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Structural basis for ADP-mediated transcriptional regulation by P1 and P7 ParA

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(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 18 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by four reviewers, whose comments are attached below. As you will see, all referees generally appreciate your study and are thus in principle supportive of its publication; nevertheless, they also raise a number of specific concerns that would have to be satisfactorily addressed before publication in The EMBO Journal may be warranted. Among those, the most salient points pertain to the results and conclusions on the effects of ATP on ParA and filamentation - this seems to require some further analysis as well as more careful interpretation and discussion (including statements in title and abstract). Furthermore, most referees also ask for a somewhat deeper investigation of the site-directed ParA mutants established and tested in vitro, for example by testing their in vivo partitioning activity or their effects on ParA polymerization.

Should you be able to satisfactorily address these main issues, as well as to adequately respond to all the other points raised, we should be happy to consider a revised manuscript for publication. I would thus like to invite you to prepare a such a revision, taking into account the various points brought up by the reviewers. Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential to diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, Dunham et al. present structural studies of the ParA plasmid partitioning protein, both in apo and ADP-bound forms. The data are presented clearly and accurately, and the work is an important addition to understanding of the mechanism of bacterial DNA segregation. Of particular signficance are observations that domain swapping occurs in the apoParA dimeric structure, and that the apoParA dimers adopt multiple configurations by utilizing a highly flexible dimer interface. Importantly, mutagenesis data support the crystallization studies. ADP binding by ParA induces major structural changes in the protein which apparently favour formation of a preferred dimeric configuration in which two new DNA binding motifs are generated that likely support the interaction of ParA-ADP with the parABS operator site. Although the main thrust of the work relates to apoParA and ParA-ADP structures, evidence is also provided that ATP induces ParA polymerization as established for various ParA homologues.

1. Page 10-11. The trypsinolysis studies nicely support the contention that ADP stabilizes ParA and reduces its susceptibility to proteolysis. However, it is unclear how the authors reach the conclusions that '...the Arg351 site in P1 apoParA (Arg352 in P7 ParA) is readily cleaved...and indeed, Lys164 is cleaved closely after or at the same time as Arg351.' Were the tryptic fragments analyzed by mass spectrometry?
2. Page 13, second paragraph. The authors state that the S370A and R351A ParA variants bind DNA 5-10 fold less well than ParA. However, Fig. 6A shows that the mutated proteins provide the same DNaseI protection patterns at 0.8 ug protein as wild-type ParA does at 0.4 ug protein.
3. Page 14, third paragraph. The section 'ATP binding leads to filament formation...that is optimal for filament formation' is highly speculative without any solid data to support it. Similarly, the following specific proposal that 'ParA filament ends are stabilized by contacts by the ParB-parS complexes' lacks compelling evidence. Finally, the last sentence on page 15 suggests that ATP binding also locks ParA into a preferred dimeric state without any convincing supporting data. The authors may wish to revise the final paragraph of the Results/Discussion section.
4. The authors describe the oligomeric state of a number of ParA mutants in Fig. 3, mutants with altered trypsin digestion patterns in Fig. 4, and DNA binding mutants in Fig. 6. However, the affects of these mutations in partioning activity in vivo are not established. Testing the mutations in partition assays would be highly informative, would fit ideally with the in vitro studies, and would further strengthen the manuscript.

Minor comments
1. Abstract, last sentence. The authors suggest that ATP binding by ParA drives plasmid segregation as if this was a novel idea. However, this is not a new proposal as a number of ParA proteins have now been shown to polymerize in response to ATP binding.
2. Page 3, first paragraph. '...while the type Ib ParA and ParB homologues are smaller...' The DNA binding proteins in type Ib systems are not ParB homologues.
3. Page 5, line 3. Radnedge et al. 1998.
4. Page 8, second paragraph. Funnell, unpublished data.
5. Page 9, second paragraph. 'While the specific DNA site bound by P7 ParA has not been well defined...' The Austin group has shown operator site protection by P7 ParA in DNase I footprinting.
6. Page 13, third paragraph. Is Moller-Jensen et al. (2003) the correct supporting reference for the observation that ParA produces 'string-like formations' in the presence of ATP?

Referee #2 (Remarks to the Author):

Review Dunham et al

Partition ATPases of a number of prototype Par systems (P1, F, RK2, ...) are distinguished by an N-terminal domain that gives them a function besides active segregation of plasmid copies - autoregulation of Par protein production via binding to the par operon promoter. Whereas the structures of certain ATPases of this family which lack the N-terminal domain, notably MinD and Soj, had been determined, the contribution of the N-terminal domain to partition ATPase structure was unknown. In this MS Dunham et al report their structure determination for two very closely related ParA ATPases, those of the P1 and P7 prophage-plasmids, and so fill this gap. The work makes two important contributions: it details the interaction of the N-ter domain with the C-ter and the formation of dimers; and it advances our understanding adenine-nucleotide modulation of P1 ParA binding to DNA reported in previous studies by one of the authors (Dr Funnell). The MS also probes the anatomy of ADP-ParA interaction.
More specifically, the structures indicate a dimerization interface based on interactions of a long N-terminal a-helix of one monomer with two quasi-parallel C-terminal helices of the other. Although interaction of N-ter and C-ter domains was not entirely unexpected - it had been suggested for partition ATPase homologues from genetic (Ravin & Lane, JMolBiol, 2003) and biophysical (Batt et al, JMolBiol 385, 1361-1374, 2009) evidence - this structural study provides a far more concrete and detailed demonstration. The study further demonstrates that ADP can transform ParA to a specific, more structured dimer form, also indicated by greater resistance to trypsin; again, ADP-induced transition was not unanticipated in view of circular dichroism and DNA-binding data from Dr Funnell's lab but the detail afforded allows proposition of a plausible model of ParA binding to the parAB promoter region. The ParA-DNA complex model is interesting because it involves not only the expected interaction of the N-ter helix-turn-helix residues in the major groove but also non-specific interaction of a C-ter region with the backbone; mutational evidence is provided that is consistent with this latter aspect of the model. The model requires an induced bend of promoter region DNA that could easily be (and should have been) tested.
The authors have also included some observations on the effects of ATP on ParA properties. These are inconclusive and detract from the quality of the MS, which would be improved by simple excision of Fig. 6B and its associated text. A better title might be: "Structural basis for ADP-mediated transcriptional regulation by P1/P7 ParA". A second general comment is that the text surprisingly does not show the care and rigour that normally characterize Dr Funnell's papers; specific problems are listed below.
A general comment regarding structure papers: since the pdb files are not made available the reviewer is forced to squint at small figures showing indistinct shapes and largely to take the authors' word for what he is supposed to be seeing. It would help if authors took the trouble to orient, highlight and blow-up the structure elements they refer to in the text. This is not a particular criticism of this paper, which does a good job in some places e.g; Fig 3A, but less so in others e;g. a view of the relationship of the wings to the hth is as absent as it is relevant, and Fig 5A is bewilderingly dense.

Specific comments:
1 - p.9, line 16-17: Not true. The P7 site was determined with much the same precision as the P1 site, by Hayes et al, Mol Mic 11, 249-260 (1994). Cite.
2 - p.9, line 22-24: How is it known that the initial binding event is to a pseudo-palindrome? A reference should be given. Is it necessarily true that the specific contact is the first one? the specific site search could be accelerated by repeated non-specific contacts which would then necessarily be the initial ones.
3 - p.11, lines 18 - : ATP contacts with ParA are described as if demonstrated, but no ATP-ParA structure means no data. These contacts should be described as presumed or supposed. The text is in any case incoherent here - a cross contact between Lys117 and the ATP phosphate is said to be responsible for dimer formation, but ParA is then said to dimerize spontaneously .... This section needs rewriting.
4 - p.12, line 18 (actually, the same point as p.9, line 22-24 above): It is not clear what this assertion of the chronology of ParA binding is based on; the Davey & Funnell paper did not claim it. If it is based on the footprinting data in this paper it is unjustified - non-specific binding may precede specific contacts but not be seen at low ParA concentrations owing to its instability.
5 - p.13 lines 16-17: the indications that the basic regions are structured by ADP to play a role in specific binding is intriguing, but the existence and importance of these regions has been documented, albeit for partition and in less detail (Hester & Lutkenhaus, PNAS 104, 20326-20331 (2007); Castaing et al, Mol Mic 70, 1000-1011), and the claim of novelty is unjustified. These

precedents should be cited.
6 - p13, "ParA-ATP mediates ... ." This title leads the reader to expect a demonstration that ParA polymerization drives partition. This might well be the case but the evidence provided here falls far short of showing it. What it does show, in the first frame of Fig 6B, is that P1 ParA in the presence of ATP and Mg2+ can be seen as filaments; by itself this is hardly an advance given the precedents of several other ParA family proteins. The second frame purports to be an in vitro demonstration of partition. It is not. No movement is demonstrated, the only element seen is ParA so the relation to ParB-parS is unknown, there are no controls without ATP or of ParA in conditions unable to polymerize. The final frame shows what is supposed to be a ParB bead stuck to the end of a ParA filament but also looks similar to a negative-stain deposition artefact.
7 - p.14, lines 19- : This concluding section treats the structural effects of ATP on ParA as demonstrated or given, but the ATP-ParA structure is unknown and thus no "insight" (line 19) is provided and there is no "data (to) suggest that .... ATP binding ... locks in a specific dimer state .." lines 24-25). The structural consequences of the supposed Lys117-gphosphate interaction for polymerization or other ParA activities are not modelled or explained. The in vitro reconstitution experiments do not indicate filament stabilization because no destabilization is shown; the ParM model seems to have been assumed uncritically to apply to some very preliminary ParA data.

Minor comments:

8 - p.3, line 1-2: The sentence should make clear that it is bacteria whose segregation is the subject.
9 - p.3, line 3: Is it really generally thought that this is the sole reason for the advantage of plasmids? Their dispensability and ease of manipulation also figure large, at least historically.
10 - p.3, lines 6-7: sentence ambiguous - As written it appears that there are other, less abundant, Type I systems with other properties, but the contrast is made with type II systems implying the authors mean Type I systems are more abundant than these.
11 - p.3, line 23: The impression given is that L & S 2003 suggested that conversion to dimer suffices for segregation function, but they only claimed that it is a pre-requisite.
12 - p.4: Much of the main paragraph is devoted to detailing ParB binding to DNA, and seems irrelevant to the main subject of the paper. The useful part of this paragraph describes the elements of the P1 par system, points out their similarity to other known systems e.g. P7 and outlines ParA's actions in partition. Lines 5-7 and 15-19 could be cut with no loss.
13 - p.8, line 14: Funnell making a personal communication to her own paper? "Unpublished data" is the usual formula.
14 - p.12, line 25: Davey & Funnell only suggested wrapping; there is no evidence for it, and "likely" misrepresents the situation.
15 - p.13, lines 11-12: "fact" or only "likely"? rewrite to remove the contradiction.
16 - p.13, lines 22-26: It is unclear why SopA is in a separate category in this context or why Soj is left out.

Referee #3 (Remarks to the Author):

Opposed to actin like segregation mechanisms in bacteria like ParM, Walker type segregation proteins are less well understood. This manuscript indeed gives new insights how DNA segregation by Walker type APTases may work and is suitable for publication in EMBO J.

Some comments:

To classify ParA as a motor may be a bit misleading for most people.
Generally myosins are called motors.
I would suggest the wording linear polymerizing motor to distinguish from classical myosin like motors. Or cytomotive motors as suggested recently in the latest review article by Jan Loewe and Linda Amos.

The authors state that ParA forms regular filaments. Yet inspecting the shown EM image, I agree that some kind of polymers are formed upon adding nucleotide but they appear to be rather ragged. Ni-coated grids were used, how were they made or where were they obtained? And were filaments also seen on conventional carbon coated grids?

Referee #4 (Remarks to the Author):

Dunham et al report a structure function analysis of ADP binding to the bacterial plasmid segregation protein ParA. ParA has dual functions. In the ATP bound state it drives plasmid segregation, while in the ADP bound state, it acts as a transcriptional regulator. The authors report a series of crystal structures, low resolution structural analysis (SAXS, EM) as well as some biochemical data on the apo as well as ADP bound conformation of ParA. All in all, I find this paper in generally well written, although some parts may be reduced to improve readability. The technical quality of the data is good and the biochemical and mutational data support the structure analysis. It remains to be shown what structure change ATP induces and the DNA binding interactions are modeled and thus speculative. However, the mutational analysis supports to modeling data. I have two points that should be revised or included.

1) The second ADP binding site is very interesting but the authors do not present any data regarding its functional relevance. Since the authors anyway do protection assays with different ParA mutants (Fig. 6), it is relatively straightforward in my opinion to extend the analysis also to mutations in this site. This would lend considerable experimental support to their discussion on page 12.

2) The authors propose that ADP induces local folding of a loop that then forms a binding site for DNA in the transcriptional functions of ParA. They demonstrate that mutations in this site prevent DNA binding to this site. Do these mutations have an effect on polymerization? If their model is correct, polymerization in the presence of ATP should still be functional.

1st Revision - point-by-point response 03 March 2009

Please find attached our revised manuscript entitled "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA". We have read the comments of the four reviewers with great care and have addressed their concerns as outlined below (our detailed responses in red). As suggested by the reviewers, primarily reviewer 2, we have greatly de-emphasized the ParA filamentation studies. Reviewer 2 even suggested changing the title to "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA" (which we have done) as the main emphasis of our studies is on the ADP-mediated DNA-binding activation of ParA. Indeed, this is the first structure of a type Ia ParA partition ATPase, which unlike the smaller ATPases, carries out the additional function of ADP-mediated DNA-binding transcription activation and our studies provide a mechanistic explanation for this activation. We have also carried out the partition assays on the mutant ParAs as suggested. However, we do not agree that these residues must be functional in only DNA-binding and not partition. There is no data to suggest this. In fact, it is not only possible but quite likely that some activities could be shared by both the partition and repressor functions of ParA as one would predict that residues on the surface and involved in structural/conformational changes mediated by ADP or ATP binding would be the ones utilized in both transcription and partition functions (see discussions below).

However, as recommended, we carried out these partition assays and indeed we found that the K375/R378 and 16ParA (missing the first 16 residues) proteins are all defective for partition while the R351A is greatly reduced in partition activity (see Table below). These data therefore, suggest that these residues are important for partition. Results from filament assays indicate that they may still form polymers, however, the efficiency of polymer formation appears affected. Indeed, we do not know enough about ParA polymerization kinetics and stability to make strong predictions. Also, and very importantly, we have now greatly reduced our emphasis on the filamentation aspects of ParA in this revision (consistent with the new title "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA"). Therefore, we feel, very strongly, that these experiments and the very speculative discussions that would be required are not really appropriate for this study, especially given the length restraints and the fact that the reviewers did not favor high speculation.

However, if you and/or the reviewers would like us to include this data and the requisite speculation we would certainly do so.

Reviewer 1:
1. Page 10-11. The trypsinolysis studies nicely support the contention that ADP stabilizes ParA and reduces its susceptibility to proteolysis. However, it is unclear how the authors reach the conclusions that '...the Arg351 site in P1 apoParA (Arg352 in P7 ParA) is readily cleaved...and indeed, Lys164 is cleaved closely after or at the same time as Arg351.' Were the tryptic fragments analyzed by mass spectrometry? This is a good point. We were remiss in leaving out the method by which we determined the sites of proteolysis, which was N-terminal sequencing of fragments. We have now included this as the following:

Limited proteolysis experiments showing that apoParA has multiple flexible regions that become stabilized upon ADP binding are consistent with the structures (Figure 4D). Although the P7 apo protein appears to be more flexible than the P1 protein, the trypsinolysis pattern is very similar between the two. N-terminal sequencing of the fragments was used to identify proteolysis sites. These data show that the initial cut sites correspond to residues just C-terminal to 1. After this cleavage, 1 likely remains bound to the other subunit of the dimer, but the C-domain becomes more exposed. These data, in combination with the observation that P1 R351A is lacking a cleavage indicate that the residue after Arg351 in P1 apoParA is cleaved following proteolysis in 1 (Figure 4D). The P1 ParA structure shows that the region from 159-171 would also become exposed and indeed, the residue after Lys164 is cleaved closely after or at the same time as that after Arg351. ADP binding leads to marked protection against all these proteolysis events as expected from our structural data (Figure 4D).

2. Page 13, second paragraph. The authors state that the S370A and R351A ParA variants bind DNA 5-10 fold less well than ParA. However, Fig. 6A shows that the mutated proteins provide the same DNaseI protection patterns at 0.8 ug protein as wild-type ParA does at 0.4 ug protein.
We agree that this experiment, specifically, the S370A binding study, does not show the maximum difference we reported because we did not test lower levels of WT ParA. Thus, we have altered these estimates towards the conservative side and now say the following:

DNase I protection assays revealed that the S370A and R351A mutant proteins bound the 150 bp parOP operator region with 2-2.5 fold and 5 fold reduced affinity relative to wild type, respectively, while the K375A/R378A double mutation essentially abrogated DNA binding (Figure 6A).

3. Page 14, third paragraph. The section 'ATP binding leads to filament formation...that is optimal for filament formation' is highly speculative without any solid data to support it. Similarly, the following specific proposal that 'ParA filament ends are stabilized by contacts by the ParB-parS complexes' lacks compelling evidence. Finally, the last sentence on page 15 suggests that ATP binding also locks ParA into a preferred dimeric state without any convincing supporting data. The authors may wish to revise the final paragraph of the Results/Discussion section.
We have extensively revised and shortened the final paragraph (including removing the sentence "ATP binding leads to filament formationÖ). We have also revised the discussion on ATP binding by ParA. The idea that ATP binding locks in a specific dimeric state is indeed, our speculation at this point and we have removed all this discussion.

revised discussion on ATP binding
Lys117 of the Walker A motif makes no contacts to ADP in the ParA-ADP structure. However, in structures of other Walker box proteins bound to ATP, the corresponding residue makes contacts to the phosphate of the ATP molecule bound in the other subunit of the dimer, favoring the formation of an ATP sandwich dimer. As ParA is already a dimer, such cross contacts, if present, would likely play a different role, such as stabilizing a specific dimer state that is active for partition. However, the structure of a ParA-ATP complex is needed to address this issue.

revised conclusion
In conclusion, our studies have shown that the type Ia cytomotive ParA proteins form dimers in their apo state and have revealed the structural architecture of these proteins. The flexibility imparted by the 1-C-domain¥ dimer interface of ParA permits binding of ADP or ATP to the Walker box motif (Figure 6C, D). As observed for other Walker type cytomotive motors, we find that ATP binding

appears to stimulate ParA polymerization. While ATP binding activates partition, ADP binding stimulates the transcription regulation function of ParA. Our structural and biochemical studies show that ADP stabilizes a specific dimer state of ParA and leads to large scale folding of several regions of ParA, including two basic motifs, which are critical for DNA binding (Figure 6D).

4. The authors describe the oligomeric state of a number of ParA mutants in Fig. 3, mutants with altered trypsin digestion patterns in Fig. 4, and DNA binding mutants in Fig. 6. However, the affects of these mutations in partioning activity in vivo are not established. Testing the mutations in partition assays would be highly informative, would fit ideally with the in vitro studies, and would further strengthen the manuscript.

We initially did not such perform partition assays on the mutant proteins as the outcome is not necessarily indicative of one function versus the other (partition vs. transcription regulation/DNA-binding). Indeed, we think it is quite possible that some activities will be shared by both the partition and repressor functions of ParA as one would predict that residues on the surface and involved in structural/conformational changes would be the ones utilized in both transcription and partition functions. However, as suggested by the reviewer we have carried out these assays and the results (listed below) indicate that the K375/R378 and 16ParA (missing the first 16 residues) proteins are actually all defective for partition while the R351A is greatly reduced in partition activity. These data therefore, suggest that these residues are important for partition. Results from filament assays indicate that they may still form polymers, however, the efficiency of polymer formation appears affected. Indeed, we do not know enough about ParA polymerization kinetics and stability to make strong predictions.

Stability tests:

parA allele	% Retention of miniP1 (pBEF246)
wild-type	87
R351A	54
S370A	84
K375A/R378A	29
parA(17-398)	27
none (pBR322)	30

Materials and methods/ partition assays
The mutations R351A, S370A, and K375A/R378A and the 16 ParA were cloned into the pEF5 context (parA-parB genes expressed under the control of the -lactamase promoter). Stability of miniP1 plasmid pBEF246 (lacking parAB) was measured in the presence of the pEF5 derivatives as described in Schumacher et al, 2007, and is reported as retention in cells after 18-20 generations of bacterial growth in the absence of selection for miniP1. The data are an average of three experiments.

Thus, in summary, although this is a very interesting question, our model does not require that this basic region must play a role in only DNA-binding and not partition/polymerization and we do not know enough about ParA polymerization kinetics and stability to make strong predictions. Also, and very importantly, we have now greatly reduced our emphasis on the filamentation aspects of ParA in this revision (consistent with the new title "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA"). Therefore, we feel that such experiments with mutants and the very speculative discussions that would be required are not really appropriate for this study, especially due to length constraints. Thus, we have not included the partition assays in the revised manuscript. However, if the reviewer feels strongly that they should be added, we will include the data and the requisite speculation(s).

Minor comments
1. Abstract, last sentence. The authors suggest that ATP binding by ParA drives plasmid segregation as if this was a novel idea. However, this is not a new proposal as a number of ParA proteins have now been shown to polymerize in response to ATP binding.
The reviewer is correct that many type I ParA proteins have been shown to form polymers/filaments. We did not mean to imply in the abstract that P1/P7 ParA is unique or that this

is the first instance where this has been observed. To rectify this we have changed the last sentence in the abstract to:
By contrast, ATP binding stimulates ParA polymerization as observed in other ParA proteins, which we propose drives plasmid separation.
2. Page 3, first paragraph. '...while the type Ib ParA and ParB homologues are smaller...' The DNA binding proteins in type Ib systems are not ParB homologues.
Our meaning was that they were functional homologues, however the reviewer is correct that homologues formally refers to proteins of a common evolutionary ancestor. So we have changed the word to "proteins" -the sentence is as follows:
Specifically, the type Ia ParA and ParB proteins contain between 321-420 and 312-342 residues respectively, while the type Ib ParA and ParB proteins are smaller, containing 192-308 and 46-131 residues, respectively.
3. Page 5, line 3. Radnedge et al. 1998.
We thank the reviewer for catching this typo, and have fixed it in the revised manuscript.
4. Page 8, second paragraph. Funnell, unpublished data.
We have changed this to "unpublished data".
5. Page 9, second paragraph. 'While the specific DNA site bound by P7 ParA has not been well defined...' The Austin group has shown operator site protection by P7 ParA in DNase I footprinting.
Indeed, this was not worded correctly. We have clarified this as follows: P7 and P1 ParA function as DNA-binding proteins to autoregulate par transcription (Hayes et al., 1994). Studies on P1 ParA have shown that it recognizes a ~40 nucleotide imperfect inverted repeat between the -10 box and ribosome binding site of the P1 par promoter (Hayes et al, 1994). ParAís DNA binding activity is very cooperative however, so that in vitro one sees extensive protection of a region of ~150 nucleotides that is centered over the inverted repeat (Davey and Funnell, 1994 and Fig. 6B). ADP binding stimulates DNA binding by ParA by more than 20 fold.

6. Page 13, third paragraph. Is Moller-Jensen et al. (2003) the correct supporting reference for the observation that ParA produces 'string-like formations' in the presence of ATP?
This reference has been removed and now the papers describing the data showing polymerization by other type I Para proteins is referenced in this context.

Referee #2 (Remarks to the Author):

The authors have also included some observations on the effects of ATP on ParA properties. These are inconclusive and detract from the quality of the MS, which would be improved by simple excision of Fig. 6B and its associated text. A better title might be: "Structural basis for ADP-mediated transcriptional regulation by P1/P7 ParA".
As recommended we have changed the title to "Structural basis for ADP-mediated transcriptional regulation by P1 and P7 ParA". We are not opposed to removing Figure 6B. We have asked the editor to guide this decision (as none of the other reviewers requested this).

A general comment regarding structure papers: since the pdb files are not made available the reviewer is forced to squint at small figures showing indistinct shapes and largely to take the authors' word for what he is supposed to be seeing. It would help if authors took the trouble to orient, highlight and blow-up the structure elements they refer to in the text. This is not a particular criticism of this paper, which does a good job in some places e.g; Fig 3A, but less so in others e;g. a view of the relationship of the wings to the hth is as absent as it is relevant, and Fig 5A is bewilderingly dense.
We apologize for the small size of some of the figures. In trying to stay within the limits of figures while displaying what we felt were important structural concepts, in several cases, we ended up with figures with many panels. We have attempted to address this. First of all, we have enlarged all the figures in the revised text. Also, as suggested, we have remade Figure 5A and have removed the side chains, which we think greatly clarifies the figure while still emphasizing the location of the nucleotide binding motifs (Walker A, B etc.) within the context of the full length protein. Close ups of the specific contacts between ADP and protein side chains are shown in separate panels (i.e. Figure 5B). In addition, we have remade Figure 6A to remove some of the "clutter". In this new figure, only the "canonically" bound ADP molecules are shown and they are now shown as sticks rather than cpk, for clarity. The basic residues that were mutated and shown to be critical for DNA binding are now shown as dot surfaces, which emphasizes their location. The basic region that contains these residues and which is largely folded upon ADP binding, is colored red. We have

removed the final panel of Figure 6C. Finally, we have also added a Supplementary Figure (Supplementary Figure 1) showing the relationship of the wing and HTH. This figure also includes a topology diagram for further clarification.

Specific comments:
1 - p.9, line 16-17: Not true. The P7 site was determined with much the same precision as the P1 site, by Hayes et al, Mol Mic 11, 249-260 (1994). Cite.
We apologize for this error. We have cited the Hayes et al paper and changed this sentence to:
P7 and P1 ParA function as DNA-binding proteins to autoregulate par transcription (Hayes et al, 1994). Studies on P1 ParA have shown that it recognizes a ~40 nucleotide imperfect inverted repeat between the -10 box and ribosome binding site of the P1 par promoter (Hayes et al, 1994). ParAís DNA binding activity is very cooperative however, so that in vitro one sees extensive protection of a region of ~150 nucleotides that is centered over the inverted repeat (Davey and Funnell, 1994 and Fig. 6B). ADP binding stimulates DNA binding by ParA by more than 20 fold.

2 - p.9, line 22-24: How is it known that the initial binding event is to a pseudo-palindrome? A reference should be given. Is it necessarily true that the specific contact is the first one? the specific site search could be accelerated by repeated non-specific contacts which would then necessarily be the initial ones.
Mutagenesis by Hayes and binding data from Davis et al and Davey & Funnell support that the 40 bp sequence is the initial contact of ParA, but the referee is correct that the order of binding has not been conclusively established. We have changed the sentence to read: "Although ParA functions by cooperatively coating a ~150 bp region of the promoter, mutagenesis data indicate that the ~40 bp imperfect inverted repeat contains the recognition site for ParA (Hayes et al, 1994)."

3 - p.11, lines 18 - : ATP contacts with ParA are described as if demonstrated, but no ATP-ParA structure means no data. These contacts should be described as presumed or supposed. The text is in any case incoherent here - a cross contact between Lys117 and the ATP γ phosphate is said to be responsible for dimer formation, but ParA is then said to dimerize spontaneously .... This section needs rewriting.
This has been revised as follows:
Lys117 of the Walker A motif makes no contacts to ADP in the ParA-ADP structure. However, in structures of other Walker box proteins bound to ATP, the corresponding residue makes contacts to the phosphate of the ATP molecule bound in the other subunit of the dimer, favoring the formation of an ATP sandwich dimer. As ParA is already a dimer, such cross contacts, if present, would likely play a different role, such as stabilizing a specific dimer state that is active for partition. However, the structure of a ParA-ATP complex is needed to address this issue.

4 - p.12, line 18 (actually, the same point as p.9, line 22-24 above): It is not clear what this assertion of the chronology of ParA binding is based on; the Davey & Funnell paper did not claim it. If it is based on the footprinting data in this paper it is unjustified - non-specific binding may precede specific contacts but not be seen at low ParA concentrations owing to its instability.
Mutagenesis by Hayes and binding data from Davis et al and Davey & Funnell support that the 40 bp sequence is the initial contact of ParA, but the referee is correct that the order of binding has not been conclusively established. We have changed the sentence to read: "Although ParA functions by cooperatively coating a ~150 bp region of the promoter, mutagenesis data indicate that the ~40 bp imperfect inverted repeat contains the recognition site for ParA (Hayes et al, 1994)."
5 - p.13 lines 16-17: the indications that the basic regions are structured by ADP to play a role in specific binding is intriguing, but the existence and importance of these regions has been documented, albeit for partition and in less detail (Hester & Lutkenhaus, PNAS 104, 20326-20331 (2007); Castaing et al, Mol Mic 70, 1000-1011), and the claim of novelty is unjustified. These precedents should be cited.
We have changed the passage as suggested and also added citations (below).
In addition to direct contacts from basic residues, the dipoles of these helices are also positioned to interact with the phosphate backbone. This finding suggested that a key role of ADP induced folding might be the creation of new DNA binding motifs. Interestingly, recent studies have revealed the importance of basic residues in DNA binding by partition ATPases. Studies on Soj showed that ATP binding leads to the creation of a nucleotide sandwich dimer in which the basic residues are positioned to contact the DNA phosphate backbone, while a basic residue in SopA was shown to be critical for non-specific DNA binding and partition (Hester and Lutkenhaus, 2007; Castaing et al,

2008).

6 - p13, "ParA-ATP mediates ... ." This title leads the reader to expect a demonstration that ParA polymerization drives partition. This might well be the case but the evidence provided here falls far short of showing it. What it does show, in the first frame of Fig 6B, is that P1 ParA in the presence of ATP and Mg2+ can be seen as filaments; by itself this is hardly an advance given the precedents of several other ParA family proteins. The second frame purports to be an in vitro demonstration of partition. It is not. No movement is demonstrated, the only element seen is ParA so the relation to ParB-parS is unknown, there are no controls without ATP or of ParA in conditions unable to polymerize. The final frame shows what is supposed to be a ParB bead stuck to the end of a ParA filament but also looks similar to a negative-stain deposition artefact.
This has been greatly de-emphasized to indicate that our data only indicate that ATP stimulates polymerization, which is similar to that observed for several other type I ParA proteins. As recommended the manuscript is now focused on the ParA structures, the dimeric nature of the apo form and the ADP-induced conformational changes and their impact on DNA binding. This is reflected in the new title, which was suggested by the reviewer.
7 - p.14, lines 19- : This concluding section treats the structural effects of ATP on ParA as demonstrated or given, but the ATP-ParA structure is unknown and thus no "insight" (line 19) is provided and there is no "data (to) suggest that .... ATP binding ... locks in a specific dimer state .." lines 24-25). The structural consequences of the supposed Lys117-gphosphate interaction for polymerization or other ParA activities are not modelled or explained. The in vitro reconstitution experiments do not indicate filament stabilization because no destabilization is shown; the ParM model seems to have been assumed uncritically to apply to some very preliminary ParA data.
This has been revised to:

revised discussion on ATP binding
Lys117 of the Walker A motif makes no contacts to ADP in the ParA-ADP structure. However, in structures of other Walker box proteins bound to ATP, the corresponding residue makes contacts to the phosphate of the ATP molecule bound in the other subunit of the dimer, favoring the formation of an ATP sandwich dimer. As ParA is already a dimer, such cross contacts, if present, would likely play a different role, such as stabilizing a specific dimer state that is active for partition. However, the structure of a ParA-ATP complex is needed to address this issue.

revised conclusion
In conclusion, our studies have shown that the type Ia cytomotive ParA proteins form dimers in their apo state and have revealed the structural architecture of these proteins. The flexibility imparted by the 1-C-domain¥ dimer interface of ParA permits binding of ADP or ATP to the Walker box motif (Figure 6C,D). As observed for other Walker type cytomotive motors, we find that ATP binding appears to stimulate ParA polymerization. While ATP binding activates partition, ADP binding stimulates the transcription regulation function of ParA. Our structural and biochemical studies show that ADP stabilizes a specific dimer state of ParA and leads to large scale folding of several regions of ParA, including two basic motifs, which are critical for DNA binding (Figure 6D).

Minor comments:

8 - p.3, line 1-2: The sentence should make clear that it is bacteria whose segregation is the subject.
9 - p.3, line 3: Is it really generally thought that this is the sole reason for the advantage of plasmids? Their dispensability and ease of manipulation also figure large, at least historically.
In response to 8 and 9, We simply meant that these systems are particularly amenable to structural studies ñ we should have made this clearerñ thus we have altered this passage to read.
In bacteria, low copy number plasmids represent excellent model systems to study partition from a structural standpoint because they require only three elements, a centromere-like DNA site, a cytomotive motor protein and a centromere-binding protein (Hayes and Barill‡, 2006; Schumacher, 2008).

10 - p.3, lines 6-7: sentence ambiguous - As written it appears that there are other, less abundant, Type I systems with other properties, but the contrast is made with type II systems implying the authors mean Type I systems are more abundant than these.
We have removed the reference to abundance.
11 - p.3, line 23: The impression given is that L & S 2003 suggested that conversion to dimer

suffices for segregation function, but they only claimed that it is a pre-requisite.
This has been changed to:
Data from other Walker type proteins reveal that they undergo an ATP induced dimerization (Lutkenhaus and Sundaramoorthy, 2003), suggesting that dimerization may be a pre-requisite for partition.

12 - p.4: Much of the main paragraph is devoted to detailing ParB binding to DNA, and seems irrelevant to the main subject of the paper. The useful part of this paragraph describes the elements of the P1 par system, points out their similarity to other known systems e.g. P7 and outlines ParA's actions in partition. Lines 5-7 and 15-19 could be cut with no loss.
These sentences have been removed as suggested.

13 - p.8, line 14: Funnell making a personal communication to her own paper? "Unpublished data" is the usual formula.
This has been changed to unpublished data.

14 - p.12, line 25: Davey & Funnell only suggested wrapping; there is no evidence for it, and "likely" misrepresents the situation.
The discussion on DNA wrapping has all been removed.

5 - p.13, lines 11-12: "fact" or only "likely"? rewrite to remove the contradiction.
Changed to:
These residues likely make direct contacts with DNA, but also may reconfigure the DNA site to permit the docking of the HTH and wing elements, which would explain the severity of their mutations.

16 - p.13, lines 22-26: It is unclear why SopA is in a separate category in this context or why Soj is left out.
These sentences have been combined and Soj is included.

Referee #3 (Remarks to the Author):

Opposed to actin like segregation mechanisms in bacteria like ParM, Walker type segregation proteins are less well understood. This manuscript indeed gives new insights how DNA segregation by Walker type APTases may work and is suitable for publication in EMBO J.

Some comments:

To classify ParA as a motor may be a bit misleading for most people.
Generally myosins are called motors.
I would suggest the wording linear polymerizing motor to distinguish from classical myosin like motors. Or cytomotive motors as suggested recently in the latest review article by Jan Loewe and Linda Amos.
We agree that this clarifies the discussion greatly. Therefore, as suggested by the reviewer, we have changed "motors" to "cytomotive motors" throughout the text.

The authors state that ParA forms regular filaments. Yet inspecting the shown EM image, I agree that some kind of polymers are formed upon adding nucleotide but they appear to be rather ragged. Ni-coated gridsADP- were used, how were they made or where were they obtained? And were filaments also seen on conventional carbon coated grids?
Filaments were observed for both. The Ni2+ grids were purchased from Electron Microscopy Services (Hatfield, PA). The Ni2+ grids are with Formvar film and treated with a solution (poly-L-lysine) to add charge to the grid before applying the sample and treating with unranyl acetate. Filaments were also observed in Cu grids, Formvar/Carbon film, treated by glow discharging (add negative charge) and Cu grids (C-flat) holey carbon treated by glow discharging. Then we added a continuous carbon film by hand. Staining was with Uranyl formate, which provided better resolution. These details are now provided in the Materials and methods. However, we also would like to point out that we have greatly de-emphasized the discussion on filament formation. There is evidence from several studies that type I ParA proteins form polymers/filaments. The main points

we are emphasizing in the manuscript are the ParA structures, the dimeric nature of the apo form and the ADP-induced conformational changes and their impact on DNA binding. This is reflected in the new title "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA".

Referee #4 (Remarks to the Author):

1) The second ADP binding site is very interesting but the authors do not present any data regarding its functional relevance. Since the authors anyway do protection assays with different ParA mutants (Fig. 6), it is relatively straightforward in my opinion to extend the analysis also to mutations in this site. This would lend considerable experimental support to their discussion on page 12.
We do not ascribe any functional relevance to this 2nd site. In fact, in the text we state that this 2nd binding site is likely not relevant in vivo. This is supported by the fact that the residues contacting the second ADP in the P1 ParA structure are not conserved in the P7 parA protein. Our paragraph reads:

The ParA-ADP structure obtained with 5 mM ADP contains a second bound ADP molecule, which is less than 10 ≈ from the primary ADP site and located in a surface exposed cleft between both subunits of the ParA dimer (Figure 5A; Supplementary Figure 2). There are numerous contacts to this ADP from each ParA subunit in the dimer ParA. Residues Lys164, Lys320¥ and His323¥ contact ADP phosphate groups while Asp280¥ interacts with both ribose hydroxyls. The ADP adenine is contacted by the side chains of Ser162, Lys164, Met159 and Phe356 (Figure 5A). Although this non-canonically bound ADP is intriguing, the fact that the in vivo concentration of ADP (~100 M) is far lower than the 5 mM used to crystallize the complex makes it unlikely that this binding site is important in vivo. Moreover, the residues that contact this ADP are not conserved between the P1 and P7 ParA proteins.

However, an interesting study (Batt et al, 2009) which was just published on the type Ia ParA protein, IncC, provides evidence for a second ADP binding site. Yet the binding affinity is quite low and likely not to be physiologically relevant. We have added this data and the paragraph now reads:

The ParA-ADP structure obtained with 5 mM ADP contains a second bound ADP molecule, which is less than 10 ≈ from the primary ADP site and located in a surface exposed cleft between both subunits of the ParA dimer (Figure 5A; Supplementary Figure 3). There are numerous contacts to this ADP from each ParA subunit in the dimer ParA. Residues Lys164, Lys320¥ and His323¥ contact ADP phosphate groups while Asp280¥ interacts with both ribose hydroxyls. The ADP adenine is contacted by the side chains of Ser162, Lys164, Met159 and Phe356 (Figure 5A). Given that the in vivo concentration of ADP (~100 M) is far lower than the 5 mM used to crystallize the complex it appears unlikely that this binding site is important in vivo. Moreover, the residues that contact this ADP are not conserved between the P1 and P7 ParA proteins. Interestingly, recent data suggests that the ParA protein, IncC, from the broad-host-range plasmid RK2 has a second, low affinity ADP binding site (Batt et al, 2009). However, the affinity of this ADP binding site was ~1000 fold lower than that of the "primary" ADP binding site in IncC (54 M) indicating that it may not be physiologically relevant.

2) The authors propose that ADP induces local folding of a loop that then forms a binding site for DNA in the transcriptional functions of ParA. They demonstrate that mutations in this site prevent DNA binding to this site. Do these mutations have an effect on polymerization? If their model is correct, polymerization in the presence of ATP should still be functional.
We actually do not agree with this statement. In fact, it appeared to us that it is quite possible that some activities could be shared by both the partition and repressor functions of ParA as one would predict that residues on the surface and involved in structural/conformational changes would be the ones likely utilized in both transcription and partition functions. In fact, we have now carried out partition assays on these mutants (below) and these experiments indicate that the K375/R378 and 16ParA (missing the first 16 residues) proteins are all defective for partition while the R351A is greatly reduced in partition activity. These data therefore, suggest that these residues are important for partition. Results from filament assays indicate that they may still form polymers yet the efficiency of polymer formation appears affected. Also, the detailed polymer structure may be affected and we have no knowledge of the exact conformation required for partition.

Stability tests:

parA allele	% Retention of miniP1 (pBEF246)
wild-type	87
R351A	54
S370A	84
K375A/R378A	29
parA(17-398)	27
none (pBR322)	30

Thus, in summary, although this is a very interesting question, our model does not require that this basic region must play a role in only DNA-binding and not partition/polymerization. Moreover, we do not know enough about ParA polymerization kinetics and stability to make strong predictions. Also, and very importantly, we have now greatly reduced our emphasis on the filamentation aspects of ParA in this revision (consistent with the new title "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA"). Therefore, we feel that such experiments with mutants and the very speculative discussions that would be required are not really appropriate for this study.

We hope that our revised manuscript has satisfied the concerns of the reviewers. If you should need any additional information, please contact me at your earliest convenience.

2nd Editorial Decision 24 March 2009

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees 2 and 4, and I am happy to inform you that both of them are largely satisfied with your response and the changes made during revision. Therefore, only a few minor points will need to be adjusted before we can proceed with publication of the manuscript (see the reports below) - and I think I should comment on two of these points:

The main point is that referee 2 still remains unconvinced that the inclusion of the partition data in Figure 6C adds any valuable novel information to the study. I realize that none of the other reviewers had voiced a similarly strong opinion on this (although they did appear to partially share this concern during the initial round of review); nevertheless, I feel that the critical referee has a point here, and given that you have indicated your willingness to replace the panel in question, I would like to suggest to either remove these data, mentioning them e.g. as 'unpublished observations', or to delegate them to the supplementary information and to shorten the pertinent part of the result section.

The second point is only a semantic one and refers to the change of 'motor' into 'cytomotive motor' introduced upon referee 3's original suggestion. Again, not wanting to be finicky I still have to agree with referee 2 that 'cytomotive motor' may not be the most fortunate expression to be used here, and indeed, the L^we & Amos review quoted by referee 3 does actually propose a slightly different term, 'cytomotive filaments'. Either this, or the originally proposed alternative 'linear polymerizing motor' should be fine.

I would thus like to ask you to incorporate these additional changes requested in a last round of revision. Once we will have received this final version, we should then be able to proceed swiftly with the formal acceptance of your paper.

I am looking forward to receiving your final version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have for the most part made serious attempts to modify the M/S in line with the reviewer's comments.

Replies to my comments:
1 - OK, but co-operativity is now introduced as the reason for coverage of flanking DNA, and I'm not sure if this is a correct use of the term as commonly understood. In any case, wouldn't non-specific DNA binding ability be important as well, or instead?
2 - The proposed replacement sentence does not appear in the actual text. But it is not missed, the text is better without it, and it does appear on p.12 where it is appropriate.
3 - OK
4 - OK
5 - OK
6 - The ATP part has indeed been de-emphasized, but it is still a largely useless appendage that for some reason the authors are trying to cling to. The only real information is given by the first two sentences (of p.13 - ATP stimulates ..), the rest could be covered by a statement that ParA forms filaments of probable importance for partition as seen for other ParA proteins, and the whole included as 'results not shown' in the concluding discussion. Shifting the notion that the figures say something solid about partition from the heading to the sentence "To address the possibility ... " does not somehow make it acceptable. And the only justification for the statement of p.14 line 13-14 is the old data from the Funnell and Austin labs, appropriate references to which should be given. Turning two-thirds of Fig 6B, which should have been excised, into Fig. 6C is not convincing. It is hard to see why the authors want to retain this blemish on an otherwise very good and defendable paper.
All minor comments - OK

Missed the first time:
p.4, bottom 2nd para - Bouet and Funnell should be 1999, as in the reference list
p.13, line 19 - why E.coli?, the plasmid is F.

Replies to other reviewers:
Reviewer 1, minor comment 1 - The objectionable part of this was the sense that the authors are themselves proposing something (" .. as if this was a novel idea .."), and it is still there in the modified sentence. As indicated above, I think the best solution is to simply dump the sentence, and to end the abstract in some other way.
Reviewer 3, comments on "motor" - I hesitate to contradict another reviewer because it risks creating confusion - nevertheless I disagree that the term motor has to be restricted to myosins if other proteins have verifiable motor properties. But surely if an alternative has to be found one can do better than "cytomotive motors". I don't think it is only falling on an English ear that makes it sound silly; it is redundant in two ways, one because we accept that to a first approximation the protein works in a cellular (cyto) environment using the properties we detect in vitro, and two because motive and motor convey the same idea, or if they do not then someone is guilty of throwing unexplained jargon into the works. I think the terminology used in the original MS was irreproachable, and if it expands the horizons of myosin folk a little so much the better.

Referee #4 (Remarks to the Author):

The authors addressed my concerns satisfactorily. I recommend publication

2nd Revision - point-by-point response 31 March 2009

Please find attached our revised manuscript entitled "Structural basis for ADP-mediated transcription regulation by P1 and P7 ParA". We have read the remaining comments from reviewer 2 and your suggestions and have addressed these concerns as outlined below (our responses in red).

Editor comments:
referee 2 still remains unconvinced that the inclusion of the partition data in Figure 6C adds any valuable novel information to the study. I realize that none of the other reviewers had voiced a similarly strong opinion on this (although they did appear to partially share this concern during the initial round of review); nevertheless, I feel that the critical referee has a point here, and given that you have indicated your willingness to replace the panel in question, I would like to suggest to either remove these data, mentioning them e.g. as 'unpublished observations', or to delegate them to the supplementary information and to shorten the pertinent part of the result section.
Indeed, we are willing to take out this panel from Figure 6. We have moved these figures (and the Materials and methods describing these studies) to Supplementary and combined them with the filament figures/data already present in Supplementary Figure 4 and Supplementary data. In addition, we have significantly shortened the discussion on the filament study, as suggested by reviewer 2.

The second point is only a semantic one and refers to the change of 'motor' into 'cytomotive motor' introduced upon referee 3's original suggestion. Again, not wanting to be finicky I still have to agree with referee 2 that 'cytomotive motor' may not be the most fortunate expression to be used here, and indeed, the Löwe & Amos review quoted by referee 3 does actually propose a slightly different term, 'cytomotive filaments'. Either this, or the originally proposed alternative 'linear polymerizing motor' should be fine.
We do not really think the term "cytomotive filament" is fully appropriate here. Indeed, it has not yet been established that all these proteins utilize filaments in partition. In fact, Lowe and Amos only tentatively place the ParA/Soj type of proteins in the cytomotive filament category. The difficulty in terminology here seems to have arisen primarily because of the findings of Popp et al. who showed that R1 ParM can use GTP as well as ATP and thus, the utilization of "ATPase" became no longer appropriate to summarize this entire family of partition proteins (i.e. ParM actin-like and ParA Walker box proteins). So because there is variation in the field in the semantics for ParAs, we changed the descriptor to the much more general "partition NTPase". This seems the most conservative approach, as it does not imply any function that has not yet been attributable to both Walker and actin-like proteins, yet is fully accurate in its description.

Referee #2 (Remarks to the Author):

The authors have for the most part made serious attempts to modify the M/S in line with the reviewer's comments.

Replies to my comments:
1 - OK, but co-operativity is now introduced as the reason for coverage of flanking DNA, and I'm not sure if this is a correct use of the term as commonly understood. In any case, wouldn't non-specific DNA binding ability be important as well, or instead?
We have simplified this sentence so that cooperativity is no longer mentioned. We now simply indicate that ParA binding extends over 150 nucleotides as follows:" Studies on P1 ParA have shown that it recognizes a ~40 nucleotide imperfect inverted repeat between the -10 box and ribosome binding site of the P1 par promoter (Hayes et al, 1994). However, the entire protected site includes ~150 nucleotides that is centered over the inverted repeat (Davey and Funnell, 1994 and Fig. 6B)."
2 - The proposed replacement sentence does not appear in the actual text. But it is not missed, the text is better without it, and it does appear on p.12 where it is appropriate.
3 - OK
4 - OK
5 - OK
6 - The ATP part has indeed been de-emphasized, but it is still a largely useless appendage that for

some reason the authors are trying to cling to. The only real information is given by the first two sentences (of p.13 - ATP stimulates ..), the rest could be covered by a statement that ParA forms filaments of probable importance for partition as seen for other ParA proteins, and the whole included as 'results not shown' in the concluding discussion. Shifting the notion that the figures say something solid about partition from the heading to the sentence "To address the possibility ... " does not somehow make it acceptable. And the only justification for the statement of p.14 line 13-14 is the old data from the Funnell and Austin labs, appropriate references to which should be given. Turning two-thirds of Fig 6B, which should have been excised, into Fig. 6C is not convincing. It is hard to see why the authors want to retain this blemish on an otherwise very good and defendable paper.
As suggested we have taken out Figure 6C and have moved the images to Supplementary Figure 4. We have also quite significantly shortened the discussion on the ATP part of the manuscript to only include a few sentences and the statement that the filaments formed by ParA are "of probable importance for partition as seen for other ParA proteins", as recommended.
All minor comments - OK

Missed the first time:
p.4, bottom 2nd para - Bouet and Funnell should be 1999, as in the reference list
We have fixed this typo and thank the reviewer for catching this.
p.13, line 19 - why E.coli?, the plasmid is F.
Changed to F plasmid

Replies to other reviewers:
Reviewer 1, minor comment 1 - The objectionable part of this was the sense that the authors are themselves proposing something (" .. as if this was a novel idea .."), and it is still there in the modified sentence. As indicated above, I think the best solution is to simply dump the sentence, and to end the abstract in some other way.
As recommended we have simply removed the last sentence.
Reviewer 3, comments on "motor" - I hesitate to contradict another reviewer because it risks creating confusion - nevertheless I disagree that the term motor has to be restricted to myosins if other proteins have verifiable motor properties. But surely if an alternative has to be found one can do better than "cytomotive motors". I don't think it is only falling on an English ear that makes it sound silly; it is redundant in two ways, one because we accept that to a first approximation the protein works in a cellular (cyto) environment using the properties we detect in vitro, and two because motive and motor convey the same idea, or if they do not then someone is guilty of throwing unexplained jargon into the works. I think the terminology used in the original MS was irreproachable, and if it expands the horizons of myosin folk a little so much the better.
We have used the simplified term "partition NTPase (see comments to editor above). We do not prefer the term cytomotive filament and because there is variation in the field in the semantics for ParAs, we changed the descriptor to the much more general "partition NTPase". This seems the most conservative approach, as it does not imply any function that has not yet been attributable to both Walker and actin-like proteins, yet is fully accurate in its description

Referee #4 (Remarks to the Author):

The authors addressed my concerns satisfactorily. I recommend publication

