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Structural and Functional Stability of the Genetic Recombinant Plasmid pCU103 in Different Water Environments

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The stability of the genetically engineered microorganisms and their recombinant plasmids released in natural environments has been regarded as one of the molecular ecological topics. In this study, the recombinant plasmids pCU103 in which the *pcbCD* genes involved in biodegradation of biphenyl and 4-chlorobiphenyl were cloned in pBluescript SK(+) vector, were examined for their structural and functional stability in different waters at 15°C by the methods of electrophoresis, Southern hybridization, quantification with fluorescent dye, and transformation. The recombinant plamids maintained their stabilities for about 30 days in sterilized distilled water (SDW), 15 days in autoclaved creek water (AW), 25 days in filtered and autoclaved non-sterile creek water (FAW), 4 days in Luria-Bertani (LB) broth, and less than one day in filtered non-sterile creek water (FW). The covalently closed circular (CCC) form of the plasmid was decreased and open circular (OC) form was increased as a function of incubation time, and then linear (L) form was produced to be ultimately degraded out. The degradation rates of the plasmid were proportionally correlated to trophic level of the water, and the biological factor such as DNases was found to be one of the most critical factors affecting structural and functional stability of the plasmid in non-sterile natural water.

Keywords: Recombinant plasmid, pCU103, persistence, transformation, water microcosm.

Genetic transformation is recognized to be one of the potential transfer mechanisms of the genes occurring among bacteria in natural environments. It is processed for recipient organisms to take up extracellular DNA released by excretion or lysis of the cells (16, 19, 26). This potential is based on the facts that natural environments contain large amount of extracellular DNAs and bacterial species which develop to be competent to acquire DNA (7, 10).

Several studies (2, 6) have reported that significant amounts of DNA are dissolved in environmental samples, and a number of studies on interaction of the DNA molecules and bacterial cells have been conducted in microcosm (15, 17, 23, 29). Transformation activity of the free DNA was studied in water environments with *Vibrio* strain (8), *Pseudomonas stutzeri* (3, 29), and *Acinetobacter calcoaceticus* (3). The frequencies of transformation were reported to be affected by environmental factors, such as amount of DNA (29), extracellular DNases (23), and minerals (4, 27).

Since the DNA recombination techniques are widely used in almost all the biological laboratories, the genetically recombinated DNAs and the genetically engineered microorganisms (GEMs) have been regarded as possible components to cause a safety problem in natural environments in terms of their sources of genetic information. The fates of them released into natural environments and their impacts to the ecosystem have not been understood completely. Kloos *et al.* (12) developed systems which could be used to study transformation involving DNA released by lysis of dying donor cells, and reported that DNA released from pDKL01-containing bacteria was degraded immediately after release in the culture broth, whereas that from pDKL02-containing bacteria persisted for days. This means that structural integrity and transformation activity of recombinant DNA are different depending upon the kinds of genes and vectors, recipient strains, and environmental factors of the

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ecosystem involved in the study.

In this study, therefore, the recombinant plasmid of pCU103 in which *pcbCD* genes were cloned in pBluescript SK(+) plasmid vector as reported previously by Kim *et al.* (11) was examined for its structural and functional stability when suspended in microcosms of different waters.

Materials and Methods

Recombinant pCU103 plasmid

The recombinant plasmid pCU103 used in this study was constructed by cloning the *pcbCD* genes responsible for meta-cleavage in degradation of biphenyl and 4-chlorobiphenyl using pBluescript SK(+) vector as previously reported by Kim *et al.* (11). The size and restriction map of pCU103 are as shown in Fig. 1. The pCU103 was isolated from the cloned cells of *E. coli* CU103 containing the recombinant plasmid by the alkaline lysis methods described by Koetsier *et al.* (13). The cells pelleted from the *E. coli* CU103 culture grown in Luria-Bertani (LB) broth for 24 hours were suspended in solution I (50 mM glucose; Tris-HCl, pH 8.0; 10 mM EDTA) and then destructed in solution II (0.2N NaOH; 1% SDS). After adding the solution III (5 M potassium acetate, pH 4.8; glacial acetic acid; H_2O) to the mixture and placing it on ice for 5 minutes, the mixture was centrifuged at 12,000×g for 15 minutes. The supernatant was mixed with 0.6 volume of 99.8% isopropanol and centrifuged at 12,000×g for 15 minutes. The pellet of the plasmid DNA was redissolved in TE buffer (pH 8.0) and stored at -20°C until use.

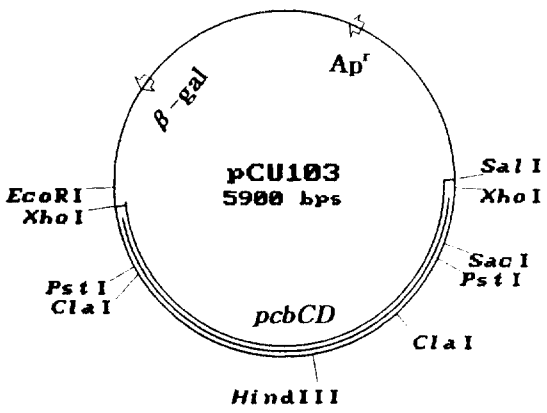

Fig. 1. The recombinant plasmid pCU103 carrying *pcbCD* genes.

Water microcosms

The water microcosms were provided as described by Park *et al.* (21). The 5 ml capped tube were filled with 4 ml of different waters: sterile distilled water (SDW), Luria-Bertani broth (LB), or Mooshimcheon creek waters in Cheongju, Korea, which were filtered through 0.2 μm Nuclepore membrane filter (FW), autoclaved (AW), or filtered and autoclaved (FAW). The microcosms were suspended with 0.3 mg/ml of pCU103 plasmid DNA. Water temperature of the microcosms was controlled to 15°C during the experiment.

Agarose gel electrophoresis

The plasmid DNA samples taken from the microcosms at appropriate time were electrophoresed on 0.8% agarose slab gel by the methods described by Sambrook *et al.* (28). If necessary, the plasmid in the sample was digested with restriction endonucleases according to the instructions of the manufacturer (POSCOCHEM, Korea). Electrophoresis was performed in TBE buffer or TAE buffer with a voltage gradient of 5 v/cm for 1.5 hours. After electrophresis, the gels were stained with ethidium bromide (0.5 μg/ml) in TBE buffer for 40 min, and then photographed with UV SLII camera system under 305 nm UV transilluminator (Spectronics Co., Westbury, N.Y., USA).

DNA hybridization

The 2.8 kb *Hin*dIII-X*ho*I fragments of pCU103 containing *pcbCD* genes were electrophoresed in 0.8% agarose gel. The fragments were eluted from the gel by using the Geneclean II kit (Bio 101 Co., La Jolla, CA, USA). The probe DNA of the fragment was nick translated with a translation system and biotin-14-dATP as described in the instructions of BRL (1). DNA hybridization was performed as described in the blotting and hybridization protocol for the Hybond membranes of Amersham Co. (1). Agarose gel electrophoresed with pCU103 plasmids was incubated in 0.25 N HCl for about 20 minutes until the bromophenol blue marker turned yellow, and then denatured with denaturation solution (1.5 M NaCl; 0.5 N NaOH). After the gel was neutralized in neutralization buffer (1 M Tris; 1.5 M NaCl, pH 7.4), plasmid DNA in the gel was transferred to a nylon membrane (Hybond-N, Amersham International plc., Amersham, UK) using 1N NaOH for 2 hours. The membrane was briefly washed with 2× standard saline citrate (SSC) solution. The membrane was baked at 80°C for 2 hours for adsorption of the plasmid DNA to the membrane. Hybridization was performed overnight at 42°C using the solution (2× SSC, 1% SDS, 0.5% fat-free milk powder. 0.5 mg/ml of denatured salmon sperm DNA, 10% dextran sulfate, and 0.1~0.5 μl/ml of denatured DNA probes) for 12 to 16 hours. The hybridized DNAs were detected by the Bluegene non-radioactive nucleic acid detection system (BRL, Gaithersberg, MD, USA).

Transformation

The competent cells of *E. coli* XL1-Blue (0.2 ml containing about 10^5 to 10^9 cells) were mixed with 1 μl of the water containing the plasmid DNA and 3 μl of dimethyl sulfoxide as described in the previous report (14). After placing the mixture on ice for 30 min and shocking at 42°C, the cells were incubated at 37°C for one hour. 200 μl of the cultures was plated on LB agar plate containing 100 μg/ml ampicillin and 15 μg/ml tetracycline for selection of transformants. The transformant cells were counted as number of the colonies turning yellow, when sprayed over the plates with 0.1 % 2,3-dihydroxybiphenyl in ethanol.

Quantification of the plasmid DNA

The quantification of pCU103 plasmid DNA suspended in waters was carried out by the method described by Paul and Myers (24). Two milliliters of SSC (standard saline citrate) containing 0.5×10^{-7} M bisbenzimide (Hoechst 33258, Polysciences, Inc. Warrington, USA) and 2 μl of water samples taken from the microcosms were mixed in a fluoresence cuvette. After reaction for 1 minute, the fluoresence was measured at wavelength of 365 nm with a TKO 100 fluoresence spectrophotometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

Results and Discussion

Structural integrity of pCU103

The pCU103 is a recombinant plasmid, as previously reported by Park *et al.* (21), which pBluescript SK(+) derivative containing the 3.0 kb DNA sequence carrying *pcbCD* genes responsible for benzene-ring cleavage of 2,3-dihydroxybiphenyl in the process of biphenyl and 4-chlorobiphenyl degradation. The pCU103 plasmid suspended in the water microcosms revealed two bands, as seen in the lane 1 of Fig. 2, the plasmids of bottom and upper bands, each of which was proved to be covalently closed circular (CCC) and open circular (OC) structures, respectively, as reported previously (14). When each band of pCU103 separately eluted from the agarose gel was electrophoresed again, the two bands were also observed in the gel. In the electrophoresis performed with each band of pCU103 which was digested with the same restriction endonucleases, exactly the same number and size of DNA fragments were produced in both samples of OC (lane 2~4) and CCC forms (lane 5~7) in Fig. 2.

Such structural conversions of the plasmids were reported in sterile and non-sterile natural waters. Paget *et al.* (20) and Gallori *et al.* (9) observed that the three forms of plasmids were produced when pBR328 and pHV14 recombinant plasmids were suspended in sterile water and buffer added with clay particles, respectively. Conversion of the supercoiled plasmid pBR322 DNA to the open-circular and then to linear form was reported by Phillips *et al.* (25) to occur within 20 min when exposed to wastewater.

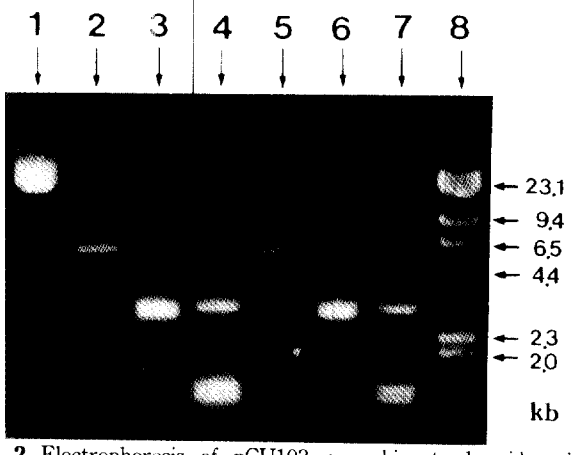

Fig. 2. Electrophoresis of pCU103 recombinant plasmid and its fragments digested with several endonucleases. Lanes: 1, pCU103; 2~4, enzymes (*Sal*I, *Xho*I, and *Hin*dIII)-digested upper band eluted from lane 1; 5~7, enzymes (*Sal*I, *Xho*I, and *Hin*dIII)-digested bottom band eluted from lane 1; 8, λ-*Hin*dIII size marker.

Persistence of pCU103 in different waters

When the pCU103 was suspended in different water microcosms, such two forms of the plasmid were observed at zero time of incubation. The structural integrity of pCU103 was changed as a function of incubation time, but the extent of structural change varied with the kinds of waters which the plasmid was suspended in. After exposure of pCU103 plasmid in different water for an appropriate period of time, the plasmids were electrophoresed in agarose gel and then hybridized with the *pcbCD* gene probe to detect existence of the pCU103 containing *pcbCD* genes. Some representative electrophresis profiles of the pCU103 plasmids are shown in the panel I of Fig. 3. The pCU103 recombinant plasmids in SDW were incubated for 40 days, in FW for 10 days. They began to degrade and to reveal the linear form in AW incubated for 20 days and then were completely lost in the water incubated for 40 days. Such electrophoresis patterns of the plasmids (panel I in Fig. 3.) resulted from their persistence in different waters were exactly coincident with those (panel II) of Southern hybridization of the gel with *pcbCD* genes.

At the time when pCU103 began to degrade, linear (L) form of the plasmid always appeared in any water

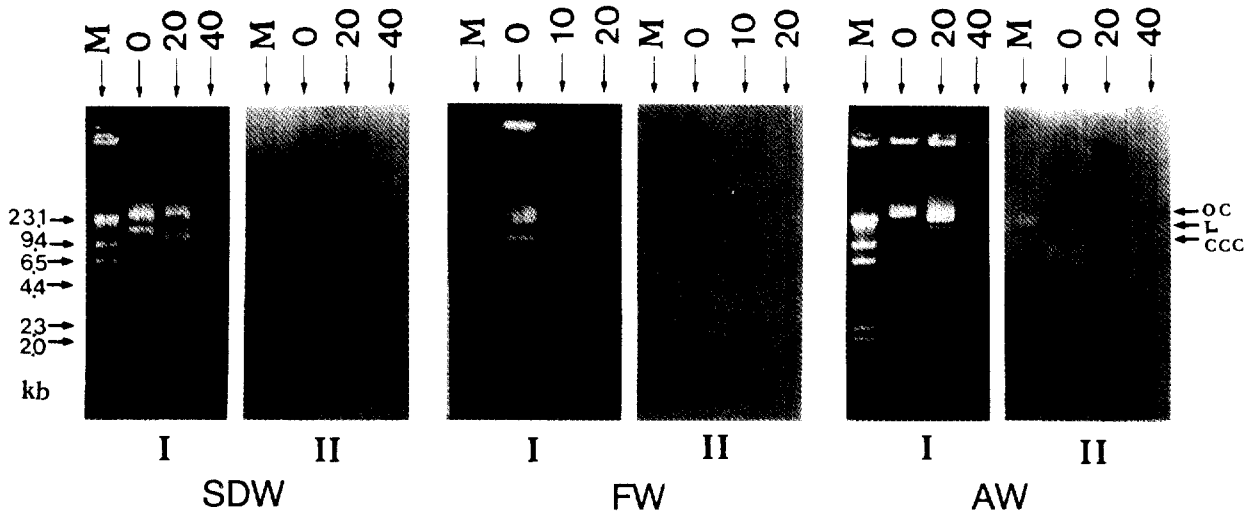

Fig. 3. Electrophoresis (I) of pCU103 plasmid suspended in different waters at 15℃ for various incubation time, and Southern hybridization (II) of the gel with DNA probe of *pcbCD* gene. Numbers indicate the period of incubation time (day). Abbreviations: M, λ-*Hin*dIII size marker; SDW, sterilized distilled water; FW, filtered river water; AW, autoclaved river water; OC, open circular; L, linear; CCC, covalently closed circular.

microcosms. But the duration for persistence of pCU103 was different depending upon the water quality (Table 1). In sterile distilled water (SDW) and filtered autoclaved creek water (FAW), pCU103 sustained its persistence for upto about 30 days. The pCU103 persisted for at least 4 and 15 days in Luria-Bertani (LB) and autoclaved creek water (AW), respectively. But the recombinant plasmid began to degrade within one day incubation in non-sterile filtered creek water (FW). At the time when pCU103 plasmid began to degrade, it was founded that the linear (L) form first appeared and the CCC form began to disappear, and then both OC and L forms were finally degraded and completely disappeared.

The combined data indicate that considerable quantity of the extracellular DNA is present in water environments (7, 18, 24) and continuously released by normal and genetically engineered microorganisms (21, 22). Extracellular DNA was reported to be degraded with half-lives of several hours in natural freshwater and marine water (18). The nucleases, particularly DNase I, have been proved to be the most crucial factor affecting the rate of DNA degradation in non-sterile natural waters (16, 20, 23). But the suspended particulates and cations in water and clay particles in soil were well recognized to adsorb DNA molecules and to protect them from degradation by the nucleases. Turk *et al.* (30) reported that DNA degradation rates were more than 10 times higher in the P-limited water than in the N-limited water. These imply that the trophic status of the water was correlated with the rate of DNA hydrolysis. Therefore, the degradation rates of the plasmids suspended in waters are thought to depend upon the environmental condition of water, such as biotic factors, particulates, trophic status, and etc.

Transformation activity of pCU103

Quantitative analysis of the recombinant pCU103 plasmid DNA suspended in different water microcosms was carried out by the bisbenzimide fluoresent method during the period of experiment. More than 1.7 ng/μl plasmid DNAs were detected until 30 days of incubation in SDW and AW as seen in Fig. 4. But the DNA was rapidly degraded down below about 1.2 ng/μl in 5 days in FW. Such a difference in persistence of the plasmid DNA resulted in different waters was very coincident with those of transformation activity of the DNA as shown in Fig. 5. These results indicated that the recombinant plasmid of pCU103 sustained its structural integrity and transformation activity for longer time in sterile water than in raw water, but the duration for its stability became shorter when the water was nutritionally enriched. On the other hand, the plasmids in non-sterile creek water such as FW were degraded out and lost their transformation activity right after 1 or 2 days' incubation.

The efficiency of transformation was known to be affected by the molecular size and double stranded conformation of the free DNA. Carlson *et al.* (4) reported that transformation frequencies were equally high for DNA of 10 and 60 kb, but decreased by more than 10-fold for DNA between 10 and 1 kb in *Pseudomonas stutzeri*. The quantitative measurement of extracellular DNA with fluorescent dyes was based on double strand-

Table 1. Summarized stability of pCU103 plasmid during incubation in different waters at 15℃

Water	Incubation Time (day)	Plasmid DNA on the gel*			
		No. of band	Form of DNA		
SDW	0	2	CCC	OC	
	1	2	CCC	OC	
	4	2	CCC	OC	
	7	2	CCC	OC	
	10	2	CCC	OC	
	15	2	CCC	OC	
	25	2	CCC	OC	
	30	2	CCC	OC	
	35	3	CCC	OC	L
	40	2		OC	L
	45	0			
LB	0	2	CCC	OC	
	1	2	CCC	OC	
	4	2	CCC	OC	
	7	3	CCC	OC	L
	10	2		OC	L
	15	2		OC	L
	25	0			
FW	0	2	CCC	OC	
	1	3	CCC	OC	L
	4	2		OC	L
	7	2		OC	L
	10	2		OC	L
	15	0			
AW	0	2	CCC	OC	
	1	2	CCC	OC	
	4	2	CCC	OC	
	7	2	CCC	OC	
	10	2	CCC	OC	
	15	2	CCC	OC	
	25	3	CCC	OC	L
	27	2		OC	L
	30	0			
FAW	0	2	CCC	OC	
	1	2	CCC	OC	
	4	2	CCC	OC	
	7	2	CCC	OC	
	10	2	CCC	OC	
	15	2	CCC	OC	
	25	2	CCC	OC	
	30	3	CCC	OC	L
	35	2		OC	L
	40	0			

*The data were analysed from the electrophoresis profiles of the plasmid suspended in water. Abbreviation: SDW, sterile distilled water; LB, Luria-Bertani broth; FW, filtered river water; AW, autoclaved river water; FAW, filtered autoclaved river water; CCC, covalently closed circular; OC, open circular; L, linear.

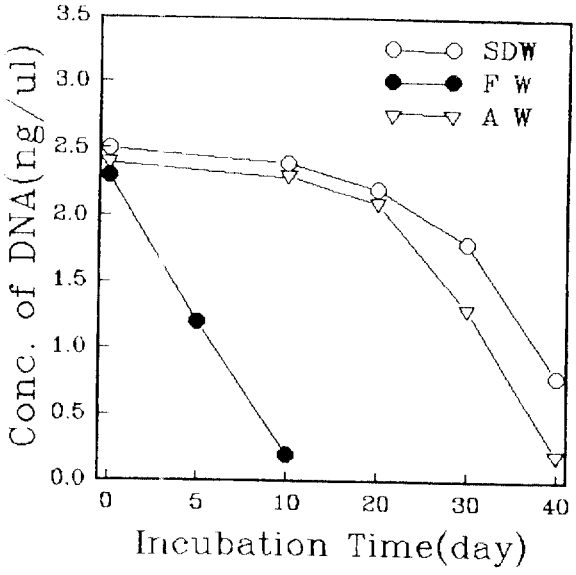

Fig. 4. Quantitative analysis of pCU103 recombinant plasmids suspended in different waters during incubation at 15℃. Abbreviations: SDW, sterilized distilled water; FW, filtered river water; AW, autoclaved river water.

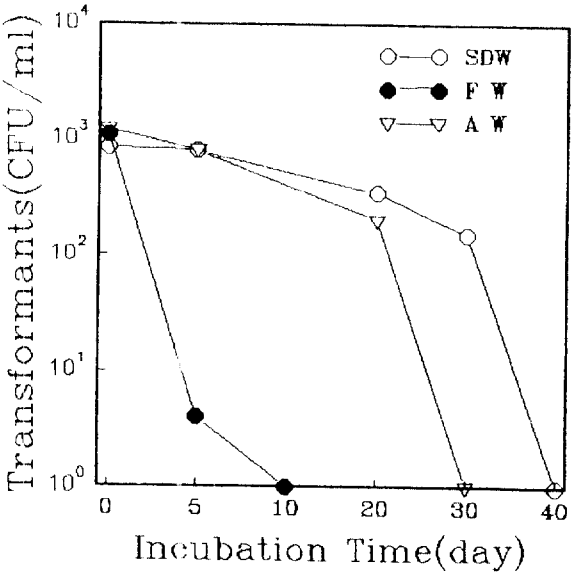

Fig. 5. Transformation activity of pCU103 recombinant plasmid suspended in different waters during incubation at 15℃. Abbreviations: SDW, sterilized distilled water; FW, filtered river water; AW, autoclaved river water.

edness of free DNA, as reported by DeFlaun *et al.* (6) and Paul & Myers (24). Therefore, our results of transformation activity with pCU103 obtained in this study indicate that the plasmids should be CCC and OC forms and double stranded in structure, and should be more than 1.7~2.0 ng/μl in concentration in order to exhibit their transformation activity in any waters.

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