A Novel Target of IscS in *Escherichia coli*: Participating in DNA Phosphorothioation

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Abstract

Many bacterial species modify their DNA with the addition of sulfur to phosphate groups, a modification known as DNA phosphorothioation. DndA is known to act as a cysteine desulfurase, catalyzing a key biochemical step in phosphorothioation. However, bioinformatic analysis revealed that 19 out of the 31 known *dnd* gene clusters, contain only four genes (*dndB-E*), lacking a key cysteine desulfurase corresponding gene. There are multiple cysteine desulfurase genes in *Escherichia coli*, but which one of them participates into DNA phosphorothioation is unknown. Here, by employing heterologous expression of the *Salmonella enterica* *dnd* gene cluster named *dptBCDE* in three *E. coli* mutants, each of which lacked a different cysteine desulfurase gene, we show that IscS is the only cysteine desulfurase that collaborates with *dptB-E*, resulting in DNA phosphorothioation. Using a bacterial two-hybrid system, protein interactions between IscS and DptC, and IscS and DptE were identified. Our findings revealed IscS as a key participant in DNA phosphorothioation and lay the basis for in-depth analysis of the DNA phosphorothioation biochemical pathway.

Introduction

Sequence and stereo specific physiological DNA phosphorothioation occurs in many bacteria [1–4]. In *Streptomyces lividans* 1326, a five-gene cluster, *dndA–E*, determines the modification [1]. Orthologs of these genes were found in 30 bacterial species and one Archaea [2]. The *dnd* genes are usually located on genomic islands that were probably acquired by horizontal gene transfer [3].

Several of these gene clusters contain *dndB-E* homologues, but lack a *dndA* homologue [2,3]. In-frame deletion of *dndA* in *S. lividans* showed that the gene is essential for DNA phosphorothioation [1,4]. DndA was then shown to be a cysteine desulfurase involved in the iron/sulfur cluster assembly for apo-Fe DndC [5].

*Salmonella enterica* serovar cerro 87 contains *dndB-E* orthologs that are called *dptB-E* [6]. There is, however, no *dndA* ortholog in the entire 20 kb genomic island that contains the *dpt* genes (Fig. 1A) [2]. Heterologous expression of *dptB-E* in *E. coli* DH10B [7] resulted in DNA phosphorothioation [8]. Since DndA is essential for DNA phosphorothioation in *S. lividans*, we hypothesized that there should be one or more genes in the *E. coli* genome that could provide the cysteine desulfurase activity known to be necessary for the modification. Searching for a putative *dndA* ortholog in *E. coli* BW25113 was easier than in *S. enterica* because of the availability of a comprehensive library of knockout mutants of all nonessential genes [9]. In *E. coli*, there are at least three different cysteine desulfurases: IscS, SuS and CsdA [10,11]. Here we show that only one of them, IscS, supports DNA phosphorothioation in *E. coli* expressing the *S. enterica* *dptB-E* gene cluster. Protein interactions, which are likely necessary for DNA phosphorothioation, were detected between IscS and both DptC and DptE.

Materials and Methods

Bacterial strains, plasmids and primers

Bacterial strains, plasmids, and primers are listed in Table 1, 2 and 3.

The *E. coli* BW25113 gene replacement mutants listed in Table 1 were obtained from Yale Coli Genetic Stock Center [9]. Among these, the iscS mutant JW2514 was not viable, and was recreated by using the gene knockout method described by Datsenko [12]. For this, the neo-FRT (FLP, recombinase recognition target) cassette was amplified using primer P1 and P2, then H1P1 and H2P2. Successful iscS deletion was confirmed by PCR using the flanking primers U and D (Fig. 2A).

Detection of DNA phosphorothioation

Phosphorothioate DNA is sensitive to double-strand cleavage by Tris-peracetic acid (TPA) [13]. The phosphorothioation was detected by incubating DNA samples for 30 min at 25°C in TAE buffer (40 mM Tris, 20 mM sodium acetate, 0.8 mM EDTA pH 7.5) supplemented with 1.0% peracetic acid. Phosphorothioate DNA, but not normal DNA, shows Dnd phenotype, producing a
E. coli becomes phosphorothioated when expressing doi:10.1371/journal.pone.0051265.g001 unlinked gene in for DNA phosphorothioation. The

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**Table 1.** Strains that are used in this study.

| STRAINS                  | CHARACTERISTICS                                                                 | REFERENCE |
|--------------------------|---------------------------------------------------------------------------------|-----------|
| Salmonella enterica Cervo 87 | Strain containing naturally S-modified DNA, source of the dptE gene cluster    | [6]       |
| E. coli DH10B             | Non-restricting host strain for gene cloning                                     | [7]       |
| E. coli BW25113           | ac\(^R\) mrr\(^R\), lacZ\(^{MCS}\), hostS14, araBAD\(^{MCS}\), rhaBAD\(^{LD70}\) strain used for creating gene knockouts | [12]      |
| BL21(DE3)pLysS            | Lacks Lon and ompT proteases Cml\(^{I}\)                                        | Novagen   |
| JW2514-4                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), lacS776::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW1670-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), lacS755::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW2781-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), lacZ738::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW2513-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), lacU775::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW3955-2                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW3956-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW2512-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), lacA774::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW2508-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), lacX770::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW0810-2                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), DmoeB262::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW3779-3                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), rph-1, DscyT752::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW3435-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), DyphPm1327::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| JW0413-1                 | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), Dthi780::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | Yale Coli Genetic Stock Center [9] |
| AXH034                   | E. coli F\(_r\), (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), &lambda\(^{}\), Dsc1191::kan, rph-1, (araD-araB)\(^{567}\), (rhaD-rhaB)\(^{568}\), hsdRS14 | This study |
| E. coli XL1-Blue MR      | Host strain for propagating pBT and pTRG recombinants Δ(mcrA)Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi1 recA1 gyrA96, relA1 lac                  | BacterioMatch II Kit (Agilent) |
| E. coli XL1-Blue MRF⁺ Kan| Derivative of XL1-Blue MR. Reporter strain for two-hybrid test using pBT and pTRG derivatives Δ(mcrA)Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi1 recA1 gyrA96 relA1 lac [F" proAB lacZΔM15 Trp+ (Kan)] | BacterioMatch II Kit (Agilent) |
Table 2. Plasmids that are used in this study.

| PLASMIDS                         | CHARACTERISTICS | REFERENCE |
|----------------------------------|-----------------|-----------|
| pKD46                            | amp rep"        | [7]       |
| pJTU3510                          | dptE from S. enterica Cerro 87, p15A origin of replication, Cm" | This study |
| pJTU3523                          | dptC from S. enterica Cerro 87, cloned in pSU7 expression vector | This study |
| pJTU3525                          | dptE from S. enterica Cerro 87, cloned in pSU7 expression vector | This study |
| pBT                              | Bait plasmid, λcI Cm", cloning between NotI and Xhol | bacterioMatch II Two-Hybrid System Vector Kit (Agilent) |
| pTRG                             | Target plasmid, Tet', cloning between BamHl and Xhol | bacterioMatch II Two-Hybrid System Vector Kit (Agilent) |
| pBT-LGF2                         | Control plasmid λcI LGF2 Cm" | bacterioMatch II Two-Hybrid System Vector Kit (Agilent) |
| pTRG-GAL11P                      | Control plasmid RNAS-x GAL11P" | bacterioMatch II Two-Hybrid System Vector Kit (Agilent) |
| pJTU3609                          | dptB cloned in pTRG with site BamHl and Xhol | This study |
| pJTU3610                          | dptC cloned in pTRG with site BamHl and Xhol | This study |
| pJTU3611                          | dptD cloned in pTRG with site BamHl and Xhol | This study |
| pJTU3612                          | dptE cloned in pTRG with site BamHl and Xhol | This study |
| pJTU3618                          | iscs cloned in pBT with site NotI and Xhol | This study |
| pET15b                            | Expression vector with His-tag Amp" | Novagen |
| pJTU3619                          | Expressing E. coli iscs (amplified using primers iscs exU/exD) in pET15b Ndel and BamHl | This study |
| pJTU3625                          | pJTU3619 derivative site mutant with C111A | This study |
| pJTU3626                          | pJTU3619 derivative site mutant with C170A | This study |
| pJTU3627                          | pJTU3619 derivative site mutant with C328A | This study |
| pJTU3622                          | dptC with TEV site insert into pGEX-6P-1 between S6m and Xhol | This study |
| pJTU3624                          | dptE with TEV site insert into pGEX-6P-1 between S6m and Xhol | This study |

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smear of DNA fragments in an agarose gel. To prevent DNA degradation during electrophoresis, 50 mM thiourea was added to the TAE electrophoresis buffer [13,14].

Bacterial two-hybrid analysis

Protein-protein interactions were investigated using the BacterioMatch II two-hybrid system (Stratagene), according to the manual [15] with some modifications. The system features a HIS3-aadA reporter cassette, whose expression allows E. coli growth in the presence of 3-AT (3-amino-1,2,4-triazole), which is a competitive inhibitor of His3 (imidazoleglycerol-phosphate dehydrogenase), and in the presence of streptomycin.

To test protein-protein interactions, in-frame gene fusions were created in the pBT (bait) or pTRG (target) vectors. PCR primers with suitable restriction sites were constructed and are listed in Table 1. IscS was fused with a bait protein, generating pBT-IscS; DndB-E were fused with target protein, generating pTRG-DptB, pTRG-DptC, pTRG-DptD and pTRG-DptE respectively. The resulting bait and target clones were co-transformed into the reporter strain E. coli XL1-Blue MRF" Kan (Stratagene/Agilent) and selected on LB agar containing 25 μg/ml chloramphenicol (to select for pBT derivatives), 12.5 μg/ml tetracycline (to select for pTRG derivatives), and 50 μg/ml kanamycin (to maintain F" proAB lacF"ZAM13 Tn5).

To test for resistance to 3-AT, single colonies were inoculated into 1 mL LB containing the three above antibiotics, and kept shaking overnight at 30°C. 500 μl of this overnight culture was then inoculated into 5 mL SOC medium and incubated for 90 min at 37°C. The cells were then spun down at 3500 rpm for 5 min at room temperature, and the supernatant was carefully removed. The cells were then re-suspended in 2 mL M9" His-drop out broth, collected by centrifugation as described above, and re-suspended in 3 mL M9" His-drop out broth [15]. After incubation for 2 hours at 37°C, three parallel ten-fold dilutions 10⁻¹–10⁻⁷ were prepared and plated 105, 10⁴, 10³ and 10² on Selective Screening Medium (SSM) containing 5 mM 3-AT and 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ on Nonselective Screening Medium (NSM) without 3-AT. Colonies were counted after 24 h incubation at 37°C. If there were no visible colonies, the plates were incubated in dark at 25°C for another 16 hours.

Putative positive interactions were verified using Dual Selective Screening Medium containing 5 mM 3-AT + 12.5 μg/mL streptomycin.

Strep and GST Pull-down

Ten milliliters of E. coli BL21 (DE3) strain (harboring Strep-iscS, or GST-DptC, or GST-DptE) was inoculated to 1 L and grew at 37°C for 3 hours with shaking (220 rpm). IPTG was then added to a final concentration of 0.2 mM (from 1000 folds stock). The culture was then moved to 16°C and grew for another 24 hours. The cells were collected by centrifuge for 10 minutes at 4°C. Cell pellet was re-suspended using Buffer S (25 mM HEPES pH 7.6, 100 mM KCl, 10% glycerol, 1 to 10 folds [w/v]) and sonicated for 30 minutes. Cell debris was removed by centrifugation at 15000 g for 20 minutes. Equal volume of the extract was mixed [IscS-GST DptC or IscS-GST DptE]. Two milliliter of the mixture was incubated with 0.1 ml Strepactin resin (Qiagen) or GST resin (Qiagen) pre-equilibrated using Buffer S. After 1 hour, the resin was spin down (400 g, 3 minutes). Supernatant was removed. The resin was wash 5 times using 2 mL Buffer S. The protein was eluted using 0.3 mL Buffer S supplemented with 2.5 mM Desthiobiotin or 20 mM Glutathione. Western blot was
Expression of *S. enterica* dptB-E in *E. coli* BW25113 results in DNA phosphorothioation

Owing to the observation that in *Streptomyces lividans*, dndA is essential for DNA phosphorothioation, we sought to find the cysteine desulfurase gene in *E. coli*. The *E. coli* genome was searched for orthologs of a cysteine desulfurase gene.

Fig. 1B shows that there are at least three cysteine desulfurase genes in the *E. coli* genome [10,11]. Fig. 1C shows that introducing pJTU3510 carrying dptB-E four genes, a low-copy plasmid, into *E. coli* BW25113 resulted in DNA S-modification (lane 3). We speculated that dptB-E, in cooperation with one or more *E. coli* desulfurase gene, leads to DNA phosphorothioation.

For DNA phosphorothioation in *E. coli* BW25113, it seemed likely that a protein similar to the cysteine desulfurase DndA was needed in addition to the *S. enterica* dptB-E gene cluster. Individual *E. coli* BW25113 knockout mutants, *iscS*, *dsuS*, and *csdA* were available from the Yale Coli Genetic Stock Center. The *iscS* mutant did not survive the transport and was reconstructed (Fig. S1).

*IscS* is responsible for the DNA phosphorothioation in *E. coli*

For DNA phosphorothioation in *E. coli* BW25113, it seemed likely that a protein similar to the cysteine desulfurase DndA was needed in addition to the *S. enterica* dptB-E gene cluster. Individual *E. coli* BW25113 knockout mutants, *iscS*, *dsuS*, and *csdA* were available from the Yale Coli Genetic Stock Center. The *iscS* mutant did not survive the transport and was reconstructed (Fig. S1).

*E. coli* BW25113 and the three cysteine desulfurase mutants were transformed with pJTU3510 expressing dptB-E, and tested for the phosphorothioation status by Dnd phenotypic assay (DNA smear, an indicator of DNA phosphorothioate modification) (Fig. 2). Only the *iscS* mutant failed to modify its DNA (lane 4), suggesting that only *E. coli* IscS, but not SuS or CsdA, was responsible for DNA phosphorothioation in *E. coli*.

To confirm that *iscS* is responsible for DNA phosphorothioation, *iscS* was cloned into pET15b, and co-transformed with a dpt gene cluster harboring low copy number plasmid pJTU3510 into BW25113.

### Table 3. Primers that are used in this study.

| PRIMERS  | SEQUENCE                     | USE                      |
|----------|------------------------------|--------------------------|
| P1       | ATCCCGGGGATCCCGTCGACC        | Amplification of neo FRT |
| P2       | TGTAGGCCTGAGGCTCTTC          | Amplification of neo FRT |
| H1P1     | GGTAGCCTGATTCTCTGATTGATGGATGTTACGGGATCCCGTCGACC | Replacement of *iscS* |
| H2P2     | ATTATAAATTCTCTCTGATTCTGATTGATGGATGTTACGGGATCCCGTCGACC | Replacement of *iscS* |
| U        | AAGTGCCTGATTGATTCTG         | Verification of *iscS* deletion |
| D        | GAGGTCTCCTGCTGTTGTT         | Verification of *iscS* deletion |
| iscS exU | GGGAATTCAATAGAATAATCCGATTATATC | To clone *iscS* with *NdeI* site |
| iscS exD | CCGGGATCCAGCAATATAAATCTCC | To clone *iscS* with *BamHI* site |
| GST-dptF R | CCGCTCAGATGTAATACCATGTTG | To clone dptF with *XhoI* site |
| GST-dptF F | TGTAGGCTGGAGCTGCTTC | To clone dptF with *XhoI* site |
| GST-dptE R | CCGCTCAGATGTAATACCATGTTG | To clone dptE with *XhoI* site |
| GST-dptE F | TGTAGGCTGGAGCTGCTTC | To clone dptE with *XhoI* site |
| C111A R | GGGAATTCAATAGAATAATCCGATTATC | To clone *iscS* with *NdeI* site |
| C111A F | CCGGGATCCAGCAATATAAATCTCC | To clone *iscS* with *BamHI* site |
| C170A R | GGGAATTCAATAGAATAATCCGATTATC | To clone *iscS* with *NdeI* site |
| C170A F | CCGGGATCCAGCAATATAAATCTCC | To clone *iscS* with *BamHI* site |
| C328A R | GGGAATTCAATAGAATAATCCGATTATC | To clone *iscS* with *NdeI* site |
| C328A F | CCGGGATCCAGCAATATAAATCTCC | To clone *iscS* with *BamHI* site |
| iscS-CMu U | ATCCGACCAATGCGGGGATCCCG | To verify *iscS* mutations |
| iscS-CMu F | CTTCGATAGTTAATACCATGTTG | To verify *iscS* mutations |
| dptBTRG U | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptB to pTRG with *BamHI* |
| dptBTRG D | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptB to pTRG with *BamHI* |
| dptCTRUG U | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptB to pTRG with *BamHI* |
| dptCTRUG D | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptB to pTRG with *BamHI* |
| dptDTRG U | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptB to pTRG with *BamHI* |
| dptDTRG D | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptB to pTRG with *BamHI* |
| dptETRG U | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptE to pTRG with *BamHI* |
| dptETRG D | CCGGGATCCAGCAATATAAATCCGATTATC | To clone dptE to pTRG with *BamHI* |
| iscBT U | ATATAAGAATTACGGCGGATCCATTATAATCCGATTATC | To clone *iscS* to pBT with *NotI* |
| iscBT D | CCGGGATCCAGCAATATAAATCCGATTATC | To clone *iscS* to pBT with *NotI* |

IscS Participates in DNA Phosphorothioation

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**Figure 2. E. coli iscS is required for DNA phosphorothioation.** Ethidium bromide-stained agarose gels containing E. coli total genomic DNA, separated in Tris-acetate EDTA (TAE) buffer. Top gel (TAE), untreated samples; bottom gel (PAA), identical DNA samples after incubation in TAE containing 1% per-acetic acid (PAA). A fluorescent smear in the lower gel indicates that the DNA was S-modified. Lanes 1–8, Dnd (DNA degradation) phenotypes of E. coli cysteine desulfurase deletion mutants (iscS, afsU, jacA) containing the S. enterica dptBCDE gene cluster cloned on pJTU3510 (lane 1–8); lanes 9–12, trans complementation of the chromosomal iscS mutation by pJTU3619 containing mutant derivatives of iscS (lanes 9–12). E. coli hosts: wt, wild type. The mutations ΔiscS, ΔafsU and ΔjacA are in the E. coli chromosome. pJTU3510: –, no plasmid; +, pJTU3510 expressing dptBCDE. pJTU3619 (compatible with pJTU3510) containing the following genes: S+, wild-type E. coli iscS; 111, 170, 328, mutant ΔiscS genes containing the aa changes Cys111Ala, Cys170Ala or Cys328Ala, respectively. –, no plasmid. TAE, gel running buffer; PAA, TAE containing per-acetic acid.

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the E. coli iscS deletion mutant. Fig. 2 lane 9 shows that DNA phosphorothioation was restored in the strain, proving that IscS, in cooperation with DptB-E, restored DNA phosphorothioation in E. coli.

Involvement of IscS in DNA phosphorothioation in E. coli was further confirmed by site-directed mutagenesis. Three conserved cysteine residues in IscS, were mutated to Ala, generating three iscS cysteine mutants (C111A, C170A, and C328A). These mutants were then co-transformed with pJTU3510 (harboring the dpt gene cluster) into the iscS deletion mutant. Fig. 2 lane 10–12 shows that only C328A abolished DNA phosphorothioation.

**IscS might participate in DNA phosphorothioation directly.**

The cysteine desulfurase IscS is a highly conserved master enzyme initiating sulfur transfer via persulphide to a range of acceptor proteins. IscS is involved in various physiological processes, including Fe-S cluster assembly, tRNA modification, and sulfur-containing cofactor biosynthesis. IscS-interacting partners, including IscU, TusA, ThiI, ThiF and MoeB are sulfur acceptors. Other proteins, such as CyaY, IscA and IscX, also bind to IscS, but their functional roles are not directly related to sulfur transfer [16].

Mutants of cyaY, iscA, iscU, moeB, tusA, thiF, thiI and thiS, proteins known to interact with IscS in E. coli, were tested for their possibility to participate into DNA phosphorothioation. Fig. 3 shows that none of these genes was required for the modification, as assayed by Dnd phenotype. This suggested that IscS in E. coli might participate directly into the modification process.

**Protein-protein interactions between IscS and Dpt proteins.**

The bacterial two-hybrid system was used to detect interactions between E. coli IscS and DptB, C, D and E. IscS was fused with the bait protein, while DptB, C, D, and E were fused with the target protein.

Strong protein-protein interactions were immediately detected between IscS and DptC (2% surviving cells on 3AT), IscS and DptE (2% surviving cells on 3AT), but not between IscS and DptB and DptD (Fig. 4A). These protein interactions were confirmed further by plating the co-transformed strains on medium containing streptomycin (Fig. 4B).

Protein-protein interaction between IscS and DptC as well as IscS and DptE were further confirmed by pull-down experiments. Fig. 4C shows that Strep tagged IscS can pull-down both GST tagged DptC and DptE. Reciprocally, GST tagged DptC and DptE can also pull-down Strep tagged IscS.

**Discussion**

IscS is a highly conserved, but functionally versatile pyridoxal-5’-phosphate (PLP)-dependent enzyme. It delivers sulfur to players within various metabolic pathways, including iron-sulfur cluster assembly, thiamine and biotin synthesis, tRNA modifications, and molybdopterin biosynthesis [16,17]. We show here that IscS can also participate in DNA phosphorothioation.

The involvement of IscS in DNA phosphorothioation could be direct or indirect. By analyzing the Dnd phenotype and the mutants (Fig. 3), we were able to rule out the possibility that IscS participates indirectly via other pathways. We hypothesized that if IscS is involved in the DNA phosphorothioation process directly, we might be able to detect protein-protein interaction between IscS and the Dnd proteins. In keeping with this hypothesis, protein interaction between IscS and DndE and DndC were detected using the bacterial two hybrid system.

There are two potential functions of IscS in the process of DNA phosphorothioation. One is Fe-S cluster assembly for the DndC protein. It is known that DndA can catalyze apo-Fe DndC to its Fe-S cluster form [5]. Another function might be to transfer sulfur from cysteine to the target DNA via protein interactions with the Dnd proteins, which is reminiscent of tRNA modification [18,19]. These hypotheses are currently under intensive investigation.

**Figure 3. IscS might participate DNA phosphorothioation directly.** Ethidium bromide-stained agarose gels. TAE (top gel), samples run in normal TAE buffer; PAA (bottom gel), samples run in TAE containing PAA. Expression of S. enterica dptB-E resulted in DNA S-modification and a fluorescent smear in all samples, except for E. coli ΔiscS. IscS was therefore the only gene that was required for DNA S-modification among the tested deletions.

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Supporting Information

Figure S1 Disruption of iscS gene. A. Replacement of iscS by PCR targeting using a neo cassette flanked by 50 bp homologous E. coli sequences. B. Ethidium bromide-stained agarose gel showing PCR products obtained from E. coli DiscS and wild-type E. coli, using flanking primers.

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Author Contributions

Conceived and designed the experiments: JDL ZXD ZJW. Performed the experiments: XHA JDL WX YY FHL. Analyzed the data: XHA JDL. Wrote the paper: XHA JDL XFZ ZXD ZJW.

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