Biometals (2007) 20:219–231
DOI 10.1007/s10534-006-9072-5

Energy-coupled outer membrane transport proteins and regulatory proteins

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Received: 20 October 2006 / Accepted: 28 November 2006 / Published online: 17 March 2007

Abstract FhuA and FecA are two examples of energy-coupled outer membrane import proteins of gram-negative bacteria. FhuA transports iron complexed by the siderophore ferrichrome and serves as a receptor for phages, a toxic bacterial peptide, and a toxic protein. FecA transports diferric dicitrate and regulates transcription of an operon encoding five ferric citrate (Fec) transport genes. Properties of FhuA mutants selected according to the FhuA crystal structure are described. FhuA mutants in the TonB box, the hatch, and the β-barrel are rather robust. TonB box mutants in FhuA FecA, FepA, Cir, and BtuB are compared; some mutations are suppressed by mutations in TonB. Mutant studies have not revealed a ferrichrome diffusion pathway, and tolerance to mutations in the region linking the TonB box to the hatch does not disclose a mechanism for how energy transfer from the cytoplasmic membrane to FhuA changes the conformation of FhuA such that bound substrates are released, the pore is opened, and substrates enter the periplasm, or how surface loops change their conformation such that TonB-dependent phages bind irreversibly and release their DNA into the cells. The FhuA and FecA crystal structures do not disclose the mechanism of these proteins, but they provide important information for specific functional studies. FecA is also a regulatory protein that transduces a signal from the cell surface into the cytoplasm. The interacting subdomains of the proteins in the FecA $\rightarrow$ FecR $\rightarrow$ FecI $\rightarrow$ RNA polymerase signal transduction pathway resulting in *fecABCDE* transcription have been determined. Energy-coupled transporters transport not only iron and vitamin B_{12}, but also other substrates of very low abundance such as sugars across the outer membrane; transcription regulation of the transport genes may occur similarly to that of the Fec transport genes.

Keywords Bacterial iron and sugar transporters · Signaling device · ECF sigma factors

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Introduction

Substrates cross the outer membrane of gram-negative bacteria by diffusion, facilitated diffusion, or energy-coupled active transport. In diffusion, the substrates are not recognized by the porin proteins through which they pass and are only discriminated by their size. For example, porins of *Escherichia coli* K-12 exclude compounds larger than ~700 Da. In facilitated

diffusion, in contrast, substrates transiently bind to the porins. The best-studied example of facilitated diffusion is that of maltodextrins by the *E. coli* LamB protein (Van Gelder et al. 2002). In energy-coupled active transport, substrates bind tightly to outer membrane proteins with K_d values in the sub-nanomolar range. Approximately ten amino acid side chains are involved in substrate binding. For release of the substrate from the binding site, the stereochemistry of the side chains must be changed, and this requires energy.

Both active transporters and porins form β-barrel structures. The pores of the porin β-barrels are always open, but those of active transporters are tightly closed by a globular domain, called the hatch, plug, or cork. The movement of the hatch domain is an additional energy-consuming process in active transport. The energy required for the movement of the hatch and the release of the substrate in active transport is provided by the proton-motive force of the cytoplasmic membrane; there is no energy source in the outer membrane.

The energy-coupling device between the cytoplasmic membrane and the outer membrane is formed by three proteins: TonB, ExbB, and ExbD (Fig. 1). All three proteins somehow react to the proton-motive force. The C-proximal region of periplasmic TonB contacts the N-terminal region, called the TonB box, of the outer membrane transporters. The TonB box becomes disordered upon binding of substrates to the transporters (Ferguson and Deisenhofer 2004; Wiener 2005). Direct experimental evidence for interaction via these regions comes from genetic suppression analyses of point mutants in TonB and the TonB box of transporters (Bell et al. 1990; Gudmundsdottir et al. 1989; Heller et al. 1988; Schöffler and Braun 1989), spontaneous in vivo disulfide cross-linking between cysteine residues introduced into TonB and the TonB box (Cadieux and Kadner 1999; Ogierman and Braun 2003), and crystal structures of C-proximal TonB fragments bound to two outer membrane transporters (Pawelek et al. 2006; Shultis et al. 2006). These results leave no doubt of the functional importance of the interaction between these two regions, yet they do not disclose how TonB harvests the proton-motive force energy of the cytoplasmic membrane, how it reacts to the proton-motive force, how it transmits the energy to the outer membrane transporters, and how the transporters react to the energy input.

It is known that energization causes conformational changes in TonB (Ghosh and Postle 2005). It is thought that TonB in the energized conformation induces a conformational change in the outer membrane transporters such that the substrates are released from their binding sites and the hatch moves either to the side or out of the β-barrel to allow diffusion of the released substrates through the pore of the β-barrel into the periplasm.

Conformational changes also arise upon binding of substrate to the transporters, yet, of the crystal structures determined for six outer membrane transporters, only that of the FecA ferric citrate (Fec) transporter shows such conformational changes great enough to close access to the substrate binding site. Upon binding of diferric dicitrate, surface loops 7 and 8 move to close the binding site; ferric citrate can then only move through FecA into the periplasm and cannot escape to the medium (Ferguson et al. 2002). Interestingly, loops 7 and 8 do not move when two molecules of citrate bind to FecA at the same site as diferric dicitrate and in a similar manner (Yue et al. 2003). In the crystal structures of the five other transporters, substrate binding causes movements of loops to different degrees, but the substrate entry site is never closed.

FhuA, An Outer Membrane Transport Protein

Probing structural requirements of transport activities using mutational analyses

FhuA serves as the transporter for the Fe^{3+}-ferrichrome, the structurally related antibiotic albomycin, and the toxic peptide microcin J25. In addition, it functions as the receptor for colicin M and the phages T1, T5, and Φ80. The multitude of ligands offers a variety of functional tests to characterize mutants. Phage T1 and Φ80 adsorption serve to define the energy-requirement since *tonB* mutants and cells de-energized by energy

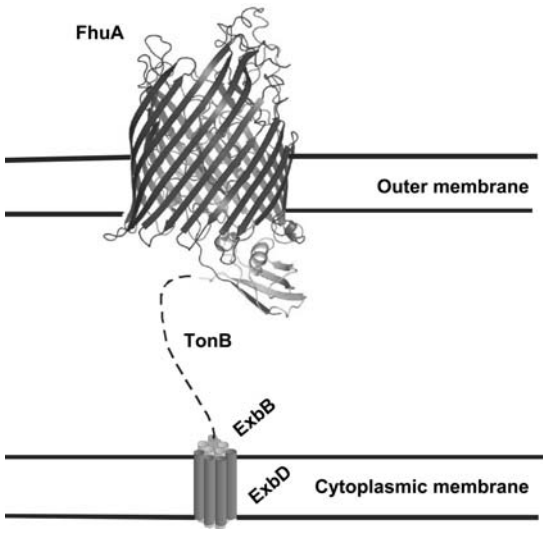

Fig. 1 Model showing the coupling of the FhuA outer membrane transport protein to the energy-transducing device consisting of the TonB, ExbB, and ExbD proteins. Only the C-terminal part of TonB was co-crystallized with FhuA (Pawelek et al. 2006). Localization of TonB and ExbD is similar. Both are anchored to the cytoplasmic membrane and extend into the periplasm. ExbB crosses the cytoplasmic membrane three times, with the N-terminus in the periplasm and most of the protein in the cytoplasm. Only the transmembrane portions of ExbB and ExbD are indicated; these portions interact with TonB

inhibitors adsorb the phages reversibly and are not infected. The analysis of heme-deficient mutants that no longer synthesize cytochromes and an ATPase mutant reveal that the cytoplasmic membrane must be energized either by the electron transport chain or by ATP hydrolysis (Hancock and Braun 1976). The analysis of FhuA and TonB mutants, however, could not take advantage of the many ligands because most mutants are more or less equally affected in transport and receptor functions.

TonB box mutants

The TonB box in the N-terminal region of FhuA contacts the C-proximal region of periplasmic TonB. Mutations in the TonB box affect FhuA activities and are suppressed by mutations in TonB. Mutation V11D (Val-11 replaced by Asp) in the TonB box (residues 7–11) inactivates FhuA in all TonB-related activities but fully retains sensitivity to phage T5, which does not require TonB for infection (Schöffler and Braun 1989; Endriß et al. 2003) (Table 1). Mutation I9P strongly reduces all FhuA activities except sensitivity to phage T5. Both mutations can be suppressed by the Q160L or the Q160K mutation in TonB, which partially restore all TonB-dependent FhuA functions (Table 2). Another mutation in TonB, only two residues away from the mutations at residue 160, R158L, partially suppresses the I9P FhuA mutation (Günter and Braun 1990).

The site on TonB that interacts with the FhuA TonB box has been determined using in vivo cross-linking of cysteine residues inserted into the TonB box with cysteine residues introduced into TonB. FhuA(I9C) and FhuA(V11C) are transport and receptor active in cells with wild-type TonB (Table 1). In cells synthesizing TonB(Q160C), FhuA(I9C) and FhuA(V11C) spontaneously form in vivo disulfide bridges with TonB(Q160C). In contrast, FhuA(I9C) does not cross-link to TonB(Y163C), and FhuA(V11C) cross-links to TonB(Y163C) to a minor extent. Unfortunately, the activities of cross-linked FhuA-TonB cannot be determined because of substantial amounts of un-cross-linked proteins. This applies also for the similar cross-links of FecA (see section on the Fec signaling pathway) (Ogierman and Braun 2003) and of BtuB (Cadieux and Kadner 1999) to cysteine residues introduced around residue 160 of TonB. The spontaneous in vivo cross-linkages formed strongly indicate that the TonB boxes interact with region 160 of TonB. They also demonstrate a rather high tolerance to alterations in the TonB box sequence without affecting TonB box function. This finding and the suppression of TonB(Q160L) and TonB(Q160K) by the same TonB box mutations indicate a minor role for specific amino acid side-chain interactions and a stronger effect of the TonB box conformation.

In the Fec transporter FecA, deletion of the DALTV TonB box (residues 80–84) inactivates the transport and induction activities (Ogierman and Braun 2003). Replacement of the DALTV FecA TonB box by the DTITV TonB box of FhuA or the DTIVV TonB box of FepA retains the induction and transport activities, although FecA(DTIVV) displays only 50% of the wild-type

Table 1 Phenotype of TonB box mutants of various TonB-dependent receptors

FhuA 7-DTITV-11		Transport	Receptor	FecA 80-DAL TV-84		Transport	Induction	FepA 12-DTI VV-16		Transport	Receptor	BtuB 6-DTL VV-10		Transport	Receptor	CirA 6-ET MVV-10		Transport	Receptor
D	D7G	a	a	D	D80C	pa	pa	D				D	D6P	a	a	E			
	D7G T8N	a	a		D80G, A81T, L82N	i	i						D6C	a	nd				
	D7G T8S	a	a																
	D7G I9N	i	pa									T	T7P	a	a	T			
													T7C	a	nd				
T	T8I	a	a	A	A81C	a	a	T											
	T8N	a	a		A81T, V84R	a	a					L	L8P	i	a	M	M8R	nd	a
	T8S	a	a										L8C	a	nd		M8V	nd	a
I	I9S	pa	pa	L	L82N	i	i	I	I14S	i	i	V	V9P	a	a	V			
	I9P	i	pa		L82P	i	i		I14P	i	i		V9C	a	nd				
	I9C	a	a		L82C	a	a						V9G	pa	nd				
	I9T	a	a																
T				T	T83C	a	a	V				V	V10G	i	nd	V	V10G	nd	i
													V10C	a	nd		V10A	nd	a
V	V11D	i	i	V	V84G	i	i	V	V16D	i	i		V10P	I	a				
	V11C	a	a		V84C	a	pa		V16L	i	pa								
	V11A	pa	pa		V84R	i	i					T	T11P	a	pa				
													T11C	a	nd				
	Δ7–11	i	i		Δ80–84	i	i												

Additional BtuB TonB box mutants display a transport-active phenotype: D6E, D6G, T7A, T7N, T7I, T7S, L8R, L8N, L8E, L8I, V9A, V9C, V9I, V9L, V9T, V10A, V10I,V10D, T11N, T11I

a, active; i, inactive; pa, partially active; nd, not determined

Table 2 TonB suppressor mutants restore transport of inactive outer membrane transport mutants

	TonB							
	Wild-type	R158L	N159C	Q160L	Q160K	Q160P	Q160C	Q162C
FhuA								
Wild-type	a	a	nd	a	a	nd	nd	nd
D7G	a	nd	nd	a	a	nd	nd	nd
D7G T8N	a	nd	nd	a	a	nd	nd	nd
D7G T8S	a	nd	nd	a	a	nd	nd	nd
D7G I9N	i	nd	nd	i	pa	nd	nd	nd
T8I	a	nd	nd	a	a	nd	nd	nd
T8N	a	nd	nd	a	a	nd	nd	nd
T8S	a	nd	nd	a	a	nd	nd	nd
I9S	pa	nd	nd	pa	pa	nd	nd	nd
I9T	a	nd	nd	a	a	nd	nd	nd
I9P	i	pa	nd	i	pa	nd	nd	nd
V11D	i	i	nd	i	i	nd	nd	nd
V11A	pa	pa	nd	pa	pa	nd	nd	nd
FecA								
Wild-type	a	a	nd	a	a	nd	nd	nd
L82P	i	i	nd	i	i	nd	nd	nd
L82N	i	i	nd	i	i	nd	nd	nd
L84G	i	i	nd	i	i	nd	nd	nd
V84R	i	i	nd	i	i	nd	nd	nd
A81T V84R	a	pa	nd	a	a	nd	nd	nd
D80G A81T L82N	i	i	nd	i	i	nd	nd	nd
BtuB								
Wild-type	a	nd	a	a	a	a	a	a
L8P	i	nd	pa	a	a	pa	a	pa
V10P	i	nd	pa	a	i	i	a	pa
V10G	i	nd	nd	a	a	a	nd	nd

a, active; i, inactive; pa, partially active; nd, not determined

transport rate. DAPTV, DALTG, DALTR, DANTV, and GTNTV TonB box derivatives of FecA are inactive, and none of these mutations are suppressed by TonB(Q160L), TonB(Q160K), or TonB(R158L) (Habeck 1998). However, FecA(DTLTR) is fully active with wild-type TonB and all TonB mutations other than TonB(R158L), which partially inactivates FecA (DTLTR) (Table 2). Replacement of the indicated FecA TonB box residues by cysteine residues retains the FecA transport and regulatory activities, except FecA(D80C) that shows 40% of the wild-type inductions level and is nearly transport inactive, and FecA(V84C) that shows 63% of the wild-type induction level.

The crystal structures of FhuA and BtuB bound to TonB(158–235) and to TonB(153–233) have been used to analyze these mutations further. Larger TonB fragments were used for the crystallization, but only these fragments diffracted well. Suppression is not allele specific. Restoration is not caused by pair-wise interaction of altered amino acid residues that fit together in the double mutant as the unaltered amino acids did in the wild-type. Rather, the FhuA and BtuB mutations apparently alter the conformations such that the disrupted side-chain interactions in these mutants are partially restored in the suppressor mutants. Detailed analyses of TonB box mutations in BtuB suppressed by mutations TonB(Q160L) and TonB(Q160K) (Table 1) have revealed conformational changes in TonB (Cadieux et al. 2000; Coggshall et al. 2001).

Hatch mutants

The hatch tightly closes the pore of the β-barrel of FhuA. In the crystal structure, residue E56 of FhuA in the hatch domain is closely oriented to residue R166 of TonB. This electrostatic

interaction could play a role in transmitting conformational energy from TonB to FhuA. However, results obtained with mutants do not support this theory. Mutants FhuA(E56A), FhuA(E56R), and FhuA(E56K) reduce ferrichrome transport only to 97, 81, and 86% of the wild-type rate, respectively. Mutants FhuA(E56R) and FhuA(E56K) fully retain sensitivity to albomycin, microcin J25, colicin M, and the phages T1, Φ80, and T5, and FhuA(E56A) yields turbid zones of growth inhibition with all ligands at all tested ligand concentrations.

If energized TonB allosterically interacts with FhuA and induces in FhuA a conformational change that (1) allows binding of phages T1 and Φ80, followed by infection; (2) releases the bound substrates from their binding site; and (3) opens the channel, then the linker between the TonB box and the hatch should be indispensable for TonB-dependent FhuA activities. Again, mutant analyses do not support this hypothesis. Deletion of residues 13–20 or 24–31, duplication of residues 23–30, and mutations affecting the conformation, e.g., P15A and P17A, have either no or only a slight effect on the FhuA transport and receptor activities (Endriß et al. 2003).

β-barrel mutants

In FhuA and other outer membrane transporters, the pore is formed by a β-barrel. The same charged residues at equivalent locations in the transporters are positioned such that they can form salt bridges between the hatch and the β-barrel. Residue R93 of FhuA bridges with residues E522 and E571, and residue R133 bridges with E522. Mutations that disrupt the salt bridges or even lead to repulsion between the side chains do not inactivate FhuA. However, mutant FhuA(E522R) is inactive in ferrichrome transport and receptor functions, and mutant FhuA(E571R) is inactive in transport but retains receptor activities. The double mutants FhuA(R93L E522R) and FhuA(R93E E522R) show the same phenotype as the single mutant FhuA(E522R), and the double mutant FhuA(R93L E571R) shows the same phenotype as the single mutant FhuA(E571R), which demonstrates that it is not the salt bridge that is important but rather that the introduced arginine residues are not tolerated (Endriß et al. 2003).

The crystal structures of all six outer membrane transporters known to date show a conserved motif in β21. In FhuA, this motif (residues 680–685) has the sequence VNNLFD (Fig. 2). In this region, starting with the sequence NLFD (residues 682–685) in FhuA, β21 is part of a loop that does not form an anti-parallel β-strand pair with β22. With the notion that this exposed structure might interact with other components of the outer membrane for FhuA membrane insertion, various deletions and amino acid replacements were constructed and the functions of the resulting FhuA derivatives were tested. Deletion of V680, N682, L683, F684, D685, or NLFD, or replacement of NLFD by AAAA reduces the ferrichrome transport rate to ~70% of the FhuA wild-type level, but does not affect the FhuA receptor activities. Excision of HVNNLF, including the sequence HVN involved in the anti-parallel strand interaction with β22, abolishes all FhuA activities and no FhuA is found in the outer membrane. This finding suggests that the region is critical for FhuA assembly and insertion into the outer membrane.

In contrast, deletions of larger regions involved in β-strand interactions are tolerated. For example, FhuAΔ322–355 and FhuAΔ335–355 result in FhuA derivatives that are inserted into the outer membrane (Killmann et al. 1996). Replacement of the ferrichrome binding F693 with A abolishes ferrichrome binding but reduces ferrichrome transport only to 76% and does not affect the receptor activities. F693 is only one of ten ferrichrome binding amino acids. Replacement of the FhuA fragment 675–724 by the homologous FecA fragment 700–741 results in a FhuA-FecA hybrid protein with no activity except that it confers full sensitivity to phage T5. A FhuA-BtuB hybrid protein (replacement with the homologous fragment 560–594 of BtuB) has no activity except full sensitivity to phages T5 and T1. The hybrid proteins apparently are mainly affected in the TonB-dependent FhuA activities. The FhuA-BtuB hybrid has a residual activity high enough to confer phage T1 sensitivity, as occasionally observed with mutants in TonB-dependent functions (Langenscheid et al. 2004).

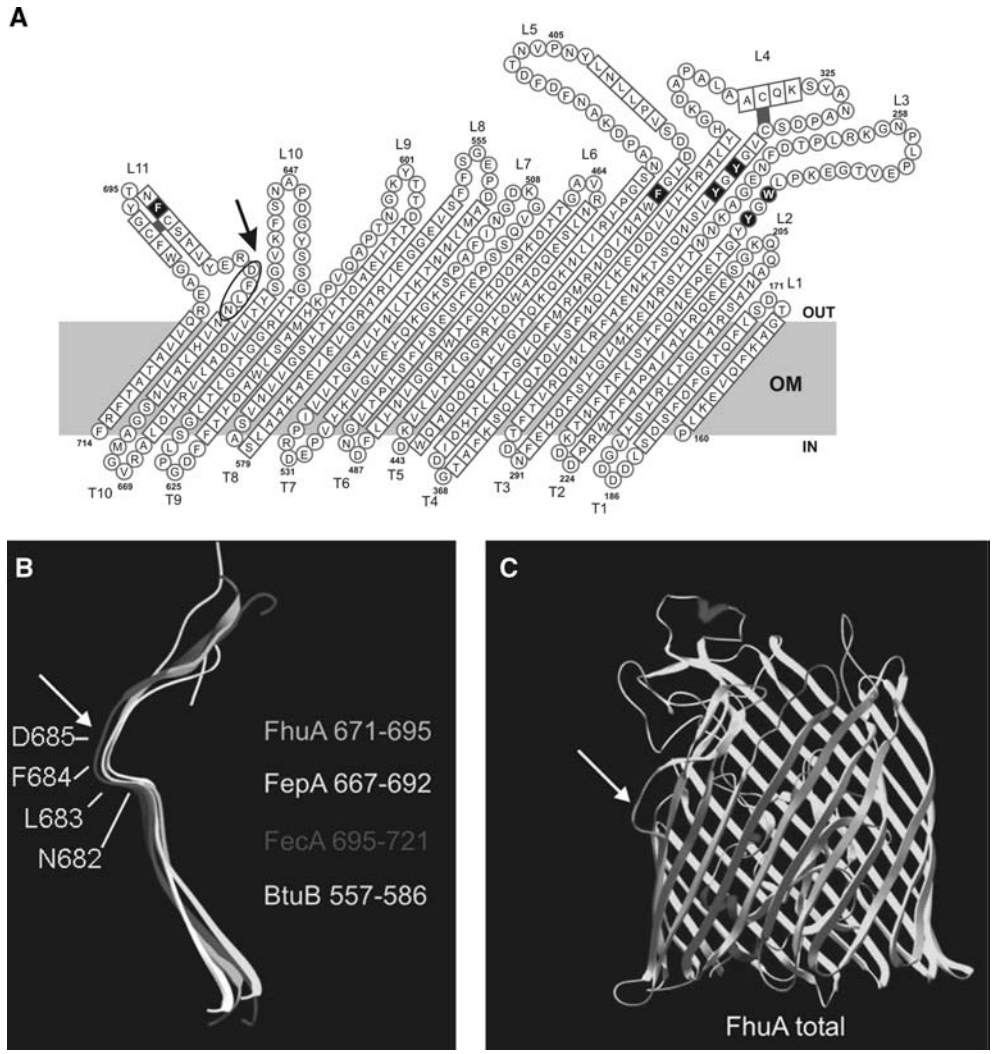

Fig. 2 (**A**) Transmembrane arrangement of the FhuA protein. The conserved domain β21, NLFD in FhuA, that forms part of a loop and does not form an anti-parallel β-strand pair with β22, is *circled* and indicated with an *arrow*. Aromatic ferrichrome binding sites are boxed in *black*, and the two disulfide bonds are shown in *gray*. (**B**) Conserved conformation of the loop in *E. coli* outer membrane transporters. (**C**) Illustration of how the loop (*arrow*) extends from the β-barrel of FhuA

Ferrichrome diffusion pathway

In LamB, a series of aromatic amino acids (''greasy slide'') facilitate diffusion of maltodextrins. Replacement of individual aromatic amino acids with alanine decreases maltose uptake to less than 10% of the wild-type level (van Gelder et al. 2002). Along these lines, it has been proposed that ferrichrome moves through FhuA from the cell surface along low-affinity binding sites (a slide) into the periplasm (Ferguson et al. 2001). To test this hypothesis, one or two of the amino acid residues along the proposed slide—N427 (close to the ferrichrome binding site), Q429, Q431, D579, D358, Q360, N299, and R297 (the latter close to the periplasm)—were replaced by glycine. Single and double mutants displayed ferrichrome transport rates between 84 and 110%

of the FhuA wild-type level. The data do not support the involvement of these residues in ferrichrome diffusion through the channel. An exception is FhuA(N299G), which has an increased ferrichrome binding of 183% and a transport rate of 128% of the wild-type level. Since N299 does not directly bind ferrichrome, conversion to glycine might affect the stereochemistry of the binding site such that binding of ferrichrome is enhanced. A strong increase in ferrichrome binding, however without alteration of the ferrichrome transport rate is also observed with FhuA(N427G) and FhuA(Q429G). All of the mutant FhuA receptor activities correspond to those of the FhuA wild-type.

Residues Y105, G108, K110, and N150 of FhuA extend into the space between the hatch and the barrel. Y105 and N150 are highly conserved among energy-coupled outer membrane import proteins. Each of these four residues were replaced by alanine to examine whether they facilitate diffusion of ferrichrome. The mutants display wild-type ferrichrome transport rates and unaltered receptor activities, except FhuA (K110A), which is resistant to microcin J25. Therefore, K110 might be specifically required for microcin J25 uptake. Mutants K110A and N150A are slightly sensitive to rifamycin at a concentration to which the wild-type is resistant, which suggests facilitation of the antibiotic permeation through the outer membrane. Replacement of the small G108 residue by a bulky tryptophan did not change the ferrichrome transport rate (Langenscheid 2003; Langenscheid et al. 2004), which indicates that the pore at the interface between the hatch and the β-barrel can be narrowed without reduction of ferrichrome transport.

Fixation of the hatch to the β-barrel

The FhuA crystal structure starts with residue 19; residues 1–18 are disordered in substrate-free and substrate-loaded FhuA. Residue 19 moves 17 Å upon binding of ferrichrome. Fixation of residue 27 to residue 533 through a disulfide bridge formed by introduced cysteine residues (T27C, P533C) abolishes transport of ferrichrome and sensitivity to albomycin and microcin J25, and these functions are restored when the disulfide bond is reduced with dithiothreitol. However, sensitivity of cells to colicin M and the phages T5 and Φ80 is largely retained in the mutant (Endriß et al. 2003). This receptor activity might arise from residual uncross-linked T27C and P533C, which would suffice for phage and colicin infection but not for transport since the number of FhuA molecules required for infection is lower than the number necessary for a discernible transport rate. When cross-linking is enhanced by oxidation with 100 μM $CuSO_4$ in the nutrient agar plates used for the assays, sensitivity to colicin M is abolished and sensitivity to phages T1 and Φ80 is reduced at least tenfold, resulting in turbid plaques. Since sensitivity to phage T5 is unaltered, fixation of the hatch to the β-barrel close to the periplasm affects only the TonB-dependent activities of FhuA. These activities seem to require a flexible N-proximal FhuA region. It remains unclear whether the C27–C533 cross-link prevents movement of the hatch out of the β-barrel.

In an attempt to prevent movement of the hatch out of the β-barrel, amino acids located inside the β-barrel were replaced with cysteine residues. Cross-linking of S137C with S499C results in turbid zones of growth inhibition in the presence of colicin M, albomycin, or microcin J25. Sensitivity to phages Φ80 and T5 is unaltered. The yield of cross-linking is increased by treatment with $CuSO_4$ but is than the yield of cross-linking obtained with C27 and C533 (nearly 100%). R93C cross-links to E522C only to a small extent; the receptor activity is unaltered and decreases only tenfold upon addition of $CuSO_4$. These data do not allow the conclusion that the hatch moves out of the barrel. Additional disulfide bonds were created between L109C and S356C and between Q112C and M383C (Eisenhauer et al. 2005). The moderate spontaneous yield in disulfide linkage was greatly increased by treatment with $CuSO_4$. Cells containing the highly cross-linked FhuA derivatives transport ferricrocin (a derivative of ferrichrome) with yields similar to those of the wild-type. Apparently, during transport residues 109–112 remain in close proximity to the inner barrel wall along the putative siderophore transport pathway.

Outer Membrane Regulatory Proteins

The Fec signaling pathway

The Fec signaling pathway has recently been described in detail (Braun et al. 2006). Therefore, the Fec regulatory model will be only briefly described, and new data on similar systems will be presented.

Ferric citrate is used as an iron source by *E. coli* K-12. Diferric dicitrate binds to the FecA outer membrane protein. This protein has two functions: it transports Fec into the periplasm, and it regulates transcription of the *fecABCDE* transport genes.

Binding of diferric dicitrate causes major structural changes in FecA. Two surface loops, loops 7 and 8, move 11 and 15 Å, respectively, and close the entry to the diferric dicitrate binding site (Ferguson et al. 2002; Yue et al. 2003). At the opposite side of FecA, exposed to the periplasm, a short α-helix is then no longer observed in the crystal structure, probably because of flexibility of the unwound helix. These structural changes are required to release diferric dicitrate from its binding site and to move the hatch in the β-barrel so that diferric dicitrate can pass through FecA into the periplasm. In addition, it is likely that the structural transitions in FecA enhance interaction with TonB, which is required for transport and regulation, and with FecR, which is required for transcription regulation.

FecR receives the initiation signal for *fecABCDE* transcription from FecA. FecR is well suited for this function since most of the protein is located in the periplasm, where it interacts with the periplasmic signaling domain of FecA. FecR transmits the signal across the cytoplasmic membrane through its transmembrane segment into the cytoplasm, where it interacts through its short cytoplasmic portion with the FecI sigma factor. FecI recruits the RNA polymerase and directs it to the promoter upstream of the *fecA* gene. This sequence of reactions was established by mutational analyses and determination of domain interactions between FecA and FecR, FecR and FecI, FecI and RNA polymerase, and FecI/RNA polymerase and the *fecA* promoter region (summarized in Braun 1997; Braun et al. 2003, 2006; Braun and Mahren 2005).

The N-terminal residues (1–79) of FecA form the signaling domain. This domain is only found in those outer membrane transporters that regulate gene transcription. The signaling domain is not seen in the FecA crystal structure because of its flexibility, as shown by NMR analysis of the isolated domain, which shows a distinct novel fold (Garcia-Herrero and Vogel 2005). Randomly generated point mutations in the FecA signaling domain are all located on one side of the signaling domain structure, which involves helices α1 and α3, and β-strands β1 and β3. In addition, two FecA mutations that suppress mutations in the C-proximal region of FecR and restore the FecR regulatory activity to 36% of the wild-type activity are both located on this side. These results indicate that this side of the FecA signaling structure forms the interface to the C-proximal region of FecR (Breidenstein et al. 2006). Unexpectedly, some of the mutations in the FecA signaling sequence that reduce transcription also strongly impair transport (Fig. 3). At first this finding was astounding since deletion of the signaling domain does not affect transport. However, there must be a strong structural coupling involving the hatch domain, the β-barrel, and the signaling domain during initiation of *fecABCDE* transcription upon binding of diferric dicitrate to FecA. It is conceivable that the structural coupling not only occurs from the hatch and β-barrel domains to the signaling domain, but also from the signaling domain to the hatch and β-barrel. Therefore, structural changes in the signaling domain not only reduce functional interaction with FecR, resulting in lower transcription initiation, but also generate structural transitions within FecA, resulting in lower transport rates.

fecA is the only regulatory gene that is induced by diferric dicitrate. The other two regulatory genes, *fecI* and *fecR*, are regulated by iron via the Fur protein. Binding of Fe^{2+} to Fur converts Fur into a repressor that inhibits *fecIR* transcription. As long as there is enough iron in the cells, the Fec transport system is not induced. Upon iron starvation, *fecIR* are transcribed, but the adjacent *fecABCDE* transport genes remain silent. Transcription of these genes requires the cognate

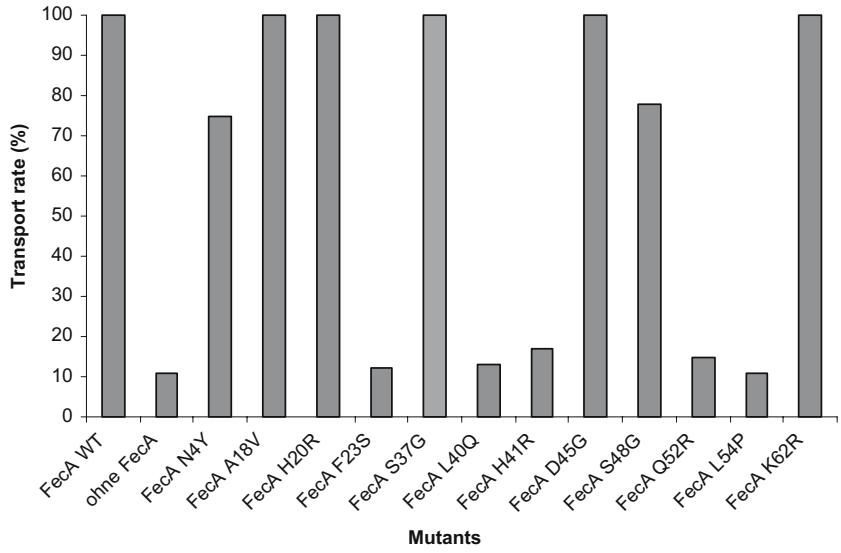

Fig. 3 Transport rates (percentage of the wild-type level) of FecA derivatives with point mutations in the predicted interface between FecA and FecR (Fig. from E. Breidenstein, Diplomarbeit, Universität Tübingen, 2006)

substrate in the medium. The signal, initiated by binding of diferric dicitrate to FecA, is transmitted via FecR to FecI and the RNA polymerase. The amount of FecA far exceeds the amount of all other Fec proteins so that there is sufficient FecA in the uninduced state to initiate induction under iron-deplete, ferric-citrate-replete conditions. When Fec has filled up the iron pool of the cells, Fe^{2+} binds to Fur, which represses *fecIR* and *fecABCDE* transcription. Repression of *fecABCDE* by Fe^{2+}-Fur leads to a rapid adaptation of iron uptake, which otherwise would require dilution of FecIR to a level that would allow induction to be terminated.

Fec-type regulation as a paradigm of similar regulatory devices in bacteria

Although the Fec transport system occurs in few bacterial species other than *E. coli* K-12, the Fec type of regulatory mechanism is more widespread. The presence of the Fec transport system in the genetically and physiologically well-known *E. coli* K-12 allowed its mode of action to be unraveled. Only 9 of 23 to date complete or partial *E. coli* and *Shigella* genome sequences encode the *fec* system. It is not found in uropathogenic *E. coli*. One out of three studied *Klebsiella pneumoniae* strains and *Photorhabdus luminescens* encode a complete Fec system (Mahren et al. 2005). *Shigella flexneri* 2a YSH6000 encodes *fecIRABCDE* on a pathogenicity island (Luck et al. 2001). The virulence plasmid pLVPK of *K. pneumoniae* encodes an incomplete *fec* operon with highly mutated *fec* genes (truncations, additions, point mutations), which signifies an accumulation of mutations in genes that are not used. An incomplete *fec* system is also found in *Aerobacter aerogenes*. The *fec* genes were disseminated between bacteria by horizontal gene transfer, as evidenced by IS (insertion) elements flanking the *fec* locus in *E. coli* K-12 and their localization on chromosomal pathogenicity islands and plasmids.

Numerous sigma factors with similarity to FecI occur in bacteria. Since FecI responds to an external signal, it is classified as a member of the extracytoplasmic sigma factors (ECF) of the σ^{70} family. Of the many ECFs classified, only a few have been shown to function similarly to FecI.

Pseudomonas putida encodes 11 FecIRA homologs. One of these, PupIR, regulates the synthesis of the PupB outer membrane protein (Koster et al. 1994). PupB transports Fe^{3+} via the siderophore pseudobactin BN8, which serves as inducer. Based on genome sequence analysis, *Pseudomonas aeruginosa* is predicted to encode eight FecIRA homologs and four FecIR homologs (Braun and Mahren 2005). One set of these is involved in ferric pyoverdine uptake. Induction

by ferric pyoverdine involves the outer membrane protein FpvA, the cytoplasmic membrane protein FpvR, and actually two FecI type sigma factors, PvdS and FpvI (Beare et al. 2003). FpvI mediates transcription of the *fpvA* gene, PvdS mediates transcription of genes for pyoverdine biosynthesis enzymes and the virulence factors exotoxin A, alkaline protease AprA, and an endoproteinase. In *Serratia marcescens*, heme uptake is mediated by the secreted HasA protein, which releases heme from hemoglobin and donates it to the HasR outer membrane transporter. Both heme and HasA must bind to HasR to induce transcription of the heme transport genes. A transport-inactive HasR mutant still induces transcription, which shows that signaling is independent of transport and initiates at the cell surface (Cwerman et al. 2006). Heme uptake into *Bordetella* strains is also regulated by a Fec-type control system. Two regulatory genes are encoded upstream of the heme transport genes, which are transcribed in response to heme in the medium. The regulatory genes show 44 and 28% sequence identity with *fecI* and *fecR*, respectively (Vanderpool and Armstrong 2003; Kirby et al. 2004).

Of the FecIR-type regulatory systems that have not been characterized experimentally, those of *Bacteroides thetaiotaomicron* are particularly noteworthy since 51 FecI type sigma factors are predicted from the genome sequence, of which 4 *fecI* homologs are linked to *fecR* homologs and 23 are linked to *fecRA* homologs (Braun and Mahren 2005). Linkage to *fecR* and *fecA* homologs suggests a regulatory mechanism similar to *fec* gene regulation. A plethora of predicted outer membrane proteins enables *B. thetaiotaomicron* to take up sugars derived from the host and the diet. Only one uptake system has been partially characterized. Uptake of maltooligosaccharides is mediated by the outer membrane proteins, designated SusC and SusD (Cho and Salyers 2001). The transporter genes are frequently clustered together with glycoside hydrolases and ECF sigma factors (Bjursell et al. 2006), which suggests that the ECF sigma factors regulate polysaccharide degradation and sugar transport. Six TonB homologs of the size of *E. coli* TonB and two larger homologs are predicted.

Sugar Versus Iron Transporters

In the above-mentioned study of starch utilization by *B. thetaiotaomicron*, SusCD-dependent growth, binding, and induction by starch and maltodextrins was determined, but transport was not measured. This has been done with a maltodextrin transport system of *Caulobacter crescentus*. The genome predicts 67 outer membrane transporters. One of them, designated MalA, is required for growth on maltodextrins larger than maltotetraose (Neugebauer et al. 2005). Deletion of the *malA* gene diminishes the maltose transport rate to 1% of the wild-type level. Deletion of two genes homologous to the *E. coli exbB exbD* genes abolishes transport. This finding suggests an energy-coupled MalA-mediated outer membrane transport. This notion is supported by the K_d of maltodextrin transport, which is 1,000-fold lower than the K_d of the *E. coli* LamB protein, which facilitates maltodextrin diffusion. However, the K_d is 100-fold higher than that of *E. coli* ferric siderophore transporters.

It is predicted that substrates distinct from ferric siderophores, heme, Fe^{3+}, and vitamin B_{12} are transported across the outer membrane of gram-negative bacteria by an energy-consuming process, provided they occur in such a low concentration in the medium that they do not support growth if they merely diffuse through the outer membrane. Tight binding to outer membrane proteins concentrates the substrates at the cell surface, from where they are actively transported across the outer membrane into the periplasm, and from there across the cytoplasmic membrane into the cytoplasm.

Concluding Remarks

Energy-coupled outer membrane transport and signaling is a fascinating scientific field that can only be approached by using a variety of techniques, such as determination of structures and the interaction of proteins and protein subdomains, and currently and most importantly, reconstitution of TonB, ExbB, and ExbD in liposomes to determine whether they form a complex and a proton-conducting channel and

how the proteins react to the proton-motive force. The next, presumably more difficult step would be to connect the reconstituted complex to an outer membrane transporter and to determine whether substrates flow through the transporters in response to an artificial membrane potential applied to the liposomes. With FhuA it would also be possible to determine the response of the transporter and receptor by measuring inactivation of phage Φ80, which requires coupling of FhuA to an energized cytoplasmic membrane (Hancock and Braun 1976). This is a very sensitive test since single phage particles can be measured.

Numerous protein complexes have been isolated from the cytoplasmic membrane, e.g., ATPase, photosynthesis reaction centers, succinate dehydrogenase, cytochrome oxidase, formate dehydrogenase/nitrate reductase, and proteins of the protein secretory system. Protein import into mitochondria is a similarly complex problem that involves an outer and inner membrane import apparatus and sorting to the outer membrane, the intermembrane space, the inner membrane, and the matrix. Highly sophisticated methods have resulted in a well-advanced understanding of mitochondria protein import (Wiedemann et al. 2006). Similar methods can be used to isolate TonB, ExbB, and ExbD, to reconstitute the complex, and to determine its mode of action. It is likely that, as in the mitochondria protein import research, proteins in addition to TonB, ExbB, and ExbD will be discovered that participate in energy transfer from the cytoplasmic membrane into the outer membrane.

Acknowledgments We thank K. Hantke for discussions and genome analyses, M. Braun for constructing some of the FhuA mutants, and K. A. Brune for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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