HHS Public Access

Author manuscript

Trends Microbiol. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Trends Microbiol. 2016 January ; 24(1): 26–34. doi:10.1016/j.tim.2015.10.001.

Game of 'Somes: Protein Destruction for *Mycobacterium tuberculosis* Pathogenesis

Marie I. Samanovic and **K. Heran Darwin***

New York University School of Medicine, Department of Microbiology, 550 First Avenue, MSB 236, New York, NY 10016

Abstract

The proteasome system of *Mycobacterium tuberculosis* is required for causing disease. Proteasomes are multi-subunit chambered proteases and, until recently, were only known to participate in adenosine triphosphate (ATP)-dependent proteolysis in bacteria. In this review, we discuss the latest advances in understanding how both ATP-dependent and ATP-independent proteasome-regulated pathways contribute to *M. tuberculosis* virulence.

Keywords

Mycobacterium tuberculosis; proteasome; pupylation; pathogenesis

Tuberculosis Infections

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), which affects nearly one-third of the world's population. If active TB is not properly treated, the disease can be fatal, making TB one of the greatest killers on earth. The latest report from the World Health Organization reported that in 2013, about nine million people developed TB and 1.5 million died from the disease [1]. Drug-resistant *M. tuberculosis* strains are on the rise, including the emergence of multi-drug resistant and extensively-drug resistant (XDR) resistant strains that are untreatable with the two most powerful anti-TB drugs (isoniazid and rifampicin), in addition to fluoroquinolones and at least one of three injectable second-line drugs. To date, cases of XDR-TB have been reported in at least 100 countries [2]. Thus, there is an urgent need to develop new drugs to battle the TB pandemic.

TB is contracted by the inhalation of air-borne droplets containing *M. tuberculosis* bacilli released from an infected individual (reviewed in [3]). Bacteria most commonly infect the lungs and reside in alveolar macrophages. If the host cannot control the infection, *M. tuberculosis* growth will result in the destruction of lung tissue and eventually the death of the host. Despite the high global mortality caused by TB, 90% of immunocompetent

*Correspondence: heran.darwin@med.nyu.edu (K.H. Darwin).

individuals harboring the tubercle bacillus are able to suppress bacterial growth, and often do not progress to active disease. Vertebrate hosts have several effective mechanisms to control microbial growth; in mammals, these include the production of reactive oxygen and reactive nitrogen species (ROS and RNS, respectively) by phagocytes (reviewed in [4, 5]). Nitric oxide (NO) is an antimicrobial free radical produced by the inducible NO synthase (iNOS or NOS2) in activated macrophages (reviewed in [6]). Among its potential activities, NO can penetrate bacterial membranes and combine with ROS such as superoxide to generate peroxynitrite, a highly toxic molecule. RNS and ROS can damage nucleic acids and proteins, and cause lipid peroxidation [7, 8]. Mice lacking iNOS are highly susceptible to *M. tuberculosis*, demonstrating a critical function for NO during infections [9]. While a role for NO in human tuberculosis is controversial, there is abundant indirect evidence suggesting NO participates in controlling bacterial growth in humans [10–15]. Relevant to this review, the investigation of NO resistance ultimately led to the identification of a bacterial proteasome as an important regulator of *M. tuberculosis* pathogenesis.

The *M. tuberculosis* Proteasome: Guardian of the Cell

For many years, Carl Nathan hypothesized that *M. tuberculosis* encodes gene products that allow it to resist NO-mediated killing in animals. To test this hypothesis, his team took a forward genetic approach and screened for *M. tuberculosis* mutants that were sensitive to NO *in vitro*, with the idea that these mutants would be attenuated in mice producing NO, but fully virulent in mice lacking iNOS. Darwin *et al*. [16] identified several NO-hypersensitive mutants with transposon insertions in genes predicted to encode components of a bacterial proteasome. The NO-hypersensitivity phenotype of these mutant strains was mimicked in wild-type (WT) *M. tuberculosis* treated with eukaryotic proteasome inhibitors, further supporting a role for proteasome-dependent proteolysis in NO resistance [16].

Proteasomes are responsible for the majority of cytosolic and nuclear proteolysis in eukaryotes [17]. In particular, the 26S proteasome is a multi-subunit, barrel-shaped protease composed of two functionally distinct sub-complexes, the 20S core particle (CP) and the 19S regulatory particle (RP). The 20S CP is composed of four stacked hetero-heptameric rings forming a cylinder, whereas the 19S RP is a complex of at least 19 proteins located at one end or both ends of the CPs (reviewed in [18]). RPs include a ring of six different ATPases that unfold and translocate substrates into CPs as well as non-ATPase subunits that function in various aspects of substrate recognition and processing. Before being targeted to the CP, most substrates need to be covalently modified with a 76-amino acid protein called ubiquitin (reviewed in [19]). Ubiquitylated proteins are recognized by the RP, and unfolded by the hexameric ATPase ring in order to deliver the protein into the 20S CP for degradation.

In bacteria, proteasomes are found in members of the order Actinomycetales [20–23]. Similar to eukaryotic proteasomes, bacterial 20S CPs are also barrel-shaped, with four stacked seven–subunit rings (two homo-heptameric β-subunit rings in between two homo-heptameric α-subunit rings), and are tightly gated to prevent unregulated proteolysis (reviewed in detail elsewhere [24]). Unlike their eukaryotic counterparts, complex RPs have not been identified with bacterial 20S CPs; however, ATPases similar to those found in

eukaryotic RPs are found in all proteasome-bearing bacteria. Interestingly, robust interactions have not been detected between bacterial ATPases and 20S CPs *in vitro*.

Over the past 10 years, we and other groups have characterized the biochemistry of proteasomal degradation in *M. tuberculosis*. Most critically, it was determined that many proteins destined for proteasomal degradation are post-translationally modified with the small protein Pup (prokaryotic ubiquitin-like protein), which functions like ubiquitin [25, 26]. Unlike ubiquitin, Pup is an intrinsically disordered protein [27, 28]. Pup is encoded upstream of the proteasome 20S CP genes, *prcBA*, and is translated with a carboxyl-terminal glutamine that must be converted to glutamate by the enzyme Dop (deamidase of Pup). Proteasome accessory factor A (PafA) then catalyzes the formation of an isopeptide bond between Pup and a lysine of a target protein [26, 29, 30]. Thereafter, Pup interacts with the mycobacterial proteasome ATPase Mpa for unfolding and translocation of the pupylated substrate into the 20S CP [26, 29, 31]. Additionally, Dop can remove Pup from substrates prior to degradation, and Mpa appears to facilitate this process [32–36] (Figure 1A). Over 60 confirmed targets of pupylation have been identified in *M. tuberculosis*, all of which require PafA for modification [37]. Unlike eukaryotes that have hundreds if not over a thousand ubiquitin ligases, PafA incredibly appears to be the only Pup ligase in bacteria.

In addition to observations that mutations in Pup-proteasome system (PPS) genes result in increased NO susceptibility in vitro, Nathan and colleagues also determined that PPS mutants are highly attenuated *in vivo* [16, 38]. PPS mutant strains do not grow to high numbers in the lungs or spleens of mice, and in the case of *prcBA* mutants, the bacteria are eventually cleared [39, 40]. Interestingly, an *mpa* mutant in the CDC1551 *M. tuberculosis* background has a conspicuous *in vitro* growth defect [41], which is not observed in the H37Rv *M. tuberculosis* background [16]. In addition, Bishai and co-workers showed that besides reduced numbers of bacteria in the lungs of the *mpa* mutant-infected mice, there are also reduced levels of interferon-γ (IFN-γ) production in the infected animals [41]. It is unclear if this is simply due to lower bacterial numbers, or if disruption of the PPS alters the immune response in another way.

While it remains to be determined if all pupylation leads to protein degradation, proteasome-dependent proteolysis is nonetheless an essential weapon in an arsenal that allows the robust growth and persistence of *M. tuberculosis* in animals.

NO: You Win or You Die

Although the Nathan lab determined that a link exists between proteasome activity and NO resistance over 10 years ago, it remained unknown how proteolysis protected against NO toxicity. There were two main hypotheses: the proteasome degrades damaged proteins that would otherwise misfold, aggregate and ultimately kill the bacteria; or, the accumulation of one or more specific proteasome substrates exacerbates NO toxicity. Recently, the question of why PPS mutants are more sensitive to NO than WT bacteria may have been at least partially answered. A suppressor screen for secondary mutations in an *mpa* mutant background that could revert its NO-hypersensitive phenotype to WT identified insertions in a previously uncharacterized gene, Rv1205. Remarkably, genetic disruption of Rv1205 not

only restores WT NO resistance to an *mpa* mutant but also partially restores virulence in mice. Rv1205 encodes a proteasome substrate that accumulates in *mpa*, *pafA*, and *prcBA* mutants [42]. Based on structural homology searches and deep bioinformatics analysis, Rv1205 was determined to be homologous to members of the LONELY GUY (LOG) family of plant enzymes that catalyze the last step of cytokinin biosynthesis [43].

Cytokinins are plant hormones that stimulate transcriptional responses via two-component regulatory systems to shape plant development (reviewed in [44, 45]). In order to produce a cytokinin, a precursor molecule, which is an adenosine monophosphate carrying either an isoprene or aromatic group at the position six nitrogen (N^6) of the adenine base, must be processed by LOG to remove the phosphoribose group; the liberated modified adenine base is the bioactive cytokinin. Because Rv1205 has phosphoribohydrolase activity like plant LOGs, Rv1205 is referred to as "Log" [42].

From these studies, it was found that WT *M. tuberculosis* secretes cytokinins. Importantly, an *mpa* mutant produces cytokinins in strikingly greater abundance, correlating with the accumulation of Log in this strain, whereas a *log* mutant shows dramatically reduced cytokinin production. Interestingly, the accumulation of cytokinins is not directly accountable for sensitizing *M. tuberculosis* to NO. A metabolomics analysis revealed that an *mpa* mutant accumulates high concentrations of a cytokinin breakdown product, *para*-hydroxybenzaldehyde (*p*HBA). Cytokinins can be broken down into adenine and aldehydes (reviewed in [46]) and while *p*HBA alone is not toxic to *M. tuberculosis*, the addition of NO with *p*HBA or another cytokinin aldehyde results in robust bacterial killing *in vitro* [42]. It is unknown how *p*HBA and NO synergize to kill; it is possible that the aldehydes react with proteins or other molecules in the mycobacterial membrane, which sensitizes it to NO or other RNS (Figure 1B). Taken together, the tight regulation of Log levels by the PPS is essential to prevent unintentional NO hypersensitivity during infections.

What is the function of Log and cytokinins in *M. tuberculosis*? This question has yet to be answered, but we speculate that cytokinins have a signaling role during infections. In addition to plants using cytokinins as signal transducers, microbial pathogens and symbionts can subvert plant development by producing cytokinins; this can include tumor formation, which promotes microbial growth in plant tissues (reviewed in [47]). Thus, it is tempting to speculate that *M. tuberculosis*, which is never found naturally outside of humans, uses cytokinins to signal transcriptional changes in mammalian cells to favor its growth in vivo. Alternatively, cytokinins could be used among the bacteria to signal specific messages during infection to alter the bacterial transcriptome and promote their survival.

It is worth discussing here a previously reported and somewhat curious observation: the complementation of an *M. tuberculosis prcBA* deletion-disruption mutant (Δ*prcBA*::*hyg*) with a *prcBA* allele encoding a catalytically inactive 20S CP can restore NO resistance to WT levels *in vitro*, but not WT persistence in mice [39]. Recent studies have shown that the production of inactive 20S CPs in *M. tuberculosis* is highly effective in trapping known proteasome substrates [64]. Thus, it is possible that catalytically inactive 20S CPs are able to trap substrates and reduce the amount of free or active protein in the bacterial cytosol. For example, trapping and inactivating Log in this way might be sufficient to prevent the

accumulation of aldehydes *in vitro*. In contrast, long-term infections of *M. tuberculosis* likely requires the proteasomal degradation of numerous substrates affecting various pathways required for successful growth in a mammalian host. Proteasomal degradation might also simply be necessary to recycle amino acids or liberate other nutrient sources such as metal co-factors during an infection.

The Metal Throne

In addition to providing resistance to host-derived NO, the proteasome regulates other pathways important for pathogenesis. As with other chambered proteases that degrade transcription factors (reviewed in [48–50]), it was hypothesized that the *M. tuberculosis* proteasome could affect gene expression. A transcriptomic analysis of *mpa* and *pafA* mutants revealed the dysregulation of several genes regulated by zinc and copper [51]. Members of the Zur (zinc uptake regulator) regulon are up regulated in *mpa and pafA* mutants compared to WT bacteria. Zur is a zinc-sensing transcriptional repressor, which under low zinc concentrations is released from the operators of eight *M. tuberculosis* promoters that control the expression of 24 genes [52]. Among them is the *esx-3* operon, which encodes an essential type VII secretion system involved in iron and zinc uptake in *M. tuberculosis* [53–56]. While we do not know how the increased expression of the Zur regulon affects pathogenesis (if at all), it seems likely that the perturbation of zinc and iron homeostasis would disrupt the physiology of *M. tuberculosis* during infections.

The transcriptomic analysis of PPS mutants also identified a copper-responsive regulon unique to pathogenic mycobacteria. The RicR (regulated in copper repressor) regulon includes five loci distributed throughout the *M. tuberculosis* chromosome and is repressed in both *mpa* and *pafA* mutants. RicR is a paralog of CsoR (copper sensitive operon repressor), which is released from its operators when bound to copper [51, 57, 58]. When the concentration of intracellular copper is low, RicR represses transcription of *ricR* itself, *mymT* (encoding a copper metallothionein), *lpqS* (encoding a putative lipoprotein), Rv2963 (encoding a putative permease), *socAB* (small ORF induced by copper A and B), and *mmcO* (mycobacterial multi-copper oxidase) [51]. Metallothioneins are small, typically cysteine-rich proteins that sequester metals to protect against metal-catalyzed toxicity. MymT binds up to six copper (Cu^{+1}) ions, and a *mymT* mutant is hypersensitive to copper *in vitro* [59]. Similarly, a mutant deficient in the multi-copper oxidase MmcO, which oxidizes Cu^{+1} to Cu^{+2}, is sensitive to copper *in vitro* [60, 61]. While single mutants of *mymT*, *mmcO*, or any other RicR-regulated gene do not have virulence defects in mice [59, 61], constitutive repression of the entire RicR regulon results in a strong growth defect *in vivo* [61]. Similarly, *mpa* and *pafA* mutants, in which the entire *ricR* regulon is partially repressed, are also hypersensitive to copper [61] (Figure 1B).

Several studies suggest that either host-restricted access to metals (e.g. iron, zinc, and manganese) or host-imposed metals (e.g. copper and zinc) can play a role in immunity against pathogens (reviewed in [62, 63]). It would thus be unsurprising if defects in metal homeostasis were at least partially responsible for the attenuated phenotype of *M. tuberculosis* PPS mutants in mice. We do not yet understand how the PPS is connected to the regulation of the Zur and RicR regulons, and there are currently no data to suggest either

regulator is a substrate of the PPS. One possibility is there is an accumulation of proteasome substrates in PPS mutants that are metal binding proteins, resulting in the increased sequestration of metals. The accumulation of a zinc- or copper-binding protein could potentially result in the induction of the Zur regulon or repression of the RicR regulon, respectively.

Different Roads Sometimes Lead to the Same 20S Proteasome

It was noted that defects in *mpa* and *pafA* result in a subtle growth defect *in vitro*, whereas a *prcBA* mutant has a dramatic growth defect in liquid and, in particular, on solid medium [39, 40]. It was thus speculated that there were additional ways for proteins to be targeted to the 20S proteasome in *M. tuberculosis*. This hypothesis was finally tested when a new proteasome activator was discovered that promotes the degradation of peptides or unfolded proteins by the mycobacterial proteasome without using ATP or Pup [64, 65]. This protein, PafE (proteasome accessory factor E; also known as Bpa for bacterial proteasome activator), forms dodecamers and is functionally homologous to several eukaryotic ATP-independent proteasome activators (reviewed in [66]). Similar to other proteasome activators, PafE caps the ends of 20S CPs and enhances proteasomal degradation, presumably by opening the entrance to the 20S CP to allow proteins to enter [64, 65].

A proteomic search for PafE-dependent proteasome substrates revealed a distinct class of proteins that so far do not appear to be targets of pupylation [64]. Among the most abundant proteins to accumulate in a *pafE* mutant is the heat shock protein repressor (HspR). In *M. tuberculosis*, HspR is encoded by the last gene of the *dnaK* (or *hsp70*) operon and represses expression from the *dnaK* promoter, as well as the promoters of two other heat-shock responsive protein chaperone genes (Figure 2). The *dnaK* operon encodes a chaperone system that is essential for protein quality control during heat shock (reviewed in [67]). Upon heat shock, it is hypothesized that HspR partially unfolds in order to detach from its promoter to allow expression of the *dnaK* operon. When first described, it was unknown if HspR itself was degraded or simply failed to bind DNA during heat shock [68]. Jastrab *et al*. showed that PafE stimulates proteasomal degradation of HspR, and at elevated temperatures HspR is even more rapidly degraded *in vitro* [68]. These data suggest that the denaturation of HspR functions to both release the repressor from the *dnaK* operator and facilitate its degradation by the PafE-proteasome (Figure 2).

The observation that the PafE-proteasome degrades unfolded proteins suggests it could help minimize proteotoxic stress during heat shock by at least two ways: to degrade any misfolded proteins before they aggregate and to increase production of the DnaK protein quality control system to minimize misfolding of newly translated proteins (Figure 2). While these may be the primary functions of PafE, future studies will determine if this road to degradation regulates other aspects of *M. tuberculosis* pathogenesis (see Outstanding Questions).

Finally, PafE and Mpa do not appear to work together on the same 20S CP. Delley *et al*. reported that PafE can prevent the degradation of a pupylated substrate by Mpa and 20S CPs *in vitro*, suggesting PafE competes with Mpa for 20S CP access [65]. Moreover, proteomics

analysis of a *M. tuberculosis* Δ*pafE*::*hyg* mutant demonstrated that the pupylome differs from the 'PafEylome' [64]. Perhaps most relevantly, PafE is exclusively found in proteasome-bearing bacteria, unlike Mpa, further suggesting an independent role for PafE in proteolysis. While we cannot rule out that 20S CPs can be simultaneously capped with PafE and Mpa, it seems more likely that these two proteasome activators function independently and for different purposes.

Concluding Remarks and Future Directions

Proteolysis should never be dismissed as 'housekeeping'. Regulated protein degradation by proteasomes and other proteases is critical for the normal function of all living organisms. For *M. tuberculosis*, proteasomal degradation affects numerous pathways that are essential for it to be a successful pathogen.

Part of the success of *M. tuberculosis* lies in its ability to survive within its host for a long or latent period without causing overt symptoms, and until waning immunity allows the bacteria to revive and cause disease. In addition to host immune effectors like RNS and ROS, bacteria face inhospitable conditions such as hypoxia and nutrient starvation during latency [69–71]. In eukaryotes, proteasomal degradation can have nutritional roles under starvation conditions through the recycling of amino acids [72, 73]. In *M. smegmatis*, a non-pathogenic, saprophytic *Mycobacterium* species, the PPS is needed for survival under conditions of nitrogen limitation and, to a lesser extent, during carbon starvation [74]. It is possible that nutrient limitation is one reason why a proteasome core protease mutant is unable to persist in mice [39, 40].

Is winter finally coming for TB? Because the proteasome is essential for *M. tuberculosis* to cause lethal infections, it is a highly attractive drug target. Considerable effort is being placed on the inhibition of the 20S CP of *M. tuberculosis* with the expectation that inhibitors specific to the *M. tuberculosis* proteasome, and ineffective against mammalian proteasomes, can be developed [75, 76]. In contrast to the 20S CP, other components of the PPS (i.e. Dop and PafA) and PafE are biochemically distinct from those of the eukaryotic proteasome system. As such, these enzymes may provide more specific targets for the development of TB drugs. In addition, a recent report identified peptide aptamers that could target Pup to prevent it from interacting with Mpa, thus inhibiting proteolysis of a pupylated protein [77]. Although this required the production of the peptide within mycobacteria, this study nonetheless showed that Pup, which has no enzymatic activity, could be targeted. In addition to providing hope for finding new TB therapeutics, the continued characterization of pathways regulated by the *M. tuberculosis* proteasome will undoubtedly reveal new insights into the physiology of one of the world's most successful pathogens.

Acknowledgments

We thank Samuel Becker and Jordan Jastrab for critical review of a draft version of this manuscript, and the anonymous reviewers for excellent suggestions. Tuberculosis proteasome research in the K.H. Darwin lab is supported by NIH grants HL92774 and AI088075. K.H.D. holds an Investigator in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund. M.I.S. received support from the Jan T. Vilcek Endowed Fellowship fund.

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Outstanding Questions

- What are the roles of cytokinins during tuberculosis infections?
- What are the sources of copper and zinc during infections?
- How is the proteasome linked to the Zur and RicR regulons?
- Does proteasome-dependent proteolysis help generate nutrients during infections?
- What are the other functions of Mpa-dependent and PafE-dependent proteasomes?

Trends

- The proteasome is a highly regulated protease that is required for the pathogenesis of *Mycobacterium tuberculosis*.
- Both ATP-dependent and ATP-independent pathways to the proteasome are needed to promote bacterial virulence.
- The *M. tuberculosis* proteasome degrades an enzyme that produces cytokinins, which break down into aldehydes that synergize with nitric oxide to kill bacteria.
- Defects in proteasomal degradation perturb metal homeostasis, including zinc and copper regulated genes required for pathogenesis.
- Proteasome function is required for a robust heat shock response.

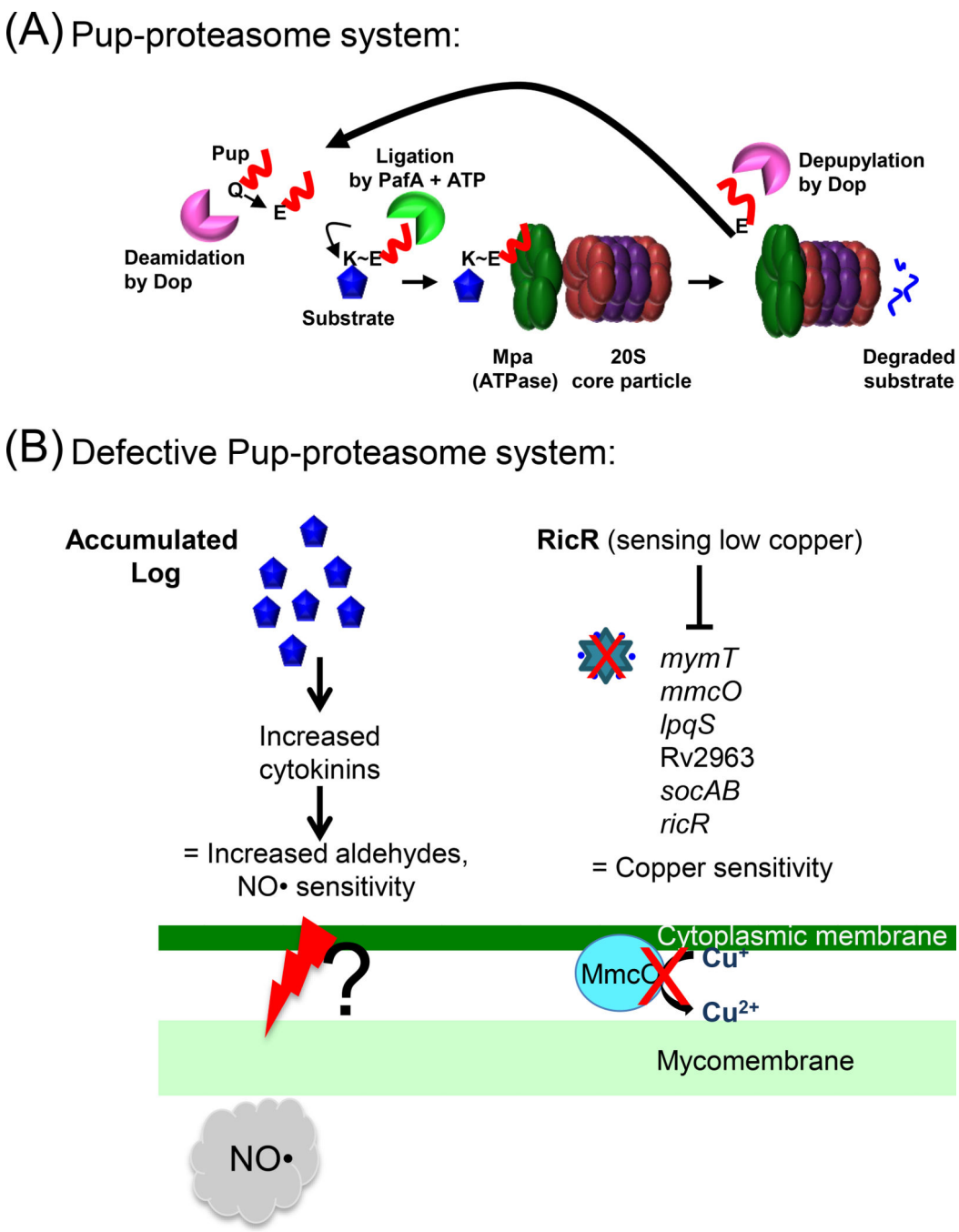

Figure 1. The Pup-proteasome System (PPS) of *M. tuberculosis*

(A) ATP-dependent degradation by the proteasome. 20S core particle subunits are colored in red (α-subunit, PrcA) and purple (β-subunit, PrcB). Pup (red line) is an intrinsically disordered protein. In *M. tuberculosis*, the last amino acid of Pup is glutamine (Gln, Q), which must be deamidated on its side chain by Dop to convert it to glutamate (Glu, E). The Glu is the phosphorylated by PafA, making it prime for attack by the amino group on the side chain of a substrate lysine (Lys, K). Although Pup is largely disordered, it forms helices upon interacting with amino-terminal coiled-coil domains of Mpa. This interaction is

essential for targeting pupylated proteins for degradation. In *M. tuberculosis*, Pup appears to be recycled by Dop in an Mpa-dependent manner, suggesting depupylation can occur just before proteins are threaded into the proteasome core particle. See text for additional details and references. (B) Disrupted proteasomal degradation results in several dysregulated pathways. Left: Accumulation of the enzyme Lonely Guy (Rv1205, Log) results in the hyperproduction of cytokinins, which can break down into aldehydes. Aldehydes synergize with NO by an unknown mechanism to kill mycobacteria. A mechanism of aldehyde-mediated toxicity might include damage (as represented by the red lightening bolt) to the mycobacterial membranes. This damage may be exacerbated in the presence of host-produced NO. Right: The RicR regulon is partially repressed in *mpa* and *pafA* mutants, leading to reduced copper (Cu) resistance. Red Xs indicate loss of MymT (Cu metallothionein) and MmcO (mycobacterial multi-copper oxidase) activities.

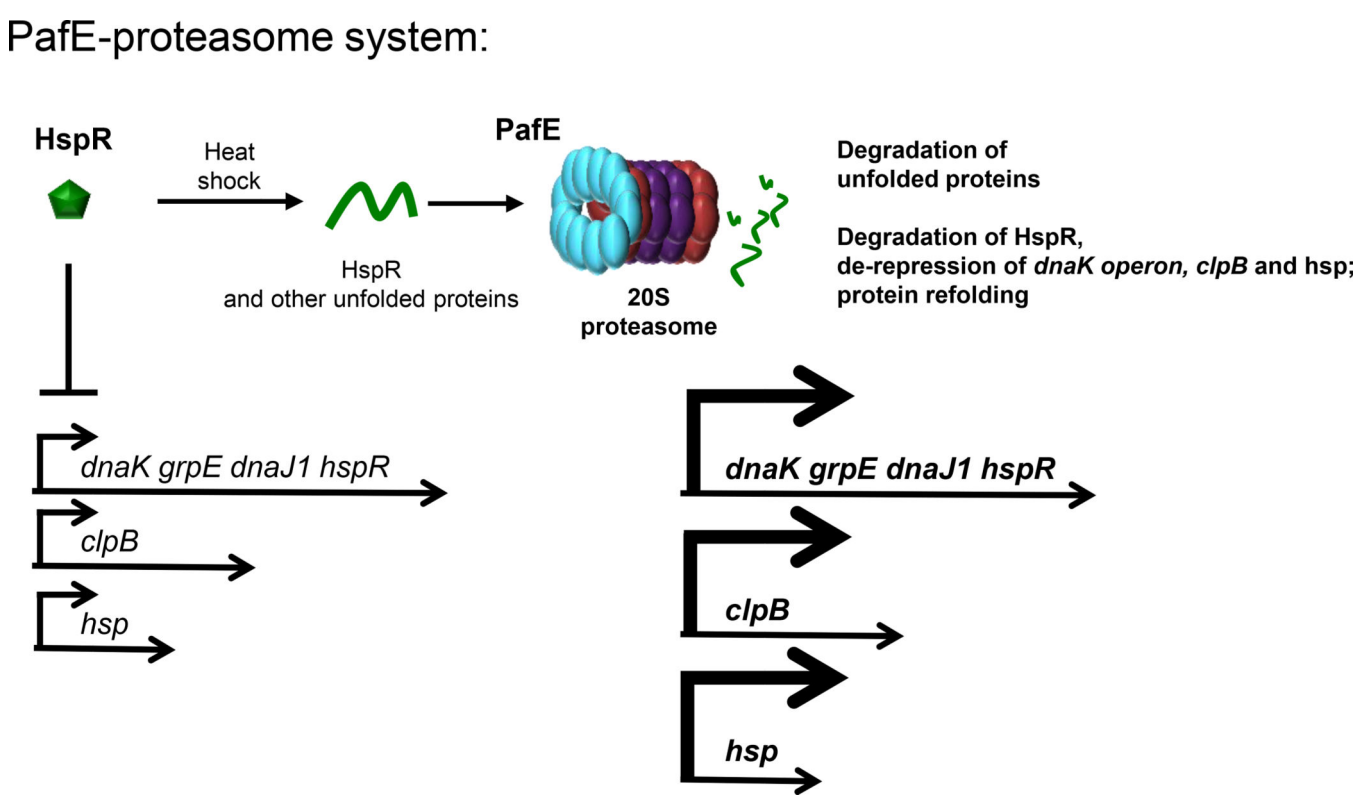

Figure 2. The PafE-proteasome System of *M. tuberculosis*

PafE (blue) forms dodecameric rings and caps 20S CPs. This allows the ATP-independent opening of 20S CPs to facilitate the degradation of peptides and denatured proteins as well as HspR. HspR represses expression of the *dnaK* operon, *clpB* and *hsp* (Rv0249c). See text for details.