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Extracellular DNAs Released from the Genetically Engineered *E. coli* CU103 During Growth in Different Liquid Media

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During growth of the genetically engineered *E. coli* CU103 in different media, extracellular DNAs released from the cells were studied. The extracellular DNAs released in the medium were concentrated by an ethanol precipitation method and then quantified by a fluorescence method using Hoechst 33258. The released extracellular DNAs were also examined by gel electrophoresis and identified by Southern hybridization for the cloned *pcbCD* genes. The chromosomal DNAs and recombinant plasmid containing the cloned genes were observed to be released in an exponential growth phase. In Luria-Bertani (LB) broth and MM2-glucose, 210 and 69 ng/ml of DNAs were detected, respectively, after 3~4 days incubation at 30°C and at pH 7.0. But the released DNAs were measured to be about 10~15 ng/ml in filtered river water (FW) and Tris-EDTA (TE). The extracellular DNAs in the MM2-glucose (pH 7.0) were increased about 2~3 times more at 30°C than at both 15°C and 4°C, but the released DNAs were more easily degraded at the higher temperature. The extracellular DNAs were produced about 2 times more at pH 7.0 than at both pH 5.0 and pH 9.0 in MM2-glucose medium at 30°C. Therefore, the extracellular DNAs were found to be released actively from the cells during growth in liquid media.

Key words: Extracellular DNA, release, genetically engineered bacteria, liquid media

Extracellular DNAs are present in considerable quantity in natural environments, such as water, sediment, and soil. Extracellular DNAs are known to be released by lysis of the cells. Lysis was reported to be enhanced by autolysin (11), various cations and phosphates (23), and chemosmotic potential of the membrane (7). Catlin (3) reported first that DNAs could be released from essentially all the bacteria during growth in standard media. Extracellular DNAs were reported to be actively excreted from the bacterial cells introduced into aquatic and soil environments (12, 17). Dorward and Garon (5) reported that chromosomal and plasmid DNAs were externalized by a formation of membrane-derived vesicles in Gram-negative bacteria, but the physiological mechanism of that release is not understood.

Extracellular DNAs in natural environments seem to be mostly of microbial origin, and they have been detected by colorimetric methods and gel electrophoresis (4, 14, 18). DNAs are well recognized to possess the genetic information which can be transferred to other microorganisms by transformation. The correlation between the amount of free DNAs and transformation frequency has been reported in natural water environments.

Since DNA recombination techniques are widely used in almost all biological laboratories, genetically engineered DNA sequences and microorganisms are increasingly introduced into natural environments both by accident and on purpose. Paul and David (17) reported that DNAs were released similarly by normal and genetically engineered microorganisms introduced into an aquatic microcosm. Since the beginning of the debate on the safety and impact of the genetically engineered microorganisms (GEMs) on the natural ecosystem, the

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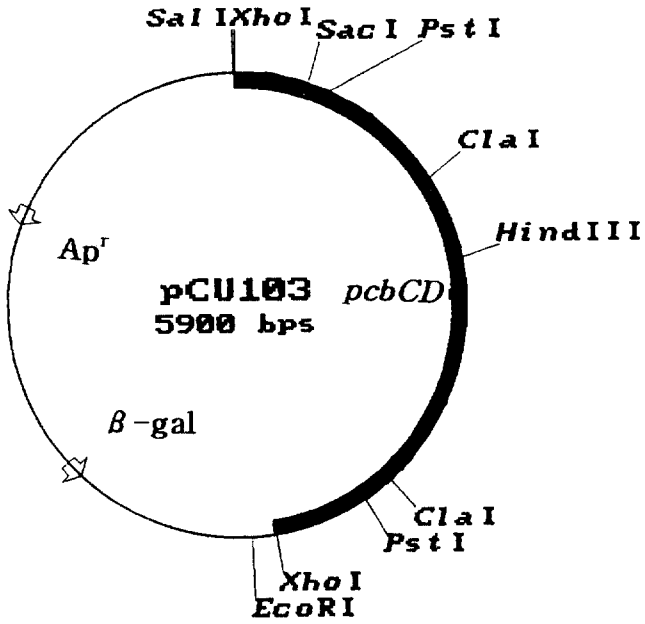

Fig. 1. Recombinant plsmid pCU103 included in *E. coli* CU103.

genetic and ecological aspects of the extracellular DNAs released from the GEMs are of growing interest to molecular ecologists.

In this study, therefore, extracellular DNAs released from the genetically engineered *E. coli* CU103 strain during growth was quantified by the method of Hoechst 33258 fluorescence, electrophoresis, and Southern hybridization. In addition, the effects of different media and water conditions on the release of the DNAs were comparatively examined.

Materials and Methods

Bacterial strain

The bacterial strain used in this study was a genetically engineered *E. coli* CU103 containing a pCU103 hybrid plasmid (Fig. 1) as described previously by Kim *et al.* (9). The hybrid plasmid was constructed by cloning the 2.8 kb genomic DNA fragment including the *pcbCD* genes in pBluescript SK(+) vector from *Pseudomonas* sp. DJ-12 degrading 4-chlorobiphenyl (8).

Experimental conditions

The liquid media used in this study were Luria-Bertani broth (LB), MM2 minimal medium supplemented with 0.1% glucose (MM2-glucose), Tris-EDTA buffer (TE), and a creek water filtered through 0.2 μm Nuclepore membrane (FW). *E. coli* CU103 was grown in 500 ml-Erlenmeyer flasks containing 30 ml of each medium on a shaking incubator as reported previously (16). The effects of water temperature on the growth of *E. coli* CU 103 and release of extracellular DNAs were investigated in an MM2-glucose medium (pH 7.0) at 30℃, 15℃, and 4℃. The effects of pH were also investigated in an MM2-glucose medium at pH 9.0, 7.0, and 5.0.

Measurement of cell growth

Growth of the bacteral cells in each liquid medium was measured for viable cells and turbidity of the culture. The viable cells were counted by colony number developed on LB agar according to the Standard methods (1). Turbidity of the culture was measured at 600 nm with a spectrophotometer (Spectronic 20, Milton Roy Co., San Francisco, CA, USA).

Quantification of extracellular DNAs

The extracellular DNAs released into the medium were quantified by the Hoechst 33258 fluorometric method as described by DeFlaun *et al.* (4). Culture solutions were passed through a 0.2 μm Nuclepore membrane filter. The DNAs in the filtrate were precipitated by the addition of two volumes of absolute ethanol and then storage at -20℃ for 48 h. The precipitate of the DNAs was collected by centrifugation at 16,000×g for 30 min. The concentrated DNAs were suspended in 2.0 ml of SSC (standard saline citrate), to which was added 1 ml of 6×10^{-7} M Hoechst 33258 disolved in SSC according to the methods described by Paul and Myers (18). Fluorescence emitting from the dye bound to the DNAs was measured at 365 nm with a TKO 100 fluorescence spectrophotometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

Electrophoresis of DNAs

The concentrated DNAs were electrophoresed on a 0.7% agarose slab gel by the methods described by Sambrook *et al.* (22) and Koetsier *et al.* (10). If necessary, the DNAs were digested with *Xho*I and *Hind*III according to the instructions of the manufacturer (POSCOCHEM Co., Seoul, Korea). Electrophoresis was performed in a TBE or TAE buffer with a voltage gradient of 5 v/cm for 1.5 hours. The DNAs in the gels were stained with ethidium bromide (0.5 μg/ml) dissolved in a TBE buffer for 40 min and then photographed.

Southern hybridization

The DNA probe was made with a *Xho*I-*Hind*III fragment (2.8 kb) of pCU103 containing *pcbCD* genes as described previously (16). The probe was labelled by a nick translation system and biotin-14-dATP as described by Walia *et al.* (24). DNA hybridization was performed according to the blotting and hybridization protocol for the

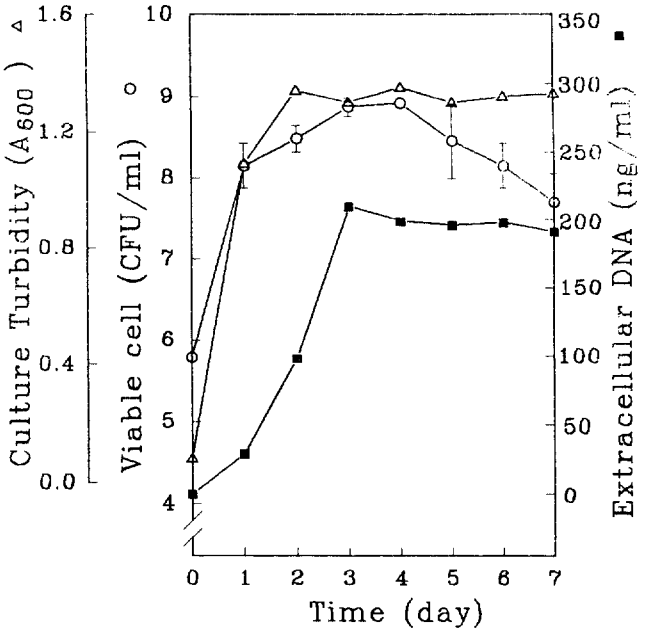

Fig. 2. Growth of *E. coli* CU103 in Luria-Bertani (LB) broth at 30℃ and release of its extracellular DNAs.

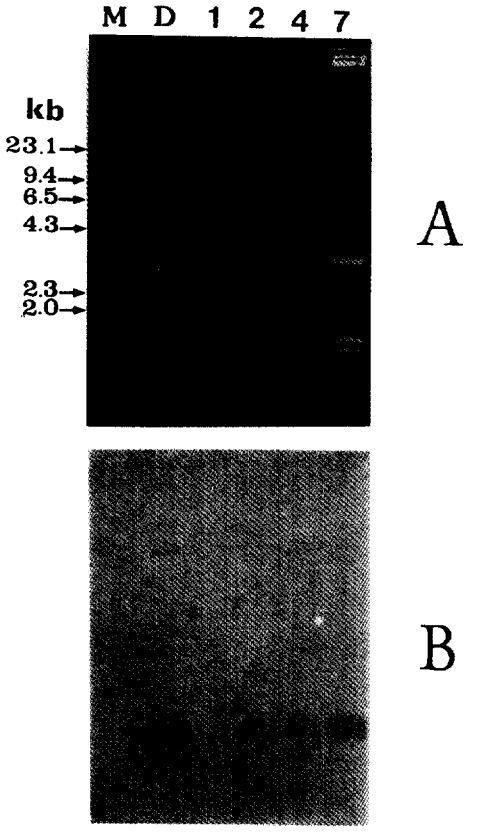

Fig. 3. Gel electrophoresis (A) of the *Xho*I-*Hin*dIII digested extracellular DNAs released from *E. coli* CU103 during growth in LB broth at 30℃ and Southern hybridization (B) of the gel with a *pcbCD* probe. M, *Hin*dIII-digested lambda size marker; D, pCU103 plasmid DNA; Lane 1 to 7, extracellular DNAs isolated from 1 to 7-day incubated cultures.

Hybond membranes (Amersham Co., Amersham, UK), as reported by Amy and Hiatt (2). The gels were incubated in 0.25 N HCl for 15 min until the bromophenol blue marker turned yellow and then denatured with a denaturation solution. After neutralization of the gel, the DNAs in the gel were transfered to a Hybond-N nylon membrane by using 1 N NaOH for 2 hours. The membrane was baked at 80℃ for 2 hours and hybridized with a hybridization solution. The hybridized DNAs were detected by the Blugene non-radioactive nucleic acid detection system (BRL, Gaithersberg, MD, USA).

Results and Discussion

Release of extracellular DNAs during growth

During cultivation of the genetically engineered *E. coli* CU103 in a Luria-Bertani (LB) medium at 30℃ for 7 days, growth curves of the cells and the extracellular DNAs released from the cells were obtained as shown in Fig. 2. After one day of exponential phase, the stationary phase was continued until the 4th day of post-incubation. The extracellular DNAs began to be measured after 1 day of incubation and then drastically increased until the 3rd day, reaching about 210 ng/ml in concentration. The concentration of the DNAs was maintained at a level of about 200 ng/ml during the remaining period after the stationary phase. The fluorometric method using Hoechst 33258 for the quantification of free DNAs in water environment was reported to be so sensitive that 0.2 ng/ml of free DNA could be measured in fresh and marine waters by DeFlaun *et al.* (4) and Paul and Meyer (18). In this study, 4 ng/ml of the extracellular DNAs could be detected from the waters, to which had been added purified DNAs, and about 85% of the DNAs was recovered.

In the electrophoresed gel of the extracellular DNAs digested with *Xho*I and *Hin*dIII endonucleases (Fig. 3A), no DNA bands were observed in the one-day old culture, but vector plasmid (3.0 kb) and two fragments (1.4 and 1.3 kb) of the inserted DNA separated from the recombinant plasmid pCU103 appeared clearly in the cultures incubated for two days and longer. Two bands of the fragments including *pcbCD* genes were identified by Southern hybridization as seen in Fig. 3B.

Extracellular DNAs were released in LB broth even in the exponential phase of growth, as seen in Fig. 4. More than 15 ng/ml of the DNAs were detected during the 2 to 4 h incubation period, and the concentration of ex-

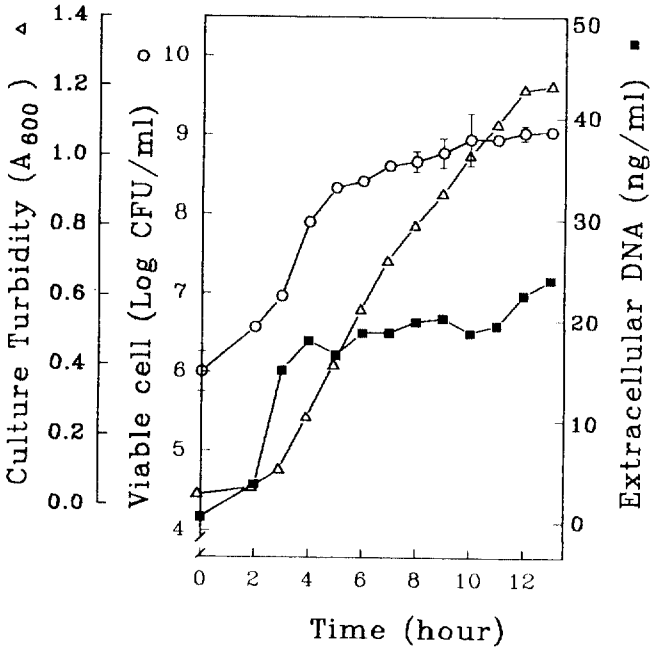

Fig. 4. Growth of *E. coli* CU103 in Luria-Bertani (LB) broth at 30°C and release of its extracellular DNA during the exponential phase.

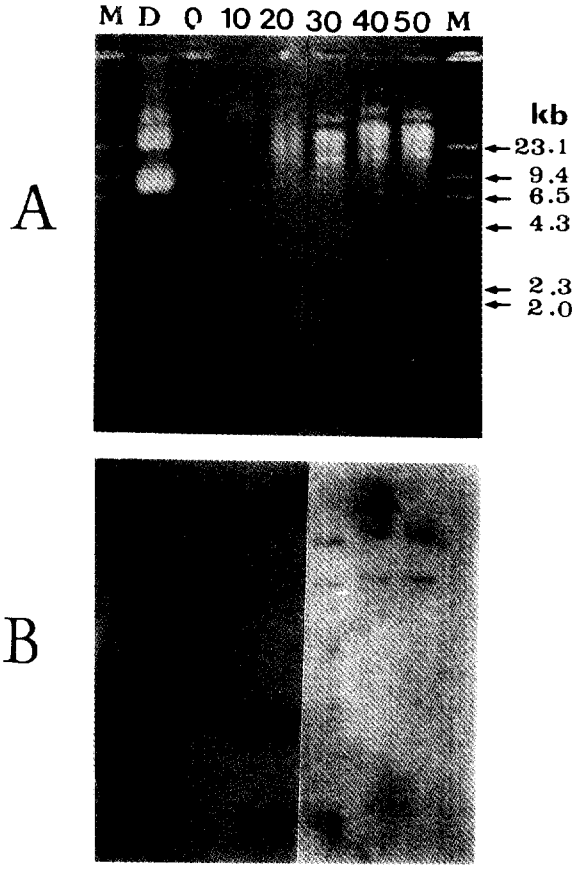

Fig. 5. Gel electrophoresis (A) of extracellular DNA released from *E. coli* CU103 during the exponential phase in LB broth at 30°C and Southern hybridization (B) of the gel with a *pcbCD* probe. M, *Hin*dIII-digested lambda size marker; D, pCU103 plasmid DNA; Lane 0 to 50, extracellular DNAs isolated from 0 to 50-hour incubated cultures.

tracellular DNAs increased to about 20 ng/ml within the 12 hours of the exponential phase. Paul and David (17) reported that extracellular DNAs were released as a normal physiological function from the genetically altered bacteria in aquatic environments. Lorenz and Wackernagel (14) indicated that extracellular DNAs were released from all bacteria by both lysis and unknown physiological functions.

Extracellular DNAs began to be detected in the gel electrophoresed with the 10 h culture (Fig. 5A), but the recombinant plasmid pCU103 was detected from after a 30 h incubation period when the gel was hybridized with *pcbCD* gene as shown in Fig. 5B. The two hybridized bands were proved to be covalently closed circular (bottom band) and open circular forms (upper band) of the pCU103, respectively, as reported in the previous paper (16). Such structural conversion of plasmids was also reported in pBR328 and pHV14 introduced into soils by Paget *et al.* (15) and Gallori *et al.* (6), respectively.

Effects of media conditions on release of extracellular DNAs

The release of extracellular DNAs was compared among different media (LB, MM2-glucose, FW, and TE) during growth of *E. coli* CU103 at 30°C for 7 days. The results together with the cell growth are shown in Fig. 6. The cells were exponentially increased in LB broth (Fig. 6A) within one day, and then a stationary phase followed for about 4 days. But extracellular DNAs were gradually increased up to about 200 ng/ml of concentration for the first 3 days, and the concentration was maintained until the 7th day of incubation. In a MM2-glucose medium (Fig. 6B), viable cells were increased until the 3rd day and then gradually decreased, but extracellular DNAs were increased to 69 ng/ml within 4 days and then slowly decreased to about 50 ng/ml on the 7th day. In both FW (Fig. 6C) and TE (Fig. 6D), the viable cells were decreased gradually without any increase for 7 days. During the period of the 7 d incubation, extracellular DNAs were found to be less than 10 ng/ml in concentration. These results mean that the metabolically retarded cells in oligotrophic waters did not release the DNAs and that non-sterile water such as FW might contain DNases degrading the extracellular DNAs. Degradation activity of the DNase I to free DNA in natural waters was measured by Paget *et al.* (15), Romanowski *et al.* (20, 21), and Paul *et al.* (17).

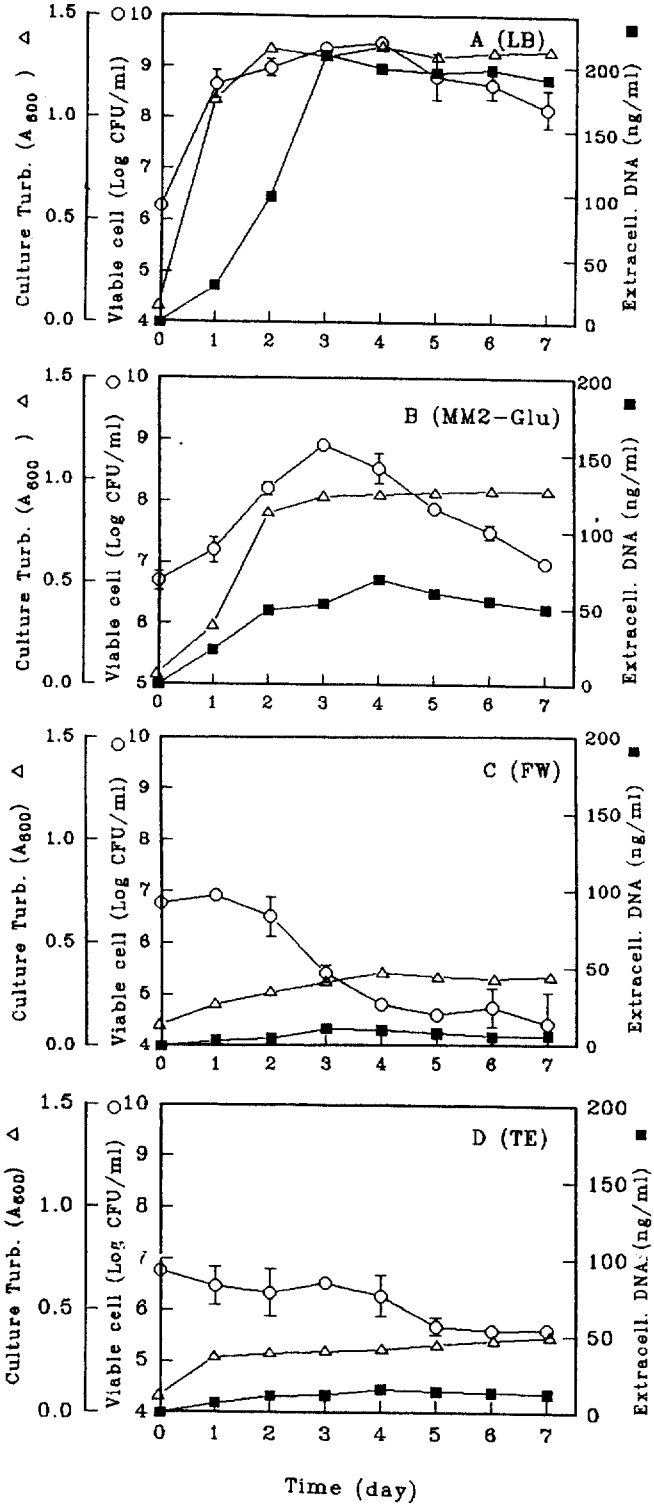

Fig. 6. Growth of *E. coli* CU103 in different liquid media (pH 7.0) at 30℃ and release of its extracellular DNAs.

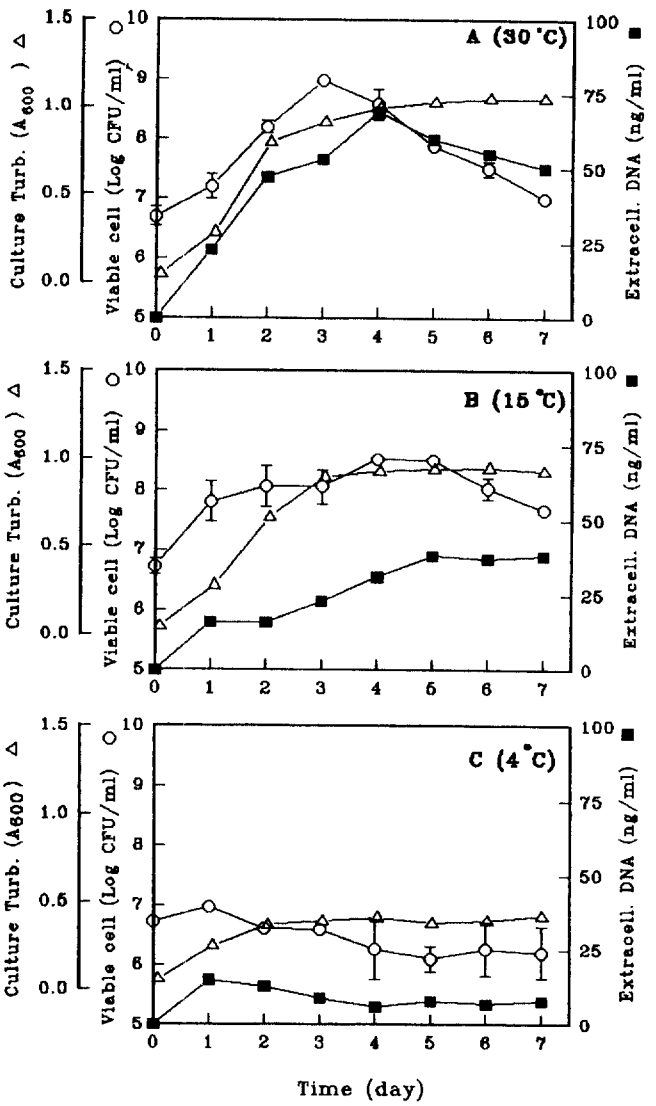

Fig. 7. Effects of water temperature on the growth of *E. coli* CU 103 in MM2-glucose at pH 7.0 and release of its extracellular DNAs.

The effects of water temperature on growth of the cells and the release of extracellular DNAs in a MM2-glucose medium (pH 7.0) were comparatively studied at 30℃, 15℃, and 4℃ as shown in Fig. 7. The viable cells as well as the release of the DNAs were slowly increased up to the 5th day of incubation at 15℃ (Fig. 7B), in comparison with the exponential growth for 3 days and increased release of the DNAs for 4 days at 30℃ (Fig. 7A). But the cells did not show any growth at 4℃ as seen in Fig. 7C. During incubation at 15℃, the extracellular DNAs were gradually increased up to 35 ng/ml within 7 days of incubation. But less than 15 ng/ml of the extracellular DNAs were detected in the medium during 7 days of incubation at 4℃. The larger amount of extracellular DNAs detected at the higher water temperature are thought to be attributed to the more active metabolic activity of the cells.

The effects of different pH values on cell growth and release of extracellular DNAs were compared in a MM2-glucose liquid medium as shown in Fig. 8. The cells did

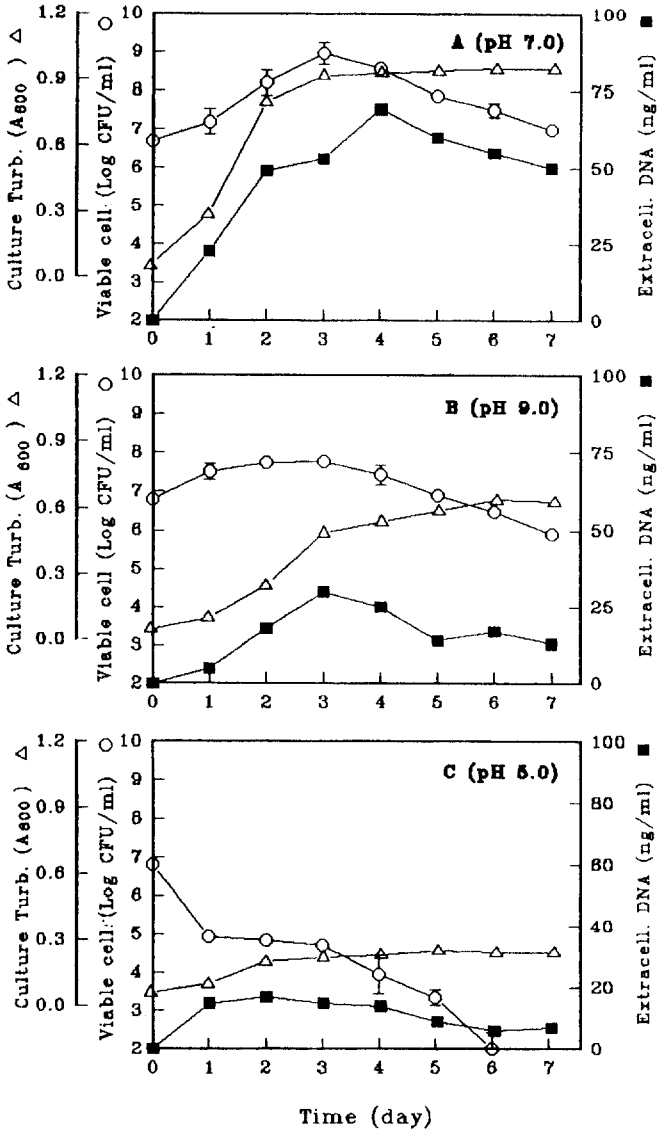

Fig. 8. Effects of pH value on the growth of *E. coli* CU103 in MM2-glucose at 30℃ and release of its extracellular DNAs.

not grow at pH 9.0, and the viable cells decreased drastically at pH 5.0. The extracellular DNAs released in the MM2-glucose medium at pH 9.0 increased up to 35 ng/ml until the 3rd day and then gradually decreased as seen in Fig. 8B. DNAs were detected to be about 15 ng/ml during the 2nd to 3rd day incubation period at pH 5.0 (Fig. 8C) and less than 15 ng/ml of the DNAs were observed afterwards. As for the results obtained in this study, Lorenz and Wackernagel (13) reported that more extracellular DNAs were released from *Pseudomonas stutzeri* introduced in soil and stimulated more natural transformation at pH 7.0 than at pH 9.0 and 5.0. Paul and David (13) also reported that the largest amount of extracellular DNAs were released from the genetically engineered *Pseudomonas cepacia* in both fresh and marine waters at pH 7.0 and at 37℃. Therefore, extracellular DNAs including the recombinant plasmid pCU103 were found to be actively released from the recombinant cells during growth in the liquid media studied in this study.

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