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Optimal Conditions for Genetic Transformation of the Cyanobacterium *Anacystis nidulans* R2

SUSAN S. GOLDEN† AND LOUIS A. SHERMAN*

Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211

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Under optimal conditions, the cyanobacterium *Anacystis nidulans* R2 was transformed to ampicillin resistance at frequencies of $>10^7$ transformants per μg of plasmid (pCH1) donor DNA. No stringent period of competency was detected, and high frequencies of transformation were achieved with cultures at various growth stages. Transformation increased with time after addition of donor DNA up to 15 to 18 h. The peak of transformation efficiency (transformants/donor molecule) occurred at plasmid concentrations of 125 to 325 ng/ml with an ampicillin resistance donor plasmid (pCH1) and 300 to 625 ng/ml for chloramphenicol resistance conferred by plasmid pSG111. The efficiency of transformation was enhanced by excluding light during the incubation or by blocking photosynthesis with the electron transport inhibitor 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) or the uncoupler carbonyl cyanide-*m*-chlorophenyl hydrazone. Preincubation of cells in darkness for 15 to 18 h before addition of donor DNA significantly decreased transformation efficiency. Growth of cells in iron-deficient medium before transformation enhanced efficiency fourfold. These results were obtained with selection for ampicillin (pCH1 donor plasmid)- or chloramphenicol (pSG111 donor plasmid)-resistant transformants. Approximately 1,000 transformants per μg were obtained when chromosomal DNA from an herbicide (DCMU)-resistant mutant was used as donor DNA. DCMU resistance was also transferred to recipient cells by using restriction fragments of chromosomal DNA from DCMU-resistant mutants. This procedure allowed size classes of fragments to be assayed for the presence of the DCMU resistance gene.

The cyanobacteria are procaryotic organisms which perform oxygenic photosynthesis similar to that of higher plant chloroplasts. These organisms provide an opportunity to combine genetic manipulations, which have been developed for bacteria, with investigation of photosynthetic parameters by biochemical and biophysical procedures (5, 6, 11). Molecular cloning technology provides a means of exploiting these organisms for genetic study in the absence of conjugation or transduction. Genetic transformation has been reported in the unicellular cyanobacterium *Anacystis nidulans* R2 (4, 16), and this strain can serve as a recipient for recombinant DNA donor molecules. The polypeptide composition of the photosynthetic membrane of this strain has been analyzed extensively (6, 7), and procedures for the isolation of photosynthesis-deficient mutants have been developed (5, 11). The availability of a transformable strain, photosynthesis-deficient mutants, and appropriate cloning vehicles will enable a variety of genetic manipulations to be performed, including the identification of specific genes by complementation experiments.

Progress in the construction of cloning vehicles for *A. nidulans* R2 has improved the outlook for molecular cloning in this organism. Derivatives of the small native plasmid pUH24 have been produced which contain transposon-borne antibiotic resistance markers (16). Some hybrid molecules containing *Escherichia coli* plasmids linked to pUH24 have been constructed; these are capable of replication in *E. coli* as well as in *A. nidulans* (3, 9, 12). Published procedures for the transformation of *A. nidulans* with plasmid DNA vary as to the concentration of donor DNA and length of incubation (9, 12, 16), but transformation conditions have not been systematically optimized. The goal of the following research was to determine optimal experimental conditions for the transformation of *A. nidulans* R2, with special attention given to the relationship of photosynthesis to the transformation process.

We have studied various parameters which affect the efficiency of *A. nidulans* transformation by plasmid pCH1 (16). which confers ampicillin resistance to transformed cells. Several alterations in incubation conditions improved the frequency of transformation. Transformation of *A. nidulans* increased with the length of time that recipient cells were incubated with donor DNA, up to 15 to 18 h. A peak of transformation efficiency (transformants/donor molecule) occurred at a low donor plasmid concentration (125 to 625 ng/ml, depending on the donor molecule), although the absolute number of transformants obtained increased with higher donor concentrations. The most significant variation from previously reported procedures was the finding that exclusion of light, or perturbation of photosynthesis by chemical inhibitors, greatly enhanced the efficiency of transformation. The growth of recipient cells in iron-deficient medium, a manipulation which causes extensive changes in the photosynthetic apparatus (7a), resulted in an additional increase in the transformation of *A. nidulans*.

MATERIALS AND METHODS

A. nidulans R2 and its pCH1-transformed derivative, R2A6, were obtained from G. van Arkel (Utrecht, The Netherlands). Cultures were grown in BG-11 medium (1) as previously described (11), with 1 μg of ampicillin per ml added to culture medium for the maintenance of *A. nidulans* R2A6. Conditioned medium refers to the supernatant fraction of a culture from which cells have been removed by

* Corresponding author.
† Present address: Department of Biophysics and Theoretical Biology, University of Chicago, Chicago, Il 60637.

centrifugation. Iron-deficient BG-11 medium was prepared by substituting ammonium citrate for ferric ammonium citrate. All glassware used for the preparation of iron-deficient medium was rinsed with EDTA, and stock solutions which did not contain divalent cations were cleared of trace iron by ion-exchange chromatography on Chelex resin (Bio-Rad Laboratories, Richmond, Calif.). For growth on solid medium, 2× BG-11 and 3% agar (in water) were prepared and autoclaved separately, mixed after cooling to 50 to 60°C, and dispensed into petri dishes in 40-ml aliquots.

Ampicillin (Polycillin N; Bristol Laboratories, Syracuse, N.Y.) was prepared in water at 100 mg/ml and stored at −20°C in 10-μl aliquots. An aliquot was thawed as needed and used once without refreezing. Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was prepared in absolute ethanol at 34 mg/ml and stored at −20°C. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) were obtained from DuPont Co. (Wilmington, Del.) and stored at −20°C in ethanol as 10^{-2} and 10^{-4} M stocks, respectively.

Plasmid pCH1 (12.9 kilobases) is a transposon insertion derivative of the native *A. nidulans* plasmid pUH24 (16). This plasmid confers ampicillin resistance to transformed recipient cells. Isolation of pCH1 from *A. nidulans* R2A6 was performed as described by van den Hondel et al. (15). Greater than 90% of the plasmid prepared in this manner was pCH1; the preparation also contained some of the native 50-kilobase plasmid pUH25 (16). Chromosomal DNA was prepared from a DCMU-resistant *A. nidulans* mutant, R2D2 (Golden and Sherman, Biochim. Biophys. Acta in press) by the same procedure. Plasmid pSG111 (3) is a 12.9-kilobase hybrid plasmid containing pUH24 joined to the *E. coli* plasmid pBR328 (14). This plasmid expresses resistance to chloramphenicol and ampicillin in *A. nidulans* and *E. coli*. Plasmid pSG111 DNA was prepared from *E. coli* by a method similar to that described by Meagher et al. (10).

Restriction endonucleases were purchased from New England BioLabs (Beverly, Ma.) and Bethesda Research Laboratories (Rockville, Md.), and cleavage reactions were carried out as suggested by Davis et al. (2).

A. nidulans cells were prepared for transformation by washing once in 10 mM NaCl and resuspending in BG-11 (or iron-deficient BG-11) at 5×10^8 cells per ml. Aliquots of cells (300 or 400 μl) were dispensed to glass test tubes or microcentrifuge tubes, and donor DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) was added to a final concentration of 30 ng to 3.5 μg/ml. Donor DNA was stored as a 20-μg/ml solution, so that no more than 50 μl was added to cells. The transformation mixtures were incubated for various times at 28 to 30°C in a constant-temperature chamber under standard conditions. The key parameter was the time of incubation of the cells with the donor DNA. In preliminary experiments, it was shown that termination of the transformation with or without several types of DNases yielded the same level of transformation (data not shown). These results indicated that *A. nidulans* cells were not transformed after plating on solid medium.

DCMU or CCCP was added to some transformation mixtures at a concentration of 10^{-6} M. The treated samples were plated in a fashion identical to that for control experiments, and no inhibitor was added to the plates. Plating for cell viability indicated that DCMU treatment had very little adverse effect on cell growth. However, CCCP treatment, under illuminated conditions, killed 90% of the cells in 75 min, and the results with CCCP were normalized to the number of viable cells. Dark incubations were achieved by wrapping the tubes securely in opaque black cloth. All tubes were incubated nearly horizontally on laboratory shakers to keep the cells in suspension. Samples were plated (100 μl per plate) in triplicate or with a series of twofold serial dilutions (through 1/64) for quantitation of transformants. Variations in donor DNA concentration and length of incubation reflect the status of protocol optimization at the time each experiment was performed.

Inoculated plates were incubated in a 30°C constant-temperature growth chamber for 3 to 12 h. At this time the agar slab was partially lifted with a sterile spatula, and 400 μl of a 100× stock of a selective agent was dispensed underneath. The agar was carefully lowered, and plates were incubated for 5 to 7 days before transformants were scored. Final concentrations of selective agents were as follows: ampicillin, 0.5 μg/ml; chloramphenicol, 7.5 μg/ml; and DCMU, 10^{-6} M. The number of colonies obtained during overnight illuminated incubation was adjusted to account for a 50% increase in cell number which occurred under those conditions. No cell division occurred when cells were incubated overnight in the dark, and the viability of dark-incubated cells remained constant.

RESULTS

Several incubation parameters of *A. nidulans* transformation were studied with plasmid pCH1 (16) as a donor for the transfer of ampicillin resistance to recipient cells. Cultures at various stages of growth were used as recipients, and no competency period was detectable. Two-week-old stock cultures varied little in transformation frequency from exponentially growing cultures, aside from a decrease in cell viability in the older cultures. Resuspension of cells in conditioned medium or fresh BG-11 gave similar results in transformation experiments. The number of transformants increased linearly with increasing cell concentration in the transformation mixture, and no critical cell concentration was observed to be necessary for transformation (data not shown).

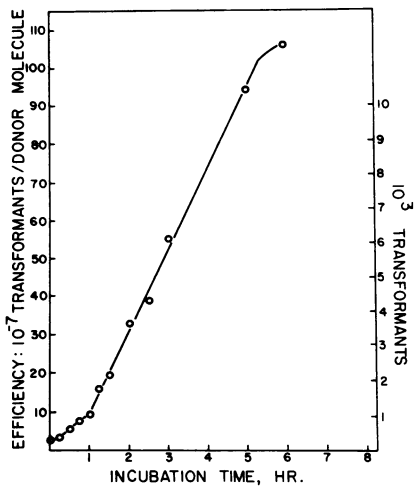

FIG. 1. Effect of length of incubation on transformation. Plasmid pCH1 was added to 300-μl aliquots of cells to a final concentration of 50 ng/ml. Incubations were performed in the dark, and a sample was plated (100 μl; in triplicate) at each of the times shown. Ampicillin (0.5-μg/ml final concentration) was added under the agar of each plate 8 h after inoculation. The line for the points between 1 to 5 h was obtained by using the least-squares method and generated a correlation coefficient of 0.993.

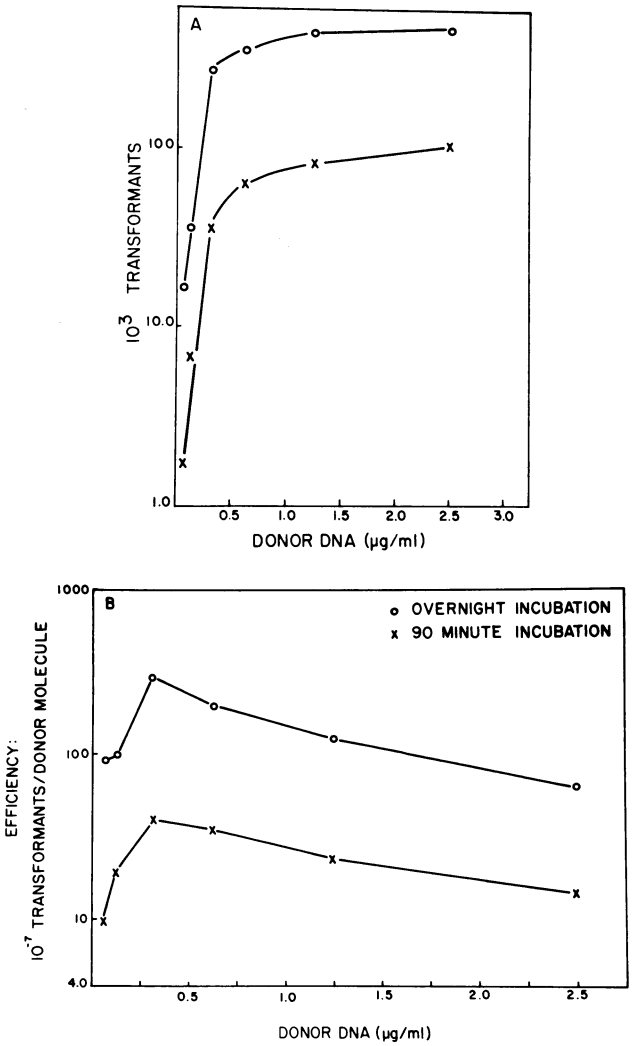

FIG. 2. Effect of donor DNA concentration on transformation. Aliquots (300 μl) of cells were incubated in the dark with various concentrations of pCH1 (60 ng to 2.5 μg/ml). After 90 min (lower curves) a 100-μl sample of each reaction was diluted serially (through 1/64) and several dilutions were plated; the remainder was incubated overnight (upper curves) before plating of diluted cells. The data were plotted as number of transformants (A) and efficiency of transformation (B).

A time course was performed to determine whether *A. nidulans* transformation is a time-dependent process. Cells were plated immediately after the addition of pCH1 to a final concentration of 50 ng/ml (ca. 10 molecules per cell) and at various time intervals thereafter. The frequency of transformation increased slowly for the first hour and then continued to rise rapidly until about 6 h (Fig. 1). Tenfold more transformants were obtained after 6 h than with a 1-h incubation. In subsequent experiments, higher levels of transformation were obtained with overnight (12 to 18-h) incubations. This continued increase suggests that little or no degradation of donor DNA occurred in the cell suspension.

The time at which the selective antibiotic was added to transformed cells also affected the yield of transformants. Identical transformation reactions were plated and incubated under standard growth conditions for various times before the addition of ampicillin under the agar. Plates which had incubated for 6 h between plating and ampicillin addition yielded twice as many transformants as those to which the antibiotic was added immediately after inoculation, or after 1 h had elapsed. No change in the transformant yield was seen when the elapsed time was increased from 6 to 22 h. Other experiments showed that 3 to 4 h were sufficient to obtain maximum transformation frequencies. The results presented here were obtained with 3 to 12 h between plating and addition of the selective agent.

A. nidulans cells were incubated with various amounts of pCH1 to determine how the concentration of donor DNA affected transformation (Fig. 2 and 3). Figure 2 shows the effect of increasing donor DNA concentrations for transformation mixtures that were incubated in the dark either for 90 min or overnight (15 h). In both cases, the number of transformants increased greatly with increasing DNA concentration until approximately 750 ng/ml (100 to 150 molecules per cell); it is evident in both Fig. 2A and 3A that short

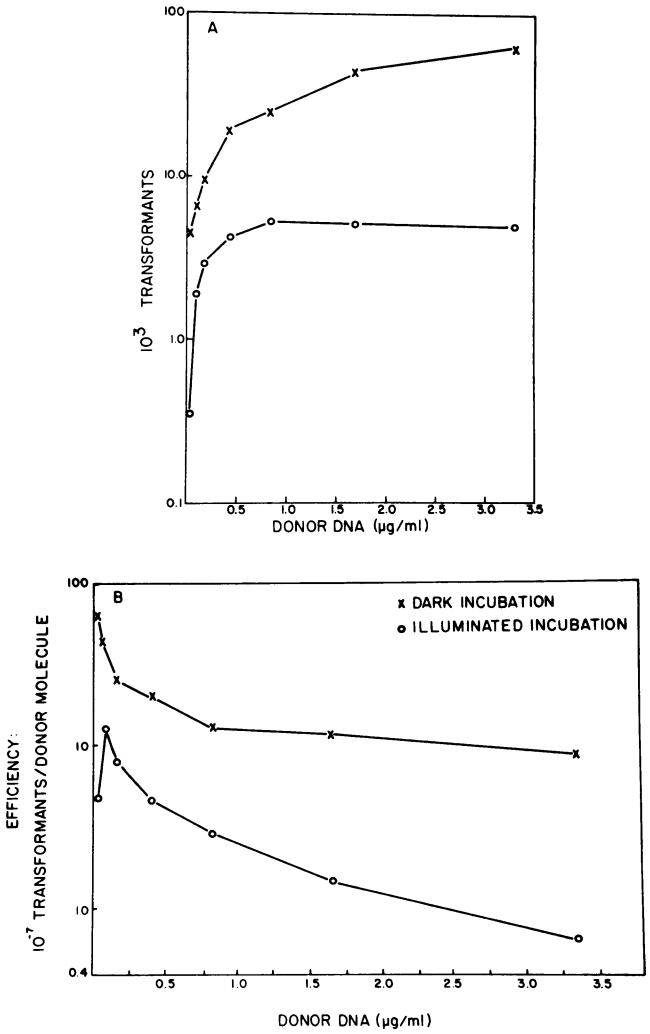

FIG. 3. Effect of illumination on transformation, tested at various donor DNA concentrations. Aliquots (300 μl) of cells were incubated with various concentrations of pCH1 (33 ng to 3.3 μg/ml) in the light (lower curve, O) or dark (upper curve, ×) for 50 min before plating of diluted reactions. The data were plotted as number of transformants (A) and efficiency of transformation (B).

incubations in the dark did not yield saturation until somewhat higher DNA concentrations. The concentration dependence shown in Fig. 2A was also obtained if the donor DNA was added at two separate times. Donor DNA was added to a concentration of 62.5 ng/ml and incubated for 4 h in the dark; an additional quantity of donor DNA (to a final concentration of 312 ng/ml) was then added to the transformation mixture and incubation was continued overnight. Under these conditions, the number of transformants was virtually identical to that obtained when 312 ng of DNA per ml was added initially (data not shown).

The peak of efficiency (transformants/donor molecule) usually occurred at 125 to 325 ng/ml; however, optimal transformation efficiency was occassionally observed at very low donor DNA concentrations (<50 ng/ml; see Fig. 3B). Although the absolute number of transformants continued to increase with higher donor concentration until at least 750 ng/ml, the rise in the number of transformants was less than the increase in donor molecules and the efficiency of the reaction decreased. Figure 2B shows that the drop in transformation efficiency with increasing DNA concentration occurred during overnight (15 h) as well as 90-min incubations. However, the overnight incubation yielded higher efficiencies than the 90-min incubation at all DNA concentrations.

The decrease in transformation efficiency at high donor DNA concentration implies competition for DNA binding sites. A series of experiments demonstrated that similar competition occurred with nontransforming DNA and that this competition was according to mass only. Nontransforming linear DNAs (*A. nidulans*, *E. coli*, or calf thymus chromosomal DNA) and circular DNAs (pBR322 and pACYC184) were added in mass ratios of 10- to 200-fold relative to the transforming DNA. All of these molecules generated similar competition curves, indicating that there was no significant effect of complexity, source, or form on binding of DNA to membrane receptors (data not shown). Calculations based on the results of Fig. 2 and 3 indicated that saturation occurred at 100 to 150 molecules of pCH1 per cell.

The central role of photosynthesis in the general metabolism of *A. nidulans*, an obligate photoautotroph, suggested that photosynthetic activity might also affect the process of transformation. To determine whether cells must be actively photosynthesizing to be competent for transformation, we prepared duplicate reactions which were incubated either under normal growth conditions or in complete darkness (Fig. 3). The cells incubated in the absence of light produced 4- to 10-fold more transformants than the illuminated samples at all concentrations of donor DNA. The effect was seen on both the number of transformants (Fig. 3A) and transformation efficiency (Fig. 3B). The efficiency of transformation was highest when low DNA concentrations were incubated with cells in the dark. Under dark conditions, we obtained 2.2×10^6 transformants per μg of DNA, and 0.15% of the cells were transformed at an input of 1 μg of DNA. These results indicated that disruption of the photosynthetic process enhanced the transformation efficiency of *A. nidulans* R2.

The enhancement of transformation by dark incubation was further investigated by preincubating cells for 15 to 18 h in darkness or standard illumination before the addition of donor DNA. Two samples of each preincubated culture were mixed with pCH1; one of each was incubated with illumination and the other was kept in darkness. Dark preincubation significantly decreased the frequency of transformation (Table 1). Dark incubation still enhanced efficiency, but there was a net decrease relative to cells preincubated in the light. The difference in transformation frequency between light-preincubated/dark-incubated and dark-preincubated/light-incubated cells was greater than 250-fold. Similar results were obtained in an experiment having slightly different incubation conditions (80 ng of pCH1 per ml and a 6-h incubation period). These results indicated that the enhancement of transformation of *A. nidulans* by dark incubation occurred during the period in which cells were in contact with donor DNA, rather than acting to predispose recipient cells to higher levels of competence.

TABLE 1. Effect of illumination on *A. nidulans* transformation by pCH1[a]

Preincubation	Incubation	No. of transformants
Dark	Light	72
Dark	Dark	585
Light	Light	3,992
Light	Dark	24,000[b]

[a] Plasmid pCH1 was added to 5×10^8 cells per ml at a final concentration of 100 ng/ml. Preincubation and incubation periods were 18 h.

[b] Estimated from plates with a very high colony population.

This enhancement of transformation by dark incubation can also be induced by treatment of cells with chemicals which inhibit photosynthesis. Figure 4 demonstrates the level of stimulation of transformation when photosynthesis was perturbed by various treatments (relative to the frequency obtained with standard illuminated incubation). Cells were incubated with 400 ng of pCH1 per ml either in the light or in darkness for 75 min. Some samples were treated with the electron transport inhibitor DCMU or the uncoupler of ATP synthesis, CCCP, during the period in which cells were incubated with DNA. Plating of cells in the absence of DCMU or CCCP diluted the compounds to noninhibitory levels. The presence of DCMU or CCCP stimulated transformation with either illuminated or dark incubations. When cells were incubated in the light with DCMU, the level of stimulation was lower than with dark incubations or in the presence of CCCP. This suggests that inhibition of photosystem II activity (which is completely inhibited by 10^{-6} M DCMU) is not sufficient to generate the maximum enhancement of transformation efficiency. CCCP greatly enhanced the proportion of viable cells which became transformed. The absolute number of transformants obtained in the presence of CCCP in the light was equivalent to the frequency obtained with DCMU in the light; however, 90% of the cells were killed by the CCCP treatment. The number of transformants obtained with cells which were incubated in the light was more variable than for other treatments. Because light-incubated samples served as the base line in these experiments, the values for enhancement by photosynthesis-perturbing treatments also varied. However, the relative enhancement by the various perturbations was reproducible between experiments. Enhancement of transformation relative to light incubations was also observed when recipient cells were treated with the ionophores nigericin and valinomycin in the presence of 150 mM KCl (data not shown).

Under conditions of iron deficiency, the photosynthetic apparatus of *A. nidulans* undergoes a reduction in the number of photosynthetic membranes, phycobilisomes, and carboxysomes and is deficient in chlorophyll and phycocyanin (7a). The growth of recipient cells in iron-deficient

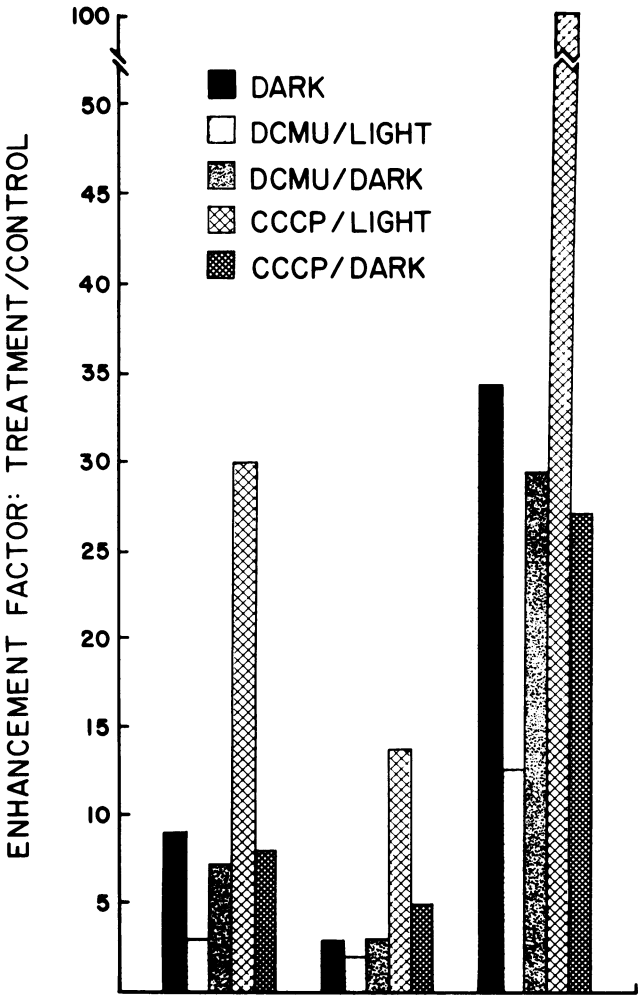

FIG. 4. Enhancement of transformation by perturbation of photosynthesis. Results are shown for three separate experiments in which aliquots (300 μl) of cells were incubated with DNA (400 ng/ml) for 75 min in the light or dark and in the presence or absence of DCMU or CCCP. The total number of transformants obtained with each treatment was divided by the number obtained with incubation under standard illuminated growth conditions; the resulting ratio (enhancement factor) is plotted for each treatment. The control values for light incubation were, from left to right, 1.4×10^3, 4.5×10^3, and 1.3×10^3 transformants per μg, respectively.

medium also enhanced transformation (Table 2). The transformation efficiency with 100 ng of pCH1 per ml was fourfold higher in iron-stressed cells relative to cells grown in BG-11. These cells yielded the highest level of transformation that we have obtained (2.9×10^7 transformants per μg), and 1.4% of the cells were transformed. The potential value of iron-stressed cells as recipients for recombinant plasmid molecules is discussed below.

Plasmid pSG111 is a hybrid cloning vector which comprises the native *A. nidulans* plasmid pUH24 and the *E. coli* plasmid pBR328 joined at their unique *Bam*HI sites (3). The effects of incubation duration, donor DNA concentration, and perturbation of photosynthesis observed with pCH1 were also obtained when pSG111 was used as the donor DNA (with chloramphenicol selection). However, the overall efficiency of transformation was 160-fold lower than with pCH1 (Table 2), and peak efficiency occurred at 300 to 625 ng/ml.

A. nidulans R2 can be transformed by chromosomal donor DNA (4), a feature which allows the direct assay of chromosomal DNA for the presence of a positively selectable gene of interest. Table 2 shows results obtained in the transformation of wild-type *A. nidulans* with chromosomal DNA from a DCMU-resistant mutant, *A. nidulans* R2D2 (Golden and Sherman, in press). Approximately 1,000 transformants per μg were obtained when mutant chromosomal DNA was added to cells. Similar values were obtained whether the DNA was cleaved with *Xho*I or *Eco*RI or added as high-molecular-weight, undigested DNA. *Eco*RI-digested DNA was separated into size classes by sucrose gradient centrifugation, and a small aliquot from each fraction was assayed for the ability to transform *A. nidulans* to DCMU resistance. A sharp peak of donor activity was present in a 5- to 13-kilobase *Eco*RI fraction, which yielded 4×10^3 transformants per μg. The transformation frequency with fractions containing larger or smaller restriction fragments was 10- to 800-fold lower under the same experimental conditions. This assay is currently being used to define subsets of restriction fragments in preparation for molecular cloning of specific genes.

DISCUSSION

A significant finding of this study is the observation that perturbation of photosynthesis improves the efficiency of the transformation process. Dark incubation is a useful manipulation for enhancing the transformation of *A. nidulans* without damaging the recipient cells. Inhibitors of photosynthesis are helpful tools for studying the nature of the enhancement of transformation by dark incubation, but most result in cell death when cells are incubated in the light for extended periods of time. For this reason they are not useful additions to the routine transformation protocol.

The role that cessation of photosynthesis plays in transformation enhancement is suggested by the effect of photosynthesis inhibitors such as DCMU, CCCP, and ionophores. All of these compounds dissipate the high energy state that is

TABLE 2. Transformation efficiencies[a] of *A. nidulans* with various donor DNA species

Donor	Selective agent	Growth medium	Transformants per μg	Efficiency (transformants/donor molecule)
pCH1 (65 ng/ml)	Ampicillin	BG-11	7.6×10^6	1.0×10^{-4}
pCH1 (65 ng/ml)	Ampicillin	Iron-deficient BG-11	2.9×10^7	4.0×10^{-4}
pSG111 (625 ng/ml)	Chloramphenicol	BG-11	1.2×10^4	6.4×10^{-7}
R2D2 chromosomal DNA	DCMU	BG-11		
High molecular wt (1 μg/ml)			1.4×10^3	
*Xho*I cleaved (1.3 μg/ml)			1.1×10^3	

[a] All incubations were carried out in the dark for 15 to 18 h.

produced in *A. nidulans* by photosynthesis and enhance the efficiency of transformation. Dissipation of proton motive force by the addition of ionophores has been shown to abolish transformation in *Bacillus subtilis* (17). This affects specifically the transmembrane transport of DNA, rather than initial DNA binding. The steps involved in transformation in *A. nidulans* have not been studied, but the enhancement of transformation by treatments which deenergize the thylakoid or plasma membranes or both indicates that the mechanism is different from many other transformable procaryotes. Perhaps a transport system that serves to bring DNA across the membrane has another primary transport role which is driven by the proton motive force. Dissipation of some component of the proton motive force may free this system for increased transport of DNA.

The alteration which occurs in the photosynthetic apparatus during iron starvation has interesting implications for manipulations using molecular genetic techniques. Upon restoration of iron to the medium, these cells undergo a sequential regeneration of photosynthetic components. If iron restoration occurs after transformation of cells with cloned photosynthesis genes, the newly synthesized thylakoids can be assembled containing the products of the cloned genes. This procedure may be important when cloned genes code for components of the photosynthetic apparatus which are not rapidly turned over. Selection of transformed cells will be dependent upon expression of the cloned gene at the time selective pressure commences. Demonstration that iron-stressed cells are transformable (in fact, more highly transformable than cells grown in the presence of iron) indicates that this approach is feasible. Temperature-sensitive photosynthesis mutants of *A. nidulans* R2, which are now available in this laboratory, will serve as the recipient cells in such experiments.

Cells grown in low-iron medium manifest a number of changes in membrane protein composition (7b). Importantly, a 34-kilodalton protein is synthesized in large amounts and inserted into a membrane fraction that has different physical properties from the photosynthetic membrane. We have speculated that the protein may be involved in iron transport and may be located in the plasmalemma (7b). Therefore, it is possible to consider that this polypeptide is somehow involved in the binding of DNA to a cell or to the transport of DNA into the cell. These possibilities will be assessed in the near future.

Unlike many other naturally transformable bacteria (for review, see reference 13), *A. nidulans* R2 has no apparent competence period. Cells can become transformed at relatively high frequency by homologous chromosomal or plasmid DNA at all phases of culture growth. The property of transformation competency is generally a transient phenomenon in bacteria, with the period of maximal competence occurring in the mid- to late-logarithmic growth phase for *E. coli*. In an extensive re-evaluation of transformation parameters for *E. coli*, Hanahan (8) obtained conditions that eliminated the sharp peak in competence with respect to growth phase. When transformation was performed in the presence of dithiothreitol and hexamine cobalt (III) chloride, very high and reproducible frequencies of transformation were obtained, with cultures ranging in cell density from 2×10^7 to $>10^8$ cells per ml. This range corresponds to the typical cell densities in our *A. nidulans* cultures. Hanahan (8) postulates that the hexamine cobalt (III) chloride may act as a competitive inhibitor of vitamin B_{12} and that vitamin B_{12} is a competitive inhibitor of plasmid transformation. This may be similar to dark incubation acting to prevent certain transport functions in *A. nidulans* which otherwise compete with DNA for binding or transport.

Chauvat et al. (1a) did not observe an enhancement of transformation by dark incubation of *A. nidulans* cells, but reported a dramatic drop in frequency after dark preincubation of cells before donor DNA addition. They found that *A. nidulans* cells were highly transformable (optimal efficiency, 2×10^{-6} transformants/donor molecule) only during the transition between the first and second phases of logarithmic growth. A difference in growth conditions of *A. nidulans* or a variation in clonal origins of the strain may account for differences in observed transformation parameters. In our laboratory, *A. nidulans* R2 sometimes produces clones which vary considerably in transformability relative to the original culture. Differences in the origin and culture conditions of stocks of this strain may account for the variation in transformation parameters. To preserve highly transformable stocks, single-colony purification of cultures is avoided; aliquots of axenic cultures, which possess the high transformation frequency phenotype, are stored at 77°K in 10% polyvinylpyrrolidone in case a new seed culture is needed. If a culture is purified by plating, isolated colonies must be assayed for transformability.

The size of donor DNA does not seem to be a critical factor in the efficiency of transformation of *A. nidulans*. Plasmids pCH1 and pSG111 are identical in size, but differ markedly in *A. nidulans* transformation efficiency (Table 2). This difference may be related to the level of expression of the antibiotic resistance genes on the two plasmids. A derivative of pSG111 which contains an *A. nidulans* rRNA operon (3) transforms *A. nidulans* as well as the parent plasmid, although it is 50% larger. A cointegrate of pBR328 and the native *A. nidulans* plasmid pUH25 (16), which is greater than 50 kilobases in size, transforms *A. nidulans* cells at a high frequency (Golden and Sherman, unpublished data). Uptake and maintenance of large derivatives of the native plasmids may be stabilized by recombination with resident plasmids. Low transformation frequency has been correlated with loss of the small native plasmid pUH24 (1a; Golden and Sherman, unpublished data). Interplasmid recombination has been observed in our laboratory and by others (9).

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