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Structure of the Bacillus subtilis hibernating 100S ribosome reveals the basis for 70S dimerization

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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 15 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies again for the extended duration of the review period. Your study has now been seen by three referees and their comments are shown below.

As you will see from the reports, all three referees express interest in the findings reported in your manuscript, although they also raise a number of concerns that you will have to address before they can support publication in The EMBO Journal.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> You'll see that Ref #1 is the most critical of the three referees and that this person questions the generality of the interaction mode described in your manuscript. Consequently, the referee finds that it would significantly strengthen the study if you were to include data on disome formation in other bacterial species relying on the long form of HPF for ribosome hibernation. I realize that this may not be a trivial thing to add but I would be happy to discuss what kind of data you could envision

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including in revised manuscript.

-> Please address/clarify all technical points from ref #1 and #3 related to sample preparation, data acquisition, and analysis.

-> The referees all make suggestions for additional discussion points and paragraphs that could be rephrased. I generally agree with these points and would encourage you to incorporate them.

REFEREE REPORTS

Referee #1:

As a response to stress bacteria down-regulate protein synthesis, which is accompanied by the formation of 70S dimers, so-called 100S ribosomes. High-resolution structures previously described the mechanism of translation inhibition by protein factors involved in binding to ribosomes under stress conditions to inhibit protein synthesis, such as HPF, YifA and pY, but only low resolution structural data is available for the 70S dimerization. Abdelshahid etal. present in this manuscript structural insights of the 100S formation mediated by the long form of HPF (LHPF), which is expressed by the majority of bacteria. They provide a cryo-EM reconstruction of the complete 100S ribosome from Bacillus subtilis at medium resolution. Furthermore, they present results of additional biochemical studies to confirm the oligomeric state of LHPF and dissect the functions of the individual domains

Although understanding bacterial stress response mechanisms through regulation of translation and the formation of 100S dimers is interesting the submitted manuscript adds only incrementally to our understanding of the process. The authors demonstrate that the C-terminal domain of B. subtilis HPF is responsible for dimerization but it is not clear why dimerization occurs. It is also not clear whether the observed relative orientation of ribosomes in 100S dimers is mechanistically relevant or an artefact of sample preparation or steric constrains imposed by the structure of the ribosome. For this manuscript to be a strong candidate for publication in EMBO J the authors would have to demonstrate that the 100S dimer structure is conserved among bacterial species in which long form of HPF leads to dimerization.

Specific comments:

1) The authors have calculated various reconstruction including the complete 70S dimer (full 100S map) and a reconstruction of 30S-70S subcomplex. However, both maps are referred to be Bs100S reconstructions and the text does not specify which map was used for the interpretation. Furthermore, overall resolutions are indicated for parts of these maps (e.g. 70S-A within the 30S-70S map), which is in some cases misleading. The authors should use unambiguous naming and clearly indicate resolution for the map that was used for the interpretation.

2) In the introduction, the authors describe the stress response of E.coli, in which HPF and RMF as well as the antagonistic-acting factor YfiA are expressed in the stationary phase. These statements imply that HPF/RMF and YfiA are simultaneously expressed. Is this really the case or are they expressed under different stress conditions?

3) In the introduction, the authors describe the homology between the CTD of long HPF and RMF as weak. Can you provide some further information (size difference and sequence identity - similarity)?

4) It should be noted that T.thermophilus does not belong to the γ-proteobacteria where ribosomes dimerize to form the 100S particle mediated by the LHPF. The complex of T.thermophilus ribosome and the factors SHPF and RMF studied by Polikanov et al. is not native, but was used due to better crystallization behaviour.

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5) Does the NTD of BsHPF influence 30S conformation? Perhaps such analysis would point to a function of the NTD of LHPF in preventing binding of mRNA, tRNAs and translation factors and/or stabilizing the 30S conformation for proper 70S dimerization.

6) From inspecting the figures it seems that the CTD of LHPF is located at a position similar to the binding site of the ribosomal protein bS1. Is there density for bS1 in the cryo-EM reconstruction? Is it possible to detect bS1 in the MS analysis of the sample? Is it possible that bS1 has to be detached for LHPF binding and successful 70S dimerization.

7) The authors propose a model for the LHPF induced 100S formation, in which LHPF binds as dimer to ribosomes, which leads to dimerization of the 70S ribosomes. If this is true it should be possible to detect LHPF in other sucrose gradient fractions in addition to the 100S peak - is this the case?

8) Has a mask been applied for the final high-resolution refinements of the 30-70S map or the full 100S map or only for the amplitude correction or not at all? If yes, it should be noted in the classification scheme in figure EV2 and in material and methods section.

9) According to the section on molecular modelling, the structures of the N- and the C-terminal domains of HPF were refined in Phenix. However, no further details about the refinement strategy neither the refinement statistics are provided. Because the local resolution at the NTD and CTD differs considerably, the refinement probably had to be adjusted to the map properties. The authors should provide a more detailed description of the refinement strategy and refinement and validation statistics of the complete model and the HPF factor.

10) The local resolution plot in Figure EV3 shows many low local resolution areas on the surface of the70S-A map. This would suggest that the applied mask is too tight (masking artefact). The authors should check, if this is the case and might use a wider mask for the local resolution estimation.

Additional minor corrections and suggestions:

Introduction
- Phylogenetic analysis have revealed...

Results
- ...with a windy and blurred density... "Windy" is not a common word for describing a cryo-EM density.
- Naming error: ... 10aa (BsHPF-LΔ10AA, lacking residues 110-119) or 20aa (BsHPF-LΔ20AA, lacking residues 105-124)...

Discussion
- ...to remove LHPF (PSRP-1) from Spinach...

Material and methods
- 'A260/ml' and 'OD260/ml' should be replaced by the molarity or g/L.

Figures
- Fig 4: The 30S-B structure is grey, although indicated as orange in the figure legend. For clarification, the surface outline of the 30S-A and the 30S-B should be differently coloured.
- Fig 5: Panels E and F are wrongly labelled. Panel F is indicated twice instead of panel G.
- Fig EV5 B-C: Indicate difference between B and C (transverse section).

Referee #2:

The manuscript entitled "Structure of the Bacillus subtilis..." by Abdelshahid et al reports the 3.7 A cryo-EM structure of hibernating 70S ribosome dimers (disomes) purified from Bacillus subtilis cells grown under stress conditions (i.e., grown into stationary phase). Given the high resolution of

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the cryo-EM map, the authors were able to unambiguously model the structure of the N-terminal domain (NTD) of the long-form hibernation promoting factor (LHPF) on the basis of its homology to two proteins of known structure, Escherichia coli- YfiA and HPF. Consistent with the previously solved structures of Escherichia coli YfiA- and HPF-bound ribosomes, the authors find that the LHPF NTD binds in the intersubunit space of the ribosome, between the head- and the body domains of the small ribosomal subunit. Because binding of the LHPF NTD and its homologs at this position would sterically block the binding of an mRNA and the anticodon stem loops of the A- and P-site tRNAs, the structure rationalizes the ability of these proteins to block translation during hibernation. The authors also find that the C-terminal domain (CTD) of LHPF protrudes through the back of the platform domain of the small ribosomal subunit. Given the high resolution of the cryo-EM map, the authors are once again able to unambiguously model the structure of the LHPF CTD on the basis of its homology to a protein of known structure, the Clostridium acetobutylicum LHFP CTD. Importantly, the authors were able to unambiguously model the dimer form of the Clostridium acetobutylicum LHFP CTD into the cryo-EM density of the LHPF CTD, demonstrating that a CTD-CTD dimerization interaction mediates formation of the disome. Based on the structures, the authors then prepared a series of LHPF mutants in which the linker between the NTD and the CTD as well as the CTD itself are mutagenized and assessed the functional activities of the mutants using sucrose gradient ultracentrifugation assays and cell growth assays. The authors conclude the manuscript by proposing a mechanism for LHPF-induced disome formation and ribosome hibernation.

The cryo-EM data reported in this manuscript are of a very high quality and high resolution, the modeling has been carefully done and is justified given the high resolution of the cryo-EM map, and the structures are carefully interpreted to generate a proposed model for the mechanism of LHPF-mediated disome formation. Moreover, the structure-based mutational and functional analyses provide a relatively strong validation of the structures and the authors interpretations of the structures. As such, the work reported here represents a significant advance in the field and in our understanding of the mechanism through which ribosomes hibernate during stress conditions. Given all of this, I would recommend publication of this manuscript in EMBO J after the authors address the following minor comment:

Can the authors comment on whether the 70S ribosomes in the disomes are in the rotated or unrotated subunt conformations? Related to this, do the authors think that intersubunit rotation might play a role in the mechanism depicted in Fig 7 and in regulating ribosome hibernation?

Referee #3:

This well-written paper describes the molecular mechanism by which a long-form hibernation-promoting factor (HPF) promotes dimerization of Bacillus subtilis 70S ribosomes into an inactive 100S disome. The authors present a 6 Å cryo-EM map of the disome (and a 3.7 Å map of a masked monosome). These structures show that the N-terminal domain of B. subtilis HPF binds at the intersubunit interface to inhibit translation (similar to the short-form HPF present in Escherichia coli), while the C-terminal domain, which is absent in the E. coli homolog, is directly involved in forming the dimer interface by forming a homo-dimer. This interface and general mechanism is distinct from the formation of 100S species in Escherichia coli, which requires an additional factor (RMF; ribosome modulation factor). The structure has implications for the development of antibiotics specific to particular bacteria.

Comments

1. In Figure EV2 it is clear that particles are initially picked as individual 70S ribosomes. This yields a final dataset of 24,516 particles, which display some density for a neighboring ribosome. These particles are extracted in a larger box size and used to generate the model of the disome. However, does this mean that the same disome may be included twice in the same dataset? Do 24,516 70S particles mean 12,258 disome particles? Would including the same data twice artificially inflate the resolution estimate? If only unique disomes are included, what is the final resolution?

2. The results of the size-exclusion chromatography (SEC) experiments are not particularly conclusive, with many of the species running at a higher molecular weight than expected and some peaks display shoulders. Could an alternative approach such as multi-angle light scattering or

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analytical ultracentrifugation provide better confirmation that B. subtilis HPF exists as a dimer in solution? Table 3G should have an additional column for the apparent stoichiometry and it should be made clear that the "actual" mass refers to that of a monomer.

3. Please include a sequence-to-structure alignment (e.g. PROMALS3D) for B. subtilis HPF NTD to PDB ID 4V8H and B. subtilis HPF CTD to PDB ID 3KA5 to help assess the quality of the homology model.

4. "electron density" should be replaced with "map density" or equivalent.

5. The description of the density for 70S-B as "windy and blurred" should be revised. Blurred would be sufficient.

6. The observation that the CTD forms a homodimer with the CTD of HPF from the second 70S ribosome is described as being "unexpected" and the presence of a dimer of Clostridium acetobutylicum HPF CTD in the crystal structure as "curious". However, does the presence of a dimer in the crystal structure not immediately suggest a possible mechanism for HPF-mediated dimerization of 70S ribosomes?

7. The first paragraph of the discussion is better suited to the introduction. The current introduction makes no mention of (p)ppGpp.

8. In the section "Binding assay for BsHPF variants with pelleted ribosomes" there is duplication of the word protein.

9. In the section "Sucrose density gradient centrifugation analysis" the reference {Akanuma, 2016 #17016} is unformatted.

10. Out of curiosity, how was the acceleration voltage of the Titan Krios calibrated at 302 kV?

11. In Figure 2D are the mRNAs/tRNAs drawn to the same scale as the proteins in panels B and C? A better way of demonstrating the overlap between the NTD of HPF and the tRNAs should be considered.

12. Figure 4A-B, actually shows 3 views of the interface, not 2.

13. Is the anti-Shine Dalgarno sequence disordered in the structure?

14. Figure 7 (the model of HPF-induced dimerization) is unnecessary as the structure presented in the manuscript tells us little about how 100S assembles or disassembles. The model is also potentially misleading - for example panel B seems to suggest that the CTDs remain undocked from the ribosome until two 70S ribosomes have been recruited. It is likely that assembly is more nuanced.

15. Are all ribosomes in the unrotated state? Were disome conformations identified during in silico classification?

16. For ease of comparison and to avoid being misleading, all ResMap figures should be shown with the same scale.

17. Do any of the NTD sidechains have well-resolved density? If so, the density and fitted model should be shown.

1st Revision - authors' response 20 March 2017

General Response to Referees:

We would like to thank all the Referees for their time and efforts in reviewing our manuscript and for their constructive criticisms. We believe this has significantly improved the manuscript and hope

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that you also find the revised version much improved. Please note that in addition to the changes below we have all updated Figure 2B to include the binding data for the F160E mutant and added additional linker mutants into Figure EV5C-E. Thanks again.

Referee #1:
As a response to stress bacteria down-regulate protein synthesis, which is accompanied by the formation of 70S dimers, so-called 100S ribosomes. High-resolution structures previously described the mechanism of translation inhibition by protein factors involved in binding to ribosomes under stress conditions to inhibit protein synthesis, such as HPF, YifA and pY, but only low resolution structural data is available for the 70S dimerization. Abdelshahid et al. present in this manuscript structural insights of the 100S formation mediated by the long form of HPF (LHPF), which is expressed by the majority of bacteria. They provide a cryo-EM reconstruction of the complete 100S ribosome from Bacillus subtilis at medium resolution. Furthermore, they present results of additional biochemical studies to confirm the oligomeric state of LHPF and dissect the functions of the individual domains

Although understanding bacterial stress response mechanisms through regulation of translation and the formation of 100S dimers is interesting the submitted manuscript adds only incrementally to our understanding of the process.

Obviously, we disagree that our manuscript adds only incrementally to our understanding of the process. We present the first structure of a 100S from a Gram-positive bacteria showing that the mechanism of dimerization is completely different to that reported for E.coli.

The authors demonstrate that the C-terminal domain of B. subtilis HPF is responsible for dimerization but it is not clear why dimerization occurs.

Rather than inducing a head rotation to promote back-to-back dimerization as proposed for E.coli HPF activity, we observe that LHPF is a dimer in solution and on the ribosome. Therefore, dimerization of ribosomes by LHPF can clearly facilitate disome formation via direct interaction between the two monomers in the LHPF dimer, each of which is tethered to separate ribosomes. This is distinct from the situation in E.coli where dimerization is proposed to occur via ribosome-ribosome interactions induced by conformational changes upon HPF and RMF binding.

It is also not clear whether the observed relative orientation of ribosomes in 100S dimers is mechanistically relevant or an artefact of sample preparation or steric constrains imposed by the structure of the ribosome.

The 100S sample was taken directly from stationary phase B. subtilis lysates, a condition where disome formation takes place, and therefore we do not see why the structure should be an artifact of sample preparation. We have previously determined structures of B. subtilis 70S ribosomes and never observed dimers so there is no reason to suspect that it is something to do with sample preparation. Therefore, we believe the observed dimerization is mechanistically relevant. Moreover, we generated BsHPF variants including a mutant in the CTD that prevents LHPF dimerization, which also prevents 100S formation in vivo, thus validating our structural findings.

For this manuscript to be a strong candidate for publication in EMBO J the authors would have to demonstrate that the 100S dimer structure is conserved among bacterial species in which long form of HPF leads to dimerization.

We respectfully disagree. The work involved in determining another structure of a 100S from a different bacterial species would represent a completely new project. We also do not believe it would significantly change the outcome of our findings since the side-to-side orientation that we observe for B. subtilis 100S is similar to that observed in negative stain images of 100S from another Gram-positive bacteria, Lactococcus lactis (Puri et al, 2014):

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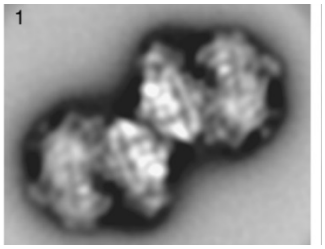

Specific comments:
1) The authors have calculated various reconstruction including the complete 70S dimer (full 100S map) and a reconstruction of 30S-70S subcomplex. However, both maps are referred to be Bs100S reconstructions and the text does not specify which map was used for the interpretation. Furthermore, overall resolutions are indicated for parts of these maps (e.g. 70S-A within the 30S-70S map), which is in some cases misleading. The authors should use unambiguous naming and clearly indicate resolution for the map that was used for the interpretation.

The reconstruction of the complete 100S is not introduced into the text until the last section of the results on page 10. Therefore, all prior text, figures and interpretation relate to the only cryo-EM reconstruction that is presented up until that point, namely, the 30S-70S subcomplex. Therefore, we did not see the need to provide distinct nomenclature, especially when the complete 100S reconstruction was performed simply to be able to compare the full orientation of the 70S monomers in the 100S with that determined previously for Ec100S or polysomes and is presented in the last section of the results.

We disagree that we are misleading with respect to the indication of resolution. We provide overall resolutions for the two cryo-EM maps and provide ResMap images showing overall local resolution Figure EV3 for the 30S-70S subcomplex and Figure EV5 for the complete 100S. Moreover, we show local resolutions for the NTD and CTD of LHPF for the 30S-70S subcomplex, which is used for interpretation in all figures. In the text on page 5, we explicitly state that while the local average resolution of the cryo-EM map is 3.7 Å, "local resolution calculations indicate that the resolution for 70S-A monomer ranges in the core between 3.5-5.0 Å, whereas, as expected, the resolution for 70S-B is worse, ranging between 5.0-10 Å". Moreover, we specifically state in the text on page 6 that the local resolution of the NTD of BsHPF is between 3.5-5 Å and on page 7 that "the limited resolution of the BsHPF-CTD (Fig EV3F-G) does not allow a detailed analysis of the contacts with the ribosomal components to be made".

2) In the introduction, the authors describe the stress response of E.coli, in which HPF and RMF as well as the antagonistic-acting factor YfiA are expressed in the stationary phase. These statements imply that HPF/RMF and YfiA are simultaneously expressed. Is this really the case or are they expressed under different stress conditions?

It could be that they are differentially expressed under different stress conditions, however, the sentence refers to stationary phase where all three are simultaneously present. This is re-emphasized by the second part of the sentence describing how YfiA is antagonistic to HPF/RMF mediated 100S formation.

3) In the introduction, the authors describe the homology between the CTD of long HPF and RMF as weak. Can you provide some further information (size difference and sequence identity - similarity)?

In the introduction, we state that the CTD of long HPF "was proposed to have weak homology with RMF (Ueta et al, 2010)". This refers to the report from Ueta et al 2010 where they provide a sequence alignment in Figure 6 (see below) showing the "weak homology". As we explain later in the manuscript, this "weak homology" is not borne out by our structure.

Figure 6 alignment from Ueta et al, 2010:

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4) It should be noted that T.thermophilus does not belong to the γ-proteobacteria where ribosomes dimerize to form the 100S particle mediated by the LHPF. The complex of T.thermophilus ribosome and the factors SHPF and RMF studied by Polikanov et al. is not native, but was used due to better crystallization behaviour.

We agree with the referee that the complex of E. coli HPF and RMF with T. thermophilus 70S ribosomes is not native and was used for crystallization because of ease, however, we still think that the authors used this system to provide insight into how E. coli SHPF and RMF dimerize 70S ribosomes and inactivate translation in γ-proteobacteria, as stated.

5) Does the NTD of BsHPF influence 30S conformation? Perhaps such analysis would point to a function of the NTD of LHPF in preventing binding of mRNA, tRNAs and translation factors and/or stabilizing the 30S conformation for proper 70S dimerization.

We do not observe subunit or head rotation as observed in the crystal structure of E.coli HPF. Just to reiterate, the mechanism reported here for LHPF mediated 100S formation is distinct from that reported for SHPF – no head movement is required but rather dimerization is mediated via direct interaction between the CTDs of the LHPF. The 30S conformation is similar to that observed for the classical B.subtilis post-translocational state ribosome reported previously (Sohmen et al., 2015). Therefore, we conclude that sterically overlapping with the position of mRNA, tRNAs etc is a more likely explanation for the observed inhibition on translation, as stated in the manuscript and illustrated in Figure 2D.

6) From inspecting the figures it seems that the CTD of LHPF is located at a position similar to the binding site of the ribosomal protein bS1. Is there density for bS1 in the cryo-EM reconstruction? Is it possible to detect bS1 in the MS analysis of the sample? Is it possible that bS1 has to be detached for LHPF binding and successful 70S dimerization.

B. subtilis S1 lacks domains 1 and 2 compared to E.coli S1 and is thus not ribosome associated like E.coli S1. Therefore, as expected, we do not observe S1 on the ribosome by cryo-EM or MS, and also did not observe S1 in our previous reconstructions of translating B.subtilis 70S ribosomes (Sohmen et al., 2015). For this reason, we do not believe that bS1 needs to detach for LHPF binding and successful dimerization.

7) The authors propose a model for the LHPF induced 100S formation, in which LHPF binds as dimer to ribosomes, which leads to dimerization of the 70S ribosomes. If this is true it should be possible to detect LHPF in other sucrose gradient fractions in addition to the 100S peak - is this the case?

Yes, LHPF has been detected also in 70S fractions (Ueta et al., 2013).

8) Has a mask been applied for the final high-resolution refinements of the 30-70S map or the full 100S map or only for the amplitude correction or not at all? If yes, it should be noted in the classification scheme in figure EV2 and in material and methods section.

Yes, a 70S-30S ribosome mask was applied for the final high-resolution refinement and this is now mentioned in the materials and methods.

9) According to the section on molecular modelling, the structures of the N- and the C-terminal domains of HPF were refined in Phenix. However, no further details about the refinement strategy neither the refinement statistics are provided. Because the local resolution at the NTD and CTD differs considerably, the refinement probably had to be adjusted to the map properties. The authors should provide a more detailed description of the refinement strategy and refinement and validation statistics of the complete model and the HPF factor.

We have now included a more detailed description of the refinement strategy in the Methods section,

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a table of statistics as well as an overfitting analysis in Figure EV3. Refinement was only performed on the higher resolution 70S-30S map and included models for the 30S and 50S subunits of the 70S-A monomer as well as the NTD of HPF. Due to the limited resolution of the CTD, we present only a rigid-body fit of a homology model of CTD dimer to the density.

10) The local resolution plot in Figure EV3 shows many low local resolution areas on the surface of the70S-A map. This would suggest that the applied mask is too tight (masking artefact). The authors should check, if this is the case and might use a wider mask for the local resolution estimation.

We thank the reviewer for pointing this out. Indeed, the mask was not aligned properly. This has been corrected and the corresponding images have been updated.

Additional minor corrections and suggestions:
Introduction
- Phylogenetic analysis have revealed...

"Analyses" is plural for analysis.

Results
- ...with a windy and blurred density... "Windy" is not a common word for describing a cryo-EM density.

We have removed the term "Windy"

- Naming error: ... 10aa (BsHPF-LΔ10AA, lacking residues 110-119) or 20aa (BsHPF-LΔ20AA, lacking residues 105-124)...

We have corrected BsHPF-LΔ10AA to BsHPF-LΔ20AA as appropriate.

Discussion
- ...to remove LHPF (PSRP-1) from Spinach...

We have corrected PRSP-1 to PSRP-1.

Material and methods
- 'A260/ml' and 'OD260/ml' should be replaced by the molarity or g/L.

We disagree. Ribosome concentration is traditionally reported as OD_{260}/ml, however, we have unified A_{260}/ml to OD_{260}/ml through-out the text to be consistent.

Figures
- Fig 4: The 30S-B structure is grey, although indicated as orange in the figure legend. For clarification, the surface outline of the 30S-A and the 30S-B should be differently coloured.

We have added a dashed outline of 30S-B to distinguish it from the 30S-A.

- Fig 5: Panels E and F are wrongly labelled. Panel F is indicated twice instead of panel G.

We have now corrected the legends to match the panels.

- Fig EV5 B-C: Indicate difference between B and C (transverse section).

Text to the legend is changed now to indicate the difference between B and C.

Referee #2:
The manuscript entitled "Structure of the Bacillus subtilis..." by Abdelshahid et al reports the 3.7 A cryo-EM structure of hibernating 70S ribosome dimers (disomes) purified from Bacillus subtilis cells grown under stress conditions (i.e., grown into stationary phase). Given the high resolution of the cryo-EM map, the authors were able to unambiguously model the structure

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of the N-terminal domain (NTD) of the long-form hibernation promoting factor (LHPF) on the basis of its homology to two proteins of known structure, Escherichia coli- YfiA and HPF. Consistent with the previously solved structures of Escherichia coli YfiA- and HPF-bound ribosomes, the authors find that the LHPF NTD binds in the intersubunit space of the ribosome, between the head- and the body domains of the small ribosomal subunit. Because binding of the LHPF NTD and its homologs at this position would sterically block the binding of an mRNA and the anticodon stem loops of the A- and P-site tRNAs, the structure rationalizes the ability of these proteins to block translation during hibernation. The authors also find that the C-terminal domain (CTD) of LHPF protrudes through the back of the platform domain of the small ribosomal subunit. Given the high resolution of the cryo-EM map, the authors are once again able to unambiguously model the structure of the LHPF CTD on the basis of its homology to a protein of known structure, the Clostridium acetobutylicum LHFP CTD. Importantly, the authors were able to unambiguously model the dimer form of the Clostridium acetobutylicum LHFP CTD into the cryo-EM density of the LHPF CTD, demonstrating that a CTD-CTD dimerization interaction mediates formation of the disome. Based on the structures, the authors then prepared a series of LHPF mutants in which the linker between the NTD and the CTD as well as the CTD itself are mutagenized and assessed the functional activities of the mutants using sucrose gradient ultracentrifugation assays and cell growth assays. The authors conclude the manuscript by proposing a mechanism for LHPF-induced disome formation and ribosome hibernation. The cryo-EM data reported in this manuscript are of a very high quality and high resolution, the modeling has been carefully done and is justified given the high resolution of the cryo-EM map, and the structures are carefully interpreted to generate a proposed model for the mechanism of LHPF-mediated disome formation. Moreover, the structure-based mutational and functional analyses provide a relatively strong validation of the structures and the authors interpretations of the structures. As such, the work reported here represents a significant advance in the field and in our understanding of the mechanism through which ribosomes hibernate during stress conditions. Given all of this, I would recommend publication of this manuscript in EMBO J after the authors address the following minor comment:

Can the authors comment on whether the 70S ribosomes in the disomes are in the rotated or unrotated subunt conformations?

The 70S ribosomes are in a classic non-rotated state as observed in the previous B.subtilis 70S ribosome structure (Sohmen et al., Nat Comm 2015). This is now mentioned on page 5.

Related to this, do the authors think that intersubunit rotation might play a role in the mechanism depicted in Fig 7 and in regulating ribosome hibernation?

Because we do not observe intersubunit rotation, nor head swivel, we do not think that these movements play a significant role in LHPF-mediated 100S formation, in contrast to E.coli HPF-mediated 100S formation where head swivel was proposed to promote dimerization. We have also mentioned this on page 5

Referee #3:
This well-written paper describes the molecular mechanism by which a long-form hibernation-promoting factor (HPF) promotes dimerization of Bacillus subtilis 70S ribosomes into an inactive 100S disome. The authors present a 6 Å cryo-EM map of the disome (and a 3.7 Å map of a masked monosome). These structures show that the N-terminal domain of B. subtilis HPF binds at the intersubunit interface to inhibit translation (similar to the short-form HPF present in Escherichia coli), while the C-terminal domain, which is absent in the E. coli homolog, is directly involved in forming the dimer interface by forming a homo-dimer. This interface and general mechanism is distinct from the formation of 100S species in Escherichia coli, which requires an additional factor (RMF; ribosome modulation factor). The structure has implications for the development of antibiotics specific to particular bacteria.

Comments
1. In Figure EV2 it is clear that particles are initially picked as individual 70S ribosomes. This yields a final dataset of 24,516 particles, which display some density for a neighboring ribosome. These particles are extracted in a larger box size and used to generate the model of

the disome. However, does this mean that the same disome may be included twice in the same dataset? Do 24,516 70S particles mean 12,258 disome particles? Would including the same data twice artificially inflate the resolution estimate? If only unique disomes are included, what is the final resolution?

The referee has raised a valid point, which we had not considered because the 100S reconstruction was simply performed to obtain the relative orientation of the two disomes to compare with the previous structures of E. coli disomes and polysomes. This map was not used otherwise for molecular interpretation therefore we were not concerned about influence of particle number on the resolution. Nevertheless, to address the reviewers concerns we have re-analyzed the particles comprising the final disome population. It turns out that 5511 particles were within close proximity of another particle so as to be potential disome particles and were as the reviewer correctly pointed out, used twice in the same dataset. After removal of these 5511 particles, we recalculated a cryo-map and observed a slight decrease in the resolution as predicted by the reviewer. Thus, if only unique disomes are included, the final resolution is 6.2 Å rather than 6.05 Å. *We have now mentioned this in the Materials and methods and updated the text and Figure EV5 to reflect this.*

2. The results of the size-exclusion chromatography (SEC) experiments are not particularly conclusive, with many of the species running at a higher molecular weight than expected and some peaks display shoulders. Could an alternative approach such as multi-angle light scattering or analytical ultracentrifugation provide better confirmation that B. subtilis HPF exists as a dimer in solution?

We agree with the reviewer that the SEC data was not particularly conclusive and therefore have now performed static light scattering (SLS) to complement the SEC data. The SLS data are now presented in Figure 3G and confirm that the CTD is a dimer in solution. Moreover, the SLS data also show that the mutation F160E in the CTD abolishes dimerization of HPF, which is consistent with our in vivo data showing the absence of 100S formation.

Table 3G should have an additional column for the apparent stoichiometry and it should be made clear that the "actual" mass refers to that of a monomer.

We have now included the apparent stoichiometries in Figure 3G as requested

3. Please include a sequence-to-structure alignment (e.g. PROMALS3D) for B. subtilis HPF NTD to PDB ID 4V8H and B. subtilis HPF CTD to PDB ID 3KA5 to help assess the quality of the homology model.

We have now included the sequence alignments as requested in a new Figure EV4.

4. "electron density" should be replaced with "map density" or equivalent.

We replaced all occurrences of electron density with map density as requested

5. The description of the density for 70S-B as "windy and blurred" should be revised. Blurred would be sufficient.

We have removed the term windy as suggested.

6. The observation that the CTD forms a homodimer with the CTD of HPF from the second 70S ribosome is described as being "unexpected" and the presence of a dimer of Clostridium acetobutylicum HPF CTD in the crystal structure as "curious". However, does the presence of a dimer in the crystal structure not immediately suggest a possible mechanism for HPF-mediated dimerization of 70S ribosomes?

The presence of a dimer for the LHPF CTD in the crystal structure does indeed raise the possibility that it might be a dimer in solution and/or on the ribosome. However, it does not prove it. In our manuscript, we prove that it is a dimer in solution as well as on the ribosome and importantly show that dimerization of the CTD is essential for 100S formation.

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7. The first paragraph of the discussion is better suited to the introduction. The current introduction makes no mention of (p)ppGpp.

We feel that the introduction is already long enough and introduces the main points necessary for interpretation of the results. The introduction of ppGpp in the discussion is to put the results into the overall context of the literature and regulation. Therefore, we would rather leave it as it is unless the referee/editors strongly disagree.

8. In the section "Binding assay for BsHPF variants with pelleted ribosomes" there is duplication of the word protein.

We have corrected the duplication

9. In the section "Sucrose density gradient centrifugation analysis" the reference {Akanuma, 2016 #17016} is unformatted.

We have formatted the reference.

10. Out of curiosity, how was the acceleration voltage of the Titan Krios calibrated at 302 kV?

This was a typo and was corrected to 300kV.

11. In Figure 2D are the mRNAs/tRNAs drawn to the same scale as the proteins in panels B and C? A better way of demonstrating the overlap between the NTD of HPF and the tRNAs should be considered.

The scale of 2D was smaller than B and C. We have now rearranged the figure to use similar sizes and the same orientation.

12. Figure 4A-B, actually shows 3 views of the interface, not 2.

We have reworded the legend to avoid this complication.

13. Is the anti-Shine Dalgarno sequence disordered in the structure?

The 3' end of the 16S rRNA containing the anti-Shine-Dalgarno sequence is ordered and can be traced to nucleotide 1551, which can be seen in panel B of Figure EV4.

14. Figure 7 (the model of HPF-induced dimerization) is unnecessary as the structure presented in the manuscript tells us little about how 100S assembles or disassembles. The model is also potentially misleading - for example panel B seems to suggest that the CTDs remain undocked from the ribosome until two 70S ribosomes have been recruited. It is likely that assembly is more nuanced.

We feel that Figure 7 is needed to present a model for LHPF mediated 100S formation based on the results from the manuscript but also to highlight the regulatory aspects that still remain to be investigated. However, we agree that panel B could be potentially misleading and have therefore simplified Figure 7.

15. Are all ribosomes in the unrotated state? Were disome conformations identified during in silico classification?

All ribosomes were in the non-rotated state. This is mentioned now page 5. We did not observe any disomes with rotated ribosome conformations.

16. For ease of comparison and to avoid being misleading, all ResMap figures should be shown with the same scale.

All ResMap figures are now shown with the same scale.

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17. Do any of the NTD sidechains have well-resolved density? If so, the density and fitted model should be shown.

Some of the large and bulky sidechains of the NTD have well-resolved density. The map density and fitted model are now shown in Figure 2B and Supplementary Figure EV3.

2nd Editorial Decision 24 March 2017

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by one of the original referees whose comments are shown below. As you will see the referee finds that all major criticisms have been sufficiently addressed and recommend the manuscript for publication, pending minor text revision. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revised version.

-> Please provide an accession code for the new structure data generated. This can be inserted as a placeholder for now and updated with the actual number at proof stage.

-> During our routine image check we noticed a splice mark in the gel depicted in fig 5A, suggesting that the bands shown were not run on the same gel. Could you please include a line separator to indicate that this is not a continuous gel? In addition, please provide us with the raw image files as source data.

-> We generally accommodate up to 5 typeset EV figures and I noticed that you currently have 6 in this manuscript. Would it be possible to combine figures EV1 and EV4? I realize that this may break up the flow of the figures slightly but since they are both fairly small and 'simple' images it should be possible to do.

-> Please provide the three EV tables as separate .docx files (currently part of the main manuscript text file)

-> We noticed that figure EV2 displays labels A-D but that the legend only describes A-C. Could either add a briefly description of 'D' in the legend or remove the letter from the figure?

REFEREE REPORTS

Referee #3:

The authors have suitably addressed my comments and I believe the paper is now ready to publish. I have just two additional comments:

1) Having read the other reviewers' comments, I have to agree with referee #1 that the nomenclature could be confusing and that the masked 70S+30S subcomplex should not be called Bs100S. Currently, the manuscript contains the sentence "subsequent refinement yielded a cryo-EM reconstruction of the Bs100S with an average resolution of 3.8A" and then later a very similar statement but with a different resolution "we were able to obtain a reconstruction of the Bs100S with an average resolution of 6.2A". This is clearly confusing.

2) Figure 7 is much improved. However, it still suggests that ribosome splitting only occurs once LHPF has dissociated. The authors acknowledge in the discussion that recycling factors may be involved, in which case the pathway could be 100S -> 50S +30S.

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2nd Revision - authors' response 26 March 2017

Response to Editor:

-> Please provide an accession code for the new structure data generated. This can be inserted as a placeholder for now and updated with the actual number at proof stage.

We will deposit the cryo-EM map in the EMDB and the molecular model in the PDB. The accession codes will be uploaded with the actual number in the proof as suggested. We have inserted a place-holder in the text on page 19.

-> During our routine image check we noticed a splice mark in the gel depicted in fig 5A, suggesting that the bands shown were not run on the same gel. Could you please include a line separator to indicate that this is not a continuous gel? In addition, please provide us with the raw image files as source data.

Although there is indeed a splice mark in the gel, the bands shown were all run on the same gel. The gel was simply spliced to remove some lanes. We have nevertheless included a line separator as requested and provided the raw image as source data.

-> We generally accommodate up to 5 typeset EV figures and I noticed that you currently have 6 in this manuscript. Would it be possible to combine figures EV1 and EV4? I realize that this may break up the flow of the figures slightly but since they are both fairly small and 'simple' images it should be possible to do.

We have now relocated EV4A,B to Figure EV1 as panel C-D as requested and adjusted the text accordingly.

-> Please provide the three EV tables as separate .docx files (currently part of the main manuscript text file)

We have now provided the three EV tables as separate .docx files as requested.

-> We noticed that figure EV2 displays labels A-D but that the legend only describes A-C. Could either add a briefly description of 'D' in the legend or remove the letter from the figure?

We have removed "D" from the figure and re-adjusted A-C accordingly.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

We now provide the source data for the blots in Figure 5A and 5B.

Response to Referee #3:

The authors have suitably addressed my comments and I believe the paper is now ready to publish. I have just two additional comments:

1) Having read the other reviewers' comments, I have to agree with referee #1 that the nomenclature could be confusing and that the masked 70S+30S subcomplex should not be called Bs100S. Currently, the manuscript contains the sentence "subsequent refinement yielded a cryo-EM reconstruction of the Bs100S with an average resolution of 3.8A" and then later a very similar statement but with a different resolution "we were able to obtain a

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reconstruction of the Bs100S with an average resolution of 6.2A". This is clearly confusing.

We have now changed the sentence to "subsequent refinement yielded a cryo-EM reconstruction of the Bs70S-30S subcomplex with an average resolution of 3.8A" to avoid any confusion. We have also changed the legends accordingly in Figure 1-4 and EV2-3 to include "Bs70S-30S subcomplex" rather than "Bs100S".

2) Figure 7 is much improved. However, it still suggests that ribosome splitting only occurs once LHPF has dissociated. The authors acknowledge in the discussion that recycling factors may be involved, in which case the pathway could be 100S -> 50S +30S.

The referee is correct. We have amended Figure 7 to include both possible pathways, 100S -> 70S -> 30S + 50S or 100S -> 30S + 50S

3rd Editorial Decision 29 March 2017

Thank you for submitting the final revision of your manuscript, I am pleased to inform you that the study has now been officially accepted for publication in The EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Daniel Wilson
Journal Submitted to:	EMBO J
Manuscript Number:	EMBOJ-2016-96189R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	

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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Accession code will be added to the proof
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

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