Metabolism and Chromosome Copy Number Control
Mutually Exclusive Cell Fates in Bacillus subtilis

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

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please excuse the delay in getting back to you with a decision, but unfortunately two of the three referee reports arrived at our office only after I had left for the holiday season. However I am pleased to now inform you that all three referees have favorably reviewed your submission, and would be supportive of publication pending adequate revision to address a number of specific issues, as detailed in the comments attached below. I am thus inviting you to prepare such a revised manuscript and a point-by-point response in light of those reports, which we should be happy to consider further for publication. When preparing your letter of response, please be reminded that our policy to allow only a single round of major revision will necessitate diligent and comprehensive answering, and also bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, please also amend the manuscript text with a brief ‘Author Contribution’ section and a ‘Conflict of Interest’ statement before resubmission. 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 reading the revised manuscript.

Yours sincerely,
Editor
The EMBO Journal
REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This submission from the Losick/Kolter labs is interesting and insightful. It sheds light on the regulation of an important developmental process in a major model organism and suggests a novel regulatory mechanism. Although I do have some comments concerning the data and the interpretations of the data, this will be a provocative and important addition to the literature.

1. The declarative nature of the title contradicts the more cautious interpretation in the discussion. Specifically, "Metabolism and chromosome copy number control...." vs "We propose that the doubling of chromosome copy number....". In my opinion some caution is merited. Although the chromosome copy number hypothesis is lovely, explanatory and quite plausible, it represents a leap from the demonstration that the system is sensitive to gene copy number. In other words there is no direct evidence that chromosome copy number is responsible for or even contributes to the down-regulation of matrix gene expression during sporulation. Perhaps the title should be muted a bit. (Note that the abstract says in the last sentence "...could contribute..." suggesting some hesitation on the part of the authors.

2. The lux data do not seem to have been corrected for growth. Much of the increase and in the sporulating cells the decrease in light output seen in the figures seems to be due to increase in cellular mass. These curves would look very different if plotted as specific activities. In Fig. 7A the decrease in light would be much less impressive if so corrected because of the decrease in OD.

3. A minor point concerns the use of "real time". The dictionary says that this means immediately or instantaneously. Taking samples at intervals for later measurement does not meet this definition.

4. The methods section does not tell us about the substrate used for the lux assay.

5. "neither the deletion mutation nor the scrambled...increased ...expression strongly (not shown)". This is an important point and should be shown because it indicates that Spo0A-P does not reach an inhibitory level in MsGG.

6. A comment on Fig. 3A. With the wt probe, the lower-shifted high affinity band disappears with higher concentrations of protein. This suggests that there is cooperative binding to the multiple operator sites so that there is a concentration regime in which the apparent affinity for the operator sites is greater than to the activator site. If I am right, this has implications that favor the model and may be worth pointing out.

7. In Fig. 3Ad, binding to the operator sites occurs with a much lower concentration of protein than in Aa or Ab. Why? Is the amount of DNA so close to being overwhelmed by protein that it is sensitive to a two-fold reduction?

8. Fig. 3B. worries me a bit. The amount of probe in the "DNA only" lane seems higher than in the other lanes, particularly the adjacent one. Given this, the protection of O1 is questionable. Can this protection be documented, perhaps by densitometry? Also, there seems to be protection of a sequence between O2 and 10, but closer to the former, at least with the highest concentration of protein. Comment?

9. Neither of the insertions in the region upstream of sinIR interrupt the two open reading frames. Do these gene products play a role in the regulation?

10. How does the promoter in front of sinR affect the regulation?

11. The references to Grossman concerning the higher copy number of origin-proximal genes might also include the seminal papers of Noburu Sueoka, who I think was the first to point this out. (About 1963 or 1964 I think in PNAS?, which dates me).

12. Fig. 6 Aa and Ad and Ba and Bd show strikingly heterogeneous expression on the single cell level. Comments?

Referee #2 (Remarks to the Author):

The authors describe a series of very interesting and important studies that investigate the control of Bacillus subtilis cell fate selection of sporulation or matrix production cell states. These alternate developmental conditions have been shown by the authors and earlier studies to be regulated by Spo0A-P levels, SinI, and catabolite repression/metabolic state. Spo0A-P levels and sinI expression
are also known from previous studies to be affected by transition-state regulators. The authors suggest that low levels of Spo0A~P favor matrix production/biofilm fates by induction of sinI, and high levels of Spo0A~P commit cells to the sporulation pathway by repression of sinI. The authors also demonstrate that two copies of sinI and sinR within the same cell block matrix production by the cooperative binding of SinR to cognate DNA targets. A very important set of experiments demonstrate that sinI and sinR function depend on chromosomal position and gene dosage. Two completely replicated chromosomes are present in early stage sporulation cells until the final internal cell division which separates the mother cell and forespore. sinI expression is shown to be controlled by a previously unknown transcriptional read-through mechanism. The authors demonstrate that the canonical Spo0A~P binding site (0A-box) upstream of the sinI promoter does upregulate sinI expression. The authors propose on somewhat weaker evidence that other 0A-like box sequences (imperfect sequence matches) found in the upstream promoter regions of sinI could be involved in repression of sinI.

The evidence presented for the SinI mediated cell fate selection mechanism is novel, convincing and of substantial interest to a wide range of developmental molecular geneticists. The authors could benefit from putting their results in a broader context of earlier studies that examined the role of chromosome replication and transition-state phenomena in determining sporulation cell fates.

The evidence for the involvement of 0A-like box sequences in a proposed sinI repression mechanism is not entirely convincing. The site-directed alterations at these sites termed O1 - O4 comprise extensive sequence changes that could have a number of effects on transcriptional regulation of the sinI promoter well beyond the binding of Spo0A~P. Furthermore, their effects on read-through transcription are unknown. The authors should consider whether the extensive amount of space taken by these experiments detracts from the other very important and salient findings reported.

Referee #3 (Remarks to the Author):

Comments
This paper concerns the response of Bacillus subtilis to nutrient limitation and the decision to be made between following the pathway to biofilm formation and that to sporulation. Both of these developmental pathways are regulated by the response regulator, Spo0A. In the case of biofilm formation, Spo0A~P exerts its effects through regulation of sinI which encodes an antagonist of the master regulator (repressor) of biofilm formation SinR. In this paper, the authors identify and analyse Spo0A~P binding sites at the sinI promoter and present evidence for a tight binding activator site and weak binding repressor sites. They argue convincingly that mild nutrient depletion and low Spo0A~P levels activate sinI, leading to SinI production, SinR inhibition and biofilm formation. More drastic nutrient limitation leads to higher concentrations of Spo0A~P and repression of sinI. The outcome here is persistent SinR repression of biofilm genes and activation of sporulation genes.

In addition to metabolic control, the authors convincingly demonstrate strong effects of chromosome position on SinR activity and the expression of biofilm genes. This is interpreted as chromosome copy number control. This rather than metabolic control, would be needed under some circumstances to down-regulate biofilm gene expression during spore formation. The paper is clearly written, well argued and addresses an important and fundamental question in regulation. I recommend publication.

Suggested revision
Cooperativity is a reasonable and even a likely explanation for the gene dosage effect but I wonder if the extent of the discussion including Hill functions is stretching the interpretation beyond what is justified by the data presented in Figure 5C. These gels do demonstrate qualitatively that there is greater sensitivity of DNA binding to changes in the SinR concentration than to changes on the SinI concentration. However, there are multiple shifted bands in these gels. As the molecular mechanisms of cooperativity are not addressed here, the discussion should be softened.

Minor points
There is alternation between OA~P and 0A~P - 0A~P (0 (zero) rather than O (Oh!)) should be used throughout.
In the gel-mobility shift experiments of Figure 3A, the authors should comment on the modest mobility shift given by binding of 0A-CTD to the activation site and the much larger mobility-shift observed upon binding the first of the repressor sites.

It would be a good idea to show an additional panel in Figure 2B with the results of the deletion/scrambled mutant Psinl-lacZ grown on MSgg, or alternatively add this to the Supplemantics. The lack of effect at intermediate levels is an important result.

1st Revision - authors' response 22 January 2011

Referee #1 (Remarks to the Author):
This submission from the Losick/Kolter labs is interesting and insightful. It sheds light on the regulation of an important developmental process in a major model organism and suggests a novel regulatory mechanism. Although I do have some comments concerning the data and the interpretations of the data, this will be a provocative and important addition to the literature.

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Agreed. We have changed the title to "Evidence that...."

2. The lux data do not seem to have been corrected for growth. Much of the increase and in the sporulating cells the decrease in light output seen in the figures seems to be due to increase in cellular mass. These curves would look very different if plotted as specific activities. In Fig. 7A the decrease in light would be much less impressive if so corrected because of the decrease in OD.

- The lux data is corrected for growth, in other words, the results represent average activities (arbitrary units) per cell. We clarified this in the Materials and Methods.

3. A minor point concerns the use of "real time". The dictionary says that this means immediately or instantaneously. Taking samples at intervals for later measurement does not meet this definition.

Actually, since the plate reader records the light signal instantaneously we think real time is ok in this context.

4. The methods section does not tell us about the substrate used for the lux assay.

We used the luciferease reporter system from Vibrio fisheri that was adapted for use by us in B. subtilis. We neglected the appropriate reference for this in the supplemental but have now done so. This multi-gene luxA-E reporter generates bioluminescence from cellular ATP and a substrate produced from fatty acid via lux gene products. In other words, no extra substrates are needed (not like the one from firefly).

5. "neither the deletion mutation nor the scrambled...increased ...expression strongly (not shown)”. This is an important point and should be shown because it indicates that Spo0A-P does not reach an inhibitory level in MsGG.
Now included in the supplemental as Fig. S5.

6. A comment on Fig. 3A. With the wt probe, the lower-shifted high affinity band disappears with higher concentrations of protein. This suggests that there is cooperative binding to the multiple operator sites so that there is a concentration regime in which the apparent affinity for the operator sites is greater than to the activator site. If I am right, this has implications that favor the model and may be worth pointing out.

- in Fig. 3A (a), we think it is also possible that the lower-shifted high affinity band disappears when 0A-CTD amounts increase simply because 0A-CTD not only binds to the activation site but also to multiple operator sites.

7. In Fig. 3Ad, binding to the operator sites occurs with a much lower concentration of protein than in Aa or Ab. Why? Is the amount of DNA so close to being overwhelmed by protein that it is sensitive to a two-fold reduction?

Yes, a high Hill coefficient due to cooperative binding to the operator sites could be sensitive to a twofold change.

8. Fig. 3B. worries me a bit. The amount of probe in the "DNA only" lane seems higher than in the other lanes, particularly the adjacent one. Given this, the protection of O1 is questionable. Can this protection be documented, perhaps by densitometry? Also, there seems to be protection of a sequence between O2 and 10, but closer to the former, at least with the highest concentration of protein. Comment?

- We agree with the reviewer that the amount of probe in the "DNA only" lane seems somewhat higher than other lanes. However, it is also obvious that the O1 region is more protected with the highest amount of 0A-CTD when compared to the right-hand two lanes, which contain lower amounts of 0A-CTD, and in these three lanes, the DNA probe was added at similar amounts. Also, some of the bands, as indicated with asterisks, showed enhanced cleavage.

In the literature, sometimes the 0A binding sequence was extended from 7-bp (which we use in the manuscript) to 10-11 bp, including extra base pairs from both ends. In other words, the protected region can be (and has been shown, for example, in Shafikhani et al., 2002) bigger than 7-bp.

9. Neither of the insertions in the region upstream of sinIR interrupt the two open reading frames. Do these gene products play a role in the regulation?

- We donít think the gene products play a role in the regulation of biofilms. We have an insertional knockout mutation in the first gene of the upstream operon (though in this mutant, expression of the antibiotic resistance gene is in the same direction with that of sinI). The mutant does not show any defect in biofilm formation if not more robust (possible due to stronger read-through from the antibiotic marker).

10. How does the promoter in front of sinR affect the regulation?

- The promoter in front of sinR is a typical sigma-A dependent promoter and our previous data (Chai et al., 2008, Mol. Micro) suggests that it is constitutively expressed.

11. The references to Grossman concerning the higher copy number of origin- proximal genes might also include the seminal papers of Noburu Sueoka, who I think was the first to point this out. (About 1963 or 1964 I think in PNAS?, which dates me).
We added the reference, thanks for pointing this out.

Fig. 6 Aa and Ad and Ba and Bd show strikingly heterogeneous expression on the single cell level. Comments?

- We have previously shown that strong yqxM expression depended on Slr, whose gene is under the negative control of SinR. Slr and SinR form a self-reinforcing double negative loop (Chai et al., 2010). This loop is also a bistable switch, which we believe contributes to heterogeneous expression of yqxM.

Referee #2 (Remarks to the Author):
The authors describe a series of very interesting and important studies that investigate the control of Bacillus subtilis cell fate selection of sporulation or matrix production cell states. These alternate developmental conditions have been shown by the authors and earlier studies to be regulated by Spo0A~P levels, SinI, and catabolite repression/metabolic state. Spo0A~P levels and sinI expression are also known from previous studies to be affected by transition-state regulators. The authors suggest that low levels of Spo0A~P favor matrix production/biofilm fates by induction of sinI, and high levels of Spo0A~P commit cells to the sporulation pathway by repression of sinI. The authors also demonstrate that two copies of sinI and sinR within the same cell block matrix production by the cooperative binding of SinR to cognate DNA targets. A very important set of experiments demonstrate that sinI and sinR function depend on chromosomal position and gene dosage. Two completely replicated chromosomes are present in early stage sporulation cells until the final internal cell division which separates the mother cell and forespore. sinI expression is shown to be controlled by a previously unknown transcriptional read-through mechanism. The authors demonstrate that the canonical Spo0A~P binding site (0A-box) upstream of the sinI promoter does upregulate sinI expression. The authors propose on somewhat weaker evidence that other 0A-like box sequences (imperfect sequence matches) found in the upstream promoter regions of sinI could be involved in repression of sinI.

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Actually, we think the case is pretty strong because we both built the deletion and the scrambled sequence mutants with the same phenotype and because the effect of inactivating the operators was seen under conditions in which Spo0A~P levels were high (DS medium) but much less so under conditions in which levels were low (MSgg medium). Nonetheless, and acknowledging the reviewer is point of view, we shortened the text as much as possible in these sections, removing close to 300 words.

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We softened the discussion by inserting "propose" or "according to our model" in three places. However, we think the formalism we present for our model is valuable in the context of our discussion of dosage compensation and sex determination in metazoans, where cooperativity may also contribute to the high sensitivity to chromosome copy number.

Minor points

There is alternation between OA–P and 0A–P - 0A–P (0 (zero) rather than O (Oh!)) should be used throughout.

- We thank the reviewer for catching the errors, which we corrected.

In the gel-mobility shift experiments of Figure 3A, the authors should comment on the modest mobility shift given by binding of 0A-CTD to the activation site and the much larger mobility-shift observed upon binding the first of the repressor sites.

Yes, this fits nicely with the idea that there is a single binding site in the activator and multiple tandem operators, and we have added a sentence to comment on this.

It would be a good idea to show an additional panel in Figure 2B with the results of the deletion/scrambled mutant PsinI-lacZ grown on MSgg, or alternatively add this to the Supplementaries. The lack of effect at intermediate levels is an important result.

We have now added this data as supplemental figure S5.