THE EMBO JOURNAL

Manuscript EMBO-2011-79781

Soj/ParA stalls DNA replication by inhibiting helix formation of the initiator protein DnaA

Graham Scholefield, Jeff Errington, Heath Murray

Corresponding author: Heath Murray, University of Newcastle

Review timeline:

Submission date:	10 October 2011
Editorial Decision:	02 November 2011
Revision received:	20 December 2011
Accepted:	22 December 2011

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 02 November 2011

Thank you again for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of three expert referees, which are copied below. I am pleased to inform you that all reviewers consider your findings of interest and in principle suited for publication in The EMBO Journal, pending adequate addressing of a number of specific points raised in the reports.

I would thus like to invite you to prepare a new version of the manuscript, revised along the lines of the referees' comments copied below. When preparing your letter of response, please be reminded that our policy to allow only a single round of major revision will necessitate diligent and comprehensive answering; please further bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication (for more details on our Transparent Editorial Process initiative, 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.

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The paper makes a significant advance in understanding regulation of replication initiation in bacteria. DnaA, which perhaps regulates replication in all bacteria, forms oligomers in vitro, the physiological relevance of which was unknown before this study. Here the authors not only show that oligomerization can happen in vivo but it can be a regulatory event: their formation was directly correlated with the initiation efficiency. The oligomerization of DnaA is implicated in the opening of origin DNA, a fundamental requirement in any DNA transaction on duplex DNA. The topic of the paper is thus likely to be of interest to a wide readership.

Some of the specific contributions of the paper are: Identification of the region of DnaA that contacts the regulator of Soj; DnaA oligomerization on both single- and double-stranded DNA. This should help narrow the models on origin opening; Inhibition of DnaA oligomerization by monomeric Soj through regulating the activity of AAA+ domain, and not through the oligomerization domain I. The authors may like to comment whether the domain I, although not necessary, can still help oligomerization, which was missed in the present analysis due to the use of cross-linking. Another pleasing aspect of the paper was the congruence seen between the in vivo and in vitro results.

Minor points:

1. L.20: Add 'normal' before cell growth. Mutants defective in control such as SeqA grow more or less fine.
2. L.80: Titration by DnaA boxes distributed throughout the chromosome is definitely a mechanism to reduce DnaA availability to origin and should be mentioned.
3. Sup Figure 1: Change DnaA bypass to DnaA suppressor (Red dot legend).
4. L.153: "Homo-oriented surface" - do you mean DnaA is homo? If so, say homo-oriented DnaA surface?
5. Figures 1C vs. 2B: Phenotypes of 341V was similar to 323D in Fig. 1C but in Fig. 2B. The same is true in Fig. S6A. May note this and comment. Similarly, try to comment how the oligomerization of R379A (Figure 4B) was so much better. I gather the ssDNA was not oriC specific DUE.
6. Figure 2D is not mentioned in Results.
7. L.480: Add Fogel & Waldor 2006 G&D 20:3269. Pulling mechanism was first proposed in this study.
8. L.512: The Discussion might include what happens to replication in WT and delta(soj-spoOJ) cells. If regulating oligomerization is such a big deal for controlling replication initiation frequency, what happens to E. coli and the likes that do not have ParA? From the known facts, how the oligomerization could be controlled without ParA would make the Discussion more comprehensive. Include the paper from the Berger Lab that just came out in Nature.

Referee #2 (Remarks to the Author):

DnaA is an essential regulator of DNA replication initiation and its function must be tightly regulated in time. This manuscript examines the mechanism by which the ParA homolog Soj inhibits DnaA function in Bacillus subtilis. The authors combined genetics and in vitro assays to convincingly show that Soj interacts with DnaA directly. Using a crosslinking assay, they showed that both double- and single-stranded DNA stimulate DnaA oligomerization. Using mutants, they found that monomeric but not dimeric Soj inhibits DnaA from forming DNA-stimulated oligomeric species, even though both forms of Soj bind DnaA. They also provided in vivo data consistent with this idea. Finally, they demonstrated that the firing frequency in vivo correlates with the extent of DnaA oligomerization in vitro, implicating the role of DnaA oligomerization in replication initiation.

Overall, this is a thorough study that provides significant conceptual advance to 1) the role of DnaA oligomerization in replication initiation, and 2) the mechanism underlying Soj-mediated DnaA regulation. I recommend publication assuming that the following comments will be addressed.

- The abstract is not very informative and should be revised.

- Fig. S8B is not very convincing in showing that monomeric Soj inhibits DnaA oligomerization without DNA.

- Also, the authors seem to imply that DnaA oligomers with and without DNA form a similar helical structure. Is that true?

- Lines 266-267: It is not clear in Fig. 5A that DnaA(A341V) shows an intermediate effect; one could even argue that it enhances the Soj(G12V) inhibition at least in terms of abundance of oligomers (at the lowest concentration of Soj(G12V))

- How is Fig. 4B compatible with the idea that DnaA binds to its boxes and oligomerizes from there since pUC18 (lacking DnaA boxes) stimulates oligomer complex as well as pBsOriC4 (carrying the origin region with DnaA boxes)? How does DnaA specifically distinguish the DNA origin regions in vivo?

- Fig. S7B is inconsistent with Felczak et al, 2005. Could it be a difference between E. coli and B. subtilis DnaA or do the authors have reasons to believe that the previous work is incorrect? An explanation should be provided.

- Discussion: It is proposed that WT Soj would primarily be in monomeric form in vivo. What would prevent Soj from dimerizing? In vitro, the dimer form is predominant in the presence of ATP; so the expectation would be for Soj to be primarily in dimeric form. What is the oligomer state of DnaAcc in wild-type cells, soj deletion cells and soj-overexpressing cells?

- Discussion: The authors seem to downplay the role of ParA in chromosome segregation in Vibrio. Does the Kadoya et al 2011 work refute the work by the Waldor's lab? If so, the authors should explain it to the readers. The ParA localization in Vibrio and its dynamics in relation to ParB localization are more consistent with that of Caulobacter than that of Bacillus subtilis, which is consistent with a role in chromosome segregation. Also, the authors argue that it is difficult to imagine how ParA could act in segregation in bacteria that have more than one replication initiation event per the cell cycle (as in Bacillus subtilis unlike in Caulobacter). However, segregation still seems to occur in Caulobacter filamentous cells that have multiple origins (Sliusarenko et al, Mol Micro 2011). So the multiplicity of origins doesn't seem to be a problem. The authors' proposal that ParA's role in DNA replication came first and that its role in segregation is limited to bacteria with particular cell cycles seem unjustified.

Minor comments:
- lines 78-79: What is the SeqA literature in Caulobacter?

-lines 108: what does "related oligomers" mean? Not clear at this stage.

- Duderstadt et al 2010 reference is not complete.

- Fig. 2D is not discussed.

- Fig. 6A: It would be good to show by western blot that there were similar amounts of overexpressed mutant proteins.

- Figure 6C and D. Axis label. % of DnaA 'in' an oligomer (note that oligomer was misspelled as oliogmer multiple times in this article).

Referee #3 (Remarks to the Author):

The manuscript by Murray and Errington builds off their earlier discovery that the activity of the B. subtilis DnaA protein, which helps mediate the initiation of DNA replication, is regulated by a ParA-class ATPase known as Soj. In this present study, the authors use a combination of biochemical and genetic assays (including a particularly clever cell-based crosslinking trick) to

convincingly show that monomeric Soj can interact with DnaA to disrupt its higher-order assembly into a helical filament both in vitro and in vivo. Analysis of DnaA suppressor mutations against a constitutively-active form of Soj pinpoint the likely site of Soj binding, and further identify new mutations in DnaA that map to subunit/subunit interfaces to stabilize higher-order initiator oligomers. The paper is clearly written and technical quality of the experiments excellent; the findings not only support the idea that replication initiators are AAA+ proteins that form helical (rather than ring-shaped) assemblies, but also define a significant and compelling new regulatory mechanism for the control of replication initiation in bacteria. Overall, the paper helps to further dispel the commonplace, but misguided, notion that bacteria are "simple" organisms with little need to regulate replicative events, and should appeal to a broad audience working on DNA replication and AAA+-dependent processes. Pending the resolution of a few minor issues, publication in the EMBO Journal would seem well warranted.

Specific comments:

a) Page 7 and Figure 2B/2C. Why do the authors think that the binding of monomeric Soj states to DnaA might be sensitive to its nucleotide state? I.e., ADP-Soj and ATP-bound SojG12V don't appear to bind equivalently to DnaA.

b) Page 11, line 260. Although the SojR189A mutant interacts with DnaA, this association does not appear as robust as the G12V substitution (despite the claim to the contrary). Can the authors comment on why this might be?

c) Page 11. If monomeric and dimeric Soj bind a similar region of DnaA, then why does only the monomeric form inhibit oligomerization? The authors briefly comment on this dichotomy later in the discussion, but an explanation is not immediately apparent.

d) Page 13, lines 310 to 313. The claim that monomeric Soj does not act by stimulating the ATPase activity of DNA is not explicitly tested in this work. Soj could override the hydrolysis deficiency of the DnaA mutant used in this assay.

e) Some of the suppressor mutations obtained against SojG12V are a little surprising. For instance, both Val323 and Leu294 are buried, and their substitution with charged amino acids would be expected to destabilize the helical subdomain in which they reside. Can the authors comment on this point?

f) Supplemental methods. Although the SPR data appear reasonable, the methods as described would imply that unbuffered NaOH was used to clear off bound Soj protein between each injection. If so, the authors should show that B. subtilis DnaA retains its wildtype ATP binding and assembly properties after such treatment. If DnaA is affect by the regeneration conditions, certain conclusions may need to be tempered or adjusted.

g) Figure 3B. The boundaries between subunits in this figure could be more clearly defined by drawing an independent surface around each monomer.

h) Several references are cited incorrectly given their sentence context, or are missing for key statements. This is particularly apparent in the introduction and with respect to acknowledging prior crosslinking studies (including disulfide engineering) that have been performed to look at DnaA assembly.

i) Supplemental Figure 4 legend. The homology model shown can't be based on the T. maritima structure as claimed, because that model doesn't contain domain IV.

j) Supplemental methods, line 112. It is not clear why the PCR protocol as described was mutagenic. Was Mn2+ used in place of Mg2+?

k) Supplemental methods, page 6. Have the authors ascertained that His-tagged DnaA behaves as the wild-type protein for ATP-binding, DNA binding, Soj binding, etc.?

1st Revision - authors' response 20 December 2011

Referee #1 (Remarks to the Author):

The paper makes a significant advance in understanding regulation of replication initiation in bacteria. DnaA, which perhaps regulates replication in all bacteria, forms oligomers in vitro, the physiological relevance of which was unknown before this study. Here the authors not only show that oligomerization can happen in vivo but it can be a regulatory event: their formation was directly correlated with the initiation efficiency. The oligomerization of DnaA is implicated in the opening of origin DNA, a fundamental requirement in any DNA transaction on duplex DNA. The topic of the paper is thus likely to be of interest to a wide readership.

Some of the specific contributions of the paper are: Identification of the region of DnaA that contacts the regulator of Soj; DnaA oligomerization on both single- and double-stranded DNA. This should help narrow the models on origin opening; Inhibition of DnaA oligomerization by monomeric Soj through regulating the activity of AAA+ domain, and not through the oligomerization domain I.

1. The authors may like to comment whether the domain I, although not necessary, can still help oligomerization, which was missed in the present analysis due to the use of cross-linking. Another pleasing aspect of the paper was the congruence seen between the in vivo and in vitro results.
We thank the reviewer for this valuable suggestion. We have added a paragraph to the Discussion where we consider the role of Domain I in DnaA oligomerization.

Minor points:
2. L.20: Add 'normal' before cell growth. Mutants defective in control such as SeqA grow more or less fine.
We have made this addition.

3. *L.80: Titration by DnaA boxes distributed throughout the chromosome is definitely a mechanism to reduce DnaA availability to origin and should be mentioned.*
We have now mentioned *datA* in *E. coli* and DBCs in *B. subtilis* within the Introduction (line 83).

4. Sup Figure 1: Change DnaA bypass to DnaA suppressor (Red dot legend).
We have corrected this Figure reference.

5. L.153: "Homo-oriented surface" - do you mean DnaA is homo? If so, say homo-oriented DnaA surface?
We have made this change.

6. Figures 1C vs. 2B: Phenotypes of 341V was similar to 323D in Fig. 1C but in Fig. 2B. The same is true in Fig. S6A. May note this and comment.
Indeed, it appears that the purified proteins can behave somewhat differently in vitro and in vivo. We have now noted in the text (line 273-275) that the A341V mutant is the least effective suppressor in both the SPR binding assay (Figure 2B) and the helix formation assay (Figure 5A).

7. Similarly, try to comment how the oligomerization of R379A (Figure 4B) was so much better.
This is a good point. We have added the following statements to the manuscript (line 251): “We note that single-stranded DNA stimulated helix formation of $DnaA^{CC,\ R379A}$ to a greater degree than $DnaA^{CC}$. This observation suggests that the arginine residue may interact with the phosphate backbone of the single-stranded substrate and inhibit the docking of Domain IV into the AAA+ domain, a requirement for single-stranded DNA binding activity (Duderstadt et al. 2010).”

8. I gather the ssDNA was not oriC specific DUE.
Correct, we utilized a synthetic oligonucleotide (oGJS159) that was predicted to lack secondary structure.

9. Figure 2D is not mentioned in Results.
The following statement regarding Figure 2D was mistakenly omitted during the draft process (line 267): "… Soj^{R189A} had little or no effect (Figure 4C) even though it was capable of interacting with DnaA (Figures 2D-E)."

10. L.480: Add Fogel & Waldor 2006 G&D 20:3269. Pulling mechanism was first proposed in this study.
The section of the Discussion containing this statement has been removed.

11. L.512: The Discussion might include what happens to replication in WT and delta(soj-spoOJ) cells. If regulating oligomerization is such a big deal for controlling replication initiation frequency, what happens to E. coli and the likes that do not have ParA? From the known facts, how the oligomerization could be controlled without ParA would make the Discussion more comprehensive. Include the paper from the Berger Lab that just came out in Nature.
Agreed. We have added a paragraph to the Discussion where we address this issue. Also, we have amended the manuscript to include the recent publication from the Berger Lab where appropriate.

Referee #2 (Remarks to the Author):

DnaA is an essential regulator of DNA replication initiation and its function must be tightly regulated in time. This manuscript examines the mechanism by which the ParA homolog Soj inhibits DnaA function in Bacillus subtilis. The authors combined genetics and in vitro assays to convincingly show that Soj interacts with DnaA directly. Using a crosslinking assay, they showed that both double- and single-stranded DNA stimulate DnaA oligomerization. Using mutants, they found that monomeric but not dimeric Soj inhibits DnaA from forming DNA-stimulated oligomeric species, even though both forms of Soj bind DnaA. They also provided in vivo data consistent with this idea. Finally, they demonstrated that the firing frequency in vivo correlates with the extent of DnaA oligomerization in vitro, implicating the role of DnaA oligomerization in replication initiation.

Overall, this is a thorough study that provides significant conceptual advance to 1) the role of DnaA oligomerization in replication initiation, and 2) the mechanism underlying Soj-mediated DnaA regulation. I recommend publication assuming that the following comments will be addressed.

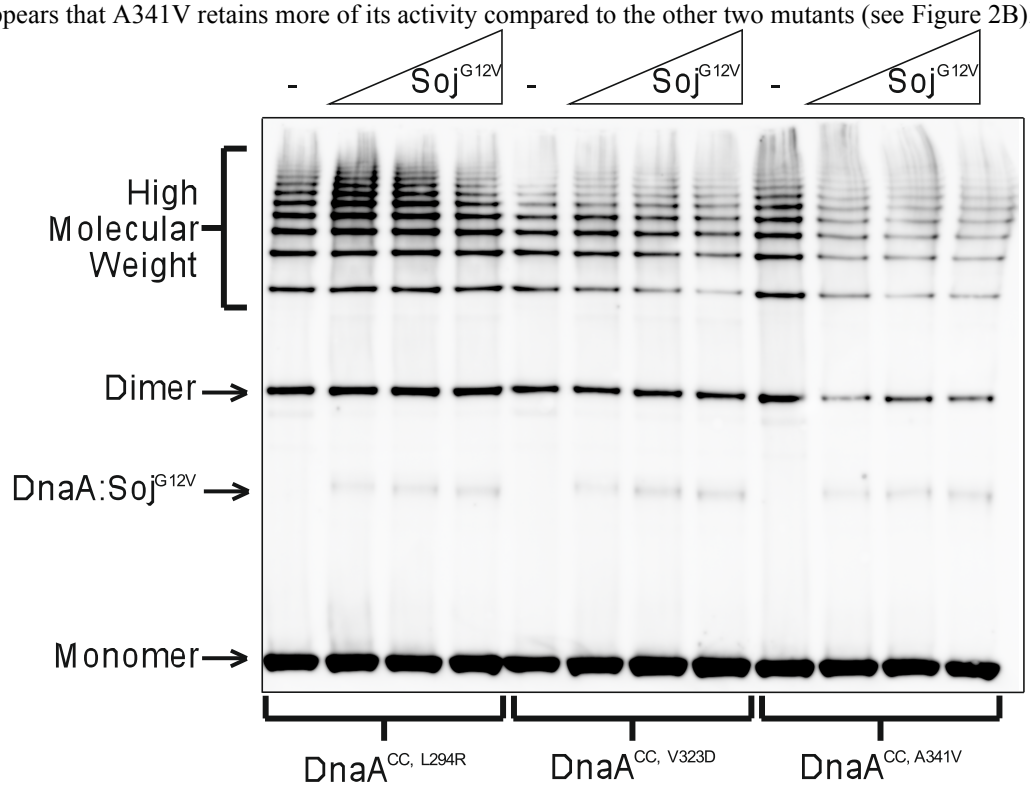
12. The abstract is not very informative and should be revised.
We have rewritten the abstract in an attempt to make it more informative.

13. Fig. S8B is not very convincing in showing that monomeric Soj inhibits DnaA oligomerization without DNA.
The issue here is that the degree of DnaA oligomerization in the absence of DNA is much less than in its presence (compare Fig 4A, lanes 3 and 5), thus the starting point for observing oligomerization is much lower. To address this issue we have (i) provided a higher contrast image of the high molecular weight complexes and (ii) quantified the amount of protein in the helix (as defined by the amount of higher order DnaA complexes) for the two proteins (see Figure S8B).

14. Also, the authors seem to imply that DnaA oligomers with and without DNA form a similar helical structure. Is that true?
As we have argued in the Discussion (line 377-387), we believe that the results of our crosslinking studies suggest that neighbouring AAA+ domains must adopt a distinct helical conformation in order for the BMOE to act on the adjacent cysteine residues, although clearly DNA stimulates/stabilizes this conformation. We cannot exclude that there are minor changes to the helical structure in the presence or absence of DNA, but both of these would still have to position the cysteine residues with the ~8Å distance required for BMOE to act, indicating that the overall helical arrangement of the AAA+ domains is maintained. The remaining Domains of DnaA are almost certainly being influenced by the presence and the nature of the nucleic acid substrate, and therefore although the overall structures will be influenced by these factors, our interpretation of the data is that the structure of the AAA+ core must be relatively fixed.

15. Lines 266-267: It is not clear in Fig. 5A that DnaA(A341V) shows an intermediate effect; one could even argue that it enhances the Soj(G12V) inhibition at least in terms of abundance of oligomers (at the lowest concentration of Soj(G12V))

One of the challenges that our helix formation assay presents is reliably detecting protein complexes of vastly different sizes across a gel by western blot. After repeating the experiment many times the description of Figure 5A outlined in the text is the trend we continuously observed. The image below is another example of the experiment shown in Figure 5A (lane constituents are identical) where again you can see a fading of the HMW complexes for DnaA$^{CC,\ A341V}$. Our conclusion regarding the A341V mutant also takes into consideration the interaction experiments where it appears that A341V retains more of its activity compared to the other two mutants (see Figure 2B).



16. How is Fig. 4B compatible with the idea that DnaA binds to its boxes and oligomerizes from there since pUC18 (lacking DnaA boxes) stimulates oligomer complex as well as pBsOriC4 (carrying the origin region with DnaA boxes)? How does DnaA specifically distinguish the DNA origin regions in vivo?

As the Reviewer correctly notes, the wild-type DnaA protein does not specifically assemble into helices on *oriC* DNA in our in vitro assay (although interestingly the DnaA^{R379A} protein does!). This is likely due to the conditions we must use in vitro where the concentration of protein is in excess of the DNA, the opposite of the situation in vivo. Furthermore, in vivo there may be additional factors that guide DnaA localization, for example in *B. subtilis* the DNA binding proteins HBsu and DnaD are potential specificity factors.

17. Fig. S7B is inconsistent with Felczak et al, 2005. Could it be a difference between E. coli and B. subtilis DnaA or do the authors have reasons to believe that the previous work is incorrect? An explanation should be provided.

In the work of Felczak et al. (2005) the authors find that a mutation in DnaA Domain I [W6A] inhibits oligomerization (as judged by gluteraldehyde crosslinking and formation of the pre-priming complex) and helicase loading. Firstly, Figure 6 in Felczak (2005) shows that the DnaA[W6A] mutant can still oligomerize, although less efficiently than wild-type. Secondly, as noted above in Response#14, our data does not speak to the overall oligomeric structure of the DnaA oligomer within the initiation complex but only focuses on the AAA+ core of the complex. Therefore, we believe that our data is not inconsistent with the results of Felczak, but rather these observations suggest that the different methodologies used to detect DnaA oligomerization provide distinct insights into the process. Whereas we (and others, see Duderstadt 2010 as one example) have found that Domain I is not required for DnaA helix formation *per se*, the work by Felczak indicates that

DnaA oligomers lacking Domain I dimerization activity are likely less stable than wild-type, and this could account for the loss in initiation activity. We have now included a paragraph in the Discussion where we address the role of Domain I dimerization in DnaA helix formation (line 424-435).

18. Discussion: It is proposed that WT Soj would primarily be in monomeric form in vivo. What would prevent Soj from dimerizing? In vitro, the dimer form is predominant in the presence of ATP; so the expectation would be for Soj to be primarily in dimeric form.
Spo0J inhibits Soj dimerization by stimulating Soj ATPase activity (see Murray and Errington 2008 and Scholefield *et al.* 2011). In the absence of Spo0J, GFP-tagged Soj co-localizes with the chromsome in a manner that requires residue Arg189, a key determinant for Soj DNA binding activity in vitro (Quisel 1999, Marston 1999, Hester 2007, Scholefield 2011).

19. What is the oligomer state of DnaAcc in wild-type cells, soj deletion cells and soj-overexpressing cells?
Regarding the *soj* deletion, please note that in Figure 6A these strains (sGJS006 and sGJS033) carry an in-frame *soj* deletion, thus when grown in the absence of the inducer xylose these strains are phenotypically Soj$^-$ (see Figure 3G in Scholefield 2011 showing a Western blot of *amyE*::*Pxyl-soj*^{R189A} in the absence and presence of inducer, including a comparison to its endogenous expression level).

Regarding the wild-type Soj, the strain shown in Figure 6B (left panel) harbours the WT gene and since it was grown in the absence of the inducer xylose, this strain is phenotypically Soj$^+$.

Regarding the last question, we would note that overexpression of wild-type Soj activates DnaA activity (Spo0J is no longer sufficient to inhibit Soj dimerization under these conditions; see Ogura *et al.* 2003), and the mechanism underlying positive regulation of DnaA by Soj is currently under investigation. However, the issue of positive activation is beyond the scope of the current study and we would prefer not to include this data as it will form the basis for a separate manuscript.

20. Discussion: The authors seem to downplay the role of ParA in chromosome segregation in Vibrio. Does the Kadoya et al 2011 work refute the work by the Waldor's lab? If so, the authors should explain it to the readers. The ParA localization in Vibrio and its dynamics in relation to ParB localization are more consistent with that of Caulobacter than that of Bacillus subtilis, which is consistent with a role in chromosome segregation. Also, the authors argue that it is difficult to imagine how ParA could act in segregation in bacteria that have more than one replication initiation event per the cell cycle (as in Bacillus subtilis unlike in Caulobacter). However, segregation still seems to occur in Caulobacter filamentous cells that have multiple origins (Sliusarenko et al, Mol Micro 2011). So the multiplicity of origins doesn't seem to be a problem. The authors' proposal that ParA's role in DNA replication came first and that its role in segregation is limited to bacteria with particular cell cycles seem unjustified.
Due to space limitations following the addition of several sections to the Discussion, as well as the speculative nature of the paragraph in question, we have removed this section from the manuscript.

Minor comments:
21. lines 78-79: What is the SeqA literature in Caulobacter?
We inadvertently referred to the transcriptional regulation of *dnaA* by methylation status in *Caulobacter* (Collier et al. 2007) as involving SeqA. We have removed this reference.

22. lines 108: what does "related oligomers" mean? Not clear at this stage.
As stated above in Response #14, we cannot be sure of the overall oligomeric conformation of DnaA, only of the AAA+ domain, and we did not want to overstate our results. However, as this issue is not yet broached at this point in the manuscript, we have removed the adjective "related" from this description.

23. Duderstadt et al 2010 reference is not complete.
We have corrected this reference.

24. Fig. 2D is not discussed.
The following statement regarding Figure 2D was mistakenly omitted during the draft process (line 266): "… Soj^{R189A} had little or no effect (Figure 4C) even though it was capable of interacting with DnaA (Figures 2D-E)."

25. Fig. 6A: It would be good to show by western blot that there were similar amounts of overexpressed mutant proteins.
We have confirmed by Western blot analysis that the two Soj proteins are overexpressed to the same extent, consistent with our previous results (see Scholefield et al 2011, Figure 2B). We have provided this data in Figure S9B and mentioned this result in the text (lines 343).

26. Figure 6C and D. Axis label. % of DnaA 'in' an oligomer (note that oligomer was misspelled as oliogmer multiple times in this article).
We have made these changes.

Referee #3 (Remarks to the Author):

The manuscript by Murray and Errington builds off their earlier discovery that the activity of the B. subtilis DnaA protein, which helps mediate the initiation of DNA replication, is regulated by a ParA-class ATPase known as Soj. In this present study, the authors use a combination of biochemical and genetic assays (including a particularly clever cell-based crosslinking trick) to convincingly show that monomeric Soj can interact with DnaA to disrupt its higher-order assembly into a helical filament both in vitro and in vivo. Analysis of DnaA suppressor mutations against a constitutively-active form of Soj pinpoint the likely site of Soj binding, and further identify new mutations in DnaA that map to subunit/subunit interfaces to stabilize higher-order initiator oligomers. The paper is clearly written and technical quality of the experiments excellent; the findings not only support the idea that replication initiators are AAA+ proteins that form helical (rather than ring-shaped) assemblies, but also define a significant and compelling new regulatory mechanism for the control of replication initiation in bacteria. Overall, the paper helps to further dispel the commonplace, but misguided, notion that bacteria are "simple" organisms with little need to regulate replicative events, and should appeal to a broad audience working on DNA replication and AAA+-dependent processes. Pending the resolution of a few minor issues, publication in the EMBO Journal would seem well warranted.

Specific comments:
27. Page 7 and Figure 2B/2C. Why do the authors think that the binding of monomeric Soj states to DnaA might be sensitive to its nucleotide state? I.e., ADP-Soj and ATP-bound SojG12V don't appear to bind equivalently to DnaA.
There are two reasons that could likely explain why the binding of the “monomeric” Soj proteins (i.e. – Soj:ADP vs. Soj^{G12V}:ATP) differ in their binding to DnaA. First, we suspect that at high protein concentrations Soj:ADP may be able to form a dimer when in complex with DnaA on the sensor surface. As Figure 2A shows, the shape of the binding curves for Soj:ADP changes with increasing protein concentration. It is important to note that while the G12V mutation is thought to inhibit Soj dimerization by acting as a steric wedge, ADP does not cause a related steric problem *per se*. Rather, ATP binding stabilizes the Soj dimer by allowing residues in one monomer to contact the gamma phosphate of the ATP bound by the adjacent monomer (Leonard et al. 2005). Second, the purified Soj^{G12V} mutant may be less active than the wild-type protein.

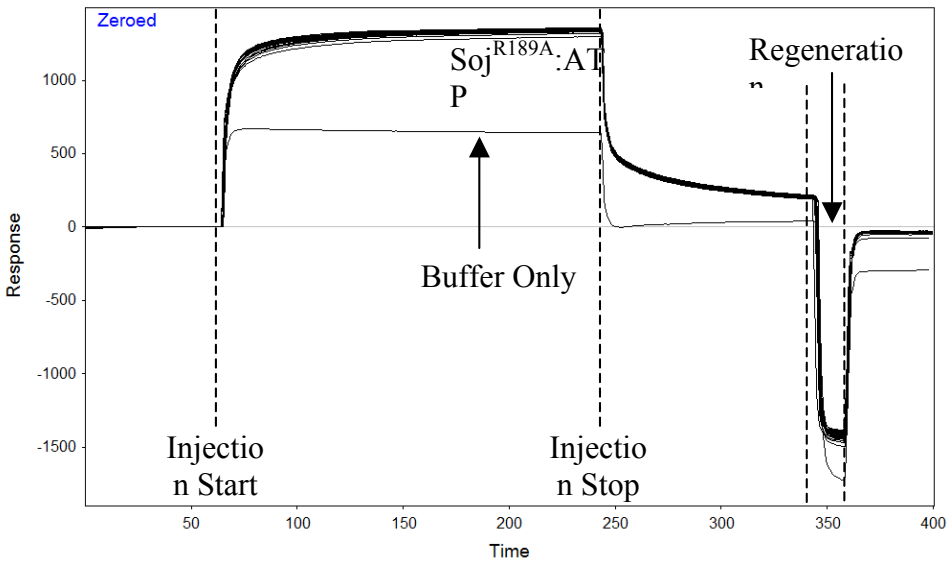
28. Page 11, line 260. Although the SojR189A mutant interacts with DnaA, this association does not appear as robust as the G12V substitution (despite the claim to the contrary). Can the authors comment on why this might be?
Unfortunately we are unclear how to interpret/address this comment. Comparing the response curves in Figure 2C with 2D, it appears that Soj^{R189A} interacts with DnaA to an equal degree compared to Soj^{G12V} (note that the RU for Soj^{R189A} is 250 and taking into account that the dimer is twice as large, this is roughly equal to the 100 RU observed for Soj^{G12V}). In addition, although we have not been able to calculate the kinetic constants describing this interaction, a qualitative assessment indicates that the on-rate of Soj^{R189A} is greater than Soj^{G12V}, and conversely the off-rate is slower, suggesting that Soj^{R189A} interacts with DnaA “better” than Soj^{G12V}. Finally, Figure 2E indicates that Soj^{R189A} and Soj^{G12V} interaction with DnaA to a similar extent. As noted in Response #9 above, we omitted the reference to Figure 2D on Page 11; we hope that the information is clearer following this correction.

29. Page 11. If monomeric and dimeric Soj bind a similar region of DnaA, then why does only the monomeric form inhibit oligomerization? The authors briefly comment on this dichotomy later in the discussion, but an explanation is not immediately apparent.
We do not currently understand this dichotomy. However, our previous in vivo studies have established that the Soj monomer inhibits DnaA while the Soj dimer activates DnaA (Murray and Errington 2008, Scholefield et al. 2011), indicating that our in vitro assay faithfully recapitulates the regulation in vivo. We have now added a paragraph to the Discussion (line 461-468) noting that the Soj dimer may have a secondary interaction site on DnaA, and we suggest that this might explain how the monomer and dimer of Soj differentially regulate DnaA.

30. Page 13, lines 310 to 313. The claim that monomeric Soj does not act by stimulating the ATPase activity of DNA is not explicitly tested in this work. Soj could override the hydrolysis deficiency of the DnaA mutant used in this assay.
We have updated Figure S8A with an experiment that directly tests the effect of Soj^{G12V} and Soj^{R189A} on the rate of ATP hydrolysis by DnaA. This data shows that Soj^{G12V} does not stimulate DnaA ATPase activity as judged by a malachite green Pi release assay. We have also added this information to the text (line 320).

31. Some of the suppressor mutations obtained against SojG12V are a little surprising. For instance, both Val323 and Leu294 are buried, and their substitution with charged amino acids would be expected to destabilize the helical subdomain in which they reside. Can the authors comment on this point?
Regarding the nature of the substitutions, although they may appear surprising, it is difficult to know exactly how they affect the structure of *B. subtilis* DnaA. The DnaA proteins appear to be fully active and normally expressed (Figures 1C, 1D, S2), thus they do not seem to appreciably destabilize this subdomain. Regarding the location of the V323 residue, it appears to be surface exposed in the ADP structure of A.a.DnaA and solvent exposed in the T.m.DnaA structure. Regarding the location of the L294 residue, it is found within a flexible region connecting alpha helices 9 and 10 in both structures, suggesting that it may become exposed when the protein is in solution. However, we will not be able to comment more meaningfully on this issue until we have a structure of B.s.DnaA.

32. Supplemental methods. Although the SPR data appear reasonable, the methods as described would imply that unbuffered NaOH was used to clear off bound Soj protein between each injection. If so, the authors should show that B. subtilis DnaA retains its wildtype ATP binding and assembly properties after such treatment. If DnaA is affect by the regeneration conditions, certain conclusions may need to be tempered or adjusted.
The Reviewer is correct in that unbuffered NaOH was used to regenerate the DnaA surface. While establishing this system we found that 20 sequential regeneration steps reduced the response units generated by Soj^{R189A}:ATP by <5% (see figure below). Therefore, these regeneration conditions do not seem to disrupt DnaA binding activity.



33. Figure 3B. The boundaries between subunits in this figure could be more clearly defined by drawing an independent surface around each monomer.
We have updated the colouring of this figure.

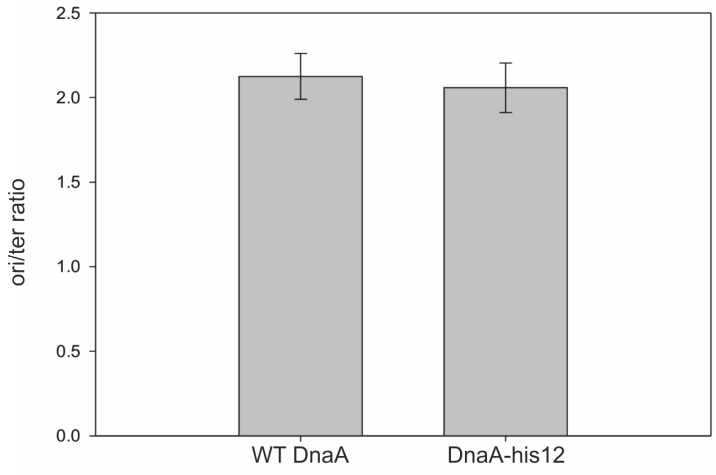
34. Several references are cited incorrectly given their sentence context, or are missing for key statements. This is particularly apparent in the introduction and with respect to acknowledging prior crosslinking studies (including disulfide engineering) that have been performed to look at DnaA assembly.
We have revised the text and attempted to reference primary literature more accurately. The DnaA cross-linking assay utilized in Felczak (2005) has been cited within the Discussion.

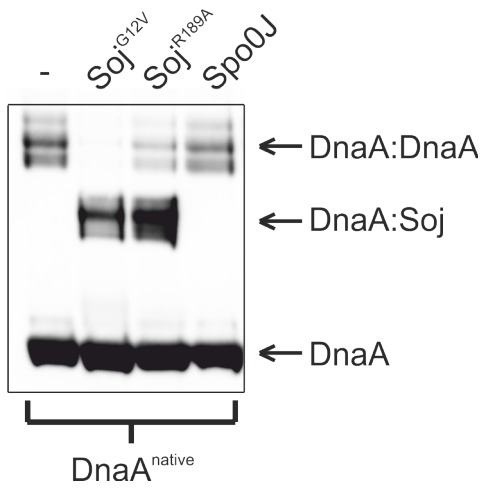
35. Supplemental Figure 4 legend. The homology model shown can't be based on the T. maritima structure as claimed, because that model doesn't contain domain IV.
Correct. Indeed we show the *A. aeolicus* crystal structure (PDB ID: 1L8Q) in Figure S4.

36. Supplemental methods, line 112. It is not clear why the PCR protocol as described was mutagenic. Was Mn2+ used in place of Mg2+?
For mutagenesis of *dnaA* we utilized Phire DNA polymerase. This enzyme has an error-rate of ~10^{-4}, so amplifying a 7793 basepair product over 20 rounds of PCR resulted in an acceptable level of mutagenesis. However, as pointed out by the Reviewer the term "mutagenic" was misleading, so we have replaced it with the term "error-prone" to distinguish it from high-fidelity PCR.

37. Supplemental methods, page 6. Have the authors ascertained that His-tagged DnaA behaves as the wild-type protein for ATP-binding, DNA binding, Soj binding, etc.?
It is possible to replace the endogenous dnaA with a C-terminal His-tagged dnaA (dnaA-his_{12}) in vivo (Figure1D). DnaA is known to require its ATP binding, DNA binding and oligomerization activities to facilitate origin unwinding, thus DnaA-His_{12} must exhibit these functions in vivo. To further quantify the functionality of DnaA-His_{12} we performed marker frequency analysis and found there to be no difference between the initiation frequency of wild-type DnaA and DnaA-his_{12} (see figure below, left panel).

We note that the C-terminal His-tag (and linker sequence) used to purify DnaA-His_6 is identical to that of the in vivo construct except it is six histidine residues shorter. In addition, during our preliminary studies we found that untagged DnaA was capable of interacting with Soj^{G12V} and Soj^{R189A}, and that Soj^{G12V} preferentially disrupted DnaA oligomers compared to Soj^{R189A}, as judged by BS^3 cross-linking (see figure below, right panel). Thus, DnaA-His_6 appears to behave like the untagged protein in regards to the Soj interaction and the inhibition of oligomerization by monomeric Soj.





Acceptance letter 22 December 2011

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, there is just one minor things I need to ask you for, a 'conflict of interest' statement to be included at the end of the manuscript text. To expedite this, please simply send this to us in the body of an email, from where we can easily copy it into the manuscript text file.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Best regards

Editor
The EMBO Journal

