A ribosome-nascent chain sensor of membrane protein biogenesis in Bacillus subtilis

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1st Editorial Decision 10 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see referees 1 and 3 are very positive about the paper and would support publication here after appropriate revision. Referee 2 also considers the manuscript as interesting in principle, but feels very strongly that the functional/physiological significance of your findings needs to be analysed in more depth. We will therefore be happy to consider a revised manuscript in which the referees' criticisms should be addressed in an adequate manner. In this respect it would indeed strengthen the paper considerably if the functional significance of your findings could be looked at in more depth.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,

Editor
The EMBO Journal

REFEEEREE REPORTS
Referee #1 (Remarks to the Author):

In this elegant and carefully executed study, Chiba and co-workers provide strong evidence that the expression of the B. subtilis yidC2 gene is regulated at the translational level by the coordinately transcribed mfiM gene. They show that MfiM is essentially an analog of E. coli SecM. Stalling of ribosomes near the 3’ end of mfiM exerts a strong effect on the expression of yidC2 in the same way that ribosome stalling near the 3’ end of secM influences the expression of the downstream secA gene. Remarkably, mfiM and secM monitor the status of two different export pathways and have no sequence homology. This study represents a significant advance in that it elucidates the mechanism by which the expression of yidC2 is regulated and expands the universe of phenomena that are regulated by ribosome stalling. Like many studies that break new ground, this one raises a lot of interesting questions! I have only a few relatively minor concerns.

1) Fig 1: The use of small squares beneath the bar graphs in parts B, E and F to indicate a deficiency in spoIIIJ seems rather unconventional and potentially confusing. The meaning of the data would be easier to grasp if the authors use symbols like "+" and "-".

2) p. 6, middle: The authors should explain why they hypothesize that the translation of mfiM and yidC2 might be coupled. Is there precedent for the coupling of the translation of two genes within an operon in B. subtilis or any other prokaryote?

3) Fig. 2B and C: In part B, what is the difference between lanes 1, 2 and 3 (and between 4, 5, and 6)? Do these represent different strains? An explanation should be provided in the Results or figure legend.

4) Fig. 2C: Data presented later in the manuscript indicates that translation stalls around amino acid 89. The authors should state whether the molecular weight of product B is compatible with stalling at this position.

5) p. 14, top: The authors should clarify the fact that a prevalence of cold-sensitive mutations has been observed specifically in the E. coli secretory pathway. Also, cold-sensitive mutations in E. coli YidC have also been reported (see Yuan, J. et al. (2007) J. Bacteriol 189: 8961).

6) p. 15, end of first paragraph: The statement that the MfiM-YidC2 system provides the "second example of a regulatory nascent chain that monitors events outside the ribosome" is misleading and should be modified. Most or all of the regulatory peptides described to date can be considered to monitor events outside the ribosome (e.g., amino acid concentration).

7) p. 16, last two lines: The statement that cofactors "alter the interaction between the nascent chain and the ribosome" is a bit too strong. This statement leads to the impression that the cofactor acts directly when, in most cases, it probably binds to the ribosome and indirectly alters the ribosome-nascent chain interaction.

8) One thing that strikes me as very odd is the fact that the MfiM-like proteins show essentially no sequence conservation (Table S2). While the degree of sequence conservation varies, there are at least a few invariant residues found in both SecM and TnaC. The lack of sequence conservation in MfiM, together with the observation that most of the key residues are fairly far away from the peptidyl transferase center, makes me wonder if there may be something unique about MfiM-based stalling. While the authors devote a lot of attention to the parallels between MfiM and other regulatory peptides, I think that they should comment at least briefly on the possible differences.

9) An untested assumption in this study is that that MfiM is inserted into the cytoplasmic membrane cotranslationally. To the best of my knowledge, it is unclear that YidC substrates are integrated in a cotranslational fashion, even in E. coli. While I do not think that the authors need to demonstrate cotranslational insertion here, they should at least state their assumption more explicitly. The observation that MfiM works only in cis (Fig. 1G-I) certainly suggests that MfiM is inserted cotranslationally, but I think that a bit more caution is warranted.

Referee #2 (Remarks to the Author):
Bacillus subtilis contains two YidC homologs, the constitutively expressed spoIIIJ (yidC1) and yqjG (yidC2) that expressed at a low level and induced in spoIIIJ mutants. For viability either spoIIIJ or yqjG suffice, although yqjG cannot restore the sporulation defect of the spoIIIJ deletion strain. The current manuscript demonstrates that yqjG induction requires the upstream gene yqzJ (mifM), and that decreased levels of SpoIIIJ (or deletion of the MifM transmembrane domain) causes the arrest of mifM translation resulting in the unfolding of a mRNA hairpin that normally blocks initiation of yqjG expression. The data suggest that MifM is a regulatory nascent chain that functions by a mechanism analogous to SecM that monitors protein export in E. coli.

Overall this is an interesting and mostly technical sound manuscript. However, there is a major issue not tackled and that concerns the functional significance of these findings. The activity of the proposed regulatory cascade has been demonstrated only in an artificial manner, i.e. mutational inactivation of SpoIIIJ or truncation or mutational inactivation of MifM. It remains unclear under what physiological conditions the regulatory system functions and to what it responds to.

The apparent similarity with SecM as outlined in Fig. 6 is suggestive but requires further experimental evidence. SecM controls the functional expression of secA upon a secretion defect that reduced the amount of functional SecA in the cell. YqzJ seems to control the production of YqjG that seems to function in addition to SpoIIIJ as YqjG is normally expressed only at a low level and it is unable to complement the sporulation defect of a spoIIIJ deletion strain. It further stress the need for evidence on the physiological function of the YqzJ. For instance, does overproduction of SpoIIIJ substrates result in a regulatory response. A critical test that can be conducted easily.

The phylogenetic distribution of mifM is puzzeling as it seems that not all Gram-positives containing two YidC homologs also have mifM-like genes. What does this say about the physiological role of MifM? Why would Bacilli need such backup system whereas it is absent in many other Gram-positive bacteria?

The authors generally assumes that SpoIIIJ and YqjG have a similar function in membrane protein insertion and folding as the E. coli YidC. However, sofar there is no experimental evidence for such a function. Rather previous deletion studies have implicated YqjG and SpoIIIJ in a late stage in protein export in B. subtilis and not in membrane protein insertion (see reference Tjalsma et al., 2003).

At Page 17, the authors state that the structure of MifM preducts that is likely to be inserted into the bilayer in a YidC-dependent manner but Sec-independent manner. There are no general rules defined yet that can be used to predict safely if a protein is inserted into the membrane via YidC or not, so the claim seems unsubstantiated. For the mechanism proposed, it is necessary to demonstrate that MifM is a SpoIIIJ substrate.

Referee #3 (Remarks to the Author):

The findings presented here give a novel aspect to the rationale of regulation a duplicated gene in B. subtilis. The authors justify their interpretation of the data with clear data. They show that yidC2 is only repressed when a stem loop structure is present and SpoIIIJ is expressed. They provide direct evidence that the translation of mifM is arrested when mifM is close to the stem loop structure.

However, a big gap in the understanding the sequence of events is that the signal is only provided when MifM is membrane-inserted. This is observed with the TM deletion mutant or when spoIIIJ is deleted. Most likely, the presence of the aminoterminal region of MimF in the cytoplasm signals to the translating ribosome to release to translational arrest. It should be made more clear (in the abstract and results) that this has still to be investigated to fully understand the sequence of events.

Fig. 6: Here it looks like MifM requires the translocon for membrane insertion whereas in the text (p. 9) it depends on SpoIIIJ. The drawing is confusing and should be changed that the schematic membrane pore at the top of the figure is lettered with SpoIIIJ or SecYEG, respectively. In addition, the authors should analyse whether MifM insertion really depends on Sec or/and on SpoIIIJ directly.
Minor changes:

Figure legend for Fig. 2B is incomplete and lanes 1-6 are not described.

p.9 / Fig. 2: I would appreciate a scheme of the domains of MifF respective to the residue number to see which residues are in the translocated domain, TM and cytoplasmic domain.

I miss an exact description of the spoI11J deletion.

p.19: It is Streptococcus mutans, not mutants!

1st Revision - Authors' Response 05 August 2009

Reviewer #1 was very enthusiastic about our paper and had a variety of helpful suggestions that we have incorporated into our revised manuscript.

1. The “-“ symbols were replaced by boxes during image conversion; we have corrected this error.

2. Translational coupling of adjacent genes in bacterial operons is well established in *E. coli* and *B. subtilis*, and plays a critical role in maintaining the appropriate stoichiometry of interacting proteins, such as those in the ribosome. Two mechanisms have been described. First, sometimes the termination codon of the upstream gene overlaps with the initiation codon of the downstream gene, which lacks a detectible Shine-Dalgarno sequence. In such cases, translation of the downstream gene depends on ribosomes loaded onto the mRNA by the SD sequence for the previous ORF. Second, sometimes the SD sequence of the downstream gene is occluded by a stem loop, as is the case here, and this stem loop must be unfolded by the ribosome translating the upstream gene. We now more simply state that induction might be due to changes in transcription or *mifM* translational initiation (page 6).

3. We have modified the results section (page 9) and the legend to Figure 2 as suggested.

4. This is a good suggestion; we have added this statement on page 11.

5. We have included a comment on page 14.

6. We have clarified this point, by contrasting arrest sequences that respond to cofactors that bind within the ribosome with SecM and MifM, which sense events outside the ribosome. Page 15 and Page 17.

7. We have modified the text to specify that the cofactors directly or indirectly modify the interaction between the regulatory nascent chain and the ribosome (bottom of page 15).

8. We now highlight this counterintuitive result in paragraphs on page 18 and in our concluding paragraph on page 21. We speculate that contrary to current views, translation represents an ongoing negotiation between the ribosome and the nascent chain, rather than an effectively blind translation of the nucleotide code into protein.

9. The best evidence for this comes from YidC homologues in mitochondria and S. mutans (page 21). We now more explicitly mention that our data suggests that at least one YidC substrate, MifM is inserted into the membrane cotranslationally (pages 19 and 21). We have expanded the paragraph addressing this on page 19 (bottom).

Reviewer #2 states that this is an interesting and technically sound manuscript and makes a variety of suggestions that highlight exciting areas for future research. Their main concern is that our manuscript does not fully address the functional significance of this regulatory system nor identify the physiological role of *yidC2*. As discussed below, we do not agree with the first concern and although the second represents an exciting area for future research it is outside the topic of the
The present manuscript, which concerns the molecular mechanism for yidC2 induction. Description of this molecular mechanism will allow studies to discriminate between shared and novel mechanisms by which the SecM and MifM nascent chains interact with the ribosome and with two different membrane protein complexes.

1. “… the functional significance of these findings…” Previous publications have established that spoIIIJ and yidC2 share an essential function and that it is possible to genetically inactivate one but not both genes. The mifM-yidC2 operon thereby provides a backup mechanism for this essential function, providing a clear advantage by allowing cells to survive an otherwise lethal event. The mechanism that we have described allows this backup function to be inducible, perhaps reducing the fitness cost associated with expressing YidC2 when it is not needed. We now more fully discuss this point (page 21).

2. “…apparent similarity with SecM…is suggestive…” The system we describe is precisely analogous to the SecM-SecA system, except that in this case the regulatory system allows induction of a second homologous gene, rather than inducing the sole homologue. The meaning of this statement is elusive and it contradicts statements in paragraph one that MifM acts in a manner analogous to SecM.

3. “evidence on the physiological function of yidC2”. It is tempting to speculate that YidC2 both provides a backup for SpoIIIJ and that is required during a particular environmental or developmental state, and we are actively investigating this possibility. However, it might be challenging or impossible to find this putative condition under laboratory conditions, which do not replicate the various environments that Bacilli are known to inhabit (soil, plant roots, mixed microbial communities) nor the diverse stresses it might experience in these environments. Furthermore, the topic of whether YidC2 performs a specialized role analogous to the role of SpoIIIJ in sporulation is unrelated to the topic of the present paper, which describes the molecular mechanism of yidC2 induction.

4. “does overproduction of SpoIIIJ substrates” induce yidC2. Overproducing a SpoIIIJ substrate is expected to titrate available SpoIIIJ and thereby induce yidC2. We have more directly tested this possibility by isolating mutations in the spoIIIJ SD sequence that reduce expression of SpoIIIJ and cause yidC2 induction. Overexpressing MifM or the putative SpoIIIJ substrate SpoIIIAE would not provide further insight into the role of YidC2 in nature, since this is an artificial condition, and it would make only a modest addition to the present study. These mutations are now discussed on page 6 and included in the supplemental section.

5. “the phylogenetic distribution of mifM is puzzling since not all bacteria with yidC2 contain mifM”. We agree that this is interesting and speculate that in these other bacterial species yidC2 might be regulated by another mechanism, although to date no studies have examined the expression of yidC1 and yidC2 in other species. We speculate that certain Gram positive bacteria inhabit a niche that exposes them to stresses that can be mitigated by a second copy of YidC, as is supported by previous findings that yidC2 is essential for stress resistance in S. mutans. We now include these previous findings into the discussion.

6. “The authors generally assume….” It is true that the precise role of SpoIIIJ and YidC2 in B. subtilis membrane biogenesis remains unclear. We now clarify this point (page 4), and discuss the findings by Tjalsma et al. in more detail and the accumulating indirect evidence that SpoIIIJ is involved in membrane protein insertion (page 21). Specifically, previous data from the Losick lab suggests that SpoIIIJ is required for activity of the SpoIIIAE membrane protein and the data in our paper demonstrates that deletion of the MifM transmembrane domain has the same effect on yidC2 induction as the spoIIIJ mutation, implying that SpoIIIJ function is similar to that of the transmembrane domain.

7. “At page 17…” The reviewer correctly points out that we here made an overstatement. We have modified this paragraph to say that the predicted topology of MifM is similar to that of E. coli proteins that are inserted into the membrane in a YidC-dependent but SecYEGA-independent manner (page 17, top).
Reviewer 3 was enthusiastic and mentioned that our model was clearly supported by the data. We have addressed their comments as described below.

1. Our data suggests that it is the engagement of the N-terminal domain of MifM with SpoIIIJ that releases the translational arrest, not the presence of this domain in the cytoplasm, since in the latter case release of the arrest would not depend on SpoIIIJ. However, it is clearly the case that the mechanism for the release of translational arrests remains much less studied than that for the arrest itself. We have clarified that the precise mechanism by which the arrest is released requires further investigation (page 20).

2. “Fig. 6…” We have modified figure 6 to make the SecYEG complex appear different from the SpoIIIJ complex and we have labeled the complexes as suggested. This is very helpful. The reviewer also suggests that we should directly determine if MifM insertion depends on SpoIIIJ and or SecYEGA. This important area of future research will require development of either a new in vitro assay for membrane protein insertion in *B. subtilis* or a new in vivo assay for this process, since previous publications have failed to show a clear phenotype when SpoIIIJ and YidC2 are depleted. This is a high priority for future research, but it is outside the scope of the present manuscript.

   Furthermore, our results provide indirect evidence supporting a role for these proteins in membrane protein insertion, since deletion of the MifM transmembrane domain has the same effect on *yidC2* induction as the *spoIIIJ* mutation so the function of SpoIIIJ is similar to that of the transmembrane domain. This indirect evidence is accumulating (see reply to reviewer #2 comment #6) and is now discussed on pages 16 and 21.

3. We have corrected the figure legend.

4. The *spoIIIJ-jag* deletion is central to our study, so we have also inserted a clarifying statement about this previously published deletion in the methods section (page 22) and we retain the reference for the strain in Table S3.

5. We corrected this mistake.

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Thank you for sending us your revised manuscript. In the meantime, I have had a chance to look into your revisions and your responses to the referees. Also, I have consulted with referee 1 once more (his/her reply is pasted below). You will be pleased to learn that we have come to the conclusion that the manuscript will now be publishable in The EMBO Journal; and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

**REFEREE REPORT**

Referee #1 (Remarks to the Author):

The authors have satisfactorily addressed all of the concerns that I raised in my original review.

This study clearly demonstrates that *yqiG* expression is controlled by the upstream *mifM* gene at the
level of translation. In my opinion, the discovery of a SecM-like translational regulatory phenomenon in B. subtilis represents a significant advance in itself. I do not think that the authors need to delve as deeply into the parallels between the B. subtilis mifM/yqjG system and the E. coli secM/secA system as referee #2 suggests. I also agree with the authors that an analysis of the physiological conditions under which yqjG is activated addresses a distinct question and is beyond the scope of their study. Given that a considerable amount of additional work would be required to prove that MifM is a SpoIIIJ substrate and that the authors are careful about their claims, I think that further experimental work on the MifM insertion mechanism does not need to be included in this study. Finally, I should note that the limited phylogenetic distribution of MifM is probably not so surprising in light of the fact that the two best studied stalling peptides in E. coli, SecM and TnaC, also show a limited phylogenetic distribution.