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CtsR, the Gram+ master regulator of protein quality control, feels the heat

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(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 25 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, we experienced severe difficulties in finding willing and suitable referees for this manuscript.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while referees 1 and 3 consider the study as interesting in principle referee 3 is more sceptical and does not offer strong support for publication of the study here. On balance I have come to the conclusion that we should be able to consider a revised version of this manuscript in which you need to address the referees' concerns in full and in an adequate manner. I see two major issues at this point. First, the two parts of the study, the thermosensitivity of CtsR and the role of McsB remain disconnected at this point and it would thus be crucial to develop the connection between these two aspects of the study by further experimentation. It will thus be very important to address points 2 and 4 of referee 2 and referee 3's point "Line337" experimentally. Second, as pointed out by all three referees the presentation, in particular the line of argumentation and flow of logic, needs considerable improvement. It will thus be indispensable to work extensively on this aspect to make the study more accessible not only to the specialist reader, but also to a more general readership. I would like to urge you to run the final version of the manuscript by a colleague outside your field before you return it to us.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript presents some important data and conclusions and will be of broad interest when published. The data is convincing. However, the presentation is confusing and the manuscript is overly long. The title is cute but uninformative. The Discussion can be cut in half at least. Two figures are numbered wrong. Often there are rambling passages when a simple statement of conclusion, like "McsB is stable when dephosphorylated" would bring immediate clarity. The entire text is in urgent need of rewriting. The message is endangered due to poor presentation.

Do the authors mean (line 125) that "CtsR phosphorylation.....is not REQUIRED..."

The stability of the McsB point mutants (line 161) should be demonstrated, at least in the supplement.

Much is made in the text of DNA-protein Kd measurements. The EMSA experiments seem to have been done with EtBr rather than radiolabled DNA. What DNA concentrations were used? Unless [DNA<<Kd) these measurements cannot be made by EMSA. Please state the concentration. Perhaps the authors should speak of "relative Kds".

Fig. S4A-state what DNA probe was used.

Show that the B.stearothermophilus protein is not permanently inactivated at 70 C. (Isn't the new name Geobacillus)

In the section starting in line 236, what were the levels of expression in the heterologous hosts?

Lines 261-262: Can the authors explain the discrepancy with the Derre results?

The model presented in Fig. 7 is interesting. However, McsB appears to bind directly to ClpC. In the figure, CtsR is shown to bind. It is well established that ClpC/ClpP cannot assemble without an adaptor. Do the authors know something the rest of us do not? Perhaps the activated McsB, with its new CtsR partner, rebinds to ClpC, delivering its partner for degradation, just as MecA does for ComK. This hints at a fascinating model. Is unphosphorylate or phosphorylated McsB degraded also under these conditions?

Referee #2 (Remarks to the Author):

In the presented manuscript, the authors investigate the mechanisms of CtsR-mediated heat shock regulation in the Gram-positive model organism Bacillus subtilis. The authors provide solid evidence that CtsR acts as a thermosensor that becomes transiently inactivated at increased temperatures. The authors also identify the region within CtsR that is responsible for temperature-sensing and demonstrate that this regulatory principle is conserved in other Gram-positive bacteria.

These data challenge a previous report (Fuhrmann et al., Science, 2009) stating that the kinase McsB regulates CtsR activity during stress conditions and, consistently, the authors demonstrate that CtsR activity during heat stress is not altered in a mcsB null mutant.
At the current stage the manuscript is a mixed bag. While the data demonstrating CtsR function as a thermosensor are largely convincing, the manuscript suffers from poor writing and bad organization. The discussion section is very chaotic and will be impossible to follow for a non-expert reader. The manuscript also includes various data and figures that are not required or are even confusing (see below). At the same time, interesting findings are only mentioned as unpublished results (see below). The addition of such data would significantly strengthen the paper. In conclusion, I support publication of the study in principle, however, the manuscript is in need of a complete revision and the addition of novel data.

Major criticism

1) Heat shock regulation is not altered in a mcsB null mutant (Figure 1). It is therefore unneccessary to investigate the effect of McsB on CtsR stability during heat shock (Figure 2) or to monitor McsB stability at high temperatures (Figure 3). Such data are irrelevant rather misleading and do disturb the flow of the paper. Along the same line, on page 6 the authors report in the interactions between McsB and ClpC or McsA at physiological and heat shock temperatures. The context of these experiments remains entirely enigmatic. It is suggested to either move such data to the supplementary section or to entirely delete them from the manuscript.

2) The authors mention in the discussion section that CtsR inactivation becomes McsB-dependent upon oxidative stress. This is a very interesting and important finding and I strongly recommend adding such data set to the manuscript as it indicates dual activity control of CtsR dependent on the actual stress condition. Furthermore, it might help to explain the differences between the reported study and the study from Fuhrmann et al.

3) The authors state that CtsR becomes dissociated from DNA upon heat shock by demonstrating weakened binding of CtsR at 50{degree sign}C. In fact, such a statement can only be concluded from order-of-addition experiments, allowing for CtsR binding to DNA at 37ºC and shifting the mixture to 50ºC. Such a setup is currently missing and has to be added to the manuscript.

4) The authors demonstrate that phosphorylation of the winged HTH region of CtsR by McsB is not crucial for heat shock regulation, in contrast to previous findings (Fuhrmann et al., 2009). The authors conclude that "CtsR phosphorylation is not required for CtsR degradation in vivo". On the other hand CtsR is stabilized in a kinase-deficient McsB mutant. It is currently not clear whether this discrepancy is based on (i) McsB mediated phosphorylation of CtsR at other sites or whether (ii) the kinase activity of McsB regulates McsB interaction with McsA or ClpC.

5) Page 11, lanes 350-366: It is impossible to follow the rationale of the authors. Lanes 376-381 should be removed from the manuscript

Further points:

1) Figures 2/3 and 5/6 are interchanged.
2) Figure 2B: ClpE is responsible for CtsR degradation in L. lactis. In B. subtilis CtsR degradation is not required for heat shock regulation. Does the same hold true for L. lactis? In other words, do clpE mutant cells exhibit an altered heat shock regulation?
3) CtsR variants that exhibit increased thermostability are not further characterized. Do such variants exhibit a temperature-sensitive growth phenotype?
4) Figure 7: The model is pretty simplistic and should be removed from the manuscript.
5) Figure 5C: The authors should provide a complete figure (some pictures at different temperatures are missing).
6) Combine Figure 4C and 4D

Referee #3 (Remarks to the Author):

The manuscript concerns with the control of heat shock regulation in Gram-positive bacteria. The authors examine role of McsB in degradation of CtsR as well as address the role of phosphorylation in the degradation of CtsR. Interestingly, some organisms lack McsB while still conducting CtsR mediated heat shock regulation and therefore, the authors look for intrinsic properties of CtsR and demonstrate the it is a temperature sensitive protein. The manuscript is in large well prepared although the figure and citations in the text are not correct and this hampers the reading somewhat. The major problem with the paper is that it appears to be composed of two stories: One is dealing with the fine tuning of CtsR regulation and the role of McsB, phosphorylation and to some extent by McsA whereas the latter half deals with temperature sensitive properties of CtsR, which is the most novel finding of the manuscript. Therefore, the manuscript lacks a focus.

Specific comments:

Figure 1 is not really necessary and can easily be explained in the text as data not shown. Further the first and the second sections of the manuscripts should be combined and shortened (sections starting line 82 and 102) as in the first section it is shown that McsB does not influence CtsR mediated activation of heat shock response and therefore, it seems obvious that the kinase activity more specifically studied in the second paragraph is not important either.

Line 128: The organism should be mentioned for which CtsR was phosphorylated.

Line 131: On which basis were these assigned to be prominent phosphorylation sites? It should be discussed somewhere in the manuscript if at all other phosphorylation sites could be relevant.

Line 118- 119 and line 123 appear contradictory - please clarify.

Lines 136-144 are not clear. Here the authors mix up degradation with DNA binding. The authors should state and show the data for the R50K mutant in terms stability of the protein rather than inferring from expression studies whether the resulting protein is there or not. It can be imagined that the absence of the phosphorylation site both stabilized the protein and resulted in a conformational change reducing DNA binding.

Paragraphs staring line 159 and 176 could be more easily understood in the context of a model of how binding/phosphylation interacts in terms of leading to CtsR activity or inactivity. The manuscript is out of focus here.

I do not see data on 70C in figure 5A as stated line 234.

Line 242 should be figure 6C.

Line 250 - 266 should be shortened substantially.

Line 275: I do not know which figure you are referring to! It does not look like 6A is the right one.

Line 337: The physiological function of McsB is still unclear as the absence of degradation does not seem to influence the heat shock regulation - and why is CtsR degradation necessary?

Line 396: What is the McsB kinase activity targeting?

Page 24 and 25: Figure 2 and 3 must have been switched.

The L. lactis data in figure 3 should be improved for better reproduction.

What were the standard deviations in figure 4C? As the data are described as average of three independent experiments SD should be indicated.

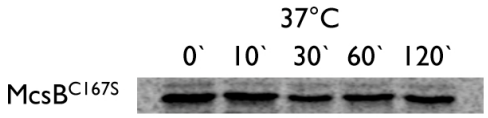
1st Revision - authors' response 19 July 2010

Answers to referee #1:

We completely re-structured the manuscript, re-wrote the entire text with better English wording, clarified statements, shortened the discussion, numbered all figures correctly (Sorry for this mistake!) and improved the overall presentation as suggested.
However, we would like to retain the title, because we think that it expresses a new phenomenon in a clear, short and self-explanatory manner.

Line 125: Yes, this was actually meant and now we use "required" as suggested (now line 237).

Line 161: The experiment for the stability of the McsB point mutant is shown in the "referee-only" supplemental figure. However, please notice that the part dealing with this mutant was removed to improve the line of argumentation (former line 161).



The criticism of referee #2 is absolutely correct, the EMSA's were done with EtBr. The conc. of the *clpC* promoter DNA fragment was 500 ng (now line 460). These assays are routinely used in lab since 1998 for the discrimination of CtsR binding/dissociation. Now we use the term "relative Kd" as suggested by the referee #2 (line 123).

Fig. S4A: A *B. subtilis clpC* promoter fragment was used. An appropriate statement was introduced into the figure legend (now Figure legend S3A). Sorry again.

Yes , the new name is *Geobacillus stearothermophilus*, but *Bacillus stearothermophilus* can still be used and now we use the new term throughout the entire text passage. *G. stearothermophilus* is still able to grow at 70°C. Accordingly, CtsR binds DNA at this temperatures, although with a reduced affinity than at lower temperatures as depicted in former Fig. 4B (now Fig. 3B). The relative binding constant of *G. stearothermophilus* CtsR at 70°C is similar to that of CtsR from other low GC, Gram+ bacteria at heat stress temperatures. Permanently inactivate CtsR would not bind DNA at all. Former fig. 4B (now Fig. 3B) convincingly demonstrates that CtsR is still active at this temperatures.

Line 236: We expressed heterologous CtsR in *trans* from the autonomously replicating pDG148 plasmid with 1mM IPTG on LB plates. From earlier studies we know that the artificially expressed heterologous CtsR level corresponds at least the heat-induced natural CtsR level under these circumstances.

Line 261-262: We measured the influence of these mutations with our *in vivo* expression system, whereas Derre *et al.* used a different experimental set-up. They over-expressed CtsR in a *ctsR* knock-out and measured CtsR activity by a reporter gene assay which possesses the disadvantage that it is also influenced by SigB and heat-activated McsB. Therefore, we prefer data reflecting the *in vivo* situation.

Fig. 7: The referee #1 criticism is absolutely correct and we changed the figure accordingly (Fig.7). McsB is released from ClpC during heat stress to activate McsB kinase (as was shown by Kirstein *et al.* EMBO J. 2005) and rebinds in its activated state to deliver CtsR for ClpCP mediated degradation. Because our data revealed that McsB appears rather unstable in a *ywlE* mutant during heat stress (see Fig. 6D), we suggest that phosphorylated McsB is targeted for degradation.

Answers to referee #2:

We completely re-structured the manuscript, improved the English wording, shortened and reorganized the discussion to make it easier for non-expert readers. We performed two additional experiments (i. 2D Western analysis for the verification of McsB auto-phosphorylation and ii. demonstration that the McsB deficient kinase mutant is unable to target CtsR) (Fig. 6A and 6E and S9, line 253-306) to show details for McsB activation/inactivation as specific adaptor protein and to develop a connection between the two major aspects of our manuscript.

Major criticism

1) Right, the flow of the paper was disturbed at least mentioning the effect of McsB on CtsR and the stability of McsB in the first part of the manuscript, however these data are important for the understanding of the regulation of McsB adaptor function during heat stress. Therefore we keep these paragraphs in our current manuscript, but changed the structure of the manuscript to avoid misinterpretations (now line 225-235 and 283-306).
Interaction of McsB with ClpC and McsA was monitored to gain insights into the transition from inactive to active McsB kinase. We realized that this part may disturb the flow of the manuscript and was thus deleted (former line 174-194).

2) In fact, McsB-dependent CtsR de-repression during oxidative stress explains the discrepancy between our *in vivo* data and previously reported *in vitro* data (Krüger et al., EMBO-J. 2001, Kirstein et al., EMBO-J. 2005, Fuhrmann et al., Science 2009). Therefore, we mentioned our findings as unpublished data in the discussion section.
Indeed, we have plenty of data demonstrating that CtsR activity is differently regulated under oxidative stress. But adding these experiments would not only disrupt the flow of logic, but would also completely overload the current manuscript. The elucidation of the mechanism for CtsR inactivation during oxidative stress is an entirely new story and needs to be published separately. The whole study involves lots of different experimental set-ups with very specific approaches (e.g., MS-based identification of oxidatively modified targets, diagonal non-reducing/reducing gels for SS protein characterization etc.) and description of this scenario would go far beyond the intention of our current manuscript. Thus, the presentation of these experiments would make our revised manuscript overly long and hard to understand for non-expert readers.

To avoid misinterpretations we deleted the section dealing with McsA in the discussion section as suggested by referee #2 point 5.

3) It is correct, that we stated in our manuscript that, "... CtsR dissociates from the *clpC* operator sequence upon a shift to heat stress conditions. ... (former Fig. S4A) (former line 204)" However, the corresponding figure (former S4A, now S3A) exactly shows the order-of-addition experiment that was demanded by the referee #2. To clarify misunderstanding of this particular experiment, the text in the manuscript was changed to (now line 125-127 "... CtsR massively dissociated from the *clpC* operator when shifted to heat stress after DNA binding had been allowed at control conditions (Fig. S3A)...") and the figure legend was adapted to ("*B. subtilis* CtsR dissociated in greater portion from the DNA after a shift to 50°C, when DNA binding had been allowed at 37°C for 30 minutes. Then the mix was divided and one half remained at 37°C and the other was shifted to 50°C for 5 min.").

4) A corresponding paragraph was introduced regarding activation of McsB adaptor function by auto-phosphorylation (line 253-269 and 282-306). In addition, two experiments with McsB were performed (i. 2D Western analysis for the verification of McsB auto-phosphorylation and ii. demonstration that the McsB deficient kinase mutant is unable to target CtsR) (Fig. 6A and 6E and S9, line 253-306). These presented results clearly demonstrate that McsB kinase is essential for McsB auto-phosphorylation, which is only in this phosphorylated form able to bind non-functional CtsR. These results also underline that CtsR phosphorylation is not needed for CtsR degradation.

5) Both sections were removed from the manuscript as suggested by referee #2 (former line 350-366 and 376-381).

Further points:

1) We are sorry for this mistake. Now, all figures are labeled correctly.

2) A *clpE* mutant in *L. lactis* shows a wild-type like induction pattern during heat stress (Varmanen et al. 2003 J. Bacteriol.) demonstrating that CtsR degradation is not required for CtsR inactivation during heat stress. A corresponding sentence was introduced in the revised manuscript (line 317-318 "... In these low GC, Gram+ bacteria CtsR degradation is neither essential for CtsR activity as it was shown previously that a *L. lactis clpE* mutant is fully inducible during heat stress ... ").

3) Such an experiment would be nice, but our manuscript deals with the regulation and degradation of CtsR and not with the physiological role of Clp proteins. Therefore, we have not performed these experiments for the current manuscript, although it would be with no doubt an interesting approach for a follow-up study.

4) Yes, the model is rather simplistic, but illustrates and focuses on the main new findings of the manuscript. Therefore, we would like to keep this scheme in the manuscript.

5) Please find a new and complete figure (now Fig. 3C) in the revised version.

6) Figure 4C and 4D were combined as suggested by referee #2 (now Fig. 2C).

Answers to referee #3:

Referee #3 is right. We totally re-structured our manuscript, performed 2 additional experiments and established a logic connection between the two major findings, thermosensitivity of CtsR and controlled CtsR degradation, which is mediated by the regulated McsB adaptor. The fate of CtsR during heat stress including inactivation and controlled degradation mediated by the regulated adaptor protein McsB was put into the focus of the manuscript.

Specific comments:

Line 82 and 102: The discovery that McsB is not involved in the regulation of CtsR activity during heat stress is a novelty and one of the central findings of the manuscript and stands in contrast to previous publications which regard McsB as the main player for CtsR activity (Kru□ger, 2001 EMBO J.; Kirstein, 2005 EMBO J.; Fuhrmann, 2009 Science).
The second section deals with the establishment of an *in vivo* expression system, which allowed us to express specifically modified proteins from the native promoter without changing the sensitive equilibrium of McsA/McsB/ClpC. This was also an important achievement for the investigation of CtsR activity and degradation.
Accordingly, we think figure 1 is required to introduce the reader into the field and to explain the following experiments.

Line 128: *G. stearothermophilus* was introduced according to the reviewer's suggestion (now line 242).

Line 131: These phosphorylation sites were all found by an *in vitro* approach (Fuhrmann, 2009 Science). It is not clear whether CtsR gets phosphorylated at all sites simultaneously *in vitro*. However, no other phosphorylation sites could be determined *in vitro.* Our results clearly show that CtsR phosphorylation is not required for degradation *in vivo*. Additionally, we performed two experiments (Fig. 6A and 6E and S9, line 253-306) which clearly demonstrate that McsB autophosphorylation instead of the earlier postulated CtsR phosphorylation is essential for CtsR degradation. Therefore, discussion of other putative phosphorylation sites is from our point of view irrelevant.

Line 118 and line 123: To date, it was only demonstrated that McsB is essential for CtsR degradation *in vivo*, but a role of the McsB kinase was only shown *in vitro*. Our result demonstrates a physiological function of the McsB kinase for the first time *in vivo*. To prevent misunderstandings, the sentence (former line 123) was deleted.

Line 136-144: Right, the paragraph may appear confusing. We demonstrated that all CtsR R-->K

point mutations, including R50K, show no influence on CtsR stability (former line 127-135, now line 239-249 and former Fig. 2A now Fig. 5). Therefore a potential stabilization of the protein cannot explain the alteration in CtsR activity. Our data convincingly show that absence of the phosphorylation sites in CtsR neither stabilize the protein nor influences CtsR activity, while CtsR activity is regulated independently of McsB kinase. Consequently, any impact of the R-->K mutations must dependent on interference of CtsR DNA binding. Nevertheless, we realized that this section could lead to misinterpretations and we deleted this section and introduced a short paragraph clarifying why phosphorylation of these residues cannot play a role for CtsR activity (line 239-240 "... Demonstrating that McsB kinase and accordingly CtsR phosphorylation is not involved in CtsR inactivation during heat stress, ... ").

Line 159 and 176: Both paragraphs deal with the activation/de-activation of McsB as a regulated adaptor protein for CtsR degradation. The paragraph starting line 159 is important to understand how heat-activated McsB is shut-down to prevent degradation of re-activated CtsR (now line 289-296). However, we realized that the part starting line 176 can lead to misinterpretations. Therefore, we deleted the part dealing with the activation of McsB kinase (former line 176-193) and re-structured the manuscript for a better understanding of how McsB adaptor is activated/inactivated during heat stress.

Line 234: Sorry, figures 5 and 6 were interchanged. In the revised manuscript all figures are correctly labeled.

Line 242: Sorry again, in the revised manuscript all figures are correctly labeled.

Line 250-266: However, this part is really essential to understand why we choose the G64P mutation to affect the thermosensitivity of CtsR. Therefore, we would like keep this paragraph unchanged.

Line 275: Sorry, figures 5 and 6 were interchanged. In the revised manuscript all figures are correctly labeled.

Line 337: Yes, CtsR degradation does not influence heat shock regulation, but we were able to show that the physiological function of McsB during heat stress is to function as a heat specific adaptor protein which specifically targets CtsR for a ClpCP mediated degradation (line 251-306). Thus McsB ensures that non-functional CtsR is removed from the heat stressed cell to prevent aggregation and to maintain protein homeostasis.

Line 396: The two new experiments (Fig. 6A and 6E and S9, line 253-306) which we added to the manuscript show that the McsB kinase is needed for auto-phosphorylation. This modification is necessary to bind non-functional CtsR.

Figure 2 and 3: Right, in the revised manuscript all figures are correctly labeled.

Figure 3: Done. (now Fig. S10)

Fig 4: We calculated the standard deviations with a variance of less than 10 % and also presented these data in the text for all relative Kd`s (now Fig. 2C) (former line 203, now line 124-125 " ... CtsR displays a substantially lower binding affinity to the *clpC* operator at 50°C (Kd=8.76±0.49µM) than at 37°C (Kd=0.93±0.091µM) (Fig. 2C) ..."). However, presentation of s.ds. in the graph would make the figure too unfocused for our opinion. Therefore we would like to keep our original figure.

2nd Editorial Decision 18 August 2010

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Still, referee 1 feels that there are two issues that need to be addressed (see below) before we ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

In their revised version the authors have nicely addressed most of my previous concerns. Especially the writing and organization of the manuscript have been vastly improved, it almost reads like a new study. There are only two issues remaining. First, it is suggested to add the supplementary figure 3A to the original figures (e.g. Fig. 2C). This temperature-upshift experiment mimics the physiological relevant events and therefore should be included. Second, I still would prefer one piece of experimental evidence that McsB is crucial for CtsR inactivation upon oxidative stress. It is clear that the incorporation of a detailed analysis of McsB role under oxidizing conditions would exceed the capacity of the manuscript, as pointed out by the authors. However, a single data set showing that CtsR inactivation upon oxidative stress depends on McsB would (i) strengthen the observation that the McsB-independent inactivation of CtsR upon heat stress relies on CtsR thermolability and (ii) clarify the discrepancies between the reported data and previously published findings (Fuhrmann et al., 2009).

Referee #3 (Remarks to the Author):

The changes made by the authors have greatly improved the manuscript and the novelty of their findings has been more clearly highlighted.

2nd Revision - authors' response 19 August 2010

Answers to referee #2:

In their revised version the authors have nicely addressed most of my previous concerns. Especially the writing and organization of the manuscript have been vastly improved, it almost reads like a new study. There are only two issues remaining. First, it is suggested to add the supplementary figure 3A to the original figures (e.g. Fig. 2C). This temperature-upshift experiment mimics the physiological relevant events and therefore should be included.

We added Figure S3A to the manuscript as suggested by referee #2 (now Fig. 2C).

Second, I still would prefer one piece of experimental evidence that McsB is crucial for CtsR inactivation upon oxidative stress. It is clear that the incorporation of a detailed analysis of McsB role under oxidizing conditions would exceed the capacity of the manuscrip, as pointed out by the authors. However, a single data set showing that CtsR inactivation upon oxidative stress depends on McsB would (i) strengthen the observation that the McsB-independent inactivation of CtsR upon heat stress relies on CtsR thermolability and (ii) clarify the discrepancies between the reported data and previously published findings (Fuhrmann et al., 2009).

According to the referee #2, we included a data set regarding McsB dependent inactivation of CtsR during disulfide stress. (now line 349-354 " We suggest that the discrepancies regarding the role of McsB between our results and former *in vitro* studies (Furhmann et al, 2009; Kirstein et al, 2005;

Krüger et al, 2001) depend on the fact that McsB is responsible for CtsR inactivation during disulfide stress (Fig. S11). Consequently, McsB is able to inactivate CtsR *in vitro*, but is not involved in heat shock inactivation of CtsR *in vivo*. We are currently investigating the specific mechanism by which CtsR becomes inactivated during disulfide stress.“ and Fig. S11).

