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# Characterization and engineering of an *o*-xylene dioxygenase for biocatalytic applications

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## HIGHLIGHTS

- The DK17 *o*-xylene dioxygenase catalyzes unique hydroxylations of aromatic compounds.
- Enzyme activity was manipulated and improved through structure-based engineering.
- These findings provide insights for the development of novel hydroxylation catalysts.

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## ABSTRACT

Depending on the size and position of the substituent groups on the aromatic ring, the *o*-xylene dioxygenase from *Rhodococcus* sp. strain DK17 possesses the unique ability to perform distinct regioselective hydroxylations via differential positioning of substrates within the active site. The substrate-binding pocket of the DK17 *o*-xylene dioxygenase is large enough to accommodate bicyclics and can be divided into three regions (distal, central, and proximal), and hydrophobic interactions in the distal position are important for substrate binding. Current molecular and functional knowledge contribute insights into how to engineer this enzyme to create tailor-made properties for chemoenzymatic syntheses.



## 1. Introduction

Almost 15 years ago, a major trade journal of the chemical industry reported that, "Fine chemical makers are increasingly using enzymatic methods to make chiral intermediates" (McCoy, 1999). Indeed, as recently reviewed by Muñoz Solano et al. (2012), the world's leading pharmaceutical and chemical companies, such as BASF – The Chemical Company, Bristol-Myers Squibb, GlaxoSmithKline, Lonza, and Merck, have used various enzymes to prepare a wide variety of chiral compounds on an industrial scale. These enzymes mostly belong to the hydrolase and oxidoreductase classes. Oxidoreductases are the largest and most diverse group of enzymes, which can be divided into four groups: oxygenases, oxidases, dehydrogenases, and peroxidases (Blank et al., 2010). Oxygenases and oxidases use molecular oxygen as a substrate and as an electron acceptor, respectively. Dehydrogenases and peroxidases typically catalyze reversible hydrogen transfer reactions and oxidative transformations of organic reactants with peroxide (usually $H_2O_2$) as the oxidant.

Oxygenases are defined as enzymes catalyzing the specific introduction of one (monooxygenase) or two (dioxygenase) oxygen atoms from molecular oxygen into the substrate. Oxygenases have a high potential for applications in the chemical and the pharmaceutical industries because such specific hydroxylation reactions can lead to the selective activation of chemically inert C–H bonds, a reaction that is often unattainable with chemical methods (Straathof et al., 2002). During approximately the past decade aromatic dioxygenases, which catalyze *cis*-dihydroxylation to arene substrates, have increasingly attracted interest. This interest results primarily from their potential application as biocatalysts for regioselective and enantioselective synthesis of vicinal *cis*-dihydrodiols and other oxygenated products such as catechols, epoxides, and phenolics (Nolan and O'Connor, 2008). Toluene and naphthalene dixoygenases are the most applied aromatic dioxygenase enzymes for producing potential industrial synthons (Table 1). The larger the toolbox of aromatic dioxygenases, the higher the probability of finding the needed biocatalysts. In this context, this review

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**Table 1**
Representative examples of *cis*-dihydrodiol synthons and their applications.

| Enzyme | Organism | Aromatic substrate | *cis*-Dihydrodiol synthon | End product from *cis*-dihydrodiol | References |
|---|---|---|---|---|---|
| Toluene dioxygenase | *Pseudomonas putida* UV4 | Toluene | Toluene 2,3-*cis*-dihydrodiol | 6C-Methyl-D-mannose | Boyd and Bugg (2006) |
| | | Chlorobenzene | Chlorobenzene 2,3-*cis*-dihydrodiol | (−)-Cladospolide A | Boyd and Bugg (2006) |
| | | Bromobenzene | Bromobenzene 2,3-*cis*-dihydrodiol | (−)-*ent*-Bengamide E | Boyd and Bugg (2006) |
| | *Pseudomonas putida* F1 | Indene | *cis*-(1S,2R)-Dihydroxyindan | Indinavir sulfate (Crixivan) | Zhang et al. (2000) |
| | | Bromobenzene | Bromobenzene 2,3-*cis*-dihydrodiol | L-Chiro-inositol dimers | Boyd et al. (2001) |
| | | 1-Bromo-4-iodobenzene | 1-Bromo-4-iodo benzene 2,3-*cis*-dihydrodiol | *ent*-7-Deoxypancrafistatin | Boyd et al. (2001) |
| | | 1-Fluoro-4-iodobenzene | 1-fluoro-4-iodobenzene 2,3-*cis*-dihydrodiol | *ent*-7-Deoxypancrafistatin | Boyd et al. (2001) |
| Naphthalene dioxygenase | *Pseudomonas putida* 9816/11 | Naphthalene | Naphthalene 1,2-*cis*-dihydrodiol | (+)-Goniodiol | Boyd and Bugg (2006) |
| Tetralin dioxygenase | *Sphingomonas macrogolitabida* TFA | Indole | *cis*-Indole-2,3-dihydrodiol | Indigo dye | Royo et al. (2005) |

specifically summarizes the molecular functional studies of a novel aromatic dioxygenase derived from an *o*-xylene-degrading *Rhodococcus* species.

## 2. *Rhodococcus* sp. strain DK17 as a source of novel aromatic dioxygenases

*Rhodococcus* sp. strain DK17 was originally isolated because it can replicate on *o*-xylene and has the capability to utilize aromatic compounds such as benzene, alkylbenzenes (toluene, ethylbenzene, isopropylbenzene, and *n*-propyl to *n*-hexylbenzenes), phenol, and phthalates as sole carbon and energy sources (Kim et al., 2002). However, despite its apparent metabolic versatility, DK17 is unable to grow on the other two xylene isomers, reinforcing a previous observation that bacteria that degrade xylenes commonly fall into two classes: those that can degrade both *m*- and *p*-xylene and those that can degrade *o*-xylene only. Only very rarely are the two abilities found together in the same organism (Barbieri et al., 1993; Davey and Gibson, 1974). Interestingly, cells of DK17 that have been grown on *o*-xylene can oxidize *m*- and *p*-xylene to 2,4-dimethylresorcinol and 2,5-dimethylhydroquinone, respectively (Kim et al., 2003). Because the addition of oxygen to the aromatic ring of *m*-xylene between the two methyl groups must be catalyzed by a monooxygenase or a very unusual dioxygenase the structural identification of 2,4-dimethylresorcinol provides direct chemical evidence for this strain's ability to perform aromatic hydroxylation with unique regioselectivity.

*Rhodococcus* sp. strain DK17 uses *o*-xylene 3,4-dioxygenase to initiate *o*-xylene degradation and produce *o*-xylene *cis*-3,4-dihydrodiol, which is subsequently channeled into *meta*-cleavage pathway via 3,4-dimethylcatechol (Kim et al., 2002). It is noteworthy that DK17 was the first example supported by direct experimental evidence of the aromatic oxidation of *o*-xylene by bacteria; although, previously, Gibson and Subramanian (1984) had proposed that a *Nocardia* sp. strain and *Rhodococcus* sp. strain C125 (originally *Corynebacterium* strain C125, renamed by van der Meer et al. (1992)) (Schraa et al., 1987) metabolize *o*-xylene through an initial aromatic dioxygenase to form a *cis*-dihydrodiol. Although the details of the metabolic pathways for *m*- and *p*-xylenes were well documented at the biochemical and molecular levels (Jindrová et al., 2002; Zylstra, 1994), little in-depth work had been reported for the degradation of *o*-xylene by bacteria at the time of the isolation of DK17. In addition, several researchers had independently reported that among the three isomers *o*-xylene is the most resistant to biodegradation (Alvarez and Vogel, 1991; Baggi et al., 1987; Bibeau et al., 2000; Smith, 1993; Solano-Serena et al., 2000). Thus, due to its metabolic versatility and the relatively scarce research on *o*-xylene degradation by bacteria, DK17 has been subjected to in-depth studies to evaluate its potential for biocatalytic applications.

## 3. Identification and functional characterization of the DK17 *o*-xylene dioxygenase

*Rhodococcus* sp. strain DK17 possesses three megaplasmids (380-kb pDK1, 330-kb pDK2, and 750-kb pDK3) and the genes encoding the *o*-xylene metabolism are present on pDK2 (Kim et al., 2002). All of the postulated genes necessary for the degradation of *o*-xylene were cloned and completely sequenced (GenBank database accession number AY502075; Kim et al., 2004). Similar to typical bacterial aromatic dioxygenases (Ferraro et al., 2005; Kweon et al., 2008), the DK17 *o*-xylene dioxygenase is a three-component enzyme system consisting of a flavoprotein reductase, a ferredoxin, and an oxygenase component containing a Rieske [2Fe-2S] center and non-heme iron ($Fe^{2+}$). The encoded reductase and the ferredoxin components form a short electron transport chain that supplies electrons from NADH to the oxygenase component, which consists of a small subunit and a large subunit. The large subunit containing the catalytic and substrate-binding domains attacks the aromatic ring by means of the transition metal iron for the activation of dioxygen ($O_2$). Binding of $O_2$ to an enzyme-coordinated iron atom causes some of the oxygen's electron density to overlap with the metal's *d*-orbitals. This process facilitates the spin conversion of $O_2$ to render it more reactive with the benzenoid aromatic ring because *d*-orbitals normally require small energy input to move electrons between energy states (Wackett and Hershberger, 2001).

One notable observation in the hydroxylation of the substrates tested is that the size and the position of the substituent groups on the aromatic ring apparently affect the regioselectivity of aromatic oxidation by the DK17 *o*-xylene dioxygenase. When expressed in *Escherichia coli* the DK17 *o*-xylene dioxygenase transformed *o*-xylene into 2,3- and 3,4-dimethylphenol. These products were apparently derived from an unstable *o*-xylene *cis*-3,4-dihydrodiol, which would readily dehydrate (Kim et al., 2004). This result indicates that there is a single point of attack of the dioxygenase on the