Jour. Microbiol. September 1995 p. 178-183 Vol. 33, No. 3

Effect of Temperature on Persistence of Recombinant Plasmid pCU103 in Different Waters

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(Received July 10, 1995/Accepted September 11, 1995)

The recombinant plasmid of pCU103 constructed by cloning *pcbCD* genes in pBluescript SK(+) was studied for the effect of temperature on its persistence in different waters by the methods of electrophoresis, Southern hybridization, quantification, and transformation. The plasmid was very rapidly degraded out in non-sterile FW water without regards to water temperature, probably due to the effect of biochemical factor such as nucleases. The pCU103 was most persistent at 4°C in any water environments, moderately persistant at 15°C, but least stable at 30°C. Such results could be explained by the facts that hydrogen bonds in double-stranded plasmid DNAs become unstable and that nucleases are activated by increasing temperature. The intact structure of pCU103 was generally observed by gel electrophoresis under the conditions which the plasmid should be 2.0 ng/μl or higher in concentration and that about 10^2 CFU/ml or more transformant cells should be recovered.

Key words: Recombinant plasmid, pCU103, persistence, temperature effect, different waters

As DNA recombination techniques have been used in general biological laboratory and the genetically engineered microorganisms (GEMs) are frequently released into natural environments, the GEMs and their recombinant genes have become the subject of environmental scientific concerns in terms of their survivals and functional impacts to indigenous microorganisms (21, 22). The extracellular DNAs in natural environment are known to be produce by not only lysis of bacterial cells, but also active release from live organisms (10). These extracellular DNAs could be utilized by various microorganisms as precursors of nucleic acids and mineral nutrients sources, such as nitrogen and phosphorous. But more important role of the extracellular DNAs is recognized to be the genetic agents which could be transferred to other microorganisms.

In cases, furthermore, such extracellular DNAs in the environments were reported to be more stable than intact bacterial cells, and be transferred more easily intra- and inter-species of bacteria (3, 4, 5, 6, 16, 17). The genetically modified *E. coli* JM83 suspended in sea water was reported to become non-culturable state after 6 days, but the recombinant plasmid pUC18 released from the cells sustained its biological activity for up to 21 days (2). From the experiments with the GEMs in aquatic microcosms, however, Paul and David (15) reported that the stable extracellular nucleic acids released from the cells could be affected by various environmental factors.

The *pcbCD* genes responsible for meta-cleavage in degradation of biphenyl and 4-chlorobiphenyl were cloned from *Pseudomonas* sp. DJ-12 in this laboratory (8) and the cloned cell of *E. coli* CU103 including recombinant plasmid pCU103 showed same stability as the host in water environments (13). In this study, the pCU103 recombinant plasmids suspended in several water microcosms with different pH values were examined for their persistence by the methods of electrophoresis, Southern hybridization, quantitation with Hoechest 33258, and transformation of the plasmids.

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Materials and Methods

Preparation of pCU103 recombinant plasmid

The recombinant plasmid of pCU103 used in this study was constructed by cloning of *pcbCD* genes responsible for degradation of 2,3-dihydroxybiphenyl in pBluescript SK(+) vector as previously reported by Kim *et al.* (8). The size and restriction map of pCU103 were shown as Fig. 1. The pCU103 was isolated from the cloned cells of *E. coli* CU103 by the alkaline lysis methods described by Sambrook *et al.* (20). The cells pelleted from the Luria-Bertani (LB) culture grown for 24 hours were suspended in solution I (50 mM glucose; Tris-HCl, pH 8.0; 10 mM EDTA) and then destroyed in solution II (0.2 N NaOH; 1% SDS). After adding the solution III (5 M potassium acetate, pH 4.8; glacial acetic acid; H_2O) to the mixture and then storing the mixture on ice for 5 minutes, the tube was centrifuged at 12,000 ×g for 15 minutes. The supernatant was mixed with 0.6 volume of isopropanol and then centrifuged at 12,000 ×g for 15 minutes. The pellet of the plasmid DNA was redissolved in TE (pH 8.0) buffer and stored at −20℃ until use.

Water environments

The water environments were provided as described by Park *et al.* (13). The 5 ml cap tubes were filled with different waters: Luria-Bertani broth (LB), sterile distilled water (SDW), or Mooshimcheon river waters in Cheongju, which were filtered through 0.2 μm Nuclepore membrane filter (FW) and autoclaved (FAW) or autoclaved (AW). The test tubes filled with 4 ml of different waters were added with 0.3 mg/ml of pCU103 plasmid DNA. Water temperature was controlled to be 4℃, 15℃, and 30℃ during the period of experiment.

Enzyme digestion and electrophoresis

The plasmid DNA samples taken from the waters were subjected to horizontal slab agarose gel electrophoresis by the methods described by Sambrook *et al.* (20). When necessary, the plasmid was digested with several restriction endonucleases according to the instructions of the manufacturer (KOSCO Co. Korea). The 0.8% agarose gel was run with TBE buffer or TAE buffer. Electrophoresis was performed with a voltage gradient of 5 V/cm for 1.5 hours. After electrophoresis, the gels were stained in TBE buffer containing ethidium bromide (0.5 μg/ml) for 40 min, and then photographed with UV II camera system under UV transillumination (Spectronics Co., Westbury, N.Y., USA).

Southern hybridization with DNA probe

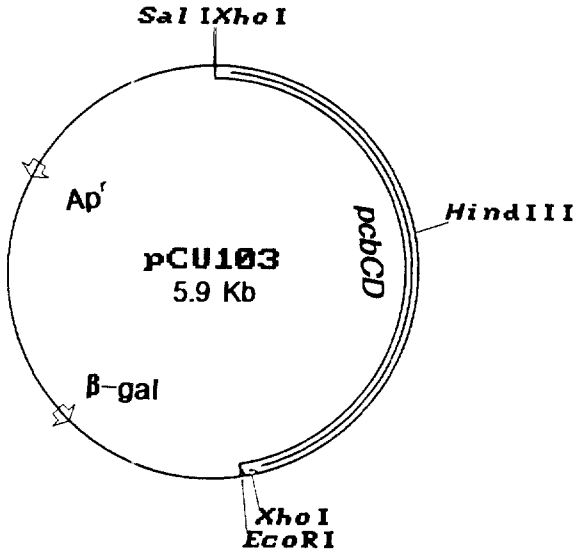

Fig. 1. Resriction map of pCU103 recombinant plasmid.

The pCU103 plasmid was digested with *Xho*I and *Hin*dIII and then electrophoresed in 0.8% agarose gel in TAE buffer. The 2.8 kb fragments containing *pcbCD* genes were eluted from the gel by using the GENECLEANE 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 (9) for the Hybond membranes of Amersham Co. Agarose gel was incubated in 0.25 N HCl for 20 minutes until the bromophenol blue marker turned yellow, denatured with denaturation solution (1.5 M NaCl; 0.5 N NaOH), and neutralized with neutralization buffer (1 M Tris; 1.5 M NaCl). After plasmid DNA was transferred to a nylon membrane (Hybond-N, Amersham International Plc., Amersham, UK) using 1 N NaOH for 2 hours according to the method described by Koetsier *et al.* (9), the membrane was briefly washed with 2×SSC solution for the membrane to be neutralized. The membrane was baked at 80℃ for the plasmid DNA to be absorbed to the membrane. Hybridization was performed overnight at 42℃ using the solution (2×SSC, 1% SDS, 0.5% fat-free milk powder, 0.5 mg/ml of denatured salmon sperm DNA and 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).

Quantification of pCU103 DNA

The method for quantitation of DNA was essentially an adaptation of the method described by Paul and My-

ers (14). Two milliliters of SSC (standard saline citrate) containing 0.5×10^{-7} M bisbenzimide fluorescent dye (Hoechst 33258) and 2 μl samples of blank or the plasmid DNA taken from the microcosms were placed in a fluorescence cuvette. After reaction for 1 minute, the fluorescence was measured at wavelength of 365 nm with a TKO 100 fluorescence spectrophotometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

Transformation

The competent cells of *E. coli* XL1-Blue (0.2 ml containing about $10^8\sim10^9$ cells) were incubated with 1 μl of the DNA suspension plus 3 μl of dimethyl sulfoxide on ice for 30 min. After shocked at 42℃, the mixtures were incubated at 37℃ for one hour. 200 μl of the cultures was spread on LB agar plate containing 100 μg/ml ampicillin and 15 μg/ml tetracycline for selection of transformants. The transformant cells were counted as the number of colonies turning yellow, when sprayed over the plate with 0.1% 2,3-dihydroxybiphenyl in ethanol.

Results and Discussion

Electrophoresis patterns of pCU103 suspended in water

The pCU103 is a recombinant plasmid, in which 2.8 kb *Eco*RI-*Sal*I fragment including *pcbCD* genes was cloned in pBluescript SK(+) vector. The recombinant plasmids suspended in the water were appeared in two forms of CCC (covalently closed circular) and OC (open circular) in electrophoresed gel as seen in Fig. 2. When each form of the plasmid eluted from the gel was electrophoresed, the two forms were reappeared. The fragments produced by digestion of each form with restriction enzymes were found to be just identical in size and number. Johnson and Grossman (7) reported that the CCC, linear (L), and OC forms of DNA with same molecular weight migrated at different rates through agarose gels. The CCC forms migrated faster than their linear counter part DNAs, and the OC molecules that lost their superhelicity migrated appreciably slower than either supercoils or linear molecules.

The recombinant plasmid pCU103 suspended in different waters at 4℃ showed both structural forms of the plasmid as seen in Fig. 3. The plasmid was quite stable keeping both forms for up to 60 days in the waters of SDW, LB, AW, FW, and FAW. But structure of the plasmid in FW water was changed to linear (L) form by incubation for 3 days, and the CCC form of the plasmid began to disappear after 30-day incubation. Such structural changes of the plasmid were also observed in the paper of Paget *et al.* (12).

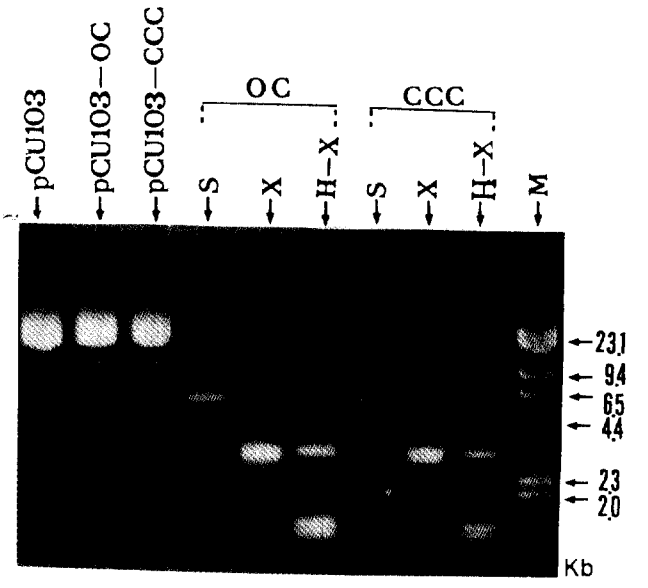

Fig. 2. Electrophoresis patterns of pCU103 recombinant plasmid and its fragments digested with several restriction endonucleases. pCU103-OC is the electrophoresis pattern of the open circular form (top band in the first lane) of pCU103 eluted from the gel and pCU103-CCC is the electrophoresis pattern of the covalently closed circular form (bottom band in the first lane) of plasmid eluted from the gel. S, *Sal*I; X, *Xho*I; H, *Hin*dIII; CCC, covalently closed circular form; OC, open circular form.

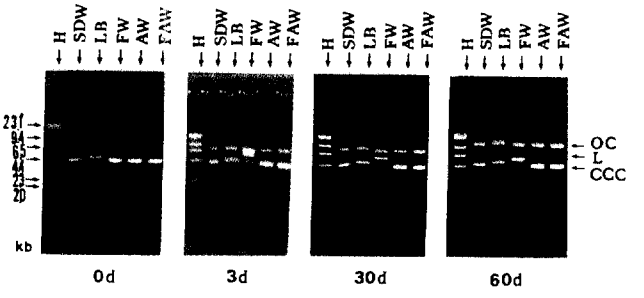

Fig. 3. Electrophoresis patterns of pCU103 recombinant plasmid DNAs suspended in different waters at 4℃. H, size marker of *Hin*dIII-digested lambda DNA. SDW, sterile distilled water; LB, Luria-Bertani broth; FW, filtered river water; AW, autoclaved river water; FAW, filtered autoclaved river water; OC, open circular form; CCC, covalently closed circular form; L, linear form.

Effect of water temperature on persistence of pCU103

The pCU103 was quite persistent in the water environments at 4℃, except for FW, for 60 days or longer period of time. Therefore, the persistence of the plasmid was comparatively studied at 15℃ and 30℃ in the waters of SDW, FW, and AW. The electrophoresis patterns and Southern hybridization of the pCU103 plasmids suspended in SDW during experiments of 40 days were shown in Fig. 4(A) and 4(B), respectively. The plasmid was observed to keep its structural integrity in the period of

20 days at 15℃, but the plasmid DNAs were disappeared in the samples of 40 days at 15℃ and 20 days at 30℃. The quantitative changes of the pCU103 suspended in SDW at 15℃ and 30℃ are shown in Fig. 5(A). About 2.0 ng/μl or higher concentration of pCU103 was maintained in SDW at 15℃ for 20 days, but it was rapidly decreased below 2.0 ng/μl after 10-day incubation at 30 ℃. Transformation activities of the plasmids suspended in SDW at 15℃ and 30℃ were compared as seen in Fig. 6(A). The plasmid lost its transformation activity more rapidly at 30℃ than at 15℃. More than 10^2 CFU/ml transformants were recovered in 20 days at 15℃, but the number of transformants at 30℃ were obtained only during the period of 10 days. The two forms of pCU103 plasmid were intactly observed only in zero time samples of FW at both 15℃ and 30℃, as seen in Fig. 7A. The *pcbCD* genes included in the plasmid were also detected by Southern hybridization in the zero time samples at 15℃ and 30℃ (Fig. 7B). The concentration of pCU103 in FW at 15℃ was rapidly decreased to 1.3 ng/μl in 10 days as seen in Fig. 5(B), and it decreased more quickly at 30℃. The transformants recovered from FW at 15℃ and 30℃ were lowered below 10^2 CFU/ml in 2 and 1 days, respectively (Fig. 6B).

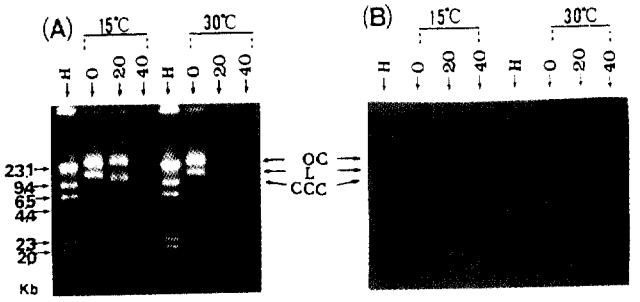

Fig. 4. Electrophoresis (A) of pCU103 suspended in SDW at 15℃ and 30℃ during incubation for various periods of time and Southern hybridization (B) of the gel with DNA probe. The numbers indicate period of incubation time in day. H, size marker of *Hin*dIII-digested lambda DNA.

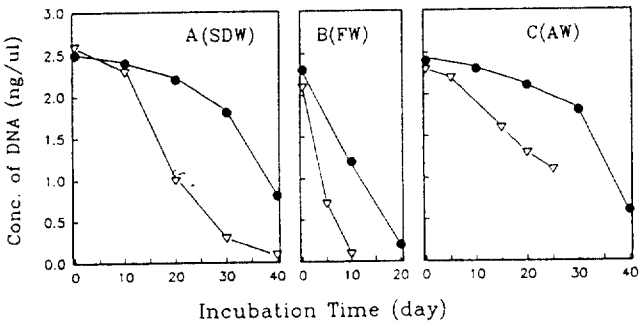

Fig. 5. Quantitative changes of pCU103 recombinant plasmid DNAs suspended in different waters during incubation at 15℃ (●--●) and 30℃ (▽--▽).

In AW water, which pCU103 was persistent at 4℃ for about 60 days, structural integrity of pCU103 was examined at 15℃ and 30℃ by electrophoresis and Southern hybridization as seen in Fig. 8A and 8B. The plasmid incubated at 15℃ for 20 days began to reform to linear (L) shape in structure, but the CCC form of the plasmid was completely disappeared in the sample incubated at 30℃ for 20 days. The result of Southern hybridization was perfectly in agreement with electrophoresis pattern. The concentration of pCU103 in AW were gradually decreased down below 2.0 ng/μl after incubation at 15℃ for 20 days and at 30℃ for 10 days as seen in Fig. 5C. The transformants of more than 10^2 CFU/ml were recovered from the AW samples incubated at 15℃ for 20 days and at 30℃ for 10 days (Fig. 6C).

Persistence of the recombinant plasmid pCU103 in water microcosms at different pH values could be generalized as shown in Table 1. The pCU103 was very persistent at 4℃ in any water environments and moderately stable at 15℃, but very vulnerable at 30℃. Mojica *et al.* (11) pointed that temperature was one of the factors affecting functional persistence and topology of extracellular plamsid DNAs in water environments, because hydrogen bonds in the double-stranded DNA could become

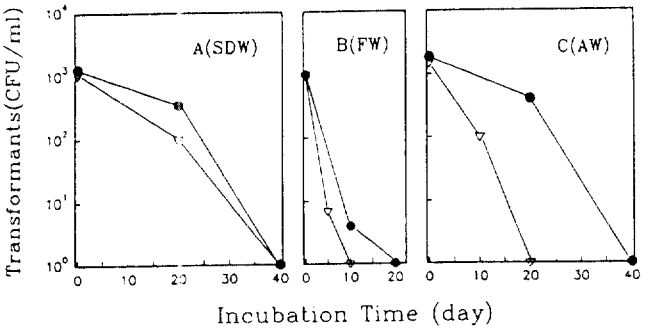

Fig. 6. Transforming activity of pCU103 recombinant plasmid DNAs suspended in different waters during incubation at 15℃ (●--●) and 30℃ (▽--▽).

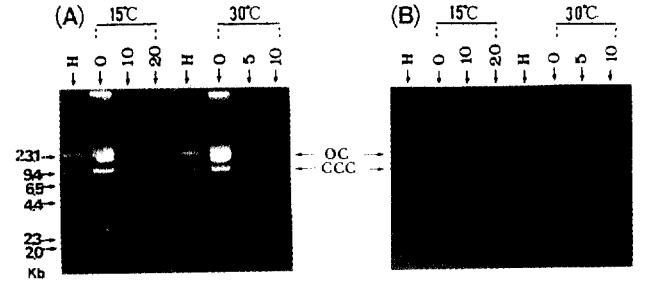

Fig. 7. Electrophoresis (A) of pCU103 suspended in FW at 15℃ and 30℃ during incubation for various periods of time and Southern hybridization (B) of the gel with DNA probe. The numbers indicate period of incubation time in day. H, size marker of *Hin*dIII-digested lambda DNA.

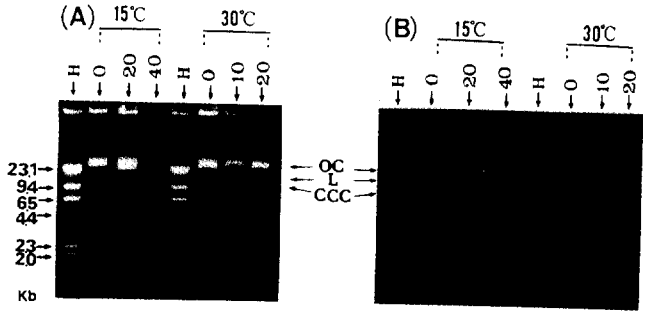

Fig. 8. Electrophoresis (A) of pCU103 suspended in AW at 15℃ and 30℃ during incubation for various periods of time and Southern hybridization (B) of the gel with DNA probe. The numbers indicate period of incubation time in day. H, size marker of *Hin*dIII-digested lambda DNA.

Table 1. Persistence of pCU103 in different waters at different temperatures.

Water Temperature (℃)	Persistence (day)		
	SDW	FW	AW
4	60	<3	<60
15	30	<2	<20
30	10	<1	<10

SDW, sterile distilled water; FW, filtered river water; AW, autoclaved river water.

unstable and various nucleases could be activated by increasing temperature. The structural integrity of pCU103 shown by gel electrophoresis was very coincident with the results of concentration and transformation activity of the plasmid in this study. In order for pCU103 plasmid to reveal its intact structure in electrophoresis and to recover the transformants of at least 10^2 CFU/ml, the concentration of the plasmid in the waters should be maintained about 2.0 ng/μl or higher.

The pCU103 plasmid was persistent for a long time in the SDW water, but its persistence in FW was last for only a few days. Romanowski *et al.* (18, 19) reported that pUC8-ISP plasmid inoculated in non-sterile soil environment was stable for only 2 to 3 days and half-time of transformation activity of the plasmid was 9.2 to 28.2 hours. In the non-sterile FW in this study, the pCU103 was persistent during only 3 days at any temperatures. Such results were in good agreement with those of Park *et al.* (13) which were obtained in non-sterile FW at 15℃. This means that the DNAses existing in the non-sterile FW might be one of the most critical factors to persistence of the recombinant plasmid pCU103, as indicated by Romanowski *et al.* (18).

Acknowledgements

The present studies were supported by the Basic Science Research Institute Program, Ministry of Education, project No. 94-4432 and in part, by grant from KOSEF (Research Center for Molecular Microbiology, SNU).

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