The Journal of Microbiology, Dec. 1996, p.349-354
Vol. 34, No. 4

Cloning and Expression in *E. coli* of the HOPDA Hydrolase Gene from *Pseudomonas* sp. P20

Jong-Chul Lim, Jong-Chan Chae, Youngsoo Kim[1], Hyong Bai Kim[2], and Chi-Kyung Kim*

Department of Microbiology and [1]Department of Pharmacy, Chungbuk National University, Cheongju 361-763, and [2]Department of Biotechnology, Korea University, Chochiwon 339-800, Korea

(Received May 30, 1996/Accepted October 17, 1996)

***Pseudomonas* sp. P20 is a natural isolate which is capable of degrading biphenyl and 4-chlorobiphenyl. From a clone of pCK1022 harboring *pcbCD* genes of *Pseudomonas* sp. P20, a *pcbD* gene encoding 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) hydrolase was subcloned in *Escherichia coli* XL1-Blue by using pBluescript SK(+) vector. The 2.8-kb *Hind*III fragment harboring the *pcbD* gene cloned in pCK1024 had a single site for each of *Xho*I, *Sal*I, *Bst*XI, and *Xba*I restriction enzymes. *Escherichia coli* CK1024 carrying pCK1024 degraded HOPDA to benzoate and 2-hydroxypenta-2,4-dienoate by HOPDA hydrolase encoded by *pcbD* gene as effectively as *E. coli* CK 1022 harboring *pcbCD* genes.**

Key words: HOPDA(2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate), HOPDA hydrolase, cloning of *pcbD*, *Pseudomonas* sp. P20

Biphenyl is a backbone structure of polychlorinated biphenyls (PCBs) that are known as one of the recalcitrant pollutant chemicals. The PCBs are mixtures of 209 different congeners that can be produced depending on the number and position of chlorine atoms on the biphenyl structure. The recalcitrant property of PCBs is recognized to be caused by the ring structures and carbon-chlorine bonds, so that biodegradation of biphenyl and chlorinated biphenyl has been focused on the cleavage reaction of benzene ring in several bacterial strains, such as *Acinetobacter* sp. (1), *Alcaligenes* sp. (1, 18), and *Pseudomonas* sp. (2, 6, 8, 20).

Degradation of biphenyl by ring cleavage reaction under aerobic condition is reported by Furukawa and Miyazaki (3) to be initiated by insertion of two atoms of oxygen at carbon positions 2 and 3 by biphenyl dioxygenase (BphA). The resulting 2,3-dihydrodiol is reduced to 2,3-dihydroxybiphenyl (2,3-DHBP) by dihydrodiol dehydrogenase (BphB) and 2,3-DHBP is further converted to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) by *meta*-cleavage reaction catalyzed by 2,3-dihydroxybiphenyl dioxygenase (BphC). The HOPDA is then hydrolyzed by HOPDA hydrolase (BphD) to produce benzoic acid and 2-hydroxypenta-2,4-dienoic acid as shown in Fig. 1 (3).

The *bphABCD* gene cluster responsible for degradation of biphenyl to benzoic acid, which was designated by Furukawa and Miyazaki (3), has been cloned from the chromosomal DNAs of *Pseudomonas putida* KF715 (4), *Pseudomonas* sp. KKS102 (11), and *Pseudomonas pseudoalcaligenes* KF707 (3). Those in *Pseudomonas putida* OU 83 (7) and *Pseudomonas* sp. DJ-12 (8) which were capable of degrading biphenyl and 4-chlorobiphenyl (4CB) were named as *cbpABCD* and *pcbABCD*, respectively.

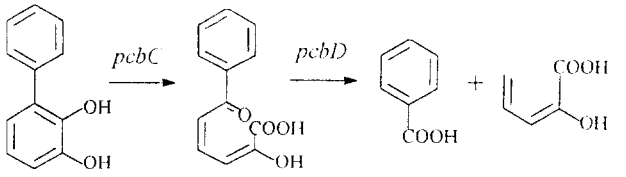

Fig. 1. Microbial conversion of 2,3-dihydroxybiphenyl (2,3-DHBP) to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) and then to benzoate and 2-hydroxypenta-2,4-dienoate. The enzymes encoded by *pcbC* and *pcbD* are 2,3-DHBP dioxygenase and HOPDA hydrolase, respectively.

* To whom correspondence should be addressed

The HOPDA hydrolase (BphD), the product of *bphD*, has been studied for its genetic structure in the strains of *Pseudomonas putida* KF715 (4), *Pseudomonas* sp. KKS102 (11), and *Pseudomonas* sp. DJ-12 (10). The HOPDA hydrolase were similar each other in the nucleotide sequence, showing about 54% similarity in their amino acid sequences (4, 10). HOPDA hydrolase purified from *Pseudomonas cruciviae* S93 B1 was reported to be about 160 kDa and composed of the subunits with about 29 kDa (15).

Pseudomonas sp. P20 as a natural isolate was reported to degrade biphenyl and 4-chlorobiphenyl (4CB) more effectively than *Pseudomonas* sp. DJ-12 (13). The *pcbCD* gene cluster in *Pseudomonas* sp. P20 which was the same as the *bphCD* genes (3) in *Pseudomonas pseudoalcaligenes* was cloned in *E. coli* XL1-Blue from the chromosomal DNA as reported previously (14). The nucleotide sequence of *pcbC* gene encoding 2,3-DHBP dioxygenase was determined (9) and the enzymatic characteristics of the 2,3-DHBP dioxygenase was also studied recently (16). In this paper, we describe the cloning and expression in *E. coli* XL1-Blue of the *pcbD* gene of *Pseudomonas* sp. P20 responsible for hydrolysis of HOPDA, the *meta*-cleavage compound produced from 2,3-DHBP by the *pcbC* gene activity.

Materials and Methods

Bacterial strains, plasmids, and growth media

The bacterial strains and plasmids used in this study including their relevant markers and references are listed in Table 1. The bacterial strains were grown in Luria-Bertani (LB) medium consisted of 1% Bacto tryptone, 0.5% yeast extract, and 0.5% NaCl supplemented with ampicillin (100 μg/ml). For selection of the cloned cells, the LB medium supplemented with 100 μg/ml of ampicillin, 15 μg/ml of tetracycline, 40 μg of 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal; Promega, U.S.A.) and 40 μg of isopropyl β-D-thiogalactopyranoside (IPTG; Sigma, U.S.A.) was used.

Cloning of *pcbD* gene

Plasmid DNA was isolated by alkaline lysis method described by Sambrook *et al.* (17). T4 DNA ligase and restriction enzymes used for genetic manipulation were purchased from POSCOCHEM (SungNam, Korea).

For cloning of the *pcbD* gene, the pCK1022 constructed by Nam and Kim (13) was digested with *Hind*III and ligated with *Hind*III-digests of pBluescript SK(+) at the ratio of 3 to 1 as shown in Fig. 2. The hybrid vector was transformed into *E. coli* XL1-Blue by $CaCl_2$-heat shock treatment according to the procedure descirbed by Sambrook *et al.* (17). Transformants were selected on LB medium supplemented with several antibiotics as descirbed above. The selected transformants were confirmed for the inserted DNA by electrophoresis and tested for HOPDA hydrolase activity by the resting cell assay as described by Furukawa and Miyazaki (3).

Activity of HOPDA hydrolase

The 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) used as substrate for HOPDA hydrolase was provided by cultivating *E. coli* AW313 with 1 mM 2,3-dihydroxybiphenyl (Wako Pure Chem., Tokyo, Japan) in phosphate buffer by the method described by Khan and Walia (6). The cells were removed from the culture by centifugation at 12,000 rpm, and then HOPDA was obtained by filtering the culture solution through 0.2 μm filter membrane.

The cloned cells harboring HOPDA hydrolase gene

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant markers	References
Strains		
Pseudomonas sp. P20	$4CB^+$, BP^+, 4CBA$^-$, $2,4DCBP^+$, Ap^r	Youn *et al.* (20)
E. coli CK1022	*E. coli* XL1-Blue carrying pCK1022, Ap^r, Tc^r	Nam and Kim (13)
E. coli CK1092	*E. coli* XL1-Blue carrying pCK1092, Ap^r, Tc^r	Nam and Kim (13)
E. coli CK1024	*E. coli* XL1-Blue carrying pCK1024, Ap^r, Tc^r	This study
E. coli AW313	*E. coli* HB101 carrying pAW313, Ap^r	Khan and Walia (6)
E. coli XL1-Blue	Host strain, Tc^r	Stratagene Ltd.
Plasmids		
pCK 1022	*pcbCD*, 4.2 kb *Eco*RI-*Hind*III fragment of pCK102 cloned in pBluescript SK(+)	Nam and Kim (13)
pCK 1092	*pcbC*, 1.95 kb *Eco*RI-XbaI fragment of pCK1022 cloned in pBluescript SK(+)	Nam and Kim (13)
pCK 1024	*pcbD*, 2.8 kb *Hind*III fragment of pCK1022 cloned in pBluescript SK(+)	This study
pAW 313	*cbpCD*, 2.4 kb *Hind*III fragment of *P. putida* OU83	Khan and Walia (6)
pBluescript SK(+)	Ap^r, cloning vector	Stratagene Ltd.

Abbreviation : 4CB, 4-chlorobiphenyl; BP, biphenyl; 4CBA, 4-chlorobenzoic acid; 2,4DCBP, 2,4-dichlorobiphenyl

were cultivated in LB broth to become about 10^9 cells/ml in density. After centrifugation of the culture at 5,000 rpm for 5 min, cells were washed with 10 mM potassium phosphate buffer (pH 7.0) three times and reacted with 1 mM HOPDA. The activity of HOPDA hydrolase was measured by examining the absorbance at 434 nm with a UV-visible spectrophotometer (Biochrom 4060, Pharmacia, Sweden) according to the method described by Omori *et al.* (15).

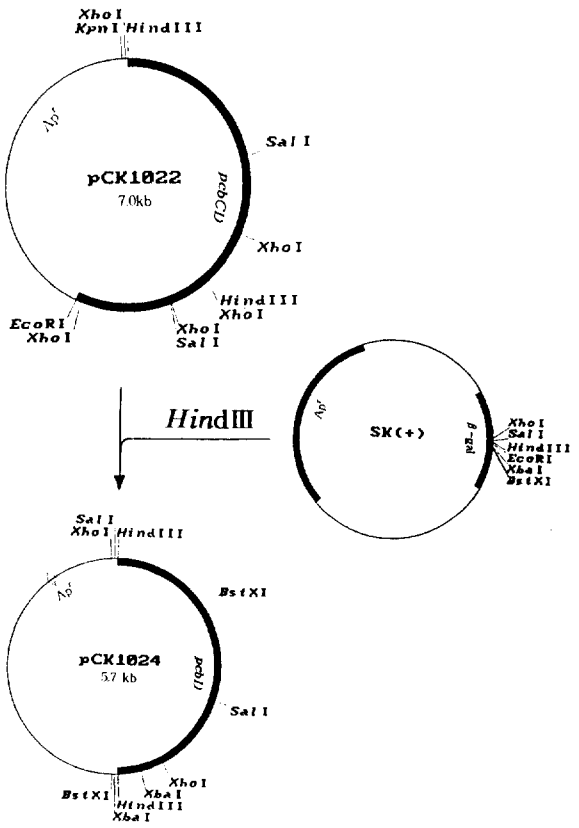

Fig. 2. Cloning of *pcbD* gene from pCK1022.

Results and Discussion

Cloning of HOPDA hydrolase gene

From the pCK1022 including the 4.2 kb insert DNA which harbors the *pcbCD* gene cluster, the *pcbD* gene was cloned by using pBluescript SK(+) vector to construct pCK1024 as shown in Fig. 2. The electrophoresis profiles of pCK1022 and pCK1024 as well as the fragments of pCK1024 digested with several enzymes are shown in Fig. 3. The insert DNA (2.8 kb in size) harboring the *pcbD* gene in pCK1024 has a single site for each of *Xho*I, *Sal*I, *Bst*XI, and *Xba*I restriction enzymes. The activity of HOPDA hydrolase was observed in the clone of pCK1024 as shown in Fig. 4. But the activity of 2,3-DHBP dioxygenase shown in pCK1022 was not observed in pCK1024. The pCK1092 did not have the HOPDA hydrolase activity as reported by Kim *et al.* (9). The results suggest that the HOPDA hydrolase gene is lo-

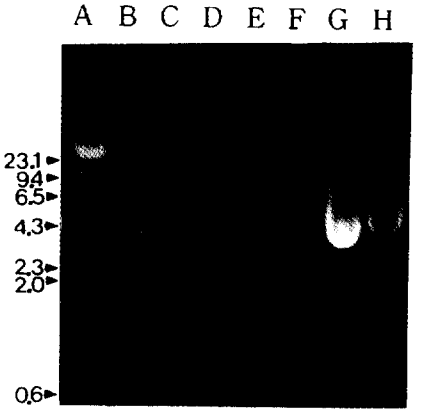

Fig. 3. Electrophoresis of recombinant plasmid pCK1024 digested with several endonucleases. Lane A, λ-*Hind*III size marker; B, pCK1024-*Bst*XI; C, pCK1024-*Hind*III; D, pCK1024-*Sal*I; E, pCK 1024-*Xho*I; F, pCK1024-*Xba*I; G, pCK1024; H, pCK1022.

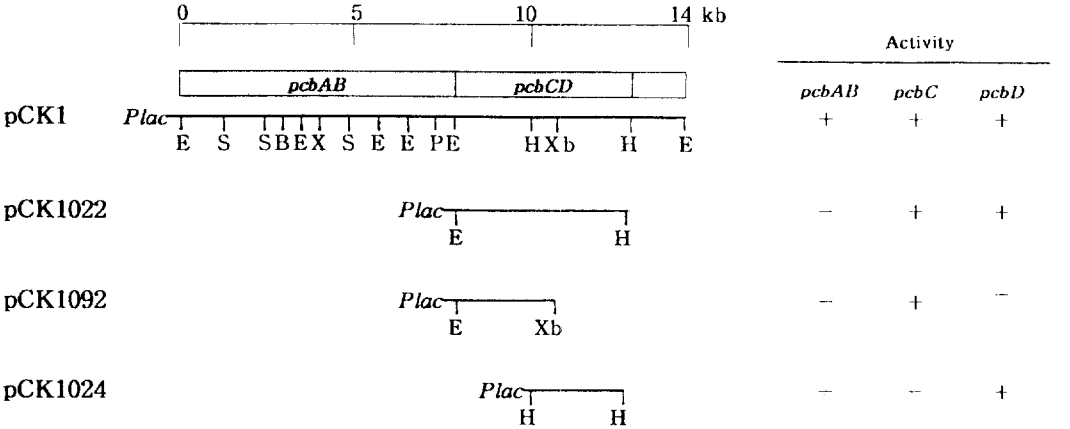

Fig. 4. Physical maps and gene expression of the recombinant plasmid of pCK1 and its subclones. Abbreviation: E, *Eco*RI; S, *Sal*I; B, *Best*XI; X, *Xho*I; Xb, *Xba*I; +, expression; -, no expression.

cated in the region between *Hind*III and *Xba*I sites in pCK1024 as shown in Fig. 4.

In general, the genes encoding for degradation of biphenyl and chlorinated biphenyl were known to be organized as a cluster (3, 7), but the order of the genes was different among bacterial strains. The *pcbCD* genes in *Pseudomonas* sp. P20 were reported to be arranged in the order *CD* as shown in Fig. 4 (14). The promoter of the *pcbC* gene was found to locate at the upstream position of *pcbC* gene (9). In the cases of *bphABCD* gene clusters in *Pseudomonas pseudoalcaligenes* KF707 (19) and *Pseudomonas* sp. LB400 (5), the *bphD* gene was separated from the *bphABC* genes. On the other hand, *bphAB* genes were placed apart from *bphCD* genes in *Pseudomonas putida* KF715 (4) and *Pseudomonas* sp. KKS102 (11). *Pseudomonas putida* OU83 (7) was shown to have a 3 kb intergenic region between *cbpA* and *cbpDCB* genes. The *pcbCD* genes of *Pseudomonas* sp. DJ-12 were arranged in the order *DC* as those of *Pseudomonas putida* OU83 (7), and a promoter-like sequence was located at the upstream position of *pcbD* (10). However, the *pcbCD* genes of *Pseudomonas* sp. P20 were organized in the order *CD* (13) which was the same as those reported in *Pseudomonas putida* KF715 (14) and *Pseudomonas* sp. KKS102 (11). A promoter-like sequence was found at the upstream of the *pcbC* gene of *Pseudomonas* sp. P20 (9). These results suggest that the expression of the *pcb* enzymes including HOPDA hydrolase can be different among the bacterial strains due to different organizations and structures of the *pcb* genes, even though they can catalyze the same reaction.

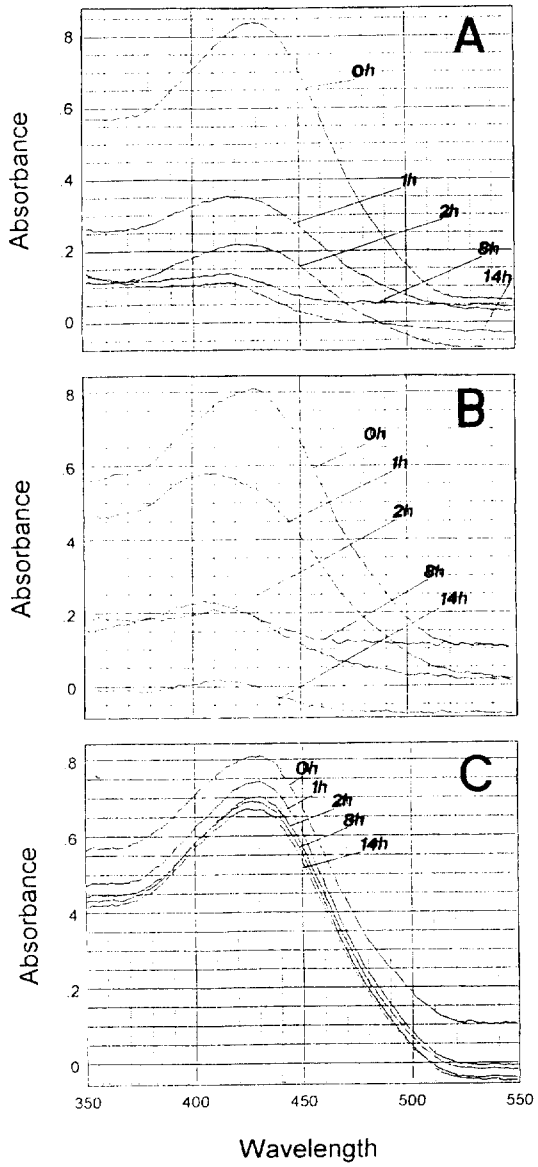

Fig. 5. Degradation of HOPDA by the cloned cells examined by resting cell assay. A, *E. coli* CK1022; B, *E. coli* CK1024; C, *E. coli* CK1092.

Expression of HOPDA hydrolase gene

The expression of the *pcbD* gene encoding HOPDA hydrolase in the cloned cell carrying pCK1024 was examined by resting cell assay described by Furukawa and Miyazaki (3). HOPDA was completely degraded to benzoate and 2-hydroxypenta-2,4-dienoate by *E. coli* CK1024 after incubation for 14 hours as shown in Fig. 5B. *E. coli* CK1022 harboring the *pcbCD* genes and *E. coli* CK1024 harboring the *pcbD* gene degraded HOPDA equally well during the reaction of 14 hours (Fig. 5A). However, *E. coli* CK1092 harboring the *pcbC* gene did not show any

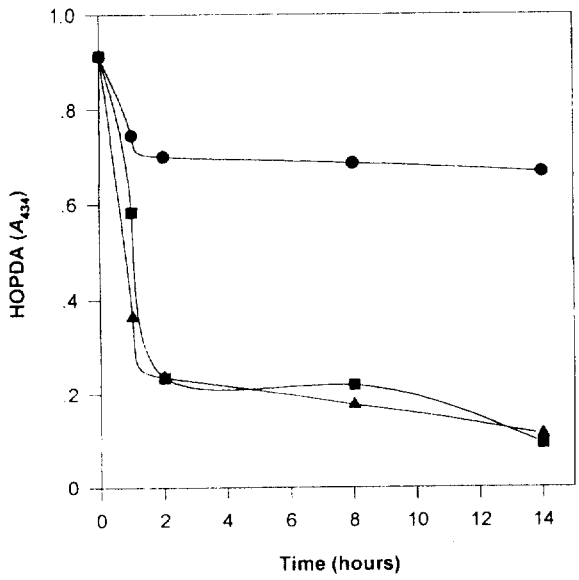

Fig. 6. Comparison of HOPDA degradation activities among the cloned cells. ●, *E. coli* CK1092; ▲, *E. coli* CK1022; ■, *E. coli* CK 1024.

significant reduction in the absorbance, except the early decrease of HOPDA during 2 h incubation coming from natural oxidation of the substrate (Fig. 6). The results indicate that degradation of HOPDA in *E. coli* CK1022 and *E. coli* CK1024 results from the activity of HOPDA hydrolase encoded by the *pcbD* gene in the cloned cells.

When the *bphCD* genes of *Pseudomonas* sp. KKS102 were cloned in *E. coli* by Kimbara *et al.* (11), they were poorly expressed in the cloned cell (*E. coli* KH131) from their own promoter located at the upstream position of *bphC* gene. However, the *bphCD* genes in the clone of KH101 which was constructed from pUC18 vector carrying *lacZ* promoter were well expressed in *E. coli* host. They suggested that the *bphCD* genes of *Pseudomonas* sp. KKS102 were not readily expressed from their own promoter in the *E. coli* host system. On the other hand, Khan and Walia (6) reported that the *cbpCD* genes of *Pseudomonas putida* OU83 were well expressed in *E. coli* host from their own promoter. For the HOPDA hydrolase of *Pseudomonas* sp. P20, it was not yet examined whether the enzyme was expressed from its own promoter or from *lacZ* promoter in the pBluescript SK(+) vector.

Acknowledgement

This work was supported by a research grant of BSRI 96-4432 from the Ministry of Education, and in part by a grant of SRC (Research Center for Molecular Microbiology at Seoul National University) from KOSEF.

References

1. **Furukawa, K., F. Matsumura, and K. Tonomura,** 1978. *Alcaligenes* and Acinetobacter strains capable of degrading polychlorinated biphenyls. *Agr. Biol. Chem.* **42**, 543-548.
2. **Furukawa, K., N. Hayase, K. Taira, and N. Tomizuka,** 1989. Molecular relationship of chromosomal genes encoding biphenyl/polychlorinated biphenyl catabolism: Some soil bacteria possess a highly conserved *bph* operon. *J. Bacteriol.* **171**, 5467-5472.
3. **Furukawa, K. and T. Miyazaki,** 1986. Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. *J. Bacteriol.* **166**, 392-398.
4. **Hayase, N., K. Taira, and K. Furukawa,** 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. *J. Bacteriol.* **172**, 1160-1164.
5. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis,** 1993. Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. *Gene* **130**, 47-55.
6. **Khan, A. A. and S. K. Walia,** 1990. Identification and localization of 3-phenylcatechol dioxygenase and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase genes of *Pseudomonas putida* and expression in *Escherichia coli*. *Appl. Environ. Microbiol.* **56**, 956-962.
7. **Khan, A. A. and S. K. Walia,** 1991. Expression, localization, and functional analysis of polychlorinated biphenyl degradation genes *cbpABCD* of *Pseudomonas putida*. *Appl. Environ. Microbiol.* **57**, 1325-1332.
8. **Kim, C.-K., T.-K. Sung, J.-H. Nam, Y.-C. Kim, and J.-K. Lee,** 1994. Cloning and expression of *pcbCD* genes in *Escherichia coli* from *Pseudomonas* sp. DJ-12. *Kor. J. Microbiol.* **32**, 40-46.
9. **Kim, C.-K., E. Kim, J.-C. Chae, and Y. Kim, 1996.** Characterization of the *pcbC* gene encoding 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. P20. *Biophy. Biochem. Res. Comm.* **226**, 15-20.
10. **Kim, E., Y. Kim, and C.-K. Kim, 1996.** Genetic structure of the genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase from biphenyl- and 4-chlorobiphenyl-degrading *Pseudomonas* sp. strain DJ-12. *Appl. Environ. Microbiol.* **62**, 262-267.
11. **Kimbara, K., T. Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano,** 1989. Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. *J. Bacteriol.* **171**, 2740-2747.
12. **Mondello, F. J.,** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. *J. Bacteriol.* **171**, 1725-1732.
13. **Nam, J.-H. and C.-K. Kim,** 1994. Cloning of *pcb* genes in *Pseudomonas* sp. P20 specifying degradation of 4-chlorobiphenyl. *Kor. J. Appl. Microbiol. Biotechnol.* **22**, 353-359.
14. **Nam, J.-H., H.-M. Oh, and C.-K. Kim,** 1995. Cloning and expression of *pcbC* and *pcbD* genes responsible for 2,3-dihydroxybiphenyl degradation from *Pseudomonas* sp. P20. *J. Microbiol. Biotechnol.* **5**, 68-73.
15. **Omori, T., K. Sugimura, H. Ishigooka, and Y. Minoda,** 1986. Purification and some properties of a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolyzing enzyme from *Pseudomonas cruciviae* S93 B1 involved in the degradation of biphenyl. *Agr. Biol. Chem.* **50**, 931-937.
16. **Park, H.-N., Y. Kim, Y.-C. Kim, C.-K. Kim, and J.-Y. Lim,** 1996. Purification and characterization of 2,3-dihydroxybiphenyl dioxygenase from recombinant *E. coli* CK 1092. *Kor. J. Appl. Micobiol. Biotechnol.* **24**, 282-289.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis,** 1989. Molecular cloning. *A laboratory manual, 2nd ed.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. **Springael, D., S. Kreps, and M. Mergeay,** 1993. Iden-

tification of a catabolic transposon, Tn4371, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus A5*. *J. Bacteriol.* **175**, 1674-1681.

19. **Taira, K., J. Hirose, S. Hayashida. and K. Furukawa,** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. *J. Biol. Chem.* **267**, 4844-4853.
20. **Youn, D.-J., J.-J. Han, C.-K. Kim, and Y. Kim,** 1992. Divergence of the *cbp* genes in 4-chlorobiphenyl catabolizing bacteria. *Kor. J. Microbiol.* **30**, 53-59.