THE EMBO JOURNAL

Manuscript EMBO-2009-71867

Fully efficient chromosome dimer resolution in E. coli cells lacking the integral membrane domain of FtsK

Nelly Dubarry and François-Xavier Barre

Corresponding author: François-Xavier Barre, CNRS

Review timeline:

Submission date:	10 July 2009
1st Editorial Decision:	29 April 2009
Rebuttal:	05 May 2009
Editor's response:	13 May 2009
Author's Response to referees:	18 May 2009
Editor's response	29 May 2009
Author's response:	29 May 2009
1st Revision received:	09 July 2009
2nd Editorial Decision:	03 September 2009
2nd Revision received:	27 October 2009
Accepted:	06 November 2009

Transaction Report:

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

1st Editorial Decision 29 April 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise that it has taken a little longer than usual to have your manuscript reviewed, three referees have evaluated your manuscript and I enclose their reports below, but as you will see the decision was not a straightforward one and I asked other people for their input. The decision unfortunately is not a positive one.

As you will see from their reports, two of the referees find the conclusion that the transmembrane domain of FtsK is not required for chromosome dimer resolution and therefore it is unlikely that a pore is formed for translocation to be interesting and important. However, as you will see from the comments of referee #2 s/he finds that this has been described in your previous Cell paper and therefore, although the finding that the length of the linker region is important for FtsK function is interesting, overall this does not provide sufficient conceptual advance to be published in the EMBO Journal. This is obviously a concern and I have read the original Cell paper and agree with this referee, I have also discussed the matter with an Editorial Advisor and the Executive Editor. Although we find that the study uses a variety of elegant experiments to demonstrate that the transmembrane domain is required and the manuscript does provide some new insight into the translocation of DNA, the study does not provide sufficient new insight over the previous work. Therefore, unfortunately we will mot be able to further consider your manuscript for publication in the EMBO Journal.

Thank you for the opportunity to consider your manuscript. I am sorry we cannot be more positive

on this occasion, but we hope nevertheless that you will find our referees' comments helpful. We also hope that this negative decision does not prevent you from considering our journal for publication of your future studies.

Yours sincerely,

Editor
The EMBO Journal

****
REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this study, Dubarry and Barre have addressed the issue of how the E. coli FtsK DNA pump interfaces with the dividing septum, and specifically whether a pore constructed from the N-terminus of FtsK, FtsKn, is necessary for DNA translocation. The simple answer is no; the authors have used a variety of creative protein constructs and indirect assays to measure DNA translocation and conclude that FtsKn is dispensable. The authors bypass the need for FtsKn in cell viability by use of an ftsA* allele, and measure translocation as a function of chromosome dimer resolution effects on growth rate (growth in this background +/- dif). They find that tethering FtsKlc to another septal protein, either FtsW or ZipA, or including an extra 50 aa at the N-L junction is sufficient to target FtsKlc to the septum, and to support chromosome dimer resolution measured in the growth assay and by a plasmid resolution assay. Together these data are convincing that FtsKlc is a machine that can use the incompletely closed septum as the doorway for DNA pumping. This result does contrast those of SpoIIIE, which is the B. subtilis homolog; studies by the Rudner lab strongly suggest that SpoIIIE pumps DNA after the septum has been completed, supporting a SpoIIIE pore model. There are differences in the genetics and structures of SpoIIIE and FtsK that could support a different architecture however, and I think the current study is important in that it challenges the field to think about alternative models for the activities of FtsK and its various domains.

1. The results however do not formally rule out that FtsKn forms a pore, or that the rest of FtsKlc could use this pore if it were available. The results say that it is dispensable for chromosome dimer resolution. Given that the FtsK - L domain is a large, 600 aa region, I could imagine that FtsKc could use available pores as well as the one normally provided by FtsKn. Even if the authors think this unlikely, they cannot rule out this possibility and it should be addressed.

2. Have the authors tested these fusions for protein stability by Westerns? It is possible that some of the results could be explained by stability not localization. For example, the incomplete septal localization of FtsKlc could be a stability thing, and the mislocalized protein represents proteolytic fragments. Differences in the plasmid assay could be due to differences in expression, and in my view, fluorescence of the YFP derivatives is a yes/no answer and is not quantitative. And these were expressed from pBAD. The authors should provide some data that the fusions are expressed at the same level from the ftsK promoter, such as with immunoblotting.

3. Fig. 3 vs Fig. 4: why is the f(%) different for FtsK and FtsW::FtsKlc in Fig. 3 (51%, 34%) compared to that in Fig. 4 (both 14%)? How is variability controlled?

4. Fig. 4B: the photo is much too small; even after a magnification on my computer screen I cannot see any resolution in these cells.

Referee #2 (Remarks to the Author):

E. coli FtsK is composed of a 210 amino acid N terminal domain (FtsKn) that localizes to the cell division septum and participates in septum formation, a long flexible linker (FtsKl), and a C terminal ATPase motor domain (FtsKc) that acts as a DNA transporter. This transport function, which has received considerable attention in the last few years, serves to move the chromosomal DNA, particularly the replication terminus region, away from the division septum and to move the termini into position for XerCD-mediated chromosome dimer resolution. In B. subtilis, SpoIIIE

mobilizes DNA after the division septum is complete, suggesting that SpoIIIE is involved in membrane fusion during completion of the prespore septum and forms a membrane channel through which DNA is transported. In this manuscript, the authors test the idea that FtsKn may form such a channel in E. coli concomitant with septal membrane fusion. They conclude, however, that chromosomal DNA is transported prior to membrane fusion and that FtsKn does not form a membrane pore. They also show that the overall length but not any specific region of the FtsKl linker is important for proper function of FtsKc in dimer resolution, consistent with the idea that FtsKn tethers FtsKc to the septal membranes via a long, flexible FtsKl domain that can reach out a significant distance to bind to DNA. Finally, they show that residues 179-210 of FtsK, which correspond to the C-terminal segment of FtsKn, are sufficient to localize FtsK to the septum and permit its full function in dimer resolution.

On their own, the data seem to support the conclusions. However, Aussel et al. (2002) showed in their first Figure (Fig. 1B) that FtsK50c, a derivative of FtsK (FtsK50lc) containing residues 172-210 fused to FtsKc without FtsKl, was fully competent for dimer resolution in vivo. (The authors refer to this experiment on page 7). Residues 172-210 were predicted (Dorazi and Dewar, 2000) to be cytoplasmic and not form transmembrane segments; this means that FtsK lacking its integral membrane domain can support dimer resolution and that a membrane pore is not required for this activity. As the current manuscript's title basically states the same concept, the work presented here does not break much important new ground. The authors do show that residues 172-210 localize to the septum, which was only suggested by the Aussel et al. paper. They also show that tethering FtsKc to the septum via another septal protein allows full function of FtsKc. This confirms the widely held notion that a main purpose of FtsKn is to tether FtsKc to the septum, and also is not surprising given that FtsK50lc localizes to the septum by itself and is functional for dimer resolution (which was previously shown by Aussel et al. anyway). Finally, they also show that the linker's length is important for optimal dimer resolution. This is interesting, but hardly surprising. Overall, while the manuscript is solid and interesting, the key points mostly confirm previously published work. Although some new findings are presented, their significance for FtsK function do not seem to be sufficiently major for publication in EMBO J.

Other comments:

1) First sentence of Results: the authors should cite earlier references that demonstrated the importance of FtsKn in cell division.

2) Suppressed ftsK- cells are known to form chains at high frequency. Were these observed? (Fig. 2C does not show them). Wouldn't the presence of such chains confound the dimer resolution data?

3) Lines 7-9, page 6: It is not surprising that FtsKlc::YFP fails to localize to the septum, as these regions have been shown previously to be dispensable for septal localization.

4) An important control seems to be missing in the experiments with fusions to other septal proteins such as ZipA and FtsW: a test to see if a non-septal membrane-localizing segment could permit FtsKlc to activate dimer resolution. This would be a one way to rule out general membrane tethering as a mechanism.

5) In Fig. 2, the proteins either are fused to GFP at the N terminus (WT FtsK) or YFP at the C terminus (all the others). It might be better to draw the GFP or YFP on the diagram for clarity. In the legend (last line on page 16), "D" needs to replace "C". For the growth competition in panel D, do these proteins have GFP/YFP on them as shown in panel B or not?

6) The diffuse localization pattern for FtsKlc could be caused by degradation of the non-YFP portion of the protein. Was this tested?

7) Fig. 2D: Were ZipA::FtsKlc, FtsK, and FtsW::FtsKlc more active in dimer resolution because they grew better? This is potentially relevant because they seem to partially complement the loss of FtsK (see comment 4).

Referee #3 (Remarks to the Author):

FtsK and SpoIIIE are membrane-anchored DNA translocases that move DNA across bacterial membranes during the resolution of dimeric chromosomes and sporulation, respectively. It is widely assumed that the C-terminal motor domain of these proteins moves DNA through a channel comprised of the N-terminal transmembrane domain. The authors here investigate the roles of the various domains of E. coli FtsK in the resolution of dimeric chromosomes. First, they demonstrate that the N-terminal transmembrane domain is dispensable for dif-recombination if the C-terminal domain is targeted to the septum by another protein, even if this protein lacks transmembrane domains. These results demonstrate that the transmembrane domain is not essential for FtsK function. Second, the authors demonstrate that FtsKLC can localize and function if 50 amino acids of the extreme C-terminal part of the transmembrane domain are included. Finally, the authors take advantage of the functionality of the FtsW-FtsKLC protein to investigate the role of the ~600 amino acid linker domain by constructing an array of similarly sized deletions at various places within the protein. The authors conclude that FtsK translocates DNA across an incompletely formed septum, when a transmembrane channel is not required, and that the linker domain allows the motor domain to reach its DNA substrate before septal biogenesis is complete.

Together these experiments represent an important contribution towards our understanding of FtsK function. One reservation is the strength with which the authors conclude that FtsK transmembrane domain does not assemble a channel for DNA translocation across the membranes. The experiments presented clearly demonstrate that dif recombination can occur in the absence of the membrane domain, but the experiments do not directly address whether the native protein assembles a transmembrane channel. In part this is because the experiments address the ability to ultimately complete dif recombination, but not the time or energy required for process. However, this reservation can be readily addressed, as suggested below. Despite this reservation, the manuscript contains well-designed experiments.

Specific comments:
1. Page 5. The competition assay provides a good estimate of the ability of a protein to support dimeric chromosome resolution, but it does not estimate the efficiency of this process since the latter requires an assessment of the time and energy required. The results demonstrate that the chimeric FtsK molecules are ultimately able to resolve all dimeric chromosomes, but does not assess the time required for each event.

2. Figure 3B. The plasmid assay for dif recombination is performed with high induction of the chimeric proteins (0.2% arabinose) and with long induction times (4 hours). This makes it impossible to determine if the chimeric proteins resolve dimeric chromosomes less rapidly than wild type proteins. The data contains hints that this might be the case, since the wild type protein supports ~50% recombination, while the chimeras supported 25-38% recombination (Fig. 3B). These results would be more convincing if the authors assessed recombination frequencies from several earlier timepoints and with lower induction, as they have done previously (Barre, 2000).

3. The discussion of B. subtilis SpoIIIE on page 3 and page 10-11 is complicated by conflicts in the Bacillus literature about whether SpoIIIE is necessary to compartmentalize B. subtilis daughter cells and membranes during sporulation, as suggested by Wu et al, 1994 and 1995, Liu et al., 2006, or is instead dispensable for compartmentalization (as suggested by Burton et al., 2007). This issue could be avoided by replacing the sentence on page 4 that ends with "acts after septal membrane fusion" with a statement that the assembly of SpoIIIE at the septum is correlated with a barrier to diffusion between the two cells (Wu, 1994, Liu, 2006 and Burton, 2007).

4. The above comment also makes it difficult to determine what the authors mean by "translocates DNA across a pre-existing opening in the septum". Perhaps this could be replaced by across an incompletely formed septum (?)

5. Page 4, Page 10. The paired channel model for SpoIIIE topology was proposed by Liu et al 2006 (Mol. Micro., 59:1097-) as well as by Burton et al., 2007. Both references should be cited.

6. The wording is a bit strong in several places (particularly in the abstract), given that neither the structure of the native TM domain nor the kinetics by which the chimeric proteins support DNA translocation have been directly investigated. For example on page 4, the authors state "FtsKN and

FtsKL do not participate in the formation of a pore". It would be more accurate to state that the formation of a pore is not essential for DNA translocation. On the bottom of page 6, one could tone down the concluding sentence by inserting "essential "..."argues against the idea that FtsKN creates a pore that is essential for the transport of DNA across lipid bilayers." It would be best if the authors considered this point as suggested by their data, rather than directly demonstrated by the data.

7. Is the 50 amino acid domain of FtsK (FtsK50) sufficient for septal localization?

8. Figure 1. The grey box in panel B is not defined in the figure legend, and it appears smaller than the bracket for FtsK50.

9. Figure 3. Scale bar size should be indicated.

10. Figure 4. Panel B should be larger.

11. Figure 5. Shows only one FtsK subunit attached to the membrane, perhaps to avoid clutter. How each subunit is proposed to be attached to the membrane should be clarified in the figure or legend.

Rebuttal 05 May 2009

I appreciate the efforts you made to reach a rapid decision about our EMBOJ-2009-70960 manuscript and I understand that the comment of Referee #2 on the novelty of our data put you in an awkward position. However, I would like to point out that the comment of Referee #2 comes from a very unfortunate misunderstanding and assure you of the novelty and importance of our results, as stated by Referee #1 ("[...] the current study is important in that it challenges the field to think about alternative models for the activities of FtsK and its various domains") and Referee #3 ("together these experiments represent an important contribution towards our understanding of FtsK function"). I hope therefore that, once the origin of the misunderstanding of Referee #2 is made clear, you will accept to reconsider your decision on our paper.

As summarized in your editorial decision, the data we report lead us to conclude that " [...] the transmembrane domain of FtsK is not required for chromosome dimer resolution and therefore it is unlikely that a pore is formed for translocation". The three referees state that the data are sufficient for this claim: "Together these data are convincing [...]" (Referee #1), "On their own, the data seem to support the conclusions." (Referee #2) and "The experiments presented clearly demonstrate that dif recombination can occur in the absence of the membrane domain [...]" (Referee #3).
Referee #2 was misled into thinking that part of the data we report in the present manuscript had been described in the Figure 1B of our previous Cell paper (Aussel at al. 2002) by an unfortunate misunderstanding. The confusion arises from the complexity of the action of FtsK in chromosome dimer resolution. Indeed, FtsK has two independent roles in chromosome dimer resolution: it functions as an activator of Xer recombination by a direct contact with the XerCD-dif nucleoprotein complex (Aussel et al. Cell 2002) and it serves to transport chromosomal DNA across the closing septum in the orientation dictated by KOPS sequences (Bigot et al. EMBO J. 2005). Early works about the role of FtsK in chromosome dimer resolution, such as in the Aussel et al. paper, focused on the mechanism of Xer recombination activation. To this aim, FtsK-dependent Xer recombination had to be monitored independently from the other roles that FtsK has in cell division and chromosomal DNA transport. We monitored therefore Xer recombination between PLASMID-borne dif sites. This can be done in vitro, using purified proteins, but also in vivo, using E. coli cells as a kind of "test tube". It has to be noted that in these experiments, no KOPS are needed for the oriented loading of FtsK and that Xer recombination activation is independent from the cell cycle, and notably cell division and homologous recombination (Barre et al. Genes and dev. 2000). Indeed, in vivo experiments on plasmids are performed in conditions of overproduction of the FtsK chimeras, which are often deleterious to the cells and lead to their death in a few hours (Barre et al. 2000; Yates et al. Mol. Mic. 2003). The experiments described in Figure 1B of the Aussel et al. paper are of this type. As a consequence, no conclusion can be made on the role of the N-terminal domain of FtsK in chromosomal DNA transport based on this figure and none was made at the time. The data in Figure 1B helped us define a truncated peptide, FtsK50C, which is able to activate Xer recombination on PLASMIDS in vitro and in vivo. The rest of the 2002 paper focused on the study

of the mechanism of Xer recombination activation. Of course, we tested the activity of FtsK50C in chromosome dimer resolution at the time and found, as clearly stated in the discussion of the 2002 paper, that FtsK50C does not resolve chromosome dimers: "expression of FtsK50C in vivo does not suppress the chromosome segregation defect of FtsKC- cells (data not shown). Since FtsK50C does not localize specifically to the septum, this supports the view that it is the spatial and temporal restriction of Xer recombination to the septum at the time of cell division that enables chromosome dimer resolution.". This is the exact contrary of what is claimed by Referee #2 ("FtsK50C, a derivative of FtsK (FtsK50lc) containing residues 172-210 fused to FtsKc without FtsKl, was fully competent for dimer resolution in vivo")!
I wish to emphasize that it took us 7 years to acquire enough knowledge on the roles of FtsK in the cell cycle and in chromosome dimer resolution in general to be able to address the question of the mode of transport of chromosomal DNA across the dividing septum. During this period, several models for the mode of transport of chromosomal DNA across the division septum have been proposed. However, no study experimentally addressed the subject, apart from the 2007 cell paper by the Rudner lab on B. subtilis SpoIIIE (Burton et al. Cell 2007). Our finding that FtsK50LC fully supports chromosome dimer resolution, which contradicts the Pore formation model of the Rudner lab, was a complete surprise to all the specialists in the field, as attested by the comments of Referee #1 and #3.

We have read carefully all the other points raised by the three referees, which we found to be very constructive. We are pleased to tell you that we should be able to answer all of them if you accept to reconsider our manuscript.

I understand that this is a difficult editorial decision and would like to suggest, in case of doubts, to contact the other two referees or a fourth referee that would know more about the subject, such as Prof David Sherratt or Prof Kit Pogliano. Also, please do not hesitate to phone or Email me at the above address. I thank you very much for your consideration and hope this issue can be solved before I meet you next week at the EMBO YIP meeting.

Editor's response 13 May 2009

I have read your letter about the differences between the two sets of experiments in your previous Cell paper and the submitted EMBO J manuscript. I can see that their is potential for misunderstanding here and I am willing to look into it in more detail.
What I suggest is that you write up a point-by-point response to referee #2, I will then seek further input from people to resolve this issue, referee #2 will be one of these but not the only one. We can then take it from there.

Response to referees 18 May 2009

here is a letter in which we answer the comments of Referee 2 point by point (AnswerReferee2.pdf) and a letter in which we answer Referee 1 and 3 points (AnswerReferee13.pdf). I hope you can make a quick decision on this.

Answer to referees 1 and 3:

Thanks again for the opportunity you give us in defending our work.

We were very pleased by the fact that all three referees and the editor appreciated our demonstration that the transmembrane domain of FtsK is not required for DNA transport across the division septum with the following statements of Referee 1 *("The authors have used a variety of creative protein constructs and indirect assays to measure DNA translocation and conclude that FtsKn is dispensable. [...] Together these data are convincing that FtsKlc is a machine that can use the*

incompletely closed septum as the doorway for DNA pumping."), Referee 2 *("On their own, the data seem to support the conclusions. [...] The manuscript is solid and interesting."),* Referee 3 *("Together these experiments represent an important contribution towards our understanding of FtsK function. [...] The manuscript contains well-designed experiments.")* and the Editor *("the study uses a variety of elegant experiments to demonstrate that the transmembrane domain is not required").*

We were also pleased by the fact that Referee 1 and referee 3 appreciated the novelty and importance of our results with statements such as: *"Dubarry and Barre have addressed the issue of how the E. coli FtsK DNA pump interfaces with the dividing septum, and specifically whether a pore constructed from the N-terminus of FtsK, FtsKn, is necessary for DNA translocation. [...] I think the current study is important in that it challenges the field to think about alternative models for the activities of FtsK and its various domains."* (Referee 1) and *"It is widely assumed that the C-terminal motor domain of these proteins moves DNA through a channel comprised of the N-terminal transmembrane domain. [...]The authors here investigate the roles of the various domains of E. coli FtsK in the resolution of dimeric chromosomes. [...] The authors conclude that FtsK translocates DNA across an incompletely formed septum, when a transmembrane channel is not required, and that the linker domain allows the motor domain to reach its DNA substrate before septal biogenesis is complete"* (Referee 3).

Please find below a brief answer to the specific points made by Referee 1 and 3.

Answer to Referee 1 comments:

Point 1 : *"FtsKc could use available pores [...] even if the authors think this unlikely, they cannot rule out this possibility and it should be addressed"* .

We agree with this point and think that this should be added in the discussion section.

Point2 : *"Have the authors tested these fusions for protein stability by Westerns?"*

See Referee 1 point 6.

First, we wish to emphasize that all the conclusions that we make in our report derive form positive results, i.e. chimeras that are active in chromosome dimer resolution. Consequently, detailed analysis of the stability of inactive chimeras is not important for the message of the paper. However, we have previously shown that Nter and Cter truncations of FtsK are stable by western blot analysis (Barre et al. Genes and dev. 2000). In addition, the stability of chimeras engineered for the present study was addressed by western blot analysis (data not shown). This could be included as a supplementary figure, if required.

Point 3: *"Fig. 3 vs Fig. 4: why is the f(%) different for FtsK and FtsW::FtsKlc in Fig. 3 (51%, 34%) compared to that in Fig. 4 (both 14%)? How is variability controlled?"*

The results of Figure 3 cited by Referee 1 correspond to the level of Xer recombination activation on PLASMID pseudo dimers whereas the results of Figure 4 correspond to chromosome dimer resolution. At time 0 of plasmid experiments, 100% of the plasmids are in the form of a pseudo dimer, which explains how high rates of recombination can be achieved. After longer period of incubation, most of the pseudo dimers are resolved. In the chromosome dimer resolution measurements made by growth competition, recombination occurs only when a dimer has been formed, at a rate of approximately 1/7 cells per generation. The maximum rate of recombination is therefore in the order of 15%. The 14% of growth fitness of FtsK and FtsW ::FtsKLC over their dif-counterparts indicate that they are fully efficient in chromosome dimer resolution, which is indicated by a +++ sign in the Figure.

Point 4: *"Fig. 4B: the photo is much too small; even after a magnification on my computer screen I cannot see any resolution in these cells."*

The format of the photo will be changed.

Answer to referee 3 comments :

Point 1 and 2: *"The competition assay provides a good estimate of the ability of a protein to support dimeric chromosome resolution, but it does not estimate the efficiency of this process since the latter requires an assessment of the time and energy required. The results demonstrate that the chimeric FtsK molecules are ultimately able to resolve all dimeric chromosomes, but does not assess the time required for each event"*

The co-culture assay we used measures the fitness of a strain expressing FtsK or a modified form in a dif - background, compared to its isogenic dif + parent. The difference in fitness between the two strains represents the rate of dimer resolution due to FtsK or to the chimeric forms (the ability of the protein) but also the efficiency of the process. Indeed, a delay in the process of chromosome dimer resolution should delay the cell cycle and thus induce an increase of the generation time of the dif+ strain, which would be easily detected. We cannot rule out that such a delay exist, but we can say that it is minor.

Point 2: *"The plasmid assay for dif recombination is performed with high induction of the chimeric proteins (0.2% arabinose) and with long induction times (4 hours). This makes it impossible to determine if the chimeric proteins resolve dimeric chromosomes less rapidly than wild type proteins. The data contains hints that this might be the case, since the wild type protein supports ~50% recombination, while the chimeras supported 25-38% recombination (Fig. 3B). These results would be more convincing if the authors assessed recombination frequencies from several earlier timepoints and with lower induction, as they have done previously (Barre, 2000)."*

In initial experiments, we played with shorter and longer time points, and also with different amount of arabinose to find conditions in which we can identify differences in the efficiency of Xer recombination. The data of the 4h time point and 0.2 % of arabinose were chosen because they allow the difference to be made.
We wish to emphasize that differences in the efficiency of Xer recombination on PLAMIDS do not directly correlate with the efficiency of chromosome dimer resolution. For instance, we previously showed that a chimera carrying the Cter of H. influenzae FtsK, which is totally inactive in Xer recombination activation on Plasmids (Yates et al. Mol Mic 2003) resolves 2/3 of the chromosome dimers (Bigot et al. Mol Mic 2004).

Point 3 *"The discussion of B. subtilis SpoIIIE on page 3 and page 10-11 is complicated by conflicts in the Bacillus literature about whether SpoIIIE is necessary to compartmentalize B. subtilis daughter cells and membranes during sporulation, as suggested by Wu et al, 1994 and 1995, Liu et al., 2006, or is instead dispensable for compartmentalization (as suggested by Burton et al., 2007). This issue could be avoided by replacing the sentence on page 4 that ends with "acts after septal membrane fusion" with a statement that the assembly of SpoIIIE at the septum is correlated with a barrier to diffusion between the two cells (Wu, 1994, Liu, 2006 and Burton, 2007)."*

In the original version of the paper, we tried to limit citations to original papers to limit the space taken by references. As the EMBO editorial policy now allows many more citations, this will be easily taken care of.

Point 4: *"The above comment also makes it difficult to determine what the authors mean by "translocates DNA across a pre-existing opening in the septum". Perhaps this could be replaced by across an incompletely formed septum (?)"*

This will be done.

Point 5: *"Page 4, Page 10. The paired channel model for SpoIIIE topology was proposed by Liu et al 2006 (Mol. Micro., 59:1097-) as well as by Burton et al., 2007. Both references should be cited."*

We agree. The citation will be added.

Point 6: *"The wording is a bit strong in several places [...]"*

We agree with the modifications proposed by Referee 3.

Point 7: *"Is the 50 amino acid domain of FtsK (FtsK50) sufficient for septal localization?"*

The 50 does not seem to be sufficient: no septal localization of a GFP ::FtsK50 fusion, of a GFP::FtsK50C fusion nor a FtsK50C::YFP fusion was observed.

Points 8 to 10:

We agree with the modification of the figures proposed.

Answer to Referee 2 comments:

Point 0a: *"Aussel et al. (2002) showed in their first Figure (Fig. 1B) that FtsK50c, a derivative of FtsK (FtsK50lc) containing residues 172-210 fused to FtsKc without FtsKl, was fully competent for dimer resolution in vivo. […] As the current manuscript's title basically states the same concept, the work presented here does not break much important new ground."*

FtsK has two independent roles in chromosome dimer resolution: it functions as an activator of Xer recombination by a direct contact with the XerCD-*dif* nucleoprotein complex (Aussel et al. Cell 2002) and it serves to transport chromosomal DNA across the closing septum in the orientation dictated by KOPS sequences (Bigot et al. EMBO J. 2005). Early works about the role of FtsK in chromosome dimer resolution, such as in the Aussel et al. paper, focused on the mechanism of Xer recombination activation. To this aim, FtsK-dependent Xer recombination had to be monitored independently from the other roles that FtsK has in cell division and chromosomal DNA transport. We monitored therefore Xer recombination between PLASMID-borne *dif* sites. This can be done *in vitro,* using purified proteins, but also *in vivo,* using *E. coli* cells as a kind of "test tube". It has to be noted that in these experiments, no KOPS are needed for the oriented loading of FtsK on DNA and that Xer recombination activation is independent from the cell cycle, and notably cell division and homologous recombination (see Barre *et al*. Genes and dev. 2000). Indeed, *in vivo* experiments on plasmids are performed in conditions of overproduction of the FtsK chimeras, which are often deleterious to the cells and lead to their death in a few hours (Barre *et al*. Genes and dev. 2000; Yates *et al*. Mol. Mic. 2003). The experiments described in Figure 1B of the Aussel et al. paper are of this type. As a consequence, no conclusion can be made on the role of the N-terminal domain of FtsK in chromosomal DNA transport based on this figure and none was made at the time. The data in Figure 1B helped us define a truncated peptide, FtsK50C, which is able to activate Xer recombination on PLASMIDS *in vitro* and *in vivo*. The rest of the 2002 paper focused on the study of the mechanism of Xer recombination activation. Of course, we tested the activity of FtsK50C in chromosome dimer resolution at the time and found, as clearly stated in the discussion section of the 2002 paper, that FtsK50C does not resolve chromosome dimers: "*expression of FtsK50C in vivo does not suppress the chromosome segregation defect of FtsKC- cells (data not shown)*."

Point 0b: *"The authors do show that residues 172-210 localize to the septum, which was only suggested by the Aussel et al. paper."*

We did not show that residues 172-210 localize to the septum! On the contrary, we know that these residues are not sufficient for septal localization. Indeed, we clearly stated that "*FtsK50C does not localize specifically to the septum"* in the discussion section of the 2002 Aussel al al. paper (unpublished data). Thus, it was a real surprise to us and to other specialists in the field to discover that the longer FtsK50LC construct targeted to the septum. We are currently investigating how FtsK50LC is recruited to the site of division.

Point 0c: *"They also show that tethering FtsKc to the septum via another septal protein allows full function of FtsKc."*

This is an oversimplification. Indeed, in the present report we show that FtsKc cannot resolve chromosome dimers when it is targeted to the septum by a fusion to ZapA (Figure 2;

ZapA::FtsKLC construct).

Point 0d: *"[The study] confirms the widely held notion that a main purpose of FtsKn is to tether FtsKc to the septum."*

We agree that it is a widely held notion that one of the purposes of FtsKn is to tether FtsKc to the septum. This notion arose form our earlier reports in Cell (Aussel et al. 2002) and Genes and dev (Barre et al. 2000). However, this was not the point of the present study. The present study addresses the widely held notion that *"the C-terminal motor domain of [FtsK/SpoIIIE/TraB] moves DNA through a channel comprised of the N-terminal transmembrane domain"* as stated by Referee 3 and more "*specifically whether a pore constructed from the N-terminus of FtsK, FtsKn, is necessary for DNA translocation"* as stated by Referee 1. Note that this is acknowledged in the summary of our results made by referee 2: "*In B. subtilis, SpoIIIE mobilizes DNA after the division septum is complete, suggesting that SpoIIIE is involved in membrane fusion during completion of the prespore septum and forms a membrane channel through which DNA is transported. In this manuscript, the authors test the idea that FtsKn may form such a channel in E. coli concomitant with septal membrane fusion*."

Point 0e: *"It is not surprising that [tethering FtsKc to the septum via another septal protein allows chromosome dimer resolution] given that FtsK50lc localizes to the septum by itself and is functional for dimer resolution (which was previously shown by Aussel et al. anyway)."*

FtsK cannot resolve chromosome dimers when tethered to the septum via ZapA (See point 0c). We never showed in the 2002 Aussel *et al.* paper that FtsK50LC could target to the septum nor resolve chromosome dimers (See point 0a).

Point 0f: *"Finally, they also show that the linker's length is important for optimal dimer resolution. This is interesting, but hardly surprising."*

Contrary to *E. coli* FtsK, most other members of the FtsK/SpoIIIE/TraB family possess relatively short linkers, ranging from 100 to 300 aa. In addition, we know that this linker is dispensable for full Xer recombination activation in vivo and in vitro (Aussel et al. Cell 2002). Therefore, it would have been more reasonable to argue that the linker might not be important for chromosome dimer resolution. We wish to emphasize that the point of Figure 4 is not only to show that the linker length is important, but also to show that no specific region within this linker plays a role in chromosome dimer resolution (and hence DNA transport). Again, we would like to stress that the aim of this report is to demonstrate that DNA is not transported through a pore made by the Nter and/or the linker region of FtsK. However, we agree with Referee 2 that the importance of the linker's length for optimal dimer resolution fits very well with the idea that FtsK functions before the end of septum closure.

Point 0g: "*while the manuscript is solid and interesting, the key points mostly confirm previously published work."*

As already answered in point 0a to 0f, there is no previously published data on the role of the Nter and linker of FtsK in DNA transport. On the contrary, results in other species suggested that *"the C-terminal motor domain of these proteins moves DNA through a channel comprised of the N-terminal transmembrane domain"* as stated by referee 3, that SpoIIIE "*forms a membrane channel through which DNA is transported*" as stated by referee 2 and that *"SpoIIIE pumps DNA after the septum has been completed, supporting a SpoIIIE pore model"* as stated by Referee 1. The data we report is both original and important, as stated by referee 1 *("[…] the current study is important in that it challenges the field to think about alternative models for the activities of FtsK and its various domains*") and by Referee #3 ("*together these experiments represent an important contribution towards our understanding of FtsK function*").

Point 1: *"First sentence of Results: the authors should cite earlier references that demonstrated the importance of FtsKn in cell division."*

In the original version of the paper, we cited reviews rather than original papers to limit the space taken by references. As the EMBO editorial policy now allows many more citations, this will be easily taken care of.

Point 2a: *"Suppressed ftsK- cells are known to form chains at high frequency. Were these observed?*

Chains were observed. As indicated in the original description of the ftsA* mutant, only 30% of the cells present a normal phenotype.

Point 2b*: "Fig. 2C does not show them [chains].*

As stated in the legend of figure 2C, the images shown were taken from AB1157 cells to avoid the complex chain and filament phenotypes of ftsK- ftsA* strains, which might confuse non-specialist readers. Note, however, that we made sure that localization studies in wild type AB1157 strains were fully consistent with studies in ftsK- ftsA* strains and ftsK(deltaLC) strains.

Point 2c: *Wouldn't the presence of such chains confound the dimer resolution data?"*

No. The efficiency of chromosome dimer resolution is monitored by comparing the growth of a ftsKftsA* strain expressing a specific chimera to the growth of its dif- version. Any other defect than chromosome dimer resolution, such as in cell division, is therefore equivalent for both strains.

Point 3: *"Lines 7-9, page 6: It is not surprising that FtsKlc::YFP fails to localize to the septum, as these regions have been shown previously to be dispensable for septal localization."*

It is true that it has been previously shown that the N-terminal 1-210 aa of FtsK are sufficient for septum recruitment. However, this did not indicate that the rest of the protein could not target to the septum via an independent domain. Indeed, we observed that FtsK50LC::YFP does localize to the septum even if it lacks all 4 transmembrane segments of FtsK.

Point 4: *"An important control seems to be missing in the experiments with fusions to other septal proteins such as ZipA and FtsW: a test to see if a non-septal membrane-localizing segment could permit FtsKlc to activate dimer resolution. This would be a one way to rule out general membrane tethering as a mechanism."*

We agree with referee 2 that it could be interesting to test such a chimera. However, we can already rule out general membrane tethering as a mechanism with the data presented in the report since ZapA::FtsK50LC and FtsK50LC, which both lack any transmembrane segment, are fully competent in chromosome dimer resolution (Figure 3).

Point 5a: *"In Fig. 2, the proteins either are fused to GFP at the N terminus (WT FtsK) or YFP at the C terminus (all the others). It might be better to draw the GFP or YFP on the diagram for clarity."*

GFP/YFP versions of the chimeras were just used for localization purposes.

Point 5b: *" In the legend (last line on page 16), "D" needs to replace "C"."*

True. Thanks!

Point 5c: *"For the growth competition in panel D, do these proteins have GFP/YFP on them as shown in panel B or not?"*

No. We engineered three series of constructs, on in which the chimeras are under the FtsK promoter on a pSC101 plasmid, one in which they are under the same promoter but fused to GFP or YFP, and one in which they are expressed from the arabinose promoter. Chimeras under the pAra promoter were used to test for Xer recombination activation on PLASMID-pseudo dimer assays. GFP/YFP

constructs for localization studies. For growth competition we chose to test chimeras under the ftsK promoter and without GFP/YFP fusions. We realize that the text of the paper should be make clearer.

Point 6: *"The diffuse localization pattern for FtsKlc could be caused by degradation of the non-YFP portion of the protein. Was this tested?"*

First, we wish to emphasize that all the conclusions that we make in our report derive form positive results, i.e. chimeras that are active in chromosome dimer resolution. Consequently, detailed analysis of the stability of inactive chimeras is not important for the message of the paper. However, we have previously shown that Nter and Cter truncations of FtsK are stable by western blot analysis (Barre et al. Genes and dev. 2000). In addition, the stability of chimeras engineered for the present study was
addressed by western blot analysis (data not shown). This could be included as a supplementary figure, if required.

Point 7a: "*Fig. 2D: Were ZipA::FtsKlc, FtsK, and FtsW::FtsKlc more active in dimer resolution because they grew better?"*

No. As stated in the answer to Point 2c, the way in which we perform the growth competition experiments allow us to exclude the possibility that the observed differences in chromosome dimer resolution are due to any alteration that the production of a chimera may have on the cell cycle of the strains.

Point 7b: "*This is potentially relevant because they seem to partially complement the loss of FtsK (see comment 4)."*

As stated in the legend of Figure 2C and in answer to point 2b, the images shown were taken from AB1157 cells to avoid the complex chain and filament phenotypes of ftsK- ftsA* strains, which might confuse a non-specialist reader.

Editor's response 29 May 2009

I have just heard back from one of the people I asked to evaluate your appeal, since this comes from referee #2 I do not see any reason to wait for the responses from the other people involved. I paste part of the email below, as you can see the referee acknowledges the misunderstanding of the previous work and states that the manuscript should be reconsidered for EMBO J. Could you please resubmit a revised version of the manuscript addressing all the concerns of the referees.

Best wishes,

Editor

Referee comments:

Thanks for sending me the authors' reply. Their arguments are correct--I mistakenly equated the plasmid dimer resolution data from Aussel et al. with the chromosome dimer resolution data in the present manuscript, and my opinions then ensued from this incorrect assumption. So I think that the paper does deserve reconsideration at EMBO J. The fact that both you and I were both led down the wrong path suggests that other readers may have the same issues; therefore, the revised manuscript will need to make the distinctions between the present and earlier work (and the protein chimeras and their activities) much more clear.

Author's response 29 May 2009

Excellent!
I agree with referee #2 that this unfortunate affair taught us that we must explain more clearly the difference between earlier work on Xer rec. activation on plasmids and the new data on

chromosome dimer resolution.

1st Revision - authors' response 09 July 2009

We were pleased by the fact that all three referees and the editor appreciated our demonstration that the transmembrane domain of FtsK is not required for DNA transport across the division septum and the importance of this result for our understanding of the late stage of chromosome segregation in bacteria with statements such as: *"It is widely assumed that the C-terminal motor domain of [FtsK/SpoIIIE/TraB] moves DNA through a channel comprised of the N-terminal transmembrane domain of [these proteins]"* (Referee 3*), "the study uses a variety of elegant experiments to demonstrate that the transmembrane domain [of FtsK] is not required [for DNA transport]"* (The Editor*), "The manuscript contains well-designed experiments" (Referee 3), "The data seem to support the conclusions"* (Referee 2), *"The data are convincing that FtsKlc is a machine that can use the incompletely closed septum as the doorway for DNA pumping." (Referee 1), "The manuscript is solid and interesting."* (Referee 2) and *"The current study is important in that it challenges the field to think about alternative models [of late chromosome segregation]"* (Referee 1).

We also appreciated the detailed comments of the referees and of the editor, which we found to be very constructive and helped us improve our manuscript. Two main issued were raised: first, referee 2 and the editor made us realize that it was difficult for a non-specialist reader to distinguish the chromosome dimer resolution data reported in the initial version of the manuscript from previously published data on the activation of Xer recombination on plasmids, which undermined the novelty and interest of the present study. We took care in the revised version of the manuscript to clearly distinguish Xer recombination and chromosome dimer resolution assays. Second, we did not comment potential differences in the time and energy required to resolve chromosome dimers in chimeric strains in the initial version of the manuscript (see point 3 of referee 1 and point 1 and 2 of referee 3). In the revised version of the manuscript, we took care to distinguish data that allow us to affirming that some chimeras promote efficacious dimer resolution (Figure 1 to 4) from data directly suggesting that the process is fully efficient (new Figure 5). Briefly, we used quantitative PCR to monitor in real time the kinetics of activation of Xer recombination on the chromosome by chimeric ftsK alleles (Figure 5B). The qPCR results clearly indicate that ZipA::FtsKLC and FtsW::FtsKLC are as efficient in Xer recombination activation on the chromosome as FtsK, suggesting that the absence of the FtsK integral membrane domain in these chimeras neither slow down DNA mobilization across the dividing septum nor the subsequent activation of Xer recombination. We also directly compared the fitness of FtsKLC - strains ectopically expressing the wild-type ftsK allele, FtsKLC, FtsW::FtsKLC, FtsK50LC, and FtsW::FtsK50LC (Figure 5A). We found no differences in the fitness of cells expressing wild type FstK, FtsW::FtsKLC, FtsK50LC, and FtsW::FtsK50LC, indicating that these alleles are as efficient as wild-type FtsK in chromosome dimer resolution. Taken together, the qPCR and growth competition results considerably strengthened the conclusions of our paper, and we sincerely believe that this revised version of our manuscript is now suitable for publication in the EMBO Journal.

Answer to Referee 1 comments:

Point 1 : *"FtsKc could use available pores [...] even if the authors think this unlikely, they cannot rule out this possibility and it should be addressed"* .

Referee 1 is right. This is why in the initial version of the manuscript we took care to write that our data indicate that FtsK can transport DNA through a "pre-existing opening", before proposing that this opening is likely to be the uncompletely sealed septum.
We took care in the revised version of the manuscript to distinguish between the direct conclusion of our results and their most likely explanation:

The Abstract now reads:

"Here, we show that truncated Escherichia coli FtsK proteins lacking all of the FtsK transmembrane segments allow for the efficient resolution of chromosome dimers if they are connected to a septal

targeting peptide via a sufficiently long linker. These results indicate that FtsK does not need to transport DNA through a pore formed by its integral membrane domain. We propose therefore that FtsK transports DNA before membrane fusion, at a time when there is still an opening in the constricted septum."

The end of the Introduction now reads:

"These results indicate that the transport of chromosomal DNA across lipid bilayers during the last stage of chromosome segregation in E. coli does not require the formation of a pore by FtsKN and FtsKL. We propose therefore that E. coli FtsK most likely transports DNA before membrane fusion, at a time when there is still an opening in the constricted septum."

The Discussion now reads:

"We conclude that chimeric FtsK can transport DNA through a "pre-existing opening" between the two future daughter cells. The nature of this "pre-existing opening" is subject to speculations. We cannot exclude that chimeric FtsK proteins use a pore created by another septal membrane protein than FtsK. However, the most likely explanation for the fully efficient chromosome dimer resolution activity of ZipA::FtsKLC, FtsW::FtsKLC, FtsW::FtsK50LC and FtsK50LC (Figure 5), is that FtsK transports DNA before the final closure of the septum, at a time when there is still a rather large opening between the two daughter cells."

Point2 : *"Have the authors tested these fusions for protein stability by Westerns?It is possible that some of the results could be explained by stability not localization"*

All the conclusions that we make in our report derive form positive results, i.e. chimeras that are active in chromosome dimer resolution. Consequently, we thinkt that a detailed analysis of the stability of inactive chimeras was not crucial for the message of the paper. In addition, we had previously shown that Nter and Cter truncations of FtsK are stable by western blot analysis (Barre et al. Genes and dev. 2000). However, we agree that speculations about protein stability could distract the reader from the main results of the paper and we decided to include a western blot demonstratimg that FtsKLC is efficiently produced from the ftsK promoter and stable (Supplementary Figure 1; See point 2a of referee 1 and point 6 of referee 2). In addition, we include as a Supplementary Figure 2 the coomassie staining of an SDS-PAGE demonstrating that our different chimeras are expressed at the same level from pBAD in the plasmid dimer resolution asay (See point 2b of referee 1).

Point2a : *"The incomplete septal localization of FtsKLC could be a stability thing, and the mislocalized protein represented proteolytic fragments"*

We now include a western blot demonstratimg that FtsKLC is as efficiently produced from the ftsK promoter as FtsK50LC, and as stable (Supplementary Figure 1). This is commented in the result section of the paper as follows:

"We previously reported that a truncated protein lacking the whole of the N-terminal domain of FtsK, FtsKLC (Figure 2B), supports dif-recombination on plasmids (Barre et al, 2000). FtsKLC is efficiently produced from a pSC101 vector under the ftsK promoter and is stable (Supplementary Figure 1). However, it provided no growth advantage to ftsA* ftsK- cells compared to ftsA* ftsK- dif- cells (Figure 2D), indicating that it is completely inactive in chromosome dimer resolution."

Point 2b: *"Differences in the plasmid assay could be due to differences in expression [...]"*

We now include as a Supplementary Figure 2 the coomassie staining of an SDS-PAGE demonstrating that the chimeras are expressed at the same level from pBAD in the course of the plasmid dimer resolution assay. This is commented in the result section of the paper as follows:

"The zapA::ftsK50LC allele yielded 25% (±7) of cassette excision compared to 11% (±8) for zapA::ftsKLC in the plasmid Xer recombination assay (Figure 3B). We checked by SDS-PAGE that this was not linked to a major change in the stability and/or production of the chimera (Supplementary Figure 2) [...]."

Point 2c: *"Fluorescence derivatives [...] were expressed from pBAD"*

No. Fluorescence derivatives were expressed from the natural ftsK promoter carried on a low copy plasmid (pSC101). This is now clearly stated in the main text of the results for the benefit of the reader:
"The expression and the localization of the different ftsK alleles were checked using fusions with green and/or yellow fluorescent proteins (GFP and YFP) expressed from a low copy pSC101 vector under the natural ftsK promoter."

Point 3a: *"Fig. 3 vs Fig. 4: why is the f(%) different for FtsK and FtsW::FtsKlc in Fig. 3 (51%, 34%) compared to that in Fig. 4 (both 14%)?"*

The results of Figure 3 cited by Referee 1 correspond to the level of Xer recombination activation on plasmidic pseudo dimers whereas the results of Figure 4 correspond to real chromosome dimer resolution. At time 0 of plasmid experiments, 100% of the plasmids are in the form of a pseudo dimer, only 51% or 34% of them being recombined in 4h. In the chromosome dimer resolution measurements made by growth competition, recombination occurs only when a dimer has been formed, at a rate of approximately 1/7 per cell per generation. The maximum rate of recombination is therefore in the order of 15%. The 15% growth advantage of FtsK and FtsW::FtsKLC dif+ cells over their difcounterparts (Figure 2D, 3D and 4A) therefore indicates that they are 100% effective in chromosome dimer resolution, which is indicated by a +++ sign in Figure 4. Thus, full length FstK and FtsW::FtsKLC are more efficacious on chromosome dimers than on plasmid pseudo dimers. This is expected since chromosome dimers are necessarily trapped in the septum, which brings them into contact with FstK, whereas plasmids can only be processed when they encounter FtsK by chance.

Point 3b: *"How is variability controlled?"*

As indicated in the text and figures, plasmid and growth competition experiments were repeated at least three times.

Point 4: *"Fig. 4B: the photo is much too small; even after a magnification on my computer screen I cannot see any resolution in these cells."*

The size of the photo has been changed.

Answer to Referee 2 comments

Point 0a: *"Aussel et al. (2002) showed in their first Figure (Fig. 1B) that FtsK50c, a derivative of FtsK (FtsK50lc) containing residues 172-210 fused to FtsKc without FtsKl, was fully competent for dimer resolution in vivo. [...] As the current manuscript's title basically states the same concept, the work presented here does not break much important new ground."*

FtsK has two independent roles in chromosome dimer resolution: it functions as an activator of Xer recombination by a direct contact with the XerCD-dif nucleoprotein complex (Aussel et al. Cell 2002) and it serves to transport chromosomal DNA across the closing septum in the orientation dictated by KOPS sequences (Bigot et al. EMBO J. 2005). Early works about the role of FtsK in chromosome dimer resolution, such as in the Aussel et al. paper, focused on the mechanism of Xer recombination activation. To this aim, FtsK-dependent Xer recombination had to be monitored independently from the other roles that FtsK has in cell division and chromosomal DNA transport. We monitored therefore Xer recombination between PLASMID-borne dif sites. This can be done in vitro, using purified proteins, but also in vivo, using E. coli cells as a kind of "test tube". It has to be noted that in these experiments, no KOPS are needed for the oriented loading of FtsK on DNA and that Xer recombination activation is independent from the cell cycle, and notably cell division and homologous recombination (see Barre et al. Genes and dev. 2000). Indeed, in vivo experiments on plasmids are performed in conditions of overproduction of the FtsK chimeras, which are often deleterious to the cells and lead to their death in a few hours (Barre et al. Genes and dev. 2000; Yates et al. Mol. Mic. 2003). The experiments described in Figure 1B of the Aussel et al. paper are of this type. As a consequence, no conclusion can be made on the role of the N-terminal domain of

FtsK in chromosomal DNA transport based on this figure and none was made at the time. The data in Figure 1B helped us define a truncated peptide, FtsK50C, which is able to activate Xer recombination on PLASMIDS in vitro and in vivo. The rest of the 2002 paper focused on the study of the mechanism of Xer recombination activation. Of course, we tested the activity of FtsK50C in chromosome dimer resolution at the time and found, as clearly stated in the discussion section of the 2002 paper, that FtsK50C does not resolve chromosome dimers: "expression of FtsK50C in vivo does not suppress the chromosome segregation defect of FtsKC- cells (data not shown)."

We wish to thank the referee and the editor for making us realize that we should have taken more care in the manuscript to differentiate between previous experiments on the activation of Xer recombinatioon on plasmids from the novel experiments we present here. We took care in the revised version of the manuscript to stress this difference.

To this aim, we added in the introduction:

"It was observed that a truncation of FtsK lacking most of FsKN and FtsKL did not support chromosome dimer resolution even if it could efficiently process plasmid dimers in vivo (Aussel et al, 2002), which fitted with the idea that FtsKN and FtsKL might be implicated in the formation of a pore for the transport of chromosomal DNA at cell division."

We also included the following modifications in the result section to clarify this point:

Page 5
"Their ability to activate Xer recombination, which reflects the formation of stable and active FtsKC hexamers on DNA, was checked using a pseudo-dimer plasmid recombination assay (Barre et al, 2000)."

Page 6
"We previously reported that a truncated protein lacking the whole of the N-terminal domain of FtsK, FtsKLC (Figure 2B), supports dif-recombination on plasmids (Barre et al, 2000). FtsKLC is efficiently produced from a pSC101 vector under the ftsK promoter and is stable (Supplementary Figure 1). However, it provided no growth advantage to ftsA* ftsK- cells compared to ftsA* ftsK- dif- cells (Figure 2D), indicating that it is completely inactive in chromosome dimer resolution."

Page 7
"We previously characterized a region of 50 amino acids (FtsK50; aa 179 to 230; Figure 1B) that increases the efficiency with which FtsK peptides carrying an intact C-terminal domain activate Xer recombination under low expression levels (Aussel et al, 2002). Only half of this region was present in our initial chimeras (aa 211 to 230; Figure 3A). We decided therefore to include the rest of this region (aa 179 to 210) in our ZapA fusion to increase its capacity to activate Xer recombination. The zapA::ftsK50LC allele yielded 25% (±7) of cassette excision compared to 11% (±8) for zapA::ftsKLC in the plasmid Xer recombination assay (Figure 3B)."

Page 9
"A 500 aa deletion of the linker led to a 70% reduction of chromosome dimer resolution (Figure 4A), even if the truncated peptide supported dif-recombination on plasmids to a similar level than full length ftsK (Figure 4B) and was efficiently recruited to the septum (Figure 4C)."

Point 0b: *"The authors do show that residues 172-210 localize to the septum, which was only suggested by the Aussel et al. paper."*

We did not show that residues 172-210 localize to the septum! On the contrary, we know that these residues are not sufficient for septal localization. Indeed, we clearly stated that "FtsK50C does not localize specifically to the septum" in the discussion section of the 2002 Aussel al al. paper (unpublished data). Thus, it was a real surprise to us and to other specialists in the field to discover that the longer FtsK50LC construct targeted to the septum. We are currently investigating how FtsK50LC is recruited to the site of division.

Point 0c: *"They also show that tethering FtsKc to the septum via another septal protein allows full function of FtsKc."*

This is an oversimplification. Indeed, in the present report we show that FtsKc cannot resolve chromosome dimers when it is targeted to the septum by a fusion to ZapA (Figure 2; ZapA::FtsKLC construct).

Point 0d: *"[The study] confirms the widely held notion that a main purpose of FtsKn is to tether FtsKc to the septum."*

We agree that it is a widely held notion that one of the purposes of FtsKn is to tether FtsKc to the septum. This notion arose form our earlier reports in Cell (Aussel et al. 2002) and Genes and dev (Barre et al. 2000). However, this is not the point of the present study. The present study addresses the widely held notion that *"the C-terminal motor domain of [FtsK/SpoIIIE/TraB] moves DNA through a channel comprised of the N-terminal transmembrane domain" as stated by Referee 3 and more "specifically whether a pore constructed from the N-terminus of FtsK, FtsKn, is necessary for DNA translocation"* as stated by Referee 1.

Point 0e: *"It is not surprising that [tethering FtsKc to the septum via another septal protein allows chromosome dimer resolution] given that FtsK50lc localizes to the septum by itself and is functional for dimer resolution (which was previously shown by Aussel et al. anyway)."*

FtsK cannot resolve chromosome dimers when tethered to the septum via ZapA (See point 0c). We never showed in the 2002 Aussel et al. paper that FtsK50LC could target to the septum nor resolve chromosome dimers (See point 0a).

Point 0f: *"Finally, they also show that the linker's length is important for optimal dimer resolution. This is interesting, but hardly surprising."*

Contrary to E. coli FtsK, most other members of the FtsK/SpoIIIE/TraB family possess relatively short linkers, ranging from 100 to 300 aa. In addition, we know that this linker is dispensable for full Xer recombination activation in vivo and in vitro (Aussel et al. Cell 2002). Therefore, it would have been more reasonable to argue that the linker might not be important for chromosome dimer resolution. We wish to emphasize that the point of Figure 4 is not only to show that the linker length is important, but also to show that no specific region within this linker plays a role in chromosome dimer resolution (and hence DNA transport). Again, we would like to stress that the aim of this report is to demonstrate that DNA is not transported through a pore made by the Nter and/or the linker region of FtsK. However, we agree with Referee 2 that the importance of the linker's length for optimal dimer resolution fits very well with the idea that FtsK functions before the end of septum closure.

Point 0g: *"while the manuscript is solid and interesting, the key points mostly confirm previously published work."*

As already answered in point 0a to 0f, there is no previously published data on the role of the Nter and linker of FtsK in DNA transport. On the contrary, results in other species suggested *that "the C-terminal motor domain of these proteins moves DNA through a channel comprised of the N-terminal transmembrane domain"* as stated by referee 3, that SpoIIIE *"forms a membrane channel through which DNA is transported"* as stated by referee 2 and that *"SpoIIIE pumps DNA after the septum has been completed, supporting a SpoIIIE pore model"* as stated by Referee 1. The data we report is both original and important, as stated by referee *1 ("[...] the current study is important in that it challenges the field to think about alternative models for the activities of FtsK and its various domains")* and by Referee #3 *("together these experiments represent an important contribution towards our understanding of FtsK function")*.

Point 1: *"First sentence of Results: the authors should cite earlier references that demonstrated the importance of FtsKn in cell division."*

We now cite the necessary references in the introduction:

"Previous structural and functional analysis indicated that E. coli FtsK is composed of an amino-terminal domain with four transmembrane segments that is essential for cell division (FtsKN, aa 1-

210; Figure 1B; (Begg et al, 1995; Diez et al, 1997; Dorazi & Dewar, 2000; Draper et al, 1998; Liu et al, 1998; Wang & Lutkenhaus, 1998; Yu et al, 1998)"

Point 2a: *"Suppressed ftsK- cells are known to form chains at high frequency. Were these observed?*

Chains were observed. As indicated in the original description of the ftsA* mutant, only 30% of the cells present a normal phenotype.

Point 2b: *"Fig. 2C does not show them [chains].*

As stated in the legend of figure 2C, the images shown were taken from AB1157 cells to avoid the complex chain and filament phenotypes of ftsK- ftsA* strains, which might confuse non-specialist readers. Note, however, that we made sure that localization studies in wild type AB1157 strains were fully consistent with studies in ftsK- ftsA* strains and ftsK(deltaLC) strains.

Point 2c: "*Wouldn't the presence of such chains confound the dimer resolution data?"*

No. The efficiency of chromosome dimer resolution is monitored by comparing the growth of a ftsKftsA* strain expressing a specific chimera to the growth of its dif- version. Any other defect than chromosome dimer resolution, such as in cell division, is therefore equivalent for both strains.

Point 3: *"Lines 7-9, page 6: It is not surprising that FtsKlc::YFP fails to localize to the septum, as these regions have been shown previously to be dispensable for septal localization."*

It is true that it has been previously shown that the N-terminal 1-210 aa of FtsK are sufficient for septum recruitment. However, this did not indicate that the rest of the protein could not target to the septum via an independent domain. Indeed, we observed that FtsK50LC::YFP does localize to the septum even if it lacks all 4 transmembrane segments of FtsK.

Point 4: *"An important control seems to be missing in the experiments with fusions to other septal proteins such as ZipA and FtsW: a test to see if a non-septal membrane-localizing segment could permit FtsKlc to activate dimer resolution. This would be a one way to rule out general membrane tethering as a mechanism."*

We agree with referee 2 that it could be interesting to test such a chimera. However, we can already rule out general membrane tethering as a mechanism with the data presented in the report since ZapA::FtsK50LC and FtsK50LC, which both lack any transmembrane segment, are competent in chromosome dimer resolution (Figure 3).

Point 5a: *"In Fig. 2, the proteins either are fused to GFP at the N terminus (WT FtsK) or YFP at the C-terminus (all the others). It might be better to draw the GFP or YFP on the diagram for clarity."*

GFP/YFP versions of the chimeras were just used for localization purposes, but not for growth competition experiments nor in the plasmid pseudo dimer assays. The positions of GFP/YFP have been added in the name tags of each of the fluorescent images (Figure 2C, 3C and 4C).

Point 5b: *" In the legend (last line on page 16), "D" needs to replace "C"."*

True. Thanks!

Point 5c: *"For the growth competition in panel D, do these proteins have GFP/YFP on them as shown in panel B or not?"*

No. We engineered four series of constructs, one in which the chimeras are under the ftsK promoter on a pSC101 plasmid, one in which they are under the same promoter but fused to GFP or YFP, one in which they are expressed from the arabinose promoter on a high copy number pBAD vector and one in which they are expressed from the arabinose promoter on a pSC101 vector. Chimeras produced from pBAD were used to test for Xer recombination activation on PLASMID-pseudo dimer assays. GFP/YFP constructs on pSC101 plasmids under the ftsK promoter for localization studies. For growth competition we chose to test chimeras under the ftsK promoter and without

GFP/YFP fusions. To make this clearer, we added the position of GFP/YFP in the name tags of each of the fluorescent images (Figure 2C, 3C and 4C). We also mentioned in the different result section w=from which palsmid and from which promoter the proteins were expressed.

Point 6: *"The diffuse localization pattern for FtsKlc could be caused by degradation of the non-YFP portion of the protein. Was this tested?"*

See point 2a of Referee 1.

Point 7a: *"Fig. 2D: Were ZipA::FtsKlc, FtsK, and FtsW::FtsKlc more active in dimer resolution because they grew better?"*

No. As stated in the answer to Point 2c, the way in which we perform the growth competition experiments allow us to exclude the possibility that the observed differences in chromosome dimer resolution are due to any alteration that the production of a chimera may have on the cell cycle of the strains.

Point 7b: *"This is potentially relevant because they seem to partially complement the loss of FtsK (see comment 4)."*

As stated in the legend of Figure 2C and in answer to point 2b, the images shown were taken from AB1157 cells to avoid the complex chain and filament phenotypes of ftsK- ftsA* strains that might confuse a non-specialist reader.

Answer to referee 3 comments :

Point 1: *"The competition assay provides a good estimate of the ability of a protein to support dimeric chromosome resolution, but it does not estimate the efficiency of this process since the latter requires an assessment of the time and energy required. The results demonstrate that the chimeric FtsK molecules are ultimately able to resolve all dimeric chromosomes, but does not assess the time required for each event"*

Referee 1 is right in writing that in the initial version of the mansucript, we did not present any data that could be used to directly assess the time and energy required by chimeras to resolve chromosome dimers. Indeed, the co-culture assay we used measure the fitness of a strain expressing FtsK or a modified form in a dif- background, compared to its isogenic dif+ parent, which represents the rate of dimer resolution due to the ftsK allele. Note, however, that a delay in the process of chromosome dimer resolution of the dif+ strain would induce an increase of its generation time, which would in turn decrease the total number of cell divisions that occurred during the time frame of the experiment. This was not observed, which made us confident about the efficiency of chromosome dimer resolution achieved by the chimeras, even if this is not obvious to non-expert readers. This is now explained in a new paragraph at the end of the result section:

"Fully efficient resolution of chromosome dimers by chimeras lacking the FtsK transmembrane segments,The competition assay between ftsA* ftsK- cells and ftsA* ftsK- dif- cells provides a good estimate of the ability of a protein to support dimeric chromosome resolution. However, it does not provide a direct estimate of the efficiency of the process since the latter requires an assessment of the time and energy that are spent to eventually achieve it. Nevertheless, we noticed in the course of our growth competition experiments that dif+ ftsA* ftsK- cells expressing FstK chimeras that were fully active in dimer resolution yielded a similar number of colony forming units after four days of growth than dif+ ftsA* ftsK- cells expressing full length FtsK, which suggested that these chimeras efficiently resolved chromosome dimers (data not shown)."

Following the referee's advice, we also decided to delete the term "efficient" or replace it by "efficacious or effective" in our description of Figure 1 to Figure 4. For instance we conclude the results presented in Figure 1 with:

"We conclude that anchoring FtsKLC in the cytoplasmic membrane and targeting it to the septum is sufficient for the resolution of chromosome dimers in E. coli, which argues against the idea that formation of a pore by FtsKN is essential for the transport of DNA across lipid bilayers."

More importantly, we decided to include two types of experiments that directly address the problem of the efficiency of the chimeras, which are presented in Figure 5. In the first type of experiment, we expressed chimeras from an inducible arabinose promoter on a low copy pSC101 plasmid in an FtsKATP - strain harbouring a dif-dif cassette at the normal dif locus. Excision of the cassette can be monitored in real time by qPCR (Kennedy et al. 2008). In the vast majority of cases, it only occurs in the presence of a chromosome dimer (Perals et al. 2001; Kennedy et al. 2008; Barre et al. 2000). The rate of excision of the cassette as a function of time therefore reflects the time required by a chimera to process the DNA of the chromosome dimer, align the dif sites and activate Xer recombination. As illustrated in Figure 5B, we did not observe any difference in the rate of cassette excision between wild type FtsK, ZipA::FtsKLC and FtsW:::FtsKLC. We also directly compared the fitness of FtsKLC - strains ectopically producing wild-type FtsK, FtsKLC, FtsK50LC, FtsW::FtsKLC and FtsW::FtsK50LC (Figure 5A). We found no differences in the fitness of strains expressing FtsK, FtsK50LC, FtsW::FtsKLC or FtsW::FtsK50LC, which indicates that these alleles are fully efficient in chromosome dimer, resolution.

A whole paragraph is dedicated to the description of these experiments in the result section of the revised manuscript:

"To further investigate the efficiency with which FtsK chimeras resolved chromosome dimers, we decided to directly compare the growth of cells expressing them with the growth of cells expressing full length FtsK. However, this must not be done in the ftsA* ftsK- background since these chimeras do not necessarily compensate for the division defect of ftsA* ftsK- cells in contrast to full length FtsK. Consequently, we compared the growth of FtsKLC - cells ectopically expressing FtsK chimeras from a low copy pSC101 plasmid under the ftsK promoter to the growth of FtsKLC - cells ectopically expressing full length FtsK (Figure 5A). FtsKLC - cells expressing full length FtsK had a growth advantage of 15.7% per cell per generation over cells expressing FtsKLC (Figure 5A), which fits with the estimated rate of dimer formation (Perals et al, 2001; Steiner & Kuempel, 1998). In contrast, FtsKLC - cells expressing full length FtsK displayed no growth advantage over cells expressing FtsW::FtsKLC, FtsW::FtsK50LC and FtsK50LC, suggesting that these three chimeras are fully efficient in chromosome dimer resolution (Figure 5B). Note that this result also indicates that the ectopic expression of these chimeras did not interfere with other cellular processes than chromosome dimer resolution such as cell division.
The phenotype of FtsKLC - cells ectopically expressing ZipA::FtsKLC or ZipA::FtsK50LC from a low copy pSC101 vector under the ftsK promoter suggested that the resulting overexpression of ZipA altered the process of division (data not shown), which would confound the growth competition result. Consequently, we had to use a second approach to assess the efficiency with which ZipA::FtsKLC processes chromosome dimers. We previously described how Xer recombination can be monitored in real time using quantitative PCR in FtsKLC - cells habouring two dif sites in direct tandem repeat at the normal dif locus (Kennedy et al, 2008). Briefly, activation of Xer recombination leads to the excision of the dif-cassette, which can be detected by PCR using two pairs of primers (Figure 5B). Previous work indicated that in the vast majority of cases, this only occurs in the presence of a chromosome dimer (Barre et al, 2000; Kennedy et al, 2008; Perals et al, 2001) and at the time of cell division (Kennedy et al, 2008). Therefore, this assay allows us to directly address the time needed for the alignment of dif sites carried on a chromosome dimer and the subsequent activation of Xer recombination. The assay is only reliable when the production of FtsK is precisely controlled (Kennedy et al, 2008). To this aim, full length FtsK, FtsW::FtsKLC and ZipA::FtsKLC were produced from a low copy pSC101 plasmid under the arabinose promoter. We observed no difference in the rate of cassette excision promoted by these constructs following the induction of their production, suggesting that ZipA::FtsKLC is as efficient as full length FtsK and FtsW::FtsKLC in chromosome dimer resolution (Figure 5B).
Taken togther, these results suggest that anchoring FtsKLC in the cytoplasmic membrane and targeting it to the septum is sufficient for the fully efficient resolution of chromosome dimers in E. coli."

We thank referee 3 for this precious comment since the qPCR and growth competition results not only considerably strengthened the conclusions of our paper, but also made it easier for us to explain why we think FtsK most likely transports DNA before the final closure of the septum in the discussion section (See point 1 of referee 1):

"We conclude that chimeric FtsK can transport DNA through a "pre-existing opening" between the two future daughter cells. The nature of this "pre-existing opening" is subject to speculations. We cannot exclude that chimeric FtsK proteins use a pore created by another septal membrane protein than FtsK. However, the most likely explanation for the fully efficient chromosome dimer resolution activity of ZipA::FtsKLC, FtsW::FtsKLC, FtsW::FtsK50LC and FtsK50LC (Figure 5), is that FtsK transports DNA before the final closure of the septum, at a time when there is still a rather large opening between the two daughter cells."

Point 2: *"The plasmid assay for dif recombination is performed with high induction of the chimeric proteins (0.2% arabinose) and with long induction times (4 hours). This makes it impossible to determine if the chimeric proteins resolve dimeric chromosomes less rapidly than wild type proteins. The data contains hints that this might be the case, since the wild type protein supports ~50% recombination, while the chimeras supported 25-38% recombination (Fig. 3B). These results would be more convincing if the authors assessed recombination frequencies from several earlier timepoints and with lower induction, as they have done previously (Barre, 2000)."*

In this comment, referee 3 tried to use the results of Figure 3B to obtain an answer on the time required by the different FtsK chimeras to resolve chromosome dimers (See the first point of the referee). This is not possible because the results of Figure 3B correspond to the level of Xer recombination activation on plasmidic pseudo dimers, which are only processed when they encounter FtsK by chance (See our answer to point 3a of referee 1). Indeed, we wish to emphasize that differences in the efficiency of Xer recombination on PLAMIDS do not directly correlate with the efficiency of chromosome dimer resolution. For instance, we previously showed that a chimera carrying the Cter of H. influenzae FtsK, which is totally inactive in Xer recombination activation on plasmids (Yates et al. Mol Mic 2003), resolves 2/3 of the chromosome dimers (Bigot et al. Mol Mic 2004). However, we could answer the point raised by referee 3 by including quantitative PCR data (See our answer to point 1 of referee 3).

Point 3 *"The discussion of B. subtilis SpoIIIE on page 3 and page 10-11 is complicated by conflicts in the Bacillus literature about whether SpoIIIE is necessary to compartmentalize B. subtilis daughter cells and membranes during sporulation, as suggested by Wu et al, 1994 and 1995, Liu et al., 2006, or is instead dispensable for compartmentalization (as suggested by Burton et al., 2007). This issue could be avoided by replacing the sentence on page 4 that ends with "acts after septal membrane fusion" with a statement that the assembly of SpoIIIE at the septum is correlated with a barrier to diffusion between the two cells (Wu, 1994, Liu, 2006 and Burton, 2007)."*

We thank the referee for this judicious remark. The introduction now reads:

"The assembly of SpoIIIE at the sporulation septum of B. subtilis (Figure 1A, (ii)) correlates with the establishment of a barrier to protein and membrane dye diffusion between the mother cell and the prespore compartments (Burton et al, 2007; Liu et al, 2006; Wu & Errington, 1994; Wu et al, 1995), which suggested that SpoIIIE transports DNA after septal membrane fusion (Becker & Pogliano, 2007; Burton et al, 2007; Liu et al, 2006)."

Point 4: *"The above comment also makes it difficult to determine what the authors mean by "translocates DNA across a pre-existing opening in the septum". Perhaps this could be replaced by across an incompletely formed septum (?)"*

We have clarified the meaning of the "pre-existing opening" by stating in the discussion that it could be a pore created by other septal membrane proteins than FstK, as suggested by referee 1 (See point 1 of referee 1). We then propose the more likely explanation that FstK transports DNA acrosss an incompletely formed septum.

The discussion now reads:

"We conclude that chimeric FtsK can transport DNA through a "pre-existing opening" between the two future daughter cells. The nature of this "pre-existing opening" is subject to speculations. We cannot exclude that chimeric FtsK proteins use a pore created by another septal membrane protein than FtsK. However, the most likely explanation for the fully efficient chromosome dimer resolution

activity of ZipA::FtsKLC, FtsW::FtsKLC, FtsW::FtsK50LC and FtsK50LC (Figure 5), is that FtsK transports DNA before the final closure of the septum, at a time when there is still a rather large opening between the two daughter cells."

In the rest of the discussion, we then use the wordings proposed by referee 3:

Page 12
"Note that the idea that FtsK transports DNA across an incompletely formed septum does not exclude the possibility that a pore created by FstKN migh be involved in another cellular process, such as membrane fusion at the end of cell division as suggested for SpoIIIE (Liu et al, 2006)."

Page 13
"However, a model in which SpoIIIE transports DNA before membrane fusion, across an incompletely formed septum, is compatible with the observation that cytoplasmic proteins and membrane dyes do not diffuse freely between the mother cell and the prespore compartments during B. subtilis sporulation. Actually, reduction of the opening during septum constriction and molecular crowding at the site of division, which involves more than a dozen proteins, could diminish the rate of diffusion of membrane dyes between these compartments. In addition, formation of the SpoIIIE motors at the periphery of the incompletely formed sporulating septum might participate in blocking the diffusion or proteins between the mother cell and pespore comparments, which could partially explain why in its absence the two compartments are no more isolated (Liu et al, 2006)."

Page 13
"Transport of chromosomes across an incompletely formed septum is also compatible with the observation that active SpoIIIE molecules are only found on the side of the septum from which DNA is exported (Becker & Pogliano, 2007; Sharp & Pogliano, 2002a; Sharp & Pogliano, 2002b)"

Page 14
"The notion that FtsK and SpoIIIE could transport DNA through an incompletely formed septum fits with the previously accumulated evidences implicating SpoIIIE in membrane fusion during sporulation (Liu et al, 2006; Sharp & Pogliano, 1999; Sharp & Pogliano, 2003)."

Point 5: *"Page 4, Page 10. The paired channel model for SpoIIIE topology was proposed by Liu et al 2006 (Mol. Micro., 59:1097-) as well as by Burton et al., 2007. Both references should be cited."*

We agree. The citation was added.

Point 6a: *"The wording is a bit strong in several places given that the structure of the TM domain [...] has not been investigated"*

The structure of the TM domain of FtsK was investigated by Dorazi and Dewar in 2000. In the revised version of the paper, we take care to cite this paper in the introduction and in the result section. In the result section, we wrote:

"It is very unlikely that FtsK50 contains a full transmembrane segment since previous characterization of the membrane topology of FtsK indicated that all the amino acid residues after 146 are in the cytoplasm (Dorazi & Dewar, 2000)."

We also decided to include a sentence in the discussion (page 12) in which we clearly state that :

"[...] the idea that FtsK transports DNA across an incompletely formed septum does not exclude the possibility that a pore created by FstKN migh be involved in another cellular process, such as membrane fusion at the end of cell division as suggested for SpoIIIE (Liu et al, 2006)."

Point 6b: *"The wording is a bit strong in several places given that [...] the kinetics by which chimeric proteins support DNA translocation have not been directly investigated"*

In the revised version of the paper, we include two sets of experiments in which we directly investigate the kinetics of chromosome dimer resolution, which considerably strengthten our conclusions (Figure 5; see our answer to point 1 of referee 3).

Poin 6c*: "on page 4, the authors state 'FtsKN and FtsKL do not participate in the formation of a pore'. It would be more accurate to state that the formation of a pore is not essential for DNA translocation."*

Dealt with. The introduction now reads:

"These results indicate that the transport of chromosomal DNA across lipid bilayers during the last stage of chromosome segregation in E. coli does not require the formation of a pore by FtsKN and FtsKL."

Point 6d: *"On the bottom of page 6, one could tone down the concluding sentence by inserting "essential "..."argues against the idea that FtsKN creates a pore that is essential for the transport of DNA across lipid bilayers." It would be best if the authors considered this point as suggested by their data, rather than directly demonstrated by the data"*

Dealt with. The sentence now reads:

"We conclude that anchoring FtsKLC in the cytoplasmic membrane and targeting it to the septum is sufficient for the efficacious resolution of chromosome dimers in E. coli, which argues against the idea that formation of a pore by FtsKN is essential for the transport of DNA across lipid bilayers."

Point 7: *"Is the 50 amino acid domain of FtsK (FtsK50) sufficient for septal localization?"*

The 50 does not seem to be sufficient: no septal localization of a GFP-FtsK50 fusion, of a GFP-FtsK50C fusion nor a FtsK50C-YFP fusion was observed (data not shown).

Point 8: *"Figure 1. The grey box in panel B is not defined in the figure legend, and it appears smaller than the bracket for FtsK50"*

Dealt with. The figure legend now reads:

"A grey rectangle indicates the part of FtsKN that belongs to a 50 amino acid region (FtsK50; aa 179 to 230) that increases the efficiency with which FtsK peptides carrying an intact C-terminal domain activate Xer recombination under low expression levels."

Point 9: *"Figure 3. Scale bar size should be indicated."*

Dealt with.

Point 10: *"Figure 4. Panel B should be larger."*

Dealt with.

Point 11: *"Figure 5. Shows only one FtsK subunit attached to the membrane, perhaps to avoid clutter. How each subunit is proposed to be attached to the membrane should be clarified in the figure or legend"*

We have clarified how each subunit is attached to the membrane in the legend to avoid clutter:

"To avoid clutter, only one of the six linker arms attaching FtsK hexamers to the membrane is drawn."

We also propose a 3D reconstitution of the closing septum with FtsK hexamers pumping chromosomes to serve as a cover illustration for the journal (septum_late3.jpg).

Additional points raised by referee 3 in his second letter to the editor:

Point 12: *"the authors data does not rule out the possiblity that the N-terminal domain of FtsK assembles a channel"*

True. We included a sentence in the discussion (page 12) of the revised paper in which we clearly state that :

"[...] the idea that FtsK transports DNA across an incompletely formed septum does not exclude the possibility that a pore created by FstKN migh be involved in another cellular process, such as membrane fusion at the end of cell division as suggested for SpoIIIE (Liu et al, 2006)."

Point 13: *"The authors data do not allow any insight into the efficiency of the process in chimeric strains."*

We added new data to answer this point. See point 1 of referee 3.

2nd Editorial Decision 03 September 2009

I apologize for the length of time that it has taken to have your new manuscript evaluated, however, one of the referees was unavailable for a time and then subsequently ill. However, given the previous issues with the manuscript it was extremely important to have both these referees assess the manuscript. I have now received the reviewers comments and as you will see that overall they recommend publication in the EMBO Journal pending changes to the figures and the addition negative control.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Best wishes,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

This is a revised version of a previously submitted manuscript that was rejected in part because the differences between plasmid dimer resolution and chromosome dimer resolution assays used here vs. previous manuscripts by the same group were not made sufficiently clear for nonspecialist readers. The authors did a good job of clarifying the necessary sections of text and figures. The other two reviewers also had a number of significant concerns about the original manuscript, and in my opinion the authors addressed these well, including the addition of a qPCR experiment that contributed additional evidence for the efficient function of the chimeras. Overall, the present results support the model in which FtsK's transmembrane region does not need to form a pore for chromosome dimer resolution to work, suggesting that this process occurs prior to septum closure.

Referee #2 (Remarks to the Author):

The authors have revised their manuscript according to my suggestions and substantially clarified the manuscript. I have only two remaining areas of concern.

First, in several places either the font size or the cells shown are too small. I suspect the final font size will be below journal specifications for most figures. In response to concerns about the size of microscopy panels, one panel was increased by just 60%. This is not sufficient. The cells should be shown at ~2x their current magnification in all the figures and scale bars are required in each figure.

Second, the q-PCR assay results (Fig. 5B) should show the actual data points at each time as well as the best-fit lines and the data should include a non-functional allele, such as FtsK-LC. The symbols used in the schematic to the right of the results should be described in the figure legend, so that the

reader can understand the figure without referring to the previous publication.

2nd Revision - authors' response 27 October 2009

We were pleased that the revised version of the manuscript "substantially clarified" our results (Referee #2) and that "the present results support the model in which FtsK's transmembrane region does not need to form a pore for chromosome dimer resolution to work, suggesting that this process occurs prior to septum closure" (Referee #1).

Answer to Referee 2 comments:

Point 1: *"in several places either the font size [...] is too small. I suspect the final font size will be below journal specifications for most figures."*

Figure 1, figure 2, figure 3 and figure 5 have been designed to be 1-column figures (with a final width of 82 mm after reduction to 2/3 of their original size). Figure 4 and figure 6 have been designed to fit in 2-columns. The final width of Figure 4 should be 130 mm (after reduction to 2/3 of its original size). The final width of Figure 6 should be 172 mm (after reduction to 2/3 of its original size). In the final printed copies of the figures, the smallest font size will be 7 pt, which is 2.47 mm high.

Point 2 : *"In response to concerns about the size of microscopy panels, one panel was increased by just 60%. This is not sufficient. The cells should be shown at ~2x their current magnification in all the figures and scale bars are required in each figure."*

The microscopic images were magnified so that in each of the figures in which cells are shown, the length of bacteria should be in the order of 1 cm in the final printed version of the figures. This is large enough to observe if the position of the fluorescent signals of the chimeras is at mid-cell or not.

Point 3: *"the [qPCR] data should include a non-functional allele, such as FtsK-LC."*

We wish to emphasize that the conclusions of our paper are not based on negative results, such as the very low activity of FtsKLC in chromosomal dif-cassette excision. This is why we did not think that such a 'negative' control was necessary. However, we now include qPCR results obtained with the FtsKLC allele (Figure 5B). These results are commented in the text as follows:

"The assay is only reliable when the production of FtsK is precisely controlled (Kennedy et al, 2008). To this aim, full length FtsK, FtsW::FtsKLC, ZipA::FtsKLC and FtsKLC were produced from a low copy pSC101 plasmid under the arabinose promoter. Under these conditions, ZipA::FtsKLC proved to be as efficient as full length FtsK and FtsW::FtsKLC (Figure 5C). In contrast, FtsKLC did not promote excision of the chromosomal dif-cassette (Figure 5C)."

Point 4: *"the q-PCR assay results (Fig. 5B) should show the actual data points at each time as well as the best-fit lines"*

For each FtsK chimera that we tested, several independent experiments were performed to monitor the efficiency of cassette excision as a function of time using qPCR. The graph of Figure 5C now shows the actual data points and best-fit lines from one typical experiment. The slopes of the best-fit lines provide a good estimate of the rate of cassette excision as a function of time, which allowed us to calculate the relative efficiency of each chimera when compared to full length FtsK in each independent experiment. We now indicate the mean relative efficiencies of cassette excision of the FtsW::LC, FtsK LC and ZipA::LC alleles on the right of the graph in Figure 5C. The new figure 5C is commented in the figure legends as follows:

"**(C)** Chromosomal *dif*-cassette excision upon ectopic production of full length FtsK or FtsK chimeras. The % of cassette excision was monitored by qPCR at different times after the induction of the production of the *ftsK* alleles. The actual data points and best-fit lines from a

typical experiment are shown on the left. The slopes of the best-fit lines provide a good estimate of the rate of cassette excision as a function of time, which allowed us to calculate the relative efficiency of cassette excision of each chimera when compared to full length FtsK. The mean relative efficiencies are indicated on the right (results from 3 independent experiments for ZipA::FtsK$_{LC}$ and FtsW::FtsK$_{LC}$ and from 2 independent experiments for FtsK$_{LC}$)."

Point 5: "*The symbols used in the schematic to the right of the [qPCR] results should be described in the figure legend, so that the reader can understand the figure without referring to the previous publication*."

We now describe the schematic in the figure legend as follows:

"(B) Scheme of the chromosomal *dif*-cassette (left drawing) and of the recombinant product (right drawing) that is used to monitor the efficiency with which FtsK chimeras induce Xer recombination at the normal *dif* locus on the chromosome. *dif* sites are represented by black triangles. Arrows indicate the positions of the primers used in the qPCR assay. Primers X/Z serve to quantify the relative number of chromosomes in which the DNA cassette between the two repeated *dif* sites is excised (right drawing). To this aim, genomic DNA needs to be digested with *Hin*dIII (dashed line, H3) to prevent contamination by larger products encompassing the cassette. Primers X/Y serve to quantify the relative number of chromosomes that still harbour the *dif*-cassette."

