Biochemical and Biophysical Research Communications **260,** 181–187 (1999)
Article ID bbrc.1999.0706, available online at http://www.idealibrary.com on IDEAL®

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

D. Barriault,* M. Vedadi,† J. Powlowski,† and M. Sylvestre*,[1]

**INRS-Santé, Institut national de la recherche scientifique, 245 Hymus Boulevard, Pointe-Claire, Québec, Canada H9R 1G6; and †Department of Chemistry and Biochemistry, Concordia University, Montreal, Quebec, Canada H3G 1M8*

Received March 17, 1999

***Pseudomonas putida* strain G7 *cis*-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase (NahB) and *Comamonas testosteroni* strain B-356 *cis*-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase (BphB) were found to be catalytically active towards *cis*-2,3-dihydro-2,3-dihydroxybiphenyl (specificity factors of 501 and 5850 s^{-1} mM^{-1} respectively), *cis*-1,2-dihydro-1,2-dihydroxynaphthalene (specificity factors of 204 and 193 s^{-1} mM^{-1} respectively) and 3,4-dihydro-3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl (specificity factors of 1.6 and 4.9 s^{-1} mM^{-1} respectively). A key finding in this work is the capacity of strain B-356 BphB as well as *Burkholderia cepacia* strain LB400 BphB to catalyze dehydrogenation of 3,4-dihydro-3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl which is the metabolite resulting from the catalytic *meta-para* hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl by LB400 biphenyl dioxygenase.**

Bacteria able to convert selected chlorobiphenyl (PCB) congeners into corresponding chlorobenzoates use the biphenyl degradation pathway. This pathway involves four enzymatic steps. The initial reaction is catalyzed by the three component biphenyl 2,3-dioxygenase (Bph dox) which catalyzes the oxygenation of two vicinal *ortho-meta* carbons of the biphenyl ring to yield a 2,3-dihydrodihydroxybiphenyl (2,3-DD-biphenyl) (1, 2). The dihydrodiol intermediate is then a substrate for BphB, an NAD^+-dependent dehydrogenase that produces 2,3-dihydroxybiphenyl for BphC, a ring fission dioxygenase. Following ring fission, hydrolytic cleavage by BphD produces benzoate and 2-hydroxypenta-2,4-dienoate.

[1] Corresponding author. Fax: 514-630-8850. E-mail: Michel.Sylvestre@INRS-Sante.Uquebec.ca.

Tolerance of the various enzymes of the biphenyl degradation pathway for chlorine substituents varies from organism to organism. A key feature of *Burkholderia cepacia* LB400 Bph dox is its capacity to catalyze the oxygenation of selected PCB congeners at the 3,4-positions, rather than at the usual 2,3-positions. For example, unlike the homologous enzyme from *Comamonas testosteroni* B-356 (3), strain LB400 Bph dox can oxygenate selected congeners, such as 2,2′,5,5′-tetrachlorobiphenyl, for which none of the *ortho-meta* positions are available for oxygenase attack (4). However, complete degradation of PCB congeners requires that such products be converted into corresponding acyclic metabolites. Preliminary results have indicated that the second enzyme of the pathway, 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase (BphB) of strain LB400, is unable to dehydrogenate *cis*-3,4-dihydro-3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl (3,4-DD-2,2′,5,5′-TCB) which is the metabolite resulting from the 3,4-dioxygenation of 2,2′,5,5′-tetrachlorobiphenyl (4, 5, 6). While Hülsmeyer *et al.* recently elucidated the structure of BphB from strain LB400, and modeled enzyme-substrate interactions, these studies were unable to explain why 3,4-DD-2,2′,5,5′-TCB is not a substrate for the enzyme (6).

Several reports have suggested that *cis*-dihydrodiol dehydrogenases involved in the metabolism of aromatic compounds have relaxed substrate specificity (7, 8, 9, 10, 11, 12). In addition, *cis*-1,2-dihydro-1,2-dihydroxynaphthalene 1,2-dehydrogenase from *Pseudomonas putida* G7 NAH7 plasmid (G7 NahB) was found to dehydrogenate 3,4-DD-2,2′,5,5′-TCB (13). Furthermore, a preliminary investigation in our laboratory using crude extracts suggested that *Comamonas testosteroni* B-356 BphB, like strain G7 NahB, can catalyze the dehydrogenation of 3,4-DD-2,2′,5,5′-TCB.

The most suitable enzymes for the development of engineered bacteria with enhanced ability to degrade

0006-291X/99 $30.00

the most persistent PCB congeners will be those that show the highest level of affinity and selectivity towards these substrates. Although the crystal structure of strain LB400 BphB is now known, identification of the major structural determinants responsible for binding and specificity of the enzyme is so far hindered by the lack of a structure for the enzyme-substrate complex (6). Given these premises, the purpose of this work was to compare the kinetic parameters of strain G7 NahB with those of strain B-356 BphB towards *cis*-2,3-dihydro-2,3-dihydroxybiphenyl (2,3-DD-biphenyl), *cis*-1,2-dihydro-1,2-dihydroxynaphthalene (1,2-DD-naphthalene) and 3,4-DD-2,2′,5,5′-TCB, and to reevaluate the capacity of LB400 BphB to catalyze the dehydrogenation of the latter substrate.

MATERIALS AND METHODS

Bacterial Strains and General Protocols

The bacterial strains used in this study were: *Escherichia coli* M15[pREP4] carrying *C. testosteroni* B-356 *bphB* or *P. putida* G7 *nahB* on pQE31 (13, 14); *C. testosteroni* B-356 (15); and *B. cepacia* LB400 (16). Most molecular biology manipulations were done according to protocols described by Sambrook *et al.* (17). Polymerase chain reactions (PCR) to amplify *nahB* from strain G7 NAH7 for DNA sequencing were performed using *Pwo* DNA polymerase according to procedures outlined previously (13). DNA Sequencing was done from subclones in M13mp18 and M13mp19 using the Pharmacia Automated Laser Fluorescent A.L.F. DNA Sequencer. Sequence analyses were performed by the DNA sequencing service at Institut Armand-Frappier, Laval, Québec, Canada. The two oligonucleotides used to amplify *nahB* for sequencing were based on the known sequences of the terminal portion of strain G7 $nahA_d$ (18), and the terminal portion of *Pseudomonas putida* OUS82 *pahB* (19) and of *nahB* carried on plasmid NPL1 of *Pseudomonas putida* BS202 (20). The oligonucleotide sequences were: *Kpn*I 5′ CGAGGTACCGATGATGATCAATATTCAAG 3′ and *Kpn*I 5′ CGACGGTACCTCACTTGCGACCGAGC 3′.

Expression and Purification of His-Tagged B-356-BphB and G7-NahB

B-356 BphB and G7-NahB were expressed and purified as fused His-tagged (ht-) proteins. Ht-B-356-BphB and ht-G7-NahB were expressed in *E. coli* and purified by affinity chromatography according to protocols described previously (13, 14).

Chemicals

2,3-DD-Biphenyl and 3,4-DD-2,2′,5,5′-TCB were produced enzymatically from biphenyl (Aldrich Chemicals, Milwaukee, WI) and 2,2′,5,5′-tetrachlorobiphenyl (ULTRA Scientific, North Kingstown, RI) according to protocols described previously (13, 14). The identities of 2,3-DD-biphenyl and 3,4-DD-2,2′,5,5′-TCB were assessed by gas chromatography-mass spectrometry (GC/MS) analysis of their butylboronate derivatives as described previously (14). Chemically synthesized 1,2-DD-naphthalene was graciously provided by D. T. Gibson and S. M. Resnick (University of Iowa, Iowa City, IA). Concentrations of 2,3-DD-biphenyl and 1,2-DD-naphthalene were calculated from the weights of purified powder used to make the stock solutions. The concentration of 3,4-DD-2,2′,5,5′-tetrachlorobiphenyl in solution was obtained using high-performance liquid chromatography (HPLC) analysis as described previously (13).

BphB and NahB Assays

The activities of purified preparations of ht-BphB and ht-NahB were measured at 37°C in Bicine buffer, 50 mM, pH 9.0.

(i) HPLC assay. The assay mixture (200 μl total) contained 2-2.5 mM NAD^+, 0.5 μg of the purified enzyme and variable concentrations (75 to 1,000 μM) of 2,3-DD-biphenyl or 1,2-DD-naphthalene. When the substrate was 3,4-DD-2,2′,5,5′-TCB, the amount of enzyme in the reaction medium was raised to 25 μg. The reaction was initiated by adding the substrate dissolved in 2 μl of acetone, and it was stopped after 1 min by addition of 400 μl acetonitrile. This mixture was centrifuged 30 s, then samples of the supernatant (50 μl) were injected onto an octyldecyl silane Hypersil II HPLC column to evaluate substrate depletion by reverse-phase chromatography. The substrate was detected using a Perkin-Elmer LC95 UV/visible detector set at 306 nm for 2,3-DD-biphenyl, 264 nm for 1,2-DD-naphthalene and 283 nm for 3,4-DD-2,2′,5,5′-TCB.

(ii) Fluorometric assay. When 2,3-DD-biphenyl was used as substrate for BphB or NahB, activity was also evaluated by monitoring continuous production of NADH using a Shimadzu RF-5000 fluorometer. The assay (400 μl total volume) contained 2.5 mM NAD^+, 0.05 μg of the purified enzyme, and varying concentrations of diol substrate. The excitation wavelength was set at 340 nm and emitted light was measured at 460 nm: wavelength dispersion was 5 nm for both excitation and emission.

Kinetic parameters, K_m^{app} and V_{max}^{app}, at 37°C were obtained from linear least-squares fitting to the Lineweaver-Burk equation. The k_{cat}^{app} values were determined according to the equation $k_{cat}^{app} = V_{max}^{app}/[E]$ where [E] is the enzyme molar concentration determined using the Lowry method (21) and the predicted molecular weight of the enzyme subunit. The specificity constant for each substrate is the k_{cat}/K_m ratio.

BphB activity toward 3,4-DD-2,2′,5,5′TCB was also determined in crude extracts of *C. testosteroni* B-356 and *B. cepacia* LB400 cells. Crude lysates were prepared from 500 ml of fresh log-phase culture grown on minimal medium no 30 (22) containing 0.1% (w/v) biphenyl as growth substrate. The harvested cell paste was washed and resuspended in 4 ml of 100 mM Bicine buffer, pH 8.0. The cell suspension was sonicated and then cleared by centrifugation. The supernatant was used directly in the enzyme assay. The reaction medium in 100 mM Bicine, pH 8.0, buffer (200 μl total volume) contained 100 μl of cell lysate, 1 mM NAD^+ and 15 nmol substrate added in 2 μl acetone to initiate the reaction. The reaction products were extracted with ethyl acetate, treated with butylboronate and analyzed by GC/MS as described previously (14).

Nucleotide Sequence Accession Number

The nucleotide sequence data in this paper has been submitted to the GenBank nucleotide sequence data bases under the accession number AF125184.

RESULTS

Catalytic Properties of BphB and NahB toward Selected Substrates

Preliminary work with crude cell extracts showed that, like *P. putida* strain G7 NahB, *C. testosteroni* strain B-356 BphB can metabolize 3,4-DD-2,2′,5,5′-TCB. These results prompted a more exhaustive comparison of the catalytic properties of both enzymes toward this substrate, as well as their natural substrates. Purified ht-BphB and ht-NahB were produced as described previously (13, 14). Under the assay con-

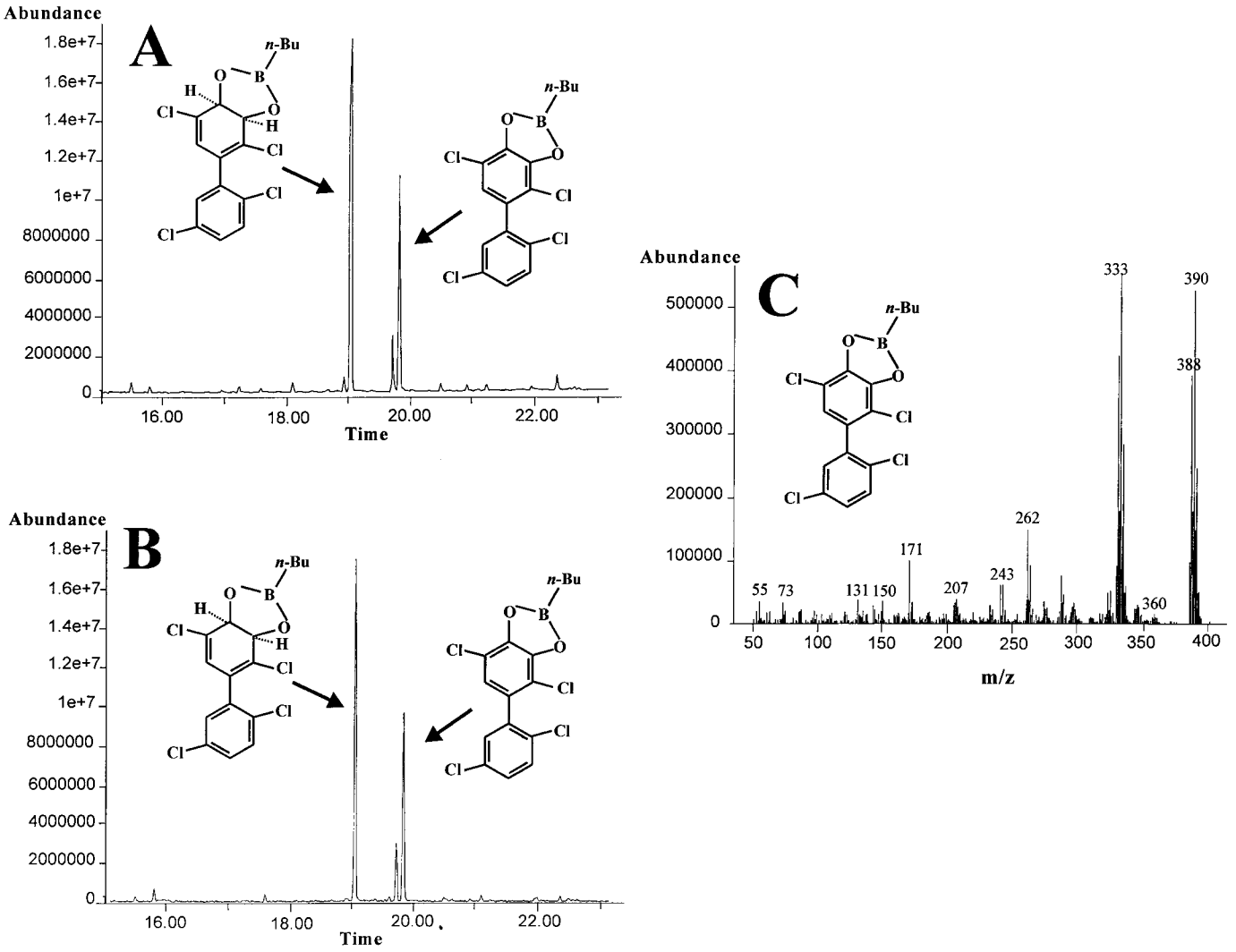

FIG. 1. Total ion chromatograms of butylboronate-derived oxidative metabolites produced from 3,4-DD-2,2′,5,5′-TCB by (A) G7 ht-NahB and (B) B-356 ht-BphB. (C) Mass spectrum of 3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl produced from 3,4-DD-2,2′,5,5′-TCB.

ditions described in Materials and Methods, purified ht-B-356 BphB or ht-G7 NahB catalyzed the dehydrogenation of 3,4-DD-2,2′,5,5′-TCB to generate 3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl (Fig. 1A and 1B). 3,4-Dihydroxy-2,2′,5,5′-tetrachlorobiphenyl was identified from the mass spectrum of its butylboronate derivative. The spectrum (Fig. 1C) is similar to one for 2,3-dihydroxy-4′-chlorobiphenyl reported by Massé *et al.* (23). The major fragmentation sequence in its mass spectrum first involves the loss of an *n*-butyl moiety from the molecular ion with subsequent elimination of one or two HCl to yield ions at *m/z* 333, *m/z* 298 and *m/z* 262. Other significant fragmentation ions are found at *m/z* = 290 (M − *n*BuBO$_2$) and *m/z* = 278 (M − *n*BuBO − CO). 3,4-DD-2,2′,5,5′-TCB was stable and remained unaltered for hours under the reaction conditions used. Furthermore, controls containing no NAD^+ did not produce this metabolite. Taken together, these results show that the catechol derivative generated in the reaction medium is the result of a catalytic dehydrogenation reaction.

Assays were run to determine the K_m^{app} and apparent maximal rate of turnover (k_{cat}) for both enzymes when either 2,3-DD-biphenyl, 1,2-DD-naphthalene or 3,4-DD-2,2′,5,5′-TCB were used as substrate (Table 1). In most cases, assays were done using an HPLC method developed previously to quantitate 3,4-DD-2,2′,5,5′-TCB (13). In the case of 2,3-DD-biphenyl, the K_m^{app} for BphB was too low to be determined by HPLC measurement of substrate depletion at 37°C, and it was therefore estimated by continuous fluorometric monitoring of NADH production. The kinetics of NahB with 2,3-DD-biphenyl were also estimated using this method, and were comparable to those obtained using the HPLC assay (data not shown). Since kinetic parameters sometimes varied after prolonged storage, the ki-

TABLE 1

Kinetic Constants of BphB and NahB Measured at pH 9.0

	2,3-DD-Biphenyl		1,2-DD-Naphthalene		3,4-DD-2,2′,5,5′-Trichlorobiphenyl	
Substrate	BphB	NahB	BphB	NahB	BphB	NahB
K_m^{app} (mM)	0.0039 ± 0.0002*	0.11 ± 0.032	0.28 ± 0.06	0.73 ± 0.16	1.5 ± 0.44	0.062 ± 0.008
k_{cat}^{app} (s^{-1})	22.8 ± 1.4*	51.7 ± 5.5	54 ± 6.6	149 ± 14	7.4 ± 1.4	0.10 ± 0.005
k_{cat}/K_m (s^{-1} mM^{-1})	5850	501	193	204	4.9	1.6

* Determined using fluorometry as described in Materials and Methods. The concentration of NAD^+ in the assay was 2.5 mM: similar results were obtained using 1.5 mM NAD^+. Assays with other substrates and enzymes were performed using HPLC and 2-2.5 mM NAD^+.

netic parameters of ht-BphB and ht-NahB reported here are those of freshly prepared and diluted enzymes.

The apparent k_{cat} values for 2,3-DD-biphenyl are within a factor of approximately 2 for both enzymes. However, the specificity of BphB toward 2,3-DD-biphenyl is 12-fold higher than NahB resulting from the fact that BphB has much greater affinity than NahB for 2,3-DD-biphenyl (Table 1). It is also interesting that the affinity and the specificity factor of NahB for 2,3-DD-biphenyl are higher than they are for its natural substrate, 1,2-DD-naphthalene. However, the maximal substrate turnover rate with 1,2-DD-naphthalene is approximately 3 fold higher when the reaction is catalyzed by NahB than by BphB. Both enzymes can dehydrogenate 3,4-DD-2,2′,5,5′-TCB. However, although the specificity of BphB toward 3,4-DD-2,2′,5,5′-TCB is three-fold that of NahB, NahB showed a much higher affinity for this substrate than BphB.

Previous data of a preliminary nature had suggested that strain LB400 BphB was unable to catalyze the dehydrogenation of 3,4-DD-2,2′,5,5′TCB (4, 5, 6). The data presented above prompted a reevaluation of the capacity of strain LB400 BphB to catalyze this reaction. Results presented in Fig. 2 show that, indeed, biphenyl-induced strain LB400 cell lysates can metabolize 3,4-DD-2,2′,5,5′TCB to produce the corresponding catechol. A similar experiment with biphenyl-induced B-356 cell extracts (not shown) showed that B-356-BphB can metabolize 3,4-DD-2,2′,5,5′TCB under the same conditions used for LB400 in Fig. 2. Thus B-356 BphB is not unique in its ability to catalyze the 3,4-dehydrogenation of 3,4-DD-2,2′,5,5′TCB.

Genetic Analysis of G7 nahB

In previous work, alignment of the amino acid sequences of bacterial aryl-dihydrodiol dehydrogenases differentiated two main clusters (14). One cluster includes the enzymes involved in the degradation of monoaryls containing a carboxyl group, for which biodegradation is initiated by class I dioxygenases. The second cluster is divided into two lineages: one of these comprises the dehydrogenases involved in the degradation of compounds for which the initial reaction is catalyzed by class II dioxygenases (e.g. biphenyl 2,3-dioxygenase), and the other lineage comprises the dehydrogenases that participate in pathways involving class III dioxygenases (e.g. naphthalene 1,2-dioxygenase). This suggested that the dihydrodiol dehydrogenases involved in degradation pathways for aromatic compounds have evolved simultaneously with the dioxygenase (14).

The nucleotide sequence of *nahB* from plasmid NAH7 has not yet been reported, so it is not clear whether this enzyme fits the previously observed pattern. Since B-356 BphB and G7 NahB can catalyze the dehydrogenation of the same range of substrates, there is a possibility of a recent substitution through recombination of G7 *nahB* by a gene encoding an enzyme phylogenetically more akin to BphB than NahB. For example, there is evidence that *Sphingomonas*

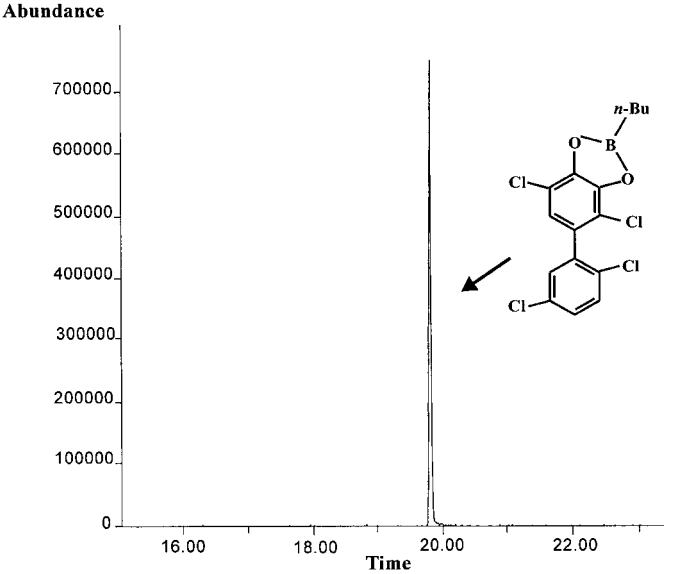

FIG. 2. Total ion chromatogram showing the butylboronate-derived oxidative metabolite produced from 3,4-DD-2,2′,5,5′-TCB by a crude lysate of biphenyl induced LB400 cells. The protocol used to prepare the cells and perform the reaction is described in Materials and Methods.

```
      nahAd
1     cgaaaattgg tccagcgatt cgtggattac ccagagcgca tacttcagac
       R  K  L   V  Q  R  F   V  D  Y   P  E  R   I  L  Q  T
51    gcacaatctg atggtctttc tgtgatccag tgaccacttt tacaaatggt
        H  N  L   M  V  F   L  *
101   gactgctacc gcggtcacca ttaatcaaaa gggaatgtaa gtgtatgggc
                                                nahB   M  G
151   aatcaacaag tcgtttcgat aaccggtgcc ggctcaggaa tcggtctcga
       N  Q  Q   V  V  S  I   T  G  A   G  S  G   I  G  L  E
201   actggttcga tcctttaagt tggccggtta ttgcgtatcc gctctcgtgc
        L  V  R   S  F  K   L  A  G  Y   C  V  S   A  L  V
251   gaaacgagga gcaagaggcg cttctttgca atgagttcaa ggacgcactc
       R  N  E  E   Q  E  A   L  L  C   N  E  F  K   D  A  L
301   gagatcgtag tgggcgatgt ccgggaccac gcgacaaatg agaagctgat
        E  I  V   V  G  D  V   R  D  H   A  T  N   E  K  L  I
351   aaagcagaca attgatagat tcggtcatct cgattgtttc atcgcaaatg
        K  Q  T   I  D  R   F  G  H  L   D  C  F   I  A  N
401   ccggtatttg ggattacatg ctgaatatcg aagagccttg ggagaaaatc
       A  G  I  W   D  Y  M   L  N  I   E  E  P  W   E  K  I
451   tcgagcagtt ttgacgaaat attcgacatc aatgtcaaga gctatttcag
        S  S  S   F  D  E  I   F  D  I   N  V  K   S  Y  F  S
501   tggcatcagt gccgccctgc ccgagctgaa aaagactaac ggatcagtgg
        G  I  S   A  A  L   P  E  L  K   K  T  N   G  S  V
551   tgatgaccgc ttcggtgtcg tcccatgcgg tcggtggtgg tggttcttgc
       V  M  T  A   S  V  S   S  H  A   V  G  G  G   G  S  C
601   tacatcgcca gcaagcatgc ggtgctgggt atggtcaagg ctttggccta
        Y  I  A   S  K  H  A   V  L  G   M  V  K   A  L  A  Y
651   cgaattggcc cccgaaattc gcgtgaacgc tgtttcgcct ggcggcaccg
        E  L  A   P  E  I   R  V  N  A   V  S  P   G  G  T
701   tgacgtctct gtgcggtccc gctagcgccg gtttcgacaa aatgcacatg
       V  T  S  L   C  G  P   A  S  A   G  F  D  K   M  H  M
751   aaagacatgc ccggcatcga cgatatgatc aaaggtctca cacctcttgg
        K  D  M   P  G  I  D   D  M  I   K  G  L   T  P  L  G
801   gtttgcagcc aagcccgaag acgtggtggc accctatctg ttgctggctt
        F  A  A   K  P  E   D  V  V  A   P  Y  L   L  L  A
851   cgcgaaagca aggaaaattc atcactggca ccgtgattag cattgatggc
       S  R  K  Q   G  K  F   I  T  G   T  V  I  S   I  D  G
901   ggtatggcgc tcggtcgcaa gtga
        G  M  A   L  G  R  K   *
```

FIG. 3. Nucleotide sequences of G7 NahB.

yanoikuyae B1 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase is involved in both the biphenyl and the naphthalene degradation pathways of this organism (9). The restriction map of the 11 Kb DNA fragment from *Pseudomonas* sp. C18 carrying the naphthalene/dibenzothiophene degradation genes is very similar to the one carrying the NAH7 naphthalene genes (24, 25) except for the portion comprising *doxE,* the gene homologous to *nahB* (24).

In order to clarify the origin of G7 *nahB,* we have sequenced the gene (Fig. 3). From the dendogram presented in Fig. 4, which is based on the alignments of the deduced amino acid sequences of a number of 1,2-DD-naphthalene 1,2-dehydrogenases and 2,3-DD-biphenyl-2,3-dehydrogenases, it is clear that G7 NahB is more closely related to 1,2-DD-naphthalene 1,2-dehydrogenases.

DISCUSSION

Sequence analysis shows that G7 NahB and B-356 BphB have evolved separately. However, in spite of the fact that G7 NahB and B-356 BphB show only 35-38% identity, both enzymes catalyze the oxidation of the same range of substrates. Previous reports have indicated that dihydrodiol dehydrogenases involved in the degradation of aromatic compounds have relaxed substrate specificities (10, 11, 12). G7 NahB and B-356 BphB are no exception to this. However, the specificity constant of NahB for its natural substrate is much lower than that of BphB for 2,3-DD-biphenyl. There is no clear explanation for this observation. Nevertheless, it is tempting to speculate that NahB reactivity toward 1,2-DD-naphthalene might have adjusted to prevent the accumulation of 1,2-dihydroxynaphthalene which is spontaneously transformed into 1,2-naphthoquinone, a dead end metabolite (12, 26).

Recent modeling studies on the three dimensional structure of LB400 BphB suggested the involvement of Asn^{143} in enzyme specificity. Asn^{143} hydrogen-bonds to the substrate hydroxyl in the modeled ternary complex (6). Asn^{143} is conserved in this position in all BphB sequences. However, in the NahB sequences this position is occupied by the hydrophobic Val (not shown). Hydrogen bonding involving Asn^{143} might therefore be the explanation for the fact that BphB showed a high affinity for 2,3-DD-biphenyl. Given the results presented here, it will be interesting to evaluate the effect of replacing Val by Asn at that position in NahB.

It is also interesting that the sequence of PhnB, which is the dihydrodiol dehydrogenase of the *Burk-*

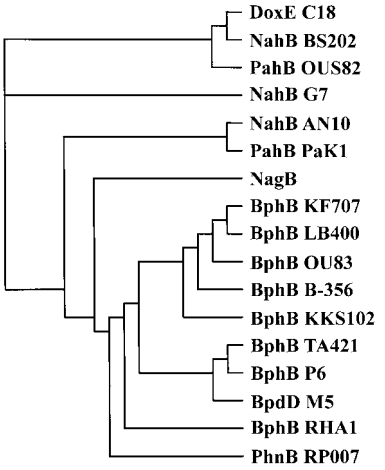

FIG. 4. Dendrogram obtained from the alignment of amino acid sequences of gene products by using the Clustal X program (30) and Drawgram program (31). Number in parentheses refers to the accession number to databases. DoxE-C18 (M60405 and Q52459) DoxE of *Pseudomonas* sp. C18 (23); NahB BS202 (AF010471) NahB of *P. putida* BS202 (Unpublished); NahB AN10 (AF039533) NahB of *Pseudomonas stutzeri* AN10 (unpublished); NagB (AF036940) NagB of *Pseudomonas* sp. U2 (unpublished); PahB-OUS82 (D16629) PahB of *Pseudomonas putida* OUS82 (19); PahB Pa1K (D84146) PahB of *Pseudomonas aeruginosa* Pa1K (unpublished); BphB-KF707 (M83673) BphB of *Pseudomonas pseudoalcaligenes* KF707 (32); BphB-LB400 (M66122) BphB of *B. cepacia* LB400 (33); BphB OU83 (Y07655) BphB of *P. putida* OU83 (34); BphB-B-356 (U57451) BphB of *C. testosteroni* B-356 (14); BphB-KKS102 (D17319) BphB of *Pseudomonas* sp. KKS102 (35); BphB TA421 (D88021) BphB of *Rhodococcus erythropolis* TA421 (unpublished); BphB-P6 (X80041) BphB of *Rhodococcus globerulus* P6 (36); BpdD-M5 (U27591) BpdD of *Rhodococcus* M5 (37); BphB-RHA1 (D32142) BphB of *Rhodococcus* sp. RHA1 (38); PhnB RP007 (AF061751) PhnB of *Burkholderia* sp. RP007 (27).

holderia sp. RP007 phenanthene degradation pathway (27), clusters with BphB of *Rhodococcus* sp. RHA1 rather than with the dihydrodiol dehydrogenases of the other naphthalene degradation pathways (Fig. 4). The amino acid sequence surrounding Asn^{143} of LB400 BphB, TIS(N^{143})AGFYP is conserved in all *bphB* products and it is replaced by the sequence TAS(V)SSHAV in all *nahB* and *pahB* products. In PhnB, this sequence is identical to the one found in the *bphB*-encoded proteins. Thus it appears the 1,2-DD-naphthalene dehydrogenase of the *Burkholderia* sp. RP007 naphthalene catabolic pathway has been substituted by a 2,3-DD-biphenyl-2,3-dehydrogenase in the course of the evolution of the pathway. Similarly, it has been suggested that BphB serves both the biphenyl and the naphthalene degradation pathways in *S. yanoikuyae* B1 (9). These observations are interesting since they support the data suggesting that BphB can catalyze the dehydrogenation of 1,2-DD-naphthalene as efficiently as NahB.

Examples of substitution of an enzyme belonging to a catabolic pathway by a homologous enzyme taken from another pathway are still rare. The reductase component of B-356 BPH dox has been suggested to be one such an example (28). However, such substitution is not likely to be a common phenomenon in the naphthalene degradation pathway. Further investigations, including the possible feedback inhibition of the enzymes by their reaction products, might bring some more insight to the understanding of why, in spite of similar kinetic properties toward the same substrate, BphB and NahB are generally preserved in their respective pathways.

A key observation made during this work was that BphB can catalyze the dehydrogenation of 3,4-DD-TCB. Since the kinetic parameters of the other BPH degrading enzymes toward 2,2′,5,5′-tetrachlorobiphenyl metabolites are unknown, it is too early yet to determine if the BphB reaction is a limiting step in the degradation of this PCB congener. However, our data clearly show that the dehydrogenase reaction need not interrupt its degradation. At this time, we have no clear explanation for the fact that under the conditions used in the present work, strain LB400 BphB was found able to dehydrogenate 3,4-DD-TCB whereas previous reports indicated the inability of this enzyme to catalyze this reaction (4, 5, 6). However, in the course of this work, we found that the detection by HPLC or GC/MS of 3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl, generated from the dehydrogenation of 3,4-DD-2,2′,5,5′-TCB, is strongly influenced by the composition of the reaction medium (not shown). The reaction medium described in *Materials and Methods* was the only one that allowed its detection. The chemistry of the dihydrodiol and dihydroxyl metabolites derived from the first catalytic steps involved in aromatic degradation has not yet been investigated thoroughly. For example, it is not clear why 2,3-dihydroxy-2′-chlorobiphenyl is generated rather than 2,3-dihydro-2,3-dihydroxy-2′-chlorobiphenyl when LB400 Bph dox catalyzes the oxygenolytic dehalogenation of 2,2′-dichlorobiphenyl (4). Similarly, B-356 Bph dox produces a quinone derivative from 3,3′-dihydroxybiphenyl (29), and 1,2-dihydroxynaphthalene is rapidly converted into 1,2-naphthoquinone when it is produced in reaction media (12, 26). Therefore, although it has yet to be demonstrated, under certain conditions, 3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl may be spontaneously transformed into a more reactive species, such as a quinone, which is not detected by the methods used.

ACKNOWLEDGMENT

This work was supported by Grant STP0193182 from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

1. Haddock, J. D., and Gibson, D. T. (1995) *J. Bacteriol.* **177,** 5834–5839.
2. Hurtubise, Y., Barriault, D., and Sylvestre, M. (1996) *J. Biol. Chem.* **276,** 8152–8160.
3. Hurtubise, Y., Barriault, D., and Sylvestre, M. (1998) *J. Bacteriol.* **180,** 5828–5835.
4. Haddock, J. D., Horton, J. R., and Gibson, D. T. (1995) *J. Bacteriol.* **177,** 20–26.
5. Nadim, L. M., Schocken, M. J., Higson, F. K., Gibson, D. T., Bedard, L. D., Bopp, L. H., and Mondello, F. J. (1987) Proceedings of the US EPA Thirteenth Annual Research Symposium on Land Disposal, Remedial Action, Incineration, and Treatment of Hazardous Waste, pp. 395–402. U.S. Environmental Protection Agency, Cincinnati, OH.
6. Hülsmeyer, M., Hecht, H. J., Niefind, K., Hofer, B., Eltis, L. D., Timmis, K. N., and Schomburg, D. (1998) *Protein Sci.* **7,** 1286–1293.
7. Eaton, S. L., Resnick, S. M., and Gibson, D. T. (1996) *Appl. Environ. Microbiol.* **62,** 4388–4394.
8. Spiess, E., and Gorisch, H. (1996) *Arch. Microbiol.* **165,** 201–205.
9. Zylstra, G. J., and Kim, E. (1997) *J. Indust. Microbiol. Biotechnol.* **19,** 408–414.
10. Patel, T. R., and Gibson, D. T. (1976) *J. Bacteriol.* **128,** 842–850.
11. Rogers, J. E., and Gibson, D. T. (1977) *J. Bacteriol.* **130,** 1117–1124.
12. Werlen, C., Kohler, H. P. E., and van der Meer, J. R. (1996) *J. Biol. Chem.* **271,** 4009–4016.
13. Barriault, D., Durand, J., Maaroufi, H., Eltis, L. D., and Sylvestre, M. (1998) *Appl. Environ. Microbiol.* **64,** 4637–4642.
14. Sylvestre, M., Hurtubise, Y., Barriault, D., Bergeron, J., and Ahmad, D. (1996) *Appl. Environ. Microbiol.* **62,** 2710–2715.
15. Ahmad, D., Massé, R., and Sylvestre, M. (1990) *Gene* **86,** 53–61.
16. Bedard, D. L., Unterman, R., Bopp, L. H., Brennan, M. J., Haberl, M. L., and Johnson, C. (1986) *Appl. Environ. Microbiol.* **51,** 761–768.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

18. Simon, M. J., Osslund, T. D., Saunders, R., Ensley, B. D., Suggs, S., Harcourt, A., Suen, W. C., Cruden, D. L., Gibson, D. T., and Zylstra, G. J. (1993) *Gene* **127,** 31–37.
19. Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Satoh, S., and Kiyohara, H. (1994) *J. Bacteriol.* **176,** 2444–2449.
20. Boronin, A. M., Tsoi, T. V., Kosheleva, I. A., Arinbasarov, M. U., and Adanin, V. M. (1989) *Genetika* **25,** 226–237.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
22. Massé, R., Messier, F., Ayotte, C., Lévesque, M-F., and Sylvestre, M. (1989) *Biomed. Environ. Mass Spectrom.* **18,** 27–47.
23. Sylvestre, M., and Fauteux, J. (1982) *J. Gen. Appl. Microbiol.* **28,** 61–72.
24. Denome, S. A., Stanley, D. C., Olson, E., and Yong, K. D. (1993) *J. Bacteriol.* **175,** 6890–6901.
25. Eaton, R. W., and Chapman, P. J. (1992) *J. Bacteriol.* **174,** 7542–7554.
26. Patel, T. R., and Gibson, D. T. (1974) *J. Bacteriol.* **119,** 879–888.
27. Laurie, A. D., and Lloyd-Jones, G. (1999) *J. Bacteriol.* **181,** 531–540.
28. Sylvestre, M., Sirois, M., Hurtubise, Y., Bergeron, J., Ahmad, D., Shareck, F., Larose, A., Barriault, D., Guillemette, I., and Juteau, J. M. (1996) *Gene* **174,** 195–202.
29. Sondossi, M., Lloyd, B. A., Barriault, D., Sylvestre, M., and Simard, M. (1995) *Acta Crystallographica* **C51,** 491–494.
30. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
31. Felsenstein University of Washington.
32. Taira, K., Hirose, J., Hayashida, S., and Furukawa, K. (1992) *J. Biol. Chem.* **267,** 4844–4853.
33. Hoffer, B., Eltis, L. D., Dowling, D. N., and Timmis, K. N. (1993) *Gene* **130,** 47–55.
34. Khan, A. A., Wang, R. F., Nawaz, M. S., and Cerniglia, C. E. (1997) *FEMS Microbiol. Lett.* **154,** 317–324.
35. Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M., and Yano, K. (1989) *J. Bacteriol.* **171,** 2740–2747.
36. Asturias, J. A., Diaz, E., and Timmis, K. N. (1995) *Gene* **156,** 11–18.
37. Wang, Y., Garnon, J., Labbé, D., Bergeron, H., and Lau, P. C. K. (1995) *Gene* **164,** 117–122.
38. Masai, E., Yamada, A., Healy, J. M., Hatta, T., Kimbara, K., Fukada, M., and Yano, K. (1995) *Appl. Environ. Microbiol.* **61,** 557–580.