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Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA

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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 02 March 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. It has now been seen by three referees, whose comments are shown below. All of the referees find the study to be interesting and they require some further experimental analysis before it can be further considered at the EMBO Journal.

In general the referees find the identification and analysis of RpoS translational regulation by ArcZ to be convincing and ask for the addition of controls. The referee's main criticisms focus on the regulation of ArcZ by ArcA/ArcB, where they require some discrepancies in the data to be resolved and for more direct evidence for the proposed regulatory mechanism. This includes describing the effect of ArcZ on endogenous ArcB mRNA levels, ArcA in directly transcriptionally regulating ArcZ and testing a role for RpoS regulation of ArcZ. Should be able to address these issues we would be happy to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The present manuscript by Mandin & Gottesman reports the discovery of the conserved, Hfq-dependent ArcZ sRNA as the third noncoding translational activator of rpoS mRNA in E. coli. This positive regulation was discovered in the course of screening a newly constructed expression plasmid library of 26 Hfq-binding sRNAs for effects on an rpoS::lacZ reporter; ArcZ activated rpoS as strongly as did DsrA and RprA, two sRNAs previously discovered by the Gottesman lab. Mutational analysis provided convincing evidence for direct base pairing of ArcZ with the rpoS mRNA, which is predicted to open an inhibitory structure around the ribosome binding site and thereby enhance translation. In addition, the authors report results showing that the ArcA/B two-component system negatively controls ArcZ under anaerobic growth. The negative regulation seems to occur at two levels: ArcA might directly bind at the arcZ promoter, and the 3' overlap of arcZ and arcB might result in mutual antisense and/or transcriptional repression. The latter indicates that ArcZ and ArcA/B act in concert to provide a novel of regulation of RpoS synthesis.
Given the vital interest in post-transcriptional regulation by Hfq-dependent sRNAs, the role of RpoS as a major stress sigma factor in many model bacteria, and the recent discovery of negatively regulated ArcZ targets in Salmonella, this is a very timely manuscript that will be interesting for many researchers. The following issues should be addressed prior to publication in EMBO J.

Major comments:

- The manuscript is very long and could easily be shortened without loss of clarity, especially by reducing redundancy between the Results and Discussion sections. Examples include page 7, lines 26-29, and the upper two paragraphs on page 10. Moreover, since the functional characterization of ArcZ is the focus of this paper, the previous work on this sRNA should be introduced earlier, i.e. at the end of Introduction rather than somewhere in the middle of Results on page 10.

- Page 7, upper para, and Figure 1. The authors determine fold-regulations of a pBAD-rpoS-lacZ fusion by individual sRNAs, as compared to expression of this fusion in the presence of the "empty" sRNA vector. However, it is not clear to me whether the sRNAs were also tested for effects on the pBAD promoter or lacZ activity alone, i.e. on a control fusion. This is relevant because some of the overexpressed sRNAs seem to greatly impact on bacterial growth, with toxicity at the extreme end. In other words, do we really know that all of these fold-regulations can be attributed to the rpoS part of the reporter?

- Page 11, lines 13-26. The description of the individual forms of ArcZ is fairly redundant with the previous reports in E. coli (Argaman 2001) and Salmonella Papenfort 2009), and could be condensed. As a result of these previous mappings, and unlike the authors' statement, the short Arcz might not be the only active form of the sRNA in the context of rpoS regulation. That is, the processed ArcZ starts with U67 (according to the authors' numbering in Figure 2B), whereas the upstream located A65 position (in the ArcZ precursor) is revelad in Figure 2C to be involved in duplex formation with rpoS mRNA. Furthermore, in Figure 4A, asterisks indicate positions of ArcZ processing. However, it is not clear how these positions were determined, especially since they do not seem to be in agreement with the previously published ArcZ data (The processed ArcZ starts with UUCCCUGG...).

- Page 15, bottom part. When investigating a putative mutual regulation by the 3' overlapping arcZ and arcB genes, the authors fail to detect the chromosomally encoded arcB mRNA on northern blots. This prompts them to strongly overexpress arcB in order to determine effects on arcZ. While my feeling is that their model in principles is correct, the experimental support for it is compromised because of the strong overexpression. If northern analysis is not sensitive enough to detect the native

arcB mRNA, why not use more sensitive techniques such as quantitative real-time PCR (qRT-PCR), or an RNase protection assay? The detection of differential expression of the native arcB mRNA in an acrZ deletion mutant would greatly increase confidence in the proposed complexity of ArcA/B/Z and RpoS regulation. In the absence of such data, the authors want to tone down their conclusion in the Abstract and Discussion (page 18).

- Page 16, third para, and Figure 6C, middle part. The level of the 55nt ArcZ species rises after 10 and 15 minutes of rifampicin addition. This is unusual and should be commented upon.

- Page 18, bottom part. The approach taken here is conceptionally similar to the co-expression of sRNAs with target fusion to a gfp reporter gene (Urban & Vogel 2007 NAR), which should be discussed.

Minor comments:
- Page 5, line 7. The reference for deep sequencing-based identification of Hfq-associated sRNAs is missing.

- Page 5, upper part: I am not certain whether the 26 sRNAs investigated should be considered a close-to-saturation set of Hfq-binding sRNAs in E. coli. Most of the present screens were limited to a couple of growth or stress conditions, and to abundant sRNAs. Moreover, the authors claim that all sRNAs are expressed from their native 5' ends. Have 5' ends have really been mapped in all of these cases, for example, for IS118? Whereas Zhang, 2003, Mol Micro proposed a size of 298 nt, the authors claim here that IS118 is 194 nt long (Table 1). Moreover, IS118 is located downstream of yfdI in the E. coli K12 genome and not yfdL, as indicated in Table 1.

- Page 13, line 17-18. The conclusion presented by this sentence is hard to understand. Do you mean titration effects by ArcA binding sites carried on the 8 plasmids? Please clarify.

- Page 16, line 25. If there indeed is clear-cut evidence for "colliding transcription of arcB and arcZ", this should be elaborated on in the Discussion section.

- Figure 6A. How exactly were arcB mRNA levels quantified? The values given below some of the lanes, especially 14 and 15 versus 19 and 20, are hard to reconcile with the presented blot images.

- Figure S1. Include size markers on the individual blots.

- Figure S2A. Please elaborate why a C561G mutation of the rpoS mRNA should impair its translation. The mutation does not interfere with the core of the ribosome rpoS binding site (AGGAGC) and the paring nucleotide on 16S rRNA would be a U, thus one would expect translation to increase rather than decrease.

Referee #2 (Remarks to the Author):

Comments for Mandin and Gottesman:
Integrating anaerobic/aerobic sensing and the general stress response via the ArcZ small RNA

Regulation by small RNAs has been highly prominent in recent years, and rpoS mRNA, which encodes the general stress sigma factor in E. coli, is certainly one of the most interesting targets for small regulatory RNA in bacteria as it is positively and negatively regulated by several such RNAs. Using a library of 26 plasmid-encoded small regulatory RNAs of E. coli, ArcZ is identified here as a third small RNA (besides the well-studied DsrA and RprA) that can stimulate rpoS translation by directly interacting with rpoS mRNA. As the other two small RNAs, ArcZ operates by relieving translational inhibition by intramolecular base-pairing of rpoS mRNA. Moreover, ArcZ is shown to be negatively regulated by the ArcB/ArcA two-component system, both by ArcB downregulating ArcZ directly by an anti-sense mechanism (as the arcB and arcZ genes converge onto each other) as well as by ArcA inhibiting arcZ transcription, probably by binding in the arcZ promoter region. This demonstrates an even greater influence of the Arc system on RpoS than previously known (repression of rpoS transcription by ArcA and promotion of RpoS degradation by ArcB via RssB was shown before), which now also includes translational control. Moreover, this regulation sets up

some intriguing feedback cycles the biological functions of which will have to be explored in future studies.

Overall, this is a scientifically sound, generally relevant and nicely presented study appropriate for publication in EMBO Journal. Nevertheless I have a few comments and suggestions for the clarification of some relevant details.

Comments and suggestions:

1. p. 6, l.12: Thirty nucleotides of coding region in a translational reporter fusion do not necessarily capture ALL effects exerted by small regulatory RNAs, which can also bind further downstream and still interfere with translation/degradation of the mRNA. So, one should keep in mind that the analysis presented here is not necessarily exhaustive even for the tested RNAs.

2. p. 6, l. 18 ff: the pBAD promoter responds to inducer in a non-linear fashion, i.e. this promoter is either OFF or ON and intermediate inducer levels result in population heterogeneity with the fraction of cells in the ON state depending on inducer concentrations (see Siegele and Hu. 1997. PNAS USA 94: 8168-72). "Basal activity" thus means that only a fraction of the cells is in the ON state. Nevertheless, the approach chosen here works because rpoS::lacZ mRNA made in these ON cells is still translationally inhibited because of intramolecular basepairing that can be affected by small RNAs. But it may be worth checking these rpoS::lacZ cells under the microscope for population heterogeneity and keep in mind that sRNAs tested could also somehow change the expression of the pBAD promoter. An important control is therefore also to check effects with a pBAD::lacZ construct not containing any rpoS sequences.

3. In general, when cells are tested in "stationary phase", what exactly does this mean? When cells have reached a certain OD or overnight cells?

4. P. 9, Fig. 3: all the data presented here were obtained with rpoS::lacZ fusions. At least the effect of the knockout mutations of arcZ and the other small RNAs involved should be shown in one experiment also for wildtype rpoS mRNA (e.g. in a Northern blot).

5. Fig. 4: Where is the terminator for the arcB gene? This is important to know in order see how much (complementary) overlap there is between ArcZ and arcB mRNA.

6. Fig. 4: The sequence supposed to be an ArcA box in the arcZ promoter region does not really match the ArcA box consensus very well. Before drawing the conclusion that ArcA binds directly, it seems safer to do either a footprint experiment in vitro or to introduce point mutations that (i) deteriorate, and (ii) improve the putative ArcA box and measure the corresponding effects on the expression of the arcZ::lacZ fusion in vivo.

7. Fig. 4C: Induction of ArcZ during transition into stationary phase suggests that ArcZ is involved in the increased rpoS translation observed during the post-exponential phase (see Lange et al. 1994. Genes & Dev. 8: 1600-12). This should be tested with the arcZ mutant (just follow rpoS::lacZ activity along the growth curve). Moreover, it would be consistent with ArcB becoming inactive not only by aerobiosis but also in energy limited cells, since ArcB is oxidized (inactivated) by oxidized quinones, which arise not only in response to high levels of oxygen (increased efflux of electron from the respiratory chain) but also in response to energy limitation (reduced influx of electron into the respiratory chain) (see Mika et al. 2005. Genes & Dev. 19: 2770-81); this impact of energy limitation (in addition to +/- oxygen) may also be integrated into Fig. 7.

8. The arcZ promoter looks as if it could also be activated by RpoS-containing RNAP (in particular, it has a -13C). If so, arcZ induction during entry into stationary phase would also be one of several positive feedbacks that make RpoS induction more robust. This could easily be checked by measuring arcZ::lacZ also in an rpoS mutant background.

9. Table II could be shown in the Supplement, as the results presented there leave a lot of open ends and the effect of ArcB and ArcA on arcZ expression is then directly shown in Fig. 5.

10. P. 15, l. 11 ff: The finding that RpoS is reduced in the arcA mutant in stationary phase, or, to put

it the other way round, that ArcA contributes to RpoS accumulation in stationary phase, has been reported and explained before (see again Mika and Hengge, 2005, Fig. 6 therein): arcA expression is further stimulated by RpoS-containing RNAP, increased ArcA scavenges phosphate from ArcB at the expense of RssB phosphorylation (because ArcA phosphorylation by ArcB is about 10fold more efficient than that of RssB), which results in increased stabilization of RpoS - in fact the arcA mutant is shown to have a shorter half-life of RpoS in stationary phase. This RpoS/ArcA effect is one of the several positive feedbacks that stabilize high RpoS levels in stationary phase.

11. The "anaerobic/aerobic sensing" mentioned in the title is the least well documented aspect of this study. I would suggest to think about this title again.

Minor comments:

1. p. 3, l. 7 from bottom: The positive role of Hfq in rpoS translation was also described by Muffler et al. (1996. Genes & Dev 10: 1143-1151) in a publication that actually appeared earlier than Brown and Elliott (1996).
2. Fig. 2A: it may be helpful to also label the ribosome binding site
3. Fig. 2C: "Actifity"
4. Fig. 5D: replace ".65" by "0.65" (in +O2, arcA-)

Referee #3 (Remarks to the Author):

In this study, Mandin and Gottesman constructed a set of plasmids expressing 26 Hfq-binding sRNAs of E.coli and tested the effect of their overexpresssion on rpoS-lacZ translational fusion. This allowed them to identify ArcZ as a third new sRNA activator for rpoS translation, along with previously known DsrA and RprA sRNAs. Genetic analyses demonstrated convincingly that AcrZ activates rpoS translation by opening the secondary structure of rpoS mRNA leader as DsrA and RprA do. Evidence was also shown that deletion of the chromosomal acrZ significantly reduces the rpoS expression. They also showed AcrZ exists as three forms in cells (the shortest one containing the base-pairing region seems to be a processed active form). Then, the authors showed that the arcZ expression is negatively regulated by ArcA/ArcB two component system first by a mulicopy library screen of factors affecting arcZ-lacZ expression and by sequence examination of arcZ promoter region. Further mutational analyses indicated that ArcA/ArcB system represses ArcZ expression under anaerobic condition and affects RpoS expression through ArcZ expression. Finally, the authors showed evidence that ArcZ expression markedly destabilize arcB mRNA expressed from the arabinose-inducible promoter.

There are two major findings regarding the function of Hfq-binding small sRNAs in E. coli. The first is identification and characterization of ArcZ as a third sRNA activator for rpoS translation. The second is existence of an autoregulatory loop between ArcZ and AcrA/ArcB system: the arcZ expression (transcription) is negatively regulated by AcrA/ArcB and ArcZ negatively regulates ArcB expression. The first conclusion is well supported by a series of elegant experiments and I have no criticism except one point regarding the active form of ArcZ. On the other hand, several points are unclear and ambiguous for me concerning the second conclusion while the results basically support the authors' claim.

Specific comments:
1) It is likely that the shortest 55 nt form of ArcZ is active because it accumulates in cells and contains the sequence complementary to the rpoS. But, it is not proved experimentally. It is easy to test this by expressing only 55 nt piece of ArcZ by deleting the 5' portion of arcZ gene.
2) It was shown clearly that ArcA/ArcB system causes a marked reduction in ArcZ expression only in anaerobic condition and that arcA deletion abolishes this repression (Fig. 5A and B). There are discrepancies between lacZ assay and Northern analysis. Under aerobic condition, the arcA knockout causes a moderate increase in AcrZ expression in lacZ assay while it seems to cause a slight reduction in Northern analysis. In addition, the ArcZ expression is significantly higher in aerobic compared to anaerobic conditions in lacZ assay while the situation is just opposite in Northern analysis.
3) The effects of arcA and/or arcZ deletion on RpoS expression are quite confusing. Although

authors claim that AcrA negatively regulates RpoS expression through ArcZ expression only under anaerobic stationary condition, Western analysis of RpoS shown in Fig. 5D indicates that AcrA down-regulates RpoS expression through ArcZ expression also under aerobic exponential growth condition.
4) I am also confused again with a discrepancy between rpoS-lacZ assay and western analysis. The arcA mutation does not affect the expression of rpoS-lacZ under aerobic stationary condition (Fig. 5C) while it reduces RpoS expression in Western analysis (Fig. 5D).
5) It was shown convincingly that ArcZ expression markedly destabilize the arcB mRNA in Fig. 6B and C. Although the authors argue that arcB mRNA expression does not affect the stability of ArcZ, the level of ArcZ is also significantly reduced by arcB expression. For me, both RNAs seem to be degraded in a coupled manner.
6) It is interesting to know whether ArcZ is also able to destabilize the arcB mRNA expression when it is provided in trans. It is possible that ArcZ expression affects the arcB mRNA expression only when two genes are overlapping on the same DNA.

1st Revision - authors' response 13 June 2010

Many thanks for providing a rapid review of our manuscript on ArcZ, and for your flexibility in allowing us a bit more time to send in the revision. We appreciated the comments of the reviewers, and have tried to clarify or add the data to address their major concerns. The major changes can be summarized as:

1) Addition of a control for the fusion data. We used pBAD-lacZ integrated at the same site as the experimental fusion. The data, shown in a new supplemental Figure (Fig. S3), was used to normalize the results in Fig. 1; the results of this normalization are shown in Fig. S3B, and make no major change in our conclusions. This has been summarized in the text (p. 7).
2) Role of RpoS in regulation. We had previously tested the possible role of RpoS in regulating ArcZ, and did not find any evidence for such a role. This is now described in the text (p11).
3) Further evidence of direct regulation by ArcA of arcZ. We tested a set of mutations in our promoter fusion (new supplemental Fig. S5); none abolished ArcZ repression and one improved it. This data led us to reevaluate our predicted ArcA binding site. However, it is clear that this data does not prove direct interaction, and we now acknowledge that in the text and summarize why we think it is still quite possibly direct (p.13). The regulatory effects of ArcA, however, are unambiguous, whether regulation is direct or indirect.
4) In response to the suggestion of reviewer 2 (point 7) about when ArcZ might be most critical, an additional set of expression experiments were done, using an rpoS-lacZ fusion that has the native promoter and is degraded as is RpoS. These result, now shown in Fig. 5D, suggest that the biggest contribution of ArcZ is in the transition out of stationary into early exponential growth and that the ArcA effect on RpoS early in exponential is dependent upon ArcZ, with a modest effect at other stages of growth. The Western blot, which is consistent with these results, has now been moved to a supplemental figure S6. The results were integrated into the previous description of Fig. 5 on p. 14, and compared to previous work by others.
5) Reviewers asked for other measures of the effect of arcZ on arcB, and we have now added qRT-PCR data to address this (Fig. S7), and compared it to the results in Fig. 6. The results confirm that ArcZ negatively regulates *arcB* mRNA levels, under anaerobic conditions, but also uncover additional levels of complexity, which have been briefly described (p. 16).
6) Change in specificity mutations used in Fig. 2: As reviewer 1 had pointed out (comment 2), and after discussions with colleagues, we realized that there was an inconsistency in the data in Fig. 2 that needed to be addressed. The issue is that the change in arcZ at position 65 is outside the processed RNA as mapped by others, and now confirmed by us. Other studies in our lab have suggested that it is the processed form that is active. When we repeated the experiment in Fig. 2, the A65G mutation was not able to activate. We believe that one of the other plasmids (DsrA or RprA) must have been used in error in the original set of experiments. We have now substituted the specificity mutants that were previously in supplemental data (new Fig. 2). The basic conclusions are the same; we are

understandably disturbed at this apparent error and thank the reviewer for bringing the issue to our attention. The data on position 65, which supports the processed form as the active ArcZ, has been included in supplemental Fig. S4 and is briefly described in a new paragraph (p.10).

7) We have shortened the text throughout in response to reviewers' comments and to try to abide by the character limit.

Specific response to reviews:

Referee 1:

1. The manuscript is very long and could easily be shortened without loss of clarity, especially by reducing redundancy between the Results and Discussion sections. Examples include page 7, lines 26-29, and the upper two paragraphs on page 10. Moreover, since the functional characterization of ArcZ is the focus of this paper, the previous work on this sRNA should be introduced earlier, i.e. at the end of Introduction rather than somewhere in the middle of Results on page 10.

Response: Part of the information on p. 10 was moved to the end of the introduction. The manuscript was shortened at various points, and redundancy reduced.

2. Page 7, upper para, and Figure 1. The authors determine fold-regulations of a pBAD-rpoS-lacZ fusion by individual sRNAs, as compared to expression of this fusion in the presence of the "empty" sRNA vector. However, it is not clear to me whether the sRNAs were also tested for effects on the pBAD promoter or lacZ activity alone, i.e. on a control fusion. This is relevant because some of the overexpressed sRNAs seem to greatly impact on bacterial growth, with toxicity at the extreme end. In other words, do we really know that all of these fold-regulations can be attributed to the rpoS part of the reporter?

Response: The effect of the fusions was measured on a pBAD-lacZ fusion, now shown in Fig. S3A. Modest effects of the plasmids were seen, and when the effects on pBAD-rpoS-lacZ were normalized to this control, the same set of plasmids showed what we consider significant effects (Fig. S3B). This is now described on p. 7.

3. Page 11, lines 13-26. The description of the individual forms of ArcZ is fairly redundant with the previous reports in E. coli (Argaman 2001) and Salmonella Papenfort 2009), and could be condensed. As a result of these previous mappings, and unlike the authors' statement, the short ArcZ might not be the only active form of the sRNA in the context of rpoS regulation. That is, the processed ArcZ starts with U67 (according to the authors' numbering in Figure 2B), whereas the upstream located A65 position (in the ArcZ precursor) is revealed in Figure 2C to be involved in duplex formation with rpoS mRNA. Furthermore, in Figure 4A, asterisks indicate positions of ArcZ processing. However, it is not clear how these positions were determined, especially since they do not seem to be in agreement with the previously published ArcZ data (The processed ArcZ starts with UUCCCUGG...).

Response: The description of ArcZ has been shortened. We thank the reviewer for pointing out the very important discrepancy in the 5' end of ArcZ. In a repeat of the experiment shown in Fig. 2B, we were unable to demonstrate regulation by the ArcZA65G mutation of the rpoS U468C mutation. Furthermore, no decrease in the activity of ArcZ65G in regulation of the wild-type fusion was seen (now in Fig. S4). We conclude that we inadvertently had used one of the other sRNAs (DsrAA11G or RprAA37G) in the original set of experiments. The new results are now fully consistent with no pairing by ArcZ at position 65 (outside the processed RNA), and are consistent with our own in vitro and in vivo data on the ability of the short ArcZ to regulated rpoS (Soper et al, PNAS 2010). We have substituted other specificity data in Fig. 2. We did repeat 5' RACE on the wild type and A65G mutant; both have the same 5' end, identical to that listed by the reviewer.

4. Page 15, bottom part. When investigating a putative mutual regulation by the 3' overlapping arcZ and arcB genes, the authors fail to detect the chromosomally encoded arcB mRNA on northern blots. This prompts them to strongly overexpress arcB in order to determine effects on arcZ. While my feeling is that their model in principles is correct, the experimental support for it is compromised

because of the strong overexpression. If northern analysis is not sensitive enough to detect the native arcB mRNA, why not use more sensitive techniques such as quantitative real-time PCR (qRT-PCR), or an RNase protection assay? The detection of differential expression of the native arcB mRNA in an acrZ deletion mutant would greatly increase confidence in the proposed complexity of ArcA/B/Z and RpoS regulation. In the absence of such data, the authors want to tone down their conclusion in the Abstract and Discussion (page 18).

Response: An experiment was added to address this point, measuring *arcB* mRNA via qRT-PCR (Fig. S7). The results suggest somewhat more complex regulatory loops (now discussed on p. 16), but the negative effect of ArcZ on *arcB* mRNA levels can be seen, at least under anaerobic conditions. We have also cited a previous publication showing an ArcA effect on arcB, which may well be due to ArcZ, judging by our results (both the original data and the new qRT-PCR data).

5. Page 16, third para, and Figure 6C, middle part. The level of the 55nt ArcZ species rises after 10 and 15 minutes of rifampicin addition. This is unusual and should be commented upon.

Response: There is no increase of ArcZ during those experiments, but rather an increase in loading as can be seen in the SsrA loading control lane. When levels were quantified using SsrA as a control, no increase of the short form of ArcZ was seen at these time points.

6. Page 18, bottom part. The approach taken here is conceptionally similar to the co-expression of sRNAs with target fusion to a gfp reporter gene (Urban & Vogel 2007 NAR), which should be discussed.

Response: Text added on p. 18 to acknowledge this similarity.

Minor comments:

- *Page 5, line 7. The reference for deep sequencing-based identification of Hfq-associated sRNAs is missing.*

Response: Reference added.

- *Page 5, upper part: I am not certain whether the 26 sRNAs investigated should be considered a close-to-saturation set of Hfq-binding sRNAs in E. coli. Most of the present screens were limited to a couple of growth or stress conditions, and to abundant sRNAs. Moreover, the authors claim that all sRNAs are expressed from their native 5' ends. Have 5' ends have really been mapped in all of these cases, for example, for IS118? Whereas Zhang, 2003, Mol Micro proposed a size of 298 nt, the authors claim here that IS118 is 194 nt long (Table 1). Moreover, IS118 is located downstream of yfdI in the E. coli K12 genome and not yfdL, as indicated in Table 1.*

Response: The basis for suggesting that this is close to saturation, even though we agree that a limited number of growth conditions have been examined, is that for those sRNAs that we know are hard to detect by Northern blot (OxyS, for instance), Hfq-immunoprecipitation significantly enriches them, suggesting that we can detect even low basal levels by this method. Thanks for catching the error in Table I. Clones do reflect mapped 5' ends. The 5' end of IS118 has been mapped in our lab; this is now indicated as a personal communication in the Table.

- *Page 13, line 17-18. The conclusion presented by this sentence is hard to understand. Do you mean titration effects by ArcA binding sites carried on the 8 plasmids? Please clarify.*

Response: The first sentence in this paragraph was expanded to make it clearer, and the final sentence deleted.

- *Page 16, line 25. If there indeed is clear-cut evidence for "colliding transcription of arcB and arcZ", this should be elaborated on in the Discussion section.*

Response: We're not sure what the referee is asking for here; we've changed the wording to indicate there is overlapping transcription (the ORF of *arcB* ends within *arcZ*; mapping of the 3' end of *arcB* showed stops within *arcZ*, now shown in Fig. 4A). We do discuss the effects of this feedback loop in the discussion (p. 21).

- *Figure 6A. How exactly were arcB mRNA levels quantified? The values given below some of the lanes, especially 14 and 15 versus 19 and 20, are hard to reconcile with the presented blot images.*

Response: As stated in the figure legend, levels of the RNA species were quantified using SsrA for normalization. One can clearly see that in the particular experiment represented in figure 6 B, less RNA was loaded in lanes 19 and 20 as compared to 14 and 15 when looking at SsrA levels, thus explaining why there is more arcB in lanes 19 and 20 than what one would think just comparing the two bands. The quantification was not always done from the gel exposure shown in the figures.

- *Figure S1. Include size markers on the individual blots.*

Response: Size markers positions have now been added to the figure.

- *Figure S2A. Please elaborate why a C561G mutation of the rpoS mRNA should impair its translation. The mutation does not interfere with the core of the ribosome rpoS binding site (AGGAGC) and the paring nucleotide on 16S rRNA would be a U, thus one would expect translation to increase rather than decrease.*

Response: We do not really know why this is, but the decreased translation is quite clear. Possibly this change leads to interactions with other parts of the rpoS mRNA, blocking the RBS.

Referee 2: Comments and suggestions:

1. p. 6, l.12: Thirty nucleotides of coding region in a translational reporter fusion do not necessarily capture ALL effects exerted by small regulatory RNAs, which can also bind further downstream and still interfere with translation/degradation of the mRNA. So, one should keep in mind that the analysis presented here is not necessarily exhaustive even for the tested RNAs.

Response: We agree. The limits of what we are measuring is now noted, with a phrase added at the end of this section.

2. p. 6, l. 18 ff: the pBAD promoter responds to inducer in a non-linear fashion, i.e. this promoter is either OFF or ON and intermediate inducer levels result in population heterogeneity with the fraction of cells in the ON state depending on inducer concentrations (see Siegele and Hu. 1997. PNAS USA 94: 8168-72). "Basal activity" thus means that only a fraction of the cells is in the ON state. Nevertheless, the approach chosen here works because rpoS::lacZ mRNA made in these ON cells is still translationally inhibited because of intramolecular basepairing that can be affected by small RNAs. But it may be worth checking these rpoS::lacZ cells under the microscope for population heterogeneity and keep in mind that sRNAs tested could also somehow change the expression of the pBAD promoter. An important control is therefore also to check effects with a pBAD::lacZ construct not containing any rpoS sequences.

Response: As noted above (referee 1, point 2), a control was added showing results with a pBAD-lacZ fusion, as suggested (Fig. S3), under the same growth conditions. The effects on the rpoS::lacZ fusion are also shown after normalizing to the control fusion.

3. In general, when cells are tested in "stationary phase", what exactly does this mean? When cells have reached a certain OD or overnight cells?

Response: We have now indicated in the figure legends what the OD was, or that overnight cultures were used.

4. P. 9, Fig. 3: all the data presented here were obtained with rpoS::lacZ fusions. At least the effect of the knockout mutations of arcZ and the other small RNAs involved should be shown in one experiment also for wildtype rpoS mRNA (e.g. in a Northern blot).

Response: The Western blot (originally in Fig. 5, now in supplemental Fig. S6), was meant to address this issue for the conditions the paper focuses on, the influence of ArcZ sRNA. We have

also now added ArcZ effects on a different fusion that is degraded and transcriptionally regulated as is *rpoS* (Fig. 5D). We have not done a Northern blot for rpoS. In other work in our lab, we do not find a good correlation between rpoS mRNA levels and RpoS translation. For instance, when mutations in ribonucleases are examined, mRNA levels are high but protein does not increase proportionately; the sRNAs are necessary for high translation. Therefore, a Northern blot would not really address the issue the reviewer is raising. The fusion allows us to isolate the effects of the sRNAs on translation from indirect effects on either the promoter or on RpoS turnover, and therefore, we think this is most useful for evaluating the relative and direct contributions of the chromosomally encoded sRNAs.

5. Fig. 4: Where is the terminator for the arcB gene? This is important to know in order see how much (complementary) overlap there is between ArcZ and arcB mRNA.

Response: We do not see a clear terminator, although possibly the stem-loop terminator of arcZ also acts as a terminator for arcB. We have now included in Fig. 4 our results from mapping the 3' end of arcB, suggesting an overlap of 25-28 nt.

6. Fig. 4: The sequence supposed to be an ArcA box in the arcZ promoter region does not really match the ArcA box consensus very well. Before drawing the conclusion that ArcA binds directly, it seems safer to do either a footprint experiment in vitro or to introduce point mutations that (i) deteriorate, and (ii) improve the putative ArcA box and measure the corresponding effects on the expression of the arcZ::lacZ fusion in vivo.

Response: We agree that this is not a particularly strong ArcA site (and based on mutational analysis, we have shifted the predicted site slightly). Mutations were made in the putative site (new Fig. S5) but did not significantly disrupt ArcA repression; one mutation improved it. The text now reflects the uncertainty about direct vs. indirect regulation by ArcA.

7. Fig. 4C: Induction of ArcZ during transition into stationary phase suggests that ArcZ is involved in the increased rpoS translation observed during the post-exponential phase (see Lange et al. 1994. Genes & Dev. 8: 1600-12). This should be tested with the arcZ mutant (just follow rpoS::lacZ activity along the growth curve). Moreover, it would be consistent with ArcB becoming inactive not only by aerobiosis but also in energy limited cells, since ArcB is oxidized (inactivated) by oxidized quinones, which arise not only in response to high levels of oxygen (increased efflux of electron from the respiratory chain) but also in response to energy limitation (reduced influx of electron into the respiratory chain) (see Mika et al. 2005. Genes & Dev. 19: 2770-81); this impact of energy limitation (in addition to +/- oxygen) may also be integrated into Fig. 7.

Response: We have added an examination of a rpoS::lacZ fusion at points throughout the growth curve, as a new panel in Fig. 5 (5D), comparing arcZ- and arcZ+ cells. We find the largest effect of ArcZ as cells emerge from stationary phase (low OD). We have moved the Western blot in this figure into supplemental material.

8. The arcZ promoter looks as if it could also be activated by RpoS-containing RNAP (in particular, it has a -13C). If so, arcZ induction during entry into stationary phase would also be one of several positive feedbacks that make RpoS induction more robust. This could easily be checked by measuring arcZ::lacZ also in an rpoS mutant background.

Response: This had been tested, both with the fusion and by looking at ArcZ sRNA; no effect of an rpoS mutant was seen. The lack of an effect is now mentioned in the text (p 11).

9. Table II could be shown in the Supplement, as the results presented there leave a lot of open ends and the effect of ArcB and ArcA on arcZ expression is then directly shown in Fig. 5.

Response: We prefer to retain this table in the text. We agree there are unanswered questions, but this shows that all of the negatively regulating plasmids act upstream of an arcA or arcB mutation, reinforcing a possible direct role of ArcA.

10. P. 15, l. 11 ff: The finding that RpoS is reduced in the arcA mutant in stationary phase, or, to put it the other way round, that ArcA contributes to RpoS accumulation in stationary phase, has been

reported and explained before (see again Mika and Hengge, 2005, Fig. 6 therein): arcA expression is further stimulated by RpoS-containing RNAP, increased ArcA scavenges phosphate from ArcB at the expense of RssB phosphorylation (because ArcA phosphorylation by ArcB is about 10fold more efficient than that of RssB), which results in increased stabilization of RpoS - in fact the arcA mutant is shown to have a shorter half-life of RpoS in stationary phase. This RpoS/ArcA effect is one of the several positive feedbacks that stabilize high RpoS levels in *stationary phase.*

Response: Thank you for pointing this out. A reference to that work has been added to the paper in place of the previous statement. We note that those effects should be seen only when the other levels of RpoS regulation (regulated degradation) are intact, true for Fig. 5D and the Western blot, not true for Fig. 5C, so our data is quite consistent with this.

11. The "anaerobic/aerobic sensing" mentioned in the title is the least well documented aspect of this study. I would suggest to think about this title again.

Response: We prefer to leave the title as is.

Minor comments:

1. p. 3, l. 7 from bottom: The positive role of Hfq in rpoS translation was also described by Muffler et al. (1996. Genes & Dev 10: 1143-1151) in a publication that actually appeared earlier than Brown and Elliott (1996).

Response: Reference added.

2. Fig. 2A: it may be helpful to also label the ribosome binding site

Response: This has been done.

3. Fig. 2C: "Actifity"

This figure has been replaced.

4. Fig. 5D: replace ".65" by "0.65" (in +O2, arcA-)

This has been corrected; the figure is now supplementary (Fig. S6).

Referee 3:

Specific comments:
1) It is likely that the shortest 55 nt form of ArcZ is active because it accumulates in cells and contains the sequence complementary to the rpoS. But, it is not proved experimentally. It is easy to test this by expressing only 55 nt piece of ArcZ by deleting the 5' portion of arcZ gene.

Response: Yes, we do believe that the short form is active, and have demonstrated this by cloning the short form, as suggested. This was reported in a recent publication (Soper et al, PNAS 2010); this result is now mentioned in the text.

2) It was shown clearly that ArcA/ArcB system causes a marked reduction in ArcZ expression only in anaerobic condition and that arcA deletion abolishes this repression (Fig. 5A and B). There are discrepancies between lacZ assay and Northern analysis. Under aerobic condition, the arcA knockout causes a moderate increase in AcrZ expression in lacZ assay while it seems to cause a slight reduction in Northern analysis. In addition, the ArcZ expression is significantly higher in aerobic compared to anaerobic conditions in lacZ assay while the situation is just opposite in Northern analysis.

Response: We do not have a simple explanation for these differences. Of course, the promoter fusion only reflects activity of the promoter of ArcZ whereas Northern blots reflect actual levels (reflecting promoter activity and stability of the sRNA), but given the stability of this sRNA, we do

not expect large differences due to this. We note that the Northern blot in Fig. 5B was not precisely quantitated (normalized to another RNA, for instance), so that it may be best to interpret the general trends (down anaerobically, up in an *arcA* mutant).

3) The effects of arcA and/or arcZ deletion on RpoS expression are quite confusing. Although authors claim that AcrA negatively regulates RpoS expression through ArcZ expression only under anaerobic stationary condition, Western analysis of RpoS shown in Fig. 5D indicates that AcrA down-regulates RpoS expression through ArcZ expression also under aerobic exponential growth condition.

Response: I think we would say that the effects are more dramatic anaerobically, but as the referee suggests, there clearly are effects of both ArcA and ArcZ aerobically as well. We have tried to indicate this by rephrasing some of the text. Additional experiments with an rpoS-lac fusion (in addition to the pBAD-rpoS-lacZ fusion) help to point out the complexity of the interactions, some of which have been noted before (Mika and Hengge, 2005).

4) I am also confused again with a discrepancy between rpoS-lacZ assay and western analysis. The arcA mutation does not affect the expression of rpoS-lacZ under aerobic stationary condition (Fig. 5C) while it reduces RpoS expression in Western analysis (Fig. 5D).

Response: This discrepancy presumably reflects the multiple levels at which ArcA appears to regulate RpoS (see Mika and Hengge, 2005). The fusion in Fig. 5C has the pBAD promoter, not the native rpoS promoter, and is not subject to RssB-dependent degradation, as RpoS itself is. Therefore, the differences between these two assays may well reflect the combination of other levels of ArcA regulation. Some additional experiments have been added in response to comments by referee 2, using a fusion that reflects all levels of regulation, and the text has been clarified to make the differences in the assays clearer, as well as to discuss the complexity of the system.

5) It was shown convincingly that ArcZ expression markedly destabilize the arcB mRNA in Fig. 6B and C. Although the authors argue that arcB mRNA expression does not affect the stability of ArcZ, the level of ArcZ is also significantly reduced by arcB expression. For me, both RNAs seem to be degraded in a coupled manner.

Response: We agree that the levels of processed ArcZ decrease in Fig. 6B. However, we do not see any evidence for turnover of the processed ArcZ RNA (Fig. 6C). One interpretation of this is that the full length ArcZ is degraded with arcB in a coupled manner, reducing the amount of precursor for the processed ArcZ. However, once processed, the ArcZ RNA is stable. We have tried to clarify this in the text.

6) It is interesting to know whether ArcZ is also able to destabilize the arcB mRNA expression when it is provided in trans. It is possible that ArcZ expression affects the arcB mRNA expression only when two genes are overlapping on the same DNA.

Response: We agree this would be of interest, and in a preliminary experiment see evidence of a trans effect, but have not pursued this further.

2nd Editorial Decision 06 July 2010

I have received the below comments from the one referee who I asked to review your revised manuscript. The referee finds that you have satisfactorily addressed all the original concerns and recommends publication in The EMBO Journal. I am happy to accept the paper for publication and you should receive the official acceptance letter in the next day or so.

Best wishes,

Editor
The EMBO Journal

Referee #2

The manuscript has now been thoroughly revised taking properly into account the comments made by all referees. Taken together, this study presents a new small RNA acting in the translation of the the important global regulator RpoS. What I particularly like about this work, is that it not only presents a biochemical mechanism but places this mechanism into a complex physiological context. Overall, I recommend publication.