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ORIGINAL PAPER

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Purification and characterization of the monooxygenase catalyzing sulfur-atom specific oxidation of dibenzothiophene and benzothiophene from the thermophilic bacterium *Paenibacillus* sp. strain A11–2

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Abstract A benzothiophene (BT) and dibenzothiophene (DBT) monooxygenase (TdsC), which catalyzes the oxidation of the sulfur atoms in BT and DBT molecules, was purified from *Paenibacillus* sp. strain A11–2. The molecular mass of the purified enzyme and its subunit were determined to be 200 kDa and 43 kDa by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis, respectively, indicating a tetrameric structure. The N-terminal amino acid sequence of the purified TdsC completely matched the amino acid sequence deduced from the nucleotide sequence of the *tds*C gene reported previously [Ishii et al. (2000) Biophys Biochem Res Commun 270:81–88]. The optimal temperature and pH for the TdsC reaction were 65°C and pH 9, respectively. TdsC required NADH, FMN and TdsD, a NADH-dependent FMN oxidoreductase, for its activity, as was observed for TdsA. FAD, lumiflavin and/or NADPH had some effect on the maintenance of TdsC activity. A comparison of the substrate specificity of TdsC and DszC, the homologous monooxygenase purified from *Rhodococcus erythropolis* strain KA2–5–1, demonstrated a contrasting pattern towards alkylated DBTs and BTs.

Introduction

Organic sulfur compounds in fossil fuels have been a major cause of environmental pollution. Among these compounds, dibenzothiophene (DBT), benzothiophene (BT) and their alkylated derivatives are resistant to conventional hydrodesulfurization (HDS) treatment, which uses hydrogen gas under severe conditions such as high temperatures and pressures. The levels of sulfur in diesel oil and gasoline are now subject to regulation, and an alternative method for sulfur removal is required. Biodesulfurization (BDS) in which microbes are used as catalysts is one candidate for a method of lowering the sulfur levels of petroleum fractions (Kilbane 1989; Foght et al. 1990). Extensive studies of microbial desulfurization have been performed with *Rhodococcus erythropolis* strain IGTS8. The plasmid-encoded *dsz* genes responsible for DBT desulfurization have been cloned and sequenced (Denome et al. 1993; Piddington et al. 1995). The product of *dsz*C catalyzes the sulfur oxidation of DBT to DBT sulfone, which is subsequently converted to 2-(2′-hydroxyphenyl)benzene sulfinate (HBPSi) by the product of the *dsz*A gene. HBPSi is finally desulfurized to 2-hydroxybiphenyl (2-HBP) and sulfite by the product of *dsz*B.

Many microorganisms have been found to remove sulfur from model compounds such as DBT and its alkylated derivatives in a C-S bond-targeted fashion to produce desulfurized hydroxyphenols such as 2-HBP and sulfate as the final products (Van Afferden et al. 1990; Izumi et al. 1994; Wang and Kraviec 1994). These bacteria are considered to desulfurize DBT through the same metabolic pathway as strain IGTS8 (Gallagher et al. 1993).

Recently, BT-desulfurizing bacteria have been isolated from several sources, e.g., *Gordonia desulfuricans* strain 213E (Gilbert et al. 1998), *G. rubropertinctus* strain T08 (Matsui et al. 2001), *Rhodococcus* sp. strain T09 (Matsui et al. 2000) and *Sinorhizobium* sp.KT55 (Tanaka et al. 2001). These bacteria have been demonstrated to desulfurize BT and several kinds of alkylated BTs. However, they cannot grow on DBT as the sole source of sulfur, suggesting the absence of a DBT-desulfurizing enzyme system.

We previously isolated *Paenibacillus* sp. strain A11–2, which can grow on both BT and DBT at 50°C

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(Konishi et al. 1997). A11–2 desulfurized BT, DBT and several methylated DBTs via the degradation pathway following cleavage of the C-S bond even at around 60°C. To date, A11–2 is the only microorganism known to desulfurize both BT and DBT. The DBT-desulfurization genes (*tds*A, *tds*B and *tds*C) (Ishii et al. 2000a) and a FMN-dependent NADH oxidoreductase gene (*tds*D) (Ishii et al. 2000b) have been cloned and sequenced. The *tds*ABC forms a plasmid-located operon and encodes TdsA, B and C enzymes. Desulfurization of DBT is considered to proceed via the 4S pathway (Gallagher et al. 1993) by the action of TdsA-C with the aid of TdsD. TdsA and TdsD have recently been purified, and some of their properties have been investigated (Konishi et al. 2000a). The use of DBT-desulfurizing enzymes, corresponding to TdsA–C, active at high temperature, might be of practical use in petroleum refining as there would be no need to cool the HDS-treated petroleum fractions to ambient temperatures.

In this paper, we describe the purification and some properties of TdsC produced by A11–2, which catalyzes the oxidation of sulfur atoms in both DBT and BT. We also compared the substrate specificity of TdsC, especially toward several methylated DBTs and BTs, with that of DszC purified from *R. erythropolis* strain KA2–5–1 (Kobayashi et al. 2000) isolated in our laboratory.

Materials and methods

Chemicals

DBT, BT and 2-HBP were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). BT sulfone was synthesized by a conventional oxidation method as described previously (Konishi et al. 2000a). The methylated DBTs were synthesized by the Nard Institute (Hyogo, Japan). 3-Methyl BT and 5-methyl BT were purchased from Lancaster Synthesis (Morecambe, UK).

Bacteria and cultivation

Paenibacillus sp. strain A11–2 was used throughout this study. Cultivation was carried out with A medium supplemented with 0.27 mM DBT as the sulfur source as described previously (Konishi et al. 1997).

Analytical methods

DBTs, BTs and corresponding sulfones were analyzed by gas chromatography (GC, Shimadzu, GC-17A) with a DB-17 fused-silica capillary column (30 m ×0.25 mm i.d., 0.25 μm film) and GC-mass spectrometry (MS) (Finnigan Mat, Magnum; San Jose, Calif.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% (w/v) polyacrylamide gel by the method of Laemmli (1970). The native molecular mass was determined by gel filtration on a Bio-Silect SEC 250–5 (300×7.8 mm) column (Bio-Rad, Hercules, Calif.). The protein concentration was determined with a Bio-Rad Protein Assay kit using bovine serum albumin as the standard. The N-terminal amino acid sequence of the purified protein was determined on a Beckman LF-3000 protein sequencer (Beckman, Palo Alto, Calif.).

Enzyme assays

The standard assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 10 μM FMN, 9 mM NADH, 0.3–0.5 μM TdsD and an appropriate amount of the TdsC preparation in a total volume of 1 ml. The reaction was initiated by the addition of 0.27 mM BT and stopped by the addition of 30 μl 6 N HCl followed by extraction with 0.4 ml ethyl acetate. The resultant organic layer was subjected to GC to determine BT sulfone formed. One unit of enzyme activity was defined as the amount that catalyzed the formation of 1 nmol of BT sulfone per minute. FMN-dependent NADH oxidoreductase (TdsD) activity was evaluated by measuring the rate of NADH oxidation at 340 nm (ε =6,220 $M^{-1}cm^{-1}$) using a JASCO V-520 spectrophotometer. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.0) and 10 μM FMN in a volume of 1 ml. The reaction was initiated by addition of 0.1 mM NADH and was terminated by addition of 0.1 ml 10% SDS. One unit of enzyme activity was defined as the amount that oxidized 1 μmol of NADH per minute.

Enzyme purification

The purification of DBT monooxygenase from *Paenibacillus* sp. strain A11–2 (TdsC) and that from *R. erythropolis* strain KA2–5–1 (DszC) was performed as follows. Frozen cells (20 g, wet weight) were suspended in 200 ml of a buffer containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (buffer A) and were disrupted by sonication for three 15 min periods followed by centrifugation at 100,000 *g* for 60 min. The resultant supernatant was applied to a column of HiLoad 26/10 Q Sepharose (Amersham Pharmacia, Uppsala, Sweden) equilibrated with buffer A. The proteins were eluted with a linear gradient from 0 to 1 M NaCl and the fractions containing BT-oxidizing activity were combined and dialyzed overnight against buffer A at 4°C. The dialyzed fraction was applied to a column of DEAE Toyopearl 650 M (Tosoh, Tokyo, Japan) equilibrated with buffer A. The proteins were eluted with a linear gradient from 0 to 1 M NaCl. The active fractions were pooled and the solution was dialyzed overnight against buffer A. Solid ammonium sulfate was added to the dialyzed solution to bring it to 30% saturation and the solution was then applied to a column of Phenyl Sepharose 16/10 (Amersham Pharmacia) equilibrated with buffer A containing 1.3 M ammonium sulfate. The proteins were eluted with a linear gradient from 1 to 0 M ammonium sulfate and the fractions containing activity were combined and concentrated with an Ultrafree 15 filter (Millipore). The concentrated protein solution was applied to a Biogel CHT-1 hydroxyapatite column (Bio-Rad) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol and 0.5 mM DTT. The proteins were eluted with an increasing gradient from 10 to 200 mM potassium phosphate buffer. The active fractions were pooled and used as the purified enzyme preparation. For purification of TdsD, frozen cells of *Escherichia coli* JM109 carrying pD1RSm1 encoding the *tds*D gene were used as the source of the enzyme preparation (Ishii et al. 2000b). TdsD was purified essentially as described previously (Konishi et al. 2000a). All operations were carried out at 4°C unless otherwise stated.

Results

Purification of TdsC

Since crude extract of A11–2 exhibited extremely low activity when DBT was used as the substrate, we used BT in the enzyme assay instead of DBT. When proteins in the crude extract of A11–2 were separated by Q Sepharose column chromatography, no single fraction catalyzed the oxidation of BT. However, addition of the fraction containing NADH oxidoreductase activity in the same Q Sepharose eluate restored BT oxidation activity. A similar

Table 1 Summary of the steps for the purification of TdsC. Assays were carried out with purified TdsD (0.3 μM) as described in Materials and Methods

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)
Cell-free extract	2,170	347	0.16	1
Q Sepharose	200	1,814	9.07	56.7
DEAE Toyopearl	94	911	9.69	60.6
Phenyl Sepharose	32	454	14.2	88.8
Biogel CHT-5	20	374	18.6	116

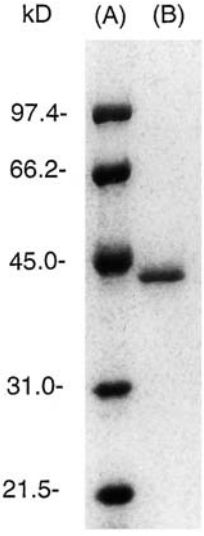

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified TdsC. The purified enzyme was electrophoresed and stained with Coomassie brilliant blue. Lanes: *A* Low range molecular weight standards (Bio-Rad), *B* purified enzyme

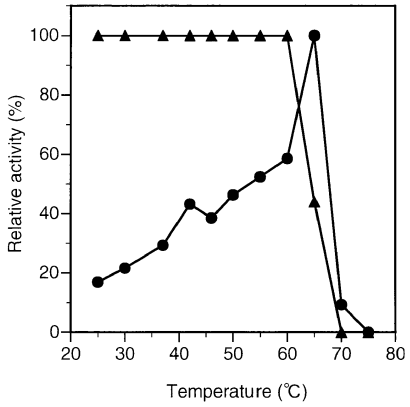

Fig. 2 Effect of temperature on the activity and stability of purified TdsC. Enzyme activity was determined under the conditions described in Materials and Methods. For measurement of stability, the purified enzyme in 50 mM Tris-HCl buffer (pH 7.0) was incubated for 30 min at various temperatures. After cooling on ice, the residual activity was determined at 55°C. *Circles* activity, *triangles* stability

phenomenon was observed in the purification of TdsA (Konishi et al. 2000a). We previously reported that TdsD was required for the detection of TdsA activity (Konishi et al. 2000a). Therefore, we concluded that the coexistence of TdsD (0.015 U mg^{-1}) was also indispensable for TdsC activity. Table 1 shows the results of the purification of the TdsC enzyme. TdsC was purified 116-fold by the subsequent chromatographies and the specific activity of the final enzyme preparation was 18.6 U mg^{-1}. The cell-free extract of A11–2 exhibited very weak activity. The activity was remarkably enhanced at the step of ion chromatography suggesting the presence of some inhibitory factor(s) in the extract. The purified enzyme showed a single protein band on SDS-PAGE (Fig. 1). The molecular mass of the purified enzyme and its subunit were calculated to be 200 kDa and 43 kDa by gel filtration and SDS-PAGE, respectively, suggesting a tetrameric structure. This value was a little smaller than that of DszC from *R. erythropolis* D-1, which was reported to be a hexamer (Ohshiro et al. 1997). The N-terminal amino acid sequence of the purified TdsC was MRTIHANSSAVRED. This sequence completely matched the amino acid sequence deduced from the nucleotide sequence of the cloned *tds*C gene from A11–2 (Ishii et al. 2000a).

Effects of temperature and pH on enzymic activity

The activity profile of TdsC was examined from 30°C to 70°C (Fig. 2). The activity was found to be highest at 65°C. The specific activity at 65°C was about 1.5 times that at 50°C. The enzyme retained more than 80% of its activity after exposure to 65°C for 30 min. However, no activity was detectable after incubation for 30 min at 70°C. TdsC showed maximum activity at pH 9.

Kinetic parameters

The apparent K_m and V_{max} for BT calculated from a Lineweaver-Burk plot were 0.36 mM and 80 U mg^{-1}, respectively. The K_m for DBT could not be calculated because of the low reactivity of TdsC toward DBT at high substrate concentrations.

Coupling and cofactor requirements of TdsC and TdsD

The influence of the concentration of TdsD on TdsC activity at a constant concentration was investigated (Fig. 3). Maximum activity was obtained at a TdsD/TdsC molar ratio of 0.5. About 70% of the original activity was obtained with only one-tenth of the concentration of TdsD compared to TdsC. This indicates high coupling efficiency of TdsD with TdsC. Purified TdsC exhibited no activity in the absence of TdsD (Table 2). To attain complete activity, the presence of both NADH and FMN was necessary. NADPH could substitute for NADH to some extent. FAD and lumiflavin had only weak activity as reducing agents.

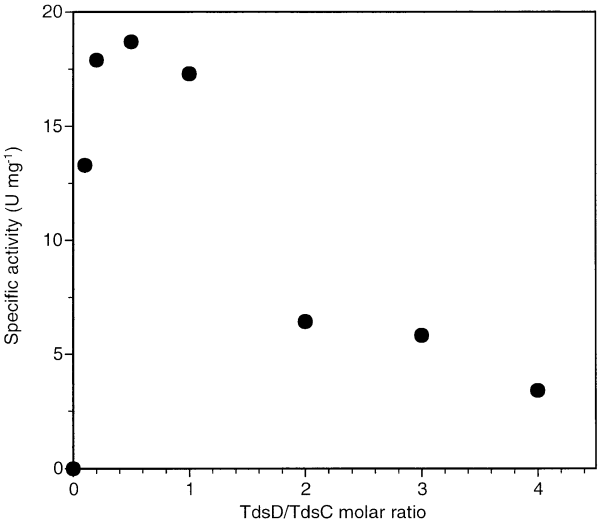

Fig. 3 Effect of the molar ratio of TdsC and TdsD on TdsC activity. The effect of TdsC/TdsD molar ratio on TdsC activity was investigated by keeping the TdsC concentration constant at 0.95 nmol and varying the TdsD concentration. The reaction temperature was 55°C. Other conditions were as described in Materials and Methods

Table 2 Requirements for the activity of TdsC. Assays were carried out with purified TdsC (1.05 μM) and TdsD (0.51 μM) as described in Materials and Methods

Conditions	Specific activity (U mg^{-1})
TdsC	0
TdsC + TdsD	0
TdsC + TdsD + NADH[a]	6.3
TdsC + TdsD + FMN[b]	0
TdsC + TdsD + NADH[a] + FMN[b]	23.7
TdsC + TdsD + NADPH[a] + FMN[b]	13.6
TdsC + TdsD + NADH[a] + FAD[b]	3.1
TdsC + TdsD + NADH[a] + lumiflavin[b]	2.5
TdsC + NADH[a] + FMN[b]	0

[a] At 3 mM; [b] At 10 μM

Effect of various compounds on enzymic activity

The effect of various compounds on the activities of TdsC and TdsD was investigated (Table 3). TdsC activity was strongly inhibited by *p*-chloromercuribenzoic acid and 5,5′-dithio-bis-2-nitrobenzoic acid, and was significantly inhibited by thiol reagents such as *N*-ethylmaleimide and iodoacetic acid. As was observed for DszC purified from *R. erythropolis* strain D-1 (Ohshiro et al. 1997), the addition of a chelating reagent caused complete inhibition of TdsC activity. These results indicated that divalent cations are essential for TdsC enzymic activity. TdsC was quite susceptible to heavy metal ions such as Zn^{2+} and Cu^{2+}, which caused a complete loss of activity. TdsD activity was hardly affected at all by these ions.

Table 3 Effects of metal ions and various compounds on the activity of TdsC and TdsD. TdsC reaction mixtures (1 ml) containing 50 mM Tris-HCl buffer (pH 7.0), 9 mM NADH, 10 μM FMN, 0.7 μM TdsC and 50 μl cell-free extract of *Escherichia coli* JM109 harboring pD1RSm1 that carries *tds*D, were preincubated at 50°C for 5 min with various metal ions and compounds. The activity of TdsD in the cell-free extract was 7.8 U ml^{-1}. The reactions were initiated by the addition of 0.27 mM benzothiophene (BT). TdsD reaction mixtures (1 ml) containing 50 mM Tris-HCl buffer (pH 7.0), 10 μM FMN and 0.51 μM TdsD were preincubated at 50°C for 5 min with various metal ions and compounds. The reactions were initiated by the addition of 0.1 mM NADH

Compound	Concentration (mM)	Relative activity (%)	
		TdsC	TdsD
None		100	100
$MgCl_2$	1	108	95
$CaCl_2$	1	84	102
$MnCl_2$	1	34	87
$ZnCl_2$	1	0	90
$CuCl_2$	1	0	117
$CoCl_2$	1	26	77
p-Chloromercuribenzoic acid	0.5	0	29
5,5′-Dithio-bis-2-nitrobenzoic acid	0.5	6	nt[a]
N-Ethylmaleimide	1	2	72
Iodoacetate	1	54	100
EDTA	1	32	100
1,10-Phenanthroline	1	0	90

[a] Not tested

Table 4 Substrate specificity of TdsC. TdsC reaction mixtures (1 ml) containing 50 mM Tris-HCl buffer (pH 7.0), 9 mM NADH, 10 μM FMN, 0.7 μM TdsC and 50 μl cell-free extract of *E. coli* JM109 harboring pD1RSm1 that carries *tds*D, were preincubated at 50°C for 5 min. The activity of TdsD in the cell-free extract was 7.8 U ml^{-1}. DszC reaction mixtures (1 ml) containing 50 mM Tris-HCl buffer (pH 7.0), 9 mM NADH, 10 μM FMN, 0.92 μM DszC and 100 μl cell-free extract of *E. coli* JM109 harboring a derivative of pUC118 carrying the *dsz*D gene were used. The activity of DszD in the cell-free extract was 0.26 U ml^{-1}. The reactions were initiated by the addition of 0.27 mM of the substrates. After 30 min, the enzymic activity was determined as described in Materials and Methods

Substrate	Specific activity (U mg^{-1})[a]	
	TdsC	DszC
Dibenzothiophene (DBT)	4.6	40.7
1-Methyl DBT	1.2	24.1
2-Methyl DBT	10.8	48.1
3-Methyl DBT	12.4	38.0
4-Methyl DBT	4.6	30.6
4,6-Dimethyl DBT	5.5	15.2
4,6-Diethyl DBT	11.5	21.4
4,6-Dipropyl DBT	0.1	17.9
4,6-Dibutyl DBT	0	0
BT	36.1	4.5
3-Methyl BT	16.4	36.1
5-Methyl BT	17.5	7.7

[a] One unit of enzyme activity was defined as the amount producing 1 nmol of corresponding sulfone per minute

Substrate specificity

BT was rapidly oxidized by TdsC to produce BT sulfone. The reaction was almost stoichiometric and no other product was detected by GC. In the case of the oxidation of DBT, both DBT sulfoxide and DBT sulfone were detected, indicating the slow oxidation step of DBT (unpublished data). Table 4 shows the reactivities of TdsC against alkylated DBT derivatives and three kinds of methylated BTs. The reactivities of DszC purified from *R. erythropolis* KA 2–5–1 were also examined for comparison. TdsC and DszC exhibited contrasting behavior towards DBT and BT. The activity of TdsC toward BT was about eight times higher than that toward DBT as expected. On the other hand, the activity of TdsC was ten times lower than that of DszC toward DBT. Alkylated BTs were poor substrates for DszC except for 3-methyl BT as previously reported (Kobayashi et al. 2000). Among the alkylated DBTs examined, TdsC exhibited extremely low activity against 1-methyl DBT and 4-methyl DBT. In contrast to DszC, TdsC showed 2.5 times higher activity against 4,6-diethyl DBT than DBT and did not oxidize 4,6-dipropyl DBT. Neither enzyme oxidized 4,6-dibutyl DBT indicating limited catalytic ability.

Discussion

Several BT-desulfurizing bacteria (Gilbert et al. 1998; Matsui et al. 2001; Tanaka et al. 2001) have been isolated and their metabolites characterized. Similar to other bacteria, BT sulfone was found as an intermediate for A11–2. However, it should be noted that none of the BT-desulfurizing bacteria reported so far grow on DBT, whereas A11–2 grows on both BT and DBT (Konishi et al. 2000b). In addition, we demonstrated in this study that TdsC exhibited activity towards both BT and DBT. Therefore, we concluded that TdsC is responsible for the first step of desulfurization of both BT and DBT by strain A11–2. TdsC shows 51.5% homology at the amino acid level with DszC of IGTS8 (Ishii et al. 2000a). This relatively low similarity may explain the difference of substrate specificity as well as thermostability between these enzymes. TdsC was extremely sensitive to SH-reagents, similar to DszC of *R. erythropolis* D-1 (Ohshiro et al. 1997). Since both TdsC and DszC have only one cysteine residue (^{159}Cys for TdsC and ^{162}Cys for DszC), we consider these sites to be potential modification sites.

A small amount of TdsD was sufficient for the activity of TdsC. TdsD is a yellowish enzyme and contains FMN as a prosthetic cofactor (unpublished result). Therefore, the high coupling efficiency of TdsD might be caused by the tightly bound FMN. The presence of a large amount of TdsD caused a decrease in TdsC activity (Fig. 3). This effect may be due to toxic reactive oxygen species generated by autooxidation of reduced FMN, as observed in *E. coli* (Gaudu et al. 1994) and *Pseudomonas putida* (Galan et al. 2000).

The strong activity of TdsC against BT compared to DBT supported the observation that strain A11–2 grew more rapidly on BT than on DBT. We also demonstrated that DszC purified from *R. erythropolis* strain KA2–5–1 exhibited the highest level of activity against 3-methyl BT among alkylated BTs examined (Kobayashi et al. 2000). This result is consistent with the observation that strain KA2–5–1 can use only 3-methyl BT among various alkylated BTs as a sulfur source (Kobayashi et al. 2000). The maximum concentration of DBT soluble in water is relatively low (in the micromolar range) and the concentration of DBT in the assay mixture for activity measurement was beyond the upper limit of solubility. However, the DszC activity of KA2–5–1 increased with the concentration of DBT within the same concentration range of DBT as TdsC (unpublished data). Thus, we consider that the low solubility of DBT is not the major cause of the low activity toward DBT compared to BT. In the case of TdsC, the position of the methyl group attached to the aromatic ring seems to have a great influence on the activity as was observed for 1-methyl DBT and 4-methyl DBT (Table 4). Furthermore, as was observed in the comparison of 4,6-diethyl DBT and 4,6-dipropyl DBT, the addition of only one methyl group markedly decreased the activity. Thus, the length of the alkyl chain attached in the vicinity of the sulfur of DBT (4- and/or 6-position) had a remarkable effect on TdsC activity. These results suggest that the substrate binding area of TdsC might be more closed-off than that of DszC. A precise explanation of these results must await further structural study of this enzyme. In addition, expanding the substrate spectrum as well as increasing specific activity of TdsC, especially toward alkylated DBTs, would be necessary for practical application.

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