Regulation of DNA phosphorothioate modification in *Salmonella enterica* by DndB

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DNA phosphorothioate (PT) modification, in which the non-bridging oxygen of the sugar-phosphate backbone is substituted by sulfur, occurs naturally in diverse bacteria and archaea and is regulated by the DndABCDE proteins. DndABCDE and the restriction cognate DndFGHI constitute a new type of defense system that prevents the invasion of foreign DNA in *Salmonella enterica* serovar Cerro 87. GAAC/GTTC consensus contexts across genomes were found to possess partial PT modifications even in the presence of restriction activity, indicating the regulation of PT. The abundance of PT in cells must be controlled to suit cellular activities. However, the regulatory mechanism of PT modification has not been characterized. The result here indicated that genomic PT modification in *S. enterica* is controlled by the transcriptional regulator DndB, which binds to two regions in the promoter, each possessing a 5′-TACGN₁₀CGTA-3′ palindromic motif, to regulate the transcription of *dndCDE* and its own gene. Site-directed mutagenesis showed that the Cys29 residue of DndB plays a key role in its DNA-binding activity or conformation. Proteomic analysis identified changes to a number of cellular proteins upon up-regulation and loss of PT. Considering the genetic conservation of *dnd* operons, regulation of PT by DndB might be widespread in diverse organisms.

Phosphorothioate (PT) modification is a unique physiological modification in the sugar-phosphate backbone of DNA, in which the non-bridging oxygen is replaced by sulfur in a sequence-selective and *R*₉ stereo-specific manner by Dnd proteins¹. The five-gene *dnd* cluster was originally identified by electrophoresis to cause a DNA degradation phenotype in *Streptomyces lividans*. The cluster has since been detected in more than 200 bacterial and archaeal strains and shows a highly conserved genetic organization²,³. Some *dnd* clusters consists of only *dndBCDE* without a linked *dndA*. DndA is a NiFS-like cysteine desulfurase that can be functionally replaced by an IscS homolog located elsewhere in the genome⁴,⁵. *dndFGHI* is a divergently transcribed cluster that was later identified in the vicinity of the *dndBCDE* operon. The DNA PT system shows similarity to the methylation-based restriction-modification (RM) system; DndABCDE enables the PT modification of ‘self’ DNA, and the restriction cognate DndFGHI discriminates and destroys non-labeled foreign DNA via double-stranded DNA breakage in *S. enterica* serovar Cerro 87⁶,⁷. A subset of *dndBCDE* clusters is not accompanied by *dndFGHI* in diverse strains. This is reminiscent of an orphan DNA methyltransferase, implying that the DNA PT system plays other physiological roles in addition to host protection⁸,⁹.

The DNA PT system is more intricate than typical RM systems and involves five Dnd proteins that sequence-selectively and *R*₉ stereo-specifically incorporate sulfur. The DndA protein is capable of

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assembling DndC as a 4Fe-4S cluster protein, and DndC shows significant homology to phosphoaden- 
osine phosphosulfate (PAPS) reductase. DndB is a functionally unknown protein with a conserved 
DGQHR sequence motif, and DndD exhibits ATPase activity and has been proposed to introduce nicks 
in DNA during sulfur incorporation. The DndE protein adopts a tetramer conformation and has bind- 
ing affinity for nicked double-stranded DNA in vitro via positively charged lysine residues on its sur-
f ace. However, lysine residue mutations do not have a significant effect on the total PT modification 
frequency under physiological conditions, implying that the dsDNA binding capacity of DndE may not 
be crucial for PT modification and/or that DndE may have other biological functions. As a newly iden-
tified component of the PT system, DndFGHI in Salmonella enterica serovar Cerro 87 has been found to induce 
double-stranded DNA breaks in non-PT-modified DNA.

The replacement of an oxygen atom with sulfur confers the PT-modified DNA backbone with resist-
ance to nucleases. Upon DNA hydrolysis, PT-linked dinucleotides are generated in addition to canoni-
cal mononucleotides. This phenomenon can be detected by a sensitive liquid chromatography-coupled 
tandem quadrupole mass spectrometry (LC-MS/MS) method that can be used to identify PT sequence 
contexts and quantify PT frequencies. PT-modified d(GpA) and d(GpT) have been identified in Escherichia coli 
B7A, which has Dnd proteins highly homologous to those in Salmonella enterica serovar Cerro 87, 
at frequencies of 370 ± 11 and 398 ± 17 per 10⁸ nt, whereas a frequency of 2,624 ± 22 of d(CpC) per 10⁸ 
nt has been detected in Vibrio cyclitrophicus FF75. Our recent application of single-molecule, 
real-time (SMRT) sequencing to characterize genomic PT sites has revealed unique features of DNA PT 
systems. The d(GpA) and d(GpT) sites in E. coli B7A are located within stringently conserved 4-bp 
GpAAAC/GpATTC sequences. However, only 12% (4855) of 40,701 GAAC/GTTC sites on the E. coli B7A 
chromosome demonstrate PT modifications, even in the presence of active DndFGHI. In contrast, PT 
mapping in V. cyclitrophicus FF75 revealed single-stranded PT modifications in CpCA motifs. The same 
finding of incomplete modification has been observed for V. cyclitrophicus FF75, in which only 14% of 
genomic CCA sites have undergone PT modifications. PT modifications have been found to be distrib-
uted across the E. coli B7A and V. cyclitrophicus FF75 chromosomes without an apparent preference for 
open reading frames, tRNA, rRNA or noncoding regions. Unexpectedly, partial PT modifications have been 
observed at given sites in the population of DNA molecules in both E. coli B7A and V. cyclitrophicus 
FF75. These highly unusual features differentiate DNA PT systems from typical RM systems and known 
DNA epigenetic mechanisms.

The PT linkage in the DNA backbone is susceptible to cleavage by an oxidative Tris derivative gener-
ated in the electrophoretic buffer adjacent to the anode, resulting in a DNA degradation (Dnd) pheno-
type. The disruption of dndA, dndC, dndD or dndE abolishes the degradation phenotype, whereas the 
mutation of dndB preserves the Dnd phenomenon in both Salmonella enterica serovar Cerro 87 and Streptomyces 
lividans 1326. The levels of genomic PT modifications vary from 3–8 per 10⁹ nt to 2-3 per 10¹⁰ nt in 
diverse bacterial strains. The low abundance of PT suggests that the modification must be controlled 
to suit cellular activities. The regulation of PT modification is important for understanding its cellular 
importance. However, the molecular mechanism of PT regulation has not been characterized.

Given the ubiquity of DNA PT systems, we decided to perform a detailed investigation of how 
genomic PT modifications are regulated. In this study, we first observed a 2-fold increased PT frequency 
in response to dndB deletion in Salmonella enterica serovar Cerro 87. Subsequent proteomic analysis revealed that 
DndCDE was the most up-regulated protein in response to the deletion of dndB. DndB, a transcriptional 
regulator, binds to its promoter region to influence the transcription of dndCDE and its own gene to 
regulate the frequency of genomic PT modification. Considering the genetic conservation of dndBCDE 
operons, regulation of PT frequency by DndB might be widely adopted in diverse organisms.

Results

PT modifications in dnd mutants. The disruption of individual dndCDE genes completely abolished 
the DNA degradation phenotype, whereas disruption of dndB in S. lividans and Salmonella enterica serovar 
Cerro 87 led to smeared DNA. These results prompted us to first compare the PT modifications in wild-type 
Salmonella enterica serovar Cerro 87 and individual dnd mutants using sensitive LC-MS/MS. The results of these 
quantifications were well-correlated with the electrophoretic phenotype, with no DNA PT modifications 
detected in the dndCDE mutants. In contrast, dptB⁻, the dndB in-frame deletion mutant, still carried PT 
modifications. Moreover, the total PT modifications in dptB⁻ occurred at a frequency of 1158 ± 192 per 10⁸ 
nt, which is twice the rate measured in wild-type Salmonella enterica serovar Cerro 87 (594 ± 11 per 10⁸ nt) 
(Table 1). However, the PT modifications in dptB⁻ still strictly occurred at d(GpA) and d(GpT) sites at 
a 1:1 ratio, suggesting that the preference for the GpAAAC/GpATTC consensus sequence was unaltered.

Global proteome analysis of dnd mutants. To understand the molecular basis of altered PT abun-
dance and the global impact of excessive PT on cellular physiology, proteomic analysis was carried out 
to identify proteins that were differentially expressed in dptB⁻ and XTG102, a dndBCDE deletion mutant 
without PT. In total, 82 and 110 proteins were differentially expressed with a fold change higher than 1.5 
or lower than 0.66 (p-value < 0.05) in dptB⁻ and XTG102 relative to wild-type Salmonella enterica serovar 
Cerro 87 (GenBank no. CP008925) (Supplementary Table S2). The 82 and 110 proteins were classified into 19 
and 18 clusters of orthologous groups (COGs), respectively, based on their predicted function (Fig. 1).
In dptB− cells, DndC (18.8-fold), DndD (9.5-fold) and DndE (15.4-fold) were induced to the greatest extents (Supplementary Table S2). In contrast, no significant changes in the restriction cognates DndFGHI were observed in dptB− following the increase in total PT. However, five genes in the replication group—the DNA gyrase GyrA, the DNA mismatch repair (MMR) protein MutS, the nucleotide excision repair (NER) protein UvrA, topoisomerase IV subunit B and DNA polymerase I—were activated in dptB− but not in XTG102 (Supplementary Table S2, Fig. 1). The transcription category represented another significant fraction of the differentially expressed members of the proteome. Four transcriptional regulators—MalT (GW13_PRO2528), NanR (GW13_PRO2351), TorR (GW13_PRO2606), and a GntR family regulator (GW13_PRO2829)—as well as a transcription associated protein (GW13_PRO2516) and an RNA polymerase associated protein (RapA; GW13_PRO3646), were significantly up-regulated. More than half of the 26 down-regulated proteins were categorized into the energy production and conversion group or the amino acid transport and metabolism groups.

In response to PT loss in XTG102, the most noticeable change was the activation of a number of SOS genes (e.g., recA, dinI, rada, ruvA, ruvB, rmuc, yebG, gyrI), as well as eight prophase genes (i.e., GW13_PRO00152, GW13_PRO00162, GW13_PRO00165, GW13_PRO00175, GW13_PRO00176, GW13_PRO00183, GW13_PRO00191, and GW13_PRO00194) (Supplementary Table S2). In addition, the expression levels of nine genes involved in replication (e.g., recN, ruvA, ruvB, recA) were elevated, many of which overlapped with the SOS genes. These features were similar to those observed in the transcriptomic profile of DNA without PT protection that suffered double-stranded cleavage and then triggered the SOS response and prophase induction6. The comparison of gene expression data in dptB− and XTG102 showed distinctive proteomic profiles. As shown in Fig. 1B, only five proteins were commonly up-regulated: GW13_PRO2474, GW13_PRO2275, and GW13_PRO0191, a phosphoribulokinase homolog, a phosphoheptose isomerase and a hypothetical protein. The commonly down-regulated proteins were DndB and GW13_PRO3998, a putative oxidoreductase.

### Table 1. Comparison of total PT in wild-type S. enterica serovar Cerro 87 and dptB−.

| Bacterium | PT modifications per 10^6 nt | p value |
|-----------|-----------------------------|---------|
| S. enterica serovar Cerro 87 | d(G2sA) | d(G2sT) | Total PT |
| dptB− | 297±4 | 297±7 | 594±11 | — |
| dptB(pWHU746) | 577±95 | 581±97 | 1158±192 | 0.036 |
| dptB(pWHU746) | 327±10 | 305±2 | 632±12 | 0.023 |

DndB binds to the promoter region of the dnd operon. Up-regulation of the DndCDE proteins and increased PT frequencies in dptB− implied that DndB might regulate genomic PT by influencing the transcription of the dnd genes. We first compared the transcript levels of dndCD by quantitative RT-PCR and observed a dramatic increase of approximately 15-fold after dndB deletion. Complementing the dptB− mutation with a plasmid (pWHU746) containing dndB lowered the transcription of dndCD and decreased the total PT to 632±12 per 10^6 nt (Table 1). EMSAs were performed to assess the direct interaction of the DndB protein with its promoter DNA. The native mass of purified DndB was determined to be 79 kDa by size exclusion chromatography, indicating that it exists as a homodimer (Supplementary Figure S1). DndB was incubated with DNA fragments spanning −460 to +38 bp with respect to the transcription start site of the dndBCDE operon. As shown in Fig. 2, the addition of DndB to the reaction mixture caused a specific shift in the mobility of DNA fragment B1, extending from position −85 bp to +38 bp. In contrast, no shift was observed for the DNA fragments B2 and B3, spanning −234 bp to −85 bp and −460 bp to −232 bp, respectively, relative to the transcription start site. The 123-bp DNA fragment B1 was PCR-fused to a promoterless lacZ gene and cloned into a pEASY-Blunt Zero plasmid, generating pWHU1809. β-Galactosidase activity was compared in wild-type S. enterica serovar Cerro 87 and dptB− strain pWHU1809, revealing that the level of β-galactosidase activity in dptB− (pWHU1809) was more than 2-fold higher than that in the wild-type strains (Supplementary Figure S2). Taken together, these results indicate that the 123-bp DNA fragment B1 harbors the essential elements for DndB binding.

DndB binds to two palindromic inverted repeat motifs. A DNase I footprinting assay was performed to identify the exact DNA sequences to which DndB binds in DNA fragment B1. The assay revealed two protected regions, BB1 and BB2 (Fig. 3A). BB1 covered a protected region of 33 nt extending from nucleotide −72 to −40 on the coding strand and encompassing 26 nt on the complementary strand. The BB2 region spanned nucleotides −66 to −49 and −14 to +4 on the coding and complementary strands, respectively. The footprinting assay further revealed a putative palindromic motif of 5′-TACGNNCGTA-3′ that was formed from two inverted repeats separated by ten nucleotides in both
the BB1 and BB2 regions (Fig. 3B). The palindromic motifs were found to be essential for DndB binding because the gel mobility assays showed that mutations in TACG in BB1 and CGTA in BB2 regions dramatically decreased the DndB-DNA interaction (Fig. 3C). The mutation of the two 5′-TACG⁻¹⁻⁰⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓…
RM systems and are thus regarded as a new type of bacterial defense system. RM systems are selfish genetic elements that kill cells that have eliminated them, leading to post-segregational cell killing\(^\text{21}\). However, the deletion of \textit{dndBCDE} as well as \textit{dndFGHI} in \textit{S. enterica} serovar Cerro 87 had no effect on cell viability\(^\text{6}\). In addition, GAAC/GTTC sites across genomes were partially modified and a given GAAC/GTTC site was not consistently PT modified in the bacterial population\(^\text{16}\). The unique feature of partial modification densities across the genome in the presence of active restriction enzymes make DNA PT systems different from the known RM systems and suggest the presence of unidentified physiological characteristics.

A total of 53 positive hits (with BLASTN \textit{e}-value = 0, identity \textit{\%} 99\% and coverage \textit{\%} 96\%) were obtained from both databases of non-redundant nucleotide collection (nr/nt) and whole genome shotgun contigs (WGS) with \textit{Salmonella} set as 'organism of selection' using the \textit{dndBCDE} operon of \textit{S. enterica} serovar Cerro 87 as the query. All strains are \textit{Salmonella enterica} subsp. enterica with diverse serotypes of Cerro, Saintpaul, Panama, Bareilly, Mbandaka and Namur. The current study in \textit{S. enterica} serovar Cerro 87 revealed that PT modifications are regulated by DndB, which functions as a transcriptional regulator to control genomic PT modifications by influencing the level of DndBCDE expression. PT modifications in \textit{dptB}\(^-\) strictly occurred at \textit{d(G PSA)} and \textit{d(GPST)} sites at a 1:1 ratio, suggesting that DndB regulates the transcription of the \textit{dnd} operon but plays no role in PT sequence selectivity. DndB bound to two regions of the \textit{dndBCDE} operon, each of which contains a 5\textquotesingle-TAGN10CGTA-3\textquotesingle palindromic box. Mutations of both boxes significantly decreased the binding affinity of DndB. DndB\(_{C29S}\) showed a stronger binding affinity for the promoter, leading to a sharp decrease in DNA PT modifications \textit{in vivo}. However, DndB\(_{C29S}\) still possessed residual DNA binding and barely detectable PT modifications. These data raise two possibilities, namely, that C29 may be involved in direct binding to the promoter or that C29 may have critical effects on DndB conformation. In the future, the availability of the DndB structure may allow for a better understanding of the interaction between DndB and its DNA substrates, and the mechanism

**Figure 2.** DndB binding sites in the \textit{dndBCDE} promoter region. (A) Localization of the DNA fragments used in the EMSAs. The numbers represent the positions of the fragments with respect to the transcription start site. (B) EMSAs were performed using serial concentrations of DndB ranging from 0 to 300 nM with the DNA fragments \(B_3\), \(B_2\) and \(B_1\).
by which the substitution of C29 alters the structure of the DndB/DNA complex could be explored. Bioinformatics pattern searches revealed that the 5'-TACGN10CGTA-3' motif is located in upstream regions, 200 bp prior to the start codon, of eleven genes—GW13_PRO0283, GW13_PRO0295, GW13_PRO0296, GW13_PRO0509, GW13_PRO0798, GW13_PRO2418, GW13_PRO2485, GW13_PRO2826, GW13_PRO3447, GW13_PRO3539 and GW13_PRO4223. Among them, only GW13_PRO3447, encoding DndB protein, harbors two 5'-TACGN10CGTA-3' motifs in the promoter region. The other ten genes were not differentially expressed upon dndB deletion.

Xie et al. reported that PT modified DNA functions as a peroxide reducing reagent, enabling the wild-type S. enterica serovar Cerro 87 to be more resistant to H2O2 than dnd gene mutants 9. However, qRT-PCR showed no significant changes of dnd cluster upon exposure to oxidants of H2O2 and HOCl. This suggests that DndB does not function as a redox-sensing transcriptional regulator. This study was unable to identify the triggering factors that act as an on/off switch for the PT system through DndB. This goal should be pursued in future studies.

Bacterial defense systems, e.g., RM systems, require the tight regulation of restriction and modification enzymes to provide effective protection. Initially, it is essential that methylation precede endonuclease restriction activity in the host cell to prevent autorestriction. Subsequently, the level of methyltransferase activity is expected to be reduced because excessive methylation may decrease the efficiency of restriction activity against viral DNA or lead to DNA mutations 22,23. Proteomic profiling identified a group of proteins in the replication COG group, including UvrA, MutS, DNA gyrase subunit A, topoisomerase IV subunit B, and DNA polymerase I, that were up-regulated in dptB-. The induction of the

Figure 3. DndB binds to two regions in the promoter of the dndBCDE operon. (A) Footprinting assays identifying two regions protected by DndB, BB1 and BB2, on both the coding and noncoding DNA strands. The palindromic motifs are shown in red letters. (B) The promoter sequence of the dndBCDE operon was analyzed using BPROM and Neutral Network Promoter Prediction online software. (C) EMSAs were performed on serial concentrations of DndB ranging from 0 to 300 nM with the DNA fragments B1 and mutated B1. The TACG in BB1 and CGTA in BB2 were replaced by GGTT and TTGG respectively, in the mutated B1 fragment. Mutation of the B1 fragment impaired the DNA-binding activity of DndB. Shifted bands were recovered when the wild-type DNA B1 fragment was added.
Figure 4. Effect of cysteine mutations on DndB regulation. (A) PT frequencies in *E. coli* DH10B harboring pJTU1238 and its derivatives. (B) EMSA was performed with serial concentrations of DndB_C29S ranging from 0 to 90 nM with the B fragment. (C) Amino acid sequence alignment of DndB homologs (cysteine residues are marked by a red asterisk) from *S. enterica* serovar Cerro 87 (GW13_PRO3447), *E. coli* B7A (AIF62361), *Vibrio tasmaniensis* 1F-267 (WP_017104390), *Enterovibrio calviensis* 1F-230 (WP_017017144), *Vibrio splendidus* ZS-139 (WP_017074154), *Pseudomonas fluorescens* pf0-1 (YP_346470), *Streptomyces lividans* (WP_003972933) and *Hahella chejuensis* KCTC2396 (WP_011400707).

Table 2. Quantification of *dnd* gene transcription by quantitative real-time PCR. Values represent the means ± SD for three analyses. The transcriptional changes of *dnd* relative to the housekeeping gene, *gapA*. Quantitative real-time PCR data analysis was performed according to the comparative threshold cycle (CT) method, also known as $2^{-\Delta\Delta C_t}$. 

| Bacterium            | Average CT ± SD | dndCD fold change relative to *gapA* |
|----------------------|-----------------|--------------------------------------|
| *S. enterica*        | 22.00 ± 0.21    | 17.76 ± 0.39                         | 1 (0.8–1.1) |
| *E. coli* (H2O2, 1mM)| 21.77 ± 0.21    | 23.67 ± 0.15                         | 0.97 (0.66–1.42) |
| *S. enterica*        | 21.56 ± 0.38    | 22.64 ± 0.45                         | 0.77 (0.35–1.71) |
SOS response (UvrA) suggests that phosphorothioation possibly affect DNA replication. DNA gyrase and topoisomerase IV are type II bacterial DNA topoisomerases that are essential for the maintenance of DNA topological homeostasis and for solving topological problems linked to DNA replication and transcription24. UvrA and MutS participate in NER and MMR, respectively. In E. coli, NER is carried out by the UvrABC complex to repair lesions caused by exogenous damage25. The MMR system recognizes and removes single-base mismatches and small nucleotide insertions or deletions that result from errors introduced during replication26. NER and MMR are closely linked and represent two primary pathways for the removal of DNA damage to maintain genomic stability. Although sulfur replacement is a minor modification compared to other DNA changes, an in vitro assay with UvrABC revealed that the PT-modified DNA backbone is recognized as a substrate by NER, albeit at a low efficiency27. The disruption of dndB resulted in 18.8-, 9.5- and 15.4-fold increases in DndC, DndD, and DndE, respectively. In contrast, the total PT sites in dptB− only increased by 2-fold, accounting for 16% of 32,795 genomic GAAC/ GTTC sites. Proteomic analysis implied that genomic GAAC/GTTC sites might have a limitation that prevents PT from being modified; otherwise, excess PT would be regarded as abnormal, inducing a massive DNA repair response. The most marked changes in protein abundance involved the dnd operon itself, the only other proteins showing at least a 5-fold change are involved in DNA repair (UvrA) or envelope stress (RcsD). These observations appear to be consistent with the idea that dnd is a selfish genetic element.

This study provides insights into the regulatory mechanisms underlying DNA PT modifications. DndB binds to two palindromic inverted repeats in the promoter region. The control of PT modification might be a consequence of an unknown environmental or cellular stimulus of DndB, acting as a switch to regulate modification frequency. Proteomic analysis identified global changes in response to dndB deletion and increased PT modifications, shedding light on PT biology. dndB is co-transcribed as a single operon with dndCDE, and dndBCDE operons are widely present in prokaryotes and have a conserved genetic organization28. The aggravated DNA degradation phenomenon was also observed in dndB-deficient mutant of S. lividans18. These findings imply that transcriptional regulation of the dnd operon by DndB might be widely employed in DNA PT systems.

Materials and Methods
Bacterial strains, plasmids and primers. All strains and plasmids used in this work are listed in Table 3. Primers are listed in Supplementary Table S1. Salmonella and E. coli strains were grown in Luria-Bertani (LB) broth. For dptB− complementation, pWHU746 was constructed by PCR amplifying the DNA fragment containing the dndB gene and the upstream promoter region using dndB-F and dndB-R as primers. The 1299bp fragment was inserted into plasmid pBluescript II SK(+) to yield pWHU746.

Protein expression and purification. The dndB gene was PCR-amplified using the S. enterica serovar Cerro 87 chromosome as a template and cloned into EcoRI and XhoI digested pSJ7, a pET43a derivative, to yield pJTU3522 (a generous gift from Dr. Jingdan Liang). pJTU3522 was transformed into E. coli BL21 (DE3) and DndB protein was expressed as a N-terminal NusA-tagged protein containing a poly-His tag and the tobacco etch virus (TEV) recognition site. Cells were grown at 37 °C in Luria broth medium to an A600 of 0.6–0.8 and then induced overnight at 16 °C with 0.5 mM IPTG. The cells were harvested by centrifugation at 6000 × g for 30 min. The supernatant was then applied to a 5 mL HisTrapTM HP chelating column (GE Healthcare). The NusA-tag and His-tag of the purified DndB protein were removed by TEV protease in phosphate-buffered saline (PBS), and the protein concentration was determined using the Bradford method29. The native molecular mass of DndB was estimated by size exclusion chromatography using a HiLoadTM 16/60 SuperdexTM 200 column (GE Healthcare). The column was equilibrated with 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and elution was performed with the same buffer at a flow rate of 0.7 mL/min. Calibration was performed with myoglobin (17 kDa) ovalbumin (44 kDa), albumin (66 kDa), phosphorylase b (97 kDa), and γ-globulin (158 kDa).

Quantification of PT modifications. The LC-MS/MS method used for PT quantification has been described previously13. Briefly, DNA hydrolytes were resolved using a Thermo Hypersil GOLD aQ column (150 × 2.1 mm, 3μm). Elution was performed at 35 °C, beginning with incubation in 97% buffer A (0.1% acetic acid in water) and 3% buffer B (0.1% acetic acid in acetonitrile) for 5 min followed by an increase in buffer B from 3% to 98% over another 30 min at a flow rate of 0.3 mL/min. The LC column was coupled to a Thermo TSQ Quantum Access MAX mass spectrometer with an electrospray ionization source in positive mode. The multiple reaction monitoring (MRM) mode was employed for the detection of daughter ions derived from precursor ions. All instrument parameters were optimized for maximal sensitivity13.

Quantitative real-time PCR. To monitor DndB regulation in response to diverse environmental stresses, cell cultures with an A600 of 0.8 were treated with 1 mM H2O2, 0.4 mM HOCl and 1 mM formaldehyde for 10 min, followed by RNA extraction. Total RNA was isolated using an RNeasy Protect
Bacteria Mini Kit (Qiagen) followed by DNase I treatment. The RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time RT-PCR was performed using a SsoFast EvaGreen Supermix with Low ROX Kit (Bio-Rad) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). To quantitatively compare transcription of the dnd operon, the primers dndB-RT-1 and dndB-RT-2 were designed according to the dndCD genes (Supplementary Table S1). The housekeeping gene gapA, which encodes GAPDH, was used for normalization. mRNA levels were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

**Site-directed mutagenesis of dndB.** Site-directed mutagenesis of the dndB gene in plasmid pJTU1238, which harbored the dndBCDE cluster of S. enterica serovar Cerro 87, was conducted using a Muta-direct Site Directed Mutagenesis Kit (SBS Genetech). The five cysteine residues were individually replaced with serine. The primers were designed according to the manufacturer's recommendations (Table S1). Mutations in pJTU1238 were verified by DNA sequencing. The derived plasmids were transformed into E. coli DH10B to detect PT modifications by LC-MS/MS.

**Electrophoretic mobility shift assay (EMSA).** Putative DNA promoter regions were PCR-amplified or commercially synthesized, purified with an Omega Gel Extraction Kit and dissolved in water. The purified DndB protein or mutated derivatives were incubated with 20 nM DNA fragments in 20 μL of binding buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 5 mM EDTA, 10 mM DTT, and 5% glycerol). The reaction mixtures were incubated at room temperature for 30 min and then loaded onto 6% native polyacrylamide gels. Electrophoresis was performed using 0.5 × TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA) at room temperature. The gel was stained with GelRed and photographed.

**β-Galactosidase assays.** To analyze the binding of DndB to DNA fragment B1, plasmid pWHU1809 was constructed for the following β-galactosidase assay. First, the 123 bp B1 fragment and promoterless lacZ gene were generated by PCR using the primer pairs OverB-LL/OverB-LR and OverB-RL/OverB-RR, respectively (Supplementary Table S1). OverB-LL and OverB-RL are chimeric primers. A mixture of two purified PCR products, which overlapped by 36 bp, served as the template for a ligation PCR using primers overB-LL and overB-RR. A 3,294 bp product was obtained and cloned into the pEasy-Blunt Zero vector (TransGen Biotech), yielding plasmid pWHU1809. β-Galactosidase activity was determined in Miller units using the protocol described by Zhang et al. Four 20-μL samples of culture were obtained at an A₆₀₀ of between 0.2 and 0.6 and mixed with 80 μL of permeabilization solution (100 mM Na₂HPO₄, 20 mM KCl, 2 mM MgSO₄, 0.8 mg/mL CTAB, 0.4 mg/mL sodium deoxycholate and 5.4 μL/mL β-mercaptoethanol). The permeabilization mixture was incubated at 30°C for approximately 30 min, and

| Strains or plasmids | Relevant properties | Source or reference |
|---------------------|--------------------|---------------------|
| **Strains**         |                    |                     |
| S. enterica serovar Cerro 87 | strain naturally contains the dndBCDE gene cluster and PT-modified G₃₉A and G₉₉T | 7                     |
| dptB⁻ | S. enterica derivative, dndB in-frame deletion mutant | 9                     |
| XTG102 | S. enterica derivative, dndB-E in-frame deletion mutant | 7                     |
| **Plasmids**        |                    |                     |
| pBluescript II SK(+) | cloning vector, Amp' | 34                   |
| pSJ7                | cloning vector, Amp' | 35                   |
| pJTU3522           | dndB from S. enterica, cloned in pSJ7 | Gift from Jingdan Liang |
| pZWHJ002           | lacZ promoterless, derivative of pXM119, Cam' | 36                   |
| pJTU1238           | dndBCDE from S. enterica, cloned in SK(+) | 7                     |
| pWHU1809          | B₁ fragment with promoterless lacZ cloned in pEASY-Blunt Zero (TransGen Biotech) | This study            |
| pWHU746            | dndB with promoter from S. enterica, cloned in SK(+) | This study            |
| pWHU751            | pJTU1238 derivative site mutant with C9S | This study            |
| pWHU752            | pJTU1238 derivative site mutant with C29S | This study            |
| pWHU753            | pJTU1238 derivative site mutant with C102S | This study            |
| pWHU754            | pJTU1238 derivative site mutant with C235S | This study            |
| pWHU755            | pJTU1238 derivative site mutant with C336S | This study            |

**Table 3. Bacterial strains and plasmids.**
600 μL of substrate solution (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 1 g/mL o-nitrophenyl-β-D-galactoside and 2.7 μL/mL β-mercaptoethanol) prewarmed to 30°C was added to initiate the reaction. After 0.5-2 h at 30°C, the reactions were terminated by the addition of 700 μL of 1 M Na₂CO₃. The rate of change in A₄₀₀/hour per A₆₀₀ unit of culture was then converted into Miller units.

**Dye primer-based DNase I footprinting assay.** DNase I footprinting assays were performed using FAM-labeled primers. For preparation of the sense-strand probe, the promoter region of dndB was PCR-amplified with Dpx DNA polymerase (TOLO Biotech) using the primers B₃-F and B₃-R (Table S1). The amplicon was then purified and further cloned into a HincII-digested pUC18H vector (TOLO Biotech). The obtained plasmid was verified by DNA sequencing and used as a template for the further preparation of fluorescent FAM-labeled probes with the primers M13F-47(FAM)/M13R-48 and M13R-48(FAM)/M13F-47. The FAM-labeled probes were purified with a Wizard® SV Gel kit and a PCR Clean-Up System (Promega) and quantified with a NanoDrop 2000C (Thermo). For each assay, 200 ng of probes were incubated with different amounts of DndB in a total volume of 40 μL. After incubation for 30 min at 25°C, 10 μL of a solution containing approximately 0.015 units of DNase I (Promega) and 100 nmol of freshly prepared CaCl₂ was added and further incubated for 1 min at 25°C. The reaction was stopped by adding 140 μL of DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% SDS). The samples were first extracted with phenol/chloroform and then precipitated with ethanol, and the pellets were dissolved in 30 μL of MilliQ water. The preparation of the DNA ladder, electrophoresis and data analysis were performed as described previously by Wang et al., with the exception that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

**Proteomic analysis.** Wild-type *S. enterica* and *dndB* mutant strains were grown in LB medium at 30°C to an A₆₀₀ of 0.8. The bacteria were centrifuged at 10,000 × g for 2 min, mixed with Laemmli sample buffer and heated at 95°C for 5 min. The denatured proteins were separated by 10% SDS-PAGE, and electrophoresis was stopped when the bromophenol blue band reached ~1 cm below the stacking gel. Sample preparation and in-gel digestion were performed as described previously by Hu et al.. The digested peptides were extracted by incubating the gels twice with 50% ACN and 5% formic acid for 30 min at 25°C. 100 nmol of freshly prepared CaCl₂ was added and further incubated for 1 min at 25°C. The reaction was stopped by adding 140 μL of DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% SDS). The samples were first extracted with phenol/chloroform and then precipitated with ethanol, and the pellets were dissolved in 30 μL of MilliQ water. The preparation of the DNA ladder, electrophoresis and data analysis were performed as described previously by Wang et al., with the exception that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

**References**

1. Wang, L. et al. Phosphorothioation of DNA in bacteria by dnd genes. *Nat Chem Biol* 3, 709–10 (2007).
2. He, X. et al. Analysis of a genomic island housing genes for DNA S-modification system in Streptomyces lividans 66 and its counterparts in other distantly related bacteria. *Mol Microbiol* 65, 1034–48 (2007).
3. Zhou, X. et al. A novel DNA modification by sulphur: DndA is a NifS-like cysteine desulfurase in *Pseudomonas fluorescens* Pf0-1. *FEBS Lett* 583, 729–33 (2009).
4. An, X. et al. A novel target of IscS in Escherichia coli: participating in DNA phosphorothioation. *PLoS One* 7, e51265 (2012).
5. Wang, L., Chen, S. & Deng, Z. Phosphorothioation: An unusual post-replicative modification on the DNA backbone. *Appl Environ Microbiol* 79, 7547–55 (2013).
6. Gan, R. et al. DNA phosphorothioate modifications influence the global transcriptional response and protect DNA from double-stranded breaks. *Sci Rep* 4, 6642 (2014).
7. Xu, T., Yao, F., Zhou, X., Deng, Z. & You, D. A novel host-specific restriction system associated with DNA backbone S-modification in *S. enterica* and *dptB*. Proteins with an average fold change of >1.5 or <0.66 were considered up- and down-regulated, respectively (p-value < 0.05, Student's t-test).
Regulation of DNA phosphorothioate modification in Salmonella enterica by DndB.

14. Cao, B. et al. Pathological phenotypes and in vivo DNA cleavage by unrestrained activity of a phosphorothioate-based restriction system in Salmonella. Mol Microbiol 93, 776–85 (2014).
15. Wang, L. et al. DNA phosphorothioation is widespread and quantized in bacterial genomes. Proc Natl Acad Sci USA 108, 2963–8 (2011).
16. Cao, B. et al. Genomic mapping of phosphorothioates reveals partial modification of short consensus sequences. Nat Commun 5, 3951 (2014).
17. Ray, T., Mills, A. & Dyson, P. Tris-dependent oxidative DNA strand scission during electrophoresis. Electrophoresis 16, 888–94 (1995).
18. Liang, J. et al. DNA modification by sulfur: analysis of the sequence recognition specificity surrounding the modification sites. Nucleic Acids Res 35, 2944–54 (2007).
19. Sun, F. et al. Protein cysteine phosphorylation of SarA/MgrA family transcriptional regulators mediates bacterial virulence and antibiotic resistance. Proc Natl Acad Sci USA 109, 15461–6 (2012).
20. Kulik, J., Toleldano, M.B., Tartaglia, L.A. & Storz, G. Mutaional analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J Bacteriol 177, 1275–84 (1995).
21. Kobayashi, I. Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. Nucleic Acids Res 29, 3742–56 (2001).
22. Bandaru, B., Gopal, J. & Bhagwat, A.S. Overproduction of DNA cytosine methyltransferases causes methylation and C –> T mutations at non-canonical sites. J Biol Chem 271, 7851–9 (1996).
23. Shen, J.C., Rideout, W.M., 3rd & Jones, P.A. High frequency mutagenesis by a DNA methyltransferase. J Biol Chem 271, 1073–80 (1992).
24. Zechiedrich, E.L. & Cozzarelli, N.R. Roles of topoisomerase IV and DNA gyrase in DNA unlinking during replication in Escherichia coli. Genes Dev 9, 2859–69 (1995).
25. Li, G.M. Mechanisms and functions of DNA mismatch repair. Cell Res 18, 85–98 (2008).
26. Truglio, J.J., Croteau, D.L., Van Houten, B. & Kisker, C. Prokaryotic nucleotide excision repair: the UvrABC system. Chem Rev 106, 233–52 (2006).
27. Braunm, M.E., Reardon, J.T. & Sancar, A. DNA repair excision nuclease attacks undamaged DNA. A potential source of spontaneous mutations. J Biol Chem 276, 25421–6 (2001).
28. Xu, T. et al. DNA phosphorothioation in Streptomyces lividans: mutational analysis of the dnd locus. BMC Microbiol 9, 41 (2009).
29. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–54 (1976).
30. Miller, J. in Experiments in Molecular Genetics (Cold Spring Harbor Laboratory, Cod Spring Harbor, NY, 1972).
31. Zhang, X. & Bremer, H. Control of the Escherichia coli rmbR P1 promoter strength by ppGpp. J Biol Chem 270, 11181–9 (1995).
32. Wang, Y., Cen, X.F., Zhao, G.P. & Wang, J. Characterization of a new GlnR binding box in the promoter of amtB in Streptomyces coelicolor inferred a PhoP/GlnR competitive binding mechanism for transcriptional regulation of amtB. J Bacteriol 194, 5237–44 (2012).
33. Hu, M., Liu, Y., Yu, K. & Liu, X. Decreasing the amount of trypsin in in-gel digestion leads to diminished chemical noise and improved protein identifications. J Proteomics 109, 16–25 (2014).
34. Alting-Mees, M.A. & Short, I.M. plplescript II: gene mapping vectors. Nucleic Acids Res 17, 9494 (1989).
35. Zhou, J. et al. The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. Proc Natl Acad Sci USA 103, 14343–8 (2006).
36. Chao, H. & Zhou, N.Y. Involvement of the global regulator GlxR in 3-hydroxybenzoate and gentisate utilization by Corynebacterium glutamicum. Appl Environ Microbiol 80, 4215–25 (2014).

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Author Contributions
L.W. and S.C. designed research. W.H., T.H. and X.W. conducted the experiments. Y.T., X.L. and Y.L. performed proteomic analysis. S.C. conducted the bioinformatic analysis. W.H., T.H., Y.T., X.W., W.C., Y.W., S.C. and L.W. analyzed data. W.H., T.H., X.L., S.C. and L.W. wrote the manuscript.

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