nature portfolio

Peer Review File

Structural basis for transcription activation through cooperative recruitment of MntR

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript describes the structure of Bacillus subtilis MntR, a manganese-sensing transcriptional regulator, bound to its DNA operator. This structure has been long awaited. While crystal structures of the iron-sensing homologs of MntR, IdeR/DtxR proteins, in complex with DNA, have been available for many years, the MntR-DNA complex has long eluded structure elucidation. I am certain that this is not due to a lack of trying, but apparently MntR-DNA complexes don't like to crystallize, so MntR had to wait until cryo-EM became a feasible option.

Despite the limited resolution of the cryo-EM maps, the authors were able to build a model that is consistent with all available data. They take the limited resolution into account in an appropriate manner and were able to construct a model that is highly likely to be correct without overinterpreting the data. The structures suggest how cooperative DNA binding is mediated across MntR dimer-dimer interfaces. The authors tested this hypothesis and were able to verify it.

The data are, as far as I am able to ascertain, solid, and the paper is well written. I only have a few requests for some minor improvements.

General comments:

- Please authorize the PDB and EMDB entries. The preprint is out there, so the entries should be released.

- In the absence of access to the PDB and EMDB entries, I want to see the validation reports.

- I would appreciate if you made it clear which MntR proteins are included in your sequence comparisons. If I am not mistaken, you have only included sequences from the subgroup without the C-terminal SH3-like domain.

- A comment on the similarities and differences to the DNA-binding mode suggested for Streptococcus MtsR would be nice.

- What about the arginine cluster? Did you mutate those residues as well? I guess you'd lose DNA binding entirely and consequently you wouldn't learn that much from these mutations, but just curious if you tried. (I am absolutely not asking you to make these mutations.)

- I'm assuming that the models were built before AlphaFold 3 was released, but just out of curiosity, does AF3 give you (more or less) the same model?

Detailed comments:

Main text:

- p. 4, l. 97-99: not a proper sentence

- p. 5, l. 107: Structural alignment of…

- p. 5, first paragraph: Meaning that 2F5C essentially captured the DNA-bound conformation, right?

- p. 5, l. 116-117: Not all MntRs have a binuclear Mn binding site. Maybe clarify that you're meaning B. subtilis MntR and its very close homologs.

- p. 6, l. 143: with each MntR dimer interacting with…

- p. 8, first paragraph: Indeed, the Pro-T interaction seems to be a common feature of DtxR family regulators, despite their otherwise large divergence regarding recognition sequences and mechanism.

- p. 10, l. 240: extended data?

- p. 11, l. 279: Is this conclusion based on the FSEC data alone?

- p. 14, l. 357-358: This seems a bit speculative to me. You've already provided some more solid reasoning for the behavior of this variant. Also, you're using both additionally and hence twice in a row in this paragraph.

- p. 30, l. 719: It's a Coulomb potential map, not an electron density map.

- Fig. 3, panel c: In the left box we see the surface of the DNA colored by charge distribution? In the right box the residues are shown both as sticks and as transparent vdW surfaces? Please amend figure legend.

Supplementary information:

Table 2:

- What are the 3 different B factors separated by slashes given for each category?

- What are the numbers in parentheses for RMS deviations? The number of outliers?

Fig. 1:

- Can you remove the spell checker underlines from the figure?

- l. 17: H26 in bold? It's orange in the figure.

Fig. 4: starting model shown in pink?

Fig. 6: the Weblogo is so compressed, it's very hard to read. Might warrant panoramic layout.

Reviewer #3 (Remarks to the Author):

On this research article the authors describe the molecular basis of the Manganese transport regulator (MntR) binding to regulatory regions of the mneP promoter and rationalize its Mn2+-dependent activation. Through structural biology, biophysical and activity assays the authors provide a deep understanding of manganese homeostasis regulation.

The manuscript has been redacted on a very clear and easy-to-follow language and the introduction and results are clear, stating the background, general hypothesis and conclusions in a very comprehensive manner. The methods are sufficiently detailed to guarantee reproducibility and interpretation of the manuscript.

The experimental design is sound, and the work supports the claims and conclusions drawn by the authors. The two three-dimensional structures described on this section have been obtained following a well-designed Cryo-EM approach, highlighting the expansion of the box size to pick large protein-DNA complex particles.

In general, the characterization of the MntR dimers and DNA features observed on the Cryo-EM structures is robust and the authors perform a thorough analysis and interpretation of the protein-protein and protein-DNA contacts observed on the Cryo-EM structures. The analyses of MntR dimers interaction through Mass Photometry and Fluorescence-based size exclusion chromatography (FSEC) experiments clearly highlight the relevance of the cooperativity process on the binding and activation of the operator.

In general, the data analysis and interpretation are correct.

As a summary, I would recommend this article for publication.

I would like to highlight a few minor comments:

1. The nomenclature of the two CryoEM structures makes a bit difficult to follow the text. It would be better to define them as Dimer and Tetramer or 2x- 4x- Complexes.

2. The presence of the 9 bp inverted repeats on the P84 DNA is not evident from the figures.

3. The comparison of both structures on Supplementary Figures 2 and 3 leads to confusion given the different scale used on both Cryo-EM maps (ie. Supp Fig 3a vs b). I would recommend homogenizing the scale of the maps to help on the interpretation of the models and maps.

4. It would also be recommended to label the A-site and C-site metal positions in Supplementary Figure 4. They are mentioned in the text but not clearly defined on the figure, which makes difficult to analyze it.

Review overall summary:

The manuscript “Structural basis for transcription activation through cooperative recruitment of MntR” by Shi, H. *et al.* submitted to Nature Communications addresses an active area of research in molecular mechanisms of transcription control and is likely of interest to a broad audience. Using a combination of approaches, the authors show that the transcriptional regulatory protein MntR functions differentially as an activator and repressor to control expression of genes encoding Mn-specific transporters. MntR cooperatively binds multiple proteins to specific regions in the operator to help activate transcription of *mneP* but only one binding-event is needed when functioning as a repressor for inhibiting *mntH* transcription. The data presented is sound and well analyzed. The data also advance the understanding of the molecular mechanism of how transcriptional regulatory proteins function globally as both a repressor and activator. It will be interesting to see how MntR binding to the DNA triggers recruitment of the RNA polymerase.

Minor comments:

I find it interesting that the bacteria cell requires a single binding event for repression of *mntH* to diminish Mn import but requires multiple events for activation of *mneP* for Mn to be exported. It is as if the cellular response needs to act with certainty that intracellular Mn level is too high before the cell will remove Mn. Why would this not be the case for Mn import? Does it have something to do with the ability to unload MntR off the DNA when Mn returns to healthy levels? How does the rate of Mn import vs. Mn export affect the transition to Mn homeostatic levels?

It appears that the binding site for MntR as a repressor has much higher affinity than that of the sites as an activator. Given this, then what would be the benefit to the cell? It would be nice to see how the cooperative binding of MntR to the *mneP* affects the rate of its transcription.

Major comments:

None. The manuscript is well written and structured.

Please find below our point-by-point responses (in blue color) organized by reviewer. Let us know if anything is unclear or if you have any further questions for us.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript describes the structure of Bacillus subtilis MntR, a manganese-sensing transcriptional regulator, bound to its DNA operator. This structure has been long awaited. While crystal structures of the iron-sensing homologs of MntR, IdeR/DtxR proteins, in complex with DNA, have been available for many years, the MntR-DNA complex has long eluded structure elucidation. I am certain that this is not due to a lack of trying, but apparently MntR-DNA complexes don't like to crystallize, so MntR had to wait until cryo-EM became a feasible option.

Despite the limited resolution of the cryo-EM maps, the authors were able to build a model that is consistent with all available data. They take the limited resolution into account in an appropriate manner and were able to construct a model that is highly likely to be correct without overinterpreting the data. The structures suggest how cooperative DNA binding is mediated across MntR dimer-dimer interfaces. The authors tested this hypothesis and were able to verify it.
We appreciate the referee's succinct and supportive summary.

The data are, as far as I am able to ascertain, solid, and the paper is well written. I only have a few requests for some minor improvements.

General comments:
- Please authorize the PDB and EMDB entries. The preprint is out there, so the entries should be released.
We have released the PDB and EMDB entries for 4x$MntR_2$-P84 (PDB ID 9C4D, EMD-45182) and 2x$MntR_2$-P84 (PDB ID 9C4C, EMD-45181) complex.

- In the absence of access to the PDB and EMDB entries, I want to see the validation reports.
We have provided the validation reports to the editor.

- I would appreciate if you made it clear which MntR proteins are included in your sequence comparisons. If I am not mistaken, you have only included sequences from the subgroup without the C-terminal SH3-like domain.
The reviewer is correct. For the sequence comparison, we have omitted those homologs of MntR that contain the C-terminal SH3-like domain. Based on the reviewer's comment, we have included the following details in the legend for Supplementary Fig. 6.
"**Supplementary Figure 6. A Weblogo highlighting sequence conservation among MntR homologs.** Sequence analysis of MntR and its homologs was based on the results of a BlastP[4] search against the *B. subtilis* MntR sequence using the refseq_select database, extending the

results to an E value of 1e-15. Furthermore, sequences less than 120 amino acids or longer than 170 amino acids were omitted to avoid partial sequences or sequences with a third C-terminal domain, which *B. subtilis* MntR lacks. The analysis resulted in 1991 sequences (listed in the extended data table) that were used to create a logo using Weblogo (http://weblogo.berkeley.edu/logo.cgi). Conservation level correlates with the diversity of residues at a specific position, with the symbol's height indicating the frequency of occurrence within the sequence. Some of the *B. subtilis* MntR amino acids of interest are highlighted with black arrows. Note strong conservation of metal-binding residues (marked with "*") typical of the binuclear Mn^{2+} site."

- A comment on the similarities and differences to the DNA-binding mode suggested for Streptococcus MtsR would be nice.
Based on the reviewer's suggestion, we have added the following text and accompanying references on the comparison between the DNA binding mode of MntR with those suggested for the Streptococcal Mn regulators (Line 388 of the revised manuscript)

"Streptococcal Mn regulators, including ScaR, SloR, and MtsR, can bind to multiple, adjacent sites in the regulatory region, like MntR, showing cooperativity through "end-to-end" interactions that appear to take place between the C-terminal FeoA-like domain that is not present in MntR[29–31]."

- What about the arginine cluster? Did you mutate those residues as well? I guess you'd lose DNA binding entirely and consequently you wouldn't learn that much from these mutations, but just curious if you tried. (I am absolutely not asking you to make these mutations.)
We agree with the reviewer that the affinity for DNA binding would likely be severely impacted by any mutation of those residues. Our goal was to investigate the role of protein-protein interactions, and not to mutationally dissect the protein-DNA interface.

- I'm assuming that the models were built before AlphaFold 3 was released, but just out of curiosity, does AF3 give you (more or less) the same model?
We were intrigued by the first referee's suggestion that Alphafold 3 (AF3) might be able to predict the $4xMntR_2$-P84 complex we observed. The predictions we obtained did not align with the experiment. In one run of Alphafold, two of five models placed an MntR dimer at site 1, but in no case were any of the other identified sites occupied as our model suggests (see example model in the **Figure** below). We believe that is because, in all five models, at least two dimers are placed on opposite faces of a short stretch of duplex DNA, mimicking the arrangement of dimers observed in complexes of DtxR and IdeR with DNA (see ref. 17-19). It seems plausible that Alphafold's modeling is biased to promote that mode of binding given its representation in the PDB. Hence, otherwise appropriate binding sites (2-4) are overlooked.

Even so, it was interesting that in each model at least one pair of dimers interact in an "end-to-end" fashion similar to what we observed in our complexes. The separation between dimers along the DNA duplex is identical to our observations and the models include Tyr22 and Asp27 at the

interface in each instance. However, the hydrogen bonding we observe with those residues is absent. The basic residues at the interface (Lys20, Arg24, and Arg58) are not in the positions we observe which necessarily disrupts the interactions that form the network shown in Figure 3.

Ultimately, we found this an interesting opportunity to explore the limits of what Alphafold 3 is capable of predicting. Perhaps the relatively limited number of protein-DNA models in the PDB (relative to the total number of proteins being used to guide protein structure predictions) creates a weaker training set, but as experimental data is gathered, undoubtedly Alphafold will continue to improve as a tool for biological structure prediction. If the reviewer is interested, we are happy to share the sequences of MntR and DNA that were used for the modeling in AF3.

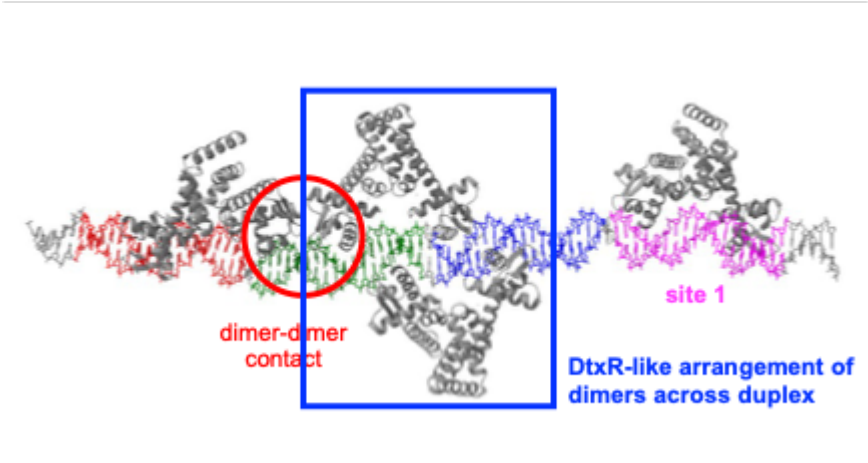

Figure. Example model produced by Alphafold 3. Binding sites 1-4 are colored as in Supplemental Figure 4). Site 1 is occupied by an MntR dimer as we observe, and one example of a dimer-dimer contact similar to those we observe is present. However the arrangement of MntR dimers across the duplex is more similar to the DtxR•DNA complex in the PDB (1C0W).

Detailed comments:

Main text:

- p. 4, l. 97-99: not a proper sentence

Corrected: Based on the reviewer's comment, we have modified that sentence as shown below (lines 96-99 of the revised manuscript)

"Despite the heterogeneity and reduced resolution caused by the flexibility of the DNA, the 4x($MntR_2$)-P84 map still offers valuable evidence regarding the stoichiometry of MntR homodimers binding to the entire regulatory region of the *mneP* promoter sequence."

- p. 5, l. 107: Structural alignment of…

Corrected: We have modified the sentence in line 104 in the revised manuscript, per the reviewer's comment.

- p. 5, first paragraph: Meaning that 2F5C essentially captured the DNA-bound conformation, right?
It is nice to see that the DNA-bound conformation of MntR is close to the metal-bound models previously deposited.

- p. 5, l. 116-117: Not all MntRs have a binuclear Mn binding site. Maybe clarify that you're meaning B. subtilis MntR and its very close homologs.
Corrected: Based on the reviewer's comment, we have modified lines 113-115 as shown below;
"A binuclear binding site has been consistently observed for manganese complexes of MntR from *B. subtilis* and its close homologs from *B. anthracis* and *Salmonella* both in crystal structures and in solution[2,14-17]."

- p. 6, l. 143: with each MntR dimer interacting with…
Corrected: Based on the reviewer's comment we have changed line 139-140 of the revised manuscript to include the following:
"The position of MntR dimers on duplex DNA is consistent with each MntR dimer interacting with nucleic acid bases across 18 bp, with 9 bp inverted repeats."

- p. 8, first paragraph: Indeed, the Pro-T interaction seems to be a common feature of DtxR family regulators, despite their otherwise large divergence regarding recognition sequences and mechanism.
Based on the reviewer's comments we have modified line 195-198 of the revised manuscript to include the following:
"Similar contacts between a proline residue and thymine methyl group, have been identified as playing significant roles in operator recognition in related, DtxR[20] and IdeR[21,22] regulators, despite their otherwise large divergence in recognition sequences and mechanisms."

- p. 10, l. 240: extended data?
Corrected: A new "Extended data 1" (an excel sheet) has been submitted with the revised manuscript. The extended data was modified to include all the protein sequences that were used to create the weblogo presented in Supplementary Fig. 6.

- p. 11, l. 279: Is this conclusion based on the FSEC data alone?
Yes. the conclusion presented in line 279 was based on the FSEC data alone. Mass Photometry could not be used for smaller-sized (less than 35 kDa) complexes between MntR and DNA.

- p. 14, l. 357-358: This seems a bit speculative to me. You've already provided some more solid reasoning for the behavior of this variant. Also, you're using both additionally and hence twice in a row in this paragraph.
Corrected: We have modified two paragraphs starting at line 327 of the revised manuscript to include the following:

"In contrast with Y22A MntR, the D27A MntR protein forms 4x$MntR_2$-P84 (Fig. 4c, f) and 4x$MntR_2$-C84 (Fig. 5c, f) complexes. It also binds the H26 duplex but fails to form a complex with P26 in the FSEC experiment (Supplementary Fig. 8c). The presence of a single peak at ~182 kDa in MP data (Fig. 4c), indicates that D27A MntR forms a higher affinity complex with P84 than WT MntR (Fig. 4a). The network of interactions involving Asp27 at the MntR dimer-dimer interface in our 2x$MntR_2$-P84 structure provides a plausible explanation for this observed increase in DNA-binding affinity (Fig. 3). Asp27 forms a salt bridge with the side chain of Lys20 on the same MntR and Arg58 on the neighboring MntR dimer (Fig. 3c). Arg58, in turn, interacts with the phosphate group of nucleotide +11 in the MntR-recognized half site (Fig. 2b). Mutation of Asp27 to alanine allows the conserved Arg58 to form stronger interactions with the backbone phosphate group on the DNA duplex, potentially increasing MntR's affinity for P84, consistent with mass photometry experiments (Fig. 4). Additionally, mutation of Asp27 to a small, non-polar residue like alanine also allows Lys20 to interact with and further stabilize the network of interactions with the Tyr22 side chain on the same MntR and the carboxylate of Glu55 on the adjacent MntR dimer, consistent with in-vitro binding studies to the P84. We suggest that these interactions cumulatively account for the increased DNA-binding affinity and might serve to compensate for any decrease in cooperativity due to the D27A substitution.
Despite the ability to form a 4x$MntR_2$-P84 complex, the D27A mutant protein is unable to activate transcription. The reasons underlying the failure of D27A MntR to activate transcription from the mneP promoter in vivo are unclear but likely reflect defects in the formation of the activation complex between MntR and RNA polymerase. The -35 recognition site for binding of the RNAP holoenzyme overlaps MntR-dimer binding site 1 on the mneP promoter sequence (Fig. 1a). One possibility is that Asp27 from the MntR subunit at site 1 could contribute to favorable binding interactions with RNAP. Alternatively, the activation complex formed between MntR and RNAP at this promoter site may involve conformational changes that are impeded by either the D27A substitution directly or as a consequence of the higher DNA affinity. Further studies are required to define the composition of the activation complex ultimately formed by MntR and RNAP at the mneP promoter."

- p. 30, l. 719: It's a Coulomb potential map, not an electron density map.
Corrected: It is indeed a Coulomb potential map. Based on the suggestions in this reference "*Beckers, Maximilian, Daniel Mann, and Carsten Sachse. Structural interpretation of cryo-EM image reconstructions. Progress in Biophysics and Molecular Biology 160 (2021): 26-36*", we have changed the "electron density map" to "**cryo-EM density map"** for the measured Coulomb potential of the scattered atoms.

- Fig. 3, panel c: In the left box we see the surface of the DNA colored by charge distribution? In the right box the residues are shown both as sticks and as transparent vdW surfaces? Please amend figure legend.
Corrected: In the left box in panel (c) of Fig. 3, we see the surface of the DNA colored by charge distribution. Based on the reviewer's comment we have modified the legend for Fig. 3 in the revised manuscript to include the following text:
"**Figure 3. Interdimer interactions observed in the ($MntR_2$)$_2$.P84 structure.** (a) A cartoon representation of the cryo-EM structure of the ($MntR_2$)$_2$-P84 complex with two MntR dimers

highlighted in green and blue, with each dimer bound to 4 Mn^{2+} ions (yellow spheres). The MntR dimers are interacting with a portion of P84 (orange). Tyr22 and Asp27 are highlighted in the cartoon. (b) A schematic representing a summary of interactions observed between the two adjacent dimers of MntR in the $(MntR_2)_2$-P84 structure. Polar interactions such as dipole-dipole interactions, H-bonds, and salt-bridges are highlighted by black lines and hydrophobic or vdW interactions are highlighted by orange lines. The dotted lines represent weaker interactions between residues that are separated by a distance ~4 – 5 Å. Note that the side chains of Tyr22 and Val 61 are interacting with the backbone of Leu60 and Lys20, respectively. (c) Expanded views of the network of interdimer contacts around the conserved Asp27 (left) and Tyr22 (right). The DNA surface in the left box is colored by charge distribution, while amino acids in the right box are shown as sticks and semi-transparent vdW surfaces. The black dotted lines represent polar interactions including salt bridges and H-bonds."

Supplementary information:
Table 2:
- What are the 3 different B factors separated by slashes given for each category?
Corrected: The 3 different B-factors are min/max/mean. We have amended Supplementary Table 2 to highlight that. We have replaced "B factors (Å^2)" with "B factors (Å^2) (Min/Max/Mean)"

- What are the numbers in parentheses for RMS deviations? The number of outliers?
Corrected: The numbers in the parentheses are outliers. We have amended Supplementary Table 2 to highlight that: "Bond lengths (Å) (# > 4σ) and "Bond angles (°) (# > 4σ)"

Fig. 1:
- Can you remove the spell checker underlines from the figure?
Corrected: We apologize for the oversight and thank the reviewer for noting it. We have made the change.

- l. 17: H26 in bold? It's orange in the figure.
Corrected: We have modified the legend for Supplementary Fig. 1 to highlight that.
"**Supplementary Figure 1. MntR binding site sequence alignments.** (a) *mneP* and *mneS* promoter sequences containing all the four observed binding sites for MntR dimers (site 1 (purple), site 2 (blue), site 3 (green), and site 4 (red)). The panel also shows P84 containing the *mneP* promoter sequence, C84 containing all four consensus sites (orange), P26 containing the *mneP* promoter site 1 (purple), and H26 containing the mntH promoter site (orange). (b) MntR binding sites from *mntA, mntH, mneP,* and *mneS* promoter sequences were aligned (left) and a sequence logo was created (right) using Weblogo (http://weblogo.berkeley.edu/logo.cgi). The overall height of each stack indicates the sequence conservation at that position (measured in bits), whereas the height of each letter within the stack reflects the relative frequency of the corresponding base at that position."

Fig. 4: starting model shown in pink?
Yes, the starting model of the MntR dimer is in pink. We have modified the legend for Supplementary Fig. 4 to highlight that.
"**Supplementary Figure 4. Structural alignments of MntR dimers with and without bound DNA.** Two MntR dimers from the 2x$MntR_2$-P84 structure (PDB: 9C4C) were aligned to the starting model of the MntR dimer (pink) without the bound DNA[2] (PDB: 2F5C) in PyMOL[3] with RSMD values of 0.8 Å (tan) and 0.9 Å (blue).

Fig. 6: the Weblogo is so compressed, it's very hard to read. Might warrant panoramic layout.
Corrected: We agree with the reviewer that it was hard to read the protein sequence previously presented in Supplementary Fig. 6. We have modified the weblogo to present sequence conservation over two lines instead of one. We hope this change makes it easier for the reader to read the weblogo.

Reviewer #2 (Remarks to the Author):

Review overall summary:
The manuscript "Structural basis for transcription activation through cooperative recruitment of MntR" by Shi, H. et al. submitted to Nature Communications addresses an active area of research in molecular mechanisms of transcription control and is likely of interest to a broad audience. Using a combination of approaches, the authors show that the transcriptional regulatory protein MntR functions differentially as an activator and repressor to control expression of genes encoding Mn-specific transporters. MntR cooperatively binds multiple proteins to specific regions in the operator to help activate transcription of mneP but only one binding-event is needed when functioning as a repressor for inhibiting mntH transcription. The data presented is sound and well analyzed. The data also advance the understanding of the molecular mechanism of how transcriptional regulatory proteins function globally as both a repressor and activator. It will be interesting to see how MntR binding to the DNA triggers recruitment of the RNA polymerase.
We appreciate the reviewer's succinct and supportive summary. In our ongoing work we are hoping to address the last point raised by the reviewer.

Minor comments:

I find it interesting that the bacteria cell requires a single binding event for repression of mntH to diminish Mn import but requires multiple events for activation of mneP for Mn to be exported. It is as if the cellular response needs to act with certainty that intracellular Mn level is too high before the cell will remove Mn. Why would this not be the case for Mn import? Does it have something

to do with the ability to unload MntR off the DNA when Mn returns to healthy levels? How does the rate of Mn import vs. Mn export affect the transition to Mn homeostatic levels?

The reviewer raises interesting questions. Both repression of Mn import and activation of efflux are triggered by changes in Mn-binding to MntR, and for both types of regulation, it is the Mn-bound form of MntR that is associated with DNA. The referee is correct that Mn export commences at levels of Mn greater than those that trigger repression of uptake so that the two processes do not both run at high levels in tandem (which would be a futile cycle). The rate of Mn import and export is governed by the number of transporters per cell, the kinetics of transport, and the level of substrate, and it requires the PMF (for MntH-dependent import and MneS/P-dependent export) or ATP.

It appears that the binding site for MntR as a repressor has much higher affinity than that of the sites as an activator. Given this, then what would be the benefit to the cell? It would be nice to see how the cooperative binding of MntR to the mneP affects the rate of its transcription.

The reviewer is correct that the activation of efflux occurs with greater cooperativity than for repression of import. Occupancy of the MntR-binding sites for repression (single sites) *vs.* activation (four dimers bound) are affected by several equilibria: the binding of Mn to MntR, MntR:Mn to DNA, and interdimer contacts between bound MntR dimers. The impact on transcription was shown previously using in vitro transcription reactions and a cooperative response to changes in Mn was observed (e.g. Fig. 8c in Huang, X., Shin, J.-H., Pinochet-Barros, A., Su, T. T. & Helmann, J. D. *Bacillus subtilis* MntR coordinates the transcriptional regulation of manganese uptake and efflux systems: *Bacillus subtilis* MntR. *Molecular Microbiology* **103**, 253–268 (2017)).

Major comments:

None. The manuscript is well written and structured.

Reviewer #3 (Remarks to the Author):

On this research article the authors describe the molecular basis of the Manganese transport regulator (MntR) binding to regulatory regions of the mneP promoter and rationalize its Mn2+-dependent activation. Through structural biology, biophysical and activity assays the authors provide a deep understanding of manganese homeostasis regulation.

The manuscript has been redacted on a very clear and easy-to-follow language and the introduction and results are clear, stating the background, general hypothesis and conclusions in a very comprehensive manner. The methods are sufficiently detailed to guarantee reproducibility and interpretation of the manuscript.

The experimental design is sound, and the work supports the claims and conclusions drawn by the authors. The two three-dimensional structures described on this section have been obtained following a well-designed Cryo-EM approach, highlighting the expansion of the box size to pick large protein-DNA complex particles.
In general, the characterization of the MntR dimers and DNA features observed on the Cryo-EM structures is robust and the authors perform a thorough analysis and interpretation of the protein-protein and protein-DNA contacts observed on the Cryo-EM structures. The analyses of MntR dimers interaction through Mass Photometry and Fluorescence-based size exclusion chromatography (FSEC) experiments clearly highlight the relevance of the cooperativity process on the binding and activation of the operator.
In general, the data analysis and interpretation are correct.

As a summary, I would recommend this article for publication.
We appreciate the reviewer's succinct and supportive summary.

I would like to highlight a few minor comments:

1. The nomenclature of the two CryoEM structures makes a bit difficult to follow the text. It would be better to define them as Dimer and Tetramer or 2x- 4x- Complexes.
In response to the reviewer's comment, we have changed the nomenclature of the two cryoEM structures throughout the revised manuscript and supplementary information to be:
4xMntR$_2$-DNA - for four MntR dimers bound to a DNA
2xMntR$_2$-DNA - for two MntR dimers bound to a DNA

2. The presence of the 9 bp inverted repeats on the P84 DNA is not evident from the figures.
Corrected: We have modified panel b in Supplementary Fig. 1 to highlight the 9 bp inverted repeat.

3. The comparison of both structures on Supplementary Figures 2 and 3 leads to confusion given the different scale used on both Cryo-EM maps (ie. Supp Fig 3a vs b). I would recommend homogenizing the scale of the maps to help on the interpretation of the models and maps.
Corrected: We have modified Supplementary Fig. 3 to homogenize the scales of the two Cryo-EM density maps.

4. It would also be recommended to label the A-site and C-site metal positions in Supplementary Figure 4. They are mentioned in the text but not clearly defined on the figure, which makes difficult to analyze it.
Corrected: We have modified Supplementary Fig. 4 to include labels for the A-site and C-site Mn^{2+} ions positions in MntR.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

All my concerns have been adequately addressed by the authors, and all my questions have been answered. After looking through the PDB validation reports and examining the cryo-EM maps in 3D, I have no concerns regarding the cryo-EM data and their interpretation. In summary, I think this paper is ready for publication.

I very much appreciate the authors taking the time to address my rather tangential question about AlphaFold3 in such detail. I find it very interesting that AF3 failed to produce a correct model in your case because I have seen astoundingly accurate results from it. I agree with your reasoning that it is likely biased by the IdeR/DtxR complex structures it has learned from, yet it seems to recognize that there is a certain likelihood for dimer-dimer interfaces being formed.

Reviewer #3 (Remarks to the Author):

All the points raised during the original review process have been addressed and they improved the interpretation of the results and conclusions. Therefore, I would recommend this article for publication.