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c-di-GMP does not bind H-NS, nor inhibits H-NS binding DNA

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Jiayu Wang[1,2], Hehan Wei[1,2] & Bin Xia[1,2,3]

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H–NS is a nucleoid-associated protein involved in chromosome organization and regulation of gene expression in proteobacteria[1]. H–NS functions as a global transcriptional repressor, particularly acting as a xenogeneic silencer to suppress foreign genes, such as virulence and antimicrobial resistance genes. However, bacteria have evolved anti-silencing mechanisms that enable the expression of beneficial foreign genes[2]. Recently, Li et al. proposed a new anti-silencing mechanism in which the bacterial second messenger c-di-GMP can directly bind to the DNA-binding domain of H–NS and prevent it from binding DNA[3]. As a result, increased c-di-GMP intracellular levels can derepress genes silenced by H–NS, such as T6SS genes, in *Salmonella enterica* serovar Typhimurium. Here, we provide several pieces of evidence that c-di-GMP does not bind H–NS and does not prevent H–NS from binding DNA; therefore, c-di-GMP does not directly derepress genes silenced by H–NS.

As Li et al. reported that the dissociation constants (K_d) between c-di-GMP and full-length H–NS or its DNA-binding domain are ~0.3 μM, we carried out NMR titration experiments to examine the interaction between c-di-GMP and the DNA-binding domain of H–NS (residues 85–137, H–NS^{ctd}) at pH 7.5. Surprisingly, we failed to observe c-di-GMP binding H–NS^{ctd} from either *Salmonella* or *E. coli*, which are highly conserved in their primary sequences (90.6% identity, 98.1% similarity) (Supplementary Fig. 1). There was no obvious chemical shift perturbation or signal intensity change for NH signals in 2D ^{1}H–^{15}N SOFAST-HMQC spectra, even with 10-fold of c-di-GMP (Fig. 1a, b). For cGMP and c-di-AMP, which Li et al. employed as negative controls for c-di-GMP binding, NMR titration results were not different from those of c-di-GMP, indicating that none of the three nucleotides binds H–NS^{ctd}.

To verify those negative results, we first validated our c-di-GMP reagent using 1D ^{1}H NMR spectrum. The ^{1}H chemical shifts and peak splitting patterns were all consistent with previous reports for c-di-GMP[4,5] (Supplementary Fig. 2). As a positive control, we also used NMR to monitor the c-di-GMP binding to the GSPII-B domain (PilF$_{159–302}$) of PilF from *T. thermophilus*, a structurally well-characterized c-di-GMP binding protein[6]. As expected, upon addition of c-di-GMP, we observed the characteristic changes in NH signals in 2D ^{1}H–^{15}N HSQC spectra of PilF$_{159-302}$[6] (Fig. 1d). Therefore, our c-di-GMP reagent had the right chemical composition and configuration.

For comparison, we titrated a double-stranded oligo DNA (3AT: 5′-CGCATATATGCG-3′) into H–NS^{ctd}, which resulted in chemical shift perturbation for NH signals even at 0.2× molar ratio; the protein was saturated by 5× DNA as NH peaks stopped moving afterwards (Fig. 1c). The K_d value between 3AT DNA and H–NS^{ctd} was determined to be 6.3 ± 0.6 μM from NMR data, at pH 7.5. NMR is known to be a very sensitive technique for probing very weak protein/ligand interactions (K_d > 1 mM)[7]. If the K_d between H–NS and c-di-GMP were indeed ~0.3 μM as reported by Li et al., it is expected that the addition of c-di-GMP at 1:1 molar ratio would result in significant NH signal perturbations, as evident from other NMR studies of c-di-GMP binding proteins such as the second messenger binding protein SmbA from *Caulobacter crescentus*[8], as well as our NMR data of PilF$_{159–302}$ (Fig. 1d). We also conducted ITC measurements, and again no binding was observed between full-length H–NS and c-di-GMP (Fig. 1e). Consistently, the K_d between the 3AT DNA and H–NS^{ctd} was 7.6 ± 0.5 μM from ITC (Fig. 1f). These results indicate that there is no direct interaction between c-di-GMP and H–NS, not even a weak interaction with the dissociation constant of a few mM.

We next conducted EMSA experiments to examine the effect of c-di-GMP on the DNA-binding ability of full-length *E. coli* H–NS, with identical experimental buffer, salt, pH, and gel conditions as reported by Li et al. (Supplementary Note 1). We first tested the promoter sequence of the *LEE5* operon (P_{LEE5}, 317-bp, AT content 73.5%) from enterohemorrhagic *Escherichia coli* (EHEC), which is known to be directly bound by H–NS[9]. As shown in Fig. 1g, H–NS binds strongly to P_{LEE5} DNA (2.5 ng/μL) with almost no free DNA band visible at 1 μM concentration. However, the addition of c-di-GMP at 80× molar ratio to H–NS had no effect on the H–NS/DNA complex bands, no matter whether c-di-GMP, P_{LEE5} DNA, and H–NS were mixed simultaneously, or either two of them were mixed first and the third one was added 20 min later. These results indicate that c-di-GMP does not interfere with H–NS binding to P_{LEE5} DNA. We next tested two DNA fragments used by Li et al.: P_{yaiU} (301-bp, AT content 74.1%) and control DNA$_{yaiU}$ (C_{yaiU}, 337-bp, AT content 55.2%). The binding of H–NS to P_{yaiU} DNA was almost the same as to P_{LEE5} DNA, as expected since their AT contents are similar. Contrary to that reported by Li et al., we observed binding of H–NS to the C_{yaiU} DNA in our EMSA experiment, although

[1]College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China. [2]Beijing Nuclear Magnetic Resonance Center, Peking University, Beijing 100871, China. [3]School of Life Sciences, Peking University, Beijing 100871, China. e-mail: binxia@pku.edu.cn

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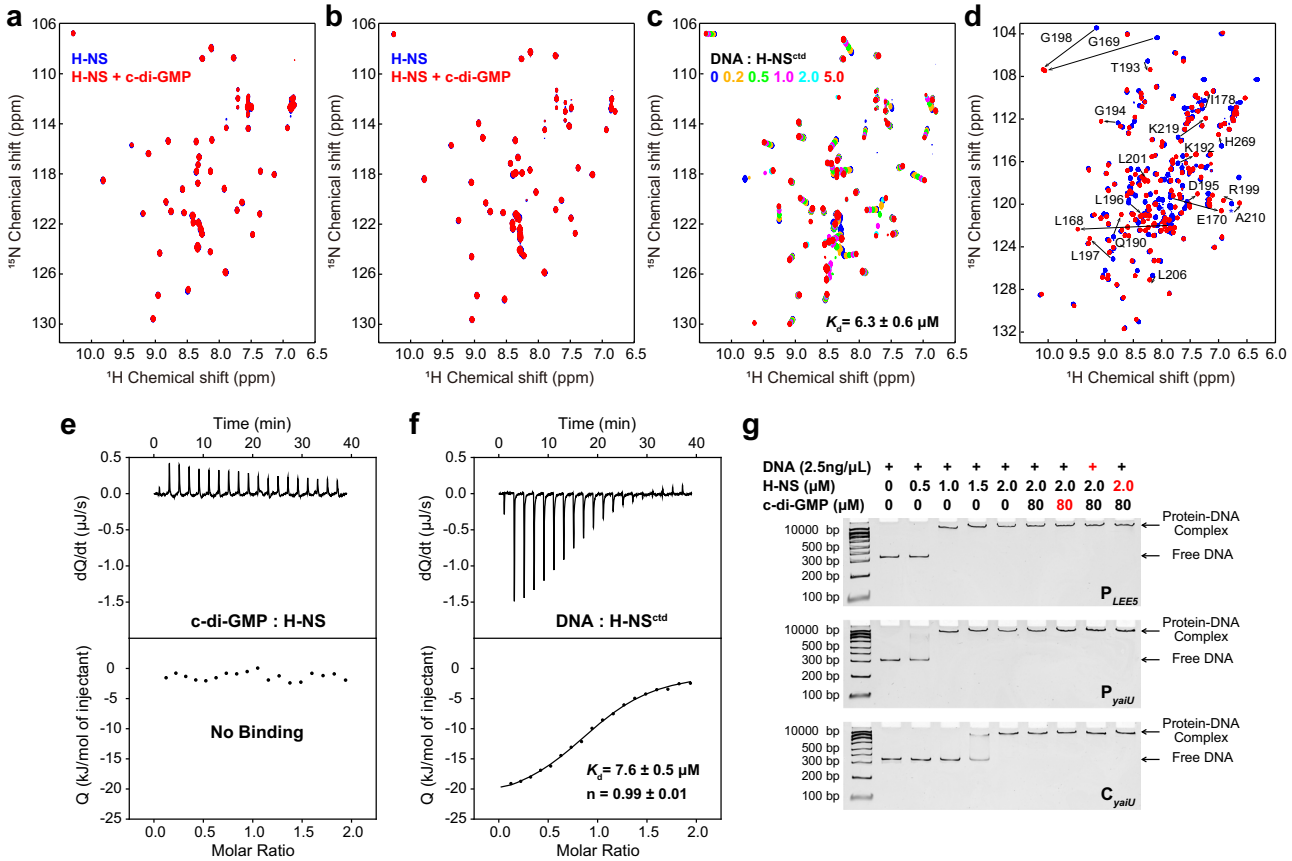

Fig. 1 | c-di-GMP does not directly bind H–NS. a, **b** Overlay of 2D ^{1}H–^{15}N SOFAST-HMQC spectra of *E. coli* (**a**) or *Salmonella* (**b**) H–NS^{ctd} with (red) and without (blue) 10× c-di-GMP. **c** Overlay of 2D ^{1}H–^{15}N SOFAST-HMQC spectra of *Salmonella* H–NS^{ctd} with the titration of 3AT DNA at indicated molar ratios. **d** Overlay of 2D ^{1}H–^{15}N HSQC spectra of free (blue) and c-di-GMP bound (red) PilF$_{159\text{-}302}$ in 50 mM Bis–Tris buffer (pH 5.8) with 200 mM NaCl. NH signals exhibiting large chemical shift differences between the free and c-di-GMP bound states are indicated by arrows, with the assignments labeled. **e** Characterization of the binding between full-length *E. coli* H–NS and c-di-GMP by ITC. **f** Characterization of the binding between *Salmonella* H–NS^{ctd} and 3AT DNA by ITC. **g** EMSAs for *E. coli* H–NS binding to P$_{LEE5}$ (top), P$_{yaiU}$ (middle), or C$_{yaiU}$ (bottom) DNA, in the presence or absence of c-di-GMP. The concentrations of H–NS, DNA and c-di-GMP are indicated. Red characters indicate samples with the corresponding ingredient added 20 min after the other two ingredients mixed, and the other samples have all ingredients mixed at the same time. The sequences of each DNA are shown in Supplementary Fig. 1. All NMR samples of H–NS contain 0.1 mM protein in 25 mM Tris-HCl buffer (pH 7.5) with 150 mM NaCl.

the binding affinity of H–NS toward C$_{yaiU}$ DNA was weaker than that toward P$_{LEE5}$ and P$_{yaiU}$. It is surprising that H–NS does not bind C$_{yaiU}$ DNA at all in the study by Li et al.[3], since C$_{yaiU}$ DNA still has a relatively high AT-content and 8 stretches with at least 5-bp consecutive AT sequences, which are preferred by H–NS[10]. The addition of c-di-GMP at an 80× molar ratio to H–NS had no effect on the complex bands of H–NS and P$_{yaiU}$ or C$_{yaiU}$ DNA, no matter how the components of the samples were mixed. Together, the results clearly demonstrate that c-di-GMP does not prevent H–NS from binding DNA.

Li et al. reported that the point mutations Y99A, D101A, K107A, and T115A result in about a two orders of magnitude reduction in the c-di-GMP binding affinity of H–NS; K107A does not affect its DNA binding; and T115A reduces its DNA-binding affinity by over 30 times[3]. Our analysis of the solution structure of the H–NS DNA-binding domain indicated that residues Y99 and T115 are involved in the hydrophobic core packing, especially the aromatic ring of Y99 is mainly buried in the hydrophobic core[11] (Fig. 2d). Indeed, the 2D ^{1}H–^{15}N SOFAST-HMQC spectrum of H–NS^{ctd} Y99A mutant shows characteristics of an unfolded protein (Fig. 2a), where mutation T115A results in global chemical shift perturbations (Fig. 2c). Notably, although sidechains of Y99, D101, and K107 are clustered together, the sidechain of T115 is separated from them by the α-helix, and it is unlikely for the sidechain of T115 to interact simultaneously with a small molecule like c-di-GMP together with the other three residues (Fig. 2d, e). The effects of mutations D101A and K107A on the spectra are quite similar: both affect NH signals of residues distant from the mutation sites, including the DNA-binding AT-hook-like motif (Fig. 2b, Supplementary Fig. 4). We also titrated K107A and T115A mutants (pH 6.0) with 3AT DNA using NMR, and their DNA-binding K_d values were determined to be 5.3 ± 1.6 μM and 4.1 ± 1.2 μM, respectively (Fig. 2f, Supplementary Fig. 5). Compared with WT protein (K_d 1.5 ± 0.5 μM, pH 6.0), both mutations have weakened the DNA-binding affinity of H–NS^{ctd} by ~3 times, and K107A mutant shows slightly lower affinity than T115A. H–NS is a small protein composed of an N-terminal oligomerization domain and a C-terminal DNA-binding domain. Its oligomerization domain enables H–NS to self-associate and form oligomers, and each oligomeric H–NS molecule has multiple DNA-binding domains, which can interact with DNA at multiple sites[1]. This multivalent interaction enables the oligomeric H–NS molecule to bind DNA tightly, even though each DNA-binding domain binds preferred AT-rich DNA sequences only with K_d of a few μM. Therefore, it is highly unlikely that K107A does not affect the DNA-binding of H–NS, but T115A reduces its DNA-binding affinity by over 30 times, as claimed by Li et al.[3].

It is known that the N-terminal or C-terminal tags of recombinantly expressed proteins may sometimes affect the protein functions, which often arises because the relevant terminal residues are directly involved in protein structure packing or inter-molecular interactions[12,13]. In our study, the full-length H–NS protein and its

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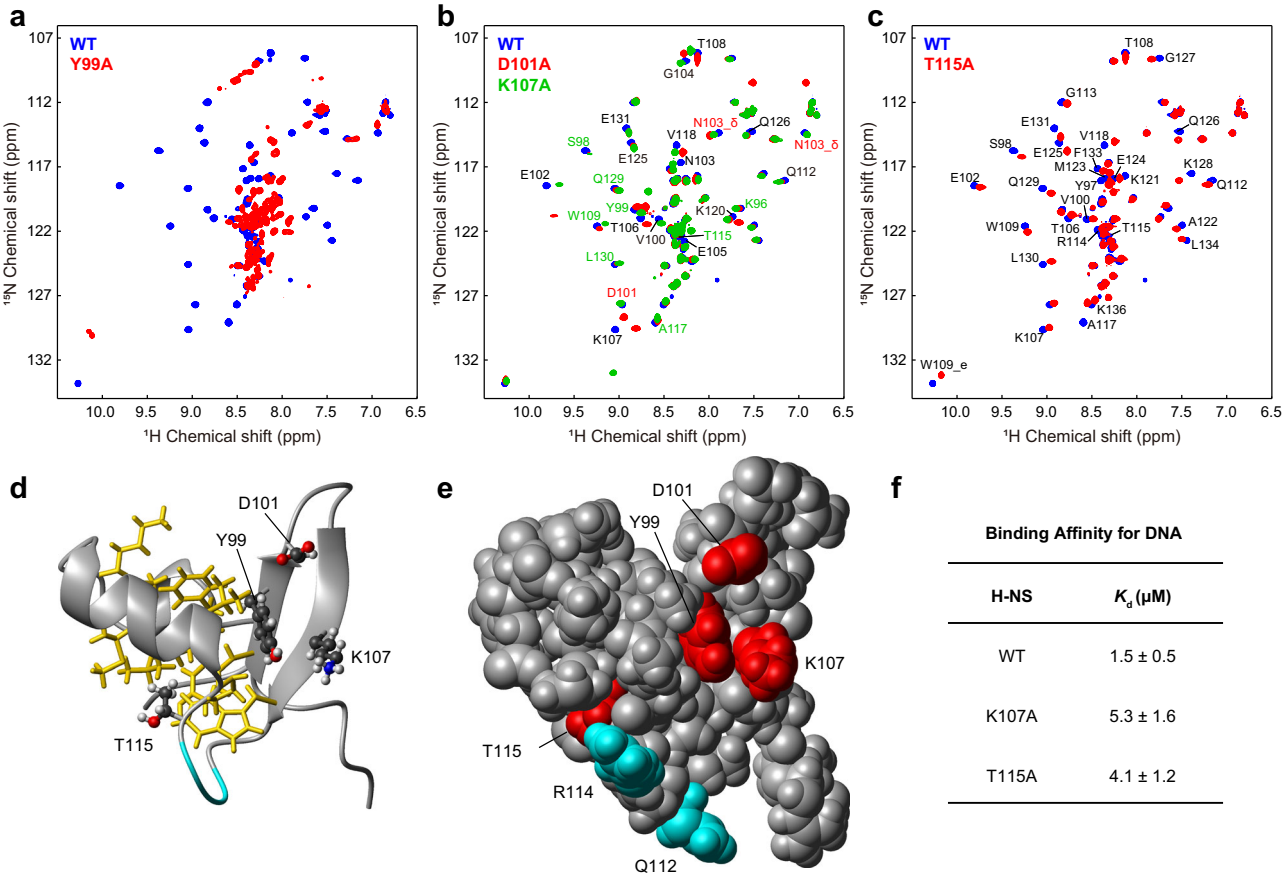

Fig. 2 | Characterization of *Samonella* H–NS^{ctd} mutants. a–c Overlay of 2D ^{1}H–^{15}N HSQC of H–NS^{ctd} (blue) with its Y99A (red) (**a**), D101A (red) and K107A (green) (**b**), or T115A (red) (**c**) mutants. Assignments of NH signals with large chemical shift changes are labeled in (**b**, **c**). **d**, **e** Ribbon (**d**) and space-filling (**e**) diagrams of the DNA-binding domain structure of H–NS (PDB ID 2L93). Sidechains of the mutated residues are shown as ball-and-sticks (**d**) or in red (**e**). The DNA-binding AT-hook-like motif residues Q112–R114 are indicated in cyan. Sidechains of the other residues participating the hydrophobic core are shown as gold sticks (**d**). **f** The dissociation constant (K_d) for WT, K107A, and T115A H–NS^{ctd} binding 3AT DNA. The NMR titration data used to fit K_d are shown in Supplementary Fig. 5. All NMR samples contain 0.1 mM protein in 50 mM sodium phosphate buffer (pH 6.0) with 50 mM NaCl.

DNA-binding domain (residues 85–137) include a His-tag with 8 non-native C-terminal residues (LEHHHHHH) (Supplementary Note 1). During our previous structure determination of the DNA-binding domain, the six histidine residues of the His-tag were omitted due to very few NOEs observed[11], which indicates that the His-tag is highly flexible in solution (Supplementary Fig. 6a). Consistently, the per-residue backbone heavy atom RMSD of the H–NS DNA-binding domain structure ensemble progressively increases starting from the last two native C-terminal residues (K136 1.5 Å, E137 3.1 Å), and the RMSD for the His-tag residue E139 reaches 10.1 Å (Supplementary Fig. 6b). The inherent flexibility of the C-terminal His-tag excludes the possibility for the tag to bind tightly at the putative c-di-GMP binding site and to occlude c-di-GMP binding (supposedly K_d ~ 0.3 μM). Also, the C-terminal tag is structurally incapable of approaching the putative c-di-GMP binding site proposed by Li et al., since the tag is positioned on the opposite side relative to the putative site and extends away from the protein (Supplementary Fig. 6c). Even the AT-hook-like DNA-binding motif, which is closer to the C-terminal tag, is not affected by the tag, as NH NMR signals from the C-terminal residues are hardly perturbed upon DNA binding (Supplementary Fig. 5a). Therefore, it is impossible for the C-terminal His-tag to interfere with our characterization data about the interaction between H–NS and c-di-GMP. However, we noticed a potentially important N-terminal tag issue in their H–NS protein expression strategy. Li et al. cloned the coding sequences of full-length H–NS and its DNA-binding domain (residues 91–137) into the pET-28a vector between the BamHI and HindIII restriction enzyme sites. Consequently, the expressed proteins should contain a 34-residue N-terminal His-tag, and even after thrombin cleavage, there would still be 17 non-native N-terminal residues (GSHMASMTGGQQMGRGS) left in all full-length or truncated H–NS protein samples used in their experiments. Since some peptides as short as 19 residues have been shown to bind c-di-GMP with sub-micromolar affinity[14], it is uncertain whether the 17 non-native N-terminal residues of the H–NS proteins used by Li et al. may promote apparent interactions between c-di-GMP and H–NS. However, this 17-aa N-terminal tag still cannot explain why H–NS could not bind control DNA$_{yaiU}$, and the K107A mutation did not affect DNA binding in the study by Li et al.

Recently, it has been reported that c-di-GMP can bind and modulate the function of the mycobacterial protein Lsr2[15], a functional analog of H–NS, but its binding site on Lsr2 was not identified. However, that study suggested that c-di-GMP binding enhances the DNA-binding ability of Lsr2, which contrasts with the effect of c-di-GMP on H–NS reported by Li et al. Although both Lsr2 and H–NS share an AT-hook-like motif for DNA binding, their primary sequences and overall folds of their DNA-binding domains are quite distinct[11], and thus the c-di-GMP binding site proposed for H–NS by Li et al. is not conserved in Lsr2 (Supplementary Fig. 7). Therefore, the report that c-di-GMP can bind Lsr2 is irrelevant to the proposed c-di-GMP binding to the DNA-binding domain of H–NS.

In conclusion, our results refute the claims that H–NS acts as a c-di-GMP effector or sensor, and that c-di-GMP derepresses H–NS-

silenced genes by directly binding H–NS and preventing its DNA binding. More studies are required to reveal the mechanism underlying the regulation of T6SS expression by c-di-GMP in *Salmonella*.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the data generated or analyzed during this study are included in this paper, its Supplementary Information and the Source Data file. Source data are provided with this paper.

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Author contributions

J.W. and H.W. performed experiments; J.W. and B.X. analyzed data and wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Bin Xia.

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