

Kohtaro Kirimura · Koji Harada · Hidekazu Iwasawa ·  
Takeomi Tanaka · Yuichiro Iwasaki · Toshiki Furuya ·  
Yoshitaka Ishii · Kuniki Kino

## Identification and functional analysis of the genes encoding dibenzothiophene-desulfurizing enzymes from thermophilic bacteria

Received: 28 November 2003 / Revised: 27 April 2004 / Accepted: 7 May 2004 / Published online: 25 June 2004  
© Springer-Verlag 2004

**Abstract** Thermophilic bacteria *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1 desulfurize dibenzothiophene (DBT) and alkylated DBTs through specific cleavage of the carbon-sulfur bonds over a temperature range up to 52°C. In order to identify and functionally analyze the DBT-desulfurization genes, the gene cluster containing *bdsA*, *bdsB*, and *bdsC* was cloned from *B. subtilis* WU-S2B. The nucleotide and amino acid sequences of *bdsABC* show homologies to those of the other known DBT-desulfurization genes and enzymes; e.g. a nucleotide sequence homology of 61.0% to *dszABC* of the mesophilic bacterium *Rhodococcus* sp. IGTS8 and 57.8% to *tdsABC* of the thermophilic bacterium *Paenibacillus* sp. A11-2. Deletion and subcloning analysis of *bdsABC* revealed that the gene products of *bdsC*, *bdsA* and *bdsB* oxidized DBT to DBT sulfone (DBTO<sub>2</sub>), converted DBTO<sub>2</sub> to 2'-hydroxybiphenyl-2-sulfinate (HBPSi), and desulfurized HBPSi to 2-hydroxybiphenyl (2-HBP), respectively. Resting cells of a recombinant *Escherichia coli* JM109 harboring *bdsABC* converted DBT to 2-HBP over a temperature range of 30–52°C, indicating that the gene products of *bdsABC* were functional in the recombinant. The activities of DBT degradation at 50°C and DBT desulfurization (2-HBP production) at 40°C in resting cells of the recombinant were approximately five times and twice, respectively, as high as those in *B. subtilis* WU-S2B. The recombinant *E. coli* cells also degraded alkylated DBTs, such as 2,8-dimethylDBT and 4,6-dimethylDBT. The nucleotide sequences of *B. subtilis* WU-S2B *bdsABC* and the corresponding genes from *M. phlei* WU-F1 were found to be completely identical to each other although the strains are genetically different.

### Introduction

Organosulfur compounds contained in fossil fuels are one of the major causes of acid rain and air pollution since the combustion of fossil fuels releases sulfur oxides into the atmosphere. During the refinery process of petroleum, sulfur is removed from petroleum by hydrodesulfurization (HDS) with chemical metal-containing catalysts under extremely high temperatures and pressures. However, heterocyclic sulfur compounds, such as dibenzothiophene (DBT) and alkylated DBTs, are recalcitrant to conventional HDS processes, and alkylated DBTs especially remain in the fraction of light gas oil. Therefore, the development of a desulfurization process for more intensive desulfurization in light of increasingly stringent environmental regulations is required. One such method, the application of a biodesulfurization process using microbial catalysts capable of desulfurizing HDS-resistant sulfur compounds, such as alkylated DBTs, has been the focus of great interest because of its reduced burden on the environment with respect to energy consumption and CO<sub>2</sub> evolution (for review, see Monticello 2000; Ohshiro and Izumi 1999, 2002; Suzuki 1999).

Various mesophilic bacteria capable of desulfurizing DBT, a model organosulfur compound recognized as a target for more intensive desulfurization, through targeted reactions at the carbon-sulfur bonds have been isolated (for review, Ohshiro and Izumi 1999, 2002; Suzuki 1999). For example, in *Rhodococcus* sp. IGTS8, genes involved in sulfur-specific desulfurization of DBT were identified, and three desulfurization genes (*dszA*, *dszB*, and *dszC*) were confirmed to constitute a single operon (Denome et al. 1993; Li et al. 1996; Piddington et al. 1995). In this bacterium, four enzymes consisting of two monooxygenases (*DszC* and *DszA*), one desulfinate (*DszB*), and one flavin reductase (*DszD*) that couples with *DszC* and *DszA*, are responsible for DBT desulfurization (Gray et al. 1996). Furthermore, in a bacterial community residing in sulfurous-oil-containing soils, the sequence homology and classification of desulfurization genes were determined using PCR (Duarte et al. 2001).

K. Kirimura (✉) · K. Harada · H. Iwasawa · T. Tanaka ·  
Y. Iwasaki · T. Furuya · Y. Ishii · K. Kino  
Department of Applied Chemistry, School of Science and  
Engineering, Waseda University,  
3-4-1 Ohkubo, Shinjuku-ku,  
Tokyo, 169-8555, Japan  
e-mail: kkohtaro@waseda.jp  
Tel.: +81-3-52863208  
Fax: +81-3-32323889

Since most DBT-desulfurizing bacteria are mesophilic, their DBT-desulfurizing activities are high at temperatures of around 30°C and lower at temperatures above 40°C (Nakayama et al. 2002; Ohshiro et al. 1997, 1999). However, HDS-treated light gas oil would be supplied to the biodesulfurization process at a temperature of more than 40°C and thus would have to first be cooled to around 30°C before being desulfurized by the bacteria (Borgne and Quintero 2003; Mcfarland 1999). Moreover, in the refinery process, to avoid solidification of the oils, the temperature of HDS-treated light gas oil is usually maintained at around 45°C even after recovery of the residual heat of the oil by the heat exchanger (COSMO Oil 2002). Therefore, the ability to carry out thermophilic biodesulfurization at 40–50°C is desirable and could be integrated more easily into the refining process, since the energy cost for cooling the HDS-treated light gas oil is thereby omitted. Another advantage of thermophilic biodesulfurization is that contamination by mesophilic bacteria would be avoided. With this goal in mind, we previously isolated two thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B (Kirimura et al. 2001) and *Mycobacterium phlei* WU-F1 (Furuya et al. 2001, 2002), which can desulfurize DBT and DBT derivatives through targeted oxidation of the carbon–sulfur bonds even at 50°C. Furthermore, we confirmed that *M. phlei* WU-F1 efficiently desulfurized HDS-treated light gas oil over a temperature range up to 50°C (Furuya et al. 2003).

The thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 was previously isolated (Konishi et al. 1997), and its genes and enzymes involved in thermophilic desulfurization were characterized (Ishii et al. 2000a,b; Konishi et al. 2000). In this bacterium, three desulfurization genes (*tdsA*, *tdsB*, and *tdsC*) constitute an operon similar to that of *Rhodococcus* sp. IGTS8, and each of the *tds* genes shows a considerable homology to the corresponding *dsz* genes (Ishii et al. 2000a). The thermal profiles of DBT desulfurization by growing and resting cells of *Paenibacillus* sp. A11-2 showed that DBT was desulfurized only at a temperature of around 50°C (Konishi et al. 1997). By contrast, since growing and resting cells of *B. subtilis* WU-S2B and *M. phlei* WU-F1 can desulfurize DBT over a temperature range from 30 to 52°C (Furuya et al. 2001; Kirimura et al. 2001), these strains might be more practical. Before the properties of *B. subtilis* WU-S2B and *M. phlei* WU-F1 can be applied as genetic resources for biodesulfurization, the DBT-desulfurization genes must be identified and functionally analyzed. Furthermore, in order to understand explicitly the molecular basis of bacterial desulfurization, a comparative analysis of the nucleotide sequences of desulfurization genes and the deduced amino acid sequences of the respective enzymes from different species of bacteria is required.

In this report, we describe the identification and functional analysis of the genes responsible for DBT desulfurization in *B. subtilis* WU-S2B. The nucleotide sequences of three open reading frames (ORFs, designated *bdsA*, *bdsB*, and *bdsC* for bacterial desulfurization) were

found to constitute a single operon, and the individual functions of these ORFs were characterized by their heterologous expression in *Escherichia coli*. The effects of temperature on the desulfurizing activity of recombinant *E. coli* cells harboring *bdsABC* and that of *B. subtilis* WU-S2B were compared. The results showed that the gene products of *bdsABC* were functional in the recombinant *E. coli* cells. In addition, we cloned and sequenced the DBT-desulfurizing genes of *M. phlei* WU-F1. Surprisingly, the desulfurization genes of the two bacteria were identical to each other. Therefore, we concluded that the desulfurizing abilities of *B. subtilis* WU-S2B and *M. phlei* WU-F1 over a temperature range up to 52°C are basically dependent on the function of *bds* gene products.

## Materials and methods

### Bacterial strains and culture conditions

*B. subtilis* WU-S2B (FERM P-17041, Kirimura et al. 2001) and *M. phlei* WU-F1 (FERM P-17717, Furuya et al. 2001) were used as sources of desulfurization genes. For isolation of total DNA, *B. subtilis* WU-S2B was cultivated as described previously (Kirimura et al. 2001) and *M. phlei* WU-F1 as described previously (Furuya et al. 2001) with the modifications of Poupin et al. (1999). *E. coli* XL1-Blue MRA(P2) and lambda DASH II phage vector (Stratagene, CA) were used for cloning of the desulfurization genes, and *E. coli* JM109 and cloning vector pGEM-T (Promega, WI) for cloning and sequencing of PCR products. *E. coli* JM109 and cloning vector pUC19 were used for subcloning of genes from the phage vector, and *E. coli* JM109 and pKK223-3 (Amersham Biosciences, NJ) for expression of the genes. *E. coli* strains were grown in either Luria–Bertani (LB) medium or NZY medium (Sambrook and Russell 2001). Recombinants were selected on LB agar plates or in liquid media containing 50 µg ampicillin/ml.

### DNA manipulation

Total DNA of *B. subtilis* WU-S2B and *M. phlei* WU-F1 was prepared with a QIAGEN Genomic-tip (QIAGEN, CA). Phage DNA was purified with a QIAGEN Lambda Midi Kit, and plasmid DNA was purified with a QIAGEN Plasmid Mini Kit. DNA fragments were purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Digestion with restriction endonucleases, ligation, and deletion experiments were carried out using standard procedures (Sambrook and Russell 2001) under the conditions recommended by the manufacturers. Automated DNA sequencing was performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). The sequence was determined by complete sequencing of both strands, with multiple sequencing of some regions. Sequence assembly and analysis were done using GENETYX-MAC ver10.1 (SDC, Tokyo, Japan). When necessary, PCR-derived fragments were sequenced to confirm that no point mutation had occurred. The GenBank and SwissProt databases were searched for similarities of nucleic acid and amino acid sequences using the FASTA program of the DNA Data Bank of Japan (DDBJ) and the BLAST program of the National Center for Biotechnology Information (NCBI). The analysis of amino acid homology by the phylogenetic tree was carried out using the ClustalW program of the DDBJ.

### Construction of a total DNA library

Total DNAs of *B. subtilis* WU-S2B and *M. phlei* WU-F1 were partially digested with *Sau3A*I and treated with bacterial 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 III Gold packaging kit (Stratagene). The packaged phages were propagated in *E. coli* XL1-Blue MRA(P2) cells.

### Oligonucleotide primers, probes, and hybridization

The nucleic acid sequences of the PCR primers were designed using conserved amino acid sequences of DBT-desulfurizing enzymes from *Rhodococcus* sp. IGTS8 and *Paenibacillus* sp. A11-2. PCR was done with the GeneAmp PCR system 9700 (Applied Biosystems Japan) as follows. The PCR reaction mixture (50  $\mu$ l), containing 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, dNTP (each species, 200  $\mu$ M), PCR primers (1  $\mu$ M each), DNA template, and *Taq* DNA Polymerase (1.25U, Nippon Roche, Tokyo, Japan), was reacted according to the following PCR program: 5 min at 95°C; 30 cycles of 1 min at 95°C, 1.5 min at 52°C, and 3 min at 72°C; followed by a final 10 min at 72°C. The PCR product was cloned in *E. coli* and sequenced.

A DNA probe was prepared by randomly labeling the PCR product with digoxigenin-11-dUTP using the DIG DNA Labeling Kit (Nippon Roche) and then used for screening clones harboring desulfurization genes. The lambda DASH II library of *B. subtilis* WU-S2B total DNA was plated on a lawn of *E. coli* XL1-Blue MRA (P2) cells grown on NZY agar plates and transferred to Hybond-N+ nylon membranes (Amersham Biosciences). The membranes were probed at 68°C in 5 $\times$ SSC, 1% (w/v) blocking reagent (Nippon Roche), 0.1% (w/v) *N*-lauroylsarcosine and 0.02% (w/v) SDS, and washed twice at 25°C in 2 $\times$ SSC and 0.1% (w/v) SDS, and at 68°C in 0.1 $\times$ SSC and 0.1% (w/v) SDS.

### Construction of the expression plasmids for desulfurization genes

In order to adjust the restriction site to the cloning site of pKK223-3 for construction of expression plasmids, a fragment from -41 to 386 bp in the direction of the start codon of *bdsA* was amplified by PCR using the primers 5'-GCC AAG CTT TGG TCT CCG GTA ACT GAT CCC-3' and 5'-GAC CAC ATT CCA CGA GAT CCG GC-3' to generate a *Hind*III site and *Bss*SI site, respectively. The amplified 0.4 kb *Hind*III-*Bss*SI fragment and a 3.4-kb *Bss*SI-*Mlu*I fragment of pU1Nh were cloned into the *Hind*III site of pKK223-3 to construct plasmid pKBDS, since *Mlu*I has the same cohesive ends as *Hind*III. The deletion plasmids pKBDSA, pKBDSB and pKBDS C were constructed from pKBDS with restriction endonuclease digestion and self-ligation.

### Chemicals

DBT, 2-hydroxybiphenyl (2-HBP), and 2,8-dimethylDBT were purchased from Tokyo Kasei (Tokyo, Japan). DBT sulfone (DBT<sub>2</sub>) and benzo[*b*]naphtho[2,1-*d*]thiophene (3,4-benzoDBT) were purchased from Aldrich (Milwaukee). 4,6-DimethylDBT, 2'-hydroxybiphenyl-2-sulfinate (HBPSi) and 2-ethylnaphtho[2,1-*b*]thiophene (2-ethylnTH) were kindly supplied by the laboratory of Japan Cooperation Center, Petroleum (Shizuoka, Japan). All other reagents were of analytical grade and commercially available.

### Thermal profile of desulfurization by resting cells of the recombinant *E. coli*

Recombinant *E. coli* JM109 cells were cultivated overnight at 30°C in LB-ampicillin medium and then diluted 100-fold with fresh LB-ampicillin medium followed by shaking at 37°C for 16 h. Cells were harvested at 4°C by centrifugation at 8,000 *g* for 5 min, washed twice and resuspended in 0.1 M potassium phosphate buffer (pH 7.0) to give an optical density at 660 nm (OD<sub>660</sub>) of 20. Fifty microliters of substrate solution, DBT and its metabolic intermediates (5.4 mM, in *n*-tridecane or ethanol), were added to 1 ml of the cell suspension in a screw-cap test tube. For measurement of desulfurizing activity in an organic/aqueous two-phase reaction system, *n*-tridecane was used as the organic phase since it dissolved DBT and showed no harmful effect on *E. coli*. The reaction was done at 50°C or at the temperatures indicated in the figures with inverted shaking in a rotating incubator at 50 rpm for 1 h. After the reaction, the bacterial suspension was acidified by adding 20  $\mu$ l of 6 M HCl and extracted with 0.5 ml ethylacetate. The extract was filtered through a 0.20- $\mu$ m polytetrafluoroethylene membrane filter (Advantec Toyo, Tokyo, Japan) and then analyzed by high-performance liquid chromatography (HPLC, type LC-10A; Shimadzu, Kyoto, Japan) using a Puresil C18 column (Waters, MA), as described by Kirimura et al. (2001).

### Substrate specificity of desulfurization by growing cells of recombinant *E. coli*

The recombinant *E. coli* JM109 cells harboring plasmids were incubated overnight at 30°C in LB-ampicillin medium. Aliquots (50  $\mu$ l) of these cultures were inoculated into 5 ml of fresh, modified M9-IPTG-ampicillin medium using MgCl<sub>2</sub> instead of MgSO<sub>4</sub>, and substrate solution (50  $\mu$ l) containing DBT or its derivatives (5.4 mM, in *n*-tridecane) was added. After cultivation with reciprocal shaking at 37°C for 72 h, the culture broth was acidified by adding 100  $\mu$ l of 6 M HCl and extracted with 2 ml ethylacetate. The resulting extract was then analyzed by gas chromatography (GC, type GC-2010; Shimadzu) equipped with a flame ionization detector, a flame photometric detector and a 30-m type DB-5 capillary column (J&W Scientific, CA). The flow rate of the nitrogen carrier gas was 15 ml/min, the column temperature was set at 210°C (240°C for 3,4-BenzoDBT), and the injector and detector temperatures were maintained at 260°C. *B. subtilis* WU-S2B was cultivated with reciprocal shaking at 45°C for 72 h using A-2 medium (Kirimura et al. 2001) instead of LB or modified M9 medium.

### Accession number

The nucleotide sequence data for the desulfurization genes of *B. subtilis* WU-S2B were submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB076745.

## Results

### Cloning of the DBT-desulfurization genes from *B. subtilis* WU-S2B

DBT-desulfurization genes constituting operons, such as *dszABC* and *tdsABC*, were previously cloned from the mesophilic bacterium *Rhodococcus* sp. IGTS8 (Denome et al. 1993; Piddington et al. 1995) and the thermophilic bacterium *Paenibacillus* sp. A11-2 (Ishii et al. 2000a), respectively. Since the conserved regions in the deduced

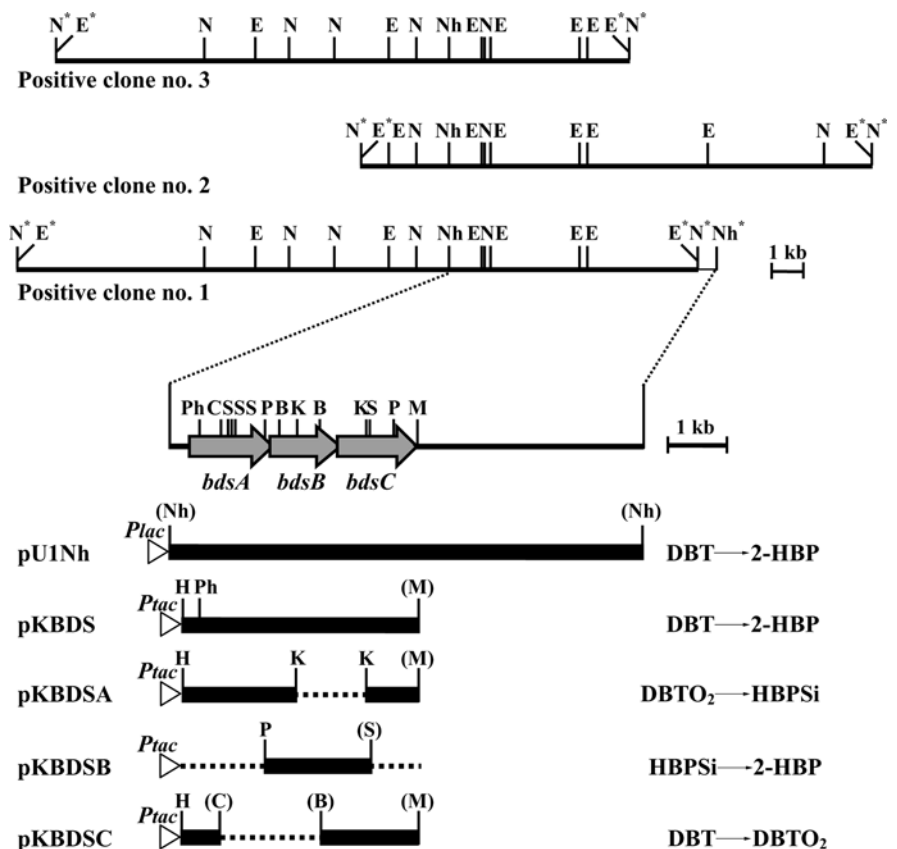
amino acid sequences of these genes were searched and identified as AEARNFG, between 429 and 447 nucleotides from the initiation codon of *dszA*, and GFDRFWR, between 99 and 116 nucleotides from the termination codon of *dszC*, they were chosen as appropriate regions for PCR primers. Based on these amino acid and nucleotide sequences, degenerated primers (5'-GCI GAR GCI MGI AAY TTY GG-3' as the sense primer and 5'-CGT IGC GCC AIA AGC GGT C-3' as the anti-sense primer; I, inosine) were synthesized and used for PCR with total DNA prepared from *B. subtilis* WU-S2B as the template. A 3.2-kb amplified DNA fragment was cloned into the pGEM-T vector, and the nucleotide sequence of this PCR product was determined. Since the amino acid sequence deduced from the nucleotide sequence showed approximately 60% homology to both *dsz* and *tds*, this PCR product was presumed to be a part of the DBT-desulfurization genes of *B. subtilis* WU-S2B. Primers for amplification of the internal region of this PCR product (5'-GCA TGA CAT CCG ATA CG-3' as the sense primer and 5'-TAG TTT GGG TGG GTT CC-3' as the anti-sense primer) were subsequently synthesized and used for another PCR with total DNA of *B. subtilis* WU-S2B. The PCR product amplified with these primers was labeled and used as probe to screen a total DNA library of *B. subtilis* WU-S2B in the lambda DASH II vector by plaque hybridization. Among approximately 8,000 plaques screened by plaque hybridization, three positive plaques were obtained. As shown in Fig. 1, restriction and Southern hybridization analyses suggested that all insert

DNAs from three positive lambda clones contained a part of the same region of the total DNA of *B. subtilis* WU-S2B and that a 7.1-kb *NheI* fragment of positive clone no. 1 covered the entire region (*bdsABC*) encoding all the DBT-desulfurizing enzymes, as described later. The 7.1-kb *NheI* fragment was then cloned into *XbaI* site of pUC19 to give pU1Nh plasmid.

#### Characterization of the nucleotide sequence of the *bds* genes

The nucleotide sequence hybridizable with the probe and the sequence adjacent to the hybridizable region of pU1Nh were determined. Three ORFs were identified to be aligned in the same direction. These ORFs show significant homology to the *dsz* operon of *Rhodococcus* sp. IGTS8 and the *tds* operon of *Paenibacillus* sp. A11-2. Each ORF homologous to *dszA*, *dszB*, and *dszC* of IGTS8 was therefore designated as *bdsA*, *bdsB*, and *bdsC*, respectively, for bacterial desulfurization. The overall nucleotide sequence of the *bds* operon, *bdsABC*, shows 61.0% homology to *dszABC* of *Rhodococcus* sp. IGTS8 and 57.8% to *tdsABC* of *Paenibacillus* sp. A11-2. In this operon structure of *bdsABC*, the end of *bdsA* has a 4-bp overlap with the translational initiation site of *bdsB*, similar to the *dsz* genes of *Rhodococcus* sp. IGTS8 and the *tds* genes of *Paenibacillus* sp. A11-2. Furthermore, *bdsB* also has a 4-bp overlap with *bdsC*, which is in contrast to the *dsz* and *tds* genes.

**Fig. 1** Physical maps of three positive lambda clones and the desulfurization genes from *Bacillus subtilis* WU-S2B. The construction of the deletion plasmids is described in "Materials and methods". The reaction catalyzed by recombinant *Escherichia coli* cells harboring each deletion plasmid is indicated on the right side of plasmid map. The restriction sites from the vector are shown with an asterisk; sites removed by blunting and ligation are shown in parenthesis. B *Bpu1102I*, C *CpoI*, E *EcoRI*, H *HindIII*, K *KpnI*, M *MluI*, N *NotI*, Nh *NheI*, P *PstI*, Ph *PshAI*, S *SacII*. *Plac* and *Ptac* (open triangles) show the *lac* and *tac* promoter directions of pUC18 and pKK223-3, respectively



The first ORF, *bdsA*, encodes a protein of 453 amino acids, and the corresponding gene product, BdsA, shows significant homology with several proteins, as determined by BLAST and FASTA programs. BdsA has 78.8 and 64.4% homologies to DszA of *Rhodococcus* sp. IGTS8 and TdsA of *Paenibacillus* sp. A11-2, respectively. Low homologies were found between BdsA and several FMNH<sub>2</sub>-dependent monooxygenases, except for DszA and TdsA. For example, BdsA shows 41.5, 41.2, and 32.2% homologies to SnaA, pristinamycin II<sub>A</sub> synthase subunit A of *Streptomyces pristinaespiralis* (Blanc et al. 1995), NtaA, nitrilotriacetate monooxygenase of *Chelatobacter heintzii* (Knobel et al. 1996), and EmoA, EDTA monooxygenase of EDTA-degrading bacterium BNC1 (Bohuslavsek et al. 2001), respectively. Alignment of the amino acid sequences of BdsA and its equivalents, DszA and TdsA, from two other desulfurizing bacteria (Fig. 2) showed that BdsA has high homology in its N-terminal region, approximately 250 amino acids, and its C-terminal region, approximately 50 amino acids, to the equivalent proteins. When compared to the FMNH<sub>2</sub>-dependent monooxygenase family, BdsA also shows high homology in the regions conserved within desulfurizing enzymes. However, BdsA shows no similarity in its N-terminal region of approximately 100 amino acids to members of this family, such as NtaA, SnaA, and EmoA (data not shown). In this family, as shown in Fig. 3a, DBT-desulfurizing enzymes could be assigned to one cluster on the phylogenetic tree, but, as shown in Fig. 3b, *bdsA* is independently located from three clusters of the partial *dszA* genes classified by Duarte et al. (2001).

The second ORF, *bdsB*, encodes a protein of 356 amino acids, and the deduced amino acid sequence shows 68.1 and 53.1% homology to those of DszB of *Rhodococcus* sp. IGTS8 and TdsB of *Paenibacillus* sp. A11-2, respectively. As shown in Fig. 2, while the N-terminal region of BdsB shows high homology to those of the equivalents, the amino acid sequence of this region has no homology to other enzymes, except to DszB and TdsB. BdsB also shows partial homology (approximately 20–30%) to SsuA (van der Ploeg et al. 1999), AsfC (Vermeij et al. 1999), and AtsR (Kahnert and Kertesz 2000), which are substrate-binding proteins responsible for sulfonate or sulfate ester utilization and are members of sulfate starvation-induced proteins.

The last ORF, *bdsC*, encodes a protein of 415 amino acids, and the deduced amino acid sequence of BdsC shows 72.9 and 51.2% homology to DszC of *Rhodococcus* sp. IGTS8 and TdsC of *Paenibacillus* sp. A11-2, respectively. BdsC also shows partially homology to acyl-CoA dehydrogenases of several microorganisms (P45857, P52042, and Q06319).

Heterologous expression of the DBT-desulfurization genes (*bdsABC*) in *E. coli*

Recombinant *E. coli* cells harboring pU1Nh, containing the entire region of *bdsABC*, degraded DBT to produce 2-

HBP (data not shown), indicating that *bdsABC*, encoding the enzymes responsible for the ability of DBT-desulfurization, could be expressed in *E. coli* cells. Subsequently, the contribution of each ORF (*bdsA*, *B* and *C*) to DBT-desulfurization was characterized. As shown in Fig. 1, the recombinant *E. coli* cells harboring pKBDSA carrying only *bdsA* but lacking *bdsB* and *bdsC* could convert DBTO<sub>2</sub> into HBPSi, but no degradation of DBT and HBPSi was observed. The recombinant *E. coli* cells harboring pKBDSB carrying only *bdsB* desulfurized HBPSi into 2-HBP, and the recombinant *E. coli* cells harboring pKBDSB carrying only *bdsC* oxidized DBT into DBTO<sub>2</sub>. These results clearly indicated that the gene products of *bdsA*, *bdsB*, and *bdsC* are responsible for the desulfurization of DBT to 2-HBP via DBTO<sub>2</sub> and HBPSi.

Temperature effects on DBT desulfurization by resting cells of recombinant *E. coli*

The effects of temperature on DBT desulfurization were investigated using resting cells of recombinant *E. coli* harboring pKBDS. Since the amounts of DBT degraded and 2-HBP produced were especially increased between 16 and 20 h of cultivation of recombinant *E. coli* cells, the desulfurizing activities of the resting cells after 16, 20, and 24 h of cultivation were measured. The activity was highest in cells cultivated for 16 h (data not shown). The resting cells of recombinant *E. coli* showed 2-HBP-producing activity over a temperature range of 30–52°C, most efficiently at 40°C (0.08 nmol/min/mg dry cell weight) (Fig. 4). In addition, resting cells degraded DBT most efficiently at 50°C (0.16 nmol/min/mg dry cell weight). These thermal profiles of desulfurizing activity in recombinant *E. coli* cells agreed with those in the original strain *B. subtilis* WU-S2B (Kirimura et al. 2001). Resting cells of recombinant *E. coli* showed DBT-degrading and 2-HBP-producing activities approximately two- and three-fold, respectively, higher than those of *B. subtilis* WU-S2B at 40°C, and showed DBT-degrading activity approximately five times as high as *B. subtilis* WU-S2B at 50°C (Kirimura et al. 2001). For the resting cell reactions, the relationship between the amount of DBT degraded and that of 2-HBP produced does not seem to be stoichiometric, especially at temperatures above 40°C, as observed for *B. subtilis* WU-S2B (Kirimura et al. 2001).

The thermal profile of BdsA was studied with resting recombinant *E. coli* cells harboring pKBDSA using DBTO<sub>2</sub> as substrate, and those of BdsB and BdsC were studied with resting recombinant *E. coli* cells harboring pKBDS using HBPSi and DBT, respectively. Although the effects of temperature on each DBT-desulfurization step were measured in resting cells of recombinant *E. coli* JM109 harboring pKBDSA, pKBDSB, and pKBDSB, cells harboring pKBDSB and pKBDSB showed low activities of BdsB and BdsC, respectively. Therefore, these two activities were instead measured using recombinant *E. coli* cells harboring pKBDS. BdsA was measured in recombinant *E. coli* cells harboring pKBDSA since the

## (A)

BdsA_WU-S2B	1	MAEQRQLHLGAGFFSAGNVTHAHGAWRHVGTNGFLTGEFYKQIARTLERGRFDLLFLPDGLAIEDSYGDNLETGVGLGGQ	80
DszA_IGTS8	1	MTQRQRMHLGAGFFSAGNVTHAHGAWRHVDASNDFLSKGYQHARTLERGRFDLLFLPDGLAVIEDSYGDNLDTCVGLGGQ	80
TdsA_A11-2	1	--ROMHLGAGFFAAGNVTHHAGAWRHPKTDNGFLSISWYQHARTLERGRFDLLFLPDGLAIWDSYGNLDAQLRFGGQ	77
BdsA_WU-S2B	81	GAVALEPTSVIATMAAVTORLGLGATVSTITYPPYHVARVFATLDNLSDGRTISWNVVTSLNDSEARNFGVDEHLEHDIIRY	160
DszA_IGTS8	81	GAVALEPASVVATMAAVTEHLGLGATISATITYPPYHVARVFATLDQLSGGRVSWNVVTSLNDSEARNFGINQHLHDIIRY	160
TdsA_A11-2	78	GAAFLLDPVPLATMAAAATERLGLGATISITITYPPYHVARVFATLDHLTKGRAAWNVTSLNNAEARNFGVEHLEHDSIRY	157
BdsA_WU-S2B	161	DRADEFLEAVKKLWSSWSEDALLLDKVGRFADPKKQVYVNERGRWLSVRGPLQVPRSRQCEPVILQAGLSPRGRRFAGR	240
DszA_IGTS8	161	DRADEFLEAVKKLWNSWDEDALVLDKAAGVFADPAKVHYVDHHEWLNVRGPLQVPRSPQCEPVILQAGLSPRGRRFAGK	240
TdsA_A11-2	158	DRADEFLEITDKLWRSWQDALLLDKKQGLFADPRKVVHYIDHSGTWFSVRGPLQVPRSPQGRPVILQAGSSARCKTFAAR	237
BdsA_WU-S2B	241	WAEAVFSVSPNLDIMRAVYQDIKAHVAAAGRDPEQTRVFTAVMPVLGETEQVARERLEYLNSLVHPEVGLSTLSSHSGLN	320
DszA_IGTS8	241	WAEAVFSLAPNLEVMQATYQGIKAEVDAAGRPDQTKITFTAVMPVLGESQAVQERLEYLNSLVHPEVGLSTLSSHITGIN	320
TdsA_A11-2	238	WAEAVFTIAPNRVAMRAFVEDLKKQVIAAGRRPEKCLPVAIPIIGDTEKEARERQEEVNVQLVPEAGLSTLSSHCGVD	317
BdsA_WU-S2B	321	LSKYPLDITKFSDIVADLGRHVPTMLQMFSAVAGGGADLTALAEGLRRYGTNVGFVPQWACTAEQIADQLISHFEAGAADC	400
DszA_IGTS8	321	LAAVPLDITPKDILRDLDQDRNVPTQLHMFAAATHSEELTLAEMGRRYGTNVGFVPQWACTCEQIADQLLRHFEGGAADC	399
TdsA_A11-2	318	FSRYPLDAPITREVLDVAGEVGGTRGLLEMVVKLTETENLTLRDLGVRYGW-VL-VPLQVGTPEQVAGELESLFNEPAADC	395
BdsA_WU-S2B	401	FIISPAYLPGIYEFVDDVVPVLLQQRGVFRTEYEGITLREHLGLAHPEVGV--SIR----	453
DszA_IGTS8	400	FIISPAFLPGSYDFVDDVVPVLLQDRGVFRTEYQGNLRLDHLGLRVPLQGGQPS-----	453
TdsA_A11-2	396	FVISPYLPGAYEFVDRVVPVLLQDRGLFRREYEGDTRQHLGLIEDVSEAEAEAVQGVSE	454

## (B)

BdsB_WU-S2B	1	-----MTTDD-VHD-VLAYSNCVPPNALLTALLESNL LAGNGISLNVLSGAQAGLHFTYDHPAYTRFGGEIPPLI	68
DszB_IGTS8	1	MTRVDPANPGSELDSARTD-LTYSNCVPPNALLTASESGFLDAAQIELDVLSGQQGTVHFTYDHPAYTRFGGEIPPLI	79
TdsB_A11-2	1	-----MSTLSAIGPTRVAYSNCVPPNALLTASRTGKLERQGVLSQIAFAQCATHFAYDHAAYTRFGGEIPPLV	69
BdsB_WU-S2B	69	SEGLRAPGRTRLLGITPLAGRQGLYVRVDSVPTSPEQLRGRRVGVSGAAITRITGELGD-YRHLDPWRQTLIALGTWEAR	147
DszB_IGTS8	80	SEGLRAPGRTRLLGITPLGRQGFVVRDSDSPITAAA DLGRRITGVSSAATIRLRGQDGD-YLELDPWRQTLVALGSWEAR	158
TdsB_A11-2	70	SEGLRAPGRTRLLGITVLPKPRQGFYVHSAGL IASPSDLRGRITGLSRAAQRLLFGHLGEBYRNLPWEQTLVALGSWEAR	149
BdsB_WU-S2B	148	GLLOTLHTGGI GVDVDELVR IESPGVDVPEERLEAAASVKGADLFPDVA-GHORDL DSCNVDALEFWLWPAAELEDLSC	226
DszB_IGTS8	159	ALLHTLEHGLGVDDELVPIESPGVDVPAEQLEESATVKGADLFPDVARG-QAAV LASGDVDALYSWLPWAGELQATG-	236
TdsB_A11-2	150	ALKHTLAAAGLRLNDVIVEDV ENPWVDVPRPKLDDSRDFSSRELFAATAVEW-QSQQLKSGQVDALFSWLPYAAELEDLQV	228
BdsB_WU-S2B	227	ARVLA DLGDDQRSR YASVWTVSAQLVDERPDLVQRLVDAAVQASRWQAHPETEYV IHAANLGVAPS AIGRGFGADFAQH	306
DszB_IGTS8	237	ARPVVDLGLDERNA YASVWTVSGLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFADFQQR	316
TdsB_A11-2	229	AKPVFAL TGE ENAWAS-VWTVS AALVERRPEI VQRLVDSVWBAASWATDHAKETIE IHALNLGVSVKAVETGFGEGFHRD	307
BdsB_WU-S2B	307	LIPRLDSDALAVDDTQQFLLDHNLLDHPVDLTQWAAPOFLTQ-STTGDQQ	356
DszB_IGTS8	317	LVPRLDHDALAL LERTQQFLLTNLLQEPVALDQWAAPEFLNN-SLNRHR-	365
TdsB_A11-2	308	LRPRLDQAALRI LEQTQQFLFDHGLIDRLVDIERWAAPEFLDNASL-----	353

## (C)

BdsC_WU-S2B	1	MTLTDTTATAQNN RNGD--PIE VARELTKRQWATVVERDKAGGSATEEREDLRASGLLSVTVPRHLGGWADWPTALEVV	78
DszC_IGTS8	1	MTLSPEKQHVPRDAADNDPVA VARGLAEKWRATAVERDRAGGSATAEREDLRASALLSLLVPREYGGWADWPTALEVV	80
TdsC_A11-2	1	--MRTIHANSSAV RE-DHRALD VATELAKTF RVTVRERERAGGTPK AERDAI RRSGLITLLISK ERGGLGESWPTVYVEAI	77
BdsC_WU-S2B	79	REIAKVDCSLGHLFGYHLSTP AVIDLWCSPEQSERLLRQLAENNNWWTGNASSENNSHILEWKVITATPTDDGGYVLTNGIKH	158
DszC_IGTS8	81	REIAAADGSLGHLFGYHLTNAPM IELIGSQEQEEHLYTQIAQNNWWTGNASSENNSHVLDWKVSATPTEDGGYVLTNGIKH	160
TdsC_A11-2	78	AEIASADASLGHFLFGYHFSNFAYV DLFASPEQKARWYQAVRER WFLGNASSENNAHVLDWRVITATPLPDGSEYIENGTKA	157
BdsC_WU-S2B	159	FSSGAKGSDLLLVFGVIEPEG SPQQGATVAAAIPITRECVQTNDDWQALGMRRTDSCITTEFHNVVVRPDEVLGKPNNAVVA	238
DszC_IGTS8	161	FCSCAKGSDLLLVFGVVQD DSPQQGATVAAAIPITSRAGVTPNDDWAAIGMRQTDSCSTDFHNVKVEPDEVLGAPNAFVLA	240
TdsC_A11-2	158	FCSCSADA DRLLVFAVTSRDPNGD GRIVAAALIPSDRACVQVNGDWDSLGMRTDSCSVTFSGVVVYPDELGLTGCQVTD	237
BdsC_WU-S2B	239	FLASGRGSLFGPIVOLFVATVYLGIAARGALETAREYTRTQARPWTAGVTOAVEDPYTIRSYGEFGIELOAGDAAAREAA	318
DszC_IGTS8	241	FIQSERGSLFRPIAQLIFANVYLGIAHGALDAAREYTRTQARPWTAGIQQATEDPYTIRSYGEFTIALQDADAAAREAA	320
TdsC_A11-2	238	FASGSKP SLWTPITQLIFTHLYLGIAARGALEBAAHYSRSHSRPPTLAGVEKATEDPYVLAITYGEFAAQLOVAEACAREVA	317
BdsC_WU-S2B	319	QLLOTAWDKGDALTPQERGELAVQVAGVKAIATKAGLDVTSRI FEVIGARGTHPN-YGDFRFRWRNIRTHLHDPVSYKIA	397
DszC_IGTS8	321	HLLOTVWDKGDALTPEDRGELMVKVSGVKALATNAALNIS SGVFEVIGARGTHPR-YGDFRFRWRNVRTSLHDPVSYKIA	399
TdsC_A11-2	318	LRVQELWERNHVT-PEQRGQLMVAQVASKIVATRLVIELTSRLYIEMGARAAAS RQFGDFRFRWRDARTHHLHDPVAYKIR	396
BdsC_WU-S2B	398	EVGNYVLNQRYPPIPGFITS	415
DszC_IGTS8	400	DVGKHTLNGQYPIPGFITS	417
TdsC_A11-2	397	EVGNWFLNHRFPTRSEFYS	414

◀ **Fig. 2** Multiple alignments of the amino acid sequence of desulfurizing enzymes from various desulfurizing bacteria. Amino acid residues identical in all the members are indicated by *white letters* in *black boxes*. Amino acid residues identical in more than half of the members are indicated by *shaded boxes*. Numbers indicate the positions of the residues in the complete amino acid sequence of each enzyme. **a** Comparison of BdsA, DBTO<sub>2</sub> monooxygenase from *B. subtilis* WU-S2B, with its equivalents from other strains. **b** Comparison of BdsB, HBPSi desulfinase from *B. subtilis* WU-S2B, with its equivalents from other strains. **c** Comparison of BdsC, DBT monooxygenase from *B. subtilis* WU-S2B, with its equivalents from other strains. DszA, DszB, and DszC are desulfurization enzymes from *Rhodococcus* sp. IGTS8. TdsA, TdsB, and TdsC are desulfurization enzymes from *Paenibacillus* sp. A11-2

activity was as high as in cells harboring pKBDS. As shown in Fig. 5, the optimal temperatures of BdsC, BdsA, and BdsB were about 50, 45, and 37°C, respectively. BdsB activity was highest at temperatures below 40°C. The relative activities of BdsC and BdsA were higher than that of BdsB at temperatures above 45°C, and the activity of BdsC was the highest of the three enzymes at temperatures above 50°C. Furthermore, DBTO<sub>2</sub> was not detected as an intermediate in the reaction of resting cells over a temperature range of 30–52°C (data not shown). HBPSi was not quantitatively extracted with ethylacetate due to its water solubility, and was detected in low amounts in the reaction mixture at 50°C. However, the amount of HBPSi could be calculated with the equation  $\text{HBPSi} = \text{initial DBT} - \text{residual DBT} - \text{DBTO}_2 - 2\text{-HBP}$ , and was 0.050 nmol in the reaction mixture at 50°C. Based on the amount of DBT degraded, 0.055 nmol in the reaction mixture, at 50°C the rate of HBPSi was calculated to be 91%. These results suggest that BdsC and/or BdsA are the rate-determining enzymes in DBT desulfurization at temperatures below 40°C, and that BdsB is the rate-determining enzyme at temperatures above 45°C. These tendencies agreed with the relationship between DBT-degrading activity and 2-HBP-producing activity (Fig. 4).

Substrate specificity of desulfurization by the recombinant *E. coli* cells

Alkylated DBT derivatives detected in light gas oil are more recalcitrant to conventional hydrodesulfurization

than DBT (Suzuki 1999), while *B. subtilis* WU-S2B can desulfurize various DBT derivatives (Kirimura et al. 2001). As shown in Table 1, 2,8-dimethylDBT, 4,6-dimethylDBT, and 3,4-benzoDBT could be degraded by growing cells of recombinant *E. coli* harboring pKBDS in modified M9 medium, and each desulfurized metabolite corresponding to 2-HBP derived from DBT was detected by GC analysis (data not shown). When cells were cultivated using LB medium instead of modified M9 medium, the recombinant *E. coli* cells grew well in LB medium supplemented with each substrate. However, the degradation rates for 4,6-dimethylDBT and 3,4-benzoDBT were very low (data not shown). By contrast, recombinant *E. coli* cells were unable to grow in modified M9 medium without supplementation of DBT derivatives as the sole source of sulfur (data not shown). The low degradation rates of 4,6-dimethylDBT and 3,4-benzoDBT by the recombinant *E. coli* cells might be related to a difference in the uptake and/or utilization of DBT derivatives.

Cloning of the gene corresponding to *B. subtilis* *bdsABC* from *M. phlei* WU-F1

Using a strategy similar to that used for cloning of *bdsABC* from *B. subtilis* WU-S2B, the corresponding gene responsible for DBT desulfurization was also cloned from the total DNA library of *M. phlei* WU-F1 (details not shown). The nucleotide sequences of *bdsABC* and the 3 kb-upstream region in *M. phlei* WU-F1 were found to be completely identical to the genes of *B. subtilis* WU-S2B, although the two strains are genetically different.

## Discussion

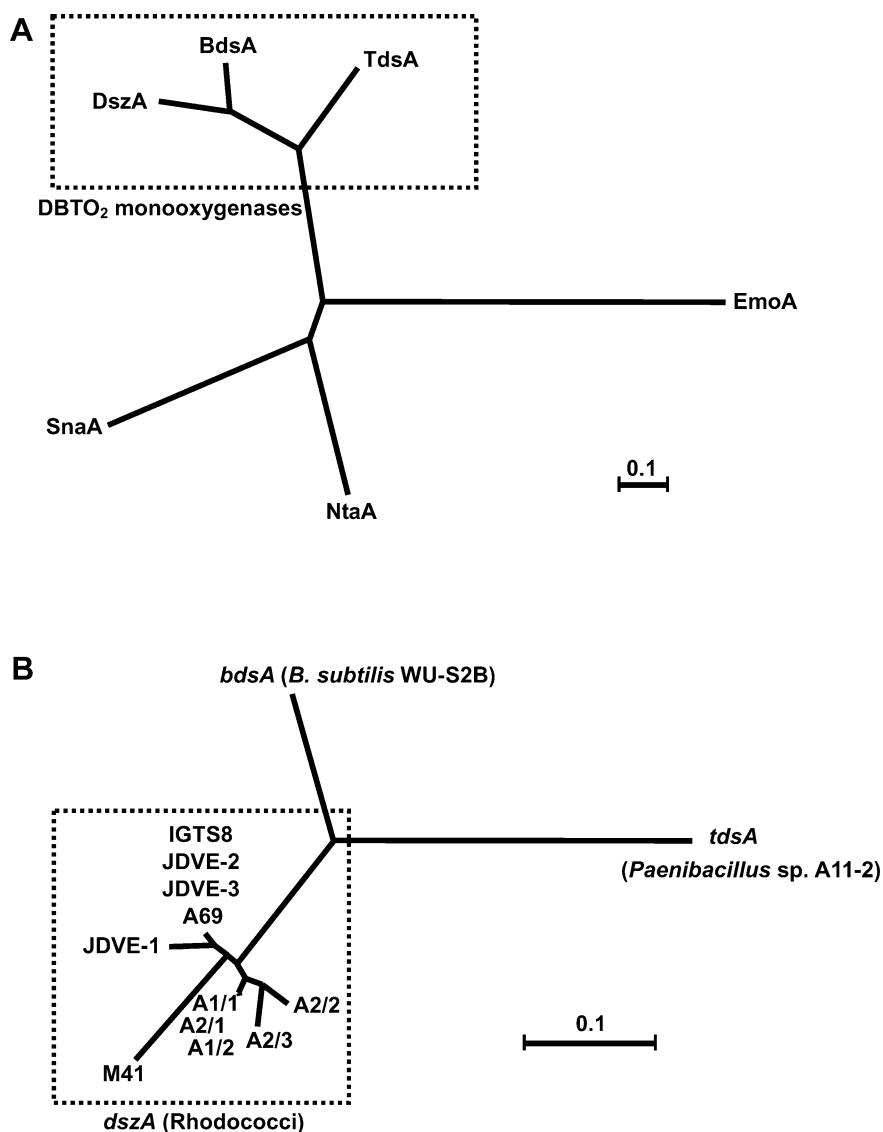
In the present study, *bdsABC*, the DBT-desulfurization genes (*bds* genes) from *B. subtilis* WU-S2B and the corresponding genes from *M. phlei* WU-F1 were cloned based on the conserved amino acid sequences between mesophilic *dsz* genes and thermophilic *tds* genes. Based on the nucleotide sequence and heterologous expression, we confirmed that the operon structure of the *bds* genes is quite similar to those of DBT-desulfurization genes, such as the *dsz* genes and the *tds* genes. Since resting cells of

**Table 1** Degradation of DBT derivatives by growing cells of recombinant *E. coli* harboring pKBDS

Substrate	<i>E. coli</i> harboring pKBDS <sup>a</sup>			<i>B. subtilis</i> WU-S2B <sup>b</sup>		
	Growth (OD <sub>660</sub> )	Degradation ratio (%)	Relative activity (%)	Growth (OD <sub>660</sub> )	Degradation ratio (%)	Relative activity (%)
DBT	2.4	81	100	3.5	80	100
2,8-DimethylDBT	2.2	30	34	3.1	55	68
4,6-DimethylDBT	2.1	44	55	3.0	52	64
3,4-BenzoDBT	2.2	48	58	3.4	66	82

<sup>a</sup>Each cultivation was done at 37°C for 72 h in modified M9 medium supplemented with 0.27 mM of substrates as the sole source of sulfur

<sup>b</sup>Each cultivation was done at 45°C for 72 h in A-2 medium (Kirimura et al. 2001) supplemented with 0.27 mM of substrates as the sole source of sulfur



**Fig. 3** **a** Unrooted phylogenetic tree of FMNH<sub>2</sub>-dependent monooxygenase family relating to BdsA and **b** classification of gene equivalents to *dszA* from several desulfurizing bacteria. Scale bars 0.1 substitution per site. **a** The positions of DBTO<sub>2</sub> monooxygenases are boxed. *BdsA* DBTO<sub>2</sub> monooxygenase of *B. subtilis* WU-S2B, *DszA* DBTO<sub>2</sub> monooxygenase of *Rhodococcus* sp. IGTS8 (L37363), *TdsA* DBTO<sub>2</sub> monooxygenase of *Paenibacillus* sp. A11-2 (AB033997), *SnaA* pristinamycin II<sub>A</sub> synthase subunit A of *Streptomyces pristinaespiralis* (U21215), *NtaA* nitrilotriacetate monooxygenase of *Chelatobacter heintzii* (U399411), *EmoA* EDTA monooxygenase of EDTA-degrading bacterium BNC1 (AF176664). **b** The group of the Rhodococci *dsz* genes is boxed. *bdsA* Gene encoding DBTO<sub>2</sub> monooxygenase of *B. subtilis* WU-S2B, *tdsA* gene encoding DBTO<sub>2</sub> monooxygenase of *Paenibacillus* sp. A11-2 (AB033997), *IGTS8* gene encoding DBTO<sub>2</sub> monooxygenase of *Rhodococcus* sp. IGTS8 (L37363), *A1/1* uncultured

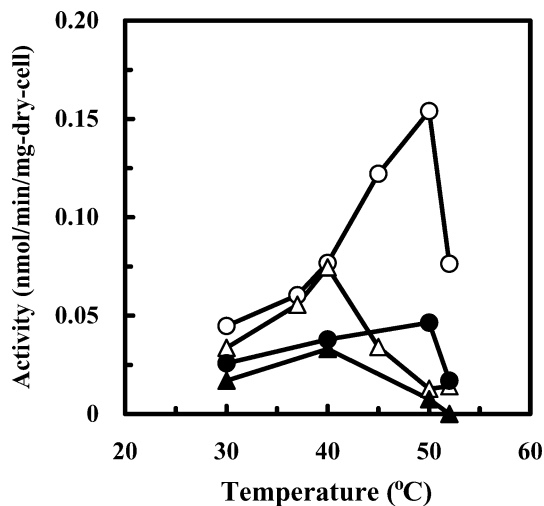
*Rhodococcus* sp. A1/1 DBT-desulfurization enzyme-like protein (*dszA*) gene (AF322033), *A1/2* uncultured *Rhodococcus* sp. A1/2 DBT-desulfurization enzyme-like protein (*dszA*) gene (AF322034), *A2/1* uncultured *Rhodococcus* sp. A2/1 DBT-desulfurization enzyme (*dszA*) gene (AF322036), *A2/2* uncultured *Rhodococcus* sp. A2/2 DBT-desulfurization enzyme-like protein (*dszA*) gene (AF322035), *A2/3* uncultured *Rhodococcus* sp. A2/3 DBT-desulfurization enzyme-like protein (*dszA*) gene (AF322037), *A69* *Rhodococcus* sp. A69 DBT-desulfurization enzyme (*dszA*) gene (AF322041), *JDVE-1* uncultured *Rhodococcus* sp. JDVE-1 DBT-desulfurization enzyme-like protein (*dszA*) gene (AF322038), *JDVE-2* uncultured *Rhodococcus* sp. JDVE-2 DBT-desulfurization enzyme (*dszA*) gene (AF322039), *JDVE-3* uncultured *Rhodococcus* sp. JDVE-3 DBT-desulfurization enzyme (*dszA*) gene (AF322040), *M41* *Rhodococcus* sp. M41 DBT-desulfurization enzyme-like protein (*dszA*) gene (AF322042)

recombinant *E. coli* cells could desulfurize DBT over temperatures ranging from 30 to 52°C as well as *B. subtilis* WU-S2B, the thermal profiles of *bdsABC* products were functionally reproducible in *E. coli* as a host. Furthermore, the recombinant *E. coli* cells harboring pKBDS desulfurized 2,8-dimethylDBT, 4,6-dimethylDBT, and 3,4-benzodBT as well as the original strain. These results

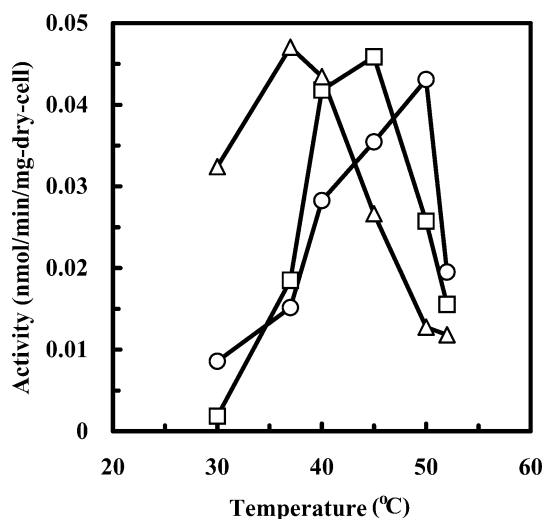
suggested that the thermal profile and substrate specificity of desulfurizing activity, i.e., the desulfurization ability, observed for *B. subtilis* WU-S2B was endowed by the gene products of *bdsABC* but not by other cellular properties of *B. subtilis* WU-S2B.

*BdsA* shows homologies to equivalents of the other known desulfurization genes and to several members of





**Fig. 4** Effects of temperature on DBT desulfurization by resting cells of recombinant *E. coli* harboring pKBDS. Resting cell reaction was carried out using *n*-tridecane solution containing DBT as substrate. DBT-degrading activities by recombinant *E. coli* (open circles) and *B. subtilis* WU-S2B (closed circles) are shown. 2-HBP-producing activities by recombinant *E. coli* (open triangles) and *B. subtilis* WU-S2B (closed triangles) are also indicated



**Fig. 5** Effects of temperature on each step of DBT desulfurization by resting cells of recombinant *E. coli*. The reaction was carried out using an ethanol solution of DBT or one of its metabolic intermediates as substrate. BdsB activity was determined by the amount of 2-HBP-production, since HBPSi as a substrate could not be quantitatively extracted with ethylacetate due to its water solubility. DBT-degrading activity (circles), DBTO<sub>2</sub>-degrading activity (squares), and 2-HBP-producing activity (triangles) are shown

FMNH<sub>2</sub>-dependent monooxygenases. It should be noted that in common these enzymes require flavin reductase, similar to BdsA, and catalyze  $\alpha$ -hydroxylation of the carbon atom next to the sulfur or nitrogen atom, although the structures of their substrates are very different (Blanc et al. 1995; Bohuslavek et al. 2001; Knobel et al. 1996; Oldfield et al. 1997). On the phylogenetic tree of the FMNH<sub>2</sub>-dependent monooxygenase family, BdsA of *B. subtilis* WU-S2B, DszA of *Rhodococcus* sp. IGTS8, and

TdsA of *Paenibacillus* sp. A11-2 form independent cluster groups (Fig. 3a). *bdsA* can be distinctly classified from many *Rhodococcus* *dszA* genes, which were grouped into three clusters by Duarte et al. (2001), on the phylogenetic tree based on the partial nucleotide sequences of the equivalents for *dszA* (Fig. 3b). Since the location on the phylogenetic tree of *bdsA* relative to genes equivalent to *dszA* (Fig. 3b) does not correspond to the location of *B. subtilis* WU-S2B relative to other bacteria based on 16S rRNA sequences (data not shown), the desulfurization genes such as *dsz* and *bds* are considered to have been transferred horizontally via plasmids, as discussed by Denis-Larose et al. (1997). In Fig. 3b, it is interesting to note that *bdsA* of *B. subtilis* WU-S2B is remotely located from *tdsA* of *Paenibacillus* sp. A11-2, which is taxonomically related to *B. subtilis*. Nevertheless, the *bds* genes of *B. subtilis* WU-S2B and the *tds* genes of *Paenibacillus* sp. A11-2 would have been independently transmitted, but not derived from identical DBT-desulfurization genes. Furthermore, the *bdsABC* genes of *B. subtilis* WU-S2B and *M. phlei* WU-F1, bacteria that have no taxonomic relation, were identical to each other. These findings strongly suggest that *B. subtilis* WU-S2B and *M. phlei* WU-F1 might have acquired *bdsABC* genes not by vertical evolution but by horizontal transmission.

The DBT-desulfurizing enzymes encoded by the *tds* genes of *Paenibacillus* sp. A11-2 showed higher activity toward benzothiophene (BT) than toward DBT. This result was supported by the observation that strain A11-2 grew more rapidly on BT than on DBT (Konishi et al. 2002). By contrast, DszC purified from *R. erythropolis* KA2-5-1 showed highest activity toward various alkylated DBT derivatives and 3-methylBT among alkylated BTs. The substrate specificity of desulfurization correlated well with that of the sulfur source for growth of strain KA2-5-1 (Konishi et al. 2002). The substrate specificity of the sulfur source for growth of *B. subtilis* WU-S2B more closely resembled that of *R. erythropolis* KA2-5-1, producing Dsz enzymes, than that of *Paenibacillus* sp. A11-2 (Kirimura et al. 2001). Therefore, it is likely that the amino acid sequences of Bds enzymes of *B. subtilis* WU-S2B are similar to those of Dsz enzymes of *R. erythropolis* KA2-5-1 but not to those of Tds enzymes of *Paenibacillus* sp. A11-2. Since the sulfur components in HDS-treated light gas oils are mainly alkylated DBT derivatives and subsequently BT derivatives, the substrate specificity of Bds enzymes of *B. subtilis* WU-S2B is superior to that of *Paenibacillus* sp. A11-2 enzymes. Furthermore, Bds enzymes of resting cells of *B. subtilis* WU-S2B showed DBT-desulfurizing activities over a temperature range of 30–52°C (Kirimura et al. 2001), a property that is superior to those of *R. erythropolis* KA2-5-1 and *Paenibacillus* sp. A11-2 enzymes. Although the correlation between the amino acid sequences of Bds enzymes and their respective thermal profiles remains unclear, the consensus sequences between Bds enzymes and Tds enzymes might be essential for thermal stability of DBT desulfurization. This topic is currently the subject of further studies. Using site-directed mutagenesis of *Rhodococcus* sp. IGTS8 genes, it was

found that the residues Gln-345 of DszA and Val-261 of DszC are related to recognition of C–S bond cleavage specificity and utilization of 5-methylBT, respectively (Arensdorf et al. 2002; Konishi and Maruhashi 2003). Although BdsC conserved the corresponding residue (Val-259 to Val-261 of DszC), *M. phlei* WU-F1 and *B. subtilis* WU-S2B could desulfurize 2-ethylNTH, which could not be desulfurized by *R. erythropolis* KA2-5-1 harboring the DszC enzyme. Therefore, it appears that the *bdsABC* genes are useful genetic sources for improvement of substrate specificity and wide-range thermal reactivities of desulfurization enzymes.

In the heterologous expression analysis, recombinant *E. coli* cells harboring *bdsC* oxidized DBT to DBTO<sub>2</sub>, those harboring *bdsA* converted DBTO<sub>2</sub> into HBPSi, and those harboring *bdsB* desulfurized HBPSi to 2-HBP. This pathway for DBT desulfurization is in accordance with the sulfur-specific pathway of DBT desulfurization reported previously (Kirimura et al. 2001) based on the analyses of metabolites. Thus, the *bds* genes could be individually expressed with the *tac* promoter of the vector and each gene product could function fully and independently. The monooxygenases of Dsz enzymes of *Rhodococci* and Tds enzymes of *Paenibacillus* sp. A11-2 require flavin reductase as a coupling enzyme for desulfurizing activity (Konishi et al. 2000; Ohshiro et al. 1994). The recombinant *E. coli* cells harboring the *bdsABC* genes could degrade DBT to 2-HBP without introduction of flavin reductase genes, indicating that BdsA and BdsC expressed in *E. coli* couple with *E. coli* oxidoreductases, such as HpaC (Galán et al. 2000a), which were reported to be able to enhance the DBT-desulfurizing activity of DszA and DszC of *Rhodococcus* sp. IGTS8 (Galán et al. 2000b).

Although the DBT-degrading activity of recombinant *E. coli* cells was highest at 50°C, the DBT-desulfurizing activity (2-HBP-producing activity) was highest at 40°C, as shown in Fig. 4. As shown in Fig. 5, this is probably due to the heat liability of BdsB, which catalyzed the reaction followed by the conversion DBTO<sub>2</sub> to HBPSi. Thus, BdsB might be the rate-determining enzyme at temperatures above 45°C. However, since HBPSi is easily dissolved in water, DBT desulfurization from the oil phase can be achieved. Moreover, it was reported that HBPSi as a byproduct of desulfurization was converted to valuable surfactants and utilized further (Mcfarland 1999). Therefore, the accumulation of HBPSi would not become an issue in the modification of process design.

The genes responsible for DBT desulfurization in *M. phlei* WU-F1 were also cloned and found to be completely identical to *bdsABC* from *B. subtilis* WU-S2B. Moreover, the nucleotide sequence for the 3-kb upstream region, containing the putative promoter sequence, of *bdsABC* was identical for the genes of *B. subtilis* WU-S2B and *M. phlei* WU-F1, suggesting that the same promoter is functioned in the two strains (details not shown). Since the DBT desulfurization profiles of *M. phlei* WU-F1, especially the thermal properties, were very similar to those of *B. subtilis* WU-S2B (Kirimura et al. 2001; Furuya et al. 2001), the fact that *B. subtilis* WU-S2B and *M. phlei*

WU-F1 have the same DBT-desulfurization genes is perhaps unusual but not irrational. Although the DBT-desulfurizing activity of resting cells of *B. subtilis* WU-S2B was lower than that of resting cells of *M. phlei* WU-F1, this difference might be due to other cellular factors, such as flavin reductases coupling with BdsA and BdsC. This possibility is the subject of current studies.

The desulfurizing activity of the recombinant *E. coli* cells used in this study was 0.08 nmol/min/mg dry cell weight, and was similar to the level reported for other *E. coli* recombinant strains harboring DBT-desulfurization genes (Ishii et al. 2000a). For example, the desulfurizing activities of recombinant *E. coli* cells without coexpression of flavin reductase was 0.025 nmol/min/mg dry cell weight (Ishii et al. 2000a). The target level of desulfurizing activity needed for practical industrial applications has been evaluated and was determined to be 20–50 nmol/min/mg dry cell weight (Monticello 2000; Maruhashi 2001). Therefore, at this stage, the desulfurizing activity achieved in this study is not sufficient for use in practical biodesulfurization. However, having determined the properties of thermally stable BdsABC enzymes, improvement of desulfurizing activity by genetic engineering, using the *bdsABC* genes and a desirable host strain such as *M. phlei* WU-F1, should be possible.

In conclusion, we identified and functionally analyzed the genes responsible for DBT desulfurization in *B. subtilis* WU-S2B and *M. phlei* WU-F1. It was confirmed that the desulfurizing activity of *B. subtilis* WU-S2B and *M. phlei* WU-F1 at temperatures of 30–52°C is basically dependent on the function of products of the *bds* genes, which are both unique and distinct from other DBT-desulfurization genes, such as *dsz* and *tds*. Genetic improvement of desulfurizing bacteria will allow their practical application to novel biodesulfurization processes.

**Acknowledgements** This work was supported in part by the Japan Cooperation Center, Petroleum subsidized by the Ministry of Economy, Trade and Industry, and by Grants-in-Aid for Scientific Research (13650859) from the Ministry of Education, Science, Sports, and Culture of Japan, and by Waseda University Grant for Special Research Projects (2001A-557 and 2001A-863).

## References

- Arensdorf JJ, Loomis AK, DiGrazia PM, Monticello DJ, Pienkos PT (2002) Chemostat approach for the directed evolution of biodesulfurization gain-of-function mutants. *Appl Environ Microbiol* 68:691–698
- Blanc V, Lagneaux D, Didier P, Gil P, Lacroix P, Crouzet J (1995) Cloning and analysis of structural genes from *Streptomyces pristinaespiralis* encoding enzymes involved in the conversion of pristinamycin II<sub>B</sub> to pristinamycin II<sub>A</sub> (PII<sub>A</sub>): PII<sub>A</sub> synthase and NADH:riboflavin 5'-phosphate oxidoreductase. *J Bacteriol* 177:5206–5214
- Bohuslavek J, Payne JW, Liu Y, Bolton H Jr, Xun L (2001) Cloning, sequencing, and characterization of a gene cluster involved in EDTA degradation from the bacterium BNC1. *Appl Environ Microbiol* 67:688–695
- Borgne SL, Quintero R (2003) Biotechnological processes for the refining of petroleum. *Fuel Process Technol* 81:155–169

- COSMO Oil (2002) Optimization of preheating process for boiler feed water (in Japanese). Annual report of energy conservation of factory, The Energy Conservation Center Japan, Tokyo
- Denis-Larose C, Labbé D, Bergeron H, Jones AM, Greer CW, Al-Hawari J, Grossman MJ, Sankey BM, Lau PCK (1997) Conservation of plasmid-encoded dibenzothiophene desulfurization genes in several *Rhodococci*. *Appl Environ Microbiol* 63:2915–2919
- Denome SA, Olson ES, Young KD (1993) Identification and cloning of genes involved in specific desulfurization of dibenzothiophene by *Rhodococcus* sp. strain IGTS8. *Appl Environ Microbiol* 59:2837–2843
- Duarte GF, Rosado AS, Seldin L, de Araujo W, van Elsland JD (2001) Analysis of bacterial community structure in sulfurous-oil-containing soils and detection of species carrying dibenzothiophene desulfurization (*dsz*) genes. *Appl Environ Microbiol* 67:1052–1062
- Furuya T, Kirimura K, Kino K, Usami S (2001) Thermophilic biodesulfurization of dibenzothiophene and its derivatives by *Mycobacterium phlei* WU-F1. *FEMS Microbiol Lett* 204:129–133
- Furuya T, Kirimura K, Kino K, Usami S (2002) Thermophilic biodesulfurization of naphthothiophene and 2-ethylnaphthothiophene by a dibenzothiophene-desulfurizing bacterium, *Mycobacterium phlei* WU-F1. *Appl Microbiol Biotechnol* 58:237–240
- Furuya T, Ishii Y, Noda K, Kino K, Kirimura K (2003) Thermophilic biodesulfurization of hydrodesulfurized light gas oils by *Mycobacterium phlei* WU-F1. *FEMS Microbiol Lett* 221:137–142
- Galán B, Díaz E, Prieto MA, García JL (2000a) Functional analysis of the small component of the 4-hydroxyphenylacetate 3-monooxygenase of *Escherichia coli* W: a prototype of a new flavin:NAD(P)H reductase subfamily. *J Bacteriol* 182:627–636
- Galán B, Díaz E and García JL (2000b) Enhancing desulfurization by engineering a flavin reductase-encoding gene cassette in recombinant biocatalysis. *Environ Microbiol* 2:687–694
- Gray KA, Pogrebinsky OS, Mrachko GT, Xi L, Monticello DJ, Squires CH (1996) Molecular mechanisms of biocatalytic desulfurization of fossil fuels. *Nat Biotechnol* 14:1705–1709
- Ishii Y, Konishi J, Okada H, Hirasawa K, Onaka T, Suzuki M (2000a) Operon structure and functional analysis of the genes encoding thermophilic desulfurizing enzymes of *Paenibacillus* sp. A11-2. *Biochem Biophys Res Commun* 270:81–88
- Ishii Y, Konishi J, Suzuki M, Maruhashi K (2000b) Cloning and expression of the genes encoding the thermophilic NAD(P)H-FMN oxidoreductase coupling with the desulfurization enzymes from *Paenibacillus* sp. A11-2. *J Biosci Bioeng* 90:591–599
- Kahnert A, Kertesz MA (2000) Characterization of a sulfur-regulated oxygenative alkylsulfatase from *Pseudomonas putida* S-313. *J Biol Chem* 275:31661–31667
- Kirimura K, Furuya Nishii Y, Ishii Y, Kino K, Usami S (2001) Biodesulfurization of dibenzothiophene and its derivatives through the selective cleavage of carbon–sulfur bonds by a moderately thermophilic bacterium *Bacillus subtilis* WU-S2B. *J Biosci Bioeng* 91:262–266
- Knobel HR, Egli T, van der Meer JR (1996) Cloning and characterization of the genes encoding nitrilotriacetate monooxygenase of *Chelatobacter heintzii* ATCC 29600. *J Bacteriol* 178:6123–6132
- Konishi J, Ishii Y, Onaka T, Okumura K, Suzuki M (1997) Thermophilic carbon–sulfur-bond-targeted biodesulfurization. *Appl Environ Microbiol* 63:3164–3169
- Konishi J, Ishii Y, Onaka T, Ohta Y, Suzuki M, Maruhashi K (2000) Purification and characterization of dibenzothiophene sulfone monooxygenase and FMN-dependent NADH oxidoreductase from the thermophilic bacterium *Paenibacillus* sp. A11-2. *J Biosci Bioeng* 90:607–613
- Konishi J, Ishii Y, Onaka T, Maruhashi K (2002) Purification and characterization of the monooxygenase catalyzing sulfur-atom specific oxidation of dibenzothiophene and benzothiophene from the thermophilic bacterium *Paenibacillus* sp. A11-2. *Appl Microbiol Biotechnol* 60:128–133
- Konishi J, Maruhashi K (2003) Residue 345 of dibenzothiophene (DBT) sulfone monooxygenase is involved in C–S bond cleavage specificity of alkylated DBT sulfones. *Biotechnol Lett* 25:1147–1150
- Li MZ, Squires CH, Monticello DJ, Childs JD (1996) Genetic analysis of the *dsz* promoter and associated regulatory regions of *Rhodococcus erythropolis* IGTS8. *J Bacteriol* 178:6409–6418
- Maruhashi (2001) Development of petroleum-refining process by biotechnology (in Japanese). *Biosci Ind* 61:561–564
- Mcfarland BL (1999) Biodesulfurization. *Curr Opin Microbiol* 2:257–264
- Monticello DJ (2000) Biodesulfurization and the upgrading of petroleum distillates. *Curr Opin Biotechnol* 11:540–546
- Nakayama N, Matsubara T, Ohshiro T, Moroto Y, Kawata Y, Koizumi K, Hirakawa Y, Suzuki M, Maruhashi K, Izumi Y, Kurane R (2002) A novel enzyme, 2'-hydroxybiphenyl-2-sulfinate desulfinate (DszB), from a dibenzothiophene-desulfurizing bacterium *Rhodococcus erythropolis* KA2-5-1: gene overexpression and enzyme characterization. *Biochim Biophys Acta* 1598:122–130
- Ohshiro T, Izumi Y (1999) Microbial desulfurization of organic sulfur compounds in petroleum. *Biosci Biotechnol Biochem* 63:1–9
- Ohshiro T, Izumi Y (2002) Desulfurization of fossil fuels. In: Bitton G (ed) *Encyclopedia of environmental microbiology*. Wiley, New York, pp 1041–1051
- Ohshiro T, Hine Y, Izumi Y (1994) Enzymatic desulfurization of dibenzothiophene by a cell-free system of *Rhodococcus erythropolis* D-1. *FEMS Microbiol Lett* 118:341–344
- Ohshiro T, Suzuki K, Izumi Y (1997) Dibenzothiophene (DBT) degrading enzyme responsible for the first step of DBT desulfurization by *Rhodococcus erythropolis* D-1: purification and characterization. *J Ferment Bioeng* 83:233–237
- Ohshiro T, Kojima T, Torii K, Kawasoe H, Izumi Y (1999) Purification and characterization of dibenzothiophene (DBT) sulfone monooxygenase, an enzyme involved in DBT desulfurization, from *Rhodococcus erythropolis* D-1. *J Biosci Bioeng* 88:610–616
- Oldfield C, Pogrebinsky O, Simmonds J, Olson ES, Kulpa CF (1997) Elucidation of the metabolic pathway for dibenzothiophene desulfurization by *Rhodococcus* sp. strain IGTS8 (ATCC53968). *Microbiology* 143:2961–2973
- Piddington CS, Kovachvich BR, Rambosk J (1995) Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of *Rhodococcus* sp. strain IGTS8. *Appl Environ Microbiol* 61:468–475
- van der Ploeg JR, Iwanicka-Nowicka R, Bykowski T, Hryniewicz MM, Leisinger T (1999) The *Escherichia coli* *ssuEADCB* gene cluster is required for the utilization of sulfur from aliphatic sulfonates and is regulated by the transcriptional activator Cbl. *J Biol Chem* 274:29358–29365
- Poupin P, Ducrocq V, Hallier-Soulier S, Truffaut N (1999) Cloning and characterization of the genes encoding a cytochrome P450 (PipA) involved in piperidine and pyrrolidine utilization and its regulatory protein (PipR) in *Mycobacterium smegmatis* mc<sup>2</sup>155. *J Bacteriol* 181:3419–3426
- Sambrook J, Russell DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor
- Suzuki M (1999) Enzymology and molecular genetics of biodesulfurization. *Recent Res Devel Microbiol* 3:515–534
- Vermeij P, Wietek C, Kahnert A, Wuest T, Kertesz MA (1999) Genetic organization of sulphur-controlled aryl desulphonation in *Pseudomonas putida* S-313. *Mol Microbiol* 32:913–926