

NOTE

Plant Terpene-Induced Expression of Multiple Aromatic Ring Hydroxylation Oxygenase Genes in *Rhodococcus* sp. Strain T104

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(Received August 25, 2003 / Accepted November 12, 2003)

Recent studies have shown that some of the PCB (polychlorinated biphenyl)-degraders are able to effectively degrade PCB in the presence of monoterpenes, which act as inducers for the degradation pathway. *Rhodococcus* sp. T104, an effective PCB degrader, has been shown to induce the degradation pathway by utilizing limonenes, cymenes, carvones, and pinenes as sole carbon sources which can be found in the natural environment. Moreover, the strain T104 proved to possess three separate oxidation pathways of limonene, biphenyl, and phenol. Of these three, the limonene can also induce the biphenyl degradation pathway. In this work, we report the presence of three distinct genes for aromatic oxygenase, which are putatively involved in the degradation of aromatic substrates including biphenyl, limonene, and phenol, through PCR amplification and denaturing gradient gel electrophoresis (DGGE). The genes were differentially expressed and well induced by limonene, cymene, and plant extract A compared to biphenyl and/or glucose. This indicates that substrate specificity must be taken into account when biodegradation of the target compounds are facilitated by the plant natural substrates.

Key words: *Rhodococcus* sp., DGGE, RT-PCR, PCBs, aromatic oxygenases, plant terpenes

Polychlorinated biphenyls (PCBs) were once widely used as very useful industrial chemicals such as solvent extenders, plasticizers, and flame retardants (Furukawa *et al.*, 1982; Abramowicz *et al.*, 1990), but they are now on the U.S. EPA list of priority pollutants because of their potential toxicity. Biphenyl has conventionally been used as a growth substrate with which to isolate and grow bacteria that degrade PCB congeners (Bedard *et al.*, 1987), and also has been used to enhance their biodegradation in soil (Focht and Brunner, 1985) and sediments (Harkness *et al.*, 1993). However, since use of biphenyl as a soil amendment is not possible due to its adverse health effects and high costs (Lewis, 1989; Robinson *et al.*, 1994), efforts have been made to find alternative natural substrates, which are nontoxic and can induce the biphenyl/PCB pathway, for the purpose of PCB decontamination (Focht, 1995). We previously reported that some of the PCB-degraders are able to degrade PCB congeners effectively in the presence of monoterpenes (*e.g.*, limonenes, cymenes, carvones, and pinenes), which act as inducers

for the PCB-degradation pathway (Park *et al.*, 1999; Jung *et al.*, 2001) (Fig. 1). More recently, we also showed that an effective PCB degrader, *Rhodococcus* sp. T104, possesses three separate oxidation pathways of limonene, biphenyl, and phenol (Kim *et al.*, 2002), and among these three, limonene induces biphenyl degradation pathway. The fact that biphenyl and limonene induced almost the same amount of catechol 1,2-dioxygenase activity indicates that limonene can induce both upper and lower pathways of biphenyl degradation by T104. The presence of multiple aromatic oxygenase genes has also been reported in *Sphingomonas yanoikuyae* B1 (Bae and Kim, 2000; Song *et al.*, 2000). The strain simultaneously carried the genes such as *bphA*, *bphB*, *bphC*, *xylL*, and *nahE*. Recently, two strains of *Rhodococcus* sp. were reported to oxidize limonene, and one of these strains, *Rhodococcus opaquus* PWD4, grown on toluene, was responsible for the conversion of limonene to *trans*-carveol. The conversion pathway was inducible (van der Werf *et al.*, 1999; Duetz *et al.*, 2001). Thus, the present work was initiated as an initial effort to elucidate the induction mechanism in more detail at the molecular level.

Recently, Kitagawa *et al.* (2001) detected five different aromatic ring hydroxylation dioxygenase genes by dena-

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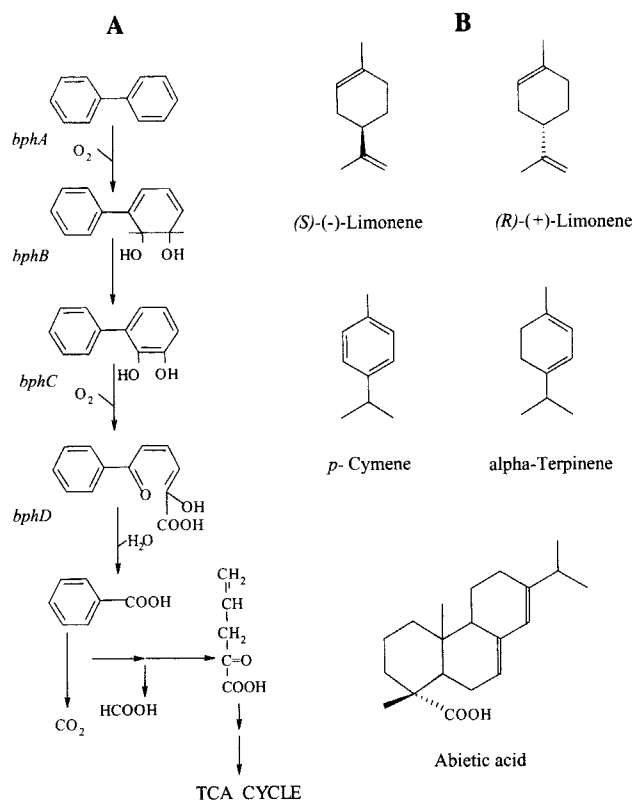


Fig. 1. Bacterial metabolic pathway of biphenyl (A) and structural similarity of various terpenes to biphenyl (B) (Adapted from Jung *et al.*, 2001). The genes, *bphA*, *bphB*, *bphC*, and *bphD*, encode biphenyl dioxygenases, dihydrodiol dehydrogenase, 2,3-dihydroxybiphenyl dioxygenases, and 2-hydroxy-6-phenylhexa-2,4-dienoic acid hydrolase, respectively.

turing gradient gel electrophoresis (DGGE) of PCR products. They were able to be amplified using degenerate primers corresponding to conserved regions in the aromatic dioxygenases from a PCB-degrading strain, *Rhodococcus* sp. RHA1. The same set of degenerate PCR primers (forward, 5'-TGCASSTWTCACGGSTGG-3'; reverse, 5'-CTCGACTCCGAGCTTCCAGTT-3') was tested against the total genomic DNA of T104. A total of 50 μ l of PCR reaction mixture contains approximately 100 ng of genomic DNA, 4.8 pmol of each primer, 0.2 mM of each dNTP, 5 μ l of 10X PCR buffer, and 2.5 units of *Taq* DNA polymerase (iNtRON Biotechnology, Korea). The amplification was carried out in a thermal cycler (MJ Research, MA) that was programmed for one cycle of 5 min at 94°C, a total of 25 cycles of 30 sec at 95°C, 30 sec at 55°C, 1 min at 72°C, and a final incubation of 7 min at 72°C followed by 4°C. Indeed, as shown in Fig. 2A, the expected size of DNA (c.a. 300 bp) was amplified. Subsequently, electrophoresis of the PCR product was performed in denatured gradient gels (10% acrylamide and denaturation gradient ranging from 45 to 75%). Gels were run for 14 h at 70 V using Dcode Universal Mutation System (Bio-Rad Laboratories, CA). The running buffer temperature was maintained at 60°C throughout the experiment.

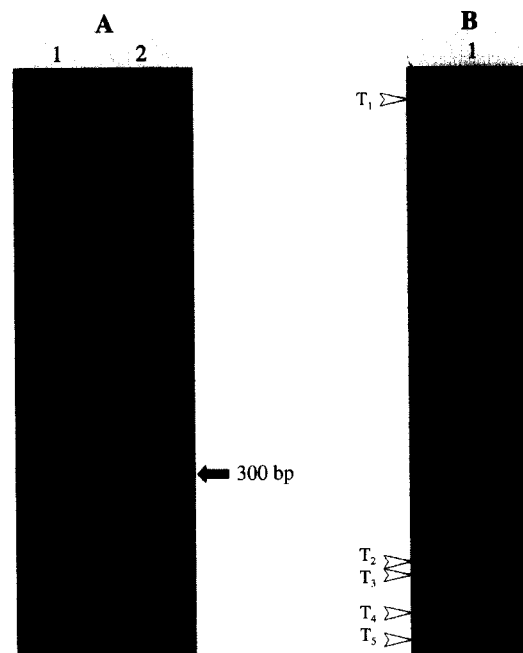


Fig. 2. PCR amplification of putative multiple aromatic ring hydroxylation dioxygenase genes in *Rhodococcus* sp. strain T104 (panel A) and DGGE separation of the putative genes (panel B). A apparent single fragment was amplified using the degenerate primers designed from multiple aromatic dioxygenases (panel A) but the fragment was separated into five distinctive fragments in the DGGE gel depending upon DNA sequence differences (panel B).

The gels were then stained by gently shaking for 15 min in 50 ml of 1X TAE containing ethidium bromide (100 ppm). The amplified DNA fragment was separated into the five distinctive bands designated as genes T₁, T₂, T₃, T₄ and T₅ (Fig. 2B). Each band was cut and eluted by freezing and thawing treatments for 5 min (repeated 3 times). Then, the eluted fragments were subjected to a second phase amplification using the same primer set, and the amplified fragments were identified on an agarose gel. The remaining PCR products were subjected to a PCR-based, direct sequence analysis performed by Takara Korea (Korea), and the sequences were comparatively analyzed in reference to the GenBank in NCBI.

Sequence analysis data of the putative dioxygenase genes has shown that they matched with the sequences of the oxygenases involved in the degradation of aromatic compounds (data not shown). Herein, the genes T₁, T₂, and T₄ were shown to have the highest similarities to a gene encoding for the oxidation enzyme of a diaromatic ring compound other than biphenyl. The three genes, despite their different mobility in the DGGE gel, turned out to be the same gene. This inaccuracy of DGGE analysis appears to be due to artificial microheterogeneity, resulting in the several bands that were generated from the single target DNA sequence (Kisand and Wikner, 2003). The genes T₃ and T₅ were shown to have the highest similarities to genes encoding oxidation enzymes of monoar-

omatic ring compounds. The gene expression induced by a few representative natural substrates was also examined. The primer sets (forward and reverse ones) were designed from the sequences of the three genes (T1, T3 and T5) using the primer design program that was made available by Bioneer, Co., Ltd. (Korea). The primer sets used for RT-PCR, in the examination of these gene transcription, were: forward 5'-CTGCCAGATCGATGAAGTAT-3' and reverse 5'-CGGT-TACGAGGATTACAAGA-3' (PCR product size 167 bp) for T1; forward 5'-CGAGGAACGTAAGCAGATCC-3' and reverse 5'-CGAGGATTACAAGAGCGAGC-3' (PCR product size 128 bp) for T3; forward 5'-GGATCGAAGTTCAC-GAAGTA-3' and reverse 5'-CGGTACGAGGATTACAAG-AG-3' (PCR product size 103 bp) for T5. For transcription analysis, the total RNA was first extracted from each late log phase culture, which was grown on the natural growth substrate (limonene, cymene, and plant extract A), biphenyl and glucose, according to the preceding report (Park *et al.*, 1999). 10 ng-2 µg of the extracted RNA was used for reverse transcription that was performed according to the protocol recommended by Super-bio Co., Ltd (Korea). Gene expression of the three genes in T104, grown in different substrates, was monitored through RT-PCR (Fig. 3). The three target genes was differentially induced by natural substrates (limonene, cymene, and plant extract A): biphenyl and glucose. Expression of the gene T1 (167 bp) was the strongest in cymene,

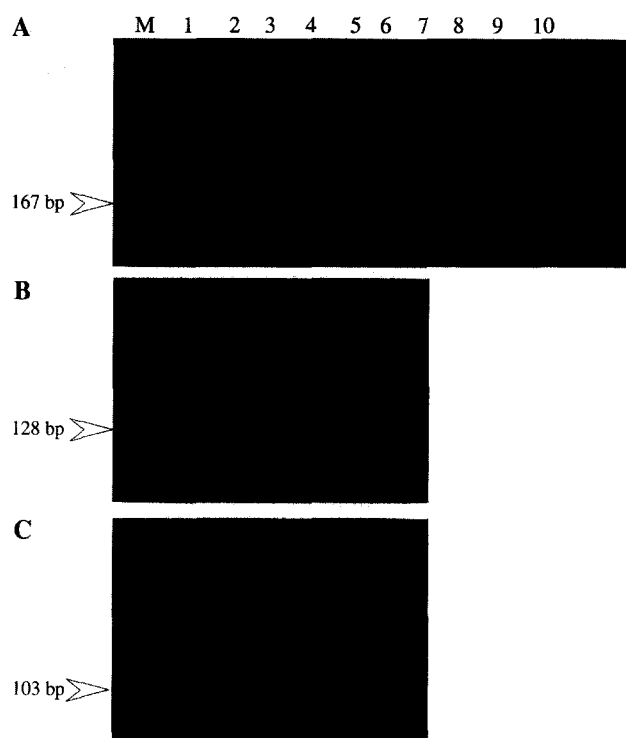


Fig. 3. Through RT-PCR was monitored the expression of multiple aromatic ring hydroxylation oxygenase genes T₁ (A), T₃ (B) and T₅ (C) in *Rhodococcus* sp. strain T104 grown in various substrates (lane 1, biphenyl; lane 2, limonene; lane 3, cymene; lane 4, Plant extract A; lane 5, glucose; lanes 6-10 negative controls of lanes 1-5 (target RNA was digested with RNase).

evenly followed by limonene and plant extract A while biphenyl was the least effective in the induction (Fig. 3A). Here, the gene was also induced by glucose and equivalently to limonene and plant extract A. There was no amplification when the total RNA treated with RNase was used as a template for RT-PCR (Fig. 3A, lanes 6-10). For gene T3 (128 bp), cymene and plant extract A appeared to be the strongest inducers, equally followed by limonene, biphenyl and glucose (Fig. 3B). The gene T5 (103 bp), however, was most strongly induced by limonene, cymene, and plant extract A compared with biphenyl and glucose (Fig. 3C). The differential expression of these genes was clearly affected depending upon the growth substrates used. Hence, the substrate specificity must be taken into account when biodegradation of the target compounds is considered. To a certain extent, glucose was also an inducer for the gene expressions tested in this study. This is not surprising because ortho-cleavage dioxygenase of catechol of T104 was induced by glucose at about one third strength of the induction level created by limonene or biphenyl (Kim *et al.*, 2002). Previous reports (Gilbert and Crowley, 1997; Tandlich *et al.*, 2001) reported that plant compounds of the p-menthane structure motif (cymene, isoprene, carvone and limonene) could promote a stronger PCB cometabolism activity than biphenyl. PCB degradation, however, appears to be significantly influenced by the enantioselectivity exerted by the degrader, the congener and the co-substrate (Singer *et al.*, 2002). Meanwhile, the plant compounds appeared to induce not only the bph gene (Park *et al.*, 1999) but also other potential aromatic oxygenase genes (Master and Mohn, 2001). This indicates that multiple aromatic oxygenase genes, identified in this study, will be useful in elucidating the mechanism of PCB and target compound degradation through enzymes other than the bph gene-encoded enzyme system. Our research team has been working on the bph gene expression of PCB degraders that utilize plant terpenes as both the only carbon source and inducer. Thus far, only a few Gram-positive bacteria of PCB-degraders were observed to induce the PCB-degradation pathway utilizing various plant mono-terpenes as co-substrates (Jung *et al.*, 2001). Also, *Rhodococcus* sp. strain T104 was proven to be one of the most effective PCB degrader that can utilize terpenes and induce potential aromatics-degradation pathway. Findings of this study will be of environmental significance because monocyclic aromatics as pollutants in a site may act as co-substrates that can influence the composition and activity of biphenyl-metabolizing communities (Abraham *et al.*, 2002). The use of plant terpenes as a growth substrate and an inducer for the various degradation pathways has clear advantages in the bioremediation of environmental pollutants: their diversity in structures, ubiquity, and non-toxicity in the environment.

Acknowledgment

We thank Dr. Suk-Kyun Han for his valuable help for DGGE analysis. This work was supported by a grant (KRF-2000-015-DP0340) from the Korea Research Foundation.

References

- Abraham, W.R., B. Nogales, P.N. Golyshin, D.H. Pieper, and K.N. Timmis. 2002. Polychlorinated biphenyl-degrading microbial communities in soils and sediments. *Curr. Opin. Microbiol.* 5, 246-253.
- Abramowicz, D.A. 1990. Aerobic and anaerobic biodegradation of PCBs: a review. *Crit. Rev. Biotechnol.* 10, 241-251.
- Bae, M.H. and E.B. Kim. 2000. Association of a common reductase with multiple aromatic terminal dioxygenases in *Sphingomonas yanoikuyae* strain B1. *J. Microbiol.* 38, 40-43.
- Bedard, D.L., M.L. Haberl, R.J. May, and M.J. Brennan. 1987. Evidence for novel mechanism of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. *Appl. Environ. Microbiol.* 53, 1103-1112.
- Duetz, W.A., A.H. Fjallman, S. Ren, C. Jourdat, and B. Witholt. 2001. Biotransformation of D-limonene to (+) trans-carveol by toluene-grown *Rhodococcus opacus* PWD4 cells. *Appl. Environ. Microbiol.* 67, 2829-2832.
- Focht, D.D. and W. Brunner. 1985. Kinetics of biphenyl and polychlorinated biphenyl metabolism in soil. *Appl. Environ. Microbiol.* 50, 1058-1063.
- Focht, D.D. 1995. Strategies for the improvement of aerobic metabolism of polychlorinated biphenyls. *Curr. Opin. Biotechnol.* 6, 341-346.
- Furukawa, K. 1982. Microbial degradation of polychlorinated biphenyls (PCBs), p. 33-57. In A.M. Chakrabarty (ed.), *Biodegradation and detoxification of environmental pollutants*. CRC Press, Inc., Boca Raton, Fla.
- Gilbert, E.S. and D.E. Crowley. 1997. Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. strain B1B. *Appl. Environ. Microbiol.* 63, 1933-1938.
- Harkness, M.R., J.B. McDermott, D.A. Abramowicz, J.J. Salvo, W.P. Flanagan, M.L. Stephens, F.J. Mondello, R.J. May, J.H. Lobos, K.M. Carroll, A.A. Bracco, K.M. Fish, G.L. Warner, P.R. Wilson, D. K. Dietrich, D.T. Lin, C.B. Morgan, and W.L. Gately. 1993. *In situ* stimulation of aerobic PCB biodegradation in Hudson River sediments. *Science*. 259, 503-507.
- Jung, K.J., E.B. 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.K., M.J. Park, S.C. Koh, J.S. So, and E.B. Kim. 2002. Three separate pathways for the initial oxidation of limonene, biphenyl, and phenol by *Rhodococcus* sp. strain T104. *J. Microbiol.* 40, 86-89.
- Kisand, V. and J. Wikner. 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. *J. Microbiol. Methods*. 54, 183-191.
- Kitagawa, W., A. Suzuki, T. Hoaki, E. Masai, and M. Fukuda. 2001. Multiplicity of aromatic ring hydroxylation dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1 demonstrated by denaturing gradient gel electrophoresis. *Biosci. Biotechnol. Biochem.* 65, 1907-1911.
- Lewis, R.J., Sr. 1989. *Saxs Dangerous Properties of Industrial Materials*, 8th ed. van Nostrand Reinhold, New York, N.Y.
- Master, E.R. and W.W. Mohn. 2001. Induction of *bphA*, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. *Appl. Environ. Microbiol.* 67, 2669-2676.
- Park, Y.I., J.S. So, and S.C. Koh. 1999. Induction by carvone of polychlorinated biphenyl(PCB)-degradative pathway in *Alcaligenes eutrophus* H850 and its molecular monitoring. *J. Microbiol. Biotechnol.* 9, 804-810.
- Robinson, G.K. and M.J. Lenn. 1994. The bioremediation of polychlorinated biphenyls (PCBs): problems and perspectives. *Biotechnol. Genet. Eng. Rev.* 12:139-188.
- Singer, A.C., C.S. Wong, and D.E. Crowley. 2002. Differential enantioselective transformation of atropisomeric polychlorinated biphenyls by multiple bacterial strains with different inducing compounds. *Appl. Environ. Microbiol.* 68, 5756-5759.
- Song, J.M., J.H. Sung, Y.M. Kim, G.J. Zylstra, and E.B. Kim. 2000. Roles of the *meta*- and the *ortho*-cleavage pathways for the efficient utilization of aromatic hydrocarbons by *Sphingomonas yanoikuyae* B1. *J. Microbiol.* 38, 245-249.
- 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.
- van der Werf, M.J., H.J. Swarts, and J.A. Bont. 1999. *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. *Appl. Environ. Microbiol.* 65, 2092-2102.