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Operon Structure and Functional Analysis of the Genes Encoding Thermophilic Desulfurizing Enzymes of *Paenibacillus* sp. A11-2

Yoshitaka Ishii,[1] Jin Konishi, Hideki Okada, Kazuaki Hirasawa, Toshimitu Onaka, and Masanori Suzuki

Bio-Refining Process Laboratory, Advanced Technology and Research Institute, Petroleum Energy Center, 1900 Sodeshi-Cho, Shimizu-Shi, Shizuoka 424-0037, Japan

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***Paenibacillus* A11-2 can efficiently cleave two carbon—sulfur bonds in dibenzothiophene (DBT) and alkyl DBTs, which are refractory by conventional petroleum hydrodesulfurization, to remove sulfur atom at high temperatures. An 8.7-kb DNA fragment containing the genes for the DBT desulfurizing enzymes of A11-2 was cloned in *Escherichia coli* and characterized. Heterologous expression analysis of the deletion mutants identified three open reading frames that were required for the desulfurization of DBT to 2-hydroxybiphenyl (2-HBP). The three genes were designated *tdsA*, *tdsB*, and *tdsC* (for thermophilic desulfurization). Both the nucleotide sequences and the deduced amino acid sequences show significant homology to *dszABC* genes of *Rhodococcus* sp. IGTS8, but there are several local differences between them. Subclone analysis revealed that the product of *tdsC* oxidizes DBT to DBT-5,5′-dioxide via DBT-5-oxide, the product of *tdsA* converts DBT-5,5′-dioxide to 2-(2-hydroxyphenyl) benzene sulfinate, and the product of *tdsB* converts 2-(2-hydroxyphenyl)benzene sulfinate to 2-HBP. Cell-free extracts of a recombinant *E. coli* harboring all the three desulfurization genes converted DBT to 2-HBP at both 37 and 50°C. *In vivo* and *in vitro* exhibition of desulfurization activity of the recombinant genes derived from a *Paenibacillus* indicates that an *E. coli* oxidoreductase can be functionally coupled with the monooxygenases of a gram-positive thermophile.** © 2000 Academic Press

Sulfur emission on the combustion of fossil fuels is a global problem because it is a cause of acid rain. Fossil fuels contain DBT and alkyl DBTs, which are refractory by conventional hydrodesulfurization (HDS) process. In several decades, many kinds of microbes that can degrade DBT as a model compound have been searched and isolated (1–3). The HDS process is carried out under severe conditions such as extremely high temperature and pressure. On the other hand, biodesulfurization (BDS) process is carried out under mild conditions and has the advantage of holding CO_2 discharged in operation down to a low level.

Various types of bacteria capable of degrading DBT via carbon-carbon (C—C)-bond-target (4, 5) or carbon-sulfur (C—S)-bond-targeted (6–12) reactions have been isolated. The C—C-bond-targeted reactions have the concomitant disadvantage of reducing thermal units of fossil fuel and usually cannot degrade DBT with alkyl substituents. In contrast with this, the C—S-bond-targeted bacteria can degrade alkyl DBTs without any loss of thermal units and are thought to be desirable for BDS.

Among the C—S-bond-targeted bacteria, *Rhodococcus* sp. IGTS8 has been investigated in detail as a representative strain (13, 14). The C—S-bond-specific desulfurization genes (*dszA*, *dszB* and *dszC*) have been cloned from only this strain and identified as a single operon by the heterologous expression in *E. coli* (15, 16). Its regulatory region and the closely located insertion sequence (IS) elements have been developed using *Pseudomonas* species as a host (17). To understand explicitly the molecular basis of bacterial desulfurization, comparative analysis of the information about nucleotide sequence of desulfurization genes and amino acid sequences of enzymes from different species of bacteria should be required. In the course of screening unique microbes capable of desulfurizing heterocyclic organosulfur compounds, we have isolated *Paenibacillus* sp. A11-2 that can degrade methyl DBTs as well as DBT even at high temperatures in a C—S-bond-specific fashion (18).

We now report the cloning and identification of the DNA sequence responsible for the thermophilic desul-

[1] To whom correspondence should be addressed. E-mail: yishii@brpl.pecj.or.jp.

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furization of A11-2. The DNA sequence contains an operon consisting of three open reading frames (ORFs, designated as *tdsA*, *tdsB* and *tdsC* for thermophilic desulfurization) that show an appreciable extent of homology with dsz genes identified in IGTS8. The individual functions of these ORFs were characterized by subclone analysis and it was proved that the gene products are involved in a reaction cascade for the conversion of DBT or alkyl DBTs to their hydroxybiphenyl forms. An *Escherichia coli* clone carrying all of these three tds genes and its cell free extracts converted DBT to 2-HBP even at 50°C. This indicates that the high-temperature capabilities of the desulfurizing enzymes of a gram-positive thermophile can be transferred in its entirety into a gram-negative desulfurization-less bacterium.

MATERIALS AND METHODS

Bacterial strains. *Paenibacillus* sp. A11-2 was used as a source of thermophilic desulfurization gene. *E. coli* XL1-Blue MRA and Lambda DASHII phage vector were used for cloning of thermophilic desulfurization gene. *E. coli* JM109 and an expression vector pKK223-3 were used for expression of genes.

Media and culture conditions. *Paenibacillus* sp. A11-2 was grown as described previously (18). *E. coli* strains were grown in Luria-Bertani (LB) medium and arranged M9 medium at 37°C. Arranged M9 medium was used $MgCl_2$ instead of $MgSO_4$ and added with 50 μg of yeast extract per ml in media. Selection was made with 50 μg of ampicillin per ml in LB agar or liquid media.

DNA methods. Genomic DNA was prepared from A11-2 with a QIAgen genomic DNA kit. Phage was purified with a QIAgen lambda kit. Plasmid DNA was purified with a QIAgen plasmid kit. DNA fragments were isolated from agarose gels with a QIAquick gel extraction kit. PCR products were purified with a QIAquick PCR Purification kit. Digestion with restriction endonucleases, ligation and deletion experiments were carried out by standard procedures (19) under conditions recommended by the manufacturers. Automated DNA sequencing was performed with a Pharmacia ALFred DNA sequencer. The sequence was determined by complete sequencing of both DNA strands, with multiple sequencing of some regions. Sequence assembly and analysis were performed with Genetyx-Mac ver9.01 (SDC, Japan). The GenBank and SwissProt database were searched for nucleonic acid and amino acid similarities with Genetyx-Mac CD No.36 (SDC, Japan) and FASTA program at DNA Data Bank of Japan (DDBJ).

Transformations. Transformations were carried out with commercially available competent *E. coli* cells. The procedures followed the protocols recommended by the manufacturers.

Construction of a genomic library. Genomic DNA of A11-2 was partially digested with *Sau*3AI and treated with calf intestine alkaline phosphatase. The DNA fragments were ligated to *Bam*HI-digested Lambda DASH II arms (Stratagene). The recombinant phage DNA was packaged into phage heads in vitro with the Gigapack II XL packaging kit (Stratagene). The packaged phages were then propagated in *E. coli* XL1-Blue MRA cells to a titer of 1×10^6 pfu/ml.

Oligonucleotide primers, probes and hybridization. Native desulfuring enzyme was subjected to N-terminal sequencing with a Beckman LF-3000 protein sequencer. The nucleic acid sequences of the degenerate PCR primers were designed from the amino acid sequences. Synthetic oligo nucleotides were purchased from Nippon Flour Mills Co. Ltd. The probe DSZ was made with the degenerate PCR primers by using the gradient PCR method as a following. A 50 μl PCR reaction mixture contained 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1.5 mM $MgCl_2$, dNTP (each species, 200 μM), PCR primers (0.5 μM each), DNA template and *Taq* DNA polymerase (1.25 U). Gradient PCR was performed with ROBO cycler gradient 96 (Stratagene) apparatus. The temperature program was used the following profile: 95°C for 3 min.; 30 cycles of the following: 95°C for 1 min., 44 to 66°C (2°C stepwise) for 1 min. and 72°C for 3 min.; add 72°C for 10 min.

DNA probes were labeled with digoxigenin-11-dUTP random labeling method by using the Boehringer DIG DNA Labeling Kit. The Lambda DASH II library of the A11-2 genomic DNA was plated on a lawn of *E. coli* XL1-Blue MRA cells grown on NZY (5 g/l of NaCl, 2 g/l of $MgSO_4 \cdot 7\ H_2O$, 5 g/l of yeast extract, 10 g/l of NZ amine, pH 7.5) agar plates and was transferred to Hybond-N+ nylon membranes (Amersham) in accordance with the manufacturer's instructions. The membranes were probed at 68°C in 5× SSC, 1% Blocking reagent, 0.1% *N*-lauroylsarcosine and 0.02% SDS, and washed twice at 25°C in 2× SSC and at 68°C in 0.1× SSC. Four positive clones were selected after secondary screening, and phage DNA was prepared from each clone by using the QIAgen Lambda Kit.

Desulfurization minus mutant. A11-2 was cultured with 2× YT (16 g/l of tryptone, 10 g/l of yeast extract, 5 g/l of NaCl, pH 7.6) medium at 50°C for 1 night. This culture 0.1 ml was inoculated into 5 ml fresh 2× YT medium containing 30 μg/ml of acridine orange at 50°C for 1 night. This culture was centrifuged, and bacterial pellet was washed with A medium. This washed bacteria was resuspended with 0.1 ml of A medium, and inoculated into fresh 2 ml of 2× YT medium and incubated at 50°C for 4 h. Bacterial culture was spread on 2× YT agar plate medium, and incubated at 50°C for 1 night. Grown colonies were inoculated into A medium containing DBT as a sole sulfur source to find the desulfurization minus mutant. Desulfurization minus mutant was checked with examination its growth on AY medium supplemented DBT or alkyl DBT derivatives. A11-2 and desulfurization minus mutant were cultured on AYD medium at 50°C for 1 night, and these culture were centrifuged and washed twice with AY medium, and then inoculated into 5 ml of AY medium and added 1 ml *n*-tetradecane containing 50 ppm sulfur concentration of organic sulfur compounds. After cultivation, these culture were added 100 μl of 6 N-HCl and extracted with 1 ml of ethylacetate, and then analyzed by gas-chromatography and gas-chromatograph/Mass spectrometry.

Heterologous expression of desulfurizing gene by growing cells in E. coli. Desulfurization assays were performed in liquid culture on growing cells in *E. coli* JM109. For these assays, cell were incubated overnight at 30°C in LB-ampicillin broth medium. These cultures were inoculated 1% volume to 5 ml of fresh LB-ampicillin broth containing the test substrate. These assay cultures were shaken at 37°C for 24 h, and added 100 μl of 6 N-HCl and extracted with 1 ml of ethylacetate, and then analyzed by gas-chromatography and gas-chromatograph/mass spectrometry.

Desulfurization activity by resting cells reaction of recombinant E. coli. Desulfurization assays were performed by resting cells reaction of recombinant *E. coli* JM109. For these assays, cells were incubated overnight at 30°C in LB-ampicillin medium. These cultures were inoculated 1% volume to fresh LB-ampicillin medium, and shaken at 37°C up to OD = 1.0. These cells were centrifuged and washed twice with 50 mM Tris-HCl buffer (pH 7.0). These cells were resuspended about OD = 20 with Tris-HCl buffer. These suspension were used as resting cells for desulfurization assay. Desulfurization assays of resting cells reaction were performed following conditions; reaction mixtures (1 ml) were added 50 μg/ml of test substrate and incubated at 37°C or 50°C for 1 h. After reaction, these assay solutions were added 20 μl of 6 N-HCl and extracted with 0.4 ml of ethylacetate, and then analyzed by gas-chromatography and gas-chromatograph/Mass spectrometry.

TABLE 1

Sequence of Primer for Desulfurizing Gene Amplification

Name	Sequence
For PCR between N-terminal of two enzymes	
Sense primer	
SM3	5′-TTY GCN GCN GGN AAY GT-3′
SM4	5′-TTY TTY GCN GCN GGN AA-3′
SM5	5′-GCN GGN TTY TTY GCN GC-3′
Antisense primer	
AM3	5′-TAN GCN ACY CTN GTN GG-3′
AM4	5′-TCR TTN ACN GCN GTY TC-3′
AM5	5′-ACY CTN GTN GGN CCD AT-3′
For PCR of probe to screen a genome library	
Sense primer	
DszA-SN	5′-CAA CAC ATC GCC CGT AC-3′
Antisense primer	
DszA-AN	5′-CCA AAT CCG GTG TAG GC-3′

Desulfurization activity by cell free extract of recombinant E. coli. Desulfurization assays were performed by cell free extract of recombinant *E. coli.* For these assays, cells were incubated overnight at 30°C in LB-ampicillin medium. These cultures were inoculated 1% volume to fresh LB-ampicillin medium, and shaken at 37°C up to OD = 1.0. These cells were centrifuged and washed twice with 50 mM Tris-HCl buffer (pH 7.0). These cells were resuspended about OD = 20 with Tris-HCl buffer. Cell suspensions were sonicated 50% duty for 10 min with ultrasonicator (BRANSON). Sonicated cell suspensions were centrifuged at 15,000*g* for 60 min to remove cell debris. These supernatants were used as cell free extraction for desulfurization assay. Desulfurization assays of cell free extraction were performed following conditions; reaction mixtures (1 ml) were contained 50 mM Tris-HCl, 10 nM NADH, 3 μM FMN, 50 μg/ml test substrate and cell free extract of recombinant and desulfurization minus mutant M18, and incubated at 37°C or 50°C for 4 h. After reaction, these assay solutions were added 20 μl of 6 N-HCl and extracted with 0.4 ml of ethylacetate, and then analyzed by gas-chromatography and gas-chromatograph/Mass spectrometry.

Accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession Number AB033997.

RESULTS

Cloning of thermophilic desulfurization genes. Paenibacillus sp. A11-2 degrades DBT to 2-HBP via DBT sulfoxide (DBTO). We examined the other bacterial metabolites of DBT by LC-MS in extracts from the A11-2 culture and identified DBT-5,5′-dioxide ($DBTO_2$) and 2-(2′-hydroxyphenyl) benzene sulfinate (HPBS) as the intermediates (data not shown). Active enzymes catalyzing the oxidation of $DBTO_2$ to HPBS and the desulfination of HPBS to 2-HBP were purified from this strain (in preparation). Their N-terminal amino acid sequences were determined to be MXQMXLAGF-FAAGNVTXXXGA and (M)TKSAIGPTRVAYSNX-PVANXL (X, unidentified residue). Based on these amino acid sequences, primers were synthesized (Table 1) and used for gradient PCR with genomic DNA of A11-2 as the template. Of all the primer combinations, two primer pairs SM3/AM5 and SM3/AM3 gave a 1.6-kb PCR product. The 1.6-kb fragment obtained with SM3/AM5 was cloned into the PCR cloning vector pCR-Script SK(+). Partial sequencing of the cloned DNA revealed that it contained regions encoding the N-terminal sequences of the two enzymes purified. However, this PCR product was found to contain the region downstream of the location of the anti-sense primer used for the PCR amplification as well. The downstream region of the PCR product was stopped with the 3′-terminal sequence complementary to the sense primer sequence used for PCR. Therefore, it is thought that the 1.6-kb PCR product results from amplification by annealing of the sense primer as an anti-sense primer with the template DNA at the downstream region. The amino acid sequences of the N-terminal regions of the two enzymes which were deduced from the partial DNA sequence of the PCR product, showed the homology of 73% and 61% to the corresponding regions of DszA and DszB, respectively, of *Rhodococcus* sp. IGTS8.

Next, the regions between 120 and 137 nucleotide from the 5′-terminal of *dszA* and between 169 and 185 nucleotide from the 5′-terminal of *dszB* of IGTS8 are chosen as the sense and anti-sense primer (Table 1), respectively, for another PCR with the A11-2 genomic DNA. The PCR fragment amplified with the primers was then used as the probe (designated as DszA probe) to screen a library of A11-2 genomic DNA in lambda DASH II by plaque hybridization. Four positive clones were obtained from a total of 2×10^3 plaques. Restriction and Southern hybridization analysis of the cloned DNAs suggested that one lambda clone p4 contained the sequence hybridizable with the DszA probe and its downstream region of about 18 kb. It was supposed that the cloned DNA was enough to cover the A11-2 desulfurization genes, if its size and arrangement were the same as IGTS8′s ones. About a 2 kb *Not*I-*Sal*I fragment of the p4 insert was hybridized with the DszA probe and the 8.7-kb *Eco*RI-*Hin*dIII fragment containing it was thus anticipated to bear the entire region encoding the desulfurization enzymes. The *Eco*RI-*Hin*dIII fragment was then cloned into pBlueScript II KS(+) to give p4EH plasmid for sequence analysis.

Characterization of the nucleotide sequences of tds genes. The nucleic acid sequence of the *Eco*RI-*Hin*dIII fragment was determined with deleted versions of p4EH plasmid. Deletion plasmids were prepared through the steps of double digestion by *Kpn*I-*Hin*dIII or *Sac*I-*Eco*RI, *Exo*III nuclease treatment of the restriction fragments, Mung bean nuclease and Klenow fragment treatments. Nucleotide sequencing with ThermoSequenase identified six open reading frames (ORFs) in the insert of p4EH. Three out of the six ORFs were aligned in the same direction (Fig. 1) and showed

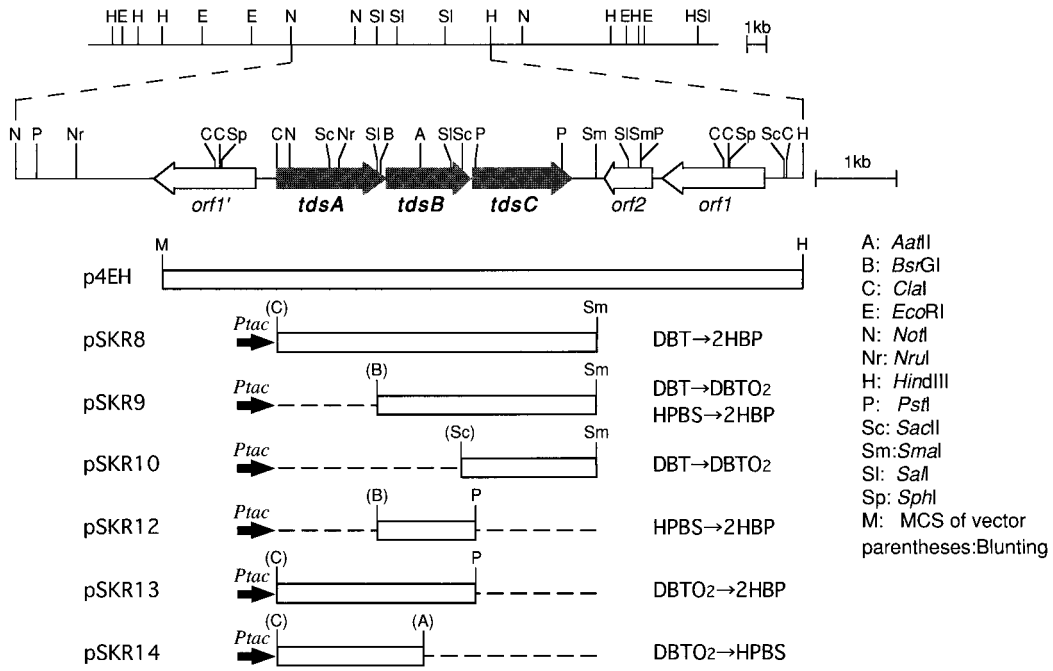

FIG. 1. Physical map of thermophilic desulfurization genes from *Paenibacillus* sp. A11-2. DBT, dibenzothiophene; DBTO, DBT-5-oxide; DBTO2, DBT-5,5′-dioxide; HPBS, 2-(2-hydroxyphenyl)benzenesulfinate; 2HBP, 2-hydroxybiphenyl. *Ptac, tac* promoter of pKK223-3 vector.

significant sequence homology to the *dsz* gene cluster of IGTS8 in BLAST search. These three ORFs homologous to *dszA, dszB* and *dszC* of IGTS8 were then designated as *tdsA, tdsB* and *tdsC,* respectively, for thermophilic desulfurization genes. The ORF (orf2 in Fig. 1) adversely located downstream of these three ORFs was similar to carbonic anhydrase (40% homology). Two ORFs (orf 1 and 1′ in Fig. 1) on both sides of these four ORFs encoded a putative transposase and were similar to a bacterial insertion sequence (IS), IS1202 (20), suggesting these ORFs might constitute a transposable unit of DNA.

The first ORF, *tdsA* (281 to 1660 nucleotide) encodes a protein of 454 amino acids with a calculated molecular mass of 50.6 kDa (Fig. 2). Homology search by FASTA program identified several proteins having significant similarity to the *tdsA* gene product or TdsA protein. DszA of IGTS8 shows 64.5% identity to TdsA. Several members of an FMNH2-dependent monooxygenase family (13, 21–23) were found homologous to TdsA (Fig. 3, Table 2). The deduced amino acid sequence of TdsA was 44.7% identical to that of NTA, one component of nitrilotriacetate monooxygenase of *Chelatobacter heintzii.* It was also 43.3% and 35.5% identical to the two components, SnaA and SnaB, of Pristinamycin IIA synthase of *Streptomyces pristinaespiralis.* The N-terminal regions of about 150 amino acids (between Ala89 and Ala235 in TdsA sequence) showed extensive conservation within these members. TdsA also showed a partial identity (approximately 27%) to Ssi6, a sulfur-starvation-induced protein (SSI protein) of *E. coli.* (24)

The end of *tdsA* shows a 4-bp overlap with the translational start site of t*dsB* (1660 to 2718 nucleotide), which encodes a protein of 353 amino acids with a molecular mass of 38.9 kDa (Fig. 2). TdsB shows 54.1% identity to DszB of IGTS8. In addition, TauA(Ssi1), which is one of the *E. coli* SSI proteins and identified to be a constituent of a gene cluster involved in the utilization of taurine as a sulfur source (25), is approximately 20% identical to TdsB. The last ORF, *tdsC* (2737 to 3978 nucleotide), encodes a protein of 414 amino acids with a molecular mass of 45.8 kDa (Fig. 2). TdsC shows 51.5% identity to DszC of IGTS8. In addition, it was approximately 25% identical to mammalian acyl-CoA dehydrogenases (26).

The tds genes can function in mesophilic gram-negative E. coli cells. Contribution of each ORF (*tdsA, B* and *C*) to desulfurization of DBT was characterized with the recombinant *E. coli* cells growing at 37°C. Conversion of DBT, DBTO, $DBTO_2$ or HPBS added as a substrate in the growth medium into their corresponding metabolites was examined. As shown in Fig. 1, the pSKR8 clone carrying all three *tds* genes can degrade DBT to generate 2-HBP, indicating that they have sufficient genetic information for conferring the desulfurization activity on *E. coli.* The plasmid pSKR9 carrying *tdsB* and *tdsC* but lacking *tdsA* conferred the capability of converting DBT and HPBS into DBTO2 and 2-HBP, respectively, but no degradation of $DBTO_2$

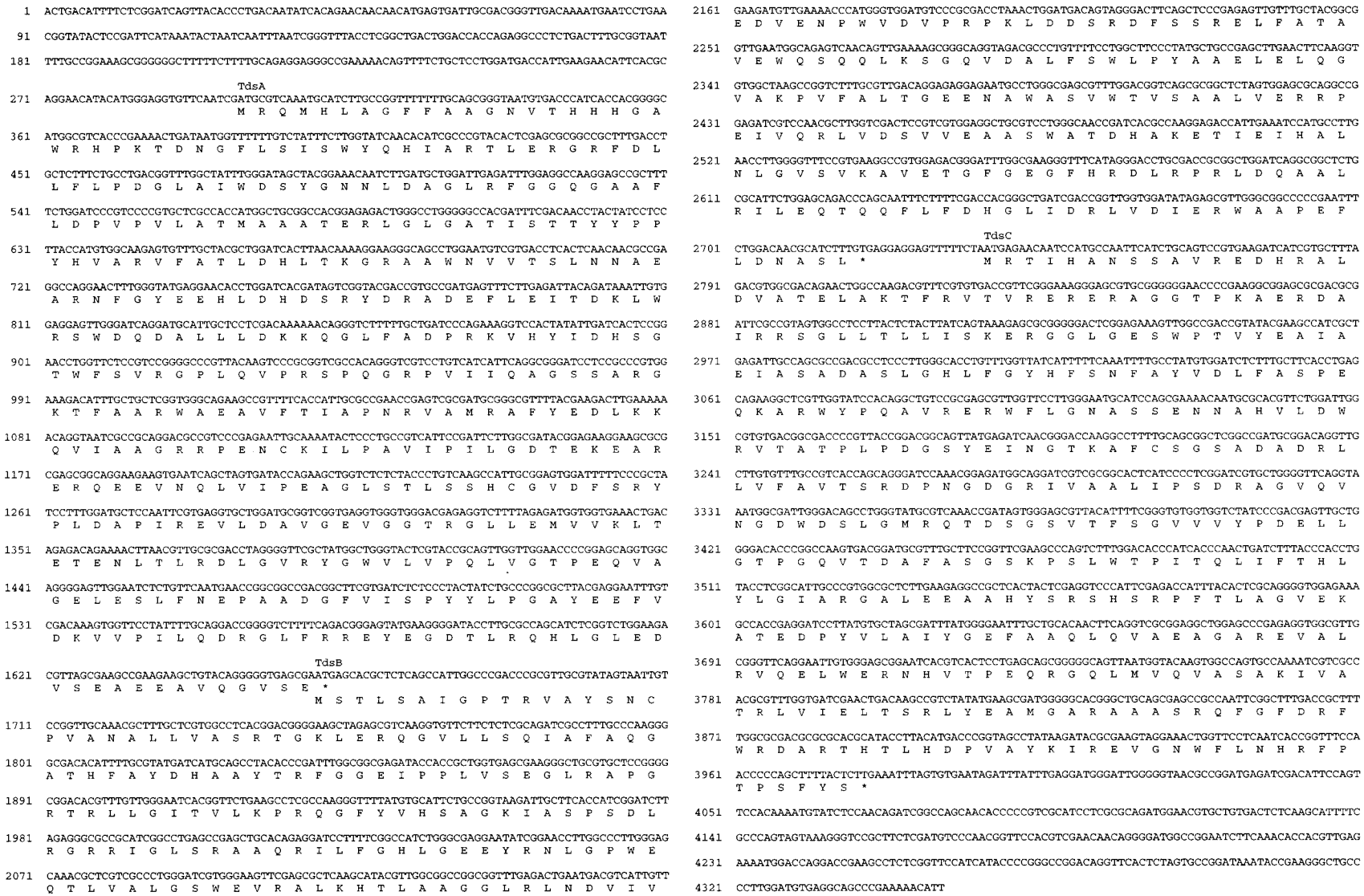

FIG. 2. Nucleic acid and amino acid sequence of thermophilic desulfurization gene from *Paenibacillus* sp. A11-2.

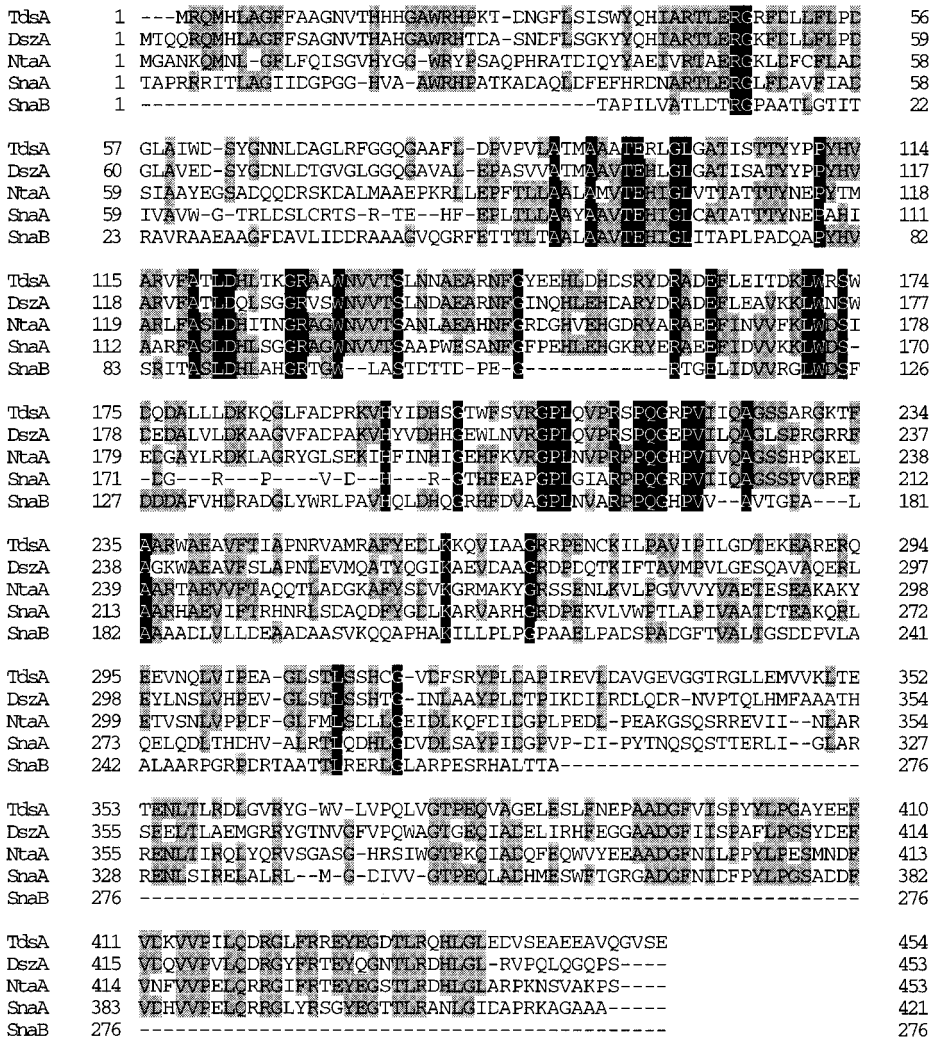

FIG. 3. Alignment of the amino acid sequence of TdsA with $FMNH_2$-dependent monooxygenase family. DszA(P54995), DBT-5,5′-dioxide monooxygenase; NtaA(P54989), nitrilotriacetate monooxygenase; SnaA(P54991), pristinamycinIIA synthase subunit A; SnaB(P54993), pristinamycinIIA synthase subunit B. Accession numbers of these enzymes are listed in Table 2.

was observed. The pSKR10 clone carrying only *tdsC* converted DBT into $DBTO_2$ and the pSKR12 clone carrying only *tdsB* did HPBS into 2-HBP. Coexpression of *tdsA* with *tdsB* was enough to convert DBTO2 into 2-HBP as shown for the pSKR13 clone. The pSKR14 clone carrying a full length of *tdsA* and a part of *tdsB* converted $DBTO_2$ into sultine (closed form of HPBS) but no further degradation to 2-HBP was detected. All of these data are consistent with the view that the gene products of *tdsA*, *tdsB* and *tdsC* are responsible for cleavage of a C—S-bond in DBTO2, desulfination of HPBS and sulfur oxidation of DBT, respectively. The *tdsA* clone (pKSR14) did not show the capability of converting DBTO to HPBS, which had been proposed earlier to modify the 4S pathway of IGTS8.

TABLE 2

List of Homologous Enzyme to TdsABC

Enzyme	% Identity	Accession No.
vs TdsA		
DszA	64.5	P54995
NtaA	44.7	P54989
SnaA	43.3	P54991
SnaB	35.5	P54993
vs TdsB		
DszB	55.2	P54997
vs TdsC		
DszC	51.7	P54998

The time course of growth and DBT desulfurization of the recombinant *E. coli* harboring pSKR8 were investigated (data not shown). Sharp changes in the amounts of DBT and 2-HBP were detected between 6 and 10 h after the start of cultivation. The rate of

TABLE 3

Activity of Desulfurization Recombinant *E. coli*

	Activity (nmol/mg/h)	
Temp. (°C)	CFE	Resting Cells
37	0.303	0.55
50	3.709	0.41

Note. Activities were determined by the amount of 2-HBP product for 1 h with mg protein for CFE or mg dry cell for resting cells, respectively.

the production of 2-HBP significantly fell at the stationary phase. Greater amounts of DBTO2 were detected as compared with HPBS during culturing, suggesting that HPBS could be easily converted into 2-HBP but the reaction rate of the cleavage of a C—S bond in DBTO2 may be relatively low. We found that HPBS tended to be spontaneously changed into 2-HBP even at very low efficiencies and this might explain at least partly the efficient degradation of HPBS.

Thermophilic DBT degradation in the extracts of recombinant E. coli cells. DBT degradation activity was also investigated with the cell free extracts and resting cells of the recombinant *E. coli* harboring pSKR8, which contained the *tdsABC* sequence. The resting cells of the recombinant degraded DBT to 2-HBP at either 37°C or 50°C, indicating that the *tdsABC* gene cluster carry information enough to confer the desulfurizing capability at even high temperature on the mesophile (Table 3). Nevertheless, the cell free extracts were not able to degrade DBT to 2-HBP at either temperature without the addition of the cell free extracts of strain M18, which was a desulfurization-minus mutant strain. Moreover, the substantial conversion of DBT into 2-HBP with the cell free extracts of the *E. coli* (pSKR8) recombinant also required the addition of both NADH and FMN.

DISCUSSION

Conservation of plasmid-encoded DBT-desulfurization genes in several Rhodococci has already been demonstrated by using Southern hybridization and PCR with the probe and primers, respectively, specific to the IGTS8′s *dsz* sequences. In the present paper, we introduce the molecular evidence for the presence of the *dsz*-like genes and Dsz-like enzymes in the bacterial species other than Rhodococci. Based on the data concerning the nucleotide sequence of the gene cluster as well as the structures and functions of the gene products, the *tds* sequence seems to be a thermophilic equivalent of the mesophilic *dsz* operon. Moreover, organization of the *tds* genes in the cluster is also quite similar to that of the *dsz* genes. Four-base overlapping (ATGA) of the termination codon of *tdsA* with the initiation codon of *tdsB* is conserved in the *dszA-dszB* junction of IGTS8, suggesting some significance in expression of these genes such as translational coupling.

The *E. coli* clone harboring the *tdsC* gene oxidizes DBT to $DBTO_2$ via DBTO. The *tdsA* clone can convert $DBTO_2$ into HPBS and the *tdsB* clone desulfurizes HPBS to 2-HBP. This pathway for DBT conversion into 2-HBP is practically the same as the 4S pathway found in IGTS8 cells. Desulfurization of DBT to 2-HBP by the *E. coli* recombinants proves that all of the *tds* genes transferred from a gram-positive thermophile can be expressed and their products can function in the gram-negative mesophile at 37°C. The experimental data on heterologous expression of the individual *tds* genes also demonstrate that each of the *tds* gene products can function independently of the other gene products. However, the fact does not rigorously exclude the mechanism that the enzymes may act cooperatively in the bacterial desulfurization process.

In either IGTS8 cells or the other Rhodococci, the *dsz* genes reside on giant plasmids. Two IS-like elements (IS1166 and IS1295) associated with the *dsz* genes have been identified on a plasmid of ca. 120 kb present in IGTS8 cells. The *dsz* operon is adjacent to IS1166 containing two ORFs on the plasmid. One of the ORFs substantially overlaps *dszC* on the opposite strand in a contrary direction and encodes a putative transposase. The *Paenibacillus tds* genes are sandwiched between two putative IS elements (orf1′ and orf1 in Fig. 1), which shows a considerable similarity to IS1202 and are located in the same direction. The *tds* genes and the two IS elements are arranged in an opposite orientation and do not overlap in either orientation with each other.

It has been demonstrated that at least six *Rhodococcus* strains carrying the *dsz* sequence contain IS1166-like sequence. The widespread co-distribution of the *dsz* sequence with IS1166-like sequence in various Rhodococci may raise the possibility of their transposition among the bacterial species. The organization of the IS elements and other genes including the *tds* genes in the pKSR8 clone is analogous to that found in Tn9. The orf2 interposed by *dszC* and orf1 seems to encode a protein like carbonic anhydrase, whose gene is usually localized on bacterial chromosome.

TdsA is significantly similar to DszA, NtaA (NmoA), SnaA and SnaB, all of which need NADH:FMN oxidoreductase such as DszD, NtaB (*nmoB*) and SnaC, respectively, for their enzymatic actions. TdsC is significantly similar to DszC, which also needs NADH: FMN oxidoreductase (DszD) for its monooxygenase action (27). From these facts, one can expect that both TdsA and TdsC may act in cooperation with some specific oxidoreductase. During cultivation of the recombinant clone, significant amounts of both DBTO and

$DBTO_2$ were recovered (data not shown). The NADH: FMN oxidoreductase activity of *E. coli* may not be fully coupled with the Tds enzymes (TdsC and TdsA).

Cell free extracts of the recombinant clone harboring the *tds* gene cluster can degrade DBT to 2-HBP at 37°C. However, the activity at 50°C is about 10 times as high as that at 37°C, demonstrating that the Tds enzymes are thermophilic. Requirement of the addition of M18 extracts for the cell free conversion of DBT suggests that some component indispensable for DBT desulfurization may be omitted from or inactivated in the *E. coli* cell free extracts. The most probable candidate for such a component may be some NADH-FMN oxidoreductase activity as a coupling enzyme.

The resting cells of the recombinant clone also degrade DBT to 2-HBP at both 37°C and 50°C. That the resting *E. coli* recombinant cells can degrade DBT to 2-HBP without introduction of any other enzyme activity indicates the existence of some coupling NADH-FMN oxidoreductase in the cells. The DBT desulfurizing activity of *E. coli* (pKSR8) clone in resting cell reaction at 50°C is lower than that at 37°C. This may reflect the heat liability of *E. coli* NADH:FMN oxidoreductase activity and/or its inadequate enzymatic coupling with TdsC and TdsA. In fact, we have demonstrated that the Rhodococcal NADH:FMN oxidoreductase (DszD) is heat-labile and less effective than the *Paenibacillus* equivalent in the enzymatic coupling with TdsC and TdsA (data not shown).

It is worth noting that the sequences of TdsA and TdsB are even slightly similar to Ssi6 and TauA (Ssi1), which is a taurine (2-aminoethanesulfonate)-binding periplasmic protein of *E. coli*, respectively. Either Ssi6 or *tauA* is known to be the members of *E. coli* SSI proteins whose gene expression is induced by sulfur starvation. Production of DszA and DszB, which are Rhodococcal homologs of TdsA and TdsB, respectively, are repressed in the presence of sulfate and in this respect analogous to SSI proteins. We also found that A11-2 hardly converted $DBTO_2$ and HPBS into HPBS and 2-HBP, respectively, when it was cultured in the medium containing sulfate as the sole sulfur source (data not shown). It is tempting to investigate the relationship between the Tds (or Dsz) enzymes and the SSI proteins in their physiological roles and the regulation of their gene expression.

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