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Functional determinants of the quorum-sensing non-coding RNAs and their roles in target regulation

Yi Shao, Lihui Feng, Steven T. Rutherford, Kai Papenfort and Bonnie Bassler

Corresponding author: Bonnie Bassler, HHMI and Princeton University

Review timeline:

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Editorial Decision:	13 May 2013
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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 13 May 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 great interest in the findings reported in your manuscript. However, they also raise a number of minor points - mainly related to the data presentation and the phrasing of specific sections - that you will need to address in full before submitting a revised version of the manuscript. I would like to emphasize that we from the editorial side agree with referees #1 and #2 that changing figures 1 and 2a to a table would make the data presented more easily accessible to the reader. Furthermore, we suggest you to follow the recommendation by referee #2 to shorten the discussion to enhance the focus on the RNAs addressed in the current study.

Given the referees' positive recommendations, I would like to invite you 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.

When preparing your letter of response to the referees' comments, please bear in mind 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, 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS:

Referee #1:

In this paper from the Bassler lab, Shao et al. have performed the first global search for new mRNA targets of the Vibrio Qrr sRNAs, followed by very comprehensive analyses of the functional architecture of these sRNAs and of base pairing requirements for successful target recognition. Using a sRNA pulse-expression approach and target validation with gfp reporter genes, they validate 16 new mRNA targets. Systematic mutational analysis of the Qrr sRNA reveals important subregions that are required for different functions, such as seed pairing with mRNA, protection against RNase E-mediated decay and association with Hfq protein.

The very similar Qrr sRNAs whereof the various Vibrio species seem to possess four to five gene copies, have become a model system in the quest to understand the general principles of post-transcriptional circuits that include regulatory small RNAs. Whereas the authors' previous work much focused on Qrr-mediated control of two crucial transcription factors in quorum sensing, and the underlying feedback loops, the new work significantly expands the set of known Qrr targets to now include many other genes (mRNAs) whose product may be directly involved in quorum sensing. The work also significantly contributes to a growing understanding of the structural constraints of regulatory small RNAs in bacteria. That is, while we used to think of these seemingly heterogeneous RNA molecules (lacking clear common denominators for length and structure) as strings of nucleotides that fortuitously select targets through extended base pairing interactions, the present work substantially supports a view that these regulatory molecules possess defined functional modules.

The data is of high quality and the manuscript is well written. I have few comments that should be addressed prior to publication.

1. Page 6, second para, validation with gfp fusions: The authors write that four candidate targets could not be validated with the fusion approach, because either these were indeed no targets or the Qrr sites lay outside the cloned regions. For clarity, tell the reader here what was included in the fusions.

2. Page 13, second para, first sentence: the authors write that one presumed advantage of sRNA regulation over protein transcription factors is a rapid response to external stimuli. With the many regulations by sRNAs investigated thus far, has there been experimental evidence that the difference in production time (a few seconds for a sRNA, perhaps a minute for a regulatory protein) is indeed relevant for stress responses? The sentence needs a reference(s) or should be rephrased.

3. Page 14, first para: I disagree with the authors' general statement that 'positive regulation by sRNAs is slow when compared to negative regulation'. At first glance, it seems true that mRNA repression can (and often does) result in faster changes of steady-state levels than does mRNA activation by passive stabilization, because the repression may involve accelerated degradation through active recruitment of RNase E. However, the important event in regulation is the change in target protein levels, and the situation here may be entirely different: one can expect the protein of

an activated mRNA to accumulate without delay, whereas how quickly the protein of a repressed target disappears will much depend on the protein's intrinsic half life. If a stable membrane protein, this may take very long (much longer than in activation), no matter how quickly the mRNA is degraded. A recent paper on sugar stress control in Salmonella indeed shows that mRNA activation may achieve a more immediate regulatory outcome than the concomitant mRNA repression by the same sRNA (Cell 153(2):426-37). The authors may want to briefly elaborate on this here or in the Discussion.

4. Page 17, line 9: correct microRNA length to ~22nt.

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Bassler and coworkers have done an extensive investigation of the broader set of genes regulated by the well-studied Qrr sRNAs, identifying a large number of new targets and defining the regions within the Qrrs necessary for regulation. This provides an important extension of the picture of the Qrr network.

1. Fig. 1, text: It was somewhat confusing to try to compare text as the Qrr4 sRNAs and Figure 1 in terms of the specific targets and how they behave. It might be better to present the parallel experiments first with Qrr4 and then the others right away, before going through the outcomes in any detail. That would allow a simpler discussion of the total range of positive and negative targets.

2. Fig 2, p. 6, p18: target 02446: This target is interesting but not really presented sufficiently for it to be clear what is happening. The data presented is that there is activation (at the level of the mRNA level) in Vibrio but repression in E. coli. On p. 18 (Discussion), this is interpreted as stabilization but sequestration, with a statement that the protein is not increased. Was that actually measured, in Vibrio, or is this conclusion from the GFP fusion in E. coli? If it was measured in Vibrio, that data needs to be included, and if it has not been measured in Vibrio, it should be before this sequestration model is presented. As is, it is not possible to distinguish between differences due to organism (a specific translational repressor in Vibrio?) or a difference between protein and mRNA.

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7. The discussion is probably longer than it needs to be; it reads a bit more like a review of sRNA regulation than is needed here. For instance, the section on stabilization by the 5' SL could be significantly shortened; while no one may have published that the 5' SL of a sRNA stabilizes, this is certainly not at all surprising.

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The manuscript by Shao and colleagues describes a very interesting study of the five small RNAs of Vibrio harveyi whose expression is induced by LuxO-P, and which activate AphA, and inhibit translation of LuxR, LuxO, LuxM, and LuxN. In the current study, sixteen new target genes were identified. The finding that these genes were regulated in E. coli indicated that regulation is direct. Direct interactions were also confirmed by making point mutations that decrease base pairing with target genes, showing defects in regulation, and the suppressing these defects with compensatory mutations in the target genes. The roles of four stem loops were explored by removing them, or by inverting the stems. Finally, the kinetics of induction of these small RNA's was compared to that of their direct and indirect targets. These data significantly advance the field, and are suitable for publication. I have only a small number of specific comments.

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1st Revision - authors' response 24 May 2013

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regulatory small RNAs in bacteria. That is, while we used to think of these seemingly heterogeneous RNA molecules (lacking clear common denominators for length and structure) as strings of nucleotides that fortuitously select targets through extended base pairing interactions, the present work substantially supports a view that these regulatory molecules possess defined functional modules.

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As suggested by the referee, we added the sequence information included in the fusions.

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As suggested by the referee, we changed “expression” to “mRNA level” to clarify.

4. Page 17, line 9: correct microRNA length to ~22nt.

As suggested by the referee, we changed to ~22nt (although it says ~23nt in the cited review).

5. What is the purpose of Figures 1 and 2A? Neither is terribly informative, whereas it would help the reader to have the corresponding information of target regulation with the native mRNAs (Fig. 1) or the fusions (Fig. 2A) listed side by side in a table in the main manuscript. The table should also include the putative gene functions and general processes these target are involved.

As suggested by all the referees, we combined Figure 1 and Figure 2A, and we also added a table to the main manuscript.

Referee #2:

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With respect to the referee, we prefer to maintain the original order since all of our analyses to determine base pairing stemmed from the Qrr4 induction array. We believe it is a more logical path to have these two sections together before going into the other Qrrs.

2. Fig 2, p. 6, p18: target 02446: This target is interesting but not really presented sufficiently for it to be clear what is happening. The data presented is that there is activation (at the level of the mRNA level) in Vibrio but repression in E. coli. On p. 18 (Discussion), this is interpreted as stabilization but sequestration, with a statement that the protein is not increased. Was that actually measured, in Vibrio, or is this conclusion from the GFP fusion in E. coli? If it was measured in Vibrio, that data needs to be included, and if it has not been measured in Vibrio, it should be before this sequestration model is presented. As is, it is not possible to distinguish between differences due to organism (a specific translational repressor in Vibrio?) or a difference between protein and mRNA.

As suggested by the referee, we clarified the discussion text regarding repression by the Qrr sRNAs and how we measured this in *E. coli* using GFP fusions. Indeed, since we have not measured protein levels in *V. harveyi*, the sequestration model needs to be further studied and so we toned down that text.

3. What constitutes a significant level of regulation? 05020 looks very slight in Fig. 2.

VIBHAR_05020-GFP is repressed ~1.5-fold. Although the fold-change is less compared to the change we observed in the microarray analysis (~3-fold repression), the level of regulation is valid since the experiments were performed in triplicates with controls.

4. Fig. 3, Fig. S4; The assumption in this discussion of Qrr3 is that there is competition for targets, and that the pairing region is conserved between Qrrs. It would be helpful to state this clearly, if it is true, and this could be a transition to Fig. 4.

We have not examined completion so we hesitate to add text stating such a mechanism.

5. Fig. 5: It would be useful to remind the reader that SL1 is the one that differs between Qrr1 and

the other sRNAs at the first mention of this deletion; as is this doesn't get discussed until panel C is presented.

As suggested by the referee, we added one sentence to remind the reader.

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As suggested by the referee, we shortened the discussion.

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Use Line Numbers!!!

Page 7, line 8. I would soften the word "eliminated" as there was a residual effect.

As suggested by the referee, we changed the word “eliminated” to “substantially reduced”.

Page 7, line 18. Start a new paragraph here?

As suggested by the referee, we began a new paragraph with this line.

Page 8. This section describes pulse expression of qrr1 2, 3, and 5 and shows that they regulate the same targets as qrr4. However, qrr1 is not described in this section. In Table S1, there are a lot of blanks for qrr1. Why is this? I suggest showing the fold induction for all genes in Table S1, not just those that are over 2 fold. Was there any reason to leave qrr1 out? Why is qrr5 left out of Fig S1A? The legend says that a northern was used because qRT-PCR wasn't sensitive enough, but this does

seem counterintuitive.

In the manuscript, Qrr1 is discussed in the paragraph immediately following the paragraph the referee refers to. Qrr1 is treated separately under its own heading because it behaves differently from the other Qrr sRNAs. Regarding Table S1, Qrr1 only regulates a small number of genes likely because its in vivo preferences for targets are different than the other Qrr sRNAs. As suggested by all the referees, we combined Figure 1 and Figure 2A, and we also added a table to the main manuscript. The reason to use Northern blot for Qrr5 is because the PCR of this Qrr was unsuccessful. We changed the figure legend to convey this point.

Page 13. It's not terribly surprising that early steps in a transcription cascade respond to a stimulus more rapidly than later steps. If a truly rapid response were needed, LuxO could directly regulate a target gene directly. I should know this by heart, but does LuxO have direct targets other than qrr1-5?

As far as we know, LuxO only binds to its own promoter in a negative feed-back loop and the *qrr*1-5 promoters.

Page 17, line 2. "are diverging" sounds overly informal. Perhaps "may continue to diverge" would work better.

As suggested by the referee, we made the change.

Figure 1. I think figure 1 and figure 2A might be regrouped as Fig 1A and 1B.

As suggested by all the referees, we combined Figure 1 and Figure 2A, and we also added a table to the main manuscript.

2nd Editorial Decision 04 June 2013

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by one of the original referees whose comments are shown below. As you will see this referee finds that all original criticisms have been sufficiently addressed, and I am therefore pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE REPORT

Referee # 2:

This revised manuscript reports a comprehensive study of the role of the Qrr RNAs in regulation in Vibrio. The results will be of general interest both to those interested in regulatory circuits in Vibrio and those interested in small regulatory RNAs and how they work. I do not see anything that requires further revision.