Research of Industrial Technology
Vol. 22, No. 1, 2004. 6

The Journal of Microbiology
Vol. 42, No. 2, June 2004

NOTE

Molecular Cloning and Identification of a Novel Oxygenase Gene Specifically Induced during the Growth of *Rhodococcus* sp. Strain T104 on Limonene

Ki Young Choi[1], Dockyu Kim[1], Sung-Cheol Koh[2], Jae-Seong So[3], Jong-Sul Kim[4] and Eungbin Kim[1,*]

[1]*Laboratory of Environmental Microbial Genomics, Department of Biology, Yonsei University, Seoul 120-749, Korea*
[2]*Division of Civil and Environmental Engineering, Korea Maritime University, Pusan 606-791, Korea*
[3]*Department of Biological Engineering, Center of Advanced Bioseparation Technology, Inha University, Incheon 402-751, Korea*
[4]*Division of Life Science, University of Ulsan, Ulsan 680-749, Korea*

(Received March 23, 2004 / Accepted June 1, 2004)

Rhodococcus **sp. strain T104 is able to utilize both limonene and biphenyl as growth substrates. Furthermore, T104 possesses separate pathways for the degradation of limonene and biphenyl. Previously, we found that a gene(s) involved in limonene degradation was also related to indigo-producing ability. To further corroborate this observation, we have cloned and sequenced a 8,842-bp genomic DNA region with four open reading frames, including one for indole oxygenase, which converts indole to indigo (a blue pigment). The reverse transcription PCR data demonstrated that the identified indole oxygenase gene is specifically induced by limonene, thereby implicating this gene in the degradation of limonene by T104.**

Key words: *Rhodococcus,* limonene, biphenyl, indole oxygenase

Terpenoids have been speculated to serve as natural substrates for induction of the biphenyl degradation pathway (Focht, 1995). The monoterpene limonene is produced by more than 300 plants, and considered to be one of the most abundant terpenoids found on earth (Burdock, 1995). Due to its structural similarity with biphenyl (Fig. 1), limonene has been used as a soil amendment, inducing the biphenyl pathway for the purpose of ameliorating polychlorinated biphenyl (PCB) decontamination. This has been shown to enhance the degradation of PCBs to an equal or greater degree than does biphenyl (Hernandez *et al.*, 1997). More recently, Tandlich *et al.* (2001) demonstrated limonene s enhancing effect on PCB biodegradation when utilized as a potential inducer of the dioxygenase metabolic pathway in *Pseudomonas stutzeri.*

Rhodococcus sp. strain T104, which was previously defined as *Corynebacterium* sp. T104, is able to grow on biphenyl or terpenoidsblankincluding limonene, cymene, pinene, and abietic acid, using them as its sole carbon and energy source (Hernandez *et al.*, 1997). Previous work has also showed that T104 grown on limonene is capable of degrading up to 30% of both 2,2-dichlorobiphenyl and 4,4-dichlorobiphenyl (Jung *et al.*, 2001). We had also previously reported that limonene is able to induce both the upper and lower pathways of biphenyl degradation in T104 (Kim *et al.*, 2002). However, the molecular genetic mechanism by which limonene induces the catabolic pathway of biphenyl degradation in T104 still remains to be elucidated, and genetic information is not currently available. In order to clarify the mechanism by which limonene induces the biphenyl/PCB-degradative pathway in T104, we examined the genes that are specifically induced during growth on limonene.

A cosmid library of *Rhodococcus* sp. strain T104 was constructed by using the pWEB::TNC™ Cosmid Cloning Kit (EPICENTRE, USA), as recommended by the manufacturer. For generating more randomly distributed DNA fragments, genomic DNA was mechanically sheared using a syringe with a small-bore needle (Hamilton HPLC/GC syringe, 50 μl, USA). The 30-45 kb DNA fragments were then selected for in-gel ligation into the cosmid vector pWEB::TNC. The ligation mixture was packaged into bacteriophage λ particles using lambda packaging extract, and transfected into cells of *E. coli* strain EPI100. It had been previously established that T104 converts indole to indigo (blue colonies), a fact which indicates the involvement of an initial aromatic oxygenase (Ensley *et al.*, 1983), following growth in the presence of limonene. In biphenyl-grown T104 cells, on the other hand, no reaction

* To whom correspondence should be addressed.
(Tel) 82-2-2123-2651; (Fax) 82-2-312-5657
(E-mail) eungbin@yonsei.ac.kr

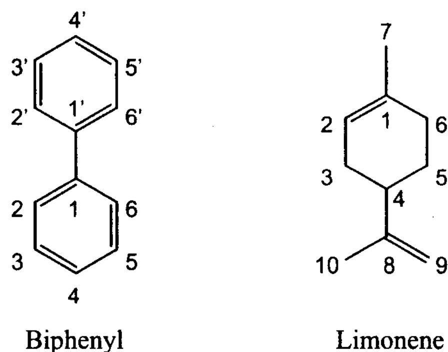
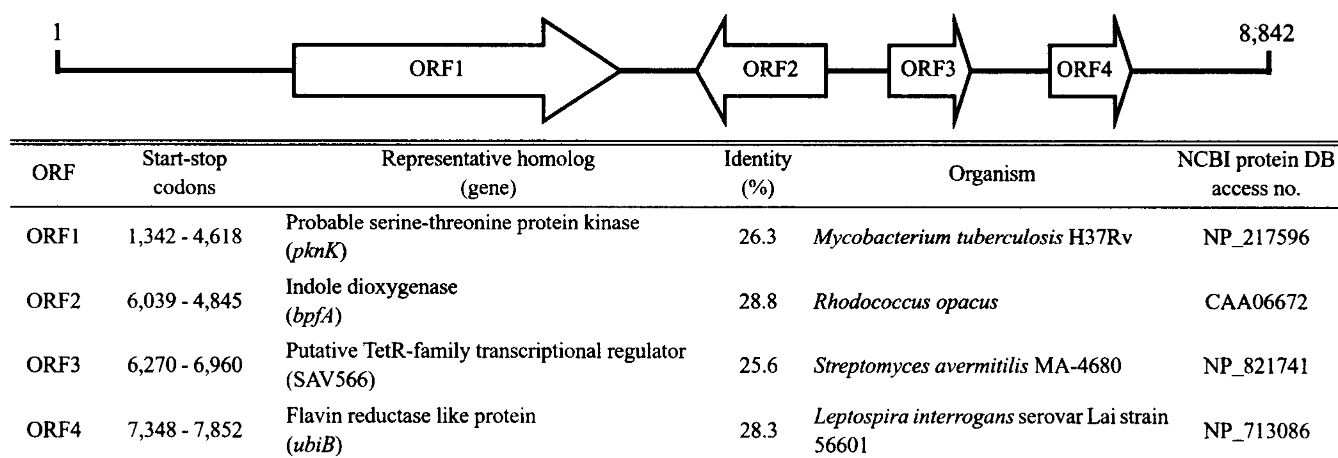


Fig. 1. Chemical structures and carbon atom numbering of biphenyl and limonene.

of this nature was observed (Kim *et al.*, 2002). Accordingly, the cosmid library was screened in *E. coli* for the ability to produce indigo during growth on LB agar plates, and one blue colony was isolated. Subsequently, the cosmid clone, designated as pKEB4001, was purified. In order to more precisely ascertain the DNA region which encodes for indigo-producing activity, pKEB4001 was digested by *Eco*RV and self-ligated, for the purpose of deleting the region between the innermost internal *Eco*RV site, and the unique *Eco*RV site on the cosmid vector. The resultant 14.6-kb plasmid pKEB4010 (8.8-kb insert plus 5.8-kb cosmid vector) retained the ability to produce indigo. This confirmed that the insert in pKEB4010 contains the oxygenase gene suspected to be involved in the degradation of limonene.

In order to further characterize the cloned DNA region, at the molecular level, the entire cloned region of pKEB4010 was sequenced. Two end sides of the insert were initially sequenced, and then primer walking was performed on the pKEB4010 to determine the complete nucleotide sequence of the insert. The obtained sequences were assembled into one contig using DNAStar Laser-gene program (DNAStar, USA). Analysis of the 8,842-bp sequenced region (Fig. 2) identified four open reading frames (ORFs), with putative ribosome-binding sites preceding each ORF. Three of them were in the same orientation (ORF1, ORF3, and ORF4), and ORF2 was in the opposite orientation. The product of ORF2 showed homology with an indole dioxygenase from *Rhodococcus opacus,* although the degree of identity was only 28.8%. This low degree of identity (less than 30%) was also characteristic of the products of the other three ORFs. The nucleotide sequences determined in this study were deposited into the GenBank database, under accession number AY575969.

In order to functionally confirm this gene, ORF2 was cloned and expressed in *E. coli.* ORF2 was amplified using PCR, and then cloned into the pGEM-T easy vector (Promega, USA), to construct the recombinant plasmid pKEB4020. PCR amplification was carried out in a PTC-150 MiniCycler (MJ Research, USA). Custom primers (forward primer, 5 -ATGACCCAAGTGATTGCC-3 ; reverse primer, 5 -TCAAATGAAGGATGTGAT-3) were supplied by Cosmo Genetech (Korea). The PCR reaction was performed in 20 μl of reaction mixture, containing approximately 100 ng of template DNA and 10 pmol of each primer, with ReadyMix *Taq* PCR Reaction Mix (Sigma, USA) according to the manufacturer s instructions. The thermal cycling program was a 10-min hot start (95°C), 30 cycles of 30 sec of denaturation (95°C), 30 sec of annealing (55°C), 1 min of extension (72°C), and a final 10 min of extension (72°C). The transformants harboring pKEB4020 formed blue colonies on the LB agar plates. One blue colony was inoculated into 100 ml of LB medium supplemented with ampicillin (100 μg/ml), and incubated at 37°C for 16 h. The culture was subsequently centrifuged at 10,000×g for 15 min, and the collected supernatant was extracted twice, with an equal volume of ethyl acetate. After



ORF	Start-stop codons	Representative homolog (gene)	Identity (%)	Organism	NCBI protein DB access no.
ORF1	1,342 - 4,618	Probable serine-threonine protein kinase (*pknK*)	26.3	*Mycobacterium tuberculosis* H37Rv	NP_217596
ORF2	6,039 - 4,845	Indole dioxygenase (*bpfA*)	28.8	*Rhodococcus opacus*	CAA06672
ORF3	6,270 - 6,960	Putative TetR-family transcriptional regulator (SAV566)	25.6	*Streptomyces avermitilis* MA-4680	NP_821741
ORF4	7,348 - 7,852	Flavin reductase like protein (*ubiB*)	28.3	*Leptospira interrogans* serovar Lai strain 56601	NP_713086

Fig. 2. Gene organization of a 8,842 base-pair region from *Rhodococcus* sp. strain T104. The direction of transcription is indicated by arrowheads. The nucleotide numbers are marked on the beginning and the end of the line. The column below the gene diagram shows representative homologous proteins retrieved by the BlastP search using the deduced amino acid sequences of the ORFs as queries.

dCollection

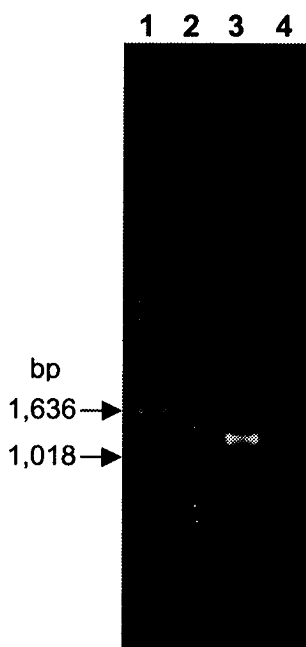


Fig. 3. Agarose gel electrophoresis of reverse transcription PCR products from total RNA. Lane 1, molecular weight markers; lanes 2, 3, and 4, *Rhodococcus* sp. T104 grown on glucose, limonene, and biphenyl, respectively.

concentration by a rotary evaporator, the residue was dissolved in a small volume of dimethylformamide (DMF). Indigo production was determined spectrophotometrically, by the method of O'Connor and Hartmans (1998). These results strongly indicated that ORF2 encodes a functional indole oxygenase.

One question that still remains is whether the identified indole oxygenase gene (ORF2) is actually expressed as a response to limonene. Reverse transcription (RT)-PCR experiments were performed to address this question. *Rhodococcus* sp. strain T104 cells reaching the exponential phase in 50 ml of mineral salts basal (MSB) medium (Stanier *et al.,* 1966), containing 20 mM glucose, were harvested and resuspended in 50 ml of fresh MSB medium. In order to induce the salient degradative genes, limonene and biphenyl were directly added to the suspension at a final concentration of 0.1% (v/v) and 100 ppm, respectively, and then further incubated at 30°C for one h. A negative control was prepared by adding glucose, up to a final concentration of 10 mM. The total RNA extraction was performed as described by Mahenthiralingam (1998). The extracted total RNA was further purified by spin column and DNase I treatments, according to manufacturer s instructions (QIAGEN, Germany), and then subjected to PCR with and without a prior reverse transcription step with the same primers, which were also used for the amplification of ORF2. RT-PCR reactions were performed in 25 μl batches containing 70 ng of total RNA, and 25 pmol of each primer, with QIAGEN OneStep RT-PCR Enzyme Mix (QIAGEN, Germany). Reverse transcription was performed at 50°C for 30 min, and was immediately followed by a denaturation step at 95°C for 15 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and finally 72°C for 10 min. As shown in Fig. 3, the PCR product of the expected size for ORF2 was amplified only from T104 cells grown on limonene, and was not amplified on glucose or biphenyl. These results clearly demonstrated that ORF2, which encodes indole oxygenase, is specifically induced by limonene. Thus, combined with the previous report that indigo formation was detectable following growth on limonene (Kim *et al.*, 2002), our study clearly shows that the identified indole oxygenase gene is implicated in the degradation of limonene by *Rhodococcus* sp. strain T104.

This work was supported by a grant from the Korea Research Foundation (KRF 2001-015-DP0443).

References

Burdock, G.A. 1995. Fenarolis handbook of flavor ingredients, 3rd ed. CRC Press, Boca Raton. Florida.

Ensley B.D., B.J. Ratzkin, T.D. Osslund, M.J. Simon, L.P. Wackett, and D.T. Gibson. 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. *Science* 222, 167-169.

Focht, D.D. 1995. Strategies for the improvement of aerobic metabolism of polychlorinated biphenyls. *Curr. Opin. Biotechnol.* 6, 341-346.

Hernandez, B.S., S.-C. Koh, M. Chial, and D.D. Focht. 1997. Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil. *Biodegradation* 8, 153-158.

Jung, K-J., E. Kim, J-S. So, and S-C. Koh. 2001. Specific biodegradation of polychlorinated biphenyls (PCBs) facilitated by plant terpenoids. *Biotechnol. Bioprocess Eng.* 6, 61-66.

Kim, D., M.J. Park, S-C. Koh, J-S. So, and E. Kim. 2002. Three separate pathways for the initial oxidation of limonene, biphenyl, and phenol by *Rhodococcus* sp. strain T104. *J. Microbiol.* 40, 86-89.

Mahenthiralingam, E. 1998. Extraction of RNA from mycobacteria. *Methods Mol. Biol.* 101, 65-75.

O'Connor, K.E. and S. Hartmans. 1998. Indigo formation by aromatic hydrocarbon-degrading bacteria. *Biotechnol. Lett.* 20, 219-223

Stanier, R.Y., N.J. Palleroni, and M. Duodoroff. 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* 43, 159-271

Tandlich, R., B. Brezna, and K. Dercova. 2001. The effect of terpenes on the biodegradation of polychlorinated biphenyls by *Pseudomonas stutzeri*. *Chemosphere* 44, 1547-1555.

dCollection