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Improvement of 4-Chlorobiphenyl Degradation by a Recombinant Strain, *Pseudomonas* sp. DJ12-C

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***Pseudomonas* sp. P20 and *Pseudomonas* sp. DJ-12 isolated from the polluted environment are capable of degrading biphenyl and 4-chlorobiphenyl (4CB) to produce benzoic acid and 4-chlorobenzoic acid (4CBA), respectively, by *pcbABCD*-encoded enzymes. 4CBA can be further degraded by *Pseudomonas* sp. DJ-12, but not by *Pseudomonas* sp. P20. However, the *meta*-cleavage activities of 2,3-dihydroxybiphenyl (2,3-DHBP) and 4-chloro-2,3-DHBP dioxygenases (2,3-DHBD) encoded by *pcbC* in *Pseudomonas* sp. P20 were stronger than *Pseudomonas* sp. DJ-12. In this study, the *pcbC* gene encoding 2,3-DHBD was cloned from the genomic DNA of *Pseudomonas* sp. P20 by using pKT230. A hybrid plasmid pKK1 was constructed and *E. coli* KK1 transformant was selected by transforming the pKK1 hybrid plasmid carrying *pcbC* into *E. coli* XL1-Blue. By transferring the pKK1 plasmid of *E. coli* KK1 into *Pseudomonas* sp. DJ-12 by conjugation, a recombinant strain *Pseudomonas* sp. DJ12-C was obtained. Expression of the *pcbC* gene encoding 2,3-DHBP dioxygenase in *Pseudomonas* sp. P20, *Pseudomonas* sp. DJ-12, and the recombinant strain of *Pseudomonas* sp. DJ12-C was comparatively examined by the growing cell and resting cell assay methods. *Pseudomonas* sp. DJ12-C readily degraded 4CB and 2,3-DHBP to produce 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), and the resulting 4CBA and benzoic acid were continuously catabolized. *Pseudomonas* sp. DJ12-C degraded 1 mM 4CB completely after incubation for 20 h, but *Pseudomonas* sp. P20 and *Pseudomonas* sp. DJ-12 showed only 90% and 75% degradation, respectively. *Pseudomonas* sp. DJ12-C had the same substrate specificity as *Pseudomonas* sp. DJ-12 had, but its degradation activity to 2,3-DHBP, 3-methylcatechol, and catechol was improved.**

Key words: 4-chlorobiphenyl, degradation, cloning of *pcbC*, intragenic recombination, *Pseudomonas* sp. DJ12-E

Polychlorinated biphenyls (PCBs) have been widely used in many kinds of industrial products by virtue of their chemical and thermodynamic stabilities. The chemicals are known as toxic pollutants due to their recalcitrance and toxicity when contaminated the environments. Therefore, various strains of bacteria, such as *Acinetobacter* (1), *Pseudomonas* (4, 5, 8, 13, 16), *Alcaligenes* (5, 17), and *Arthrobacter* (5, 19) have been isolated as PCB-degrading microorganisms and their degrading characteristics of the chemicals have been intensively studied. Microbial conversion of PCBs as well as biphenyl which is the aromatic structure of PCBs was found to be conducted by sequential activities of four enzymes encoded by *bphABCD* gene cluster (or *pcbABCD* in other strains) to form corresponding benzoates (9). That is, biphenyl dioxygenase (BphA), dihydrodiol dehydrogenase (BphB), 2,3-dihydroxybiphenyl (2,3-DHBP) dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) hydrolase (BphD).

Among the upper four-step pathway of PCBs and biphenyl degradation, the extradiol (*meta*) cleavage of benzene ring by 2,3-DHBP dioxygenase (2,3-DHBD) is most crucial step (21). So that the *pcbC* gene has been intensively studied for genetic structure and regulation of the gene expresion by using different vectors in many bacterial strains. Mondello (15) and Nam *et al.* (16) cloned *pcbC* gene from *Pseudomonas* strains by using pMMB34 cosmid and pBluescript SK(+) vectors, respectively, and over-expressed 2,3-DHBD gene in *E. coli*. Kimbara *et al.* (13) cloned *bphC* by using broad host-range cosmid vector in *Pseudomonas putida* and constructed a recombinant strain having the improved activity of 2,3-DHBD than parent strains. Andreyeva *et al.* (2) also reported that 2,3-DHBD activities were improved when *bphC* gene of *Pseudomonas putida* SU83 strain was cloned in *Pseudomonas putida* and *Pseudomonas aeruginosa* rather than in *E. coli*, by using broad host-range RSF

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1010 vector.

Adams *et al.* (1) constructed recombinant *Pseudomonas* sp. strain CB15 capable of growing on 3-chlorobiphenyl (3CB) from *Pseudomonas* sp. HF1 and *Acinetobacter* sp. P6 strains which grow on 3-chlorobenzoate and biphenyl, respectively, using continuous amalgamated culture apparatus. Hirose *et al.* (8) constructed a hybrid gene cluster with *bph* and *tod* genes showing the improved degradation of biphenyl as well as toluene and increased substrate range. Furukawa *et al.* (6) also reported that the hybrid gene clusters constructed with the toluene metabolic *tod* operon and the biphenyl metabolic *bph* operon greatly enhanced the biodegradation rate of trichloroethylene. Dowling *et al.* (4) described the development and construction of a DNA module encoding *bph* genes for the metabolism of PCBs and integrating stably into chromosome of Gram negative bacteria. Recently, Ogawa and Miyashita (17) demonstrated an effective conjugal recombination of a 3-chlorobenzoate (3CBA) catabolic pENH91 plasmid from *Alcaligenes euthrophus* NH9 to 3CBA negative strains.

Pseudomonas sp. P20 and *Pseudomonas* sp. DJ-12 strains isolated as 4-chlorobiphenyl (4CB) degraders were reproted to degrade 4CB and biphenyl through *meta*-cleavage pathway in the previous work (16). *Pseudomonas* sp. DJ-12 could further degrade 4-chlorobenzoate (4CBA) produced from 4CB, but *Pseudomonas* sp. P20 could not. However, the *meta*-cleavage activity of the dioxygenase encoding by *pcbC* gene of *Pseudomonas* sp. P20 was stronger than that of *Pseudomonas* sp. DJ-12. The nucleotide sequences and genetic structures of *pcbC* genes of both *Pseudomonas* sp. P20 and DJ-12 strains were already studied in the previous reports (9, 12). In this study, therefore, we constructed a recombinant strain, *Pseudomonas* sp. DJ12-C, which had stronger dioxygenase activity for 4CB and 2,3-DHBD degradation, by intrageneric recombination of the *pcbC* genes in *Pseudomonas* sp. DJ-12.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* sp. DJ-12 (11) and *Pseudomonas* sp. P20 (24) are natural isolates which were previously reported on their characteristics. *E. coli* CK102 is a recombinant strain containing *pcbCD* in pBluescript SK(+) vector which was reported by Nam *et al.* (16). *E. coli* XL1-Blue and broad host-range plasmid, pKT230 (3), were used as host strain and cloning vector, respectively.

Media and cell growth

MM2 minimal medium was used for growth of *Pseudomonas* sp. DJ-12 and *Pseudomonas* sp. P20 (24). Biphenyl (1 mM) or 4CB (1 mM) was added to the medium as a carbon and energy source. *E. coli* CK102 and *E. coli* KK1 were grown in Luria-Bertani (LB) medium (Bacto tryptone, 10 g/l; Bacto yeast extract, 5 g/l; NaCl, 5 g/l; pH 7.0) containing ampicillin (100 μg/ml) and kanamycin (30 μg/ml), respectively. *Pseudomonas* sp. DJ12-C was cultured in LB medium added with ampicillin (100 μg/ml) and kanamycin (30 μg/ml). *Pseudomonas* strains were cultivated at 30°C and *E. coli* strains at 37°C.

Enzymes and chemicals

T4 DNA ligase and restriction enzymes were purchased from POSCO Co. (Seoul, Korea). Biphenyl, 4CB, and 2,3-DHBP were purchased from Wako

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant markers	Sources
Bacterial strains		
Pseudomonas sp. DJ-12	Natural isolate, 4CB$^+$, 2,3-DHBP$^+$, 4CBA$^+$	11
Pseudomonas sp. P20	Natural isolate, 4CB$^+$, 2,3-DHBP$^+$, 4CBA$^-$	24
E. coli CK102	Recombinant strain, 2,3-DHBP$^+$, HOPDA$^+$	16
E. coli KK1	Recombinant strain, 2,3-DHBP$^+$, HOPDA$^-$	This study
Pseudomonas sp. DJ12-C	Recombinant strain, 4CB$^+$, 2,3-DHBP$^+$, 4CBA$^+$	〃
E. coli XL1-Blue	Host strain, F$'$	Stratagene Ltd.
Plasmids		
pCK102	*pcbCD*, subclone containing 6.3 kb *Eco*RI fragment of pCK1, Ap^r	16
pKK1	*pcbC*, 1.95 kb *Eco*RI fragment of pCK102/SK(+), Km^r	This study
pBluescript SK(+)	Cloning vector, Ap^r	Stratagene Ltd.
pKT230	Cloning vector, Mob$^+$, Sm^r, Km^r	3

Abbreviation: 4CB, 4-chlorobiphenyl; 4CBA, 4-chlorobenzoate; 2,3-DHBP, 2,3-dihydroxybiphenyl; HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Mob$^+$, *mob* gene.

Chemical Ind., Ltd. (Osaka, Japan). Catechol was acquired from Sigma Chemical Co. (St. Louis, MO, USA), and 3-methylcatechol, 4-methylcatechol and 4-chlorocatechol were obtained from Tokyo Chemical Co. (Tokyo, Japan).

Cloning of *pcbC*

Plasmid DNA samples were prepared by alkaline-SDS method and subjected to horizontal slab gel electrophoresis as described by Sambrook *et al.* (20). After electrophoresis, agarose gel was stained with 0.5 μg/ml ethidium bromide for 40 min and photographed on UV-transilluminator (305 nm). Elution of plasmid DNA from the gel was performed by GENECLEAN II kit (Bio 101 Inc., La Jolla, CA, USA). For cloning of the *pcbC* gene encoding 2,3-DHBP dioxygenase (2,3-DHBD), pCK102 was completely digested with *Eco*RI and *Xba*I, and then mixed with pBluescript SK(+) vector digested with the same enzymes. This recombinant plasmid was redigested with *Eco*RI and inserted into pKT230 vector in ligation buffer with T4 DNA ligase at room temperature as seen in Fig. 1. The recombinant plasmids were then transformed into *E. coli* XL1-Blue according to the procedures described by Sambrook et al. (20). The transformants were selected on the LB agar medium containing kanamycin (30 μg/ml), as the colony turned yellow when sprayed with 2,3-DHBP solution (0.1%) over the colonies.

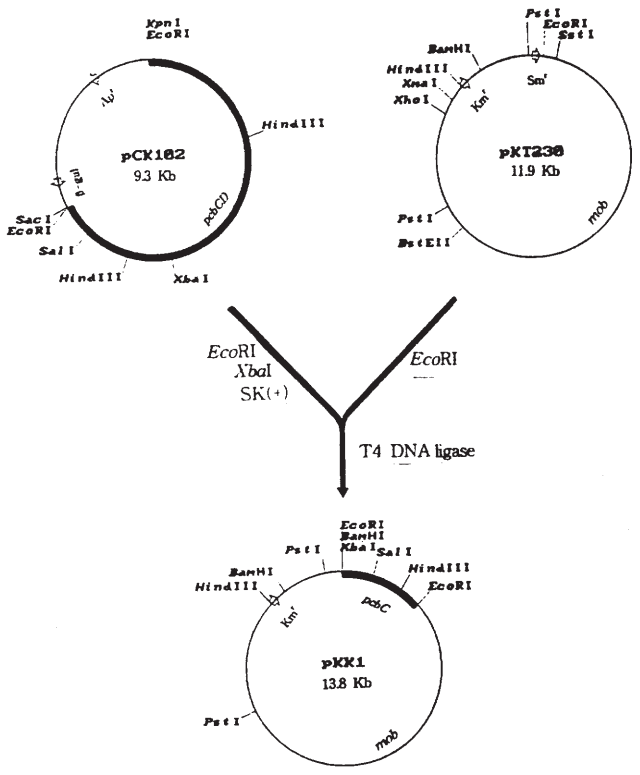

Fig. 1. Cloning strategy of *pcbC* gene from pCK102.

Conjugation

The recombinant plasmid of pKK1 which carried *pcbC* gene in pKT230 plasmid was transferred into *Pseudomonas* sp. DJ-12 from *E. coli* KK1 by conjugation procedure described by Top *et al.* (23). *E. coli* KK1 and *Pseudomonas* sp. DJ-12 used as donor and recipient cells, respectively, were grown in LB medium. Kanamycin (30 μg/ml) was added to the culture of donor cells. One ml portions of overnight-grown cultures of the donor and recipient cells were mixed in an Eppendorf tube. The mixture was filtered through a sterile filter disk (0.2 μm pore size) and the disk was put on an LB agar plate for incubation at 30°C. The filter disk was resuspended in 2 ml of saline by vigorous agitation with a vortex mixer. The resuspended cells were cultivated on LB agar medium containing ampicillin (100 μg/ml) and kanamycin (30 μg/ml) at 30°C. The transconjugants which had acquired pKK1 were selected by yellow coloring when sprayed with 2,3-DHBP solution over the colonies as described above. The numbers of donor and recipient cells were determined by a serial plate count method on LB medium. Conjugation efficiency was calculated by the number of transconjugants divided by the number of recipient cells. In order to confirm the conjugal transfer of pKK1 plasmid, the donor cells treated with DNase at concentration of 50 μg/ml or killed by heat at 72°C for 2 h were compared with untreated donor cells for the efficiency of transconjugation.

Southern hybridization

The DNA probe for Southern hybridization was made with a *Eco*RI-*Xba*I fragment (1.95 kb) carrying *pcbC* from pCK102. The *Eco*RI-*Xba*I fragment was eluted from agarose gel electrophoresed with a GENECLEAN II kit (BIO 101 Inc., La Jolla, U.S.A.). The plasmid DNAs isolated from the organisms were digested with *Eco*RI and electrophoresed. The gel was incubated in 0.25 M HCl for 10~20 min and denatured with denaturation solution (1.5 M NaCl; 0.5 N NaOH) for 45 min as described by Sambrook *et al.* (20), and then neutralized twice with neutralizing buffer (1 M Tris [pH 7.4]; 1.5 M NaCl) for 15 min. The DNAs in the gel were transferred onto a Hybond-N membrane (Amersham International plc., Amersham, UK) by using 1 M NaOH for 2 h (22). The transferred DNAs were hybridized with the DNA probe which was labelled with peroxidase by ECL system (Amersham, Buckinghamshire, England) and detection was performed according to the manufacturer's instructions.

SDS-PAGE of 2,3-DHBD

The proteins of 2,3-DHBD produced by natural isolates and recombinant strains were examined by sodium dodecyl sulfate (SDS)-PAGE by the procedure described by Sambrook *et al.* (20) The cells grown in LB broth were harvested and washed with 10 mM potassium phosphate buffer (pH 7.0). The cells were suspended in the same buffer and broken with a sonicator (Fisher, Springfield, U.S.A). After centrifugation of the suspension at 14,240 × g for 10 min, supernatant was used for detection of soluble protein. The cell debris was washed with lysis buffer (phenylmethylsulfonyl fluoride (PMSF), 0.1 mM; urea, 8 M) and added with 50 mM KH_2PO_4. After centrifugation of the mixture at 14,240 x g for 15 min, supernatant was used for assay of insoluble protein (20). SDS-PAGE was performed by using 12.5% polyacrylamide gel and 4% stacking gel by the method described by Lee *et al.* (15). Electrophoresis was carried out in continuous buffer system at constant current of 50 mA. The gel was stained for 2 h with a staining solution (Coomassie brilliant blue R-250, 1.0 g; methanol, 450 ml; glacial acetic acid, 100 ml; H_2O, 450 ml), and destained in the mixture (5:1:4 in volume) of methanol, glacial acetic acid, and H_2O.

Assay for 4CB and 2,3-DHBP degrading activities

Degradation activites of the organisms to 4CB and 2.3-DHBP were measured by growing cell and resting cell assay methods. The growth of the organisms in MM2 medium containing 1 mM 4CB or 2.3-DHBP were measured by optical density of the culture at 600 nm. The cells collected at appropriate time were mixed with the same volume of ethanol as the culture, and then the aqueous solution was measured for remnants of 4CB at 254 nm. For resting cell assay, the cells (about 10^8 CFU/ml) grown in LB broth were harvested and washed with 10 mM phosphate water (pH 7.0). The cells suspended in 1/10 volume of the same buffer were incubated with 1 mM 4CB or 2,3-DHBP at 30°C as described by Nam *et al.* (16). The cell-free supernatants were analyzed for degradation activity of the organisms with a spectrophotometer (Pharmacia LKB, Bromma, Sweden) at 254 nm for 4CB and 430 nm for HOPDA produced from 2,3-DHBP.

Test for substrate specificity

The substrate specificity of the organisms for several aromatic hydrocarbons was examined by the resting cell assay method described by Hirose (7). The *meta*-cleavage product (MCP) produced from the substrates were assayed with a spectrophotometer. The MCPs produced from catechol (ε=33,000 $cm^{-1}M^{-1}$) and 3MC (ε=13,400 $cm^{-1}M^{-1}$) were measured at 375 nm and 388 nm, respectively. Those from 4MC (ε=28,100 $cm^{-1}M^{-1}$) and 4CC (ε=36,100 $cm^{-1}M^{-1}$) were measured at 379 nm.

Results and Discussion

Construction of a recombinant strain degrading 4-chlorobiphenyl

The *pcbC* gene harboring in *Eco*RI fragment (1.95 kb) of pCK102 which was originated from *Pseudomonas* sp. P20 was cloned by using a broad host-range pKT230 vector, and a recombinant plasmid of pKK1 (13.8 kb) was constructed as seen in Fig. 1. When pKK1 was transformed into *E. coli* XL1-Blue, a transformant cell obtained was named as *E. coli* KK1 (10).

By transferring the pKK1 plasmids of *E. coli* KK1 to *Pseudomonas* sp. DJ-12 by conjugation, a transconjugant of *Pseudomonas* sp. DJ12-C was selected which mineralizes 4CB, 2,3-DHBP, and 4CBA. In order to confirm the conjugal transfer of pKK1, the efficiency of transconjugation was compared between the donor cells treated with DNase or killed by heat and untreated donors according to the method described by Paul (18). The transfer frequency with *E. coli* KK1 treated with DNase was 1.34×10^{-3} and it was as high as that obtained with the untreated donor cells as seen in Table 2.

Table 2. Conjugal transfer of the recombinant plasmid pKK1 from *E. coli* KK1 to *Pseudomonas* sp. DJ-12

Donors (*E. coli* KK1)	Recipients (*Pseudomonas* sp. DJ-12)	Tansconjugants (*Pseudomonas* sp. DJ12-C)	Frequency of transconjugation[a]
Viable cells	2.60×10^{8}[b]	3.5×10^{5}	1.34×10^{-3}
Viable cells treated with DNase	4.28×10^{7}	2.5×10^{4}	5.84×10^{-4}
Heat killed cells	1.12×10^{7}	ND[c]	0
Heat killed cells filtered through 0.2 μm filter	1.65×10^{8}	ND	0

[a] Frequency was calculated the numbers of transconjugant divided by the numbers of recipient cells.
[b] The numbers are expressed as CFU/ml.
[c] ND: not detected.

However, no transconjugant was detected in the case of heat-killed donor cells. Paul (18) described that the recombinant cells obtained from DNase-treated donor cells should be transconjugants, because conjugation could be conducted by a contact-mediated process between viable donor and recipient cells which were resistant to DNase.

The pKK1 recombinant plasmid transferred into *Pseudomonas* sp. DJ-12 from *E. coli* KK1 was also confirmed by gel electrophoresis (Fig. 2A) and Southern hybridization (Fig. 2B). The pKK1 plasmids (1.95 kb) were observed in *Eco*RI-digested plasmids isolated from both *E. coli* KK1 donor (lane 4) and *Pseudomonas* sp. DJ12-C transconjugant cells (lane 6), but not from *Pseudomonas* sp. DJ-12 used as recipient cells (lane 2). The SDS-PAGE patterns of the proteins produced by *Pseudomonas* sp. DJ-12, *Pseudomonas* sp. P20, and *Pseudomonas* sp. DJ12-C are shown in Fig. 3. The proteins of 2,3-DHBDs produced by the three strains are observed to be about 33 kDa in molecular weight as indicated by an arrow. The 2,3-DHBD encoded by *pcbC* gene was overproduced in *Pseudomonas* sp. DJ12-C recombinant strains (Fig. 3) comparing to the natural isolates of *Pseudomonas* sp. DJ-12 and *Pseudomonas* sp. P20. The molecular weight of 2,3-DHBD produced by *Pseudomonas* sp. DJ12-C was also 33 kDa as those reported in both *Pseudomonas pseudoalcaligenes* KF 707 and *Pseudomonas paucimobilis* Q1 (5).

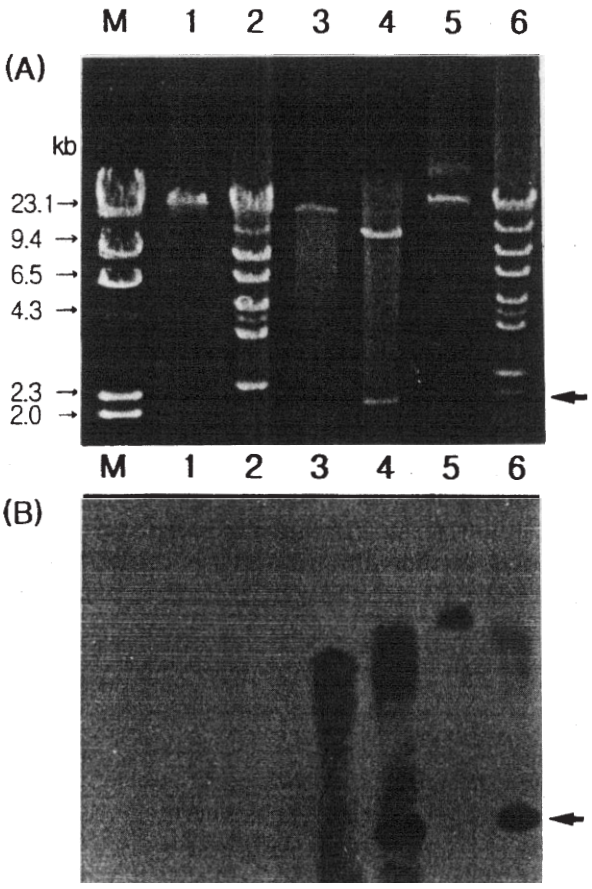

Fig. 2. Agarose gel electrophoresis (A) and Southern hybridization (B) of the plasmid DNAs isolated from *Pseudomonas* sp. DJ-12, *E. coli* KK1 and *Pseudomonas* sp. DJ12-C. Arrows show *pcbC* gene of pKK1. Lanes: M, λ-*Hind*III size marker; 1, plasmid DNA isolated from *Pseudomonas* sp. DJ-12; 2, *Eco*RI-digested plasmid DNA from *Pseudomonas* sp. DJ-12; 3, plasmid DNA from *E. coli* KK1; 4, *Eco*RI-digested plasmid DNA from *E. coli* KK1; 5, plasmid DNA from *Pseudomonas* sp. DJ12-C; 6, *Eco*RI-digested plasmid DNA from *Pseudomonas* sp. DJ12-C.

Degradation of 4CB and 2,3-DHBP by *Pseudomonas* sp. DJ12-C

Degradation avtivities of *Pseudomonas* sp. DJ-12, *Pseudomonas* sp. P20, and *Pseudomonas* sp. DJ12-C to 4CB are shown with their growth curves in Fig. 4. The natural isolates of *Pseudomonas* sp. P20 (Fig. 4A) and *Pseudomonas* sp. DJ-12 (Fig. 4B) degraded 82% and 69% of 4CB added initially, respectively, after 16 h cultivation in MM2 broth medium containing 1 mM 4CB. On the other hand, the recombinant strain of *Pseudomonas* sp. DJ12-C degraded almost all the 4CB within 14 h (Fig. 4C). Growth patterns of the three strains were very coincident to those of 4CB degradation. These results indicate that *pcbABCD* genes were more effectively expressed in the recombinant strains to utilize 4CB as a sole carbon and energy source. The results examined by the resting cell assay (Fig. 5) shows the comparative activity of 4CB degradation between *Pseudomonas* sp. DJ-12 and the recombinant strain *Pseudomonas* sp. DJ12-C. *Pseudomonas* sp. DJ12-C degraded almost all the 4CB after 20 h incubation compairing to only 76% degradation by *Pseudomonas* sp. DJ-12.

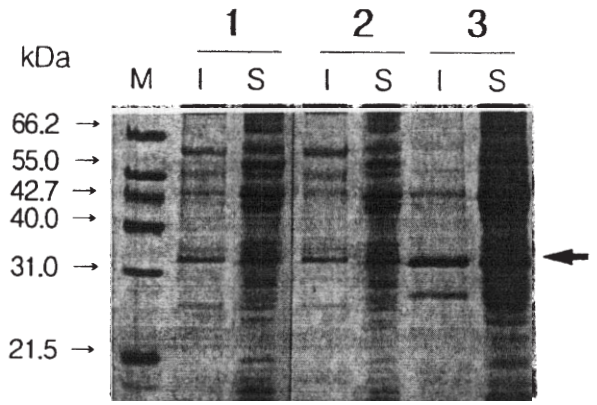

Fig. 3. SDS-PAGE of 2,3-DHBD produced by natural isolates and recombinant strain. 1, *Pseudomonas* sp. DJ-12; 2, *Pseudomonas* sp. P20; 3, *Pseudomonas* sp. DJ12-C Abbreviation: M, molecular weight markers; I, insoluble protein; S, soluble protein

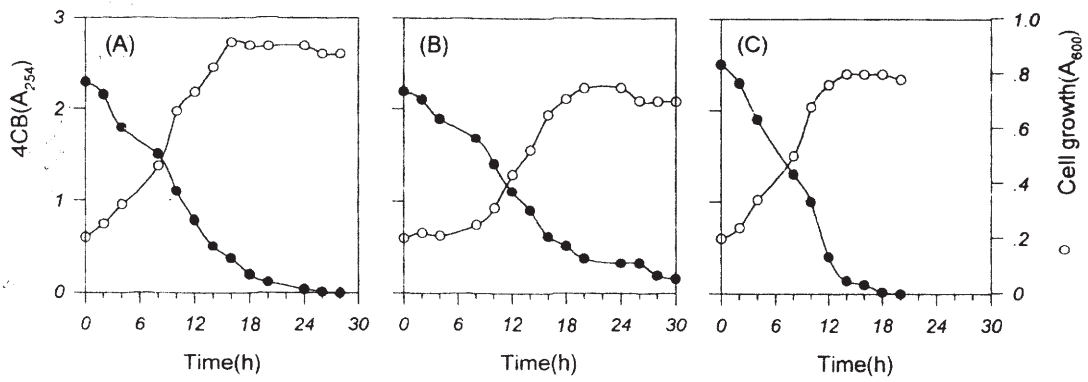

Fig. 4. Growth and degradation of natural isolates and recombinant strain for 4CB. The growing cell assay was conducted with the culture taken at appropriate time. (A) *Pseudomonas* sp. P20. (B) *Pseudomonas* sp. DJ-12. (C) *Pseudomonas* sp. DJ 12-C.

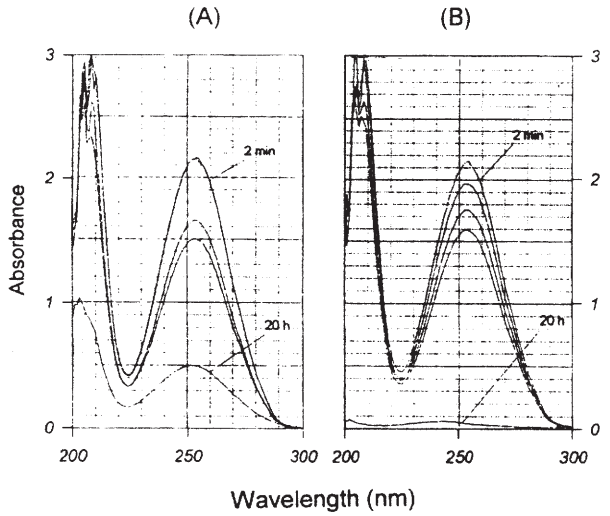

Fig. 5. Comparison of 4CB degradation between *Pseudomonas* sp. DJ-12 and *Pseudomonas* sp. DJ12-C, The resting cell assay was conducted in 10 mM potassium phosphate buffer containing 1 mM 4CB. (A) *Pseudomonas* sp. DJ-12. (B) *Pseudomonas* sp. DJ12-C.

In the MM2 medium supplemented with 1 mM 2, 3-DHBP, *Pseudomonas* sp. P20 degraded 2,3-DHBP (marked by 1) to BA (marked by 3) which was appeared after 17 h incubation (Fig. 6A), but *Pseudomonas* sp. DJ-12 (Fig. 6B) degraded 2,3-DHBP to BA which was further degraded without accumulation as reported by Kim *et al.* (10). The recombinant strain of *Pseudomonas* sp. DJ12-C (Fig. 6C) carrying *pcbABCD* genes, *pcbC* of which was originated from *Pseudomonas* sp. P20, showed increased activity of 2,3-DHBD (marked by 2) without accumulation of BA, compairing to two other strains. These are similiar to the results reported by Lee *et al.* (14) that a hybrid strain recombinated the *tod* and *tol* pathways mineralized benzene, toluene, and p-xylene without accumulation of any metabolic intermediates.

Specific activity of 2,3-DHBP dioxygenase of the recombinant strain, *Pseudomonas* sp. DJ12-C, was compared to those of natural isolates in Table 3. The 2,3-DHBD activities of both *Pseudomonas* sp.

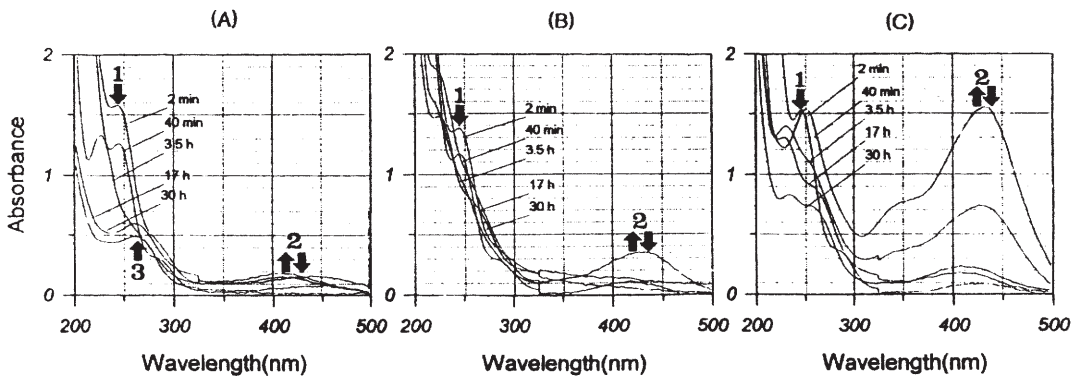

Fig. 6. Degradation of 2,3-DHBP and production of HOPDA and BA by the strains. The resting cell assay was conducted in 10 mM potassium phosphate buffer containing 1 mM 2,3-DHBP. (A) *Pseudomonas* sp. P20. (B) *Pseudomonas* sp. DJ-12. (C) *Pseudomonas* sp. DJ12-C. Compounds: 1, 2,3-dihdroxybiphenyl (2,3-DHBP); 2, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA); 3, benzoate (BA)

DJ12-C and *E. coli* KK1 which carried the pKK1 plasmids harboring *pcbC* gene were about the same, but the activities of *Pseudomonas* sp. P20 and *Pseudomonas* sp. DJ-12 were approximately 12.6% and 4.2% of that shown by *Pseudomonas* sp. DJ12-C. This means that 2,3-DHBD activity of *Pseudomonas* sp. DJ12-C constructed in this study was markedly improved comparing with those of *Pseudomonas* sp. P20 and *Pseudomonas* sp. DJ-12 strains. Andreyeva *et al.* (2) used a broad host-range vector, RSF1010, for cloning of 2,3-DHBD gene from *Pseudomonas putida* SU83 in *E. coli*, *Pseudomonas putida*, and *Pseudomonas aeruginosa*. The 2,3-DHBD activities were found to increase 5.3 fold in *Pseudomonas putida* and 9 fold in *Pseudomonas aeruginosa*, comparing with that in *E. coli*. Kimbara *et al.* (13) also cloned *bphCD* from PCB-degrading *Pseudomonas* sp. KKS102 by using the broad host-range cosmid vector, pCP13, and constructed a recombinant plasmid pKS13. When *bphCD* genes of pKS13 were transformed into *E. coli* and *Pseudomonas putida, bphC* encoding 2,3-DHBD was expressed only in *Pseudomonas putida*. On the other hand, *bphC* gene was expressed in *E. coli* when *bphCD* was cloned by using pUC18. These results indicated that 2,3-DHBD gene could be expressed in *Pseudomonas putida* by using its own promoter. Therefore, over-expression of *pcbC* encoding 2,3-DHBD in *Pseudomonas* sp. DJ12-C is thought to be accelerated by both promoters in the *pcbC* genes of *Pseudomonas* sp. DJ-12 and the transferred pKK1 plasmid originally cloned from *Pseudomonas* sp. P20.

Degradation of other catecholic compounds by *Pseudomonas* sp. DJ12-C

Table 3. Specific activity and substrate specificities of dioxygenases for 2,3-DHBP and other substrates by natural isolates and recombinant strains

Strains	Specific activity(mU/mg)[a] with:				
	2,3-DHBP	Catechol	3MC	4MC	4CC
Pseudomonas sp. P20 (NI)[b]	76.5	3.2	10.7	0	1.1
Pseudomonas sp. DJ-12 (NI)	25.5	4.4	25.6	0	2.1
E. coli KK1 (RS)[c]	675	2.7	51.3	0	2.9
Pseudomonas sp. DJ12-C (RS)	605	15.4	121.9	0	2.2

[a] Units are expressed in micromoles of the *meta*-cleavage products formed per minute at 25°C.
[b] Natural isolate.
[c] Recombinant strain.
Abbreviation: 2,3-DHBP, 2,3-dihydroxybiphenyl; 3MC, 3-methylcatechol; 4MC, 4-methylcatechol; 4CC, 4-chlorocatechol.

The *meta*-cleavage (dioxygenase) activities of natural isolates and recombinant strains for catechol, 3-methyl catechol (3MC), 4-methyl catechol (4MC) and 4-chlorocatechol (4CC) degradation were compared in Table 3. Two recombinant strains showed the same substrate specificity as the natural isolates had. The specific dioxygenase activities of *Pseudomonas* sp. DJ12-C for catechol and 3MC degradation were increased 4.8 and 11.4 folds, respectively, comparing to those of *Pseudomonas* sp. P20, and 3.5 and 4.7 folds to *Pseudomonas* sp. DJ-12. These results indicate that *Pseudomonas* sp. DJ12-C kept to possess the same substrate specificity of *Pseudomonas* sp. DJ-12 and *Pseudomonas* sp. P20, but the degrading activities to the substrates were improved. Furukawa *et al.* (6) and Hirose *et al.* (8) also reported that the recombinant strains constructed with *bph* and *tod* hybrid gene cluster showed better degrading activity and wider substrate range than the host strains.

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