THE EMBO JOURNAL

The SiaA/B/C/D signaling network regulates biofilm formation in *Pseudomonas aeruginosa*

Gukui Chen, Jianhua Gan, Chun Yang, Yili Zuo, Juan Peng, Meng Li, Weiping Huo, Yingpeng Xie, Yani Zhang, Tietao Wang, Xin Deng and Haihua Liang

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 7th Oct 2019

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

As you will see from the comments, both reviewers appreciate the work and the topic. However, they also raise a number of concerns that need to be addressed before they can support publication here. From my side, I judge the referee comments to be generally reasonable, therefore, based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of both referees. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

REFEREE REPORTS:

Referee #1:

The authors investigate a c-di-GMP regulatory system in Pseudomonas aeruginosa, components of which had been implicated previously in cell aggregation and biofilm formation. The particular focus of the study is on the siaABCD operon. Phenotypes and function associated with siaA and siaD, encoding a phosphatase and diguanylate cyclase, respectively, had been reported before but the relevance and role of the central genes siaB and siaC remained poorly defined. Here, genetic experiments coupled with phenotypic readouts (i.e., biofilm formation and aggregation) reveal the hierarchy of the operon's components. Their interplay on the protein level is shown by bacterial two-hybrid assays that were confirmed by pulldowns from cell lysates and in vitro protein-protein interaction studies. Enzyme assays report on activity of the proteins studied here. The resulting data indicate that the diguanylate cyclase SiaD requires SiaC for activity. SiaC in turn is inversely regulated by SiaA (a phosphatase) and SiaB (a kinase). The switch from an active SiaC (i.e., SiaD-

stimulating) to an inactive SiaC is controlled by phosphorylation of a threonine residue at position 68 in SiaC. The authors go on to present a crystal structure of a SiaC-SiaB complex that reveals the mechanism by which SiaB phosphorylates SiaC. The proposed mechanism, supported by key experimental data, is distinct from other mechanisms that regulate diguanylate cyclases, which adds to the significance of this study.

Overall, the individual experiments are well controlled. In particular, the genetic dissection of the system by using various mutant backgrounds and complementation strategies clearly demonstrate the interplay and hierarchy of the siaABCD system. Biochemical and structural studies add mechanistic insight into the individual steps. While the aggregated data describe a new mode of c-di-GMP signaling regulation that will be of interest to the field, there are a few major points the authors are encouraged to consider. They pertain mainly to the biochemical characterization and target a better integration of the individual steps that were described here.

Major points:

1. Please comment on the variability in the cell-based assays. For example, the siaA deletion appears more pronounced in Figure 1A than in Figure 2G. What could be reasons for the apparent variabilities?

2. Both, SiaA and SiaB interact directly with SiaC, and their complexes appear to be rather stable based on the apparent stoichiometric amounts in the pull downs and gel filtration analysis. Is their binding mutually exclusive? Is there an affinity difference for the SiaA-SiaC and SiaB-SiaC interactions? Do SiaA and/or SiaB preferentially bind to phosphorylated or unphosphorylated SiaC? Another question concerns the interaction between SiaC and SiaD - is it maintained when SiaA or SiaB act on SiaC? Do SiaA-SicC-SiaD or SiaB-SiaC-SiaD complexes form? These questions are important since addressing them should shed light on the interplay of the signaling components and inverse regulation of SiaD by phosphorylation of SiaC.

3. The authors use mutational analyses to corroborate structural findings. Since most of the phenotypes are loss-of-function (and in particular the SiaB-L110A-F174A mutant that is insoluble in E. coli; see lines 386-387), it is imperative to test whether these proteins are made in the expression host (Pseudomonas aeruginosa for the biofilm assays). Otherwise, it is difficult to discern between (less-specific) protein folding defects and (more-specific) effects due to the disruption of interfaces without impacting protein stability.

4. The question of metal specificity for SiaB kinase activity remains unanswered. Many ATPases rely on divalent cations (usually Mg2+ or Mn2+), which includes the structural homologs of SiaB discussed in this study. However, the chelating agent EDTA had no effect on catalytic activity of SiaB. From the structure, there appears to be a monovalent ion at the active site. It is not clear whether this is sufficient to support catalysis. A related question is whether this ion is required for catalysis.

5. The model in Figure 6 suggests that SiaD dissociates from SiaC, however there is no evidence presented in support for this event. If anything, SiaD activity requires SiaC, suggesting activity arises from the complex.

Minor points:

6. Line 57: It is not clear why the Chen et al. 2016 reference is included here. The study does not describe PDE activity of oligoribonuclease.

7. Lines 58-82: The relevance of this paragraph for the following study is not clear. It sounds more like a review of selected works in the field. It would be appropriate to move this section to the Discussion and compare these modes of diguanylate cyclase regulation to the SiaC-SiaD system, where control is achieved through a heterologous complex.

8. Lines 94-99: The reference format is off in this paragraph.

9. Line 180: 'FALG' should be 'FLAG'.

10. Line 186: Consider changing 'promoted' to 'prompted'?

11. Line 205: 'regulate DGC activity' and 'c-di-GMP production' are redundant statements.

12. Lines 243, 245 and 246: Figure references should point to Figures S3A, S3E, and S3F, respectively.

13. Figures S3C and S3F: Please show elution profiles for the individual proteins for comparison. This is an important control since peak elution volumes are not only dependent on proteins size but also hydrodynamic radius.

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16. Line 285: Maybe '..., which is in contrast to the phenotype observed in wild-type.'

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18. Line 326: 'Fig. 2F' should be 'Fig. 3F'.

19. Line 328: Maybe 'DGC reaction mixture' would be more appropriate here (instead of 'c-di-GMP reaction mixture).

20. Figure 3A: The description of how ATPase level data were handled is fairly cryptic. Please describe explicitly how the data was normalized.

21. Figure 3G: Please mention explicitly that ATP was added to the reactions in order to assess the effect of protein phosphorylation. While it is mentioned in the Materials and Methods section, it is an important detail of the experimental setup and should be mentioned more visibly.

22. Line 339: 'Per' should be 'The'.

23. Figures in the main text and supplement use labels 'WT' or 'PAO1' interchangeably. I would suggest choosing the same label for all to be consistent throughout.

24. Page 14 (and throughout): For references to specific residues, use either single-letter or three-letter code, not both.

25. Figure 4D: I assume the second half of the graph should indicate that experiments were conducted in the delta-siaA background (not delta-siaC, as shown).

26. Figure 5A: Instead of showing a 2Fo-Fc map, please show a Fo-Fc omit map. The latter would have less model bias.

27. Line 405: Please report the scores for the top hits from the Dali search.

28. Lines 423-424: 'Might be due to...,' sounds a bit awkward and should probably rephrased.

29. Lines 494, 499, 508: 'one' should be 'a'.

30. Line 668-669: This description is redundant with lines 663-664.

31. Throughout: The work 'noselective' is used several times and should probably be replaced with 'nonselective'.

32. Figures (in general): Some of the panels (i.e., the aggregation images) and panel labeling are hard to read on printed copies.

Referee #2:

Review of "Structural and functional insights into a novel signalling network regulating biofilm formation" by Chen et al.
The manuscript describes the characterisation of four proteins, SiaABCD from Pseudomonas, whose interplay regulates classical biofilm/aggregation phenotypes via cyclic-di-GMP levels. On the whole, the work is competent, novel* and well-described, but several concerns have to be addressed to warrant publication in EMBO J.
*I note that a partly overlapping study exists on Biorxiv by the Klebensberger lab; for the purposes of review I have looked at this "blind" in comparison to that.

Major points to be addressed:
-The researchers present a model for interplay/regulation that needs to be verified by further experimentation, namely they must check (using purified protein) if SiaB-phosphorylated SiaC is able to pull-down or interact with SiaD.
-In the enzyme assays, measuring SiaD DGC activity, did the researchers mutate the I-site to inhibit negative feedback? This may help in signal detection which they otherwise claim is weak (line 212). If they didn't I would recommend assessing the effect of doing this.
-I am a little surprised at the section (lines 291-333) headed "SiaB functions as a protein kinase to phosphorylate SiaC at T68". This is written as true discovery, when in fact it should be written as confirmation - it is already known that SiaC is phosphorylated on this residue - (Ravichandran, A., Sugiyama, N., Tomita, M., Swarup, S. & Ishihama, Y. Ser/Thr/Tyr phosphoproteome analysis of pathogenic and non-pathogenic Pseudomonas species. Proteomics 2009). I would rewrite this part to reflect that. Of course, the authors are discovering that SiaB is the exact kinase responsible, but I felt the omission of a reference to the above was either an oversight or misleading.
-does a SiaC T68D mutant possess any phenotype as a putative phosphomimic?
-lines 320-321 - SiaCT68A interacts with SiaB....this could occur indirectly via a third interaction with SiaD (or any other component). Why has this not been performed with purified proteins rather than (multifactorial) lysate? Or, use lysate from SiaD KO strains. [an example can be seen in fig2e in which SiaA indirectly pulls down SiaB]
-lines 389-390 "these residues...are essential for function of SiaB in vivo". Or for protein folding this has not been demonstrated conclusively and needs further experimentation or rewording.

Minor points:
-I would rewrite abstract to introduce the proteins and their functions more clearly
-please remove and replace any references to the retracted work by the Ryan group (e.g. HDGYP reference)
-lines 58-82 are overlong, over-detailed with respect to the actual subject of this study, I would condense this section; possibly expanding section lines 83-93 in its place
-end of introduction would benefit from a description of several known examples of phosphorylation in cyclic-di-GMP signalling (non-histidine kinase) e.g. move WspR reference from discussion to here, include DgcB from Bdellovibrio bacteriovorus (doi: 10.1038/s41467-019-12051-6) and MtT from Moorella thermoacetica (https://doi.org/10.1016/j.str.2012.01.003)
-I would not use the term "chaperone" e.g. line 101 to describe SiaC UNLESS you can show it acts in a true manner of a chaperone to modulate protein folding. Binding partner yes, chaperone less-likely
-line 112 denoting SiaA as a putative phosphatase should go into introduction
-line 115 standardize - sia first, PA#### in brackets
-similarly, throughout, standardize proteins vs genes, capital letters, italics etc, currently messy
-line 121 state which (KO or wildtype) "behaves similarly"
-line 131/2 "restored their biofilm formation" - it appears that SiaB complementation in fig1A reduces biofilm formation - please explain this apparent discrepancy
-line 139-140 - did researchers ever measure [cdg] levels in cell lysates?
-line 148 I would phrase this as "suggest" rather than "demonstrated"

-line 223 needs to read "promotes bacterial aggregation"
-line 269-270, please add clarity as to nature of siaA386 and siaA337
-I'm not sure their metal ion is Na (line 401-404) and would recommend looking at papers by Majorie Harding or others to investigate if co-ordination informs on a different metal
-lines 396 onward; The idea that K72A is unphosphorylated needs to be mentioned before results that rely on this interpretation
-line 405 - provide rmsd statistics for fold matches, also at lines 435-436
-line 431-432 - are these changes a result of crystal packing?
-lines 508-509 - the authors may want to use the common term "substrate-mediated catalysis" to explain this phenomenon
[typos/errors need to be fixed in all figures and their legends]
-fig1 - what are the values in 1b normalized in reference to?
-fig2 - a and d - where is the SiaA vs SiaB data?
-fig 6 - if main part labels SiaA and B directly, then why not do this for C and D also - i.e. why put them in key? Left-hand side should read "high [c-di-GMP]", right-hand side "low [c-di-GMP]" rather than relying on star graphic abundance.
-does phosphorylation of SiaC block GTP binding of SiaD? This could be measured, maybe even with a catalytic dead version of SiaD to simplify the experiment.

1st Revision - authors' response 10th Dec 2019

Response to the Reviewers' comments

The authors would like to thank for the Reviewers' constructive comments concerning our manuscript entitled "Structural and functional insights into a novel signaling network regulating biofilm formation" (ID: EMBOJ-2019-103412). These comments are all valuable and help us improve our manuscript. We have done a series of experiments and incorporated all the corrections and suggestions raised by the Reviewers' in the revised manuscript. The point-to-point responses to the Reviewer's comments are as following:

Referee #1:
The authors investigate a c-di-GMP regulatory system in Pseudomonas aeruginosa, components of which had been implicated previously in cell aggregation and biofilm formation. The particular focus of the study is on the siaABCD operon. Phenotypes and function associated with siaA and siaD, encoding a phosphatase and diguanylate cyclase, respectively, had been reported before but the relevance and role of the central genes siaB and siaC remained poorly defined. Here, genetic experiments coupled with phenotypic readouts (i.e., biofilm formation and aggregation) reveal the hierarchy of the operon's components. Their interplay on the protein level is shown by bacterial two-hybrid assays that were confirmed by pulldowns from cell lysates and in vitro protein-protein interaction studies. Enzyme assays report on activity of the proteins studied here. The resulting data indicate that the diguanylate cyclase SiaD requires SiaC for activity. SiaC in turn is inversely regulated by SiaA (a phosphatase) and SiaB (a kinase). The switch from an active SiaC (i.e., SiaD-stimulating) to an inactive SiaC is controlled by phosphorylation of a threonine residue at position 68 in SiaC. The authors go on to present a crystal structure of a SiaC-SiaB complex that reveals the mechanism by

which SiaB phosphorylates SiaC. The proposed mechanism, supported by key experimental data, is distinct from other mechanisms that regulate diguanylate cyclases, which adds to the significance of this study.

Overall, the individual experiments are well controlled. In particular, the genetic dissection of the system by using various mutant backgrounds and complementation strategies clearly demonstrate the interplay and hierarchy of the siaABCD system. Biochemical and structural studies add mechanistic insight into the individual steps. While the aggregated data describe a new mode of c-di-GMP signaling regulation that will be of interest to the field, there are a few major points the authors are encouraged to consider. They pertain mainly to the biochemical characterization and target a better integration of the individual steps that were described here.

Response: We sincerely thank the Reviewer's positive evaluation for our manuscript. The constructive comments and suggestions have helped us improve the quantity of our manuscript.

Major points:
1. Please comment on the variability in the cell-based assays. For example, the siaA deletion appears more pronounced in Figure 1A than in Figure 2G. What could be reasons for the apparent variabilities?

Response: Thanks for your comment. The variabilities in cell-based assays in Fig 1A and Fig 2G were due to the deviation during quantification of crystal violet stained biofilm by ethanol elution. We have repeated biofilm assays strictly under the same conditions and the results were shown in Fig 1A and Fig 2H.

2. Both, SiaA and SiaB interact directly with SiaC, and their complexes appear to be rather stable based on the apparent stoichiometric amounts in the pull downs and gel filtration analysis. Is their binding mutually exclusive?

Response: Thanks for the helpful comments. To address this, we performed a GST pull down assay using GST-SiaA$_{386}$ (GST-SiaA was not expressed) and SiaC-His proteins. After SiaC-his binding, DH5α lysates containing SiaB-FLAG was then added. In principle, if SiaA-SiaC and SiaB-SiaC bindings are mutually exclusive, SiaA$_{386}$-SiaC-SiaB complex would not form. However, our results showed that the SiaA$_{386}$-SiaC-SiaB complex is formed (Appendix Figure 5C). This result indicates that the SiaA-SiaC and SiaB-SiaC bindings are not mutually exclusive. Therefore, we speculate that SiaA and SiaB might bind to SiaC at separate sites.

Is there an affinity difference for the SiaA-SiaC and SiaB-SiaC interactions?

Response: To test the binding affinity for SiaA-SiaC and SiaB-SiaC interactions, we performed SPR (Surface Plasmon Resonance) assays using these purified proteins. The SPR data showed that the binding affinity of SiaA$_{386}$-SiaC (Fig EV2C, K_D=19.2 nM) is slightly higher than that of SiaB-SiaC (Fig 2C: K_D=40.1 nM).

Do SiaA and/or SiaB preferentially bind to phosphorylated or unphosphorylated SiaC?

Response: Thanks. To address this question, we performed SPR and GST pull-down assays. These data showed that: 1) both SiaA$_{386}$ and SiaB interact with SiaC (Fig EV2C and Fig 2C); 2) SiaA$_{386}$ could also interact with SiaC$_P$ in the presence of Mg^{2+} (Fig EV2C, D). The binding affinity (K_D = 19.2 nM) between SiaA$_{386}$ and SiaC was slightly higher than that (K_D = 46.5 nM) between SiaA$_{386}$ and SiaC$_P$. However, SiaB was unable to bind to SiaC$_P$ in the absence of ADP (Adenosine diphosphate) (Fig EV4A, B), suggesting that SiaB preferentially binds to unphosphorylated SiaC.

Another question concerns the interaction between SiaC and SiaD - is it maintained when SiaA or SiaB act on SiaC? Do SiaA-SicC-SiaD or SiaB-SiaC-SiaD complexes form? These questions are important since addressing them should shed light on the interplay of the signaling components and inverse regulation of SiaD by phosphorylation of SiaC.

Response: Thanks for the helpful suggestions. To test whether SiaC-SiaD binding is maintained when SiaA acts on SiaC, a GST pull down assay was performed. First, immobilized GST-SiaA$_{386}$ was incubated with SiaC-His for 2 hours. Then, the DH5α lysate containing SiaD-Flag was added and incubated for 2 additional hours before washed by reaction buffer. In principle, if SiaC-SiaD interaction maintained when SiaA acts on SiaC, the SiaA-SiaC-SiaD ternary complex would retained in the pull down sample. However, western blot analysis showed that the SiaA-SiaC-SiaD complex is not formed (Appendix Figure S5D). This result suggests that SiaA binding will disrupt the interaction between SiaC and SiaD.

In addition to SiaA, we also performed pull down assay using GST-SiaB. Similarly, no SiaB-SiaC-SiaD complex formation was observed (Appendix Figure S3D), suggesting that the binding of SiaB will also disrupt the interaction between SiaC and SiaD.

3. The authors use mutational analyses to corroborate structural findings. Since most of the phenotypes are loss-of-function (and in particular the SiaB-L110A-F174A mutant that is insoluble in E. coli; see lines 386-387), it is imperative to test whether these proteins are made in the expression host (Pseudomonas aeruginosa for the biofilm assays). Otherwise, it is difficult to discern between (less-specific) protein folding defects and (more-specific) effects due to the disruption of interfaces without impacting protein stability.

Response: Thanks for the helpful comments. To test whether these SiaB mutants were expressed, the protein levels of SiaB-Flag and its mutants from biofilms formed by strains PAO1/EV (EV represents empty vector), Δ*siaB*/EV, Δ*siaB*/p-*siaB*-Flag, Δ*siaB*/p-*siaB*$_{L110A-F174A}$-Flag and Δ*siaB*/ p-*siaB*$_{E61-E64A-E67A}$-Flag were determined by western blot assays. Although all proteins were expressed during biofilm formation, the abundance of SiaB$_{L110A-F174A}$-Flag was lower than that of SiaB-Flag (Appendix Figure S8), suggesting that L110A-F174A mutation might affect the expression of SiaB.

4. The question of metal specificity for SiaB kinase activity remains unanswered. Many ATPases rely on divalent cations (usually Mg2+ or Mn2+), which includes the structural homologs of SiaB discussed in this study. However, the chelating agent EDTA had no effect on catalytic activity of SiaB. From the structure, there appears to be a monovalent ion at the active site. It is not clear whether this is sufficient to support catalysis. A related question is whether this ion is required for catalysis.

Response: Thanks. The identity of Na^+ is mainly determined by the electron density and the crystallization condition in our study. As suggested by reviewer #2, we have carefully studied the coordination of Na^+ in the literatures. In consistent with the literatures, Na^+ coordinates with the side chain of Asn65 of SiaB and the O atoms of the phosphate groups of ADP and TPO68, which could mimic the main-chain carbonyl O atom in coordination.

In addition to the regular ATPase assay buffer, we also measured the ATPase activity of SiaB in ammonium phosphate buffer, which lacks Na^+ ion. Our new results showed that SiaB is still active in the ammonium phosphate buffer (Appendix Figure S6E), suggesting that Na^+ is not strictly required for catalysis. However, cations (Na^+, $NH4^+$, or others) could still play certain role in the function of SiaB, such as, stabilizing the SiaB-SiaC complex.

5. The model in Figure 6 suggests that SiaD dissociates from SiaC, however there is no evidence presented in support for this event. If anything, SiaD activity requires SiaC, suggesting activity arises from the complex.

Response: Thanks for the good suggestion. We have modified this model in order to better present our findings.

Minor points:
6. Line 57: It is not clear why the Chen et al. 2016 reference is included here. The study does not describe PDE activity of oligoribonuclease.
Response: We have deleted this reference.

7. Lines 58-82: The relevance of this paragraph for the following study is not clear. It sounds more like a review of selected works in the field. It would be appropriate to move this section to the Discussion and compare these modes of diguanylate cyclase regulation to the SiaC-SiaD system, where control is achieved through a heterologous complex.
Response: Thanks. We have moved this section to the Discussion part and rearranged this paragraph (lines: 504-513).

8. Lines 94-99: The reference format is off in this paragraph.
Response: We have corrected it.

9. Line 180: 'FALG' should be 'FLAG'.
Response: We have corrected it.

10. Line 186: Consider changing 'promoted' to 'prompted'?
Response: We have replaced “promoted” by “prompted”.

11. Line 205: 'regulate DGC activity' and 'c-di-GMP production' are redundant statements.
Response: Thanks. We have rewritten this sentence as: “The interaction between SiaD and SiaC prompted us to speculate that SiaC acts as a modulator to regulate DGC activity of SiaD.” (line: 200-201)

12. Lines 243, 245 and 246: Figure references should point to Figures S3A, S3E, and S3F, respectively.
Response: Figure references were corrected according to the comment.

13. Figures S3C and S3F: Please show elution profiles for the individual proteins for comparison. This is an important control since peak elution volumes are not only dependent on proteins size but also hydrodynamic radius.
Response: Thanks. The elution profiles for the individual proteins were shown in Figure EV1A and Figure EV2B for comparison.

14. Figure 2C: Lanes 5 and 6 are labeled identically but have different phenotypes. Please verify and correct labeling.

Response: We are very sorry for the mistake. Lane 6 should be "Δ*siaB*Δ*siaC*/p-*siaB*". We have corrected it in this figure (Fig 2D in the revised manuscript).

15. Line 257: Remove 'the' preceding 'similar'. Also, since aggregation and biofilm formation are not necessarily the same mechanism, maybe referring to 'trends' instead of 'results' would be a better wording.
Response: Thanks for your comments. We have rewritten this sentence as ".....by these strains and similar trends were observed (Appendix Figure S3E) ." (line:257).

16. Line 285: Maybe '..., which is in contrast to the phenotype observed in wild-type.'
Response: We have corrected this grammatical mistake.

17. Line 294: 'Confidence' instead of 'confidences'.
Response: We have revised it.

18. Line 326: 'Fig. 2F' should be 'Fig. 3F'.
Response: We have corrected it.

19. Line 328: Maybe 'DGC reaction mixture' would be more appropriate here (instead of 'c-di-GMP reaction mixture).
Response: Thanks. We have replaced "c-di-GMP reaction mixture" with "DGC reaction mixture".

20. Figure 3A: The description of how ATPase level data were handled is fairly cryptic. Please describe explicitly how the data was normalized.
Response: Thanks. We have rephrased the description for measuring ATPase level (line:751-757). The residual ATP concentration in the reaction buffer was determined by mixing the buffer with luciferase reagent. The emitted light was measured using a microplate luminometer. The amount of ATP hydrolyzed during reaction for each sample represents the activity for each sample. The relative activity of each sample was normalized to that of the sample using SiaB and SiaC.

21. Figure 3G: Please mention explicitly that ATP was added to the reactions in order to assess the effect of protein phosphorylation. While it is mentioned in the Materials and Methods section, it is an important detail of the experimental setup and should be mentioned more visibly.
Response: We have rewritten the Figure legends for Fig 3G (line:774-777).

22. Line 339: 'Per' should be 'The'.
Response: We have corrected it.

23. Figures in the main text and supplement use labels 'WT' or 'PAO1' interchangeably. I would suggest choosing the same label for all to be consistent throughout.
Response: Thanks. We have used labels "WT" for all figures.

24. Page 14 (and throughout): For references to specific residues, use either single-letter or three-letter code, not both.
Response: We have corrected this in the text.

25. Figure 4D: I assume the second half of the graph should indicate that experiments were conducted in the delta-siaA background (not delta-siaC, as shown).
Response: Sorry for the mistake. The second half of this figure (Fig 4C in the revised manuscript) represents experiments conducted in the delta-*siaA* background. We have corrected it.

26. Figure 5A: Instead of showing a 2Fo-Fc map, please show a Fo-Fc omit map. The latter would have less model bias.
Response: We have replaced the "2Fo-Fc map" with "Fo-Fc omit map" in the revised manuscript.

27. Line 405: Please report the scores for the top hits from the Dali search.
Response: Thanks for the suggestion. SiaB is most similar to SpoIIAB proteins from *Bacillus stearothermophilus* and *Bacillus subtilis*. In addition to the Z-scores, we also provided the rmsd values between SiaB and the two SpoIIAB proteins in the revised manuscript (lines:427-431).

28. Lines 423-424: 'Might be due to...,' sounds a bit awkward and should probably rephrased.
Response: 'Might be due to...,' has been rephrased as "Owing to" in the revised manuscript.

29. Lines 494, 499, 508: 'one' should be 'a'.
Response: We have corrected these mistakes.

30. Line 668-669: This description is redundant with lines 663-664.
Response: Thanks. We have rearranged this sentence (line: 694-696).

31. Throughout: The work 'noselective' is used several times and should probably be replaced with 'nonselective'.
Response: We have replaced "noselective" with "nonselective" in the text.

32. Figures (in general): Some of the panels (i.e., the aggregation images) and panel labeling are hard to read on printed copies.
Response: Thanks. We have arranged these figures and moved all the aggregation images to Appendix Figures for better read on printed copies.

Referee #2:

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Response: Thanks for the constructive suggestions. We have performed SPR and GST-pull down assays using purified proteins (GST-SiaD, SiaD and phosphorylated SiaC) and found that the phosphorylated SiaC was unable to interact with SiaD (Fig 1G and Figure EV1B).

-In the enzyme assays, measuring SiaD DGC activity, did the researchers mutate the I-site to inhibit negative feedback? This may help in signal detection which they otherwise claim is weak (line 212). If they didn't I would recommend assessing the effect of doing this.

Response: Thanks. We have purified SiaD$_{R130G}$ protein (I-site mutant protein [ref: PMID 26955366]). *In vitro* DGC activity assay showed that this mutated

protein also exhibited weak activity and SiaC still enhanced its activity (Appendix Figure S2D).

-I am a little surprised at the section (lines 291-333) headed "SiaB functions as a protein kinase to phosphorylate SiaC at T68". This is written as true discovery, when in fact it should be written as confirmation - it is already known that SiaC is phosphorylated on this residue - (Ravichandran, A., Sugiyama, N., Tomita, M., Swarup, S. & Ishihama, Y. Ser/Thr/Tyr phosphoproteome analysis of pathogenic and non-pathogenic Pseudomonas species. Proteomics 2009). I would rewrite this part to reflect that. Of course, the authors are discovering that SiaB is the exact kinase responsible, but I felt the omission of a reference to the above was either an oversight or misleading.

Response: Thanks for your comments. We have rewritten this section accordingly and cited the reference above (lines:294-319).

-does a SiaC T68D mutant possess any phenotype as a putative phosphomimic?

Response: Thanks for the suggestive comment. We created a plasmid expressing SiaC^{T68D}. Phenotypic assays showed that overexpression of SiaC^{T68D} in Δ*siaC* and Δ*siaA* promoted biofilm formation (Appendix Fig S6D), which is similar to that of SiaA^{T68A}. Therefore, SiaC^{T68D} mutant can't mimic SiaC phosphorylation.

-lines 320-321 - SiaCT68A interacts with SiaB....this could occur indirectly via a third interaction with SiaD (or any other component). Why has this not been performed with purified proteins rather than (multifactorial) lysate? Or, use lysate from SiaD KO strains. [an example can be seen in fig2e in which SiaA indirectly pulls down SiaB]

Response: Thanks. We have determined the SiaC^{T68A}-SiaB interaction by GST pull down assay using purified SiaC^{T68A}-His and GST-SiaB proteins. Our results showed that SiaC^{T68A} interacts with SiaB directly (Figure EV2E).

-lines 389-390 "these residues...are essential for function of SiaB in vivo". Or for protein folding this has not been demonstrated conclusively and needs further experimentation or rewording.

Response: Thanks for the good suggestion. The protein levels of SiaB-Flag and its mutants from biofilms formed by strains PAO1/EV (EV represents empty vector), Δ*siaB*/EV, Δ*siaB*/p-*siaB*-Flag, Δ*siaB*/p-*siaB*$^{L110A-F174A}$-Flag and Δ*siaB*/ p-*siaB*$^{E61-E64A-E67A}$-Flag were determined by western blot assays. Although all proteins were expressed during biofilm formation, the abundance of SiaB^{L110A-}

^{F174A}-Flag was lower than that of SiaB-Flag. Based on these observations, we have rewritten the sentence as: "All together, these results indicated that the residues Leu110 and Phe174 are important for the function of SiaB."

Minor points:
-I would rewrite abstract to introduce the proteins and their functions more clearly
Response: Thanks. We have rewritten abstract accordingly.

-please remove and replace any references to the retracted work by the Ryan group (e.g. HDGYP reference)
Response: We have removed this reference.

-lines 58-82 are overlong, over-detailed with respect to the actual subject of this study, I would condense this section; possibly expanding section lines 83-93 in its place
Response: Thanks. We have condensed and moved "line 58-82" to discussion (line: 504-513) and expanded lines 83-93 (lines:63-72).

-end of introduction would benefit from a description of several known examples of phosphorylation in cyclic-di-GMP signalling (non-histidine kinase) e.g. move WspR reference from discussion to here, include DgcB from Bdellovibrio bacteriovorus (doi: 10.1038/s41467-019-12051-6) and MtT from Moorella thermoacetica (https://doi.org/10.1016/j.str.2012.01.003)
Response: We have added three known examples (WspR, DgcB, and MtT) of phosphorylation in c-di-GMP signaling (non-histidine kinase) in the introduction part (lines: 83-90).

-I would not use the term "chaperone" e.g. line 101 to describe SiaC UNLESS you can show it acts in a true manner of a chaperone to modulate protein folding. Binding partner yes, chaperone less-likely
Response: Thanks. We have replaced "chaperone" with "binding partner".

-line 112 denoting SiaA as a putative phosphatase should go into introduction
Response: We have revised it (lines: 66-67).

-line 115 standardize - sia first, PA#### in brackets
Response: We have corrected it.

-similarly, throughout, standardize proteins vs genes, capital letters, italics etc, currently messy
Response: Thanks. We have standardized them in text.

-line 121 state which (KO or wildtype) "behaves similarly"

Response: Thanks. We have revised this sentence as "Similar to the Δ*siaA* and Δ*siaD* mutants, phenotypic characterization of the Δ*siaC* mutant during growth in M9 salt with SDS as the sole carbon source revealed decreased formation of macroscopic aggregates compared to the PAO1 strain."

-line 131/2 "restored their biofilm formation" - it appears that SiaB complementation in fig1A reduces biofilm formation - please explain this apparent discrepancy

Response: Sorry for the confusion. The statement of this sentence was not accurate. We have rewritten it as "Indeed, expression of *siaC* and *siaB in trans* in the corresponding mutant exhibited increased or reduced biofilm formation compared to that of wild type strain, respectively (Fig 1A)."

-line 139-140 - did researchers ever measure [cdg] levels in cell lysates?

Response: Yes. We have measured c-di-GMP levels in cell lysates by LC/MS/MS and the result was shown in Fig 1B.

-line 148 I would phrase this as "suggest" rather than "demonstrated"

Response: We have revised it.

-line 223 needs to read "promotes bacterial aggregation"

Response: We have revised it.

-line 269-270, please add clarity as to nature of siaA386 and siaA337

Response: Thanks. We have rephrased this sentence as "...the free-standing PP2C-like phosphatase domain (SiaA protein lacking the N-terminal 385 amino acids, SiaA$_{386}$) or both the HAMP and PP2C-like phosphatase domains (SiaA protein lacking the N-terminal 336 amino acids, SiaA$_{337}$)." line: 269-271.

-I'm not sure their metal ion is Na (line 401-404) and would recommend looking at papers by Majorie Harding or others to investigate if co-ordination informs on a different metal

Response: We sincerely thank the reviewer for the wonderful suggestion. We have carefully studies the papers published by Majorie Harding and others for cation coordination. As summarized in the table below and in Table 2 in the literature (The architecture of metal coordination groups in Proteins, Marjorie M. Harding, *Acta Cryst.* 2004, D60:849-859, DOI: 10.1107/S0907444904004081), Na^+ mainly coordinates with the main-chain carbonyl O atoms, which could be mimicked by the O atoms of the phosphate groups of ADP and TPO68 in our complex structure. In addition, the side chains of some amino acids, especially Asn and Asp, could also coordinate with Na^+. In our complex structure, Na^+ coordinates with the side chain of Asn65 of SaiB, which is consistent with the literature.

(*a*) Numbers of occurrences of different kinds of donor groups (from amino-acid side chains) in metal coordination groups with two or more protein donors. M.ch. O stands for main-chain carbonyl O atom is a donor.

	D, N	E, Q	S, T	H	C	M	K, R	Y	M.ch. O	All
Ca	339	127	34	3	—	—	—	1	309	813
Mg	88	42	38	3	—	—	1	2	54	228
Mn	51	30	3	22	1	—	—	—	6	113
Fe	12	30	—	60	18	3	—	5	7	135
Cu	2	3	3	77	26	10	—	1	4	126
Zn	63	50	1	179	206	1	3	—	10	517
Na	22	12	6	—	—	—	1	—	93	135
K	16	17	18	—	—	—	—	—	79	130

-lines 396 onward; The idea that K72A is unphosphorylated needs to be mentioned before results that rely on this interpretation.

Response: Thanks. We have revised these sentences as "The structural data suggested that Lys72 of SiaC may play an important role in SiaC phosphorylation. To further confirm this observation, we performed biofilm assay and found that $SiaC_{K72A}$ restores the ability of Δ*siaA* to form biofilm, indicating that the mutant protein is present *in vivo* predominantly in the unphosphorylated state (Fig 4C)."

-line 405 - provide rmsd statistics for fold matches, also at lines 435-436

Response: Done as suggested.

-line 431-432 - are these changes a result of crystal packing?

Response: No, the conformational changes are not caused by crystal packing. Arg103 is completely disordered in the apo SiaC structure, whereas it interacts with Asp54 of SiaB in the complex structure. We believed that the conformational changes of the 101-104 region of SiaC are mainly due to the interactions between Arg103 of SiaC and Asp54 of SiaB.

-lines 508-509 - the authors may want to use the common term "substrate-mediated catalysis" to explain this phenomenon

Response: Thanks for the wonderful suggestion. We have replaced the sentences with "Taken together, we conclude that SiaB is a unique kinase and it may follow a substrate-mediated mechanism in catalysis" in the revised manuscript.

-fig1 - what are the values in 1b normalized in reference to?

Response: Thanks. The values in Fig 1B (Appendix Figure S1B in the revised manuscript) are normalized to the mRNA level of wild type PAO1.

-fig2 - a and d - where is the SiaA vs SiaB data?

Response: Thanks. Fig 2A showed that SiaB failed to interact with SiaA$_{337}$. Additionally, Fig 2E (Fig 2F in the revised manuscript) actually represents the interaction between GST-SiaC and SiaA-Flag. Sorry for this mistake in figure labeling and we have corrected it.

-fig 6 - if main part labels SiaA and B directly, then why not do this for C and D also - i.e. why put them in key? Left-hand side should read "high [c-di-GMP]", right-hand side "low [c-di-GMP]" rather than relying on star graphic abundance.

Response: Thanks. We have modified the model according to the Reviewer's comment.

-does phosphorylation of SiaC block GTP binding of SiaD? This could be measured, maybe even with a catalytic dead version of SiaD to simplify the experiment.

Response: Thanks for the constructive comment. Both SPR and GST pull down results showed that phosphorylated SiaC was unable to interact with SiaD (Fig 1G and Figure EV1B). Therefore, it is likely that SiaB-mediated phosphorylation of SiaC inhibits SiaD activation by preventing SiaC-SiaD interaction, but not blocking GTP binding of SiaD.

2nd Editorial Decision 13th Jan 2020

Thank you for submitting a revised version of your manuscript. I sincerely apologise for the delay in handling of your manuscript over the holiday period. Your study has now been seen by both of the original referees, who find that their main concerns have been addressed and are now in favour of publication of the manuscript. There now remain only a few editorial issues that have to be addressed before I can extend formal acceptance of the manuscript.

REFEREE REPORTS:

Referee #1:

The authors have addressed all the points that were raised during the review of the original submission. The additional experiments and argumentation strengthen the overall conclusions of this interesting study.

There are only a few, very minor textual changes that should be introduced in the final text:

Page 2, Abstract, line 32: "...a signaling network THAT regulates...".

Page 2, Abstract, line 40: :...may facilitate antimicrobial drug development" or "...may facilitate the development of antimicrobial drugs".

Page 3, line 71: "during DUAL-species biofilm...".

Page 10, line 265: "...formation, which prompted us to...".

Page 11, line 280: "...formed A complex...".

Page 11, line 295: "A previous study,...at residue Thr68...".

Page 12, line 328: "...we also constructed A T68D mutant".

Page 13, line 356: "Conformations of the (space) b-sheet...".

Page 16, line 441: "...phosphorylation of A Ser residue...".

Page 20, line 553: "...should be important for the ...".

Referee #2:

The researchers have addressed my concerns adequately - one small alteration to improve may be that the 3 examples of protein phosphorylation and DGCs (in introduction) be preceded by a statement that mentions they are examples of known cdg/phosphorylation crosstalk

2nd Revision - authors' response 21st Jan 2020

The authors performed the requested editorial changes.

3rd Editorial Decision 3rd Feb 2020

Thank you for addressing the final minor issues. I am now pleased to inform you that your manuscript has been accepted for publication.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Haihua Liang
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2019-103412

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
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  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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

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1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
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