# Chapter 2
# The Tricky Ways Bacteria Cope with Iron Limitation

**Volkmar Braun and Klaus Hantke**

**Abstract** Iron is an essential element for many key redox systems. It is difficult to acquire for cells under oxic conditions, since $Fe^{3+}$ forms insoluble hydroxides. In the human host, iron is tightly bound to proteins. Bacteria invented iron transport systems which solubilize external $Fe^{3+}$ by secreted low-molecular weight compounds, designated siderophores, or directly from the human proteins. Gram-negative bacteria contain an intricate energy-coupled iron transport mechanism across the outer membrane which lacks an energy source. The electrochemical potential of the cytoplasmic membrane delivers the energy. Transport across the cytoplasmic membrane is most frequently achieved by ABC transporters in Gram-positive and Gram-negative bacteria. Under anaerobic conditions, iron is the soluble $Fe^{2+}$ form and transported different to $Fe^{3+}$. Iron transport and intracellular iron concentrations are controlled by transcription regulation of iron transport genes. Transcription is turned on under iron-limiting growth conditions which usually exist in natural environments.

**Keywords** Bacterial iron transport · Siderophores · Human iron binding proteins

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## 2.1 Introduction

For almost all bacteria, iron is an essential nutrient since it is contained in the redox centers of many enzymes of the respiratory chains, photosyntheses, and intermediary metabolism. Bacteria are confronted with a variety of iron limitation conditions. At an anaerobic stage, usually under reducing conditions, enough soluble $Fe^{2+}$ is present to cope with the iron demands. At an aerobic stage, under oxidative conditions, $Fe^{3+}$ is present, which at pH 7 is completely insoluble. In addition, in the human host, most iron exists as free heme, heme bound to hemoglobin, hemopexin, and hemoglobin-haptoglobin. The human host fulfills its own iron requirement by synthesizing the proteins transferrin and lactoferrin, which very tightly bind iron. Free iron in equilibrium with these compounds is orders of magnitude below the concentration ($\sim$0.1 μM) required to sustain bacterial growth. To cope with iron deprivation, under these conditions, bacteria were highly inventive and developed a number of intricate mechanisms to fulfill their iron requirements. In the following, these mechanisms will be discussed because they form the basis of a huge variety of individual solutions that certain bacteria, under their specific environmental conditions, have developed (Cornelis and Andrews 2010). This becomes especially apparent in the microbial synthesis of a great number of siderophores. A compilation of siderophore structures known since 2009 lists more than 279 siderophores (Hider and Kong 2010) and more have been added since then. Siderophores are synthesized and secreted into the medium where they solubilize the $(Fe^{3+}OOH)_n$ precipitate and transport the $Fe^{3+}$ siderophore complex into the cells, where $Fe^{3+}$ is released by reduction and enters the metabolism. In heme transport and transport of iron delivered by transferrin and lactoferrin, siderophores are usually not involved (see Chap. 3).

This chapter only touches iron transport and iron regulatory systems of *Pseudomonas* which are described in depth in a separate chapter in this book.

## 2.2 Iron Transport into Gram-Negative Bacteria

In Gram-negative bacteria, iron must be transported across the outer membrane and the cytoplasmic membrane. Both transport processes occur independently of each other (Fig. 2.1).

### *2.2.1 Transport Across the Outer Membrane*

Transport across the outer membrane involves transport proteins with 22 antiparallel $\beta$ strands that form a $\beta$ barrel which is tightly closed by a globular plug domain (Noinaj et al. 2010). Regardless of whether iron is delivered by

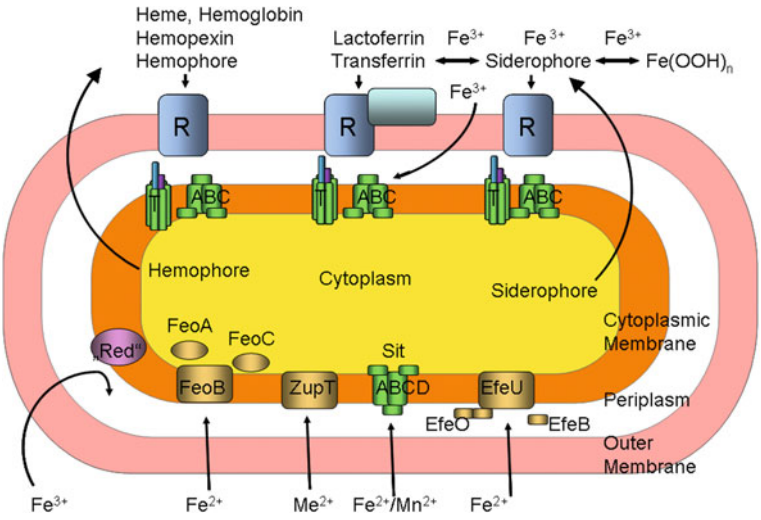


**Fig. 2.1** Transport systems of $Fe^{2+}$ and $Fe^{3+}$ into Gram-negative bacteria. R denotes receptor proteins; ABC denotes ATP-binding cassette transporter, T indicates the Ton complex. The heme proteins release the heme at the cell surface which is taken up into the cytoplasm. Transferrin and lactoferrin release iron at the cell surface which is taken up into the cytoplasm. $Fe^{3+}$ siderophores are taken up into the cytoplasm, but some enter only the periplasm. $Fe^{2+}$ diffuses across the outer membrane and is transported by various systems across the cytoplasmic membrane. Red indicates a reduction of $Fe^{3+}$ to $Fe^{2+}$. Hemophores and siderophores are synthesized in the cytoplasm and released by specific export systems. See text for details

siderophores, heme, hemoglobin, transferrin, or lactoferrin (Fig. 2.1), the iron ligands bind tightly ($K_m \sim 1$ nM) to surface-exposed regions of the transporters. From there, they must be released by changes in the conformation of the binding site and the plug must move so that the $Fe^{3+}$ siderophores, heme, or $Fe^{3+}$ can move into the periplasm. Energy is required to drive these movements which is not generated in the outer membrane but is derived from the proton motive force (pmf) of the cytoplasmic membrane. Coupling of the outer membrane to the cytoplasmic membrane requires a protein complex which consists of the proteins TonB, ExbB, and ExbD. Their location and transmembrane topology are shown in Fig. 2.2. TonB, ExbB, and ExbD are found in cells at a ratio of 1:7:2 (Higgs et al. 2002), but it is unclear whether the proteins are all in the complex or whether some still exist in a soluble form not yet assembled. Detergent-solubilized ExbB forms a hexamer and copurified ExbD forms an $ExbB_6$-$ExbD_1$ complex. TonB has not been copurified in a stoichiometric ratio to ExbB and ExbD. It is assumed that $ExbB_6$ forms the platform on which the final complex is assembled (Pramanik et al. 2010, 2011). Interaction of the three proteins was shown in various ways: ExbB stabilizes TonB and ExbD, and formaldehyde cross-links TonB dimers, ExbD dimers, TonB-ExbB dimers, and ExbB-ExbD dimers (Postle and Larsen 2007). The yield of cross-linked products is too low to unravel larger oligomers. TonB contains in the hydrophobic transmembrane segment a single charged residue, histidine,

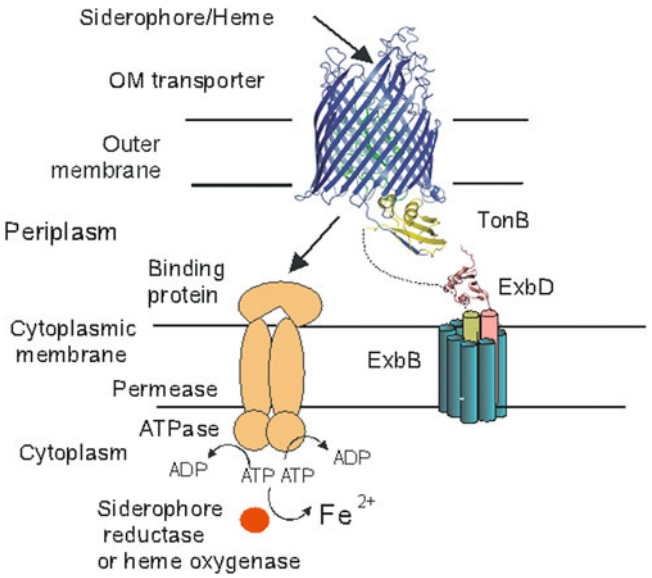


**Fig. 2.2** Model of siderophore and heme transport across the outer membrane and the cytoplasmic membrane of Gram-negative bacteria. TonB binds to a specific region, TonB box, of the outer membrane transporter which is exposed to the periplasm. The iron compounds are transported across the outer membrane at the expense of energy provided by the proton motive force of the cytoplasmic membrane mediated by the TonB-ExbB-ExbD protein complex. Once in the periplasm, a binding protein binds the iron compounds and delivers them to the ABC transporter in the cytoplasmic membrane. The ABC transporter consists of the transmembrane permease to which an ATPase is attached in the cytoplasm. $Fe^{3+}$ is released from the siderophores by reduction to $Fe^{2+}$ and from heme by heme oxygenase. Heme is also incorporated into heme proteins. See text for details

which was supposed to be the amino acid that responds to the pmf. However, this attractive hypothesis had to be abandoned when it was shown that His20 can be replaced by Asn, while still retaining full TonB activity (Swayne and Postle 2011). Other important amino acids in transmembrane regions are Asp25 in ExbD (Braun et al. 1996) and Glu176 in ExbB (Braun and Herrmann 2004a, b). Asp/Asn and Glu/Ala mutants are completely inactive. These negatively charged amino acids are reasonable candidates for a protonation/deprotonation cycle in response to the pmf across the cytoplasmic membrane, as observed in membrane-bound $H^+$-ATPases. Protonation/deprotonation may change the conformation of the complex. It is conceivable that TonB in the energy-rich conformation allosterically changes the conformation of the outer membrane transporters. During this process, TonB is "deenergized" and is subsequently reenergized by the pmf to reenter the cycle. TonB physically interacts with outer membrane transporters (Fig. 2.2), as shown by the suppression of point mutants in the N-terminal region (TonB box) of

outer membrane transporters by mutations in TonB. The same region was cysteine cross-linked and shown by crystal structures to interact (Pawelek et al. 2006; Shultis et al. 2006). Three early stages in TonB energization were revealed by proteinase K sensitivity and formaldehyde cross-linking assays (Ollis and Postle 2012). To access TonB with proteinase K, spheroplasts were used in which TonB and ExbD were cross-linked depending on the proton motive force, as is the case in cells. TonB was degraded by proteinase K in mutants lacking ExbD, expressing an inactive ExbD(L132Q) or TonB(H20A). No formaldehyde cross-links were formed, which was also the case in an ExbD(D25N) mutant. However, in the latter case, TonB was degraded by proteinase K. In wild-type spheroplasts, TonB was degraded by proteinase K and cross-linked with formaldehyde. In spite of these data, it remains unclear how TonB and the entire complex react to the pmf and how they interact with the outer membrane transporters in a way that changes their conformation, releases the bound substrates, and opens the pore.

A bioinformatic analysis reveals that all Gram-negative bacteria contain one or up to three TonB proteins (Chu et al. 2007). The carboxy-terminal region with which TonB interacts with the outer membrane transporters and receptors assumes a conserved fold. They are either redundant in that they can replace each other or they interact specifically with a transporter. In Vibrio strains, TonB1 is associated with ExbB1 and ExbD1, TonB2 is associated with ExbB2 and ExbD2, and TonB3 is associated with ExbB3 and ExbD3. In addition, vibrios contain TtpC proteins associated with the second and third TonB system. TtpC is a 49 kDa protein that is predicted to span the cytoplasmic membrane three times with its C-terminus in the cytoplasm and the majority of the protein in the periplasm. TtpC is essential for TonB-mediated iron transport systems; however, its mode of action has not been resolved. TonB2 and TonB3 are shorter than TonB1, which does not need a TtpC. It has been hypothesized that TtpC is required for the shorter TonBs to contact the outer membrane transporters (Kustusch et al. 2011).

### *2.2.2 FhuA Transports Ferrichrome*

FhuA is a multifunctional transporter that not only transports ferrichrome across the outer membrane, but also the structurally similar antibiotic albomycin, the structurally diverse antibiotic CGP4832, a synthetic rifamycin derivative, the peptide antibiotic microcin J25, and colicin M. It also serves as a receptor of the phages T1, T5, and Φ80. For all of these functions, FhuA is essential. FhuA of *Salmonella* strains also transports ferrichrome and albomycin, but the phage receptor specificity differs (Killmann et al. 1998). FhuA was the first outer membrane transport protein whose crystal structure was determined (Ferguson et al. 1998; Locher et al. 1998). Its basic design is exemplary for all of the other outer membrane transporters whose crystal structures were solved (Table 2.1).

**Table 2.1** Crystal structures of TonB-dependent outer membrane transporters

| Protein | Ligand[a] | PDB ID |
|---|---|---|
| FhuA, *E. coli* | – | 1BY5 |
| | Ferrichrome | 1FCP |
| | Ferrichrome/LPS | 2FCP |
| | CGP4832/LPS | 1F11 |
| | Albomycin | 1QKC |
| | TonB/ferricrocin | 2GRX |
| FecA, *E. coli* | – | 1KMO |
| | – | 1PNZ |
| | Diferric dicitrate | 1KMP |
| | Diferric dicitrate | 1PO3 |
| | Dicitrate | 1POo |
| FepA, *E. coli* | – | 1FEP |
| Cir, *E. coli* | | 2HDF |
| BtuB, *E. coli* | | 1NQE |
| | TonB, vitamin $B_{12}$ | 2GSK |
| | $Ca^{2+}$ | 1NQG |
| | $Ca^{2+}$ vitamin $B_{12}$ | 1NQH |
| FpvA, *P aeruginosa* | – | 2O5P |
| | pyoverdine | 2W16 |
| | Ferric pyoverdine | 2IAH |
| | Ferric pyoverdine | 1XKH |
| FptA, *P. aeruginosa* | Pyochelin | 1XKW |
| FauA, *B. pertussis* | – | 3EFM |
| HasR, *S. marcescens* | HasA/heme | 3CSL |
| | HasA | 3CSN |
| ShuA, *S.dysenteriae* | | 3FHH |
| TbpA, *N. meningitidis* | Human transferrin | 3V8X |
| FyuA, *Y. pestis* | Yersiniabactin | – |
| YiuR, *Y. pestis* | | – |

[a] Not all structures and ligands are listed. The *Yersinia pestis* FyuA protein was recently published (Lukacik et al. 2012), YiuR was not published (Noinaj et al. 2010)

#### 2.2.2.1 Reconstitution of FhuA

FhuA was in vivo reconstituted from the $\beta$ barrel domain and the plug domain, both equipped with a signal sequence for secretion across the cytoplasmic membrane (Braun et al. 2003a, b). The yield of active protein was rather high (45 %). This suggests that the $\beta$ barrel folds independent of the plug and that the plug is inserted after the $\beta$ barrel has formed. It is not clear whether folding is completed in the periplasm or whether final stages occur during insertion into the outer membrane. To yield an active FhuA, no precise plug structure (residues 1–160) is required as a much larger fragment (residues 1–357) also forms an active FhuA.

FhuA has also been reconstituted in a functional state in planar lipid bilayer membranes (Udho et al. 2009). FhuA does not conduct ions across planar lipid

bilayer membranes (Braun et al. 2002a, b), since the plug tightly closes the pore in the $\beta$ barrel. However, the addition of 4 M urea to the *cis* compartment to which FhuA was added results in a continuous rise of conductance for tens of minutes with occasional single channel events of 0.1–1 nS. Upon removal of urea, conductance through FhuA fell 60–90 % and could be repeated several times by the addition and removal of urea. The addition of 4 M urea or 3 M glycerol to the *trans* solution abolished conductance which was established upon removal of urea or glycerol from the trans solution. Urea exerts two functions: it unfolds the plug and it establishes an osmotic pressure gradient across the membrane. If phage T5 was added to either the *cis* or the *trans* solution, no channels were formed. However, when 3 M glycerol was added to the *cis* solution and subsequently perfused out, T5 added to the *cis* or the *trans* solution caused the appearance of numerous 0.1–1nS channels. The glycerol gradient oriented FhuA in the membrane such that phage binding sites faced the *cis* as well as the *trans* compartment. Ferrichrome added to the *trans* side stopped and partially reversed the 4 M urea-induced conductance of FhuA, showing that the ferrichrome binding site was intact in the reconstituted FhuA. To some extent, urea mimics the TonB-dependent reorientation of the plug within the barrel or movement of the plug out of the barrel.

### 2.2.3 FepA Transports $Fe^{3+}$ Enterobactin

FepA transports cyclic $Fe^{3+}$ enterobactin and its linear forms. It also serves as a receptor for colicins B and D. $Fe^{3+}$ enterobactin transport by FepA has been studied in most detail and unraveled the essential parameters of all these types of transporters (Newton et al. 2010). Although the number of FepA molecules in fully induced cells (35,000 per cell) far exceeds the number of TonB molecules per cell (1000), all FepA molecules are engaged in transport with a maximum turnover number of approximately 5/min and an activation energy of 33-35 kcal/mol. Accumulation of $Fe^{3+}$ enterobactin in the periplasm requires the FepB periplasmic binding protein. In the absence of FepB $Fe^{3+}$, enterobactin escapes through TolC into the medium. FepB does not interact with FepA. FepA was saturated with $Fe^{3+}$ enterobactin with a $K_D$ of 0.2 nM. Simultaneous transport of $Fe^{3+}$ enterobactin via FepA and ferrichrome via FhuA does not change the $K_m$ and $V_{max}$ of $Fe^{3+}$ enterobactin transport, but reduces the $V_{max}$ of ferrichrome transport by $\sim$50 %. TonB does not seem to be a decisive limiting factor in a simultaneous cotransport of various $Fe^{3+}$ siderophores. The rather high activation energy presumably results from movement of the plug to get $Fe^{3+}$ enterobactin through the FepA pore. The intracellular iron concentration has been determined to be in the range of 0.1 to 0.25 mg/g dry weight, which translates into 0.66–1.7 mM or 0.6–1.6 $\times 10^6$ ions per cell. If this quantum is transported per cell cycle, it would cost 2.4–6 $\times 10^6$ ATP equivalents.

*E. coli* expresses additional outer membrane transporters, Cir, Fiu, and IroN, which recognize with different affinities the iron complexes of cyclic enterobactin, its linear forms (2,3 dihydroxybenzoylserine)$_{1\text{-}3}$, and salmochelin and its linear forms. They also bind microcins with catecholate moieties (Müller et al. 2009).

### 2.2.4 FecA Transports Ferric Citrate and Elicits a Transcription Regulatory Signal

A TonB-dependent iron transport system exists in *E. coli* that is mediated by citrate. Determination of the crystal structure revealed diferric dicitrate bound in a cavity at the cell surface (Ferguson et al. 2002; Yue et al. 2003). Comparison with the unloaded FecA protein reveals a strong movement of two surface loops so that they occlude the entry of the cavity. If ferric citrate is released from the FecA binding site by interaction of FecA with energized TonB, it cannot escape into the medium but diffuses vectorially across the opened pore in FecA into the periplasm. Since citrate is not cotransported with iron into the cytoplasm, it is likely that iron is transported across the cytoplasmic membrane by the FecBCDE ABC transporter (see also Sect. 6.1). FecA was the first case where it was shown that it not only transports ferric citrate, but initiates the transcription of the *fecABCDE* transport genes. Binding of ferric citrate to FecA elicits a signal that is conferred across the outer membrane to the FecR regulatory protein in the cytoplasm which transfers the signal across the cytoplasmic membrane to the FecI sigma factor. In contrast to outer membrane transporters which do not regulate transcription, FecA and all signaling transporters contain a N-terminal extension that extends into the periplasm and serves to interact with FecR (summarized in Braun 2010; Braun et al. 2003a, b, 2006; Braun and Mahren 2005, 2007; Ferguson et al. 2007; Noinaj et al. 2010). A genome survey identified the presence of signal receptors/transducers of the FecA type in a variety of Gram-negative bacteria (Koebnik 2005).

### 2.2.5 TbpA/TbpB of Neisseria meningitidis Transport Transferrin Iron

Neisseria do not synthesize siderophores but they can use siderophores of other organisms, called xenosiderophores, such as aerobactin, enterobactin, salmochelin, a diglycosylated derivative of enterobactin (Bister et al. 2004), and dimers and trimers of dihydroxybenzoylserine which are derived from enterobactin. Their major iron source is human transferrin that binds to the receptor TbpA and its coreceptor TbpB (Fig. 2.1). TbpA is a rather large outer membrane protein of 100 kDa that belongs to the TonB-dependent transporters. The structure of TbpB differs from the structure of TbpA (Moraes et al. 2009), it is smaller (80 kDa), and

it is attached by a lipid anchor to the outer membrane. TbpA binds iron-loaded and unloaded transferrin with similar affinities, whereas TbpB binds only iron-loaded transferrin. TbpA is sufficient to transport iron but iron uptake is more efficient in the presence of TbpB. To unravel how tightly bound iron ($K_a = 10^{23}$ $M^{-1}$) is transferred from transferrin to TbpA, the crystal structure of TbpA with unloaded transferrin was determined (Noinaj et al. 2012). A special feature of the structure is a long plug loop that protrudes $\sim$25 Å above the cell surface, whereas in the other transporters the plug is buried in the $\beta$ barrel. With this plug loop, TbpA interacts with the C1 subdomain of transferrin. This interaction induces a partial opening of the cleft in the transferrin C-lobe that destabilizes the iron coordination site, and thereby facilitates the release of iron and its transfer to TbpA. In addition, the $\alpha$-helix of the extracellular loop 3 of TbpA is inserted into the cleft between the C1 and C3 subdomains of the transferrin C-lobe. The latter interface contains residues found only in human transferrin which may explain the specificity of Neisseria TbpA for human transferrin. X-ray and SAXS analysis of TbpB with iron-loaded transferrin revealed binding of TbpB to the C-lobe of transferrin, but at sites that differ from the sites TbpA binds to the C-lobe. Iron bound to the N lobe of transferrin does not affect the binding of transferrin to TbpA and TbpB. All three proteins form an enclosed chamber with a volume of $\sim$1000 $Å^3$ which is located directly above the plug domain of TbpA. The released iron is guided to the pore of TbpA and loss by diffusion into the medium is prevented. Steered molecular dynamics were applied to obtain an idea of how iron may diffuse from the cell surface through TbpA into the periplasm. In the ground state, a large, highly negatively charged transmembrane cavity is located between the barrel and the plug whose access is restricted by a loop from the extracellular side and by a helical gate of the plug from the periplasmic side. When force is applied to the plug domain, that might mimic the action of TonB (Gumbart et al. 2007), first the helical gate and then the restriction loop are removed, producing an unobstructed pathway from the extracellular space to the periplasm.

### 2.2.6 *Ferredoxins Enhance Growth of Pectobacterium spp Under Iron-Limiting Conditions*

*P. carotovorum* and *P. atrosepticum* form colicin-type bacteriocins, which consist of two domains, a N-terminal [2Fe-2S] ferredoxin with 60 % identity to spinach ferredoxin and a C-terminal toxin with 46 % identity to the activity domain of colicin M (Grinter et al. 2012). The two toxins, known as pectocin M1 and pectocin M2, display an increased activity against certain *P. carotovorum* and certain *P. atrosepticum* strains when these are grown under iron-limiting conditions, suggesting the synthesis of an outer membrane receptor for the ferredoxin moiety that serves as an iron source. Spinach ferredoxin prevents the cytotoxicity of M1, suggesting competition with binding to the transporter. Growth enhancement and sequence homology is specific for ferredoxins of plants on which the pectobacteria

thrive. Until now, the receptor protein has not been identified and no iron transport measurements were performed.

Replacement of Asp222 by Ala, which is equivalent to the Asp225 essential for the activity of colicin M (Helbig and Braun 2011) inactivates M1. Another *P. carotovorum* strain produces a third bacteriocin, named pectocin P that consists of an N-terminal ferredoxin and a C-terminal domain that is 41 % identical to the activity domain of pesticin of *Yersinia pestis* (Grinter et al. 2012). Pesticin—like all colicin-type bacteriocins—consists of three domains of which the N-terminal region was experimentally fused to lysozyme (gene e product) of phage T4, resulting in a hybrid protein that killed cells (Patzer et al. 2012). M1, M2, and pectocin P are further examples of the evolution of bacteriocins by the assembly of DNA fragments that encode receptor binding, translocation, and activity domains (Braun et al. 2002a, b).

## *2.2.7 Heme Uptake by Bacteria*

### 2.2.7.1 Heme Uptake Via Hemophores: The HasR/HasA System of *Serratia marcescens*

The particular feature of heme uptake by *S. marcescens* is the participation of a secreted protein, named HasA hemophore (Fig. 2.1), which was discovered in studies of type I protein secretion of *S. marcescens* (Létoffé et al. 1994). HasA tightly binds heme ($K_d = 18$ pM) or heme from hemoglobin, leghemoglobin, hemopexin, and myoglobin without forming stable complexes with these proteins (Wandersman 2010; Wandersman and Stojiljkovic 2000) (Fig. 2.1). HasA binds to the TonB-dependent outer membrane transporter HasR in the heme loaded and the heme unloaded form with a $K_d$ of 5 nM. Two large HasR extracellular loops contact HasA. Transfer of heme from its high-affinity site in HasA to the low-affinity site in HasR against a $10^5$ affinity gradient is not energy-driven and occurs in vitro. The crystal structure of HasA bound to HasR reveals a possible mechanism of heme transfer from HasA to HasR (Krieg et al. 2009). Ile 671 in an extracellular loop of HasR contacts heme in HasA resulting in the disruption of the HasA-Tyr75 heme coordination. Replacement of Ile 671 by Gly abolishes heme transfer from HasA to HasR. TonB and pmf are required to release heme-free HasA from HasR. A specific TonB variant, HasB, binds to HasR with high affinity ($\sim$10 nM), which suggests that the two proteins do not dissociate during the energization/denergization cycle of heme transport across HasR and release of HasA from HasR. Hemophore-mediated heme transport has also been found in *Yersinia pestis*, *Yersinia enterocolitica*, *Erwinia carotovora*, *Pseudomonas aeruginosa,* and *Pseudomonas fluorescens*.

#### 2.2.7.2 HxuC/HxuA of *Haemophilus influenzae* Transport Hemopexin Heme

*H. influenzae* is a heme auxotroph that takes up heme from various sources of its human host such as hemoglobin, hemoglobin/haptoglobin, hemopexin, and serum albumin via several TonB-dependent outer membrane transporters. HuxC/HuxA is required for using heme from hemopexin, which binds heme with extreme affinity ($K_d$ below picomolar). HxuC is a TonB-dependent outer membrane transport protein, HxuA is secreted by HxuB and a part of it, depending on the *H. influenzae* strain, is released into the medium (hemophore). In *E. coli*, 95 % of HuxA are found to be cell-associated. The *hxuABC* genes were cloned in a heme auxotrophic strain of *E. coli* and the transport system reconstituted (Fournier et al. 2011). In addition, the *H. influenzae* TonB-ExbB-ExbD complex is required for efficient hemopexin heme uptake by *E. coli*. Heme acquisition from hemopexin requires the HxuC transporter and HxuA, which could also be added from outside of the cells. The replacement of two cysteine residues by serine increased the amount of HxuA in the supernatant. It was with this derivative that in vitro studies were performed. HxuA formed a 1:1 complex with heme-free and heme-loaded hemopexin; however, the $\Delta H$ values differed by –71 kJ $mol^{-1}$. The binding of HxuA to hemopexin changes the heme environment; no binding of heme to HxuA could be found. The binding of HxuA to hemopexin also releases heme, which can even be transferred to HasR of *S. marcescens*. The occurrence of the Hxu system in most clinical isolates of *H. influenzae* supports the importance of this heme uptake system for heme acquisition.

## 2.3 TonB-Independent Iron Uptake Across the Outer Membrane

An ABC transporter encoded by the *sfuABC* genes of *Serratia marcescens* transports iron into a TonB, ExbB, and ExbD mutant (Angerer et al. 1990, 1992; Zimmermann et al. 1989) (Fig. 2.1). It was the first of this category that was characterized. No siderophores and no outer membrane proteins could be related to this iron transport system. $FeCl_3$ and $Fe^{3+}$ citrate supported growth of an *E. coli* K-12 mutant devoid of enterobactin synthesis that had been transformed with the *sfuABC* genes. In strong iron deficiency conditions, growth was stimulated by citrate, which was dependent on active transport by the FecA outer membrane receptor and TonB. Under these conditions, the diffusion of iron across the outer membrane was no longer sufficiently fast to support growth. However, SfuABC transported iron delivered by citrate across the cytoplasmic membrane in a *fecCDE* mutant required for citrate-mediated iron transport in *E. coli*. Apparently, iron was dissociated from citrate and was taken over by SfuABC. Although the experiments were performed with ferric iron under aerobic conditions, the possibility that ferric

iron was reduced to ferrous iron prior to uptake across the outer membrane and the cytoplasmic membrane cannot be excluded. A very similar system was described for all *Yersinia* species pathogenic for humans, called YfuABC (Saken et al. 2000). *Haemophilus influenzae* contains an ABC transporter for iron which does not seem to be coupled to a TonB-dependent outer membrane transporter (Adhikari et al. 1995).

In the *Neisseria gonorrhoeae* strain FA19, the FbpABC proteins are required for ferric iron transport removed from transferrin and lactoferrin through the periplasm and across the cytoplasmic membrane. This system also transports iron by enterobactin, its linear monomeric product dihydroxybenzoylserine and the linear form of salmochelin designated S2, each provided in 10 μM concentrations (Strange et al. 2011). *N. gonorrhoeae* does not produce siderophores, but can use xenosiderophores.

In *Vibrio cholerae,* the VctPDGC ABC cytoplasmic membrane transport system promotes iron uptake by vibriobactin, a catecholate siderophore, but also transports iron independent of a siderophore and TonB (Wyckoff and Payne 2011).

In *Haemophilus influenzae*, iron is taken up by the HitABC transporter (Adhikari et al. 1995). No TonB-dependent outer membrane transporter was related to this system.

*Legionella pneumophila* forms the siderophore legiobactin, which is taken up through the outer membrane protein LbtU. *In silico* analysis of LbtU reveals a typical outer membrane protein with long surface loops and short periplasmic turns. However, in contrast to the 22-stranded ß barrels of TonB-dependent transporters, LbtU consists of only 16 ß strands. *L. pneumophila* does not contain a TonB protein. Therefore, the iron complex of legiobactin is taken up across the outer membrane via LbtU in a TonB-independent manner (Chatfield et al. 2011).

## 2.4 Hemophore-Type Heme Uptake in Gram-Positive Bacteria

The former assumption that heme diffuses across the cell wall of Gram-positive bacteria until it reaches the cytoplasmic membrane, where the heme transport systems reside, suffers from the fact that free heme is rather insoluble and usually very tightly bound to proteins such as hemoglobin, hemoglobin/haptoglobin, and hemopexin. To cope with this situation, Gram-positive bacteria express intricate hemophore-type heme acquisition systems (Fig. 2.3).

### *2.4.1 Heme Uptake into Staphylococcus aureus*

*S. aureus*, in which most of the original investigations have been performed (Mazmanian et al. 2003; Hammer and Skaar 2011), transports heme vectorially

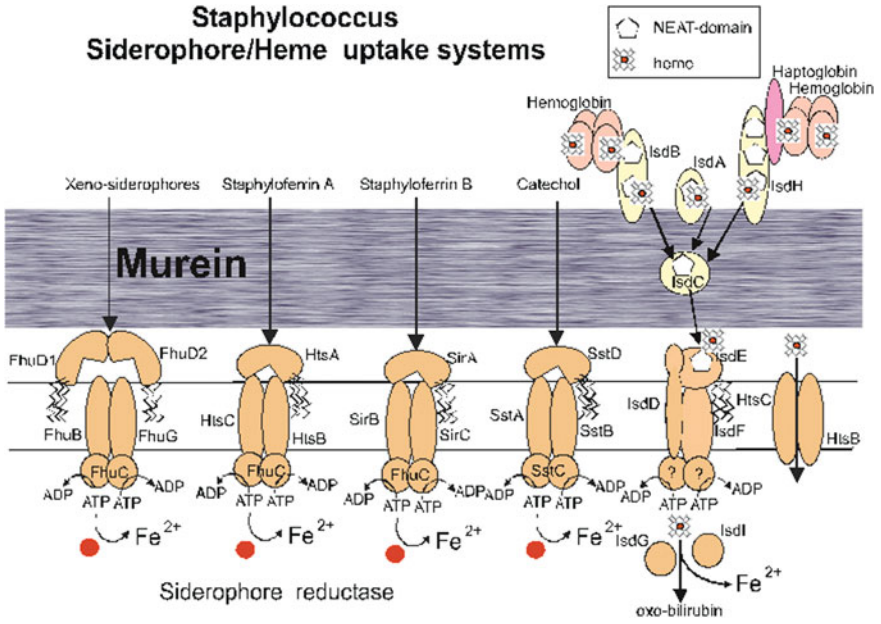


**Fig. 2.3** $Fe^{3+}$ siderophore and heme transport into *S. aureus* as an example for Gram-positive bacteria. $Fe^{3+}$ siderophores and $Fe^{3+}$ xenosiderophores are supposed to diffuse unspecifically across the cell wall composed of the murein, wall teichoic acids, teichuronic acids, and proteins to the cytoplasmic membrane, through which they are transported by specific ABC transport systems. In contrast, for heme, a specific diffusion cascade exists through the cell wall along the indicated proteins. In the cytoplasm, $Fe^{3+}$ is mobilized from the siderophores by reduction to $Fe^{2+}$ and from heme by conversion to oxobilirubin. See text for details

across the thick cell wall by a cascade of proteins which are thought to be positioned such that heme moves within single proteins and from one protein to the next protein until it reaches the cytoplasmic membrane. A portion of these proteins is released into the medium like the hemophores of Gram-negative bacteria. The proteins are designated Isd from *i*ron-regulated *s*urface *d*eterminant. They are covalently anchored to the peptidoglycan by sortase A, except IsdC, which is anchored by sortase B. They contain up to three structurally conserved NEAT domains of 125 amino acids that bind heme. Transcription of the *isd* genes is controlled by iron through a Fur repressor. From a number of in vivo and in vitro studies, the following model of heme uptake has been derived. Heme from hemoglobin-haptoglobin is mobilized by NEAT 1 of IsdH and then transferred to NEAT 2 and NEAT 3 of IsdH (Fig. 2.3). Heme from hemoglobin is mobilized by NEAT1 of IsdB and transferred to NEAT 2 of IsdB. Heme is then transferred from IsdH and IsdB either directly to the IsdE binding protein of the transport system in the cytoplasmic membrane, or through IsdA and IsdC. IsdE then transfers heme to the IsdF permease, which—together with IsdD—transports heme across the

cytoplasmic membrane. Heme transfer from IsdH/IsdB, IsdA, IsdC, and IsdE is unidirectional and complete. Knockout mutants of *isdDEF* still show reduced heme uptake because a second ABC transporter encoded by *htsBC* transports heme. Once inside the cytoplasm, iron must be mobilized from heme. Heme degradation is catalyzed by two heme oxygenases which are very similar structurally and are termed IsdG and IsdI. These heme oxygenases display the special properties that cause the product formed to not be biliverdin, carbon monoxide, and iron (which are usually created by heme oxygenases), but a mixture of $\beta$- and $\sigma$-isomers of oxobilirubin, called staphylobilin (Hammer and Skaar 2011).

### 2.4.2 Heme Uptake by Listeria monocytogenes

Acquisition of free heme and heme incorporated into hemoglobin by *L. monocytogenes* also involves proteins covalently anchored to the peptidoglycan. They are designated Hbp1 and Hbp2 (Xiao et al. 2011). Hbp2 is the primary acceptor for heme and presumably transfers heme to Hbp1, which then delivers heme to the heme-binding HupD lipoprotein ($K_D = 26$ nM), which is part of the ABC transporter in the cytoplasmic membrane. Hbp1 and Hbp2 are only required when the heme concentration is lower than 50 nM. At higher heme concentrations, enough heme diffuses across the cell wall to be directly transported by the HupDGC transporter into the cytoplasm. The overall $K_M$ for heme uptake is 1 nM with a $V_{max} = 23$ pmol $10^9$ cells$^{-1}$ min$^{-1}$. A second heme uptake system accounts for the residual transport of heme in *hupDGC* mutants.

## 2.5 Mobilization of Transferrin Iron by Staphyloferrins and Catecholamines

*S. aureus* grows on transferrin as a sole iron source. No protein cascade as shown for heme uptake and no receptor/transporter as present in *N. meningitidis* are involved in transferrin iron uptake by *S. aureus*. Rather, siderophores mobilize transferrin iron and donate it to siderophore-specific ABC transporters in the cytoplasmic membrane. Staphyloferrin A and B and also host catecholamine hormones support growth of *S. aureus* on iron-loaded transferrin. The latter reduce transferrin $Fe^{3+}$ to $Fe^{2+}$ for which transferrin has a low affinity. Staphyloferrin iron is taken up via the HtsABC and SirABC systems, whereas catecholate iron is taken up by the newly identified SstABCD transport system (Beasley et al. 2011).

## 2.6 Iron Transport Across the Cytoplasmic Membrane

### *2.6.1 ABC Transporters*

ABC transporters are the prevailing systems for $Fe^{3+}$ siderophores, heme, and iron transport across the cytoplasmic membrane. The name ABC is derived from an ATP-binding cassette, which indicates that an ATPase associated from the cytoplasmic side to the transmembrane transport protein, is an essential component of the transport system. The transport proteins consist of one or two different subunits to which two ATPases are usually bound. The substrates are delivered by binding proteins which are either in Gram-negative bacteria free in the periplasm or in Gram-positive bacteria anchored by a lipid moiety of the murein lipoprotein type to the cytoplasmic membrane. The binding proteins determine the substrate specificity of the transport systems. The iron transport proteins usually consist of 20 transmembrane segments, as has first been determined for FhuB of the *E. coli* ferrichrome transport system (Groeger and Köster 1998). In this chapter, 42 known transport proteins of $Fe^{3+}$ siderophore transport systems were in silico analyzed and shown to be arranged in the cytoplasmic membrane like FhuB. Early mutant analyses suggesting active sites in FhuB, the FhuD periplasmic binding protein, the FhuC ATPase, and the interaction sites between these proteins were summarized by Braun et al. (1998) and Köster (2005). After the transfer of iron, $Fe^{3+}$ siderophores, or heme across the outer membrane by the TonB-dependent transporters, the iron compounds bind to periplasmic binding proteins which deliver the iron compounds to the permease in the cytoplasmic membrane. Based on a considerable number of crystal structures and amino acid sequences, the bacterial binding proteins were ordered in a number of classes (Chu and Vogel 2011). The periplasmic binding proteins for $Fe^{3+}$ siderophores belong to the type III structures, as was first determined with FhuD (Clarke et al. 2000). They adopt two independently folded amino- and carboxy-terminal domains with the particular feature of a single long α-helix of approximately 20 amino acids that connects the two domains. The α-helix restricts movement of the two domains relative to each other as is observed in other binding proteins which undergo a "Venus fly trap" movement when they bind their substrates in the cleft between the two domains. Consequently, the substrate of FhuD remains exposed to the surface; for example, the antibiotic part of albomycin remains flexible and is not seen when it is bound via its $Fe^{3+}$ hydroxamate moiety to FhuD (Clarke et al. 2002). The small structural change observed upon substrate binding raises the question of how substrate-loaded FhuD is recognized by FhuB. Molecular dynamics simulations indicate that the C-terminal domain closes 6° upon substrate release, which is considered to be a sufficiently large change to be seen by FhuB (Krewulak et al. 2005).

The crystal structure of a cytoplasmic membrane component of an ABC transporter related to iron transport has not been determined. The closest relative of iron transporters is the vitamin $B_{12}$ transporter, which in fact was the first ABC transporter that was crystallized and the structure determined (Locher et al. 2002).

Vitamin $B_{12}$ is transported across the outer membrane by the high-affinity TonB-dependent BtuB transporter. In the periplasm, vitamin $B_{12}$ is bound by the BtuF binding protein. The ABC transporter consists of two transmembrane spanning subunits (BtuC) which provide 20 transmembrane helices that form a translocation pathway. Two identical ATPases (BtuD) are bound to BtuC at the inner side of the cytoplasmic membrane. The three proteins form a stable, high-affinity complex ($K_d \sim 10^{-13}$). Vitamin $B_{12}$ accelerates the BtuCD-BtuB complex dissociation rate $10^7$-fold, which is further destabilized by ATP (Lewinson et al. 2010). Exposed to the cytoplasm is a large water-filled channel. Based on the crystal structures, quantitative binding determinations and electron spin resonance spectroscopy, the following transport reaction cycle is proposed (Joseph et al. 2011). BtuF loaded with vitamin $B_{12}$ binds to the apo or ADP state of BtuCD. Vitamin $B_{12}$ is released to the translocation channel. ATP binding to the ATPases inwardly opens the translocation channel and vitamin $B_{12}$ diffuses into the cytoplasm. Concomitantly, the periplasmic BtuC gate is closed. Excess vitamin $B_{12}$ in the periplasm and ATP bound to BtuD promotes dissociation of BtuF from BtuCD and ATP is hydrolyzed. BtuCD is restored to an outward-facing conformation, ready to interact with another vitamin $B_{12}$-loaded BtuF. The mechanism proposed for vitamin $B_{12}$ transport across the cytoplasmic membrane may apply to most iron transport systems.

The mechanism of vitamin $B_{12}$ transport differs from the mechanism of maltose transport, which is the model system for bacterial type I ABC transporters. These importers are composed of 10–14 helices and alternate from an ATP-bound outward-facing conformation where the periplasmic binding protein releases its substrate to the low-affinity binding site of the transmembrane protein, to an ADP-bound inward-facing conformation where the substrate is released into the cytoplasm. In the crystal structure, in the absence of maltose binding protein, $MalFGK_2$ (MalFG transmembrane proteins, MalK ATPase) forms an inward-facing conformation with the transmembrane maltose binding site exposed to the cytoplasm. The crystal structure $MalFGK_2$ with unloaded maltose binding protein and ATP shows that closure of the nucleotide binding domains of MalK is concomitant with the transfer of maltose from the binding protein to the MalFG transmembrane domains. Interactions of maltose-loaded binding protein induce partial closure of the MalK dimer in the cytoplasm. ATP binding to this conformation promotes progression to the outward-facing state (Oldham and Chen 2011).

## 2.7 Transport of $Fe^{2+}$

### *2.7.1 The Feo System*

Under anaerobic/microaerophilic conditions and/or low pH, iron may occur in the $Fe^{2+}$form, which has a much higher solubility at pH 7 (0.1 M) than $Fe^{3+}$ ($10^{-18}$ M). In 1987, an $Fe^{2+}$ transport system was discovered in *E. coli* (Hantke 1987) and in the

meantime found widely distributed in bacterial genomes (Cartron et al. 2006). The *feo* operon consists of three genes—*feoA*, *feoB*, *feoC*—arranged in this order and transcribed from a $Fe^{2+}$-Fur-regulated promoter upstream of *feoA*. Only FeoB is essential for $Fe^{2+}$ transport. FeoB is an unusual bacterial transport protein that consists of three domains, an N-terminal GTPase (Marlovits et al. 2003) exposed to the cytoplasm which is linked by a spacer to the C-proximal transmembrane domain. The spacer may function as a GDP dissociation inhibitor (Eng et al. 2008). This kind of structure resembles eukaryotic regulatory GTPases. The crystal structures of the cytosolic domains of Feo from *E. coli* (Guilfoyle et al. 2009), *Methanococcus jannaschii* (Köster et al. 2009), and *Thermotoga maritima* (Hattori et al. 2009) were determined. They elucidate the GTP binding site. The GDP dissociation inhibitor and the GTPase form a large interface with a hydrophobic and two polar interactions. GDP binding to the GTPase domain is stabilized by the interaction of the GTPase with the GDP dissociation inhibitor. It is speculated that this represents the "off state" of the FeoB transporter; the GTP bound state would be the "on state." Any model must take into account that the GTP hydrolysis rate is much too slow (50 % hydrolysis after 6 h at 37 °C) to serve as an energy source to drive iron import. In contrast, dissociation of GDP is much too fast to regulate the open/closed state. Therefore, additional proteins are most likely involved in FeoB activity regulation, one of which could be FeoA that contains a SH3 domain (Su et al. 2010) which in eukaryotes is involved in protein–protein interactions. It seems that a GTP/GDP cycle regulates the activity of the transporter which resides in the transmembrane portion that makes up most of the protein. No structural or functional studies have been reported on this portion of FeoB. The energy source for $Fe^{2+}$ transport is another open question. In *Helicobacter pylori,* ATP hydrolysis has been claimed to drive $Fe^{2+}$ transport on the experimental basis that FCCP (a protonophore), DCCD (an ATP synthesis inhibitor), and orthovanadate, an ATP hydrolysis inhibitor, abolish high-affinity $Fe^{2+}$ uptake ($Ks = 0.54\ \mu M$) (Velayudhan et al. 2000). However, the structure of FeoB does not resemble any ATPase, nor does it contain an ATP but rather a GTP binding motif.

$Fe^{2+}$ uptake via FeoB is a major pathway for *H. pylori* Fe acquisition as *feoB* mutants are unable to colonize the gastric mucosa of mice, which is a low pH, low-oxygen environment. The virulence of other bacteria also depends on the Feo iron transport system. For example, *feoB* mutants of *E. coli* show an attenuated ability to colonize the mouse intestine (Stojiljkovic et al. 1993). The $LD_{50}$ of a *feoB* mutant of *Streptococcus suis* was tenfold higher than the $LD_{50}$ of the *feoB* wild-type strain (Aranda et al. 2009). Investigation of the role of iron transport for the virulence of a particular strain is hampered by the formation of redundant iron transport systems, such as, for example, enterobactin, salmochelin, yersiniabactin, aerobactin, and the Feo system in extraintestinal pathogenic *E. coli*. To study the contribution of one system, all of the other systems must be inactivated. In the case of the TonB-dependent $Fe^{3+}$ transport systems deletion of TonB is sufficient to evaluate the contribution of iron to virulence.

### 2.7.2 The EFE System

Under aerobic conditions, *E. coli* and *Shigella* strains express a $Fe^{2+}$ transport system that is encoded by the *efeUOB* operon (Grosse et al. 2006). This system resembles oxidase-dependent iron transporters in yeast, since EfeU is homologous to Ftr1p of yeast. It resides in the cytoplasmic membrane with seven transmembrane helices and two REXXE motifs that are required for iron transport. The periplasmic EfE belongs to the heme peroxidase family and contains a *b*-type heme.

### 2.7.3 The Sit System

Besides the common Feo system, all Shigella strains and numerous pathogenic Gram-negative bacteria express a second $Fe^{2+}$ transport system (Fisher et al. 2009) composed of the *sitABCD* genes which form an ABC transporter. SitA is a periplasmic binding protein, and SitB is the ATPase that is attached to the SitCD transmembrane proteins. Sit is not very specific for $Fe^{2+}$ but also transports $Mn^{2+}$(Kehres et al. 2002). Although Sit transports $Fe^{2+}$in addition to $Mn^{2+}$, transcription of the *sit* genes in *S. flexneri* is unexpectedly induced under aerobic conditions and is repressed under anaerobic conditions. Sit is an important virulence factor as it enhances growth of *S flexneri* in cultured epithelial cells and for virulence in a mouse lung model.

### 2.7.4 *VciB of* Vibrio cholerae

VciB was discovered as a growth stimulatory factor in low-iron medium of *E. coli* and *S. flexneri* transformed with *vciB* (Mey et al. 2008). VciB enhances iron uptake via the Feo or the Sit $Fe^{2+}$ transport systems; it does not catalyze iron uptake by itself. It resides in the cytoplasmic membrane with a large loop in the periplasm. It is not known how it achieves an increase in $Fe^{2+}$ transport; however, the following possibilities are discussed: 1) VciB acts as a $Fe^{3+}$ reductase that increases the local concentration of $Fe^{2+}$ for transport by the Feo or Sit $Fe^{2+}$ transport systems, 2) VciB binds $Fe^{2+}$ and delivers it to the Feo or Sit transport proteins, and 3) VciB stimulates the activities of the transporters. Since VciB would have to interact with Feo and the Sit transporters, lack of protein specificity argues against a direct interaction with the Feo and Sit proteins. VciB does not stimulate $Fe^{3+}$ transport by Fbp of *V. cholerae* or Hit of *Haemophilus influenzae*.

## 2.8 Iron Uptake of Marine Bacteria

$Fe^{3+}$ concentrations in oceanic surface waters are as low as 0.02–1 nM, with more than 99 % bound to mostly undefined organic ligands. This extremely low concentration is caused by the virtual insolubility of $Fe^{3+}$ at the slightly basic pH of seawater and the low iron input. Iron is a growth-limiting nutrient in seawater for the phytoplankton. To cope with iron limitation, marine bacteria synthesize siderophores and $Fe^{3+}$ siderophore transport systems. Since cultivation of marine bacteria is usually difficult or impossible, data on iron transport genes and siderophore biosynthesis gene clusters are mostly derived from genomic and metagenomic approaches (Hopkinson and Barbeau 2011) and siderophores may be identified without relating them to a particular strain. TonB-dependent outer membrane transporters (TBTs) are predicted for $Fe^{3+}$ hydroxamates, $Fe^{3+}$ catecholates, and heme. $Fe^{3+}$ hydroxamate and heme transporters are found in almost half of the prokaryotes, and $Fe^{3+}$ catecholate transporters are found in about one-third of the genomes. TBTs are not evenly distributed among marine bacteria. On average, 2.5 TBT genes per genome are encountered but many genomes encode more than 30 TBTs. Some marine bacteria may encode up to 117 TonB-dependent transporters, which certainly do not take up only iron compounds but other metal ions, as has been found for $Ni^{2+}$ (Schauer et al. 2007, 2008), $Zn^{2+}$ (Stork et al. 2010), and in particular for sugars (Blanvillain et al. 2007; Bjursell et al. 2006; Eisenbeis et al. 2008; Lohmiller et al. 2008; Neugebauer et al. 2005). $Fe^{3+}$ ABC transporters are found in nearly all members of the *Cyanobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria,* but only in one member of the *Bacteroidetes*. In contrast, examination of the genomes of known abundant, free-living marine picocyanobacteria and *Pelagibacter ubique* revealed only very rarely $Fe^{3+}$ siderophore transport genes; they may take up inorganic iron. There is a negative correlation between the occurrence of $Fe^{3+}$ transport systems and the FeoA/FeoB $Fe^{2+}$ transport system. Marine prokaryotes tend to have only one of these systems, but not both. For example, picocyanobacteria have the $Fe^{3+}$ ABC system but no FeoA/FeoB and the *Bacteriodetes* have FeoA/FeoB but no $Fe^{3+}$ ABC transporter, with one exception: *feoA* is more frequently encountered than *feoB* because *feoA* occurs often in multiple copies per genome. This finding is unexpected as studies with *E. coli* and other bacteria assign the $Fe^{2+}$ transport function to FeoB and FeoA is dispensable.

## 2.9 Single Protein Transporters Across the Cytoplasmic Membrane

In addition to the ABC transporters—which consist of three to four proteins—there exist iron transporters across the cytoplasmic membrane which are composed of only a single protein. These are less well-studied than the ABC transporters. In

*P. aeruginosa*, $Fe^{3+}$ pyochelin is taken up across the outer membrane by the TonB-dependent FptA transporter and across the cytoplasmic membrane by the FptX protein (Reimmann 2012). $Fe^{3+}$ pyoverdine is transported by *P. aeruginosa* across the outer membrane by the TonB-dependent FpvA transport proteins. In the periplasm, $Fe^{3+}$ is released from pyoverdine by reduction and pyoverdine is secreted unmodified to the extracellular space by the PvdRT-OmpQ efflux pump, which also secretes newly synthesized pyoverdine, which is, in turn, used for the next round of $Fe^{3+}$ transport. $Fe^{3+}$ pyoverdine is not taken up into the cytoplasm (Greenwald et al. 2007). How iron enters the cytoplasm is not known.

## 2.10 Iron Release from Siderophores in the Cytoplasm

In cytoplasmic fractions of bacteria, reduction of $Fe^{3+}$ siderophores to $Fe^{2+}$ can usually be encountered. However, few studies specifically related reduction to the use of $Fe^{3+}$ siderophores. Evidence that reductases are specifically involved in iron acquisition comes from genomic localization of putative reductase genes adjacent to $Fe^{3+}$ siderophore transports genes, derepression of reductase gene transcription by iron limitation, and substrate specificity. Examples are FhuF of *E. coli* (Matzanke et al. 2004), YqjH of *E. coli* (Miethke et al. 2011a, b), and FchR of *Bacillus halodurans* (Miethke et al. 2011a, b). FhuF is a 2Fe-2S protein that reduces $Fe^{3+}$ hydroxamates; in particular, ferrioxamine B. *fhuF* mutants show reduced growth on plates with ferrioxamine B as the sole source of iron. Although iron uptake via ferrioxamine B, ferrichrome, and coprogen is unimpaired, iron removal is reduced. In contrast, YqjH catalyzes the NADPH-dependent release of iron from a variety of siderophores, most effectively from $Fe^{3+}$ 2,3 dihydroxybenzoylserine which is formed by intracellular hydrolysis of $Fe^{3+}$ enterobactin. $Fe^{3+}$ dicitrate serves as another substrate for YqjH. *fchR* clusters with a ferric citrate-hydroxamate uptake system. It catalyzes iron removal from $Fe^{3+}$ schizokinen, produced by *B. halodurans*, supplied $Fe^{3+}$ dicitrate, $Fe^{3+}$ aerobactin, and ferrichrome. A *fchR* deletion mutant shows a strongly impaired growth but accumulates iron, indicating that the $Fe^{3+}$ siderophores are not efficiently metabolized. In vivo, FchR is inhibited by redox-inert siderophore mimics, supporting $Fe^{3+}$ siderophore-specific reduction which may be a target for future antibiotics.

The iron-free siderophores are degraded, as has been shown for enterobactin (Greenwood and Luke, 1978), modified like ferrichrome which is acetylated in *E. coli* (Hartmann and Braun, 1980) and *P. aeruginosa,* or secreted as pyoverdine (Hannauer et al. 2010a). Cells must get rid of the siderophores to avoid iron sequestration from cell metabolism.

## 2.11 Secretion of Siderophores

Siderophores synthesized in the cytoplasm are secreted to the external medium where they scavenge iron and transport it into cells. Only a very few studies are devoted to siderophore export. Enterobactin secretion was the first siderophore secretion system to be characterized. Enterobactin is secreted across the cytoplasmic membrane by the EntS protein (Furrer et al. 2002) and across the outer membrane by the TolC protein (Bleuel et al. 2005). In *P. aeruginosa*, the protein PvdE secretes a precursor of pyoverdine, called ferribactin, across the cytoplasmic membrane. The precursor is then further processed to pyoverdine, which is exported by PvdRT-OpmQ across the outer membrane (Hannauer et al. 2010b). Pyoverdine synthesis is a complex, not entirely resolved process. Precursors contain myristic or myristoleic acid (Hannauer et al. 2012), which may attach the siderophore to the membrane and prevent its diffusion in the cytoplasm and the periplasm where the siderophore may bind iron and withdraw it from synthesis of iron-containing redox enzymes. The PvdRT-OpmQ complex is involved in the secretion of imported pyoverdine after the release of iron in the periplasm. PvdR functions as an adapter between PvdT, a predicted inner membrane protein, and OpmQ. PvdRT-OpmQ is an ABC transporter related to the TolC containing ABC transporters of *E. coli*. In the cyanobacterium *Anabaena* PCC 7120, SchE, a putative cytoplasmic membrane protein, has been implicated in HgdD, a TolC homolog, mediated secretion of schizokinen, a siderophore synthesized by *Anabaena* (Nicolaisen et al. 2010).

## 2.12 Examples of Newly Discovered Siderophores

To the huge variety of known siderophores (Carvalho et al. 2011; Hider and Kong 2010), each year new siderophores are added. A few examples of these new siderophores will be discussed. Streptomycetes frequently produce desferrioxamines but some synthesize in addition catechol-type siderophores. The first discovered example is griseobactin, of which the genes for biosynthesis, secretion, uptake, and degradation have been identified. Sequencing and mutant analysis defined the biosynthesis pathway. The antibiotic was isolated and its siderophore function characterized. It consists of a cyclic and, to a lesser extent, linear trimeric ester of 2,3-dihydroxybenzoyl-arginyl-threonine (Patzer and Braun, 2010). The versatile capacity of *Streptomycetes* to synthesize a large variety of compounds is supported by the finding that the Gram-positive *Streptomyces scabies* 87–22 synthesizes an active *pyochelin* that has hitherto only been found in Gram-negative *Pseudomonas* strains (Seipke et al. 2011).

A unique mixed-type catecholate-hydroxamate siderophore has been isolated from *Rhodococcus jostii* RHA1. It contains an unusual ester bond between an L-$\delta$-N-formyl-$\delta$-N-hydroxyornithine moiety and the side chain of a threonine residue (Bosello et al. 2011).

Siderophore synthesis genes of the nonribosomal peptide type (NRPS) are found in 27 % of the marine prokaryotic genomes and of the NRPS-independent pathway in 10 % of the genomes (Hopkinson and Barbeau 2011). However, assignment to siderophore biosynthesis is not unequivocal, since other secondary metabolites are synthesized by these pathways.

Marine siderophores have the specific properties of being amphiphilic. For example, the acetyl group at the lysine residue in aerobactin found in many pathogenic Gammaproteobacteria is in a marine *Vibrio* species replaced by fatty acids that range in size from C8 to C12 (Gauglitz et al. 2012). This siderophore belongs to the class of citrate-based amphiphilic siderophores. Marine bacteria form a second class of amphiphilic siderophores which are peptide-based (Vraspir and Butler 2009). The amphiphilic siderophores are associated with the bacterial membranes which may prevent loss of siderophores by diffusion into the ocean.

## 2.13 Therapeutic Use of Siderophores

Currently, there are three siderophores used for therapeutic purposes (Chu and Vogel 2011). For more than 40 years, desferrioxamine B (DFO, Desferal®) (produced by *Streptomyces pilosus*) has been administered to reduce iron overload in patients who suffer from severe anemia caused by the hereditary disease ß-thalassemia. DFO binds $Fe^{3+}$ tightly in serum and is secreted into bile ($\sim$70 %) and urine ($\sim$30 %). However, the preferred treatment using DFO, suffers from poor absorption if taken orally, so it must be injected intravenously or infused subcutaneously for many hours. New chemotherapeutics such as deferiprone (tradename Ferriprox) and deferasirox (trade name Exjade) are active when taken orally. Deferiprone requires three daily doses, whereas deferasirox requires only one. Both compounds still require long-term inspection to monitor side effects.

Another therapeutic approach is the use of active siderophore transport systems to bring antibiotics into cells into which they are poorly taken up (Trojan Horse concept). Most antibiotics enter cells by diffusion through the cell envelope for which their structure may not be well suited. In particular, the outer membrane of Gram-negative bacteria forms a permeability barrier that prevents the generation of a sufficiently high intracellular concentration of antibiotics to kill cells. Attempts to transport antibiotics chemically coupled to siderophores carriers into bacteria were experimentally successful, but did not sufficiently meet the therapeutic requirements. One well-studied example is albomycin, which is composed of a ferric hydroxamate carrier to which the antibiotic thioribosyl pyrimidine is attached. Albomycin is synthesized by the *Streptomyces* species and is taken up by an energy-coupled transport across the outer membrane and the cytoplasmic membrane of bacteria that contain a transport system for ferrichrome. This applies to most Enterobacteriaceae, *Staphylococcus aureus,* and *Streptococcus pneumoniae* (Pramanik and Braun 2006; Pramanik et al.,2007). Inside cells, the antibiotic must be released from the siderophore carrier by peptidases in order to be active (Braun

et al. 1983). In a screening for antibiotics that interfere with RNA metabolism, the antibiotic moiety of albomycin was isolated and shown to inhibit in vitro t-RNA synthetases at a minimal inhibitory concentration ($MIC_{50}$) of 8 ng/ml (Stefanska et al. 2000). This is also the $MIC_{50}$ of albomycin against cells, in contrast to the 256 mg/ml required of the antibiotic uncoupled from the siderophores carrier. In contrast to diffusion of the antibiotic, energy-coupled transport of the siderophores-antibiotic conjugate reduces the $MIC_{50}$ 32,000-fold.

Another antibiotic, CGP 4832, a semisynthetic rifamycin derivative, is >200-fold more active than rifamycin against *E. coli* and *Salmonella,* since it is taken up across the outer membrane by the energy-coupled ferrichrome transport system (Pugsley et al. 1987). Although it differs structurally from ferrichrome and albomycin (Ferguson et al. 2001), it binds at the same site to the outer membrane FhuA transporter (Ferguson et al. 2000). The ferrichrome transport system across the cytoplasmic membrane does not accept CGP 4832. It is sufficient to overcome the permeability barrier of the outer membrane to strongly reduce the $MIC_{50}$.

Antibiotics with catechol siderophores occur naturally, e.g. certain microcins, and have been chemically synthesized. They exhibit a 100–10,000-fold higher activity than the antibiotics (compiled in Braun 1999; Braun et al. 2009; Ji et al. 2012). To render *E. coli* more susceptible to the aminocoumarin antibiotic clorobiocin, a gyrase inhibitor, a 3,4-dihydroxybenzoyl moiety was added by modifying the biosynthetic gene cluster. The resulting adduct was taken up via catechol siderophore transporters (Alt et al. 2011).

## 2.14 Host Defense Mechanisms, Nutritional Immunity

Bacterial hosts bind iron to proteins for several reasons. Iron binding proteins serve to establish iron homeostasis, solubilize $Fe^{3+}$, serve as iron carriers, prevent concentrations of soluble iron that are too high and which would elicit the formation of oxygen radicals that damage DNA, proteins, and membranes, and withdraw iron from bacteria to control their multiplication. The proteins that serve these purposes are transferrin, lactoferrin, ferritin, hemoglobin, haptoglobin, hemopexin, and siderocalin (NGAL lipocalin). Iron withdrawal by these proteins, also called nutritional immunity, is very effective for bacteria that are unable to mobilize the iron sources deposited in these proteins. However, as described in previous sections, commensal and pathogenic bacteria have developed systems with which they can cope with iron limitation. They withdraw the extremely tightly bound iron from transferrin and lactoferrin ($K_D = 10^{-23}$ $M^{-1}$), and they mobilize heme from hemoglobin, hemopexin, and the hemoglobin-haptoglobin complex. Although bacteria use the protein-bound iron sources, the constant fight for iron limits their proliferation. For example, only 30–40 % of the transferrin molecules are loaded with iron, leaving a substantial capacity to withdraw various forms of iron from bacteria. Lactoferrin is released from neutrophil granules at sites of inflammation, inhibiting the growth of infecting pathogens by directly

sequestering iron. It also prevents *P. aeruginosa* from forming biofilms by stimulating surface mobility and renders them more susceptible to endogenous and exogeneous antimicrobials in the planktonic state (Singh et al. 2002).

Another protein that is secreted by host cells at sites of infection is siderocalin, which contains bound enterobactin when it is expressed in *E. coli.* This accidental observation resulted in intense investigation of the occurrence and the binding properties of siderocalins (reviewed by Correnti and Strong, 2012). Siderocalins often bind siderophores with subnanomolar affinities. Scn (also known as NGAL) binds enterobactin-like siderophores and carboxymycobactin. Scn knock-out mice are more susceptible to infections with bacteria that use these siderophores for iron acquisition. Interestingly, diglucosylated enterobactin known as salmochelin, that has been first found in *Salmonella enterica* (Bister et al. 2004) is not bound by siderocalin (Fischbach et al. 2006; Floh et al. 2004; Valdebenito et al. 2007). *S. enterica* and other bacteria avoid growth inhibition by iron deficiency exerted by siderocalin in that they form the glucosylated salmochelin.

The binding of vibriobactin, a siderophore of the catechol type, to the ferric vibriobactin periplasmic binding protein of *Vibrio cholerae* differs from the binding of enterobactin to transport proteins. The three catechol moieties donate five, rather than six, oxygen atoms as iron ligands. The sixth iron ligand is provided by a nitrogen atom from the second oxazoline ring. This particular iron coordination may explain the failure of vibriobactin to bind to siderocalin, thus evading the siderocalin-mediated innate immune response (Li et al. 2012).

Bacterial infections elicit an increased biosynthesis of the hepcidin peptide hormone in the liver that is released into the serum. It inhibits iron uptake by the gut and prevents the release of iron from iron stores in the liver and in macrophages into the circulatory system. The resulting iron deprivation reduces bacterial multiplication to a level that the innate and acquired immune system can eradicate the infection (Ganz 2006).

## 2.15 Regulation of Bacterial Iron Homeostasis

### *2.15.1 The Fur Protein Family*

The first function observed for the Fur protein in *E. coli* was **f**erric iron **u**ptake **r**egulation. Fur, loaded with $Fe^{2+}$, repressed transcription of iron uptake genes. This general regulatory activity of Fur is crucial for many bacteria to achieve iron homeostasis in the cell. Fur regulates a variety of cellular functions which are directly or indirectly related to iron metabolism. For example, in *Anabaena* sp PCC 7120, Fur is essential as no *fur* mutants were isolated. Proteomic analysis revealed Fur targets that belonged to photosynthesis, energy metabolism, redox regulation, oxidative stress, and signal transduction, and thus link these processes to iron metabolism (Gonzales et al. 2011). Proteins of the Fur family are also involved in

regulating the uptake of other divalent cations like zinc (Zur) (Patzer and Hantke 1998), manganese (Mur) (Diaz-Mireles et al. 2004; An et al. 2009), and nickel (Nur) (An et al. 2009). PerR of *Bacillus subtilis* is also a member of the Fur family. Its major task is the response to oxidative stress (Faulkner et al. 2012). A special heme-dependent variant of Fur is the Irr protein, which regulates via heme iron homeostasis in *Bradyrhizobium* (Small et al. 2009). The various Fur homologs make it difficult to ascribe a function that predicts Fur proteins in a newly sequenced organism without further physiological studies. Moreover, as observed for many general regulators, Fur proteins also have the opposite functions as they not only repress gene transcription, but directly or indirectly activate genes.

The Fur and Fur-like proteins have an N-terminal winged helix-turn-helix DNA binding domain and a C-terminal domain responsible for dimerization and binding of divalent metal ions. The crystal structure of the Fur protein from Pseudomonas was the first to be published (Pohl et al. 2003). In the meantime, the structures of the *Vibrio cholerae* Fur, *Mycobacterium tuberculosis* Zur, the *Bacillus subtilis* PerR, and the *Streptomyces coelicolor* Nur reveal a similar overall structure of this protein family. The iron binding site has been a matter of debate as discussed in the recent paper on the *Helicobacter pylori* Fur (Dian et al. 2011) and in a recent review on iron-containing transcription factors (Fleischhacker and Kiley 2011). It seems that the metal binding site 2, which connects the C-terminal dimerization domain with the N-terminal DNA binding domain is the iron binding domain (Dian et al. 2011)

### *2.15.2 General Regulators of Iron Metabolism Other Than Fur*

In most bacteria, the Fur protein has been described as a major regulator of iron metabolism. However, in certain bacterial groups, other regulators have taken over this essential function.

In the Rhizobiales and the Rhodobacterales, both belonging to the alphaproteobacteria, the RirA protein operates as the general iron regulator and Fur homologs like Irr, Mur, and Zur have more specialized regulatory functions (Johnston et al. 2007). The RirA protein is a member of the Rrf2 proteins which contain an Fe–S cluster. The Rrf2 family has very divergent functions in regulating the response to NO (e.g. NsrR in *Neisseria*), cysteine metabolism (e.g. CymR in *Bacillus*), and Fe–S cluster biogenesis (e.g. IscR in *E. coli*).

RirA does not dominate iron regulation in all alphaproteobacteria. For example, in *Caulobacter*, another member of the alphaproteobacteria, there is evidence for a Fur protein as general iron regulator (da Silva Neto et al. 2009).

Firmicutes, the low GC Gram-positive bacteria (e.g. *Bacillus* species, *Staphylococcus* species, and others) use Fur as a general iron regulator (Ollinger et al. 2006), whereas in the high GC Gram-positive bacteria, Corynebacteria, Mycobacteria, and Streptomycetes, iron metabolism is regulated by DtxR (**d**iphtheria

**t**oxin **r**egulator) (Wennerhold and Bott 2006; Günter-Seeboth and Schupp 1995). DtxR has an overall architecture similar to Fur, although its sequence is not homologous to Fur.

Like Fur, DtxR consists of an N-terminal DNA binding site with a helix-turn-helix motif and a C-terminal dimerization and metal binding domain. In other bacteria, MntR, a DtxR homolog, regulates manganese uptake (Patzer and Hantke 2001).

In addition to DtxR, four Fur homologs are found in *Streptomyces coelicolor*. One of them functions as a Nur nickle regulator (Ahn et al. 2006), and another one as a Zur zinc regulator (Shin et al. 2007).

In the phylum Spirochaetes, some species like *Treponema pallidum* and the *Borrelia* group do not encode Fur homologs. The TroR protein, a member of the DtxR family, is suggested to regulate iron and manganese levels in *T. pallidum* (Brett et al. 2008). However, another member of this phylum, *Leptospira denticola*, has four Fur homologs, but it is not clear if one or some of them are regulating iron transport (Louvel et al. 2006). A more recent publication suggests a role in oxidative stress response for one of these genes (Lo et al. 2010).

A DtxR homolog has also been found in *Deinococcus radiodurans* (Deinococcus thermus group) to inhibit manganese uptake and stimulate iron uptake.

In the cyanobacterium *Anabaena*, FurA regulates iron homeostasis (Gonzalez et al. 2011) and in cyanobacterial genomes, up to four members of the Fur protein family are observed. The toxin microcystin is regulated among others by Fur in the freshwater cyanobacterium *Microcystis aeruginosa*.

A *fur* homolog was seen in the genome of *Thermococcus kodakaraensis*; however, the gene seemed not to be expressed due to a frameshift mutation. A DtxR homolog may regulate in this organism iron supply (Louvel et al. 2009).

*Thermotoga maritima* and *Chloroflexus aurantiaca* contain Fur homologs; however, nothing is known about their function.

Very little is known about the regulation of iron homeostasis in archaea except *Halobacterium salinarum* in which two DtxR-like proteins Idr1 and Idr2 take care of iron homeostasis. Gene expression profiles and DNA binding of the regulators were analyzed under iron-rich and iron-limiting conditions. These data were used to define the regulons of Idr1 and Idr2. Both regulators bind mainly in the presence of iron to DNA and many of the affected genes are involved in iron metabolism. A certain subset of genes was regulated in concert by Idr1 and Idr2 (Schmid et al. 2011).

## *2.15.3 Indirect Regulation by Fur*

### 2.15.3.1 Small RNAs

A major principle of indirect regulation by Fur is exerted by small RNAs. In *E. coli*, the small RNA (sRNA) RyhB is 90 nucleotides long and its synthesis is repressed by $Fe^{2+}$Fur. In iron deficiency, the production of this RNA is derepressed and about 20 genes are downregulated. With Hfq as a cofactor for the pairing of

sRNA and mRNA, the degradation of RhyB and the bound mRNA is triggered. The products of the regulated genes often need high amounts of iron for maturation as e.g. succinate dehydrogenase. Their downregulation helps the cell to spare iron. However, it has also been observed that a few genes are upregulated (Prevost et al. 2007). For example, *shiA* encoding a shikimate transporter is upregulated by RhyB. The induction of *shiA* may help the cells to obtain more of the precursor for the synthesis of enterobactin, the siderophore which allows a better iron supply. In addition, RyhB is necessary for good expression of the enterobactin biosynthesis gene cluster *entCEBAH* (Salvail et al. 2010). The amount of active Hfq oligomers is regulated by the stringent response regulator RelA. The production of ppGpp by RelA seems not to be involved in the regulation of Hfq activity (Argaman et al. 2012). However, ppGpp production by SpoT is increased in iron deficiency and this high ppGpp level stimulates the expression of iron uptake genes in an unknown way (Vinella et al. 2005). RhyB is also involved in the regulation of the virulence factor IscP in *Shigella* (Africa et al. 2011). IscP is an outer membrane protease which degrades IscA, the outer membrane protein responsible for actin polymerization on the surface of the bacterium. This polymerization results in an actin tail which moves the bacterium within cells and into neighboring cells. This regulation allows the bacterium to lower the synthesis of IscP inside colonic cells where the available iron is low and actin-based movement aids infection.

#### 2.15.3.2 ECF Sigma Factors

Bacteria are able to respond specifically to the presence of certain siderophores in their environment by the induction of specific transport systems. Recognition of these siderophores is often dependent on an extracytoplasmic function (ECF) sigma factor. In the Fec system of *E. coli*, the sigma factor is called FecI and the cognate signaling protein FecR (Braun and Mahren 2005). The expression of FecI and FecR is regulated by ferric citrate iron and Fur. The outer membrane transporter FecA has to bind the substrate $Fe^{3+}$-dicitrate and in a TonB-dependent manner, this binding is recognized by a structural change of FecR, which interacts with an N-terminal domain of the transporter. The structural change of FecR, which is anchored by one transmembrane helix in the cytoplasmic membrane, leads to proteolytic degradation in the cytoplasmic membrane by the site-2 protease RseP. The resulting cytoplasmic FecR fragment binds to FecI which is activated. Active FecI recruits the RNA-polymerase to the promoter of the *fec* operon. In *Pseudomonas*, several similar systems are found which have been analyzed carefully (Draper et al. 2011). The activation of the ECF sigma factor HurI in *Bordetella bronchiseptica* is also dependent on the predicted site-2 protease HurP—this system regulates the uptake of heme. An N-terminal elongation of the receptor protein by about 80 residues and the presence of a FecIR type pair is a good indication of ECF sigma factor regulation. This type of regulation is found in many Gram-negative bacteria.

Regarding the Fec system, an interesting second level of regulation has recently linked iron uptake to carbon metabolism. Bioinformatic methods were used to explore more than 400 microarray data sets of *E. coli* to find new regulatory interactions (Faith et al. 2007). PdhR, a pyruvate sensing repressor, was shown to regulate FecA expression. Both the activated FecI and the activated PdhR are necessary for maximal expression of the *fec* operon.

#### 2.15.3.3 AraC- and LysR-Type Regulators

The first $Fe^{2+}$Fur regulated AraC-like regulators were identified in pyochelin, yersiniabactin, and alcaligin biosynthesis and transport. In all of these cases, the corresponding siderophores act as coinducers in the system as it is known for many AraC-like regulators. With a catechol as a coinducer, DhbR acts as an activator in catechol biosynthesis of *Brucella abortus* (Anderson et al. 2008). The uptake of the xenosiderophore ferrioxamine B is induced in *Vibrio furnissii* via the AraC-like regulator DesR (Tanabe et al. 2011).

An AraC-like regulator, MpeR, was found in pathogenic *Neisseria gonorrhoeae* to stimulate expression of the FetA enterobactin outer membrane transporter (Hollander et al. 2011) under iron-limiting conditions. Interestingly, MpeR had been found previously to act as a repressor of the Mtr transport system under iron-limiting conditions. The Mtr transport system exports structurally divergent hydrophobic antibiotics and detergents. It was not possible to identify a coinducer like those found with most other AraC-like regulators.

LysR-type regulators belong to the most common prokaryotic regulators which control a functionally very diverse set of genes. The OxyR proteins regulating the response to oxidative stress belong to the LysR protein family. They act in line with their oxidative stress response and also on iron metabolism, since $Fe^{2+}$ reacting with oxygen is a major source of toxic radicals (Faulkner and Helmann 2011). In addition to the induction of repair enzymes, the distribution of metal ions in the cell is changed through OxyR. A major aim for the cells is to lower the ferrous iron concentration in the cytosol. Therefore, it is not surprising that there are also LysR-type regulators which control specific iron uptake genes. An example of this is the LysR-type activator HmuB from *Vibrio mimicus*, which stimulates the expression of heme uptake genes (Tanabe et al. 2010).

## 2.16 Concluding Remarks

Iron is an essential element, since it is contained in many redox enzymes. Although available in large quantities on earth, its insolubility in the $Fe^{3+}$ state makes it difficult for all aerobic organisms to acquire enough iron for their cell metabolism. To cope with this problem, all organisms invented intricate iron transport systems. Since microbes live under very diverse environmental

conditions, they had to develop a large variety of iron uptake mechanisms to get enough iron into the cells. Competition for iron is a means of eukaryotic cells to keep microbial invaders under control. Microbes on the other hand developed systems to overcome iron limitation imposed by eukaryotes. Moreover, they had to find ways to control the intracellular iron concentrations to avoid formation of damaging oxygen radicals by a surplus of iron. The control mechanisms go beyond maintenance of iron homeostasis and affect pathways that are directly or indirectly related to iron metabolism. The iron regulatory network is one of the central control mechanisms of cell metabolism.

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