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Circular plasmid DNAs from the red alga *Gracilaria chilensis*

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Summary. Total cellular DNA extracted from eight red algal species (from the genera *Gracilaria, Gracilariopsis, Porphyra* and *Gymnogongrus*) was centrifuged on Hoechst dye/CsCl gradients. In five species, plasmid-like DNAs banded with the A+T rich organellar DNAs in the CsCl gradients. Based on their electrophoretic migration in different agarose gels, the plasmid-like DNAs are circular. This is the first report of putative plasmid DNAs in the red algae outside the genus *Gracilaria*. Two similar *Gracilaria chilensis* plasmid-like DNAs of 3.8 and 3.4 kb (GC2 and GC3) were cloned in pUC19. The cloned GC2 DNA did not hybridize to either the organellar or nuclear genomes of *G. chilensis*, suggesting that GC2 is a true plasmid. GC2 did hybridize to the plasmid of one other red algal species, *Gracilaria sordida*.

Key words: Rhodophyta – *Gracilaria* – Eukaryotic plasmids

Introduction

Several plants and fungi, as well as a slime mold, contain small linear or circular DNAs sharing no homology with sequences in the principal genome of the cellular compartment (nucleus or mitochondria) in which they are found (Guerineau et al. 1971; Collins et al. 1981; Gunge et al. 1981; Metz et al. 1983; Pring and Lonsdale 1985; Erickson et al. 1985; Bailey-Serres et al. 1987; Smith et al. 1987; Wahleithner and Wolstenholme 1987; Crouzillat et al. 1989). To be maintained in the cell, these DNAs, here referred to as plasmids, must contain sequences that can be utilized by the replication mechanism of the principal genome. Other than replication and recombination (Armstrong et al. 1988), little is known about eukaryotic plasmid function. Even though certain plasmids have been found in eukaryotic organisms with specific phenotypes (Gunge et al. 1981; Pring and Lonsdale 1985; Turpen et al. 1988; Shikanai and Yamada 1988), the relationships between plasmids and phenotypes are not understood.

Even less is known of plasmids in the algae. Several circular plasmids have been observed in three species of the red algal genus *Gracilaria* (*Gracilaria lemaneiformis, G. pacifica* and *G. robusta*) collected from the west coast of North America (Goff and Coleman 1988a, b). These DNAs are of unknown cellular location, range in size between 2 to 8 kb and comigrate with A+T rich plastid DNA in Hoechst dye/CsCl gradients. Plasmids such as these might be useful as eukaryotic vectors for introducing recombinant genes into the red algae. This paper reports the occurrence of plasmid DNA in eight red algal species as well as the cloning and the characterization of two similar plasmids from the red alga *G. chilensis*.

Materials and methods

Plant materials. Gracilaria tikvahiae was collected from the shore of Nova Scotia and grown in the laboratory in sterile sea water. *Gracilaria* cf *verrucosa, Gracilaria sordida* and *Gracilaria chilensis* (originally collected from Argentina, New Zealand and Chile, respectively) were grown in filtered sea water at the Atlantic Research Laboratory, Aquaculture Research Station at Sandy Cove, Nova Scotia. *Porphyra linearis, Porphyra miniata* and *Gymnogongrus* sp were collected from the shore of Nova Scotia. A putative species of *Gracilariopsis* was collected from the coast of England.

DNA extraction of red algae. Protocol I. Algal tissues (2–5 g) were cleaned by shaking vigorously in a bag with sea water, ground in liquid nitrogen and thawed in 50 ml of lysis buffer [20 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) sodium n-lauryl sarcosinate, 10 mM β-mercaptoethanol]. The extract was swirled on ice for 1 h, frozen at –70 °C for 1–2 h, and then thawed and centrifuged at 3000 *g* for 15 min. The pellet was ground in liquid nitrogen and combined with the supernatant. The extract was treated with 50 µg/ml of proteinase K (Sigma, St. Louis, Mo, USA) for 1 h at 37 °C with gentle agitation and extracted once with phenol and once with phenol/chloroform/isoamyl alcohol (50/49/1). The final aqueous phase was made 2.5 M in ammonium acetate and DNA was precipitated at –20 °C with 0.6 volume of isopropanol. Following centrifugation at 8000 *g* for 20 min, the pellet was dissolved in

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TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA (200–500 µg) was centrifuged at 40,000 rpm for 40 h in a Beckman 70.1 Ti rotor in a CsCl gradient at a homogeneous density of 1.60 with 40 µg/ml of Hoechst dye no. 33258 (Sigma; see Aldrich and Cattolico 1981). Presumptive mitochondrial and plastid DNAs (upper band) and nuclear DNA (lower band; Goff and Coleman 1988a) were visualized with ultraviolet light and removed from the tube by puncturing with a needle and removing bands with a syringe. The Hoechst dye was extracted with isopropanol equilibrated against NaCl-saturated H_2O, and the DNA solution was diluted three-fold and precipitated at –20 °C with two volumes of ethanol.

Protocol II. Tissues (5–100 g) were cleaned and ground as described above, thawed in solution A [0.23 M sodium phosphate pH 7.0, 10 mM EDTA, 1% (w/v) SDS (sodium dodecyl sulfate), 8 M urea] at 3 ml per gram of tissue, and extracted with phenol/chloroform/isoamyl alcohol. The aqueous phase was diluted with 3–5 volumes of solution B (8 M urea, 0.23 M sodium phosphate, pH 7.0). The extract was mixed with hydroxyapatite (Bio-Rad, Mississanga, Ontario, Canada, DNA grade, Bio-gel HTP) equilibrated with solution B (1 g of hydroxyapatite per g of tissues) and allowed to stand for 2–16 h at room temperature. The supernatant was decanted and the hydroxyapatite was washed with 200–500 ml of solution B and then 1–5 l of 0.1 M sodium phosphate, pH 7.0. DNA was eluted with 5–15 ml of 0.5 M sodium phosphate, pH 7.0. After an overnight dialysis against TE buffer, DNA was made 0.3 M in sodium acetate and precipitated with 2 volumes of ethanol at –20 °C. DNA was resuspended in TE buffer and centrifuged in a CsCl gradient as described above.

Electron microscopy. DNA was prepared for electron microscopy using the Kleinschmidt method (Coggins 1987). DNA spread on a cytochrome C monolayer (Sigma type V) was picked up on Butvar-coated 300 mesh EM grids. The grids were then stained with uranyl acetate, rotary shadowed with platinium/paladium (80/20) and examined with a JEOL 100 C electron microscope at 60 kv accelerating voltage. The circular plasmid vector pUC19 was used as a reference (Davis et al. 1986).

Enzymes and molecular cloning. The restriction endonucleases were from Pharmacia Piscataway, NJ, USA, and the T4 DNA ligase from Bethesda Research Laboratories (BRL, Burlington, Ontario, Canada). The *Escherichia coli* strains CES 201 and DH5α were obtained from Stratagene, La Jolla, CA, USA, and BRL, respectively. Cloning procedures, and the purification and analysis of recombinant DNAs were performed according to standard procedures (Maniatis et al. 1982; Davis et al. 1986).

Electrophoresis and hybridization. Electrophoresis was performed with 0.5–1.4% (w/v) agarose gels using TBE (89 mM Tris-base, pH 8.3, 89 mM boric acid, 2 mM EDTA) or TAE (40 mM Tris-acetate, pH 7.7, 2 mM EDTA) as buffers. *Sty*I restriction fragments of bacteriophage lambda DNA were used as a DNA molecular weight markers. DNAs were transferred to Zeta Probe membranes (Bio-Rad) (Southern 1975). Prehybridization was performed at 65 °C for 1–2 h in 0.5% (w/v) skim milk powder, 2 × SSPE (360 mM NaCl, 20 mM NaH_2PO_4, pH 7.4, 2 mM EDTA), 1% SDS, and 200 µg/ml of sonicated and denatured salmon sperm DNA. Hybridization was at 65 °C for 12–16 h in the same solution plus 10^6–10^7 cpm/ml of ^{32}P-labelled DNA probe. The probes were prepared by the random priming technique (Feinberg and Vogelstein 1983) at more than 10^8 cpm/µg. Membranes were washed with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS for 30 min at room temperature, and then twice for 30 min at 60 °C in 0.1 × SSC and 0.1% SDS, with agitation. The membranes were exposed (2 h to 7 days) to Kodak X-Omat AR film.

Results

Identification of red algal plasmid-like DNAs

Eight red algae (*Gracilaria chilensis*, *Gracilaria sordida*, *Gracilaria tikvahiae*, *Gracilaria* cf *verrucosa*, *Gracilariopsis* sp, *Gymnogongrus* sp, *Porphyra linearis* and *Porphyra miniata*) were examined for the presence of plasmid-like DNAs. Organellar (plastid and mitochondrial) DNAs from these algae were separated from nuclear DNA by Hoechst dye/CsCl gradient centrifugation and subjected to agarose gel electrophoresis. Plasmid-like DNA bands were detected in five of these species. In *Gymnogongrus* sp, two plasmid-like DNAs were observed (Fig. 1 A, lane 1). *Gracilaria chilensis* has four plasmid-like DNAs which were named GC1, GC2, GC3 and GC4, respectively (Fig. 1 B, lane 1). *Gracilaria sordida* has one major plasmid-like DNA (Fig. 1 B, lane 2), *Gracilaria tikvahiae* has a minimum of three (Fig. 1 B, lane 3) and *Porphyra miniata* has three (Fig. 1 B, lane 6). No plasmid-like DNAs were detected in *Porphyra linearis*, *Gracilaria* cf *verrucosa*, or *Gracilariopsis* sp DNAs (Fig. 1 A, lane 2; Fig. 1 B, lanes 4 and 5). This is the first indication that putative plasmids occur in red algae other than *Gracilaria*. These DNAs are A+T rich as all of them band in Hoechst dye/CsCl gradients close to the A+T rich organellar DNA (Aldrich and Cattolico 1981; Goff and Coleman 1988a; Manuelidis 1977).

The red algal plasmid-like DNAs are circular

The electrophoretic mobility of circular DNA molecules relative to linear ones is dependent upon the conditions of electrophoresis. In fact, agarose concentration, the ionic strength of the running buffer, and the strength of applied current can modify the mobility of circular DNA relative to linear DNA (Johnson and Grossman 1977). Results of the initial electrophoretic examination of DNA extracts for the occurrence of plasmid-like DNAs (Fig. 1 B) were obtained with 0.5% agarose gels and Tris-borate buffer (TBE). To determine whether these DNAs are circular or linear, a 1.2% agarose gel with a Tris-acetate buffer (TAE) was used (Fig. 1 C). All plasmid-like DNAs demonstrated mobilities (relative to the linear DNA markers) that were different from those observed in the 0.5% gels. For the *G. chilensis* and *G. tikvahiae* DNAs, the species which migrated at 3.8 kb in the 0.5% gel (Fig. 1 B, lanes 1 and 3), migrated at 4.4 kb in the 1.2% gel (Fig. 1 C, lanes 1 and 3). The difference is more evident with the larger DNAs. The major species found in the *G. sordida* DNA presumably migrated with high molecular weight DNA in the 1.2% agarose gel (Fig. 1 C, lane 2), instead of at 8.2 kb in the 0.5% gel (Fig. 1 B, lane 2). The plasmid-like DNAs in *G. tikvahiae* and *P. miniata*, which migrated at 5.5 and 6.7 kb respectively in the 0.5% gel (Fig. 1 B, lanes 3 and 6), migrated at 7.2 and 12 kb in the 1.2% gel (Fig. 1 C, lanes 3 and 6). The same phenomenon was observed with *Gymnogongrus* sp (data not shown). Based upon gel electrophoresis behavior, all the plasmid-like DNAs found in these algae are circular.

Further evidence for the circular nature of these DNAs comes from visualization by electron microscopy. A mixture of plasmid-like DNAs from *G. chilensis* (GC1–GC4) were purified by gel electrophoresis and electroelution and were examined by electron microscopy. Circular molecules were clearly visible (Fig. 2). Measurement of approximately 30 molecules revealed that they varied in size between 3.5 and 4.0 kb.

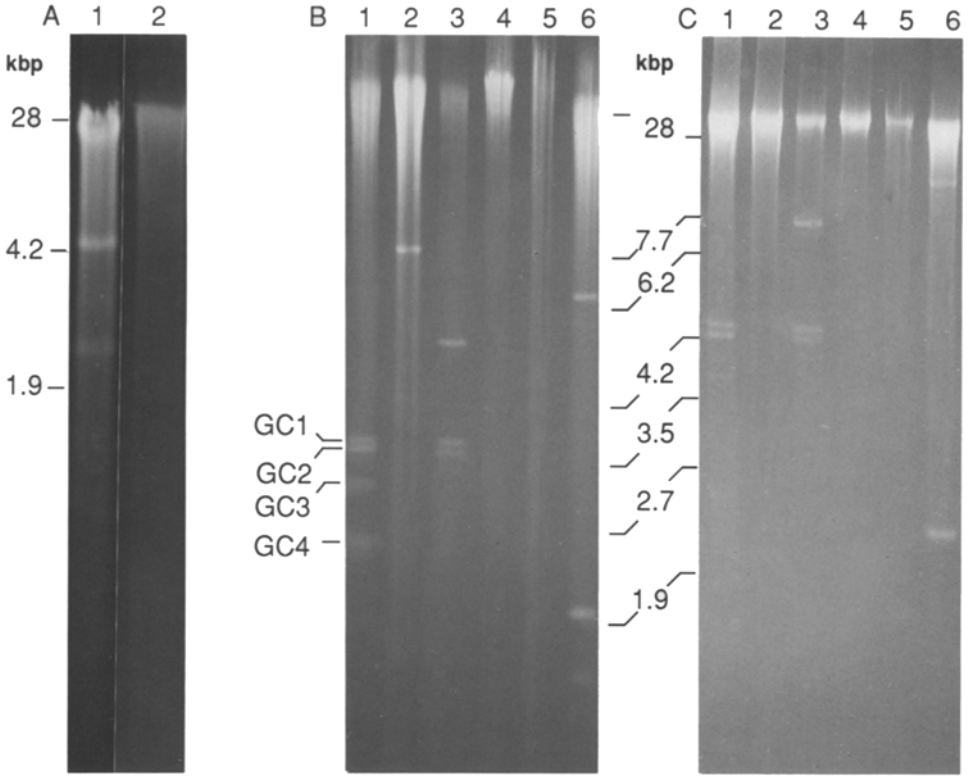

Fig. 1. Identification of plasmid-like DNAs in different red algae. Undigested organellar DNAs were subjected to agarose gel electrophoresis and stained with ethidium bromide. Gels were 0.5% agarose in TBE buffer (*A, B*), or 1.2% agarose in TAE buffer (*C*). *Panel A: lane 1, Gymnogongrus* sp.; *lane 2, Porphyra linearis. Panels B* and *C: lane 1, Gracilaria chilensis; lane 2, Gracilaria sordida; lane 3, Gracilaria tikvahiae; lane 4, Gracilaria* cf. *verrucosa; lane 5, Gracilariopsis* sp.; *lane 6, Porphyra miniata*

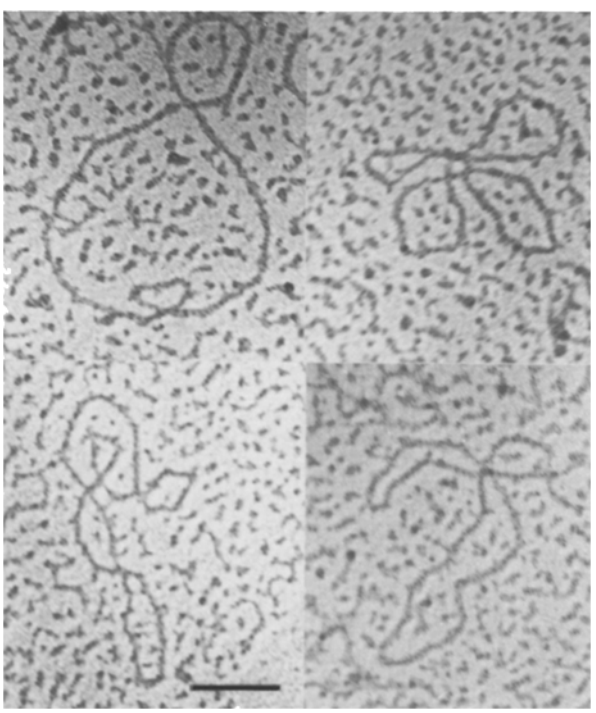

Fig. 2. Electron micrographs of *G. chilensis* plasmid-like DNAs. Bar = 100 nm

Molecular cloning of plasmid-like DNAs from Gracilaria chilensis

As a first step toward constructing a eukaryotic vector for red algae, it was decided to clone at least one of the putative plasmids described above. *G. chilensis* was chosen as the experimental organism due to the presence of multiple plasmid-like DNAs and ready tissue availability. In order to test these plasmids for restriction enzyme sites useful for cloning, it was necessary to first purify the DNAs by electroelution from agarose gels. Due to the difficulty of extracting DNA from red algae (because of polysaccharide contamination) and the inefficiency of electroelution, this procedure yielded only small amounts of purified DNA. Only 10–20 ng each of GC1 and GC2 and even less of GC3 and GC4 could be recovered from 10 g of *G. chilensis*. Also, the size difference between GC1 and GC2 is too small to resolve these two DNA species on agarose gels and electroelute them separately. For these reasons, restriction endonuclease digestions were made on the four electroeluted plasmid-like DNAs pooled together. Ten restriction endonucleases (*Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sph*I, *Sma*I, *Xba*I) were tested using 20–40 ng of DNA per digestion (data not shown). GC1 did not reveal to have any sites for these ten endonucleases; however, *Xba*I cleaved GC2 once, generating a 3.8 kb fragment, while *Bgl*II produced two fragments of 3.0 and 0.8 kb. The linear length of GC2 is in agreement with the length estimated from electron microscopy. Too little GC3 and GC4 were present for conclusive analysis.

In order to clone GC2, the electroeluted DNA preparation containing the four plasmid-like DNAs was digested with *Xba*I to linearize GC2 and then ligated into a *Xba*I-digested pUC19 vector. Two kinds of clones were obtained. When the type represented by pGC38 was digested with *Xba*I, only one fragment of 6.5 kb was gener-

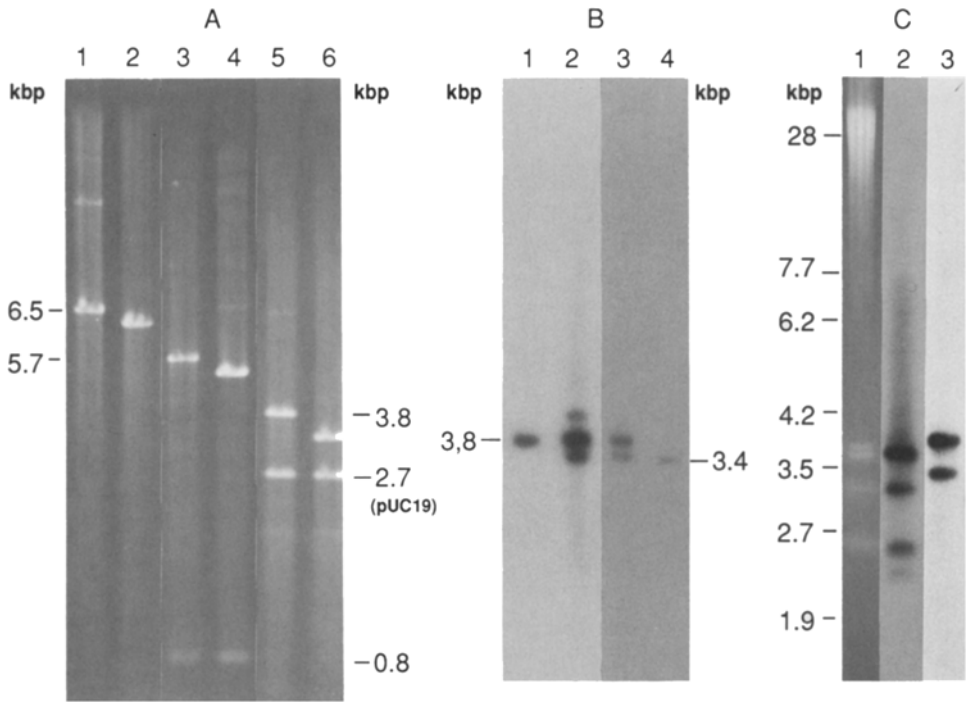

Fig. 3. Characterization of two recombinant plasmids carrying distinct *Gracilaria chilensis* plasmid-like DNA inserts. *Panel A:* agarose gel electrophoresis of restriction endonuclease digestions of pGC38 and pGC34. *Lanes 1, 3 and 5,* pGC38; *lanes 2, 4 and 6,* pGC34. DNAs were digested with *Xba*I (*lanes 1 and 2*), *Bgl*II (*lanes 3 and 4*) and *BamH*I + *Xba*I (*lanes 5 and 6*). *Panel B:* co-migration of the pGC38 and pGC34 inserts and restricted *Gracilaria chilensis* plasmid DNAs. pGC38 and pGC34, digested with *Xba*I + *BamH*I (*lanes 1 and 4*) to release insert DNAs, and electroeluted GC1-GC4 digested with *Xba*I (*lanes 2 and 3*), were subjected to agarose gel electrophoresis, transferred to a membrane and hybridized with the ^{32}P-labelled 3.8 kb insert from pGC38. The largest fragment in *lane 2* is probably undigested *G. chilensis* plasmid DNA. *Panel C:* undigested and digested *G. chilensis* organellar DNAs were subjected to agarose gel electrophoresis and transferred to a membrane. *Lane 1,* undigested DNA stained with ethidium bromide. *Lanes 2 and 3,* undigested and *Xba*I-digested DNAs respectively hybridized with the ^{32}P-labelled pGC38 insert

ated, instead of the expected 3.8 kb insert and the 2.7 kb linear pUC19 (Fig. 3A, lane 1). When digested with *Bgl*II, pGC38 generated at 5.7 kb fragment as well as the 0.8 kb fragment characteristic of GC2 (Fig. 3A, lane 3). Finally, when pGC38 was digested with *Xba*I and *BamH*I, the two expected fragments at 3.8 and 2.7 kb were generated (there is a *BamH*I site in the multiple cloning site of pUC19 and no *BamH*I sites in GC2; Fig. 3A, lane 5). It appeared, at this point, that a full length copy of GC2 was indeed present in pGC38, but that one of the two *Xba*I sites was resistant to digestion. DNA sequence analysis around the two *Xba*I sites in pGC38 revealed that both are intact but one of them overlaps a GATC sequence (data not shown). This site would be methylated when pGC38 is propagated in *Escherichia coli* DH5α and CES201 since these strains contain the *dam* methylase which methylates the adenine in the GATC sequence. A *Xba*I site (TCTAGA) which overlaps a methylated GATC sequence is known to be resistant to *Xba*I cleavage (Marinus and Morris 1973; Geier and Modrich 1979).

The second type of clone obtained, represented by pGC34, has some of the same characteristics as pGC38 (i.e., one of the *Xba*I sites is apparently methylated, and a 0.8 kb fragment is released upon digestion with *Bgl*II). However the pGC34 insert is 400 bp shorter than that of pGC38 (Fig. 3A, lanes 2, 4 and 6).

Analysis of pGC38 and pGC34

In order to clarify the relationship of pGC38 and pGC34 to each other and with the GC1-GC4 DNAs, Southern hybridization experiments were carried out. The 3.8 kb insert from pGC38 was hybridized to *Xba*I/*BamH*I-digested pGC38 (Fig. 3B, lane 1), *Xba*I-digested, electroeluted GC1-GC4 (Fig. 3B, lanes 2 and 3) and *Xba*I/*BamH*I-digested pGC34 (Fig. 3B, lane 4). The pGC38 insert hybridizes to itself, to the pGC34 insert and to two bands in the electroeluted DNA preparation. These two bands comigrate with the inserts from pGC38 and pGC34. These results indicate that *G. chilensis* contains at least two related plasmid-like DNA species of different sizes that may correspond to GC2 and GC3. Hybridization of the pGC38 insert to the undigested organellar fraction DNA from *G. chilensis* (Fig. 3C, lane 2) reveals that, in fact, four plasmid-like DNAs share homology with this insert. The first three hybridizing species, with apparent sizes of 3.7, 3.3 and 2.6 kb, co-migrate with GC2, GC3 and GC4 (Fig. 3C, lane 1) whereas the remaining species migrates as a 2.4 kb linear DNA. When *G. chilensis* organellar DNA is digested with *Xba*I and probed with the 3.8 kb insert from pGC38, only two fragments of 3.8 and 3.4 kb give positive signals (Fig. 3C, lane 3). These data indicate that the 2.6 and 2.4 kb species are probably the supercoiled versions of GC2 and GC3. These results are

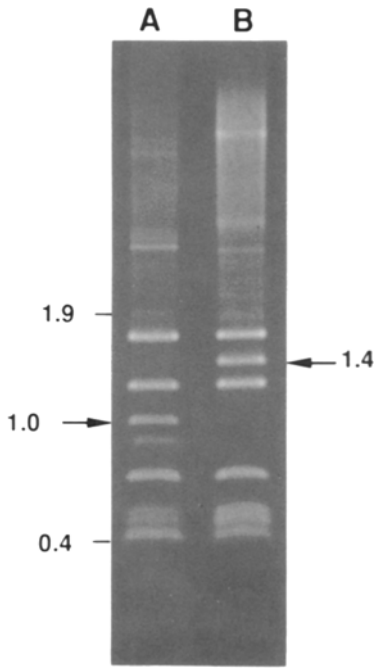

Fig. 4. Localization of the 400 bp deletion/addition difference between pGC38 and pGC34. 0.5 µg of pGC34 and pGC38 was digested with *Dra*I and subjected to agarose gel electrophoresis. *Lane A, Dra*I-digested pGC34; *lane B, Dra*I-digested pGC38. Numbers in kb. Fragment at 0.9 kb in *lane A* is the result of partial DNA digestion

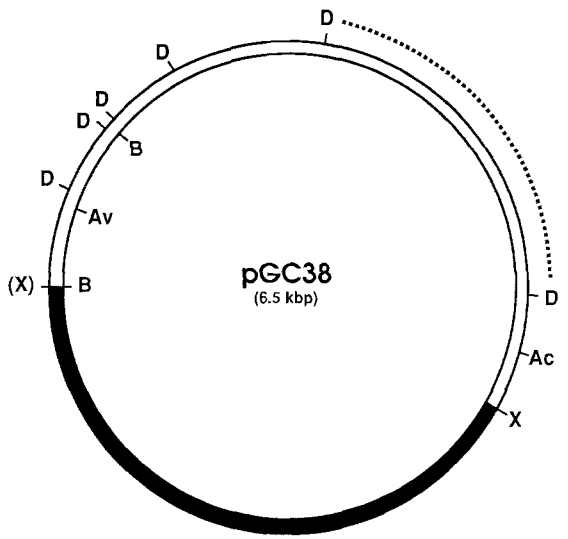

Fig. 5. Restriction endonuclease map of pGC38. *Filled area:* the 2.7 kb pUC19 vector. *Open area:* the 3.8 kb insert. *Dotted line* indicates the *Dra*I fragment that contains the 400 bp deletion in pGC34. *Ac: Acc*I, *Av: Ava*II, *B: Bgl*II, *D: Dra*I, *X: Xba*I, *(X) Xba*I site, presumably methylated. The restriction sites of pUC19 are not shown. The following restriction enzymes do not cleave pGC38: *Apa*I, *BamH*I, *Bcl*I, *Bgl*I, *Cla*I, *EcoR*I, *EcoR*V, *Hind*III, *Kpn*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Sph*I, *Stu*I, *Sty*I, *Xho*I

also consistent with the absence of any circular molecules less than 3.5 kb in the electron micrographs.

A *Dra*I digestion of clones pGC34 and pGC38 was done to determine in which part of the two clones the 400 bp difference is located (Fig. 4). The only substantial difference between pGC34 and pGC38 in the restriction pattern bands is a 1.0 kb fragment found in the *Dra*I-digested pGC34 instead of the 1.4 kb fragment found in *Dra*I-digested pGC38. Figure 5 shows the location of this 1.4 kb fragment on the restriction endonuclease map of pGC38.

Search for G. chilensis plasmid-like DNA sequences in the organellar and nuclear fractions of this alga and five other red algal species

Although GC2, GC3, GC4 and the 2.4 kb DNA of *G. chilensis* may be autonomously replicating molecules, it is possible that these DNAs are derived from a chromosomally-located master copy. Consequently, undigested organellar and nuclear DNA fractions from *G. chilensis* were probed with the pGC38 insert (Fig. 6). Even with long exposure times (1 week), no signals other than those to the plasmid-like DNAs were detected for either organellar (Fig. 6 B, lane 1) or nuclear DNA (Fig. 6 C, lane 2). Small amounts of contaminating plasmid-like DNAs appear to be present in the nuclear DNA fraction (Fig. 6 C, lane 2). As the hybridization conditions employed should be able to reveal a single copy in the high molecular weight genomes, it is unlikely that sequences homologous to the pGC38 insert are integrated into any of these genomes. Thus, GC2, GC3, GC4 and the 2.4 kb DNA of *G. chilensis* are likely true plasmids.

To determine whether the *G. chilensis* plasmids are related to the plasmid-like DNAs observed in *G. sordida, G. tikvahiae* and *P. miniata*, undigested organellar DNA fractions of these algae were hybridized with the pGC38 insert. The major plasmid-like DNA found in *G. sordida* strongly hybridized with the pGC38 insert (Fig. 6 B, lane 2), while the weakly hybridizing bands are probably other forms of this circular DNA. No hybridizing sequences were found in the two remaining species when high stringency conditions were used (Fig. 6 B, lanes 3 and 6). This hybridization was repeated at low strigency (42 °C hybridization, with washes in 2 × SSC at room temperature), and again, no hybridization to the plasmid-like DNAs of *G. tikvahiae* and *P. miniata* was seen (data not shown). In addition, no hybridization to the *Gymnogongrus* plasmid-like DNAs could be detected (data not shown). It is also noteworthy that the 3.8 kb probe failed to hybridize to the high molecular weight organellar DNA from any of these algae, including two species that lack plasmid-like DNAs (*G.* cf *verrucosa* and *Gracilariopsis* sp) (Fig. 6 B). The same results were observed with nuclear DNA from these red algae (data not shown).

Discussion

Agarose gel electrophoresis of undigested DNA from five red algal species revealed several different DNA species that migrated faster than the high molecular weight DNAs. These low molecular weight DNAs, which may be plasmids, were found in three *Gracilaria* species (*G. chilensis, G. tikvahiae* and *G. sordida*) and two other red algae (*Porphyra miniata* and *Gymnogongrus* sp). This is

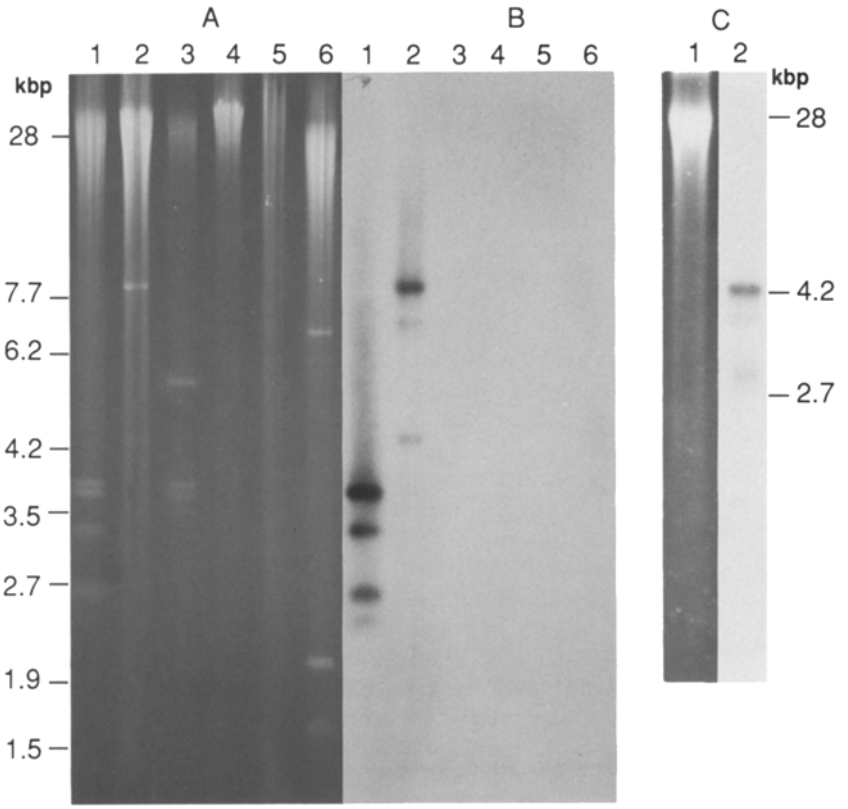

Fig. 6. Search for GC2 related sequences in organellar and nuclear *G. chilensis* DNAs and other red algal organellar DNAs. Undigested organellar and nuclear DNAs from *G. chilensis* and undigested organellar DNA from five other red algae were subjected to agarose gel electrophoresis and transferred to a membrane. *Panels A and B,* organellar DNA: *lane 1, Gracilaria chilensis; lane 2, Gracilaria sordida; lane 3, Gracilaria tikvahiae; lane 4, Gracilaria* cf *verrucosa; lane 5, Gracilariopsis* sp; *lane 6, Porphyra miniata. Panel C:* nuclear *G. chilensis* DNA. *Panel A, lanes 1–6* and *panel C, lane 1:* DNA stained with ethidium bromide. *Panel B, lanes 1–6* and *panel C, lane 2:* DNA hybridized with the pGC38 insert. *Panel A:* 0.7% agarose gel. *Panel C:* 1% agarose gel

the first observation of putative plasmids in red algal species other than *Gracilaria*. Based on their migration under different electrophoresis conditions, and the results of electron microscopy, all of these plasmid-like DNAs were shown to be circular. In addition, these plasmid-like DNAs banded at a high position in Hoechst dye/CsCl gradients, migrating close to the A+T rich organellar DNA. Thus, the nucleotide sequences of these DNAs must have a higher percentage of A+T nucleotides than the nuclear DNA. The observation that these DNAs migrated to positions close to those of organellar DNAs in CsCl gradients does not mean that they originate from these organelles. These plasmid-like DNAs could be A+T rich and still be located in the nucleus. Unfortunately, our DNA extraction methods do not allow the isolation of intact organelles or nuclei, and thus the organellar location of these plasmids has not been determined.

Agarose gel electrophoresis of *G. chilensis* DNA revealed at least four plasmid-like DNA entities (GC1, GC2, GC3 and GC4). An additional 2.4 kb plasmid-like DNA was detected by hybridization. Restriction digestion and hybridization experiments indicate that some of these DNA species represent different conformations of the same DNA molecule. GC4 and the 2.4 kb DNA are likely to be the supercoiled forms of GC2 and GC3, respectively. Thus, only three independent plasmid-like DNAs, GC1, GC2 and GC3, have been identified in *G. chilensis*. GC2 and GC3 were cloned in pUC19 as the recombinant plasmids pGC38 and pGC34, respectively. The inserts of these two plasmids are 3.8 and 3.4 kb long, and are highly similar since they strongly cross-hybridize. In fact, pGC34 shows the same restriction digestion pattern as pGC38, except that the insert in pGC34 is 400 bp shorter. Apparently, the *G. chilensis* plasmid-like DNAs belong to two distinct families, represented by GC1 and the pair of related plasmids GC2 and GC3. These two families must have completely different nucleotide sequences since no cross-hybridization was found between them. Unfortunately, no restriction sites were identified that would allow the cloning of GC1.

No evidence was found that the organellar or nuclear genomes of *G. chilensis*, and seven other red algae, contain sequences related to pGC38. Since GC2, GC3 and their supercoiled forms are unlikely to originate from those genomes, they must replicate autonomously to be propagated in the red algal cells. Therefore, they should have their own origin of replication, and may be considered true plasmids.

The *G. chilensis* plasmids GC2 and GC3 are not related to the putative plasmids found in other red algae, except that of *G. sordida*. Strong cross-hybridization was observed between the unique *G. sordida* plasmid-like DNA and pGC38. This result may not be unexpected since cross-fertility studies and restriction enzyme digestion analysis of plastid DNA suggest that *G. sordida* and *G. chilensis* belong to the same species (manuscript in preparation). The structural relationship between the cross-hybridizing *G. chilensis* and *G. sordida* plasmids has not been studied. It is possible that the larger plasmid-like DNA of *G. sordida* consists of both of the two types of *G. chilensis* plasmids (GC1 and GC2).

The plasmids identified in this study have characteristics similar to those found in three *Gracilaria* species studied by Goff and Coleman (1988 a). They are all circular, A+T rich molecules that migrate in agarose gels as if they were linear DNAs of 2–10 kb in size. Preliminary results from the same researchers revealed other similarities with this study (Goff and Coleman 1988 b). Two different plasmids cloned from *G. lemaneiformis* did not show any similarities with other *Gracilaria* plasmids or organellar and nuclear genome of different *Gracilaria* species. The presence of plasmids in *Gracilaria* species seems to be a common characteristic of this genus, although no such molecules were detected in *Gracilaria* cf *verrucosa*.

Further characterization of red algal plasmids is required to understand their function. These plasmids may give us the opportunity to isolate and characterize genes specific to red algal plasmids, and also to find and study a red algal origin of replication. With such an origin, it will be feasible to derive a vector that can be used to introduce recombinant genes into the red algae.

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