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Regulation of DNA phosphorothioate modifications by the transcriptional regulator DptB in *Salmonella*

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Summary

DNA phosphorothioate (PT) modifications, with one non-bridging phosphate oxygen replaced with sulfur, are widely but sporadically distributed in prokaryotic genomes. Short consensus sequences surround the modified linkage in each strain, although each site is only partially modified. The mechanism that maintains this low-frequency modification status is still unknown. In *Salmonella enterica* serovar Cerro 87, PT modification is mediated by a four-gene cluster called *dptBCDE*. Here, we found that deletion of *dptB* led to a significant increase in intracellular PT modification level. In this deletion, transcription of downstream genes was elevated during rapid cell growth. Restoration of *dptB* on a plasmid restored wild-type levels of expression of downstream genes and PT modification. *In vitro*, DptB directly protected two separate sequences within the *dpt* promoter region from DNase I cleavage. Each protected sequence contained a direct repeat (DR). Mutagenesis assays of the DRs demonstrated that each DR was essential for DptB binding. The observation of two shifted species by gel-shift analysis suggests dimer conformation of DptB protein. These DRs are conserved among the promoter regions of *dptB* homologs, suggesting that this regulatory mechanism is widespread. These findings demonstrate that PT modification is regulated at least in part at the transcriptional level.

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Introduction

DNA phosphorothioate (PT) modification, with sulfur replacing one nonbridging phosphate oxygen in a sequence- and stereo-specific manner (Wang *et al.*, 2007; 2011), is involved in a restriction–modification (R–M) system that is used as a novel cell defense mechanism by some bacteria including *Salmonella* and *Escherichia* (Xu *et al.*, 2010; Cao *et al.*, 2014b). The *dpt* gene clusters from *Salmonella enterica* or the *dnd* gene cluster from *Streptomyces lividans*, both containing five genes, are responsible for PT modification (Zhou *et al.*, 1988; 2005). So far, PT modifications and/or PT-modifying genes have been found in many taxonomically unrelated bacterial and archaeal genomes (Evans *et al.*, 1994; Dyson and Evans, 1998; Romling and Tummler, 2000; Murase *et al.*, 2004; Zhang *et al.*, 2004; Zhou *et al.*, 2005; He *et al.*, 2007; Wang *et al.*, 2011; Hu *et al.*, 2012; Barbier *et al.*, 2013; Howard *et al.*, 2013). Over the years, studies of PT modifying enzymes have provided further insights into the biochemistry of PT modifications. DndA acts as a cysteine desulphurase and assembles the 4Fe–4S cluster of DndC (You *et al.*, 2007; Chen *et al.*, 2012). In some bacteria, DndA is not present but functionally replaced by endogenous cysteine desulphurase, such as IscS in *Escherichia coli* (An *et al.*, 2012). A DndD homolog in *Pseudomonas fluorescens* Pf0-1, SpfD, has ATPase activity that is possibly required for the alteration or nicking of DNA structure during the process of sulfur incorporation (Yao *et al.*, 2009). Crystal structure of C-terminal truncated DndE from *E. coli* indicates that DndE is a tetramer conformer and is a nicked dsDNA-binding protein (Hu *et al.*, 2012).

Our recent study of genomic mapping of PT sites across bacterial genomes reveals highly unusual features of PT modificaton. In *E. coli* B7A, the double-stranded PT modifications occured in G_{ps}AAC/G_{ps}TTC motifs, but only 12% of these consensus sequences are modified (Cao *et al.*, 2014a). Considering the fact that a PT-dependent restriction system is present in *E. coli* B7A, the partial modification of consensus sequences suggests a regulation of PT modification frequency. Previous observation that deletion of *dndB* in *S. lividans* led to enhanced levels of DNA

degradation phenotype indicates an increase in PT modification (Liang *et al.*, 2007; Xu *et al.*, 2009). However, the mechanism of regulation of PT modification levels by DndB remained unknown.

In *S. enterica* serovar Cerro 87, *dptBCDE* gene and *iscS* are responsible for PT modification of the G_{ps}AAC/G_{ps}TTC motifs, while the PT-dependent restriction system involves additional three genes *dptF–H* (Xu *et al.*, 2010; An *et al.*, 2012). In this study, the physiological role of DptB was characterized as a negative transcriptional regulator for the *dptBCDE* cluster. DptB is demonstrated as a dimer and DptB binds two pairs of direct repeats in the promoter region. Moreover, a regulatory model was proposed for DptB-mediated regulation of the bacterial PT modification frequencies.

Results

Disruption of dptB *increases the level of* in vivo *PT modification*

In order to study the role of *dptB* in PT modification, we constructed its in-frame deletion mutant (Δ*dptB*, Fig. S1) in *S. enterica* serovar Cerro 87. DNA PT modifications were then quantitatively analyzed in both the wild-type strain and Δ*dptB* mutant. By using the iodine-induced PT-specific cleavage assay, which was developed in our previous study (Cao *et al.*, 2014a), a significant increase in the cleavage efficiencies was found in the genomic DNA of Δ*dptB* in comparison with that of the wild-type strain (Fig. 1). This observation is similar to the previous observation that disruption of *dndB* in *S. lividans* aggravated its DNA degradation phonotype (Liang *et al.*, 2007; Xu *et al.*, 2009) and therefore suggested that the absence of *dptB* probably led to an increase in PT modifications. To verifiy this interpretation, the PT-linked dinucleotides were then quantified in Δ*dptB* mutant by liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) method as previously described (Wang *et al.*, 2011). Meanwhile, to determine the effects of *dptB* deletion on sequence specificity of PT modification, 16 possible PT-linked dinucleotides were monitored in its early-, late-exponential and stationary growth phases. Results showed that, same as the wild-type strain, PT modification occurred at the G_{ps}A and G_{ps}T sites in Δ*dptB* mutant (Fig. S2), suggesting that *dptB* deletion did not alter the sequence specificity of PT modification. However, PT modifications of Δ*dptB* mutant in the G_{ps}T and G_{ps}A sites increased about twofold in comparison with the wild-type strain, *i.e.* ~ 1200 G_{ps}A and G_{ps}T sites per 10^6 nt in the mutant *versus* ~ 600 sites per 10^6 nt in the wild type. Notably, the frequencies of PT modification were relatively stable throughout the different growth phases in both strains (Fig. 1C).

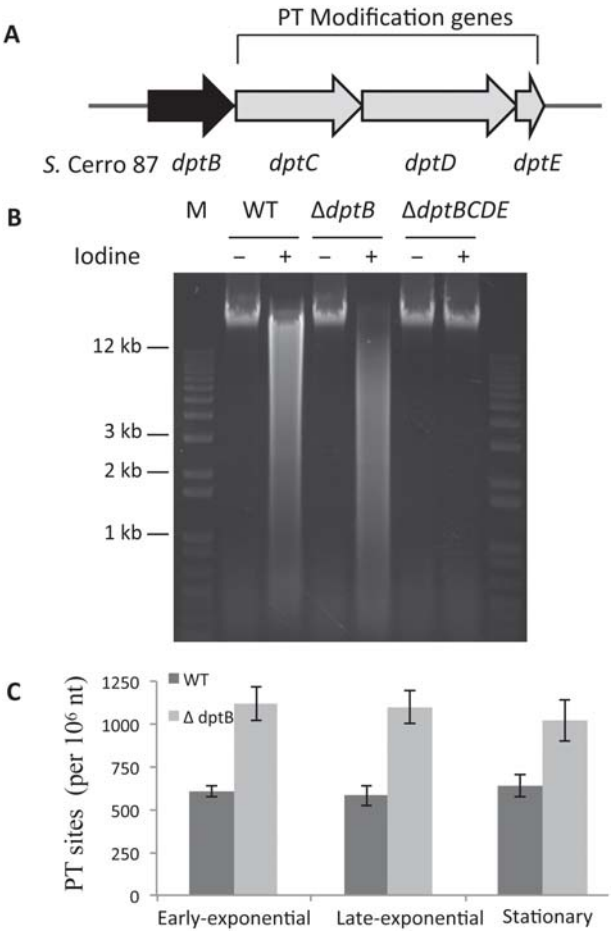

Fig. 1. The organization of the PT modifying genes and quantitative analysis of PT modifications.
A. The organization of the PT modifying genes of *S. enterica* serovar Cerro 87.
B. Iodine cleavage of genomic DNA from wild-type, Δ*dptB* and Δ*dptBCDE* mutant strain of *S. enterica* serovar Cerro 87.
C. PT modifications (G_{ps}A and G_{ps}T) per 10^6 nt in wild-type and Δ*dptB* mutant strain of *S. enterica* serovar Cerro 87 at different growth phases. Data represent mean ± SD for three biological replicates.

DptB negatively regulates the transcription of dptBCDE *operon*

One model to account for the increased level of PT modification observed earlier is that deletion of DptB relieves repression of the expression or activity of the enzymes that insert the modification. Thus, we examined the level of transcriptional expression of genes coding for the PT modifying enzymes. In order to determine the mechanism of the increased frequencies of PT modification in Δ*dptB* mutant, the expression of PT modifying genes was then investigated in this strain. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of wild-type strain of *S. enterica* serovar Cerro 87 confirmed that *dptB*, *C*, *D* and *E* genes were co-transcribed from the same

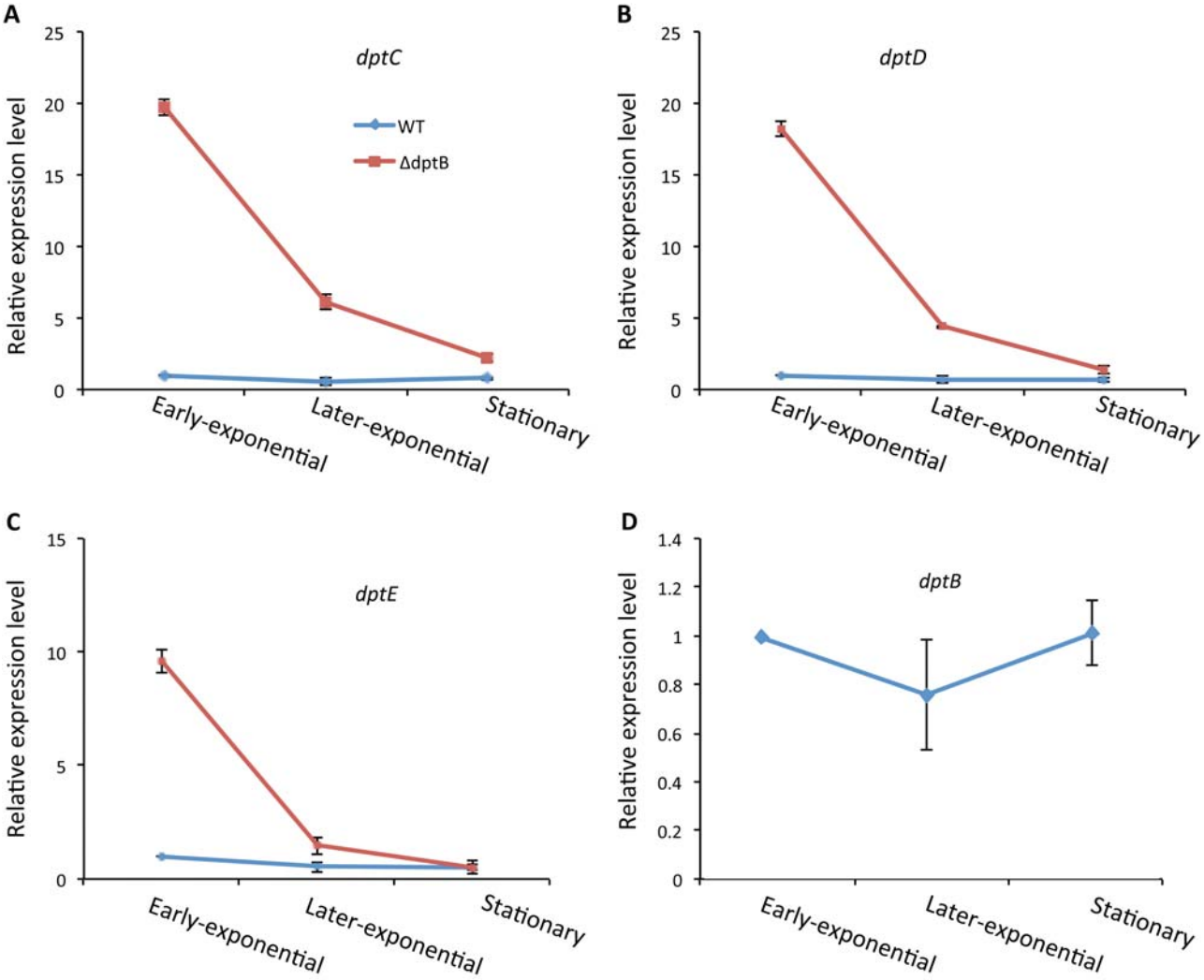

Fig. 2. Quantitative real-time PCR analysis of the transcription of *dptC* (A), *dptD* (B), *dptE* (C), *dptB* (D) in wild-type and Δ*dptB* mutant strains of *S. enterica* serovar Cerro 87.

promoter upstream of *dptB*, thus forming an operon (*dpt* operon) (Fig. S3). Subsequent quantitative real-time PCR revealed that all of these four *dpt* genes maintained similar transcriptional level across all three growth phases in the wild-type strain (Fig. 2). However, in Δ*dptB*, a significantly higher level (10- to 20-fold) of *dptCDE* transcription was found at its early-exponential phase (Fig. 2), which was in agreement with its higher *in vivo* PT modification frequencies. Complementation of Δ*dptB* mutant with plasmid carrying the intact *dptB* and its own promoter region resulted in about 10-fold decrease in the transcription of *dpt* operon and 1/3 decrease in PT modifications at its early-exponential phase (Fig. S4). These results clearly suggested that DptB functioned as a negative transcriptional regulator to control the transcription of *dpt* operon. Unexpectedly, although the PT modification frequency was maintained at constant, higher level in Δ*dptB* mutant than in the wild-type strain throughout the growth phases, the transcription of *dptCDE* genes in Δ*dptB* mutant decreased dramatically to a similar level to that in the wild-type strain at the late-exponential phase (Fig. 2).

DptB binds to two regions in the upstream of dpt *operon*

His_6-tagged DptB of *S. enterica* serovar Cerro 87 was produced in *E. coli* BL21(DE3) and purified by Ni-chelating chromatography (Fig. S5A). Gel filtration chromatography analysis revealed DptB protein with a molecular weight of ~ 80 kDa (Fig. S5B). Considering the ~ 40 kDa molecular mass analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. S5A), DptB protein appeared to be in a dimer conformation. To test whether DptB was able to directly bind to the promoter region of *dpt* operon, the purified DptB and a 393-bp DNA fragment from

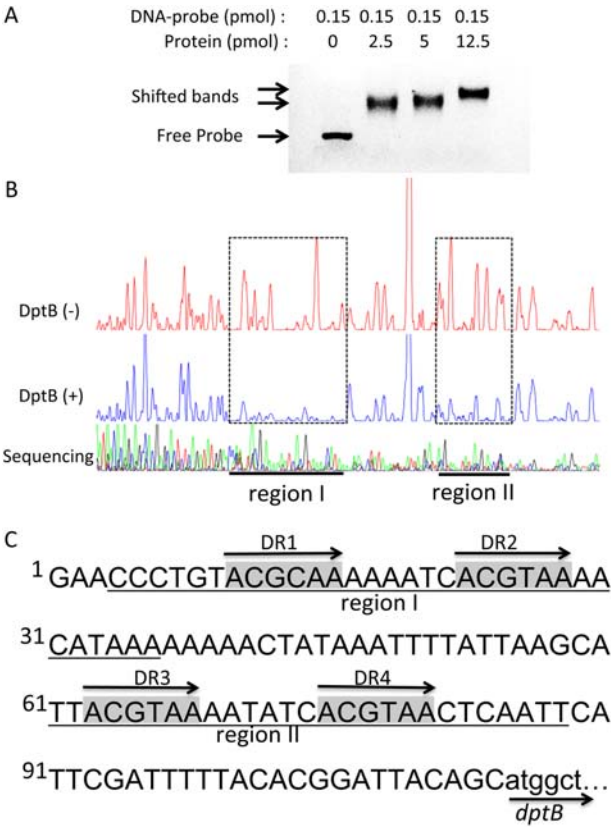

Fig. 3. Characterization of the DptB-binding region in the *dptBCDE* promoter.
A. EMSA is performed with His-tagged DptB protein and FAM-labeled *dptBCDE* promoter region. Sheared salmon sperm DNA is added to prevent non-specific binding.
B. DNase I footprinting analysis of the DptB binding to the *dptBCDE* promoter region. The DNA fragment from the *dptBCDE* promoter region is labeled with FAM dye on the sense strand, incubated with DptB (blue line) or without DptB (red line). DNA sequencing of the promoter region is shown at the bottom. The regions (regions I and II) protected by DptB from DNase I cleavage is indicated with dashed black boxes.
C. The DNA sequence of the *dptBCDE* promoter region. DptB protected sequences are labeled with underlines for sense strand. Conserved direct repeats (DR1–DR4) are indicated with gray backgrounds and black arrows.

the upstream region of *dpt* operon were employed for the electrophoretic mobility shift assay (EMSA). As shown in Fig. 3A, DptB bound to this region in a concentration-dependent manner and shifted bands were observed with the increase in DptB. DNase I footprinting assay was subsequently applied to determine the accurate binding sequence of DptB within the *dpt* upstream region. Consistent with the EMSA results, two separate regions (regions I and II; Fig. 3B, Fig. S6) were identified to be protected by DptB from DNase I digestion, employing either labeled sense DNA strand (Fig. 3B) or labeled antisense of DNA strand (Fig. S6). Sequence analysis of regions I and II revealed that each region contained a pair of imperfect 6 nt direct repeats, termed as DR1, DR2 in region I and DR3, DR4 in region II (ACGTAA for DR2-4, ACGCAA for DR1; Fig. 3C), which were separated by 6 nt, suggesting the DptB-binding motif as 'DR–N_6–DR'. Considering the dimer conformation of DptB, each of these two regions is probably bound by one subunit of DptB protein.

The DRs are recognition sequence by regulator DptB

To test this hypothesis, individual mutagenesis of these four direct repeats (DRs) in *dpt* promoter was performed (Fig. 4A), and the mutated promoters were then used to test the DptB-binding activities. As shown in Fig. 4B, the overall signals of the DR sites were weak probably due to the DNA secondary structures in the mutated promoter regions (Koo and Crothers, 1988; Yoon *et al.*, 2010). To facilitate the comparison between the mutated and the wild-type sites, signals of the DR sites were highlighted (Fig. 4B). As predicted, all mutated DRs showed obviously reduced binding affinities by DptB, thus suggesting each DR as a basic unit for DptB binding.

Discussion

DNA molecules are made up of four nucleotides of A, T, C and G, which are linear polymers with the phosphodiester bond connected to each other. Epigenetic modifications on DNA have been found to play important roles in cellular physiologies without alterations to the nucleic acid sequence. The most well studied DNA modifications are methylation on the bases, such as m^6A, m^4C, m^5C, which often regulate gene expression or participate the R–M system (Murphy *et al.*, 2013; Loenen and Raleigh, 2014). As a novel epigenetic modification on DNA backbone, PT modifications exist in many bacteria and archaea with a highly partial modification phenomenon in short consensus sequences, despite the presence of R–M system (Cao *et al.*, 2014a,b). This unusual PT-dependent R–M raises a question about the regulation of low frequency of PT modification of consensus sequences and its relationship with the restriction activity. Here, we focus on the modification frequency.

Our recent *in vitro* study showed that PT-modifying enzymes from *S. enterica* serovar Cerro 87 were able to modify any GAAT/GTTC sites, even though PT occurred in only a small fraction of these sites, suggesting a regulation of PT levels *in vivo* by controlling the concentration of PT-modifying enzymes instead of the sequence recognition. In this study, we demonstrated that PT modification in *S. enterica* serovar Cerro 87 was regulated by DptB through depressing the transcription of the DNA PT-modifying gene cluster, *dptBCDE* operon. Under the negative regulation by DptB, the transcription of *dpt* operon was found to be stringently controlled and thus PT modification remained in an appropriate level. *In vitro*, DptB

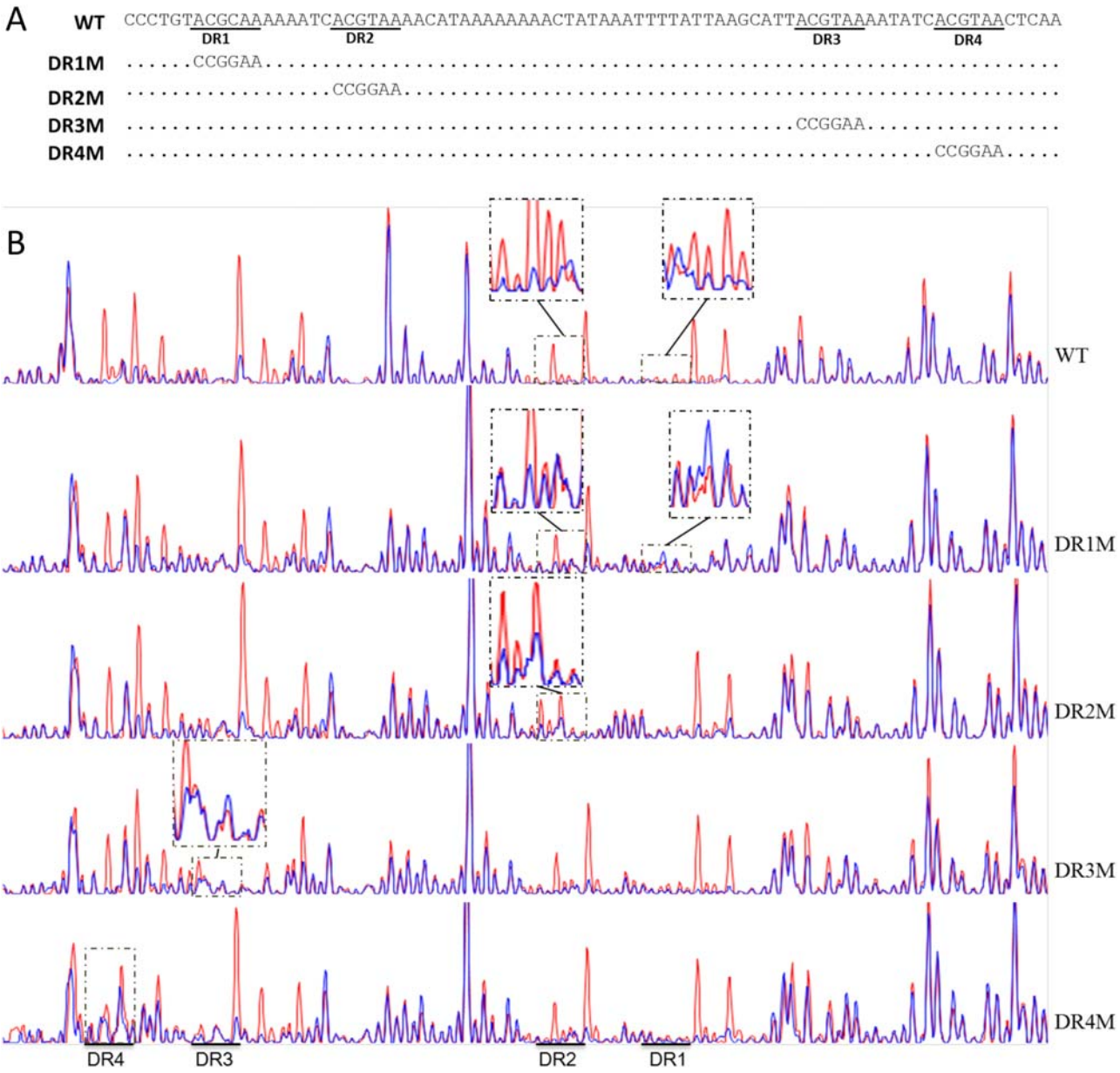

Fig. 4. Mutagenesis analysis of the DptB-binding sites.
A. Site-directed mutagenesis of the DRs. Mutated sequences of DR1M–DR4M are indicated.
B. Effects of mutated DR1–DR4 on DNase I footprinting of DptB. The DNA fragment from the mutated DR1–DR4 are labeled with FAM dye on the antisense strand, incubated with 25 pmol DptB. The mutated region is indicated with dashed boxes. The DNA fragment from wild-type *dptBCDE* promoter is used as a positive control (WT). For each probe, red line (DptB–) and blue line (DptB+) are merged and regions with different peak values showed the DptB-protected sequences. The DR1-4 sites are indicated with dashed black boxes. To facilitate the comparison between the mutated and the wild-type sites, signals from DRs are highlighted in dashed boxes.

migrated as a dimer in gel filtration columns and bound to two regions of the promoter of *dpt* operon. We infer that, *in vivo*, this controls the transcription of *dptBCDE* and prevents the accumulation of large amounts of PT modification enzymes (Fig. 5). Unexpectedly, we found that a 100-fold increase in *dptB* transcription (Fig. S7A) led only to a 10% decrease in *dpt*C/D/E transcription (Fig. S7B) and a slight decrease (~ 5%) of PT modification in the cell (Fig. S7C). It is possible that the basal transcription of *dptB* in wide-type strain is sufficient to repress the *dpt* genes transcription and to maintain the PT modification level in the cell. Interestingly, the transcription of the *dpt* operon in the Δ*dptB* mutant increased in early-exponential phase, but then decreased to a level similar to that in the wild-type strain in late-exponential phase. There are several possible explanations for this instability. First, it is possible that the

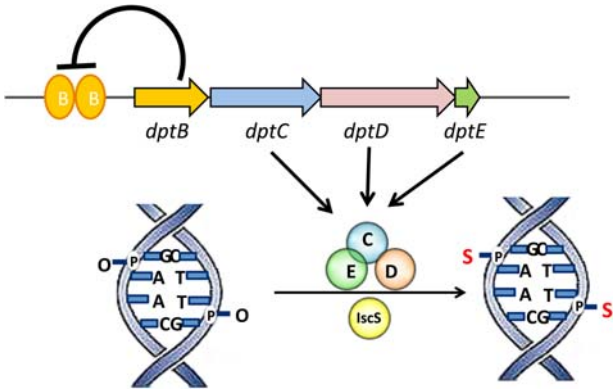

Fig. 5. Schematics of DptB-mediated regulation of PT modification.

decrease in *dpt* transcripts detected by quantitative real-time PCR is the result of decreased mRNA stability in the late growth phase. It is also possible that accumulating PT modifying proteins and/or PT modifications provide feedback inhibition on *dpt* gene transcription. Finally, the transcription of *dpt* genes could be affected by bacterial growth factors in the mutant, such as Rpos (Hengge-Aronis, 2002). Indeed, the levels of both *dpt* gene transcription and PT modifications remain relatively constant in the wild-type strain throughout its growth cycle, while the absence of *dptB* results in a growth-dependent increase in *dpt* gene transcription and increased level of PT modifications. This would suggest a more complicated mechanism controlling PT modifications than DptB simply acting as a negative transcriptional regulator.

This study reveals a DptB-mediated negative regulation of the bacterial PT modification frequency, but it is still unknown that whether DptB responds to a cellular signal. One possibility is that DptB is feedback controlled by the PT modification level within the cell. To test this hypothesis, we constructed a PT-lacking mutant with *dptE* disruption (Fig. S8) and examined whether the absence of PT led to increase in the transcription of the *dpt* operon. The result of real-time PCR showed that the genes *dptB*/*C*/*D* in the Δ*dptE* mutant presented a similar transcription level with that in the wide-type strain (data not shown), indicating that the regulatory activities of DptB are not likely to be controlled by the PT modification level within the cell.

This regulation of PT modification is probably essential for its physiological function. Similar to the traditional R–M systems, bacteria adopt the PT-dependent R–M system to distinguish and prevent invasion by foreign DNAs. However, our previous work showed several unusual features of PT R–M system distinct from traditional ones (Cao *et al.*, 2014b). In addition, the frequency of PT modification also significantly affected the protecting efficiency of this restriction system. When using the efficiency of transformation (e.o.t.) of unmodified DNAs *versus* PT-modified DNAs into the competent cells, we found that the e.o.t. was 0.0098 ± 0.0026 in the wild-type strain and 1.03 ± 0.13 in the R–M minus strain (Δ*dptBCDEFGH*). Interestingly, the approximately twofold increase in PT modification in the Δ*dptB* mutant results in a higher e.o.t. of unmodified DNA than the wild-type strain. One explanation for this lower restriction efficiency is likely to be that: when the concentration of PT modification enzymes is not under control by DptB, the excessive enzymes increase the PT modification sites of the host DNA; on the other hand, the invading foreign DNA is also modified by the excessive modification enzymes so that restriction sites are protected, resulting in an escape of restriction.

The *dptB* homologs exist in almost all of PT modification gene clusters among bacteria, suggested widespread of regulation of PT modifications by *dptB*-like negative regulators. In this study, DptB has been confirmed to bind to the four DRs in its promoter region. Sequence analysis of homologs of the *dpt* promoter reveals a wide distribution of DRs and some bacteria even harbored the same DR–N_6–DR sequences (Fig. 3C), *e.g.* several *Salmonella* strains, several *E. coli* strains and *Cedecea neteri* ND14a. Meanwhile, imperfect DRs exist in some bacteria containing more diverged DptB, *e.g.* only one conserved DR–N_6–DR motif and a single DR were found in *Photorhabdus luminescens* LN2 and *Alteromonas australica* H17 (Fig. S9A). In addition, with a phylogenetic analysis of the DptB protein sequences, a strong correlation was found between the DptB protein sequence phylogeny and the distribution of DptB-binding motif in the promoter regions (Fig. S9B). Since the frequencies and sequence specificity of PT modification vary among bacterial strains, *e.g.* from ~ 280 $G_{ps}A$ per 10^6 nt in *H. chejuensis* to ~ 3000 $C_{ps}C$ per 10^6 nt in *Vibrio* (Wang *et al.*, 2011), it is possible that the binding activity of DptB-like regulators to the promoter regions controls the modification frequency that is essential for the physiological functions of PT.

Experimental procedures

Materials, bacterial strains and culturing conditions

Enantiomerically pure d($G_{ps}A$) and d($G_{ps}T$) in R_p and S_p configuration were obtained from Sangon Biotech Co. Ltd. (Shanghai). The following kits and reagents were purchased from New England BioLabs (Ipswich, MA): Phosphatase, Quick Blunting Kit, Quick Ligation Kit, Klenow Fragment (3′→5′exo-) and dATP solution. Custom oligodeoxynucleotides were ordered from Sangon Biotech Co. Ltd. (Shanghai) (sequences shown in Supplementary Table S1). Iodine and Na_2HPO_4 were purchased from Sigma-Aldrich (St. Louis, MO).

Strains used in this study, *S. enterica* serovar Cerro 87, and its derivative XTG102 were constructed by this laboratory and described in our previous work (Xu *et al.*, 2010). Cells were grown at 37°C on Luria–Bertani (LB) plate or in LB liquid medium supplemented with appropriate antibiotics. To

obtain cells from different growth phases, overnight grown bacterial cultures were diluted into 5 ml of LB medium at a concentration of 5000 cells ml^{-1} and the cell growth was monitored by the optical density 600 nm (OD_{600}). The cells reached OD_{600} of 0.8, 2.0 and 3.5, which were considered to be at their early-exponential, late-exponential and stationary phases, respectively, were harvested for further analyses.

Construction of the ΔdptB *and* ΔdptE *mutants in* S. enterica *serovar Cerro 87*

The *ΔdptB* and *ΔdptE* mutants were constructed by homologous recombination using the thermo- and sucrose-sensitive plasmid pKOV-Kan (Lalioti and Heath, 2001). Plasmid pJTU1238 (Wang *et al*., 2007) was used as a template to amplify the left and right arms of *dptB* and *dptE*, with primers for the left arm (SdndBLL and SdndBLR for *dptB*; SdndELL and SdndELR for *dptE*) and right arm (SdndBRL and SdndBRR for *dptB*; SdndERL and SdndERR for *dptE*) (primer sequence in Supplementary Table S1 with *Bam*HI and *Sal*I sites underlined). With a 40 nt overlapping, the left and right arms were amplified together with primers (SdndBLL and SdndBRR for *dptB*; SdndELL and SdndERR for *dptE*) and the resulted recombinant fragment was introduced with *Bam*HI and *Sal*I sites at the termini. The entire fragment was then cloned into *Bam*HI and *Sal*I site of the plasmid pKOV-Kan, replacing the original *dptB* or *dptE* gene on the plasmid and generating pJTU3846 and pJTU5802, respectively. Plasmid was then introduced into *E. coli* DH10B (pJTU1238) to allow for phosphorothioation of pJTU3846 or pJTU5802DNA, which was then introduced into *S. enterica* serovar Cerro 87. The transformant was first grown at 30°C and then the temperature was elevated to 43°C to select the single crossover intermediate. Finally, 15% sucrose of final concentration was used to select the double crossover (Δ*dptB* or Δ*dptE*) at 43°C, which was verified with PCR and DNA sequencing using primers (BT-f and BT-r for *dptB*; ET-f and ET-r for *dptE*) (primer sequences in Supplementary Table S1) (Figs. S1 and S2).

Complementation and overexpression of dptB

To complement the Δ*dptB* mutant YF10, plasmid pBluScript SK (+) containing the complete *dptB* gene under the control of its own promoter was used. The gene *dptB* under its own promoter was amplified from *S. enterica serovar* Cerro 87 using the primers dndB-conmF/R (Table S1). The PCR product was purified, cloned into pBluScript SK (+) vector and verified by DNA sequencing. Then, it was introduced into YF10 for complementation of *dptB* or into *S. enterica serovar* Cerro 87 for overexpression of *dptB*. In both cases, the corresponding strains containing vector pBluScript SK (+) were used as a control for RT-PCR or PT analysis.

Iodine cleavage at genomic PT sites

A 30 mM iodine solution in ethanol was freshly prepared and reactions were then setup in a 20 μl system in PCR tubes as follows: 2 μg genomic DNA (gDNA), 50 mM Na_2HPO_4 (pH 9.0) and 3 mM I_2. Reactions were heated to 65°C for 15 min and then slowly cooled down to 4°C with the rate of 0.1°C s^{-1}. PT modifications of gDNAs from *S. enterica* serovar Cerro 87 (wild type), YF10 (*ΔdptB*) and XTG102 (*ΔdptB-E*) were subject to iodine cleavage and samples were run on a 0.7% agarose gel buffered with 0.5 × Tris–acetate–EDTA buffer.

Quantitative determination of PT modification in DNA

Phosphorothioate modifications in *S. enterica* serovar Cerro 87 and YF10 were quantified by LC-coupled, time-of-flight mass spectrometry. The hydrolyzation of gDNA and sample preparation were the same as previously described (Wang *et al*., 2011). The digestion mixture containing PT dinucleotides was resolved on a Poroshell120 SB-AQ column (3.0 × 150 mm, 2.7 μm) with a flow rate of 0.4 ml min^{-1} with the following parameters: column temperature: 35°C; solvent A: 0.2% ammonium acetate; solvent B: 0.2% ammonium acetate in acetonitrile; gradient: 5% B for 1 min, 5–20% B over 20 min, and 20–95% B over 1 min. The high-performance LC (HPLC) column was then coupled to an Agilent 6410 Triple Quad LC–MS spectrometer with an electrospray ionization source in positive mode with the following parameters: gas flow, 10 l min^{-1}; nebulizer pressure, 30 psi; drying gas temperature, 325°C and capillary voltage, 3100 V. Multiple reaction monitoring mode was used for detection of product ions derived from the precursor ions, with the optimized retention time in min: $d(G_{ps}A)$, 13.19; $d(G_{ps}T)$, 13.6; $d(C_{ps}G)$, 6.54; $d(C_{ps}C)$, 3.88; $d(G_{ps}G)$, 11.77; $d(C_{ps}A)$, 8.68; $d(C_{ps}T)$, 8.83; $d(A_{ps}G)$, 18.8; $d(T_{ps}G)$, 15.2; $d(G_{ps}C)$, 8.91; $d(A_{ps}A)$, 16.02; $d(T_{ps}A)$, 15.78; $d(A_{ps}C)$, 11.49; $d(T_{ps}C)$, 10.37; $d(A_{ps}T)$, 17.4; and $d(T_{ps}T)$, 17.85. Other instrument parameters, including precursor ion m/z, product ion m/z, fragmentor voltage and collision energy, were the same as previously described (Wang *et al*., 2011).

RNA preparation and real-time PCR

Total RNA was isolated with usage of a Qiagen RNeasy Protect Bacteria Mini Kit, following the manufacturer's protocol. To synthesize cDNA, 2 μg of purified total RNA was amplified in a 20 μl reaction volume using RevertAid™ H Minus Reverse Transcriptase and Random Hexamer Primer (Thermo). cDNA (25 ng) was used as the template for real-time PCR analysis using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo) and an Applied Biosystems 7500 fast real-time PCR system. The 16S rRNA was used as the internal reference. Primers used were shown in Supplementary Table S1. Comparative Ct method was used and the relative transcription of each target gene was shown as the ratio of the samples in different growth phases to early-exponential phase of the wild type, which was assigned a value of 1.0 for each gene. All the quantitative real-time PCR assays were carried out using triplicate independent cultures.

Production of recombinant DptB

The *dptB* gene was PCR amplified with dptB-f and dptB-r and inserted into the N-terminal His6-tagged expression vector pET-28a. The *dptB* expression plasmid was transformed into

E. coli BL21(DE3) for production of DptB protein. Transformants were grown at 37°C in 1 l of LB medium containing selective antibiotics (50 mg ml^{-1}, kanamycin for pET-28a) until the OD_{600} reached 0.8–1.0. Cells were then induced with isopropyld–thiogalactoside (0.6 mM) and allowed to continue to grow at 16°C for another 12 h before cells being harvested by centrifugation. Cells were re-suspended in 40 ml of lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole) and then sonicated on ice. Soluble fraction was collected by centrifugation (15,000 *g*, 30 min at 4°C) and applied onto a HisTrap HP column (GE Healthcare, 1 ml). Proteins were eluted with a linear gradient of buffer B (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 500 mM imidazole) with an AKTA fast protein LC system (GE Healthcare). Eluted fractions were analyzed by SDS–PAGE and His-tagged DptB was dialyzed in the desalting buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl).

EMSA

The promoter region of the *dpt* operon was PCR amplified employing primer pair of s87-1001F and s87-1393R (Table S1). Labeling of the probe with 6-carboxyfluorescein (FAM) was conducted through a second round of PCR employing M13R-FAM and M13F-47. The binding of His-tagged DptB to labeled probes was performed at room temperature in a total volume of 20 μl containing 10 mM Tris–HCl (pH 8.0), 25 mM KCl, 2.5 mM $MgCl_2$, and 1.0 mM dithiothreitol. To prevent nonspecific binding, sheared salmon sperm DNA was added to a final concentration of 100 ng μl^{-1}. After 20 min of incubation, the fragments were separated by a 2% agarose gel buffered with 0.5 × Tris-borate-EDTA buffer. Gels were scanned with the ImageQuant™ LAS 4000 mini (GE Healthcare).

DNase I footprinting assay

The DNase I footprinting experiments were carried out using FAM-labeled probes, following the protocol previously described by Wang *et al*. (2012). Labeling of the probes was the same as that described in EMSA. Purification of the probe was performed using the Wizard SV Gel and PCR Clean-Up system (Promega). About 250 ng of probe was incubated with 2.5–25 pmol DptB protein in a total volume of 40 μl in the same buffer as EMSA described earlier. After 30 min incubation at room temperature, 10 μl solution containing 0.015 units DNase I (Promega) and 100 nmol freshly prepared $CaCl_2$ was added, followed by 1 min incubation. Reaction was stopped by addition of 140 μl stop solution containing 200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% SDS (Le *et al*., 2011). Phenol/chloroform extraction was adopted to remove the proteins before the digested DNA was precipitated with ethanol. Pellets were dissolved in 10 μl MiniQ water for further analysis. The fmol DNA Cylce Sequencing System (Promega) was used for preparation of the DNA sequencing ladder, using the FAM-labeled primer of M13R and following the manufacturer's instructions. The sequencing samples were precipitated with ethanol and dissolved in 5 μl Mini-Q water. For the digested DNA fragments and the sequencing products, 1 μl of each sample was added to 8.5 μl HiDi formamide and 0.5 μl GeneScan-LIZ500 size standard (Applied Biosystems), and the mixture was then analyzed with 3130 DNA Analyzer and Peak Scanner software v1.0 (Applied Biosystems).

The promoter region of *dpt* was cloned into the pMD18-T vector (Takara), which was subsequently used as the template for site-directed mutagenesis of DR sites with primer pairs of box1-F/-R, box2-F/R, box3-F/-R and box4-F/-R. Obtained plasmids were then used as templates for preparation of mutated probes for DNase I footprinting assay, using two rounds of amplification the same as described earlier. Following procedures for DNase I footprinting assay of the mutated probes was the same as that for the wild type.

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