Published in final edited form as:
Future Microbiol. 2010 September ; 5(9): 1403–1412. doi:10.2217/fmb.10.90.

The TonB energy transduction systems in *Vibrio* species

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Abstract

Studying the organization and conservation of the TonB systems across the genus *Vibrio*, we can tease out trends in gene arrangement and function that lead to clues about the evolution and necessity of the proteins in multiple TonB systems. The TonB2 systems, with additional TtpC proteins, are in general more promiscuous regarding their interactions with many different TonB-dependent transporters in the outer membrane. Studies show that the TtpC protein spans the periplasmic space, suggesting that it can be the connection between the energy from the proton motive force and the outer membrane protein receptors, which the shorter TonB2 cannot provide. As an earlier system, the combination of the TtpC protein and a TonB2 system must have been necessary for the function of the smaller TonB2 protein and to transduce energy in a medium that can have osmotic challenges.

Keywords

iron transport; outer membrane protein receptor; pathogenic *Vibrio*; proton motive force; siderophore; TonB protein; virulence

Iron is a precious resource for most bacterial species. Iron ions are essential for cellular processes, acting as electron donors and acceptors in redox reactions and are also used as co-factors for a number of enzymes [1–3]. The availability of free, unbound iron is low in most environments, especially in the mammalian host where it is chelated by heme groups, transferrin and other iron-binding proteins. In order to survive in low-iron environments, the expression of siderophores and iron-uptake machinery in microorganisms is increased through derepression by the master regulator, Fur [4].

Small-molecular-weight compounds called siderophores have a high affinity for iron, more so than many host iron-binding proteins and are secreted by the bacteria to scavenge iron from the surrounding environment. Iron bound by siderophore complexes is internalized via an energy-dependent process. In Gram-negative species, the iron–siderophore complex is bound on the surface of the outer membrane by receptor proteins, internalized into the periplasmic space and then transported through the inner membrane to the cytosol. The active transport of the iron–siderophore compounds across the outer membrane requires energy that is transduced to the outer membrane via a complex of proteins called the TonB energy-transduction system [5–7]. The process of iron transport in *Escherichia coli* has been

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Financial & competing interests disclosure
The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

studied and is quite well understood. However, these processes in organisms belonging to the family Vibrionaceae, as well as other aquatic bacteria, still pose unanswered questions.

In Gram-negative organisms, the cytoplasm of the cell is separated from the environment by two lipid bilayers – the inner and outer membranes. The inner membrane is the site of cellular energy production, where the cell actively pumps protons generated during respiration into the periplasmic space between the inner and outer membranes. This proton pumping results in the generation of a proton gradient across the inner membrane with the majority of the protons in the periplasm. The potential energy created by the proton gradient across the inner membrane is known as the proton motive force. This potential energy is converted to the energy currency of the cell, ATP, by inner-membrane proteins termed ATP synthases. The outer membrane contains many membrane-bound proteins, some of which can sense changes in the surrounding environment or transport substrates into and out of the cell. However, there is no energy production in the outer membrane. If proteins in the outer membrane require energy, it must be transferred from the inner membrane proton motive force by a system of proteins that, in *E. coli*, consist of a TonB and its accessory proteins [5–7].

In this bacterium, in addition to the energy-transducing protein TonB, the TonB system includes two accessory proteins, ExbB and ExbD. This system has been well studied in the contexts of iron and vitamin B12 transport [8,9]. ExbB is necessary to stabilize the TonB protein in the inner membrane as TonB interacts with a ‘TonB box’ region on the periplasmic domain of the TonB-dependent transporter in the outer membrane [10]. TonB and ExbD pass through the inner membrane once with their carboxy-terminal domains (CTDs) in the periplasmic space, while ExbB has three transmembrane (TM) domains with its carboxy terminus in the cytoplasm [11–15]. Interaction between TM domains has been proven for TonB and ExbD in *E. coli* via formaldehyde crosslinking and western blot analysis [16].

Two models for the mechanism of TonB-mediated energy transduction in *E. coli* have been proposed. In the ‘shuttle’ model, the energized form of the TonB protein leaves the cytoplasmic membrane and traverses the periplasmic space to interact with the TonB box region of the outer membrane transporter [17,18]. In the ‘pulling’ model, the TonB protein remains imbedded in the inner membrane through interaction with ExbB and ExbD, but spans the periplasm to interact with the outer membrane transporter, pulling the plug domain of the TonB-dependent transporter resulting in a conformational change of the plug domain or displacement of the plug from the barrel of the transporter [19–24]. How these proteins convert the potential energy of the proton motive force into conformational changes is still unknown.

TonB proteins in *Vibrio* species

Vibrio spp. are Gram-negative, oxidase-positive organisms characterized by a requirement for 1–3% salt in defined growth media. Morphologically, they are short, curved rods with polar and, in some species, lateral flagella.

Vibrio species are abundant in the marine environment and are common pathogens for several marine organisms including oysters, eels and fish. *Vibrio* species can also cause significant morbidity and mortality when they become opportunistic pathogens of humans by ingestion of contaminated seafood or drinking water, or through direct contact with open wounds. A collection of virulence factors aid in colonization of the host including toxin production, biofilm formation and the ability to bind and actively transport iron across the cell membranes [25–30].

Vibrio species usually possess two chromosomes and unlike *E. coli* with its single TonB-ExbB–ExbD complex, they have multiple TonB systems in their genomes. These TonB systems and iron uptake proteins are depicted in Figure 1. Occhino *et al.* first identified the existence of multiple TonB systems in a single organism, *Vibrio cholerae*, in 1998 [31]. In subsequent work, Seliger *et al.* reported that the two TonB systems of *V. cholerae* are not completely redundant in regard to facilitating uptake of several different iron sources including ferrichrome, hemin, vibriobacin, enterobactin and schizokinen [32]. Multiple TonB systems have been characterized in other *Vibrio* species, including *Vibrio anguillarum*, *Vibrio vulnificus*, *Vibrio alginolyticus* [27,33,34] and *Vibrio parahaemolyticus* [Kuehl C, Crosa J, Unpublished Data].

It was observed that, with some exceptions, the genes of the TonB1 systems in *Vibrio* spp. are present in the smaller chromosome and consist of the proteins ExbB1, ExbD1 and TonB1, which are associated with heme transport genes and intervene in heme and ferrichrome transport [27,29,34–36], while the genes of the TonB2 systems, arranged as *ttpC*, *exbB2*, *exbD2* and *tonB2*, found in the larger chromosome of *V. cholerae*, *V. vulnificus* and *V. anguillarum*, are more promiscuous, supplying energy for transport of all siderophore-bound iron sources such as endogenous and exogenous siderophores. In *V. anguillarum*, TonB2 is essential for the transport of the endogenous siderophores anguibactin and vanchrobactin, as well as of the exogenously produced sources such as enterobactin and ferrichrome. This is also the case for *V. cholerae* [32,37] in which the TonB2 system can also energize heme transport through the HasR outer membrane receptor [31].

In *V. cholerae*, strains lacking TonB1 are unable to compete with the wild-type classical strain CA401 or a mutant in *tonB2* using an *in vitro* competition assay with heme as the sole iron source [32,38]. Conversely, the *tonB2* mutant, as well as the TonB1-deficient strain, complemented with a clone expressing TonB1, did not show a growth disadvantage compared with the wild-type strain. These results suggest a preferential role for TonB1 in *V. cholerae* hemin uptake.

Payne's laboratory proposed that the role of TonB1 in heme–hemoglobin uptake in medium mimicking sea water provides a physiological explanation for the presence of a second TonB. Furthermore, TonB2 could not use hemin at increased NaCl concentrations [29,32]. Seliger *et al.* demonstrated that the *Vibrio* TonB2 proteins lack the extended proline-rich sequence in the periplasmic spanning region found in Vibrio TonB1 proteins and *E. coli* TonB [32,39]. Considering that the periplasmic space expands when cells are grown in media with increased osmolarity [40], the authors postulated that the TonB2 protein could not interact with the heme receptor in the outer membrane under high salt conditions because of the expanded periplasmic space. This hypothesis was supported by the evidence that a strain expressing the TonB1 protein lacking 35 amino acids of the nonessential proline-rich region, as characterized by Larsen *et al.* in *E. coli* [39], was also unable to energize hemin transport at NaCl concentrations above 250 mM. The ability of *V. cholerae* TonB1, but not TonB2 or this TonB1 deletion to interact with the hemin receptor at high salt concentrations may be a function of the extended periplasmic domain of TonB1. However, this hypothesis needs further testing because the strains expressing TonB2 or the shortened TonB1 mutant were still able to grow in the presence of ferrichrome at high salt concentrations [32].

Receptor specificities of the TonB1 & TonB2 systems

While part of chimeric TonB proteins constructed using portions of *E.coli* and *V. cholerae* TonB proteins, the carboxy terminal one-third of either the *V. cholerae* TonB1 or *E. coli*

TonB confers the receptor specificities associated with the full-length protein. Single-amino-acid substitutions near the carboxy terminus of *V. cholerae* TonB1 were used to determine this specificity [41]. The TonB membrane topology is conserved in TonB2s of both *V. cholerae* and *V. anguillarum*, with the CTD in the periplasm where it could theoretically interact with the periplasmic loops of TonB2-dependent transporters in the outer membrane [42]. *In silico* observations indicate that this is also true in the other pathogenic *Vibrio* spp. [Kuehl C, Crosa J, Unpublished Data] [34].

TonB-dependent receptors have conserved TonB boxes, short peptide sequences in the amino terminal and periplasmically located loops, that interact with specific TonB proteins. Mey *et al.* investigated this TonB specificity by switching conserved domains of TonB box peptides between *E. coli* and *V. cholerae* [41]. The TonB specificities of the receptors were not changed.

In *V. cholerae*, both the TonB1 and the TonB2 systems can energize the heme receptors HutA and HutR, although maximum efficiency of heme uptake through these receptors is only observed when the TonB1 system is present [43]. Mey and Payne reported that the amino acid sequences of HutA and HutR are homologous to each other and to other heme receptors [31]. They found that a double *hutA–hutR* mutant had a significantly decreased ability to utilize hemin when it was present as the sole iron source. A third heme receptor, HasR, was most similar to non-*Vibrio* heme receptors and can only be energized by the TonB2 system. HasR possesses a ‘TonB box’ unlike the TonB-binding regions found in other *Vibrio* heme receptors [31]. This result may be due to the role played by the TtpC proteins in all of the observed TonB2 systems [44], which is discussed in the next section.

TonB1-mediated transport of heme is a trend that continues in *V. alginolyticus* (Table 1). Wang *et al.* demonstrated that heme and hemoglobin support *V. alginolyticus* growth only for strains that express TonB1, in contrast to their evidence using liquid growth assays that showed the endogenous siderophore vibrioferrin uses both the TonB1 and TonB2 systems [34].

In the case of *V. anguillarum*, functional analysis of the CTD of the TonB2 protein revealed that a deletion of the final two amino acids did not change ^{55}Fe–anguibactin transport efficiency, but that larger deletions resulted in *V. anguillarum* strains that were no longer capable of Fe–anguibactin transport [42]. In the same study, alanine substitution mutations at the far carboxy terminus showed that the length of the protein, as opposed to the specific amino acids involved at positions 204–206, was important for function in ^{55}Fe–anguibactin transport. Similar mutations were made at positions 201–203, which demonstrated the essentiality of those residues for transport. These observations verified that TonB2 was essential for iron transport of some iron–siderophore complexes and that the organization of the carboxy terminus of the *V. anguillarum* TonB2 was significantly different from that of the *E. coli* TonB.

Another player in the game

In *Vibrio* species and Vibrionaceae family members, a second and occasionally a third TonB system are observed. These TonB2 (and TonB3) systems (Figure 2) consist of the classic ExbB2, ExbD2 and TonB2 (or ExbB3, ExbD3 and TonB3) proteins as well as a fourth protein TtpC [37].

It is remarkable that the 49 kDa TtpC protein is essential for TonB2-mediated iron transport in *V. anguillarum* and *V. cholerae* and has since been identified upstream of the TonB2 and TonB3 gene clusters in all *Vibrio* and other aquatic species examined (Table 2). TtpC is predicted to span the membrane three times with a carboxy-terminal distribution of TM

domains highly similar to the ExbB proteins, leaving the majority of the protein, including the amino terminal signal sequence, predicted to be in the periplasm (Figure 3). Work is currently underway to determine the actual membrane topology of the TtpC protein.

It is possible that TtpC could aid the shorter TonB2 to span the periplasmic space; however, there is another potential reason that could have motivated the evolutionary appearance of TtpC. The *V. anguillarum* TonB2 CTD has two significant differences in tertiary structure as compared with the solution structure of the *E. coli* TonB CTD [42]. General observations show that the *V. anguillarum* TonB2-CTD is less basic overall than the *E. coli* TonB-CTD. The TonB boxes of many TonB-dependent receptors contain primarily hydrophobic and acidic residues and this difference in the composition of the electrostatic surface between TonB2 and TonB may affect recruitment of TonB box regions to the TonB2 CTD. In fact, this appears to be the case since no *in vitro* binding was observed between the ten amino acid TonB box peptide of the *E. coli* enterobactin receptor FepA (EDTITVTAAP) or *V. anguillarum* anguibactin receptor FatA (ESITVYGEA) and the CTD of TonB2 [42]. However, binding was observed between the TonB box peptides and the *E.coli* TonB CTD [42].

In the *V. anguillarum* TonB2 protein, the loop extending from the α2 helix to the β3 strand is significantly longer than the corresponding region in *E. coli* TonB. The β4 strand present in the *E. coli* TonB CTD is absent from the *V. anguillarum* protein. Functional complementation between *E. coli* TonB and *V. anguillarum* TonB2 in a *V. anguillarum tonB2*-deletion background demonstrated that *E. coli* TonB is capable of substituting for *V. anguillarum* TonB2 in ferric-anguibactin transport through FatA; however, neither enterobactin nor vanchrobactin supported growth in bioassays with this strain. Conversely, TonB2, as well as chimeric *V. anguillarum* TonB2 proteins that possessed the missing β4 strand present in *E. coli* TonB, failed to complement enterobactin uptake in a TonB deletion mutation in *E. coli*, although this same chimeric TonB2-β4 was able to support Fe–anguibactin transport in *V. anguillarum* at wild-type levels [42]. This evidence underscores the dependence of *V. anguillarum* TonB2 on its accessory protein, TtpC.

The necessity of the novel TtpC protein for iron transport mediated by the TonB2 system was first identified by a Tn10 transposon mutagenesis screen in *V. anguillarum* [37]. The TtpC protein of *V. anguillarum* was originally annotated as TolR owing to its sequence homology to the 457 amino acid *V. cholerae* protein (also TolR) encoded upstream of *V. cholerae exbB2*. This *V. cholerae* protein was annotated as TolR because the carboxy terminal half of the protein had highest sequence homology to the much shorter TolR protein of the TolQRAB system [37]. The annotation as TolR was discovered to be misleading upon further molecular characterization of the protein. Although the carboxy terminal TM domain region, specifically the region from predicted TM domain 2 to TM domain 3, is highly similar to the MotA/TolQ/ExbB family; the amino-terminal portion of the protein has no significant homology to any characterized or predicted protein except for other TtpC proteins. Thus, it is in a class by itself.

Stork *et al.* identified that the TtpC protein was present in four multiprotein complexes formed when proteins in *V. anguillarum* cells were crosslinked with 1% formaldehyde [37]. The multiprotein complexes were absent and TtpC became unstably expressed in the membrane fraction of cells deleted for the TonB2 protein. These data suggest that TonB2 and TtpC interact with each other and that TonB2 expression stabilizes TtpC in the membrane [37]. Very-high-molecular-weight complexes of more than 150kDa were also identified to contain TtpC. The authors proposed that these complexes might contain outer membrane receptor proteins specific for ferric siderophores or other iron sources.

The TtpC proteins upstream of the *tonB2* gene clusters in several pathogenic *Vibrio* species are highly similar with amino acid sequence similarities between 73 and 80%. The *V. cholerae* TtpC amino acid sequence has 66% identity to that of *V. anguillarum* using the align function on the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST)p interface [45]. The TtpC proteins are also essential for the TonB2-mediated transport of enterobactin, vibriobactin and hemin in *V. cholerae*, vulnibactin in *V. vulnificus* and likely, vibrioferrin in *V. parahaemolyticus* and *V. alginolyticus* [Kuehl C, Crosa J, Unpublished Data] [46].

The enterobactin receptors in both *V. anguillarum* and *V. cholerae* are energized by their respective TonB2 energy transduction systems [27,32]. Stork *et al.* used ferric-enterobactin utilization bioassays to show that the TtpC protein from *V. anguillarum* was unable to complement a mutation in the TtpC protein of the *V. cholerae* TonB2 system and that growth around ferric-enterobactin as an iron source was only restored when the entire *V. anguillarum* TonB2 system was present in the complementing plasmid, indicating that slight differences at the level of amino acid sequence may abrogate physical interactions or other involvement between the TtpC–TonB2 protein complex [37].

Interspecies complementation of Δ*ttpC* deletion mutations in *V. anguillarum* and *V. cholerae* TonB2-dependent iron uptake with the TtpC proteins of other *Vibrio* spp. is currently being explored.

Virulence attributes of the multiple TonB systems

The role of iron transport in virulence has been studied in several *Vibrio* species using a variety of infection models, while in some cases the natural host–pathogen interaction can be examined. The marine fish pathogen *V. anguillarum* requires an active iron uptake mechanism mediated by the siderophore anguibactin to be able to cause an infection in a vertebrate fish host [27,47,48]. However, it can also acquire iron via transport of heme and siderophores secreted by other microorganisms, such as ferrichrome and enterobactin. Once bound to iron, ferric anguibactin is transported back into the cell cytosol through the specific outer membrane receptor FatA [49–51]. TonB2, but not TonB1, functions in the transport of anguibactin and enterobactin, while both TonB proteins can operate in the transport of ferrichrome and heme. *tonB2* mutants are severely attenuated in virulence by more than 100-fold, while the *tonB1* mutants show only a tenfold decrease in virulence [27]. Complementation of the *tonB2* and *tonB1–tonB2* mutants with the wild-type *tonB2* gene results in restoration of virulence to a level close to that of the wild-type. These results demonstrate that a functional *tonB2* system rather than *tonB1* is essential for ferric-anguibactin transport and virulence of *V. anguillarum* in the natural vertebrate host.

In a similar vein, another fish pathogen that possesses two sets of TonB systems, *Vibrio alginolyticus*, can infect zebrafish [34]. However, in this case, when inoculated intraperitoneally, mutants of either of the two TonB systems demonstrate a marked attenuation in virulence, indicating that both systems are essential for the virulence of this bacterium [34]. As in *V. anguillarum*, the two systems were arranged as TonB1–ExbB1–ExbD1 and TtpC–ExbB2–ExbD2–TonB2, respectively. The TonB1 system specifically contributed to hemin and hemoglobin uptake and both of the TonB systems support iron uptake mediated by ferrichrome and vibrioferrin, the endogenous siderophore of *V. alginolyticus* [34].

Virulence of the human pathogens *V. cholerae* and *V. vulnificus* has also been studied with respect to TonB1 or TonB2 requirement, using the suckling mouse model for *V. cholerae* and the iron-overloaded subcutaneous infection mouse model for *V. vulnificus* [46]. Mouse colonization assays carried out in Payne's laboratory using *V. cholerae* mutants in the

TonB1 or TonB2 systems indicate a role for both TonB systems and mutations in either system resulted in reduced ability to compete with the wild-type *in vivo* [32]. *V. vulnificus* possesses three *tonB* systems and multiplies rapidly in host tissues under iron-overloaded conditions. The TonB1 and TonB2 systems involved in vulnibactin transport are essential for virulence in the iron-overloaded mice inoculated subcutaneously. However, these genes were induced under iron-limiting conditions, indicating that active iron transport is important in infection by this bacterium, even under high-iron conditions. Expression of the TonB3 cluster occurs only when the bacterium grows in human serum and does not show any relevance to pathogenesis [46]. TonB3 could play a role in transport processes associated with metabolic or energetic steps still unidentified.

In summary, except for the absolute necessity for the TonB2 system in *V. anguillarum* virulence, the TonB1 and TonB2 systems are equally responsible for virulence in all the other members of the Vibrionaceae that were examined.

Molecular machinery: comparing TonB2 cluster proteins with torque-generating MotA/B subunits of the flagellar motor

The carboxy terminal TM domain region in the TtpC proteins is most similar to members of the MotA/TolQ/ExbB family of proteins. In *E. coli*, turning the flagellar motor requires the conversion of H^+ ion flux through a proton channel in the inner membrane into a mechanical force applied to the flagellar rotor through the cytoplasmic region of the MotA protein. Ions pass through the inner membrane via a channel composed of four MotB proteins and the TM domain of two MotA proteins [52]. This ion flux is thought to cause a conformational change in MotA, allowing it to move from one FliG subunit of the rotor to the next FliG subunit, thereby turning the rotor [53].

In *V. parahaemolyticus*, the flagellar motor is powered by Na^+ ion flux across the inner membrane. Jaques *et al.* identified MotA and MotB homologs in screens for mutants that could swim in the presence of phenamil, a Na^+-channel inhibitor [54]. The flagellar motor of *V. alginolyticus* is also powered by Na^+ ion flux. Sato and Homma showed that purified *V. alginolyticus* PomAB complexes (MotAB homologs) reconstituted in liposomes allowed Na^+ flux, demonstrating that PomA and PomB formed the Na^+ channel [55]. They later demonstrated that a fused dimer of PomA along with PomB allowed motility and that a single functional PomA unit in the complex was not sufficient, as two functional PomA subunits are required to interact with PomB [56]. The functional characteristics of the closest homologs to the CTD of *Vibrio* TtpC proteins is intriguing and may offer some insight into the molecular mechanisms involved in TonB2-mediated energy transduction.

The high peptide sequence similarity between the TM domains of the MotA, ExbB2 and TtpC proteins point toward similar structure and function of the proteins [57]. This leads us to hypothesize that ExbB2 proteins and the TM domains of TtpC proteins in the TonB2 complex could be arranged as part of a proton channel, making them key players in the transduction of energy from the inner membrane proton motive force to the outer membrane receptors via interaction with TonB2 and, possibly, the amino-terminal region of TtpC. A similar mechanism of TM domain interaction was described using the *E.coli* TonB system [57] and may be similar to the arrangement of the TonB system proteins present in the *Vibrio* spp.

Conclusion

Studying the organization and conservation of the TonB systems across the genus *Vibrio*, we can tease out trends in gene arrangement and function that lead to clues about the evolution

and necessity of the proteins in multiple TonB systems. The TonB2 systems with the additional TtpC proteins are, in general, more promiscuous regarding their interactions with many different TonB-dependent transporters in the outer membrane (Table 1). Often, the TonB2 system is associated with transducing energy for uptake of endogenously produced siderophores that are essential for virulence. In some species, such as *V. alginolyticus*, these virulence-determining siderophores are also transported with energy supplied by the TonB1 system. Studies by Crosa *et al.* show that the TtpC protein spans the periplasmic space, suggesting that it can be the connection between the energy from the proton motive force and the outer membrane protein receptors, which the shorter TonB2 cannot provide. However, this process requires TonB2 because in its absence the TtpC protein is unstable and degrades rapidly. This is where TtpC must play a role in bridging the problem of a short TonB2. As an earlier system, the combination of the TtpC protein and a TonB2 system must have been necessary for the function of the smaller TonB2 protein and to transduce energy in a medium that can have osmotic challenges [32]. It is an evolutionary mystery as to why the TtpC–TonB2 systems have evolved and remained as an important energy transduction system in *Vibrio* spp. and other aquatic bacteria, while the acquisition of the TonB1 systems appears to have occurred more recently in the evolution of these bacteria, possibly motivated by the concomitant carriage of the heme uptake genes.

Future perspective

It appears that the challenge for the next few years will be to understand the exact mechanism by means of which the TtpC and TonB2 proteins join forces to transduce the energy of the proton motive force to the outer membrane receptor proteins. The form of energy being transfered, be it ATP or potential energy released in a conformational change of TtpC or TonB2, is yet unknown and is crucial to defining the mechanism of energy transfer.

Executive summary

Iron transport

- The process of iron transport in *Escherichia coli* has been studied and is quite well understood. However, these processes in organisms belonging to the Vibrionaceae family, as well as other aquatic bacteria, still pose unanswered questions.
- The inner membrane is the location of cellular energy production known as the proton motive force.
- There is no energy production in the outer membrane. If proteins in the outer membrane require energy, it must be transferred from the inner membrane proton motive force by a system of proteins that consist of TonB and its accessory proteins.

TonB proteins in *Vibrio* species

- The *Vibrio* species are abundant in the marine environment and are common pathogens for humans and several marine organisms including oysters, eels and fish.
- *Vibrio* species usually possess two chromosomes and, unlike *E. coli* with its single TonB–ExbB–ExbD complex, they have multiple TonB systems in their genomes.

- The two TonB systems of *Vibrio cholerae* and *Vibrio anguillarum* are not completely redundant in regard to facilitating uptake of several different iron sources including ferrichrome, hemin, vibriobacin, enterobactin and schizokinen.
- The TonB2 systems are more promiscuous than the TonB1 systems, supplying energy for transport of all siderophore-bound iron sources such as endogenous and exogenous siderophores.
- TonB2 shows significant differences at the carboxy-terminal end from the TonB1 proteins and TonB from *E. coli*. These differences might have led to the evolutionary appearance of the 49 kDa TtpC protein.

Requirement of TtpC in the TonB2 system

- The necessity of the novel TtpC protein for iron transport mediated by the TonB2 system was first identified by a Tn10 transposon mutagenesis screen in *V. anguillarum*.
- The TtpC protein is essential for TonB2-mediated iron transport in *V. anguillarum* and *V. cholerae* and has since been identified upstream of the TonB2 and TonB3 gene clusters in all *Vibrio* and other aquatic species examined.
- TonB1 is longer than TonB2. This could be the reason for the discrepancy in heme uptake between the two TonB systems in *V. cholerae* under high salt concentrations, similar to the marine environment. It is possible that TtpC could aid the shorter TonB2 to span the periplasmic space.
- Except for the absolute necessity for the TonB2 system in *V. anguillarum* virulence, the TonB1 and TonB2 systems are equally responsible for virulence in all the other members of the Vibrionaceae examined.
- The carboxy-terminal transmembrane domain region in the TtpC proteins is most similar to members of the MotA/TolQ/ExbB family of proteins, which provide the mechanical force to turn the flagellar rotor.
- ExbB2 proteins and the transmembrane domains of TtpC proteins in the TonB2 complex could be arranged as part of a proton channel, making them key players in the transduction of energy from the inner membrane proton motive force to the outer membrane receptors via interaction with TonB2 and, possibly, the amino-terminal region of TtpC.

Acknowledgments

The research from our laboratory presented in this review was funded by NIH grants 5RO1-AI 019018 and 5RO1-AI 065981. Carole J Kuehl was supported by a graduate student training grant fellowship from the NIH 5T32-AI 007472-13.

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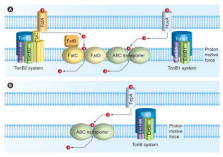

Figure 1. *Vibrio anguillarum* and *Escherichia coli* TonB energy transduction systems

Heme or ferric iron–siderophore complexes are shown as red circles with arrows depicting their transport through the outer membrane TonB-dependent transporters into the periplasm and eventually into the cytoplasm where the iron can be used in cell processes. **(A)** The *V. anguillarum* TonB systems 1 and 2 are depicted as a model for *Vibrio* TonB systems and are compared with **(B)** the *Escherichia coli* TonB system.

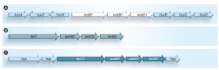

Figure 2. Genetic arrangement of the *tonB* gene clusters in *Vibrio* spp

(A) The *tonB1* operon and surrounding heme uptake genes of *Vibrio anguillarum* are shown as a representation of *Vibrio tonB1* gene clusters. **(B)** Conserved arrangement of the *Vibrio tonB2* gene cluster. **(C)** The *tonB3* gene cluster surrounded by conserved hypothetical proteins is found in several pathogenic *Vibrio* spp. and other marine organisms, as noted in Table 2.

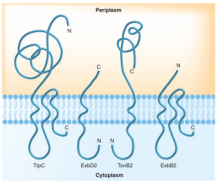

Figure 3. Predicted membrane topologies of the proteins in the *Vibrio* TonB2 energy transduction systems

The membrane topologies of *Escherichia coli* ExbB, ExbD and TonB are known, as is the membrane topology of *Vibrio anguillarum* TonB2, which has one transmembrane domain, with its amino terminus in the cytoplasm. Based on amino acid sequence homologies and *in silico* transmembrane domain predictions, the predicted membrane topologies of the *Vibrio* TonB2 system proteins in the inner membrane are shown.

Table 1

TonB system specificities for siderophore transport.

Species	Siderophores produced	Siderophores used	TonB system used	Ref.
Vibrio anguillarum	Anguibactin	Anguibactin	TonB2	[27]
	Vanchrobactin	Vanchrobactin	TonB2	[58]
		Enterobactin	TonB2	[27]
		Ferrichrome	TonB1 and 2	[27]
		Hemin	TonB1	[27]
Vibrio cholerae	Vibriobactin	Vibriobactin	TonB1 and 2	[29]
		Enterobactin	TonB2	[29]
		Agrobactin	Unknown	[59]
		Fluvibactin	Unknown	[60]
		Ferrichrome	TonB1 and 2	[29]
		Hemin	TonB1 and 2	[29]
Vibrio vulnificus	Vulnibactin	Vulnibactin	TonB1 and 2	[Alice, Unpublished Data] [61]
	Hydroxamate type	Hydroxamate-type	TonB1 and 2	[Alice, Unpublished Data] [62]
		Aerobactin	Unknown	[63]
		Hemin	TonB1 and 2	[35]
Vibrio parahaemolyticus	Vibrioferrin	Vibrioferrin	Unknown	[64]
		Ferrichrome	Unknown	[64]
		Aerobactin	Unknown	[64]
		Hemin	Unknown	[36]
Vibrio alginolyticus	Vibrioferrin	Vibrioferrin	TonB1 and 2	[34]
		Ferrichrome	TonB1 and 2	[34]
		Hemin	TonB1	[34]

Table 2

Marine species with TtpC-TonB2 system homologs.

TtpC2–TonB2	TtpC3–TonB3
Vibrio anguillaram	
Vibrio cholerae	
Vibrio coralliilltticus	
Vibrio furnissii	
Vibrio metschniovi	
Vibrio mimicus	
Vibrio orientalis	
Vibrio shilonii	
Vibrio splendidus	
Vibrio alginolyticus	*V. alginolyticus*
Vibrio angustum	*V. angustum*
Vibrio fischeri	*V. fischeri*
Vibrio harveyi	*V. harveyi*
Vibrio parahaemolyticus	*V. parahaemolyticus*
Vibrio vulnificus	*V. vulnificus*
Aliivibrio salmonicida	*A. salmonicida*
Photobacterium profundum	*P. profundum*
Teredinibacter turnerae	*T. turnerae*
Aeromonas hydrophilia	
Aeromonas salmonicida	
Photobacterium damselae	
Pseudomonas mendocina	
Pseudomonas stutzeri	
Shewanella halifaxenis	
Shewanella putrefaciens	