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Isolation and Characterization of Benzene-Tolerant *Rhodococcus opacus* Strains

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Twenty-two benzene-utilizing bacteria were isolated from soil samples. Among them, three isolates were highly tolerant to benzene. They grew on benzene when liquid benzene was added to the basal salt medium at 10-90% (v/v). Taxonomical analysis identified the benzene-tolerant isolates as *Rhodococcus opacus*. One of the benzene-tolerant isolates, designated B-4, could utilize many aromatic and aliphatic hydrocarbons including benzene, toluene, styrene, xylene, ethylbenzene, propylbenzene, *n*-octane and *n*-decane as sole sources of carbon and energy. Strain B-4 grew well in the presence of 10% (v/v) organic solvents that it was capable of using as growth substrates. Genetic analysis revealed the benzene dioxygenase pathway is involved in benzene catabolism in strain B-4. A deletion-insertion mutant defective in the benzene dioxygenase large and small subunits genes (*bnzA1* and *bnzA2*) was as tolerant to organic solvents as the wild-type strain B-4, suggesting that utilization or degradation of organic solvents is not essential for the organic solvent tolerance of *R. opacus* B-4.

[**Key words:** solvent-tolerant bacterium, *Rhodococcus opacus*, benzene-oxidizing bacterium, two-phase system, benzene dioxygenase gene mutation]

Biotransformation offers an environmentally compatible and efficient route for the production of industrially important chemicals. Selective catalysis is becoming a requirement for the chemical industry, and it is expected that the biotransformation process will be extended to the synthesis of not only specialty chemicals and polymers, but also bulk chemicals (1). Interesting conversions include the reduction of prochiral ketones, the oxidation of alcohol and the hydroxylation of nonactivated carbon atoms (2). Since cofactors and their regeneration are required for oxidation and reduction reactions, whole cells are favored as biocatalysts. In the chemical industry, especially in the petrochemical industry, substrates of chemical syntheses are often apolar compounds such as petroleum hydrocarbons and their derivatives. These substrates and reaction products are highly toxic to the biocatalysts and the toxicity has limited the application of biotransformation in the chemical industry. A possible solution to this problem is the use of solvent-tolerant bacteria as host strains for biocatalysts.

We are investigating the bioconversion of benzene to phenol, because phenol is one of the most important commodity chemicals in industry: world production exceeded 6.6 megatons in 2000. The toxicity of a solvent correlates with its hydrophobicity as expressed by the logarithm of the partition coefficient of the compound in a mixture of 1-octanol and water (log P_{ow}) (3). Organic solvents with log P_{ow} values between 1 and 5 are highly toxic to microorganisms. Several bacteria are known to be highly tolerant to toluene (log P_{ow} = 2.3) (4-7). Benzene with a log P_{ow} value (2.0) lower than that of toluene is extremely toxic, and there are very limited reports on bacteria able to grow in the presence of high concentrations of benzene. We have been screening soil, sediment and activated sludge samples for benzene-tolerant bacteria that are required for the development of a bioconversion process from benzene to phenol. In this study, we report the isolation and characterization of benzene-tolerant bacteria isolated from gasoline-contaminated soil.

MATERIALS AND METHODS

Growth media and culture conditions The mineral salt basal (MSB) medium consisted of 4.3 g of K_2HPO_4, 3.4 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.34 g of $MgCl_2 \cdot 6H_2O$, 0.001 g of $MnCl_2 \cdot 4H_2O$, 0.006 g of $FeSO_4 \cdot 7H_2O$, 0.026 g of $CaCl_2 \cdot 2H_2O$, 0.02 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg of $ZnCl_2 \cdot 7H_2O$, 0.01 mg of $CoCl_2 \cdot 6H_2O$, 0.01 mg of $CuSO_4$, 0.001 mg of $NiSO_4 \cdot 6H_2O$ and 0.001 mg of Na_2SeO_4 per liter of deionized water. Tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) was used as a complete medium. For solid media, 2% agar was added to the MSB and TSB media.

Hydrocarbons used for growth substrate analysis were provided in the vapor phase or directly added to MSB medium at 10% (v/v). Growth in the presence of organic solvents was also measured in TSB with 10% (v/v) solvent. The total volume of medium plus solvent was 20% that of a 50-ml screw-capped vial. Each vial was incubated at 28˚C with shaking (120 rpm). Growth was measured by the increase in turbidity

at 600 nm and the total number of viable cells per milliliter as determined by microscopic enumeration in a counting chamber. When water-soluble organic compounds were used as the sole carbon source, they were usually supplied at a final concentration of 10 g/*l*.

Isolation of benzene-metabolizing bacteria Soil samples were taken from chemical plants and roadsides in Hiroshima, Japan. Approximately 5 g of soil was incubated in a 50-ml screw-capped vial containing a small tube with benzene for 1 week at 28˚C and acclimated to benzene. After acclimation, 1 g of soil was dispersed in 4 ml of sterile water and allowed to stand for 30 min. One milliliter of this soil suspension was added to 9 ml of MSB medium and 0.5 ml of benzene was added to the small tube inside the screw-capped vial. The vial was incubated with shaking (120 rpm) at 28 ˚C for 5 d. Subsequently, 1 ml of the culture was transferred to 9 ml of fresh MSB medium for another 2-day incubation. Serial dilutions of the second culture were plated out on MSB agar plates and incubated in a desiccator with a beaker containing liquid benzene. Colonies obtained were reinoculated into the liquid medium to confirm the utilization of benzene. Pure cultures utilizing benzene as a sole carbon source were kept on TSB agar plates.

Identification of strains B-4, B-9 and B-10 Isolates B-4, B-9 and B-10 were characterized with phenotypic and morphological analyses. All biochemical tests were performed as described previously (8). The G+C content of DNA was determined by HPLC according to Johnson (9). Electron microscopic observations were done with a JSM5900 scanning electron microscope (JEOL, Tokyo). The microorganisms were fixed with 1 to 2% glutaraldehyde, dried and given a metal coating with Pt (10).

Amplification and sequencing of 16S rRNA gene (rDNA) Genomic DNA was isolated using standard bacterial procedures (11). The following primers were used for PCR amplification of the 16S ribosomal DNA: 63f (5’-CAGGCCTAACACATGCAAGTC-3’), 1387r (5’-GGGCGGWGTGTACAAGGC-3’) (12). The PCR mixtures (50 µl) contained 25 pmoles of each primer, 200 µM each of deoxynucleoside triphosphate, TaKaRa EX-Taq PCR buffer (Takara Bio, Ohtsu), 0.5 U of TaKaRa EX-Taq (Takara Bio) and 10 ng of DNA per µl. The thermocycling conditions consisted of a denaturation step at 96˚C for 3 min, 25 amplification cycles of 96˚C for 30 s, 50˚C for 1 min and 72˚C for 2 min, and a final polymerization for 4 min with a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA). PCR products were visualized on 1.0% agarose gels, and the products were excised and purified with glassmilk (Gene Clean; Bio 101, Vista, CA, USA) following the manufacturer’s instructions. The PCR products were cloned in a pGEM-T easy vector (Promega, Madison, WI, USA) by following the manufacturer’s instruction. Several clones containing a 1.5-kb insert were isolated, and some of them were sequenced completely. Both strands of the 16S sequences were sequenced by the dideoxynucleotide method (13). Nucleotide sequence similarities were determined using BLAST (National Center for Biotechnology Information databases).

Cloning of benzene dioxygenase operon of strain B-4 Standard procedures were used for plasmid

DNA preparations, restriction enzyme digestions, ligation, transformations and agarose gel electrophoresis (14). A strain B-4 genomic library was constructed by ligating 3- to 6-kb partially *Pst*I cleaved DNA fragments to the *Pst*I site of pUC118 (Takara Bio). The ligated DNA fragments were used to transform the host strain *Escherichia coli* MV1184. Transformants were screened for catechol 2,3-dioxygenase (C23O) activity by spraying catechol dissolved in acetone onto transformant colonies and selecting yellow colonies. A cosmid genomic library of strain B-4 was constructed using a SuperCos I cosmid vector kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. Southern hybridization (14) was carried out using the *R. opacus* B-4 C23O gene as a probe to select cosmid clones containing the benzene dioxygenase operon.

Construction of the *bnzA1 bnzA2* mutant of *R. opacus* B-4 The *bnzA1 bnzA2* deletion-insertion mutant of *R. opacus* B-4 was constructed by the direct gene replacement technique (15). A 3.5-kb *Eco*RI-*Not*I fragment containing *bnzA1*, *bnzA2* and *bnzA3* was cloned into pBluescript II KS+ (Stratagene) to construct pKSN2 (Fig. 3). A *Hin*cII-flanked *kan* (conferring kanamycin resistance [Km^r]) cassette from pUC4K (Amersham) was inserted between the *Bal*I and *Eco*RV sites of pKSN2 to disrupt *bnzA1* and *bnzA2*. *R. opacus* B-4 was transformed with the resulting plasmid pKSN3 by electroporation (16) to transfer the mutation into the genome of *R. opacus* B-4. The deletion-insertion was confirmed by Southern blot analysis.

Nucleotide sequence accession numbers The DDBJ accession numbers of the *bnzA1A2A3A4CB* genes of B-4 and the 16S rDNA sequences of B-4, B-9 and B-10 are AB193045, AB192962, AB192963, and AB192964, respectively.

RESULTS AND DISCUSSION

Isolation of benzene-tolerant bacteria Twenty-two bacteria that grew on benzene as a sole carbon and energy source were isolated from soil samples. Of twenty-two isolates, three isolates, designated B-4, B-9 and B-10, grew on benzene when liquid benzene was added to MSB medium at 10-90% (v/v).

Isolates B-4, B-9 and B-10 were Gram-positive, non-motile, strictly aerobic rods (Fig. 1) which grew at 33˚C, but not at 42˚C. Partial sequencing of 16S rDNA indicated that B-4, B-9 and B-10 were distinct and were strains of *Rhodococcus*. The 16S rDNA sequences determined for these isolates were compared to previously published near-complete 16S rRNA gene sequences for the Gram-positive bacteria and related microorganisms. Comparison of the 16S rDNA sequences of B-4, B-9 and B-10 with those in the GenBank, EMBL and DDBJ databases revealed a consistently high similarity (more than 95% similarity) with species of *Rhodococcus*. B-4, B-9 and B-10 are especially closely related to *R. opacus*, with more than 98% similarity to other *R. opacus* strains including *R. opacus* DSM 43206^T (accession no. X80631), *R. opacus* SAO101 (accession no. AB032565), *R. opacus* 1CP (accession no. Y11893) and *R. opacus* DNP14-5

(accession no. AY027585).

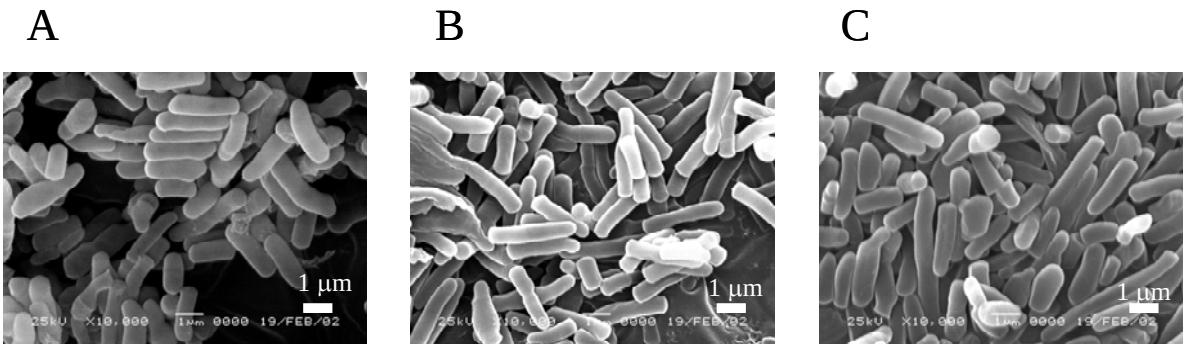

FIG. 1. Scanning electron micrographs of isolates B-4 (A), B-9 (B) and B-10 (C). Bars: 1 μm.

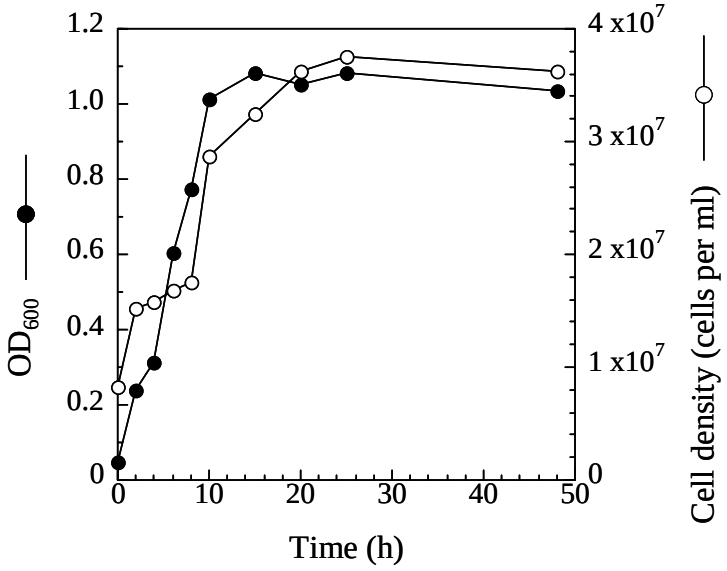

FIG. 2. Growth of *R. opacus* B-4 on TSB in the presence of 10% (v/v) benzene. Growth was measured by the increase in turbidity at 600 nm (closed circles) and the total number of viable cells per milliliter (open circles) as determined by microscopic enumeration in a counting chamber.

Identification of benzene-tolerant bacteria To verify identification of B-4, B-9 and B-10 as *R. opacus*, these strains were characterized with phenotypic analysis. They were not acid-fast and were negative for the Voges-Proskauer reaction. B-4 and B-9 were negative for oxidase, while B-10 was positive. All three strains were positive for catalase and urease. They did not hydrolyze casein, starch, elastin or gelatin. Their predominant menaquinones were MK-8(H_2). The moles % G+C of the B-4, B-9 and B-10

DNAs were 66.8%, 67.7% and 66.5%, respectively. These characteristics of the three strains are consistent with those of *R. opacus* (17). The three strains then were grown with a variety of substrates as sole sources of carbon for comparison with reported characteristic strains of *Rhodococcus rhodochrous*, *Rhodococcus rhodnii*, *Rhodococcus erythropolis* and *R. opacus* (17). They utilized the following carbon sources: D-fructose, D-galactose, D-glucose, *myo*-inositol, D-arabitol, L-rhamnose, sucrose and D-turanose. *R. erythropolis*, *R. rhodnii* and *R. rhodochrous* do not utilize D-turanose. *R. rhodnii* and *R. rhodochrous* do not use *myo*-inositol as a growth substrate. Most strains of *R. opacus* do not utilize L-rhamnose as the sole carbon source, whereas Uz *et al*. reported that *R. opacus* M213 grew on L-rhamnose (18). These data support classification of B-4, B-9 and B-10 as *R. opacus*.

Hydrocarbon utilization and tolerance of *R. opacus* B-4 *R. opacus* B-4 was selected for further studies because it was the fastest growing isolate among 3 benzene-tolerant *R. opacus* strains. *R. opacus* B-4 used as sole sources of carbon and energy a broad spectrum of organic solvents including benzene, toluene, styrene, *o*-xylene, *m*-xylene, *p*-xylene, ethylbenzene, propylbenzene, dimethylphthalate, diethylphthalate, cyclohexane, hexane, *n*-octane, and *n*-decane. It grew well on these organic solvents when they were supplied in the vapor phase or added directly to the medium at 10% (v/v) (Table 1). Phenol, catechol and naphthalene were also used as growth substrates by *R. opacus* B-4. *R. opacus* B-4 was unable to grow on *n*-heptanol, chloroform, *n*-butanol and anthracene.

TABLE 1. Organic solvent utilization and tolerance of *R. opacus* B-4

Organic solvent	(log P_{ow})	Utilization	Tolerance
n-Decane	(5.6)	+	+
n-Octane	(4.5)	+	+
Propylbenzene	(3.6)	+	+
n-Hexane	(3.5)	+	+
Diethylphthalate	(3.3)	+	+
Cyclohexane	(3.2)	+	+
Ethylbenzene	(3.1)	+	+
o-Xylene	(3.1)	+	+
m-Xylene	(3.1)	+	+
p-Xylene	(3.1)	+	+
Styrene	(3.0)	+	+
Toluene	(2.5)	+	+
n-Heptanol	(2.3)	-	-
Benzene	(2.0)	+	+
Chloroform	(2.0)	-	-
n-Butanol	(0.8)	-	-

+, Utilizable, tolerant; -, nonutilizable, sensitive.

R. opacus B-4 could grow in MSB medium when benzene was supplied in a two-phase system where benzene was present at 10% to 90% (v/v). Figure 2 shows typical growth of *R. opacus* B-4 with 10 % (v/v) benzene. Growth was observed without an appreciable lag phase and maximum growth was obtained after 25 h. Turbidity paralleled the increase in viable cells. The highest cell density was 3.6×10^7 cells per ml. When *R. opacus* B-4 was grown on TSB, turbidity and viable cell measurements were also parallel and the cell density reached 3×10^8 cells per ml (data not shown).

The ability of B-4 to grow on TSB in the presence of a range of organic solvents at 10% (v/v) was tested (Table 1). Solvents are listed in Table 2 in decreasing order of hydrophobicity expressed as log P_{ow}. *R. opacus* B-4 grew well in the presence of 10% (v/v) organic solvents that *R. opacus* was capable of using as growth substrates. No growth was observed when *n*-heptanol, chloroform or *n*-butanol were added to TSB.

Paje *et al.* reported isolation of a benzene-tolerant *Rhodococcus* strain (19). This *Rhodococcus* strain grew on benzene when liquid benzene was added to medium at 2% (vol/vol). The tolerance to and utilization of other organic solvents by this strain have not been characterized. The 16S rDNA sequence of this strain (accession no. U20791) showed only 88% sequence similarity with *R. opacus* B-4, B-9 and B-10, indicating that B-4, B-9 and B-10 are distinct from this *Rhodococcus* strain.

Several *Pseudomonas putida* strains with increased tolerance to organic solvents have been isolated (4-7, 20). Physiological investigation of the organic tolerant *P. putida* strains revealed a correlation between solvent toxicity and log P_{ow} value. The lower the log P_{ow} of the solvent, the higher its toxicity. The tolerance level of each *P. putida* strain is represented by the index solvent. The index solvent is the solvent with the lowest log P_{ow} among those to which the strain is tolerant. Each strain is tolerant to organic solvents with log P_{ow} values greater than that of the index solvent. However, this is not the case with *R. opacus* B-4, which is tolerant to benzene with a log P_{ow} value of 2.0, but not to *n*-heptanol having a log P_{ow} value (2.3) greater than 2.0. In contrast to the case with *R. opacus* B-4, the organic solvents that can be tolerated by *P. putida* strains are not always their growth substrates. *R. opacus* B-4 may have molecular mechanisms of solvent tolerance different from those of *P. putida* strains.

Characterization of benzene metabolic pathway in *R. opacus* B-4 To determine the metabolic pathway of benzene in *R. opacus* B-4, we first tested C23O activity. The rapid spot test described by Pankhurst (21) was carried out to assess C23O activity. Catechol dissolved in acetone was sprayed onto 2-day-old B-4 colonies grown on benzene vapor plates. The spot test revealed bright yellow colonies, indicative of C23O activity against catechol. It is known that the C23O gene often forms an operon together with genes involved in the oxidation of benzene to catechol. To investigate the benzene oxidation pathway in *R. opacus* B-4, the C23O gene was cloned from the B-4 genomic DNA. A *R. opacus* B-4 genomic library was created with pUC118 and *E. coli* MV1184, and screened for C23O activity as described in MATERIALS AND METHODS. Three positive clones were obtained. All of the recombinant plasmids were shown to contain an identical 3.0-kb *Pst*I insert fragment. One of the plasmids was

designated pKSN1 (Fig. 3).

DNA sequence analysis of the pKNR1 insert revealed that the 3.0-kb *Pst*I fragment contained part of two open reading frames (ORFs) (*bnzA4* and *bnzB*) and one whole ORF (*bnzC*). The predicted product of *bnzC* had 100% amino acid identity with the product of *ipbC* from *R. erythropolis* strain BD2 (22). The truncated *bnzA4* and *bnzB* genes were highly similar to the 3'-portion of *ipbA4* and the 5'-portion of *ipbB* from *R. erythropolis* strain BD2, respectively. The *ipbA4*, *ipbC* and *ipbB* genes, together with *ibpA1*, *ipbA2* and *ipbA*3, form the isopropylbenzene dioxygenase operon (22). The *ipbA1*, *ipbA2*, *ipbA3*, *ipbA4*, *ipbC* and *ipbB* genes encode isopropylbenzene dioxygenase large subunit, isopropylbenzene dioxygenase small subunit, ferredoxin, ferredoxin reductase, isopropylcatechol 2,3 dioxygenase, and isopropylbenzene glycol dehydrogenase, respectively. These results suggest that strain B-4 possesses the benzene dioxygenase operon.

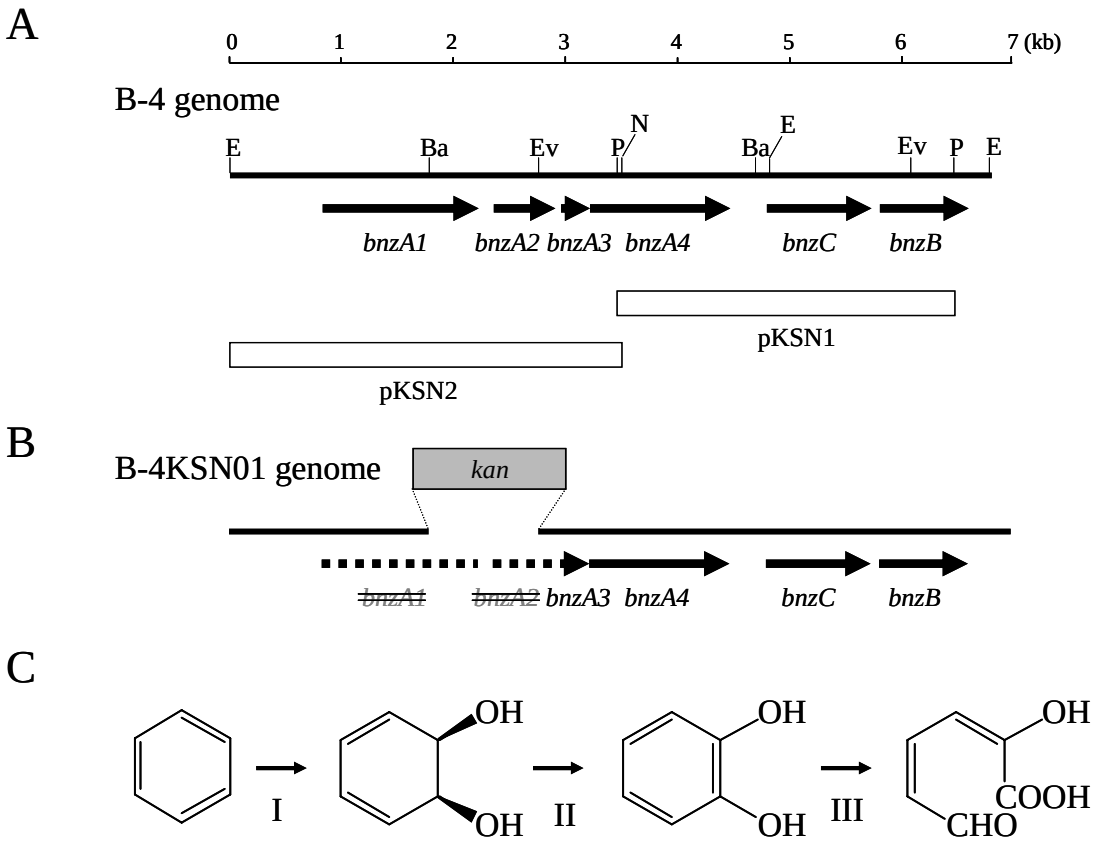

FIG. 3. Physical maps of the benzene dioxygenase operon region of *R. opacus* B-4 (A) and B-4KSN01 (B), and proposed catabolic pathway for benzene by *R. opacus* B-4 (C). (A) The locations and orientations of the *bnzA1*, *bnzA2*, *bnzA3*, *bnzA4*, *bnzC* and *bnzB* genes are shown by horizontal arrows below the map. Open bars indicate *R. opacus* B-4 genomic DNA fragments subcloned into vector plasmids. Restriction sites: Ba, *Bal*I; E, *Eco*RI; Ev, *Eco*RV; N, *Not*I; P, *Pst*I. Only restriction sites used to construct recombinant plasmids are indicated. (B) The *bnzA1* and *bnzA2* were disrupted by inserting a *kan* cassette into the genomic genes of *R. opacus* B-4. (C) I, Benzene dioxygenase; II, benzene glycol dehydrogenase; III, catechol 2,3-dioxygenase.

To confirm the presence of the benzene dioxygenase operon in strain B-4, cloning of DNA sequences upstream of *bnzA4* and downstream of *bnzB* was conducted. We constructed a cosmid genomic library from *R. opacus* B-4 and screened it using the 3.0-kb *Pst*I insert fragment of pKSN1 as a probe. One positive cosmid clone, designated pB4cos51, was obtained. To subclone target DNA sequences, Southern hybridization to the *Eco*RI digests of pB4cos51 was performed with the pKNS1 insert as a probe. The 4.9- and 1.9-kb *Eco*RI fragments hybridizing with the probe were subcloned into pUC118 and completely sequenced. DNA sequence analysis revealed that the 4.9- and 1.9-kb fragments overlapped with the 3.0-kb *Pst*I insert of pKNS1 and were adjacent to each other (Fig. 3). Six ORFs (*bnzA1*, *bnzA2*, *bnzA3*, *bnzA4*, *bnzC* and *bnzB*) were found in the 6.8-kb region. The putative products of *bnzA1*, *bnzA2*, *bnzA3*, *bnzA4* and *bnzB* showed more than 92% similarities to IpbA1, IpbA2, IpbA3, IpbA4 and IpbB from *R. erythropolis* strain BD2. These results strongly suggest that strain B-4 possesses the benzene dioxygenase pathway (Fig. 3).

Construction and characterization of the *bnzA1 bnzA2* mutant of *R. opacus* B-4 To confirm that *bnzA1A2A3A4CB* genes are involved in benzene catabolism, the *bnzA1* and *bnzA2* genes were disrupted by inserting a *kan* cassette into the wild-type genes in the *R. opacus* B-4 genome. Since the predicted products of *bnzA1* and *bnzA2* were 99% identical to the isopropylbenzene dioxygenase large and small subunits of *R. erythropolis* BD2, it is assumed that the BnzA1 and BnzA2 proteins are involved in the initial oxidation step of the benzene dioxygenase pathway (Fig. 3). The resulting Km^r mutant, designated B-4KSN01 (Fig. 3), was examined for its growth on benzene. The *bnzA1 bnzA2* mutant B-4KSN01 did not grow on benzene when it was supplied in the vapor phase. B-4KSN01 was also unable to grow on other aromatic compounds. These results demonstrate that *bnzA1A2* genes are involved in benzene catabolism.

Results in Table 1 suggest the possibility that utilization or degradation plays a crucial role in solvent tolerance in *R. opacus* B-4. To assess the possibility, B-4KSN01 was examined for solvent tolerance. B-4KSN01 grew as well as the wild-type B-4 in TSB medium with 10% (v/v) benzene. B-4KSN01 was also tolerant to other organic solvents that are tolerated by B-4. These results suggest that utilization or degradation is not essential for organic solvent tolerance of *R. opacus* B-4. More studies need to be undertaken to elucidate the molecular mechanisms of solvent tolerance in *R. opacus* strains.

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