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Aromatic Hydroxylation of Indan by *o*-Xylene-Degrading *Rhodococcus* sp. Strain DK17▽

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The metabolically versatile *Rhodococcus* sp. strain DK17 utilizes indan as a growth substrate via the *o*-xylene pathway. Metabolite and reverse transcription-PCR analyses indicate that *o*-xylene dioxygenase hydroxylates indan at the 4,5 position of the aromatic moiety to form *cis*-indan-4,5-dihydrodiol, which is dehydrogenated to 4,5-indandiol by a dehydrogenase. 4,5-Indandiol undergoes ring cleavage by a *meta*-cleavage dioxygenase.

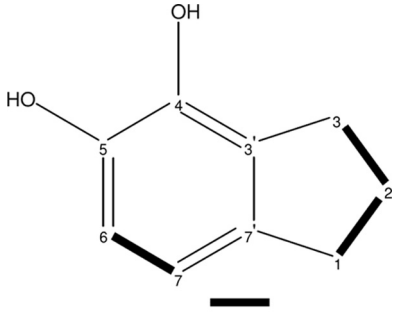
Rhodococcus sp. strain DK17 is capable of metabolizing various alkylbenzenes, such as *o*-xylene, toluene, and ethylbenzene, which are degraded through a common pathway initiated by a ring-oxidizing dioxygenase (*o*-xylene dioxygenase) (4, 6). One interesting aspect of alkylbenzene metabolism in DK17 is a unique regioselective hydroxylation that depends on the position(s) of the substitution group on the aromatic ring. More recently, we showed that the size and the position of the substitution groups on the aromatic ring affect the regioselectivity of aromatic oxidation by the DK17 *o*-xylene dioxygenase (5).

Indan is a bicyclic compound containing one aromatic ring plus one cyclopentane ring, the structure of which is similar to that of *o*-xylene, which is substituted at the two adjacent carbon atoms on the aromatic ring by a bulky side group (two methyl groups or a second alicyclic ring). Up to now, little in-depth work has been reported for bacterial metabolism of indan, although some pseudomonads are known to oxidize indan to 1-indanol by benzylic hydroxylation (1, 2, 3, 10). It was recently reported that some fungal species can perform enantioselective benzylic hydroxylation of indan to (1*R*)-indanol (8). Taken together, the currently available experimental data suggest benzylic oxygenation is a common initial step in indan degradation by microorganisms.

The ability of *Rhodococcus* sp. strain DK17 to grow on indan was determined by the rate of colony formation on mineral salts basal (MSB) plates (9) in the presence of indan (provided in the vapor phase in a glass bulb) as the sole carbon source. DK17 grew well on indan, excreting a yellow-colored compound into the media around the colonies and forming 1-mm-size colonies in less than 5 days, while there was no detectable growth on MSB plates without indan. The structural similarity between indan and *o*-xylene led us to investigate the possibility that the proteins catalyzing the initial steps in *o*-xylene metabolism are involved in indan degradation. To address this question, we examined the ability of the mutant strain DK180, which is unable to utilize *o*-xylene as a growth substrate due to the lack of the *meta*-cleavage dioxygenase enzyme (AkbC) (6), to grow with indan as the sole carbon source and found that it is unable to grow on indan.

Since DK180 accumulated brown metabolites from indan when grown on glucose as a second carbon source, attempts were made to identify the structure of the indan metabolite. DK180 cells were inoculated into MSB media containing 20

TABLE 1. ^{1}H (CD_3OD, 300 MHz) and ^{13}C (CD_3OD, 75 MHz) chemical shifts and HMBC (CD_3OD, 500 MHz) correlations of 4,5-indandiol



^{1}H - ^{1}H COSY

Position	Chemical shift[a]		HMBC[b]
	^{1}H	^{13}C	
C-1	2.78 (m)	33.4	2, 3, 3′, 7, 7′
C-2	2.02 (m)	26.9	1, 3, 3′, 7′
C-3	2.78 (m)	30.3	1, 2, 3′, 4, 7′
C-3′		131.5	
C-4		142.3	
C-5		144.3	
C-6	6.55 (d, J = 7.8)	114.6	4, 7′
C-7	6.50 (d, J = 7.8)	115.9	3′, 5
C-7′		137.5	

[a] Chemical shifts δ in ppm (multiplicity, J in Hz).
[b] Carbons coupled to the corresponding H atom.

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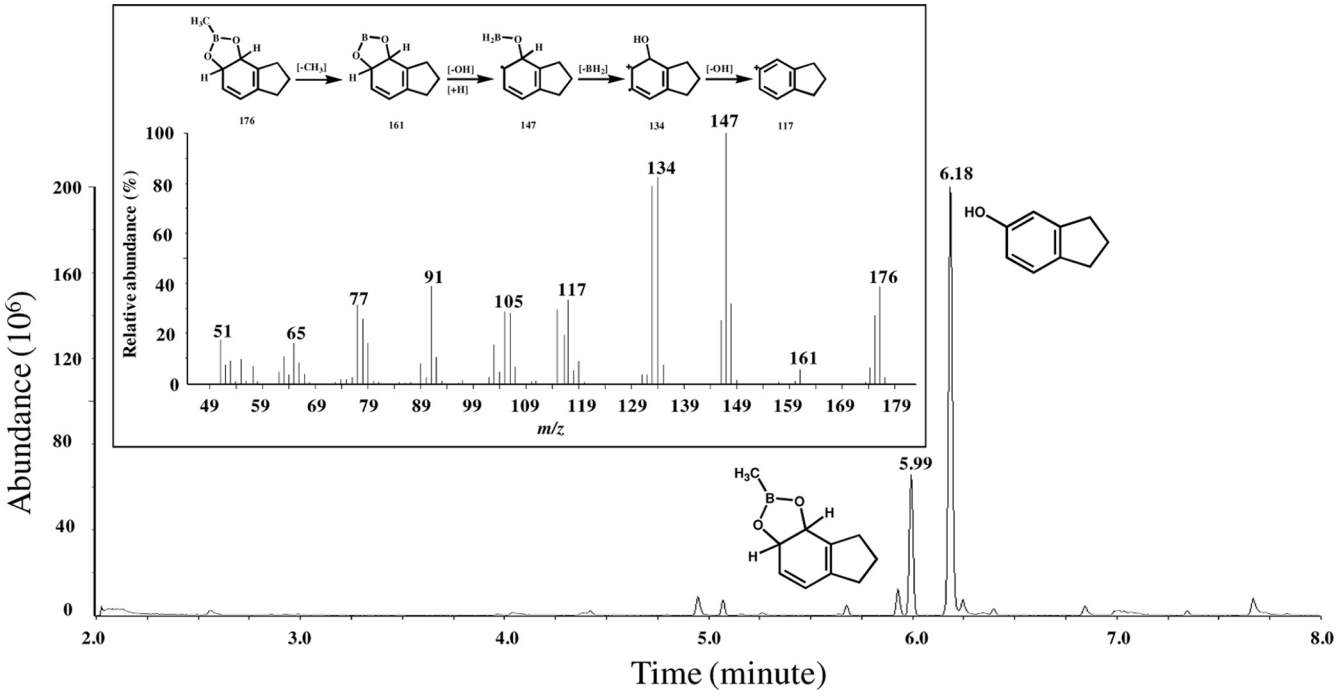

FIG. 1. Total ion chromatogram of metabolites formed during indan bioconversion by *E. coli* BL21(DE3) expressing *o*-xylene dioxygenase. The inset shows the electron ionization mass spectrum and the fragmentation pattern of the metabolite at a retention time (T_r) of 5.99 min.

mM glucose and incubated overnight at 30°C with shaking. A 10-ml sample of the overnight culture was used to inoculate 400 ml of fresh MSB plus 20 mM glucose medium with indan provided in the vapor phase and was incubated for 20 h. After removal of cells by centrifugation, the indan metabolites in the supernatant were extracted with ethyl acetate and analyzed by gas chromatography-mass spectrometry (GC-MS) using an HP 5972 mass detector/5890 gas chromatograph. The following conditions were used for GC: 1 ml He/min, on-column injection mode; oven temperature of 100°C for 1 min; thermal gradient, 10°C/min to 300°C and then holding at 300°C for 10 min. Analysis by GC-MS revealed one major peak for the indan (molecular weight, 118.18) metabolite that eluted at 8.63 min on the total ion chromatogram. When the 8.63-min metabolite was fragmented, a molecular ion (M^+) at *m/z* 150 (base peak ion) and a series of prominent ions due to the fission of M^+ at *m/z* 133 $[M\text{-}OH]^+$, 132 $[M\text{-}(OH, H)]^+$, and 131 $[M\text{-}(OH, 2H)]^+$ were produced, suggesting that the metabolite is a dihydroxylated indan.

The metabolite was purified by high-performance liquid chromatography, dissolved in perdeuterated methanol (CD_3OD), and analyzed by one-dimensional (^1H and ^{13}C nuclear magnetic resonance [NMR], Varian Unity-300) and two-dimensional (^1H-^1H correlated spectroscopy [COSY], heteronuclear single quantum coherence [HSQC], and heteronuclear multiple bond coherence [HMBC], Varian Unity-500) NMR techniques. Table 1 shows all of the ^1H and ^{13}C chemical shifts and coupling constants for the dihydroxylated indan and the ^1H-^{13}C heteronuclear multiple bond correlations, upon which the unambiguous assignment of all carbons and hydrogens was based. The protons on carbons 1, 2, 3, 6, and 7, which could be easily assigned on the basis of their chemical shifts and multiplicities, enabled the assignment of the carbons to which they are attached by direct correlation. Two cross peaks in the ^1H-^1H COSY implied the two protons on C-6 and C-7 are coupled to each other. Six protons on the alicyclic ring were detected at δ 2.02 (m, 2H) and 2.78 (m, 4H), and two aromatic protons that are reciprocally coupled were absorbed at δ 6.50 (d, J = 7.8 Hz) and δ 6.55 (d, J = 7.8 Hz), proving that the aromatic protons were attached at C-6 and C-7. Thus, based on the GC-MS and NMR data, the indan metabolite was clearly identified as 4,5-indandiol. This result confirms that the *meta*-cleavage dioxygenase encoded by the *akbC* gene is responsible in vivo for the degradation of indan by DK17.

The rigorous structural identification of 4,5-indandiol means that the initial oxidation of indan had occurred at the 4,5 positions on the aromatic ring carbon. Thus, to determine whether the DK17 *o*-xylene dioxygenase is responsible for the regioselective hydroxylation (dioxygenation), indan biotransformation experiments were performed under the same conditions previously described (4). In this study, *Escherichia coli* BL21(DE3) expressing the DK17 *o*-xylene dioxygenase (4) was cotransformed with a chaperon plasmid, pKJE7 (TaKaRa Bio Inc, Ohtsu, Japan), which provided higher expression and stable maintenance of the *o*-xylene dioxygenase enzyme, and the expression of *o*-xylene dioxygenase was induced by isopropyl-β-D-thiogalactopyranoside (1.0 mM) and arabinose (0.002%). The bioconversion product in the reaction supernatant was extracted with ethyl acetate, derivatized to a methaneboronate (6), and analyzed using a Perkin Elmer Clarus 500 MS/Clarus 500 gas chromatograph, following the same GC conditions as described above. Two significant indan metabolite peaks were detected, at 5.99 and 6.18 min on the total ion chromatogram (Fig. 1). The 5.99-min metabolite has a molecular ion at *m/z*

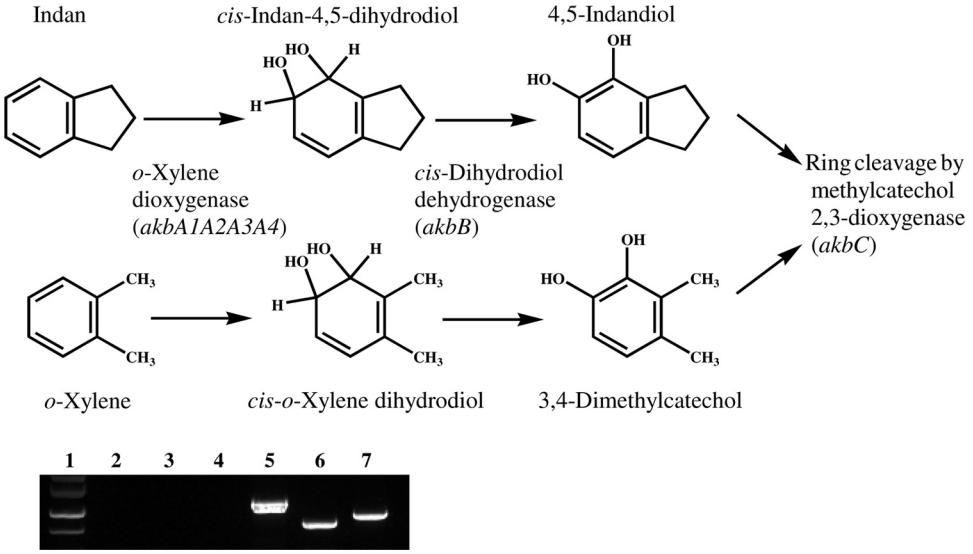

FIG. 2. Implication of *o*-xylene-degrading enzymes in indan biotransformation by *Rhodococcus* sp. strain DK17. Proposed pathways for early steps in indan and *o*-xylene degradation by *Rhodococcus* sp. strain DK17 are shown in the upper panel. Gene designations are shown in parentheses. The lower panel shows agarose gel electrophoresis of RT-PCR products for the oxygenase component large subunit of *o*-xylene dioxygenase (*akbA1*), *cis*-dihydrodiol dehydrogenase (*akbB*), and methylcatechol 2,3-dioxygenase (*akbC*) grown on glucose (lanes 2, 3, and 4) and indan (lanes 5, 6, and 7), respectively. Lane 1 was loaded with molecular weight markers (750, 1,000, and 1,500 bp).

176 and a base ion at *m*/*z* 147 [M-(CH_3, OH) + H]$^+$, suggesting that the metabolite is a methaneboronate derivative and the original form is a vicinally dihydroxylated indan (Fig. 1, inset). In addition, a major metabolite having a molecular ion at *m*/*z* 134 eluted at 6.18 min; the mass fragmentation patterns of this compound matched with those of 5-indanol in the GC-MS library database (NIST MS Search 2.0). One plausible explanation for the formation of a 5-indanol metabolite is that *cis*-indan-4,5-dihydrodiol would dehydrate to 5-indanol due to the electron-donating nature of the hydrogens bound to carbons 1 and 3. In fact, we previously observed that when expressed in *E. coli*, the *o*-xylene dioxygenase transformed *o*-xylene into 2,3- and 3,4-dimethylphenol, which were derived from an unstable *o*-xylene *cis*-3,4-dihydrodiol for the same reason. Accordingly, the apparent biotransformation of indan to *cis*-indan-4,5-dihydrodiol suggests that the *o*-xylene dioxygenase initiates the indan breakdown pathway in DK17.

Based on all the above results, one can reasonably propose that the indan breakdown pathway in DK17 is initiated via aromatic oxidation, leading to the formation of 4,5-indandiol, which undergoes ring cleavage by the *meta*-cleavage dioxygenase (Fig. 2, upper panel). This postulation is further corroborated by two other experimental observations: (i) the indan-specific induction of the genes encoding *o*-xylene dioxygenase, *cis*-dihydrodiol dehydrogenase, and *meta*-cleavage dioxygenase as confirmed by reverse transcription-PCR (RT-PCR) analysis (Fig. 2, lower panel) and (ii) the inability of the regulatory mutant strain DK183, which lacks the functional sensor kinase for the induction of *o*-xylene dioxygenase (7), to utilize indan as a growth substrate.

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