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Dear Josh:

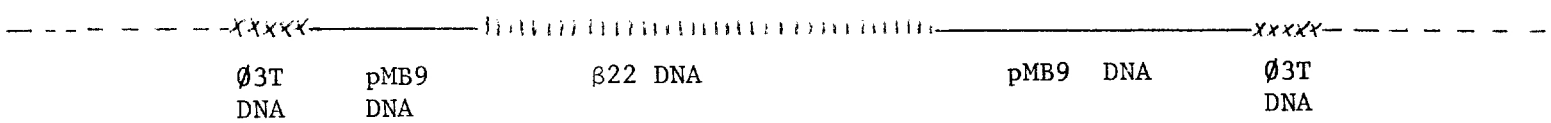
Thanks for the review of the manuscript that we submitted for publication to PNAS. We have added an additional experiment, revised some of the manuscript to conform with suggestions made by the reviewers and wish to explain to you the essence of our rebuttal to the alternative hypothesis raised by Reviewer #1. Each of the comments will be addressed separately. Reviewer #1 suggested interesting experiments that we have attempted ourselves but due to negative results we did not include them. To facilitate your review of the revisions, all changes are marked in yellow in the original manuscript.

Experiment 1. "See if a recE4 mutant of B. subtilis can be transformed to Thy^+ with pCD4". Because rec genes influence recombination we concentrated on this experiment early during the course of these studies. Regretfully, neither recA, recC nor recE are stable in a Thy^- background. Two methods were used to construct Thy^-Rec^- strains. First, we made the rec strains Thy^- by isolating cells that are resistant to trimethoprim. Second, we transferred the rec mutations by congression into thyA thyB strains. In either case the rec mutation is unstable resulting in reversion of the rec locus in 20-60% of the cells even after extensive subculture. This instability was not seen in the parent rec^- strains or in auxotrophs other than Thy^-. Therefore, the experiment must be done with a gene other than the one encoding thymidylate synthetase. The insertion of bacteriophage Ø3T is rec independent for all of the rec genes. This observation will be explored further in a subsequent study.

Experiment 2. "Demonstrate that the Thy^+ pCD4 transformed clones are stable Thy^+ after growth in a rich medium". This experiment has been done with both the Thy^+ transformants obtained with the E. coli gene encoding thymidylate synthetase and the Thy^+ transformants obtained by transformation with pCD4. In both cases the reversion frequency is about 10^{-3}. We have not included this result because a simple statement about reversion is not sufficient. The key experiment is the analysis of Thy^- revertants for the presence of pMB9 and the β22 thy gene. Reversion could be due to excision of the entire plasmid from the chromosome, excision of the Thy gene only, a point mutation, or inactivation of the enzyme by an intracellular

protein. We do not think the latter two possibilities are very plausible, yet they are possible. The only way to correctly test these alternatives is to determine what, if any, fragment(s) of the plasmid is present in the revertants by Southern hybridization. While this experiment is currently being performed as part of a continued analysis of the fate of foreign DNA incorporated into the chromosome, we do not believe it should be part of this manuscript. It will only add more information to an already complex manuscript.

Experiment 3. "Show that the total cell DNA from a Thy^+, pCD4 cell does indeed transform Thy^- subtilis to Thy^+ at a much higher frequency than the initial transformation with plasmids". We have done this experiment previously and have inserted it briefly into the manuscript. The reisolated DNA does transform Thy^- cells to Thy^+ but not at an increased frequency on a μg/ml level. There is some increase in relative efficiency, however, the increase is not significant when viewed in comparison with the usual increase obtained with intergenotic transformation. Since the integration of the plasmid would produce the following chromosomal structure, it is apparent that there are major known regions of non-homology.



The real test is to clone the Thy^+ gene from the chromosome of the intergenote transformed with pCD4 in another vector and then analyze the chimeric plasmid for the presence of chromosomal DNA. This experiment is also too detailed for inclusion in this manuscript in our opinion. Nevertheless, we have added the more trivial information requested by the reviewer. The comprehensive experiments are in progress.

Minor comments

A: We do not feel it is premature to suggest a "Campbell-like model" to explain integration. This is based not only on the lack of pCD4 transformants with linearized DNA, but also by the Southern blots that clearly show DNA added to the chromosome.

B: Change made.

C: These are complicated experiments. We do not know how to present them more clearly.

D: Plasmid pCD2 hybridizes the B. subtilis chromosome and pMB9 does not. We have included this statement in the text.

F: Addition made.

Reviewer 2

We have changed the usage of the word transformation throughout the text and have also changed the title with this suggestion in mind.

Further Comments

1. Change made.

2. We divided the first paragraph but somewhat differently than suggested by the reviewer. We changed the sentence on p.2 line 18 to clarify the query made by the reviewer.

3. Change made.

4. Change made.

5. The inclusion of a new figure addresses this point and makes our argument stronger.

I hope that these revisions will meet with your approval and will enable the manuscript to be published in PNAS. Thank you for your consideration.

Sincerely,

FEY/n
Enc.