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

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Summary

DNA phosphorothioate (PT) modi“cations, with one non-bridging phosphate oxygen replaced with sulfur, are widely but sporadically distributed in prokaryotic genomes. Short consensus sequences surround the modi“ed linkage in each strain, although each site is only partially modi“ed. The mechanism that maintains this low-frequency modi“cation status is still unknown. In Salmonella enterica serovar Cerro 87, PT modi“cation is mediated by a four-gene cluster called dptBCDE . Here, we found that deletion of dptB led to a signi“cant increase in intracellular PT modi“cation 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 modi“cation. 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 “ndings demonstrate that PT modi“cation is regulated at least in part at the transcriptional level.

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Introduction

DNA phosphorothioate (PT) modi“cation, with sulfur replacing one nonbridging phosphate oxygen in a sequence- and stereo-speci“c manner (Wang et al., 2007; 2011), is involved in a restriction…modi“cation (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 “ve genes, are responsible for PT modi“cation (Zhou et al., 1988; 2005). So far, PT modi“cations 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 modi“cations. DndA acts as a cysteine desulphurase and assembles the 4Fe…4S cluster of DndC (Youet 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 ”uorescens 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 modi“caton. In E. coli B7A, the double-stranded PT modi-“cations occured in $G_{ps}AAC/G_{ps}TTC$ motifs, but only 12% of these consensus sequences are modi“ed (Cao et al., 2014a). Considering the fact that a PT-dependent restriction system is present in E. coli B7A, the partial modi“cation of consensus sequences suggests a regulation of PT modi“cation frequency. Previous observation that deletion of dndB in S. lividans led to enhanced levels of DNA

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

In S. enterica serovar Cerro 87, dptBCDE gene and iscS are responsible for PT modi“cation 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 modi“cation frequencies.

Results

Disruption of dptB increases the level of in vivo PT modi“cation

In order to study the role of dptB in PT modi“cation, we constructed its in-frame deletion mutant (dptB, Fig. S1) in S. enterica serovar Cerro 87. DNA PT modi“cations were then quantitatively analyzed in both the wild-type strain and dptB mutant. By using the iodine-induced PT-speci“c cleavage assay, which was developed in our previous study (Cao et al., 2014a), a signi“cant increase in the cleavage efficiencies was found in the genomic DNA of dptB in comparison with that of the wild-type strain (Fig. 1). This observation is similar to the previous obser-vation 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 modi“cations. To veri“y this interpretation, the PT-linked dinucleotides were then quanti“ed in 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 speci“city of PT modi“cation, 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 modi“cation occurred at the $G_{ps}A$ and $G_{ps}T$ sites in dptB mutant (Fig. S2), suggesting that dptB deletion did not alter the sequence speci“city of PT modi“cation. However, PT modi“cations of 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 modi“cation were relatively stable throughout the different growth phases in both strains (Fig. 1C).

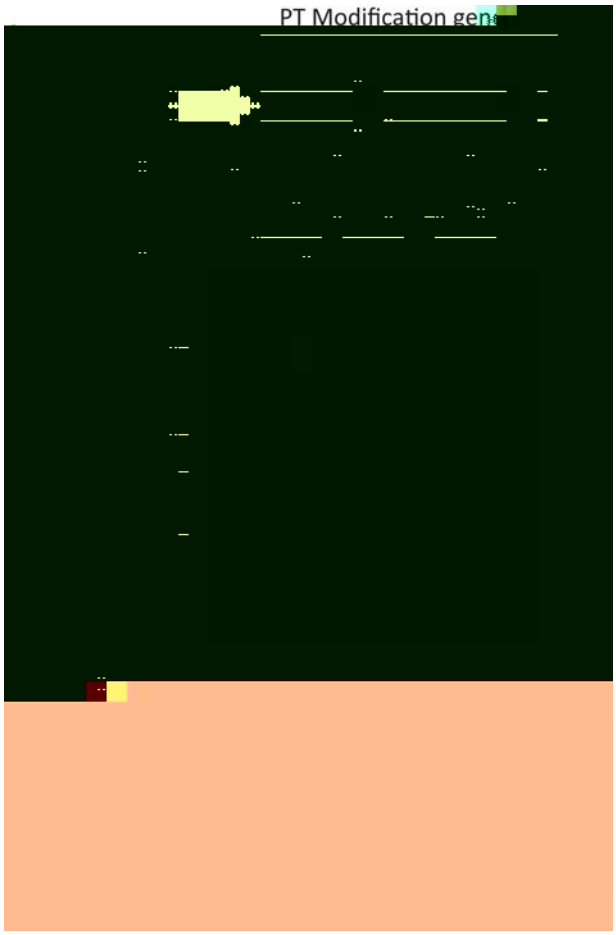

Fig. 1. The organization of the PT modifying genes and quantitative analysis of PT modi“cations.
A. The organization of the PT modifying genes of S. enterica serovar Cerro 87.
B. Iodine cleavage of genomic DNA from wild-type, dptB and dptBCDE mutant strain of S. enterica serovar Cerro 87.
C. PT modi“cations ($G_{ps}A$ and $G_{ps}T$) per 10^6 nt in wild-type and 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 modi-“cation observed earlier is that deletion of DptB relieves repression of the expression or activity of the enzymes that insert the modi“cation. 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 modi“cation in 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 con“rmed that dptB, C, D and E genes were co-transcribed from the same

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 dptB, a sig-ni“cantly 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 modi-“cation frequencies. Complementation of 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 modi“cations 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 modi“cation fre-

dptCDE

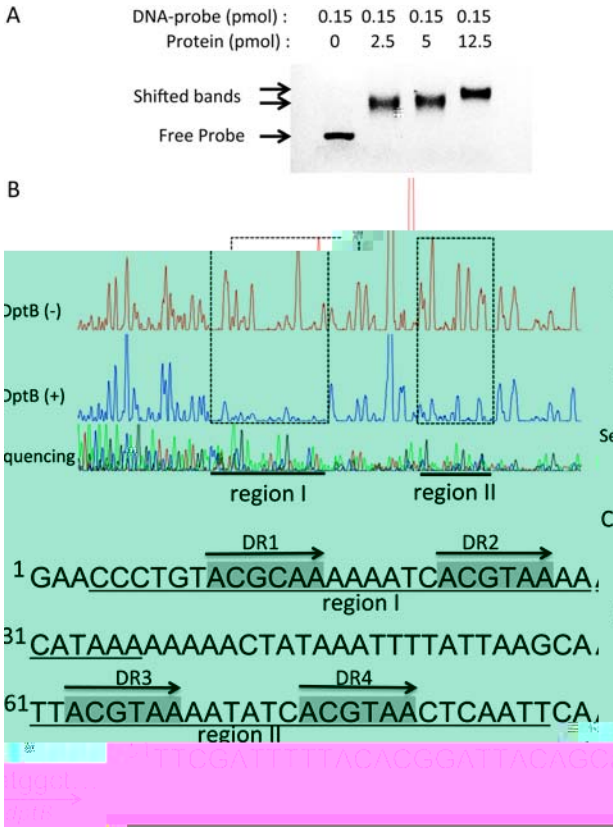

Fig. 3. Characterization of the DptB-binding region in the dptBCDE promoter.
A. EMSA is performed with His-tagged DptB protein and FAM-labeled dptBCDE promoter region. Sheared salmon sperm DNA is added to prevent non-speci“c binding.
B. DNase I footprinting analysis of the DptB binding to the dptBCDE 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 identi“ed 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 modi“cations on DNA have been found to play important roles in cellular physiologies without alterations to the nucleic acid sequence. The most well studied DNA modi“cations are methylation on the bases, such as m^{6}A, m^{4}C, m^{5}C, which often regulate gene expression or participate the R…M system (Murphy et al., 2013; Loenen and Raleigh, 2014). As a novel epigenetic modi“cation on DNA backbone, PT modi“cations exist in many bacteria and archaea with a highly partial modi“cation 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 modi“cation of consensus sequences and its relationship with the restriction activity. Here, we focus on the modi“cation 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 modi“cation 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 modi“cation remained in an appropriate level. In vitro, DptB

Fig. 4.

migrated as a dimer in gel “ltration 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 modi“cation enzymes (Fig. 5). Unexpectedly, we found that a 100-fold increase in dptB transcription (Fig. S7A) led only to a 10% decrease in dptC/D/E transcription (Fig. S7B) and a slight decrease (5%) of PT modi“cation 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 modi“cation level in the cell. Interestingly, the transcription of the dpt operon in the 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

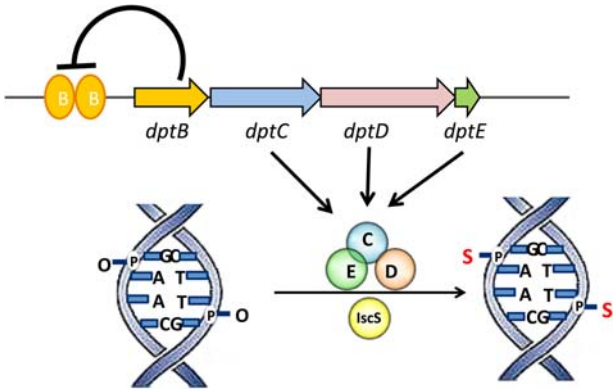

Fig. 5. Schematics of DptB-mediated regulation of PT modi“cation.

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 modi“cations 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 modi“cations 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 modi“cations. This would suggest a more complicated mechanism controlling PT modi“cations than DptB simply acting as a negative transcriptional regulator.

This study reveals a DptB-mediated negative regulation of the bacterial PT modi“cation 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 modi“cation 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 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 modi“cation level within the cell.

This regulation of PT modi“cation 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 modi“cation also signi“cantly affected the protecting efficiency of this restriction system. When using the efficiency of transformation (e.o.t.) of unmodi“ed DNAs versus PT-modi“ed 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 (dptBCDEFGH). Interestingly, the approximately twofold increase in PT modi“cation in the dptB mutant results in a higher e.o.t. of unmodi“ed DNA than the wild-type strain. One explanation for this lower restriction efficiency is likely to be that: when the concentration of PT modi“cation enzymes is not under control by DptB, the excessive enzymes increase the PT modi“cation sites of the host DNA; on the other hand, the invading foreign DNA is also modi“ed by the excessive modi“cation enzymes so that restriction sites are protected, resulting in an escape of restriction.

The dptB homologs exist in almost all of PT modi“cation gene clusters among bacteria, suggested widespread of regulation of PT modi“cations by dptB-like negative regulators. In this study, DptB has been con“rmed 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$_8$…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$_8$…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 speci“city of PT modi“cation 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 modi“cation 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 con“guration 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 dptB and dptE 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 BamHI and SalI sites underlined). With a 40 nt overlapping, the left and right arms were ampli“ed together with primers (SdndBLL and SdndBRR for dptB; SdndELL and SdndERR for dptE) and the resulted recombinant fragment was introduced with BamHI and SalI sites at the termini. The entire fragment was then cloned into BamHI and SalI 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 “rst grown at 30°C and then the temperature was elevated to 43°C to select the single crossover intermediate. Finally, 15% sucrose of “nal concentration was used to select the double crossover (dptB or dptE) at 43°C, which was veri“ed 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 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 ampli“ed from S. enterica serovar Cerro 87 using the primers dndB-conmF/R (Table S1). The PCR product was puri“ed, cloned into pBluScript SK (+) vector and veri“ed 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 l system in PCR tubes as follows: 2 g 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 modi“cations 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 modi“cation in DNA

Phosphorothioate modi“cations in S. enterica serovar Cerro 87 and YF10 were quanti“ed by LC-coupled, time-of-”ight 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 m) with a ”ow 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 ”ow, 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 g of puri“ed total RNA was ampli“ed in a 20 l 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 ampli“ed 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 ampli“ed employing primer pair of s87-1001F and s87-1393R (Table S1). Labeling of the probe with 6-carboxy”uorescein (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 l containing 10 mM Tris…HCl (pH 8.0), 25 mM KCl, 2.5 mM $MgCl_2$, and 1.0 mM dithiothreitol. To prevent nonspeci“c binding, sheared salmon sperm DNA was added to a “nal concentration of 100 ng l ^{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. Puri“cation 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 l in the same buffer as EMSA described earlier. After 30 min incubation at room temperature, 10 l 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 l 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 l 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 l Mini-Q water. For the digested DNA fragments and the sequencing products, 1 l of each sample was added to 8.5 l HiDi formamide and 0.5 l 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 ampli“cation 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.

Acknowledgements

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