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Development of a Genetic Transformation System for Benzene-Tolerant *Rhodococcus opacus* Strains

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Rhodococcus opacus **B-4 and B-9 are tolerant to various organic solvents including benzene, toluene, ethylbenzene, xylenes and styrene, and are suitable bacterial hosts for the production of chemical products from hydrophobic substrates. A 4.4-kb endogenous plasmid (pKNR01) was isolated from *R. opacus* B-4 and sequenced completely. Plasmid pKNR01 encodes proteins that share similarity to replication proteins from the enteric bacterial and actinomycete θ-replication plasmids. A 7.4-kb chimeric plasmid, designated pKNR01.1, was constructed by fusing *Xho*I-digested pKNR01 and *Escherichia coli* vector pSTV28. Plasmid pKNR01.1 had the ability to replicate in B-4 and B-9. A protocol for transformation of B-9 by electroporation was optimized employing pKNR01.1. Frequencies of 4.1×10^5 transformants per μg of plasmid DNA were obtained for B-9 cells, whereas B-4 harboring naturally occurring pKNR01 was transformed at lower frequencies (approximately 1×10^4 transformants per μg of plasmid DNA). Deletion analysis of pKNR01.1 showed that the 1.9-kb *Sph*I-*Xho*I region containing the *repA* and *repB* genes and the 0.6-kb region upstream of *repA* was essential for plasmid maintenance in *R. opacus* strains.**

[**Key words:** genetic transformation, *Rhodococcus opacus*, shuttle vector, solvent-tolerant bacterium]

There is considerable interest in the development of technology for the production of commercially valuable chemicals from hydrophobic substrates such as petroleum hydrocarbons by using biocatalysts. In particular, enantiospecific oxidation and reduction of aromatic and aliphatic hydrocarbons have attracted much interest, because many important chemicals can be produced by these reactions. Since cofactors and their regeneration are required for oxidation and reduction reactions, whole cells are favored as biocatalysts. For the bioproduction of chemical products from hydrophobic substrates, the reactions have to be carried out in a two-phase (organic-aqueous) system. Low-molecular-weight aromatic hydrocarbons including benzene, toluene, styrene and xylenes are important substrates for a variety of chemical products, however, they are extremely toxic to microorganisms. Therefore, solvent-tolerant bacteria as host strains are required for the development of bioproduction processes in the two-phase system. The establishment of a genetic transformation system for solvent-tolerant bacterial hosts is also necessary for strain improvement.

We recently isolated 22 benzene-oxidizing bacteria from soil samples. Among them, strains B-4 and B-9 were highly tolerant to organic solvents (1). B-4 and B-9 were identified as *Rhodococcus opacus* by 16S rRNA, physiological and chemotaxonomic analyses. These strains were able to grow on various organic solvents such as benzene, toluene, ethylbenzene, xylenes and styrene when they were supplied at 10% (vol/vol). These benzene-tolerant *R. opacus* strains are potential bacterial hosts for the production of chemical products from hydrophobic substrates. This report describes the isolation and characterization of a 4.4-kb plasmid and successful use of the plasmid in the construction of *Escherichia coli–Rhodococcus* shuttle vectors. An optimized electroporation protocol for *R. opacus* B-4 and B-9 was also established.

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MATERIALS AND METHODS

Bacterial strains and plasmids *R. opacus* strains B-4 and B-9 are solvent-tolerant benzene-oxidizing bacteria (1). Strain B-4 was used as the source of the 4.4-kb pKNR01 and the rhodococcal host, and strain B-9 was also used as a host strain. 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 $(NH_4)_2SO_4$, 0.16 g of $MgCl_2\cdot6H_2O$, 0.001 g of $MnCl_2\cdot4H_2O$, 0.006 g of $FeSO_4\cdot7H_2O$, 0.026 g of $CaCl_2\cdot2H_2O$, 0.02 mg of $Na_2MoO_4\cdot2H_2O$, 0.01 mg of $ZnCl_2\cdot7H_2O$, 0.01 mg of $CoCl_2\cdot6H_2O$, 0.01 mg of $CuSO_4$, 0.001 mg of $NiSO_4\cdot6H_2O$ 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 complete medium. For solid media, 2% agar was added. *R. opacus* cells were grown at 28°C with shaking in TSB. The MSB medium with 2% glucose was used for the preparation of *R. opacus* cells

for fluorescence determination.

E. coli MV1184 (2), which was used for plasmid construction and DNA manipulation, was grown at 37°C with shaking in 2x YT medium (3) supplemented with appropriate antibiotics.

Isolation of plasmid DNA from *Rhodococcus* strains Plasmid extractions were performed by the alkaline lysis method (3) with some modifications. Before the alkaline lysis step, cells were incubated with lysozyme (10 mg/ml) at 37°C for 2 h.

DNA manipulation and sequencing Standard procedures were used for preparation of plasmid DNA from *E. coli* strains, restriction enzyme digestions, ligations, transformations and agarose gel electrophoresis (3). To determine the complete nucleotide sequences of plasmids pKNR01 and pKNR02 on both strands, overlapping fragments were subcloned in pUC118 (2) and subjected to double-stranded DNA sequencing using a CEQ2000 automated sequencer (Beckman Coulter, Fullerton, CA, USA). Sequence analysis was performed with the BLAST programs (4) of the National Center for Biotechnology Information (Bethesda, MD, USA) and the DNASIS package (Hitachi Software Engineering, Tokyo). Multiple sequences were aligned using the CLUSTAL W program (DDBJ).

Construction of plasmids The 4.4-kb *Xho*I and 4.2-kb *Sal*I fragments from pKNR01 were cloned into the *Sal*I site of pSTV28 (Takara Bio, Tokyo) to construct pKNR01.1 and pKNR01.2, respectively. Plasmids pKNR01.3 and pKNR01.4 were generated by ligating the 1.9-kb *Sph*I/*Xho*I and 1.6-kb *Sph*I/*Eco*RI fragments from pKNR01 with *Sph*I/*Sal*I- and *Sph*I/*Eco*RI-digested pSTV28, respectively. The 1.4-kb *Pst*I/*Xho*I fragment from pKNR01 was cloned between the *Pst*I and *Sal*I sites of pSTV28 to obtain pKNR01.5. Plasmids pKNR02.1, pKNR02.2 and pKNR02.3 were constructed by subcloning the *Pst*I-, *Sal*I- and *Xho*I-digested pKNR02 into pSTV28, respectively. A 1.1-kb *Sac*I/*Pst*I fragment containing the promoter region of the benzene dioxygenase operon from *R. opacus* B-4 (1) and a 0.8-kb *Pst*I/*Hin*dIII (destroyed by blunting with T4 DNA polymerase [Takara Bio]) fragment containing the promoterless *gfp*(*mut3*) gene from the green fluorescent protein (GFP) expression vector pMRP9-1 (5) were ligated with the backbone of *Sac*I/*Sma*I-digested pKNR01.3 to construct pKNR01.6.

Electroporation Plasmids were introduced into strains of *R. opacus* by electroporation using an Electro Cell Manipulator 620 (BTX Inc., San Diego, CA, USA). The following optimized protocol was devised from different experiments described in the Results and Discussion section. To obtain electrocompetent cells of *R. opacus*, 10 ml of TSB supplemented with 0.5% (w/v) glycine in a 50-ml screwed-capped vial was inoculated with 0.1 ml of an overnight TSB preculture and grown at 28°C for 24 h. Cells were harvested, washed twice with ice-cold HS buffer containing 7 mM HEPES and 252 mM sucrose (pH 7.0) and concentrated 10-fold in ice-cold HS buffer. Immediately before electroporation, 400 μl of competent cells were preincubated at 40°C for 10 min and mixed with DNA (final concentration 0.1–1 μg/ml). The electroporation was performed in electrocuvettes (BTX Inc.) with gaps of 2 mm and the following settings: 6.5 kV/cm, 725 Ω and 50 μF. Pulsed cells were immediately diluted with 4 ml of TSB and regenerated at 28°C for 24 h before they were plated on TSB agar plates supplemented with 50 mg per liter of chloramphenicol.

Determination of plasmid copy number The average copy number of pKNR01.1 in B-9 harboring pKNR01.1 was estimated based on the molar ratio of plasmid DNA to a 2.0-kb chromosomal DNA fragment as described by Yamagata *et al*. (6). A 2.0-kb *Xho*I fragment of the B-9 chromosome was cloned into pUC118 to construct pUC9X2.0. The 2.0-kb insert fragment of pUC9X2.0 and the 1.9-kb *Eco*RI fragment from pKNR01 were labeled with digoxigenin to generate the probes for Southern hybridization. The 2.0-kb *Xho*I fragment probe did not hybridize with pKNR01.1. The total DNA of B-9 (pKNR01.1) was digested with *Xho*I and resolved by agarose gel electrophoresis, blotted onto a nylon membrane and hybridized with the digoxigenin-labeled probes. The densities of the DNA-blotted regions of the membrane were determined with a gel scanner (Nihon Bio Image, Tokyo).

GFP quantification Bacterial cells, grown for 24 h in the MSB medium with glucose, were inoculated into 10 ml of fresh MSB medium with glucose in a 50-ml screwed-capped vial (a 5% inoculum). When necessary, hydrocarbons were provided in the vapor phase. After 24 h of incubation, cells were harvested by centrifugation (10,000×*g*, 5 min, 4°C) and suspended in 50 mM phosphate buffer (pH 7.0). The optical density at 600 nm (OD_{600}) of all cell suspensions was adjusted to 0.5. The fluorescence of cells was measured using a fluorescence spectrometer (ARVO 1420 Multilavel Counter; Wallac, Turku, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Nucleotide sequence accession numbers The complete sequences of pKNR01 and pKNR02 of *R. opacus* strain B-4 have been assigned GenBank/EMBL/DDBJ accession nos. AB193159 and AB193160, respectively.

RESULTS AND DISCUSSION

Isolation and characterization of cryptic plasmids

R. opacus strains B-4 and B-9 were isolated from an enrichment culture in which benzene was the sole source of carbon and energy (1). They were able to grow on benzene when liquid benzene was added to MSB medium at 10–90% (vol/vol). They grew on a broad spectrum of organic solvents including toluene, ethylbenzene, *o*-, *m*-, and *p*-xylenes, styrene, *n*-octane and *n*-decane when these organic solvents were supplied in a two-phase system where they were present at 10% (vol/vol) (1).

Two small plasmids, designated pKNR01 (4.4 kb) and pKNR02 (2.8 kb), were detected in *R. opacus* strain B-4 (Fig. 1A). In strain B-9, no small indigenous plasmid was detected. Plasmid DNA purified from B-4 was separated in a 1% agarose gel and pKNR01 and pKNR02 were purified individually from the gel. Plasmids pKNR01 and pKNR02 were digested with various restriction enzymes to determine physical maps (Fig. 1B). Plasmid pKNR01 had unique *Xho*I and *Sac*I sites, while *Bam*HI, *Bgl*II, *Pst*I, *Sal*I, *Sac*I and *Xho*I sites were unique in pKNR02.

The complete nucleotide sequences of pKNR01 and pKNR02 were determined as described in Materials and Methods. Plasmids pKNR01 and pKNR02 were 4369 and 2774 bp in length, respectively, with G+C contents of 64.5% and 63.8%. The G+C content of the *R. opacus* strain B-4 chromosome is 66.8% (1). Nucleotide sequence analysis revealed that pKNR01 contained six potential open reading frames (ORFs) (*repA*, *repB*, ORF1, ORF2, ORF3 and ORF4) (Fig. 1B). A computer-assisted homology search showed that the protein sequence deduced for RepA showed significant similarity to replication proteins encoded by plamids including pFAJ2600 from *R. erythropolis* NI86/21 (7), p545 from *Propionibacterium freudenreichii* (8), and pRBL1 from *Brevibacterium lines* (9), and θ-replicases encoded by the ColE2-type plasmids including ColE2-P9 from *Shigella* sp. (10) and pAsal1 from *Aeromonas salmonicida* subsp. *salmonicida* (11). The alignment of the different RepA sequences (Fig. 2A) revealed that the highest identity (35%)