Desulfurization of Dibenzothiophene and Diesel Oil by Metabolically Engineered *Escherichia coli*

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Abstract The desulfurization genes (*dszABC*) were cloned from *Gordonia nitida*. Nucleotide sequences similarity between the *dszABC* genes of *G. nitida* and those of *Rhodococcus rhodochrous* IGTS8 was 89%. The similarities of deduced amino acids between the two were 86% for DszA, 86% for DszB, and 90% for DszC. The *G. nitida dszABC* genes were expressed in several different *Escherichia coli* strains under an inducible *trc* promoter. Cultivation of these metabolically engineered *E. coli* strains in the presence of 0.2 mM dibenzothiophene (DBT) allowed the conversion of DBT to 2-hydroxybiphenyl (2-HBP), which is the final metabolite of the sulfur-specific desulfurization pathway. The maximum conversion of DBT to 2-HBP was 16% in 60 h. Recombinant *E. coli* was applied for the deep desulfurization of diesel oil supplemented into the medium at 5% (v/v). Sulfur content in diesel oil was decreased from 250 mg sulfur/l to 212.5 mg sulfur/l, resulting in the removal of 15% of sulfur in diesel oil in 60 h.

Key words: Desulfurization, dibenzothiophene, diesel oil, *Gordonia nitida*, metabolic engineering, *Escherichia coli*

Sulfur oxides released from the combustion of fossil fuels are a major cause of air pollution that results in acid rain. To remove sulfur compounds from fossil fuels, hydrodesulfurization (HDS) process is currently employed. However, HDS is costly and energy-intensive, since hydrogen gas is used at high temperature and pressure. Furthermore, many complex organic sulfur compounds are known to be difficult to remove by the HDS process.

Biodesulfurization (BDS) employing a biocatalyst has been suggested as a possible alternative for HDS. Since

dibenzothiophene (DBT) is a typical recalcitrant organic sulfur compound in fossil fuels, DBT has been a widely accepted model compound in developing a BDS process. Selective removal of sulfur is desirable because valuable combustible carbons should be retained. Several microorganisms such as *Rhodococcus erythropolis* D1 [6], *R. rhodochrous* IGTS8 [8], and *Corynebacterium* sp. strain SY1 [10] have been reported to specifically remove the
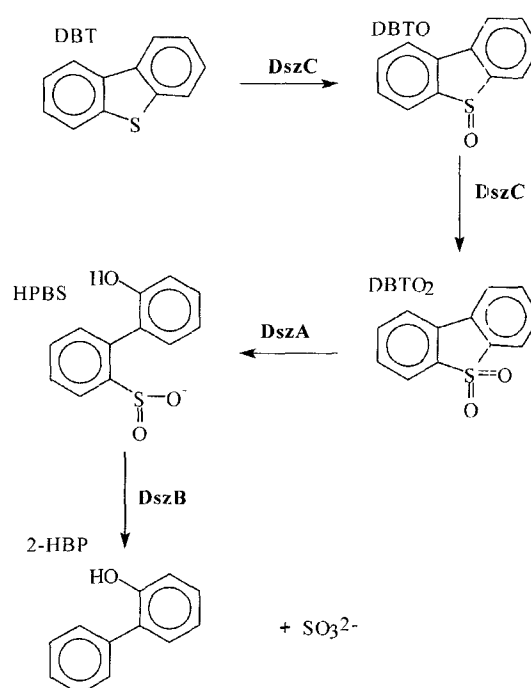
Fig. 1. Sulfur-specific DBT desulfurization pathway (4-S pathway).
Abbreviations: DBT, dibenzothiophene; DBTO, dibenzothiophene-sulfoxide; DBTO₂, dibenzothiophene-5,5-dioxide; HBPS, 2-(2-hydroxybiphenyl)-benzenesulfinate; 2-HBP, 2-hydroxybiphenyl.

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sulfur from DBT without breaking the carbon-carbon bond, converting DBT to 2-hydroxybiphenyl (2-HBP) through the sulfur-specific pathway called the 4-S pathway (Fig. 1). Molecular cloning and analyses of genes involved in the desulfurization of DBT in *R. rhodochrous* IGTS8 have been reported [3, 12].

Several recombinant microorganisms containing the *R. rhodochrous dszABC* genes have been constructed and examined for their abilities to convert DBT to 2-HBP [3, 5, 7, 12, 13]. The operon consisting of the *dszABC* genes is regulated at the transcriptional level, in which sulfate and sulfur-containing amino acids strongly repress the transcription of the *dszABC* genes [9]. The desulfurization pathway in *R. rhodochrous* IGTS8 is as follows: DszC converts DBT to dibenzothiophene-5,5-dioxide (DBTO$_2$), DBTO$_2$ converts to 2-(2-hydroxybiphenyl)-benzenesulfinate (HBPS) by DszA, and finally, DszB converts HBPS to 2-HBP as a dead-end metabolite (Fig. 1).

Recently, it has been reported that the newly isolated *Gordonia nitida* efficiently removed sulfur atoms from DBT and diesel oils through the 4-S pathway [14]. Recombinant *Escherichia coli* strains have been generally applied for the production of recombinant proteins and biotransformation [15, 16]. In this study, we report the cloning of the

desulfurization genes from *G. nitida* and the use of these genes for the development of metabolically engineered *E. coli* strains that are capable of desulfurizing DBT.

MATERIALS AND METHODS

Bacterial Strains

Bacterial strains used in this work are listed in Table 1. *G. nitida* was previously reported to be able to desulfurize DBT [14]. *E. coli* XL1-Blue was used as a host strain for general cloning work. Recombinant *E. coli* strains transformed with the plasmid containing the *G. nitida* desulfurization genes were examined for their abilities to desulfurize DBT.

Cloning of Desulfurization Genes from *G. nitida*

Chromosomal DNA of *G. nitida* was prepared as described by Stoecker *et al.* [18]. All DNA manipulations were carried out by following the standard procedures [17]. The desulfurization genes were cloned from *G. nitida* by polymerase chain reaction (PCR) performed with the PCR Thermal Cycler MP (Takara Shuzo Co., Ltd., Shiga, Japan) using Expand™ High Fidelity PCR System (Roche Molecular Biochemicals, Manheim, Germany).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
XL1-Blue	*recA1, endA1, gyrA96, thi, hsdR17, suppE44, relA1, l⁻, lac⁻, F' [proAB lacI^q lacZΔM15, Tn10 (tet)ʳ]*	Stratagene[a]
W3110	*F⁻ mcrA mcrB IN(rrnΔ⁻rrnE)1λ*	Lab stock
BL21(DE3)	*F⁻ ompT hsdS_B (r_B⁻ m_B⁻) gal dcm (DE3)*	Novagen[b]
BL21(DE3) pLysS	*F⁻ ompT hsdS_B (r_B⁻ m_B⁻) gal dcm (DE3) pLysS*	Novagen[b]
C600	*F⁻[e14⁻(McrA⁻) or e14⁺(McrA⁺)] thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21*	Lab stock
DH5α	*SupE44ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab stock
HB101	*F⁻Δ(gpt-proA(62) leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20(Strʳ)xyl-5 mtl1 recA13*	Lab stock
JM101	*F traD36 lacI^q Δ(lacZ)M15 proA⁺B'/supE thi Δ(lac-proAB)*	Lab stock
Gordonia nitida	Wild-type strain, *dsz⁺*	[14]
Plasmids		
pTrc99A	Ap^r; *trc* promoter; protein expression vector	Pharmacia[c]
pUC19	Ap^r; *lacZ*; Cloning vehicle	
pBluescript SK(-)	Ap^r; *lacZ*; Cloning vehicle	Stratagene
pACYC184	Tc^r; Cm^r; Cloning vehicle	New England Biolab[d]
pS1AB	pUC19 derivative; *dszAB*	This study
pS1C	pUC19 derivative; *dszC* fused with partial *dszB*	This study
pACYCS1AB	pACYC184 derivative; *dszAB*	This study
pBlueS1C	pBluescript SK(-) derivative; *dszC*	This study
pBlueS1ExABC	pBluescript SK(-) derivative; *dszABC*	This study
pTrcS1ExABC	pTrc99A derivative; *dszABC*	This study

[a]Stratagene Cloning System, La Jolla, CA, U.S.A.
[b]Novagen, Inc., Madison, WI, U.S.A.
[c]Pharmacia Biotech, Uppsala, Sweden.
[d]New England Biolabs, Berverly, MA, U.S.A.

Table 2. List of primers used in the PCR reactions[a].

Primer	Primer sequence	Target gene	Template used
Primer1	5'-CCCAAGCTTCATGACTCAACAACGACAACTGCATCTGGCC	dszAB	Chromosomal DNA of G. nitida
Primer2	5'-CCCAAGCTTTCCTATCGGTGACGGTTGAGGCTG		
Primer3	5'-CCCAAGCTTCTGCAGGAGCCCGTCGCC		
Primer4	5'-CCCAAGCTT GCATGACTCTGTCCGTTGAAAAGC	dszC	Chromosomal DNA of G. nitida
Primer5	5'- TGCTCTAGATCATCATCAGGAGGTGAAGCCGGG		

[a]Restriction enzyme sites are shown in bold or underlined.

DNA sequencing was carried out with the Bigdye terminator cycle sequencing kit (Perkin-Elmer Co., Boston, MA, U.S.A.) and Taq polymerase using the ABI Prism™ 377 DNA sequencer (Perkin-Elmer Co.). The available DNA sequences of desulfurization genes from R. rhodochrous IGTS8 [3] and PCR amplified fragments of G. nitida were used to design primers for the amplifications of dszAB and dszC genes. The PCR primers for the dszAB and dszC genes are listed in Table 2. The original six base pairs between the dszB and dszC genes were substituted with the HindIII recognition site for the convenient construction of an artificial operon consisting of dszAB and dszC (Fig. 2). Using the chromosomal DNA of G. nitida as a template, PCR yielded a 2.5 kb fragment for the dszAB genes and a 1.3 kb fragment for the dszC gene. The dszAB and dszC genes were cloned into the HindIII-digested pACYC184 and the HindIII/XbaI-digested pBluescript SK(-), yielding pACYCS1AB and pBlueS1C, respectively. Plasmid pBlueS1ExABC containing an artificial dszABC operon was constructed by the insertion of the dszAB genes from pACYCS1AB into the HindIII site of pBlueS1C (Fig. 3).

To construct pTrcS1ExABC, in which the expression of the dszABC genes is controlled by an inducible trc promoter, the dszABC genes were excised from pBlueS1ExABC by digesting with BspHI and XbaI, and the genes were then inserted into the NcoI/XbaI-digested pTrc99A (Fig. 3).

Culture Conditions
Luria-Bertani (LB) medium (containing per liter: 10 g tryptone, 5 g yeast extract, and 5 g NaCl) was used for the routine cultivation of recombinant E. coli strains. The defined media used were R/2 [11] and BSM [3] media supplemented with 10 g/l glucose. Casamino acids (0.1 g/l)

and thiamine (5 mg/l) were added to the BSM medium when necessary (see the genotypes of E. coli strains in Table 1). Ampicillin (Ap) was added at the concentration of 50 mg/l.

Recombinant E. coli strains were grown to the OD_{600} of 1 in tubes containing 10 ml LB medium in a shaking incubator at 30°C and 200 rpm. Protein expression was induced by adding IPTG to 0.5 mM at the OD_{600} of 0.4. Cells were harvested by centrifugation and washed twice with BSM medium. Washed cells were used to inoculate fresh BSM medium (10 ml) containing 0.2 mM DBT.

Analytical Procedures
As reported previously [14], the desulfurization of DBT was analyzed by high-performance liquid chromatography (HPLC). The culture broth was acidified to pH 2.0 with 5 N HCl and extracted with an equal volume of ethyl acetate. After extraction, 10 µl of upper ethyl acetate layer was analyzed by using a reverse-phase HPLC equipped with a UV-VIS detector (Model L-6200, Hitachi, Japan) and Nova-Pak C18 column (3.9×150 mm). The wavelength of the UV-VIS detector was set at 280 nm. The mobile phase consisted of pure methanol or 70% (v/v) acetonitrile, and the flow rate was 0.6 ml/min.

Sulfur content in diesel oil was determined by a gas chromatograph equipped with a sulfur chemiluminescence detector as previously described [1].

RESULTS AND DISCUSSION

Cloning of the Desulfurization Genes from G. nitida
G. nitida was reported to be able to desulfurize DBT following the 4-S pathway [14], which is comman in many
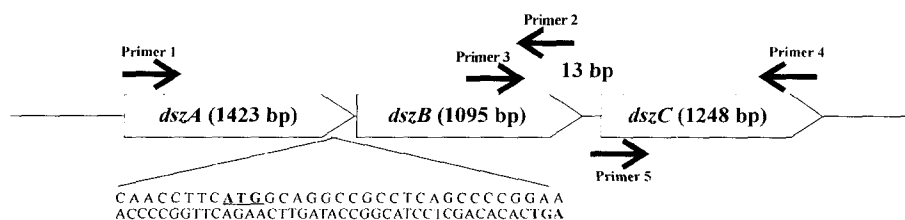
Fig. 2. Organization of G. nitida dszABC operon.
The sequences of overlapping 64 bp between the dszA and dszB genes are shown (the start codon of the dszB gene and the termination codon of the dszA gene are shown in bold). Primers used in the cloning of the dszAB and dszC genes are also shown (Primers 1- 5).
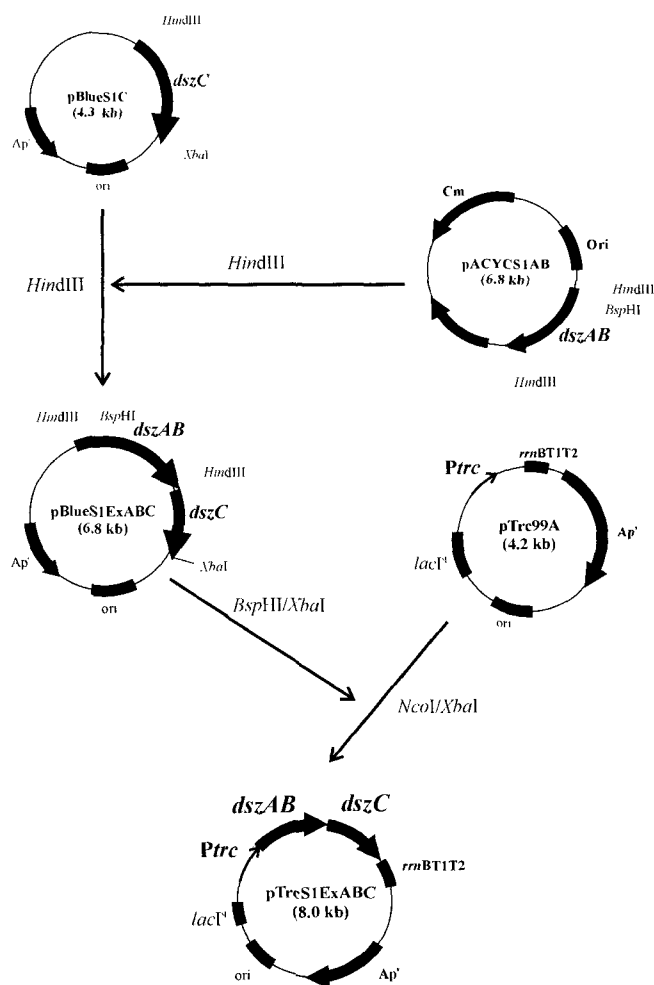
Fig. 3. Schematic diagram for the construction of plasmids used in this study.

soil bacteria that are able to degrade DBT [2, 4]. Since the *dszABC* operon involved in the desulfurization of DBT through the 4-S pathway has been cloned from *R. rhodochrous* IGTS8 [3], we decided to clone the desulfurization genes from *G. nitida* by PCR using the primers designed based on the reported DNA sequence (primers are specified in Fig. 2). The 2.5 kb *dszAB* genes were successfully obtained by PCR using primers 1 and 2. However, the *dszC* gene could not be amplified using the primers designed based on the *R. rhodochrous* IGTS8 *dszC* gene.

First of all, plasmid pS1AB containing the amplified *G. nitida dszAB* genes was constructed by the insertion of the *dszAB* genes into the *Hin*dIII site of pUC19. After sequencing the *dszAB* genes in pS1AB, a new forward primer for the *dszC* gene was designed based on the sequence of the *dszB* gene (primer 3). The PCR amplified *dszC* gene (1.4 kb) that was fused with a partial fragment (100 bp) of the *dszB* gene was cloned into the *Hin*dIII/ *Xba*I-digested pUC19 to make pS1C. Based on the DNA

sequence of newly cloned *G. nitida dszABC* genes in pS1AB and pS1C, modified primers were designed as shown in Table 2. These new primers were used to clone the *dszAB* genes (primers 1 and 2) in pACYCS1AB and the *dszC* gene (primers 4 and 5) in pBlueS1C, which were subsequently used to construct pBlueS1ExABC and pTrcS1ExABC.

Molecular Characterization of Desulfurization Genes

The sequences of nucleotides and deduced amino acids of the *G. nitida dszABC* genes were compared with those of *R. rhodochrous* IGTS8 [3]. The *dszABC* operon of *G. nitida* was very similar to that of IGTS8 (Fig. 2). The DNA sequence similarity between the *G. nitida dszABC* genes and the *R. rhodochrous* IGTS8 genes was 89%. The deduced amino acid sequences of *G. nitida* DszA, DszB, and DszC shared 86%, 86%, and 90% similarity, respectively, with those proteins of *R. rhodochrous* IGTS8. The *dszA* gene consists of 1,423 bp and encodes a protein of 474 amino acids (M_r of 51,906), while the *dszB* gene consists of 1,095 bp and encodes a protein of 365 amino acids (M_r of 39,339). The *dszC* gene consists of 1,248 bp and encodes a protein of 416 amino acids (M_r of 44,989). The *dszA* and *dszB* genes overlapped by 64 bp (Fig. 2). The *G. nitida dszC* gene lacked one amino acid compared with that of *R. rhodochrous* IGTS8 at the N-terminus. There was a 13 bp gap between the stop codon of the *dszB* gene and the start codon of the *dszC* gene.

Biodesulfurization of DBT and Diesel Oil by Metabolically Engineered *E. coli* Strains

The transcription of *dszABC* genes by their native promoter is known to be strongly repressed in the presence of sulfate and sulfur-containing amino acids [9]. Therefore, we replaced the native promoter with an inducible *trc* promoter. Thus, plasmid pTrcS1ExABC was constructed for the expression of the *dszABC* genes in *E. coli*. Eight different *E. coli* strains, listed in Table 1, were transformed with this plasmid, and their abilities to desulfurize DBT in BSM medium were compared. The results of test tube cultures are summarized in Fig. 4. All the recombinant *E. coli* strains containing the *dszABC* genes showed desulfurization activities with varying efficiencies. Among them, metabolically engineered *E. coli* HB101 and W3110 strains converted DBT into 2-HBP with relatively high efficiencies of 16% and 15%, respectively. We have previously reported desulfurization of DBT by recombinant *Klebsiella* sp. and recombinant *E. coli* harboring the *dszABC* genes from *R. rhodochrous* IGTS8 [7]. The conversion efficiency of DBT to 2-HBP was quite low as only 1% of DBT was converted to 2-HBP [7]. This was due to the inefficient expression of the *dszABC* genes because the *dszABC* gene cluster was fused to the *lac* promoter approximately 4 kb upstream (including native *dsz* promoter) of the coding
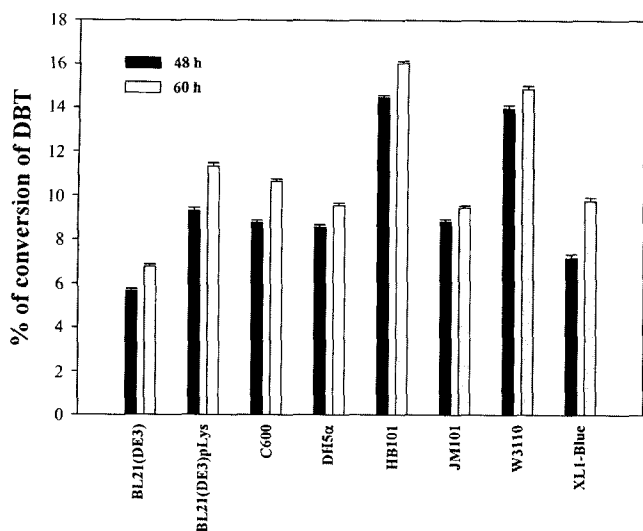
Fig. 4. Test tube cultures of various metabolically engineered *E. coli* strains harboring the *G. nitida dszABC* genes in BSM medium containing 0.2 mM DBT.

sequence [3, 12]. As mentioned above, the native *dsz* promoter is known to be repressed by the presence of sulfur-containing compounds [9]. In this study, this problem was overcome by the optimization of the *dszABC* expression system using the *trc* promoter and an artificial *dszABC* operon.

Since *E. coli* HB101 is the auxotroph for several amino acids, recombinant *E. coli* W3110 was selected for further examination of its desulfurization ability in a flask culture. Flask culture of recombinant *E. coli* W3110 (pTrcS1ExABC) was carried out in R/2 medium supplemented with 10 g/l of glucose at 30°C. DBT (0.2 mM) was added to the medium in 6 h after the induction with 0.5 mM IPTG at the OD_{600} of 0.7. Recombinant *E. coli* W3110 (pTrcS1ExABC) converted 8.7% and 11.4% of DBT to 2-HBP after 48 h and 60 h, respectively, which are similar to the results obtained in test tube cultures. Also, diesel oil treated by HDS process containing 250 mg sulfur/l was examined for the desulfurization (SK Corp., Daejeon, Korea). Diesel oil was supplemented into the medium at 5% (v/v) in 6 h after the induction with 0.5 mM IPTG at OD_{600} of 0.7. Recombinant *E. coli* W3110 (pTrcS1ExABC) could remove 15% of sulfur in diesel oil, decreasing sulfur content from 250 to 212.5 mg/l in diesel oil in 60 h.

In this study, we have demonstrated that metabolically engineered *E. coli* strains harboring the new desulfurization genes from *G. nitida* were able to efficiently desulfurize DBT and diesel oil. Deregulation of the transcription repression site by employing the inducible *trc* promoter and an artificial *dszABC* operon resulted in a relatively high efficiency of DBT conversion to 2-HBP. Use of these metabolically engineered *E. coli* strains for the removal of DBT in a large fermentor is currently under investigation.

Acknowledgments

REFERENCES

1. Chang, J. H., S.-K. Rhee, Y. K. Chang, and H. N. Chang. 1998. Desulfurization of diesel oils by a newly isolated dibenzothiophene degrading *Nocardia* sp. strain CYKS2. *Biotechnol. Prog.* **14:** 851–855.

2. Denis-Larose, C., D. Labbe, H. Bergeron, A M. Jones, C. W. Greer, J. al-Hawari, M. J. Grossman, B. M. Sankey, and P. C. Lau. 1997. Conservation of plasmid-encoded dibenzothiophene desulfurization genes in several *Rhodococci*. *Appl. Environ. Microbiol.* **63:** 2915–2919.

3. Denome, S. A., C. Oldfield, L. J. Nash, and K. D. Young. 1994. Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. *J. Bacteriol.* **176:** 6707–6716.

4. Duarte, G. F., A. S. Rosado, L. Seldin, W. de Araujo, and J. D. van Elsas. 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.

5. Gallardo, M. E., A. Ferrandez, V. De Lorenzo, L. L. Garcia, and E. Diaz. 1997. Designing recombinant *Pseudomonas* strains to enhance biodesulfurization. *J. Bacteriol.* **179:** 7156–7160.

6. Izumi, Y., T. Oshiro, H. Ogino, Y. Hine, and M. Shimao. 1994. Selective desulfurization by *Rhodococcus erythropolis* D-1. *Appl. Environ. Microbiol.* **60:** 223–226.

7. Jeong, K. J., H. S. Lee, S. Y. Lee, and Y. K. Chang. 1998. Development of recombinant bacteria for the degradation of dibenzothiophene. *Ann. N. Y. Acad. Sci.* **864:** 375–378.

8. Kilbane, J. J. and K. Jackowski. 1992. Biodesulfurization of water-soluble coal-derived material by *Rhodococcus rhodochrous* IGTS8. *Biotechnol. Bioeng.* **40:** 110^7–1114.

9. Li, M. Z., C. H. Squires, D. J. Monticello, and J. D. Childs. 1996. Genetic analysis of the *dsz* promoter and associated regulatory regions of *Rhodococcus erythropolis* IGTS8. *J. Bacteriol.* **178:** 6409–6418.

10. Omori, T., L. Monna, Y. Saiki, K. Kasuga, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1. *Appl. Environ. Microbiol.* **58:** 911–915.

11. Park, S. J., G. Georgiou, and S. Y. Lee. 1999. Secretory production of recombinant protein by a high cell density culture of a protease negative mutant *Escherichia coli* strain. *Biotechnol. Prog.* **15:** 164–167.

12. Piddington, C. S., B. R. Kovacevich, and J. Rambosek. 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.

13. Reichmuth, D. S., J. L. Hittle, H. W. Blanch, and J. D. Keasling. 2000. Biodesulfurization of dibenzothiophene in *Escherichia coli* is enhanced by expression of a *Vibrio harveyi* oxidoreductase gene. *Biotechnol. Bioeng.* **67:** 72–79.

14. Rhee, S. K., J. H. Chang, Y. K. Chang, and H. N. Chang. 1998. Desulfurization of dibenzothiophene and diesel oils by a newly isolated *Gordona* strain, CYKS1. *Appl. Environ. Microbiol.* **64:** 2327–2331.

15. Kim, M.-D., W.-J. Lee, K.-H. Park, K.-H. Rhee, and J.-H. Seo. 2002. Two-step fed-batch culture of recombinant *Escherichia coli* for production of *Bacillus licheniformis* maltogenic amylase. *J. Microbiol. Biotechnol.* **12:** 273–278.

16. Kwon, M.-J., S.-L. Park, S.-K. Kim, and S.-W. Nam. 2002. Overproduction of *Bacillus macerans* cyclodextrin glucanotransferase in *E. coli* by coexpression of GroEL/ES chaperone. *J. Microbiol. Biotechnol.* **12:** 1002–1005.

17. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.

18. Stoecker, M. A., R. P. Herwig, and J. T. Staley. 1994. *Rhodococcus zopfii* sp. nov., a toxicant-degrading bacterium. *Int. J. Syst. Bacteriol.* **44:** 106–110.