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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.

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## 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 (Strathof 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

aromatic ring. Attack of the DK17 *o*-xylene dioxygenase on toluene and ethylbenzene resulted in the formation of two different *cis*-dihydrodiols that resulted from an oxidation at the 2,3 and the 3,4 positions on the aromatic ring in the ratios of 8:2 and 9:1, respectively. However, all of the larger substrates tested (biphenyl, naphthalene, indan, tetralin, indene, and 1,2-dihydronaphthalene) were transformed exclusively into each corresponding *cis*-dihydrodiol (Kim et al., 2007, 2011). These data suggest that the DK17 *o*-xylene dioxygenase possesses the ability to perform distinct regioselective hydroxylations in which the size and position of the substituent group determines the number and position of the dihydroxylation on the aromatic ring. This hypothesis is further supported by the profiles of oxidation products of *m*- and *p*-xylenes: *m*-xylene was transformed into 3-methylbenzylalcohol and 2,4-dimethylphenol in a 9:1 ratio while *p*-xylene was oxidized to *cis*-*p*-xylene dihydrodiol (Kim et al., 2010b Kim et al., 2007).

As described in Section 2, *Rhodococcus* sp. strain DK17 oxidizes *m*-xylene into 2,4-dimethylresorcinol, which is somewhat surprising because an oxygen atom must be added to the aromatic ring of *m*-xylene between the two methyl groups. Kim et al. (2003) proposed that this product is achieved either through the action of a dioxygenase, forming a dihydrodiol that subsequently dehydrates to a phenolic compound, or through the action of a monooxygenase that directly hydroxylates, or from a combination of the two types of monooxygenase. This earlier observation, combined with the detection of 2,4-dimethylphenol in the biotransformation experiments with the DK17 *o*-xylene dioxygenase, favors the hypothesis of two successive monohydroxylations, and shows that this enzyme is also able to carry out a monohydroxylation reaction on the methyl substituent or aryl ring on *m*-xylene.

## 4. Structure-based engineering of the DK17 *o*-xylene dioxygenase

### 4.1. Molecular modeling

Recent molecular modeling studies of the oxygenase component large subunit of the DK17 *o*-xylene dioxygenase (Kim et al., 2010a,b; Yoo et al., 2011) revealed that (1) the active site is large enough to accommodate bicyclics, (2) the catalytic pocket can be divided into three regions (distal, central, and proximal), depending on the distance to the mononuclear iron atom (Jakoncic et al., 2007) and in case of a substrate containing two ring moieties, the one located in the proximal site is to be attacked for oxidation, and (3) hydrophobic interactions in the distal position play an important role in substrate binding, largely via the stacking of benzene ring structures from the substrate with the specific amino acid residues at the active site.

According to molecular modeling, the DK17 *o*-xylene dioxygenase can hold aromatic substrates at a kink region between α6 and α7 helices of the active site and α9 helix covers the substrates. *m*-Xylene is unlikely to locate at the active site with a methyl group facing the kink region because this configuration would not fit within the substrate-binding pocket. The *m*-xylene molecule can flip horizontally to expose the *meta*-position methyl group to the catalytic motif. In this configuration, 3-methylbenzylalcohol could be formed, presumably due to the *meta* effect, which states that a substituent at a *meta*-position on a benzene ring does not allow binding and catalysis of arene *cis*-dihydroxylation of the toluene dioxygenase from *Pseudomonas putida* UV4 (Boyd et al., 2006). Alternatively, the *m*-xylene molecule can rotate counterclockwise, which would allow the catalytic motif to hydroxylate at C-4, yielding 2,4-dimethylphenol.

Likewise, indan and its derivatives such as indene, tetralin, and 1,2-dihydronaphthalene can be positioned in two ways. In the first position, each molecule exposes its alicyclic moiety to the catalytic motif. Alternatively, each molecule can rotate 180° from the first position to expose its aromatic moiety to the catalytic motif. Based on the effect of hydrophobic interactions in substrate binding, the first position is likely to be much more favored than the second. In the first configuration, neither indan nor tetralin can be oxygenated because they lack double bonds, while indan-1,2-diol, and *cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene would be formed as products from indene and 1,2-dihydronaphthalene, respectively. In contrast, the second configuration would allow all the substrates to be oxygenated at their aromatic rings. This explanation not only answers the earlier question as to why DK17 is unable to grow on 1,2-dihydronaphthalene and indene despite their structural similarities to indan and tetralin, respectively, but suggests that aromatic hydroxylation is a prerequisite for proper DK17 degradation of bicyclics with aromatic and alicyclic rings. Also, it should be noted that before the discovery of the DK17 *o*-xylene dioxygenase, benzylic hydroxylation was predominantly known for microbial indan metabolism (Boyd et al., 1991; Brand et al., 1992; Gibson et al., 1995; Limberger et al., 2007; Wackett et al., 1988).

### 4.2. Generation and characterization of engineered *o*-xylene dioxygenases

Based on the molecular modeling data, a total of four engineered enzymes (A218L, D262L, L266F, and V297L) were generated by directed evolution (Wang et al., 2012). When expressed in *E. coli* in the presence of *m*-xylene, D262L produced 3-methylbenzylalcohol and 2,4-dimethylphenol in a 7:3 ratio, while L266F produced them in a ratio similar to that produced by the native enzyme. One plausible explanation for the increased formation of 2,4-dimethylphenol is that in D262L the hydrophobic side chain of the leucine allows a closer approach of a methyl group of *m*-xylene and promotes the production of 2,4-dimethylphenol. However, a bulkier hydrophobic side chain in L266F seems not to affect *m*-xylene positioning (Kim et al., 2010b).

The same four enzymes were also expressed in *E. coli* in the presence of biphenyl and examined for metabolites by gas chromatography–mass spectrometry. The culture supernatants were acidified and heat-treated to dehydrate the produced *cis*-2,3-biphenyl dihydrodiol to either 2- or 3-hydroxybiphenyl (Omori et al., 1991). It is more reliable to measure the latter two compounds than to measure *cis*-2,3-biphenyl dihydrodiol directly because they are commercially available and can be used as standards for the quantification. Interestingly, L226F, which was found not to affect *m*-xylene positioning, produced approximately 24 (2.43 vs. 0.1 μg/L) and 66 (1.97 vs. 0.03 μg/L) times as much 2-hydroxybiphenyl and 3-hydroxybiphenyl as the wild type, respectively (Yoo et al., 2011). The results would be different with biphenyl because the phenyl ring of mutant enzyme L266F is positioned almost perpendicular to the aromatic ring of biphenyl distal to α6 helix. Because such a configuration apparently enhances the hydrophobic interaction between the two ring structures the substitution of the leucine at position 266 to phenylalanine is likely to increase binding stability between L266F and biphenyl via hydrophobic interactions. To corroborate this hypothesis, an additional engineered enzyme was generated by replacing L266 with tyrosine. As expected, the total amount of biphenyl metabolites produced by the engineered enzyme L266Y was determined to be 72% and 2% of those produced by the wild-type and the L266F enzymes, respectively. This result, combined with the fact that tyrosine is structurally similar to phenylalanine except for the addition of a hydroxyl group to the phenyl ring, strongly suggests that the introduction of a hydroxyl group at position 266 hinders the hydrophobic interactions for

optimal substrate binding, and results in the dramatic change of the L266F mutant's activity.

### 4.3. Potential application examples of the DK17 o-xylene dioxygenase

Indan and its derivatives are frequently used as starting materials for the preparation of synthons for pharmaceutical and fine chemical synthesis. In addition to the oxidation of indene to *cis*-(1*S*,2*R*)-dihydroxyindan (Table 1), indan and tetralin were transformed to a variety of indandiols for chiral drug by enantioselective benzylic microbial hydroxylation (Priefert et al., 2004). For the past decade there have also been research efforts to obtain patentable melatonin receptor ligands including synthesis of tricyclic indan derivatives for the development of new melatonin receptor agonists (Pandi-Perumal et al., 2007; Uchikawa et al., 2002). Accordingly, the capability for the aromatic and alicyclic hydroxylation of indan derivatives makes the wild type and engineered DK17 *o*-xylene dioxygenases promising biocatalysts for the development of new fine chemicals.

## 5. Conclusions and future research

Molecular functional characterization studies have revealed that the DK17 *o*-xylene dioxygenase catalyzes unique hydroxylations of aromatic compounds and produces products that do not result from hydroxylations by other dioxygenases (Hudlicky et al., 1999; Nolan and O'Connor, 2008). For example, to our knowledge, 2,4-dimethylphenol has not previously been detected in the oxidation of *m*-xylene. Based on the present findings, future research will concentrate on the development of stable enzyme and/or whole cell biocatalysts derived from DK17. This work will contribute to address a major challenge in current biocatalysis engineering, the cost-effective production of robust biocatalysts (Illanes et al., 2011).

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