Transformation of the Cyanobacterium *Synechococcus* PCC 6301 Using Cloned DNA

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Stable ampicillin-resistant transformants of *Synechococcus* PCC 6301 were isolated using as donor fragments chromosomal DNA cloned into ColE1-derived vectors. Using dark-incubated recipient cells, transformation was achieved reproducibly at frequencies of approximately 10^{-5} per cell and 3×10^2 per μg of donor DNA. These frequencies were 10^2 - to 10^4 -fold lower than those reported previously by other workers for the closely related strain PCC 7942 but only 5- to 10-fold lower than found by us in parallel experiments with both strains. Different donor fragments of PCC 6301 DNA gave different characteristic transformation frequencies which were greatly reduced by short internal deletions. The results are most simply explained by a model of integration of circular plasmid DNA into the recipient chromosome by a single cross-over event.

INTRODUCTION

Transformation of some strains of the unicellular cyanobacterium *Synechococcus*, associated with incubation of cells in the presence of homologous DNA, is documented (Shestakov & Khyen, 1970; Orkwiszewski & Kaney, 1974; Grigorieva & Shestakov, 1976; Grigorieva, 1985). In some cyanobacteria it is possible to follow the fate of the presumed transforming DNA with genetic markers (Astier & Joset-Espardelier, 1976; Stevens & Porter, 1980; Williams & Szalay, 1983) or radiolabelling (Shestakov *et al.*, 1982). Recombination between plasmid-borne chromosomal fragments and the homologous region in the chromosome of the recipient cell occurs at high frequency in strains such as *Synechococcus* PCC 7942 and *Synechococcus* PCC 7002 (Williams & Szalay, 1983; Buzby *et al.*, 1983).

The work described in this paper was done with *Synechococcus* PCC 6301, a strain for which a great deal of biochemical and genetic information has been amassed (Herdman, 1982). Transformants of this strain have been isolated following incubation of cells with chromosomal DNA (Herdman 1973; Orkwiszewski & Kaney, 1974), but integrative transformation of PCC 6301, detected by selection for donor antibiotic-resistance markers, has not been reported. It has been suggested that during laboratory culture this strain has lost the ability to take up, assimilate or maintain exogenous DNA (Kuhlemeier, 1984). We now report the stable and reproducible transformation of *Synechococcus* PCC 6301 using donor chimaeric plasmids carrying inserts of *Synechococcus* chromosomal DNA. A simple protocol for transformation is described, involving incubation of recipient cells with donor DNA in darkness followed by selection for the donor ampicillin-resistance marker.

METHODS

Strains and plasmids. Strains of *Synechococcus* and *Escherichia coli* are listed in Table 1. Plasmids are described in Table 1 and Fig. 1.

Media and growth. *E. coli* CLR207 *recA* was grown at 37 °C on L-broth and L-agar plates containing 0.2% (w/v) glucose. Ampicillin (20 $\mu\text{g ml}^{-1}$) and tetracycline (15 $\mu\text{g ml}^{-1}$) were added when required. The strain was periodically selected on tetracycline and tested for UV-sensitivity to confirm Tn10 inactivation of *recA*.

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Fig. 1. Linear restriction maps of plasmids used for transformations. Panel (a) shows the recombinant plasmids containing random genomic inserts. Panel (b) shows pDN1 and its derivatives. Sites shown are *Bam*HI (B), *Bst*EII (T), *Eco*RI (E), *Hind*III (H), *Sma*I (S) and *Xho*I (X).

Table 1. *Relevant characteristics of strains and plasmids used*

Strain	Source or reference	Relevant characters		
<i>E. coli</i> CLR207 <i>recA</i>	Mattaj <i>et al.</i> (1982)	Tc, <i>recA</i>		
<i>Synechococcus</i> PCC 6301	N. G. Carr	Wild-type		
<i>Synechococcus</i> PCC 7942	C. Kuhlemeier	Wild-type		
Plasmid*	Nature of insert DNA	Size of plasmid (kbp)	Vector†	Relevant characters
pGO1	Native chromosomal	9.5	pBR322 (H)	Ap
pGO4	Native chromosomal	10.1	pBR322 (H)	Ap
pGO3	Native chromosomal	8.9	pRK9 (B)	Ap
pDN1	Native chromosomal	13.5	pBR322 (B)	Ap
pDS1	Duplication of pDN1 sequences	13.0	pRK9 (B)	Ap
pNH1	Deletion of pDN1	7.0	pBR322 (B, H)	Ap
pCI3	Native chromosomal	7.0	pRK9 (B)	Ap
pHB1	Internal deletion of 1.3 kbp	5.7	pBR322 (B, H, B)	Ap
pHX1	Internal deletion of 1.0 kbp	6.0	pBR322 (B, H, X)	Ap
pNS1	Internal deletion of 1.5 kbp	12.0	pBR322 (B, N, S)	Ap
pBR322	None	4.3		Ap, Tc
pRK9	None	4.1		Ap

* All plasmids except pBR322 and pRK9 were constructed in this work (see text for details).

† Letters in parenthesis indicate the restriction site in the vector used for cloning; the second of two indicates a vector site used to construct deletions of the chimaeric plasmids and the third of three indicates the pair of sites in the insert used to construct internal deletions (B, *Bam*HI; H, *Hind*III; N, none; S, *Sma*I; X, *Xho*I).

Medium BG-11 (Rippka *et al.*, 1979) was used for growth of all cyanobacterial strains. The components of the medium were made up separately, at 200 times the working concentration, in double-distilled water and sterilized by autoclaving. Medium containing agar was made by sterilizing 0.85% (w/v) Difco agar in double-distilled water and adding medium components after cooling to 55 °C. Ampicillin was added at 2 µg ml⁻¹. The source of ampicillin was very important: Amphipen (sodium ampicillin BP, for intramuscular use), as supplied by Brocades

Ltd, was satisfactory indefinitely if stored dry at -20°C or for one month in solution at -20°C . Liquid cultures were incubated at 30°C , with 10 W m^{-2} fluorescent white light, and vigorous aeration. Agar plates were incubated at 30°C , with 3 W m^{-2} white light in a Gallenkamp illuminated incubator.

Cloning strategies. A gene library was constructed from *Synechococcus* PCC 6301 chromosomal DNA and two plasmid vectors, pBR322 and the derived expression vector pRK9 (Schechtman & Yanofsky, 1983). The chromosomal DNA was either partially digested to an average length of 3 kbp with *Sau*3A and ligated into the *Bam*HI site of both plasmid vectors or digested to completion with *Hind*III and ligated into the *Hind*III site of pBR322. Recombinant plasmids were transformed into the multiply-auxotrophic *E. coli* strain CLR207 *recA* (Mattaj *et al.*, 1982). *E. coli* transformation was done as described by Hanahan (1983). Plasmid DNA was isolated by the method of Ish-Horowitz & Burke (1981). Restriction maps (Fig. 1) were constructed from single and double digests separated by electrophoresis on 0.8% agarose gels.

Construction of deleted derivatives from hybrid plasmids. The deleted derivative pNH1 (Fig. 1) was produced from pDN1 by digestion of plasmid DNA with *Hind*III followed by religation and transformation into *E. coli*. The internal deletions (Fig. 1) were constructed by restriction endonuclease digestion of the plasmids pDN1 and pNH1 with enzymes known to cleave twice within the DNA of cyanobacterial origin but not the vector, followed by religation at low DNA concentration. *Bam*HI, *Xho*I and *Sma*I were used to produce the derivatives pHBI, pHX1 and pNS1 respectively. Plasmid pDS1 was constructed by ligation of a partial *Sau*3A digest of pDN1 DNA into the *Bam*HI site of pRK9. Restriction analysis showed that multiple fragment insertions occurred, producing a tandem repeat structure (Fig. 1).

***Synechococcus* transformation protocol.** Typically a liquid culture (50 ml) was grown to an OD_{660} of 1.0 (approximately 2×10^8 c.f.u. ml^{-1}). Cells were centrifuged (8000 g, 10 min) and resuspended in half the volume of fresh BG-11 medium. Samples (1 ml) were transferred to polycarbonate tubes (Sterilin) containing 0.3, 1.0 or 10 μg of the transforming DNA. The tubes were wrapped in aluminium foil to exclude light and incubated at 30°C overnight before being incubated for a further 6 h in the light (3 W m^{-2}). Samples of up to 0.1 ml were plated directly onto fresh, undried, BG-11 plates containing ampicillin. The plates were incubated for 4 h before inverting to allow the surface of the plate to dry. Incubation was continued for 20–30 d to allow large colonies to form. The Ap^r phenotype of putative transformed colonies was verified by restreaking on fresh BG-11 plates containing ampicillin. After incubation these plates were used to inoculate liquid cultures (50 ml) containing ampicillin. Total DNA was extracted from these cells by the method of Mazur *et al.* (1980) for analysis by Southern hybridization (Southern, 1975).

RESULTS AND DISCUSSION

Transformation of recipient cells incubated in the light

Duplication of the original transformation protocol for *Synechococcus* PCC 6301 (Herdman, 1973) failed to produce any Ap^r colonies with either pBR322 or pDN1 DNA, despite elevated DNA concentrations (to $20\text{ }\mu\text{g ml}^{-1}$). A 24 h growth period on non-selective medium facilitated the recovery of a few transformants, probably by permitting the expression of the incoming foreign gene. Seven stable Ap^r colonies were isolated from cells treated with pDN1 DNA, but none could be detected from the parallel pBR322 transformations. Controls of untreated cells or DNA alone failed to produce Ap^r colonies; since titrations of treated cells on media lacking antibiotic showed that 3×10^8 c.f.u. were plated (10^8 c.f.u., three plates), the spontaneous mutation rate to this level of Ap^r was less than 3×10^{-8} per cell. The transformation frequency detected was 4.2×10^{-7} per cell or one transformant per μg DNA. Transformation of PCC 6301 by this method thus gave unsatisfactorily low frequencies.

Transformation of recipient cells incubated in the dark

A increase of at least 100-fold in the transformation frequency of strain PCC 6301 was observed when the cells were incubated with DNA in darkness, similar to that reported by Golden & Sherman (1984) with strain PCC 7942. Table 2 shows the frequencies with which stable Ap^r colonies were obtained from samples of cultures of PCC 6301 treated in parallel with one of a number of plasmids. Between two and eight independent transformation experiments were made with each plasmid. Transformants were obtained only from donor plasmids carrying inserts of *Synechococcus* DNA. No stable Ap^r colonies were detected from cells treated with pBR322, corresponding to a transformation frequency of less than 10^{-9} per cell, or less than 0.02 transformants per μg DNA of this vector.

Table 2. *Comparison of transformation frequencies of chimaeric plasmids*

Plasmid	Size of insert (kbp)*	Mean no. $\pm$ SD of Ap ^r colonies per μ g DNA†	Total of DNA used in all experiments (μ g)
pGO1	5.2	615 $\pm$ 15 (2)	2
pGO4	5.9	506.5 $\pm$ 6 (2)	2
pGO3	4.8	320 $\pm$ 20 (2)	2
pDN1	9.2	162.3 $\pm$ 44 (8)	26
pDS1	8.9	151 $\pm$ 11 (2)	2
pNH1	3.2	147 $\pm$ 24 (3)	3
pCI3	3.0	1 $\pm$ 0 (2)	2
pHB1	1.9	0.35 $\pm$ 0.5 (4)	22
pHX1	2.2	0.40 $\pm$ 1 (4)	22
pNS1	7.7	0.25 $\pm$ 0.5 (4)	22
pBR322	—	< 0.02 (8)	44
pRK9	—	< 0.04 (4)	22

* The nature of the insert DNA is shown in Table 1.

† Values in parenthesis indicate the number of experiments performed.

Table 3. *Comparison of transformation frequencies of strains PCC 6301 and PCC 7942*

The results are from a single representative experiment.

Plasmid	DNA (μ g)	No. of Ap ^r colonies per μ g DNA	
		Strain 6301	Strain 7942
pDN1	1	137	1200
pHB1	10	1	3
pHX1	10	1	5
pNS1	10	1	7
pBR322	1	0	0

Effect of size and continuity of donor DNA on transformation

Donor plasmids carrying different inserts of native *Synechococcus* chromosomal DNA gave characteristic transformation frequencies varying from 1 to 615 transformants per μ g of donor DNA (Table 2). The size of the homologous DNA seemed to exert an effect within the range tested (3–10 kbp), with optimal recovery of transformants being obtained from plasmids with inserts in the 5–6 kbp range. Internal deletions of the insert, which interrupted the linear sequence of the cyanobacterial DNA, decreased the transformation efficiencies more than 100-fold, indicating that transformation required structural integrity of the homologous DNA. Transformation with these deleted derivatives required elevated DNA concentrations, 10-fold higher (10 μ g ml⁻¹) than that found to be saturating in transformations with pDN1.

Parallel transformation of PCC 6301 and PCC 7942

The same plasmids were used to transform two separate *Synechococcus* strains in parallel experiments (Table 3). Strain PCC 7942 shows high transformation frequencies with homologous DNA sequences (Williams & Szalay, 1983; Golden & Sherman 1984). Internal deletions within the homologous DNA resulted in decreases in transformation frequencies for both PCC 7942 and PCC 6301, although the frequencies were 3- to 10-fold higher overall with PCC 7942 as recipient (Table 3).

State of transforming DNA

A number of stable Ap^r colonies derived from pDN1 transformations with both light- and dark-treated cells of strain PCC 6301 were shown to contain plasmid DNA by Southern hybridization with probes prepared from plasmid sequences. Fig. 2 shows a typical

Fig. 2. Hybridization of total genomic DNA to plasmid probes. Total genomic DNA, from a strain of *Synechococcus* PCC 6301 transformed to ampicillin resistance by incubation with pDN1 (transformant 2.2), and supercoiled pDN1 DNA, purified from *E. coli*, were digested with *Eco*RI and separated by electrophoresis in 0.8% (w/v) agarose at loadings of 3 μ g and 3 ng respectively. Ethidium bromide staining showed that all samples were digested to completion. After Southern blotting, they were hybridized successively with probes constructed from pBR322 DNA, then pDN1 DNA, each hybridization being followed by washes at high stringency and autoradiography. Lane A contains DNA from transformant 2.2. Lane B contains pDN1 DNA. Sizes of fragments were measured by comparison with *Hind*III fragments of bacteriophage λ DNA.

hybridization pattern obtained with restriction digests of DNA from one of these stable Ap^r strains (transformant 2.2). A single fragment of similar size (7.9 kbp) to that containing the majority of the pBR322 DNA in the transforming plasmid (Fig. 1) hybridized strongly with a probe constructed from pBR322 (Fig. 2); the second fragment produced by *Eco*RI digestion of pDN1 DNA (5.6 kbp) hybridized weakly, as it only contains 380 bp of sequence from pBR322. Use of pDN1 as a probe detected these two fragments and two additional fragments (20 and 6.7 kbp) at similar intensities which were the same size as fragments of recipient chromosome detected by probing untransformed *Synechococcus* PCC 6301 DNA with pDN1 (data not shown). No hybridization was detected in probings of DNA from untransformed *Synechococcus* PCC 6301 with pBR322 (data not shown). This result does not formally distinguish between the hypotheses of (a) integration of donor DNA by a single homologous crossover, and (b) maintenance of a single-copy autonomously replicating plasmid form of the donor DNA.

The following observations strongly support the integrative model. Probing uncut total DNA of transformant 2.2 with pBR322 established that hybridizing sequences derived from this plasmid were present in the high-molecular-mass DNA (50–100 kbp, data not shown). Additionally, transformation of *E. coli* with the supercoiled fraction of DNA isolated from transformant 2.2 by CsCl density-gradient centrifugation gave no Ap^r colonies. The equivalent amount of control pDN1 DNA gave 10^7 Ap^r colonies. pDN1 is not known to contain a cyanobacterial origin. Further, autonomous maintenance does not provide a plausible explanation for the drastic reduction in transformation frequency caused by internal deletions of the PCC 6301 DNA in the donor plasmids, which very probably reflects an integration mechanism involving homologous recombination.

Conditions affecting transformation

The use of dark conditions for incubation of recipient cells with DNA dramatically increased transformation frequencies of strain PCC 6301 with hybrid plasmids. The reason for this is unknown. Golden & Sherman (1984) hypothesized that a membrane-bound component involved in photosynthetic ion exchange, and required for DNA uptake, is sequestered during illumination. Shestakov *et al.* (1982) invoked a similar mechanism to explain the light-dependence of transformation. It seems equally likely that the depressed metabolism of darkened cells might favour uptake, maintenance or stable integration of DNA, or expression of the foreign resistance gene. Differences in our method which might also influence successful

transformation of strain PCC 6301 include the absence of CO₂ enrichment during selection for Ap^r colonies, and the use of a different medium. Other factors which might affect transformation include the period of incubation of cells with DNA (Daniell & McFadden, 1986), the genotype of clonal isolates (Williams & Szalay, 1983) and the source of water (Hanahan, 1985).

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