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Minireview

Iron transport and signaling in *Escherichia coli*

Volkmar Braun*, Michael Braun

Microbiology/Membranephysiology, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

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Abstract Bacteria solve the iron supply problem caused by the insolubility of Fe^{3+} by synthesizing iron-complexing compounds, called siderophores, and by using iron sources of their hosts, such as heme and iron bound to transferrin and lactoferrin. *Escherichia coli*, as an example of Gram-negative bacteria, forms sophisticated Fe^{3+}–siderophore and heme transport systems across the outer membrane. The crystal structures of three outer membrane transport proteins now allow insights into energy-coupled transport mechanisms. These involve large long-range structural transitions in the transport proteins in response to substrate binding, including substrate gating. Energy is provided by the proton motive force of the cytoplasmic membrane through the activity of a protein complex that is inserted in the cytoplasmic membrane and that contacts the outer membrane transporters. Certain transport proteins also function in siderophore-mediated signaling cascades that start at the cell surface and flow to the cytoplasm to initiate transcription of genes encoding proteins for transport and siderophore biosynthesis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Substrate-gated transport protein

1. Introduction

The environment of all aerobically growing organisms, including bacteria, can be hostile with respect to iron through: (i) iron starvation caused by the insolubility of Fe^{3+} in the environment, and (ii) toxicity of intracellular Fe^{2+} through radical formation. The free Fe^{3+} concentration in equilibrium with environmental Fe^{3+}–hydroxide polymer is in the order of 10^{-9} M [1], which is far below the concentration of 10^{-7} M required to support bacterial growth. Hydroxyl radicals are formed from Fe^{2+} and H_2O_2 in the Fenton reaction and from oxygen radicals and H_2O_2 in the Haber–Weiss reaction. Bacteria avoid both hazards by regulating iron uptake through active transport mechanisms [2]. Under conditions of iron starvation, bacteria synthesize iron-complexing molecules, called siderophores, and Fe^{3+}–siderophore transport systems. If the intracellular iron concentration is sufficient, siderophore and transport protein synthesis is shut off by inhibition of transcription of the related genes through the binding of Fe^{2+} to two proteins, Fur and DtxR, which act in the iron-loaded form as transcriptional repressors [3].

In addition to siderophores synthesized by a particular strain, the strain can use siderophores produced and released into the extracellular medium by other bacteria and even fungi. The spectrum of siderophores a particular strain can use is determined by the specificity of the Fe^{3+}–siderophore transport systems it forms. Moreover, bacteria that infect humans and animals may use iron sources of their hosts, such as heme, hemoglobin, hemopexin, and iron bound to transferrin and lactoferrin [4]. In Gram-negative bacteria, which contain an outer membrane, all iron sources are bound to highly specific proteins that serve as transporters across the outer membrane [5]. In Gram-positive bacteria, which contain a permeable cell wall, the iron sources gain direct access to the cytoplasmic membrane. As far as it is known, transport across the cytoplasmic membrane is catalyzed by ABC (ATP binding cassette) transporters [6], which are the most frequently occurring transport systems for many different nutrients in Gram-positive and Gram-negative bacteria [7].

2. Energy-coupled active transport across the outer membrane of Gram-negative bacteria

Cellular iron supply via Fe^{3+}–siderophores, heme, and iron-binding proteins requires special mechanisms of Fe^{3+} transport. In Gram-negative bacteria, this is achieved through binding of the Fe^{3+}-carrying compounds to proteins in the outer membrane that function as transporters. Fe^{3+}–siderophores and heme are transported into the cytoplasm. Heme is released from hemoglobin at the transporter or by soluble proteins (hemophores) that are secreted into the external medium [8]. Fe^{3+} is released from transferrin and lactoferrin by the transporters by an unknown mechanism. After binding of the Fe^{3+}–siderophores and heme to the Fe^{3+} transporters, the Fe^{3+} complexes are released from the binding sites and vectorially transported across the outer membrane into the periplasm, where they bind to binding proteins. The binding proteins deliver Fe^{3+} and the Fe^{3+} compounds to the ABC transporters in the cytoplasmic membrane. The K_d of siderophores bound to the transporters is in the nanomolar range [9]. Release from the binding site and transport across the outer membrane consumes energy. Provision of energy poses a problem since the outer membrane contains no energy source – the high protein-mediated diffusion permeability of the outer membrane for most nutrients prevents formation of a membrane potential, and energy-rich compounds such as

*Corresponding author. Fax: (49)-7071-295834.
E-mail address: volkmar.braun@mikrobio.uni-tuebingen.de (V. Braun).

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ATP, GTP, and PEP are lacking in the outer membrane. Therefore, bacteria developed a device that energizes outer membrane transport by the proton motive force of the cytoplasmic membrane. The device consists of three known proteins, TonB, ExbB, and ExbD (the Ton system) in a molar ratio of 1:7:2 [10]. These proteins interact with each other [11–14] and presumably form a complex that responds to the proton motive force (Fig. 1). Most of the TonB protein is located in the periplasm with the N-terminus anchored to the cytoplasmic membrane. The hydrophobic membrane-spanning region contains a histidine residue (His20), which, when converted to an arginine residue, renders the TonB mutant transport-deficient [13]. This histidine residue probably responds to the proton motive force by binding a proton. Binding of the proton causes a structural change in TonB that converts TonB into an energized form. The energized form interacts with the outer membrane transporters and changes their conformation such that Fe^{3+} and the Fe^{3+} complexes dissociate from their binding sites and diffuse through the channel that is opened at the same time. Another residue that may be important for energy transduction is Asp25, which is located at the border or within the single transmembrane segment of ExbD. Replacement of Asp25 by Asn abolishes TonB-dependent activities [15]. It is conceivable that Asp25 serves as proton donor or proton acceptor of His20 in the reaction cycle of activation and deactivation of TonB. In ExbB, which spans the cytoplasmic membrane three times, with most of the protein located in the cytoplasm [16], Glu176 is entirely conserved in all annotated ExbB and TolQ sequences (TolQ is homologous to ExbB [16] and is part of a second energy-transducing system from the cytoplasmic membrane to the outer membrane) and is contained in a highly conserved sequence of hydrophobic residues. Glu176 seems to be a functionally important charged residue in a transmembrane segment (residues 162–194) of ExbB. Another highly conserved charged residue, Lys24 in the first transmembrane segment (residues 16–39), may play an important functional role, whereas residues His18 and Asp20 are less conserved.

3. The crystal structures of three active outer membrane transporters provide insights into the transport mechanisms

The crystal structures of FhuA [17,18], FepA [19], and FecA [20] have been determined. FhuA transports ferrichrome, a siderophore of the hydroxamate type; FepA transports Fe^{3+}–enterobactin, a siderophore of the catecholate type; and FecA transports ferric citrate. The basic design of the three proteins is the same – a β-barrel completely closed by a globular domain, which for this reason is designated cork or plug (Fig. 2). The β-barrel in each protein is formed by 22 antiparallel β-strands connected by short turns in the periplasm and external loops of various size that extend far (45 Å) above the cell surface. The crystal structures of FhuA and FecA in the free form and loaded with substrates were determined. Upon binding of their substrate, both proteins undergo major structural changes, which, however, do not open a channel. In FecA, loops 7 and 8 are translated by 11 and 15 Å, respectively, and bend over the external cavity through which ferric citrate gains access to the binding site. The cavity is closed, and ferric citrate can no longer escape into the external milieu. FecA therefore functions as a substrate-gated transporter. In the structure of both crystal forms of ferrichrome-loaded FhuA [17,18] no such loop movement is observed, but this does not mean that it does not occur in FhuA embedded in the outer membrane. Constraints imposed by the crystal structure may prevent the structural transition.

Another major structural transition observed in FhuA and FecA involves a short α-helix (residues 24–29 in FhuA) that is exposed to the periplasm. Upon binding of the substrate, the α-helix is unwound and assumes a flexible extended conformation, which in FhuA results in a transition of residues Glu19, Ser20, and Trp22 by 17 Å. The N-proximal end of FhuA (residues 1–18), FecA (residues 1–79; see Section 6 for a discussion of the extra long N-terminus), and FepA (residues 1–10) does not assume a fixed structure, as deduced from the crystal structures. It is thought that substrate-induced mobilization of the N-proximal region facilitates interaction of the transporters with the TonB protein. Genetic [21] and biochemical [22] evidence indicates that a region in the N-proximal end, the TonB box (residues 6–11 in mature FhuA, residues 11–16 in FepA, and residues 80–84 in FecA), interacts with region 160 of the TonB protein. For example, single point mutations in the TonB box of FhuA (Ile9 → Pro, Val11 → Asp) that inactivate FhuA are suppressed by point mutations Gln160 → Leu or Lys in TonB. Cysteine residues inserted into the TonB box of the BtuB outer membrane protein, which transports vitamin B_{12} by a mechanism similar to that of the transport of iron by iron transporters, form in vivo disulfide bridges to cysteine residues inserted into region 160 of TonB. The disulfide bridge formation is enhanced by binding vitamin B_{12} to BtuB [22]. These results clearly demonstrate that the TonB box of outer membrane transporters interacts with TonB. Additional data in favor of a substrate-induced interaction have been obtained by cross-linking with formaldehyde, which results mainly in dimers formed between TonB and FhuA. Dimer formation is enhanced upon binding of ferrichrome [23].

4. The β-barrel of FhuA functions as an active transporter

As described in the Section 3, ample evidence exists for the interaction of the TonB box of the transporters with TonB. The TonB box precedes the globular domain (in the following called cork) that closes the β-barrel. Removal of the cork along with the TonB box should abolish TonB-dependent activities and open the channel of the β-barrel, resulting in a porin-like protein through which substrates diffuse across the outer membrane into the periplasm. This prediction has been examined by deleting residues 5–160 of mature FhuA [24]. FhuAΔ5–160 is present in the outer membrane in amounts lower than wild-type FhuA which may arise from proteolytic degradation, impaired export across the cytoplasmic membrane, or difficult incorporation into the outer membrane. However, FhuAΔ5–160 displays all FhuA activities, such as transport of ferrichrome, the structurally related antibiotic albomycin, and the unrelated antibiotic rifamycin CGP 4832, and the receptor function for colicin M and the phages T1, T5, and ϕ80. The only activity missing is transport of microcin J25. All these functions, except infection by phage T5, require the Ton system. Contrary to the expectation, these results indicate that the β-barrel is an active transporter that responds to the Ton system. Moreover, interaction of FhuA with TonB occurs not only through the TonB box, but also through the β-barrel. Interaction of TonB with the β-barrel

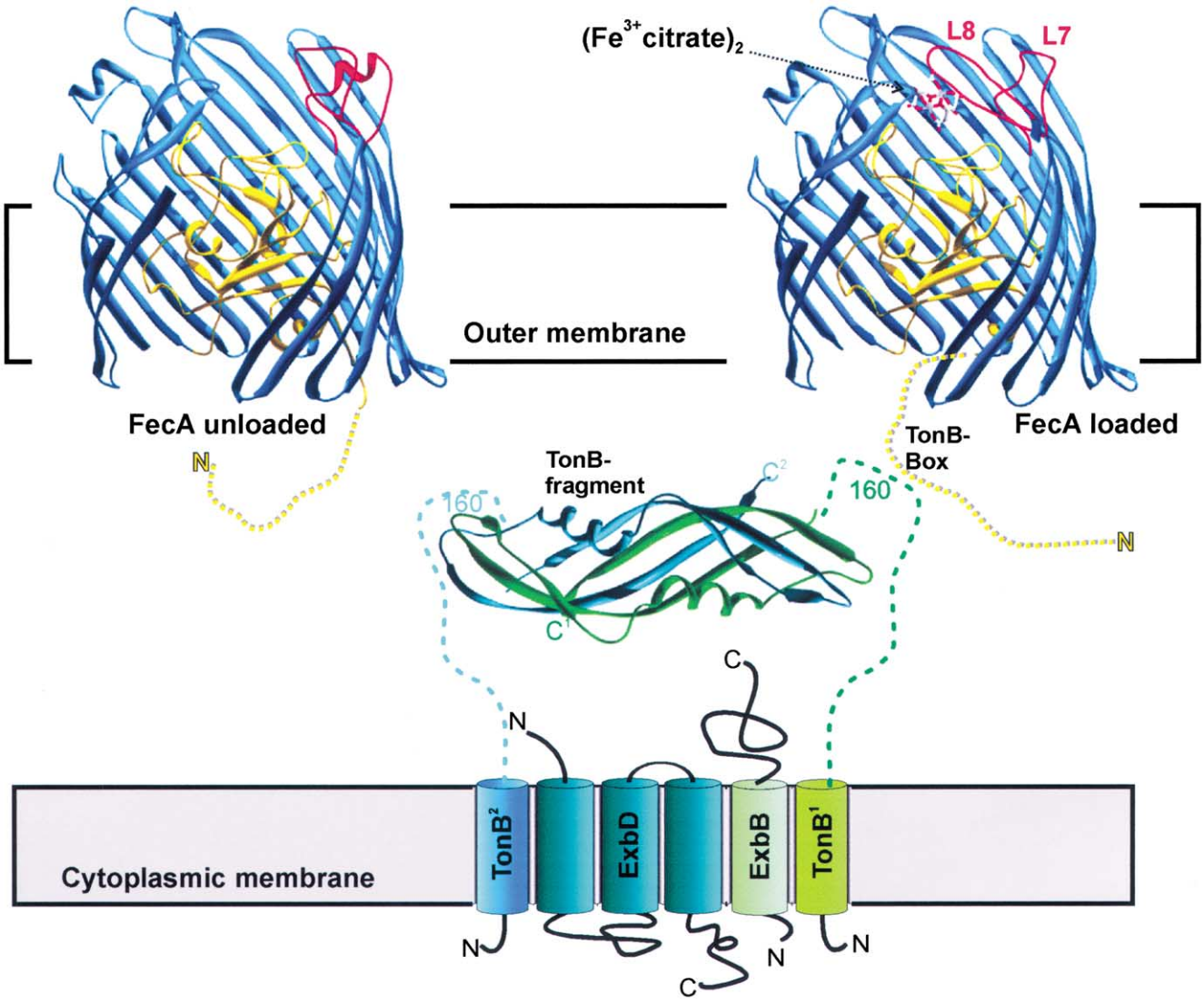

Fig. 1. Transport of $(Fe^{3+}$–citrate$)_2$ across the outer membrane of *E. coli*. The crystal structures of the FecA outer membrane transport protein unloaded and loaded with $(Fe^{3+}$–citrate$)_2$ disclose the large structural transitions of surface loops 7 and 8 and of the region exposed to the periplasm. The crystal structure of a C-terminal dimeric fragment of TonB (residues 164–239) is shown on the same scale as FecA, which demonstrates that only one region 160 at a time is able to interact with the TonB box of FecA.

must cause a structural change in FhuA that changes the geometry of the amino acid residues that bind ferrichrome (six out of 10 binding sites are located in the β-barrel), resulting in the release of ferrichrome and diffusion into the periplasm. Interaction with TonB must also change the conformation of loop 4, the largest loop at the surface, to which phages T1 and ϕ80 bind [25]. Binding to loop 4 triggers a structural change in the phages that causes release of the DNA from the phage head and passage of DNA through the phage tail [25]. T1 and ϕ80 infection only occurs in energized $TonB^+$ cells, which suggests a conformation of loop 4 that differs from that of $TonB^-$ cells and unenergized cells [26]. Since TonB is located in the periplasm and presumably interacts with sites of FhuA exposed to the periplasm, interaction with TonB must cause a long-range (70 Å) structural transition through the entire β-barrel.

Purified FhuAΔ5–160 incorporated into artificial lipid bilayer membranes forms pores through which ions such as those of KCl diffuse, in contrast to wild-type FhuA, which does not increase the permeability of ions. However, the conductance increase rarely shows discrete steps indicative of single channels; rather, the recordings of the current had a high degree of noise (M. Braun, H. Killmann, E. Maier, R. Benz, V. Braun, unpublished results). In vivo, FhuAΔ5–160 somewhat increases sensitivity of cells to the antibiotics novobiocin, erythromycin, rifamycin, and vancomycin, which have molecular masses larger than 600 Da and therefore diffuse only slowly through the porins that render the outer membrane of *Escherichia coli* permeable for hydrophilic compounds [24]. FhuAΔ5–160 also increases the diffusion of maltodextrins through the outer membrane of a mutant lacking the maltodextrin-specific porin LamB [24]. However, cells remain resistant to SDS; this, together with the other permeability data, suggest that FhuAΔ5–160 restricts diffusion and does not form a permanently open channel.

In wild-type FhuA, the cork must move to open a channel.

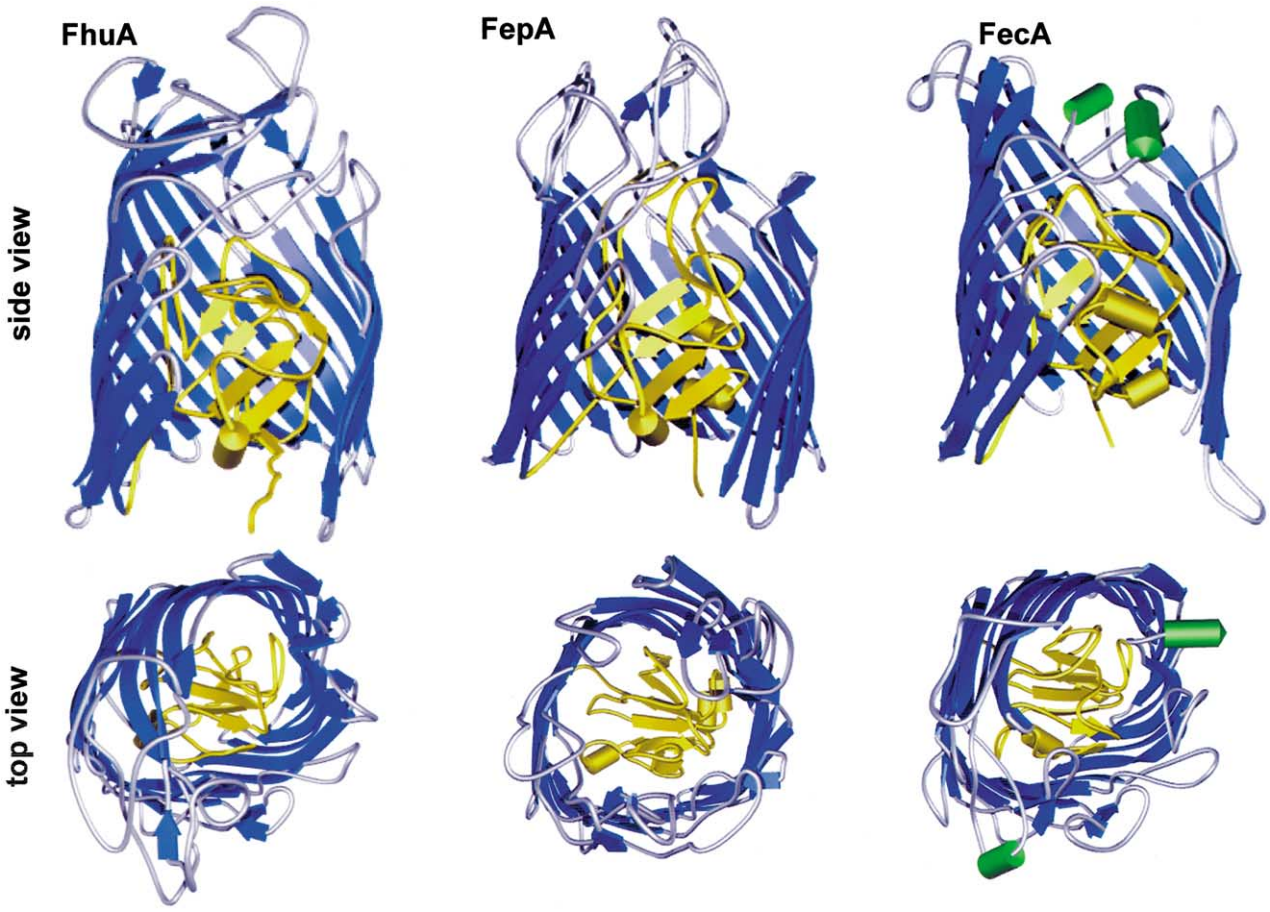

Fig. 2. Comparison of the FhuA, FepA, and FecA crystal structures. In the side views, a portion of the β-strands (blue) was deleted to improve the view on the globular central domain (cork; yellow). The view from outside the cell (top view) illustrates the closure of the β-barrel channel by the cork domain. The N-terminal segments of the proteins, FhuA1–18, FepA1–10, FecA1–79 are not seen in the crystal structure which suggests a flexible structure.

It is conceivable that movement is triggered by interaction of energized TonB (potential energy stored in a certain TonB conformation) with the cork through the TonB box and with the β-barrel. The cork and the β-barrel change conformations, thereby loosening interaction of ferrichrome to its binding sites at the cork and the β-barrel and interactions between the cork and the β-barrel. This results in dissociation of ferrichrome from FhuA, opening of the β-barrel channel, and subsequent diffusion of ferrichrome into the periplasm. Ferrichrome could diffuse along amino acid side chains that line the channel, as has been demonstrated for the facilitated diffusion of maltodextrins through the LamB porin [27]. The external cavity of FhuA is connected to the periplasmic cavity by a 10-Å aqueous channel [28], which requires only small movements of amino acid side chains to be opened and to form a continuous channel. It is less likely that the entire cork is pulled out of the β-barrel, which would require much energy [29], since more than 60 hydrogen bonds are involved in binding the cork to the β-barrel and the buried surface area between the inner barrel wall and the cork is in the range of 5000 $Å^2$.

A gene fragment encoding only the cork of the FepA protein has been cloned. The isolated gene-fragment product, FepA(1–148), is predominantly unfolded, as shown by circular dichroism and NMR [30]. It still binds Fe^{3+}–enterobactin, but only with an affinity ($K_d \sim 5$ μM) 100-fold lower than the affinity of complete FepA. This result suggests that if the cork comes out of the β-barrel, it will unfold. The question is then whether the cork can be incorporated again into the β-barrel for the next round of ferrichrome transport. The activity of corkless FhuAΔ5–160 indicates that the β-barrel does not require the cork for proper folding (24). It is therefore likely that the β-barrel folds independently of the cork and that the cork is subsequently incorporated into the β-barrel. Under this premise, the cork could move in and out of the β-barrel during transport. Even if this is a slow process, it may be sufficient to meet the iron requirement. An *E. coli* cell needs approximately 10^5 iron ions per generation. Under iron-limiting growth conditions, the cell synthesizes 10^4–10^5 FhuA molecules per cell. This means that on average one FhuA molecule has to transport only one to 10 ferrichrome molecules. Under experimental conditions, a ferrichrome transport rate of 4 molecules per min per FhuA molecule has been determined [31].

5. Crystal structures of FhuA with bound antibiotics

The first crystal structures of antibiotics bound to a trans-

porter were obtained with FhuA [32,33]. Albomycin is an antibiotic highly efficient against a variety of bacteria. The minimal inhibitory concentration for *E. coli* K-12 is 100-fold lower than that of ampicillin. The reason lies in the uptake of albomycin by an active transport system, in contrast to diffusion of ampicillin. Albomycin consists of a Fe^{3+}–siderophore carrier similar to ferrichrome and an antibiotically active portion, a thioribosyl pyrimidine moiety, that inhibits the serine t-RNA synthetase [34]. In order to be active, the antibiotic has to be cleaved from the carrier by peptidases [35,36]. The crystal structure of albomycin bound to FhuA [32] shows that it occupies the same position as ferrichrome. The antibiotic portion is fixed to amino acid residues in the external cavity and assumes two different conformations – a compact form and an extended form [32]. The structural changes observed upon binding of ferrichrome are also observed upon binding of albomycin. Transport across the outer membrane requires the proton motive force of the cytoplasmic membrane transmitted by the Ton system. Further transport into the cytoplasm occurs via the FhuBCD transport proteins as ferrichrome [6].

A rifamycin derivative, designated CGP 4832, is 200 times more active than rifamycin since it is transported across the outer membrane by FhuA [37], in contrast to diffusion of unmodified rifamycin into cells. Since the chemical structure of CGP 4832 does not resemble that of ferrichrome or albomycin, it was difficult to envisage how it is recognized and transported by FhuA. The crystal structure of FhuA with bound CGP 4832 shows a binding site that largely overlaps with that of ferrichrome [33]. The two moieties with which CGP 4832 differs from rifamycin substantially contribute to CGP 4832 binding to FhuA.

6. FecA not only functions as a transporter, but also as a signaling protein involved in transcription initiation

Compared to FhuA and FepA, FecA contains an extra N-proximal sequence [38]. For this reason, the TonB box is not close to the N-terminal end as in most other TonB-dependent outer membrane transporters, but is further inside between residues 80 and 85 (Fig. 1) [38]. In addition to its function in the transport of ferric citrate, FecA is part of a new kind of regulatory device that was discovered in this system. Ferric citrate serves as inducer of the transcription of the *fecABCDE* transport genes, which encode the ferric citrate transport proteins [39,40]. For transcription initiation, binding of ferric citrate to FecA is sufficient without further transport into the periplasm [41,42]. FecA, FecR, and FecI comprise the regulatory device [43–45]. FecR interacts in the periplasm with the N-terminal extension of FecA [46]. Deletion of the FecA N-terminal extension abolishes transcription initiation, but does not affect FecA transport activity [42]. FecR contains a single transmembrane segment in the cytoplasmic membrane with which it transmits the signal received from FecA upon loading with ferric citrate into the cytoplasm [47]. There, the N-proximal sequence of FecR interacts with FecI, which is converted into an active sigma factor that directs the RNA polymerase to the promoter upstream of the *fecA* gene, thereby initiating transcription of the *fecABCDE* operon [44,45,48]. The *fecI* and *fecR* regulatory genes are located upstream of *fecABCDE* [43]. They are transcribed under iron limitation, during which the Fur protein does not function as a repressor. The multi-faceted regulation of the entire system is elegant. First, the cell recognizes iron deficiency and synthesizes a few copies of the regulatory proteins. FecI and FecR only become active when the proper Fe^{3+}–siderophore, ferric citrate, is in the external medium. Ferric citrate binds to FecA, which is by far the most abundant protein of the Fec system, being present in sufficient amounts under all conditions. Binding initiates a signal that is transmitted across the outer membrane by FecA, through the periplasm by the N-proximal end of FecA and the C-proximal part of FecR, and through the cytoplasmic membrane by the hydrophobic transmembrane sequence 84–100 of FecR [47]. We assume that the conformational change observed in the FecA crystal upon binding of ferric citrate triggers a conformational change in FecR that either imposes a conformational change in FecI so that it functions as a sigma factor, or leads to FecI, associated with FecR in an inactive form, dissociating from FecR.

The transmembrane transcription initiation unraveled in the *fec* system of *E. coli* K-12 has recently been found in *Pseudomonas putida* [49], *Pseudomonas aeruginosa* [50,51], *Bordetella pertussis* and *Bordetella bronchiseptica* [52]. In these species, outer membrane transporters with N-terminal extensions similar to the one in FecA and genes homologous to *fecI* and *fecR* have been found. The amounts of the outer membrane transporters are regulated by the *fecIR* homologs. In *P. aeruginosa*, the pyoverdin siderophore regulates its own synthesis and the synthesis of exotoxin A and an endoprotease [50]. Specific interaction of two sets of FecI and FecR homologs in *P. aeruginosa* has been demonstrated using a bacterial two-hybrid system [53]. Fec-like regulatory devices seem to be widely distributed: examination of published genome sequences has revealed more than 70 *fecIR* homologs in a variety of different bacterial species.

7. Various initiation mechanisms for transport of Fe^{3+}–siderophores across the outer membrane

Fe^{3+}–siderophores bind to amino acid residues located at the entrance of the cavity in which the specific high-affinity binding site is located. The entrance to the cavity of the FepA and FhuA proteins is lined with aromatic residues, that of the FecA protein with positively charged residues. Aromatic amino acids as binding sites of Fe^{3+}–enterobactin, a cyclic trimer of dihydroxybenzoyl serine, and of the uncharged ferrichrome, and the positively charged residues as binding sites of the negatively charged $(Fe^{3+}\text{–citrate})_2$ molecule make sense. In the high-affinity binding site of FhuA, seven out of 10 residues are aromatic; in the high-affinity binding site of FecA, there are three positively charged residues and no aromatic residues. Since Fe^{3+}–enterobactin is not seen in the crystal structure and it is unclear whether the crystal represents the loaded or unloaded form [19,54], the residues that bind Fe^{3+}–enterobactin are not known. A kinetic analysis with a fluorescent probe bound to an introduced Cys280 residue has revealed a two-step binding [55]. Apparently, the Fe^{3+}–siderophores are extracted from the external medium by multiple binding to residues in the entrance of the cavity before they are finally fixed deeper in the transporter molecules at the high-affinity binding sites. In most cases the bound Fe^{3+}–siderophores are taken up into the cytoplasm where iron is released by reduction to Fe^{2+} with an affinity

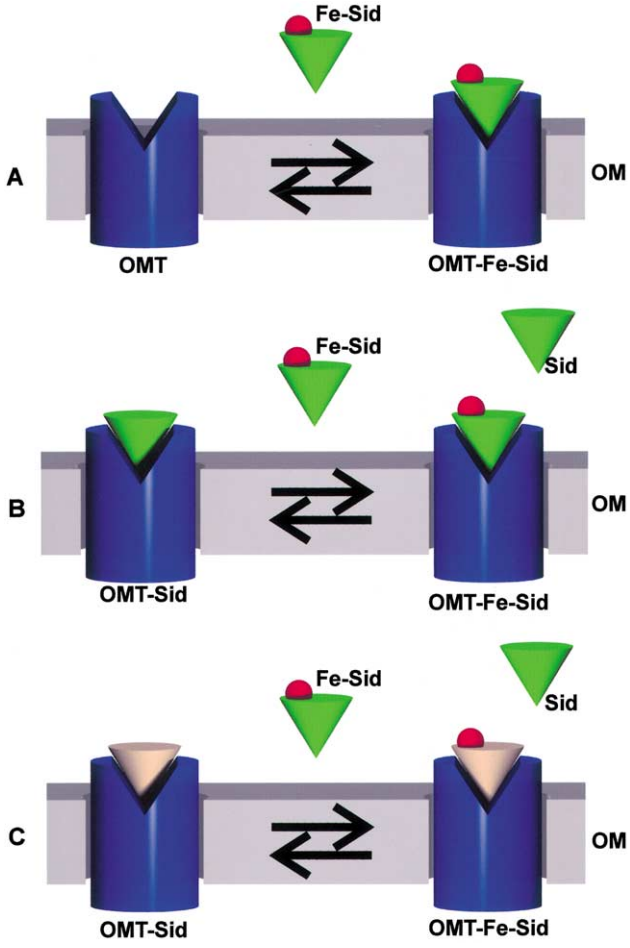

Fig. 3. Illustration of the three mechanisms of ferric siderophore transport initiation mechanisms. A: Ferric (red) siderophore (green) (Fe-Sid) binds to the outer membrane transport protein (blue) (OMT) and is transported as such across the outer membrane. B: Ferric pyoverdin displaces pyoverdin at the FpvA transport protein of *P. aeruginosa* [59]. C: Different ferric siderophores supply iron to the siderophore bound to the transport protein as proposed for amonabactin-mediated iron transport of *A. hydrophila* [62]. OMT-Fe-Sid, ferric siderophore bound to an outer membrane transport protein.

to siderophores orders of magnitude lower than Fe^{3+} (Fig. 3A).

7.1. Transport of Fe^{3+}–pyoverdin by the FpvA protein of *P. aeruginosa*: siderophore displacement

Pyoverdin is a siderophore formed specifically by *P. aeruginosa*. Free pyoverdin fluoresces owing to the 2,3-diamino-6,7-dihydroxyquinoline group. Iron-loaded pyoverdin does not fluoresce. After excitation of Trp residues in FpvA at 290 nm, pyoverdin bound to FpvA emits light at 447 nm [56]. FpvA purified from a strain that synthesizes pyoverdin contains pyoverdin [57]. This novel finding prompted a study of the transport initiation mechanism using fluorescence resonance energy transfer. In less than 7 min ($t_{1/2}$), half of the pyoverdin and Fe^{3+}–pyoverdin is bound to FpvA in cells [56]. Pyoverdin bound to FpvA is displaced in less than 4 min by Fe^{3+}–pyoverdin. Since cells under iron-limiting growth conditions synthesize a surplus of pyoverdin, FpvA is always occupied with pyoverdin, which is not transported into the cells. In order to get iron into the cells, pyoverdin is replaced by Fe^{3+}–pyoverdin (Fig. 3B). Radiolabeled pyoverdin enters cells along with radiolabeled iron. The kinetics of iron uptake resemble the kinetics of the replacement of pyoverdin by Fe^{3+}–pyoverdin at the FpvA transporter. A particularly interesting observation is the lack of replacement of pyoverdin by Fe^{3+}–pyoverdin in a *tonB1 tonB2* double mutant [57] (*P. aeruginosa* contains two *tonB* genes) since it demonstrates involvement of TonB in initial binding and replacement and not only in transport subsequent to binding. Binding of pyoverdin is not affected by TonB, which indicates that binding of Fe^{3+}–pyoverdin requires a geometry of the binding site that differs from binding of pyoverdin and that is induced by TonB, presumably in response to the proton motive force of the cytoplasmic membrane. The TonB-induced geometry of FpvA favors dissociation of pyoverdin and association of Fe^{3+}–pyoverdin to FpvA. A TonB-dependent binding step has been shown previously: inhibition of phage T5 binding to the FhuA protein by ferrichrome is strongly affected by TonB [58]. $TonB^{+}$ cells cannot be completely inhibited even when a ferrichrome concentration 1000-fold higher than required for inhibition of $TonB^{-}$ cells is used. In addition, release of DNA from the head of phages T1 and ϕ80 is only triggered when FhuA is coupled to TonB and the proton motive force [26].

Isolated FpvA behaves differently than FpvA incorporated in the outer membrane. Pyoverdin bound to FpvA can be loaded with iron supplied as ferric citrate, which does not take place in vivo. The exchange of pyoverdin by Fe^{3+}–pyoverdin takes about 50 h ($t_{1/2} = 24$ h) [56]. Such findings are important to bear in mind when properties of isolated membrane proteins are extrapolated to properties of the proteins in cells.

After transport of Fe^{3+}–pyoverdin into the cytoplasm, Fe^{3+} is released and pyoverdin is secreted into the external medium and bound to FpvA [59]. This is similar to Fe^{3+}–aerobactin transport, in which aerobactin is recycled and used several times. [60]. It differs from ferrichrome transport, in which, after intracytoplasmic mobilization of iron, deferri-ferrichrome is inactivated by acetylation of the hydroxamate moiety [61], and from Fe^{3+}–enterobactin transport, after which enterobactin is hydrolyzed in the cytoplasm [54].

7.2. Transport of Fe^{3+}–amonabactin by Aeromonas hydrophila: siderophore-iron exchange mechanism

In contrast to all studied iron transport systems, a large variety of structurally different, naturally occurring and synthetic Fe^{3+}–siderophores serve as iron sources and are thought to be transported by a single transporter across the outer membrane of *A. hydrophila* [62]. Based mainly on competition experiments, it has been concluded that structurally unrelated Fe^{3+}–siderophores bind to the same transporter as the genuine iron complex Fe^{3+}–amonabactin and deliver iron to the bound unloaded siderophore. The iron-free siderophore and a structurally unrelated iron-loaded siderophore bind at the same time to the transporter, and iron must be transferred from one siderophore to the other siderophore without reduction, which usually releases iron from the siderophore in the cytoplasm (Fig. 3C). Further proof of the unusually broad specificity of the transporter and the unusual transport initiation mechanism awaits identification of the transporter and the isolation of mutants that lack the transporter and mutants that allow the various transport steps to be dissected.

8. Outlook

The most important unresolved questions of iron transport concern how the proton motive force of the cytoplasmic membrane activates the transporters in the outer membrane. How does the TonB–ExbB–ExbD complex respond to the proton gradient of the cytoplasmic membrane, how is the energy transferred to the outer membrane, and how do the outer membrane transporters react to the binding of the 'energized' TonB protein? It is also not certain whether TonB alone or also ExbB and in particular ExbD contact the transporters. The stoichiometry of ExbB:ExbD:TonB of 7:2:1 and the interaction of the proteins points to a protein complex of unknown size in the cytoplasmic membrane. A complex similar in function seems to be formed by TolQ, TolR, and TolA (TolQ:TolR = 3:1) [63]; TolQ and TolR can partially replace the functions of ExbB and ExbD, respectively, and vice versa [64]. Isolation of the predicted complexes formed by the TonB, ExbB, and ExbD proteins and the TolQ, TolR, and TolA proteins or reconstitution of the complexes with the isolated proteins will be necessary to understand the structural basis of energy transfer. Amino acid alignment discloses conserved amino acids between ExbB and TolQ, and ExbD and TolR, in particular in the transmembrane regions [16,65–67]. Similar sequence similarities are also observed in the MotA and MotB proteins, which form the motor that drives bacterial flagella. MotB has the same subcellular location and transmembrane arrangement as ExbD and TolR and contains at the same position (Asp23) as ExbD (Asp25) an aspartate residue; when the Asp residue is changed to asparagine, MotB is inactivated [68]. MotA is the equivalent of ExbB and TolQ [69] and together with MotB forms the stator of the flagellar motor. Protons flow through MotA MotB [69,70]; therefore, perhaps also ExbB ExbD and TolQ TolR also form proton-conductive devices (see Section 2). Whether ExbB ExbD and TolQ TolR form the stator of a motor with TonB and TolA, respectively, as the rotating proteins, like, for example, the γ subunit in the center of the F_1F_0 ATPase, is an interesting idea that is worth examining. If TonB forms a dimer as seen in the crystal of a TonB fragment [71], a rotating TonB might interact alternatively with the TonB box of the cork and the barrel, or with different transporters. Since there are many more copies of transporters than of TonB, TonB is likely to interact transiently with various transporters. However, the known rotating molecular motors contain a ring of proteins that allow stepwise rotation. Such a ring of proteins has not been identified in the Ton and Tol systems.

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