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Ongoing Analysis of the Plasmid Genome from *Rhodococcus* sp. Strain DK17: an Effort to Convert the Soil Bug into an Industrially Versatile Catalyst

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Members of the genus *Rhodococcus* demonstrate a remarkable ability to utilize a wide variety of natural organic and xenobiotic compounds including aliphatic, aromatic, and alicyclic hydrocarbons (Finnerty, 1992; Warhurst and Fewson, 1994; see also the special issue of Antonie Van Leeuwenhoek, volume 74). Besides the ability to degrade a broad spectrum of chemical compounds, many rhodococcal strains are known to catalyze the stereoselective oxidation of structurally different compounds such as indene (Treadway *et al.*, 1999), monoterpene (van der Werf *et al.*, 1999), aliphatic alkenes (Smith *et al.*, 1999), and phenylpropionitrile (Gilligan *et al.*, 1993). Accordingly, rhodococci have the great potential to synthesize valuable chemical synthons, and of particular interest is the incorporation of molecular oxygen into the aromatic nucleus to form vicinal arene *cis*-diols (Bell *et al.*, 1998; O'Brien *et al.*, 2002). For example, an aromatic dioxygenase from *Rhodococcus* sp. strain I24 was used for the oxidation of indene to *cis*-(1*S*,2*R*)-dihydroxyindan, which could serve as a precursor for a new anti-HIV drug indinavir (Treadway *et al.*, 1999).

To date, several gene clusters involved in the degradation of aromatics have been cloned from *Rhodococcus* spp. These genes are namely degradative genes for biphenyl from *Rhodococcus* sp. strain M5 (Wang *et al.*, 1995), *Rhodococcus globerulus* P6 (Asturias *et al.*, 1995), and *Rhodococcus* sp. strain RHA1 (Sakai *et al.*, 2003), isopropylbenzene-degrading genes from *Rhodococcus erythropolis* BD2 (Kesseler *et al.*, 1996), and benzoate dioxygenase genes from *Rhodococcus* sp. strain 19070 (Haddad *et al.*, 2001). However, no in depth genetic work has been reported for the abilities of *Rhodococcus* strains to degrade *o*-xylene.

We previously reported that the oxidation of *o*-xylene/toluene in *Rhodococcus* sp. DK17 is initiated by a ring-oxidizing oxygenase to form 3,4-dimethylcatechol/3- and 4-methylcatechols, and the genes encoding the initial steps in alkylbenzene metabolism are present on a 330-kb megaplasmid pDK2 (Kim *et al.*, 2002). More recently, we showed that the same initial oxygenase genes are apparently implicated in the regiospecific oxidation of *m*- and *p*-xylene to 2,4-dimethylresocinol and 2,5-dimethylhydroquinone, respectively (Kim *et al.*, 2003).

The genes encoding alkylbenzene degradation were cloned in a cosmid clone (designated

pKEB2002) and sequenced completely to reveal 35 ORFs. Among the ORFs were gene clusters encoding subunits of the terminal dioxygenase (*akbA1A2*) and ferredoxin (*akbA3*) of a putative three-component aromatic dioxygenase system. Subsequently, *akbA1A2A3* were amplified together by PCR, and the PCR product was cloned into the *E. coli* expression vector pCR®T7/CT-TOPO to generate a recombinant plasmid, pKEB051. The cells of *E. coli* BL21(DE3) harboring pKEB051 were incubated with *o*-xylene or ethylbenzene after induction by IPTG, and subsequently, the culture supernatant was extracted with ethyl acetate. Potential metabolites were stabilized by acetylation and analyzed by capillary GC-MS. *o*-Xylene was transformed to 2,3- and 3,4-dimethylphenol. In the case of ethylbenzene, *cis*-2,3-ethylbenzene dihydrodiol and a regioisomer (apparently *cis*-3,4-ethylbenzene dihydrodiol) were identified as oxidation products. It is already known that the initial oxidation reaction of alkylbenzenes is catalyzed by a common oxygenase in DK17. This, coupled with the rigorous structural identification of *cis*-dihydrodiols during ethylbenzene oxidation by AkbA1A2A3, strongly suggests that the metabolism of alkylbenzene in DK17 is initiated by a dioxygenase-catalyzed reaction. Considering that *o*-xylene dihydrodiol is extremely unstable and no catecholic compounds were detected despite thorough examination of all the significant peaks of the total ion chromatogram of *o*-xylene metabolites, the formation of 2,3- and 3,4-dimethylphenol from *o*-xylene by AkbA1A2A3 could be explained by spontaneous loss of one molecule of water from *o*-xylene dihydrodiol.

Another cosmid clone (designated pKEB2003) containing the genes for phthalate degradation was identified by colony blot hybridization with an aromatic oxygenase probe. The nucleotide sequence of the cloned DNA was completely determined. Extensive molecular analysis of the 40,643-bp sequenced region of pKEB2003 reveals that it contains a total of 36 ORFs, apparently divided into four functional categories: structural genes for phthalate metabolism, regulation, transposition, and miscellaneous or unknown functions. Among the ORFs were discovered two different sets of genes for an iron sulfur protein showing 77% and 68% identity with those of phthalate and terephthalate dioxygenases from *Arthrobacter keyseri* 12B and *Delftia* sp. T7, respectively. In order to determine the location of the genes for phthalate degradation in the genome a Southern blot was performed with PFGE-separated DNA from DK17 and pKEB2003 as a probe. Interestingly, DK17 has two hybridizing bands, namely chromosome and pDK2. Close examination of the PFGE gel revealed that the intensity of the DK17 chromosome band is unusually strong, suggesting coexistence of other genetic elements at the same location in the gel. PFGE under different conditions resolved approximately a 750-kb megaplasmid designated pDK3 from the band, which hybridized specifically to the pKEB2003 probe. To determine whether the entire cloned region in pKEB2003 hybridizes to both pDK2 and pDK3, smaller DNA fragments were utilized as probes in additional Southern blots. The probes derived from either end of the cloned region hybridized only to pDK3. This means that the cloned region in pKEB2003 originated from pDK3 and that DK17 has two copies of genes in the central region of the cosmid clone.

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