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Metabolism of dibenzofuran and dibenzo-*p*-dioxin by the biphenyl dioxygenase of *Burkholderia xenovorans* LB400 and *Comamonas testosteroni* B-356

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Abstract We examined the metabolism of dibenzofuran (DF) and dibenzo-*p*-dioxin (DD) by the biphenyl dioxygenase (BPDO) of *Comamonas testosteroni* B-356 and compared it with that of *Burkholderia xenovorans* LB400. Data showed that both enzymes oxygenated DF at a low rate, but *Escherichia coli* cells expressing LB400 BPDO degraded DF at higher rate (30 nmol in 18 h) compared with cells expressing B-356 BPDO (2 nmol in 18 h). Furthermore, both BPDOs produced dihydro-dihydroxy-dibenzofuran as a major metabolite, which resulted from the lateral oxygenation of DF. 2,2′,3-Trihydroxybiphenyl (resulting from angular oxygenation of DF) was a minor metabolite produced by both enzymes. Deuterated DF was used to demonstrate the production of 2,2′,3-dihydroxybiphenyl through angular oxygenation of DF. When tested for their ability to oxygenate DD, both enzymes produced as sole metabolite, 2,2′,3-trihydroxybiphenyl ether at about the same rate, indicating similar catalytic properties toward this substrate. Altogether, although LB400 and B-356 BPDOs oxygenate a different range of chlorobiphenyls, their metabolite profiles toward DF and DD are similar. This suggests that co-planarity influences the regiospecificity of BPDO toward DF and DD to a higher extent than the presence of an *ortho* substituent on the molecule.

Introduction

Aryl-hydroxylating dioxygenases are of considerable interest as they are potentially capable of initiating the degradation of numerous aromatic pollutants. Recent investigations provided evidence that these enzymes can be engineered to further increase their catalytic activity toward selected targeted pollutants, such as polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (Brühlmann and Chen 1999; Kumamaru et al. 1998; Parales et al. 2000; Suenaga et al. 2002).

Among the aryl-hydroxylating dioxygenases, biphenyl dioxygenase (BPDO; Broadus and Haddock 1998; Haddock and Gibson 1995; Haddock et al. 1997; Hurtubise et al. 1996) has been investigated thoroughly. It catalyzes the first step of the biphenyl catabolic pathway to generate *cis*-(2*R*,3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene(*cis*-biphenyl-2,3-dihydrodiol). The substrate specificity of BPDO is crucial, because it limits the range of compounds that are degradable by the catabolic pathway (Haddock et al. 1995). BPDO is a three-component enzyme (Fig. 1). The first component is an oxygenase, which is an iron–sulfur protein (ISP_{BPH}) that catalyzes the addition of molecular oxygen. The second and third components are flavoprotein reductase (RED_{BPH}) and ferredoxin (FER_{BPH}) that transfer electrons from NADH to ISP_{BPH}, which then activates molecular oxygen for insertion into the aromatic substrate. BPDO components are encoded by *bphA* (α-subunit of ISP_{BPH}), *bphE* (β-subunit of ISP_{BPH}), *bphF* (FER_{BPH}) and *bphG* (RED_{BPH}) in *Burkholderia* sp. strain LB400 (Erickson and Mondello 1992), which was recently renamed *B. xenovorans* LB400 (Denef et al. 2004), and in *Comamonas testosteroni* B-356 (Sylvestre et al. 1996). Several evolved BPDOs have been reported which exhibit extended catalytic activity toward PCBs (Barriault et al. 2002; Brühlmann and Chen 1999; Kumamaru et al. 1998; Suenaga et al. 2002), trichloroethylene (Maeda et al. 2001), alkylbenzene (Suenaga et al. 2001b), dibenzo-*p*-dioxin (DD) or dibenzofuran (DF; Suenaga et al. 2001a). Of particular interest are DD and DF, as their chlorinated derivatives are of concern for the environment. Furthermore, DF and DD can be regarded as analogues of symmetric doubly *ortho*-substituted biphenyls and diphenyl ethers (Fig. 2); and 2,2′-dichlorobiphenyl is an example. In

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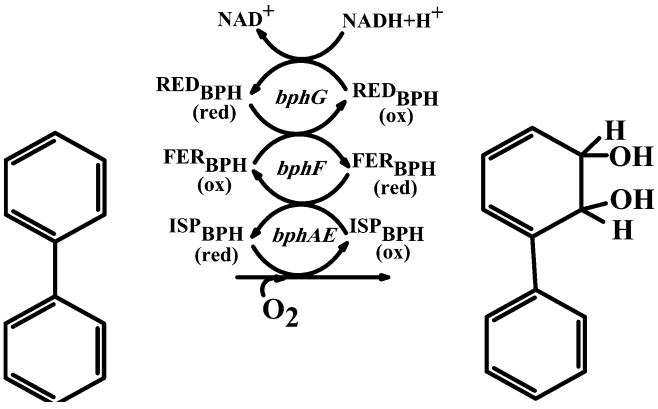

Fig. 1 The BPDO reaction

addition, the bonds between the oxygen atom and the two *ortho*-carbons lock both phenyl rings into a co-planar configuration that characterizes some of the most toxic PCB congeners, e.g. 3,3′,4,4′-tetrachlorobiphenyl. In this respect, DF and DD share features of both co-planar and *ortho*-substituted PCB congeners. Therefore, it is of interest to use these compounds as models to better understand how these structurally distinct analogues are recognized by the enzyme active center and what orientations they can occupy inside the catalytic pocket of the enzyme.

Several bacterial isolates able to degrade DF and DD have been described (Kimura and Urushigawa 2001; Nojiri and Omori 2002). As noted above, the first reaction in the degradation process involves the introduction of molecular oxygen at lateral or angular carbons by a dioxygenase. The possible sites of oxygenation are shown in Fig. 2. Angular oxygenation is the most desirable reaction, as a concomitant carbon–oxygen bond-cleavage occurs to generate 2,2′,3-trihydroxybiphenyl (from DF) or 2,2′,3-trihydroxydiphenyl ether (from DD). These two metabolites can be further metabolized through, respectively, the biphenyl or the diphenyl ether catabolic pathway, whereas the degradation of the metabolites produced from lateral oxygenation of DD and DF has not been demonstrated. Several dioxygenases such as *dbfA1A2* (Kasuga et al. 2001), *DxnA1A2* (Armengaud et al. 1998) and *CarAa* (Nojiri et al. 2001) are known to catalyze exclusively via angular oxygenation of DF and/or DD. However, based on amino acid sequence alignment, these dioxygenases do not belong to the same phylogenetic lineages as the BPDOs (Nojiri and Omori 2002). Angular attack of DF by the DF dioxygenase of *Brevibacterium* strain DPO1361 has been inferred from the observation that fluorene is transformed to a stable intermediate identified as 1,10-dihydro-1,10-dihydroxyfluoren-9-one (Engesser et al. 1989).

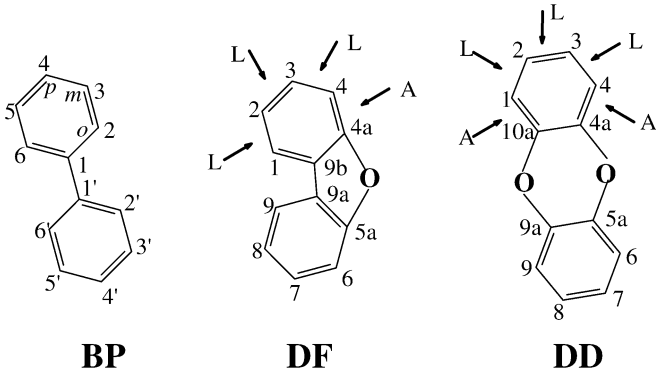

Fig. 2 Schematic representation of DF and DD, their possible sites of oxygenation (*A* angular oxygenation, *L* lateral oxygenation) and a comparison with the structure of biphenyl (*BP*)

Seeger et al. (2001) examined the metabolites generated from DD and DF by LB400 BPDO. This enzyme is among the most-characterized BPDO and it oxygenates a broad range of chlorobiphenyl congeners (Mondello et al. 1997). The identification of 2,2′,3-trihydroxybiphenyl as one of the metabolites suggested this enzyme is capable of hydroxylating angular carbons. However, because LB400 BPDO is distantly related to the oxygenases listed above and because, unlike the latter oxygenases, LB400 BPDO oxygenates DF at more than one position (Seeger et al. 2001), we cannot exclude the possibility that 2,2′,3-trihydroxybiphenyl is produced from the rearrangement of an unstable dihydro-dihydroxy metabolite resulting from lateral oxygenation of DF. Metabolites generated by rearrangement of unstable dihydro-dihydroxybiphenyls were recently identified among the metabolites obtained from the catalytic oxygenation of hydroxybiphenyls by B-356 BPDO (Sondossi et al. 2004) and chloro-hydroxybiphenyls (Francova et al. 2004). Before we can conclusively determine the structural features responsible for changing the regiospecificity of BPDOs to favor angular oxygenation of DF, it is mandatory that angular oxygenation be unambiguously demonstrated.

Unlike LB400 BPDO, B-356 BPDO oxygenates 2,2′-dichlorobiphenyl (2,2′-CB) poorly (Barriault et al. 1997) and is unable to oxygenate naphthalene (Hurtubise et al. 1995). Since DF is structurally analogous to both naphthalene and 2,2′-CB, it was of interest to compare the metabolites generated from DF by B-356 and LB400 BPDOs.

The purpose of the present investigation was to examine the metabolite profile obtained from catalytic oxygenation of DF and its analogue DD by B-356 BPDO and to compare it with that obtained with LB400 BPDO. Furthermore, we used DF deuterated on all carbons (DF_{d8}) to demonstrate unambiguously that LB400 BPDO catalyzes angular oxygenation of DF.

Materials and methods

Bacterial strains, plasmids, chemicals and general protocols

Escherichia coli DH11S (Lin et al. 1992) was used in this study. Plasmids pDB31[LB400-*bphAE*] and pDB31[B-356-*bphAE*] were described by Barriault et al. (2002). To construct pQE31[LB400-*bphFG*], *bphFG* was amplified as a 1,555-bp *Bam*HI/*Kpn*I fragment from LB400 DNA (using antisense primers 5′-GCGGGATCCTATGAAATTT ACCAGAGTTTG-3′, 5′-CTGGTACCGCTTCACCTTT

CA-3′) and then cloned into the *Bam*HI/*Kpn*I-digested pQE31. DNA general protocols were done according to Sambrook et al. (1989). The other plasmids used (pQE31[B-356-*bphAE*], pQE31[LB400-*bphAE*], pQE31[B-356-*bphF*], pQE31[LB400-*bphF*], pQE31[LB400-*bphG*]) to produce the purified His-tagged components of LB400 and B-356 BPDOs were described by Barriault et al. (2002) and Hurtubise et al. (1996).

Chemicals

The chemicals used in this work were of the highest grade available commercially. Dibenzofuran (99% minimum purity) was from Aldrich Chemicals (Milwaukee, Wis.) and dibenzo-*p*-dioxin (98% minimum purity) was from Accu-Standard (New Haven, Conn.). DF_{d8} (99% purity) was obtained from CDN Isotope (Pointe-Claire, Québec, Canada).

Assays to identify metabolites and quantify catalytic activity

Metabolites were analyzed from whole-cell suspensions of *E. coli* [pQE31*bphFG*] + [pDB31*bphAE*] induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown in LB broth (Sambrook et al. 1989) to reach an optical density at 600 nm (OD_{600}) of 1.0 and were then induced for 3 h with 0.5 mM IPTG. Induced cells were harvested by centrifugation, washed and suspended to an OD_{600} of 2.0 in M9 medium (Sambrook et al. 1989) containing 0.5 mM IPTG. This cell suspension was distributed by portions (2 ml) among 7-ml glass tubes covered with Teflon-lined screw caps. Each tube received 2 µl of a 50 mM acetone solution of the appropriate substrate. They were incubated overnight at 37°C with shaking. Cell suspensions were extracted at neutral pH with ethyl acetate. Metabolites were identified by gas chromatographic–mass spectrometric (GC-MS) analysis of their *n*-butylboronate (*n*BuB) or trimethylsilyl (TMS) derivatives (Barriault et al. 1999, 2002). All analyses were done in triplicate.

Metabolites were also analyzed from catalytic conversion of DF by His-tagged purified enzymes. In this case, the enzyme components were purified by affinity chromatography on Ni-nitrilotriacetic acid resin, according to the protocols of Hurtubise et al. (1995, 1996). Enzyme assays were performed in a volume of 200 µl in 100 mM morpholinoethanesulfonic acid buffer, pH 6.0 (Hurtubise et al. 1996). The reactions were initiated by adding 100 nmol of substrate dissolved in acetone. Metabolites were extracted at pH 6.0 with ethyl acetate and treated with *n*BuB or TMS for GC-MS analysis.

Results

Metabolism of DD and DF by LB400 and B-356 BPDOs

Using GC-MS analysis, Seeger et al. (2001) examined the TMS-derived metabolites produced from DF by *E. coli* cells expressing LB400 BPDO. They found five metabolites, two of which represented respectively 2–5% and 5–10% of the total metabolites and were identified as monohydroxy-dibenzofuran (monohydroxy-DF). Two other metabolites, representing respectively 55–65% and 10–20% of total metabolites were identified as dihydro-dihydroxy-DF. The last metabolite represented 10–15% of total metabolites and was identified as 2,2′,3-trihydroxybiphenyl.

Under our experimental conditions, IPTG-induced cells of *E. coli* pDB31[LB400 *bphAE*] +pQE51[*bphFG*] oxygenated 30 nmol of DF in 18 h. Five TMS-derived metabolites were detected from GC-MS analysis of these cultures. Based on the peak surface of the total ion chromatogram, 2,2′,3-trihydroxybiphenyl represented approximately 5% (or 1.5 nmol) of the substrate converted. It was

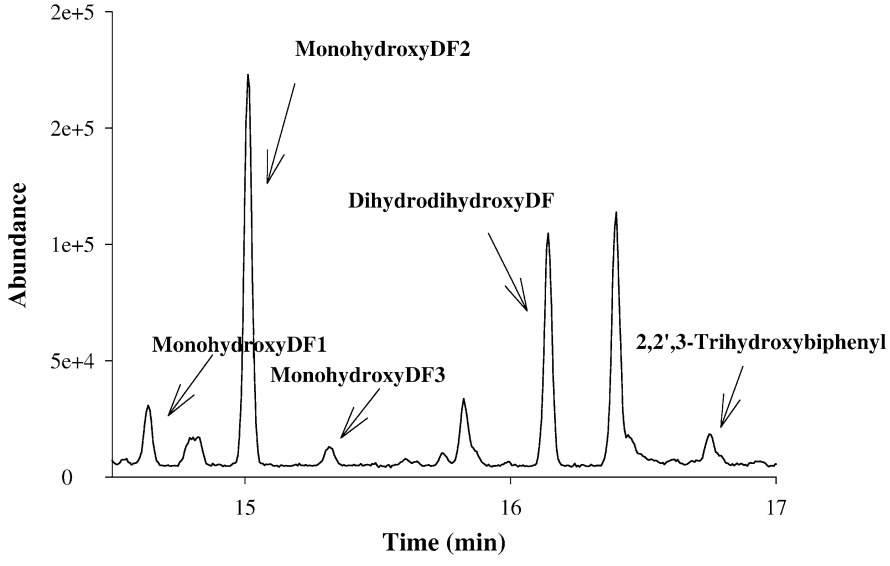

Fig. 3 Total ion chromatogram showing the peaks of TMS-derived metabolites produced from DF by *E. coli* cells expressing LB400 BPDO. The unlabeled peaks are metabolites of *E. coli* produced in the absence of DF

identified on the basis of: (1) its mass spectral fragmentation pattern (data not shown) that comprised ions at *m/z* 418 (M^+), *m/z* 403 (M^+–CH_3) and *m/z* 315 [M^+–CH_3–$(CH_3)_4Si$; where the loss of a molecule of tetramethylsilane from the molecular ion is characteristic of a catechol moiety (Massé et al. 1989)] and (2) by comparison with authentic 2,2′,3-trihydroxybiphenyl produced by catalytic oxygenation of 2-hydroxybiphenyl by *E. coli* cells expressing LB400 BPDO. Three other metabolites exhibited mass spectral features (molecular ion at *m/z* 256, prominent ions at *m/z* 241, 225) that were identical to those reported by Seeger et al. (2001) for monohydroxy-DF. Based on the peak surface of the total ion chromatogram, they represented 5, 5 and 50% of the total metabolites produced, respectively (Fig. 3). One last metabolite exhibited mass spectral features identical to those reported for dihydro-dihydroxy-DF (molecular ion at *m/z* 346, prominent ions at *m/z* 256, 241, 184, 168, 156, 147, 139) by Seeger et al. (2001). This metabolite represented 35% of the total metabolites. Data confirmed that 2,2′,3-trihydroxybiphenyl was a minor metabolite of DF oxygenation. There was an apparent discrepancy between our results and those of Seeger et al. (2001) about the relative amount of monohydroxy-DF and dihydro-dihydroxy-DF. However, this can be explained on the basis of differences in extraction procedures, whereby the procedure we followed has most likely favored the dehydration of the dihydro-dihydroxy metabolites.

When DF metabolites produced by *E. coli* cells expressing LB400 BPDO were derived with butylboronate, two metabolites (metabolites 1, 2 in Fig. 4) were detected on GC-MS chromatograms but were not detected in a control culture that did not express the enzyme. Both metabolites exhibited a molecular ion at *m/z* 268 (Fig. 4). Based on their molecular ions and mass spectral fragmentation pattern, they could have been butylboronate-derived dihydro-dihydroxy-DF or 2,2′-3-trichlorobiphenyl. However, authentic butylboronate-derived 2,2′,3-trihydroxybiphenyl obtained by catalytic conversion of 2-hydroxybiphenyl by LB400 BPDO was not detected under our chromatographic conditions (data not shown). Furthermore, the fact that the molecular ion of both butylboronate-derived metabolites obtained from catalytic conversion of DF_{d8} by LB400 BPDO was at *m/z* 276 (Fig. 5) conclusively demonstrated that metabolites 1 and 2 were isomers of dihydro-dihydroxy-DF. If one of these metabolites had been 2,2′,3-trihydroxybiphenyl generated from an angular attack of DF_{d8}, the mass spectral pattern of its butylboronate derivative should have exhibited a molecular mass at *m/z* 275 instead of *m/z* 276 because of the replacement of one deuterium by a hydroxyl group in the trihydroxybiphenyl metabolite (Fig. 5).

When *E. coli* cells expressing B-356 BPDO were used to catalyze the oxygenation of DF, the reaction was much less efficient than for cells expressing LB400 BPDO. Based on the peak surface of substrate in the total ion chromatogram,

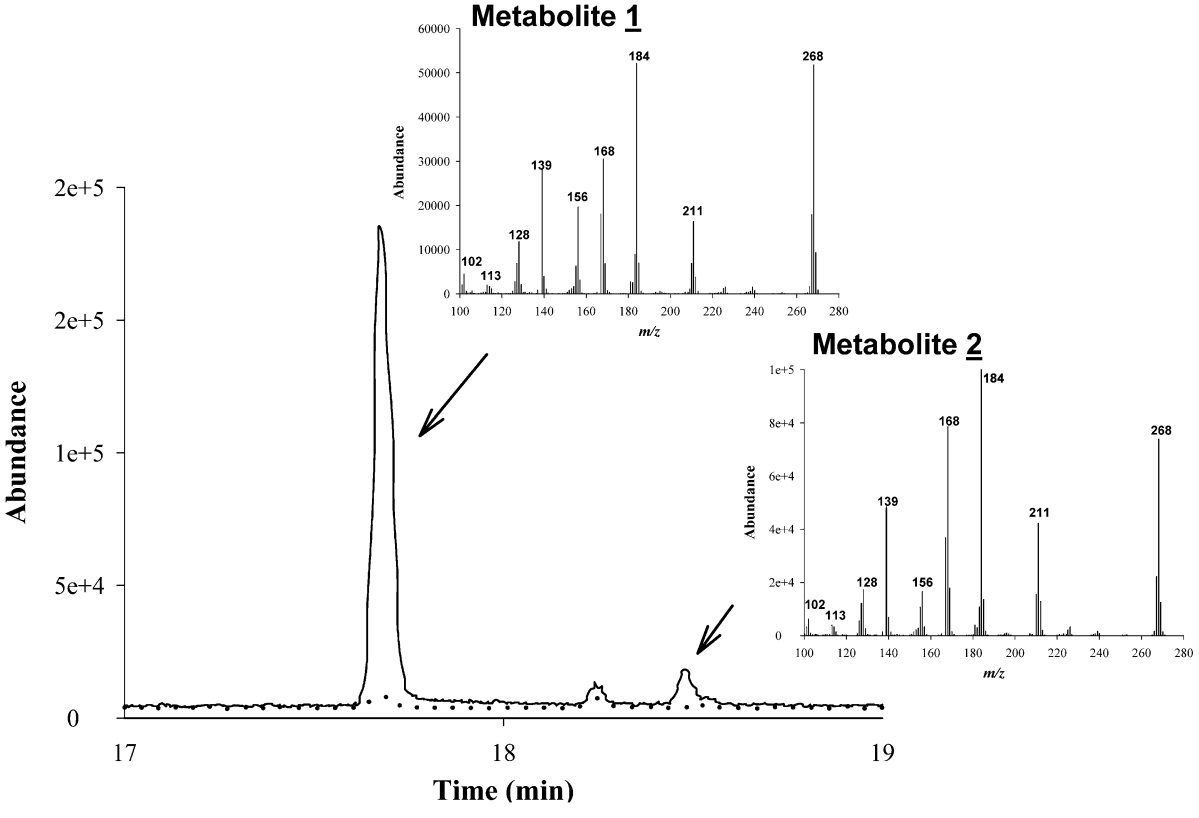

Fig. 4 Total ion chromatograms showing the peaks and mass spectra of the butylboronate-derived metabolites produced from DF by *E. coli* cells expressing LB400 BPDO (*solid line*) and B-356 BPDO (*dotted line*)

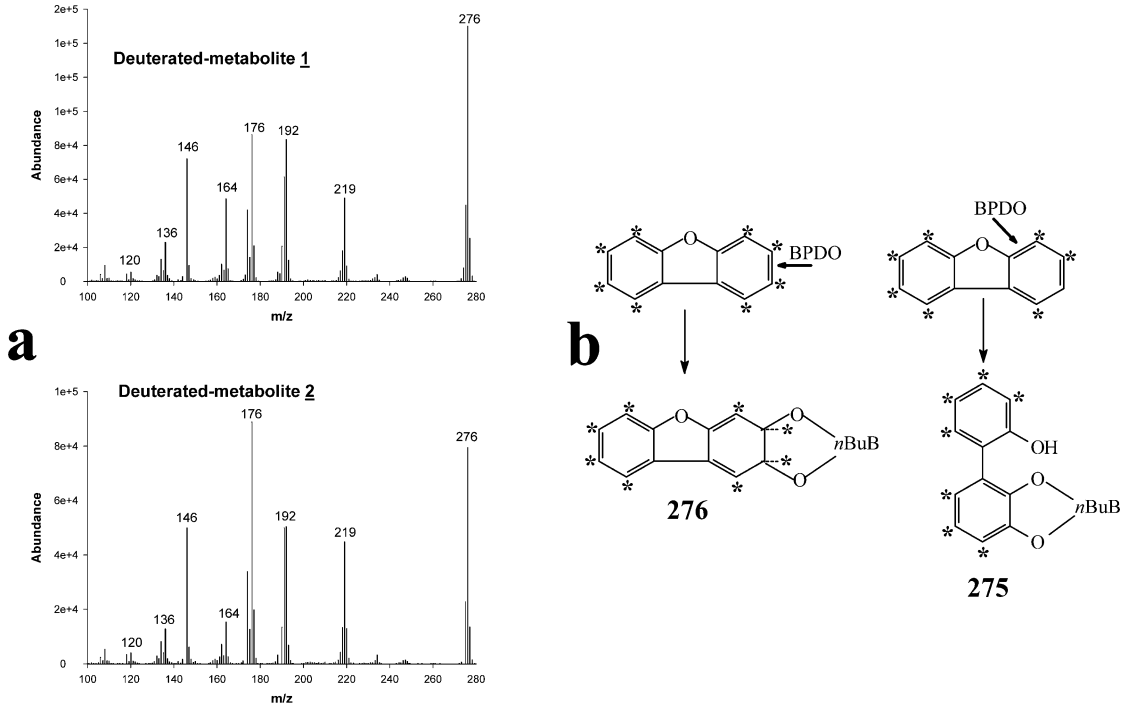

Fig. 5 **a** Mass spectra of butylboronate-derived metabolites 1 and 2 produced from DF_{d8}. **b** Schematic representation showing the difference in molecular mass of the butylboronate-derived dihydro-dihydroxy-DF and 2,2′,3-trihydroxybiphenyl produced from DF_{d8}

B-356 BPDO transformed 2.0±0.3 nmol of the substrate after 18 h of incubation, compared with 30.0±2.0 nmol by cells expressing LB400 BPDO. A small amount of metabolite 1 was detected when the metabolites were derived with butylboronate (Fig. 4) and only traces of dihydro-dihydroxy-DF and monohydroxy-DF were detected when the metabolites were derived with TMS (data not shown). However, when a purified preparation of His-tagged B-356 BPDO was used to catalyze the oxygenation of DF, the same metabolite profile as that obtained with purified LB400 BPDO was observed (Fig. 6), including 2,2′,3-trihydroxybiphenyl that represented approximately 10% of the substrate converted. Thus, DF metabolism by B-356 BPDO was similar to that by LB400 BPDO, except that the rate of transformation was slower. At this time, there is no clear explanation why both purified enzymes produced similar amounts of each metabolite after 15 min of incubation, whereas *E. coli* cells expressing these enzymes metabolized DF at considerably different rates. Because the metabolite production was determined at a single time-

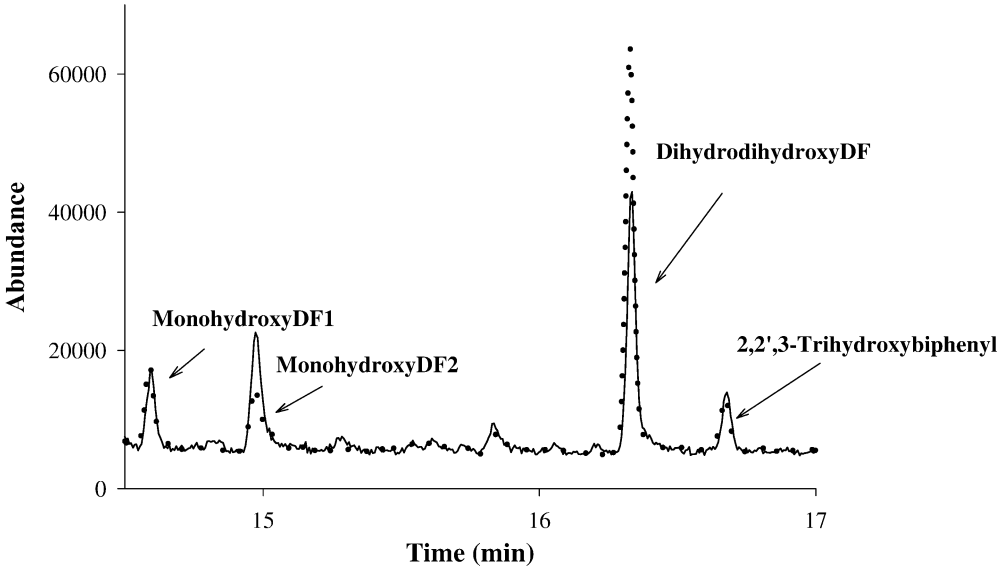

Fig. 6 Total ion chromatograms showing the peaks of TMS-derived metabolites produced from DF by purified preparation of His-tagged LB400 BPDO (*solid line*) and B-356 BPDO (*dotted line*)

point, it was not possible to determine whether higher sensitivity of LB400 BPDO to environmental conditions or product inhibition could have explained this difference. The rate of conversion of 4-chlorobiphenyl, a substrate that both enzymes oxygenate equally well, was similar for *E. coli* cells expressing B-356 or LB400 BPDO at respectively 4.2 nmol h^{-1} and 4.4 nmol h^{-1}. This suggests that the level of expression of BPDO is similar for the two *E. coli* recombinant clones. Nevertheless, data show that the regiospecificity of B-356 BPDO toward DF resembles that of LB400 BPDO where the orientation of the substrate inside the catalytic pocket favors a lateral rather than angular oxygenation.

Use of DF_{d8} to demonstrate angular oxygenation by BPDO

Because LB400 BPDO oxygenated DF at more than one position, we could not exclude the possibility that 2,2′,3-

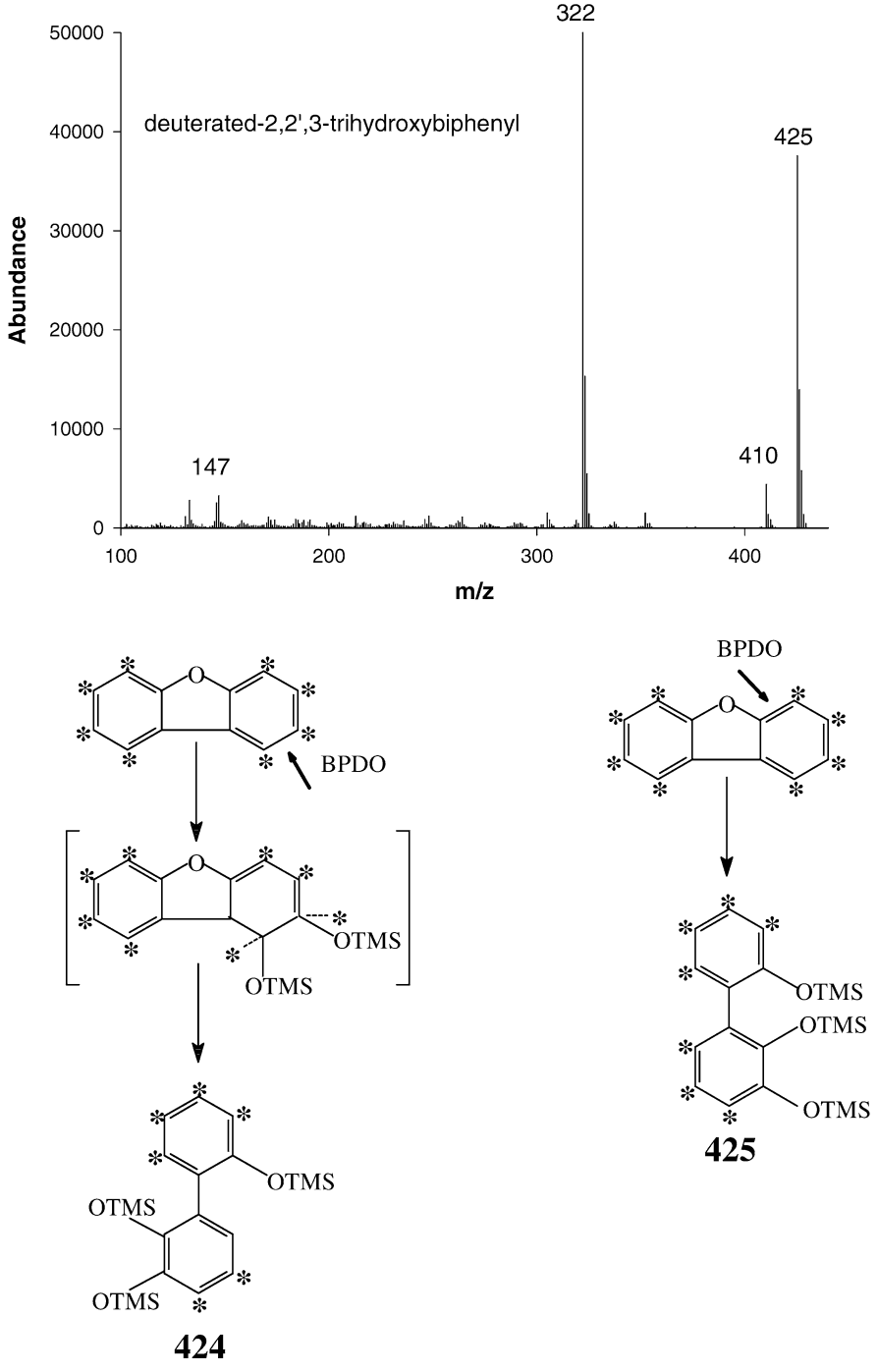

Fig. 7 Mass spectra of TMS-derived 2,2′,3-trihydroxybiphenyl generated by catalytic oxygenation of DF_{d8} and schematic representation to explain the difference in molecular mass of the TMS-derived 2,2′,3-trihydroxybiphenyl, depending on the pathway used for its formation from DF_{d8}

trihydroxybiphenyl was produced from the rearrangement of 1,2-dihydro-1,2-dihydroxy-DF. In order to unambiguously show that 2,2′,3-trihydroxybiphenyl was generated from angular oxygenation of DF, we used DF_{d8} as substrate. The mass spectra of the non deuterated 2,2′,3-trihydroxybiphenyl-TMS exhibited a molecular ion at *m*/*z* 418, with prominent ions at *m*/*z* 403 (M^+–CH_3, or 418-15) and *m*/*z* 315 (M^+–$(CH_3)_4Si$–CH_3, or 418-88-15; data not shown). The mass spectral fragmentation pattern of the deuterated 2,2′,3-trihydroxybiphenyl-TMS (Fig. 7) was identical to that of the non-deuterated compound, except that the fragmentation ions were displaced by a value of *m*/*z* = +7. Prominent ions were at *m*/*z* 410 (M^+–CH_3, or 425-15) and *m*/*z* 322 (M^+–$(CH_3)_4Si$–CH_3, or 425-88-15) and the molecular ion was at *m*/*z* 425.

This shows that 2,2′,3-trihydroxybiphenyl was produced from an angular attack. Mass spectrometry is a very sensitive and precise method to determine the molecular mass of chemicals. If 2,2′,3-trihydroxybiphenyl had been produced from the rearrangement of 1,2-dihydro-1,2-dihydroxy-DF resulting from a lateral attack on carbons 1 and 2, the expected fragmentation ions of its TMS-derivative would have been displaced by a value of *m*/*z* = +6. The molecular ion would have been at *m*/*z* 424 instead of *m*/*z* 425 (Fig. 7).

Metabolism of DD by LB400 and B-356 BPDOs

Seeger et al. (2001) showed that, unlike DF, DD was preferentially dioxygenated at the angular position by LB400 BPDO. Our data confirmed that LB400 BPDO generated a single metabolite from DD that exhibited the mass spectral features reported by Seeger et al. (2001) for 2,2′,3-trihydroxy-diphenyl ether-TMS (molecular ion at *m*/*z* 434, prominent ions at *m*/*z* 419, 331). B-356 BPDO generated the same metabolite from DD (data not shown). However, the rate of DD oxygenation by both strains was much lower than for DF. Cells of *E. coli* expressing LB400 BPDO or B-356 BPDO transformed approximately 2 nmol of DD in 18 h when assayed under the conditions described in the Materials and methods.

Discussion

Suenaga et al. (2001a, b) showed that residue T^{376} of *Pseudomonas pseudoalcaligenes* KF707 BPDO (corresponding to N^{377} of LB400 BPDO) was critical to enhance the catalytic activity of the enzyme toward DF and DD. Replacing residue T^{376} of KF707 BPDO by N or V increased significantly the catalytic activity of the evolved enzyme toward DF and DD. Residue 377 of LB400 BPDO was also found to be important for substrate recognition and regiospecificity toward 2,2′,5,5′-CB, allowing oxygenation onto *meta–para* carbons (Suenaga et al. 1999). Suenaga et al. (2002) found that this residue was located in the vicinity of the iron active center on a three-dimensional model of KF707 BPDO structure that was based on the crystallographic coordinates of naphthalene dioxygenase. The recently published crystal structure of *Rhodococcus* sp. RHA1 BPDO confirmed the proximity of this residue to the enzyme active center (Furusawa et al. 2004).

The fact that LB400 BPDO oxygenated DF poorly suggests that residue N^{377} of LB400 BPDO is not the only one responsible for these enzyme features. Furthermore, the observation that LB400 BPDO favored the lateral rather than the angular reaction suggests that co-planarity rather than *ortho* substitution determines the orientation that DF can take within the catalytic pocket of LB400 BPDO. DF can be regarded as an analogue of symmetrical doubly *ortho*-substituted 2,2′-CB which is oxygenated principally onto carbons 2 and 3 by LB400 BPDO (Haddock et al. 1995). Therefore, if the *ortho* substitution had determined the orientation of DF in the catalytic pocket, the angular oxygenation that corresponds to an oxygenation of carbons 2 and 3 of 2,2′-CB would have been preferred. It is noteworthy that, although their residue 377 differs (N in LB400 BPDO, T in B-356 BPDO), both enzymes catalyze the oxygenation of DF and DD similarly. We still need to study both enzymes' crystal structures to understand precisely the contribution of residue 377 to the enzyme catalytic activity, substrate recognition, turnover rate of oxidation and regiospecificity.

Many questions remain to be answered to understand which protein structural features determine regiospecificity and how they interact with the substrate to influence its orientation within the catalytic pocket. Seeger et al. (2001) presented the five possible orientations that DF could occupy in the enzyme catalytic pocket and the three orientations that DD could occupy. LB400 BPDO allows only the angular attack plus two of the four possible lateral oxygenation reactions of DF; and it allows only the angular oxygenation of DD. Based on the alignment of the conformation of DF with that of biphenyl, Seeger et al. (2001) made the observation that the two orientations of DF that are most similar to that of biphenyl should lead to an oxygenation on carbons 1 and 2 (lateral) or 4 and 4a (angular) of DF. The metabolism of DF by the biphenyl degrader *Beijerinckia* sp. B1 (now called *Sphingomonas yanoikuyae* B1; Gibson 1999) was examined by Cerniglia et al. (1979). They reported the production of two metabolites tentatively identified as *cis*-1,2-dihydro-1,2-dihydroxydibenzofuran and *cis*-2,3-dihydro-2,3-dihydroxydibenzofuran. The identification was based indirectly on the identification of the monohydroxylated derivatives obtained after acidification of the dihydro-dihydroxy metabolites. Oxygenation of carbons 1 and 2 was also the preferred site of attack of DF by the naphthalene dioxygenase of *Pseudomonas* sp. NCIB 9816-4 (Resnick and Gibson 1996), where 50–60% of the substrate was converted into *cis*-1,2-dihydro-1,2-dihydroxydibenzofuran. *Cis*-3,4-dihydro-3,4-dihydroxydibenzofuran was the second most abundant metabolite, representing 30–40% of the substrate converted. The precise position of hydroxylation of DF by BPDOs can only be determined from direct identification of the resulting dihydro-dihydroxy-DF. Although we have not identified precisely all the positions of attack of DF during BPDO oxygenation,

our results using DF_{d8} demonstrated unambiguously that 2,2′,3-trihydroxybiphenyl generated from the catalytic oxygenation of DF by LB400 resulted from an angular attack of the molecule.

References

Armengaud J, Happe B, Timmis KN (1998) Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J Bacteriol 180:3954–3966

Barriault D, Plante MM, Sylvestre M (2002) Family shuffling of a targeted *bphA* region to engineer biphenyl dioxygenase. J Bacteriol 184:3794–3800

Barriault D, Vedadi M, Powlowski J, Sylvestre M (1999) *Cis*-2, 3-dihydro-2,3-dihydroxybiphenyl dehydrogenase and *cis*-1,2-dihydro-1,2-dihydroxynaphathalene dehydrogenase catalyze dehydrogenation of the same range of substrates. Biochim Biophys Res Commun 260:181–187

Barriault D, Pelletier C, Hurtubise Y, Sylvestre M (1997) Substrate selectivity pattern of *Commamonas testosteroni* strain B-356 towards dichlorobiphenyls. Int Biodeterior Biodegrad 39:311–316

Broadus RM, Haddock JD (1998) Purification and characterization of the NADH:ferredoxin BPH oxidoreductase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. Arch Microbiol 170:106–112

Brühlmann F, Chen W (1999) Tuning biphenyl dioxygenase for extended substrate specificity. Biotechnol Bioeng 63:544–551

Cerniglia CE, Morgan JC, Gibson DT (1979) Bacterial and fungal oxidation of dibenzofuran. Biochem J 180:175–185

Denef VJ, Park J, Tsoi TV, Rouillard JM, Zhang H, Wibbenmeyer JA, Verstraete W, Gulari E, Hashsham SA, Tiedje JM (2004) Biphenyl and benzoate metabolism in a genomic context: outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl Environ Microbiol 70:4961–4970

Engesser KH, Strubel V, Christoglou K, Fischer P, Rast HG (1989) Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1, 10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran. FEMS Microbiol Lett 53:205–209

Erickson BD, Mondello FJ (1992) Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J Bacteriol 174:2903–2912

Francova K, Mackova M, Macek T, Sylvestre M (2004) Ability of bacterial biphenyl dioxygenases from *Burkholderia* sp LB400 and *Comamonas testosteroni* B-356 to catalyse oxygenation of *ortho*-hydroxychlorobiphenyls formed from PCBs by plants. Environ Pollut 127:41–48

Furusawa Y, Nagarajan V, Tanokura M, Masai E, Fukuda M, Senda T (2004) Crystal structure of the terminal oxygenase component of biphenyl dioxygenase derived from *Rhodococcus* sp. strain RHA1. J Mol Biol 342:1041–1052

Gibson DT (1999) *Beijerinckia* sp strain B1: a strain by any other name. J Ind Microbiol Biotechnol 23:284–293

Haddock JD, Gibson DT (1995) Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol 177:5834–5839

Haddock JD, Horton JR, Gibson DT (1995) Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2, 3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol 177:20–26

Haddock JD, Pelletier DA, Gibson DT (1997) Purification and properties of ferredoxin BPH, a component of biphenyl 2,3-dioxygenase of *Pseudomonas* sp strain LB400. J Ind Microbiol Biotechnol 19:355–359

Hurtubise Y, Barriault D, Powlowski J, Sylvestre M (1995) Purification and characterization of the *Comamonas testosteroni* B-356 biphenyl dioxygenase components. J Bacteriol 177: 6610–6618

Hurtubise Y, Barriault D, Sylvestre M (1996) Characterization of active recombinant his-tagged oxygenase component of *Comamonas testosteroni* B-356 biphenyl dioxygenase. J Biol Chem 271:8152–8156

Kasuga K, Habe H, Chung JS, Yoshida T, Nojiri H, Yamane H, Omori T (2001) Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the Gram-positive dibenzofuran-degrader *Terrabacter* sp. strain DBF63. Biochim Biophys Res Commun 283:195–204

Kimura N, Urushigawa Y (2001) Metabolism of dibenzo-p-dioxin and chlorinated dibenzo-p-dioxin by a gram-positive bacterium, *Rhodococcus opacus* SAO 101. J Biosci Bioeng 92:138–143

Kumamaru T, Suenaga H, Mitsuoka M, Watanabe T, Furukawa K (1998) Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. Nat Biotechnol 16:663–666

Lin JJ, Smith M, Jessee J, Bloom F (1992) DH11s: an *E. coli* strain for preparation of single-standed DNA from phagemid vectors. BioTechniques 12:718–721

Maeda T, Takahashi Y, Suenaga H, Suyama A, Goto M, Furukawa K (2001) Functional analyses of Bph-Tod hybrid dioxygenase, which exhibits high degradation activity toward trichloroethylene. J Biol Chem 276:29833–29838

Massé R, Messier F, Ayotte C, Lévesque M-F, Sylvestre M (1989) A comprehensive gas chromatographic/mass spectrometric analysis of 4-chlorobiphenyl bacterial degradation products. Biomed Environ Mass Spectrosc 18:27–47

Mondello FJ, Turcich MP, Lobos JH, Erickson BD (1997) Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl Environ Microbiol 63:3096–3103

Nojiri H, Habe H, Omori T (2001) Bacterial degradation of aromatic compounds via angular dioxygenation. J Gen Appl Microbiol 47:279–305

Nojiri H, Omori T (2002) Molecular bases of aerobic bacterial degradation of dioxins: Involvement of angular dioxygenation. Biosci Biotechnol Biochem 66:2001–2016

Parales RE, Lee K, Resnick SM, Jiang HY, Lessner DJ, Gibson DT (2000) Substrate specificity of naphthalene dioxygenase: effect of specific amino acids at the active site of the enzyme. J Bacteriol 182:1641–1649

Resnick SM, Gibson DT (1996) Regio-and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4. Appl Environ Microbiol 62:4073–4080

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Seeger M, Camara B, Hofer B (2001) Dehalogenation, denitration, dehydroxylation, and angular attack on substituted biphenyls and related compounds by a biphenyl dioxygenase. J Bacteriol 183:3548–3555

Sondossi M, Barriault D, Sylvestre M (2004) Metabolism of 2,2′- and 3,3′-dihydroxybiphenyl by the biphenyl catabolic pathway of *Comamonas testosteroni* B-356. Appl Environ Microbiol 70:174–181

Suenaga H, Goto M, Furukawa K (2001a) Emergence of multifunctional oxygenase activities by random priming recombination. J Biol Chem 276:22500–22506

Suenaga H, Mitsuoka M, Ura Y, Watanabe T, Furukawa K (2001b) Directed evolution of biphenyl dioxygenase: emergence of enhanced degradation capacity for benzene, toluene, and alkylbenzenes. J Bacteriol 183:5441–5444

Suenaga H, Nishi A, Watanabe T, Sakai M, Furukawa K (1999) Engineering a hybrid pseudomonad to acquire 3,4-dioxygenase activity for polychlorinated biphenyls. J Biosci Bioeng 87:430–435

Suenaga H, Watanabe T, Sato M, Ngadiman, Furukawa K (2002) Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering. J Bacteriol 184:3682–3688

Sylvestre M, Sirois M, Hurtubise Y, Bergeron J, Ahmad D, Shareck F, Barriault D, Guillemette I, Juteau JM (1996) Sequencing of *Comamonas testosteroni* strain B-356-biphenyl/chlorobiphenyl dioxygenase genes: Evolutionary relationships among Gram-negative bacterial biphenyl dioxygenases. Gene 174:195–202