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Recombinant *Rhodococcus* sp. Strain T09 Can Desulfurize DBT in the Presence of Inorganic Sulfate

Toru Matsui,[1] Ken-ichi Noda,[1] Yasuhiro Tanaka,[1,2] Kenji Maruhashi,[1] Ryuichiro Kurane[3]

[1]Bio-Refining Process Laboratory, Japan Cooperation Center Petroleum (JCCP), 1900 Sodeshi-cho Shimizu-shi, Shizuoka 424-0037, Japan
[2]National Institute of Advanced Industrial Science and Technology (AIST), 1-1 Higashi Tsukuba-shi, Ibaraki, 305-8566, Japan
[3]Biotechnology Research Center, Kubota Co., 5-6 Koyodai Ryugasaki-shi, Ibaraki, 301-0852, Japan

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Abstract. The putative *Rhodococcus rrn* promoter region was cloned from the benzothiophene desulfurizing *Rhodococcus* sp. strain T09, and the dibenzothiophene desulfurizing gene, *dsz*, was expressed under the control of the putative *rrn* promoter in the strain T09 using a *Rhodococcus–E.coli* shuttle vector. Strain T09 harboring the expression vector, pNT, could desulfurize dibenzothiophene in the presence of inorganic sulfate, methionine, or cysteine, while the Dsz phenotype was completely repressed in recombinant cells carrying the gene under the control of the native *dsz* promoter under the same conditions. Among the sulfur sources examined, no intermediates were detected and only the final desulfurized product, 2-hydroxy-biphenyl, was produced using ammonium sulfate as the sulfur source.

Sulfur compounds in petroleum are released as sulfur oxyacids during combustion and cause environmental problems such as acid rain. Diesel oil contains various organosulfur compounds such as alkylated dibenzothiophene (alkyl-DBT), alkylated benzothiophene (alkyl-BT), sulfide, thiol, and other thiophene compounds, some of which have been reported to be difficult to remove by conventional physicochemical desulfurizing methods.

Most research into Biological Desulfurization Systems (BDS) uses DBT [5, 11] or alkyl-DBT [5] as the model compound. DBT desulfurizing enzymes are encoded as a cluster in the *dsz* operon, which is expressed under the control of the *dsz* promoter. The Dsz phenotype was shown to be strictly repressed by various sulfur compounds such as inorganic sulfate, methionine (Met) and cysteine (Cys) [7]. Also, expression control by sulfur compounds was extensively studied in *E. coli* [14]. To avoid sulfur regulation, Dsz enzymes have been expressed in *E. coli*, or use *E. coli* promoter has been examined [12, 13]. Two-stage cultivation including a growth phase and Dsz phenotype induction phase by sulfur starvation was also examined with wild *Rhodococcus erythropolis* IGTS8 [3]. As nocardioform bacteria such as *Rhodococcus* are considered feasible for petroleum desulfurization [10], construction of self-cloned recombinant nocardioform would be required for practical oil-desulfurization.

In this study, we cloned the putative *rrn* promoter element in the 16SrRNA region from benzothiophene desulfurizing nocardioform *Rhodococcus* sp. strain T09 and expressed the Dsz phenotype in this strain, that could desulfurize DBT even in the presence of inorganic sulfate, Met or Cys. This would make it possible to desulfurize sulfur-containing oil in one-step with a simple culture technique.

Materials and Methods

Bacterial strains and plasmids. *Rhodococcus* sp. strain T09 [8] was used as the host bacterium. pDsz/KS (Fig.1) contains the promoterless *dsz* operon (*dsz* A, B, and C) and *dsz* D genes cloned into the *Xba*I/*Hin*dIII sites of pBluescript II KS+ (Stratagene Co., CA). The *Rhodococcus–E. coli* shuttle vector pRHK1 was used to transform strain T09 [9]. pR16AP and pR16AP2 contained 1.4 kb *Bam*H1 and 0.7 kb *Bgl*II/*Xba*I fragments, respectively, of partial *tyr*S and *rrn* in pBluescript II KS+. The recombinant plasmid pNT contained *dsz* A, B, C, and D genes downstream from the putative *rrn* promoter fragment from strain T09 (Fig.1). The plasmid, pRKPP, containing the *dsz* operon under the control of the *dsz* promoter, was used as a reference [9].

Recombinant DNA techniques. Chromosomal DNA and plasmid DNA were exracted from *Rhodococcus* sp. strain T09 as described previously [4, 9]. *Rhodococcus* sp. strain T09 was transformed by

Correspondence to: T. Matsui; *email:* tmatsui@brpl.jccp.or.jp

electroporation using a Gene Pulser II (Bio-Rad Co., CA). The probe DNA for colony hybridization was amplified by PCR using the strain T09 chromosomal DNA template and primers 5′-CGTGCCAGCAGC-CGC GGTAAT-3′ and 5′-AAGGAGGTGATCCAGCCGA-3′.

Cultivation and resting cell reaction. Medium A containing 0.5% glucose (AG), prepared as described previously [8], was used as the basal medium. Cultivation was performed at 30°C in Erlenmeyer flasks with rotation at 150 rpm. DBT was added as *N,N′*-dimethyl formamide solution or n-tetradecane solution. The desulfurizing activity was determined by measuring the amount of desulfurized product.

Analysis. Cellular growth was estimated by measuring the optical density at 660 nm. Product formation and substrate consumption were analyzed by gas chromatography (GC) after extracting the reaction solution with ethyl acetate at pH below 1. GC-MS analysis was performed as described previously [5].

Chemical. DBT sultine (dibenz[*c,e*][1, 2] oxathiin-6-oxide), and DBT sultone (dibenz[*c,e*][1, 2] oxathiin-6,6-dioxide) were synthesized by and obtained from Nard Co., Hyogo, Japan. Dibenzothiophene (DBT) and dibenzothiophene S,S dioxide (DBTO2) were supplied by Tokyo Kasei Kogyo Co., Tokyo, Japan. All other materials were of the highest purity commercially available and were used without further purification.

Sequencing. The 1.5 kb *Pma*C1/*Bam*HI fragment was cloned into the *Eco* RV site of pUC18 after blunting the fragment and its sequence was determined on both strands by primer walking with an ABImodel 310 and Big Dye terminator kit (Applied Biosystems Inc. CA) according to the manufacturer's instructions.

Alignment of sequences. Computer-aided analysis of the alignment of nucleotide sequences of the gene encoding *rrn* regions was achieved by means of the BLAST program at the National Center for Biotechnology Information (NCBI).

Nucleotide sequence accession number. The 1.4 kb sequence of the fragment from *Rhodococcus* sp. Strain T09 has been assigned DDBJ/EMBL/GenBank accession no. AB074048.

Results

Cloning of putative *rrn* operon fragment from strain T09. A genomic DNA library from strain T09 was probed with a 0.65 kb PCR-amplified fragment of a partial 16SrRNA region from strain T09. A specific region for nocardioform 16SrRNA (*R. fascians* accession no.Y11196) was selected as the probe for colony hibridization to avoid hybridization with the 16SrRNA region of the host (*E. coli*). An 8.0-kb *Bam*HI positive fragment was obtained by colony hybridization using the 0.65-kb probe and was further digested with *Pma*CI and *Bam*HI to obtain a 1.4-kb fragment followed by blunting and insertion into the *Eco* RV site of pBluescript II KS+ (Fig. 1, pR16AP) for sequencing.

Sequence analysis indicated that the 5′-end of this fragment comprised a partial region encoding a protein 68.5% homologous to Tyr-tRNA synthase (*tyr*S) from *Mycobacterium tuberculosis* (accession no. Z98268) [1], and the 16SrRNA sequence started downstream of *tyr*S. Putative promoter elements were detected between the two regions, which were aligned by comparison with those from *M. smegmatis* [2] (Fig. 2). From the above assignments, the 0.7-kb *Bgl*II–*Xba*I fragment was used to construct the *dsz* gene expression plasmid.

Effects of various sulfur sources in the medium on *dsz* gene expression. A *dsz* expression vector with the putative *rrn* promoter was constructed as shown in Fig. 1. In addition to the *dsz* operon, the flavin oxidoreductase gene, *dsz* D, was introduced to enhance the desulfurization activity, as reported previously [6]. The transformant obtained by electroporating pNT into strain T09, T09/pNT, was confirmed to have pNT as an independent replicon and grew with DBT as the sole sulfur source (data not shown).

T09/pNT was cultivated with various sulfur sources (0.4 mM) in the presence of DBT to examine the desulfurization activity and compared with T09/pRKPP carrying the *dsz* gene under the control of the native *dsz* promoter for Dsz phenotype expression (Table 1). Strain T09/pNT could significantly degrade DBT even when inorganic sulfate, Met, or Cys was used as the sulfur source, while T09/pRKPP could not degrade DBT at all. With the use of Met, Cys, $MgSO_4 \cdot 7H_2O$, or Na_2SO_4 as the sulfur source, significant levels of intermediates such as DBTO2 and DBT sultine were detected resulting in a decreased yield of desulfurized product, 2-hydroxy biphenyl (2HBP). On the other hand, using $(NH_4)_2SO_4$ as the sulfur source resulted in a good yield of 2HBP with no production of intermediates.

For application in oil desulfurization, organic solvent-water, bi-phase cultivation was examined using n-tetradecane as the model oil fraction. Production of 2HBP was increased by addition of n-tetradecane, as shown in Table 2.

Discussion

In this study, we constructed a recombinant strain that could desulfurize DBT even in the presence of Met, Cys, or inorganic sulfate as the sulfur source by using the putative *Rhodococcus rrn* promoter region. A partial 16SrRNA region was cloned from the BT desulfurizing nocardioform *Rhodococcus* sp. strain T09. Southern blotting of *Eco*RI-digested *Rhodococcus* sp. T09 total DNA with the 0.65-kb PCR-amplified probe containing a partial 16SrRNA region of strain T09 detected four positive bands with estimated sizes of 10.0, 6.0, 3.0, and 2.5 kb (data not shown). It seems, therefore, that at least four copies of the rRNA operon are present in the genome of this strain. From the sequence and alignment of the *Bam*H1 fragment

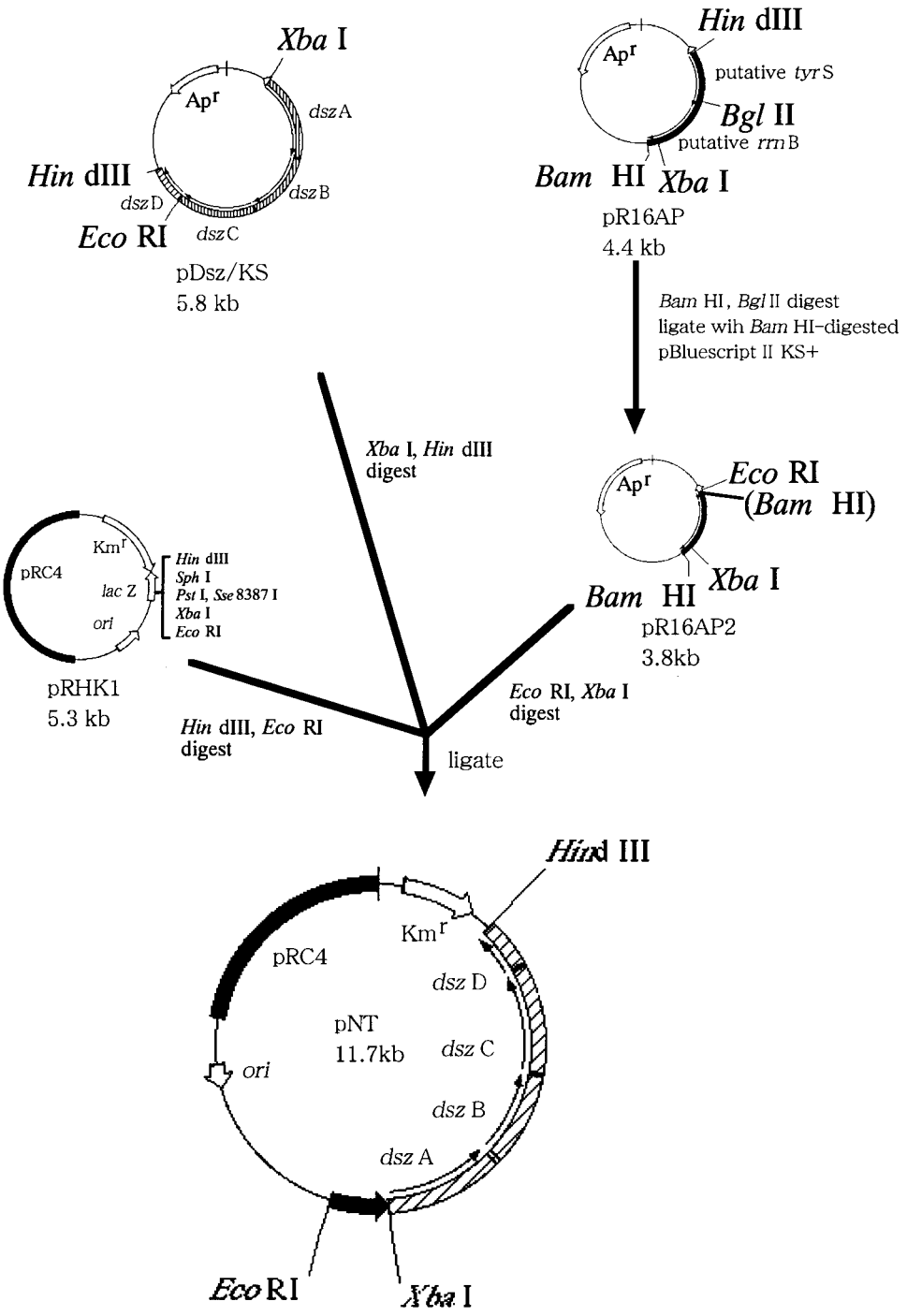

Fig. 1. Construction of *dsz* gene expression vector under the control of the putative *rrn* promoter. Abbreviations: Ap^r, ampicillin resistance; Km^r, kanamycin resistance; *tyr*S, downstream region of tyrosine tRNA synthetase; *rrn* B, upstream region of 16SrRNA containing promoter element; pRC4, cryptic plasmid from *R. rhodochrous* [10].

obtained from colony hybridization, with the BLAST program, a putative promoter element was found. Genetic analyses such as primer extention are required to characterize the detailed function based on these alignments.

The putative *rrn* promoter was replaced upstream from the Dsz enzyme coding region to construct a Dsz enzyme expression vector for the strain T09 using the *Rhodococcus–E.coli* shuttle vector pRHK1. Although

Table 1. DBT desulfurization by T09/pNT (NT) and T09/pRKPP (PP) in the presence of various sulfur sources

Sulfur compound	Strain	Growth (OD660)	Residual DBT (%)	Product		
				DBTO2 (μM)	DBT-sultine (μM)	2HBP (μM)
Organic sulfur						
DBT	NT	1.52	38.2	0	0	103.3
	PP	3.19	18.5	0	0	317.3
Dimethyl Sulfoxide	NT	2.61	13.6	111.1	20.0	205.1
	PP	2.63	63.9	0	0	177.8
S-containing amino acid						
Taurine	NT	2.69	15.5	84.7	19.8	221.5
	PP	2.68	96.6	0	0	0
Methionine	NT	2.40	2.70	457.8	9.5	5.3
	PP	2.50	99.8	0	0	0
Cystein	NT	2.41	1.78	364.1	15.4	8.8
	PP	2.56	100	0	0	0
Inorganic sulfate						
$MgSO_4 \cdot 7H_20$	NT	2.10	17.5	57.8	4.3	5.46
	PP	2.69	100	0	0	0
Na_2SO_4	NT	2.82	11.8	15.9	2.0	5.6
	PP	2.96	97.1	0	0	0
$(NH_4)_2SO_4$	NT	2.33	15.31	0	0	166.8
	PP	3.08	100	0	0	0

Cultivations were carried out at 30°C for four days.

Table 2. Effects of *n*-tetradecane on DBT desulfurization by T09/pNT

S source	TD[1]	Residual DBT (%)	DBTO2 (mM)	DBT-sultine (mM)	2HBP (mM)
Na_2SO_4	×	11.8	15.9	2.0	5.6
Na_2SO_4	○	0	0	21.3	171.9
$(NH_4)_2SO_4$	×	15.31	0	0	166.8
$(NH_4)_2SO_4$	○	0	0	23.2	309.6

Cultivations were carried out at 30°C for four days.
[1] TD; 1 ml of *n*-tetradecane was added to 1 ml of medium.

M. smegmatis

$_{36}$GTCTTGACTCCCAGTTTCCAAGGACGTAACTTATTCCAGGTCA$_{78}$
*

Rhodococcus sp.T09

$_{806}$GATTTGACGCTCCGTTTCCCGTCGCGTAACTTATTCCAGGTCA$_{848}$

Fig. 2. Alignment of the promoter sequence of *rrn* Bf (P1) from *M. smegmatis* (top sequence) [3] with the upstream region of 16SrRNA from *Rhodococcus* sp. strain T09 (bottom sequence). Putative promoter elements [−35 (former) and −10 (latter) boxes] are underlined. The asterisk denotes the transcriptional start site.

the obtained recombinant strain T09/pNT could degrade DBT irrespective of the sulfur source used in this study, product formation profiles were dependent on the sulfur source. T09/pRKPP, in which the Dsz phenotype was controlled by the *dsz* promoter, could only desulfurize DBT with DBT or dimethyl sulfoxide (DMSO) as the sulfur source, as reported previously [7]. Dsz phenotye expression in the presence of taurine may be due to a mechanism similar to that reported for *E. coli* [14]. Among the sulfur sources tested, T09/pNT produced 2HBP when grown with $(NH_4)_2SO_4$ to the same level as the case of growth with DBT. This was also confirmed by comparing Dsz enzymes expression using Western blotting analysis (data not shown).

Increased 2HBP production was observed with ad-

dition of *n*-tetradecane. This might have been due to the facilitated contact by *n*-tetradecane between DBT and cells as DBT is water-immiscible [9, 11]. We are currently optimizing the culture conditions using this recombinant cell.

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