molecular systems biology

Active Regulation of Receptor Ratios Controls Integration of Quorum Sensing Signals in Vibrio harveyi

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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 13 March 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who accepted to evaluate the study. As you will see, the referees find the topic of your study of potential interest. They raise however a series of concerns and make constructive suggestions for modifications, which we would ask you to carefully address in a revision of the present work.

In addition to our capacity to host datasets in our supplementary in formation section, we provide a functionality on our website, which allows readers to directly download the 'source data' associated with selected figure panels (eg <http://tinyurl.com/365zpej>), for the purpose of alternative visualization, re-analysis or integration with other data. These files are separate from the traditional supplementary information files and are directly linked to specific figure panels.

In the case of this study, we would kindly ask you to submit the 'key quantitative experimental data displayed in the figures panels of the present study as individual 'source data files'*. We provide below some general guidelines with regard to the format of such data tables.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the

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scientific community. More information about this initiative is available in our Instructions to Authors.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor

Molecular Systems Biology

REFEREE REPORTS:

Reviewer #1 (Remarks to the Author):

This manuscript explores how feedback shapes the response to two quorum sensing signals in Vibrio harveyi. In the first part of the paper, the authors identify a feedback loop where the Qrr sRNA represses the expression of LuxM and LuxN. Overall, the results here are compelling and the discussion is easy to follow. In the second part of the paper, the authors explore the integrated response to two quorum sensing signals, AI-1 and AI-2, in different feedback mutants, including the newly identified LuxN loop. The data presented in this section are particularly rich and a number of interesting observations are made. The key points are that these loops affect signal integration by expanding the input range and compressing the output range through altered receptor ratios.

Even though I do not actively follow the quorum sensing field, I found the problem to be very interesting and the results significant. I suspect these results will also be interesting to others outside the field. My major criticism is that some of the arguments are difficult to follow, particularly in the modeling section. I suspect that these issues can easily be addressed with some editing and reorganization.

Comments:

1. Why was the Qrr4 the only sRNA investigated? Was this the only one where a complementary sequence is found? A short justification would be useful.

2. You might comment on Page 7 that LuxOD47E is a phosphorylation mimic. While you point this out on the previous page, you also remind the reader on page 7 that phosphorylation of LuxO is necessary for Qrr expression.

3. Each panel in Figure 4 contains 5x5 experiments. I question how much information can be obtained from the contours from such a coarse set of measurements. In particular, how strongly are the contours affected by the interpolation algorithm? It might be helpful to provide the "raw" data as a 3D bar graph.

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4. You should also comment of how reproducible, from day to day, these data in Figure 4 are? Ideally, this information would be provided as errorbars say in the 3D bar graph mentioned above. The same goes for Figure 5.

5. Swap Figures 5 and 6?

6. On page 15, is it really reasonable to say a Hill coefficient of 2 represents a "sharp on-off switch". There is really no need to exaggerate your arguments here.

7. Top of page 18, "Figure 4N" should be "Figure 4O"

8. Page 18, the arguments regarding the positive feedback here are confusing to me. I get what you are saying after multiple reads. I just think that you could state it more clearly.

9. I found the arguments in the modeling section to be confusing. Why not just plot the inferred receptor ratios in Figure 7 instead of having the reader attempt to divine them from the contour slopes?

10. I did not understand the arguments on pages 24-26? For one, I do not see from Figure 4B how you can state "the cells pay more attention to AI-2 than AI-1 at low cell density and then pay more attention to AI-1 than to AI-2 at high cell density". The response looks symmetric to me.

Reviewer #2 (Remarks to the Author):

This paper is a remarkably thorough study of the effects of several feedback loops on quorum sensing in V. harveyi. Four feedback loops were previously described for the system. This paper starts out by identifying a fifth loop, in which the luxMN operon is repressed by Qrr sRNAs. Using a previously developed technique to quantify fluorescent protein levels in growing cells, the authors characterize the input-output relations for a "wild-type" strain and mutants that lack various feedback loops. They also show that the data can be interpreted in terms of a simple model of the quorum sensing network. Impressively, reparametrization of the input variables produces approximately linear contours with slope -1, as predicted by the model. The primary conclusion from this work is that the feedback loops regulate the input and output dynamic ranges. However there are many additional interesting observations, including the effects of these loops on noise. Overall I found the results clearly presented and convincing. This work will be of broad interest to the systems biology community and should be published in Molecular Systems Biology.

Specific Comments

1) A new feedback loop controlling luxMN expression is demonstrated using Qrr4. The text seems to imply that the loop is controlled by all of the Qrrs (e.g. end of first paragraph on p. 11). Has this actually been shown for the other Qrrs or this assumed to be the case based on sequence similarity?

2) p. 13 bottom. Why is the kinase activity of the constitutive LuxN reduced only at very high AI-1 levels (10-100uM)? It is stated in the text that 1 uM of AI-1 is saturating (the KI is estimated to be 6.9 nM on p. 21). At 1 uM AI-1, shouldn't essentially all of the LuxN be in the phosphatase state?

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3) On p. 14 it is stated that "LuxR ... is slowly diluted by cell growth" but on p.28 it is stated that this was shown for HapR and "Assuming the same behavior for LuxR in V. harveyi". Has it been shown in V. harveyi? And if not, shouldn't this be easy to do with the LuxR-mCherry fusion?

4) p. 18 second paragraph - it is stated that when LuxN is a kinase, disruption of the LuxN loop increases noise in the five loop mutant compared with the LuxOLuxR loop mutant. However it looks like the noise in the lower left corner of FIg. 4S is lower than the noise in the lower left corner of Fig. 4K.

5) p.18 bottom - For the low AI-1 limit, it is noted that a negative feedback effect on noise is not observed when comparing the LuxN loop mutant to the WT loop strain. Is there an explanation for this? Does this call into question the interpretation higher up on the page for the case of high AI-1, where it was argued that the noise is explained by a positive feedback loop?

6) p.29 top "positive feedback leads to phenotypic heterogeneity". The referenced paper is a theoretical work. However experimental studies of positive feedback in at least some two-component systems have demonstrated that these systems do not show phenotypic heterogeneity.

7) Figure 2B, right most column - why did deletion of qrr1-5 cause almost a 2-fold drop in luxN mRNA for the strain with a BP- mutation?

8) p. 25 VarS/VarA system affects activity of LuxO - Is there a reference for this?

9) p. 9 - reference to Figure S4 should be S3?

10) p.10 middle "Figure 4C generalizes a specific result exhibited in Figure 4C"

should be "Figure 4C generalizes a specific result exhibited in Figure 3C"?

11) p.18 bottom - references to Figures 5S, 5K, 5O, and 5C should be 4S, 4K, 4O 4C?

Reviewer #3 (Remarks to the Author):

This manuscript describes the impact of several feedback loops on the QS circuitry of V. harveyi. In particular, they describe using knockout mutants and a very simple mathematical model, the impact of the regulatory architecture on the suppresion of noise and the fidelity of the ultimate signal. This system is very interesting as there are several signal inputs that converge on a singular well-described output. Thus, it is relevent to understand how the signal transduction process is regulated as stimulated independently and simultaneously using the two autoinducers, AI1 and AI2.

The manuscript is interesting, the work is well done, and the conclusions seem to be consistent with the data. The model supports the conclusions, hence the outcomes are reasonable and explain QS phenomena in this system.

This reviewer has several concerns, however, that when addressed will strengthen this manuscript:

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1. Introduction: Discussion of insulin signaling as an example of signaling, second messenger, and neurodevelopment is not relevant to QS systems. Suggest modifying signaling introduction to QS or other bacterial systems. There are enough examples of bacterial systems to recast the introduction so that readers will understand better how this work fits into the greater context of bacterial signaling.

2. Page 6, because they did not find an independent start site for luxN does not suggest it is monocistronic. This should be relatively easily found. This should be discussed or dropped.

3. Page 7, the efforts to demonstrate a repressive feedback loop between Qrr sRNAs and luxMN transcript are well designed and carefully executed. Nice work.

4. Throughout - it is important to characterize the fluorescence intensity over time. How long does the response take and what sensitivity is there towards process time? My guess is the AI-1 and AI-2 pathways may control noise and signal fidelity as suggested, but this is not conclusive without discussion of the mCherry data over time. That is, does the AI-1 signal change with different time constants than the AI-2 system? This is not addressed at all. Instead, the authors describe mCherry expression as a fixed output of the AI-1 and AI-2 concentrations. This is not at all clear and is likely not the case.

5. Page 14 - transition from HCD to LCD? Under what circumstances is this a natural system or germane to the QS concept? This should be justified with real examples or circumstances. Cells that are expelled from a biofilm are likely radically modified due to other factors than QS systems and signaling. Instead basic nutrient levels are likely to dominate behavior. I'm not sure of the value of this section or of the results using this transition. Please justify or remove.

6. Page 16 - Noise. It would be interesting to validate that the fluorescence measurement for a cell in an optical image is constant and not dependent on focal plane or other factors.

7. Page 25 - I like the discussion of the relative weight of the AI-1 and AI-2 signaling pathways.

1st Revision - authors' response 18 April 2011

We are submitting a revised version of our manuscript entitled, ‘Active Regulation of Receptor Ratios Controls Integration of Quorum Sensing Signals in Vibrio harveyi.’

First, we would like to thank you for obtaining the very thoughtful and useful reviews. We would also like to thank the reviewers for their careful reading of our manuscript. We believe that their detailed comments and extensive questions reflect the general interest of our work. We have carefully read and considered each of the comments and questions of the reviewers, and have addressed all of their points both below and in the text. With these improvements, we believe that the manuscript is suitable for publication in Molecular System Biology.

Overall, the response of the reviewers was very positive. The first reviewer notes "*the problem to be very interesting and the results significant", and states that "these results will also be interesting to others outside the field."* The second reviewer found *"the results clearly presented and convincing.",*

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and he/she "found this work will be of broad interest to the systems biology community." The third reviewer notes that he/she "found the paper is interesting, the work is well done, and the conclusions seem to be consistent with the data."

As noted in your letter, the reviewers requested that we provide source data and re-plot some of the existing data. To address the concerns of Reviewer #1, we have included the supplemental source data of Figure 4 and included a new Figure S8 to demonstrate that the reliability of the interpolation and reproducibility of the experiment. We also have included the best fits for the inferred receptor ratios as a new Figure S9 in the SI to help readers easily discern these ratios.

Below we reply to each point raised by the reviewers and indicate, where appropriate, how the manuscript has been revised. We hope you agree that the consensus of the reviewers was very positive, and that our response and revisions are thorough and complete. We believe these changes have improved the accessibility and impact of the manuscript. We hope that given the positive timbre of all three reviews, and the relatively simple changes required, that you will now find that the manuscript is suitable for publication in Molecular System Biology without the additional delays associated with return to the reviewers.

In the end of this document, we highlight the main findings of our study in the 'Article Highlights' section and we summarize the study in the "Standfirst Text' section. Thank you again for your helpful remarks.

Response to Reviewers

Reviewer 1-

1) Why was the Qrr4 the only sRNA investigated? Was this the only one where a complementary sequence is found? A short justification would be useful.

We have tested the ability of all of the Qrrs to regulate luxMN, and they all behave similarly. Qrr4 is the most strongly expressed of the set of Qrrs. Importantly, the Qrrs are redundant in their functions. Thus, we routinely use Qrr4 as the representative example for the Qrrs. We have noted in the text (pg. 7) that Qrrs1-3 also repress LuxM.

2) You might comment on Page 7 that LuxOD47E is a phosphorylation mimic. While you point this out on the previous page, you also remind the reader on page 7 that phosphorylation of LuxO is necessary for Qrr expression.

As suggested, we have included a reminder about LuxOD47E.

3) Each panel in Figure 4 contains 5x5 experiments. I question how much information can be obtained from the contours from such a coarse set of measurements. In particular, how strongly are the contours affected by the interpolation algorithm? It might be helpful to provide the "raw" data as a 3D bar graph.

We have uploaded all raw data to the MSB source data section, and given a pointer to this data in the caption to Figure 4 and in Sec. A.7 of Supplemental Information.

4) You should also comment of how reproducible, from day to day, these data in Figure 4 are? Ideally, this information would be provided as errorbars say in the 3D bar graph mentioned above. The same goes for Figure 5.

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We have added a section A.7 in SI to explicitly address interpolation and day-to-day variation.

5) Swap Figures 5 and 6?

We kept the order for Figure 5 and 6 for the following reason. We focused on the demonstration of dose-response curves (Figure 5) just after describing the input-output relationship (Figure 4) for the same five strains. After completing the discussion of these five strains, we introduced two more strains with receptors constitutively expressed (Figure 6). As a result, it will be logical to introduce Figure 5 before Figure 6.

6) On page 15, is it really reasonable to say a Hill coefficient of 2 represents a "sharp on-off switch". There is really no need to exaggerate your arguments here.

In page 15, we have changed ‘sharp on-off switch’ to 'on-off switch'.

7) Top of page 18, "Figure 4N" should be "Figure 4O"

We have changed the reference to Figure 4N to Figure 4O.

8) Page 18, the arguments regarding the positive feedback here are confusing to me. I get what you are saying after multiple reads. I just think that you could state it more clearly.

As suggested, we have rewritten the text on p. 18 to state the point more clearly.

9) I found the arguments in the modeling section to be confusing. Why not just plot the inferred receptor ratios in Figure 7 instead of having the reader attempt to divine them from the contour slopes?

We have included the best fits for the inferred receptor ratios as a new figure in the SI. While the qualitative trends in receptor ration are self-evident in the data, quantification of the exact receptor ratio is difficult due to noise and undersampling in the data. The receptor ratios are inferred from the slopes of lines of constant contours in Figure 7. In general, this involves computing derivatives of a noisy function and leads to quantitative errors. Nonetheless, general trends such as whether receptor ratios are increasing or decreasing are still easily discernable from Figure 7 and the new figure. For this reason, we have kept Figure 7 in the main text and added a new figure with a best fit for receptor ratios.

10) I did not understand the arguments on pages 24-26? For one, I do not see from Figure 4B how you can state "the cells pay more attention to AI-2 than AI-1 at low cell density and then pay more attention to AI-1 than to AI-2 at high cell density". The response looks symmetric to me.

We have added a note to remind the reader that our argument is base on Figure S9. Specifically, we use the Results subsection ‘Modeling the Network’ to support the argument for the regulation of the receptor ratios.

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Reviewer 2-

1) A new feedback loop controlling luxMN expression is demonstrated using Qrr4. The text seems to imply that the loop is controlled by all of the Qrrs (e.g. end of first paragraph on p.11). Has this actually been shown for the other Qrrs or is this assumed to be the case based on sequence similarity?

We have tested the ability of all of the Qrrs to regulate luxMN, and they all behave similarly. Qrr4 is the most strongly expressed of the set of Qrrs. Importantly, the Qrrs are redundant in their functions. Thus, we routinely use Qrr4 as the representative example for the Qrrs. We have noted in the text (pg. 7) that Qrrs1-3 also repress LuxM.

2) p. 13 bottom. Why is the kinase activity of the constitutive LuxN reduced only at very high AI-1 levels (10-100uM)? It is stated in the text that 1 uM of AI-1 is saturating (the KI is estimated to be 6.9 nM on p. 21). At 1 uM AI-1, shouldn't essentially all of the LuxN be in the phosphatase state?

While the value of KI for a single LuxN receptor is indeed a constant, estimated to be 6.9 nM, the effective KI to inhibit the total kinase activity of all LuxN is an increasing function of LuxN copy number. The copy number of LuxN is much higher in "WTLoop LuxN-on" strain than in "WT Loop" strain. As a result, the phosphorylation of LuxO via the kinase activity of LuxN is only reduced at a very high AI-1 level (~10uM) in "WTLoop LuxN-on" strain, compared to the low AI-1 level required in the "WTLoop" strain (7nM).

3) On p. 14 it is stated that "LuxR ... is slowly diluted by cell growth" but on p.28 it is stated that this was shown for HapR and "Assuming the sam behavior for LuxR in V. harveyi". Has it been shown in V. harveyi? And if not, shouldn't this be easy to do with the LuxR-mCherry fusion?

It was previously reported that, indeed, V. harveyi LuxR decreases slowly after removal of autoinducers, consistent with dilution by growth. We now write this more explicitly on p. 14 in the main text and include a reference citing the earlier finding.

4) p. 18 second paragraph - it is stated that when LuxN is a kinase, disruption of the LuxN loop increases noise in the five loop mutant compared with the LuxOLuxR loop mutant. However it looks like the noise in the lower left corner of FIg. 4S is lower than the noise in the lower left corner of Fig. 4K.

The point is well taken ñ there is no significant difference in noise levels between Fig. 4S and 4K where LuxN acts as a kinase and we have rewritten the discussion on p. 18 accordingly.

5) p.18 bottom - For the low AI-1 limit, it is noted that a negativefeedback effect on noise is not observed when comparing the LuxN loop mutant to the WT loop strain. Is there an explanation for this? Does this call into question the interpretation higher up on the page for the case of high AI-1, where it was argued that the noise is explained by a positive feedback loop?

As suggested, we have rewritten the discussion on p. 18.

6) p.29 top "positive feedback leads to phenotypic heterogeneity". The referenced paper is a theoretical work. However experimental studies of positive feedback in at least some two-component systems have demonstrated that these systems do not show phenotypic heterogeneity.

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To our knowledge there is currently no other experimental data except ours to show that a two-component system acts as a positive feedback mechanism. We think it is still valuable to mention the theoretical work.

7) Figure 2B, right most column - why did deletion of qrr1-5 cause almost a 2-fold drop in luxN mRNA for the strain with a BP- mutation?

We do not yet know why the RNA levels modestly decrease when the BP mutation is present. We are beginning experiments now to understand this observation.

8) p. 25 VarS/VarA system affects activity of LuxO - Is there a reference for this?

We now cite a reference regarding the VarS/VarA system on p. 25 of the main text.

9) p. 9 - reference to Figure S4 should be S3?

We have changed the reference to Figure S4 to S3.

10) p.10 middle "Figure 4C generalizes a specific result exhibited in Figure 4C" should be "Figure 4C generalizes a specific result exhibited in Figure 3C"?

The second reference to Figure 4C should be to Figure 1C, and we have changed it.

11) p.18 bottom - references to Figures 5S, 5K, 5O, and 5C should be 4S, 4K, 4O 4C?

We have corrected these references.

Reviewer #3:

1) Introduction: Discussion of insulin signaling as an example of signaling, second messenger, and neurodevelopment is not relevant to QS systems. Suggest modifying signaling introduction to QS or other bacterial systems. There are enough examples of bacterial systems to recast the introduction so that readers will understand better how this work fits into the greater context of bacterial signaling.

As suggested, we have added a relevant bacterial example. We prefer to keep the other examples to indicate the broader context of our study.

2) Page 6, because they did not find an independent start site for luxN does not suggest it is monocistronic. This should be relatively easily found. This should be discussed or dropped.

As suggested by the referee, we have removed this sentence since our data are suggestive but they do not conclusively prove the idea.

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3) Page 7, the efforts to demonstrate a repressive feedback loop between Qrr sRNAs and luxMN transcript are well designed and carefully executed. Nice work.

4) Throughout - it is important to characterize the fluorescence intensity over time. How long does the response take and what sensitivity is there towards process time? My guess is the AI-1 and AI-2 pathways may control noise and signal fidelity as suggested, but this is not conclusive without discussion of the mCherry data over time. That is, does the AI-1 signal change with different time constants than the AI-2 system? This is not addressed at all. Instead, the authors describe mCherry expression as a fixed output of the AI-1 and AI-2 concentrations. This is not at all clear and is likely not the case.

In this work, we studied the WT Loop and various signaling mutants exposed to defined levels of exogenous autoinducers for 14 hours prior to measurement. The phosphorelay system is fast, as is the Qrr production rate, so consequently LuxR concentration essentially follows AI concentration and approaches steady state on a timescale much faster than 14 hours. Moreover, it was shown in Long et al. that the AI-1 and AI-2 systems feed into the phosphorelay in a strictly additive way, so differential dynamics could only occur on the very fast timescales of ligand binding/unbinding and receptor switching. However, as the reviewer suggests the general dynamics of the network is indeed important and interesting. These questions, including how feedback onto receptor ratios and the temporal profile of AI signals affects the dynamics, will be central topics of a future study.

5) Page 14 - transition from HCD to LCD? Under what circumstances is this a natural system or germane to the QS concept? This should be justified with real examples or circumstances. Cells that are expelled from a biofilm are likely radically modified due to other factors than QS systems and signaling. Instead basic nutrient levels are likely to dominate behavior. I'm not sure of the value of this section or of the results using this transition. Please justify or remove.

It is a good question how other inputs like nutrition and oxygen impact the quorum-sensing response. Quantitative separation of the impact of nutrition level and quorum-sensing signaling will be the subject of future studies. Previously, it was demonstrated that quorum sensing promotes Vibrio cholerae exit from a biofilm, which is a basic feature of V. cholerae's survival strategy of rapid cycles of colonization and dispersal (Zhu and Mekalanos, 2003). As a result, the transition from high to low cell density is likely to be a fast process following disruption of a biofilm. We have included the reference for the example of V. cholerae on p.14.

6) Page 16 - Noise. It would be interesting to validate that the fluorescence measurement for a cell in an optical image is constant and not dependent on focal plane or other factors.

In the section Experimental Procedures (p. 32), we have added a description of the fluorescence calibration.

7) Page 25 - I like the discussion of the relative weight of the AI-1 and AI-2 signaling pathways.

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