JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 648-652
0021-9193/79/01-0648/05$02.00/0

Vol. 137, No. 1

Covalently Closed Circular DNAs in Closely Related Unicellular Cyanobacteria

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Received for publication October 1978

Plasmids of *Synechococcus cedrorum* and two *Anacytsis nidulans* strains were characterized physically, and a probable instance of spontaneous "curing" is described.

Several reports of the existence in cyanobacteria (blue-green algae) of covalently closed circular (CCC) DNAs, universally presumed to be plasmids, have appeared (1, 11; D. Heaton and E. W. Frampton, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, I117, p. 131; A. V. Morey and L. O. Krampitz, Abstr. Annu. Meet. Am. Soc. Microbiol., 1978, H123, p. 124; R. D. Simon, Abstr. Annu. Meet. Am. Soc. Microbiol., 1977, I105, p. 172). Only one of these reports (11) provides extensive physical characterization of such DNAs. None has identified plasmid-borne genetic determinants, and none has described either spontaneous or induced plasmid "curing." Techniques for genetic manipulation of cyanobacteria are not well developed (see reference 3 for review), and initial attempts to identify cyanobacterial plasmid function may perforce rely on the correlation of CCC DNA content and phenotypic variables such as antibiotic resistance (9), heavy-metal resistance, toxin production, and gas vacuolation (5, 19), all of which seem likely to be plasmid determined. It is therefore important that CCC DNA characterization be sufficiently complete to allow unambiguous identification of plasmids that appear to be common to a number of species. Here we present data on CCC DNAs in three *Synechococcus*-like (16) cyanobacterial strains that confirm their structural homology, and we document what appears to be an instance of spontaneous curing.

Anacystis nidulans strains WT and IU625 both derive from the isolate characterized by Kratz and Myers (8), now described as TX-20 by Myers, who considers it identical to the *Synechococcus* 6301 of Stanier et al. (16; J. A. Myers, personal communication). The former has been maintained at Dalhousie since 1967; the latter is kept independently in the Indiana University Culture Collection (17) and was obtained from E. W. Frampton, who identified in it a plasmid estimated, by sucrose density centrifugation, to be of 35.5×10^6 to 38×10^6 daltons (D. Heaton and E. W. Frampton, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, I117, p. 131; see also reference 1). *Synechococcus cedrorum* (IU1911) was obtained from M. M. Allen; it is virtually identical to *A. nidulans* in morphology, DNA guanosine-plus-cytosine (G+C) content (55.6 versus 55.1 mol%), and the ability to propagate cyanophage AS-1 (12, 16), but it does differ from *A. nidulans* in the kinetics of its absorption of this virus (14; M. M. Allen, personal communication).

Late-exponential-phase cells of each strain (15) were harvested by centrifugation, washed twice in TES {50 mM tris(hydroxymethyl)-aminomethane [Tris]-hydrochloride-5 mM ethylenediaminetetraacetate [EDTA]-50 mM NaCl, pH 8.0}, suspended in 50 mM Tris-hydrochloride (pH 8.0) containing 25% sucrose and 1 mM EDTA, treated with lysozyme (Worthington, 1.0 mg/ml) at 37°C for 1 h, chilled on ice for 5 min, and lysed by adding EDTA to 50 mM and sodium dodecyl sulfate to 1.0%. Lysates were made 1.0 M in NaCl, stored overnight at 4°C, and centrifuged for 35 min at 4°C and 17,300 × *g*. CCC DNAs were precipitated from the supernatant with polyethylene glycol (7), suspended in TES, clarified by low-speed centrifugation after addition of CsCl to 7.7 M (7), adjusted to 4.6 M CsCl and 160 μg of ethidium bromide per ml, and centrifuged at 36,000 rpm for 44 h at 20°C in a Beckman Ti-50 rotor. DNAs from all three strains showed two bands of UV-fluorescent material, the lower of which was recovered, desalted on columns of Sephadex G-25 equilibrated with TES, and freed of ethidium bromide by isoamyl-alcohol extraction before ethanol precipitation.

CCC DNA preparations were subjected to electrophoresis through 0.7% agarose horizontal gel slabs in a solution of 50 mM Tris-hydrochloride (pH 8.05), 2 mM EDTA, and 18 mM NaCl, using coliphages PM2 and ΦX174 RFI and plasmids RR1, 4A9, pMB9, pSC101, CKΔII, pML21, and pCR1 (gifts of D. Stoltz, O. Andrésson, and F. Fuller) as mobility markers. All three strains

exhibited plasmids determined by this method to be of approximately 27.5×10^6 daltons. CCC DNAs from *A. nidulans* IU625 and *S. cedrorum*, but not *A. nidulans* WT, showed in addition a second plasmid of 5.3×10^6 to 5.4×10^6 daltons.

Electron microscopic examination of CCC DNAs from the three strains, using methods of Davis et al. (2), revealed supercoiled and relaxed circular molecules, with no (<1%) contamination by linear fragments of chromosomal DNA. Contour-length measurements of relaxed circles (calibrated against DNA from phage PM2 [4]) yielded values corresponding to molecular weights of $30.5 \pm 0.8 \times 10^6$ and $30.3 \pm 0.5 \times 10^6$ for the large plasmids of the two *A. nidulans* strains and *S. cedrorum*, respectively. (Although differential stretching in formamide may introduce some error in comparisons of large plasmid to PM2 DNA, we nevertheless feel these values to be more accurate than those determinable by

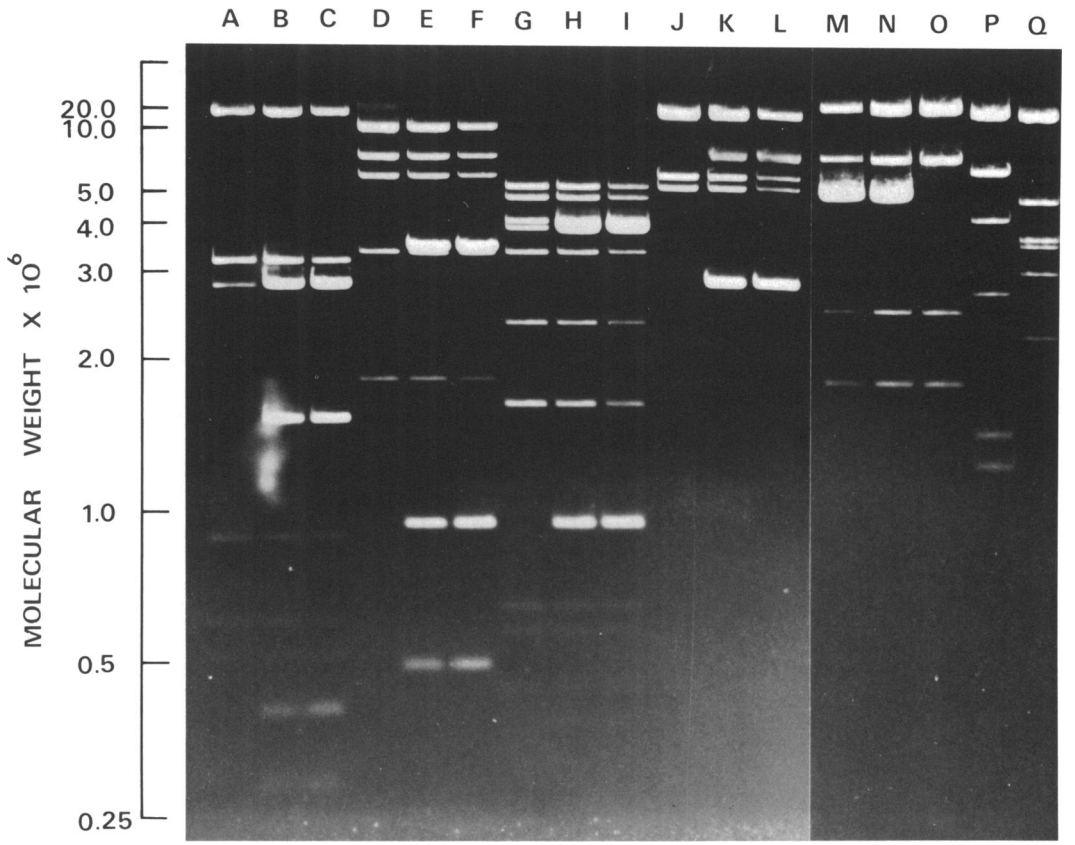

FIG. 1. *Products of digestion of cyanobacterial CCC DNAs by each of five restriction endonucleases. Digestion products were resolved on a 1.0% agarose slab gel, together with HindIII and EcoRI digests of λ DNA. A, B, and C show digests of A. nidulans WT, A. nidulans IU625, and S. cedrorum DNAs, respectively, and contain fragments of 15.5 × 10⁶, 3.15 × 10⁶, 2.75 × 10⁶, 0.9 × 10⁶, and 0.6 × 10⁶, and 0.5 × 10⁶ (sum 23.4 × 10⁶) daltons from the large plasmid and 2.85 × 10⁶, 1.55 × 10⁶, 0.4 × 10⁶, and 0.3 × 10⁶ (sum 5.1 × 10⁶) daltons from the small plasmid. C, D, and E show KpnI digests of the same DNAs, and contain fragments of 11.5 × 10⁶, 7.4 × 10⁶, 5.9 × 10⁶, 3.35 × 10⁶, and 1.75 × 10⁶ (sum 29.9 × 10⁶) daltons from the large plasmid and 3.5 × 10⁶, 0.9 × 10⁶, and 0.5 × 10⁶ (sum 4.9 × 10⁶) daltons from the small plasmid. G, H, and I show HindIII digests of the same DNAs, and contain fragments of 5.4 × 10⁶, 4.9 × 10⁶, 4.15 × 10⁶, 3.9 × 10⁶, 3.3 × 10⁶, 2.3 × 10⁶, 1.65 × 10⁶, 0.65 × 10⁶, 0.6 × 10⁶ and smaller (sum >26.9 × 10⁶) daltons from the large plasmid and 3.9 × 10⁶ and 0.95 × 10⁶ (sum 4.85 × 10⁶) daltons from the small plasmid. J, K, and L show SalI digests of the same DNAs, and contain fragments of 16.0 × 10⁶, 5.9 × 10⁶, and 5.2 × 10⁶ (sum 27.1 × 10⁶) daltons from the large plasmid. The small plasmid appears to contain no SalI sites, although some open circular molecules were generated during digestion. M, N, and O show XhoI digests of A. nidulans IU625, S. cedrorum, and A. nidulans WT, respectively, and contain fragments of 16.0 × 10⁶, 7.15 × 10⁶, 2.35 × 10⁶, and 1.65 × 10⁶ (sum 27.2 × 10⁶) daltons from the large plasmid and 4.8 (single site) × 10⁶ daltons from the small plasmid. P and Q show HindIII and EcoRI digests of λ DNA.*

gel electrophoresis of intact CCC DNA or summation of restriction endonuclease fragment sizes [Fig. 1].)

A. nidulans IU625 and *S. cedrorum* also showed small circles of lengths corresponding to $5.08 \pm 0.07 \times 10^6$ and $5.11 \pm 0.06 \times 10^6$ daltons, respectively. No comparable small molecules were seen in any preparation from *A. nidulans* WT, and it seems most likely that this strain has lost its small plasmid during repeated subculturing in our laboratory.

Restriction endonucleases *Bgl*I, *Kpn*I, *Hin*dIII, *Sal*I, and *Xho*I (purchased from New England Biolabs and handled as recommended by them) were used to digest completely CCC DNAs from each of the three strains, and the resultant products were resolved on 1.0% horizontal agarose slab gels together with *Eco*RI (6) and *Hin*dIII (18) digests of λ DNA as molecular weight markers (Fig. 1). Digestion patterns with each of the five nucleases for CCC DNAs of *A. nidulans* IU625 and *S. cedrorum* were identical,

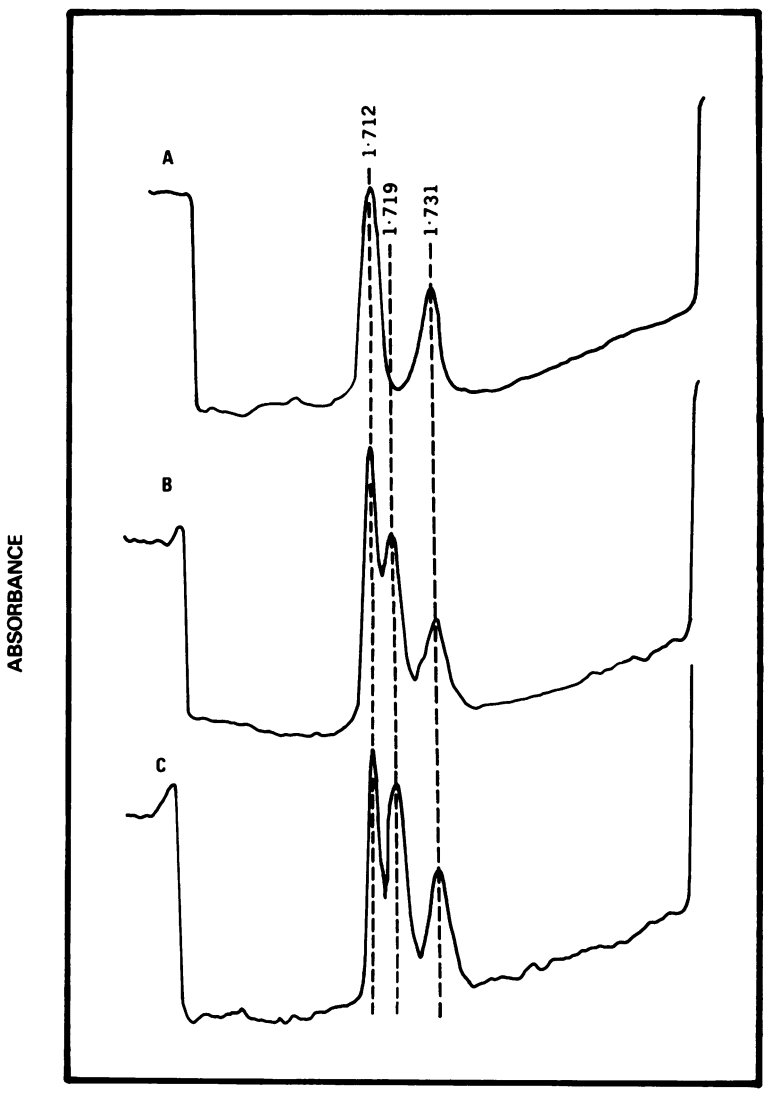

FIG. 2. *Analytical CsCl equilibrium centrifugation profiles, in the absence of ethidium bromide, of purified plasmid DNAs from A. nidulans WT (A), A. nidulans IU625 (B), and S. Cedrorum (C). M. lysodeikticus DNA (1.731 g/cm³) was included as a density marker.*

and it is reasonable to conclude that large and small plasmids in the two strains are homologous in sequence as well as in size. Digests of *A. nidulans* WT CCC DNA showed no new fragments but lacked certain bands observed with DNAs from the other two strains. We therefore assign these to the small (5×10^6 dalton) plasmid, and assume the large plasmid to be homologous to those of *A. nidulans* IU625 and *S. cedrorum*. Restriction fragment molecular weights and their sums are presented in the legend to Fig. 1. Sums are in most cases close to molecular weights determined by electrophoresis of intact plasmids or contour-length measurements, although possible loss of very small fragments or overlapping of fragments of similar mobilities can of course result in underestimation of molecular weights in such summations.

CCC DNAs from the three strains were analyzed by CsCl equilibrium centrifugation in the absence of ethidium bromide (Fig. 2), with *Micrococcus lysodeikticus* DNA ($\rho = 1.731$ g/cm^3; Sigma Chemical Co., St. Louis, Mo.) as standard. Buoyant densities and G+C contents were calculated from microdensitometer tracings of photographic negatives of UV-absorbing material (10, 13). Chromosomal DNAs from *A. nidulans* and *S. cedrorum* have buoyant densities of 1.714 to 1.715 g/cm^3 (16, and confirmed by us) and do not significantly contaminate the preparations analyzed here. *A. nidulans* IU625 and *S. cedrorum* show CCC DNAs of $\rho = 1.719$ (G+C content approximately 60.2 mol%) and $\rho = 1.712$ g/cm^3 (G+C content approximately 53.1 mol%), whereas CCC DNAs from *A. nidulans* WT contain only material of the latter density, which we therefore assume to correspond to the large plasmid present in all three strains.

These data provide the first extensive characterization of the CCC DNAs known for some time to exist in strains of *A. nidulans* (1) and show for the first time that a second, closely related, cyanobacterial species harbors plasmids homologous if not identical in structure. More interestingly, they suggest that our strain of *A. nidulans* (WT) has spontaneously lost a 5×10^6-dalton plasmid. We can as yet detect no difference in growth characteristics between the two *A. nidulans* strains, nor is there any difference in their sensitivities to salts of As, Cd, Co, Cr, Fe, Hg, Mg, Mn, Mo, Ni, Pb, Te, U, or Zn. That the small plasmid appears inessential for growth under laboratory conditions and contains only a limited number of sites for a variety of restriction endonucleases (Fig. 1) suggests that it might be a suitable vector for "self-cloning" in *A. nidulans*, one of the few cyanobacteria in which transformation has been unambiguously demonstrated (3). Even should our continuing attempts to identify a selectable phenotypic trait controlled by plasmid genetic determinants fail, it should be possible to select transformants bearing recombinant plasmids with inserted *A. nidulans* chromosomal DNA by complementation of any of a number of auxotrophic mutations already characterized for this strain (3).

This work was supported by grants from the Medical Research Council of Canada and by a research agreement with the Water Resources Research Support Program, Fisheries and Environment Canada.

We thank D. Stoltz for extensive help with electron microscopy, and C. W. Helleiner and S. Sangster for assistance with the analytical ultracentrifuge.

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