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Replication termination and chromosome dimer resolution in the archaeon Sulfolobus solfataricus

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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 25 August 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It was sent to three expert reviewers, however due to the summer holiday season it had taken somewhat longer than usual to find three suitable experts that were available - please accept my apologies for the resulting delay in the manuscript's evaluation. At this stage we are still waiting for the third set of comments, but as I am myself going to be away from the office for some days now, and as the two reports we received so far are both generally positive, I have chosen to contact you at this point with a preliminary decision on your manuscript, in order to avoid unnecessary further loss of time. As you will see from the comments copied below, both reviewers 1 and 2 consider your findings on archaeal replication termination and dimer resolution interesting and important, but raise a number of specific issues mostly related to the discussion/interpretation/presentation of the data and conclusions. We should thus be happy to consider the manuscript further for publication pending satisfactory revision, and I am thus inviting you to start working on such a revision in light of the referees' comments and suggestions. I nevertheless have to stress that this is a preliminary decision and thus still subject to change should the last, missing report bring up serious additional concerns. Once we will have heard back from the third, outstanding referee, we shall contact you as soon as possible to transmit his/her comments and finalize the decision.

I should add that it is EMBO Journal policy to allow a single round of revision only, and that it is thus essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community 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). Please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, the authors investigate the interplay between DNA replication and chromosome dimer resolution in the archaeon Sulfolobus solfataricus. This is an interesting organism with an eukaryotic mode of multi-origin replication, and bacteria-like circular chromosome and Xer recombinase. The authors first showed data suggesting that replication termination does not take place at specific terminus. They then identified the dif site and characterized how it interacts with the Xer recombinase to complete chromosome dimer resolution. Interestingly, the dif site is not at the replication fork fusion zones. They conclude that replication and chromosome dimer resolution are separate in Sulfolobus. This reviewer is particularly impressed with the nice data and controls identifying the dif site and characterizing Xer function, and the evolutionary implication of the coexistence of eukaryotic mode of replication and prokaryotic mode of chromosome resolution in an archaeon. It is an interesting and well-written paper, and I have only minor comments:

1. The authors introduced in detail the connection of chromosome replication and segregation in other organisms. Clearly, their results have a significant impact on understanding this connection and they should elaborate on this in the discussion. For example, in the Introduction, the authors described how FtsK, the major factor facilitating chromosome translocation, also facilitates chromosome dimer resolution in other bacteria, but fell short in discussing whether such chromosome translocation system exists in S. solfataricus. If the FtsK system does not exist, does Sulfolobus rely on an alternative eukaryotic mode of chromosome segregation? Is this connected to the observation of extended postreplicative sister chromosome pairing?
2. The ChIP-chip uses only one oligo probe per open reading frame, is this sufficient to cover all Xer-binding sites? The figure shows many smaller peaks in addition to the strong dif site; are they significant? If the authors delete the DYAD2 site, will they observe phenotypes similar to the delta xer mutant?
3. The observation that Xer in Sulfolobus is sufficient to carry out recombination in vitro, while other bacteria require help from FtsK, is intriguing. Is there any distinct sequence motif in Xer in Sulfolobus that might explain this difference?
4. The 2D gel data can benefit from having a clear schematic annotation of the replication forks. The authors' previous work has a very nice illustration. Perhaps something similar can be added here.
5. Figure 5A, the monomer band should be labeled. In B, the grey triangles (DYAD1) are confusing as they are not labeled in the schematics.
6. 4th page, first paragraph: solfataricus instead of sofataricus.

Referee #2 (Remarks to the Author):

This manuscript examines the results of investigations into how replication termination occurs in the archaeal organism Sulfolobus. In addition, the authors examine how chromosome dimers that have arisen as a result of replication are resolved. This work builds on previous work from this group and uses a wide range of techniques to address the questions being asked. In general the data presented are of a high quality and offer clear-cut answers to the questions posed. I found the data concerning the mapping of termination regions the most difficult to interpret, and suggest some ways in which this could be overcome below.

Specific comments
1. A large number of 2D agarose gels are presented in figure 1. This is a rather specialist technique, and can be rather difficult for the non-expert to interpret. The authors clearly describe what they

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expect to see in the case of defined or random termination sites (e.g. a spot of greater intensity on the "Y-arc"), but do not explain what a Y-arc is. It would be very useful to provide a cartoon of an idealised 2D gel showing the Y arc, and more importantly showing how this might be expected to vary with the different outcomes described (i.e. specific vs non-specific termination).
2. There are a large number of 2Ds in which there are very prominent spots on the "linear" arc - that is the base-line between unreplicated and completely replicated DNA - e.g. Fig. 1A (d), (e), (h) etc. Such spots are normally indicative of breakage of the DNA. The fact that these seem to be occurring at specific sites on the DNA is of interest and should be commented on. Why should particular regions of the DNA be more prone to breakage unless there are particular sites for termination? Undoubtedly there could be other explanations, but I think this merits some discussion, and possibly further investigation.
3. There are a number of 2Ds that do not appear to display a Y arc at all (e.g. Fig 1C (g), (i), (o), (w), (y). While this may be the quality of the image available for review, this should be confirmed and addressed, either by replacing or removing the images.
4. The figure legend for Fig 1 is not particularly useful. It is not clear to me how this could be used to reproduce the data. Additional supplementary information would be very useful here.
5. Complex 1 and complex 2 are noted on figure 4B. Two complexes are also seen in Fig 4A for the dif3 fragment. Is it expected that these differently shifted complexes are similar? The proportion of supershifted dif3 appears to be much larger than that amount of complex 2 produced from binding DYAD2, even though the molecular ratio of protein to DNA is higher in the latter than the former. Does this imply that additional sites are required within the DNA for effective Xer binding?
6. It appears that at least one multimer can form in the presence of Xer whether or not DYAD 1 or 2 are present. Is this correct (Fig 5A substrate1, Xer +)? If so, perhaps this should be mentioned in the text. Is it possible that there are DYAD2-like sequences in the rest of the plasmid?
7. I find the cartoon in Fig 5B confusing. Surely there should also be grey triangles in the circular plasmids? Otherwise, where is DYAD1 coming from in the digested products? What exactly do the substrates tested consist of - the full length dif3 fragment, or individual dyads? I think that some clarification of this figure and how the experiment was carried out is required in order for the reader to understand it.

what are the major claims and how significant are they?
That replication termination in Sulfolobus occurs over a fork fusion zone and not at specific sites. That chromosome dimer resolution occurs through a bacterial-like Xer/dif system that is physically and temporally separated from chromosome replication termination. Both of these conclusions provide novel insight into archaeal chromosome biology.

are the claims novel and convincing?
The claims are novel. I would like more discussion of the 2D results (see comment 1-3) in order to be entirely convinced of the conclusions drawn. The Xer/dif results are convincing, but Fig. 5D needs clarifying.

are the claims appropriately discussed in the context of earlier literature?
Yes

is the study of interest to more than a specialised audience?
Yes. The archaea are a major group of organisms. Understanding how they function is of interest not just to those working with these species, but to the wider field of DNA metabolism.

does the paper stand out in some way from the others in its field?
This is a high quality manuscript using a range of techniques to address the questions posed, as expected from this group, which is one of the leaders in the field. To the best of my knowledge, this is the first manuscript to address the question of replication termination and dimer resolution in archaea.

are there other experiments that would strengthen the paper?
Primer extension assays could probably be used to support the random fork fusion result (producing a smear as a result), but this is likely not necessary if the discussion of the results presented can be clarified.

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Additional correspondence 27 August 2010

We have just received the 3rd referee report and I have copied it below.

Yours sincerely,
Editor
The EMBO Journal

referee 3

Remarks to the Author:

In this work potential roles for an archaeal Xer recombinase homolog were characterized. This protein has 31% amino acid sequence identity to the E. coli XerD protein. A palindromic binding site on the S. solfataricus chromosome was identified, and the protein was shown to recombine at this site in vitro without aid of accessory proteins. Deletion of the xer gene caused cells to become larger and changed the DNA distribution. The work is interesting and technically well done. It is suggested that archael chromosomes stay cohesed for some time after replication is completed and that the dif recombination is not coupled to completion of replication as in bacteria.
My only major comment is whether it would be possible to extract more information from in vivo experiments with the xer deletion strain. The cell volume experiments (fig 2C) show that cells become a little larger and more heterogenous indicating that cells filament slightly when dimers are not resolved. It would have been nice to see a microscopy image with stained DNA of these cells to see whether cells are guillotined as the authors suggest or whether instead DNA less cells are found. It is assumed that the small population of cells with low fluorescence in the flow cytometry histogram (fig 2D) is just debris. However, this is unlikely since it has a higher scatter signal than normal cells. (In figure 2D a change of the scatter axis from logarithmic to linear on the dot-plots should be made to make it easier to see a doubling in value.) A fluorescence microscopy study will show whether these cells are DNA less cells. Looking at the cells in the microscope might also give additional infomation relevant to cohesion, segregation, and xer recombination.

1st Revision - authors' response 28 October 2010

Referee #1 (Remarks to the Author):

In this manuscript, the authors investigate the interplay between DNA replication and chromosome dimer resolution in the archaeon Sulfolobus solfataricus. This is an interesting organism with an eukaryotic mode of multi-origin replication, and bacteria-like circular chromosome and Xer recombinase. The authors first showed data suggesting that replication termination does not take place at specific terminus. They then identified the dif site and characterized how it interacts with the Xer recombinase to complete chromosome dimer resolution. Interestingly, the dif site is not at the replication fork fusion zones. They conclude that replication and chromosome dimer resolution are separate in Sulfolobus. This reviewer is particularly impressed with the nice data and controls identifying the dif site and characterizing Xer function, and the evolutionary implication of the coexistence of eukaryotic mode of replication and prokaryotic mode of chromosome resolution in an archaeon. It is
an interesting and well-written paper, and I have only minor comments:

1. The authors introduced in detail the connection of chromosome replication and segregation in other organisms. Clearly, their results have a significant impact on understanding this connection and they should elaborate on this in the discussion. For example, in the Introduction, the authors described how FtsK, the major factor facilitating chromosome translocation, also facilitates chromosome dimer resolution in other bacteria, but fell short in discussing whether such chromosome translocation system exists in S. solfataricus. If the FtsK system does not exist, does Sulfolobus rely on an alternative eukaryotic mode of chromosome

segregation? Is this connected to the observation of extended postreplicative sister chromosome pairing?

We have expanded this section of the discussion (the second last paragraph of the Results and Discussion section). As the referee anticipates, we believe that the period of post-replicative pairing possibly enforces a temporal disconnection between replication and segregation. There is no FtsK homolog in Sulfolobus, and precisely what the mechanism of segregation of Sulfolobus chromosoomes entails is a question of great interest to us ñ particularly so given the lack of tubulin and actin superfamily proteins in this organism.

2. The ChIP-chip uses only one oligo probe per open reading frame, is this sufficient to cover all Xer-binding sites? The figure shows many smaller peaks in addition to the strong dif site; are they significant? If the authors delete the DYAD2 site, will they observe phenotypes similar to the delta xer mutant?

The numerous additional peaks are part of the background signal. Any additional specific sites would most likely have been detected since the distance between adjacent oligos is quite consistent (~1 kb), and both adjacent ORFs to the identified dif site showed a significant signal. Our further studies on the identified locus characterized DYAD3 as a dif site and it is unique in the genome. Finally, we believe that having more than one dif site in the genome is very likely to be hazardous, as genome rearrangements could occur. There are additional complications arising due to a need for coordination of dif site usage, which further argue that more than one dif site would be cumbersome.

We also agree with the reviewer in that deletion of dif would give a similar phenotype to the deletion of Xer. In the present study, our aim was to inactivate the system in vivo, and we are confident that deletion of Xer has satisfactorily achieved this. The subject of future studies could be to analyse deletion or other mutations in dif, and to examine the details of the Xer-dif interaction.

3. The observation that Xer in Sulfolobus is sufficient to carry out recombination in vitro, while other bacteria require help from FtsK, is intriguing. Is there any distinct sequence motif in Xer in Sulfolobus that might explain this difference?

Sulfolobus Xer does not have any obvious additional sequence features when compared to the XerD family. However, we expect that an alternative mechanism of chromosome segregation exists, distinct from the FtsK mechanism of E. coli. This is currently under investigation.

4. The 2D gel data can benefit from having a clear schematic annotation of the replication forks. The authors' previous work has a very nice illustration. Perhaps something similar can be added here.

We have added a detailed explanatory section to Fig. 1.

5. Figure 5A, the monomer band should be labeled. In B, the grey triangles (DYAD1) are confusing as they are not labeled in the schematics.

We have corrected the figure and the legend.

6. 4th page, first paragraph: solfataricus instead of sofataricus.

Thanks for spotting that!

Referee #2 (Remarks to the Author):

This manuscript examines the results of investigations into how replication termination occurs in the archaeal organism Sulfolobus. In addition, the authors examine how chromosome dimers that have arisen as a result of replication are resolved. This work builds on previous work from this group and uses a wide range of techniques to address the questions being asked. In general the data presented are of a high quality and offer clear-cut answers to the questions posed. I found the data concerning the mapping of termination regions the most difficult to interpret, and suggest some

ways in which this could be overcome below.

Specific comments
1. A large number of 2D agarose gels are presented in figure 1. This is a rather specialist technique, and can be rather difficult for the non-expert to interpret. The authors clearly describe what they expect to see in the case of defined or random termination sites (e.g. a spot of greater intensity on the "Y-arc"), but do not explain what a Y-arc is. It would be very useful to provide a cartoon of an idealised 2D gel showing the Y arc, and more importantly showing how this might be expected to vary with the different outcomes described (i.e. specific vs non-specific termination).

Many thanks for the suggestion, we have added explanatory cartoons in Fig. 1A, and a description of the Y-arc.

2. There are a large number of 2Ds in which there are very prominent spots on the "linear" arc - that is the base-line between unreplicated and completely replicated DNA - e.g. Fig. 1A (d), (e), (h) etc. Such spots are normally indicative of breakage of the DNA. The fact that these seem to be occurring at specific sites on the DNA is of interest and should be commented on. Why should particular regions of the DNA be more prone to breakage unless there are particular sites for termination? Undoubtedly there could be other explanations, but I think this merits some discussion, and possibly further investigation.

The species are linear DNAs that are the result of very weak non-specific binding of probe to the large quantity of linear DNA on the membrane, or they may occasionally represent incomplete digestion by the restriction enzymes. This effect is observed to varying degrees in almost all 2D gels, and we have added an illustration of the background linear species in Fig. 1A.

3. There are a number of 2Ds that do not appear to display a Y arc at all (e.g. Fig 1C (g), (i), (o), (w), (y). While this may be the quality of the image available for review, this should be confirmed and addressed, either by replacing or removing the images.

The faintness is often due to the technical difficulty in detecting moving replication forks in whole genomic DNA. We have repeated these particular 2D gels so as to provide clearer examples in which the Y-arc is dark enough to be clearly seen.

4. The figure legend for Fig 1 is not particularly useful. It is not clear to me how this could be used to reproduce the data. Additional supplementary information would be very useful here.

We have added to the supplementary data file a table that lists the coordinates of the restriction sites and probe sequences. We have also expanded the remaining Fig. 1 legend to describe the experiment more clearly.

5. Complex 1 and complex 2 are noted on figure 4B. Two complexes are also seen in Fig 4A for the dif3 fragment. Is it expected that these differently shifted complexes are similar? The proportion of supershifted dif3 appears to be much larger than that amount of complex 2 produced from binding DYAD2, even though the molecular ratio of protein to DNA is higher in the latter than the former. Does this imply that additional sites are required within the DNA for effective Xer binding?

This is an excellent observation from the reviewer. We have expanded the data in response and have been able to more precisely define the dif sequence (required for giving the double-shift). Indeed, several extra base-pairs to DYAD2 are needed in order to give the complete double-shift: we found that the DYAD2 site overlaps significantly with a third dyad, DYAD3, which, although symmetrically weaker, clearly gives the double-shift product in isolation (data shown in Fig. 4 for all three dyads). We have also included an additional panel in this figure that contains information about the sequence features of DYAD3, including some similarities to bacterial dif sites.

6. It appears that at least one multimer can form in the presence of Xer whether or not DYAD 1 or 2 are present. Is this correct (Fig 5A substrate1, Xer +)? If so, perhaps this should be mentioned in the text. Is it possible that there are DYAD2-like sequences in the rest of the plasmid?

Yes, although the level of the first multimer seen is only very low, suggesting a very low level of

non-specific multimerization. Any non-specific multimerization evident in the new Fig. 5A is essentially undetectable, and so we believe this is unimportant.

7. I find the cartoon in Fig 5B confusing. Surely there should also be grey triangles in the circular plasmids? Otherwise, where is DYAD1 coming from in the digested products? What exactly do the substrates tested consist of - the full length dif3 fragment, or individual dyads? I think that some clarification of this figure and how the experiment was carried out is required in order for the reader to understand it.

We revised this figure in light of the new EMSA data described above and have more fully described the cartoons legend and main text. The referee may wonder (as did we) why we saw recombination previously with the DYAD2 element. We believe the reasons for this are 2-fold:
1) DYAD3 and DYAD2 overlap substantially and we had to do the new targeted mutations in the new Fig. 5A to tease out their relative contributions.
2) As shown below, the sequence context of the pBluescript backbone into which DYAD2 was cloned in fact extends the similarity to DYAD3 beyond the cloned oligonucleotides. Uppercase signifies cloned oligonucleotide sequences, lowercase are plasmid-derived sequences. Dyad elements are indicated with chevrons.

Referee #3 (Remarks to the Author):

In this work potential roles for an archaeal Xer recombinase homolog were characterized. This protein has 31% amino acid sequence identity to the E. coli XerD protein. A palindromic binding site on the S. solfataricus chromosome was identified, and the protein was shown to recombine at this site in vitro without aid of accessory proteins. Deletion of the xer gene caused cells to become larger and changed the DNA distribution. The work is interesting and technically well done. It is suggested that archael chromosomes stay cohesed for some time after replication is completed and that the dif recombination is not coupled to completion of replication as in bacteria.
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We have removed the comment in the text referring to guillotining, as we agree that this is a description of an individual cellís phenotype rather than of the whole population that our data represents. We have also provided microscope images and statistical analysis in Fig.2 and in supplementary Fig S2. We have identified the various cell phenotypes and compared the two strains by counting the various phenotypes observed. The data are consistent with the flow cytometry and Coulter data.

Regarding the scatter signal in the flow cytometry, we agree that the signals we have described as "debris" could have some spherical form to them (anucleate cells), so we have generalized our statement accordingly in the text in accordance with the reviewerís point. Furthermore the microscopy now included in Fig. 2 does indicate a correlation between increased anucleate cells and the originally noted "debris" in the flow cytometry data.

Acceptance letter 29 November 2010

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. We will send you a formal letter of acceptance shortly and should then be able to swiftly proceed with production of the manuscript!

Yours sincerely,
Editor
The EMBO Journal

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