Bacterial Proteasomes: Mechanistic and Functional Insights

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Published 14 December 2016

Citation Becker SH, Darwin KH. 2017. Bacterial proteasomes: mechanistic and functional insights. *Microbiol Mol Biol Rev* 81:e00036-16. <https://doi.org/10.1128/MMBR.00036-16>.

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SUMMARY Regulated proteolysis is essential for the normal physiology of all organisms. While all eukaryotes and archaea use proteasomes for protein degradation, only certain orders of bacteria have proteasomes, whose functions are likely as diverse as the species that use them. In this review, we discuss the most recent developments in the understanding of how proteins are targeted to proteasomes for degradation, including ATP-dependent and -independent mechanisms, and the roles of proteasome-dependent degradation in protein quality control and the regulation of cellular physiology. Furthermore, we explore newly established functions of proteasome system accessory factors that function independently of proteolysis.

KEYWORD proteasome

INTRODUCTION

Proteolysis is a fundamental process in all cells, playing essential roles in posttranslational regulation, responses to environmental changes and intracellular stress, and removal of unfolded proteins. The proteasome, a proteolytic machine present in all three domains of life, is a barrel-shaped protease complex that performs both ATP-dependent and -independent proteolysis (reviewed in reference 1). In many cases, proteins are targeted to the proteasome through specific posttranslational modifications; in eukaryotes, a small protein called ubiquitin (Ub) tags proteins for

ATP-dependent proteasomal degradation. Three classes of enzymes carry out ubiquitylation: a Ub-activating enzyme (E1) uses ATP to adenylate the C-terminal glycine (Gly) of Ub, priming it for attack by a cysteine (Cys) in the E1 enzyme to form a Ub-E1 thioester bond. Ub is then transferred to a Ub-conjugating enzyme (E2), which allows a Ub ligase (E3) to catalyze the formation of an isopeptide bond between the Ub C terminus and a substrate lysine (Lys) residue. Substrates are selected through recognition by dedicated E3 ligases, of which there are up to several hundred representatives in a given eukaryotic genome (reviewed in reference 2). As the major pathway of ATP-dependent proteolysis in the eukaryotic cytosol and nucleus, the Ub-proteasome system is essential for eukaryotes and is involved in numerous cellular processes (reviewed in reference 3).

The protease component of all proteasomes, called the 20S core particle (20S CP) based on its sedimentation coefficient, is gated to prevent nonspecific entry of proteins; substrates must be fed into the proteolytic core by a proteasome activator (4–6). In eukaryotes, the 19S regulatory particle (RP) caps the 20S CP, where it functions to both recognize and unfold ubiquitylated proteins prior to degradation (7–10). Additionally, the 19S RP removes Ub from substrates as a recycling mechanism (11, 12). While the 19S RP uses ATP hydrolysis to provide energy for protein unfolding, several ATP-independent proteasome activators have also been described for eukaryotes (13, 14), although their substrate specificities and biological functions are poorly understood.

Proteasomal genes are represented in nearly every lineage of the bacterial orders *Actinomycetales* and *Nitrospirales*, whose members include the human pathogen *Mycobacterium tuberculosis* and a major source of natural antibiotics, *Streptomyces coelicolor* (reviewed in reference 15). Although proteasomes perform critical physiological functions in all proteasome-bearing bacteria characterized to date, they are not absolutely essential as in eukaryotes. To target proteins to a proteasome, bacteria employ a mechanism similar to ubiquitylation, called pupylation. Several roles for pupylation in bacterial physiology have been identified, and most notably, the degradation of pupylated substrates is required for the full virulence of *M. tuberculosis*. Beyond pupylation, the study of bacterial proteasomes has also revealed the importance of ATP-independent proteasomal degradation for the normal physiology of *M. tuberculosis*, and possibly for other species.

20S PROTEASOME CORE PARTICLES

Structure and Assembly

In all domains of life, 20S CPs are 28-subunit complexes consisting of four stacked rings (16–19) (Fig. 1A). Two identical outer rings, each constituting a heptamer of α -subunits, provide the entry point at which substrates are delivered. Likewise, two identical inner rings, each a heptamer of β -subunits, form the active site of the protease (19–22). The β -subunits belong to the amino (N)-terminal nucleophilic hydrolase family of proteins (23). Notably, eukaryotic 20S CPs are formed from 14 unique gene products, while archaea and proteasome-bearing bacteria encode only a single type each of the α - and β -subunits (16, 17, 20, 24) (Fig. 1B). The only known exception to this is a single sequenced strain of *Rhodococcus erythropolis* (strain NI86/21) that encodes two functionally interchangeable variants of each subunit (20).

In bacteria, assembly of a 20S CP is preceded by the formation of a half-proteasome consisting of an α - and a β -ring (25). All β -subunits are produced with an N-terminal propeptide; autocatalytic processing of β -subunits proceeds upon β -ring-to- β -ring joining of two half-proteasomes (25, 26). Removal of the propeptides is catalyzed by β -subunit threonines (Thr), which become the N-terminal catalytic residues of the assembled holoproteasome. Initial insight into the function of propeptides was elucidated upon studies of 20S CP assembly in *R. erythropolis*, which encodes α - and β -subunits called PrcA and PrcB (proteasome components A and B), respectively. Production of a propeptideless PrcB subunit significantly lowers the rate of 20S CP assembly; furthermore, supplementation of PrcA and propeptideless PrcB with propeptides in *trans* results in functional proteasomes that assemble more rapidly than their

FIG 1 Structure of a bacterial proteasome and proteasome gene loci in select actinobacteria. (A) (Top) Crystal structure of the 20S CP from *M. tuberculosis* (PDB entry 2FHG). An individual subunit from each ring is shown in a darker tint. (Bottom) Top-down view of the 20S CP. (B) Proteasome gene loci in five representative actinobacterial species. *C. glutamicum* does not carry *prcBA*.

wild-type counterparts (25). These observations suggest that in *R. erythropolis*, the propeptide promotes structural maturation of the proteasome. In contrast, the propeptide of *M. tuberculosis* PrcB is dispensable for the assembly of functional 20S CPs; in fact, production of PrcB lacking catalytic residues for processing halts assembly at the half-proteasome stage (24, 26). Because propeptide processing appears to be a thermodynamic barrier to *M. tuberculosis* 20S CP assembly (24), we speculate that the

propeptide is conserved in *M. tuberculosis* PrcB in order to reduce unregulated peptidase activity that may have detrimental effects on bacterial physiology.

Catalytic Activity

The mechanism of proteolysis by 20S CPs appears to be universal among proteasomes (27). The β -subunit active site facilitates hydrolysis of both its own propeptide and protein substrates entering the proteasome. In the preprocessed state, Thr1 (numbered according to its position in the processed β -subunit) hydrolyzes the amide bond linking it to the adjacent -1 residue, thereby releasing the propeptide; Thr1 subsequently hydrolyzes substrates in an identical manner. Several conserved residues participate in this reaction: an aspartate (Asp) or glutamate (Glu) orients Thr1 toward the substrate, and a lysine (Lys) is responsible for deprotonating the terminal amine of Thr1 to carry out hydrolysis (19, 27, 28). Together, Thr, Lys, and Asp/Glu form a set of catalytic residues that is conserved in proteasomes from all domains of life. In mature bacterial proteasomes, all 14 β -subunits are processed and catalytically active (24); this is in contrast to the hetero-oligomeric proteasome of eukaryotes, in which only three of seven β -subunits harbor a functional catalytic Thr (16).

While the proteasome active site residues are universally conserved, the peptide sequences targeted for proteolysis are variable. Proteolytic specificity is determined by the chemical nature of the pocket surrounding the active site. In the 20S CPs of eukaryotes, the three active β -subunits each have a unique specificity for hydrophobic, basic, or acidic sequences (29). Because prokaryotes, for the most part, carry a single allele each for the α - and β -subunits, specificity is predictably more limited; indeed, 20S CPs in both *R. erythropolis* and *Thermoplasma acidophilum* have an overall hydrophobic central chamber and thus appear to selectively process hydrophobic substrates (19, 20). Remarkably, however, the 20S CP of *M. tuberculosis* has a highly unique active site pocket with both hydrophobic and hydrophilic surfaces (19). This affords the *M. tuberculosis* proteasome the ability to efficiently degrade all three peptide sequence types (24).

Gating

The α -subunits of 20S CPs harbor an N-terminal extension that prevents substrates, including most peptides, from freely entering the central protease chamber (4, 24, 26). In *M. tuberculosis* PrcA, this extension consists of several hydrophobic residues, and the N termini of PrcA monomers stack atop one another to occlude the CP entrance (24). This structure is capable of a wide range of movement, which is a requisite for the large positional shifts it must undergo upon binding to proteasome activators that allow the entry of proteins into a 20S CP (26). Many studies using N-terminally truncated α -subunits, which result in the assembly of so-called “open gate” proteasomes, have demonstrated that the absence of a gate allows for the degradation of peptides and unfolded proteins without the need for a proteasome activator (4, 24, 30). Moreover, activators appear to associate more strongly with 20S CPs in the absence of a gate (31, 32), altogether indicating that the gate acts as a barrier to substrate translocation.

ATP-DEPENDENT PROTEASOME ACTIVATION

Proteasomal ATPases

ATPase proteasome activators are present in all domains of life. In bacteria, they perform three essential functions: specific recognition of substrates, ATPase-driven unfolding, and engagement with 20S CPs to induce gate opening. The two best-characterized bacterial ATPase activators are *M. tuberculosis* Mpa (mycobacterial proteasome ATPase) and its orthologue, *R. erythropolis* ARC (AAA ATPase forming ring-shaped complexes). Mpa and ARC form homohexameric rings, highly similar to the previously characterized archaeal proteasome activator PAN (proteasome-activating nucleotidase) (32–34) (Fig. 2A). The eukaryotic 19S RP, which consists of at least 19 different gene products, includes a base subcomplex that is structurally and functionally analogous to the prokaryotic ATPase activators (10, 35). It therefore appears that

FIG 2 ATP-dependent degradation requires a hexameric chaperone. (A) Crystal structure of a hexamer composed of Mpa₁₋₂₃₄ monomers from *M. tuberculosis* (PDB entry 3M9D). Shading is used to mark individual subunits; two domains of Mpa are indicated. (B) Crystal structure of an Mpa coiled-coil domain (green shades) bound to Pup₂₁₋₆₄ (orange), demonstrating binding-induced folding of Pup by Mpa.

the general features of ATP-dependent delivery of proteins into a 20S CP are evolutionarily conserved from bacteria to eukaryotes.

Mpa and ARC consist of at least the following three domains that, in an assembled hexamer, form a channel: an N-terminal coiled-coil domain, an interdomain, and an ATPase domain (32, 34). Atop the entrance to the channel, the N-terminal helices of each monomer pair up to form three coiled coils, which extend from the hexamer (32, 34) (Fig. 2A). In Mpa, the coiled-coil domain is responsible for substrate recognition (32). In the absence of comprehensive studies, little is known about the substrate selection of ARC or other bacterial proteasome activators, but considering that the coiled-coil domain is a conserved feature of proteasomal ATPases and is absent from nonproteasomal protease activators (reviewed in reference 36), it is likely that this domain is required for substrate recognition in most or all other bacterial proteasomal ATPases.

The interdomain of Mpa and ARC harbors two oligosaccharide/oligonucleotide-binding-fold (OB) subdomains. Despite this connotation, the only known function of the interdomain is to promote hexamer assembly and stability. In accordance with the high degree of structural rigidity and intersubunit contacts found within the OB subdomains, the interdomain can uniquely self-assemble (32, 37).

Mpa and ARC are representative of a class of enzymes called AAA ATPases (ATPases associated with various cellular activities) (38, 39). The AAA domain powers substrate delivery into the 20S CP. The initial characterization of the role of AAA ATPases in

protein degradation was performed on the ATPases of nonproteasomal bacterial proteases, such as ClpP and HslV (ClpQ) (reviewed in references 40 and 41). The study of both nonproteasomal and proteasomal activators has proven to be mutually beneficial, as these are thought to carry out highly similar mechanisms of protein unfolding and translocation. The defining feature of AAA ATPase unfoldases is a conserved aromatic-hydrophobic-glycine (Ar- Φ) motif, commonly referred to as the pore loop, which extends into the channel of the ATPase and makes direct contact with substrates (31, 32, 38, 42). Through mechanistic studies of ClpX, an AAA ATPase activator of ClpP, a model has been proposed whereby individual pore loops “grab” an exposed peptide and pull the substrate in the direction of the protease opening, an action driven by ATP hydrolysis (42). Furthermore, a recent investigation into PAN and the 19S RP put forth a mechanism by which ATP hydrolysis (and thus pore loop mobilization) cycles in a counterclockwise fashion to facilitate the sequential grabbing and pulling of polypeptides (43). This is thought to be a mechanism utilized by all degradation-associated ATPases; indeed, Mpa mutants lacking conserved pore loop residues are unable either to unfold proteins or to facilitate protein degradation in *M. tuberculosis* (31, 32).

As discussed previously, 20S CPs have gated entries, formed by N-terminal helices extending from each subunit of the α -subunit ring, which must be opened to allow for proteolysis. To accomplish this, PAN and several eukaryotic activators have C-terminal extensions that are required to open proteasome gates in order to facilitate proteolysis (5, 6, 33). The C-terminal three amino acids in these activators are a hydrophobic amino acid, tyrosine, and any amino acid (HbYX motif) (6). Structural and biochemical studies have demonstrated that these C-terminal residues insert into grooves between α -subunits, inducing rigid-body movements that widen the 20S CP entrance (5, 6). Although such scrutinizing experiments have not been performed specifically with bacterial proteasome activators, it is nonetheless reasonable to hypothesize that gate opening in mycobacteria occurs through a similar mechanism. In support of this hypothesis, Mpa/ARC hexamers also have C-terminal extensions, ending with the sequence glycine-glutamine-tyrosine-leucine (GQYL), which are required for engaging 20S CPs (44–46). By sequence and location, the GQYL motif is similar to the HbYX motif; indeed, truncation or amino acid substitutions within the GQYL sequence render Mpa nonfunctional in *M. tuberculosis* (45, 47–49). Furthermore, *M. tuberculosis* PrcA has a Lys residue that is highly conserved among proteasomes, and this residue in other proteasomes allows the HbYX motif of a proteasome activator to open the 20S CP (44). Collectively, these data suggest that the GQYL motif inserts between α -subunits in order to open the *M. tuberculosis* 20S CP gate.

Despite harboring a C-terminal GQYL motif, Mpa is unable to activate the proteolysis of even small peptides by wild-type 20S CPs *in vitro*. Furthermore, several reports have demonstrated that the degradation of substrates by use of Mpa and 20S CPs *in vitro* is much slower than the degradation observed in bacteria, a phenomenon that has long confounded the field (31, 50, 51). The failure of Mpa to facilitate robust *in vitro* degradation is likely explained by the fact that Mpa has very poor *in vitro* affinity for the 20S CP (32); however, the structural explanation for this phenomenon has yet to be defined.

Pupylation Targets Proteins for ATP-Dependent Degradation

To begin to understand how doomed proteins are targeted to a bacterial proteasome, Pearce et al. performed a genetic screen to identify proteins that interact with Mpa; this led to the discovery of prokaryotic ubiquitin-like protein (Pup). Pup covalently links to several known proteasome substrates in mycobacteria, and this “pupylation” is essential for their degradation (51, 52). Around the same time, the authors of another study noted that a gene, *orf7*, found directly upstream of the 20S CP genes of mycobacteria and other members of the *Actinomycetes* (21), was predicted to encode a small protein with a diglycine motif near its C terminus, a feature found in Ub. Hypothesizing that this gene might encode a degradation tag, an epitope-tagged Orf7 protein (later named Pup) was produced in *Mycobacterium smegmatis*, resulting in the

FIG 3 ATP-dependent degradation requires pupylation. (A) From left to right, Pup is deamidated at its C terminus by Dop, producing Pup_{Glu}; PafA ligates Pup_{Glu} to a substrate; and a pupylated substrate can either be depupylated by Dop or be degraded by the proteasome. (B) Proposed mechanisms of deamidation and pupylation. Starting from top left, the Pup C-terminal Gln is attacked by a nucleophilic Asp in Dop, forming a Pup-Dop intermediate; Pup undergoes a nucleophilic attack by water, activated by Dop, to yield Pup_{Glu}; PafA uses ATP to phosphorylate the γ -carboxylate of Pup_{Glu}; and nucleophilic attack by a substrate Lys residue results in the covalent linkage of Pup to a substrate. Another mechanism of Pup deamidation has been proposed, in which the Dop Asp residue coordinates a water molecule and nucleophilic attack by this activated water results in deamidation (66). (C) Depupylation of a pupylated substrate in *M. smegmatis* requires an intact Pup N terminus as well as Mpa. The top labels indicate the *M. smegmatis* strain background, as follows: WT, wild-type strain; $\Delta prcBA$, deletion mutant lacking the *prcBA* genes; and *mpa*, a strain in which the gene has been disrupted with an integrated plasmid. Lanes: vec., empty vector; Pup, overproduced full-length Pup; Pup91, overproduced truncated Pup missing 30 N-terminal amino acids. Immunoblotting (IB) was performed on cell lysates from the indicated strains by using an antibody to *M. tuberculosis* Ino1 (inositol-1-phosphate synthetase), a known pupylated substrate. Bands corresponding to Ino1 and its Pup or Pup91 conjugate are indicated at the right. (Reprinted from reference 50 with permission from Elsevier.)

visualization of pupylation of numerous proteins (53). The “pupylomes” of *M. smegmatis* and *M. tuberculosis* include at least 50 proteins in which a pupylated site has been identified (52, 54, 55).

Pup is ligated to substrates via a covalent linkage between its C-terminal residue and a substrate Lys residue (51, 53, 56). This is comparable to the linkage of Ub, whose C terminus also attaches to Lys. Remarkably, however, Pup itself has no structural similarity to Ub: rather than harboring the β -grasp fold characteristic of Ub and its related modifiers, free Pup is mostly unstructured (57, 58).

In mycobacteria, Pup is produced with a C-terminal glutamine (Gln), yet mass spectrometry and nuclear magnetic resonance (NMR) studies revealed that Pup attaches to a substrate via a C-terminal glutamate (Glu), which is suggestive of a deamidation event on the Gln side chain (51, 53, 56). It was discovered that two enzymes, Dop (deamidase of Pup) and PafA (proteasome accessory factor A), are required to carry out the pupylation reaction. Dop catalyzes the ATP-dependent deamidation of the C-terminal Gln of Pup, generating Pup_{Glu} (59, 60); PafA subsequently phosphorylates the C-terminal Glu, rendering it available for nucleophilic attack by the ϵ -amino group of a specific Lys residue in a substrate protein (59, 61) (Fig. 3A and B). Reconstitution of the mycobacterial pupylation system in *Escherichia coli*, which lacks pupylation, demonstrated that Dop and PafA are necessary and sufficient for pupylation of known mycobacterial substrates as well as *E. coli* proteins (62). Notably, the requirement of Dop for pupylation can be bypassed upon production of Pup_{Glu}, a variant found in several actinobacteria, including *S. coelicolor* (60–63).

While bacterial 20S CPs and ATP-dependent proteasome activators are homologous to their eukaryotic counterparts, Dop and PafA are completely unrelated to the E1, E2,

and E3 enzymes required for the attachment of Ub to proteins in eukaryotes. Dop and PafA are in the glutamine synthetase (GS) family of proteins, whose members carry out condensation reactions between Glu and amino groups (61, 64). GS proteins primarily use ATP to phosphorylate the γ -carboxylate of Glu, preparing it for attack by an amino group, as is the case with PafA (65). In contrast, Dop performs deamidation of Pup without ATP hydrolysis, rather using ATP as a cofactor (59). Because Dop and PafA share high sequence and structural identities (64, 66), one might hypothesize that one of the two proteins arose from a genetic duplication event in order to provide a second activity in the pupylation pathway.

Efforts to define the regions of Pup that are recognized by Dop and PafA have established that the C-terminal half of Pup is sufficient for deamidation and substrate ligation (67, 68). The crystal structure of PafA bound to the C-terminal fragment of Pup revealed that PafA binding causes Pup to adopt two short, helical folds, allowing it to be positioned correctly within the ligase active site (69). As Dop harbors the same Pup-stabilizing pocket, it is predicted to associate with Pup in a similar manner. In line with this observation, the Pup C terminus is highly conserved, while the N-terminal half of Pup is variable among bacterial genera and species (58).

Another major difference between the Ub and Pup systems is that while polyubiquitylation is generally required for substrate recognition and degradation in eukaryotes (70), polypupylation has been observed only *in vitro* or in bacteria overproducing Pup (71–73). While we cannot rule out the occurrence of polypupylation in a natural setting, the fusion of a single Pup to a protein is sufficient to target it for efficient proteasomal degradation (68). Thus, it is generally accepted that a single Pup is necessary and sufficient to doom a protein to a bacterial proteasome.

While a consensus pupylation motif has not been identified in substrates, a comparison of the pupylation of free Lys versus that of a known mycobacterial substrate, PanB (ketopantanoate hydroxymethyltransferase) (49), demonstrated that while both occur through the same reaction, PanB pupylation proceeds more quickly (65). Moreover, PafA has an affinity for PanB that is approximately 3 orders of magnitude higher than that for free lysine, suggesting that intrinsic features of substrate proteins significantly influence their pupylation by PafA (65). Additionally, once a protein is pupylated, it appears to have a weaker affinity for PafA, suggesting that it will not be subjected to additional rounds of pupylation (71).

Pup Interacts with Mpa/ARC To Target Substrates for Proteolysis

In mycobacteria, substrates are targeted to the proteasome through the interaction of Pup with the N-terminal coiled-coil domains of Mpa (31, 37). The crystal structure of the Pup-Mpa complex revealed that, similar to that of PafA and Dop, Mpa binding causes Pup to adopt a partially helical structure, which in this case orients the N terminus toward the ATPase channel (Fig. 2B). Mutations that disrupt Pup-Mpa helical interactions abolish degradation of a model substrate in *M. tuberculosis* (37).

Numerous studies collectively suggest that the flexible nature of Pup allows it to be manipulated by Mpa in order to initiate the process of substrate unfolding. Using in-cell NMR analysis, the disordered N-terminal region of Pup can be observed within the central channel of Mpa when in complex with a 20S CP (74). Because the N terminus of Pup is required both for unfolding by Mpa and for substrate degradation (31, 60), it is hypothesized that headfirst translocation of Pup through the ATPase channel of Mpa is required for degradation of pupylated substrates. It is not known how Mpa interacts with substrates themselves once Pup is pulled through the hexameric channel. In the case of *E. coli* ClpXP and other proteases, association of substrates with the protease complex is enhanced by adaptors that bind to both the protease and the degradation tag (reviewed in reference 41); it is possible that similar adaptors function for Mpa.

Dop Is a Depupylase

There have been several observations suggesting that Dop might have a function beyond deamidation of Pup. First, several bacterial species containing Dop encode

Pup_{Glu} rather than Pup_{Gln}, thus obviating the need for a deamidase. Second, evidence for the existence of depupylation emerged during the phenotypic characterization of an *M. tuberculosis* *dop* mutant. An attempt was made to rescue pupylation in a *dop* mutant by ectopically expressing *pup_{Glu}*; however, the amount of pupylated proteins was only partially restored, and free Pup was undetectable. It was proposed that pupylation had been rescued but that the vast majority of Pup was degraded along with substrates, suggesting a possible requirement of Dop for the removal and recycling of Pup prior to proteasomal degradation. To test this hypothesis, the strain was treated with a proteasome inhibitor, which restored the pupylome (61). It was subsequently determined that Dop indeed catalyzes the removal of Pup from pupylated substrates (50, 75).

The mechanisms of deamidation and depupylation by Dop are proposed to be similar; both processes involve the hydrolysis of an amide bond at the C terminus of Pup (66, 76). The use of a molecular trap to capture a Dop-Pup intermediate revealed that an aspartate (Asp) in the Dop active site could be a direct nucleophile. Based on these studies, it is proposed that the Asp residue in Dop attacks the side chain amide of the Pup C-terminal Gln residue, causing the exit of ammonia (in the case of deamidation) (Fig. 3B) or the substrate Lys residue (in the case of depupylation) (76).

Additional observations led to the conclusion that Mpa participates in depupylation. In wild-type *M. smegmatis*, overproduction of Pup results in the disappearance of the proteasome substrate Ino1 (inositol-1-phosphate synthetase), likely driven by increased pupylation; in contrast, overproduction of Pup in an *mpa* mutant results in the robust accumulation of pupylated Ino1 (Pup~Ino1) (Fig. 3C). This was surprising considering that in a strain lacking 20S CP genes, only Ino1, not Pup~Ino1, is detectable. These data suggest that, in bacteria, depupylation occurs robustly only in the presence of Mpa. Supporting this hypothesis, production of an N-terminally truncated Pup variant ('Pup) which lacks several key Mpa-interacting residues results in the accumulation of 'Pup~Ino1 in both wild-type and *mpa* mutant *M. smegmatis* strains (50) (Fig. 3C). Taken together, these observations strongly suggest that in the absence of proteasome activity, substrates are depupylated rather than maintained in a pupylated state. Furthermore, depupylation by Dop appears to require the N-terminal half of Pup as well as Mpa *in vitro* and *in vivo* (Fig. 3C) (75).

The apparent necessity of Mpa for efficient depupylation might be explained by the ability of Mpa to partially unfold Pup or its bound substrate, which may allow easier access of Dop to the isopeptide bond formed between Pup and its substrate. Notably, another study demonstrated that Mpa enhances the *in vitro* pupylation activity of PafA, with Mpa, Pup, and PafA forming a ternary complex (77); Dop may likewise form such a complex, as it is structurally similar to PafA. Further studies are needed to define the mechanism by which Mpa facilitates depupylation.

In contrast to the proven necessity for Ub recycling in eukaryotes (11), the importance of Pup recycling in bacteria is unclear. Unlike in *M. tuberculosis*, ectopic expression of *pup_{Glu}* in an *M. smegmatis* *dop* mutant appears to fully restore the pupylome (60, 61). Additionally, an *M. tuberculosis* *dop* mutant expressing *pup_{Glu}* grows normally (our unpublished data). However, there may be certain conditions in which depupylation confers an overall advantage to the bacterium. It remains to be determined whether Pup recycling occurs or is important in other bacterial species.

The current model of substrate degradation indicates that the N terminus of Pup is threaded through the Mpa pore (31). What is unclear is whether or not Pup is then threaded into the 20S CP and how Dop fits into this picture. A possibility is that Dop, with assistance from Mpa, depupylates substrates independently of proteasome engagement; this is supported by the observation that Pup~Ino1 is undetectable in an *M. smegmatis* 20S CP mutant (50) (Fig. 3C). However, because Mpa is itself a proteasome substrate (49), it is possible that this result is artificial: if Mpa facilitates depupylation, then the accumulation of Mpa in a 20S CP mutant may result in hyperdepupylation regardless of proteasome association. Further experiments are needed to determine if depupylation is coupled to proteasomal degradation.

Degradation-Independent Functions of Pupylation

In several bacterial species, pupylation components are not always encoded by 20S CP subunit genes. One such example is *Corynebacterium glutamicum*, a relative of mycobacteria, which expresses *pup_{Glu}*, *pafA*, *arc*, and *dop* but lacks *prcBA* (Fig. 1B) (78). This bacterium has generated interest in the hope of establishing a degradation-independent role for Pup. A recent study of *C. glutamicum* found that ferritin, a protein that assembles into multimeric complexes to sequester ferrous iron (Fe^{2+}), is a pupylation substrate; engagement of pupylated ferritin by ARC causes complex disassembly, presumably to release Fe^{2+} . It is hypothesized that ferritin disassembly by pupylation and ARC unfolding is a requisite for survival during iron starvation, as a Pup-deficient mutant of *C. glutamicum* grows poorly under Fe-depleted conditions (79).

Interestingly, the ferritin homologue BfrB in *M. smegmatis* is also pupylated, and it is potentially pupylated in *M. tuberculosis* (52, 55). While it is unknown if *prcBA*, *mpa*, and *pafA* mutants are more sensitive to Fe limitation, *M. tuberculosis* mutants are more resistant to peroxide, for reasons that have never been determined (47, 80). It is possible that *M. tuberculosis* mutants that cannot effectively degrade or disassemble ferritin complexes have lower levels of exchangeable Fe^{2+} , which could otherwise catalyze Fenton chemistry with peroxide to generate highly toxic reactive oxygen species (81). Supporting the notion that BfrB levels contribute to peroxide sensitivity, an *M. tuberculosis bfrB* null mutant is more sensitive to Fe-generated oxidative stress (82).

S. coelicolor is a relative of *Mycobacterium* spp. that has a Pup-proteasome system and a sizeable pupylome (21, 63). Although proteasomal degradation appears to be functional in this species, several phenotypes have been identified for *S. coelicolor* that depend exclusively on pupylation rather than degradation. *S. coelicolor pup* and *pafA* mutants, but not 20S CP mutants, cannot properly sporulate, and they undergo changes in the production of several secondary metabolites (63, 83). It is unknown how pupylation regulates these processes.

It is notable that several proteins that were identified as confirmed or putative pupylated substrates in *M. tuberculosis* do not accumulate in *mpa* or *pafA* mutants under routine culture conditions (52). While it is plausible that these proteins are not targeted to the 20S CP for degradation, it is also possible that these proteins are degraded only under specific conditions that remain to be determined. Thus, further work is needed to establish whether pupylation has functions independent of proteolysis in mycobacteria and other proteasome-bearing bacteria.

ATP-INDEPENDENT PROTEASOMAL ACTIVATION

In *M. tuberculosis*, disruption of *mpa* results in only a slight growth defect in culture; however, chemical inhibition of proteasome activity or deletion of *prcBA* results in marked growth defects under all conditions tested (47, 80). This observation suggests that the proteasome provides functions that are independent of pupylation. To identify new proteins that interact with the *M. tuberculosis* proteasome, a catalytically inactive 20S CP was used to “trap” potential proteasome cofactors. This approach resulted in the discovery of a novel, non-ATPase proteasome activator, proteasome accessory factor E (PafE) (45), also known as Bpa (bacterial proteasome activator) (46), that is not genetically linked to the Pup proteasomal genes (*pafE* is annotated Rv3780 for *M. tuberculosis*). PafE assembles into an unprecedented dodecameric ring with 12-fold symmetry (Fig. 4), though monomers within the ring adopt helical structures similar to those of the eukaryotic ATP-independent 11S activators PA26 and PA28, which form heptamers (44). Notably, PafE, like Mpa, contains a C-terminal GQYL motif required for 20S CP gate opening. This feature also allowed for the independent identification of PafE by a homology search (46). Both studies found that PafE could stimulate the degradation of short peptides and a model unfolded protein (45, 46).

Rather than acting as an unfoldase to deliver proteins into a 20S CP, PafE seems to function simply as a gate opener. In line with this, the PafE-proteasome efficiently degrades unfolded proteins *in vitro* without ATP (45, 46). The PafE ring is lined with hydrophobic residues that may allow unfolded proteins, which tend to expose normally

FIG 4 PafE/Bpa is an ATP-independent proteasome activator. (Top) Crystal structure of dodecameric PafE/Bpa from *M. tuberculosis* (PDB entry 5IET). Dashed lines approximate the extended C termini of PafE in contact with the 20S CP α -ring. (Bottom) Top-down view of PafE.

buried hydrophobic amino acids, to slip into the 20S CP (44). Furthermore, proteomic analysis of a *pdfE* mutant identified an inherently unstable transcriptional repressor, HspR (heat shock protein repressor), as a PafE-proteasome substrate. Remarkably, native HspR can be degraded robustly by PafE and 20S CPs in the absence of ATP; this experiment presented the first example of rapid and robust degradation of an intrinsic substrate by an intact prokaryotic proteasome *in vitro*. Because recombinant HspR produced in a heterologous system (*E. coli*) can be degraded efficiently *in vitro*, it does not appear that a mycobacterium-specific posttranslational modification is required for degradation by the PafE-proteasome; this suggests that PafE-proteasome substrates have inherent features that target them for degradation (45). The identification of additional PafE-proteasome substrates should ultimately reveal features in common among these proteins, which may indicate the presence of a PafE-dependent degron.

CONTRIBUTIONS OF PROTEASOMES TO BACTERIAL PHYSIOLOGY

Proteasomes degrade potentially dozens, if not hundreds, of different bacterial proteins and are thus essential for the normal physiology of all proteasome-bearing bacteria studied to date. Here we focus on the pathways that have so far been determined to be affected either directly or indirectly by proteasomal degradation in both nonpathogenic and pathogenic bacterial species.

Nitric Oxide Resistance

The study that reinvigorated the bacterial proteasome field described a screen for *M. tuberculosis* mutants hypersensitive to nitric oxide (NO). Nathan and colleagues discovered that Mpa and PafA, which were uncharacterized at the time, are essential for NO

resistance in *M. tuberculosis* (47). This and other studies further demonstrated that NO resistance is substantially dependent on proteasomal degradation (47, 80, 84). *M. tuberculosis* is the causative agent of the human disease tuberculosis, which is currently the global leading cause of death by a single infectious agent (85). *M. tuberculosis* is contracted by the inhalation of aerosolized droplets released from an infected individual by coughing or sneezing. In the lungs, *M. tuberculosis* is phagocytosed by macrophages and dendritic cells, which are sentinels of the immune system (reviewed in reference 86). An important mechanism by which phagocytes control pathogen growth is through the production of NO by inducible nitric oxide synthase (iNOS or NOS2) (87). The importance of NO for tuberculosis has been demonstrated most clearly in iNOS-deficient mice, which succumb rapidly to *M. tuberculosis* infection (88). However, despite its obligate residence within macrophages, *M. tuberculosis* persists, avoiding clearance, often for the lifetime of the host. The identification of proteasome activity in *M. tuberculosis* as a protective factor against NO toxicity suggested that proteolysis of one or more proteins serves to defend this bacterium against host immunity.

NO can damage proteins, lipids, and nucleic acids to inhibit microbial growth (reviewed in reference 89). While it was often presumed that the Pup-proteasome system was needed to degrade NO-damaged proteins, this was never demonstrated. Furthermore, as previously mentioned, *mpa* and *pafA* mutants are more resistant to peroxide stress and have wild-type resistance to heat shock, suggesting that the Pup-proteasome system is not required for a general proteotoxic stress response (47, 80). It would be over a decade before the mechanism of Pup-proteasome-mediated NO protection was elucidated. A screen for suppressor mutants of the NO hypersensitivity of an *mpa* mutant identified a new Pup-proteasome substrate, Log (Lonely guy), whose accumulation renders bacteria hypersensitive to NO (90). Log is an orthologue of a plant enzyme, LONELY GUY, which catalyzes the production of adenine-based hormones called cytokinins (91). Intriguingly, Samanovic et al. determined that *M. tuberculosis* secretes cytokinins and that cytokinin production is significantly increased in an *mpa* strain (90). Cytokinins can be broken down into toxic aldehydes (92); indeed, at least one aldehyde, *para*-hydroxybenzaldehyde, accumulates in an *mpa* mutant. Addition of this or another aldehyde, 2-methyl-3-butanal, along with NO effectively kills wild-type *M. tuberculosis*. Because aldehydes and NO are unlikely to react with each other, it is proposed that aldehyde-induced cellular damage sensitizes *M. tuberculosis* to nitrosative stress through a mechanism that has yet to be elucidated (Fig. 5A). Notably, disruption of Log leads to a partial restoration of virulence in an *mpa* mutant, indicating that NO sensitivity resulting from cytokinin accumulation is a significant but not exclusive cause of the attenuated *in vivo* phenotype of this strain (90).

It is notable that treatment of an *mpa* mutant with a proteasome protease inhibitor can exacerbate the NO-sensitive phenotype of the strain (47). Barring off-target effects of the proteasome inhibitor, this suggests that there may be additional, proteasome-dependent, Pup-independent mechanisms of NO resistance in *M. tuberculosis*.

Copper Resistance

A transcriptional analysis of genes misregulated in *pafA* and *mpa* mutants led to the discovery of a group of copper-inducible genes whose expression is controlled by a single transcriptional repressor, RicR (regulated in copper repressor), thus linking proteasome-dependent degradation to copper (Cu) homeostasis (93). Cu is both an essential nutrient and a potent source of toxicity; it is a cofactor for several enzymes involved in aerobic respiration and the oxidative stress response, but it can also be detrimental to cells by nonspecifically binding to and destabilizing proteins and may promote the generation of reactive oxygen species by Fenton-like chemistry (94). In wild-type bacteria, the RicR regulon is activated under conditions of elevated Cu, as Cu⁺ induces the dissociation of RicR from its respective promoters, thereby alleviating repression of gene expression (Fig. 5B). Several proteins encoded within the RicR regulon have been characterized functionally, including MmcO (mycobacterial multicopper oxidase), which oxidizes Cu⁺ to the less reactive species Cu²⁺ (95), and MymT

FIG 5 Contributions of proteasomes to the physiology of *M. tuberculosis*. (A) Proteasomal degradation of the cytokinin synthase Log keeps levels of cytokinins and aldehydes low. Accumulation of Log in a Pup-proteasome mutant causes increased cytokinin production. Cytokinins break down into aldehydes, which cause cytotoxicity in the presence of NO. (B) In wild-type bacteria, the transcriptional repressor RicR binds Cu⁺, causing its dissociation from promoters controlling the expression of Cu homeostasis genes at five loci on the *M. tuberculosis* chromosome. In the absence of proteasomal degradation, RicR appears to sense low intracellular Cu levels and binds to DNA, repressing expression of these genes. In the presence of elevated Cu levels, the RicR regulon may not be induced fully in a Pup-proteasome mutant. It is unclear if it is the weak induction of this regulon or another unidentified pathway that results in the hypersensitivity of Pup-proteasome system mutants to Cu. (C) The heat shock-responsive transcriptional repressor HspR, which controls expression of the protein chaperone-encoding genes *dnaK*, *clpB*, and *hsp*, is degraded by the PafE-proteasome. The abundance of misfolded proteins is kept low by DnaK, ClpB, and Hsp, and possibly by direct degradation by the PafE-proteasome. When degradation is disrupted, it is presumed that the failure to degrade HspR causes incomplete derepression of chaperone genes, leading to an accumulation of toxic unfolded proteins. See the text for proteasome-regulated pathways in other bacterial species.

(mycobacterial metallothionein), which sequesters Cu⁺ ions from other cellular components to mitigate toxicity (96). There are four additional genes of unknown function that are also regulated by RicR (Fig. 5B). While disruption or deletion of any single RicR-regulated gene does not affect the virulence of *M. tuberculosis*, simultaneous repression of the entire regulon leads to Cu hypersensitivity and attenuation in mice (97). Notably, only *Mycobacterium* species that infect mammals are known to have the RicR regulon in its entirety (93).

Where does *M. tuberculosis*, which is naturally found only in humans, encounter Cu? In macrophages, Cu is imported through a plasma membrane transporter, Ctr1 (reviewed in reference 98), while an intracellular Cu transporter, ATP7A, traffics Cu into intracellular vesicles (99). Ctr1 and ATP7A expression is induced during hypoxia (100, 101), a defining feature of *M. tuberculosis* lung infection; additionally, proinflammatory stimulation of macrophages induces localization of ATP7A to phagosomal vesicles, a mechanism required for antimicrobial activity *in vitro* (99). It has been proposed that the host Cu transport machinery is induced and mobilized during *M. tuberculosis* infections (reviewed in reference 102). Perhaps supporting this hypothesis, Niederweis and colleagues showed in a guinea pig infection model that Cu concentrations are significantly increased at sites of *M. tuberculosis* infection compared to healthy lung tissue and that supplementation of food with Cu promotes increased control of bacterial growth (103). Interestingly, *M. tuberculosis*-infected macrophages accumulate intraphagosomal copper to a degree that is not observed in cells infected with nonpathogenic mycobacteria (104).

The RicR regulon is repressed in *pafA* and *mpa* null mutants, and the induction of this regulon in response to Cu is impaired in these strains (93; our unpublished observations). Consistent with this observation, both *pafA* and *mpa* mutants are markedly more sensitive to Cu than wild-type *M. tuberculosis* (97), presumably due to a failure to fully induce expression of the RicR regulon. While the simplest model

suggests that RicR itself is a Pup-proteasome substrate, RicR levels are not conspicuously different between wild-type and *mpa* strains (93). Importantly, we cannot rule out that there are RicR-independent reasons for the Cu hypersensitivity of Pup-proteasome mutants. Thus, a molecular link remains to be established between proteasomal degradation and Cu homeostasis.

Cu is somewhat unique among physiologically relevant metals in that free Cu is essentially nonexistent in cells; rather, it is passed between proteins or other small-molecule ligands (105–107). Because both the RicR regulon and other independently regulated Cu-inducible genes are repressed in Pup-proteasome strains (93, 108), it is possible that the accumulation of an unknown Cu-binding proteasome substrate results in a decrease in bioavailable Cu in the cytoplasm, which could explain the failure of RicR and other Cu-sensing repressors to disassociate from DNA. Furthermore, we cannot yet rule out that pupylation affects Cu homeostasis independently of proteasomal degradation, as described for iron homeostasis in *C. glutamicum* (79).

Metabolism

In eukaryotes, proteolysis is an essential source of amino acids for the synthesis of new proteins, and proteasome activity increases when amino acids are withheld from growth media (109–111). Several lines of evidence suggest that the same may be true for bacteria. In *M. smegmatis*, mutants in the Pup-proteasome system have survival defects in carbon- or nitrogen-limited media, and a model Pup-proteasome substrate in *M. smegmatis* is degraded more rapidly when bacteria are grown under nitrogen starvation than when they are grown in standard media (73). To explain how proteolysis for amino acid recycling might be regulated, Gur and colleagues suggested that in *M. smegmatis*, PafA preferentially pupylates high-molecular-weight proteins, while Dop preferentially depupylates low-molecular-weight proteins. This may constitute a mechanism whereby proteolysis for amino acid recycling is optimized: to obtain the same amount of recycled material, degradation of a few large proteins would be preferential to the degradation of many small proteins in order to avoid disruption of essential cellular systems (112). While this is a reasonable hypothesis, it remains unclear if the defective growth of *M. smegmatis* Pup-proteasome mutants during nutrient starvation is directly and only caused by a failure to recycle proteins or if other metabolic processes required for growth under starvation are regulated by proteasomal degradation. Along these lines, the Gur group found that complementation of a $\Delta prcSBA$ mutation (*prcS* is another name for *pup*) with *prcS* alone could restore part of the growth defect observed in this strain (73). In light of the observation that *C. glutamicum* uses pupylation to disassemble ferritin complexes under low-iron conditions (79), it is thus possible that pupylation, independently of degradation, regulates one or more pathways needed for growth of mycobacteria under nutrient-limiting conditions.

Deletion of *pup* in *S. coelicolor* results in drastic defects in cell morphology and division. Additionally, the production of specific antibiotics is abolished in this strain, demonstrating roles for pupylation in distinct cellular processes in this species (83). Notably, in contrast to the increased peroxide resistance of *M. tuberculosis* Pup-proteasome mutants, degradation-defective *S. coelicolor* is hypersensitive to peroxide (63, 83). Thus, defects in Pup-proteasome system components can result in markedly different phenotypes among bacterial genera.

Proteotoxic Stress Response

A role for ATP-independent proteasomal degradation was established with the identification of the *M. tuberculosis* PafE-proteasome substrate HspR (45). HspR is a repressor of several genes encoding proteins involved in the proteotoxic stress response, including the protein chaperones DnaK (Hsp70), ClpB, and Hsp (Acr2) (113). Based on the observation that this regulon is required for heat shock resistance, it was presumed that the failure to degrade HspR in a *pafE* mutant would contribute to increased heat sensitivity in this strain. Indeed, a *pafE* null mutant is hypersensitive to heat shock and is also slow to grow under normal conditions (45). HspR itself is a

somewhat unstable protein, a feature that may allow it to act as a sensor of proteotoxic stress: Das Gupta and colleagues proposed that unfolding of HspR contributes to derepression of the *clpB*, *dnaK*, and *hsp* gene promoters (114). It was thus proposed that PafE-proteasome-dependent degradation of HspR enhances the proteotoxic stress response by preventing HspR from prematurely rebinding to these promoters before all proteotoxic stress has been alleviated (Fig. 5C). In support of this hypothesis, we found that under certain conditions, a *pafE* mutant will acquire spontaneous suppressor mutations to restore wild-type-like growth. Most of the suppressor mutants have loss-of-function mutations in *hspR*, resulting in complete derepression of the HspR regulon (J. Jastrab, M. Samanovic, and K. H. Darwin, unpublished data). These suppressor mutants are also as resistant to heat shock as the parental *pafE*⁺ strain. Thus, these results suggest that the inability of a *pafE* mutant to fully derepress the *hspR* regulon both slows the growth of a *pafE* mutant under routine culture conditions and hinders recovery after heat shock.

An *M. tuberculosis* *pafE* mutant was significantly attenuated in mice, suggesting that bacteria encounter proteotoxic stress *in vivo* (45). This stress may include not only damage induced by phagocyte-derived reactive oxygen and nitrogen species but also dramatic changes in temperature when an infected host releases bacteria in aerosolized droplets into the environment; transitions from body temperature (~37°C) to room temperature (~25°C) and back into a new host may potentially induce protein misfolding.

It is important that we cannot rule out other pathways affected by PafE-dependent protein degradation that are unrelated to the proteotoxic stress response but are nonetheless important for the pathogenesis of *M. tuberculosis*. Several proteins accumulate in a *pafE* mutant, and it remains to be determined if some or all of them are proteasome substrates and if any contribute to the pathogenesis of tuberculosis.

REMAINING QUESTIONS

While much has been learned in the past decade about proteasomal degradation in bacteria, there remain significant gaps in our current knowledge. Below are several of the most pertinent questions in the field.

What Are the Requirements for Mpa/ARC-Mediated Degradation in Bacteria?

An issue that has frequently complicated the study of bacterial proteasome function is that ATP-dependent proteasomal degradation is very inefficient *in vitro*; recombinant Mpa associates poorly (if at all) with wild-type 20S CPs and therefore fails to induce gate opening (32). How, then, does this proteasome maintain a stable association with Mpa in bacteria? While it is possible that optimal *in vitro* reaction conditions have simply not yet been established, another possibility is that there are other accessory factors that bind to or otherwise promote a closer association between Mpa and 20S CPs. It may also be that additional proteins are required to maintain substrates in an unfolded state. The lack of these elements *in vitro* may result in the instability of the complex during proteolysis.

How Are Substrates Selected for Pupylation, Depupylation, and Degradation?

To date, it is unknown how proteins are selected for pupylation in any species. Production of an *M. tuberculosis* substrate in *E. coli* results in the pupylation of several different Lys residues rather than one specific Lys residue as observed in mycobacteria. Furthermore, the production of *M. tuberculosis* PafA and Pup_{Glu} in *E. coli* results in the pupylation of numerous *E. coli* proteins, most of which do not overlap the mycobacterial pupylomes (62). While it might seem that the mere presence of an exposed lysine would be sufficient to destine proteins for pupylation, this is not the case, as not all proteins with lysines are necessarily PafA targets. Taken together, these observations suggest the existence of other factors as well as intrinsic features of substrates that target them for pupylation. The structures of intermediate complexes of PafA, Pup, and

substrates will hopefully reveal the physical interactions among the proteins that facilitate pupylation.

A possible mechanism of selection or negative regulation is Lys acetylation, an emerging phenomenon in *M. tuberculosis* that is known to directly affect the activities of several enzymes (115–117). It is possible that Lys acetylation protects exposed lysines on nonsubstrates, as well as nonideal lysines on proteins that are pupylated at another, preferred site. It is also possible that phosphorylation or other posttranslational modifications affect interactions between substrates and PafA.

Relatedly, how are pupylated substrates selected for degradation? We know of several pupylated proteins, for example, several enzymes of the *M. tuberculosis* fatty acid synthase complex II pathway, that do not seem to be robustly degraded under routine culture conditions (52). This particular example may offer a potential clue: if Pup modifications are sheltered within a complex, Mpa might be unable to access them or to otherwise unfold them in the presence of multiple interacting proteins.

How Do Bacteria Balance Degradation and Depupylation?

It is intriguing to imagine that the processes of degradation and depupylation are dynamically regulated in response to signals such as environmental stress or nutrient starvation, as suggested previously (73). While we have not observed any changes in the relative abundances of pupylation system components in *M. tuberculosis* under different culture conditions (our unpublished observations), studies of *M. smegmatis* have shown that the levels of Mpa, Dop, PafA, and PrcA oscillate during stationary growth phase and upon nitrogen starvation (73, 112). It is possible that the activities of PafA, Dop, and Mpa change in the presence of cofactors or posttranslational modifications that have yet to be identified. It has additionally been proposed that phosphorylation of the 20S CP regulates degradation of pupylated substrates. In *M. smegmatis*, overproduction of *M. tuberculosis* protein kinase B (PknB) leads to phosphorylation of PrcA and is associated with the increased degradation of a pupylated substrate (118). However, these data must be taken with caution, as the overproduction of PknB was required to observe this phenomenon.

Do PafE and Mpa/ARC Compete for Binding to the 20S CP?

PafE inhibits the degradation of pupylated substrates by the Mpa-proteasome *in vitro* (46). This observation, along with the apparently stronger *in vitro* binding of 20S CPs to PafE than to Mpa (44), suggests that PafE may compete with Mpa-mediated degradation in bacteria. While this is a reasonable hypothesis, overproduction of a 20S CP trap in wild-type and *mpa* mutant *M. tuberculosis* strains showed that PafE binding to CPs was not enriched in the *mpa* mutant, as would be expected if PafE and Mpa competed for access to 20S CPs (45). However, we cannot rule out the possibility that there are conditions in which the activity or interaction of either activator with 20S CPs is altered based on need.

What Are the Roles of Proteasomal Degradation in Broader Bacterial Physiology?

While several pathways affected by proteasomal degradation have been identified, there are dozens, if not hundreds, of other proteins reliant on ATP-dependent and -independent proteasomal degradation. *M. tuberculosis* strains with a deletion or knockdown of *prcBA* are viable but unable to grow normally *in vitro* (80, 84); notably, because a high-density transposon screen predicted that *prcBA* is essential in *M. tuberculosis* (80, 119), we propose that the *prcBA* deletion mutant harbors one or more suppressor mutations that allow it to survive in culture. Curiously, while deletion of 20S CP activity impairs *M. tuberculosis* growth under almost all conditions tested, it appears to have a minimal effect on *M. smegmatis* growth under most conditions, with the exception of nitrogen and carbon limitation (53, 73, 120). *S. coelicolor* is also viable upon deletion and disruption of its proteasome genes (83). Thus, it may be that the proteasome has more specialized functions in environmental bacterial strains while

being essential for the normal physiology of *M. tuberculosis*, which is found only in humans.

CONCLUDING REMARKS

In addition to revealing several new contributions of regulated proteolysis to bacterial physiology, the study of bacterial proteasomes may potentially translate broadly to the understanding of proteasomal degradation in general. For one, despite extensive structural and mechanistic studies of eukaryotic ATP-independent proteasomal activators, the physiological role of ATP-independent protein degradation in eukaryotes has remained elusive. Ongoing studies of PafE in bacteria may finally reveal functions of ATP-independent degradation that are relevant to eukaryotes as well as other prokaryotes. The study of proteasome-dependent degradation has also revealed insights into the challenges faced by bacterial pathogens, such as *M. tuberculosis*, during infections. From proteotoxic stress responses to copper resistance, there is little doubt that the study of bacterial proteasome-regulated pathways will reveal new avenues of study for years to come.

ACKNOWLEDGMENTS

We are grateful to Huilin Li for providing the images presented in Fig. 4. We thank Jordan Jastrab and Marie Samanovic for reviewing a draft version of this review. We are grateful to the anonymous reviewers, who made several critical corrections and helpful suggestions.

Proteasome research in the Darwin lab is supported by NIH grants HL92774 and AI088075. K.H.D. holds an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund. S.H.B. received support from the Jan T. Vilcek Endowed Fellowship Fund and is supported by NIH grant T32AI007180-34.

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