AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

MINIREVIEW

Bacterial Carboxyl-Terminal Processing Proteases Play Critical Roles in the Cell Envelope and Beyond

Alexis G. Sommerfield,[a] Andrew J. Darwin[a]

[a]Department of Microbiology, NYU Grossman School of Medicine, New York, New York, USA

ABSTRACT Proteolysis is essential throughout life, and as more proteases are characterized, our understanding of the roles they play continues to expand. Among other things, proteases are critical for protein turnover and quality control, the activation or inactivation of some enzymes, and they are integral components of signal transduction pathways. This review focuses on a family of proteases in bacteria known as the carboxyl-terminal processing proteases, or CTPs. Members of this family occur in all domains of life. In bacteria, CTPs have emerged as important enzymes that have been implicated in critical processes including regulation, stress response, peptidoglycan remodeling, and virulence. Here, we provide an overview of the roles that CTPs play in diverse bacterial species, and some of the underlying mechanisms. We also describe the structures of some bacterial CTPs, and their adaptor proteins, which have revealed striking differences in arrangements and mechanisms of action. Finally, we discuss what little is known about the distinguishing features of CTP substrates and cleavage sites, and speculate about how CTP activities might be regulated in the bacterial cell. Compared with many other proteases, the study of bacterial CTPs is still in its infancy, but it has now become clear that they affect fundamental processes in many different species. This is a protease family with broad significance, and one that holds the promise of more high impact discoveries to come.

KEYWORDS carboxyl-terminal processing protease, cell envelope, stress response, gene regulation, cell wall hydrolase

Bacterial cells contain many proteases, belonging to different families, and are associated with a wide range of functions. One widely conserved family is the carboxyl-terminal processing proteases (CTPs), which are assigned as the S41A family of serine proteases in the MEROPS peptidase database (1). Two of their distinguishing features are a serine-lysine catalytic dyad, and a PDZ domain that is thought to be important for substrate recognition. Another common feature is that all bacterial CTPs are known or predicted to be located outside of the cytoplasm. This fits with the fact that they are ATP-independent proteases. However, there are also some differences between them. The MEROPS database assigns some CTPs into subgroups based on homology and domain organization. For example, *Escherichia coli* Prc/Tsp, was assigned to the C-terminal processing peptidase-1 group. Members of this group are larger than some other CTPs and have additional domains at their N- and C-terminal ends.

Some bacterial CTPs have been identified only by association with a phenotype, but how the CTP affects the phenotype has not been determined in many cases (Table 1). The number of CTP substrates that meet the gold standard of confirmation by both *in vivo* and *in vitro* assays is low. Nevertheless, in recent years some bacterial CTPs have been characterized in detail and their substrates identified (Table 1). The picture emerging is one in which CTPs play diverse roles, including protein processing, signal transduction, and quality control. In Gram-negative bacteria, an exciting role has emerged in the degradation of cell wall hydrolases, and it is likely to be a widely conserved phenomenon (2, 3).

Editor Conrad W. Mullineaux, Queen Mary University of London

Address correspondence to Andrew J. Darwin, andrew.darwin@med.nyu.edu.

The authors declare no conflict of interest.

Published 16 March 2022

TABLE 1 Bacterial C-terminal processing proteases in the literature

Species and CTPs[a]	Notable null phenotype[b]	Known or proposed substrates(s)	Selected references
Acinetobacter baumannii			
Ctp WP_000776302.1	Reduced virulence	None	(23, 24)
Bacillus subtilis			
CtpA WP_003231186.1	DNA damage sensitivity	YneA	(41, 42)
CtpB WP_003228041.1	Sporulation defect	SpoIVFA	(39, 40, 74–76)
Bartonella bacilliformis			
CtpA WP_005766244.1	Not tested	None	(77)
Bordetella Bronchiseptica			
CtpA WP_033446625.1	Defective FHA processing	FhaB[c]	(27)
Borrelia burgdorferi			
CtpA WP_002656708.1	Reduced growth rate	P13[c], BB0323[c], OspC[c]	(29, 30)
Brucella suis			
CtpA WP_004690508.1	Reduced virulence	None	(20)
Burkholderia mallei			
CtpA WP_004198008.1	Reduced virulence	None	(21)
Burkholderia pseudomallei			
Prc WP_004534131.1	Meropenem sensitivity	None	(22)
Chlamydia trachomatis			
CT441 WP_010725198.1	Not tested	NF-κB p65 subunit (host protein)[c,d]	(31, 32, 35)
Escherichia coli K-12			
Prc WP_032199908.1	Thermosensitive at low osmolarity	FtsI, MepM, MepS, MltG, MltB[c], DigH[c]	(6, 9, 50, 62–64, 67, 78)
Legionella pneumophila			
Tsp WP_010946247.1	Thermal stress sensitivity	None	(15, 79)
Paenibacillus lautus			
CtpA WP_015737691.1	Not tested	None	(80)
Pseudomonas aeruginosa			
CtpA WP_003096067.1	Reduced virulence	MepM, PA1048, PA1198, PA1199, PA4404	(3, 68, 69)
Prc WP_003091593.1	Suppresses mucoid phenotype	MucA22[c], FoxR[c], FiuR[c]	(47, 55, 59, 81)
Pseudomonas putida			
Prc WP_164721827.1	Altered cell surface signaling	IutY[c], PP_0161 (FoxR)[c], PP_0351 (FiuR)[c]	(47, 49)
Staphylococcus aureus			
CtpA WP_000342125.1	Reduced virulence	SosA[c]	(43, 82)
Synechocystis sp. PCC 6803			
CtpA WP_010873417.1	Photosynthesis defect	D1	(12, 14)
Vibrio cholerae			
Tsp WP_000650135.1	Altered TcpP cleavage	TcpP[c]	(46)
Xanthomonas campestris			
Prc WP_014509001.1	Osmostress sensitivity	VgrS	(44, 45)

[a]Listed alphabetically by genus. The name of each protease as first reported in the literature is followed by a National Center for Biotechnology Information RefSeq nonredundant protein identification number.
[b]In some cases, multiple phenotypes have been reported for protease null strains (see references) but only one of the notable phenotypes is listed.
[c]Not confirmed to be directly cleaved/degraded by the purified protease *in vitro*.
[d]Cleavage of p65 by CT441 has been called into question (33, 34).

EARLY DISCOVERIES OF BACTERIAL CTPS

The first bacterial CTP was reported in *E. coli*. The transpeptidase Penicillin-Binding Protein 3 (PBP3 or FtsI) is made as a precursor (4). A processing event was found to remove a short C-terminal region from PBP3, and an *E. coli* strain was identified with a mutation that prevented it (5). The mutation was named *prc*, to reflect the defect in processing the C-terminus of PBP3 (5). Subsequent work identified the *prc* gene, which encoded a periplasmic protein with no homology to any known proteins at the time (6). The Prc-dependent cleavage site in PBP3 was also identified, and later work confirmed direct cleavage of PBP3 by Prc *in vitro* (7, 8).

Another group was interested in understanding why C-terminal amino acids affected protein half-lives in *E. coli* (9). Wild type λ repressor has a half-life of over 10 h, whereas a variant with the five C-terminal polar amino acids replaced by nonpolar residues has a half-life of only 15 min (10). An *E. coli* protein that rapidly degraded the mutant λ repressor *in vitro*, but not the wild type, was purified and named Tsp for Tail-specific protease (9). Tsp and Prc are the same protein, and both names persist in the literature. However, it was apparent that Tsp/Prc was not responsible for degrading λ repressor *in vivo*, because Tsp/Prc is periplasmic whereas λ repressor is in the cytoplasm (11). Even so, Prc might degrade cell envelope proteins with aberrant C termini, as described later.

Shortly after the discovery of Prc, a CTP involved in the functioning of photosystem II (PSII) was identified in the cyanobacterium *Synechocystis* sp. PCC 6803 (12). This species is used as a model to investigate the PSII complex, two components of which make up the reaction core, the D1 and D2 proteins. In most oxygenic photosynthetic organisms, the D1 protein has a short C-terminal extension that must be removed to make it functional (13). To find proteins crucial for PSII function, spontaneous photosynthesis-deficient *Synechocystis* sp. PCC 6803 mutants were isolated (12). One mutation introduced a premature stop codon into a gene that was named *ctpA* (carboxyl-terminal processing protease) (12). The CtpA protein had a predicted signal sequence to direct it across the thylakoid membrane, and a mature portion that was 28% identical and 50% similar to *E. coli* Prc (12). Further characterization found that CtpA was responsible for processing the D1 protein into its mature form by removing the C-terminal extension (14).

Since these early discoveries, several other CTPs have been identified in bacteria (Table 1). For example, the *Legionella pneumophila* Tsp protein is important for survival under thermal stress and inside amoebae (15). *Rhizobium leguminosarum* CtpA is important for desiccation tolerance, biofilm formation, and cell envelope integrity (16–19). *Brucella suis* and *Burkholderia mallei* CTP-defective mutants have disrupted envelopes and reduced virulence, and a *Burkholderia pseudomallei* CTP mutant has increased sensitivity to meropenem (20–22). An *Acinetobacter baumannii ctp* null mutant has reduced motility, cell envelope integrity, and virulence, along with increased autolysis (23, 24). These and other examples show that CTPs are important for stress tolerance, cell envelope functions, and virulence, but the substrates of these CTPs are unknown. However, there are CTPs for which much more is known, some of which are described in the following sections.

LIVING UP TO THEIR NAME: CTPS PROPOSED TO PROCESS C-TERMINAL REGIONS

One role played by some CTPs is to process proteins by removing C-terminal regions. Two examples came from the earliest discoveries outlined above: processing of PBP3 (FtsI) and protein D1. The importance of CtpA-dependent processing for D1 function is clear, and it occurs widely in oxygenic photosynthetic organisms (25). In contrast, the significance of PBP3 processing by Prc in *E. coli* is unclear, although it has been implicated in the filamentous phenotype displayed by both *prc* and *ftsI* mutants (5). Three other interesting examples of proposed CTP-dependent processing in bacteria are described below.

A complex example of C-terminal processing occurs in the Gram-negative pathogen *Bordetella bronchiseptica*. An important virulence factor is the filamentous hemagglutinin (FHA), which is required for adhesion, manipulating cytokine signaling, and for persistence in the host. FHA is synthesized as a massive $\sim$ 3,700 amino acid protein FhaB, of which the C-terminal $\sim$ 1,200 amino acids are a prodomain. Removal of this prodomain involves three different proteases, and controls the timing of FHA maturation and release. The N-terminal region of FhaB is exported across the outer membrane, with the C-terminal prodomain remaining in the periplasm. DegP initiates prodomain degradation by removing the extreme C-terminus (ECT) in response to an unknown inducing signal (26). ECT removal by DegP generates a new C-terminus that is recognized by CtpA, which removes most of the remaining prodomain (27). Finally, the remainder of the prodomain is removed by a third protease, SphB1, which results in release of mature FHA from the cell surface (28).

Another CTP named CtpA was identified by searching for proteins with potential C-terminal processing capabilities in the etiological agent of Lyme disease, *Borrelia burgdorferi* (29).

Proteomic analysis identified potential CtpA substrates, all of which were predicted to be outer membrane proteins (29). One was the surface exposed antigen P13, an outer membrane channel-forming protein, which was shown to have its C-terminal region processed by CtpA (29, 30). In addition, CtpA was also shown to remove the C-terminus of the surface exposed lipoprotein OspC, a virulence factor required for the establishment of mammalian infection (30). The authors speculated that C-terminal processing of *B. burgdorferi* surface-exposed lipoproteins by CtpA might be part of a quality control mechanism (30). This is an interesting idea, which is discussed later.

A questionable example of proposed C-terminal processing by a CTP in the literature comes from the obligate intracellular pathogen *Chlamydia trachomatis*. The *C. trachomatis* CTP named CT441 was detected in middle and late stages of infection, which corelated with the cleavage of the host cell p65 subunit of NF-κB (31, 32). A putative cleavage site within p65 was identified, suggesting that CT441 removes its C-terminal region (31, 32). Mutation of the p65 cleavage site, or of the CT441 serine/lysine catalytic dyad, prevented p65 cleavage. However, these studies did not demonstrate direct cleavage of p65 by purified CT441, and the cleavage of p65 by CT441, or in fact by any chlamydial protease, has since been called into question (33, 34). Later, a yeast two-hybrid experiment identified the host protein SRAP1, a co-activator of estrogen receptor α, as an interaction partner of the PDZ domain of CT441, and SRAP1 also interacted with CT441 *in vitro* (35). Although the authors did not find evidence that CT441 cleaved SRAP1, they did report that CT441 interferes with SRAP1 function in mammalian cells (35). However, that conclusion replied on the use of artificial expression constructs in cultured cells, which does not closely mimic a chlamydial infection. Another study found that CT441 cleaved SRAP1 *in vitro*, but did not find any evidence that it did so *in vivo* during the intracellular developmental cycle of *C. trachomatis* (36). There is also no evidence that CT441 is exported from *C. trachomatis* cells, which would be required for it to access the host proteins p65 and/or SRAP1. Therefore, although CT441 was proposed to process the C-terminus of p65, its role and the identity of its substrates await unequivocal confirmation.

FLIPPING THE SWITCH: CTPS THAT PARTICIPATE IN REGULATORY PATHWAYS

CTPs have also been implicated in regulatory pathways. A well characterized example occurs during sporulation in *Bacillus subtilis*. Sporulation is a complex process that has been reviewed by others (37). It begins with the formation of a septum close to one pole that gives rise to a sporangium made up of the smaller forespore and the larger mother cell. The mother cell engulfs the forespore so that it is enclosed in the mother cell cytoplasm. The final steps of spore development are controlled by the mother cell specific sigma factor σ^K, which is produced as the inactive membrane-associated pro-σ^K. Release of σ^K requires pro-σ^K to be cleaved by the membrane-embedded protease SpoIVFB (4FB), which in turn is inhibited by the transmembrane protein, SpoIVFA (4FA; [38]). The N-terminus of 4FA is in the mother cell cytoplasm, whereas the C-terminus is in the interspace separating mother cell and forespore (Fig. 1A). This sets up a system for forespore signaling to be transmitted across the membrane to the mother cell cytoplasm. The inhibition of 4FB by 4FA is relieved by the sequential action of two proteases on the 4FA C-terminus in the interspace, the second of which is a CTP named CtpB (Fig. 1A). The first protease removes the folded C-terminus of 4FA, which protects it from CtpB. The truncated 4FA C-terminus interacts with and activates CtpB, which removes more of the C-terminal segment of 4FA, including the part that inhibits 4FB (39). The uninhibited 4FB then cleaves the membrane-associated pro-σ^κ, which releases active σ^K into the mother cell cytoplasm (Fig. 1A). Intriguingly, *ctpB* expression is controlled by both mother cell and forespore specific sigma factors, and CtpB is produced in both cells (40). However, it is the forespore-specific CtpB that is important for σ^K activation and the completion of sporulation. Nevertheless, if *ctpB* is overexpressed in the mother cell only, it triggers sporulation (40). This suggests that CtpB could play additional roles in the mother cell involving unidentified substrates, although this also might be an artifact of *ctpB* overexpression causing cleavage of non-physiological substrates.

B. subtilis has a second CTP called CtpA (41). CtpA was discovered before CtpB but 2 decades passed before anyone identified a role for CtpA, which is controlling the process of

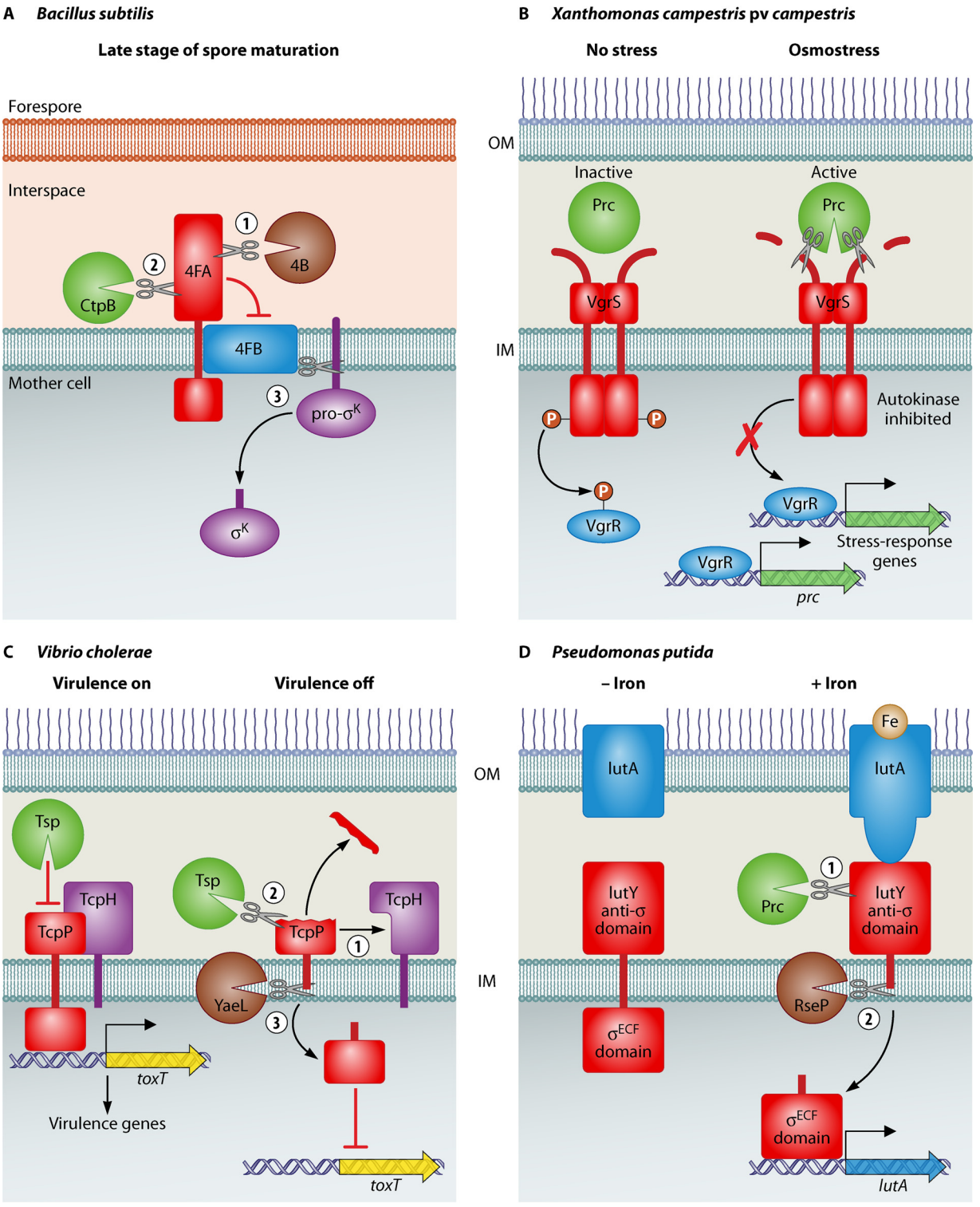

FIG 1 CTPs involved in regulation. (A) In the final steps of *B. subtilis* spore development, the SpoIVB protease (4B) removes the C-terminus of SpoIVFA (4FA), which then allows CtpB to remove the 4FA domain that inhibits the SpoIVFB (4FB) protease. 4FB then cleaves pro-sK, releasing active sK into the mother cell cytoplasm. (B) In *X. campestris* pv. *campestris*, osmostress activates Prc to cleave the N-terminal periplasmic domain of VgrS. This reduces phosphotransfer to VgrR, which alters its global promoter binding profile, allowing it to induce expression of stress-response genes, and of *prc* itself, providing a positive feedback loop. (C) In conditions that do not favor *V. cholerae* virulence gene expression, TcpH dissociates from TcpP, allowing Tsp to cleave close to the C-terminus of TcpP. The truncated TcpP is then further degraded by the YaeL protease. This sequential cleavage of TcpP by Tsp and YaeL inactivates TcpP, so that *toxT* and the virulence genes controlled by ToxT, are no longer expressed. (D) In *P. putida* iron-loaded aerobactin (brown circle) binds to the IutA receptor. This causes the IutA signaling domain to interact with IutY, triggering IutY cleavage by Prc. The transmembrane domain of the truncated IutY is then cleaved by RseP (an ortholog of *V. cholerae* YaeL), which releases the σ^{ECF} domain into the cytoplasm to induce *iutA* expression.

DNA damage checkpoint recovery (42). CtpA, and an unrelated protease YlbL, are membrane anchored proteins that degrade the extracellular portion of the transmembrane cell division inhibitor YneA. When DNA damage occurs, *yneA* expression increases, and the amount of YneA protein overwhelms the proteases and allows it to enforce a checkpoint, which delays cell division. Once DNA repair is complete, *yneA* expression decreases, the YibL and CtpA proteases clear YneA from the cell, and cell division proceeds (42). An analogous phenomenon has also been reported in *Staphylococcus aureus*, involving degradation of a cell division inhibitor by CtpA (43).

The plant pathogen *Xanthomonas campestris* pv. *campestris* provides another example of a regulatory role, albeit one in which the protease does not live up to the CTP name. A *prc* deletion mutant had reduced virulence in cabbage, was more susceptible to antibiotics, and had decreased survival under osmostress conditions (44, 45). The reduced osmostress tolerance was attributed to the role played by Prc in regulating an osmostress response (44). Osmostress activates Prc to cleave the N-terminal periplasmic domain of the histidine kinase sensor protein VgrS (Fig. 1B). This reduces VgrS autophosphorylation, which in turn reduces transfer of phosphate to its response regulator partner, VgrR. The reduced phosphorylation of VgrR alters its DNA-binding affinity and changes its global promoter binding profile. Some VgrR-bound promoters control genes important for osmostress-tolerance, and a low level of VgrS-VgrR phosphorylation is optimal to promote bacterial resistance to osmostress (44) (Fig. 1B). The inhibition of VgrS autokinase activity by Prc proteolysis is an effective mechanism to achieve this. VgrR also controls *prc* expression directly, providing a positive feedback loop (44).

In *Vibrio cholerae*, a Prc homolog using the alternate Tsp name has been implicated in shutting off the regulatory cascade controlling production of the virulence factors cholera toxin and the toxin-co-regulated pilus (46). TcpP is a transmembrane protein with a C-terminal domain in the periplasm and an N-terminal cytoplasmic DNA binding domain that induces the expression of *toxT*, encoding a transcriptional activator of virulence factors (Fig. 1C). In conditions favoring virulence factor expression, another transmembrane protein, TcpH, binds to TcpP and protects it from proteolysis. In non-inducing conditions, TcpH dissociates from TcpP, and Tsp is activated to cleave a small C-terminal region from TcpP. This generates a truncated TcpP protein that is a substrate for the inner membrane zinc metalloprotease YaeL, which further degrades TcpP (Fig. 1C). The sequential cleavage of TcpP by Tsp and YaeL inactivates TcpP, so that *toxT* and the genes encoding cholera toxin and the toxin-co-regulated pilus are no longer expressed (46).

Prc orthologs have been implicated as site-1 proteases in cell surface signaling (CSS) systems of *Pseudomonas* species. In CSS systems, a ligand, which is often a siderophore, binds to a receptor protein in the outer membrane. The receptor interacts with a transmembrane anti-sigma factor that extends from the periplasm into the cytoplasm, where it controls the activity of a sigma factor of the extracytoplasmic function class (σ^{ECF}). However, a *Pseudomonas putida* CCS system, which responds to the siderophore aerobactin, has a hybrid protein called IutY with both transmembrane anti-sigma factor and cytoplasmic σ^{ECF} domains (47). Genetic experiments led to a model in which extracellular iron-loaded aerobactin binds to the outer membrane receptor protein IutA, which then interacts with IutY and triggers cleavage of the IutY C-terminal periplasmic domain by Prc (47, 48) (Fig. 1D). The truncated IutY is now a substrate for RseP, an ortholog of *V. cholerae* YaeL, which cleaves the transmembrane domain of IutY and releases the σ^{ECF} domain into the cytoplasm (47). Prc was also implicated in regulating classical CSS systems in which the sigma and antisigma factors are separate proteins. These were the ferrioxamine (Fox) and ferrichrome (Fiu) responsive systems in both *P. putida* and *P. aeruginosa* (47, 49). Therefore, it was suggested that Prc is involved in the control of multiple CSS systems in *Pseudomonas* species.

The examples above suggest that *E. coli* Prc orthologs are site 1 proteases in regulated intramembrane proteolysis cascades in *V. cholerae*, *P. putida*, and *P. aeruginosa*, which suggests that it might be a common Prc role (Fig. 1B to D). However, in all cases the involvement of Prc was deduced from genetics and Prc was not shown to cleave the regulatory proteins directly (46–49) (Table 1). These genetic experiments strongly

support the involvement of Prc, but leave open the possibility that Prc itself does not cleave the regulatory proteins. For example, if changes in Prc activity causes envelope stress, that might affect the ability of other proteases to cleave the regulatory proteins. On balance though, the case for direct involvement of Prc in these proteolytic cascades is compelling.

DEALING WITH THE TRASH: CTPS IMPLICATED IN PROTEIN QUALITY CONTROL

Some CTPs have been implicated in protein quality control within the bacterial cell envelope. When translation is stalled on the ribosome in *E. coli*, the 11-amino acid SsrA tag (AANDENYALAA) is added to the C-terminus in a co-translational mechanism (50, 51). This tag marks the mistranslated protein for proteolytic degradation, and it is a widely conserved phenomenon in bacteria (52, 53). In the *E. coli* cytoplasm, at least four different proteases degrade proteins with SsrA tags (reviewed in [53]). However, data suggests that Prc degrades SsrA-tagged proteins in the periplasm. This was demonstrated with an engineered derivative of cytochrome b_{562} with an SsrA-tag added to its C-terminus. The tagged cytochrome had a half-life below 5 min in a *prc*$^+$ strain, but over 1 h in a *prc* null mutant (50). However, the significance of Prc in degrading naturally SsrA-tagged proteins *in vivo* is unclear (50). Intriguingly though, in *Streptomyces coelicolor and Streptomyces lividans*, the gene encoding Prc is located immediately upstream of the two genes required to SsrA-tag proteins (54). When this observation is combined with the data from *E. coli*, it supports the possibility of a functional link between Prc and SsrA-tagged proteins.

Another Prc might also degrade proteins with aberrant C termini. *Pseudomonas aeruginosa* has an apparent ortholog of *E. coli* Prc, which has been named Prc, but also AlgO due to its impact on production of the polysaccharide alginate (55). A critical control point in the regulation of alginate biosynthesis is the inhibition of the AlgU/T sigma factor by the anti-sigma factor MucA (reviewed in [56]). During lung colonization in individuals suffering from cystic fibrosis, *P. aeruginosa* mutants arise that constitutively produce alginate. These mucoid strains frequently have mutations in the *mucA* gene, the most common of which is the *mucA22* frameshift allele (57, 58). This mutation truncates MucA and adds three arginines to its C-terminus, destabilizing the anti-sigma factor and causing constitutive alginate production. Screens for mutations that suppress the *mucA22* phenotype isolated null mutations in *prc* (55, 59). It was suggested that Prc degrades MucA22, due to its aberrant C-terminus, in conditions that are normally unfavorable for alginate biosynthesis (55, 59, 60). While this is the most likely explanation, Prc has not been shown to degrade MucA22 directly, which leaves open the possibility of an indirect effect. Nevertheless, if Prc does degrade MucA22, it would be another example of protein quality control, and raise the possibility that Prc might degrade other proteins with aberrant C termini.

A third and somewhat different implication of a CTP playing a role in quality control arose from a study of the C-terminal processing of *B. burgdorferi* surface-exposed lipoproteins by CtpA (30). In the proposed model, low level C-terminal processing of these lipoproteins by CtpA, before the mature protein is inserted into the outer membrane, might be a quality control system for surface lipoprotein translocation. The authors speculated that detection of the released C-terminal peptides might report that proper translocation of surface lipoproteins is occurring (30). Disruption of proper translocation might increase the level of CtpA-dependent processing, raising the level of the C-terminal peptides and triggering a stress response to mitigate the problem. This is an intriguing model, although it is highly speculative and in need of experimental interrogation.

The studies above outline the current support for an involvement of CTPs in bacterial cell envelope protein quality control. It is a particularly attractive hypothesis that C-terminal processing proteases like Prc might target proteins with aberrant C termini. However, the evidence is limited and more work is needed in order to understand how significant this is, and how widespread it might be.

AN EMERGING ROLE IN CELL WALL HYDROLASE DEGRADATION AND THE DISCOVERY OF CTP ADAPTOR PROTEINS

Almost all bacteria have a peptidoglycan cell wall, which determines cell shape and provides resilience against turgor pressure. Peptidoglycan is made up of glycan strands

with attached stem peptides. The peptides of parallel glycan chains cross-link via peptide bonds to form a mesh like sacculus surrounding the cell. In addition to enzymes for peptidoglycan synthesis, a number of hydrolases cleave the various bonds in the polymer to facilitate cell growth and division. These hydrolases must be controlled, but the mechanisms by which this is achieved have only begun to emerge recently. Two different CTPs, in two different species, have been found to constrain the activities of some cell wall hydrolases by degrading them. These studies also led to the discovery of CTP adaptor proteins, which are needed for the degradation of some CTP substrates (2, 3).

The first discovery came from *E. coli*, where the cell wall cross-link hydrolase MepS is more abundant in exponential phase than stationary phase (2). A genetic screen identified two null mutations that increased and equalized MepS abundance at all growth stages (2). One was a *prc* null mutation, suggesting that Prc degraded MepS. Indeed, a *mepS* (formerly *spr*) null mutation was identified originally because it suppressed the thermosensitivity of a *prc* null mutant at low osmolarity (61). Subsequent experiments confirmed that Prc degrades MepS directly (2). Studies in the last couple of years have also shown that *E. coli* Prc degrades another cross-link hydrolase, MepM, and the lytic transglycosylase MltG, which cleaves the glycan chains of peptidoglycan (62, 63). The peptidoglycan hydrolases MltB and DigH have also been proposed as likely Prc substrates, although they have not yet been confirmed to be cleaved directly by Prc (64).

As mentioned above, a genetic screen identified two null mutations that stabilized MepS, and one of these affected *prc*. The second mutation affected *nlpI*, encoding an outer membrane lipoprotein with tetratricopetide repeat (TPR) motifs (2). In addition to stabilizing MepS, *nlpI* and *prc* null mutants also share other common phenotypes (6, 65). It was originally proposed that Prc processes the C-terminus of NlpI, but this was not supported by further investigation (2, 66). Instead, NlpI is an adaptor required for Prc-dependent degradation of MepS *in vivo* and *in vitro* (2). It achieves this by binding to Prc and MepS independently, acting as a scaffold to bring them together (2, 67). However, NlpI inhibits the processing of PBP3 by Prc *in vitro* (7). It has also been suggested that NlpI might not be required for Prc to degrade the cell wall hydrolase substrates MepM and MltG *in vivo* (62, 63). However, this is unclear for MepM, because although a *nlpI* null mutation did not cause MepM accumulation *in vivo*, NlpI massively accelerated Prc-dependent degradation of MepM *in vitro* (63). Regardless, all these observations suggest the possibility that NlpI might be a substrate-specific adaptor protein.

A similar phenomenon was uncovered in *P. aeruginosa*, which unlike *E. coli*, has two CTPs. One is an apparent ortholog of *E. coli* Prc, but remarkably, it is the other one that has been found to work with an adaptor protein to degrade cell wall hydrolases. This CTP, named CtpA, was found to be important for type III secretion system function and virulence (68). CtpA interaction partners and substrates were identified by biochemical and genetic approaches (3). This identified four CtpA substrates, all of which are predicted cell wall cross-link hydrolases. Two are members of the LytM/M23 peptidase family (MepM and PA4404) and two are members of the NlpC/P60 peptidase family (PA1198 and PA1199, which are homologs of *E. coli* MepS). Therefore, CtpA degrades proteins that are homologous to the *E. coli* Prc substrates MepS and MepM. The study also identified an adaptor protein that was named LbcA and was required for CtpA to degrade all four substrates *in vivo* and *in vitro* (3). Like *E.coli* NlpI, LbcA is an outer membrane lipoprotein with TPR motifs. However, LbcA is approximately twice the size of NlpI and their primary sequences are not similar. A more recent study identified a fifth CtpA substrate that is also degraded in an LbcA-dependent manner (69). This substrate, PA1048, is not a predicted cell wall hydrolase, but it has an OmpA-like C-terminal region predicted to bind to peptidoglycan non-covalently. In *P. aeruginosa* no LbcA-independent CtpA substrate has been identified so far.

The similarities between the *E. coli* NlpI-Prc and *P. aeruginosa* LbcA-CtpA systems are obvious. Both consist of a CTP in complex with an outer membrane lipoprotein adaptor, and both degrade cell wall hydrolases. In both cases, the adaptor protein acts as a scaffold that binds to the protease and substrate independently (2, 69). However, while these two systems are similar, they are not orthologs. Prc and CtpA have been assigned to different

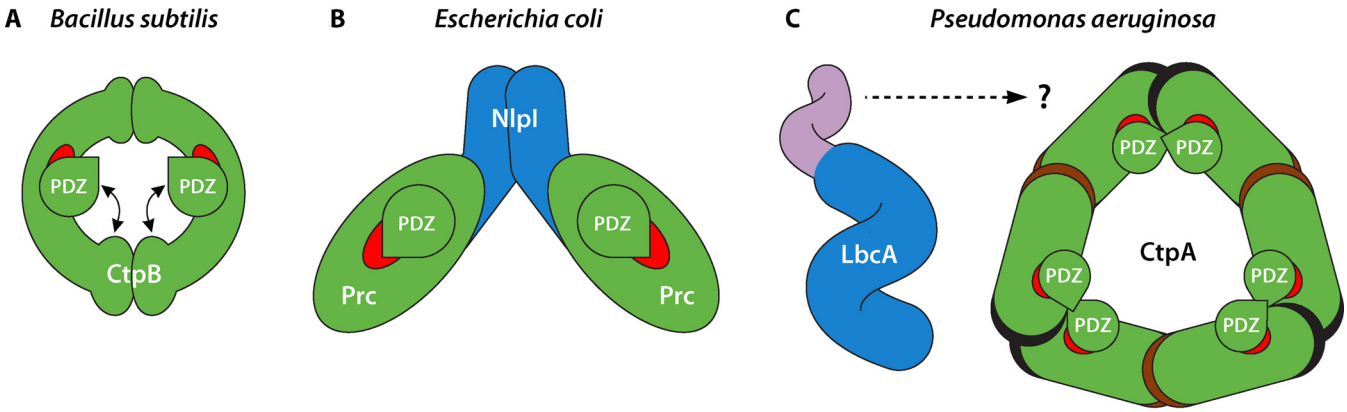

FIG 2 Contrasting arrangements of bacterial CTPs. (A) *B. subtilis* CtpB forms a ring-like dimer via N-terminal to N-terminal and C-terminal to C-terminal interactions. The positioning of the PDZ domain controls access to a narrow protease tunnel (red). CtpB switches between inactive and active forms, defined by whether or not the PDZ domain blocks the protease tunnel. (B) *E. coli* Prc is a monomer that forms a self-compartmentalized bowl-like structure, with part of the protease domain comprising a vault (red), and a lid-like PDZ domain. Upon interaction with a substrate, the PDZ domain shifts position causing a productive repositioning of the active site residues (not shown). The adaptor protein NlpI is a dimer, with each subunit interacting with one Prc monomer to form the tetrameric arrangement shown. NlpI is required for Prc to degrade MepS, but not for the degradation of any other Prc substrates *in vivo*. (C) *P. aeruginosa* CtpA forms a ring-like hexamer, arranged as a trimer of dimers mediated by N-terminal to N-terminal (black) and C-terminal to C-terminal (brown) interactions. The LbcA adaptor protein is a monomer with 11 TPR motifs forming a spiral structure (blue), and a separate N-terminal helical domain (purple) that is required for interaction with CtpA. LbcA is required for CtpA to degrade all five of its known substrates. The CtpA hexamer is inactive in the absence of LbcA because the active site residues (red) are misaligned (not shown). Studies to understand exactly how LbcA interacts with and activates CtpA are in progress.

CTP subgroups, and NlpI and LbcA are not homologous. The differences between them are further emphasized by structural analysis (see below). Therefore, two different CTPs interact with two different adaptor proteins, in two evolutionary divergent species, to achieve the same function. This raises the possibility that this is a widely conserved mechanism used to constrain bacterial cell wall hydrolases. In fact, many CTP-defective mutants of Gram-negative species have been reported to have defective cell envelopes and other phenotypes that might be explained by accumulating cell wall hydrolases (Table 1).

STRUCTURES OF CTPS AND ADAPTOR PROTEINS

The structures of some bacterial CTPs and adaptor proteins have been solved, which has provided valuable insights into their mechanisms of action. However, these studies have also uncovered striking differences, especially in their stoichiometric arrangements.

B. subtilis CtpB has a PDZ domain that adopts a similar fold as other PDZ domains, two protease subdomains (cap and core), and dimerization motifs at its N- and C-terminal ends (39). The dimerization domains interact to assemble a ring-like dimer (Fig. 2A). A narrow tunnel between the protease cap and core subdomains is wide enough for an unfolded substrate to enter. The PDZ domain is the most flexible part of the protein and its positioning controls access to the protease tunnel. CtpB switches between inactive and active forms, with the PDZ domain blocking the protease tunnel in the former (Fig. 2A). Once the PDZ domain interacts with a substrate it locks into the active position, allowing the substrate to access the protease tunnel for cleavage to occur. This inhibitory role of the PDZ domain is demonstrated by the fact that removal of the PDZ domain from CtpB yields a constitutively active protease (39).

The next bacterial CTP structure reported was CT441 from *C. trachomatis* (36). It has a novel N-terminal domain (NTD) that appears to be important for CT441 folding and/or solubility. This NTD is also important for a chaperone activity of CT441 that was uncovered during the structural analysis, and might play a role in protein quality control (36). The NTD is connected to the PDZ domain by a highly flexible region, which prevented the PDZ domain from being defined in the crystal structure. The PDZ domain is followed by a C-terminal domain that mediates the formation of homodimers and contains the active site. However, although CT441 formed dimers in the crystal, it purified as a monomer, meaning that the physiological significance of the dimers is uncertain. The authors speculated that dynamic dimerization might play a role *in vivo*, perhaps affecting the activation status of the protease. They also

hypothesized that movement of the PDZ domain might be important for protease activation (36). However, unlike the PDZ domain of CtpB, the CT441 PDZ domain was required for activity and so it is not only an inhibitor.

E. coli Prc is a monomer that forms a self-compartmentalized bowl-like structure, with a lid-like PDZ domain (67) (Fig. 2B). The PDZ domain of Prc is not an inhibitor, but instead it functions as an activator that is required for protease activity (7, 67). In the inactive state, the catalytic residues of Prc are misaligned. However, when a substrate interacts with the PDZ domain, it changes its position, and this movement triggers a structural change that aligns the active site residues correctly (7). Therefore, in both CtpB and Prc, the PDZ domain plays an important regulatory role, but those roles are different. The structure of *E. coli* Prc was solved in complex with its NlpI adaptor protein (67). NlpI is a dimer with each subunit of the dimer interacting with one Prc monomer to assemble a 2:2 tetramer (Fig. 2B). NlpI has four TPR motifs, which are all on the outside edge of the dimer. NlpI interacts with Prc mainly via TPR2, whereas TPR1 was implicated in binding to the MepS substrate. Once MepS is bound to NlpI, it is thought that the PDZ domain of Prc interacts with the MepS C-terminus, prompting the activating shift to occur in the Prc catalytic residues. Rotational movement of the substrate-bound PDZ domain might drive translocation of the MepS polypeptide through the protease active site (67). This lever-like mechanism could explain how Prc completely degrades MepS.

The *P. aeruginosa* LbcA-CtpA system is functionally analogous to the *E. coli* NlpI-Prc system, but their protease and adaptor protein components are quite different. Structural analysis has revealed that the differences between these systems are even more striking, and that CtpA itself is very different to the other CTPs described above. CtpA consists of a PDZ domain, two protease subdomains (cap and core), and dimerization motifs at its N- and C-terminal ends (70). The N-terminal dimerization domain of CtpA is similar to that of *B. subtilis* CtpB, but the C-terminal dimerization domain is different. The N termini of two CtpA molecules dimerize in the same way as CtpB. However, instead of N-N and C-C interactions forming a ring-like dimer, the six C-terminal domains of three CtpA dimers interact to form a triangular trimer-of-dimers in the crystal (Fig. 2C). This oligomeric state is supported by the estimated mass of a hexamer from the gel filtration profile of CtpA (70). The CtpA hexamer is inactive because the catalytic residues are beyond hydrogen-bonding distance. This is consistent with the observation that CtpA alone was inactive in substrate degradation *in vivo* and *in vitro* (3). Therefore, unlike the *B. subtilis* CtpB dimer, which fluctuates between active and inactive forms and can be activated by a protein substrate, the CtpA hexamer is locked in an inactive conformation and requires LbcA for activation. The LbcA adaptor is a monomer, in contrast to the dimeric NlpI adaptor of *E. coli* Prc (67, 70). LbcA contains only α-helices and connecting loops, with 22 of these α-helices comprising the 11 TPR motifs. These 11 TPRs form a spiral, which has the potential to wrap around a substrate for delivery to CtpA (70) (Fig. 2C). Outside of the TPR spiral are four additional N-terminal α-helices that form an extension partially capping the spiral ring. The first of these helices is essential for binding to CtpA (70). Ongoing studies are characterizing the LbcA-CtpA complex. Preliminary indications suggest that up to three LbcA molecules might bind to one CtpA hexamer to assemble a giant, active protease complex (Hao-Chi Hsu, Andrew J. Darwin and Huilin Li, unpublished data).

Although few bacterial CTP structures have been solved, a common theme is that the PDZ domain is a mobile regulatory component, although exactly how it achieves this function differs. The PDZ domain of *B. subtilis* CtpB is an inhibitor that blocks access to the proteolytic site. In contrast, the PDZ domain of *E. coli* Prc is a substrate-responsive activator that promotes reconfiguration of the active site residues. These studies have also revealed some other contrasts. For example, *E. coli* Prc and *C. trachomatis* CT441 are monomers in solution, whereas *B. subtilis* CtpB is a dimer, and *P. aeruginosa* CtpA is a hexamer. Also, although both *E. coli* Prc and *P. aeruginosa* CtpA partner with adaptors to degrade cell wall hydrolases, their macromolecular arrangements are completely different (Fig. 2).

FINDING THEIR TARGET: CTP SUBSTRATE AND CLEAVAGE SITE RECOGNITION

What features allow CTPs to recognize their substrates and cleavage sites? Very few studies have examined this, and no clear consensus has emerged. Early attention focused

on the importance of the substrate C-terminus, and suggested that non-polar residues might be required for recognition by *E. coli* Prc. Prc degrades λ repressor when the non-polar sequence WVAAA is added to its C-terminus, but does not degrade the wild-type protein that has RSEYE at its C-terminus (9). The C-terminal SsrA tag that also renders λ repressor susceptible to cleavage by Prc ends with YALAA, which is similar to WVAAA. In addition, changing the final two non-polar amino acids of the SsrA tag from AA to DD protected it from Prc (71). Additional analysis of λ and Arc repressors with altered C termini supported a requirement for non-polar residues at the C-terminus, as well as a free α-carboxyl group (72). However, a free α-carboxyl group cannot be a universal requirement, because *X. campestris* Prc cleaves the N-terminus of VgrS, and is separated from its C-terminus by the cytoplasmic membrane (44).

It was suggested that *E. coli* Prc prefers substrates that are not stably folded (72). This might be a key finding, because it is possible that a disordered tail is the critical factor for recognition by a CTP, rather than a non-polar tail. In support of this, the *E. coli* Prc substrates MltG, MepM, and FtsI have polar residues at or very close to their C termini. The C-terminal tails of *P. aeruginosa* CtpA substrates, which are critical for degradation, also contain polar residues (73).

CTP cleavage sites have also been investigated, and are usually located some distance from the terminus of the substrate. Prc cleaves PBP3 (FtsI) at a Val-Ile bond (8). Systematic analysis suggested that *E. coli* Prc prefers small, uncharged residues, or residues with aliphatic side chains, on the N-terminal side of the cleavage site (72). There was a similar preference on the C-terminal side, with the exception that no preference for small residues was found (72). CTP cleaved bonds have also been identified in other species. *B. burgdorferi* CtpA cleaves an Ala-Leu bond in P13 and an Ala-Glu bond in OspC (30). *X. campestris* Prc cleaves an Ala-Gln bond in VgrS (44). These findings support a preference for a small amino acid on the N-terminal side of the cleavage site. It is also interesting to consider cleavage site preference when substrates are completely degraded. Perhaps only the first cleavage site has sequence specificity, with subsequent cleavages driven by movement of the substrate through a protease tunnel (67).

KEEPING THINGS UNDER CONTROL: IS CTP ACTIVITY REGULATED?

Another aspect of CTPs that is not clear is whether or not their activities are regulated, and if so, how. An obvious mechanism is to change expression of the CTP-encoding gene. *B. subtilis ctpB* expression is controlled by mother cell and forespore specific sigma factors, and *X. campestris prc* expression is directly controlled by VgrR (40, 44). However, there is also evidence that proteolytic activity might be controlled by other mechanisms. The *E. coli* Prc substrate MepS is degraded more in the stationary growth phase than in exponential phase, whereas MltG appears to be degraded at the same rate regardless of growth phase (2, 62). This suggests a substrate-specific mechanism of regulation. Interestingly, MepS degradation requires the NlpI adaptor protein, whereas MltG degradation does not. NlpI binds to MepS directly and might sequester some of it away from Prc. A more interesting possibility is that NlpI regulates Prc activity when in complex with it, perhaps in response to a growth-rate dependent signal such as the rate of peptidoglycan synthesis. Recently, increased intracellular aromatic amino acid concentration was suggested to affect the level of MepS in a Prc-dependent manner (63). The authors noted that aromatic amino acids are high-cost amino acids, and speculated that their presence could reduce the burden of amino acid biosynthesis and increase the biosynthesis of peptidoglycan precursors (63). Increased peptidoglycan biosynthesis would also require increased levels of hydrolases, such as MepS. Regardless of the mechanism, the growth-rate dependent control of MepS degradation by the NlpI-Prc complex is intriguing. Something similar might also occur in *P. aeruginosa*, where the levels of CtpA and its LbcA adaptor protein appear constant, regardless of growth phase, but the levels of the substrates are not (3). Therefore, adaptor-dependent control of CTP activity might emerge as a conserved phenomenon.

CONCLUDING REMARKS

CTPs regulate developmental processes, stress responses, and virulence; they are implicated in protein quality control; and they degrade cell wall hydrolases. Even so, there is much

we don't know. Beyond how they recognize substrates, and how their activities are controlled, many other areas also need more investigation. We must identify more CTP substrates, and when possible, confirm them with direct *in vitro* assays. Substrate identification will help to explain the various *ctp* null mutant phenotypes (Table 1). Increasing the known substrates should also reveal common themes spanning bacterial genera. For example, do Prc-like CTPs participate broadly in regulated intramembrane proteolysis cascades? Is degradation of cell wall hydrolases a widely conserved phenomenon? CTP adaptor proteins have come to light in the last few years, with the discovery of *E. coli* NlpI and *P. aeruginosa* LbcA. It is fascinating that these adaptor proteins share no significant sequence similarity. However, the true significance of this requires more CTP-adaptor systems to be identified and characterized. Are these adaptor proteins limited to Gram negative bacteria, or do Gram positive CTPs have adaptors as well? Perhaps it is also time to reconsider the name of these proteases now that C-terminal processing is clearly not their only role. Some degrade their substrates completely, and *X. campestris* Prc processes the N-terminus of its substrate. Regardless, the links of these proteases to stress responses, gene regulation, the cell wall, and virulence, means that their continued study provides the potential for broad impacts on our understanding of fundamental processes in all bacterial cells.

ACKNOWLEDGMENTS

CTP research in our laboratory is supported by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health, under Award Numbers R01AI136901 and R21AI51097 to A.J.D., and award number T32AI007180 also supported A.G.S. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank Patrick Lane of ScEYEnce Studios for graphical enhancement of the figures.

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