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IRON UPTAKE BY *ESCHERICHIA COLI*

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1. ABSTRACT

Ferric iron is transported into *Escherichia coli* by a number of chelating compounds. Iron transport through the outer membrane by citrate, ferrichrome, enterobactin, aerobactin, yersiniabactin, and heme is catalyzed by highly specific proteins and across the cytoplasmic membrane by ABC transport systems with lower specificity. Transport across the outer membrane requires energy, which is provided by the proton motive force of the cytoplasmic membrane and transmitted to the outer membrane via the TonB-ExbB-ExbD proteins. Binding of substrates induces large long-range structural changes in the transport proteins, but does not open the channel. It is thought that the channel is opened by energy input from the cytoplasmic membrane. Although a basic understanding of how the transport proteins might function has been obtained from the crystal structures of three outer membrane proteins of *E. coli* and from many genetic and biochemical experiments, numerous fundamental questions still remain open. Transcription of the transport protein genes is regulated by the Fur protein, which when loaded with ferrous iron functions as a repressor. Fur also positively regulates genes of iron-containing proteins by repressing synthesis of an anti-sense RNA. Regulation of ferric citrate transport genes via a transmembrane device has become the paradigm of the regulation of a variety of systems, including the hypersensitivity response of plants to bacterial infections.

2. INTRODUCTION

Bacteria use a variety of iron sources. The insoluble Fe^{3+}, which at the physiological pH of 7 forms a hydroxy-aquo polymer with a free Fe^{3+} concentration of approximately 10^{-9} M (1), requires solubilizing components for transport: siderophores of bacteria and fungi, and proteins of higher organisms. Siderophores are low-molecular-weight compounds that bind Fe^{3+} very tightly with high specificity (2). Siderophores are synthesized by microorganisms and secreted into the surroundings, where they complex Fe^{3+}. Bacteria use their own siderophores and also those synthesized by other bacterial species or even fungi. The Fe^{3+}-siderophores are actively transported into the cells, where iron is released, usually by reduction to Fe^{2+}. The siderophores are degraded, modified, or secreted for the next cycle of Fe^{3+} transport. Many bacteria can use heme, heme bound to hemoglobin, hemoglobin-haptoglobin, myoglobin, and hemopexin of their hosts (3). Certain bacteria use iron bound to transferrin and lactoferrin (4). Iron and heme are mobilized from the host proteins at the bacterial cell surface. *Serratia marcescens* and *Pseudomonas aeruginosa* secrete proteins, called hemophores that release heme from hemoglobin and deliver the heme to transport proteins in the outer membrane (3).

In gram-negative bacteria, Fe^{3+}, Fe^{3+}-siderophores, and heme are actively transported across the outer membrane (Figure 1). In gram-positive bacteria, which lack an outer membrane, the iron sources are transported across the cytoplasmic membrane by the same mechanism as in gram-negative bacteria. Transport is energized by ATP hydrolysis (5, 6) (see section 4). Transport across the outer membrane is energized by the proton motive force of the cytoplasmic membrane (7, 8). Three proteins — TonB, ExbB, and ExbD (designated as the Ton system) — couple the membrane potential of the

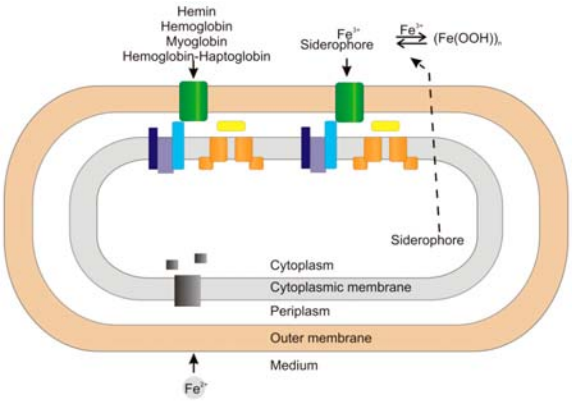
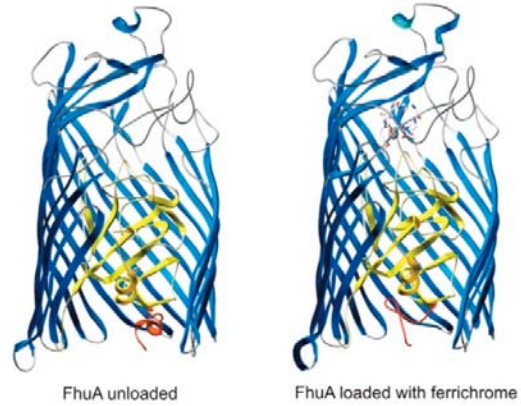


Figure 1. Subcellular location of Fe3+ transport proteins encoded by the genes shown in Figure 3. The location of the TonB-ExbB-ExbD proteins which couple the proton motive force of the cytoplasmic membrane to active transport across the outer membrane is also shown



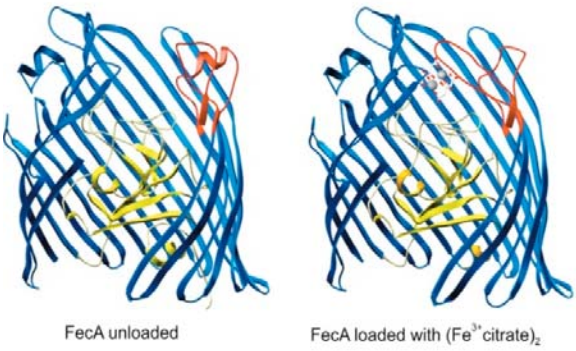


Figure 2. Comparison of the crystal structures of the substrate-loaded and unloaded FhuA and FecA proteins. Note the strong structural changes upon binding of the substrate of the periplasmic switch helices in both proteins and the strong translation of loops 7 and 8 in FecA, which has not been observed in FhuA

cytoplasmic membrane to the outer membrane transport proteins. A 1:7:2 molar ratio has been determined for TonB, ExbB, and ExbD proteins (9), and they form a complex of unknown size (10, 11, 12). It is thought that they respond to the proton gradient across the cytoplasmic membrane. Energy stored as conformational energy in TonB might be transmitted to the outer membrane transport proteins. Interaction of TonB with outer membrane transport proteins has been demonstrated both genetically (13, 14) and biochemically (15-18). Binding of TonB is enhanced when FhuA is loaded with ferrichrome (15).

3. COMMON FEATURES OF THE OUTER MEMBRANE FERRIC-SIDEROPHORE TRANSPORT PROTEINS

The crystal structures of three outer membrane proteins — FecA, FepA, and FhuA — that transport ferric siderophores have been determined. The proteins are composed of 22 antiparallel beta-strands that form a beta-barrel (Figure 2). The N-proximal portions have entirely different structures. They form globular domains that tightly fit into the beta-barrels and completely close the channels formed by the beta-barrels, This domain is therefore called the cork or plug. For the transport of ferric-siderophores, the cork has to move relative to the beta-barrel to open a channel through which the ferric-siderophores move into the periplasm, the compartment between the outer membrane and the cytoplasmic membrane. Although binding of ferric-siderophores to the outer membrane transport proteins triggers long-range structural transitions in the transport proteins that extend across the entire outer membrane, the beta-barrel channels are not opened. It is thought that interaction with energized TonB opens the channel. The structural change in the transport proteins releases the ferric-siderophores from their binding sites. Interaction of TonB with the transport proteins also triggers long-range structural transitions in the corks and the beta-barrels, as will be discussed below.

3.1. The FecA protein transports ferric citrate and regulates gene transcription

The FecA protein exerts two independent activities. It functions as the transporter for dinuclear ferric citrate [$(Fe^{3+}citrate)_2$] across the outer membrane, and it serves as the sensor and signal transmitter for the initiation of transcription of the ferric citrate transport genes (19). Ferric citrate binds to FecA (20), which is essential for growth of *E. coli* on low concentrations of ferric citrate as sole iron source. At higher concentrations, ferric citrate (mol. wt. 488) diffuses fast enough through the porins to support growth (21). The entire ferric citrate transport system is composed of five proteins: the FecA outer membrane transport protein, the FecB periplasmic binding protein, the FecC-FecD cytoplasmic membrane transport proteins, and the FecE ATPase associated with the inner side of the cytoplasmic membrane (22-24). Transcription of the *fecABCDE* genes (Figure 3) is controlled by the promoter upstream of *fecA*. It is assumed that upon binding of ferric citrate to FecA, a conformational change occurs in FecA that is transmitted to the FecR protein, a transmembrane protein (25) that interacts in the periplasm with the N-proximal end of FecA (26, 27). FecR transmits the signal received from FecA across the cytoplasmic membrane into the periplasm, where it converts the FecI protein into an active sigma factor (28-34). FecI recruits the

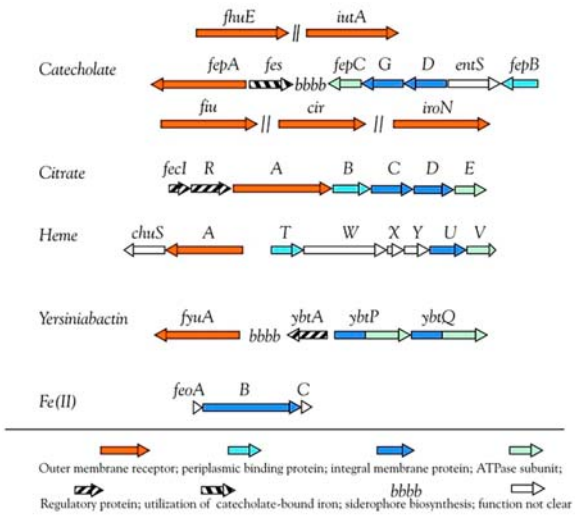


Figure 3. Genes for transport of Fe^{3+} complexed by hydroxamate and catecholate siderophores, citrate, heme, and yersiniabactin into E. coli. Genes encoding additional outer membrane proteins that transport ferric-hydroxamates (FhuE and IutA) and ferric-catecholates (Fiu, Cir, and IroN) are listed because the substrates are transported across the cytoplasmic membrane by the FhuBCD and FepBCDG proteins, respectively. IroN was originally found in S. typhimurium (110) and later in E. coli (111). entS encodes a function for the secretion of enterobactin (106). bbbb indicates the number, 4, of enterobactin biosynthesis genes located within the transport gene cluster

RNA polymerase, and the complex binds to the promoter upstream of *fecA* and initiates transcription of *fecA* and the adjacent *fecBCDE* transport genes. FecA is larger than ferric siderophore transport proteins of *E. coli*. FecA contains an N-terminal extension that does not occur in outer membrane proteins that only transport ferric siderophores and play no role in transcription regulation. Removal of the N-proximal end of FecA fully retains the transport activity, but entirely disrupts the inducing activity (26).

The concept of how FecA functions, as derived from genetic and biochemical studies, is fully supported by the recently determined crystal structures of FecA with and without bound $(Fe^{3+}citrate)_2$ (Figure 2) (35). Fe^{3+} and citrate can form a variety of different chemical compounds in solution (36-38). Which ferric citrate compound functions as ferric siderophore was elucidated from the crystal structure. Upon binding of $(Fe^{3+}citrate)_2$, large structural changes occur in the cork and the beta-barrel domain of FecA. Various portions of the cork move 0.5 to 2 Angstroms toward $(Fe^{3+}citrate)_2$ and the periplasm. Most notably, loop 7 of the beta-barrel is translated up to 11 Angstroms and loop 8 is translated up to 15 Angstroms, with the result that bound $(Fe^{3+}citrate)_2$ is no longer accessible to solvent. The channel from the surface to the $(Fe^{3+}citrate)_2$ binding site located deep inside FecA is closed, and $(Fe^{3+}citrate)_2$ can no longer escape into the medium. For the next step of $(Fe^{3+}citrate)_2$ uptake, it is assumed that energized TonB causes a structural transition in the entire FecA molecule so that $(Fe^{3+}citrate)_2$ is released from its binding site, the channel of the beta-barrel is opened, and $(Fe^{3+}citrate)_2$ diffuses into the periplasm, where it binds to the FecB protein. The envisaged transport mechanism of $(Fe^{3+}citrate)_2$ through FecA involves a bipartite gating. Interaction of FecA with TonB might be facilitated by the unwinding of a short helix, the switch helix, which is exposed to the periplasm. Unwinding is caused by binding of $(Fe^{3+}citrate)_2$ to FecA. Residues T138, Q176, Q178, and S180 of the cork and R365, L369, R380, R438, Q570, D573, and N721 of the beta-barrel serve as binding site for the negatively charged $(Fe^{3+}citrate)_2$; the OH groups of the citrate molecules are dissociated, resulting in a double negatively charged $(Fe^{3+}citrate)_2$ molecule.

The site of interaction of TonB with the cork of FecA resides in residues 80–85 (DALTV, TonB box); the point mutations DAPTV, DALTG, DALTR, DANTV, and GTNTV abolish FecA transport and induction activities (18). Interaction of the DALTV box with TonB was demonstrated by spontaneous in vivo disulfide formation between cysteine residues introduced into the DALTV box and cysteine residues introduced into region 160 of TonB (18). This result shows that the TonB box of FecA interacts with the region around residue 160 of TonB, as has been found for FhuA, Cir, and BtuB (13, 14, 17, 39-41). In the latter proteins, the TonB box is close to the N-terminus; this is consistent with the lack of transcription regulation of these proteins. In the crystal structures of FecA, FhuA and FepA the N-proximal sequences are not seen, indicating that they are flexible.

Regulatory devices of the FecIRA type are observed in many other organisms (42) such as in *Shigella flexneri* (encoded on a pathogenicity island) (42), *Pseudomonas putida* (43), *Pseudomonas aeruginosa* (44), *Bordetella pertussis* (45), *Bordetella bronchiseptica* (46), and *Bordetella avium* (47). The genome of *Caulobacter crescentus* also reveals homologs of FecIR (48).

A particularly interesting case in *Ralstonia solanacearum* has been described (49-51). *R. solanacearum* is pathogenic for host plants and elicits a hypersensitive reaction on nonhost plants. Induction of the hypersensitivity reaction requires direct contact of the bacteria to plant cells, from which is inferred that no diffusible substance is involved. A FecAIR-type mechanism is particularly suited for elicitation of such a reaction since a bacterial outer membrane receptor protein has to contact only a receptor on the plant cell to initiate a signaling cascade that finally induces transcription of the relevant bacterial genes. Indeed, transcription induction of hypersensitivity genes involves the outer membrane protein PrhA and two regulatory proteins homologous to FecI and FecR, named PrhI and PrhR. Mutations in the *prhA*, *prhI*, and *prhR* genes display a low and delayed pathogenic reaction on *Arabidopsis* and tomato plants. Like the *fecIR* genes, the *prhIR* genes are not autoregulated (50). Expression of *prhIR* is independent of *prhAIR*; *fecIR* expression is independent of *fecAIR*. In contrast to *fecIR* transcription, *phrIR* transcription is not regulated by iron,

but requires co-cultivation with plant cells. The signal that induces *phrIR* transcription must differ from the signal that induces hypersensitivity gene transcription. A scheme has been proposed in which PrhI acts as a sigma factor that initiates transcription of the *prhJ* gene; PrhJ in turn activates transcription of the *hrpG* gene, and HrpG activates transcription of the *hrpB* gene (50). *hrpB* affects expression of the *hrp*, *hrc,* and *pop* genes. In this model, the *prhAIR* genes stand at the beginning of a regulatory hierarchy that controls synthesis of regulatory proteins, a type III secretion system, and several effector proteins.

FecI and the FecI-like proteins together form a subgroup of sigma factors that have been designated as extracytoplasmic sigma factors since they receive their signals from the cells' surroundings (52).

3.2. FhuA is a multifunctional protein that transports ferrichrome

The FhuA protein transports ferrichrome, the structurally related antibiotic albomycin, and the structurally unrelated antibiotic rifamycin CGP4832 (a synthetic derivative of rifamycin), and serves as receptor for the phages T1, T5, phi80, and UC-1, and for colicin M (a toxic protein) and microcin J25 (a toxic peptide). All functions require TonB activity except infection by phage T5. The multifunctional properties render FhuA particularly suitable for studies of structure-function relationships. Comparison of the crystal structures of FhuA loaded with ferrichrome and unloaded FhuA (Figure 2) reveals major long-range structural changes (53, 54). Upon binding of ferrichrome, the switch helix, equivalent to the switch helix of FecA (section 3.1), is unwound and residues E19, S20, and W22 are translated 17 Angstroms from their former alpha-carbon position. This movement exposes the TonB box of FhuA (residues 7–11 of the mature protein) to the periplasm, where most of TonB resides. The TonB box is not seen in the crystal structure and therefore is probably flexible. The I9P and V11D mutations inactivate FhuA, but do not affect the TonB-independent infection by phage T5 (14). The mutation Q160K in TonB partially restores transport of ferrichrome by FhuA(I9P). Addition of ferricrocin, a naturally occurring derivative of ferrichrome, enhances the yield of FhuA cross-linked with formaldehyde to TonB (15). The N-proximal region has to be flexible since its fixation by a disulfide bride formed between the genetically introduced cysteine residues at position S27 of the cork and S533 of the barrels inactivates FhuA transport activity but retains its receptor activity for the phages and colicin M. This result could mean that the cork must no leave the barrel for phage infection and colicin M uptake. However, it is not excluded that a small uncross-linked fraction of FhuA molecules remains which functions as receptors (55).

The strong translation of surface loops as observed in FecA upon binding of $(Fe^{3+}citrate)_2$ is not seen in FhuA when it is loaded with ferrichrome. Two different crystal forms do not reveal closure of the surface-exposed cavity. It is possible that crystal forces favor the open conformation. A fluorescence label linked to residue 336 of loop 4 reveals quenching upon binding of ferrichrome (56). Additional evidence for conformational changes in surface loops comes from binding of phages T1 and phi80 to loop 4 (57), which only occurs irreversibly, accompanied by DNA release from the phage heads, in energized $TonB^+$ cells (58). This finding implies that loop 4 assumes in response to the proton motive force of the cytoplasmic membrane, mediated by TonB to FhuA, a conformation that differs from the unenergized conformation to which the phages bind reversibly without triggering the DNA release. The activity of FhuA involves strong long-range structural changes that take place throughout the molecule from the periplasm, where TonB interacts with FhuA, to the most prominent loop 4 at the cell surface, and in the opposite direction from the ferrichrome binding site 20 Angstroms above the outer lipid boundary of the outer membrane to the periplasm, where the switch helix unwinds and interaction with TonB may be facilitated.

3.2.1. The beta-barrel of FhuA forms an open channel but displays no FhuA-specific activity

Removal of the cork should convert FhuA into an open channel through which substrates flow into the periplasm. This prediction was examined by excision of residues 5 to 160 (59). Purified FhuA delta5-160 incorporated into artificial lipid bilayer membranes increases the conductance, as determined by recording the current with 1 M KCl, but it rarely forms stable channels that display clearly discernable single-channel conductance. Wild-type FhuA does not enhance conductance through lipid bilayers (61). This *in vitro* finding agrees with the *in vivo* increase of the permeability of the outer membrane. Ferrichrome stimulates growth of a TonB mutant on an iron-restricted medium and a mutant that lacks the LamB maltoporin grows on maltotriose, maltotetraose, and maltopentaose, which indicates diffusion through FhuA delta 5-160. The FhuA deletion derivative increases sensitivity to antibiotics larger than 600 Da which slowly diffuse through the outer membrane (bacitracin, erythromycin, rifamycin).

Previous claims (59-61) that FhuA delta 5-160 transports ferrichrome, albomycin, rifamycin CGP 4832, and serves as receptor for colicin M and the phages T1, T5 and phi80 resulted from complementation of the barrel by the cork of an inactive FhuA protein synthesized by the strain used in the FhuA delta5-160 activity studies (63). Reconstitution involves the highly unlikely incorporation of the cork of full-length but inactive FhuA which carries several mutations in the barrel into FhuA delta 5-160. For steric reasons it is unlikely that the cork is incorporated into FhuA delta 5-160 while it is still covalently bound to inactive FhuA. Rather, it may be proteolytically released from inactive FhuA prior to incorporation into FhuA delta 5-160. A second strain used which carries a *fhuA* deletion synthesizes an N-terminal FhuA fragment of 357 residues (and 27 residues unrelated to FhuA) which also complements FhuA delta 5-160 (62). Also this fragment may be proteolytically trimmed to a size similar to the cork (residues 1-160) before it is incorporated into FhuA delta 5-160. However, it is not excluded that the cork portion is incorporated into FhuA delta 5-160 and the rest of the 384 fragment hangs out of the barrel into the periplasm since

the linkage between the cork and the barrel is exposed to the periplasm.

Separately synthesized cork is also incorporated into separately synthesized barrel. To prevent separation of the two domains during SDS-PAGE they were cross-linked with formaldehyde or spontaneous in vivo disulfide formation between cysteine residues inserted at site 27 of the cork and site 533 of the barrel. For reconstitution to occur the cork and the barrel must be endowed with a signal sequence which indicates that reconstitution occurs in the periplasm or while the barrel is inserted into the outer membrane. The barrel is formed and inserted into the outer membrane without the help of the cork since the separately synthesized barrel enhances the permeability of the outer membrane. For the assembly of wild-type FhuA these results suggest that the barrel is formed prior to incorporation of the cork .

Among *E. coli*, *Salmonella paratyphi* B*, Salmonella enterica serovar Typhimurium and Pantoea agglomerans* the corks and barrels of the FhuA proteins can mutually be exchanged resulting in active hybrid proteins (61). For example, the cork of *E. coli* incorporated into the beta-barrel of *S. enterica* (FhuAEcCSeB) results in a reconstituted hybrid FhuA that exerts 84% of the ferrichrome transport activity of genuine *S. enterica* FhuA. The receptor specificity is determined by the barrel as hybrid proteins with the *E. coli* and *S. typhi* barrels display *E. coli* and *S. paratyphi* specific phage sensitivities. However, infection by phage ES18 requires the cork and the barrel of *S. enterica* since cells expressing the hybrid proteins are ES18 resistant and cells synthesizing FhuAEcBSeC are resistant to phage T1 and only slightly sensitive to phage phi80, indicating a contribution of the cork to receptor activity of the barrel. The corkless derivatives of *E. coli*, *S. paratyph*i and *S. enterica* show transport activities of 35, 14, and 21 % of the wild-type FhuA proteins which result from FhuA complementation by the *E. coli* strain in which the activities are measured. This *E. coli* test strain synthesizes full-length but inactive FhuA protein. The unlikely complementation occurs even in the heterologous constructions. The corks of FepA and FecA do not complement FhuA delta 5-160 (62).

In FhuA, TonB has to trigger not only release of ferrichrome from its binding site, but also movement of the cork so that a continuous channel is formed between the outer cavity and the periplasmic cavity for ferrichrome, the antibiotics albomycin and rifamycin CGP 4832, and microcin J25 to diffuse into the periplasm. If colicin M (mol. wt. 27 000) also crosses the outer membrane through the FhuA channel, it is likely that the entire cork has to be expelled into the periplasm. The same holds true if the DNA of phages T1, T5, phi80, and UC-12 passes through the FhuA beta-barrel channel. Since a few colicin M molecules suffice to kill a cell and a single phage DNA molecule is sufficient to infect a cell, inactivation of FhuA by expelling the cork would probably not disrupt the outer membrane since cells are still able to support multiplication of the phages. However, the cork is firmly incorporated in the beta-barrel through approximately 60 hydrogen and van der Waals contacts. A rather large energy barrier would have to be overcome to release the cork from the beta-barrel. This could occur stepwise: water molecules occupy the amino acid side chains and form hydrogen bonds, which replace the hydrogen bonds between the amino acids. Even if this is a rather slow process, one has to take into account the low transport rate of FhuA of less than 10 ferrichrome molecules (FhuA molecule)$^{-1}$ min^{-1}. This is more than sufficient to satisfy the iron requirement of a cell in the order of 10^5 iron ions generation^{-1} (30 min) through approximately 10^4 FhuA molecules under iron-limiting conditions. However, this calculation assumes that all FhuA molecules at a time participate in transport which in fact is not the case since the number of TonB molecules is far less (9) than the number of FhuA molecules (under iron-limiting conditions 1 000 / 10 000 molecules). In addition, the resolution of the crystal structure (2.5 Å) is not sufficient to reveal whether water molecules bridge hydrogen bonds and are intercalated between salt bridges. If this holds true, the energy barrier would be much lower. Unless release of the cork is disproved experimentally, this possibility has to be considered.

3.3. The FepA protein transports ferric-enterobactin

The FepA protein transports ferric-enterobactin across the outer membrane and serves as receptor for colicins B and D. The crystal structure has been determined, but it is not clear whether it is composed of a mixture of FepA loaded with ferric-enterobactin and the unloaded form (63, 64). Ferric-enterobactin is not seen in the crystal, and it seems that it disrupts the crystal of the unloaded form. Thus, the FepA crystal does not disclose structural changes upon binding of ferric-enterobactin. However, biochemical and biophysical data indicate structural transitions caused by binding of ferric-enterobactin to FepA.

A spin-label probe bound to an introduced cysteine residue at position 280 in mature FepA, which is located at the boundary of loop 3 to beta-strand 6, has been used as a tool to study structural changes (65). Binding of ferric-enterobactin decreases fluorescence with biphasic kinetics — an initial rapid step and a subsequent slower step. This finding is supported by site-directed mutagenesis of aromatic amino acids that suggests the presence of two ferric-enterobactin binding sites: an outermost site with aromatic amino acids to which the aromatic portion of enterobactin binds and a second site deeper in the outer cavity with charged and aromatic side chains to which the aromatic and the triple negatively charged ferric-enterobactin binds (66). Loop-deletion analysis has revealed the importance of surface loops in FepA transport activity. Deletions in nine loops decrease or eliminate ferric-enterobactin affinity and transport rate (67). Of particular interest are the deletions in loops 7 and 8 because they close the surface cavity of FecA upon binding of ferric-citrate (section 3.1). These loop deletions, and only these loop deletions, completely eliminate FepA transport activity. However, the deletions are not precisely confined to the loop regions, which raises some doubts about the conclusions. The deletion in loop 7 excises two residues of beta-strand 13 and the deletion in loop 8 removes nine

residues of beta-strand 16. All beta-strand deletions eliminate FepA activities (68). Electron spin resonance spectroscopy with a spin label bound to Cys280 discloses two distinct conformations, one related to binding of ferric-enterobactin and one subsequent to ferric-enterobactin binding (68). The observed structural changes are TonB-and energy-dependent. A TonB-independent conformational change upon binding of ferric-enterobactin is observed with a spin label linked to residue 338 of FepA (69), which the crystal structure localizes in loop 4. These data indicate multiple structural changes in FepA upon binding of ferric-enterobactin and structural changes that depend on energy coupling by TonB. They are probably representative of equivalent structural changes that occur in the other active outer membrane transport proteins.

FepA mediated ferric enterobactin transport can be monitored by measuring fluorescence changes of fluorescein maleimide linked to cysteine residues introduced at certain positions (C271, C397) in surface loops of the barrel which are selected according to the FepA crystal structure (70). Ferric enterobactin binding quenches fluorescence and the derived K_d is very low (0.2 nM) which agrees with the K_d obtained by radiolabeling. Ligand binding and transport are accompanied by conformational changes in the loops. The proton ionophore CCCP and the electron transport inhibitors cyanide and azide inhibit transport but unexpectedly also the phosphate analog arsenate reduces transport.

Hybrid proteins consisting of the cork of FhuA and the barrel of FepA, and of the cork of FepA and the barrel of FhuA have been constructed (71) They exhibit TonB-coupled barrel specific ferric enterobactin and ferrichrome transport activities, respectively, which are much lower than those of wild-type FepA [V_{max} 1.6 and 9.8 versus 411/ 87 (FepA / FhuA) pmol min^{-1} $(10^9$ cells$)^{-1}$; k_3 0.9 and 6.5 versus 6.4 / 4 molecules transported min^{-1}]. The capacity goes down from 64 / 23 to 1.6 pmol ferric enterobactin / ferrichrome bound $(10^9$ cells$)^{-1}$. The *fepA* and *fhuA* mutations in the test strain are not characterized by DNA sequencing which makes it possible that both hybrids are complemented by a chromosomally encoded homologous N-terminal fragment carrying no mutation. The low activity may arise from the covalently linked heterologous cork which inhibits incorporation of the homologous N-terminal fragment into the barrel. This conclusion is supported by the higher ferrichrome transport rate of the FhuA barrel (V_{max} 25) into which in the absence of the covalently linked FepA cork, the chromosomally encoded FhuA N-terminus could more easily incorporate. As outlined in the FhuA chapter, the transport and receptor activity of corkless FhuA comes from the incorporation of the chromosomally encoded cork derived from an inactive 714 residues FhuA or a 357 residues FhuA fragment. Homologous reconstitution of the heterologous FepA / FhuA hybrid proteins would be a highly interesting event and shed light on FepA / FhuA assembly. The failure to obtain active FepA mutants lacking the entire cork and cork fragments in another *fepA* mutant test strain than the one used in (71) led Vakaharia and Postle to suggest that interprotein complementation by two nonfunctional proteins restores TonB-dependent activity (72).

The FepA cork has been cloned separately and the structure of the isolated protein studied by NMR and circular dichroism (73). The isolated cork does not assume a structure similar to the cork incorporated in the beta-barrel; instead, it is predominantly unfolded. It exhibits a residual ferric-enterobactin binding (1% of that of wild-type FepA). These data suggest that if the cork moves out of the beta-barrel during transport, the globular conformation unfolds. This results in an increase in conformational entropy, which lowers the energy costs of exit from the beta-barrel, but costs enthalpy owing to the loss of internal interactions. Changes in bound water interactions will occur but difficult to assess.

3.4. The IutA protein transports ferric-aerobactin

Aerobactin is a siderophore synthesized by certain naturally isolated strains of *E. coli* (74). The outer membrane transport protein is encoded by the *iutA* gene (75), which is located downstream of the aerobactin biosynthesis genes *iucABCD* (76- 78), originally designated *aerDBCA* (78), and transcribed in this order. *iutA* (Figure 3) together with the *iucABCD* genes form an operon that is repressed by iron through the Fur repressor protein (79-81). The genes are encoded on plasmids, usually on ColV plasmids, or on the chromosome (74). Further transport of ferric-aerobactin across the cytoplasmic membrane is mediated by the FhuBCD transport system of ferrichrome and other ferric-hydroxamate siderophores (81). The K_m for binding of ferric-aerobactin to the periplasmic binding protein FhuD is the lowest of all ferric hydroxamates (82). Transport of ferric aerobactin depends on the Ton system. Iron supply via ferric-aerobactin contributes to the virulence of *E. coli* pathogens (74, 83).

3.5. The ChuA protein transports heme

Hemin, hemoglobin, myoglobin, and hemoglobin-haptoglobin are the most abundant iron sources in mammalian hosts and can be used by certain pathogenic strains of *E. coli*. The bacteremic *E. coli* 0157:H7 (84) and the uropathogenic *E. coli* CFT073 (85) transport heme via a heme transport system across the outer membrane and the cytoplasmic membrane (Figs. 1, 3). The heme transport system contributes to the virulence of *E. coli* CFT073 in mice. In a competition assay between a $ChuA^-$ mutant and its $ChuA^+$ parent, the mutant was less able to colonize bladders and kidneys of infected mice than the wild-type strain (85).

3.6. The FyuA protein transports ferric yersiniabactin

Yersiniabactin is a siderophore found in the spent medium of pathogenic *Yersinia* species, e.g., *Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica* (86). The transport system (Figure 3) is usually encoded on a high pathogenicity island that is also found in *E. coli* strains, most frequently in enteroaggregative *E. coli* and in *E. coli* blood culture isolates (86). FyuA serves as receptor also for the toxic pesticin of *Y. pestis* (87).

4. TRANSPORT ACROSS THE CYTOPLASMIC MEMBRANE BY ABC TRANSPORTERS

In the periplasm, the ferric-siderophores bind to binding proteins that deliver the ferric-siderophores to the transport proteins in the cytoplasmic membrane (Figure 1). The transport system in the cytoplasmic membrane does not transport the ferric-siderophores in the absence of the periplasmic binding proteins. This holds true also for gram-positive bacteria, in which the binding proteins are anchored to the cytoplasmic membrane by a lipid of the murein lipoprotein type (88). Transport of ferric-siderophores across the outer membrane is independent of transport across the cytoplasmic membrane. There is no evidence for an inteaction of outer membrane transport proteins and cytoplasmic membrane transport proteins. The binding proteins trap the ferric-siderophores in the periplasm, but they do not prevent their escape since, for example, a surplus of non-radioactive ferrichrome chases out radiolabeled [$^{55}Fe^{3+}$]ferrichrome from the periplasm but not from the cytoplasm. The genes involved in transport across the cytoplasmic membrane are listed in Figure 3. They encode a binding protein, one or two polypeptides which form the permease that translocates iron, ferric siderophores and heme across the cytoplasmic membrane and two copies of an ATPase which are associated with the inside of the cytoplasmic membrane. The ATPase contains the two Walker motifs of ATP binding which give the transport systems their name, ABC (ATP binding cassette) transporters. ATP binding and subsequent hydrolysis trigger the structural change. Work on iron ABC transporters of *E. coli* is summarized in (5, 89, 90).

A breakthrough in understanding of the structure of ABC transporters was achieved by the crystal structure determination of the vitamin B_{12} BtuCD transporter (91). This structure is particularly representative of the iron transport systems since vitamin B_{12} transport is in all respects very similar to iron transport. Although the crystal structure reveals only one state, presumably the ground state, in the sequence of structural changes that must occur during vitamin B_{12} transport, one can draw conclusions about the most likely transport mechanism and exclude alternatives (91, 92). In BtuCD the two ATP molecules (in the crystal ATP is replaced by cyclotetravanadate) are located in the BtuD dimer interface. BtuCD form a cavity that is large enough to accomodate vitamin B_{12}. In addition, the crystal structure of the periplasmic vitamin B_{12} binding protein BtuF was determined (93). It is similar to FhuD (90) in the sense that it contains an α-helical backbone that prevents large hinge and twist movements of one lobe relative to the other when the substrate is bound, as has been observed in most binding proteins. Sequence alignment to binding proteins of iron transporters reveal two conserved glutamate residues which are positioned such that they can form salt bridges to conserved arginine residues in the two permease subunits. Binding of the substrate-loaded binding proteins to the permeases triggers ATP hydrolysis (6, 92). It is likely that triggering of ATP hydrolysis and release of ADP and P_i cause two opposite movements in BtuD which through the tight structural coupling of BtuD to BtuC trigger movement of BtuC and cause opening / closing of the BtuC/D channel.

5. Fe^{2+} TRANSPORT SYSTEM IN *E. COLI* K-12

E. coli also grows under anoxic conditions, which predominate in the gut. In a reducing environment, iron is present as Fe^{2+}, which has a much higher solubility than Fe^{3+}. A transport system for Fe^{2+} has been characterized genetically (94, K. Hantke, personal communication). Three genes, *feoABC* (Figure 3), are required for Fe^{2+} uptake. *feoB* encodes an 84-kDa protein located in the cytoplasmic membrane (Figure 1). FeoB seems to be the transport protein. The function of the smaller *feoA* gene (7-kDa encoded protein) and *feoC* gene (9-kDa encoded protein) is not known. The promoter of *feoB* contains a Fe^{2+}-Fur and an Fnr binding site. Fnr functions as a transcriptional activator of anaerobically expressed genes. Fe^{2+}-Fur represses *feoB* transcription, and Fnr activates *feoB* transcription.

Fe^{2+} can also be taken up by the Mg^{2+} transport system and may accumulate to toxic concentrations (95). A Mn^{2+} transport system encoded by the *mntH* gene also transports Fe^{2+} with low affinity. *mntH* is repressed by Fe^{2+}-Fur and Mn^{2+}-MntR, a specific repressor of *mntH* transcription (96).

6. REGULATION OF IRON TRANSPORT SYSTEMS

The intracellular iron concentration is measured by the Fur protein (95-97), which is loaded by free Fe2+ not bound to or incorporated into proteins. Fe2+-Fur serves as repressor and binds to the consensus sequence (Fur box) composed of tandemly arranged GATAAT hexamers (98). Fur contains two domains, an unusually structured helix-turn-helix motif in the N-terminus and two metal bindings sites in the C-terminus, one of which is occupied by Zn2+ (99, 100). All iron transport systems of *E. coli* are negatively regulated by Fur. Fur is widely distributed among gram-negative bacteria and is also found in some gram-positive bacteria (100).

Fur also acts positively on the expression of a number of proteins (101). For example, *fur* mutants do not grow on succinate as sole carbon source (97). This phenotype was not understood until it was shown that Fur represses synthesis of RyhB, which acts as an antisense RNA (102). RyhB is complementary to a segment of the *sdhCDAB* operon encoding succinate dehydrogenase. The strongest complementarity is at the end of the *sdhC* gene just before the start of the *sdhD* gene. RyhB is a 90-nucleotide long RNA that belongs to the family of small RNAs. Other genes positively regulated by Fur — *sodB*, *acnA*, *fumA*, *bfr*, and *ftn* — are all subject to regulation by RyhB. Binding of the RyhB RNA to theses mRNAs is stimulated by the Hfq protein, which in turn inhibits translation. RyhB and Hfq regulate intracellular iron usage. This system together with regulation of iron acquisition enables cells to guarantee proper iron supply for growth and to avoid iron overload, which is toxic owing to oxygen radical formation by surplus iron (103).

7. PERSPECTIVES

Although it is thought that a basic understanding of iron transport across the outer membrane and the

cytoplasmic membrane has been gained, much needs to be done to unravel the transport mechanisms. This begins with the elucidation of how heme is released from hemoglobin and myoglobin at the cell surface by the transport proteins. The role of cell surface loops of the transport proteins must also be delineated. Do they close the entrance to the high-affinity binding sites upon binding of the substrates, as has been observed in FecA when ferric citrate is bound, and does closing prevent escape of the substrates into the external milieu when they are released from the high-affinity binding sites for transport through the channels of the beta-barrels? Energy input from the cytoplasmic membrane for outer membrane transport is required. How do the TonB-ExbB-ExbD proteins measure the proton gradient, and how do they respond? Only few data on the conformational changes in TonB and in outer member transport proteins exist. Fluorescence techniques could be applied to monitor these processes, and a reconstituted system may be required. We do not know whether binding of substrates is required for the structural transitions in the outer membrane transport proteins in response to the proton motive force and the Ton system since TonB interacts also with the unloaded transport proteins. With regard to the established interaction of TonB with the TonB box at the N-proximal end of the cork, it will be important to determine at which stage of the multi-step transport process this interaction is required and what it achieves. Is the interaction important for the movement of the cork within the beta-barrel and the release of the substrates from the amino acid side chains at the corks and the beta-barrels to which they are attached? Does the cork move out of the channel, and if so, can it insert again into the beta-barrel, or can each transport protein only transport a single substrate molecule? There is no electrophoretic evidence for a mixture of intact FhuA and FhuA with released cork or of FhuA and corkless FhuA, which might be formed by proteolytic degradation of the released cork (F. Endriß, her unpublished results). Are the mechanisms of transport of small ferric siderophores identical to that of colicin proteins and phage DNA? For example, does colicin M move through FhuA, and why does it require a TonB box in addition to FhuA [both of which have to be intact for colicin M to kill cells (104)]. Is the phage DNA translocated through the beta-barrel of FhuA (105), and if so, is the cork pushed out of the beta-barrel with the help of the osmotic pressure in the phage heads?

Few data on the transport of iron through the cytoplasmic membrane have been collected (5, 89). It is generally assumed that the insights gained mainly with the ABC transporters of maltose and histidine also apply for the iron transport systems. This might be true in principle, but the mechanisms may differ in instructive details.

Transport of ferrous iron across the cytoplasmic membrane is even less understood than transport of ferric iron. The FeoB membrane protein contains a nucleotide binding site that resembles a GTP binding site rather than an ATP binding site. It is not known whether GTP is hydrolyzed and drives active transport or whether GTP regulates FeoB activity. The functions of the two soluble small proteins FeoA and FeoC is also unknown. The Feo system contributes to the iron supply also under oxic conditions since Fur-regulated genes are derepressed in *feo* mutants (101). In addition, Fe^{2+} is transported by transport systems of divalent metal ions, and it is not known how these transport systems contribute to the bacterial iron supply, especially in natural environments.

Siderophores are released from the producing cells. They are usually charged molecules that cannot simply diffuse through the cytoplasmic membrane, and diffusion across the outer membrane is also restricted by the size of the pores formed by the porins. Active secretion systems specific for siderophores must exist or systems with a broad substrate spectrum of the type found for the secretion of antibiotics that confer resistance to the antibiotics must be used. The first identified gene related to an siderophore export system is *entS* (Figure 1); EntS exports enterobactin (106) and belongs to a large family of export pumps that contain 12 transmembrane segments. It will be interesting to determine its substrate specificity and its mode of action. The coupling of secretion and synthesis of other siderophores, e.g., of *E. coli* aerobactin, should be studied to understand why internally synthesized siderophores do not inhibit growth of cells or even kill them by withdrawal of iron.

The use of active ferric-siderophore transport systems as carriers for antibiotics sounds attractive. This concept gained a new impetus by the determination of the crystal structure of FhuA with bound albomycin (107) and the synthetic rifamycin derivative CGP 4832 (108). Active transport of antibiotics compared to diffusion into cells increases their efficacy by reducing the minimal inhibitory concentration by a factor of more than 100 (109). Naturally occurring sideromycins, derivatives of siderophores, and synthetic sideromycins clearly demonstrate a high inhibitory potential of this class of compounds and a broad spectrum of action for gram-negative and gram-positive bacteria. Since bacteria cannot afford to lose a transport system required in a certain environment of their hosts, the development of resistance *in vivo* might not occur as frequently as is observed on nutrient agar plates. Resistance of bacteria to antibiotics is a serious problem and every effort to combat it is worthwhile. Sideromycins offer the potential for new drugs composed of an iron carrier and an antibiotically active moiety.

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