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Isolation and Characterization of a *Rhodococcus* Species Strain Able to Grow on *ortho*- and *para*-Xylene

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***Rhodococcus* sp. strain YU6 was isolated from soil for the ability to grow on *o*-xylene as the sole carbon and energy source. Unlike most other *o*-xylene-degrading bacteria, YU6 is able to grow on *p*-xylene. Numerous growth substrate range experiments, in addition to the ring-cleavage enzyme assay data, suggest that YU6 initially metabolizes *o*- and *p*-xylene by direct aromatic ring oxidation. This leads to the formation of dimethylcatechols, which was further degraded largely through *meta*-cleavage pathway. The gene encoding *meta*-cleavage dioxygenase enzyme was PCR cloned from genomic YU6 DNA using previously known gene sequence data from the *o*-xylene-degrading *Rhodococcus* sp. strain DK17. Subsequent sequencing of the 918-bp PCR product revealed a 98% identity to the gene, encoding methylcatechol 2,3-dioxygenase from DK17. PFGE analysis followed by Southern hybridization with the catechol 2,3-dioxygenase gene demonstrated that the gene is located on an approximately 560-kb megaplasmid, designated pJYJ1**

Key words: *Rhodococcus*, xylene isomers, catechol 2,3-dioxygenase, megaplasmid

Many bacterial strains have been isolated for their abilities to grow on three xylene isomers [ortho (*o*-), meta (*m*-) and para (*p*-)] as the sole carbon and energy sources. Bacteria that degrade xylenes frequently fall into two classes: those that can degrade both *m*- and *p*-xylene and those that can degrade *o*-xylene only. Since the two abilities are very rarely found together in the same organism, the positions of the methyl groups on the aromatic ring have major influences in the selection of bacteria's ability to grow on xylene isomers (Davey and Gibson, 1974; Barbieri *et al.*, 1993; Kim *et al.*, 2002). Simplified pathways for *o*- and *p*-xylene degradation are depicted in Fig. 1. In *Rhodococcus* sp. strain DK17 (Kim *et al.*, 2002; Kim *et al.*, 2003; Kim *et al.*, 2004; Kim *et al.*, 2005a) and *Pseudomonas stutzeri* OX1 (Baggie *et al.*, 1987; Bertoni *et al.*, 1996; Bertoni *et al.*, 1998; Arenghi *et al.*, 1999; Arenghi *et al.*, 2001; Sazinsky *et al.*, 2004) *o*-xylene is metabolized through a dioxygenation and two successive monooxygenation of the aromatic ring through the corresponding dimethylcatechols, respectively. These strains are two of the best studied *o*-xylene-degrading strains representing gram-positive and gram-negative bacteria. A methyl-group oxidation pathway for *o*-xylene degradation was also reported in *Rhodococcus* sp. strain B3 (Bickerdike *et al.*, 1997). *p*-Xylene is metabolized using a classic TOL plasmid pathway, through the oxidation of the methyl group to *p*-toluate (Worsey and Williams, 1975; Burlage *et al.*, 1989). Alternatively, *p*-xylene degradation proceeds through the oxygenation and dehydrogenation to form 3,6-dimethylcatechol (Gibson *et al.*, 1974; Zylstra and Gibson, 1989).

Until now, no wild type bacterial strains growing on *o*--xylene (as the sole carbon and energy sources), have been reported to additionally utilize *m*- and/or *p*-xylene as growth substrates. The one exception is a mutant strain originally derived from the *o*-xylene-degrading *P. stutzeri* OX1 (Di Lecce *et al.*, 1997). In this study, our research isolated a new wild type *Rhodococcus* strain, with the ability to grow on both *o*-xylene and *p*-xylene. Biological characteristics were also investigated into the new isolate.

Materials and Methods

Isolation of bacteria and growth conditions

A standard enrichment method was used to isolate *o*-

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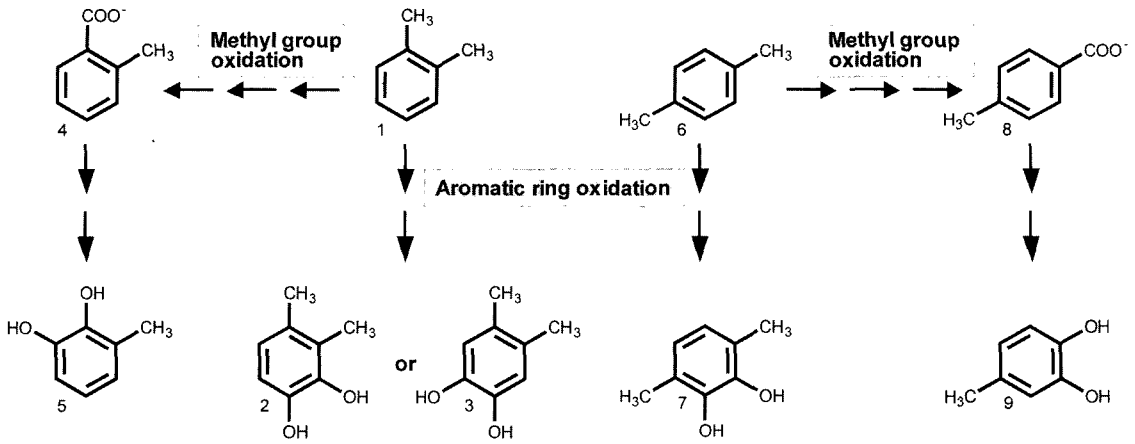

Fig. 1. Catabolic pathways for the aerobic degradation of *o*- and *p*-xylene. For simplicity, only selected metabolites are shown. The compounds are indicated as follows: 1, *o*-xylene; 2, 3,4-dimethylcatechol; 3, 4,5-dimethylcatechol; 4, 2-methylbenzoate (*o*-toluate); 5, 3-methylcatechol; 6, *p*-xylene; 7, 3,6-dimethylcatechol; 8, 4-methylbenzoate (*p*-toluate); 9, 4-methylcatechol.

xylene degraders from a plant site in Yeochon, South Korea contaminated with crude oil as previously described (Kim *et al.*, 2002). The *Rhodococcus* sp. strain YU6 was grown on a mineral salts basal (MSB) medium (Stainier *et al.*, 1966) each containing growth substrates at 30°C. Alkylbenzenes including xylene isomers, benzene, and phenol were all provided in the vapor phase. Directly added to the MSB medium were 2,3- and 3,4-dimethylphenol without dilution at the final concentration between 0.01 and 0.1% (v/v). 2- and 4-Methylbenzoate were dissolved in distilled water and added to the MSB medium to give a final concentration of 5 mM.

DNA manipulation, polymerase chain reaction, and nucleotide sequencing

Total DNA from *Rhodococcus* sp. strain YU6 was prepared according to the method of Asturias and Timmis (1993). Plasmid DNA was purified using a plasmid spin kit (Genenmed, Korea). Agarose gel electrophoresis was performed in a TAE buffer. Agarose plugs containing genomic DNA were prepared as previously described for the analysis of pulsed field gel electrophoresis (PFGE) (Kim *et al.*, 2002). The PFGE was performed using a Bio-Rad Laboratories CHEF DRIII system. Gels [1.0% agarose in 0.5 x TBE buffer (pH 8.0)] were run at 6 V/cm at 14°C. The pulse duration increased from 50 to 100 s during an 18 h run. The transfer of DNA from agarose gels to Hybond-N+ membranes (GE Healthcare Bioscience, USA) was carried out using a TurboBlotter transfer system as recommended by the supplier (Schleicher & Schuell, Germany). Southern hybridization was performed as recommended by the supplier for the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim, Germany). PCR amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, USA). The PCR reaction was carried out in 20 ml of reaction mixture, containing approximately 100 ng of template DNA and 10 pmol each of primer with ReadyMix *Taq* PCR Reaction Mix (Sigma, USA), according to the instructions from the manufacturer. The thermal cycling program was a 10 min hot start (95°C), with 30 cycles of the following: 30 sec of denaturation (95°C), 30 sec of annealing (55°C), 1 min of extension (72°C), and 10 min of extension (72°C). DNA sequencing was performed as previously described (Choi *et al.*, 2004).

Ring-cleavage dioxygenase assays

Rhodococcus sp. strain YU6 was cultured in 1 l Erlenmeyer flasks containing 200 ml of the medium. It was provided with *o*-xylene, *p*-xylene, or benzene and harvested at the mid-exponential growth phase. The enzyme solution was prepared as previously described (Kim *et al.*, 2002). Catechol 2,3-dioxygenase (C23O) activity was spectrophotometrically assayed by measuring the absorbance increases of corresponding wavelengths for each *meta*-cleavage product. Each individual *meta*-cleavage product was formed from the following substrates: catechol, λ_{max} = 375 nm and ε = 33,400 $cm^{-1}M^{-1}$; 3-methylcatechol, λ_{max} = 388 nm and ε = 13,800 $cm^{-1}M^{-1}$; 4-methylcatechol, λ_{max} = 382 nm and ε = 28,100 $cm^{-1}M^{-1}$ (Bayly *et al.*, 1966). The reaction mixture contained a 100 mM phosphate buffer (pH 7.4) and an appropriate substrate using a final concentration of 0.4 mM. The activity of catechol 1,2-dioxygenase (C12O) was also spectrophotometrically assayed by measuring absorbance increases of corresponding wavelengths for corresponding wavelengths of each *ortho*-cleavage product formed from the following substrates: catechol, λ_{max} = 260 nm and ε = 16,800 $cm^{-1}M^{-1}$; 3-methylcatechol, λ_{max} = 260 nm and ε = 18,000 $cm^{-1}M^{-1}$; 4-methylcatechol, λ_{max} = 255 nm and ε = 14,300 $cm^{-1}M^{-1}$ (Dorn and Knackmuss, 1978). The reaction mixture contained 50 mM of Tris-HCl (pH 8.0), 1.3 mM of EDTA, and an appropriate

substrate using a final concentration of 0.4 mM. Protein content was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Results and Discussion

Isolation, identification, and growth characterization of strain YU6

A new bacterial strain (designated YU6) was isolated from a factory site in Yeochon, South Korea contaminated with crude oil, for the ability to grow on *o*-xylene as the sole sources of carbon and energy via enrichment culture. The YU6 strain is a gram-positive bacterium with an optimum growth temperature of 30-35°C. In order to taxonomically identify the YU6 strain, its 16S rRNA gene was amplified by PCR using the 27F and 1522R primers, and the PCR product directly sequenced with internal primers: forward primers, 27F, 357F, 704F, 926F, and 1242F; reverse primers, 321R, 685R, 907R, 1220R, and 1522R (Johnson, 1994). Analysis of the nucleotide sequence using the GenBank database (Altschul *et al.*, 1990) revealed that the YU6 16S rRNA sequence has high levels of identity (c.a. 99%) with those from several different *Rhodococcus* species. Thus, YU6 was positively identified as a strain of *Rhodococcus* sp. The *Rhodococcus* sp. strain YU6 16S rRNA gene sequence was deposited into the GenBank with the accession number DQ011232.

The ability of YU6 to grow on different monocyclic aromatic compounds including the other xylene isomers was determined by the rate of colony formation on MSB plates in the presence of each substrate as the sole carbon source (Table 1). YU6 grew on *o*- and *p*-xylene, forming 1 mm sized colonies in less than five and seven days, respectively. This occurred despite the fact *m*-xylene was not used as a growth substrate by YU6. The growth substrate range experiments also demonstrated that YU6 has the ability to grow on benzene, monoalkyl benzenes (from toluene to *n*-hexylbenzene), and phenol. In order to characterize the catabolic pathways for *o*- and *p*-xylene degradation, the ability of YU6 to grow on several commercially-available metabolites of *o*- and *p*-xylene was analyzed. As summarized in Table 1, it was discovered that YU6 was unable to grow on all of the potential metabolic intermediates tested. Bickerdike *et al.* (1997) previously reported that the growth failure of the *o*-xylene-degrading *Rhodococcus* sp. strain B3 on 2-methylbenzylalcohol, 2-methylbenzylaldehyde, and 3,4-dimethylphenol is mainly due to cell toxicity because the strain's growth on glucose was completely inhibited by these compounds. The potential toxicity of 2,3-dimethylphenol, 3,4-dimethylphenol, 2-methylbenzoate (*o*-toluate), and 4-methylbenzoate (*p*-toluate) to YU6 was thus examined by monitoring the growth of YU6 on glucose in the presence of each substrate. Indeed, 2,3- and 3,4-dimethylphenol were found to completely inhibit the growth of YU6 on glucose when provided at 0.1%.

Table 1. Growth characteristics of *Rhodococcus* sp. strain YU6 on various monocyclic aromatic hydrocarbons

Compound	Growth	Compound	Growth
o-Xylene	+++[a]	Benzene	++++
m-Xylene	-[b]	Toluene	++
p-Xylene	++	Ethylbenzene	+++
Phenol	+++	Isopropylbenzene	+++
2,3-Dimethylphenol	-	*n*-Propylbenzene	+++
3,4-Dimethylphenol	-	*n*-Butylbenzene	+++
2-Methylbenzoate	-	*n*-Hexylbenzene	+
4-Methylbenzoate	-	Benzoate	++

[a]Four, three, two, and one plus signs indicate the formation of 1.0 mm-in-diameter colony within 3, 5, 7, and 9 days, respectively.
[b]A minus sign denotes that no growth was detected after 9 days of incubation.

This observation suggests that the inability of YU6 to grow on 2,3- and 3,4-dimethylphenol does not preclude the possibility that they are intermediates in the degradation of *o*-xylene by YU6. In contrast, toxicity may not be the reason YU6 failed to grow on 2- and 4-methylbenzoate, because the YU6 growth on glucose was not inhibited by these compounds at the concentrations of 5 to 10 mM. Instead, it is apparent that 2- and 4-methylbenzoate are not intermediates in the degradation of *o*- and *p*-xylene by YU6, respectively. In this context, YU6 is unlikely to metabolize *o*- and *p*-xylene via methyl group oxidation pathways.

Induction of meta- and ortho-cleavage dioxygenases in YU6

Catechol and its derivatives are key metabolic intermediates in the aerobic degradation of aromatic hydrocarbons by bacteria. Hence, catechol dioxygenases play a major role in metabolizing aromatic hydrocarbons. Catechol dioxygenases can be divided into two major groups: 1) cleave the aromatic ring between the vicinal diols (the *ortho*-cleavage dioxygenase group); and 2) cleave the ring to one side of the vicinal diols (the *meta*-cleavage dioxygenase group) (Broderick, 1999; Jung *et al.*, 2003). In the well researched *o*-xylene-degrading *Rhodococcus* sp. strain DK17, *o*-xylene degradation is initiated by a ring-oxidizing dioxygenase pathway through 3,4-dimethylcatechol. This is subsequently cleaved exclusively using a C23O (Kim *et al.*, 2002; Kim *et al.*, 2004; Kim *et al.*, 2005b).

Since the growth test results mentioned above suggest that YU6 metabolizes *o*- and *p*-xylene through a ring-oxidizing oxygenase pathway, YU6 was analyzed to discern the type of catechol dioxygenase produced physiologically during the growth on *o*-xylene, *p*-xylene, or benzene. A significant amount of C23O (also called *meta*-

cleavage dioxygenase) activity was detected in cells of YU6 grown on *o*-xylene, *p*-xylene, or benzene, as summarized in Table 2. The detected C23O indicated maximal activity against 3-methylcatechol and significant activity against 4-methylcatechol (approximately 25% of that against 3-methylcatechol). However, the enzyme showed very low levels of activity against catechol (less than 2% of that against 3-methylcatechol). It is apparent that the same C23O enzyme was induced in the presence of *o*-xylene, *p*-xylene, and benzene because the ratios of activities on the other three substrates are the same under all the three conditions. The C12O activity (called *ortho*-cleavage dioxygenase) was also detected in the *o*- or *p*--xylene-grown cells although the induction levels were much lower than that in the YU6 cells grown in the presence of benzene (Table 2). These enzyme assay data indicate that both *meta*- and *ortho*-cleavage pathways are being used for the degradation of *o*- and *p*-xylene by YU6 although the former pathway plays a major role. This is unlike DK17 where C12O is induced only in the growth phase in the presence of benzene (Kim *et al.*, 2002).

Cloning and sequencing of the C23O gene from YU6

It is remarkable that the enzyme activity profile of C23O from YU6 was not significantly different from that of C23O (AkbC), which is specifically involved in alkylbenzene metabolism by *Rhodococcus* sp. strain DK17 (Kim *et al.*, 2002; Kim *et al.*, 2005b). These results raise the possibility that the C23O enzyme from YU6 has a close molecular relationship to the DK17 AkbC. Thus, in order to address this possibility, attempts were made to amplify the putative coding sequence of the YU6 C23O gene using nucleotide sequence of the DK17 *akbC* gene as a guide. Indeed, the application of the primers (forward, 5'-ATGGCAAAAGTGACCGAA-3'; reverse, 5'-TTATGCGGGGATGTCGAG-3') which correspond to the beginning and end of the DK17 *akbC* gene, in a PCR reaction with total genomic DNA from YU6, amplified the PCR products of the desired size. Subsequent cloning and sequencing of the 918-bp PCR product (GenBank accession number DQ011233) revealed a 98% identity (with no gaps), to the entire *akbC* gene from DK17 (Kim *et al.*, 2004; Kim *et al.*, 2005b).

Table 2. Comparison of catechol 2,3-dioxygenase (C23O) and catechol 1,2-dioxygenase (C12O) activities in cell extracts of *Rhodococcus* sp. strain YU6 grown on *o*-xylene, *p*-xylene, or benzene

Enzyme	Inducer	Enzyme activity (U/mg of protein)[a] against		
		3-Methyl-catechol	4-Methyl-catechol	Catechol
C23O	Glucose	ND[b]	ND	ND
	o-Xylene	679.8 ± 12.0	169.8 ± 1.7	11.7 ± 2.3
	p-Xylene	566.9 ± 15.3	131.5 ± 10.2	8.5 ± 1.1
	Benzene	253.1 ± 4.3	67.7 ± 5.1	2.4 ± 0.3
C12O	Glucose	ND	ND	ND
	o-Xylene	71.3 ± 2.4	66.8 ± 1.3	104.6 ± 6.0
	p-Xylene	74.7 ± 2.3	68.5 ± 7.0	108.4 ± 2.3
	Benzene	979.1 ± 55.6	847.9 ± 11.8	1,461.5 ± 67.7

[a]Enzyme activities are the averages from at least three independent experiments. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of one micromole of product per minute.
[b]Not detected

Location of the C23O gene in theYU6 genome

It is generally known that most *Rhodococcus* strains harbor one to several megaplasmids (50-1,000 kb) (Pisabarro *et al.*, 1998). Rhodococcal plasmids usually possess linear topology and frequently encode catabolic genes. This enables their hosts to multiply on various aromatic compounds including biphenyl (Masai *et al.*, 1997), isopropylbenzene (Stecker *et al.*, 2003), and *o*-xylene (Kim *et al.*, 2002). PFGE analysis of the YU6 genomic DNA was performed to separate large plasmids from each other and from the chromosome. For comparison purposes, DK17 genomic DNA embedded in an agarose plug was also loaded in the same gel because the *akbC* gene encoding C23O in DK17 is located on the 330-kb megaplasmid pDK2 (Kim *et al.*, 2004). As shown in Fig. 2A, *Rhodococcus* sp. strain YU6 contains at least one megaplasmid, designated pJYJ1 (approximately 560 kb). A Southern blot was performed with the PFGE-separated DNA using

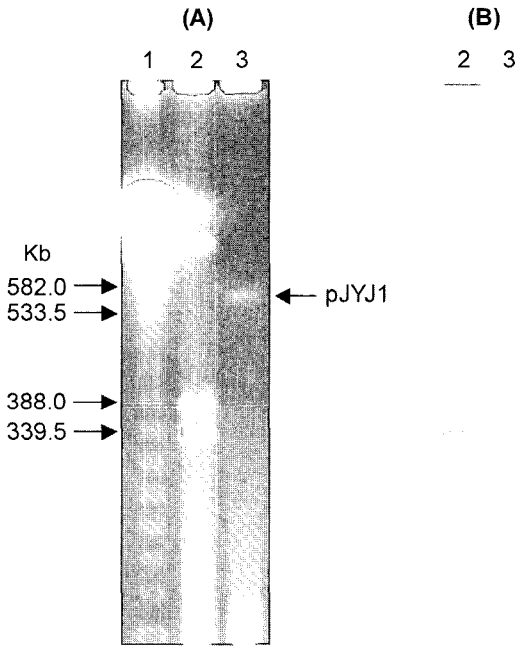

Fig. 2. Genomic locations of the YU6 C23O gene. (A) PFGE separation of genomic DNAs prepared from *Rhodococcus* sp. strains DK17 and YU6. (B) Southern blot of the gel shown in panel A with the YU6 C23O gene probe. Lane 1, λ ladder standard; lane 2, *Rhodococcus* sp. strain DK17; lane 3, *Rhodococcus* sp. strain YU6.

the PCR cloned YU6 C23O gene as a probe. Indeed, the probe hybridized to pJYJ1 in the YU6 and to pDK2 in the DK17 lanes, respectively (Fig. 2B). Results from these observations confirm that the YU6 C23O gene is derived from pJYJ1.

In conclusion, the ability of *Rhodococcus* sp. strain YU6 to grow on both *o*- and *p*-xylene makes it an exception to the general rule that *o*-xylene, *m*-xylene, and *p*-xylene catabolic pathways cannot be expressed in the same strain (Davis *et al.*, 1968; Barbieri *et al.*, 1993; Kim *et al.*, 2002). Based on the data obtained from these growth substrate experiments, coupled with ring-cleavage enzyme assays, it can reasonably be expected that the degradation of *o*-and *p*-xylene by YU6 is initiated by direct aromatic ring oxidation successively leading to the formation of the corresponding dimethylcatechol, which are further degraded mainly through a *meta*-cleavage pathway.

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