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Power plays: iron transport and energy transduction in pathogenic vibrios

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Abstract

The Vibrios are a unique group of bacteria inhabiting a vast array of aquatic environments. Many Vibrio species are capable of infecting a wide assortment of hosts. Some of these species include V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. anguillarum, and *V. cholerae*. The ability of these organisms to utilize iron is essential in establishing both an infection in their hosts as well as surviving in the environment. Bacteria are able to sequester iron through the secretion of low molecular weight iron chelators termed siderophores. The iron-siderophore complexes are bound by specific outer membrane receptors and are brought through both the outer and inner membranes of the cell. The energy needed to drive this active transport is achieved through the TonB energy transduction system. When first elucidated in *E. coli*, the TonB system was shown to be a three protein complex consisting of TonB, ExbB and ExbD. Most *Vibrio* species carry two TonB systems. The second TonB system includes a fourth protein; TtpC, which is essential for TonB2 mediated iron transport. Some *Vibrio* species have been shown to carry a third TonB system that also includes a TtpC protein.

Keywords

Vibrio; Iron; TonB; TtpC

Introduction

Vibrios are Gram-negative gammaproteobacteria and facultative anaerobes that require 1–3% salt in defined growth media (Faruque 2008; Ray 2003; Thompson et al. 2004). To date, all species within the genus Vibrio carry two chromosomes with some species carrying additional large plasmids (Heidelberg et al. 2000; Makino et al. 2003; Thompson et al. 2004). Morphologically, members of this genus are ~1 μm wide by 2–3 μm in length, curved shaped rods, and are motile due to at least one polar sheathed flagellum (Austin and Zhang 2006; Hickman et al. 1982). *Vibrio* species are ubiquitous in most aquatic habitats including riverine, estuarine, and marine environments (Martinez et al. 2003; Thompson et al. 2006). The majority of vibrios are nonpathogenic, however, the genus does include several pathogenic species (Janda et al. 1988).

There are four significant human pathogenic species of vibrio that include, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*. Infections caused by these organisms are usually associated with ingestion of raw or undercooked shellfish or exposure of wounds to seawater. *Vibrio cholerae*, the causative agent of cholerae in humans, was first described

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by Pacini in 1854 and later studied in more detail by Robert Koch in 1883 (Wachsmuth et al. 1994). To date, it has been the most studied vibrio pathogen. In the environment it is a free-living bacterium associated with plankton, fish, and shellfish. Once ingested by humans, the bacteria pass the acid barrier of the stomach and penetrate the epithelial cells in the intestine (Hornick et al. 1971). During the colonization of the small intestine several virulence factors are induced including cholerae toxin (CT) (Thompson et al. 2006). The result of the CT on the epithelial cells in the small intestine is what is responsible for the massive watery diarrhea. During this process, large amounts of bacteria are flushed from the body into the environment (Thompson et al. 2006). This profuse diarrhea can lead to death within hours if untreated. The World Health Organization (WHO) estimates that there are 3–5 million cases a year with over 100,000 deaths. Recent outbreaks in Iraq 2007–2008, Zimbabwe 2008–2009, and Haiti 2010, emphasize this threat to public health.

Vibrio parahaemolyticus is ubiquitous in marine environments and is the leading cause of seafood-associated gastroenteritis (Daniels et al. 2000; Joseph et al. 1982). This *Vibrio* species is often associated with oysters and disease can occur in humans when contaminated oysters are consumed raw. Although there have been extensive studies on this bacterium, the exact route of its pathogenic action has not been entirely found (Thompson et al. 2006).

V. alginolyticus was originally classified as *V. parahaemolyticus*, however, under closer examination this species was found to be distinctly different based on sucrose fermentation and other phenotypic characteristics (Sakazaki 1968). *V. alginolyticus* has been found to be the predominant vibrio in many environmental surveys in various parts of the world (Barbieri et al. 1999; Cavallo and Stabili 2002; Chan et al. 1989; Matte et al. 1994). This species was first recognized as a human pathogen in 1973 when it was isolated from tissue infections (Zen-Yoji et al. 1973). *V. alginolyticus* has since been isolated from wound and ear infections but not often from blood infections (Schmidt et al. 1979).

The bacterium *V. vulnificus* is part of the normal flora in both estuarine and coastal waters and can often be found in oysters and clams. *V. vulnificus* is considered the most significant human pathogen naturally found in seawater (Thompson et al. 2006). In the United States it is the number one cause of seafood-borne death with fatality rates as high as 50–60% when infected raw or undercooked seafood is consumed (Hlady et al. 1993). Furthermore, this organism produces fatal wound infections in people who acquire injuries while in seawater. *V. vulnificus* can be fatal to persons with hepatic diseases and other compromised conditions, such as hemochromatosis, beta thalassemia, and liver cirrhosis (Gulig et al. 2005). The most susceptible individuals appear to be ones with elevated iron levels in their serum (Gulig et al. 2005; Hor et al. 2000).

The vibrios are also responsible for causing infections in many different aquatic animals. One of the most well studied animal pathogens in the genus Vibrio is *Vibrio anguillarum*. This fish pathogen is the causative agent of vibriosis, a highly fatal hemorrhagic septicemic disease in salmonid fish (Harbell et al. 1979; Thompson et al. 2006). The disease caused by this bacterium is very similar to septicemic disease in humans (Actis et al. 1999). Vibriosis caused by *V. anguillarum* has been recognized as a major obstacle for salmonid marine culture.

Iron uptake and use

One of the most important factors in the growth and invasion of bacteria is their ability to compete for and acquire iron from both their environment and their host (Crosa et al. 2004). Iron is an essential element for almost all living organisms since it is used in many cellular processes ranging from signaling to metabolism. Iron is the key component of many enzymes including cytochrome proteins (redox reactions) and ribonucleotide reductase

(DNA biosynthesis) (Wandersman and Delepelaire 2004). In fact iron is highly versatile because it can act as both an electron donor as well as an acceptor (Crosa et al. 2004). Regulation of iron is critical; too little and certain reactions cannot proceed, too much and an abundance of free radicals can build up leading to cell death (Crosa et al. 2004). However, iron in vertebrates is bound by a number of different complexes including transferrin or lactoferrin and iron-porphyrin complexes in hemoglobin (Bullen et al. 1978). In order for microorganisms to establish an infection they depend heavily on their ability to use iron from these host-complexes.

The key feature that allows vibrios to survive and cause infection within their host is their iron-sequestering systems (Crosa 1980, 1984; Tolmasky et al. 1988). These systems center upon the production of iron-scavenging compounds known as siderophores and the subsequent transport of the ferric-siderophore complex back into the cell cytosol (Actis et al. 1999; Crosa 1997; Koster et al. 1991). Siderophores are small molecular weight, high affinity iron-binding compounds (Crosa 1989; Griffiths et al. 1984). In Gram-negative bacteria, iron-siderophore complexes first bind to receptor proteins in the outer membrane. The siderophore is then internalized into the periplasmic space and transported through the inner membrane to the cytosol. Siderophores are brought into the cell through active transport that requires energy. Unlike the inner membrane that has access to an established ion gradient or ATP, the outer membrane has no energy production. Proteins in the outer membrane rely on energy generated from the proton motive force (PMF) in the inner membrane that is then transduced to the outer membrane (Crosa et al. 2004). The PMF is created through respiration. The pumping of protons from the cytosol through the inner membrane to the periplasm creates potential energy within this membrane (Braun 1995; Crosa et al. 2004). The periplasmic space between the two membranes presents a challenge to Gram-negative bacteria; how to move the energy from the production factory to the site that needs it. This is solved through a complex of proteins that make up the TonB energy-transduction system (Braun 1995; Crosa et al. 2004; Henderson and Payne 1994).

E. coli TonB

The TonB energy-transduction system was originally dissected in *E. coli*. In this organism there are three proteins involved in the system; TonB, ExbB, and ExbD. *E. coli* TonB is a 239-amino-acid protein (~26 kDa) with the majority of the protein in the periplasmic space. This protein only has one transmembrane (TM) domain and its carboxy-terminus (C-terminus) lies in the periplasmic space (Thompson et al. 2006). The TonB protein interacts with TonB-dependent receptors through the "TonB Box" sequence. This short peptide sequence is located in the amino-terminal periplasmic loop of the receptors and only allows for specific TonB protein interactions (Thompson et al. 2006). Through this mechanism, the TonB protein couples the cytoplasmic membrane PMF to the active transport of substrates through the outer membrane. This system has been well studied in both its ability to transport vitamin B12 as well as iron bound substrates such as siderophores (Fischer et al. 1989; Hantke and Braun 1975). ExbB and ExbD are necessary to stabilize TonB in the inner membrane (Ahmer et al. 1995). ExbB has three TM domains with its C-terminus in the cytoplasm; whereas ExbD has only one TM domain with its C-terminus in the periplasm (Thompson et al. 2006). Interactions between the TM domains of TonB and ExbD have been demonstrated in *E. coli* through both formaldehyde cross-linking and Western blot analysis (Ollis et al. 2009).

Over the years, many different models explaining how the potential energy in the inner membrane is transduced to the outer membrane using TonB have been described. Current evidence leans towards two possibilities, the “shuttle model” and the “pulling model”. In this first model, TonB is thought to disassociate from the inner membrane, transverse the

periplasm and bind to the TonB-dependent receptor. In this model, the potential energy is stored in TonB and brought to the outer membrane (Larsen et al. 2003; Letain and Postle 1997). The second “pulling model” has TonB imbedded and staying in the inner membrane. TonB is then thought to span the periplasmic space and interact with the outer membrane receptor (Crosa et al. 2004). Further study is needed to elucidate how exactly TonB is capable of transducing the energy to the outer membrane and allowing transport of bound substrate siderophores into the cell.

TonB systems and TtpC in *Vibrio* species

Unlike *E. coli*, all of the *Vibrio* species studied to date have at least two TonB systems. The TonB1 system in vibrios shows similarity to the TonB system *E. coli*. The *exbB1*, *exbD1*, and *tonB1* genes are found within an operon and are always associated with the heme uptake system, similar to the genetic arrangement in *E. coli* (Stork et al. 2004; Wang et al. 2008, 1996; Wyckoff et al. 2007). Other TonB systems within *Vibrio* species contain four genes in each cluster; *tonB2*, *exbD2*, *exbB2* and *ttpC*. The fourth gene, *ttpC*, is essential in all of the TonB2-medated iron transport systems tested to date (Stork et al. 2004). TtpC is a 49 kDa protein that is predicted to span the inner membrane 3 times with its C-terminus in the cytosol and the majority of the protein located within the periplasm (Kuehl and Crosa 2010).

Vibrio species are not the only bacteria to encode the fourth accessory protein TtpC. As shown in Table 1, many other bacteria possess TtpC. When TtpC2 from either *V. anguillarum* or *V. cholerae* is used in a BLAST analysis against the other bacterial species listed, every vibrio as well as *A. salmonicida*, *P. profundum* and *P. damselae* show a high percentage of similarity, ~60%. The rest of the species have approximately 40% similarity to either of the TtpC2 proteins tested. Six of the *Vibrio* species listed here contain a third TonB system that includes TtpC3. When TtpC3 from either *V. parahaemolyticus* or *V. vulnificus* is used in this analysis, all of the *Vibrio* species, save *V. fischeri*, show a very high degree of similarity, ~90%, to each of them. It is interesting to note that although TtpC2 from *V. fischeri* shows a high degree of similarity to either of the TtpC2 proteins tested, the third TtpC shows a very low percentage of identity. This phenomenon continues in the other two bacterial species that contain a third TtpC, *P. profundum* and *T. turnerae*. It is very interesting to note that these three species show a very high degree of similarity between TtpC2 and TtpC3 within their own genome, while the other *Vibrio* species show a low degree of identity between these two proteins, ~40%. When comparing TtpC2 or TtpC3, the region with the most homology in all of the species tested is the carboxy terminal end. This end of the protein contains the transmembrane domains and is planted inside of the inner membrane.

Receptor specificities of the TonB systems

Vibrios can not only produce their own siderophores but also have the ability to use siderophores produced from other *Vibrio* species and even other bacterial and fungal species. In a recent review, Kuehl and Crosa (2010) have outlined which TonB system is responsible for transporting siderophores in different *Vibrio* species. With the exception of *V. alginolyticus*, both TonB1 and TonB2 are redundant in their ability to uptake both heme and ferrichrome in vibrios (Kuehl and Crosa 2010). Only in *V. anguillarum* do the siderophores naturally produced by this species have specificity to the TonB2 system (Stork et al. 2004). In other *Vibrio* species studied, it appears that either TonB1 or TonB2 can be used to power the uptake of its naturally produced siderophore (Kuehl and Crosa 2010). Enterobactin, the siderophore produced by *E. coli*, has been shown to only enter *V. cholerae* and *V. anguillarum* through the TonB2 system (Stork et al. 2004; Wyckoff et al. 2007). It is interesting to note that the TonB1 protein in vibrios is on average about 15% longer than its

TonB2 homologue. As mentioned previously, the TonB2 system requires a fourth accessory protein, TtpC, to function (Stork et al. 2007). The possibility exists that while the longer TonB1 is perfectly capable of making contact with its receptor protein in the outer membrane, its homologue, TonB2, is not. The accessory protein TtpC may in fact be doing the binding in its place and transducing the energy from the PMF to the outer membrane.

Virulence and the TonB systems in vibrio

To date, iron has been shown to be a critical component in different *Vibrio* species to effectively cause infection in their host. Virulence has been studied in multiple infection models as well as in their natural host-pathogen interaction. Both *V. anguillarum* and *V. alginolyticus* have been studied for their ability to infect fish, their natural host. Specifically, which TonB systems are needed for a successful infection has been elucidated. It has been shown that *Vibrio anguillarum* requires its naturally produced siderophore, anguibactin, to cause infection in fish (Crosa 1980; Crosa et al. 1980; Wolf and Crosa 1986). Unlike ferrichrome and heme, anguibactin can only be transported through the use of TonB2 and its receptor FatA (Stork et al. 2004). Infection assays have shown that while a *tonB1* mutation causes a 10-fold attenuation in virulence, a *tonB2* mutation had a 100-fold attenuation. A double mutation in both *tonB* genes resulted in a ~200-fold reduction in virulence. This additive effect may have been due to the ability of TonB1 to uptake heme. When complemented with the wild type TonB2 protein, both the *tonB1* and *tonB1/tonB2* mutants were restored to a level close to wild type. These results demonstrate that a functional TonB2 system is needed for virulence as well as a successful anguibactin transport system in *V. anguillarum* (Stork et al. 2004).

Vibrio alginolyticus has also been examined for virulence in its natural host. Throughout Europe and South East Asia, *V. alginolyticus* has been linked to high mortality outbreaks of vibriosis in farmed fish (Balebona et al. 1998; Xie et al. 2005). The siderophore-mediated iron-uptake system of *V. alginolyticus* includes vibrioferrin, its naturally produced siderophore, as well as its receptor PvuA (Wang et al. 2008; Yamamoto et al. 1994). To determine which TonB system was used to power this system, zebra fish infection studies were performed (Wang et al. 2008). When inoculated intraperitoneally, mutants in either the TonB1 or TonB2 system showed ~10-fold attenuation in virulence. When both systems were mutated a ~25-fold attenuation was seen. These results suggest that both systems are essential for virulence in *V. alginolyticus* (Wang et al. 2008).

Virulence work has also been investigated using non-natural hosts of vibrio. The suckling mouse model has been used to compare each of the two TonB systems in *V. cholerae*. Shelley Payne's laboratory has performed competitive indexes by infecting mice orally with equal amounts of two strains, wild type or a *tonB* mutant, and determining their ability to colonize and compete with each other. Single mutants, either in *tonB1* or *tonB2*, both showed a moderate reduction in its ability to compete with the wild type strain. A double mutant (*tonB1/tonB2*), showed the greatest defect in colonization, about a two fold reduction compared to either single mutation (Seliger et al. 2001). These results suggest an in vivo role in infection for each of the two TonB systems in *V. cholerae*.

Studies examining virulence of *V. vulnificus* and the use of its different TonB systems have also been performed. *V. vulnificus* is an opportunistic marine pathogen that has been shown to cause fatal septicemic disease in both eels and humans (Gulig et al. 2005). This *Vibrio* species has been shown to possess three TonB systems (Alice et al. 2008). The TonB1 system is homologous to the one found in *E. coli*, it contains only *tonB1*, *exbB*, and *exbD*, and is associated with the genes required for heme uptake. The TonB2 and TonB3 system both have the fourth accessory protein, TtpC. *V. vulnificus* produces two distinct

siderophores, vulnibactin and a hydroxymate-type. Both TonB1 and TonB2 are used in the uptake process of each of these siderophores. The two TonB systems are required for virulence in the iron-overloaded mouse model inoculated by subcutaneous injection (Alice et al. 2008). In this study the tonB genes were induced under iron-limiting conditions, demonstrating that active iron transport is crucial in *V. vulnificus* infections (Alice et al. 2008). Expression of the third TonB system only occurs when the bacterium is grown in the presence of human serum. Further study is needed to determine which metabolic and, or energetic steps the TonB3 system is involved in (Alice et al. 2008).

Conclusion

The possession of multiple TonB systems imbued the vibrios and other aquatic bacteria with foolproof mechanisms to ensure that the microorganisms efficiently take up the iron from hemoglobin and/or from siderophore complexes. Some, like *V. cholerae* and *V. anguillarum*, have two *tonB* systems, *tonB1* and *tonB2*, whereas other vibrios and aquatic bacteria can have three. Uptake can be either redundant or specific for the different *tonB* systems depending on the iron sources. Another hallmark of these systems is the universal presence of a novel protein, TtpC, which is part of the TonB2 and TonB3 systems but not of the TonB1 cluster. TtpC plays an essential role in TonB2 mediated iron transport in at least two of these organisms, *V. cholerae* and *V anguillarum*, and might also be needed for iron transport for the other vibrios and aquatic bacteria. It is likely that its requirement for TonB2 or TonB3 mediated iron transport must be a reflection of the molecular properties of these TonB-like proteins as well as the evolutionary complexity of the ecological niches of the *Vibrio* species.

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Table 1

Similarity of TtpC in different bacterial species

Species with TtpC2 and/or TtpC3	TtpC2		TtpC3		% similarity of TtpC2-TtpC3
	V. anguillarum[a] % similarity	*V. cholerae*[b] % similarity	*V. parahaemolyticus*[c] % similarity	*V. vulnificus*[d] % similarity	
Vibrio anguillarum	100	67	NA	NA	NA
Vibrio cholerae	67	100	NA	NA	NA
Vibrio coralliilyticus	66	66	NA	NA	NA
Vibrio furnissii	67	67	NA	NA	NA
Vibrio metschniovi	67	67	NA	NA	NA
Vibrio mimicus	90	90	NA	NA	NA
Vibrio orientalis	67	67	NA	NA	NA
Vibrio shilonii	54	54	NA	NA	NA
Vibrio splendidus	68	68	NA	NA	NA
Vibrio alginolyticus	68	68	95	89	39
Vibrio angustum	62	62	84	87	35
Vibrio fischeri	60	61	37	38	62
Vibrio harveyi	68	68	93	87	39
Vibrio parahaemolyticus	66	65	100	87	38
Vibrio vulnificus	58	58	87	100	37
Aliivibrio salmonicida	62	62	36	37	61
Photobacterium profundum	59	59	37	37	72
Teredinibacter turnerae	36	36	44	44	41
Aeromonas hydrophilia	41	41	NA	NA	NA
Aeromonas salmonicida	41	41	NA	NA	NA
Photobacterium damselae	61	61	NA	NA	NA
Pseudomonas mendocina	38	38	NA	NA	NA
Pseudomonas stutzeri	38	38	NA	NA	NA
Shewanella halifaxenis	41	41	NA	NA	NA
Shewanella putrefaciens	40	40	NA	NA	NA

Percent identity was found through the align function of the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST)p interface (Altschul et al. 1997)

NA not applicable. These strains only have two TonB systems

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[a] *Vibrio anguillarum* strain 775, TtpC2 (GenBank: AAV48774) was used for the BLAST analysis

[b] *Vibrio cholerae* strain CA401, TtpC2 (GenBank: AAC69453) was used for the BLAST analysis

[c] *Vibrio parahaemolyticus* strain RIMD, TtpC3 (GenBank: BAC58429) was used for the BLAST analysis

[d] *Vibrio vulnificus* strain CMCP6, TtpC3 (GenBank: AAO09347) was used for the BLAST analysis