Molecular Microbiology (2015) 97(5), 822–831 ■
doi:10.1111/mmi.13069
First published online 30 July 2015

Regulated intramembrane proteolysis of the virulence activator TcpP in *Vibrio cholerae* is initiated by the tail-specific protease (Tsp)

Wei Ping Teoh,[1] Jyl S. Matson[2] and Victor J. DiRita[1]*
[1]*Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA.*
[2]*Department of Medical Microbiology and Immunology, University of Toledo, Toledo, OH 43614, USA.*

Summary

Vibrio cholerae **uses a multiprotein transcriptional regulatory cascade to control expression of virulence factors cholera toxin and toxin-co-regulated 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-co-regulated 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 *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-co-regulated pilus (DiRita and Mekalanos, 1991).

Cellular TcpP levels are regulated at multiple levels of expression (Matson and DiRita, 2005; Matson *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 *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 *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 *Escherichia 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

Accepted 20 May, 2015. *For correspondence. E-mail vdirita@umich.edu; Tel. (+1) 734 936 3804; Fax (+1) 734 764 3562.

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 *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 *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 *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 *et al.*, 2005; Qiu *et al.*, 2007; Pulcrano *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) and 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.

Table 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

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 *et al.*, 1989; 1991; Nagasawa *et al.*, 1989). 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 1).

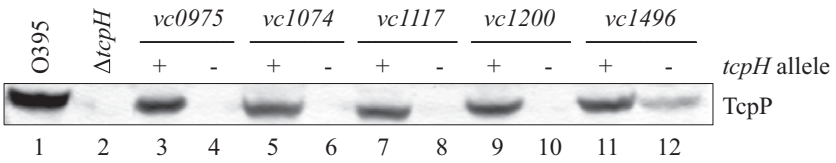

Fig. 1. Effect of transposon insertion in candidate protease genes on TcpP stability. The following transposon-inserted alleles were transduced from C6706 into O395 wild type (lanes 3, 5, 7, 9 and 11) and O395 Δ*tcpH* (lanes 4, 6, 8, 10 and 12) backgrounds: *vc0975* (lanes 3 and 4), *vc1074* (lanes 5 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.

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 *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. 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 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. S1). 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 (data not shown). 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). 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). In contrast, within 30 min of inducing *tsp* in the triple mutant lacking TcpH, a lower molecular weight species corresponding in size to TcpP*

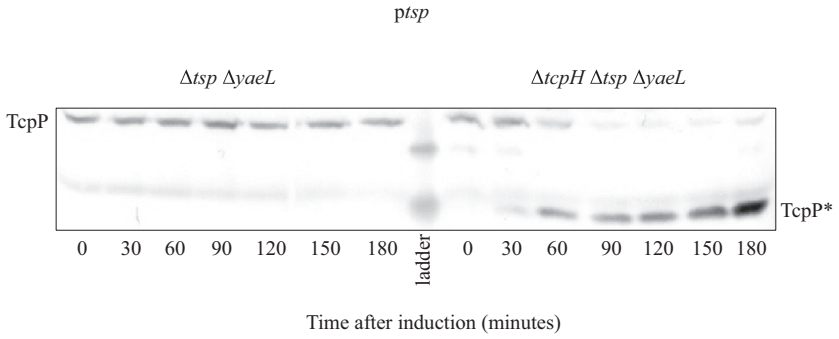

Fig. 2. TcpP* accumulation as a function of *tsp* expression. Overnight cultures of Δ*tsp* Δ*yaeL* and Δ*tcpH* Δ*tsp* Δ*yaeL* cells containing pBAD18-Kan-*tsp* were subcultured 1:50 and grown to midlogarithmic phase in pH 6.5 LB at 30°C. Arabinose was then added (final concentration, 0.1%) and grown 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.

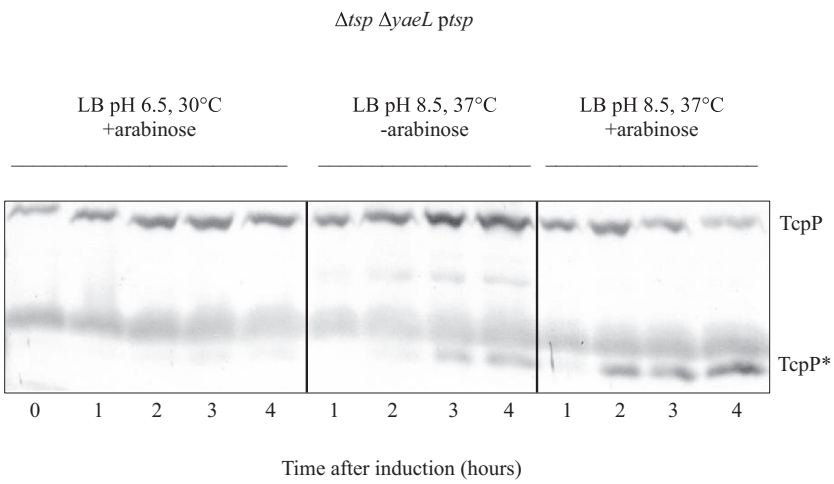

Fig. 3. TcpP proteolysis in the presence of TcpH. Overnight cultures of O395 Δ*tsp* Δ*yaeL*/*p*BAD18-Kan-*tsp V. cholerae* were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. Cell culture was split into two equal portions for harvest by centrifugation and resuspended either 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.

accumulated (right panel in Fig. 2). By 3 h, TcpP* had accumulated to high levels, and full-length TcpP was barely 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) 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. 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). Lack of TcpP* accumulation at pH 6.5/30°C even when *tsp* is overexpressed (Fig. 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. 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. 1, lane 12), and a corresponding decrease in *toxT-lacZ* expression relative to wild type in the Δ*tcpH vc1496*::TnFGL3 mutant (Table 1). This is reminiscent of what has been reported in the RIP 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 *et al.*, 2004). We hypothesized that YaeL can similarly attack TcpP when the first protease (Tsp) and the coexpressed protease inhibitory protein (TcpH) are missing.

To test this, we inducibly expressed wild-type *yaeL* and a mutant *yaeL* allele with a lesion in a predicted key

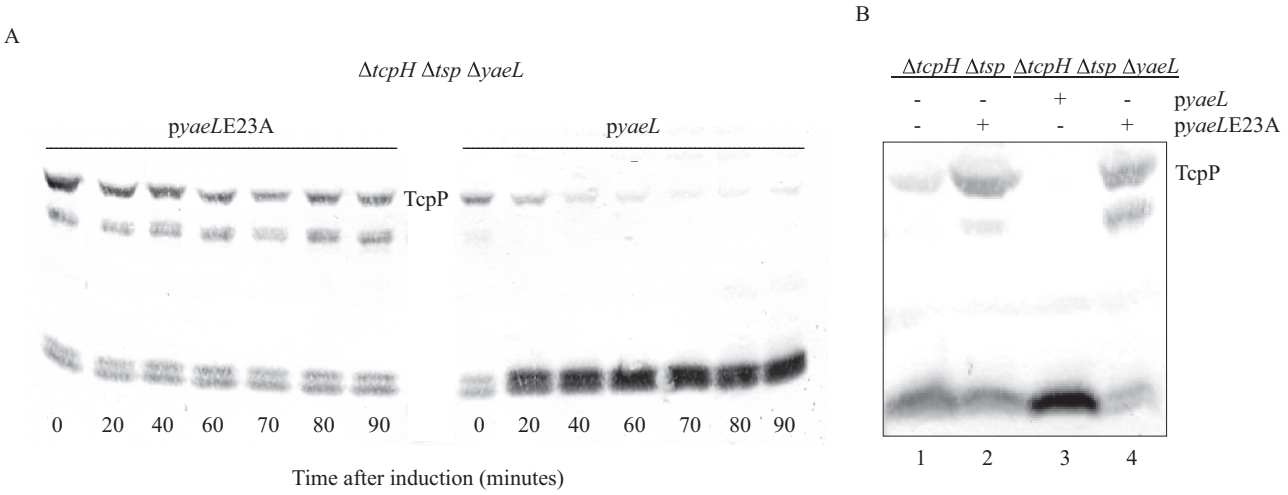

Fig. 4. Effects of *yaeL* expression on TcpP stability in the absence of TcpH and Tsp.
A. TcpP stability in Δ*tcpH* Δ*tsp* Δ*yaeL* cells with *yaeL*E23A or *yaeL* ectopically expressed from a plasmid copy. Overnight cultures were subcultured 1:50 and grown to mid-logarithmic phase in pH 6.5 LB at 30°C. 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.

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 min (right panel of Fig. 4A; Fig. 4B lane 3). In contrast, inducing *yaeL*E23A resulted in far less degradation of full-length TcpP over time (left panel in Fig. 4A; Fig. 4B 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. 4B, 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 *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 Δ*tcpH* cells under these conditions (Fig. 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. 5, lane 12), similar to what we observed with full-length TcpP (Fig. 5, lane 4). However, when expressed in Δ*tcpH* Δ*yaeL* cells, $TcpP_{190}$ was processed to TcpP*, which accumulated to high levels (Fig. 5, lane 10), again similar to what we observed with full-length TcpP (Fig. 5, lane 2). In contrast, $TcpP_{169}$ accumulated to similar levels irrespective of whether or not *tsp* is expressed in the cell (compare Fig. 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. 5, lane 11).

Insertions of the pentapeptide CGRTG using a transposon-based approach (Hallet *et al.*, 1997) guided

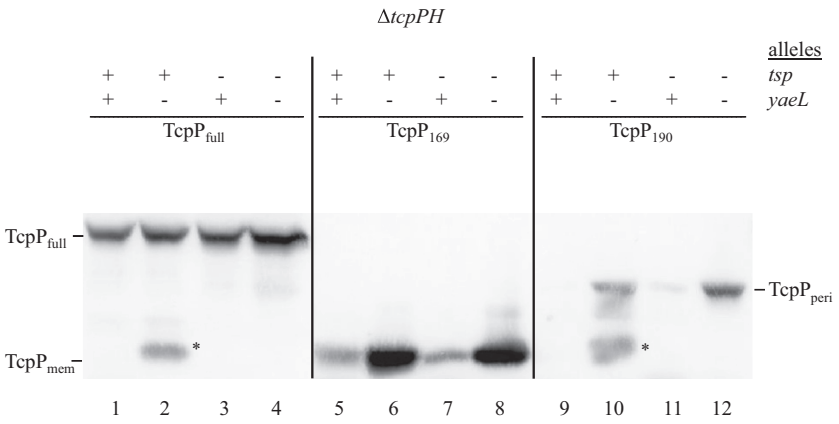

Fig. 5. Stability of TcpP truncations in the presence and absence of Tsp. The following plasmids were expressed in O395 Δ*tcpPH* (lanes 1, 5 and 9), O395 Δ*tcpPH* Δ*yaeL* (lanes 2, 6 and 10), O395 Δ*tcpPH* Δ*tsp* (lanes 3, 7 and 11) and O395 Δ*tcpPH* Δ*tsp* Δ*yaeL* (lanes 4, 8 and 12): pBAD18-Kan-P_{full} (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.

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.

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 (Fig. 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 *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

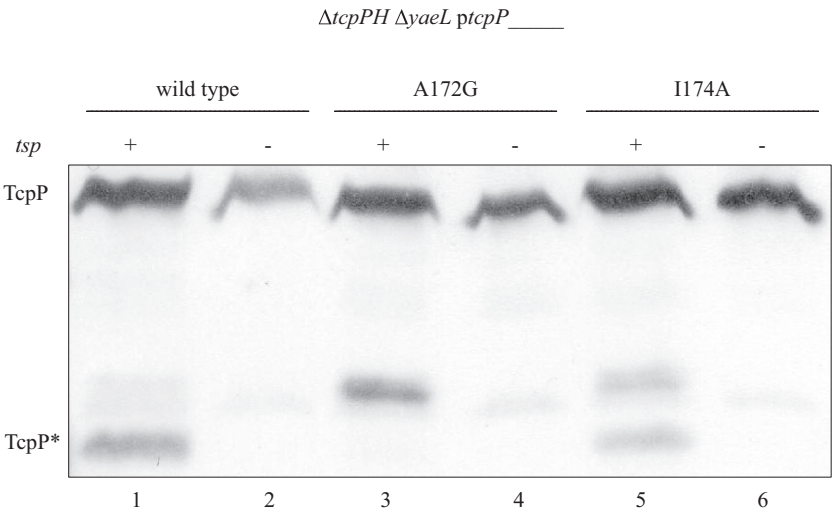

Fig. 6. Effects of point mutations in TcpP region hypothesized to contain Tsp cleavage site. The following plasmids were expressed in O395 Δ*tcpPH* Δ*yaeL* cells (lanes 1, 3 and 5) and Δ*tcpPH* Δ*tsp* Δ*yaeL* cells (lanes 2, 4 and 6): pBAD18-Kan-*tcpP* (lanes 1 and 2), pBAD18-Kan-*tcpP*A172G (lanes 3 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.

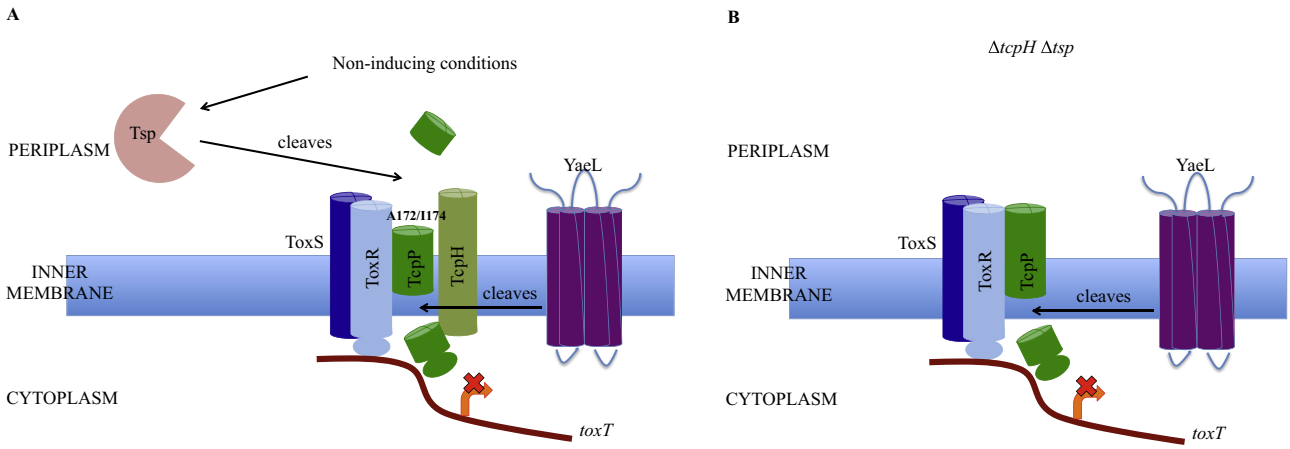

Fig. 7. Two modes of TcpP degradation.
A. Wild-type: Tsp-dependent proteolysis of TcpP. When conditions no longer favor virulence gene expression, Tsp is activated to initiate proteolysis of TcpP, a process that requires residues A172 and I174 of TcpP for proper localization. YaeL can then cleave the 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.

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. 7A). 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. 7B), 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 *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 *et al.*, 1980; Hayashi *et al.*, 1988; Hara *et al.*, 1989; 1991; Nagasawa *et al.*, 1989). Also in *E. coli*, Tsp can degrade a periplasmic protein via the SsrA peptide-tagging system that targets proteins expressed from damaged mRNA (Keiler *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 *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 (Figs 2 and 3, and Fig. S2) and the accumulation of a slightly smaller-than-full-length TcpP species in C6706 *tcpH*/*tsp* mutants (Fig. S1) 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 *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 *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 *et al*., 2007; Sohn *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 *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 *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 *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 *et al*., 2015). The authors determined that ToxR proteolysis occurs when *V. cholerae* enters a dormant state called viable but non-culturable (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, 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

Vibrio 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 Luria–Bertani (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 *et al*., 1999), and the arabinose-inducible expression vector pBAD18-Kan (Guzman *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^{-1}; kanamycin, 50 μg ml^{-1}; and streptomycin, 100 μg ml^{-1}, except when selecting for loss of plasmid integrants, when it was used at 1 mg ml^{-1}. X-Gal (Invitrogen) was used in LB agar at 40 μg ml^{-1}.

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 *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 *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^{-1} carbenicillin. Resolution of the cointegrate was selected on LB plates containing 1 mg ml^{-1} 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 poly-

merase (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 *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 h at 30°C. Arabinose was added to the culture medium at the time of subculture or 4–5 h after subculture for strains containing pBAD18 or pBAD18-Kan. One milliliter of midlogarithmic culture was pelleted by centrifugation and resuspended in 1 × 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. One hundred microliters of culture samples in duplicates were used to measure β-galactosidase activity as described in 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.

Acknowledgements

This work was supported by AI045125 to V.J.D. from NIAID. The authors declare that there are no conflicts of interest.

References

Almagro-Moreno, S., Kim, T.K., Skorupski, K., and Taylor, R.K. (2015) Proteolysis of virulence regulator ToxR is associated with entry of *Vibrio cholerae* into a dormant state. *PLoS Genet* **11:** e1005145.

Bastiaansen, K.C., Ibanez, A., Ramos, J.L., Bitter, W., and Llamas, M.A. (2014) The Prc and RseP proteases control bacterial cell-surface signalling activity. *Environ Microbiol* **16:** 2433–2443.

Beck, N.A., Krukonis, E.S., and DiRita, V.J. (2004) TcpH influences virulence gene expression in *Vibrio cholerae* by inhibiting degradation of the transcription activator TcpP. *J Bacteriol* **186:** 8309–8316.

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* **100:** 391–398.

Cameron, D.E., Urbach, J.M., and Mekalanos, J.J. (2008) A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **105:** 8736–8741.

Chaba, R., Alba, B.M., Guo, M.S., Sohn, J., Ahuja, N., Sauer, R.T., and Gross, C.A. (2011) Signal integration by DegS and RseB governs the sigmaE-mediated envelope stress response in *Escherichia coli*. *Proc Natl Acad Sci USA* **108:** 2106–2111.

Damron, F.H., and Goldberg, J.B. (2012) Proteolytic regulation of alginate overproduction in *Pseudomonas aeruginosa*. *Mol Microbiol* **84:** 595–607.

DiRita, V.J., and Mekalanos, J.J. (1991) Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. *Cell* **64:** 29–37.

Grigorova, I.L., Chaba, R., Zhong, H.J., Alba, B.M., Rhodius, V., Herman, C., and Gross, C.A. (2004) Fine-tuning of the *Escherichia coli* sigmaE envelope stress response relies on multiple mechanisms to inhibit signal-independent proteolysis of the transmembrane anti-sigma factor, RseA. *Genes Dev* **18:** 2686–2697.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177:** 4121–4130.

Hallet, B., Sherratt, D.J., and Hayes, F. (1997) Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein. *Nucleic Acids Res* **25:** 1866–1867.

Hara, H., Nishimura, Y., Kato, J., Suzuki, H., Nagasawa, H., Suzuki, A., and Hirota, Y. (1989) Genetic analyses of processing involving C-terminal cleavage in penicillin-binding protein 3 of *Escherichia coli*. *J Bacteriol* **171:** 5882–5889.

Hara, H., Yamamoto, Y., Higashitani, A., Suzuki, H., and Nishimura, Y. (1991) Cloning, mapping, and characterization of the *Escherichia coli prc* gene, which is involved in C-terminal processing of penicillin-binding protein 3. *J Bacteriol* **173:** 4799–4813.

Hase, C.C., and Mekalanos, J.J. (1998) TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **95:** 730–734.

Hasselblatt, H., Kurzbauer, R., Wilken, C., Krojer, T., Sawa, J., Kurt, J., *et al*. (2007) Regulation of the sigmaE stress response by DegS: how the PDZ domain keeps the protease inactive in the resting state and allows integration of different OMP-derived stress signals upon folding stress. *Genes Dev* **21:** 2659–2670.

Hava, D.L., and Camilli, A. (2001) Isolation and characterization of a temperature-sensitive generalized transducing bacteriophage for *Vibrio cholerae*. *J Microbiol Methods* **46:** 217–225.

Hayashi, S., Hara, H., Suzuki, H., and Hirota, Y. (1988) Lipid modification of *Escherichia coli* penicillin-binding protein 3. *J Bacteriol* **170:** 5392–5395.

Heinrich, J., Hein, K., and Wiegert, T. (2009) Two proteolytic

modules are involved in regulated intramembrane proteolysis of *Bacillus subtilis* RsiW. *Mol Microbiol* **74:** 1412–1426.
Keiler, K.C., and Sauer, R.T. (1996) Sequence determinants of C-terminal substrate recognition by the Tsp protease. *J Biol Chem* **271:** 2589–2593.
Keiler, K.C., Silber, K.R., Downard, K.M., Papayannopoulous, I.A., Biemann, K., and Sauer, R.T. (1995) C-terminal specific protein degradation: activity and substrate specificity of the Tsp protease. *Protein Sci* **4:** 1507–1515.
Keiler, K.C., Waller, P.R., and Sauer, R.T. (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271:** 990–993.
Kovacikova, G., and Skorupski, K. (1999) A *Vibrio cholerae* LysR homolog, AphB, cooperates with AphA at the *tcpPH* promoter to activate expression of the ToxR virulence cascade. *J Bacteriol* **181:** 4250–4256.
Martinez-Hackert, E., and Stock, A.M. (1997) Structural relationships in the OmpR family of winged-helix transcription factors. *J Mol Biol* **269:** 301–312.
Matson, J.S., and DiRita, V.J. (2005) Degradation of the membrane-localized virulence activator TcpP by the YaeL protease in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **102:** 16403–16408.
Matson, J.S., Withey, J.H., and DiRita, V.J. (2007) Regulatory networks controlling *Vibrio cholerae* virulence gene expression. *Infect Immun* **75:** 5542–5549.
Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Miller, M.B., Skorupski, K., Lenz, D.H., Taylor, R.K., and Bassler, B.L. (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* **110:** 303–314.
Nagasawa, H., Sakagami, Y., Suzuki, A., Suzuki, H., Hara, H., and Hirota, Y. (1989) Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*. *J Bacteriol* **171:** 5890–5893.
Pulcrano, G., Iula, D.V., Raia, V., Rossano, F., and Catania, M.R. (2012) Different mutations in *mucA* gene of *Pseudomonas aeruginosa* mucoid strains in cystic fibrosis patients and their effect on *algU* gene expression. *New Microbiol* **35:** 295–305.
Qiu, D., Eisinger, V.M., Rowen, D.W., and Yu, H.D. (2007) Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **104:** 8107–8112.
Reiling, S.A., Jansen, J.A., Henley, B.J., Singh, S., Chattin, C., Chandler, M., and Rowen, D.W. (2005) Prc protease promotes mucoidy in *mucA* mutants of *Pseudomonas aeruginosa*. *Microbiology* **151** (Part 7): 2251–2261.
Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., and Mekalanos, J.J. (1999) In vivo transposition of mariner-based elements in enteric bacteria and mycobacteria. *Proc Natl Acad Sci USA* **96:** 1645–1650.
Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Skorupski, K., and Taylor, R.K. (1996) Positive selection vectors for allelic exchange. *Gene* **169:** 47–52.
Sohn, J., Grant, R.A., and Sauer, R.T. (2009) OMP peptides activate the DegS stress-sensor protease by a relief of inhibition mechanism. *Structure* **17:** 1411–1421.
Tamura, T., Suzuki, H., Nishimura, Y., Mizoguchi, J., and Hirota, Y. (1980) On the process of cellular division in *Escherichia coli*: isolation and characterization of penicillin-binding proteins 1a, 1b, and 3. *Proc Natl Acad Sci USA* **77:** 4499–4503.
Weski, J., Meltzer, M., Spaan, L., Monig, T., Oeljeklaus, J., Hauseke, P., *et al*. (2012) Chemical biology approaches reveal conserved features of a C-terminal processing PDZ protease. *Chembiochem* **13:** 402–408.
Wood, L.F., Leech, A.J., and Ohman, D.E. (2006) Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. *Mol Microbiol* **62:** 412–426.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.