Optimal Condition for Efficient DNA Transfer in Filamentous Cyanobacteria by Electroporation

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Filamentous cyanobacteria are an ecologically important group of bacteria because they are able to provide both organic carbon and fixed nitrogen that can support the nutritional requirements for other microorganisms. Because of their prokaryotic nature, they can also be used as potentially powerful model systems for the analysis of oxygenic photosynthesis and nitrogen fixation. Gene transfer is an indispensable procedure for genetic analysis of filamentous cyanobacteria. Electroporation was used to introduce foreign DNA into cyanobacterial cells. In experiments designed to optimize the electroporation technique, the effects of the field strength (amplitude of pulse) and time constant (duration of pulse), DNA concentration and host restriction/modification of DNA on the efficiency of electro-transformation were investigated. The results of this research revealed that a high voltage pulse of short duration was effective for the electro-transformation of *Anabaena* sp. M131. The maximal number of transformants was obtained at 6 kV/cm with a pulse duration of 5 msec. The efficiency of electro-transformation was also sensitive to concentration of DNA; even small amounts of DNA (0.01 μg/ml) were able to give a large number of transformants (1.0×10^3 cfu/ml).

Key words: Cyanobacteria, electroporation, host restriction

Cyanobacteria are a widely distributed group of photosynthetic prokaryotes. Cyanobacteria are able to convert light energy to chemical energy (ATP) in a process known as oxygenic (oxygen-producing) photosynthesis just like higher plants (3). Although mechanisms for ATP synthesis are basically the same in all photoautotrophs, there are very important differences in the photosystems between cyanobacteria and anaerobic photosynthetic bacteria. Photosynthesis of cyanobacteria is performed via two-stage photosynthetic pathways known as photosystem I and II (3, 17).

Cyanobacteria can be considered as a potentially powerful model system for the analysis of higher plant processes such as oxygenic photosynthesis. The structure and biochemistry of the photosynthetic reaction center of chloroplasts and cyanobacteria are very similar (17). However, because of their prokaryotic nature, cyanobacteria are more amenable to a variety of genetic and molecular biological manipulation than are plants.

To study biological processes such as photosynthesis, nitrogen fixation and differentiation, genetic exchanges between cyanobacterial cells are often required. Analysis of cyanobacterial genes and their mutations would not be possible without gene transfer between mutant strains. Until recently, the genetic analysis of cyanobacteria was hindered because the transfer of genetic material was not possible, either within a given species and between different cyanobacterial species. There is another method to transfer DNA into bacterial strains-electroporation. Electroporation could be as effective a method as conjugation for the gene exchange of heterocystous cyanobacteria. DNA transfer occurs via transient permeabilization (pores) of the cell membrane induced by an electric field pulse (2).

In this study, we optimized this technique as a method for genetic transformation of filamentous cyanobacteria using *Anabaena* sp. strain M131 and the shuttle vector, pRL6 plasmid which is the choice of cell strain and plasmid DNA for electro-transformation because of the high efficiency of transfer of the pRL6 plasmid into this strain by conjugation, stable replication of pRL6 in this strain, and the large amount of pRL6 extractable available from this strain (5).

In experiments designed to optimize the electroporation technique, the effects of the field strength and time constants (duration of pulse) as electrical variables, DNA concentration, and host restriction/modification on the efficiency of transformation were studied.

Materials and Methods

Growth of filamentous cyanobacteria

The filamentous cyanobacterial *Anabaena* sp. strain M131 was grown in an eightfold dilution of 50 ml of liquid medium as described in Allen and Arnon (1) supplemented with 5 mM nitrate (AA/8+ nitrate) in a 125-ml flask on a reciprocal shaker at 100 rpm under cool-white fluorescent lights in an environmental chamber of 65% relative humidity. Difco agar was added to BG-11 medium at 1.5% (8).

Preparation of plasmid from *E. coli.*

HB101 (*recA-*, *mcrB-*) and CPB 1321 (*recA+*, *mcrB-*) *E. coli* strains were used for preparing plasmid. These *E. coli* strains were grown in LB broth, supplemented as appropriate with antibiotics. The plasmids used in these experiments were shuttle vectors pRL6 and the helper plasmid pRL528 which were supplied by C. P. Wolk. Large-scale isolation of plasmid DNA from *E. coli* was performed by alkaline lysis and purification of plasmid DNA by cesium chloride-ethidium bromide gradients (7).

Electroporation

Four to eight day old cyanobacterial cell cultures were used and the density of the cultures was approximately 2.0×10^7 cells/ml. Cells were washed by centrifugation at $12,000 \times g$ for 10 min. Pellets were washed twice with about 10 ml of 1 mM Hepes, pH 7.2 and resuspended in the same buffer to concentrate cells.

Cells and a sterile plastic cuvette, which has a 2 mm gap between electrodes, were chilled on ice. DNA was added to cells in a tube at a final concentration of 0-10 µg/ml. A 40 µl aliquot of the mixture of cells and DNA was placed in the cuvette and electroporated with a single pulse with field strengths ranging 0~12 kV/cm at a resistance of 100 or 200 Ω (time constant 2.0-5.0 msec).

The BioRad Gene Pulser electroporation device was equipped with a pulse controller to vary the field strength. In most experiments, the capacitor was 25 µF and the actual time constant (T.C.) was controlled by varying the resistor (100 Ω for a time constant of about 2.5 msec, 200 Ω for a time constant of about 5.0 msec). Electroporated cells were immediately diluted out of the cuvette with 10 ml of AA/8+nitrate (5 mM) and centrifuged in a bench top centrifuge for 5 min. Pellets were suspended in 10 ml of AA/8+nitrate in a sterile 25 ml flask and incubated overnight as described above. Next day, the cells were serially diluted and 100 µl aliquotes were spread on agar-solidified BG-11 medium to determine cell viability and also on BG-11 plates with neomycin at 15 µg/ml to select the cells which had been transformed with plasmid. Plates were incubated in the culture room and the number of colonies were counted after 5~10 days.

Variation of electro-transformation condition

The standard protocol described above was modified to vary several parameters that might affect viability and electro-transformation efficiency of cyanobacterial cells. Varying the field strength from 0 to 12.0 kV/cm, electroporation was performed with plasmid pRL6 isolated from *Anabaena* sp. M131 or pRL6 and pRL528 isolated from *E. coli* HB101 strain. The concentration of these plasmid was about 10 µg/ml and the resistance was 100 Ω (T.C.=2.5 msec).

To investigate the effect of the concentration of plasmid DNA on electro-transformation efficiency, electroporation was performed by adding the appropriate amount of DNA (0 to 10 µg/ml) directly to cells in the cuvette. The field strength was 8 kV/cm and the resistance was 100 Ω. Plasmid pRL6 isolated from *Anabaena* sp. M131 was used.

The effect of restriction/modification on the efficiency of electro-transformation was observed using plasmid DNA at 1, 5, or 10 µg/ml from various sources, *Anabaena* sp. M131 (pRL6), *E. coli* HB101 (pRL6 pRL528), *E. coli* HB101 (pRL6) for electroporation. The field strength was 10 kV/cm and resistance was 100 Ω.

Cyanobacterial plasmid isolation

Large scale preparation of plasmid pRL6 from transformed cells was performed. Transformed cyanobacteria cells were grown in growth medium of AA/8+nitrate with neomycin at 10 µg/ml for about one week and collected by centrifugation. The pellet was washed, resuspended with 20 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in 50-ml polypropylene centrifuge tubes, and centrifuged in the benchtop centrifuge. The pelleted cells were suspended in 2.0 ml 25% sucrose, 50 mM Tris, pH 8.0, 100 mM EDTA and 0.2 ml of freshly prepared lysozyme (10 mg/ml in 50 mM Tris-HCl pH 8.0) were added to each tube. Afterwards, each tube was incubated at 37°C for 30-60 min. To this suspension, 4 ml of 0.75% sarcosyl (N-lauryl sarcosinate sodium salt) were added and mixed gently. The mixture was incubated at 37°C until it looked transparent and became viscous. After lysis occurred, the mixture was centrifuged at 18,000 rpm for 30 min at 4°C. Supernatants were gently poured into 15-ml polypropylene centrifuge tubes and pellets were discarded.

Supernatants were extracted with an equal volume of Tris-saturated (50 mM, pH 8.0) phenol:

chloroform: isoamyl alcohol (25:24:1) by gently vor-texing and centrifuged in a benchtop centrifuge for 10 min. The aqueous phase was removed and saved. Approximately 4 ml of 50 mM Tris, 50 mM EDTA, pH 8.0 was added to the aqueous phase which was extracted twice more with phenol: $CHCl_3$, and then it was extracted twice with $CHCl_3$-isoamyl alcohol (24:1).

Approximately 0.1 volume of 3 M sodium acetate, and 2 volumes of ethanol were added into the mixture. This mixture was incubated at -20°C over-night and centrifuged at 12,000 rpm for 30 min at 4°C. Supernatants were poured off and pellets were resuspended in TE (0.2-0.5 ml). RNase was added to a final concentration of 50 μg/ml and incubated at 37°C for 30 min. This solution was transferred to microfuge tubes, extracted with phenol-$CHCl_3$, $CHCl_3$, and ethanol precipitated as described above. Pellets were resuspended in a total volume of a-bout 1.0 ml of TE buffer. A small amount of the plasmid DNA samples was loaded on an agarose gel to estimate the amount of DNA present.

Results and Discussion

Field strength and capacitor

It was important to determine the viability of the cyanobacteria after electroporation. A high ef-ficiency of electro-transformation would be difficult to determine if the viability were extremely low be-cause the actual number of transformants would be very low. The viabilities and electro-transfor-mation efficiencies of cyanobacterial cells at vari-ous field strength and time constants were in-vestigated using *Anabaena* sp. strain M131 (University of Tokyo) and plasmid pRL6 isolated from *Anabaena* sp. M131 or pRL6 and pRL528 plasmids from *E. coli* HB101 (Fig. 1).
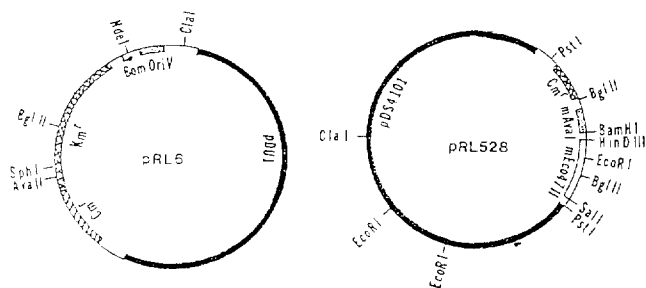

Fig. 1. Maps of plasmids pRL6 and pRL528. The portions derived from pDU1, RSF1010, pDS4101 are solid bars, the km[R] fragment is from transposon Tn5 (pBR322), the Cm[R] fragment is from pBR328. oriV; the origin of vegetative re-plication, nic; the origin of transfer replication, bom; the basis of mobility gene.

The effects of two variables-pulse amplitude (field strength) and duration (capacitor or time constant) on the viability and electro-transfor-mation of *Anabaena* sp. M131 were investigated. As field strength increased, the viability of cells generally decreased (approximately 40 times low-er at 8 kV/cm than 0 kV/cm (control)) (Fig. 2). In-creased capacitance which produces a longer pulse duration also caused the increased number of dead cells. The cells exposed to the large capa-citor (or longer pulse duration) of 960 μF (101 msec) showed about 100 times more fatality than those exposed to the small capacitor (25 μF, time constant; 4.3 msec) at a field strength of 2.25 kV/cm (data not shown).

The effects of electrical variables on the ef-ficiencies of transformation were also tested. The ef-ficiency of transformation was calculated as the number of colony forming units (cfu) of transfor-mants divided by the number of total colony form-ing units that survived electroporation. For field strengths between 6 and 12 kV/cm, the efficiency in-creased significantly (Table 1A) when cells were electroporated at a time constant of 2.4-2.5 msec (resistance of 100 Ω). The time constant was ad-justed by the pulse controller (resistors placed between the capacitor and sample). The efficiency at 8 kV/cm was approximately 10 times greater than that at 6 kV/cm. In contrast, longer time con-stants (larger capacitor) with relatively low field strengths (4 or 6 kV/cm) decreased the efficiency of electro-transformation (Table 1B). These data in-dicated that high field strength with short pulse du-ration was most effective for obtaining the max-imum efficiency of electro-transformation.
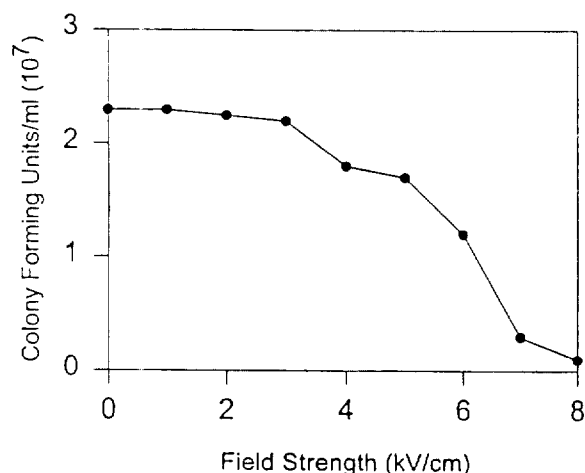

Fig. 2. Effect of field strength on viability of cells. *An-abaena* sp. strain M131 was electroporated in 8 mM Hepes, 4 mM NaCl without DNA. Time constant ranged from 3.9 to 7.9 msec.

Table 1. Effect on efficiency of electro-transformation with high field strength and small capacitor (time constant) and with low field strength and large capacitor

A. Electro-transformation efficiency with high field strength and small capacitor

Field Strength (kV/cm)	Time constant (msec)	Efficiency (10^{-3})
6	2.5	0.11
8	2.4	1.00
10	2.4	1.30
12	2.4	2.60

B. Electro-transformation efficiency with low filed strength and large capacitor

Field Strength (kV/cm)	Time constant (msec)	Efficiency (10^{-3})
4	9.1[a]	0.20
4	15.3[a]	0.18
6	8.3[b]	0.19
6	13.8[b]	0.28

Electro-transformation efficiency was the number of colony forming units (cfu) of transformants divided by the number of viable cfu.
[a] Time constant (pulse duration) was adjusted with resistor, 400 Ω.
[b] Time constant (pulse duration) was adjusted with resistor, 800 Ω.

Because of the lethality of electroporation, a maximum number of transformants of 2.06×10^4 colony forming units per ml was found at 8 kV/cm with a pulse duration of 2.4-2.5 msec (a resistance of 100 Ω) (Fig. 3). At 200 Ω, a maximum number of transformants (2.26×10^4 cfu/ml) was obtained at 6 kV/cm. Field strengths lower than 6 kV/cm were required for optimum elctro-transformation when higher resistances (400 or 800 Ω) were used for longer pulse duration (7.4-15.3 msec) (data not shown). As control experiments, cells were electroporated without DNA or not electroporated with DNA. There were no antibiotic-resistant transformants found in these experiments.

Optimization of the pulse amplitude (field strength) and pulse duration (capacitance) was important for obtaining the maximum number of viable cells receiving plasmid DNA. The field strength must be high enough to create pores in the cell membrane without high lethality of the cells. When *Anabaena* sp. strain M131 was electroporated at very high field strength, the observed leakage of the blue proteinaceous pigment was a sign of the cell lysis.

A rapid and high electric pulse efficiently transformed the *Anabaena* sp. strain M131. The maximum number of transformants (2.09×10^4 cfu/
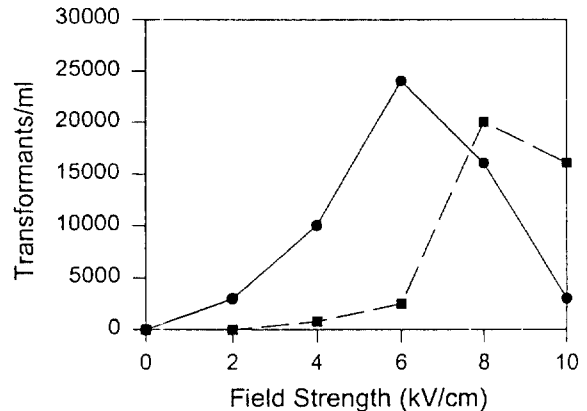

Fig. 3. Effect of field strength on the efficiency of electro-transformation. *Anabaena* sp. strain M131 was electrporated at a resistance of 100 Ω (- ■ -) or 200 Ω (- ● -) in the presence of pRL6 and pRL528 (10 µg/ml). 1 mM Hepes was used for the electroporation buffer.

ml) was obtained at a 6 kV/cm with a pulse duration of 5 msec (200 Ω resistance) (Fig. 3). Under these conditions, 30-50% of the cells were killed. At 8 kV/cm with a pulse duration of 2.5 msec (100 Ω resistance) almost the same number of transformants (2.0×10^4 cfu/ml) were obtained with about the same the lethality. High resistance (200 Ω) resulted in higher lethality than low resistance (100 Ω) at the same field strength. These results showed that longer pulse durations required lower field strengths for the maximum efficiency of electro-transformation

Generally, the optimal field strength for electro-transformation depends on the size of a cell. The size of a cyanobacterial cell is intermediate between that of *E. coli* and *Saccharamyces cerevisiae*. *E. coli* cells generally require a field strength of more than 12 kV/cm (2) and yeast cells require only 2.5 kV/cm (4) for optimum electro-transformation. Cyanobacterial cells required intermediate field strengths (6~8 kV/cm) for optimum electro-transformation.

Electroporation medium

The electroporation medium was Hepes buffer (1 mM, pH 7.4). The effects of ionic strength (which was adjusted by the concentration of NaCl) on the viability of *Anabaena* sp. M131 and on the efficiency of electro-transformation were also tested using plasmid pRL6 DNA extracted from *Anabaena* sp. M131. The effect of the concentration of Hepes in electroporation buffer on the viability of cells was also investigated.

The viability of cells increased as the concentration of NaCl was increased when the time constant was varied by changing the concentration
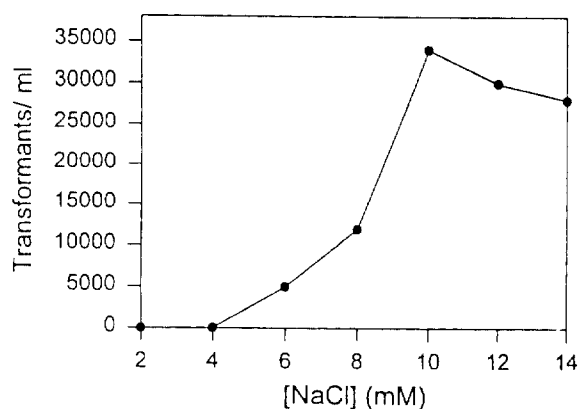
Fig. 4. Effect of the concentration of NaCl on the efficiency of Electro-transformation. Cells (*Anabaena* sp. strain M131) were electroporated at 10 kV/cm with the capacitance of 25 μF with plasmid pRL6 extracted from the same strain (10 μg/ml). 8 mM Hepes buffer was used for electroporation medium with various concentration of NaCl.
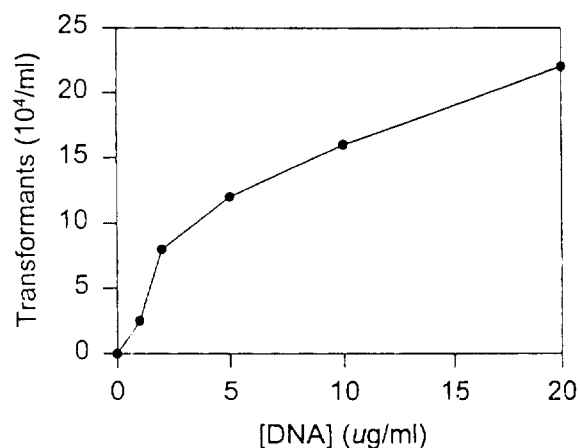

Fig. 5. Effect of the concentration of DNA on the efficiency of electro-transformation. *Anabaena* sp. strain M131 was electroporated in 1 mM Hepes with various concentrations of pRL6 isolated from the same strain. Field strength was 8 kV/cm and pulse duration was about 2.5 msec (100 Ω resistor).

of NaCl. Time constants ranged from 13.8 to 2.4 msec as the concentration of NaCl was varied from 0 to 15 mM. This result was consistent with the decrease in cell viability when the pulse duration was adjusted via the capacitor. Thus it was probably the long pulse duration rather than the actual salt concentration that affected the cell viability. The efficiency of electro-transformation was maximal at approximately 2.5-4.0 msec (8-15 mM NaCl) and the maximum number of transformants was obtained with 8 mM Hepes buffer with 8 mM NaCl (T.C.=3.8 msec) (Fig. 4). Cells of *Anabaena* sp. M131 were also electroporated in the media of different concentrations of Hepes (0-8 mM) without DNA. Lower concentration of Hepes (1 and 4 mM) gave relatively greater cell density than high concentration of Hepes (8 mM). At 0 mM of Hepes (distilled water), however, cell density decreased most as field strength increased.

The ionic strength of the electroporation buffer was altered with the various concentration of NaCl. The optimum concentration was 1 to 4 mM NaCl giving a time constant of 2.5-4.5 msec. The optimum time constant found by changing the capacitance was 4.0-4.5 msec (data not shown). The optimum time constant value found by altering ionic strength was very similar to the optimum time constant obtained by changing the capacitance. Therefore, the optimum ionic strength is probably only a function of the optimum time constant (pulse duration). At 15 mM NaCl, however, less viability was observed than at 10 mM NaCl even though the pulse duration was shorter. High concentrations of NaCl (higher than 15 mM) might be a cause of cell death.

DNA Concentration

The effect of plasmid DNA (pRL6 from the M131 strain) concentration on the total number of electro-transformants of *Anabaena* sp. strain M131 was examined. The number of transformants was generally increased with increasing concentration of DNA (0-20 μg/ml) (Fig. 5). However, the increase in the number of electro-transformants obtained at each concentration was not directly proportional to the amount of DNA added. The highest transformation frequency (1.05×10^4 transformants per μg of DNA) was obtained at low concentrations (0.01 μg/ml) of pRL6 DNA (Table 2). However, the maximum number of transformants was obtained with high concentrations of DNA.

Table 2. Effect of DNA concentration on electro-transformation frequency[a]

[DNA] (μg/ml)	Transformation frequency[b] (# of transformants/μg of DNA)
0	0
0.01	105,000
0.1	100,000
0.5	56,400
1.0	33,000
2.0	42,500
5.0	21,000
10.0	16,000
20.0	10,000

[a] *Anabaena* sp. M131 (concentration; 4×10^7/ml) were electroporated at 10 kV/cm with 100 Ω of resistance with pRL6 isolated from same strain.
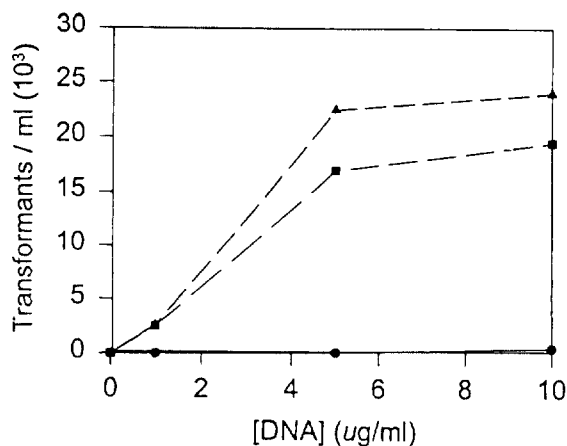[b] Frequency=number of transformants/μg of DNA, T.C=2.4-2.5 msec.

Fig. 6. Modification and restriction affect the efficiency of electro-transformation of *Anabaena* sp. strain M131. Cells were electroporated with plasmid DNAs from three sources-pRL6 isolated from the same strain (▲), pRL6 and pRL 528 from *E. coli* HB101 (■) and pRL6 from *E. coli* HB 101 (●) (1, 5, and 10 μg/ml). Field strength was 10 kV/cm and pulse duration was 2.5 msec.

When *Anabaena* sp. M131 was electroporated with pRL6 isolated from the same strain at a final concentration of 0-20 μg/ml the number of transformants increased as the concentration of DNA increased and showed signs of saturation at a high concentration of DNA (10-20 μg/ml). Natural transformation of unicellular strains of cyanobacteria generally shows saturation at lower DNA concentrations of approximately 1-2 μg/ml (*Synechocystis* PCC 6803) (11). This may indicate that the optimal conditions for gene transfer by electroporation of cyanobacteria may be different from those for the natural transformation of unicellular strains of cyanobacteria.

Restriction and modification

The effect of restriction and modification on efficiency of electro-transformation was examined by electro-transforming plasmid pRL6 extracted from the following cells; *Anabaena* sp. strain M131 (pRL 6), *E. coli* HB101 (pRL6, pRL528), and *E. coli* HB 101 (pRL6). *Anabaena* sp. strain M131 contain two known restriction enzymes-*Ava*I and *Ava*II and modifies these sites. The *E. coli* plasmid pRL528 has genes encoding methylases which modify *Ava*I and *Ava*II sites. Thus *E. coli* HB101 (pRL6, pRL 528) modifies *Ava*I and *Ava*II sites in pRL6. There was no significant difference in the number of transformants obtained by electroporation with pRL6 from *Anabaena* sp. strain M131 or pRL6 (containing the modifying plasmid pRL528) from *E. coli* HB101. However, approximately 100 times fewer number of electro-transformants was obtained

when cells of *Anabaena* sp. strain M131 were electroporated with pRL6 isolated from *E. coli* HB101 without prior modification of *Ava*I and *Ava*II sites (Fig. 6). This result shows that restriction of DNA might be a serious problem for high efficiency electro-transformation of DNA between different species.

Possible restriction of unmodified DNA in cyanobacteria was one of the most important parameters tested in determining the optimum condition for electro-transformation. Plasmid pRL6 isolated from *E. coli* (unmodified plasmid) was approximately 100 times less efficient in electro-transformation than pRL6 plasmid isolated from the cyanobacterial strain or pRL6 plasmid in which *Ava*I and *Ava*II sites were previously modified by the helper plasmid pRL528.

References

1. **Allen, M.B. and D.I. Arnon.** 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol.* **30**, 366-372.
2. **Calvin, N.M. and P.C. Hanawalt.** 1988. High-efficinecy transformation of bacterial cells by electroporation, *J. Bacteriol.* **170**, 2796-2801.
3. **Carr, N.G. and B.A. Whitton.** 1982. The Biology of cyanobacteria, Botanical monographs, vol. 19, p. 263-305. Blackwell Science Publications. Oxford.
4. **Delorme, E.** 1989. Transformation of *Saccharamyces cerevisiae* by electroporation. *Appl. Environ. Microbiol.* **55**, 2242-2246.
5. **Elhai, J. and C.P. Wolk.** 1988. Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol.* **167**, 747-754.
6. **Fiedler, S. and R. Wirth.** 1988. Transformation of bacteria with plasmid DNA by electroporation. *Anal. Biochem.* **170**. 38-44.
7. **Maniatis, T., J. Sambrook, and E.F. Fritsch.** 1989. Molecular cloning. Cold Spring Harbor, N. Y.
8. **Porter, R.D.** 1987. Transformation of cyanobacteria. *Crit. Rev. Microbiol.* **13**, 111-132.
9. **Reiser, J., V. Glumoff, U.A. Ochsner, and A.J. Fiechter.** 1994. Molecular analysis of the *Trichosporon cutaneum* DSM 70698 *argA* gene and its use for DNA-mediated transformations. *J. Bacteriol.* **176**, 3021-3032.
10. **Shigekawa, K. and W.J. Dower.** 1988. Electroporation of eukaryotes and prokaryotes: A general approach to the introduction of macromolecules into cells. *Biotechniques* **6**, 742-751.
11. **Summers, D.K. and H.L. Withers.** 1990. Electrotransfer: direct transfer of bacterial plasmid DNA by electroporation. *Nucl. Acid Res.* **18**, 2192-2197.
12. **Thiel, T. and M. Leone.** 1986. Effect of glutamine on growth and heterocysts differentiation in the cyanobacterium *Anabaena variabilis*. *J. Bacteriol.* **168**, 769-774.
13. **Thiel, T. and C.P. Wolk.** 1987. Conjugal transfer of plasmids to Cyanobacteria. *Methods Enzymol.* **153**, 232-243.

14. **van der Plas, J., H. Hegeman, G. de Vrieze, M. Tuyl, M. Borrias, and P. Weisbeek.** 1990. Genomic integration system based on pBR322 sequences for the cyanobacterium *Synechococcus* sp. PCC7942: transfer of genes encoding plastocyanin and ferredoxin. *Gene* **95**, 39-48.

15. **Watts, J.W., J.M. King, and N.J. Stacey.** 1987. Inoculation of protoplasts with viruses by electroporation. *Virology* **157**, 40-46.

16. **White, M.A., J.C. Bailey, G.C. Cannon, and C. Heinhorst.** 1997. Partial purification and characterization of the DNA polymerase from the cyanelles of Cyanophora paradoxa. *FEBS Lett.* **410**, 509-514.

17. **Wolk, C.P.** 1980. Cyanobacteria (blue-green algae). In: The Biochemistry of plants. vol. 1, p. 659-684, Academic Press Inc., N.Y.