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Original paper (short paper)

Development of Unmarked Gene Modification System in Organic Solvent-Tolerant *Rhodococcus opacus* Strain B4

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Unmarked gene modification system in organic solvent tolerant *Rhodococcus opacus* strain B4 has been developed. Upstream and downstream regions of a target site for deletion are tandemly cloned into pG19II, a suicide plasmid which was originally constructed for gene disruption in *Pseudomonas aeruginosa* and contains gentamycin resistant gene as a selective marker and *sacB* as a counter-selective marker. Unmarked deletion mutant of strain B4 can be obtained after two homologous recombination events between the resulting plasmid and the B4 genome. This unmarked gene modification system can be used for unmarked insertion mutation in *R. opacus* strain B4.

Key words: unmarked gene modification, solvent-tolerant bacterium, *Rhodococcus opacus*

Rhodococcus opacus strain B4, isolated from a soil sample, tolerates various organic solvents including benzene, toluene, xylene, ethylebenzene, *n*-octane, and *n*-decane[1]. This strain has a high hydrophobicity and exhibits a high affinity for hydrocarbons. Strain B4 not only survived for at least 5 days in organic solvents, but also exhibited toluene dioxygenase activity in anhydrous organic solvents[2]. This solvent-tolerant and hydrophobic strain is a potential host for the production of chemical products from hydrophobic substrates. To construct biocatalytic cells for bioproduction, the establishment of a genetic transformation system for strain B4 is necessary. In the previous study, we constructed *E. coli-Rhodococcus* shuttle plasmid vectors using a 4.4-kb endogenous plasmid in strain B4 and established a genetic transformation system by electroporation[3]. In the industrial bioproduction process, the genome modification (deletion of genomic genes and insertion of exogenous genes into the genome) are favored over the genetic modification using plasmid vectors due to its genetic stability. Especially, unmarked genomic modification is a technology desirable for the construction of biocatalytic cells because unlimited times of genomic modifications are possible theoretically. Although van der Geize *et al.* reported application of unmarked gene modification technique to deletion of 3-ketosteroid Δ^1-dehydrogenase gene (*kstD*) in *Rhodococcus erythropolis* SQ1[7], there was quite limited reports about the construction of unmarked gene deletion mutants in the genus *Rhodococcus*. In this report, we describe development of an unmarked gene modification system in solvent-tolerant *R. opacus* strain B4.

R. opacus strains were grown in tryptic soy broth (TSB) medium (Difco) at 28°C with shaking. TSB agar was prepared by adding 20 g/l of agar to TSB medium. Whenever necessary, TSB medium and TSB agar were supplemented with 5 mg/l of gentamycin or 25 mg/l of chloramphenicol. Plasmid pG19II[4] was used for the unmarked gene modification in *R. opacus* strain B4. pG19II was originally constructed for the insertion-deletion mutation of chromosomal genes in *Pseudomonas aeruginosa*. This plasmid contains gentamycin resistant (Gm^r) gene as a selectable marker, *sacB* (conferring sucrose sensitive phenotype) as a counter-selectable marker, the multi-cloning sites, an *Escherichia coli* replication origin, and an origin of transfer. Plasmids were introduced into *R. opacus* strains by electroporation as described by Na and colleagues[3]. Polymerase chain reactions (PCR) were carried out using KOD DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer's instructions.

HS buffer (252 mM sucrose, 7 mM HEPES [pH 7.0]) was used for cell suspension buffer in the previous study[3]. In this study, buffer containing no sucrose was examined for cell suspension buffer of electroporation because pG19II contains sucrose sensitive determinant (*sacB*). HG buffer (0.3 M glycerol, 7 mM HEPES [pH 7.0]) was used for cell suspension buffer instead of HS buffer, and strain B4 was transformed with *E. coli-Rhodococcus* shuttle vector pKNR01.1[3]. Since the transformation efficiency (approximately 0.7×10^4 transformants per μg of plasmid DNA) was comparable to that with HS buffer, we used HG buffer as cell suspension buffer for electroporation.

Since pG19II contains only the *E. coli* replication origin but not the *Rhodococcus* replication origin, it should not

be replicated in *R. opacus* B4. As expected, no Gm^r transformants were obtained when strain B4 was transformed with pG19II. pG19II derivatives harboring genomic fragments of strain B4 are expected to be integrated into the B4 genome by homologous recombination and it generates Gm^r transformants of strain B4. Actually, Gm^r transformants were obtained when strain B4 was transformed with pG19 derivative harboring a 1.5-kb upstream region of benzene dioxygenase (*bnz*) operon (accession number AB193045) of strain B4 (Fig. 1). We then investigated effects of lengths of B4 genomic DNA fragments cloned in pG19II derivatives on transformation efficiency of strain B4. PCR was conducted to amplify 0.2-, 0.5-, 1.0-, 1.5-, and 2.0-kb upstream regions of *bnz* operon and the PCR products were cloned into the *Sma*I site of pG19II to construct pG19-0.2 to pG19-2.0 (Fig. 1). Strain B4 was transformed with the

Table 1. Oligonucleotides used for PCR

Primer	Sequence (5' to 3')
bnzA-200-F	TGGAAGCTTAGCCGTTGGTATCGAACC
bnzA-500-F	TGGAAGCTTCCATCGCTGGAAGTACC
bnzA-1000-F	TGGAAGCTTCGTAGTCGTGGTCAAGTC
bnzA-1500-F	TGGAAGCTTAGTGACAACTCGACCAGG
bnzA-2000-F	TGGAAGCTTATCCGACCCAACACCTG
bnzA-H-R1	ACGTTTAAACAACCAATATGGTCGTGATCG
bnzA1500-R	GTGGTTCGGATTGGCATG
bnzS0-2F	AGAAGACTGCAGAACAACAGCGAACGACACCAGG
bnzS0-2R	AGAAGAAGCTTTCGCGTTTCCTCCATCGCTATC
bnzA-kp-F12	ACGAAGCTTGGTCATCAATCCGATCATTGC
bnzA-kp-R12	ACTGGTACCATGCAGGTGATGGCGTG
bnzA-kp-F2	ACGTCTAGACACTAACGACCGTACAAGTG
bnzA-kp-R2	ACGTTTAAAGTTCTGTTCAGGTGGAGAC
pk-F	ACTGGTACCTGTGGAATTGTGAGCGGATAAC
pk-R	ACGTCTAGAGTATTACTGTTTATGTAAGCAGACAG
bnzA-RT-F	TGTACGAACAGGAACTGGAG
bnzA-RT-R	ATCCTCGCCCATGTAGTTC
gyrB-RT-F	GTCTACAGCTTCGAGACG
gyrB-RT-R	TGACGACCTCTTCGGTG

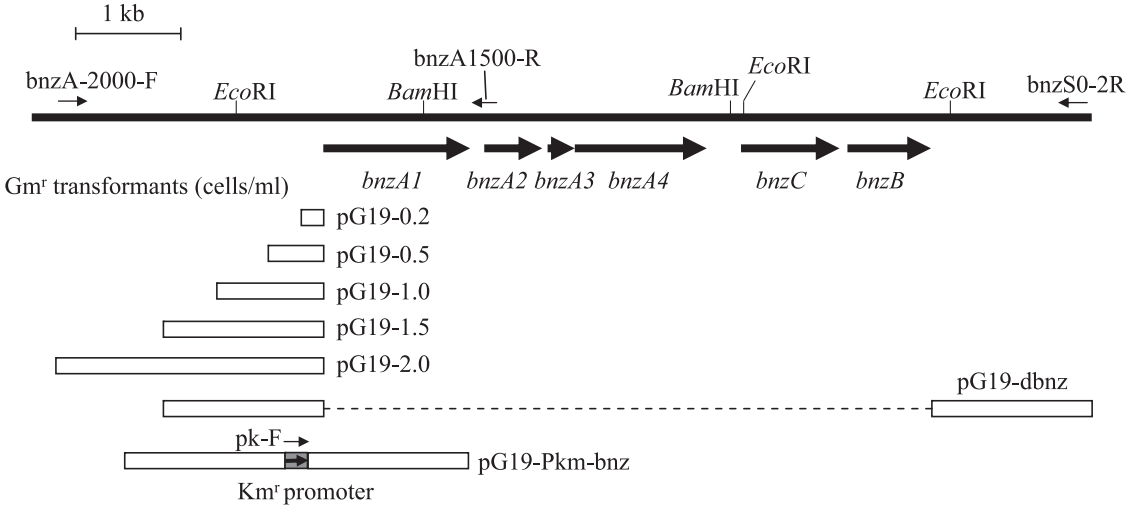

Fig. 1. Physical map of the *bnz* operon region of *R. opacus* strain B4 and pG19II derivatives used in this study.
Upstream regions of *bnz* operon were amplified from the *R. opacus* strain B4 genome by PCR. PCR primer sets bnzA-200-F/bnzA-H-R1, bnzA-500-F/bnzA-H-R1, bnzA-1000-F/bnzA-H-R1, bnzA-1500-F/bnzA-H-R1, and bnzA-2000-F/bnzA-H-R1 (Table 1) were used to amplify 0.2-, 0.5-, 1.0-, 1.5-, and 2.0-kb regions upstream of *bnz* operon, respectively. The PCR products were cloned into the *Sma*I site of pG19II to construct pG19-0.2 to pG19-2.0. A region downstream of *bnz* operon was amplified with a primer set bnzS0-2F/bnzS0-2R. The 1.5-kb product was cloned into pG19-1.5 after digestion with *Pst*I and *Hin*dIII to construct pG19-dbnz. Upstream and downstream regions of the *bnz* operon promoter and the Km^r gene promoter from pUC4K were amplified with PCR primer sets bnzA-kp-F12/bnzA-kp-R12, bnzA-kp-F2/bnzA-kp-R2, and pk-F/pk-R, respectively. The amplified Km^r promoter region was digested with *Kpn*I and *Xba*I, while the amplified upstream and downstream regions were digested with *Hin*dIII and *Kpn*I, and *Xba*I and *Dra*I, respectively. These digested PCR products were ligated with the backbone of *Hin*dIII-*Sma*I-digested pG19II to obtain pG19-Pkm-bnz. The locations of PCR primers used for confirmation of unmarked gene modifications are indicated by thin arrows.

resulting plasmids and spread on TSB agar plates with gentamycin. Transformation efficiency was determined by counting Gm^r colonies. Although pG19II derivatives (pG19-0.5 to pG19-2.0) contained different lengths of genomic fragments of strain B4, transformation efficiency was almost same (15–20 transformants per μg of plasmid DNA). When pG19-0.2 was used for transformation, transformants were obtained but its transformation efficiency (approximately 5 transformants per μg of plasmid DNA) was somewhat lower than that by pG19-0.5 to pG19-2.0. Therefore, we used 1.5–2-kb B4 genomic DNA fragments to construct pG19II derivatives for unmarked gene modification.

We examined concentrations of sucrose for counter-selection by *sacB* in strain B4. Gm^r transformant cells obtained by transforming strain B4 with pG19-1.5 were streaked on TSB agar plates with gentamycin and 0–20% sucrose and the plates were incubated at 28°C for 2 days. The transformant showed normal growth on TSB agar containing up to 10% sucrose, while 20% sucrose severely inhibited colony formation of the transformant (data not shown). Growth of the wild-type B4 was not affected by 20% sucrose. Therefore, we used 20% sucrose for counter-selection by *sacB* in strain B4.

The scheme of unmarked gene deletion is shown in Fig. 2. DNA regions upstream and downstream of the target site for deletion are cloned in tandem into pG19II to construct the suicide plasmid for unmarked gene deletion. Single crossover recombination between homologous regions of genomic DNA and the plasmid results in integration of the plasmid into the genome. Phenotype of cells containing the plasmid in their genome should be Gm^r and sucrose sensitive and they can be selectively obtained as Gm^r cells. The second single crossover recombination generates two types of cells. When the recombination occurs between regions "a" (Fig. 2), cells return to the wild type cells. The recombination between regions "b" generates unmarked deletion mutant cells. After the second single crossover recombination, phenotype of cells is Gm^s and sucrose resistant and they can be selected by growth in the presence of 20% sucrose.

To delete the whole *bnz* operon, we constructed pG19-dbnz by cloning upstream and downstream regions of *bnz* operon into pG19II (Fig. 1). Strain B4 was transformed with pG19-dbnz and Gm^r and sucrose-sensitive transformant cells were obtained. Transformation efficiency was 20 transformants per μg of plasmid DNA. The transformant cells were cultivated in TSB medium with shaking and time course of occurrence of sucrose resistant cells was examined. Six hour incubation in TSB medium was enough to obtain sucrose resistant cells (data not shown). One hundred sucrose resistant colonies were randomly selected to examine for occurrence of the second single crossover recombination. All of them were Gm^s. About half of colonies were able to grow on toluene as a sole source of carbon, while the remaining colonies failed to grow on toluene. PCR analysis using PCR primers specific to upstream and downstream sites of *bnz* operon confirmed that colonies able to grow on toluene contained the wild type *bnz* operon and that *bnz* operon was deleted in colonies which could not grow on toluene (Fig. 3). These results clearly demonstrate that unmarked gene deletion is possible in *R. opacus* strain B4.

Unmarked gene modification depicted in Fig. 2 may be used not only for unmarked deletion mutation but also for unmarked insertion mutation. pG19II derivatives used for unmarked insertion mutation should contain an inserted fragment consisting of upstream region-a fragment to be inserted-downstream region. To confirm that pG19II can be

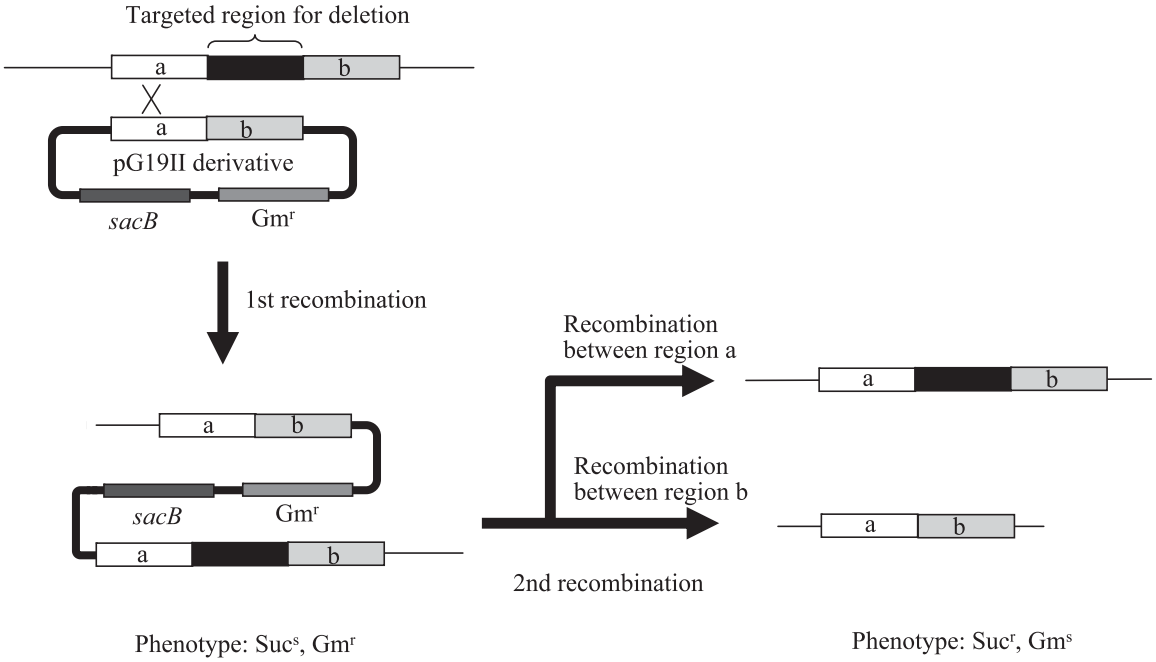

Fig. 2. Scheme of unmarked deletion mutation.
See the text for details.

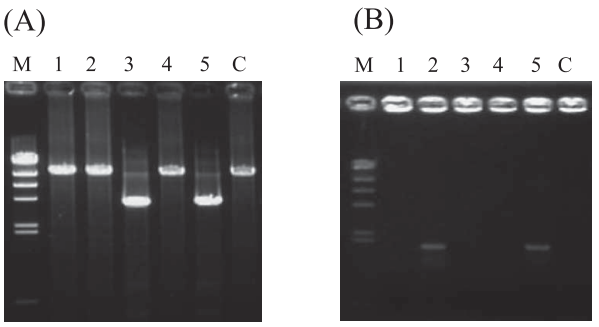

Fig. 3. PCR analysis of unmarked deletion and insertion mutants of *R. opacus* strain B4.
(A) Unmarked deletion of genomic *bnz* operon was performed for strain B4 using pG19-dbnz. Resulting Gm^s and sucrose resistant colonies after the second recombination were subjected to PCR analysis with primers specific to sequences upstream and downstream of *bnz* operon (bnzA-2000-F and bnzS0-2R [Fig. 1]). M, λ/*Hin*dIII size marker; lanes 1–5, Gm^s sucrose resistant colonies; C, parental strain. Colonies No. 1, 2, 4, and the wild type B4 could grow on toluene, while colonies 3 and 5 failed to grow on toluene. A PCR product 6.6 kb shorter than that from the wild type B4 was obtained from the colonies which could not grow on toluene. (B) Unmarked insertion of the Km^r gene promoter into the upstream region of *bnz* operon was performed for strain B4 with pG19-Pkm-bnz. Resulting Gm^s and sucrose resistant colonies after the second recombination were subjected to PCR analysis with primers specific to sequences of Km^r promoter and *bnzA2* (pk-F and bnzA1500-R [Fig. 1]). This PCR should produce 1.8-kb amplified product from the insertion mutant strain, but not from the wild type strain. Colonies No. 2 and 5 were expected to be insertion mutant strains.

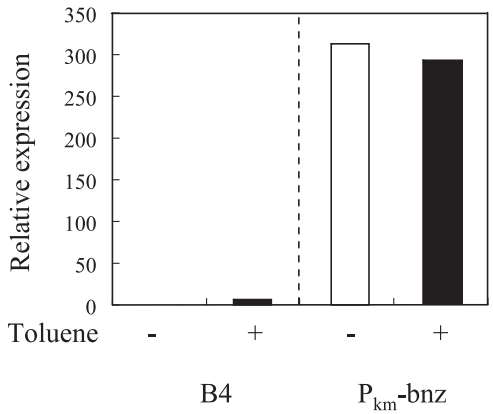

Fig. 4. Transcription of the *bnzA1* gene in the unmarked insertion mutant and parental strains of *R. opacus* B4.
The Km^r promoter insertion mutant and parental strains were cultivated in TSB with and without toluene in the vapor phase, and total RNA was extracted from cells by an RNeasy kit (QIAGEN, Inc., Valencia, CA). A One Step SYBR PrimeScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan) was used for cDNA generation and quantitative RT-PCR (qRT-PCR). Quantitative RT-PCR was carried out using a LightCycler system (Roche Diagnostics). For qRT-PCR analysis of the *bnzA1* mRNA, a primer set bnzA-RT-F/bnzA-RT-R was used. The *gyrB* mRNA was also measured as the standard using primer set gyrB-RT-F/gyrB-RT-R and used for normalization of *bnzA1* transcription.

used for unmarked insertion mutation, we tried to exchange the *bnz* promoter with the promoter of kanamycin resistant (Km^r) gene from pUC4K[5]. The *bnz* promoter is inducible and toluene, benzene, and ethylbenzene are inducers[3], while the Km^r promoter shows constitutive expression. Therefore, unmarked insertion mutant strain harboring the Km^r gene promoter-*bnz* oepron is expected to constitutively express *bnz* operon. To obtain the unmarked insertion mutant, we constructed pG19-Pkm-bnz by cloning the upstream region of the *bnz* promoter region, the promoter region of Km^r gene from pUC4K, and the downstream region of the *bnz* promoter region into pG19II as shown in Fig.1. We performed the procedures shown in Fig. 2 with pG19-Pkm-bnz and obtained Gm^s and sucrose resistant colonies where the second recombination was supposed to have occurred. Genomic DNA was extracted from five Gm^s and sucrose-resistant colonies and subjected to PCR analysis. PCR analysis revealed that two of the five colonies harbored insertion of the Km^r gene promoter in the region upstream of *bnz* operon (Fig. 3). To further confirm chromosomal insertion of Km^r gene promoter, we measured *bnzA1* transcription in the insertion mutant and the wild type strains by quantitative reverse transcription PCR (Fig. 4). As described in our previous study, transcription of *bnzA1* was induced by toluene in the wild type strain, whereas the *bnzA* gene was constitutively transcribed in the Km^r gene promoter insertion mutant. These results confirmed that pG19II can be used for unmarked insertion mutation in strain B4.

In summary, we have developed unmarked gene modification system for *R. opacus* strain B4 using the *Pseudomonas* suicide vector pG19II. It was demonstrated that this system can be used for introduction of both unmarked deletion and insertion mutation in the *R. opacus* strain B4 genome. This technology developed in this paper will enable not only future cell engineering of the strain, but also protein tagging for easy purification of any given protein and signal sequence fusion to secrete any give protein in *R. opacus* strain B4.

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