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Optimum Conditions for Transformation of *Synechocystis* sp. PCC 6803

Xiaonan Zang, Bin Liu, Shunmei Liu, K.K.I.U. Arunakumara, and Xuecheng Zhang*

College of Marine Life Sciences, Ocean University of China, Qingdao 266003, P. R. China

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This study was conducted to determine the optimal conditions for introduction of exogenous DNA into *Synechocystis* sp. PCC 6803. Of the three transformation techniques studied, electroporation, ultrasonic transformation and natural transformation, natural transformation showed the highest efficiency. Additionally, this study demonstrated that the higher plasmid concentration and longer homologous recombining fragments resulted in a greater number of transformants. For successful transformation, the lowest concentration of plasmid was 0.02 μg/ml, and the shortest homologous recombining fragment was 0.2 kb. Use of *Synechocystis* sp. PCC 6803 in the logarithmic growth phase resulted in two-fold higher transformation rate than that of the same organism when cells in the latent phase or the plateau phase were used for transformation. Pretreatment of the host strain, *Synechocystis* sp. PCC 6803, with EDTA (2 mM) for two days prior to transformation increased the transformation efficiency by 23%. Additionally, incubation of the cells and DNA for 5 h under light conditions increased the transformation efficiency by two orders of magnitude. Moreover, recovery treatment of the cells before they were plated onto antibiotic medium also increased the transformation efficiency.

Keywords: Cyanobacterium, electroporation, *Synechocystis* sp. PCC 6803, ultrasonic

Synechocystis sp. PCC 6803 is a cyanobacterium that is spontaneously transformable and able to integrate foreign DNA into its genome through homologous recombination (Grigorieva and Shestakov, 1982; Williams, 1988). This system has been used successfully to study the function of the photosynthetic apparatus (Chauvat *et al.*, 1989). Effective modification and engineering of biochemical pathways have been reported in this system (Wu and Vermass, 1995). This system has also been used to express functional proteins, such as human liver metallothionein (Song *et al.*, 2001) and mouse liver metallothionein (Wang *et al.*, 2000). Although the application of this system has become more widespread, it still could not satisfy many genetic manipulations, such as genetic mapping and genomic function study. Therefore, it is necessary to optimize the transformation conditions of this system to obtain the maximal number of transformants using a limited amount of exogenous DNA.

It has been reported that DNA concentration (Porter, 1986; Kufryk *et al.*, 2002), length of homologous recombination fragments (Williams, 1988; Labarre *et al.*, 1989), physiological condition of the cyanobacterium, the use of linear or circle DNA (Barten and Lill, 1995), and details in the transformation procedure (Kufryk *et al.*, 2002) can effect transformation efficiency in *Synechocystis* sp. PCC 6803. However, changes in the transformation conditions have not been studied systematically to date. Additionally, new transformation techniques such as electroporation and ultrasonic transformation have not been employed in *Synechocystis* sp. PCC 6803. Therefore, this study was conducted to apply electroporation and ultrasonic transformation to *Synechocystis* sp. PCC 6803, as well as to ameliorate the entire transformation procedure. Information regarding the factors that affect transformation may lead to mechanistic insight in the transformation phenomenon in *Synechocystis* sp. PCC 6803.

* To whom correspondence should be addressed.
(Tel) 86-532-8203-2789; (Fax) 86-532-8590-2708
(E-mail) xczhang@ouc.edu.cn

Materials and Methods

Strains and growth condition

Wild-type axenic *Synechocystis* sp. Strain PCC 6803 was obtained from the Institute of Hydrobiology, Academia Sinica of China. The cyanobacterium was cultured in BG11 medium at 25-30°C under illumination of approximately 50 μmol photons/m^2/s in a 12 h photoperiod. Transformants were grown on modified BG11 solid medium containing either 5 μg/ml chloromycetin or 10 μg/ml kanamycin.

Plasmids

Plasmid pKW1188 (Williams, 1988) was obtained from the Institute of Hydrobiology, Academia Sinica of China. Plasmids pGSL, pDAK, pRM1, and pRM2 were constructed by cloning a 2.8 kb fragment of the *gro*ESL gene (Lehel *et al.*, 1993; Genbank accession no. D12677), 0.8 kb fragment of the *des*A gene (Wada *et al.*, 1990; Genbank accession no. X53508), and 1.6 kb and 1.5 kb random fragments from the *Synechocystis* sp. PCC 6803 genome into the pMD18-T vector. Then, Km^r was inserted into each of the aforementioned plasmids at the *Xho*I site (Table 1). The Cm^r plasmid, pDAC, was constructed by inserting a 0.8 kb fragment of the *des*A gene (Wada *et al.*, 1990; Genbank accession no. X53508) obtained from the genome of *Synechocystis* sp. PCC 6803 into pUC18

that had been digested with *Eco*RI and *Hin*dIII. Construction of the Cm^r plasmid was followed by ligation of a Cm^r *Bam*HI fragment into the plasmid (Table 1). Plasmid pGL was reconstructed from pGSL by kicking out one side of the homologous fragment (Table 1).

Transformation

The optimal transformation conditions of *Synechocystis* sp. PCC 6803 were determined by comparison of the experimental results with those of the natural transformation, which was performed as follows: Five ml of an exponentially growing culture ($1\text{-}3\times10^8$ cells/ml) were centrifuged at room temperature at 6,000×g for 15 min. The pellet was suspended in fresh BG11 medium at a density of 1×10^9 cells/ml and then mixed with exogenous DNA to a final concentration of 10 μg/ml. Next, the mixture of cells and DNA was incubated for 5 h, and then spread onto membrane filters resting on BG11 agar plates amended with antibiotic.

The following variations to the transformation parameters were tested:

1. The addition of electroporation and ultrasonic treatment. Electroporation was exerted by an instruments-gene pulser and controller plus (Bio-rad Laboratories). Ultrasonic treatment was performed using an Ultrasonic Processor (Sonics & Materials Inc).
2. The use of various plasmids, including the Km^r plasmids pGSL, pRM1, pDAC, pRM2, pGL, and the Cm^r plasmid pDAK.
3. Variation of the amount of DNA added from 0 μg/ml to 50 μg/ml.
4. Variation of the growth phase of the cyanobacterium. ($OD_{730}<0.5$, latent growth phase; $0.5\leqq OD_{730}\leqq1.0$, logarithmic growth phase; $OD_{730}>1.0$, plateau growth phase)
5. Cultivation of the cyanobacteria in EDTA-BG11 medium before transformation.
6. Variation of the length of time for which the cell suspension was incubated with DNA before plating (from 1 h to 10 h).
7. Darkness vs. lightness of the incubation period.
8. Recovery treatment vs. non-recovery treatment. After incubation with DNA, the cells were spread onto antibiotic-free medium for recovery before being transferred to BG11 plates containing antibiotic.

Table 1. Effect of various plasmids on transformation efficiency

Plasmid	Structure	Transformation efficiency
pKW1188	1300 bp Km^r 1700 bp	1.008×10^{-5}
pGSL	1300 bp Km^r 1500 bp	8.667×10^{-6}
pRM1	900 bp Km^r 700 bp	1.242×10^{-6}
pDAK	400 bp Km^r 400 bp	9.865×10^{-7}
pDAC	400 bp Cm^r 400 bp	9.696×10^{-7}
pRM2	1300 bp Cm^r 200 bp	4.962×10^{-7}
pGL	1300 bp Cm^r	0

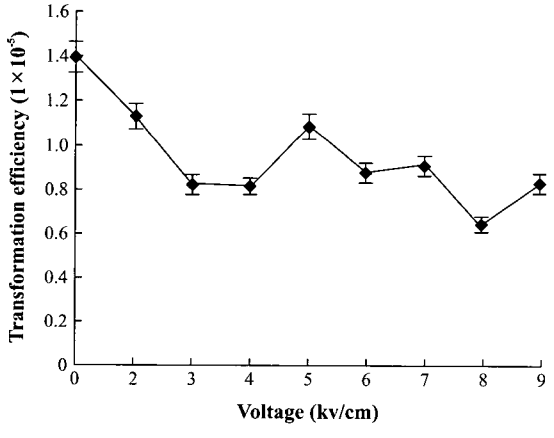

Fig. 1. Effect of voltage of electroporation on transformation efficiency. Results are expressed as the mean of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

Analysis of the transformation efficiency

Transformants grew after approximately 10 days when plated onto BG11 agar plates containing either 10 μg/ml kanamycin or 5 μg/ml chloromycetin. The number of transformants was then counted and the transformation efficiency was calculated using the following equation: Transformation efficiency (%) = the number of transformants / the total number of cells before transformation ×100.

Results

Effect of methods on transformation efficiency

Two transformation methods, electroporation and ultrasonic treatment, were performed using plasmid pKW1188 (10 μg/ml) as the donor and *Synechocystis* sp. PCC 6803 as the recipient. Neither electroporation nor ultrasonic treatment improved the transformation efficiency compared to that of natural transformation. The transformation efficiency decreased as the voltage increased in the electroporation experiment, with the highest voltage (9 kv/cm) resulting in a 41% decrease compared to the efficiency at 0 kv/cm. (Fig. 1). Ultrasonic treatment resulted in a greater decrease in transformation efficiency than electroportation. When ultrasonic treatment (3 W) was conducted for 5 sec, there were 38% less transformants than were obtained via natural transformation. Further, when ultrasonic treatment was conducted for 10 seconds, nearly all of the cells died (Fig. 2).

Effect of different plasmids on transformation efficiency

When the transformation efficiency of the Km^r plasmids was compared it was found that use of plasmids with a longer region of homology resulted in a greater number of

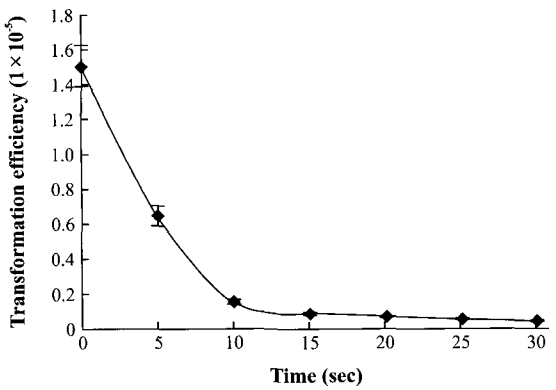

Fig. 2. Effect of pulse time of ultrasonic treatment on transformation efficiency. Results are expressed as the means of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

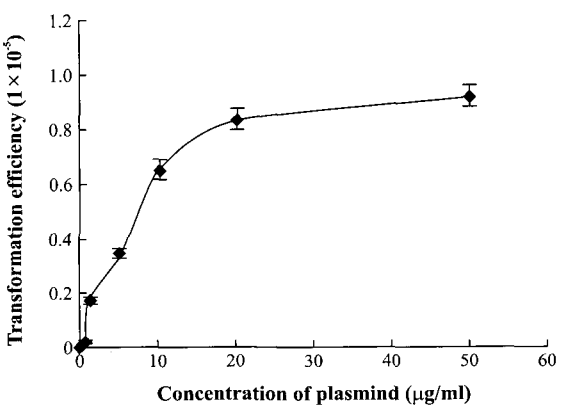

Fig. 3. Effect of plasmid concentration on transformation efficiency. Results are expressed as the means of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

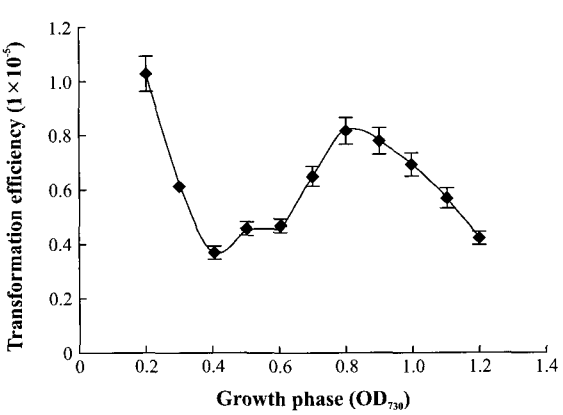

Fig. 4. Effect of growth phase of *Synechocystis* sp. PCC 6803 on transformation efficiency. Results are expressed as the means of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

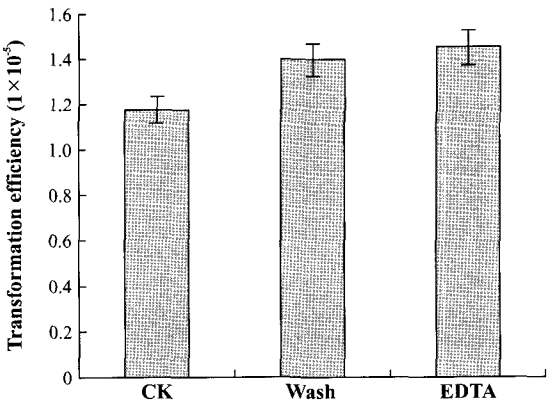

Fig. 5. Effect of treatment before transformation on transformation efficiency. Results are expressed as the means of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

tranformants. When the homologous segments were reduced to 0.4 kb (pDAK), the efficiency dropped to 10% of that of plasmid pKW1188. Additionally, the transformation efficiency dropped markedly for molecules with shorter homologous segment on one side (pRM2). Further, when there was homology on only one side (pGL), no transformants were obtained (Table 1).

When the transformation efficiency of the Km^r plasmid, pDAK, was compared to that of the Cm^r plasmid, pDAC, which differed only in their selective markers, there was no obvious difference in transformation efficiency observed. However, the transformants on the plates containing kanamycin grew 4-6 days earlier than those on the plates containing chloromycetin.

Effect of plasmid concentration on transformation efficiency

In order to determine how the concentration of donor DNA affected transformation, the cells were incubated with plasmid pKW1188 at a series of concentrations ranging from 0 to 50 μg/ml. The number of transformants increased greatly with increasing DNA concentration to a concentration of approximately 20 μg/ml, over which point no obvious increase in transformation efficiency was observed (Fig. 3). Conversely, transformants were observed even at the lowest donor DNA concentration, 0.02 μg/ml (Fig. 3).

Effect of growth phase of Synechocystis sp. PCC 6803 on transformation efficiency

The OD_{730} of cultures was used to determine the stage of growth the cells were in. When transformations were conducted using cells obtained from cultures with OD_{730} values from 0.4 to 1.2, transformation efficiency peaked when cells in mid log phase (OD_{730} of 0.8) were used (Fig. 4). The transformation efficiency of cells at mid log phase (OD_{730}= 0.8) was two fold higher than the efficiency obtained using cells in latent growth phase (OD_{730}=0.4). However, the transformation efficiency in the early latent phase (OD_{730}=0.2-0.3) was rather high (Fig. 4).

Effect of pretreatment on transformation efficiency

It has been reported that a Ca-dependent nuclease located in or on the cytoplasmic membrane can degrade exogenous DNA during uptake (Barten and Lill, 1995). Therefore, in this study, two methods were used to limit the activity of the nuclease. The first method was washing the cells with fresh BG11 medium before transformation and the second method was culturing the cells in BG11 medium modified with EDTA (2 mM) for two days. EDTA treatment improved the transformation efficiency by 23% (Fig. 5).

Effect of incubation time on transformation efficiency

The amount of incubation time between when the DNA was added to the cultures and when cells from the cultures were plated onto selective medium had a critical effect on transformation efficiency (Fig. 6). A sharp peak in transformation efficiency occurred when the incubation time was 5 h.

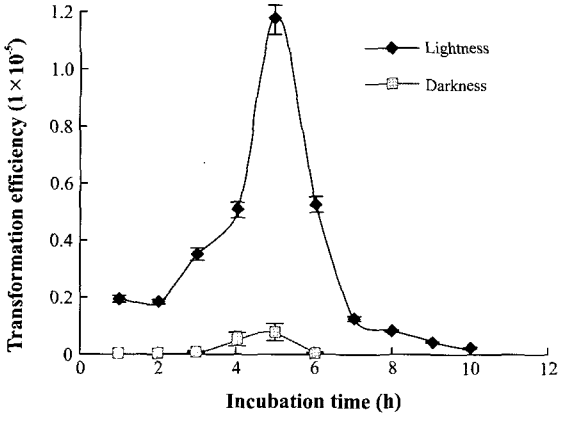

Fig. 6. Effect of time of incubation on transformation efficiency. Results are expressed as the means of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

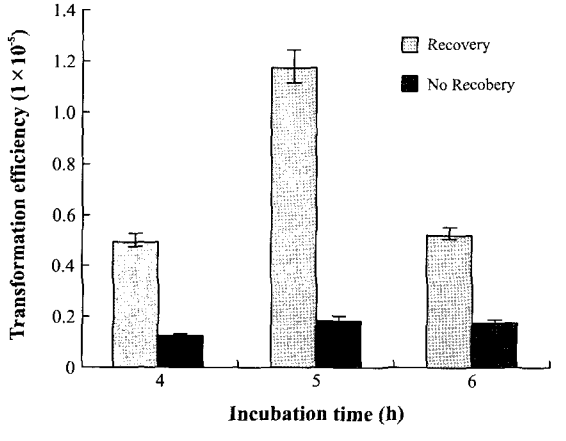

Fig. 7. Effect of recovery treatment on transformation efficiency. Results are expressed as the means of three independent determinations. The bar denotes the standard deviation (SD). When the error bar cannot be seen, the deviation is less than the size of the symbol.

Additionally, a longer incubation period resulted in a marked decrease in transformation efficiency, with only 2% of the efficiency observed after a 5 h incubation period being observed after a 10 h incubation period.

Moreover, incubation in darkness also decreased transformation efficiency (Fig. 6). For example, the transformation efficiency of cells incubated under light conditions was 1.185×10^{-5}, whereas the transformation efficiency of cells incubated in darkness was only 0.075×10^{-5}.

Effect of recovery treatment on transformation efficiency

Approximately 7 times fewer transformants were recovered from cells plated directly onto BG11 plates amended with antibiotic than from those plated onto antibiotic free medium, regardless of incubation time (4 h, 5 h or 6 h) (Fig. 7).

Discussion

Since transformation was first introduced, many new transformation techniques, such as electroporation and ultrasonic treatment, have been developed. In the case of cyanobacteria, many species, such as *Anabaena* sp. (Thiel and Poo, 1989), *Synechococcus* sp. (Matsunaga *et al.*, 1990), *Fremylla diplosiphon* (Chiang *et al.*, 1992) and *Plectonema boryanum* (Vachhani *et al.*, 1993) have been transformed by electroporation. Additionally, ultrasonic treatment has been successfully used to transform exogenous DNA into yeast (Wyber *et al.*, 1997) and maize (Wang *et al.*, 1996). Therefore, we conducted this study to determine if the transformation efficiency of *Synechocystis* sp. PCC 6803 could be improved by electroportation or ultrasonic treatment, neither of these methods resulted in an increase in efficiency. These results may indicate that *Synechocystis* sp. PCC 6803 absorbs almost all exogenous DNA through natural transformation, therefore any method that resulted in decreased viability lead to a decline in transformation efficiency.

In *Synechocystis* sp. PCC 6803, it has been reported that the full saturation concentration of transformation was occurred at about 50 μg/ml (Porter, 1986). However, in most genetic analyses, such as gene mapping, it would not be possible to provide a concentration of DNA that large, therefore the lowest concentration of DNA necessary to exert transformation should be known. It has also been reported that the transformation efficiency drops markedly for molecules with shorter homologous segments (Labarre *et al.*, 1989), therefore the shortest homologous recombination fragment necessary for an efficient transformation to occur should also be known. This paper showed that the lowest concentration of plasmid necessary for transformation to occur was 0.02 μg/ml, and that the shortest homologous fragment necessary was 0.2 kb if there was no absence of homology on either side. The optimal concentration of the plasmid and length of the homologous segments could not be provided here, however, because the transformation efficiency improved continuously as plasmid concentration and homology length increased. Moreover, it has been reported that site specific recombination exists in many species of cyanobacteria (including *Synechocystis* PCC 6803) and that the mechanism by which this recombination occurs is based on a palindromic element of the genome. (Akiyama *et al.*, 1998). This property

results in transformation occurring relatively easily at some "hot spots" in the genome. Comparing the transformation efficiency of the different plasmids used in this research revealed no unexpected increases in efficiency, therefore no "hot spots" were identified in this study.

Unlike *Anacystis nidulans* R2 (Golden and Sherman, 1984), transformation efficiency varied greatly in *Synechocystis* sp. PCC 6803 at different growth phases. There were two transformation efficiency peaks during the mid-logarithm growth phase (OD_{730}=0.8) and during the latent phase (OD_{730}=0.2-0.3). The peak during the latent phase (OD_{730}=0.2-0.3) was rather greater than that during the mid-logarithm growth phase (OD_{730}=0.8). This result may indicate that cells should not be transformed immediately upon reaching the logarithm phase. One or two days in fresh medium at a low concentration (OD_{730}=0.2-0.3) may improve the transformation efficiency.

A Ca-dependent nuclease located in or on the cytoplasmic membrane of *Synechocystis* sp. PCC 6803 was found to degrade one of the two strands of DNA during uptake (Barten and Lill, 1995). This nuclease was not present in *Synechocystis* sp. PCC6714, which is much less competent (Barten and Lill, 1995). It has also been reported that deletion of the *sll1354* gene, which encodes the single-stranded-DNA-specific exonuclease RecJ from *Synechocystis* sp. PCC 6803 increased the transformation efficiency by two orders of magnitude (Kufryk *et al.*, 2002). In this study, *Synechocystis* sp. PCC 6803 was incubated in BG11 medium containing 2 mM EDTA for two days and then washed prior to transformation. EDTA chelated Ca thereby limiting the activity of the nuclease. Washing with fresh BG11 medium removed some of the nucleases from the cytoplasmic membrane. This process resulted in a 23% increase in transformation efficiency.

In addition to the factors listed above, many transformation parameters had an effect on transformation efficiency. For instance, there was greater transformation efficiency if the mixture of cells and exogenous DNA was incubated for 5 h under light condition than under dark condition. Additionally, seven times more transformants survived when they were grown in BG11 medium containing no antibiotics for one day prior to being transmitted to BG11 medium containing an antibiotic.

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