THE EMBO JOURNAL

Manuscript EMBO-2013-84565

Antagonistic functions between the RNA chaperone Hfq and a sRNA regulate sensitivity to the antibiotic colicin

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Review timeline:

Submission date:	22 January 2013
Editorial Decision:	05 March 2013
Revision received:	16 June 2013
Editorial Decision:	15 July 2013
Revision received:	29 July 2013
Accepted:	14 August 2013

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.)

Editor: Anne Nielsen

1st Editorial Decision 05 March 2013

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript but also raise a number of critical concerns that you will have to address in full before they can support publication of a revised manuscript.

A clear issue raised by all three refs is the need to substantially restructure and shorten the manuscript to focus it on the core message of antagonistic regulation of CirA by Hfq and RyhB. In addition, ref #1 and #3 point to a number of inconsistencies between the presented data and the suggested model and ask you to further clarify the mechanism underlying activation of translation as well as the requirement for Hfq in this process. Another issue relates to the 'functional translatability' of the in vitro assays as exemplified by the concerns raised about the toe-printing assay.
Regarding the broader functional implications of your work, we would ask you to either provide additional experimental evidence for the role for RyhB in regulation of colicin sensitivity as requested by ref #1 or to demonstrate the existence of a Fur, RyhB, cirA feed-forward loop as suggested by ref #2. In addition, all three refs raise a number of additional concerns that need to be addressed in full.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

REFEREE REPORTS:

Referee #1:

Savail and co-workers report that the cirA mRNA of E. coli is post-transcriptionally activated by a mechanism that involves the activities of the iron-responsive RyhB sRNA and the RNA-binding protein Hfq. CirA has been known as an important siderophore transporter that also happens to be the receptor for colicin, and the present study also suggests a mechanism for iron-dependent susceptibility of strains that express CirA.

This is a well-conceived study that builds on the previous successful work in the MassÈ lab on RybB. The data represent a tremendous amount of work, the experiments are carefully performed, and the manuscript is well-written for the most part (except for an overuse of the phrase "Results showed that ..." which is occurs 17 times, three times alone on page 10).

Nonetheless, the mechanism underlying the activation of translation is not entirely clear. The authors propose that binding of RyhB to cirA mRNA dislodges Hfq from sites close to the start codon/Shine Dalgarno region, and so relieves translational inhibition. However, several details of the experiments do not fully fit the model, and should be discussed more carefully.

Major criticism:

1. Pages 2-6: The introduction is comprehensive but not terribly focused; for example, throughout page 3 the reader is exposed to mechanistic details of colicin action that are of little importance for the present story. It should be shortened and focused on the main scientific questions that are addressed in the subsequent Results section.

2. Figs. 1C and S1: The authors address expression of cirA in different mutants and at different phases over growth. Deletion of the Fur repressor results in higher RyhB levels (Fig. 1C, lanes 7-9), and an increase in cirA promoter activity (Fig. S1). However, although RyhB is strongly induced under all tested growth phases in a fur mutant, and cirA promoter activity is highest at early exponential growth, no cirA mRNA is detected under this condition (Fig. 1C, lane 7-9). This observation cannot be explained by the model proposed by the authors, and claims that RyhB expression was essential, but not sufficient to induce cirA. How are other targets of RyhB affected in this situation?

3. What is the reason for higher promoter activity of cirA in a Δfur ΔryhB compared to a Δfur mutant (Fig. S1B)?

4. Fig. 4A: it is unclear why in the absence of degradosome assembly, an increase in RyhB but not in cirA mRNA levels is observed.

5. Fig. 4B: Do we know whether the observed upregulation of cirA mRNA in lane 8 (no RyhB, RNase E inactivated) is due to transcript stabilization or increased transcription from the cirA promoter?

6. Fig. 5: The choice of the "transcriptional" and the "translational" cirA fusions as reporters is misleading. The same insert comprising the upstream region of the gene and the first 32 nt of the coding sequence (cf. M&M part, ll. 1066-1069) is ligated into two different reporter plasmids. The pFRΔ-based construct is not optimal to monitor transcriptional activity. For a true transcriptional reporter, the fusion should not depend or contain the translation initiation site of the gene tested. In any case, if the fusion reported solely transcriptional changes, the results would be inconsistent with the experiments described in Fig. S1. The same fusions are again used in Fig. 6.

7. Fig. 7: These in vitro experiments are meant to support the model that RyhB dislodges Hfq from the cirA mRNA, and so increases ribosome binding. While the results obtained are technically clean and can be interpreted, in principle, as fully supporting the authors' model, they do not make much sense with respect to a regulation in vivo without a further discussion of numbers. For example, in panel 7A, it takes 200 nM Hfq to suppress 30S subunit binding to the cirA mRNA but what is the concentration of the latter? The M&M section gives say 2 pmol mRNA but gives no reaction volume so it is difficult to understand the stochiometry of Hfq and mRNA.

8. Fig. S4: In the absence of Hfq, the authors observe a reduction in toeprint formation dependent on RyhB binding. While the authors claim that RyhB cannot further enhance 30S association in the absence of Hfq (ll. 424 ff.), it is unclear why RyhB (but not the mutant RyhB1) can influence toeprint formation from the distance. How does this result fit with the observation that RyhB can activate cirA in the absence of Hfq in vivo?

9. Fig. 8: With regulations so small (in the 1.3-fold range), it would be important to show that Hfq and RyhB do not regulate a non-cognate mRNA in this toeprinting assay.

10. Fig. 9: The role of RyhB in the regulation of colicin sensitivity is not addressed in much detail. The authors observe a growth defect in cells (presumably) expressing the sRNA, however these cultures recover at later stages of growth. The authors can exclude a suppressor mutation, thus it would be helpful to determine the levels of RyhB/cirA at some critical points during this experiment.

Minor points:

- Paginate the manuscript.

- Fig. 1C: decay should be plotted logarithmically over linear time.

- Fig. 2A: drawing of the figure should be improved. Use different fonts and colour.

- Fig. 2A: can the structure drawn be supported by a conservation/covariation analysis of the suggested single-stranded and paired regions?

- Fig. 4B: is there an explanation for the strong effect of temperature on the regulation of cirA by RyhB? In ll. 1/2, sRNA levels are constant, but stabilization is only observed at lower temperature. Is there a difference in promoter activity? If yes, the authors should consider a reporter under the control of a temperature-insensitive promoter.

- Fig. 7: Hfq concentrations should be consistently given as nanomolar or micromolar.

- ll. 54/55: something is missing here. The authors start to describe the composition of a colicin gene cluster, but the term "immunity protein" is missing.

- l. 60: "colicins" should read "colicin"

- l. 109: the listed references look like a reading list, and it is unclear which of them supports which point in the relevant sentence.

- l. 113: "condtions" should read "conditions"

- ll. 138-9: Which 'active role' was confirmed? Rephrase the sentence for clarity.

- l. 144: correct to 'quantitative Real Time-PCR (qRT-PCR)'

- l. 201: "proceed" should read "proceeded"

- l. 240: "Fusions activity" should read "Fusion activity"

- l. 250: I do not think that these RNase E mutants "were engineered" in this study.

- l. 261: "thermosenstive" should read "thermosensitive".

- ll. 262-6: The logic, results and conclusions of this experiment must be better explained.

- l. 386: correct "it could expected"

- l. 390: correct "it could then be expect"

- ll. 382-393: this paragraph is really hard to read. On the one hand, the authors claim that region -40 to -12 is not cleaved by RNase I due to the formation of a stem-loop, but on the other hand susceptible to PbAc treatment due to instability of the base-pairing. The authors should try to make a clear point here.

- ll. 453-456: The observed "increased cleavage of nucleotides -41 to -38 and -34 to -31" (Fig. 8B) occurs also independent of Hfq if only RyhB was present.

- l. 573: correct "elemen t"

- l. 573: correct "Shine-Darlgarno"

Referee #2:

In this study Salvail et al. examined the ability of the RyhB small RNA to activate translation of the cirA mRNA. Unlike other examples of positive regulation where the small RNA acts to prevent the formation of an inhibitory secondary structure, RyhB pairing promotes changes in the cirA secondary structure that relieve the inhibitory binding of the RNA chaperone Hfq. The manuscript represents a significant amount of work and the results are interesting. However, the authors need to address the following.

1. The text of the manuscript should be shortened by at least one-third throughout. The current manuscript is unnecessarily difficult to read due to the lengthy descriptions. For example, while there is a detailed description of colicins in the introduction, this section is not very clear.

2. The authors point out that Fur, RyhB and cirA are in an interesting feedforward loop and speculate about the possible physiological consequences of the loop. The study would be a more substantial contribution if the authors actually tested their predictions. (The rationale for delaying cirA expression when cells experience iron starvation to express cirA only when absolutely necessary makes sense, but what are the benefits of delaying repression upon a shift from iron-poor to iron-rich conditions.) This data could replace the section on colicin Ia sensitivity since this last section is a bit of an outlier from the rest of the data presented in the paper.

3. Better or additional data should be shown for some figures:
--Figure 1B: Size markers should be shown for the first cirA Northern presented.
--Figure 2: This figure is not optimally clear. I suggest presenting 2A as supplemental data with two RNAs indicated in different colors. Figure 2B should be part of Figure 3. The authors should also comment on the region of RyhB involved in base pairing with cirA compared to the RyhB regions involved in base pairing with other targets.
--Figure 3B: The cirA Northern is not very convincing. The OmrA and OmrB panels in Figure 6A also are not optimal.
--Figure 7A: The authors should examine the effects of a control protein in the toe print assay (to demonstrate that the loss of ribosome binding is a specific effect).

4. Given that evidence for Hfq binding to sites II and III is subtle, the authors should assay lacZ fusions in which the Hfq binding sites have been mutated. The prediction from the model presented in the paper is that expression from these mutants should not be changed in an hfq deletion background.

More minor comments:

5. The authors need to be more explicit about the following:
--They should specify that "shiA" not regulated by Fur in the introduction.
--They need to acknowledge that some of the unexplained regulation of cirA could be due to the action of another small RNA given that that many mRNAs are regulated by multiple small RNAs.
--The type of lacZ fusions being assayed should be spelled out (the "tic" is easy to miss).

6. There are a number of typographical errors (not a complete list):
--Lines 54-55: "...encode a colicin, which is a protein that protects..."
--Line 106: "stimulate"
--Line 113: "condtions"
--Lines 516-517: "series of experiment"
--Line 575: "Shine-Dalargno"

7. Strains and plasmids table should be given as Supplementary Data.

Referee #3:

This paper reports the study on post-transcriptional regulation of cirA by an Hfq-binding small RNA RyhB in E. coli. The authors first showed that RyhB is required for the expression of cirA mRNA encoding CirA, a siderophore receptor involved in ferric ion uptake and the cirA mRNA is stabilized by RyhB. They showed evidence that RyhB promotes CirA expression through pairing with cirA mRNA and protects the cirA mRNA from RNase E degradation. The data suggest that RyhB pairing with cirA mRNA leads to increased translation resulting in the protection and accumulation of full-length cirA mRNA. In addition, the authors showed evidence that Hfq represses cirA mRNA translation in the absence of RyhB by preventing ribosome binding and RyhB pairing with cirA mRNA stimulates ribosome binding. Finally, the authors showed that RyhB increases the sensitivity to colicin Ia through cirA activation.

This work demonstrates convincingly that the Fur-regulated cirA gene is positively regulated by RyhB at post-transcriptional level, revealing the presence of an intriguing Fur-RyhB-cirA regulatory circuit. Furthermore, the proposed mechanism by which RyhB activates the cirA mRNA is quite interesting. The findings expanded our view regarding how bacterial Hfq-binding sRNAs act in bacterial cells. I have criticisms/comments on the data and the organization of the manuscript.

Comments:
1) First of all, the manuscript is too long and the argument is too diverse. Some data seem to be less informative. I think that the manuscript should be reorganized by focusing on the cirA regulation by RyhB and by trimming drastically throughout. The followings can be deleted or described more concisely: a) the argument concerning colicin/CirA and the detailed data regarding the colicin

sensitivity test because it is already known that CirA is a target of colicin Ia; b) some data and description on lacZ fusion experiments are less informative and could be deleted; c) the results of Figs. 7 and 8 could be described more concisely.
2) The data show overall a good correlation between RyhB expression and cirA expression in the absence of Fur. However, the cirA is little expressed while RyhB well expressed at OD600=0.3 in cells lacking fur gene (Fig. 1C, lane 7). This is quite confusing.
3) The cirA mRNA stabilization by RyhB (Fig. 1D) is too moderate to explain the dramatic activation of cirA mRNA by RyhB shown in Fig 1B and 1C.
4) Fig. 6A shows that the cirA mRNA is highly expressed in the absence of Hfq even without RyhB, suggesting that RyhB promotes the cirA expression simply through relieving Hfq repression. On the other hand, the Fig. 6C shows the cirA mRNA expression require RyhB even in the absence of Hfq. Thus, two experiments appear to be inconsistent each other.
5) RyhB-cirA pairing can activate the cirA expression by antagonizing Hfq repression and/or by increasing the ribosome accessibility to the cirA mRNA. It is frustrating that the data are confusing and not matured yet to make this important point clear. One simple important but experiment is to examine the effect of hfq deletion on CirA protein expression.
6) The authors propose antagonistic function between Hfq and RyhB. I think this proposal is not appropriate because Hfq is essential for RyhB to base pair with cirA to activate the cirA translation.

1st Revision - authors' response 16 June 2013

Referee #1:

Savail and co-workers report that the cirA mRNA of E. coli is post-transcriptionally activated by a mechanism that involves the activities of the iron-responsive RyhB sRNA and the RNA-binding protein Hfq. CirA has been known as an important siderophore transporter that also happens to be the receptor for colicin, and the present study also suggests a mechanism for iron-dependent susceptibility of strains that express CirA.

This is a well-conceived study that builds on the previous successful work in the Massé lab on RybB. The data represent a tremendous amount of work, the experiments are carefully performed, and the manuscript is well-written for the most part (except for an overuse of the phrase "Results showed that ..." which is occurs 17 times, three times alone on page 10).

Nonetheless, the mechanism underlying the activation of translation is not entirely clear. The authors propose that binding of RyhB to cirA mRNA dislodges Hfq from sites close to the start codon/Shine Dalgarno region, and so relieves translational inhibition. However, several details of the experiments do not fully fit the model, and should be discussed more carefully.

Major criticism:

1. Pages 2-6: The introduction is comprehensive but not terribly focused; for example, throughout page 3 the reader is exposed to mechanistic details of colicin action that are of little importance for the present story. It should be shortened and focused on the main scientific questions that are addressed in the subsequent Results section.

Authors' answer. This is a very good comment. We have shortened the introduction, especially the section on colicin to make it more focused.

2 Figs. 1C and S1: The authors address expression of cirA in different mutants and at different phases over growth. Deletion of the Fur repressor results in higher RyhB levels (Fig. 1C, lanes 7-9), and an increase in cirA promoter activity (Fig. S1). However, although RyhB is strongly induced under all tested growth phases in a fur mutant, and cirA promoter activity is highest at early exponential growth, no cirA mRNA is detected under this condition (Fig. 1C, lane 7-9). This observation cannot be explained by the model proposed by the authors, and claims that RyhB expression was essential, but not sufficient to induce cirA. How are other targets of RyhB affected in this situation?

Authors' answer. The in vivo concentration of Hfq is highest during early log phase (Ali-Azam 1999). This might help the binding of Hfq on cirA mRNA and explain the strong repression of cirA mRNA level because no translation is present. In any case, we have changed the figures related to these data (Fig 1C and Fig S1B) to remove the problematic point (OD_{600} 0.3) It is worth to mention that most of the Northern and b-galactosidase assays in the paper have been performed at this OD_{600} of 0.6.

3. What is the reason for higher promoter activity of cirA in a Δfur ΔryhB compared to a Δfur mutant (Fig. S1B)?

Authors' answer. A previous study has shown an iron-independent activation pathway that depends on CRP-cAMP complex (Griggs, 1990, J Bacteriol). Interestingly, a study by Zhang *et al.* published in J Bacteriol. in 2005 ("Functional interactions between the carbon and iron utilization regulators, CRP and Fur, in Escherichia coli") showed that in a D*fur* background, CRP inactivation (D*fur* D*crp*) results in a 3.5 fold decrease of *cirA* mRNA levels as compared with D*fur* background, which is consistent with data provided by Griggs (1990), suggesting a CRP-cAMP- mediated activation of *cirA* promoter.

Considering that some of the negative targets of RyhB are Krebs cycle enzymes (e.g. *fumA*, *sdhC* and *acnB*), we could hypothesize RyhB inactivation in D*fur* background to result in decreased levels of available glucose as compared to D*fur* cells (RyhB inactivation –> Increased levels of Krebs cycle enzymes -> decreased glucose at the top of the glycolysis pathway), and then, in increased levels of cAMP. CRP would then be more active in D*fur* D*ryhB* background, thereby explaining the higher promoter activity of *cirA*.

4. Fig. 4A: it is unclear why in the absence of degradosome assembly, an increase in RyhB but not in cirA mRNA levels is observed.

Authors' answer. [Fig 4A is now Fig S2A] According to the data published by Masse *et al.* in Genes and Dev in 2003, RyhB would be codegraded with its mRNA targets upon post-transcriptional repression. Considering that many RyhB negative targets are degraded less efficiently in a *rne131* background (e.g. *sodB*, *fumA* and *iscSUA*, see Massé *et al.*, 2003; Desnoyers *et al.*, 2009 and Prévost *et al.*, 2011), there might be less RyhB codegradation as well, thereby explaining increased RyhB accumulation in *rne131* background.

The level of *cirA* mRNA observed in WT background at the OD tested (Figure S2A, lane 1) might correspond to maximum expression for *cirA* and the level of RyhB expressed in the same conditions seems sufficient to promote this level of expression, thereby explaining why there is no increased *cirA* accumulation in *rne131* background, despite increased RyhB expression. In other words, past a certain level of RyhB expression (WT levels, lane 1), *cirA* mRNA levels cannot be further increased by expressing more RyhB (Figure S2A, lane 3).

5. Fig. 4B: Do we know whether the observed upregulation of cirA mRNA in lane 8 (no RyhB, RNase E inactivated) is due to transcript stabilization or increased transcription from the cirA promoter?

Authors' answer. [Fig 4B is now Fig S2B] Since the incubation at 44°C with (compare lanes 5 and 6) or without (compare lanes 1 and 2) RNase E inactivation both result in a decrease of *cirA* accumulation in WT backgrounds (*ryhB*+ cells), we doubt that RNase E inactivation (and/or temperature switch to 44°C) promotes an increase of *cirA* promoter activity even if it has not been addressed experimentally.

We show in our manuscript that the stabilization of *cirA* by RyhB is promoted by translational activation. Moreover, RNase E has been shown by Guillier *et al.*, 2008 (Mol Microbiol) to be involved in the rapid turnover of *cirA* upon translational repression by OmrA/B. Thus, we are quite confident that it is RNase E that promotes the rapid degradation of *cirA* when RyhB is not expressed, thereby explaining the increased accumulation of *cirA* upon RNase E inactivation in a D*ryhB* background.

6. Fig. 5: The choice of the "transcriptional" and the "translational" cirA fusions as reporters is misleading. The same insert comprising the upstream region of the gene and the first 32 nt of the coding sequence (cf. M&M part, ll. 1066-1069) is ligated into two different reporter plasmids. The pFRΔ-based construct is not optimal to monitor transcriptional activity. For a true transcriptional reporter, the fusion should not depend or contain the translation initiation site of the gene tested. In any case, if the fusion reported solely transcriptional changes, the results would be inconsistent with the experiments described in Fig. S1. The same fusions are again used in Fig. 6.

Authors' answer. [Fig 5 is now Fig 4] The pFRD and pRS1551 have been proved to be reliable genetic tools in previous studies from our lab to distinguish between transcriptional and translational effects of RyhB-mediated or riboswitch-based regulations (Prévost *et al.*, Mol Microbiol., 2007, Desnoyers *et al.*, Genes and Dev, 2012; Caron *et al.*, PNAS, 2012). Removing *cirA* translation initiation site from our transcriptional fusion, as proposed in point 6, would make the fusion insensitive to RyhB *trans*-regulation, given the fact that RyhB interaction site is located in this region. Using such a fusion would be useful to determine if the RyhB effect is direct or indirect (e.g. effect on the promoter), which is addressed in a convincing way in Figure 4C and D.

The point of using both transcriptional and translational fusions was to determine if the stabilization of *cirA* mRNA by RyhB was occurring through translational activation or through a translation-independent mechanism. Since the transcriptional fusion harbours two Shine-Dalgarno sequences (one for *cirA* and one for *lacZ*) and the translational fusion harbours only one Shine-Dalgarno sequence for *cirA* and *lacZ*, the translation of *lacZ* totally depends on *cirA* translation. Therefore, if *cirA* activation occurs through translation activation, we should expect a higher RyhB effect on *cirA* translational fusion than on *cirA* transcriptional fusion, which is the case, as shown in Figure 4A and B. The same rationale was applied to demonstrate that RyhB activates *shiA* through translational activation (Prévost *et al.*, Mol Microbiol., 2007). There was a stronger RyhB effect on the *shiA* translational fusion (constructed with pRS1551) as compared to the transcriptional fusion (constructed with pFRD).

7. Fig. 7: These in vitro experiments are meant to support the model that RyhB dislodges Hfq from the cirA mRNA, and so increases ribosome binding. While the results obtained are technically clean and can be interpreted, in principle, as fully supporting the authors' model, they do not make much sense with respect to a regulation in vivo without a further discussion of numbers. For example, in panel 7A, it takes 200 nM Hfq to suppress 30S subunit binding to the cirA mRNA but what is the concentration of the latter? The M&M section gives say 2 pmol mRNA but gives no reaction volume so it is difficult to understand the stochiometry of Hfq and mRNA.

Authors' answer. [Fig 7 is now Fig 6] The concentration of *cirA* used in the assay is 0.2 mM. Therefore, it takes equimolar amounts of Hfq and *cirA* (Hfq:*cirA* molar ratio, 1:1) to observe 2.2-fold reduction of 30S binding. To make it clearer, we have added the *cirA* mRNA final concentration in the figure's legend.

8. Fig. S4: In the absence of Hfq, the authors observe a reduction in toeprint formation dependent on RyhB binding. While the authors claim that RyhB cannot further enhance 30S association in the absence of Hfq (ll. 424 ff.), it is unclear why RyhB (but not the mutant RyhB1) can influence toeprint formation from the distance. How does this result fit with the observation that RyhB can activate cirA in the absence of Hfq in vivo?

Authors' answer. [Fig S4 is now Fig S6] The fact that RyhB, but not RyhB1, represses 30S binding indicates that the effect depends on RyhB specific pairing to *cirA* instead of being a sequestration of the ribosome away from *cirA* mRNA through non-specific interactions of the 30S with the added sRNA (RyhB or RyhB1). Since RyhB represses 30S binding to *cirA* only when added in excess amounts (0.4 mM; 2-fold and 0.8 mM; 4-fold), it might be an *in vitro* artefact which might not reflect what is really happening *in vivo*. Indeed, we observe in Figure 6C that RyhB increases *cirA* mRNA levels in the absence of Hfq, which does not fit with our *in vitro* results (if RyhB would really prevent 30S binding *in vivo*, when expressed in the absence of Hfq, we would expect *cirA* destabilization instead of increased accumulation as observed in Figure 5C).

We could expect this increased accumulation of *cirA* mRNA upon RyhB expression in the absence of Hfq to occur via a stabilization independent of translation activation. Nevertheless, this RyhB

positive effect on *cirA* in the absence of Hfq remains elusive for now. However, it is worth to mention that sustained expression of RyhB through induction of pBAD-*ryhB* with 0.1% arabinose, as it was done for Figure 5C, does not represent homeostatic amounts of RyhB accumulating in an iron-dependent manner as it is the case for experiments performed in iron-free M63 minimum medium (e.g. Figures 1C; S2; 4B and D and 5A and B). Indeed, the levels of RyhB accumulating through induction with 0.1% arabinose are far higher than the levels observed in iron-free medium. We can therefore expect nonspecific effects, especially in a background that is far for WT conditions (i.e. D*fur* D*ryhB* D*hfq*). The fact that removing *hfq* in a D*ryhB* background results in WT levels of *cirA* translational fusion (Figure 5B) reinforces our main hypothesis that RyhB activates *cirA* expression mainly, if not solely, by counteracting Hfq negative regulation in homeostatic conditions. This Hfq counteraction was reproduced in toeprinting in Figure 7A using Hfq and RyhB/RyhB1 concentrations that are below the equimolar amount with *cirA* mRNA (150 nM Hfq; 150 nM RyhB/RyhB1; 200 nM *cirA*).

9. Fig. 8. With regulations so small (in the 1.3-fold range), it would be important to show that Hfq and RyhB do not regulate a non-cognate mRNA in this toeprinting assay.

Authors' answer. [Fig 8 is now Fig 7] This is an excellent comment. To address this, we have performed a toeprint on *lpp* mRNA that has been previously used as a non-target of Hfq (Vytvytska *et al.*, Genes and Dev., 2000). (NEW Fig S4A and B).

The toeprinting of Figure 7 has been repeated with the *lpp* mRNA that is used as a non-Hfq target in Vytvytska's paper. It is shown in the paper that Hfq does not repress 30S binding in toeprinting even at high molar ratios (up to 1:30 *lpp*:Hfq molar ratios). Of note, there is no RyhB effect on *lpp* according to the microarray data from Massé *et al.*, 2005. The *lpp* mRNA should then be a good non-cognate mRNA.

10. Fig. 9: The role of RyhB in the regulation of colicin sensitivity is not addressed in much detail. The authors observe a growth defect in cells (presumably) expressing the sRNA, however these cultures recover at later stages of growth. The authors can exclude a suppressor mutation, thus it would be helpful to determine the levels of RyhB/cirA at some critical points during this experiment.

Authors' answer. [Fig 9 is now Fig 8] We agree with the reviewer and we would like to point out that conditions of culture used for this experiment were the same as for Figure 1C (Iron-free minimal medium). The Northern shows that RyhB as well as *cirA* mRNA are expressed in WT conditions at the OD_{600} for which we observe the colicin killing effect. The fact that removing RyhB results in a colicin resistance phenotype that is basically the same as the D*cirA* strain, together with all the results presented in the manuscript showing that RyhB is essential for *cirA* mRNA accumulation as well as for CirA expression, strongly suggests that RyhB is responsible for promoting colicin Ia sensitivity upon iron starvation. We have added the sentence : "For a determination of the levels of RyhB and *cirA* RNAs performed in the same growth conditions, please refer to Fig 1C" in the legend of Fig 8.

As explained in the manuscript (last section of the Results), the fact that WT culture recover from the treatment at late stages of growth might be explained by cells that were not contacted by the colicin at the beginning of the treatment. For example, if the amount of colicin Ia we add to the flask contacts only two-thirds of the cells, the one-third remaining that is not exposed to colicin Ia will keep dividing. However, the other cells will be killed rapidly. If two-thirds of the cells are killed and only one-third is dividing, the OD_{600} will indeed increase slowly over time as compared to untreated WT cells in which all the cells are dividing normally. The growth recovery we observe for treated WT cells at the later stages of growth might be the cells that were not initially contacted by the colicin Ia that keep dividing.

Minor points:

Paginate the manuscript.

Authors' answer. Done.

Fig. 1C: decay should be plotted logarithmically over linear time.

Authors' answer. Done.

Fig. 2A: drawing of the figure should be improved. Use different fonts and colour.

Authors' answer. We improved the Figure 2A by including colour and different fonts.

Fig. 2A: can the structure drawn be supported by a conservation/covariation analysis of the suggested single-stranded and paired regions?

Authors' answer. Protein BLAST suggests that CirA is mostly restricted to *E. coli* and *Shigella* species, which makes sense assuming that the main substrate of the permease is the colicin Ia, for which the killing action is mainly restricted to *E. coli* and closely related species. The sequence (so the structure) of *cirA* 5'-UTR is pretty much the same for *E. coli* and *Shigella,* which is in itself not surprising considering the fact that both species have very similar genomes. It is then difficult to support the structure prediction by any conservation/covariation analysis. Of note, CirA is present in *Salmonella*. Despite the fact that *cirA* 5'-UTR sequence in this species resembles the one from *E. coli* and *Shigella*, it seems to have some differences. However, because no transcriptional start sites have been determined for *Salmonella cirA*, no reliable structure prediction, and then no conservation/covariation analysis, can be performed.

- Fig. 4B: is there an explanation for the strong effect of temperature on the regulation of cirA by RyhB? In ll. 1/2, sRNA levels are constant, but stabilization is only observed at lower temperature. Is there a difference in promoter activity? If yes, the authors should consider a reporter under the control of a temperature-insensitive promoter.

Authors' answer. [Fig 4B is now Fig S2B] The negative effect of high temperature (44°C) on *cirA* expression remains elusive. As explained above in point 5, it is probably not resulting from increased RNase E activity, since this effect is also observed in *rne*-3071 background. We could indeed expect a negative effect of high temperature on *cirA* promoter activity. However, we consider further investigation of the temperature effect on *cirA* expression, and therefore the use of a reporter under the control of a temperature-insensitive promoter, as unnecessary since all the required controls and conditions were included in Figure 4B to be able to distinguish between RNase E inactivation and high temperature effects (see point 5 for a complete analysis of Figure 4B). In other words, the point here is not to determine the cause of the temperature effect on *cirA* expression, but instead to consider this effect in our analysis of Figure 4B.

Fig. 7: Hfq concentrations should be consistently given as nanomolar or micromolar.

Authors' answer. [Fig 7 is now Fig 6] Done.

- ll. 54/55: something is missing here. The authors start to describe the composition of a colicin gene cluster, but the term "immunity protein" is missing.

Authors' answer. Done.

- l. 60: "colicins" should read "colicin"

Authors' answer. Done.

- l. 109: the listed references look like a reading list, and it is unclear which of them supports which point in the relevant sentence.

Authors' answer. Done.

- l. 113: "condtions" should read "conditions"

Authors' answer. Done.

- ll. 138-9: Which 'active role' was confirmed? Rephrase the sentence for clarity.

We have rephrased the sentence to "Moreover, our results confirmed the role of RyhB in gene activation in addition to its role as a gene silencer".

- l. 144: correct to 'quantitative Real Time-PCR (qRT-PCR)'

Authors' answer. Done.

l. 201: "proceed" should read "proceeded"

Authors' answer. Done.

l. 240: "Fusions activity" should read "Fusion activity"

Authors' answer. Done.

l. 250: I do not think that these RNase E mutants "were engineered" in this study.

Authors' answer. We rephrased the sentence to "two mutants of RNase E were used".

l. 261: "thermosenstive" should read "thermosensitive".

Authors' answer. Done.

ll. 262-6: The logic, results and conclusions of this experiment must be better explained.

Authors' answer. We have rewritten the section to make it clearer: "The other mutant (*rne-3071*) was a thermosensitive allele where RNase E becomes inactive at non-permissive temperature (44°C for 15 min) (McDowall *et al*., 1993). In these experiments, *cirA* transcript accumulated to levels similar to WT in the absence of RyhB expression when RNase E was inactivated following a 15 min-exposure of the *rne-3071* Δ*ryhB* strain at 44°C (Fig 4B, lanes 6 and 8). These data led us to the conclusion that RNase E was responsible for the turnover of *cirA* mRNA in the absence of RyhB".

l. 386: correct "it could expected"

Authors' answer. Done.

- l. 390: correct "it could then be expect"

Authors' answer. Done.

- ll. 382-393: this paragraph is really hard to read. On the one hand, the authors claim that region -40 to -12 is not cleaved by RNase I due to the formation of a stem-loop, but on the other hand susceptible to PbAc treatment due to instability of the base-pairing. The authors should try to make a clear point here.

Authors' answer. Very good observation from the reviewer. To answer to this point properly, we have added the following lines of explanation at the end of the paragraph: "Despite that both PbAc and RNase I have a preference of cleavage for unstructured RNA, RNase I activity is generally considered as more sensitive to secondary structures than PbAc (Pan, 2001; Ziehler and Engelke, 2001). It then explains higher cleavage of nucleotides -33 to -31 by PbAc than RNase I".

- ll. 453-456: The observed "increased cleavage of nucleotides -41 to -38 and -34 to -31" (Fig. 8B) occurs also independent of Hfq if only RyhB was present.

Authors' answer. [Fig 8B is now Fig 7B] We have added the following sentence: "Notably, these secondary structure changes are also observed when RyhB is added in the absence of Hfq (compare lanes 5 and 6), suggesting they do not require Hfq to occur."

- l. 573: correct "elemen t"

Authors' answer. Done.

- l. 573: correct "Shine-Darlgarno"

Authors' answer. Done.

Referee #2:

In this study Salvail et al. examined the ability of the RyhB small RNA to activate translation of the cirA mRNA. Unlike other examples of positive regulation where the small RNA acts to prevent the formation of an inhibitory secondary structure, RyhB pairing promotes changes in the cirA secondary structure that relieve the inhibitory binding of the RNA chaperone Hfq. The manuscript represents a significant amount of work and the results are interesting. However, the authors need to address the following.

1. The text of the manuscript should be shortened by at least one-third throughout. The current manuscript is unnecessarily difficult to read due to the lengthy descriptions. For example, while there is a detailed description of colicins in the introduction, this section is not very clear.

Authors' answer. This is a very good comment that resonated with reviewer #1, comment #1. As mentioned above, we have shortened the introduction, especially the section on colicin to make it more focused on the content of the manuscript.

2. The authors point out that Fur, RyhB and cirA are in an interesting feedforward loop and speculate about the possible physiological consequences of the loop. The study would be a more substantial contribution if the authors actually tested their predictions. (The rationale for delaying cirA expression when cells experience iron starvation to express cirA only when absolutely necessary makes sense, but what are the benefits of delaying repression upon a shift from iron-poor to iron-rich conditions.) This data could replace the section on colicin Ia sensitivity since this last section is a bit of an outlier from the rest of the data presented in the paper.

Authors' answer. The editor asked us to either provide additional experimental evidence for the role of RyhB in the regulation of colicin sensitivity or to demonstrate the existence of a Fur, RyhB, cirA feed-forward loop. We chose the first option as several results were already addressing this point.

It is worth to mention that a comprehensive study of the feedforward loop would be quite difficult to achieve given the fact that RyhB is essential for CirA expression upon iron starvation. It would then be difficult to assess the contribution of RyhB to the dynamic of induction by removing the sRNA from the loop as Beisel *et al.* did it with Spot42 in Mol. Cell in 2011. In other words, since *ryhB* mutant strain expresses really low levels of CirA protein upon iron starvation, it will be difficult to monitor how many time it takes for D*ryhB* cells to fully express CirA upon addition of a iron chelator (e.g. 2,2-dipyridyl) as compared to WT cells.

Potential benefits of delaying *cirA* repression upon a shift from iron-poor to iron-rich conditions are briefly discussed in the Discussion section (lines 649-651). We suggest that it would be a way for bacteria to turn off CirA expression (and then DHB and Fe^{3+}-DHBS uptake) only when the intracellular iron starvation is totally relieved and not only in response to transient increases of intracellular iron concentration. Assuming the potential benefits of colicin sensitivity upon iron

starvation as discussed in the Discussion, we could also hypothesize that interrupting rapidly colicin sensitivity in response to transient iron pulses could be detrimental for a cell population experiencing iron scarcity.

3. Better or additional data should be shown for some figures:

--Figure 1B: Size markers should be shown for the first cirA Northern presented.

Since only full-length *cirA* mRNA is monitored in the Northern blot analysis, we do not consider necessary to include a size marker to the Figure.

--Figure 2: This figure is not optimally clear. I suggest presenting 2A as supplemental data with two RNAs indicated in different colors.

Authors' answer. We thank the reviewer for this comment. We have improved Figure 2 by adding colored symbols to the different sites with increased cleavage in the presence of RyhB.

-Figure 2B should be part of Figure 3. The authors should also comment on the region of RyhB involved in base pairing with cirA compared to the RyhB regions involved in base pairing with other targets.

Authors' answer. We have move Fig 2B into Fig 3 and we have added the following sentence: "Interestingly, the region of RyhB potentially involved in the base pairing with *cirA* (41-51) is part of the sRNA central loop, which is involved in the base pairing with all the other RyhB mRNA targets characterized so far (Peer and Margalit, 2011).

--Figure 3B: The cirA Northern is not very convincing.

Authors' answer. [Fig 3B is now Fig 3C] We agree that the *cirA* bands in lanes 5 and 6 are not that sharp. However, it nonetheless recapitulates pretty well the pBAD-*ryhB*-mediated activation of *cirA* in D*fur* D*ryhB* cells as shown in Figure 1B. It is worth to mention that the point of Figure 3C is to show the absence of RyhB1-mediated activation of *cirA* expression, which is demonstrated, in our opinion, in a really convincing way by the Northern blot analysis, but also by the b-galactosidase assay in Figure 3D. The pBAD-*ryhB* condition (lanes 4-6) was included in Figure 3C to better contrast the RyhB-mediated activation of *cirA* with the absence of RyhB1-mediated activation of *cirA*.

-The OmrA and OmrB panels in Figure 6A also are not optimal.

Authors' answer. [Fig 6A is now Fig 5A] We do not expect OmrA/B to be strongly expressed under these conditions as OmrA/B expression levels in M63 minimal medium are really low (Guillier and Gottesman, 2006). Northern membranes then need to be exposed for a long period of time in order to detect a signal, thus explaining the granular background of the membrane. Also, the poor OmrA/B expression in the absence of Hfq (see lanes 3 and 4) correlates with previous reports (Guillier and Gottesman, 2008; Holmqvist *et al.*, 2010).

--Figure 7A: The authors should examine the effects of a control protein in the toe print assay (to demonstrate that the loss of ribosome binding is a specific effect).

Authors' answer. [Fig 7A is now Fig 6A] We consider this an excellent suggestion. As a control, we have performed toeprinting on *lpp* mRNA, which was shown to be not regulated by Hfq (Vytvytska, 2000), in the presence of increasing Hfq concentration. As shown in Fig S4, while the *lpp* mRNA is only slightly affected by 200 nM Hfq (repressed to 90%), the *cirA* mRNA is repressed to 45%.

4. Given that evidence for Hfq binding to sites II and III is subtle, the authors should assay lacZ fusions in which the Hfq binding sites have been mutated. The prediction from the model presented in the paper is that expression from these mutants should not be changed in an ?hfq deletion background.

Authors' answer. This is a good point. To address this, we have performed additional lacZ assay that are presented in a new figure (Fig S9). We have added the following text to describe the new results: "Remarkably, there was a 50% decrease of *cirA'-'lacZ* translational fusion activity when mutations were introduced in Hfq binding site III, suggesting that this region is important for efficient *cirA* translation (Fig S9, compare *cirA'-'lacZ* WT with *cirAMHfqIII'-'lacZ* WT). Hfq binding to sites II and III would then be sufficient to inhibit *cirA* translation and to promote transcript destabilization".

More minor comments:

5. The authors need to be more explicit about the following:
--They should specify that "shiA" not regulated by Fur in the introduction.

Authors' answer. We have rephrased a sentence in the Intro: "…RyhB activates translation of the Fur-independent *shiA* mRNA that encodes a transporter of shikimate…".

--They need to acknowledge that some of the unexplained regulation of cirA could be due to the action of another small RNA given that that many mRNAs are regulated by multiple small RNAs.

Authors' answer. We agree with the reviewer that the positive effect of removing *hfq* in a D*ryhB* background on *cirA* expression could be due to decreased expression of a not yet identified sRNA that post-transcriptionally represses *cirA* expression. However, we could discard this hypothesis based on two lines of evidences.

First, we were able to reproduce by toeprinting the direct negative effect of Hfq on 30S binding to *cirA* mRNA as well as the coregulation of *cirA* by Hfq and RyhB, thereby discarding that additional regulators would be required for both the Hfq-mediated repression and the RyhB-activation of *cirA* to occur.

Second, the fact that *rne131* D*ryhB* cells do not accumulate increased levels of *cirA* mRNA as compared to D*ryhB* cells discards the hypothesis that a sRNA depending on the degradosome assembly with RNase E and/or on Hfq would repress *cirA* in the culture conditions used in our study (LB, M63 iron-free minimal medium).

We could also explain activation of *cirA* by RyhB in a D*fur* D*ryhB* D*hfq* background to result from a potential counteraction of OmrA/B repression, considering the pairing of RyhB and OmrA/B to *cirA* to be potentially mutually exclusive. Even if these two sRNAs are not much expressed in the absence of Hfq, I have unpublished Northern blot results showing a 1.5-fold increase of *cirA* levels in D*hfq* D*ryhB* D*omrAB* cells as compared to D*hfq* D*ryhB* cells. The same fold increase is observed with a CirA'-'LacZ translational fusion when comparing the fusion activity in both backgrounds. These results suggest that there is still a small contribution of OmrA/B to the repression of *cirA* mRNA, even in a D*hfq* strain. However, in WT conditions most of the *cirA* activation would occur through the counteraction of the Hfq repressing effect through RyhB, as *omrAB* inactivation in D*ryhB* cells does not result in increased expression of *cirA* as compared to D*ryhB* cells.

--The type of lacZ fusions being assayed should be spelled out (the "tic" is easy to miss).

Authors' answer. We have added the type of fusion for many constructs in the text.

6. There are a number of typographical errors (not a complete list):
--Lines 54-55: "...encode a colicin, which is a protein that protects..."
--Line 106: "stimulate"
--Line 113: "condtions"
--Lines 516-517: "series of experiment"
--Line 575: "Shine-Dalargno"

Authors' answer. These typographical errors and others were corrected.

7. Strains and plasmids table should be given as Supplementary Data.

Authors' answer. We agree with the reviewer and this section will be resubmitted in the Supplementary Materials section.

Referee #3:

This paper reports the study on post-transcriptional regulation of cirA by an Hfq-binding small RNA RyhB in E. coli. The authors first showed that RyhB is required for the expression of cirA mRNA encoding CirA, a siderophore receptor involved in ferric ion uptake and the cirA mRNA is stabilized by RyhB. They showed evidence that RyhB promotes CirA expression through pairing with cirA mRNA and protects the cirA mRNA from RNase E degradation. The data suggest that RyhB pairing with cirA mRNA leads to increased translation resulting in the protection and accumulation of full-length cirA mRNA. In addition, the authors showed evidence that Hfq represses cirA mRNA translation in the absence of RyhB by preventing ribosome binding and RyhB pairing with cirA mRNA stimulates ribosome binding. Finally, the authors showed that RyhB increases the sensitivity to colicin Ia through cirA activation.

This work demonstrates convincingly that the Fur-regulated cirA gene is positively regulated by RyhB at post-transcriptional level, revealing the presence of an intriguing Fur-RyhB-cirA regulatory circuit. Furthermore, the proposed mechanism by which RyhB activates the cirA mRNA is quite interesting. The findings expanded our view regarding how bacterial Hfq-binding sRNAs act in bacterial cells. I have criticisms/comments on the data and the organization of the manuscript.

Comments:

1) *First of all, the manuscript is too long and the argument is too diverse. Some data seem to be less informative. I think that the manuscript should be reorganized by focusing on the cirA regulation by RyhB and by trimming drastically throughout. The followings can be deleted or described more concisely: a) the argument concerning colicin/CirA and the detailed data regarding the colicin sensitivity test because it is already known that CirA is a target of colicin Ia; b) some data and description on lacZ fusion experiments are less informative and could be deleted; c) the results of Figs. 7 and 8 could be described more concisely.*

a) [Fig 7 and 8 are now Fig 6 and 7, respectively] This is an interesting point. Despite the fact that the paper is mostly focused on the molecular mechanism by which RyhB regulates *cirA*, the colicin sensitivity phenotype demonstrates in a very straightforward manner the physiological consequence of *cirA* regulation by RyhB, in addition to further support that RyhB is essential for CirA protein production in iron starvation and, therefore, for colicin sensitivity.

   We consider this part of the paper in which we study the effect of RyhB expression on colicin Ia sensitivity to be enough concise and straightforward, thereby not requiring further trimming.

b) We consider all the b-galactosidase data presented at Figure 4 as crucial to assess if RyhB-mediated stabilization of *cirA* mRNA (Figure 1D) results from translational activation.

c) The description of both figures 7 and 8 have been shortened.

2) The data show overall a good correlation between RyhB expression and cirA expression in the absence of Fur. However, the cirA is little expressed while RyhB well expressed at OD600=0.3 in cells lacking fur gene (Fig. 1C, lane 7). This is quite confusing.

Authors' answer. We agree with the reviewer. While these results can be explained with increased intracellular Hfq concentration at earlier time points, we removed the OD_{600} of 0.3 to reduce confusion.

3) The cirA mRNA stabilization by RyhB (Fig. 1D) is too moderate to explain the dramatic activation of cirA mRNA by RyhB shown in Fig 1B and 1C.

Authors' answer. RyhB increases *cirA* half-life from 2 to 8 minutes (4-fold stabilization) (Figure 1D) and increases *shiA* half-life from ~1 minutes to 3 minutes (3-fold stabilization) (see Prévost *et al.*, 2007). These data are consistent with the qRT-PCR data of Figure 1A showing that RyhB promotes a 2.3 to 2.6-fold increase of *shiA* transcript levels and a 4.7-fold increase of *cirA* mRNA levels.

4) Fig. 6A shows that the cirA mRNA is highly expressed in the absence of Hfq even without RyhB, suggesting that RyhB promotes the cirA expression simply through relieving Hfq repression. On the other hand, the Fig. 6C shows the cirA mRNA expression require RyhB even in the absence of Hfq. Thus, two experiments appear to be inconsistent each other.

Authors' answer. [Fig 6 is now Fig 5] This is a good observation. We have addressed this in our answer to Referee #1, point 8.

5) RyhB-cirA pairing can activate the cirA expression by antagonizing Hfq repression and/or by increasing the ribosome accessibility to the cirA mRNA. It is frustrating that the data are confusing and not matured yet to make this important point clear. One simple important but experiment is to examine the effect of hfq deletion on CirA protein expression.

Authors' answer. Excellent point. To address this, we have performed an additional experiment (shown in Fig 1E) in which we have compared CirA protein expression in a D*hfq* D*fur* D*ryhB* strain as compared with a D*fur* D*ryhB* strain (as well as D*fur* and D*fur* D*cirA* strains). The data suggest that in absence of Hfq the expression if CirA protein increases significantly.

6) The authors propose antagonistic function between Hfq and RyhB. I think this proposal is not appropriate because Hfq is essential for RyhB to base pair with cirA to activate the cirA translation.

Authors' answer. It is worth to mention that we do not mention at any time in the manuscript that "Hfq is essential for RyhB to base pair with *cirA* to activate its translation". In contrast, we have observed results that support the idea that Hfq is not absolutely essential for RyhB to base pair with *cirA* to activate its translation.

1) Despite the fact that Hfq increases pairing efficiency of RyhB to *cirA* mRNA *in vitro* (Figure S11), some of our *in vitro* experiments show that RyhB is pairing pretty well with *cirA* in the absence of Hfq (e.g. Figure 3B; Figure 7B, compare lanes 5 and 6; Figure S6, see RyhB-induced reverse transcription block). However, it is important to notice that in these experiments, RyhB was added in excess amount, which might not necessarily reflect the *in vivo* reality.

2) In Figure 5C, we show that a 10-minutes expression of RyhB is sufficient to result in an increase of *cirA* mRNA expression in the absence of *hfq*, suggesting that RyhB may be able to base-pair with *cirA* mRNA in the absence of Hfq to promote its translation *in vivo*. As discussed above, this RyhB positive effect on *cirA* expression in the absence of Hfq remains elusive.

On the other hand, we cannot totally exclude Hfq to be required for RyhB to base-pair with *cirA in vivo*. Binding of Hfq to both RyhB and Hfq binding site I would be necessary to promote RyhB pairing to *cirA* mRNA and therefore dislodge Hfq from its repressing sites (i.e. Hfq II and Hfq III binding sites). However, considering that it is not thoroughly demonstrated in our study that Hfq is required for RyhB interaction with *cirA*, it is accurate to define the functions of Hfq and RyhB in *cirA* regulation as being antagonistic, since one regulator (Hfq) is repressing *cirA* expression while the other (RyhB) activates *cirA* expression by antagonising the first one (Hfq).

2nd Editorial Decision 15 July 2013

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by all three original referees whose comments are shown below.

As you will see, while the referees acknowledge your efforts to strengthen the conclusions made in your study, and are consequently broadly in favour of publication, they all emphasize the need for you to substantially shorten and clarify the manuscript text (either directly in their comments as provided below or in their recommendations to the editorial office).

An extensive shortening of the manuscript was already requested in the initial decision letter and as you will notice the absence of such shortening in the current version has caused substantial irritation with ref #3, who at this stage does not support publication of the revised manuscript. The EMBO Journal guidelines furthermore limit manuscript length to around 55.000 characters (including spaces) and since your manuscript currently contains almost 90.000 characters it is vital that you perform a substantial shortening of all sections (at least 25% reduction of current manuscript length) before we can take any further steps towards publication.

In addition, I have to ask you to address the following minor editorial issues:

-> include a conflict of interest statement and an outline of author contributions

-> provide information on the nature of error bars as well as the number of replicates underlying statistical calculation (n≥3) in the legend for the following figures: 1A+D, 3D, 4A-D, 5B+D, 6D and 7A as well as for all relevant supplementary figures.

- we now also encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data".

In light of the recommendations from the referees I would invite you to submit a revised version of your manuscript addressing all of the issues outlined above. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS:

Referee #1:

The revised version of this manuscript has sufficiently addressed the majority of the previous points of criticism. There are a few typos here and there that need to be fixed still. Figures S8 and S9 would be good to have in the main manuscript as they present important data but I will leave this to the authors' discretion.

Referee #2:

The authors have made a reasonable effort to address the reviewers' comments, which has improved this study. I think further effort to improve the readability of the manuscript would be appreciated by general readers.
--The text could be condensed even further.
--Long sections could be subdivided with additional subheadings.
--Paragraphs could be more focused with clear topic sentences (as just one example, the sentence beginning on line 102 "RyhB also promotes..." should be in the subsequent paragraph on RyhB function as an activator).

Referee #3:

Unfortunately, the revised manuscript does not meet my major concerns although authors have addressed minor points. The revised manuscript is still too long and the argument is too diverse. The manuscript is not easy to read and digest. The data are not fully convincing to support the proposed mechanism.

2nd Revision – authors' response 29 July 2013

We respectfully resubmit our revised version of our manuscript "Antagonistic functions between the RNA chaperone Hfq and a sRNA regulate sensitivity to the antibiotic colicin".

As suggested in the second round of review, we have substantially reduced the length of all sections of the manuscript (overall 25%) and we have added the statistical description in the legend of the figures. We also added a source data file of our figures.

We believe that we have fully addressed every concerns raised by the editor and reviewers and, as a result, we feel that the manuscript is clearly improved.