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Cloning and Nucleotide Sequence of the 2,3-Dihydroxybiphenyl Dioxygenase Gene from the PCB-Degrading Strain of *Pseudomonas paucimobilis* Q1†

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ABSTRACT: The *bphC* gene encoding 2,3-dihydroxybiphenyl dioxygenase was cloned from biphenyl-degrading and chlorinated biphenyl-degrading *Pseudomonas paucimobilis* Q1, and its complete nucleotide sequence was determined. The DNA-derived protein sequence provides the primary structure of 298 amino acids. Polyclonal antibodies raised against this protein from *P. paucimobilis* Q1 failed to cross-react with the previously isolated 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 [Furukawa, K., & Arimura, N. (1987) *J. Bacteriol. 169*, 924-927. Furukawa, K., Arimura, N., & Miyazaki, T. (1987) *J. Bacteriol. 169*, 427-429], despite the close similarities of these proteins in terms of their native as well as subunit molecular weights, cofactor, and enzymatic activities. The sequence homology of the 2,3-dihydroxybiphenyl dioxygenase from the two different sources is examined.

2,3-Dihydroxybiphenyl dioxygenase (23OHBPO)[1] catalyzes the oxidative ring cleavage of 2,3-dihydroxybiphenyl to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (Figure 1). This is one of the key reactions in the metabolism of the widespread pollutant polychlorinated biphenyls (PCBs). Although the metabolic importance of PCB-degrading microorganisms has inspired extensive research on such organisms over the past decade, only quite recently have these systems become amenable to molecular studies (Furukawa, 1982, 1986).

We have recently cloned a gene cluster (*bphA*, *bphB*, *bphC*) encoding PCB-degrading enzymes from *Pseudomonas pseudoalcaligenes* KF707, and a major catabolic pathway of PCBs has been proposed as in Figure 1 (Furukawa & Miyazaki, 1986). In its first catabolic mechanistic stage a molecular oxygen is introduced at the 2,3-position of the nonchlorinated or less chlorinated ring to produce a dihydrodiol (compound II in Figure 1) by the action of a biphenyl dioxygenase

† This paper is dedicated to Dr. Akio Sato, the former Director General of FRI, for his 60th birthday as well as for his contribution to FRI until his retirement on Dec 1, 1987.

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[1] Abbreviations: 23OHBPO, 2,3-dihydroxybiphenyl dioxygenase; 34OHBP, 3,4-dihydroxybiphenyl; *bphC*, a gene that encodes 23OHBPO; Q1, *Pseudomonas paucimobilis* Q1; KF707, *Pseudomonas pseudoalcaligenes* KF707; 23OHBPO(Q1), 23OHBPO of Q1 origin; *bphC*-(KF707), *bphC* of KF707 origin; PCB, polychlorinated biphenyl; BP, biphenyl; kb, kilobase; SDS, sodium dodecyl sulfate; SD, Shine-Dalgarno sequence; Km, kanamycin; Sm, streptomycin; DEAE, diethylaminoethyl.

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(product of gene *bphA*). The dihydrodiol is then dehydrogenated to a 2,3-dihydroxybiphenyl (III) by a dihydrodiol dehydrogenase (product of gene *bphB*). The 2,3-dihydroxybiphenyl is then cleaved at the 1,2-position by the 23OHBPO (product of gene *bphC*) to yield IV, which is eventually hydrolyzed to the corresponding chlorobenzoic acid, V, by a hydrolase (product of gene *bphD*). The *bph* operon consists of at least three genes, *bphA*, *bphB*, and *bphC*, in the case of *P. pseudoalcaligenes* KF707.

The *bphC* gene product, 23OHBPO, from either *P. pseudoalcaligenes* KF707 or *P. paucimobilis* Q1, is colorless and contains the ferrous form of iron as the sole cofactor, which is the typical iron state for the extradiol-cleaving enzymes (Nozaki, 1979). These enzymes possess many features in common such as molecular weight (ca. 260 000), subunit structure ($[\alpha Fe(II)]_8$), and substrate specificity. They are specific for only 2,3-dihydroxybiphenyl; thus, its isomer, 3,4-dihydroxybiphenyl, is resistant to the enzymatic degradation. Despite these close similarities, the 23OHBPO isolated from the Q1 cells is immunologically most different from the corresponding 23OHBPO obtained from the KF707 cells. Among over 20 23OHBPOs, obtained from various strains and immunologically tested by utilizing antibodies raised against 23OHBPO of the KF707 origin, the enzyme from Q1 belonged to one of the few cases where no cross-reaction could be detected. Pertinent to this finding is the observation that a *bphC* DNA probe of the KF707 origin failed to function during a search for the corresponding *bphC*(Q1) gene. All of those 23OHBPOs that exhibited immunological similarities were from strains isolated from various places in Japan. The unique *bphC*(Q1) was the only gene from a strain isolated outside Japan.

Thus, in order to find the exact difference between these immologically antagonistic *bphC*s at the level of DNA as well as amino acids, we have cloned the *bphC*(Q1) gene, and the complete nucleotide sequence is reported here.

Materials and Methods

Bacterial Strains, Plasmids, and Cloning of bphC(Q1). The *P. paucimobilis* strain Q1 was isolated from soil in Chicago, IL, by enrichment culture with biphenyl (BP) as the sole source of carbon and energy (Furukawa et al., 1983). The plasmid vector pKF330 [12.6 kilobases (kb)] was derived from pKT230, which had been obtained from K. Timmis at University of Geneva, Switzerland (Bagdasarian & Timmis, 1982), but it contained an additional small *Pst*I fragment (0.7 kb) derived from RSF1010. Chromosomal DNA from strain Q1 was prepared essentially as described (Marmur, 1961). The plasmid vector pKF330 as well as the chromosomal DNA (Q1) were subjected to *Xho*I digestion followed by ligation of those *Xho*I fragments by utilizing T4 DNA ligase. *P. aeruginosa* PAO1161 was then transformed according to the procedure of Bagdasarian and Timmis (1982). Transformants were selected on basal salts agar medium containing succinate (1 g/L) and streptomycin (300 μg/mL). Clones expressing *bphC* genes were identified by spraying colonies with 2,3-dihydroxybiphenyl (III in Figure 1) solution (1 g/L). Only those colonies possessing the *bphC* gene turned yellow, resulting from the conversion of III to the yellow meta-cleavage compound IV (Furukawa et al., 1979).

Subcloning and Sequencing of the bphC(Q1). The plasmid pMFQ1 (15.2 kb), in which the 2.6-kb *Xho*I fragment carrying the *bphC*(Q1) gene had been inserted into pKF330 (Figure 2), was digested by *Xho*I, and the resulting *bphC*(Q1) fragment (2.6 kb) was subcloned into the *Xho*I site of pHSG396 (2.24 kb; Takara Shuzo, Kyoto), yielding pAF396. The

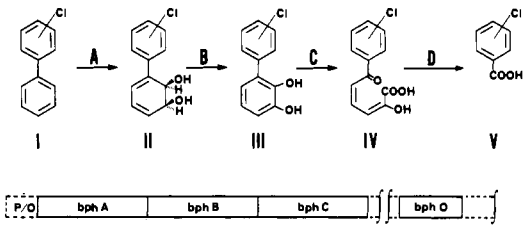

FIGURE 1: Catabolic pathway for degradation of biphenyl and chlorinated biphenyls. The *bph* operon in *P. pseudoalcaligenes* KF707 consists of at least three genes (*bphA*, *bphB*, and *bphC*).

plasmid pAF396 was digested by both *Kpn*I and *Xba*I followed by a treatment with exonuclease III so that depolymerization from the 3′-terminus of the *Xba*I site initiated toward the direction of the *bphC*(Q1) insert. The reaction was then stopped at an interval of ca. 1 min by heating a sample mixture at 65 °C for 5 min, and the samples were treated with mung bean nuclease, followed by Klenow fragment with appropriate components necessary for polymerization. The resulting blunt-end DNAs were ligated, treated with *Xba*I to remove any unreacted pAF396, and transformed into *Escherichia coli* JM83 (Yanisch-Perron et al., 1985) to produce various deletion mutants.

The chain-termination procedure of Sanger et al. (1977) was used for sequencing the above-mentioned deletion mutants, employing an M13 primer, d(GTTTTCCCAGTCACGAC). Once the sequence of one strand had been established, that of the opposite strand was determined by employing several small synthetic primers (17-mers prepared by DNA synthesizer; Applied Biosystems Model 380B) designed on the basis of the predetermined sequence. Thus, the nucleotide sequence was determined on both strands of the 1-kb DNA fragment containing the *bphC*(Q1) gene.

All the restriction enzymes utilized in this work were purchased from Takara Shuzo Co., Kyoto.

Enzymatic and Immunological Studies. Purification of 23OHBPO from strain Q1 was performed according to the procedure described earlier (Furukawa & Arimura, 1987b). Polyacrylamide gel electrophoresis of the native enzyme was done with polyacrylamide gradient gel PAA 4/30 (Pharmacia, Uppsala, Sweden). Polyacrylamide gel electrophoresis of the denatured enzyme, treated with 2.5% sodium dodecyl sulfate (SDS) followed by heat treatment (5 min at 95 °C), was also performed. The amino acid sequence of the NH_2-terminal portion of the enzyme was analyzed by an automated protein sequencer (Applied Biosystems, Foster City, CA, Model 470A). The extinction coefficient at 434 nm of the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (IV) at pH 9.0 was determined experimentally to be 22 000 M^{-1} cm^{-1}. This value was used for the calculation of the enzyme activity at 25 °C in 50 mM phosphate buffer. The enzymatic reaction product (IV) was confirmed by GC–MS analysis on trimethylsilyl derivatives. The purified 23OHBP dioxygenases encoded by KF707 and Q1 were tested with rabbit antisera prepared against these purified enzymes.

Results

Cloning of the bphC Gene. Two streptomycin-resistant colonies were found, among ca. 12 000 candidates, that immediately turned yellow upon spraying with 2,3-dihydroxybiphenyl (III in Figure 1) solution, indicating the conversion of III to the meta-cleavage compound 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (IV). These clones harbored the same hybrid plasmid, pMFQ1 (15.2 kb), that contained a 2.6-kb DNA insert in the unique *Xho*I site of pKF330 (Figure 2). Southern blot experiments confirmed that the 2.6-kb *Xho*I

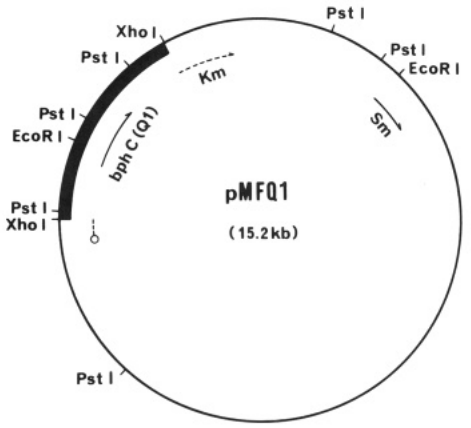

FIGURE 2: Schematic presentation of plasmid pMFQ1 carrying the *bphC* gene. The thick line (2.6 kb) contains the cloned *bphC*(Q1) gene derived from *P. paucimobilis* Q1 chromosomal DNA and inserted slightly downstream of the kanamycin promoter (small circle). The thin line represents the pKF330 vector (12.6 kb) possessing a streptomycin-resistant determinant. The arrow inside the *bphC* region indicates the direction of transcription.

fragment was derived from Q1 genomic DNA; that is, the Q1 strain harbors three plasmids, however, the cloned 2.6-kb *Xho*I fragment did not hybridize to *Xho*I digests of these three plasmids, confirming that the 2.6-kb *Xho*I fragment originated from the chromosome of strain Q1.

Immunological and Enzymatic Studies with 2,3-Dihydroxybiphenyl Dioxygenase (23OHBPO). Shown in Figure 3 are the results of immunological cross-reactivity of rabbit antisera of which one is prepared against the purified 23OHBPO(Q1) and the other against the counterpart of KF707 origin. As evidenced from the precipitation patterns, both antibodies against the Q1 enzyme [a-Q1; II-immu, antiserum taken 2 weeks after injection of 23OHBPO(Q1), and III-immu, taken 3 weeks after injection] cross-reacted with the 23OHBPO(Q1). Similarly, the 23OHBPO(KF707) and its antisera are immunologically cross-reactive. In the figure "preimmu" denotes serum, which was collected as a control

Table I: Enzymatic Properties of *bph*C Gene Products in Strains KF707 and Q1

	23OHBPO (KF707)	23OHBPO(Q1)
induction	BP	BP
mol wt	260 000	260 000
subunit mol wt	33 000	33 000
cofactor	Fe(II)	Fe(II)
structure	$[\alpha Fe(II)]_8$	$[\alpha Fe(II)]_8$
V_{max} (s^{-1})		
subst		
23OHBP	330	150
34OHBP	<1	<1
catechol	~3	33

before the injection of each 23OHBPO, and as expected it did not cross-react with either enzyme.

More importantly, the antibodies (a-Q1) raised against the Q1 enzyme did not cross-react with the 23OHBPO from the KF707, and antibodies (a-707) against the 707 enzyme also failed to cross-react with the Q1 enzyme. Thus, the 23OHBPO(Q1) is immunologically far from the 23OHBPO-(707) despite their close similarities in many other aspects as described below.

Shown in Table I are some of the enzymatic properties of these 23OHBPOs. The enzyme activity was completely lost upon Sepharose CL gel filtration. The preparation could be reactivated by incubation with ferrous iron in the presence of cysteine as described previously for catechol 2,3-dioxygenase (Nakai et al., 1983a,b). The maximum activity was gained when ferrous iron concentration was maintained at 10^{-6} M. Addition of ferric iron (10^{-5} M final concentration) readily inactivated the enzyme.

The molecular weights of native 23OHBPOs from Q1 and KF707 were determined by gradient polyacrylamide gel electrophoresis on the basis of relative mobility with respect to standard proteins (Furukawa & Arimura, 1987b). Both enzymes showed a molecular weight of about 260 000. Polyacrylamide gel electrophoresis of the enzymes, treated with 2.5% SDS followed by a heat treatment (95 °C for 5 min), produced a single band, and the molecular weight was esti-

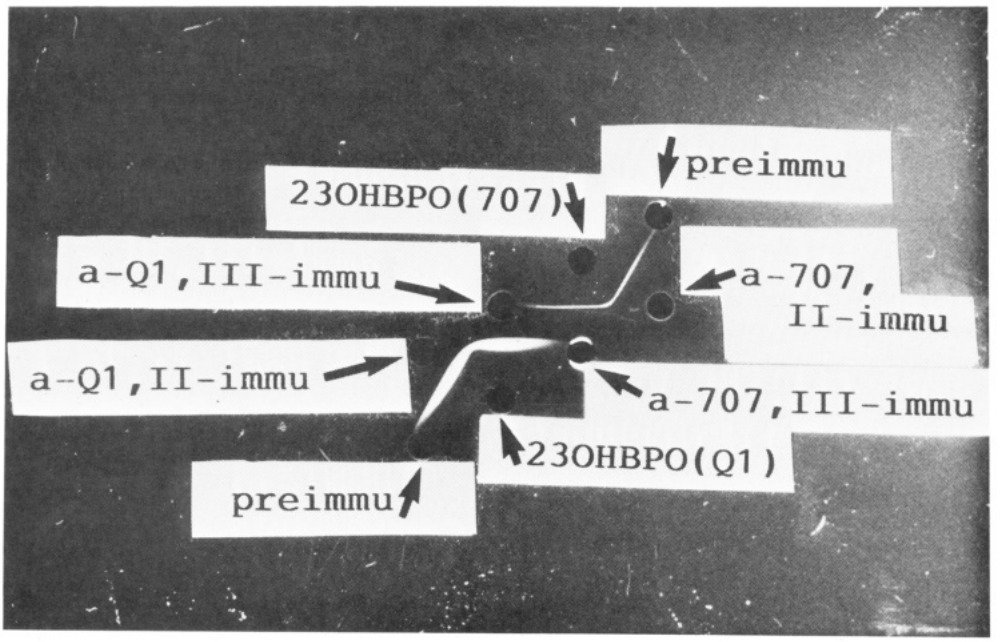

FIGURE 3: Immunoprecipitation pattern of purified 23OHBPOs from KF707 and Q1 with antibodies prepared against these enzymes. Wells containing the pure 23OHBPOs are indicated by 23OHBPO(707) and 23OHBPO(Q1) for the KF707 and Q1 enzymes, respectively. The control serum taken from a rabbit before the 23OHBPO was injected is indicated by "preimmu". Antisera taken 2 and 3 weeks after the 23OHBPO(Q1) injection are shown by "a-Q1, II-immu" and "a-Q1, III-immu", respectively. The "a-707, II-immu" and "a-707, III-immu", also represent antisera of 2 and 3 weeks raised against the KF707 enzyme.

Table II: Comparison of Codon Usage and Deduced Amino Acid Structures

	23OHBPO(Q1)	23OHBPO(KF707)	cat. oxyg		23OHBPO(Q1)	23OHBPO(KF707)	cat. oxyg
Gly	32	27	24	Thr	11	17	17
GGC	12	14	13	ACC	8	12	13
GGG	9	7	2	ACG	2	4	1
GGA	6	3	2	ACA	1	1	1
GGU	5	3	7	ACU	0	0	2
Ala	33	33	21	Asp	21	25	27
GCC	14	14	10	GAC	13	18	19
GCG	10	9	3	GAU	8	7	8
GCA	5	7	4	Glu	18	13	21
GCU	4	3	4	GAA	11	7	12
Val	19	27	21	GAG	7	6	9
GUG	9	12	13	Asn	8	3	9
GUC	6	6	4	AAC	7	2	6
GUU	2	7	2	AAU	1	1	3
GUA	2	2	2	Gln	10	9	8
Leu	30	26	34	CAG	9	6	7
CUG	13	15	18	CAA	1	3	1
CUC	11	4	8	Lys	8	10	14
UUG	3	7	5	AAG	8	4	10
CUU	3	0	1	AAA	0	6	4
CUA	0	0	2	Arg	17	18	19
UUA	0	0	0	CGC	12	10	10
Ile	11	10	8	CGG	4	2	3
AUC	6	7	6	CGU	1	2	4
AUU	4	3	2	CGA	0	0	2
AUA	1	0	0	AGA	0	2	0
Pro	14	12	12	AGG	0	2	0
CCG	6	8	8	His	13	11	15
CCC	5	0	3	CAC	6	8	10
CCU	2	3	0	CAU	7	3	5
CCA	1	1	1	Cys	3	2	3
Phe	11	18	16	UGC	3	2	1
UUC	10	10	12	UGU	0	0	2
UUU	1	8	4	Met			
Tyr	14	8	12	AUG	10	9	11
UAC	10	4	5	End			
UAU	4	4	7	UGA	1	0	1
Trp				UAG	0	1	0
UGG	6	5	5	UAA	0	0	0
Ser	10	20	10				
UCG	4	7	1	overall G+C (%)	62	60	57
UCC	3	3	5	third base G+C (%)	77	72	72
AGC	2	4	1	amino acid residues	298	302	307
AGU	1	3	2	calcd mol wt	32964	33074	35155
UCU	0	2	1	mol wt of holoenzyme	264000	265000	141000
UCA	0	1	0	structure	$[\alpha Fe(II)]_8$	$[\alpha Fe(II)]_8$	$[\alpha Fe(II)]_4$

mated to be 33000. These results suggest that the 23OHBPOs from both Q1 and KF707 are similar not only in their native sizes but also in subunit structures: The enzyme consists of eight identical subunits.

Both 23OHBPOs are specific for 2,3-dihydroxybiphenyl, and they do not oxidize a positional isomer such as 3,4-dihydroxybiphenyl. Despite the above-mentioned close similarities between these enzymes, there exists some discrepancy in terms of the substrate specificity; that is, although the 23OHBPO(Q1) can oxidize catechol with a catalytic efficiency of almost one-quarter ($^1/_4V_{max}$) with respect to the natural substrate, 2,3-dihydroxybiphenyl, the catechol is nearly inert to the 23OHBPO(KF707). It is thus interesting to determine the complete nucleotide sequence of the *bphC*(Q1) gene that encodes the 23OHBPO(Q1), possessing an active site that can accommodate both catechol and 2,3-dihydroxybiphenyl, and compare its sequence with that of the *bphC*(KF707) gene as well as the catechol 2,3-dioxygenase gene encoded by *xylE* on the TOL plasmid pWW0 (Nakai et al., 1983b).

Nucleotide Sequence of the bphC(Q1). By use of the dideoxy chain termination method of Sanger (1977), the complete nucleotide sequence of the structural gene of 23OHBPO(Q1) has been determined, and the results are presented in Figure 4. Analysis of the sequence by a computer-programmed analytical system reveals one open reading frame of 900 base pairs, which includes the initiation codon ATG and the termination codon TGA ending at position 900, of sufficient length to accommodate 23OHBPO(Q1). Automated Edman degradation of the enzyme has established the NH_2-terminal sequence of 15 amino acids, Val-Ala-Val-Thr-Glu-Leu-Gly-Tyr-Leu-Gly-Leu-Thr-Val-Thr-Asn, with the removal of the NH_2-terminal methionine. This sequence is in perfect agreement with the predicted amino acid sequence of the NH_2 terminus shown in Figure 4. The molecular weight of the processed protein with 298 amino residues is calculated, from the predicted amino acid sequence, to be 32964 (Table II), which is in excellent agreement with that of the Q1 subunit component of 33000 (Table I) determined by SDS–polyacrylamide gel electrophoresis.

There is a purine-rich region, 5′-GAGAAGG-3′, nearly complementary to the sequence of the 3′ end of the *P. aeruginosa* 16S rRNA, which is regarded as the ribosome-binding sequence (SD sequence; Shine and Dalgarno, 1975) for the 23OHBPO. Upstream of the SD sequence, there are two sequential termination codons, TGA, in the same reading frame, which may be the 3′-terminal portion of a putative *bphB*

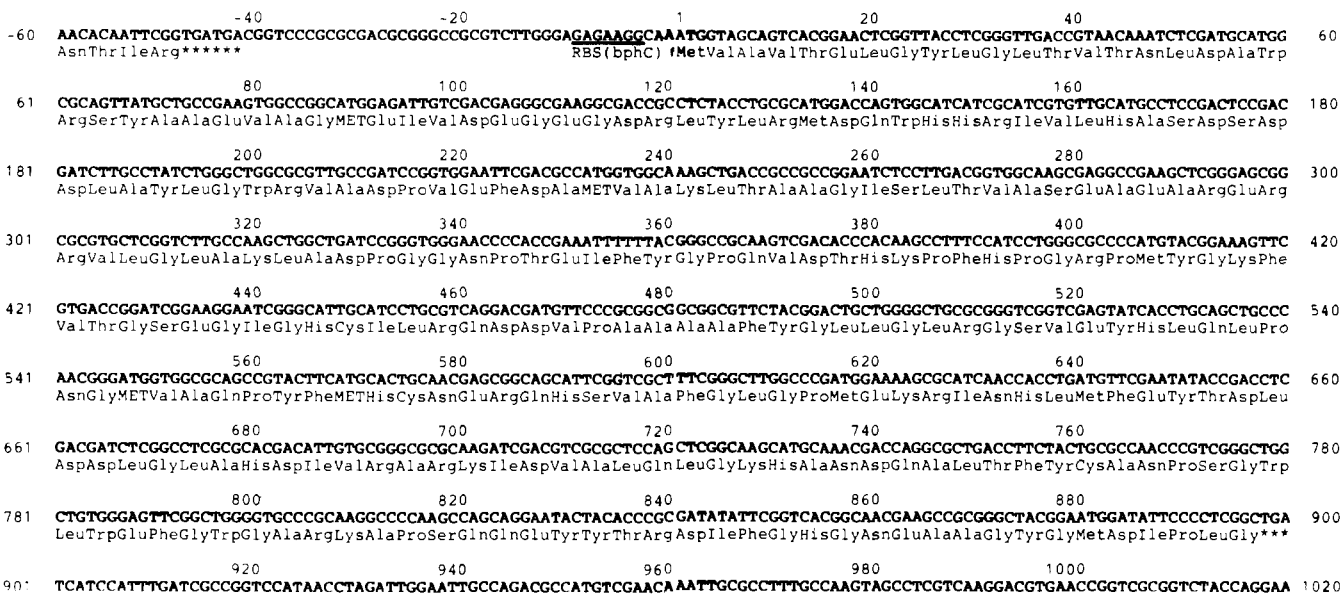

FIGURE 4: Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase (23OHBPO) gene of *P. paucimobilis* Q1 and corresponding amino acid sequence. The possible ribosome binding site (RBS) is underlined. The asterisks denote termination codons.

FIGURE 5: Comparison of amino acid sequences of 23OHBPO(707), 23OHBPO(Q1), and catechol 2,3-dioxygenase, represented by KF707, Q1, and Catechol, respectively. Identical amino acids are indicated by colons, and the double asterisks represent conserved amino residues among the three dioxygenases.

gene encoding dihydrodiol dehydrogenase as in the operon for the KF707 gene (Furukawa et al., 1987a). In agreement with this idea, neither a promoter-like sequence, upstream of the 5′-region, nor a sequence capable of forming a stem-and-loop with terminal Us in the downstream region of the 3′-end is found.

As summarized in Table II, the DNA sequence for 23OHBPO has a G+C content of 62 mol %, which is close to those of *P. putida* (60–63%) and *P. pseudoalcaligenes* (62–64%) (Normore, 1976). In accord with the finding of Nozaki and Nakazawa's group (Nakai et al., 1983b), the preferential usage of C- and G-terminated codons has contributed to the high G+C content of the gene; the frequencies of the G or C appearing in the third position of codons in the *bphC*(Q1) gene is calculated to be as high as 77%, compared to the overall 62% G+C content (Table II). As recognized previously (Nakai et al., 1983b; Brown et al., 1983), the relatively high G+C content of the gene refelects its origin from the genus *Pseudomonas*.

Comparison of the Deduced Amino Acid Sequences of the bphC(Q1) with Those of the bphC(KF707) and the Catechol 2,3-Dioxygenase Genes. The amino acid sequence for the *bphC*(Q1) gene deduced from the nucleotide sequence is presented in Figure 5, together with, for comparison, the amino acid sequences for the *bphC*(KF707) gene (Furukawa, 1987b) as well as for the catechol 2,3-dioxygenase gene encoded by *xylE* on the TOL plasmid, pWW0, originated from *P. putida* mt-2 (Nakai et al., 1983b). In the figure, dashes represent missing amino acids when the sequences are maximally aligned. Colons indicate identical amino acids, and double asterisks in place of colons are utilized when amino acids are strictly conserved among the three species. The overall homology between *bphC*(Q1) and *bphC*(KF707) at the level of amino acids is 38%, and that between *bphC*(Q1) and the

catechol 2,3-dioxygenase gene is 24%.

Discussion

2,3-Dihydroxybiphenyl dioxygenase (23OHBPO) is an extradiol-type dioxygenase, catalyzing an oxidative cleavage at the 1,2-position of an aromatic ring. The 23OHBPO(KF707) is encoded by the *bphC*(KF707) gene, a component of the *bph* operon, found from the chromosomal DNA of polychlorinated biphenyl degrading *P. pseudoalcaligenes* KF707 (Furukawa & Miyazaki, 1986). We have isolated over 20 23OHBPOs from various strains including *Pseudomonas* species capable of utilizing biphenyls as the sole carbon source. Studies with antibodies have confirmed that most of these 23OHBPOs are immunologically similar (Furukawa, unpublished results). However, the 23OHBPO(Q1) isolated from the *P. paucimobilis*(Q1) is an exception. As shown in Figure 3, 23OHBPO purified from Q1 does not show any cross-reactivity with the antibodies prepared against 23OHBPO(KF707) despite the following similarities between these enzymes: Both enzymes (a) exhibit a native molecular weight of 260 000, (b) contain the ferrous form of ion, (c) are compared of eight identical subunits, (d) are inhibited by the ferric form of ion, and (e) *cannot* catalyze the oxidative cleavage of the substrate's positional isomer, 3,4-dihydroxybiphenyl.

There is, however, one significant difference between these enzymes isolated from strains Q1 and KF707: The enzyme from Q1 can utilize catechol as a substrate, whereas the enzyme of KF707 origin does not show any significant enzymatic activity toward the catechol. Amino acid sequences of catechol 2,3-dioxygenase, which clearly catalyzes an oxidative extradiol cleavage of catechol, and 23OHBPO(KF707) have already been deduced from the determined nucleotide sequences by Nakai et al. (1983b) and Furukawa et al. (1987a), respectively. We have thus cloned the *bphC*(Q1) gene, and the complete nucleotide sequence has been determined as reported in Figure 4. It is now possible to examine these enzymes not only with respect to their nucleotide sequences but, more importantly, in terms of possible substrate binding sites from the deduced amino acid sequences. By so doing, the comparison may provide basic information for understanding of the enzymatic mechanism of these dioxygenases.

The *bphC*(Q1) gene (Figure 4) exhibits approximately 60% homology with the *bphC*(KF707) gene (Furukawa et al., 1987a) when their nucleotide sequences are optimally aligned. There are some regions of high identity, e.g., in the sequence between 561 and 577 (Q1 nucleotide numbering), 20 of 21 nucleotides are identical. The regions of higher homology are found more frequently toward the middle of the gene but not at the NH_2- or CO_2H-terminal domains (data not shown). Reflecting the origin from *Pseudomonas*, all three genes possess high G+C contents of 62, 60, and 57 mol % for the 23OHBPO(Q1), 23OHBPO(KF707), and catechol 2,3-dioxygenase, respectively. The G+C contents are even higher for the third "wobble" positions with values of 77, 72, and 72 mol % (Table II). These results are in accord with the previous finding (Nakai et al., 1983b; Brown et al., 1983; Misra et al., 1985). The preferential codon usage appears to be common, as seen in Table II, with a few exceptions: For almost all the amino acids, G or C is preferentially utilized as the third base, accounting for the high G+C contents. The only exception is for the glutamic acid, in which case GAA is more frequently utilized than GAG in all three *Pseudomonas* genes. For leucine, CUC is preferentially utilized over UUG for the Q1 and catechol genes, but the preference is reversed for the KF707 gene. Similarly, CCC for proline is favored over CCU in the case of Q1 and catechol genes but not for the KF707 gene. The termination codon for the Q1 and catechol genes is UGA, whereas it is UAG for the KF707 gene. Thus, in terms of the codon usage, 23OHBPO(Q1) is closer to the catechol 2,3-dioxygenase gene, despite the much lower nucleotide homology between these genes compared to that between 23OHBPO(Q1) and 23OHBPO(KF707) genes.

The nucleotide sequence is translated into amino acid sequence, and the deduced NH_2-terminal sequence is in perfect agreement with that determined by Edman degradation. Alignment of the two 23OHBPO sequences with one another and with the sequence of catechol 2,3-dioxygenase provides some common conserved regions that may be of functional significance. The 23OHBPO(Q1) exhibits merely 24% homology with the catechol dioxygenase; however, the sequence homology extends to 38% with its counterpart from KF707, 23OHBPO(KF707). The homology of 38% between these 23OHBPOs is not considered to be high, judging from the fact that both of these enzymes are of *Pseudomonas* origin. Binding studies with synthetic peptides as well as X-ray crystallographic data on the antigen binding site of immunoglobulin molecules indicate that about six or seven amino acid residues will constitute an antigenic determinant in the primary sequence of a protein (Atassi, 1975; Ratnam et al., 1986). Notwithstanding the fact that there are some short segments of identity between the two 23OHBPOs (Figure 5), antibodies rasied against the 23OHBPO(Q1) did not cross-react with the KF707 enzyme, and vice versa (Figure 3). Thus, a sequence of high homology such as residues 185–194 (KF707 numbering), Tyr-Phe-Leu-His-Cys-Asn-Glu-Arg-His-His, despite its relatively high hydrophilicity, may line the binding pocket and confer substrate specificity and/or comprise the catalytic system. Otherwise, those regions of high homology could be exposed and targeted as antigenic recognition sites. It is interesting to note Ratnam et al.'s observation (1986) that all the antibodies raised against native human dihydrofolate reductase (DHFR) could bind as effectively to its denatured counterpart, in spite of the fact that antigenic sites on proteins could be conformational determinants (Atassi, 1975). This is in agreement with the observation that antibodies to synthetic peptides cross-react at a high frequency with native proteins containing these sequences (Niman et al., 1983).

The catechol 2,3-dioxygenase exhibits 24 and 26% homology with the 23OHBPOs from Q1 and KF707 sources, respectively. In one interpretation these values are not high enough to be considered significant, inasmuch as structurally as well as functionally unrelated enzyme, carboxypeptidase G2 encoded by a *Pseudomonas* gene (Minton et al., 1984), which was arbitrarily chosen for comparison, could exhibit 23% homology with the 23OHBPO(Q1). Nevertheless, in some cases, primary sequences do not reflect their ternary structures: The *Lactobacillus casei* and *E. coli* dihydrofolate reductases have less than 30% homology, while there is little similarity in primary sequence; however, the X-ray crystallographic structures of these native enzymes are strikingly similar (Blakley, 1984). The conserved amino acids in the dihydrofolate reductase have been shown to be of functional significance: Asp-27 (*E. coli* numbering) is found to be responsible for protonation of a substrate (Villafranca et al., 1983) and Phe-31 for binding of dihydrofolate as well as antifolate drugs (Taira & Benkovic, 1988), just to name a few. It is thus conceivable that, among the three dioxygenases, some of those conserved amino acids indicated by double asterisks (Figure 5) would be functionally important since all these enzymes utilize the ferrous form of iron in common as the sole cofactor. Tampering with those residues by means of site-directed

mutagenesis should guide us into a clearer understanding of the role of each invariant amino acid in the enzymatic mechanism.

The regions of higher homology between the 23OHBPOs occur more frequently toward the middle of the gene. At the CO_2H-terminal sequence 266–302, there are only four identical residues in these two enzymes. During a preparation of deletion mutants, a plasmid corresponding to a 66 amino acid deletion from the C terminus was isolated. Since a colony harboring this plasmid did not turn yellow upon spraying with 2,3-dihydroxybiphenyl, the 23OHBPO(Q1) with this much deletion is apparently inactive.

The catechol 2,3-dioxygenase is composed of four identical subunits. On the other hand, the 23OHBPOs consist of eight identical subunits. The former enzyme carrying three cysteines could in principle form intersubunit disulfide linkages. However, the latter enzyme, especially the 23OHBPO(KF707) possessing only two cysteines, cannot form octamer merely by disulfide bonds. Two of three cysteines in the Q1 enzyme locate at positions 149 and 189, and at or near these locations the two cysteines of the Q1 enzyme are also found. Together with the fact that those regions are highly conserved domains between these two enzymes, the two cysteines appear to be functionally important.

In conclusion, because of the pivotal role in degrading environmental pollutants, PCB-degrading microorganisms have been subjected to close scrutiny in recent years. We have cloned a gene, *bphC*(Q1), encoding 2,3-dihydroxybiphenyl dioxygenase, and the complete nucletoide sequence has been determined. Comparison of the deduced amino acid sequence with the reported sequences of functionally similar enzymes enables us to identify potential catalytically and/or structurally important amino residues. It is hoped that accumulation of nucleotide sequences of various dioxygenases and ongoing protein engineering on these enzymes augur well for the future understanding of the enzymatic mechanism.

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