nature portfolio

Peer Review File

Formation of a membraneless compartment regulates bacterial virulence

Corresponding Author: Professor Ilan Rosenshine

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)
The manuscript by Aroeti et al. investigates how the RNA-binding protein CsrA contributes to regulation of virulence gene expression in pathogenic E. coli. By fusing CsrA to fluorescent proteins and study its expression by microscopy, the authors observe that CsrA forms specific foci within the bacterial cell, a phenomenon that appears to be conserved in several enterobacterial species. The CsrA foci become more prevalent in stationary phase, and their formation seems to depend on the presence of the small RNA CsrB, previously well described as an antagonist of CsrA activity. Isolation of CsrA foci suggest that they, in addition to CsrA and CsrB, contain components of the RNA degradosome, as well as a defined set of mRNAs (e.g. virulence mRNAs) and small RNAs. The authors suggest that the outcome of CsrA activity may differ depending on if CsrA is freely diffusing in the cytoplasm or as part of a condensate-like focus.

The findings are novel, exciting, and suggest a new and unexpected facet of CsrA-dependent regulation. That said, several important issues need to be addressed before the manuscript can be considered for publication. A number of essential control experiments are missing, and the interpretations of the results are sometimes not well supported by the presented data.

Major points:

1) The authors state that the CsrA foci are “orchestrated by the assembly of a membraneless compartment via a process resembling, yet different from, typical liquid-liquid phase separation”. This needs to be rephrased and toned down since no information about the physical properties of the CsrA condensates is presented in the manuscript. Moreover, the authors claim that the foci serve as a regulatory hub for T3SS expression. What is clear from the data is that the CsrA-GFP/ -mScarlett proteins form foci that also contain a specific set of proteins and RNA. However, the evidence for regulation within these compartments is weak. Several alternative interpretations that also fit well with the data should be considered. For instance, are these compartments used to store molecules rather than to regulate them, or do they function as RNA and/ or protein degradation centers? Are they simply RNA/ protein aggregates without a specific function? These issues should be clearly addressed in the manuscript.

2) The authors convincingly show that expression of CsrA fused to GFP or mScarlett results in foci formation. However, the manuscript lacks evidence for native CsrA forming foci. This is a very important point since fusion proteins involving fluorescent proteins have been shown to result in aggregation, although not aggregating when expressed separately (e.g. Pandey et al. JMB 2024, this is just one recent example of several similar studies). To corroborate this major finding in the manuscript, the authors should test whether native CsrA forms foci. A direct way to do this is detection of native CsrA by immunofluorescence. If antibodies against native CsrA are not available, the CsrA-3xFLAG protein could be used. This should be a straightforward experiment considering the expertise of the Rosenshine group in this technique.

3) Figure 4A: Two important points need to be addressed here. I) Only some of the cells are GFP positive and the authors explain this to be the result of an “unstable plasmid”. This issue needs to be fixed and should be easily solved by expressing CsrB-MS2 from a stable plasmid or by integrating the construct on the chromosome. II) The experiment lacks an essential control where the MS2 aptamer is expressed alone or as a fusion to an RNA which CsrA does not bind. Without this control(s) it is simply not possible to judge whether the distribution of the fluorescent signals is specific to CsrB.

4) Figure 5B: The authors interpret the result to mean that CsrA foci colocalizes with RNase E. However, since RNase E puncta are found throughout the cell periphery, the overlap between CsrA-mScarlett and RNase E-mNeon signals could as well happen by chance. Indeed, there are several cells in which the signals do not overlap. The authors need to provide quantitative data on a large number of cells and test whether the suggested co-localization is indeed statistically significant.

5) The transcriptomic analysis (Fig. 5C) was not sufficiently described. How did the authors perform differential expression analysis, how was enrichment determined, what was compared to what? Please include a detailed description in Materials and methods, and provide sufficient information in Results and the figure legend so this becomes clear for the reader.

6) Statistical analysis has been performed in some figures (ex. Fig. 6C) but not in others (e.g. Fig. 2B-C, Fig. 3E, Fig 4C, and Fig. S3C-D). Please provide statistical analyses for every result described as “significant” in the text, and describe what type of statistical analysis was performed.

7) In the legend to Fig. 2B the authors state that “The graph represents one out of three biological repeats, all show the same trend”. Please include data for all replicates in the graph, including variation between the replicates.

Minor points:

1) In the summary the authors state that mechanisms underlying activation and repression of virulence “remain ill-understood”. Considering the large amount of knowledge gathered by many different groups over many years, describing both transcriptional, post-transcriptional, and post-translational regulation of virulence gene expression, this statement is rather odd.

2) Purification of CsrA foci: To strengthen their conclusions the authors should include WB detection of a control protein (not associated with the condensates) after affinity purification. Alternatively, the authors could provide a Coomassie stained gel of the total protein and of the purified fraction. This is important for the reader to confirm the success of the foci purification.

3) In their results the authors state that ‘At late growth phase, the LEE1 and LEE7 transcripts are recruited to the enlarging foci’. Nevertheless, the authors do not present evidence about recruitment of these mRNAs into the foci. How are these mRNAs recruited? Do they have evidence about reduced cytoplasmic concentration of LEE1 and LEE7 after the focus formation?

4) Based on their microscopy data, it seems that there are differences in the number of bacteria that have foci formation when the system is expressed in different species. To make it more clear for the reader, quantification of the data presented in Fig. 1E and Fig. S2 is suggested.

5) In Fig. 6F an empty plasmid negative control (deltaCsrB, pempty) is missing.

6) In Fig. 3C the scale bar is missing.

7) Fig. 1Aa: how much of the csrA ORF is included in the translational fusion? Please clarify in the text and Table S2.

8) Fig. S3C: labeling of the x-axis is misaligned.

9) Introduction: please mention the role of CsrA for flagellar expression/ formation, since this is important for CsrA’s role in infection.

10) Introduction: “It functions by binding to mRNAs containing two or more motifs, consisting of a GGA sequence within a stem-loop structure”. Please clarify that high affinity binding by CsrA requires the GGA sequence to be in the single-stranded loop.

11) ‘CsrA foci was detected under infection conditions (Fig 1E)’. Please rephrase to ‘…conditions mimicking infection’.

12) In Results the authors state: “During cell division, this focus was inherited by one daughter cell, while the other regenerated a new focus”. While the inheritance of the focus is clear (Fig. S1) the generation of a new one is not.

13) Results: “CsrA-GFP and CsrA-FLAG were biologically functional”. Functionality was only shown for CsrA-GFP but not CsrA-FLAG. The authors should include data verifying that CsrA-FLAG is functional as well.

14) From the interpretation of the data it is not clear how the authors explain the co-existence of CsrA, CsrB, CsrC and CsrA-regulated RNAs in the same hub. Is it a balance between free CsrA and inhibited CsrA? Is it a matter of localisation of CsrB/ CsrC in the focus, or something else? If all CsrA molecules are bound to CsrB or CsrC then CsrA would not be able to regulate its targets.

15) In Results the authors state: “Notably, at the late growth phase, the production of Ler was repressed in a CsrA-dependent manner, consistent with the recruitment of LEE1 and LEE7 mRNAs to the repressing focus.” However, in Fig. 6B there is CsrA complementation only for OD=0.3 (exponential phase). The authors should include CsrA complementation for OD=0.9

(stationary phase) as well.

16) In Results the authors state: “We found that at the late growth phase, the T3SS is no longer functional, as reflected by the fading EPEC infectivity (Fig. 6C and 6D), suggesting that CsrA is required for repressing virulence at late growth phase.” To strengthen this claim, they should use either a csrA deletion strain or a strain carrying the csrAR44A in comparison to the WT.

17) I agree that from Fig. 6E you see stronger repression of Ler when CsrB is expressed. However, delta csrB has no effect to Ler levels, compared to WT. How do the authors explain that? The WB experiment seems to be performed for bacteria at OD=0.3 where the foci are not as many compared to stationary phase. Also, what is the OD of the bacteria that were used for infections in Fig. 6F-G? Did the authors try double mutants of CsrA and CsrB with the respective complementation? Did the authors check the mRNA levels of ler in these mutants? This could help understand further the levels of regulation.

18) In the results the authors claim: ”we monitored the relative concentrations of CsrA-GFP in these two compartments”. How was the relative concentration measured? If it was extrapolated by GFP intensity, then it should be stated as relative intensity rather than relative concentration.

19) “Of note, the degradosome controls the decay rate of CsrB, and thus its activity likely impacts the size of the CsrA-CsrB focus.” This is very speculative and should be rephrased.

20) The authors claim that “CsrB overexpression led to rapid formation of large foci”. However, there is no information about time in Fig. S3B.

21) From the data presented in Fig. S5 it is not clear how the authors connect foci formation with activation and repression of NleA and EspD, respectively.

22) In the results the authors state that ‘Yet, in the focus context CsrA and CsrB appear to cooperate and regulate genes’, even though in the manuscript there are data only for one mRNA. Please rephrase.

23) The csrA strain used in Fig. 6B is not listed in the strain list. Since CsrA is an essential gene in many bacterial species, including strains of E. coli, it is important to specifically state whether the strain used here is a full or partial deletion.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Formation of a membraneless compartment orchestrates bacterial virulence
CsrA is a widely conserved RNA-binding protein (RBP) which post-transcriptionally regulates target transcripts via binding to a short, GA-rich sequence motif. Whereas the CsrA represses the majority of bound transcripts, few mRNAs are stabilized by the RBP. The Csr system is balanced through the activity of small, regulatory RNAs (in E. coli and related species: CsrB, CsrC) that antagonize CsrA binding, providing numerous recognition sites for the RBP.
In this manuscript, Aroeti, Elbaz and colleagues provide insight into the involvement of CsrA in the regulation of virulence gene expression in enteropathogenic E. coli (EPEC). In particular, the authors seek to understand how localization of CsrA and certain RNAs to membraneless foci is connected to an observed decrease of infectivity in late phases of bacterial growth. The authors use different versions of CsrA to demonstrate the necessity of RNA association for the localization, and introduce a methodology for the selective purification of CsrA-containing foci.
The concept of CsrA inactivation via relocalization is compelling, however the data require more systematic analyses to not appear anecdotal. In my opinion, a number of control experiments are required to bolster the obtained results.

My specific comments are the following:
1) It remains unclear what really drives foci formation. A clean quantification of the major players involved could clarify whether there is a concentration-dependent mechanism. At different places of the manuscript there is Western blot data regarding CsrA expression and quantification of fluorescent CsrA fusion proteins, but it would be helpful to comprehensively determine the expression patterns of CsrA (+mutants), CsrB/C over growth/under the relevant conditions in this organism. E.g. the shift in foci/cytoplasm ratio based on fluorescence intensity measurements (which would correspond to an overall increase in RBP) is not reflected in the equal (total) expression of CsrA at low/high cell density as displayed in 4D.
2) Do the authors have an explanation for the peculiar, polar localization pattern of the CsrA-containing foci? It rather resembles the localization/exclusion (Yersinia) pattern of some components of the cell division machinery.
3) I understand that the authors consider RNase E and/or the degradosome as one of the co-localizing factors, however the decay machinery has a much broader distribution. Is the degradosome required for foci formation, or just recruited? How does CsrA localize in an rne701 mutant lacking the C-term of RNase E?
4) How does the number of foci-less cells develop over time (is it solely dependent on division rates)? These have been

excluded from the analysis.
5) Regarding the RNA-content of the purified foci: How do known CsrA substrates distribute? I would be interested to see a more global analysis of known and unknown ligands instead of highlighting a few. The data is a bit hidden in the supplement. Binding motifs and repertoires for CsrA have been determined, is there a pattern to be observerd which RNAs are enriched (affinity, number of sites, …?)? Which RNAs have antagonizing function? E.g., in Salmonella, the highly expressed fim operon has been implicated in CsrA deactivation (PMID 24056837), and a stable processing product of the fim operon of E. coli/Salmonella likewise interferes with the protein's activity (PMID 36354005).
6) The experimental evidence for "cooperation" between CsrA and CsrB on gene regulation is not obvious. As discussed in point 1, all factors in question need to be shown (How much CsrB is made? What is the difference in Ler protein, when considering the non-specific band for quantification?) What would be the mechanistic basis?
7) l. 126: "CsrA-GFP and CsrA-FLAG were biologically functional (Fig. S1)": Fig. S1 only shows data for CsrA-GFP.

Reviewer #4

(Remarks to the Author)
Overall, this manuscript from Aroeti et al. provides compelling new evidence that CsrA/B/C form a membraneless condensate (CSRsome) that acts to regulate virulence gene expression. This study provides a key step forward in the understanding of CSR regulation, by observation of a foci in vivo with CsrB/C RNA, CsrA, and the RNA degradosome complex. Overall, this helps to explain the complex regulation of virulence gene expression at different growth phases. This has a potential for broad readership, to both molecular biologists and pathogenic microbiologists. While this is very exciting, there are a few concerns that if addressed, would improve the manuscript.

Major Concerns

1) GFP is known to impact localization of multimeric proteins [10.1038/nmeth.1955]. To control for this, can the authors perform immunofluorescence to explore CsrA localization in the absence of a fluorescent protein?

2) Are the CsrB/C in the CsrA foci stable from RNase E degradation, or sensitive to RNase E degradation? Perhaps rif-treating the cells and imaging will show whether the foci disassemble.

3) The dimerization model is complicated, as these cells each contain multiple types of CsrA homo and hetero-complexes of unknown stoichiometry. It seems odd that a hetero-dimer with one monomer binding RNA and another mutated, would not bind to a wild-type homo-dimer and enter these foci. Could the authors run a split GFP experiment to examine the localization of only heterodimeric CsrA? In addition, it would be useful to colocalize both WT and mutant in the same cells.

Minor Concerns
1) Is csrA and csrB/C sufficient to drive foci assembly, or does CsrA foci assembly require the CTD of RNase E? There appears to still be CsrA foci even in the delta csrB/C mutant. Can CsrB form foci independent of CsrA? RNase E is known to phase separate, forming a BR-body, might these CsrB foci be specialized BR-bodies?

2) What do the authors mean by condensate like? Biomolecular condensates are not only driven by liquid-liquid phase separation as implied by the authors; and this appears to be a biomolecular condensate.

3) No scale drawn on Fig 3C.

4) Can the authors speculate on what is unique about Y. pseudotuberculosis CsrA as to why it doesn't form foci?

5) Please provide statistical tests on the localization bar graphs presented.

6) CsrA appears to be enriched from cells, but the CsrA foci are not "purified". Please change the nomenclature to denote that they have been enriched, but clearly aren't pure.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all points raised in a satisfactory manner.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
My major comments regarding the manuscript by Aroeti and colleagues on the investigation of CsrA-dependent foci formation in pathogenic E. coli have been mostly addressed in the revised version.

Regarding comment 1, I still miss a comparison between CsrA-GFP as measured in Fi. 2C/D and native CsrA determined in 4E by Western blot analysis. Based on the quantification of the fluorescence microscopy, cytoplasmic (free) CsrA levels are constant over growth, while foci (complexed CsrA) increase in signal. This would result in an overall increase in CsrA protein over growth. How much? The authors state that there is a “small increase” in CsrA protein levels over growth (line 171) – please add a quantification of the blot since that conclusion is not obvious from the image.

Minor comments:
l. 98: Vibrio Cholerae should read Vibrio cholera
l. 275: choice of references does not fit with the species mentioned
Fig. 6E: just as a remark: McaS has been copurified with CsrA before (PMID 23666921)

Reviewer #4

(Remarks to the Author)
Overall, I find that most comments were properly addressed. The manuscript has been much improved, and I think that it provides interesting new findings about CsrA system, and its role in virulence gene regulation. I only have a few minor comments that deal only with some of the writing in the manuscript which could be rather easily clarified in the text.:

From the new data, since both CsrA-mut-GFP and CsrA-mScarlet colocalize, I think this sentence should be tempered: "These results signify that RNA interaction with divalent CsrA dimers is the driving force for foci assembly."

Instead of "the driving force", perhaps "the major driving force".

The lack of dissolution of the CsrA foci by rifampicin is interesting. This suggests that the RNase E colocalization may not be coordinating decay of the RNAs within. It could be an interesting area for follow up studies, and would be useful to add a sentence in the discussion about possible roles for the RNA degradosome localization to CsrA foci.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response to the referees' comments

In the new version we addressed all the concerns raised by the reviewers as detailed by our point-by-point response. Accordingly, the revised version includes refinement of the results and their statistical significance, as well as additional data shown in the main body of the manuscript and the supplemented material. This includes:

- Figures that were refined and improved as suggested include; **2B, 4A, 6A, 7E, S1A, S9B, S9C** and **S9E**. The table that was part of old Fig 5 is shown now as a separate table (Table 1).
- The new data is included in figures **1F, 4E, 4F, 5B-G and 6B-E**, **S1D, S2B, S5, S6, S7D, S11, S12**, **S13,** and matching texts

In all cases, the corresponding text was adjusted as described in our point-by-point response.

In addition, based on the comment, we improved the clarity and accuracy of the text.

We feel that the review process improved the manuscript and wish to thank the reviewers for their constructive comments.

Please find below also our Point-by-point response to all the comments

Point-by-point response

Reviewer #1 (Remarks to the Author):

The manuscript by Aroeti et al. investigates how the RNA-binding protein CsrA contributes to regulation of virulence gene expression in pathogenic *E. coli*. By fusing CsrA to fluorescent proteins and study its expression by microscopy, the authors observe that CsrA forms specific foci within the bacterial cell, a phenomenon that appears to be conserved in several enterobacterial species. The CsrA foci become more prevalent in stationary phase, and their formation seems to depend on the presence of the small RNA CsrB, previously well described as an antagonist of CsrA activity. Isolation of CsrA foci suggest that they, in addition to CsrA and CsrB, contain components of the RNA degradosome, as well as a defined set of mRNAs (e.g. virulence mRNAs) and small RNAs. The authors suggest that the outcome of CsrA activity may differ depending on if CsrA is freely diffusing in the cytoplasm or as part of a condensate-like focus.

The findings are novel, exciting, and suggest a new and unexpected facet of CsrA-dependent regulation. That said, several important issues need to be addressed before the manuscript can be considered for publication. A number of essential control experiments are missing, and the interpretations of the results are sometimes not well supported by the presented data.
We appreciate the positive comments of the reviewer.

Major points:

1) The authors state that the CsrA foci are “orchestrated by the assembly of a membraneless compartment via a process resembling, yet different from, typical liquid-liquid phase separation”. This needs to be rephrased and toned down since no information about the physical properties of the CsrA condensates is presented in the manuscript.
Thank you for this comment. We rephrased the text as suggested, and as proposed by reviewer #4 we termed the CsrA foci structures as “foci”, or “condensates”.

Moreover, the authors claim that the foci serve as a regulatory hub for T3SS expression. What is clear from the data is that the CsrA-GFP/ -mScarlett proteins form foci that also contain a specific set of proteins and RNA. However, the evidence for regulation within these compartments is weak. Several alternative interpretations that also fit well with the data should be considered. For instance, are these compartments used to store molecules rather than to regulate them, or do they function as RNA and/ or protein degradation centers? Are they simply RNA/ protein aggregates without a specific function? These issues should be clearly addressed in the manuscript.
We agree with the reviewer as to the alternative interpretation and addressed this issue in Fig. 8 of the revised version. This Figure shows the model emerging from our results and our perspective of how the foci might function (See legend of Fig. 8B).

2) The authors convincingly show that expression of CsrA fused to GFP or mScarlett results in foci formation. However, the manuscript lacks evidence for native CsrA forming foci. This is a very important point since fusion proteins involving fluorescent proteins have been shown to result in aggregation, although not aggregating when expressed separately (e.g. Pandey et al. JMB 2024, this is just one recent example of several similar studies). To corroborate this major finding in the manuscript, the authors should test whether native CsrA forms foci. A direct way to do this is detection of native CsrA by immunofluorescence. If antibodies against native CsrA are not available, the CsrA-3xFLAG protein could be used. This should be a straightforward experiment considering the expertise of the Rosenshine group in this technique.
Thank you for this comment. As suggested, in the revised version, we include new data demonstrating foci formation by immunofluorescent microscopy (see Fig. S2B and associated text).

3) Figure 4A: Two important points need to be addressed here.
I) Only some of the cells are GFP positive and the authors explain this to be the result of an “unstable plasmid”. This issue needs to be fixed and should be easily solved by expressing CsrB-MS2 from a stable plasmid or by integrating the construct on the chromosome.
The reduced plasmid stability was caused since two plasmids carrying the same ori reside in the same bacterium. In the new version, we refine the results by constructing new plasmids eliminating the issue of incompatibility. The new data is in the revised version (See Fig. 4A, S6, and associated text).

II) The experiment lacks an essential control where the MS2 aptamer is expressed alone or as a fusion to an RNA which CsrA does not bind. Without this control(s) it is simply not possible to judge whether the distribution of the fluorescent signals is specific to CsrB.
The requested control was added, including the expression of untagged CsrB and the MS2 aptamer. In both cases, colocalization between the MS2 protein and the CsrA focus do not occur (See Fig. S6).

4) Figure 5B: The authors interpret the result to mean that CsrA foci colocalizes with RNase E. However, since RNase E puncta are found throughout the cell periphery, the overlap between CsrA-mScarlett and RNase E-mNeon signals could as well happen by chance. Indeed, there are several cells in which the signals do not overlap. The authors need to provide quantitative data on a large number of cells and test whether the suggested co-localization is indeed statistically significant.
We thank the reviewer for this comment. In the revised version two independent quantitative statistical analyses were added. Both indicating that co-localization of the CsrA foci with the RNase E puncta is

statistically significant and exclude the case that the colocalizations are random events. For the latter, we developed an automated assay testing in each biological repeat 400-2000 individual bacteria (See Fig. 5C-G, S11 and S12).

5) The transcriptomic analysis (Fig. 5C) was not sufficiently described. How did the authors perform differential expression analysis, how was enrichment determined, what was compared to what? Please include a detailed description in Materials and methods, and provide sufficient information in Results and the figure legend so this becomes clear for the reader.
The revised version includes a more detailed description of the analysis (See Materials and legend for Fig. 6).

6) Statistical analysis has been performed in some figures (ex. Fig. 6C) but not in others (e.g. Fig. 2B-C, Fig. 3E, Fig 4C, and Fig. S3C-D). Please provide statistical analyses for every result described as "significant" in the text, and describe what type of statistical analysis was performed.
These analyses and related data were added in the revised version.

7) In the legend to Fig. 2B the authors state that "The graph represents one out of three biological repeats, all show the same trend". Please include data for all replicates in the graph, including variation between the replicates.
Thank you for this note. As suggested all the data was unified into one graph in the revised version (See Fig. 2B).

Minor points:

1) In the summary the authors state that mechanisms underlying activation and repression of virulence "remain ill-understood". Considering the large amount of knowledge gathered by many different groups over many years, describing both transcriptional, post-transcriptional, and post-translational regulation of virulence gene expression, this statement is rather odd.
"Ill" was replaced by "partially".

2) Purification of CsrA foci: To strengthen their conclusions the authors should include WB detection of a control protein (not associated with the condensates) after affinity purification. Alternatively, the authors could provide a Coomassie stained gel of the total protein and of the purified fraction. This is important for the reader to confirm the success of the foci purification.
As suggested, we supplemented the revised version with the images of the total protein and of the purified fractions. We used Stain-Free gels (Bio-Rad), which is equivalent to Coomassie stained gels (Fig. S9).

3) In their results the authors state that 'At late growth phase, the LEE1 and LEE7 transcripts are recruited to the enlarging foci'. Nevertheless, the authors do not present evidence about recruitment of these mRNAs into the foci. How are these mRNAs recruited? Do they have evidence about reduced cytoplasmic concentration of LEE1 and LEE7 after the focus formation?

- We thank the reviewer for this comment indicting to us that the phrasing was misleading, suggesting that the LEE1 and LEE7 are recruited **only** in the late log phase. These transcripts are likely colocalized to the foci as the foci evolve, even to the few foci that appear at early log phase and not recruited specifically at late log. However, the consequence of repression of these transcripts by CsrA on the Ler steady state level is evident in the late log phase. This is likely due to the combination of increase in foci development, and the balance between the decay in Ler production and rate of Ler degradation, and dilution due to growth. We apologize for the confusion and corrected the text accordingly (lines 233-234, 250-251, 289-292, 576-580).

- Previous reports indicate that LEE1 and LEE7 contain at least one CsrA binding site[1,2] that likely contribute for their recruitment to the foci. Our RNA seq data indicate, with very high probability (Padj > 10^{-15}), that the LEE1 and LEE7 transcripts are enriched in the foci (Fig. 6A).
- We couldn't obtain reliable results as to the concentration of these transcripts in the cytoplasmic fraction, possibly due to the contamination of this fraction with small foci that couldn't be removed by the ultracentrifugation. Thus, we are not including this data.

4) Based on their microscopy data, it seems that there are differences in the number of bacteria that have foci formation when the system is expressed in different species. To make it more clear for the reader, quantification of the data presented in Fig. 1E and Fig. S2 is suggested.
Quantification of the data was added (See Fig. 1E). It should be said that we do not make any claims as to the significance of these foci in the case of the none-EPEC bacteria, but we simply show that the foci assembly is likely a general phenomenon, not unique to EPEC. Of note, in some cases the CsrA is ectopically expressed. Thus, the significance of CsrA foci in other species remain to be tested and we hope that our results will stimulate such studies.

5) In Fig. 6F an empty plasmid negative control (deltaCsrB, pempty) is missing.
We feel this is not needed as we used a control we believe is superior to that of an empty vector, i.e., the same plasmid but without IPTG for expression.

6) In Fig. 3C the scale bar is missing.
Thank you for noticing! This is now corrected (See Fig. 3C).

7) Fig. 1Aa: how much of the csrA ORF is included in the translational fusion? Please clarify in the text and Table S2.
The entire ORF is included to preserve the CsrA biological function. In the new version, it is more clearly indicated (Fig. 1A legend).

8) Fig. S3C: labeling of the x-axis is misaligned.
Sorry about it and thank you for noting. It is now corrected.

9) Introduction: please mention the role of CsrA for flagellar expression/ formation, since this is important for CsrA's role in infection.
We apologize for omitting this point. The impact on motility is indicated in the revised version (line 58).

10) Introduction: "It functions by binding to mRNAs containing two or more motifs, consisting of a GGA sequence within a stem-loop structure". Please clarify that high affinity binding by CsrA requires the GGA sequence to be in the single-stranded loop.
Sorry for omitting this important fact. This is now indicated as suggested (lines 49-50).

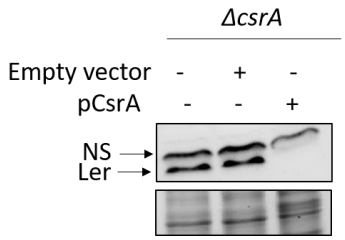
11) 'CsrA foci was detected under infection conditions (Fig 1E)'. Please rephrase to '…conditions mimicking infection'.
We agree and this is now rephrased as requested (lines 82-83).

12) In Results the authors state: "During cell division, this focus was inherited by one daughter cell, while the other regenerated a new focus". While the inheritance of the focus is clear (Fig. S1) the generation of a new one is not.
The text was corrected accordingly. We eliminated conjecture about the foci re-generation (lines 92-94).

13) Results: “CsrA-GFP and CsrA-FLAG were biologically functional”. Functionality was only shown for CsrA-GFP but not CsrA-FLAG. The authors should include data verifying that CsrA-FLAG is functional as well.
The requested data is included in the new version (See Fig. S1A and S1D, lines 88-91 and corresponding figure legends).

14) From the interpretation of the data it is not clear how the authors explain the co-existence of CsrA, CsrB, CsrC and CsrA-regulated RNAs in the same hub. Is it a balance between free CsrA and inhibited CsrA? Is it a matter of localisation of CsrB/ CsrC in the focus, or something else? If all CsrA molecules are bound to CsrB or CsrC then CsrA would not be able to regulate its targets.
Our interpretation is shown in the model (new Fig. 8) and its legend. The data indicate that the backbone of the foci is composed of bivalent CsrA cross bridged by multivalent CsrB. Additional mRNAs and sRNAs containing CsrA binding sites are also recruited based on the affinity to CsrA, number of CsrA binding sites/per RNA and abundance. We cannot exclude that some RNAs are recruited based on base pairing, which is not included in the model for simplicity. We revised the legend of the model to better clarify these points (See legend of Fig. 8B).

15) In Results the authors state: “Notably, at the late growth phase, the production of Ler was repressed in a CsrA-dependent manner, consistent with the recruitment of LEE1 and LEE7 mRNAs to the repressing focus.” However, in Fig. 6B there is CsrA complementation only for OD=0.3 (exponential phase). The authors should include CsrA complementation for OD=0.9 (stationary phase) as well.
Thank you for your comment. The major point in this experiment was to show that CsrA is positively regulating EspD and Repressing Ler. In OD 0.9, EspD is no longer expressed anyway so adding



complementation at 0.9 would be confusing and thus we prefer not to show the complementation for OD 0.9.. Yet, to satisfy the reviewer curiosity please see the blot below, showing Ler levels in the relevant strains reaching stationary phase.

16) In Results the authors state: “We found that at the late growth phase, the T3SS is no longer functional, as reflected by the fading EPEC infectivity (Fig. 6C and 6D), suggesting that CsrA is required for repressing virulence at late growth phase.” To strengthen this claim, they should use either a csrA deletion strain or a strain carrying the csrAR44A in comparison to the WT.
We cannot perform the suggested experiment since CsrA is also essential for biogenesis of functional T3SS during the early log phase as previously reported[1,2] and as shown in Fig. 7A and 7B. We attempted to make it clearer in the revised version.

17) I agree that from Fig. 6E you see stronger repression of Ler when CsrB is expressed. However, delta csrB has no effect to Ler levels, compared to WT. How do the authors explain that? The WB experiment seems to be performed for bacteria at OD=0.3 where the foci are not as many compared to stationary phase. Also, what is the OD of the bacteria that were used for infections in Fig. 6F-G? Did the authors try double mutants of CsrA and CsrB with the respective complementation? Did the authors check the mRNA levels of ler in these mutants? This could help understand further the levels of regulation.

- The minor effect of the deletion of CsrB on Ler levels is related to the nature of Ler, which is an autorepressor, forming a negative feedback loop that prevents its expression above a certain threshold[3,4]. This is better explained in the revised version.
- The requested additional information is included in the revised version (quantification of WB fig. 7E and RT-PCR of CsrB fig. S7C).

18) In the results the authors claim: "we monitored the relative concentrations of CsrA-GFP in these two compartments". How was the relative concentration measured? If it was extrapolated by GFP intensity, then it should be stated as relative intensity rather than relative concentration.
The reviewer is right, the relative intensity was determined. This is now better stated in the revised version.

19) "Of note, the degradosome controls the decay rate of CsrB, and thus its activity likely impacts the size of the CsrA-CsrB focus." This is very speculative and should be rephrased.
This was rephrased in the revised version. The rephrasing was done also in light of the results of an experiment suggested by Reviewer #4: to test the foci stability upon treatment with Rif and Cm. In both cases the foci remain stable during one-hour treatments. (See Fig. S13)

20) The authors claim that "CsrB overexpression led to rapid formation of large foci". However, there is no information about time in Fig. S3B.
The "rapid" is deleted in the revised version.

21) From the data presented in Fig. S5 it is not clear how the authors connect foci formation with activation and repression of NleA and EspD, respectively.
This blot is just a control to show that the classical function of CsrB as anti-CsrA is maintained. It also enhances the novelty of our findings since increased CsrB levels lead to foci formation, to anti-CsrA activity related to some genes (e.g., NleA and EspD) and to pro-CsrA activity related to Ler (Fig 6E).

22) In the results the authors state that 'Yet, in the focus context CsrA and CsrB appear to cooperate and regulate genes', even though in the manuscript there are data only for one mRNA. Please rephrase.
This is now rephrased as requested.

23) The csrA strain used in Fig. 6B is not listed in the strain list. Since CsrA is an essential gene in many bacterial species, including strains of *E. coli*, it is important to specifically state whether the strain used here is a full or partial deletion.
The entire CsrA ORF was deleted as previously reported[1,2]. So, CsrA is not essential for EPEC, but the mutant grow at a lower rate. This data is included in the new version (See Table S1).

Reviewer #2 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We thank reviewer #2 for contributing to the improvement of our manuscript

Reviewer #3 (Remarks to the Author):
Formation of a membraneless compartment orchestrates bacterial virulence
CsrA is a widely conserved RNA-binding protein (RBP) which post-transcriptionally regulates target transcripts via binding to a short, GA-rich sequence motif. Whereas the CsrA represses the majority of bound transcripts, few mRNAs are stabilized by the RBP. The Csr system is balanced through the activity of small, regulatory RNAs (in E. coli and related species: CsrB, CsrC) that antagonize CsrA binding, providing numerous recognition sites for the RBP. In this manuscript, Aroeti, Elbaz and colleagues

provide insight into the involvement of CsrA in the regulation of virulence gene expression in enteropathogenic E. coli (EPEC). In particular, the authors seek to understand how localization of CsrA and certain RNAs to membraneless foci is connected to an observed decrease of infectivity in late phases of bacterial growth. The authors use different versions of CsrA to demonstrate the necessity of RNA association for the localization, and introduce a methodology for the selective purification of CsrA-containing foci.The concept of CsrA inactivation via relocalization is compelling, however the data require more systematic analyses to not appear anecdotal. In my opinion, a number of control experiments are required to bolster the obtained results.
Thank you for finding the concept interesting.

My specific comments are the following:
1) It remains unclear what really drives foci formation. A clean quantification of the major players involved could clarify whether there is a concentration-dependent mechanism. At different places of the manuscript there is Western blot data regarding CsrA expression and quantification of fluorescent CsrA fusion proteins, but it would be helpful to comprehensively determine the expression patterns of CsrA (+mutants), CsrB/C over growth/under the relevant conditions in this organism. E.g. the shift in foci/cytoplasm ratio based on fluorescence intensity measurements (which would correspond to an overall increase in RBP) is not reflected in the equal (total) expression of CsrA at low/high cell density as displayed in 4D.
Thank you for your comment. As suggested, a clean quantification of CsrA and CsrB was added to Fig. 4D, 4E and 4F.

2) Do the authors have an explanation for the peculiar, polar localization pattern of the CsrA-containing foci? It rather resembles the localization/exclusion (Yersinia) pattern of some components of the cell division machinery.
The foci are localized to the cell periphery, occasionally, but not exclusively, to the poles. The mechanism of the peripheral localization remains to be elucidated.

3) I understand that the authors consider RNase E and/or the degradosome as one of the co-localizing factors, however the decay machinery has a much broader distribution. Is the degradosome required for foci formation, or just recruited? How does CsrA localize in an rne701 mutant lacking the C-term of RNase E?
Thank you for bringing this up. Following the reviewer's suggestion, we constructed EPEC *rne* mutant (*rne*701) lacking the C-terminus of RNase E. Furthermore, we fused mNeon to the C-terminus of the truncated RNase E. This mutant still formed CsrA foci (Fig. S12A), indicating that the RNase E C-terminus is dispensable to create the CsrA foci. Testing for colocalization of the truncated RNase E and CsrA foci was more challenging since the steady-state levels of the mutated RNase E were found to be higher than those of the wild-type protein, thus, increasing the chance for colocalization due to random events. We thus used a methodology to automate the colocalization analysis of a large number of bacteria (Fig. S12B), and used it to address this issue. We show that CsrA foci signicantly colocalize with condensates of the truncated RNase E (Fig. S12). This new data is included in the revised version.

4) How does the number of foci-less cells develop over time (is it solely dependent on division rates)? These have been excluded from the analysis.
We think that the division rates are an important factor, but we cannot exclude the involvement of additional mechanisms.

5) Regarding the RNA-content of the purified foci: How do known CsrA substrates distribute? I would be interested to see a more global analysis of known and unknown ligands instead of highlighting a few. The data is a bit hidden in the supplement.
As suggested the revised version provides a more global view of the results (see Fig. 6).

Binding motifs and repertoires for CsrA have been determined, is there a pattern to be observerd which RNAs are enriched (affinity, number of sites, …?)? Which RNAs have antagonizing function? E.g., in Salmonella, the highly expressed fim operon has been implicated in CsrA deactivation (PMID 24056837), and a stable processing product of the fim operon of E. coli/Salmonella likewise interferes with the protein's activity (PMID 36354005).

To address this comment, we compared our finding to the only other reported comprehensive study of CsrA-RNA interaction in *E. coli* that we know of[5]. The new analysis is shown in Fig. 6 and described in the text. This study, by the Romeo group, reports the results of CLIP-seq of CsrA in *E. coli* K12 grown in LB. Accordingly, we clearly indicated in the text that the two studies used different strains (K12 and EPEC), grown in different medium (LB and DMEM) and employed two different methodologies (CLIP-seq of total lysate and RNA-seq of enriched foci fraction). Despite these differences this comparison turns out to be revealing, particularly in the case of the sRNAs (Fig. 6D and 6E). Finally, unfortunately, as far as we know, even for *E. coli* K12 very little is known about the exact binding sites of most of the mRNAs, including their number and affinity to CsrA. Interestingly, the study of Romeo group indicates that in most cases the significant of CsrA-mRNA interaction remain elusive[5]. I.e., the binding by CsrA showed no impact on translation or RNA stability.

6) The experimental evidence for "cooperation" between CsrA and CsrB on gene regulation is not obvious. As discussed in point 1, all factors in question need to be shown (How much CsrB is made? What is the difference in Ler protein, when considering the non-specific band for quantification?) What would be the mechanistic basis?

As suggested, the amounts of all the factors are shown in the revised version (Fig. 4D-F, S7C-D). As to the involved mechanism, We feel that elucidating this mechanism is more appropriate for a follow-up investigation. We suppose that this issue might be challenging. Our results indicate that in addition to its anti-CsrA function, CsrB plays a key role in the assembly of the foci backbone, leading a local crowding of mRNAs, regulatory sRNAs, and CsrA. We speculate that multiple dynamic protein-RNA and RNA-RNA and protein-protein interactions are taking place within the foci forming intricate and dynamic networks, leading to regulatory consequences. This issue is currently under investigation in our group.

7) l. 126: "CsrA-GFP and CsrA-FLAG were biologically functional (Fig. S1)": Fig. S1 only shows data for CsrA-GFP.

We apologize for unintentionally omitting this data. The revised version includes the data related to CsrA-FLAG (See Fig. S1A and S1D). On note, the FLAG tagging was shown also by others to have little impact, if any on CsrA function[6].

Reviewer #4 (Remarks to the Author):

Overall, this manuscript from Aroeti et al. provides compelling new evidence that CsrA/B/C form a membraneless condensate (CSRsome) that acts to regulate virulence gene expression. This study provides a key step forward in the understanding of CSR regulation, by observation of a foci in vivo with CsrB/C RNA, CsrA, and the RNA degradosome complex. Overall, this helps to explain the complex regulation of virulence gene expression at different growth phases. This has a potential for broad readership, to both molecular biologists and pathogenic microbiologists. While this is very exciting, there are a few concerns that if addressed, would improve the manuscript.

Thank you for the positive feedback.

Major Concerns

1) GFP is known to impact localization of multimeric proteins [10.1038/nmeth.1955]. To control for this, can the authors perform immunofluorescence to explore CsrA localization in the absence of a fluorescent protein?

In the revised version we include new data demonstrating foci formation by immunofluorescence microscopy (See Fig. S2B).

2) Are the CsrB/C in the CsrA foci stable from RNase E degradation, or sensitive to RNase E degradation? Perhaps rif-treating the cells and imaging will show whether the foci disassemble.
We performed the proposed experiment and the results are included in the revised version (Fig. S13), showing that 60 min treatment with either Rif, or Cm, show no significant impact on foci stability.

3) The dimerization model is complicated, as these cells each contain multiple types of CsrA homo and hetero-complexes of unknown stoichiometry. It seems odd that a hetero-dimer with one monomer binding RNA and another mutated, would not bind to a wild-type homo-dimer and enter these foci. Could the authors run a split GFP experiment to examine the localization of only heterodimeric CsrA? In addition, it would be useful to colocalize both WT and mutant in the same cells.
We assume that whereas divalent CsrA-dimers efficiently integrate into the foci, this efficiency drops in the case of mono-valent CsrA-heterodimers. This likely accounts for our inability to detect the heterodimers of CsrA alleles, expressed at relatively low levels via the native promoters, as shown in Fig 3C and 3D. Given that the native expression levels are relatively low, we assumed that increasing the concentration of the monovalent heterodimers should compensate for their mono-valency. Thus, to address this comment, we generated an EPEC strain expressing CsrA-mScarlet via the native promoter (i.e., relatively low expression levels), supplemented with a plasmid expressing $CsrA_{R44A}$-GFP via a strong IPTG-regulated promoter. We then allow the bacteria to form foci by growing them in DMEM to reach the early exponential phase and afterwards, induce overexpression of $CsrA_{R44A}$-GFP. As the reviewer predicted, the data show that overexpressed $CsrA_{R44A}$-GFP is recruited to the foci formed by the wild type CsrA-mScarlet. The new data is included in the revised version (Fig. S5).

Minor Concerns
1) Is csrA and csrB/C sufficient to drive foci assembly, or does CsrA foci assembly require the CTD of RNase E? Following the reviewer's suggestion, we constructed an EPEC *rne* mutant deleted of its (CTD) (i.e., *rne*701 mutation). This mutant was still able to form CsrA foci and this new data is included in the revised version (Fig. S12).
There appears to still be CsrA foci even in the delta csrB/C mutant. This is correct, but much fewer and fainter foci. Those rare foci might be promoted by other sRNAs that are also very enriched in the foci (Fig 6)
Can CsrB form foci independent of CsrA? In Fig. 4A and other fields, CsrB foci independent of CsrA were rarely observed, if at all, even though CsrB is overexpressed from a plasmid. This suggests that the formation of CsrB foci independent of CsrA is unlikely. However, it is challenging to completely rule out the possibility of rare CsrA-independent CsrB foci, as this requires genetic manipulation of EPEC with a deleted *csrA* gene. Our attempts to further manipulate this strain were largely unsuccessful, and transforming it with plasmids, other than those expressing CsrA, proved difficult.

RNase E is known to phase separate, forming a BR-body, might these CsrB foci be specialized BR-bodies? Notably, the C-term of the *E. coli* RNase E is shorter than that of *C. crescentus* and this likely accounts for the small puncta formed by the *E. coli* RNase E, whereas that of *C. crescentus* forms large BR-bodies.

2) What do the authors mean by condensate like? Biomolecular condensates are not only driven by liquid-liquid phase separation as implied by the authors; and this appears to be a biomolecular condensate.
We adjusted the text as suggested, and used the terms “condensates” or “foci”.

3) No scale drawn on Fig 3C.
Thank you for noticing! This is now corrected.

4) Can the authors speculate on what is unique about Y. pseudotuberculosis CsrA as to why it doesn't form foci?
We think that under the used condition Yp is not expressing CsrB. This assumption is based on the reports of Heroven *et. al.*[7,8], indicating that in Yp CsrB is expressed primarily in the infected animal and not in cultures. This is indicated in the revised version.

5) Please provide statistical tests on the localization bar graphs presented.
This is provided in the revised version.

6) CsrA appears to be enriched from cells, but the CsrA foci are not "purified". Please change the nomenclature to denote that they have been enriched, but clearly aren't pure.
The text was adjusted as requested.

Reviewer #5 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We appreciate the input of reviewer #5 for the enhancement of our manuscript

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Point-by-point response to the comments

Reviewer #1 (Remarks to the Author):

The authors have addressed all points raised in a satisfactory manner.
We thank the reviewer for helping to improve our manuscript

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We thank the reviewer for helping to improve our manuscript

Reviewer #3 (Remarks to the Author):

My major comments regarding the manuscript by Aroeti and colleagues on the investigation of CsrA-dependent foci formation in pathogenic E. coli have been mostly addressed in the revised version.
We thank the reviewer for helping to improve our manuscript

Regarding comment 1, I still miss a comparison between CsrA-GFP as measured in Fi. 2C/D and native CsrA determined in 4E by Western blot analysis. Based on the quantification of the fluorescence microscopy, cytoplasmic (free) CsrA levels are constant over growth, while foci (complexed CsrA) increase in signal. This would result in an overall increase in CsrA protein over growth. How much? The authors state that there is a "small increase" in CsrA protein levels over growth (line 171) – please add a quantification of the blot since that conclusion is not obvious from the image.
A quantification was added as required (see Fig 4E)

Minor comments:
l. 98: Vibrio Cholerae should read Vibrio cholera
Thank you for the comment. As far as we know "Cholera" is the name of the disease, not the bacterium itself, which should be *Vibrio Cholerae*

l. 275: choice of references does not fit with the species mentioned
We are sorry that instead of citing the original papers we used ref #21, a review that summarizes the vast knowledge of CsrA involvement in the virulence of many pathogens. We typically prefer to respect and cite the original papers, but in this case, we felt that the number of original papers supporting the statement is just too large.

Fig. 6E: just as a remark: McaS has been copurified with CsrA before (PMID 23666921)
We are aware of this report by Jørgensen, yet, the Jørgensen report is not a comprehensive analysis of CsrA-bound sRNAs. Fig 6E compares our results to those of the CsrA CLIP-seq analysis published by the Romeo group. McaS expression might be very low under the conditions used in Romeo study and thus was not re-identified by this group as CsrA-bound sRNA.

Reviewer #4 (Remarks to the Author):

Overall, I find that most comments were properly addressed. The manuscript has been much improved, and I think that it provides interesting new findings about CsrA system, and its role in

virulence gene regulation. I only have a few minor comments that deal only with some of the writing in the manuscript which could be rather easily clarified in the text.:
We thank the reviewer for helping to improve our manuscript

From the new data, since both CsrA-mut-GFP and CsrA-mScarlet colocalize, I think this sentence should be tempered:
"These results signify that RNA interaction with divalent CsrA dimers is the driving force for foci assembly."

Instead of "the driving force", perhaps "the major driving force".
We agree and the text is corrected

The lack of dissolution of the CsrA foci by rifampicin is interesting. This suggests that the RNase E colocalization may not be coordinating decay of the RNAs within. It could be an interesting area for follow up studies, and would be useful to add a sentence in the discussion about possible roles for the RNA degradosome localization to CsrA foci.
Thank you for this good idea! We will compare the foci transcriptome before and after the rif treatment to obtain a global view of differential RNA stability within the enriched foci. A sentence about it was added in the fig legend of the model.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We thank the reviewer for helping to improve our manuscript