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Transformation of a Filamentous Cyanobacterium by Electroporation

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The filamentous cyanobacterium *Anabaena* sp. strain M131 was transformed with the shuttle vector pRL6 by electroporation. Optimum conditions for electroporation required relatively high field strengths with short time constants. Restriction significantly lowered the efficiency of transformation. A plasmid containing a single unmodified *Ava*II restriction site transformed cells with about 100-fold-lower efficiency than did the same plasmid with a modified restriction site.

Cyanobacteria are photosynthetic procaryotes with many characteristics that make them useful for the study of a variety of biological processes. The photosynthetic apparatus of cyanobacteria is very similar to that of higher plants, including both photosystem I and photosystem II (7). Many filamentous cyanobacteria have heterocysts, which are specialized cells in the filament that fix nitrogen during aerobic growth (6, 21). Heterocysts form in a semiregular pattern within a filament and are potential models for the study of cell-to-cell communication (21). Genetic techniques for the study of photosynthesis, nitrogen fixation, cell differentiation, and pattern formation in filamentous cyanobacteria have developed slowly. There is no reproducible transformation procedure for filamentous cyanobacteria as there is for several strains of unicellular cyanobacteria (14, 16). The only well-documented method for gene transfer in filamentous cyanobacteria is conjugative transfer of shuttle vectors from *Escherichia coli* by using a broad-host-range plasmid such as RP4 (3, 4, 20, 22; J. Elhai, T. Thiel, and H. B. Pakrasi, *in* S. Gelvin and R. Schilperoort, ed., *Plant Molecular Biology Manual*, in press).

Electroporation appears to be a general technique for the introduction of macromolecules into a variety of cells, both eucaryotic and procaryotic (17). Electroporation is the reversible permeabilization of cell membranes by a high-voltage potential across the membrane (10, 11). It has been used to introduce DNA into plant, animal, and bacterial cells (17). We report here optimum conditions for the transformation of a plasmid into a filamentous cyanobacterium by electroporation.

The shuttle vector pRL6 (22) can be readily introduced into *Anabaena* sp. strain M131 by conjugative transfer from *E. coli* by using a broad-host-range plasmid such as RP4 for mobilization. We chose this plasmid and this cyanobacterial strain for three reasons. (i) *Anabaena* sp. strain M131 is receptive to foreign DNA. The frequency of transfer of plasmids (including pRL6) by conjugation to this strain is high, and it is one of the best hosts for cyanophages (8, 22). (ii) pRL6 replicates stably in this strain, expressing both the neomycin (Nm^r) and chloramphenicol (Cm^r) resistance genes. (iii) pRL6 can be isolated from *Anabaena* sp. strain M131 (after transfer by conjugation) in sufficient quantities to allow electroporation with DNA isolated from the cyanobacterial host. This would eliminate possible problems of restriction, which could have made detection of low-efficiency electroporation difficult.

Anabaena sp. strain M131 (University of Tokyo) was grown in 50 ml of an eightfold dilution of the medium of Allen and Arnon (AA) (1) with 5.0 mM $NaNO_3$ (AA/8 plus nitrate) in a 125-ml flask. Cultures were incubated at 30°C on a reciprocal shaker at 100 rpm under cool-white fluorescent lights at an intensity of approximately 50 microeinsteins m^{-2} s^{-1} to a density of about 2×10^7 cells ml^{-1}.

The two *E. coli* strains used in these experiments were HB101 (*recA mcrB*) (15) and CPB1321 (*recA*$^+$ *mcrB*) (15). *E. coli* strains were grown in L broth, supplemented as appropriate with kanamycin at 50 μg ml^{-1} and Cm at 25 μg ml^{-1}. The primary plasmid used in these experiments was a shuttle vector, pRL6 (22), very kindly provided by C. P. Wolk. This plasmid has the replication origin of pBR322 and the replicon from an endogenous plasmid, pDU1, from *Nostoc* sp. strain PCC 7524. It encodes resistance to neomycin (derived from transposon Tn*5*) and chloramphenicol (derived from pBR328) (22). It replicates well in *E. coli* and in several strains of cyanobacteria, including *Anabaena* sp. strain M131, after transfer to the cyanobacterium by conjugation (4, 20, 22). In some experiments, a second plasmid, pRL528, was also present in the *E. coli* cells used to propagate pRL6; pRL528 carries the gene for *Eco*47II methylase. This methylase protects against restriction by *Ava*II, a restriction enzyme produced by *Anabaena* sp. strain M131 (3).

Plasmid DNA isolation from *E. coli* strains, digestion with restriction endonucleases, and visualization after agarose gel electrophoresis were done by standard recombinant DNA techniques (12). Plasmid DNA was isolated from *Anabaena* sp. strain M131 by the method of Simon (18). Plasmid stocks for electroporation were dissolved in 1.0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2).

For electroporation, cells of *Anabaena* sp. strain M131 at a density of about 2×10^7 cells ml^{-1} were washed twice in 1.0 mM HEPES, pH 7.2, and suspended in the same buffer at a concentration of about 10^9 cells ml^{-1}. Cells were chilled on ice, and DNA (in 1.0 mM HEPES) was added at the concentration indicated in the figure legends (generally 1 to 10 μg ml^{-1}). Cells were electroporated in 40-μl volumes in a chilled, sterile cuvette with a 2-mm gap between the electrodes at the field strengths and time constants indicated in each figure legend. The electroporation device was a Bio-Rad Gene Pulser equipped with a pulse controller to vary the resistance (and hence the time constant). For all data given here, the 25-μF capacitor was used and the time constant

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was varied by changing the resistors (100 ohms for a time constant of 2.5 ms, 200 ohms for a time constant of 5 ms). The field strength was equal to the voltage applied divided by the gap distance of the cuvette (2 mm). Cells were given a single pulse and then were immediately diluted and rinsed out of the cuvette with 2.0 ml of AA/8 plus nitrate. Cells were washed once with 5 ml of AA/8 plus nitrate, suspended in 10 ml of the same medium in a 25-ml flask, and incubated under the growth conditions described above. After 18 to 24 h, cells were harvested by centrifugation, suspended in 1.0 ml of AA/8-nitrate, and serially diluted in the same medium, and 0.1-ml portions were spread on petri plates containing agar-solidified BG-11 medium (2) (1.5% agar [Difco or Scott] with 1.0 mM thiosulfate [5]) with neomycin at 15 μg ml^{-1} to select for the presence of the plasmid. Plates were incubated at 30°C under lights as described above until colonies appeared, in about 5 days. Liquid cultures of *Anabaena* sp. strain M131 containing pRL6 were grown in AA/8 plus nitrate with neomycin at 10 μg ml^{-1}.

Electroporation experiments with plasmid pRL6 isolated from *Anabaena* sp. strain M131 with a variety of capacitors from 2.5 to 900 μF (time constants were in the range of 1 to 50 ms) at relatively low field strengths (<2 kV cm^{-1}; high field strengths cannot be used with large capacitors in the Bio-Rad apparatus) indicated that low field strengths with short time constants gave very few transformants. In addition, long time constants (i.e., large capacitors) at low field strengths gave high killing but no transformants (data not shown). In contrast, shorter time constants with high field strengths reproducibly yielded transformants. Using the pulse controller (i.e., resistors) to regulate time constants, we established optimum field strengths and time constants for electroporation. The results of a representative experiment are given in Fig. 1. At a time constant of 2.5 ms (100-ohm resistance), the maximum number of transformants occurred at a field strength of 8 kV cm^{-1}; however, because of the greater killing at field strengths of 10 and 12 kV cm^{-1}, the transformation efficiency (number of transformants per viable CFU) was greatest at a field strength of 12. Similarly, at a time constant of 5 ms (200-ohm resistance), the greatest number of transformants was at 6 kV cm^{-1}, whereas the highest efficiency was at 8 kV cm^{-1}. At time constants above 5 ms (10 to 15 ms), the total number of transformants and the efficiencies were 10 to 100-fold lower than the values given in Fig. 1, and killing was greater, particularly at high field strengths (data not shown). The field strength values for maximum numbers of transformants were much lower than the 12.5 kV cm^{-1} reported for *E. coli* (17), but were much larger than those reported for plant and animal cells, which typically require low field strengths and long time constants (17). The intermediate size of cyanobacterial cells (in general, larger than bacteria but smaller than eucaryotic cells) may dictate these intermediate conditions.

The frequency of transformation was dependent on the concentration of DNA (Fig. 2). Although relatively high concentrations of DNA were required to give large numbers of transformants, this was not a significant problem. Electroporation was done in a volume of only 40 μl and hence, small amounts of DNA were required. Also, DNA isolated from *E. coli* transformed *Anabaena* sp. strain M131 well (with modification of restriction sites; see discussion below).

Presumptive transformants which grew well in the presence of neomycin were tested further to determine whether the plasmid was present and replicating in the cells. Several Nm^r colonies, streaked on medium containing chloramphenicol at 25 μg ml^{-1} (Cm^r is also encoded by pRL6), were also

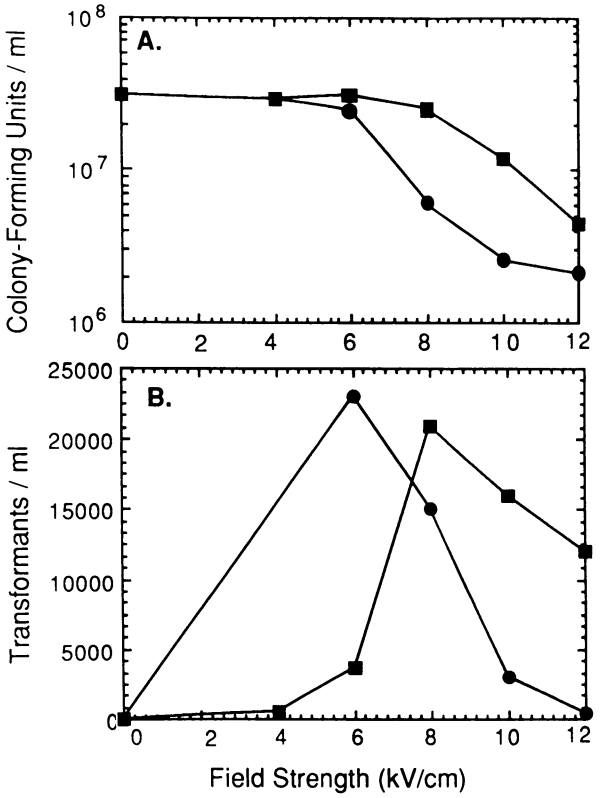

FIG. 1. Optimum conditions for electroporation of *Anabaena* sp. strain M131 with pRL6. Plasmid pRL6 at a concentration of 5 μg ml^{-1} was used to transform cells of *Anabaena* sp. strain M131 as described in the text. (A) Viability of cells as a function of field strength. (B) Number of transformants as a function of field strength. Time constants: 2.5 ms (■) and 5.0 ms (●).

Cm^r. Plasmid extracted from putative *Anabaena* sp. strain M131 transformants was found to give restriction fragments that were the same size as restriction fragments of pRL6 (Fig. 3). It thus appears that the plasmid recovered from the transformants was identical to the plasmid provided and that the plasmid replicated autonomously.

Restriction of transforming DNA isolated from a heterologous source has been reported to cause decreased frequencies of transformation by electroporation in *Campylobacter jejuni* (13). Cyanobacteria are a rich source of restriction endonucleases; *Anabaena* sp. strain M131 contains *Ava*I and *Ava*II restriction enzymes (19). The plasmid pRL6 has no

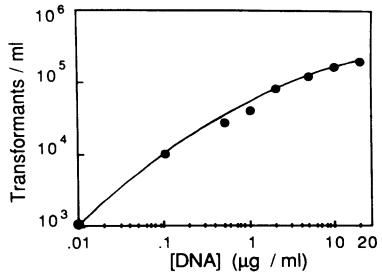

FIG. 2. Effect of DNA concentration on transformation frequency. Cells of *Anabaena* sp. strain M131 were transformed as described in the text with various concentrations of plasmid pRL6 at a field strength of 8 kV cm^{-1}, with a time constant of 2.5 ms.

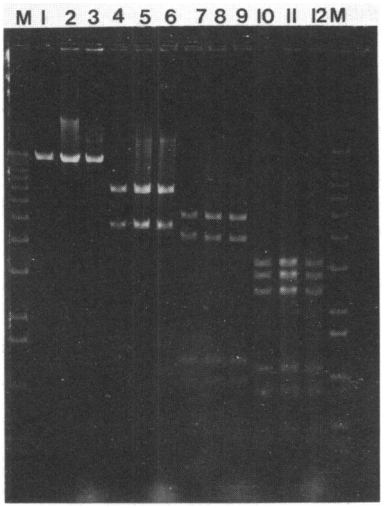

FIG. 3. Plasmid DNA was extracted from cells of *E. coli* HB101(pRL6) (lanes 1, 4, 7, 10), *Anabaena* sp. strain M131(pRL6) (the plasmid was transferred into the cells by conjugation) (lanes 2, 5, 8, 11), and from *Anabaena* sp. strain M131(pRL6) (the plasmid was transformed into the cells by electroporation) (lanes 3, 6, 9, 12). Plasmid was digested with restriction enzymes *Bgl*II (lanes 1, 2, 3), *Eco*RI (lanes 4, 5, 6), *Eco*RV (lanes 7, 8, 9), or *Hin*dIII (lanes 10, 11, 12) prior to electrophoresis in a 0.7% agarose gel. Lanes marked M contain molecular size markers of approximately 1 kilobase difference in size, with 12 kilobases at the top.

*Ava*I sites, but has one *Ava*II site (22). The possible effect of restriction of unmodified DNA in *Anabaena* sp. strain M131 on the frequency of transformation of this strain with pRL6 was examined. Plasmid pRL6 was isolated from *Anabaena* sp. strain M131(pRL6), *E. coli* HB101(pRL6, pRL528), *E. coli* CPB1321(pRL6, pRL528), and *E. coli* HB101(pRL6). The plasmid pRL528 carries the gene for *Eco*47II methylase, which protects against restriction by *Ava*II (3). Plasmid from *E. coli* HB101(pRL6, pRL528) (*recA mcrB*) and *E. coli* CPB1321(pRL6, pRL528) (*recA*$^+$ *mcrB*) was tested to determine whether possible multimers of the plasmid that could be formed in a *recA*$^+$ strain (9) affected the transformation frequency. pRL6, isolated from the four strains described above, was used to electroporate *Anabaena* sp. strain M131 at a concentration of 5 to 10 μg ml^{-1}. The field strength was 10 kV cm^{-1}, and the time constant was 2.5 ms. The frequency of transformants (the number of Nm^r transformants divided by the total number of CFU) for DNA isolated from *Anabaena* sp. strain M131(pRL6), *E. coli* HB101 (pRL6, pRL528), and *E. coli* CPB1321(pRL6, pRL528) was in the range of 1.2×10^{-4} to 8.5×10^{-4} (three to four experiments), whereas the frequency for pRL6 isolated from *E. coli* HB101(pRL6) was 0.9×10^{-6} to 2.2×10^{-6} (four experiments). There was no significant difference in the transformation frequency of *Anabaena* sp. strain M131 with DNA extracted from the three strains that modify *Ava*II sites, nor did the possible presence of multimers of plasmid from *Anabaena* sp. strain M131 or from the *recA*$^+$ strain of *E. coli* affect transformation frequency. However, plasmid from the *E. coli* strain that did not modify *Ava*II sites transformed *Anabaena* sp. strain M131 with about 100-fold-lower efficiency than plasmid that had been modified. It appears, therefore, that even a single unmodified restriction site can significantly lower the efficiency of transformation by electroporation. While circular plasmid transformed well, pRL6 restricted with *Cla*I (there is a single *Cla*I site in a nonessential region of pRL6) gave no transformants.

In addition to the data presented for transformation of *Anabaena* sp. strain M131 with pRL6, we also obtained antibiotic-resistant colonies when a derivative of the broad-host-range plasmid RSF1010, which can replicate in *Anabaena* sp. strain M131 after transfer by conjugation (T. Thiel and J. Elhai, unpublished), was transformed into this strain by electroporation. Successful preliminary attempts to transform other cyanobacterial strains (e.g., *Anabaena* sp. strain PCC 7120, *Nostoc* sp. strain MAC, and *Synechococcus* sp. strain PCC 7942) by electroporation with replicative plasmids suggest that the conditions described here for *Anabaena* sp. strain M131 may be generally applicable for the transfer of DNA into many other cyanobacteria.

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