REGULATED INTRAMEMBRANE PROTEOLYSIS OF THE VIRULENCE ACTIVATOR TCPP IN *VIBRIO CHOLERAE*

by

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This thesis is dedicated to my best friend, supporter, and wife, Huey Shann. Your unwavering love, support, and encouragement have been a constant throughout my graduate studies.

"*Try not. Do, or do not. There is no try.*"

-Yoda

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ABSTRACT

In *Vibrio cholerae*, direct activation of genes that encode cholera toxin (CT) and toxin-coregulated pilus (TCP) requires the ToxT transcription activator, whose expression is regulated by a complex of membrane regulatory proteins including ToxR and TcpP. TcpP, with a cytoplasmic domain similar to that found in OmpR and a periplasmic domain that may respond to specific environmental signals, is degraded in a two-step proteolytic pathway known as regulated intramembrane proteolysis (RIP), which eliminates the expression of CT and TCP. Under specific conditions, degradation is inhibited by another membrane-bound periplasmic protein, TcpH. I applied forward and reverse genetic approaches to determine the site-1 protease of TcpP. Through these analyses, I found that a C-terminal protease called Tsp catalyzes TcpP degradation to a truncated yet still active species that we termed TcpP*, which is the substrate for the previously identified site-2 protease, YaeL. My results also indicate a role of TcpH in protecting full-length TcpP from spurious degradation by YaeL. Moreover, I investigate the possibility of RIP regulating steady-state levels of TcpH and present evidence that TcpP confers stability to TcpH. Furthermore, *in vivo* studies in infant mice suggest that YaeL, but not Tsp, is required for intestinal colonization. These results expand our knowledge about RIP and the unorthodox mechanism of gene activation by membrane-bound transcription factors.

CHAPTER 1

INTRODUCTION

Vibrio cholerae General Background

Vibrio cholerae is a Gram-negative, comma-shaped bacterium that causes the human diarrheal disease cholera (Ayre 1849). Outside its human host, *V. cholerae* is commonly found in coastal waters of many parts of the world, where it either exists planktonically in brackish water and estuaries, or colonizes the chitinous exoskeleton of shellfish and zooplanktons (Colwell 1996). Transmission of *V. cholerae* to humans occurs through the oral-fecal route, primarily via ingestion of contaminated food or drinking water. Once the bacterium reaches the upper small intestine, it secretes cholera toxin (CT), which binds to and enters intestinal epithelial cells (Angelichio, Spector et al. 1999). The endocytosed CT activates a pathway that results in constitutive cyclic AMP (cAMP) production, which in turn leads to secretion of water and ions into the lumen of the small intestine (Angelichio, Spector et al. 1999). Rapid dehydration ensues, and is manifested in the form of a voluminous watery diarrhea, otherwise colloquially referred to as rice-water stool (Sanchez and Holmgren 2011). In severe cases, a person may lose as much as one liter of fluid an hour and die if not treated with a simple and inexpensive oral rehydration therapy (ORT) (Hirschhorn, Kinzie et al. 1968, Guerrant, Carneiro-Filho et al. 2003). The World Health Organization (WHO) estimates that there are three to five million cholera cases and 100,000 to 120,000 deaths due to cholera every year, the highest incidence being in sub-Saharan

African and South Asian countries, where water and sanitation facilities are suboptimal.

The ToxR/TcpP/ToxT Virulence Cascade

In *V. cholerae*, the expression of the two principal virulence factors – CT, the protein complex responsible for the diarrheal symptoms of cholera, and toxin-coregulated pilus (TCP), the hairlike appendage on the outer membrane surface that allows successful intestinal colonization – are the result of a multiprotein regulatory cascade known as the ToxR regulon, named after a key positive regulator of virulence described below (Peterson and Mekalanos 1988, Skorupski and Taylor 1997). Activation of ToxT – the direct activator of genes encoding CT and TCP – is controlled by two unusual inner membrane-bound transcription activators, ToxR and TcpP (DiRita 1992, Higgins and DiRita 1994, Thomas, Williams et al. 1995, Hase and Mekalanos 1998, Krukonis, Yu et al. 2000, Crawford, Krukonis et al. 2003)

ToxR and TcpP are type-II single pass transmembrane proteins with N-terminal winged helix-turn-helix DNA-binding/transcription activator cytoplasmic domains found in response regulators of the OmpR/PhoB family (Martinez-Hackert and Stock 1997, Martinez-Hackert and Stock 1997), and C-terminal periplasmic domains of unknown function. For optimal ToxR activity, another inner membrane protein, ToxS, is hypothesized to interact with ToxR in the periplasmic space to provide stability and promote ToxR dimerization (Miller, DiRita et al. 1989, DiRita and Mekalanos 1991). Likewise, TcpP requires a cognate inner membrane protein, TcpH, for stability and wild-type activity (Beck, Krukonis et al. 2004). Single-molecule fluorescence microscopy suggests that ToxR recruits TcpP to the *toxT* promoter to activate transcription (Haas, Matson et al. 2014). However, TcpP overexpression can partially bypass the requirement

for ToxR in this process, but not vice versa, indicating that ToxR plays an accessory role in *toxT* activation (Krukonis, Yu et al. 2000). Further characterization of ToxR indicates that this role is contingent on its wing domain, which contributes to ToxR/TcpP interaction (Morgan, Felek et al. 2011).

ToxT is a cytoplasmic protein that belongs to the AraC/XylS family of transcriptional regulators with the two helix-turn-helix DNA-binding motifs at its C-terminus (Higgins, Nazareno et al. 1992, Gallegos, Schleif et al. 1997, Martin and Rosner 2001). On the other hand, the N-terminus contains dimerization and regulatory elements that may respond to positive (bicarbonate) and negative (bile and unsaturated fatty acids) effectors (Abuaita and Withey 2009, Childers, Cao et al. 2011). After ToxR and TcpP initiate transcription from the *toxT* promoter, ToxT is produced and activates transcription of the CT genes (*ctxAB*) and the *tcp* operon by binding to a region called toxboxes – a degenerate 13-base pair DNA sequence located upstream of all known ToxT-activated genes (Withey and DiRita 2006). Interestingly, the *toxT* gene is also located in the *tcp* operon. Therefore, an autoregulatory loop is established when ToxT produces more of itself, independent of ToxR and TcpP, by activating transcription from the *tcp* promoter (Yu and DiRita 1999).

Under *in vitro* virulence-repressing conditions, ToxT, TcpP, and ToxR have been shown to undergo proteolysis, resulting in the discontinuation of CT and TCP production (Matson and DiRita 2005, Abuaita and Withey 2011, Almagro-Moreno, Kim et al. 2015). These evidence support a model for terminating *V. cholerae* virulence gene expression late in infection prior to escape from the human host.

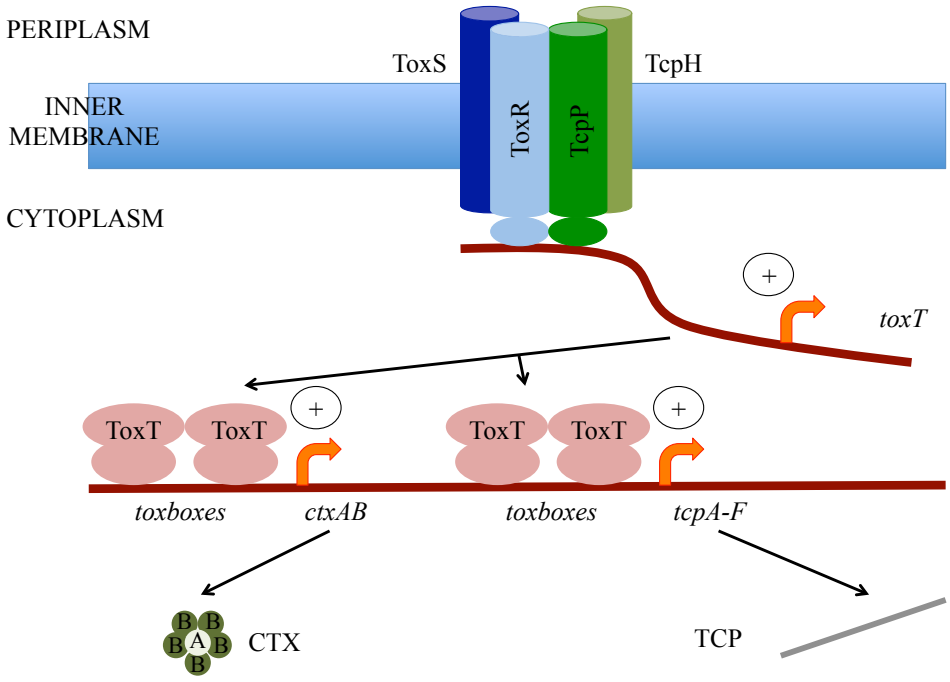

Figure 1.1. The ToxR Regulon. Expression of genes that encode cholera toxin (CTX) and toxin-coregulated pilus (TCP) is induced when the master virulence regulator ToxT binds to a region called *toxboxes* upstream of the *ctx* and *tcp* operons. *toxT* transcription is in turn activated by two unusual membrane-bound transcription activators – ToxR and TcpP, which interact with two other membrane-bound proteins – ToxS and TcpH, respectively.

Membrane-Bound Transcription Factors

Bacteria sense environmental stimuli via various cell surface expressed receptors, which in turn transduce the signals to cognate transcription factors that initiate expression of genes required for adaptation and survival in changing environments. Sensory transduction is conventionally achieved by a phosphorelay cascade that begins with the autophosphorylation of the membrane-bound sensor kinase, which then transfers the phosphoryl group to the corresponding response regulator in the cytoplasm (Figure 1.2). The regulatory output of this mechanism is collectively termed two-component signal transduction. In the context of pathogens, sensing the host environment is crucial in triggering virulence gene expression and evading the host immune response. Given that DNA and other components of the transcription machinery reside in the cytoplasm, it has generally been thought that transcription factors will all be in the same subcellular location.

Discovery of ToxR in *V. cholerae* changed this assumption due to its localization to the membrane, where it is evidently capable of binding DNA and activating transcription (Miller, Taylor et al. 1987). More important, ToxR lacks evidence of the histidine kinase or phosphoryl-acceptor domains found in canonical two-component systems. In recent years, emerging evidence has shown that this mechanism is not unique to *V. cholerae*. Here I discuss features of membrane-bound transcription factors in several important pathogens, using the ToxR regulon in *V. cholerae* to illustrate the potential signal sensor/response regulator bifunctionality of this unorthodox class of proteins to induce virulence.

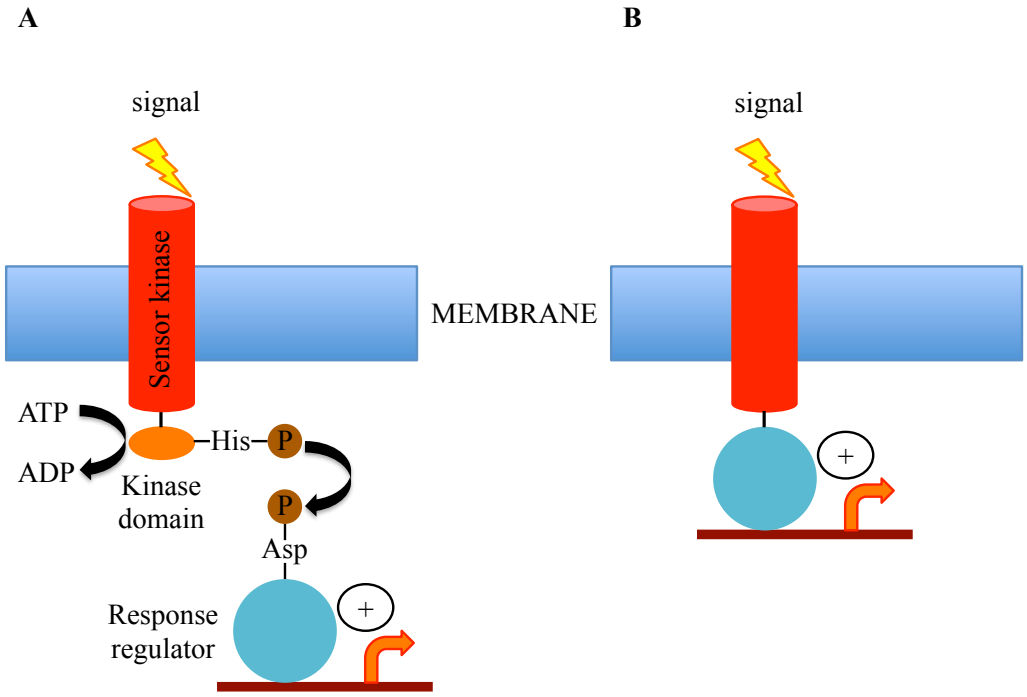

Figure 1.2. Comparison Between the Prototypical Bacterial Two-Component Signal-Transduction System and the Atypical One-Component System. (A) In the prototypical two-component signal-transduction system, the kinase domain of the membrane-bound sensor kinase autophosphorylates at a conserved histidine residue upon recognition of a specific environmental signal by the input domain. The phosphate is then transferred to a conserved aspartate residue within the receiver domain of the response regulator (RR). This phosphorelay activates the output domain of the RR, resulting in DNA binding and transcriptional regulation of genes encoding components that respond to the signal. (B) In the one-component system, a transmembrane protein binds DNA and activates transcription in a phosphorelay-independent manner when the sensor domain encounters a triggering environmental cue.

ToxR/TcpP

As previously discussed, *toxT* transcription is activated by the membrane-bound transcription factors ToxR and TcpP. Although the periplasmic domains of these two proteins have yet to be functionally characterized, evidence of osmolarity, pH and temperature of the growth medium influencing expression of CT and TCP led to the speculation that the transmembrane nature of ToxR and TcpP allows monitoring of extracellular cues. This strategy could either involve physical interaction of the periplasmic domains with outer membrane proteins that undergo conformational changes under specific conditions or sensing of molecules that enter the cell from the environment. One example of such is the dimerization of TcpP by intermolecular disulfide bond formation in the presence of bile salts (Yang, Liu et al. 2013).

Besides its role in *toxT* activation, ToxR is also known to directly activate and repress the transcription of two major outer membrane porin encoding genes – *ompU* and *ompT* respectively, in a TcpP-independent manner (Miller and Mekalanos 1988, Crawford, Kaper et al. 1998, Li, Crawford et al. 2000). Tight regulation of OmpU and OmpT levels confers resistance to antimicrobial peptides, bile and other natural detergents in the host (Provenzano and Klose 2000, Mathur and Waldor 2004). Interestingly, membrane localization of ToxR is required for TcpP-dependent *toxT* transcription, but dispensable for the regulation of *ompU* and *ompT* gene expression (Crawford, Krukonis et al. 2003).

Under conditions that do not favor virulence expression or in the absence of TcpH, TcpP is subjected to a two-step proteolytic cleavage known as regulated intramembrane proteolysis (RIP) (Matson and DiRita 2005), which will be the main focus of this dissertation. Despite the

resemblance with other well-studied RIP systems involving sigma/anti-sigma factor complexes, the outcome is different, as proteolysis of anti-sigma factors results in the liberation of sigma factors to bind RNA polymerase and initiate transcription, whereas proteolysis of TcpP culminates in the loss of *toxT* transcription and hence CTX and TCP expression. More recently, Almagro-Moreno *et al.* showed that ToxR also undergoes YaeL-dependent RIP under nutrient starvation at alkaline pH (Almagro-Moreno, Kim et al. 2015). However, the role of ToxS in ToxR proteolysis was not addressed in that study. Previously, removal of ToxS has been shown to eliminate the ability of ToxR to activate transcription while the protein's DNA binding function remains intact (Pfau and Taylor 1998).

TfoS

Outside its human host, *V. cholerae* lives in aquatic environments, where it is often found to colonize the chitinous exoskeleton of zooplanktons (Huq, Small et al. 1983). This association not only improves survival but also induces natural competence in the bacterium (Meibom, Blokesch et al. 2005, Nahar, Sultana et al. 2011). When chitin oligomers are present, TfoS, a membrane-bound transcriptional regulator, activates *tfoR* transcription (Dalia, Lazinski et al. 2014, Yamamoto, Mitobe et al. 2014). TfoR then interacts with the translational repressor Hfq and the stem-loop structure in the 5' UTR of *tfoX* mRNA (Yamamoto, Izumiya et al. 2011). These interactions expose the Shine-Dalgarno sequence of the *tfoX* mRNA to allow ribosome to be recruited for translation initiation, ultimately resulting in the activation of competence genes by TfoX.

Currently, the mechanism by which TfoS function is regulated by chitin is a subject of debate. One hypothesis holds that chitin is sensed and bound by the periplasmic chitin oligosaccharide-binding protein (CBP), which binds to an orphan histidine kinase ChiS in the absence of chitin (Yamamoto, Mitobe et al. 2014). Sequestration of CBP by chitin allows ChiS to stimulate TfoS in a phosphorelay-independent manner. The collaboration of ChiS and TfoS for *tfoR* transcription is not unlike that of ToxR and TcpP for *toxT* transcription, although ChiS lacks a DNA binding domain and does not appear to be a transcription activator. Another hypothesis proposes that chitin oligomers interact with and dimerize TfoS to allow activation of *tfoR* transcription in a ChiS-independent manner (Dalia, Lazinski et al. 2014).

CadC

Another member of the ToxR-like family of transcriptional regulators is the CadC protein, which has been characterized in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae*, and *Vibrio vulnificus* (Merrell and Camilli 2000, Kuper and Jung 2005, Rhee, Kim et al. 2005, Lee, Kim et al. 2007). CadC activates the *cadBA* operon in response to low pH and lysine to trigger the acid tolerance response (ATR) (Watson, Dunyak et al. 1992, Neely and Olson 1996, Lee, Kim et al. 2007). CadA catalyzes decarboxylation of lysine to produce cadaverine, whereas CadB serves as the lysine-cadaverine antiporter that secretes the cadaverine to neutralize external pH. Like the canonical sigma/anti-sigma factor signaling pathway, full-length CadC has to be cleaved to generate a biologically active N-terminal fragment that contains the DNA-binding domain (Lee, Kim et al. 2008). However, rather than being released into the cytoplasm like other membrane-bound sigma factors do after cleavage, truncated CadC fragments accumulate in the inner membrane to activate its target genes.

Subsequent effort to identify the genetic factors required for the proteolytic activation of CadC in *S. enterica* did not result in the discovery of the responsible protease. Instead, a putative phosphotransferase system permease was found to be required for CadC proteolysis (Lee, Kim et al. 2013). Lee *et al.* also showed that the low pH signal alone was sufficient to trigger degradation. On the other hand, a lysine signal activates ATR by inducing conformational changes in LysP, a lysine-specific permease that inhibits CadC via transmembrane and periplasmic interactions (Tetsch, Koller et al. 2008). Coupled with concomitant protonation of periplasmic amino acids in both proteins under acidic conditions, LysP inhibition is relieved, hence rendering CadC susceptible to proteolytic activation.

BcrR

Because they lack an outer membrane, Gram-positive bacteria can use membrane-bound transcription factors to directly perceive extracellular stimuli. One such example is BcrR of *Enterococcus faecalis*. This commensal species is known to be resistant to a wide range of antibiotics, and thus is one of the leading causes of nosocomial infections. BcrR imparts bacitracin resistance to *E. faecalis* by activating transcription of the bacitracin resistance operon – *bcrABD*, upon direct interaction with extracellular or membrane-associated Zn^{2+}-bound bacitracin (Gebhard, Gaballa et al. 2009). The genes *bcrA* and *bcrB* encode, respectively, the ATP-binding domains and the membrane-spanning domains of a homodimeric ATP-binding cassette (ABC) transporter that is responsible for expelling bacitracin from the cell (Manson, Keis et al. 2004). Similar to ToxR regulation of *toxT* transcription, BcrR requires membrane localization for activity (Gauntlett, Gebhard et al. 2008). However, ToxR only has one membrane-spanning domain whereas BcrR has four. Therefore, membrane localization is viewed

as playing a more prominent role for BcrR function. In addition, BcrR does not appear to use any ToxS/TcpH-like auxiliary proteins for function, nor is its activity inhibited by a LysP/anti-sigma factor-like protein.

CpsA

During the course of systemic dissemination, streptococcal pathogens encounter various host immune responses such as phagocytosis and complement deposition (Marques, Kasper et al. 1992, Locke, Colvin et al. 2007). As a strategy, streptococci produce polysaccharide capsules for immune evasion to ensure survival prior to reaching the site of colonization. Genetic evidence has shown that capsule levels are transcriptionally regulated by the membrane-bound CpsA protein (Cieslewicz, Kasper et al. 2001, Lowe, Miller et al. 2007). Functional analysis in the group B streptococcus *Streptococcus agalactiae* and the aquatic pathogen *Streptococcus iniae* established that CpsA activates transcription of the capsule operon by binding to promoter regions upstream of the *cpsA* and *cpsE* genes (Hanson, Lowe et al. 2011, Hanson, Runft et al. 2012). Unlike ToxR and TcpP, CpsA is a multiple-pass transmembrane protein with a short (20+ amino acids) N-terminal cytoplasmic domain, three transmembrane domains, and a large C-terminal extracellular domain (>400 amino acids) that presumably detects host environment cues. The cytoplasmic N terminus by itself is sufficient to bind DNA in *S. agalactiae* but not in *S. iniae*. The cytoplasmic loop between the second and third transmembrane domains is required for DNA binding in *S. iniae* and also for conferring binding specificity in conjunction with the extracellular domain in *S. agalactiae*.

Outlook

Given the breadth of functions regulated by bacterial membrane-bound transcription factors, this class of proteins has the potential to become prime targets for antimicrobial therapeutic compounds. Of particular interest, considering the emergence of multidrug resistant bacteria, is to determine the existence of other BcrR-like proteins that could confer resistance to other classes of antibiotics. Development of small molecules that disrupt either the sensing capacity or the DNA-binding function of such proteins should render a resistant bacterium susceptible again. The same strategy could also be used to inhibit the activity of other membrane-bound transcription factors, therefore reducing a bacterial pathogen's virulence or ability to survive in a specific environment (Anthouard and DiRita 2013). Identifying the signals that trigger gene activation by these unique transcription factors would also yield new insights into devising methods to control signal availability and access to the proteins. Moreover, the advent of single-molecule fluorescence microscopy has opened up the possibility to visualize interactions between membrane-bound transcription factors and other proteins in high resolution (Haas, Matson et al. 2014). This technology allows identification of novel interactive partners and elucidation of the mechanism that links signal sensing to transcription activation.

Regulated Intramembrane Proteolysis (RIP)

As noted in the section above, membrane-bound transcription activators TcpP, ToxR, and CadC are subjected to RIP under certain conditions. This mechanism has been extensively studied in eukaryotic systems (Brown, Ye et al. 2000, Urban and Freeman 2002, Wolfe and Kopan 2004), such as the controlled release of mammalian sterol regulatory element-binding

proteins (SREBPs) and epidermal growth factor receptor (EGFR) ligands. Moreover, RIP is implicated in Alzheimer's disease via the production of amyloidogenic Aβ peptides from cleavage of the β-amyloid precursor protein. More recently, overwhelming evidence has shown RIP to be a major contributor in bacterial transmembrane signaling processes involved in a wide gamut of cellular functions. These include stress response, biofilm formation, nutrient uptake, cell division, sporulation, cell-cell communication, and virulence.

As its name suggests, RIP involves cleavage of substrate proteins in the transmembrane domain by intramembrane cleaving proteases (I-CLiPs) (Weihofen and Martoglio 2003). In most, if not all known cases, intramembrane cleavage is preceded by another proteolytic event in the extracytoplasmic domain of the substrate protein, also generically termed site-1 proteolysis. This step creates the substrate for I-CLiPs in a subsequent process logically named site-2 proteolysis. In addition, cytoplasmic proteases may also participate in the further trimming of substrates, eventually resulting in a cellular response to the extracytoplasmic stimulus.

Based on the evidence so far, the RIP-inducing stimuli and their cognate site-1 proteases (S1P) vary among different processes. In contrast, the site-2 proteases (S2Ps) share a higher degree of similarity with each other. For bacteria, virtually all described RIP systems employ S2Ps of the zinc metalloprotease family. This family of proteases contains conserved HExxH and LDG motifs that permit binding of the catalytic zinc atom (Wolfe 2009). Studies to determine the substrate requirements of this enzyme uncovered a sequence independent ability to cleave at different intramembrane sites depending on the substrate, as long as the cleavage-susceptible

region possesses residues of low helical propensity (Akiyama, Kanehara et al. 2004), a feature that serves to stabilize the enzyme-substrate complex (Koide, Ito et al. 2008).

σ^E-Dependent Stress Response Pathway in *Escherichia coli*

No discussion of RIP is complete without considering the σ^E pathway in *E. coli*, arguably the best characterized RIP system in bacteria (Ades 2004, Alba and Gross 2004, Ehrmann and Clausen 2004). The cascade is activated when the bacterium is subjected to envelope stress, which causes outer membrane proteins (OMPs) to unfold and expose their C-terminal tails in the periplasm. This allows the C-terminal PDZ domain of the single-pass transmembrane S1P – DegS, to bind to exposed OMP residues that bear the consensus sequence YxF (Walsh, Alba et al. 2003, Wilken, Kitzing et al. 2004, Hasselblatt, Kurzbauer et al. 2007). As a consequence, the catalytic site of DegS, normally concealed by the PDZ domain, is uncovered and activates cleavage between amino acids Val-148 and Ser-149 in the periplasmic domain of the σ^E-sequestering transmembrane anti-sigma factor RseA (Cezairliyan and Sauer 2007). The truncated RseA species is subsequently cleaved between amino acids Ala-108 and Cys-109 in the transmembrane domain by the multispanning membrane S2P – YaeL, releasing the σ^E-bound RseA fragment into the cytoplasm (Akiyama, Kanehara et al. 2004). Cytoplasmic proteases, including ClpXP (Flynn, Levchenko et al. 2004, Chaba, Grigorova et al. 2007), completely remove the remaining RseA fragment from σ^E, which is thereby able to join the transcription machinery to activate expression of σ^E-regulated genes. These include the σ^E-encoding *rpoE*, heat-shock factor σ^{32}-encoding *rpoH*, envelope chaperone genes (*dsbC*, *fkpA*, *skp*, and *surA*), periplasmic protease gene *degP*, and also *yaeL* (Dartigalongue, Missiakas et al. 2001).

Another periplasmic protein – RseB, encoded in the same operon as RseA and σ^E, protects RseA from DegS cleavage by binding to a periplasmic region in RseA that does not overlap with the DegS recognition sequence (Missiakas, Mayer et al. 1997). Instead of responding to the C-terminal YxF motif of OMPs that activates DegS, RseB protection is alleviated by sequence elements upstream of the motif and also periplasmic LPS fragments, which level increases in the event of envelope stress (Cezairliyan and Sauer 2007, Chaba, Alba et al. 2011). In the absence of RseB and DegS, YaeL is able to cleave full-length RseA directly (Grigorova, Chaba et al. 2004). This is presumably due to the relief in inhibition by one of the two periplasmic PDZ domains of YaeL that plays a role in preventing premature cleavage of full-length RseA by the S2P (Kanehara, Ito et al. 2003).

σ^W Regulon in *Bacillus subtilis*

The Gram-positive bacterium *B. subtilis* is known to encode seven extracytoplasmic function (ECF) sigma factors, one of which is σ^W. Approximately 30 promoters, including that preceding the gene encoding σ^W (*sigW*), are regulated by the transcription factor (Huang, Fredrick et al. 1998). As the integral component of an antibiosis regulon that protects the bacterium from various antimicrobial compounds (Cao, Wang et al. 2002, Pietiainen, Gardemeister et al. 2005, Butcher and Helmann 2006, Helmann 2006), σ^W has also been shown to be activated by phage infection and alkaline shock (Wiegert, Homuth et al. 2001). In the absence of activating signals, σ^W is sequestered by the anti-sigma factor RsiW. But the presumed sensing of antimicrobial compounds by the multispanning membrane S1P PrsW, which belongs to the MEM-superfamily of proteases, activates proteolysis between Ala-168 and Ser-179 of RsiW (Ellermeier and Losick 2006, Heinrich and Wiegert 2006). This removes 40

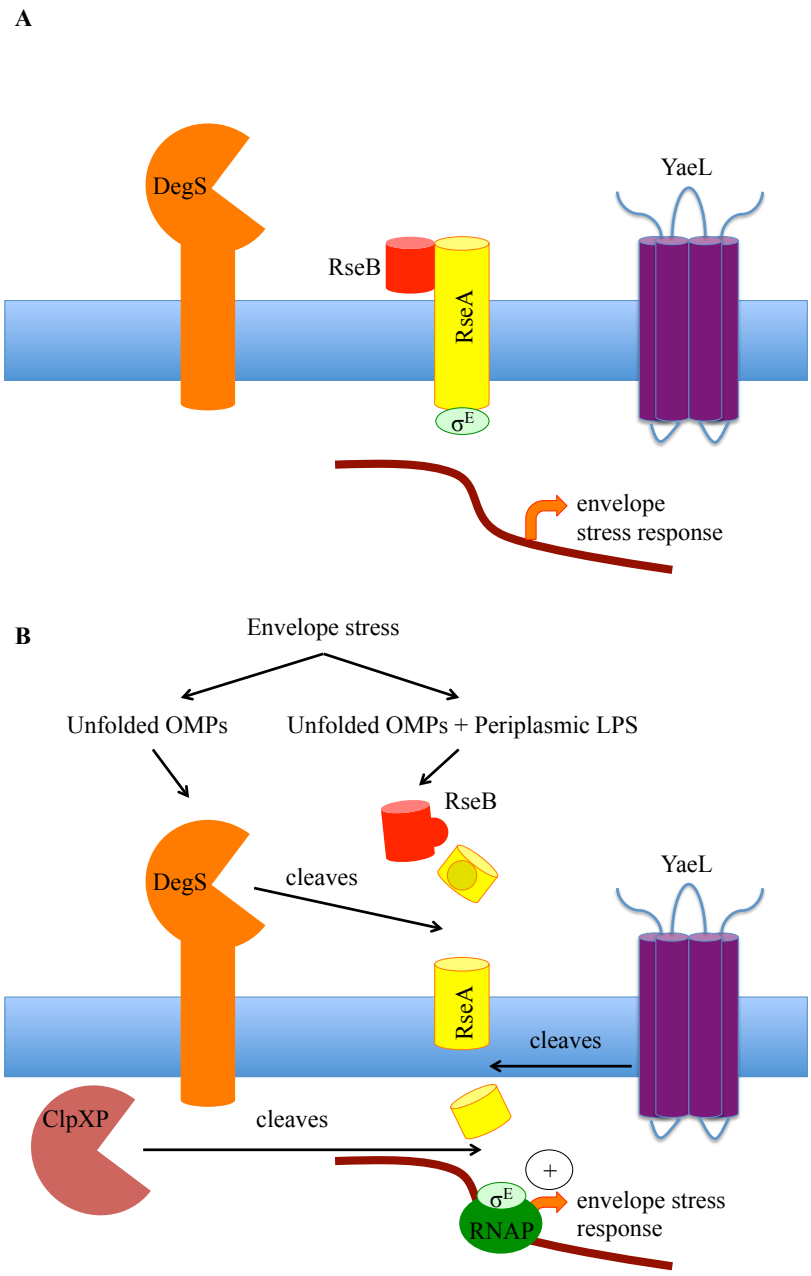

Figure 1.3. σ^E-Dependent Stress Response Pathway in *E. coli*. (A) In the absence of envelope stress, σ^E is sequestered to the inner membrane by the anti-sigma factor protein RseA. (B) Envelope stress results in the unfolding of OMPs and elevation of periplasmic LPS levels. These molecular signals relieve RseB protection of RseA. Exposed YxF-motif containing C-termini of unfolded OMPs activate DegS, which cleaves RseA in the periplasmic domain. YaeL then cleaves RseA in the transmembrane domain to release σ^E into the cytoplasm. Further processing by ClpXP removes any remaining RseA fragment still attached to σ^E, therefore liberating σ^E to bind to RNAP and initiate transcription of σ^E-controlled genes.

extracytoplasmic amino acids from the C-terminus to allow other extracytoplasmic proteases including Tsp to remove most of the rest of the extracytoplasmic domain (Heinrich, Hein et al. 2009). The action by the PrsW-Tsp proteolytic module renders RsiW susceptible to the second proteolytic module, which consists of RasP and ClpXP (Heinrich, Hein et al. 2009). Like the S2P of RseA in *E. coli*, RasP cleaves RsiW in its transmembrane domain to release σ^W into the cytoplasm, revealing a cryptic proteolytic tag in the process. This tag is recognized by cytoplasmic proteases such as ClpXP, which cleaves off the remaining RsiW fragment still attached to σ^W. The liberated σ^W is then able to bind to core RNAP and initiate transcription of genes in the σ^W regulon. Unlike RseA in the σ^E pathway, RsiW does not have an RseB-like protein that protects the anti-sigma factor from proteolysis.

AlgU-Dependent Alginate Biosynthesis in *Pseudomonas aeruginosa*

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen that is commonly implicated as a cause of morbidity and mortality in cystic fibrosis (CF) patients. The bacterium generates copious amounts of alginate, a polysaccharide that contributes to the mucus buildup in the lungs and intestine as a result of biofilm formation. The mucoid phenotype allows *P. aeruginosa* to endure host defenses and antibiotic challenge.

The ECF sigma factor AlgU (also known as AlgT), a σ^E ortholog, is responsible for activating transcription of the *algD-algA* alginate biosynthesis operon. In the absence of activating signal, AlgU is tethered to the inner membrane by the anti-sigma factor MucA, which is in turn bound by the periplasmic protein MucB. However, once LPS fragments sequester MucB away from MucA, the anti-sigma factor becomes exposed to RIP (Lima, Guo et al. 2013).

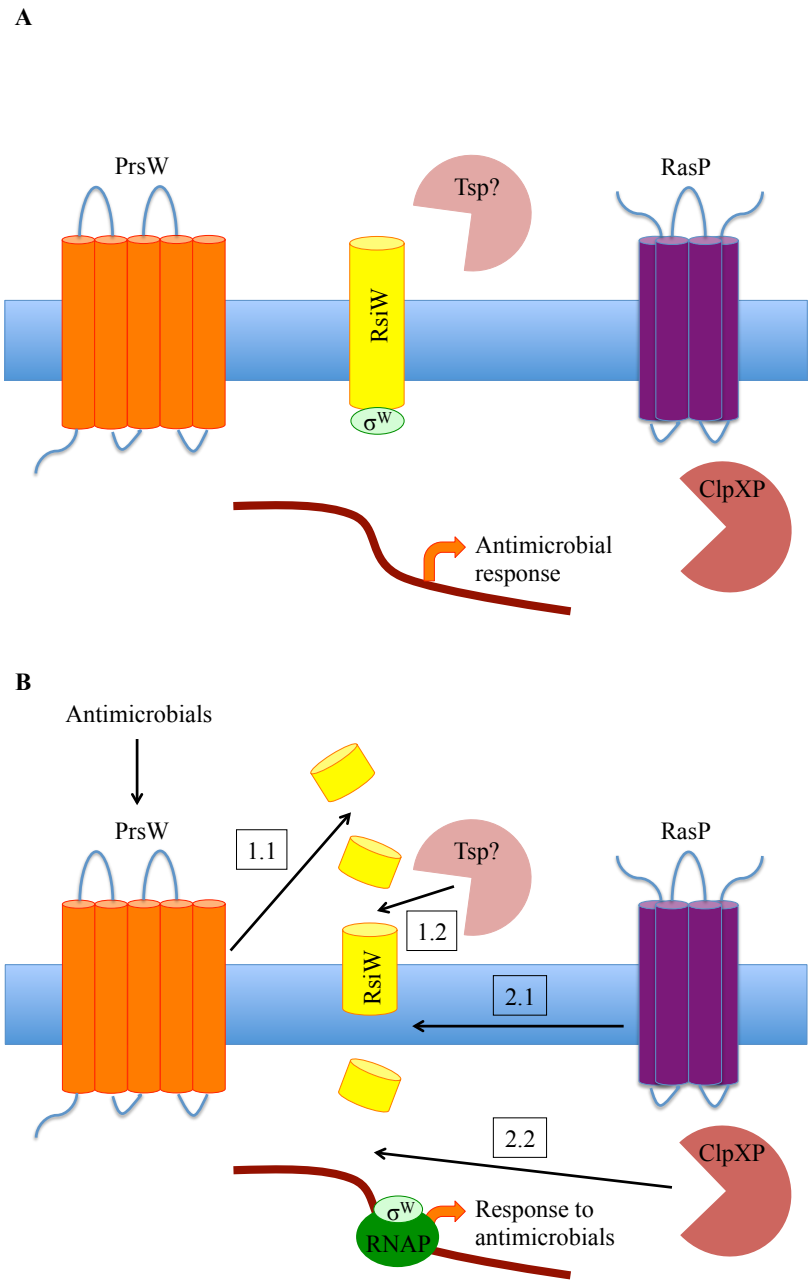

Figure 1.4. σ^W Regulon in *Bacillus subtilis*. (A) In the absence of antimicrobials, σ^W is sequestered to the membrane by the anti-sigma factor protein RsiW. (B) Antimicrobials trigger cleavage of RsiW in the extracellular domain by proteolytic module I – first by PrsW (1.1), followed by other extracytoplasmic proteases such as Tsp (1.2). The remaining RsiW fragment then becomes the substrate for proteolytic module II – the first protease being the S2P RasP, which cleaves in the transmembrane domain (2.1) to release σ^W into the cytoplasm. Further processing by ClpXP removes any remaining RsiW fragment still attached to σ^W, therefore liberating σ^W to bind to RNAP and initiate transcription of σ^W-controlled genes.

Accumulation of MucE, a small (9.5 kDa) likely outer membrane protein, triggers MucA proteolysis via AlgW (DegS) and MucP (YaeL) (Qiu, Eisinger et al. 2007). Like the signal in the σ^E pathway, the C-terminal motif (WVF) of MucE is critical for relieving inhibition of AlgW by its PDZ domain. Another more recently identified signal that activates AlgW is the periplasmic accumulation of the adhesive protein CupB5, which is normally secreted with facilitation of the CupB4 chaperone (de Regt, Yin et al. 2014). CupB5 appears to act synergistically with MucE, but instead relies on an internal TVV motif for AlgW activation.

Interestingly, the highly mucoid phenotype of clinical isolates from CF patients has been demonstrated to be the consequence of mutations in *mucA*, notably ones that generate premature stops and severely truncated forms of MucA (Pulcrano, Iula et al. 2012). Instead of AlgW, Tsp is the protease that acts on these variants, most likely in a MucE-independent manner (Reiling, Jansen et al. 2005). In contrast, processing of full-length MucA does not require Tsp (Qiu, Eisinger et al. 2007).

Another important regulator of alginate biosynthesis is MucD, encoded by a member of the AlgU-activated *algU mucABCD* operon. In *E. coli*, the MucD homolog (HtrA/DegP) is hypothesized to eliminate misfolded proteins that can activate DegS. MucD appears to play the same role in regulating AlgW activity (Qiu, Eisinger et al. 2007), in the process inhibiting the direct proteolysis of MucA by MucP independently of AlgW (Damron and Yu 2011).

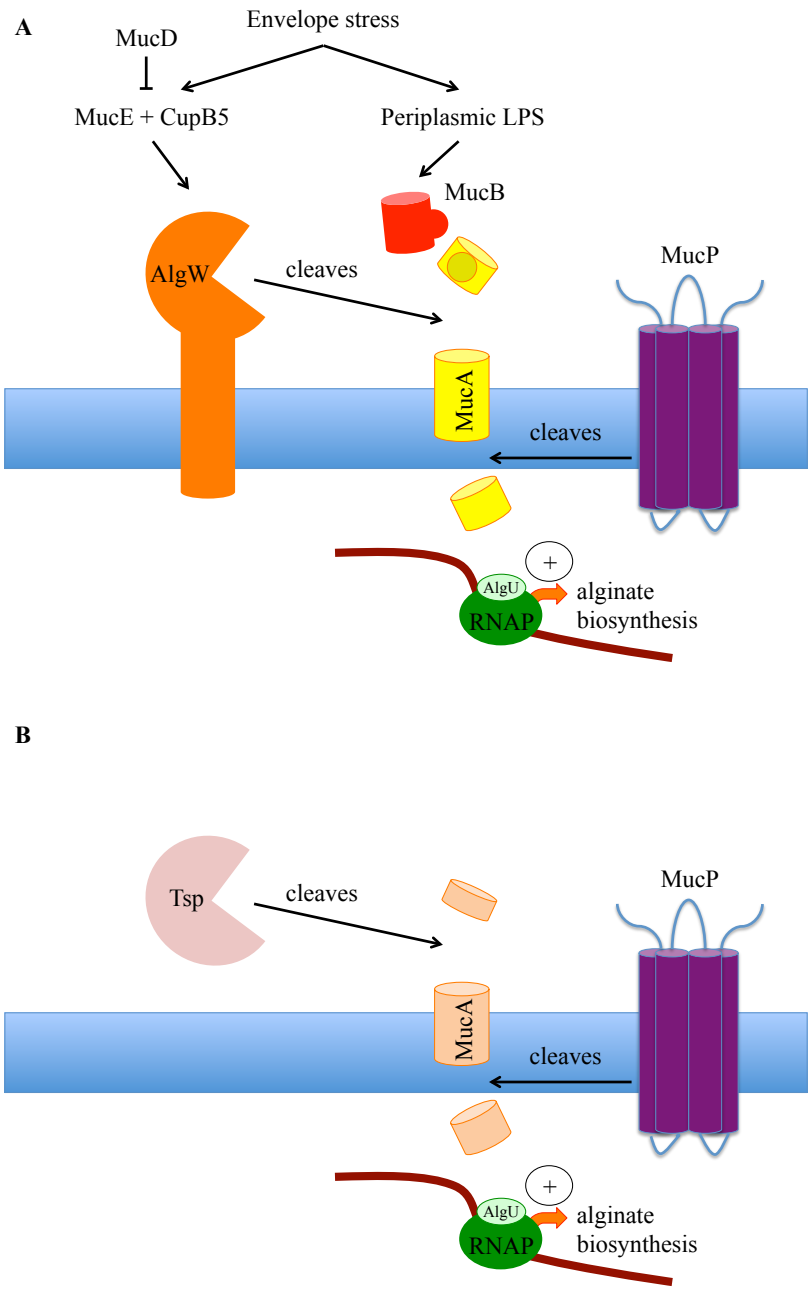

Figure 1.5. Two Modes of MucA Degradation in *Pseudomonas aeruginosa*. (A) Envelope stress results in accumulation of LPS fragments in the periplasm, sequestering MucB away from the anti-sigma factor MucA. Simultaneously, accumulation of MucE and CupB5 activates AlgW, which then cleaves MucA to create a substrate for further processing by MucP. This releases the sigma factor AlgU into the cytoplasm to bind RNA polymerase and activate transcription of alginate biosynthesis genes. MucD inhibits alginate biosynthesis by reducing accumulation of MucE and CupB5. (B) *mucA* mutant cells, commonly found in cystic fibrosis patients, produce truncated MucA proteins that are subjected to proteolysis by Tsp, instead of AlgW, prior to MucP processing.

Regulators of TcpP Stability

Previously, Beck et al. (2004) demonstrated that a mutant of *V. cholerae* lacking TcpH produced barely detectable levels of TcpP, even though *tcpP* transcription remained intact. This suggested that TcpP is subjected to degradation, perhaps as a means to regulate virulence gene expression under unfavorable conditions. A genetic screen to identify transposon mutants which TcpP levels were restored in the absence of TcpH uncovered a mutant with a transposon insertion in *yaeL* – the gene encoding the S2P of anti-sigma factors in known RIP systems. *tcpH*/*yaeL* mutants accumulate a TcpP species that is of lower molecular weight than the full-length protein, indicating there is at least one other protease that cleaves TcpP prior to YaeL. However, the identity of this protease was not revealed in the screen. Deletion of genes that encode serine proteases (DegS, DegP, Vc0157, and Vca0803) in a *tcpH*/*yaeL* mutant showed that none of these candidate proteases is responsible for the initial cleavage of TcpP (Matson and DiRita 2005).

The primary objective of work in this thesis was to identify and characterize the initial protease that contributes to TcpP degradation prior to YaeL. As discussed in the previous section, initial proteases of other RIP systems often detect molecular signals, upon which site-1 cleavage of the respective substrates are activated. Knowing which protease acts on TcpP first is imperative for establishing the molecular signal that triggers RIP of the membrane-bound transcription activator.

This dissertation also seeks to address the interaction between TcpP and TcpH, where TcpH has always been thought of as protecting TcpP from degradation – in the same way that

RseB and MucB inhibit proteolysis of RseA and MucA respectively. My findings suggest a more reciprocal relationship and demonstrate the possible role of RIP in regulating TcpH levels.

Furthermore, I attempt to identify other regulators of TcpP stability in light of what is already known in homologous RIP systems described in the previous section. In line with this effort, I also scrutinize the hypothesis that downstream gene products in the ToxR regulon may regulate TcpP levels via a negative feedback loop. Briefly, I examine the *in vivo* significance of the two known proteases of TcpP using an infant mouse model of colonization. Finally, I end this dissertation by discussing how all these results fit into the existing body of research on RIP and pave the way for future studies that will further elucidate the mechanism by which virulence is regulated in *V. cholerae*.

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CHAPTER 2

REGULATED INTRAMEMBRANE PROTEOLYSIS OF THE VIRULENCE ACTIVATOR TCPP IN *VIBRIO CHOLERAE* IS INITIATED BY THE TAIL-SPECIFIC PROTEASE (TSP)

SUMMARY

Vibrio cholerae uses a multiprotein transcriptional regulatory cascade to control expression of virulence factors cholera toxin and toxin-coregulated pilus. Two proteins in this cascade are ToxR and TcpP – unusual membrane-localized transcription factors with relatively undefined periplasmic domains and transcription activator cytoplasmic domains. TcpP and ToxR function with each other and two other membrane-localized proteins, TcpH and ToxS, to activate transcription of *toxT*, encoding the direct activator of toxin and pilus genes. Under some conditions, TcpP is degraded in a two-step proteolytic pathway known as regulated intramembrane proteolysis (RIP), thereby inactivating the cascade. The second step in this proteolytic pathway involves the zinc metalloprotease YaeL; *V. cholerae* cells lacking YaeL accumulate a truncated yet active form of TcpP termed TcpP*. We hypothesized that a protease acting prior to YaeL degrades TcpP to TcpP*, which is the substrate of YaeL. In this study, we demonstrate that a C-terminal protease called Tsp degrades TcpP to form TcpP*, which is then acted upon by YaeL. We present evidence that TcpH and Tsp serve to protect full-length TcpP from spurious proteolysis by YaeL. Cleavage by Tsp occurs in the periplasmic domain of TcpP, and requires residues TcpPA172 and TcpPI174 for wild-type activity.

INTRODUCTION

Expression of virulence factors cholera toxin and toxin-coregulated pilus in *Vibrio cholerae* is the result of a multiprotein transcription regulatory cascade that includes membrane-associated transcription factors, ToxR and TcpP. These bitopic membrane proteins have carboxy-terminal periplasmic domains and amino-terminal cytoplasmic DNA binding/transcription activator domains similar to activators of the OmpR/PhoB family (Martinez-Hackert and Stock 1997). ToxR and TcpP work in conjunction with other membrane proteins, ToxS and TcpH, respectively, which are less well characterized but likely interact with the cognate activator within the periplasmic space to regulate either its activity or stability (Hase and Mekalanos 1998, Beck, Krukonis et al. 2004). This complex of membrane proteins activates transcription of *toxT*, encoding the direct transcription activator of the two principal virulence factors of *V. cholerae*, cholera toxin and toxin-coregulated pilus (DiRita and Mekalanos 1991).

Cellular TcpP levels are regulated at multiple levels of expression (Matson and DiRita 2005, Matson, Withey et al. 2007). Two activators, AphA and AphB, control transcription, which in most strains is subject to a quorum-sensing pathway involving numerous small, regulatory RNAs (Kovacikova and Skorupski 1999, Miller, Skorupski et al. 2002). Post-translational regulation of TcpP involves its degradation by a process with the hallmarks of regulated intramembrane proteolysis (RIP), a mechanism conserved from bacteria to humans (reviewed in Brown, Ye et al. 2000). In the general form of the mechanism, a membrane protein is degraded in two sequential steps by proteases generically termed site-1 and site-2 proteases. The site-1 protease makes an initial cleavage in the target protein, revealing the substrate for the site-2 protease, which cleaves the target protein again to remove it from the membrane

altogether. In many well-studied examples, RIP leads to gene activation through destruction of a transcription inhibitor that sequesters an activator to the membrane. Upon sequential cleavage by the site-1 and site-2 proteases, the activator is liberated from the membrane and becomes free to interact with the basal transcription apparatus. For example, in *E. coli*, the transmembrane protein RseA binds to a sigma factor, σ^E, keeping it in the membrane unable to activate transcription. In response to envelope stress, RseA is sequentially degraded by the action of two proteases: DegS (site-1 protease) makes an initial cleavage and produces the substrate for YaeL (site-2 protease, also called RseP), which subsequently cleaves RseA and removes it from the membrane. In the process, σ^E is released and becomes associated with the transcription apparatus to activate envelope stress response genes (Chaba, Alba et al. 2011). RseA is protected from degradation by a small periplasmic protein called RseB. Although the process of RIP that removes RseA from the cell is typically an ordered, two-step process with YaeL acting only after DegS, in cells that lack DegS and RseB by mutation, YaeL can act on full length RseA (Grigorova, Chaba et al. 2004). An emerging example of RIP is in the regulation of alginate biosynthesis in *Pseudomonas aeruginosa*; alginate contributes to the mucoid phenotype of clinical strains from cystic fibrosis (CF) patients (reviewed in Damron and Goldberg 2012). Degradation of the transmembrane anti-sigma factor MucA leads to release of the extracytoplasmic sigma factor σ^{22} (AlgU/T), resulting in alginate overproduction as a response to membrane stress (Wood, Leech et al. 2006). AlgW and MucP, orthologues of DegS and YaeL, respectively, act in sequence to cleave MucA, although another protease, AlgO, can act on mutant MucA proteins, which are often expressed in CF isolates (Reiling, Jansen et al. 2005, Qiu, Eisinger et al. 2007, Pulcrano, Iula et al. 2012).

Degradation of TcpP occurs in two steps similar to RIP pathways described above, albeit with a different outcome because TcpP is an activator, rather than an anti-activator like RseA and MucA. RIP of TcpP is more readily observed in cells lacking TcpH, suggesting a protective role for TcpH similar to that of RseB, although processing can occur even in the presence of TcpH under specific conditions unfavorable for virulence gene activation (Matson and DiRita 2005). The instability of TcpP in cells lacking TcpH prompted a genetic screen to identify the protease(s) responsible for TcpP degradation (Matson and DiRita 2005). This approach identified an insertion in *yaeL*, and further characterization demonstrated that *yaeL* mutant bacteria cleave TcpP into an active, though truncated, form (TcpP*) that accumulates in the absence of YaeL. We hypothesized that TcpP* is the product of the site-1 protease acting prior to YaeL. Because DegS acts before YaeL on RseA in *E. coli*, we tested *degS/yaeL* mutant *V. cholerae*. However, that mutant continues to accumulate TcpP*, therefore ruling out DegS as the site-1 protease of TcpP (Matson and DiRita 2005). We attribute the fact that our screen did not identify the site-1 protease to one of the following reasons: i) it is essential; ii) our mutagenesis was not saturating; iii) we were overly stringent in scoring the level of ß-galactosidase activity that mutants in our screen needed to express in order to be called a potential hit.

To identify the site-1 protease that provides the YaeL substrate to control TcpP levels, we took two approaches: i) refining and extending our earlier genetic screen and ii) testing mutants lacking putative proteases. Both approaches yielded the same protease, the C-terminal "tail-specific" protease Tsp. We demonstrate that Tsp degrades TcpP to produce the YaeL substrate TcpP*. We also localize the region of TcpP where Tsp acts, and identify residues in TcpP

required for Tsp cleavage. Finally, we demonstrate that TcpH and Tsp inhibit spurious, direct degradation of TcpP by YaeL.

RESULTS

Genetic and reverse genetic screens identify *vc1496* as encoding a candidate TcpP site-1 protease. In our original screen, we exploited the fact that Δ*tcpH toxT-lacZ* cells appear as light blue colonies on X-gal media, reflecting low levels of *toxT* transcription due to the instability of TcpP in cells lacking TcpH. Mutants with insertions in *yaeL* express TcpP*, which is stable and active in the *tcpH*/*yaeL* mutant background. We did not uncover the site-1 protease in this screen originally, suggesting one of two possibilities: i) there was a technical flaw in the screen, perhaps being non-saturating or having too stringent a cutoff for scoring potential mutants, or ii) the gene encoding this protease is essential. Insertions into *yaeL* exhibited *toxT-lacZ* expression levels close to wild type in a *tcpH* mutant background; these colonies arose as dark blue on X-gal. In retrospect we reasoned that the nearly wild type levels of *toxT-lacZ* in cells with TcpP* may be due to the fact that this species is active and accumulates in the *yaeL* mutant.

We rescreened the insertion library for mutants that produce lighter blue colonies to avoid identifying insertions in *yaeL* again. Two such colonies carried insertions in *vc1496*, encoding a protein annotated as a "tail-specific protease" (Tsp). In *E. coli*, Tsp cleaves an 11-residue C-terminal peptide from the precursor form of penicillin-binding protein 3 (PBP3) and is hypothesized to protect cells from thermal and osmotic stresses (Hara, Nishimura et al. 1989, Nagasawa, Sakagami et al. 1989, Hara, Yamamoto et al. 1991). The Δ*tcpH/toxT-lacZ* strain carrying insertions in the *vc1496* strain expressed approximately 100 units of ß-galactosidase,

well above background, but only about 30% of that expressed either in the wild-type *toxT-lacZ* strain or in the Δ*tcpH toxT-lacZ* strain with a mutation in *yaeL* (Table 2.1).

Table 2.1. *toxT-lacZ* Expression in Wild Type and *Mariner*-Induced Protease Mutants

Strain	**Miller Units**
O395 *toxT-lacZ*	342.3 +/- 35.6
O395 Δ*tcpH toxT-lacZ*	54.0 +/- 2.9
O395 Δ*tcpH toxT-lacZ*/*yaeL*::TnFGL3	339.0 +/- 94.6
O395 Δ*tcpH toxT-lacZ*/*vc1496-1*::TnFGL3	89.7 +/- 13.7
O395 Δ*tcpH toxT-lacZ*/*vc1496-2*::TnFGL3	107.3 +/- 4.7

To expand our identification of potential site-1 proteases further, we carried out a candidate gene approach to test specific genes annotated as proteases, emphasizing those predicted to be membrane or periplasmic proteins. We used a collection of ordered transposon-insertion mutants (Cameron, Urbach et al. 2008); insertions were introduced into wild-type strain O395 or O395 Δ*tcpH* using transducing phage CP-T1ts (Hava and Camilli 2001). Immunoblotting was then used to assess TcpP stability in the absence of TcpH. We reasoned that a strain deficient for a TcpP-specific protease would exhibit full-length TcpP in the absence of TcpH, unlike wild type and any other mutants lacking insertions in the TcpP protease, which would still degrade TcpP in the absence of TcpH.

One mutant carrying the transposon in *vc1496* exhibited partial recovery of full-length TcpP in the absence of TcpH, suggesting that RIP is not fully functional in that mutant background (Fig. 2.1, lane 12). In contrast, mutants lacking other predicted proteases produced wild type levels of full-length TcpP in the presence of TcpH, but significantly lower levels in its

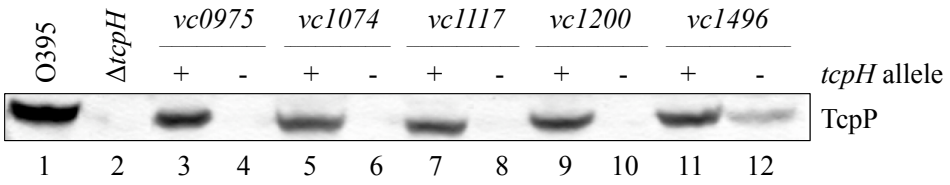


Fig. 2.1. Effect of Transposon Insertion in Candidate Protease Genes on TcpP Stability. The following transposon-inserted alleles were transduced from C6706 into O395 wild type (lanes 3, 5, 7, 9 and 11) and O395 Δ*tcpH* (lanes 4, 6, 8, 10 and 12) backgrounds: *vc0975* (lanes 3 and 4), *vc1074* (lanes 5 and 6), *vc1117* (lanes 7 and 8), *vc1200* (lanes 9 and 10) and *vc1496* (lanes 11 and 12). Overnight cultures were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP.

absence, indicating that proteolysis through the RIP pathway remained intact in those mutants. This is consistent with our genetic screen in which mutants with mariner transposon insertions exhibited partially restored (30% of wild type) levels of *toxT-lacZ* expression. Loss of *tcpH* in El Tor *V. cholerae* strain C6706 resulted in diminished steady state levels of TcpP, similar to what we observed in the classical strain O395. Wild type levels were restored in a strain carrying a mutation in *vc1496*, indicating a role in stability of TcpP in the El Tor background as well, although in the absence of *vc1496*, TcpP may be processed slightly, based on its increased mobility in the gel (Fig. S2.1). This suggests another protease that may cleave TcpP prior to Tsp, which is discussed further below. We introduced mutations into several other predicted protease genes to determine whether any of these might contribute to this slight decrease in size of TcpP in cells lacking *vc1496*, but were unable to identify any of them as playing such a role (Fig. S2.2). Given that *vc1496* arose in both the genetic and reverse genetic screens, and contributes to instability of TcpP in both classical and El Tor backgrounds, we explored its activity further.

Vc1496 (Tsp) activity on TcpP provides the YaeL substrate TcpP*. To examine whether Tsp produces TcpP*, the substrate for YaeL, we compared TcpP production in a Δ*tsp* Δ*yaeL* mutant versus a Δ*tsp* Δ*yaeL* Δ*tcpH* mutant after ectopic induction of *tsp* gene expression (Fig. 2.2). In the double mutant, which retains TcpH, TcpP remained at full length after *tsp* induction, consistent with earlier work indicating a role for TcpH in protecting TcpP from degradation by the first-site protease acting before YaeL (Matson and DiRita 2005) (left panel in Fig. 2.2). In contrast, within thirty minutes of inducing *tsp* in the triple mutant lacking TcpH, a lower molecular weight species corresponding in size to TcpP* accumulated (right panel in Fig. 2.2). By three hours, TcpP* had accumulated to high levels and full-length TcpP was barely

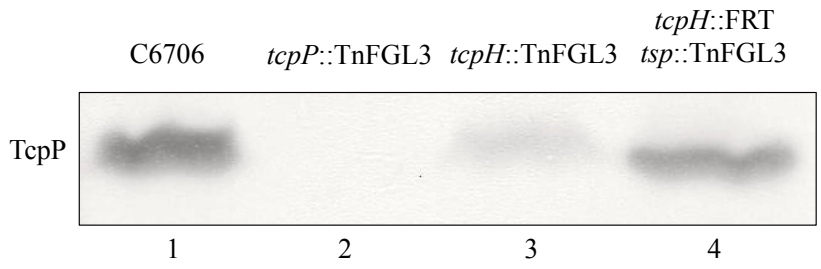


Fig. S2.1. Effect of *tsp* Mutation on TcpP Stability in the El Tor Biotype. The transposon-inserted *tsp* allele (*tsp*::TnFGL3) was transduced into a C6706 *tcpH* mutant that had its kanamycin resistance cassette removed by FLP recombinase-mediated excision (*tcpH*::FRT). Overnight cultures were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP.

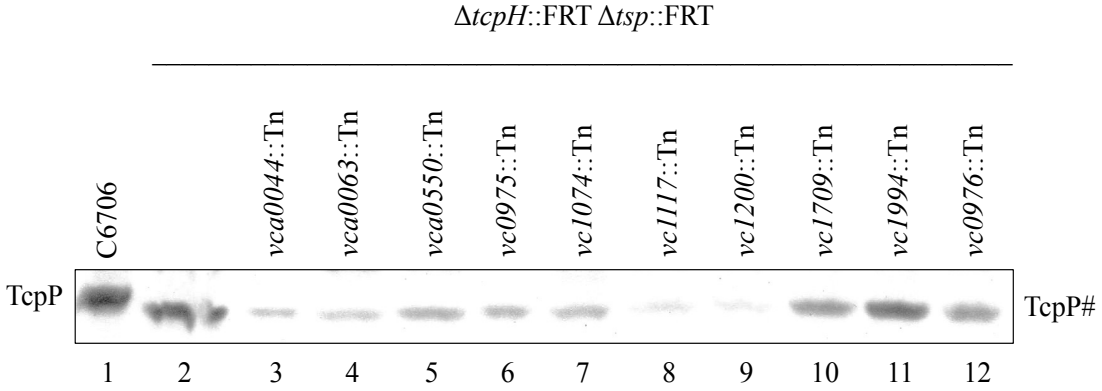


Fig. S2.2. Effect of Transposon Insertion in Candidate Protease Genes on TcpP Stability of an El Tor *tcpH*/*tsp* Mutant. The following transposon-inserted alleles were transduced into a C6706 *tcpH*/*tsp* mutant (lanes 3 to 12): *vca0044* (lane 3), *vca0063* (lane 4), *vca0550* (lane 5), *vc0975* (lane 6), *vc1074* (lane 7), *vc1117* (lane 8), *vc1200* (lane 9), *vc1709* (lane 10), *vc1994* (lane 11), and *vc0976* (lane 12). Overnight cultures were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP.

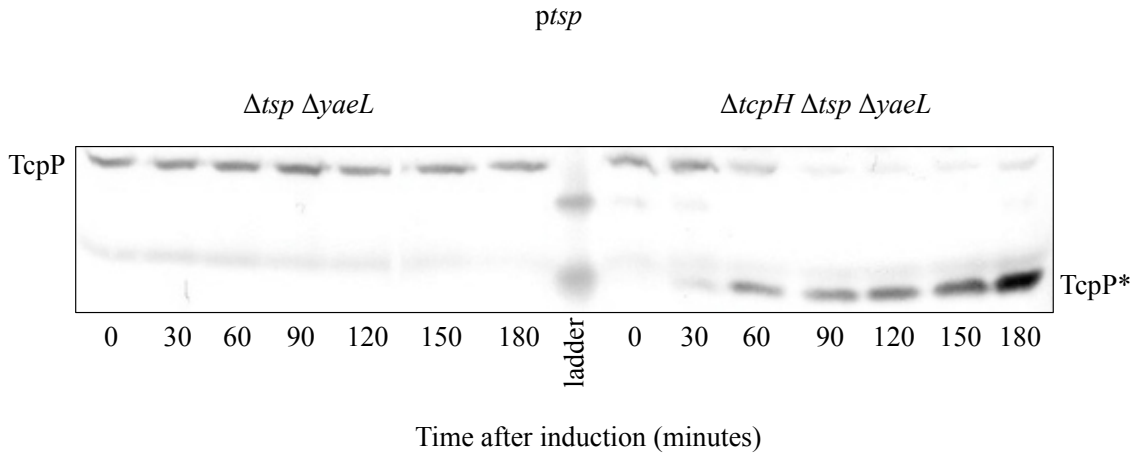


Fig. 2.2. TcpP* Accumulation as a Function of *tsp* Expression. Overnight cultures of Δ*tsp* Δ*yaeL* and Δ*tcpH* Δ*tsp* Δ*yaeL* cells containing pBAD18-Kan-*tsp* were subcultured 1:50 and grown to midlogarithmic phase in pH 6.5 LB at 30°C. Arabinose was then added (final concentration, 0.1%) and grown at the same temperature for an additional 3 h. Samples were harvested for Western blot analysis before the addition of arabinose (time 0), and at various time points. Samples were separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP.

detectable. Considering that i) mutation of *vc1496* (*tsp*) results in increased stability of full-length TcpP in the absence of TcpH and ii) induced expression of *tsp* caused accumulation of the YaeL substrate TcpP* in cells lacking *tcpH* but not in such cells expressing *tcpH*, we conclude that Tsp acts prior to YaeL in the RIP pathway regulating TcpP levels, and that TcpH blocks the action of Tsp in degrading TcpP. The presence of an intermediate band beneath the full-length protein in some of the lanes (Fig. 2.2) suggests that rare activity by other proteases may also cleave TcpP under these conditions.

Tsp overcomes the TcpH blockade to degrade TcpP. TcpP cleavage by the site-1 protease occurs conditionally in cells expressing both *tcpP* and *tcpH*, with much less occurring at pH 6.5/30˚C than at pH 8.5/37˚C (these conditions have been used as "toxin inducing" and "toxin non-inducing" respectively, in classical strains of *V. cholerae*) (Matson and DiRita 2005). To establish whether this is a feature of Tsp-dependent site-1 proteolysis of TcpP, we analyzed TcpP production in Δ*tsp* Δ*yaeL* cells in these two conditions by expressing *tsp* under arabinose induction in toxin inducing and toxin non-inducing conditions. Cultures were grown overnight at pH 6.5/30˚C, and then split into two cultures, one at pH 6.5/30˚C, and the other at pH 8.5/37˚C. Arabinose was added to induce *tsp* expression and TcpP/TcpP* levels were analyzed by immunoblotting over time. We used cells lacking YaeL (Δ*yaeL*) because doing so readily reveals the action of the first protease via accumulation of TcpP*, which does not get processed further in the absence of YaeL.

Tsp had little effect on TcpP at pH 6.5/30˚C – toxin inducing conditions – as evidenced by the lack of TcpP* production. Shifting the cultures to pH 8.5/37˚C without inducing *tsp*

resulted in small amounts of TcpP* production at the later time points, while significantly more TcpP* was observed after inducing Tsp with arabinose (Fig. 2.3). The limited TcpP* production without arabinose induction is likely from leakiness at the arabinose promoter in the plasmid expressing *tsp*, as we do not observe TcpP* at pH 8.5/37˚C simply by culturing Δ*tsp* Δ*yaeL* cells in those conditions (Fig. S2.3). Lack of TcpP* accumulation at pH 6.5/30˚C even when *tsp* is overexpressed (Fig. 2.3) confirms that TcpH blocks Tsp under conditions favorable for virulence gene expression. As was seen in Fig. 2, an intermediate band accumulated underneath the full-length protein in cultures grown at pH 8.5/37˚C in the absence of arabinose, but not when arabinose was present (Fig. 2.3). With our earlier findings, these results are consistent with a model in which TcpP degradation occurs in at least two steps, with Tsp cleaving prior to YaeL, and perhaps another protease acting before Tsp. TcpH serves to conditionally block the pathway.

Tsp and TcpH prevent access to TcpP by YaeL. We observed diminished levels of full length TcpP in cells that lack Tsp and TcpH but express *yaeL* (Fig. 2.1, lane 12), and a corresponding decrease in *toxT-lacZ* expression relative to wild type in the Δ*tcpH vc1496*::TnFGL3 mutant (Table 2.1). This is reminiscent of what has been reported in the regulated intramembrane proteolysis of the *E. coli* anti-sigma factor RseA by DegS and YaeL (RseP). RseA is normally protected from degradation by RseB, but when the first protease (DegS) and RseB are both lost due to mutation, YaeL (RseP) can access RseA and degrade it directly (Grigorova, Chaba et al. 2004). We hypothesized that YaeL can similarly attack TcpP when the first protease (Tsp) and the co-expressed protease inhibitory protein (TcpH) are missing.

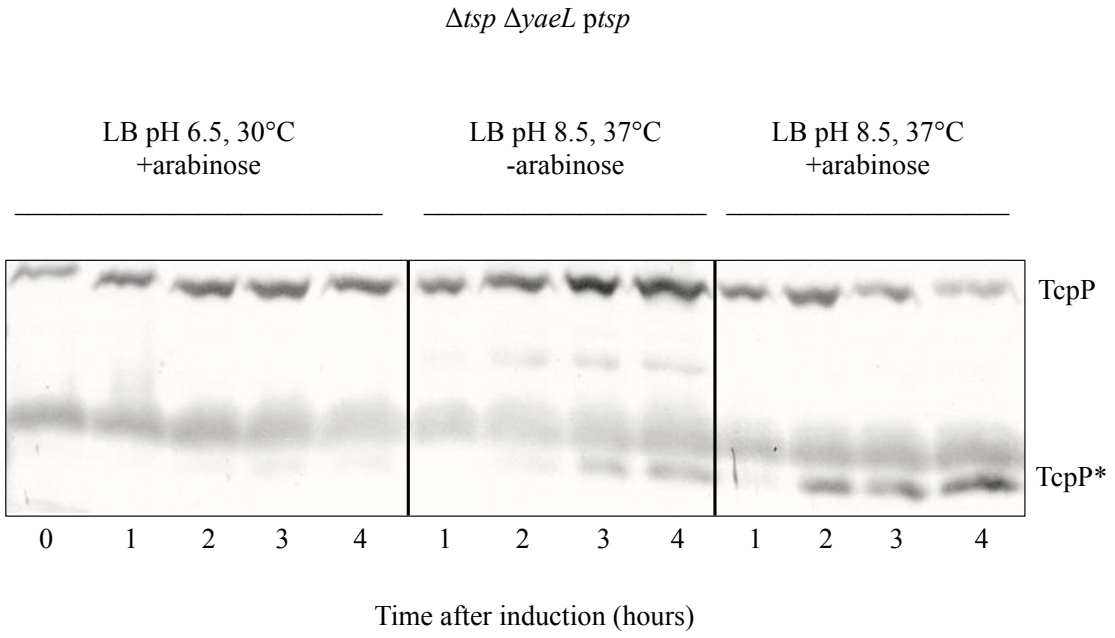


Fig. 2.3. TcpP Proteolysis in the Presence of TcpH. Overnight cultures of O395 Δ*tsp* Δ*yaeL/p*BAD18-Kan-*tsp V. cholerae* were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Cell culture was split into two equal portions for harvest by centrifugation and resuspended either in pH 6.5 LB and grown at 30°C or in pH 8.5 LB and grown at 37°C for an additional 4 h, both in the presence of arabinose. Samples were harvested for Western blot analysis before resuspension in new media (time 0), and at each hour after resuspension for both conditions. Samples were separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP. Ghost protein bands that were slightly bigger than TcpP* were present in all lanes.

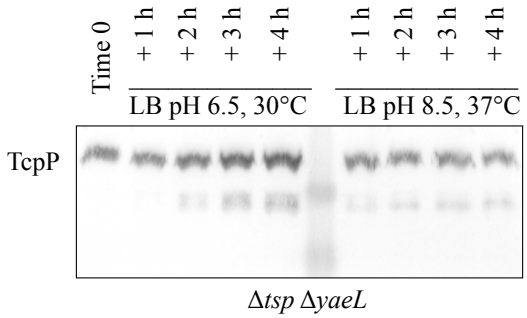
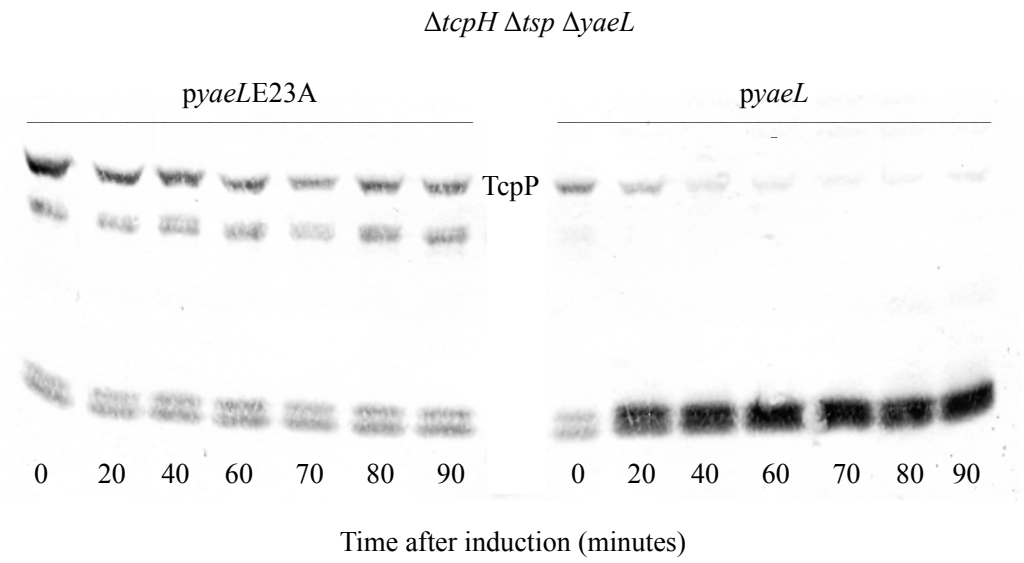


Fig. S2.3. TcpP Proteolysis in Δ*tsp* Δ*yaeL* Cells. Overnight cultures of O395 Δ*tsp* Δ*yaeL V. cholerae* were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Cell culture was split into two equal portions for harvest by centrifugation and resuspended either in pH 6.5 LB and grown at 30°C or in pH 8.5 LB and grown at 37°C for an additional 4 h. Samples were harvested for Western blot analysis before resuspension in new media (time 0), and at each hour after resuspension for both conditions. Samples were separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP. Detection of smaller TcpP-antibody reactive bands indicates possible nonspecific degradation of TcpP.

A



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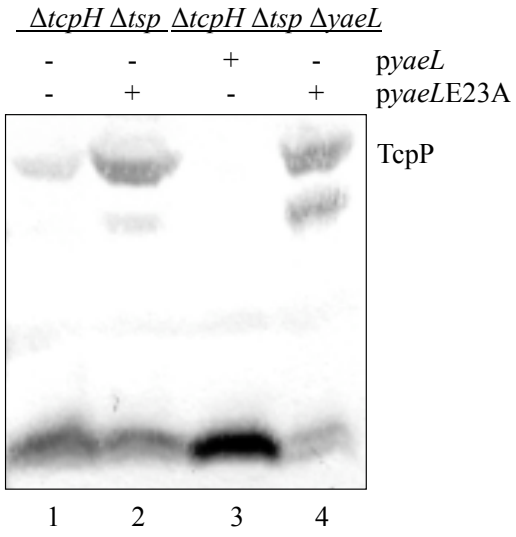

Fig. 2.4. Effects of *yaeL* Expression on TcpP Stability in the Absence of TcpH and Tsp. (*A*) TcpP stability in Δ*tcpH* Δ*tsp* Δ*yaeL* cells with *yaeL*E23A or *yaeL* ectopically expressed from a plasmid copy. Overnight cultures were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Arabinose was then added to both subcultures (final concentration, 0.1%) and grown at the same temperature for an additional 1.5 h. Samples were harvested for Western blot analysis before the addition of arabinose (time 0), and at various time points. Samples were separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpP. (*B*) Relative TcpP stability in strains Δ*tcpH* Δ*tsp* containing pBAD18-Kan (lane 1) and pBAD18-Kan-*yaeL*E23A (lane 2), and Δ*tcpH* Δ*tsp* Δ*yaeL* containing pBAD18-Kan-*yaeL* (lane 3) and pBAD18-Kan-*yaeL*E23A (lane 4). Overnight cultures were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE, and analyzed by immunoblotting with antibodies against TcpP.

To test this, we inducibly expressed wild-type *yaeL* and a mutant *yaeL* allele with a lesion in a predicted key active-site residue (*yaeL*E23A) (Matson and DiRita 2005). Inducing wild-type *yaeL* in Δ*tcpH* Δ*tsp* Δ*yaeL* cells resulted in steady degradation of TcpP over 90 minutes (right panel of Fig. 2.4*A*; Fig. 2.4*B* lane 3). In contrast, inducing *yaeL*E23A resulted in far less degradation of full length TcpP over time (left panel in Fig. 2.4*A*; Fig. 2.4*B* lane 4); the alteration in the active site resulted in spurious, incomplete cleavage, as we previously observed (Matson and DiRita 2005). Expression of the mutant allele in cells encoding a chromosomal copy of wild type *yaeL* led to diminished degradation of TcpP, suggesting that the mutant protein may inhibit wild type protein activity (Fig. 2.4*B*, lanes 3 and 4).

These results confirm that YaeL can access and cleave full-length TcpP provided that other TcpP interaction partners. – TcpH and the site-1 protease Tsp – are absent from the cell. This is consistent with earlier observations regarding RseA cleavage by YaeL (Grigorova, Chaba et al. 2004).

Tsp cleaves TcpP between H169 and Q190 and requires A172 and I174 for wild type activity. To localize the site on TcpP where Tsp cleaves, we analyzed plasmid-encoded alleles of *tcpP* encoding truncated proteins that terminate at residue H169 ($TcpP_{169}$), lacking the entire periplasmic domain, or at residue Q190 ($TcpP_{190}$), lacking approximately the C-terminal half of the periplasmic domain. We predicted that cleavage by Tsp of either of these truncated proteins in cells lacking YaeL would lead to accumulation of detectable TcpP*.

Steady state levels of full-length TcpP were not appreciably reduced in *ΔtcpH* cells under these conditions (Fig. 2.5, lane 1), which we attribute to excess TcpP from the plasmid relative to the chromosomally-encoded proteases Tsp and YaeL. In Δ*tcpH* Δ*tsp* Δ*yaeL* cells, we observed accumulation of $TcpP_{190}$ with no evidence of degradation (Fig. 2.5, lane 12), similar to what we observed with full-length TcpP (Fig. 2.5, lane 4). However, when expressed in Δ*tcpH* Δ*yaeL* cells, $TcpP_{190}$ was processed to TcpP* which accumulated to high levels (Fig. 2.5, lane 10), again similar to what we observed with full-length TcpP (Fig. 2.5, lane 2). In contrast, $TcpP_{169}$ accumulated to similar levels irrespective of whether or not *tsp* is expressed in the cell, (compare Fig. 2.5, lanes 6 and 8) indicating that Tsp has no effect on its stability. We conclude that the Tsp cleavage site in TcpP is C-terminal to residue H169 and N-terminal to residue Q190, consistent with our previous observation when we originally identified YaeL in this system (Matson and DiRita 2005). We also observe further evidence that YaeL can attack TcpP directly given the opportunity in Δ*tcpH* Δ*tsp* cells, as $TcpP_{190}$ was barely detectable in that background (Fig. 2.5, lane 11).

Insertions of the pentapeptide CGRTG using a transposon-based approach (Hallet, Sherratt et al. 1997) guided us further to the region bounded by residues A172 through G186, as peptide insertions within this region disrupted cleavage by Tsp (data not shown). We analyzed this stretch of amino acids further using site specific mutagenesis, incorporating single-base substitutions in the *tcpP* coding sequence to change codons of interest to alanine codons (except the codon for residue A172, which was mutated to a glycine codon). Site-directed mutagenesis was performed on the arabinose-inducible plasmid that contains the *tcpP* gene. Mutant constructs were then transformed into Δ*tcpPH* Δ*yaeL* cells to assess their cleavage by Tsp.

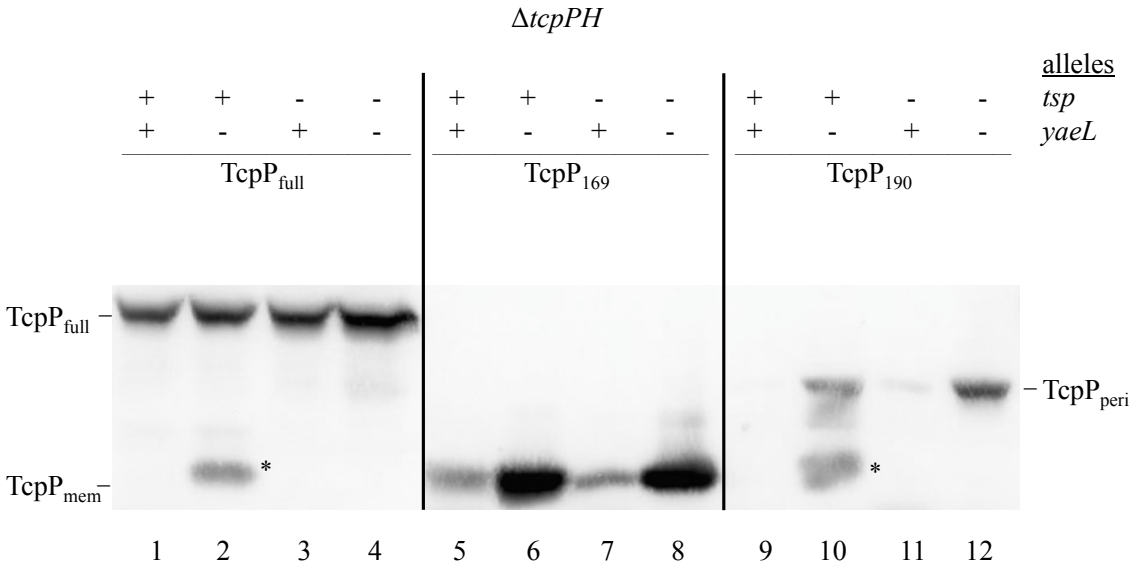

Fig. 2.5. Stability of TcpP Truncations in the Presence and Absence of Tsp. The following plasmids were expressed in O395 Δ*tcpPH* (lanes 1, 5 and 9), O395 Δ*tcpPH* Δ*yaeL* (lanes 2, 6 and 10), O395 Δ*tcpPH* Δ*tsp* (lanes 3, 7 and 11) and O395 Δ*tcpPH* Δ*tsp* Δ*yaeL* (lanes 4, 8 and 12): pBAD18-Kan-P_{full} (amino acids 1-222; lanes 1-4), pBAD18-Kan-P_{169} (amino acids 1-169; lane 5-8), and pBAD18-Kan-P_{peri} (amino acids 1-190; lanes 9-12). Overnight cultures were subcultured 1:50 and grown in the presence of 0.1% arabinose to mid-logarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE, and analyzed by immunoblotting with antibodies against TcpP.

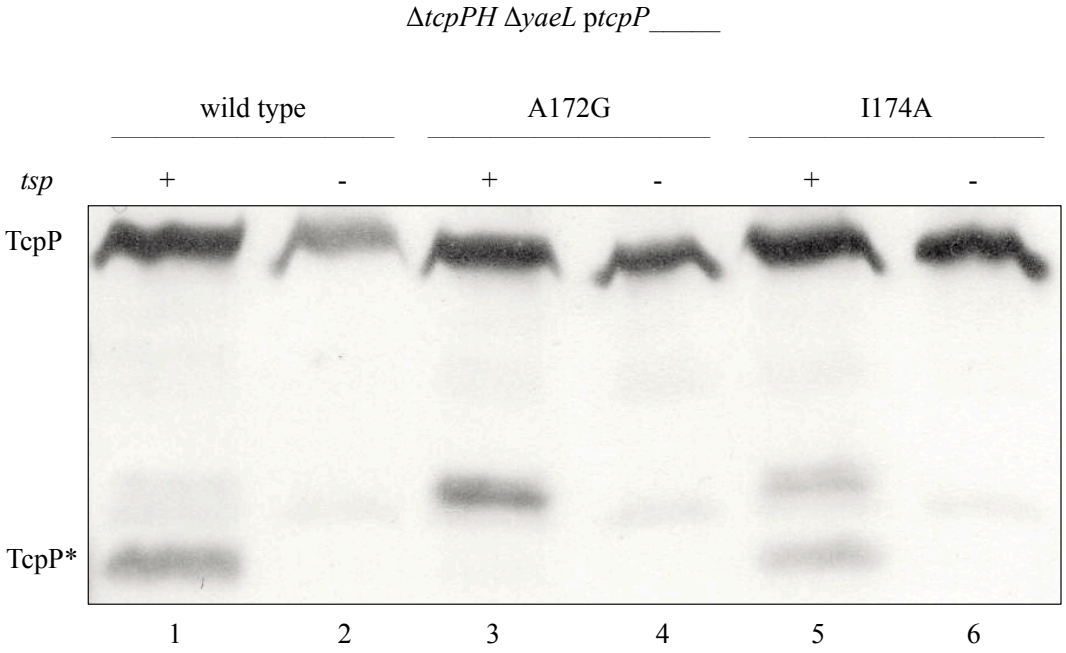

Fig. 2.6. Effects of Point Mutations in TcpP Region Hypothesized to Contain Tsp Cleavage Site. The following plasmids were expressed in O395 Δ*tcpPH* Δ*yaeL* cells (lanes 1, 3 and 5) and Δ*tcpPH* Δ*tsp* Δ*yaeL* cells (lanes 2, 4 and 6): pBAD18-Kan-*tcpP* (lanes 1 and 2), pBAD18-Kan-*tcpP*A172G (lanes 3 and 4), and pBAD18-Kan-*tcpP*I174A (lane 5 and 6). Overnight cultures were subcultured 1:50 and grown in the presence of 0.1% arabinose to mid-logarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE, and analyzed by immunoblotting with antibodies against TcpP.

Point mutations altering residues A172 and I174 resulted in permuted cleavage by Tsp, leading to a TcpP cleavage product of higher molecular weight than TcpP* in both mutants (Figure 2.6, lane 3 and 5); alanine substitutions at other residues in this region did not reproducibly disrupt the ability of Tsp to cleave TcpP (data not shown). In the case of the A172G variant, there was no evidence of cleavage at the wild type site leading to TcpP* accumulation, whereas with the I174A variant, there was evidence that cleavage was equivalently carried out at the wild type site and at another site. We conclude that changing the residues at these two positions alters the site specificity of Tsp, or reveals another Tsp cleavage site that is usually far less favored by Tsp in cleaving wild type TcpP. This latter possibility would be consistent with the ability of *E. coli* Tsp to cleave its substrates at more than one site (Keiler, Silber et al. 1995). Therefore, these residues of TcpP appear to be important for substrate recognition by Tsp to initiate degradation.

DISCUSSION

In this study, we identified the protease Tsp (Vc1496) as acting prior to YaeL in the two-step degradation of virulence regulator TcpP. Previous work had ruled out DegS as producing the substrate for YaeL, despite its role in producing the YaeL (RseP) substrate in the degradation of the anti-sigma factor RseA to regulate σ^E levels in *E. coli*. Two different approaches led us to Tsp: i) examining specific candidate protease genes and ii) screening a random transposon library for mutants that maintained TcpP activity in cells lacking the degradation-blocker TcpH. Both approaches led us to Tsp.

This and previously published work (Matson and DiRita 2005) has led us to a model for the degradation of TcpP (Fig. 2.7*A*). Under conditions that favor virulence gene expression,

TcpH remains associated with TcpP, keeping it inaccessible to proteolysis by Tsp and YaeL and maintaining *toxT* expression. Under non-favorable conditions, dissociation of TcpH from TcpP exposes the periplasmic domain of TcpP to degradation by Tsp, which requires amino acids A172 and I174 for proper site selection. TcpP* is then subjected to further inactivating proteolysis by YaeL. Under some circumstance, TcpP can be degraded directly by YaeL (Fig. 2.7*B*), consistent with previous work in the *E. coli* σ^E signal transduction pathway, which is arguably the most extensively studied RIP system in bacteria. In that system, YaeL can cleave intact RseA in cells lacking DegS and RseB, a factor analogous in some respects to TcpH in the *V. cholerae* system (Grigorova, Chaba et al. 2004).

Tsp is implicated in other systems of protein quality control and gene regulation. It is responsible in *E. coli* for processing the C-terminal region of penicillin-binding protein 3 (PBP3) a lipoprotein involved in peptidoglycan synthesis (Tamura, Suzuki et al. 1980, Hayashi, Hara et al. 1988, Hara, Nishimura et al. 1989, Nagasawa, Sakagami et al. 1989, Hara, Yamamoto et al. 1991). Also in *E. coli*, Tsp can degrade a periplasmic protein via the SsrA peptide-tagging system that targets proteins expressed from damaged mRNA (Keiler, Waller et al. 1996). In *Bacillus subtilis*, a RIP system responsible for processing the anti-sigma factor RsiW requires cleaving by a site-1 protease (PrsW) to produce RsiW-S1 (analogous to TcpP*), which is then trimmed by Tsp prior to subsequent cleavage by a site-2 protease RasP (Heinrich, Hein et al. 2009). Our work supports a role for Tsp as the site-1 protease of TcpP but also suggests that the mechanism of degradation may involve other proteases that function at lower frequency. Detection of an intermediate band in O395 *tsp*/*yaeL* mutants (Fig. 2.2, Fig. 2.3, and Supplemental Fig. 2.2) and the accumulation of a slightly smaller-than-full-length TcpP species

in C6706 *tcpH*/*tsp* mutants (Supplemental Fig. 1) point to the existence of an earlier proteolytic step before Tsp processing, similar to what is observed with RsiW proteolysis in *B. subtilis*. The only example we are aware of in which Tsp acts as a *bona fide* sole site-1 protease is in the Iut (iron uptake) system of *Pseudomonas putida* leading to expression of the aerobactin receptor IutA. Under iron-limiting conditions, the hybrid sigma/anti-sigma protein IutY is cleaved consecutively by Tsp and RseP to release the sigma domain, which then activates *iutA* transcription (Bastiaansen, Ibanez et al. 2014).

Unfettered access of YaeL in the absence of Tsp and TcpH led to degradation of TcpP. In *E. coli,* YaeL (RseP) access to the RseA substrate requires a C-terminal hydrophobic residue that is revealed after cleavage by DegS. However, as with our demonstration that YaeL can attack TcpP when TcpH and Tsp are absent in *V. cholerae*, YaeL (RseP) can similarly degrade RseA when DegS and RseB are missing (Grigorova, Chaba et al. 2004). This perhaps suggests that when the periplasmic protease-blockers like TcpH and RseB are missing, target hydrophobic residues of TcpP and RseA may become revealed, enabling YaeL to access its substrate for degradation.

An unanswered question in the TcpP/Tsp/YaeL degradation pathway is what the specific signals are that induce initiation of TcpP cleavage by Tsp. Our work demonstrates that under *in vitro* conditions unfavorable for expression of toxin and pilus, proteolysis is initiated by Tsp. In the RIP system that degrades RseA, DegS is activated when three C-terminal amino acids of misfolded outer membrane proteins directly interact with its PDZ domain (Hasselblatt, Kurzbauer et al. 2007, Sohn, Grant et al. 2009). Studies on Tsp *in vitro* support a model of

sequence-dependent activation in which a specific C-terminal tetrapeptide motif [L(I)RV] substrate activates Tsp, and that activators do not necessarily act as substrates (Weski, Meltzer et al. 2012). In terms of mechanism of action, Tsp recognizes determinants at the C-terminus of substrates and cleaves at a discrete number of sites upstream with rather broad primary sequence specificity (Keiler, Silber et al. 1995, Keiler and Sauer 1996). A dipeptidic site in TcpP at Ala-183 and Arg-184, matches one of the several previously reported cleavage sites (Nagasawa, Sakagami et al. 1989, Keiler and Sauer 1996). However, the three C-terminal residues of TcpP (Thr-219-Lys-220-Asn-221) do not conform to the pattern of sequence determinants for Tsp recognition, i.e., small, uncharged residues (Ala, Cys, Ser, Thr, Val) are preferred at the C-terminal position; whereas non-polar residues are also preferred at the second and third positions (Keiler and Sauer 1996). Our results, based on analyzing truncated forms of TcpP and site-specific mutants demonstrate that cleavage occurs between H169 and Q190, and that amino acid residues A172 and I174 in TcpP are critical for Tsp activity. Whether the region between residues 172-174 is the actual cleavage site or simply a region that activates Tsp is unclear.

Recently, Almagro-Moreno *et al.* showed that ToxR also undergoes YaeL-dependent RIP during stationary phase of growth (Almagro-Moreno, Kim et al. 2015). The authors determined that ToxR proteolysis occurs when *V. cholerae* enters a dormant state called viable but nonculturable (VBNC) – a condition in which the bacterium is postulated to be commonly found in the aquatic environment. Such a regulatory mechanism presumably allows cells to shut down the virulence cascade when they are prepared to leave the host or to conserve energy when they are in a nutrient-poor environment. This study, together with our work on the regulation of TcpP,

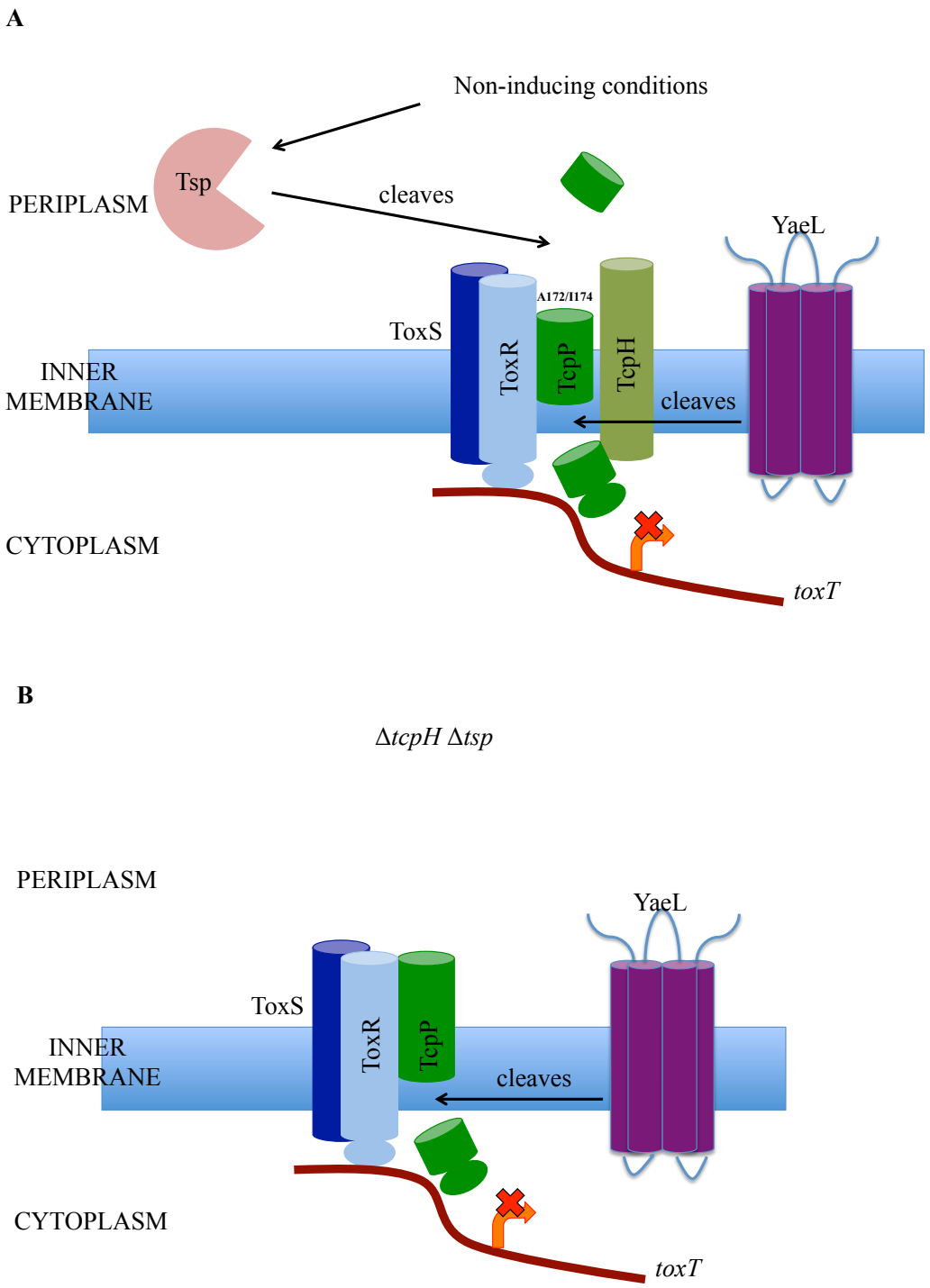

Fig. 2.7. Two modes of TcpP degradation. (*A*) Wild-type: Tsp-dependent proteolysis of TcpP. When conditions no longer favor virulence gene expression, Tsp is activated to initiate proteolysis of TcpP, a process that requires residues A172 and I174 of TcpP for proper localization. YaeL can then cleave the TcpP fragment (TcpP*) generated by Tsp cleavage. (*B*) Δ*tcpH* Δ*tsp*: YaeL can initiate cleavage of the full-length TcpP in the absence of TcpH and Tsp.

highlights a new perspective on how levels of membrane-bound transcription factors are modulated in response to environmental signals.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions. *V. cholerae* classical strain O395 and El Tor strain C6706 were used as indicated for various experiments in this study. The *E. coli* strains JM101, DH5α, and DH5αλpir were used for cloning, and SM10λpir was used for conjugation of plasmids into *V. cholerae*. *V. cholerae* was cultured at 30°C in pH 6.5 LB to activate expression of virulence genes and cultured at 37°C in pH 8.5 LB as indicated. Plasmids used in this study include the suicide vector pKAS32 (Skorupski and Taylor 1996), the mariner transposon suicide vector, pFD1 (Rubin, Akerley et al. 1999), and the arabinose-inducible expression vector pBAD18-Kan (Guzman, Belin et al. 1995). Expression of transposase from pFD1 was induced by the addition of isopropyl-D-thiogalactopyranoside (Invitrogen) to a final concentration of 1 mM, and expression of pBAD was induced by the addition of L-arabinose to 0.1%. *E. coli* strains were transformed by standard methods (Sambrook and Russell 2001), and plasmid DNA was introduced into *V. cholerae* by electroporation or by filter conjugation with SM10λpir. Antibiotics were used at the following concentrations: carbenicillin, 50 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 100 μg/ml, except when selecting for loss of plasmid integrants, when it was used at 1 mg/ml. X-Gal (Invitrogen) was used in LB agar at 40 μg/ml.

Transposon Insertion Library Screening. Fourteen open reading frames (ORFs) were identified by searching the *V. cholerae* genome database for membrane proteases and metalloproteases, using the search tool on the National Microbial Pathogen Data Resource

Center (http://www.nmpdr.org/FIG/wiki/view.cgi). Returned from the search were *yaeL, degS, degP, vca0044*, *vca0063*, *vca0550*, *vc0975*, *vc1074*, *vc1117*, *vc1200*, *vc1496*, *vc1709*, *vc1994* and *vc0976*. Using the library of transposon insertions in all non-essential ORFs of *V. cholerae* strain C6706 generously provided by the Mekalanos lab (Cameron, Urbach et al. 2008), the 11 mutant alleles with *vc*/*vca* designations were transduced from the C6706 strain into the O395 Δ*tcpH* background. The phage CP-T1ts was used for this purpose for transducing markers between biotypes.

Strain Construction. The O395 Δ*tcpH* (Beck, Krukonis et al. 2004) and O395 Δ*tcpH* Δ*yaeL* (Matson and DiRita 2005) strains used in this study have been described in the indicated references. Strains containing deletions of *tsp* (*vc1496*) were constructed by using PCR (Sambrook and Russell 2001) to amplify a region of DNA spanning 500 bp upstream of the *tsp* start codon to 500 bp downstream of the stop codon containing an internal deletion of the *tsp* gene. This fragment was then cloned into the suicide plasmid pKAS32 (Skorupski and Taylor 1996), and the resulting recombinant plasmid was introduced into the *E. coli* strain SM10λpir by electroporation. This strain was then mated with the *V. cholerae* strains O395, O395 Δ*tcpH*, O395 Δ*tcpH* Δ*yaeL*, O395 Δ*tcpPH*, and O395 Δ*tcpPH* Δ*yaeL* by filter conjugation. Integration of the plasmid into the *V. cholerae* chromosome was selected for by plating on TCBS (thiosulfate-citrate-bile-sucrose) plates (Difco) containing 50 μg/ml carbenicillin. Resolution of the cointegrate was selected on LB plates containing 1 mg/ml streptomycin. Recombination and loss of the wild-type allele was confirmed by PCR using primers flanking the deletion.

Plasmid Construction. Full-length *yaeL* and *tsp* were amplified from *V. cholerae* O395 chromosomal DNA by using Expand Hi-Fidelity polymerase (Roche Molecular Biochemicals). After amplification, the PCR products were digested with EcoRI and XbaI and ligated into the arabinose-inducible expression vector pBAD18-Kan (Guzman, Belin et al. 1995). The YaeL active-site mutant (E23A) was constructed by performing site-directed mutagenesis on pBAD18-Kan-YaeL. Site-directed mutagenesis was performed with *PfuTurbo* DNA polymerase by using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions.

Protein Electrophoresis and Immunodetection. Overnight cultures of *V. cholerae* were subcultured 1:100 in pH 6.5 LB and grown for 4-5 hours at 30°C. Arabinose was added to the culture medium at the time of subculture or 4-5 hours after subculture for strains containing pBAD18 or pBAD18-Kan. One milliliter of midlogarithmic culture was pelleted by centrifugation and resuspended in 1X sample buffer. Proteins were separated by SDS/PAGE using 15% (weight/volume) polyacrylamide gels, and loading volumes were adjusted to normalize for culture OD_{600}. Proteins were then transferred to nitrocellulose membranes and probed with rabbit anti-TcpP antibodies (generated by Rockland Immunochemicals). Blots were probed with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Cell Signaling Technology) and then visualized by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich).

Measurement of *toxT-lacZ* Activation. Overnight cultures of *V. cholerae* were subcultured 1:40 in pH 6.5 LB and grown at 30°C for 3 h. 100 μl of culture samples in duplicates were used to measure β-galactosidase activity as described in reference 25 (Miller 1972).

Peptide Insertion Mutagenesis of TcpP. Linker scanning mutagenesis was performed to generate random 15 bp insertions in *tcpP* using the Mutation Generation System Kit (Life Technologies) according to the manufacturer's instructions.

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CHAPTER 3

RECIPROCAL INTERACTION WITH TCPP IS ESSENTIAL FOR STABILITY OF TCPH IN *VIBRIO CHOLERAE*

SUMMARY

TcpH is a membrane-bound protein involved in the virulence cascade that leads to the production of cholera toxin (CT) and toxin-coregulated pilus (TCP) in *Vibrio cholerae*. It presumably interacts with its cognate activator TcpP, which is an unusual membrane-associated transcription factor that activates transcription of the master regulatory gene *toxT*. *toxT* in turn encodes the direct transcription activator of CT and TCP. Previous studies on TcpP regulation demonstrate that TcpH is able to inhibit regulated intramembrane proteolysis (RIP) of TcpP, which is a posttranslational regulatory mechanism that shuts down the virulence cascade under some conditions. In this study, analysis of the TcpH requirement for inhibiting TcpP proteolysis unexpectedly revealed a role for YaeL in TcpH stability. Moreover, we determined that TcpH requires TcpP for stability. Evidence of gradual loss of TcpH protein under *in vitro* conditions that are noninducing for virulence gene expression lends credence to a model for phase variation in *tcpH* proposed in a previous study.

INTRODUCTION

The eponymous Gram-negative bacterium that is the causative agent of cholera, *Vibrio cholerae*, triggers an acute watery diarrhea that may lead to death by severe dehydration if not treated immediately. The secreted cholera toxin (CT), which is responsible for the symptom, together with the toxin-coregulated pilus (TCP) that is essential for intestinal colonization, are encoded by genes whose expression are regulated by a multiprotein transcriptional cascade known as the ToxR regulon (Peterson and Mekalanos 1988, Skorupski and Taylor 1997). Transcription of CT and TCP genes are activated by the master virulence regulator, ToxT, whose encoding gene expression is in turn activated by an unusual protein complex comprising four inner membrane proteins, ToxRS and TcpPH (DiRita 1992, Higgins and DiRita 1994, Thomas, Williams et al. 1995, Hase and Mekalanos 1998, Krukonis, Yu et al. 2000, Crawford, Krukonis et al. 2003).

While *toxRS* appears to be constitutively expressed, the *tcpPH* genes seem to be only transcribed under permissive conditions for virulence expression by two transcription activators AphA and AphB (Kovacikova and Skorupski 1999). In addition, cellular TcpP levels are subjected to posttranslational regulation in the form of regulated intramembrane proteolysis (RIP) (Matson and DiRita 2005), a mechanism widely conserved in prokaryotes and eukaryotes (Brown, Ye et al. 2000). In bacteria, RIP is responsible for the release of sigma factors from the membrane in order for them to bind RNA polymerase and activate gene expression in response to environmental cues. The anti-sigma factor, which normally tethers the sigma factor to the membrane, undergoes RIP when the site-1 protease (S1P) is activated by a molecular signal. The S1P cleaves the anti-sigma factor to generate the substrate for the site-2 protease (S2P), which

then cleaves the intermediate in the membrane domain to liberate the sigma factor into the cytoplasm.

The most well known example of RIP in bacteria is the extensively studied σ^E pathway in *Escherichia coli* (Ades 2004, Alba and Gross 2004, Ehrmann and Clausen 2004). When cells experience envelope stress, outer membrane proteins (OMPs) unravel and expose their C-terminal tails to activate the S1P DegS, which cleaves the anti-sigma factor RseA in its periplasmic domain to produce the substrate for the S2P YaeL (Cezairliyan and Sauer 2007). YaeL then cleaves in the membrane domain of the TcpP intermediate to remove it from the inner membrane (Akiyama, Kanehara et al. 2004). In the process, σ^E is released to join the basal transcription apparatus, which activates genes required for countering the envelope stress. RseA proteolysis is inhibited by a small periplasmic protein called RseB, without which YaeL can act directly on full length RseA (Missiakas, Mayer et al. 1997, Grigorova, Chaba et al. 2004). Previous studies have shown that the combination of unfolded OMPs and elevated levels of lipopolysaccharide (LPS) fragments in the periplasm sequesters RseB away from RseA, thereby exposing it to RIP (Cezairliyan and Sauer 2007, Chaba, Alba et al. 2011). A homologous system can also be found in the medically important bacterium *Pseudomonas aeruginosa*, where the RIP-dependent alginate biosynthesis pathway contributes to the mucoid phenotype of clinical strains isolated from cystic fibrosis (CF) patients. This system employs the same components as that of the σ^E pathway – AlgW (DegS), MucP (RseP), and MucB (RseB), to regulate the anti-sigma factor MucA (RseA) (Qiu, Eisinger et al. 2007).

In *V. cholerae*, RIP of TcpP proceeds in at least two steps, of which the periplasmic cleavage is catalyzed by the tail-specific protease (Tsp) (Teoh, Matson et al. 2015), while the subsequent intramembrane cleavage is triggered by YaeL (Matson and DiRita 2005). In contrast to other RIP systems, TcpP is an activator, rather than an anti-activator like RseA and MucA. Therefore, destruction of TcpP terminates *toxT* transcription and hence results in the loss of CT and TCP production. Like RseB and MucB, TcpH, the presumed interaction partner of TcpP, acts as a negative regulator of TcpP proteolysis. Previous studies showed that *tcpH* mutants are defective in infant mice colonization (Carroll, Tashima et al. 1997) as a consequence of diminished *toxT* and downstream *tcp* expression (Yu and DiRita 1999).

Recent studies have focused on how TcpP levels are regulated but the mechanism by which TcpH levels are maintained, especially during RIP of TcpP, has been less well characterized. Thus, in this study, we seek to identify regulators of TcpH stability. A truncation study of TcpH to determine structural requirements for inhibition of TcpP proteolysis unexpectedly uncovers a role for YaeL in TcpH stability. Moreover, TcpH appears to require TcpP for stability. These findings represent a new view of RIP and shed further light on the intricacies of the interaction between TcpP and TcpH.

RESULTS

Overexpressed TcpH and its C-terminal truncation variants are more stable in the absence of YaeL. Our investigation of TcpH stability grew out of studies on regulators of TcpP levels, where we recently identified Tsp as the protease that cleaves TcpP to provide the substrate for the S2P YaeL (Teoh, Matson et al. 2015). We provided evidence that TcpH may serve to inhibit

Tsp activity and also to protect full-length TcpP from direct proteolysis by YaeL (Teoh, Matson et al. 2015). An effectively identical function was described previously as well for RseB, which blocks proteolysis of anti-sigma factor RseA in *E. coli* (Missiakas, Mayer et al. 1997, Cezairliyan and Sauer 2007).

We sought to determine the structural requirements for functional TcpH-dependent stabilization of TcpP by constructing truncated forms of TcpH, shown in Fig. 3.1*A*. Both truncation variants – $TcpH_{-5aa}$ and $TcpH_{-10aa}$, with deletion of five and ten carboxy-terminal amino acid residues, respectively, are significantly less stable than the full-length protein (Fig. 3.1*B*, lanes 1 to 3). This impairment is well reflected by their diminished TcpP levels (Fig. 3.1*B*, lanes 7 to 9). Interestingly, $TcpH_{-10aa}$ is more stable than $TcpH_{-5aa}$ (compare Fig. 3.1*B*, lanes 3 and 2), suggesting that the final five amino acid residues somehow antagonize the final six to ten residues – the former conferring stability to TcpH while the latter imparting susceptibility to proteolysis.

Given these observations, we wondered whether YaeL might regulate TcpH levels in the same way it does with TcpP. To address this question, we examined the stability of TcpH and its C-terminal truncation variants in cells lacking YaeL. We observed an increase of protein levels when full-length TcpH and its truncation variants were expressed in cells lacking YaeL (Fig. 3.1*B*, lanes 4, 5 and 6). The enhanced stability of TcpH is manifested by the greater accumulation of full-length TcpP, despite of higher TcpP intermediate (TcpP*) buildup as a result of Tsp cleavage in cells producing the truncated variants (Fig. 3.1*B*, lanes 11 and 12).

Thus, the seemingly increased stability of TcpH and its truncation variants in the absence of YaeL led us to hypothesize that TcpH is subjected to the same RIP mechanism as TcpP.

Steady-state levels of TcpH are dependent on TcpP. A *tcpH* mutant was previously shown to exhibit barely detectable levels of TcpP and, consequently, to produce significantly reduced amounts of *toxT* transcripts and cholera toxin (Beck, Krukonis et al. 2004). The instability of TcpP in this background was later attributed to RIP, involving proteases Tsp and YaeL (Matson and DiRita 2005, Teoh, Matson et al. 2015). Loss of TcpP stability has also been demonstrated to occur in wild-type cells expressing chromosomal *tcpH* (Matson and DiRita 2005, Teoh, Matson et al. 2015). Hence, we hypothesized that TcpH ceases to interact with TcpP under conditions that are not favorable for virulence gene activation, thereby allowing proteases to access the cleavage sites in TcpP. One plausible explanation for the loss of interaction is the loss of TcpH stability. This could very well be the result of a frameshift mutation in a string of nine G residues near the amino terminus within *tcpH* (Carroll, Tashima et al. 1997). Similar slipped-strand mutations in *tcpH* have been shown to arise during overnight growth and accumulate in the majority of cells eventually.

Even so, compared to its interactive partner, TcpH is still poorly understood in terms of how its levels are regulated. To identify other factors that regulate TcpH stability, we assessed the role of TcpP in TcpH function by examining the phenotype of a *V. cholerae tcpP* mutant. We determined the steady-state levels of TcpH in wild-type cells and the *tcpP* mutant by immunoblotting (Fig. 3.2). This experiment showed that steady-state levels of TcpH are

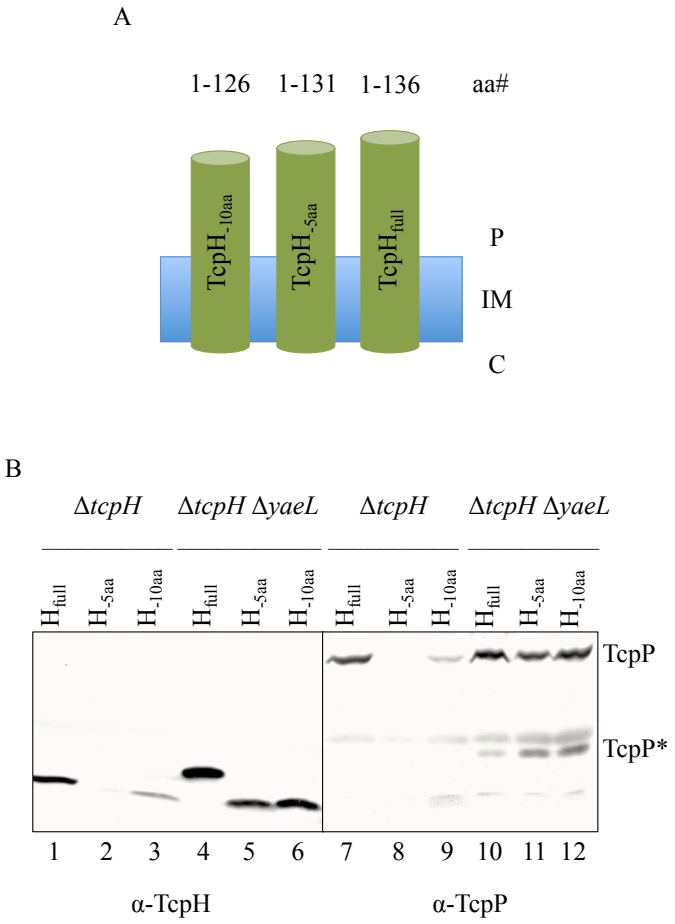

Fig. 3.1. Stability of TcpH Truncation Variants in the Presence and Absence of YaeL. (*A*) Schematic diagram of the TcpH truncation variants constructed. The genes encoding the variants were expressed from the inducible plasmid pBAD18. (*B*) The following plasmids were expressed in either O395 Δ*tcpH* (lanes 1-3, 7-9) or Δ*tcpH* Δ*yaeL* (lanes 4-6, 10-12): pBAD18-*tcpH*$_{full}$ (amino acids 1-136; lanes 1, 4, 7, and 10), pBAD18-*tcpH*$_{-5aa}$ (amino acids 1-131; lanes 2, 5, 8, and 11), and pBAD18-*tcpH*$_{-10aa}$ (amino acids 1-126; lanes 3, 6, 9 and 12). Overnight cultures were subcultured 1:100 and grown in the presence of 0.1% arabinose to midlogarithmic phase in pH 6.5 LB at 30°C. Samples were harvested, separated by SDS/PAGE, and analyzed by immunoblotting with antibodies against TcpH (lanes 1-6) and TcpP (lanes 7-12).

dependent on expression of *tcpP*, as the *tcpP* mutant strain accumulated virtually undetectable levels of TcpH after growth to the mid-logarithmic phase in LB at pH 6.5 and 30°C, conditions that stimulate toxin and pilus production in wild-type *V. cholerae* (Fig. 3.2, lane 2). Complementation by ectopic expression of *tcpP* under an arabinose-inducible promoter (p_{BAD}) restores TcpH to partially wild-type levels (Fig. 3.2, lane 3).

One explanation to account for diminished levels of TcpH in the *tcpP* null strain is that *tcpP* deletion ablates *tcpH* transcription. To test this possibility, we performed qRT-PCR with *tcpH* specific primers on RNA from mid-logarithmic phase cultures grown under inducing conditions. No difference in *tcpH* transcript levels between wild-type and *tcpP* mutant cultures was observed (data not shown). These findings rule out any profound influence of TcpP on *tcpH* transcription and suggest that TcpP contributes either to translation of the *tcpH* transcript or to the stability of the protein in the same way that TcpH stabilizes TcpP.

Having observed that TcpH and its C-terminal truncation variants are more stable in the absence of YaeL (Fig. 3.1, lanes 4 to 6), we wondered if eliminating YaeL from the *tcpP* mutant would restore TcpH levels. Interestingly, no difference in TcpH levels between *tcpP* and *tcpP*/*yaeL* mutants was observed (compare Fig. 3.2, lanes 2 and 4). Likewise, the *tcpP*/*yaeL* mutant did not appear to accumulate more TcpH than the *tcpP* mutant when both cells were complemented by ectopic expression of *tcpP* under an arabinose-inducible promoter (compare Fig. 3.2, lane 5 and 3). Taken altogether, these results suggest that TcpP plays an essential role in conferring stability to TcpH and that YaeL only exerts an effect on stability when *tcpH* is overexpressed.

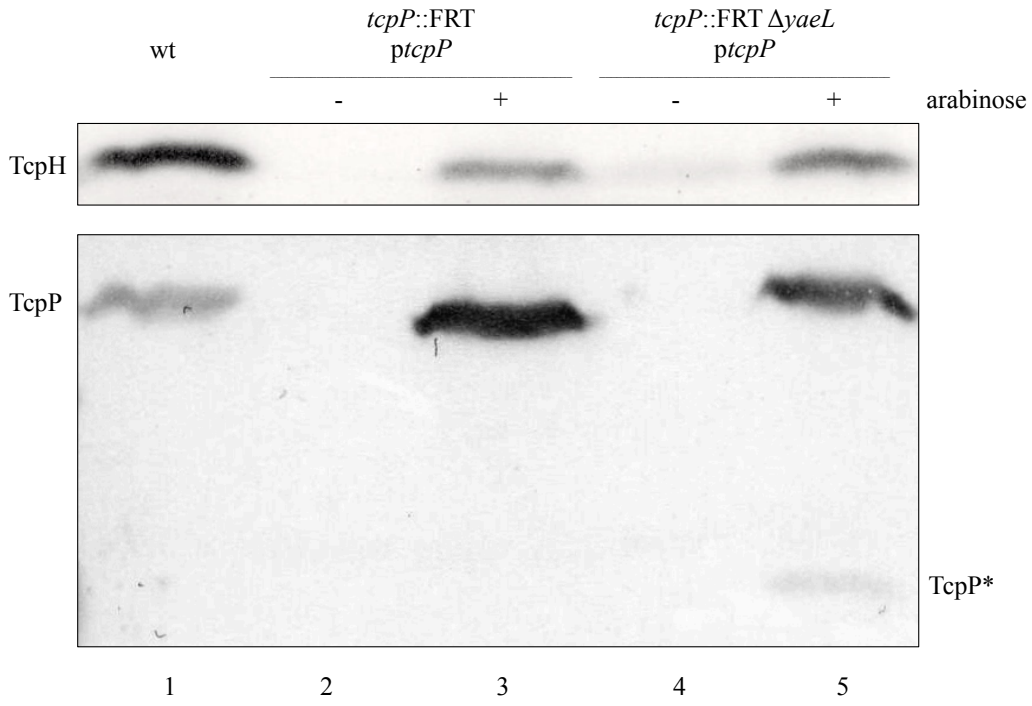

Fig. 3.2. Effects of TcpP and YaeL on Endogenous TcpH Stability. Overnight cultures of the following strains were subcultured 1:100 and grown in the absence or presence of 0.1% arabinose, where stated: O395 wild-type (wt, lane 1); *tcpP*::FRT containing pBAD18-Kan-*tcpP* (lanes 2 and 3); and *tcpP*::FRT Δ*yaeL* (lanes 4 and 5). Cells were harvested after growth to midlogarithmic phase in pH 6.5 LB at 30°C. Samples were separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpH and TcpP.

Conditional degradation of TcpH in the Presence of TcpP. We previously observed the accumulation of TcpP* over time in Δ*yaeL* cells under *in vitro* conditions that are noninducing for virulence gene expression (pH 8.5 LB at 37°C) (Matson and DiRita 2005). This was later determined to be the result of Tsp activity, which generates the substrate for the missing site-2 protease YaeL. We hypothesized that under these conditions, gradual degradation of full-length TcpP to TcpP* results in the disruption of TcpP/TcpH interaction interface, therefore exposing TcpH to concurrent degradation. To determine whether TcpH degradation can occur in the presence of TcpP, we cultured *V. cholerae* strain Δ*yaeL* under virulence inducing conditions to express *tcpP*. After growth in these conditions, an aliquot of the cultures was shifted to the same inducing conditions, or to noninducing conditions. Samples were taken over time, prepared for and separated by SDS/PAGE, and then immunoblotted with anti-TcpH antibodies.

Conforming to our predictions, we detected a loss of TcpH over time in Δ*yaeL* cells grown under noninducing conditions (Fig. 3.3, right of top panel), which corresponds to the gradual accumulation of TcpP* (Fig. 3.3, right of bottom panel). In contrast, TcpH remained stable in cells grown under inducing conditions (Fig. 3.3, left of top panel), and no TcpP* accumulation was detected after four hours (Fig. 3.3. left of bottom panel). These results indicate that TcpH and TcpP play reciprocal roles in virulence modulation of *V. cholerae*, as both require each other to stabilize the transcription activator complex that activates *toxT* expression. Moreover, loss of endogenous TcpH under noninducing conditions appears to be independent of YaeL, and could perhaps be better explained by the accumulation of frameshift mutations during phase variation in *tcpH*.

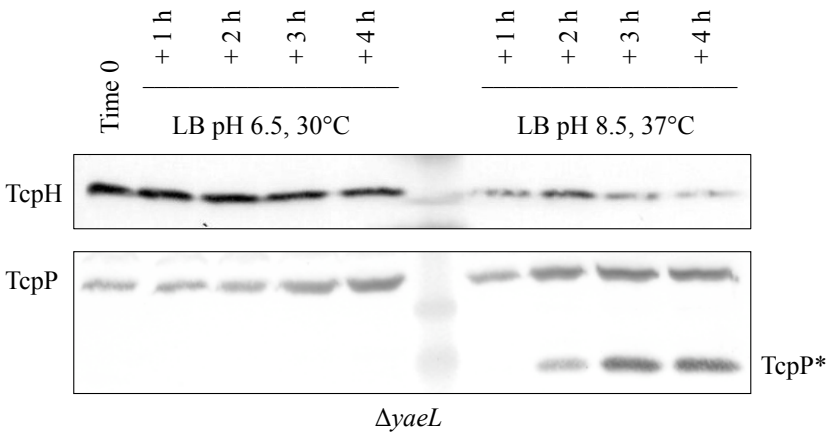

Fig. 3.3. TcpH Degradation in the Presence of TcpP. Overnight culture of Δ*yaeL V. cholerae* were subcultured 1:100 and grown to midlogarithmic phase in pH 6.5 LB at 30°C. The cells were harvested by centrifugation, and equal portions of the sample were resuspended either in pH 6.5 LB and grown at 30°C or in pH 8.5 LB and grown at 37°C for an additional 4 h. Samples were harvested for Western blot analysis before resuspension in new media (time 0), and at each hour after resuspension for both conditions. Samples were separated by SDS/PAGE and analyzed by immunoblotting with antibodies against TcpH and TcpP.

DISCUSSION

In this study, an investigation to determine the structural requirements that confer function to TcpH, a membrane protein hypothesized to interact with and stabilize TcpP, uncovered a role for YaeL in TcpH stability (Fig. 3.1). YaeL was previously identified as the site-2 protease involved in the RIP of TcpP, and TcpH serves to inhibit its ability to cleave full-length TcpP directly (Matson and DiRita 2005). Further characterization of the regulation of TcpH stability demonstrated that TcpP stabilizes TcpH in a reciprocal manner and YaeL appears to exert a homeostatic effect on aberrant levels of TcpH. We also observed a gradual loss of TcpH protein that is independent of YaeL when *V. cholerae* is grown under *in vitro* conditions that are noninducing for virulence gene expression, a result consistent with the previously proposed model of phase variation in *tcpH* (Carroll, Tashima et al. 1997).

The findings of this report add to an already complex array of mechanisms employed by *V. cholerae* to regulate expression of CT and TCP. To our knowledge, this is the first study to find a reciprocal protective association between an RIP substrate and the protein that protects it from degradation. In other Gram-negative RIP systems, functional homologs of TcpH are sequestered away from the RIP substrates by a combination of unfolded OMPs and LPS fragments in the periplasm (Cezairliyan and Sauer 2007, Chaba, Alba et al. 2011). Moreover, the susceptibility of TcpH to YaeL regulation when produced at high levels has not been described in other homologous systems. Perhaps, this could be attributed to the fact that TcpH is peripherally bound to the membrane whereas its functional homologs in other systems are predominantly periplasmic (De Las Penas, Connolly et al. 1997, Mathee, McPherson et al. 1997). Therefore, TcpH could be one of the many membrane substrates of YaeL that do not

share any sequence homology with each other (Akiyama, Kanehara et al. 2004, Saito, Hizukuri et al. 2011), as long as there exist residues of low helical propensity that stabilize the YaeL-substrate interaction (Koide, Ito et al. 2008).

In the TcpH truncation studies, we observed that TcpH$_{-10aa}$ is more stable than the TcpH$_{-5aa}$ variant (compare Fig. 3.1*B*, lanes 3 and 2), suggesting that the stretch of amino acid residues 132-136 and 127-131 somehow antagonize each other – the former conferring stability to TcpH while the latter imparting susceptibility to proteolysis. The instability of the TcpH$_{-5aa}$ variant is rescued in the absence of YaeL (Fig. 3.1*B*, lane 5), even though the protease is only known to cleave in the membrane (Akiyama, Kanehara et al. 2004). We hypothesize that the stretch of residues 132-136 acts an inhibitory domain that conceals a protease recognition sequence within residues 127-131. Removal of the latter stretch renders proteolysis impossible, thereby stabilizing the TcpH$_{-10aa}$ variant (Fig. 3.1*B*, lanes 3 and 6).

The finding that TcpP confers stability to TcpH, just as TcpH does to TcpP, illustrates the need to revise the relationship between TcpH and TcpP from that of protector and protected to that of mutual/recriprocal partnership. Consequently, this raises the question of whether the membrane-bound nature of TcpH has an influence on its own stability and thereby of TcpP. The simultaneous degradation of both TcpP and TcpH under *in vitro* virulence noninducing conditions led us to speculate about the probability of the same molecular signals triggering both proteolytic events. The periplasmic domains of TcpP and TcpH could potentially allow monitoring of extracellular cues, either through physical interactions with OMPs that undergo conformational changes under specific conditions or sensing of molecules that enter the cell from

the extracellular environment. In fact, the latter has been described in a study showing dimerization of TcpP by intermolecular disulfide bond formation in the presence of bile salts (Yang, Liu et al. 2013). All in all, our findings in this study has certainly given us more details to consider in our quest to elucidate the complex mechanisms of virulence regulation in *V. cholerae*.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and culture conditions. *V. cholerae* classical strain O395 was used throughout this study. *V. cholerae* was cultured at 30°C in pH 6.5 LB to activate expression of virulence genes and cultured at 37°C in pH 8.5 LB as indicated.. Plasmids used in this study are the arabinose-inducible expression vectors pBAD18 and pBAD18-Kan (Guzman, Belin et al. 1995). Expression of p_{BAD} was induced by the addition of $_L$-arabinose to 0.1%. Plasmid DNA was introduced into *V. cholerae* by electroporation. Antibiotics were used at the following concentrations: carbenicillin, 100 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 100 μg/ml.

Strain construction. The O395 Δ*tcpH* (Yu and DiRita 1999), Δ*yaeL* and Δ*tcpH* Δ*yaeL* (Matson and DiRita 2005) strains used in this study have been described in the indicated references. *tcpP* mutants were constructed by transduction of the *tcpP* mutant allele from the C6706 transposon insertion library into the O395 wild-type and Δ*yaeL* background. The phage CP-T1ts was used for this purpose of transducing markers between biotypes (Hava and Camilli 2001). FLP-mediated recombination at the flanking FLP recombinase target (FRT) sites of the transposon excised the kanamycin resistance cassette, leaving behind a 192-bp scar in the *tcpP* allele, whose remaining FRT site enables subsequent transformation of pBAD18-Kan-*tcpP*, which contains a kanamycin resistance cassette, into these mutants (Cameron, Urbach et al. 2008).

Plasmid construction. The pBAD18-*tcpH* (Matson and DiRita 2005) and pBAD18-Kan-*tcpP* (Beck, Krukonis et al. 2004) plasmids used in this study have been described in the indicated references. The TcpH C-terminal truncation variants were constructed by performing site-directed mutagenesis on pBAD18-*tcpH*. Site-directed mutagenesis was performed with *PfuTurbo* DNA polymerase by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Protein electrophoresis and immunodetection. Overnight cultures of *V. cholerae* were subcultured 1:100 in pH 6.5 LB and grown for 4-5 h at 30°C. Arabinose was added to the culture medium at the time of subculture for strains containing pBAD18 or pBAD18-Kan unless stated otherwise. One milliliter of midlogarithmic culture was pelleted by centrifugation and resuspended in 1X sample buffer. Proteins were separated by SDS/PAGE using 15% (wt/vol) polyacrylamide gels, and loading volumes were adjusted to normalize for culture OD_{600}. Proteins were then transferred to nitrocellulose membranes and probed with rabbit anti-TcpH or anti-TcpP antibodies (generated by Rockland Immunochemicals). Blots were probed with goat anti-rabbit alkaline phosphatase-*conjugated* secondary antibody (Cell Signaling Technology) and then visualized by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (Sigma-Aldrich).

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CHAPTER 4

GENERAL DISCUSSION

This work follows up on the initial discovery that RIP was involved in the proteolysis of the membrane-bound virulence regulator TcpP under certain conditions. The finding that YaeL is the S2P of TcpP in the initial study was significant as it was the first report of proteolysis as a means to regulate virulence factor production in *V. cholerae*. Given that the initial proteases in other known RIP systems respond most directly to the environmental signals that trigger the proteolytic cascade, it became our primary goal to uncover the protease that acts prior to YaeL in the RIP of TcpP. Using both forward and reverse genetic approaches, we identified a C-terminal protease called Tsp as being responsible for generating the substrate for the S2P YaeL. We also discovered that YaeL is able to cleave full-length TcpP directly in a Tsp-independent manner. Our attempt to further characterize the role of TcpH in the RIP of TcpP unexpectedly revealed a role for YaeL in TcpH stability. Moreover, steady state levels of TcpH appear to be TcpP-dependent. All in all, these findings have led to a new paradigm for the extent to which RIP is able to regulate virulence in *V. cholerae* and possibly in other bacterial pathogens.

DegP

In the same study that demonstrated the role of TcpH as a stability determinant of TcpP, Beck *et al.* sought to identify the protease responsible for the destruction of TcpP in the absence

of TcpH (Beck, Krukonis et al. 2004). Their attention was focused on DegP, a protease-chaperone that is ubiquitous across bacterial species (Johnson, Charles et al. 1991, Boucher, Martinez-Salazar et al. 1996, Pedersen, Radulic et al. 2001, Cortes, de Astorza et al. 2002, Purdy, Hong et al. 2002, Lyon and Caparon 2004). Despite evidence showing that DegP does not degrade TcpP (data not shown), further investigation was carried out to characterize the role of the protein in *V. cholerae* given its involvement in multiple prokaryotic and eukaryotic metabolic processes. Phenotypic assays of a *degP* mutant did not exhibit any defects characteristic of a *degP* homolog mutant, other than an increased sensitivity to bile salts (Beck, unpublished). Hence, DegP appears to play a different role from other known DegP homologs. In a later study, the role of DegP was further elucidated in a proteomic analysis of *V. cholerae* outer membrane vesicles (OMVs). Altindis *et al.* found that DegP dictates the incorporation of proteins into OMVs, besides being required for intestinal colonization, optimal type II secretion activity, and biofilm matrix assembly (Altindis, Fu et al. 2014).

As we have discussed in Chapter 1, DegP homologs in *E. coli* (HtrA) and *P. aeruginosa* (MucD) are hypothesized to eliminate misfolded proteins that can activate their respective S1Ps DegS and AlgW. In *P. aeruginosa*, MucD is found to inhibit the direct proteolysis of MucA by MucP independently of AlgW (Damron and Yu 2011). Therefore, based on these findings, we conjectured the possibility that DegP could disrupt TcpP proteolysis by inhibiting Tsp/YaeL activity. In order to test this hypothesis, we monitored TcpP* accumulation (a reporter of Tsp activity) over time in Δ*yaeL* cells that overexpressed *degP* under virulence noninducing conditions (LB pH 8.5, 37°C). We observed a loss of TcpP* accumulation in these cells (Fig. 4.1*A*, lanes 7 to 10), in contrast to Δ*yaeL* cells grown under the same conditions without *degP*

overexpression (Fig. 4.1*A*, lanes 3 to 6). This result suggests that high levels of DegP could interfere with Tsp function. We also tested two out of the three hypothetical active site residues of DegP (H110 and D140) by site-directed mutagenesis to determine their requirement for inhibition of Tsp activity. There was no recovery of TcpP* accumulation in Δ*yaeL* cells that overexpress the mutant *degP* alleles ($degP_{H110A}$ and $degP_{D140A}$) after four hours of growth under virulence noninducing conditions (Fig. 4.1.*B*, lanes 9 and 13). Therefore, active site residues H110A and D140A are not required for DegP inhibition of Tsp activity. Next, we examined whether DegP could inhibit direct proteolysis of TcpP by YaeL independently of Tsp. Hence, we compared TcpP levels between Δ*tcpH* Δ*tsp* cells that overexpress *degP* and ones that do not. Ectopic expression of *degP* failed to restore TcpP back to wild-type levels (Fig. 4.1*C*, lane 3), indicating that DegP does not inhibit YaeL activity in the same way that MucD inhibits MucP proteolysis of MucA. These new findings further reinforce the difference in function between DegP and other known DegP homologs.

Sequence Determinants of TcpP Recognition by Tsp

Work by Silber *et al.* demonstrated that *E. coli* Tsp specifically recognizes a C-terminal tripeptide in its substrate, in which the third residue from the C-terminal end is preferably alanine or leucine, the penultimate residue is preferably alanine or tyrosine, and the final residue is preferably alanine (Keiler and Sauer 1996). Tsp then cleaves at a variable distance from the C-terminus at one or more sites depending on the substrate. However, the C-terminus of TcpP does not fit the criteria, as the final five residues are glutamic acid, cysteine, threonine, lysine, and asparagine, respectively. In any case, we determined if the C-terminal residues of TcpP are required for Tsp proteolysis.

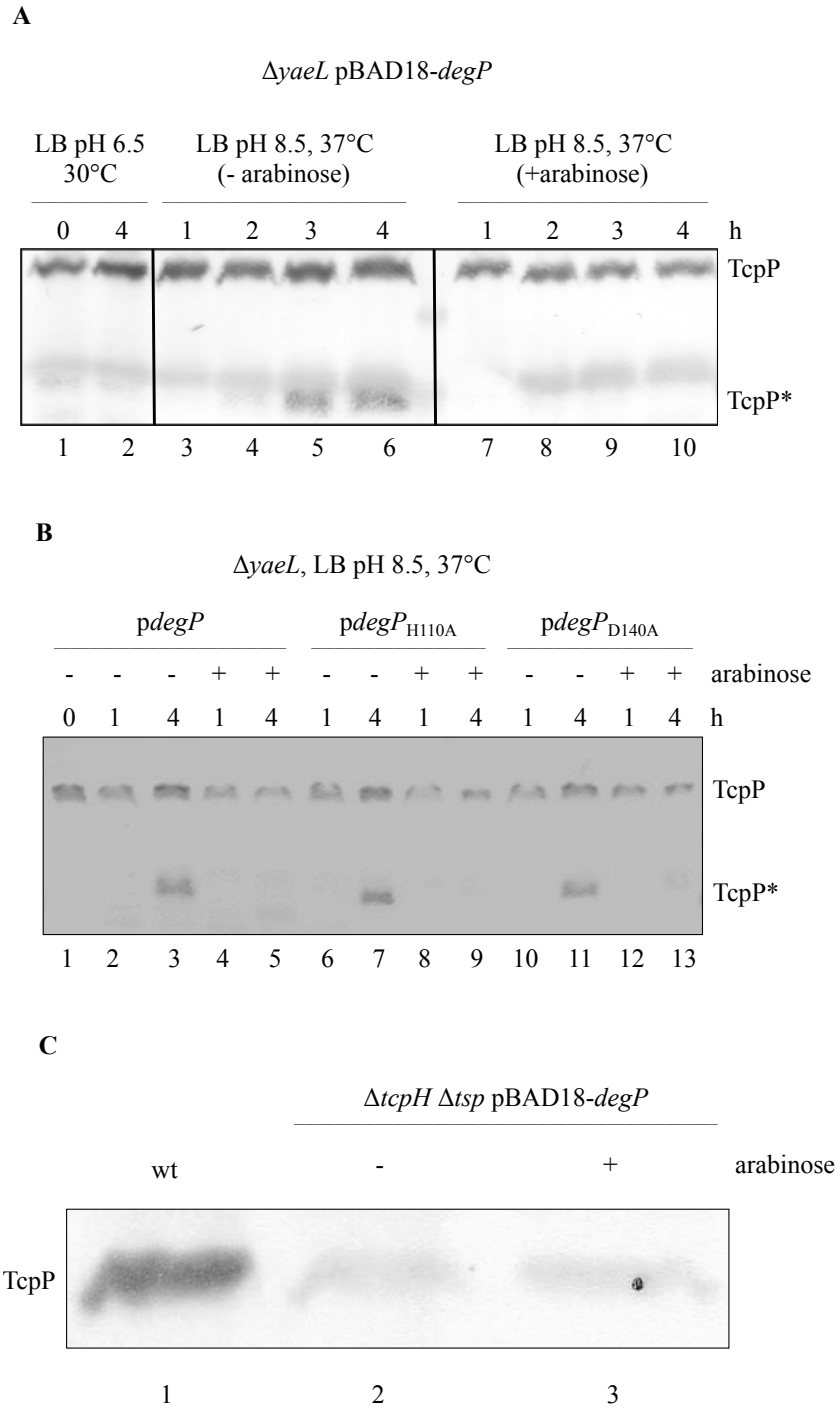

Figure 4.1. *degP* Overexpression Inhibits Function of Tsp but not of YaeL. (*A*) TcpP stability in Δ*yaeL* cells under virulence noninducing conditions (LB pH 8.5, 37°C) when *degP* is overexpressed. (*B*) TcpP stability in Δ*yaeL* cells under virulence noninducing conditions when active-site mutant *degP* alleles ($degP_{H110A}$ and $degP_{D140A}$) are overexpressed. (*C*) TcpP stability in Δ*tcpH* Δ*tsp* cells under virulence noninducing conditions when *degP* is overexpressed.

To address this question, we introduced stop codons by site-directed mutagenesis at the 3' end of *tcpP* to create short truncation variants and tested their stability in cells lacking YaeL. Deletion of two to four C-terminal residues generated another TcpP intermediate of higher molecular weight than TcpP* besides TcpP*, as we have seen when residues A172 and I174A of TcpP were altered in Chapter 2, suggesting Tsp cleavage at another site (Fig. 4.2, lanes 5, 7, and 9). In the case of the $TcpP_{-1aa}$ variant, the decrease in TcpP* accumulation compared to wild type led us to deduce that the last amino acid of TcpP (asparagine) is required for full Tsp activity (Fig. 4.2, compare lane 3 to 1). We could not make any conclusion based on the $TcpP_{-5aa}$ variant due to the overall decrease in TcpP levels, possibly the result of compromised protein stability (Fig. 4.2, lanes 11 and 12). Regardless, these observations corroborate the *in silico* findings, suggesting that either Tsp recognizes a different sequence than its homolog in *E. coli*, or that Tsp actually exerts its effect on TcpP stability via another protease.

Role of ToxT-Regulated Gene Products in TcpP Stability

One of the most important questions that has remained unanswered in the regulation of TcpP levels is what molecular signal triggers TcpP proteolysis. We described in Chapter 1 the various signals that activate known RIP pathways, and we hoped that the discovery of Tsp as the protease that acts prior to YaeL would lead us one step closer to identifying the signal that precipitates RIP of TcpP. One hypothesis we had was that the accumulation of ToxT-regulated gene products, i.e. CT and TCP, would stimulate a negative-feedback decrease in TcpP levels by activating RIP to keep virulence expression at a homeostatic state (Fig. 4.2). To test our hypothesis, we knocked out *toxT* in a Δ*yaeL* mutant, which we know accumulates TcpP* as a

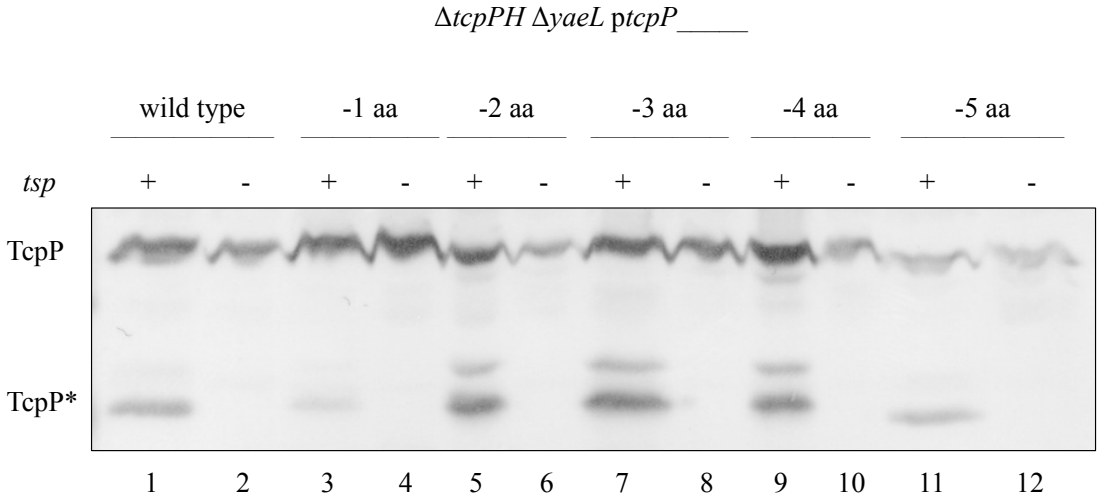

Fig. 4.2. Requirement of C-Terminal Residues of TcpP for Tsp Activity. Expression of full-length and truncated *tcpP* in Tsp-deficient cells was used to confirm the Tsp-dependence of lower molecular weight TcpP species accumulation in *tsp*-expressing cells (lanes 2, 4, 6, 8, 10, and 12). See text for details.

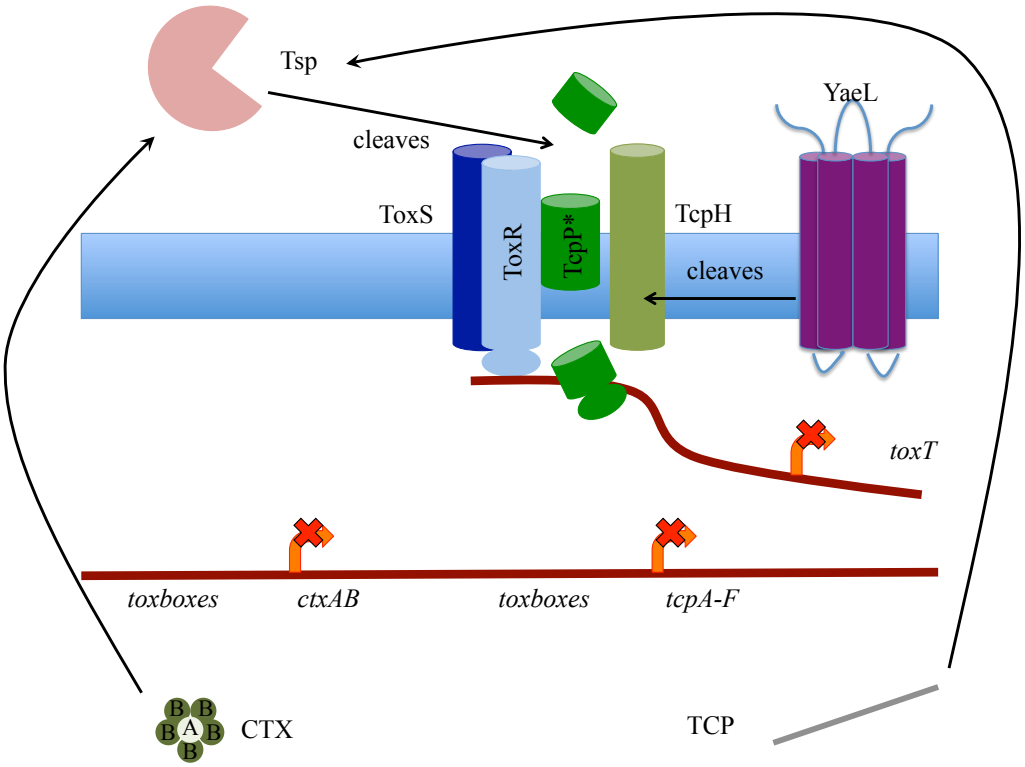

Fig. 4.3. CTX and TCP Could Trigger RIP of TcpP. See text for details.

result of Tsp activity under virulence noninducing conditions. If the hypothesis holds up, the negative feedback loop would be shut down and TcpP degradation would not occur under virulence noninducing conditions. We also introduced a *toxT* expressing plasmid into the same strain background and predicted that unregulated *toxT* expression would enhance the negative feedback loop and therefore result in TcpP degradation even when cells are grown under virulence inducing conditions.

TcpP* still accumulated to wild-type levels in a *toxT*/*yaeL* mutant grown under virulence noninducing conditions (Fig. 4.3, lane 5) whereas no TcpP* accumulation was detected in *toxT*-overexpressing cells grown under inducing conditions (Fig. 4.3 lane 4). These results led us to conclude that neither ToxT nor the products of the genes it regulates have an effect on TcpP regulation.

The Role of Extracytoplasmic Stress on TcpP Stability

As we have discussed in Chapter 1, the σ^E pathway is involved in the response to extracytoplasmic stress and plays a role in the virulence of a variety of bacterial pathogens including *P. aeruginosa* and *V. cholerae* (Firoved, Boucher et al. 2002, Kovacikova and Skorupski 2002). A *yaeL* null mutant shows a growth phenotype in LB containing 3% ethanol, a stress inducer, leading us to surmise whether the same signals that induce extracytoplasmic stress can induce RIP of TcpP.

To test our hypothesis, we grew Δ*yaeL* cells in the presence of 3% ethanol in pH 6.5 LB at 30°C and monitored for TcpP* accumulation over time. We observed a slight accumulation of

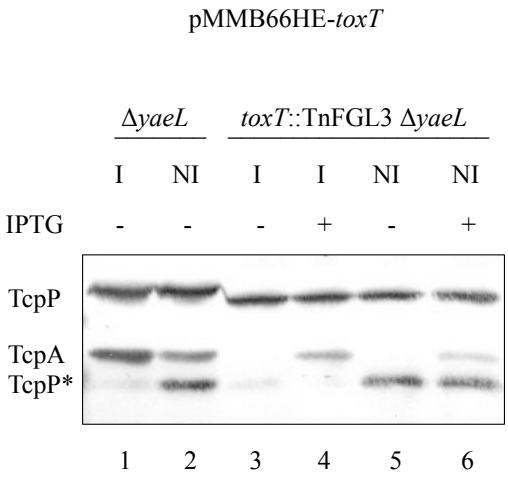

Fig. 4.4. Effect of ToxT on TcpP Stability. I denotes virulence inducing conditions (LB pH 6.5, 30°C). NI denotes virulence noninducing conditions (LB pH 8.5, 37°C). See text for details.

TcpP* towards the four-hour time point (Fig. 4.5*A*, lane 5), even though the levels are below cells that are grown in pH 8.5 LB at 37°C (Fig. 4.5*A*, lane 6). We also examined the effects of other inducers of oxidative stress (hydrogen peroxide and paraquat) on TcpP stability, but did not detect any TcpP* accumulation (Fig. 4.5*B*, lanes 8 to 11, Fig. 4.5*B*). Therefore, we concluded that extracytoplasmic stress does not play a role on TcpP proteolysis.

In Vivo Phenotype of Protease Mutants

The results supporting the role of Tsp and YaeL in TcpP degradation in this dissertation thus far have been generated from cells grown *in vitro* in LB broth. But what we are really interested in is to understand the importance of both proteases in cells grown *in vivo*. Before we moved into an *in vivo* model to address our question, we examined the *in vitro* competitive fitness of the Lac^+ protease mutants by growing them with Lac^- wild-type cells in LB broth for 18 hours. We then performed a blue-white screen to enumerate the number of cells from each strain background in every competitive group. We observed that the Δ*tsp* mutant grew as well as wild type whereas the Δ*yaeL* and Δ*tsp* Δ*yaeL* mutants were outcompeted by approximately three to fourfold (Fig. 4.6*A*). We also plated the mutants by themselves and showed that none of the mutants exhibited any plating deficiencies (data not shown). Given that the common denominator of both competition-defective mutants is the lack of YaeL, we deduced that YaeL is required for competitive viability in a population.

To assess the *in vivo* competitive fitness of the protease mutants, we coinoculated each protease mutant with wild type in 5 to 6 day old specific pathogen free (SPF) CD-1 mice. The Δ*tsp* mutant grew as well as wild type but we failed to recover any Δ*yaeL* and Δ*tsp* Δ*yaeL*

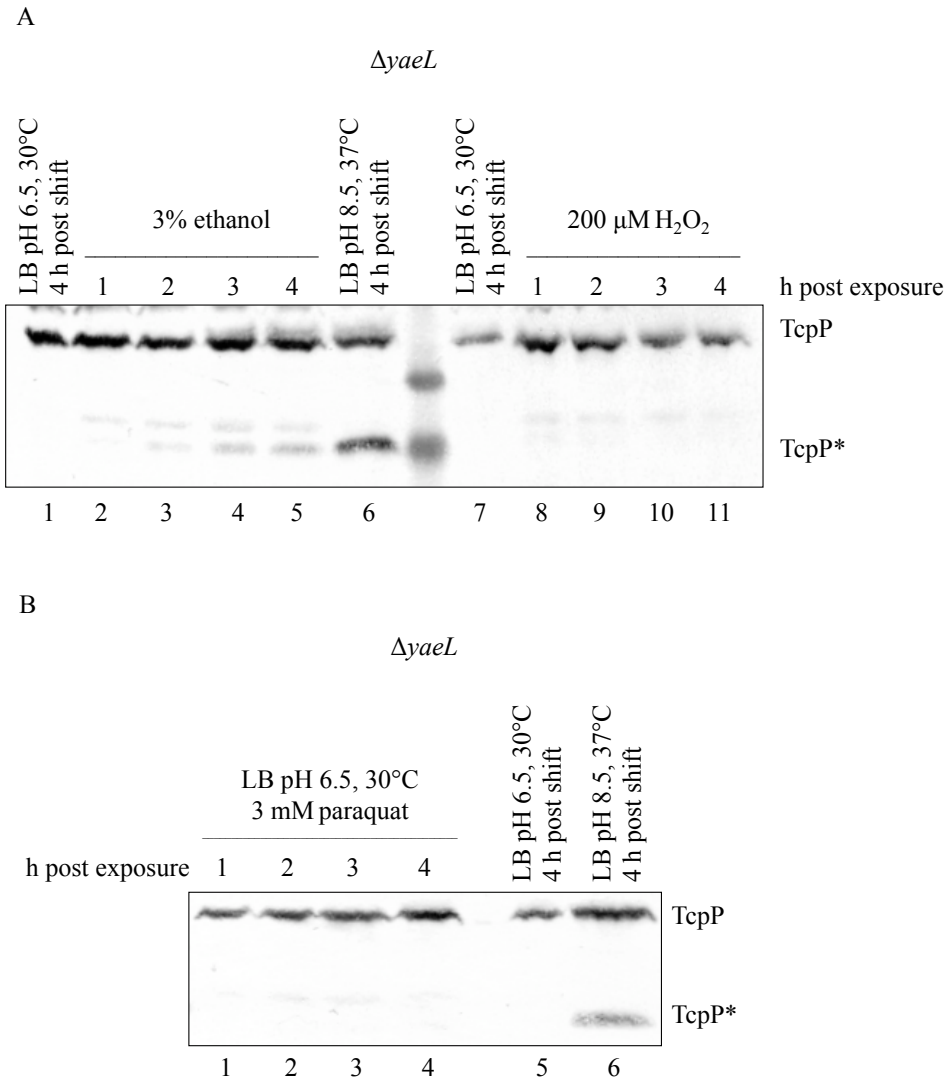

Fig. 4.5. Effect of Extracytoplasmic Stress on TcpP Stability. (*A*) Effect of 3% ethanol and hydrogen peroxide (H_2O_2) on TcpP stability over time. (*B*) Effect of paraquat on TcpP stability over time.

mutants (data not shown), reminiscent to what we observed in the *in vitro* competition. Therefore, we performed a monoculture inoculation to evaluate the colonization efficiency of each protease mutant in comparison to that of wild type. Again, there was no significant difference between the Δ*tsp* mutant and wild type whereas both Δ*yaeL* and Δ*tsp* Δ*yaeL* mutants exhibited significantly lower colonization efficiency (Fig. 4.6*B*). This suggests to us that YaeL could play a role in the response against the host immune system or microbiota, reflective of its function in the σ^E stress response pathway.

Real-Time Visualization of TcpP Proteolysis

To better understand the dynamics of TcpP proteolysis, it is imperative that we examine the interaction of TcpP with other components of the ToxR regulon (TcpH, ToxRS, and *toxT* promoter) and the proteases (Tsp and YaeL) that degrade it. Our current method of visualizing TcpP levels by immunoblotting is inadequate, as crucial time and conditions are lost during preparation of cell lysate. The advent of single-molecule fluorescence microscopy has opened a new avenue for addressing our aim in live cells. For example, this technique revealed that ToxR recruits TcpP to the *toxT* promoter (Haas, Matson et al. 2014). An ongoing project in the lab seeks to understand by two-color super-resolution imaging how the interaction between TcpP and ToxR are affected by other constituents of the ToxR regulon.

For the purpose of characterizing TcpP proteolysis, this technique would allow us to track different populations of TcpP moving in live cells with different mobilities. We predict that degraded TcpP would move faster and eventually accumulate in the cytoplasm. We could also

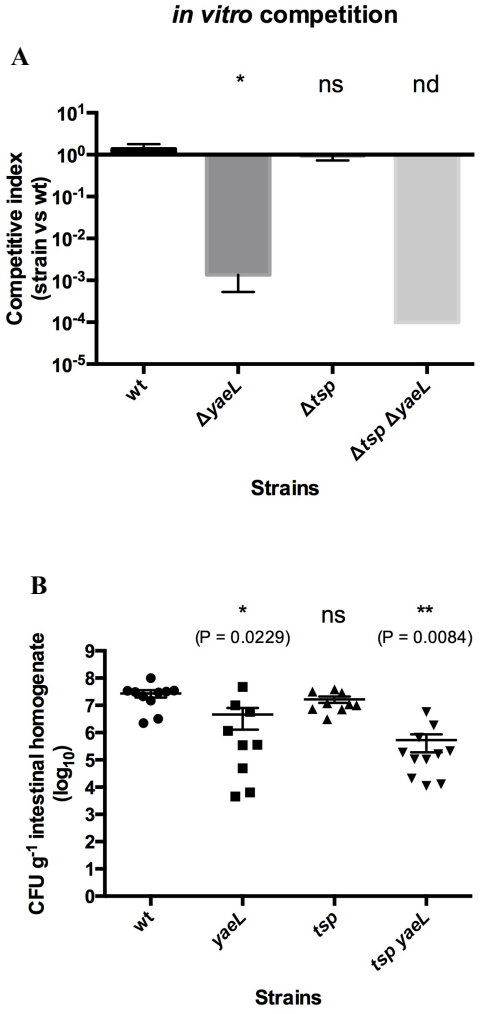

Fig. 4.6. *In Vitro* and *In Vivo* Fitness of Protease Mutants. Asterisks denote the statistical significance of a group relative to the wild-type control. ns, not significant; nd, not determined; *, $P < 0.05$; **, $P < 0.01$. (*A*) *In vitro* competition between wild type and protease mutants. See text for details. (*B*) Number of *V. cholerae* organisms recovered from mice orogastrically inoculated with 10^5 cells.

utilize this technique to study the colocalization of TcpP and Tsp/YaeL proteases. Ultimately, we hope to detect TcpP proteolysis in live animal models such as infant mice or infant rabbit, and human rice-water stool samples, once the technology is available. Our goal is to identify the environmental signal that triggers RIP of TcpP and to determine the *in vivo* relevance of proteolysis.

One of the major challenges of this study is to generate a fluorescently labeled TcpP protein that is both functional and degradable. Previous attempts to tag TcpP in the periplasmic C terminus have resulted in a hyperstable species that is resistant to Tsp degradation (data not shown). Tagging TcpP in the N terminus would also be infeasible, as doing so would disrupt its DNA-binding function. Therefore, our only option was to generate an internally tagged TcpP. We tested a TcpP variant with an internal citrine fluorescent protein tag that was kindly provided by Eric Krukonis for its function and ability to be degraded. Unfortunately, the absence of TcpA accumulation and TcpP intermediate in a YaeL-deficient cell indicate that the variant is neither functional (Fig. 4.7, lane 3 to 5) nor degradable (Fig. 4.7, lane 5). Further structural studies will need to be performed to determine the optimal strategy for tagging TcpP.

Future Directions

This dissertation consists of two relevant stories that are connected by the interdependence of two membrane-bound proteins, each of which forms the subject of focus in the respective narratives. The beginning of this dissertation describes how we uncovered the

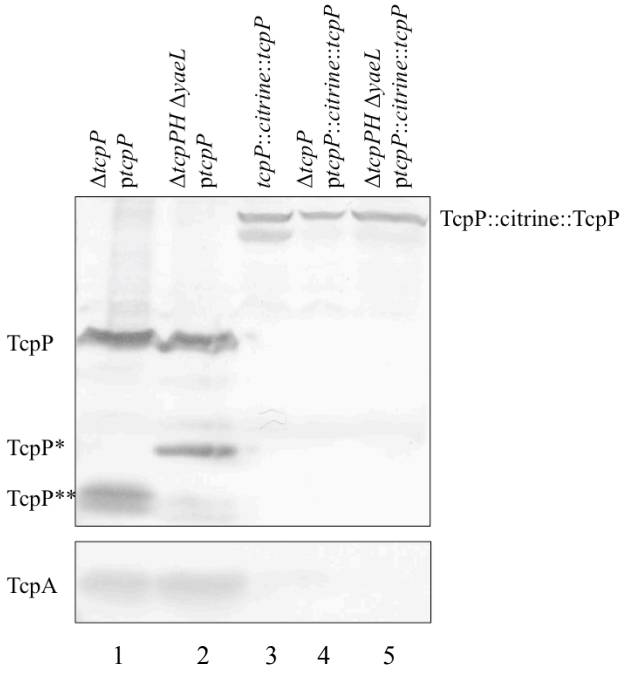

Fig. 4.7. Stability and Function of TcpP Variant with Internal Citrine Tag. See text for details.

protease that provides the TcpP intermediate substrate for the previously identified site-2 protease YaeL (Matson and DiRita 2005). To establish a full-fledged model of how TcpP levels are regulated, it is of paramount importance that we address the following major questions (1) How are TcpH levels regulated and (2) what molecular signals trigger RIP of TcpP?

A study to determine the structural requirements for TcpH inhibition of TcpP proteolysis unexpectedly led us to uncover a role for YaeL in TcpH stability. This naturally suggests that TcpH is also subjected to RIP, which to our understanding is the first known RIP inhibitor to experience a similar fate as the protein that it supposedly protects. Moreover, we also discovered that TcpH requires TcpP for stability. In order to gain a better understanding of TcpP/TcpH interaction and the extent to which RIP plays a role in the regulation of virulence in *V. cholerae*, the following questions should be resolved: (1) is there a protease acting prior to YaeL in TcpH proteolysis, just as Tsp is the protease acting prior to YaeL in TcpP proteolysis; (2) what are the molecular signals that trigger TcpH proteolysis: more specifically, are they the same as the ones that trigger TcpP proteolysis, or can high levels of LPS fragments in the periplasm sequester TcpH away from TcpP in the same way that those signals do to RseB and MucB (Chaba, Alba et al. 2011, Lima, Guo et al. 2013); (3) are there other proteases besides Tsp that cleave TcpP prior to YaeL just as the case in the σ^W pathway of *B. subtilis* (Heinrich, Hein et al. 2009); (4) is there a link between TcpP proteolysis and the entry of *V. cholerae* into an environmentally persistent state as have been established with ToxR proteolysis (Almagro-Moreno, Kim et al. 2015); (5) does TcpP proteolysis occur in the host environment?

Final Comments

In pursuing these questions concerning TcpP regulation, it will be important to remain open-minded about alternative routes that lead to the proteolysis of the membrane-bound transcription factor. As we have discussed in Chapter 1, AlgW is responsible for the site-1 cleavage of full-length MucA in the alginate biosynthesis pathway of *P. aeruginosa* (Qiu, Eisinger et al. 2007). However, high incidences of *mucA* mutations, which in turn led to the translation of truncated MucA variants, were discovered in isolates from CF patients (Pulcrano, Iula et al. 2012). These variants are cleaved by a Tsp homolog – AlgO, instead of the conventional S1P AlgW (Reiling, Jansen et al. 2005). That being said, the *P. aeruginosa* population in CF lungs is a heterogeneous one, as both wild type and *mucA* mutants coexist in the same environment (Pulcrano, Iula et al. 2012). Likewise, a similar scenario could play out in the intestinal lumens of cholera patients, where phase variation in *tcpH* (Carroll, Tashima et al. 1997) leads to some cells cleaving TcpP in an orderly fashion via the Tsp/YaeL pathway, while others cleave full-length TcpP directly through YaeL. In fact, a previous report has shown that *tcpA* expression is heterogeneous in luminal fluid at later stages of infection (Nielsen, Dolganov et al. 2010), suggesting that *V. cholerae* could display a bifurcation phenotype through various regulatory mechanisms. Therefore, one should always remember that the host environment is far from a homogeneous one and bacteria employ an "end justifies the means" strategy to ensure survival and dissemination to the next host.

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