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Metabolic Diversity of BTX-Degrading Bacteria: Focus on *o*-Xylene Degraders

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Methylbenzenes, which have been used in various industrial processes including the production of drugs, paints and enamels (Patty, 1963; Fishbein, 1985), consist of widespread environmental contaminants (Barbieri et al., 1993). Up to now many bacterial strains have been reported that have the capability to grow on different methylbenzenes including three xylene isomers as the sole sources of carbon and energy. During the past three decades much research has centered on determining the metabolism of *m*- and *p*-xylenes, and details of these metabolic pathways have been elucidated at the biochemical and molecular levels (for reviews see Assinder and Williams, 1990; Zylstra, 1994). In contrast, only a few reports have appeared on *o*-xylene-degraders, and the metabolic pathways for *o*-xylene degradation have not been clarified yet. In addition no *m*- or *p*-xylene degrading bacteria are known to be able to grow on *o*-xylene, which suggests the importance of the position of the methyl substituent on the aromatic ring in the selection of bacteria able to grow on the xylenes (Davis et al., 1968; Barbieri et al., 1993).

Currently, three different metabolic pathways for *o*-xylene degradation have been proposed (Fig. 1). *Nocardia* sp. (Raymond et al., 1967) and *Corynebacterium* sp. strain C125 (Schraa et al., 1987) are thought to metabolize *o*-xylene through an initial dioxygenase reaction on the aromatic ring to form *cis*-dihyrodiol although there is no direct evidence for the presence of a dioxygenase in both strains. In *Pseudomonas stutzeri* OX1, *o*-xylene is degraded initially through two successive monooxydations of the aromatic ring by a monooxygenase to form 2,3-dimethylphenol and 3,4-dimethylphenol simultaneously, which are converted into 3,4-dimethylcatechol and supposedly into 4,5-dimethylcatechol, respectively (Baggi et al., 1987;

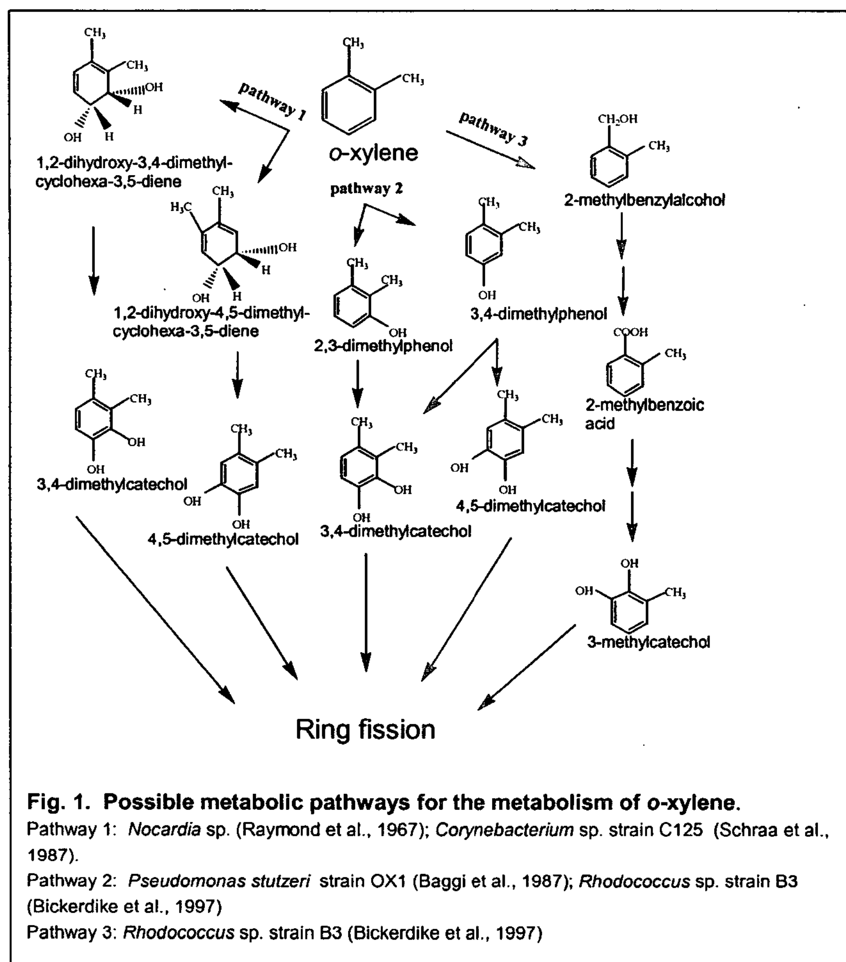


Fig. 1. Possible metabolic pathways for the metabolism of *o*-xylene.
Pathway 1: *Nocardia* sp. (Raymond et al., 1967); *Corynebacterium* sp. strain C125 (Schraa et al., 1987).
Pathway 2: *Pseudomonas stutzeri* strain OX1 (Baggi et al., 1987); *Rhodococcus* sp. strain B3 (Bickerdike et al., 1997)
Pathway 3: *Rhodococcus* sp. strain B3 (Bickerdike et al., 1997)

Bertoni et al., 1996). In *Rhodococcus* strain B3, two pathways are found to operate simultaneously, which are initiated by a monooxygenase. One pathway involves the oxidation of a methyl group to form 2-methylbenzylalcohol, which is oxidized via 2-methylbenzoic acid to 3-methylcatechol. The other pathway involves the oxidation of the aromatic ring to form 2,3-dimethylphenol, but the product of the followed oxidation, dimethylcatechol, is not yet identified (Bickerdike et al., 1997). Functional duplication of pathways for degrading of aromatic substrates is fairly common amongst bacteria which degrade methylbenzene derivatives (Barbieri et al., 1993; Johnson et al., 1997; Bolognese et al., 1999). For example toluene can be degraded by five different catabolic pathways, which are initiated by either mono- or dioxygenase (Zylstra, 1994). So it is not entirely unexpected that bacteria employ more than one catabolic pathway for *o*-xylene degradation. In this paper we report the isolation *Rhodococcus* sp. strain DK17 that utilizes *o*-xylene as sole carbon and energy

sources for growth and the structural identification of 3,4-dimethylphenol and 3,4-dimethylcatechol as key metabolites during *o*-xylene degradation by this strain.

1. Isolation and growth characteristics

A gram-positive bacterial strain was isolated from a crude oil-contaminated plant site in Yeochon, Korea by enrichment culture for the ability to grow on *o*-xylene as the sole source of carbon and energy. Homology searches, which were conducted with the GenBank database using Blast algorithm (Altschul et al., 1990), revealed that the 16S rRNA gene sequence of the strain had high levels of identity with *Rhodococcus* spp. For example, the 16S rRNA gene shows 96% identity with that of *Rhodococcus opacus* (Bell et al., 1999; Uz et al., 2000). Therefore, this strain was tentatively classified as *Rhodococcus* sp. Strain DK17.

Rhodococcus sp. strain DK17 grew on on *o*-xylene producing yellow pigment around the colonies. Subsequently, it was also found to grow on *p*-xylene, toluene, and benzene, but not on *m*-xylene. Subsequently, in order to obtain initial information about the catabolic pathway for *o*-xylene degradation, strain DK17 was grown on potential *o*-xylene intermediates including 2-methylbenzylalcohol, 2,3-dimethylphenol, 3,4-dimethylphenol, *cis*-benzenedihydrodiol (an analogous compound for 1,2-dihydroxy- 3,4-dimethylcyclohexa-3,5-diene or 1,2-dihydroxy-4,5-dimethylcyclohexa-3,5-diene), and 2-methylbenzoic acid. Only *cis*-benzenedihydrodiol was found to support the growth of strain DK17. However, the good growth on *cis*-benzenedihydrodiol and the absence of growth on other tested compounds do not necessarily mean that this strain initiates *o*-xylene degradatin through a dioxygenase attack on the aromatic ring to form *cis*-dihyrodiol because 2-methylbenzylalcohol and 2,3- and 3,4-dimethylphenols are known to be too toxic for many bacteria to grow on. In fact Bickerdike et al. (1997) reasoned that the inability of *Rhodococcus* sp. srain B3 to grow on these compounds was mainly due to toxicity to cells by confirming the complete growth inhibitions for *Rhodococcus* sp. strain B3 on glucose.

2. Construction and characterization of *o*-xylene-negative mutant strains

Two mutant strains, DK176 and DK180, which are unable to grow on *o*-xylene as sole

carbon and energy sources, were constructed by treating glucose-grown DK17 with UV light. One mutant strain DK176 lost simultaneously the ability to grow on toluene and benzene although it can still grow on a putative metabolic intermediate of benzene or *cis*-benzenedihydrodiol. Also, DK176 was unable to oxidize indole to indigo (blue colony), when the mutant was exposed to indole after growth in the presence of *o*-xylene, which is indicative of the presence of initial aromatic oxygenase (Ensley et al., 1983). This observation indicates that the same oxygenase may be involved in the initial oxidation of the BTX compounds tested. The other mutant strain DK180 was unable to grow on *o*-xylene and toluene but retained the ability to grow on benzene and *cis*-benzenedihydrodiol. These characteristics of DK176 and DK180 suggest that wild type DK17 possesses at least two different pathways for the degradation of (alkyl)benzenes although the initial oxidation reactions is catalyzed by a common oxygenase.

3. Identification of metabolites

After trimethylsilylation, the ethyl acetate soluble fraction obtained from the culture supernatant of DK180 incubated with *o*-xylene was analyzed by a capillary GC-MS. Two peaks for *o*-xylene metabolite-like compounds were detected at 12.66 (**I**, trace metabolite) and 20.61 min (**II**, main metabolite) on total ion chromatogram, repectively. Metabolite I showed the same GC retention time and mass spectrum as that of authentic 3,4-dimethylphenol trimethylsilyl (TMSi) ether (Table 1). Therefore the trace metabolite **I** was identified to be 3,4-dimethylphenol. Metabolite **II** gave a molecular ion at *m/z* 282 and prominent ions due to fission of TMSi at *m/z* 193 (M+-OTMSi-H)and 105 (M+-2OTMSi-H), suggesting metabolite **II** carried two hydroxyls connected to *o*-xylene. Derivatization of metabolite **II** with methaneboronic acid and 9-phenanthreneboronic acid gave mass spectra for **II** methaneboronate and phenanthreneboronate, respectively, indicating that two hydroxyls are vicinal. Thus, metabolite **II** was assumed to be ether 1,2-dihydroxy-2,3-dimethyl-benzene (3,4-dimethylcatecol) or 1,2-dihydroxy-4,5-dimethyl-benzene (4,5-dimethylcathecol). Since metabolite **II** in the ethyl acetate soluble fraction was very unstable at room temperature it was acetylated and, subsequently, purified by a normal phase HPLC for the further ^{1}H-NMR

Table 1. GC-MS data for the metabolites I, II, and 3,4-dimethylphenol

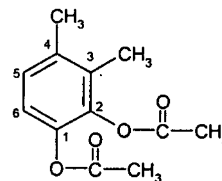
Compound	Derivative*	Retention Time on GC (min)	Prominent Ions (*m/z*, intensity %)
3,4-dimethylphenol	TMSi	12.66	194 (M+,56), 179 (100), 163 (7), 149 (9), 105 (19), 73 (8)
metabolite I	TMSi	12.66	194 (M+,59), 179 (100), 163 (6), 149 (3), 105 (23), 73 (12)
metabolite II	TMSi	20.61	282 (M+,65), 267 (14), 194 (22), 179 (17), 73 (100)
	MB	12.08	162 (M+,68), 161 (94), 147 (100), 91 (18), 77 (9)
	PB	27.94	324 (M+,100), 309 (22), 204 (18), 154 (18), 91 (8), 77 (3)

* TMSi, MB and PB indicate trimethylsilyl ether, methanboronate and phenanthreneboronate, respectively

analysis. As summarized in Table 2 acetate of metabolite **II** gave a singlet at 2.27 (6H), which was assignable for two equivalent methyls in diacetyls. Two methyls originated from *o*-xylene were detected at 2.08 (s, 3H) and 2.32 (s, 3H). Two aromatic protons which were reciprocally coupled, were absorbed at 7.05 (d, J=8.3Hz) and 6.92 (d, J=8.3Hz). Together with GC-MS data, this indicated thatthe aromatic protons were attached at C-5 and C-6. Therefore, the main metabolite **II** was identified to be 3,4-dimethylcatechol. In conclusion, 3,4-dimethylphenol (**I**) and 3,4-dimethylcatechol (**II**) were successfully identified as metabolites during the degradation of *o*-xylene by DK17. Based on the current physiological and chemical data the metabolic pathway for *o*-xylene degradation by DK17 is proposed as summarized in Fig. 2.

Table 2. 300MHz 1H-NMR data (TMS internal standard) of Metabolite II acetate.

Proton(s)	ppm
2CH3-1, 2 OAc	2.27 (s)
CH3-3	2.32 (s)
CH3-4	2.08 (s)
H-5 / H- 6	6.92 / 7.05
	(d, *J*=8.3Hz) / (d, *J*=8.3Hz)



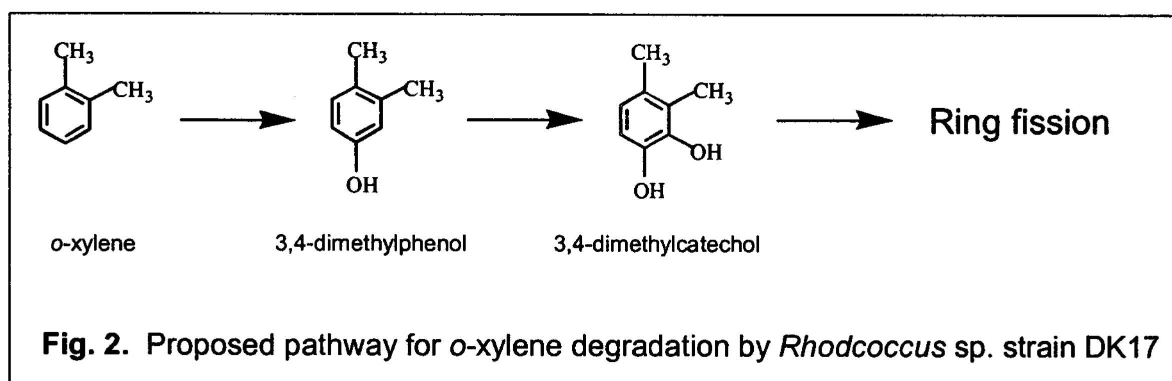


Fig. 2. Proposed pathway for *o*-xylene degradation by *Rhodcoccus* sp. strain DK17

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