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Métabolisme du 1,1,1-trichloro-2,2-*bis*(4-chlorophenyl)éthane (DDT), du dibenzofuranne et du dibenzo-*p*-dioxine par certaines dioxygénases du biphényle

par

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RÉSUMÉ

De multiples substances toxiques, tant pour la faune que pour la flore sont présentes dans l'environnement. Parmi celles-ci compte plusieurs analogues du biphényle, dont les biphényles polychlorés (BPC), les dérivés chlorés du dibenzofuranne (DF), la dibenzo-*p*-dioxine (DD) ainsi que le 1,1,1-trichloro-2,2-*bis*(4-chlorophényl)éthane (DDT) qui sont des polluants persistants dans l'environnement. Leur persistance est attribuée à l'absence de voies cataboliques microbiennes efficaces pour leur dégradation. Cependant certaines bactéries isolées de l'environnement tels *Comamonas testosteroni* B-356, *Burkholderia xenovorans* LB400 et *Cupriavidus necator* A5 possèdent une voie catabolique du biphényle capable de dégrader partiellement, par co-métabolisme, certains de ces polluants persistants. La voie catabolique du biphényle est initiée par la dioxygénase du biphényle (BPDO) qui introduit deux atomes d'oxygène sur deux carbones adjacents du noyau biphényle. Cette enzyme comporte trois composantes, dont l'oxygénase interagissant directement avec le substrat pour catalyser l'oxygénation, la ferrédoxine et la réductase de la ferrédoxine étant deux composantes qui catalysent le transport d'électrons du NADH à l'oxygénase. Chez les souches B-356 et LB400, les gènes *bphA* et *bphE* codent pour les deux sous-unités qui formant l'oxygénase. Les gènes *bphF* et *bphG* codent respectivement pour la ferrédoxine et la réductase de la ferrédoxine. Le développement de BPDO douées de capacités cataboliques accrues envers les analogues persistants du biphényle permettra, éventuellement de construire des bactéries recombinantes portant des voies cataboliques plus efficaces pour la dégradation de ces polluants. Plusieurs travaux récents ont permis d'obtenir, par ingénierie génétique, des BPDO plus performantes capables de dégrader un éventail plus grand de congénères de BPC que les BPDO isolées de souches sauvages. C'est le cas en particulier des BPDO variantes II-9, III-17, III-34, III-37 et III-52 qui ont été obtenues par recombinaison *in*

vitro aléatoire (« DNA-Shuffling ») entre les gènes *bphA* des souches B-356 et LB400.

Les objectifs de ce travail étaient 1-) de vérifier le potentiel catabolique des BPDO parentales de LB400 et B-356 envers le DF et le DD 2-) de comparer les propriétés catalytiques des BPDO variantes II-9, III-17, III-34, III-37 et III-52 envers le DDT et des BPDO parentales de LB400 et B-356. Dans le cas du DF, nous avons observé qu'une souche recombinante d'*Escherichia coli* produisant la dioxygénase de LB400 pouvait dégrader le DF à un taux plus élevé (30 nmol en 18 H) comparativement à une souche exprimant la dioxygénase de B-356 (2 nmol en 18 H). Cependant, les deux dioxygénases ont produit le même dihydro-dihydroxy-dibenzofuranne comme métabolite majeur, et de faibles quantités de 2,2',3-trihydroxybiphényle. Lorsque le DD était utilisé comme substrat, le 2,2',3-trihydroxybiphényle éther était le seul métabolite détecté et il était produit à un taux similaire pour les deux enzymes, indiquant une attaque similaire pour ce composé. Ces résultats montrent que la régiospécificité de ces deux dioxygénases envers le DF et le DD est semblable, même si pour plusieurs analogues du biphényle, les BPDO de B-356 et LB400 ont fréquemment un patron de métabolisme très différent. Ces résultats suggèrent que la coplanarité du DF et du DD influence la régiospécificité de la dioxygénase d'une manière plus importante que leurs congénères de BPC ayant des substituants en position *ortho*. L'utilisation de DF marqué au deutérium a permis de valider la production de 2,2',3-trihydroxybiphényle par oxygénation angulaire du DF.

Dans le cas du DDT, nous avons démontré que la BPDO de B-356 est aussi efficace que la BPDO de *Cupriavidus necator* A5, une dioxygénase connue pour sa capacité à oxygéner le DDT efficacement. Cependant, contrairement à la BPDO de A5 qui ne produit qu'un seul métabolite, celle de B-356 produit un deuxième

métabolite dihydrodiol en petite quantité. D'autre part, la BPDO de LB400 catalyse la dioxygénation du DDT très inefficacement. Nous avons comparé les propriétés catalytiques des variants II-9, III-17, III-34, III-37 et III-52 envers le DDT étant des hybrides résultants de recombinaisons génétiques entre *bphA* de LB400 et de B-356. Les résultats démontrent que la régiospécificité ainsi que l'efficacité à catalyser la réaction varient grandement selon le variant utilisé. Le variant III-37 était le moins efficace. L'efficacité des variants III-52, III-17 et II-9 à oxygéner le DDT était semblable pour ces enzymes, et ces variants produisaient principalement un métabolite qui était identique au métabolite mineur produit par la BPDO de B-356. Enfin, l'efficacité du variant II-34 était moindre que celle des trois autres variants, mais cet enzyme produisait exclusivement un métabolite qui correspondait au métabolite majeur produit par la BPDO de B-356. Des analyses RMN de ces métabolites purifiés ont permis d'identifier les deux métabolites comme étant deux diastéréoisomères de 1,1,1-trichloro-2,(4-chlorophényl-2,3-dihydroxy-4,6-cyclohexadiène)-2-(4'-chlorophényl)éthane (DDT-2,3-dihydrodiol). Ces résultats montrent que certaines modifications structurales de la BPDO permettent d'altérer, non seulement la régiospécificité, mais la stéréospécificité de l'enzyme envers les analogues du substrat. Nous avons comparé les séquences primaires en acides aminés des BphA des variants avec celles des BphA parentales. Cette comparaison a permis de montrer qu'un segment de 7 acides aminés de l'enzyme, appelé région III, influence significativement la capacité de la dioxygénase à catalyser l'oxygénation du DDT efficacement. Cependant, la stéréospécificité envers le DDT est déterminée par un (ou quelques) acides aminés compris entre les résidus 237 et 261 de BphA de LB400 qui sont communs pour le variant III-34 et les parents B-356 et A5 qui produisent principalement le même métabolite du DDT.

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TABLE DES MATIÈRES

LISTE DES FIGURES

CHAPITRE 2

(Article 1)

CHAPITRE 3

(Article 2)

LISTE DES TABLEAUX

CHAPITRE 3

(Article 2)

ABRÉVIATIONS

µL	Microlitre
BPC	Biphényle polychlorés
BPDO	Dioxygénase du biphényle
CB	Chlorobiphényles
CDDs	Polychlororinated dibenzo-p-dioxins
CDF	Polychlororinated dibenzofurans
DD	Dibenzo-p-dioxine
DDD	Dichlorodiphényldichloroéthane
DDE	Dichlorodiphényldichloroéthylène
DDT	Dichlorodiphényltrichloroéthane
DF	Dibenzofuranne
DF_{d8}	Dibenzofuranne deutéré
DNA	Adénosine désoxyribonucléotide
FER_{BPH}	Composé férrédoxine de la BPDO
GC-MS	Chromatographie en phase gazeuse-spectromètre de masse
h	Heure
HPLC	Chromatographie liquide haute performance
IPTG	Isopropyl-β-D-thiogalacto-pyranoside
ISP_{BPH}	Protéine fer-soufre
MES	Morpholinoethanesulfonic
mL	Millilitre
NADH	Nicotinamide adénine dinucleotide
nBuB	Acide n-butylboronique
Ni-NTA	Nickel-Nitrilotriacetic Acid
nmol	Nanomoles
NMR	Résonance magnétique nucléaire
OD	Densité optique
PAHs	Hydrocarbones polyaromatiques
POPs	Persistent organic pollutants
RED_{BPH}	Composé réductase de la BPDO
TMS	Tétraméthylsilane

CHAPITRE 1

REVUE DE LITTÉRATURE

INTRODUCTION

Les enzymes possèdent un pouvoir catabolique impressionnant. En fait, plusieurs bactéries ont acquis des propriétés intéressantes par sélection naturelle au cours de milliers d'années. Ces milliers d'années de spécialisation leur ont permis d'acquérir des propriétés inégalées au niveau de la dégradation de certains composés persistants (Sylvestre, 2004). Plusieurs polluants toxiques présents dans l'environnement sont des analogues du biphényle, ceci inclus les biphényles polychlorés (BPC) et divers congénères chlorés du dibenzofuranne (DF) ou de la dibenzo-*p*-dioxine (DD) qui proviennent d'activités humaines comme les rejets industriels. Quelques bactéries sont capables de dégrader certains de ces composés en utilisant la voie catabolique du biphényle. La dioxygénase du biphényle (BPDO), qui est la première enzyme de la voie de dégradation du biphényle, a été modifiée génétiquement pour augmenter ses capacités cataboliques envers certains de ces analogues toxiques. Les bactéries recombinantes produisant ces enzymes modifiées sont capables de dégrader plus efficacement les BPC et plusieurs autres molécules apparentées (Sylvestre, 2004).

Parmi les isolats naturels, dont les BPDO sont très bien caractérisées, on compte *Burkholderia xenovorans* LB400 et *Comamonas testosteroni* B-356. Les deux enzymes ont une spécificité distincte envers les congénères de BPC. La dioxygénase de B-356 a été cristallisée (Imbeault *et al.*, 2000) mais la structure de la protéine n'a pas encore été publiée. La BPDO de B-356 catalyse la dioxygénation des congénères de BPC comme le 3,3'-dichlorobiphényle qui compte un atome de chlore en position *meta* sur chacun des noyaux du biphényle. Cependant la BPDO de B-356 dégrade le 2,2'-dichlorobiphényle inefficacement.

B. xenovorans LB400 est l'une des rares souches capables de dégrader les biphényles polychlorés (BPC) portant des substitutions *ortho* (Bédard *et al.*, 1986). LB400 peut également oxygéner les positions 3 et 4 de divers BPC (Barriault *et al.*, 2004). Les séquences déduites en acides aminés des oxygénases de B-356 et de LB400 présentent une analogie de séquence de l'ordre de 75%. Certains domaines de l'enzyme sont reconnus pour leur implication dans la spécificité de l'enzyme, mais l'identification précise des résidus responsables des propriétés catalytiques de l'enzyme envers les analogues du biphényle reste à déterminer.

Caractéristiques de la BPDO et des autres enzymes de la voie catabolique du biphényle

La BPDO est la première enzyme de la voie catabolique du biphényle (Haddock *et al.* 1995) ; (Hurtubise *et al.*, 1995). Cette dernière comprend trois composantes : une oxygénase fer-soufre (ISP_{BPH}) constituée de deux sous-unités : α (50 000 Da) et β (22 000 Da), une ferrédoxine (FER_{BPH}) (12 000 Da) et une ferrédoxine réductase (RED_{BPH}) (Haddock *et al.*, 1995) ; (Hurtubise *et al.*, 1995). Le composant principal est un hétérohexamère comprenant trois sous-unités α et trois β qui s'assemblent pour former la dioxygénase proprement dit qui interagit directement avec le substrat. La sous-unité α comprend le centre fer-soufre, le centre Rieske qui reçoit les électrons de la ferrédoxine pour les transmettre au fer mononucléaire qui se trouve au centre catalytique de l'enzyme, aussi situé sur la sous-unité α.

En ce qui concerne les dioxygénases provenant des souches LB400 et B-356, les gènes codant sont : *bphA* (ISP_{BPH} sous-unité α), *bphE* (ISP_{BPH} sous-unité β), *bphF* (FER_{BPH}) et *bphG* (RED_{BPH}) (Erickson et Mondello, 1992) ; (Sylvestre *et al.*, 1996). La réaction catalysée par cette enzyme permet l'ajout d'une molécule d'O_2 sur le

noyau du biphényle portant le moins d'atome de chlore. Les composants BphF et BphG servent au transport des électrons entre le NADH et l'oxygénase.

La deuxième enzyme de la voie catabolique du biphényle est la 2,3-dihydro-2,3-dihydroxybiphényle-2,3-déshydrogénase (BphB). Cette dernière est une déshydrogénase qui rétablit l'aromaticité du cycle benzénique en catalysant l'ajout d'une molécule d'hydrogène (H_2). La réaction suivante dans la séquence de dégradation du biphényle (chlorobiphényle) est catalysée par la 2,3-dihydroxybiphényle-2,3-dioxygénase (BphC) qui est responsable de l'ouverture du cycle aromatique pour générer un métabolite de couleur jaunâtre : le composé de *meta*-clivage.

Sommaire de la problématique des BPC

Les BPC sont des polluants toxiques très persistants dans l'environnement et par le fait même très difficiles à dégrader. Comme nous l'avons indiqué ci-dessus, il existe des souches bactériennes comme LB400 et B-356 étant capables de dégrader certains congénères de BPC par co-métabolisme (Ahmad *et al.*, 1991) et (Sylvestre, 2004). Plusieurs autres isolats naturels capables de dégrader certains congénères de BPC ont aussi été rapportés (Furukawa et Matsumura, 1976) ; (Ahmad *et al.*, 1990) ; (Khan *et al.*, 1988) ; (Sylvestre *et al.*, 1982) ; (Furukawa et Miyazaki, 1986) ; (Bédard *et al.*, 1986) ; (Parsons *et al.*, 1998) et (Massé *et al.*, 1984). Cependant, de façon générale, les BPC sont dégradés inefficacement et ils s'accumulent dans l'environnement (Barriault *et al.*, 1997).

Sommaire de la problématique des DF et DD chlorés

La plupart des DF et des DD chlorés sont produits au cours de l'incinération de divers herbicides et pesticides. À cause de leur liposolubilité, ces composés s'accumulent chez les organismes vivants. Leur effet est très néfaste (Nojiri et Omori, 2002). Ces produits, à l'instar des BPC sont dégradés inefficacement dans l'environnement (Habe *et al.*, 2001). Pour le moment, la seule méthode « économique » de destruction des dérivés chlorés du DF et du DD est l'incinération. Même si plusieurs autres moyens ont été investigués, il n'en reste pas moins que ce procédé est très dispendieux et non applicable sur une grande échelle. D'où l'intérêt d'approches faisant appel à des bactéries ayant le potentiel de dégrader ces polluants.

Sommaire de la problématique du DDT

Ce composé a été utilisé pendant cinq décennies comme insecticide, surtout pour le contrôle de la malaria et autres maladies des pays en voie de développement. Sa très grande stabilité, son coût réduit et son rayon d'action très grand lui ont conféré une place de choix dans notre écosystème. Le DDE et le DDD sont des sous-produits dérivant du DDT et leur accumulation dans la chaîne trophique est très néfaste tant pour les écosystèmes terrestres que marins (Ahuja et Kumar, 2003) ; (Harris *et al.*, 2000) ; (Attaran *et al.*, 2000). Du à l'usage intensif sur plusieurs décennies, de nombreux sites demeurent contaminés. Tout comme pour les BPC, le DF et le DD, le traitement des sols est très couteux. C'est pour cette raison que plusieurs laboratoires à travers le monde tentent de modifier certaines isolats naturels afin d'accroître leur potentiel de dégradation dans un but ultime de pouvoir les utiliser pour des procédés de biorestauration de sites contaminés.

Mes travaux de recherche visaient à examiner les propriétés catalytiques de certaines BPDO d'origine naturelle ou obtenues par ingénierie génétique, envers le DF, le DD et le DDT. Ces travaux ont été entrepris dans le but de vérifier la capacité des dioxygénases à dégrader des analogues du substrat naturel et éventuellement d'identifier certains domaines de la séquence primaire de la sous-unité α qui pourrait être responsable des propriétés catalytiques de l'enzyme envers ces analogues du biphényle.

CHAPITRE 2

(Article 1)

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

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RÉSUMÉ

Nous avons examiné le métabolisme du dibenzofuanne (DF) et de la dibenzo-*p*-dioxine (DD) par la dioxygénase du biphényle (BPDO) de *Comamonas testosteroni* B-356 et nous avons comparé ses propriétés catalytiques à celles de la BPDO de *Burkholderia xenovorans* LB400. Les données démontrent que les deux enzymes ont oxygéné le DF à un taux peu élevé. Les souches recombinantes d'*Escherichia coli*, produisant la dioxygénase de LB400, ont dégradé le DF à un taux plus élevé (30 nmol en 18 h) comparativement aux souches exprimant la dioxygénase de B-356 (2 nmol en 18 h). Les deux dioxygénases ont produit un dihydro-dihydroxy-dibenzofuranne comme métabolite majeur, qui résultait d'une oxygénation latérale du DF. Le 2,2',3-trihydroxybiphényle, résultant de l'oxygénation angulaire, était un métabolite mineur produit par les deux enzymes. Le DF deutéré a été utilisé afin de valider la production de 2,2',3-trihydroxybiphényle par oxygénation angulaire du DF. La capacité des deux enzymes à dégrader le DD a été évaluée. Les deux BPDO produisent le 2,2',3-trihydroxybiphényle éther comme seul métabolite, et à un taux similaire, indiquant une attaque semblable pour ce composé. Même si B-356 et LB400 ont des patrons très différent envers une multitude de substrats divers, leurs patrons de métabolisme envers le DF et la DD sont similaires. Ceci suggère que la coplanarité du substrat influence la régiospécificité de la dioxygénase d'une manière plus importante envers le DF et la DD que la présence d'un substituant en position *ortho*.

ABSTRACT

We examined the metabolism of dibenzofuran (DF) and dibenzo-*p*-dioxin (DD) by the biphenyl dioxygenase (BPDO) of *Comamonas testosteroni* B-356 and we compared it to that of *Burkholderia xenovorans* LB400. Data showed that both enzymes oxygenated DF at low rate, but *Escherichia coli* cells expressing LB400 BPDO degraded DF at higher rate (30 nmol in 18 h) compared to cells expressing B-356 BPDO (2 nmol in 18 h). Furthermore, both BPDOs produced as major metabolite, a dihydro-dihydroxy-dibenzofuran that resulted from 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 chorobiphenyls, 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 have 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 (PAHs) (Brühlmann and Chen, 1999) ; (Kumamaru *et al.*, 1998) ; (Parales *et al.*, 2000) ; (Suenaga *et al.*, 2002).

Among 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*-(2R,3S)-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) [recently renamed *Burkholderia xenovorans* LB400 (Denef *et al.*, 2004)] and in *Comamonas testosteroni* B-356 (Sylvestre *et al.*, 1996). Several evolved BPDOs exhibiting 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) or toward dibenzo-*p*-dioxin (DD) or dibenzofuran (DF) (Suenaga *et al.*, 2001a) have been reported. Of particular interest are the DD and DF as their chlorinated derivatives are of concern for the environment. Furthermore, DF and DD can be regarded as analogs of symmetrical doubly *ortho*-substituted biphenyls and diphenyl ethers (Fig. 2); 2,2'-dichlorobiphenyl is an example. In 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 analogs are recognized by the enzyme active center and what are the orientations they can occupy inside the catalytic pocket of the enzyme.

Several bacterial isolates able to degrade DF and DD have been described (Hiroshi *et al.*, 2001) ; (Kimura and Urushigawa, 2001) ; (Nojiri and Omori, 2002). As noted above, the first reaction in the degradation process involves the introduction of molecular oxygen onto 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 an angular oxygenation of DF and/or DD. However, based on

amino acids 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 was transformed to a stable intermediate identified as 1,10-dihydro-1,10-dihydroxyfluoren-9-one (Engesser et al., 1989).

Seeger *et al.*, (2001) have 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 that this enzyme was 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 oxygenated DF at more than one positions (Seeger *et al.*, 2001), we cannot exclude the possibility that 2,2′,3-trihydroxybiphenyl was produced from the rearrangement of an unstable dihydro-dihydroxy metabolite resulting from lateral oxygenation of DF. Metabolites generated by rearrangement of unstable dihydro-dihydroxybiphenyls have recently been identified among the metabolites obtained from 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 of its analog DD, by B-356 BPDO and compare it to 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 previously (Barriault et al., 2002). To construct pQE31[LB400-*bphFG*], *bphFG* was amplified as a 1555 bp *Bam*HI/*Kpn*I fragment from LB400 DNA using antisense primers 5'GCGGGATCCTATGAAATTTACCAGAGTTTG3' and 5'CTGGTACCGCTTCACCTTTCA3'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*] and pQE31[LB400-*bphG*]) to produced the purified His-tagged components of LB400 and B-356 BPDOs have been described previously (Barriault *et al.* 2002) ; (Hurtubise *et al.* 1996).

Chemicals

The chemicals used in this work were of the highest grade available commercially. Dibenzofuran (minimum purity, 99%) was from Aldrich Chem. (Milwaukee, WI., USA) and dibenzo-*p*-dioxin (minimum purity, 98%) was from AccuStandard (New Haven, CT). Dibenzofuran deuterated on all carbons (DF_{d8}) (purity of 99%) was obtained from CDN Isotope (Pointe-Claire, Québec, Canada).

Assays to identify the metabolites and quantify the catalytic activity

Metabolites were analyzed from whole cell suspensions of *E. coli* [pQE31*bphFG*] + [pDB31*bphAE*] induced with isopropyl-β-D-thiogalacto-pyranoside

(IPTG). Cells were grown in LB broth (Sambrook *et al.*, 1989) to reach an OD of 1.0 at 600 nm and were then induced for 3 h with 0.5 mM IPTG. Induced cells were harvested by centrifugation, washed and suspended to an optical density at 600 nm of 2.0 in M9 medium (Sambrook *et al.*, 1989) containing 0.5 mM IPTG. This cell suspension was distributed by portions of 2 ml in 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 chromatography-mass spectrometry (GC-MS) analyses of their *n*-butylboronate (*n*BuB) or trimethylsilyl (TMS) derivatives (Barriault *et al.*, 2002) ; (Barriault *et al.*, 1999). All analyses were done in triplicate experiments.

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 protocols published previously (Hurtubise *et al.*, 1995, 1996). Enzyme assays were performed in a 200 µl volume 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 and collaborators (Seeger *et al.*, 2001) have examined the TMS-derived metabolites produced from DF by *E. coli* cells expressing LB400 BPDO. They found five metabolites. Two metabolites, representing respectively 2-5 and 5-10% of the total metabolites were identified as monohydroxydibenzofuran (monohydroxy-DF). Two other metabolites, representing respectively 55-65 and 10-20% of total metabolites were identified as dihydro-dihydroxydibenzofuran (dihydro-dihydroxy-DF). The last metabolite representing 10-15% of total metabolites 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 peaks surface of the total ion chromatogram, 2,2′,3-trihydroxybiphenyl represented approximately 5% (or 1.5 nmol) of the substrate converted. It was identified based on its mass spectral fragmentation pattern (not shown) that comprised ions at *m/z* 418 (M^+), 403 (M^+ - CH_3) and 315 (M^+ - CH_3 - $(CH_3)_4Si$) where the loss of a molecule of tetramethylsilane from the molecular ion is a characteristic of catechol moiety (Massé *et al.* 1989), and by comparison to 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 and prominent ions at *m/z* 241 and 225) that were identical to those reported by Seeger and his collaborators (Seeger *et al.*, 2001) for monohydroxy-DF. Based on the peaks 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 with prominent ions at *m/z* 256, 241, 184, 168, 156, 147 and 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 have 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 and 2 on Fig 4) were detected on GC-MS chromatograms that 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-trihydroxybiphenyl. 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 (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 276 because of the replacement of one deuterium by an hydroxyl

group in the trihydroxybiphenyl metabolite (Fig. 5). It is noteworthy that based on the analysis of these spectras, the ratios of m/z 275/276 intensities for metabolites I and II, derived from conversion of DF_{d8}, were respectively 0.31 ± 0.02 and 0.32 ± 0.01 compared to ratios of m/z 267/268 intensities of 0.32 ± 0.01 and 0.31 ± 0.00 for metabolite I and II derived from non deuterated of DF.

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 surface of the peak of substrate on the total ion chromatogram, B-356 BPDO transformed 2 ± 0.3 nmol of the substrate after 18 h of incubation, compared to 30 ± 2 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 of monohydroxy-DF were detected when the metabolites were derived with TMS (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 of 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 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 and 4.4

nmol.hour^{-1}. This suggests that the level of expression of BPDO was 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-trihydroxybiphenyl was produced from the rearrangement of 1,2-dihydro-1,2-dihydroxyDF. In order to unambiguously show that 2,2′,3-trihydroxybiphenyl was generated from angular oxygenation of DF, we have 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 at *m/z* 315 (M^+-$(CH_3)_4Si$-CH_3 or 418-88-15) (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 at *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-dihydroxyDF resulting from a lateral attack on carbon 1,2, 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 425 (Fig. 7).

Metabolism of DD by LB400, B-356 BPDOs

Seeger *et al.*, (2001) have shown 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 and prominent ions at *m/z* 419 and 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 section.

DISCUSSION

Suenaga *et al.*, (2001a) 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 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 has also been 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 the 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 favoured the lateral rather than the angular reaction suggests that co-planarity more than the *ortho* substitution of the biphenyl ring determines the regiospecificity.

DF can be regarded as an analog 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 and T in B-356 BPDO) both enzymes catalyze the oxygenation of DF and DD similarly. We

will need to study the 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 in 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 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 as *cis*-2,3-dihydro-2,3-dihydroxydibenzofuran.

The identification was based indirectly from the identification of the monohydroxylated derivatives obtained after acidification of the dihydro-dihydroxy metabolites. Oxygenation of carbon 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-dihydroxyDF. 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.

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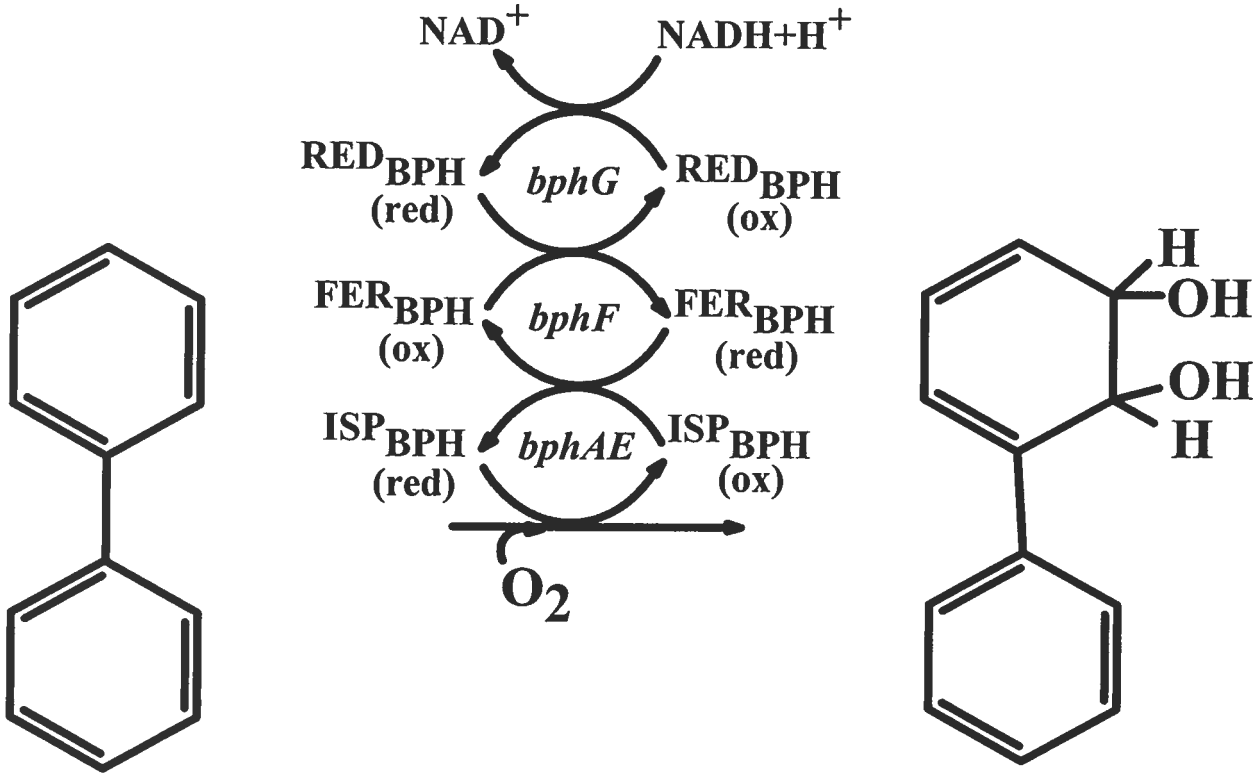
Figure 1 : Réaction de la dioxygénase du biphényle.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NADH+H+
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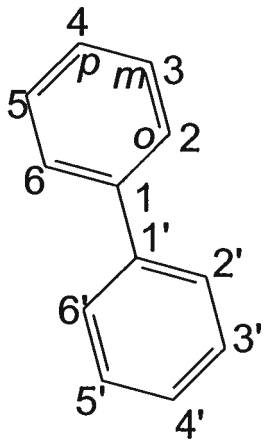
bphG
REDBPH (ox)
FERBPH (ox)
bphF
FERBPH (red)
ISPBPH (red)
bphAE
ISPBPH (ox)
O2
H
OH
OH
H

Figure 2 : Représentation schématique du dibenzofuranne (DF) et de la dibenzo-*p*-dioxine (DD) et leurs possibles sites d'oxygénation (A, angulaire; L, latérale) en comparaison avec la structure du biphényle.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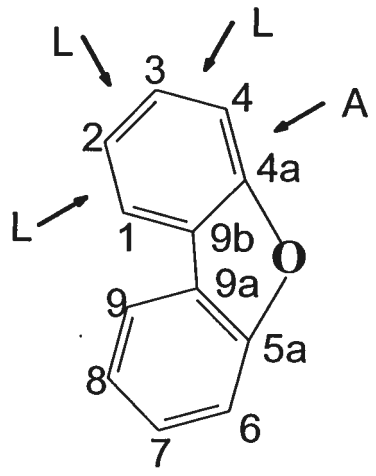
BP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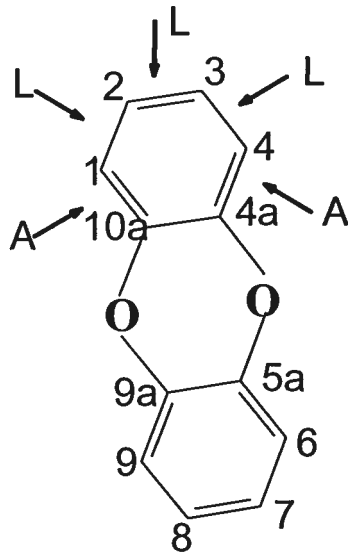
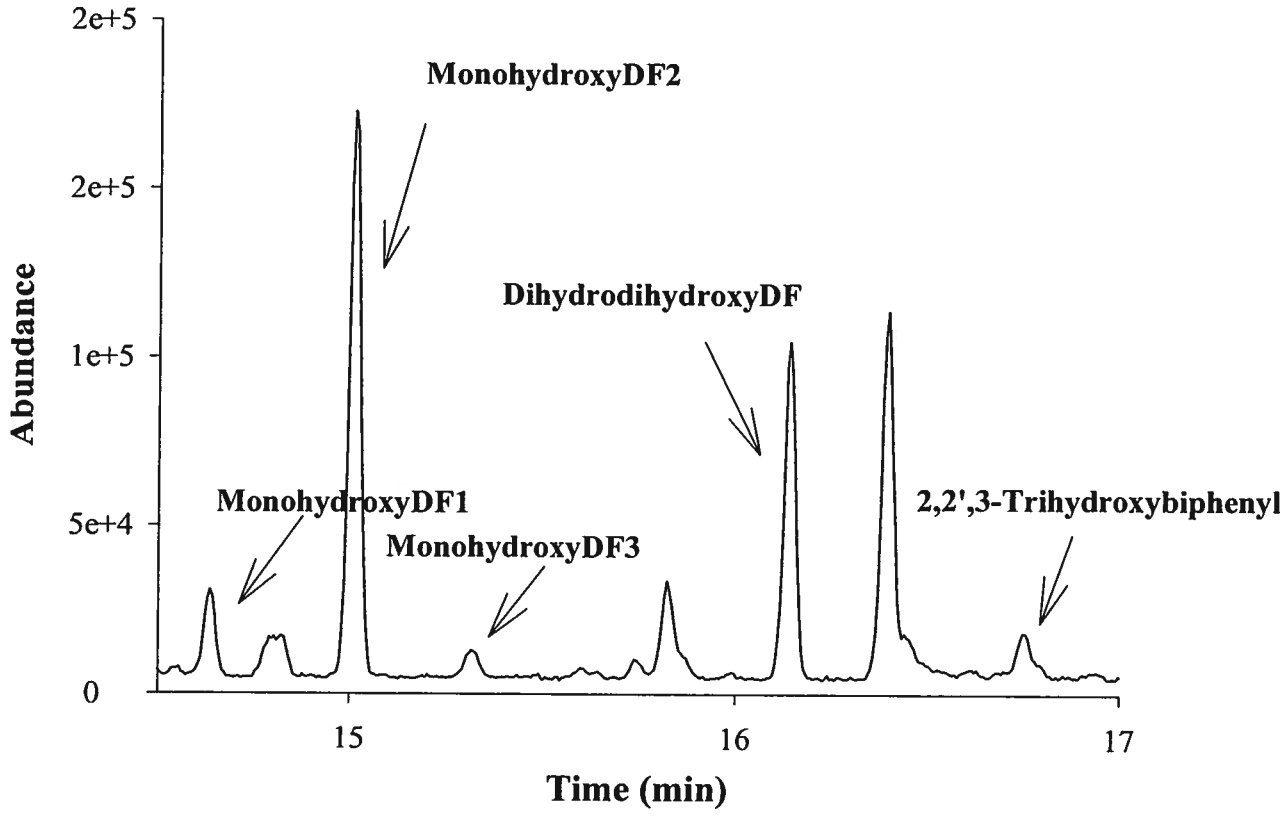
DF


L
L
2
3
L
4
1
A
10a
4a
A
O
O
9a
5a
6
9
7
8

DD

Figure 3 : Chromatogramme ionique total démontrant les métabolites du DF, dérivés au TMS, produits par *E. coli* exprimant la dioxygénase de LB400. Les pics non identifiés sont les métabolites produits par *E. coli* en absence de DF.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2e+5
1e+5
5e+4
0
Abundance
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DihydrodihydroxyDF
MonohydroxyDF1
MonohydroxyDF3
2,2',3-Trihydroxybiphenyl
15
16
17
Time (min)

Figure 4 : Chromatogramme ionique total démontrant les pics des dérivés au butylboronate du DF produits par *E. coli* exprimant LB400 (ligne pleine) et B-356 (ligne pointillée) ainsi que leur spectre de masse.

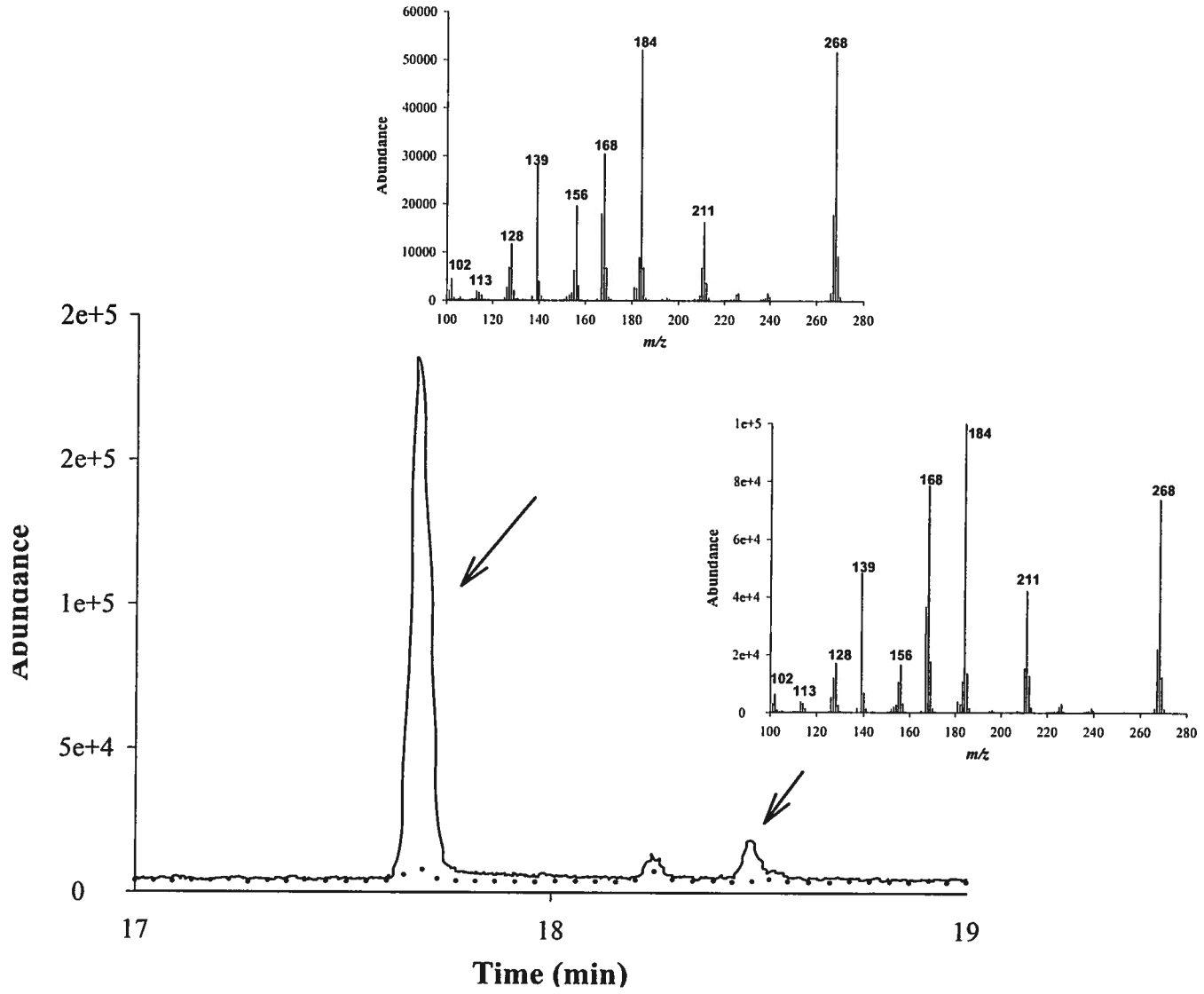
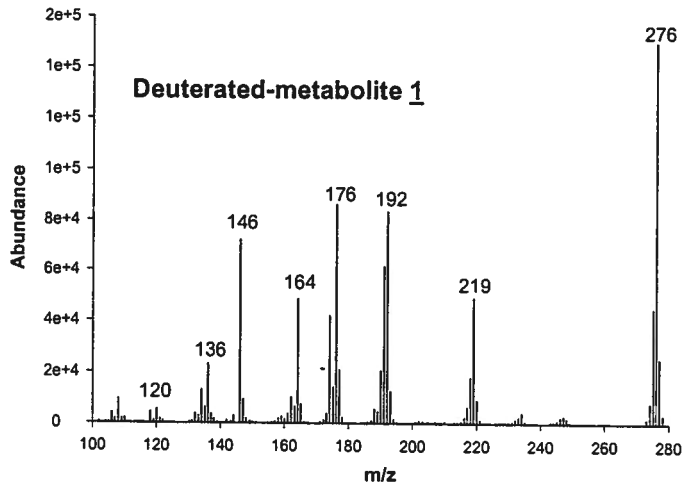
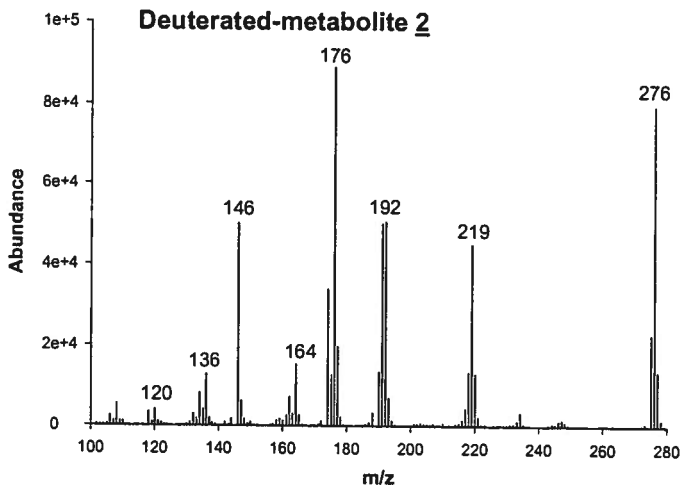

Abundance
2e+5
2e+5
1e+5
5e+4
0
17
18
19
Time (min)
Abundance
m/z
102
113
128
139
156
168
184
211
268

Figure 5 : (A) Spectre de masse des métabolites 1 et 2 du DF_{d8} dérivés au butylboronate. (B) Représentation schématique illustrant la différence de masse moléculaire entre le dérivé au butylboronate du dihydro-dihydroxyDF et le 2,2′,3-trihydroxybiphényle produits à partir du DF_{d8}.


Deuterated-metabolite 1
Abundance
m/z
276
176
192
146
164
219
136
120

A


Deuterated-metabolite 2
Abundance
m/z
176
276
146
192
219
136
164
120

B

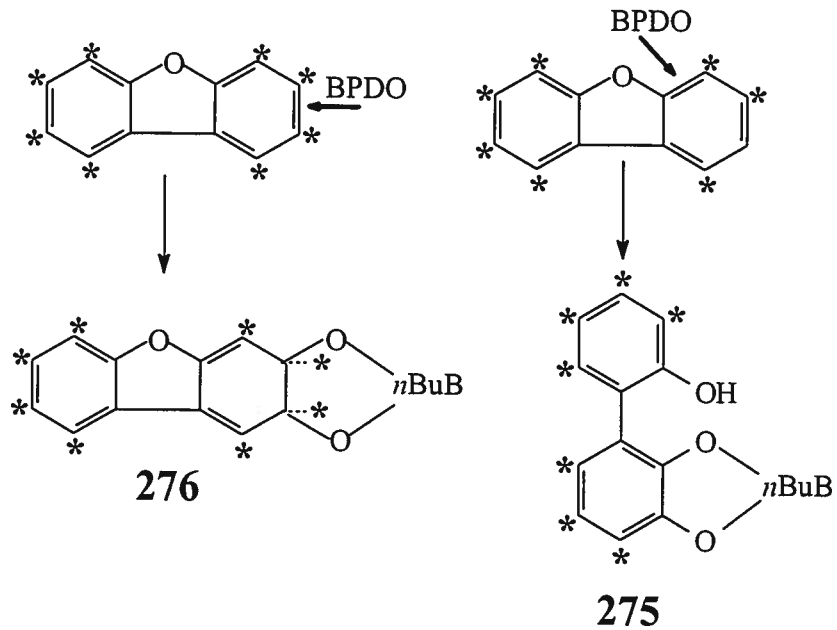

BPDO
BPDO
nBuB
OH
nBuB
276
275

Figure 6 : Chromatogramme ionique total illustrant les pics des métabolites du DF dérivés au TMS d'une préparation purifiée de dioxygénase provenant de LB400 (ligne pleine) ou de B-356 (ligne pointillée) portant une queue His.

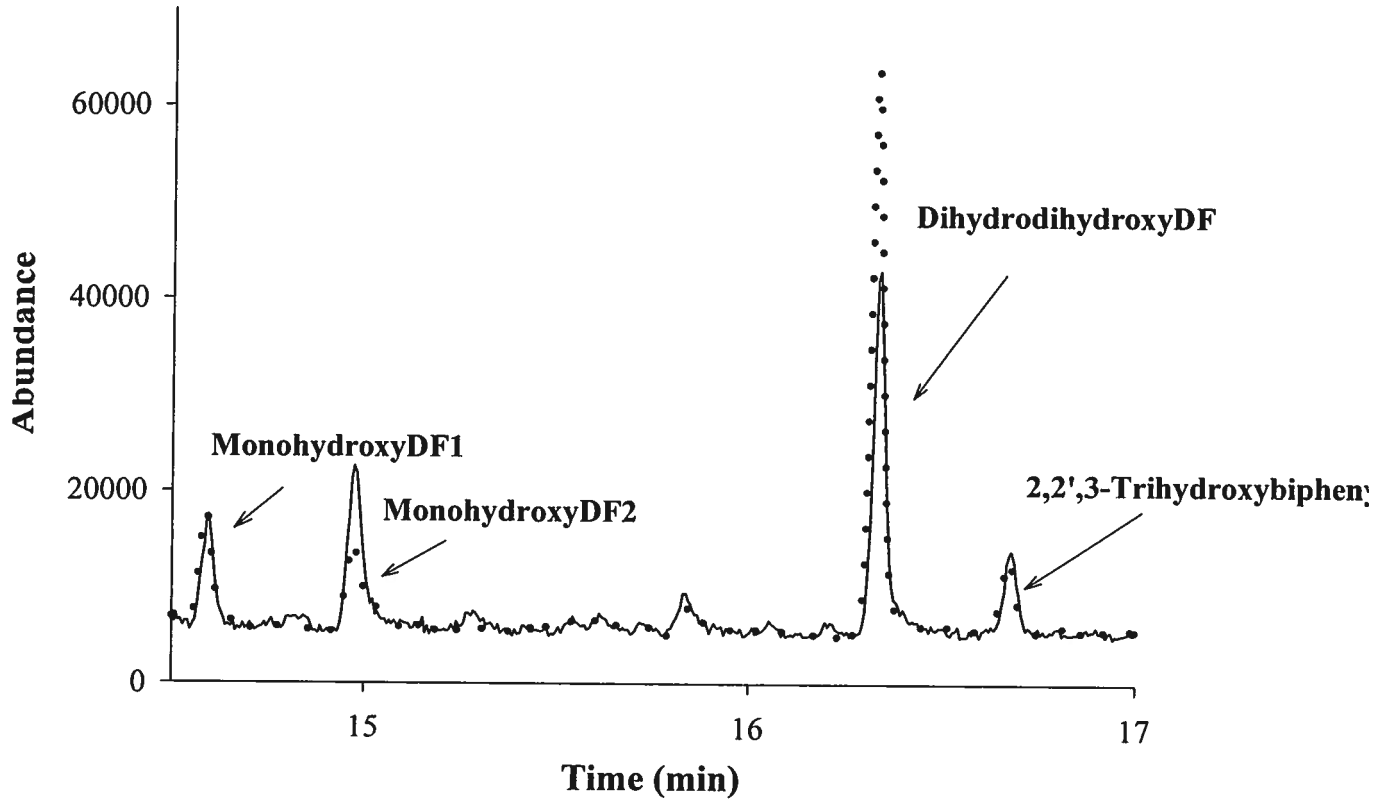
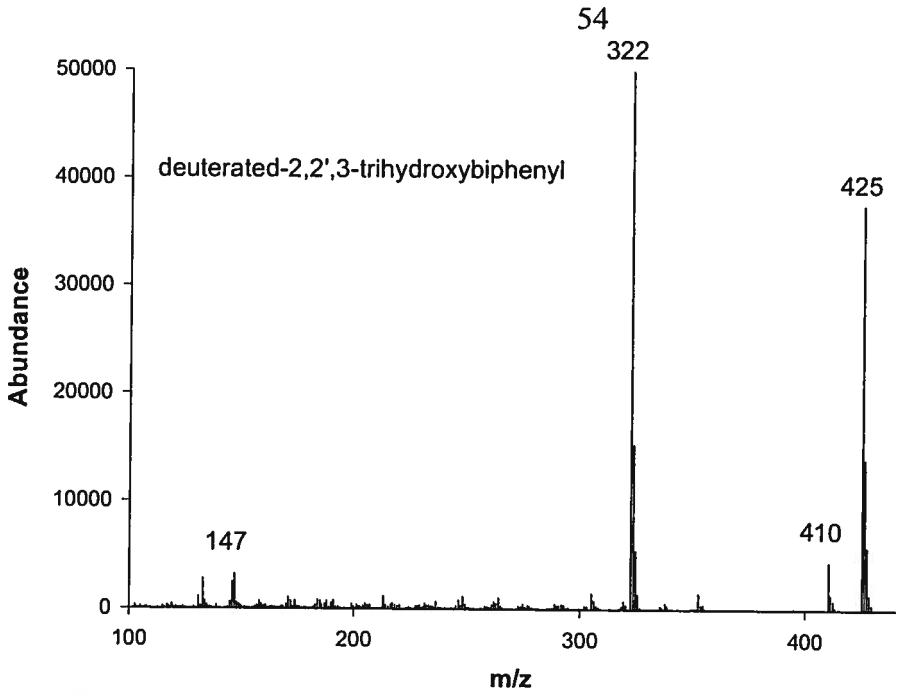
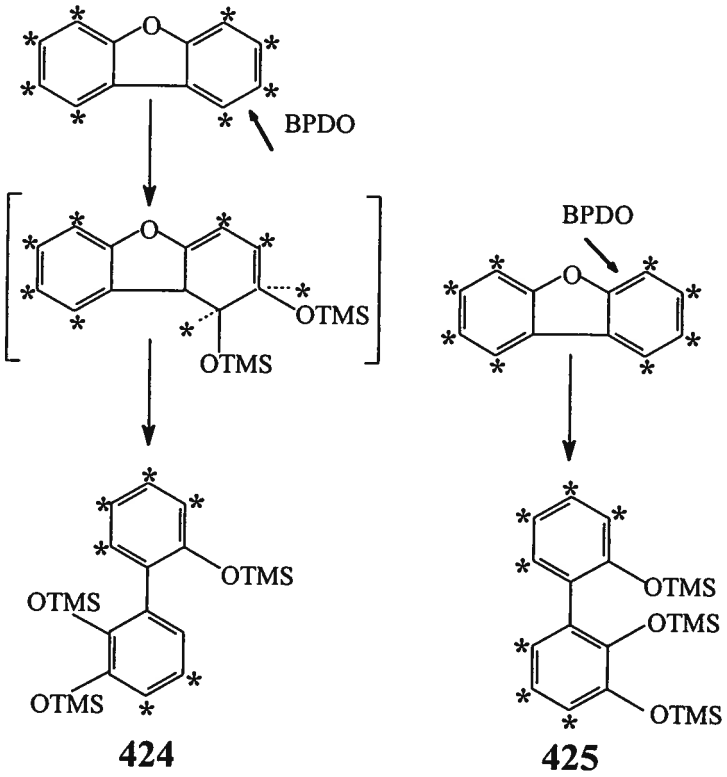

60000
40000
20000
0
Abundance
MonohydroxyDF1
MonohydroxyDF2
DihydrodihydroxyDF
2,2',3-Trihydroxybiphen
15
16
17
Time (min)

Figure 7 : Spectre de masse du 2,2′,3-trihydroxybiphényle dérivé au TMS généré par l'oxygénation catalytique du DF_{d8}. La représentation schématique explique également la différence de masse moléculaire du dérivé 2,2′,3-trihydroxybiphényle-TMS dépendemment de la voie utilisée à partir du DF_{d8}.


deuterated-2,2',3-trihydroxybiphenyl
Abundance
m/z
322
425
147
410
50000
40000
30000
20000
10000
0
100
200
300
400


BPDO
OTMS
OTMS
OTMS
OTMS
OTMS
BPDO
OTMS
OTMS
OTMS
424
425

CHAPITRE 3

(Article 2)

Metabolism of 1,1,1-trichloro-2,2-*bis*(4-chlorophenyl)ethane (DDT) by evolved and wild-type biphenyl dioxygenases

(non-publié)

Written by

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Institut National de Recherche Scientifique (INRS-IAF)

RÉSUMÉ

Ce travail visait à comparer les propriétés catalytiques des dioxygénases du biphényle (BPDO) de *Comamonas testosteroni* B-356 et de *Burkholderia xenovorans* LB400 envers le DDT avec les propriétés catalytiques d'enzymes hybrides dérivées par recombinaison génétique *in vitro* entre *bphA* de LB400 et de B-356. Les résultats démontrent que la BPDO de B-356 catalyse l'oxygénation du DDT de façon similaire à la réaction catalysée par la BPDO de *Cupriavidus necator* A5 dont l'efficacité avait été démontrée dans un rapport précédent. Cependant, la BPDO de B-356 produit en faible quantité, un second dihydrodiol que la BPDO de A5 ne produit pas. Les résultats confirment que la BPDO de LB400 ne dégrade pas le DDT efficacement. Cependant, tous les variants hybrides analysés ont été capables de dégrader le DDT, y compris le variant II-9 qui a été obtenu en remplaçant les acides aminés de la région III de BphA de LB400 par ceux de *bphA* de B-356. Ceci démontre que les acides aminés de la région III influencent considérablement la capacité de la dioxygénase à oxyder le DDT. Cependant, deux métabolites dihydrodiols étaient produits par les variants et les concentrations respectives de ces métabolites différaient considérablement selon le variant. Les deux métabolites ont été identifiés comme étant des diastéréoisomères du 1,1,1-trichloro-2-(4-chlorophényl-2,3-dihydroxy-4,6-cyclohexadiène)-2-(4'chlorophényl) éthane. Un alignement des séquences primaires en acides aminés des BphA des enzymes hybrides avec celles des BphA de B-356, LB400 et A5 suggère qu'un ou quelques acides aminés d'un segment compris entre les résidus 237 et 261 de BphA de LB400 seraient responsables de la stéréospécificité de la dioxygénase.

ABSTRACT

The purpose of this investigation was to compare the catalytic properties of the biphenyl dioxygenases (BPDOs) of *Comamonas testosteroni* B-356 and of *Burkholderia xenovorans* LB400 toward DDT with those of hybrid BPDO derived by shuffling LB400 and B-356 *bphA*. Data showed that B-356 BPDO oxygenated DDT similarly to *Cupriavidus necator* A5 BPDO which was previously demonstrated to degrade DDT. However, B-356 BPDO produced as minor metabolite, a second dihydrodiol that A5 BPDO did not produce. Data confirm a previous report showing that LB400 BPDO is unable to oxygenate DDT, however, all the variant BPDO tested were able to oxygenate DDT, including variant II-9 that was obtained by replacing region III amino acids of LB400 BphA by those of B-356. Therefore, region III amino acids significantly influence the capacity of the enzyme to degrade DDT. However, two dihydrodiol metabolites were produced from the reaction and their ratio differed considerably among the variants. Both metabolites were identified by NMR analysis as being two diastereoisomers of 1,1,1-trichloro-2-(4-chlorophenyl-2,3-dihydroxy-4,6-cyclohexadiene)-2-(4'-chlorophenyl)ethane. Primary amino acids sequence alignment of the variant enzymes with those of LB400, B-356 and A5 BphA suggested that one or several amino acids comprised on a segment between residue 237 and 261 of BphA are responsible for the stereospecificity of the enzyme toward DDT.

INTRODUCTION

Numerous concerns have arisen over the past decades about the adverse impacts of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) on the environment and human health (Walker and Ricciardone, 2003). Its use was banned in many countries more than 20 years ago. However, in spite of being listed among the 12 persistent organic pollutants (POPs) for which the Stockholm Convention has provided a framework for an international action, DDT will still be around for many years to come. This insecticide remains one of the most powerful and cost effective tools for disease vectors control (No authors listed, 1995 : Vector control for malaria and other mosquito-borne disease). By 1999, 23 countries still used DDT routinely for malaria control.

Lack of efficient microbial systems to catalyze their degradation explains the persistence of POPs in the environment. Since many of them are halogenated aromatic compounds, there has been a considerable interest over the last decades for the aryl hydroxylating dioxygenases (AHOs) as they are seen as potentially capable of initiating their degradation. These enzymes can catalyze the oxygenation of a broad range of substrate analogs including POPs such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and polychlororinated dibenzo-*p*-dioxins (CDDs) and dibenzofurans (CDF) (Brühlmann and Chen, 1999) ; (Kumamaru *et al.*, 1998) ; (Parales *et al.*, 2000) ; (Suenaga *et al.*, 2002). Furthermore, AHOs can be engineered to broaden the range of substrate they can oxygenate.

To date, no specific biochemical pathway has yet been detected in bacteria to break down DDT. However, many reports have shown that biphenyl induced

bacteria can transform DDT and its breakdown products 1,1-dichloro-2,2-*bis*(4-chlorophenyl)ethylène (DDE) and 1,1-dichloro-2,2-*bis*(4-chlorophenyl)ethane (DDD) (Hay and Focht, 1998), (Aislabie *et al.*, 1999), (Nadeau *et al.*, 1994), (Nadeau *et al.*, Sayler, 1998), (Massé *et al.*, 1989). From the identification of the metabolites produced, Nadeau *et al.*, (1994) has obtained evidence that the initial oxygenation reaction of DDT is catalyzed by the biphenyl catabolic pathway of *Cupriavidus necator* A5 (previously called *Alcaligenes eutrophus* and *Ralstonia eutropha* A5) (Nadeau *et al.*, 1998). However, *Burkholderia xenovorans* LB400 (previously called *Burkholderia sp.* LB400) which is the most efficient PCB degrading bacteria isolated from the environment was unable to transform DDE (Hay and Focht, 1998). The biphenyl dioxygenase (BPDO) of strain LB400 is known to catalyze poorly the *para*-substituted chlorobiphenyl which might explain in part, its inability to oxygenate DDE (Nadeau *et al.*, 1998). However, other structural features of the molecule that distinguish it from biphenyl could also have explained these results.

LB400 BPDO is among the best characterized oxygenase and the one exhibiting the highest PCB degrading potency among the other BPDOs of natural occurrence. *Pseudomonas pseudoalcaligenes* KF707 BPDO is closely related to LB400 BPDO but their PCB degrading potency differs considerably from each other. Among the differences, KF707 BPDO oxygenates 4,4'-dichlorobiphenyl much more efficiently than LB400 BPDO. BPDOs are multicomponent enzymes comprised of the iron-sulfur oxygenase (ISP_{BPH}) made up of an α (M_r = 51,000) and a β (M_r = 22,000) subunits, the ferredoxin (FER_{BPH}, M_r = 12,000) and the ferredoxin reductase (RED_{BPH}, M_r = 43,000). The encoding genes for *B. xenovorans* LB400 BPDO are *bphA* (ISP_{BPH} α subunit), *bphE* (ISP_{BPH} β subunit), *bphF* (FER_{BPH}) and *bphG* (RED_{BPH}); for *P. pseudoalcaligenes* KF707 BPDO they are *bphA1* (ISP_{BPH} α subunit), *bphA2* (ISP_{BPH} β subunit), *bphA3* (FER_{BPH}) and *bphA4* (RED_{BPH}). Minor structural variations on the C-terminal portion of the α subunit of the oxygenase component are responsible

for the phenotypic differences between these two strains. Mondello *et al.*, (1997) have replaced a stretch of seven amino acids (called region III) of the C-terminal portion of LB400 BPDO α subunit with that of KF707. Data showed that this domain strongly influences the capacity of the enzyme to catalyze the oxygenation of 4,4'-dichlorobiphenyl (Mondello *et al.*, 1997) plus a wide range of other PCB congeners.

Recently, Barriault *et al.*, (2002), have obtained variant BPDOs exhibiting an extended PCB degrading ability by shuffling the C-terminal of the more distantly related *Comamonas testosteroni* B-356 and LB400 *bphA* genes. Data emphasized the fact that region III was critical to extend the BPC degrading potency of the evolved BPDOs. Region III was also found to influence the capacity of BPDO to oxygenate chlorodibenzofurans (Mohammadi and Sylvestre, 2005).

On the other hand, Suenaga *et al.*, (2001) found that residue T^{376} of KF707 BphA1 (corresponding to region IV) was also critical to confer the capacity of KF707 BPDO to oxygenate dibenzofuran and dibenzo-*p*-dioxin (Suenaga *et al*, 2001). This residue is also critical to confer the capacity to catalyze oxygenation of chlorobiphenyls on *meta-para* carbons (Suenaga *et al.*, 2001). However, the catalytic oxygenation of DDT by evolved BPDOs has never been reported. Engineering efficient biocatalysts for the degradation of biphenyl analogs will require that we alter the turnover rate of reaction and regiospecificity of the enzyme toward these substrates. These features are determined through interactions between amino acid residues and the carbons of the aromatic ring or the ring substituents. The configuration of the molecule such as co-planarity or stereoisomerism should also influence the catalytic reaction. Identification of the amino acids that interact with the substrate molecule and their mode of interaction should help design strategies to engineer evolved enzymes exhibiting desirable catalytic features.

Preliminary data showed that strain B-356 BPDO oxygenated DDT more efficiently that LB400 BPDO. On the other hand, in a previous report, BphA hybrids resulting from shuffling LB400 *bphA* with B-356 *bphA* were found to have extended their PCB degrading capacity (Barriault *et al.* 2002). In order to identify some of the structural features of the biphenyl dioxygenase that confer the ability of B-356 BPDO to oxygenate DDT, we have assessed the capacity of these hybrids BPDO to degrade DDT and characterized their metabolic pattern toward it.

MATERIALS AND METHODS

Bacterial strains, plasmids, chemicals and general protocols

Escherichia coli DH11S was used in this study. Plasmids pDB31[LB400-*bphAE*] and pDB31[B356-*bphAE*] have been described previously (Barriault *et al.*, 2002). The replacement in plasmid pDB31[LB400-*bphAE*] of the 864 bp MluI/AvrII fragment of *bphA* by a library of MluI/AvrII fragments obtained by shuffling LB400 *bphA* with B-356 *bphA* and *Rhodococcus globerulus* P6 *bphA1* created *bphA* variant II-4, II-9, II-10, III-17, III-34, III-37 and III-52 that were described previously (Barriault *et al.*, 2002). *E. coli* strains bearing these plasmids expressed His-tagged (ht)ISP_{BPH} (Barriault *et al.*, 2002) carrying the His-tag on BphA. To construct pQE31[LB400-*bphFG*], *bphFG* was amplified as a 1555 bp BamHI/KpnI fragment from LB400 DNA using primers 5'GCGGGATCCTATGAAATTTACCAGAGTTTG3' and 5'CTGGTACCGCTTCACCTTTCA3' and then cloned into the BamHI/KpnI digested pQE31. To construct pET-14b[A5-*bphAE*], *bphAE* was amplified from *C. necator* A5 (Nadeau *et al.*, 1998) total DNA using primers 5'CGGGATCCCATGGGAATACATTCGGA3' and 5'CCCCAAGCTTTCAGAAAAACACGCTG3'. They were cloned as BamHI/HindIII fragment into pET-14b. DNA protocols were done according to Sambrook *et al.*, (1989). B-356, A5 and III-52 *bphAE* were cloned in the vector pET-14b (Novogen, Madison, WI) and expressed in *E. coli* C41(DE3) to obtain higher levels of expression. 1,1,1-trichloro-2,2-*bis*(4-chlorophenyl)ethane (*p,p* DDT) (99% + pur) ; 1,1-dichloro-2,2-*bis*(4-chlorophenyl)ethane (*p,p* DDD) (99% + pur) ; *o,p* DDT (99% + pur) were from Aldrich Chemicals.

Assays to identify the metabolites and quantify the catalytic activities

Metabolites were analyzed from suspensions of isopropyl-β–D-thiogalatopyranoside (IPTG)-induced whole cells of *E. coli* [pQE51*bphFG*] + [pDB31*bphAE*]. In this case, log phase cells grown in LB broth were induced for 3 H with 0.5 mM IPTG, and then washed and suspended to an optical density at 600 nm of 2.0 in M9 medium (Sambrook *et al.*, 1989), containing 0.5 mM IPTG. The cell suspension was distributed by portions of 2 ml in 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. The ethyl acetate was evaporated under a stream of nitrogen and the residues were directly used for gas chromatography-mass spectrometry (GC-MS) analysis of their butylboronate (*n*BuB) or N,O-*bis*(trimethylsilyl)trifluoroacetamide to produce the trimethylsylil (TMS) derivatives (Massé *et al.*, 1989).

DDT metabolites produced by *E. coli* pQE31[III-34-*bphAE*] + pDB31[LB400-*bphFG*], pQE31[III-52-*bphAE*] + pDB31[LB400-*bphFG*], were also purified by High Performance Liquid Chromatography (HPLC) for nuclear magnetic resonance (NMR) analysis. In this case, the ethyl acetate extract from 500 ml of IPTG-induced culture was evaporated and the residues were dissolved in a mixture of water : acetonitrile : methanol (50:25:25 v/v). This solution was injected into a semi-preparative Zorbax-ODS reverse-phase column (9.4 mm by 25 cm). The column was equilibrated with water-acetonitrile-methanol (50:25:25). The compounds were eluted with a linear gradient to acetonitrile-methanol (80:20) in 20 min at 0.7 ml/min. The Agilent model 1100 variable wavelength detector was set at 238 nm. Since each of III-52 and III-34 BPDO produces only one metabolite from DDT, the peak of metabolite was collected, the solvent phase was evaporated in vacuum and

the residual aqueous phase was extracted with ethyl acetate. The identity and purity of the metabolites were confirmed by GC-MS analyses. NMR spectra were obtained at the NMR spectrometry center of INRS-Institut Armand-Frappier with a Brucker 500-mHz spectrometer. The analyses were carried out in deuterated acetonitrile at room temperature.

In some experiments, the metabolites were analyzed from catalytic oxygenation using reconstituted BPDOs prepared from His-tagged purified enzymes components that were obtained following protocols published previously (Hurtubise *et al.*, 1996). However, in this case, B-356, II-9, III-52 and A5 BphAE were cloned into pET-14b (Novagen, Madison, WI) and expressed in *E. coli* C41(DE3) to obtained higher levels of expression. The purified enzyme assays were performed in 50 mM morpholineethanesulfonic (MES) buffer pH 6.0 at 37°C as described previously (Hurtubise *et al.*, 1996). Catalytic activities were determined according to protocols described previously from the measurement of substrate depletion recorded by GC-MS analysis (Hurtubise *et al.*, 1998), or by recording the oxygen consumption rates using a Clarke-type Hansatech model DW1 oxygraph (Mohammadi and Sylvestre, 2005).

Wild-type *C. testosteroni* B-356 and A5 cells were also tested for their capacity to oxygenate DDT. In this case, cells were grown to an OD_{600nm} of 1.0 in M9 medium containing 0.5% biphenyl as sole growth substrate. The cells were harvested and resuspended to an OD_{600} of 2.0 in M9 medium containing no carbon source except 50 nmol/ml of DDT. These cell suspensions were incubated overnight at 29°C with shaking. The metabolites were then extracted as indicated above.

Catalytic activity of BphB and BphC on DDT and DDD metabolites

The capacity of 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase (BphB) and of 2,3-dihydroxybiphenyl 2,3-dioxygenase (BphC) the second and third enzymes of the biphenyl catabolic pathway to oxidize further, the metabolites produced from catalytic oxygenation of DDT was evaluated with purified enzyme preparations. His-tagged BphB and His-tagged BphC were produced and purified according to published protocols (Barriault *et al.*, 1999) ; (Hein *et al.*, 1998). The BphB assay to assess its capacity to oxidise 1,1,1-trichloro-2-(4-chlorophenyl)-2,3-dihydro-4,6-cyclohexadiene-2-(4′-chlorophenyl)-ethane (DDT-2,3-dihydrodiol) was performed by measuring NAD consumption, as described previously (Barriault *et al.*, 1999). The capacity of BphC to oxidise DDT-2,3-dihydrodiol to the yellow colored *meta*-cleavage metabolite 1,1,1-trichloro-2-(4-chloro-2,3-dihydrophenyl)-2-(4′-chlorophenyl)-ethane was evaluated in a coupled reaction involving BphB and BphC, according to the protocol described previously to assess the capacity of BphC to oxidise chlorobiphenyl metabolites by spectrophotometric measurement of its *meta*-cleavage metabolite (Barriault *et al.*, 2004).

RESULTS AND DISCUSSION

Metabolism of DDT by parent and variant BPDOs

Resting cells of *C. necator* A5 grown in M9 medium and biphenyl as substrate were able to transform DDT to a metabolite that exhibited mass spectral features identical to those published for 1,1,1-trichloro-2,(4-chlorophenyl-2,3-dihydroxy-4,6-cyclohexadiene)-2-(4'-chlorophenyl)ethane (DDT-2,3-dihydrodiol) (Fig. 2A) (Nadeau *et al.*, 1998). *C. testosteroni* B-356 grown under identical conditions produced two metabolites from DDT, one of which exhibited a GC retention time and mass spectrum features identical to the DDT-2,3-dihydrodiol produced by strain A5 (Fig. 2A, E) where the *n*BuB derivative exhibited diagnostically important fragmentation ions at *m/z*=454, 335 (M-$HCCl_3$), 299 (M-$HCCl_4$). The second one exhibited identical mass spectrum features but its GC retention time was slightly different. (See Fig. 2E for the MS spectra)

IPTG-induced cells of *E. coli* pQE31[B356 *bphAE*] + pDB51[*bphFG*] produced the same two metabolites than the wild B-356 strain when grown in the presence of DDT (Fig. 2B). On the other hand, IPTG-induced cells of *E. coli* pQE31[LB400 *bphAE*] + pDB51[*bphFG*] metabolized DDT very poorly, which confirms previously reported data (data not shown) (Nadeau *et al.*, 1998).

Five *E. coli* strains expressing either one of the previously described variant II-9, III-17, III-34, III-37 or III-52 *bphA* (Barriault *et al.*, 2002) were tested for their ability to metabolize DDT. Of these, the strain that expressed variant III-37 was unable to metabolize DDT. Based on the areas of metabolite peaks produced in the culture media after 18 h of incubation, the rate of metabolism of DDT varied among the strains. This variation can be due to intrinsic variation in the

activitiesof the hybrid enzymes, but we cannot exclude variation that could result from variation of enzyme expression in these clones. Nevertheless, the ratio of metabolite I to metabolite II varied significantly between the variants. Thus variant III-34 generated almost exclusively metabolite II whereas in the case of variants II-9, III-17 and III-52 over 80% of the DDT transformed was converted to metabolite I (see Fig. 2C and 2D).

We have excluded the possibility that one of the two metabolites resulted from the dioxygenation of *o,p'*-DDT which is often a contaminant of *p,p*-DDT preparations. *E. coli* cells expressing variant III-52 BPDO was able to metabolize *o,p'*-DDT. Two metabolites were produced that exhibited mass spectral features similar to the one produced from *p,p'*-DDT but their retention time differed significantly (data not shown).

The *cis*-dioxygenation of achiral *p,p*-DDT generates a dihydrodihydroxy metabolite with a chiral center yielding potentially four diastereoisomers (Fig. 3). Therefore, the two major peaks of metabolites produced from variants III-17, III-52 and II-9 were likely two diastereoisomers of DDT-2,3-dihydrodiol.

In order to show that the production of two DDT metabolites by *E.coli* cells producing each enzyme was not due to differences in expression levels, we have determined, the metabolite profile toward DDT of His-tagged purified preparations of each enzyme. Results showed that the same metabolite ratios were observed than when *E. coli* cells were used to catalyze the reaction (data not shown).

NMR analysis of DDT metabolites

Attempts were made to resolve metabolite I from metabolite II by HPLC chromatography. Several HPLC conditions were tested to resolve the two metabolites, including various sets of solvent mixtures and of elution gradient on the ODS reverse phase column. However, the efforts were unsuccessful. Nevertheless, we were able to obtain a pure preparation of metabolite I from a culture of *E. coli* cells expressing III-52 *bphAE*. A purified preparation of metabolite II was similarly obtained from a culture of *E. coli* cells expressing III-34. The purity of each metabolite that was produced by the different cultures was assessed by GC-MS analysis. At this time, we have no explanation, but when IPTG-induced cells of *E. coli* pQE31[III-52-*bphAE*] + pDB31[LB400-*bphFG*] grown in large volume were exposed to DDT, metabolite I only was produced. Each of these metabolites was purified by HPLC using the protocol described in the Materials and Methods section. The purified preparations were then analyzed by NMR. The NMR features of metabolite I and II in acetone d_6 was identical to those reported for DDT-2,3-dihydrodiol produced by biphenyl-induced cells of *C. necator* A5 (Nadeau *et al.*, 1994) (Fig. 4). The proton assignments shown in Fig. 4 were confirmed by heteronuclear multiple quantum correlation (HMQC) experiments that showed a Overhauser Effect (NOE) between methine proton at C_5 at 5.07 ppm and the doublet at 7.59 ppm and between the multiplet at 3.98 ppm and the doublet at 6.60 ppm.

The signal pattern of metabolite I was similar to that of metabolite II although the signals were slightly displaced. Thus the α-hydroxy proton was displaced by 0.69 ppm (from 3.98 to 4.67 ppm) and the methine proton was displaced from 5.07 to 5.22 ppm. A 2D-COSY analysis confirmed the structure of the compound to be a *cis*-2,3-dihydro-2,3-dihydroxy-1,1,1-trichloro-2,2-*bis*(4-

chlorophenyl)ethane. Data confirmed that both metabolite I and metabolite II are diastereoisomers resulting from stereoselectivity of the enzymes that catalyzed the oxygenation of DDT.

Catalytic properties of purified BPDO preparations toward DDT and DDD

Biphenyl-induced cells of wild-type *C. necator* A5 oxygenated very efficiently DDT to generate a single one of the four possible diastereoisomers. *C. testosteroni* B-356 produced the same metabolite, but in addition small amounts of a second diastereoisomer was generated. Hybrid BPDOs produced by shuffling LB400 *bphA* with B-356 *bphA* produced variable ratios of the two diastereoisomers (Fig. 2). It is noteworthy that variant III-52 BPDO produced preferentially the diastereoisomer that was a minor metabolite of B-356 BPDO. For this reason, we have evaluated the kinetic parameters of B-356 and III-52 BPDOs. It was also interesting that variant III-34 BPDO was producing exclusively the same diastereoisomer produced by A5. However, the purified enzyme oxygenated DDT too poorly to be allow reliable measurements of the initial rate of oxygenation.

The steady state kinetic parameter of B-356 and III-52 BPDOs were evaluated toward DDT and DDD by measuring the oxygen consumption measurements using a Clark-type oxygraph. The kcat values toward biphenyl, at substrate saturation, for the B-356 BPDO preparation used in the assay was 1.8 sec^{-1} and the kcat for the III-52 BPDO preparation was 1.0 sec^{-1}. The steady state parameters toward DDT and DDD for these two enzymes are shown in Table 1. Data show that III-52 exhibited a slightly higher specificity toward both substrates than B-356 BPDO, this is due to a higher affinity of the variant enzyme compared to the parent enzyme. Experiments were conducted to determine the kinetic parameters of A5 BPDO toward DDT and DDD. However, none of our

preparations of A5 were active enough toward DDT to give reliable results. It is likely that the His-tagged A5 oxygenase was less stable than the other BPDO used. However, the most likely explanation would be that the oxygenase component of strain A5 did not function well with the recombinant ferredoxin component (BphF) used in this investigation and which was obtained from strain B-356.

Conversion of DDT and DDD-2,3-dihydrodiol

A previous report (Nadeau *et al.*, 1994) indicated that biphenyl-induced cells of *C. necator* A5 were able to oxidize DDT to benzoic acid. Another report (Hay and Focht, 2000) showed that biphenyl-induced cells of *C. necator* A5 transformed DDD to the corresponding *meta*-cleavage metabolite. It was therefore of interest to evaluate the capacity of the second and third enzymes of the biphenyl catabolic pathway (BphB and BphC) to further oxidize the dihydrodiol metabolites produced from DDT and DDD. All attempts made to oxidize both DDT-2,3-dihydrodiol diastereoisomers by purified preparations of His-tagged B-356 BphB were unsuccessful, suggesting that this enzyme is unable to further metabolize DDT. This was confirmed by the absence of metabolites other than DDT-2,3-dihydrodiol in culture media of biphenyl-induced wild-type cells of strain B-356. It is noteworthy that we did not find any likely DDT metabolites other than DDT-2,3-dihydrodiol in cultures of biphenyl-induced cells of strain A5 suggesting that BphB of the A5 biphenyl pathway is also unable to oxidize DDT-2,3-dihydrodiol. We cannot exclude the possibility that other genes involved in DDT degradation were not expressed under the conditions we used or the sub-strain we used had lost the genes required for DDT degradation. The fact that the biphenyl pathway genes are located on a large transposon in strain A5 might explain the lost of genes involved in DDT degradation. It is noteworthy that strain A5 carried initially all

genes required to mineralize 4-chlorobiphenyl (Pettigrew *et al.*, 1990) but it had loss part of the pathway (Burlage *et al.*, 1990).

On the other hand, a yellow-colored metabolite was produced when a coupled reaction involving the B-356 BPDO with BphB plus BphC was used to oxidize DDD (data not shown). This confirms the observation reported by Hay and Focht, (1998) whereby the biphenyl catabolic enzymes BphB and BphC can further metabolize the oxidation product resulting from catalytic oxygenation of DDD.

Sequence analysis

Fig. 5 compares the major structural features of the BphA variants to that of LB400 and B-356 BphA and *C. necator* A5 BphA1. As first observation, B-356 BphA and A5 BphA1 share 84% homology compared to 74% with LB400 BphA. In a previous report, the replacement of a stretch of amino acid $T^{335}F^{336}N^{337}N^{3338}I^{339}R^{340}I^{341}$ of LB400 BphA called region III by the corresponding segment $G^{335}I^{336}N^{337}T^{3338}I^{339}R^{340}T^{341}$of B-356 BphA was shown to create a more relaxed variant called II-9 BPDO that exhibited an extended PCB degrading potency (Barriault *et al.* 2002). The enzyme was also able to oxygenate efficiently other biphenyl analogs such as naphthalene and toluene. In this work, we showed that the replacement of region III of LB400 BphA by that of B-356 BphA enhanced the capacity of variant II-9 to oxygenate DDT. However based on the rate of metabolism of DDT by IPTG-induced cells of *E. coli* producing either one of the variant II-9, III-17 or III-52, concomitant replacement of a stretch of LB400 BphA that include residues 166 to 211 did not change significantly the activity of the enzyme toward DDT as seen by comparing the ratio of metabolites and rate of metabolism of DDT. On the other hand, the catalytic properties of variant III-34 and III-37 toward DDT were significantly different than those of the other variants.

Variant III-37 that exhibited a fairly broad PCB degrading potency (Barriault *et al.*, 2002) only poorly oxygenated DDT. The only structural variations between III-37 and III-52 concern the replacement of $T^{237}T^{238}$ of LB400 BphA by $M^{237}S^{238}$ and the presence of I^{326} instead of V as in III-52. These two residues are located in a stretch of amino acids that, based on the crystal structure of naphthalene dioxygenase (NDO) (Parales *et al.*, 2000) and of *Rhodococcus sp.* RHA1 BPDO (Furusawa *et al.*, 2004), are likely involved in the coordination of the active iron center. In RHA1 BphA1, these residues are located between His^{224} and His^{230} that are located on each side of the mononuclear iron atom involved in catalytic oxygenation. It is thus likely, that either residue 237 or 238 or both affect significantly the binding and or recognition of DDT as substrate. Unlike variant III-37, III-34 oxygenated DDT but at lower rate than variant III-52. This variant differed from III-37 by the fact that an additional stretch of LB400 BphA (from residues 243 to 267) were replaced by that of B-356 BphA and that I^{326} was replaced by V of B-356 BphA. Thus if any of residues 237, 238 or 326 influences the activity toward DDT, the portion of BphA between residues 243 and 267 contains residues that can counteract the influence of these amino acids on enzyme activity. It is also noteworthy that III-34 is the only one of the five variants that produced only one of the two DDT-2,3-dihydrodiol diastereoisomers. Thus, the stretch of amino acids between 237 and 267 influenced considerably the regiospecificity of the enzyme toward DDT.

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Table 1 : Steady-state kinetics of B-356 and III-52 BPDOs toward DDT and DDD.

Substrate		B-356	III-52
DDT	Km app (μM)	14.2	1.6
	kcat (sec^{-1})	0.13	0.1
	kcat/Km app ($10^3 M^{-1} sec^{-1}$)	9	62
DDD	Km app (μM)	24.6	5.3
	kcat (sec^{-1})	0.45	0.19
	kcat/Km app ($10^3 M^{-1} sec^{-1}$)	18	35

Figure 1 : Réaction de la dioxygénase du biphényle.

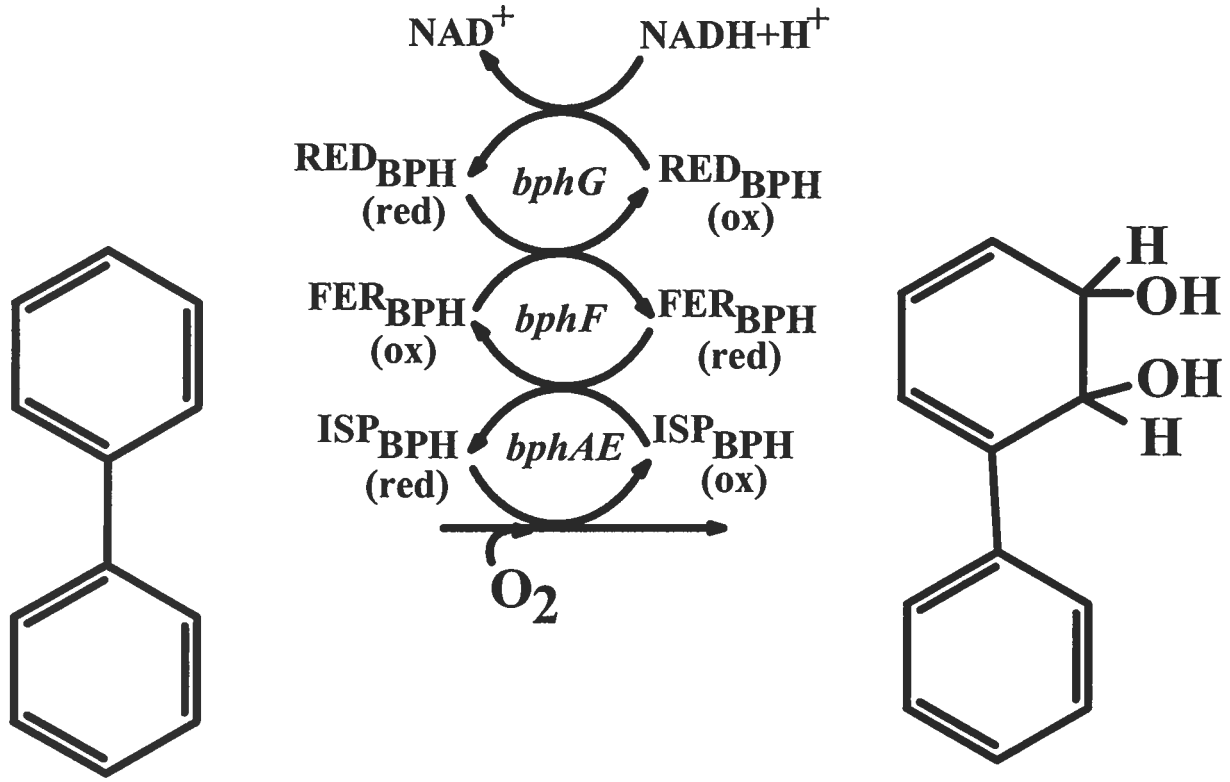

NAD+
NADH+H+
REDBPH
(red)
bphG
REDBPH
(ox)
FERBPH
(ox)
bphF
FERBPH
(red)
ISPBPH
(red)
bphAE
ISPBPH
(ox)
O2
H
OH
OH
H

Figure 2 : Chromatogramme ionique total des métabolites du DDT produits par des cultures sauvages (wt) en phase logarithmique de *C. testosteroni* B-356 et de *C. necator* A5 (wt) (A) ou par certains variants (C,D) de même que par *E. coli* et certains variants produisant BphAE de B-356 (B). Spectre de masse du métabolite I et II produits par la BPDO de B-356 (E).

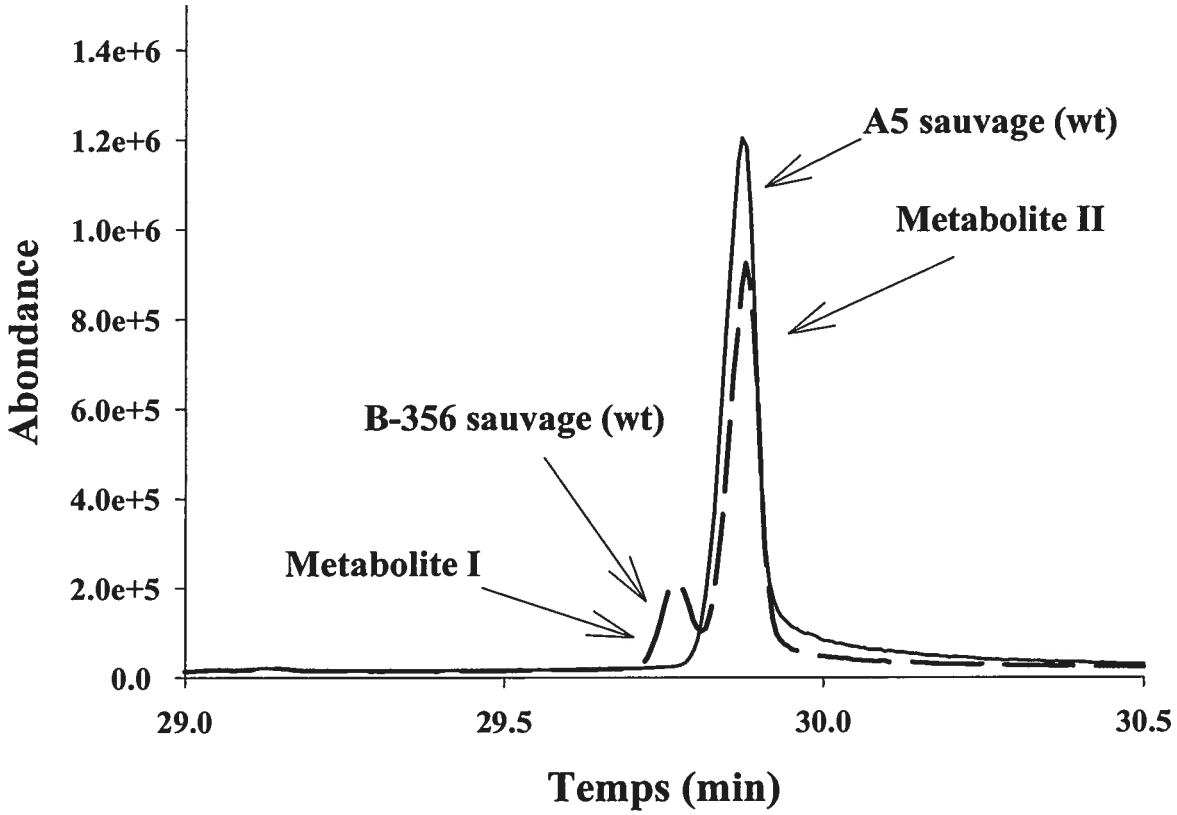

1.4e+6
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
Abondance
A5 sauvage (wt)
Metabolite II
B-356 sauvage (wt)
Metabolite I
29.0
29.5
30.0
30.5
Temps (min)

Fig. 2A

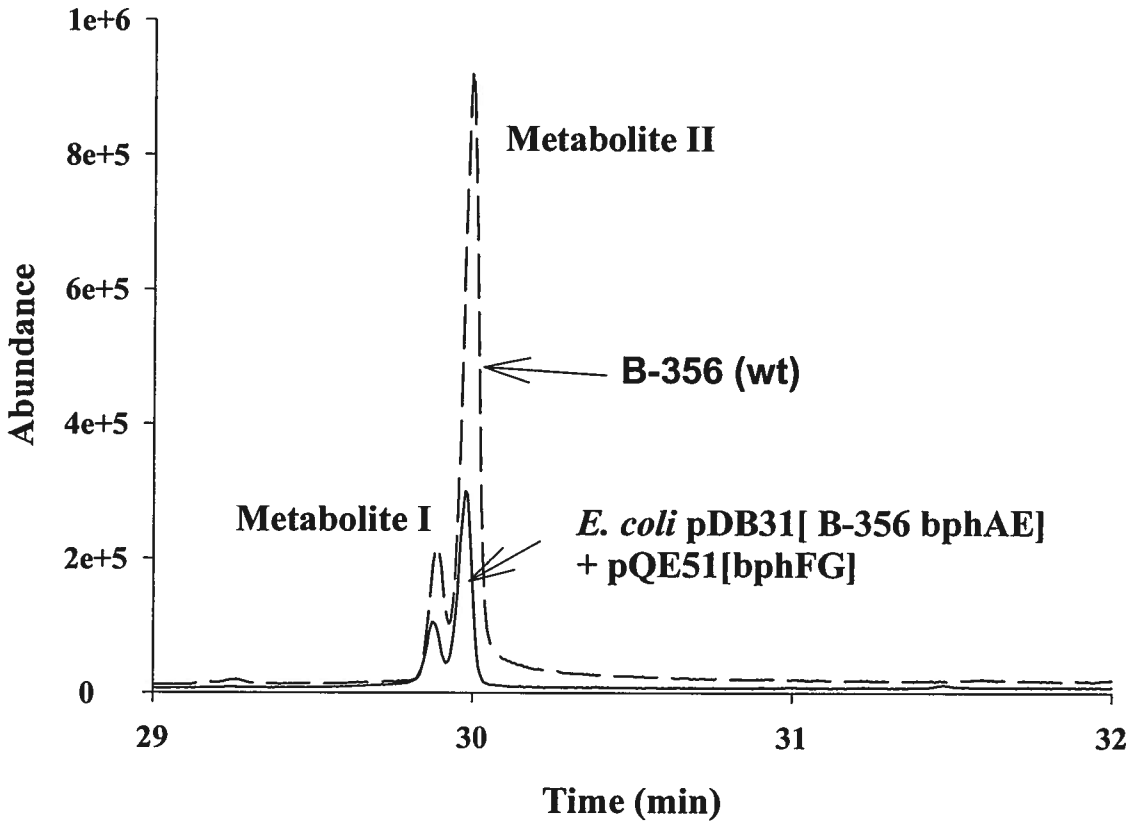

1e+6
8e+5
6e+5
4e+5
2e+5
0
Abundance
Metabolite II
B-356 (wt)
Metabolite I
E. coli pDB31[B-356 bphAE]
+ pQE51[bphFG]
29
30
31
32
Time (min)

Fig. 2B

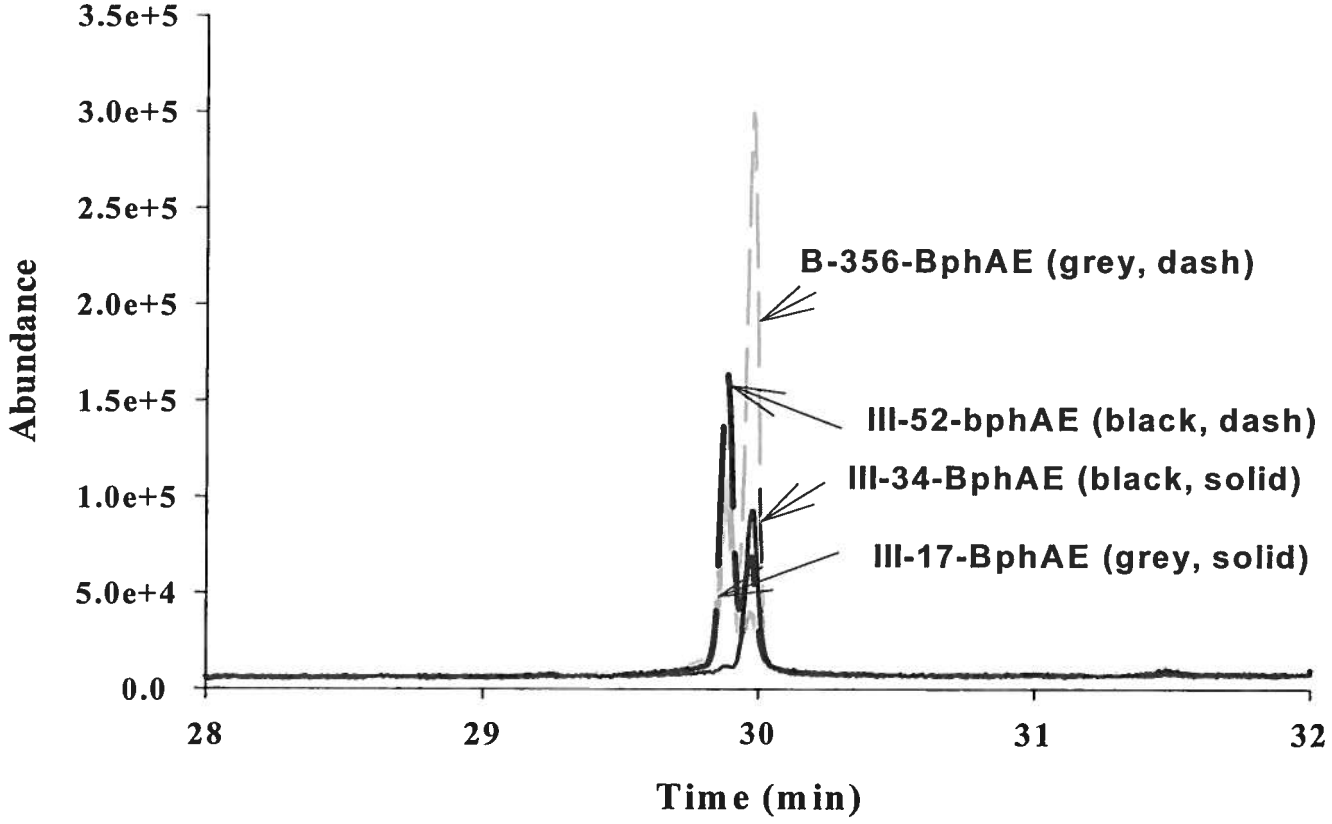

3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
Abundance
B-356-BphAE (grey, dash)
III-52-bphAE (black, dash)
III-34-BphAE (black, solid)
III-17-BphAE (grey, solid)
28
29
30
31
32
Time (min)

Fig. 2C

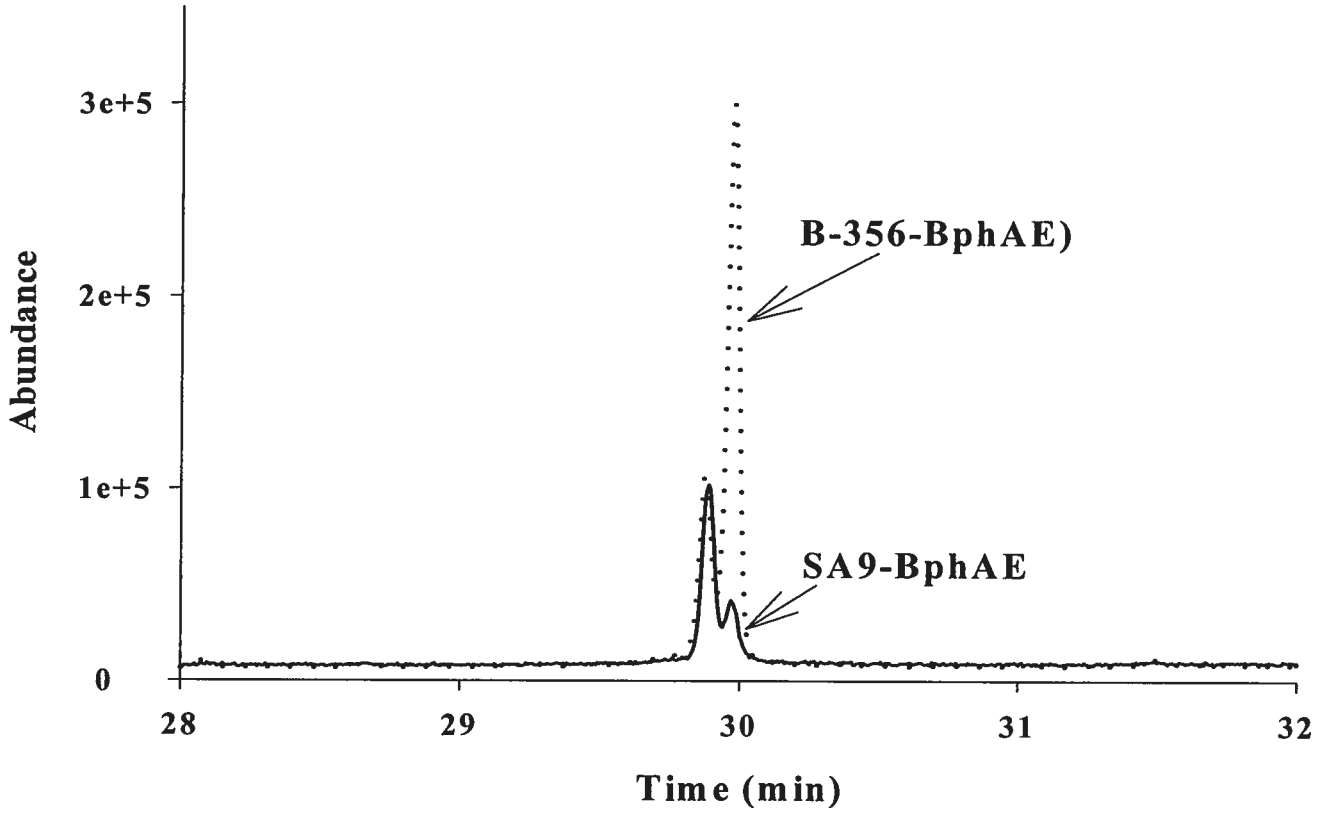

3e+5
2e+5
1e+5
0
Abundance
B-356-BphAE)
SA9-BphAE
28
29
30
31
32
Time (min)

Fig. 2D

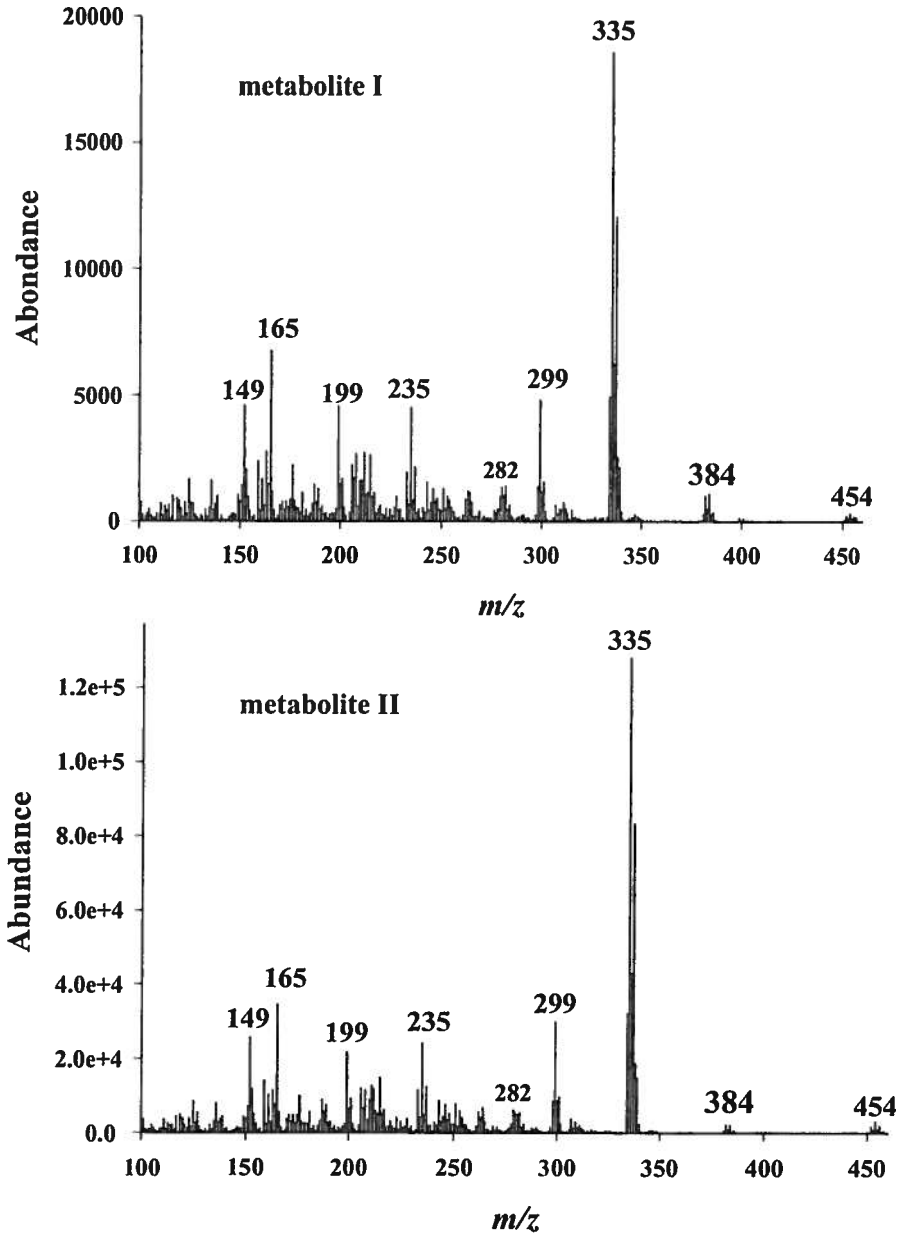
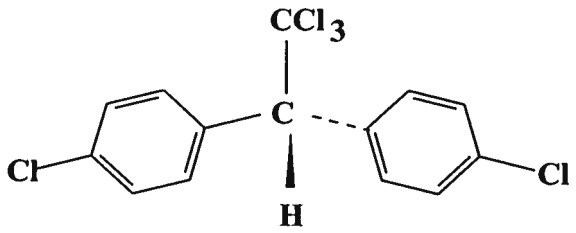
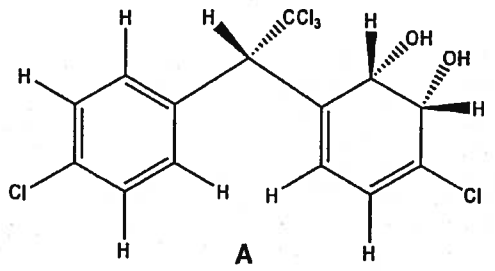
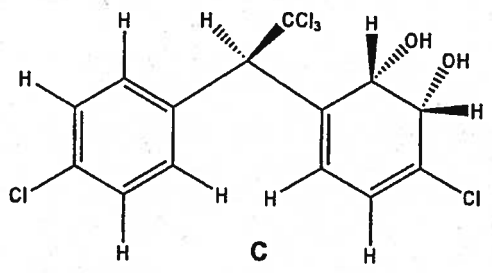

metabolite I
Abondance
20000
15000
10000
5000
0
335
165
149
199
235
299
282
384
454
100
150
200
250
300
350
400
450
m/z
metabolite II
Abundance
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
335
165
149
199
235
299
282
384
454
100
150
200
250
300
350
400
450
m/z

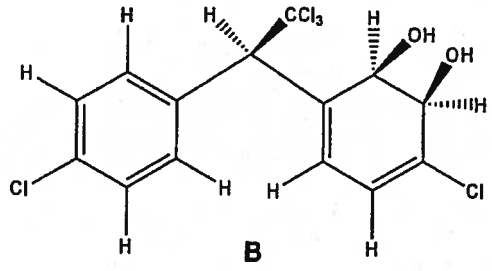
Fig. 2E

Figure 3 : Schéma démontrant les quatre diastéréoisomères du DDT-2,3-dihydrodiol étant produits par l'oxygénation catalytique du DDT.


CCl3
C
Cl
Cl
H


A


C


B

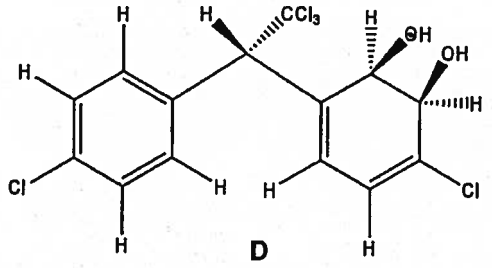
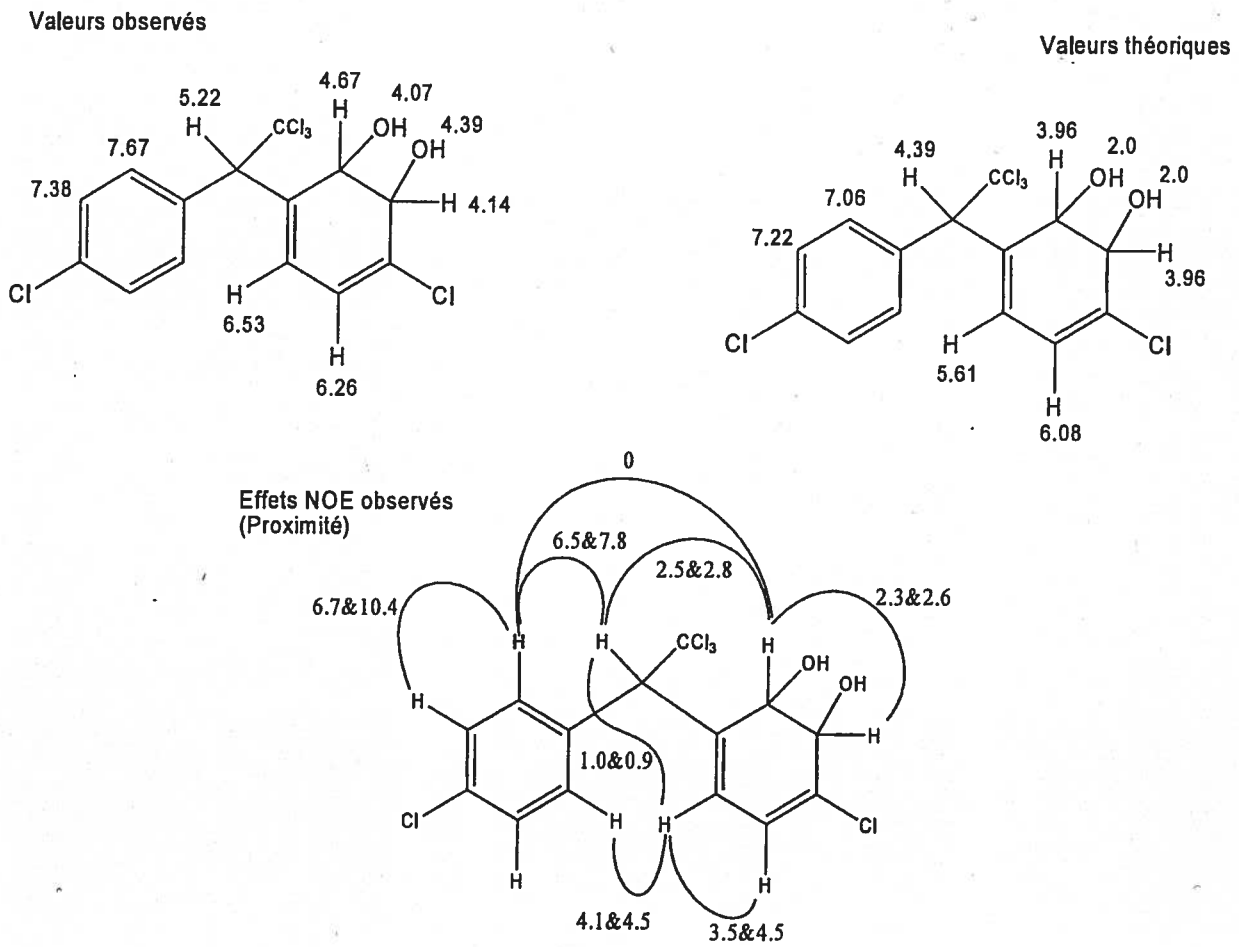

D

Figure 4 : Spectre de résonance magnétique nucléaire des deux diastéréoisomères du DDT-2,3-dihydrodiol produit par l'oxygénase du biphényle. Valeurs de couplage observées pour le métabolite I (A) et II (B) ainsi que les interactions NOE entre les différents hydrogènes.

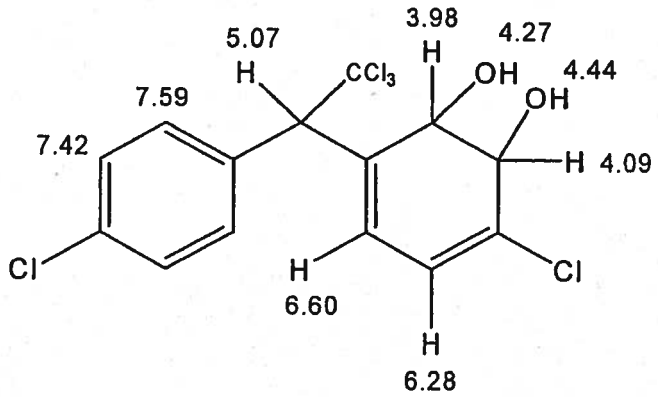
	Metabolite I		Metabolite II	
Protons	Blindage (ppm)	Caractéristique	Blindage (ppm)	Caractéristique
H_1 or H_2	4.67	m, 1H	3.98	m, 1H
H_1 or H_2	4.14	m, 1H	4.09	m, 1H
H_3	6.26	d, J=6.4 Hz, 1H	6.28	d, J=6.4 Hz, 1H
H_4	6.53	dd, J=1.5 Hz, J=6.4 Hz, 1H	6.60	d, J=6.4 Hz, 1H
H_5	5.22	s, 1H	5.07	s, 1H
H_6	7.67	d, J=8.5 Hz, 2H	7.59	d, J=8.5 Hz, 2H
H_7	7.38	d, J=8.5 Hz, 2H	7.42	d, J=8.5 Hz, 2H

(A) Metabolite I

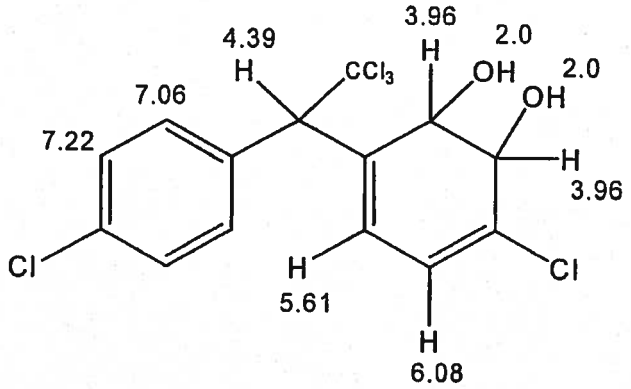


(B) Metabolite II

Valeurs observés



Valeurs théoriques



Effets NOE observés
(Proximité)

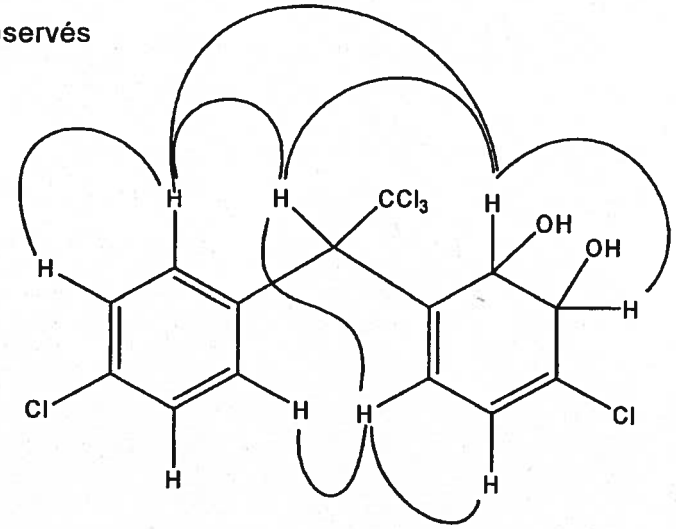


Figure 5 : Alignement des séquences de BphA de LB400 avec les BphA de B-356, III-17, III-34, III-37 and III-52. La séquence de la BphA1 d'A5 est également illustrée. Seuls les résidus de protéine BphA se différenciant de BphAE de LB400 sont inscrits.

	166	172	178	179	184	188	191	202	206	211	237	238	243	247	254	256	258	259	260	261	267	277	278	279	280	282	283	292	298	304	306	307	309	310	311
LB400	a	v	v	q	e	g	r	p	v	m	t	t	i	i	s	a	i	p	t	k	a	y	v	d	e	g	s	v	e	l	e	q	l	g	h
17	e	.	p	e	k	s	m	e	e	i	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
34	e	.	p	e	k	s	m	e	e	i	m	s	v	l	t	i	l	s	k	n	s	.	.	.	.	.	.	.	.	.	.	.	.	.	.
52	.	.	p	e	k	s	m	e	e	i	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
37	e	.	p	e	k	s	m	e	e	i	m	s	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B-356	e	.	p	e	k	s	m	e	e	i	m	s	v	l	t	i	l	s	k	n	s	f	i	n	d	s	i	i	q	k	a	r	-	-	v
A5	e	i	a	e	k	s	m	e	t	.	m	s	v	l	t	v	m	s	k	n	.	f	t	n	d	a	i	i	q	k	a	k	.	-	-

	312	313	316	317	318	320	326	335	336	338	341	350	356	364	365	370	373	377	397	402	407	410	412	416	417	418	419	420	421	422	428	429	430	446	455
LB400	t	g	v	r	r	v	i	t	f	n	i	i	t	a	e	y	h	n	k	y	q	n	q	g	r	s	q	t	g	h	n	v	g	m	a
17	.	.	.	.	.	.	v	g	i	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
34	.	.	.	.	.	.	v	g	i	t	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
52	.	.	.	.	.	.	v	g	i	t	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
37	.	.	.	.	.	.	.	g	i	t	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B-356	p	q	i	l	d	f	v	g	i	t	t	v	v	e	d	f	q	t	r	h	t	c	k	n	v	p	n	k	n	n	k	t	a	s	d
A5	n	q	t	q	t	y	v	g	i	t	s	v	v	e	d	f	q	t	r	h	a	c	h	n	v	p	n	k	s	n	k	t	a	a	d

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