THE ROLE OF THE TtpC PROTEIN IN ENERGY TRANSDUCTION IN THE TonB2 SYSTEMS OF PATHOGENIC VIBRIOS

By

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For surely I know the plans I have for you, says the Lord, plans for your welfare and not for harm, to give you a future with hope.
Jer. 29:11

School of Medicine

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

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Abstract

The ability to scavenge and transport iron from the environment and inside the vertebrate host is essential for survival of most bacterial species. The TonB-ExbB-ExbD energy transduction system is conserved through many Gram-negative species and is used to transduce energy from the proton motive force (PMF) in the inner-membrane to the outer-membrane iron-siderophore transporters. Unlike E. coli, the *Vibrio* species have multiple TonB systems. In these bacteria, the TonB2 systems also include a fourth protein, TtpC that is essential for energy transduction and iron transport. The TtpC proteins are highly conserved yet display specificity towards certain outer-membrane transporters – suggesting that the TtpC protein, in conjunction with, or in place of TonB2, could be transducing the energy to the outer-membrane transporters. Most TonB proteins contain a conserved SxxxH motif that is involved in converting the energy of the PMF to conformational changes in the TonB periplasmic domain. The SxxxH motif does not exist in the *vibrio* TonB2 proteins, but is instead found in the *vibrio* TtpC proteins. The conserved histidine is essential for Fe-ferrichrome transport in V. cholerae and supports the hypothesis that TtpC is the energy-transducing protein in the *vibrio* TonB2 energy transduction systems. The discovery of a fourth component to the TonB2 system is of significant interest because it suggests a variation or unique mechanism that is used by marine organisms to transduce energy to the outer membrane receptor proteins. Although the *vibrio* TonB2 systems are similar in organization and protein homology to other Gram-negative TonB systems, the addition of the TtpC protein, and the proof of its essentiality presented here indicate a more complex mechanism of energy transduction present in the periplasm of the *Vibrio* species.

List of Abbreviations

PMF: proton motive force

EDDA: ethylenediamine-di-o-hydroxyphenylacetic acid

DHBA: 2,3-dihyroxybenzoic acid

CCCP: carbonylcyanide *m*-chlorophenylhydrazone

PMSF: phenylmethylsulfonyl fluoride

TCA: Trichloracetic Acid

DSP: Dithiobis [succinimidyl propionate]

IPTG: Isopropyl β-D-1-thiogalactopyranoside

DMSO: Dimethyl sulphoxide

FAC: Ferric ammonium citrate

TMD: Trans-membrane domain

DAS: Dense Alignment Surface

SOE: Splicing by overlap extension

bp: base pair

NTA: Nirilotriacetate

DTT: Dithiothreitol

SDS-PAGE: Sodium dodecyl sulfide polyacrylamide gel electrophoresis

MOPS: Morpholinepropanesulfonic acid

Fur: Ferric ion uptake regulator

OM: Outer-membrane

IM: Inner-membrane

Preface

Studying the organization and conservation of the TonB energy transduction systems across the genus *Vibrio*, we can tease out trends in gene arrangement and function that provide 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. Our 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. The *vibrio tonB2* systems are located with many of the housekeeping genes on the larger chromosome 2, whereas the *tonB1* systems, which are associated with genes required for heme uptake, are located on the smaller, chromosome 1 in *V. anguillarum* and *V. cholerae* (Naka, H et al 2011, Mey and Payne, 2003). It is likely that the *tonB1* system was acquired via horizontal transfer along with other genes necessary to survive in various environments such as the host intestinal tract and the marine environment. 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 the necessity to transduce energy in a medium that can have osmotic fluctuations.

The TtpC protein is an essential part of the TonB2 system of the pathogenic *vibrio* species. While the essentiality of this protein is known, its function as part of

the TonB2-energy transduction system is unknown. I hypothesize that the trans-membrane domains of the TtpC protein, possibly in conjunction with the trans-membrane domains of other TonB2-system proteins, function as a proton channel and that the TtpC protein, or a combination of the TtpC and TonB2 proteins, transfer the energy derived from the proton-motive-force to the iron-siderophore transporters located in the outer membrane.

Chapter 1 will present a review of what is known about the TonB systems in general, and particularly in *vibrio*s. It is essential to understand as much about the three previously studied proteins of the TonB systems, TonB, ExbB and ExbD, before we determine the functions of the additional fourth protein. I will discuss iron transport and its importance in virulence, the multiple TonB energy transduction systems in the *vibrio* species as compared to the single well studied TonB system present in *E. coli*, the newly discovered TtpC protein in the *vibrio* TonB2 and TonB3 systems, and its similarity to other molecular motors found in Gram-negative bacterial species.

Chapter 2 concerns the conserved HxxxS motif found in all non-*vibrio* TonB proteins and in the *vibrio* TonB1 proteins, the HxxxS motif is only present in the TtpC proteins of the *vibrio* TonB2 systems. I will present mutational analysis of this motif in the TtpC proteins of several *vibrio* species, showing the essentiality of the Histidine residue for iron transport mediated by the TonB2 system and for the ability of the TtpC protein to respond to the presence of the proton motive force.

Chapter 3 will focus on the similarities of the TtpC proteins found in several pathogenic *vibrio* species and their ability, or lack thereof, to complement *ttpC*

deletions across species. I will also present information that further characterizes the TtpC protein in terms of functional domains, membrane orientation, and outer-membrane receptor specificity. I will argue that in the *vibrio* TonB2 systems, TtpC, or a combination of TtpC and TonB2 could be interacting with the outer-membrane receptor proteins as opposed to the previously established notion that TonB2 was the sole energy-transducing protein.

Chapter 4 will be a discussion of the conclusions I can draw about, the function of the TtpC protein in the *vibrio* TonB2 energy transduction systems. I will argue that the TtpC protein of the marine TonB2 systems is either the sole energy-transducing protein, or transduces the energy from the proton motive force in conjunction with TonB2. I will also argue that, unlike the previously studied TonB system in *E. coli* and the TonB1 system in *V. cholerae*, the TtpC protein, rather than the TonB2 protein, specifically interacts with the outer membrane transporters to induce ferric-siderophore transport through the outer membrane. I will also present some future directions that I think will enhance understanding in the field of energy transfer between membranes in Gram-negative species.

Finally, in Chapter 5 I will present my materials and methods related to Chapters 2 and 3.

Chapter 1. Introduction

Some tables and portions of the text in Chapter 1 were previously published in the September 2010 issue of the journal *Future Microbiology*, published by Future Medicine Ltd. The content is used by permission from the publisher.

A. Iron and Virulence in the *Vibrio*s

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 (Imlay, 2008; Jordan and Reichard, 1998; Roux et al., 2009). 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 the low-iron environments, microorganisms increase the expression of siderophore biosynthesis and iron-uptake machinery through de-repression from the master-regulator, Fur (Hantke, 1981). Ferric iron Uptake Regulator (Fur) is responsible for the regulation of iron acquisition genes in many Gram-negative bacteria. Fur is an iron-responsive transcriptional repressor of the genes that encode the proteins responsible for iron uptake and iron utilization. These genes have a sequence within their promoter region called the "Fur box" that binds to the Fur protein when the protein is also bound to its co-repressor, Fe^{2+}. When iron is scarce, Fur can no longer bind to the Fur boxes in the absence of its co-repressor, and de-repression of these genes is induced.

Small molecular weight compounds called siderophores have a high affinity for iron, which is greater than that of many host iron-binding proteins. For example, the *E. coli* siderophore enterobactin, has an affinity for Fe (III) of 10^{51} M^{-1} (Carrano and Raymond, 1979), compared to that of the host iron-binding protein transferrin, which is 10^{24} M^{-1} at pH 7.4 (Aisen et al., 1978). Siderophores are secreted by bacteria and fungi to scavenge iron from the surrounding environment. In bacteria, 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 (Braun, 1995; Crosa et al., 2004; Postle and Larsen, 2007). The process of iron transport in *E. coli* has been studied and is well understood. However, these processes in species belonging to the family *Vibrionaceae*, as well as other aquatic bacteria, still pose unanswered questions.

In the fish pathogen *Vibrio anguillarum*, the 65Kbp plasmid pJM1 is necessary for virulence (Crosa, 1980). Notably, this plasmid carries genes encoding one set of iron uptake machinery specific for the endogenously produced siderophore, anguibactin (Alice et al., 2005; Di Lorenzo et al., 2003). The biosynthetic genes for anguibactin are also encoded on the pJM1 plasmid (Di Lorenzo et al., 2003). The production of

anguibactin and the ferric-anguibactin transport system are essential for *V. anguillarum* virulence. Likewise, the TonB2 protein complex that energizes the ferric-anguibactin receptor in the outer membrane is also essential for virulence. In other pathogenic *vibrio* species such as *Vibrio cholerae* and *Vibrio alginolyticus*, both the TonB1 and the TonB2 systems supply energy to transport the endogenously produced siderophores and both TonB systems are necessary for wild-type virulence levels (Henderson and Payne, 1994; Wang et al., 2008).

B. TonB1 and TonB2 systems of iron transport

In Gram-negative organisms, the cytoplasm of the bacterium is separated from the environment by two lipid bilayers – the inner and outer membranes. Bacterial energy production occurs at the inner membrane 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 (PMF). This potential energy is converted to the energy currency of the cell, ATP, by inner-membrane proteins called 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 (Braun, 1995; Crosa et al., 2004; Postle and Larsen, 2007).

In *E. coli*, the TonB system includes the TonB protein as well as two accessory proteins, ExbB and ExbD. This system has been well studied in the contexts of iron and vitamin B12 transport and is shown in the top panel of Figure 1 (Hantke and Braun, 1975; Fischer et al., 1989). ExbB is necessary to stabilize the TonB protein in the inner membrane as TonB interacts with a "TonB box" region on the periplasmic face of the TonB-dependent transporter, located in the outer membrane (Ahmer et al., 1995). The "TonB box" is a 7-8 amino acid consensus sequence at the far amino-terminus of the outer-membrane receptor that interacts with the periplasmic domain of the energy-transducing TonB protein (Gudmundsdottir et al., 1989, Mey and Payne 2003). TonB and ExbD pass through the inner membrane once with their carboxy terminal domains in the periplasmic space, while ExbB has three trans-membrane domains with its carboxy terminus in the cytoplasm (Hannavy et al., 1990; Jaskula et al., 1994; Kampfenkel and Braun, 1992; Kampfenkel and Braun, 1993; Karlsson et al., 1993a). Interaction between the trans-membrane domains of *E.coli* TonB and ExbD was demonstrated via formaldehyde cross-linking and western blot analysis (Ollis et al., 2009).

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 (Larsen et al., 2003; Letain and Postle, 1997). 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 (Chimento et al., 2005; Devanathan and Postle, 2007; Gumbart et al., 2007; Ma et al., 2007; Pawelek et al., 2006; Shultis et al., 2006). How these proteins convert the potential energy of the proton motive force into conformational changes is still unknown.

i. TonB Proteins in *Vibrio* Species

The *vibrio*s 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.

The *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 (Kim et al., 2003b; Lee et al., 2004; Stork et al., 2004; Faruque et al., 2006; Wyckoff et al., 2007; Childers and Klose, 2007).

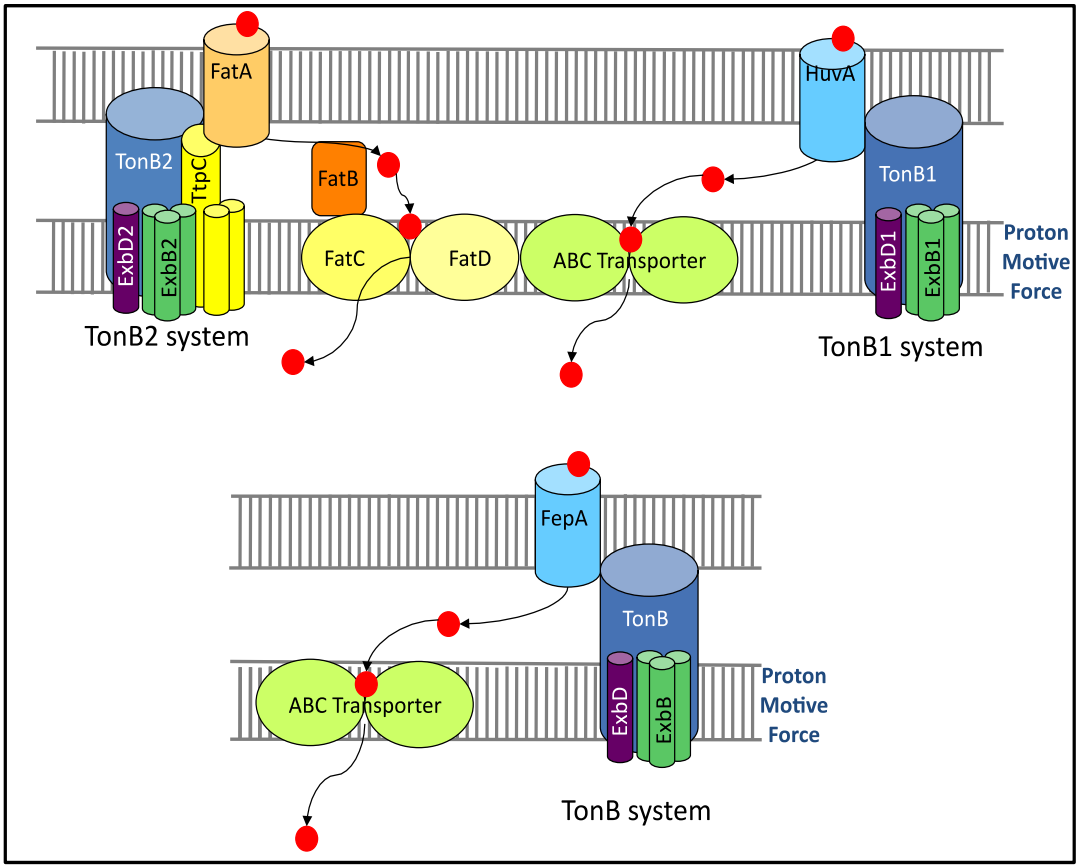
The *Vibrio* species possess two chromosomes, and unlike *E. coli* with its single TonB-ExbB-ExbD complex, they have multiple TonB systems encoded in their genomes. These TonB systems and iron uptake proteins are depicted in Figure 1-1. Occhino *et al.* (Occhino et al., 1998) first identified the existence of multiple TonB systems in a single organism, *V. cholerae*, in 1998. In subsequent work, Seliger *et al.* (Seliger et al., 2001) 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, vibriobactin, enterobactin and schizokinen. Multiple TonB systems have been characterized in other *vibrio* species, including *V. anguillarum*, *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus* (O'Malley et al., 1999; Stork et al., 2004; Wang et al., 2008).

With some exceptions, the TonB1 systems in the *vibrio*s are present in the smaller chromosome and consist of the proteins *exbB1, exbD1* and *tonB1.* These genes are associated with heme transport genes and intervene in heme and ferrichrome transport (Litwin and Byrne, 1998; Stork et al., 2004; Wang et al., 2008; Wong et al., 1996; Wyckoff et al., 2007). The TonB2 systems, arranged as *ttpC, exbB2, exbD2* and *tonB2* are found in the larger chromosome of at least *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 for the exogenously produced sources such as enterobactin and ferrichrome (Stork et al., 2007).

Figure 1-1.

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



The TonB2 system of *V. cholerae* is similarly responsible for energizing the receptor specific for its endogenously produced siderophore, vibriobactin, and can energize enterobactin transport through both VctA and IrgA and heme transport via HasR (Mey and Payne, 2001; Mey et al., 2002). In *V. cholerae*, strains lacking TonB1 were unable to compete with the wild-type classical strain CA401 or a TonB2 mutant using an *in vitro* competition assay with heme as the sole iron source (Seliger et al., 2001). Conversely, the *tonB2* mutant, as well as the TonB1-deficient strain, complemented with a clone expressing TonB1, did not show a growth disadvantage as compared with the wild-type strain (Seliger et al., 2001). These results suggest a preferential role for TonB1 in *V. cholerae* heme uptake.

Shelley 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 heme at increased NaCl concentrations (Wyckoff et al., 2007; Seliger et al., 2001). When grown in media with increased osmolarity, the periplasmic space expands (Stock et al., 1977a). Seliger *et al.* demonstrated that the *vibrio* TonB2 proteins, lacking an extended proline-rich region (PRR) in the periplasmic spanning region, are shorter than the TonB1 proteins(Seliger et al., 2001). Based on this observation, they postulated that they 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 non-essential PRR, as characterized by Larsen *et al.* in *E. coli*

(Larsen et al., 1993) was, like the TonB2 protein, also unable to energize heme transport at NaCl concentrations above 250 mM. The ability of *V. cholerae* TonB1, but not TonB2 or the TonB1-PRR deletion mutant protein to interact with the heme receptor at high salt concentrations may be a function of the extended periplasmic domain of TonB1. However, this hypothesis needs further testing because the strain expressing TonB2 or the shortened TonB1 mutant were still able to grow in the presence of ferrichrome at high salt concentrations (Seliger et al., 2001).

ii. Receptor specificities of the TonB1 and TonB2 systems

In *V. cholerae* the carboxy-terminal one-third of either TonB confers the receptor specificities associated with the full-length TonB. Single-amino-acid substitutions near the carboxy terminus of *V. cholerae* TonB1 determined this specificity (Mey and Payne, 2003). The TonB membrane topology is conserved in TonB2s of both *V. cholerae* and *V. anguillarum*, with the carboxy terminal domain in the periplasm, where it theoretically interacts with the periplasmic loops of TonB2-dependent transporters in the outer membrane (López et al., 2009). *In silico* observations indicate that this is also true in the other pathogenic *vibrio*s (Wang et al., 2008 and Kuehl and Crosa, unpublished).

The regions of the TonB-dependent receptors involved in specificity for a particular TonB protein were investigated in experiments involving domain switching between *V. cholerae* and *E. coli* receptors exhibiting different TonB specificities. Switching the

conserved TonB box heptapeptides at the amino termini of these receptors did not alter their TonB specificities.

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 (Mey and Payne, 2001). Mey and Payne reported that the amino acid sequences of HutA and HutR are homologous to each other and to other heme receptors. (Mey and Payne, 2001) 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 those of the TonB-binding regions found in other *vibrio* heme receptors (Mey and Payne, 2001). This result may be due to the role played by the TtpC proteins in all of the observed TonB2 systems (Kuehl and Crosa, 2009) as will be discussed in the next section.

Consistent with observations in *V. cholerae*, heme transport by *V. alginolyticus* is mediated by TonB1. Wang *et al.* demonstrated that heme and hemoglobin support growth of *V. alginolyticus* strains that express TonB1, whereas liquid growth assays showed the endogenous siderophore *vibrio*ferrin uses both the TonB1 and TonB2 systems (Wang et al., 2008).

López *et al.* performed structure and function analysis of the *V. anguillarum* TonB2 protein. Their study demonstrated that deletion of the final two amino acids of TonB2 did not change the ^{55}Fe-anguibactin transport efficiency, but that larger deletions resulted in *V. anguillarum* strains that were no longer capable of Fe-anguibactin transport (López et al., 2009). 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 which has an extra β-sheet in the carboxy-terminus of the protein as compared to the carboxy-terminus of the *V. anguillarum* TonB2 (López et al., 2009).

In the studies published by López et al., the *V. anguillarum* TonB2 mutant was complemented with the *E. coli* TonB and efficiency of Fe-anguibactin transport assessed. Heterogenous expression of *E. coli* TonB energized Fe-anguibactin transport through the FatA receptor. ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) analysis demonstrated the soluble carboxy-terminal periplasmic domain of *E. coli* TonB directly interacts with TonB-box peptides from the periplasmic loops of FatA. The same method detected interactions between the FatA TonB-box peptides with the purified soluble carboxy-terminal domain of TonB2.

However the carboxy-terminal domain of *V. anguillarum* TonB2 protein and the periplasmic loops of FatA do not directly interact (López et al., 2009). It is well established that a direct interaction between the TonB protein or a TonB homolog and the periplasmic loops of the outer membrane transporter is essential for energy transduction from the PMF to the outer membrane transporter. Based upon published data and my work that shows TtpC plays a key role in TonB2-mediated transport, I hypothesize that this lack of direct interaction between TonB2 and FatA in *V. anguillarum* could be overcome by the N-terminal periplasmic domain of the TtpC protein. Alternatively, our data is also consistent with the model where the TtpC protein allows a conformational change in the TonB2 protein, thereby allowing it to interact directly with the TonB-box peptides of FatA.

In addition, it is important to note that during the HSQC studies, only TonB or TonB2 and the FatA peptides were included. A study by K. Postle et al. demonstrated that the structure of the *E. coli* TonB C-terminal domain is not representative of the structure of the full-length protein embedded in the inner membrane (Postle et al., 2010). It is highly possible that the structure of the TonB2 C-terminal domain could be altered in the presence of its accessory proteins, ExbB2, ExbD2 and TtpC. This possibility is supported by the observation that the stability of the TonB protein in *E. coli* is drastically decreased in an ExbB deletion mutant strain (Ahmer et al., 1995). Direct interactions between TonB proteins and other OM receptors have been shown in *E. coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Skare et al., 1993; Paquelin et al., 2001; Lefevre et al., 2008; Gaisser and Braun, 1991).

iii. Virulence attributes of the *Vibrio* TonB systems

The role of iron transport in virulence has been studied in several *vibrio* species using a variety of infection models, and in some cases the natural host-pathogen interaction can be examined. The marine fish-pathogen *V. anguillarum* requires an active iron uptake via the siderophore anguibactin for infection of a vertebrate fish host (Di Lorenzo et al., 2004; Di Lorenzo et al., 2008; Stork et al., 2004). The bacterium 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 bacterial cytosol through the specific outer membrane receptor FatA (López and Crosa, 2007; Actis et al., 1985; Actis et al., 1988). TonB2, but not TonB1, is essential for the transport of anguibactin and enterobactin, while both TonB proteins can function in the transport of ferrichrome and heme. *V. anguillarum tonB2* mutants are severely attenuated in virulence, whereas the *tonB1* mutant shows mild attenuation, (more than 100-fold and 10-fold increase in LD_{50}) in the rainbow trout infection model (Stork et al., 2004). Complementation of the *tonB2* and *tonB1-tonB2* mutants with the wild-type *tonB2* gene restores 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, salmonid fish.

In a similar vein, another fish pathogen that possesses two sets of TonB systems,

Vibrio alginolyticus can infect the model organism *Danio rerio* (zebrafish) (Wang et al., 2008). However in this case, when inoculated intraperitoneally, mutants of either of the two TonB systems showed a marked attenuation in virulence, indicating that both systems are essential for the virulence of this bacterium (Wang et al., 2008). As in *V. anguillarum*, the two systems were arranged as *tonB1-exbB1-exbD1* and *ttpC-exbB2-exbD2-tonB2*, respectively. TheTonB1 system specifically contributed to hemin and hemoglobin uptake, and both of the TonB systems support iron uptake mediated by ferrichrome and vibrioferrin (Wang et al., 2008). Vibrioferrin is the endogenous siderophore of *V. alginolyticus* (Stoebner et al., 1992).

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* (Seliger et al., 2001), and the iron-overloaded subcutaneous infection mouse model for *V. vulnificus* (Alice et al., 2008). Mouse colonization assays carried out in Shelley Payne's laboratory (Seliger et al., 2001) using *V. cholerae* mutants in the TonB1 or TonB2 systems indicated a role for both TonB systems. Mutations in either system resulted in reduced ability to compete with the wild type *in vivo*. *V. vulnificus* possesses three *tonB* systems and multiplies rapidly in host tissues under iron-overloaded conditions. The TonB1 and TonB2 systems facilitate vulnibactin transport and are essential for virulence in the iron-overloaded mice. Active iron transport is important in infection by this bacterium, even under high-iron conditions however, transcription of these genes was induced under iron-limiting conditions. Expression of the TonB3 cluster occurs only when the bacterium grows

in human serum and does not show any relevance to pathogenesis (Alice et al., 2008). 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 for the host fish, the TonB1 and TonB2 systems are equally responsible for virulence in all the other members of the Vibrionaceae examined.

C. The TtpC protein – Another Player in the Game.

i. Essentiality in *V. anguillarum* and *V. cholerae* for TonB2-mediated Fe-uptake

All *Vibrio* species have a second, and some contain a third TonB system. These TonB2 (and TonB3) systems (Figure 1-2) consist of the classical ExbB2, ExbD2 and TonB2 (or ExbB3, ExbD3 and TonB3) proteins as well as a fourth protein TtpC, for TonB2 (or 3) complex Transport associated Protein C (Stork et al., 2007).

The 45 kDa TtpC protein is essential for TonB2-mediated iron transport in *V. anguillarum* and *V. cholerae*, and is present upstream of the TonB2 and TonB3 gene clusters in all *vibrio* and other aquatic species examined (Table 1-1). TtpC is predicted to span the membrane three times with a carboxy-terminal distribution of trans-membrane domains highly similar to the ExbB proteins. The majority of the protein, including the amino-terminal signal sequence, is predicted to be in the

Figure 1-2.

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 1-2. Gene Arrangement of the Three *Vibrio* TonB Systems

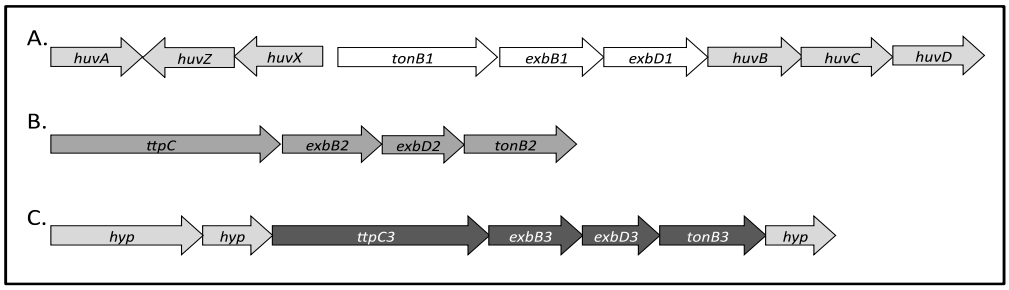


Table 1-1. Marine Species With TtpC-TonB2 System Homologues

Marine species with TtpC-TonB2 system homologues	
TtpC2-TonB2	**TtpC3-TonB3**
Vibrio anguillarum	
Vibrio cholerae	
Vibrio coralliilyticus	
Vibrio furnissii	
Vibrio metschnikovi	
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 hydrophila	
Aeromonas salmonicida	
Photobacterium damselae	
Pseudomonas mendocina	
Pseudomonas stutzeri	
Shewanella halifaxensis	
Shewanella putrefaciens	

periplasm (Figure 1-3). Experimental determination of the *V. anguillarum* TtpC membrane topology is presented in Chapter 2.

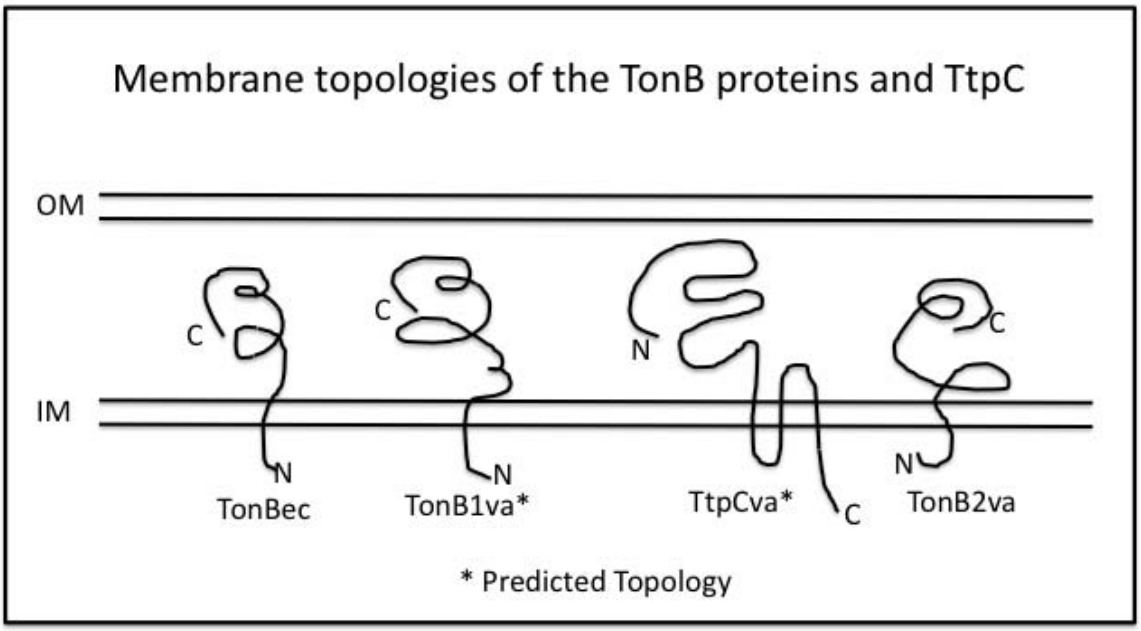
TtpC may allow the shorter TonB2 to span the periplasmic space; however there is another potential reason that could have motivated the evolutionary appearance of TtpC. The *V. anguillarum* TonB2 carboxy-terminal domain (CTD) has two significant differences in tertiary structure as compared to the solution structure of the *E. coli* TonB CTD (López et al., 2009). 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 support of this model, 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 (López et al., 2009). However, binding was observed between the TonB-box peptides and *E. coli* TonB CTD (Lopez et al., 2009).

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 showed that *E. coli* TonB is capable of

Figure 1-3.

The membrane topologies of *E. coli* ExbB, ExbD and TonB are known, as is the membrane topology of *V. 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. TonBec: *E. coli* TonB, TonB1va, TtpCva, TonB2va; *V. anguillarum* TonB1, TtpC and TonB2, respectively.

Figure 1-3. Predicted Membrane Topologies of the Proteins in the *Vibrio* TonB2 Energy Transduction Systems



substituting for *V. anguillarum* TonB2 in ferric-anguibactin transport through FatA, but 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 to supported ^{55}Fe-anguibactin transport in *V. anguillarum* at wild-type levels (Lopez et al., 2009). 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* (Stork et al., 2007). This protein was originally annotated as TolR because of its sequence homology with a 457 amino acid *V. cholerae* protein that was annotated as TolR because of its sequence homology to the much shorter TolR of its *tolQRA* (Stork et al., 2007). The TolQRA system is a homologous energy transduction system in Gram-negative bacteria that is necessary to maintain the integrity of the outer membrane (Lloubès et al., 2001). The annotation as TolR was discovered to be misleading upon further molecular characterization of the protein. Although the carboxy-terminal trans-membrane (TM) domain region, specifically the region from predicted TM domain 2-TM domain 3, is highly similar to the

MotA/TolQ/ExbB family of inner membrane proton channels, 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.

ii. Protein expression and membrane localization

Stork *et al.* (Stork et al., 2007) demonstrated that the TtpC protein was present in four multi-protein complexes formed when proteins in *V. anguillarum* cells were cross-linked with 1% formaldehyde. The multi-protein 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 (Stork et al., 2007). Very high molecular weight complexes >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.

iii. Sequence conservation across the *vibrio*s

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 (Altschul et al., 1997). 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* (Alice et al., 2008) (*and to be published elsewhere).*

The enterobactin receptors in both *V. anguillarum* and *V. cholerae* are energized by their respective TonB2 energy transduction systems (Seliger et al., 2001; Stork et al., 2004). Stork *et al.* (Stork et al., 2007) 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. Growth around ferric-enterobactin as an iron source was only restored when the entire *V. anguillarum* TonB2 system was present in the complementing plasmid. These data suggest that slight differences at the level of amino acid sequence may abrogate physical interactions or other involvement between the TtpC-TonB2 protein complex. Interspecies complementation of Δ*ttpC* deletion mutations in *V. anguillarum* and *V. cholerae* TonB2-dependent iron uptake with the TtpC proteins of other *vibrio*s is currently being explored

D. Molecular Machinery: Comparing TonB2 cluster proteins to torque-generating MotA/B subunits of the Flagellar Motor

i. MotAB rotor/stator complex

The carboxy-terminal trans-membrane domain of in the TtpC proteins is most similar to that of MotA/TolQ/ExbB family proteins. Members of the MotA/TolQ/ExbB protein family are comprised of integral membrane proteins that form proton channels, (EMBL http://www.ebi.ac.uk). In the *E. coli* flagellar rotation

system, the transmembrane domains of two MotA proteins, together with four MotB proteins form a proton channel in the inner membrane (Nakamura et al., 2009). The ion flux through the channel is thought to cause a conformational change in the periplasmic domain of MotA, allowing it to move from one FliG subunit of the flagellar rotor to the next FliG subunit, thereby turning the rotor (Berg, 2008).

There are functional homologs of the MotA/B proteins in *V. parahaemolyticus* that utilize Na+ ion flux rather than proton flux across the inner membrane to power flagellar rotation. Jaques *et al.* (Jaques et al., 1999) identified MotA and MotB homologues in screens for mutants that could swim in the presence of phenamil, a Na^+-channel inhibitor. The flagellar rotor of *V. alginolyticus* is also powered by Na^+ ion flux. Sato and Homma (Sato and Homma, 2000a) showed that purified *V. alginolyticus* PomAB complexes (MotAB homologs) reconstituted in liposomes allowed Na^+ flux, demonstrating that PomA and PomB formed the Na^+ channel. 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 needed to interact with PomB (Sato and Homma, 2000b). The functional characteristics of the closest homologues to the carboxy-terminal domain of *vibrio* TtpC proteins is intriguing and may offer some insight into the molecular mechanisms involved in TonB2-TtpC-mediated energy transduction.

ii. Homologous systems

The high peptide sequence similarity between the trans-membrane domains of the ExbB, TolQ, MotA, ExbB2 and TtpC proteins point toward similar structure and function of the proteins (Zhai et al., 2003; Germon et al., 2001; Cascales et al., 2007). This leads me to hypothesize that ExbB2 proteins and the trans-membrane 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 PMF to the outer membrane receptors via interaction with TonB2 and possibly, the amino-terminal region of TtpC. A similar proposal of TM-domain interaction was described by (Zhai et al., 2003) using the *E. coli* TonB system and may be similar to the arrangement of the TonB system proteins present in the *vibrio*s. These TM-domain interactions were also seen in the *E. coli* TolQRA system. To demonstrate the mechanistic similarity between the TolQR and ExbBD proteins in *E. coli*, Volkmar Braun swapped the TolQR proteins for the ExbBD proteins in *E. coli* and showed by colicin susceptibility assay that the TonB protein was still able to energize the OM receptors when it used TolQ and TolR as accessory proteins (Braun, 1989).

E. TonB *E coli* conformational changes and functional domains of TonB *E. coli* and PMF-induced Conformational Changes.

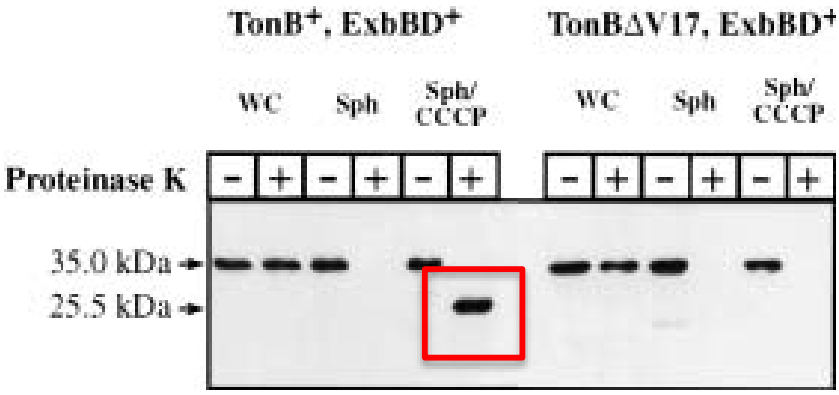
Similar to the conformational change in MotA driving the rotation of the flagellar motor, the TonB and TolA proteins are also thought to transfer energy from the PMF to the OM receptors via a conformational change and direct interaction with the OM receptors.

Differential proteinase K susceptibility in the presence or absence of the PMF is used as an assay to determine if a conformational change in a given protein occurs (Larsen et al., 1999; Germon et al., 2001). In *E. coli*, the wild-type TonB protein adopts a proteinase K-resistant conformation in the presence of the proton motive force inhibitor CCCP. This PMF-dependent conformational change is dependent on a number of factors including the presence of a conserved SxxxH motif in the single trans-membrane domain of TonB. This motif is conserved in the homologous protein, TolA and the TonB1 proteins of the *vibrio* species (Wang et al., 2008; Germon et al., 2001). When the spacing between the TonB trans-membrane domain residues S16 and H20 is shifted by one amino acid as shown in Figure 1-4, the ΔV17 TonB is no longer able to adopt the proteinase K-resistant conformation, indicating that the correct positioning of the SxxxH motif is essential for the PMF-induced conformational change in TonB (Larsen et al., 1999). Further, deletion of accessory proteins ExbB and ExbD or point mutations in TonB, S16L and H20L also result in the absence of the proteinase K-resistant conformation of TonB (Larsen et al., 1999). Recently, Swayne et al. performed mutational analysis on the TM domain of the *E. coli* TonB protein to determine the residues responsible for responding to the PMF and allowing a conformational change in the carboxy-terminal (periplasmic) domain of the TonB protein (Swayne and Postle, 2011). The authors discovered that only one H20 mutation was permissible and allowed TonB function, as assessed by colicin susceptibility and *in-vivo* formaldehyde cross-linking capability with the ExbD protein.

Figure 1-4

Full length and degradation products of the E. coli TonB protein from whole cells (WC), spheroplasts (sph) and spheroplasts treated with CCCP to collapse the PMF (sph/CCCP) are shown by western blot analysis with the monoclonal 4F1 anti-TonB antibody. A proteinase K-resistant degradation product in the CCCP-treated spheroplasts is highlighted with a red box. This proteinase K-resistant fragment is not detected when the position of the conserved H20 residue is changed by deletion of the V17 residue. (Larsen et al., 1999).

Figure 1-4. Wild type *E.coli* TonB adopts a proteinase K-resistant conformation in the presence of PMF inhibitor CCCP



Larsen,R *et al.* Mol. Micro. 1999

A previous theory for TonB function posited that the H20 residue, which is protonatable, was active in shuttling protons through the TonB complex proton channel formed by the TM domains of TonB, ExbB and ExbD (Ollis et al., 2009). Surprisingly, an H20N mutation was the only mutant TonB found by Swayne *et al*. that retained function (Swayne and Postle, 2011). The asparagine residue is similar in bulk to the histidine side-chain but is non-protonatable, disproving the theory that the H20 residue of TonB was directly involved in sensing the PMF.

This PMF-induced conformational change is also observed in the TonB homolog TolA. TolA is required, in conjunction with its accessory proteins TolQ and TolR, for outer membrane integrity and the translocation of F-specific filamentous (Ff) bacteriophage DNA (Karlsson et al., 1993b). TolA and TonB are homologous in their N-terminal TMD, where a conserved SXXXH motif is found (Larsen and Postle, 2001; Germon et al., 2001). In both proteins, the histidine is essential for a conformational change to occur. Similar to the TonB protein, TolA has been shown to adopt a PMF-dependent proteinase K-susceptible conformation that is only partially degraded by the protease in the presence of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP). This partial degradation pattern is evident in the presence of the PMF in *tolQR* strains as well as in strains with mutations in the conserved H22 and S18 residues (Germon et al., 2001). These results provide evidence that the conformation of TolA depends on the PMF, TolQ, TolR and the trans-membrane domain of TolA, specifically, the conserved SxxxH motif. The common observation between the TonB and TolA systems is that both proteins

change conformation in response to the PMF and therefore, have a different proteinase K degradation pattern with and without energy production in the inner membrane.

F. HxxxS motif and its essentiality in the *Vibrio* TonB systems.

The *E. coli* TonB and *Vibrio* TonB1 proteins are very similar, with conserved locations of the single trans-membrane domain, and are predicted to have the same topology in the inner membrane with the amino-terminus in the cytoplasm. A key conserved feature of these two proteins is an SxxxH motif in the amino-terminal trans-membrane domain. In *E. coli*, this histidine is the only amino acid in the trans-membrane domain that is essential for function of the TonB protein. This conserved histidine residue is also essential for cross-linking of TonB with ExbD and the ability of TonB to respond via conformational shift, to the proton motive force (Germon et al., 2001; Larsen et al., 2007; Larsen and Postle, 2001; Ollis et al., 2009).

One might expect that this conserved motif would be present in the TonB2 protein and that it might be essential for TonB2 function and interaction with its accessory proteins. Surprisingly, no such motif exists in the TonB2 proteins. The motif does, however, exist in the TtpC proteins and is highly conserved in the third trans-membrane domain of all TtpC proteins studied. It is intriguing that the motif is in the opposite order from the motif found in the *E. coli* TonB, TolA and the *Vibrio* TonB1 proteins. It is also in the carboxy-terminal region of the protein, rather than the N-terminal domain. This arrangement conserves the positioning of the HxxxS in

the trans-membrane domain. In all four proteins, the histidine is closest to the periplasm, whereas the serine is closest to the cytoplasm. Chapter 2 will focus on the essentiality of the HxxxS motif in the *vibrio* TtpC proteins and the role of the conserved motif in translating energy from the PMF into conformational changes in TtpC and TonB2.

G. Hypothesis for the function of TtpC in the TonB2 system

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 1-2) (Kuehl and Crosa, 2010).

The TonB2 system is often 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. As mentioned in the earlier section B.i, the *V. cholerae* TonB2 protein was incapable of energizing hemin transport when the periplasmic space was increased under higher concentrations of NaCl, consistent with the TonB2 proteins being shorter in primary amino acid sequence than the TonB1 proteins (Larsen et al., 1993; Seliger et al., 2001). The experimental results presented in Chapter 3, show that the TtpC protein spans the periplasmic space and interacts with OM receptor proteins. These data suggest that

Table 1-2. TonB System Specificities for Siderophore Transport

Species	Siderophores produced	Siderophores used	TonB system used
V. anguillarum	Anguibactin	Anguibactin	TonB2
	Vanchrobactin	Vanchrobactin	TonB2
		Enterobactin	TonB2
		Ferrichrome	TonB1 and 2
		Hemin	TonB1
V. cholerae	Vibriobactin	Vibriobactin	TonB1 and 2
		Enterobactin	TonB2
		Agrobactin	Unknown
		Fluvibactin	Unknown
		Ferrichrome	TonB1 and 2
		Hemin	TonB1 and 2
V. vulnificus	Vulnibactin	Vulnibactin	TonB1 and 2
	Hydroxamate-type	Hydroxamate-type	TonB1 and 2
		Aerobactin	Unknown
		Hemin	TonB1 and 2
V. parahaemolyticus	Vibrioferrin	Vibrioferrin	Unknown
		Ferrichrome	Unknown
		Aerobactin	Unknown
		Hemin	Unknown
V. alginolyticus	Vibrioferrin	Vibrioferrin	TonB1 and 2
		Ferrichrome	TonB1 and 2

it can be the connection, which the shorter TonB2 cannot provide, between the energy from the PMF and the outer membrane protein receptors. However, this process requires TonB2 because in its absence, the TtpC protein is unstable and degrades rapidly (Stork et al., 2007). As an earlier system, the combination of the TtpC protein and a TonB2 system was likely necessary for the function of the smaller TonB2 protein, especially in the context of osmotic challenges (Seliger et al., 2001). It is an evolutionary mystery why the TtpC-TonB2 systems have evolved and remained as an important energy transduction system in the *vibrios* and other aquatic bacteria, while the acquisition of the TonB1 systems appears to have occurred more recently in the evolution of these bacteria, possibly in conjunction with the acquisition of the heme uptake genes, *hutDCB and hutWXZ* (Wyckoff et al., 2004).

This dissertation dissects the role of the TtpC protein in energy transduction and recognition of the OM ferric-siderophore receptors in the Vibrio species. I hypothesize that the TtpC protein, or a combination of the TtpC and TonB2 proteins, interacts with the iron-siderophore transporters located in the outer membrane via a conformational change allowing interaction with the OM transporters. This interaction may be the method of energy transduction from the PMF in the inner membrane to the OM transporters.

Chapter 2. Conservation of the HxxxS motif in *Vibrio* TonB2 systems

The cytoplasm of Gram-negative bacterial cells is separated from the environment by two lipid bilayers - the inner and outer membranes (IM and OM respectively). The OM is the site of interaction with the environment and contains a myriad of proteins involved in binding and active transport of scarce minerals and nutrients such as iron, vitamins and carbohydrates; selective and non-selective porins; secretion apparatus; and efflux pumps. The IM is the site of respiration that results in the generation of the proton motive force (PMF) and energy production in the form of adenosine tri-phosphate (ATP). The IM accordingly houses proteins responsible for transducing the energy generated by the PMF to the energy-dependent OM transport proteins.

The energy transducing protein complex has been well studied in *E. coli* in the context of colicin, cobalamin, iron and vitamin B12 transport, and consists of the integral membrane proteins TonB, ExbB and ExbD (Bassford et al., 1976; Bradbeer and Woodrow, 1976; Braun et al., 1996). The TonB protein complexes are highly conserved across the Gram-negative species. The *vibrios* and other marine bacterial species have a similar TonB system consisting of TonB1, ExbB1 and ExbD1 that transduces the energy for heme and ferricrhome transport. The *vibrio*s possess a second and, in some cases, a third TonB system that consists of ExbB2, ExbD2, TonB2 and TtpC (Alice et al., 2008; Kuehl and Crosa, 2010; Kustusch et al., 2011; Stork et al., 2007; Wang et al., 2008). The second TonB system is more promiscuous, transducing energy from the PMF to a much broader range of TonB-

dependent transporters (Kuehl and Crosa, 2009; Kuehl and Crosa, 2010; Seliger et al., 2001; Wang et al., 2008).

TtpC is highly conserved in all pathogenic *vibrio* species (Stork et al., 2007; Kuehl and Crosa, 2010) and shows homology to the smaller ExbB-related proteins and members of the MotA/TolQ/ExbB family, but only in the carboxy-terminal domain (CTD). This CTD anchors the protein in the membrane and contains three transmembrane domains (TMD), whereas the remaining sequence is periplasmic. In spite of its ubiquity in the *vibrio*s, little is known of its mechanism of action (Kuehl and Crosa, 2010).

Multiple protein complexes with similar structure and function to the TonB energy transduction complex have the conserved SxxxH motif, which highlights its importance in the function of energy transduction (Wang et al., 2008). In *E. coli*, TonB forms a complex with ExbB and ExbD to mediate energy transfer of the electrochemical potential (Postle and Larsen, 2007). A homologous system to the *E. coli* TonB energy transduction system is the TolQ,TolR,TolA system. TolQ is required together with TolR, and TolA, all of which are inner membrane proteins, to maintain the stability of the outer membrane (Germon et al., 2001). These proteins share homology with ExbB, ExbD, and TonB, respectively. TolA operates in energy transduction between the inner membrane (IM) and the OM, and its conformation depends on the PMF (Germon et al., 2001). TolA and TonB are homologous in their N-terminal TMD, where a conserved SXXXH motif is found (Larsen and Postle, 2001; Germon et al., 2001). In both proteins the histidine is essential for a conformational change to occur. The serine is required for the conformational change in TolA but not in TonB (Postle and Larsen, 2007; Germon et al., 2001). The TonB H20 amino acid intervenes in complex formation with ExbB and ExbD rather than in the direct recruitment of the PMF, a role that

is probably played by ExbD (Swayne and Postle, 2011).

I identified a similar SxxxH motif in *V. cholerae* TonB1 but not in TonB2. The *vibrio* TonB1 proteins are very similar to *E. coli* TonB, with conserved locations of the single trans-membrane domain, and are predicted to have the same topology in the inner membrane with the N-terminus in the cytoplasm, a flexible proline-rich linker region between the trans-membrane domain, and a periplasmic carboxy-terminal domain (Larsen et al., 1993; Seliger et al., 2001). Since TonB2 operates together with TtpC in iron transport (Stork et al., 2007; Kuehl and Crosa, 2010) I searched for the motif in TtpC, identifying it in the third TMD. Interestingly, the conserved motif was found in the third predicted TMD and in the opposite orientation in the primary amino acid sequence, HxxxS. This change in orientation is predicted to preserve the orientation of the motif in the inner membrane because the topology of the TtpC protein was predicted as opposite that of the *E. coli* TonB and *vibrio* TonB1 proteins with its amino-terminus in the periplasm rather than the cytoplasm.

In this chapter, I will present experiments and results used to examine the essentiality of the *V. cholerae* TtpC residue His414 for energy transduction mediated by the *V. cholerae* TonB2 complex in the context of ferric iron-siderophore transport, and the ability of the TonB2 complex to respond to the presence of the PMF via conformational shift.

Materials and Methods used in this chapter are presented in detail in Chapter 5 and are referenced throughout the work.

A. HxxxS motif is found in TtpC.

The similarity in the major domains of the $TonB_{ec}$ and $TonB1_{vib}$ proteins is conserved in the *vibrio* TonB2 proteins except for the presence of the SxxxH motif in the amino-terminal trans-membrane domain as seen in Figure 2-1. The TonB2 complexes of the *vibrio*s are similar to the *E. coli* TonB and *vibrio* TonB1 complexes in that they transduce energy to the outer-membrane receptors and that the TonB2, ExbB2 and ExbD2 proteins are all essential for energy transduction to the outer membrane, but the TonB2 complexes contain a fourth essential protein, TtpC (Kuehl and Crosa, 2009; Stork et al., 2007).

The absence of the SxxxH motif in the *vibrio* TonB2 proteins and the necessity of a fourth protein in the TonB2 complex prompted a search for the motif in the other proteins: ExbB2, ExbD2 and TtpC. Interestingly, the conserved motif was found in the third predicted TMD and in the opposite orientation in the primary amino acid sequence, HxxxS. This difference in orientation of the motif in the primary amino acid sequence is predicted to preserve the orientation of the motif in the inner membrane because the topology of the TtpC protein is predicted as opposite that of the *E. coli* TonB and *vibrio* TonB1 proteins with its N-terminus in the periplasm rather than the cytoplasm. The HxxxS motif in the predicted TtpC TMD-3 is conserved across all *vibrio* species studied.

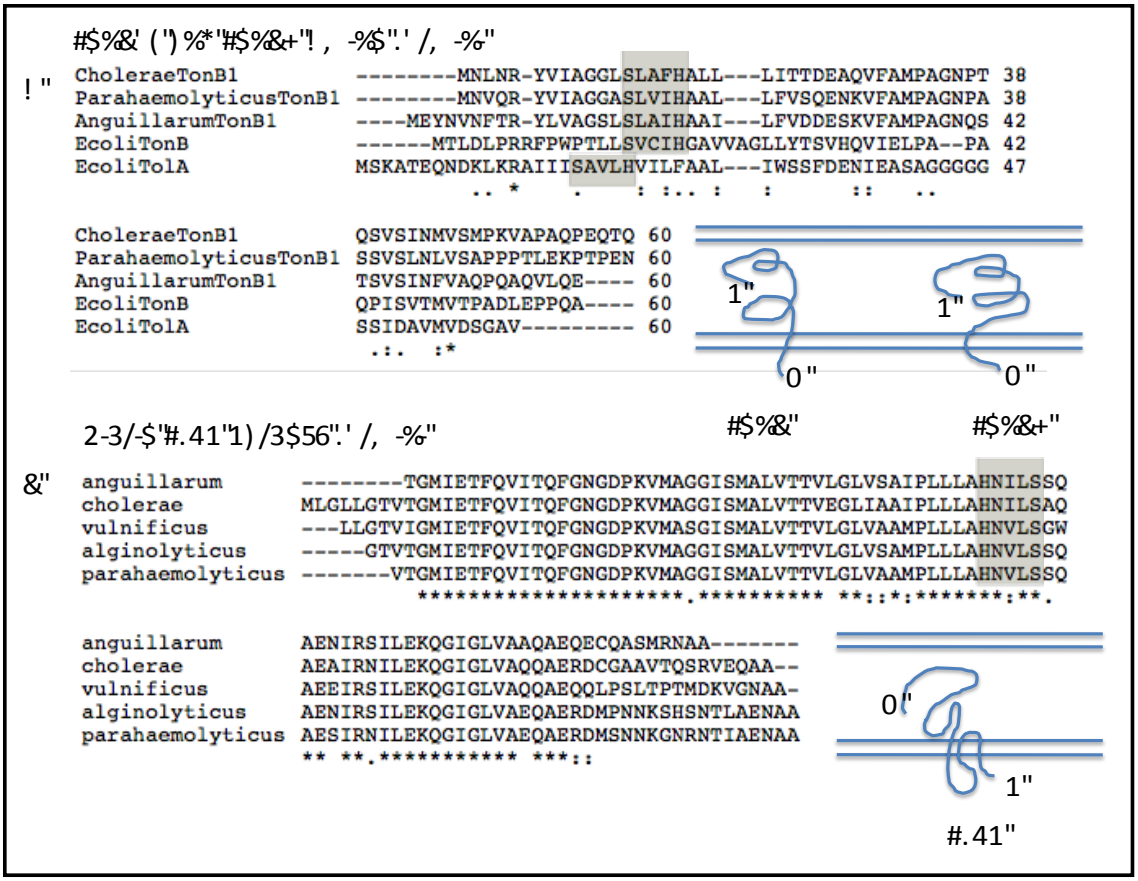
B. Determination of TtpC membrane topology.

Sucrose density gradient analysis of total membrane proteins in the original characterization of the TtpC protein by Stork *et al.* (Stork et al., 2007) indicated that the TtpC protein is located in the inner membrane fraction. Because of the amino acid sequence similarity of the TtpC C-terminal half to the sequence of the three TMD-containing ExbB proteins (65% similarity) (Altschul et al., 1997), I hypothesized that the C-terminal half of

Figure 2-1.

A. Clustal W amino acid sequence alignment of the N-terminal 60 amino acids of *vibrio* TonB1 proteins with *E. coli* TonB, and TolA. Conserved SxxxH motif highlighted in yellow. The membrane topography of the *E. coli* TonB and the *vibrio* TonB1 proteins is shown schematically. B. Clustal W alignment of the C-terminus of the TtpC protein from five pathogenic *vibrio* species. The conserved HxxxS motif is highlighted in yellow. The membrane topography of the TtpC proteins is shown schematically.

Figure 2-1. Conservation of the HxxxS motif in the TonB, TonB1 and TttpC proteins.



the TtpC protein would pass through the membrane three times as well. I used several *in silico* trans-membrane domain prediction algorithms to predict the membrane topology and the number of TMDs in the TtpC protein (Cserzö et al., 1997; Hofmann and Stoffel, 1993; Sonnhammer et al., 1998; Tusnady and Simon, 2001). The topology profiles of the *V. anguillarum* and *V. cholerae* TtpC proteins created with the Dense Alignment Surface (DAS) (Cserzö et al., 1997) TMD prediction algorithm are shown in Figure 2-2. To verify the *in silico* prediction of three trans-membrane domains in the C-terminal end of the TtpC proteins as well as the predicted topology of the protein, with the N-terminus in the periplasm, I created a set of TtpC-alkaline phosphatase C-terminal reporter fusions.

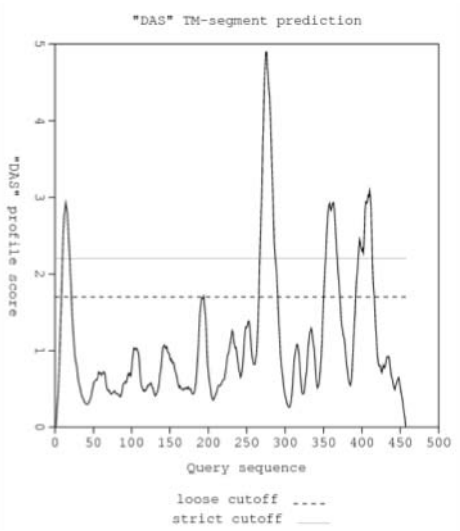
The *V. anguillarum* TtpC-PhoA fusions were expressed with 1mM IPTG induction from the pLac promoter on the pMMB206 vector in *E. coli* CC118 (Manoil, 1991; Morales et al., 1991). All of the fusions correctly localized to the membrane fraction as determined by preparation of total membrane proteins and detection by western blot with a monoclonal antibody to the *E. coli* PhoA protein, Figure 2-3 B. The trans-membrane domains of the TtpC protein as predicted by the DAS TMD prediction algorithm placed the first TMD at amino acids 261-278, thus a second fusion was placed at $TtpC_{va}295$ (p*ttpC*$_{va}$295*phoA*). The cytoplasmic localization of this region was confirmed with baseline levels of alkaline phosphatase activity. The second TMD was predicted to fall between amino acids 346-359. To verify a second passage through the inner membrane, the third fusion was engineered at residue 359 (p*ttpC*$_{va}$359*phoA*). This fusion resulted in increased alkaline phosphatase activity. The third TMD was predicted to fall between amino acids 386-405, therefore, the final fusion was created as a full-length TtpC fusion protein with the junction placed at $TtpC_{va}443$ (p*ttpC*$_{va}$Full*phoA*). The full-length fusion confirmed a third passage through the inner

Figure 2-2.

TtpC proteins are predicted to have three carboxy-terminal TMD. A. DAS (Cserzö et al., 1997) trans-membrane segment prediction profile of *V. cholerae* TtpC protein (left), *V. cholerae* TtpC amino acid residues corresponding to the predicted secretion signal sequence and the three transmembrane domains (right). Two stringency cut off values (1.7 – less stringent and 2.2 – more stringent) are listed for each TMD prediction. B. DAS trans-membrane segment prediction profile of *V. anguillarum* TtpC protein (left), *V. anguillarum* TtpC residues corresponding to the predicted secretion signal sequence and the three trans-membrane domains (right).

Figure 2-2. Predicted transmembrane domain locations in TtpC.

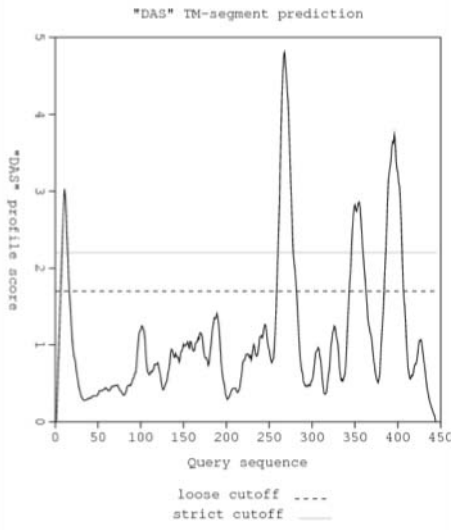
A. *V. cholerae* TtpC



Potential transmembrane segments

Start	Stop	Length	~	Cutoff
9	21	13	~	1.7
10	19	10	~	2.2
194	194	1	~	1.7
266	290	25	~	1.7
268	287	20	~	2.2
351	371	21	~	1.7
353	367	15	~	2.2
392	416	25	~	1.7
396	413	18	~	2.2

B. *V. anguillarum* TtpC



Potential transmembrane segments

Start	Stop	Length	~	Cutoff
7	16	10	~	1.7
8	14	7	~	2.2
259	281	23	~	1.7
261	278	18	~	2.2
344	362	19	~	1.7
346	359	14	~	2.2
385	406	22	~	1.7
386	405	20	~	2.2

membrane with a return to baseline levels of alkaline phosphatase activity. Representative results from a complete set of TtpC-PhoA fusions assayed in duplicate are shown in Figure 2-3 A.

The results from the alkaline phosphatase fusion assays in *V. anguillarum* confirmed the predicted periplasmic location of the mature N-terminus (after cleavage of the secretion signal sequence) and the presence of three trans-membrane domains with the carboxy terminus in the cytoplasm. This orientation in the inner membrane conserves the positioning of the HxxxS motif in the TtpC protein as compared to the *E. coli* TonB and the *vibrio* TonB1 proteins with the conserved histidine residue closer to the periplasm.

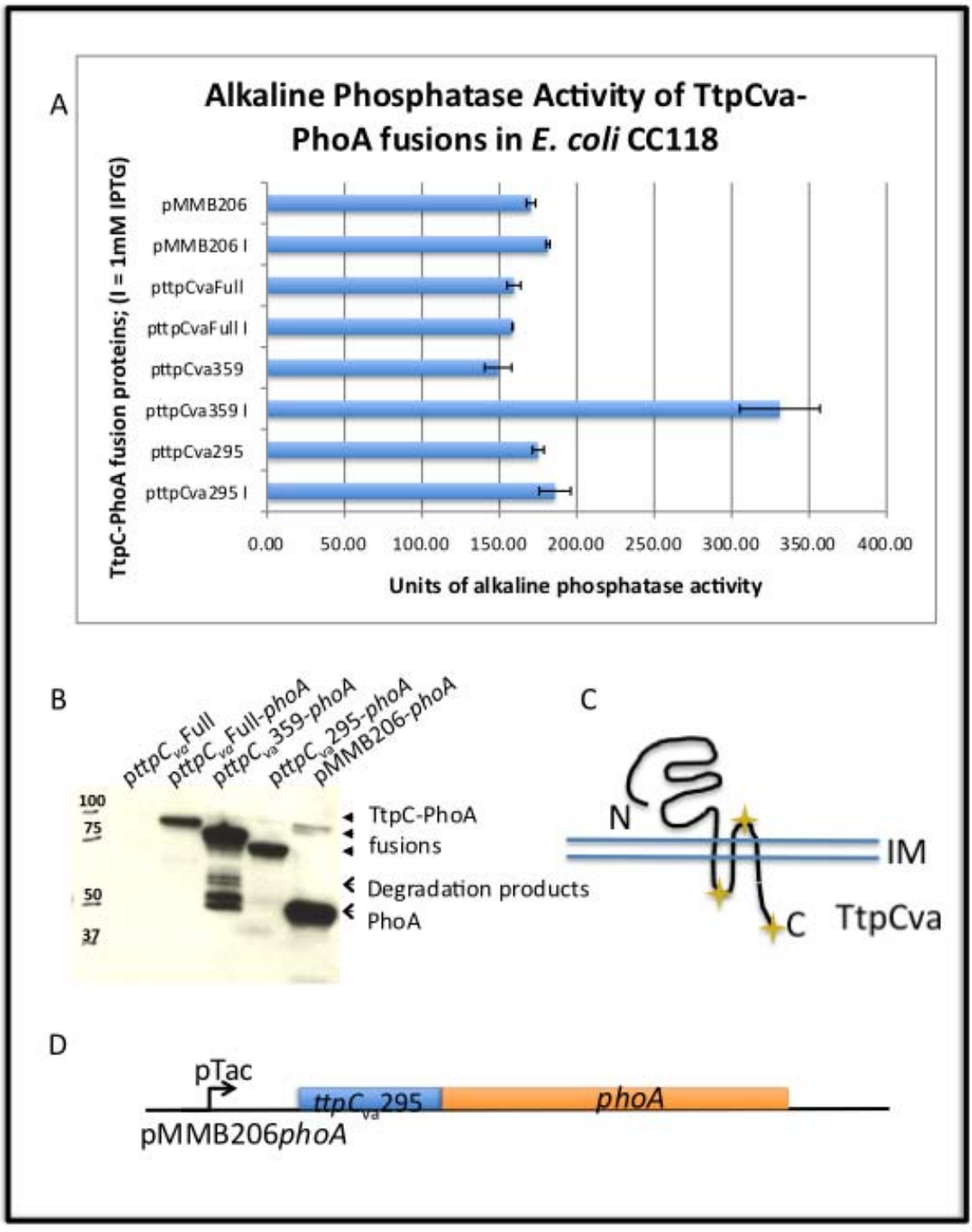
C. Mutations in the HxxxS motif abolish iron transport mediated by the TonB2 system.

After determining that the TtpC HxxxS motif was conserved, not only in sequence, but in intra-membrane positioning, I set out to determine if it was essential for the function of the *vibrio* TonB2 systems as it is for the *E. coli* TonB. Site-directed mutagenesis was employed to mutate the conserved H414 and S418 to alanine residues in the *V. cholerae* TtpC. These point mutations were cloned into an IPTG-inducible, low copy-number plasmid, pMMB208 for use in complementing a chromosomal deletion of *ttpC* in *V. cholerae* strain AMV527, which lacks vibriobactin production and has a transposon inserted in *tonB1*, inactivating the TonB1 energy transduction system (Mey and Payne, 2001; Morales et al., 1991). The single point mutations of the *V. cholerae ttpC* gene are expressed from plasmids pVcHxxxA and pVcAxxxS. pVcAxxxA was also constructed and is a double H414A, S418A mutant. Expression of all plasmid-encoded point mutants was induced with 1mM IPTG and the

Figure 2-3.

$TtpC_{va}$ amino-terminal periplasmic location determined by alkaline phosphatase fusion analysis. A. Relative units of phosphatase activity normalized to Miller units from host strain *E. coli* CC118 without the pMMB206*phoA* plasmid set to zero. Expression was induced with 1mM IPTG. B. Western blot of $TtpC_{va}$-PhoA fusion proteins. Primary antibody anti-PhoA. $TtpC_{va}$-PhoA fusions are designated with solid arrows and degradation products are designated with open arrows. C. Cartoon of the *V. anguillarum* TtpC protein in the inner membrane. Yellow stars designate the locations of the $TtpC_{va}$-PhoA fusions. D. Schematic of the pMMB206 *$ttpC_{va}$-phoA* fusion constructs.

Figure 2-3. Membrane Topology of the *V. anguillarum* TtpC Protein.



retention of the complementing plasmid was ensured by growth in 10μg/ml chloramphenicol.

I tested the ability of the TtpC point mutations to function in TonB2-mediated energy-transduction to various OM transport proteins using bioassays with $CM9_{E.coli}$ (see Chapter 5.G). Expression of all plasmid-encoded TtpC point mutations was determined by western blot analysis using the αTolR (TtpC)-specific antibody on membrane fractions as shown in Figure 2-4. The ability to grow around various iron sources is shown in Table 2-1 (representative results from one experiment, of a total of 5 biological replicates). While the S418A point mutation had no effect on robustness of the growth halos around the iron sources tested, mutants expressing the H414A and the double mutant, were unable to utilize any of the iron sources aside from the positive control, 10mg/ml ferric ammonium citrate, indicating that these mutations rendered the TtpC protein non-functional for TonB2-mediated iron transport.

Bioassays are highly sensitive, but they deliver only an end-point reading. To determine if the H414A and the double mutation were less efficient in TonB2-mediated iron transport, or if they were completely incapable of supplying energy for iron transport, I tested the kinetics of iron uptake using radioactive ^{55}Fe-ferrichrome assays. The transport of ferric-ferrichrome can be energized by either TonB system in *V. cholerae*. To test the efficiency of the TtpC point mutations, the same complemented AMV527Δ*ttpC* strains were used. AMV527Δ*ttpC* complemented with wild-type *ttpC* was used as the positive control. The same strain treated with 2mM KCN as well as AMV527Δ*ttpC* complemented with the empty vector were used as negative controls. KCN binds to cytochrome oxidase, permanently

Table 2-1. *V. cholerae* bioassays to test the function of TtpC HxxxS mutations.

V. cholerae Bioassays to test the function of TtpC HxxxS mutations

V. cholerae Strain	Ferric Ammonium Citrate	Ferrichrome	Enterobactin	Hemin	Vibriobactin
CA401/ pMMB208	+	+	+	+	+
AMV527ΔT/ pMMB208	+	-	-	-	-
AMV527ΔT/ pMMB*ttpCvc*	+	+	+	+	+
AMV527ΔT/ pMMBHxxxAvc	+	+	+	+	+
AMV527ΔT/ pMMBAxxxSvc	+	-	-	-	-
AMV527ΔT/ pMMBAxxxAvc	+	-	-	-	-

Transcription of the plasmid-encoded *ttpCvc* genes was induced with 1mM IPTG. + or - indicates growth or lack of growth around the specified iron source. 1µl of each iron source was spotted on the surface of the plates in the following concentrations; Ferric Ammonium Citrate: 10mg/ml; Enterobactin: 1.0mg/ml; Anguibactin: 1.0mg/ml; Vibriobactin1.0mg/ml; Ferrichrome 5.0 mM; Hemin 2µM.

Figure 2-4

Western blot of plasmid-encoded *V. cholerae* TtpC proteins with HxxxS motif point mutations in *V. cholerae tonB1:Tn10, ΔttpC,* vibriobactin ⁻ genetic background. TtpC expression is identified with the anti – TtpC C terminal domain antibody.

Figure 2-4 Expression of plasmid-encoded *V. cholerae* TtpC point mutations

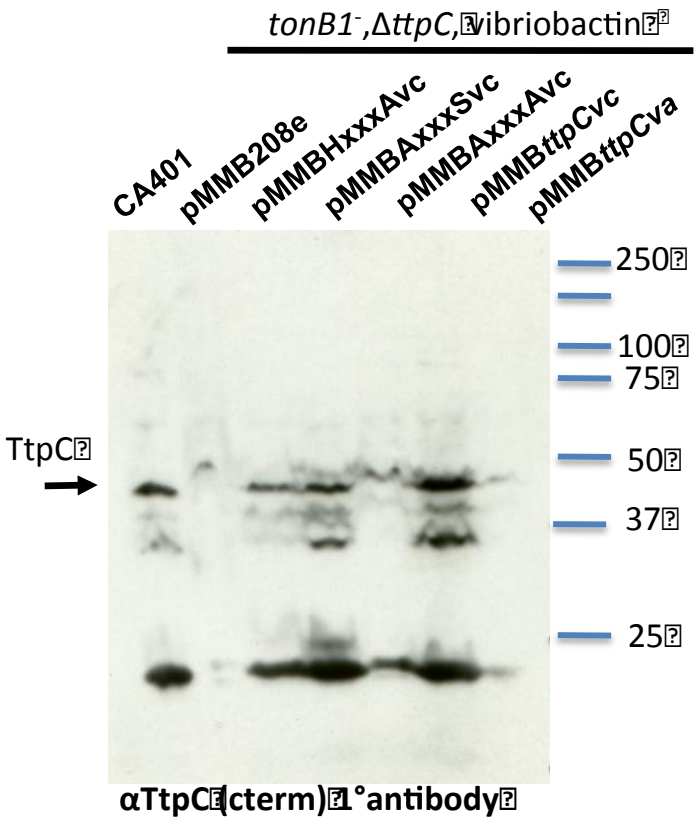
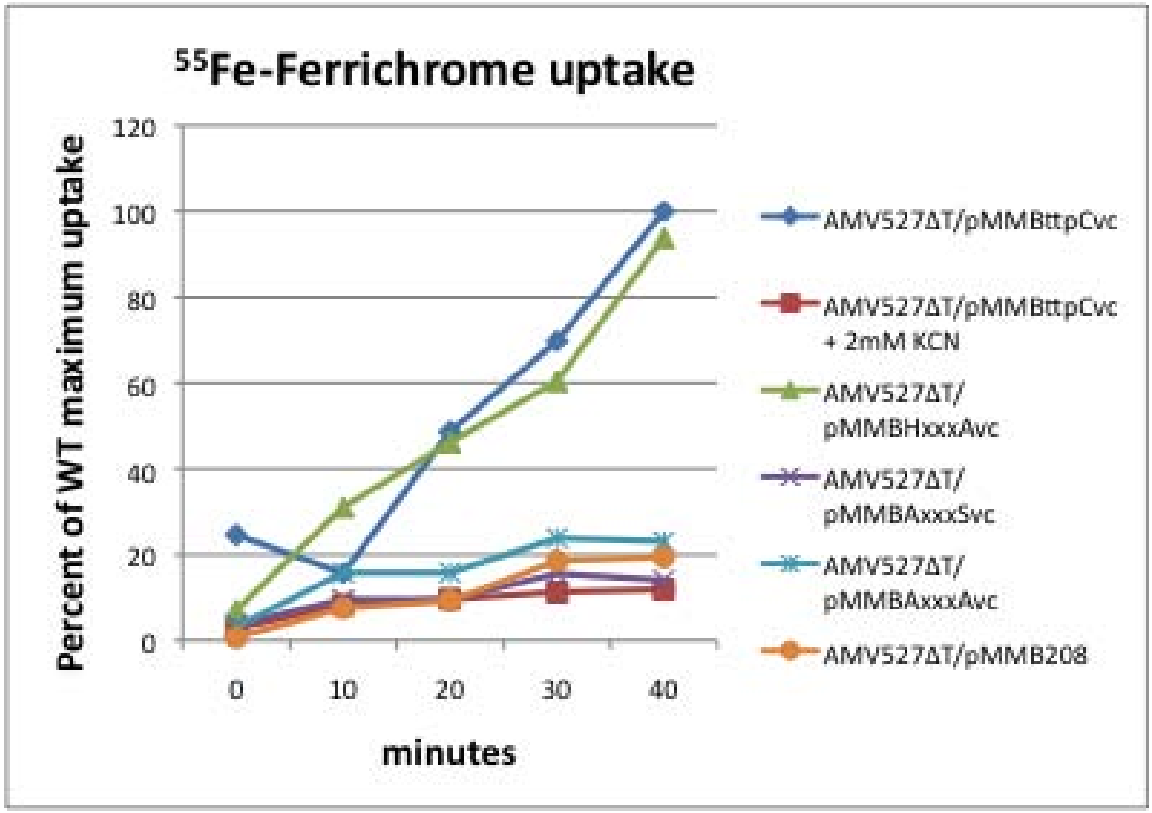


Figure 2-5.

V. cholerae histidine 414 is essential for ^{55}Fe-ferrichrome transport. Representative data set of percent uptake of ^{55}Fe-ferrichrome uptake. Cpm (counts per minute) of *V. cholerae* AMV527ΔT (CA40130N tonB1:Km, Δ*ttpC*) complemented with wild-type *V. cholerae ttpC* at 40 minutes after addition of radio-labeled iron set as maximum. Cpm readings of HxxxS point mutant-complemented strains at ten minute intervals are reported as percent of the maximum. n=3

Figure 2-5. ^{55}Fe-Ferrichrome uptake



inhibiting energy production regardless of the electron-donating substrate (Illingworth, J 2010).

^{55}Fe-ferrichrome uptake kinetics from one representative experiment, (n=3) are shown in Figure 2-5. The S418A point mutation behaves similarly to the strain complemented with wild-type *V. cholerae* TtpC whereas the H414A and double mutation show an uptake pattern similar to the two negative control strains. These results confirm the essentiality of H414 for TonB2-mediated iron uptake.

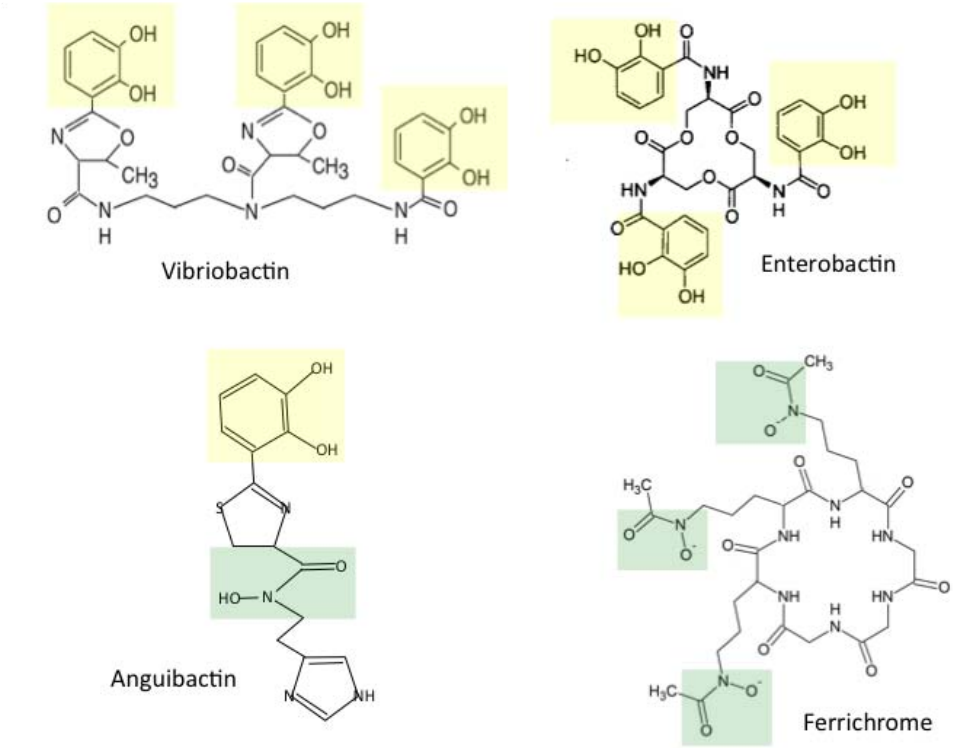
This assay was attempted with several siderophores. I first attempted the ^{55}Fe-uptake assay by complexing the radio-labeled iron with enterobactin because the enterobactin receptor can only be energized by the TonB2 system. I hypothesized that using enterobactin would make the assay as specific for testing the function of the TonB2 system as possible. However, I was unable to create a stable ^{55}Fe-enterobactin complex for use in the uptake assay. Next, I attempted creating a ^{55}Fe-vibriobactin complex because vibriobactin is the endogenously produced siderophore of *V. cholerae* and iron-free HPLC-purified vibriobactin is commercially available from EMC biosciences, (Germany). I again followed the protocol our lab developed for complexing the *V. anguillarum* siderophore, anguibactin with the $^{55}FeCl_3$. Once again, I was unable to create a stable iron-siderophore complex.

^{55}Fe-Ferrichrome uptake assays have been used to measure the efficiency of the TonB system in *E. coli* (Larsen and Postle, 2001). Both anguibactin and ferrichrome have hydroxamate-type active groups and enterobactin and vibriobactin are both strictly catechol-type siderophores as shown in Figure 2-6 (Pollack and Neilands, 1970; O'Brien and Gibson, 1970; Jalal et al., 1989; Griffiths et al., 1984). I hypothesized that the Fe-

Figure 2-6.

Iron-binding groups of siderophores used by *V. anguillarum* and *V. cholerae* TonB2 systems. Yellow boxes highlight the catechol groups and green boxes highlight the hydroxamate groups in the structures for Vibriobactin (Griffiths et al. 1984), Enterobactin (Pollack and Nielands, 1970; O'Brien and Gibson, 1970), Anguibactin (Actis et al 1986; Jalal et al 1989) and Ferrichrome (Van Der Helm et al 1980).

Figure 2-6. Catechol-type and hydroxamate-type siderophores used by the *vibrios*.



hydroxamate complexes are more stable under the conditions routinely used in the uptake assay and that a ^{55}Fe-Ferrichrome complex would be stable and produce consistent uptake kinetics with *V. cholerae*. The ^{55}Fe-ferrichrome-complex was formed with iron-free ferrichrome from Sigma following the protocol presented in Postle (Larsen and Postle, 2001) in a 6.7:1 $^{55}FeCl_3$ to ferrichrome ratio in 0.01M HCl. The uptake reaction was quenched with 0.1M LiCl, which allowed the bound but not internalized ^{55}Fe-ferrichrome to be washed away. Details of this complex formation are also presented in the Materials and Methods section Ch. 5.H.

D. TtpC HxxxS motif is not essential for PMF-dependent conformational change.

In *E. coli*, the conserved H20 is the only amino acid in the TonB TM domain that is essential for the energy-transducing function of the TonB protein, cross-linking of TonB with ExbD, and the ability of TonB to respond to the PMF via conformational shift (Swayne and Postle, 2011; Larsen et al., 2007; Larsen and Postle, 2001). An energy non-responsive mutation of this histidine to an alanine residue can be suppressed by corresponding mutations in ExbB (A39E) allowing the TonB protein to assume the energized conformation as assessed by proteinase K susceptibility assays, but preventing it from cycling back to the non-energized conformation. This conformation allows only limited iron transport (Larsen et al., 2003; Larsen et al., 1999).

It is hypothesized that the PMF-responsive conformational shift seen in the *E. coli* TonB protein is the mechanism by which energy is transduced from the PMF in the inner membrane where it is stored in the "energized" TonB conformation (Swayne and Postle, 2011; Postle and Larsen, 2007; Ollis et al., 2009; Larsen et al., 2007; Larsen et al., 2003;

Larsen and Postle, 2001; Larsen et al., 1999; Larsen et al., 1993; Kuehl and Crosa, 2009; Higgs et al., 1998; Germon et al., 2001). The PMF energy is then transferred to the OM transport proteins when TonB changes conformation and physically interacts with the OM transport proteins. This model is supported by the HSQC interaction studies by Vogel (López et al., 2009). Similar studies were performed with the periplasmic portion of the *V. anguillarum* TonB2 protein and no interaction with OM transport protein peptides was observed (López et al., 2009). It is possible then, that the TtpC protein could be changing conformation in response to the PMF and energizing the OM transport proteins in place of, or in concert with the TonB2 protein in the *vibrio*s.

Protease susceptibility assays were performed in *V. cholerae* spheroplasts to identify PMF-responsive conformational changes of the TtpC and TonB2 proteins. Wild-type *V. cholerae* with a chromosomally encoded carboxy-terminal 6XHis affinity tagged TtpC protein (*V. cholerae* strain TtpC 6-His) was used to generate the spheroplast samples by the method of Ahn *et al.* 2005 (Ahn et al., 2005). Briefly, the cells were washed and resuspended in 0.2M Tris-Cl pH 8.0 with 1M sucrose, lysozyme (0.04 mg/ml) and EDTA (0.2mM), then incubated with gentle agitation at room temperature for 15 min to create spheroplasts (covered in detail in the materials and methods chapter 5.K iii). The 6XHis-tagged strain was used for ease in detection of the TtpC degradation products because the anti-6XHis monoclonal antibody is more robust and has less cross-reactivity than the polyclonal anti *V. cholerae* TtpC antibody.

To test the effect of the PMF on the conformation of the TtpC protein, cells were grown in CM9*vibrio* minimal iron media to mid-log phase. Half of the cells were treated with 50μM of carbonyl m-chlorophenylhydrazone (CCCP), an un-coupler of cellular respiration, before

spheroplasts were generated and treated with 25μg/ml proteinase K or 90μg/ml trypsin. Two proteases were tested to confirm the conformational changes in the TtpC protein in response to the PMF. Western blots of the degradation products using trypsin treatment and proteinase K treatment are presented in Figure 2-7; panels A and B respectively. A western blot with anti-6XHis monoclonal antibody against the protein samples from the non His-tagged wild-type *V. cholerae* strain CA401 treated with CCCP and trypsin is shown in panel A as a negative control for the anti-6XHis antibody. As seen in Figure 2-7, panels A, and B, the TtpC 6-His degradation patterns in the spheroplasted cells treated with protease differed between the presence or absence of the PMF inhibitor, CCCP. This change in degradation pattern indicates a PMF-responsive conformational change in the TtpC protein similar to that observed in the *E. coli* TonB (Larsen et al., 1999). The altered degradation pattern is indicated with an arrow in each panel.

To assess the effect of the HxxxS point mutations on the ability of the TtpC protein to respond to the PMF via conformational change, spheroplasts and whole cells of the non-functional *V. cholerae* strains TtpC AxxxS 6-His and TtpC AxxxA 6-His were treated with 90μg/ml trypsin in the presence or absence of CCCP. The HxxxS mutants were chromosomally encoded TtpC point mutants in the HxxxS motif with a carboxy-terminal 6XHis tag. Treatment with 50μM CCCP resulted in collapse of the PMF prior to splitting the samples into whole cells and spheroplasts. The trypsin degradation patterns of the *V. cholerae* strain TtpC 6-His and the wild-type *V. cholerae* CA401 are shown in Figure 2-8, panels A and B. The HxxxS point mutants are shown in Fig 2-8, panels C and D.

There is one aspect of this data set that should be noted prior to analysis. The trypsin degradation pattern of the TtpC-6XHis strain does not show the previously identified

Figure 2-7.

PMF-dependent protease degradation of *V. cholerae* TtpC. Western blots of *V. cholerae* strain TtpC-6His whole cells or spheroplasts treated with or without CCCP, then exposed to 90μg/ml Trypsin in panel A, or 25μg/ml Proteinase K in panel B. Blue arrows indicate unique degradation products that appear depending on the presence or absence CCCP, which collapses the PMF. Molecular weight standards in the Kaleidoscope Precision Plus protein standard are indicated in kDa.

Figure 2-7. Spheroplasts with Trypsin or Proteinase K

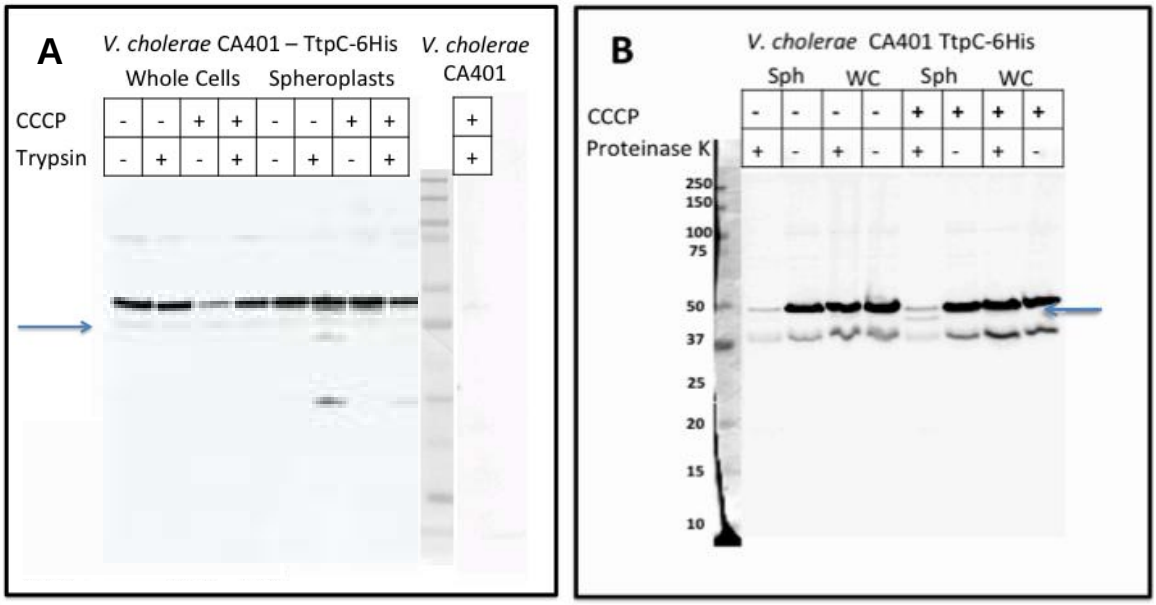
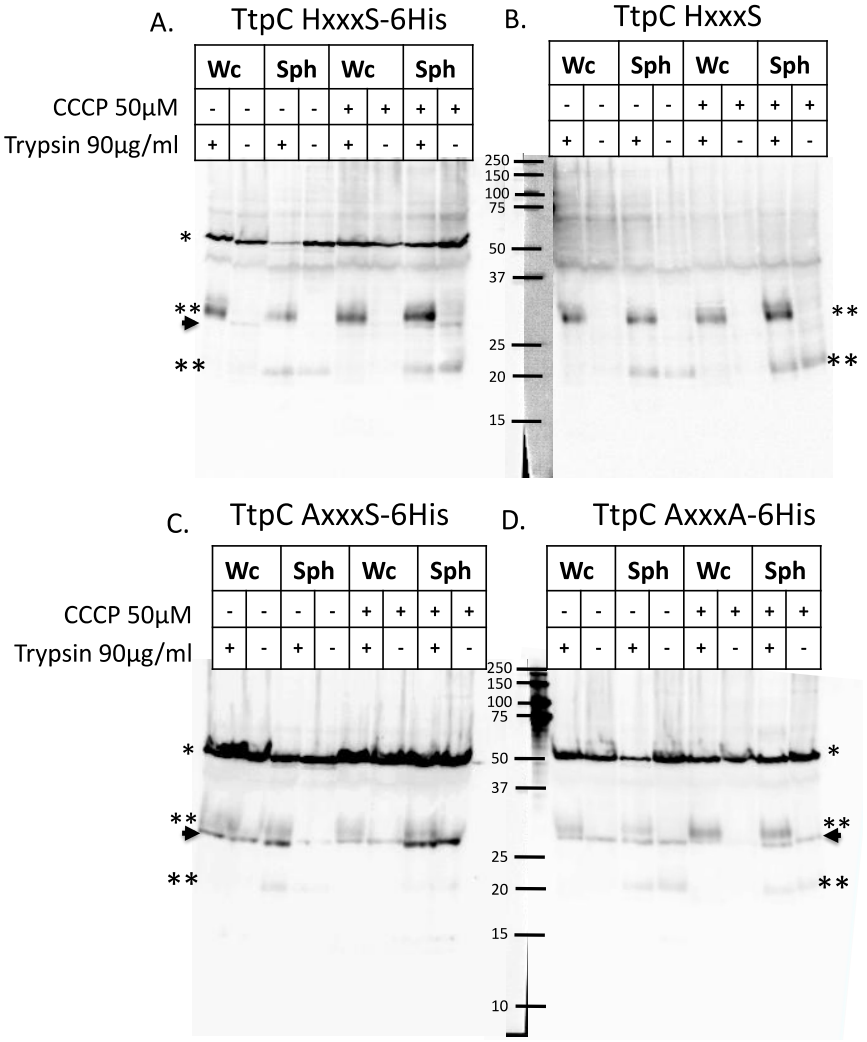


Figure 2-8.

Western blots of TCA precipitated total proteins from *V. cholerae* whole cells (Wc) or spheroplasts (Sph) after treatment with CCCP and Trypsin as indicated. (*) indicates the full-length TtpC 6XHis protein. (▶) indicates a TtpC-6His degradation product consistent among all samples. (**) indicate cross-reacting proteins. A. *V. cholerae* strain TtpC-6His, B. All samples generated from *V. cholerae* wild-type strain CA401, C. *V. cholerae* strain TtpC AxxxS-6His, D. *V. cholerae* strain TtpC AxxxA-6His. Molecular weight standards in the Kaleidoscope Precision Plus protein standard are indicated in kDa.

Figure 2-8 *V. cholerae* H414A mutation does not effect the conformation of the TtpC protein



degradation pattern seen in Figure 2-7, panel A. The pattern observed in Figure 2-7 was observed in two other sample sets of the *V. cholerae* strain TtpC 6-His treated with a 30µg/ml trypsin for 15 minutes and for 30 minutes, thus I am confident that the degradation pattern in Figure 2-7 is valid. There are no detectable 35 or 20 kDa degradation products in the trypsin-treated spheroplast samples as observed before. There are however, consistent degradation products in each sample at roughly 27 kDa that are detectable with the anti-6XHis monocolonal antibody in each of the His-tagged strains (indicated by black arrow heads in Figure 2-8). In this sample set, the TtpC degradation pattern for either point mutant (AxxxS-6 His or AxxxA-6 His) in the presence or absence of the PMF does not change compared to that of the wild-type TtpC-6His. These results indicate that either the H414 is not necessary for the TtpC protein to respond to the PMF via conformational change despite the lack of iron transport detected in *V. cholerae* strains expressing H414A point mutations, or the H414A mutation allows a conformational change that does not alter the proteolytic cleavage pattern. Because the degradation pattern of the trypsin-treated strain TtpC 6-His in Figure 2-8 does not show an altered degradation pattern in conditions with the PMF compared to conditions without the PMF as seen in Figure 2-7, further testing of the HxxxS point mutants in response to CCCP and trypsin digestion should be pursued.

It is possible that other proteins in the *V. cholerae* TonB2 system change their conformation in response to the presence or absence of the PMF. To assess if, as in the case of the *E. coli* TonB protein, TonB2 was changing conformation in response to the PMF, I identified TonB2 proteinase K degradation products from spheroplasts and whole cells treated with or without 50µM CCCP. Full length and degradation products were detected using a polyclonal antibody directed toward the periplasmic region of the *V. cholerae* TonB2 protein. The

western blot of the TonB2 degradation products is shown in Figure 2-9. While there are many cross-reacting bands visible, the full length TonB2 protein (based on previous identification of wild-type *V. cholerae* TonB2 in total membrane protein samples) is visible at 40kDa. Several degradation products at roughly 28, 25 and 17kDa are visible except in the spheroplast samples treated with proteinase K. Based on these results, the *V. cholerae* TonB2 protein does not change conformation in response to the PMF, as is the case in *E. coli*, because there is no change in the TonB2 degradation pattern between the CCCP treated and non-treated spheroplast samples.

A chromosomal deletion mutant of the *V. cholerae* TonB2 protein does not yet exist. An important control for this identification of TonB2 degradation products would be to identify the degradation products produced by proteinase K treatment of the deletion strain with the anti-*V. cholerae* TonB2 antibody to identify which bands are non-specific.

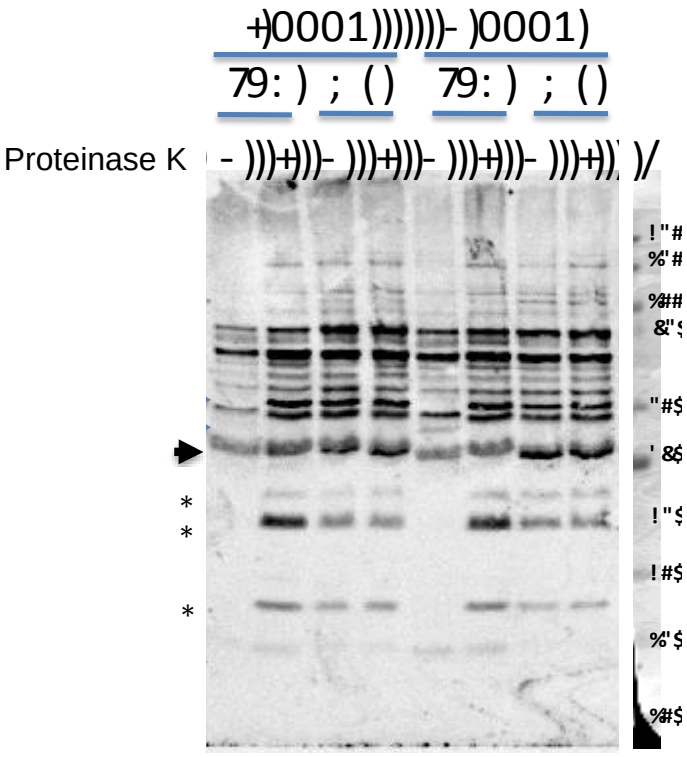
E. Mutation of Histidine 414 does not result in altered protein-protein interactions.

In *E. coli,* PMF-non-responsive mutations of the conserved H20 residue result in an inability of the TonB protein to form formaldehyde-linked complexes with the ExbD protein (Swayne and Postle, 2011). I hypothesized that conformational changes in TtpC in response to the PMF seen in Figure 2-7 would lead to altered protein-protein interactions with other proteins in the TonB2 energy transduction system. To visualize potential changes in protein-protein interactions, I used *in vivo* formaldehyde cross-linking with 1% formaldehyde or 0.5% formaldehyde followed by western blot of total proteins with anti-6XHis monoclonal antibody to identify all of the TtpC-containing complexes. I hypothesized that lowering the concentration of formaldehyde would decrease the amount of non-

Figure 2-9.

The protease susceptibility of *V.* cholerae TonB2 is not effected by changes in the PMF. Western blot of *V. cholerae* CA401 spheroplasts (sph) and whole cells (wc) treated with or without 50μM CCCP prior to treatment with 25μg/ml Proteinase K. Molecular weight standards are noted in kDa. The *V. cholerae* TonB2 protein and TonB2 degradation products are identified with the anti-TonB2vc polyclonal antibody. The black arrow indicates the full-length TonB2 protein (40 kDa), which migrates more slowly than its molecular weight predicts it should. (*) indicate possible TonB2 degradation products. Molecular weight standards in the Kaleidoscope Precision Plus protein standard are indicated in kDa

Figure 2-9 PMF-induced conformational changes in *V. cholerae* TonB2



specific cross-linking. The TtpC-containing complexes of the non 6XHis-tagged TtpC, TtpC 6-His, TtpC AxxxS 6-His and TtpC AxxxA 6-His are shown in Figure 2-10. The cross-linking patterns of the TtpC protein in strains harboring the TtpC HxxxS point mutations were identical to those of the wild-type *V. cholerae*. The cross-linking pattern is specific, clearly identifying the TtpC monomer at roughly 49kDa, and based on molecular weight predictions in the Kaleidoscope Precision Plus protein standard (Bio-Rad), a TtpC dimer between 75 and 100kDa and a TtpC tetramer close to 150kDa. Non-specific cross-linking would show a TtpC tetramer or larger complexes. Fainter bands in both western blots may indicate complexes of TtpC with other proteins where the ratio of TtpC to other membrane proteins is nota s high as in the TtpC dimer and tetramer complexes.

Although I saw no difference between the complexes formed by wild-type and HxxxS-mutated TtpC proteins, I hypothesized that perhaps the PMF-dependent conformational changes in the TonB2 system proteins would not be detectable in cross-linked complexes in the presence of the PMF. To test the effect of the PMF on the ability of the HxxxS mutant TtpC proteins to form cross-linked complexes with other proteins in the TonB2 system, I added 50μM CCCP to the bacterial cultures prior to cross-linking and observed complexes containing the ExbB2 protein. As seen in Figure 2-11 panels A and B, collapse of the PMF by CCCP did not alter the cross-linking pattern in any of the strains tested. I also examined the effect of collapsing the PMF on the cross-linked complexes formed by the TtpC 6-His strain using the anti-6XHis monoclonal antibody. The cross-linking pattern was not altered in the presence of CCCP, which leads me to conclude that the portion of the TtpC protein that is interacting with other proteins in the TonB2 system is either not the portion of the protein that changes conformation in response to the PMF or the conformational changes seen in Figure 2-7 do not affect protein-protein interactions. It is also possible that the changes in

the protein-protein interactions are too subtle to be detected by the formaldehyde cross-linking method. It is most likely that the TtpC-containing complexes are formed via interactions between the trans-membrane domains of the TonB2 system proteins.

Figure 2-10.

Formaldehyde cross-linked protein complexes containing V. cholerae TtpC-6His or 6 His-tagged HxxxS point mutations. A. complexes observed with 1% formaldehyde for 0 to 5 minutes; B. complexes observed with 0.5% formaldehyde for 0 to 5 minutes. Molecular weight of Kaleidoscope Precision-Plus protein standard is noted in kDa. Exposure to formaldehyde is noted in minutes for each sample and strain names are indicated along the top of each sample set.

Figure 2-10. Formaldehyde Cross-linking - HxxxS Motif Mutants Do Not Have Altered Protein-Protein Interaction Patterns.

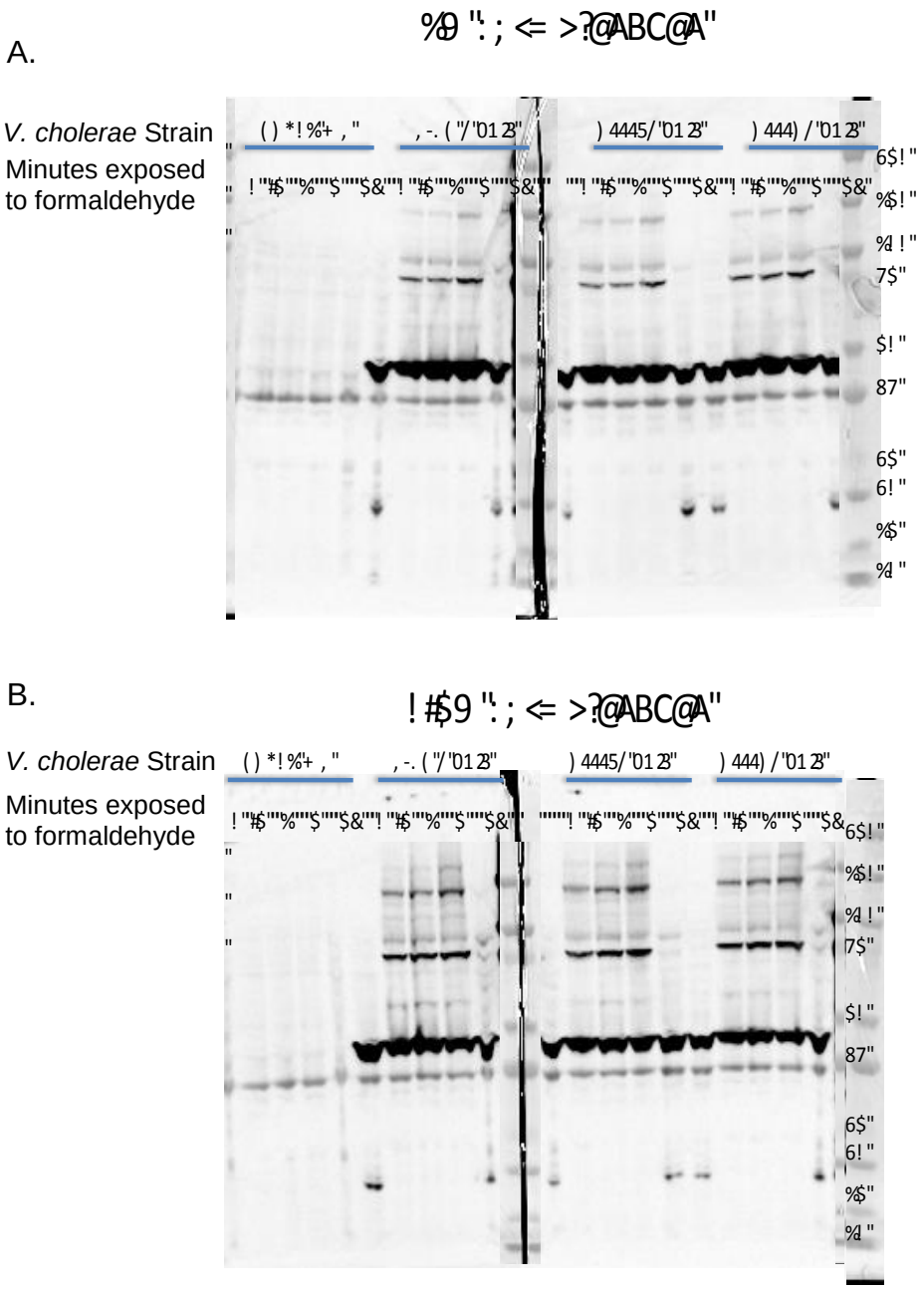
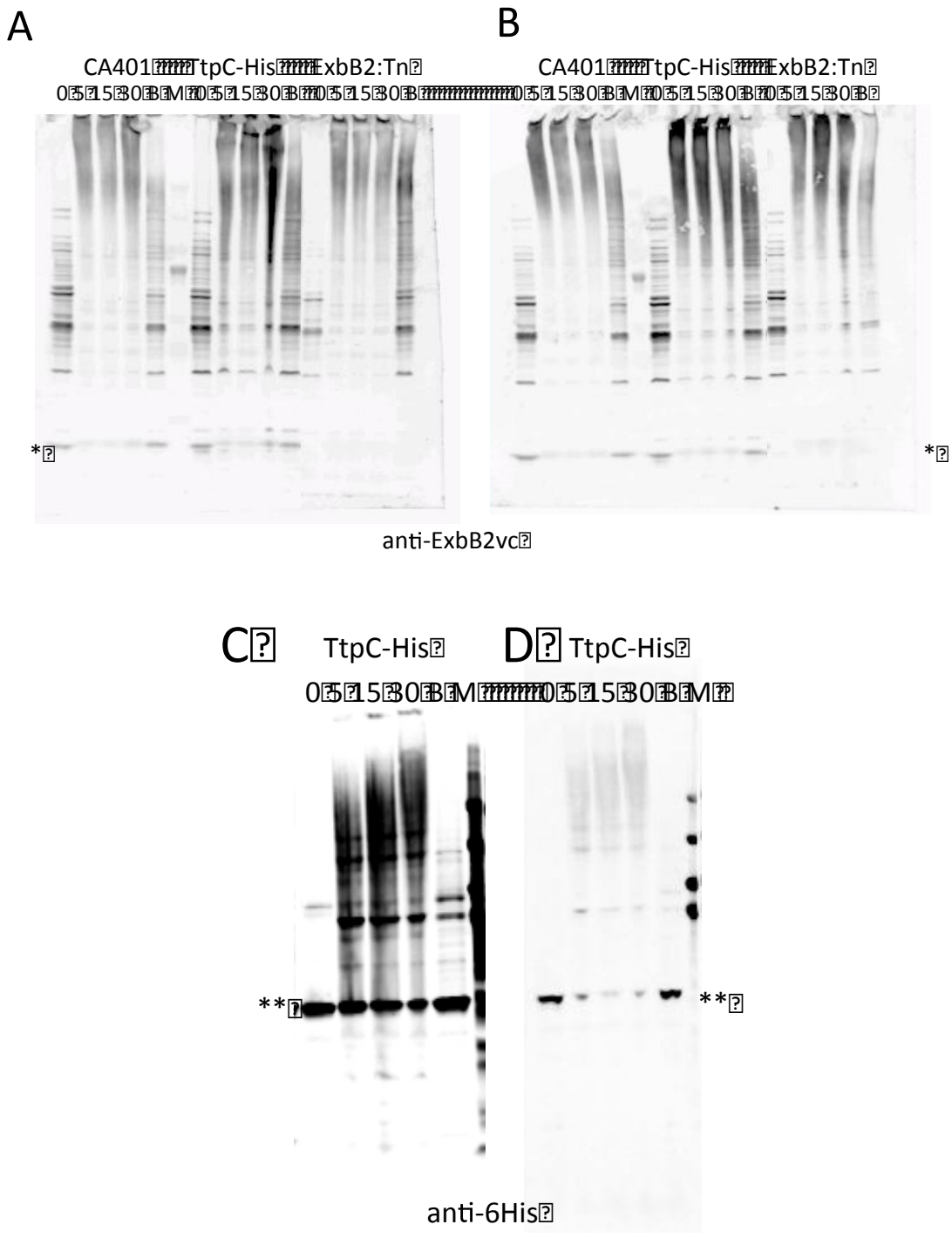


Figure 2-11.

Collapse of the PMF does not alter the protein-protein interactions of *V. cholerae* TtpC or ExbB2. A. Cross-linked complexes containing the ExbB2 protein in *V. cholerae* CA401, TtpC-6His and AMV257 (Tn10 insertion in ExbB2) treated with 50μM CCCP prior to cross-linking with 1% formaldehyde for 0 to 30 minutes. B. No CCCP added prior to crosslinking. * indicates the ExbB2 monomer. C. Cross-linked complexes containing the TtpC-6His protein in *V. cholerae* TtpC-6His treated with 50μM CCCP prior to cross-linking. D. No CCCP added prior to cross-linking. ** indicates the TtpC-6His monomer. "B" indicates a 30 minute cross-linked sample boiled for 5 minutes prior to loading on an SDS-PAGE gel for separation.

Figure 2-11 Effect of CCCP on TonB2 system complex formation



Strains used in Chapter 2

Strain	Genotype	Plasmid	Reference
E. coli CC118	F2 D(ara-leu)7697 araD139 D(lac)X74 phoAD20 galE galK thi rpsE rpoB argE(Am) recA1	pMMB206phoAempty	This work
		pttpC295phoA	This work
		pttpC359phoA	This work
		pttpCfullphoA	This work
V. cholerae CA401			
V. cholerae AMV527	*tonB1:Tn10, vib-*		Mey et al 2001
V. cholerae AMV527ΔT	*tonB1:Tn10, ΔttpC, vib-*	pMMB208	This work
		pMMBV*cttpC*HxxxA	This work
		pMMBV*cttpC*AxxxS	This work
		pMMBV*cttpC*AxxxA	This work
		pMMBV*attpC*	This work
V. cholerae CA401	*ttpC-6His*		This work
V. cholerae CA401	*ttpCAxxxS-6His*		This work
V. cholerae CA401	ttpCAxxxA-6His		This work
V. cholerae DOV221	*exbB2:Tn10*		Occhino et al 1998

Chapter 3. Characterization of the TtpC protein and its role in the TonB2 energy transduction system

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Pathogenic bacteria possess virulence factors that allow them to invade a vertebrate host and colonize the host while evading the innate and adaptive immune systems. One of these virulence factors is the ability to compete with the host cells for iron. Iron is an essential element for nearly all species and vertebrate hosts have developed mechanisms to sequester free iron in efforts to deter the growth of pathogenic bacteria. In biological fluids, iron is always bound in a complex with iron-binding proteins such as transferrin, lactoferrin or as part of the oxygen-carrying heme group in red blood cells. Thus, bacterial pathogens scavenging for iron must also possess iron binding proteins and mechanisms to internalize bound iron in order to establish an infection. Some species express outer-membrane receptors specific for iron binding host proteins like transferrin or heme. Alternatively, bacteria can synthesize siderophores that are used to scavenge iron from eukaryotic iron-binding proteins. Siderophores are low molecular weight compounds that have an extremely high affinity for iron, in some cases more so than host iron-binding proteins, and are secreted by the bacterium to scavenge iron from the surrounding environment. In Gram-negative species, the iron-siderophore complexes are bound on the surface of the

outer membrane by receptor proteins. Most bacterial species express several outer-membrane receptors, each one specific for a different ferric iron-siderophore complex.

Depending on the outer membrane transport protein and inner membrane permeases being used, the ferric iron or the iron-siderophore complex is internalized into the periplasmic space and then transported through the inner membrane to the cytosol where it can be used by the cell as a cofactor in oxidation–reduction reactions and as a transcriptional co-repressor with the master-regulator Fur (Ernst et al., 1978; Hantke, 1981). The energy required to power iron uptake is derived from the proton motive force in the inner membrane of the Gram-negative cell. This energy is harvested and transduced to the iron-siderophore complex-binding proteins in the outer membrane via a complex consisting of a TonB-family protein, and accessory energy harvesting proteins, ExbB and ExbD (Brinkman and Larsen, 2008). ExbB is necessary to stabilize TonB (Fischer et al., 1989; Postle and Kadner, 2003; Skare and Postle, 1991). The TonB protein interacts with a ‘‘TonB box’’ region on the periplasmic domain of the outer membrane receptor (Crosa et al., 2004). Much of the elucidation of energy transduction between the cellular membranes of Gram-negative bacteria has been determined in *E. coli*. *E. coli* has one classical TonB system whereas the pathogenic *vibrio* species possess at least two, and in some species, three TonB systems that appear to be specific for the transport of various iron sources (Fig. 3-1) (Stork et al., 2004; Seliger et al., 2001; Kustusch et al., 2011; Alice et al., 2008).

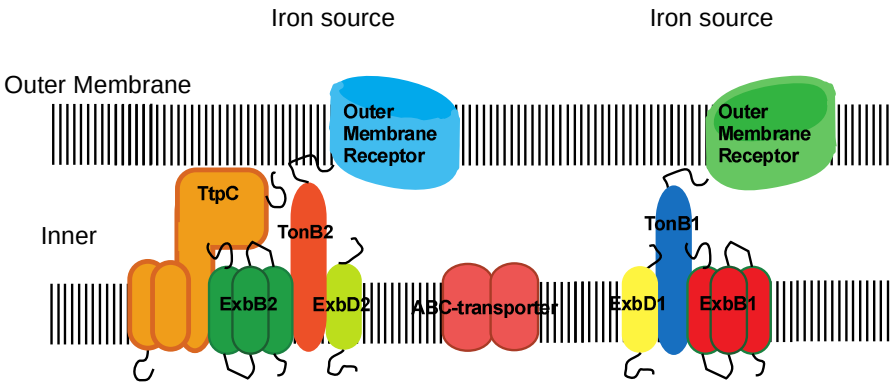
A. Requirement of a fourth protein

The presence of TonB along with the accessory proteins ExbB and ExbD is conserved across many Gram-negative species. In *V. anguillarum* however, a fourth protein was discovered to be necessary for ferric anguibactin transport mediated by the TonB2 system

Figure 3-1.

Cartoon diagram of the *V. anguillarum* TonB1 and TonB2 systems. The iron sources transported with energy transduced by each system are listed under the respective TonB systems.

Figure 3-1 The TonB systems of *V. anguillarum*



TonB2 system-dependent Iron sources in *V. anguillarum*:
Anguibactin
Vanchrobactin
Enterobactin
Ferrichrome
Hemin

TonB1 system-dependent Iron sources in *V. anguillarum*:
Ferrichrome
Hemin

(Stork et al., 2007). This protein, TtpC, is encoded in the *tonB2* gene cluster of *V. anguillarum*. Using sucrose-density gradients, it was shown that TtpC is located in the inner membrane. Through the use of bioassays to test iron-source utilization, it was shown that TtpC is necessary for ferric-anguibactin transport mediated by the TonB2 system in the fish pathogen *V. anguillarum* (Table 3-1) (Stork et al., 2007). In addition, the TtpC homologue in the human pathogen *V. cholerae* is essential for enterobactin transport mediated by the *V. cholerae* TonB2 system. Although *V. anguillarum* has two TonB energy transduction systems, the TtpC protein is specific for the TonB2 system and not necessary for iron transport mediated by the TonB1 system in *V. anguillarum.* (Table 3-1)

B. Homology of TtpC proteins in several pathogenic *vibrio* species

The data presented in Table 3-1 show that TtpC is necessary in the TonB2 system of both *V. anguillarum* and *V. cholerae* and that the TonB2 system from *V. anguillarum* can support iron transport of TonB2-specific iron sources in *V. cholerae*. These results raise the question of how similar the TtpC proteins are to one another. To compare the proteins, I used the T-coffee server to perform a multiple sequence alignment of the amino acid sequences of the TtpC protein from five pathogenic *vibrio* species. The T-coffee server uses the lalign algorithm to perform several pair-wise alignments between input sequences to create a series of local alignments, then creates a multiple sequence alignment using either a BLOSUM matrix or a PAM matrix, which give a higher score to an alignment that is more likely to be related biologically than by chance (Henikoff and Henikoff, 1992). A BLOSUM matrix was used to create the multiple sequence alignment shown in Figure 3-2. The Clustal W-formatted alignment and the score are shown in Figure 3-2. The multiple sequence alignment produced an overall score of 66 indicating that the proteins were highly similar (Poirot et al., 2003). Individual alignments of the *V. anguillarum* TtpC sequence to the TtpC

Table 3-1 TtpC is necessary for Ferric-siderophore transport mediated by the TonB2 system in *V. anguillarum* and *V. cholerae*

Strains	Iron Sources[c]				
	Ferric Ammonium Citrate	Anguibactin	Enterobactin	Ferrichrome	Heme
V. anguillarum					
775 Wild Type	+[a]	+	+	+	+
775 *tonB1*⁻, *ttpC*⁻	+	-	-	-	-
775 *tonB1*⁻, *ttpC*⁻/ p*exbB2,exbD2,tonB2*$_{(va)}$	+[d]	-	-	-	-
775 *tonB1*⁻, *ttpC*⁻/ p*ttpC,exbB2,exbD2,tonB2*$_{(va)}$	+	+	+	+	+
775 *ttpC*⁻	+	-	-	+	+
V. cholerae					
CA401 Wild Type	+	ND[b]	+	+	+
CA401 *exbB2*⁻	+	ND	-	+	+
CA401 *exBB2*⁻ p*exbB2,exbD2,tonB2*$_{(va)}$	+	ND	-	+	+
CA401 *exBB2*⁻ p*ttpC,exbB2,exbD2,tonB2*$_{(va)}$	+	ND	+	+	+
CA401 *ttpC*⁻	+	ND	-	+	+

a. + or - indicates growth or lack of growth around the specified iron source.
b. ND; Not determined
c. 5 l of each iron source was spotted on the surface of the plates in the following concentrations; Ferric Ammonium Citrate: 500 g/ml; Enterobactin: 1.0mg/ml; Anguibactin: 1.0mg/ml; Ferrichrome: 1.0mg/ml; Hemin: 20 g/ml.
d. Mutations in chromosomally-encoded genes were complemented by genes expressed from the low copy-number expression vector pACYC177.

Figure 3-2.

(*) indicate amino acid residues conserved across all five pathogenic *Vibrio* species. (:) indicate amino acids with similar chemical properties that are conserved across all five species. (.) indicate amino acids with similar shapes that are conserved across all five species.

Figure 3-2 Clustal W alignment of the TtpC protein from pathogenic *Vibrio* species

```
Alginolyticus     --mmkkwlsvalistaalmpyttf------ssdallqkaqqenrqqqshnvaresgfkqt
Parahaemolyticus  --mikkwlsvalistaammphttf------asdtllqkaqqenrqqqthnasresgfkkt
Anguillarum       --mvfslsslcl----tllafnsf------astelvsnakvenkqqqqhniereagfkqt
Cholerae          mtlrkttlalalslpfafvsvan-------asnslvqqatqekaqqqqhnqqreagfvqt
Vulnificus        ---mkgfktiaaallaslfmvssahaeapkslnellknvksesiveskenkareqeflad
                         ::.     ::.   .       :   *:.:.  *.  :. .*  **  *

Alginolyticus     eqdlqaiknklvaeraalqaeadslsvtfgeneaelaqleeklrletgslgelfgvvrqn
Parahaemolyticus  eqelqaiknklvaeraalqaeadtlsvtfseneaelaqleeklrletgslgelfgvvrqn
Anguillarum       eqqlrrakaeleaqrqdlqaqadklstqfsnnenrlarleeqlrletgslgelfgvvrqn
Cholerae          aqelqaakaellaernrlqkeadqlssqfsdnentlarleetlrletgslgemfgvvrqn
Vulnificus        rnkqaellkqakaqlkaetalgeqlkatfddndkklteltetlrqrsgtlgemfgvvrqy
                   :.      :  *:       .: *.  *.:*:  *:.* * ** .:*:***:******

Alginolyticus     akeleselkssvtgvdanayqkdidaivaakslptltqlqamwrsmeeqikasgemanvs
Parahaemolyticus  akeleselkhavtgvdansyqkdieaivaakslptlkqlqamwrsmeeqvtasgelakvs
Anguillarum       aa-lsselessitavdrhqytsrvqeivaakslpsmpqltglwhsyveqiiasgelsrvq
Cholerae          akelqseldqsvtgveprahqqsiddvvaaktlpsmaqlrglwqamseeirasgqvktte
Vulnificus        agefkglfnasqnavqfpqrdalltklaeskelpsteelesfwhtvlqqiivsgetsttp
                  *  :.. :. : ..*:       :  :. :* **:  :* .:*::  ::: .**:   .

Alginolyticus     ftllngegreqtvsgvrlgsmallddtgyvkwngqrgdavnylrqpesgpt--an---ti
Parahaemolyticus  ftlldgegkeqtvngvrlgamallddngyvkwngqrgdavnylrqpengpt--as---af
Anguillarum       iafingdgvtqnvaayrigtf-lvteqgyvkwngdkqaataylkqpdngpi--fs---gl
Cholerae          iqwlngqgetqtvpalrlgslgliseqgyvkwdnarqqalsyqqlpsdfpt--fshirtl
Vulnificus        atvvygegkeavrdvtlvgefnaiadgkyvtyvpqtgkfeelsrqpranitrhvagfes-
                     : *:*         :* :  : :  **.:           : *

Alginolyticus     ssgdidalvidpsrgilleqlansptladrlnaggvvgkiilgllaigllialvrgaslm
Parahaemolyticus  tngeldslvidpsrgilleqlansptlkdrlnaggvvgqiilvllaigliialvrgvslf
Anguillarum       ntgeieslvvdpsrgimleqlaltptlaerlqmggavgniilvllgigliiafyrgfalv
Cholerae          vdgdvvtmkvdpsrgvlleqlaltptfsqrlqaggvignvilvllgvgliialyrgaila
Vulnificus        tsssyeplfidpsrgvilsllvqsptvkeridqggivgyvilamgalgalialfcylrll
                    ..  .: :*****::*. *. :**. :*:: ** :* :** : .:* :**:     *

Alginolyticus     isrqkimkqlktpaqpgnnplgrvlavyqkdkhrsvealelrlleavvdeqthlekglsm
Parahaemolyticus  iarqkiakqlknptkaednplgrvlsvyqndkhrsvealelrlleavvdeqthlekglsm
Anguillarum       mthhkikaqlrdvqkignnplgrvlsvynkeqnrsvealelrlleavvdeqahlekglsm
Cholerae          tlrqkikaqlknpeqpgnnplgrilavynkeqqrsvealelrlleavvdeqnhletglsm
Vulnificus        viggkmrkqaksdaviegnplgeviqayqdhkgdnledleakldeiilrnapkierfivs
                      *:  * :      .****.:: .*:..:  .:* ** :* * :: :  ::*  :

Alginolyticus     lkllaalapmlgllgtvtgmietfqvitqfgngdpkvmaggismalvttvlglvsampll
Parahaemolyticus  lkllaalapmlgllgtvtgmietfqvitqfgngdpkvmaggismalvttvlglvaampll
Anguillarum       lkllaaiapmlgllgtvtgmietfqvitqfgngdpkvmaggismalvttvlglvsaipll
Cholerae          lkllaalapmlgllgtvtgmietfqvitqfgngdpkvmaggismalvttvegliaaipll
Vulnificus        iklfasvapllgllgtvmgmigtfqaitlfgtgdpklmaggisealvttmlglvvaipll
                  :**:*::**:******* *** ***.** **.****:****** *****: **: *:***

Alginolyticus     lahnvlssqaenirsilekqgiglvaeqaerdmpnnkshsntlaenaa
Parahaemolyticus  lahnvlssqaesirnilekqgiglvaeqaerdmsnnkgnrntiaenaa
Anguillarum       lahnilssqaenirsilekqgiglvaaqaeqecqas-------mrnaa
Cholerae          lahnilsaqaeairnilekqgiglvaqqaerdc--gaavtqsrveqaa
Vulnificus        ffytlvhskgrrlvqtleeqsagfiaryqeklhtae-----------r
                  : :.:: ::.. : . **:*. *::*   *:
```

sequence of other pathogenic *vibrio*s by BLAST-P reveal amino acid sequence similarity between 73 and 80% (Altschul et al., 1997). Most of the high sequence similarity between the TtpC proteins from different species is located in the carboxy-terminal half of the proteins shown in Figure 3-2. This region encodes three transmembrane domains that anchor the TtpC proteins in the inner membrane and are thought to participate in protein-protein interactions with other proteins in the TonB2 system and may participate in harvesting the energy from the proton motive force.

C. Interspecies complementation

The amino acid sequence of the TtpC proteins in the pathogenic *vibrio* species are highly similar (Figure 3-2). They are so similar that it is possible for the TtpC protein from one species to complement a deletion of the TtpC protein in another *Vibrio* species. Cross-species complementation of a chromosomal deletion in the *V. anguillarum ttpC* with the *ttpC* genes from *V. cholerae* and *V. vulnificus* was tested using bioassays. I cloned the *ttpC* genes from *V. anguillarum* 775, *V. cholerae* CA401, and the *ttpC2* gene from *V. vulnificus* CMCP6 were cloned individually into the IPTG-inducible pMMB208 expression vector. I observed by bioassays in CM9*Vibrio* plus the iron chelator EDDA that *V. cholerae ttpC* and *V. vulnificus ttpC2* can complement a Δ*ttpC* mutation in *V. anguillarum* for the uptake of ferric anguibactin and for ferrichrome. *V. cholerae ttpC* was the only complementing gene that could not restore growth on all tested iron sources (Table 3-2). The lack of growth of the *V. anguillarum* Δ*tonB1,* Δ*ttpC*, Δ*angA*/p$ttpC_{vc}$ strain around enterobactin as an iron source indicates that while the TtpC proteins from the different species are highly similar, slight differences may impede interactions with specific outer membrane receptors in *V. anguillarum*.

Table 3-2 Cross species complementation of *V. anguillarum* TtpC mutation

Strains[c]	Iron Sources[b]				
	Ferric Ammonium Citrate	Enterobactin	2,3-DHBA	Vanchrobactin	Hemin
V. anguillarum					
775/pMMB208	+[a]	+	+	+	+
775 Δ*ttpC*,Δ*angA*	+	-	-	-	+
775 Δ*ttpC*,Δ*angA*/ pMMBV*attpC*	+	+	+	+	+
775 Δ*ttpC*,Δ*angA*/ pMMBV*cttpC*	+	-	+	-	+
775 Δ*ttpC*,Δ*angA*/ pMMBV*vttpC*	+	+	+	+	+

a. + or - indicates growth or lack of growth around the specified iron source.

b. 1µl of each iron source was spotted on the surface of the plates in the following concentrations; Ferric Ammonium Citrate: 10mg/ml; Enterobactin: 1.0mg/ml; 2,3-DHBA: 3.0mg/ml; Vanchrobactin: 1.0mg/ml; Hemin: 20µg/ml.

c. Mutations in chromosomally-encoded genes were complemented by genes expressed from the low copy-number expression vector pMMB208. Expression was induced with 1mM IPTG

D. Isolating the functional regions of the TtpC protein in *V. anguillarum*.

The TtpC protein contains three transmembrane domains, as demonstrated in Chapter 2, Figure 2-3 that anchor the protein in the inner membrane. It is not known whether these transmembrane domains serve only to anchor the protein in the inner membrane while the periplasmic domain performs the functions necessary for TonB2-mediated iron uptake, or if the transmembrane domains play a greater role in the function of the protein. To determine if the periplasmic domain and all three transmembrane domains were essential for energy transduction by the *V. anguillarum* TonB2 system, I constructed a series of carboxy-terminal truncations based on the locations of the three transmembrane domains as predicted by the DAS transmembrane domain prediction algorithm as mentioned in Chapter 2 (Cserzö et al., 1997).

TtpC has a predicted secretion signal peptide in the first 19 amino acids. To ensure proper insertion of the protein in the inner membrane, I made carboxy-terminal truncations rather than deletions at the amino-terminus, which may risk mis-localization of the protein. A version of the TtpC protein containing an internal deletion from amino acids 30-240 was constructed to determine if the periplasmic domain of the protein was essential for function, but the altered protein was not expressed as determined by western blot with the anti- TolR (TtpC) antibody (data not shown).

The first two carboxy-terminal truncations, at 45 and 242 amino acids, did not include any of the predicted transmembrane domains. These truncations allowed me to determine if the transmembrane domains were essential for function. The third truncation at 295 amino acids included the periplasmic region and the first predicted transmembrane domain to

determine if the periplasmic domain simply needed to be anchored in the inner membrane for proper localization and function in the TonB2 system. The fourth and fifth truncations at 359 and 408 amino acids added one and two predicted transmembrane domains respectively. Each of the truncations was cloned into the low copy number vector pMMB208 and expressed in *V. anguillarum* strain Δ1TA, which lacks the TonB1, TtpC and VabA proteins. This strain was used for bioassay analysis of TonB2 system function. The VabA deletion results in a strain that is incapable of converting 2,3-dihydro-2-3, di-hydroxy benzoic acid to 2-3, di-hydroxy benzoic acid (DHBA), an essential step in the production of the *V. anguillarum* siderophore, anguibactin (Alice et al., 2005; Wyckoff et al., 1997) This lack of anguibactin production greatly reduces the background growth in the bioassay plate.

Expression of the truncations was confirmed by western blot with a polyclonal antibody specific to the KVENKQQQQHNIERE (amino acids 28-42) peptide in the N-terminal region of the *V. anguillarum* protein. This epitope is located in the periplasmic region of the protein, prior to the TtpC45 truncation. The bioassay results of the TtpC truncation functional analysis are presented in Table 3-3, panel A. The bioassays show that, while most of the truncations were expressed in the *V. anguillarum* cell, except for the TtpC45 truncation, which was not detected, the entire TtpC protein is essential for proper function of the TonB2 system and growth around all iron sources tested. The western blot for truncation expression is shown in Figure 3-3.

It has been presented in *E. coli* that altered stoichiometry of the proteins in the TonB complex which can occur as a result of plasmid-based over expression of one of the three

Table 3-3 Carboxy-terminal truncations of the *V. anguillarum* TtpC protein cannot complement a Δ*ttpC* mutation.

A. Effect of TtpC truncation on TonB2-mediated iron uptake

V. anguillarum Strain	Ferric Ammonium Citrate	Anguibactin	Enterobactin
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB208	+	-	-
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$	+	+	+
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$*45*	+	-	-
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$*242*	+	-	-
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$*295*	+	-	-
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$*359*	+	-	-
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$*408*	+	-	-

B. Effect of TtpC truncations on wild-type TtpC function with theTonB2 system

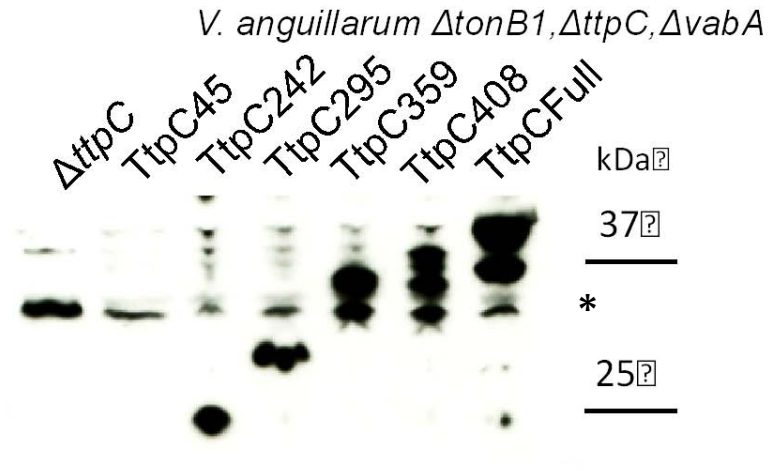
V. anguillarum Strain	Ferric Ammonium Citrate	Anguibactin	Enterobactin
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB208	+	-	-
Δ*tonB1*,Δ*ttpC*, Δ*vabA*/pMMB*ttpC*$_{va}$	+	+	+
Δ*tonB1*,Δ*vabA*/pMMB*ttpC*$_{va}$*45*	+	+	+
Δ*tonB1*,Δ*vabA*/pMMB*ttpC*$_{va}$*242*	+	+	+
Δ*tonB1*,Δ*vabA*/pMMB*ttpC*$_{va}$*295*	+	+	+
Δ*tonB1*,Δ*vabA*/pMMB*ttpC*$_{va}$*359*	+	+	+
Δ*tonB1*,Δ*vabA*/pMMB*ttpC*$_{va}$*408*	+	+	+

Expression of the truncated TtpC proteins does not result in a dominant negative phenotype for iron uptake mediated by the TonB2 system in *V. anguillarum*. + or - indicates growth or lack of growth around the specified iron source. 1µl of each iron source was spotted on the surface of the plates in the following concentrations; Ferric Ammonium Citrate: 10mg/ml; Enterobactin: 1.0mg/ml; Anguibactin: 1.0mg/ml.

Figure 3-3.

Western blot of plasmid-encoded V. anguillarum TtpC truncations expressed in the *V. anguillarum* Δ*tonB1, ΔttpC, ΔvabA* genetic background. The truncation products are identified with the anti TtpCNterm antibody. The TtpC45 truncation was not identified, despite containing the anti TtpCNterm epitope. * indicates a cross-reacting protein present in all samples.

Figure 3-3 Expression of *V. anguillarum* TtpC Carboxy-terminal truncations



αTtpCNterm 1:20,000

Predicted sizes of truncated TtpC proteins:
TtpC45 – 5kDa
TtpC242 – 26.6kDa
TtpC295 – 32.5kDa
TtpC359 – 39.5kDa
TtpC408 – 45 kDa
TtpCfull – 49kDa

proteins, resulted in a dominant negative phenotype, inhibiting transport of ferrichrome (Higgs et al., 1998; Crosa et al., 2004). *In vivo*-formaldehyde crosslinking analysis between the ExbD protein and TonB show that interactions between the transmembrane domains of the proteins are essential for proper conformation of the TonB periplasmic domain and for function. To determine if the TtpC truncations were non functional because they were altering the stoichiometry of the TonB2 system, resulting in a dominant negative phenotype, I expressed them in a *V. anguillarum* ΔvabA, ΔtonB1 background with a wild-type TonB2 system. All of the truncations were expressed in the wild-type TonB2 system background and, as determined by bioassay using iron sources with receptors specific for the TonB2 system, were determined not to inhibit energy transduction of the native TonB2 system. The results from these bioassays are presented in Table 3-3, panel B.

E. TtpC interacts with outer membrane receptors

In *E. coli*, the C-terminal periplasmic domain of the TonB protein directly interacts with the periplasmic loops of the outer membrane ferric-siderophore receptors (Braun, 1995; Devanathan and Postle, 2007; Gudmundsdottir et al., 1989; Koebnik et al., 1993; Lopez et al., 2009). These interactions were shown by multiple methods including *in vivo* formaldehyde crosslinking followed by western blot analysis and HSQC interaction mapping. The Crosa laboratory previously attempted to identify interactions between the *V. anguillarum* TonB2 protein and the periplasmic loops of the ferric-anguibactin receptor, FatA via HSQC.

The Crosa laboratory demonstrated by bioassay and ^{55}Fe-anguibactin uptake assays that the *E. coli* TonB can complement a *V. anguillarum* Δ*tonB2* deletion allowing ferric-anguibactin transport (López and Crosa, 2007). The *E. coli* TonB protein is also capable of energizing the FatA receptor in the absence of both the TonB2 and TtpC proteins as shown by bioassay

analysis in Tables 3-4 and 3-5. This is evidence that the *E. coli* TonB protein is capable of physically interacting with the FatA receptor without the aid of the TtpC protein allowing energy transfer from the PMF to the ferric-anguibactin receptor. Based on this evidence, I may conclude that while the function of the of *V. anguillarum* TonB1 or *E. coli* TonB proteins do not require TtpC, the TtpC protein is required for TonB2 function in *V. anguillarum* (Tables 3-4 and 3-5).

F. Requirement of TtpC dependent on TonB2

By testing several purified siderophores as iron sources in *V. anguillarum* bioassays, I observed that TtpC is necessary for growth surrounding iron sources that utilize only the TonB2 system for transport through the outer membrane. To determine if TtpC was necessary for transport of iron sources that utilize both TonB systems in *V. anguillarum*, I performed bioassays using ferrichrome as an iron source capable of being taken up by both TonB systems and forced transport to occur through the TonB2 system by using *tonB1* deletion mutant strains as shown in Table 3-3. I observed that *V. anguillarum, V. vulnificus* and *V. cholerae* TtpC proteins can complement a Δ*ttpC* mutation in *V. anguillarum* for the uptake of ferrichrome mediated by the TonB2 system.

From these results, I conclude that outer membrane receptors that receive energy from the TonB1 system where TtpC is not required for transport must have a TtpC protein present to receive energy from the TonB2 system (Table 3 -4).

These biological analyses of TonB–FatA interactions were the basis for using the interactions between the carboxy-terminal domain of *E. coli* TonB and periplasmic loop peptides as a positive control in the HSQC peptide interaction analysis. When López *et al*. examined the interactions between the *V. anguillarum* TonB2 carboxy-terminal domain and

Table 3-4 TtpC is necessary for TonB2-mediated iron transport

Strains[c]	Iron sources[b]			
	TonB1- and TonB2-mediated transport		TonB2-mediated transport	
	Ferric ammonium citrate	Ferrichrome	Enterobactin	2,3-DHBA
V. anguillarum				
775/pMMB208	+[a]	+	+	+
775 Δ*tonB1*, Δ*ttpC*, Δ*angA*	+	−	−	−
775 Δ*tonB1*, Δ*ttpC*, Δ*angA*/p*ttpC*va	+	+	+	+
775 Δ*tonB1*, Δ*ttpC*, Δ*angA*/p*ttpC*vc	+	+	−	+
775 Δ*tonB1*, Δ*ttpC*, Δ*angA*/p*ttpC*vv	+	−	−	−

a. + or - indicates growth or lack of growth around the specified iron source.

b. 1µl of each iron source was spotted on the surface of the plates in the following concentrations; Ferric Ammonium Citrate: 10mg/ml; Enterobactin: 1.0mg/ml; 2,3-DHBA: 3.0mg/ml, Ferrichrome: 1.0mg/ml

c. Mutations in chromosomally-encoded genes were complemented by genes expressed from the low copy-number expression vector pMMB208. Expression was induced with 1mM IPTG.

Table 3-5 *E. coli* TonB does not require TtpC to mediate transport of ferric-anguibactin in *V. anguillarum*

Strains[c]	Iron sources[b]		
	Ferric ammonium citrate	Enterobactin	Anguibactin
V. anguillarum			
775/pMMB208	+[a]	+	+
CSL68: Δ*tonB1*, Δ*tonB2*/pMMB208	+	−	−
CSL64: Δ*tonB1*, Δ*tonB2*/p*ton8*$_{E. coli}$	+	−	+
775 Δ*ttpC*/pMMB208	+	−	−
775 Δ*tonB1*, Δ*tonB2*, Δ*ttpC*/pMMB208	+	−	−
775 Δ*tonB1*, Δ*tonB2*, Δ*ttpC*/p*tonB*$_{E. coli}$	+	−	+

a. + or - indicates growth or lack of growth around the specified iron source.

b. 1µl of each iron source was spotted on the surface of the plates in the following concentrations; Ferric Ammonium Citrate: 10mg/ml; Enterobactin: 1.0mg/ml; 2,3-DHBA: 3.0mg/ml, Ferrichrome: 1.0mg/ml

c. Mutations in chromosomally-encoded genes were complemented by genes expressed from the low copy-number expression vector pMMB208. Expression was induced with 1mM IPTG.

the periplasmic loop peptides of FatA, no interactions were detected (Lopez et al., 2009). This lack of interactions led me to propose that the TtpC protein, or perhaps a combination of the TtpC protein and the TonB2 protein, are interacting with and allowing energy transfer to the outer membrane receptors.

Because the TtpC protein is known not to be functional with only the periplasmic domain expressed, using the purified amino-terminal periplasmic domain for interaction studies with FatA peptides by HSQC analysis was not an option for determining if the TtpC protein interacts with the outer membrane receptors. Instead, I used *in vivo* formaldehyde cross-linking followed by western blot analysis with antibodies specific for TtpC and the FatA protein to identify cross-linked protein complexes that contained both proteins. I performed this analysis using log-phase liquid cultures from three strains of *V. anguillarum*: wild-type strain 775, a Δ*ttpC* chromosomal deletion mutant, and a strain with a chromosomally encoded C-terminal 6XHis tag on the TtpC protein. The western blot analysis of the cross-linked proteins is shown in Figure 3-4. FatA is identified with a polyclonal antibody to the protein in the top panel (A) and TtpC is identified with the polyclonal TolR antibody in the bottom panel (B). For a comparison, two independent cross-linking experiments identifying the complexes that contain the TtpC protein in are shown in Figure 3-5 with formaldehyde cross-linked complexes identified on the right and dithiobis [succinimidyl propionate] (DSP) cross-linked complexes identified on the right.

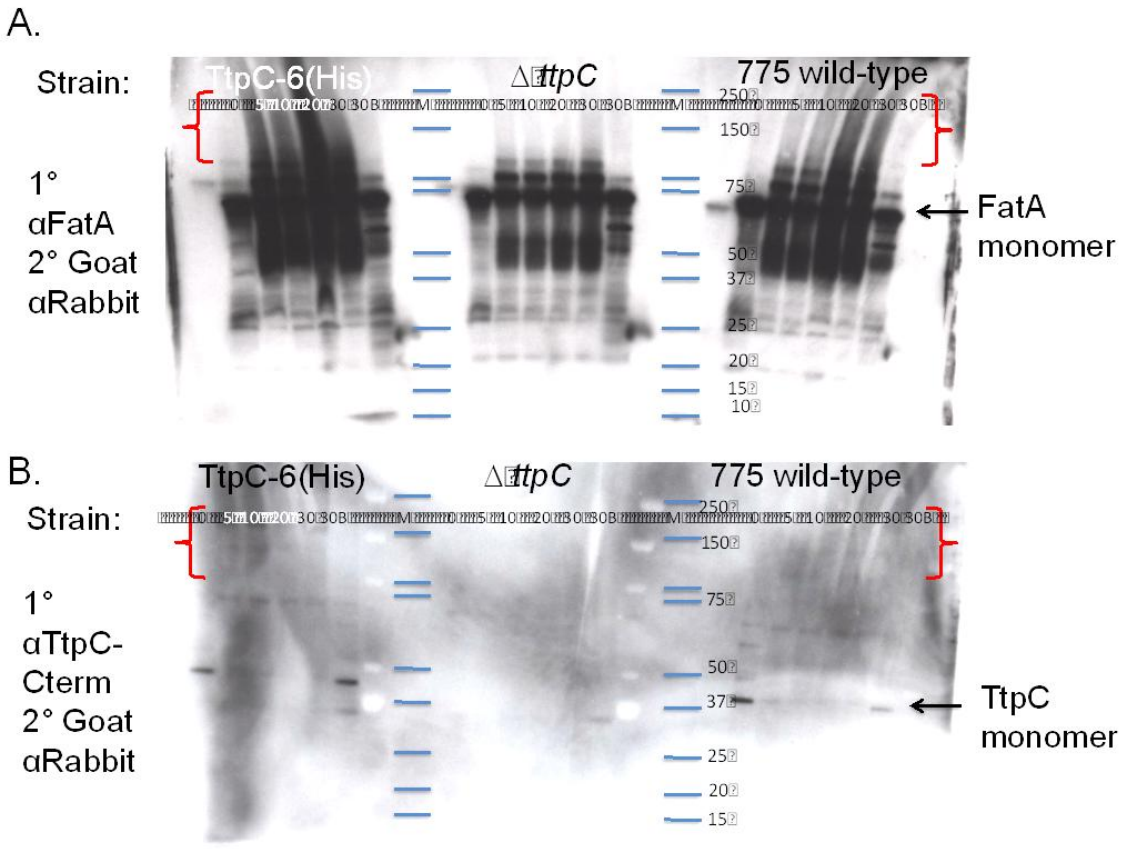
Of note in Figure 3-4, several high molecular weight cross-linked complexes containing FatA are absent in the Δ*ttpC* strain indicating that TtpC is necessary for these complexes to form and that TtpC may be part of those complexes. The bottom panel faintly shows several high molecular weight complexes that contain the TtpC protein. Compared to the molecular

Figure 3-4

Western blot analysis of *in-vivo* formaldehyde crosslinked TtpC-FatA complexes in *V. anguillarum.* A. Western blot detecting FatA-containing protein complexes present in the *V. anguillarum* TtpC-6His strain on the left, the *V. anguillarum* Δ*ttpC* chromosomal deletion strain in the center and the wild-type *V. anguillarum* strain 775 on the right. B. Western blot detecting V. anguillarum TtpC. Samples are identical to those in panel A.

Molecular weights of the Kaleidoscope Precision Plus protein standard are indicated with blue lines in lanes marked M. Length of exposure to 1% formaldehyde is noted in minutes at the top of each lane. 30B indicates that the sample was boiled for 5 minutes after cross-linking to remove cross-links.

Figure 3-4 *V. anguillarum* TtpC and FatA proteins co-migrate in formaldehyde-linked complexes



weight standard, it is apparent that these complexes, designated with red brackets, are the same as the high molecular weight FatA-containing complexes. Since the molecular weight of the overlapping complexes is greater than the expected molecular weight of one TtpC protein and one FatA protein ~ 120 kDa, it is likely that other proteins are associated with the FatA-TtpC complexes. This interpretation is supported by the evidence from previous *in-vivo* formaldehyde cross-linking studies, which show that TtpC becomes very unstable and that several TtpC-containing complexes are undetectable in the absence of the TonB2 protein (Stork, 2007). Based on the western blots in Figure 3-4 and Figure 3-5, it is not known if the TonB2 protein is also present in these complexes or if it is directly interacting with FatA.

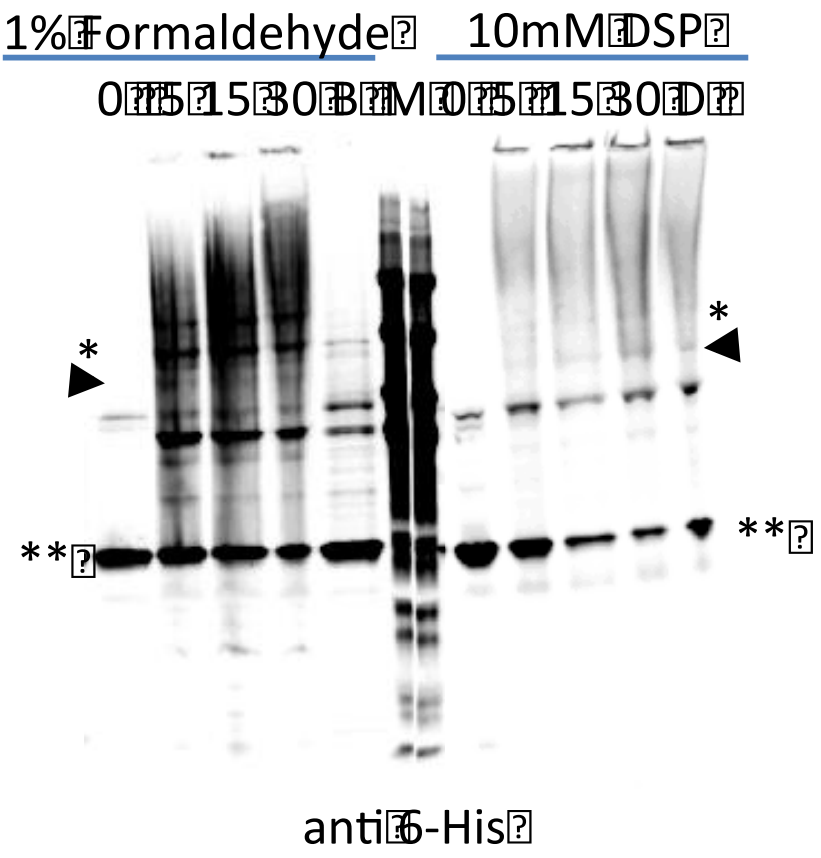
G. Screen for enterobactin uptake gain of function mutations in *V. cholerae* TtpC expressed in *V. anguillarum*.

The *V. anguillarum* and *V. cholerae* proteins are 66% similar at the amino acid level. To determine which amino acids in the *V. cholerae* TtpC prevent it from energizing the FetA enterobactin receptor in *V. anguillarum*, I used growth on enterobactin as the sole iron source to positively select for spontaneous point mutations in *V. cholerae* TtpC that resulted in a gain of function mutation for enterobactin transport. The *V. anguillarum* Δ*tonB1*, Δ*ttpC*, Δ*vabA* strain was complemented with *V. cholerae ttpC* expressed from the pMMB208 vector with IPTG induction. This strain was not able to grow around enterobactin as its sole iron source, therefore growth on enterobactin-supplemented minimal media acted as a very strong selective pressure to isolate gain-of-function mutations that allowed transport of Fe-enterobactin and growth on the iron-chelated media.

Figure 3-5.

V. anguillarum strain TtpC 6-His cross-linked with either 1% formaldehyde or 10mM Dithiobis [succinimidyl propionate] for 0 to 30 minutes. “B” indicates that the 30 minute cross-linked sample was boiled for 5 minutes to remove cross-links. “D” indicates that cross-links were reversed by addition of Dithiothreitol prior to SDS-PAGE separation to remove cross-links in the 30 minute DSP cross-linked sample. ** indicates the TtpC-6His monomer. ◀Indicates a conserved ~125kDa cross-linked complex, and * indicates a ~150kDa cross-linked complex.

Figure 3-5 Similar protein complexes are identified with multiple chemical cross-linkers



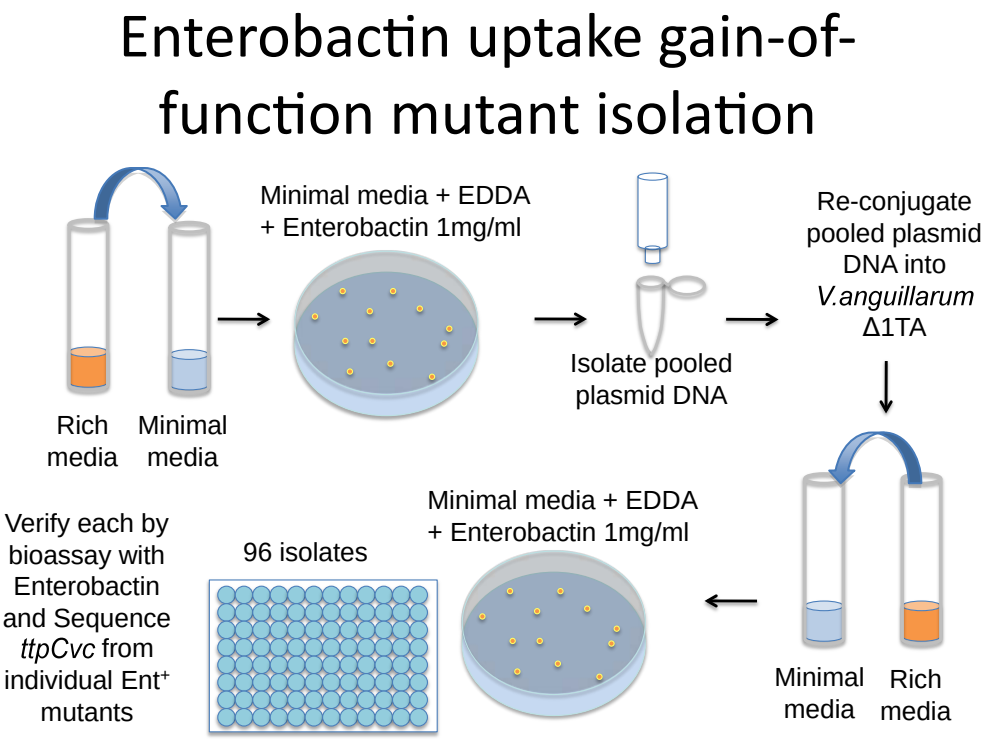
After the initial selection for gain-of-function mutants, I isolated and pooled the plasmid DNA from the *V. anguillarum* colonies. I transformed this plasmid DNA back into *E. coli* S17-λpir, then conjugated the pMMB208-V*cttpC-ent+* mutants *en masse* into the *V. anguillarum* Δ*tonB1*, Δ*ttpC*, Δ*vabA* recipient strain again. I selected for ex-conjugants that possessed a gain of function mutation for enterobactin uptake by once again selecting on iron-chelated media with enterobactin as the sole iron source. I reasoned that this second selection would isolate the plasmid-encoded *V. cholerae ttpC* mutants that contained a mutation rather then strains that had a mutation somewhere in the *V. anguillarum* genome. A cartoon of the selection process for the enterobactin gain-of-function mutations can be seen in Figure 3-6.

After the second selection, I grew and froze 96 isolates for further verification of the positive enterobactin uptake phenotype. I tested the strains for growth around enterobactin, anguibactin, and ferric ammonium citrate on bioassay plates. Each of the isolates grew around all of the iron sources tested. To determine the mutation that allowed for the growth around enterobactin, I isolated the plasmid DNA and sequenced the *V. cholerae ttpC* encoded on the pMMB208 vector (Morales et al., 1991). To my surprise, none of the 35 isolates that were sequenced contained a mutation in the *V. cholerae ttpC*. It appears that the use of enterobactin as the sole iron source was a very good selective pressure for isolating gain-of-function mutations, but, the mutations were located in other proteins, such as FetA or TonB2, rather than the plasmid-encoded *V. cholerae ttpC*. The use of growing bacteria in iron-chelated media with a single iron source to select for gain-of-function mutations in the *V. cholerae* outer membrane receptor for heme was successfully used in the past by Wyckoff *et al.* (Wyckoff et al., 2004). There are several proteins involved

Figure 3-6.

Diagram of enterobactin uptake gain-of-function mutant isolation scheme. The mutants were double selected with growth on enterobactin as the sole iron source. Mutations were then identified by sequencing the *ttpCvc* gene carried on the pMMB208 plasmid.

Figure 3-6 Procedure for isolating *V. cholerae* TtpC enterobactin uptake gain of function mutants



in the transport of enterobactin in *V. anguillarum* that could have been mutated to allow for enterobactin uptake, such as the FetA enterobactin receptor or any member of the TonB2 energy transduction system. These proteins are a logical substitute for mutations in the *V. cholerae* TtpC protein because it has been shown in the *E. coli* TonB system that TonB interacts with the periplasmic loops of the outer-membrane ferric-siderophore transport proteins (Braun, 1995; Devanathan and Postle, 2007; Gudmundsdottir et al., 1989; Koebnik et al., 1993; Lopez et al., 2009) and that trans-membrane domain interactions with both ExbB and ExbD are essential to maintain the conformation of the TonB periplasmic domain (Ollis et al., 2009).

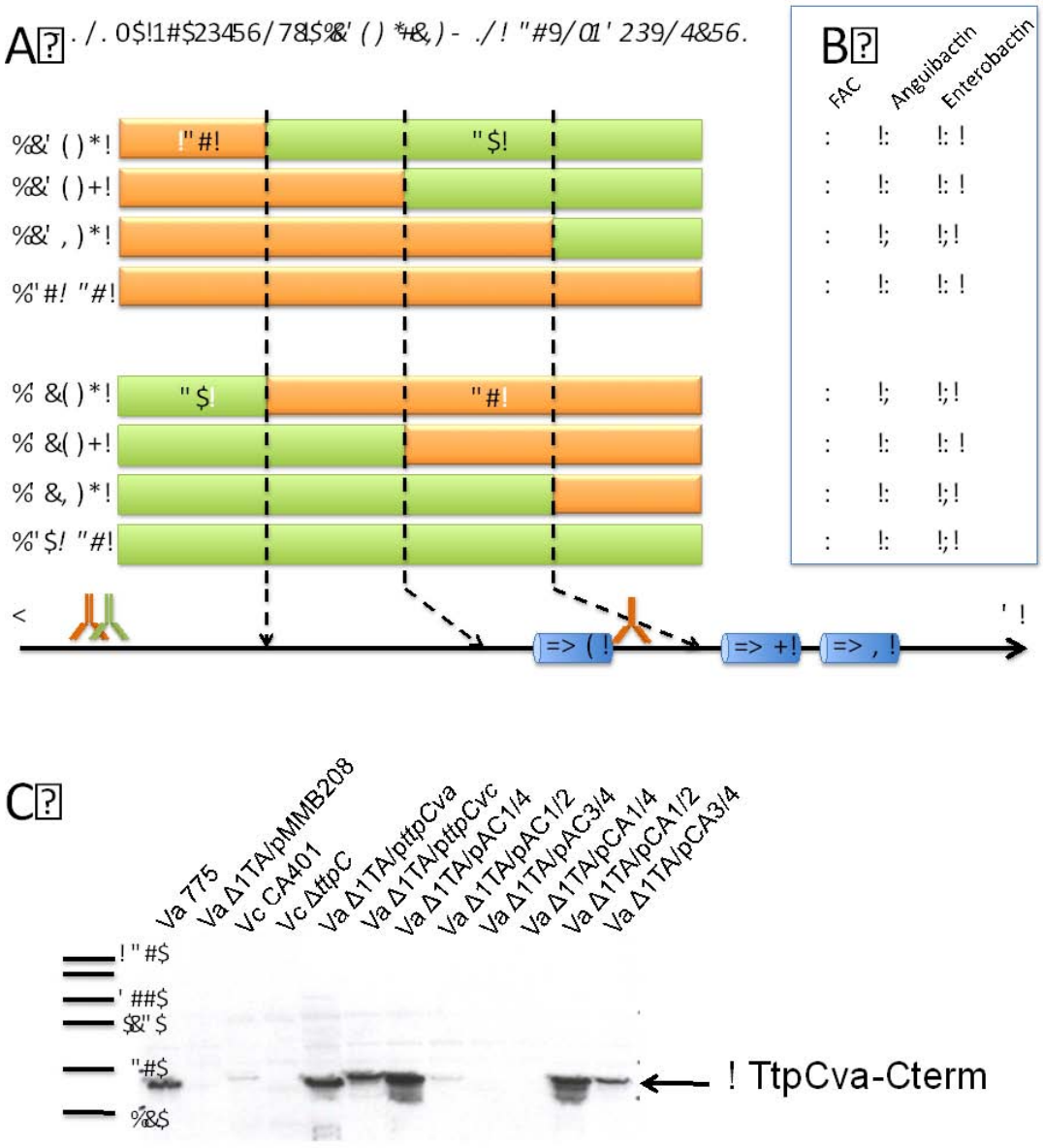
H. *V. anguillarum* – *V. cholerae* chimeric TtpC proteins.

In another approach to determine the region of the *V. anguillarum* TtpC protein that allows for energy transduction to the *V. anguillarum* FetA enterobactin receptor, as compared to the *V. cholerae* TtpC protein which does not, I constructed a series of chimeric TtpC proteins. The chimeric proteins were expressed from pMMB208 in *V. anguillarum* Δ*tonB1*, Δ*ttpC*, Δ*vabA* to assay for growth of the strain around enterobactin as an iron source. The chimeric *ttpC* genes were constructed using splicing by overlap extension (SOE) polymerase chain reaction (PCR) (Horton et al., 1989). A diagram of the different chimeric TtpC proteins and the locations that various polyclonal antibodies bind to the full-length protein for detection by western blot is presented in Figure 3-7, panel A. The genes were split into quarters to create six chimeric TtpC proteins, which were compared to the function of the two full-length wild-type proteins for growth around anguibactin, enterobactin, and ferric ammonium citrate (FAC) in bioassays. The results of the bioassays are presented in Figure 3-7, panel B and a western blot confirming the expression of the chimeras with the C-terminal end *V. anguillarum* TtpC-specific polyclonal antibody (Lampire) in panel C. All of

Figure 3-7.

A. Schematic diagram of the *V. anguillarum* and *V. cholerae* ttpC chimeric genes. Dotted lines indicate the relative position of the three joint locations in the full-length TtpC protein indicated at the bottom of the figure. Orange antibody cartoons indicate *V. anguillarum* anti-TtpC N and C-term antibody binding regions. Green antibody cartoon indicates *V. cholerae* anti TtpC antibody binding region. B. Bioassay results for each of the chimeric TtpC proteins expressed in the V. anguillarum Δ*tonB1*, Δ*ttpC*, Δ*vabA* genetic background. + or – indicates growth or lack of growth around the indicated iron source. FAC 10mg/ml, enterobactin 1.0mg/ml, anguibactin 1.0mg/ml. C. Western blot of the total membrane fraction of each of the assayed strains in panel B. Wild type and chimeric TtpC proteins were detected with the anti-*V. anguillarum* TtpC Cterm antibody which cross-reacts with the *V. cholerae* TtpC C-terminal region.

Figure 3-7 *V. anguillarum : V. cholerae* chimeric TttpC proteins



the chimeric proteins that expressed in the *V. anguillarum* cell were able to complement the Δ*ttpC* mutation for growth around anguibactin and all but one of the chimeric proteins that expressed was capable of complementing growth around enterobactin. The chimera that could not support growth around enterobactin as an iron source was composed of the N-terminal ¾ of the *V. cholerae* TtpC protein joined to the C-terminal ¼ of the *V. anguillarum* protein.

Intriguingly, both ½ and ½ chimeras, the AC1/2 with the N-terminal portion of *V. anguillarum* TtpC and the C-terminal portion of *V. cholerae* TtpC, and the CA1/2 with the N-terminal portion of *V. cholerae* TtpC and the C-terminal portion of *V. anguillarum* TtpC, were capable of energizing the transport of anguibactin and enterobactin. The joint between the two regions of the protein in the ½ and ½ chimeras is located 5' to the region that encodes for the first trans-membrane domain. The chimera that was incapable of energizing transport of enterobactin, but was functional for the transport of anguibactin, was the CA3/4 with the N-terminal ¾ of *V. cholerae* and the C-terminal ¼ of *V. anguillarum*. The joint between the two regions in this chimera lies in between the region encoding trans-membrane domain 1 and trans-membrane domain 2. The opposite chimera, AC3/4 was constructed three times and moved into the *V. anguillarum* Δ*tonB1*, Δ*ttpC*, Δ*vabA* background but was never expressed in the *V. anguillarum* cell.

I cannot draw any decisive conclusions about which region(s) of the TtpC protein are essential for allowing enterobactin transport in *V. anguillarum* based on the results from the chimera bioassays, except that the three transmembrane domains must be from the same species to allow for enterobactin uptake. This observation could be related to the importance of the HxxxS motif in the TtpC transmembrane domain 3 and its essentiality for

energy transduction to the outer membrane receptors. I hypothesize that the three trans-membrane domains are essential for proper conformation and function of the TtpC protein, and that the three transmembrane domains from *V. anguillarum* allow the TonB2 system to be more efficient than the three transmembrane domains of the *V. cholerae* TtpC at harnessing the energy of the PMF in conjunction with the *V. anguillarum* TonB2 system proteins.

I. The TonB3 systems of pathogenic *Vibrios*.

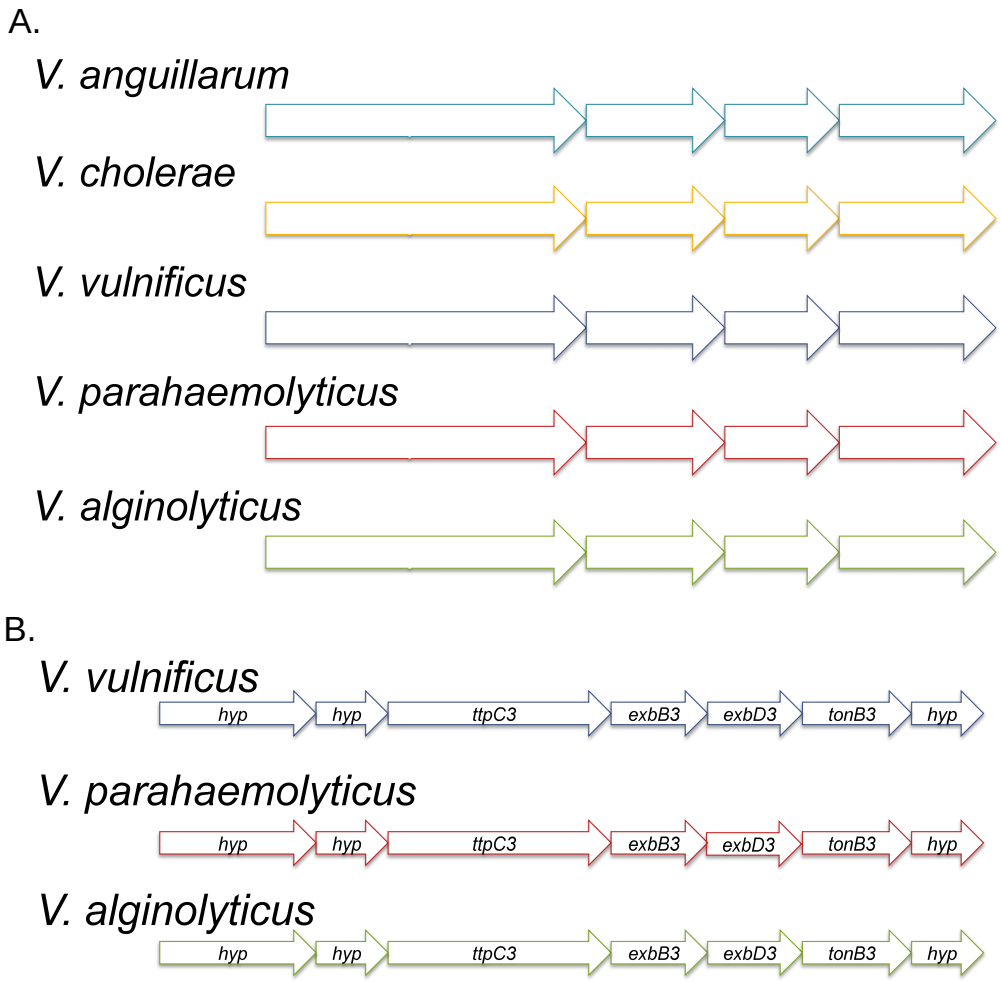
As shown in Table 1-1 (TonB-TtpC containing bacteria), several pathogenic *vibrio* species possess three TonB systems, including the human pathogens *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*. The TonB1 systems in these pathogens are similar to the TonB1 systems in *V. anguillarum* and *V. cholerae*. They are composed of the ExbB1, ExbD1 and TonB1 proteins and transduce energy to outer membrane ferric-siderophore receptors. The TonB2 systems are also similar in that they are composed of TtpC2, ExbB2, ExbD2 and TonB2 proteins and energize a distinct subset of the outer membrane receptors (Alice et al., 2008; Kuehl and Crosa, 2010; Kustusch et al., 2011; Seliger et al., 2001; Stork et al., 2004; Wang et al., 2008). Unlike *V. anguillarum* and *V. cholerae*, these three species also encode a third TonB system, which looks very much like the TtpC-TonB2 systems, containing a TtpC3 protein followed by an ExbB3, ExbD3 and TonB3. The gene arrangement of the three TonB systems is presented in Figure 3-8.

The TtpC3 proteins are similar to the TtpC2 proteins in sequence, with 55% similarity (Altschul et al., 1997) and in their predicted membrane topography, with a large periplasmic domain followed by three transmembrane domains with the carboxy-terminus of the protein predicted to be in the cytoplasm (Cserzö et al., 1997). It was shown in *V.*

Figure 3-8.

Schematic diagram of the genes in the TonB2 operon A of five pathogenic *vibrio* species. Similar gene arrangement is observed in the TonB3 operon B found in a subset of the pathogenic *vibrio* species. *Hyp* refers to an annotated hypothetical protein.

Figure 3-8 Three TonB systems in pathogenic *vibrios*



vulnificus that expression of the TonB3 operon is only induced under growth in human serum and is not regulated by iron concentration, implying that the TonB3 system of *V. vulnificus* is responsible for energy transduction to a completely different subset of outer membrane transporters (Alice et al., 2008). Although the expression of the TonB2 and TonB3 systems is regulated by different environmental conditions, the proteins themselves are similar enough that I hypothesized that the TonB3 system proteins would be able to complement corresponding deletions in the TonB2 system. To test this hypothesis, I cloned the *V. vulnificus* TtpC3 protein into the pMMB208 vector and expressed it in the *V. anguillarum* Δ*tonb1, ttpC, vabA* genetic background. I assayed the strain for growth around several iron sources to see if the TtpC3 could complement the *V. anguillarum* TtpC2 mutation as the *V. vulnificus* TtpC2 could. The bioassay results of this strain are presented in Table 3-3 and show that despite the *V. vulnificus* TtpC2 protein being able to fully complement the *V. anguillarum* Δ*ttpC* mutation, the TtpC3 cannot complement the TonB2 system to allow growth around any of the iron sources tested. There are several possible explanations for this lack of function. While TtpC2 and TtpC3 proteins are similar and the other proteins in the TonB2 system are similar to those of the TonB3 system, it is likely that one or many sequence-specific interactions need to occur to allow proper energy transduction to the outer membrane transporters. Despite the observation that all of the *vibrio* TonB systems studied to-date transduce energy to more than one outer membrane receptor, the *V. vulnificus* TtpC3 appears to be non-promiscuous in its interactions, and specific for interactions with outer membrane proteins other than those involved in iron transport in *V. anguillarum*.

J. Lack of anguibactin uptake or lack of anguibactin production?

When 2,3-DHBA is spotted on the surface of the *V. anguillarum* bioassay plates as an iron source, the Δ*ttpC*, Δ*angA* mutant strains complemented with either *V. anguillarum* or *V. cholerae ttpC* are able to grow, but when 2,3-DHBA is supplied to the Δ*ttpC*, Δ*angA* mutant strain complemented with the *V. vulnificus ttpC3*, no growth is observed (Table 3-2). Because of this observation, I asked the question: Is the lack of growth seen in the mutant complemented with *V. vulnificus ttpC3* due to a lack of iron transport mediated by the TonB2 system or is it due to that particular strain not being able to produce anguibactin from the 2,3-DHBA? To answer this question, I used the anguibactin-indicator strains *V. anguillarum* CC9-8 (Walter et al., 1983), which cannot produce or transport anguibactin and *V. anguillarum* CC9-16 which cannot produce anguibactin but is proficient in transport. 3 µl of overnight, IPTG-induced culture of the complemented Δ*ttpC* , Δ*vabA* mutants grown in CM9 minimal media were used as the iron sources in the bioassay (Table 3-6). When 2,3-DHBA was added to the bioassay plate, CC9-16 (Walter et al., 1983), was able to grow around all of the supernatants tested indicating that all strains were producing anguibactin when grown on 2,3-DHBA.

K. Complicated specificities of the TonB2 system protein interactions.

The genetic complementations of the ΔttpC mutation in *V. anguillarum* show that the *V. vulnificus* TtpC3 is unable to complement the Δ*ttpC* mutant to restore growth around the TonB2-specific iron sources despite the conservation of basic protein domains and features. These results illuminate a potential mechanism by which TtpC could be working. Previous data from M. Stork shows that protein–protein interactions occur between TtpC and TonB2 in *V. anguillarum* (Stork et al., 2007). Based on these observations, we postulate that the interactions between the TonB2 protein and the *V. vulnificus* TtpC3 in the *V. anguillarum*

Table 3-6 Growth around Anguibactin-producing *V. anguillarum* strains

Growth around anguibactin-producing *V. anguillarum* strains

Indicator strains	Anguibactin-producing strains[b]					
	775 Wild type	Δ*ttpC*	Δ*ttpC*, Δ*angA*/ *pttpC*$_{va}$	Δ*ttpC*, Δ*angA*/ *pttpC*$_{vc}$	Δ*ttpC*, Δ*angA*/ *pttpC*$_{vv}$	Ferric ammonium citrate
CC9-8	–[a]	–	–	–	–	+
CC9-16	+	+	–	–	–	+
CC9-8 + DHBA	–	–	–	–	–	+
CC9-16 + DHBA	+	+	+	+	+	+

a. + or - indicates growth or lack of growth around the specified iron source.

b. 3µl of each anguibactin-producing strain was spotted on the surface the plate

Δ*tonB1,* Δ*ttpC* , Δ*angA* mutant strain are either not occurring all together or a necessary interaction between the two proteins, or possibly, with the accessory proteins ExbB2 and ExbD2, is unstable and does not allow for energy transduction by the TonB2 system. Based on the results from Tables 3-2 , 3-4, and 3-5 , as well as figure 3-4, the TtpC protein may be essential for interactions between the TonB2 system and the outer-membrane receptor. This theory is supported by the observation that the *V. cholerae* TtpC is able to complement growth around anguibactin and ferrichrome as iron sources, but is unable to complement growth around vanchrobactin or enterobactin as iron sources. This specificity leads me to conclude that these potential interactions are very specific due to the high similarity between the *V. anguillarum* and *V. cholerae* TtpC protein sequences.

Strains Used in Chapter 3

Strains	Genotype	Plasmid	Reference
V. anguillarum 775			Crosa, JH 1980
V. anguillarum 775		pMMB208	Lopez et al 2008
	ΔttpC		Kuehl et al 2009
	ΔttpC, ΔvabA	pMMB208	Kuehl et al 2009
	ΔttpC, ΔvabA	pMMBVa*ttpC*	Kuehl et al 2009
	ΔttpC, ΔvabA	pMMBVc*ttpC*	Kuehl et al 2009
	ΔttpC, ΔvabA	pMMBVv*ttpC*	Kuehl et al 2009
	ΔttpC, ΔvabA	pMMB*ttpC45*	This Work
	ΔttpC, ΔvabA	*pMMBttpC242*	This Work
	ΔttpC, ΔvabA	pMMB*ttpC295*	This Work
	ΔttpC, ΔvabA	pMMB*ttpC359*	This Work
	ΔttpC, ΔvabA	pMMB*ttpC408*	This Work
	ΔtonB1, ΔttpC	pACYC-Va*exbB2,exbD2,tonB2*	Stork, et al 2007
	ΔtonB1, ΔttpC	pACYC-Va*ttpC,exbB2,exbD2,tonB3*	Stork, et al 2007
	ΔtonB1, ΔttpC		Kuehl et al 2009
	ΔtonB1, ΔttpC, ΔvabA		Kuehl et al 2009
	ΔtonB1, ΔttpC, ΔvabA	pMMB208	Kuehl et al 2009
	ΔtonB1, ΔttpC, ΔvabA	pMMBVa*ttpC*	Kuehl et al 2009
	ΔtonB1, ΔttpC, ΔvabA	pMMBVc*ttpC*	Kuehl et al 2009
	ΔtonB1, ΔttpC, ΔvabA	pMMBVv*ttpC*	Kuehl et al 2009
	ΔtonB1, ΔttpC, ΔvabA	pMMB*ttpC45*	This Work
	ΔtonB1, ΔttpC, ΔvabA	*pMMBttpC242*	This Work
	ΔtonB1, ΔttpC, ΔvabA	pMMB*ttpC295*	This Work
	ΔtonB1, ΔttpC, ΔvabA	pMMB*ttpC359*	This Work
	ΔtonB1, ΔttpC, ΔvabA	pMMB*ttpC408*	This Work
V. anguillarum CSL68	*ΔtonB1,ΔtonB2*	pMMB208	Lopez et al 2008
V. anguillarum CSL64	*ΔtonB1,ΔtonB2*	pMMB*EcolitonB*	Lopez et al 2008
	ΔtonB1,ΔtonB2, ΔttpC	pMMB208	Kuehl et al 2009
	ΔtonB1,ΔtonB2, ΔttpC	pMMB*EcolitonB*	Kuehl et al 2009
V. anguillarum CC9-8			Walter et al 1983
V. anguillarum CC9-16			Walter et al 1983
V. cholerae CA401			Gardner et al 1964
V. cholerae DOV221	*exbB2:Tn10*		Occhino et al 1998
	exbB2:Tn10	pACYC-Va*exbB2,exbD2,tonB2*	Stork, et al 2007
	exbB2:Tn10	pACYC-Va*ttpC,exbB2,exbD2,tonB3*	Stork, et al 2007
V. cholerae CA401	*ΔttpC*		This Work

Chapter 4. Discussion

While much is known about the TonB system of *E. coli*, which is made up of the three proteins, TonB, ExbB and ExbD, the knowledge about the mechanics of the system can only be applied loosely to the *Vibrio* TonB2 system. With the fourth essential protein, TtpC, the *vibrio* TonB2 systems have a more complicated distribution of functions to achieve energy transduction from the inner membrane PMF to the outer membrane receptors than the *Vibrio* TonB1 systems of the *E. coli* TonB systems do.

Why do the *vibrio* species possess multiple TonB systems?

The *vibrio* species are found in marine environments as well as infecting animal and human hosts. In order to survive in such varied conditions, each species must be able to utilize a variety of iron sources under a variety of environmental conditions. The laboratory of Shelley Payne first studied the two TonB systems of *V. cholerae* (Seliger et al., 2001). In their analysis of iron source usage under environmental and infection conditions, they observed that some outer membrane receptors were specific for either TonB1 or TonB2. They also noted that the TonB1 protein is longer than TonB2 in *V. cholerae* and that TonB1, but not TonB2 can interact with the heme receptor. Seliger *et al.* (Seliger et al., 2001) hypothesized that this may be due to the heme receptor being induced in higher salinity, which causes the cytoplasm to shrink and therefore, increases the distance between the inner and outer membrane (Stock et al., 1977b). This distance may be increased too much for the TonB2 protein to span the outer membrane to interact with the heme receptor. Work by M. Stork also brought up the idea that the TonB1 is longer than the TonB2 in the fish pathogen, *V.*

anguillarum and this may explain the need for a fourth accessory protein to span the distance of the periplasmic space (Stork et al., 2007).

In Figure 2-4, I presented topological analysis of the *V. anguillarum* TtpC protein showing that the N-terminal half of the protein, around 220 amino acids, is located in the periplasm. Although the structure of this periplasmic domain is not known, it is equal in size to the entire TonB2 protein (206 amino acids), whereas the TonB1 protein is 268 amino acids (Naka et al., 2011). The TtpC protein cannot take the place of the TonB2 protein though, as all four proteins in the TonB2 energy transduction system are essential for iron transport in *V. anguillarum* and *V. cholerae* (Stork et al., 2007).

In the *Vibrio* TonB2 systems, it may be that the N-terminal periplasmic domain of the TtpC protein or TonB2 protein and the TtpC together interact with the outer membrane receptor proteins. López et al (Lopez et al., 2009) were unable to detect interactions between the purified periplasmic domain of *V. anguillarum* TonB2 and peptides from the outer membrane anguibactin receptor, FatA. This result may have been due to the absence of the TtpC periplasmic domain in those experiments. One role of TtpC may be to influence the conformation of TonB2. The lack of TtpC as well as ExbB2 and ExbD2 in this experiment could have resulted in the TonB2 periplasmic region being in the wrong conformation to interact with the peptides from the outer membrane receptor protein. Another possibility is that the TtpC protein itself is the link between the TonB2 system and the outer membrane transport proteins. This possibility is supported by Figure 3-4 showing that the *V. anguillarum* FatA protein and the TtpC protein co-migrate in formaldehyde cross-linked complexes but could be elucidated further by using an antibody to identify the TonB2 protein in the samples. While I have a polyclonal antibody against *V. anguillarum* TonB2, it

still cross-reacts with too many proteins to be used for cross-linking analysis (Stork et al., 2007). Further affinity purification of the antibody or creating a chromosomally encoded epitope tag on the TonB2 protein would aid in this proposed analysis.

Additional support for the role of the TtpC protein as the energy-transducing protein to the outer membrane transporters is the demonstration of receptor specificity in the *V. cholerae* and *V. vulnificus* TtpC cross-species complementation presented in Chapter 3. While the *V. vulnificus* TtpC protein could function with the other members of the *V. anguillarum* TonB2 system for transport of all iron sources tested, the *V. cholerae* TtpC protein could not complement the lack of enterobactin transport in the *V. anguillarum* Δ*ttpC* strain.

It is clear from the bioassay results presented in Chapter 3, Table 3-4 that the requirement for the TtpC protein in TonB2-system energy transduction is not a requirement of the outer-membrane receptor, but of the TonB2 system. The *V. anguillarum* TonB1 system, which has no TtpC protein associated with it, is capable of energizing the ferrichrome receptor in the absence of TtpC. Growth around ferrichrome is not observed however when the TonB2 system is used exclusively (in a *tonB1*$^{-}$ genetic background) when the TtpC protein is absent.

In this dissertation, I have presented the results of several experiments that have expanded our knowledge about the TtpC protein and its role in the TonB2 energy-transduction systems of the *vibrio* species. I have shown that the TtpC protein interacts with outer membrane ferric-siderophore receptors and that TtpC, rather than TonB2 harbors an essential HxxxS motif. The conserved histidine residue in the HxxxS motif is essential for Fe-ferrichrome transport in *V. cholerae* (Fig 2-6). Another observation that highlights the role of TtpC as the energy-transducing protein of the *vibrio* TonB2 systems is that the HxxxS

motif is found in the energy-transducing proteins of similar systems, for example the TonB and TolA proteins of their respective systems (Germon et al., 2001; Larsen et al., 2007; Larsen and Postle, 2001; Larsen et al., 1994).

HxxxS motif is essential to allow PMF-induced conformational changes in TolA, TonB and TtpC.

Swayne, C *et al* have shown that this conserved histidine residue is not involved in proton shuttling by the *E. coli* TonB system because it could be replaced by a non-protonatable asparagine residue and continue to function to transduce energy to the ferrichrome receptor in the outer membrane (Swayne and Postle, 2011). The *E. coli* TonB SxxxH motif may not be involved in harnessing the PMF, but it does appear to be involved in transferring the energy of the PMF to the conformational change in the periplasmic region of the TonB protein.

K. Postle observed that formaldehyde cross-links between *E. coli* ExbD and TonB are dependent on the PMF (Ollis et al., 2009). Similarly, *E. coli* TolA changes conformations in response to the PMF, but the same conformational changes are not present in the absence of either accessory protein TolQ or TolR, which may be playing a similar role to ExbD in harnessing the PMF (Germon et al., 2001; Goemaere et al., 2007).

Recently, Ollis *et al.* have elucidated the early steps of *E. coli* TonB energy transduction via PMF-induced conformational changes of the TonB protein and of the ExbD protein (Ollis et al., 2009). These conformational changes in TonB do not occur in the absence of ExbD or in strains where key residues in the trans-membrane domain and in the periplasmic region of the ExbD protein are mutated which prevent ExbD from sensing the PMF or interacting with

the periplasmic domain of TonB (Ollis and Postle, 2011). This observation of periplasmic residues in ExbD being necessary for stabilizing and conveying the energy from the PMF to TonB is consistent with and reinforces my observations that C-terminal truncations of the *V. anguillarum* TtpC protein are non-functional, most likely because the truncated proteins are incapable of receiving energy from the PMF via ExbD2. The non-functional *V. anguillarum* ΔNterm TtpC protein, which retained the native secretion signal sequence but lacked the periplasmic region was not expressed in the membrane fraction. This lack of expression was possibly due to extreme instability of this truncated protein despite maintaining the integrity of the HxxxS motif in the third trans-membrane domain.

V. vulnificus has three TonB systems. In the TtpC switching experiment presented in Chapter 3, I tested the ability of the *V. vulnificus* TtpC3 to complement the Δ*ttpC* mutation in *V. anguillarum*. The TtpC3vv was non-functional in the *V. anguillarum* TonB2 system despite having 55% similarity to the *V. anguillarum* TtpC protein (Altschul et al., 1997). Upon further sequence analysis of the TonB3 systems, I discovered that the HxxxS motif essential to the function of *E. coli* TonB and *V. cholerae* TtpC, is not present in the *vibrio* TtpC3 proteins. In the TonB3 systems of *V. vulnificus*, *V. alginolyticu*s, and *V. parahaemolyticus*, the HxxxS motif is located in the predicted transmembrane domain of the TonB3 protein. I propose that the lack of this motif in the TtpC3vv-complemented *V. anguillarum* TonB2 system was the reason for the lack of iron transport because while the PMF may have been harvested by the ExbD2 and ExbB2 proteins, there was no HxxxS motif to transfer the PMF energy to the TtpC protein (Ollis and Postle, 2011). The lack of energy transfer resulted in no conformational change in the periplasmic domains and no transfer of energy to the outer membrane transporters.

The TtpC periplasmic domain is essential for recognition and energy transfer to the outer membrane receptors by the TonB2 system.

Based on the bioassay data from the *V. anguillarum: V. cholerae* chimeric TtpC experiments (Figure 3-7), the three transmembrane domains from *V. ch*olerae TtpC can still function with the other proteins of the *V. anguillarum* TonB2 system to harness the energy of the PMF. One explanation for the ability of the AC 1/2 chimera to grow around enterobactin as its sole iron source may be that the N-terminal region of the *V. anguillarum* TtpC, either by itself or in conjunction with the TonB2 protein, has a more stable interaction with the periplasmic domain of the FetA enterobactin receptor than does the N-terminal region of the *V. cholerae* TtpC protein (Balado et al., 2009).

One way to test for a difference in efficiency of energy transduction of the two chimeric TtpC proteins would be to determine the rate of Fe-enterobactin transport in the two strains using ^{55}Fe-labeled enterobactin. This experiment however, has been very difficult to perform using *V. anguillarum*. Surface plasmon resonance could be used to test for a difference in the strength of interactions between the N-terminal regions of the *V. anguillarum* and *V. cholerae* TtpC proteins with the periplasmic domain of the FetA enterobactin receptor (Bober et al., 2011). The FvtA receptor could be purified and mounted on a chip under non-denaturing conditions using a non-ionic detergent such as Triton X-100. The binding efficiency of the *V. anguillarum* and *V. cholerae* TtpC periplasmic domains could then be determined using the Biacore by adding the purified periplasmic domains in solution to the chip with purified FetA. Measuring binding affinity with the Biacore would allow determination of the constants of association and dissociation. One potential downfall of this approach could be that the periplasmic region of the TtpC protein may not be in the correct conformation without the transmembrane domains (Postle et al.,

2010). Co-immuno precipitation of the TtpC proteins with other probable binding partners could be used to test protein-protein interactions as well, but these interactions would still need to be studied in the context of a detergent to solubilize the membrane proteins.

I expected that the *V. anguillarum* TtpC protein periplasmic truncations (Table 3-3) might interact with the outer membrane receptors, preventing the wild-type TonB2 system proteins from interacting with the receptors. As shown in the truncation competition bioassays though, this was not the case. An explanation for the lack of inhibition of the intact TonB2 system when the TtpCva truncations were expressed is that the truncations, especially those with fewer than three transmembrane domains, do not adopt the correct conformation.

In *E. coli*, the proper conformation of the TonB periplasmic domain is dependent on the presence of the TonB transmembrane domain and its interaction with the transmembrane domains of the ExbB and ExbD proteins (Larsen et al., 1999; Larsen et al., 1994; Ollis et al., 2009). The largest truncation, that with the N-terminal 408 amino acids, is most likely not functional because the truncation is located in the middle of the HxxxS motif. While the serine residue was shown not to be essential for iron transport and function of the TtpC protein in *V. cholerae*, it is still part of the highly conserved motif. Based on the DAS transmembrane domain predictions (Figure 2-2), the 408th amino acid in the *V. anguillarum* TtpC was predicted to be in the cytoplasm, even by the lower threshold prediction, but I later identified the HxxxS motif in residues 406 to 410. In *E. coli* TonB and TolA this motif is known to be located in the transmembrane domain therefore it is most likely located in the third transmembrane domain of the TtpC protein and a truncation in the membrane may

not maintain proper interactions with the transmembrane domains of the other TonB2 system proteins (Cascales et al., 2001; Germon et al., 2001; Larsen and Postle, 2001).

In conclusion, a challenge for the future 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 transferred, be it ATP or more likely, potential energy released in a conformational change of the TtpC or TonB2 proteins, is yet unknown and is crucial to defining the mechanism of energy transfer.

Chapter 5. Materials and Methods

A. Bacterial cell culture

i. *E. coli*

E.coli strains S17-λpir (TpR, SmR, *recA, thi, pro, hsdR-M* + RP4:2-Tc:Mu:Km T7 λpir) and DH5α (*fhuA2* Δ(argF-lacZ) U169 *phoA, glnV44,* ϕ80 Δ(lacZ) M15 *gyrA96 recA1, relA1, endA1, thi-1, hsdR17*) were used for genetic manipulations and cloning. The strains were grown in Luria Burtani (Bertani, 1951) medium, shaking at 37°C, or on Luria Burtani agar (1.5%) at 37°C. Antibiotic concentrations used were chloramphenicol 30μg/ml, ampicillin 100μg/ml, kanamycin 50μg/ml.

ii. *V. anguillarum*

V. anguillarum strain 775 harboring the pJM1 plasmid (Crosa et al., 1977) and derivatives were grown in tryptic soy broth supplemented with 1.5% NaCl [final], shaking at 25°C, or on tryptic soy agar (1.5%) supplemented with 1.5% NaCl [final]. Antibiotic concentrations used were chloramphenicol 10μg/ml, Ampicillin 750μg/ml, Rifampicin 100μg/ml.

Iron-restricted conditions were achieved by growing *V. anguillarum* strains in $CM9_{vibrio}$ [1X M9*vibrio* salts (60g Na_2HPO_4, 30g KH_2PO_4, 50g NaCl, 10g NH_4Cl per liter, pH 7.4), 0.2% Casamino Acids, 0.5% glucose, 10μM $CaCl_2$, 100μM $MgSO_4$] at 25°C.

iii. *V. cholerae*

V. cholerae strain CA401 (Griffiths et al., 1984) and derivatives were grown in Luria Burtani medium, shaking at 37°C, or on Luria Burtani agar (1.5%) at 37°C. Antibiotic concentrations used were chloramphenicol 10µg/ml and kanamycin 20µg/ml.

Iron-restricted conditions were achieved by growing *V. cholerae* strains in $CM9_{e.coli}$ [1X $M9_{E.coli}$ salts (60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 10g NH_4Cl per liter, pH 7.4), 0.2% Casamino Acids, 0.5% glucose, 10µM $CaCl_2$, 100µM $MgSO_4$, 0.004% Tryptophan, 0.01% Thiamine] at 37°C.

iv. *V. vulnificus*

V. vulnificus strain CMCP6 (Kim et al., 2003a) was grown in tryptic soy broth supplemented with 1.5% NaCl [final], shaking at 37°C, or on tryptic soy agar 91.5%) supplemented with 1.5% NaCl [final].

B. Chromosomal mutation strategies and plasmid-based complementation

i. Conjugation

Plasmid DNA was moved into *V. anguillarum* via conjugation. An overnight lawn of the *V. anguillarum* recipient strain from one agar plate was scraped with a glass Pasteur pipette and combined with the bacteria harvested from an overnight lawn of the *E. coli* donor strain, and when needed, an equal amount of strain carrying the mobilization helper plasmid pRK2013 (Simon et al., 1983). Recipient, donor and helper strains were combined to homogeneity and mounded into a "pastita" in the center of a non-selective plate, tryptic soy agar plus salt (TSAS) overnight at 25°C. Ex-conjugants were selected by plating with a

cotton-tipped applicator on TSAS plus antibiotics. Rifampicin^r, chloramphenicol^r colonies were then transferred to a new TSAS rifampicin100µg/ml, chloramphenicol10µg/ml plate and the oxidase test was performed to verify that the ex-conjugants were of the *Vibrio sp.* For mobilizing pDM4 (Milton et al., 1996), ex-conjugants were selected on TSAS plus rifampicin 100µg/ml and 10µg/ml. For mobilizing pMMB208 (Morales et al., 1991), ex-conjugants were selected on identical plates, but the pastita mix was streaked to isolated colonies.

ii. Selection of homologous recombinants

First recombinants with one cross-over event to integrate the pDM4 plasmid into the *vibrio* chromosome were selected on TSAS Rifampicin 100µg/ml, chloramphenicol 10µg/ml for *V. anguillarum* and TCBS (Kobayashi et al., 1963) supplemented with 2µg/ml chloramphenicol for *V. cholerae* and *V. vulnificus*. A second cross-over event and loss of the pDM4 plasmid was selected for by growing isolated first recombinants in non-selective media (TSBS for *V. anguillarum* and *V. vulnificus*, or LB for *V. cholerae*) shaking overnight. The overnight culture was plated to single colonies on TSAS or LBA with 15% Sucrose (filter sterilized) to select against any ex-conjugants that retained the pDM4 plasmid, which encodes the *sacB* gene from *Bacillus subtilis*. Colonies that grew on sucrose-supplemented media were replica plated on TSAS rifampicin 100µg/ml, chloramphenicol 10µg/ml, followed by TSAS sucrose 15%, followed by TSAS for *V. anguillarum*, TCBS chloramphenicol 2µg/ml, followed by TSAS sucrose 15%, followed by TSAS for *V. vulnificus*, or TCBS chloramphenicol 2µg/ml, followed by LBA sucrose 15%, followed by LBA for *V. cholerae*.

Chloramphenicol sensitive, sucrose resistant colonies were screened for gene deletion using colony PCR with primers specific to the regions 700 base pairs upstream and downstream of the deleted gene.

iii. Chemically competent cell production

Competent cells were prepared from a modified protocol based on that of Hanahan D, *et al.* (Hanahan et al., 1991) All cultures were grown in detergent-free glassware. The detergent was removed autoclaving glassware filled to ¾ total volume with de-ionized water. The autoclaved water was then dumped out and the cultures were grown in clean flasks. Seed stocks used in preparing competent cells were prepared by streaking the desired cell line a Super Optimal Broth (SOB) (Hanahan, 1983) plate to single colonies and allowing the cultures to grow at room temperature. The single colonies were then picked into 2ml SOB liquid medium and grown at room temperature overnight with shaking. After overnight growth, glycerol was added to 15% and 1ml samples were aliquoted into Eppendorf tubes and flash frozen in a dry ice/ ethanol bath for 5 minutes. The seed stocks were then stored at -80°C.

To prepare competent cells, 250ml of SOB liquid medium was inoculated with 1ml of seed stock and the culture was grown at 20°C to an OD_{600} of 0.3 (roughly 16 hours). The 16 hour culture was harvested by centrifugation at 4°C. The cells were resuspended in 80ml of ice cold CCMB80 buffer (Per Liter:10mM KOAc pH 7.0 (10 ml of a 1M stock), 80 mM $CaCl_2 \bullet 2H_2O$ (11.8g/L), 20mM $MnCl_2 \bullet 4H_2O$ (4.0 g/L), 10 mM $MgCl_2 \bullet 6H_2O$ (2.0 g/L), 10% glycerol. pH can be adjusted down to 6.4 with 0.1N HCl if necessary. CCMB80 buffer should be sterile filtered and stored at 4°C. A dark precipitate will not affect its function) and incubated on ice for 20 minutes. The cells were harvested by centrifugation again at 4°C and resuspended in 10 ml of ice cold CCMB80 buffer. The OD of the cells was tested by mixing 200 µl SOB media with 50µl of the resuspended cells and the volume of the resuspended cells was adjusted with ice cold CCMB80 buffer until the OD_{600} of the test cells was between 1.0 and 1.5. The resuspended cells were then incubated on ice for 20 minutes before being aliquoted into

chilled 1.5 ml Eppendorf tubes (400 µl cells/tube). The cells were then transferred quickly to storage at -80°C. Flash freezing is not necessary.

iv. Transformation

Competent cells were thawed on ice, then 1/40th cell volume of plasmid DNA was added to the cells and incubated on wet ice for 30 minutes, transferred to a 42°C water bath for 45 seconds, then transferred back to the wet ice for 2 minutes. After heat shock, 500µl SOC broth was added and the cells were incubated at 37°C for 30 minutes prior to plating on selective media.

C. DNA preparation

i. Genomic DNA

Genomic DNA was isolated using the Bio-Rad AquaPure genomic DNA isolation kit. Briefly, the cell pellet from 1ml of overnight bacterial culture was lysed in a lysozyme EDTA solution and a 5 minute incubation at 80°C. RNA was degraded by addition of RNase A and cell debris and proteins were precipitated and removed by centrifugation. DNA from the supernatant was precipitated by addition of isopropanol and centrifuged at 13kRPM in a desk-top centrifuge to pellet the DNA. Salts were removed from the DNA pellet with a 70% ethanol wash and DNA was re-hydrated in purified, autoclaved H_2O.

ii. Plasmid DNA

Plasmid DNA in *E. coli* was harvested using the Qiagen mini-prep spin kit (Qiagen Germantown, MD). 3ml of bacterial culture was used as the starting volume for strains harboring high copy number plasmids, such as pCR2.1. 4.5ml of bacterial culture was use as the starting volume for strains harboring low copy number plasmids, such as pMMB208 or

pDM4. Cells were lysed and cell debris was removed by centrifugation. The supernatant was loaded onto the column and the protocol was performed exactly as stated in the technical manual except that DNA was eluted from the column with 40µl purified, autoclaved H_2O rather than Tris:EDTA buffer.

It is difficult to produce a high yield of clean plasmid DNA from *vibrio sp* using a column purification method. To purify plasmid DNA from *V. anguillarum* and *V. cholerae*, cells from 1.5 ml overnight CM9 culture was harvested by centrifugation then re-suspended in 150µl Solution I (25mM Tris-HCl, 10mM EDTA, 50mM Glucose with 10µg/ml RNase). To the bacterial suspension, 300µl Solution II (0.2N NaOH, 1% SDS) and 300µl Solution III (3M potassium acetate (CH_3CO_2K), pH 5.3 with glacial acetic acid) and 10µl Alkaline Protease solution (Promega Madison, WI) were added, then the solution was incubated on ice for 20 min. Cell debris was collected by centrifugation and supernatant was removed to a new Eppendorf tube to which, 400µl of a mixture of 25:24:1 phenol: chloroform: isoamyl alcohol was added. The phases were separated by centrifugation at 13,000 RPM and the upper phase was transferred to a new Eppendorf tube. 1/10 volume of 3M sodium acetate, pH5.2 was added and the DNA was precipitated by addition of 3 volumes of cold isopropanol. The solution was incubated at -80°C for 40 minutes or -20°C overnight. The DNA was pelleted with a 30-minute centrifugation at 13,000 RPM and the pellet was dried at room temperature for 10 minutes. DNA was re-suspended in 25µl H_2O

D. Restriction digests and DNA electrophoresis

New England Biolabs (Ipswich, MA) restriction enzymes were used exclusively. Digests were performed for 2-4 hours at the indicated temperature in the recommended buffers.

For diagnostic restriction digests, 5 µl of the digest was analyzed on a 1% agarose gel with 0.5µg/ml Ethidium Bromide with a Kodak Gel Logic 100 imaging system. For gel purification, 20µl of the digest was separated on 1% agarose gel with 0.5µg/ml Ethidium Bromide and desired bands were removed with a clean razor blade. DNA fragments were purified from the agarose gel using the Qiagen Gel Extraction kit (Qiagen Germantown, MD). The DNA was eluted with 40µl purified, autoclaved H_2O.

E. Polymerase Chain Reaction

i. PCR

PCR was performed in a Bio-Rad thermocycler (Bio-Rad, Hercules, CA). Almost all reactions were performed with a 0.3°C incremental decrease in annealing temperature starting at 63°C and continuing for 30 cycles. If no amplification was achieved, a more specific, single annealing temperature was used based on the predicted melting temperature of the primers. A two minute extension step at 72°C was used for all DNA fragments less than 2kb. A final concentration of 0.5µM of each primer was used in the reactions with a final concentration of 0.08µM dNTP mix. NEB Taq polymerase (0.025 units/50µl reaction) and ThermoPol buffer were used for diagnostic reactions. NEB Vent high fidelity polymerase (0.008 Units/50µl reaction) and Thermo Pol buffer were used for cloning.

ii. Colony PCR

Colonies of *E. coli* were re-suspended in 50µl H2O. 2µl of the colony suspension was used as the template in the PCR reaction. For all colony PCR, a combination of NEB Taq and NEB Vent polymerases was used (0.1µl Vent + 0.15µl Taq/50µl reaction volume). *Vibrio sp*. colonies were re-suspended in 50µl H_2O and boiled for 15 minutes in the thermocycler

prior to using 2µl of the boiled bacterial suspension as the template in the PCR reaction. Immediately following the PCR reaction, agarose gel electrophoresis of these samples was performed to analyze the PCR fragments before they are degraded by DNases from the boiled *Vibrio sp*.

iii. Site Directed Mutagenesis by SOE PCR

Site directed mutagenesis was carried out using the technique of Splicing by Overlap Extension (SOE) PCR. Two 30-40bp complementary primers were designed with the desired point mutation(s) at the center of the DNA oligo flanked by at least 9bp of homology to the surrounding region. These primers were used individually in the first step of cloning along with a primer at the 5' or 3' end of the gene being amplified. The two resulting PCR products with overlapping region were then used at an equi-molar ratio as the template in a second PCR reaction using the two outside primers for SOE. For plasmid complementation, the mutated sequence of the SOE product was verified and the product was cloned into the pMMB208 vector. For chromosomal mutation, the SOE product was cloned into the pDM4 vector and used for homologous recombination to replace the wild-type gene.

F. Inverse Polymerase Chain Reaction

This method was used to sequence the genomic DNA upstream of the *V. anguillarum ttpC* and downstream of the *V. anguillarum tonB2* genes.

Overnight DpnI (NEB) digests of *V. anguillarum* 775 genomic DNA in multiple dilutions (1, 1:10, 1:25, 1:50, 1:100) were performed to cut the genomic DNA into roughly 1.5 – 2kb segments. Digested DNA was analyzed on an agarose gel to confirm digestion. DpnI was heat inactivated and the digestions were diluted by half and 1:10 with purified water. The

diluted digestions were ligated with T4 DNA ligase (NEB) overnight at 16°C. Heat-inactivated ligations were used as templates for PCR reactions using opposing primers inside the *ttpC* or *tonB2* genes. The reverse primer should be 10-100bp from the beginning of the known sequence. The forward primer should be no more than 100bp upstream of the restriction enzyme cut site. The PCR fragments were then sequenced at the MMI Sequencing Core and the sequence output was analyzed using the Sequencher program (GeneCodes, Ann Arbor, MI).

G. Bioassay for iron-source utilization

i. *V. anguillarum*

V. anguillarum strains were grown overnight in TSBS plus antibiotic(s) to maintain any plasmids. The culture was then diluted 1:100 in $CM9_{vibrio}$ plus antibiotic(s) and grown overnight at 25°C. For bioassay plates, 10ml of molten 3% agar was added to 10ml 2X $CM9_{vibrio}$ in a 50ml glass culture tube with 20 or 40μM EDDA for iron chelation. The molten agar + media was kept in a 47° water bath, then 100μl of overnight *V. anguillarum* culture in $CM9_{vibrio}$ media was added. After addition of the bacterial culture, the contents were gently vortexed to mix and poured into a plastic petri dish. Individual samples in petri dishes were cooled to room temperature and, once set, iron sources were spotted on the surface of the plates.

ii. *V. cholerae*

V. cholerae strains were grown overnight in LB plus antibiotic(s) to maintain any plasmids or transposon insertions. For bioassay plates, 10ml of molten 3% agar was added to 10ml 2X $CM9_{E.coli}$ in a 50ml glass culture tube with 20 or 40μM EDDA for iron chelation. 100μl of

LB overnight bacterial culture was added to the agar-media mix and poured into plastic petri dishes as described above.

iii. Iron Sources

Iron sources were spotted onto the surface of bioassay plates. 0.5µl iron-free enterobactin 1mg/ml in methanol (EMC biosciences, Germany), 0.5µl iron-free vibriobactin 1mg/ml in methanol (EMC bioscience, Germany), 1.0µl iron-free ferrichrome 5mM in 50% methanol (Sigma, St. Louis, MO), 1.0µl Hemin 2µM (Sigma) in 10mM NaOH, 2.0µl Ferric Ammonium Citrate 10mg/ml in water. Other non-purified siderophores were tested by streaking a producing strain on the surface of the plate. Observing a halo of growth inside the plate around the bacterial growth on the surface, for example, *V. anguillarum* 775 was used to test for growth on anguibactin and *V. vulnificus* CMCP6 was used to test for growth on vulnibactin. The plates were then incubated 1-2 days and growth around various iron sources was visualized.

H. ^{55}Fe-Ferrichrome uptake assay

V. cholerae cells were grown in $CM9_{E.\ coli}$ with 10µg/ml chloramphenicol and 1mM IPTG to induce plasmid-encoded gene expression to OD_{600} 0.3, harvested by centrifugation and re-suspended to $2X10^8$/ml in $M9_{ecoli}$ salts plus 100µM Nitrilotriacetate(NTA) followed by incubation with shaking for 90 min at 37°C to deplete intracellular iron pools. The transport assay was initiated by the addition of 37.3 pmol ^{55}Fe:Ferrichrome complex (generated by preincubation of deferriferrichrome (Sigma) with $^{55}FeCl_3$ at a 6.7:1 molar ratio in 10mM HCl at 37°C for 15 minutes (Larsen and Postle, 2001) to 5ml iron-depleted bacterial culture with continued incubation with shaking at 37°C. 1ml samples were harvested at the indicated

time points by vacuum filtration onto 0.45μm nitrocellulose HATF filters (Millipore, Billerica, MA). To measure ^{55}Fe:Ferrichrome internalization rather than binding, binding to the ferrichrome receptor was quenched by washing with 2 X 10ml 0.1M LiCl. The filters were dried and the amount of internalized ^{55}Fe was determined by liquid scintillation counting. As a negative control, 2mM KCN was added to a duplicate of the positive control sample 15 minutes prior to the initiation of the transport assay.

I. *In vivo* formaldehyde crosslinking

Bacterial cultures were grown in iron-limiting media overnight, then subcultured in iron-limiting media and grown to OD_{600} 0.6. Cells were harvested by centrifugation and washed once in 0.1M sodium phosphate buffer pH 6.8 and re-suspended in $NaPO_4$ buffer to OD_{600} 0.5. The culture was then separated to 1ml aliquots. Proteins were cross-linked at room temperature by addition of formaldehyde (Thermo Pierce, 16% formaldehyde in methanol 1ml ampules) (Thermo, Waltham, MA) to a final concentration of 1% for the desired amount of time. Crosslinking was quenched by addition of 200mM glycine [final]. The cells were pelleted by centrifugation at 13,000 PRM in a desktop centrifuge for 1 minute then re-suspended in phosphate buffered saline pH 7.2 with 1mM phenylmethanesulfonylfluoride (PMSF) or the indicated amount of Cømplete EDTA-Free protease inhibitor cocktail (Roche).

i. Membrane Protein Preparation

Samples were sonicated on ice 5-6 x 10seconds with 10 seconds on ice in between. Whole cells were removed by 1-minute centrifugation at 13,000 RPM. The supernatant was then transferred to a new 1.5ml centrifuge tube and membranes were pelleted by centrifugation at 15,000 RPM in a Sorvall SS-34 rotor at 4°C for 40 minutes. Supernatant was removed and lipids were extracted by washing the pellet with 500μl cold acetone for 10 minutes on ice.

Acetone was removed by aspiration and the pellet was re-suspended in 2X SDS-PAGE running buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 4% sodium dodecyl sulfide (SDS), 3.1% dithiothreitol, 0.1mg/ml bromophenol blue).

ii. Total protein preparation

Total proteins were precipitated immediately after quenching by addition of 10% [final] trichloroacetic acid (TCA) and incubation on wet ice for 10 minutes. Precipitated proteins were pelleted by centrifugation at 13,000 RPM for 10 minutes at 4°C. Supernatant was removed and TCA was extracted by washing the pellet with cold acetone on ice for 10 minutes. The acetone was removed by aspiration and the remainder was allowed to evaporate for 10 minutes. Total proteins were re-suspended in 2X SDS-PAGE running buffer. Samples were loaded onto the gel for SDS-polyacrylamide gel electrophoresis (PAGE) analysis immediately or stored at -80°C.

J. Dithiobis [succinimidyl propionate] (DSP) crosslinking analysis

V. cholerae cells grown in $CM9_{Ecoli}$ to OD_{600} 0.4 were harvested by centrifugation, then re-suspended in sodium phosphate buffer pH 6.8 to the original volume. The cells were aliquoted 1ml/Eppendorf tube and DSP (in DMSO) was added to a final concentration of 1mM. Cells were incubated with DSP or carrier for the indicated time points at room temperature, then the cross-linking reactions were quenched by adding 2M glycine to a final concentration of 200mM. Total proteins were TCA precipitated by adding 1/10 volume stock TCA and incubated on wet ice for 10 minutes. Supernatant was removed and TCA was extracted by washing the pellet with cold acetone on ice for 10 minutes. The acetone was removed by aspiration and the remainder was allowed to evaporate for 10 minutes. Total

precipitated proteins were harvested by centrifugation at 13000 RPM at 4°C and re-suspended in 2X SDS-PAGE running buffer. Samples were loaded onto the gel for SDS-polyacrylamide gel electrophoresis (PAGE) analysis immediately or stored at -80°C. DSP-cross-linked proteins were un-cross-linked in the control sample by the addition of fresh DTT solution prior to loading the sample on the gel.

K. Protease accessibility assay

i. Proteinase K susceptibility

Cells were grown to OD_{600} 0.5 and spheroplasts were prepared by the method of Ahn *et al.* (described in detail in section 4.L). The effect of protonophores on intact spheroplasts or whole cells was examined by addition of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 50μM or an equal volume of carrier, ethanol. Samples were then incubated with or without Proteinase K (25μg/ml) for 15 minutes at 4°C, then treated for 2 minutes with 1mM PMSF to inactivate the proteinase K. Total proteins were prepared by TCA precipitation as described above.

ii. Trypsin susceptibility

Trypsin susceptibility assays were carried out in the manner of the proteinase K susceptibility assays, using 90μg/ml trypsin for 5 minutes at 4°C. The trypsin was inactivated for 5 minutes by addition of 1mM PMSF and 90μg/ml soybean trypsin inhibitor. Total proteins were prepared by TCA precipitation as described above.

iii. Spheroplast production

Spheroplasts of *V. cholerae* were prepared in the method of Ahn *et al* (Ahn et al., 2005). Briefly, 10 ml of *V. cholerae* was grown in $CM9_{E.\ coli}$ to OD_{600} 0.6 and harvested by centrifugation at 4°C. The pellet was re-suspended in 0.5 ml of 0.2M Tris-HCl pH 8.0. The following solutions were added in sequence to 0.25ml of the re-suspended cells and gently mixed with the cells: 0.5ml of 0.2M Tris-HCl (pH8.0), 1 M sucrose, 0.05 ml of 10 mM EDTA (pH8.0), 0.05 ml of lysozyme (2mg/ml), and 1.6 ml of H_2O. The cells were then kept for 10 minutes at room temperature on a rocking platform to convert most cells to spheroplasts. Whole cells were obtained by adding 2.2ml 0.2M Tris-HCl (pH8.0). Formation of spheroplasts and integrity of the whole cells was determined by phase contrast microscopy.

L. Alkaline phosphatase and β-galactosidase plate assay for fusion protein expression

Alkaline phosphatase activity in strains harboring TtpC-PhoA fusion proteins was detected by streaking to isolation on E-medium (0.2g of $MgSO_4 \cdot 7H_2O$, 2g of citric acid-$1H_2O$, 10g of K_2HPO_4, 3.5g of $NaHNH_4PO_4 \cdot 4H_2O$, 0.4% glucose, 1.5% casamino acids, 1mg vitamin B1, 1.5% agar per liter) containing 1mM IPTG and 100µg/ml XP reagent (5-bromo-4-chloro-3-indolyl phosphate) (Croxatto et al., 2007). Strains that grew blue on the E-medium, XP plates were selected for sequencing and further analysis by liquid alkaline phosphatase assay described in section 4.N.

β-galactosidase activity in strains harboring TtpC-LacZ fusion proteins was detected by streaking to isolation on TSAS or LB with 1mM IPTG and 0.004% X-gal (bromo-chloro-indolyl-galactopyranoside) (Roche Basel, Switzerland).

M. Alkaline phosphatase assay

Exponential phase cell cultures (*E. coli* CC118 Δ(ara-leu) araD ΔlacX74, *gale, galK, phoA20, thi-1, rpsE, rpoB, argE(Am)* recA1) harboring the pMMB206-*ttpCva-phoA* were induced with 1mM IPTG for 30 minutes prior to harvesting 1ml of the culture by centrifugation for 5 minutes at 4°C. Cell pellets were washed in 10mM Tris-HCl pH 8.0, 10mM $MgSO_4$. The final pellet was re-suspended in 1ml cold 1M Tris-HCl pH 8.0 and 0.1ml cells were diluted into 0.4ml cold 1M Tris-HCl pH 8.0. The OD600 was recorded and 0.1ml washed cells were added to 0.9ml 1M Tris-HCl pH 8.0, 0.1mM $ZnCl_2$ in a borosilicate glass tube. To the tube, 50µl of 0.1% SDS and 50µl chloroform were added and mixed, then incubated at 37°C for 5 minutes, followed by 5 minutes incubation on ice. The colorimetric assay was started with the addition of 0.1ml 0.4% p-nitrophenylphosphate in 1M Tris-HCl, pH 8.0 and incubated at 37°C. The tubes were incubated until a pale yellow color developed, then the reaction was stopped by addition of 120µl stop solution (1:5 0.5M EDTA, pH 8.0: 1M KH_2PO_4). The reaction time, the OD_{550}, and OD_{420} were recorded and used to calculate the alkaline phosphatase activity with the equation: alkaline phosphatase activity = (OD_{420}-OD_{550})*1000/(reaction time in minutes)(OD_{600})

N. Custom antibody production

Custom polyclonal chicken antibodies were designed against immunogenic peptide sequences (determined by Aves Labs, Tigard,OR) from the proteins: *V. anguillarum* TtpC (N-terminus) CZKVENKQQQQHNIERE, ExbB2 CHLLELKLEKSLRSLR, ExbD2 CZRRQPRNDEAQIDLTS, *V. cholerae* ExbB2 CRGLEMKLEQALRSQS, ExbD2 CZRRTSKQEEAQIDLTS and TonB2 CZEQEQDVQRRQRSVPEQ. Custom polyclonal rabbit

antibodies were designed against immunogenic peptide sequences (determined by Lampire, Pipersville, PA) from the proteins: *V. anguillarum* TtpC (C-terminus) CSVYNKEQNRSVEALE, TonB2 EQEHDLQRRQRSVPEC, *V. cholerae* TtpC VQQATQEKAQQQHNQQREC, and *V. vulnificus* TtpC2 KATQESRQQQAENQQRENAC. The chicken antibodies were affinity purified by Aves Labs. The rabbit antibodies were received as serum samples and cross-reacting antibodies were adsorbed with acetone powder of chromosomal deletion strains (protocol follows in section 4.O).

O. Adsorption of cross-reacting antibodies

i. Acetone Powder

Cross-reacting antibodies in the rabbit anti-*V. an*guillarum TtpC Cterm, anti-*V. cholerae* TtpC and anti-*V. vulnificus* TtpC were removed using adsorption with Δ*ttpC* acetone powder specific to the species. Chromosomal Δ*ttpC* mutants from each of the three *vibrio* strains listed above were grown in 500ml iron-limiting media ($CM9_{Vibrio}$ or $CM9_{E.coli}$) overnight to induce expression of proteins induced in the experimental growth conditions. The pellets from the cultures were suspended on 2ml 0.1M NaCl per gram of cells, to which 4 volumes of ice-cold acetone was added. The mixture was stored on ice for 1 hour. The cells were then harvested by centrifugation and re-suspended in 4 volumes of ice-cold acetone and stored on ice for 10 minutes. The cells were again harvested by centrifugation and the pellet was transferred to a piece of Saran Wrap (Dow Chemical Co. Midland, MI) and the acetone was allowed to evaporate overnight.

ii. Adsorption

Acetone powder was added to anti-TtpC antiserum at a final concentration of 1% (w/v). Cross-reacting antibodies were adsorbed by shaking the solution overnight on a rocking platform at 4°C. The powder was removed by centrifugation at 4°C and the supernatant was filter-sterilized and stored in 0.25ml aliquots at -20°C.

P. Western blot protein analysis

Protein samples were mixed with 2x SDS-PAGE buffer, and the proteins were separated on 4-20% gradient Bis-Tris criterion XT precast gels in 1x XT morpholinepropanesulfonic (MOPS) acid buffer (Bio-Rad) for cross-linking studies or 16% Tris-Tricine Criterion precast gels in 1X Tricine buffer (Bio-Rad) for protease susceptibility analysis. All other SDS-PAGE experiments were carried out on 12% Bis-Tris Criterion XT precast gels in 1x XT MOPS Buffer (Bio-Rad). Gels were soaked for 15 min in transfer buffer (0.3% Tris, 1.44% glycine, and 20% methanol) and transferred to 0.45µm nitrocellulose membrane (Bio-Rad) in transfer buffer for 1.5 h at 400 mA in a Transblot cell (Bio-Rad). Membranes were blocked for 1 h in 5% skim milk in phosphate-buffered saline containing 0.5% Tween 20 (PBST). After the blocking step, membranes were incubated with the primary antibody diluted in PBST with 5% bovine serum albumin fraction V on a rocking platform at 4°C for 16 h. The antibody concentrations used were as follows:

Antibody	Species	Buffer	Dilution
Va TtpC N-term	chicken	PBST	1:50,000
Va TtpC C-term	rabbit	PBST	1:20,000
Va ExbB2	chicken	TBST	1:5000
Va ExbD2	chicken	TBST	1:5000
Va TonB2	rabbit	TBST	1:2000
Vc TtpC	chicken	PBST	1:20,000
Vc ExbB2	chicken	TBST	1:5000
Vc ExbD2	chicken	TBST	1:5000
Vc TonB2	chicken	PBST	1:2000
Va FatA	rabbit	PBST	1:15,000
Vv TtpC	rabbit	PBST	1:50,000
Ec PhoA (Pierce)	mouse	PBST	1:20,000
6His (Pierce)	mouse	PBST	1:1000
anti rabbit IgG (Pierce)	goat	PBST	1:10,000
Anti chicken IgY (Pierce)	donkey	PBST	1:100,000

Membranes were washed four times for 10 min in PBST and incubated for 1 h in the secondary antibody conjugated to horseradish peroxidase. Membranes were washed four times for 10 min each, then incubated for 1 min in Millipore Luminata Forte chemiluminescent substrate according to the manufacturer's recommendations. Signal was detected on a GE ImageQuant CCD-based imaging system and analyzed with GE ImageQuant TL software.

Q. EDDA (ethylenediamine-di-o-hydroxyphenylacetic acid) purification

Commercially available EDDA contains more iron than is acceptable for use in the assays presented in this thesis. 5g of EDDA powder was dissolved in 95ml boiling 1N HCl. This was performed in a round bottom flask in a semi-spherical heating mantel with a water-cooled condensation tube on top until the EDDA was dissolved. The solution was cooled to room

temperature then filtered through a 2MM filter paper in a large porcelain funnel. The filtered solution was diluted to 700ml with acetone and the pH was adjusted to 6.0 with NaOH. The solution was incubated at 4°C overnight and the deferrated EDDA was allowed to precipitate. The precipitate and supernatant was again filtered through a 2MM filter paper and washed with acetone then allowed to dry at room temperature. The deferrated EDDA was a very light yellow powder and was collected with a plastic spoon and stored in a 50ml plastic conical tube, protected from light at -20°C.

A stock solution of EDDA was prepared by dissolving 10g in 150ml of 1N NaOH. The pH was adjusted to 9.0 with HCl and the volume increased to 200ml. The volume was then adjusted to result in a 10mM stock solution. 15ml aliquots were stored in plastic conical tubes wrapped in foil and stored at -20°C. The frozen stock solution was thawed in a 37°C water bath prior to use.

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