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Small RNA-induced differential degradation of a polycistronic mRNA iscRSUA

Guillaume Desnoyers, Audrey Morissette, Karine Prevost

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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 30 January 2009

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now evaluated your manuscript and their comments directly for authors are provided below. As you can see there is an interest in your study. However, referees #1 and 3 also raise technical concerns with the analysis and find that the present analysis does not provide strong enough support for the proposed model. The issues raised have to be fully resolved in order for further consideration here. Therefore, should you be able to address the concerns in full, we would consider a revised manuscript. I would like to add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important that you fully resolve the points raised if you wish the manuscript ultimately to be accepted.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this study, Desnoyers et al. report that the small RNA RyhB directs the differential degradation of

the iscRSUA mRNA leading to a stable iscR fragment and an unstable iscSUA fragment. The authors propose an interesting model that should be of general interest, however I think the experimental support for the model may not be up to the standards of EMBO J.

Some Northerns have high levels of background bands and the relative levels of the main bands vary significantly between blots of the same types of samples (for example, Figure 1B compared to Figure 3A). This made me wonder how effects of the different mutations and constructs were quantitated. For example, looking at the Northerns in Figure 2, although the levels of the iscRSUA transcript are different at time zero, it is not obvious that the decrease in the top band is significantly faster for the wild-type strain. Is there any estimation of the variation in this experiment? Ideally, a defined amount of iscR transcript would serve as an internal control for the Northern blots.

If generation of the iscR fragment is RyhB-dependent, why are the levels so high in the ryhB mutant strain (or hfq and rne mutant strains)? What is the 3' end of the iscR fragment generated in the ryhB mutant strain?

There is no direct evidence to rule out the presence of another promoter in the iscR-iscS intergenic region. Does the region downstream of the iscR transcription start have any promoter activity? Can the presumed RyhB-dependent cleavage be transferred to a heterologous gene? The authors should map the 5' end of the iscSUA fragment (this should be possible even if the band cannot be detected by the Northern analysis, particularly in RNase mutant strains).

It would be nice to have see controls such as the size markers for at least one of the Northern blots and a Northern showing the levels of RyhB in at least one of the experiments (to be able to correlate the amount of cleavage product with the amount of RyhB).

Referee #2 (Remarks to the Author):

This article presents a very well thought out case for ryhB sRNA regulation of the iscRSUA transcipt, which encodes for proteins involved in the biosynthesis of iron-sulfur clusters. Interestingly, the authors find evidence that ryhB recruits the RNA chaperone, Hfq, and RNA degradosome to the isc transcript, initiating degradation of iscSUA mRNA. Simultaneously, the 3' end of iscR is stabilized by REP secondary structure. While two previous examples of discoordinated sRNA regulation have been reported, this work represents a third novel mechanism of regulation through direct interaction between the sRNA and polycistronic mRNA. Additionally, it adds another layer of understanding to our knowledge of conditions that promote the expression of iron-sulfur cluster biosynthetic machinery. Discoordinated regulation of the isc transcript explains how expression of iron-sulfur machinery maybe downregulated even under conditions of iron starvation when IscR is in an apoform. These results also emphasize the importance of a stabilized iscR transcript, which presumably would allow for increased IscR regulation of other cellular targets during iron-limited conditions.

Comments on the Manuscript
Add something to the title to include the isc transcript. For example, "Small RNA induced differential degradation of iscRSUA, a polycistronic mRNA"
Switch order of sentences line 65 and 66 page 3.

General Comments on the Figures and Figure Legend
A description of "ryhB cells" or "iscR cells" is not clear to the reader. Since a strain table has been included, authors show list specifically the strains used in the figure legend. Figures should be more clear; ryhB for example should be written as ryhB and iscR, as iscR.

Figure 6B legend. Translational start site is boxed (not highlighted in gray)

Figure 8. Is this working model only applicable for Enterobacteria where the REP 111nt iscRS intergenic region is conserved? If so, please title "Working model of RyhB in Enterobacteria..."
Figure would be enhanced with inclusion of Hfq and Fur proteins and their role in RyhB regulation.

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Referee #3 (Remarks to the Author):

The E. coli small RNA RyhB is induced under Fe depletion and regulates, mostly negatively, a group of mRNAs encoding Fe-related proteins. The polycistronic mRNA iscRSUA encoding proteins required for biosynthesis Fe-S clusters is one of the targets of RyhB. Under Fe depletion the isc operon transcription is also induced because the transcriptional repressor of this operon, IscR, is no longer active in this condition. Previously the authors' group showed that RyhB promotes the degradation of the downstream iscSUA RNA without affecting the upstream iscR RNA. In this work, the authors investigated the mechanism of this RyhB-mediated differential degradation of the iscRSUA mRNA. The major findings are: 1) RyhB reduces the full-length iscRSUA RNA and increases the short iscR RNA by promoting the degradation of the iscSUA but not iscR RNA under iron starvation; 2) both RNase E (retaining the scaffold region) and Hfq are required for this regulation; 3) the intergenic region between iscR and iscS forms a stable REP-like structure that is responsible for the RyhB-mediated accumulation of iscR RNA; 4) RyhB base-pairs with the translation initiation region of iscS RNA; 5) RyhB represses the expression of IscS but not IscR proteins resulting in an increased IscR/IscS ratio. Based on these results, the authors propose a model of RyhB-mediated discoordinated regulation of the polycistronic iscRSUA mRNA and its physiological significance.

This is an interesting paper that reports a novel mechanism of sRNA-induced differential degradation of a polycistronic mRNA. While the major data and arguments are convincing, there are several ambiguous points that should be clarified before publication of this manuscript.

Specific comments:
1) A significant amount iscRSUA RNA is stably expressed in M63 medium without iron in cells lacking RyhB presumably Apo-IscR no longer represses the iscRSUA transcription (Fig. 1C). It should be mentioned why iscRSUA RNA is decreased even in ryhB cells sometime later after iron depletion in LB medium (Fig. 1B).
2) I do not see a significant difference at least by my eye between wild-type and ryhB cells regarding the stability of the iscRSUA mRNA (Fig. 2A).
3) The iscRSUA RNA level decreases 15-20 min later after the dip addition in cells expressing the wild-type RNase E while it is still well expressed in rne131 cells (Fig. 3A, B). Similarly, the reduction of iscRSUA RNA level is no longer observed 20 min later after the dip addition in hfq cells though the overall iscRSUA RNA level is significantly reduced (Fig. 3C). I am wondering why this happens (related question to comment 1).
4) It is reasonable to assume the G100 to U104 region of iscS mRNA base-pairs with RyhB because this region is also complementary to RyhB. I think it is difficult to obtain the experimental evidence for base-pairing by footprinting since this region is resistant to the cleavage by PbAc independently from RyhB/Hfq.
5) The authors argue that RyhB inhibits IscS expression (probably IscU and IscA together) but not IscR by modulating the stability of mRNAs. However, RyhB could down-regulate IscS by inhibiting directly its translation because it base-pairs with iscS mRNA at near the translation initiation region as in the case of other RyhB targets such as sodB mRNA. This point should be discussed properly. Nevertheless, I am surprised that the IscS protein expression is only weakly inhibited by RyhB (Fig. 7A, B). What are the possible reasons for this weak effect of RyhB. Could this weak effect of RyhB on IscS expression be physiologically relevant?

1st Revision - point-by-point response 06 March 2009

Referee #1 (Remarks to the Author):

In this study, Desnoyers et al. report that the small RNA RyhB directs the differential degradation of the iscRSUA mRNA leading to a stable iscR fragment and an unstable iscSUA fragment. The authors propose an interesting model that should be of general interest, however I think the experimental support for the model may not be up to the standards of EMBO J.

Reviewer's query: Some Northerns have high levels of background bands and the relative levels of the main bands vary significantly between blots of the same types of samples (for example, Figure 1B compared to Figure 3A). This made me wonder how effects of the different mutations and constructs were quantitated. For example, looking at the Northerns in Figure 2, although the levels of the iscRSUA transcript are different at time zero, it is not obvious that the decrease in the top band is significantly faster for the wild-type strain. Is there any estimation of the variation in this experiment? Ideally, a defined amount of iscR transcript would serve as an internal control for the Northern blots.

Authors' answer: We thank the reviewer for pointing out this issue. We have addressed this question by doing better Northern blots.
We also incorporated error bars calculated from three independent experiments (Fig 2B). As mentioned in the Materials and Methods, we quantitated the bands using a software specialized in this (ImageQuant).

Reviewer's query: If generation of the iscR fragment is RyhB-dependent, why are the levels so high in the ryhB mutant strain (or hfq and rne mutant strains)?

Authors' answer: This is an excellent observation. We added the following paragraph in the Discussion to make it clearer. "Furthermore, the REP-dependent discoordination of the rxcA mRNA explains the generation of a small amount of iscR fragment in the absence of RyhB (Fig 1B and C and Fig 3A and B). As the normal turnover of the iscRSUA transcript (independently from RyhB) may start downstream of the REP sequence, in the iscSUA section, the progression of the 3'-5' exonuclease of the RNA degradosome will stop at the REP structure, thereby generating an iscR fragment. Indeed, we have mapped an iscR fragment, extracted from ryhB cells, with the same 3' end as the one described in Fig 4B. Thus, this suggests that although the 3' end of iscR is not absolutely dependent on RyhB, it requires RyhB to accumulate to a significant amount" (line 345-353)

Reviewer's query: What is the 3' end of the iscR fragment generated in the ryhB mutant strain?

Authors' answer: This is related to and partially answered by the previous comment. We did a 3'-RACE experiment to address this question and found that the 3'-end of iscR in the ryhB mutant is the same as in the wild-type. This demonstrates that the REP sequence is sufficient to protect a fraction of the iscR transcript even in the absence of RyhB, which increases dramatically when RyhB is expressed. Thus, this suggests that although the 3'-end of iscR is not absolutely dependent on RyhB, it requires RyhB to accumulate to a significant amount.

Reviewer's query: There is no direct evidence to rule out the presence of another promoter in the iscR-iscS intergenic region. Does the region downstream of the iscR transcription start have any promoter activity?

Authors' answer: This is a very interesting point to address. Indeed in 2001, Schwartz et al (P.N.A.S. 98: 14895-14900) have convincingly shown that no promoter existed in that specific area of the isc gene. The author of that study used a lacZ reporter gene to verify if any promoter activity was detectable downstream of the iscR promoter (including the iscR-iscS intergenic region) and found no significant B-galactosidase activity. In addition, none of our results suggested that such a promoter existed.

Reviewer's query: Can the presumed RyhB-dependent cleavage be transferred to a heterologous gene?

Authors' answer: This is a point that we have partly addressed in the Discussion. However, because the focus of this paper is not on the cleavage site as much as the polycistronic discoordination, we will address this question into much more details in an upcoming article.

Reviewer's query: The authors should map the 5' end of the iscSUA fragment (this should be possible even if the band cannot be detected by the Northern analysis, particularly in RNase mutant strains).

Authors' answer: This is a very relevant comment. We used different methods to quantify different section of the iscRSUA transcript. The quantitative real-time PCR (reduced 10-fold), the Northerns, and the microarrays (reduced 4-6 fold; Masse et al, 2005, J of Bacteriol, 187: 6962-6971) demonstrated that the iscSUA section of the transcript is reduced dramatically. These results suggest that mapping the 5' of iscSUA is most likely impossible because there is no RNA fragment to map. These results are supported by the actual model of mRNA degradation, which suggests that when an mRNA carries a 5í monophosphorylated end (like iscSUA would), it is rapidly degraded in vivo (Mackie, Nature, 395: 720).
Finally, we hybridized the transcript with an iscS-specific probe (Fig S1), which should indicate a second band of lower molecular weight if there were an iscSUA fragment. As the Fig S1 shows, there is no lower band that suggests an iscSUA fragment even in the presence of RyhB.

Reviewer's query: It would be nice to have see controls such as the size markers for at least one of the Northern blots and a Northern showing the levels of RyhB in at least one of the experiments (to be able to correlate the amount of cleavage product with the amount of RyhB).

Authors' answer: Excellent observation. We have addressed this point with RNA size markers (Fig S1C) and Northern blots on RyhB levels (Fig 1B and C).

Referee #2 (Remarks to the Author):

This article presents a very well thought out case for ryhB sRNA regulation of the iscRSUA transcipt, which encodes for proteins involved in the biosynthesis of iron-sulfur clusters. Interestingly, the authors find evidence that ryhB recruits the RNA chaperone, Hfq, and RNA degradosome to the isc transcript, initiating degradation of iscSUA mRNA. Simultaneously, the 3' end of iscR is stabilized by REP secondary structure. While two previous examples of discoordinated sRNA regulation have been reported, this work represents a third novel mechanism of regulation through direct interaction between the sRNA and polycistronic mRNA. Additionally, it adds another layer of understanding to our knowledge of conditions that promote the expression of iron-sulfur cluster biosynthetic machinery. Discoordinated regulation of the isc transcript explains how expression of iron-sulfur machinery maybe downregulated even under conditions of iron starvation when IscR is in an apoform. These results
also emphasize the importance of a stabilized iscR transcript, which presumably would allow for increased IscR regulation of other cellular targets during iron-limited conditions.

Comments on the Manuscript
Reviewer's query: Add something to the title to include the isc transcript. For example, "Small RNA induced differential degradation of iscRSUA, a polycistronic mRNA"

Authors' answer: We have changed the title of the article to "Small RNA-induced differential degradation of the polycistronic mRNA iscRSUA".

Reviewer's query: Switch order of sentences line 65 and 66 page 3.

Authors' answer: We have switched the order of the sentences and it now reads better.

General Comments on the Figures and Figure Legend
Reviewer's query: A description of "ryhB cells" or "iscR cells" is not clear to the reader. Since a strain table has been included, authors show list specifically the strains used in the figure legend. Figures should be more clear; ryhB for example should be written as ΔryhB and iscR, as ΔiscR.

Authors' answer: Thank you for noticing. We have made changes to the figures to make them clearer and more informative.

Reviewer's query: Figure 6B legend. Translational start site is boxed (not highlighted in gray)

Authors' answer: We have made the specific change in Fig 6B legend.

Reviewer's query: Figure 8. Is this working model only applicable for Enterobacteria where the REP 111nt iscRS intergenic region is conserved? If so, please title "Working model of RyhB in Enterobacteria..."

Authors' answer: We have made the specific change to Fig 8 title: "Working model of RyhB-induced partial degradation of the iscRSUA polycistron in Enterobacteriacea and its physiological significance".

Reviewer's query: Figure [8] would be enhanced with inclusion of Hfq and Fur proteins and their role in RyhB regulation.

Authors' answer: This is a great suggestion. For the sake of clarity however, we omitted the Hfq and Fur proteins. We believe the figure to be sufficiently charged as it is and we also wish to emphasize on the discoordinated regulation of the iscRSUA polycistron.

Referee #3 (Remarks to the Author):

The E. coli small RNA RyhB is induced under Fe depletion and regulates, mostly negatively, a group of mRNAs encoding Fe-related proteins. The polycistronic mRNA iscRSUA encoding proteins required for biosynthesis Fe-S clusters is one of the targets of RyhB. Under Fe depletion the isc operon transcription is also induced because the transcriptional repressor of this operon, IscR, is no longer active in this condition. Previously the authors' group showed that RyhB promotes the degradation of the downstream iscSUA RNA without affecting the upstream iscR RNA. In this work, the authors investigated the mechanism of this RyhB-mediated differential degradation of the iscRSUA mRNA. The major findings are: 1) RyhB reduces the full-length iscRSUA RNA and increases the short iscR RNA by promoting the degradation of the iscSUA but not iscR RNA under iron starvation; 2) both RNase E (retaining the scaffold region) and Hfq are required for this regulation; 3) the intergenic region between iscR and iscS forms a stable REP-like structure that is responsible for the RyhB-mediated accumulation of iscR RNA; 4) RyhB base-pairs with the translation initiation region of iscS RNA; 5) RyhB represses the expression of IscS but not IscR proteins resulting in an increased IscR/IscS ratio. Based on these results, the authors propose a model of RyhB-mediated discoordinated regulation of the polycistronic iscRSUA mRNA and its physiological significance.

This is an interesting paper that reports a novel mechanism of sRNA-induced differential degradation of a polycistronic mRNA. While the major data and arguments are convincing, there are several ambiguous points that should be clarified before publication of this manuscript.

Specific comments:
Reviewer's query: 1) A significant amount iscRSUA RNA is stably expressed in M63 medium without iron in cells lacking RyhB presumably Apo-IscR no longer represses the iscRSUA transcription (Fig. 1C). It should be mentioned why iscRSUA RNA is decreased even in ryhB cells sometime later after iron depletion in LB medium (Fig. 1B).

Authors' answer: This is an interesting observation that demonstrates the difference in iron content of the iron-free M63 medium compared to LB medium. In Fig 1B, we add an iron chelator to LB medium to induce RyhB and the isc operon. While the chelator starves it from iron, the cell fully expresses iron acquisition systems to compensate (siderophores, which have a stronger affinity to iron than chelators, and their cellís surface receptors). After a certain period, the intracellular iron increases back to normal levels, a situation that reactivates the IscR-dependent repression of isc operon. We also think that, during iron starvation, the suf machinery can efficiently promote the formation of Fe-S clusters, thereby forming Holo-IscR and repressing the isc operon (this is addressed in the last paragraph of the Discussion)
In contrast to LB, the M63 medium does not contain sufficient iron to feed the cells even though the iron acquisition system is fully expressed.
Therefore to clarify this, we add the following sentence to the text: "Whether in wild-type or ryhB cells, the level of iscRSUA transcript declines after 10-20 min, presumably due to the recovery of

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intracellular iron homeostasis and activated Holo-IscR repression (see Discussion for details)" (lines 158-160) and "Contrary to the results obtained in LB medium, iscRSUA expression is very stable in ryhB cells grown in M63 without iron (compare Fig 1B and C). This suggests that IscR remains under the Apo- form and does not repress the isc operon" (lines 169-171).

Reviewer's query: 2) I do not see a significant difference at least by my eye between wild-type and ryhB cells regarding the stability of the iscRSUA mRNA (Fig. 2A).

Authors' answer: Indeed, the previous figure was not the best we could have done. To correct this, we have performed a new Northern blot, which is visually clearer than the previous one.

Reviewer's query: 3) The iscRSUA RNA level decreases 15-20 min later after the dip addition in cells expressing the wild-type RNase E while it is still well expressed in rne131 cells (Fig. 3A, B). Similarly, the reduction of iscRSUA RNA level is no longer observed 20 min later after the dip addition in hfq cells though the overall iscRSUA RNA level is significantly reduced (Fig. 3C). I am wondering why this happens (related question to comment 1).

Authors' answer: This is a very good observation. We added some text to correct this: "In addition, these results indicate that when RyhB is absent (ryhB) or non-functional (rne131 and hfq) the isc operon does not self-repress efficiently as in wild-type. This suggests that RyhB promotes the formation of Holo-IscR through iron-sparing. Eventually, this will result in transcriptional repression of the isc operon (see Discussion for details)" (line 209-213).
and
"We notice that when dip is used to induce the expression of both RyhB and isc operon, the expression of iscRSUA reaches a peak after 10 min, which is followed by a rapid decrease (Fig 1B and 3A). In contrast to this, in the absence of RyhB (ryhB) or its protein partners (hfq, rne131), the iscRSUA transcript accumulates significantly for a longer time period (Fig 1B and 3A, B, and C). Aside from the direct effect of RyhB on the iscRSUA transcript, a second mechanism can partly explain the decrease in iscRSUA transcript in wild-type cells. When expressed, RyhB generates free intracellular iron through iron-sparing (MassÈ et al 2005; Jacques et al 2006), which would help the formation of Holo-IscR. In wild-type cells treated with dip, RyhB expression reduces the expression of non-essential iron-using proteins, leaving the available iron to essential proteins (such as IscR, repressing the iscRSUA transcription after 15-20 min). In the rne131 and hfq mutants however, even though it is expressed, RyhB cannot function normally. We believe that in the absence of functional RyhB, the intracellular iron will be sequestered by non-essential proteins, which results in iron shortage for essential iron-dependent proteins, such as IscR. Contrary to wild-type cells, where IscR becomes active after 15-20 min of dip addition, the ryhB, rne131 and hfq mutants still express the isc operon because IscR lacks sufficient iron to act as a repressor (Apo-IscR)" (lines 438-454).

Reviewer's query: 4) It is reasonable to assume the G100 to U104 region of iscS mRNA base-pairs with RyhB because this region is also complementary to RyhB. I think it is difficult to obtain the experimental evidence for base-pairing by footprinting since this region is resistant to the cleavage by PbAc independently from RyhB/Hfq.

Authors' answer: We agree with the reviewer and added a sentence to the text to "Although it is likely that the region between G100 and U104 of iscS interacts with RyhB, there is no such evidence as this region seems resistant to Pb+ cleavage." (lines 265-267).

Reviewer's query: 5) The authors argue that RyhB inhibits IscS expression (probably IscU and IscA together) but not IscR by modulating the stability of mRNAs. However, RyhB could down-regulate IscS by inhibiting directly its translation because it base-pairs with iscS mRNA at near the translation initiation region as in the case of other RyhB targets such as sodB mRNA. This point should be discussed properly.

Authors' answer: This is an excellent observation. We added a section in the Discussion to address this point in detail (lines 354-361): "An additional point to consider is the negative effect of RyhB on iscS translation initiation, which could be sufficient to initiate cleavage or transcriptional stop through the transcriptional terminator Rho. Indeed, RyhB was shown to block translation in the absence of the RNA degradosome (Morita et al, 2006). However, our results in Fig 3B demonstrate that even though RyhB is expressed, there is no accumulation of the iscR fragment in the absence of

the RNA degradosome. Therefore, while RyhB may block the translation initiation of iscS, it is not sufficient to induce a transcript cleavage (or termination) that would generates an iscR fragment."

Reviewer's query: Nevertheless, I am surprised that the IscS protein expression is only weakly inhibited by RyhB (Fig. 7A, B). What are the possible reasons for this weak effect of RyhB. Could this weak effect of RyhB on IscS expression be physiologically relevant?

Authors' answer: While the effect is small on the IscS protein it is sufficient to alter the activity of the IscR transcriptional factor as shown in Fig 7C. In this figure, the repression activity of IscR on an iscR-lacZ fusion is reduced 2 fold. As our data suggest that IscR switches from the Holo- form to the Apo- form in the presence of RyhB, we believe that it is the state of IscR that is the most relevant observation. Thus, we agree with the reviewer that this is an important point as we will address this question in full detail in an upcoming article.

We also describe in the Discussion (section physiological significance) that under iron starvation, not only RyhB is expressed but the isc operon itself (because of reduced Fe-S clusters). Contrary to the iscRSUA transcript, most targets of RyhB are not induced by iron starvation. The increase in target mRNA may explains the small effect of RyhB on IscS decreases. We added a sentence to the Discussion to explain this observation: "To our knowledge, this is the first description of a system where the expression of both the sRNA and its target mRNA increases in the same conditions (iron starvation), although with distinct mechanisms" (lines 435-437).

2nd Editorial Decision 27 March 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee # 1 and 3 to review the revised manuscript and I have now received their comments. As you can see below, both referees find that the revised version has satisfactorily addressed their specific concerns. Referee # 3 is still hesitant if the observed RyhB mediated downregulation of IscS protein levels is physiological relevant, but this referee is nevertheless supportive of publication of your study in the EMBO Journal. Given these opinions, I am pleased to proceed with the acceptance of your study for publication in the EMBO Journal. Referee #1 has a comment regarding the wording of the title. I will leave it up to you if you want to change it and if so then you can change it at the proof stage. You will receive the formal acceptance letter shortly.

Thank you for submitting your manuscript to The EMBO Journal

Sincerely

Editor
The EMBO Journal.

REFEREE REPORTS

Referee #1

The manuscript by Desnoyers et al. has been improved by the substitution of some panels with better Northern figures and discussion of why the iscR fragment is still detected in the ryhB mutant strain.

Given that the differential degradation is still observed in the ryhB mutant strain, I think "promoted" or "stimulated" should be substituted for "induced" in the title.

Referee #3

I think that the manuscript has been significantly improved by responding properly to the reviewers' comments. I am still not perfectly convinced with the physiological relevance of a weak down-regulation of IscS by RyhB