds (500 to 1500 Daltons) with high affinity for ferric ions (Lankford, 1973; Thulasiraman *et al*., 1998; Winkelmann, 2002).

The capacity to synthesize siderophores is not limited to aerobic bacteria, they are also secreted by facultative anaerobic bacteria (Neilands, 1995).

The siderophores are synthesized inside the cell, carried across the cell membrane and cell wall into the surrounding medium, where the siderophores bind ferric iron and the complex is transported into the bacteria through membrane receptors by active transport mechanisms. Most siderophores belong to one of the three chemical types: hydroxamates, catecholates or carboxylates, but mixed types are also found (Fig. 1).



Catecholate **Hydroxamate** **Hydroxycarboxylic acid**

Fig. 1. Complexing groups of catecholate, hydroxamate and hydroxycarboxylate type siderophores.

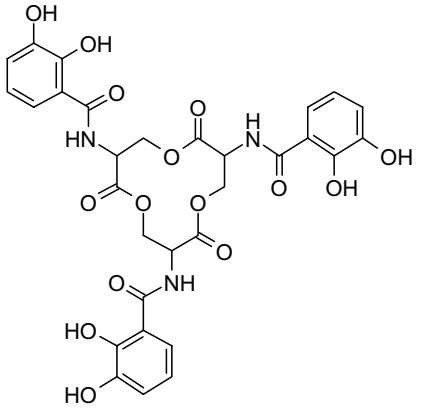
Among the siderophores, enterobactin (also called enterochelin) is a characteristic siderophore secreted by most *E. coli* strains and other Enterobacteriaceae (Fig. 2). Enterobactin, a triscatechol derivative of a cyclic triserine lactone, has been isolated from *Salmonella typhimurium* (Pollack and Neilands, 1970) and *E. coli* strains (O'Brien and Gibson, 1970; Young and Gibson, 1979). This iron chelator is the strongest ferric iron ligand known with an estimated dissociation constant (K_d) of 10^{-49} M (10^{-35} M at physiological pH) (Harris *et al*., 1979).

Around three years ago, glucosylated enterobactin derivatives were isolated by Hantke *et al*. (2003). These siderophores contain C-glucosylated 2,3-dihydroxybenzoyl-L-serine residues connected in a linear (mono-, di- , trimeric) or cyclic form. These siderophores were named salmochelins and were isolated from *S. enterica* and some uropathogenic *E. coli* strains.

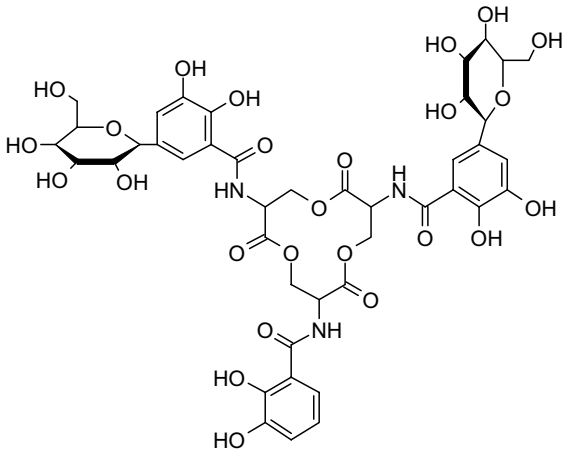
Some iron acquisition systems are virulence markers for example, the aerobactin system in invasive *E. coli* – UTI (Johnson, 1991; Silveira *et al*., 2001). Aerobactin is a

hydroxamate siderophore, which is a conjugate of 6-(N-acetyl-N-hydroxylamine)-2-aminohexanoic acid and citric acid. Its production is often encoded by ColV plasmids. Aerobactin forms an octahedral complex with ferric iron. This hydroxamate-siderophore possesses high affinity toward Fe^{3+} with a stability constant (K_s) of 10^{30} (Raymond *et al*., 1984).

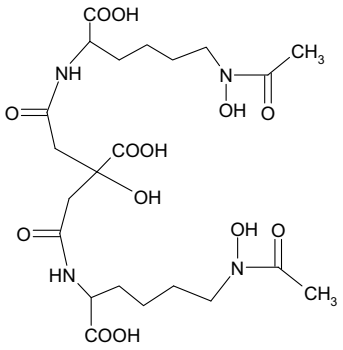
The existence of a number of pathogenicity islands in *Yersinia enterocolitica* that encode an iron uptake system was reviewed by Carniel (1999). Yersiniabactin is the iron-scavenging molecule synthesized by *Yersinia*, which contains a salicylate residue and three five-membered thiazole heterocycles that serve as iron ligands.



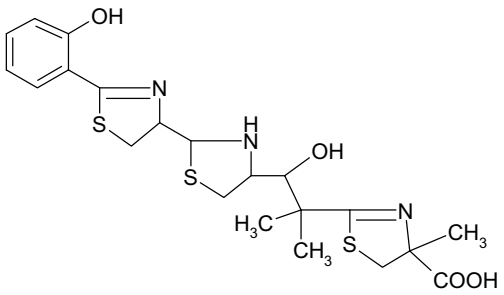
Enterobactin



Salmochelin S4



Aerobactin



Yersiniabactin

Fig. 2. Siderophores produced by some uropathogenic *E. coli* strains.

1.3 Siderophore transport systems in Enterobacteriaceae

Transport systems allow the uptake of essential nutrients and ions, excretion of end products of metabolism and of damaging substances, and communication between cells and the environment.

In gram-negative bacteria, three different transport mechanisms for carrying substrates through the outer membrane are described (Braun, 1995):

A. Type I porin-channels. These porins do not show specificity for substrates, although some are specific for anions or cations, and transport hydrophilic compounds not larger than 600 Da (Benz, 1994).
B. Type II porin-channels. These porins facilitate non-specific diffusion of small compounds, although they are also permeable for some specific substrates, such as LamB (specificity for maltodextrins) (Benz, 1994), Tsx (specificity for nucleosides) (Benz, 1994), Scr Y (specificity for sucrose) (Schülein *et al*., 1991). They allow diffusion of the cognate substrates with a higher rate than the type I porin-channels.
C. Type III of outer membrane proteins. Receptors. These transport mechanisms are used when the substrates are too large for the channels Type I and Type II. Most of these receptors are multifunctional. They are utilized for the uptake of vitamin B_{12}, colicins and ferric siderophores. Here the intact Fe^{3+}-siderophore complex is taken up into the cell with posterior reduction of the ferric- to ferrous-iron, and egress of the ligand (Earhart, 1987; Matzanke, 1987; Crowley *et al*., 1991). The other possibility to incorporate the iron into the cell is that the Fe^{3+}-siderophore complex is taken up without entrance of the ligand molecule, where the iron may be reduced in the periplasm (Emery, 1987; Matzanke, 1987; Crowley *et al*., 1991).

The iron-siderophore complexes in enteric bacteria are bound by specific active transporter proteins in the outer membrane, which retrieve the complexes inside the cell (Neilands, 1981b). The synthesis of an endogenous siderophore requires the expression of cognate transport systems, but many enteric bacteria are also able to express transport systems recognizing siderophores produced by other microorganisms (exogenous siderophores) and transport them through the outer membrane, e. g., the outer membrane receptor FhuE in *E. coli* binds and transports coprogen (Hantke, 1983; Sauer *et al*., 1987), a siderophore secreted by *Neurospora*

crassa, FhuA that binds and transports ferrichrome, a siderophore secreted by *Aspergillus* spp and some phytopathogenic fungi. Fig. 3 summarizes the membrane receptors detected in *E. coli*-K12 (Wagegg and Braun, 1981; Grewal *et al*., 1982; Hantke, 1983; Pierce *et al*., 1983; Köster and Braun, 1986; Coulton *et al*., 1987; Köster and Braun, 1990; Furrer *et al*., 2002). The secretion of high amounts of different siderophores is considerably advantageous because bacteria can prevent other bacteria from using the available iron and further they obtain iron with minimum expenditure of energy.

In *E. coli* the transport of siderophores through the outer membrane into the cytoplasm requires energy which is provided by the activation of TonB and the ABC (ATP binding cassette) transporter. TonB is a cytoplasmic membrane protein which transduces the proton electrochemical potential of the cytoplasmic membrane to the outer membrane receptors thus providing the energy source required for the import of iron-siderophore complexes and vitamin B12 across the outer membrane.

The ABC transporter is the active transport system of the cell. ABC transporters are an important family in the primary, shock-sensitive system energized directly by the hydrolysis of ATP (Saier, 2000; Higgins, 2001), which provides the energy to pump substrates from the periplasm across the inner membrane into the cell cytosol.

The expression of most proteins involved in iron acquisition is regulated at the transcriptional level by a global iron-binding repressor protein named Fur, ferric uptake regulation (Hantke, 1981; Braun and Hantke, 1991). The Fur protein is a negative regulator of several siderophore-mediated, high-affinity iron transport systems in *E. coli* acting as a repressor, that in iron-rich conditions, uses ionic Fe(II) as a corepressor, i. e., Fe^{2+}-Fur complexes bind to promoters containing a Fur box (Fur binding sequence) and repress transcription. The *E. coli* Fur protein exists in a dimeric form containing a structural zinc binding site. This form is able to bind DNA with high affinities but only after metal activation. However, under conditions of iron limitation, in addition to the Fur-mediated negative regulation there is a concurrent positive regulation of iron transport and siderophore biosynthetic genes. Fur-like proteins have been also found in Gram-positive bacteria, such as *Bacillus subtilis* and St*aphylococcus* (Bsat *et al*., 1998; Heidrich *et al*., 1996).

Heme is an iron source, in enteric bacteria which is taken up by two principal pathways. One involves the direct contact between heme or heme-containing proteins and specific bacterial cell surface receptors. The second requires the secretion of hemophores. These proteins present in several Gram negative bacteria such as *Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas fluorescens, Yersinia pestis* and *Yersinia enterocolitica*, capture free heme or extract heme from heme carrier proteins, owing to their higher affinity for heme, and return it to hemophore-specific outer membrane receptors.

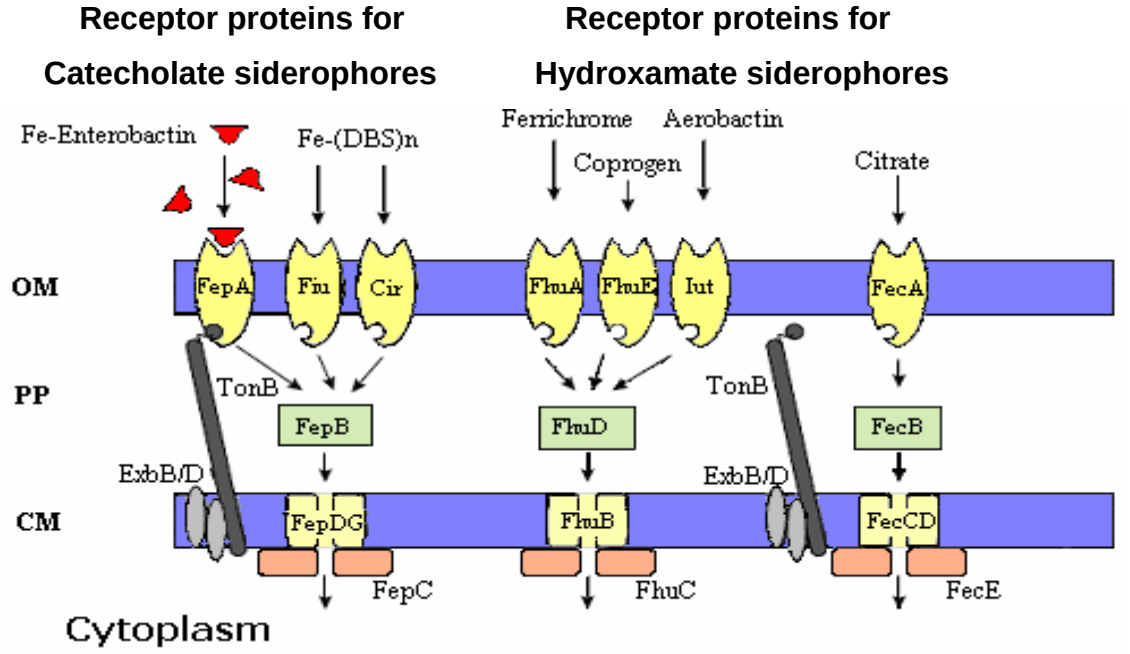


Fig. 3. Schematic representation of iron transport systems of *E. coli* (Reproduced with permission from Braun, 1997).

1.4 Bacterial competence for iron acquisition

To overcome iron restriction, mammals have high-affinity iron-binding glycoproteins such as transferrin and lactoferrin that help to solubilize and deliver iron to host cells (Weinberg, 1999).

The solution that nature has evolved to meet the iron problem is a set of iron storage proteins that shield iron and prevent it from damaging other molecules, yet allow it to

be released when needed. Bacteria, like *Escherichia coli*, have two types of ferritins: heme-containing bacterioferritins and heme-free ferritins (Andrews *et al*., 1990; Andrews, 1998).

Enteric bacteria are able to secrete highly specific iron chelators with their cognate transport systems in response to iron deficiency. Inhibition of this iron acquisition process by scavenging microbial siderophores is a powerful mechanism in the defense against microbial infections. Thus, two human proteins have been found to interfere with microbial siderophores so far. The first one found was serum albumin, which was described to inhibit the transfer of ferric enterobactin to *E. coli*, but it did not bind aerobactin (Konopka and Neilands, 1984).

The other one is NGAL lipocalin (neutrophil gelatinase-associated lipocalin), a 25 kDa glycoprotein also known as siderocalin or lipocalin 2 (see Fig. 4). NGAL lipocalin is a member of the lipocalin family of extracellular proteins that function as transporters of small, hydrophobic molecules like lipids, retinol or lipophilic siderophores. However, while antimicrobial proteins such as lactoferrin and transferrin simply bind to and sequester free iron, NGAL is specific for iron already earmarked for bacterial use as Fe^{3+}-siderophore complexes. This protein is produced by neutrophiles and epithelial cells during inflammation and enhanced cell proliferation. Goetz *et al*. (2002) demonstrated that during an infection, NGAL lipocalin binds enterobactin and inhibits bacterial growth by removing available iron. The crystal structure shows an eight-stranded antiparallel beta-barrel, typical of the lipocalin family albeit with an unusually large and atypically polar binding site, or calyx. One end of the barrel is open, providing access to the binding site within the barrel cavity, while the other is closed by a short helix. The properties of NGAL lipocalin represent a novel and important iron-depleting antimicrobial defence-strategy of higher organisms (Flo *et al*., 2004; Cowland *et al*., 2003).

Many *E. coli* strains make a second iron-scavenging molecule known as aerobactin, which causes those strains to be particularly virulent. Aerobactin has a distinct structure from enterochelin and does not associate with NGAL lipocalin. Since NGAL lipocalin does not bind aerobactin, a strain that produces aerobactin is resistant to the antibacterial action of this protein (Goetz *et al*., 2002).

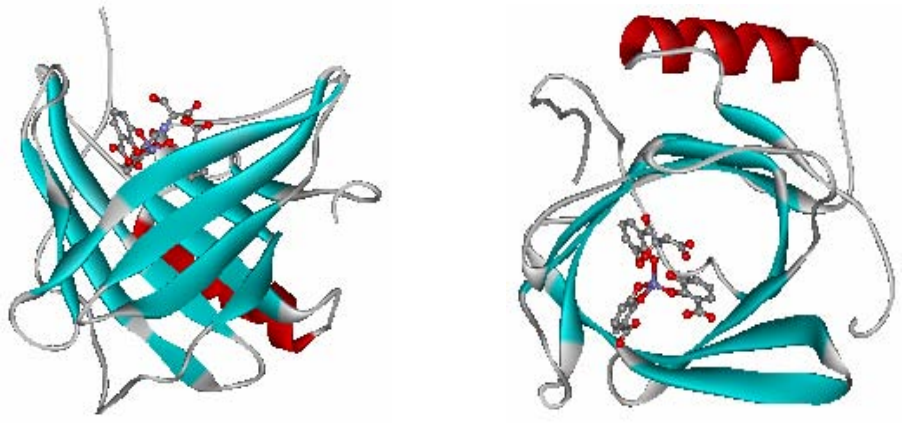


Fig. 4. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Goetz *et al*., 2002. Mol. Cell. 10: 1033-1043.

2 Objectives

2.1 General Objectives:

The purpose of this dissertation was to make a broad investigation of salmochelins and study their properties, i. e., structure, uptake and detection systems.

The presence of *iro* gene cluster in some *Salmonella* and *E. coli* strains allowed the preparation of sufficient amounts of salmochelins.

2.2 Specific Objectives:

- To elucidate the chemical structure of salmochelins.
- To find out a simple method for the detection of salmochelins in some uropathogenic *E.coli* strains (when yersiniabactin is also synthesized).
- To assay the enzymatic properties of IroD and IroE for both catalytic efficiency and regioselectivity of cleavage.