Review

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New substrates for TonB-dependent transport: do we only see the 'tip of the iceberg'?

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TonB-dependent transport is a mechanism for active uptake across the outer membrane of Gram-negative bacteria. The system promotes transport of rare nutrients and was thought to be restricted to iron complexes and vitamin B_{12}. Recent experimental evidence of TonB-energized transport of nickel and different carbohydrates, in addition to bioinformatic-based predictions, challenges this notion and reveals that the number and variety of TonB-dependent substrates is underestimated. It is becoming clear that the chemical nature of the substrates, the energetic requirements for transport and the subsequent translocation across the cytoplasmic membrane can differ from those of the well-studied systems for iron complexes and vitamin B_{12}. These findings question the understanding of TonB-dependent uptake and provide insights into the adaptation of bacteria to their environments.

What is TonB-dependent transport and what are the associated substrates?

In addition to a cytoplasmic membrane (CM), which is common to all organisms, Gram-negative bacteria possess an outer membrane (OM), which hinders the uptake of essential nutrients. In contrast to small molecules that cross the OM by passive diffusion through transmembrane porins, substrates that are either poorly permeable through porins (those greater than ~600 Da) or are present at very low concentrations require energized transport for their translocation across the OM [1]. No obvious energy source is present at the OM because ATP-hydrolyzing proteins are locally lacking and no proton gradient can be established across the outer lipid bilayer. It was realized ~30 years ago that three factors are required for active transport across the OM: (i) the proton motive force (pmf) of the CM; (ii) a cytoplasmic transmembrane complex composed of the proteins TonB, ExbB and ExbD that spans the periplasm (the TonB complex); and (iii) a specific TonB-dependent transporter (TBDT) in the OM [2–4] (Figure I, Box 1). The TonB complex transduces the pmf of the CM to energize substrate transport through a specific TBDT across the OM [5–13] (Box 1).

Until recently, TonB-dependent transport was exclusively shown for the physiological uptake of iron complexes and vitamin B_{12} (cobalamin). In most microbial habitats, the predominant iron species is ferric iron (Fe^{3+}), which is found in almost-insoluble oxide hydrate complexes (Fe_2O_3–nH_2O) in the environment or is tightly sequestered to proteins in host organisms; thus, free iron concentrations are extremely low (10^{-18}–10^{-24} M) [14]. Therefore, both environmental and pathogenic microorganisms are often restricted in their iron uptake. Gram-negative bacteria scavenge Fe^{3+} by TonB-energized transport through specific TBDTs and via adaptor molecules that strongly bind to Fe^{3+}. Different adaptor molecules have been recognized (Box 2), including host iron-binding proteins (such as transferrin) or hemoproteins, heme, citrate and siderophores [15–17]. The rare compound vitamin B_{12} (2–4 pM in sea and fresh water [18]), which is essential for many organisms, contains a Co^{2+} ion in a corrin ring and is transported by the specific TBDT BtuB [13]. In addition, several phages, colicins and naturally occurring antibiotics (sideromycins) use the TonB-dependent machinery to gain access to the periplasm of bacteria [19–21]. The fact that the phage *T one* (T1) does not infect *Escherichia coli* bacteria that are deficient in either the *tonA* or the *tonB* gene, gave rise to the name for the TonB complex. The *tonA* gene encodes a TBDT that was renamed to FhuA. Importantly, TonB-dependent transport is of high medical relevance because the survival of pathogenic bacteria in their respective hosts depends on their ability to compete for micronutrients such as iron [16,22]. Therefore, this topic has attracted tremendous interest, and numerous studies have generated data on the mechanistic aspects of TonB-dependent transport and on the structures of TBDTs alone or in complex with their substrates [8,12,13,23]. Despite this level of understanding, recent findings indicate that our knowledge of the full range of substrates for TonB-dependent transport is actually quite limited. As had been predicted for many years [24], it has now been demonstrated that the uptake of iron complexes and vitamin B_{12} is only one of the many potential functions of the TonB complex. Indeed, nutrients not previously associated with this system have recently been shown to require TonB-energized transport, indicating that, by analyzing the uptake of the iron complex and vitamin B_{12}, we

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Box 1. Characteristics of TBDTs and mechanistic aspects of TonB-dependent iron and vitamin B_{12} uptake – what is known?

Structural analyses revealed features that clearly distinguish TonB-dependent transporters (TBDTs) from porins, through which substrates pass by diffusion. TBDTs contain a C-terminal membrane-spanning barrel domain, comprising 22 antiparallel β-strands, that is bigger than the barrel domain of porins (reviewed in Ref. [60]). The N terminus of the TBDT forms a plug domain (cork or hatch), which occludes the barrel and prohibits the passage of solutes [60]. Substrate binding occurs at the plug domain and the external loops of the barrel and does not require TonB-mediated energy [33]. By contrast, substrate release into the periplasm depends on a functional TonB complex for substrate translocation across the outer membrane (OM). Substrate passage through the center of the TBDT requires structural changes of the plug domain. Transport studies using mutants of selected residues, disulfide crosslinking, endogenous labeling and, finally, crystal structure analyses established that, besides several contact sites between the TBDT and TonB, an eight-amino-acid segment termed the 'TonB box' is the central element in the interaction between the plug and the C-terminal domain of TonB (reviewed in Refs [3,4]). The precise mechanism of energy transduction is not understood.

Two mechanistic models for the function of TonB are proposed. In the 'shuttle' model, the energized form of TonB entirely exits the cytoplasmic membrane (CM), crosses the periplasmic space and transduces its energy to the TBDT [9,10]. In the 'pulling' model, TonB remains membrane bound and spans the periplasmic space to connect to the TBDT. It is hypothesized that conformational changes of TonB result in a pulling force on the plug domain of the TBDT that results in either a conformational change of the plug that remains in the barrel or a delocalization of the plug [6,7,11–13,36]. Anchoring of TonB to the membrane is accomplished through a transmembrane complex of ExbB and ExbD. The cellular ratio of TonB, ExbB and ExbD in *Escherichia coli* has been determined to be 1:7:2, respectively [61]. These proteins convert alterations in the proton motive force (pmf) into conformational changes by a mechanism that is still to be understood. Subsequent transport of iron complexes and vitamin B_{12} through the periplasm and across the CM is accomplished by ATP-binding-cassette (ABC) transporters [60,62]. These are primary transporters that are composed of a periplasmic-binding protein (PBP), two integral membrane components and two ATPases. Once in the periplasm, the substrate is bound by the PBP, which transmits the substrate to the membrane components of the ABC transporter. Substrate translocation into the cytoplasm is energized by ATP hydrolysis [63,64].

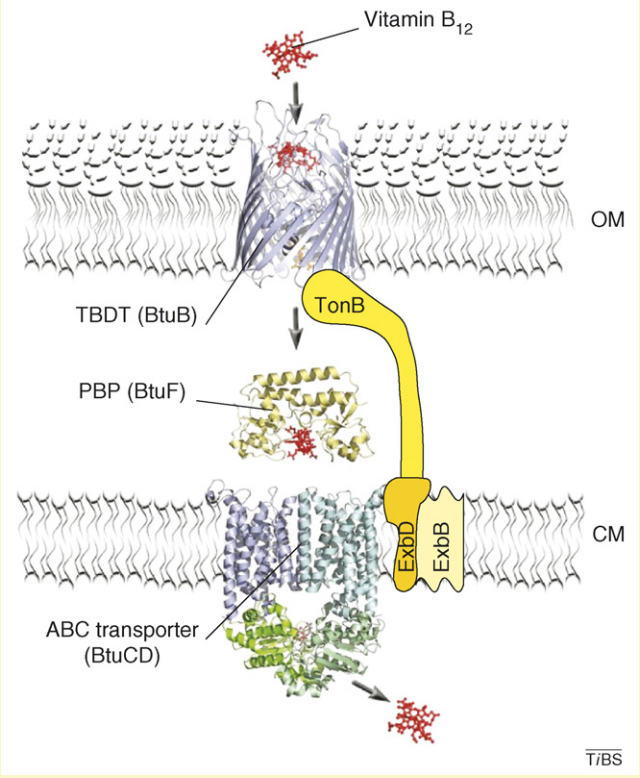

Figure I. Model of the TonB-dependent uptake of vitamin B_{12}. In Gram-negative bacteria, the TonB-dependent machinery enables transport of scarce or weakly permeable substrates through the OM. Vitamin B_{12} (red) binds to the specific TBDT BtuB (blue ribbon). The TonB–ExbB–ExbD-complex (yellow) transduces the proton gradient of the CM to induce a conformational change of the TBDT. This causes a release of the bound vitamin B_{12} into the periplasmic space, initiating substrate transport across the OM. After capture of vitamin B_{12} by the PBP BtuF (yellow ribbon), it is transmitted to the membrane-bound ABC-transporter BtuCD (the two transmembrane domains of BtuCD are shown in blue, the two ATP-binding domains are in green). Subsequent transport across the CM is accomplished by ATP-dependent transport through BtuCD. Figure image created by C. Kandt and used with permission.

have only studied the 'tip of the iceberg'. Thus, here, we review the expanding repertoire of TonB-dependent substrates and highlight the biological and biochemical implications that are resulting from these findings.

New substrates for TonB-dependent transport

The expansion of TonB-dependent substrates beyond that of iron complexes and vitamin B_{12} has recently been confirmed by experimental data of novel TonB-dependent substrates. We and others have shown that nickel and carbohydrates such as maltodextrins and sucrose require TBDTs and energy to cross the OM [25–27] (Table 1).

In the human pathogen *Helicobacter pylori*, the expression of the TBDT FrpB4 has been found to be strongly regulated by nickel ions [28–30]. Nickel is the co-factor of the highly abundant virulence factor urease, which is required for the resistance of the bacterium at low pH and is, therefore, essential for the survival of *H. pylori* in the acidic environment of the human stomach. FrpB4 was predicted to be involved in iron uptake but lacked any regulation by iron. This is surprising because transporters are usually under the regulation of their substrates [31]. Moreover, nickel represses the expression of the TonB–ExbB–ExbD complex stronger than did iron [27]. We analyzed the function of TonB–ExbB–ExbD and FrpB4 and found that both Δ*frpB4* and Δ(*exbB*/*exbD*/*tonB*) deletion mutants were deficient in nickel transport at low pH. This demonstrated that nickel acquisition in *H. pylori* requires a specific TBDT and the TonB complex [27].

Similarly, one of the OM proteins, MalA, of the environmental bacterium *Caulobacter crescentus* was induced under conditions in which maltodextrins were the sole carbon source [26]. MalA exhibits all the structural features of a TBDT and has no sequence similarity with the LamB porin of *E. coli* [26]. Maltodextrins larger than maltotetraose (667 Da) strictly required MalA for their uptake, and MalA enhanced maltose transport tenfold. Uptake was dependent on ExbB and ExbD from the TonB inner-membrane machinery, indicating an energy-dependent transport mechanism. Because a Δ*tonB* deletion mutant unexpectedly presented a wild-type transport phenotype, it was hypothesized that the product of a second *tonB* gene found in the genome of *C. crescentus* might specifically be required for maltodextrin transport or substitute for TonB function in the Δ*tonB* mutant. A mutant strain in which the second *tonB* gene is deleted is still under investigation [26].

Box 2. Adaptor molecules and their specific TBDTs

Host iron-binding proteins

In mammalian host organisms, iron is tightly bound to transferrin and lactoferrin. Transferrin and lactoferrin are transport proteins of the body's circulation system that are found in serum and lymph or milk, respectively. Because these proteins are too large (both are ~80 kDa) to cross the bacterial outer membrane (OM), iron is removed from them at the bacterial surface by a not yet entirely understood mechanism prior to transport of the iron ions. Specific TonB-dependent transporters (TBDTs) are used to bind to transferrin (e.g. TbpA of *Neisseria* ssp.) and lactoferrin (e.g. LbpA of *Neisseria* ssp.). These iron sources are used by pathogenic bacteria such as *Neisseria meningitidis* [22] and *Hemophilus influenzae*.

Heme, hemoproteins and hemophores

Heme is abundantly found as a prosthetic group in enzymes and is used as an iron source by many Gram-negative bacteria. Heme is acquired either alone or bound to hemoproteins. Several hemoproteins including hemoglobin, haptoglobin-hemoglobin and hemopexin have been described (reviewed in Ref. [17]). Hemoglobin is the oxygen transporter of red blood cells that is used as an iron donor for the TBDTs HmbR and HpuA by *Neisseria* spp. and HemR by *Yersinia enterocolitica*. Haptoglobin-hemoglobin is transported by the TBDT HpuA of *N. meningitidis* and the host protection-protein hemopexin serves as an iron source for TonB-dependent transport through HxuB in *H. influenzae*. Although heme is scarcely found, it can be transported by a variety of TBDTs such as HasR of *Serratia marcescens*, HemR of *Y. enterocolitica* and HmbR of *Neisseria* spp. Furthermore, bacteria secrete hemophores (e.g. HasA of *S. marcescens* and HxuA of *H. influenzae*) (reviewed in Ref. [65]). These extracellular carrier proteins capture heme by high-affinity binding ($K_D = 10^{-11}$ M [66]) and deliver it to specific TBDTs. Whereas hemoproteins and hemophores are not transported across the OM, heme crosses the OM and the cytoplasmic membrane (CM).

Siderophores

With some exceptions, siderophores are usually <1-kDa iron-chelating molecules composed from non-ribosomal-synthesized amino acid analogs (reviewed in Ref. [16]). They are synthesized by many microorganisms under iron-limited conditions. Because siderophores have very high affinities for iron ions ($K_D = 10^{-22}$–10^{-49} M) they can scavenge iron from oxide hydrate complexes or iron-binding proteins. More then 500 different siderophores have been identified to date. Siderophores are transported by specific TBDTs. The crystal structures of the *Escherichia coli* ferrichrome, diferric-dicitrate and ferric enterobactin TBDTs FhuA, FecA and FepA, respectively, and those of the *P. aeruginosa* ferric pyoverdin and ferric pyochelin TBDTs FpvA and FptA have been solved (reviewed in Ref. [60]). Siderophores are transported across the OM and the CM, and iron is released from the iron–siderophore complex in the cytoplasm. Several bacteria can use exogenous siderophores (xenosiderophores) that they have not synthesized [16].

Vitamin B_{12}

Vitamin B_{12} (cobalamin) and cyanocobalamin, both of which contain a Co^{2+} ion in a corrin ring, are transported by the specific TBDT BtuB of *E. coli* [13]. Both substrates are internalized in the cytoplasm as a whole.

In the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*), a recent global bioinformatic analysis (see later) indicated that, out of a total number of 48 OM proteins exhibiting the structural features of TBDTs, only seven were likely to be involved in iron uptake [25]. Detailed characterization of one pathogenicity-related locus of *Xcc* revealed that it contains a gene encoding a sucrose-specific TBDT, and that sucrose transport requires the pmf. Owing to the redundancy of the predicted TonB proteins in *Xcc* (there are eight homologs) it was not possible to demonstrate a direct involvement of TonB for sucrose uptake.

Taken together, these results demonstrate that nickel and two different carbohydrates are actively transported by specific TBDTs, and that transport is mediated by the TonB complex in at least two cases. Similar to iron, the TonB-dependent acquisition of maltodextrins and sucrose is critical for *in vivo* fitness and virulence of the *C. crescentus* and *Xcc*, respectively [25,26]. The fact that non-metal substrates such as carbohydrates are transported by TBDTs indicates that chemically diverse molecules can be scavenged by a TonB-dependent transport mechanism.

Unexpected features of the novel TBDTs

Experiments using radioactively labeled maltodextrins and sucrose revealed that concentration-dependent transport through the respective TBDTs exhibits biphasic kinetics that are similar to those measured for the transport of vitamin B_{12} and iron complexes [25,26,32,33]. The fast rate is assumed to mainly represent substrate binding to the TBDT, and the slow rate to reflect the energy-dependent release of a substrate (such as vitamin B_{12}) from a membrane-binding site into the interior of the cell [32]. Despite common transport kinetics, the affinities of the newly identified substrates to their respective TBDTs are 10–100 times lower (between 33 and 220 nM [25,26]) than the binding of substrates to iron-TBDTs and BtuB (which possess nanomolar or subnanomolar K_d values [33–35]). This could reflect the higher bioavailability of nickel and carbon sources in the environment relative to vitamin B_{12} and soluble iron. Perhaps more surprising is the possibility that, in contrast to previous observations, binding of maltodextrins to MalA could be strongly reduced by the pmf inhibitor carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP) [26]. Although this observation requires more robust experimental verification, it was proposed that energy is needed to induce a state of MalA that is able to bind maltodextrins [26]. The converse occurs in the well-studied systems of iron complexes and vitamin B_{12}, where substrate binding to their respective TBDTs is pmf-independent (no inhibition by CCCP), although substrate release requires energy (blocked by CCCP) [2]. If it is proven to be the case, these findings indicate that, depending on the substrate, TonB-dependent transport could differ in detail. Three other recent publications support this notion, demonstrating that different substrates use non-overlapping residues of the same TBDT for binding and translocation [36,37], and that substrate-induced structural transitions of TBDTs do not follow a common mechanism [8]. These findings raise questions as to why the transport mechanisms are different and whether these differences depend on the mechanisms of substrate capture in the periplasm and translocation across the CM. Indeed, it was shown for iron complexes and vitamin B_{12} that they are translocated across the CM by ATP-dependent ATP-binding-cassette (ABC) transporters (TC 3.A.1.14) [38]. By contrast, no ABC transporters are required for nickel, maltodextrin or sucrose transport [25–27]. The CM transporters for nickel, maltodextrin and sucrose belong to the class of secondary transporters that require an electrochemical potential for transport but are not known to use a

Table 1. Novel substrates of TBDTs

Substrates	TBDTs[a]	Phylogenetic distribution[b]	Evidence[c]	Refs
Maltodextrins	CC2287 (MalA)	*Caulobacter crescentus*	X	[26]
Nickel	HP1512 (FrpB4)	*Helicobacter pylori* (ε)	X	[27,44]
	Bll6948	*Bradyrhizobium japonicum* (α)	R (NikR)	
	Daro_3944, Rgel01002199	β (*Dechloromonas aromatica, Rubrivivax gelatinosus*)	C	
Sucrose	XCC3358 (SuxA)	*Xan (Xcc)*	X	[25]
	Sfri_3988	Alt (*Shewanella* spp.)	R (ScrR), C	
Cobalt	Saro02000109	*Novosphingomonas aromaticivorans* (α)	R (*B12*), C	[44]
	Daro_1684	*D. aromatica* (β)		
Thiamin	SO2715, CPS_0067, XCC0674	Alt (*Shewanella, Colwellia* spp.), Xan	R (*THI*), C	[31,47]
	BF0615	CFB (*Bacteroides fragilis*)		
	GOX1347	α (*Gluconobacter oxydans*)		
	Bcep18194_B2436	β (*Burkholderia cepacia*)		
Chito-oligosaccharides	SO3514 (ChiPII), Sden_2708, CPS_1021	Alt (*Shewanella, Colwellia* spp.)	R (NagR), C	[45]
	XCC2944, CC0446	Xan, some α (*C. crescentus*)		
Cobalamin (vitamin B_{12})	VC0156, PA1271, PA2911	Vib (*Vibrio cholerae*), Pse (*Pseudomonas aeruginosa*)	R (*B12*), C	[67]
	SO0815, XCC3067	Alt (*Shewanella* spp), Xan		
	CC1750	α (*C. crescentus, Rhodobacter sphaeroides,*		
	RSP_2402, RPA0407	*Rhodopseudomonas palustris*)		
	RS02718, BPSL0976	β (*Ralstonia solanacearum, Burkholderia pseudomallei*)		
Copper	NosA, OprC	Pse (*Pseudomonas stutzer, Pseudomonas putida,*	O	[50–53]
		Pseudomonas aeruginosa)		

[a]Standard gene or protein identificators are from GenBank. Designated names are given in brackets.
[b]Abbreviations of taxonomic groups of γ-proteobacteria are: Alt, Altermonadales; CFB, *Chlorobium/Bacteroides* group; Pse, Pseudomonadales; Vib, Vibrionales; Xan, Xanthomonadales. Other taxonomic groups: α, β and ε correspond to α-proteobacteria, β-proteobacteria and ε-proteobacteria.
[c]Genome context evidences are: X, experimentally demonstrated; R, co-regulation by a conserved regulatory motif that is either a candidate binding site of transcription factors or a metabolite-sensing riboswitch; C, conserved co-localization of the TBDT genes with the specific metabolic pathway genes on the chromosome; O, observation of regulation. Names of regulators or riboswitches and of co-localized gene loci are given in parenthesis. Predicted regulators of genes encoding TBDT are nickel repressor NikR, carbohydrate-utilization regulators NagR (N-acetylglucosamine) and ScrR (sucrose). RNA regulatory elements are cobalamin-responsive riboswitch (*B12*) and thiamin pyrophosphate-responsive riboswitch (*THI*).

periplasmic-binding protein (PBP). In the case of *H. pylori*, the only demonstrated CM nickel transporter is NixA, an eight transmembrane domain, single component secondary transporter for nickel and cobalt ions (i.e. a member of the NiCoT family) [27,39]. Deletion of *nixA* abolished nickel transport under the tested conditions [27].

Xcc scavenges sucrose by the CM transporter SuxC that belongs to the Na^+-melibiose and H^+-sugar co-transporter family and that is encoded in the divergent locus with *suxA* [25]. In *C. crescentus*, a decrease in ATP pools and subsequent ABC-transport inhibition does not reduce maltodextrin uptake, demonstrating that no ABC transporter is involved [26]. A putative transporter, CC2283, that is encoded in the same genomic locus as MalA and that belongs to the Na^+-melibiose and H^+-sugar co-transporter family was hypothesized to accomplish maltose transport across the CM [26]. Because these secondary transporters lack a PBP for substrate transport across the periplasmic space, we propose that they require a different mechanism for substrate propagation from the TBDT to the CM transporter. Interestingly, Carter *et al.* [5] demonstrated that TonB interacts with the PBP for ferrichrome FhuD. They suggest a mechanism in which TonB is recruiting FhuD beneath the lumen of the TBDT FhuA, thus acting as a scaffold to accomplish unidirectional flow of substrates from the OM to the CM [5]. Therefore, different requirements for the substrate capture in the periplasm could provide an explanation for the observed differences in the kinetics of binding to and release from TBDTs for maltodextrins and iron complexes or vitamin B_{12}. Although a lack of the requirement for a PBP clearly needs to be proven, it will be interesting to understand what distinguishes the different mechanisms of transport: is it the chemical structure of the substrate, its bioavailability, the amount of energy required for substrate uptake, a combination of the mentioned factors or even other characteristics?

Genomics-based predictions of TBDTs for novel substrates

The experimental expansion of TonB-dependent substrates correlates with bioinformatic predictions. Homology-based methods are difficult to apply to TBDTs because the sequences of this transporter class vary substantially. To assign a functional role to a hypothetical TBDT, comparative analyses of genomes using chromosomal clustering are feasible [40,41]. For example, many carbohydrate-utilization clusters contain genes encoding both catabolic enzymes and transporters for the uptake of the corresponding sugars [42]. Analysis of metabolic regulons is another efficient method for functional annotation of hypothetical transporter genes [31]. This type of genome context analysis is based on the identification and cross-genome comparison of shared regulatory sites, such as transcription-factor-binding sites, or RNA regulatory elements, such as riboswitches [43]. Thus, both co-localization and co-regulation evidences can be effectively used to predict substrates for hypothetical genes encoding TBDTs (Table 1).

The prediction of novel nickel and cobalt (Ni/Co) TBDTs in α- and β-proteobacteria was performed on the basis of genomic linkage of the genes encoding putative TBDTs to Ni/Co CM transporters, Ni/Co metabolic enzymes and the presence of upstream regulatory elements [44]. In the case of cobalt TBDT, the prediction relied on regulation by the vitamin B_{12} riboswitch, the co-localization with genes encoding cobalt transporters and/or enzymes of the vitamin B_{12} biosynthesis pathway [44]. Nickel TBDTs

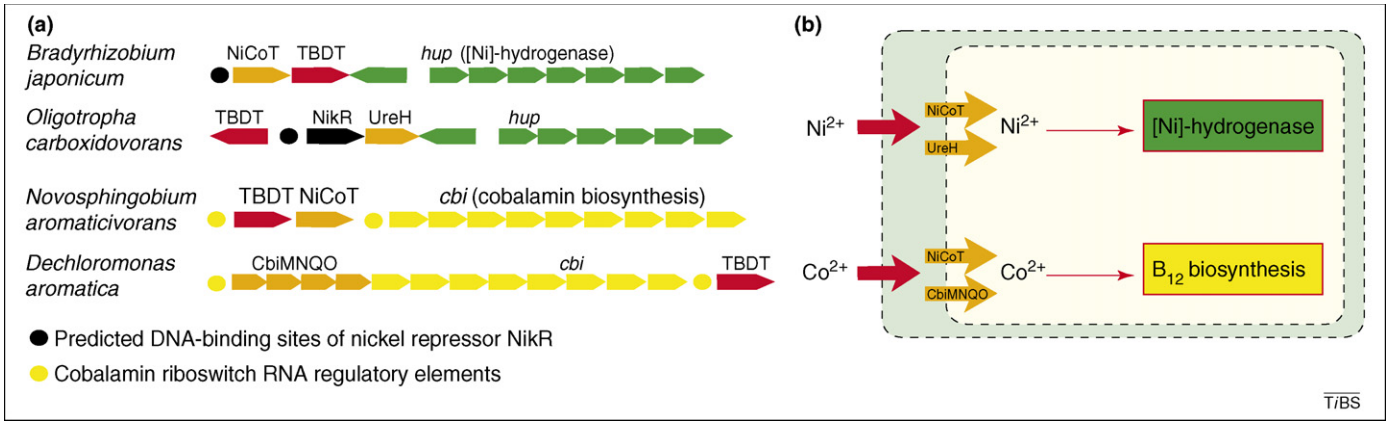

Figure 1. Genomics-based prediction of Ni/Co TBDTs. An example of genome context analyses that demonstrates the functional prediction of hypothetical TBDTs using chromosomal clustering is shown. The prediction of Ni/Co TBDTs is based on genomic co-localization of TBDTs with genes encoding [Ni]-hydrogenase gene clusters (*hup;* green) or Ni/Co transporters (NiCoT, UreH, CbiMNQO; orange) and co-regulation by the transcription factor NikR (black) or vitamin B_{12} riboswitches (yellow). Ni/Co TBDTs were predicted in *Bradyrhizobium japonicum, Oligotropha carboxidovorans pHCG3, Novosphingobium aromaticivorans* and *Dechloromonas aromatica*. **(a)** Arrows of different colors in each line show clusters of co-localized genes. Full circles denote predicted regulatory elements located in the same chromosomal loci. Genes that encode transcription factors and DNA-binding sites are shown by black arrows and circles, respectively. **(b)** Schematic representations of the cellular localization of the proteins encoded in the loci presented in (a). Color code is the same as for (a). The cytoplasm is represented in pale yellow and the periplasm is shown in pale green. Membranes are represented as broken lines.

were predicted based on co-regulation by the nickel repressor NikR and co-localization with genes encoding nickel transporters (UreH and NiCoT families) and/or [Ni]-hydrogenase enzymes (Figure 1). The predicted Ni/Co TBDTs form a distinct subfamily that shows low overall sequence homology to TBDTs for iron complexes and vitamin B_{12}, and lack sequence homology in the TonB-box motif [44]. However, it has been demonstrated that the overall secondary structure of the TonB box (β strand) is the main feature required for interaction with TonB, and not the precise sequence of this motif [13,37]. The predicted Ni/Co TBDTs exhibit a C-terminal β-barrel domain and an N-terminal plug domain [44]. Maximum likelihood phylogenetic analyses of known and predicted TBDTs place the Ni/Co TBDTs identified in α- and β-proteobacteria in a branch separate from that of the *H. pylori* TBDT for nickel uptake (FrpB4) and that of the known vitamin B_{12} TBDT (BtuB) (Figure 2). We suggest that different nickel and cobalt carriers are transported in a TonB-dependent manner, similarly to the known variety of iron complexes (e.g. iron-binding proteins and iron chelators; Box 2). Alternatively, different TBDTs for the same substrate could have evolved separately. Finally, it can be hypothesized that variations in TBDTs could result from the requirement to escape host-recognition mechanisms in pathogenic bacteria by changing the loops of the TBDTs that are localized on the bacterial surface and that are recognized by the host immune system.

Comparative genomic analyses of the N-acetylglucosamine (GlcNAc) utilization pathway and regulons in *Shewanella oneidensis* predicted a TBDT, Chip-II, that is specific for chito-oligosaccharides [45]. This conjecture was based on co-regulation by NagR, the GlcNAc-sensing transcription factor and on co-localization with genes involved in the utilization of chitin and N-acetylglucosamine. In addition, novel TBDTs for chito-oligosaccharides were predicted to exist in Alteromonadales (e.g. in the *Shewanella* spp.), Xanthomonadales and in *C. crescentus* [45].

A comparative genomics study of the TBDTs in *Xcc* revealed that 24 out of the 48 predicted TBDTs are encoded in the vicinity of regulatory elements, metabolic enzymes and CM transporters involved in polysaccharide metabolism [25]. This clustering enabled the prediction of pectin, xylan and maltose- or maltodextrin-specific TBDTs [25]. Furthermore, in the same study, it was observed that the majority of predicted *Xcc* TBDTs (35 out of 72 TBDTs) displayed strong similarities with TBDTs from γ-proteobacteria [25]. Notably, the experimentally verified sucrose TBDT SuxA from *Xcc* forms a single subfamily on the phylogenetic tree with the predicted sucrose TBDTs from the *Shewanella* spp. [25] (Figure 2). An independent bioinformatic study identified the total number of TBDTs in the genomes of 208 eubacterial species by applying two hidden Markov models of the Pfam database (PF07715.1 for the N-terminal plug domain and PF00593.10 for the C-terminal membrane-spanning β-barrel domain). This study found that the predicted TBDTs of the *Bacteroides* species are often encoded in the same genomic loci as genes involved in the processing of dietary polysaccharides [46].

Candidate thiamin-specific TBDTs were identified in the comparative genomic analysis of genes regulated by the thiamin pyrophosphate (TPP) riboswitch in bacterial genomes [47]. The TPP-regulated TBDTs were found in various species from diverse taxonomic groups. Each of these TBDT candidate genes was part of an operon with a gene encoding a CM transporter for thiamin (PnuT). Notably, the only known thiamin ABC transporter ThiBPQ is absent from all PnuT-encoding genomes, therefore the predicted PnuT–TBDT pair could replace the missing thiamin transporter [31,47].

Distribution of putative TBDTs in bacterial genomes

Blanvillain *et al.* [25] performed a systematic search for genes encoding candidate TBDTs in 226 sequenced Gram-negative genomes. The TBDTs were detected by screening the genomic databases using the two Pfam domains cited earlier. Overall, 27% of the genomes analyzed lacked

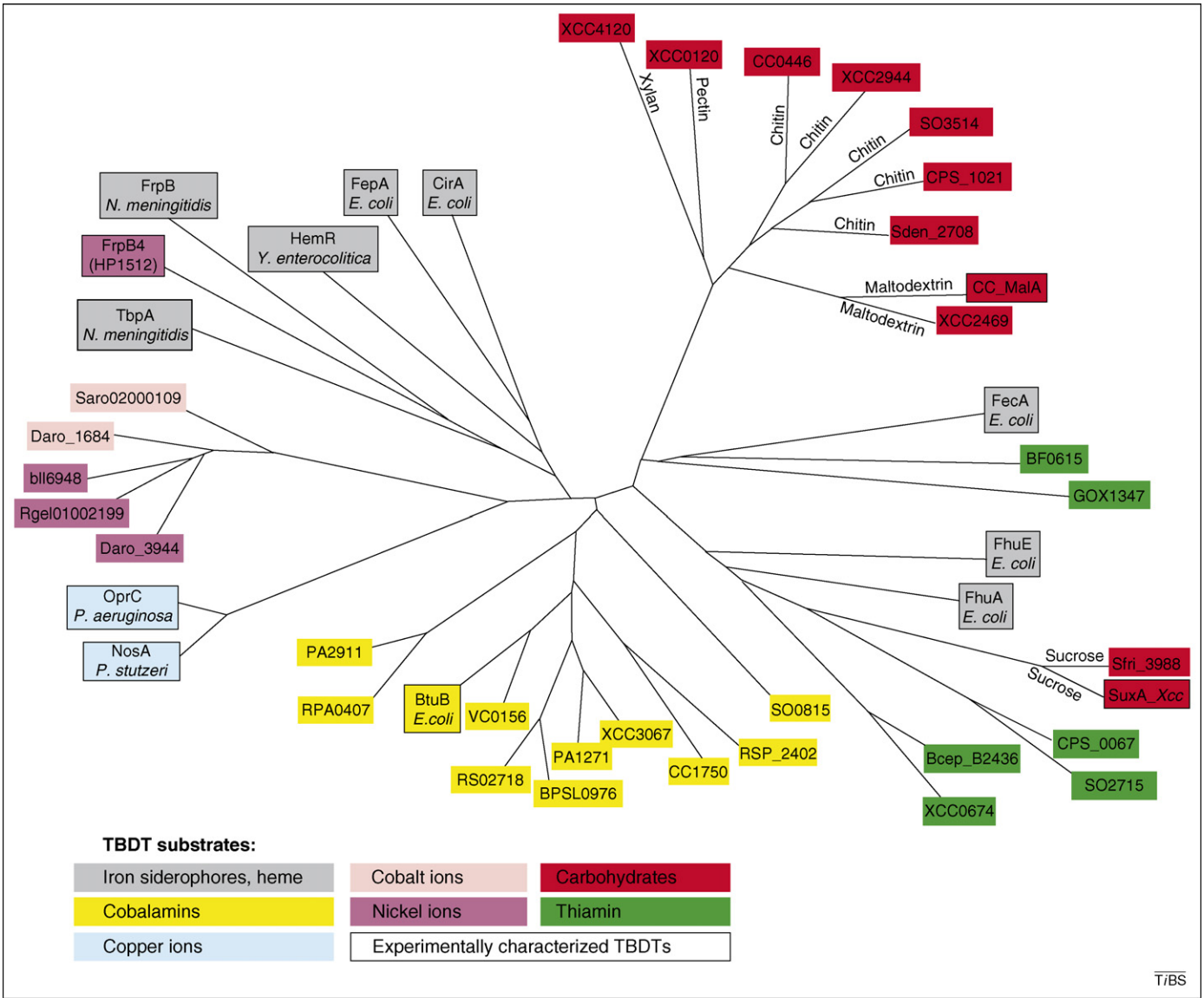

Figure 2. Phylogenetic tree of known and predicted TBDTs in Gram-negative bacteria. The phylogenic analysis of TBDTs for which the function was either predicted or experimentally demonstrated gives a glance at the expansion of the substrate repertoire. The tree was constructed using the maximum likelihood method with the PHYLIP package [59] and colored according to TBDT substrates: gray, iron siderophores and heme; yellow, vitamin B_{12} (cobalamin); blue, copper ions; pink, cobalt ions; purple, nickel ions; red, carbohydrates; green, thiamin. Various carbohydrate substrates are indicated on the tree branches. Proteins are named by genomic identifiers from GenBank (see Table 1 for the list of bacterial genomes). Experimentally characterized TBDTs are boxed.

TBDT genes, 43% contained 1–14 TBDTs and 15% had >30 TBDTs [25]. Using these data, we plotted the number of predicted TBDTs per genome against the total number of open reading frames (ORFs), which served as an indicator of genome size in bacteria (Figure 3). The estimated number of TBDTs varies greatly among different taxonomic groups, species and even strains of a same species. For instance, the pathogenic *E. coli* strain O157:H7 is predicted to have twice as many TBDTs (n = 18) as the non-pathogenic *E. coli* strain K12 (n = 9). There is no correlation between the number of predicted TBDTs and the genome size, which is in contrast to what has been observed for CM transporters [48] and transcriptional factors [43]. Instead, the number of TBDTs seems to depend on the ecological niche of a species and of its physiology. For instance, the ability to efficiently use complex carbohydrates, as the *Bacteroides* species does, which constitutes the most abundant members of the intestinal microflora of mammals, could provide a selective advantage in densely populated microbial niches or in a nutrient-poor environment [49]. Predicted TBDT-encoding genes are impressively over-represented (>30 TBDT per genome) in several taxonomic groups and species of Gram-negative bacteria, including the *Bacteroides* species (*Bacteroides thetaiotaomicron* potentially has 120 TBDT genes), γ-proteobacteria (Xanthomonadales, Altermonadales and Pseudomonadales groups), α-proteobacteria (e.g. *C. crescentus*), some β-proteobacteria (*Nitrosomonas europaea*) and cyanobacteria (*Anabaena* sp.) [25,46]. This TBDT over-representation provides the potential for a huge diversity of uncharacterized substrates that await discovery.

More (un)usual suspects

Re-inspecting old data could reveal TBDTs for novel substrates that have been overlooked. Similar to the nickel-dependent regulation of an OM protein, it was observed that the NosA OM proteins of *Pseudomonas stutzeri*

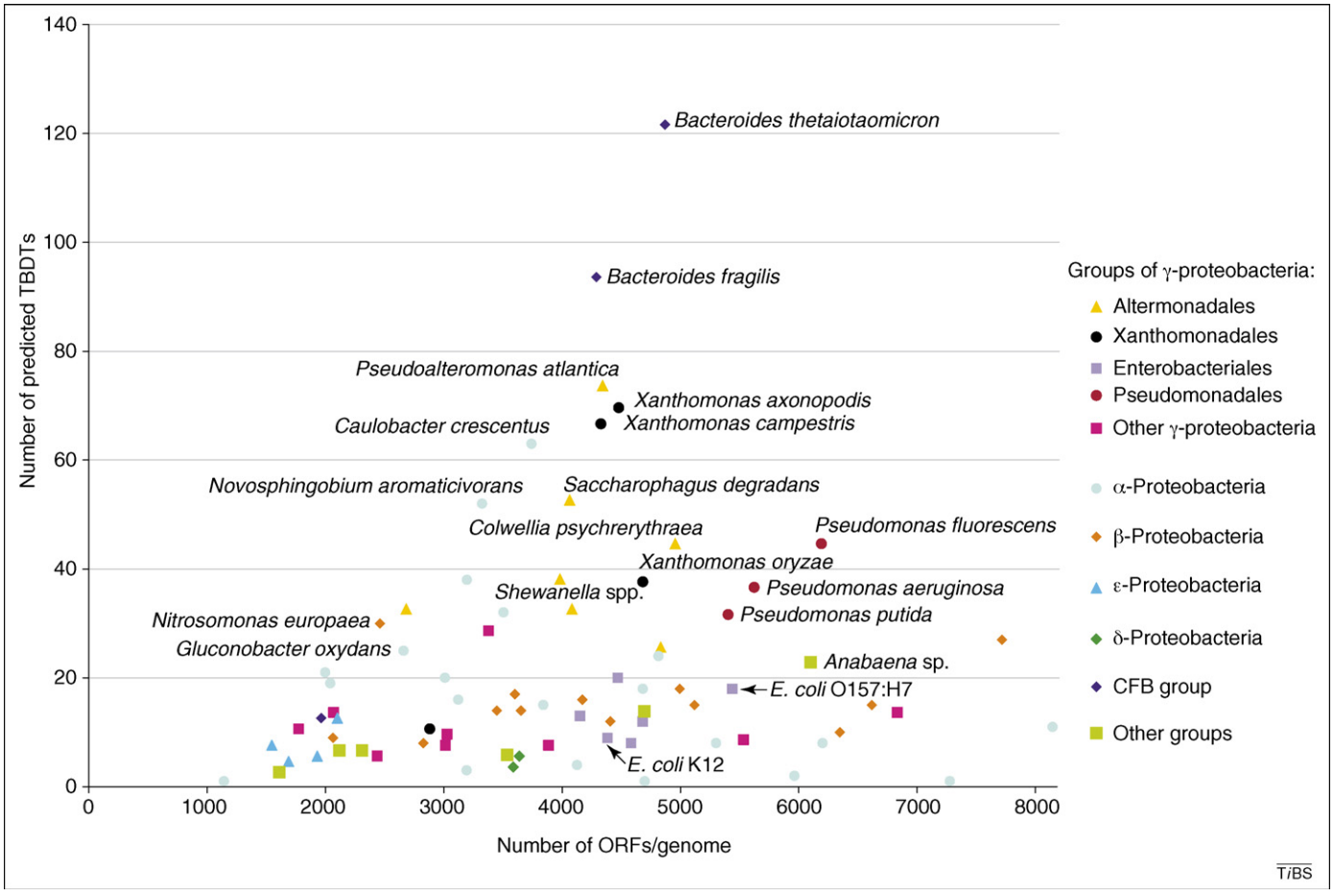

Figure 3. Predicted number of TBDTs in bacterial genomes against the total number of known ORFs per genome. A considerable variation exists in the number of predicted TBDTs that is found in the genomes of Gram-negative bacteria. A spectacular over-representation of TBDTs is predicted in some organisms (up to 120 in *Bacteroides thetaiotaomicron*), pointing towards a huge variety of new TBDT substrates. The plot shows the selection of 80 genomes with non-zero number of predicted TBDTs according to Blanvillain *et al.* [25]. Data used, with permission, from Ref. [25].

[50,51], *Pseudomonas putida* [52] and *Pseudomonas aeruginosa* (OprC) [53] are strongly repressed in the presence of exogenous copper and de-repressed in copper-limiting conditions [54]. NosA was found to bind copper and to be involved in copper uptake. Interestingly, NosA shows an N-terminal plug domain and a C-terminal β-barrel domain comprising 22 sheets, which is characteristic of TBDTs [51,54]. No studies were performed to relate NosA to the TonB-energizing machinery. In *Neisseria* spp., TdfH was annotated as an iron-TBDT but was not involved in iron uptake [55], indicating that TdfH transports a substrate different from iron complexes. Furthermore, it was found that xylose induces the expression of putative TBDTs in *B. thetaiotaomicron* [56], which revealed an over-representation of predicted TBDTs (see earlier). Finally, TBDTs and TonB complexes are differentially expressed in several transcriptome and proteome studies that monitored the genome-wide bacterial response during growth in various media or to stresses that are not related to iron, such as sulfur-containing compounds [57,58]. Thus, transcriptome analyses in the presence of different nutrients could disclose specific TBDTs for different substrates.

Unanswered questions

The identification of novel TBDT-dependent substrates and the finding that their transport mechanisms potentially differ from the uptake of iron complex and vitamin B_{12} raises several general questions (Table 2). First, what is the quantity and diversity of TonB-dependent substrates? What are their common characteristics and what are their respective TBDTs? Experimental confirmation of the substrate specificity of the predicted TBDTs will help to answer these questions. Second, what is the mechanism of substrate capture? Are adaptor molecules such as carrier proteins or siderophore-like molecules involved? Is direct binding of the substrate to a TBDT, as found for carbon sources, an alternative mechanism? Third, how is TonB-dependent transport integrated in the entire process of substrate uptake, including transport across the OM, the periplasmic space and the CM? Is a PBP, in combination with an ABC transporter, required for substrate channeling towards the cytoplasm or are there other mechanisms that use secondary transporters? What is the role of the TonB complex during this process for different substrates? Detailed characterization and comparison of the transport mechanisms of different substrates should lead to a better understanding of TonB-dependent transport. Finally, can the presence of specific TBDTs in microbial genomes provide a better understanding of virulence and general adaptation of bacteria to their specific environments by demonstrating their need for the active uptake of selective substrates? Can we learn about the environment in which

Table 2. Differences observed between classical and novel TonB-dependent transport[a]

	Classical characteristics of TonB-dependent transport	Novel characteristics of TonB-dependent transport	Emerging questions
Substrates	Iron complexes, vitamin B_{12}	Maltodextrins, nickel and sucrose	How many TonB-dependent substrates exist? How can they be classified?
Affinity of substrate binding	Nanomolar or sub-nanomolar K_d	K_d = 33–220 nM	Does the affinity of substrate binding correlate with the bioavailability of substrates?
Substrate capture	Direct and via, for example, siderophores and hemophores	Unknown	Do different capture mechanisms exist? Are adaptor molecules needed or is direct receptor binding sufficient?
TonB-dependent step	Substrate release?	Substrate binding?	How does TonB energize transport through TBDTs?
PBP	Yes	None identified	Are PBPs required for TonB-dependent transport? What is the subsequent transport mechanism through the periplasm?
CM transporter	ABC transporter	Secondary transporter that requires the pmf or smf	Which transporters can be used?
Energy source for cytoplasmic transport	ATP hydrolysis	pmf or smf	Does the substrate chemistry or concentration correlate with the energy source for transport across the CM?

[a]Abbreviations: CM, cytoplasmic membrane; PBP, periplasmic-binding protein; pmf, proton motive force; smf, sodium motive force.

a given organism resides by understanding the full range of substrates transported by its TBDTs and by analyzing their regulation?

Concluding remarks and future perspectives

A limited number of experimental studies, together with genome-wide bioinformatic analyses, indicate a diversity of TBDTs and respective substrates that largely extends beyond those previously known. This expansion challenges our knowledge of TonB-dependent transport because it shows characteristics that have not been observed previously. Thus, each stage of TonB-dependent transport deserves further investigation to eventually establish sub-families of TBDTs. Furthermore, the structural diversity of TBDTs needs to be examined, as does their recognition specificity or eventual versatility, and their phylogeny must be analyzed. Likewise, TonB is often encoded in multiple copies in a single organism and it has been suggested that each molecule energizes selective substrate uptake. The dissection of the specificity of TonB for TBDTs will complete our understanding of substrate-uptake mechanisms. Finally, the uncovered diversity of TBDTs needs to be examined in the context of bacterial ecotypes, the adaptation of a bacterium to a specific or multiple niches and, in the case of pathogens, their multiplication within the host and, thus, their virulence.

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