

Molecular mechanism of proteolytic cleavage-dependent activation of CadC-mediated antiacid response in *E. coli*



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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Acid resistance (AR) is an important stress adaptation strategy of enteric bacteria. In this manuscript, the authors elucidated the activation mechanism of the lysine-mediated decarboxylation (CAD) system in *E. coli* which is important for adaptation to mild acid stress. Combining a series of genetic and biochemical approaches, they elucidated that the transcription factor CadC is activated by successive steps of reduction mediated by DsbC and protease degradation by DegP and DegQ. The study shed light on the acid resistance of *E. coli*. The manuscript is well executed. I have a few minor points.

1. Based on the data and the model the authors presented, CadC remains as a membrane protein (1-184) following disulfide bond reduction and protease degradation. How does it travel to the cytosol to bind the promoter of *cadAB*?
2. The authors used "fragment" to indicate the protease degradation product of CadC in the figures. It is ambiguous. Please indicate the fragment, i.e. CadC 1-184 or CadC 185-512.
3. It is not clear what does "a", "b", "c" presented on the top of columns in Fig 1 a-e; Fig 2 d-e; Fig 5 a-b mean.
4. There are some sentence construction problems across the text, authors should carefully proofread and edit the manuscript when submitting the revision:
 - 1) L75 (periplasmic pH sensing domain) and L82 (cytoplasmic pH-sensing domain)??
 - 2) L115, but also
 - 3) L144, the role of CadC
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 - 9) L282-3, The dsbC mutant failed to activate ...
 - 10) L285-6, strain showed an inability of CadC...
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Reviewer #2/#3 (Remarks to the Author):

Chen et al. have investigated the potential mechanism of acid resistance by lysine/cadaverine pathway in *E. coli*. They suggest that two factors, DegP/DegQ and

DsbC, may be involved in the pathway. They propose that the LysP-mediated CadC inhibition is alleviated by DsbC reduction of an intramolecular disulfide bond in CadC, which is followed proteolytic cleavage of CadC by DegP/DegQ. And the cleaved CadC then goes on to activate the transcription of the CadAB operon. The subject is important and interesting. But some of the data presented are not convincing, undercutting the overall claim of the manuscript.

Major Concerns

1. CadC, full length or cleaved form, contains a transmembrane region. In the Method section, the authors report collecting the supernatant rather than the membrane fraction where the protein should reside, and they do not mention if detergent was used to solubilize the membrane.
2. The cleaved CadC retains the intact transmembrane region; the authors did not discuss how the membrane anchored protein gain access to the cadAB promoter region that may be buried deep inside the chromosome.
3. In the absence of LysP, there should be no inhibition on CadC, and CadC should be readily cleaved. However, Fig. 5d suggests that the presence of Lys is important and in the absence of LysP, the intracellular Lys is too low to induce a conformational change in CadC. This can be proven by using C272S and C208S mutants. These mutants will likely undergo cleavage in Δ lysP constructs.
4. The authors should perform spot assay on low pH agar plates to check the survival/growth under acid stress in the presence of lysine for the various knock out strains of CadC_R184Q, CadC_Cys mutants, Δ DegP, Δ DegQ, Δ DsbC, and Δ LysP?
5. Line 124 and Fig. 1e-f: "It is noteworthy that the full-length CadC band decreased rapidly under acidic pH conditions, implying that the proteolytic cleavage at the specific site resulted in fast degradation". This is inaccurate. At neutral pH the cleavage is very similar to the acidic pH. Also, the CadC band doesn't decrease rapidly. It is considerable up to 40 min. At 20 min of pH 7 condition the expression of CadC looks as high as in pH 5.8. But the stacked bar graph doesn't indicate the same. May be presenting as percentage will make it clearer. Further, a stacked graph with uncleaved CadC as the primary category would make the plot easier to follow. At 0 min, the uncleaved CadC should be very similar for all conditions.
6. Fig. 2d-e is not clearly explained. For example: i) Why was CadA expression in Fig. 2d so much higher than in Fig 1c? Were the two experiments done in a similar way? ii) Why did Δ CadC_pCadC condition induce CadA and CadB expression more than wild type? iii) Why was the peak expression time shifted to 2 hr for CadB and CadA in the wild-type strain as compared to 0.5 and 1hr in Fig. 1d-e? Mutant expression may be slower under stress condition, but what may have caused the slower expression of CadB/A in the wild type?
7. The CadB expression in DegP/Q double knockout condition is half of the wildtype. There is some CadC cleavage in DegP/Q double knockouts in Fig. 2f SDS gel. Is it

possible that a protease other than DegP/DegQ mediate the cleavage?

8. Line 186: “While the mRNA levels of *cadA* and *cadB* were moderately upregulated in Δ degP or Δ degQ, they”. This is inaccurate. The expression of CadA and CadB in Δ DegP or Δ DegQ is several folds higher than wild type. It is severely reduced only in the case of CadA. For CadB it reduces to half of wild type level (Fig. 2e). The expression of CadA and B seems to be decoupled, which would make sense because CadB is an antiporter and may be needed to maintain cellular homeostasis.

9. It is stated that CadC forms aggregates with DegP/Q, however, this is not evident in SEC of Fig. 3b-c. In the SDS gel (Fig. 3a) with purified proteins, cleavage of CadC occurs in 3 hr, but in SEC CadC elutes as a single peak with DegP/Q. Condition used for size exclusion and incubation times for the complex should be clearly described.

10. The negative stain EM images (Fig 3d) are not convincing, because no structural details can be recognized.

Minor Comments:

1. Fig. 1: The mRNA levels of CadC decrease after 0.5 hr despite the continued acid stress. Why?

2. Fig. 1c-d: the *cadB* mRNA expression peaks at 0.5 hr while that of *CadA* peaks at 1 hr. Any thoughts?

3. Fig. 1d-e: It appears that DegQ may compensate for loss of DegP. If this is the case, mention it when describe Fig. 2d-e.

4. Line 250: Zinc is important for DNA binding, but so is Magnesium. How did the authors choose Zinc but not magnesium? Please explain.

1 **Reviewers' comments:**

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3 Acid resistance (AR) is an important stress adaptation strategy of enteric
4 bacteria. In this manuscript, the authors elucidated the activation
5 mechanism of the lysine-mediated decarboxylation (Cad) system in *E. coli*
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7 of genetic and biochemical approaches, they elucidated that the
8 transcription factor CadC is activated by successive steps of reduction
9 mediated by DsbC and protease degradation by DegP and DegQ. The study
10 shed light on the acid resistance of *E. coli*. The manuscript is well executed.
11 I have a few minor points.

12 **Response:** Thank you for your careful reading of our manuscript and
13 professional advice, we have carefully analyzed your comments and
14 revised the manuscript.

15

16 **Question 1.** Based on the data and the model the authors presented, CadC
17 remains as a membrane protein (1-184) following disulfide bond reduction
18 and protease degradation. How does it travel to the cytosol to bind the
19 promoter of *cadAB*?

20 **Response:** CadC₁₈₄ protein in the activated state has been determined to be
21 localized on the membrane by subcellular localization experiments in
22 *Escherichia coli*, the results of which are shown in Fig. 3d and as follows.

23 There are three ways that membrane-bound transcription factors bind to
24 DNA. One scenario is that the DNA-binding domain is proteolytically
25 cleaved, such that it can search for its specific binding site in the same
26 manner as a regular cytoplasmic transcription factor¹. Another way is that
27 co-transcriptional translation and protein translocation (“transertion”^{2,3})
28 tethers the DNA locus of the one-component system to the membrane and,
29 at the same time, places the protein in the vicinity of its binding site on the
30 DNA (which is typically close to the gene encoding the one-component
31 system). The third scenario is a diffusion and capture mechanism, whereby
32 the one-component system diffuses within the membrane and captures
33 conformational fluctuations that bring the DNA close to the membrane¹.
34 Our data confirmed that CadC₁₈₄ remains as a membrane protein following
35 disulfide bond reduction and protease degradation, which suggests CadC₁₈₄
36 adopts the diffusion and trapping mechanism. We have also updated the
37 experimental methods for subcellular fractionation in the manuscript as
38 well.

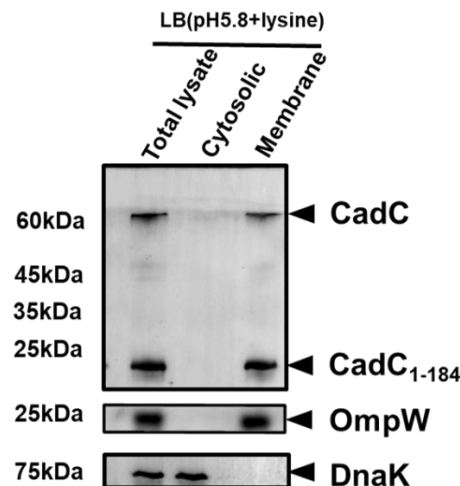


Fig. 3d, Results of subcellular fractionation of CadC protein. In the presence of acid and lysine stimulation, CadC protein undergoes proteolytic cleavage and the CadC₁₈₄ protein is localized to the membrane. DnaK and OmpW served as a control for immunoblotting analysis of total protein.

- 1 Brameyer S, Rösch TC, El Andari J, Hoyer E, Schwarz J, Graumann PL, Jung K. DNA-binding directs the localization of a membrane-integrated receptor of the ToxR family. Commun Biol. 2019 Jan 4;2:4.
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- 3 Woldringh CL. The role of co-transcriptional translation and protein translocation (transertion) in bacterial chromosome segregation. Mol Microbiol. 2002 Jul;45(1):17-29.

Question2. The authors used "fragment" to indicate the protease degradation product of CadC in the figures. It is ambiguous. Please indicate the fragment, i.e. CadC 1-184 or CadC 185-512.

Response: CadC₁₋₁₈₄ and CadC₁₈₅₋₅₁₂ have been relabeled in Fig. 2a, Fig. 2f, Fig. 5e and Fig. 5g in addition to the Fig. 1e which fragment size is unknown. The rest of the modified Fig shown below.

Fig. 2a

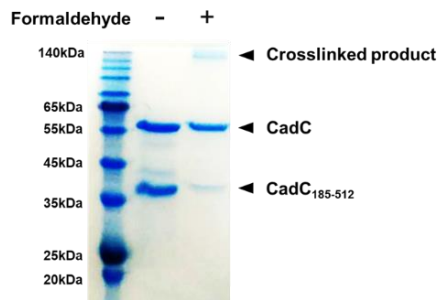


Fig. 2f

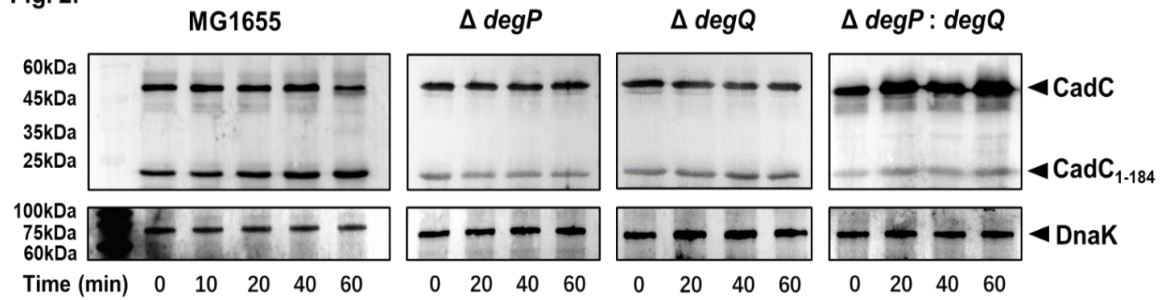


Fig. 5e

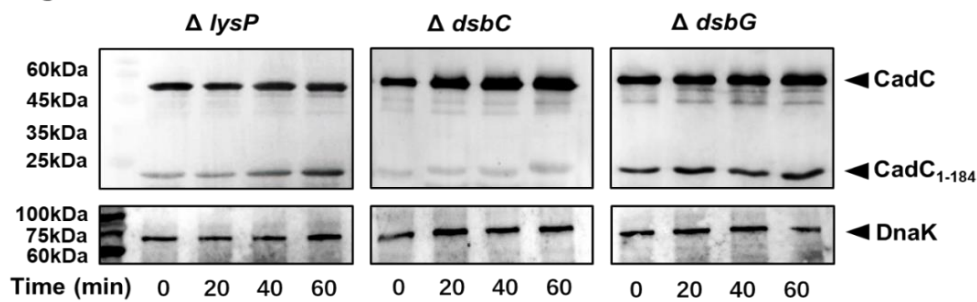
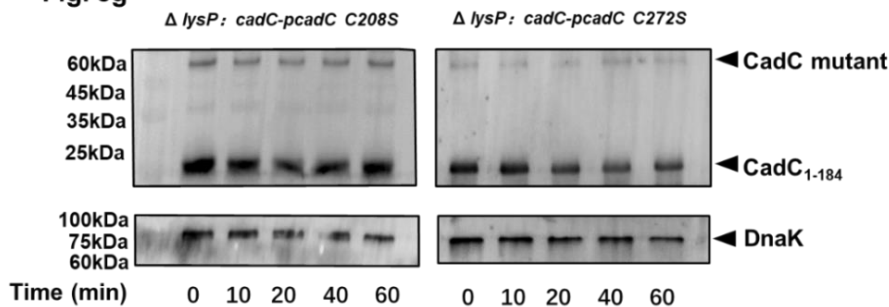


Fig. 5g



Question3. It is not clear what does "a", "b", "c" presented on the top of columns in Fig 1 a-d; Fig 2 d-e; Fig 5 a-b mean.

Response: "a", "b", "c" in Fig 1 a-d; Fig 2 d-e; Fig 5 a-b is a representation of significant difference. Because of the large number of groups to be

compared, we used Duncan's multiple range test for multiple comparisons between the data, using "a", "b", "c" to indicate significant differences between groups.

Question4. There are some sentence construction problems across the text, authors should carefully proofread and edit the manuscript when submitting the revision:

Response: Thank you for pointing out these problems. We have edited the manuscript carefully as shown below.

1) L75 (periplasmic pH sensing domain) and L82 (cytoplasmic pH-sensing domain)??

Response: "periplasmic pH sensing C-terminal domain" is right.

2) L115, but also

Response: The sentence has been changed to: not only did the expression of *cadC* significantly increase, but also the *cadA* gene expression also exhibited 145.3 and 183.6-fold up-regulation in wild-type and *cadC* complement strains.

3) L144, the role of CadC

Response: To figure out how CadC is activated, we constructed the expression plasmid of pET29b*cadC* (C-terminal His tag) for CadC expression in *E. coli* BL21.

4) The location of should be "R184 is located between the TM and ..."

Response: R184 is located between the TM and pH-sensing domains, in close proximity to the TM region, which suggested ...

5) L169, precise should be exact

Response: Addressed as suggested.

6) L191-192, Western blot analysis of??

Response: western blot analysis of CadC was conducted for $\Delta degP:degQ$, $\Delta degP$, and $\Delta degQ$ strains.

7) L219, combined should be mixed

Response: Addressed as suggested

8) L267, opening should be reducing

Response: Addressed as suggested.

9) L282-3, The *dsbC* mutant failed to activate ...

Response: In the $\Delta dsbC$ strain, the *cadBA* operon can not be activated, suggesting that *dsbC* is responsible for reducing the disulfide bonds that stabilize inactive CadC.

10) L285-6, strain showed an inability of CadC...

Response: Notably, CadC was unable to activate the *cadBA* operon in the $\Delta lysP$ strain.

11) L298-299 Sentence should be reconstructed

Response: This suggests that cleavage-dependent CadC activation is dependent on the presence of the LysP protein and reduction of the disulfide bond.

Reviewer #2/#3 (Remarks to the Author):

Chen et al. have investigated the potential mechanism of acid resistance by lysine/cadaverine pathway in *E. coli*. They suggest that two factors, DegP/DegQ and DsbC, may be involved in the pathway. They propose that the LysP-mediated CadC inhibition is alleviated by DsbC reduction of an intramolecular disulfide bond in CadC, which is followed proteolytic cleavage of CadC by DegP/DegQ. And the cleaved CadC then goes on to activate the transcription of the *CadAB* operon. The subject is important and interesting. But some of the data presented are not convincing, undercutting the overall claim of the manuscript.

Response: Thank you for your careful reading of our manuscript and pointing out the problems that need to be addressed, we have carefully analyzed your comments and revised the manuscript.

Major Concerns

Question 1. CadC, full length or cleaved form, contains a transmembrane region. In the Method section, the authors report collecting the supernatant rather than the membrane fraction where the protein should reside, and they do not mention if detergent was used to solubilize the membrane.

Response: Thank you for pointing out this problem. The description of the purification of full length CadC and the cleaved form was incomplete. We did purify them from the membrane fraction by using 0.1% DDM (n-

dodecyl- β -D-maltoside) for solubilization. Details about protein purification have been included in the updated manuscript.

Question2. The cleaved CadC retains the intact transmembrane region; the authors did not discuss how the membrane anchored protein gain access to the *cadAB* promoter region that may be buried deep inside the chromosome.

Response: CadC₁₈₄ in the activated state has been determined to be localized on the membrane by subcellular localization experiments in *Escherichia coli*, the results of which are shown in Fig. 3d and as follows.

There are three ways that membrane-bound transcription factors bind to DNA. One scenario is that the DNA-binding domain is proteolytically cleaved, such that it can search for its specific binding site in the same manner as a regular cytoplasmic transcription factor¹. Another way is that co-transcriptional translation and protein translocation (“transertion”^{2,3}) tethers the DNA locus of the one-component system to the membrane and, at the same time, places the protein in the vicinity of its binding site on the DNA (which is typically close to the gene encoding the one-component system). The third scenario is a diffusion and capture mechanism, whereby the one-component system diffuses within the membrane and captures conformational fluctuations that bring the DNA close to the membrane¹. Our data confirmed that CadC₁₈₄ remains as a membrane protein following disulfide bond reduction and protease degradation, which suggests CadC₁₈₄

adopts the diffusion and trapping mechanism. We have included the above discussion in the revised manuscript and updated the experimental methods for subcellular fractionation as well.

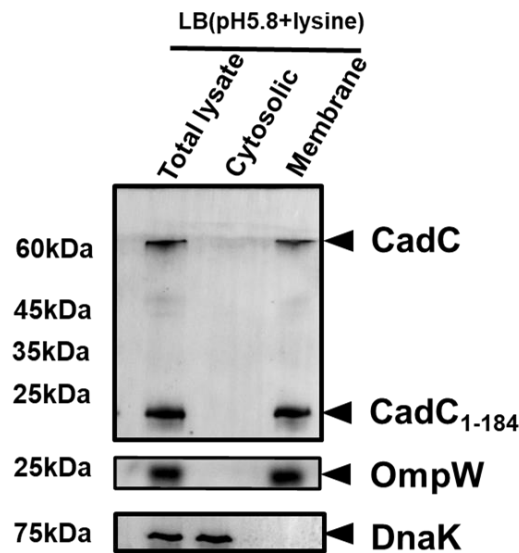


Fig. 3d, Results of subcellular fractionation of CadC protein. In the presence of acid and lysine stimulation, CadC protein undergoes proteolytic cleavage and the CadC₁₈₄ protein is localized to the membrane. DnaK and OmpW served as a control for immunoblotting analysis of total protein.

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Question3. In the absence of LysP, there should be no inhibition on CadC, and CadC should be readily cleaved. However, Fig. 5d suggests that the presence of Lys is important and in the absence of LysP, the intracellular

Lys is too low to induce a conformational change in CadC. This can be proven by using C272S and C208S mutants. These mutants will likely undergo cleavage in $\Delta lysP$ constructs.

Response: Thank you for your good advice. At beginning we also expected that in $\Delta lysP$ strain, inhibition of CadC should be relieved. However, Figures 5a, 5b, and 5e show that CadC undergoes proteolytic cleavage in this case but could not upregulate transcription of *cadA* and *cadB* genes. We speculated the reason might be that the lysine concentration in cytoplasm was too low to induce a conformational change in CadC, thus blocked its disulfide bond reduction. We then analyzed the degradation CadC mutants in $\Delta lysP$: *cadC* p-CadC C208S and $\Delta lysP$: *cadC* p-CadC C272S strains by Western blotting. As shown in fig. 5g, compared with the wild type protein, the degradation of CadC mutants was more significant and independent of external stimuli. Meanwhile, the results of qRT-PCR showed in fig. 5h that *cadA* and *cadB* genes were activated in these mutant strains. These results suggest that LysP is necessary for CadC disulfide bond reduction and proteolytic cleavage activation.

Fig. 5a

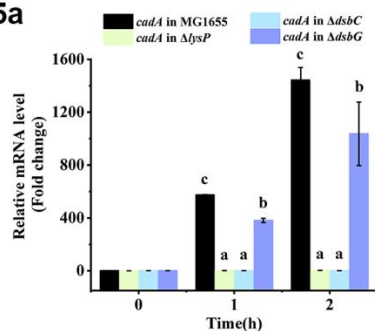


Fig. 5b

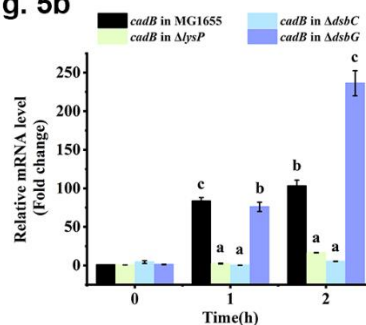


Fig. 5e

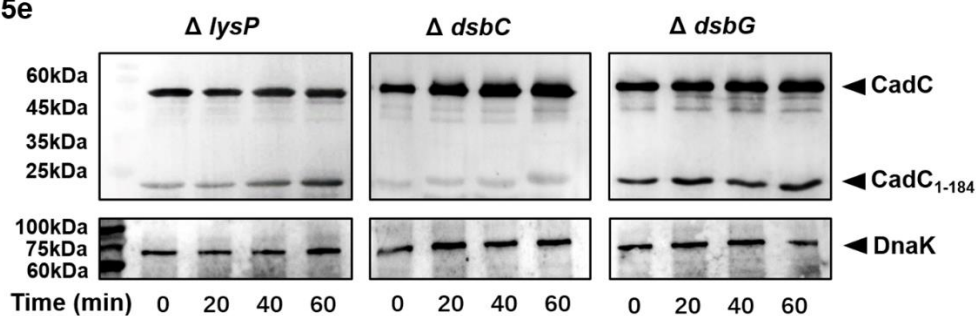


Fig. 5g

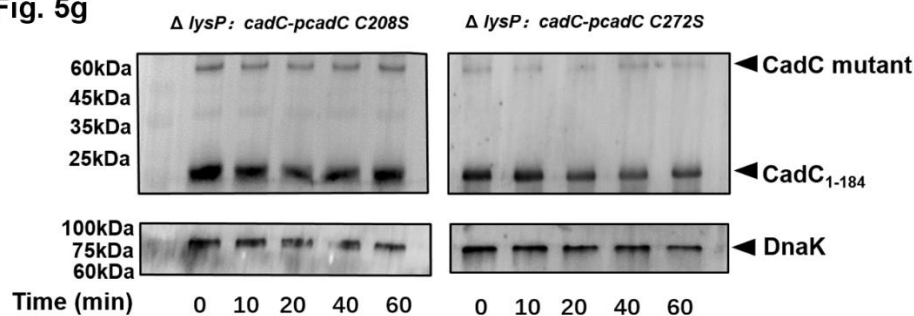


Fig. 5g, Western blot assay examining the protein levels of CadC in MG1655 Δ lysP: *cadC-pcadC* C208S, MG1655 Δ lysP: *cadC-pcadC* C272S strain. DnaK served as a control for immunoblotting analysis of total protein.

Fig. 5h

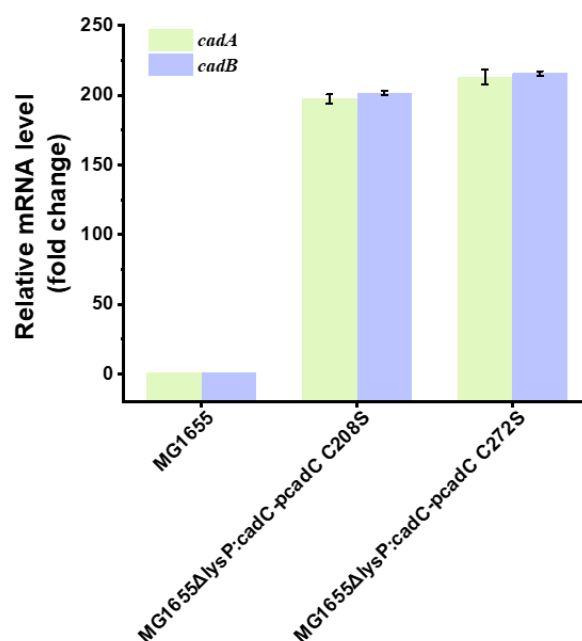


Fig. 5h qRT-PCR was used to detect the expression of *cadBA* gene in mutant strains MG1655ΔlysP: *cadC-pcadC* C208S and MG1655ΔlysP: *cadC-pcadC* C272S.

Question4. The authors should perform spot assay on low pH agar plates to check the survival/growth under acid stress in the presence of lysine for the various knock out strains of CadC_R184Q, CadC_Cys mutants, ΔDegP, Δ DegQ, ΔDsbC, and ΔLysP?

Response: As you suggested, we performed spot assay on low pH agar plates with strains including MG1655, MG1655ΔdegP: *degQ*, MG1655ΔdegP, MG1655ΔdegQ, MG1655ΔlysP, MG1655ΔdsbC; MG1655ΔcadC-pcadC C208S, MG1655ΔcadC-pcadC C272S, MG1655ΔcadC, MG1655ΔcadC-pcadCR184Q, MG1655ΔcadC-pcadC and MG1655ΔlysP: *cadC-pcadC* C208S, MG1655ΔlysP: *cadC-pcadC* C272S. The results are shown in fig. 5i and analyzed. Details have been

included in the revised manuscript line 316-325.

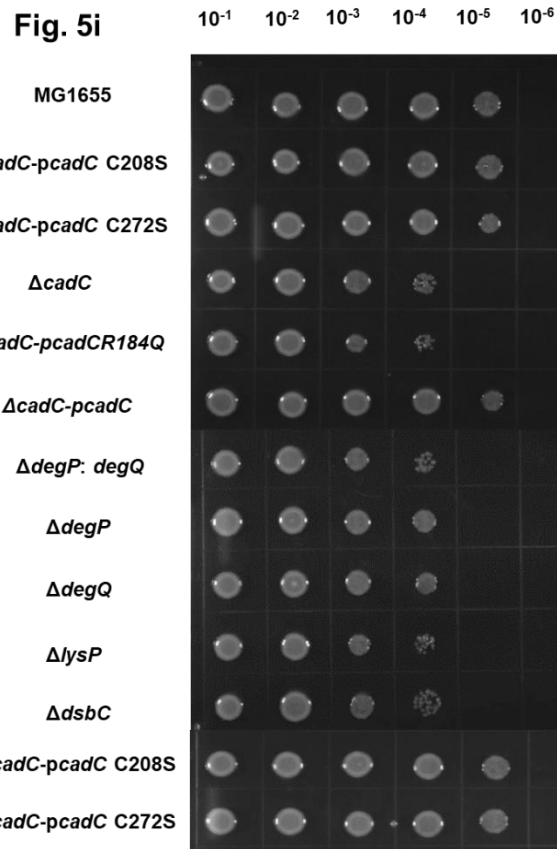
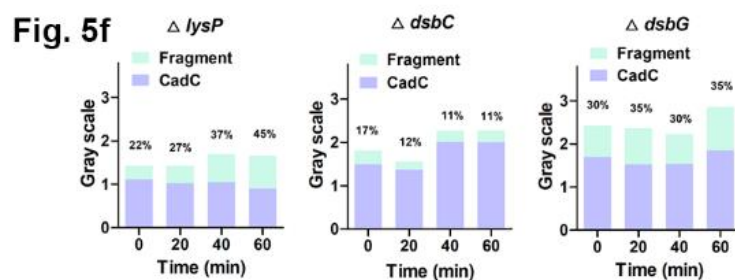
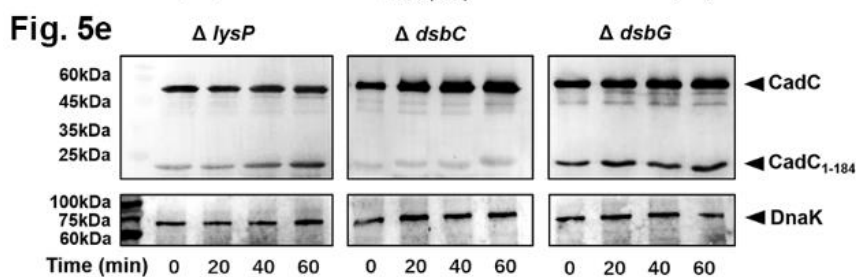
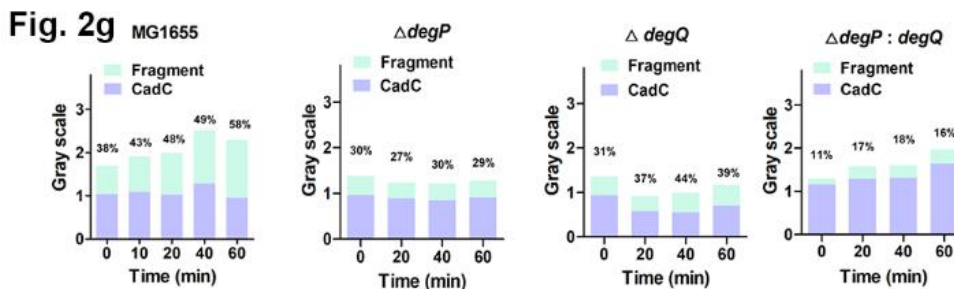
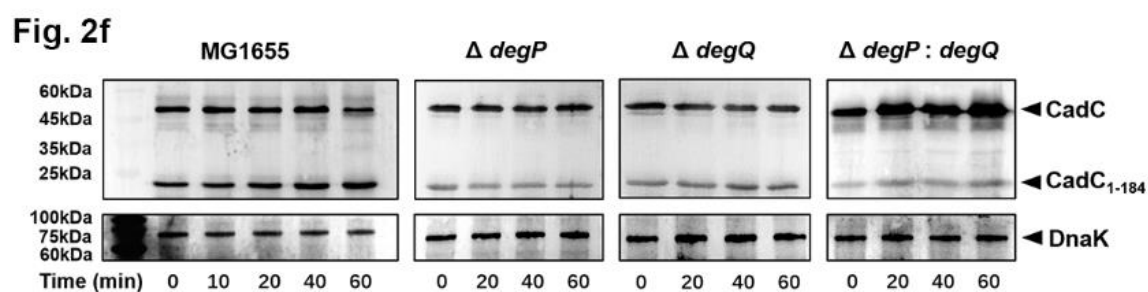
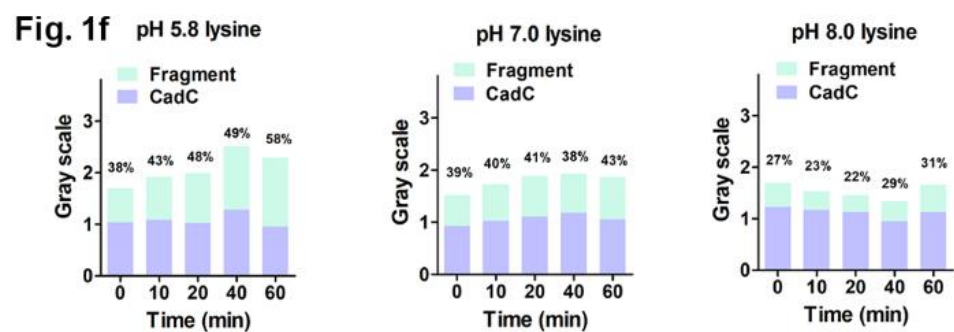
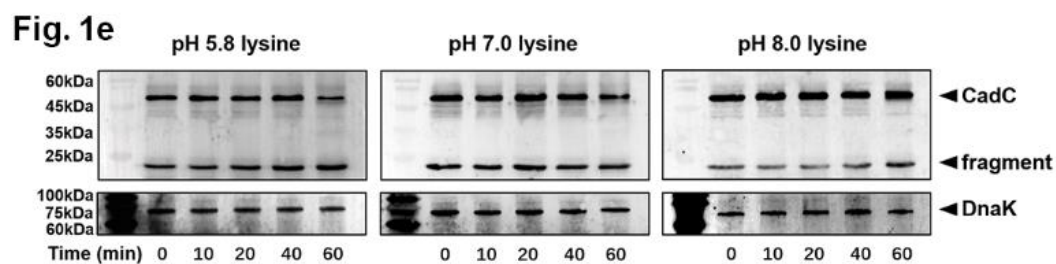


Fig. 5i, A spot assay on low pH agar is performed to evaluate the survival/growth of MG1655, MG1655 $\Delta degP$: *degQ*, MG1655 $\Delta degP$, MG1655 $\Delta degQ$, MG1655 $\Delta lysP$, MG1655 $\Delta dsbC$; MG1655 $\Delta cadC$ -*pcadC* C208S, MG1655 $\Delta cadC$ -*pcadC* C272S, MG1655 $\Delta cadC$, MG1655 $\Delta cadC$ -*pcadCR184Q*, MG1655 $\Delta cadC$ -*pcadC* and MG1655 $\Delta lysP$: *cadC*-*pcadC* C208S, MG1655 $\Delta lysP$: *cadC*-*pcadC* C272S strains.

Question 5. Line 124 and Fig. 1e-f: “It is noteworthy that the full-length CadC band decreased rapidly under acidic pH conditions, implying that the proteolytic cleavage at the specific site resulted in fast degradation”. This is inaccurate. At neutral pH the cleavage is very similar to the acidic pH. Also, the CadC band doesn’t decrease rapidly. It is considerable up to 40 min. At 20 min of pH 7 condition the expression of CadC looks as high as

in pH 5.8. But the stacked bar graph doesn't indicate the same. May be presenting as percentage will make it clearer. Further, a stacked graph with uncleaved CadC as the primary category would make the plot easier to follow. At 0 min, the uncleaved CadC should be very similar for all conditions.

Response: Thank you for your professional advice, we have revised the description in the manuscript. “Notably, the full-length CadC band decreased gradually with time under acidic pH conditions, implying that proteolytic cleavage at specific sites leads to progressive degradation of the CadC protein”. At 0 min, thickness of full-length CadC bands under different conditions were similar. In the revised manuscript, we therefore redrew the stacking diagram to include full-length CadC as a major category, expressed as the percentage of CadC₁₈₄ proteins to the sum of full-length CadC and CadC₁₈₄. The results are shown in Figure 1e, Figure 1f, Figure 2f, Figure 2g, Figure 5e and Figure 5f.



Question 6. Fig. 2d-e is not clearly explained. For example: i) Why was CadA expression in Fig. 2d so much higher than in Fig 1c? Were the two experiments done in a similar way? ii) Why did Δ CadC_pCadC condition induce CadA and CadB expression more than wild type? iii) Why was the peak expression time shifted to 2 hr for CadB and CadA in the wild-type strain as compared to 0.5 and 1hr in Fig. 1d-e? Mutant expression may be slower under stress condition, but what may have caused the slower expression of CadB/A in the wild type?

Response: Thank you for pointing out these problems. i) We scrutinized the experiment records. The experiment shown in Fig. 1c was done in December 2022, and that in Fig. 2d was done in June 2023, six months later. The experimental methodology is the same, but Luria-Bertani medium and chemicals may not be the same batch. It is not easy to make sure that cells still grew under the exact same conditions. Although the experimental data have some quantitative difference, these data still consistently support the mechanism. ii) Δ *cadC*-*pcadC* carries a multi copy plasmid containing *cadC* gene thus has higher level CadC inside cells which can induce CadA and CadB expression more than the wild type. iii) Although *cadA* and *cadB* are arranged in the same single operon, the peak expression time still have 0.5 hr's time difference in Fig. 1d-e. It seems the peak expression time is easily affected by some environmental factors. Considering the six-month time interval between the experiments there

could be some uncontrollable factors such as LB medium, chemicals or cell growth states cause the peak shift. Fortunately, these data still consistently support the mechanism.

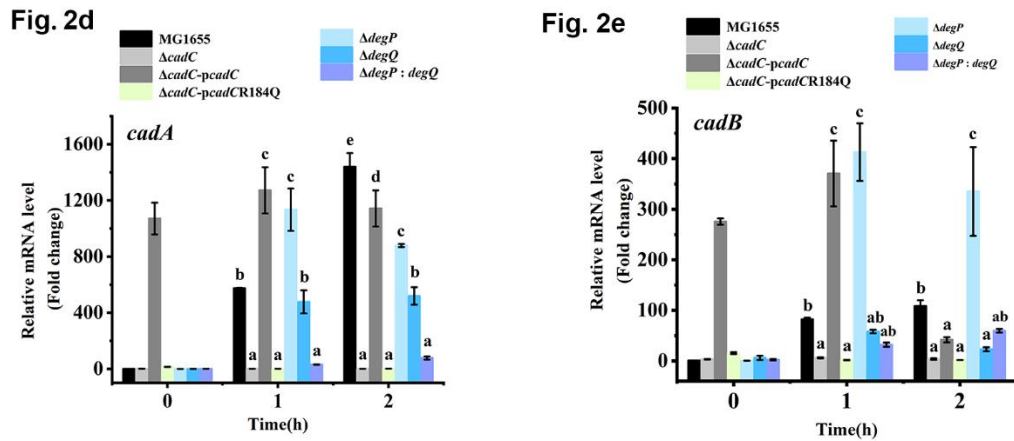


fig. 2d-2e, Transcription levels of *cadA* (d) and *cadB* (e) genes in MG1655 and its mutant strains post acid and lysine stimulation, detected by qRT-PCR. Various letters signify significant differences among samples at the same detection time, as per Duncan's test ($p < 0.05$, $n = 3$).

Question 7. The CadB expression in DegP/Q double knockout condition is half of the wild type. There is some CadC cleavage in DegP/Q double knockouts in Fig. 2f SDS gel. Is it possible that a protease other than DegP/DegQ mediate the cleavage?

Response: It is indeed possible that a protease other than DegP/DegQ can also cleave CadC. But this protease, if exists, may only play a very little role in the CadC signaling pathway since the cleavage band is very weak in the double knockout strain and the *cadBA* promoter is not activated. Although *cadA* and *cadB* are contained in a single operon *cadBA*, the *cadB* expression in DegP/Q double knockout condition is half of the wild type

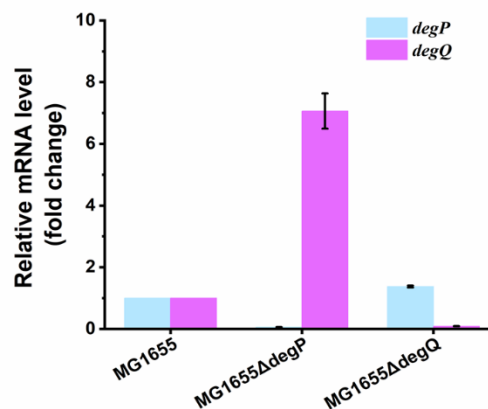
and higher than *cadA*. It may be because *cadB* is upstream of *cadA*, *cadB* mRNA is transcribed earlier but degraded later than *cadA*. As you pointed out in the question8, the expression of CadA and B may also be decoupled, since CadB is needed to maintain cellular homeostasis.

Question8. Line 186: “While the mRNA levels of *cadA* and *cadB* were moderately upregulated in $\Delta degP$ or $\Delta degQ$, they”. This is inaccurate. The expression of CadA and CadB in $\Delta degP$ or $\Delta degQ$ is several folds higher than wild type. It is severely reduced only in the case of CadA. For CadB it reduces to half of wild type level (Fig. 2e). The expression of CadA and B seems to be decoupled, which would make sense because CadB is an antiporter and may be needed to maintain cellular homeostasis.

Response: Thank you for pointing out this problem. In the revised manuscript we have changed the description to: “In the $\Delta degP$ strains, the *cadA* and *cadB* were moderately up-regulated. In contrast, in the $\Delta degQ$ strain, both *cadA* and *cadB* genes were downregulated, which seems to imply that the DegQ protein plays a major role in the activation of *cadBA*. qRT-PCR was then used to examine the expression of the *degQ* gene in $\Delta degP$ strain and *degP* gene in $\Delta degQ$ strain. The results indicated that *degQ* was significantly upregulated but *degP* only slightly increased (Fig. S3). This was consistent with the *cadBA* operon transcription results. Intriguingly, only *cadA* expression was severely reduced. As for *cadB*, it

was reduced to half of the wild-type level (Fig. 2e). The expression of *cadA* and *cadB* seems to be decoupled, which is reasonable, since CadB is an anti-transporter that may be needed to maintain cellular homeostasis. Additionally, the complementary expression of CadCR184Q mutant in MG1655 $\Delta cadC$ strain failed to restore the normal response to the acidic stress (Fig. 2d-e).”

Fig. S3



Supplementary Fig. S3. The expression of the *degP* and *degQ* gene varies in the MG1655 $\Delta degP$ strain and MG1655 $\Delta degQ$ strain. In the $\Delta degP$ strains, *degQ* gene was up-regulated. In the $\Delta degQ$ strains, *degP* gene expression was stable.

Question 9. It is stated that CadC forms aggregates with DegP/Q, however, this is not evident in SEC of Fig. 3b-c. In the SDS gel (Fig. 3a) with purified proteins, cleavage of CadC occurs in 3 hr, but in SEC CadC elutes as a single peak with DegP/Q. Condition used for size exclusion and incubation times for the complex should be clearly described.

Response: As you suggested, the relevant information has been included in the revised manuscript. *In vitro* digestion: “CadC proteins were

incubated with proteases DegP and DegQ in buffer (25 mM Tris-HCl, pH 8.0, 200 mM NaCl), respectively, at 37°C. Samples were taken at 0h, 3h, and 6h and examined by SDS-PAGE.”

In SEC: “To characterize the interaction of CadC and DegP/DegQ, CadC was mixed with DegP and DegQ respectively with a molar ratio 1:1 and incubated at 4°C for 10 minutes. CadC- DegP and CadC-DegQ complexes were then purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare) equilibrated in buffer (10 mM Tris-HCl, pH 8.0 and 100 mM NaCl). Subsequently, the elution peak of the complex was sampled out and subject to size exclusion chromatography side by side with CadC and DegP/DegQ under the same conditions to compare their peak positions. All images were processed using Origin 8.0 software (Originlab).”

Question10. The negative stain EM images (Fig 3d) are not convincing, because no structural details can be recognized.

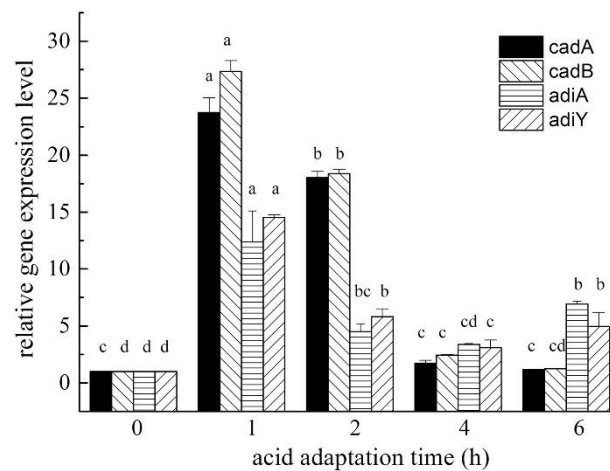
Response: We agree that the negative stain EM images did not give more information on structural details. We have decided to remove this part from the revised manuscript.

Minor Comments:

Question1. Fig. 1: The mRNA levels of CadC decrease after 0.5 hr despite

the continued acid stress. Why?

Response: This phenomenon is also observed in the case of *Salmonella enterica* antiacid response where *cadA/cadB* (Lysine decarboxylation system) and *adiA/adiY* (Arginine decarboxylation system) transcription reached a peak in 1 hour and then decreased gradually (shown as follows). CadC is a one-component system protein in response to non-lethal acidic environments. This membrane-integrated system facilitates rapid signaling transmission and which could be activated within 0.5 hours in response to acid stress. However, bacteria have many antiacid mechanisms and later on other mechanisms may also be activated and replace part of the roles of CadC.



1. Liu J, Zhai L, Lu W, Lu Z, Bie X. Amino acid decarboxylase-dependent acid tolerance, selected phenotypic, and virulence gene expression responses of *Salmonella enterica* serovar Heidelberg. Food Res Int. 2017 Feb;92:33-39. doi: 10.1016/j.foodres.2016.12.006. Epub 2016 Dec 16.

Question2. Fig. 1c-d: the *cadB* mRNA expression peaks at 0.5 hr while

that of *cadA* peaks at 1 hr. Any thoughts?

Response: Although *cadB* and *cadA* genes belong to the same single operon *cadBA*, they have different sequences and are translated successively. Their mRNAs may have different lifetime. Decoupled expression of *cadA* and *cadB* seems to be a favorable factor, since CadB is an anti-transporter that may be needed to maintain cellular homeostasis.

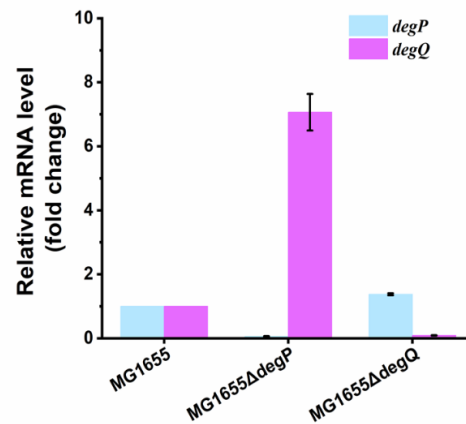
Question3. Fig. 2d-e: It appears that DegQ may compensate for loss of DegP. If this is the case, mention it when describe Fig. 2d-e.

Response: We revised this part as follows:

“In contrast, in the $\Delta degQ$ strain, both *cadA* and *cadB* genes were downregulated, which seems to imply that the DegQ protein plays a major role in the activation of *cadBA*. qRT-PCR was then used to examined the expression of the *degQ* gene in $\Delta degP$ strain and *degP* gene in $\Delta degQ$ strain. The results indicated that *degQ* was significantly upregulated but *degP* only slightly increased (Fig. S3). This was consistent with the *cadBA* operon transcription results. Intriguingly, only *cadA* expression is severely reduced. As for *cadB*, it was reduced to half of the wild-type level (Fig. 2e). The expression of *cadA* and *cadB* seems to be decoupled, which is reasonable, since CadB is an anti-transporter that may be needed to maintain cellular homeostasis. Additionally, the complementary expression of CadCR184Q mutant in MG1655 $\Delta cadC$ strain failed to

395 restore the normal response to the acidic stress. (Fig. 2d-e).

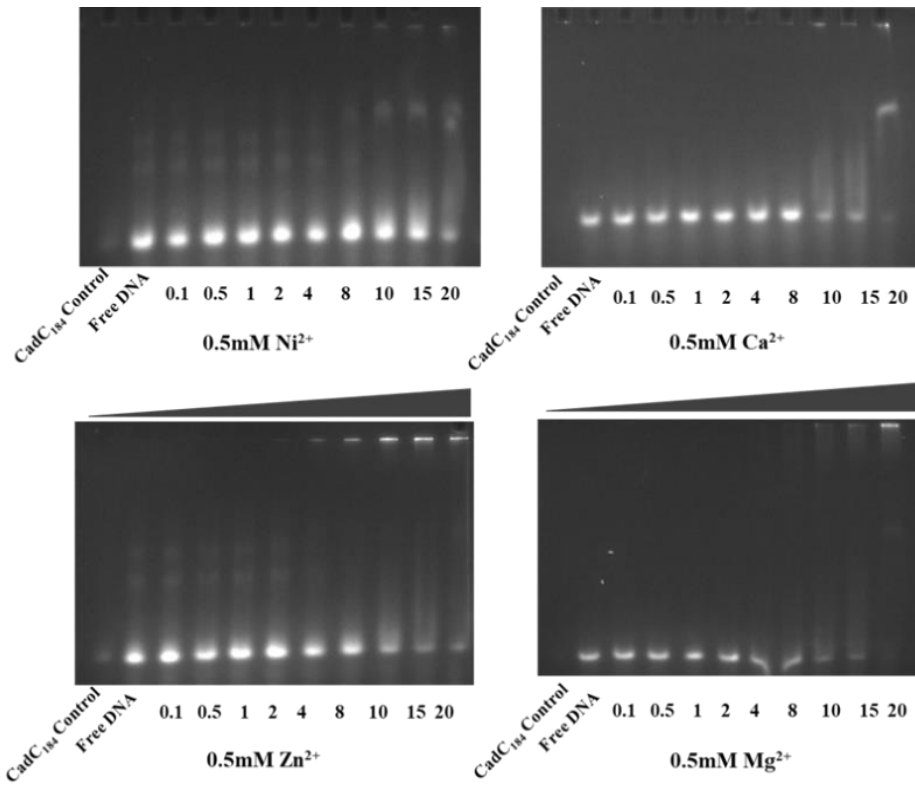
Fig. S3



396
397 **Supplementary Fig. S3.** The expression of the *degP* and *degQ* gene varies in the MG1655Δ*degP*
398 **strain and MG1655Δ*degQ* strain.** In the Δ*degP* strains, *degQ* gene was up-regulated. In the Δ*degQ*
399 strains, *degP* gene expression was stable.

400
401 **Question4.** Line 250: Zinc is important for DNA binding, but so is
402 Magnesium. How did the authors choose Zinc but not magnesium? Please
403 explain.

404 **Response:** Structure of the DNA binding domain of CadC has been solved
405 (5JU7), and Zn^{2+} was observed as a ligand. We also tested the affinity of
406 CadC₁₈₄ and *cadBA* promotor in the presence of Ni^{2+} , Ca^{2+} , Zn^{2+} , Mg^{2+}
407 respectively, and the strongest affinity was observed when Zn^{2+} was added
408 as a ligand (as shown below).



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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed my points adequately.

Reviewer #2/3 (co-reviewers; Remarks to the Author):

The authors have carried out additional experiments that have largely addressed our concerns. List below are a few minor points that may further improve the manuscript.

1. The wild-type gel image in Fig. 1e pH 5.8 condition is the same as in Fig. 2f MG1655 condition. Reusing a data needs to be clearly noted.
2. The authors revised their text to include the use of 0.1% DDM to solubilize the membrane and during protein purification and SEC in the methods section. But they did not mention using any detergent in SEC buffer (line 567) and the assays (line 559). Please clarify.
3. In Fig. 3b, the authors mentioned incubating CadC with DegP and DegQ for 10 min, yet no cleavage is observed in the SEC profile. As opposed to this, considerable cleavage of CadC can be seen in Figs. 1e and 2f wild type conditions.
4. Fig 5i: The spot assay does not look convincing. Were the dilutions consistent for all strains? The survivability of delLysP and Cys mutants of CadC is as good as the wild type. If Cys mutants are unable to upregulate CadA and B genes as mentioned in line 183 of the rebuttal, then what causes the wild type like survivability? Are there perhaps additional mechanisms at play?
5. Fig 225: The tile should be reworded as: CadC is degraded by DegP and DegQ in vitro
6. Line 271: spelling of activate.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed my points adequately.

Reviewer #2/3 (co-reviewers; Remarks to the Author):

The authors have carried out additional experiments that have largely addressed our concerns. List below are a few minor points that may further improve the manuscript.

Question 1. The wild-type gel image in Fig. 1e pH 5.8 condition is the same as in Fig. 2f MG1655 condition. Reusing a data needs to be clearly noted.

Response: Thank you for your reminding, we have added the explanatory note in the Fig. 2f legend: The results used for MG1655 strain in Fig. 2f are consistent with Fig. 1e.

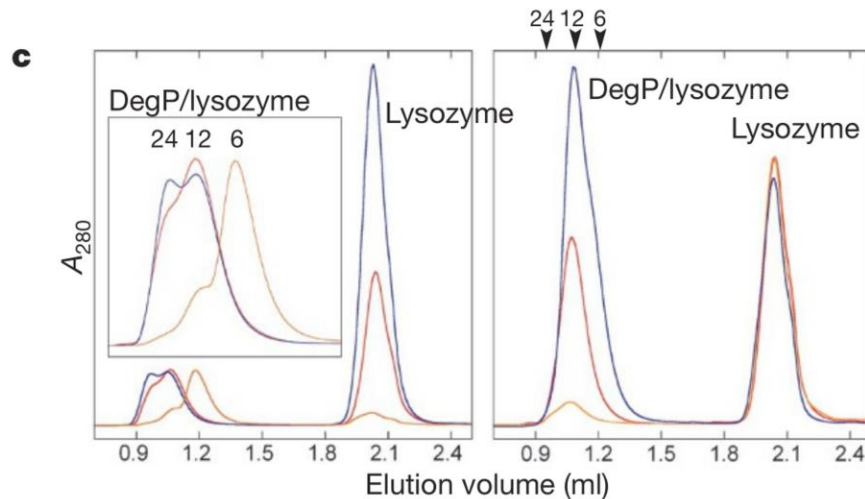
Question 2. The authors revised their text to include the use of 0.1% DDM to solubilize the membrane and during protein purification and SEC in the methods section. But they did not mention using any detergent in SEC buffer (line 567) and the assays (line 559). Please clarify.

Response: We have added descriptions of the use of 0.1% DDM in the SEC buffer (line 567) and the *in vitro* digestion assays (line 559).

Question 3. In Fig. 3b, the authors mentioned incubating CadC with DegP and DegQ for 10 min, yet no cleavage is observed in the SEC profile. As opposed to this, considerable cleavage of CadC can be seen in Figs. 1e and 2f wild type conditions.

Response: There are two reasons may explain the difference in Fig. 1e, Fig. 2f and Fig. 3b-c. Firstly, the western blot shown in Fig. 1e and Fig. 2f were performed using *E. coli* samples cultured at 37°C, but the SEC assays were performed at 4°C. Based on previous research, DegP and DegQ were more active at 42°C than at 37°C, and no activity was even detected at 4°C.^{1,2,3} Similarly, no degradation product signal has been observed in the SEC results of DegP with its substrate lysozyme (shown as follows) even cultured

31 at 37°C for 10 min.⁴ Secondly, the western blot results reflect the activity of DegP and
 32 DegQ *in vivo* and the SEC results show their interaction *in vitro*. Our data have shown
 33 that transcription of *cadA* and *cadB* was upregulated within 0.5 hours during acid
 34 induction (fig. 1c-d), but in the *in vitro* digestion assays, cleavage of CadC was clearly
 35 observed within 3 hours (fig. 3a). That is to say, the DegP and DegQ have higher activity
 36 *in vivo*.



37
 38 Figure 2. Regulation of protease activity by oligomer reassembly. **c**, Denatured
 39 lysozyme and DegP₆ were incubated in different ratios and the resulting complexes
 40 were analysed by SEC. Left: incubation of different amounts of lysozyme (orange, 30
 41 mM; red, 300 mM; blue, 600 mM) with DegP₆ (15 mM). Right: incubation of different
 42 amounts of DegP₆ (orange, 3 mM; red, 15 mM; blue, 65 mM) with lysozyme (170
 43 mM).⁴

44
 45 1 Zhang, Shuang et al. "Degp degrades a wide range of substrate proteins in *Escherichia*
 46 *coli* under stress conditions." *The Biochemical journal* vol. 476,23 (2019): 3549-3564.
 47 doi:10.1042/BCJ20190446

48 2 Ge, Xi et al. "DegP primarily functions as a protease for the biogenesis of β -barrel
 49 outer membrane proteins in the Gram-negative bacterium *Escherichia coli*." *The FEBS*
 50 *journal* vol. 281,4 (2014): 1226-40. doi:10.1111/febs.12701

51 3 Bai, Xiao-chen et al. "Characterization of the structure and function of *Escherichia*
 52 *coli* DegQ as a representative of the DegQ-like proteases of bacterial HtrA family

proteins.” *Structure* vol. 19,9 (2011): 1328-37. doi: 10.1016/j.str.2011.06.013

4 Krojer, Tobias et al. “Structural basis for the regulated protease and chaperone function of DegP.” *Nature* vol. 453,7197 (2008): 885-90. doi:10.1038/nature07004

Question 4. Fig 5i: The spot assay does not look convincing. Were the dilutions consistent for all strains? The survivability of Δ lysP and Cys mutants of CadC is as good as the wild type. If Cys mutants are unable to upregulate CadA and B genes as mentioned in line 183 of the rebuttal, then what causes the wild type like survivability? Are there perhaps additional mechanisms at play?

Response: In spot assays, the dilutions were consistent across all strains. According to the line 183 of the rebuttal, in Δ lysP and Δ dsbC strain, the *cadA* and *cadB* cannot be transcriptionally activated. Which was because the absence of LysP hinders the disulfide bond reduction of CadC by DsbC, thus affecting the enzymatic digestion activation of CadC. But in the CadC mutant strains (C208S and C272S), CadC naturally remained in the reducing state. Thus the Δ cadC-*pcadC* C208S, Δ cadC-*pcadC* C272S, Δ lysP: *cadC*-*pcadC* C208S, and Δ lysP: *cadC*-*pcadC* C272S strains have a similar survivability as the wild-type strain.

Question 5. Fig 225: The tile should be reworded as: CadC is degraded by DegP and DegQ *in vitro*

Response: We have addressed the title as suggested.

Question 6. Line 271: spelling of activate.

Response: The spelling has been revised.