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Genetic Rearrangement Strategy for Optimizing the Dibenzothiophene Biodesulfurization Pathway in *Rhodococcus erythropolis*▽

Guo-qiang Li,[1] Shan-shan Li,[2] Ming-lu Zhang,[2] Jun Wang,[2] Lin Zhu,[1] Feng-lai Liang,[2] Ru-lin Liu,[2] and Ting Ma[2]*

College of Environment Sciences and Engineering[1] and Tianjin Key Laboratory for Microbial Functional Genomics, College of Life Sciences,[2] Nankai University, Tianjin 300071, China

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Dibenzothiophene (DBT) and its derivatives can be microbially desulfurized by enzymes DszC, DszA, and DszB, which are encoded by the operon *dszABC* and contribute to the conversion in tandem. We investigated the expression characteristics of the *dsz* operon. Our results revealed that the levels of transcription and translation of *dszA*, *dszB*, and *dszC* decreased according to the positions of the genes in the *dsz* operon. Furthermore, the translation of *dszB* was repressed by an overlapping structure in the *dsz* operon. In order to get better and steady expression of the Dsz enzymes and optimize the metabolic flux of DBT, we rearranged the *dsz* operon according to the catalytic capabilities of the Dsz enzymes and expressed the rearranged *dsz* operon, *dszBCA*, in *Rhodococcus erythropolis*. After rearrangement, the ratio of *dszA*, *dszB*, and *dszC* mRNAs in the cells was changed, from 11:3.3:1 to 1:16:5. Western blot analysis revealed that the levels of expression of *dszB* and *dszC* had been enhanced but that the expression of *dszA* had decreased. The desulfurization activity of resting cells prepared from *R. erythropolis* DRB, which carried the rearranged *dsz* operon, was about 12-fold higher than that of resting cells of *R. erythropolis* DRA, which carried the original operon in a similarly constructed vector.

The combustion of sulfur-containing fossil fuels contributes to environmental pollution. Steady increases in the average sulfur content of petroleum and stricter environmental regulations concerning the sulfur content and CO_2 emissions have promoted studies of biodesulfurization (BDS) to upgrade fossil fuels. BDS can potentially provide a solution to the need for improved and expanded fuel-upgrading processes worldwide, since BDS does not require hydrogen and produces far less CO_2 than the traditional thermochemical processes (7). Dibenzothiophene (DBT) and its derivatives constitute a broad range of sulfur heterocyclic compounds found in petroleum that are recalcitrant to desulfurization via the traditionally applied hydrodesulfurization method but can easily be desulfurized via BDS (5).

Most of the reported strains can remove sulfur from DBT and its derivatives in a sulfur-specific manner without affecting the carbon skeleton by following the 4S pathway (3, 5, 7, 11). The 4S pathway proceeds via two cytoplasmic monooxygenases (DszC and DszA) and a desulfinase (DszB), which are encoded by an operon (*dszABC*). The pathway is supported by flavin reductase (DszD). DBT monooxygenase (DszC) catalyzes the sequential conversion of DBT into DBT sulfoxide and DBT sulfone. DBT sulfone monooxygenase (DszA) catalyzes the transformation of DBT sulfone into DBT sulfinate with a reaction rate 5- to 10-fold higher than that of the conversion catalyzed by DszC. DszB, an aromatic sulfinic acid hydrolase, effects a nucleophilic attack of a base-activated water molecule on the sulfinate sulfur to form 2-hydroxybiphenyl (2-HBP), with a reaction rate about 20% of that of the reaction catalyzed by DszC (13, 4).

The main goal of BDS research is to develop a commercial process for petroleum desulfurization, and it has been estimated that a successful commercial process would require a biocatalyst with a desulfurization activity of 1.2 to 3 mmol of DBT/g (dry weight) of cells/h (7). However, the desulfurization activity of naturally occurring bacterial cultures is low in comparison to these estimated requirements. In order to achieve higher desulfurization rates, genetic manipulations have been used to increase the levels of expression of the *dsz* genes. These approaches include measures such as supplying multiple copies of the *dsz* genes in *Rhodococcus erythropolis* KA 2-5-1 (6, 8), placing the genes under the control of alternative promoters (2, 12, 14), enhancing the level of expression of *dszB* by mutating its 5′ untranslated region, and removing the gene overlap regions in the *dsz* operon (9). The desulfurization rate can also be increased using directed-evolution methods such as DNA shuffling and gene rearrangement. The first approach was applied to alter the *dszC* gene by a new in vitro DNA recombination method called random chimeragenesis on transient templates (1), by which both the rate and the substrate utilization extent of catalysis by DszC were improved. However, all the efforts discussed above have achieved a maximum metabolic flow of only about 250 μmol of DBT/g (dry weight) of cells/h, which is still low in comparison to the requirements of a commercial process.

The rate of an enzyme catalytic reaction is determined by the catalytic activity and the quantity of the enzyme and the substrate concentration. In prokaryotes, several functionally related genes are often clustered and transcribed into polycistronic mRNAs with different lengths, and the transcription will potentially terminate at any termination codons or secondary

* Corresponding author. Mailing address: College of Life Sciences, Nankai University, Tianjin 300071, China. Phone: 86-022-23505967. Fax: 86-022-23508800. E-mail: meor@nankai.edu.cn.
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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
R. erythropolis 4.1491	Wild type; *dsz* null	CGMCC
R. erythropolis DS-3	Wild type; dsz^+ Cm^r	16
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- r_B^-)$ *gal dcm* (DE3)	Novagen
E. coli DH5α	*hsdR17*($r_K^- m_K^+$) *recA1 endA1* (*lac-proAB*) *gyrA96 thi-1 relA1 supE44* [F′ *traD36 proAB lacI*^{q}*ZΔM15*]	Takara
Plasmids		
pMD 18-T simple vector	Clone vector carrying *lac* promoter and f1 *ori*; Amp^r	Takara
pRHK1	Shuttle vector carrying arabinose promoter and p15A *ori*; Kan^r	6
pRABC	Carries *dsz* operon (*dszABC*) from *R. erythropolis* DS-3 on pRHK1; Kan^r	This study
pRBCA	Carries reconstructed *dsz* operon (*dszBCA*) from *dsz* operon on pRHK1; Kan^r	This study

structures in the polycistronic mRNAs that are unfavorable to transcription. Therefore, the levels of transcription of these genes usually decrease with increasing distance from the regulatory elements. This phenomenon is known as polar transcription. Gene expression is controlled first and foremost at the level of transcription for the simultaneous transcription and translation and the very short half-life of the mRNA in prokaryotes. Higher levels of mRNA are the precondition for higher levels of the encoded protein. Therefore, rearranging these genes according to the catalytic capabilities of the enzymes and their reaction orders could not only balance the catalytic capabilities but also increase the substrate concentrations for the enzymes. In this paper, we introduce a genetic rearrangement strategy for optimizing the metabolic pathway of DBT. By using recombinant PCR, the *dsz* operon of *R. erythropolis* DS-3 was rearranged according to the catalytic capabilities of the Dsz enzymes and their reaction orders in the 4S pathway. The rearranged *dsz* operon can also be recombined into its native position by a double crossover in any subsequent work.

MATERIALS AND METHODS

Chemicals. DBT, 2-hydroxybiphenyl-2-sulfinic acid (HBPS), and 2-HBP were purchased from Acoros (NJ). All other reagents were of analytical grade and were obtained commercially.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *R. erythropolis* DS-3, a DBT- and DBT derivative-desulfurizing strain isolated from soil (16) that carries the same *dsz* operon as *Rhodococcus erythropolis* IGTS8, was used throughout the study. *R. erythropolis* CGMCC 4.1491 (*dsz* null), which normally is not able to desulfurize DBT, was purchased from the China General Microbiological Culture Collection Center. *Escherichia coli* strains were cultured in Luria-Bertani medium. Antibiotics were added in order to select plasmids, as follows: ampicillin, 100 μg/ml, and kanamycin, 34 μg/ml.

Rearrangement of the *dsz* operon by overlap PCR. The rearranged *dsz* operon was created by three steps of PCR amplification (Fig. 1). In the first round, fragments with overlapping regions, the 400-bp 5′ upstream segment and the 400-bp 3′ downstream segment of *dszABC* and the *dszA* and *dszBC* segments, were produced by PCR with primers U1 and U2, CA and A22, UB and AC, and AD and D22 (Table 2). Each reaction mixture contained 15 pmol of each deoxynucleoside triphosphate, 25 μl of GC buffer (Takara, Otsu, Shiga, Japan), 20 pmol of each primer, 2.5 U of PrimeSTAR hot-start DNA polymerase, and 15 ng of the *R. erythropolis* DS-3 genome. A hot start was initiated with 5 min of denaturation at 95°C, followed by 30 amplification cycles (95°C for 30 s, 56°C for 45 s, and 72°C for 3 min). PCR products from each reaction were gel purified with an agarose gel DNA purification kit (Takara, Otsu, Shiga, Japan) and used in subsequent amplifications. The second-round PCR mixture was the same as that for the first round, except that 10 ng of each segment of the 5′ upstream and *dszBC* segments and 10 ng of the *dszA* and 3′ downstream segments from the first-round PCR, together with 20 pmol of primers U1 and AC or CA and D22, were included. PCR conditions were the same as those for the first round, except that the extension time was 4 min at 72°C. The third-round PCR mixture was the same as that for the first round, except that 10 ng of the ligated 5′ upstream-*dszBC* segment and 10 ng of the ligated *dszA*-3′ downstream segment from the second-round PCR, together with 20 pmol of primers U1 and D22, were added. PCR conditions were the same as those for the first round, except that the extension time was 5 min at 72°C. The native *dsz* operon was also created by PCR amplification with primers U1 and D22. The PCR products were ligated into pMD 18-T simple vector for sequence analysis.

Transformation of *Rhodococcus* by electroporation. To obtain electrocompetent cells of *R. erythropolis* 4.1491 (*dsz* null), 100 ml of basal salt medium (BSM) supplemented with 2.5% (wt/vol) glucose and 0.5% yeast extract in a 250-ml baffled Erlenmeyer flask was inoculated with 4 ml of precultured broth and the culture was grown at 30°C to an optical density of 0.6 at 600 nm. The cells were harvested, washed twice with 0.3 mol of ice-cold sucrose/liter, and concentrated 40-fold in 0.5 mol of ice-cold sucrose/liter. Competent cells were either used directly for electroporation or stored at −70°C. The native *dsz* operon and the rearranged *dsz* operon fragment were ligated into the EcoRI and XbaI sites of pRHK1 to yield pRABC and pRBCA. Plasmids were introduced into *R. erythropolis* 4.1491 (*dsz* null) by electroporation using a Micropulser electroporator (Bio-Rad, CA) as described previously (6) to yield *R. erythropolis* DRA and *R. erythropolis* DRB.

Quantitative analysis of transcription of the natural and rearranged *dsz* operons by real-time PCR. The total RNA of *R. erythropolis* DS-3, *R. erythropolis* DRA, and *R. erythropolis* DRB was prepared with a UNIQ-10 spin column total RNA isolation kit (Sangon, Shanghai, China). RNA samples were treated with DNase I (Takara, Otsu, Shiga, Japan) to eliminate any genomic DNA contamination. First-strand cDNA was synthesized from *R. erythropolis* DS-3, *R. erythropolis* DRA, or *R. erythropolis* DRB total RNA by using SuperScriptII RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA) with primers A1 and A2, B1 and B2, and C1 and C2 for 30 min at 42°C in an incubation mixture containing 1 mg of total RNA, 10 pmol of primers, 200 U of SuperScriptII RNase H^- reverse transcriptase, 1× reverse transcriptase buffer, and 10 mmol of each deoxynucleoside triphosphate. The quality of single-stranded cDNA was monitored by the reverse transcriptase PCR method, as described by Michalski and Weil (10). Real-time PCRs were carried out with an Opticon2 continuous fluorescence detection system (MJ Research, Waltham, MA) with primers A1 and A2, B1 and B2, and C1 and C2 and SYBR green I master mix (PE Biosystems, Foster City, CA). The PCR conditions were 5 min of denaturation at 95°C, followed by 40 amplification cycles (95°C for 30 s, 52°C for 30 s, and 72°C for 1 min). Fluorescence was detected at the end of every 72°C extension phase. cDNA primers were designed from *dsz* gene sequences from DS-3 with Primer Express software (version 1.0; PE Applied Biosystems, Foster City, CA). All primer sets had a calculated annealing temperature of ≥58°C (by the nearest-neighbor method). The primer sequences for internal controls are shown in Table 2.

Western blot analysis. Western blot analysis was done as described previously (15). Antibodies against DszA, DszB, and DszC of *R. erythropolis* DS-3 were raised in rabbits by subcutaneous injection of purified DszA, DszB, and DszC, respectively. The source of DszA, DszB, and DszC was recombinant *E. coli* BL21, which carries the recombinant plasmid pET 28a-*dszA*, pET 28a-*dszB*, or pET 28a-*dszC*. This work was done by Jingmei Biotechnology, Tianjin, China.

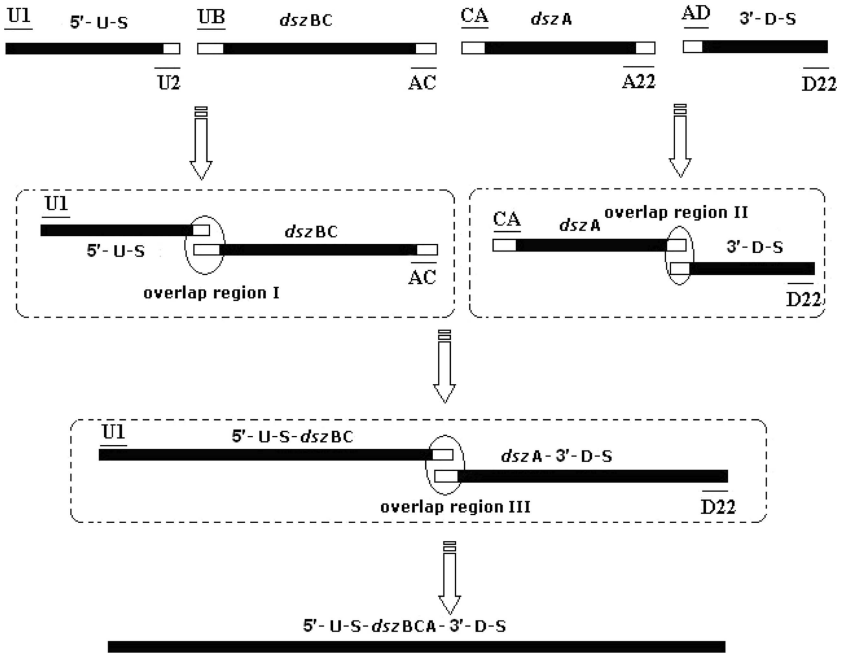

FIG. 1. Rearrangement of the *dsz* operon by overlap PCR. Fragments of the 400-bp 5′ upstream segment (5′-U-S) and the 400-bp 3′ downstream segment (3′-D-S) of *dszABC* and the *dszA* and *dszBC* segments, including the overlap regions, were yielded by PCR, then the ligated 5′ upstream-*dszBC* segment (5′-U-S-*dsz*BC) and the ligated *dszA*-3′ downstream segment (*dsz*A-3′-D-S) were produced by overlap PCR via their overlap regions, and finally, the 5′ upstream-*dszBC* segment and the *dszA*-3′ downstream segment were linked together by overlap PCR via their overlap region to yield the reconstructed *dsz* operon. Black bars represent genes, and white bars represent overlap regions.

Desulfurization by resting cells. Recombinant *R. erythropolis* strains were cultured in BSM with shaking for 48 h with DBT as the sole sulfur source. The resting-cell suspensions were prepared according to the method described previously (17). Samples of 25 ml of the suspension and an equal volume of *n*-hexadecane containing 0.5 mmol of DBT/liter were transferred into 250-ml flasks with baffles. Resting-cell reactions were performed with shaking (160 rpm) at 30°C.

Substrate and product analysis. Before analysis, DBT and intermediates in the aqueous phase were extracted with a volume of ethyl acetate equal to that of

TABLE 2. Specific primers used in this study

Primer	Gene	Sequence
Specific PCR primers used for overlap PCR		
U1	5′ upstream segment	5′-GACGAATTCTAACTGACTAGACGTGTGTGGTG-3′
U2	5′ upstream segment	5′-TCCTAAATGTCAATCATGGCTAGA-3′
CA	*dszA*	5′-CCCGGCTTCACCTCCTAGGAACATCCGCATGACT CAACAACGACAAATGC-3′
A22	*dszA*	5′-CCTCATGAAGGTTGTCCTTGCA-3′
UB	*dszBC*	5′-TCTAGCCATGATTGACATTTAGGAACATCCTCAT GACAAGCCGCGTCGAC-3′
AC	*dszBC*	5′-GCATTTGTCGTTGTTGAGTCATGCGGATGTTCCT AGGAGGTGAAGCCGGG-3′
AD	3′ downstream segment	5′-TGCAAGGACAACCTTCATGAGGATCTGAGGCGCT GATCG-3′
D22	3′ downstream segment	5′-GGTACCCTCTAGAGCACTGGATCCACTTG-3′
Specific PCR primers used for quantitative real-time PCR		
A1	*dszA*	5′-GCGCGGCAAGTTCGATCTGT-3′
A2	*dszA*	5′-TCCCGCAGGATGTCCTTGATC-3′
B1	*dszB*	5′-GCAGGGCACGGTTCATTTCAC-3′
B2	*dszB*	5′-ACGGGTTCCTGCAGCAAGTTG-3′
C1	*dszC*	5′-TCGGCTCGCAGGAACAAGAAG-3′
C2	*dszC*	5′-GTCGGCGATCTTGTAGGACACC-3′
N1	NADH gene	5′-TGGGCGGATTGCTCGAAGGTA-3′
N2	NADH gene	5′-GCGCCACCGTCGACGTAGATG-3′

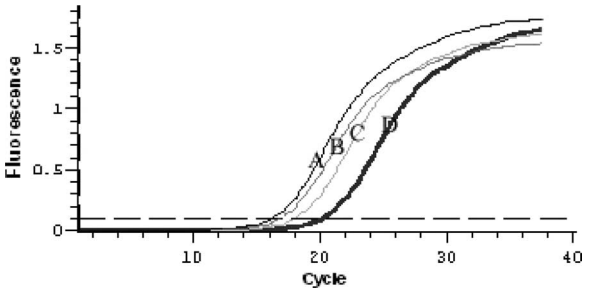

FIG. 2. Plot depicting the amplification of the native *dsz* operon. The plot shows the accumulation of *dszA*, *dszB*, and *dszC* PCR products in real-time PCR as detected by the MJ Opticon2 continuous fluorescence detection system. *dszA*, *dszB*, and *dszC* cDNAs were used as the template. The threshold line for the calculation of the C_T was set at 0.1. A, *dszA*; B, internal control gene (NADH gene); C, *dszB*; D, *dszC*.

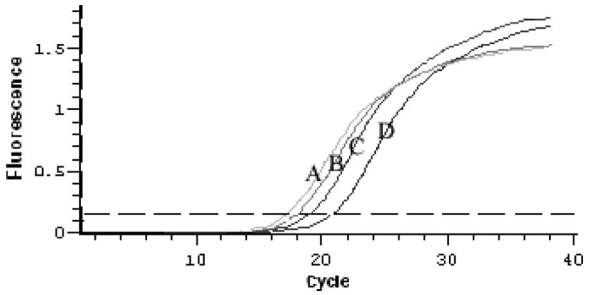

FIG. 3. Plot depicting the amplification of the reconstructed *dsz* operon. The plot shows the accumulation of *dszA*, *dszB*, and *dszC* PCR products in real-time PCR as detected by the MJ Opticon2 continuous fluorescence detection system. *dszA*, *dszB*, and *dszC* cDNAs were used as the template. The threshold line for the calculation of the C_T was set at 0.15. A, *dszB*; B, internal control gene (NADH gene); C, *dszC*; D, *dszA*.

the extracts. Products in resting-cell reaction systems were centrifuged (8,000 × *g*; 10 min), and the oil-phase supernatants were collected for analysis. DBT, HPBS, and 2-HBP were analyzed by high-performance liquid chromatography (HPLC) according to the method described previously (18).

RESULTS

Transcriptional characteristics of the native *dsz* operon. We investigated the transcriptional characteristics of the native *dsz* operon by analyzing the quantities of mRNA from the transcription of *dszA*, *dszB*, and *dszC* in cells of *R. erythropolis* DS-3 by a real-time quantitative PCR assay. The analysis fragment of each gene was about 800 bp. The amplification efficiencies of the primers used in real-time quantitative PCR were detected with the *dsz* operon by real-time PCR assays. The results indicated that each pair of primers had equivalent amplification efficiencies. The mRNA quantitative analysis revealed that the ratio of *dszA*, *dszB*, and *dszC* mRNAs was about 11:3.3:1, based on the threshold cycle (C_T) values of each gene (Fig. 2). The results indicated that the transcription levels of the desulfurization enzyme genes decreased according to the positions of the genes in the *dsz* operon. This finding indicated that we could change the transcriptional levels of the *dsz* genes by changing their positions in the *dsz* operon.

Rearrangement of the *dsz* operon. The rate of desulfurization is limited by the last enzyme in the 4S pathway, DszB, due to its low expression level and low level of activity. In our previous work, we enhanced the level of expression of DszB and increased the DBT metabolic velocity by reconstructing the ribosome binding site of *dszB* (9). However, the DBT metabolic rate still did not reach the optimal rate because the activity of DszA was not maximal due to low substrate concentrations. In order to enhance the substrate concentration for DszA, the catalytic capabilities of DszB and DszC must be increased simultaneously. An alternative method to accomplish the same goal is increasing the specific production of DszB and DszC in the cell. In the present work, we rearranged the order of the *dsz* genes in the operon to generate operon *dszBCA*. We also reconstructed the ribosome binding site of *dszB* by replacing it with the 13-bp string of nucleotides that was located between *dszB* and *dszC*. The reconstructed *dsz* operon was generated by overlap PCR, as described in Materials and Methods. To ensure the absence of insertions or mutations, any fragment generated by PCR was ligated into the pMD 18-T simple vector for sequence analysis.

Expression characteristics of the *dsz* operon. The *Rhodococcus-E. coli* shuttle vector was used to express the reconstructed *dsz* and native *dsz* operons in *R. erythropolis* CGMCC 4.1491 (*dsz* null). The native and reconstructed *dsz* operons were inserted separately into the EcoRI and XbaI sites of the pRHK1 vector to construct expression plasmids pRABC and pRBCA, respectively. These plasmids were then introduced into *R. erythropolis* 4.1491 by electroporation to yield *R. erythropolis* DRA and *R. erythropolis* DRB, respectively. A real-time quantitative PCR assay indicated that the quantities of *dszA*, *dszB*, and *dszC* mRNAs in *R. erythropolis* DRA were not greatly increased compared to the quantities detected in *R. erythropolis* DS-3. However, the quantities of *dszB* and *dszC* mRNAs in *R. erythropolis* DRB were considerably higher than those in DS-3, and the quantity of *dszA* mRNA decreased; the ratio of these mRNAs was about 1:16:5 (*dszA* mRNA to *dszB* mRNA to *dszC* mRNA) (Fig. 3). Western blot analysis revealed that *R. erythropolis* DRB produced more DszB and DszC than did *R. erythropolis* DRA and *R. erythropolis* DS-3, whereas the production of DszA in *R. erythropolis* DRB decreased sharply compared to that in *R. erythropolis* DRA and *R. erythropolis* DS-3 (Fig. 4).

Desulfurization by resting cells of recombinant strains. To test the desulfurization activities of recombinant strains *R. erythropolis* DRA and *R. erythropolis* DRB, resting cells (25 ml) were placed into a 250-ml Erlenmeyer flask, together with an equal volume of *n*-hexadecane containing 0.5 mmol of DBT/liter. The mixture was incubated at 30°C with shaking at 160 rpm. At different intervals, sample aliquots (1 ml) were withdrawn and quenched in an Eppendorf tube with 6 mol of HCl/liter, and the oil phase was separated by centrifugation. When the oil-phase samples were examined by HPLC, no intermediate products were detected, only DBT and 2-HBP. However, the intermediate product HPBS was detected in cell lysates of *R. erythropolis* DRA and *R. erythropolis* DS-3 and not in cell lysates of *R. erythropolis* DRB. The time courses for 2-HBP production in the resting-cell reaction systems catalyzed by the two recombinant strains, *R. erythropolis* DRA and *R. erythropolis* DRB, are shown in Fig. 5. As a result of the reactions, 96% of DBT (0.48 mmol of DBT/liter) was desul-

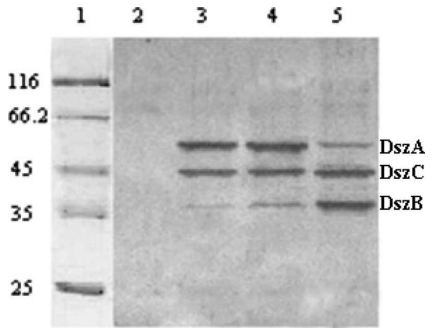

FIG. 4. Western blot analysis of Dsz enzymes in cells grown with antibody specific to DszA, DszB, and DszC. Lanes: 1, marker; 2, *R. erythropolis* 4.1491 (*dsz* null); 3, *R. erythropolis* DS-3 (*dsz*$^+$); 4, *R. erythropolis* DRA; and 5, *R. erythropolis* DRB. *R. erythropolis* DS-3, *R. erythropolis* DRA, and *R. erythropolis* DRB were cultured in BSM with DBT as the sole source of sulfur, and the control strain *R. erythropolis* 4.1491 (*dsz* null) was cultured in BSM with yeast extract as the sulfur source.

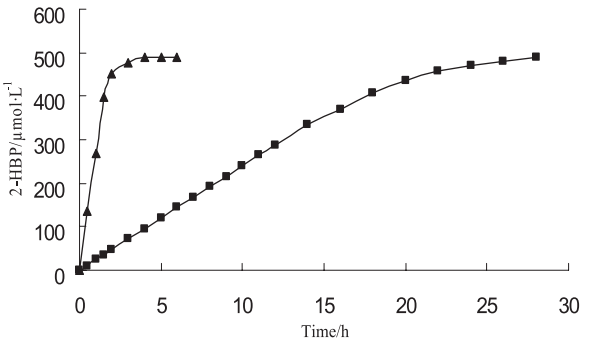

FIG. 5. Time course of the conversion of DBT into 2-HBP by resting cells of *R. erythropolis* DRA and DRB. Cells were grown to the end of the exponential phase in BSM with DBT as the sole source of sulfur, washed twice with 1.0 liter of 50-mmol/liter potassium phosphate buffer (pH 7.2), and finally resuspended in the same buffer. The suspension was portioned into 25-ml aliquots, and an equal volume of *n*-hexadecane containing 0.5 mmol of DBT/liter was added. At each time point, aliquots were withdrawn for analysis by HPLC, as described in Materials and Methods. ▲, *R. erythropolis* DRB; ■, *R. erythropolis* DRA.

furized after 3 h, and the maximum desulfurization rate was 320 μmol of DBT/g (dry weight) of cells/h. The same amount of DBT was desulfurized after 24 h by *R. erythropolis* DRA resting cells, and the desulfurization rate in this case was only about 26 μmol of DBT/g (dry weight) of cells/h. Thus, the maximum resting-cell desulfurization rate of *R. erythropolis* DRB was about 12-fold higher than that of *R. erythropolis* DRA.

DISCUSSION

The microbial conversion of DBT into 2-HBP is accomplished by the 4S pathway, consisting of two monooxygenases (DszC and DszA) and one desulfinase (DszB), which are encoded by the *dsz* operon. Sequence and subcloning analyses revealed that the three genes, *dszA*, *dszB*, and *dszC*, were transcribed and expressed in the same orientation. The termination codon for *dszA* and the initiation codon for *dszB* overlap, and there is a 13-bp gap between *dszB* and *dszC*. Potential ribosome binding sites are also present upstream of each putative ATG initiation codon (13). We investigated the expression characteristics of the *dsz* operon in the present work. The *dsz* genes (*dszA*, *dszB*, and *dszC*) were transcribed as a polar model, and the ratio of *dszA*, *dszB*, and *dszC* mRNAs was about 11:3.3:1 in *R. erythropolis* DS-3, which carries the original *dsz* operon. Western blot analysis revealed that the translational levels of DszA, DszB, and DszC appeared to be controlled by the quantities of corresponding mRNAs (Fig. 4, lane 5). However, the expression level of *dszB* was far lower than that of *dszC* (Fig. 4, lanes 3 and 4). In our prior work, we have shown that the expression level of *dszB* is determined by two factors (9). First, due to the location of *dszB* in the *dsz* operon, the quantity of *dszB* mRNA is only about 30% of that of *dszA* mRNA because of the polar transcription of the *dsz* operon. Second, there is an overlapping region in the termination codon for *dszA* and the initiation codon for *dszB*. The expression level of *dszB* is lower than that of *dszC* for this reason, even though the quantity of *dszC* mRNA is lower than that of *dszB* mRNA (Fig. 2). This result indicates that there is, in fact, a baffle structure before the initiation codon of *dszB*.

Gray et al. did a kinetic analysis of the DBT desulfurization reactions in the 4S pathway. Their results showed that the ratio of the activities of the *dsz* enzymes DszA, DszB, and DszC is about 25:1:5 (4). Under conditions in which *dszB* expression is not baffled by the overlapping structure, the ratio of the catalytic capabilities of the *dsz* enzymes is about 5:275:3.3, according to an analysis of the quantities of mRNAs and the activities of the *dsz* enzymes. To enhance the DBT metabolic flux, the intrinsic catalytic properties or the specific production of DszB and DszC in the cell must be improved. Here, we have reported a gene-rearranging strategy that enhanced the expression levels of *dszB* and *dszC*. Real-time PCR analysis and Western blot analysis with specific antibodies for the *dsz* enzymes (Fig. 3 and 4) confirmed that the recombinant strain exhibited higher levels of transcription of *dszB* and *dszC* than *R. erythropolis* DS-3 and *R. erythropolis* DRA. The levels of translation of *dszB* and *dszC* were also enhanced.

Gray et al. proved that HBPS accumulates in the reaction system catalyzed by cell extracts of *R. erythropolis* IGTS8 (4), which carries the same *dsz* operon as *R. erythropolis* DS-3. However, we did not detect any intermediates except for DBT and 2-HBP in the cell lysate of *R. erythropolis* DRB, whereas HBPS was detected in the cell lysates of *R. erythropolis* DRA and *R. erythropolis* DS-3. These results indicate that there are not any intermediates accumulated in the cells of *R. erythropolis* DRB, which carries the rearranged *dsz* operon. The desulfurization experiment was conducted using resting cells with 0.5 mmol of DBT/liter in *n*-hexadecane as a model diesel oil. The DBT desulfurization rate of *R. erythropolis* DRB was about 12-fold higher than that of *R. erythropolis* DRA, which carries the native *dsz* operon. This finding indicates that the enhanced expression levels of DszC and DszB increase the metabolic rate for DBT and HBPS in the cells and contribute to the improved desulfurization rate of *R. erythropolis* DRB.

Function-related genes are often arranged in tandem in an operon and transcribed in polar models in prokaryotes. The Dsz enzymes have different catalytic activities. Therefore, the rearrangement of the corresponding genes according to the

catalytic capabilities of the enzymes can balance these catalytic abilities and breach the metabolic bottleneck. Furthermore, the rearranged *dsz* operon could easily be recombined into its native organization by a double-crossover event to avoid losing the exogenous gene in the process of subculture.

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