

## Gene Transfer to the Desiccation-Tolerant Cyanobacterium *Chroococcidiopsis*

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**The coccoid cyanobacterium *Chroococcidiopsis* dominates microbial communities in the most extreme arid hot and cold deserts. These communities withstand constraints that result from multiple cycles of drying and wetting and/or prolonged desiccation, through mechanisms which remain poorly understood. Here we describe the first system for genetic manipulation of *Chroococcidiopsis*. Plasmids pDUCA7 and pRL489, based on the pDU1 replicon of *Nostoc* sp. strain PCC 7524, were transferred to different isolates of *Chroococcidiopsis* via conjugation and electroporation. This report provides the first evidence that pDU1 replicons can be maintained in cyanobacteria other than *Nostoc* and *Anabaena*. Following conjugation, both plasmids replicated in *Chroococcidiopsis* sp. strains 029, 057, and 123 but not in strains 171 and 584. Both plasmids were electroporated into strains 029 and 123 but not into strains 057, 171, and 584. Expression of P<sub>psbA</sub>-*luxAB* on pRL489 was visualized through in vivo luminescence. Efficiencies of conjugative transfer for pDUCA7 and pRL489 into *Chroococcidiopsis* sp. strain 029 were approximately 10<sup>-2</sup> and 10<sup>-4</sup> transconjugants per recipient cell, respectively. Conjugative transfer occurred with a lower efficiency into strains 057 and 123. Electrotransformation efficiencies of about 10<sup>-4</sup> electrotransformants per recipient cell were achieved with strains 029 and 123, using either pDUCA7 or pRL489. Extracellular deoxyribonucleases were associated with each of the five strains. Phylogenetic analysis, based upon the V6 to V8 variable regions of 16S rRNA, suggests that desert strains 057, 123, 171, and 029 are distinct from the type species strain *Chroococcidiopsis thermalis* PCC 7203. The high efficiency of conjugative transfer of *Chroococcidiopsis* sp. strain 029, from the Negev Desert, Israel, makes this a suitable experimental strain for genetic studies on desiccation tolerance.**

Desiccation damages membranes, proteins, and nucleic acids and is lethal to the majority of organisms. Some organisms, the anhydrobiotes, withstand the physiological constraints which result from multiple cycles of drying and wetting and/or prolonged desiccation, and they resume metabolism when water becomes available. How they do so poses provocative questions (7). Desiccation tolerance of cyanobacteria is of particular interest because these phototrophs produce intracellular oxygen that can generate reactive oxygen species (30). Two cyanobacteria, *Nostoc commune* and *Chroococcidiopsis*, are the subjects of studies aimed at an understanding of desiccation tolerance (29). *Chroococcidiopsis* is characteristically the dominant photosynthetic form in microbial habitats of extreme arid cold and hot deserts, and in the most extreme of these environments, it is the sole photosynthetic microorganism. These microbial communities live in airspaces of porous rocks or in microscopic fissures of weathering rocks or form biofilms at the stone-soil interface under pebbles in desert pavements (16). Most of the time, the cells are desiccated or frozen. Long-term measurements show that in the ice-free Ross desert of Antarctica, cells are wetted and metabolically active for a total of 500 to 800 h per year (17). In the most arid areas of hot deserts such as the Negev Desert, Israel, the

number of metabolically active hours per year is probably even less than this (E. I. Friedmann and C. P. McKay, personal communication).

Despite an interest in the strategies employed by anhydrobiotes, desiccation tolerance is still poorly understood. What is clear is that desiccation tolerance reflects numerous different structural, physiological, and molecular mechanisms (3, 7, 29, 30). One mechanism shared by anhydrobiotes is the accumulation of trehalose and sucrose, two nonreducing sugars which replace the structural water of cellular components, thus circumventing lethal damage during drying (7). The production of both these compounds has been reported for several cyanobacteria, including *Chroococcidiopsis* spp. (22) and *Nostoc commune* (29). *Chroococcidiopsis* spp. and *N. commune* share several features that may account for their extreme tolerance of desiccation. Both produce abundant exocellular polysaccharides which may play a central role in desiccation tolerance of cells by regulating the loss and uptake of water (8, 23, 30). Ultrastructural and cytological studies of laboratory- and field-dried cells of *Chroococcidiopsis* spp. suggested that the amounts of acid-, sulfate-, and beta-linked polysaccharides in the cell envelope increase in response to water deficit (18, 20). In dried cells of *N. commune* the extracellular polysaccharide (a complex glycan) provides an immobilization matrix in which secreted enzymes remain active (36) and where UV-absorbing pigments accumulate for photoprotection (29, 30). D-Ribose and 3-O-lactyl glucuronic acid in the glycan of *N. commune* DHR1 are thought to influence the rheological properties of the extracellular matrix upon desiccation and rehydration (21).

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TABLE 1. *Chroococcidiopsis* sp. strains

Strain no. <sup>a</sup>	Strain history
029 (CCMEE N6904)	<i>Chroococcidiopsis</i> sp. strain; cryptoendolithic, found in airspaces of porous rocks, forming in a zone a few millimeters wide under the rock crust in Nubian sandstone, Negev Desert, Makhtesh Ramon, Israel
057 (CCMEE S 6e)	<i>Chroococcidiopsis</i> sp. strain; chasmoendolithic, found in microscopic fissures of weathering rocks, near the surface in granite, Sinai Desert, Egypt
123 (CCMEE CH-7)	<i>Chroococcidiopsis</i> sp. strain; chasmoendolithic, found in granite boulder, coastal desert, Guanaqueros Peninsula, Coquimbo, Chile
171 (CCMEE A789-2)	<i>Chroococcidiopsis</i> sp. strain; cryptoendolithic; found in Beacon sandstone, Ross Desert (McMurdo Dry Valleys) University Valley, Antarctica
584 (CCMEE Gobi 91-19)	<i>Chroococcidiopsis</i> sp. strain; hypolithic; found under pebbles of a desert pavement, forming biofilms at the stone-soil interface under stones of desert pavement, Gobi Desert near the Tian Shan mountain range of Mongolia
ATCC 27900	<i>C. thermalis</i> (see reference 31), from soil in East Germany (PCC 7203)

<sup>a</sup> For the five strains obtained from the CCMEE, the CCMEE reference number is given in parentheses.

The presence of abundant Fe-superoxide dismutase in dried cells of *N. commune* CHEN1986 (36) and in a desert strain of a *Chroococcidiopsis* sp. (19) suggests that an important mechanism in desiccation tolerance is the minimization of the risk of hydroxyl radical formation (30). A capacity to withstand  $\gamma$ -radiation is thought to be an incidental consequence of the ability to repair DNA damage that results from desiccation (30). The ability of desert strains of *Chroococcidiopsis* to withstand exposure to 5 kGy of X-rays (1 kGy = 0.1 megarad), with survival reduced by 1 or 2 orders of magnitude, emphasizes their capacity for DNA repair (1).

It is likely that desiccation tolerance involves the action of a large number of genes possibly acting in parallel pathways. Understanding the molecular basis for desiccation tolerance is therefore a significant challenge. Although sophisticated genetic systems are available for the analysis of cyanobacteria, only a few strains are currently the subject of genetic manipulations (37); none of these strains shows vigor in response to extremes of water deficit. It is not yet clear whether this situation is attributable to the successes achieved with these strains or the fact that many strains are simply unsuitable for genetic manipulation (37). We investigated the possibility of gene transfer via conjugation and electroporation in different strains of *Chroococcidiopsis* spp. isolated from geographically remote deserts. *Chroococcidiopsis* sp. strain 029 from the Negev Desert, Israel, was identified as a conjugally efficient strain, and the possibility of using this strain to represent desert populations was assessed through phylogenetic analysis (based upon variable regions V6 to V8 of 16S rRNA genes [32]).

## MATERIALS AND METHODS

**Microorganisms and growth conditions.** Five *Chroococcidiopsis* sp. strains from hot and cold deserts were obtained from the Culture Collection of Microorganisms from Extreme Environments (CCMEE) at Florida State University (now located at the University of Oregon, Eugene) (Table 1). *Chroococcidiopsis* sp. strain PCC 7203 was obtained from the American Type Culture Collection (ATCC 27900). *Anabaena* sp. strain PCC 7120 (from Jeff Elhai) was used for control purposes.

All cyanobacterial strains were grown at room temperature, in liquid BG-11 medium (31), with a photon flux density of 90  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by fluorescent cool-white lamps. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (34) supplemented with ampicillin (100  $\mu\text{g ml}^{-1}$ ), kanamycin sulfate (20  $\mu\text{g ml}^{-1}$ ), or chloramphenicol (25  $\mu\text{g ml}^{-1}$ ), as appropriate.

**Conjugation.** Cargo plasmids pDUCA7 and pRL489 (Table 2) intended for transfer to the cyanobacterial host were first replicated in an *E. coli* strain, HB101, bearing the helper plasmid pRL528, which carries genes encoding DNA methylases (Table 2). Triparental mating was performed as follows: *E. coli* strain HB101 bearing a conjugative plasmid and *E. coli* strain HB101 bearing both a cargo plasmid and helper plasmid were grown overnight. Cells from 1-ml aliquots (1 ml for each mating) were washed twice with one volume of LB medium (without antibiotics) and resuspended in 200  $\mu\text{l}$  of LB medium. One-milliliter aliquots of each *Chroococcidiopsis* sp. culture (ranging in age from 4 weeks to 2 months;  $10^6$  to  $10^7$  cells  $\text{ml}^{-1}$ ) were centrifuged, and the cells were resuspended in 100  $\mu\text{l}$  of BG-11 medium. A 100- $\mu\text{l}$  volume of the *E. coli* mixture (prepared as described above) was added to 100  $\mu\text{l}$  of a cyanobacterial cell suspension, and 5- $\mu\text{l}$  aliquots were spotted on Nuclepore REC-85 filters resting on BG-11 agar (1.5%, wt/vol). The donor 1-donor 2-recipient cell ratio was approximately 10:10:1. Alternatively, recipient cyanobacterial cells were diluted serially ( $1:10^4$ ) before the *E. coli* mixture was added. Matings were carried out for 48 h under conditions used for growth of *Chroococcidiopsis* spp. Triparental spot matings were also performed as described above, using *E. coli* cells bearing pDUCA7 but lacking the helper plasmid pRL528. In other controls, spot matings were performed using  $10^6$  cells of each *Chroococcidiopsis* sp. strain mixed with  $10^5$  cells of *E. coli* bearing either the conjugative plasmid or the cargo plasmid.

TABLE 2. Plasmids

Plasmid(s)	Relevant characteristics	Source (reference)
pRL528	Helper plasmid ( $\text{Cm}^r$ ), ColK <i>oriV</i> , carries the genes for M.AvaI and M.Eco47II and <i>mob</i> gene from ColK for the mobilization of pMB1-based plasmid <sup>a</sup>	J. Elhai (12)
RK2 and RP4	Conjugative plasmids ( $\text{Ap}^r$ $\text{Km}^r$ $\text{Tc}^r$ ), RK2 <i>oriV</i> , RK2 <i>oriT</i> , provide <i>tra</i> genes and nicking function from RK2; IncP $\alpha$	J. Elhai (39)
pRL443	Spontaneous $\text{Km}^s$ mutant of RP4 ( $\text{Ap}^r$ $\text{Tc}^r$ )	J. Elhai (12)
pRK2013	RK2 derivative ( $\text{Km}^r$ ), colE1 <i>oriV</i> replaces RK2 <i>oriV</i>	J. Elhai (15)
pDUCA7	Cargo plasmid ( $\text{Km}^r$ ), RK2 <i>oriV</i> , RK2 <i>oriT</i> , pDU1 replicon <sup>b</sup>	J. C. Meeks (4)
pRL489 <sup>c</sup>	Cargo plasmid ( $\text{Km}^r$ ), pMB1 <i>oriV</i> , pMB1 <i>oriT</i> , pDU1 replicon, <sup>b</sup> P <sub>psbA</sub> - <i>luxAB</i>	J. Elhai (9)

<sup>a</sup> Derivatives of pMB1, a ColE1-like plasmid, retain *bom* site (basis of mobility or *oriT*) but not the *mob* gene, which encodes a DNA-nicking function. These derivatives can be transferred only when a *mob* product is provided in *trans*, by a helper plasmid.

<sup>b</sup> Cyanobacterial origin of replicon from pDU1, a plasmid from *Nostoc* sp. strain PCC 7524.

<sup>c</sup> P<sub>psbA</sub> is the promoter of photosystem II gene *psbA*, from *A. hybridus*.

Selection of *Chroococcidiopsis* transconjugants was performed by transferring the filters to BG-11 agar containing neomycin ( $400 \mu\text{g ml}^{-1}$ ) under a photon flux density of  $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Purification of transconjugants was performed by counterselecting *E. coli* through the use of BG-11, which does not support its growth (38). One-month-old single green colonies of putative transconjugants were restreaked twice on selective medium before transfer to liquid BG-11 containing neomycin ( $100 \mu\text{g ml}^{-1}$ ). Aliquots of the liquid cultures were plated on LB medium to detect the presence of *E. coli*.

**Electroporation.** One-milliliter aliquots were harvested from 1-month-old cultures of each *Chroococcidiopsis* sp. strain. Cells were collected, washed twice with cold 1 mM HEPES buffer, pH 7.4, and resuspended in 100  $\mu\text{l}$  of the same buffer (about  $10^8$  total cells). Cargo plasmids (Table 2) were methylated *in vivo* using *E. coli* carrying helper plasmid pRL528 (Table 2). Plasmid DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, Wis.) and added to 100  $\mu\text{l}$  of the cyanobacterial suspension at a final concentration of approximately of 2.5  $\mu\text{g ml}^{-1}$ . The mixture was placed between the electrodes (0.1-mm gap) of a cold electroporation cuvette and pulsed once in a Gene Pulser Controller (Bio-Rad Laboratories, Richmond, Calif.) at 13 kV  $\text{cm}^{-1}$  (25  $\mu\text{F}$  and 200  $\Omega$  settings; time constant, ca. 3.5). After electroporation, cyanobacterial cells were resuspended in 2 ml of BG-11 medium and allowed to grow for 24 h. After centrifugation and resuspension in 100  $\mu\text{l}$  of BG-11 medium, 5- $\mu\text{l}$  aliquots were spotted on Nuclepore REC-85 filters and incubated until green colonies appeared. Electrotransformants were used to inoculate liquid BG-11 medium containing neomycin ( $100 \mu\text{g ml}^{-1}$ ). Control electroporations were performed in the absence of plasmid DNA.

**Extracellular deoxyribonuclease assay.** One-milliliter aliquots were harvested from 1-month-old cultures of each cyanobacterial strain, and cells were collected and streaked on BG-11 agar (1.5%, wt/vol) containing 0.3 mg of DNA-methyl green (Sigma Chemical Co., St. Louis, Mo.) per ml and  $0.05 \times \text{SSC}$  ( $1 \times \text{SSC}$  is 0.15 M NaCl plus 0.015 M sodium citrate) as described previously (41). Petri plates were incubated under cyanobacterial growth conditions.

**Extraction of genomic DNA.** Total DNAs were extracted from *Chroococcidiopsis* sp. cells as described previously (2), except that genomic DNA was not purified through cesium chloride buoyant density ultracentrifugation.

**Extraction of plasmid DNA.** Cells from 15-ml aliquots of axenic cultures of transconjugants of *Chroococcidiopsis* sp. strain 029 and electrotransformants obtained from strains 029 and 123 were washed with 1 ml of TE buffer (1 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 7.4). Cells ( $10^7$  cells) were subjected to 30 cycles of freezing in liquid nitrogen and thawing at  $50^\circ\text{C}$ , and the mixture was then used directly for plasmid DNA extraction using the Wizard Plus Minipreps DNA Purification System (Promega). After the centrifugation of the cell lysate, the supernatant fraction was mixed with 1 volume of binding mix, prepared by resuspending 250 mg of silica (Sigma) in 100 ml of a solution containing 5 M guanidine thiocyanate and 4% (wt/vol) Triton X-100. After two washes with 50% ethanol and drying in a speed vacuum concentrator, plasmid DNA was eluted from silica gel particles with 30  $\mu\text{l}$  of sterile water. Since the yield of plasmid DNAs was undetectable with agarose gel electrophoresis, transformation of *E. coli* was used routinely to assay plasmids from *Chroococcidiopsis* sp. transconjugants and electrotransformants. Typically, 10- $\mu\text{l}$  aliquots were used to electroporate  $2 \times 10^9$  cells of ElectroMAX *E. coli* DH10B  $\text{ml}^{-1}$  (Novagen-LTI, Rockville, Md.). About 10 colonies of *E. coli* transformants were selected on LB agar plates containing 20  $\mu\text{g}$  of Km  $\text{ml}^{-1}$  (cargo plasmids carry the neomycin phosphotransferase gene, which confers resistance to kanamycin and neomycin).

**DNA dot blotting and Southern hybridization.** In order to characterize axenic *Chroococcidiopsis* sp. transconjugants, plasmid pDUCA7 was labeled by nick translation using alkali-labile digoxigenin (DIG)-11-dUTP (Boehringer Mannheim GmbH) and used as probe. Dot blots were performed using genomic DNAs from transconjugants of strains 029, 057, and 123, and parental strains were used as controls. Southern analyses were performed using genomic DNAs extracted from *Chroococcidiopsis* sp. 029 transconjugants before and after digestion with *Pst*I. Genomic DNA from wild-type *Chroococcidiopsis* sp. 029 and authentic pDUCA7 were used as controls. Hybridizations were performed according to protocols specified in the Genius System User's Guide for Membrane Hybridization (Boehringer Mannheim GmbH) using the DIG Easy Hyb Granules System (Boehringer Mannheim GmbH). Hybridization blots were visualized with anti-DIG alkaline phosphatase and the chemiluminescent detection system of Boehringer Mannheim GmbH.

**Luciferase detection.** Light emission resulting from the oxidation of *n*-decanal and catalyzed by luciferase was detected *in vivo* in cells bearing plasmid pRL489 (Table 2). Colonies were exposed for 5 min to the vapor of several  $\mu\text{l}$  of *n*-decanal spread on the inner surface of the top of the petri dish. Bioluminescence was recorded as digital images using the television camera of a 400 Alpha

Innotech ChemImager low-light imaging system operated with Alphase 3.3 software, a Dell Pentium computer, and an Optiquet color monitor.

**Amplification of 16S rRNA genes and DNA sequencing.** Genomic DNAs extracted from *Chroococcidiopsis* sp. strains were used with different sets of primers to amplify the almost complete 16S rRNA genes. The positions in *E. coli* 16S rRNA that are equivalent to these primer sequences are provided for reference. The forward primer was either the universal primer F2C (5'-AGA GTT TGA TCA/C TGG CTC-3') or the cyanobacterium-specific primer CYA106F (28) corresponding to *E. coli* nucleotides 8 to 25 and 106 to 127, respectively. The universal primer C (5'-ACG GGC GGT GTG TAC-3') corresponding to *E. coli* positions 1406 to 1392 was used as the reverse primer. Partial sequences were obtained by using the forward universal primer AC (5'-CAG CCG CGG TAA TAC-3') corresponding to *E. coli* positions 552 to 536 and the reverse universal primer C. Conditions for the PCR assay were 30 cycles of annealing for 1 min at  $40^\circ\text{C}$  and elongation for 3 min at  $72^\circ\text{C}$ ; amplifications were initiated with a 5-min denaturation at  $95^\circ\text{C}$  and ended with a 7-min extension at  $72^\circ\text{C}$ . PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc., Chatsworth, Calif.) and used as templates in sequencing reactions with the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Ready reaction kit (Perkin-Elmer). Sequencing reactions were obtained by using the forward primers F2C, CYA106F, and AC and the reverse universal primer C and then were analyzed by using an Applied Biosystem 377 DNA sequencer (Perkin-Elmer).

**Phylogenetic reconstruction.** The 16S ribosomal DNA (rDNA) sequences of *Chroococcidiopsis* sp. strains were aligned manually, and sequences from nucleotides 1 to 480 (corresponding to the numbering of the *E. coli* 16S rRNA) were used for the analysis.

**Phylogenetic analysis.** DNA sequences were first aligned using the MEGALIGN feature of version 4.0 of the LaserGene software (DNASTAR Inc., Madison, Wis.). Phylogenetic trees were constructed based upon parsimony analysis (ordinary parsimony) and distance methods using the Phylogenetic Inference Package (PHYLIP, version 3.57c) obtained from J. Felsenstein, Department of Genetics, University of Washington, Seattle. SEQBOOT was used to produce 100 data sets from bootstrap resampling (14). Majority rule strict consensus analysis was performed with CONSENSE, with the *Anabaena* sp. strain NIVA-CYA 281 sequence arbitrarily designated as the outgroup. Distance matrices were obtained with the Kimura two-parameter model using the default transition/transversion ratio (26) and calculated with the DNADIST and NEIGHBOR (33) programs of PHYLIP (14). Unrooted trees were plotted in DRAWTREE or DRAWGRAM and edited in Adobe Illustrator version 9.0.

**Nucleotide sequence accession numbers.** DNA sequences and annotations of *Chroococcidiopsis* sp. strains 029, 057, 123, and 171 were deposited in GenBank under accession numbers AF279107, AF279108, AF279109, and AF279110, respectively.

## RESULTS

**Conjugative transfer in *Chroococcidiopsis* spp.** Conjugative transfer of pDUCA7 and pRL489 (Table 2) was achieved in strains 029, 057, and 123 but not in strains 171 and 584 (Table 1). Mobilization of pDUCA7 via pRK2013 yielded green colonies of presumptive transconjugants from *Chroococcidiopsis* sp. strains 029, 057, and 123, visible on top of a yellow basal deposit of dead cells, about 4 weeks after the start of antibiotic selection (Fig. 1A). The efficiency of conjugation with *Chroococcidiopsis* sp. strains 057 and 123 was about  $10^{-4}$  transconjugants per recipient cell, i.e., about 20 to 30 neomycin-resistant colonies out of  $10^5$  cells per spot mating (Fig. 1A; Table 3). The efficiency of conjugative transfer of *Chroococcidiopsis* sp. strain 029 was estimated as about  $10^{-2}$  transconjugants per recipient cell after serial dilutions of the recipient. No transconjugants were observed in strains 171 and 584 when the number of recipient cells per spot was increased from  $10^5$  (Fig. 1A) to  $10^6$ . The conjugative efficiencies of *Chroococcidiopsis* sp. strains 029, 057, and 123 were unaffected by the use of *E. coli* lacking the helper plasmid but bearing plasmid pDUCA7 (Fig. 1B). Plasmid pDUCA7 has an RK2 *oriT* and requires only the mobilization functions supplied by the conjugative

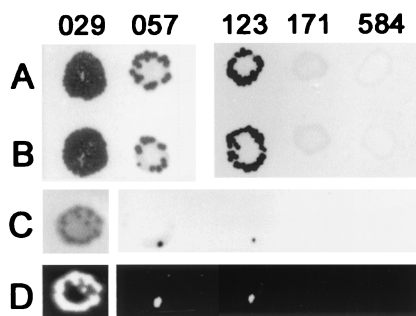


FIG. 1. Typical result of spot matings using *Chroococcidiopsis* sp. strains 029, 057, 123, 171, and 584. Rows: A, mobilization of plasmid pDUCA7 using conjugative plasmid pRK2013 in the presence of helper plasmid pRL528; B, mobilization of plasmid pDUCA7 using conjugative plasmid pRK2013 in the absence of helper plasmid pRL528; C, presumptive transconjugants of *Chroococcidiopsis* cells bearing plasmid pRL489 obtained using conjugative plasmid pRL443 and helper plasmid pRL528; D, luminescence was observed in colonies (see Fig. 1C) obtained from strains 029, 057, and 123 and not in the areas where strains 171 and 584 were used as the recipients. Filters shown were collected after 30 days of incubation.

plasmid. Using the same experimental conditions, no neomycin-resistant colonies were obtained from *Anabaena* sp. strain PCC 7120. Dot blot analysis using pDUCA7 DNA as the probe and total DNA extracted from wild-type *Chroococcidiopsis* spp. and from axenic liquid cultures obtained from transconjugants of strains 029, 057, and 123 confirmed the presence of pDUCA7 in the transconjugants (not shown). Neomycin-resistant colonies of *Chroococcidiopsis* sp. strains were never obtained in control experiments in which  $10^5$  recipient cells per spot were mixed with  $10^6$  *E. coli* cells bearing either the conjugative plasmid pRK2013 or cargo plasmid pDUCA7. Matings via pRL443 yielded frequencies of transconjugants of *Chroococcidiopsis* sp. strains 029, 057, and 123 that were 1 order of magnitude lower than those observed using pRK2013. Transconjugants of strain *Chroococcidiopsis* sp. strain 029 were subjected to further investigation (see below).

Plasmid pRL489 (Table 2) was mobilized via pRL443 into *Chroococcidiopsis* sp. strains 029, 057 and 123 but not into strains 171 and 584 (Fig. 1C; Table 3). The efficiency of conjugative transfer of pRL489 into *Chroococcidiopsis* sp. strain 029 was about  $10^{-4}$  transconjugants per recipient cell, i.e., 10-fold higher than that into strains 057 and 123 (Fig. 1C; Table 3). When 1-month-old conjugation plates were tested for

luciferase activity, luminescence was detected in green neomycin-resistant colonies from strains 029, 057, and 123 (Fig. 1D). Luminescence was not detected in corresponding spots of growth when cells of strain 171 and 584 were used as the recipient cells (Fig. 1D). Neomycin-resistant colonies of *Chroococcidiopsis* sp. strains were never observed in matings via pRL443 using *E. coli* cells bearing plasmid pRL489 but lacking plasmid pRL528. Plasmid pRL489 replicates via a pMB1 *oriT* and needs DNA nicking function encoded by the helper plasmid for its mobilization. Aliquots of liquid cultures derived from presumptive transconjugants of *Chroococcidiopsis* sp. strains 029, 057, and 123 were judged to be axenic after plating on LB medium and incubation at 37°C overnight.

Conjugative transfer of pDUCA7 and pRL489 into *Chroococcidiopsis* sp. strains 029, 057, and 123 was unaffected by the age (up to 2 months) of the cyanobacterial cells used as the recipients.

**Electrotransformation of *Chroococcidiopsis* spp.** Gene transfer of pDUCA7 and pRL489 into *Chroococcidiopsis* sp. strains 029 and 123 but not strains 057, 171, and 584 was achieved via electroporation (Table 3). Green colonies of electrotransformants appeared on neomycin-containing BG-11 medium within one month (approximately 10 generations) and were purified further through single-colony isolation. Neomycin-resistant colonies of *Chroococcidiopsis* strains 029 and 123 were never obtained following electroporation in the absence of plasmid DNA. Electrotransformants of *Chroococcidiopsis* sp. strains 029 and 123 were subjected to further investigation (see below).

A summary of the efficiencies of transfer of pDUCA7 and pRL489 into *Chroococcidiopsis* sp. strains via conjugation and electroporation is shown in Table 3. Gene transfer frequencies were evaluated with each cell aggregate or single cell of *Chroococcidiopsis* sp. considered to be 1 CFU. Though this method may be biased, it is unavoidable due to the complex life cycle of *Chroococcidiopsis* spp., which is characterized by the occurrence of single cells and multicellular aggregates (see reference 1).

**Analysis of *Chroococcidiopsis* sp. strain 029 transconjugants.** Transconjugants from *Chroococcidiopsis* sp. strain 029 were investigated further because this strain exhibited the highest efficiency of conjugative transfer (Table 3). Two independent isolates (*Chroococcidiopsis* sp. strains CH91B1 and CH91B2) were obtained following mobilization of plasmid pDUCA7 via

TABLE 3. Gene transfer efficiencies<sup>a</sup>

Recipient strain <sup>b</sup>	Conjugative efficiency with:		Electroporation efficiency with:	
	pDUCA7 <sup>c</sup> and pRK2013 <sup>d</sup>	pRL489 <sup>c</sup> and pRL443 <sup>d</sup>	pDUCA7 <sup>c</sup>	pRL489 <sup>c</sup>
029 (CCMEE N6904)	$(3 \pm 1.2) \times 10^{-2}$	$(4 \pm 0.9) \times 10^{-4}$	$(3 \pm 0.7) \times 10^{-4}$	$(5 \pm 0.4) \times 10^{-4}$
057 (CCMEE S6e)	$(5 \pm 0.6) \times 10^{-4}$	$(3 \pm 0.5) \times 10^{-5}$	ND	ND <sup>8</sup>
123 (CCME CH-7)	$(3 \pm 0.4) \times 10^{-4}$	$(4 \pm 0.3) \times 10^{-5}$	$(5 \pm 0.4) \times 10^{-4}$	$(3 \pm 0.7) \times 10^{-4}$
171 (CCME A789-2)	ND	ND	ND	ND
584 (CCMEE Gobi 91-19)	ND	ND	ND	ND
ATCC 27900	ND	ND	—	—

<sup>a</sup> Gene transfer efficiencies are expressed as the numbers of transconjugants or electrotransformants per recipient cell. ND, the result was  $<10^{-6}$  for conjugative efficiencies and  $<10^{-8}$  for electroporation efficiencies; —, not determined. Values are the averages of three or five independent experiments ( $\pm$  the standard error).

<sup>b</sup> For the five strains obtained from the CCMEE, the CCMEE reference number is given in parentheses.

<sup>c</sup> Cargo plasmid.

<sup>d</sup> Conjugative plasmid.

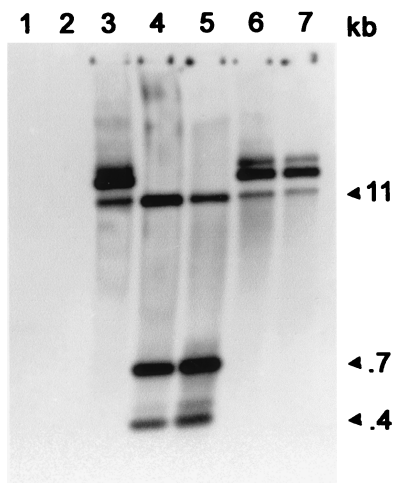


FIG. 2. Southern analysis of *Chroococcidiopsis* sp. strain 029 transconjugants (isolate CH91B1) obtained after mobilization of pDUCA7. The probe was labelled pDUCA7. Lanes: 1, 1-kb DNA ladder (LTI); 2, total DNA from *Chroococcidiopsis* sp. strain 029 (wild type); 3, total DNA from *Chroococcidiopsis* strain 029 isolate CH91B1; 4, total DNA from *Chroococcidiopsis* strain 029 isolate CH91B1 digested with *Pst*I; 5, authentic pDUCA7 digested with *Pst*I; 6, undigested pDUCA7 from *E. coli* DH10B transformed with plasmid DNA extracted from *Chroococcidiopsis* sp. strain 029 isolate CH91B1; 7, authentic pDUCA7.

pRK2013 and were analyzed through Southern analysis using pDUCA7 as the probe. Comparable results were obtained in independent trials with each of these isolates. Southern analysis was performed on total DNAs extracted from *Chroococcidiopsis* sp. strain CH91B1 and *Chroococcidiopsis* sp. strain 029 (wild type) and plasmid DNA from *E. coli* strain DH10B transformed with DNA extracted from *Chroococcidiopsis* sp. strain CH91B1. Hybridization signals were detected from total DNA of *Chroococcidiopsis* sp. strain CH91B1 (Fig. 2, lane 3), from plasmid DNA of *E. coli* strain DH10B transformed with DNA extracted from CH91B1 (Fig. 2, lane 6), and from authentic pDUCA7 (Fig. 2, lane 7). DNA extracted from wild-type cells of *Chroococcidiopsis* sp. strain 029 did not hybridize with the probe (Fig. 2, lane 2). After digestion with *Pst*I the same hybridization pattern was present in total DNA extracted from CH91B1 (Fig. 2, lane 4) and in the authentic pDUCA7 (Fig. 2, lane 5).

After digestion with *Pst*I the same pattern of bands was observed in the authentic plasmid pDUCA7 and plasmid extracted from *E. coli* strain DH10B transformed with extracts obtained from CH91B1 and CH91B2 (not shown). Restriction analysis and Southern analysis confirmed that pDUCA7 replicated autonomously and had not undergone deletion or rearrangement in transconjugants of *Chroococcidiopsis* sp. strain 029.

**Analysis of electrotransformants of *Chroococcidiopsis* sp. strains 029 and 123.** Identical restriction patterns were found for plasmid pDUCA7 (digested with *Pst*I) and pRL489 (digested with *Bam*HI) replicating in *E. coli* before and after passage through electrotransformants of *Chroococcidiopsis* sp. strains 029 and 123 (two independent isolates for each plasmid and strain were analyzed). Restriction analysis confirmed that pDUCA7 and pRL489 replicated in their original form in electrotransformants of *Chroococcidiopsis* sp. strains 029 and 123.

**Extracellular nucleases in *Chroococcidiopsis* spp.** Nuclease activities were detected in the extracellular fluids of all five *Chroococcidiopsis* sp. strains (Table 1) as a zone of clearing in agar medium containing DNA-methyl green. After a 10-day incubation, an almost complete clearing was produced by *Chroococcidiopsis* sp. strain 584 and by *Anabaena* sp. strain PCC 7120, used as the control, while weak nuclease activities were detected in *Chroococcidiopsis* sp. strains 029, 057, 123, and 171.

**Morphology of *Chroococcidiopsis* spp.** Despite an overall morphological similarity, the five desert strains of *Chroococcidiopsis* spp. (Table 1) differed in cell size, sheath thickness, and number of cells per aggregate. *Chroococcidiopsis* sp. strains 029 and 123 showed a similar morphology, while each of the *Chroococcidiopsis* sp. strains 057, 171, and 584 exhibited different morphologies (not shown). A thick, multilayered envelope surrounding the cells (Fig. 3) characterized 2-month-old cells of *Chroococcidiopsis* sp. strain 029.

**16S rRNA variable regions V6 to V8 and phylogeny of *Chroococcidiopsis* spp.** Nearly complete and partial 16S rDNA genes were amplified from *Chroococcidiopsis* sp. strains 029, 057, 123, and 171 using the primer combinations F2C-C, CYA106-C, and AC-C. High quality sequences were obtained from all the PCR products with sequence similarity to nucleotides 1 to 480 of 16S rRNA of *E. coli*; these were used for the phylogenetic analysis. The partial 16S rRNA gene sequences of the *Chroococcidiopsis* sp. strains were compared to those of *Chroococcidiopsis thermalis* PCC 7203 (EMBL accession no. z82789) and other cyanobacteria. The bottom of the unrooted tree (Fig. 4) is defined by the partial 16S rDNA sequence from *Chroococ-*

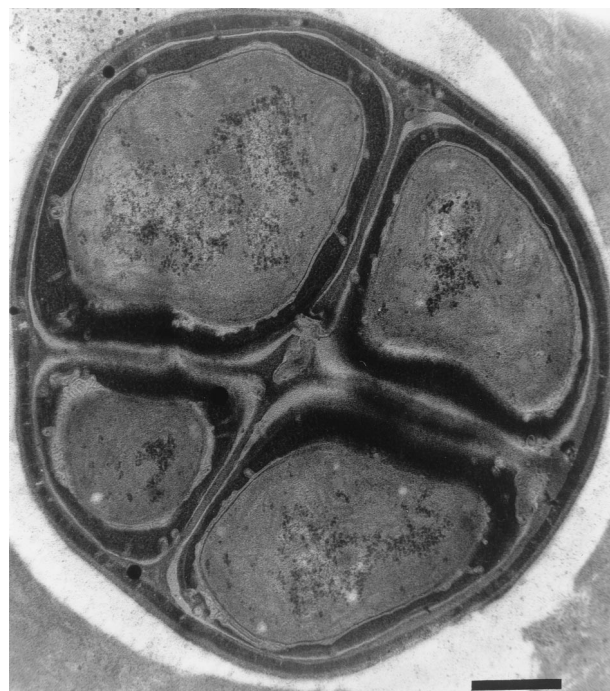


FIG. 3. Electron micrograph of ultrathin section of 2-month-old *Chroococcidiopsis* strain 029 showing multicellular aggregate containing cells of different sizes. An electron-translucent layer (white) and a fibrillar electron-dense envelope of capsular polysaccharide (grey) surround the aggregate. Bar = 0.5  $\mu$ m.

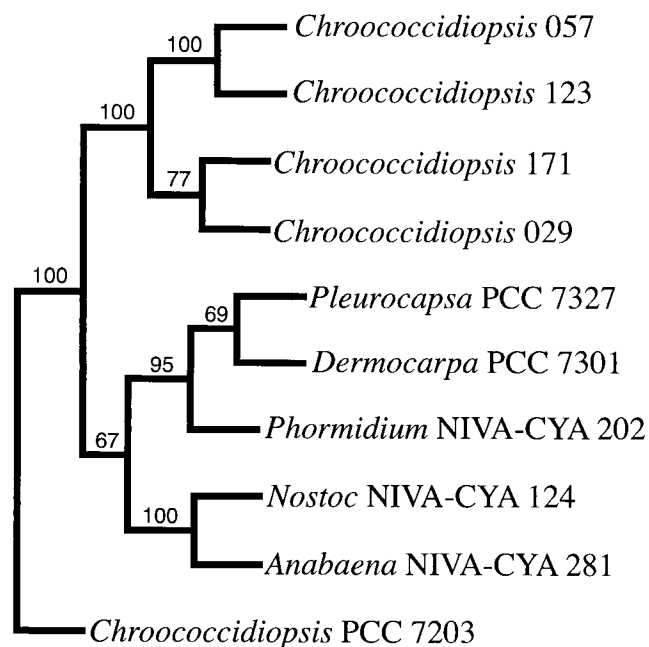


FIG. 4. Phylogenetic analysis of partial 16S rDNA. An unrooted consensus tree is shown. The numbers at the tree forks indicate the number of times out of 100 data sets that the strains to the right of the fork clustered (only numbers greater than 50 are shown).

*cidopsis* sp. strain PCC 7203. In contrast, the four *Chroococcidiopsis* sp. strains from the desert form a coherent, distant grouping, which was present in all of the 100 data sets.

## DISCUSSION

We provide the first report of genetic manipulation of *Chroococcidiopsis* spp. from hot and cold deserts and the first evidence that pDU1-based replicons can replicate in cyanobacteria of taxonomic section II (31). Until now vectors that include the pDU1 replicon of a plasmid of *Nostoc* sp. strain PCC 7524 (taxonomic section IV) had not been shown to replicate in strains other than *Anabaena* and *Nostoc* strains (35); IncQ plasmids such as pKT210 and pKT230 have been reported to replicate in a variety of unicellular cyanobacteria (37).  $P_{psbA}$  is a chloroplast promoter from *Amaranthus hybridus* that shares high sequence similarity with the consensus *E. coli*  $\sigma^{70}$  promoter and functions as a strong promoter in *Anabaena* sp. strain PCC 7120 (9). If *luxAB* gene expression in *Chroococcidiopsis* spp. is being driven by  $P_{psbA}$ , this promoter may have utility for expressing foreign genes in this cyanobacterium.

It is well known that a major problem in the genetic manipulation of cyanobacteria is the widespread distribution of restriction endonucleases (24). For several cyanobacteria, this problem is overcome by using *E. coli* strains which carry methylases to first modify plasmid DNA (12). Among the *Chroococcidiopsis* sp. strains that were amenable to conjugative transfer, strain 029 exhibited the highest frequency of transconjugation. Compared to *Chroococcidiopsis* sp. strain 029, the conjugative transfer of pDUCA7 via pRK2013 in strains 057 and 123 was 100-fold lower, while that of pRL489 via PRL443, was 10-fold lower. When pDUCA7 was mobilized by PRL443 instead of pRK2013 the efficiency of conjugative transfer

was reduced by at least 1 order of magnitude. The higher efficiency of the conjugative transfer of pDUCA7 via pRK2013 might reflect the compatibility between ColE1 *oriV* present on pRK1023 and RK2 *oriV* on pDUCA7. This allows both the conjugative and the cargo plasmids to replicate in the same *E. coli* donor and probably enhances the transfer frequency. The mobilization of pDUCA7 into *Nostoc* sp. strain PCC 73102 was at least 50-fold improved (relative to that of pRL443) by the use of either pRK2013 or pRK2073 (a  $Km^s$   $Sp^r$  derivative of pRK2013 [5, 6]). Despite the compatibility between the origins of replication of conjugative plasmid pRK443 and cargo plasmid pRL489, the efficiencies of its transfer in *Chroococcidiopsis* sp. strains 029, 057, and 123 were lower than those of pDUCA7 mobilized via pRK2013. Differences in the conjugative transfer of different plasmids in the same *Chroococcidiopsis* sp. strain and of the same cargo plasmid in different *Chroococcidiopsis* sp. strains are to be expected since the efficiency of gene transfer depends on both the cargo plasmid and the recipient strain (13, 38).

A correlation between the morphology of a given strain and its efficiency of conjugative transfer was not apparent; *Chroococcidiopsis* sp. strains 123 and 029 are morphologically identical but differ significantly in their transformability. The reduced conjugative efficiencies of *Chroococcidiopsis* sp. strains 057 and 123 may reflect the presence of host-specific restriction specificities. The low yield of plasmid DNAs extracted from *Chroococcidiopsis* transconjugants (see Materials and Methods) prevented us from testing whether it was possible to obtain electrotransformants from strain 057 using plasmid DNA extracted from transconjugants of this strain or to improve the transformation efficiencies of strains 029 and 123 by using plasmid DNAs extracted from the correspondent transconjugants. In *Anabaena* sp. strain PCC 7120, which contains isochizomers of *AvaI*, *AvaII*, and *AvaIII*, the efficiency of conjugative transfer decreases as an exponential function of the number of unprotected sites (11). The fact that conjugative transfer of pDUCA7 into *Chroococcidiopsis* sp. strains 029, 057, and 123 was unaffected by the absence of the helper plasmid pRL528 may suggest that *AvaI* and *Eco47II* restriction activities are not present in these three strains. Restriction did not pose a problem in the transformation of *Synechocystis* sp. strain PCC 6803, *Synechococcus* sp. strain PCC 7942 (10), or *Nostoc* sp. strain PCC 73102 (5, 6).

The role of cell envelope composition and structure has never been investigated as a possible barrier for conjugative transfer in cyanobacteria, although selection of conjugation-deficient recipient cells of *E. coli* yielded mutants which exhibited defects in the outer-membrane *OmpA* or in lipopolysaccharides (25). Cyanobacterial cell walls, despite their overall gram-negative structure, are characterized by a thicker peptidoglycan layer, by lipopolysaccharides containing a small amount of bound phosphate and often lacking ketodeoxyoctonate, and by the presence of external layers which differ in composition and structure (23). Extracellular polysaccharides and wall layers may prevent efficient conjugation by hindering cell-to-cell contacts. However, aged cells of *Chroococcidiopsis* spp. are characterized by a thickening of the cell envelope, yet no lowering of conjugative efficiencies was observed when late-stationary-phase 2-month-old (Fig. 3) *Chroococcidiopsis* sp. strains 029, 057, and 123 were used as recipients. The failure to

obtain transconjugants in *Chroococidiopsis* sp. strains 171 and 584 might be due to the presence of host-specific restriction endonucleases. Strains 171, 584, and 057 also failed to be transformed via electroporation, which may be due to the production of extracellular nucleases that may represent a barrier (see below).

Unlike conjugation, transformation through electroporation is prone to the problem of extracellular nucleases. The screening of over 150 strains of nostocacean cyanobacteria revealed that the great majority exhibited nuclease activity as demonstrated by the production of halos of clearing in agar medium containing DNA-methyl green (41). Moreover, a sugar-nonspecific nuclease was detected in cultures of *Anabaena* sp. strain PCC 7120 (27). In the present study, electrotransformation of *Chroococidiopsis* sp. strains 029 and 123 with either pDUC7 or pRL489 occurred at about  $10^{-4}$  per recipient cell, while no electrotransformants were obtained in strains 057, 171 and 584. The data presented here on the electrotransformation frequencies of *Chroococidiopsis* spp. may not be optimal because the same conditions were used for all the strains; settings of field strength and time constant which maximize DNA uptake while minimizing cell killing should be established for each cyanobacterial strain (10). The fact that all five *Chroococidiopsis* sp. strains are a source of extracellular deoxyribonuclease(s) suggests that further studies on gene transfer of these forms should rely on conjugation only. The inability to achieve gene transfer in *Chroococidiopsis* sp. strains 171 and 584 via electroporation and conjugation emphasizes that successful genetic manipulation of some cyanobacterial strains can be time-consuming; some cyanobacterial strains may indeed be recalcitrant to in vitro gene transfer. The evolutionary consequences of such recalcitrance in field populations of cyanobacteria remain poorly understood.

Through parsimony analysis of 16S rDNA sequences the strains of *Chroococidiopsis* (taxonomic section II [31]) from hot and cold deserts represent a coherent group that is divergent from representative strains of sections III (LPP group) and IV (Fig. 4). These *Chroococidiopsis* strains also appear to be distinct from *Chroococidiopsis* sp. strain PCC 7203, the proposed type strain of species *C. thermalis*. *Chroococidiopsis* sp. strain PCC 7203 was received at the Pasteur Culture Collection as *Myxosarcina chroococcoides* CCAP (1451/1) but was subsequently assigned to *Chroococidiopsis* on the basis of the production of immotile, not motile, baeocytes; in fact it grouped with heterocystous cyanobacteria in one phylogenetic analysis (40). The taxonomic status of *Chroococidiopsis* sp. strain PCC 7203 thus remains questionable (Fig. 4B). *Chroococidiopsis* sp. strain PCC 7203 is morphologically identical to *Chroococidiopsis* sp. strain 584 and conjugative gene transfer failed in both strains (not shown). Since *Chroococidiopsis* sp. strain 584 had the greatest sensitivity to ionizing radiation in a previous survey of 10 *Chroococidiopsis* strains, including strains 029, 057, and 171 (1), and was resistant to gene transfer, it was not studied further. The taxonomic assignment of *Chroococidiopsis* sp. strain 584 is currently under question. Notwithstanding, we observed no correlation between either morphology or position in a phylogenetic tree and capacity for gene transfer. Sequence analysis revealed 95.6% nucleotide conservation in *Chroococidiopsis* sp. strains 057 and 123, two strains amenable to conjugative gene transfer, but only the former was

transformable via electroporation. *Chroococidiopsis* sp. strains 029 and 123 were morphologically similar, showed 86.9% nucleotide conservation and exhibited a comparable suitability to electrotransformation, while efficiency of conjugative transfer of strain 123 was 10- to 100-fold reduced. In contrast, *Chroococidiopsis* sp. strains 029 and 171 shared 92.5% nucleotide conservation but only the former was suitable to gene transfer via conjugation and electroporation.

In conclusion, *Chroococidiopsis* sp. strain 029 from the Negev Desert, Israel, exhibited the highest efficiency in conjugative gene transfer and electrotransformation. Phylogenetic analysis suggests that this strain is representative of populations of *Chroococidiopsis* spp. from hot and cold deserts, and it offers promise as an experimental strain for the elucidation of mechanisms of desiccation tolerance.

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