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Characterization of the *pcbD* Gene Encoding 2-Hydroxy-6-Oxo-6-Phenylhexa-2,4-Dienoate Hydrolase from *Pseudomonas* sp. P20

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Abstract 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase catalyzes the hydrolytic cleavage of HOPDA to benzoate and 2-hydroxypenta-2,4-dienoate (HPD) during microbial catabolism of biphenyl and polychlorinated biphenyls. A HOPDA hydrolase gene (*pcbD*) was isolated from the genomic library of *Pseudomonas* sp. P20 and designated as pCNU1201; a 7.5-kb *Xba*I DNA fragment from *Pseudomonas* sp. P20 was inserted into the pBluescript SK(+) *Xba*I site. *E. coli* HB101 harboring pCNU1201 exhibited HOPDA hydrolase activity. The open reading frame (ORF) corresponding to the *pcbD* gene consisted of 855 base pairs with an ATG initiation codon and a TGA termination codon. The ORF was preceded by a ribosome-binding sequence of 5'-TGGAGC-3' and its G+C content was 55 mol %. The *pcbD* gene of *Pseudomonas* sp. P20 was located immediately downstream of the *pcbC* gene encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase, and approximately 4-kb upstream of the *pcbE* gene encoding HPD hydratase. The *pcbD* gene was able to encode a polypeptide with a molecular weight of 31,732 containing 284 amino acid residues. The deduced amino acid sequence of the HOPDA hydrolase of *Pseudomonas* sp. P20 exhibited high identity (62%) with those of the HOPDA hydrolases of *P. putida* KF715, *P. pseudoalcaligenes* KF707, and *Burkholderia cepacia* LB400, and also significant homology with those of other hydrolytic enzymes including esterase, transferase, and peptidase.

Key words: *Pseudomonas* sp. P20, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase, *pcbD* gene, nucleotide sequence, expression

Polychlorinated biphenyls (PCB) are a class of human-made compounds with exceptional stability, consisting of 209 possible congeners that differ from one another in the number and position of the chlorine substituents on their aromatic rings. A number of bacterial strains that can degrade biphenyl and PCB have been isolated. The microbial catabolism of biphenyl and PCB proceeded initially by the sequential activities of biphenyl dioxygenase, *cis*-biphenyl dihydrodiol dehydrogenase, 2,3-dihydroxybiphenyl (2,3-DHBP) 1,2-dioxygenase, and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase to form chlorinated benzoates and 2-hydroxypenta-2,4-dienoate (HPD). The genes responsible for the biphenyl and PCB catabolism have been cloned from the chromosomes and plasmids of several strains, and designated as *bphABCD*, *cbpABCD*, or *pcbABCD* [4, 8, 10, 11, 13, 16-18, 22, 23]. Some biphenyl-degrading bacteria are unable to further metabolize the chlorinated benzoates, but can catabolize HPD to become acetyl-CoA by the sequential activities of HPD hydratase, 4-hydroxy-2-oxovalerate aldolase, and acetaldehyde dehydrogenase [14, 15].

Pseudomonas sp. P20 is a soil bacterium that can grow in biphenyl or 4-chlorobiphenyl as the sole carbon and energy sources [15]. This strain is unable to degrade the 4-chlorobenzoate resulting from the catabolism of the 4-chlorobiphenyl [15]. In this study, a *pcbD* gene encoding HOPDA hydrolase was cloned from the chromosomal DNA of *Pseudomonas* sp. P20, and its nucleotide sequence and expression were analyzed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used and prepared in this study are described in Table 1. The *Escherichia coli* HB101, used as the recipient strain of the recombinant plasmids, was grown in an LB medium [21]. For antibiotic selection, the medium was supplemented with ampicillin (50 μg/ml as the final concentration). The *Pseudomonas* sp. P20 was grown in an MM2 medium containing 10 mM of biphenyl

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Table 1. Bacterial strains and plasmids used and prepared in this study.

Strain or plasmid	Description
Strains	
E. coli HB01	*supE*44 *hsdS*58($r_B^- m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *respL*20 *xyl*-5 *mtl*-1
Pseudomonas sp. P20	Soil isolate which can grow in biphenyl or 4-chlorobiphenyl as the sole carbon and energy sources
Plasmids	
pBluescript SK(+)	Cloning vector carrying *lacZ* promoter
pCNU1201	Plasmid clone selected from genomic library of P20, a 7.5-kb *Xba*I fragment of P20 inserted into the same site of pBluescript SK(+)
pCNU1202	7.5-kb *Apa*I fragment, deletion derivative of pCNU1201 lacking one *Apa*I fragment
pCNU1204	4.8-kb *Eco*RI fragment, deletion derivative of pCNU1201 lacking one *Eco*RI fragment
pCNU1209	5.5-kb *Eco*RI-*Xba*I fragment of pCNU1201 inserted into the same site of pBluescript SK(+)
pCNU1222	6.6-kb *Sac*I fragment, deletion derivative of pCNU1202 lacking two *Sac*I fragments

or 4-chlorobiphenyl as the sole carbon and energy sources [15]. pBluescript SK(+) was used as the cloning vector.

DNA Manipulations

The plasmid was isolated using the alkali lysis method, and the chromosomal DNA from *Pseudomonas* sp. P20 using the SDS-proteinase K method [21]. The DNA was resolved by electrophoresis on a 0.7% or 1% agarose gel, stained with 0.5 µg/ml ethidium bromide, and visualized using UV irradiation [21]. DNA fragmentation with restriction endonucleases and ligation of the DNA fragments were carried out according to the conditions recommended by the supplier (Boehringer Mannheim). DNA transformation was performed by the calcium chloride method [21]. The nucleotide sequencing was carried out with dideoxy-chain termination kit (Pharmacia Biotech) using an Applied Biosystems DNA Sequencer (ALFexpress II DNA analysis system).

Assay of HOPDA Hydrolase Activity

The enzyme activity was measured spectrophotometrically by following disappearance of HOPDA at 434 nm [20] at 23°C in 50 mM phosphate buffer (pH 7.4) containing an enzyme source and 0.5 mM HOPDA as the substrate. One unit of the enzyme activity was defined as the amount of 1-µmol HOPDA converted to benzoate and HPD per min. The molar extinction coefficient (ε) of HOPDA to its products under the assay conditions was taken to be 19,000 $M^{-1}cm^{-1}$. The relative activity of the enzyme to the other *meta*-cleavage compounds was determined using ε=36,000 $M^{-1}cm^{-1}$ at 375 nm for 2-hydroxymuconic semialdehyde, ε=15,000 $M^{-1}cm^{-1}$ at 386 nm for 2-hydroxy-3-methylmuconic semialdehyde, ε=32,000 $M^{-1}cm^{-1}$ at 381 nm for 2-hydroxy-4-methylmuconic semialdehyde, and ε= 41,000 $M^{-1}cm^{-1}$ at 380 nm for 2-hydroxy-4-chloromuconic semialdehyde [6]. The specific activity of the enzyme was defined as the unit(s) of enzyme per mg of protein, where the protein concentration was determined by the Bradford method [2].

As HOPDA hydrolase sources, crude lysates were prepared from *E. coli* HB101 harboring each recombinant plasmid grown to a log phase in LB medium containing 50 µg/ml ampicillin. The substrates were *meta*-cleavage compounds that had been prepared using the resting cells of *E. coli* HB101 harboring pCNU1204 or pCNU413, where pCNU1204 contained the *pcbC* gene encoding 2,3-DHBP 1,2-dioxygenase from *Pseudomonas* sp. P20 and pCNU413 contained the C23O gene encoding catechol 2,3-dioxygenase from *Alcaligenes* sp. KF711 [19]. The *E. coli* HB101 harboring pCNU1204 or pCNU413 was grown for 12 h in an LB medium containing 50 µg/ml ampicillin. After washing twice with 50 mM phosphate buffer (pH 7.4), the cells were resuspended in the same buffer containing 1 mM of all the following dihydroxylated aromatics: 2,3-DHBP in *E. coli* HB101 harboring pCNU1204 and catechol, 3-methylcatechol, 4-methylcatechol or 4-chlorocatechol in *E. coli* HB101 harboring pCNU413. After incubation at room temperature for 2 to 5 h, the cells were centrifuged at 10,000 ×g for 20 min and the supernatant of the resting cell culture was used as the source of *meta*-cleavage compound.

Spectrophotometric Detection of Metabolites

As the sources of 2,3-DHBP 1,2-dioxygenase and HOPDA hydrolase from *Pseudomonas* sp. P20, crude lysates prepared from *E. coli* HB101 harboring pCNU1201, pCNU1204, or pCNU1222 were used. Each of the enzyme sources was mixed with 50 mM phosphate buffer in the presence of 2,3-DHBP or HOPDA, and the changes of absorbance at wavelengths between 200 nm and 550 nm were then monitored at 2-min intervals.

RESULTS AND DISCUSSION

Cloning and Expression of the *pcbD* Gene

The chromosomal DNA from *Pseudomonas* sp. P20 was digested with *Xba*I and then ligated with pBluescript

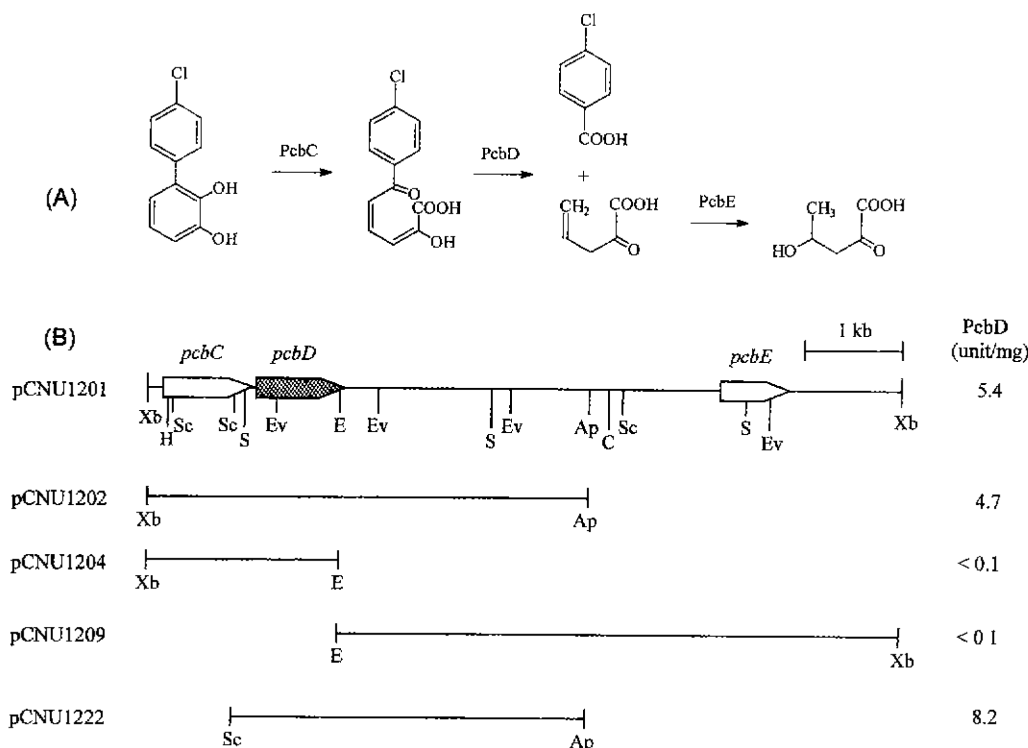

Fig. 1. 4-Chlorobiphenyl catabolism and physical map of pCNU1201.
Enzymes involved in the 4-chlorobiphenyl catabolism are 2,3-DHBP 1,2-dioxygenase (PcbC), HOPDA hydrolase (PcbD), and HPD hydratase (PcbE) (panel A). Restriction endonucleases are *Apa*I (Ap), *Cla*I (C), *Eco*RI (E), *Eco*RV (Ev), *Hin*dIII (H), *Sac*I (Sc), *Sal*I (S), and *Xba*I (Xb) (panel B). The genes responsible for the 4-chlorobiphenyl catabolism are located within an arrowed box. The HOPDA hydrolase expressed from each clone is indicated by its specific activity as unit(s) per mg of proteins.

SK(+) digested with the same endonuclease. The ligation mixture was transformed to *E. coli* HB101 to create a genomic library. A yellow clone was selected from the genomic library of *Pseudomonas* sp. P20 using ampicillin resistance followed by 2,3-DHBP spray. A recombinant plasmid with a 7.5-kb *Xba*I fragment from *Pseudomonas* sp. P20 which was inserted into the same site as the pBluescript SK(+) was present in the yellow clone, and designated as pCNU1201 (Fig. 1). *E. coli* HB101 harboring pCNU1201 exhibited HOPDA hydrolase activity in addition to 2,3-DHBP 1,2-dioxygenase activity.

To localize the *pcbD* gene in pCNU1201, several subclones were prepared as described in Table 1. *E. coli* HB01 harboring pCNU1201, pCNU1202, or pCNU1222 exhibited HOPDA hydrolase activity, however, *E. coli* HB01 harboring pCNU1204 or pCNU1209 did not (Fig. 1). The specific activity of the HOPDA hydrolase in *E. coli* HB101 harboring pCNU1201 was the same as that in *E. coli* HB101 harboring pCNU1202, yet at a significantly lower level than that in *E. coli* HB101 harboring pCNU1222.

E. coli HB101 harboring pCNU1201 metabolized both 2,3-DHBP and HOPDA (Fig. 2). HOPDA was not detected as an intermediate in the presence of 2,3-DHBP used as a substrate. This meant that both the *pcbC* and *pcbD* genes in pCNU1201 were expressed as functional proteins with corresponding enzyme activities in *E. coli* HB101, and that the HOPDA formed by 2,3-DHBP 1,2-dioxygenase seemed to be consumed immediately as the substrate of HOPDA hydrolase. *E. coli* HB101 harboring pCNU1204 metabolized 2,3-DHBP, but not HOPDA. *E. coli* HB101 harboring pCNU1222 metabolized HOPDA, but not 2,3-DHBP. These results are in support of the fact that *E. coli* HB101 harboring pCNU1222 exhibited HOPDA hydrolase activity whereas *E. coli* HB101 harboring pCNU1204 did not (Fig. 1). As for the substrate specificity, the HOPDA hydrolase from *Pseudomonas* sp. P20 catalyzed the hydrolytic cleavage of HOPDA, but not 2-hydroxymuconic semialdehyde or its derivatives.

Nucleotide Sequence of the *pcbD* Gene

The nucleotide sequence of the *pcbD* gene from *Pseudomonas* sp. P20 is represented in Fig. 3. The *pcbD* gene starts with an ATG codon at position 1 and terminates with a TGA codon at position 855. A purine-rich region of 5'-GGAG-3' regarded as the ribosome-binding sequence (RBS) was identified at about 10 nucleotides upstream of the initiation codon of the *pcbD* gene. The G+C content of the *pcbD* gene was 55 mol %.

The *pcbD* gene was located immediately downstream of the *pcbC* gene, and both genes appeared to be expressed as

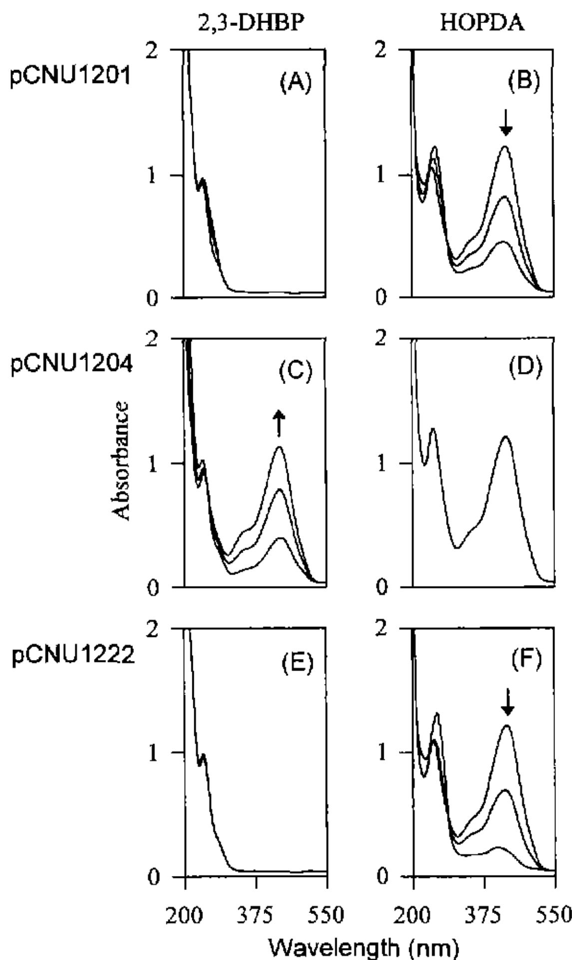

Fig. 2. Spectrophotometric identification of metabolites.
Crude lysates prepared from *E. coli* HB101 harboring pCNU1201 (A and B), pCNU1204 (C and D), or pCNU1222 (E or F) were used as the enzyme sources. Absorbance changes at wavelengths between 200 nm and 550 nm in the presence of 2,3-DHBP (A, C, and E) or HOPDA (B, D, and F) were monitored at 2-min intervals.

an operon (Fig. 1). The nucleotide sequence corresponding to the *pcbE* gene was also identified at about 4-kb downstream of the *pcbD* gene, but those encoding other enzymes involved in the PCB catabolism were not included in the pCNU1201. The *bphCDE* genes responsible for the PCB catabolism in *P. pseudoalcaligenes* KF707, *Pseudomonas* sp. LB400, and *Pseudomonas* sp. KKS102 are known to be present as clusters [2, 8, 10, 11, 14]. The *bphC* and *bphDE* gene clusters are located on different plasmids in *Rhodococcus* sp. RHA1 [17]. Accordingly, the organization of the *pcbD* gene responsible for the biphenyl and 4-chlorobiphenyl catabolism in *Pseudomonas* sp. P20 seems to be different from those of other PCB-degrading bacteria.

Sequence Comparison of the *pcbD* Gene Product with Other Hydrolases

The *pcbD* gene can encode a HOPDA hydrolase with predicted molecular weight of 31,732 containing 284 amino

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-96  GCA ATG AAC CCA CCA CCA GAA GCC ACA ACG TCT CCT ACA CGT TGA
     Ala Met Asn Pro Pro Pro Glu Ala Thr Thr Ser Pro Thr Arg ***
-51  AGCACATGAGTCCTTCTTTCATCAATACACCCACCAATGGAGCTACCAAA
                                           RBS
1    ATG ACT CTT ACC GAA GCC GGA ACC AGC AAG TTC GTG ACT ATT AAC
     Met Thr Leu Thr Glu Ala Gly Thr Ser Lys Phe Val Thr Ile Asn
46   GAA CCC GGT CTG GAG AAT TTC AAG ATC CAC GTC AAC GAT GCG GGA
     Glu Pro Gly Leu Glu Asn Phe Lys Ile His Val Asn Asp Ala Gly
91   AGT GGT CCC GCA ATC ATC ATG CTG CAC GGA GGC GGT CCT GGT GCC
     Ser Gly Pro Ala Ile Ile Met Leu His Gly Gly Gly Pro Gly Ala
136  AGT GGT TGG AGC AAC TAC TAC CGC AAC ATT GAG GTA CTG GTG AAT
     Ser Gly Trp Ser Asn Tyr Tyr Arg Asn Ile Glu Val Leu Val Asn
181  GCC GGA TAT CGA GTG CTG CTG ATC GAC AGC CCA GGA TTT AAC AAG
     Ala Gly Tyr Arg Val Leu Leu Ile Asp Ser Pro Gly Phe Asn Lys
226  TCC GCG GAG ATC CTC ACA GAT ATT CCC CGC CCG CTG ATC AAC GCA
     Ser Ala Glu Ile Leu Thr Asp Ile Pro Arg Pro Leu Ile Asn Ala
271  CGT GCA AGC AAG GGC GTG ATG GAC GCA CTC GGC ATC GAC CAA GCC
     Arg Ala Ser Lys Gly Val Met Asp Ala Leu Gly Ile Asp Gln Ala
316  CAC TTT GTC GGC AAC TCG ATG GGC GGA GCC TCT GCC ATG AGC TTC
     His Phe Val Gly Asn Ser Met Gly Gly Ala Ser Ala Met Ser Phe
361  TCC CTC GAG TTC CCT GAG CGC ATG GGC CGC CTG GTG CTT ATG GGA
     Ser Leu Glu Phe Pro Glu Arg Met Gly Arg Leu Val Leu Met Gly
406  CCA GGA GCA CAA GGT CCG AGC ATC TTC CAG CTG AGC GAA GGC GTC
     Pro Gly Ala Gln Gly Pro Ser Ile Phe Gln Leu Ser Glu Gly Val
451  AAA CGC ATG ATG AGG CTG TAC GCA GAG CCG AAT CAC GAG CCA CAA
     Lys Arg Met Met Arg Leu Tyr Ala Glu Pro Asn His Glu Pro Gln
496  AAC TTC AAT GCC ATG TTG GAG GTA TTT GTT TAC GCA CCT CAG GCT
     Asn Phe Asn Ala Met Leu Glu Val Phe Val Tyr Ala Pro Gln Ala
541  ATT ACA GAG GAG CTG CGT CAA GGG CGG TGG AAC AAC ATC CAA TCG
     Ile Thr Glu Glu Leu Arg Gln Gly Arg Trp Asn Asn Ile Gln Ser
586  AAC TTG ACT CAC CTG AAG AAT TTC GTC GAA AGC TCA AGA CTG TGC
     Asn Leu Thr His Leu Lys Asn Phe Val Glu Ser Ser Arg Leu Cys
631  CCT CAC AGC AAG TGG GAT CTG ACA GCT CGC TTC CCA GAA ATT GCT
     Pro His Ser Lys Trp Asp Leu Thr Ala Arg Phe Pro Glu Ile Ala
676  CAC AAG ACG CTC ATC ACC TGG GGC CGT GAC GAC CGC TTT GTT CCG
     His Lys Thr Leu Ile Thr Trp Gly Arg Asp Asp Arg Phe Val Pro
721  ATC GAC CAT GGC CTG CGA ATG GTC AAC ACC TTC CAA GAT TCT CGG
     Ile Asp His Gly Leu Arg Met Val Asn Thr Phe Gln Asp Ser Arg
766  CTT CAT ATC TTT GCG AAG TGC GGG CAC TGG GCT CAG TGG GAG CAC
     Leu His Ile Phe Ala Lys Cys Gly His Trp Ala Gln Trp Glu His
811  GCA GAG GAA TTC AAT CAG TTG CTG ATT GCG TTC CTG AAA GAC TGA
     Ala Glu Glu Phe Asn Gln Leu Leu Ile Ala Phe Leu Lys Asp ***
856  GAT CAA ACA GCA AAA AAA GAC ATA ACG GAG ACA TAC ATG CTG TCC
901  AGT CCT CAG TCC AGA TCA AGC CAG CGC ATT GCA AGC AGG TTG CAA
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Fig. 3. Nucleotide and deduced amino acid sequences of the *pcbD* gene from *Pseudomonas* sp. P20.
The *pcbD* gene encoding HOPDA hydrolase starts at position 1 and terminates at position 855, where the termination codon is indicated by ***. The ribosome-binding sequence (RBS) is underlined. Another open reading frame at positions -96 to -52 corresponds to the 3'-end of the *pcbC* gene encoding 2,3-DHBP 1,2-dioxygenase.

acid residues (Fig. 3). The deduced amino acid sequence of the HOPDA hydrolase from *Pseudomonas* sp. P20 exhibited the highest identity (62%) with those of the HOPDA hydrolases from *P. putida* KF715, *P. pseudoalcaligenes* KF707, and *Burkholderia cepacia* LB400 [8, 10, 11]. The HOPDA hydrolase from *Pseudomonas* sp. P20 exhibited 49% identity with the 2-hydroxy-6-ketonona-2,4-dienoate (HKND) hydrolase from *E. coli* CS520, 28–34% identity with the 2-hydroxymuconic semialdehyde hydrolases from *Rhodococcus* sp. RHA1 and *P. putida* mt-2, 33% identity with the 2-hydroxy-6-oxohepta-2,4-dienoate (HOHD) hydrolase from *P. fluorescens* IP01, and 17% to 31%

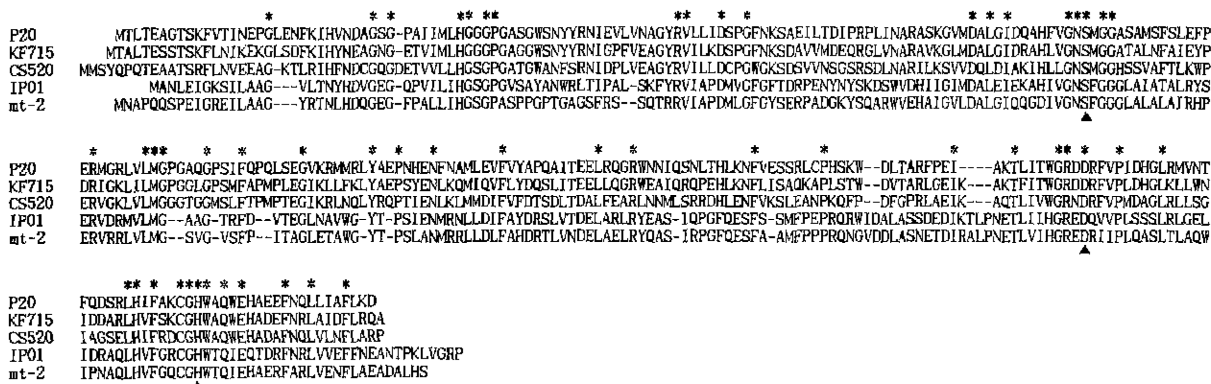

Fig. 4. Multiple alignment of amino acid sequences of aromatic hydroxylases.

Enzymes are the HOPDA hydrolases from *Pseudomonas* sp. P20 (P20) and *P. putida* KF715 (KF715), the HKND hydrolase from *E. coli* CS520 (CS520), the HOHD hydrolase from *P. fluorescens* IP01 (IP01), and the 2-hydroxymuconic semialdehyde hydrolase from *P putida* mt-2 (mt-2). The sequences were aligned using the multiple alignment program Clustal V. Identical resides aligned among the aromatic hydrolases are indicated by an asterisk. The catalytic triad in the 2-hydroxymuconic semialdehyde hydrolase from *P. putida* mt-2 is indicated by a triangle.

identity with the other hydrolytic enzymes of esterase from *Archaeoglobus fulgidus*, transferase from *P. putida* PpG2, and peptidase from *Lactobacillus delbruekii* DSM7290 [7, 9].

Recently, it has been pointed out that the hydrolases cleaving the C-C bonds in *meta*-cleavage compounds of dihydroxylated aromatics belong to the α/β hydrolase-fold family of enzymes [5, 12]. These aromatic hydrolases are known to have a catalytic triad, in the order of nucleophile-acid-histidine which is responsible for hydrolytic cleavage of a *meta*-cleavage compound [1, 3]. The HOPDA hydrolase from *Pseudomonas* sp. P20 was well aligned with other aromatic hydrolases (Fig. 4). As the catalytic triad in the HOPDA hydrolase from *Pseudomonas* sp. P20, the Ser^{111} residue within the sequence of Gly-Asn-Ser-X-Gly-Gly corresponded to the catalytic nucleophile, and Asp^{236} and His^{264} corresponded to other catalytic residues.

Abbreviations: 2,3-DHBP, 2,3-dihydroxybiphenyl; HKND, 2-hydroxy-6-ketonona-2,4-dienoate; HOHD, 2-hydroxy-6-oxohepta-2,4-dienoate; HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; HPD, 2-hydroxypenta-2,4-dienoate; ORF, open reading frame; PCB, polychlorinated biphenyls

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