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TonB-dependent transporters: regulation, structure, and function

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Abstract

TonB-dependent transporters (TBDTs) are bacterial outer membrane proteins that bind and transport ferric chelates called siderophores, as well as vitamin B_{12}, nickel complexes, and carbohydrates. The transport process requires energy in the form of protonmotive force and a complex of three inner membrane proteins, TonB-ExbB-ExbD, to transduce this energy to the outer membrane. The siderophore substrates range in complexity from simple small molecules such as citrate to large proteins like serum transferrin and haemoglobin. Because iron uptake is vital for almost all bacteria, expression of TBDTs is regulated in a number of ways that include metal-dependent regulators, σ/anti-σ factor systems, small RNAs, and even a riboswitch. In recent years many new structures of TBDTs have been solved in various states, resulting in a more complete picture of siderophore selectivity and binding, signal transduction across the outer membrane, and interaction with TonB-ExbB-ExbD. However, the transport mechanism is still unclear. In this review, we summarize recent progress in understanding regulation, structure and function in TBDTs and questions remaining to be answered.

Keywords

TonB-dependent transporter; outer membrane protein; siderophore; active transport; signal transduction

Introduction

Transport into Gram-negative organisms is initiated by passage of the transported species across the outer membrane and into the periplasmic space prior to inner membrane translocation. The uptake of iron is particularly important for bacterial growth (71) and synthesis of outer membrane iron transporters (called TonB-dependent transporters, TBDTs) is therefore regulated in a variety of ways. While iron complexes constitute the majority of substrates for TBDTs, vitamin B_{12}, nickel chelates, and carbohydrates are also transported by this mechanism (75). These transporters show high affinity and specificity for metal chelates called siderophores and require energy derived from the protonmotive force across the inner membrane to transport them (33, 87). To tap this energy source, TBDTs must interact with an inner membrane protein complex consisting of TonB, ExbB, and ExbD (72).

The first crystal structures of two *Escherichia coli* TonB-dependent transporters, ferrichrome transporter (FhuA) (34, 54) and ferric enterobactin transporter (FepA) (10), showed that TBDTs use a 22-stranded β-barrel to span the outer membrane with an

unanticipated ‘plug’ domain folded into the barrel interior. The plug domain functions to bind a specific metal chelate at the extracellular side of the membrane and to interact with TonB-ExbB-ExbD at the periplasmic side of the outer membrane. In these ‘ground state’ structures, the plug domain completely occludes the barrel pore, revealing an unexpected complexity for siderophore transport. There has been significant recent progress in structure determination of TBDTs, with a total of 45 structures solved to date, representing 12 unique transporters. In this review, we summarize new data on the complex regulation of TBDTs, structural similarities and differences, and new functional data pertaining to the transport mechanism. We will focus on *E. coli*, but include information on other Gram negative bacteria where appropriate.

Synthesis of TBDTs is Regulated at Multiple Levels

Genes encoding the seven TBDTs in *E. coli* are scattered throughout the chromosome. Several of them, *btuB*, *fhuE*, and *cirA*, are transcribed as monocistronic units. In contrast, *fecA* and *fhuA* are the first genes of multicistronic operons, *fecABCDE* and *fhuACDB* respectively. In these cases, the downstream genes encode ABC transporters that transport the siderophores across the cytoplasmic membrane. Downstream of *fepA* is the gene *entD*, involved in the synthesis of the enterobactin siderophore. Finally, *fiu* is followed by the *ybiX* and *ybiI* genes, but whether or not they form an operon has not been investigated. Expression of all of these genes is highly regulated both at the transcriptional and post-transcriptional levels. These controls can limit the synthesis of TBDTs when they are not needed, which could be beneficial since some of these outer membrane proteins are also used by phages and colicins to enter the bacterial cell (12).

Fur repressor regulates transcription of TBDTs for ferric siderophores

Although iron is essential for most living organisms, iron accumulation can be toxic because it may lead to production of reactive radicals (46) and it is therefore crucial to keep cellular iron levels under tight control. In *E. coli*, the Fur (Ferric Uptake Regulator) transcriptional repressor plays a key role in this process by regulating expression of genes involved in iron homeostasis as a function of cellular iron concentration (43). In the presence of iron, Fur binds DNA sequences referred to as Fur boxes using Fe^{2+} as a cofactor and thereby represses expression of dozens of genes (3). When iron is limiting, Fur cannot bind DNA, leading to derepression of genes that encode iron transporters and proteins involved in siderophore biosynthesis and iron metabolism, but also other cellular functions (13, 80). Somewhat surprisingly, several genes were reported to be positively regulated by Fur. This apparent puzzle was solved when Massé and Gottesman identified a regulatory small RNA, RyhB, whose transcription is repressed by Fur and which, in turn, negatively regulates expression of numerous genes (58).

Consistent with their role in iron transport, all TBDTs for ferric siderophores are controlled by Fur and their expression is therefore repressed when iron reaches a certain level (Figure 1). Fur binds *in vitro* to the promoter regions of *fepA-entD* (45), *fecABCDE* (2), *fhuACDB* (13) and *cirA* (39). In addition, Fur boxes were identified not only in these promoter regions, but also upstream of *fhuE* and *fiu*genes (13 and references therein, 64). Interestingly, Fur also directly represses transcription of the *tonB* gene (1, 89) as well as the *exbB-exbD* operon by binding upstream of *exbB* (13).

Transcription of *fecA* requires an ECF σ factor

Transcription of the *fecABCDE* operon is dependent on the minor σ factor FecI (σ19). FecI belongs to the group of ECF (Extracytoplasmic Function) σ factors, also known as group IV. ECF σ factors are present in virtually all bacteria and regulate the expression of many genes,

including genes for periplasmic or outer membrane proteins, hence the name ECF (79). A common feature of the ECF σ factors is that their activity is regulated by an anti-σ factor, which is usually coexpressed with its cognate σ factor. In general, the anti-σ factor sequesters the σ factor under non-activating conditions and this inhibition is relieved under specific activating conditions.

There is a signal transduction cascade that leads from extracellular siderophore binding to FecA to the activation of FecI and subsequent transcription of *fecABCDE* genes (44). Upon binding ferric citrate, FecA transduces a signal across the outer membrane to FecR, an inner-membrane protein. FecR then transmits the signal across the inner membrane to FecI which directs RNA polymerase to transcribe the *fecABCDE* operon. As for other ECF σ factors, the activity of FecI is regulated by its anti-σ factor, which is FecR. However, FecR is not a 'classical' anti-σ factor because FecR is required for activation of *fecABCDE* by FecI (68). Interactions between FecA and FecR, and FecR and FecI were analyzed *in vivo* : experimental data support a model where the periplasmic N-terminal region of FecA and C-terminal region of FecR interact, while the cytoplasmic region of FecR interacts with FecI (Figure 1) (26). While structures exist for the FecA transporter (32, 90) and the FecA signalling domain that interacts with FecR (36), the details of the signal transduction cascade are not understood.

Additional transcriptional controls

Fur and FecI are probably not the only regulators affecting the transcription of genes for TBDTs. Expression of *fecA, fepA, cirA* and *fiu* was found to be increased in a mutant for the global transcriptional regulator Crp (cAMP receptor protein), both by a transcriptomic approach and by RT-PCR (92). Synthesis of these 4 TBDTs is therefore expected to be modulated not only in response to iron availability, but also to the carbon status of the cell. However, even though these effects are independent of Fur since these experiments were done in a *fur* mutant, experimental data are still lacking to discriminate between direct or indirect effects.

In addition, *fecA* expression is also increased by pyruvate, because it is regulated by dhR, a transcriptional regulator of genes involved in the energy production pathway (69). In the absence of pyruvate, PdhR represses the expression of its target genes by binding to the promoter region; when the level of pyruvate is sufficiently high however, PdhR no longer binds to DNA and repression is relieved (69, 73). PdhR was identified as a potential regulator of *fecA* expression by an algorithm developed to determine transcriptional regulatory interactions in *E. coli* on the basis of multiple microarray expression profiles (27). Furthermore, ChIP experiments indicate that PdhR could directly bind to the *fecA* promoter. Consistent with this dependence of *fecA* expression on PdhR, *fecA* expression was found to be highest in the presence of both pyruvate and citrate by real-time quantitative PCR (27).

A riboswitch controls the expression of *btuB*

Riboswitches are RNA elements that can change conformation upon specific binding of a small molecule (57, 60). Typically, they are located at the 5′ end of mRNAs and the ligand-induced conformational change directly affects, either positively or negatively, transcription or translation of the downstream gene(s). Genes controlled by riboswitches are often involved in the uptake or metabolism of the ligand, which could be a vitamin, amino acid, or nucleotide (67). Over the years, a number of experimental data suggested the existence of a riboswitch controlling the synthesis of BtuB, the transporter for vitamin B_{12} (cyanocobalamin (CnCbl)). Indeed, it has long been known that *btuB* expression is repressed when cells are grown in the presence of vitamin B_{12} (47), yet no repressor protein has been identified. Mapping of the 5′ end of *btuB*mRNA revealed a 241 nt untranslated leader (55)

that is necessary for repression of *btuB* expression by vitamin B_{12}. However, mutants defective in the production of adenosylcobalamin (AdoCbl), a downstream product of vitamin B_{12} metabolism, were known to constitutively express *btuB*, suggesting that AdoCbl, not vitamin B_{12}, may have a direct role in repression. AdoCbl was later shown to inhibit ribosome binding to *btuB* mRNA (66). Further experiments demonstrated that AdoCbl binding to the *btuB* leader induced a structural change (62). This conformational change is likely to be responsible for translational control of *btuB* expression by stabilizing a structure inhibitory for translation.

Two redundant sRNAs modulate the synthesis of CirA, FecA and FepA

Some RNA-mediated post-transcriptional controls are exerted by regulatory small RNAs (sRNAs). In the last decade, sRNAs have been recognized as major regulators of gene expression (37, 85). In most cases, bacterial sRNAs are short RNA molecules (< 250 nt), which are synthesized as discrete transcripts and act as post-transcriptional regulators. A large group of sRNAs bind the RNA chaperone Hfq, that can, among other roles, facilitate RNA-RNA interactions (6, 83). Accordingly, all sRNAs of this group have the ability to pair with one or several mRNAs, and thereby regulate their translation and/or stability.

OmrA and OmrB are two Hfq-binding sRNAs conserved in several enterobacteria. They are encoded by two adjacent genes and display almost identical 5′ and 3′ ends but a rather distinct central region. Even though OmrA and OmrB could theoretically bind different targets through their central regions, only targets that are common to both OmrA and OmrB have been identified so far and are all negatively regulated by OmrA/B. They encode several outer membrane proteins (OmpT, Cir, FecA and FepA) as well as the GntP inner membrane transporter and the EnvZ-OmpR two-component system, which itself activates transcription of *omrA* and *omrB* (40, 41, 82). Preliminary data from microarray analyses also indicate that genes downstream of *fecA* or *fepA* (ie *fecBCDE* and *entD* respectively) could be regulated by OmrA/B. This may be due to a change in mRNA stability in the presence of OmrA/B and/or to translational coupling between the different genes of a single operon.

As mentioned above, OmrA/B repress the synthesis of at least three TBDTs, Cir, FecA and FepA. *cirA* was shown to be a direct target of OmrA/B, but whether this is true as well for *fecA* and *fepA* remains to be experimentally tested (41).

OmrA/B are synthesized in response to the activation of the EnvZ-OmpR two component system (40). Although the physiological signal for this activation remains unclear, the levels of phosphorylated OmpR change as a function of the osmolarity of the external medium. Consequently, several genes regulated by EnvZ-OmpR, such as the ones for the major porins OmpC and OmpF, as well as *omrA* and *omrB*, are differentially expressed at different osmolarities. The importance of down-regulating several TBDTs for siderophores in response to changes in osmolarity is not entirely clear.

Regulation in other bacteria

Virtually all Gram negative bacteria have TBDTs that are involved in the uptake of iron and vitamin B_{12}, as well as nickel, carbohydrates, and probably other substrates (75). The total number of TBDTs is highly variable among bacterial genomes: while *E. coli* synthesizes just 7 TBDTs, *Pseudomonas aeruginosa* makes 34 TBDTs (81) and *Caulobacter crescentus* makes 65 TBDTs (65). The current knowledge about the regulation of TBDT synthesis in these and other bacteria is limited.

Expression of genes for TBDTs involved in iron uptake is regulated by Fur in numerous bacteria (53). Somewhat similarly to this regulation of iron uptake genes by Fe^{2+} via Fur, synthesis of *Helicobacter pylori* FrpB4, a TonB-dependent nickel transporter (74), is

repressed by the nickel-sensing transcriptional regulator NikR (23). In *C. crescentus*, the outer membrane protein MalA, which is likely a TBDT and required for maltose uptake, is induced in presence of maltose, but the mechanism for this regulation is still unknown (63).

FecIR-type regulation is also present in numerous bacteria (5, 50). Interestingly, several of these 'anti-σ' factors were shown to be required for full activation of their cognate σ factors, just like FecR (see above). This is the case for *Bordetella avium* RhuR (49) and *Pseudomonas aeruginosa* FoxR and FiuR involved in the regulation of the uptake of desferrioxamine and ferrichrome siderophores, respectively (59).

The control of *btuB* expression by a vitamin B_{12}-responsive riboswitch is most likely widespread in Gram negative bacteria. Indeed, two independent phylogenetic analyses identified a similar conserved RNA motif not only in the 5′ UTR of *btuB* homologs from numerous Gram negative bacteria, but more generally in the 5′ UTR of genes involved in the metabolism or transport of vitamin B_{12}, as well as some other genes, both in Gram positive and Gram negative bacteria (61, 84). When this was looked at, for instance for the elements upstream of *btuB* and *cob* genes of *Salmonella typhimurium*, these RNA motifs were shown to efficiently and selectively bind vitamin B_{12} (61).

OmrA and OmrB are conserved in most enterobacteria, even though one or even both of them can be absent in some species (40). A direct base-pairing interaction between *cirA* mRNA and OmrA/B was shown to control *cirA* expression in *E. coli* and a similar interaction is predicted in other enterobacteria (41). However, whether *cirA* (and also *fecA* and *fepA*) are really regulated by OmrA/B in other species remains to be investigated. In addition, it would also be interesting to determine whether other post-transcriptional events control the synthesis of these TBDTs in bacteria lacking OmrA/B.

Structure and Function of TBDTs

In 2005, Chimento et al. published a comprehensive structural analysis (17) of the four TBDT structures published at that time (10, 18, 32, 34, 54, 90). Since then, the structures of eight more TBDTs have been determined (8, 9, 19-21, 51). In addition, structures were solved for TBDTs with various ligands bound (7, 30, 35, 38, 52, 76, 88), in complex with the periplasmic domain of TonB (70, 77), and one TBDT crystallized from a lipidic cubic phase (15), giving us 45 crystal structures to compare now (Table 1; Supplemental Figure 1). An analysis of the original four TBDTs showed that all of them have the same domain architecture: a 22-stranded transmembrane β-barrel encloses a globular plug domain (Figure 2). Ligand binding sites are formed from residues on the extracellular side of the plug domain, as well as from residues on the walls and extracellular loops of the β-barrel. The TonB box is found at the N-terminus of the plug domain, and in some structures protrudes into the periplasm. In others, the TonB box is tucked up into the plug domain within the barrel or is disordered and not visible in the structures. A structure-based sequence alignment revealed conserved motifs in the plug and barrel which are close to one another and interact. Finally, an analysis of water molecules located at the plug-barrel interface revealed that the plug is highly solvated, resembling a transient protein complex and suggesting conformational change and/or movement of the plug within the barrel during transport. In the following sections, we will outline some of the significant structural and functional studies done with TBDTs in recent years.

The twelve TBDTs are structurally similar

The 22-stranded β-barrel with inserted plug domain is conserved for all known transporters and very likely represents the architecture of all TBDTs. The ligand binding sites are customized for the cognate siderophore or colicin. For example FhuA uses aromatic residues

to bind ferrichrome (31, 54) while the binding pocket on FecA contains several arginine residues to bind the negatively charged diferric dicitrate (32, 90). Two heme transporters coordinate their 'siderophore' through conserved histidine residues residing in the plug and an extracellular loop (21, 51). Only three structures have been solved for TBDTs bound to the receptor binding domain of various colicins (9, 52, 76), and binding differs substantially from siderophores, although the binding sites for colicins and siderophores appear to overlap (12). Interfacial water molecules were analyzed for Cir (9) and agreed with previous results reported by Chimento et al. in 2005 for BtuB, FepA, FecA, and FhuA (17) – that the plug is highly solvated inside the barrel pore. For this review, we performed a structure-based sequence alignment for the twelve unique TBDTs to ask how many of the conserved features identified by Chimento et al. (17) remain conserved in this larger group representing TBDTs from a variety of Gram negative bacteria (Supplemental Figure 2). Interestingly, many of the conserved motifs identified in the four original TBDT crystal structures in 2005 are also observed in the twelve currently known TDBT crystal structures. Conserved motifs include the TEE, PGV, IRG box, LIDG box, RP box, and the Hβ4 motifs, which are all located within the plug domain. Additionally, we observed significant conservation for most of the β-strand sequences with many of the β-strands having one or more signature residues that were found completely conserved, which may have implications for structure prediction of other TBDTs and possibly even other families of β-barrel proteins. Figure 2d shows FhuA (colored gold) with residues which were found to be at least 50% conserved among all 12 unique TBDTs with known structures indicated in blue. We found that 32% of these conserved residues were located within the plug domain (27% of total plug domain residues) and the other 68% were located within the core β-strands of the β-barrel domain (16% of total β-barrel domain residues). None of the indicated conserved residues were observed within the extracellular loops, further emphasizing their evolutionary divergence.

Lipidic cubic phase crystallization yields the highest resolution TBDT structure

While almost all TBDT structures were crystallized from detergent/precipitant mixtures (4), Caffrey and colleagues crystallized the apo form of BtuB from a lipidic cubic phase (11) instead of detergent, yielding the highest resolution structure for this family of transporters (Table 1) (15). Crystals grown *in meso* exhibit denser packing than those grown in detergent, resulting in conformational differences in extracellular loops between the two apo BtuB structures (15, 16). Since these loops are unrestrained *in vivo* and probably move continuously, both structures depict physiologically relevant states of the protein. Otherwise the two structures are remarkably similar, with backbone RMSD values of less than 1.5 Å over 82% of all residues. This work confirms that lipidic cubic phase crystallization could be as useful for the crystallization of β-barrel outer membrane proteins as it is for α-helical inner membrane proteins (14).

Siderophore binding transduces a signal across the outer membrane

The binding of a siderophore to its TBDT transduces a signal across the outer membrane that results in a disordering (also called unfolding or undocking) of the TonB box, as described below. The nature of the transduced signal is not completely clear, but for some TBDTs it appears to involve large conformational changes in extracellular loops which fold in over the top of the TBDT when siderophore binds, sequestering the ligand and contributing new residues to the binding site. This type of induced fit mechanism has been observed for FecA (32, 90), ShuA (21), and FyuA (Lukacik et al., unpublished). Ligand binding also induces smaller conformational changes in the plug domain (observed in many TBDT structures) but exactly how binding of a small molecule at the extracellular surface results in disordering of the TonB box is not completely clear.

Crystallography is not the best way to monitor TonB movements

Table 1 shows that the TonB box, generally located near the N-terminus of TBDTs, adopts a variety of conformations ranging from ordered to disordered that does not seem to correlate well with siderophore binding. Since this stretch of five residues is an essential part of the transporter (transport will not happen without it), it is important to understand its location and mobility in apo and siderophore bound TBDTs. The most definitive work in this area has been done by Cafiso and colleagues using site-directed spin labelling and electron paramagnetic resonance spectroscopy (EPR) to determine position and mobility of the TonB box. They showed that siderophore binding to BtuB results in an unfolded TonB box (termed disordered by crystallographers), whereas the apo structure exhibits a folded (or ordered) TonB box (28). They also showed that reagents used in protein crystallization can inhibit this transition (29), explaining the highly variable results seen in the crystal structures. It now seems clear that siderophore binding transduces a signal across the outer membrane that ultimately results in unfolding (or increased mobility) of the TonB box, which signals to TonB-ExbB-ExbD that a particular transporter is ligand-loaded and primed for transport. However, while FecA undergoes the same order/disorder transition seen for BtuB, the TonB box of FhuA was found to be constitutively unfolded (48), suggesting either that interactions between FhuA and TonB are constitutive or not regulated by the TonB box configuration. Clearly tools in addition to crystallography and EPR will be required to elucidate the signal transduction and transport mechanisms.

TBDTs associate with TonB through β-strand pairing

When a TBDT has bound its siderophore and signalled to TonB-ExbB-ExbD, the next step appears to be a physical association between the TonB box of the TBDT and the C-terminal (periplasmic) domain of TonB. Structures of this complex have been described by Wiener and colleagues for BtuB-TonB (77) and by Coulton and colleagues for FhuA-TonB (70). In both structures TonB assumes an alpha-beta fold containing a 3-stranded β-sheet. The TonB box of either transporter adopts a β-strand conformation that pairs with the existing β-sheet of TonB. Association through strand pairing has been observed for many protein complexes and although details differ for the two complexes described here, we can conclude that the binding interface is relatively small. In both structures the plug domain still resides inside the β-barrel just like in all the other ground state structures of TBDTs. Presumably energy in the form of protonmotive force, as well as a full-length TonB-ExbB-ExbD complex, would be needed to visualize the transporter in action.

Plug domain movements occurring upon transport are still unclear

It is widely accepted that the plug domain of TBDTs must undergo some form of conformational change in order to transport either siderophores or larger cargo such as colicins (12, 33, 87). However, the extent of the conformational change and whether or not the plug domain completely exits the β-barrel is a topic of debate. It has been postulated that upon binding of siderophores, the plug domain could undergo a conformational change that creates a small pore between the plug domain and the inner wall of the barrel whereby transport may occur (10, 34, 54). The observations that the plug domain is highly solvated (9, 17) and fairly loosely packed inside the β-barrel suggest that minimal rearrangement of the plug domain could in fact lead to a pore capable of allowing siderophore passage.

While pore formation through TBDTs via conformational change in the plug domain could be a feasible mechanism for transport of smaller ligands such as siderophores, this mechanism does not explain how much larger protein cargo such as colicins, which range in size from 29 kDa (91) to 69 kDa (86), are transported across the outer membrane (Supplementary Figure 3). A narrow pore might allow siderophore transport but near

complete unfolding would be required in order for a colicin to pass through the same pore, which would be highly unlikely given the energy barrier for such a mechanism.

Several experiments to determine whether the plug exits the barrel during substrate transport used pairs of cysteine mutants to tether the plug to the β-barrel (Figure 3). When the tether was located near the N-terminus of the plug domain, both FhuA (25) and FepA (56) were inactivated. In the case of FhuA, transport was restored upon reduction of the disulfide. However, when disulfides tethered the middle of the plug domain to the β-barrel, siderophore transport still occurred, albeit at a reduced rate (24). One explanation for the discrepancy could be that disulfides were formed less efficiently in the middle of the plug domain compared to those located at the N-terminus, which is exposed to the oxidizing environment of the periplasm.

Recently, two groups attempted to label cysteine residues in the plug domain with reagents located in the periplasm to demonstrate plug domain movement. Li et al. introduced cysteine residues into the plug domain of FepA and observed differential labelling with fluorescein maleimide for G54C during transport of ferric enterobactin (56). G54C is located in the middle of the plug domain and is weakly labelled by the periplasmically located fluor in the ground state, but is more strongly labelled during transport. This suggests that the plug may partially exit the barrel during transport of the siderophore. Similarly, Devanathan and Postle introduced cysteine residues into the FepA plug domain and used biotin maleimide to probe conformational changes occurring upon translocation of colicin B (22). They observed increased labelling for N-terminal regions of the plug domain, particularly S46C, with much smaller increases in labelling residues in the C-terminal portion of the plug domain, also suggesting plug domain movement out of the β-barrel. However, Smallwood et al. found the opposite result for FepA and colicin B; they did not detect structural changes in the FepA plug domain upon interaction with colicin B using a different labelling reagent, different bacterial strains, and different colicin concentrations (78). Because of the variations in experimental approaches used, it may not be possible yet to determine whether the plug domain exits the barrel (or becomes more exposed to the periplasm) when colicin B interacts with FepA.

Taking a computational approach, Gumbart et al. used steered molecular dynamics (42) to simulate what happens when force is applied to the BtuB-TonB crystal structure (77). They found that force can be transmitted from TonB to BtuB without disruption of the β-strand interactions linking the two proteins, supporting a mechanical mode of coupling. When pulling simulations were performed, part of the BtuB plug domain unfolded, corresponding to periplasmic exposure of those residues. These results, and most of the experiments described above, suggest that some movement of the plug domain occurs upon interaction with TonB. However, the details and extent of this domain movement (or unfolding) and the precise transport mechanism remain to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

TonB box	A semi-conserved stretch of five amino acids near the N-terminus of a TBDT which is the signature sequence for this family of transporters and the region that interacts with TonB.
Siderophores	small molecules secreted by bacteria and fungi that have high affinity and selectivity for Fe^{3+}. Siderophores are among the tightest iron-binding complexes known.
Colicin	*E. coli* protein toxin synthesized in response to stress in order to kill neighboring bacteria. Colicins bind to the outer membrane of a target bacterium through TBDTs. Related proteins from other Gram negative bacteria are called bacteriocins.
Lipidic cubic phase crystallization	a crystallization method for membrane proteins that substitutes a lipid (typically monoolein) for detergent. Appropriate quantities of protein, lipid, and water are mixed to form a transparent three dimensional

array with interconnected aqueous channels. Precipitant solutions can diffuse through the channels to induce three dimensional ordering of the target membrane protein.

Acronyms

TBDT	TonB-dependent transporter
ECF	extracytoplasmic function
AdoCbl	adenosylcobalamin
CnCbl	cyanocobalamin
sRNA	small RNA
apo transporter	transporter with no siderophore bound
EPR	electron paramagnetic resonance spectroscopy

Summary Points

1. Synthesis of TBDTs is regulated in multiple ways, involving metal-dependent regulators, σ/anti-σ factors, small RNAs, a riboswitch, and possibly other mechanisms not yet detected. Multiple regulatory mechanisms allow bacteria to tailor expression of TBDTs on the cell surface to their changing environment.
2. All TBDTs share the same domain architecture, with a 22-stranded β-barrel spanning the outer membrane and a plug domain inserted inside the barrel that contributes specificity for siderophores (also colicins and phages) and interacts with TonB to initiate transport.
3. Significant progress has been made in determining the transport mechanism, but molecular details must be worked out in the future using structural, biochemical, and genetic experiments.

Future Issues

1. Which regulatory mechanisms control expression of TBDTs under various conditions? What are the molecular details of the signal transduction mechanism across the outer and inner membranes for σ/anti-σ factor regulation?
2. At which steps in the transport process is energy required, how much energy is needed, and what happens to the transporter and TonB-ExbB-ExbD?
3. How is a siderophore transported? Does the plug domain exit the barrel, does it unfold, or perhaps both? How is the TBDT reassembled?
4. How do colicins use TBDTs to cross the outer membrane? Is the mechanism similar to that for siderophores?

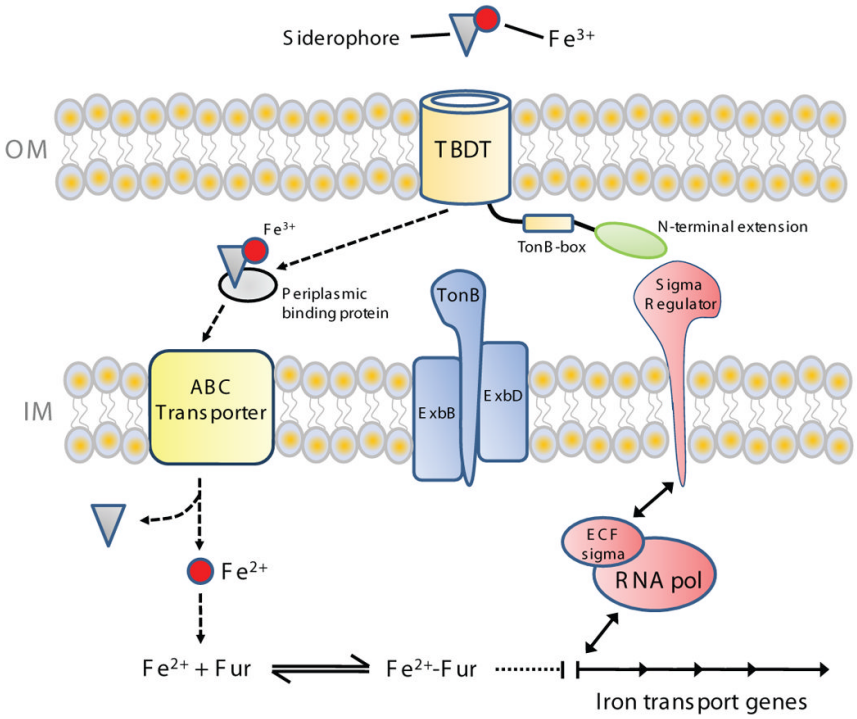

Figure 1. Transport and regulation of siderophores

Transport of ferric siderophores across the outer membrane derives energy from the inner membrane protonmotive force. This requires an energy-transducing TonB complex in the inner membrane (blue), consisting of TonB, ExbB and ExbD proteins. TonB interacts with outer membrane transporters (TBDT) at the TonB-box motif. Transport of ferric siderophores across the inner membrane requires a periplasmic binding protein and an ABC transporter. Once the ferric siderophore enters the cytoplasm, ferric ion (Fe^{3+}) is reduced to ferrous ion (Fe^{2+}), which is destined for storage or incorporation into enzymes. Excess Fe^{2+} (which could induce the formation of radicals harmful to the cell) binds to the repressor protein Fur, which in turn binds target promoters (P_{fur}) and inhibits transcription of siderophore transport genes. Some TBDTs, such as *E. coli* FecA are additionally regulated by σ/anti-σ factor systems. In addition to transporting diferric dicitrate, FecA regulates the expression of *fecABCDE* transport genes initiated by the binding of ferric citrate to FecA. This involves the N-terminal extension of FecA (green), the inner membrane regulator FecR (σ regulator, pink), and the cytoplasmic sigma factor FecI (ECF σ factor, pink). Both transport and induction require energy transduction from the TonB-ExbB-ExbD complex in the inner membrane.

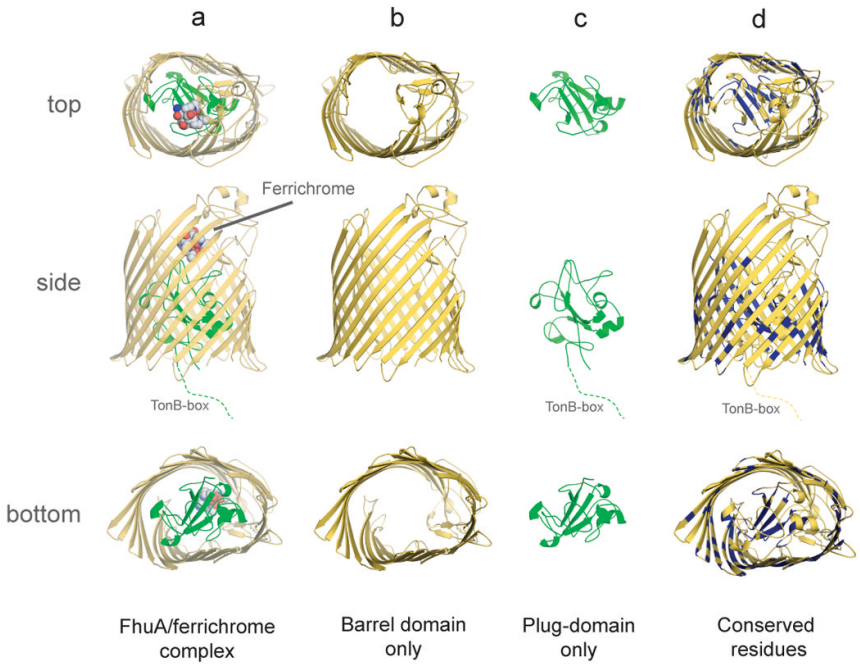

Figure 2. The structure of the (prototype) TBDT FhuA

TBDTs have an N-terminal plug domain that sits inside a C-terminal 22-stranded β-barrel domain. The conserved TonB box is found near the N-terminus of the plug domain facing the periplasm and is generally thought to remain sequestered inside the β-barrel domain in the absence of ligand. Upon binding ligand, a conformational change leads to exposure of the TonB box and subsequent interaction with TonB and siderophore transport. Panel a represents the FhuA-ferrichrome crystal structure (1BY5) with FhuA shown in ribbon and ferrichrome in spacefill model, panel b represents only the beta-barrel domain, panel c represents only the plug domain, and panel d shows the FhuA apo structure (1BY3) with those residues with at least 50% conservation highlighted in blue. Top view represents the extracellular view, side view represents the membrane view, and bottom represents the periplasmic view. The TonB box was found disordered in both structures and is represented by dashed lines.

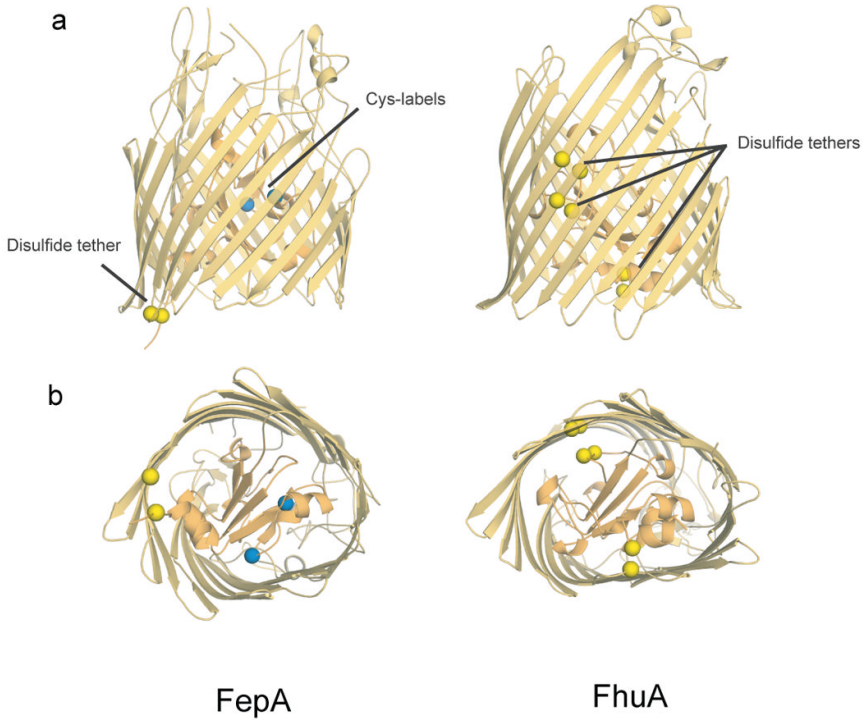

Figure 3. Role of the plug domain in siderophore transport

It is generally accepted that the plug domain of TBDTs must undergo some form of conformational change to facilitate sideophore transport. In panel a, studies with FepA have shown that engineered cysteines (S46C/G54C) within the plug domain (indicated by blue spheres) become labeled by periplasmic thio-reactive reagents only during transport of ligand. Other studies have shown that engineered disulfides which tether the plug domain to the inner face of the barrel domain (indicated by yellow spheres) in both FepA (I14C/G300C) and in FhuA (panel b, C27/C533, L109C/S356C and Q112C/M383C) significantly reduce or eliminate sideophore transport. Together, these studies provide evidence that partial or full ejection of the plug domain from the β-barrel may be required for siderophore and/or colicin transport.

TABLE 1

Summary of all known TonB-dependent transporter crystal structures.

Name	SeqID (%)/RMSD (Å)	Organism	Detergent	Resolution (Å)	Ligand	TonB-box ordered?	Reference	PDB ID
BtuB	17/6.85	*E. coli*	C_8E_4	2.00	-	Yes	Chimento et al., 2003	1NQE
		E. coli	C_8E_4	2.7	-	Yes	Chimento et al., 2003	1NQF
		E. coli	Monoolein	1.95	-	Yes	Cherezov et al., 2006	2GUF
		E. coli	LDAO/C_8E_4	2.10	TonB/vit-B_{12}	Yes	Shultis et al., 2006	2GSK
		E. coli	C_8E_4	3.31	Calcium	Yes	Chimento et al., 2003	1NQG
		E. coli	C_8E_4	3.10	Calcium/vit-B_{12}	Yes	Chimento et al., 2003	1NQH
		E. coli	LDAO	2.75	Colicin E3-R domain	Yes	Kurisu et al., 2003	1UJW
		E. coli	LDAO	3.50	Colicin E2-R domain	Yes	Sharma et al., 2007	2YSU
Cir	12/7.67	*E. coli*	LDAO/C_8E_4	2.65	-	Yes	Buchanan et al., 2007	2HDF
		E. coli	LDAO/C_8E_4	2.50	Colicin Ia- R domain	Yes	Buchanan et al., 2007	2HDI
FauA	17/2.63	*B. pertussis*	C_8E_4	2.33	-	No	Brillet et al., 2009	3EFM
FecA	14/2.57	*E. coli*	LDAO	2.00	-	Yes	Ferguson et al., 2002	1KMO
		E. coli	LDAO	2.50	-	Yes	Yue et al., 2003	1PNZ
		E. coli	LDAO	2.50	Iron-dicitrate	No	Ferguson et al., 2002	1KMP
		E. coli	LDAO	2.15	Iron-free dicitrate	Yes	Yue et al., 2003	1PO0
		E. coli	LDAO	3.40	Iron-dicitrate	No	Yue et al., 2003	1PO3
FepA	11/11.32	*E. coli*	LDAO	2.40	-	Yes	Buchanan et al., 1999	1FEP
FhuA	reference	*E. coli*	OPOE/OHES	2.74	-	No	Locher et al., 1998	1BY3
		E. coli	OPOE/OHES	2.60	Ferrichrome	No	Locher et al., 1998	1BY5
		E. coli	DDAO	2.70	Ferrichrome/LPS	No	Ferguson et al., 1998	1FCP
		E. coli	DDAO	2.50	LPS	No	Ferguson et al., 1998	2FCP
		E. coli	DDAO	2.90	CGP 4832/LPS	No	Ferguson et al., 2001	1FI1
		E. coli	LDAO	3.30	TonB/ferricrocin	Yes	Pawelek et al., 2006	2GRX
		E. coli	DDAO	2.70	Ferrichrome	No	Ferguson et al., 2000	1QFF
		E. coli	DDAO	2.50	LPS	No	Ferguson et al., 2000	1QFG
		E. coli	DDAO	2.95	Phenylferricrocin	No	Ferguson et al., 2000	1QJQ
		E. coli	DDAO	3.10	Albomycin	No	Ferguson et al., 2000	1QKC
FptA	17/2.43	*P. aeruginosa*	LDAO	2.00	Pyochelin	No	Cobessi et al, 2005	1XKW

Name	SeqID (%)/RMSD (Å)	Organism	Detergent	Resolution (Å)	Ligand	TonB-box ordered?	Reference	PDB ID
FpvA	17/1.98	*P. aeruginosa*	C_8E_5	2.90	-	No	Greenwald et al., 2009	2W75
		P. aeruginosa	C_8E_5	2.77	-	Yes/No[1]	Brillet et al., 2007	2O5P
		P. aeruginosa	C_8E_5	2.71	PVDI	No	Greenwald et al., 2009	2W16
		P. aeruginosa	C_8E_5	2.90	$PVD_{DSM50106}$	No	Greenwald et al., 2009	2W6T
		P. aeruginosa	C_8E_5	3.00	PVD_{G173}	No	Greenwald et al., 2009	2W6U
		P. aeruginosa	C_8E_4	2.73	PVD-Fe	No	Wirth et al., 2007	2IAH
		P. aeruginosa	C_8E_5	2.80	PVD_{Pa6}	No	Greenwald et al., 2009	2W76
		P. aeruginosa	C_8E_5	2.90	$PVD_{Pfl18.1}$	No	Greenwald et al., 2009	2W77
		P. aeruginosa	C_8E_5	3.00	$PVD_{ATCC13535}$	No	Greenwald et al., 2009	2W78
		P. aeruginosa	C_8E_5	3.60	PVD	No	Cobessi et al., 2005	1XKH
FyuA	14/4.03	*Y. pestis*	LDAO/C_8E_4	3.20	-	No	Lukacik et al., UNPUB	-
		Y. pestis	LDAO/C_8E_4	3.30	Yersiniabactin	No	Lukacik et al., UNPUB	-
HasR	14/11.35	*S. marcescens*	C_8E_4	2.70	HasA/heme	No	Krieg et al., 2009	3CSL
		S. marcescens	C_8E_4	3.00	HasA	No	Krieg et al., 2009	3CSN
		S. marcescens	C_8E_4	2.80	HasA/heme	No	Krieg et al., 2009	3DDR
ShuA	16/5.78	*S. dysenteriae*	OG	2.60	-	Yes	Cobessi et al., 2009	3FHH
YiuR	15/6.35	*Y. pestis*	LDAO	2.65	-	No	Noinaj et al., UNPUB	-

Abbreviations: LDAO (lauryldimethylamine-oxide), C_8E_4(n-octyltetraoxyethylene), OPOE (octyl-polyoxyethylene), OHES (n-octyl-2-hydroxyethylsulfoxide), DDAO (N,N-dimethyldecylamine-N-oxide), C_8E_5 (3,6,9,12,15-pentaoxatricosan-1-ol), OG (octyl-glucoside), LPS (lipopolysaccharide), PVD (pyoverdine), *E. coli* (*Escherichia coli*), *B. pertussis* (*Bordetella pertussis*), *P. aeruginosa* (*Pseudomonas aeruginosa*), *Y.pestis* (*Yersinia pestis*), *S. marcescens* (*Serratia marcescens*), *S. dysenteriae* (*Shigella dysenteriae*), SeqID (sequence identity to FhuA), RMSD (root mean square deviation to 1BY3), UNPUB (unpublished results).

[1]Two molecules found in asymmetric unit with one TonB-box found ordered and the other disordered.