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NOTE

Three Separate Pathways for the Initial Oxidation of Limonene, Biphenyl, and Phenol by *Rhodococcus* sp. Strain T104

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***Rhodococcus* sp. strain T104, which is able to grow on either biphenyl or limonene, was found to utilize phenol as sole carbon and energy sources. Furthermore, T104 was positively identified to possess three separate pathways for the degradation of limonene, phenol, and biphenyl. 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 for biphenyl degradation by T104.**

Key words: *Rhodococcus*, limonene, biphenyl, phenol, ring-cleavage dioxygenase

Polychlorinated biphenyls (PCBs), once a very useful industrial chemical, are on the U.S. EPA list of priority pollutants because of their potential toxicity. In the natural environment, anaerobic dechlorination typically occurs first with reduction of higher chlorinated PCBs, and then these less chlorinated congeners are aerobically degraded to chlorobenzoates (Quensen III *et al.*, 1988; Brown *et al.*, 1989; Furukawa, 1994). 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 to enhance their biodegradation in soil (Focht and Brunner, 1985) and sediments (Harkness *et al.*, 1993). However, since use of biphenyl as a soil additive is not possible due to its adverse health effects and cost (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).

Terpenoids, which have structural similarities with biphenyl, were suspected to serve as natural substrates for the induction of the biphenyl degradation pathway (Focht, 1995), and subsequently shown to enhance PCB degradation to an equal or greater extent than biphenyl (Hernandez *et al.*, 1997; Park *et al.*, 1999). Limonene is produced by more than 300 plants and considered one of the most abundant terpenoids found on earth (Burdock, 1995). Previously, we showed that *Rhodococcus* sp. strain T104 (originally *Corynebacterium* strain T104 [Hernandez *et al.*, 1997]) grown on limonene is capable of degrading 2,2'-dichlorobiphenyl (2,2'-DCBp) and 4,4'-dichlorobiphenyl (4,4'-DCBp) up to 30% (Jung *et al.*, 2001). Although limonene is known to induce the biphenyl-degradative pathway in T104, however, the exact induction mechanisms still remain to be elucidated. Thus, the present work was initiated to examine how the degradative pathways of limonene and biphenyl are related to each other and to gain an insight into how the biphenyl-degradative pathway in T104 is induced by limonene.

Previous work showed that *Rhodococcus* sp. strain T104 is able to grow on biphenyl or various terpenoids including limonene, cymene, pinene, and abietic acid as sole carbon and energy sources (Hernandez *et al.*, 1997). Since previous test substrates did not include other aromatic hydrocarbons, the ability of T104 to grow on different aromatic compounds was determined by the rate of colony formation on MSB plates in the presence of each substrate as the sole carbon source (Table 1). T104 grew well on phenol forming 1.0 mm sized colonies in less than 5 days. However, 3-, 4-chlorobenzoates are not used as growth substrates by T104 although benzoate can serve as the sole carbon and energy sources. Considering the potential toxicity of chlorobenzoates to bacteria (Park *et al.*, 2001) this observation raises the possibility that chlo-

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Table 1. Comparison of growth characteristics of *Rhodococcus* sp. strains T104 and DK210

	Biphenyl	Limonene	Phenol	Benzoate	3-chlorobenzoate	4-chlorobenzoate
T104	+	+++	++	+	-	-
DK210	-	+++	++	+	-	-

The ability of bacterial strains to use the volatile carbon sources biphenyl, limonene, and phenol was determined by providing the source in the vapor phase for cells that were inoculated on MSB agar (Stainer *et al*, 1966). Plates were placed in screw-capped jars and were incubated at 30°C. Biphenyl crystals were placed directly in the lids of inoculated plates. A cotton-stoppered glass tube containing either limonene or phenol was placed in the lids of uninoculated plates. (Chloro) benzoates were provided at the concentration of 5 mM. Three, two, and one plus signs indicate the formation of a 1.0 mm-in-diameter colony within 3, 5, and 7 days, respectively. A minus sign denotes the compound could not serve as the sole carbon and energy source.

robenzoates might be bottlenecks of PCB degradation by T104. It should also be noted that T104 was able to convert indole to indigo (blue colonies), which is indicative of an initial oxygenase (Eaton and Timmis, 1986; Ensley *et al.*, 1983), following growth in the presence of limonene while biphenyl- or phenol-grown cells of T104 failed to perform such a reaction. This means that the initial oxygenase for limonene is distinct from those for biphenyl and phenol.

Jung *et al.* (2001) reported that the initial biphenyl-degradative pathway of T104 is induced by either biphenyl or limonene by showing that the limonene-induced cells of T104 transform 2,2-DCBp and 4,4-DCBp to the corresponding ring cleavage product (compound IV in Fig. 1). Benzoate, a central intermediate during biphenyl degradation, is converted to catechol, which can be channeled into either the *meta*- or *ortho*-cleavage pathway. Accordingly, questions were raised whether T104 possesses either or both ring-cleavage pathways and whether the pathways are induced by either biphenyl or limonene. In order to address this question, the activities of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) were examined in cells of T104 grown in the presence of biphenyl or limonene. Bacterial cells reaching the exponential phase on 20 mM glucose were harvested and resuspended in 200 ml of fresh MSB medium. In order to induce the ring-cleavage pathway, limonene (0.1%) or biphenyl (500 ppm) was directly added to the suspension and further incubated at 30°C for three days. The induced cells were harvested, washed in a half volume of 1X PBS (phosphate-buffered saline; 140 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, and 1.8 mM KH_2PO_4 [pH 7.4]), suspended in 5 ml of 50 mM MOPS buffer (pH 7.8) containing 1 mM ascorbic acid, 10% acetone, 10% glycerol, and 100 μM $FeSO_4$, and disrupted by sonication. Unbroken cells and cell debris were removed by centrifugation at 10,000 x *g* for 30 min. The resulting supernatant was used as the enzyme solution. C12O and C23O activity was assayed spectrophotometrically by measuring the increase in the absorbance at the corresponding wavelength of each ring-cleavage product formed from each substrate. As summarized in Table 2, almost an equal amount of C12O activity was detected in both biphenyl- and limonene grown cells. In contrast, no C23O activity was measured in the same samples (data not shown). These results indicate that limonene is able to induce the downstream pathway of biphenyl degradation and benzoate is exclusively metabolized via the *ortho*-ring cleavage pathway. In general, catechol 1,2-dioxygenases cleave catechol at a high rate and accept 4-chlorocatechol as a poor substrate while chlorocatechol 1,2-dioxygenases

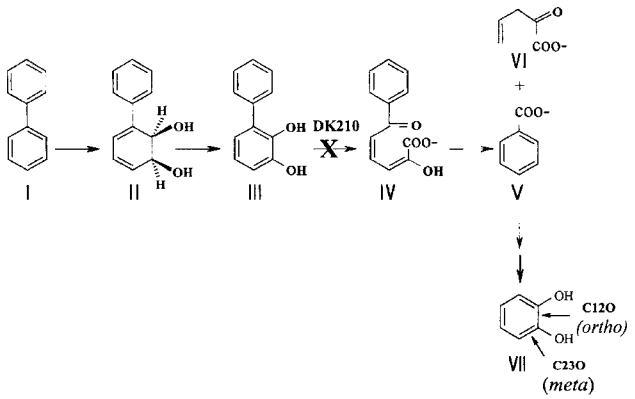

Fig. 1. Proposed catabolic pathway for the degradation of biphenyl by *Rhodococcus* sp. strain T104. I, biphenyl; II, (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-phenylcyclohexa-3,5-diene (*cis*-biphenyl dihydrodiol); III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; V, benzoate; VI, 2-oxo-penta-enoate; VII, catechol.

Table 2. Activities of *ortho*-cleavage dioxygenase (C12O) in cell extracts of T104 grown in the presence of biphenyl or limonene

Inducer	Enzyme activity (U/mg of protein)[a] against			
	Catechol	4-Chloro-catechol	3-Methy-catechol	4-Methy-catechol
Glucose	33.4 ± 2.5	20.0±2.6	17.6 ±2.7	22.5±2.5
Biphenyl	96.6 ± 5.5	123.1±2.3	67.9±4.7	68.8 ±2.9
Limonene	94.2± 2.9	103.8±2.2	65.9±1.7	90.9±13.5

[a]One unit of enzyme activity is defined as the formation of 1 μmol of product per min per mg of protein. Molar absorption coefficients are those reported by Dorn and Knackmuss (1978); ε = 16,800 $cm^{-1}M^{-1}$ at 260 nm for *cis,cis*-muconate, ε = 12,400 $cm^{-1}M^{-1}$ at 260 nm for 3-chloromuconate, ε = 18,000 $cm^{-1}M^{-1}$ at 260 nm for 2-methyl-*cis,cis*-muconate, and ε = 14,300 $cm^{-1}M^{-1}$ at 255 nm for 3-methyl-*cis,cis*-muconate. The reaction mixture contained 50 mM Tris/HCl (pH 8.0), 1.3 mM EDTA, and an appropriate substrate at a final concentration of 0.4 mM. Protein content was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

have a preference for chlorocatechols (Reineke, 1998). In this context, C12O in T104 is better classified as a member of chlorocatechol 1,2-dioxygenases because it has maximal activity toward 4-chlorocatechol. Also, this result supports the hypothesis that chlorobenzoates might be bottlenecks of PCB degradation by T104.

In order to analyze the T104 catabolic pathway for limonene and biphenyl in more depth, UV mutagenesis was performed according to the method of Carlton *et al.* (1981) with slight modification. Overnight-grown cells were harvested, washed twice with 0.1 M $MgSO_4$ by centrifugation, and resuspended in the same solution. After serial dilutions with 0.1 M $MgSO_4$ each cell suspension was transferred to sterile glass petri plates and exposed to 254 nm UV light positioned 22 cm above the open petri plates for 90 sec. The treated cell suspensions were plated out on MSB agar plates containing 20 mM glucose (master plates) and incubated at 30°C in the dark. Toothpicks were used to transfer colonies from the master plates onto MSB plates, which were subsequently incubated under a saturated atmosphere of limonene or biphenyl and monitored for the loss of ability to grow on limonene or biphenyl as sole carbon and energy sources. After screening approximately 1,500 colonies one mutant strain, designated DK210, was found to be unable to grow on biphenyl while retaining the ability to grow on limonene and phenol (Table 1). Combined with the above indigo formation data, these results indicate that T104 possesses three separate initial pathways for the degradation of limonene, phenol, and biphenyl. Since DK210 is still able to grow on benzoate (Table 1) the mutation was suspected to occur in the gene(s) responsible for degrading biphenyl to benzoates. The formation of the yellow ring fission product of biphenyl (compound IV in Fig. 1), which is obvious in the wild-type strain, was not detected when the culture supernatant was monitored spectrophotometrically at 434 nm during the growth of DK210 on glucose in the presence of biphenyl. Also, a colorimetric test was performed to detect phenolic intermediates, such as 2,3-dihydroxybiphenyl (compound III in Fig. 1), in the culture supernatant according to the method of Omori *et al.* (1991). A reddish purple color, indicative of the presence of phenolic compounds, developed on addition of two drops of a solution containing 0.85% 4-aminoantipyrine and 1.4% $NaHCO_3$ and one drop of 5.4% $K_3[Fe(CN)_6]$ solution to 1.0 ml of the culture supernatant of DK210 grown on glucose in the presence of biphenyl. This result, together with the growth test and metabolite analysis data, indicates that the gene encoding 2,3-dihydrxybiphenyl dioxygenase is disrupted in DK210.

In conclusion, *Rhodococcus* sp. strain T104 possesses three separate catabolic pathways for limonene, biphenyl, and phenol and limonene can induce both the upper and lower pathways for biphenyl degradation.

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