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Nucleotide Sequencing and Transcriptional Mapping of the Genes Encoding Biphenyl Dioxygenase, a Multicomponent Polychlorinated-Biphenyl-Degrading Enzyme in *Pseudomonas* Strain LB400

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The DNA region encoding biphenyl dioxygenase, the first enzyme in the biphenyl-polychlorinated biphenyl degradation pathway of *Pseudomonas* species strain LB400, was sequenced. Six open reading frames were identified, four of which are homologous to the components of toluene dioxygenase from *Pseudomonas putida* F1 and have been named *bphA*, *bphE*, *bphF*, and *bphG*. From this comparison, biphenyl dioxygenase was found to be a multicomponent enzyme containing a two-subunit iron-sulfur protein, a ferredoxin, and a reductase. Comparison of the large subunit of the iron-sulfur protein and the ferredoxin with other multicomponent dioxygenases identified amino acid sequences similar to Rieske iron-sulfur proteins for binding a [2Fe-2S] cluster. Sequences have also been identified in the reductase component that match the consensus sequence for FAD or NAD binding. Transcription of the biphenyl dioxygenase region was examined, and three transcription initiation sites were identified. Transcription initiating at the site furthest upstream is greatly increased when the LB400 cells are grown on biphenyl as the sole carbon source.

Polychlorinated biphenyls (PCBs) are a group of man-made compounds composed of biphenyl molecules containing from 1 to 10 chlorines. Depending on the number and position of the chlorines, it is possible to produce 209 different PCB congeners. The vast majority of PCBs in the environment are derived from commercial mixtures (e.g., Aroclors) which contain 60 to 80 different congeners (24). Bacteria able to degrade PCBs are relatively common; however, in most cases, these organisms have a narrow substrate specificity which allows them to degrade only a small number of lightly chlorinated PCBs (1, 3, 7, 10, 16, 26, 28). Such strains are of limited usefulness for bioremediation as they are unable to degrade many of the congeners found in commercial PCB mixtures. *Pseudomonas* sp. strain LB400 is a potentially valuable organism as it is able to oxidize a wide variety of PCB congeners up to and including hexachlorobiphenyls (4).

The major pathway for PCB and biphenyl degradation by strain LB400 is a four-step process initiated by the insertion of two atoms of oxygen at carbon positions 2 and 3 by biphenyl dioxygenase (a 2,3-dioxygenase). The resulting 2,3-dihydrodiol is then converted by dihydrodiol dehydrogenase to 2,3-dihydroxybiphenyl, which is transformed by 2,3-dihydroxybiphenyl dioxygenase to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid via a *meta*-cleavage reaction. A hydrase reaction converts this compound to the corresponding benzoic acid (20).

Unlike nearly all other PCB-degrading bacteria, LB400 also has a 3,4-dioxygenase activity resulting in the insertion of oxygen atoms at the 3 and 4 carbon positions. Thus far, this ability has only been demonstrated against 2,5,2′,5′-tetrachlorobiphenyl, a congener with no unchlorinated 2,3 sites (20). Since mutations which inactivate the 2,3-dioxygenase also eliminate 3,4-dioxygenase activity, it is possible that both originate from a single enzyme. Alternatively, it may be that these activities are due to different enzymes which are coregulated or that share subunits.

It has been demonstrated that differences in PCB congener specificity among bacteria reflect differences in the genes encoding their PCB-degrading enzymes (the *bph* genes). In a previous report, we have described the cloning and characterization of the *bph* genes from strain LB400 (19). Southern hybridization experiments were used to test a variety of PCB-degrading bacteria for the presence of DNA sequences similar to any of the LB400 *bph* genes. No significant hybridization was detected to strains exhibiting congener specificity different from that of LB400 (30). This suggests the existence of at least two analogous 2,3-dioxygenase pathways encoded by significantly different gene sets. A bacterium (*Alcaligenes eutrophus* H850) with PCB-degrading ability very similar to that of LB400 contained *bph* genes which hybridized strongly to those of LB400 (30).

Biphenyl/PCB dioxygenase plays a critical role in PCB degradation by catalyzing the first step in the oxidative pathway and hence determining congener specificity. The exceptional activity of the biphenyl dioxygenase of strain LB400 has led us to study the structure and function of the genes encoding this enzyme. In this report, we describe the nucleotide sequence of these genes and their relationship to those of other dioxygenases. In addition, we used S1 nuclease mapping to examine the transcription of the biphenyl dioxygenase genes under a variety of conditions.

MATERIALS AND METHODS

Nucleotide sequencing. Two adjacent DNA fragments encompassing the biphenyl-PCB dioxygenase genes were submitted to LARK Sequencing Technologies, Dallas, Tex., for nucleotide sequencing. These were the 2.85-kb *Eco*RI fragment from pGEM453.1 and the 2.7-kb *Xma*I fragment of pGEM454 (19). The additional sequencing beyond the *Xma*I (*Sma*I) site was obtained by using a Sequenase kit (U.S. Biochemical Corp.) and α-^{35}S-dATP (Du Pont, NEN Re-

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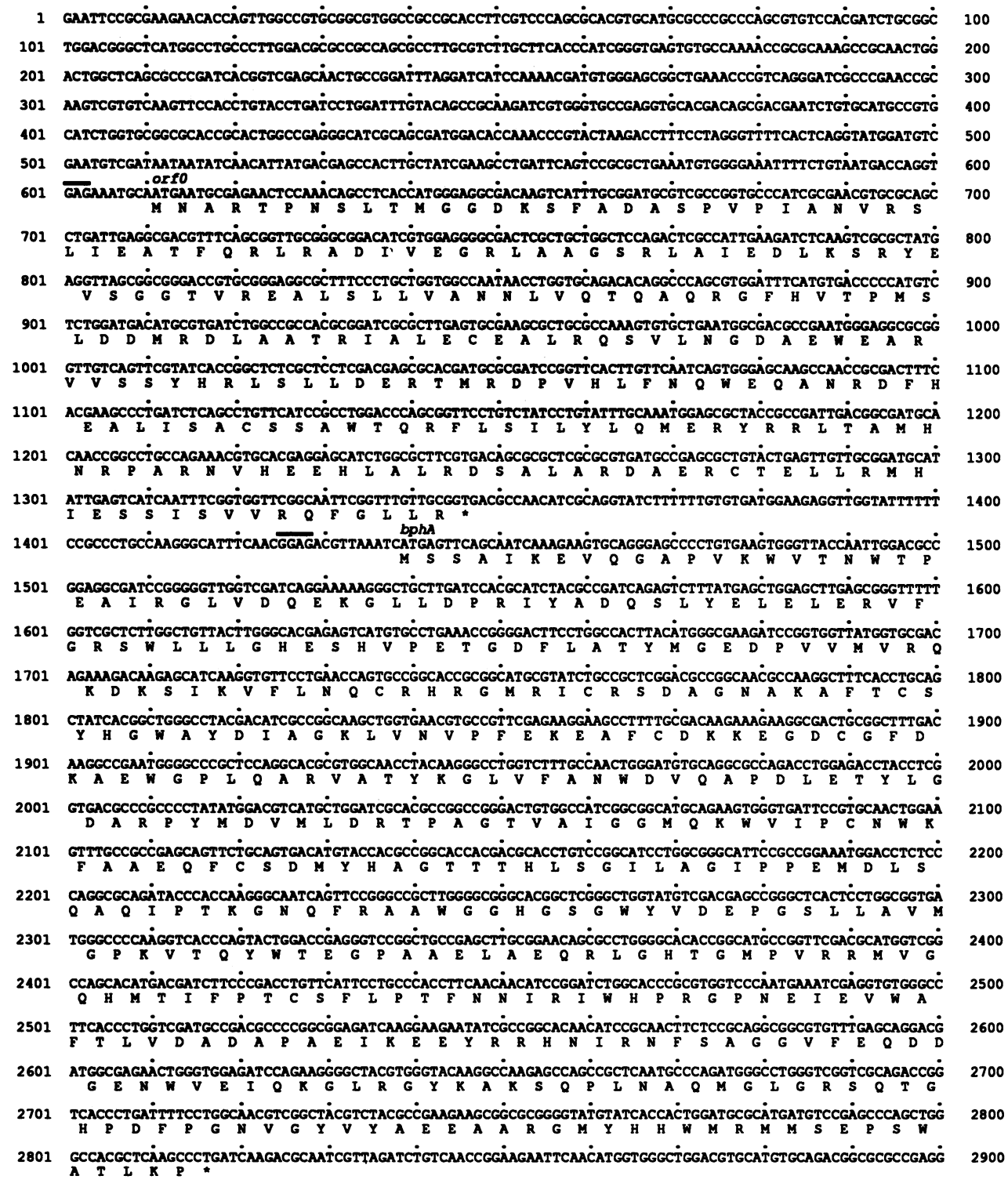

FIG. 1. Nucleotide sequence of the biphenyl dioxygenase region from *Pseudomonas* species strain LB400 and predicted amino acid sequences of the ORFs. The gene names are indicated above the initiation codons. Potential ribosome-binding sequences are indicated by horizontal lines above the nucleotide sequence.

search Products), according to the manufacturer's instructions.

Sequence analysis. The nucleotide sequence was analyzed by using the GCG Sequence Analysis Software Package (6). The programs used were SEQED for sequence loading, MAP and MAPSORT for restriction site identification, MAP for open reading frame identification, TRANSLATE for amino acid sequence determination, WORDSEARCH and

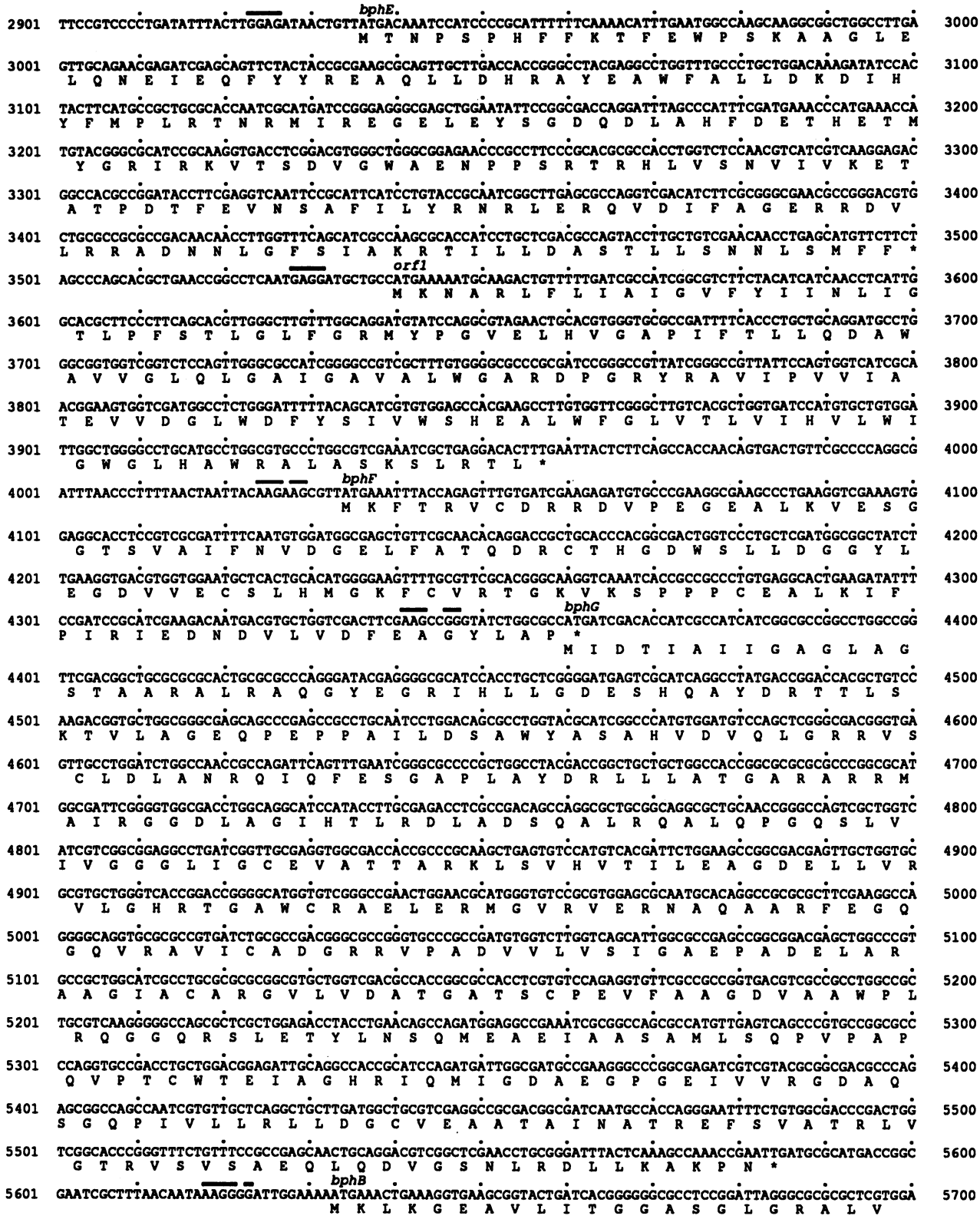

FIG. 1—*Continued.*

STRINGS for data base searching, GAP and BESTFIT for sequence alignments, and PRETTY and PUBLISH for sequence presentation.

S1 nuclease mapping. Whole-cell RNA for S1 mapping was prepared by the hot-phenol extraction method of Salser et al. (25). Cultures for the inoculum were grown to high density at 30°C in pseudomonas minimal medium (18), with biphenyl as the carbon source. Fresh cultures containing the appropriate

TABLE 1. Characteristics and comparisons of biphenyl and toluene dioxygenase components

Protein component	Gene	Nucleotides	Amino acids	Mol wt	Charge	pI	% Nucleotide identity	% Amino acid identity
Iron-sulfur protein large subunit	*bphA*	1,380	459	51,513	−8	6.4	67.6	65.6
	todC1	1,353	450	50,930	−17	5.2		
Iron-sulfur protein small subunit	*bphE*	567	188	22,085	−5	6.0	61.7	57.2
	todC2	564	187	22,013	−5	5.5		
Ferredoxin	*bphF*	330	109	11,981	−7	4.5	58.9	55.1
	todB	324	107	11,900	−8	4.5		
Reductase	*bphG*	1,227	408	42,970	−5	6.2	57.3	52.1
	todA	1,233	410	42,942	−9	5.0		

carbon source were inoculated to a starting optical density at 600 nm of 0.05 and were grown at 30°C until harvesting at an optical density at 600 nm of 0.5 to 0.8. RNA preparations from 200 ml of culture medium yielded 1 to 3 mg of RNA.

S1 nuclease mapping was performed essentially as previously described (2, 5). Analysis was performed on 20 to 40 μg of RNA with the addition of yeast tRNA to a final concentration of 100 μg of RNA per reaction. Probes were prepared by digesting pGEM453.1 (pUC18 containing the 2.85-kb *Eco*RI fragment) with the indicated restriction enzyme and then treating with calf intestinal alkaline phosphatase (Boehringer Mannheim) and labeling with T4 polynucleotide kinase (New England BioLabs) and crude [γ-^{32}P]ATP (Du Pont, NEN Research Products). The probes were then purified by electrophoresis on a 6% nondenaturing acrylamide gel and eluted by using a UEA electroeluter (International Biotechnologies, Inc.). Hybridizations were done at 45 to 48°C for 3 to 4 h. Samples were then incubated with 200 U of S1 nuclease (Boehringer Mannheim) for 30 min at 37°C; this was followed by ethanol precipitation. After precipitation, the samples were electrophoresed on a 5% acrylamide-bisacrylamide (19:1)–8 M urea sequencing gel and visualized by autoradiography.

Nucleotide sequence accession number. The nucleotide sequence of a 5,700-bp region of *Pseudomonas* species strain LB400 DNA containing the genes for biphenyl dioxygenase has been deposited in GenBank under the accession number M86348.

RESULTS

Nucleotide sequencing and sequence analysis of biphenyl dioxygenase. The nucleotide sequence of a 5,700-bp region of DNA encoding the LB400 biphenyl dioxygenase was determined. Computer analysis identified six complete open reading frames (ORFs), whose nucleotide and predicted amino acid sequences are shown in Fig. 1. The initiation codon of each ORF was preceded by a putative ribosome-binding site, suggesting that translation of these regions was possible. The TGA stop and ATG start codons for ORFs 5 and 6 overlap, which may indicate that these regions are translationally coupled. Similar arrangements have been observed for other multicomponent catabolic enzymes such as benzoate 1,2-dioxygenase from *Acinetobacter calcoaceticus* (21) and the toluene dioxygenase from *Pseudomonas putida* F1 (34).

A partial ORF preceded by a ribosome-binding site is located 43 bp downstream of the termination codon of ORF 6. The partial nucleotide sequence of this region is virtually identical to that of the *bphB* gene (encoding biphenyl 2,3-dihydrodiol dehydrogenase) from *Pseudomonas pseudoalcaligenes* KF707 (8). These data suggest that the 3′ end of the biphenyl dioxygenase sequence is complete and that the sequence presented extends into the LB400 gene for dihydrodiol dehydrogenase. In addition, a sequence fragment from the biphenyl dioxygenase region of KF707 (9) matches the beginning of ORF 2 of LB400, with 58 of 60 nucleotides and 19 of 20 amino acids identical.

The GenBank and EMBL sequence data banks were searched for sequences similar to those of the biphenyl dioxygenase ORFs. This search identified the sequences for toluene dioxygenase from *P. putida* F1 (34) and benzene dioxygenase from a *P. putida* isolate (14). The reported toluene and benzene dioxygenase sequences are essentially identical, with the exception of several frameshifts that may be the result of sequencing errors. Four of the ORFs from the biphenyl dioxygenase region showed striking similarity to the genes encoding toluene dioxygenase subunits when the nucleotide and amino acid sequences were compared. Characteristics of the genes and gene products and the percent identities of the amino acid and nucleotide comparisons of the biphenyl and toluene dioxygenase components are shown in Table 1. Toluene dioxygenase in *P. putida* F1 has a relatively broad substrate specificity and is able to oxidize a wide variety of aromatic hydrocarbons (11, 33). The enzyme has been well characterized and consists of a reductase, a ferredoxin, and a tetrameric iron-sulfur protein with two large and two small subunits ($\alpha_2\beta_2$) (27, 31). The coding regions of LB400 that correspond with the toluene dioxygenase components have been named *bphA*, *bphE*, *bphF*, and *bphG*. On the strength of the sequence comparisons, we propose that *bphA* and *bphE* are homologous to *todC1* and *todC2* and encode the large and small subunits of the iron-sulfur component of biphenyl dioxygenase, respectively. *bphF* encodes the ferredoxin and *bphG* encodes the reductase, and they are homologous to *todB* and *todA*, respectively. The amino acid identities vary from 65.5% for the large iron-sulfur subunits to 52.1% for the reductases. Most of the predicted characteristics for these two enzymes are very similar, except for the charge and pI of the large iron-sulfur protein subunits and the reductases.

The alignment and comparison of the *bph* and *tod* amino acid sequences are shown in Fig. 2. The amino acid identities are not distributed randomly throughout the sequence but show distinct clustering. For the BphA-TodC1 comparison, the first 35 residues at the amino terminus and several internal regions show little sequence conservation. In contrast, there are highly conserved regions at residues 100 to 128 (28 of 29), 212 to 246 (34 of 35), and 378 to 396 (19 of 19). The BphE-TodC2 comparison again shows poor sequence conservation in the amino terminus, while several clusters at residues 46 to 59 (12 of 14), 97 to 177 (18 of 21), and 132 to 158 (24 of 27) are more highly conserved. BphF and TodB show little sequence identity in the amino or carboxyl termini, but the middle of the protein from residues 31 to 78

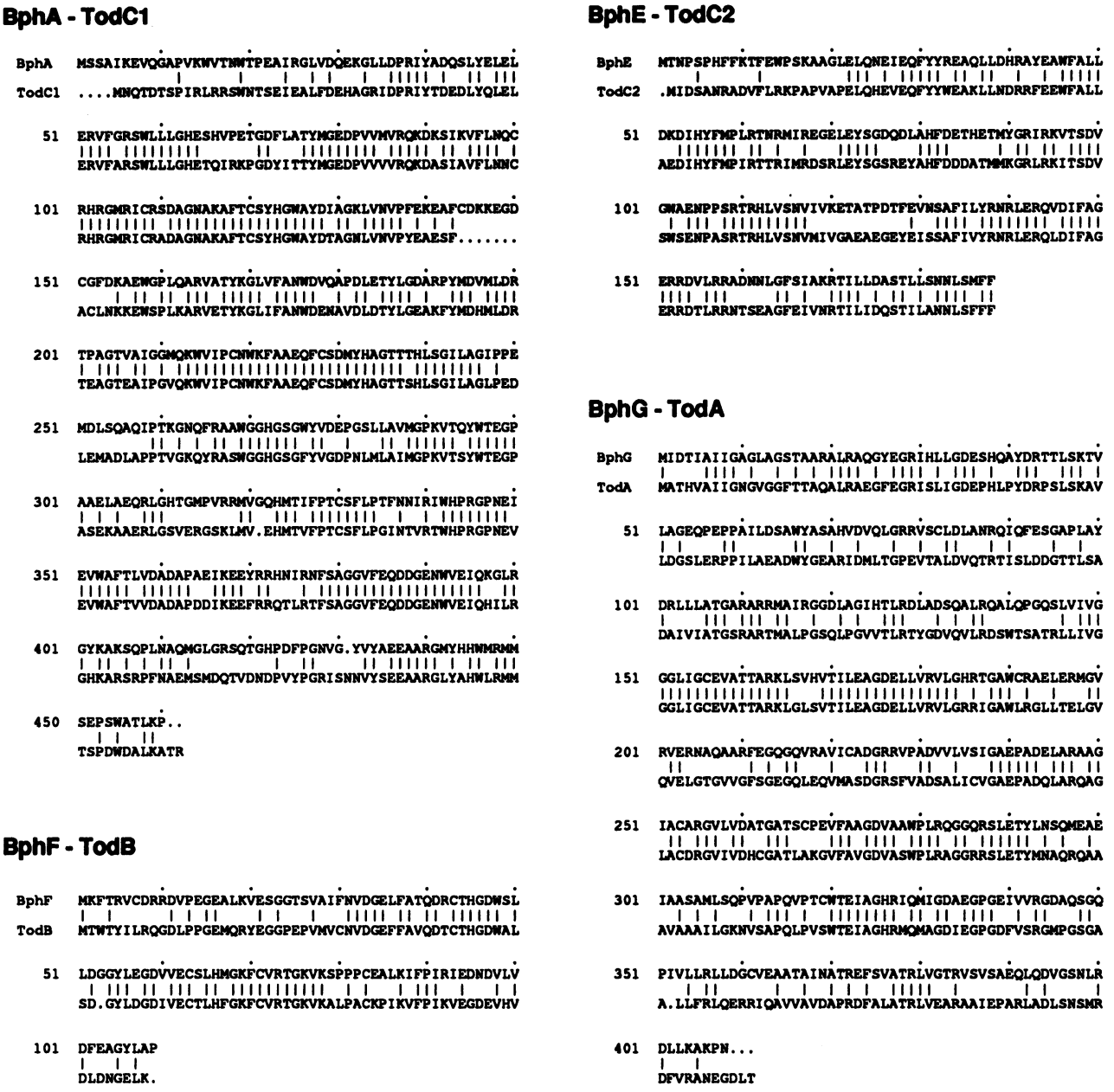

FIG. 2. Comparison of the proposed components of *Pseudomonas* species strain LB400 biphenyl dioxygenase with the components of toluene dioxygenase from *Pseudomonas putida* F1 (34). Program parameters were adjusted to suppress the labeling of similar amino acid residues. Identical residues are indicated by vertical bars.

has 38 of 48 (47 in TodB) residues conserved. Sequence conservation is lowest for BphG and TodA, and the amino acid identities are spread more evenly across the sequences. The most conserved region is from amino acids 148 to 184, with stretches of 18 and 16 identical residues separated by three nonidentical residues.

Figure 3 is a representation of the similarities in the biphenyl and toluene dioxygenase DNA regions, with the shaded areas connecting homologous coding areas. The arrangements of the genes encoding the subunits of both dioxygenases are identical except for the presence of *orf0* upstream of *bphA* and *orf1* between *bphE* and *bphF*. The nucleotide sequences of *orf0* and *orf1* do not correspond with other toluene dioxygenase sequences, although these regions are transcribed (see below). It is possible that one or both of these reading frames encode additional components of biphenyl dioxygenase, but this has yet to be demonstrated.

Transcription of biphenyl dioxygenase genes. S1 nuclease mapping studies were used to determine which ORFs were transcribed, to locate the RNA initiation sites, and to examine the effect of growth substrates on transcription. The RNA used in these experiments was extracted from LB400 cultures that had been grown on biphenyl or succinate as the

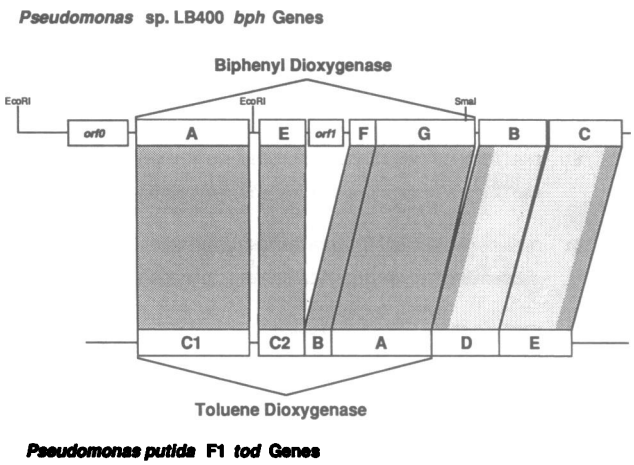

FIG. 3. Comparison of the DNA regions that contain the genes for biphenyl and toluene degradation. Open boxes indicate the genes and are labeled with the gene letter. Shaded regions connect homologous sequences. Dark shading represents comparisons of the *tod* sequences with LB400 *bph* sequences, while light shading represents comparisons with *P. pseudoalcaligenes* KF707 *bphBC*. Restriction sites relevant to the fragments for sequencing are labeled.

sole carbon source or on a medium containing both biphenyl and succinate.

DNA upstream of the *bphA* coding region was probed with a series of 5′-radiolabeled fragments. The highest-resolution results were obtained with *Hin*fI and *Msp*I fragments of 263 and 295 bp, respectively. These fragments overlap and cover the region from within the 5′ end of the *bphA* structural gene up to 150 to 250 bp upstream of the initiation codon. S1 nuclease mapping with the *Hin*fI probe produced several signals (Fig. 4). Two major clusters of shortened protected fragments of approximately 175 and 200 nucleotides were seen. This suggests the existence of two potential transcription start sites located approximately 45 bp (p1) and 70 bp (p2) upstream of the *bphA* initiation codon. In addition, full-length protection of the *Hin*fI probe was seen, indicating transcription coming from a third location (p3) further upstream. Experiments with the *Msp*I probe showed corresponding bands. The protected bands in the *Hin*fI experiment that are slightly shorter than full length are probably artifactual, as the corresponding bands in the *Msp*I experiment were barely visible.

Previous work indicated that PCB degradation by LB400 was enhanced when the cells were grown with biphenyl as the sole carbon source (19). This may be the result of increased transcription of the *bph* genes under these conditions. Transcription from p3 was significantly reduced in LB400 cells grown with succinate, as shown by the reduced intensity of the band representing full-length protection of the probe. No decrease was observed in the intensity of the bands corresponding to shortened protected fragments, demonstrating that these conditions did not significantly reduce transcription from the two sites proximal to the *bphA* start codon. RNA obtained from the culture grown on succinate and biphenyl gave an intermediate level of full-length signal (Fig. 4).

To determine the location of the transcription start site p3, we probed RNA preparations with radiolabeled *Dde*I and *Bgl*II-*Eco*RI fragments of 629 and 778 bp, respectively. An intense band corresponding to a protected fragment of approximately 600 nucleotides was obtained when the *Dde*I probe was hybridized with RNA extracted from biphenyl-grown LB400 cells. A much weaker band of the same size was observed with RNA from LB400 grown on succinate. Similar results were obtained with the *Bgl*II-*Eco*RI probe. The band intensity for a 260-nucleotide protected fragment was much greater when using RNA from biphenyl-grown versus succinate-grown cells. These data suggest that p3 is located approximately 95 bp upstream of the *orf0* initiation codon. Transcription from this promoter is induced (or derepressed) in the presence of biphenyl, since significant transcription from this site occurs only when LB400 is grown under these conditions.

In addition to the signal from p3, Fig. 4 shows apparent full-length protection of the *Dde*I and *Bgl*II-*Eco*RI probes, suggesting RNA transcripts originating outside of the sequenced region. The difference in relative intensity of the full-length and cut-down signals for the two probes suggests that this is an artifact of the S1 mapping technique. One such artifact, the formation of triplex structures (5), involves the rehybridization of the displaced probe strand to the RNA-DNA hybrid molecule, causing full-length protection. Increasing the hybridization temperature to 50°C greatly decreased this full-length protection. In addition, S1 mapping experiments with a 255-bp *Dde*I probe fragment located within the sequenced region upstream of the 629-bp *Dde*I fragment showed no protection of the probe (data not shown). This indicates that there is no transcription coming into the *bph* region from upstream of the sequenced DNA.

S1 nuclease mapping with 5′-labeled probes identifies the 5′ ends of RNA molecules. These ends generally are the result of transcription initiation but can arise from posttranscriptional processing. To confirm that the RNA 5′ ends at p1 and p2 (*bphA* proximal) were due to promoter activity, we cloned the 263-bp *Hin*fI fragment and the 295-bp *Msp*I fragment into a promoter-probe vector. The vector used was a modified pKO1 plasmid (17) which contains the promoterless *galK* gene encoding galactokinase. Both fragments showed promoter activity when cloned into the promoter-probe vector by complementing a *galK* mutation in the *Escherichia coli* host (data not shown). To confirm that the fragments have promoter activity in *Pseudomonas* species, they will be cloned into broad-host-range promoter probe vectors and tested in strain LB400.

DISCUSSION

The nucleotide sequence of the DNA region encoding the biphenyl dioxygenase of *Pseudomonas* species strain LB400 was determined. Six ORFs were identified, four of which were very similar to those encoding the components of toluene dioxygenase of *P. putida* F1. The corresponding *bph* and *tod* subunits showed amino acid identities ranging from 52.1 to 65.5% and nucleotide identities ranging from 57.3 to 67.6%. These data suggest that, like toluene dioxygenase, the LB400 biphenyl-PCB dioxygenase is a multicomponent enzyme made up of at least four different proteins. Based on the sequence comparison, the genes for these proteins have been designated *bphA* and *bphE* (large and small subunits of the iron-sulfur protein, respectively), *bphF* (ferredoxin), and *bphG* (reductase).

The enzymatic pathway for toluene and benzene degradation in *P. putida* F1 is similar to that for biphenyl and PCBs in LB400 (Fig. 5). Additional similarities in other *bph* and *tod* genes suggest that these degradation pathways are evolutionarily related. Dihydrodiol dehydrogenase and catechol

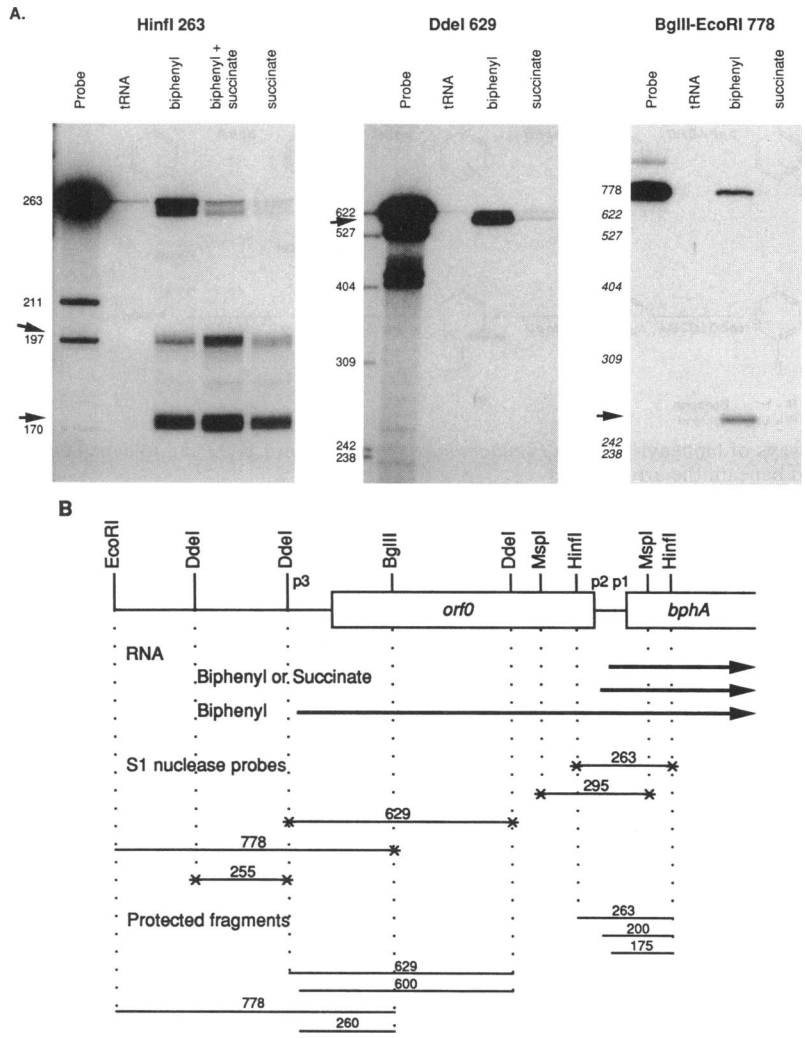

FIG. 4. S1 nuclease mapping of the transcription start sites in the *bph* region of LB400. (A) Autoradiograms of S1 nuclease reactions. The probe used in each reaction is listed at the top of each frame. Probe and tRNA control lanes are labeled appropriately. The remaining lanes are experimental reactions and are identified by the carbon source used to grow the LB400 prior to the RNA preparation. Protected fragments representing transcription start sites are indicated by arrows. Size marker positions are labeled (in base pairs). (B) Schematic diagram of the S1 mapping experiments. The locations of promoters and relevant restriction sites are indicated above the diagram of the DNA region. The arrows represent RNA transcripts and are identified with the carbon source(s) present when the transcripts are visible. DNA fragments used as S1 nuclease probes as described in the text or used in panel A are shown. Asterisks represent the radiolabeled termini of the probe fragments. Bands protected from S1 nuclease digestion in panel A are indicated at the bottom.

dioxygenase in LB400 are encoded by the *bphB* and *bphC* genes. Portions of these genes have been sequenced (Fig. 1; data not shown) and found to be nearly identical to the *bphB* and *bphC* genes in *P. pseudoalcaligenes* KF707 (8). KF707 BphB and BphC show 58.1 and 54.6% amino acid identity with the corresponding TodD and TodE proteins. The structural homologies between *bph* and *tod* gene products are reflected in a limited functional similarity, as the toluene degradation enzymes are capable of degrading biphenyl and some mono- and dichlorobiphenyls to the corresponding dihydrodiols and catechols (11, 33). It has yet to be determined whether the biphenyl/PCB degradation pathway is active against toluene or chlorotoluenes.

Homologous proteins generally show sequence conservation in regions necessary for structure or function. Since toluene dioxygenase is a well-characterized enzyme, it may be possible to identify areas involved in substrate oxidation. Several regions with likely structure-function roles are shown in Fig. 6. Recently, Neidle et al. (21) have reported the conservation of two cysteine-histidine pairs in TodC1 and the large iron-sulfur protein subunits from a variety of other multicomponent dioxygenases. These cysteine-histidine pairs are also conserved in the BphA subunit and may bind a [2Fe-2S] center similar to the Rieske iron-sulfur proteins (12, 21, 23). A similar cysteine-histidine arrangement, conserved in the ferredoxin components of TodB, NdoA, and the proposed ferredoxin component of toluene-4-monooxygenase, TmoC (32), was also found in BphF.

The reductase component of toluene dioxygenase is a flavoprotein whose function is to catalyze the transfer of electrons from NADH to the ferredoxin. The binding of NAD and FAD involves protein sequences capable of folding into a βαβ structure. Wierenga et al. (29) have identified an 11-amino-acid fingerprint sequence for regions forming

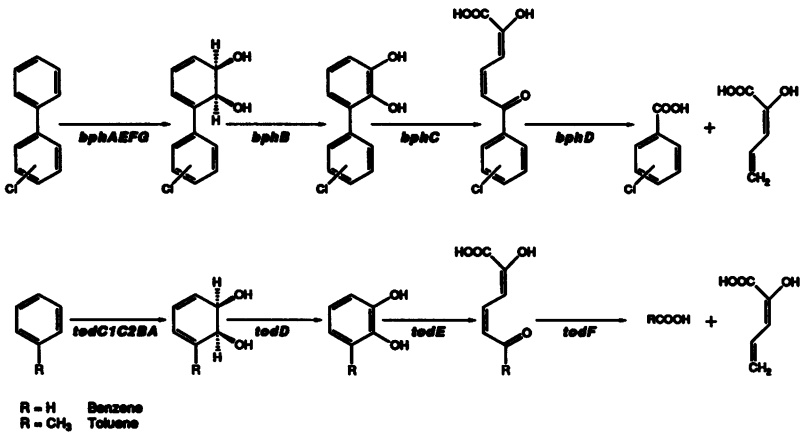

FIG. 5. Degradation pathways of biphenyl-PCBs in *Pseudomonas* species strain LB400 and toluene-benzene in *P. putida* F1. The gene names for each step are listed beneath the arrows.

these structures, and Hanukoglu and Gutfinger (13) have aligned multiple sequences to a proposed consensus sequence for NAD-FAD binding. The reductase subunits of biphenyl and toluene dioxygenase (BphG and TodA, respectively), as well as putidaredoxin reductase (CamA) (22), each contain two regions matching the consensus sequence (Fig. 6). These may represent the site of interaction of both the flavin moiety and the NADH electron donor.

Iron-Sulfur Protein Large Subunits

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BphA(90-125)   DKSIKVFLNQ C R H RGMRICRSDAGNAKAFT C SY H GW
TodC1(86-121)  DASIAVFLNN C R H RGMRICRADAGNAKAFT C SY H GW
NdoB(71-106)   DGSIRAFLNV C R H RGKTLVSVEAGNAKGFV C SY H GW
BenA(85-120)   NGELNAMINA C S H RGAQLLGHKRGNKTTYT C PF H GW
XylX(80-115)   DGELNAFVNA C S H RGATLCRFRSGNKATHT C SF H GW
Consensus      --------N- C - H RG--------GN----- C -- H GW
```

Ferredoxins

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BphF(32-76)    VDGELFATQDR C T H GDWSLLDGGYLEGDVVE C SL H MGKFCVRTGK
TodB(32-75)    VDGEFFAVQDT C T H GDWALSD.GYLDGDIVE C TL H FGKFCVRTGK
NdoA(34-77)    VEGEIYATDNL C T H G.SARMSDGYLEGREIE C PL H QGRFDVCTGK
Consensus      V-GE--A----- C T H G--------GYL-G---E C -L H -G-F-V-TGK
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Reductases

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                ****  *   *  * *    *   *     * *
BphG(1-40)     MIDTIAII G A G LA G STA A RALRAQGYEGRIHLLGDESHQA
BphG(142-181)  PGQSLVIV G G G LI G CEV A TTARKLSVHVTILEAGDELLVR
TodA(1-40)     MATHVAII G N G VG G FTT A QALRAEGFEGRISLIGDEPHLP
TodA(142-181)  SATRLLIV G G G LI G CEV A TTARKLGLSVTILEAGDELLVR
CamA(3-42)     ANDNVVIV G T G LA G VEV A FGLRASGWEGNIRLVGDATVIP
CamA(148-187)  ADNRLVVI G G G YI G LEV A ATAIKANMHVTLLDTAARVLER
NAD/FAD Consens. +•••• G   G  • G •   A +••± G ±  ••
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FIG. 6. Comparison of specific regions of multicomponent dioxygenase subunits. The sequences are separated by dioxygenase component and identified by name and amino acid range shown. In the Iron-Sulfur Protein and Ferredoxins sections, highly conserved C and H residues thought to be involved in the interaction of the peptide chain with the [2Fe-2S] center are boxed. The lines labeled Consensus show the residues conserved in all the sequences listed. In the Reductases section, the NAD-FAD consensus binding sequence (13) is given with the conserved amino acid residues indicated as follows: +, positively charged residue; •, hydrophobic residue; ±, charged residue. The most highly conserved residues in the known NAD-FAD-binding proteins are boxed. Asterisks indicate locations where both ranges of the BphG sequence match the consensus sequence. The proteins used for the comparisons are BphA, BphF, and BphG (this study); TodC1, TodB, and TodA (34); NdoB and NdoA (15); BenA and XylX (21); and CamA (22).

S1 nuclease mapping was used to identify transcription start sites in the region encoding biphenyl dioxygenase. Three such sites were identified, suggesting that biphenyl dioxygenase is transcribed from multiple promoters. Two of the sites, p1 and p2, are located approximately 45 and 70 nucleotides upstream of the *bphA* start codon, respectively. The third 5′ RNA end maps to approximately 95 bp upstream of the *orf0* start codon at a site designated p3. While none of these sites strongly match the consensus sequences for *E. coli* promoters, the p1-p2 region shows promoter activity in *E. coli*.

It has previously been reported that PCB degradation by strain LB400 is greatest when the cells are grown with biphenyl as the sole source of carbon and energy. Growth of LB400 on succinate or a combination of succinate and biphenyl reduces PCB-degrading ability in general and completely eliminates activity against double-*para*-substituted congeners (19). Our data indicate that transcription site p3 is much more active in biphenyl-grown cells than in those grown on succinate. The increased transcription of the *bph* genes from this site may account for the greater PCB-degrading ability of biphenyl-grown cells. Another possibility is that in the absence of biphenyl, little or no *orf0* gene product is formed because of a lack of transcription from p3. This product may affect the activity or congener specificity of biphenyl dioxygenase, allowing the enzyme to attack double-*para*-substituted PCBs.

We have no clear indication of a specific function for the *orf1* gene product, although the location of this gene within the *bph* cluster suggests that it is involved in PCB degradation. This is also supported by evidence that *orf1* is present in the *bph* cluster of *A. eutrophus* H850, an organism which is similar to LB400 in PCB-degrading ability. Previous studies with DNA-DNA hybridization showed a strong conservation of restriction sites (16 of 16) between the *bph* regions of LB400 and H850 (30). One of these corresponds to a *Pst*I site located within *orf1*, showing that this region has been retained by both strains and may therefore have a function. Future studies will be directed toward examining the roles of Orf0 and Orf1 in the degradation of PCBs.

DNA sequence analysis clearly shows that the LB400 biphenyl dioxygenase is one of a family of homologous multicomponent oxygenases. This family includes systems

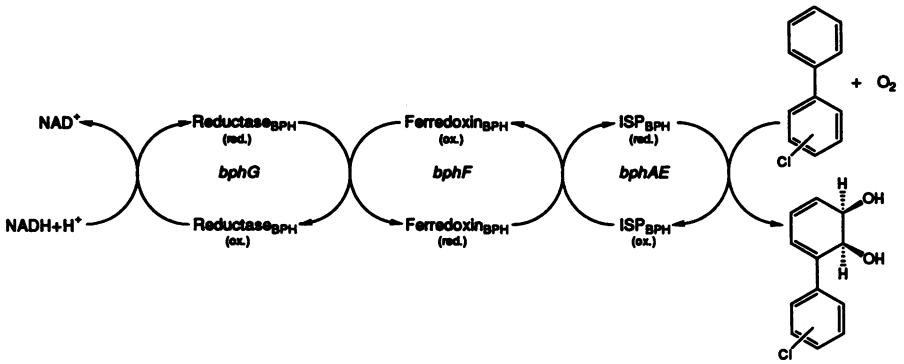

FIG. 7. Proposed electron transport chain for the LB400 biphenyl dioxygenase. The enzyme components in the chain are indicated, as are the genes encoding each protein. The redox state of each component is labeled (ox., oxidized; red., reduced).

with three components such as toluene, benzene, and naphthalene dioxygenase and those with two components such as the benzoate and toluate 1,2-dioxygenases. In the three-component systems, reducing equivalents are transported from NAD(P)H through a reductase and a ferredoxin to the terminal oxygenase, while in two-component enzymes, the reductase and ferredoxin activities are contained within a single polypeptide. It is apparent from the sequence comparisons that biphenyl dioxygenase is a three-component enzyme system, and we propose that its electron transport proceeds as shown in Fig. 7. The high degree of sequence identity between the genes involved in biphenyl and toluene degradation shows that these pathways are closely related and illustrates how bacteria are developing systems capable of degrading xenobiotic compounds such as PCBs through modification of existing degradative pathways.

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REFERENCES

1. **Ahmed, M., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of Achromobacter. Can. J. Microbiol. **19**:47–52.
2. **Barry, G., C. Squires, and C. L. Squires.** 1980. Attenuation and processing of RNA from the *rplJL-rpoBC* transcription unit of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:3331–3335.
3. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51**:761–768.
4. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1**:23–29.
5. **Burton, Z. F., C. A. Gross, K. K. Watanabe, and R. R. Burgess.** 1983. The operon that encodes the sigma subunit of RNA polymerase also encodes ribosomal protein S21 and DNA primase in *E. coli* K12. Cell **32**:335–349.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
7. **Furukawa, K.** 1982. Microbial degradation of polychlorinated biphenyls, p. 33–57. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press, Inc., Boca Raton, Fla.
8. **Furukawa, K., N. Arimura, and T. Miyazaki.** 1989. Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **169**:427–429.
9. **Furukawa, K., N. Hayase, and K. Taira.** 1990. Biphenyl/polychlorinated biphenyl catabolic gene (*bph* operon): organization, function, and molecular relationships in various pseudomonads, p. 111–120. *In* A. M. Chakrabarty, B. Iglewski, S. Kaplan, and S. Silver (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
10. **Furukawa, K., F. Matsumura, and K. Tonomura.** 1978. *Alcaligenes* and *Acinetobacter* strains capable of degrading polychlorinated biphenyls. Agric. Biol. Chem. **42**:543–548.
11. **Gibson, D. T., G. J. Zylstra, and S. Chauhan.** 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1, p. 121–132. *In* A. M. Chakrabarty, B. Iglewski, S. Kaplan, and S. Silver (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
12. **Gurbiel, R. J., C. J. Batie, M. Sivaraja, A. E. True, J. A. Fee, B. M. Hoffman, and D. P. Ballou.** 1989. Electron-nuclear double resonance spectroscopy of ^{15}N-enriched phthalate dioxygenase from *Pseudomonas cepacia* proves that two histidines are coordinated to the [2Fe-2S] Rieske-type clusters. Biochemistry **28**:4861–4871.
13. **Hanukoglu, I., and T. Gutfinger.** 1989. cDNA sequence of adrenodoxin reductase. Identification of NADP-binding sites in oxidoreductases. Eur. J. Biochem. **180**:479–484.
14. **Irie, S., S. Doi, T. Yorifugi, M. Takagi, and K. Yano.** 1987. Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*. J. Bacteriol. **169**:5174–5179.
15. **Kurkela, S., H. Lehvaeslaiho, E. T. Palva, and T. H. Teeri.** 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. Gene **73**:355–362.
16. **Masse, R., F. Messier, L. Peloquin, C. Ayotte, and M. Sylvestre.** 1984. Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl. Environ. Microbiol. **47**:947–951.
17. **McKenney, K., H. Shimatake, D. Court, U. Schmeissner, and M. Rosenberg.** 1981. A system to study promoter and terminator signals recognized by *Escherichia coli* RNA polymerase, p. 383–415. *In* J. Chirikjan and T. Papas (ed.), Gene amplification and analysis, vol. II. Elsevier/North-Holland Publishing Co., New York.
18. **Miller, R. V., and C.-M. C. Ku.** 1978. Characterization of *Pseudomonas aeruginosa* mutants deficient in the establishment of lysogeny. J. Bacteriol. **134**:875–883.
19. **Mondello, F. J.** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171**:1725–1731.

20. **Nadim, L., M. J. Schocken, F. J. Higson, D. T. Gibson, D. L. Bedard, L. H. Bopp, and F. J. Mondello.** 1987. Bacterial oxidation of polychlorinated biphenyls, p. 395–402. *In* Proceedings of the 13th Annual Research Symposium on Land Disposal, Remedial Action, Incineration, and Treatment of Hazardous Waste. EPA/600/9-87/015. U.S. Environmental Protection Agency, Cincinnati, Ohio.
21. **Neidle, E. L., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik, and S. Harayama.** 1991. Nucleotide sequences of the *Acinetobacter calcoaceticus benABC* genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. J. Bacteriol. **173**:5385–5395.
22. **Peterson, J. A., M. C. Lorence, and B. Amarneh.** 1990. Putidaredoxin reductase and putidaredoxin. J. Biol. Chem. **265**:6066–6073.
23. **Rieske, J. S., D. H. MacLennan, and R. Coleman.** 1964. Isolation and properties of an iron-sulfur protein from the (reduced coenzyme Q)-cytochrome C reductase complex of the respiratory chain. Biochem. Biophys. Res. Commun. **15**:338–344.
24. **Rochkind, M. L., J. W. Blackburn, and G. W. Sayler.** 1986. Chlorinated biphenyls, p. 129–137. *In* Microbial decomposition of chlorinated aromatic compounds. EPA/600/2-86/090. U.S. Environmental Protection Agency, Cincinnati, Ohio.
25. **Salser, W., R. F. Gestland, and A. Bolle.** 1967. *In vitro* synthesis of bacteriophage lysozyme. Nature (London) **215**:588–591.
26. **Shields, M. S., S. W. Hooper, and G. S. Sayler.** 1985. Plasmid-mediated mineralization of 4-chlorobiphenyl. J. Bacteriol. **163**: 882–889.
27. **Subramanian, V., T.-N. Liu, W.-K. Yeh, and D. T. Gibson.** 1979. Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography. Biochem. Biophys. Res. Commun. **91**:1131–1139.
28. **Sundstrom, G., and O. Hutzinger.** 1976. The metabolism of chlorobiphenyls—a review. Chemosphere **5**:267–298.
29. **Wierenga, R. K., P. Terpstra, and W. G. J. Hol.** 1986. Prediction of the occurrence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. **187**:101–107.
30. **Yates, J. R., and F. J. Mondello.** 1989. Sequence similarities in the genes encoding polychlorinated biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H850. J. Bacteriol. **171**:1733–1735.
31. **Yeh, W.-K., D. T. Gibson, and T.-N. Liu.** 1977. Toluene dioxygenase: a multicomponent enzyme system. Biochem. Biophys. Res. Commun. **78**:401–410.
32. **Yen, K.-M., M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen.** 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173**:5315–5327.
33. **Zylstra, G. J., S. Chauhan, and D. T. Gibson.** 1990. Degradation of chlorinated biphenyls by *Escherichia coli* containing cloned genes of the *Pseudomonas putida* F1 toluene catabolic pathway, p. 290–302. *In* Proceedings of the Sixteenth Annual Hazardous Waste Research Symposium. Remedial action, treatment, and disposal of hazardous waste. EPA/600/9-90 037. U.S. Environmental Protection Agency, Cincinnati, Ohio.
34. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264**:14940–14948.