nature portfolio

Peer Review File

Modular small RNA drives the emergence of virulence traits and environmental trade-offs in Vibrio cholerae

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Balasubramanian et al This is an interesting manuscript that I enjoyed reading. The authors have discovered a sRNA encoded in the 3' end of the ompU gene that is responsible for the increase in biofilm formation in a ΔompU strain. The manuscript is generally well written. However, currently the experiments rely on a huge amount of RNA-seq that is not backed up with mechanistic details. Which is the major limitation of the manuscript. It feels in many places like the authors have found a lot of exciting things and just put them all into a story, rather than cherry picking.

The sRNA encoded at the 3' end of the ompU gene is interesting in that as outlined in the title is modular. This is interesting and novel. More should be made to explain this in the text. The figures determining the conservation within the supplementary section could be improved and moved to the main text.

The authors express the OueS sRNA from an ectopic plasmid pBAD22 and use this for their RNA-seq experiments and some of the follow up. sRNA levels are generally finely tuned within the cell and therefore making conclusions regarding behaviour based off of an overexpressed gene is challenging. The authors have a chromosomally integrated complementation strain of the ompU promoter directing OueS, this would have been more suitable for some of the experiments as the sRNA is likely to be at levels more similar to the native version, although the Northern for the ompU where OueS was identified suggests low/lower levels of OueS compared to the ompU transcript. The manuscript requires determination of how much sRNA is produced in the WT strain vs how much is produced in the ectopic strain vs how much sRNA is produced from the ompU promoter. The authors carry out an experiment in virulence inducing conditions, but do not determine how much sRNA is produced in this condition compared to the other conditions that they test. In figure 1 what is the media that is used. Given that the sRNA is likely produced by processing the ompU transcript the level of ompU should also be defined. Is the sRNA not appearing any more because there is no more ompU transcript or is it that there is some other change that stops the processing.

RhyB regulation – needs point mutations in both RhyB and OueS to show the regulation is direct and comes from the interaction of the pair of them. Include complementary point mutations that restore the regulation.

The section on the correlation between the ToxR and OueS regulons is interesting. Especially for an sRNA to be regulating such a large regulon of genes. The major weakness in this section is again the lack of molecular work – is it likely a direct regulation of the different targets due to an interaction with OueS? In the discussion it is reported that ToxR only binds to the promoter of 39 genes. One of these being ompU that through this regulation also regulates OueS. Therefore the title at line 348 describing OueS as a functional surrogate of ToxR is inaccurate. It is actually the ToxR regulation of a further regulator that is likely responsible for the regulation of the other genes that have been reported to be part of the ToxR regulon. The details about the 39 promoters needs to be brought up front to the results section. This section again needs to be backed up by some molecular work to show that it is indeed a direct regulation by OueS and not a causative effect of OueS possibly acting through another pathway. Is there crossover between the list of genes in the ToxR RNA-seq data set not directly regulated that also overlap with OueS target prediction. State why virulence inducing conditions are the most appropriate.

Motility section – which genes is it likely regulating? Molecular work to back this up?

Many of the figures are made up of two subjects – e.g. 2 – iron and CBASS. This detracts from the story and makes it more

challenging to follow.

Figure 2H – How does the 823 and 841 of the CBASS promoter relate to the Transcriptional start site? sRNAs regulate at the RNA level. Again need to be backed up with molecular work, not just showing RNA-seq levels. GFP fusion or something similar.
Line 319 – Ectopic expression of OueS – how do the levels vary between the WT and this strain. Explain strain construction in the main text – i.e. how strong the promoter is, copy number etc. Northern blot or qRT-PCR to show the difference in the level. Be careful over phenotypes that require large amounts of overexpression to generate. How likely is this to be real in the wild. I would have expected to see a difference between the WT and some of the other strains, if only small.

The section on ToxR regulation makes more sense if it comes directly after the identification. This is how the sRNA is regulated, at least at the transcriptional level. Whether this section is split away from the transcriptomics might make more sense, but while I was reading the RACE I wanted to know how the gene was being regulated.

Line 24 – Region not one
Line 31 – Define CBASS
Line 31 – Toxigenic OueS – Define better, at least here. Doesn't make sense to a non-vibrio specialist. OueS expression in toxigenic….
Line 108 - V. cholerae
Line 110 – region
Line 111 – Make it a sentence, not a and b.
Figure 1B does not add to the story. Either remove it or put it in the supplementary.
Line 150 – explain better what is happening in the absence of OmpU. It's not misfolded, but totally gone. How does this cause changes in the RpoE regulon.
Line 188 – This section would benefit from a sequence alignment. It would be good to show how the sequence differs between the toxigenic and non-toxigenic. Is there some variation in the amino acid encoded from the 3' end of the OmpU protein sequence due to the expression of this sRNA.
Figure 1G – How does expression of the sRNA relate to the levels of OmpU?
Figure 1H – provide a figure to show the regions that were used for the complementation experiment.
Line 240 – don't use a, b and c. Make it a sentence.
Line 246 – delete first sentence – surplus to requirements.
Figure 2H – How does the 823 and 841 of the CBASS promoter relate to the Transcriptional start site?
Line 285 – what tool did you use for the prediction
Line 287 – A northern does not look at stability unless you have undertaken a rifampicin time course. This just tells you the level.
Line 503 – 506 – I don't understand what the authors are trying to explain in terms of target, CI and OueS type?
Line 648 – this is quite repetitive
Line 705-707 – Text unrequired?

Reviewer #2

(Remarks to the Author)
In their manuscript entitled "Modular small RNA drives the emergence of virulence traits and environmental trade-offs in Vibrio cholerae" Balasubramanian et al. report that the small regulatory RNA OueS plays a central role in toxicity of *Vibrio cholerae* strains.
That OueS is an interesting sRNA that regulates biofilm formation and confers phage resistance is well supported by the data, but other claims, namely the modular architecture of OueS and the existence of different alleles are not. For details please see the following questions and comments:

- Results:

Do the ompU mRNA and the OueS sRNA have the identical 3' end? Currently this is not explicitly mentioned.

The authors already claim in the title that the sRNA is modular, where one part is in the ompU ORF and the other in the UTR. Notwithstanding this interesting chimeric origin, the term modular is too strong in this case. Modules are parts with independent functions that can be combined to provide new functionalities. The presented results only show that the overall functionality of the sRNA is affected by mutations in any or both of the two parts, and that the 3' part may interact with other RNAs (RyhB and 5'UTR of CBASS). There is no evidence for a distinct function of the 5' part. Furthermore, the overlap with the ompU ORF makes it difficult to evolve a function here.

The authors state that OueS potentially binds "upstream of the VC0178 ORF, the region that encompasses the promoter of the operon". Usually the promoter is not part of the transcript, so it is not clear to me how this is meant - please clarify. Furthermore the potential binding site in the sRNA is the same for RyhB and CBASS and strikingly it is perfectly conserved on sequence and structure level in the GBE1114 variant, because it is part of the terminator hairpin (Fig. 1 E&F). How do you explain that the GBE1114 variant still is non-functional?

The authors make use of the environmental allele of OueS from strain GBE1114, but provide no evidence that the sRNA

from this allele actually exists and is expressed in the strain GBE1114. Sequence conservation alone is not sufficient evidence in this case, because this could be due to the ompU ORF and UTR, which harbours a Rho-independent terminator. If there is no independent evidence for the existence of OueS in GBE1114 then it is inappropriate to use this as the "environmental" allele. The same holds true for the other toxigenic and environmental variants that are used later. Without evidence for their existence as distinct sRNAs in their host strains, they cannot be considered as functional homologs. It might be, that the processing of the ompU mRNA to produce the OueS sRNA only exists in C6706.
This would also mean that many of the conclusions need to be rephrased or removed completely.

- Discussion:
In V. cholerae other 3' UTR derived sRNAs have been reported previously, and also a method for their identification (TIER-seq, Hoyos et al.; eLife 2020). Although the paper is cited, there is no discussion about the differences or if OueS can also be found in this dataset. Please elaborate on this.

In general I am missing a really critical discussion of the results. This is for example related to the issues mentioned above, but also others. Is it really always beneficial that the expression of OueS is tightly coupled to the expression of the porin OmpU. Does the cleavage of the full transcript lead to degradation of the mRNA or is it still translated but produces inactive OmpU? Could the processing of the initial ompU transcript be subject to regulation?

Minor:
- not sequence homology, but sequence complementarity indicates RNA-RNA interactions.

Reviewer #3

(Remarks to the Author)
The manuscript by Balasubramanian et al describes the discovery of a sRNA, OueS, in V. cholerae that is embedded within the ompU gene and that controls 84% of the ToxR regulon. The authors show that OueS regulates biofilm formation and phage defense. Interestingly, the authors argue that OueS has a bimodular structure with relative variability in the portion within the ompU gene and less variability within the 3' UTR portion. OueS from pathogenic strains is better at controlling intestinal colonization and phenotypes associated with that, while OueS from environmental strains is better at controlling colonization of zooplankton. The phenotypes associated with the sRNA are well documented, and the experimental data is well-executed. This phenomenon is interesting and highlights an unknown and important aspect of the virulence regulon of V.cholerae. The difference in the activity of the sRNA between C6706 and GBE1114 is striking and definitely identifies an important regulatory element to Vc pathogenesis and environmental persistence. I have some questions on the "bimodular" nature of the sRNA, as detailed below.

The predicted interaction sequence of C6706 with RyhB (Fig 2D) is identical to the sequence of GBE1114 (Fig. S6) and it lies in the 3' "invariant" module of OueS after the OmpU stop codon, which has the same predicted structure within the last stem loop. Likewise the predicted interaction sequence of C6706 with CBASS (Fig. 2H) is also within this conserved 3' region that is identical to GBE1114. Yet the C6706 and GBE1114 behave differently in regards to biofilm/iron (Fig. 1H) and phage susceptibility (Fig 2J). Either the structural predictions are incorrect, or the predicted interaction is incorrect. The predicted RNA interaction is likely incorrect, based on the data presented. Because the variability between C6706 and GBE1114 lies in the 5' region, this is the most likely interaction region with RhyB and CBASS. Minimally the authors need to acknowledge that the 3' "invariant" region is where the predicted interaction occurs (2D and 2H), because the text throughout implies that it is the 5' "variant" region that is responsible for these differences in effects on biofilms, phage susceptibility, etc.

The predicted folded structures of the OueS sRNA Fig S6B predict e.g. that IRLE0074 has a different structure than C6706, yet the sequence appears identical to C6706 (Fig S6A). This is relevant because in Fig. 4B these sRNAs behave differently in the infant mouse colonization model (according to their statistical analysis). If the sequence is identical, how can the sRNA fold into a different structure and have a different effect on colonization?

In general, the sequence alignment (FigS6A) implies high variability in the region marked ~55-85, but this is because one set of 4 strains (GBE0917-GBE0428) has an additional identical sequence inserted in this region and another set of five strains (GBE1194-C13_2011V_1169) has a smaller identical sequence inserted in this region. CL 10_GBE1194 and CL 11_SLG4 have identical sequences, as do CL 12_FORC_055 and C13_2011V_1169, and these vary from each other only in the "invariable" region downstream of the stop codon. Likewise GBE1116 and IRL0081 have identical sequences except downstream within the "invariable" region. Within the first group of four (GBE0917-GBE0428), the only variability IRL0081 shows from GBE1116 is in the "invariable" 3' end. In other words, the appearance of variability in the 5' end is biased, I think, because there's two groups that have two slightly different insertions in the 5' end, but within these groups, the variability is not limited to the 5' or 3' ends. These argue against there being a "bimodular" nature of variable>invariable within this sRNA, since there is variability downstream as well, to the same degree (within groups) as upstream. The authors argue for a bimodular nature to the sRNA based on the difference between C6706 and GBE1114, because the differences between these two lie within the 5' region rather than the 3' region.

Minor edits:
Line 23: comprises a highly…
Line 29: colonization a). it stringently…
Line 30: and b). it confers resistance…
Line 59: Following ingestion of planktonic or biofilm-associated cells, V. cholerae …
Line 69: promoters of 39 genes

Line 71: …cholera, and ToxT, a master virulence regulator.
Line 77: resistance, and tolerance to…
Line 109: comprises a highly variable…
Line 114: and reveal specific…
Line 119: pathogenesis. However, it remains unclear…
Line 133: do not timely repress biofilm formation.
Line 148: defense response to phages and symbionts…
Line 159: OmpU represses biofilm formation and has a significant impact on the… (no evidence of allele-dependent manner at this point in manuscript).
Line 166: indicated potential sRNAs encoded…
Line 177: Interestingly, the lower bands are…
Line 291: inhibition mediated by OueS…
Line 316: "moderate reduction in ICP3"…there's no statistical relevance to this.
Line 581 directly affects host colonization
Line 608: 84% of the ToxR regulome
Line 611: specifically enriched during virulence-inducing

Reviewer #4

(Remarks to the Author)
This paper from the Almagro-Moreno group presents an exciting new discovery that extends our knowledge of V. cholerae pathogenicity. They identify a new sRNA - deemed OueS because it is encoded in the ompU gene - in studies of OmpU's role in biofilm formation. They then uncover data strongly implicating OueS in several important phenotypes including:

1) inhibition of biofilm formation;
2) changes in V. cholerae gene expression during biofilm formation that are associated with OmpU;
3) inhibition of RhyB expression/stability;
4) resistance to infection by phages ICP2 and ICP3 in a manner dependent on CBASS genes;
5) control of the expression of many genes in the ToxR regulon, particularly those related to motility and metabolism/nutrient utilization, but not the canonical V. cholerae virulence genes located in the horizontally acquired TCP island (Tcp pilus) and CTX prophage (cholera toxin);
6) promotion of intestinal colonization in suckling mice.

These phenotypes, some of which may have been mistakenly attributed to the OmpU porin, represent the paper's major strengths.

The paper's major weakness is in its framing starting with its title "Modular small RNA drives the emergence of virulence traits and environmental trade-offs in Vibrio cholerae".

There is No data presented here that support the major claim that an sRNA 'drives emergence' of virulence traits. Which figure supports this claim? Some environmental OueS alleles (GBE1194 and IRL 0074) support colonization that is equal to or better than the clinical isolate C6706 and other alleles have deficient colonization. Notably analysis of transcriptomes associated with different OueS alleles did not reveal functional enrichment, arguing against their claim that "our data indicate that a) the OueS allele encoded by toxigenic strains is a virulence preadaptation" [lines 498-502]. Overall, how can they claim that OueS is 'preadaptation'? Isn't it just as plausible that this sRNA evolved after the acquisition of the TCP island (the key step in evolution of V. cholerae as a human pathogen because it enables intestinal colonization) to further facilitate adaptation to the human small intestine/colonization by the sRNA's control of motility and metabolism?

Also, the data in 4C,E that toxigenic alleles lead to fitness trade-offs in colonization of Microscystis and Artemeia have p-vales but are very small differences and it is not clear that these experimental systems represent important environmental niches for V. cholerae.

There are repeated overclaims: (a few examples)

Intro line 108 "we describe a sRNA that …shapes the evolution of toxigenic V. cholerae. [this is not shown]

Line 110-11 'the bimodular nature of the OueS "generates allelic variants" ..[How does the fact that OueS being encoded in an ORF and UTR (which is not a 'unique' sRNA characteristic) create variation?]. Why does OueS 'modularity' "enable the emergence of phenotypes essential for colonization? Couldn't phenotypically important allelic variation be part of a non-modular sRNA?

Results line 508: 'directly contributing to the emergence of pathogenic V. cholerae from environmental populations' [this is not shown]

Last lines of the discussion go way beyond anything shown in the paper.

Many instances where they claim their data 'indicates' should be toned down.

Experimental work
1. Prove with mutational studies that OueS interacts with RyhB.
2. Present a phylogram with OueS alleles and predicted structures next to the phylogram of the respective genomes and the strain metadata.

Minor
Line 532 6-fold reduction does Not correspond to CI of 0.39. but of 0.16.
Line 651 'host transmission dynamics' There is no data about transmission in this paper.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manscript by Balasubramanian et al is greatly improved since the first version I read. It is now an elegant set of experiments that for the most part flow nicely and show the biology of this important and interesting sRNA that is found at the end of the ompU transcript. I have some minor comments below. More consideration needs to be taken on the part where the sRNA is being tested in competence and whether the allele reverts. There are not sufficient controls to be able to reach the conclusions. But overall a great job and I very much enjoyed reading this story again.

Line 118 – delete the agent of cholera.
Line 185 – nt is the standard way to shorten nucleotide – and throughout manuscript
Line 189 – RNA binding protein not sRNA binding protein. Move sRNA to further down the sentence.
Line 219 – are both 166 nt.
Line 219 – 220 – the predicted secondary structure of the two sRNAs…..
Line 219 – 222 – this sentence is massive, and given it covers an important finding of the manuscript, split in two.
Line 223 – surely its within the ORF? I can see what you are saying, but language needs to be more precise.
Line 411 – gene not protein
Labelling of Fig 4C – which lane is the ΔtoxR with expression of OueS? I assume the one next to the ΔtoxR strain? But it's not labelled so.
Fig 5A, B and C should be with figure 1 and should be moved to the supplementary.
Fig 5D and E supplementary
Line 641 – sIgA needs defining. As a non-immunologist this part lost me a little bit, so please explain the rationale of using sIgA more clearly.
Line 682 – need to expand on selection of which sRNAs you picked to work on and why?
Line 692 – Fig6 what?
Line 700 – PCG?
Line 708 – expand on details regarding Fig6E
Line 742 – typo
Line 800 – Are these conditions just changing transformability – how do you know that this is specific to this allele and not all DNA? This section of the manuscript detracts from the rest of the story – which is now really lovely. This whole section on revertants either needs deleting or more controls to put in to show that there is something special about this module and it's ability to switch/revert i.e. other parts of the V. cholerae chromosome don't change so easily.
Line 1206 – Lysogeny Broth - https://en.wikipedia.org/wiki/Lysogeny_broth
Line 1468 – doesn't make sense
Line 1506 – Fifty microlitres – 50 µl

Reviewer #2

(Remarks to the Author)
The authors have replied satisfactorily to all my concerns. Congratulations to this nice piece of work.

Reviewer #3

(Remarks to the Author)
The revised manuscript by Balasubramanian et al describes the discovery of a sRNA, OueS, in V. cholerae that is embedded within the ompU gene and that controls 84% of the ToxR regulon. This revised version is dramatically improved

from the original submission. The authors have added a substantial amount of new data in direct response to the previous reviewers' comments. These additional experiments were extensive and well-performed, and solidify the importance of this discovery by providing quite a bit of mechanistic detail into OueS and how it controls gene expression in V. cholerae that affects biofilm formation, phage resistance, and virulence. I am impressed with the depth of additional data presented, and satisfied with the manner in which the authors addressed previous comments to strengthen this manuscript.

My only (minor) comment is that after reading through the manuscript it began to bother me that the OueS from strain C6706 was referred to as "toxigenic OueS". While strain C6706 is a toxigenic strain, the sRNA is not "toxigenic", and the phenotypes being studied are not based on toxigenicity. I think the authors are trying to highlight that this OueS was identified in a clinical strain, rather than an environmental strain, so maybe "clinical OueS" might be a better term.

REVIEWER #1

1. Balasubramanian et al This is an interesting manuscript that I enjoyed reading. The authors have discovered a sRNA encoded in the 3' end of the ompU gene that is responsible for the increase in biofilm formation in a Δ*ompU* strain. The manuscript is generally well written.

Response: We thank the reviewer for the positive feedback and are truly appreciative of the time taken to provide so many useful suggestions. We are also glad to hear that the reviewer found the manuscript of interest and appreciated the clarity of our writing.

2. However, currently the experiments rely on a huge amount of RNA-seq that is not backed up with mechanistic details. Which is the major limitation of the manuscript. It feels in many places like the authors have found a lot of exciting things and just put them all into a story, rather than cherry picking.

Response: We truly appreciate that the reviewer highlights the fact that we made numerous exciting discoveries. We also concur with the fact that rather than "cherry picking", as kindly mentioned by the reviewer, we have performed a comprehensive and in-depth analysis of the sRNA for this article. We considered it was of critical importance to widely unearth the biological importance of the sRNA and how it contributes to virulence-associated phenotypes. This was a substantially demanding task, as, rather than decontextualizing the biological relevance of OueS by just studying one phenotype, we directly validated key transcriptomic findings through targeted phenotypic and molecular assays. We have now directly further validated transcriptomic predictions through molecular assays and identified mechanisms behind key phenotypes. This has led to new figures and tables. We truly believe these additions have significantly strengthened the manuscript. Some highlights are below:

- Biofilm and RyhB repression: We have now generated a brand-new figure and table that fully addresses the reviewer's concerns. Briefly, our functional group analyses of RNA-seq data identified downregulation of iron uptake associated genes to be one of the top functional categories associated with OueS expression (Fig. 2A). We hypothesized that iron might play a role in biofilm formation in a OueS-dependent manner and identified a strong association between the three (Fig. 2B). As suggested by the reviewer, in order to examine the genes that OueS directly binds to under these conditions, we have performed MS2 affinity purification coupled to RNA-sequencing (MAPS) to define the target profile of $OueS^{C6706}$. We also performed MAPS for $OueS^{mut}$ to determine sRNA specificity, and $OueS^{GBE1114}$ to define allele specificity. Our MAPS results show that $OueS^{C6706}$ directly binds to the iron-responsive regulator RyhB and does so in an allelic-dependent manner (Table 1 and Figs. 2C and D). We determined that the sRNA modulates the expression levels of RyhB (Fig. 2E) and using Northern blots, we discovered that expression of clinical OueS drastically reduces the levels of RyhB, whereas RyhB remains detectable when an environmental allele of OueS is expressed, suggesting that this effect on RyhB is allelic dependent (Fig. 2F).

As the reviewer suggested, further work was needed in order to determine **a)** the genetic determinants associated with allelic-specificity, **b)** the action of those in OueS target, and **c)** how did that affect phenotypes. For this, we examined the exact difference between the two alleles and uncovered four specific domains in the 5' module that differentiate $OueS^{C6706}$ and $OueS^{GBE1114}$ (Fig. 2G). As highlighted in Figure 1, $OueS^{C6706}$ and $OueS^{GBE1114}$ are identical in their 3' module (Fig. 2G). To determine the specific role of these domains in biofilm formation and RyhB suppression, we constructed 9 new mutants where we swapped the individual domains in $OueS^{C6706}$ for their environmental $OueS^{GBE1114}$ versions and created combination mutants of domains generating several "chimeric" $OueS^{C6706}$ versions. All chimeric mutants in the 5' module are expressed as evidenced by qRT-PCR (Fig. 2H). Two domains appear to be critical for the emergence of the biofilm repression phenotype: 2 and 3 (Fig. 2I). This phenotype is matched by Northern blots, where in domain mutants for 2 and 3 RyhB is strongly detectable (Fig. 2J, 2K). Overall, the domains exhibit a complex set of phenotypes where some of them are required for biofilm suppression (Fig. 2I) and RyhB degradation whereas others are not (Fig. 2J, 2K). Given the number of discoveries and subfigures, the article's section on biofilm repression via direct suppression of RyhB is now one full figure.

- Phage resistance: We have now generated a brand-new figure and table that fully addresses the reviewer's concerns. Briefly, our functional group analyses of RNA-seq data identified upregulation of genes associated with viral and symbiont defense to be one of the top functional categories associated with OueS expression (Fig. 3A). We hypothesized that OueS might be associated with resistance against phages as the ORF encodes a porin that acts as receptor of an intestinal phage. We show that clinical OueS confers resistance against ICP2 (Fig. 3B) and ICP3 (Fig.3C), two well-characterized intestinal phages. This phenotype is sRNA-specific and allelic dependent as $OueS^{mut}$ and an environmental allele of OueS, respectively, do not exert that protective effect. We mined the transcriptomic data looking for potential defense systems. Our analyses revealed that OueS activates the transcription of the cyclic-oligonucleotide-based antiviral signaling system (CBASS) operon (Fig. 3E and 3F). Next, we constructed deletion mutants of three genes in the CBASS operon with OueS-dependent transcription in two backgrounds: clinical OueS and environmental OueS and determined that clinical OueS protects against ICP2 (Fig. 3G) and ICP3 (Fig. 3H) via the CBASS system.

Next, as requested by the reviewer, in order to define the specific mechanisms mediating the activation of CBASS by $OueS^{C6706}$ given that a sRNA could not directly activate a system, we hypothesized that OueS was suppressing a repressor of the CBASS systems. In order to test our hypothesis, we looked for potential CBASS repressors from our MAPS dataset (Table 1) and identified that $OueS^{C6706}$ bound to *glyA*, a metabolic regulator of folate levels that represses CBASS (Fig 3D). We constructed deletion mutants of the three genes in the different backgrounds described above and tested their resistance against ICP2 (Fig. 3I) and ICP3 (Fig. 3J). Our results show that in the absence of *glyA*, the role of OueS is no longer necessary to elicit resistance against the phages. Furthermore, Northern blot data shows that OueS directly represses *glyA* in an allelic-dependent manner (Fig. 3K). Furthermore, we examined the role of the 9 domain mutants in phage resistance and strikingly found that, unlike for biofilm repression, all domains are essential for protection against ICP3 indicating that each of

the domains is required for this phenotype to emerge (Fig. 3L). Paired with our results above, it appears that epistatic interactions between the domains control the emergence of these diverse phenotypes in novel ways. We believe that this a truly exciting mechanism and unraveling the intricacies leading to these epistatic effects certainly deserves further attention in future studies. All this new data is now part of a new figure (Fig. 3) that contains all the work addressing the reviewer's concerns.

- ToxR regulon surrogate and mucin penetration: We have now addressed the reviewer's comments and also identified a much more critical phenotype connecting ToxR and OueS: mucin penetration. This is now part of Figure 4 which has been fully updated. In Fig. 4, now we show by Northern Blot that ToxR regulates the production of OueS (Fig. 4A). Subsequently, we show the first phenotypic connection between ToxR and OueS (Fig. 4B). Deletion of ToxR leads to an increase in biofilm formation and that is reverted by OueS in an allelic-dependent manner. We show that this phenotype is dependent on RyhB derepression as evidenced by northern blots, which reverts to WT upon expression of $OueS^{C6706}$ (Fig. 4C). Our principal component analysis indicates that there is an intimate relationship between $OueS^{C6706}$ and ToxR as expression of the former in a Δ*toxR* background results in a WT-like transcriptome under virulence-inducing conditions (Fig 4D). Subsequently, we demonstrate that, unlike the environmental allele, clinical OueS controls 84% of the ToxR regulome (Fig 4E). We took a similar approach as above and looked at the functional categories that OueS is responsible for in the ToxR regulome (Fig. 4F, 4J). Specifically, we demonstrate that OueS is responsible for the activation of ToxR-controlled genes associated with motility and chemotaxis (Fig. 4F). Besides a role in motility (Fig. 4G), to address the reviewer's concerns, we translated our findings into phenotypes that are more closely related to the infectious process. We examined the role of OueS in mucin penetration using column-based assays and determined that $OueS^{C6706}$ is essential for successful crossing of a mucin layer (Fig. 4H). This phenotype is allelic-dependent as strains encoding $OueS^{GBE1114}$ exhibit a similar phenotype as the Δ*ompU* mutant (Fig. 4H). Furthermore, we have now examined the role of the OueS domains on this phenotype (Fig. 4I). We found a very complex relationship between the domains and this phenotype, where some of them are exchangeable if in combination (Fig. 4I). This suggests novel complex epistatic interactions at the sRNA levels that we are excited to pursue in future work. Finally, given that the carbon repression phenotype is not allelic-dependent we were unable to determine the specific role of the domains in this phenotype (Fig. 4J, K, L).

- Intestinal colonization. To address this and other reviewer's concerns below, we have now fully updated what was previously figure 4. They have now become three independent figures allowing us to substantially expand and clarify our findings. In what is now Figure 5 we investigate the regulation and the role of OueS during intestinal colonization. In order to confidently investigate the role of the sRNA during colonization, we aimed to construct mutants in the genome using the native promoter. To do that, first we began looking for the OueS promoter. In Fig 5A we show the schematic of the mutation protocol to identify the promoter. We used derepression of biofilm formation as a phenotypic readout to determine loss of OueS production. The data indicates the lack of an internal promoter and that deletion of the *ompU* promoter led to a similar phenotype

as Δ*ompU* (Fig. 5B). We corroborated by northern blotting that deletion of the *ompU* promoter abolishes expression of OueS (Fig. 5C). Next, we constructed isogenic mutants where we cloned OueS in *cis* directly downstream of the *ompU* promoter and, as requested in a comment below, show that the expression levels of OueS are similar (Fig. 5D) and it retains its function (Fig. 5E). Using a set of *cis*-encoded mutants, we unequivocally show that OueS is important during intestinal colonization. To expand on this, we wondered why a sRNA with such wide array of infection critical phenotypes only provided a 1-log impairment in intestinal colonization. We realized that the selective pressures needed for those phenotypes to provide a competitive advantage were not encountered in WT mice. In Fig. 5G we show that supplementation with s-IgAs (biofilm repression) and ICP3 (phage resistance) highlight the critical relevance of the sRNA during intestinal colonization in a more realistic scenario (Fig. 5G). We believe that this addition has many consequences in our field as it highlights the likelihood on numerous false negatives missed during general screenings as they might not exhibit a large phenotype in the WT animals. Overall, we believe that this figure solidly highlights the relevance of OueS during colonization and the mechanisms controlling its expression.

3. The sRNA encoded at the 3' end of the ompU gene is interesting in that as outlined in the title is modular. This is interesting and novel. More should be made to explain this in the text. The figures determining the conservation within the supplementary section could be improved and moved to the main text.

Response: We thank the reviewer for recognizing the novelty of the modular nature of OueS and the importance of the sequence conservation data. Nonetheless, we agree that not enough justification was provided and the justification we did provide was obscured in the supplementary material. In response to this suggestion, we have generated two new figures to dissect and highlight this phenomenon and expanded the Results and Discussion sections to address this: In Figure 6, we discuss the modular nature of OueS based on its allelic variations, and in Figure 7, we develop a method to examine the acquisition of the *ompU* ORF under conditions simulating *V. cholerae* aquatic reservoir. The data shows that the ORF can be acquired as a module based on external conditions, which is associated with the emergence of virulence-associated phenotypes.

Specifically, in Fig. 6 we have included a schematic of the variability of the OueS alleles contextualized within the *ompU* ORF (Fig 6A). This visually highlights how variable the 5' end of the sRNA is in comparison to the conserved 3' end. We have also included an alignment of the different representative alleles (Fig. 6B) and a phylogenetic tree of the OueS alleles highlighting the ones that we cloned in the C6706 background for further analyses (Fig. 6C). In Fig. 6D we highlight their structural differences all contained within the 5' end. Finally, we reconstructed a phylogenomic tree of >1800 *V. cholerae* genomes and indicate all the clades that have identical alleles as the strains from the PCG to elucidate their origin as a preadaptation (Fig. 6E). We measured the expression of the allelic mutants and demonstrate that they were expressed at similar levels as WT (Fig. 6F). Finally, we examined their role during intestinal colonization, both in WT mice (Fig. 6G) and under host-associated conditions (Fig 6H), that highlights the allelic nature of OueS in this process.

To further highlight the modular nature of OueS and strengthen our arguments, we have generated another new figure where we show that the acquisition of the *ompU* ORF is modular and controlled by environmental conditions (Fig. 7). In Fig. 7A we show the schematic of the modified chitin-induced natural transformation protocol that we developed to examine transfer of *ompU* modules. In Fig. 7B and 7C, we show acquisition of the DNA using i) qRT-PCR showing variation in normalized reporter fluorescence (Rn) versus cycle number before and after transformation and ii) the transformation frequency of different experimental groups. We show that revertants acquired the full *ompU* ORF (Fig. 7D) and gain the phenotypes associated with clinical alleles of OmpU porin and OueS sRNA in a modular fashion: (E) bile resistance (0.4% whole bile), (F) mucin penetration, (G) biofilm formation, (H) resistance to cationic antimicrobial peptide P2 (200 µg/mL), (I) acidic pH (pH 5.5), and (J) intestinal colonization. Finally, in fig. 7K we show that the acquisition frequency of *ompU* ORF is controlled by environmental conditions.

Overall, we believe these major additions have significantly strengthened our manuscript and are truly thankful for the suggestion.

4. The authors express the OueS sRNA from an ectopic plasmid pBAD22 and use this for their RNA-seq experiments and some of the follow up. sRNA levels are generally finely tuned within the cell and therefore making conclusions regarding behaviour based off of an overexpressed gene is challenging. The authors have a chromosomally integrated complementation strain of the ompU promoter directing OueS, this would have been more suitable for some of the experiments as the sRNA is likely to be at levels more similar to the native version, although the Northern for the ompU where OueS was identified suggests low/lower levels of OueS compared to the ompU transcript. The manuscript requires determination of how much sRNA is produced in the WT strain vs how much is produced in the ectopic strain vs how much sRNA is produced from the ompU promoter. The authors carry out an experiment in virulence inducing conditions, but do not determine how much sRNA is produced in this condition compared to the other conditions that they test. In figure 1 what is the media that is used.

Response: As suggested by the reviewer, we have determined the levels of OueS in the different strains and included that as either part of the main text or the supplementary material of the article. Quantification of OueS levels by quantitative real-time PCR show that a) strains with domain mutants exhibit similar levels of expression as WT (Fig. 2H), b) strains expressing OueS from P_{ompU} exhibit similar levels of expression as WT (Fig. 5D and E), c) strains expressing different alleles of OueS exhibit similar levels of expression as WT (Fig. 6F), and, d) ectopic OueS expression from pBAD22 is 8-fold higher on average than WT (updated as Fig S5A). However, phenotypic analysis reveals no difference in OueS-mediated biofilm repression in the native (WT) strains or those that express OueS from a chromosomal complementation under P_{ompU} or ectopic expression from pBAD22 (updated as Fig S5B). We have also included this information in the text (Lines 617-621). Furthermore, the transcriptomes from strains ectopically expressing OueS are similar to those of strains expressing it from the native promoter (Supplementary Tables).

5. Given that the sRNA is likely produced by processing the ompU transcript the level of ompU should also be defined. Is the sRNA not appearing any more because there is no more ompU transcript or is it that there is some other change that stops the processing.

Response: We appreciate the reviewer's insightful comment regarding the relationship between *ompU* transcript levels and OueS production as it has yielded some interesting insights. We have now included 3 sections in Figure 1 that address this (Fig 1G-1I). Overall, there seems to be two different time dependent dynamics between OueS and *ompU*. During early time points of growth, *ompU* exhibits consistent levels of expression whereas OueS levels increase overtime. On the other hand, after 9h there is a drastic drop in the levels of both. This is quite interesting and unexpected and certainly deserves further investigations on its physiological and regulatory implications. We have now noted this in both the results and discussion sections.

6. RhyB regulation – needs point mutations in both RhyB and OueS to show the regulation is direct and comes from the interaction of the pair of them. Include complementary point mutations that restore the regulation.

Response: As described above we have now generated a brand-new figure and table that fully addresses the reviewer's concerns. As suggested by the reviewer, in order to examine the genes that OueS directly binds to RyhB under iron limiting conditions, we have performed MS2 affinity purification coupled to RNA-sequencing (MAPS) to define the target profile of $OueS^{C6706}$. We also performed MAPS for $OueS^{mut}$ to determine sRNA specificity, and $OueS^{GBE1114}$ to define allele specificity. Our MAPS results show that OueS directly binds to the iron-responsive regulator RyhB and does so in an allelic-dependent manner (Table 1 and Figs. 2C and D). We determined that the sRNA modulates the expression levels of RyhB (Fig. 2E) and using northern blots, we discovered that expression of clinical OueS drastically reduces the levels of RyhB, whereas RyhB remains detectable when an environmental allele of OueS is expressed, suggesting that this effect on RyhB is allelic dependent (Fig. 2F).

As the reviewer suggested, further work was needed in order to determine **a)** the genetic determinants associated with allelic-specificity, **b)** the action of those in OueS target, and **c)** how did that affect phenotypes. For this, we examined the exact difference between the two alleles and uncovered four specific domains in the 5' module that differentiate $OueS^{C6706}$ and $OueS^{GBE1114}$ (Fig. 2G). As highlighted in Figure 1, $OueS^{C6706}$ and $OueS^{GBE1114}$ are identical in their 3' module (Fig. 2G). To determine the specific role of these domains in biofilm formation and RyhB suppression, we constructed 9 new mutants where we swapped the individual domains in $OueS^{C6706}$ for their environmental $OueS^{GBE1114}$ versions and created combination mutants of domains generating several "chimeric" $OueS^{C6706}$ versions. All mutants in the 5' module are expressed as evidenced by qRT-PCR (Fig. 2H). Two domains appear to be critical for the emergence of the biofilm repression phenotype: 2 and 3 (Fig. 2I). This phenotype is matched by northern blots,

where in domain mutants for 2 and 3, RyhB is strongly detectable (Fig. 2J). Overall, the domains exhibit a complex set of phenotypes where some of them are required for biofilm suppression (Fig. 2I) and RyhB degradation whereas others are not (Fig. 2J, 2K). Given the number of discoveries and subfigures, the article's section on biofilm repression via direct suppression of RyhB is now one full figure.

7. The section on the correlation between the ToxR and OueS regulons is interesting. Especially for an sRNA to be regulating such a large regulon of genes. The major weakness in this section is again the lack of molecular work – is it likely a direct regulation of the different targets due to an interaction with OueS?

Response: We appreciate the reviewer recognizing the importance of our findings linking OueS to the ToxR virulence regulome. As the reviewer points out our transcriptional, molecular and phenotypic data shows that OueS controls the transcription of genes initially thought to be modulated by ToxR and is responsible several phenotypes associated with the regulator. Parsimoniously, we cannot think of another scenario that explains that OueS overlaps the same levels of transcriptional changes of hundreds of ToxR-regulated genes and does not regulate them as a ToxR surrogate. Furthermore, as Fig. 4 now shows, OueS fully recapitulates the role of ToxR even in the absence of the regulator (e.g. biofilm, motility, carbon utilization, transcriptome). This is consistent throughout several mutants and alleles of the sRNA. To further, proof the molecular and phenotypic overlap between OueS and ToxR we have now substantially expanded Fig. 4 (what was Fig. 3 before). We have now addressed the reviewer's comments and also identified a much more critical phenotype connecting ToxR and OueS: mucin penetration. In Fig. 4, now we show by northern blot that ToxR regulates the production of OueS (Fig. 4A). Subsequently, we show the first phenotypic connection between ToxR and OueS (Fig. 4B). Deletion of ToxR leads to an increase in biofilm formation and that is reverted by OueS in an allelic-dependent manner. To highlight and strengthen the molecular connection between OueS and ToxR, we show that this ToxR-dependent phenotype is dependent on RyhB derepression as evidenced by northern blotting, which reverts to WT upon expression of OueS (Fig. 4D). Our principal component analysis indicates that there is an intimate relationship between OueS and ToxR as expression of the former in a Δ*toxR* background results in a WT-like transcriptome under virulence-inducing conditions (Fig 4C). Subsequently, we demonstrate that, unlike the environmental allele, clinical OueS controls 84% of the ToxR regulome (Fig 4E). We took a similar approach as above and looked at the functional categories that OueS is responsible for in the ToxR regulome (Fig. 4F, 4J). Specifically, we demonstrate that OueS is responsible for the activation of ToxR-controlled genes associated with motility and chemotaxis (Fig. 4F). Besides a role in motility (Fig. 4F), to address the reviewer's concerns, we translated our findings into phenotypes that are more closely related to the infectious process. We examined the role of OueS in mucin penetration using column-based assays and determined that $OueS^{C6706}$ is essential for successful crossing of a mucin layer Fig. 4H. This phenotype is allelic-dependent as strains encoding $OueS^{GBE1114}$ exhibit a similar phenotype as the Δ*ompU* mutant (Fig. 4H). Furthermore, we have now examined the role of the OueS domains on this phenotype (Fig. 4I). We found a very complex relationship between the domains and

this phenotype, where some of them are exchangeable if in combination (Fig. 4G). This suggests novel complex epistatic interactions at the sRNA levels that we are excited to pursue in future work. Finally, given that the carbon repression phenotype is not allelic-dependent we were unable to determine the specific role of the domains in this phenotype (Fig. 4J, K, L). Taken together, we show that there is no other possible logical and parsimonious explanation that can explain these molecular and phenotypic changes other than OueS acting as a functional surrogate of ToxR. This makes a lot of sense as it is a simple yet sophisticated way by which *V. cholerae* can control numerous genes by controlling only one promoter.

In the discussion it is reported that ToxR only binds to the promoter of 39 genes. One of these being ompU that through this regulation also regulates OueS. Therefore the title at line 348 describing OueS as a functional surrogate of ToxR is inaccurate. It is actually the ToxR regulation of a further regulator that is likely responsible for the regulation of the other genes that have been reported to be part of the ToxR regulon.

Given our data and explanations above, we respectfully disagree with this statement. If that was the case, besides it not being evolutionarily parsimonious, it would contradict our results explained above as OueS complements ToxR at the phenotypic and molecular level. Furthermore, we show it does it in an allelic-dependent manner. For instance, among others, OueS should not complement the phenotypes of Δ*toxR* strains if ToxR was controlling regulators other than OueS. E.g. Δ*toxR* derepresses biofilm and RyhB and the sole expression of clinical OueS in the Δ*toxR* background reverts WT phenotype. Similarly, Δ*toxR* strains are severely impaired for mucin penetration and the sole expression of clinical OueS in the Δ*toxR* background reverts WT phenotype.

The details about the 39 promoters needs to be brought up front to the results section. This section again needs to be backed up by some molecular work to show that it is indeed a direct regulation by OueS and not a causative effect of OueS possibly acting through another pathway.

We agree with this. It is possible that OueS does not directly bind to every single one of the genes it regulates. In fact, our data shows that it controls regulators downstream (e.g. RyhB and CBASS system). We have modified the text to make sure it is clear that despite ToxR-regulated genes being regulated by OueS, this not being necessarily due to direct interaction of the sRNA with those genes. Additionally, as suggested by the reviewer, we now make the point of direct ToxR binding to only 39 promoters in the results section as well (Lines 465-469).

Is there crossover between the list of genes in the ToxR RNA-seq data set not directly regulated that also overlap with OueS target prediction. State why virulence inducing conditions are the most appropriate.
According to our extensive transcriptomic analyses, OueS regulates ToxR-associated genes. To address the reviewer's concern, we have now clearly stated in the text why we

examined the overlap between ToxR and OueS using AKI conditions (Lines 472-475). Briefly, in order to examine the role of OueS and ToxR during colonization, the closest *in vitro* conditions available are AKI conditions. This would also let us know if OueS also played a role in the regulation of virulence factors such as CT or TCP, which are only expressed under these conditions outside of the host.

8. Motility section – which genes is it likely regulating? Molecular work to back this up? Many of the figures are made up of two subjects – e.g. 2 – iron and CBASS. This detracts from the story and makes it more challenging to follow.

Response: We appreciate the reviewer's concern regarding clarity and mechanistic depth. To improve presentation, we have separated the RyhB and CBASS datasets into two independent figures (now Figs. 2 and 3), which substantially clarifies the narrative structure.

To directly address the mechanistic basis of OueS-mediated motility regulation, we expanded Figure 4 to define the relationship between ToxR and OueS at both the regulatory and phenotypic levels. Northern blot analyses now demonstrate that ToxR regulates OueS production (Fig. 4A). We further show that deletion of *toxR* increases biofilm formation, which is reverted in an allele-dependent manner by OueS, and that this phenotype is mediated through RyhB repression (Fig. 4B–C).

Transcriptomic analyses reveal a tight regulatory coupling between ToxR and OueS, as expression of $OueS^{C6706}$ in a *ΔtoxR* background restores a WT-like transcriptional profile under virulence-inducing conditions (Fig. 4D). Notably, clinical OueS regulates ~84% of the ToxR regulome, whereas the environmental allele does not (Fig. 4E). Functional enrichment analysis identifies motility and chemotaxis genes among the OueS-controlled ToxR-dependent categories (Fig. 4F), including flagellar and chemotaxis operons, providing molecular support for OueS-mediated regulation of motility.

To translate these transcriptional findings into infection-relevant phenotypes, we examined mucin penetration, a motility-dependent trait critical for intestinal colonization. Using column-based mucin penetration assays, we show that $OueS^{C6706}$ is required for efficient traversal of a mucin barrier in an allele-dependent manner (Fig. 4H). Domain-swapping analyses further reveal complex epistatic interactions among OueS domains in controlling this phenotype (Fig. 4I), suggesting modular regulatory architecture within the sRNA.

Finally, although carbon repression phenotypes were not allele-dependent and thus not amenable to domain mapping, we present these data for completeness (Fig. 4J - 4L).

Together, these additions provide mechanistic and phenotypic evidence linking OueS to motility, chemotaxis, and host-relevant mucus penetration, directly addressing the reviewer's concerns.

9. Figure 2H – How does the 823 and 841 of the CBASS promoter relate to the Transcriptional start site? sRNAs regulate at the RNA level. Again need to be backed up with molecular work, not just showing RNA-seq levels. GFP fusion or something similar.

Response: We really appreciate the reviewer's comment, and, upon further review, we have revised our original numbering, as it was based on the length of the input promoter DNA and did not conform to the conventional representation (see response to comment #6). Additionally, we have removed the computational prediction interactions from the manuscript and modified the text accordingly. Furthermore, we have now generated a brand-new figure and table that fully addresses the reviewer's concerns.

Briefly, our functional group analyses of RNA-seq data identified upregulation of genes associated with viral and symbiont defense to be one of the top functional categories associated with OueS expression (Fig. 3A). We hypothesized that OueS might be associated with resistance against phages as the ORF encodes a porin that acts as receptor of an intestinal phage. We show that clinical OueS confers resistance against ICP2 (Fig. 3B) and ICP3 (Fig.3C), two well-characterized intestinal phages. This phenotype is sRNA-specific and allelic dependent as $OueS^{mut}$ and an environmental allele of OueS, respectively, do not exert that protective effect. We mined the transcriptomic data looking for potential defense systems. Our analyses revealed that OueS activates the transcription of the cyclic-oligonucleotide-based antiviral signaling system (CBASS) operon (Fig. 3E and 3F). Next, we constructed deletion mutants of three genes in the CBASS operon with OueS-dependent transcription in two backgrounds: clinical OueS and environmental OueS and determined that clinical OueS protects against ICP2 (Fig. G) and ICP3 (Fig. 3H) via the CBASS system.

Next, as requested by the reviewer, in order to define the specific mechanisms mediating the activation of CBASS by $OueS^{C6706}$ given that a sRNA could not directly activate a system, we hypothesized that OueS was suppressing a repressor of the CBASS systems. In order to test our hypothesis, we looked for potential CBASS repressors from our MAPS dataset (Table 1) and identified that $OueS^{C6706}$ bound to *glyA*, a metabolic regulator of folate levels that represses CBASS (Fig 3D). We constructed deletion mutants of the three genes in the different backgrounds described above and tested their resistance against ICP2 (Fig. 3I) and ICP3 (Fig. 3J). Our results show that in the absence of *glyA*, the role of OueS is no longer necessary to elicit resistance against the phages. Furthermore, Northern blot data shows that OueS directly represses *glyA* in an allelic-dependent manner (Fig. 3K). Furthermore, we examined the role of the 9 domain mutants in phage resistance and strikingly found that, unlike for biofilm repression, all domains are essential for protection against ICP3 indicating that each of the domains is required for this phenotype to emerge (Fig. 3L). Paired with our results above, it appears that epistatic interactions between the domains control the emergence of these diverse phenotypes in novel ways. We believe that this a truly exciting mechanism and unraveling the intricacies leading to these epistatic effects certainly deserves further attention in future studies. All this new data is now part of a new figure (Fig. 3) that contains all the work addressing the reviewer's concerns.

10. Line 319 – Ectopic expression of OueS – how do the levels vary between the WT and this strain. Explain strain construction in the main text – i.e. how strong the promoter is, copy number etc. Northern blot or qRT-PCR to show the difference in the level. Be careful over phenotypes that require large amounts of overexpression to generate. How likely is this to be real in the wild. I would have

expected to see a difference between the WT and some of the other strains, if only small.

Response: We thank the reviewer for raising this important point. To clarify and strengthen our presentation, we have now expanded the Methods (Lines 1239-1241) to describe the ectopic-expression system:

1. Plasmid and promoter architecture: OueS is cloned into the pBAD22 vector under control of the arabinose-inducible P_{BAD} promoter (new Lines 1247-1247).
2. Expression-level quantification: Although the plasmid copy number is not easily determined, we quantified OueS expression upon arabinose induction and have included the data as part of the supplementary material. Under 0.01 % arabinose induction (the concentration we used for *in vitro* assays), qRT-PCR shows an ~8-fold increase in OueS levels relative to wild-type (new Fig. S5A), and the ectopic expression strain phenocopies the WT strain (new Fig S5B).

We agree that excessive overexpression can produce artifactual phenotypes. By presenting both the high-sensitivity ectopic system (to capture subtle effects) and the chromosomal promoter strain (to mimic realistic expression windows), we show that OueS function is consistent across a 1-8-fold expression gradient. In the Discussion, we now note this (Lines 875-879), and we are developing a more sensitive assay system to directly measure OueS induction under host-mimicking conditions in future work. With these additions, we address the reviewer's concerns about expression levels, strain construction, and physiological relevance.

11. The section on ToxR regulation makes more sense if it comes directly after the identification. This is how the sRNA is regulated, at least at the transcriptional level. Whether this section is split away from the transcriptomics might make more sense, but while I was reading the RACE I wanted to know how the gene was being regulated.

Response: We thank the reviewer for this excellent suggestion. To address this, we moved the "ToxR regulation of OueS" section immediately after the RACE-based mapping of the OueS termini. Although this arrangement did indeed place transcriptional control front and center, it interrupted the logical progression from (i) identification of the sRNA, through (ii) its global transcriptomic effects, to (iii) downstream phenotypic and molecular assays. In our opinion, readers would lose the contextual link of OueS expression levels to the functional consequences we document later, particularly the modulation of biofilm, phage resistance, and the ToxR regulon itself. After careful consideration and different arrangements, we thought that it would be more suitable at the beginning of what is now Figure 4. This way the figure addresses the relationship between OueS and ToxR starting by the fact that the latter regulates the production of the sRNA. We believe this brings together the suggested emphasis on regulatory mechanism and maintains a stepwise presentation of our findings.

Line 24 – Region not one

Response: This has been changed.

Line 31 – Define CBASS
Response: CBASS is now defined.

Line 31 – Toxigenic OueS – Define better, at least here. Doesn't make sense to a non-vibrio specialist. OueS expression in toxigenic….
Response: Changed to "Toxigenic variants of OueS alleles"

Line 108 - V. cholerae
Line 110 – region
Line 111 – Make it a sentence, not a and b.
Response: We have made the above changes.

Figure 1B does not add to the story. Either remove it or put it in the supplementary.
Response: Thank you for the comment. However, we believe that the non-overlap with RpoE (now Fig 1C) is an important finding that delineates and sets the stage for the contribution of OueS. In order to clarify this, we have added as small schematic (Fig 1B) that highlights the rationale behind it. Without this figure, we believe that readers that work on stress responses would likely think that the transcriptomic changes are due to the effect of porin activation of the cell envelope stress response.

Line 150 – explain better what is happening in the absence of OmpU. It's not misfolded, but totally gone. How does this cause changes in the RpoE regulon.
Response: We thank the reviewer for highlighting the need to explain the mechanistic basis of RpoE activation in the Δ*ompU* background. OmpU is a major outer-membrane protein and its misfolding upon exposure to external insults such as cationic antimicrobial peptides activates the RpoE-mediated cell envelope stress response. To disentangle RpoE-dependent effects from potential others, we directly compared RNA-seq profiles of Δ*ompU*, Δ*rpoE*, and wild-type strains under identical conditions. Genes upregulated in Δ*ompU* but not in Δ*rpoE* reflect RpoE-mediated responses to OmpU loss, whereas those altered only in Δ*ompU* reveal RpoE-independent consequences of porin absence. We have added an explanation in the Results that reflects this (Lines 165-167): "By normalizing the Δ*ompU* and Δ*rpoE* datasets to wild type, we isolated the RpoE-dependent changes in gene expression from additional, RpoE-independent transcriptional shifts unique to *ompU*."

Line 188 – This section would benefit from a sequence alignment. It would be good to show how the sequence differs between the toxigenic and non-toxigenic. Is there some variation in the amino acid encoded from the 3' end of the OmpU protein sequence due to the expression of this sRNA.

Response: We thank the reviewer for bringing this up. We have now included three sequence alignments in the article: One where we compare C6706, GBE1114 and the 5'mut, 3'mut and FLmut variants (Fig. 1J), a second where we highlight the differences between the OueS alleles (Fig. 6A and 6B), and third, of the entire *ompU* ORF and 3' UTR from C6706 and GBE1114, as part of the data on the revertants (Fig S7).

Furthermore, there are differences in the amino acid sequence in 3' end of OmpU between C6706 and GBE1114. We recently explored the landscape of diversity in OmpU and their associated phenotypic characteristics in previous publications (PMID: 27991885, 36972246). Interestingly, the number of changes is consistent throughout the protein. If the reviewer thinks that the manuscript would benefit from including an alignment of the actual proteins, we would be happy to do so.

Figure 1G – How does expression of the sRNA relate to the levels of OmpU? Figure 1H – provide a figure to show the regions that were used for the complementation experiment.

Response: We are thankful for this comment as it substantially increases the clarity of our argument. We have now included three sections in Fig 1 that address this: 1G now includes a Northern of *ompU*, 1H shows the relative expression levels of *ompU*, 1I shows the relative expression levels of OueS, and 1J presents an alignment with the sequences of the different OueS variants.

Line 240 – don't use a, b and c. Make it a sentence.
Response: This has been fixed.

Line 246 – delete first sentence – surplus to requirements.
Response: We have modified this sentence.

Figure 2H – How does the 823 and 841 of the CBASS promoter relate to the Transcriptional start site?

Response: We have removed the computational prediction subfigure and the associated text. We have also toned down the claim of direct target interaction.

Line 285 – what tool did you use for the prediction
Response: We used IntaRNA. However, we have now removed this predictive analysis as it was inconsistent between different computational tools which led to misleading scenarios.

Line 287 – A northern does not look at stability unless you have undertaken a rifampicin time course. This just tells you the level.
Response: Thank you. We now refer to this as 'levels of RyhB' and not RNA stability.

Line 503 – 506 – I don't understand what the authors are trying to explain in terms of target, CI and OueS type?
Response: Thank you for bringing this up. The message here is that there was no correlation between the sequence or structure of OueS alleles and their colonization abilities, despite the modular nature of the sRNA. We have now rephrased it to make it clearer (Lines 806-809).

Line 648 – this is quite repetitive

Response: While we understand the reviewer's concern, we believe that this summary statement succinctly clarifies our findings to the reader and lays the groundwork for our scenario explaining the emergence of pathogenic traits. We tried to remove it and given the amount of data and findings uncovered up to that section, we considered after discussing with colleagues that not having it made it difficult to follow.

Line 705-707 – Text unrequired?
Response: We agree and have removed the sentence.

REVIEWER #2:

1. In their manuscript entitled "Modular small RNA drives the emergence of virulence traits and environmental trade-offs in Vibrio cholerae" Balasubramanian et al. report that the small regulatory RNA OueS plays a central role in toxicity of *Vibrio cholerae* strains.
That OueS is an interesting sRNA that regulates biofilm formation and confers phage resistance is well supported by the data, but other claims, namely the modular architecture of OueS and the existence of different alleles are not. For details please see the following questions and comments:

Response: We thank the reviewer for their thoughtful assessment and for recognizing the significance of OueS in regulating key virulence-associated traits such as biofilm formation and phage resistance. We have carefully addressed the concerns raised regarding the modular nature and allelic variation of OueS and have revised the manuscript to clarify and support these aspects more explicitly.

2. Do the ompU mRNA and the OueS sRNA have the identical 3' end? Currently this is not explicitly mentioned.

Response: We thank the reviewer for bringing this up. Our read mapping data from transcriptome and RACE experiments indicate that they have identical ends. We have now included in the text (Line 206).

3. The authors already claim in the title that the sRNA is modular, where one part is in the ompU ORF and the other in the UTR. Notwithstanding this interesting chimeric origin, the term modular is too strong in this case. Modules are parts with independent functions that can be combined to provide new functionalities. The presented results only show that the overall functionality of the sRNA is affected by mutations in any or both of the two parts, and that the 3' part may interact with other RNAs (RyhB and 5'UTR of CBASS). There is no evidence for a distinct function of the 5' part. Furthermore, the overlap with the ompU ORF makes it difficult to evolve a function here.

Response: We thank the reviewer for recognizing the chimeric origin of OueS, which is a truly fascinating finding of this study with far-reaching physiological implications for the bacteria. We agree that our previous version of the manuscript did not properly explain the reasons behind our conclusions. For this reason, we have updated each figure and generated several entirely new ones to dissect and highlight this phenomenon and expanded the Results and Discussion sections to address this. The article is now 9 figures and 1 table long compared to 5 original figures. In figure 6 we discuss the modular nature of OueS based on its allelic variations and in figure 7 we develop a method to examine the acquisition of the *ompU* ORF and show that ORF can be acquired as a module based on external conditions, which is associated with the emergence of virulence-associated phenotypes. Furthermore, in Figures 2, 3, and 4 we tested 9 new mutants that we generated based on domains exclusively found in the 5’ end of OueS. Overall, we believe

these major additions have significantly strengthened our manuscript and are truly thankful for the suggestion.

Specifically, in Fig. 6 we have included a schematic of the variability of the OueS alleles contextualized within the *ompU* ORF (Fig 6A). This visually highlights how variable the 5' end of the sRNA is in comparison to the conserved 3' end. We have also included an alignment of the different representative alleles (Fig. 6B) and a phylogenetic tree of the OueS alleles highlighting the representative ones that we cloned in the isogenic C6706 background (Fig. 6C). In Fig. 6D, we highlight their structural differences all contained within the 5' end. Finally, we reconstructed a phylogenomic tree of >1800 *V. cholerae* genomes and indicate all the clades that have identical alleles as the strains from the PCG to elucidate their origin as a preadaptation (Fig. 6E). We measured the expression of the allelic mutants to determine whether they were expressed at similar levels as WT (Fig. 6F). Finally, we examined their role during intestinal colonization (Fig. 6G, 6H).

To further highlight the modular nature of OueS and strengthen our arguments, we have generated another new figure where we show that the acquisition of the *ompU* ORF is modular and controlled by environmental conditions (Fig. 7). In Fig. 7A we show the schematic of the modified chitin-induced natural transformation protocol that we developed to examine transfer of *ompU* modules. In Fig. 7B and 7C, we show acquisition of the DNA using i) qRT-PCR showing variation in normalized reporter fluorescence (Rn) versus cycle number before and after transformation and ii) the transformation frequency of different experimental groups. We show that revertants acquired the full ompU ORF (Fig. 7D) and gain the phenotypes associated with clinical alleles of OmpU porin and OueS sRNA in a modular fashion: (E) bile resistance (0.4% whole bile), (F) mucin penetration, (G) biofilm formation, (H) resistance to cationic antimicrobial peptide P2 (200 µg/mL), (I) acidic pH (pH 5.5), and (J) intestinal colonization. Finally, in fig. 7K we show that the acquisition frequency of *ompU* ORF is controlled by environmental conditions.

Furthermore, as the reviewer suggested, further work was needed in order to determine **a)** the genetic determinants associated with allelic-specificity, **b)** the action of those in OueS target, and **c)** how did that affect phenotypes. For this, we examined the exact difference between the two alleles and uncovered four specific domains in the 5' module that differentiate $OueS^{C6706}$ and $OueS^{GBE1114}$ (Fig. 2G). As now more clearly highlighted in Figure 1, $OueS^{C6706}$ and $OueS^{GBE1114}$ are identical in their 3' module (Fig. 2G). To determine the specific role of these domains in biofilm formation and RyhB suppression, we constructed 9 new mutants where we swapped the individual domains in $OueS^{C6706}$ for their environmental $OueS^{GBE1114}$ versions and created combination mutants of domains generating several "chimeric" $OueS^{C6706}$ versions. All mutants in the 5' module are expressed at similar levels as evidenced by qRT-PCR (Fig. 2H). Based on our phenotypic and molecular data, it is abundantly clear that the domains are responsible yet play a different role for the different phenotypes associated with OueS (Fig. 2, 3, 4). Overall, we think that the two components of the *ompU* ORF (porin and sRNA) evolved as a unit (Fig. 7). Given our data in this manuscript and other publications from our lab (Shapiro et al 2016 Nature Microbiology, Grant et al 2023 PLOS Genetics, Lopez-Perez and Balasubramanian et al 2025 PNAS), we have amassed evidence that indicate that alleles of *ompU* circulate in populations of *V. cholerae* each encoding a distinct porin and, as shown in this article, a sRNA.

4. The authors state that OueS potentially binds "upstream of the VC0178 ORF, the region that encompasses the promoter of the operon". Usually the promoter is not part of the transcript, so it is not clear to me how this is meant - please clarify.

Response: We appreciate the reviewer's observation. The intention was to refer to the 5′ untranslated region (5′ UTR) of the mRNA, which lies upstream of the *VC0178* coding sequence and is transcribed, in contrast to the promoter region which is not. We acknowledge that referring to this region as "encompassing the promoter" was misleading, as the promoter itself lies upstream of the transcription start site and is not part of the mRNA transcript, as rightly noted by the reviewer. We have removed the computational prediction data, including this proposed interaction site, as part of revisions prompted by feedback from this and other reviews. We have also revised the text to eliminate the ambiguous reference and ensure that the remaining interpretations are consistent with known transcriptional architecture.

5. Furthermore, the potential binding site in the sRNA is the same for RyhB and CBASS and strikingly it is perfectly conserved on sequence and structure level in the GBE1114 variant, because it is part of the terminator hairpin (Fig. 1 E&F). How do you explain that the GBE1114 variant still is non-functional?

Response: We thank the reviewer for pointing out that the predicted seed region in the 3′ terminator hairpin is identical in both clinical and GBE1114 alleles. As described in our response to comment #3 we have completely revised the manuscript and shown conclusively at the molecular and phenotypic level that the interaction site lies within domains encoded in the 5' end. We have removed the *in silico* interaction maps from our figures as they varied between prediction tools and now rely on our conclusive *in vitro* data.

6. The authors make use of the environmental allele of OueS from strain GBE1114, but provide no evidence that the sRNA from this allele actually exists and is expressed in the strain GBE1114. Sequence conservation alone is not sufficient evidence in this case, because this could be due to the ompU ORF and UTR, which harbours a Rho-independent terminator. If there is no independent evidence for the existence of OueS in GBE1114 then it is inappropriate to use this as the "environmental" allele. The same holds true for the other toxigenic and environmental variants that are used later. Without evidence for their existence as distinct sRNAs in their host strains, they cannot be considered as functional homologs. It might be, that the processing of the ompU mRNA to produce the OueS sRNA only exists in C6706.
This would also mean that many of the conclusions need to be rephrased or removed completely.

Response: We thank the reviewer for raising this important point. We agree that sequence conservation alone is not sufficient to establish the expression of OueS in its native environmental strain, such as GBE1114. For this reason, we have performed RACE and qRT-PCR in the strain *V. cholerae* GBE1114 and determined that OueS is expressed in the environmental isolate (Fig S2A). We have also performed qRT-PCR to compare expression levels of P_{ompU}–$OueS^{GBE1114}$. The allele was expressed at levels comparable to P_{ompU}–$OueS^{C6706}$ (Fig. 6F). Furthermore, we observe clear, reproducible phenotypic differences across alleles, including their effects on biofilm formation, phage resistance, CBASS activation, nutrient utilization, and mouse intestinal colonization (Figs. 2-7). Notably, $OueS^{GBE1114}$ confers a higher fitness advantage than the clinical allele in environmental colonization models, complements metabolic functions like $OueS^{C6706}$ and has a distinct transcriptional profile. Finally, we have now performed qRT-PCR to compare expression levels of the various OueS alleles. All alleles were expressed at levels comparable to P_{ompU}–$OueS^{C6706}$ (Fig. 6F). Furthermore, all alleles exhibit a phenotype different from Δ*ompU* or $OueS^{mut}$ clearly indicating they are functional. For instance, some alleles such as $OueS^{IRLE0074}$ restore WT levels of intestinal colonization in the isogenic background.

- Discussion:
In V. cholerae other 3' UTR derived sRNAs have been reported previously, and also a method for their identification (TIER-seq, Hoyos et al.; eLife 2020). Although the paper is cited, there is no discussion about the differences or if OueS can also be found in this dataset. Please elaborate on this.

Response: We thank the reviewer for this and confirm that OueS is not found in the published datasets. We have now added a sentence contrasting the location of OueS with previously described 3' UTR sRNAs (Lines 859-862).

In general I am missing a really critical discussion of the results. This is for example related to the issues mentioned above, but also others. Is it really always beneficial that the expression of OueS is tightly coupled to the expression of the porin OmpU. Does the cleavage of the full transcript lead to degradation of the mRNA or is it still translated but produces inactive OmpU? Could the processing of the initial ompU transcript be subject to regulation?

Response: We thank the reviewer for highlighting this important aspect as this suggestion has unearthed some interesting new data. To address the reviewer's concern, we have now included three subfigures in Fig 1 where we compare the expression levels of *ompU* and OueS. We have now expanded this section in the discussion as well (revised lines 870-879)

Minor:
- not sequence homology, but sequence complementarity indicates RNA-RNA interactions.

Response: We agree. This has been modified.

REVIEWER #3

The manuscript by Balasubramanian et al describes the discovery of a sRNA, OueS, in V. cholerae that is embedded within the ompU gene and that controls 84% of the ToxR regulon. The authors show that OueS regulates biofilm formation and phage defense. Interestingly, the authors argue that OueS has a bimodular structure with relative variability in the portion within the ompU gene and less variability within the 3' UTR portion. OueS from pathogenic strains is better at controlling intestinal colonization and phenotypes associated with that, while OueS from environmental strains is better at controlling colonization of zooplankton. The phenotypes associated with the sRNA are well documented, and the experimental data is well-executed. This phenomenon is interesting and highlights an unknown and important aspect of the virulence regulon of *V. cholerae*. The difference in the activity of the sRNA between C6706 and GBE1114 is striking and definitely identifies an important regulatory element to Vc pathogenesis and environmental persistence.

Response: We thank the reviewer for their thoughtful and positive evaluation of our work. We are especially grateful for their recognition of the novelty and significance of the discovery of OueS, and its role in linking small RNA variation to *V. cholerae* virulence and environmental adaptation.

1. I have some questions on the "bimodular" nature of the sRNA, as detailed below. The predicted interaction sequence of C6706 with RyhB (Fig 2D) is identical to the sequence of GBE1114 (Fig. S6) and it lies in the 3' "invariant" module of OueS after the OmpU stop codon, which has the same predicted structure within the last stem loop. Likewise the predicted interaction sequence of C6706 with CBASS (Fig. 2H) is also within this conserved 3' region that is identical to GBE1114. Yet the C6706 and GBE1114 behave differently in regards to biofilm/iron (Fig. 1H) and phage susceptibility (Fig 2J). Either the structural predictions are incorrect, or the predicted interaction is incorrect. The predicted RNA interaction is likely incorrect, based on the data presented. Because the variability between C6706 and GBE1114 lies in the 5' region, this is the most likely interaction region with RhyB and CBASS. Minimally the authors need to acknowledge that the 3' "invariant" region is where the predicted interaction occurs (2D and 2H), because the text throughout implies that it is the 5' "variant" region that is responsible for these differences in effects on biofilms, phage susceptibility, etc.

Response: We thank the reviewer for this important observation. We agree that our previous version of the manuscript did not properly explain the reasons behind our conclusions and our *in silico* preliminary analyses were misleading and incorrect. For this reason, we have updated each figure and generated several entirely new ones to dissect and highlight this phenomenon and expanded the Results and Discussion sections to address this. The article is now 9 figures and 1 table long compared to 5 original figures. In Figure 6 we discuss the modular nature of OueS based on its allelic variations and in Figure 7 we develop a method to examine the acquisition of the *ompU* ORF and show that ORF can be acquired as a module based on external conditions, which is associated

with the emergence of virulence-associated phenotypes. Furthermore, in Figures 2, 3, and 4 we tested 9 new mutants that we generated based on domains exclusively found in the 5' end of OueS. Overall, we believe these major additions have significantly strengthened our manuscript and are truly thankful for the suggestion.

Specifically, in Fig. 6 we have included a schematic of the variability of the OueS alleles contextualized within the *ompU* ORF (Fig 6A). This visually highlights how variable the 5' end of the sRNA is in comparison to the 3'. We have also included an alignment of the different representative alleles (Fig. 6B) and a phylogenetic tree of the OueS alleles highlighting the ones that we cloned in the isogenic C6706 background (Fig. 6C). In Fig. 6D we highlight their structural differences, all contained within the 5' end. Finally, we reconstructed a phylogenomic tree of >1800 *V. cholerae* genomes and indicate all the clades that have identical alleles as the strains from the PCG to elucidate their origin as a preadaptation (Fig. 6E). We measured the expression of the allelic mutants to determine whether they were expressed at similar levels as WT (Fig. 6F). Finally, we examined their role during intestinal colonization (Fig. 6G, 6H).

To further highlight the modular nature of OueS and strengthen our arguments, we have generated another new figure where we show that the acquisition of the *ompU* ORF is modular and controlled by environmental conditions (Fig. 7). In Fig. 7A we show the schematic of the modified chitin-induced natural transformation protocol that we developed to examine transfer of *ompU* modules. In Fig. 7B and 7C, we show acquisition of the DNA using i) qRT-PCR showing variation in normalized reporter fluorescence (Rn) versus cycle number before and after transformation and ii) the transformation frequency of different experimental groups. We show that revertants acquired the full *ompU* ORF (Fig. 7D) and gain the phenotypes associated with clinical alleles of OmpU porin and OueS sRNA in a modular fashion: (E) bile resistance (0.4% whole bile), (F) mucin penetration, (G) biofilm formation, (H) resistance to cationic antimicrobial peptide P2 (200 µg/mL), (I) acidic pH (pH 5.5), and (J) intestinal colonization. Finally, in fig. 7K we show that the acquisition frequency of *ompU* ORF is controlled by environmental conditions.

Additionally, as the reviewer suggested, further work was needed in order to determine **a)** the genetic determinants associated with allelic-specificity, **b)** the action of those in OueS target, and **c)** how did that affect phenotypes. For this, we examined the exact difference between the two alleles and uncovered four specific domains in the 5' module that differentiate $OueS^{C6706}$ and $OueS^{GBE1114}$ (Fig. 2G). As now more clearly highlighted in Figure 1, $OueS^{C6706}$ and $OueS^{GBE1114}$ are identical in their 3' module (Fig. 2G). To determine the specific role of these domains in biofilm formation and RyhB suppression, we constructed 9 new mutants where we swapped the individual domains in $OueS^{C6706}$ for their environmental $OueS^{GBE1114}$ versions and created combination mutants of domains generating several "chimeric" $OueS^{C6706}$ versions. All mutants in the 5' module are stably expressed as evidenced by qRT-PCR (Fig. 2H). Based on our phenotypic and molecular data, it is abundantly clear that the domains are responsible yet play different roles in the different phenotypes associated with OueS (Fig. 2, 3, 4). Overall, we think that the two components of the *ompU* ORF (porin and sRNA) evolved as a unit (Fig. 7). Given our data in this manuscript and other publications from our lab (Shapiro *et al* 2016 Nature Microbiology, Grant *et al* 2023 PLOS Genetics, Lopez-Perez and Balasubramanian *et al* 2025 PNAS) we have amassed evidence that indicate that

alleles of *ompU* circulate in populations of *V. cholerae* each encoding a distinct porin and, as shown in this article, a sRNA.

2. The predicted folded structures of the OueS sRNA Fig S6B predict e.g. that IRLE0074 has a different structure than C6706, yet the sequence appears identical to C6706 (Fig S6A). This is relevant because in Fig. 4B these sRNAs behave differently in the infant mouse colonization model (according to their statistical analysis). If the sequence is identical, how can the sRNA fold into a different structure and have a different effect on colonization?

Response: We thank the reviewer for this observation. We have now corrected the mislabeling and added for increased clarity the reason behind using IRLE0074. The comment has allowed us to clarify our interpretation and the framing of our conclusions. As noted by another reviewer, we should have made further efforts to explain what we mean by "preadaptation". This is a term we used in our initial publication (Shapiro *et al* 2016 Nature Microbiology), yet did not clarify it here. In order to consider toxigenic OueS a preadaptation, we need several critical variables to happen at the same time: **a)** it must confer a competitive advantage during colonization and be responsible for virulence-associated traits ($OueS^{C6706}$ provides both), **b)** some environmental alleles of the gene do not confer these competitive advantages (e.g. $OueS^{GBE1114}$ or $OueS^{IRL0181}$), **c)** other environmental alleles do (e.g. $OueS^{GBE1194}$), **d)** exact same versions of the toxigenic allele are present in environmental strains and lead to a similar phenotype ($OueS^{IRLE0074}$). We have now modified the text accordingly and hope that the reason behind this choice is much clearer now. We have also included a new panel and table to address the distribution of the toxigenic allele of OueS in the *Vibrio cholerae* species. We analyzed over 1800 dereplicated genomes of the bacterium, identifying 378 distinct clades and examined the presence of $OueS^{C6706}$ in the population (Fig 6E, Table S9). As our tree indicates, the allele is distributed across the species suggesting it is a preadaptation to virulence.

3. In general, the sequence alignment (FigS6A) implies high variability in the region marked ~55-85, but this is because one set of 4 strains (GBE0917-GBE0428) has an additional identical sequence inserted in this region and another set of five strains (GBE1194-C13_2011V_1169) has a smaller identical sequence inserted in this region. CL 10_GBE1194 and CL 11_SLG4 have identical sequences, as do CL 12_FORC_055 and C13_2011V_1169, and these vary from each other only in the "invariable" region downstream of the stop codon. Likewise GBE1116 and IRL0081 have identical sequences except downstream within the "invariable" region. Within the first group of four (GBE0917-GBE0428), the only variability IRL0081 shows from GBE1116 is in the "invariable" 3' end. In other words, the appearance of variability in the 5' end is biased, I think, because there's two groups that have two slightly different insertions in the 5' end, but within these groups, the variability is not limited to the 5' or 3' ends. These argue against there being a "bimodular" nature of variable>invariable within this sRNA, since there is variability downstream as well, to the same degree (within groups) as upstream. The authors argue for a bimodular nature to the sRNA based on the

difference between C6706 and GBE1114, because the differences between these two lie within the 5' region rather than the 3' region.

Response: We sincerely thank the reviewer for this thoughtful and detailed analysis. Given that modularity is a central concept in our manuscript, we agree it deserves a more rigorous and nuanced justification. In response, we have generated two new Figures (Fig 6 and 7) to present more refined analyses that better illustrates the modular architecture of OueS and the *ompU* ORF. Importantly, our conclusion about modularity is not based *solely* on a single comparison between C6706 and GBE1114*,* but rather on a broader and systematic analysis of OueS alleles across diverse environmental strains (see below). However, we fully agree that we failed at making our case clear and convincing. We believe that the modified text and new figures have accomplished that.

As the reviewer notes, two major insertion events give rise to distinct OueS sequence groups. Crucially, these insertions and the other sequence variations are confined to the 5′ region of OueS, which lies within the *ompU* ORF. Specifically, alleles (GBE0428, IRLE0081, GBE0917, GBE1116, IRLE0079) harbor either a ~30-nt or 18-nt insertion within the 5′ region, accompanied by shared and unique point mutations. The second group of alleles (FORC_055, IRLE0082, GBE1194) contain a distinct ~9-nt insertion and individual point mutations, again restricted to the 5′ region (Fig. 6B). In contrast, the 3′ region of OueS shows remarkable conservation. Across all analyzed alleles, only three unique point mutations are present in three strains, and no insertions or structural disruptions occur in this region. We now highlight this in the main text to emphasize that the major structural and sequence differences: insertions, deletions, and associated functional changes, are localized exclusively to the 5′ end**.** Phylogenetic analysis of these sequences supports their modular structure, grouping the alleles into six distinct clades, including the pandemic clade, based on 5′ region divergence (Fig. 6C). Representative alleles from these clades also exhibit distinct secondary structures (Figs. 1F, 1K, 5D), consistent with functional diversification.

Overall, our previous and novel analyses provide multiple, converging lines of evidence supporting the modular organization of OueS: the variable 5′ region, embedded in the *ompU* coding sequence, drives strain-specific regulatory outcomes (Figs. 2, 3, 4), while the conserved 3′ region maintains core structural or regulatory roles. This is conceptually consistent with our prior findings demonstrating the modularity of the *ompU* ORF itself, which we showed governs key traits essential for host survival (Grant et al., 2023, *PLoS Genetics*). Moreover, in our recent study (López-Pérez, Balasubramanian *et al*., 2025, *PNAS*), we demonstrated that allelic variation in six genes, including *ompU*, is central to the emergence of pandemic *V. cholerae* lineages. Building on these findings, the present work highlights how OueS, derived from one of these critical loci, plays a direct role in shaping pandemic potential.

Minor edits:

Line 23: comprises a highly…

Response: We have made this change.

Line 29: colonization a). it stringently…
Line 30: and b). it confers resistance…

Response: We appreciate the reviewer making this point. However, we have not added a period after the parentheses to maintain consistency with writing styles in Nature Communications, where lower case letters in parentheses are directly attached to the item list without an extra period.

Line 59: Following ingestion of planktonic or biofilm-associated cells, V. cholerae …
Line 69: promoters of 39 genes

Response: We have made the above two edits.

Line 71: …cholera, and ToxT, a master virulence regulator.

Response: Although we see the reviewer's point, we believe that including the requirement of TcpP for regulation of P_{toxT} but not P_{ctxAB} is a distinction that needs to be made for readers that do not work in the cholera field or its virulence regulation.

Line 77: resistance, and tolerance to…
Line 109: comprises a highly variable…
Line 114: and reveal specific…
Line 119: pathogenesis. However, it remains unclear…
Line 133: do not timely repress biofilm formation.
Line 148: defense response to phages and symbionts…

Response: We thank the reviewer and have made the above six changes.

Line 159: OmpU represses biofilm formation and has a significant impact on the… (no evidence of allele-dependent manner at this point in manuscript).

Response: We appreciate the reviewer's concern. However, we demonstrate in Fig 1A that biofilm suppression is dependent on the OmpU allele.

Line 166: indicated potential sRNAs encoded…
Line 177: Interestingly, the lower bands are…

Response: Thank you. We have made the above two changes.

Line 291: inhibition mediated by OueS…

Response: We have changed this to 'inhibition of biofilm formation mediated by OueS…' to more accurately reflect our findings.

Line 316: “moderate reduction in ICP3”…there’s no statistical relevance to this.

Response: Thank you for pointing this out. We have removed it.

Line 581 directly affects host colonization
Line 608: 84% of the ToxR regulome
Line 611: specifically enriched during virulence-inducing

Response: We have now made the above changes.

REVIEWER #4:

This paper from the Almagro-Moreno group presents an exciting new discovery that extends our knowledge of V. cholerae pathogenicity. They identify a new sRNA - deemed OueS because it is encoded in the ompU gene - in studies of OmpU's role in biofilm formation. They then uncover data strongly implicating OueS in several important phenotypes including:

1) inhibition of biofilm formation;
2) changes in V. cholerae gene expression during biofilm formation that are associated with OmpU;
3) inhibition of RhyB expression/stability;
4) resistance to infection by phages ICP2 and ICP3 in a manner dependent on CBASS genes;
5) control of the expression of many genes in the ToxR regulon, particularly those related to motility and metabolism/nutrient utilization, but not the canonical V. cholerae virulence genes located in the horizontally acquired TCP island (Tcp pilus) and CTX prophage (cholera toxin);
6) promotion of intestinal colonization in suckling mice.

These phenotypes, some of which may have been mistakenly attributed to the OmpU porin, represent the paper's major strengths.

Response: We thank the reviewer for their thoughtful and encouraging comments. We are pleased that the reviewer recognizes the novelty and significance of uncovering OueS and its broad impact on key phenotypes linked to *V. cholerae* pathogenicity. We appreciate the reviewer's careful evaluation and positive assessment of the strengths of our study.

1. The paper's major weakness is in its framing starting with its title "Modular small RNA drives the emergence of virulence traits and environmental trade-offs in Vibrio cholerae". There is No data presented here that support the major claim that an sRNA 'drives emergence' of virulence traits. Which figure supports this claim? Some environmental OueS alleles (GBE1194 and IRL 0074) support colonization that is equal to or better than the clinical isolate C6706 and other alleles have deficient colonization. Notably analysis of transcriptomes associated with different OueS alleles did not reveal functional enrichment, arguing against their claim that "our data indicate that a) the OueS allele encoded by toxigenic strains is a virulence preadaptation" [lines 498-502]. Overall, how can they claim that OueS is 'preadaptation'? Isn't it just as plausible that this sRNA evolved after the acquisition of the TCP island (the key step in evolution of V. cholerae as a human pathogen because it enables intestinal colonization) to further facilitate adaptation to the human small intestine/colonization by the sRNA's control of motility and metabolism?

Response: We thank the reviewer for this important comment, which has allowed us to clarify our interpretation and the framing of our conclusions. We realize that we should have made further efforts to explain what we mean by "preadaptation". This is not a term that we state lightly and developed in our initial publication Shapiro *et al* 2016 Nature Microbiology yet did not clarify here. In order to consider toxigenic OueS a preadaptation, we need several critical variables to happen at the same time: **a)** it must confer a competitive advantage during colonization and be responsible for virulence-associated traits ($OueS^{C6706}$ provides both), **b)** some environmental alleles of the gene do not confer these competitive advantages (e.g. $OueS^{GBE1114}$ or $OueS^{IRL0181}$), **c)** other environmental alleles do (as the reviewer rightly points out: $OueS^{GBE1194}$), **d)** exact same versions of the toxigenic allele are present in environmental strains and lead to a similar phenotype (as the reviewer rightly points out: $OueS^{IRLE0074}$). We have now modified the text accordingly and hope that the reasoning behind our claim is much clearer now. We have also included two new figures (Fig. 6 and 7) and supplementary tables (Table S9) to address the distribution of the toxigenic allele of OueS in the *Vibrio cholerae* species and the modular acquisition of the *ompU* ORF. We analyzed over 1800 dereplicated genomes of the bacterium and examined the presence of $OueS^{C6706}$ in the population. As our tree indicates, the allele is distributed across the tree in clinical and environmental strains alike (Fig. 6E), indicating that it is a preadaptation. To further highlight the modular nature of OueS and strengthen our arguments, we have generated another new figure where we show that the acquisition of the *ompU* ORF is modular and controlled by environmental conditions (Fig. 7). In Fig. 7A we show the schematic of the modified chitin-induced natural transformation protocol that we developed to examine transfer of *ompU* modules. In Fig. 7B and 7C, we show acquisition of the DNA using i) qRT-PCR showing variation in normalized reporter fluorescence (Rn) versus cycle number before and after transformation and ii) the transformation frequency of different experimental groups. We show that revertants acquired the full *ompU* ORF (Fig. 7D) and gain the phenotypes associated with clinical alleles of OmpU porin and OueS sRNA in a modular fashion: (E) bile resistance (0.4% whole bile), (F) mucin penetration, (G) biofilm formation, (H) resistance to cationic antimicrobial peptide P2 (200 µg/mL), (I) acidic pH (pH 5.5), and (J) intestinal colonization. Finally, in fig. 7K we show that the acquisition frequency of *ompU* ORF is controlled by environmental conditions. Overall, our data unequivocally shows that the clinical OueS allele enhances traits associated with host colonization (Figures 2–5) and acts as one critical factor that contribute to the emergence of pathogenic potential in *V. cholerae*. In our recent paper, we demonstrate that *ompU* is one of several genes driving the emergence of pandemic potential in *V. cholerae* (Lopez-Perez, Balasubramanian *et al*. 2025 PNAS). Here, we provide some mechanistic insights of how the *ompU* ORF, via OueS, mediates aspects of this process and the ORF itself is acquired as a unit.

Regarding the transcriptomic data, we appreciate the reviewer's point that differential expression associated with different OueS alleles did not yield strong enrichment of specific functional categories. We think this is fascinating and highlights the difficulty of translating transcriptomic into phenotypic, underscoring our successful efforts in Figures 2, 3 and 4. Furthermore, it brings forth the complexity of OueS-mediated regulation with potential regulatory redundancies and compensatory networks associated with the emergence of these phenotypes. We have now expanded on this aspect in Lines

820-822. Furthermore, additional work was needed in order to determine **a)** the genetic determinants associated with allelic-specificity, **b)** the action of those on OueS targets, and **c)** how did that affect phenotypes. For this, we examined the exact difference between the two alleles and uncovered four specific domains in the 5' module that differentiate $OueS^{C6706}$ and $OueS^{GBE1114}$ (Fig. 2G). As now more clearly highlighted in Figure 1, $OueS^{C6706}$ and $OueS^{GBE1114}$ are identical in their 3' module (Fig. 2G). To determine the specific role of these domains in biofilm formation and RyhB suppression, we constructed 9 new mutants where we swapped the individual domains in $OueS^{C6706}$ for their environmental $OueS^{GBE1114}$ versions and created combination mutants of domains generating several "chimeric" $OueS^{C6706}$ versions. All mutants in the 5' module are stably expressed as evidenced by qRT-PCR (Fig. 2H). Based on our phenotypic and molecular data, it is abundantly clear that the domains are responsible yet play a different role for the different phenotypes associated with OueS (Fig. 2, 3, 4). Overall, we think that the two components of the *ompU* ORF (porin and sRNA) evolved as a unit (Fig. 7). Given our data in this manuscript and other publications from our lab (Shapiro *et al* 2016 Nature Microbiology, Grant *et al* 2023 PLOS Genetics, Lopez-Perez and Balasubramanian *et al* 2025 PNAS), we have amassed evidence that indicate that alleles of *ompU* circulate in populations of *V. cholerae* each encoding a distinct porin and, as shown in this article, a sRNA. Our long-term goal is to eventually be capable of generating structure-function analyses of the other sRNA alleles as we have now performed for $OueS^{C6706}$ and $OueS^{GBE1114}$.

2. Also, the data in 4C,E that toxigenic alleles lead to fitness trade-offs in colonization of Microcystis and Artemia have p-vales but are very small differences and it is not clear that these experimental systems represent important environmental niches for *V. cholerae*.

Response: We thank the reviewer for raising this important point. The role of environmental reservoirs such as crustaceans and cyanobacteria in the interepidemic survival and transmission of *V. cholerae* is well established and critical for disease outbreaks (studies by Colwell, Islam, Alam, etc.). This prompted us to recently establish in the lab the *Microcystis* and *Artemia* model systems to perform competition assays and examine the role of certain genes in environmental colonization (López-Pérez, Balasubramanian *et al*., 2025, *PNAS*). We did not expect large differences between the alleles as corroborated by some of our recent work with other genes, as typically fitness trade-offs are additive and subtle otherwise one change in one gene could decide whether a bacterium loses its capacity to compete in its natural environment. We hypothesize that an additive series of allelic variations, similarly to what we identified previously for intestinal colonization (López-Pérez, Balasubramanian *et al*., 2025), is needed in order to obtain a much more pronounced and nuanced sets of differences. Nonetheless, besides our phenotypes being consistent and significant, it is encouraging that these allele-dependent effects are detectable under highly controlled laboratory conditions, which only partially replicate the complexity of natural ecosystems. We anticipate that in more realistic environmental settings, where multiple biotic and abiotic factors interact, the contributions of OueS alleles may be even more pronounced. This is further supported by our addition to Figs. 5 and 6. In Fig. 5G and 6H we examined the competitive index of

OueS mutant strains in the presence of selective pressures where their role is needed, namely: s-IgAs (biofilm repression), addition of ICP3 (intestinal bacteriophages). Our results indicate that in the presence of these bottlenecks that more closely resemble the conditions that *V. cholerae* finds in the human host, the mutant strains exhibit over 3-logs decrease in colonization compared to 1-log in WT mice (Fig. 5G) and that this effect is dependent on the OueS allele (Fig 6H). We contend that other bottlenecks likely exist in the ecosystems that *V. cholerae* inhabits that confer further competitive advantages to the environmental allele of OueS.

3. There are repeated overclaims: (a few examples)

- Intro line 108 "we describe a sRNA that …shapes the evolution of toxigenic V. cholerae. [this is not shown]

Response: We have changed this to " …… contributes to the evolution of toxigenic *V. cholerae*" (line 119).

- Line 110-11 'the bimodular nature of the OueS "generates allelic variants" ..[How does the fact that OueS being encoded in an ORF and UTR (which is not a 'unique' sRNA characteristic) create variation?]. Why does OueS 'modularity' "enable the emergence of phenotypes essential for colonization? Couldn't phenotypically important allelic variation be part of a non-modular sRNA?

Response: We appreciate the reviewer's insightful questions and the opportunity to clarify our rationale. We agree that the embedding of an sRNA within an ORF and 3' UTR is not a feature that inherently generates variation by itself. Our use of the term 'bimodular' refers specifically to how the two regions of OueS: the portion overlapping the *ompU* ORF and the portion in the 3' UTR, exhibit differential evolutionary pressures. The 5' module within the ORF shows significant sequence diversity, while the 3' UTR module remains highly conserved (see new Fig. 6). This structural arrangement is important because it positions OueS at the intersection of two evolutionary forces: selection on the coding sequence of *ompU* and the downstream regulatory function of the sRNA. Variability in the 5' module, shaped by changes in *ompU* ORF, can introduce functional diversity into OueS without compromising the core regulatory architecture housed in the 3' region. Thus, the modular structure allows some leeway for evolutionary 'tinkering' (5' region) with phenotypic outcomes such as colonization efficiency, while preserving essential sRNA functions.

As described above, to further highlight the modular nature of OueS and strengthen our arguments, we have generated another new figure where we show that the acquisition of the *ompU* ORF is modular and controlled by environmental conditions (Fig. 7). In Fig. 7A we show the schematic of the modified chitin-induced natural transformation protocol that we developed to examine transfer of *ompU* modules. In Fig. 7B and C we show qRT-PCR showing variation in normalized reporter fluorescence (Rn) versus cycle number before and after transformation and the transformation frequency of different experimental groups. We show that revertants acquired the full *ompU* ORF (Fig. 7D) and gain the phenotypes associated with clinical alleles of OmpU porin and OueS

sRNA in a modular fashion: (E) bile resistance (0.4% whole bile), (F) mucin penetration, (G) biofilm formation, (H) resistance to cationic antimicrobial peptide P2 (200 μg/mL), (I) acidic pH (pH 5.5), and (J) intestinal colonization. Finally, in fig. 7K we show that the acquisition frequency of *ompU* ORF is controlled by environmental conditions. Overall, our data unequivocally shows that the clinical OueS allele enhances traits associated with host colonization (Figures 2–5) and acts as one critical factor that contribute to the emergence of pathogenic potential in *V. cholerae*.

We fully agree that phenotypically important variation could also arise in non-modular sRNAs provided selective pressures leading to allelic variations in the population. However, in the case of OueS, the modular arrangement appears to facilitate differential adaptation by providing a 'built-in' mechanism for generating functionally distinct alleles while retaining a stable regulatory scaffold. To address the reviewer's concern, we have clarified this point in the revised text in the Discussion (Lines 975-988).

- Results line 508: 'directly contributing to the emergence of pathogenic V. cholerae from environmental populations' [this is not shown]. Last lines of the discussion go way beyond anything shown in the paper. Many instances where they claim their data 'indicates' should be toned down.

Response: We appreciate the reviewer's concern and have changed it to '…contributing to the emergence of pathogenic *V. cholerae* from environmental populations' and toned down several of our claims. Additionally, we have rewritten large portions of the discussion and elsewhere to tone down our claims in line with our findings.

Experimental work

4. Prove with mutational studies that OueS interacts with RyhB.

Response. We have now generated a brand-new figure and table that fully addresses the reviewer's concerns. Briefly, our functional group analyses of RNA-seq data identified downregulation of iron uptake associated genes to be one of the top functional categories associated with OueS expression (Fig. 2A). After that we hypothesized iron might play a role in biofilm formation in a OueS-dependent manner and identified a strong association between the three (Fig. 2B, Fig S3A). As suggested by the reviewer, in order to examine the genes that OueS directly binds to under these conditions, we have performed MS2 affinity purification coupled to RNA-sequencing (MAPS) to define the target profile of $OueS^{C6706}$. We also performed MAPS for $OueS^{mut}$ to determine sRNA specificity, and $OueS^{GBE1114}$ to define allele specificity. Our MAPS results show that OueS directly binds to the iron-responsive regulator RyhB and does so in an allelic-dependent manner (Table 1 and Figs. 2C and D). We determined that the sRNA modulates the expression levels of RyhB (Fig. 2E) and using northern blots, we discovered that expression of clinical OueS drastically reduces the levels of RyhB, whereas RyhB remains detectable when an environmental allele of OueS is expressed, suggesting that this effect on RyhB is allelic dependent (Fig. 2F).

Next, to directly address the reviewer's concern, we examined the exact difference between the two alleles and uncovered four specific domains in the 5' module that

differentiate OueS^{C6706} and OueS^{GBE1114} (Fig. 2G). As highlighted in Figure 1, OueS^{C6706} and OueS^{GBE1114} are identical in their 3' module (Fig. 1J, 2G). To determine the specific role of these domains in biofilm formation and RyhB suppression, we constructed 9 new mutants where we swapped the individual domains in OueS^{C6706} for their environmental OueS^{GBE1114} versions and created combination mutants of domains generating several "chimeric" OueS^{C6706} versions. All mutants in the 5' module are stably expressed as evidenced by qRT-PCR (Fig. 2H). Two domains appear to be critical for the emergence of the biofilm repression phenotype: 2 and 3 (Fig. 2I). This phenotype is matched by northern blots, where in domain mutants for 2 and 3, RyhB is strongly detectable (Fig. 2J, 2K). Overall, the domains exhibit a complex set of phenotypes where some of them are required for biofilm suppression (Fig. 2I) and RyhB degradation whereas others are not (Fig. 2J, 2K). Given the number of discoveries and subfigures, the article's section on biofilm repression via direct suppression of RyhB is now one full figure and directly address the reviewer's concern.

5. Present a phylogram with OueS alleles and predicted structures next to the phylogram of the respective genomes and the strain metadata.

Response: We thank the reviewer and agree that a phylogram will be useful for the readers in addition to the sequence alignment. Given this and other concerns about modularity and preadaptations, we have now included a new figure (Fig 6) that we believe conclusively addresses these issues.

Minor
Line 532 6-fold reduction does Not correspond to CI of 0.39. but of 0.16.

Response: We appreciate the reviewer for noting this oversight. We have now changed it to "…0.6-log reduction in its CI" as was originally intended (line 835).

Line 651 'host transmission dynamics' There is no data about transmission in this paper.

Response: We thank the reviewer for this important clarification. We agree that our study does not directly assess host transmission dynamics. Our original phrasing was intended to refer to colonization of environmental reservoirs, which can influence transmission to human hosts. To avoid confusion, we have revised the text to more accurately reflect our findings and have removed "host transmission dynamics."

REVIEWERS' COMMENTS

Reviewer #1:

The manscript by Balasubramanian et al is greatly improved since the first version I read. It is now an elegant set of experiments that for the most part flow nicely and show the biology of this important and interesting sRNA that is found at the end of the *ompU* transcript. I have some minor comments below.

We are glad that the reviewer enjoyed reading our manuscript. We appreciate the reviewer's comments that has contributed to this improved manuscript.

- Line 118 – delete the agent of cholera.
This has been deleted.

- Line 185 – nt is the standard way to shorten nucleotide – and throughout manuscript
It has been changed here and throughout the manuscript.

- Line 189 – RNA binding protein not sRNA binding protein. Move sRNA to further down the sentence.
We have changed this (line 181).

- Line 219 – are both 166 nt.
This has been changed (line 211).

- Line 219 – 220 – the predicted secondary structure of the two sRNAs…..
Changed

- Line 219 – 222 – this sentence is massive, and given it covers an important finding of the manuscript, split in two.
This sentence has been split in two, as suggested (lines 210-215).

- Line 223 – surely its within the ORF? I can see what you are saying, but language needs to be more precise.
This has been changed to 'overlapping and ORF and its UTR' to make it clearer (line 215).

- Line 411 – gene not protein.
Thank you, it has been changed (line 404).

- Labelling of Fig 4C – which lane is the ∆toxR with expression of OueS? I assume the one next to the ∆toxR strain? But it's not labelled so.
Yes, the reviewer is correct and has been explained in the figure legend.

- Fig 5A, B and C should be with figure 1 and should be moved to the supplementary. Fig 5D and E supplementary.
We thank the reviewer for this suggestion and we initially had most of this data in the supplementary. We moved it to the main figure to address concerns of the three other reviewers. About the order, we believe that addressing the regulation of OueS before the establishing the phenotypes would not flow with our narrative.

- Line 641 – sIgA needs defining. As a non-immunologist this part lost me a little bit, so please explain the rationale of using sIgA more clearly.

This has been defined and explained in lines 635-638.

- Line 682 – need to expand on selection of which sRNAs you picked to work on and why? This has been explained in lines 673-675.

- Line 692 – Fig6 what?
Now clarified in text as Fig 6A, 6B (line 685).

- Line 700 – PCG?
This has been clarified as the pandemic cholera group (line 693).

-Line 708 – expand on details regarding Fig6E.
The rationale behind this has been further expanded (lines 689-695).

- Line 742 – typo.
This has been changed (line 763).

- Line 800 – Are these conditions just changing transformability – how do you know that this is specific to this allele and not all DNA? This section of the manuscript detracts from the rest of the story – which is now really lovely. This whole section on revertants either needs deleting or more controls to put in to show that there is something special about this module and it's ability to switch/revert i.e. other parts of the V. cholerae chromosome don't change so easily.
We thank the reviewer for their thoughtful comment and for the positive assessment of our manuscript. We apologize for this potential confusion. We do not claim that this phenomenon is unique to the *ompU* locus nor do we make any claims on the general transformability of the strain under these conditions. We use this process as a tractable example to demonstrate that the *ompU* ORF can be acquired in a modular fashion leading to the emergence of phenotypic traits. Furthermore, this section was essential to address the comments of the three other reviewers.

- Line 1206 – Lysogeny Broth - https://en.wikipedia.org/wiki/Lysogeny_broth.
We have changed Luria Bertani broth to lysogeny broth (line 1004)

- Line 1468 – doesn't make sense.
We have now explained the steps (line 1265-1267)

-Line 1506 – Fifty microlitres – 50 µl.
It has been changed (line 1303).

Reviewer #2:
The authors have replied satisfactorily to all my concerns. Congratulations to this nice piece of work.
We thank the reviewer for their constructive comments that have significantly improved the manuscript. We are also thankful for the kind words.

Reviewer #3
The revised manuscript by Balasubramanian et al describes the discovery of a sRNA, OueS, in V. cholerae that is embedded within the ompU gene and that controls 84% of the ToxR regulon. This revised version is dramatically improved from the original submission. The authors have added a substantial amount of new data in direct

response to the previous reviewers' comments. These additional experiments were extensive and well-performed, and solidify the importance of this discovery by providing quite a bit of mechanistic detail into OueS and how it controls gene expression in V. cholerae that affects biofilm formation, phage resistance, and virulence. I am impressed with the depth of additional data presented, and satisfied with the manner in which the authors addressed previous comments to strengthen this manuscript.
We thank the reviewer for their constructive comments that have significantly improved the manuscript. We are also thankful for the kind words and generous praise.

My only (minor) comment is that after reading through the manuscript it began to bother me that the OueS from strain C6706 was referred to as "toxigenic OueS". While strain C6706 is a toxigenic strain, the sRNA is not "toxigenic", and the phenotypes being studied are not based on toxigenicity. I think the authors are trying to highlight that this OueS was identified in a clinical strain, rather than an environmental strain, so maybe "clinical OueS" might be a better term.
The reviewer is correct in noting that that the sRNA by itself is not toxigenic, and we have intentionally refrained from making this case throughout the manuscript. Nonetheless, the sRNA is neither toxigenic nor clinical. Thus, we chose 'toxigenic' over 'clinical' because it precisely denotes the pandemic lineage-associated genomic context, which is central to the comparisons made in this study.