THE EMBO JOURNAL

Manuscript EMBO-2013-87076

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

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Review timeline:

Submission date:	04 October 2013
Editorial Decision:	29 October 2013
Revision received:	27 April 2014
Editorial Decision:	22 May 2014
Revision received:	29 May 2014
Accepted:	04 June 2014

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 October 2013

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the slight delay in getting back to you, but I am currently away from the office. I have now received the comments from the referees and they are provided below.

As you can see below the referees find the analysis interesting. However, significant concerns are also raised that would have to be worked out in order to consider publication here. I will not repeat them all here as they are clearly indicated below. A major issue is to provide better support for that the pup-proteasome system promotes survival of M. smegmatis via amino acid recycling. Should you be able to address the concerns raised then we could offer to consider a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the concerns raised at this stage. I can extend the revision time up to 6 months if needed.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

Elharar and colleagues investigated the role of the pup/proteasome system (PPS) during nutrient starvation in M. smegmatis. The authors show that a mutant deficient in PPS displayed a survival defect in stationary phase and during carbon or nitrogen starvation. Following nitrogen starvation, the amount of pupylated proteins increased rapidly but then decreased again, which the authors interpret as evidence that their degradation results in the generation of amino acids that can serve as carbon and nitrogen sources. Concomitantly, the amount of the proteins of the pupylation system (PafA, Dop, Mpa) decreased, which the authors take as evidence for autoregulation.

This manuscript addresses an interesting topic and the authors provide good evidence that the PPS is important for survival of M. smegmatis in nutrient limited conditions. However, the presented data are not sufficient to support the authors' conclusions that (i) the mechanism by which PPS supports survival of M. smegmatis involves amino acid recycling for use as carbon or nitrogen sources and (ii) the PPS autoregulates its activity via degradation of its components. In addition, they fail to discuss previous literature, which demonstrated that the proteasome system plays a crucial role in cell survival through amino acid recycling in yeast and mammalian cells.

There is no clear demonstration that the PPS is used as recycling machinery in M. smegmatis by degrading pupylated proteins for use as carbon and energy sources. The authors only provide indirect evidence, i.e. pupylated proteins accumulate and then disappear which is associated with a PPS mutant suffering from a survival defect during nutrient starvation. Experiments that link these two observations mechanistically are missing.

This manuscript could be strengthened by experiments that investigate whether degradation of pupylated proteins is required for survival during starvation. The authors could use a prcBA mutant or the prcSAB mutant complemented with prcS or proteasome inhibitors to determine whether proteasome-mediated protein degradation is required during nutrient starvation or if pupylation itself is sufficient.

The authors propose that the PPS is negatively autoregulated during nitrogen starvation. However, they do not show which impact PPS autoregulation has following prolonged nitrogen starvation. If protein recycling via the PPS is necessary for survival, it would be informative to determine the level of pupylated proteins at later time points (beyond 11 days). Is the PPS functioning in cycles (induction/degradation) during starvation to maintain a minimal amount of available carbon/nitrogen, yet avoiding uncontrolled degradation? Experiments in Fig6A/6C show that almost 100% of the pupylated proteins, which accumulated in response to starvation, disappeared after 4 to 6 days of starvation, which correlates with the time at which the bacteria lacking the PPS die. Does this suggest that pupylated proteins are used for the transition from rich to limited nutrient conditions rather than during starvation itself? It would be interesting to determine if the level of pupylated proteins increases again at later time points-as the bacteria continue to survive (but not replicate) upon prolonged nitrogen limitation (Fig5A).

The authors point out "up until now, however, amino acid recycling was not conceived as a role played (so necessarily) by the PPS". While this is true for mycobacteria, there is ample evidence in other systems, such as yeast and mammalian cells, that amino acid recycling via the proteasome is essential for cell survival during conditions of nutrient starvations (for example see: Vabulas and Hartl, Science 2005, 310, 1960-3; Sugaweera et al. Molecular Cell 2012, 48, 1-12). The authors should mention/discuss these reports.
Similarly, the sentence "PPS-deficient mutants of M. tuberculosis and M. smegmatis multiply as fast as do the parental wild type strains (line 25-26) is incorrect. Gandotra et al. demonstrated a replication defect in liquid culture and on agar plates of M. tuberculosis lacking the proteasome (Gandotra et al. PLOS Pathogens 2010).

Specific comments

- In Fig2B levels of pupylated proteins and expression of PafA along the growth curve of M.

smegmatis are determined by Western blot; a loading control that ensures equal amounts of proteins have been run on the SDS PAGE is absent. This is true for all Western blots in the manuscript and must be corrected.
- Pupylome analysis: the authors identified 88 and 235 proteins that were pupylated in exponential and stationary phase, respectively. They did not mention any quantification of the pupylated proteins in the specific conditions they used for pupylome purification. Did they compare the amount of pupylated proteins?
Only 21 proteins are common between the two time points, although PafA does not seem to show any substrate specificity. Does this mean that pupylation is so random that each pupylome analysis will give different results (especially in exponential phase, when a small amount of proteins are pupylated)?
- In Fig3D the authors used E. coli to show that the abundance of pupylated proteins of high molecular weight was due to more efficient pupylation of these proteins rather than more active degradation of the small ones. This analysis might be confounded by the use of a heterologous system. The authors could instead use the M. smegmatis prcSAB mutant complemented with prcS (that can pupylate but not degrade the pupylated proteins).
- Fig4B: the authors should comment on the pupylation bands present in the prcSBA mutant lane.
- Fig5A: CFUs increased by 2 orders of magnitude between days 0 and 4 of carbon limitation, but the turbidity of these cultures decreased. Can the authors please comment on how to explain this discrepancy?
- Fig5D: The levels of PPS proteins during exponential growth should be determined and compared to protein levels during nutrient limitation. This would distinguish between an increase of pupylated proteins in nitrogen starvation and a decrease in carbon starvation. A loading control is essential.
- The authors propose that "during exponential growth, expression of both PafA and 20S is not induced (to justify this conclusion a loading control must be included in Figure 6A), resulting in slow pupylation and degradation rates, respectively. However they have not compared levels of pupylated in exponential growth to levels during nitrogen starvation (Fig6C). They should determine the amounts of pupylated proteins in the prcSBA mutant complemented with only prcS in exponential phase. This would answer the question whether there is no protein pupylation/degradation, or inefficient use of the PPS in exponential/rich medium growth.
- In Figs 6A and 6D, Mpa, PafA and Dop amounts are analyzed during nitrogen starvation. These proteins are not detectable in the complemented prcSBA mutant on day 4, but detectable up to day 11 in wild type M. smegmatis. Can the authors comment on this difference?
- The authors discuss PafA and Dop as having opposite functions (page 9, line 19), i.e. PafA is required for pupylation, and Dop for de-pupylation. However, Dop is also required for pupylation (conversion of Pup glutamine into glutamate). This needs to be clarified. In addition, the significance of a 2 fold difference in cytoplasmic concentrations of Dop and PafA (Fig. 6B, and text page 9, line 21-13) is unclear.
- Some figures numbers are miss-annotated in the text, for example page 6, line 13, Fig 2B should be Fig 3B.
- Several Figures are redundant and should be removed: Fig. 4D shows the same data as Fig 4E; Fig 5B and 5C are both redundant with Fig. 5A.

Referee #2

The manuscript by Elharara et al describes the pup-proteasome system (PPS) system in the saprophytic non-pathogenic bacteria Mycobacterium smegmatis. They show for the first time that this primitive protein degradation machinery is involved in amino acids recycling during starvation through degradation of high molecular mass proteins by the proteasome system. They demonstrate the PPS system is essential for survival under nitrogen limitation but whether the amino acids are the factors involved in not conclusively provided, yet. The work is novel and advances our knowledge of this primitive ubiquitin-proteasome system in various bacterial species. While the data are appealing, there are few issues that need to be addressed prior to publication to strengthen the conclusions.

1. Previous studies on eukaryotic cells have shown that proteasome inhibition leads to cell death due to the limited amino acids generated, and that cell death in blocked upon supplementation of amino acids (Suraweera et al, Mol. Cell, 2012, 48:242-243. Studies on Legionella, cited by the authors, have shown that the requirement of host proteasomal degradation for growth of the bacteria in the

host cells is bypassed by a supplement of amino acids. Therefore, it would be prudent on the authors to show that amino acids are the direct factor involved in the PPS system under nitrogen starvation, and would mimic genetic complementation of the PPS mutant. The authors should supplement amino acids for all the data shown in the figures to show reversibility and confirm the role of amino acids as the main factor involved. This control will enhance the studies and substantiate the conclusions dramatically.
2. A pafA mutant need to be included as a control in various studies, but in particular Fig. 1 and 2.
3. Fig. 2 needs a constitutively expressed protein as a control.
4. A discussion of the evolutionary biology of this primitive system and its role in bacterial physiology need to be contrasted to the studies in eukaryotic cells (Suraweera et al, Mol. Cell, 2012, 48:242-243) and the role of the host proteasome in Legionella growth in host cells.
5. Validation of some of the studies inn M. tuberculosis would greatly enhance the significance of the findings to infectious diseases.

Referee #3

General summary and opinion about the principle significance of the study, its questions and findings:

Overall, the manuscript provides a new model as to why the Pup-proteasome system is needed for full virulence and persistence of Mycobacterium in its infected host. The authors provide evidence that suggests the Pup-proteasome system is important for amino acid recycling during conditions of nutrient limitation.

Specific major concerns essential to be addressed to support the conclusions:

The discussion of how the pupylome is biased toward proteins of high molecular weight is not convincing. Please provide the reader with enough controls, experimental details and statistical analyses to verify that the mass spectrometry and immunoblot approaches were not biased toward large proteins. Assume more than one peptide hit was required for positive identification, thus, suggesting small proteins may not be as readily detected with high probability compared to large proteins. If missed trypsin cleavage is prevalent , this would add further bias to large vs, small proteins. In addition, small proteins may be more difficult to detect after separation by SDS-PAGE and Western Blot than large proteins. For example, Millipore Immobilin P membranes are only recommended for proteins greater than 20 kDa. Serial dilution of internal controls (e.g., purified pupylated proteins of known quantity that are large vs. small) on the Western blot would strengthen the conclusions. Note that the diversion to this discussion seems somewhat distracting from the theme of the manuscript and is not well integrated. The authors state that the average molecular weight of the pupylome was calculated to be 52 vs. 34 kDa for the complete proteome. Was the complete proteome determined by MS or is it theoretical based on deduced proteome? If an MS-based dataset was used, the argument becomes more convincing and needs to be clearly explained with appropriate citation of the shotgun proteome dataset used for calculation. If not, one could argue that the large protein bias is in the sampling technique and is not biological.

Important mass spectrometry details are missing from this document. Only the gene number, accession number, description, gene name, gene locus tag number and molecular mass of the protein deduced from the genome sequence are reported for the pupylomes. MS probability scores, % coverage, peptide sequences detected with a quantitative comparison of the theoretical and actual observed masses, etc. are not in the supplemental data. Please report false discovery rate (FDR), type of target decoy and other important details of MS-based analysis within the methods. What was the percentage of pupylome with lysine modification sites identified vs. not identified? How much of the pupylome detected by mass spectrometry is not actually pupylated but instead simply associated with the pupylated proteins?

How do the authors know that the disappearance of the pupylated proteins by immunoblot is due to proteasome-dependent degradation of the pupylome and not simply depupylation of the protein target? Pup is the direct signal detected by this approach (and the MS analysis of Pup pull-downs) and not the protein target. Pup is a small protein that is likely difficult to detect at low levels via immunoblot in its free form vs. its protein conjugated form due to the problems associated with

immunoblot of small proteins. While the model developed by the authors seems logical based on a long history of our understanding of amino acid recycling during starvation for all types of cells (from bacteria to humans), the results of this report do not directly demonstrate the stated conclusions. One could counter with an alternative model that the pupylome is depupylated by Dop during long term stationary phase and that 20S proteasomes are needed for this Dop activity (e.g. degradation of an inhibitor of Dop) which appears to occur around day 2-3 after starvation when Dop levels are still detected.

Minor concerns that should be addressed:

p3 ln 2-3: statement implies that functional proteasomes that degrade pupylated proteins are demonstrated in Nitrospira, yet citations do not reflect this assumption

P3 ln 6: The claim that only single pup molecules are conjugated to substrates is not properly cited and appears based on absence of data. MtPup has 3 internal lysine residues and an N-terminal primary amine group that have the potential for pupylation.

P4 ln 25: under standard laboratory conditions? Please define what you mean within the text.

P5 ln 23: please clarify whether this strain produces native levels of Pup or whether this protein modifier is at higher levels than wild type. If at higher levels, please clarify how the pupylome identified by this approach compares to wild type.

P7 ln 5, Fig 2D is actually Fig3D

P7 ln 18: Please address the specificity of the antibody used to detect Pup and clarify why bands are detected at ~37 and 80 kDa in the delta prcSBA lane. Fig 4B -please note which blot is with anti-Pup and which is with anti-20S alpha on the figure (the legend states this info is indicated - however, this it is missing from the figure).

P8 ln 9: Enjoyed the 1905 citation. However, did this early report determine that all types of cells enter stationary phase when nutrients are limiting as was generally stated in the text? Please clarify.

P9 ln 10: at this point in the discussion, your argument that the level of pupylated proteins appeared low is not convincing due to the absence of a positive control that demonstrates the contrast on the same blot.

P9 ln 20-21: Many details are missing from the quantitative immunoblot analysis method, data, standard deviation values and figure. Thus, the reader is not convinced that the cytoplasmic concentration of Dop to PafA varies as was concluded by the authors. Please clarify what is meant in Fig. 6B by relative levels of PafA and Dop (relative to what)?

Fig 2B: Need to analyze the pupylome of cells inoculated from steady state vs. stationary phase to confirm that the high level of pupylated proteins at t=0 is due to carry over from stationary phase as was concluded by the authors.

Fig.4. Was prolonged incubation at stationary phase needed to observe the reduction in cfu/ml for the pup-proteasome deletion strain (only present OD600 for stationary phase cells not cfu/ml)? Was deletion of both pup and proteasome necessary for the reduction in cfu/ml in Fig 4D/E or is this same phenotype observed for single pup and proteasome deletion strains?

Fig.5. Please comment in text on the observed increase in CFU/ml observed after 4 days of starvation?
Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion):

P2 Ln2: use of word possesses seems to humanize Mycobacterium

P3 ln 18-19: it should be noted is an unnecessary phrase

P7 ln 16: arrival seems like a humanizing word choice

Fig 6B, time after starvation in days?
References: italicize species names in titles

Fig 6B please include error bars and increase the size of the xy scatter plot (inset) relative to the bar graph (since the latter only has two bars).

1st Revision - authors' response 27 April 2014

Point-by point responses:

Referee #1:

Elharar and colleagues investigated the role of the pup/proteasome system (PPS) during nutrient starvation in M. smegmatis. The authors show that a mutant deficient in PPS displayed a survival defect in stationary phase and during carbon or nitrogen starvation. Following nitrogen starvation, the amount of pupylated proteins increased rapidly but then decreased again, which the authors interpret as evidence that their degradation results in the generation of amino acids that can serve as carbon and nitrogen sources. Concomitantly, the amount of the proteins of the pupylation system (PafA, Dop, Mpa) decreased, which the authors take as evidence for autoregulation.

This manuscript addresses an interesting topic and the authors provide good evidence that the PPS is important for survival of M. smegmatis in nutrient limited conditions. However, the presented data are not sufficient to support the authors' conclusions that (i) the mechanism by which PPS supports survival of M. smegmatis involves amino acid recycling for use as carbon or nitrogen sources and (ii) the PPS autoregulates its activity via degradation of its components. In addition, they fail to discuss previous literature, which demonstrated that the proteasome system plays a crucial role in cell survival through amino acid recycling in yeast and mammalian cells.
Point (i) is addressed in the revised version according to the reviewer's suggestion (see below).
Point (ii) is addressed through expansion of the section concerning auto-regulation. New data are presented and exciting new conclusions are drawn (see below).

There is no clear demonstration that the PPS is used as recycling machinery in M. smegmatis by degrading pupylated proteins for use as carbon and energy sources. The authors only provide indirect evidence, i.e. pupylated proteins accumulate and then disappear which is associated with a PPS mutant suffering from a survival defect during nutrient starvation. Experiments that link these two observations mechanistically are missing.

This manuscript could be strengthened by experiments that investigate whether degradation of pupylated proteins is required for survival during starvation. The authors could use a prcBA mutant or the prcSAB mutant complemented with prcS or proteasome inhibitors to determine whether proteasome-mediated protein degradation is required during nutrient starvation or if pupylation itself is sufficient.
The revised Fig. 5D now presents a complementation assay in which Pup was expressed in the *prcSBA* deletion mutant, according to the reviewer's suggestion. Neither Pup alone nor the 20S particle alone could fully complement the survival defects of the mutant, indicating that proteasome-mediated degradation of pupylated proteins is indeed a crucial factor under conditions of nitrogen starvation. On the other hand, partial complementation following Pup expression in the mutant was observed. This finding could indicate that either pupylation alone plays an important role under starvation, or that pupylated proteins can be degraded to some extent even in the absence of the 20S particle. Data presented in Fig. 6B and 7B of the revised manuscript support the second possibility. This issue is addressed in the Discussion of the revised manuscript (P. 15, lines 9-20).

The authors propose that the PPS is negatively autoregulated during nitrogen starvation. However, they do not show which impact PPS autoregulation has following prolonged nitrogen starvation.
PPS negative auto-regulation is addressed more thoroughly in the revised text. Its impact is now made much clearer as we provide evidence to support the idea that through negative auto-regulation, oscillatory expression of PPS components is maintained (Fig. 7, pp. 11-13 & 15-16).

If protein recycling via the PPS is necessary for survival, it would be informative to determine the level of pupylated proteins at later time points (beyond 11 days).
As requested, new data are presented in revised Fig. 6B.

Is the PPS functioning in cycles (induction/degradation) during starvation to maintain a minimal amount of available carbon/nitrogen, yet avoiding uncontrolled degradation?
Yes! Support for this hypothesis is provided in the new experiments presented in Fig. 7. It is also broadly addressed in the Discussion (pp. 11-13 & 15-16).

Experiments in Fig6A/6C show that almost 100% of the pupylated proteins, which accumulated in response to starvation, disappeared after 4 to 6 days of starvation, which correlates with the time at which the bacteria lacking the PPS die. Does this suggest that pupylated proteins are used for the transition from rich to limited nutrient conditions rather than during starvation itself?
This hypothesis is reasonable and the study of Vabulas and Hartl (2005) cited by the reviewer indeed demonstrated that in starved mammalian cells, proteasome activity is most important during the early phase of amino acid starvation. Nonetheless, the data presented in the revised version of our manuscript do not point in that direction. Pupylated proteins are indeed hardly detected after a few days of starvation, yet low levels of pupylated proteins indicate either a low pupylation rate or a rapid degradation/depupylation rate (this point is now addressed in p. 10, lines 9-11 and in p. 16, lines 24-25 & p. 17, lines 1-2 of the revised text). The data presented in the revised manuscript suggest that both pupylation and degradation are accelerated throughout starvation to support nutritional requirements. In other words, the PPS functions more effectively during prolonged starvation.

It would be interesting to determine if the level of pupylated proteins increases again at later time points-as the bacteria continue to survive (but not replicate) upon prolonged nitrogen limitation (Fig5A).
The data in the revised version of Fig. 6B suggests that this is not the case.

The authors point out "up until now, however, amino acid recycling was not conceived as a role played (so necessarily) by the PPS". While this is true for mycobacteria, there is ample evidence in other systems, such as yeast and mammalian cells, that amino acid recycling via the proteasome is essential for cell survival during conditions of nutrient starvations (for example see: Vabulas and Hartl, Science 2005, 310, 1960-3; Sugaweera et al. Molecular Cell 2012, 48, 1-12). The authors should mention/discuss these reports.
Done.

Similarly, the sentence "PPS-deficient mutants of M. tuberculosis and M. smegmatis multiply as fast as do the parental wild type strains (line 25-26) is incorrect. Gandotra et al. demonstrated a replication defect in liquid culture and on agar plates of M. tuberculosis lacking the proteasome (Gandotra et al. PLOS Pathogens 2010).
This sentence has been revised according to the reviewer's comment.

Specific comments

- In Fig2B levels of pupylated proteins and expression of PafA along the growth curve of M. smegmatis are determined by Western blot; a loading control that ensures equal amounts of proteins have been run on the SDS PAGE is absent. This is true for all Western blots in the manuscript and must be corrected.
To address the reviewer's concern, the membranes used in the Western blots throughout our studies have been Coomassie-stained and their scanned images are now presented in the revised Fig. S2. This methodology for presenting loading controls is advantageous, in our case, over the standard methodology of re-blotting using antibodies against a control protein for three reasons. (1) It is hard to anticipate which "control" protein does not change its cytoplasmic level throughout prolonged starvation. (2) This methodology allows for assessment of the total protein load transferred to the membrane. (3) It is simpler.

- Pupylome analysis: the authors identified 88 and 235 proteins that were pupylated in exponential and stationary phase, respectively.

The list has been revised according to the concerns raised by the 3rd reviewer (see below) and now includes 42 and 93 protein species in exponential and stationary phase pupylomes, respectively.

They did not mention any quantification of the pupylated proteins in the specific conditions they used for pupylome purification. Did they compare the amount of pupylated proteins?
Such a comparison is presented in Fig. S1B and indicates that the protein concentration in the stationary phase pupylome is much higher than that of the exponential phase pupylome. Therefore, many more copies of each identified protein species are pupylated in stationary phase. This issue is now considered in the text (p. 6, lines 5-7).

Only 21 proteins are common between the two time points, although PafA does not seem to show any substrate specificity. Does this mean that pupylation is so random that each pupylome analysis will give different results (especially in exponential phase, when a small amount of proteins are pupylated)?
We interpret the data differently. Pupylation is not random and PafA does present substrate specificity (unpublished data). While substrate specificity is broad, some substrates are preferentially pupylated by PafA, whereas others are poor substrates. As such, pupylome analyses should be repetitive. Indeed, our data are in good agreement with previous pupylome analyses (Poulsen *et al,* 2010; Watrous *et al,* 2010; Festa *et al,* 2010)

- In Fig3D the authors used E. coli to show that the abundance of pupylated proteins of high molecular weight was due to more efficient pupylation of these proteins rather than more active degradation of the small ones. This analysis might be confounded by the use of a heterologous system. The authors could instead use the M. smegmatis prcSAB mutant complemented with prcS (that can pupylate but not degrade the pupylated proteins).
As this section has been removed from the revised version (see our response to reviewer #2), this point is no longer relevant.

- Fig4B: the authors should comment on the pupylation bands present in the prcSBA mutant lane.
We now address this issue in the revised text (p. 6, lines 24-26 and p. 7, lines 1-7), as well as in the new Fig. S3.

- Fig5A: CFUs increased by 2 orders of magnitude between days 0 and 4 of carbon limitation, but the turbidity of these cultures decreased. Can the authors please comment on how to explain this discrepancy?
Morphological adaptations of bacterial cells (e.g., change in cell size) in response to changes in the growth conditions (e.g., starvation) can affect the optical properties of the culture. At this stage, we do not have enough information that will allow for a more specific response. However, as this is a minor issue in the paper, we only comment on it succinctly in the revised text (p. 8, lines 15-16).

- Fig5D: The levels of PPS proteins during exponential growth should be determined and compared to protein levels during nutrient limitation. This would distinguish between an increase of pupylated proteins in nitrogen starvation and a decrease in carbon starvation. A loading control is essential.
This figure has been removed in the revised manuscript as it is misleading. Realizing that the PPS is highly dynamic, we see that presenting a single time point following starvation could confuse readers. As such, we address the reviewer's concerns regarding the level of pupylated proteins during exponential phase vs. starvation conditions in the new Fig. 6A&B.

- The authors propose that "during exponential growth, expression of both PafA and 20S is not induced (to justify this conclusion a loading control must be included in Figure 6A), resulting in slow pupylation and degradation rates, respectively. However they have not compared levels of pupylated in exponential growth to levels during nitrogen starvation (Fig6C). They should determine the amounts of pupylated proteins in the prcSBA mutant complemented with only prcS in exponential phase. This would answer the question whether there is no protein pupylation/degradation, or inefficient use of the PPS in exponential/rich medium growth.
The new Fig. 6B and 6C address these concerns. We show that levels of pupylated proteins increase following nitrogen starvation both in the wild type and in a 20S-deficient strain. This finding indicates that pupylation is induced following nitrogen starvation. We also found that pupylated proteins accumulated to a much higher level in a 20S-deficient strain, as compared to their accumulation in the wild type, suggesting that proteasomal degradation of pupylated proteins

prevented their extensive accumulation following starvation in the wild type. The new experiment portrayed in Fig. 6C indicates that like pupylation, proteasomal degradation is also induced following nitrogen starvation. In conclusion, as the reviewer hypothesized, there is inefficient use of the PPS in exponentially growing cells and accelerated use of the PPS in nitrogen-starved cells.

- In Figs 6A and 6D, Mpa, PafA and Dop amounts are analyzed during nitrogen starvation. These proteins are not detectable in the complemented prcSBA mutant on day 4, but detectable up to day 11 in wild type M. smegmatis. Can the authors comment on this difference?

The reviewer's point prompted us to perform new experiments (Fig. 7B-C) that revealed the oscillatory expression of PPS components. The new data (Fig. 7D&E) now explain the observed disappearance and reappearance of PPS components.

- The authors discuss PafA and Dop as having opposite functions (page 9, line 19), i.e. PafA is required for pupylation, and Dop for de-pupylation. However, Dop is also required for pupylation (conversion of Pup glutamine into glutamate). This needs to be clarified. In addition, the significance of a 2 fold difference in cytoplasmic concentrations of Dop and PafA (Fig. 6B, and text page 9, line 21-13) is unclear.

This section has been omitted from the revised version. Our current understanding is that regulation of the PPS is much more complex than thought previously. Accordingly, in order to avoid presenting over-simplistic PPS regulatory models, we prefer to address the interplay between PafA and Dop as part of a separate study addressing the regulation of the PPS. Note that removal of this section from the revised version does not affect the main issues discussed in the manuscript (i.e., the essential role of the PPS, the dynamics of PPS activity and PPS negative auto-regulation under nitrogen starvation).

- Some figures numbers are miss-annotated in the text, for example page 6, line 13, Fig 2B should be Fig 3B.

Some of the figures have been rearranged in the revised version. We have paid careful attention to their designation in the text.

- Several Figures are redundant and should be removed: Fig. 4D shows the same data as Fig 4E; Fig 5B and 5C are both redundant with Fig. 5A.

On this point, we disagree with the reviewer. Fig. 4D in not really redundant with Fig. 4E. Indeed, both describe the same phenomenon, yet differently. Whereas the spot test in Fig. 4D provides qualitative evaluation of survival under starvation, Fig. 4E presents quantitative evaluation. These assays support each other and strengthen our conclusions. We, therefore, wish to keep both figures. The same argument applies to the apparent redundancy between Fig. 5A and C and between Fig. 5B and C. As for the concerns regarding Fig. 5A and 5B, we chose to include both, since unlike in Fig. 5A, where the cfu concentration is described, Fig. 5B presents normalized cfu concentrations according to culture turbidity. Such representations of the data provide alternative approaches for comprehending the results and as such, we prefer to keep them.

Referee #2:

The manuscript by Elharara et al describes the pup-proteasome system (PPS) system in the saprophytic non-pathogenic bacteria Mycobacterium smegmatis. They show for the first time that this primitive protein degradation machinery is involved in amino acids recycling during starvation through degradation of high molecular mass proteins by the proteasome system. They demonstrate the PPS system is essential for survival under nitrogen limitation but whether the amino acids are the factors involved in not conclusively provided, yet. The work is novel and advances our knowledge of this primitive ubiquitin-proteasome system in various bacterial species. While the data are appealing, there are few issues that need to be addressed prior to publication to strengthen the conclusions.

1. Previous studies on eukaryotic cells have shown that proteasome inhibition leads to cell death due to the limited amino acids generated, and that cell death in blocked upon supplementation of amino acids (Suraweera et al, Mol. Cell, 2012, 48:242-243. Studies on Legionella, cited by the authors, have shown that the requirement of host proteasomal degradation for growth of the bacteria in the host cells is bypassed by a supplement of amino acids. Therefore, it would be prudent on the authors

to show that amino acids are the direct factor involved in the PPS system under nitrogen starvation, and would mimic genetic complementation of the PPS mutant. The authors should supplement amino acids for all the data shown in the figures to show reversibility and confirm the role of amino acids as the main factor involved. This control will enhance the studies and substantiate the conclusions dramatically.

While the reviewer's concerns are justified, their suggestion that we "supplement amino acids for all the data shown in the figures" is problematic. Addition of amino acids to the carbon-starved cells described in Fig. 2 and 4 is irrelevant, as amino acids are primarily a nitrogen source. Such addition is also not relevant to the pupylome analysis reported in Fig. 3. As for the survival assays performed under nitrogen starvation conditions that are described in Fig. 5, addition of amino acids certainly reverses the phenotype simply because it cancels starvation, rather than by-passing proteasome deficiency. Unlike in the reports on eukaryotic systems cited by the reviewer, the addition of amino acids to starved cells in our case would not prove the point. For instance, in yeast, as reported by Suraweera *et al*, consumption of peptone (a nitrogen source) normally present in the rich media used to grow yeast (YPD) depends on processing by the proteasome. As a result, proteasome-deficient mutants are unable to grow on rich media, with the addition of amino acids reversing this phenotype. In our case, proteasome activity is non-essential for proper growth in non-starved cells such that addition of a nitrogen source to nitrogen-starved cells, where PPS activity is essential, would simply cancel starvation.

However, in light of the reviewer's concern, we did include re-supplementation of a nitrogen source to experiments described in the revised manuscript that focus on PPS dynamics and negative auto-regulation (Fig. 6C & 7D). In doing so, we demonstrated that proteasome activity is induced under nitrogen starvation conditions and that the oscillatory expression of PPS components depends on nitrogen starvation. In addition, by following the suggestion of reviewer #1, we further strengthen the correlation between proteasome activity and survival under conditions of nitrogen starvation. We now demonstrate in the revised Fig. 5D that expression of Pup alone, without the 20S particle, is insufficient to fully complement the survival defects of a *prcSBA* deletion mutant under starvation. 20S expression, without Pup, is also insufficient to support survival of the mutant under stress. Accordingly, degradation of pupylated proteins, not pupylation alone, is the important factor under starvation.

2. A pafA mutant need to be included as a control in various studies, but in particular Fig. 1 and 2.

We do not understand the reviewer's point. Fig. 1 is a diagram while Fig. 2 describes dynamics in the levels of pupylated proteins during growth. We have included analysis of a *pafA* mutant in the revised submission and Fig. S3 now shows that no pupylation is observed in this mutant.

3. Fig. 2 needs a constitutively expressed protein as a control.

To address the reviewer's concern, the membranes used in the Western blots throughout our studies have been Coomassie-stained and their scanned images are now presented in the revised Fig. S2. This methodology for presenting loading controls is advantageous, in our case, over the standard methodology of re-blotting using antibodies against a control protein for three reasons. (1) It is hard to anticipate which "control" protein does not change its cytoplasmic level throughout prolonged starvation. (2) This methodology allows for assessment of the total protein load transferred to the membrane. (3) It is simpler.

4. A discussion of the evolutionary biology of this primitive system and its role in bacterial physiology need to be contrasted to the studies in eukaryotic cells (Suraweera et al, Mol. Cell, 2012, 48:242-243) and the role of the host proteasome in Legionella growth in host cells.

These studies are cited in the Discussion of the revised manuscript (p. 14, lines 10-11; p. 18, lines 5-6).

5. Validation of some of the studies inn M. tuberculosis would greatly enhance the significance of the findings to infectious diseases.

While the reviewer is correct, and although a paragraph in the Discussion is dedicated to the implications of our findings to *M. tuberculosis*, this study is focused primarily on the essential role of the Pup-proteasome system in mycobacterial species, regardless of their pathogenicity. Indeed, our lab is not equipped to work with this pathogen.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings:
Overall, the manuscript provides a new model as to why the Pup-proteasome system is needed for full virulence and persistence of Mycobacterium in its infected host. The authors provide evidence that suggests the Pup-proteasome system is important for amino acid recycling during conditions of nutrient limitation.

Specific major concerns essential to be addressed to support the conclusions:
The discussion of how the pupylome is biased toward proteins of high molecular weight is not convincing. Please provide the reader with enough controls, experimental details and statistical analyses to verify that the mass spectrometry and immunoblot approaches were not biased toward large proteins. Assume more than one peptide hit was required for positive identification, thus, suggesting small proteins may not be as readily detected with high probability compared to large proteins. If missed trypsin cleavage is prevalent , this would add further bias to large vs, small proteins. In addition, small proteins may be more difficult to detect after separation by SDS-PAGE and Western Blot than large proteins. For example, Millipore Immobilin P membranes are only recommended for proteins greater than 20 kDa. Serial dilution of internal controls (e.g., purified pupylated proteins of known quantity that are large vs. small) on the Western blot would strengthen the conclusions. Note that the diversion to this discussion seems somewhat distracting from the theme of the manuscript and is not well integrated.
Like the reviewer, we also felt, that this section distracts the reader from the main issues. Therefore, we decided not to include it in the revised manuscript.

The authors state that the average molecular weight of the pupylome was calculated to be 52 vs. 34 kDa for the complete proteome. Was the complete proteome determined by MS or is it theoretical based on deduced proteome? If an MS-based dataset was used, the argument becomes more convincing and needs to be clearly explained with appropriate citation of the shotgun proteome dataset used for calculation. If not, one could argue that the large protein bias is in the sampling technique and is not biological.
This concern is no longer relevant in light of the previous comment.

Important mass spectrometry details are missing from this document. Only the gene number, accession number, description, gene name, gene locus tag number and molecular mass of the protein deduced from the genome sequence are reported for the pupylomes. MS probability scores, % coverage, peptide sequences detected with a quantitative comparison of the theoretical and actual observed masses, etc. are not in the supplemental data. Please report false discovery rate (FDR), type of target decoy and other important details of MS-based analysis within the methods.
We have re-visited the data and re-analyzed it according to harsher criteria. These criteria, which include identification of at least two high-confidence peptides per protein, are described in the Materials & Methods section, which was revised according to the reviewer's suggestions and now reports a FDR rate <0.01 and a choice of the reverse *M. smegmatis* database as a target decoy. Our lists in the revised manuscript present 42 and 93 protein species in the exponential phase and stationary phase pupylomes, respectively. Supplementary Tables S1 & S2 now includes accession numbers of identified proteins, the MSMEG number, score and coverage. In addition, for each identified protein species, a list of all the peptides that were sequenced is provided, including the mass of each peptide, the deviation from the theoretical mass (Dppm) and the XCorr.

What was the percentage of pupylome with lysine modification sites identified vs. not identified? How much of the pupylome detected by mass spectrometry is not actually pupylated but instead simply associated with the pupylated proteins?
Only three proteins in our lists were found to contain verified pupylation sites, based on MS-MS identification of modified lysines (see Supplementary tables). It is indeed possible, as the reviewer noted, that our lists contain proteins that simply associated with pupylated proteins. We note this possibility in the text (p. 6, lines 8-9) but also note that many of the proteins in our lists were identified in previous *M. smegmatis* and *M. tuberculosis* pupylome analyses (Poulsen *et al,* 2010; Watrous *et al,* 2010; Festa *et al,* 2010). To be more precise, ten out of the 42 proteins we identified as belonging to the exponential phase pupylome and 21 out of the 93 proteins of the stationary phase pupylome were also identified in these earlier studies. This concern is highly relevant to our analysis of the PPS auto-regulation. There, we observed that Mpa, PafA, Dop and, as now included in the

revised version, the 20S particle are degraded by the proteasome. It is known that Mpa is a pupylation substrate and we rationalized, based on our pupylome analysis, that this is also the case for PafA, Dop and the 20S particle. However, these proteins are known to interact with Pup and could therefore co-elute with it in the samples that were used for pupylome analysis. In other words, there is a real concern that identification of these proteins in our pupylome analysis does not truly reflect their being pupylated. Therefore, we directly confirmed PafA and 20S pupylation in the revised manuscript via an *in vitro* assay that is now presented in Fig. 7B. Such an assay could not be performed for Dop, due to its depupylase activity.

How do the authors know that the disappearance of the pupylated proteins by immunoblot is due to proteasome-dependent degradation of the pupylome and not simply depupylation of the protein target? Pup is the direct signal detected by this approach (and the MS analysis of Pup pull-downs) and not the protein target. Pup is a small protein that is likely difficult to detect at low levels via immunoblot in its free form vs. its protein conjugated form due to the problems associated with immunoblot of small proteins. While the model developed by the authors seems logical based on a long history of our understanding of amino acid recycling during starvation for all types of cells (from bacteria to humans), the results of this report do not directly demonstrate the stated conclusions. One could counter with an alternative model that the pupylome is depupylated by Dop during long term stationary phase and that 20S proteasomes are needed for this Dop activity (e.g. degradation of an inhibitor of Dop) which appears to occur around day 2-3 after starvation when Dop levels are still detected.

Our answer to this concern is two-fold:

A- As the reviewer noted, we presented the simpler, more sensible and parsimonious explanation.

B- The revised version of the manuscript presents a new experiment that strongly supports our conclusions (Fig. 6C). In this experiment, we followed the fate of a model protein encoded by a genetic fusion of *prcS* to *zur*. The resulting chimera cannot be depupylated by Dop, based on previous analysis of Pup fusions (Impkamp *et al*, 2010b). Nevertheless, it disappears in 20S-containing cells, but not in 20S-deficient cells, as observed via detection of the Zur moiety using antibodies against an included polyhistidine tag, rather than against Pup. Strikingly, disappearance of Pup-Zur was accelerated in response to nitrogen starvation. We regard these findings as a clear demonstration of proteasomal degradation and it strengthens our original conclusion regarding the fate of pupylated proteins in response to nitrogen starvation.

Minor concerns that should be addressed:

p3 ln 2-3: statement implies that functional proteasomes that degrade pupylated proteins are demonstrated in Nitrospira, yet citations do not reflect this assumption

This sentence has been rephrased and proper citations have been included.

P3 ln 6: The claim that only single pup molecules are conjugated to substrates is not properly cited and appears based on absence of data. MtPup has 3 internal lysine residues and an N-terminal primary amine group that have the potential for pupylation.

We agree and have rephrased the sentence accordingly. A proper citation (Festa *et al,* 2010) is now added.

P4 ln 25: under standard laboratory conditions? Please define what you mean within the text.

We could not find this phrase in our text.

P5 ln 23: please clarify whether this strain produces native levels of Pup or whether this protein modifier is at higher levels than wild type. If at higher levels, please clarify how the pupylome identified by this approach compares to wild type.

This strain probably produces higher levels of Pup than does the wild type, as a strong promoter (i.e., the acetamidase promoter) is used for Pup expression. Even if expressed at levels comparable to those in the wild type, one could argue that the Strep and polyhistidine tags found at the N-terminus of this Pup variant could affect its ability to be recognized by Mpa and be degraded, thus altering the pupylome. Nonetheless, comparison to the wild type is of little relevance here. The more important comparison is between exponential and stationary phase cultures of the same strain. Moreover, we find that despite the presumably altered expression levels of the dually-tagged Pup, the higher pupylation level seen at stationary phase remains much like what we observed in the wild

type (Fig. S1B). Furthermore, as already mentioned, there is good agreement among our data and those reported in previous pupylome analyses. As such, we do not find this concern justified.

P7 ln 5, Fig 2D is actually Fig3D
This point is no longer relevant in the revised manuscript.

P7 ln 18: Please address the specificity of the antibody used to detect Pup and clarify why bands are detected at ~37 and 80 kDa in the delta prcSBA lane. Fig 4B -please note which blot is with anti-Pup and which is with anti-20S alpha on the figure (the legend states this info is indicated - however, this it is missing from the figure).
This issue is addressed in the revised text (p. 6, lines 24-26 and p.7, lines 1-7) and in Fig. S3.

P8 ln 9: Enjoyed the 1905 citation. However, did this early report determine that all types of cells enter stationary phase when nutrients are limiting as was generally stated in the text? Please clarify.
We are glad this was noticed. This early report discussed for the first time in writing the concept of a limiting factor.

P9 ln 10: at this point in the discussion, your argument that the level of pupylated proteins appeared low is not convincing due to the absence of a positive control that demonstrates the contrast on the same blot.
Loading controls for the blots in this work are now included and are presented in Fig. S2. A detailed explanation of this issue can be found in our response to reviewers 1 and 2.

P9 ln 20-21: Many details are missing from the quantitative immunoblot analysis method, data, standard deviation values and figure. Thus, the reader is not convinced that the cytoplasmic concentration of Dop to PafA varies as was concluded by the authors. Please clarify what is meant in Fig. 6B by relative levels of PafA and Dop (relative to what)?
This figure has been removed from the revised version of the manuscript.

Copied from our response to a concern of reviewer #1: Our current understanding is that regulation of the PPS is much more complex than presented in the original version of the paper. Accordingly, we wish to avoid presentating over-simplistic PPS regulatory models and prefer to address the interplay between PafA and Dop as part of a separate study addressing the regulation of the PPS. Note that removal of this section from the revised version did not weaken the main issues discussed in the manuscript (i.e., the essential role of the PPS, the dynamics of PPS activity and PPS negative auto-regulation under nitrogen starvation).

Fig 2B: Need to analyze the pupylome of cells inoculated from steady state vs. stationary phase to confirm that the high level of pupylated proteins at t=0 is due to carry over from stationary phase as was concluded by the authors.
The high level at t=0 is not due to carry-over from stationary phase; it instead represents the level of pupylated proteins in the first inoculation of the culture, as the cells adapt from growth on solid media to growth in liquid media. This point is better explained in the revised text (p. 5, lines 11-13).

Fig.4. Was prolonged incubation at stationary phase needed to observe the reduction in cfu/ml for the pup-proteasome deletion strain (only present OD600 for stationary phase cells not cfu/ml)? Was deletion of both pup and proteasome necessary for the reduction in cfu/ml in Fig 4D/E or is this same phenotype observed for single pup and proteasome deletion strains?
Survival as a function of time is described in Fig. 5A. As for single deletion strains, the revised Fig. 5D addresses this issue.

This point was raised also by reviewer #1 and our response there was: The revised Fig. 5D now presents a complementation assay in which Pup was expressed in the *prcSBA* deletion mutant, according to the reviewer's suggestion. Neither Pup alone nor the 20S particle alone could fully complement the survival defects of the mutant, indicating that proteasome-mediated degradation of pupylated proteins is indeed a crucial factor under conditions of nitrogen starvation. On the other hand, partial complementation following Pup expression in the mutant was observed. This finding could indicate that either pupylation alone plays an important role under starvation, or that pupylated proteins can be degraded to some extent even in the absence of the 20S particle. Data presented in

Fig. 6B and 7B of the revised manuscript support the second possibility. This issue is addressed in the Discussion of the revised manuscript (P. 15, lines 9-20).

Fig.5. Please comment in text on the observed increase in CFU/ml observed after 4 days of starvation?
Done. Please see p. 8, lines 12-15.

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion):
P2 Ln2: use of word possesses seems to humanize Mycobacterium
We feel that the text here is properly phrased.

P3 ln 18-19: it should be noted is an unnecessary phrase
Agreed. This phrase has been deleted.

P7 ln 16: arrival seems like a humanizing word choice
Here too, we feel that the sentence is properly phrased

Fig 6B, time after starvation in days?
Yes

References: italicize species names in titles
Done

Fig 6B please include error bars and increase the size of the xy scatter plot (inset) relative to the bar graph (since the latter only has two bars).
No longer relevant in the revised manuscript.

2nd Editorial Decision 22 May 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been re-reviewed by the three referees.

Both referees #1 and 2 are happy with the revised paper and support publication in the EMBO Journal. Referee #2 has a few minor remaining comments that can be easily addressed. Referee #3, however, is more uncertain and raises new concerns. Some of the concerns arose due to the inclusion of new experiments.

I would like to move forward with the paper for publication here, but would also like to see if we can find a constructive way to address the remaining concerns raised by referee #3 as some of them are relevant. Some of concerns can be addressed with appropriate text changes and a better discussion of the open questions. I think it will be easiest to discuss how to proceed with the revisions with a phone call. Let me know when is a good time for you.

REFEREE REPORTS

Referee #1:

The authors addressed most of my critiques.

Referee #2:

The revised manuscript is much stronger than the first submission. This is a nice and solid paper that will enhance our knowledge. However, it is largely written to the specialist than the generalist, and this needs to be revised for the EMBO readership. The abstract, in particular, should be revised to be addressed to the generalist, as explained below. This would make the paper more appealing to the generalist and get the reader to more appreciative of the unique biology involved compared to other

systems. Few minor issues remain and should be addressed as outlined below.
1. Abstract needs to be revised for the general reader rather than the current mycobacterial reader. Mention eukaryotic proteasomes and pup as a primitive system. Also mention while Legionella utilizes host proteasomes as a source of amino acids, your data show that Mycobacterium has its own system. This would broaden the readership of your paper and would highlight novelty of your system compared to what is known. Also consider revising the title to make it more appealing to the generalist.
2. In the introduction, discuss the role of the eukaryotic proteasomes from yeast to mammals in providing "recycling" amino acids for protein synthesis (Suraweera et al, 2012, MOl.Cell, 48:242), similar to what you found in the prokaryotic system. This can be also included in the discussion, where many currently rehashed statements from the results can be deleted.
3. P3, L13-20, refer to Fig. 1.
4. P3, last paragraph should start with a contrast to human proteasomes and their functions in protein degradation and amino acid generation for protein synthesis in and their role in Legionella nutrition, then highlight the novelty of the mycobacterial system.
5. In Fig. 2B, the data need to be supported by an additional but simple experiment in which Log bacteria (18-24h) is washed and shifted to be incubated in filtered spent media from stationary growth (42-48h). This will strengthen the conclusion from the current data.

Referee #3:

Overall, the manuscript presents interesting findings regarding the potential for mycobacteria to use proteasome-mediated amino acid recycling for survival during nutrient-limited conditions. However, the authors provide no definitive evidence for this claim. Furthermore, many of the experiments are lacking appropriate controls to enable the authors to draw firm conclusions regarding the molecular mechanisms responsible for the observed phenomena.

Comments:
P4 ln 14/26: The majority of the conclusions stated in the final paragraph of the introduction (designed to summarize the manuscript and its findings) are not supported by the data presented within the results section of the manuscript. For example, the proteasome-pupylation system (PPS) is not demonstrated to be essential for survival of Mycobacterium smegmatis under conditions of nitrogen starvation. A PPS-deficient mutant is not shown to fail to survive starvation. The authors do show that a PPS-deficient mutant has a reduction in CFU/ml when exponential cells are centrifuged, resuspended in media lacking nitrogen, incubated for a period of time and then plated. A portion of the cells do survive (thus, the PPS system is not essential). Furthermore, no control with nitrogen is included to provide evidence that nitrogen starvation may be the cause of this reduction, and the Pup and proteasome systems are not separately analyzed through complementation to confirm that both systems are involved in this process. In the introduction, the authors also state that their work reveals a dramatic, yet delicately regulated induction of pupylation and degradation of pupylated substrates is involved in enabling mycobacteria to overcome nutrient limitation. This type of molecular detail is not even attempted in the manuscript. The claim that the study shows the proteasome mediates degradation of PPS components is not supported by evidence. The authors show that a synthetic Pup-Zur fusion has a longer half-life in a mutant strain deficient in both Pup and proteasomes compared to wild type, but Pup-Zur is not pupylated by a ubiquitin-like isopeptide bond, is most likely artificially unfolded, and is not a native PPS component.

The biochemistry of Fig. 1 was already published and reviewed by others in the field and does not reflect the findings of this study.

The methods used to generate the growth curve and analyze the levels of pupylated proteins in Fig. 2A/B were not properly performed. The growth curve of Fig. 2A has too many variables (e.g., stationary phase cells are transitioning to fresh medium as well as from one type of growth medium to another). Standard microbiological technique using an inoculum of steady state (log phase) cells grown on the same medium for growth curve analysis would benefit this experiment. In addition, inclusion of appropriate controls with a prcS deletion grown under similar conditions, complementation of the deletion strains, and feedback of nutrient are needed. The described quantification of the pupylome by dot blot analysis of protein fractions using anti-Pup antibodies does not necessarily provide an accurate estimate the pupylome as assumed by the authors. The

pupylome was purified by His-Strep tandem affinity chromatography from strains overexpressing His-Strep-Pup and, thus, includes a mixture of free Pup, pupylated proteins and proteins non-covalently associated with Pup. Since free Pup was not separated from the samples prior to dot blot analysis, the % of pupylated proteins estimated in relationship to total protein in Fig. 2A is a reflection of both the pupylome and the artificially expressed free form of His-Strep-Pup (which may be differentially degraded/expressed based on growth condition). This problem may partially explain why the % of pupylated proteins presented in Fig. 2A does not correlate with the immunoblot of Fig. 2B. For example, the pupylated protein detected at 48 h is at its nearly highest % total in Fig. 2A while at the lowest in the immunoblot of Fig. 2B.

The authors do not provide direct evidence that the majority of the pupylome claimed to be detected by mass spectrometry in this study is actually pupylated and not simply associated with Pup or with pupylated proteins. The lengthy discussion in the response to reviewer comments to provide a rationale for the claim that this study detected the pupylome by MS/MS yet only found three proteins in the list to contain verified pupylation sites is not acceptable for publication in this caliber of a journal. The inclusion of a pie chart to represent the pupylome under different culture conditions in Fig. 3 is not appropriate.

P7 ln 7: How does analysis of the pafA deletion strain provide evidence that the prcSBA deletion strain lacks pupylated proteins? This argument is not logical. The lengthy discussion of use of a pafA deletion strain to demonstrate that the anti-Pup antibody is specific for Pup and pupylated proteins is distracting and does not provide direct evidence for their argument. The authors need to focus on the analysis of the prcS (Pup) deletion strain to demonstrate that the anti-Pup antibody is specific since this strain is presumably deficient in production of the Pup protein. The detection of cross-reactive bands in the Pup mutant strain complicates analysis and necessitates use of this strain as a control for all experiments/immunoblots that incorporate the anti-Pup antibody (which was not performed). The Pup mutant strain should be grown under the various growth conditions of this study and included on the same blots as the test strains used for analysis of the pupylome by anti-Pup antibody (since the proteome is anticipated to be composed of different anti-Pup antibody cross-reactive proteins based on growth condition).

The authors do not provide a list of the wild type and mutant strains used in this study including genotype and reference/source that describes construction. Thus, it is difficult to review the validity of strain construction (e.g., one would assume that appropriate methods were used to generate and verify that the prcSBA deletion strain is devoid of the prcS gene). However, the authors present a lengthy discussion of the pafA (and not prcS) deletion strain to justify the specificity of their anti-Pup antibody suggesting they do not trust their prcS (Pup) deletion strain. Was the prcS deletion strain examined only by PCR without follow up by Southern blotting to confirm validity? On a similar topic, the authors mention complementation of the prcSBA deletion by use of chromosomally-integrated plasmids without defining the site of integration. If the site is at the locus of interest, the authors will not be able to distinguish whether the prcSBA deletion has a polar effect on expression of downstream genes or operons.

P9 ln 8-11: The authors do not provide any direct evidence that nitrogen starvation is the signal for the observed phenomena. Furthermore, the authors should be careful to provide additional evidence before making the bold speculation that pupylated proteins are degraded in the absence of 20S proteasomes. The manuscript theme is that the Pup-proteasome system plays an important role under nitrogen starvation. However, based in the evidence provided in Fig 5D the proteasome does not appear so important under the conditions claimed to mimic nitrogen starvation and no phenotype is presented for the pafA deletion strain which mediates pupylation.

P9 ln 14: The authors start this section by stating that the phenotype of a PPS-mutant is considerably more severe under conditions of nitrogen-deprivation. However, the authors provide evidence in Fig. 5D that Pup alone is responsible for the observed phenotype. The prcSBA mutation is complemented by prcS alone and is not complemented by prcBA under the growth conditions tested in this study.

P11 ln 14: Are the N-terminal and penultimate residues of the Zur and Pup-Zur proteins similar in composition and overall structural fold? One could argue that an N-end rule that is independent of Pup-targeting is responsible for the observed phenomena. Alternatively, the Pup-Zur is artificially

unstable and, thus, degraded.

Fig. 6C. Need to complement the prcSBA deletion with prcBA to argue that 20S proteasomes are responsible for this phenomenon.

Fig. 7B. Claim that PafA and 20S proteasomes are pupylated in vitro but provide no direct evidence for the formation of isopeptide bonds or that this reaction requires ATP or Pup.

Fig. 7C. loading control?

The evidence to support the ability to specifically detect PafA, Dop, 20S proteasome alpha and Mpa proteins by immunoblotting is not provided.

Fig. 8/9 are premature models based on the evidence provided in this manuscript (particularly since Pup may function alone independent of 20S proteasomes).

Minor:
P3 ln 21/22: The authors state that "Bacterial proteasomes are simpler than their eukaryotic counterparts, containing only single types of alpha and beta subunits in the 20S core particle." However, bacterial proteasomes are described in which two different alpha- and two different beta-type subunits form a single 20S particle [Tamura et al. (1995). The first characterization of a eubacterial proteasome: the 20S complex of Rhodococcus. Curr Biol. 5:766-74].

P8 ln 4: Cells = Bacterial cells? please define within the manuscript on ln 4 what type of cells were studied by Blackman, 1905

P10 ln 2: However, whereas = awkward

2nd Revision - authors' response 29 May 2014

Point-by point response to the reviewers (second round):

Referee #2:

The revised manuscript is much stronger than the first submission. This is a nice and solid paper that will enhance our knowledge. However, it is largely written to the specialist than the generalist, and this needs to be revised for the EMBO readership. The abstract, in particular, should be revised to be addressed to the generalist, as explained below. This would make the paper more appealing to the generalist and get the reader to more appreciative of the unique biology involved compared to other systems. Few minor issues remain and should be addressed as outlined below.
1. Abstract needs to be revised for the general reader rather than the current mycobacterial reader. Mention eukaryotic proteasomes and pup as a primitive system. Also mention while Legionella utilizes host proteasomes as a source of amino acids, your data show that Mycobacterium has its own system. This would broaden the readership of your paper and would highlight novelty of your system compared to what is known. Also consider revising the title to make it more appealing to the generalist.
We have revised the abstract according to the reviewer's suggestion, without including comparison to *Legionella*.

2. In the introduction, discuss the role of the eukaryotic proteasomes from yeast to mammals in providing "recycling" amino acids for protein synthesis (Suraweera et al, 2012, MOl.Cell, 48:242), similar to what you found in the prokaryotic system. This can be also included in the discussion, where many currently rehashed statements from the results can be deleted.
The Introduction has been revised accordingly (p. 3, lines 1-10). We prefer to leave the Discussion in its current state.

3. P3, L13-20, refer to Fig. 1.
Done

4. P3, last paragraph should start with a contrast to human proteasomes and their functions in protein degradation and amino acid generation for protein synthesis in and their role in Legionella nutrition, then highlight the novelty of the mycobacterial system.
In the revised version, we begin the Introduction with a description of the roles of eukaryotic proteasomes, including amino acid recycling under starvation. We prefer to avoid repeating this information later in the Introduction. In addition, we prefer to keep the comparison between *M. tuberculosis* and *Legionella* in the Discussion.

5. In Fig. 2B, the data need to be supported by an additional but simple experiment in which Log bacteria (18-24h) is washed and shifted to be incubated in filtered spent media from stationary growth (42-48h). This will strengthen the conclusion from the current data.
In principle, the reviewer suggests a controlled starvation experiment, using conditioned medium. It is not clear to us what would be the benefit of such an experiment. In addition, a more informative starvation experiment is already described in Fig. 5.

Referee #3:

Overall, the manuscript presents interesting findings regarding the potential for mycobacteria to use proteasome-mediated amino acid recycling for survival during nutrient-limited conditions. However, the authors provide no definitive evidence for this claim. Furthermore, many of the experiments are lacking appropriate controls to enable the authors to draw firm conclusions regarding the molecular mechanisms responsible for the observed phenomena.
Comments:
P4 ln 14/26: The majority of the conclusions stated in the final paragraph of the introduction (designed to summarize the manuscript and its findings) are not supported by the data presented within the results section of the manuscript. For example, the proteasome-pupylation system (PPS) is not demonstrated to be essential for survival of Mycobacterium smegmatis under conditions of nitrogen starvation. A PPS-deficient mutant is not shown to fail to survive starvation. The authors do show that a PPS-deficient mutant has a reduction in CFU/ml when exponential cells are centrifuged, resuspended in media lacking nitrogen, incubated for a period of time and then plated. A portion of the cells do survive (thus, the PPS system is not essential).
The data in Fig. 5A-C demonstrate that the survival difference between the wild type and the mutant increases gradually and reaches a ~150-fold level after 15 days of nitrogen starvation. We find it unnecessary to extend the starvation period beyond this point as death curves are normally non-linear and asymptotic towards the end. It is most likely that if we had waited a month, for example, a higher difference between the wild type and the mutant would have been observed. Accordingly, we believe our conclusions to be reasonable and justified.

Furthermore, no control with nitrogen is included to provide evidence that nitrogen starvation may be the cause of this reduction, and the Pup and proteasome systems are not separately analyzed through complementation to confirm that both systems are involved in this process.
This is incorrect. We show that, when not starved, the mutant grows as well as does the wild type. The only component missing under the nitrogen starvation conditions is a nitrogen source. Indeed, this is the perfect control. As for the complementation assays, these are presented in Fig. 5D.

In the introduction, the authors also state that their work reveals a dramatic, yet delicately regulated induction of pupylation and degradation of pupylated substrates is involved in enabling mycobacteria to overcome nutrient limitation. This type of molecular detail is not even attempted in the manuscript. The claim that the study shows the proteasome mediates degradation of PPS components is not supported by evidence.
We believe these concerns to be unjustified. Fig. 7 addresses auto-regulation by proteasomal degradation. We specifically show that PPS components are pupylated, are stable in the mutant, and disappear in the wild type. These findings provide strong evidence in support of our conclusions.

The authors show that a synthetic Pup-Zur fusion has a longer half-life in a mutant strain deficient in both Pup and proteasomes compared to wild type, but Pup-Zur is not pupylated by a ubiquitin-like isopeptide bond, is most likely artificially unfolded, and is not a native PPS component.
It is incorrect to assume that Pup-Zur is most likely unfolded. Does the reviewer assume that any fusion protein is unfolded unless proven otherwise? Pup-Zur is an excellent model protein for

studying proteasomal degradation, as it is not subjected to either pupylation or depupylation. The same fusion was elegantly used by Burns et al. to study the PPS (*J. Bacteriol.* 192: 2933-2935, 2010), and we now show it is stable in the mutant and degraded in the wild type, with the degradation occurring much faster in response to nitrogen starvation. These findings provide evidence for a very important point in our paper.

The biochemistry of Fig. 1 was already published and reviewed by others in the field and does not reflect the findings of this study.
This is an introductory figure. We thought that as the PPS is not sufficiently well known to readers outside our field, this figure makes the paper easier to read.

The methods used to generate the growth curve and analyze the levels of pupylated proteins in Fig. 2A/B were not properly performed. The growth curve of Fig. 2A has too many variables (e.g., stationary phase cells are transitioning to fresh medium as well as from one type of growth medium to another). Standard microbiological technique using an inoculum of steady state (log phase) cells grown on the same medium for growth curve analysis would benefit this experiment.
Only one type of medium was used in this experiment and the text does not indicate otherwise. As for the transition from stationary to exponential phases, we do not understand what problem does this presents. We wanted to demonstrate a change in the level of pupylated proteins during this transition, as well as throughout growth.

In addition, inclusion of appropriate controls with a prcS deletion grown under similar conditions, complementation of the deletion strains, and feedback of nutrient are needed.
The complementation assays are already presented in Fig. 5C. As for feedback of nutrients, we explained in our previous response to the comments initially raised by reviewer #2 why this would not be an informative experiment.

The described quantification of the pupylome by dot blot analysis of protein fractions using anti-Pup antibodies does not necessarily provide an accurate estimate the pupylome as assumed by the authors. The pupylome was purified by His-Strep tandem affinity chromatography from strains overexpressing His-Strep-Pup and, thus, includes a mixture of free Pup, pupylated proteins and proteins non-covalently associated with Pup. Since free Pup was not separated from the samples prior to dot blot analysis, the % of pupylated proteins estimated in relationship to total protein in Fig. 2A is a reflection of both the pupylome and the artificially expressed free form of His-Strep-Pup (which may be differentially degraded/expressed based on growth condition). This problem may partially explain why the % of pupylated proteins presented in Fig. 2A does not correlate with the immunoblot of Fig. 2B. For example, the pupylated protein detected at 48 h is at its nearly highest % total in Fig. 2A while at the lowest in the immunoblot of Fig. 2B.
There has been a misunderstanding here. We explain in the legend to Fig. S1A that the blank sample (designated "B" in the figure), is a lysate of a pupylation-deficient strain. It is actually a lysate of the *pafA* deletion mutant, which has free Pup in its cytoplasm, a point we made clearer in the second revision. Importantly, no signal is detected in the blank, indicating that free Pup does not contribute to our quantification of the pupylome. This is because free Pup is not well bound to PVDF membranes, as also reflected from our many Western blots. As such, there is no concern that detection of free Pup biased our quantification. In addition, all samples, including the blank, are composed of lysates but these do not generate "noise", as seen in the blank sample. Regarding the inconsistency between the graph and the gel, the two show exactly the same trend. They are indeed not identical, but variations in biological repeats are to be expected. For precisely this reason, the quantification was performed in triplicates, as presented in the graph. In conclusion, our measurements were based on a proper methodology. We have now revised the Supplementary Text to clear the confusion.

The authors do not provide direct evidence that the majority of the pupylome claimed to be detected by mass spectrometry in this study is actually pupylated and not simply associated with Pup or with pupylated proteins. The lengthy discussion in the response to reviewer comments to provide a rationale for the claim that this study detected the pupylome by MS/MS yet only found three proteins in the list to contain verified pupylation sites is not acceptable for publication in this caliber of a journal.
We addressed this point in our previous response, but it seems we did not satisfy the reviewer's concerns. We would like to point out that:

A- Rather than ignoring the problem, we explain that some of the proteins detected may be due to non-specific association with the pupylome (p.7, lines 8-9).
B- Our proteomic results are in a very good agreement with previous similar analyses. This is also mentioned in the text.
C- The major justification to include the proteomic analysis in the manuscript is to demonstrate that no dramatic shift in PafA specificity occurs in response to starvation.

The inclusion of a pie chart to represent the pupylome under different culture conditions in Fig. 3 is not appropriate.
This figure demonstrates that PafA maintains its characteristic broad specificity whether at exponential or stationary phase. This conclusion still holds true even if some of the proteins identified are associated with the pupylome, rather than being pupylated. Accordingly, we prefer to include this figure while better explaining its scientific value and the caveats of our analysis (see p. 7, lines 14, 15).

P7 ln 7: How does analysis of the pafA deletion strain provide evidence that the prcSBA deletion strain lacks pupylated proteins? This argument is not logical. The lengthy discussion of use of a pafA deletion strain to demonstrate that the anti-Pup antibody is specific for Pup and pupylated proteins is distracting and does not provide direct evidence for their argument. The authors need to focus on the analysis of the prcS (Pup) deletion strain to demonstrate that the anti-Pup antibody is specific since this strain is presumably deficient in production of the Pup protein. The detection of cross-reactive bands in the Pup mutant strain complicates analysis and necessitates use of this strain as a control for all experiments/immunoblots that incorporate the anti-Pup antibody (which was not performed). The Pup mutant strain should be grown under the various growth conditions of this study and included on the same blots as the test strains used for analysis of the pupylome by anti-Pup antibody (since the proteome is anticipated to be composed of different anti-Pup antibody cross-reactive proteins based on growth condition).
The data presented in Fig. S3 clearly support the logic of our arguments.

The authors do not provide a list of the wild type and mutant strains used in this study including genotype and reference/source that describes construction. Thus, it is difficult to review the validity of strain construction (e.g., one would assume that appropriate methods were used to generate and verify that the prcSBA deletion strain is devoid of the prcS gene). However, the authors present a lengthy discussion of the pafA (and not prcS) deletion strain to justify the specificity of their anti-Pup antibody suggesting they do not trust their prcS (Pup) deletion strain. Was the prcS deletion strain examined only by PCR without follow up by Southern blotting to confirm validity? On a similar topic, the authors mention complementation of the prcSBA deletion by use of chromosomally-integrated plasmids without defining the site of integration. If the site is at the locus of interest, the authors will not be able to distinguish whether the prcSBA deletion has a polar effect on expression of downstream genes or operons.
We reported on the generation of the *prcSBA* deletion mutant in a previous work (Shenkerman et al., 2013. Gene 533: 374-378) that includes the genetic analysis mentioned by the reviewer. This publication is cited in the text (p. 7, line 24). In contrast, the *pafA* mutant is reported here for the first time, and, therefore, we provide detailed description for its construction. Complementation assays were conducted using plasmid pMV306. This is an integrative plasmid commonly used in mycobacterial research and it enters the chromosome at a defined location (see Hong et al. 1991, *Proc Natl Acad Sci USA* 88: 3111-3115).

P9 ln 8-11: The authors do not provide any direct evidence that nitrogen starvation is the signal for the observed phenomena.
We do show that nitrogen starvation results in the observed phenomena. How nitrogen starvation is sensed by the PPS is beyond of the scope of this manuscript.

Furthermore, the authors should be careful to provide additional evidence before making the bold speculation that pupylated proteins are degraded in the absence of 20S proteasomes.
This speculation is explained more carefully in the revised version (p. 16, lines 17-25).

The manuscript theme is that the Pup-proteasome system plays an important role under nitrogen starvation. However, based in the evidence provided in Fig 5D the proteasome does not appear so

important under the conditions claimed to mimic nitrogen starvation and no phenotype is presented for the pafA deletion strain which mediates pupylation.
The evidence presented in Fig. 5D indicates that complementation of Pup alone, without the 20S, is not sufficient for full complementation. This strain presents ~10-fold worse survival, in comparison to the fully complemented strain and to the wild type, indicating that the proteasome is indeed important. Our explanation for this partial complementation is that pupylated proteins can be degraded to some extent in a 20S-independent manner. We address this point in the revised version (p. 16, lines 20-25).

P9 ln 14: The authors start this section by stating that the phenotype of a PPS-mutant is considerably more severe under conditions of nitrogen-deprivation. However, the authors provide evidence in Fig. 5D that Pup alone is responsible for the observed phenotype. The prcSBA mutation is complemented by prcS alone and is not complemented by prcBA under the growth conditions tested in this study.
Pup alone does not fully complement the mutant deficiencies. Please refer to our response to the previous point and to page 16, lines 20-25 of the revised version.

P11 ln 14: Are the N-terminal and penultimate residues of the Zur and Pup-Zur proteins similar in composition and overall structural fold? One could argue that an N-end rule that is independent of Pup-targeting is responsible for the observed phenomena. Alternatively, the Pup-Zur is artificially unstable and, thus, degraded.
As explained above, we show that the degradation of Pup-Zur is 20S-dependent and is accelerated under nitrogen starvation. As such, the N-end rule is irrelevant in this case.

Fig. 6C. Need to complement the prcSBA deletion with prcBA to argue that 20S proteasomes are responsible for this phenomenon.
We find the comparison with the wild type convincing enough, especially since degradation is accelerated in response to nitrogen starvation conditions.

Fig. 7B. Claim that PafA and 20S proteasomes are pupylated in vitro but provide no direct evidence for the formation of isopeptide bonds or that this reaction requires ATP or Pup.
These controls were performed and are now presented in the revised Fig. 7.

Fig. 7C. loading control?
The controls of Fig. 7 are presented in Fig. S2 but were designated incorrectly. We have now corrected this mistake.

The evidence to support the ability to specifically detect PafA, Dop, 20S proteasome alpha and Mpa proteins by immunoblotting is not provided.
We show in Fig. 4 and in Fig. S3 that the specific bands detected by the anti-20Sa and by the anti-PafA antibodies are not detected in D*prcSBA* and in D*pafA* mutants, respectively, yet reappear in the complemented strains. The specificity for Mpa is partly demonstrated in Fig. 7C. While offering further evidence for both Dop- and Mpa-specific antibodies is possible, we thought it is unnecessary to present these technical subtleties in this paper.

Fig. 8/9 are premature models based on the evidence provided in this manuscript (particularly since Pup may function alone independent of 20S proteasomes).
As explained above, Pup alone cannot function to fully complement the phenotype of the D*prcSBA* mutant. The revised version explains (p. 16, lines 17-25) how this piece of data fits into the overall model presented in the Discussion.

Minor:
P3 ln 21/22: The authors state that "Bacterial proteasomes are simpler than their eukaryotic counterparts, containing only single types of alpha and beta subunits in the 20S core particle." However, bacterial proteasomes are described in which two different alpha- and two different beta-type subunits form a single 20S particle [Tamura et al. (1995). The first characterization of a eubacterial proteasome: the 20S complex of Rhodococcus. Curr Biol. 5:766-74].
The text was revised accordingly (p. 4, line 1).

P8 ln 4: Cells = Bacterial cells? please define within the manuscript on ln 4 what type of cells were studied by Blackman, 1905

Blackman studied plants and was the first to present the idea of a "growth-limiting factor".

P10 ln 2: However, whereas = awkward

The clause has been rephrased.

