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Functional Genomic Studies on Alkylbenzene Degradation by *Rhodococcus* sp. Strain DK17

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Rhodococcus sp. strain DK17 was isolated from soil for the ability to grow on *o*xylene as the sole carbon and energy source. Although DK17 cannot grow on *m*- and *p*-xylene it is capable of growth on benzene, toluene, phenol, phthalates, benzoates, ethylbenzene, isopropylbenzene, and other alkylbenzene isomers. One UV-generated mutant strain DK176 simultaneously lost the ability to grow on *o*xylene, ethylbenzene, isopropylbenzene, benzene, and toluene although it can still grow on phenol and phthalate. The mutant strain is also unable to oxidize indole to indigo following growth in the presence of *o*xylene. This observation suggests the loss of an oxygenase that is involved in the initial oxidation of the (alkyl)benzenes tested. Another mutant strain, DK180, isolated for the inability to grow on *o*-xylene retains the ability to grow on benzene but is unable to grow on alkylbenzenes due to loss of a *meta*-cleavage dioxygenase needed for metabolism of methyl-substituted catechols. Further experiments showed that DK180 as well as the wild type strain DK17 has an *ortho*-cleavage pathway which is specifically induced by benzene but not by *o*xylene. These results indicate that DK17 possesses two different ring-cleavage pathways for the degradation of aromatic compounds although the initial oxidation reactions may be catalyzed by a common oxygenase. Gas chromatography-mass spectrometry (GC-MS) and 300 MHz proton nuclear magnetic resonance spectrometry clearly show that DK180 accumulates 3,4dimethylcatechol from *o*-xylene and both 3- and 4-methylcatechol from toluene. This means that there are two initial routes of oxidation of toluene by the strain. Pulsed field gel electrophoresis (PFGE) analysis demonstrated the presence of two large megaplasmids in the wild type strain DK17, one of which (pDK2) was lost in the mutant strain DK176. Since several other independently derived mutant strains unable to grow on alkylbenzenes are also missing pDK2 the genes encoding the initial steps in alkylbenzene metabolism (but not phenol, phthalate, and benzoate metabolism) appear to be present on this approximately 330-kb plasmid.

Although DK17 is unable to grow on *m*- and *p*-xylene, this strain could transform these two xylene isomers to some extent after induction by *o*xylene. The major compounds accumulating during the degradation of *m*- and *p*-xylene by DK17 were isolated by high-pressure liquid chromatography and identified by gas chromatography-mass spectrometric and ^{1}H nuclear magnetic resonance spectral techniques. Both xylene isomers were transformed to dihydroxylated compounds by what must be two

successive hydroxylation events: *m*-xylene was converted to 2,4-dimethylresorcinol and *p*xylene was converted to 2,5-dimethylhydroquinone. The rigorous structural identification of 2,4-dimethylresorcinol and 2,5-dimethylhydroquinone demonstrates that DK17 can perform distinct regioselective hydroxylations depending on the position of the substituent groups on the aromatic ring.

The wild-type strain DK17 was confirmed to possess at least two megaplasmids, designated pDK1 (~380 kb) and pDK2 (~330 kb), respectively. As described above, pDK2 is involved in the degradation of alkylbenzenes because a mutant strain DK176 missing pDK2 is unable to grow on alkylbenzenes. A question that thus arises is whether a pDK2-encoded oxygenase(s) is involved in the regioselective oxidation of *m*- and *p*-xylene. In order to address this question, the pDK2-cured mutant strain DK176 was examined for the ability to oxidize *m*- and *p*-xylene. No oxidized products were detected when DK176 was exposed to *m*- or *p*-xylene following growth on glucose in the presence of *o*xylene. Thus, the genes encoding the regiospecific oxidation of *m*- and *p*xylene also appear to be present on pDK2.

In order to study the genetic basis of the pathway in more detail, the genes responsible for degrading alkylbenzenes were cloned in a cosmid clone (pKEB2002). Subsequently, the nucleotide sequence of a 37,218-bp pDK2 plasmid DNA region, which possesses the alkylbenzene-degradative genes, was completely determined. Extensive molecular analysis of the region revealed that it contains a total of 36 ORFs. Database homology searches with the deduced amino acid sequences identified thirteen ORFs as putative genes for alkylbenzene degradation (named *akb* for alkylbenzene). Based on the homology analysis, the ORFs were apparently divided into three functional categories - structural genes for alkylbenzene metabolism (11 ORFs), regulatory functions (2 ORFs), and miscellaneous or unknown functions (23 ORFs) - although these genes are not organized into operons based on catabolic segments. RT-PCR experiments imply that two putative *akb* operons for alkylbenzene metabolism are transcribed as a corresponding transcriptional unit, respectively, and that transcription is specifically induced by alkylbenzenes.

Among the ORFs were gene clusters encoding subunits of a terminal dioxygenase (*akbA1A2*) and ferredoxin (*akbA3*) of a putative three-component aromatic dioxygenase system. Subsequently, *akbA1A2A3* were cloned into an *E. coli* expression vector to generate a recombinant plasmid, pKEB051. The cells of *E. coli* BL21(DE3) harboring pKEB051 were incubated with three xylene isomers (*o*-, *m*-, and *p*-xylene) or ethylbenzene after induction by IPTG, and subsequently, the potential metabolites were analyzed by 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, 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. This hypothesis is further supported by the fact that incubation of the expression clone with *m* and *p*-xylene leads to the formation of different dihydrodiols. Thus, regioselective bioconversion of three xylene isomers (*o*-, *m*-, and *p*-xylene) and ethylbenzene into dihydrodiols demonstrated that the pDK2-derived *akbA1A2A3* genes encode a novel three-component alkylbenzene dioxygenase.

A homolog (*akbC*) to ring-cleavage dioxygenase genes was analyzed to empirically confirm that it is the bona-fide *meta*-cleavage dioxygenase for alkylbenzene degradation by DK17. The *akbC* gene was cloned in an expression vector and the specific activities of AkbC produced by the resulting construct, pKEB052, against (methyl)catechols were assayed. The substrate specificities of AkbC matched those of the corresponding *meta*-cleavage dioxygenase present in the DK17 cells, which was induced by *o*xylene, toluene, or benzene.

The putative genes for a two-component signal transduction system (*akbS* and *akbT*) were detected near the putative aromatic dihydrodiol dehydrogenase gene (*akbB*). AkbS has the conserved sequence blocks, which are most common in histidine kinases from other aromatic-degrading *Rhodococcus* spp. AkbT possesses a typical response regulator motif. Based on homology search and transcriptional expression of the *akb* genes, *akbST* appear to be involved in the regulation of the *akb* genes for alkylbenzene metabolism by DK17.

Overall, the present work provides physiological and molecular evidence that the degradation of alkylbenzenes by *Rhodococcus* sp. strain DK17 is initiated by a three-component enzyme alkylbenzene dioxygenase, consisting of a reductase, a ferredoxin, and an iron sulfur protein, with the formation of corresponding *cis*-dihydrodiols. The latter compounds are subsequently dehydrogenated by a dehydrogenase to yield catecholic compounds, which is further metabolized through a *meta*-cleavage pathway.

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