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Iron transport in *Escherichia coli*: All has not been said and done

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Abstract

During recent years new systems involved in iron transport were identified in the old workhorse *Escherichia coli* (and in other enterobacteria). This came as a bit of a surprise because one might think transport of this essential trace element was already thoroughly studied. Moreover, it appears that iron homeostasis consists not only of uptake but also of efflux of this potentially toxic redox-active metal. New findings in *E. coli* will be discussed and compared to the situation in other bacteria.

Introduction

Iron uptake in the enterobacterium *Escherichia coli* is accomplished by a multitude of independent systems. This involves metal permeases such as FeoB (Kammler *et al.* 1993), MntH (Makui *et al.* 2000) or ZupT (Grass *et al.* 2005a). When one thinks of iron homeostasis in bacteria, however, most probably siderophores cross one's mind. In *E. coli* utilization of siderophores is accomplished through specific outer membrane receptors. For the laboratory strain K12 these encompass FepA, FhuA, FhuE, FecA and several other receptors found in pathogenic or apathogenic *E. coli* strains. This review, however, will not deal with (ferric)-siderophore uptake through cognate receptors involved. Intrigued readers are encouraged to look elsewhere for an overview on this prolific field (*e.g.*, Braun & Hantke 2001; Klebba 2003). While several xenosiderophores can be utilized *E. coli* itself also produces siderophores of different biochemical properties. This variety of siderophore dependent iron-uptake systems is not surprising because acquisition of iron is most efficient when siderophores are involved.

This is due to the intrinsic properties of this element. Amongst the biological relevant transition metals (iron, zinc, copper, manganese, cobalt, nickel, molybdenum and tungsten) iron challenges bacteria the most to meet their demands. This is not because iron is too scarce to obtain in sufficient quantity but rather that iron predominantly occurs as ferric iron [Fe(III)] under aerobiosis. Fe(III) as iron hydroxide is poorly soluble in aqueous solution (as low as 10^{-18} M at pH 7.0), rendering it basically unavailable for the cells (Neilands 1981). Organic iron ligands overcome this limitation, especially the multidentate small compounds known as siderophores. Siderophores include hydroxamate, catecholate,α-hydroxycarboxylate or mixed ligands that bind to the six coordination sites of ferric ions by forming hexadentate Fe(III) complexes. These groups of siderophores form distinct families (Carrano *et al.* 2001; Winkelmann 2002).

Under anaerobiosis, reducing or acidic conditions, the iron equilibrium shifts from the ferric Fe(III) to the ferrous Fe(II) form that is more easily available for microorganisms. This allows several permeases of different protein families to contribute to iron uptake, a great benefit for the organisms in that no diffusible compound (such as a siderophore) has to be secreted, and which might end up meeting a competitor's demand for iron.

Excess iron produces oxidative damage ultimately up to the point of cell death. Most likely

this damage is caused by the ability of ferrous iron to catalyze the decomposition of hydrogen peroxide generating the highly reactive hydroxyl radical, most likely via an oxoiron(IV) intermediate, a dismutation known as the Fenton reaction($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ (Fenton 1876)). Iron resembles another redox-active metal, copper, in this property. Lately, connections of bacterial iron- and copper homeostasis have emerged that have some resemblance to those of eukaryotic systems. Permeases but probably more importantly siderophores play a major part (Dubbels *et al*. 2004; Grass *et al*. 2004).

Since iron as Fe(III) is almost insoluble, it is also hardly available to aerobic microorganisms. Under iron limited conditions, these bacteria excrete siderophores in order to sequester iron. One of the most effective ferric iron chelating compounds known is enterobactin (Pollack & Neilands 1970) also known as enterochelin (O'Brien & Gibson 1970), the catecholate type siderophore of *E. coli* and of several other bacteria. Enterobactin is a cyclic triester of 2,3-dihydroxy-*N*-benzoyl-L-serine (DHBS) with a very high stability constant for Fe(III) binding (Albrecht-Gary & Crumbliss 1998; Raymond *et al*. 2003). Genes responsible for enterobactin biosynthesis and ferric-enterobactin uptake are found in a variety of members of the enterobacteriaceae such as *Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*. These *ent* and *fep* genes are localized on the chromosome within a locus of approximately 20 kb. Expression occurs from several promoters as a consequence of iron deprivation governed by the global iron homeostasis regulator Fur (Ozenberger *et al*. 1987; Shea & McIntosh 1991).

While the molecular processes involved in ferric-enterobactin uptake by TonB-energized outer membrane receptor proteins such as FepA (recently reviewed by Klebba 2003) were studied in great detail over the last decades, we are only beginning to understand how iron-free enterobactin leaves the cell after biosynthesis. In addition to newly identified iron homeostasis systems in *E. coli*, novel ways of gene regulation have also been elucidated, for instance involving the small regulatory RNA, *ryhB* (see reviews by Andrews 1998; Masse & Arguin 2005). The scope of this review is on new findings of *E. coli* iron transport. The participating systems will be discussed and compared to the situation in other bacteria.

Salmochelin, a family of C-glucosylated catecholate type siderophores

Hantke *et al*. (2003) isolated salmochelins serendipitously when studying microcins M and H47. Following a catecholate siderophore preparation procedure, salmochelins were found predominantly in the aqueous phase, while enterobactin was known to partition into the organic ethyl acetate phase. This difference suggested that the new siderophores were more hydrophilic than enterobactin, probably caused by glycosylation (Hantke *et al*. 2003; Figure 1).

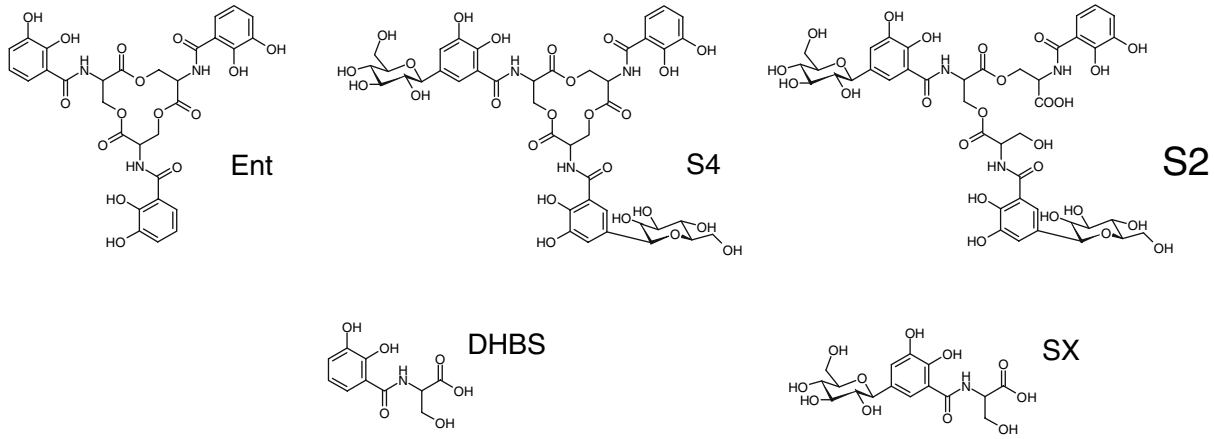

Figure 1. Structures of enterobactin, salmochelin and prominent degradation products. Shown are the catecholate siderophores enterobactin (Ent), diglucosylated salmochelin S4 and its linear derivative S2. Monomeric DHBS and its glycosylated counterpart SX (also known as pacifarinic acid) represent the esterase-hydrolyzed end-products that are also of biological importance.

When the outer membrane receptor IroN from *Salmonella* was initially investigated, IroN was thought to constitute an additional route for enterobactin uptake into *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) (Rabsch *et al.* 1999). Differences in recognition of the xenosiderophores corynebactin or myxochelin through IroN or FepA, the long known enterobactin receptor, were noted (Rabsch *et al.* 1999).

The *iroN* gene and the *iroBCDE* genes were found to be part of the *S. enterica iroA* locus that forms a functional unit. DNA-binding sites of Fur were identified in the *iroN* and *iroB* promoter regions and expression of both, *iroN* and of *iroBCDE* are iron regulated (Bäumler *et al.* 1996). While the *iroN* gene is probably transcribed as a monocistronic mRNA, a putative transporter, IroC and three enzymes IroB, D and E are encoded on a multicistronic transcript. In contrast to *e.g.*, the yersinabactin locus from several *Yersinia* and some pathogenic *E. coli* strains no gene for an additional transcriptional regulator is encoded within *iroA*. In this respect the *iroA* locus resembles the *iut*/*iuc* loci for aerobactin biosynthesis (Williams & Warner 1980).

The true role of the IroN receptor was only then elucidated when the catecholate siderophores of *S. enterica* serotype Typhimurium LT2 and of *E. coli* strain CA 46 were further investigated (Hantke *et al.* 2003). In the same study it was shown that beside enterobactin a family of related siderophores was produced. These novel siderophores were termed salmochelins owing to the fact that they were characteristic for *Salmonella* strains. Nevertheless, *iroA*-loci also occur in several *E. coli* strains that probably also produce salmochelins. However, different species produce salmochelin to different extents and *S. enterica* serotype Paratyphi B IHS1319 is probably suited best for high yield salmochelin extraction (Hantke *et al.* 2003). Interestingly, salmochelins were not new compounds. Already in 1985 a structure corresponding to SX (Figure 1) was named pacifarinic acid while pacifarins were known even longer (Wawszkiewicz *et al.* 1971; Wawszkiewicz & Schneider 1975). Pacifarin activity by enterobactin was observed by an increased survival rate of mice infected by *Salmonella enterica* serovar Typhimurium (Wawszkiewicz *et al.* 1971). It is probable that salmochelin (SX) and pacifarinic acid are identical (Bister *et al.* 2004).

Since salmochelins are aryl-glycosylated derivative siderophores of enterobactin, the initial steps of salmochelin-biosynthesis are identical to that of enterobactin (Woodrow *et al.* 1979). In short, the EntB protein synthesizes 2,3-dihydroxybenzoyl-S-pantetheinyl-EntB while EntE activates L-serine by adenylation. After condensation, EntF generates the linear enterobactin trimer through elongation and finally condensates the trilactone ring which is released from EntF, as reviewed recently (Crosa & Walsh 2002). It is important to keep in mind that cyclic enterobactin but no precursors are released.

In contrast to enterobactin salmochelins are characterized as carbohydrate containing catecholate siderophores where the DHBS moieties are C-glycosylated through C1′ from glucose to C5 of the aromatic ring of the benzoate (Bister *et al.* 2004; Figure 1). From *S. enterica* seven compounds of the salmochelin family were described. They were termed SX, S0, S1, S2, S3, S4 and S5 and represent mono- (SX) or multimers (S1–S4) of glycosylated DHBS where the numbers, however, do not correlate to the DHBS units present. All naturally occurring salmochelins are either single or two-fold β-C-glycosylated, no derivative with three glucosyl-moieties could be identified *in vivo*. The most thoroughly investigated Salmochelin is S4 which was found to be a two-fold β-C-glycosylated enterobactin analogue (Bister *et al.* 2004; Figure 1).

For biosynthesis of salmochelin from enterobactin, IroB was found to be essential (Bister *et al.* 2004). Deduced from its primary sequence IroB was suggested to be a glycosyltransferase related to family members that are involved in antibiotic biosynthesis (Bister *et al.* 2004) such as for erythromycin (Fischbach *et al.* 2005) and also to proteins necessary for microcin production (Hantke *et al.* 2003). Enzymatic studies demonstrated that IroB indeed functions as a glycosyltransferase in salmochelin biosynthesis from enterobactin and UDP-activated glucose (Fischbach *et al.* 2005) as previously suggested (Bister *et al.* 2004). Determination of the kinetic parameters indicated that both enterobactin and UDP-glucose were substrates of IroB (Fischbach *et al.* 2005). Multiple glycosylation of enterobactin occurs consecutively after the mono-glycosylated enterobactin has sufficiently accumulated in order to serve as substrate for a second catalytical round. Finally, *in vitro*

fully tri-glycosylated enterobactin formed (Fischbach *et al*. 2005). In contrast, *in vivo* only di-glycosylated enterobactin (salmochelin S4, Figure 1) but no tri-glycosylated salmochelin was detected (Bister *et al*. 2004). It remains to be seen, whether triglycosyl-enterobactin is excreted by cells under any circumstances. Interestingly, IroB glycosylated enterobactin at least 2×10^4 times more efficient than the DHBS monomer indicating that the SX salmochelin DHBS monomers detected (Hantke *et al*. 2003) were probably esterase degradation products of multimeric salmochelins (Fischbach *et al*. 2005).

The enzymatic functions of IroD and IroE were recently clarified (Lin *et al*. 2005; Zhu *et al*. 2005). Both proteins are involved in salmochelin utilization. IroD is a cytoplasmic protein, related to Fes, the esterase involved in enterobactin hydrolysis (Hantke *et al*. 2003). Fes hydrolyzes (ferric)-enterobactin resulting in tri-, di- or monomeric DHBS (Bryce & Brot 1972; Langman *et al*. 1972; Brickman & McIntosh 1992; Figure 2). Kinetic analysis showed that ferric-enterobactin rather than apo-enterobactin is the physiological substrate of Fes with catalytic efficiencies(k_{cat}/K_m) for ferric-enterobactin of $>90\ \mathrm{min^{-1}\,\mu M^{-1}}$ and $4\ \mathrm{min^{-1}\,\mu M^{-1}}$ for apo-enterobactin (Lin *et al*. 2005). Both Fes and IroD share 27% amino acid sequence identity. Therefore the *iroD* gene was expressed in a *fes* mutant strain of *E. coli*. In an enterobactin utilization bioassay IroD was able to restore an siderophore-dependent growth defect of a *fes* mutant as was McmK an IroD homologue of a gene locus encoding the microcins M and H47 (Zhu *et al*. 2005). Thus, several esterases from different biosynthetic pathways that are all linked to enterobactin modification might substitute for each other. Deletion of *iroD* from the *iroA*-locus resulted in excretion of mainly salmochelin S4 (di-glucosylated enterobactin, Figure 1) and DHBS (Hantke *et al*. 2003).

IroD was heterologously expressed in *E. coli*, purified and its enzymatic activity against several salmochelins assayed *in vitro*. IroD degraded salmochelin S4 (the cyclic di-glucosylated triester) completely, yielding S1 (mono-glycosylated DHBS

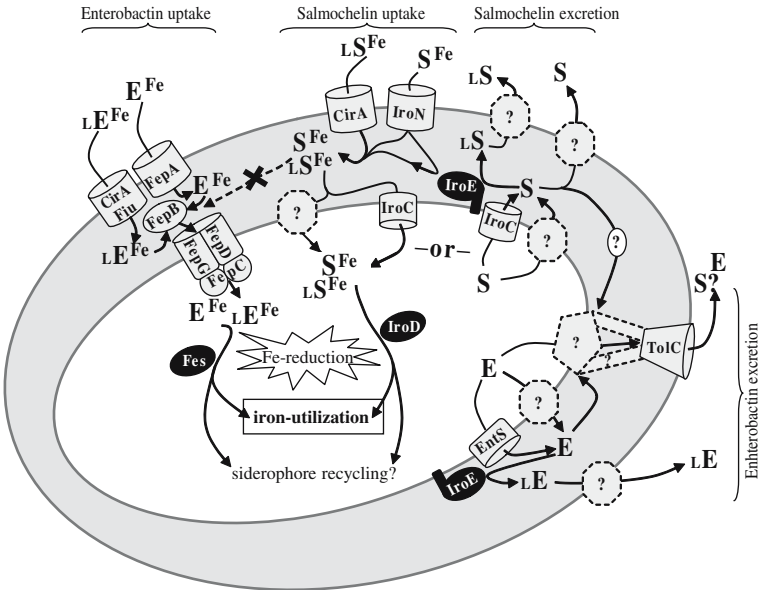

Figure 2. Model of catecholate siderophore trafficking in *E. coli* or *S. enterica* strains harboring the *iroA* locus. Several *E. coli* and *S. enterica* strains produce catecholate siderophores, enterobactin and its glucosylated derivative salmochelin. After biosynthesis enterobactin and probably also salmochelin are transported by EntS to the periplasm and consecutively to the outside by an efflux-complex including the outer membrane channel TolC. IroC might be involved in salmochelin but not in enterobactin efflux. Alternatively, IroC functions as a specific (ferric)-salmochelin uptake transporter. The periplasmic esterase IroE hydrolyses cyclic apo-siderophores to linear apo-siderophores which are released from the cell by an unknown mechanism. Ferric-siderophores are recognized by the outer membrane receptors FepA, CirA or Fiu, respectively. Enterobactin but not salmochelin enters the cytoplasm through the ABC-system FepBCDG. Within the cytoplasm the esterases Fes or IroD hydrolyze the ferric-siderophores with concomitant reduction and release of bound Fe(II). E, (E^{Fe}) and S, (S^{Fe}) represent (ferric) enterobactin and (ferric) salmochelin and LE, (LE^{Fe}) and LS, (LS^{Fe}) linear salmochelin degradation products, respectively. Black icons denote esterases (Fes, IroD or IroE).

dimer) and S2 (linear di-glycolsyated DHBS trimer, Figure 1) as well as minor amounts of the monomeric SX. The latter became more predominant after longer incubation time and also the corresponding non-glycosylated DHBS. For apo-salmochelin S4 the K_m of IroD was determined to be 1.24 mM (Zhu *et al.* 2005) in striking contrast to 120 μM by (Lin *et al.* 2005). Similarly, smaller degradation products were obtained with S1 salmochelin as a substrate (Zhu *et al.* 2005). Ultimately the function of IroD could be the fragmentation of salmochelins (Figure 2) to allow reduction and removal of iron: Concomitantly, hydrolysis of *e.g.*, Salmochelin S4 to smaller DHBS-mers would reduce the affinity towards iron. This process might be supported by parallel reduction of the complexed ferric iron resulting in diminished affinity of ferrous iron towards the siderophore (O'Brien *et al.* 1971) and thus facilitated iron utilization. Interestingly, ferric-salmochelins were stable and not cleaved by IroD as were Ga(III)-laden salmochelins (Zhu *et al.* 2005). This is in total contrast to a parallel study (Lin *et al.* 2005) where IroD was found to prefer ferric-siderophores. Likewise, enterobactin was degraded by IroD to linear oligomers of DHBS but not ferric-enterobactin (Zhu *et al.* 2005). Again Lin *et al.* (2005) found that ferric-enterobactin is a much better substrate for IroD. A possible explanation for this discrepancy is given below.

As IroD, IroE is also related to Fes. In contrast to IroD and Fes, however, IroE is a periplasmic protein (Lin *et al.* 2005; Zhu *et al.* 2005). Why does a bacterium that produces salmochelin need three related esterases? Why is IroE located in the periplasm? The purified IroE protein hydrolyzed di-glucosyl enterobactin (S4, Figure 1) to its linear derivative S2 and S3 (probably a biological inactive oxidation derivative of S2) but not further to monomeric forms as IroD does (Zhu *et al.* 2005). Substrates of IroE were also enterobactin, mono- and tri-glucosyl enterobactin but enterobactin and mono-glucosyl enterobactin were apparently preferred (Lin *et al.* 2005). The affinity of IroE towards di-glucosylated S4 was low, K_m 2.6 mM (Zhu *et al.* 2005) or 39 μM, respectively (Lin *et al.* 2005), a 100-fold difference. Conflicting data from both studies were obtained for hydrolysis of apo- or ferric-salmochelins. Zhu *et al.* (2005) found that IroE degraded ferric-salmochelin almost as well as apo-salmochelin in contrast to IroD. While cyclic enterobactin was cleaved into its linear form, Ga(III)-salmochelin was not degraded at all. Lin *et al.* (2005) claimed that IroD hydrolyzes either apo- or ferric-forms but IroE prefers apo-siderophores with catalytic efficiencies of about 20-fold higher for the later.

A reason for the existence of two salmochelin esterases might be, that IroD and IroE constitute a two step process of salmochelin degradation for iron utilization, where IroE performs an initial cleavage of the high molecular weight cyclic salmochelin in the periplasm to a linear form and cytoplasmic IroD then degrades the linear multimers to monomers from which the iron could be more easily removed through reduction (Zhu *et al.* 2005).

Alternatively, because Fes was unable to hydrolyze the glycosylated cyclic salmochelins (Lin *et al.* 2005), a second esterase might be necessary for glucosylated siderophore-specific hydrolysis in bacteria that utilize salmochelin (Lin *et al.* 2005).

Why produce salmochelin, when you already excrete enterobactin? One reason for salmochelin production in addition to enterobactin biosynthesis might be that the latter is inferior in host serum because it would adsorb to hydrophobic sites of serum proteins, *e.g.*, immunoglobulin or albumin. The binding constant for both enterobactin and ferric enterobactin to albumin was estimated to be in the range of 1×10^4 to 1.2×10^5 M^{-1} (Konopka & Neilands 1984). And in fact it was demonstrated that in growth promotion tests in the presence of albumin salmochelins were more efficient than enterobactin. Furthermore, owing to its more hydrophilic nature, the salmochelins had better diffusion properties in the aqueous agar matrix used for a biotest (Bister *et al.* 2004).

It was noted by Zhu *et al.* (2005) that several bacteria possess (putative) periplasmic siderophore esterases. It might therefore be that a two step degradation of siderophores with high-iron affinity to ones with lower affinity is advantageous for the organisms. Those bacteria could thus fine-tune their iron supply and have a transient storage of iron that is directed down a decreasing gradient of affinity and thus improved iron-availability.

Fischbach *et al.* (2005) suggested that salmochelin was superior to enterobactin for it would not as easily be sequestered by siderocalin (also known as NGAL, Kjeldsen *et al.* 2000), a neutrophil-derived antibacterial lipocalin (Goetz *et al.*

2002; Fischbach *et al.* 2005) that causes iron deprivation of bacterial invaders. This difference between enterobactin and salmochelin would provide a convenient means to differentiate bacterial strains that produce salmochelin from those that only excrete enterobactin.

What might be the ecology of salmochelin? It is still not understood how pacifarins (salmochelin, enterobactin) take effect but in an early study on enterobactin acting as pacifarin, it was noted that 'in an alkaline environment such as that which results, for example, from growth of the bacteria on lactate, enterobactin is rapidly hydrolyzed to DHBS under these conditions, in iron-limited media, certain strains of *Aerobacter* (*Enterobacter*) produce pacifarins other than enterobactin' (Wawszkiewicz *et al.* 1971). Therefore, it could now be tested if salmochelins are superior under such conditions.

Likewise, Valdebenito *et al.* (2005) pointed out that while the hydroxamate siderophore aerobactin is a good iron binder in acidic environments, under such conditions catecholate siderophores are unstable and would lose the complexed iron. On the other hand it was also suggested that salmochelin might provide an ecological advantage in neutral to alkaline conditions for instance for colonization of the urinary tract by uropathogenic *E. coli, Salmonella, Proteus or Klebsiella.* The latter can also degrade urea and thereby raise the pH. Accordingly, it was noted that in *Salmonella* (*enterica* serovar Typhimurium) expression of the salmochelin locus *iroA* was induced under high pH (Foster *et al.* 1994) while other iron regulated genes were responsive under low pH (Valdebenito *et al.* 2005).

If this is true, uropathogenic *E. coli* strains that have the possibility to produce hydroxamate type siderophores (aerobactin), mixed type siderophores (yersiniabactin) or the catecholate type siderophores enterobactin and salmochelin have advanced capabilities to respond to environmental stress such as pH change with the best-suited siderophore while passing a host animals gastrointestinal tract (Valdebenito *et al.* 2005). However, this has not been experimentally proven and it remains to be seen if ferrous iron permeases such as FeoB (Kammler *et al.* 1993), MntH (Makui *et al.* 2000), or ZupT (Grass *et al.* 2005a) are better suited for iron provision within the anaerobic environment of the gut where ferrous iron is plentiful but ferric iron is scarce (Valdebenito *et al.* 2005). The available data is inconclusive. While it was demonstrated that a *feoB* mutant of *E. coli* was deficient in its ability to colonize the mouse intestine (Stojiljkovic *et al.* 1993), a *feoB* mutant of *S. typhimurium* was not attenuated for mouse infection (Tsolis *et al.* 1996). However, colonization does not equal infection and for *S. typhimurium* it was observed that a wild-type strain out-competed its *feoB*-lacking mutant in mixed gut colonization (Tsolis *et al.* 1996).

How are siderophores secreted?

Over many years immense effort has been made to elucidate the mechanisms underlying (ferric)-siderophore recognition by their cognate receptors and subsequent uptake into the cell (reviewed in Braun & Hantke 2001; Klebba 2003). Likewise, biosynthesis of siderophores within the cytoplasm is understood in great detail (reviewed in Crosa & Walsh 2002). Remarkably however, our knowledge on how apo-siderophores are secreted by bacteria is still in its infancy. This situation is quite surprising given that so much work has been invested in siderophore uptake.

Probably the first report on siderophore efflux does not come from the enterobacteriaceae but from *Mycobacterium smegmatis* (Table 1; Zhu *et al.* 1998). The ExiT protein was suggested to be responsible for exochelin (a formylated pentapeptide siderophore with three hydroxamic acid groups for iron binding) efflux across the cytoplasmic membrane. ExiT is a member of the ABC-transporter superfamily, however, in contrast to the well known siderophore uptake ABC-transporter systems such as Fep, Fec or Fhu, ExiT belong to another sub-family. Here a permease domain is tethered to the ABC (ATP-binding cassette) domain (Linton & Higgins 1998), the hallmark of this superfamily, which is an adenosine triphosphate (ATP) binding cassette (ABC) that serves as nucleotide binding domain (Fath & Kolter 1993; Young & Holland 1999). The deletion of the *exiT* gene resulted in loss of exochelin excretion. Intracellular siderophore-accumulation was not observed. Probably, siderophore biosynthesis is coupled (Zhu *et al.* 1998) or accumulation of siderophore within the cytoplasm is prevented by degradation.

Table 1. Cytoplasmic membrane siderophore transporters from entero- and other bacteria.

Transporter family[a]	Transporter name (organism)	(Putative) transport of	Direction	Reference
MFS	EntS (*E. coli* and others)	Enterobactin	Out	Furrer *et al.* (2002)
	YbtX (*Y. pestis* and others)	(Yersiniabactin)		Furrer *et al.* (2002)
	P45 (*Acinetobacter baumanni*)	Unknown catechol siderophore		Dorsey *et al.* (2003)
	CiuF (DIP0587) (*Corynebacterium diphtheriae*)	'Corynebactin'	Out?	(Kunkle & Schmitt (2005)
ABC	FepCDG (*E. coli* and others)	Enterobactin	In	Chenault & Earhart (1991)
	FecABCDE (*E. coli* and others)	Citrate[b]	In	Staudenmaier *et al.* (1989)
	FhuBCD (*E. coli* and others)	Hydoxamates		Fecker & Braun (1983)
MDR-ABC	YbtP/YbtQ (*Y. pestis*)	Yersiniabactin	In?	Fetherston *et al.* (1999)
	C2420/21 (*E. coli* CFT073)	(Yersiniabactin)	In?	
	IroC (*S. enterica*, *E. coli*)	Salmochelin	In?	Zhu *et al.* (2005)
			Out?	Bäumler *et al.* (1996), Grass *et al.*, unp., Hantke *et al.* (2003), Bister *et al.* (2004), Lin *et al.* (2005)
	ExiT (*M. smegmatis*)	Exochelin		Zhu *et al.* (1998)

[a]MFS, major facilitator superfamily; ABC, three part polypeptide ATP-driven uptake system; MDR-ABC, Multidrug resistance efflux pump with nucleotide binding domain.[b]In contrast to the other siderophores mentioned citrate does not enter the cell with iron but rather functions as an iron-shuttle.

Other genes of permease-ABC transporters supposedly involved in siderophore transport can be identified within the *ybt* (yersiniabactin)-locus or within the *iroA*-locus of several enterobacteria (Table 1). In these instances, however, clues from experimental data suggest that direction of transport is rather from the periplasm to the cytoplasm. Some *Yersinia pestis*, *E. coli* and other enterobacterial strains produce the siderophore yersiniabactin (Heesemann *et al.* 1993). Yersiniabactin is a polyketide-non-ribosomal peptide that also contains a phenolic group. A single genetic locus is responsible for its biosynthesis and transport (Bearden *et al.* 1997). Within the *ybt*-determinant two genes, *ybtQ* and *ybtP* code for related ABC-transporters that comprise a membrane spanning permease domain and an ABC domain. The transmembrane domain likely forms a pathway in the inner membrane and the ATP-binding domain is responsible for energy generation (Linton & Higgins 1998).

Usually members of this family are composed of four such domains, two membrane spanning and two ABC cassettes. YbtQ and YbtP possably constitute two half-transporters that form a functional heterodimer (Fetherston *et al.* 1999). Analyses of mutant strains of *Y. pestis* defective in YbtQ or YbtP indicated that yersiniabactin was produced and secreted but those strains were unable to internalize yersiniabactin. Thus, strains without YbtQ or YbtP were growth deficient under iron-deficient conditions (Fetherston *et al.* 1999). When the *ybt*-locus from *Yersinia* was heterologously expressed in *E. coli*, only the biosynthesis proteins but not the transporters YbtQ and YbtP were necessary for yersinabactin-production, indicative for yersiniabactin uptake through YbtQ and YbtP (Pfeifer *et al.* 2003).

However, it was noted that another gene of the *ybt*-locus, *ybtX*, encodes a member of the large major facilitator superfamily (MFS) of membrane bound transporters that could possibly be involved in yersiniabactin efflux across the inner membrane (Table 1; Furrer *et al.* 2002). MFS transporters are energized by the proton motive force and transport a wide variety of substrates from metals to antibiotics (Pao *et al.* 1998). While YbtX does not appear to contribute any crucial function to yersiniabactin biosynthesis or utilization (Fetherston *et al.* 1999), it still is conserved within several *ybt*-loci from *Yersinia* or *E. coli*. It might be that another protein that is also capable of yersiniabactin transport is encoded elsewhere on the chromosome of those bacteria.

The IroC permease-ABC transporter is related to YbtQ and YbtP but its size equals a fusion of both half-transporters. Since all these proteins are also related to the well understood P-glycoprotein, a multidrug resistance (MDR) protein from humans (Juranka *et al.* 1989) a number of studies predicted salmochelin efflux function for IroC (Table 1; Hantke *et al.* 2003; Bister *et al.* 2004; Lin *et al.* 2005). IroC would be responsible for efflux (Figure 2) as described for many related ATPases such as HlyB (alpha-hemolysin translocation), CydCD (glutathione efflux), CvaB (colicin V secretion), MchF (microcin H47 secretion).

Recently however, contrary evidence was provided. A strain expressing the *iroA*-locus but lacking FepB, the periplasmic enterobactin binding protein for uptake, was still able to utilize external salmochelins while a *fepB iroC* double mutant could not (Zhu *et al.* 2005). This strongly argues for IroC-mediated salmochelin uptake from the periplasm (Figure 2), very similar to what is suggested for YbtQ/YbtP. This issue can probably only resolved, when IroC protein is reconstituted in liposomes and transport of salmochelin directly measured.

For *E. coli* a mechanism for enterobactin efflux across the cytoplasmic membrane into the periplasm was discovered recently. Here not an ABC transporter but a MFS transporter, EntS (Table 1; Chenault & Earhart 1991; Shea & McIntosh 1991), was demonstrated to be necessary for effective export (Figure 2; Furrer *et al.* 2002). The gene encoding EntS, *ybdA* is encoded within the 20 kb enterobactin locus of *E. coli*. Cells deleted in *entS* excreted very little enterobactin into the surrounding medium but degradation products of enterobactin (mono-, di- and triester of DHBS) were released. This finding was recently corroborated (Bleuel *et al.* 2005). However, those DHBS-mers act themselves also as efficient siderophores and were still used. Therefore, strains lacking *entS* suffered no iron depletion (Furrer *et al.* 2002; Bleuel *et al.* 2005).

Because enterobactin is too big at more than 600 Da to diffuse through the porins of the outer membrane, the question arose, how does enterobactin leave the periplasm after transport by EntS? Published reports and genome data bases provided several hints on how siderophores might be excreted. It was suggested that pyoverdine, the major siderophore of *Pseudomonas aeruginosa*, is exported by the MexA-MexB-OprK resistance nodulation cell division (RND, Saier *et al.* 1994) complex and it was speculated that AcrA and AcrB of *E. coli* were involved in the secretion of enterobactin and/or its metabolites (Poole *et al.* 1993). Likewise, in the study on the cytoplasmic membrane enterobactin transporter EntS of *E. coli* it was postulated that AcrAB or the major facilitator EmrB and its associated membrane fusion protein EmrA (Lomovskaya & Lewis 1992) might pump out excess enterobactin under low iron conditions (Furrer *et al.* 2002).

Experimental data demonstrated that transport systems of the RND type may transport substrates from the periplasm (or from the cytoplasmic membrane in case of hydrophobic substances) rather than from the cytoplasm to the outside (Nikaido *et al.* 1998; Nikaido & Zgurskaya 2001; Lomovskaya *et al.* 2002; Legatzki *et al.* 2003). RND type protein-complexes transport a very broad variety of substances including antibiotics, dyes, detergents and heavy-metal cations (Nikaido 1996; Nikaido *et al.* 1998; Nikaido & Zgurskaya 2001; Lomovskaya *et al.* 2002; Nies 2003). RND proteins are typical transporters of Gram-negative bacteria and function as a huge (about 10^6 Da) protein-complex spanning from the cytoplasmic membrane to the outer membrane. These protein complexes are composed of an RND inner-membrane protein, a membrane fusion protein and an outer membrane channel-tunnel protein (Koronakis *et al.* 2001) also termed outer membrane factor (OMF, Paulsen *et al.* 1997). The capability of periplasmic efflux would make resistance nodulation cell division complexes ideal candidates to accomplish the second step of enterobactin efflux, namely export from the periplasm to the outside.

At least for copper and cobalt, experimental data indicated that efflux is probably a two step process involving different transporters of the cytoplasmic membrane and a RND protein-complex for efflux of the periplasmic metal cations (Rensing & Grass 2003; Munkelt *et al.* 2004). Likely, the same applies to zinc and cadmium efflux (Legatzki *et al.* 2003).

Further observations pointed toward RND proteins that might be required for excretion of siderophores. Firstly, mutants of *P. aeruginosa* defective in *mexA-mexB-oprK* showed a decreased ability to grow in the presence of the iron chelator

2,2′-dipyridyl (Poole *et al.* 1993) marking this trans-envelope efflux system a likely candidate for siderophore export. Secondly, in *Acinetobacter baumannii* 8399 a gene for an RND transporter related to MexB from *P. aeruginosa* or AcrB from *E. coli* and an EntS orthologue were found to be part of a locus for siderophore biosynthesis and transport (Dorsey *et al.* 2003). Third, the *cusA* (*ybdE*) gene of *E. coli* was initially and misleadingly annotated as iron efflux system (Accession No. BAB34036) and is located in vicinity to *entS* (*ybdA*) (Chenault & Earhart 1991) on the chromosome. This genetic arrangement is fairly reminiscent to the siderophore locus of *A. baumanii* 8399 (Dorsey *et al.* 2003).

In *E. coli* there are seven described RND transport proteins [AcrB, AcrD, AcrF, MdtB (YegN), MdtC (YegO), MdtF (YhiV) and CusA (YbdE)]. Likewise, *E. coli* possesses eight known (AcrA, AcrE, CusB MdtA, MdtE MacA, EmrA and EmrK) and seven putative membrane fusion proteins (YjcR, YhcQ, YdhJ, YiaV, YibH, YhiI, YbhG). Only two outer membrane factors, TolC (Whitney 1971) and CusC have been shown to be involved in efflux in *E. coli*. CusC is the exclusive outer membrane channel-tunnel protein for the Cus-system that is responsible for copper-efflux, probably from the periplasm (Franke *et al.* 2003). The second outer membrane channel-tunnel protein of *E. coli*, TolC, is needed for transport mediated by the other six RND transporters (Nishino & Yamaguchi 2001) but not for Cus (Franke *et al.* 2003). TolC also functions in export complexes comprised of ABC-transporters or MFS-proteins (Koronakis 2003). This makes TolC an Achilles' heel of transenvelope efflux (Saier *et al.* 1994) since these exporters share TolC as the outer membrane channel-tunnel protein. Therefore, an ideal access point for dissecting enterobactin export from the periplasm is inactivation of TolC (Bleuel *et al.* 2005).

To identify the transporter responsible for periplasmic enterobactin efflux, genes of *tolC* or all seven RND genes, respectively, were deleted from the chromosome of *E. coli* and their effects on enterobactin excretion were determined. The outer membrane factor TolC but none of the RND proteins were necessary for enterobactin export across the outer membrane (Figure 2). Furthermore, deletion of *tolC* resulted in diminished growth of *E. coli* under iron deprivation (Bleuel *et al.* 2005), probably caused by inactivation of the enterobactin efflux machinery. TolC does probably not function with one RND transporter alone for enterobactin export (Bleuel *et al.* 2005). There appears to be redundancy and several RNDs may collectively contribute to enterobactin efflux all using TolC. In this scenario deletion of a single RND gene can be compensated for by the others.

For the time being probably the best hypothesis is that TolC interacts with an uncharacterized MFP (Furrer *et al.* 2002). Involvement of EntS as part of a MFS–MFP–TolC complex transporting enterobactin from the cytoplasm to the outside is rather unlikely given the observation that only a strain without EntS but not a strain without TolC still secreted some enterobactin (Bleuel *et al.* 2005). Therefore, an EntS independent, second pathway must be postulated. This notion is supported by the observation that introduction of a Δ*entS* or a Δ*tolC* mutation in the genome of an *E. coli* mutant strain that can only use enterobactin or its derivatives for iron acquisition yielded dissimilar iron-dependent phenotypes (Bleuel *et al.* 2005). Since a *tolC* mutant was growth deficient and secreted no enterobactin, EntS is probably not able to recruit membrane fusion proteins (Furrer *et al.* 2002) to constitute an EntS–MFP–TolC tripartite transenvelope efflux system. Thus, EntS is not identical with the hypothetical MFS protein of the suggested MFS–MFP–TolC enterobactin export pathway (Figure 2).

In the current working model EntS transports enterobactin into the periplasm but does not interact with MFPs and TolC for further export (Figure 2). Another MFS–MFP–TolC pathway might export cytoplasmic substrates directly to the outside similar to other MFS–MFP–TolC-type or ABC–MFP–TolC-type efflux complexes do. Such interactions of TolC with transporters such as the ATP-driven ABC-system MacAB (Kobayashi *et al.* 2001) or the major facilitator drug efflux systems EmrAB or EmrKY (Lomovskaya & Lewis 1992) is possible but appears unlikely in the light that these transporters translocate their substrates directly from the cytoplasm to the outside (Nishino & Yamaguchi 2001). On the other hand this could, at least partially, compensate for a missing EntS, for a Δ*entS* strain still excretes some enterobactin (Furrer *et al.* 2002; Bleuel *et al.* 2005). Whatever the inner membrane pumps and connecting MFP is, it seems that TolC forms the only

duct for enterobactin to leave the periplasm to the outside.

Because the *entS* and the *tolC* mutants still secreted enterobactin breakdown products (Furrer *et al*. 2002; Bleuel *et al*. 2005), other transporter(s) might be able to export DHBS and its di- or triester, which in turn serve as siderophores. From the periplasm the smaller degradation products might leave the cell through porins. The growth defect observed for a *tolC* mutant under iron deprivation assumedly stems from the clogging of the periplasm with apo-enterobactin. All incoming ferric-DHBS-mers would accordingly compete with high-affinity apo-enterobactin for iron binding eventually leading to iron starvation. It would be interesting to see if expression of the periplasmic salmochelin/enterobactin esterase IroE (Lin *et al*. 2005; Zhu *et al*. 2005) is able to attenuate the Δ*tolC* growth defect by hydrolysis of periplasmic enterobactin. The resulting linear degradation products might then leave the periplasm TolC-independently and function for iron-acquisition.

New permeases involved in iron transport in E. coli

Apart from iron acquisition through siderophores *E. coli* possesses several other means to meet its demand for this essential metal. Previously, FeoB and MntH were shown to function in iron uptake. Participation of other permeases such as the low-affinity Mg(II) transporter CorA (Hantke 1997) was controversial and it was recently demonstrated that CorA does not transport ferrous iron (Papp & Maguire 2004). Nevertheless, *corA* mutants of *E. coli* or *S. typhimurium* accumulated less iron in the log phase of growth and where more susceptible to ferrous iron-mediated killing under low Mg(II) conditions than their parental wild-type strains (Hantke 1997). Possibly the observed differences can be attributed to different growth conditions since Papp & Maguire (2004) used stationary phase cells. Therefore, the connection of iron-homeostasis and CorA remains to be elucidated.

MntH (manganese transport, H^+-stimulated) is a member of the NRAMP (natural resistance associated macrophage protein) family of high-affinity manganese uptake permeases of Pro- and Eukaryotes that also transports ferrous iron with lower affinity. Consequently, the apparent affinity for ferrous iron transport was low, approximately 100 times lower than for Mn(II) and the apparent K_m for Fe(II) was 100–200 μM (Kehres *et al*. 2000). For MntH the topology was experimentally determined and several essential amino acid residues could be identified within the polypeptide that spans the cytoplasmic membrane 11 times (Courville *et al*. 2004).

The FeoB protein is probably the most exotic ferrous iron transporter known. Initially it was thought that FeoB was energized by ATP-hydrolysis (Kammler *et al*. 1993). Now it appears that the large soluble N-terminal domain of FeoB constitutes a GTP-binding and -hydrolysis domain (Marlovits *et al*. 2002). As pointed out by a recent review by Hantke (2003) we still do not know how FeoB functions but three models were presented: FeoB could be a membrane-potential-driven permease with the GTPase necessary for regulation, FeoB may not be a transporter *per se* but regulates activity of another unknown transporter, or transport by FeoB is energized directly through GTP-hydrolysis (Hantke 2003). Only transport-assays with reconstituted FeoB protein will tell us which of these hypotheses is correct.

The *feoB* gene is part of an operon *feoABC* of which *feoA* was earlier identified alongside *feoB* (Kammler *et al*. 1993) but *feoC* (*yhgG*) was only recently identified (Taudte 2003). Both small peptides (FeoA: 8.4 kDa, FeoC 8.7 kDa) are necessary for full function of FeoB but their specific contribution to iron transport or regulation is unclear (Taudte 2003). FeoA might be involved in regulation (Andrews 1998). In other organisms the *feoB* gene can be found alongside *feoA*, *feoC* or both, indicative of a role for FeoA or FeoC, respectively.

Unexpectedly, in *Porphyromonas gingivalis* one of two FeoB paralogues plays a role in manganese accumulation while the second is involved in iron-homeostasis (Dashper *et al*. 2005). It will be interesting to see how the difference in substrate-specificity is determined in FeoB-proteins given that FeoB is indeed a transporter.

In plants and animals, proteins of the ZIP (ZRT- and IRT-like proteins) are major participants in cellular Zn(II) and Fe(II) homeostasis (Guerinot 2000). No member of this family was characterized in bacteria until recently. The first report of a bacterial ZIP transporter comes from *E. coli*'s ZupT (zinc uptake transporter) and it was initially demonstrated that cells expressing *zupT*

from a plasmid exhibited increased uptake of Zn(II) (Grass *et al*. 2002) and deletion mutants of *zupT* and *znuABC*, the high-affinity Zn(II) ABC-uptake system of *E. coli* (Patzer & Hantke 1998), responded stronger to zinc-deprivation than a *znuABC* single mutant (Grass *et al*. 2002).

The substrate spectrum of ZupT was found to be quite broad. Besides Zn(II), Fe(II), Co(II) and probably Mn(II) or Cd(II) are transported (Kwon *et al*. 2004; Grass *et al*. 2005a, b). In this, ZupT resembles its eukaryotic orthologues that also have broad substrate specificity (Gaither & Eide 2000; Guerinot 2000). In contrast to eukaryotes, however, in *E. coli* expression of the *zupT* gene is not induced by iron- (or other metal-) depletion but expression is constitutive and on a low level (Grass *et al*. 2005a). ZupT-mediated transport is efficient, because overexpression of ZupT causes severe metal-toxicity. In many bacterial and most eukaryotic ZIP family members a histidine-rich domain can be found between putative transmembrane helix III and IV. This feature is missing in ZupT. One can imagine that this domain might be responsible for regulation of metal-import (Guerinot 2000).

A great unsolved mystery of ZIP transporters is how proteins of this family are energized. Little data are available. For human hZIP2 zinc uptake was not energy-dependent and it was also independent of the membrane potential. Treatment with several different inhibitors of electron transport or of oxidative phosphorylation did not decrease zinc uptake activity. However, HCO_3 stimulated hZip2 activity (Gaither & Eide 2000). Experiments are currently underway to determine the driving force for ZupT transport.

As with siderophores, data on iron uptake permeases are plentiful but on the other hand almost nothing is known about iron efflux and environmental conditions when efflux occurs. Owing to the particular properties of iron solubility most bacteria suffer iron deficiency. However, accessible iron in the duodenal lumen of mice fed a standard diet yielded a concentration of up to 60 μM (Simpson & Peters 1990). Therefore, *E. coli* might be exposed to relatively high iron concentrations in its natural habitat, the gut. Here, stabilized under anaerobic conditions, it would occur in the ferrous form. Being on short supply some of the time, it might nevertheless be beneficial for *E. coli* to possess an iron efflux system when iron is abundant and the iron storage proteins are overwhelmed.

Clues for iron-efflux in *E. coli* came from two independent directions. First, the *yiiP* gene was found to be inducible not only by Zn(II) (Grass *et al*. 2001) but also by Fe(II) (Grass *et al*. 2005b). YiiP belongs to a subfamily of the ubiquitous cation diffusion facilitator (CDF) protein family (Paulsen & Saier 1997; Nies 2003). CDF proteins are antiporters mainly energized by the proton motive force but also by a K^+ gradient (Guffanti *et al*. 2002). Second, in magnetotactic bacteria such as *Magnetospirillum gryphiswaldense*, CDF proteins of the same subfamily, MamB and MamM, were found to be involved in magnetosome biosynthesis (Grünberg *et al*. 2001). In this organism the respective CDFs probably transport iron from the cytoplasm to the magnetosome for further magnetite crystallization. This transport would translate to *E. coli* transport from the cytoplasm to the periplasm (Grass *et al*. 2005b). Preliminary results of heterologous *yiiP* expression in *M. gryphiswaldense* suggest that proteins of this CDF sub-group are functionally equivalent and may substitute each other (D. Schüler, personal communication). This is an important prerequisite for further functional inter-species studies.

Other studies assigned YiiP a function in Zn(II) detoxification (Chao & Fu 2004; Wei *et al*. 2004; Wei & Fu 2005), contrary to observations of the initial study on YiiP which clearly showed that YiiP does not mediate zinc resistance *in vivo*, and in contrast to the second CDF from *E. coli*, ZitB (Grass *et al*. 2001). However, YiiP is able to mediate Zn(II) transport *in vitro* using inside-out membrane vesicles (Grass *et al*. 2005b). This transport utilized a NADH-energized proton gradient over the cytoplasmic membrane demonstrating the proton-dependency of YiiP-mediated transport.

In vivo studies support the idea that YiiP is an iron efflux pump. Therefore YiiP was named FieF (ferrous iron efflux; Grass *et al*. 2005b). Not only did expression of *fieF* lead to decreased accumulation of iron in *E. coli* cells, FieF was also useful for iron detoxification in Δ*fur* cells suffering deregulated iron uptake. The FieF protein was successfully reconstituted in proteoliposomes and FieF mediated iron transport could be measured *in vitro*. Thus, FieF is probably a cytoplasmic membrane iron efflux transporter utilizing the proton

gradient that is generated by the respiratory chain (Grass *et al*. 2005b).

Conclusions and outlook

The last several years have shed new light on the wide range of means *E. coli* has for iron acquisition. It remains to be seen whether further studies unearth additional players in the complex network of iron-homeostasis. For instance it is not clear if the putative YcdN protein constitutes a functional transporter in *E. coli*. YcdN is a member of the FTR high-affinity ferric-iron permease family, known from fungi and lower plants (Kosman 2003). Involvement in iron uptake for a related transporter from a magnetotactic bacterium was proposed recently (Dubbels *et al*. 2004). In *E. coli* K12 there is a frameshift mutation close to the 5′ end of the *ycdN* gene rendering the YcdN transporter inactive (Grass and Große, unpublished observations). Is YcdN an additional iron transporter in other enterobacteria?

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