0273-2289/90/2425—0151$02.00

Characterization of Cryptic Plasmids from Marine Cyanobacteria and Construction of a Hybrid Plasmid Potentially Capable of Transformation of Marine Cyanobacterium, *Synechococcus* sp., and its Transformation

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ABSTRACT

Among forty strains of marine cyanobacteria isolated in our laboratory, five strains had 1-3 different plasmids. The unicellular marine cyanobacterium, *Synechococcus* sp. NKBG 042902, contains at least three plasmids (pSY09, pSY10, and pSY11). However, these plasmids are cryptic. Therefore, a hybrid plasmid pUSY02 containing the 1.4-kb *Hin*dIII fragment of pSY11 and *Escherichia coli* plasmid pUC18 was constructed. The plasmid pUSY02 transformed both marine *Synechococcus* sp. NKBG042902-YG1116, which is a cured strain, and fresh water *Anacystis nidulans* R2 by dark incubation or Ca^{2+} treatment. However, the plasmid pSG111 constructed from the plasmid DNA of *A. nidulans* R2 failed to transform marine *Synechococcus* sp. Electroporation was also applicable to transformation of marine *Synechococcus* sp. and fresh water *A. nidulans* R2. The plasmid pUSY02 was rapidly introduced into marine *Synechococcus* sp.

Index Entries: Cyanobacteria; hybrid plasmid; transformation; electroporation.

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INTRODUCTION

Recently, considerable interest has arisen in marine biotechnology. Marine microalgae, especially marine cyanobacteria, have received much attention. The advantages of marine biotechnology are many. The most apparent is the availability of solar energy and seawater. Seawater abounds with minerals that are essential for the growth of microalgae. Moreover, some marine microalgae produce useful chemicals and fuels. However, a gene cloning system has not been developed in marine microalgae.

Gene manipulation of cyanobacteria has developed in fresh water cyanobacteria, *Anacystis nidulans* (*Synechococcus* sp.), *Synechocystis* PCC6803, and other filamentous cyanobacteria (*1–3*). At first, *A. nidulans* R2 was shown to be naturally competent to take up added DNA (*4*), so this strain was generally transformed under incubation with plasmid DNA. Transformation was performed with a shuttle vector capable of replication in both *E. coli* and cyanobacterium. A transformation system in *A. nidulans* R2 was almost established, and replication of a foreign gene using a shuttle vector in *A. nidulans* R2 has been reported (*5,6*). Marine cyanobacterium *Synechococcus* sp. has almost the same morphological characteristics as the fresh water cyanobacterium, *A. nidulans*. However, there has been no report of transformation in marine cyanobacteria.

This report describes the isolation and partial characterization of a plasmid from marine cyanobacteria, the construction of a hybrid plasmid from the *E. coli* plasmid pUC18 and the marine cyanobacterial plasmid, and transformation of marine *Synechococcus* sp. and fresh water *A. nidulans* R2 by dark incubation, Ca^{2+} treatment, and electroporation.

MATERIALS AND METHODS

Strains and Plasmids

Marine cyanobacteria including *Synechococcus* sp. NKBG042902 were isolated in our laboratory from samples of seawater collected from coastal areas in Japan. Fresh cyanobacterium, *Anacystis nidulans* R2, and its shuttle vector, pSG111 (Ap^r, Cm^r), were kindly donated by Susan S. Golden (*7*), and cured strain *A. nidulans* R2-SPc and pECAN8(Ap^r) were obtained from the Institute of Applied Microbiology, University of Tokyo. *Escherichia coli* JM109 and plasmid pUC18 were purchased from TAKARA Co. (Kyoto).

Culture of Cyanobacteria and *E. coli*

Axenic cultures of *A. nidulans* were grown at 30°C in BG11 medium (*8*). Marine *Synechococcus* sp. NKBG042902 was grown on medium (BG11) without NaCl and supplemented with 3% NaCl (i.e., sea water concentra-

tion). The cells were illuminated with approximately 36 μEinstein/m^2/s from fluorescent cool white light and were grown with air bubbling. *E. coli* K12 strain JM109 was used as a recipient cell for transformation and was grown aerobically at 37°C in LB medium (10 g/L of bacto-typtone, 5 g/L bacto-yeast extract, and 5 g/L NaCl; pH 7.4).

Preparation and Analysis of Plasmid

Isolation of plasmid DNA from cyanobacteria was performed by the modified procedure of Chauvat et al. (*9*). Isolation of plasmid DNA from *E. coli* was accomplished by the alkaline extraction method (*10*) followed by cesium chloride–ethidium bromide centrifugation on a large scale. Electrophoresis of digested DNA was carried out by 0.7% agarose gel in TBE buffer (0.089*M* Tris-Borate, 0.089*M* boric acid, 0.002*M* EDTA; pH 8.0).

Preparation of Cured Marine *Synechococcus* sp.

The cured strain, *Synechococcus* sp. NKBG042902-YG1116, was obtained by ethidium bromide treatment. The cured cells were grown in medium containing ethidium bromide, plated out on BG11-agar medium containing 1% NaCl, and colonies were picked up.

Transformation

Transformation of *E. coli* was performed by the method of Cohen et al. (*11*). Cyanobacteria transformation conditions used by Van den Hondel (*12*) and Golden (*13*) were modified as follows: Exponentially growing cells were centrifuged, washed once in medium, and resuspended in medium at 1×10^8 cells/mL. Samples of 1 mL in vol were incubated in 1 μg of plasmid DNA at 30°C under dark conditions overnight (12–18 h). After incubation, 0.1-mL samples were suspended in 5 mL of medium containing 0.5% agar and were plated on BG11-agar medium containing 1 μg/mL ampicillin or 5 μg/mL chloramphenicol. Ca^{2+} treatment as used for *E. coli* was also carried out for transformation of cyanobacteria.

Electroporation

Electroporation was carried out in EP buffer (10 m*M* Tes, 1 m*M* $MgCl_2$, 272 m*M* sucrose; pH 7.5) using a Gene Pulser™ (Bio-Rad Laboratories, Richmond, CA) (*14,15*). Cyanobacteria were harvested by centrifugation, washed once with EP buffer, and suspended in the same buffer at 1×10^9 cell/mL. Samples of 1 mL in vol were mixed with 1 mg of DNA, and 0.8 mL or 0.4 mL of these suspensions were transferred into Gene Pulser cuvets. The cells were subjected to a single pulse electroporation. Then 0.1-mL samples were suspended in 5 mL of medium containing 0.5% agar and were plated on BG11-agar containing 1 mg/mL ampicillin or 5 mg/mL chloramphenicol.

Table 1
Numbers and Size of Plasmid in Marine Cyanobacteria

Strains	No. of plasmids	Size(Kbp)
Synechococcus sp.		
NKBG 031301	2	3.2, 12.5
NKBG 040607	1	16.8
NKBG 041302	2	15.0, 19.5
NKBG 041902	2	10.5, 21.0
NKBG 042902	3	2.3, 2.7, 10.0 <

RESULTS AND DISCUSSION

Isolation and Characterization of Plasmids from Marine Cyanobacteria

As shown in Table 1, five strains of marine *Synechococcus* sp. have 1-3 different plasmids among 40 strains of marine cyanobacteria. However, these strains were sensitive to penicillin, ampicillin, tetracycline, streptomycin, chloramphenicol, erythromycin, gentamycin, and neomycin, indicating that the plasmids do not specify resistance to those antibiotics. The plasmids were cryptic. *Synechococcus* sp. NKBG042902 has more than three plasmids, pSY09, pSY10, and pSY11 (Fig. 1). A band of higher-molecule DNA was often obtained from electrophoresis. This may show a large plasmid. The plasmid pSY11 (2.3 kb) had three cleavage sites of restriction endonuclease *Hin*dIII, two sites of *Eco*RI and *Hin*cII, and one site of *Acc*I. pSY10 (2.7 kbp) had three sites of *Hin*dIII, two sites of *Eco*RI and *Acc*I and one site of *Hin*cII. pSY09 and a large plasmid were not yet characterized.

Construction of a Hybrid Plasmid Between Marine *Synechococcus* sp. and *E. coli*

The plasmids pSY10 and pSY11 were cryptic. Therefore, an antibiotic resistance gene had to be introduced into the plasmids. The three *Hin*dIII fragments of pSY11 were inserted into the multi-cloning site of the cloning vector pUC18, which encoded ampicillin resistance gene. The hybrid plasmids were introduced in *E. coli* K12 strain JM109. Plasmids were reisolated from the ampicillin-resistant colonies (ampicillin concentration: 25 μg/mL). These hybrid plasmids were used for transformation into *Synechococcus* sp. NKBG042902-YG1116, which was obtained by ethidium bromide treatment and lost pSY10, 11. Among these hybrid plasmids, only pUSY02, which has 1.4 kb fragments of pSY11 and pUC18, trans-

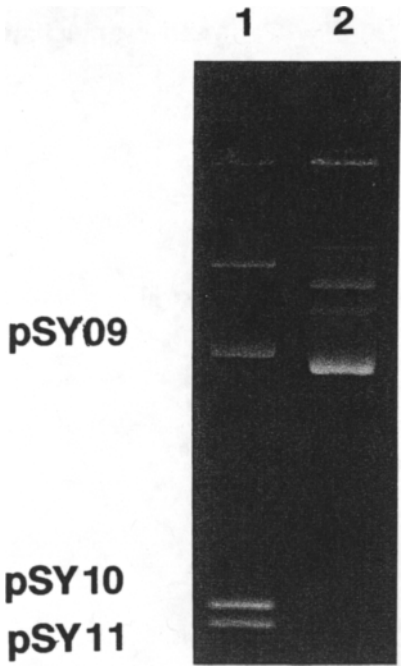

Fig. 1. Gel electrophoresis of marine *Synechococcus* sp. NKBG 042902 and its cured strain, NKBG 042902-YG 1116. Lane 1, *Synechococcus* sp. NKBG 042902. Lane 2, Cured strain, *Synechococcus* sp. NKBG 042902-YG1116.

formed the strain YG1116 to ampicillin resistance (1 μg/mL). The construction of the hybrid plasmid pUSY02 and the physical map are shown in Fig. 2.

Transformation of Marine *Synechococcus* sp. and Fresh Water *A. nidulans* R2

The plasmid pUSY02 was employed for transformation of marine *Synechococcus* sp. The strain *Synechococcus* sp. NKBG042902, harboring pSY10 and 11, were not transformed by the plasmid pUSY02, whereas pUSY02 transformed the cured strain NKBG042902-YG1116.

Before transformation, marine *Synechococcus* sp. was grown in media containing various concentrations of NaCl. This strain easily adapted to high and low salinity. However, marine *Synechococcus* sp. produced a lot of slime around the cell wall when the cell was incubated in medium containing 3–8% NaCl. The plasmid pUSY02 could not transform the strain surrounded with slime. When marine *Synechococcus* sp. was grown in the medium without NaCl, little slime was produced around the cell wall, and the strain YG1116 was transformed by the plasmid pUSY02. Therefore, the cured strain *Synechococcus* sp. NKBG042902-YG1116 grown in the medium without NaCl was used for further experiment.

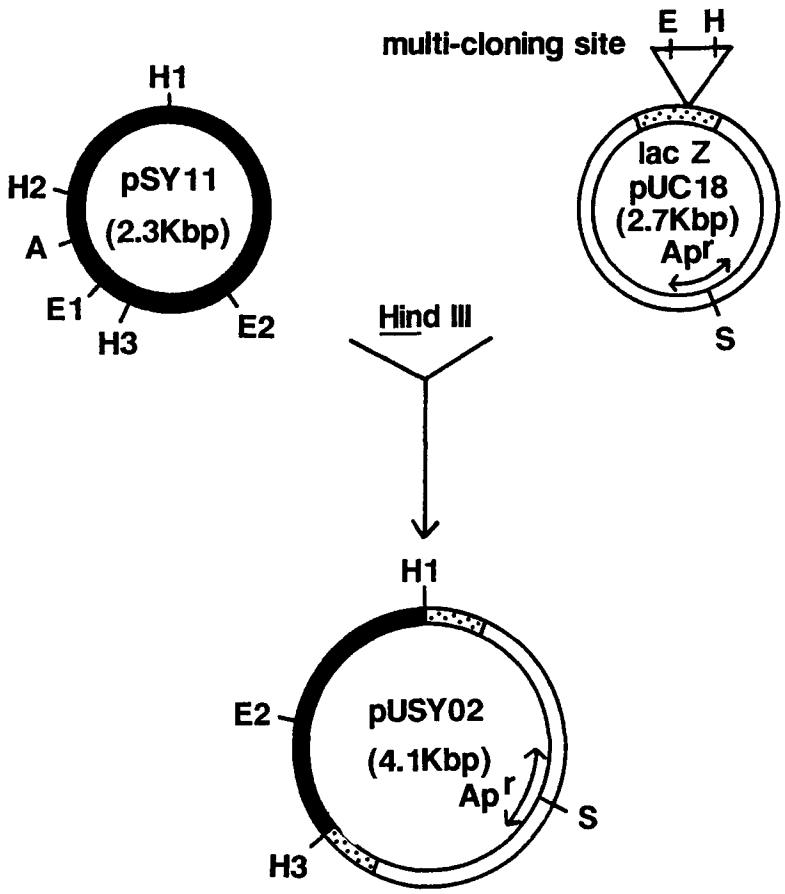

Fig. 2. Construction of the hybrid plasmid from pSY11 and pUC18. ▬▬; marine cyanobacterial sequence. ═══; pUC18 sequence. A;*Acc*I, E;*Eco*RI, H;*Hin*dIII, S;*Sca*I.

Table 2 shows the transformation efficiency of marine *Synechococcus* sp. and fresh water *A. nidulans* R2 by dark incubation or Ca^{2+} treatment. In addition to pUSY02, the plasmid pSG111, constructed from the plasmid DNA of *A. nidulans* R2, was employed for the experiment. Incubation was according to the method of Van den Hondel. Transformation efficiency was obtained by the methods of Chauvat (*9*) and Golden (*13*). The cells of marine *Synechococcus* sp. and fresh water *A. nidulans* R2 were incubated in the dark with pUSY02 and pSG111, respectively. It took more than 16 h for the strains to take up the plasmid DNA. Transformants made colonies on the BG11-agar plate containing the antibiotics. On the other hand, non transformants did not make colonies on the plate. The plasmid pUSY02 transformed both marine *Synechococcus* sp. NKBG042902-YG1116 and fresh water *A. nidulans* R2 at the efficiency of 6.0×10^{-6}–10^{-5} and 3.2×10^{-6} ampicillin-resistant transformants/viable cells, respectively. After trans-

Table 2
Transformation Efficiency of Cyanobacteria

Methods	Recipient strains	Transformation efficiency	
		pUSY02	pSG111
Dark incubation (16hr)	Synechococcus sp. NKBG 042902	N.D	—
	NKBG 042902-YG1116	6.0×10^{-6}-10^{-5}	N.D
	Anacyatis nidulans R2	3.2×10^{-6}	1.0×10^{-5}
Ca^{2+} treatment (1.5hr)	NKBG 042902-YG1116	8.8×10^{-6}	N.D
	R2	N.D	N.D

Transformation efficiency; Transformants /viable cells
N.D; No transformant was obtained.

formation, the plasmid pUSY02 was recovered from the transformed marine *Synechococcus* sp. and detected by gel electrophoresis.

On the other hand, pSG111 failed to transform marine *Synechococcus* sp. pSG111 transformed only fresh water *A. nidulans* at the efficiency of 10^{-5} chloramphenicol-resistant transformants/viable cells. For Ca^{2+} treatment, the strains were incubated with the plasmids at 0°C for 30 min, followed by 1 h of dark incubation. However, pSG111 could not transform both marine *Synechococcus* sp. and fresh water *A. nidulans*. pUSY02 transformed only marine *Synechococcus* sp. at the efficiency of 8.8×10^{-6} ampicillin-resistant transformants/viable cells. *A. nidulans* R2 were not transformed by Ca^{2+} treatment. *A. nidulans* R2 may be more sensitive to low temperature than marine *Synechococcus* sp.

Transformation by Electroporation

Dark incubation and Ca^{2+} treatment required a long incubation, leading to contamination. Therefore, electroporation was used for transformation of marine *Synechococcus* sp. and fresh water *A. nidulans*. Electroporation uses a high-voltage current through a cell suspension to induce pores in the cell membrane through which plasmid DNA enters the cell. An optimum voltage, time constant, and plasmid DNA concentration must be found. For example, high voltage and long time constant result in excessive cell death from disruption of the cell membrane. The factors affecting the transformation efficiency were investigated by using fresh water *A. nidulans* R2-SPc and the plasmid pECAN8.

Figure 3 shows the effect of time constant on the survival ratio at 6.25 kV/cm and 12.5 kV/cm. The survival ratio of *A. nidulans* decreased with increasing time constant, although ampicillin-resistant transformants/surviving cells increased. The survival ratio of 6.25 kV/cm is higher than that at 12.5 kV/cm. Further experiments were carried out at a time constant of 3–5 ms and an electric field strength of 6.25 kV/cm, since highest ampi-

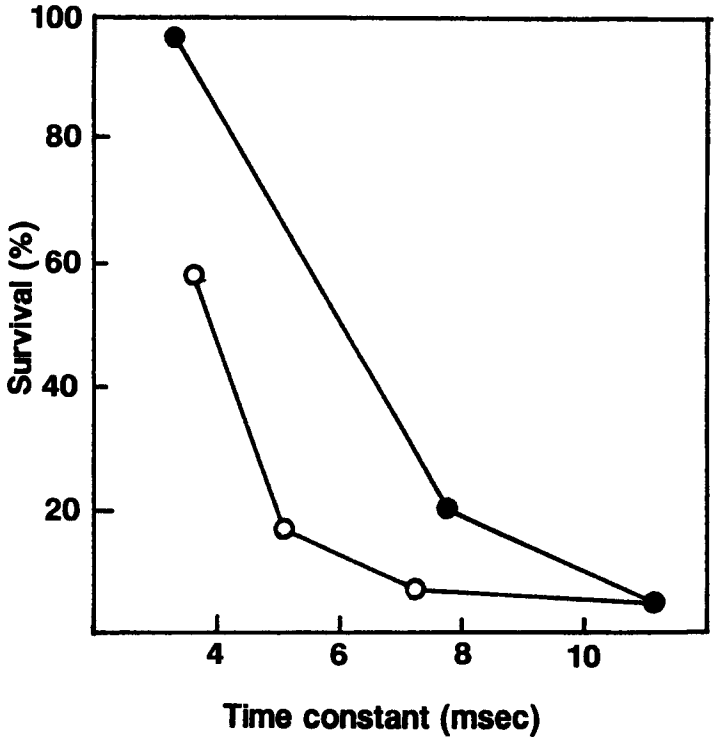

Fig. 3. Effect of time constant on survival ratio of *A. nidulans* R2-SPc. Electric field strength; 6.25 kV/cm (●), 12.5 kV/cm (○). Capacitance; 25 μF.

cillin-resistant transformants/total cells were obtained. Figure 4 shows the effect of plasmid DNA concentration on the transformation efficiency. The transformation efficiency of *A. nidulans* increased with increasing plasmid DNA concentration. Time constant changed little when plasmid DNA concentration varied.

Electroporation was applied to transformation of marine *Synechococcus* sp. Table 3 shows the transformation efficiency of marine *Synechococcus* sp. and fresh water *A. nidulans*. The plasmid pUSY02 transformed marine *Synechococcus* sp. by electroporation. The transformation efficiency was 3.0×10^{-7} and 1.2×10^{-7} ampicillin-resistant transformants/viable cells at the electric field strengths of 2.5 and 6.25 kV/cm, respectively. Marine *Synechococcus* sp. was efficiently transformed at low electric voltage. These results show that rapid transformation of marine *Synechococcus* sp. and fresh water *A. nidulans* is possible by electroporation.

The major contribution of this paper is the construction of a vector system for marine cyanobacteria. Since fresh water *A. nidulans* was shown to take up added DNA, several vector systems have been reported for *A. nidulans*. *A. nidulans* have been transformed mainly by the incubation method of Van den Hondel. Golden et al. reported that dark incubation is a useful manipulation for enhancing the transformation of *A. nidulans*, because perturbation of photosynthesis improves the efficiency of the

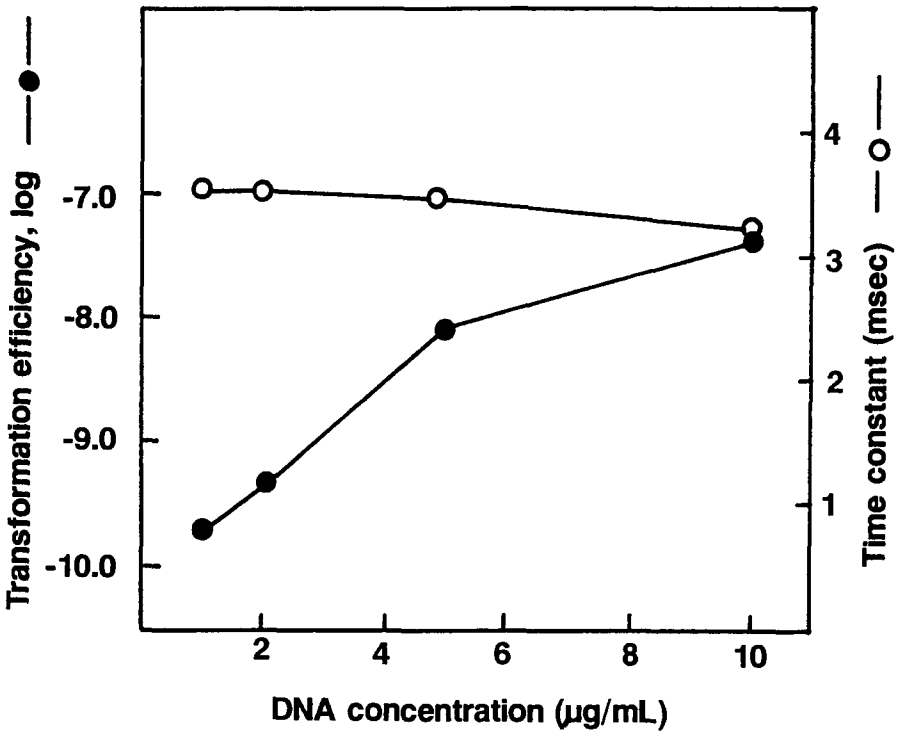

Fig. 4. Effect of DNA concentration on transformation efficiency. *Anacystis nidulans* R2-SPc was mixed with plasmid DNA pECAN8 in EP buffer at 10^9 cells/mL. Electric field strength; 6.25 kV/cm. Capacitance; 25 μF. Transformation efficiency; transformants/CFU (colony forming unit).

Table 3
Transformation Efficiency of Cyanobacteria in Various Electric Field Strength

Recipient strains	Electric field strength (kV/cm)		
	2.5	6.25	12.5
Anacystis nidulans R2-SPc (Fresh water)	N.D	2.8×10^{-8}	1.2×10^{-7}
Synechococcus sp. NKBG 042902-YG1116(Marine)	3.0×10^{-7}	1.2×10^{-7}	N.D

Transformation efficiency; Transformants / viable cells
N.D; No transformant was obtained.
Cells were subjected to a single pulse electroporation under 25 μF capacitance.

transformation process. Therefore, we have employed the dark incubation method for the transformation of marine *Synechococcus* sp. pSG111 derived from the plasmid DNA of fresh water *A. nidulans* could not transform marine *Synechococcus* sp. On the other hand, pUSY02 derived from the plasmid DNA of marine *Synechococcus* sp. transformed both marine *Synechococcus* sp. and fresh water *A. nidulans*. Ca^{2+} treatment as used for

E. coli is also applicable to transformation of pUSY02 into marine *Synechococcus* sp. Dark incubation and Ca^{2+} treatment require a long incubation time. Therefore, electroporation, which has recently found application in the transformation of eucaryotic cells including plant, yeast cell protoplasts, and B-lymphocytes, was employed for the transformation of marine *Synechococcus* sp. As a result, marine *Synechococcus* sp. was successfully transformed by electroporation using the plasmid DNA pUSY02. This method has potential for greatly facilitating research on the molecular genetics of marine cyanobacteria.

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