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Abstract

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Disciplines

Dentistry

Transformation of the cyanobacterium *Anacystis nidulans* 6301 with the *Escherichia coli* plasmid pBR322

(gene transfer/blue-green algae/β-lactamase/permeaplasts/ribulose-bisphosphate carboxylase)

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ABSTRACT   *Anacystis nidulans* 6301 has been transformed in the light to ampicillin resistance with the plasmid pBR322. Permeaplasts prepared by 2-hr treatment of cells with lysozyme and EDTA are transformed with a 50-fold higher efficiency than that observed for cells. β-Lactamase is present in *A. nidulans* transformed either with pBR322 or the plasmid pCH1 as evidenced by hydrolysis of the β-lactam ring of Nitrocefin in extracts of transformants. β-Lactamase also can be immunoprecipitated from extracts of [^{35}S]methionine-labeled pBR322 transformants and coprecipitates with ribulose-bisphosphate carboxylase. Expression of the carboxylase is apparently amplified in pBR322 transformants as is that for several soluble proteins in pCH1 transformants. Chromosomal DNA per cell is increased about 6-fold after transformation of *A. nidulans* 6301 with either pBR322 or pCH1. A 4.3-kilobase-pair plasmid can be isolated from pBR322 transformants in addition to the endogenous plasmids pUH24 and pUH25.

Cyanobacteria (blue-green algae) are Gram-negative prokaryotic organisms that constitute the most diverse group of photosynthetic prokaryotes, possessing a photosynthetic system remarkably similar to that of chloroplasts of eukaryotic algae and of higher plants. The use of cyanobacteria for genetic studies has been hampered by the lack of genetic transfer between individuals and the fact that all naturally occurring cyanobacterial plasmids are phenotypically cryptic. Although transfer of plasmids between different groups of bacteria has been demonstrated, a plasmid of a heterologous source that can be established and maintained in a cyanobacterium has not yet been found (1).

Anacystis nidulans 6301 is a unicellular cyanobacterium containing two endogenous plasmids pUH24 or pANS [8.0 kilobase pairs (kbp)] and pUH25 or pANL (48.5 kbp) (2, 3). To date, attempts to transfer plasmids from *Escherichia coli* into cyanobacteria have been unsuccessful (1, 4). In order to circumvent this, Van den Hondel *et al.* (5) constructed a plasmid, pCH1 (12.9 kbp), that contains a transposon insertion in the native *A. nidulans* plasmid pUH24. However, neither the resultant pCH1 nor its deletion derivative pUC1 could be used to transform *E. coli* (1). In order to achieve successful transformation by a plasmid in either host, a number of different *E. coli*–*A. nidulans* shuttle cloning vectors have been developed recently (1, 6–12). However, most of these hybrid plasmids suffer from one or more of the following disadvantages: (*i*) they are relatively large (10–14 kbp), thereby reducing the size of potential inserts; (*ii*) they carry few unique restriction sites for cloning; (*iii*) they lack an easy screening system for detecting the presence of DNA inserts; and (*iv*) they must be recombined with the endogenous plasmid pUH24 to potentiate transformation. Quite recently, Lau and Straus (13) reported construction of relatively smaller shuttle vectors (7.3–7.8 kbp) by utilizing the cyanobacterial origin of replication of the endogenous plasmid pUH24 from *A. nidulans*. In yet another approach, *Synechococcus* R_2 has been transformed to antibiotic resistance by chimeric DNA molecules consisting of *Synechococcus* R_2 chromosomal DNA linked to antibiotic resistance genes from *E. coli* (14, 15).

We report here the successful transformation of *A. nidulans* 6301 by the *E. coli* plasmid pBR322, using either intact cells or permeaplasts. The plasmid pBR322 (4.3 kbp), an extremely versatile cloning vector, is a ColE1 type with ampicillin resistance and tetracycline resistance as selective markers, and the complete nucleotide sequence of this plasmid is also known.

MATERIALS AND METHODS

Culture of *E. coli* and Cyanobacterial Strains. *E. coli* strain HB101 harboring pBR322 was a gift of Ray Reeves and was grown in LB broth at 37°C (16). *A. nidulans* PCC strain 6301, also designated UTEX625 (17), was used throughout the present studies and was a gift of W. Rayburn. *A. nidulans* was grown in Kratz and Myers medium (18) with shaking at 32°C under cool white fluorescent light.

Preparation of Permeaplasts. Permeaplasts were prepared as described by Ward and Myers (19). Freshly prepared Kratz and Myers medium (150 ml) was inoculated with 20 ml of a young culture to read $OD_{600} = 0.2$ after inoculation, and the cells were grown overnight under standard growth conditions. Cells were centrifuged, washed once in 50 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) (pH 7.3 at 25°C), and resuspended in 15 ml of buffer (pH 7.3) containing 50 mM Tes, 1 mM EDTA, and 2 mg of lysozyme per ml. The cells were incubated at 36°C in a 125-ml Erlenmyer flasks at 60 oscillations per min in the light. At indicated times, 2.0-ml samples were transferred to 20 ml of cold 50 mM Tes (pH 7.3) and centrifuged immediately. Control untreated samples were manipulated identically except that the incubation medium did not contain EDTA and lysozyme. The pellet was resuspended in 10 ml of fresh growth medium to read $OD_{730} = 0.5$ (2×10^8 cells per ml) and used for transformation.

Transformation. Cells or permeaplasts prepared by 2-hr treatment with lysozyme/EDTA were used for transformation immediately. To 1.0 ml of permeaplasts or cells, 1 µg of donor DNA (in 10 mM Tris/1 mM EDTA, pH 8.0 at 25°C) was added, and the suspension was incubated for different durations in sterile culture tubes on an illuminated horizontal test tube shaker at 32°C. Samples were plated in triplicate with a series of 2-fold serial dilutions to quantify transformants.

Transformants were selected by one of the two following methods: (*i*) inoculation on agar plates containing 10 mM

Abbreviation: kbp, kilobase pair(s).
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sodium acetate and 0.5 μg of ampicillin per ml in Kratz and Myers medium after growing the transformants in the presence of ampicillin (0.25 μg/ml) in fresh growth medium for 2–3 days or (*ii*) incubation of the inoculated plates in the absence of antibiotic at 32°C for 24 hr; 400 μl of an aqueous solution of ampicillin (50 μg/ml) was then dispensed underneath the agar slab, which had been partially lifted with a sterile spatula. All plates were incubated at 32°C for 7 days before transformants were scored.

Assay for β-Lactamase. β-Lactamase was assayed by a spectrophotometric method (20) in which the β-lactam Nitrocefin (λ_{max}, 390 nm) is hydrolyzed to the ring-opened product (λ_{max}, 490 nm). Nitrocefin was obtained from Glaxo Group Research (London). Two-day-old *A. nidulans* cells, untransformed or transformed with pBR322 or pCH1, were broken by sonic treatment at 0°C in 50 mM 4-morpholine-propanesulfonic acid containing 10 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol (pH 7.3) for 3 min (six treatments for 30 sec each) with intermittent cooling of the probe in a freezing mixture of ice and salt. The homogenate was centrifuged at 25,000 × *g* for 15 min, and the clear supernatant was used for β-lactamase assays (or for electrophoretic analysis of soluble proteins). Absorption spectra were recorded at 20°C in a DMS 80 Varian UV/visible spectrophotometer after incubation in the presence or absence of various sonic extracts. Nitrocefin was prepared by dissolving 5 mg of the compound in 0.5 ml of dimethyl sulfoxide and diluting the resultant mixture with 9.5 ml of 0.1 M sodium phosphate (pH 7.0). This stock preparation was stable up to 15 days at 0°C in the dark. Dilution of 25 μl of Nitrocefin stock solution in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.0) gave an approximate absorbance of 0.7 at 395 nm (light path: 1.0 cm). Assays for β-lactamase were carried out in 1.0 ml of 0.1 M sodium phosphate (pH 7.0) supplemented with 25 μl of the stock solution of Nitrocefin and 25 μl of sonic extract. Absorption spectra were recorded (scan time, 2 min) between 300 and 700 nm at 0 time and 20 min after incubation at 22°C.

Immunoprecipitation of β-Lactamase and Ribulose-Bisphosphate Carboxylase. Fresh growth medium (200 ml) was inoculated with 20 ml of young *A. nidulans* cells, untransformed or transformed with pBR322, which were grown under standard growth conditions in the presence of 200 μCi of [^{35}S]methionine (0.1 μM) for 48 hr (1 Ci = 37 GBq). Cells were centrifuged and broken as described earlier. Sonic extracts (100–200 μl) were mixed with 1 ml of 1% Triton X-100/10 mM Tris, pH 7.8 at 25°C. The mixture was clarified by 15-min centrifugation in a Beckman Microfuge B at 9000 × *g*. Protein A-Sepharose (CL-4B) that had been swollen in deionized H_2O for 2 hr at 20°C was incubated with anti-β-lactamase antiserum (a gift of G. W. Ross, Glaxo Group Research) or spinach ribulose-bisphosphate carboxylase antiserum (prepared in rabbits by José Torres-Ruiz of our laboratory) for 3 hr at 0°C with intermittent mixing. The adsorbed antigen–antibody complex on protein A-Sepharose was then washed once with 1 ml of 10 mM Tris (pH 7.8 at 25°C) and incubated with the clarified sonic extract for 3–4 hr at 0°C with intermittent mixing. After incubation, the complex was washed sequentially in 10 mM Tris, pH 7.8 at 25°C/0.5% Triton X-100/5 mM EDTA containing various concentrations of NaCl (0.15–0.5 M). Finally, the protein A-Sepharose antigen–antibody complex was washed once in 1 ml of 10 mM Tris (pH 7.8 at 25°C) and resuspended in 60 μl of gel-electrophoresis sample buffer [200 mM Tris, pH 8.8 at 25°C/0.5 M sucrose/5 mM EDTA/4% (wt/vol) NaDod-SO₄/1%(wt/vol) methionine/10 mM dithiothreitol/0.01% bromophenol blue]. The mixture was incubated in a water bath at 60°C for 60 min and at 100°C for 3 min. After centrifugation at 6500 × *g*, the supernatant was subjected to gel electrophoresis.

Gel Electrophoresis and Fluorography. ^{35}S-labeled proteins or immunoprecipitates were resolved by gel electrophoresis in the presence of 0.1% NaDodSO₄ and polyacrylamide polymerized from 12.5% Bio-Rad acrylamide (21). Each sample containing 100 μg of protein was subjected to electrophoresis in a 1-mm-thick slab gel for 10 hr at 15 mA (22). The gels were stained with 0.2% Coomassie brilliant blue R-250 in water/methanol/acetic acid, 5:5:1 (vol/vol), and destained by successive washings in water/methanol/acetic acid 5:5:1. The gels were then soaked in 200 ml of EN-³HANCE (from New England Nuclear) for 1 hr, washed in H_2O for 1 hr, vacuum dried, and fluorographed with Kodak X-Omat film at −80°C (23).

Isolation of Plasmids. The plasmid pBR322 was isolated as described by Maniatis *et al.* (16). Plasmid DNA from transformed *A. nidulans* cells was isolated as described by Van den Hondel *et al.* (2) with suitable modifications. Cells grown for 6–10 days to a high optical density in a 5-liter liquid culture were harvested by centrifugation, washed once in 0.12 M NaCl/0.05 M EDTA, pH 8.0, resuspended in 68 ml of lysis buffer (25% sucrose/0.1 M EDTA/0.05 M Tris, pH 8.0 at 25°C), and transferred into four 30-ml Corex tubes. After incubating the cell suspension with 2 mg of lysozyme per ml for 1 hr at 37°C, 3 ml of 10% NaDodSO₄ was added and mixed gently into the suspension, which was incubated for 1 hr at 37°C. Five milliliters of 5 M NaCl was added to each tube with thorough mixing, and the mixture was kept at 2°C overnight. Most of the chromosomal DNA was removed by centrifugation at 15,000 rpm for 30 min at 4°C in a Sorvall SS 34 rotor. Solutes in the clear lysate were concentrated by adding 0.5-vol of 30% polyethylene glycol (PEG 8000) and kept at 4°C overnight. The precipitate was harvested by low-speed centrifugation (3000 × *g* for 5 min) and resuspended in 5.0 ml of cold 50 mM Tris, pH 8.0 at 25°C/5 mM EDTA/50 mM NaCl. Plasmids were banded at 37,500 rpm for 72 hr at 15°C in a Ti 70.1 rotor using a Beckman L8 ultracentrifuge in a solution containing 1 g of CsCl and 0.8 ml of ethidium bromide (5 mg/ml) per ml of original lysate. After centrifugation, the gradients were viewed in UV light and photographed. The lower fluorescent band containing plasmid DNA was collected, extracted with butanol, desalted by dialysis, and precipitated with ethanol (16).

Isolation and Estimation of Chromosomal DNA. Chromosomal DNA was prepared by equilibrium centrifugation (14). DNA was estimated by the diphenylamine method (24).

Gel Electrophoresis of Plasmid DNA. Electrophoresis of DNA was performed on horizontal slab gels of 0.7% agarose submerged in 80 mM Tris/20 mM sodium acetate/2 mM EDTA, pH 8.3 at 25°C. Electrophoresis was conducted at 1 V/cm for 16 hr at 22°C. Photographs of the gels were made under UV light.

RESULTS

Permeaplast Preparation. In the course of our attempts to standardize optimal conditions for transformation of *A. nidulans* with plasmids from *E. coli*, we investigated the capacity of permeaplasts of *A. nidulans* to regenerate cell wall and subsequently divide. Treatment of *A. nidulans* cells with lysozyme and EDTA for different durations resulted in an increase in OD at 540 nm, an indication of increased light scattering probably caused by swelling of the cells (Fig. 1). Subsequent cell division was observed in cells that had been treated with lysozyme and EDTA for 1 or 2 hr. Treatment for longer than 2 hr resulted in a subsequent OD decrease for up to 30 hr and little or no apparent cell repair after 30 hr.

Transformation. Transformation of cells or permeaplasts of *A. nidulans* with the *E. coli* plasmid pBR322 was studied as a function of period of contact with the donor DNA (Fig.
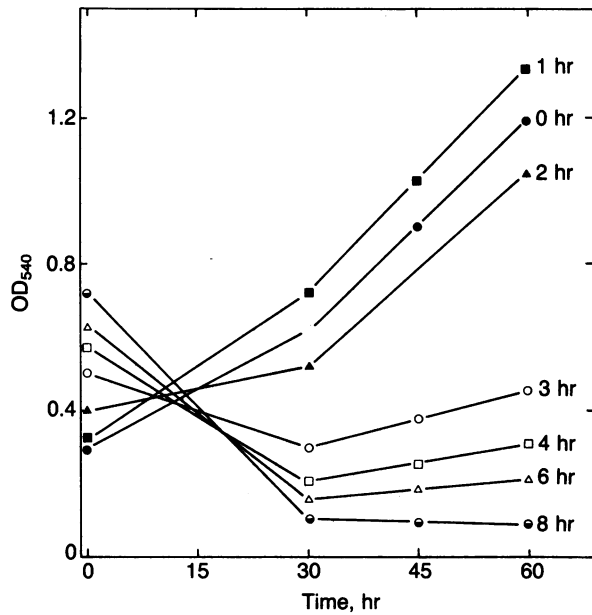
FIG. 1. Effect of EDTA/lysozyme treatment on *A. nidulans*. Permeaplasts were prepared as described by incubation with EDTA/lysozyme for the duration shown. After incubation, cells were washed once in cold Tes (50 mM, pH 7.3), resuspended in growth medium, and incubated from time zero under standard growth conditions.

2). In contrast to earlier reports of transformation of cyanobacteria with derivatives of their endogenous plasmids, wherein transformants were observed after contact periods of <1 hr, transformants with the *E. coli* plasmid pBR322 were observed only after a minimum period of 18 hr of contact with donor DNA (Fig. 2). Maximum transformation was observed after 28 hr of contact between the donor DNA and cells or permeaplasts and thence declined rapidly during prolonged

contact (Fig. 2). Inclusion of sodium acetate in the plates favored faster division and growth of the transformants, and large dark-green colonies were observed on these plates. Such a significant difference in growth was not observed when sodium acetate was included in liquid growth medium.

The efficiency of transformation was enhanced by about 50-fold in permeaplasts compared to cells (Fig. 2). Transformants have been stably maintained for over 8 months by repeated subculturing in the presence of ampicillin at 1 μg/ml. Plating of transformants on LB broth established that there was no bacterial contamination.

Assay of β-Lactamase. Ampicillin is a derivative of penicillin that kills growing cells by interfering with a terminal reaction in cell-wall synthesis. The resistance gene *bla* carried by plasmid pBR322 specifies a periplasmic enzyme, β-lactamase, that catalyzes cleavage of the β-lactam ring of the antibiotic. This enzyme has been reported in *E. coli* harboring the pBR322 plasmid based upon a spectrophotometric assay in which hydrolysis of the β-lactam of Nitrocefin is measured, but it could not be detected in transformed higher plant cells (25). Fig. 3 shows the absorption spectrum of Nitrocefin in aqueous solution with a broad peak around 390 nm. Addition of sonic extracts of *A. nidulans* cells that had been transformed with pBR322 or pCH1 resulted in the disappearance of this peak because of hydrolysis of the amide bond in the β-lactam ring of Nitrocefin and the formation of a new broad peak around 490 nm during the 20-min incubation with Nitrocefin. Sonic extracts of untransformed *A. nidulans* cells did not exhibit this spectral shift (Fig. 3).

Immunoprecipitation of β-Lactamase and Analysis of Proteins. The presence of β-lactamase was further investigated by using β-lactamase antiserum to immunoprecipitate this enzyme from sonic extracts of *A. nidulans* cells. Interestingly, immunoprecipitation of sonic extracts of transformed cells not only showed the presence of β-lactamase (Fig. 4 *Left*) but also coprecipitation of large and small subunits of ribulose-bisphosphate carboxyase. The latter was confirmed by detection of the large and small subunit bands after immunoprecipitation with anti-ribulose-bisphosphate carboxyase antiserum (data not shown). Presumably, the coprecipitation of the carboxyase observed with the β-lactamase antiserum reflects the fact that the carboxylase is a major protein in pBR322-transformed cells. Indeed, a comparison of soluble proteins by Coomassie blue staining after NaDodSO₄/polyacrylamide gel electrophoresis showed the amplification of large and small subunits of ribulose-
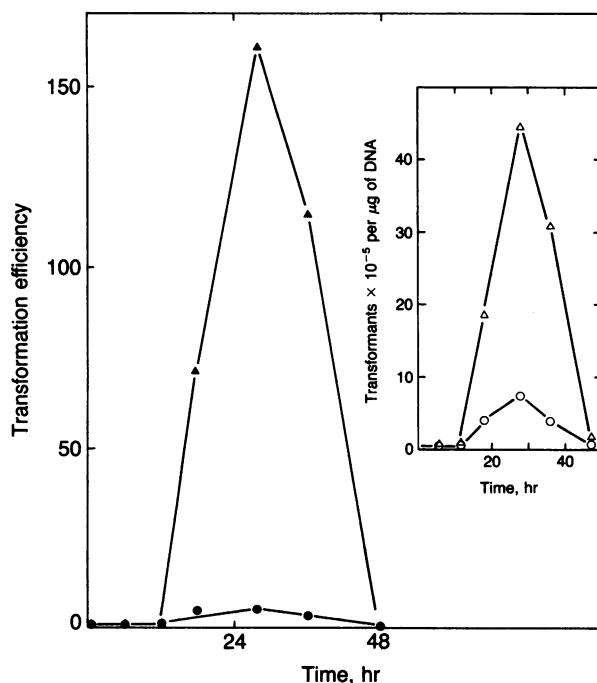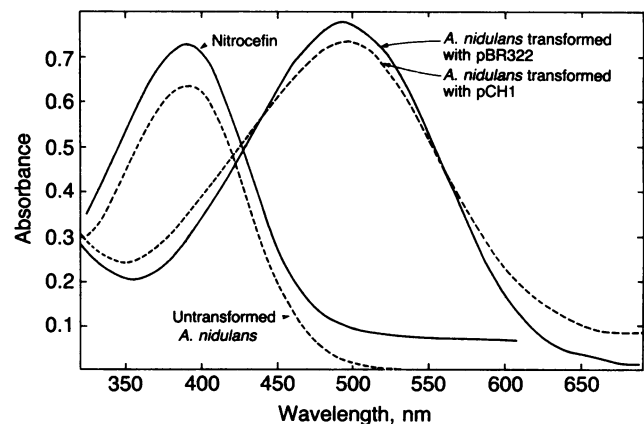

FIG. 2. Effect of length of contact between *A. nidulans* cells (○, ●) or permeaplasts (△, ▲) and pBR322 plasmid DNA on transformation to ampicillin resistance. Transformation efficiency expresses transformants per μg of donor DNA per 10^3 viable cells.

FIG. 3. Assay of β-lactamase based on hydrolysis of the amide bond in the β-lactam ring of Nitrocefin in extracts of *A. nidulans*. Cells, untransformed or transformed with pBR322 or pCH1, were broken, and the supernatant was prepared and used in β-lactamase assays as described. The spectral shift of Nitrocefin (λ_{max}, 390 nm) after hydrolysis by β-lactamase (λ_{max}, 490 nm) establishes completion of the reaction in the 20-min incubation at 22°C.
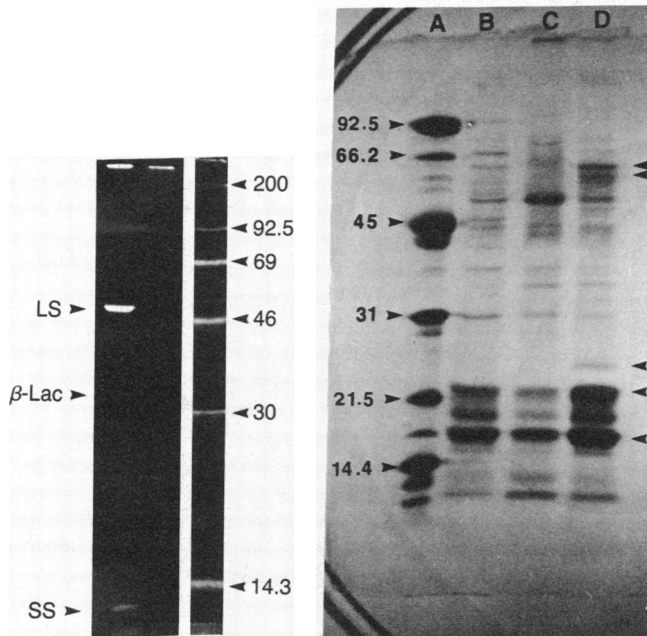
FIG. 4. (*Left*) Reverse-contrast print of the fluorograph reflecting immunoprecipitation of β-lactamase. [^{35}S]methionine-labeled *A. nidulans* cells, untransformed (middle lane) or transformed with pBR322 (left lane), were broken by sonic treatment; the soluble proteins (100 μg) were complexed with β-lactamase antiserum that had been conjugated to protein A-Sepharose and then analyzed by electrophoresis as described. A [^{14}C]methylated protein mixture (right lane) contained myosin (200 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). LS and SS, large and small subunit of ribulose-bisphosphate carboxylase, respectively; β-Lac, β-lactamase. (*Right*) Polypeptide profiles of soluble proteins in the supernatant from the centrifugation of 25,000 × *g* (see the assay for β-lactamase) of *A. nidulans* untransformed (lane B) or transformed with plasmids pBR322 (lane C) or pCH1 (lane D). The soluble proteins (50 μg per lane) were electrophoretically resolved in a NaDodSO$_4$-containing gel polymerized from 12.5% acrylamide and stained with Coomassie blue R-250. Soluble proteins amplified in pCH1 transformants compared to those in untransformed cells are indicated by arrowheads. Lane A contains protein standards phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

bisphosphate carboxylase in cells transformed with pBR322 but not in untransformed cells (Fig. 4 *Right*). In cells transformed with pCH1, amplification of several soluble proteins with molecular masses of 17, 22, 25, 63, and 66 kDa was evident. Analysis of membrane proteins did not show any significant difference between the untransformed and transformed cells (data not shown), and the pigment composition and profiles of all other soluble proteins were identical in untransformed cells and in those transformed with pBR322.

Analysis of Plasmid and Chromosomal DNA. *A. nidulans* contains two endogenous plasmids pUH24 (8 kbp) and pUH25 (48.5 kbp) that carry no phenotypic markers (5). Isolation of chromosomal and plasmid DNA from untransformed *A. nidulans* cells and cells transformed with pBR322 showed significant amplification of chromosomal DNA in transformed cells. While untransformed cells yielded 5.7 ng of chromosomal DNA per 10^8 cells, cells transformed with pBR322 and pCH1 yielded 32.9 and 29.2 ng of chromosomal DNA per 10^8 cells, respectively. Agarose gel electrophoresis of plasmid DNA in untransformed cells showed the presence of the two endogenous plasmids. The presence of an addi-
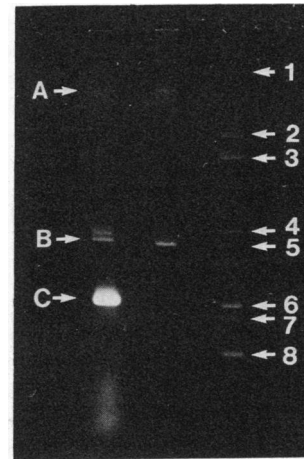

FIG. 5. Agarose gel electrophoresis of plasmid DNA isolated from *A. nidulans* cells untransformed or transformed with pBR322. Samples containing 1 μg of DNA were subjected to electrophoresis at 1 V/cm for 16 hr at 22°C on a 0.7% agarose gel submerged in gel buffer as described. Plasmids isolated from cells transformed with pBR322 (left lane) show the following bands: A, the endogenous large plasmid pUH25; B, the endogenous small plasmid pUH24; and C, pBR322-like plasmid. Plasmids isolated from untransformed cells (middle lane) reveal large and small endogenous plasmids. Standards (New England Nuclear) (right lane) contain the following covalently closed circular DNAs: 1, pJC74 (replicative form II); 2, pBR313 (form II); 3, pJC74 (form I); 4, pBR313 (form I); 5, pBR322 (form II); 6, pBR322 (form I); 7, pUK2 (form II); and 8, pUK2 (form I). Sizes of replicative form I DNAs are as follows: pUK2, 245 kbp; pBR322, 4.362 kbp; pBR313, 9.7 kbp; and pJC74, 17.5 kbp.

tional plasmid (4.3 kbp) in transformed cells (Fig. 5) indicated the presence of a pBR322-like plasmid.

DISCUSSION

Isolated protoplasts of higher plants have been used extensively in genetic experiments. Numerous attempts have been made to isolate protoplasts from cyanobacteria because of interest in (*i*) uptake or fusion of cyanobacterial protoplasts with higher plant protoplasts to study the transfer of N$_2$ fixation capacity, (*ii*) cyanophage infections, or (*iii*) their use as cloning vehicles in uptake and transformation by foreign DNA. Cyanobacteria have simpler growth requirements than bacteria, are nonpathogenic and, therefore, are ideal for genetic studies. However, presently there is no method available to isolate viable protoplasts; cells with partially digested cell walls are called spheroplasts (26). Unfortunately, spheroplasts are not suitable for genetic studies because of their poor capacity for cell-wall regeneration and cell division (27). In the present study, we have used permeaplasts (19), which are highly permeable cells that have a high efficiency of cell-wall regeneration and subsequent division, to study DNA uptake and genetic transformation. The more than 50-fold higher transformation efficiency observed for permeaplasts as compared to cells reflects an enormous increase in DNA uptake. Prolonged contact of donor DNA with recipient cells is also essential in the transformation of *A. nidulans*, and little attention has been paid to this by previous workers (for a comparison of various procedures of cyanobacterial transformation, see ref. 28).

Previous reports of cyanobacterial transformation have involved the use of modified endogenous plasmids (1, 5), chimeric plasmids constructed from cyanobacterial and *E. coli* plasmids (6–12), or chimeric DNA molecules consisting of cyanobacterial chromosomal DNA linked to antibiotic resistance genes from *E. coli* (14, 15). In all of these cases, homology between the chromosome and plasmid facilitated

transformation, although the precise mechanism of integration or plasmid establishment was not elucidated. In other organisms, chromosomal integration of plasmid DNA has been accomplished recently by constructing replicon-defective plasmids carrying a segment of DNA homologous to the chromosome of the recipient organism (29–31). Additionally, homology between plasmid and host chromosome DNA greatly enhances transformation (32). Exchange of genes between plasmid and host chromosome during transformation has also been well documented (32). In a related study, it has been found that, when a plasmid is established via homologous recombination with the chromosome, plasmids isolated from transformants may be of the same size as the plasmid used for initial transformation. Moreover, the reisolated plasmids contain segments of the host chromosome (33). In the present research, the mechanism of establishment of plasmids in transformants is unknown. Nevertheless the observations of amplification of large and small subunits of ribulose-bisphosphate carboxylase and of chromosomal DNA suggest an interaction between plasmid and host chromosomal DNA. Indeed, recombination between host and plasmid DNA may be required because pBR322 does not contain a cyanobacterial replicon. In this connection, we have recently detected hybridization of nick-translated [^{32}P]pBR322 with restriction fragments of chromosomal DNA derived from pBR322 transformants of *A. nidulans* 6301 (H.D. and B.A.M., unpublished observation).

Regardless of the mechanism, successful transformation of cyanobacteria with the versatile cloning vector pBR322 opens a new approach to genetic engineering of these organisms. It should be noted that genes of interest in photosynthesis or nitrogen fixation have been cloned in pBR322 (or its derivatives) but not as yet in any of the cyanobacterial shuttle vectors.

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