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Structure of the *pcbC* Gene Encoding 2,3-Dihydroxybiphenyl Dioxygenase of *Pseudomonas* sp. P20

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Pseudomonas sp. P20 is a soil bacterium growing in biphenyl or 4-chlorobiphenyl as the sole carbon and energy source, where the initial catabolism of biphenyl to form benzoate is catalyzed by four enzymes encoded in the *pcbABCD* gene cluster. The nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene corresponding to the *pcbC* gene was determined. The *pcbC* gene was composed of 921 base pairs with an ATG initiation codon and a TGA termination codon, which can encode a polypeptide of molecular mass 34 kDa containing 306 amino acids. A promoter-like sequence and ribosome-binding sequence were identified upstream of the initiation codon. The deduced amino acid sequence of 2,3-dihydroxybiphenyl dioxygenase exhibited 47% identity with that of corresponding enzyme of *Pseudomonas* sp. DJ-12, and less than 35% identity with those of other extradiol-type dioxygenases.

Polychlorinated biphenyls have been recognized as a group of recalcitrant aromatic pollutants in the environment [1]. However, several bacterial strains have been identified to degrade chlorinated biphenyls [2]. Microbial catabolism of biphenyl or chlorinated biphenyls is initiated to form corresponding benzoates by sequential activities of four enzymes encoded in *pcbABCD* gene cluster as shown in Fig. 1 [3-8]. The first step involves in the conversion of biphenyl to 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (biphenyl dihydrodiol) by biphenyl dioxygenase. The dihydrodiol is reduced by dihydrodiol dehydrogenase to 2,3-dihydroxybiphenyl, which undergoes extradiol (*meta*) cleavage of the aromatic ring substituted with two hydroxyl groups by 2,3-dihydroxybiphenyl dioxygenase. The *meta* cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) is converted to benzoate and 2-hydroxypenta-2,4-dienoate by a hydrolase.

Pseudomonas sp. P20 is a natural isolate which can grow in biphenyl or 4-chlorobiphenyl as the sole carbon and energy source. In this study, nucleotide sequence of 2,3-dihydroxybiphenyl dioxygenase gene corresponding to *pcbC* gene in chromosomal DNA of *Pseudomonas* sp. P20 was determined and analyzed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Escherichia coli XL1-Blue was used as a host strain, and pBluescript SK(+) as a cloning vector. *E. coli* XL1-Blue was grown in Luria-Bertani (LB) medium, and the strain harboring each of the plasmids in LB medium containing ampicillin, isopropylthio-β-D-galactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) [9].

DNA manipulations. Plasmid was isolated by alkali lysis method, and transformation performed by calcium chloride method [9]. DNA digestion with restriction enzyme(s) and ligation with T_4 ligase were accomplished at reaction conditions recommended by the supplier (Boehringer Mannheim Biochemicals). For nucleotide sequencing, nucleotides in insert DNA were sequentially deleted by using exonuclease III and S1 nuclease supplied as a kit of the Erase-a-

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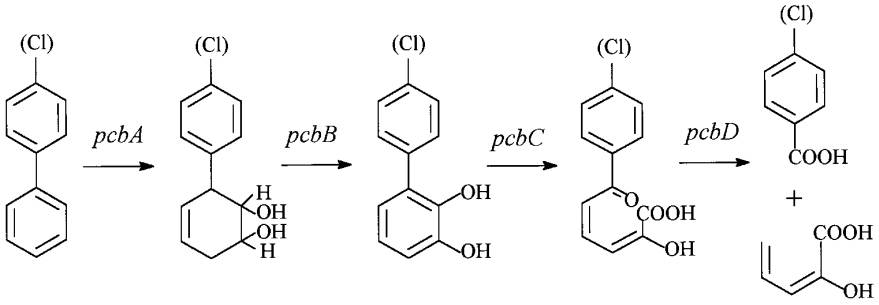

FIG. 1. Microbial catabolism of biphenyl or 4-chlorobiphenyl to forms corresponding to benzoate and 2-hydroxypenta-2,4-dienoate. Enzymes are biphenyl dioxygenase (*pcbA*), dihydrodiol dehydrogenase (*pcbB*), 2,3-dihydroxybiphenyl dioxygenase (*pcbC*), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (*pcbD*).

Base System (Promega), and its sequence was determined by dideoxy chain termination method with Sequenase version 2.0 kit (U.S. Biochemical). Nucleotide sequence was analyzed by using the DNASIS and PROSIS softwares, and alignments of amino acid sequences by the Clustal V software.

Activity assays of 2,3-dihydroxybiphenyl dioxygenase and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase. Activity of 2,3-dihydroxybiphenyl dioxygenase was measured by increase in absorbance at 434 nm, and that of HOPDA hydrolase by decrease in absorbance at the same wavelength. As the enzyme sources, crude lysates were prepared from *E. coli* XL1-Blue harboring each of the plasmids grown in LB medium containing ampicillin in the presence or absence of IPTG by sonication followed by centrifugation. Activity assays of 2,3-dihydroxybiphenyl dioxygenase were performed by addition of 20 μl of enzyme source to 980 μl of 50 mM phosphate buffer (pH 7.4) containing 0.5 mM 2,3-dihydroxybiphenyl. One unit of the enzyme activity was defined as oxidation of 1 μmol of 2,3-dihydroxybiphenyl per minuter, and calculated by using the extinction coefficient of 13,200 M^{-1} cm^{-1} [10]. Assay mixture of HOPDA hydrolase consisted of 20 μl crude lysate, 0.1 μM substrate, 50 mM phosphate buffer (pH 7.4) in 1-ml final volume. One unit was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of HOPDA per minute. Molar extinction coefficient of 13,000 M^{-1} cm^{-1} was taken to calculate the enzyme activity under the assay condition [11]. Specific activities of both 2,3-dihydroxybiphenyl dioxygenase and HOPDA hydrolase were defined as unit(s) per mg of protein. Protein concentration was determined by the Lowry method [12].

RESULTS AND DISCUSSION

Pseudomonas sp. P20 was isolated from an industrial area in Korea as a degrader of biphenyl and 4-chlorobiphenyl. The strain catabolizes biphenyl or 4-chlorobiphenyl to form corresponding benzoate and 2-hydroxypenta-2,4-dienoate. The resulting 2-hydroxypenta-2,4-dienoate is further catabolized to intermediates of glycolysis and TCA cycle which are utilized as carbon and energy sources for growth of the strain, but benzoate seems to be accumulated within the strain. A 4.1-kb *Eco*RI-*Hin*dIII fragment containing *pcbC* and *pcbD* genes encoding 2,3-dihydroxybiphenyl dioxygenase and HOPDA hydrolase in degradation of biphenyl or 4-chlorobiphenyl of *Pseudomonas* sp. P20 was obtained as a recombinant plasmid pCK1022.

To analyze the *pcbC* gene encoding 2,3-dihydroxybiphenyl dioxygenase at molecular level, several subclones were constructed from pCK1022 as described in Table 1, and their physical maps are shown in Fig. 2. *E. coli* XL1-Blue harboring pCK1022 exhibited 2,3-dihydroxybiphenyl dioxygenase and HOPDA hydrolase activities. *E. coli* XL1-Blue harboring pCK1092 exhibited 2,3-dihydroxybiphenyl dioxygenase activity but not HOPDA hydrolase activity. However, *E. coli* XL1-Blue harboring pCK1023, pCS104 or pCS33 did not have any enzyme activity at all. The 2,3-dihydroxybiphenyl dioxygenase gene in pCK1022 or pCK1092 was not inducible in the presence of IPTG. The *pcbD* gene encoding HOPDA hydrolase was located in upstream the *pcbC* gene. A putative promoter of *pcbC* gene from chromosomal DNA of *Pseudomonas* sp. P20 seems to be contained in the 2.0-kb *Xba*I-*Eco*RI fragment of pCK1092, and thus *pcbC* and *pcbD* genes would be transcribed by different promoters in *Pseudomonas* sp. P20.

TABLE 1
Bacterial Strains and Plasmids Used and Prepared in This Study

Strain or plasmid	Description
Strains	
E. coli XL1-Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *relA*1 *lac*$^-$ *F′*[*proAB*$^+$ *lacI*^{q} *lacz*ΔM15 Tn10]
Pseudomonas sp. P20[a]	4CB$^+$, BP$^+$ and 4CBA$^-$
Plasmids[b]	
pBluescript SK(+)	Cloning vector, Ap^r
pCK1022	A clone selected from a genomic library of *Pseudomonas* sp. P20, a 4.1-kb *Eco*RI-*Hin*dIII fragment of the P20 inserted into the *Eco*RI and *Hin*dIII sites of pBluescript (SK)(+), Ap^r
pCK1092	A 2.0-kb *Eco*RI-*Xba*I fragment of pCK1022 inserted into the *Eco*RI and *Xba*I sites of pBluescript SK(+), Ap^r
pCK1023	An 1.7-kb *Eco*RI-*Hin*dIII fragment of pCK1092 inserted into the *Eco*RI and *Hin*dIII sites of pBluescript SK(+), Ap^r
pCS104	An 1.1-kb *Eco*RI-*Sac*I fragment of pCK1092 inserted into the *Eco*RI and *Sac*I sites of pBluescript SK(+), Ap^r
pCS33	An 1.0-kb *Sal*I-*Xba*I fragment of pCK1092 inserted into the *Sal*I and *Xba*I sites of pBluescript SK(+), Ap^r

[a] *Pseudomonas* sp. P20 can grow in 4-chlorobiphenyl (4CB$^+$) or biphenyl (BP$^+$) but not in 4-chlorobenzoic acid (4CBA$^-$) as the sole carbon and energy source.
[b] Ampicillin resistance (Ap^r).

A nucleotide sequence corresponding to *pcbC* gene and its flanking region was determined and analyzed as shown in Fig. 3. The *pcbC* gene encoding 2,3-dihydroxybiphenyl dioxygenase was composed of 921 base pairs with ATG initiation codon at position 153 and TGA termina-

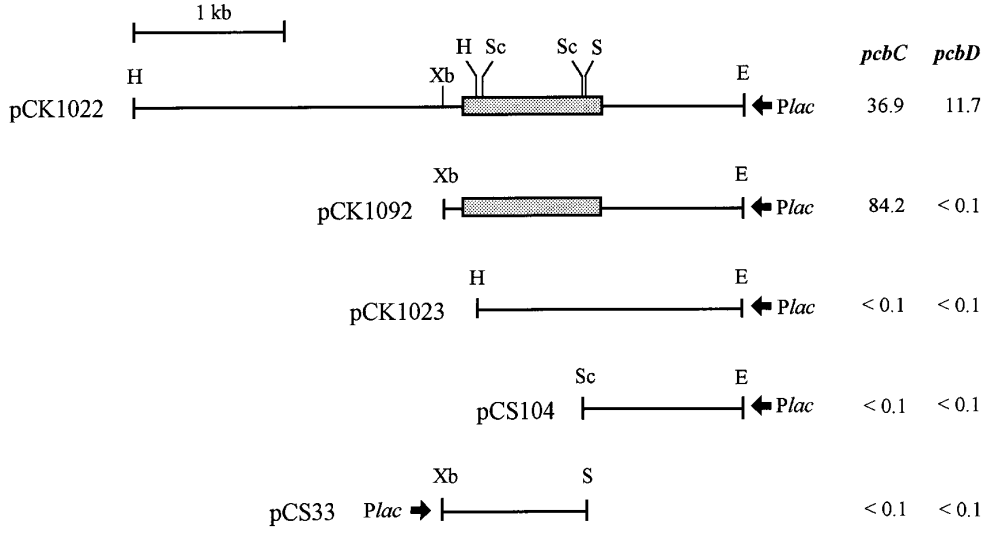

FIG. 2. Physical maps of pCK1022 and its subclones. Restriction enzymes are *Eco*RI (E), *Hin*dIII (H), *Sac*I (Sc), *Sal*I (S), and *Xba*I (Xb). Orientation of *lac* promoter (P*lac*) in pBluescript SK(+) is indicated by an arrow. The *pcb*C gene encoding 2,3-dihydroxybiphenyl dioxygenase is localized within a box. Enzyme activity of 2,3-dihydroxybiphenyl dioxygenase (*pcbC*) or 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (*pcbD*) was indicated by units per mg of protein.

FIG. 3. The nucleotide sequence of *pcbC* gene and its flanking regions. An open reading frame corresponding to *pcbC* gene is capitalized. A promoter-like sequence (PR) and a ribosome-binding sequence (RBS) are underlined. The nucleotide sequence was deposited in DDBJ/EMBL/GenBank DNA databases under the accession number D83328.

tion codon at position 1073 which can encode a polypeptide of molecular mass 34 kDa containing 306 amino acids. A potential ribosome-binding sequence (RBS) of 5′-AGGAGAAA-3′ was identified at approximately 10 nucleotides upstream the initiation codon. A putative promoter (PR) exhibiting extensive sequence homology with P3 promoter in pTN8 [13], a mutant plasmid of a RP4-TOL recombinant, was identified at positions 65 to 100. The pCK1023 contains the *pcbC* gene with deletion in 5′-terminal 63 nucleotides, and pCS33 has the gene with deletion in 3′-terminal 93 nucleotides.

The deduced amino acid sequence of 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. P20 exhibited 47% identity with that of corresponding enzyme from *Pseudomonas* sp. DJ-12, and 32 to 35% identity with those of other 2,3-dihydroxybiphenyl dioxygenases from *P. paucimobilis* Q1, *P. pseudoalcaligenes* KF707, *P. putida* KF715, *Pseudomonas* sp. KKS102 and *P. cepacia* LB400 [3,8,14-17]. The *Pseudomonas* sp. P20 and DJ-12 strains were isolated in Korea, and the other strains in Japan, USA, and europe. The higher sequence identity between 2,3-dihydroxybiphenyl dioxygenases from *Pseudomonas* sp. P20 and DJ-12 strains than those between the enzymes from *Pseudomonas* sp. P20 and other bacterial strains would reflect some regional relationships of the genetic tracts among bacterial isolates with the same metabolic function.

The amino acid sequence of 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. P20 was well aligned with those of corresponding enzymes from six different *Pseudomonas* strains (Fig. 4). As indicated by asterisks, 49 amino acids were identified to be conserved among the seven 2,3-dihydroxybiphenyl dioxygenases. 2,3-Dihydroxybiphenyl dioxygenase is known to require ferrous ion for its catalytic activity [10,18,19]. Recently an X-ray crystallography of 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 was reported [20]. Ferrous ion as a cofactor of the enzyme from *P. cepacia* LB400 is coordinated with two histidine residues, one glutamic acid residue and two water molecules where the amino acid residues are corresponded to His^{146}, His^{210}, and Glu^{260}. The last two amino acid residues, His^{210} and Glu^{260}, were

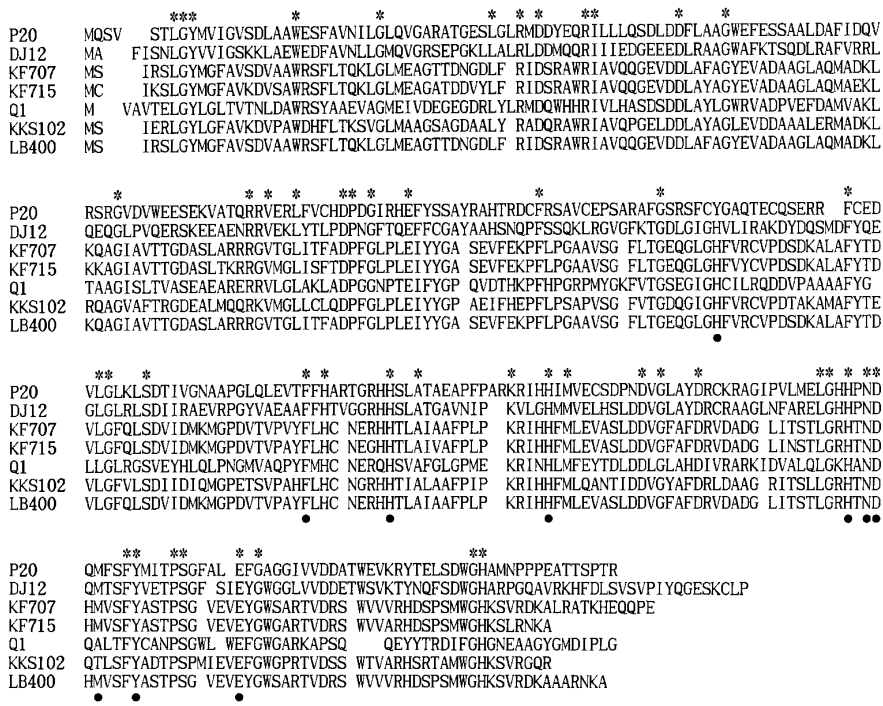

FIG. 4. Comparison of amino acid sequences of 2,3-dihydroxybiphenyl dioxygenase of *Pseudomonas* sp. P20 with those of corresponding enzymes from other *Pseudomonas* strains. Amino acid sequences of 2,3-dihydroxybiphenyl dioxygenases from *Pseudomonas* sp. P20, *Pseudomonas* sp. DJ-12, *P. pseudoalcaligenes* KF707, *P. putida* KF715, *P. paucimobilis* Q1, *Pseudomonas* sp. KKS102, and *P. cepacia* LB400 were maximally aligned. Identical amino acids among the enzymes are indicated by asterisks. Essential amino acids to interact with ferrous ion and to define the active site of 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 are indicated by solid circles.

well conserved in other 2,3-dihydroxybiphenyl dioxygenases. The His^{146} of 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 was replaced by Tyr in corresponding enzyme from *Pseudomonas* sp. P20, but well conserved in other enzymes. Other essential amino acid residues (Phe^{187}, His^{195}, His^{241}, Asn^{243}, Asp^{244}, Met^{246}, and Tyr^{250}) defined the active site pocket of 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 were well conserved in other corresponding enzymes except that Met^{246} was replaced by Thr or Ala in 2,3-dihydroxybiphenyl dioxygenases from *Pseudomonas* sp. KKS102 and *P. paucimobilis* Q1.

The pCK1023 can express the 2,3-dihydroxybiphenyl dioxygenase with deletion in N-terminal 21 amino acid residues and pCS33 does the enzyme with deletion in C-terminal 30 amino acid residues (Figs. 2 and 3). The N-terminal 21 amino acid residues of 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. P20 contain 4 amino acids (Leu^{7}, Gly^{8}, Tyr^{9}, and Trp^{20}) conserved among other corresponding enzymes, and C-terminal 31 amino acid residues contain 2 conserved amino acids corresponding to Gly^{291} and His^{292} (Fig. 4). *E. coli* harboring pCK1023 or pCS33, deletion mutants of the *pcbC* gene, did not exhibit 2,3-dihydroxybiphenyl dioxygenase activity (Fig. 2). Therefore, the N-terminal and C-terminal amino acid sequences of 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. P20 seem to play some important roles in the enzyme activity for aromatic ring fission of 2,3-dihydroxybiphenyl. Functional roles of the conserved amino acids and N′- and C′-terminal amino acid sequences in the catalytic activity of 2,3-dihydroxybiphenyl dioxygenase would be elucidated by a future site-directed mutagenesis study.

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REFERENCES

1. Furukawa, K., Tomizuka, N., and Kamibayashi, A. (1979) *Appl. Environ. Microbiol.* **38,** 301–310.
2. Abramowicz, D. A. (1990) *Crit. Rev. Biotechnol.* **10,** 241–251.
3. Hofer, B., Eltis, L. D., Dowling, D. N., and Timmis, K. N. (1993) *Gene* **130,** 47–55.
4. Khan, A., and Walia, S. (1991) *Appl. Environ. Microbiol.* **57,** 1325–1332.
5. Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M., and Yano, K. (1989) *J. Bacteriol.* **171,** 2740–2747.
6. Ahmad, D., Masse, R., and Silvestre, M. (1990) *Gene* **86,** 53–61.
7. Mondello, F. J. (1989) *J. Bacteriol.* **171,** 1725–1732.
8. Hayase, N., Taira, K., and Furukawa, K. (1990) *J. Bacteriol.* **172,** 1160–1164.
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *in* Molecular Cloning: A Laboratory Manual, Vols. 1–3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
10. Eltis, L. D., Hofmann, B., Hecht, H.-J., Lunsdorf, H., and Timmis, K. N. (1993) *J. Biol. Chem.* **268,** 2727–2732.
11. Omori, T., Sugimura, K., Ishigooka, H., and Minoda, Y. (1986) *Agric. Biol. Chem.* **50,** 931–937.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
13. Inouye, S., Asai, Y., Nakazawa, A., and Nakazawa, T. (1986) *J. Bacteriol.* **166,** 739–745.
14. Kim, E., Kim, Y., and Kim, C.-K. (1996) *Appl. Environ. Microbiol.* **62,** 262–267.
15. Taira, K., Hayase, N., Arimura, N., Yamashita, S., Miyazaki, T., and Furukawa, K. (1988) *Biochemistry* **27,** 3990–3996.
16. Taira, K., Hirose, J., Hayashida, S., and Furukawa, K. (1992) *J. Biol. Chem.* **267,** 4844–4853.
17. Kikuchi, Y., Yasukochi, Y., Nagata, Y., Fukuda, M., and Takagi, M. (1994) *J. Bacteriol.* **176,** 4269–4276.
18. Khan, A. A., Wang, R.-F., Nawaz, M. S., Cao, W.-W., and Cerniglia, C. E. (1996) *Appl. Environ. Microbiol.* **62,** 1825–1830.
19. Furukawa, K., and Arimura, N. (1987) *J. Bacteriol.* **169,** 924–927.
20. Han, S., Eltis, L. D., Timmis, K. N., Muchmore, S. W., and Bolin, J. T. (1995) *Science* **270,** 976–982.