Protein Deacetylase CobB Interplays with c-di-GMP

Zhaowei Xu[+], Hainan Zhang[+], Xingrun Zhang[+], Chengxi Liu, Hewei Jiang, Fanlin Wu, Lili Qian, Daniel M. Czajkowsky, Shujuan Guo, Lijun Bi, Shihua Wang, Haitao Li, Minjia Tan, Lei Feng, Jingli Hou, Sheng-ce Tao*

[*] Correspondence address. Email: taosc@sjtu.edu.cn (S.C.-Tao)

[+]These authors contributed equally to this work

Supplemental results

Determine the binding affinity of *S. typhimurium* CobB with c-di-GMP

To determine the binding affinity of *Salmonella* CobB with c-di-GMP, we performed the ITC and biolayer interference assay (BLI) assay. As shown in the binding curves of ITC, the former 8 injections show a consecutive increase in caloricity release that indicate a protein conformation or oligomer-state change, while their interaction is revealed by the remaining injections which show a successive decrease. Elution volume in gel filtration exhibits a highly aggregated protein form, and asymmetric UV absorption curve indicates its poly-conformational state, which are consistent with properties of the ITC binding curve **(Supplementary Fig. 10a and 10b)**. Hence, the depolymerization of *Salmonella* CobB would release caloricity and affect the ITC result. Because of this, we re-analyzed the affinity of *Salmonella* CobB for c-di-GMP using BLI that does not depend on caloricity change. In BLI assay, we determined two affinity constants of c-di-GMP/CobB 15.5 μM and 15.6 nM **(Supplementary Fig. 10c)**, possibly because *Salmonella* CobB have diversified state of multimer. Additionally, in *E. coli* and *Salmonella* CobB, the c-di-GMP binding sites *i.e.,* R8, R17, E21 are consistent, hence, we speculated that the *E. coli* and *Salmonella* CobB have similar affinity with c-di-GMP. Therefore, 15.5 μM is more likely to be the affinity constant of c-di-GMP to *Salmonella* CobB.

Supplemental methods

MST assay

We used the Cy3 label kit (GE Healthcare, Pittsburgh, USA) to label the purified CobB (0.5 mg/mL, 16.13 μM) and used desalt spin columns (Pierce, MA, USA) for removing free dye. Microscale Thermophoresis assay (MST assay)[1] is a method by measuring the molecular movement in micro-temperature gradient field to assay the moleculars' interaction. In MST assay, the serially diluted c-di-GMP, *i.e.,* 0.49, 0.97, 1.95, 3.91, 7.81, 15.63, 32.25, 62.50, 125 and 250 μM were incubated with 0.1 mg/mL (3.23 μM) of Cy3-labeled CobB in 20 μL deacetylation buffer for 5 minutes. The sample was loaded into the NanoTemper glass capillaries (NanoTemper, München, Germany) and micro-thermophoresis was carried out using 40% LED power and 80% MST laser power to producing a local temperature gradient. The K_d were calculated using the mass action equation via the NanoTemper software (NanoTemper, München, Germany) from duplicate reads of triplicate experiments.

Biolayer Interference (BLI) assay

BLI assay is a method based on the Biolayer interference principle to assay the moleculars' interaction, and performed as described previously[2]. 50 μg/mL (42.6 μM) biotin-c-di-GMP was loaded on

streptavidin (SA) tips (ForteBio/Pall Life Sciences, CA, USA) for 300 s. His-CobB$^{S.\ typhimurium}$ was prepared with serial dilution (31.25, 62.5, 125, 250, 500, 1000 and 2500 nM) and bound to the c-di-GMP-saturated tips for 600 s and then dissociated in the buffer (1xPBS, 0.02% tween20, 0.1% BSA) for 600 s. The results were recorded and processed by Octet Software v7.x from ForteBio system (ForteBio/Pall Life Sciences, CA, USA).

Supplemental figure legends

Supplementary Figure 1. ITC titration curves of wild-type CobB titrated with c-di-GMP, cGMP and c-di-AMP, and mutant CobB R8A, R17A and E21A titrated with c-di-GMP. The titration curves of wild-type CobB protein titrated with 1.5 mM c-di-GMP (a), cGMP (b) and c-di-AMP (c) are shown in blue. The titration curves of mutant CobB R8A (d), R17A (e) and E21A (f) protein titrated with c-di-GMP also are shown in blue. All control curves of titrated buffer titrated with ligands used in curve fitting of each titration assays are colored in black.

Supplementary Figure 2. The binding of c-di-GMP and CobB was confirmed by MST analysis. Serially diluted c-di-GMP, *i.e.,* from 0.1 μM to 50 mM were incubated with 0.1 mg/mL (3.23 μM) of

fluorescently-labeled CobB **(a)** and equal cGMP was included as negative controls **(b)** The binding was determined by micro-thermophoresis in 40 % LED power and 80 % laser power. The fluorescent coefficients and substrate concentrations were fitted to a curve to determine the K_d and their associated errors, using the NanoTemper software.

Supplementary Figure 3. The polymer states of CobB and $CobB_S$. (a) The polymer states of CobB and $CobB_S$ were determined by molecular sieve. CobB and $CobB_S$ were determined by OD_{260} (red curve) and OD_{280} (blue curve). **(b)** The polymer states of CobB and $CobB_S$ were determined by SDS-PAGE after cross-linking.

Supplementary Figure 4. c-di-GMP inhibits CobB deacetylase activity in vitro. (a) CobB activity assay was performed using Acs as substrate. The loss of the acetylation level of Acs indicates the CobB's deacetylase activity and it was detected by the pan anti-acetyl antibody. (three preparations; $^{**}P < 0.01$, two-tailed Student's t-test). CobB activity assay was also performed using CheY **(b)** (three preparations; $^{*}P < 0.05$, two-tailed Student's t-test), and NhoA **(c)** (three preparations; $^{**}P < 0.01$, two-tailed Student's t-test) as substrate.

Supplementary Figure 5. The growth curves of the 6 strains were

determined. These bacterial strains were cultured in the Vogel-Bonner medium with 30 mM acetate **(a)** or 30 mM **(b)** propionate and the growth was measured at 8, 12, 16, 20, 24 and 32 h.

Supplementary Figure 6. The mass spectra of representative peptide in the SILAC experiment. (a, b) The mass spectra show high light (*ydeH*$^{+}$) signal and significant low heavy (WT) signal for 2 peptides. **(c, d)** The mass spectra show high light signal vs undetectable heavy signal for another 2 peptides.

Supplementary Figure 7. The Gene Ontology (GO) analysis of the c-di-GMP-upregulated (a) and the known CobB-regulated acetylated proteins (b). The ordinates show the functional classification, and the histogram show protein amounts in each group (blue) and the fold enrichment (red).

Supplementary Figure 8. c-di-GMP blinds to CobB mutants. (a) The c-di-GMP concentration of the 5 strains are determinate by UPLC-IM-MS with three replicates. **(b-d)** The growth curves of the 5 strains were determined**.** These bacterial strains were cultured in the Vogel-Bonner medium with 10 mM propionate **(b)**, 30 mM acetate **(c)** or 30 mM propionate **(d)** and the growth was measured at 8, 12, 16, 20, 24 and 32

h with three replicates.

Supplementary Figure 9. The evolutionary tree of CobB. CobB protein sequences of a list of representative bacteria were downloaded from NCBI and aligned using CLUSTALW from EMBL-EBI.

Supplementary Figure 10. c-di-GMP binds *S. typhimurium* CobB *in vitro.* **(a)** ITC analysis of the binding of c-di-GMP and *S. typhimurium* CobB. The titration curves of *S. typhimurium* CobB titrated with 1.5 mM c-di-GMP is shown in blue and the buffer titrated with ligand is colored in black. **(b)** Gel filtration assay of *S. typhimurium* CobB. The observed protein size is 608KDa. **(c)** BLI assay of the affinity of *S. typhimurium* CobB and c-di-GMP. The BLI assay were assay under different CobB concentrations (31.5 to 2,500 nM).

Supplementary Figure 11. MS analysis to determine the K170 (a) and K277 (b) acetylation of YdeH that could be deacetylated by CobB.

Supplementary Figure 12. LC-MS analysis to determine the acetylation sites on Era (a) and YegE (b).

Supplementary Figure 13. The protein stability of overexpressed YdeH

mutants. After incubation, these samples were centrifuged at 12,000 g for 10 min to separate precipitation. These samples were separated at 0.5 h, 1.5h and 2.5 h and the protein levels were determined by the anti-His antibody. The bar graph showed the quantitation of the protein level of YdeH with three replicates.

Supplementary Figure 14. The K4 acetylation stoichiometry of overexpressed and affinity purified YdeH. The mass spectra show AQUA quantification of the acetylation of overexpressed YdeH K4 using AQUA peptide.

Supplementary Figure 15. The drop in soluble YdeH (shown in Fig 7d) is well described according to a simple exponential decay.

Supplementary Figure 16. The K4 acetylation stoichiometry of endogenous YdeH in stationary growth. The mass spectra show AQUA quantification of endogenous YdeH K4 acetylation of WT cells **(a)** and CobB defect cells **(b)**. **(c)** The level of the endogenous YdeH in supernatant and sediment. The protein levels were determined by the anti-FLAG antibody and GroEL was applied as the loading control. The bar graph showed the quantitation of the protein level of supernatant YdeH with three replicates.

Supplementary Figure 17. The model of YdeH K4 acetylation reducing the DGC activity directly. (a) The *E.coli* YdeH structure and K4 residue was highlighted in red. YdeH dimer are distinguished by color (PDB code: 4H54). **(b)** Structure of the GGDEF dimer and two GTPαS substrates are not in reacting distance (red arrows) leads to explain the misalignment. **(c)** Structure of a catalytically competent GGDEF/GTP dimer. Two GGDEF domains have been shifted into the reaction orientation. **(d)** Mechanistic model of YdeH activity regulation by K4-dependent structure alignment.

Supplementary Table 1. The statistical analysis results (*p* value). The statistical analysis results of growth curves at 8 h or 24 h in Fig. 2f. (three preparations; two-tailed Student's t-test).

References

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2. Zhang, H.-N. *et al.* Cyclic di-GMP regulates Mycobacterium tuberculosis resistance to ethionamide. *Scientific Reports* **7** (2017).

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