THE EMBO JOURNAL

A conserved RNA seed-pairing domain directs small RNA-mediated stress resistance in enterobacteria

Nikolai Peschek, Mona Hoyos, Roman Herzog, Konrad U. Förstner and Kai Papenfort

Review timeline:

Submission date:	28 January 2019
Editorial Decision:	27 February 2019
Revision received:	8 May 2019
Editorial Decision:	30 May 2019
Revision received:	31 May 2019
Accepted:	9 June 2019

Editor: Stefanie Boehm

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.)

1st Editorial Decision 27 February 2019

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you will see, the reviewers appreciate your study and find that it has overall been well conducted. Nonetheless, they do raise some points, most of which however can likely be addressed by textual changes or adding to the discussion of a revised version. I would therefore like to invite you to prepare and submit a revised manuscript.

REFEREE REPORTS

Referee #1:

Papenfort and coworkers identify and characterize a new Sigma E-dependent sRNA RNA in Vibrio cholerae, here named MicV. Like a previous Sigma E-dependent Vibrio sRNA, VrrA, this sRNA represses a set of OMPs, and the two sRNAs overlap for some targets and each have unique targets. A conserved seed pairing domain is found in each of these sRNAs, explaining some aspects of the target overlap. Deletion of the two sRNAs leads to a number of phenotypes, including sensitivitiy to the sigma E-inducing treatment with ethanol. In an interesting approach, they carry out an evolution experiment on the seed region of a synthetic sRNA similar to MicV, selecting for ethanol resistance in the absence of Sigma E, and based on the results, provide evidence that repression of OmpA is the critical sRNA target for resistance. The work is generally clearly presented, and mostly complete. While the results do not really break new ground in our understanding of sRNA function, the global approaches, the use of an evolutionary selection, and the roles of this stress response in pathogens such as Vibrio all contribute to very nice piece of work.

1. Lines 133-136, Table S1: With respect to the identification of the Sigma E regulon in Vibrio, more information would be useful, some in a legend and some in methods. Is something listed as an "orphan" here, a site without an ORF nearby? Does that suggest multiple additional sRNAs (where is VrrA in this table?), to be investigated in the future? What were the criteria for associating the TSS data with a sigma E motif (distance, degree of match, any other information)? How many of these motifs are there independent of considering the start sites? Is it possible to add to Table S1 references for previously identified sigma E genes (in Vibrio, and possibly in other bacteria)?
2. Lines 262-263: For the genes in which there was no post-transcriptional control discovered, is there a reason to think that the promoters might be directly or indirectly controlled by Sigma E (and feedback control of Sigma E when the sRNAs are expressed).
3. The title of the paper and much of what is included emphasizes the similarity in seed region of VrrA, MicV, and RybB (of E. coli, Salmonella). However, I found it striking that while 8 targets were identified (Fig. 2) in which both sRNAs regulate, of these, there is really overlap in the pairing region for only three (ompT, vca0951, and rpoE). For the others, regions of pairing to the target are either distinct or overlap only by a couple of nucleotides. This would seem to suggest (assuming predicted pairing is accurate) that there are multiple ways of repressing these targets effectively but that repressing by both sRNAs is selected. Further comment on this may be appropriate (particularly in the context of the evolution experiments).
4. Selection experiments and OmpA: Please include in Fig. S7F the region of pairing of MicV and VrrA. Some of this section leaves holes that should be further addressed to better understand the power of this approach. As presented, the authors jump from showing that the top 15 sequences all give ethanol resistance to identifying OmpA as a target. The only really strong information to show this is the critical target that leads to ethanol resistance is that deleting ompA suppresses the sensitivity of deleting rpoE, and it is not possible to know if deleting any other OMP would do the same. For instance, is there something special about OmpA, or is it abundance of OMPs, with OmpA being a particularly abundant one? Would all of these sRNAs also down-regulate ompT, or only a subset of them? Possibly testing this set of 15 plasmids with some of the other targets in Fig.2 would provide a better sense of to what extent the selection process really picks out ompA specifically. Finally, is it feasible to make a change in the ompA pairing region in the native ompA gene and show that the appropriate evolved sRNAs that pair there no longer give ethanol resistance? Given that Fig. S7F shows multiple pairing regions, predictions should be fairly strong for such an experiment. What does such an ompA mutant act like in the wild-type rpoE+ context?
5. Is MicV the RybB homolog for Vibrio? Given a similar seed in the same region of the sRNA, can anything be said about whether these sRNAs likely evolved from a common ancestor?

Referee #2:

This work describes a very complete analysis of the functions of one small non coding RNA MicV in the pathogen Vibrio cholerae. Although many works have highlighted the roles played by sRNAs in response to envelop stress (ESR) in several Enterobacteriaceae, this study is highly original in the

sense that it has combined several in vivo approaches to gain knowledge on the regulatory networks involving two redundant sRNAs in response to ESR and for the first time laboratory selection experiments were developed to unravel specific sRNA signature used for regulatory functions. The authors showed the following data. (i) Vibrio cholerae MicV sRNA belongs to the Sigma E regulon; (ii) Target identification reveals that MicV shares many mRNA targets with the well known VrrA sRNA including mRNAs encoding Omp proteins and SigE creating a negative feedback loop. (iii) Selection experiments using library of sRNAs reveal a specific motif conserved in various SigE-dependent sRNAs that confer the resistance of cells to ethanol stress; (iv) This latter experiment reveals that sRNA-dependent repression of OmpA is a key regulatory event that confers ethanol resistance.
These data reveal the complexity of the regulatory networks that control OMPs in response to envelope stress. The two SigE-dependent sRNAs are of particular interest since they carry the same seed sequence, act through very similar mechanism (inhibition of translation followed by rapid degradation), but are not totally redundant. They probably resulted from convergent evolution. This work will certainly be of interest for the whole community working on gene regulation in bacteria.

I have only minor comments to be addressed:
1- Figure S2B: A previous study has shown that VrrA sRNA negatively regulated ompA expression. How do the authors explain why the lack of VrrA sRNA expression did not enhance OmpT yields? This is also surprising that at OD 1.0 where VrrA is strongly enhanced in ΔmicV mutant strain, the levels of OmpT are even enhanced. The authors should also explain why the deletion of MicV causes a strong enhanced expression of VrrA while the contrary was not observed. Could it be that the stability of VrrA might be dependent on MicV? Measurements of the half-lives of MicV and VrrA in the various WT and mutant strains might be useful.
2- Figure 2: Only overexpression of VrrA in the double mutant strain strongly decreases ompT synthesis. These data seem to indicate that under rich medium conditions of growth, the expression of VrrA is not sufficiently abundant for efficient regulation. A quantification of the VrrA and MicV sRNA would be of interest.
3- Because MicV and VrrA share very similar seed sequences, it is surprising that VrrA seems to be less efficient than MicV for several targets (i.e. OmpT, OmpA, rpoE...). Perhaps the structural context of the seed sequence is of importance. In MicV, the seed sequence is just at the 5' end highly accessible to bind efficiently to the target mRNAs while in VrrA, the seed sequence is located just after a large hairpin motif that might perhaps alter its accessibility. This might be discussed in the manuscript.
4- Experiments showing the expression of VrrA and MicV under ethanol exposure are missing. Because only VrrA causes a growth phenotype under ethanol exposure, and that both RNAs are able to regulate ompA expression, it might be possible that additional specific VrrA-dependent targets are also responsible of this phenotype.
5- Figure S7: difficult to appreciate the base pairing schemes involving the selected sRNA and ompA. I assume that it involves the seed sequences but also part of the scaffold RNA.
6- The authors should better exploit the data obtained with the selected sRNAs (Figure S7). Indeed sRNAs 3, 5, 8 and 15 seems to be more efficient than the WT sequence. Most of the sRNAs also bind with a preference to +3 to +18 of ompA mRNA. Could it be that this region is highly exposed and more appropriate for efficient binding? The authors should present a secondary structure model of ompA mRNA that would perhaps help to better understand this bias.

Referee #3:

Review Peschek et al., EMBO J.

The authors describe MicV sRNA as a new member of the σE regulon in Vibrio cholerae. MicV was identified by searching for the σE consensus motif upstream of 7,240 experimentally identified TSS in V. cholerae. The expression of MicV and the functionally overlapping VrrA sRNAs are shown to be dependent on rpoE expression. MicV stability is also Hfq-dependent in V. cholerae. MicV and VrrA expression is activated under ethanol stress in V. cholerae, but only VrrA confers ethanol resistance. Primarily MicV suppresses OmpT levels. In the micV knockout, OmpT levels rise and increase σE activity resulting in elevated VrrA levels (Fig. S2B); MicV and VrrA seem to act redundantly in dampening σE activation. Both, MicV and VrrA cause decreased ompT mRNA

levels when overproduced. Using a GFP-based reporter system, the authors confirmed post-transcriptional control of 16 target mRNAs, eight regulated by MicV and VrrA (including ompT), five specifically by MicV, and three by VrrA only. Several of these target genes are predicted to localize to the outer membrane or periplasmic space of V. cholerae. The post-transcriptional down-regulation of omp, ushA and lpp by MicV and/or VrrA via masking the 5'-UTR was verified by mutational analysis using GFP fusions. The authors noticed a conserved seed in MicV, VrrA and RybB and showed that all three sRNA induce a strong decrease in OmpT levels in V. cholerae. Likewise, overexpression of each of the three sRNAs decreased OmpA and OmpC levels in E. coli. Seed selection experiments (within a RybB scaffold) revealed that the seed pairing domain of σE-dependent sRNAs is strongly enriched under membrane-damaging (EtOH stress) conditions and that repression of OmpA is key for sRNA-mediated stress relief.
The presented comprehensive study discovered a novel sRNA, MicV, in the σE-dependent envelope stress response of V. cholerae and provides novel and deepend mechanistic insight into this regulatory network. In addition, a selection approach to validate the biological relevance of seed-pairing sequences in non-coding RNAs has been elaborated. It is a scientifically sound piece of work that I predict to be of broad interest to readers of The EMBO Journal.

Major comments:

1) p. 3, line 74-76: the authors state that seed sequences are ~6-12 nt in length and "Hfq-dependent sRNAs have been reported to carry up to three seed pairing domains". I do not think that these rules apply to all bacterial organisms. The authors should add that these rules were inferred from studies in gamma-proteobacteria.

2) In Fig. S1B and C, length markers would be informative to assess the size of mature and premature MicV RNA.

3) p. 5, lines 149-152: the argumentation to delete vchM before deletion of rpoE to avoid suppressor mutations is not clear to me; better explain to the reader.

4) p. 7, line 208: the authors state that a double knockout (KO) of micV and vrrA results in more than 12-fold higher OmpT levels; please give the fold difference also for the micV single KO for comparison. Is the difference in OmpT levels between the micV single KO and double KO reproducibly seen? In Fig. S2C, there is essentially no effect of the vrrA single KO on PmicV promoter utilization; how do the authors explain the additive effect of the double KO?

5) Generally: in growth experiments, the authors withdrew cells at time points "2.0 + 6h" and "2.0 + 18h". What is the OD at these points? A remark on this issue would be helpful.

6) p. 8, last paragraph: give rationale here why you selected MicV-ushA and VrrA-lpp as models and not prtV or oppA and pal or acfA which represent the most efficiently downregulated targets of MicV and VrrA, respectively?

7) p. 8, line 278 & Fig. 3A: I would prefer to speak about a 16-bp long interaction, not 17-bp; I doubt that the terminal G-U pair next to three A-U base pairs really forms most of the time. Furthermore, in Fig. 3A-F and S5D and text I propose to indicate the mutations as "M1" in the sRNA and "M1*" in the target RNA (or vice versa) because those are not the same mutations. I also suggest to indicate the black circles not only as "WT" (which is misleading) in Fig. 3G-I, but rather "WT (drpoE/pBAD-rpoE)"; alternatively, a minimal solution would be put WT in quotation marks in Fig. 3G-I.

8) p. 9, 3rd paragraph: How do the authors explain the still substantial decrease in ompT levels in the MicV/VrrA double KO? Based on Fig. 4, RybB seems to be responsible for this effect. Provide a link to to RybB and Fig. 4 at this point, assuming that readers will ask themselves the same question.

9) p. 10, line 326: why is this termed a reciprocal experiment? In this paragraph, there is a rapid leap from MicV/VrrA/RybB effects on OmpT in V. cholerae to effects on OmpA/C in E. coli. This may confuse readers. Please explain here why you switched from OmpT to OmpA/C and why you didn't look at OmpA/C effects in V. cholerae.

10) p. 11, line 373: in the parentheses, add: "(Figs. 4A and 5C; for more details on enriched variants, see below and Fig. S7)." This is helpful as the reader will be curious to know at this point which sequences beyond the native one became enriched.

11) p. 11, last paragraph: here the authors again immediately focus on OmpA; for readers not as deeply concerned with outer membrane stress as the authors, it is not fully clear why focussing exclusively on OmpA, considering that the authors suggest before that various OMPs are involved in this process. Please better introduce the reader here (even if the central role of OmpA in EtOH stress resistance will become evident in Fig. 6D).

12) Fig. 4: explain the ompA/C::kanR genotypes in the legend. Add y-axis lettering in panel D.

Minor comments:

p. 3
- line 68: "... bacterial RNAs are called ..."
- line 69: delete comma before "Hfq"
- line 78: "... how these interactions discriminate against offtarget interactions involving non-canonical G-U base-pairs (Papenfort et al., 2012)."
- line 90: "... associates with the core RNA polymerase ..."

p. 5
- line 147: replace "drastically" with "strongly"

p. 6
- line 168: redundant information; "and that MicV is a σE-dependent sRNA" could be deleted.
- line 180: "... we treated exponential cultures (OD600 of 0.2) of wild-type ..."
- line 182: "... the CFU ..."
- line 185, rewrite: "... membrane perturbations in V. cholerae, but only VrrA mediates (or induces, triggers, confers) ethanol resistance."
- line 200: "... sRNAs to OmpT repression."

p. 8
- line 242, rewrite: "These observations prompted us to design an experimental setup for the identification of MicV and VrrA target mRNA candidates at a genome-wide level."

p. 9
- line 279: "... bp long consecutive ..."
- line 280: "... duplex was also ..."
- line 281: ".. (10 bp) ..."
- line 286: " Next, we tested these predictions by mutational analysis (Figs. 3D-F and S5B)."
- line 288: "Combination of the two dinucleotide mutations restored ..."
- line 290: "... which was restored by the compensatory change in the ushA mRNA ..."
- line 296: "... validating the predicted RNA duplex formation."
- line 312: "... compensatory bp exchange ..."

p. 10
- line 317: replace "(Figs. 3A, 4A and S5E)" with "(Fig. S4A)"
- line 336, rewrite: "... phenotype, with VrrA and RybB supporting cell survival ~10-fold more efficiently than MicV."

p. 11
- line 378: "... To investigate the molecular basis of ..."

p. 12
- line 416: " Our results indicated ..."

p. 13

- line 434, simplify: " At the same time, global analyses of bacterial transcriptomes by RNA-Seq revealed the positions of thousands of TSS and their association with promoter elements (Colgan et al., 2017)."
- line 441: write out "ECF" here again (although done in the Introduction, but the paper is long ...)

Referee #1:

Papenfort and coworkers identify and characterize a new Sigma E-dependent sRNA RNA in Vibrio cholerae, here named MicV. Like a previous Sigma E-dependent Vibrio sRNA, VrrA, this sRNA represses a set of OMPs, and the two sRNAs overlap for some targets and each have unique targets. A conserved seed pairing domain is found in each of these sRNAs, explaining some aspects of the target overlap. Deletion of the two sRNAs leads to a number of phenotypes, including sensitivitiy to the sigma E-inducing treatment with ethanol. In an interesting approach, they carry out an evolution experiment on the seed region of a synthetic sRNA similar to MicV, selecting for ethanol resistance in the absence of Sigma E, and based on the results, provide evidence that repression of OmpA is the critical sRNA target for resistance. The work is generally clearly presented, and mostly complete. While the results do not really break new ground in our understanding of sRNA function, the global approaches, the use of an evolutionary selection, and the roles of this stress response in pathogens such as Vibrio all contribute to very nice piece of work.

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As requested by the reviewer, we have added additional information regarding the bioinformatics analyses to the Appendix Materials and Methods section and the legend of Appendix Table S1. Also, we have highlighted *vrrA* and *micV* in this table. We identified a total of 626 motif matching sites in our analysis of which 73 could be linked to a transcriptional start site. Also, we added the requested information on previously identified σ^E-dependent genes to Appendix Table S1. Finally, we have uploaded the script underlying these bioinformatics analyses to an open source platform: https://doi.org/10.5281/zenodo.2543422

2. Lines 262-263: For the genes in which there was no post-transcriptional control discovered, is there a reason to think that the promoters might be directly or indirectly controlled by Sigma E (and feedback control of Sigma E when the sRNAs are expressed).

We thank the reviewer for this comment. Currently, we do not have evidence that these genes are controlled by σ^E. Instead, we believe that potential target genes that could not be verified by qRT-PCR (*i.e. vca0996*, *vc2240*, and *vca0845*, Appendix Fig. S2) indicate biological noise and therefore might not be controlled by MicV or VrrA. For genes displaying the expected changes in our qRT-PCR-based validation experiments, we hypothesize that our reporter gene fusion lacked the relevant sequences for VrrA/MicV base-pairing. We have added this information on page 8 of our revised manuscript.

3. The title of the paper and much of what is included emphasizes the similarity in seed region of VrrA, MicV, and RybB (of E. coli, Salmonella). However, I found it striking that while 8 targets were identified (Fig. 2) in which both sRNAs regulate, of these, there is really overlap in the pairing region for only three (ompT, vca0951, and rpoE). For the others, regions of pairing to the target are either distinct or overlap only by a couple of nucleotides. This would seem to suggest (assuming predicted

pairing is accurate) that there are multiple ways of repressing these targets effectively but that repressing by both sRNAs is selected. Further comment on this may be appropriate (particularly in the context of the evolution experiments).

Following the reviewer's suggestion, we have revisited and revised our previous base-pairing predictions (Appendix Fig. S3 of the revised manuscript). We now find overlapping base-pairing for all of these targets.

4. Selection experiments and OmpA: Please include in Fig. S7F the region of pairing of MicV and VrrA. Some of this section leaves holes that should be further addressed to better understand the power of this approach. As presented, the authors jump from showing that the top 15 sequences all give ethanol resistance to identifying OmpA as a target. The only really strong information to show this is the critical target that leads to ethanol resistance is that deleting ompA suppresses the sensitivity of deleting rpoE, and it is not possible to know if deleting any other OMP would do the same. For instance, is there something special about OmpA, or is it abundance of OMPs, with OmpA being a particularly abundant one? Would all of these sRNAs also down-regulate ompT, or only a subset of them? Possibly testing this set of 15 plasmids with some of the other targets in Fig.2 would provide a better sense of to what extent the selection process really picks out ompA specifically. Finally, is it feasible to make a change in the ompA pairing region in the native ompA gene and show that the appropriate evolved sRNAs that pair there no longer give ethanol resistance? Given that Fig. S7F shows multiple pairing regions, predictions should be fairly strong for such an experiment. What does such an ompA mutant act like in the wild-type rpoE+ context?

Following the reviewer's suggestion, we have included the base-pairing positions of MicV and VrrA in Fig. EV5C of our revised manuscript. Also, we have added additional experiments to justify our focus on OmpA as an important factor for ethanol sensitivity of the *rpoE* mutant strain. Specifically, we performed mass-spec analysis on additional bands shown in Fig. 6A. We find that the abundance of the two major OMPs OmpT and OmpU does not change during the course of the laboratory selection experiments. In addition, we isolated total RNA from the selected libraries and tested the expression of MicV/VrrA target mRNAs encoding major OMPs (*ompT*, *lpp*, *ompA*, *ompU*, and *pal*). According to our hypothesis, only the expression of *ompA* decreased significantly. We show these new data in Fig. EV4A of our revised manuscript. In addition, we constructed a chromosomal *ompA* mutant in which we introduced several mutations in codons 2-5 of *ompA*. The mutant was designed in a way that the encoded amino acid sequence remained unchanged, while base-pairing with 10 of the 15 selected sRNAs candidates would be disrupted (selected sRNAs #1, #3, and #8 pair outside the CDS, sRNAs #2 and #14 are predicted to still interact with the mutated *ompA* variant). This mutation would also block base-pairing with the MicV and VrrA sRNAs. Indeed, we found that in a *rpoE* mutant strain this mutation abrogated rescue of ethanol sensitivity by the 10 selected sRNAs and increased ethanol sensitivity of wild-type *V. cholerae* by ~10-fold, which is similar to what we discovered for *V. cholerae* cells lacking *vrrA* and *micV* (Fig. 1F). These new data are presented in Figs. EV5D and E and page 15 of our revised manuscript.

5. Is MicV the RybB homolog for Vibrio? Given a similar seed in the same region of the sRNA, can anything be said about whether these sRNAs likely evolved from a common ancestor?

We currently do not have strong evidence to believe that *rybB* and *micV* are homologous sRNAs. Gene synteny analyses show that the *E. coli rybB* gene is located in the intergenic region of *rcdA* (encoding a TetR family transcriptional regulator) and *ybjL* (encoding a putative transport protein). In contrast,

micV of *V. cholerae* is located between *vc2640* (encoding a small hypothetical protein) and *vc2641* (encoding argininosuccinate lyase, also see Fig. EV1A). In addition, *micV* is significantly shorter (68 nts) than *rybB* (81 nts) and although both sRNAs share the highly conserved 5' end (Fig. 4A), alignment of the *micV* and *rybB* scaffold elements (nts 16-73 of *rybB* and nts 17-59 of *micV*) revealed only limited sequence identity (~44 %). For comparison, alignment of the homologous Spot 42 sRNAs from *V. cholerae* and *E. coli* revealed ~84% identity.

Referee #2:

This work describes a very complete analysis of the functions of one small non coding RNA MicV in the pathogen Vibrio cholerae. Although many works have highlighted the roles played by sRNAs in response to envelop stress (ESR) in several Enterobacteriaceae, this study is highly original in the sense that it has combined several in vivo approaches to gain knowledge on the regulatory networks involving two redundant sRNAs in response to ESR and for the first time laboratory selection experiments were developed to unravel specific sRNA signature used for regulatory functions. The authors showed the following data. (i) Vibrio cholerae MicV sRNA belongs to the Sigma E regulon; (ii) Target identification reveals that MicV shares many mRNA targets with the well known VrrA sRNA including mRNAs encoding Omp proteins and SigE creating a negative feedback loop. (iii) Selection experiments using library of sRNAs reveal a specific motif conserved in various SigE-dependent sRNAs that confer the resistance of cells to ethanol stress; (iv) This latter experiment reveals that sRNA-dependent repression of OmpA is a key regulatory event that confers ethanol resistance. These data reveal the complexity of the regulatory networks that control OMPs in response to envelope stress. The two SigE-dependent sRNAs are of particular interest since they carry the same seed sequence, act through very similar mechanism (inhibition of translation followed by rapid degradation), but are not totally redundant. They probably resulted from convergent evolution. This work will certainly be of interest for the whole community working on gene regulation in bacteria.

I have only minor comments to be addressed:
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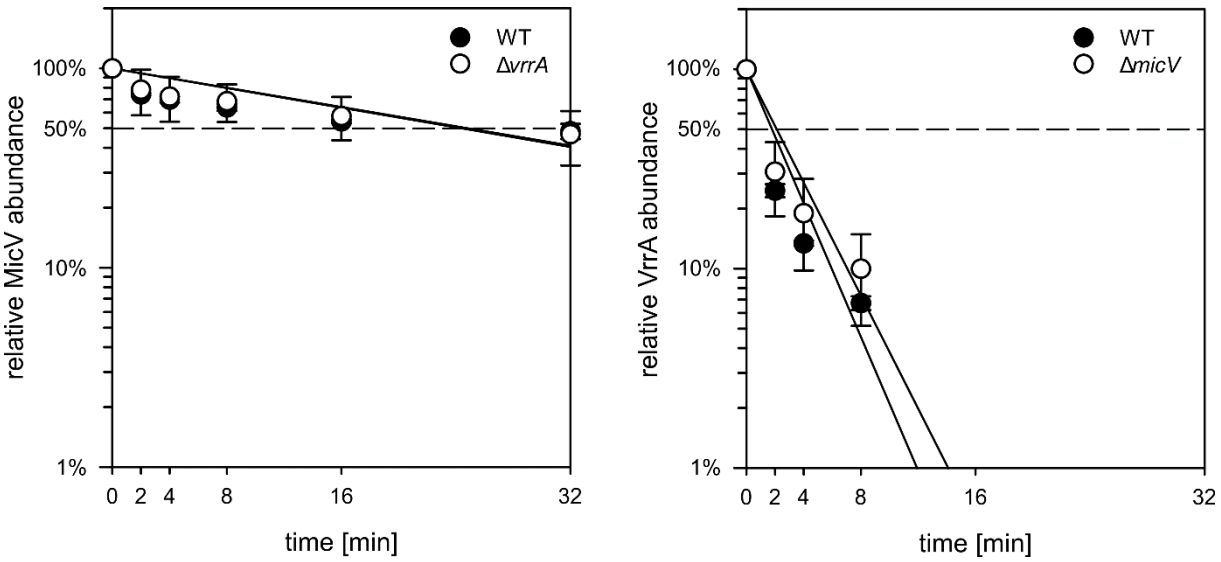
We believe that the reviewer was referring to the previous study of Song et al, 2010 (J Mol Biol. 2010 Jul 23;400(4):682-8) showing that VrrA inhibits OmpT production. However, this study also showed that mutation of *vrrA* did not result in a significant increase in OmpT levels (~ 1.1-fold; Figs. 1B and D of Song et al, 2010), which is in line with our results (Appendix Fig. S1B). Consequently, we propose that under the tested conditions MicV is the main repressor of OmpT. This hypothesis is supported by two additional observations. First, the predicted RNA-duplex formed between *ompT* and MicV is significantly more stable than the VrrA-*omp*T interaction (Appendix Fig. S3A) and second, repression of the *ompT::gfp* reporter by MicV is stronger (~10-fold, Fig. 3D) when compared to VrrA (~3-fold, Fig. EV2E). In addition, we also performed the requested transcript stability experiments. Although we did not find a significant difference in VrrA stability between wild-type and *micV*-deficient *V. cholerae*, our analyses show that VrrA and MicV half-lives are drastically different: under the tested conditions $t_{1/2}$ of MicV is ~26 min, while $t_{1/2}$ of VrrA is only ~2 min (see Fig. EV1D and below). This stark difference

could add to the different potency of the two regulators with respect to *ompT* repression (Appendix Fig. S1B) and overall impact on the activity of the σ^E-response (Appendix Fig. S1C). We added the data showing VrrA stability in wild-type and Δ*hfq* cells to Fig. EV1D of our revised manuscript. For comparison, we also provide the relative stabilities of MicV and VrrA in the respective mutant strains below.



V. cholerae wild-type and the indicated sRNA mutant strains were cultivated to an OD_{600} of 1.0. Cells were treated with rifampicin to terminate transcription. Total RNA samples were collected at the indicated time points and MicV or VrrA transcript levels were monitored on Northern blots. The data represent the mean ± SD of three independent replicates.

2- Figure 2: Only overexpression of VrrA in the double mutant strain strongly decreases ompT synthesis. These data seem to indicate that under rich medium conditions of growth, the expression of VrrA is not sufficiently abundant for efficient regulation. A quantification of the VrrA and MicV sRNA would be of interest.

As requested by the reviewer, we quantified and compared the relative levels of VrrA and MicV expressed from their chromosomal loci with pBAD-controlled overexpression. Indeed, we find that the pBAD constructs produce ~18-fold and ~7-fold higher levels of VrrA and MicV, respectively (see new Fig. 2A, legend of Fig. 2A, and Source data), when compared to the chromosomal sRNA expression levels. This might well explain the efficient repression of *ompT* mRNA by pBAD-driven VrrA expression.

3- Because MicV and VrrA share very similar seed sequences, it is surprising that VrrA seems to be less efficient than MicV for several targets (i.e. OmpT, OmpA, rpoE...). Perhaps the structural context of the seed sequence is of importance. In MicV, the seed sequence is just at the 5' end highly accessible to bind efficiently to the target mRNAs while in VrrA, the seed sequence is located just after a large hairpin motif that might perhaps alter its accessibility. This might be discussed in the manuscript.

As requested by the reviewer, we have added this information to our discussion section (Page 14). As pointed out above (comment #1, reviewer 3), we also discovered significant differences in the stability of MicV and VrrA (new Fig. EV1D), which could add to the higher efficiency of MicV in target mRNA regulation.

4- Experiments showing the expression of VrrA and MicV under ethanol exposure are missing. Because only VrrA causes a growth phenotype under ethanol exposure, and that both RNAs are able to regulate

ompA expression, it might be possible that additional specific VrrA-dependent targets are also responsible of this phenotype.

We performed the requested experiments and added the results to our revised manuscript (Figs. 1D/E and Page 6 of the revised manuscript). Also, we agree with reviewer's notion that additional VrrA targets could add to the role of VrrA in ethanol resistance.

5- Figure S7: difficult to appreciate the base pairing schemes involving the selected sRNA and ompA. I assume that it involves the seed sequences but also part of the scaffold RNA.

We addressed this issue in Fig. EV5C of our revised manuscript. We now indicate the sequence elements involved in base-pairing with *ompA*. In addition, we added all base-pairing prediction involving the selected sRNA candidates and *ompA* to the source data, which will be provided together with manuscript.

6- The authors should better exploit the data obtained with the selected sRNAs (Figure S7). Indeed sRNAs 3, 5, 8 and 15 seems to be more efficient than the WT sequence. Most of the sRNAs also bind with a preference to +3 to +18 of ompA mRNA. Could it be that this region is highly exposed and more appropriate for efficient binding? The authors should present a secondary structure model of ompA mRNA that would perhaps help to better understand this bias.

As requested by the referee, Fig. EV5C now includes a structure model of the *ompA* mRNA segment involved in base-pairing with the selected sRNAs. This analysis suggests that the translation initiation region of *ompA* is likely single stranded and therefore available for base-pairing. In addition, a closer look at the *ompA* sequence required for translation initiation is rich in adenosines, which could complicate efficient base-pairing with potential sRNAs. Seed pairing by sRNAs frequently relies on stable G-C pairs and it seems that several of the selected sRNAs use the guanosine and cytosine residues located between position +10 and +20 of *ompA* as an 'anchor' to initiate base-pairing. Therefore, we hypothesize that selecting efficient sRNA regulators for OmpA repression relies on the local nucleotide composition of the mRNA sequence involved in translation initiation.

Referee #3:

The authors describe MicV sRNA as a new member of the σE regulon in Vibrio cholerae. MicV was identified by searching for the σE consensus motif upstream of 7,240 experimentally identified TSS in V. cholerae. The expression of MicV and the functionally overlapping VrrA sRNAs are shown to be dependent on rpoE expression. MicV stability is also Hfq-dependent in V. cholerae. MicV and VrrA expression is activated under ethanol stress in V. cholerae, but only VrrA confers ethanol resistance. Primarily MicV suppresses OmpT levels. In the micV knockout, OmpT levels rise and increase σE activity resulting in elevated VrrA levels (Fig. S2B); MicV and VrrA seem to act redundantly in dampening σE activation. Both, MicV and VrrA cause decreased ompT mRNA levels when overproduced. Using a GFP-based reporter system, the authors confirmed post-transcriptional control of 16 target mRNAs, eight regulated by MicV and VrrA (including ompT), five specifically by MicV, and three by VrrA only. Several of these target genes are predicted to localize to the outer membrane or periplasmic space of V. cholerae. The post-transcriptional down-regulation of omp, ushA and lpp by MicV and/or VrrA via masking the 5'-UTR was verified by mutational analysis using GFP fusions. The authors noticed a conserved seed in MicV, VrrA and RybB and showed that all three sRNA induce a strong decrease in

OmpT levels in V. cholerae. Likewise, overexpression of each of the three sRNAs decreased OmpA and OmpC levels in E. coli. Seed selection experiments (within a RybB scaffold) revealed that the seed pairing domain of σE-dependent sRNAs is strongly enriched under membrane-damaging (EtOH stress) conditions and that repression of OmpA is key for sRNA-mediated stress relief.
The presented comprehensive study discovered a novel sRNA, MicV, in the σE-dependent envelope stress response of V. cholerae and provides novel and deepend mechanistic insight into this regulatory network. In addition, a selection approach to validate the biological relevance of seed-pairing sequences in non-coding RNAs has been elaborated. It is a scientifically sound piece of work that I predict to be of broad interest to readers of The EMBO Journal.

1) p. 3, line 74-76: the authors state that seed sequences are ~6-12 nt in length and "Hfq-dependent sRNAs have been reported to carry up to three seed pairing domains". I do not think that these rules apply to all bacterial organisms. The authors should add that these rules were inferred from studies in gamma-proteobacteria.

As requested by the reviewer, we have revised our manuscript indicating that this these rules are only relevant for the γ-Proteobacteria (Page 3 of the revised manuscript).

2) In Fig. S1B and C, length markers would be informative to assess the size of mature and premature MicV RNA.

As requested by the referee, we added size marker to Figs. EV1B and EV1C of the revised manuscript.

3) p. 5, lines 149-152: the argumentation to delete vchM before deletion of rpoE to avoid suppressor mutations is not clear to me; better explain to the reader.

As for several other enterobacterial species, *rpoE* is an essential gene in *V. cholerae*. However, mutants of *rpoE* can be achieved if additional suppressor mutations occurred. Two known genomic alterations that allow deletion of *rpoE* are mutation of the gene encoding methylcytosine methyltransferase, *vchM* (Chao et al., 2015, PLoS Genetics) and mutation of *ompU* (Davis and Waldor, 2009, NAR), which encodes a major porin. Since we did not intend to change OMP protein composition in the *rpoE* mutants, we preferred to use the Δ*vchM* background for all following mutant construction involving *rpoE*. We have added an additional sentence to our revised manuscript to clarify this issue (Page 5).

4) p. 7, line 208: the authors state that a double knockout (KO) of micV and vrrA results in more than 12-fold higher OmpT levels; please give the fold difference also for the micV single KO for comparison. Is the difference in OmpT levels between the micV single KO and double KO reproducibly seen? In Fig. S2C, there is essentially no effect of the vrrA single KO on PmicV promoter utilization; how do the authors explain the additive effect of the double KO?

As requested by the referee, the changes in OmpT protein levels in the Δ*micV* strain are now stated in the text. Regarding the contribution of MicV and VrrA to *micV* promoter activity, our interpretation of the data is that MicV is the major regulator of σ^E activity under the tested conditions and sufficiently abundant to repress OMP production in the absence of *vrrA*. This interpretation is in accordance with the rather constant MicV expression levels determined in Appendix Fig S1B (bottom panel). However, in the absence of *micV*, OmpT levels increase, the σ^E response is triggered (Appendix Fig. S1C), and *vrrA* is upregulated (Appendix Fig. S1B, bottom), which contributes to the repression of OmpT (Appendix Fig. S1B, top). It should be noted, however, that this observation might only be valid under the tested conditions where balanced OmpT levels are crucial for σ^E activity. As we have shown in other

parts of the manuscript (*e.g.* Fig. 1F), VrrA is the more relevant regulator when cells are stressed with ethanol. Here, repression of OmpA is most important for cellular survival and although both VrrA and MicV control OmpA production (Fig. 2C), only deletion of *vrrA* displays a significant phenotype. In summary, it seems that although VrrA and MicV share a large set of target mRNAs, each sRNA has a preference to regulate certain targets more efficiently than others. How this 'sorting' of target mRNAs occurs in the cell is certainly an interesting topic that will require further investigation.

5) Generally: in growth experiments, the authors withdrew cells at time points "2.0 + 6h" and "2.0 + 18h". What is the OD at these points? A remark on this issue would be helpful.

We have now used this nomenclature for several years because *V. cholerae* cells stop growing significantly at ~ OD_{600} of 2.0. Therefore, the optical density readings of cells grown for 12h do not differ significantly from cells that were grown for 24h although their physiological state is very different. For example, the OD_{600} readings for time points "2.0 + 6h" and "2.0 + 18h" were both ~2.5. Therefore, we prefer the indicated nomenclature. To clarify this point, we have added additional information to the methods section of the revised manuscript.

6) p. 8, last paragraph: give rationale here why you selected MicV-ushA and VrrA-lpp as models and not prtV or oppA and pal or acfA which represent the most efficiently downregulated targets of MicV and VrrA, respectively?

We selected MicV-*ushA* and VrrA-*lpp* because our base-pairing predictions (Appendix Fig. S3) indicated that these would form thermodynamically stable interactions. The VrrA-*acfA* RNA duplex is similarly strong (in fact, the duplex is predicted to be slightly more stable than the VrrA-*lpp* interaction), however, since *acfA* is a virulence-associated gene, which is only expressed under very specific conditions, we decided to focus on the VrrA-*lpp* interaction.

7) p. 8, line 278 & Fig. 3A: I would prefer to speak about a 16-bp long interaction, not 17-bp; I doubt that the terminal G-U pair next to three A-U base pairs really forms most of the time. Furthermore, in Fig. 3A-F and S5D and text I propose to indicate the mutations as "M1" in the sRNA and "M1*" in the target RNA (or vice versa) because those are not the same mutations. I also suggest to indicate the black circles not only as "WT" (which is misleading) in Fig. 3G-I, but rather "WT (drpoE/pBAD-rpoE)"; alternatively, a minimal solution would be put WT in quotation marks in Fig. 3G-I.

We agree with the referee and changed the text accordingly. We also changed the nomenclature of the mutations in the figures.

8) p. 9, 3rd paragraph: How do the authors explain the still substantial decrease in ompT levels in the MicV/VrrA double KO? Based on Fig. 4, RybB seems to be responsible for this effect. Provide a link to to RybB and Fig. 4 at this point, assuming that readers will ask themselves the same question.

Regarding the effects observed in Figs. 3 G-I, one possible explanation is that pBAD-driven over-production of σ^E could titrate σ^{70} from RNA polymerase and thereby indirectly reduce the expression of σ^{70}-dependent promoters. A similar effect was previously observed for σ^E-dependent sRNAs form Salmonella (Papenfort et al, Mol. Micro, 2006). With respect to *rybB*, we would like to point out that *rybB* is specific to enterobacterial species such as *Salmonella* and *E. coli* and that *V. cholerae* does not

encode a chromosomal copy of this sRNA gene. We have clarified this issue in the revised version of our manuscript (Page 10).

9) p. 10, line 326: why is this termed a reciprocal experiment? In this paragraph, there is a rapid leap from MicV/VrrA/RybB effects on OmpT in V. cholerae to effects on OmpA/C in E. coli. This may confuse readers. Please explain here why you switched from OmpT to OmpA/C and why you didn't look at OmpA/C effects in V. cholerae.

Again, we would like to apologize for the confusion. As pointed out above, *V. cholerae* does not encode the *rybB* sRNA gene. The experiments shown in Fig. 5B were performed in *V. cholerae*, while the experiments in Fig. 5C were performed in *E. coli* (this is why we consider this a reciprocal experiment). Although, *V. cholerae* and *E. coli* express similar OMPs, their abundance and nomenclature is different. For example, OmpT is a major (abundant) porin in *V. cholerae*, while in *E. coli* OmpC and OmpA are more abundant.

10) p. 11, line 373: in the parentheses, add: "(Figs. 4A and 5C; for more details on enriched variants, see below and Fig. S7)." This is helpful as the reader will be curious to know at this point which sequences beyond the native one became enriched.

Revised as suggested by the referee.

11) p. 11, last paragraph: here the authors again immediately focus on OmpA; for readers not as deeply concerned with outer membrane stress as the authors, it is not fully clear why focussing exclusively on OmpA, considering that the authors suggest before that various OMPs are involved in this process. Please better introduce the reader here (even if the central role of OmpA in EtOH stress resistance will become evident in Fig. 6D).

We thank the reviewer for raising this important point and apologize for not being clear enough. We actually tested for different abundance of the major OMPs in Fig. 6A. In our revised Fig. 6A we now indicate the bands corresponding to OmpU and OmpT for which we observed no significant changes in protein levels in the ethanol selection experiments. To further expand on this important point, we performed qRT-PCR experiments comparing *ompT*, *ompU*, *lpp, pal* and *ompA* mRNA levels in strains carrying the sRNA libraries. Here we observed significant changes only for *ompA* mRNA levels (see new Fig. EV4A). We also addressed this issue in the text of our revised manuscript (Page 12, please also see response to comment #4 of referee #1).

12) Fig. 4: explain the ompA/C::kanR genotypes in the legend. Add y-axis lettering in panel D.

Revised as suggested by the referee.

Minor comments:

We would like to thank the referee for the close reading of our manuscript. We have addressed all points and revised our manuscript accordingly.

p. 3
- line 68: "... bacterial RNAs are called ..."

- line 69: delete comma before "Hfq"
- line 78: "... how these interactions discriminate against off target interactions involving non-canonical G-U base-pairs (Papenfort et al., 2012). "
- line 90: "... associates with the core RNA polymerase ..."

p. 5
- line 147: replace "drastically" with "strongly"

p. 6
- line 168: redundant information; "and that MicV is a σE-dependent sRNA" could be deleted.
- line 180: "... we treated exponential cultures (OD600 of 0.2) of wild-type ..."
- line 182: "... the CFU ..."
- line 185, rewrite: "... membrane perturbations in V. cholerae, but only VrrA mediates (or induces, triggers, confers) ethanol resistance."
- line 200: "... sRNAs to OmpT repression."

p. 8
- line 242, rewrite: "These observations prompted us to design an experimental setup for the identification of MicV and VrrA target mRNA candidates at a genome-wide level."

p. 9
- line 279: "... bp long consecutive ..."
- line 280: "... duplex was also ..."
- line 281: ".. (10 bp) ..."
- line 286: " Next, we tested these predictions by mutational analysis (Figs. 3D-F and S5B)."
- line 288: "Combination of the two dinucleotide mutations restored ..."
- line 290: "... which was restored by the compensatory change in the ushA mRNA ..."
- line 296: "... validating the predicted RNA duplex formation."
- line 312: "... compensatory bp exchange ..."

p. 10
- line 317: replace "(Figs. 3A, 4A and S5E)" with "(Fig. S4A)"
- line 336, rewrite: "... phenotype, with VrrA and RybB supporting cell survival ~10-fold more efficiently than MicV."

p. 11
- line 378: "... To investigate the molecular basis of ..."

p. 12
- line 416: " Our results indicated ...

p. 13
- line 434, simplify: " At the same time, global analyses of bacterial transcriptomes by RNA-Seq revealed the positions of thousands of TSS and their association with promoter elements (Colgan et al., 2017)."
- line 441: write out "ECF" here again (although done in the Introduction, but the paper is long ...)

2nd Editorial Decision 30 May 2019

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees (see comments below). I am pleased to say that the referees find that their comments have in principle been sufficiently addressed and now support publication. Nonetheless referee #3 still raises some specific points, which may require textual changes in the revised final version.

REFEREE REPORTS

Referee #1:

The revised manuscript very nicely addresses the previous comments, and provides clear evidence for important and complementary roles for two sRNAs in regulation of OMPs in Vibrio. The evolutionary approach used here will be of particularly general interest.

Referee #2:

The authors have made additional experiments which strengthened their message and clarified certain aspects of their study. Although the mechanism of regulation is not highly original, the combination of in vitro evolutionary selection and in vivo study clearly showed that sRNA-dependent regulation of ompA in vibrio is responsible for the ethanol resistance phenotype. This is an elegant study that fully deserves publication for EMBO J.

Referee #3:

Review Peschek et al., Version R1

The manuscript has substantially improved in terms of clarity and coherence, and is now much easier to conceive despite the wealth of data. I have only a few final minor suggestions:

1) p. 12, line 392: the two OmpA binds in Fig. 6A are barely "major bands" when compared with those for OmpT and OmpU; delete "major".

2) p. 13, Discussion: the first paragraph of the Discussion can be omitted (redundant with the first Results part). The paper has a strong focus on the novel methodology. To place the (fascinating) biological findings somewhat more in the foreground, the authors may consider (e.g. on p. 14, top) to somewhat expand the discussion of their biological findings illustrated in the model of Fig. 7: aspects I find of interest are (a) that the dampening of sigmaE expression by MicF and VrrA also mitigates RseA/B/C expression; maybe the latter effect is a major purpose of downregulating expression of the rpoE operon by the two sRNAs; (b) sigmaE activation may be considered as the more global response to envelope stress, while sRNAs like MicF and VrrA cooperatively modulate this response toward more specific responses, such as VrrA in ethanol stress.

3) Regarding my previous comment 5 ("Generally: in growth experiments, the authors withdrew cells at time points "2.0 + 6h" and "2.0 + 18h". What is the OD at these points? A remark on this issue would be helpful."). I propose to incorporate the authors' response to my comment in M. & M., paragraph "Bacterial strains and growth conditions".

4) Last sentence of the Discussion (p. 16, lines 552-554) is a little cryptic/elusive and can be omitted.

5) In Fig. 4B, Fig. 6B and text (p. 18, lines 623 & 624; p. 24, line 908; p. 26, line 950), better change RnaP to RpoA to avoid confusion with "RNase P"

- p. 8, line 276, in parentheses: also refer here to Appendix Table S2
- p. 10, line 325: "... (R=purine) ..."

- p. 15, line 522, rewrite: "... as evident from increased sE activity in DmicV relative to wild-type and DvrrA cells (Appendix Fig. S1C)."
- p. 25, line 912, rewrite: "... For comparison, we included the E. coli insertional mutant strains ompA::kanR and ompC::kanR for specific assignment of OmpA and OmpC bands."

2nd Revision - authors' response 31 May 2019

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1) p. 12, line 392: the two OmpA binds in Fig. 6A are barely "major bands" when compared with those for OmpT and OmpU; delete "major".
Revised as requested by the referee.

2) p. 13, Discussion: the first paragraph of the Discussion can be omitted (redundant with the first Results part). The paper has a strong focus on the novel methodology. To place the (fascinating) biological findings somewhat more in the foreground, the authors may consider (e.g. on p. 14, top) to somewhat expand the discussion of their biological findings illustrated in the model of Fig. 7: aspects I find of interest are (a) that the dampening of sigmaE expression by MicF and VrrA also mitigates RseA/B/C expression; maybe the latter effect is a major purpose of downregulating expression of the rpoE operon by the two sRNAs; (b) sigmaE activation may be considered as the more global response to envelope stress, while sRNAs like MicF and VrrA cooperatively modulate this response toward more specific responses, such as VrrA in ethanol stress.
As requested by the referee, we have shortened the first paragraph of this section and added a discussion of points a) and b) (lines 493-495 and 539-541 of the revised manuscript, respectively).

3) Regarding my previous comment 5 ("Generally: in growth experiments, the authors withdrew cells at time points "2.0 + 6h" and "2.0 + 18h". What is the OD at these points? A remark on this issue would be helpful."). I propose to incorporate the authors' response to my comment in M. & M., paragraph "Bacterial strains and growth conditions".
We have added an additional sentence to the Materials and Methods section of the manuscript to address this point (line 582).

4) Last sentence of the Discussion (p. 16, lines 552-554) is a little cryptic/elusive and can be omitted.
We have removed the last sentence of our discussion.

5) In Fig. 4B, Fig. 6B and text (p. 18, lines 623 & 624; p. 24, line 908; p. 26, line 950), better change RnaP to RpoA to avoid confusion with "RNase P"
We thank the referee for this comment. We have now changed our nomenclature from “RnaP” to “RNAPα”. We did not use “RpoA” to avoid confusion with “RpoE”, which we use frequently in this manuscript.

- p. 8, line 276, in parentheses: also refer here to Appendix Table S2
Revised as requested by the referee.

- p. 10, line 325: "... (R=purine) ..."
Revised as requested by the referee.

- p. 15, line 522, rewrite: "... as evident from increased σE activity in ΔmicV relative to wild-type and ΔvrrA cells (Appendix Fig. S1C)."
Revised as requested by the referee.

- p. 25, line 912, rewrite: "... For comparison, we included the E. coli insertional mutant strains ompA::kanR and ompC::kanR for specific assignment of OmpA and OmpC bands.
Revised as requested by the referee.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Kai Papenfort
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-101650R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No estimation of statistical power was used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	n.a.
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For animal studies, include a statement about randomization even if no randomization was used.	n.a.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	n.a.
4.b. For animal studies, include a statement about blinding even if no blinding was done	n.a.
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, normal distribution of compared groups were tested using Kolmogorov-Smirnov tests.
Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	yes, equal variance was tested using Brown-Forsythe tests.

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The catalogue numbers of used antibodies are provided in the Materials & Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	n.a.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No animal models were used.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n.a.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	n.a.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n.a.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n.a.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n.a.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n.a.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n.a.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n.a.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n.a.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All accession codes are listed in the Data availability section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Used scripts are listed in the Data availability section or Appendix Supplementary Material & Methods section, and available at Github or Zenodo.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n.a.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n.a.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no