Regulation of DNA phosphorothioate modifications by the transcrip
tional regulator DptB in Salmonella

Qiuxiang Cheng,¹ Bo Cao,¹ Fen Yao,¹ Jinli Li,¹ Zixin Deng¹ and Delin You¹,²*
¹State Key Laboratory of Microbial Metabolism, and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China.
²Joint International Research Laboratory of Metabolic and Developmental Sciences, Shanghai Jiao Tong University, Shanghai 200240, China.

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 during the process of sulfur incorporation (Yao 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 P0-1, 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).

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 P0-1, 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 modification. In E. coli B7A, the double-stranded PT modifications occurred in GpaAAC/GpaTTTC 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 GpsA/GpsT/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 phenotype (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 verify 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 GpsA and GpsT sites in Δ*dptB* mutant (Fig. S2), suggesting that *dptB* deletion did not alter the sequence specificity of PT modification. However, PT modifications ofΔ*dptB* mutant in the GpsT and GpsA sites increased about twofold in comparison with the wild-type strain, i.e. ~1200 GpsA and GpsT 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).

**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
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 ΔdptB, 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 ΔdptB 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 ΔdptB mutant than in the wild-type strain throughout the growth phases, the transcription of dptCDE genes in ΔdptB 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

His6-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

© 2015 John Wiley & Sons Ltd, Molecular Microbiology, 97, 1186–1194
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 dptBCDE promoter region. Consistent with the EMSA results, two separate regions (regions I and II) protected by DptB from DNase I cleavage is indicated with dashed black boxes.

The DRs are recognition sequence by regulator DptB

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.

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 m5A, m5C, m6C, 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 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.
migrated as a dimer in gel filtration columns and bound to two regions of the promoter of \textit{dpt} operon. We infer that, in vivo, this controls the transcription of \textit{dpt}BCDE and prevents the accumulation of large amounts of PT modification enzymes (Fig. 5). Unexpectedly, we found that a 100-fold increase in \textit{dpt}B transcription (Fig. S7A) led only to a 10\% decrease in \textit{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 \textit{dpt}B in wide-type strain is sufficient to repress the \textit{dpt} genes transcription and to maintain the PT modification level in the cell. Interestingly, the transcription of the \textit{dpt} operon in the \textit{ΔdptB} 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
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 dptBCID in the ΔdptE 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 DNA. 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 DNA vs.

![Fig. 5. Schematics of DptB-mediated regulation of PT modification.](image-url)
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 \(\Delta dptB\) and \(\Delta dptE\) mutants in \textit{S. enterica serovar Cerro 87}**

The \(\Delta dptB\) and \(\Delta dptE\) 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 SdndDDR for \(dptE\)) (primer sequences in Supplementary Table S1 with BamHI and SalI sites underlined). With a 40 nt overlapping, the left and right arms were amplified together with primers (SdndBLL and SdndBLR for \(dptB\); SdndELL and SdndERL for \(dptE\)) and the resulting 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 \textit{E. coli} DH10B (pJTU1238) to allow for phosphorothioation of pJTU3846 or pJTU5802DNA, which was then introduced into \textit{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 (\(\Delta dptB\) or \(\Delta dptE\)) at 43°C, which was verified with PCR and DNA sequencing using primers (\(B\)T-f and \(B\)T-r for \(dptB\); \(E\)T-f and \(E\)T-r for \(dptE\)) (primer sequences in Supplementary Table S1) (Figs. S1 and S2).

**Complementation and overexpression of \(dptB\)**

To complement the \(\Delta dptB\) 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 \textit{S. enterica serovar Cerro 87} using the primers dndB-commF/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 \textit{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 \(\mu\)l system in PCR tubes as follows: 2 \(\mu\)g genomic DNA (gDNA), 50 mM Na\(_2\)HPO\(_4\) (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 \textit{S. enterica serovar Cerro 87} (wild type), YF10 (\(\Delta dptB\)) and XTG102 (\(\Delta dptB\)-E) were subject to iodine cleavage and samples were run on a 0.7% agarose gel buffered with 0.5 \(\times\) Tris–acetate–EDTA buffer.

**Quantitative determination of PT modification in DNA**

Phosphorothiate modifications in \textit{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 \(\times\) 150 mm, 2.7 \(\mu\)m) 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(\(G\)C\(_2\)A, 13.19; d(\(G\)A\(_2\)T, 13.6; d(\(C\)G\(_2\)C, 6.54; d(\(G\)G\(_2\)C, 3.88; d(\(G\)C\(_2\), 11.77; d(\(C\)C\(_2\), 8.68; d(\(C\)T\(_2\), 8.83; d(\(C\)G\(_2\), 18.8; d(\(T\)C\(_2\), 15.2; d(\(G\)G\(_2\), 8.91; d(\(A\)C\(_2\), 16.02; d(\(G\)T\(_2\), 15.78; d(\(A\)C\(_2\), 11.49; d(\(T\)C\(_2\), 10.37; d(\(A\)T\(_2\), 17.4; and d(\(T\)T\(_2\), 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 RNaseasy Protect Bacteria Mini Kit, following the manufacturer’s protocol. To synthesize cDNA, 2 \(\mu\)g of purified total RNA was amplified in a 20 \(\mu\)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 amplified with \(dptB\)-f and \(dptB\)-r and inserted into the N-terminal His\(_6\)-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 isopropylid–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, 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 following the manufacturer’s instructions. The sequencing samples were precipitated with ethanol and dissolved in 5 μl of 10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 1 μg/ml RNase A, 0.1 μg/ml RNase T1 at 37°C. 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 CaCl₂ 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 Cycce 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.

**Acknowledgements**

The authors thank Shanghai TOLO Biotech. Co. Ltd. for their support in the DNase I footprinting assay. This work was supported by grants from the National Science Foundation of China (30570400, 31170085, 31070058, 31470183 and 31400029); the Ministry of Science and Technology (2006AA022224, 2012CB721004 and 2009ZX09501-008); Shanghai Pujiang Program from the Shanghai Municipal Council of Science and Technology (12PJ0021); and China Postdoctoral Science Foundation.

**References**


© 2015 John Wiley & Sons Ltd, Molecular Microbiology, 97, 1186–1194