Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Kazuaki HIRASAWA, Yoshitaka ISHII, Morio KOBAYASHI, Kenichi KOIZUMI & Kenji MARUHASHI (2001) Improvememt of Desulfurization Activity in Rhodococcus erythropolis KA2-5-1 by Genetic Engineering, Bioscience, Biotechnology, and Biochemistry, 65:2, 239-246, DOI: 10.1271/bbb.65.239

To link to this article: http://dx.doi.org/10.1271/bbb.65.239

Biosci. Biotechnol. Biochem., **65** (2), 239-246, 2001

Improvement of Desulfurization Activity in *Rhodococcus erythropolis* KA2-5-1 by Genetic Engineering

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Received May 18, 2000; Accepted October 5, 2000

***Rhodococcus erythropolis* KA2-5-1 can desulfurize dibenzothiophene (DBT) into 2-hydroxybiphenyl. A cryptic plasmid, pRC4, which was derived from *R. rhodochrous* IFO3338, was combined with an *Escherichia coli* vector to construct an *E. coli-Rhodococcus* shuttle vector. The complete nucleotide sequence of 2582-bp pRC4 was analyzed. Based on the characteristics of its putative replication genes, pRC4 was assigned to the family of pAL5000-related replicons. The desulfurization gene cluster, *dszABC*, and the related reductase gene, *dszD*, cloned from KA2-5-1, were reintroduced into KA2-5-1 and efficiently expressed. The DBT desulfurization ability of the transformant carrying two *dszABC* clusters and one *dszD* on the vector was about 4-fold higher than that of the parent strain, and the transformant also showed improved desulfurization activity for light gas oil (LGO). Sulfur components in LGO before and after the reaction were analyzed with gas chromatography-atomic emission detection.**

Key words: desulfurization; dibenzothiophene; *Rhodococcus*; plasmid; light gas oil

Organic sulfur in fossil fuels is one of the causes of environmental pollution.[1] Refineries remove organic sulfur from fuels by hydrodesulfurization (HDS), which is done with metallic catalysts in the presence of hydrogen gas at high temperature and high pressure. With the depletion of low-sulfur-containing petroleum reserves and increasingly stringent regulations, adequate desulfurization by HDS alone is becoming more difficult.[2] Biodesulfurization (BDS) offers the potential for an effective method for lowering the sulfur content of petroleum products. The BDS process has the advantage of reducing the load on the environment, because it is done under mild conditions leading to lower energy consumption and lower CO_2 emission.

Dibenzothiophene (DBT) and its alkylated derivatives are known to constitute the majority of organic sulfur compounds refractory to conventional HDS processes.[3] *Rhodococcus erythropolis* KA2-5-1 was isolated previously from soil samples as an effective and stable bacterium that can desulfurize DBT, alkyl DBTs, and some of the alkyl benzothiophenes, through the specific cleavage of C-S bonds.[4] The pathway of DBT desulfurization by KA2-5-1 may be the same as that established for the *Rhodococcus* sp. strain IGTS8 (Fig. 1).[4,5] DBT is stepwise S-oxidized by DszC, first to DBT-5-oxide (DBTO) and then to DBT-5,5′-dioxide ($DBTO_2$). DszA catalyzes the conversion of $DBTO_2$ to 2-(2′-hydroxyphenyl) benzene sulfinate (HPBS), which opens the thiophenic ring. HPBS is then desulfinated by DszB to produce 2-hydroxybiphenyl (2-HBP) with the release of inorganic sulfur. DszD, a flavin reductase, is required for the first three reactions to regenerate cofactors. The desulfurization gene cluster, *dszABC*, and the flavin reductase gene, *dszD*, have been cloned from *Rhodococcus* sp. strain IGTS8 and sequenced.[6-8] DNA sequencing of the PCR clone of desulfurization gene cluster from KA2-5-1 has shown that it is practically identical with the *dszABC* cluster from IGTS8.[4]

To develope a commercially feasible BDS process, it is necessary to improve the biocatalytic activity and to make the process financially competitive with the HDS process. In our collection of wild type desulfurizing microorganisms, KA2-5-1 has the highest activity.[4] To create a prominent strain, we considered self-cloning was more effective than heterogeneous recombination because gene expression and DBT permeation could be surely done. Overexpression of the IGTS8 *dszC* gene in *E. coli* cells has been reported and the DszC enzyme purified from the recombinants has been found to be in a homodimer struc-

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Abbreviations: DBT, dibenzothiophene; 2-HBP, 2-hydroxybiphenyl; HDS, hydrodesulfurization; BDS, biodesulfurization; DBTO, DBT-5-oxide; $DBTO_2$, DBT-5,5′-dioxide; HPBS, 2-(2′-hydroxyphenyl) benzene sulfinate; DCW, dry cell weight; LGO, light gas oil; GC-AED, gas chromatography-atomic emission detection

ture.[9] This finding is inconsistent with another report that native DszC purified from *Rhodococcus* sp. IGTS8 is a tetramer.[5] There might be different patterns of polypeptide multimerization in different bacterial species. In this paper, we describe the development of a host-vector system for *Rhodococcus* spp., improvement of the desulfurization ability of *Rhodococcus erythropolis* KA2-5-1 by genetic engineering, and application of the recombinant to desulfurize light gas oil (LGO).

Materials and Methods

Bacterial strains, plasmids, and chemicals. *Rhodococcus rhodochrous* IFO3338 was obtained from Institute for Fermentation Osaka (Osaka). *E. coli* JM109 competent cells, and plasmids pUC118 and pHSG298 were purchased from Takara Shuzo Co., Ltd. (Kyoto). Lambda DASH II and pBluescript II KS(+) were from Stratagene. Enzymes for recombinant DNA techniques were from Takara Shuzo. DBT was purchased from Kanto Chemical Co., Inc. (Tokyo). LGO was supplied by a petroleum company in Japan.

Recombinant DNA techniques. DNA manipulations with *E. coli* were done as described by Sambrook *et al.*[10] Plasmid DNA was isolated from *Rhodococcus* strains as described by Denis-Larose *et al.*[10]

Transformation of Rhodococcus by electroporation. KA2-5-1 cells were grown in 100 ml of LB at 30°C with shaking, and harvested at late log phase (OD_{660} 0.9–1.2). The cells were washed once with ice-cold water and twice with ice-cold 10% glycerol before being concentrated 40-fold. These cells were stored in small portions at −80°C. Portions of 80 μl of ice-cold cells were mixed with plasmid DNA in electrocuvettes with a 1-mm gap (Bio-Rad) and given a 1.5-kV electric pulse from a Gene Pulser II (Bio-Rad) connected to a pulse controller (25 μF capacitor, 400 Ω external resistance). Pulsed cells were diluted immediately with 0.42 ml of SOC and incubated for 3 h at 30°C with shaking, after which the cultures were spread on LB plates containing 100 μg of kanamycin per ml. Transformants were scored after 3–5 days.

DNA sequencing and analysis. Automated DNA sequencing was done with an ABI PRISM 310 DNA sequencer. The complete sequence of both DNA strands was identified by the primer walking method, with multiple sequencing of some regions. Sequence assembly and analysis were done with a Genetyx-Mac ver9.01 (SDC, Japan). The GenBank and SwissProt databases were searched for nucleic acid and amino acid similarities with Genetyx-Mac CD No. 36 (SDC, Japan), the FASTA program at DNA Data Bank of Japan (DDBJ), and the BLAST program at the National Center for Biotechnology Information (NCBI).

Nucleotide sequence accession number. The nucleotide sequence data of pRC4 will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB040101.

Cloning of the desulfurization genes. Based on the sequences of *dszABC* and *dszD* of *R. erythropolis* IGTS8,[6–8] primers were synthesized and used for PCR with genomic DNA of KA2-5-1.[4] The PCR fragments amplified with the primers were used as probes to screen the genomic DNA libraries in lambda DASH II (Stratagene) by plaque hybridization. Genomic DNA preparation, library construction, and plaque hybridization screening were done by the method described by Ishii *et al.*[12]

Construction of dsz recombinant plasmids. The 3.1-kb *Bam*HI DNA fragment from a lambda clone containing the entire *dszD* was subcloned into the *Bam*HI site of pUC118 to yield plasmid pDSZD. The 2.9-kb *Eco*RI fragment from pDSZD was ligated into the *Eco*RI site of the *E. coli-Rhodococcus* shuttle vector pRHK1, resulting in the plasmid pRKBE, which included *dszD* with a 1.1-kb fragment upstream and a 1.2-kb fragment downstream of the structural gene (Fig. 5). The lambda DASH II clone containing the *dszABC* cluster was digested with *Pvu*I, and the ends were made blunt with a DNA Blunting Kit (Takara Shuzo). The 4.5-kb DNA fragment spanning the *dszABC* cluster with the promoter and associated regulatory regions was subcloned into *Sma*I-digested pBluescript II KS(+) to yield the plasmid pBKPP. Then, the 4.5-kb *Eco*RI-*Xba*I DNA fragment from pBKPP was ligated with *Eco*RI-*Xba*I-digested pRHK1 yielding the shuttle plasmid pRKPP, which contained *dszABC* with 0.4-kb fragments upstream and downstream of the structural genes (Fig. 5). Plasmid pDSZD was digested with *Bgl*I, and the ends were made blunt. The 1.2-kb fragment containing *dszD* with 0.45 kb upstream and 0.1 kb downstream of the structural gene was ligated into the *Sna*BI site of pRKPP, resulting in the plasmid pRKPPBB, containing both *dszABC* and *dszD* (Fig. 5). Plasmid pBKPP was double-digested with *Eco*RI and *Spe*I, and the ends were made blunt as described above. The 4.5-kb DNA fragment was ligated into *Sna*BI-digested pRKPPBB, resulting in the shuttle plasmid pRKPBP, containing two *dszABC* clusters and one *dszD* with their own promoter and regulatory regions (Fig. 5).

Measurement of DBT desulfurization activity. KA2-5-1 transformants were cultured in medium A[13]

containing 25 mg/l DBT and 50 mg/l kanamycin sulfate at 30°C with shaking for 2 days, and then cultured with a 3% inoculum into medium A with DBT as the sole sulfur source without kanamycin sulfate at 30°C with shaking for 20 h. These cultures were harvested by centrifugation at 10,000 × *g* for 15 min, and suspended in 0.1 mM potassium phosphate buffer (pH 7.0) to OD_{660} 15 (5.85 g DCW/l). Samples of 5 ml of the suspension were transferred to 100-ml flasks with baffles. An equal volume of *n*-tetradecane containing 3000 mg/l DBT was added to the flask and the contents were shaken (180 rpm) at 30°C for 1 h. After the reaction, 80 μl of 6 N HCl were added, and the samples were centrifuged at 10,000 × *g* for 5 min to separate the oil phase from the aqueous phase. The oil phases were analyzed by gas chromatography (GC)[14] for HBP and $DBTO_2$ content. The aqueous phases were extracted with a 20% volume of ethyl acetate, and analyzed by GC for HPBS content.

Desulfurization of LGO. KA2-5-1 and one of its transformants were cultured as described above, harvested by centrifugation, and suspended in 0.1 mM potassium phosphate buffer (pH 7.0) to OD_{660} 50 (19.5 g DCW/l). Portions of 500 ml of these suspensions were transferred to 2-liter stirred vessels. An equal volume of LGO was added to the vessel, agitated (400 rpm), and aerated (500 ml/min) at 30°C. Samples were withdrawn at defined times and centrifuged at 10,000 × *g* for 20 min. The oil phases were analyzed with ANTEK 7000V (Antek, Texas) to determine total sulfur concentration. Sulfur components in LGO were analyzed with gas chromatography-atomic emission detection (GC-AED) as described by Onaka *et al.*[14]

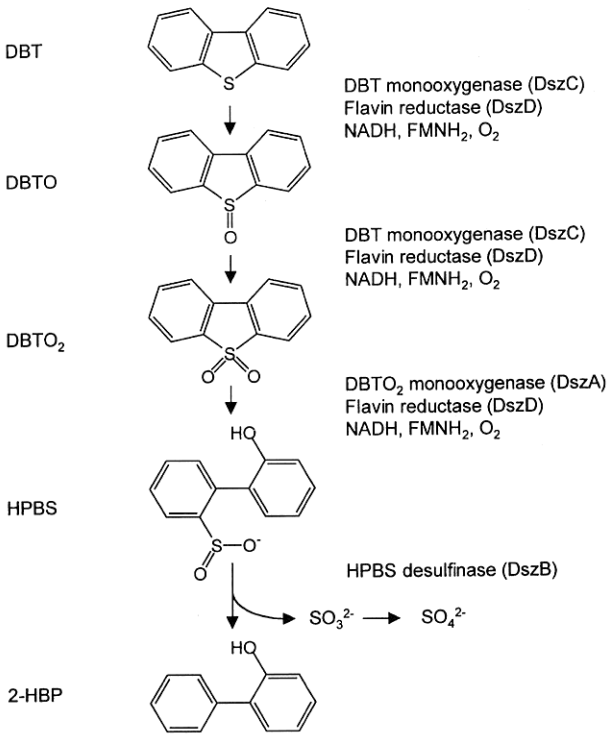

Fig. 1. Proposed Pathway of the Metabolism of DBT by *Rhodococcus* sp. IGTS8.[5]

Results and Discussion

Construction of an E. coli-Rhodococcus shuttle vector

Two plasmids were used for *Escherichia coli-Rhodococcus* shuttle vector construction. pRC4 is a cryptic plasmid in *Rhodococcus rhodochrous* IFO 3338.[15] pHSG298 is a vector plasmid for *E. coli* with a kanamycin resistance gene.[16] Isolated pRC4 was digested with *Cla*I, ligated with *Stu*I-digested pHSG298 and introduced by transformation into *E. coli* JM109. The 5.3-kb fusion plasmid obtained in this way was designed as pRHK1 (Fig. 2).

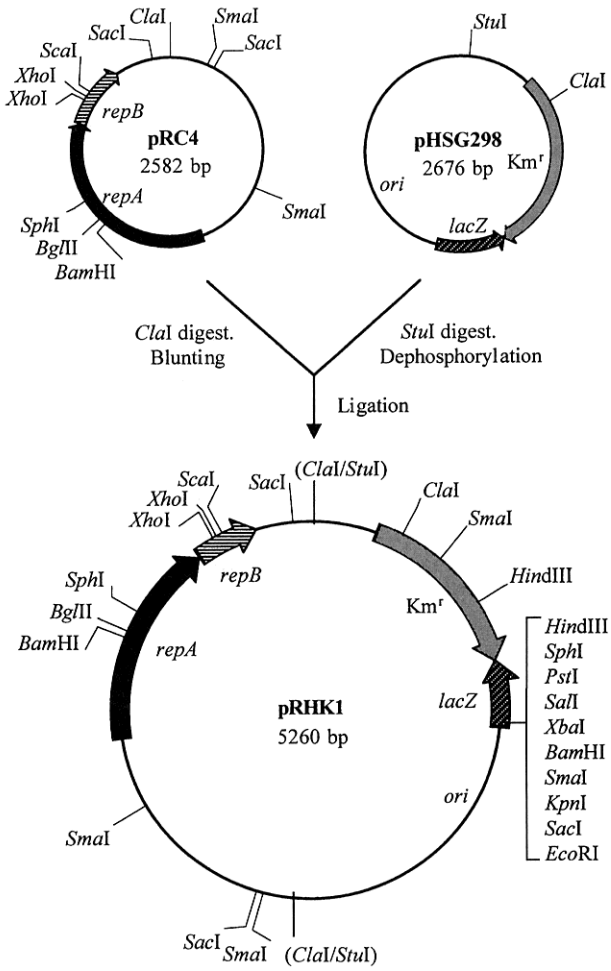

Fig. 2. Construction of *E. coli-Rhodococcus* Shuttle Vector pRHK1.

The following apply for all plasmids shown: Km^r, kanamycin resistance gene; *ori*, *E. coli* origin of replication; *lacZ*, 3′-truncated β-galactosidase gene. Parentheses indicate restriction sites removed due to ligation.

Transformation of Rhodococcus with plasmid pRHK1

The chimeric plasmid pRHK1 was examined for its ability to replicate in *R. erythropolis* KA2-5-1. The plasmid pRHK1 was put into KA2-5-1 cells by electroporation as described in Materials and Methods. Restriction analysis showed that the plasmids isolated from the kanamycin-resistant colonies were identical to pRHK1. The transformation frequency was 6×10^5 transformants per μg of plasmid DNA. The *E. coli-Rhodococcus* shuttle vector pRHK1 is more useful than pK4 constructed by Hashimoto *et al.*,[15] because pRHK1 has an intact *lacZ* gene and thus the color selection of recombinants is possible.

Replication genes in pRC4

The complete nucleotide sequence (2582 bp) of plasmid pRC4 was analyzed to locate the restriction sites and to facilitate construction of *dsz* recombinant plasmids. Based on the sequence, the replicon was identified. Two open reading frames were found in the same orientation with an overlap of 8 bp. Plasmid pAL5000 from *Mycobacterium fortuitum* and pFAJ2600 from *Rhodococcus erythropolis* NI86/21 have a 1-bp overlap between the two open reading frames, and pMB1 from *Bifidobacterium longum* has a 4-bp overlap.[17-19] These observations suggested their translational coupling. BLAST and FASTA homology searches showed that the deduced amino acid sequences of the two *orfs* were related to the products, Rep protein, of similarly organized genes in a number of cryptic plasmids from various bacteria (Fig. 3). These *orfs* were then designated as *repA* and *repB* for the replication genes (Fig. 2). The pRC4-encoded RepA showed the highest similarity (66.6%) to the putative theta replicase from pKA22,[20] and considerable similarities to the Rep proteins from ColE2-related plasmids, as noted by Hiraga *et al.* (Fig. 3(A)).[21] The pRC4-encoded RepB showed the highest level of similarity (53.3%) to RepB of pFAJ2600,[17] and considerable similarities to ORF4 of plasmid pXZ10142 from *Corynebacterium glutamicum* and ORF1 of pMB1 from *Bifidobacterium longum* (Fig. 3(B)).[19]

For pMB1, pAL5000, and pFAJ2600 it has been demonstrated that both *repA* and *repB* are required for replication.[17-19,22] Hashimoto *et al.* reported that transformation with chimeric plasmids obtained by combining pHSG299 and pRC4 via the *Bam*HI, *Sph*I, or *Xho*I sites of pRC4 failed to give kanamycin-resistant *Rhodococcus* colonies.[15] KA2-5-1 was not transformed with a plasmid disrupted within *Bgl*II in pRHK1 (data not shown). These observations suggested that both *repA* and *repB* of pRC4 are required for its replication (Fig. 2).

There are two sets of direct repeats in the upstream region of *repA*. In the upstream region of *repA* of pRC4 there is a sequence, 5′-AAACATCTGACTTGG-3′, similar to the conserved 15-bp sequence in the promoter region of the *repA* of pAL5000-related plasmids.[17] The structure of two sets of direct repeats and the conserved 15-bp sequence is similar to that of the minimum defined 435-bp *ori* region of pAL5000.[23] These observations suggested that the replicon of pRC4 belongs to that of pAL5000-related plasmids, and that pRC4 replicates by a theta-type mechanism.

Cloning of the desulfurization genes from KA2-5-1

The PCR fragment amplified with the primers was used as a probe to screen a library of KA2-5-1 genomic DNA in lambda DASH II by plaque hybridization. Two positive clones were obtained with the *dszABC* PCR fragment. Restriction analysis of the cloned DNA showed that two lambda clones contained the entire region of *dszABC*. All the restriction sites that were analyzed were the same as those of IGTS8 (data not shown).

The entire region of *dszD* encoding the flavin reductase was also cloned from a KA2-5-1 genomic library. The restriction site map of *dszD* of KA2-5-1 coincided with that of IGTS8.

Desulfurization of DBT by recombinant KA2-5-1

To examine the effects of amplification of the desulfurization genes in KA2-5-1 and to improve their biocatalytic activity, several recombinant plasmids were constructed as described in Materials and Methods (Fig. 4). *Rhodococcus erythropolis* KA2-5-1 was transformed by electroporation with pRKBE, pRKPP, pRKPPBB, or pRKPBP. As a control, KA2-5-1 was also transformed with the vector, pRHK1. Restriction enzyme analyses indicated that the plasmids isolated from these transformants were identical to the plasmids from *E. coli*.

The activity of DBT desulfurization by whole cells was examined for individual transformants. In reaction mixtures of all the transformants, HPBS and 2-HBP were detected by GC analysis after a 1-h reaction, but no other peaks of intermediates of DBT degradation were observed. These *dsz* recombinants showed higher activities than the control (Fig. 5). Especially, the DBT desulfurization activity of KA2-5-1 transformed with pRKPBP (KA2-5-1/pRKPBP) carrying two *dszABC* clusters and one *dszD* on the vector plasmid was about 4-fold higher than that of control KA2-5-1/pRHK1 that did not carry any *dsz* genes on the vector but carried only the original *dsz* genes in its genome.

Large amounts of HPBS were observed for KA2-5-1/pRKBE carrying only *dszD* on the vector. This can be explained by the stimulation by DszD of DszC and DszA proteins catalyzing the first three steps of the DBT desulfurization pathway (Fig. 1)[5] resulting in accumulation of HPBS. KA2-5-1/pRKPBP carrying two *dszABC* clusters and one

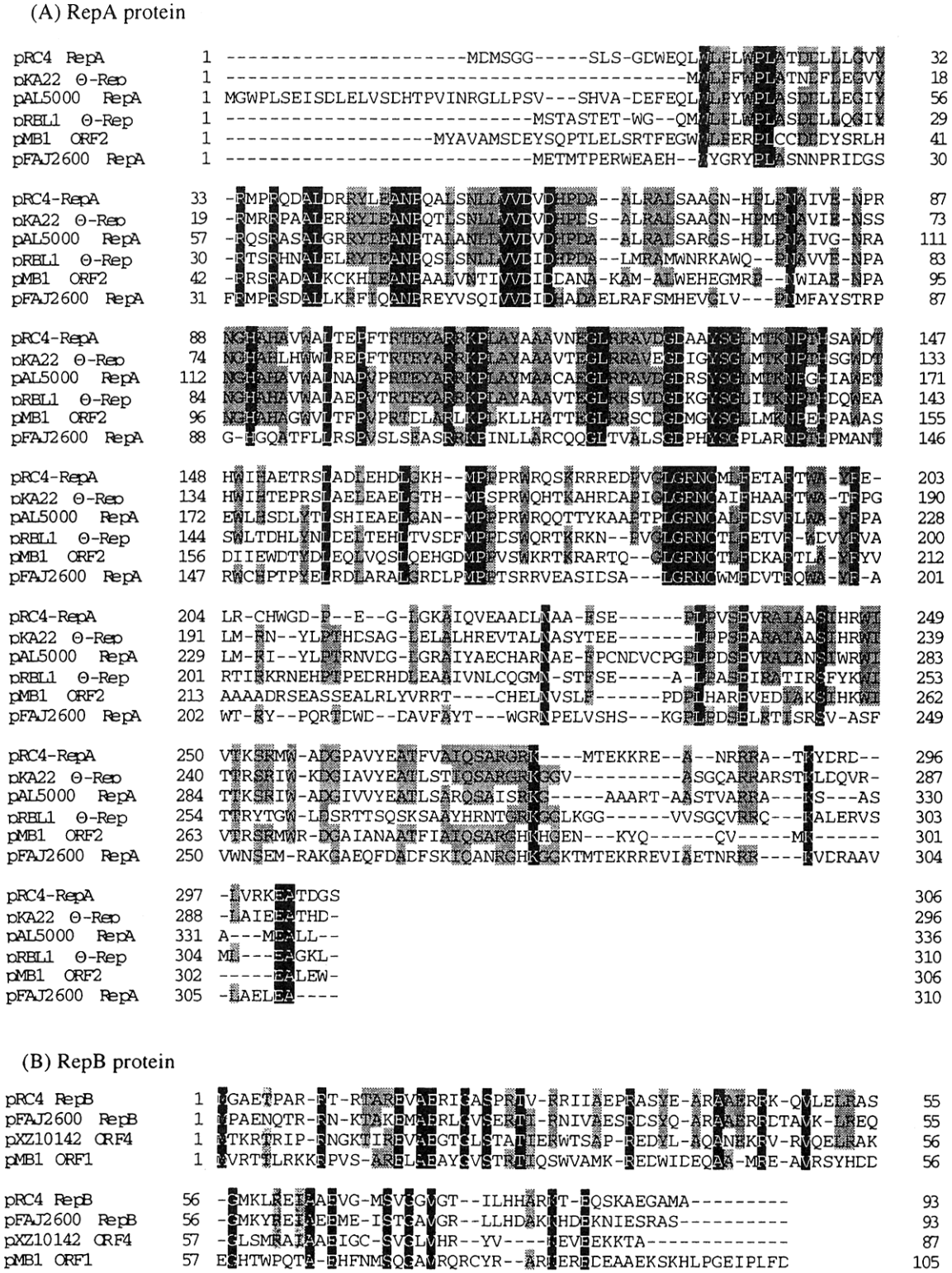

Fig. 3. Multiple Alignments of the Amino acid Sequence of Replication Proteins Encoded by Cryptic Plasmids from Various Bacteria.
Numbers indicate the positions of the residues in the complete amino acid sequence of the protein. (A) Comparison of pRC4-encoded RepA with pKA22-encoded theta-Rep from *Rhodococcus rhodochrous* NCIMB13064,[20] pAL5000-encoded RepA from *Mycobacterium fortuitum*,[18] pRBL1-encoded Rep from *Brevibacterium linens* (U39878-1), pMB1-encoded ORF2 from *Bifidobacterium longum* (X84655-1), and pFAJ2600-encoded RepA from *Rhodococcus erythropolis* NI86/21.[17] (B) Comparison of pRC4-encoded RepB with pFAJ2600-encoded RepB from *Rhodococcus erythropolis* NI86/21, pXZ10142-encoded ORF4 from *Corynebacterium glutamicum* (X72691-4), and pMB1-encoded ORF1 from *Bifidobacterium longum* (X84655-2).

dszD on the vector plasmid produced only a small amount of HPBS and a large amount of 2-HBP. It seems that well-balanced expression of *dszABC* and *dszD* is necessary for complete desulfurization of

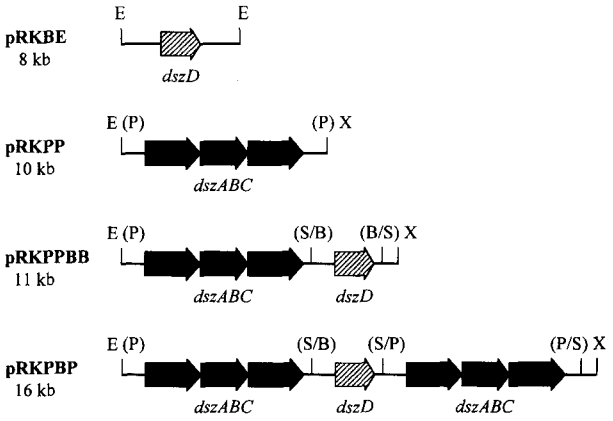

Fig. 4. Maps of DNA Fragments Ligated into the Multiple Cloning Site in *E. coli-Rhodococcus* Shuttle Plasmid pRHK1.

Total sizes of the recombinant plasmids are given under their names. Restriction sites removed by blunting and ligation are shown in parentheses. E, *Eco*RI; X, *Xba*I; P, *Pvu*I; S, *Sna*BI; B, *Bgl*I.

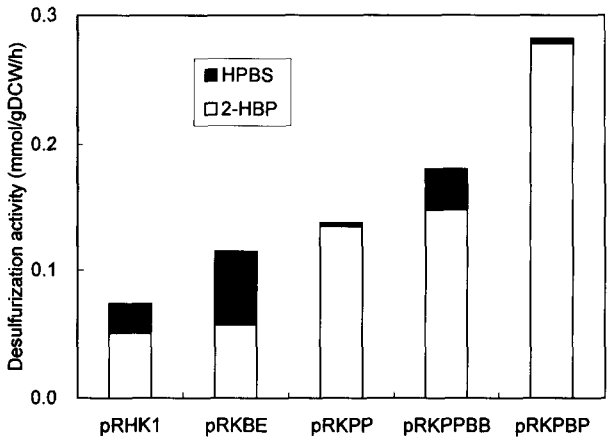

Fig. 5. DBT Desulfurization Activity of KA2-5-1 Transformants.

DBT desulfurization activities by whole cell reaction of KA2-5-1 transformed with pRHK1, pRKBE, pRKPP, pRKPPBB, or pRKPBP were measured as described in Materials and Methods.

DBT to 2-HBP.

A 26-kb recombinant plasmid containing four *dszABC* clusters and two *dszD* genes was also constructed. However, we obtained only KA2-5-1 transformants with plasmids of smaller (about half) sizes (data not shown). These results suggested that DNA deletion can occur readily in such a large plasmid with repeated sequences.

Desulfurization of LGO by recombinant KA2-5-1

We also examined the BDS activity for LGO. KA2-5-1/pRKPBP showing the highest desulfurization activity for DBT and KA2-5-1 as a control were cultured in medium A, and desulfurization reactions were done with LGO containing 390 ppm sulfur. KA2-5-1/pRKPBP showed a higher initial desulfurization rate than the control (Fig. 6). These results indicated that amplification of the desulfurization genes in *Rhodococcus erythropolis* KA2-5-1 can improve the ability to desulfurize LGO as well as DBT.

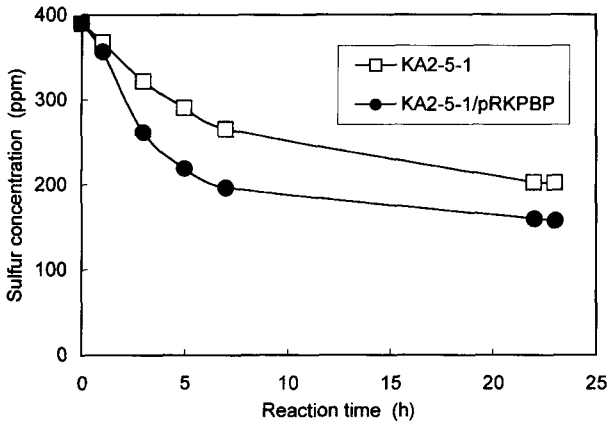

Fig. 6. Desulfurization of LGO by KA2-5-1 and its Recombinant.

Desulfurization reactions for LGO by KA2-5-1 and KA2-5-1/pRKPBP were done as described in Materials and Methods.

At early times in the reaction, the desulfurization rate was high, but decreased with time (Fig. 6). We repeated the reaction with fresh cell suspensions, but we failed to reduce the sulfur level to below approximately 100 ppm (data not shown). These results suggested that the LGO contained sulfur compounds refractory to desulfurization by the recombinant of KA2-5-1. Sulfur components in the LGO were analyzed with GC-AED. A variety of alkylated DBT derivatives including 4-methyl DBT, 4,6-dimethyl DBT, and 3,4,6-trimethyl DBT were detected in the LGO before the reaction, but no DBT were detected (Fig. 7(A)). After the 23-h whole cell reaction with KA2-5-1/pRKPBP, 4-methyl DBT, 4,6-dimethyl DBT, and 3,4,6-trimethyl DBT had almost disappeared (Fig. 7(B)). The LGO after a 23-h reaction with KA2-5-1 showed the same GC-AED pattern as with KA2-5-1/pRKPBP. The GC-AED pattern suggested that DBT derivatives with large alkyl groups remained in the LGO after the reaction with both the recombinant and the parent. It has been reported that KA2-5-1 cells can desulfurize monomethyl, dimethyl, monoethyl, trimethyl, and tetramethyl DBT but the desulfurization efficiencies decrease in that order.[4] It is speculated that the activities for alkylated DBT derivatives with more than C5 may be very low or missing. It is not clear whether the desulfurization enzymes have no activity for such sulfur compounds, or the microorganisms have no permeability for them, or both. We are now examining the activities for some kinds of DBT derivatives in cell-free or whole-cell reactions.

Repression of desulfurization activity by sulfate

High levels of inorganic sulfur in the medium were

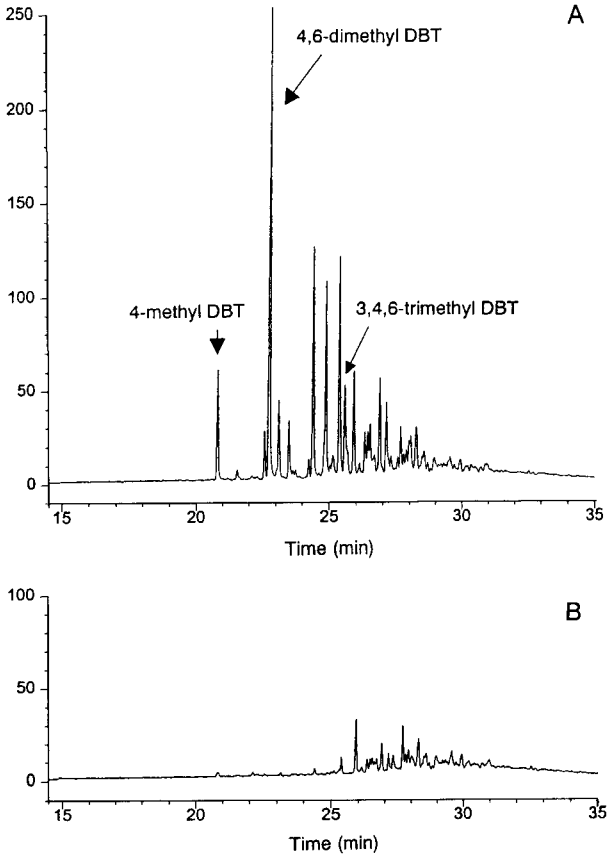

Fig. 7. GC-AED Sulfur Detection Chromatograms of LGO Before (A) and After (B) the 23-h Whole Cell Reaction by KA2-5-1/pRKPBP.

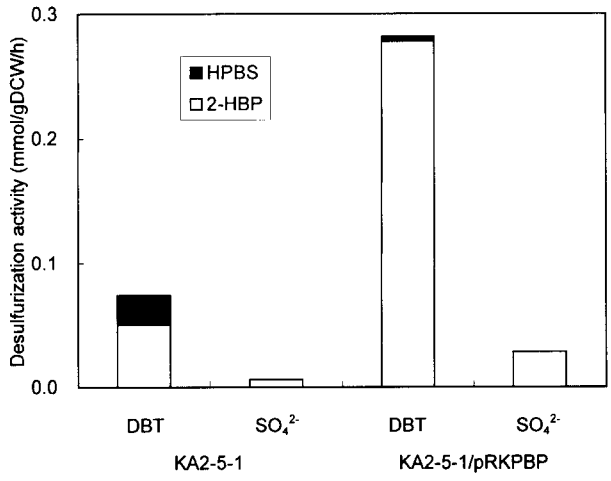

Fig. 8. Effects of Sulfur Sources in Culture Medium on DBT Desulfurization Activity of KA2-5-1 and its Recombinant.

KA2-5-1 and KA2-5-1/pRKPBP were grown in medium A with either 0.136 mM DBT or 0.136 mM Na_2SO_4, and DBT desulfurization activities were measured as described in Materials and Methods.

reported to repress the desulfurization activity of *Rhodococcus* sp. strain IGTS8.[2] Sulfur regulation in KA2-5-1 and its recombinant were examined. Strain KA2-5-1 and KA2-5-1/pRKPBP were grown in medium A with either 0.136 mM DBT or 0.136 mM Na_2SO_4, and DBT desulfurization activities were measured. The results showed that inorganic sulfur severely repressed the desulfurization activity in both the parent strain and the recombinant (Fig. 8), which suggested that the *dsz* genes on the vector plasmid were expressed under the control of its native promoter and associated regulatory regions. We are currently planning experiments to screen for alternative promoters that cannot be affected by levels of inorganic sulfur but can produce higher levels of constitutive expression of the desulfurization genes.

Acknowledgments

We thank the staff of the Bio-Refining Process Laboratory for useful discussions. This work was supported by the Ministry of International Trade and Industry, Japan.

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