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DndEi Exhibits Helicase Activity Essential for DNA Phosphorothioate Modification and ATPase Activity Strongly Stimulated by DNA Substrate with a GAAC/GTTC Motif*

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Phosphorothioate (PT) modification of DNA, in which the non-bridging oxygen of the backbone phosphate group is replaced by sulfur, is governed by the DndA-E proteins in prokaryotes. To better understand the biochemical mechanism of PT modification, functional analysis of the recently found PT-modifying enzyme DndEi, which has an additional domain compared with canonical DndE, from *Riemerella anatipestifer* is performed in this study. The additional domain is identified as a DNA helicase, and functional deletion of this domain *in vivo* leads to PT modification deficiency, indicating an essential role of helicase activity in PT modification. Subsequent analysis reveals that the additional domain has an ATPase activity. Intriguingly, the ATPase activity is strongly stimulated by DNA substrate containing a GAAC/GTTC motif (*i.e.* the motif at which PT modifications occur in *R. anatipestifer*) when the additional domain and the other domain (homologous to canonical DndE) are co-expressed as a full-length DndEi. These results reveal that PT modification is a biochemical process with DNA strand separation and intense ATP hydrolysis.

Phosphorothioate (PT)[3] modification of DNA, in which the non-bridging phosphate is replaced by sulfur (1), is widespread in prokaryotes with diverse sequence contexts (2). In terms of physiological function, PT modifications are found to participate in preventing foreign DNA introduction via a restriction-modification system (3, 4).

PT modifications are governed by a large family of five-gene clusters termed as *dndA-E* (Fig. 1). DndB is a negative transcriptional regulator for the PT-modifying genes (5), whereas it is not indispensable for PT modification and is absent in some PT-containing bacteria (5–7). DndA and DndC-E are all essential for PT modification identified by *in vivo* studies (7, 8). DndA is a cysteine desulfurase capable of assembling DndC as an iron-sulfur cluster protein (9, 10). In some cases, DndA is functionally replaced by the cysteine desulfurase IscS (11). DndD was predicted to be an ABC transporter ATP-binding protein (8) and DndE was reported to be a DNA-binding protein (12); nevertheless, their specific functions in PT modification are still obscure. Remarkably, there are two kinds of DndE protein, one is named DndE and the other is named DndEi (Fig. 1). DndEi is a recently found PT-modifying enzyme that has an additional putative helicase domain compared with canonical DndE (13). The putative helicase domain belongs to the AAA+ family of ATPases (13), a large class of ATPases that include several other helicases such as the MCM protein involved in DNA replication and RuvB protein involved in homologous recombination (14). The AAA+ family of proteins also includes dynein, chaperone proteins, transcriptional regulators, vesicular fusion proteins, and other proteins involved in additional diverse functions (14). The additional domain probably endows PT-modifying enzyme with helicase activity that has never been found in PT modification; however, no functional study of DndEi has ever been reported.

Bioinformatic analysis has revealed that a *dndCDEi* gene cluster exist in the genome of *Riemerella anatipestifer* strain ATCC 11845 (13). In this study, the enzymatic activities of DndEi from this strain were characterized. DndEi has a DNA helicase activity that is proved to be essential for PT modification; meanwhile, it has an ATPase activity that is strongly stimulated by double-stranded DNA containing a GAAC/GTTC motif.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were routinely grown in Luria broth (LB) at 37 °C and *R. anatipestifer* strains were cultured in tryptic soybean broth (Difco Laboratories, Detroit, MI) at 37 °C under 5% CO_2.

Gene Cloning and Plasmid Construction

E. coli strain DH5α was used for plasmid construction.

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[3] The abbreviations used are: PT, phosphorothioate; gDNA, genomic DNA; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside.

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Construction of plasmids used for overexpression of DndEi, DndEih, DndEi (K200A), and DndEi (D411A)—The gene encoding DndEi (Riean_0555) was amplified from *R. anatipestifer* ATCC 11845 genomic DNA, using KOD polymerase (Toyobo), with the sense primer A1F (CCGAGACATATGCAAATTAACATAAGAACATC) and the antisense primer A1R (TACTTAGTCGACGTTGTTCTTCTTTATGGGGT). Amplified PCR products were digested with NdeI and SalI (Fermentas), and then ligated into pET-28a, creating the plasmid pT1. The coding region of DndEih was amplified with A2F (GGGTGTCATATGTTCGATTTTTTAACTGAACAT) and A1R primers. Amplified PCR products were digested with NdeI and SalI, and ligated into pET-28a, creating the plasmid pT2. To obtain the DndEi K200A mutant, pT3 was constructed by PCR using pT1 as a template and A3F (AGGAAGTTCAGGAACAGGAGCAACACAATTTGCAT) and A3R (GCTCCTGTTCCTGAACTTCCTGCAACTGCTATG) as primers. To obtain the DndEi D411A mutant, pT4 was constructed by PCR using pT1 as a template and A4F (CGTTATGTTTTGTTAATTGCTGAAGCACATGT) and A4R (GCAATTAACAAAACATAACGCATAGCACGATA) as primers. All constructed plasmids were sequenced completely to ensure that no errors had been introduced.

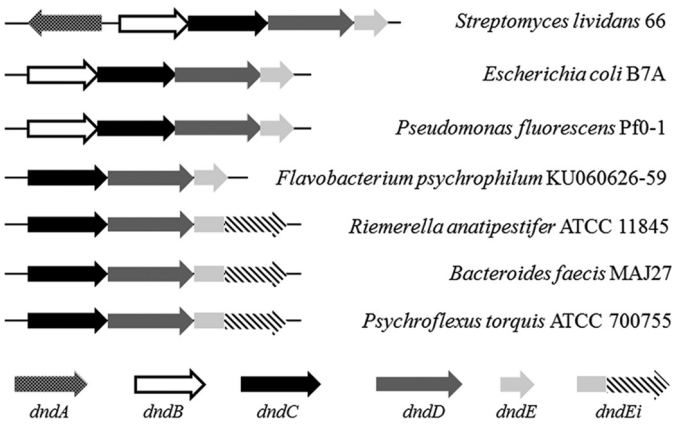

FIGURE 1. **Genomic organization of the PT-modifying genes in 7 members of bacteria.** Strains and GenBank™ accession numbers: *S. lividans* 66 (CM001889.1); *E. coli* B7A (CP005998.1); *Pseudomonas fluorescens* Pf0–1 (CP000094.2); *Flavobacterium psychrophilum* KU060626-59 (KF241852); *R. anatipestifer* ATCC 11845 (CP003388.1); *Bacteroides faecis* MAJ27 (NZ_AGDG01000022.1); *Psychroflexus torquis* ATCC 700755 (CP003879.1).

Construction of Plasmids Used for Heterologous Expression of PT-modifying Genes in R. anatipestifer HXb2—The coding region of DndCD and their promoter region (−715 to −1) were amplified from *R. anatipestifer* ATCC 11845, with the A5F (AAGAGTCTCGAGTCACGGTAGAAGCGGCAT) and A5R (TCGAGGTCTAGATTAATTTGCATAAAGCTCGT) primers. PCR products were digested with XbaI and XhoI (Fermentas), and then ligated into pBluescript SK(+) to obtain the plasmid SK1 for sequencing. The XbaI/XhoI-digested fragment from SK1 was inserted into the corresponding sites of pReS0 (15), generating pJ1. The coding region of DndCDEie and their promoter region were amplified with the A5F and A6R (CGTCGGTCTAGATTAAGTTTTTTCATCTAACTTATTTCC) primers. The stop codon TAA was introduced to the 3′ end of the *dndEie* gene after amplification. PCR products were ligated into pBluescript SK(+) to obtain SK2 for sequencing and then inserted into pReS0, generating pJ2. The coding region of DndCDEi and their promoter region were amplified with the A5F and A7R (GACTGCTCTAGACCAGCGGAAGTAAGGTAT) primers. PCR products were ligated into pBluescript SK(+) to obtain SK3 for sequencing and then inserted into pReS0, generating pJ3. SK4 plasmid was constructed by PCR using SK3 as a template and A3F and A3R as primers. The XbaI/XhoI-digested fragment from SK4 was inserted into pReS0, generating pJ4. SK5 plasmid was constructed by PCR using SK3 as a template and A4F and A4R as primers. The XbaI/XhoI-digested fragment from SK5 was inserted into pReS0, generating pJ5.

Expression and Purification of the N-terminal His-tagged DndEi, DndEih, DndEi (K200A), and DndEi (D411A)

E. coli BL21(DE3) harboring the corresponding expression plasmid was first grown at 37 °C to an optical density at 600 nm (A_{600}) of 0.8 and subsequently induced at 22 °C for 10 h with 0.4 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested by centrifugation, and the pellets were re-suspended in buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). Re-suspended cells were subjected to three freeze-thaw cycles and then sonically

TABLE 1
Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
E. coli		
DH5α	F⁻ Δlacu169 (φ80lacZΔM15), recA1, endA1, hsdR17	Biomedal
BL21(DE3)	hsdS gal, chromosomal insertion of T7 pol	Novagen
S17-1	lpir hsdR pro thi; chromosomal integrated RP4–2 Tc::Mu Km::Tn7	Biomedal
R. anatipestifer		
ATCC 11845	Wild-type, PT⁺	15
HXb2	Wild-type, PT⁻	15
Plasmids		
pBluescript SK(+)	Bla, lacZ, orif1, ColE, Ap^r	Stratagene
pET-28a	Expression vector, pBR322 replicon, P_{T7}, His_6 tag, Km^r	Novagen
pT1	Based on pET-28a, with DNA region coding DndEi inserted at NdeI-SalI sites	This study
pT2	Based on pET-28a, with DNA region coding DndEih inserted at NdeI-SalI sites	This study
pT3	Based on pT1, with A (the 599th nucletide in *dndEi* gene) mutated to C	This study
pT4	Based on pT1, with A (the 1232nd nucletide in *dndEi* gene) mutated to C	This study
pReS0	ColE1 ori, Ap^r, *E. coli-R. anatipestifer* shuttle plasmid	15
pJ1	Based on pReS0, with *dndCD* genes and their promoter region inserted at XbaI-XhoI sites	This study
pJ2	Based on pReS0, with *dndCDEie* genes and their promoter region inserted at XbaI-XhoI sites	This study
pJ3	Based on pReS0, with *dndCDEi* genes and their promoter region inserted at XbaI-XhoI sites	This study
pJ4	Based on pJ3, with A (the 599th nucletide in *dndEi* gene) mutated to C	This study
pJ5	Based on pJ3, with A (the 1232nd nucletide in *dndEi* gene) mutated to C	This study

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lysed. After centrifugation (10,000 × *g* for 60 min at 4 °C), the supernatant was loaded onto a Ni^{2+}-nitrilotriacetic acid-agarose column (Invitrogen). Elution was carried out with buffer B (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 200 mM imidazole). Fractions containing targeted protein were pooled and concentrated to 2.5 ml with Amicon Ultra Centrifugal Filter 10,000 MWCO (Millipore). PD-10 columns (GE healthcare) were then used for imidazole elimination and buffer exchange according to the manufacturer's recommendation. Expression and purification of the His-tagged protein were analyzed by 12% SDS-PAGE and the concentration was determined by the Bradford method with bovine serum albumin (BSA) as the standard.

Conjugation Assay

E. coli S17-1 was used for conjugative transfer of plasmids into *R. anatipestifer*. Donor *E. coli* S17-1 and recipient *R. anatipestifer* strains were grown to mid-log phase, respectively, and plasmid was transferred into the recipient strains by biparental mating as described previously (16).

Iodine Cleavage of PT-modified Genomic DNA

PT-modified DNA can be cleaved by iodine at the modified sites (17). A 30 mM iodine solution was freshly prepared and reactions (20 μl) were then setup in PCR tubes as follows: genomic DNA (2 μg), 50 mM Na_2HPO_4, pH 9.0, 3 mM I_2. Reactions were heated to 65 °C for 10 min and then slow cooled (0.1 °C/s) to 4 °C using a thermal cycler (Eppendorf Mastercycler pro S). The iodine-cleaved genomic DNA was run on a 0.8% agarose gel in 1× TAE containing 50 μM thiourea.

LC-MS Analysis of PT-containing Dinucleotides

20 μg of genomic DNA was hydrolyzed with 4 units of nuclease P1 (Sigma) and subsequently dephosphorylated by 10 units of alkaline phosphatase (Fermentas), essentially as described elsewhere (2). The digested DNA samples were dried and re-suspended in 50 μl of deionized water for analysis by liquid chromatography-coupled, time-of-flight mass spectrometry. The sample was resolved on an Agilent ZORBAX SB-C18 column (2.1 × 150 mm, 3.5-μm bead size) with a flow rate of 0.3 ml/min and the following parameters: column temperature: 35 °C; solvent A: 0.1% acetic acid in H_2O; solvent B: 0.1% acetic acid in acetonitrile; gradient: 4% B for 10 min, 4 to 16% B over 15 min, and 16 to 100% B over 1 min. The HPLC column was coupled to an Agilent 6230 TOF mass spectrometer with an electrospray ionization source in positive mode with the following parameters: gas flow, 10 liters/min; nebulizer pressure, 30 psi; drying gas temperature, 325 °C; and capillary voltage, 3,000 V.

Fluorescent Helicase Assay

DNA substrates (Table 2) used for helicase assay were prepared by annealing an Alexa Fluor 488-labeled oligonucleotide to a black hole quencher-1-labeled oligonucleotide at a 1:1.5 molar ratio. A typical fluorescence helicase assay was performed as described previously (18) with a slight modification. The assay was performed in 30 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.075% Triton X-100, 20 nM DNA substrate, 1 mM ATP, and 250 nM capture strand. The unwinding reaction was started by addition of 1 nmol of helicase enzyme and was carried out in a 200-μl volume at 37 °C for up to 60 min. The fluorescent signal increase was registered every 30 s using a Fluorescence Reader (Biotek synergy 2). The fluorophore was excited at 485 nm and the helicase activity was measured at 528 nm. The enzyme activity was calculated as the initial reaction velocity from the linear part of the progress curve using the linear regression method. A linear equation $Y = AX + B$ was fitted to experimental data, where Y is the enzyme activity expressed in fluorescence units, X is the reaction time, and A is the initial velocity.

Single-stranded DNA Translocation Assay—Translocation activity was investigated by measuring the ability of DndEih to displace streptavidin from a biotinylated probe at the 5′ or 3′ end of ssDNA. A 6-FAM-labeled 50-mer oligodeoxythymidylates with a biotin label on the end was incubated at a final concentration of 30 nM with 1 μM streptavidin in helicase buffer containing 1 mM ATP at 37 °C for 10 min to allow the streptavidin to bind to the DNA. Biotin (30 μM) was then added as a streptavidin trap, and the reaction was initiated by the addition of 5 μM DndEih. After 30 min, 10-μl aliquots of the reaction mixture were removed and added to 2 μl of stop solution (1 M NaCl and 200 mM EDTA, pH 8.0) together with 10 μM non-biotinylated oligodeoxythymidylates to bind to the protein and to reduce band shifting. The samples were analyzed on a 10% native acrylamide:Tris borate-EDTA gel.

ATPase Activity Assay

The ATPase activity was assayed using the EnzCheck pyrophosphate assay kit (Life Technologies) based on a previously reported procedure (19). The standard assay was performed in a 200-μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM sodium azide, 0.2 mM 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), 1 unit of purine nucleoside phosphorylase, 1 mM ATP, and 1 nmol of DndEi or DndEih in the absence or presence of 150 nM 34-bp length dsDNA substrates. Inorganic phosphate (P_i) created by ATP hydrolysis activates the enzymatic conversion of MESG to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase. Conversion of MESG results in a shift in absorbance maximum from 330 to 360 nm, which is monitored by the Biotek synergy 2 plate reader. The absorbance at 360 nm was measured and corrected with a control reaction lacking protein. The relationship between the absorbance at 360 nm and P_i concentration was established by using KH_2PO_4 as a standard. The enzyme activity was calculated as the initial reaction velocity from the linear part of the progress curve.

Results

PT Modifications Occur at $G_{ps}AAC/G_{ps}TTC$ Sites in R. anatipestifer ATCC 11845—Previous bioinformatic analysis has revealed that a *dndCDEi* gene cluster exists in *R. anatipestifer* ATCC 11845 (13). To confirm the PT phenotype of this strain, we performed the PT-specific iodine cleavage assay as previously described (17). As expected, the genomic DNA (gDNA) from *R. anatipestifer* ATCC 11845 was cleaved into small fragments by iodine, whereas the gDNA from *R. anatipestifer* HXb2

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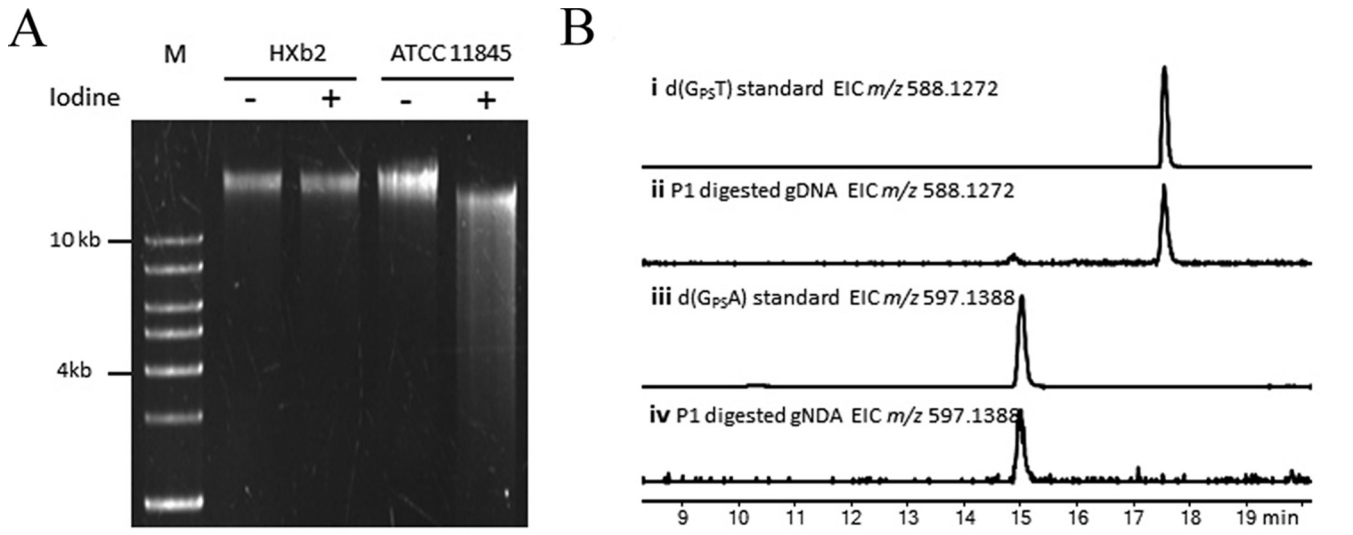

FIGURE 2. **Analysis of the PT modification in *R. anatipestifer* ATCC 11845.** *A,* electrophoresis pattern of the iodine-cleaved genomic DNA from *R. anatipestifer* ATCC 11845 (*lanes 4* and *5*), with *R. anatipestifer* HXb2 (lacking PT-modifying genes; *lanes 2* and *3*) as a negative control. *M,* DNA ladder. *B,* LC-MS analysis of the PT-linked dinucleotides from *R. anatipestifer* ATCC 11845.

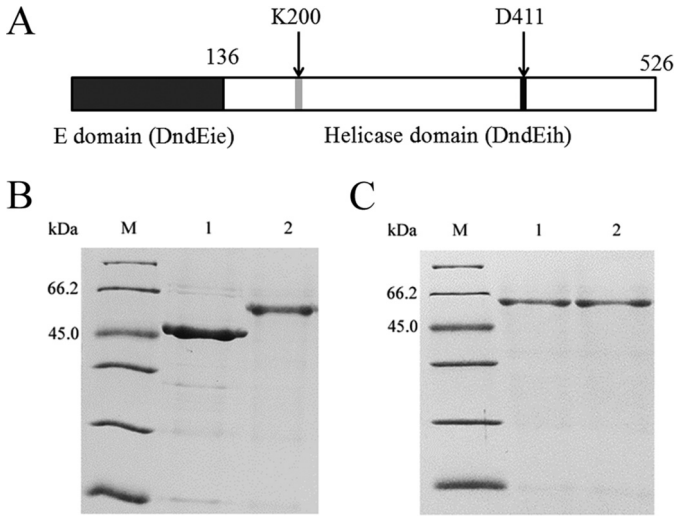

FIGURE 3. **Purification of DndEih, DndEi, and DndEi mutants.** *A,* domain organization of the DndEi protein. The positions of the two conserved motifs are indicated by *gray* and *black boxes*, respectively. The positions of the two point mutations are indicated by *arrows*. *B,* SDS-PAGE analysis of purified DndEih and DndEi stained by Coomassie Blue. Lanes: *M*, molecular mass marker; *1*, DndEih; *2*, DndEi. *C,* SDS-PAGE analysis of purified DndEi mutants stained by Coomassie Blue. Lanes: *M*, molecular mass marker; *1*, DndEi (K200A); *2*, DndEi (D411A).

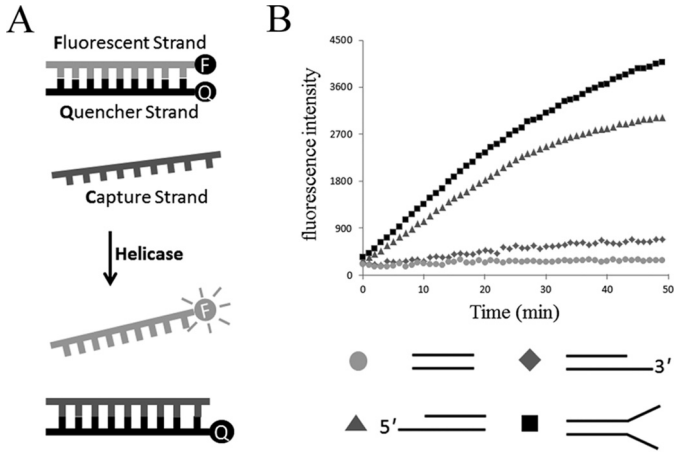

FIGURE 4. **DNA helicase activity of DndEih using various DNA substrates.** *A*, schematic representation of the fluorescence helicase assay based on FRET. The fluorescent strand is labeled with a fluorophore (*F*) and quencher strand is labeled with a quencher (*Q*). Annealing of the fluorophore strand to the quencher strand results in the fluorescence signal from the Alexa Fluor 488 dye to be quenched. When the double-stranded DNA substrate is unwound by the helicase, the fluorophore emits light upon its release from the quencher. The capture strand, which is complementary to the quencher strand, prevents the reannealing of the unwound duplex. *B,* time course of the helicase reaction of DndEih using 3′-overhang (*diamonds*), 5′-overhang (*triangles*), blunt-ended (*circles*), and forked (*squares*) DNA substrates in the presence of ATP.

(closely related to ATCC 11845, but lacking PT-modifying genes) was not cleaved (Fig. 2*A*). This result revealed that PT modification indeed existed in *R. anatipestifer* ATCC 11845, and it also revealed that PT modification occurred on both strands of gDNA, because single strand-modified gDNA cannot be cleaved into small fragments by iodine (17). LC/MS analysis was subsequently performed to investigate the PT sequence context in *R. anatipestifer* ATCC 11845 by the method of detection of the nuclease P1-resistent PT-containing dinucleotides (2). As shown in Fig. 2*B*, only d(G_{ps}A) and d(G_{ps}T) dinucleotides were detected in *R. anatipestifer* ATCC 11845. PT sequencing has revealed that PT modifications occur at G_{ps}AAC/G_{ps}TTC sites in *E. coli* B7A (17), which has the same iodine-cleaved pattern and the same PT-linked dinucleotides as *R. anatipestifer* ATCC 11845. Taken together, these results indicated that gDNA is PT modified on both strands of GAAC/GTTC motifs in *R. anatipestifer* ATCC 11845.

DndEi Unwinds dsDNA Substrates with a 5′ Single-stranded Region—The *dndEi* gene from *R. anatipestifer* ATCC 11845 encodes a hybrid protein that is composed of an E domain (DndEie, homologous to canonical DndE) and an additional putative DNA helicase domain (DndEih) (Fig. 3*A*). To investigate the helicase activity of DndEi, both the putative helicase domain and the full-length protein were expressed and purified (Fig. 3*B*). Purification of DndEi mutant proteins, whose use is described later, was also performed as shown in Fig. 3*C*.

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TABLE 2
DNA substrates used in helicase assay in this study

Substrates	Characteristics	Sequence	Modification
S1		strandF: 5'-GTAACGCCAGGGTTCTCCCAGTCAC-3'	3'BHQ-1
		strandR: 5'-GTGACTGGGAGAACCCTGGCGTTAC-3'	5'Alexa Fluor 488
S2	3′	strandF: 5'-GTAACGCCAGGGTTCTCCCAGTCAC-3'	3'BHQ-1
		strandR: 5'-GTGACTGGGAGAACCCTGGCGTTACCCAACTTAATCGCCT-3'	5'Alexa Fluor 488
S3	5′	strandF: 5'-GTAACGCCAGGGTTCTCCCAGTCAC-3'	5'BHQ-1
		strandR: 5'-TCGTTTTACAACGTCGTGACTGGGAGAACCCTGGCGTTAC-3'	3'Alexa Fluor 488
S4		strandF: 5'- GTAACGCCAGGGTTCTCCCAGTCACTTTTTTTTTTTTTTT-3'	5'BHQ-1
		strandR: 5'-TCGTTTTACAACGTCGTGACTGGGAGAACCCTGGCGTTAC-3'	3'Alexa Fluor 488
S5	no GAAC/GTTC motif	strandF: 5'-GTAACGCCAGGGTTTTCCCAGTCAC-3'	5'BHQ-1
		strandR: 5'-TCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTAC-3'	3'Alexa Fluor 488
S6	with a $G_{ps}AAC/G_{ps}TTC$ motif	strandF: 5'-GTAACGCCAGGG_{ps}TTCTCCCAGTCAC-3'	5'BHQ-1
		strandR: 5'-TCGTTTTACAACGTCGTGACTGGGAG_{ps}AACCCTGGCGTTAC-3'	3'Alexa Fluor 488

A previously reported fluorescence resonance energy transfer (FRET) assay (18, 20) was performed to detect the DNA strands separation in real time. In this assay, the fluorescent strand of the duplex DNA was modified with an Alexa Fluor 488 fluorescent dye, whereas the quencher strand was modified with a spectrally paired quencher dye, black hole quencher. Annealing of the two strands brought the two dyes close together, resulting in efficient fluorescence quenching. Duplex unwinding was monitored by observing an increase in Alexa Fluor 488 fluorescence following strand separation. Re-annealing of the fluorescent strand to the quencher strand was suppressed by the presence of a 12-fold molar excess of a capture strand that is complementary to the quencher strand (Fig. 4*A*). DNA substrates with a 3′-overhang containing a 15-nucleotide ssDNA region and a 25-bp duplex region, a 5′-overhang containing a 15-nucleotide ssDNA region and a 25-bp duplex region, or blunt ends containing a 25-bp duplex region (Table 2) were tested in this assay. DndEih unwound the 5′-overhang but not 3′-overhang or blunt DNA substrates in the presence of ATP (Fig. 4*B*). The fluorescence intensity had a slight increase in the helicase assay using 3′-overhang DNA substrates, but the increase was not due to the helicase activity of DndEih because the fluorescence curve did not change when the DndEih concentration changed (data not shown). These results revealed that DndEih requires a 5′ single-stranded region to unwind dsDNA, which is consistent with the observation that DndEih translocated along the ssDNA from 5′ to 3′ (Fig. 5). Forked substrates are relevant intermediates in DNA metabolic processes, therefore DNA substrates with single-stranded 5′ and 3′ arms were tested in helicase assay (Fig. 4*B*). DndEih unwound forked DNA substrates more effectively than 5′-overhang DNA substrates, indicating a preference of DNA substrates with the

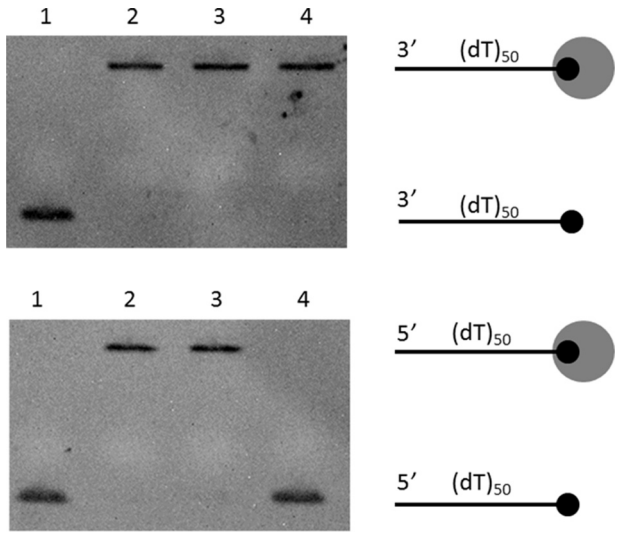

FIGURE 5. **Single-stranded DNA translocation activity of DndEih.** Translocation activity was investigated by measuring the ability of DndEih to displace streptavidin from a biotinylated probe at the 5′ or 3′ end of ssDNA. The *black circle* indicates the biotin-labeled DNA end, with the *larger circle* representing the streptavidin. *Lane 1*, biotinylated DNA; *lane 2*, biotinylated DNA bound to streptavidin; *lane 3*, streptavidin-bound DNA incubated with DndEih for 30 min in the absence of ATP; *lane 4*, streptavidin-bound DNA incubated with DndEih for 30 min in the presence of ATP.

fork structure. As DndEih is implicated in PT modification that occurs at the GAAC/GTTC sites in *R. anatipestifer* ATCC 11845, the helicase activities of DndEih to unwind DNA substrates with a GAAC/GTTC site, a PT-modified GpsAAC/GpsTTC site, or no GAAC/GTTC site were tested. As shown in Fig. 6*A*, the helicase activities (initial velocities) to unwind the three kinds of DNA substrate were at the same level. Subsequently, the full-length DndEi was tested in the helicase assay

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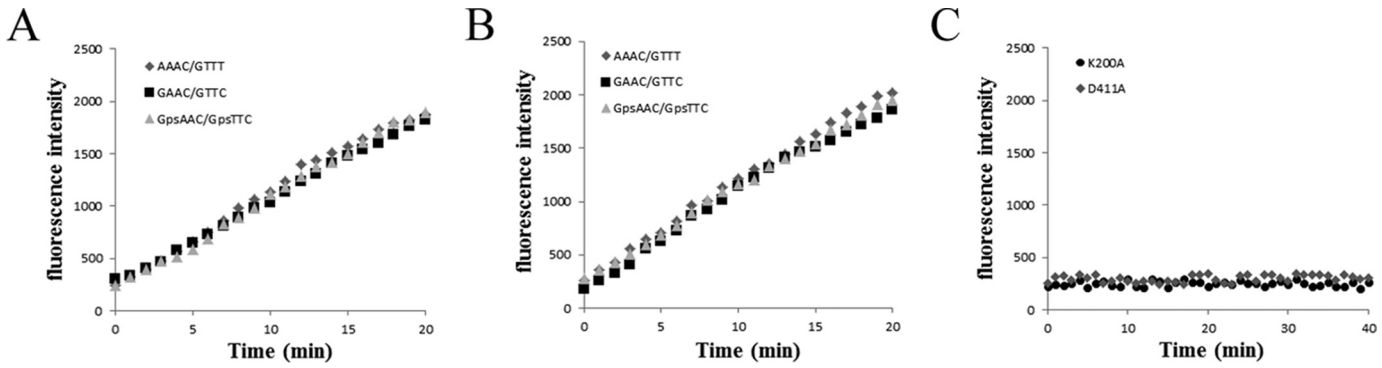

FIGURE 6. **DNA helicase activity of DndEih, DndEi, and DndEi mutants.** *A* and *B*, time course of the helicase reaction of DndEih (*A*) and DndEi (*B*) using 5′-overhang DNA substrates with different sequence motif as indicated. *C*, time course of the helicase reaction of DndEi mutants using 5′-overhang DNA substrates.

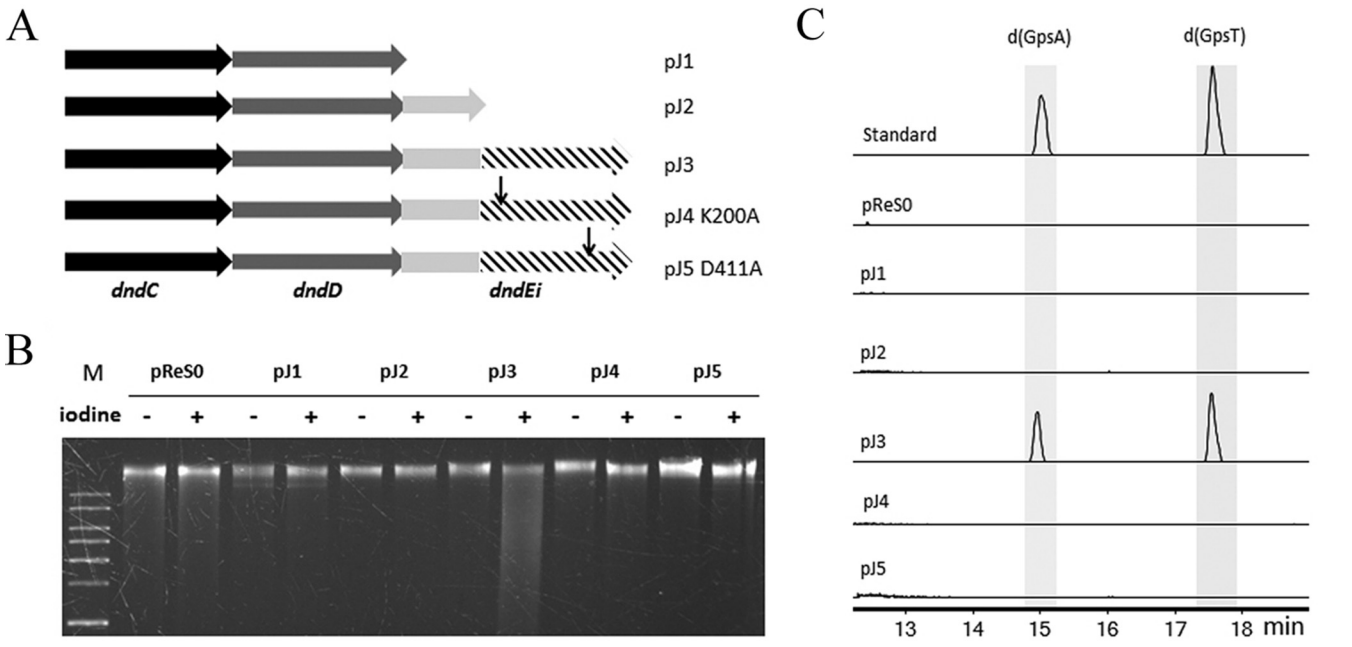

FIGURE 7. **Analysis of the PT modification in *R. anatipestifer* HXb2 harboring a pReS0-based plasmid (pJ1–5), which has the PT-modifying genes from *R. anatipestifer* ATCC 11845.** *A*, schematic diagram of the PT-modifying genes in pReS0-based plasmids (Table 1). *B*, electrophoresis pattern of the iodine-cleaved genomic DNA from *R. anatipestifer* HXb2 (pJ1) (*lanes 4* and *5*), HXb2 (pJ2) (*lanes 6* and *7*), HXb2 (pJ3) (*lanes 8* and *9*), HXb2 (pJ4) (*lanes 10* and *11*), HXb2 (pJ5) (*lanes 12* and *13*), and HXb2 (pReS0 empty vector) (*lanes 2* and *3*). *M*, DNA ladder. *C*, LC-MS analysis of the PT-linked dinucleotides from *R. anatipestifer* HXb2 harboring a pReS0-based plasmid. Electrospray ionization: m/z $[M + H]^+$ modes for LC-MS: d(G_{ps}A), 597.1388; d(G_{ps}T), 588.1272.

(Fig. 6*B*), and it had the same performance as DndEih, indicating that the E domain had no effect on the helicase activity of the helicase domain.

Sequence alignment analysis of the helicase domain of bacterial DndEi homologues revealed two conserved motifs (13) that are also conserved in other helicases (21). Motif I (G*XXXX*GKT, where *X* is any residue) at the N-terminal is supposed to be involved in nucleotide phosphate binding and hydrolysis (21), and Motif II (DEAH) is supposed to be responsible for coupling of ATPase and helicase activity (21, 22). To elucidate the functional roles of these motifs, we introduced a point mutation in motif I of DndEi, replacing the conserved lysine with alanine (K200A) and a point mutation in motif II, replacing the conserved aspartate with alanine (D411A) (Fig. 3*A*). K200A and D411A mutant proteins were then overproduced in *E. coli* and purified (Fig. 3*C*) using the same procedure as for the wild-type protein. The purified K200A and D411A proteins did not exhibit any detectable helicase activity (Fig. 6*C*), indicating that both Lys-200 and Asp-411 are essential residues for DNA helicase activity.

The Helicase Domain of DndEi Is Essential for PT Modification—Because the DNA helicase domain is not found in all of the DndE homologues, we next identified whether the helicase domain is required for PT modification in *R. anatipestifer*. Five plasmids (pJ1–5, Fig. 7*A*) containing the PT-modifying genes or its derivatives from *R. anatipestifer* ATCC 11845 were constructed based on the *E. coli-R. anatipestifer* shuttle plasmid pReS0. The recombinant plasmids were transferred into *R. anatipestifer* HXb2 (lacking PT-modifying genes) by conjugation. Genomic DNA of the transformants was then extracted and analyzed by a iodine cleavage assay (Fig. 7*B*) and LC/MS (Fig. 7*C*). The gDNA from HXb2 with *dndCDEi* (pJ3) was cleaved into small fragments (Fig. 7*B*) and PT-linked dinucleotides were detected in HXb2 (pJ3) by LC/MS (Fig. 7*C*),

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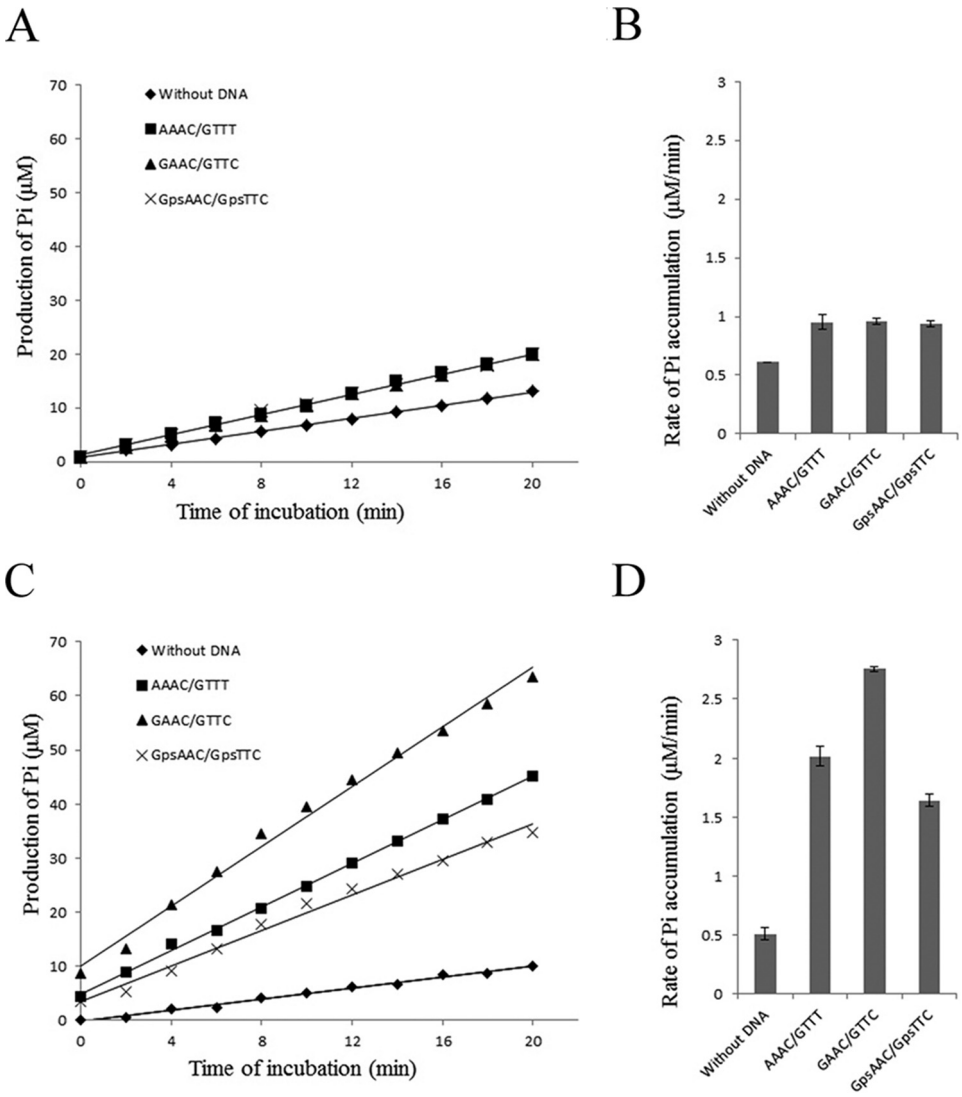

FIGURE 8. **ATPase activity of DndEih (*A* and *B*) and DndEi (*C* and *D*).** *A* and *C*, representative time courses of ATP hydrolysis by DndEih and DndEi. The ATPase assay was performed in the absence of DNA or in the presence of different kinds of dsDNA substrate (with no GAAC/GTTC motif; with a GAAC/GTTC motif; with a PT-modified $G_{ps}AAC/G_{ps}TTC$ motif). The amount of P_i produced was calculated based on a standard KH_2PO_4 curve. Each data point represents the mean of triplicate experiments. *B*, rates of P_i accumulation in the ATPase assay performed in *A*. The data are presented as mean ± S.D. *D*, rates of P_i accumulation in the ATPase assay performed in *C*. The data are presented as mean ± S.D.

indicating that the intact *dndCDEi* conferred PT modification in the heterologous host HXb2. Meanwhile, absence of the DNA helicase domain (pJ2) resulted in loss of its PT modification activity (Fig. 7, *B* and *C*), indicating that the helicase domain of DndEi is essential for PT modification. Furthermore, HXb2 (pJ4, DndEi K200A) and HXb2 (pJ5, DndEi D411A), in which DndEi was not deleted but the helicase activity was inactivated, did not have any detectable PT phenotype (Fig. 7, *B* and *C*), suggesting that the helicase activity is implicated in PT modification.

DndEi Has an ATPase Activity Strongly Stimulated by the GAAC/GTTC Motif—Helicases disrupt the hydrogen bonds that hold the two strands of duplex DNA together in a reaction that is coupled with the hydrolysis of a nucleoside 5′-triphosphate, and thus nearly all helicases are also DNA-dependent nucleoside 5′-triphosphatases (23). We therefore examined the ATPase activity of the helicase domain of DndEi by using the pyrophosphate assay kit. It was assayed by measuring an increase in inorganic phosphate as a result of ATP hydrolysis as described previously (19). As shown in Fig. 8*A*, DndEih exhibited an ATPase activity that was stimulated by double-stranded DNA, and DNA substrates with different sequence context had the same effect on the stimulation of the ATPase activity (Fig. 8*B*).

Because the helicase domain and the E domain (had no ATPase activity; data not shown) form a hybrid protein *in vivo*, the ATPase activity of the full-length DndEi was subsequently detected (Fig. 8*C*) to see whether the E domain had an effect on ATPase activity of the helicase domain. DndEi also exhibited an ATPase activity that was stimulated by double-stranded DNA as DndEih. However, DNA fragments containing a GAAC/GTTC motif stimulated ATPase (5.4-fold) greater than DNA fragments without this motif (3.9-fold) and DNA fragments containing a PT-modified $G_{ps}AAC/G_{ps}TTC$ motif (3.2-fold) (Fig. 8*D*). These results indicated that the E domain can affect the ATPase activity of the helicase domain by recognizing dif-

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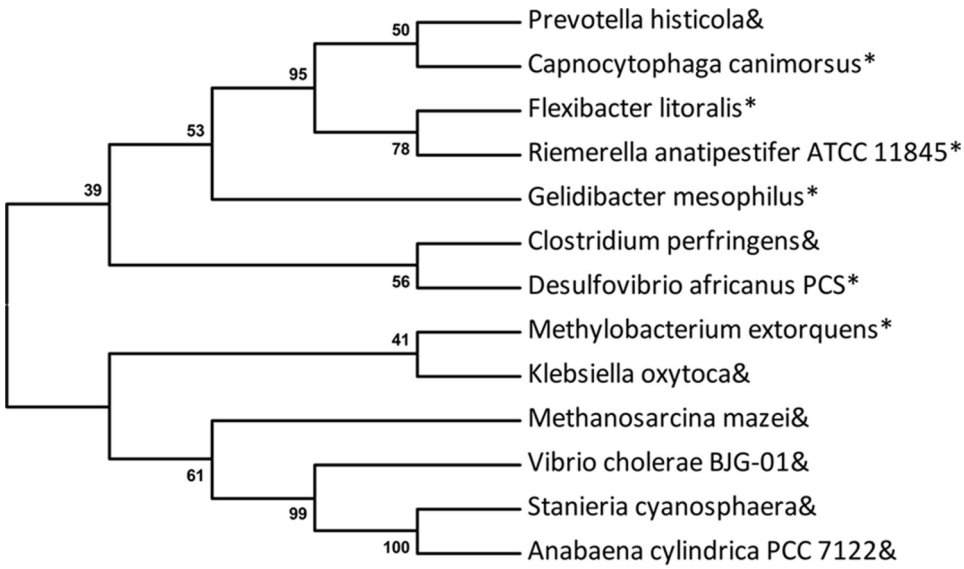

FIGURE 9. **Phylogenetic analysis of DndE homologues, using MEGA v5.0 with 500 bootstrap replicates.** *Ampersand*: DndE; *asterisk*: the E domain of DndEi (DndEie). GenBank™ accession numbers of the DndE homologues are: *C. canimorsus* (WP_041913577.1); *R. anatipestifer* ATCC 11845 (ADQ81723.1); *Vibrio cholerae* BJG-01 (EGS71244.1); *Gelidibacter mesophilus* (WP_027127205); *Methylobacterium extorquens* (WP_012754233); *Klebsiella oxytoca* (WP_046878204.1); *C. perfringens* (WP_003471805); *Desulfovibrio africanus* (PCS EMG37157); *Methanosarcina mazei* (KKI02757.1); *S. cyanosphaera* (WP_041620172.1); *P. histicola* (WP_036868676.1); *Flexibacter litoralis* (WP_041264540); and *Anabaena cylindrica* PCC 7122 (AFZ60204.1).

ferent DNA substrates. Considering the DNA fragment with a GAAC/GTTC motif is the substrate of PT-modifying enzymes, the strongly stimulated ATPase activity is probably relevant to DNA PT modification.

Discussion

DNA helicases are a class of enzymes vital to all living organisms (23). They are molecular motor enzymes that use the energy of ATP hydrolysis to separate duplex DNA into single strands (21). Nearly all DNA metabolic processes, such as DNA replication (24), recombination (25), and repair (26), involve the separation of DNA strands that necessitates the use of DNA helicases. In this study, DNA helicase was shown to have a novel biological function (*i.e.* participating in DNA PT modification). The PT-modifying enzyme DndEi from *R. anatipestifer* was identified as a 5′ to 3′ DNA helicase with a preference of forked DNA substrates. Both deletion of the helicase domain and inactivation of the helicase activity of DndEi can lead to PT modification deficiency, indicating that DNA helicase activity is necessary for PT modification in *R. anatipestifer*.

As other DNA helicases, DndEi has an ATPase activity. It has been reported that the activity of ATPase is strongly stimulated by specific motifs in some nucleic acid metabolic processes (27, 28). For example, transcription factor δ has an associated DNA-dependent ATPase activity that is strongly stimulated by the TATA region of promoters, and the stimulated ATPase activity is required for activation of run-off transcription (27). This site-specific stimulation of ATPase activity was also found in DndEi by the GAAC/GTTC motif, which is consistent with the fact that G_{ps}AAC/G_{ps}TTC is the PT modification site in *R. anatipestifer*. Interestingly, more ATP was hydrolyzed by DndEi in the presence of DNA substrates with a GAAC/GTTC motif than in the presence of DNA substrates with a PT-modified G_{ps}AAC/G_{ps}TTC motif; meanwhile, the two kinds of DNA substrates were unwound by DndEi at the same speed. In contrast, DndEi without the E domain unwound the two kinds of DNA substrates consuming equal amounts of ATP. Taken together, these results indicate that, except for helicase working, additional ATP hydrolysis is catalyzed by DndEi in PT modification. The additional ATP hydrolysis is predicted to be used for sulfur incorporation into the phosphate backbone, which presents an energetically uphill proposition (29).

The E domain can affect the ATPase activity of the helicase domain by recognizing different DNA substrates. Therefore, the E domain of DndEi may play an important role in sequence recognition in DNA PT modification. Previous study has revealed that DndCDE proteins from *Salmonella enterica* form a stable complex *in vitro* (30). Taken together, it is possible that DndEi recognizes the modification sites and unwind DNA duplex at the initiation of PT modification, and then recruits other PT-modifying proteins to incorporate sulfur into the DNA backbone. Nevertheless, more functional studies need to be done to further understand the role of helicase in DNA PT modification.

Bacteria obtain PT-modifying genes via horizontal gene transfer facilitated by genomic islands (31). However, PT-modifying enzymes lack the helicase domain in some bacteria, such as *Prevotella histicola*. Phylogenetic analysis revealed that DndE from *P. histicola* had a closer relationship with the E domain of DndEi from *Capnocytophaga canimorsus* than with DndE from *Stanieria cyanosphaera* (Fig. 9), implying that DndE and DndEi have the same ancestor. Presumably, DndE (*P. histicola*) had the helicase domain initially and lost it in evolution because there was another DNA helicase having the same function. Similarly, there is no *dndA* gene in *S. enterica*, and the *iscS* gene encoding a similar cysteine desulfurase plays the role as *dndA* in DNA PT modification (11). Therefore, it is possible that DNA helicase is also indispensable for PT modification in bacteria lacking the helicase domain. DNA helicase encoded by

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another gene, which is not adjacent to PT-modifying genes, may participate in DNA PT modification.

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References

1. Wang, L., Chen, S., Xu, T., Taghizadeh, K., Wishnok, J. S., Zhou, X., You, D., Deng, Z., and Dedon, P. C. (2007) Phosphorothioation of DNA in bacteria by *dnd* genes. *Nat. Chem. Biol.* **3,** 709–710
2. Wang, L., Chen, S., Vergin, K. L., Giovannoni, S. J., Chan, S. W., DeMott, M. S., Taghizadeh, K., Cordero, O. X., Cutler, M., Timberlake, S., Alm, E. J., Polz, M. F., Pinhassi, J., Deng, Z., and Dedon, P. C. (2011) DNA phosphorothioation is widespread and quantized in bacterial genomes. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 2963–2968
3. Xu, T., Yao, F., Zhou, X., Deng, Z., and You, D. (2010) A novel host-specific restriction system associated with DNA backbone S-modification in *Salmonella*. *Nucleic Acids Res.* **38,** 7133–7141
4. Cao, B., Cheng, Q., Gu, C., Yao, F., DeMott, M. S., Zheng, X., Deng, Z., Dedon, P. C., and You, D. (2014) Pathological phenotypes and *in vivo* DNA cleavage by unrestrained activity of a phosphorothioate-based restriction system in *Salmonella*. *Mol. Microbiol.* **93,** 776–785
5. Cheng, Q., Cao, B., Yao, F., Li, J., Deng, Z., and You, D. (2015) Regulation of DNA phosphorothioate modifications by the transcriptional regulator DptB in *Salmonella*. *Mol. Microbiol.* **97,** 1186–1194
6. Ou, H. Y., He, X., Shao, Y., Tai, C., Rajakumar, K., and Deng, Z. (2009) dndDB: a database focused on phosphorothioation of the DNA backbone. *Plos One* **4,** e5132
7. Xu, T., Liang, J., Chen, S., Wang, L., He, X., You, D., Wang, Z., Li, A., Xu, Z., Zhou, X., and Deng, Z. (2009) DNA phosphorothioation in Streptomyces lividans: mutational analysis of the dnd locus. *BMC Microbiol.* **9,** 41
8. Zhou, X., He, X., Liang, J., Li, A., Xu, T., Kieser, T., Helmann, J. D., and Deng, Z. (2005) A novel DNA modification by sulphur. *Mol. Microbiol.* **57,** 1428–1438
9. Chen, F., Zhang, Z., Lin, K., Qian, T., Zhang, Y., You, D., He, X., Wang, Z., Liang, J., Deng, Z., and Wu, G. (2012) Crystal structure of the cysteine desulfurase DndA from *Streptomyces lividans* which is involved in DNA phosphorothioation. *Plos One* **7,** e36635
10. You, D., Wang, L., Yao, F., Zhou, X., and Deng, Z. (2007) A novel DNA modification by sulfur: DndA is a NifS-like cysteine desulfurase capable of assembling DndC as an iron-sulfur cluster protein in *Streptomyces lividans*. *Biochemistry* **46,** 6126–6133
11. An, X., Xiong, W., Yang, Y., Li, F., Zhou, X., Wang, Z., Deng, Z., and Liang, J. (2012) A novel target of IscS in *Escherichia coli*: participating in DNA phosphorothioation. *Plos One* **7,** e51265
12. Hu, W., Wang, C., Liang, J., Zhang, T., Hu, Z., Wang, Z., Lan, W., Li, F., Wu, H., Ding, J., Wu, G., Deng, Z., and Cao, C. (2012) Structural insights into DndE from *Escherichia coli* B7A involved in DNA phosphorothioation modification. *Cell Res.* **22,** 1203–1206
13. Barbier, P., Lunazzi, A., Fujiwara-Nagata, E., Avendaño-Herrera, R., Bernardet, J. F., Touchon, M., and Duchaud, E. (2013) From the *Flavobacterium* genus to the phylum *Bacteroidetes*: genomic analysis of dnd gene clusters. *FEMS Microbiol. Lett.* **348,** 26–35
14. Enemark, E. J., and Joshua-Tor, L. (2008) On helicases and other motor proteins. *Curr. Opin. Struct. Biol.* **18,** 243–257
15. Hu, Q., Miao, S., Ni, X., Lu, F., Yu, H., Xing, L., and Jiang, P. (2013) Construction of a shuttle vector for use in *Riemerella anatipestifer*. *J. Microbiol. Methods* **95,** 262–267
16. Hu, Q., Han, X., Zhou, X., Ding, C., Zhu, Y., and Yu, S. (2011) OmpA is a virulence factor of *Riemerella anatipestifer*. *Vet. Microbiol.* **150,** 278–283
17. Cao, B., Chen, C., DeMott, M. S., Cheng, Q., Clark, T. A., Xiong, X., Zheng, X., Butty, V., Levine, S. S., Yuan, G., Boitano, M., Luong, K., Song, Y., Zhou, X., Deng, Z., Turner, S. W., Korlach, J., You, D., Wang, L., Chen, S., and Dedon, P. C. (2014) Genomic mapping of phosphorothioates reveals partial modification of short consensus sequences. *Nat. Commun.* **5,** 3951
18. Boguszewska-Chachulska, A. M., Krawczyk, M., Stankiewicz, A., Gozdek, A., Haenni, A. L., and Strokovskaya, L. (2004) Direct fluorometric measurement of hepatitis C virus helicase activity. *FEBS Lett.* **567,** 253–258
19. Yao, F., Xu, T., Zhou, X., Deng, Z., and You, D. (2009) Functional analysis of *spfD* gene involved in DNA phosphorothioation in *Pseudomonas fluorescens* Pf0–1. *FEBS Lett.* **583,** 729–733
20. Tani, H., Fujita, O., Furuta, A., Matsuda, Y., Miyata, R., Akimitsu, N., Tanaka, J., Tsuneda, S., Sekiguchi, Y., and Noda, N. (2010) Real-time monitoring of RNA helicase activity using fluorescence resonance energy transfer *in vitro*. *Biochem. Biophys. Res. Commun.* **393,** 131–136
21. Tuteja, N., and Tuteja, R. (2004) Unraveling DNA helicases: motif, structure, mechanism and function. *Eur. J. Biochem.* **271,** 1849–1863
22. Tanner, N. K., and Linder, P. (2001) DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Mol. Cell* **8,** 251–262
23. Matson, S. W., and Kaiser-Rogers, K. A. (1990) DNA helicases. *Annu. Rev. Biochem.* **59,** 289–329
24. Waga, S., and Stillman, B. (1998) The DNA replication fork in eukaryotic cells. *Annu. Rev. Biochem.* **67,** 721–751
25. Sung, P., and Klein, H. (2006) Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nat. Rev. Mol. Cell Biol.* **7,** 739–750
26. Brosh, R. M. (2013) DNA helicases involved in DNA repair and their roles in cancer. *Nat. Rev. Cancer* **13,** 542–558
27. Conaway, R. C., and Conaway, J. W. (1989) An RNA polymerase II transcription factor has an associated DNA-dependent ATPase (dATPase) activity strongly stimulated by the TATA region of promoters. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 7356–7360
28. Okanami, M., Meshi, T., and Iwabuchi, M. (1998) Characterization of a DEAD box ATPase/RNA helicase protein of *Arabidopsis thaliana*. *Nucleic Acids Res.* **26,** 2638–2643
29. Eckstein, F. (2007) Phosphorothioation of DNA in bacteria. *Nat. Chem. Biol.* **3,** 689–690
30. Cao, B., Zheng, X., Cheng, Q., Yao, F., Zheng, T., Ramesh Babu, I., Zhou, H., Dedon, P., and You, D. (2015) *In vitro* analysis of phosphorothioate modification of DNA reveals substrate recognition by a multiprotein complex. *Sci. Rep.* **5,** 12513
31. He, X., Ou, H. Y., Yu, Q., Zhou, X., Wu, J., Liang, J., Zhang, W., Rajakumar, K., and Deng, Z. (2007) 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,** 1034–1048

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DndEi Exhibits Helicase Activity Essential for DNA Phosphorothioate Modification and ATPase Activity Strongly Stimulated by DNA Substrate with a GAAC/GTTC Motif

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