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Improved Degradation of 4-Chlorobiphenyl, 2,3-Dihydroxybiphenyl, and Catecholic Compounds by Recombinant Bacterial Strains

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Abstract The *pcbC* gene encoding (4-chloro-)2,3-dihydroxybiphenyl dioxygenase was cloned from the genomic DNA of *Pseudomonas* sp. P20 using pKT230 to construct pKK1. A recombinant strain, *E. coli* KK1, was selected by transforming the pKK1 into *E. coli* XL1-Blue. Another recombinant strain, *Pseudomonas* sp. DJP-120, was obtained by transferring the pKK1 of *E. coli* KK1 into *Pseudomonas* sp. DJ-12 by conjugation. Both recombinant strains showed a 23.7 to 26.5 fold increase in the degradation activity to 2,3-dihydroxybiphenyl compared with that of the natural isolate, *Pseudomonas* sp. DJ-12. The DJP-120 strain showed 24.5, 3.5, and 4.8 fold higher degradation activities to 4-chlorobiphenyl, catechol, and 3-methylcatechol than DJ-12 strain, respectively. The pKK1 plasmid of both strains and their ability to degrade 2,3-dihydroxybiphenyl were stable even after about 1,200 generations.

Keywords: biodegradation, recombinant DNA, 4CBP, 2,3-DHBP, catecholic compounds

INTRODUCTION

Chlorinated aromatic hydrocarbons are major environmental pollutants, because they are toxic and resistant to degradation and accumulate in sediment and biota [1]. Among them, the polychlorinated biphenyls (PCBs), chlorobenzene, and chlorophenoxyacetate have been extensively studied in terms of their biodegradability by many soil and aquatic microorganisms [1-3]. Catechol and catecholic compounds, which are common intermediate metabolites produced via the degradation pathway of the aromatics, have been extensively studied in terms of the biodegradation by microorganisms [4,5].

Most of the naturally occurring microorganisms are reported to metabolize limited groups of aromatic pollutants, and these organisms show stringent specificities for particular steps of the degradative pathways [6]. Therefore, attempts to develop strains capable of degrading efficiently a wide variety of the pollutants have been made by molecular recombination using their existing genes to target individual pollutants [7-9]. Recently, Ogawa and Miyashita [10] demonstrated an effective conjugal recombination of a 3-chlorobenzoate (3CBA) catabolic pENH91 plasmid from *Alcaligenes euthrophus* NH9 and 3CBA negative strains. The recombinant strains effectively degraded test compounds under laboratory conditions, but failed to execute the task of decontamination under natural conditions [1].

In the upper four-step pathway of PCB and biphenyl degradation, the extradiol (*meta*) cleavage of the benzene ring by 2,3-dihydroxybiphenyl (2,3-DHBP) dioxygenase represents the most crucial step [11]. For this reason, the *pcbC* gene has been intensively studied for its genetic structure and the regulation of the gene expression using different vectors in many bacterial strains. *Pseudomonas* sp. DJ-12 and *Pseudomonas* sp. P20 strains isolated as 4-chlorobiphenyl (4CBP) degraders from contaminated soil were found to be capable of degrading 4CBP to 4-chlorobenzoate (4CBA) via a *meta*-cleavage pathway, as reported previously [8,12,13]. *Pseudomonas* sp. DJ-12 further degraded 4CBA, but *Pseudomonas* sp. P20 could not. However, the *meta*-cleavage activity of dioxygenase encoded by the *pcbC* gene of *Pseudomonas* sp. P20 was higher than that of *Pseudomonas* sp. DJ-12. The nucleotide sequences of the *pcbC* genes of *Pseudomonas* sp. P20 have already been studied [14]. In this study, therefore, we constructed two recombinant strains, *E. coli* KK1 and *Pseudomonas* sp. DJP-120, from the above natural isolates in order to improve their degradation activity on 2,3-DHBP and 4CBP, respectively, and catecholic compounds.

MATERIALS AND METHODS

Bacterial Strains and Growth

Pseudomonas sp. DJ-12 and *Pseudomonas* sp. P20 are

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Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant markers	Sources
Bacterial strains		
Pseudomonas sp. P20	Natural isolate, 4CBP$^+$, 2,3-DHBP$^+$, 4CBA$^-$	13
Pseudomonas sp. DJ-12	Natural isolate, 4CBP$^+$, 2,3-DHBP$^+$, 4CBA$^+$	13
E. coli XL1-Blue	Host strain, F "	Stratagene Ltd.
E. coli KK1	Recombinant strain, 4CBP$^+$ 2,3-DHBP$^+$, 4CBA$^-$	This study
Pseudomonas sp. DJP-120	Recombinant strain, 2,3-DHBP$^+$, 4CBA$^+$	This study
Plasmids		
pBluescript SK(+)	Cloning vector, Ap$^+$	Stratagene Ltd.
pKT230	Cloning vector, Mob$^+$, Sm$^+$, Km$^+$	4
pCK1	*pcbABCD*, 14 kb *Eco*RI fragment of *Pseudomonas* sp. P20 cloned in pBluescript SK(+), Ap$^+$	16
pCK102	*pcbCD*, subclone containing 6.3 kb *Eco*RI fragment of pCK1, Ap$^+$	16
pKK1	*pcbC*, 1.95 kb *Eco*RI fragment of pCK102/SK(+), Km$^+$	Stratagene Ltd.

bacterial strains that were isolated from contaminated soils, as shown in Table 1. Their ability to biodegrade some aromatic hydrocarbons is shown in Table 2. The organisms were grown in MM2 minimal medium containing 1 mM of 4CBP as a carbon and energy source [9,12]. Cloned cells were grown in Luria-Bertani (LB) medium containing 100 μg/mL of ampicillin and 30 μg/mL of kanamycin.

Construction of Recombinant Strains

Chromosomal DNA isolated from *Pseudomonas* sp. P20 was digested with *Eco*RI and cloned in pBluescript SK(+) vector using T4 DNA ligase, according to the methods described by Sambrook *et al.* [15]. Plasmid DNA was prepared by the alkaline-SDS method and subsequent DNA manipulation was performed as described by Nam *et al.* [16]. The plasmids used and constructed for this study are shown in Table 1. The subclones of pCK102, pCK1022, and pCK1024 were constructed with various endonucleases in the manner shown in Fig. 1. The *pcbC* gene encoding 4-chloro-2,3-dihydroxybiphenyl (4C-2,3DHBP) dioxygenase was cloned again from pCK102 using pKT230 vector to construct pKK1 recombinant plasmid. By transforming the pKK1 into *E. coli* XL1-Blue, *E. coli* KK1 was selected as a recombinant strain showing potential dioxygenase activity to 4C-2,3DHBP. The pKK1 plasmid carrying the *pcbC* gene was transferred into *Pseudomonas* sp. DJ-12 from *E. coli* KK1 using the filter disk conjugation procedure described by Top *et al.* [17]. The cells resuspended from the filter disk were cultivated on LB agar medium containing antibiotics at 30°C. *Pseudomonas* sp. DJP-120 were selected as a transconjugant by its yellow color when sprayed with 2,3-DHBP solution as described by Nam *et al.* [16].

Table 2. Biodegradability of 4-chlorobiphenyl and catecholic compounds by two natural isolates

Strain	Biodegradability							
	4CBP	4CBA	BP	BA	Cat	3MC	4MC	4CC
Pseudomonas sp. DJ-12	++	++	+++	++	+	++	–	+
Pseudomonas sp. P20	+++	–	+++	–	+	+	–	+

Abbreviation: 4CBP, 4-chlorobiphenyl; 4CBA, 4-chlorobenzoate; BP, biphenyl; BA, benzoate; Cat, catechol; 3MC, 3-methylcatechol; 4MC, 4-methylcatechol; 4CC, 4-chlorocatechol. +++, 100% degradation of 1 mM substrate after 24 h; ++, 80%; +, 50%; –, no degradation.

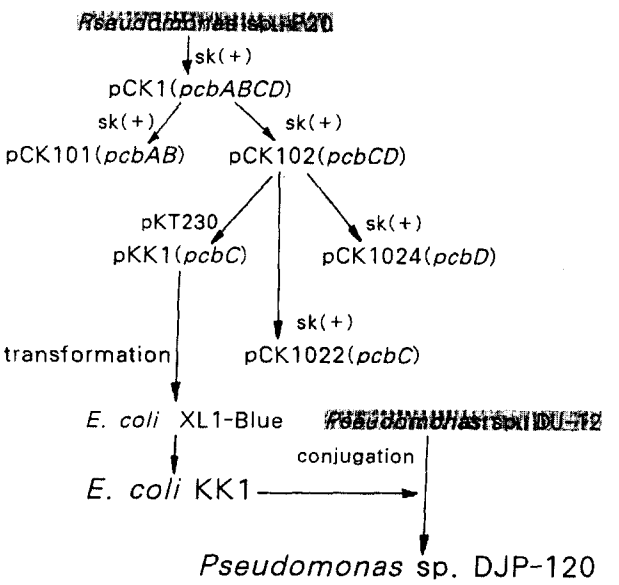

Fig. 1. Construction flow of the recombinant plasmids and strains. SK(+), pBluescript SK(+) vector.

Assay for Degradation Activity

The organisms were cultivated in MM2 medium containing 1 mM of each compound. The cells, collected at the appropriate time, were mixed with the same volume of ethanol as the culture, and the aqueous solution was then examined for remaining amounts of the compounds. For the resting cell assay, the cells (about 10^8 CFU/mL) grown in LB broth were harvested and washed with 10 mM of phosphate buffer (pH 7.0). The cells were then suspended in 1/10 volume of the same buffer and incubated with 1 mM of each compound at 30°C as described by Nam *et al.* [16]. The cell-free supernatants were analyzed with a spectrophotometer

Table 3. Degradation of 2,3-dihydroxybiphenyl by two parents and two recombinant strains, and their growths

Strain	2,3-DHBP (A_{430}) after incubation for(h)			Growth (A_{600}) after incubation for(h)		
	0	12	24	0	12	24
Parent strain						
Pseudomonas sp. DJ-12	2.2	1.2 (46)*	0.4 (82)*	0.6	1.0 (40)†	2.1 (71)†
Pseudomonas sp. P20	2.3	1.3 (44)*	0.1 (96)*	0.6	1.8 (67)†	2.6 (77)†
Recombinant strain						
E. coli KK1	2.4	0.1 (96)*	0 (100)*	0.6	2.6 (77)†	2.8 (79)†
Pseudomonas sp. DJP-120	2.5	0.2 (92)*	0 (100)*	0.6	2.4 (75)†	2.5 (76)†

*: The numbers in parenthesis indicate the percentage degradation of 2,3-DHBP concentration relative to the initial concentration

†: The numbers in parenthesis indicate the percentage of growth relative to the cell density at zero time

Table 4. Specific degradation activity of the parents and recombinant strains to 2,3-dihydroxybiphenyl and catecholic compounds

Strain	Specific degradation activity (mU/mg)				
	4CBP	2,3-DHBP	Cat	3MC	4CC
Parent strain					
E. coli XL1-Blue	0	0	0	0	0
Pseudomonas sp. DJ-12	24.6	25.5	4.4	25.6	2.1
Recombinant strain					
E. coli KK1	0 (0)	675 (26.5)	2.7 (0.6)	51.3 (2.0)	2.9 (1.4)
Pseudomonas sp. DJP-120	602 (24.5)	605 (23.7)	15.4 (3.5)	121.9 (4.8)	2.2 (1.1)

Specific activity was determined by reacting 1 mg of crude extract with 25 μM of substrate at 25°C as described by Hirose *et al.* [8]. Units are expressed in micromoles of HOPDA (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) produced per minute. The numbers in parenthesis indicate the fold increases of the specific activities relative to those of *Pseudomonas* sp. DJ-12.

(Pharmacia LKB, Bromma, Sweden) to determine their degradation activity on the compounds. The substrate specificity of the organisms for several aromatic hydrocarbons was examined using the resting cell assay as described by Hirose *et al.* [8]. The *meta*-cleavage products (MCPs) produced from the substrates were measured using a spectrophotometer [12,14].

Stability Test for pKK1 and Degradation Activity

The recombinant strains were cultivated on LB agar medium without antibiotics for a period of 30 days and the organisms were transferred onto fresh medium every day. The organisms sampled on each day were cultivated again on LB agar medium with and without antibiotics, and the stability of the recombinant plasmid pKK1 calculated by counting the colonies developed on both plates. The degradation activity of the strains upon 2,3-DHBP was examined during the experiment using the methods described above, and calculated as the percentage degradation relative to that of zero time.

RESULTS AND DISCUSSION

Biodegradation of Pollutant Chemicals

The degradation activities of the recombinant strains and their parents upon 2,3-DHBP are shown in Table 3. The parent strains degraded 44-46% of 2,3-DHBP after 12 h incubation, and 82-96% after 24 h. On the other hand, the recombinant strains degraded 92-96% of 2,3-DHBP after 12 h incubation and 100% after 24 h. The growth patterns of the recombinant strains were also slightly improved as compared to those of their parents, but their growth rates were not as notable as their degradation rates. The specific dioxygenase activities of the recombinant strains to 4CBP, 2,3-DHBP and catecholic compounds are compared with those of natural isolates in Table 4. The degradation activities of recombinant strains upon 2,3-DHBP were increased to 23.7-26.5 fold that of *Pseudomonas* sp. DJ-12. In particular, *Pseudomonas* sp. DJP-120 showed the improved degradation activity on 4CBP as high as that on 2,3-DHBP.

These results are similar to those of Lee *et al.* [18] that a hybrid strain combined the *tod* and *tol* pathways and mineralized actively benzene, toluene, and *p*-xylene without accumulating any metabolic intermediates. Kimbara *et al.* [19] cloned *bphCD* from PCB-degrading *Pseudomonas* sp. KKS102 using the broad host-range cosmid vector, pCP13, to construct a recombinant plasmid pKS13. When the *bphCD* genes of pKS13 were transformed into *E. coli* and *Pseudomonas putida*, *bphC* encoding 2,3-DHBP dioxygenase was expressed only in *Pseudomonas putida*. On the other hand, the *bphC* gene was expressed in *E. coli* when *bphCD* was cloned using pUC18. These results indicate that the dioxygenase gene could be expressed in *Pseudomonas putida* by using its own promoter. Therefore, the over-expression of *pcbC* encoding 2,3-DHBP dioxygenase in *Pseudomonas* sp. DJP-120 is thought to be accelerated by both promoters in the *pcbC* genes of *Pseudomonas* sp. DJ-12 and pKK1 plasmid originally cloned from *Pseudomonas* sp. P20.

The *meta*-cleavage (dioxygenase) activity of *E. coli* KK1 on 3-methylcatechol (3MC) was increased to twice that of *Pseudomonas* sp. DJ-12, but the activities of *Pseudomonas* sp. DJP-120 to catechol and 3MC showed

3.5 and 4.8 fold increases, respectively. This means that the recombinant DNA technique using the broad host-range vector, pKT230, allowed improvement in the degradation activity of the strains without altering their substrate specificity. Furukawa *et al.* [9] and Hirose *et al.* [8] also reported that the recombinant strains constructed with *bph* and *tod* hybrid gene cluster showed a better degrading activity and a wider substrate range than the host strains.

Stability of Degradation Activity

The stability of pKK1 plasmids carrying the *pcbC* gene in the recombinant strains was quite persistent as shown in Fig. 2. About 70% of *E. coli* KK1 was observed to retain the pKK1 plasmid after about 1,200 generations, and *Pseudomonas* sp. DJP-120 showed about 55% retention. Moreover, both recombinant strains retained 100% dioxygenase activity to 2,3-DHBP.

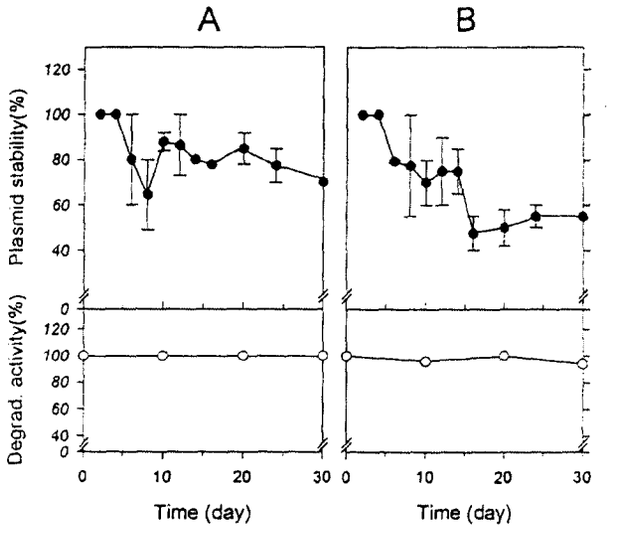

Fig. 2. Stability of the recombinant plasmids and the biodegradation activity of the recombinant strains. Degrading activity was examined for 2,3-dihydroxybiphenyl dioxygenase, and plasmid stability was determined as a percentage of the CFUs grown on antibiotic LB medium over those of the antibiotic-free medium. A. *E. coli* KK1; B, *Pseudomonas* sp. DJP-120.

CONCLUSION

The recombinant strains, *E. coli* KK1 and *Pseudomonas* sp. DJP-120, which were constructed by molecular cloning of the *pcbC* gene using pKT230 vector, revealed about 23.7 and 26.5 fold increases in degradation activity on 2,3-DHBP, respectively, compared with that of *Pseudomonas* sp. DJ-12 parent strain. *Pseudomonas* DJP-120 also showed 24.5, 3.5, and 4.8 fold increased degradation activities on 4CBP, catechol, and 3-methylcatechol compared to DJ-12 strain, respectively. The degradation activity of the strains on 2,3-DHBP and the pKK1 recombinant plasmid carrying *pcbC* gene were quite stable even after about 1,200 generations. This means that the recombinant DNA technique using a broad host-range pKT230 vector has the ability to improve the biodegrading activity of microbial isolates on aromatic pollutants.

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