

Survival of mycobacteria depends on proteasome-mediated amino acid recycling under nutrient limitation

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Abstract

Intracellular protein degradation is an essential process in all life domains. While in all eukaryotes regulated protein degradation involves ubiquitin tagging and the 26S-proteasome, bacterial prokaryotic ubiquitin-like protein (Pup) tagging and proteasomes are conserved only in species belonging to the phyla Actinobacteria and Nitrospira. In *Mycobacterium tuberculosis*, the Pup-proteasome system (PPS) is important for virulence, yet its physiological role in non-pathogenic species has remained an enigma. We now report, using *Mycobacterium smegmatis* as a model organism, that the PPS is essential for survival under starvation. Upon nitrogen limitation, PPS activity is induced, leading to accelerated tagging and degradation of many cytoplasmic proteins. We suggest a model in which the PPS functions to recycle amino acids under nitrogen starvation, thereby enabling the cell to maintain basal metabolic activities. We also find that the PPS auto-regulates its own activity via pupylation and degradation of its components in a manner that promotes the oscillatory expression of PPS components. As such, the destructive activity of the PPS is carefully balanced to maintain cellular functions during starvation.

Keywords mycobacteria; nitrogen limitation; proteasome; proteolysis; pupylation

Subject Categories Microbiology, Virology & Host Pathogen Interaction

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Introduction

The eukaryotic ubiquitin-proteasome system participates in a large variety of cellular processes and maintains homeostasis of protein folding through the degradation of regulatory and misfolded proteins (Schmidt & Finley, 2014). Proteasomal degradation also serves nutritional roles under starvation conditions through the

recycling of amino acids (Vabulas & Hartl, 2005; Suraweera *et al*, 2012). In bacteria, proteasomes are only found in species belonging to the phyla Actinobacteria and Nitrospira, where they are strictly conserved (Lupas *et al*, 1997; De Mot, 2007; Valas & Bourne, 2008); other bacterial species rely on smaller and less complex proteases for regulated protein degradation (Gur *et al*, 2011). In *Mycobacterium tuberculosis*, an actinobacterial species, it was demonstrated that the bacterial proteasome degrades protein targets conjugated to a prokaryotic ubiquitin-like protein (Pup) (Fig 1) (Pearce *et al*, 2008; Burns *et al*, 2009). Pup is a natively unfolded 64 residue-long protein, and, in contrast to ubiquitylation where poly-ubiquitin chains are added to target proteins, no evidence for poly-Pup chains has been presented yet (Pearce *et al*, 2008; Chen *et al*, 2009; Liao *et al*, 2009; Festa *et al*, 2010). The enzyme responsible for attaching Pup to protein substrates, proteasomal accessory factor A (PafA), catalyzes the ATP-dependent formation of isopeptide bonds between the Pup C-terminal glutamate γ -carboxylate and the side chain of protein substrates lysine residues (Fig 1) (Pearce *et al*, 2008; Guth *et al*, 2011). However, in many proteasome-containing species, Pup is translated with a C-terminal glutamine, rather than a glutamate. As such, a deamidase of Pup (Dop) converts this terminal glutamine into glutamate, thus allowing PafA-mediated Pup conjugation to protein substrates (i.e., pupylation; Striebel *et al*, 2009). Not only does Dop catalyze the deamidation reaction to promote substrate pupylation, it can also depupylate substrates, namely detach Pup from proteins already pupylated (Fig 1) (Burns *et al*, 2010b; Imkamp *et al*, 2010b).

Pup recognition by the proteasome regulatory subunit, termed Mpa in mycobacteria, results in its translocation, beginning at the Pup N-terminal end, into the proteolytic chamber of the 20S core proteasomal particle (Pearce *et al*, 2008; Sutter *et al*, 2009; Striebel *et al*, 2010; Wang *et al*, 2010). Bacterial proteasomes are simpler than their eukaryotic counterparts, containing only one or two types of α and β subunits in the 20S core particle and relying on a simpler regulatory particle comprising a homo-hexameric AAA+ protein (Wolf *et al*, 1998; Darwin *et al*, 2005; Hu *et al*, 2006). Mpa itself is a pupylation substrate, and it was demonstrated *in vitro* that

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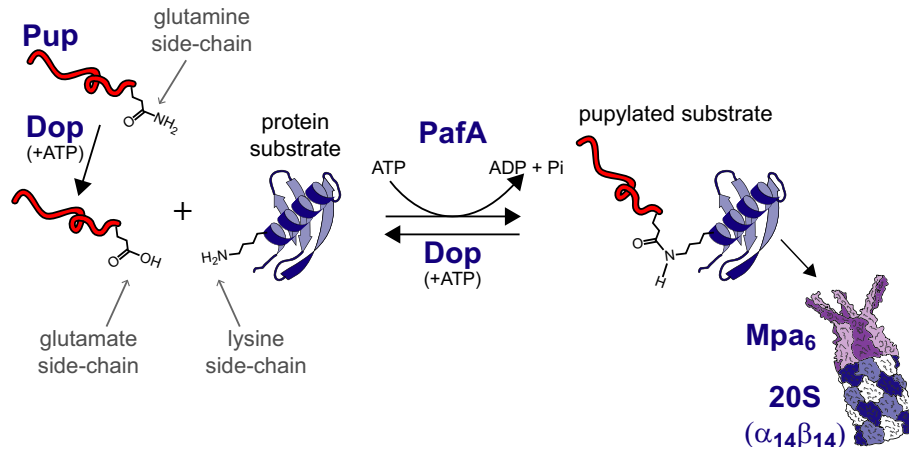


Figure 1. The Pup-proteasome system.

Protein degradation by the PPS starts with deamidation of Pup by Dop. Deamidated Pup can be conjugated to lysine side chains of protein substrates by PafA at the expense of one ATP molecule per conjugation. A pupylated protein can either be degraded by the proteasome or depupylated by Dop.

proteasome function can be negatively regulated by Mpa pupylation (Delley *et al*, 2012).

The physiological role of bacterial proteasomes has been unclear since their discovery, almost two decades ago (Tamura *et al*, 1995). In *M. tuberculosis*, the Pup-proteasome system (PPS) is essential for full virulence and persistence in the host (Darwin *et al*, 2003; Darwin 2009; Cerda-Maira *et al*, 2010; Gandotra *et al*, 2010). However, as the vast majority of PPS-containing bacteria are non-pathogenic, it would seem that the PPS plays a fundamental role in bacterial physiology, rather than serving a direct function in virulence. Nonetheless, no clear explanation for the PPS physiological role was presented thus far. On the one hand, whereas growth defects of *M. tuberculosis* PPS mutants were reported by Gandotra *et al* (2010), other studies indicated that pupylation-deficient mutants multiply as rapidly as do wild-type cells (Darwin *et al*, 2003; Cerda-Maira *et al*, 2010; Imkamp *et al*, 2010a; Küberl *et al*, 2014). An additional layer of complexity was introduced when proteomics studies failed to reveal a clear substrate specificity of PafA or defining traits for pupylation substrates (Festa *et al*, 2010; Poulsen *et al*, 2010; Watrous *et al*, 2010; Cerda-Maira *et al*, 2011). Rather, hundreds of different protein species were found to be pupylated, with no obvious PafA recognition motif detected.

In this study, we report that the PPS is essential for the survival of *M. smegmatis*, a mycobacterial model organism, under conditions of nitrogen starvation and, to a lesser extent, under conditions of carbon starvation. Whereas a PPS-deficient *M. smegmatis* mutant fails to survive starvation, the wild-type strain remains viable and responds by a dramatic, yet delicately regulated, induction of pupylation and degradation of pupylated substrates. We also find that the proteasome mediates the degradation of PPS components. Such negative auto-regulation leads to oscillatory expression of the PPS in response to starvation. Overall, our findings point to a model by which under starvation conditions, the PPS facilitates amino acid recycling so as to generate protein building blocks and a carbon source, thereby allowing for the maintenance of basal metabolic activities and survival under prolonged nutrient limitation. In considering PPS substrates merely as sources for amino acids, this

model provides a simple explanation for the broad substrate specificity of PafA, the Pup ligase.

Results

Pupylation dynamics during *M. smegmatis* growth

To understand the physiological role of the PPS, we considered whether the level of pupylated proteins in *M. smegmatis* changes during growth. As such, stationary-phase *M. smegmatis* cultures were diluted into fresh liquid media, and growth was assessed over time by measuring turbidity until the cultures once again reached stationary phase (Fig 2A). Aliquots were collected at various intervals for Western blot analysis using anti-Pup antibodies (Fig 2B, upper panel). In addition, the level of pupylated proteins in each sample was quantified (Fig 2A and Supplementary Fig S1A) as a percentage of the total protein mass using, as standard, a purified pupylome, namely the isolated pool of pupylated proteins, generated as described in Materials and Methods. The results indicated that the level of pupylated proteins changed in correlation with changes in growth phase. Specifically, the level of pupylated proteins at the beginning of the experiment, while the cells were still adjusting to growth in liquid media, accounted for approximately 2% (w/w) of the total protein population (Fig 2A). This level, however, eventually dropped to approximately 0.3% as the cultures resumed exponential growth (Fig 2A). Notably, as the bacteria re-entered stationary phase following 36 h of growth from the point of dilution, a sharp increase in the level of pupylated proteins, followed by an additional reduction in their level, was noted as the cells remained at stationary phase (Fig 2A and B). The increased level of pupylated proteins appearing at stationary phase correlated with an increase in PafA levels (Fig 2B, lower panel), suggesting an elevated pupylation rate at stationary phase. Loading controls for Fig 2B, as well as for other Western blot analyses in this study, are presented in Supplementary Fig S2.

To compare the pupylomes of exponential and stationary-phase *M. smegmatis* cultures, we adopted the methodology described by

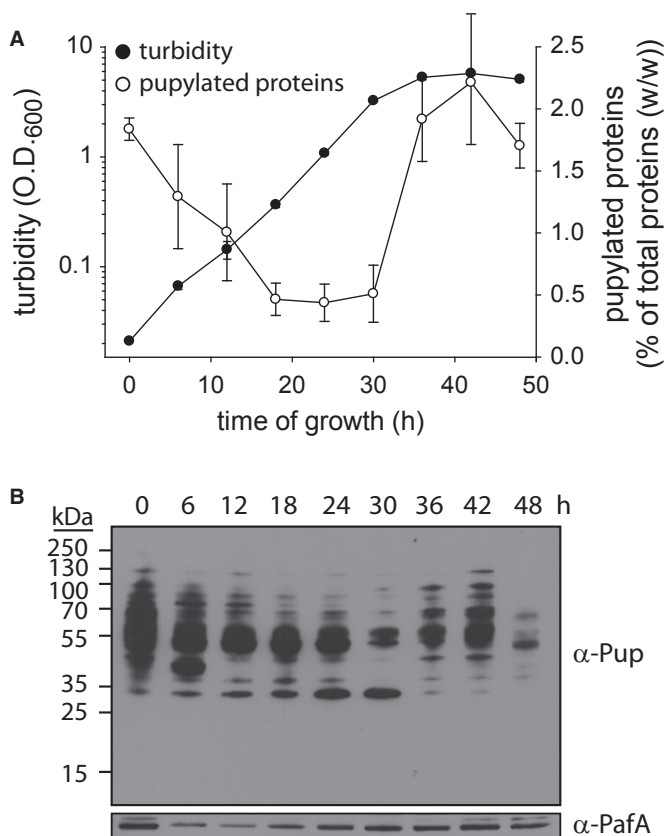


Figure 2. Elevated pupylation is observed at stationary phase.

A Three stationary-phase *Mycobacterium smegmatis* cultures were diluted into fresh media, and growth at 30°C was monitored by measuring turbidity. In addition, culture samples were collected every 6 h for quantification of pupylated proteins as described in Materials and Methods and in the legend to Supplementary Fig S1A. Data are presented as mean \pm SD; $n = 3$.

B Western blot analysis of aliquots removed at the indicated intervals was carried out using anti-Pup and anti-PafA antibodies.

Festa *et al* (2010) for pupylome purification. In short, simultaneously polyhistidine- and Strep-tagged Pup was expressed in *M. smegmatis*, and pupylomes were purified from exponential- and stationary-phase cultures via tandem purification procedures using Ni²⁺-NTA and Strep-Tactin affinity chromatography (Supplementary Fig S1B and C). As controls, cultures not expressing the dually tagged Pup were used. Proteins purified from all cultures were identified by mass spectrometry, and a protein was considered to be part of the pupylome only if it was detected in extracts prepared from cultures that expressed the dually tagged version of Pup and not in the control culture. We detected 42 and 93 protein species (excluding Pup itself) in the pupylomes of exponential- and stationary-phase cultures, respectively (Fig 3 and Supplementary Tables S1 and S2). Of these, 19 were detected in both pupylomes. Comparison of protein levels in each pupylome indicated that the protein concentration in the stationary-phase pupylome was much higher than that of the exponential-phase pupylome (Supplementary Fig S1B). Therefore, many more copies of each identified protein species were pupylated during stationary phase. There is a concern that some of the proteins detected were not pupylated,

but rather associated with the pupylome. Nonetheless, many proteins in our list were also identified in previous *M. smegmatis* pupylome analyses (Poulsen *et al*, 2010; Watrous *et al*, 2010), and proteins homologous to those included in our list were also identified in the *M. tuberculosis* pupylome (Festa *et al*, 2010). For instance, both model pupylation substrates PanB and InoI (Pearce *et al*, 2006; Burns *et al*, 2010b; Ofer *et al*, 2013) were detected in our analysis. Importantly, although more pupylated proteins were detected in stationary phase, and although some of these proteins merely associated with the pupylome rather than undergoing pupylation, the markedly broad specificity of PafA, a hallmark of protein pupylation, manifested itself at both growth stages (Fig 3).

Phenotypic examination of a pupylation-deficient mutant

We next considered the possibility that the PPS plays an important role in *M. smegmatis* physiology during stationary phase. To test this hypothesis, the survival of a PPS-deficient mutant ($\Delta prcSBA$) following arrival at stationary phase was examined. As *prcS* encodes Pup and *prcBA* encodes the 20S β and α subunits, respectively (Fig 4A), the $\Delta prcSBA$ mutant cannot pupylate proteins and lacks intact proteasomes (Shenkerman *et al*, 2013 and Fig 4B). Indeed, when Western blot analysis was performed using antibodies against the 20S α subunit, no bands were detected in the mutant strain (Fig 4B, lower panel). When similar analysis was conducted using anti-Pup antibodies, multiple bands representing pupylated proteins were detected in the wild-type strain, whereas only two bands were detected in the mutant (Fig 4B, upper panel). Through the use of a $\Delta pafA$ mutant (Supplementary Fig S3A–C), we found that these two bands represent antibody-cross-reactive proteins, rather than corresponding to pupylated proteins. Despite the absence of PafA, known to be essential for pupylation (Pearce *et al*, 2008), the two proteins in questions were nonetheless detected by Western blot analysis using anti-Pup antibodies (Supplementary Fig S3D). Furthermore, expression and tandem purification of polyhistidine- and FLAG-tagged Pup in this mutant resulted in the elution of the dually tagged Pup while the cross-reacting proteins were washed away during the purification steps (Supplementary Fig S3E and F). As such, it can be concluded the $\Delta prcSBA$ mutant completely lacks pupylated proteins. Expression of the *prcSBA* operon from a chromosomally integrated plasmid fully complemented the pupylation and proteasome deficiencies of this mutant (Fig 4B). Both the wild-type and the mutant grew equally well at 30°C during the exponential phase, with a generation time of about 4 h (Fig 4C). These findings are consistent with previous reports showing that PPS-deficient mutants of *M. tuberculosis* and *M. smegmatis* multiply as fast as do the parental wild-type strains (Darwin *et al*, 2003; Cerda-Maira *et al*, 2010; Imkamp *et al*, 2010a). By contrast, after prolonged incubation at stationary phase (17 days after inoculation), 10-fold fewer $\Delta prcSBA$ cells were alive, in comparison with wild-type cells (Fig 4D and E). As both strains reached a similar cell density upon entrance into stationary phase (Fig 4C), this phenotype reflects the reduced survival of the PPS-deficient mutant at stationary phase. This phenotype was fully complemented upon expression of the *prcSBA* operon from a chromosomally integrated plasmid, indicating that the reduced survival of the mutant results from the lack of intact PPS rather than because of a polar effect of the deletion mutation

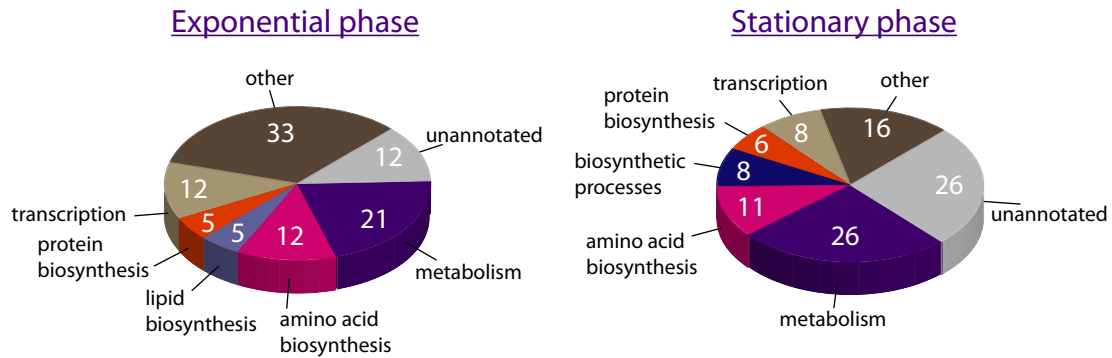


Figure 3. Pupyome analysis.

Classification of the exponential- and stationary-phase pupyomes according to protein function. The numbers are the percentage of each functional group out of the total pupyome (42 proteins at exponential phase and 93 at stationary phase). Functional groups comprising less than 3% of the pupyomes were included in the group designated as "other".

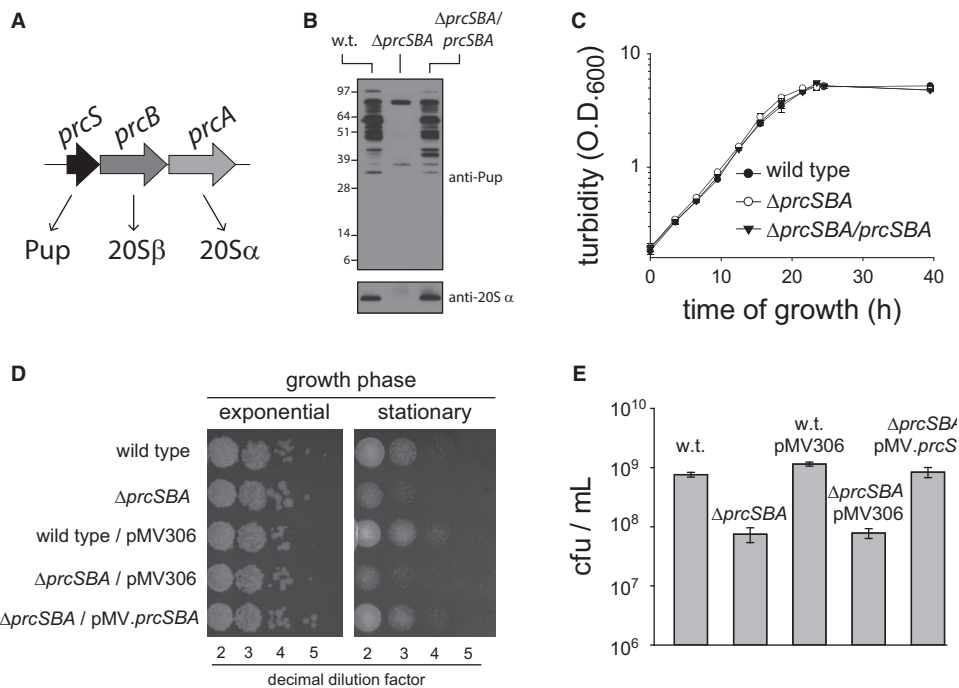


Figure 4. Starvation sensitivity of a PPS-deficient mutant.

- A The *prcSBA* operon.
 B Western blot analysis using anti-Pup and anti-20S α antibodies, as indicated.
 C Exponentially growing cultures were diluted into fresh media, and turbidity was measured ($n = 3$) at the indicated time points.
 D Exponentially growing cultures were diluted into fresh media (day 0), and samples were collected during the exponential phase at day 18. All samples were diluted to an OD₆₀₀ of 0.5, serial dilutions were carried out, and 10 μ l of the indicated dilutions were plated on solid media.
 E The concentration of live cells in 17-day-old liquid cultures was determined via colony counts ($n = 3$) following sample plating on solid media.

(Fig 4D and E). Moreover, integration of an empty vector into either the wild-type or the mutant chromosome did not affect the ability of these cells to survive during stationary phase. Collectively, the data presented thus far indicate that the elevated levels of pupylated proteins noted in stationary-phase cultures correlate with the reduced survival of a PPS-deficient mutant after prolonged incubation at stationary phase.

Carbon versus nitrogen limitation

Cells enter stationary phase when nutrients become limiting (Blackman, 1905). In the standard *M. smegmatis* growth medium (7H9 containing 0.05% (v/v) Tween-80 and 0.4% (v/v) glycerol), such as used in the experiments described in Figs 2, 3 and 4, the carbon source, glycerol, is a growth-limiting factor (Supplementary

Fig S4). At higher glycerol concentrations, however, the amount of available nitrogen becomes limiting (Supplementary Fig S4). Hence, to examine the importance of the PPS at stationary phase in more depth, the survival of wild-type and $\Delta prcSBA$ strains was monitored under conditions of either carbon or nitrogen limitation. To this end, cultures that grew exponentially in defined minimal medium were centrifuged and resuspended in a similar medium lacking either carbon or nitrogen sources. These cultures were kept at 30°C with aeration, and cell viability was monitored over the course of 15 days (Fig 5A). To account for changes in cell density during the prolonged period of incubation, turbidity measurements were carried out and viability was normalized accordingly (Fig 5B and C). At the first time point considered after either carbon or nitrogen starvation, we detected an increase in the number of cells for both the wild-type and the mutant strains, probably due to consumption of internal nutrient reservoirs. At the same time, the culture turbidity under carbon starvation decreased mildly, possibly reflecting changes in cell size under starvation. Importantly, in response to carbon or nitrogen limitation, the PPS-deficient mutant presented significant survival defects in comparison with the wild-type strain. Under carbon limitation, a approximately 10-fold decrease in the viability of the mutant cells was measured following 15 days, whereas that of the wild type was hardly compromised. These findings are consistent with the observations reported in Fig 4D and E showing a 10-fold decrease in survival of the mutant following prolonged incubation in stationary phase. Strikingly, under nitrogen

limitation, the differences between the wild type and the mutant culminated up to approximately 150-fold. It seems, therefore, that the PPS-deficient mutant is hypersensitive to nitrogen limitation, and, to a lesser extent, to carbon limitation. Expression of the *prcSBA* operon from a chromosomally integrated plasmid fully complemented the mutant phenotype in the face of nitrogen limitation (Fig 5D), indicating that the PPS deficiency is indeed responsible for the acute phenotype observed under these conditions. Interestingly, whereas *prcBA* expression in the mutant did not restore survival of the mutant under nitrogen limitation, expression of *prcS* alone resulted in partial complementation of the mutant phenotype (Fig 5D). Indeed, whereas the mutant presents an approximately 150-fold decrease in survival following nitrogen starvation, the *prcS*-complemented strain is only approximately 10-fold less viable than either the wild-type or the fully complemented strain. These findings indicate that either pupylation alone, without degradation, plays an important role under nitrogen starvation or pupylated proteins can be degraded in the absence of the 20S complex, albeit less efficiently than in the presence of this particle. Results presented below support the latter possibility.

PPS induction in response to nitrogen deprivation

Since the phenotype of a PPS-deficient mutant is considerably more severe under conditions of nitrogen deprivation, as compared to carbon deprivation, we focused our attention on the response of the

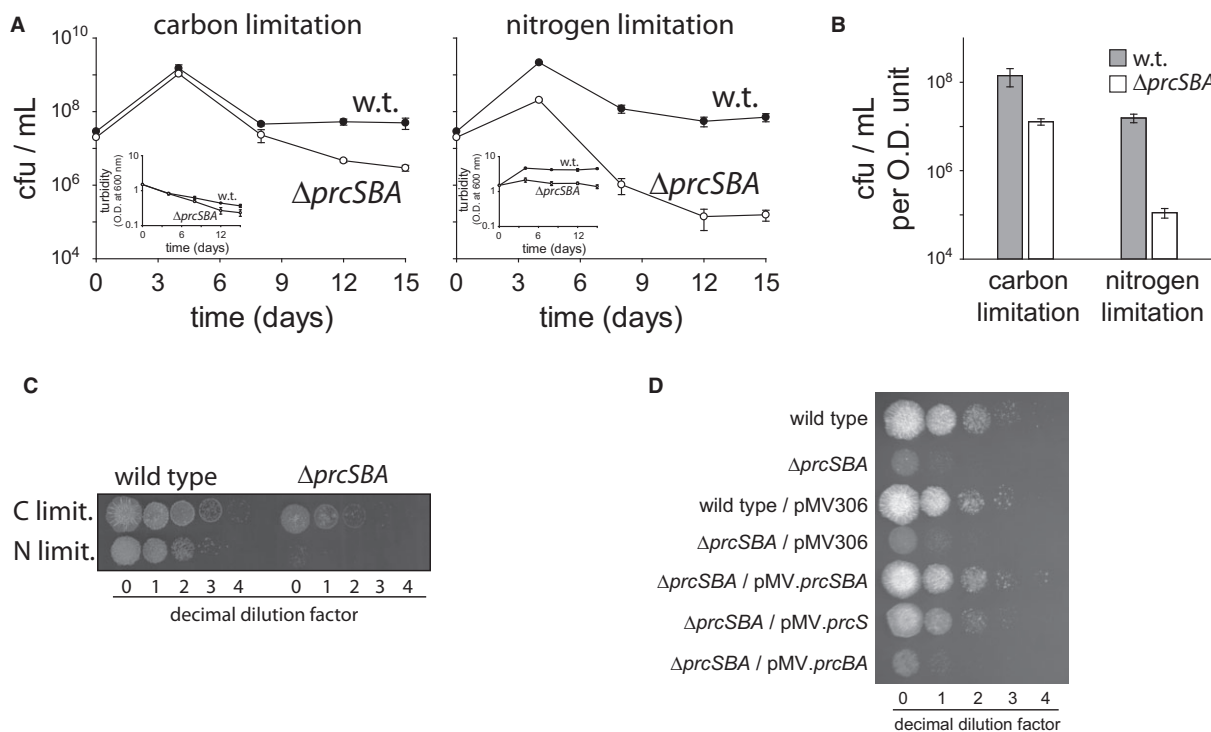


Figure 5. Hypersensitivity of a PPS-deficient mutant to nitrogen starvation.

A, B Exponentially growing cultures ($OD_{600} = 0.5$) were centrifuged and resuspended in media lacking either carbon (left) or nitrogen (right) sources. Following resuspension (time = 0), aliquots were collected at the indicated time points for determination of live cell concentration ($n = 3$).
 C Spot test of the cultures shown in (A) following 15 days of nutrient limitation. Cell density of all cultures was normalized to OD_{600} of 0.5 prior to serial dilution.
 D Spot test of the indicated cultures following 11 days of nutrient limitation. Cell density of all cultures was normalized to OD_{600} of 0.5 prior to serial dilution.

PPS in the face of nitrogen limitation. To follow the dynamics of pupylation and PPS induction in response to restricted access to nitrogen, samples were collected from a wild-type culture at time points from the onset of nitrogen starvation and subjected to Western blot analysis using antibodies against Pup. Such analysis showed that the level of pupylated proteins increased 30 min following the onset of nitrogen limitation, continued to increase throughout additional 1 h and a half, and remained high for at least 24 h (Fig 6A, upper panel). Next, in the time frame spanning days 1–6, most of the pupylated proteins disappeared. The temporally correlated dramatic increase in the level of the 20S α subunit (hereafter, 20S α), as observed via Western blot analysis using antibodies against this protein (Fig 6A, lower panel), suggested that the disappearance of the pupylated proteins resulted from proteasomal degradation. This was indeed supported by a comparison of the level of pupylated proteins in proteasome-deficient and proteasome-expressing strains. In both cases, pupylated proteins accumulated during the first day following nitrogen starvation (Fig 6B). However, these proteins almost completely disappeared at later time points in the wild-type strain, while their levels remained high in the proteasome-deficient strain (Fig 6B). This is the first indication of a

robust proteasome-dependent degradation of the entire pupylome in response to an external stimulus. We did, however, notice some reduction in the level of pupylated proteins on day 13, even in that strain lacking a 20S particle. Since equal protein amounts were loaded in each lane of the gel used for the Western blot analysis (see loading control in Supplementary Fig S2), this reduction in the levels of pupylated proteins can be interpreted as either reflecting depupylation or 20S-independent degradation. We also noticed that pupylated proteins accumulated to a much higher level in the 20S-deficient strain as early as the first day of nitrogen starvation (Fig 6B). We considered whether the reduced accumulation in the wild type could be the result of 20S-dependent degradation of pupylated proteins at early stages of nitrogen starvation. Addressing this issue is somewhat complex, as the levels of pupylated proteins are affected at each time point by three distinct processes, namely pupylation, depupylation, and degradation. To analyze the degradation of pupylated proteins at the early stages of nitrogen starvation, we designed an experimental system that is unaffected by either pupylation or depupylation. To this end, we relied on the use of a previously described Pup-Zur chimeric protein (Burns *et al*, 2010a). Msm Zur lacks lysines and, therefore, is not a PafA substrate. Moreover,

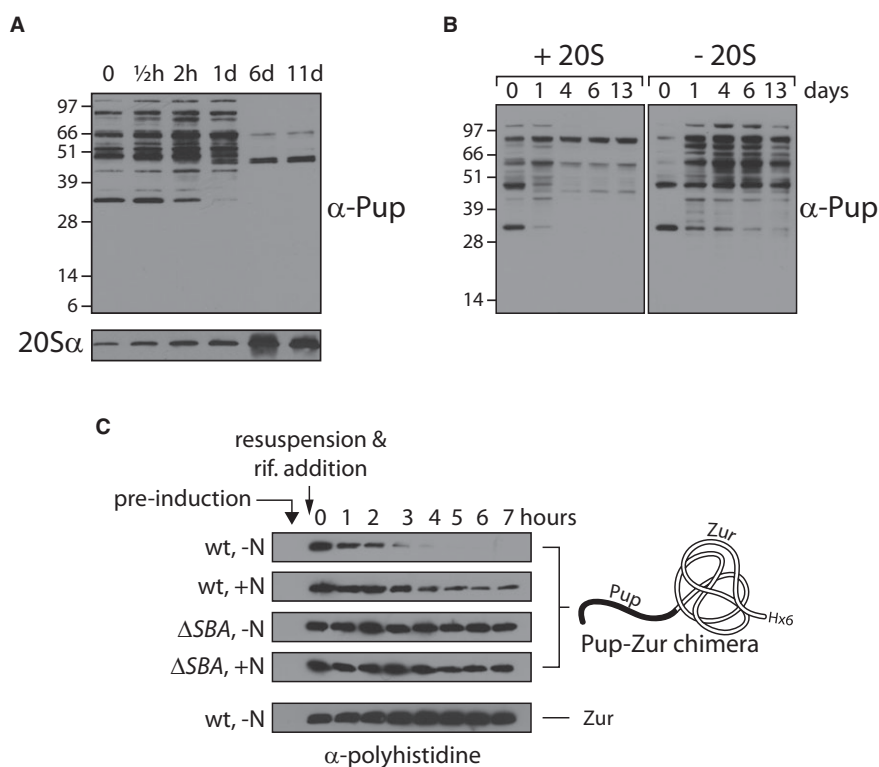


Figure 6. PPS induction under nitrogen starvation.

- A Exponentially growing cultures ($OD_{600} = 0.5$) of wild-type *Mycobacterium smegmatis* were starved for nitrogen, and samples were collected at the indicated time points from the onset of starvation ($t = 0$) for Western blot analysis
- B As in (A), except that in addition to the wild-type strain (designated +20S), a $\Delta prcSBA$ mutant that expresses the *prcS* gene from a chromosomally integrated plasmid was used (designated -20S).
- C *M. smegmatis* cultures carrying a plasmid encoding either *Zur* or a *prcS-zur* fusion, as indicated, under the transcriptional control of the acetamidase promoter were grown to exponential phase ($OD_{600} = 0.5$) before 0.2% (w/v) acetamide was added. Three hours later, the cultures were centrifuged and resuspended in media lacking acetamide and either lacking or containing a nitrogen source, as indicated. Rifampicin (200 $\mu\text{g}/\text{ml}$) was added to the cultures, and samples were collected at the indicated time points for Western blot analysis using antibodies against a polyhistidine tag.

Data information: Loading controls for all the blots in this figure are presented in Supplementary Fig S2.

Pup-Zur presents Pup as a N-terminal fusion to Zur, rather than isopeptide-bonded to a lysine side chain. As such, Pup-Zur, and in general, Pup fusions, cannot serve as substrates for depupylation by Dop (Imkamp *et al*, 2010b), although they serve as proteasome substrates (Burns *et al*, 2010a; Striebel *et al*, 2010). Conveniently, Zur presents five consecutive histidines, allowing its detection using anti-polyhistidine antibodies. Nonetheless, to facilitate sensitive detection, we cloned a genetic fusion that encodes six histidines at the C-terminus of Pup-Zur. In our experimental system, *prcS-zur* is cloned into plasmid pJV53 (Van Kessel *et al*, 2008) under the transcriptional control of the acetamidase promoter. As such, chimera expression can be induced upon addition of acetamide to the growth medium. To examine degradation of Pup-Zur following nitrogen starvation, the fusion protein was initially expressed in exponentially growing wild-type and $\Delta prcSBA$ cells. We found expression by the acetamide promoter to be tightly controlled, as no Pup-Zur was detected prior to the induction step, as determined by Western blot analysis using anti-polyhistidine antibodies (Fig 6C). Next, the cells were starved for nitrogen via multiple steps of centrifugation and resuspension in nitrogen- and acetamide-depleted media. Control cultures were resuspended in medium depleted of acetamide yet

containing nitrogen. Rifampicin was added to ensure complete arrest of Pup-Zur expression, and aliquots were removed over time for Western blot analysis using anti-polyhistidine antibodies. We found that Pup-Zur disappeared in the wild-type strain as early as 5 h following starvation, whereas Pup-Zur concentration remained largely constant in the $\Delta prcSBA$ mutant (Fig 6C), indicating that the chimeric protein is indeed degraded by the proteasome. Importantly, degradation was much faster in the wild-type following nitrogen starvation than in non-starved cells (Fig 6C). This degradation was Pup dependent, as Zur, when not fused to Pup, was not proteolyzed under similar conditions (Fig 6C, lower panel). These results indicate that proteasomal degradation of pupylated proteins is accelerated in *M. smegmatis* following nitrogen starvation. Altogether, our findings indicate that both tagging and degradation of proteins by the PPS are induced in response to nitrogen starvation.

PPS-negative auto-regulation

Induced 20S expression, as reflected in Fig 6A, was accompanied not only by a disappearance of pupylated proteins but also by a reduction in Mpa, PafA, and Dop levels (Fig 7A). Mpa is known to

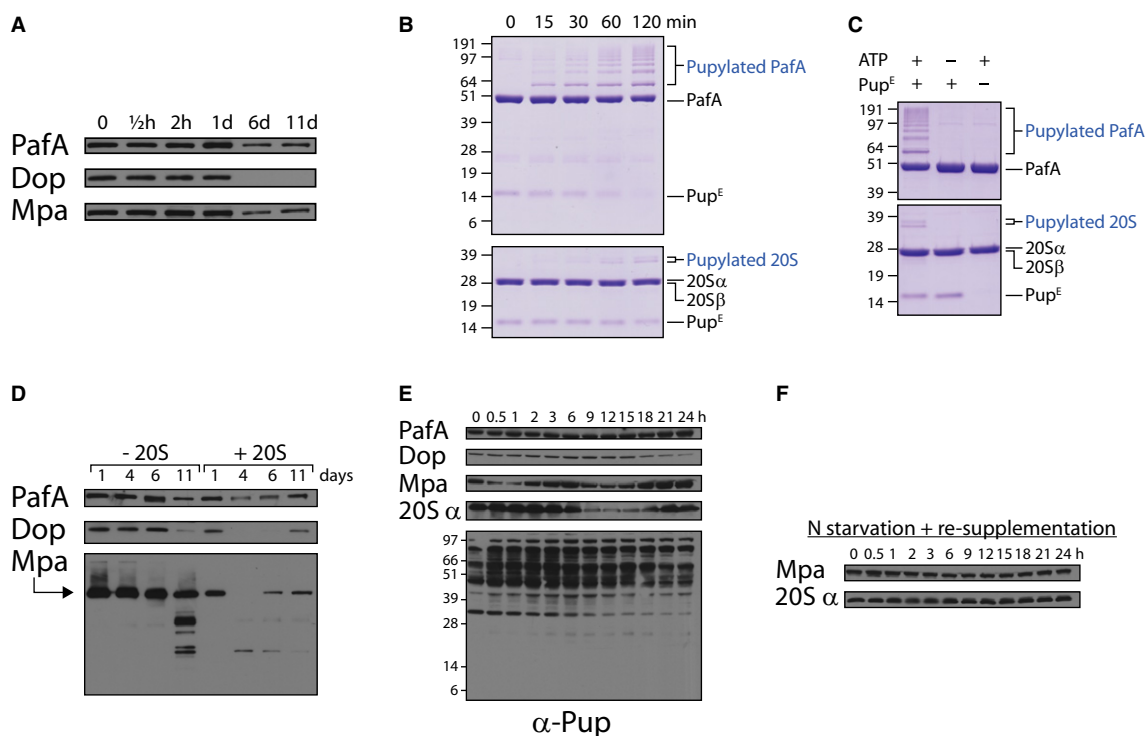


Figure 7. PPS-negative auto-regulation.

- A As in Fig 6A, except that antibodies against the indicated proteins were used in the Western blot analysis.
- B 20 μ M Pup^E were incubated in pupylation buffer at 30°C together with 5 μ M PafA (upper panel) or with 1 μ M PafA and 5 μ M 20S (lower panel). The first sample was removed ($t = 0$), ATP (2 mM) was added to start the reactions, and additional samples were removed at the indicated time points for SDS-PAGE followed by Coomassie Brilliant Blue staining.
- C Reactions were mixed as in (B) with or without ATP or Pup^E, as indicated. Following a 2-h incubation at 30°C, aliquots were collected and subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining.
- D As in (A), except that a $\Delta prcSBA$ mutant was used that expresses from a chromosomally integrated plasmid either the *prcS* gene (designated -20S) or the *prcSBA* operon (designated +20S).
- E Western blot analysis using antibodies against the indicated proteins was performed on samples collected at the indicated time points during a nitrogen starvation experiment that was performed as described in Fig 6A.
- F As in (D), except nitrogen source was re-supplemented following an hour of starvation.

be a pupylation target and a proteasome substrate (Delley *et al*, 2012; Forer *et al*, 2013). As such, the loss of Mpa in response to 20S induction is expected. By contrast, the disappearance of PafA and Dop came as a surprise, as pupylation of PafA and Dop has not been previously documented. Nevertheless, like Mpa, PafA and Dop were identified in the *M. smegmatis* pupylome (Supplementary Tables S1 and S2), suggesting that these PPS components are pupylated prior to their degradation by the proteasome. To obtain more direct evidence for PafA pupylation, an *in vitro* assay was conducted by mixing purified PafA in pupylation buffer together with a purified Pup variant presenting glutamate at its C-terminus (Pup^E). Following addition of ATP, pupylation products accumulated over time (Fig 7B, upper panel). No pupylation was observed in the absence of either ATP or Pup^E (Fig 7C, upper panel). Strikingly, PafA underwent multiple pupylation events in a manner reminiscent of the pattern observed upon poly-ubiquitylation. Due to its depupylase activity, a similar assay could not be performed for Dop.

Next, PafA, Mpa, and Dop levels were compared in 20S-positive and 20S-negative cells. Following the first few days of nitrogen limitation, PafA and Dop levels, as well as those of Mpa, decreased in proteasome-expressing cells, yet remained stable in the proteasome-deficient strain (Fig 7D and Supplementary Fig S5A). After 11 days of starvation, however, the levels of Mpa, Dop, and PafA decreased even in 20S-deficient cells (Fig 7D), as already noted for pupylated proteins (Fig 6B). In the case of Mpa, what appear to be degradation intermediates were observed at day 11 of nitrogen starvation (Fig 7D). It is possible, therefore, that Mpa, PafA, and Dop are degraded by an alternative route in the absence of the 20S, albeit much less efficiently than in the presence of the particle.

Following degradation of PPS components in 20S-positive cells, we noticed that these often reappear at later time points. For instance, whereas both Dop and Mpa disappeared by the fourth day following starvation, Dop was detected again at day 11, while Mpa already reappeared by the sixth day of starvation. A similar tendency was observed for PafA, albeit less dramatically and less consistently (Fig 7D and Supplementary Fig S5A). These findings raise the possibility that PPS proteins oscillate during starvation. This was found to indeed be the case when the levels of PPS proteins were monitored at short intervals over the course of the first day following starvation (Fig 7E). Whereas PafA levels hardly changed, remarkable oscillations were observed in the levels of Mpa and 20S α . Dop levels oscillated as well, albeit with an amplitude that appeared to be lower than those noted for Mpa and the 20S α subunit. Moreover, these proteins oscillated with different periods. Whereas Mpa levels peaked 0, 6, and 24 h following starvation, Dop levels peaked only once, 6 h following starvation, during the 24 h of the experiment. The 20S α was maintained at a stable level at the beginning of starvation and started to oscillate only 6 h later. 20S α levels then initially decreased for 12 h, at which point they again increased throughout the rest of the experiment. The finding that the 20S α oscillates indicates that this protein, like other PPS components, is proteolyzed *in vivo*. In an agreement, 20S subunits were previously identified in the *M. smegmatis* pupylome (Poulsen *et al*, 2010), and we could show in an *in vitro* pupylation assay similar to the one performed for PafA that the 20S is indeed a pupylation substrate (Fig 7B and C, lower panels). Despite the oscillatory expression of PPS components, no dramatic

oscillations in the levels of pupylated proteins were detected (Fig 7E).

Given our ability to detect oscillations of PPS components following nitrogen starvation, it follows that starved cells should be synchronized as otherwise oscillations would not be observable, considering that our measurements detect the average level of each examined component. Yet, a week following starvation, oscillations of PPS components were much less obvious over the course of the next 24 h (Supplementary Fig S5B). Presumably, cells in the starved cultures gradually desynchronize from the moment of starvation and, concomitantly, synchronization.

Finally, we wondered whether the oscillatory expression of PPS components is triggered by nitrogen starvation or whether oscillations occur constantly yet were only detected in our experiments after cell synchronization. To differentiate between these two possibilities, we starved cells for nitrogen for an hour and then replenished culture nitrogen sources. After an hour of starvation, the cells should be synchronized, as in the absence of any source of nitrogen, Mpa oscillations were already detected (Fig 7E). By contrast, following addition of a nitrogen source, the levels of both Mpa and the 20S α , those two proteins for which oscillations were most obvious in our experiments, appeared stable over the course of the subsequent 24 h from the moment of nitrogen re-supplementation (Fig 7F). These findings thus indicate that nitrogen starvation induces oscillatory expression of Mpa, Dop, and the 20S particle.

Discussion

The work presented here provides the first link between the PPS and bacterial physiology. It also provides the first evidence for a PPS response to external stimuli. The data indicate that the PPS plays a crucial role in *M. smegmatis* physiology under starvation. This conclusion is supported both by phenotypic analysis of a PPS-deficient mutant and by analysis of the PPS response to nutrient limitation. We find the phenotype of a PPS-deficient mutant, the broad substrate specificity, and the delicate regulation of the PPS under nitrogen limitation to be consistent with the model illustrated in Fig 8. This model proposes that the PPS acts as an amino acid recycling machinery for the generation of protein building blocks and a carbon source under conditions in which *de novo* synthesis of amino acids is restricted. Amino acid recycling under starvation is not a novel concept (Lilly *et al*, 1991; Vabulas & Hartl, 2005; Suraweera *et al*, 2012). Up until now, however, it was not conceived as a role played necessarily by the PPS.

An amino acid recycling machinery

The response of *M. smegmatis* to nitrogen limitation was recently characterized at the genomic and transcriptomic levels. Such analyses found that, among mycobacterial species examined thus far, *M. smegmatis* carries the highest number of genes involved in nitrogen metabolism (Amon *et al*, 2009; Jenkins *et al*, 2013; Williams *et al*, 2014). As such, *M. smegmatis* is well equipped to withstand nitrogen stress. As the nitrogen source becomes a growth-limiting factor for *M. smegmatis*, a reduction in metabolic activity is accompanied by a global adaptation at the level of gene expression. More than 100 genes responsible for nitrogen scavenging, accelerated

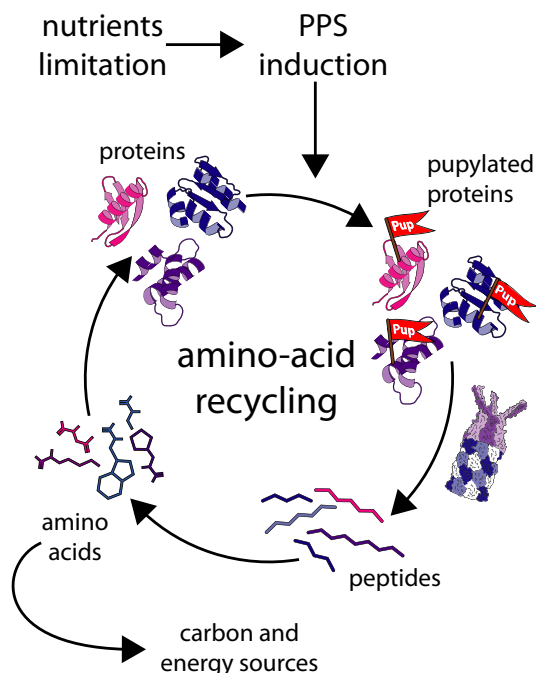


Figure 8. Protein recycling by the PPS in response to nutrient starvation.

In response to nutrient limitation, PPS induction leads to accelerated protein pupylation and degradation. The resulting protein building blocks can be used to remodel the proteome in response to changing environmental conditions.

nitrogen transport into the cell, and efficient assimilation of various nitrogen sources are induced in the first few hours of nitrogen limitation (Jenkins *et al*, 2013; Williams *et al*, 2013). However, these mechanisms are only efficient up to a certain point. Below a minimum of extracellular nitrogen concentration, *M. smegmatis* must rely on internal nitrogen sources to maintain minimal metabolic activity. We propose that the PPS answers this requirement. By degrading proteins, the PPS generates protein building blocks that cannot support an increase in biomass, yet allow adaptation to environmental changes via the *ad hoc* synthesis of proteins as required by the organism in light of these changes. For example, as a saprophytic soil bacterium, *M. smegmatis* experiences periodic changes, such as the temperature fluctuations that accompany day/night cycles, in its natural habitat. Adaptation to such fluctuations often requires flexibility at the proteome level. The PPS could provide *M. smegmatis* the flexibility to remodel its proteome even in the absence of an external nitrogen source. In view of the role suggested here for the PPS, the extreme broad specificity of PafA makes perfect sense. Indeed, if the PPS functions primarily to generate protein building blocks, substrate specificity is of little importance. We find that Pup expression in a $\Delta prcSBA$ mutant partially complements the survival deficiencies of this mutant under conditions of nitrogen starvation. Does this mean that pupylation alone plays an important role in starved *M. smegmatis* cells or that pupylated proteins are degraded to some extent even in the absence of the 20S? While not excluding the former possibility, our data reinforce the latter, as we observe modest disappearance of pupylated proteins in 20S-deficient cells (Fig 6B). This disappearance could be attributed to depupylation, rather than degradation, of the pupylated proteins. However,

PafA, Mpa, and Dop were found to be degraded in these cells with the same timing as the disappearance of pupylated proteins in 20S-deficient cells, the former events albeit occurring much less efficiently than in wild-type cells. As PafA, Mpa, and apparently Dop are pupylation substrates, their slow degradation, together with the simultaneous slow disappearance of other pupylated proteins in the absence of the 20S particle, suggests that pupylated proteins can be degraded, inefficiently as it may be, by an alternative route. This possibility indeed explains why expression of Pup alone can partially complement the nitrogen sensitivity of the $\Delta prcSBA$ mutant. It is noteworthy that alternative degradation routes were reported in parallel proteolytic systems (Kanemori *et al*, 1997; Lies & Maurizi, 2008). For instance, SsrA-tagged proteins are degraded in *Escherichia coli* primarily by ClpXP, yet can also be degraded less efficiently by other cytoplasmic proteases (Lies & Maurizi, 2008).

PPS dynamics and negative auto-regulation

Due to their destructive nature, intracellular proteolytic systems are carefully regulated so as to prevent unnecessary degradation of cellular proteins (Gur *et al*, 2011). Our data indicate that this principle also applies to the PPS. During exponential growth, both pupylation and proteasomal degradation are not induced, resulting in slow pupylation and degradation rates, respectively. In response to nitrogen starvation, PPS activity is induced, leading to accelerated pupylation and degradation of pupylated proteins. Intriguingly, whereas pupylated proteins accumulated during the early stages of stress, these proteins almost completely disappeared at later stages following starvation. These findings indicate that during the early stages of starvation, pupylation dominates over degradation, as if the cell “prepares the setting” for degradation (together with inducing nitrogen-scavenging mechanisms), while accelerating the irreversible destructive step of proteasome-mediated degradation only later during starvation, as a last resort.

The proteasome degrades Mpa, PafA, and Dop, and seemingly also the 20S α subunit, thereby reducing the rates of both protein pupylation and degradation. In other words, the PPS negatively auto-regulates its own activity via self-degradation of its components. As a result, a negative feedback loop is formed in which accelerated pupylation facilitates degradation of PPS components, which, in turn, reduces the pupylation rate and, concomitantly, proteolysis of PPS components (Fig 9A and B). As such, this negative feedback loop prevents uncontrolled pupylation and degradation of cellular proteins. Such negative auto-regulation, rather than ensuring stable steady-state levels of the PPS components, promotes the oscillatory expression of these proteins in response to nitrogen starvation. Indeed, negative feedback loops are often essential components of oscillatory systems, alongside proper time-scale balancing, as dictated by the kinetics of those enzymes involved and their rates of synthesis (Novák & Tyson, 2008). It appears that these kinetic requirements are satisfied in the case of the PPS following nitrogen starvation, when both pupylation and proteasomal degradation are accelerated. At present, however, in-depth understanding of the factors that promote PPS oscillations is limited by the availability of information regarding the regulatory mechanisms that control PPS gene expression and challenges to describing the kinetics of PPS activity *in vivo*. Despite the oscillatory expression of

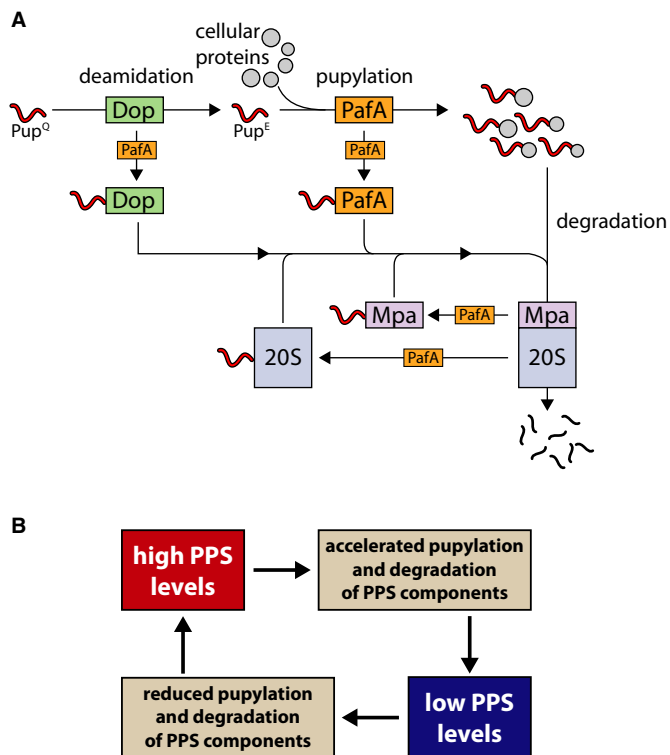


Figure 9. PPS auto-regulation through negative feedback loops.

- A Like many other cellular proteins, PPS components are subjected to pupylation and proteasomal degradation. For simplicity, depupylation is ignored in the illustration presented.
- B As a result of the interactions illustrated in (A), a negative feedback loop is formed that prevents uncontrolled tagging and degradation of cellular proteins.

PPS components, modest fluctuations in the levels of pupylated proteins were observed. This outcome reflects how the levels of pupylated proteins are determined by three parallel processes at every time point considered (i.e., pupylation, depupylation, and degradation). As such, an increased pupylation rate, for instance, would not lead to an increase in pupylated protein levels if balanced by a similar increase in the rate of degradation.

What purpose could such oscillations of PPS components serve? One reasonable answer is the fine-tuning of amino acid recycling so as to balance nutritional requirements under starvation with the need to prevent unnecessary proteolysis. By acting in pulses, the PPS generates a transient pool of amino acids that allows the cell to reassess its nutritional needs before another cycle of degradation is activated. By analogy, the PPS can be considered to act as an oscillating thermostat that avoids over-heating the system via controlled release of short pulses of heat, all the while measuring the temperature. Still, how nitrogen starvation is sensed by the PPS remains an open question.

General implications

The PPS is conserved in Actinobacteria and Nitrospira, two bacterial phyla that together comprise thousands of species. As many of these species are, like *M. smegmatis*, saprophytic soil microorganisms

encountering fluctuating environmental conditions, it is highly likely that protein recycling under starvation conditions is a general role of the PPS. Still, there exist actinobacterial species, such as *Corynebacterium glutamicum*, that encode an incomplete PPS in their genomes. Specifically, they lack the 20S-coding genes. In these species, protein pupylation may thus play a regulatory role, rather than functioning as a protein recycling machinery. Alternatively, pupylated proteins may be degraded by a non-proteasomal protease in these species. Finally, it remains to be determined to what extent our model applies to parasitic mycobacteria, such as *M. tuberculosis*. Undoubtedly, such species encounter conditions and stresses that differ markedly from those encountered by saprophytic species. Accordingly, the PPS in such bacteria may have evolved to respond to the unique conditions encountered in their hosts. It appears, however, that a necessity for the PPS under starvation conditions is conserved between *M. smegmatis* and *M. tuberculosis*. Indeed, a proteasome-deficient *M. tuberculosis* mutant presents compromised survival at stationary phase and reduced persistence in host tissues (Gandotra *et al*, 2010). These observations, therefore, suggest that our findings represent a general property of the PPS that applies both to saprophytic species and to mycobacterial pathogens, such as *M. tuberculosis*. As a rule, bacterial pathogens must acquire nutrients within their hosts in order to persist and replicate (Appelberg, 2006; Rohmer *et al*, 2011). Here, an enthralling comparison can be made between *M. tuberculosis* and *Legionella pneumophila* (both are intracellular pathogens). Whereas the latter depends on host proteasome-mediated protein degradation to replenish its nitrogen and carbon supply (Price *et al*, 2011), *M. tuberculosis* relies on its own proteasome during infection, probably for the same reason.

Materials and Methods

Strains, plasmids, and growth conditions

Unless stated otherwise, *M. smegmatis* MC²155 (wild-type and mutant) cultures were grown at 30°C in Middlebrook 7H9 broth containing 0.05% (v/v) Tween-80 and 0.4% (v/v) glycerol. For nitrogen and carbon starvation experiments, cultures were grown in defined minimal medium adapted from Davis and Mingioli (1950) containing 40 mM K₂HPO₄, 22 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 1.7 mM sodium citrate, 0.4 mM MgSO₄, 0.4% glycerol (v/v), and 0.05% Tween-80 (v/v). Exponentially growing cultures were harvested, centrifuged, washed thrice, and resuspended in similar media lacking either (NH₄)₂SO₄ (to induce nitrogen starvation) or glycerol (to induce carbon starvation). Solid media were prepared using Middlebrook 7H10 supplemented with 0.4% glycerol. *E. coli* ER2566 (New England Biolabs) was routinely used for all cloning procedures and was grown using typical procedures on LB broth and plates at 37°C. Plasmid pMV306 (Stover, 1991) was used in complementation assays to integrate a cloned *prcSBA* operon into the chromosome of a *M. smegmatis* Δ*prcSBA* mutant. Plasmid pMV206 (Stover, 1991) was used for cloning and expression of PafA. A polyhistidine-strep-pup fusion and *zur* variants were cloned into plasmid pJV53 (Van Kessel *et al*, 2008) instead of genes Che9c 60-61, under the transcriptional control of the acetamidase promoter (Parish *et al*, 1997). Plasmid pET11a was used for cloning and expression of *prcBA*.

Protein purification

For purification of the *M. smegmatis* pupylome, expression of a polyhistidine-strep-pup fusion was induced in *M. smegmatis* cells, in mid-exponential phase at 37°C by addition of 0.2% (w/v) acetamide to the growth medium. The cultures were harvested 3 and 24 h later, and pupylome purification was carried out as previously described (Festa *et al.*, 2010). Cultures that did not express the dually tagged Pup served as controls.

PafA and Pup were purified as previously described (Ofer *et al.*, 2013). For 20S purification, *E. coli* BL21 cells carrying plasmid pET.prcBA were grown in LB medium containing ampicillin (100 µg/ml) at 37°C. At OD₆₀₀ = 0.7, 20S expression was induced for 18 h by the addition of IPTG (0.5 mM). The cells were harvested and resuspended in 25 mM Tris-HCl, pH 8.0, 1 mM DTT (hereafter, Tris-DTT). Following sonication, the lysate was centrifuged (12,000 g, 4°C, 20 min) and the clear supernatant was loaded onto a HiTrap QFF column (GE Healthcare) pre-equilibrated with Tris-DTT buffer. Elution was performed using a 0–1 M linear NaCl gradient. 20S-rich fractions were pooled, concentrated, and loaded on a Superose 6 (GE Healthcare) size-exclusion column, pre-equilibrated with Tris-DTT buffer containing 100 mM NaCl. Following elution, 20S-containing fractions were pooled and loaded onto a Mono Q column (GE Healthcare) pre-equilibrated with Tris-DTT buffer containing 200 mM NaCl and elution was performed using a linear NaCl gradient (200–800 mM). 20S-containing fractions were pooled and loaded on a Superose 6 column, pre-equilibrated with Tris-DTT buffer containing 600 mM NaCl. Following elution, 20S-containing fractions were pooled, and the buffer was exchanged for 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT and 10% glycerol. The purified protein was stored in aliquots at –80°C.

Western blot analysis of *M. smegmatis* lysates

M. smegmatis lysates were prepared by sonication of cell pellets in microcentrifuge tubes containing 0.5 ml of 1 mM Tris-HCl, pH 8.0, 1 mM EDTA. Cell debris was removed by centrifugation (20,000 g, 4°C). After determining protein content in each sample by Bradford assay, equal protein amounts were loaded onto SDS-PAGE for electrophoretic separation, followed by transfer onto PVDF membranes and immuno-detection using standard procedures. As a final step after completion of immunodetection, probed membranes were stained by Coomassie Brilliant Blue to verify the equal loading and transfer of proteins in each lane.

Polyclonal antibodies against Pup, Mpa, Dop, and the 20S core particle were produced by Covance, while anti-PafA antibodies were produced by Adar Biotech. Full-length proteins served as antigens for immunization, except in the case of Dop, where a synthetic peptide was used. All antibodies were affinity purified.

Deletion of the *paf* operon

DNA fragments flanking the *Msm paf* operon were amplified by PCR and cloned into the suicide plasmid, pYUB854 (Bardarov *et al.*, 2002), upstream and downstream of the hygromycin resistance cassette. Wild-type *Msm* cells were then transformed with the cloned plasmid and spread onto plates containing hygromycin (150 µg/ml). To identify *paf* deletion mutants, colonies were doubly

screened by Western blotting using anti-Pup and anti-PafA antibodies. For complementation assays, the *Msm pafA* open reading frame and a region 207 bp upstream of the translation start site were cloned into the mycobacterial shuttle vector, pMV206.

Southern blot analysis

Chromosomal DNA was purified from wild-type and $\Delta pafA$ strains using the Qiagen RNeasy Mini kit. DNA probes were labeled with a PCR DIG Probe Synthesis kit (Roche), and band detection was carried out with an anti-DIG alkaline phosphatase antibody (Roche cat# 1093274) and a CDP-star substrate solution (Roche cat# 1759051). Hygromycin-specific probe preparation was performed with primers 5'-ccctgtactctcgacc-3'/5'-cagcagttccggaaga-3', whereas preparation of the external probe was performed with the primer pairs 5'-cgtcgacgaagtagtgct-3'/5'-tcaacgaactcggctca-3'.

In vitro pupylation

Pupylation assays were carried out at 30°C in a buffer containing 50 mM HEPES, pH 7.5, 100 mM KCl, 20 mM MgCl₂, and 10% glycerol (v/v). Following preparation of reaction mixtures as described in the text, pupylation was initiated by the addition of ATP at a final concentration of 2 mM.

Pupylated protein quantification

The *M. smegmatis* lysates to be examined were prepared at a total protein concentration of 1 µg/ml. As a blank, lysates from a pupylation-deficient mutant were similarly prepared, whereas a purified pupylome, diluted to a final concentration of 0.1 µg/ml and added to Pup-lacking lysates at a total protein concentration of 1 µg/ml, provided standards. Serial dilutions were prepared from each sample, and dot blot analysis was carried out using anti-Pup antibodies (Supplementary Fig S1A). Dot intensities were measured using ImageJ software (NIH), and a standard curve was constructed to calculate the concentration of pupylated proteins in each sample.

LC/MS analysis

MS analysis was performed using an Eksigent nano-HPLC connected to the LTQ Orbitrap XL (Thermo Fisher Scientific). Reverse-phase chromatography of peptides was performed using an homemade C-18 column (15 cm long, 75 µm ID) packed with Jupiter C18, 300 Å, 5 µm beads (Phenomenex). Peptides were separated by a 70-min linear gradient, starting with 100% buffer A (5% acetonitrile, 0.1% formic acid) and ending with 80% buffer B (80% acetonitrile, 0.1% formic acid), at a flow rate of 300 nl/min. A full scan, acquired at 60,000 resolution, was followed by CID MS/MS analysis performed for the five most abundant peaks, in the data-dependent mode. Fragmentation (with minimum signal trigger threshold set at 1,000) and detection of fragments were carried out in the linear ion trap. Maximum ion fill time settings were 300 ms for the high-resolution full scan in the Orbitrap analyzer and 100 ms for MS/MS analysis in the ion trap. The AGC settings were 5'10⁵ and 1'10⁴ for Orbitrap and linear ion trap analyzers, respectively.

Bioinformatics

Following acquisition of MS data, proteins were identified on the basis of their precursor mass and the sequence information included in their fragmentation spectra, by using the Proteome Discoverer 1.4 software package (Thermo Fisher Scientific). The acquired spectra were searched against NCBI-derived *M. smegmatis* protein database, using the SEQUEST search engine. The following search parameters were used: enzyme specificity is trypsin; maximum two missed cleavage sites; maximum 10 ppm or 0.8 Da error tolerance for the full scan and MS/MS analysis, respectively; static modification of cysteine carbamidomethylation; and dynamic modification of methionine oxidation and lysine pup-modification (GGE). Threshold criteria for protein identification were defined as having at least two peptides of high confidence as calculated by the SEQUEST algorithm and a false discovery rate (FDR) *P*-value < 0.01 acquired by the Percolator node. The reverse *M. smegmatis* database was chosen as a target decoy. The biological function of each identified protein was extracted from the UniProt-SwissProt protein database.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

YE performed most of the experiments described, analyzed the data, and helped writing the manuscript. ZR performed mass spectrometry analysis and helped writing the manuscript. IH constructed the *pafA* deletion strain and analyzed pupylation in stationary-phase *M. smegmatis* cells. AM analyzed pupylation in stationary-phase *M. smegmatis* cells. GP analyzed the cross-reactivity of anti-Pup antibodies. YS constructed the *prcSBA* deletion strain and performed experiments. MV helped to construct deletion strains and purified proteins. IK performed mass spectrometry analysis, and EG performed experiments, analyzed the data, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Amon J, Titgemeyer F, Burkovski A (2009) A genomic view on nitrogen metabolism and nitrogen control in mycobacteria. *J Mol Microbiol Biotechnol* 17: 20–29
- Appelberg R (2006) Macrophage nutritive antimicrobial mechanisms. *J Leukoc Biol* 79: 1117–1128
- Bardarov S, Bardarov S Jr, Pavelka MS Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, Jacobs WR Jr (2002) Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology* 148: 3007–3017
- Blackman FF (1905) Optima and limiting factors. *Ann Bot* 19: 282–295
- Burns KE, Liu WT, Boshoff HI, Dorrestein PC, Barry CE III (2009) Proteasomal protein degradation in Mycobacteria is dependent upon a prokaryotic ubiquitin-like protein. *J Biol Chem* 284: 3069–3075
- Burns KE, Pearce MJ, Darwin KH (2010a) Prokaryotic ubiquitin-like protein provides a two-part degron to *Mycobacterium* proteasome substrates. *J Bacteriol* 192: 2933–2935
- Burns KE, Cerda-Maira FA, Wang T, Li H, Bishai WR, Darwin KH (2010b) “Depupylation” of prokaryotic ubiquitin-like protein from mycobacterial proteasome substrates. *Mol Cell* 39: 821–827
- Cerda-Maira FA, Pearce MJ, Fuortes M, Bishai WR, Hubbard SR, Darwin KH (2010) Molecular analysis of the prokaryotic ubiquitin-like protein (Pup) conjugation pathway in *Mycobacterium tuberculosis*. *Mol Microbiol* 77: 1123–1135
- Cerda-Maira FA, McAllister F, Bode NJ, Burns KE, Gygi SP, Darwin KH (2011) Reconstitution of the *Mycobacterium tuberculosis* pupylation pathway in *Escherichia coli*. *EMBO Rep* 12: 863–870
- Chen X, Solomon WC, Kang Y, Cerda-Maira F, Darwin KH, Walters KJ (2009) Prokaryotic ubiquitin-like protein pup is intrinsically disordered. *J Mol Biol* 392: 208–217
- Darwin KH, Ehart S, Gutierrez-Ramos JC, Weich N, Nathan CF (2003) The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. *Science* 302: 1963–1966
- Darwin KH, Lin G, Chen Z, Li H, Nathan CF (2005) Characterization of a *Mycobacterium tuberculosis* proteasomal ATPase homologue. *Mol Microbiol* 55: 561–571
- Darwin KH (2009) Prokaryotic ubiquitin-like protein (Pup) proteasomes and pathogenesis. *Nat Rev Microbiol* 7: 485–491
- Davis BD, Mingioli ES (1950) Mutants of *Escherichia coli* requiring methionine or vitamin B12. *J Bacteriol* 60: 17–28
- De Mot R (2007) Actinomycete-like proteasomes in a Gram-negative bacterium. *Trends Microbiol* 15: 335–338
- Delley CL, Striebel F, Heydenreich FM, Özcelik D, Weber-Ban E (2012) Activity of the mycobacterial proteasomal ATPase Mpa is reversibly regulated by pupylation. *J Biol Chem* 287: 7907–7914
- Festa RA, McAllister F, Pearce MJ, Mintseris J, Burns KE, Gygi SP, Darwin KH (2010) Prokaryotic ubiquitin-like protein (Pup) proteome of *Mycobacterium tuberculosis*. *PLoS ONE* 5: e8589
- Forer N, Korman M, Elharar Y, Vishkautzan M, Gur E (2013) Bacterial proteasome and PafA, the Pup ligase, interact to form a modular protein tagging and degradation machine. *Biochemistry* 52: 9029–9035
- Gandotra S, Lebron MB, Ehart S (2010) The *Mycobacterium tuberculosis* proteasome active site threonine is essential for persistence yet dispensable for replication and resistance to nitric oxide. *PLoS Pathog* 6: e1001040
- Gur E, Biran D, Ron EZ (2011) Regulated proteolysis in Gram-negative bacteria-how and when? *Nat Rev Microbiol* 9: 839–848
- Guth E, Thommen M, Weber-Ban E (2011) Mycobacterial ubiquitin-like protein ligase PafA follows a two-step reaction pathway with a phosphorylated pup intermediate. *J Biol Chem* 286: 4412–4419
- Hu G, Lin G, Wang M, Dick L, Xu RM, Nathan C, Li H (2006) Structure of the *Mycobacterium tuberculosis* proteasome mechanism of inhibition by a peptidyl boronate. *Mol Microbiol* 59: 1417–1428
- Imkamp F, Rosenberger T, Striebel F, Keller PM, Amstutz B, Ser P, Weber-Ban E (2010a) Deletion of *dop* in *Mycobacterium smegmatis* abolishes pupylation of protein substrates *in vivo*. *Mol Microbiol* 75: 744–754

- Imkamp F, Strieler F, Sutter M, Ozcelik D, Zimmermann N, Ser P, Weber-Ban E (2010b) Dop functions as a depupylase in the prokaryotic ubiquitin-like modification pathway. *EMBO Rep* 11: 791–797
- Jenkins VA, Barton GR, Robertson BD, Williams KJ (2013) Genome wide analysis of the complete GlnR nitrogen-response regulon in *Mycobacterium smegmatis*. *BMC Genomics* 14: 301
- Kanemori M, Nishihara K, Yanagi H, Yura T (1997) Synergistic roles of HslIV and other ATP-dependent proteases in controlling *in vivo* turnover of sigma32 and abnormal proteins in *Escherichia coli*. *J Bacteriol* 179: 7219–7225
- Küberl A, Fränzel B, Eggeling L, Polen T, Wolters DA, Bott M (2014) Pupylated proteins in *Corynebacterium glutamicum* revealed by MudPIT analysis. *Proteomics* 14: 1531–1542
- Liao S, Shang Q, Zhang X, Zhang J, Xu C, Tu X (2009) Pup a prokaryotic ubiquitin-like protein is an intrinsically disordered protein. *Biochem J* 422: 207–215
- Lies M, Maurizi MR (2008) Turnover of endogenous SsrA-tagged proteins mediated by ATP-dependent proteases in *Escherichia coli*. *J Biol Chem* 283: 22918–22929
- Lilly WW, Wallweber GJ, Higgins SM (1991) Proteolysis and amino acid recycling during nitrogen deprivation in *Schizophyllum commune*. *Curr Microbiol* 23: 27–32
- Lupas A, Zühl F, Tamura T, Wolf S, Nagy I, De Mot R, Baumeister W (1997) Eubacterial proteasomes. *Mol Biol Rep* 24: 125–131
- Novák B, Tyson JJ (2008) Design principles of biochemical oscillators. *Nat Rev Mol Cell Biol* 9: 981–991
- Ofner N, Forer N, Korman M, Vishkautzan M, Khalaila I, Gur E (2013) Allosteric transitions direct protein tagging by PafA the prokaryotic ubiquitin-like protein (Pup) ligase. *J Biol Chem* 288: 11287–11293
- Parish T, Mahenthalingam E, Draper P, Davis EO, Colston MJ (1997) Regulation of the inducible acetamidase gene of *Mycobacterium smegmatis*. *Microbiology* 143: 2267–2276
- Pearce MJ, Arora P, Festa RA, Butler-Wu SM, Gokhale RS, Darwin KH (2006) Identification of substrates of the *Mycobacterium tuberculosis* proteasome. *EMBO J* 25: 5423–5432
- Pearce MJ, Mintseris J, Ferreyra J, Gygi SP, Darwin KH (2008) Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis*. *Science* 322: 1104–1107.
- Poulsen C, Akhter Y, Jeon AH, Schmitt-Ulms G, Meyer HE, Stefanski A, Stühler K, Wilmanns M, Song YH (2010) Proteome-wide identification of mycobacterial pupylation targets. *Mol Syst Biol* 6: 386
- Price CT, Al-Quadan T, Santic M, Rosenshine I, Abu Kwaik Y (2011) Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. *Science* 334: 1553–1557
- Rohmer L, Hocquet D, Miller SI (2011) Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. *Trends Microbiol* 19: 341–348
- Schmidt M1, Finley D (2014) Regulation of proteasome activity in health and disease. *Biochim Biophys Acta* 1843: 13–25
- Shenkerman Y, Elharar Y, Vishkautzan M, Gur E (2013) Efficient and simple generation of unmarked gene deletions in *Mycobacterium smegmatis*. *Gene* 533: 374–378
- Stover CK, et al (1991) New use of BCG for recombinant vaccines. *Nature* 351: 456–460
- Strieler F, Imkamp F, Sutter M, Steiner M, Mamedov A, Weber-Ban E (2009) Bacterial ubiquitin-like modifier Pup is deamidated and conjugated to substrates by distinct but homologous enzymes. *Nat Struct Mol Biol* 16: 647–651
- Strieler F, Hunkeler M, Summer H, Weber-Ban E (2010) The mycobacterial Mpa-proteasome unfolds and degrades pupylated substrates by engaging Pup's N-terminus. *EMBO J* 29: 1262–1271
- Suraweera A, Münch C, Hanssum A, Bertolotti A (2012) Failure of amino acid homeostasis causes cell death following proteasome inhibition. *Mol Cell* 48: 242–253
- Sutter M, Strieler F, Damberger FF, Allain FH, Weber-Ban E (2009) A distinct structural region of the prokaryotic ubiquitin-like protein (Pup) is recognized by the N-terminal domain of the proteasomal ATPase Mpa. *FEBS Lett* 583: 3151–3157
- Tamura T, Nagy I, Lupas A, Lottspeich F, Cejka Z, Schoofs G, Tanaka K, De Mot R, Baumeister W (1995) The first characterization of a eubacterial proteasome: the 20S complex of *Rhodococcus*. *Curr Biol* 5: 766–774
- Vabulas RM, Hartl FU (2005) Protein synthesis upon acute nutrient restriction relies on proteasome function. *Science* 310: 1960–1963
- Valas RE, Bourne PE (2008) Rethinking proteasome evolution: two novel bacterial proteasomes. *J Mol Evol* 66: 494–504
- Van Kessel JC, Marinelli LJ, Hatfull GF (2008) Recombineering mycobacteria and their phages. *Nat Rev Microbiol* 6: 851–857
- Wang T, Darwin KH, Li H (2010) Binding-induced folding of prokaryotic ubiquitin-like protein on the *Mycobacterium* proteasomal ATPase targets substrates for degradation. *Nat Struct Mol Biol* 7: 1352–1357
- Watrous J, Burns K, Liu WT, Patel A, Hook V, Bafna V, Barry CE III, Bark S, Dorrestein PC (2010) Expansion of the mycobacterial “PUPylome”. *Mol Biosyst* 6: 376–385
- Williams KJ, Bryant WA, Jenkins VA, Barton GR, Witney AA, Pinney JW, Robertson BD (2013) Deciphering the response of *Mycobacterium smegmatis* to nitrogen stress using bipartite active modules. *BMC Genomics* 14: 436
- Wolf S, Nagy I, Lupas A, Pfeifer G, Cejka Z, Müller SA, Engel A, De Mot R, Baumeister W (1998) Characterization of ARC a divergent member of the AAA ATPase family from *Rhodococcus erythropolis*. *J Mol Biol* 277: 13–25