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Characterization of two circular plasmids from the marine diatom *Cylindrotheca fusiformis*: plasmids hybridize to chloroplast and nuclear DNA

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Summary. This paper reports the discovery and initial characterization of two small plasmids, pCf1 and pCf2, in the marine diatom *Cylindrotheca fusiformis*. Extracted diatom DNA separates into two bands in CsCl-Hoechst 33258 dye gradients. Upon agarose gel electrophoresis of a sample of the upper band of the gradient we observed, in addition to high molecular weight (genomic) chloroplast and mitochondrial DNA, pairs of lower molecular weight bands. These bands contained two species of circular plasmid DNA molecules, as shown by electron microscopy. The nucleotide composition of the plasmids, and chloroplast and mitochondrial DNAs is similar, as indicated by their co-banding in the gradients. They were cloned, and their restriction maps determined, showing that pCf1 is 4.27 and pCf2 4.08 kb in size. By hybridization analysis, we showed that pCf1 and pCf2 share regions of similarity, but not identity. Neither plasmid hybridizes with mitochondrial DNA. Both plasmids hybridize with chloroplast DNA, and pCf2 also hybridizes with nuclear DNA.

Key words: Diatoms – *Cylindrotheca fusiformis* – Plasmids – Hybridization

Introduction

Plasmids have been identified in a large number of eucaryotic organisms (see Esser et al. 1986 for a review). Many eucaryotic plasmids have been localized in organelles, or share hybridizing sequences with organellar genomic DNA (Belcour et al. 1981; Bertrand et al. 1980; Ebert et al. 1985; Green 1976; Heizmann et al. 1982; Kemble and Mans 1983; Lazarus et al. 1980; Levings et al. 1980; Turmel et al. 1986). However, this most commonly occurs with mitochondrial DNA, and only rarely with chloroplast DNA. The presence of plasmid-hybridizing sequences in organellar genomic DNA may have occurred by integration of the plasmid sequences into the genome, or the plasmids may have arisen from genomic sequences, leaving a duplicate portion of the sequence in the genome. These events would require recombination or transposition. Such processes are essential for shaping genome structure. A thorough characterization of organellar plasmids and their interactions with genomic DNA should be valuable for understanding the evolution of organellar genomes.

We have studied silicon metabolism in the marine pennate diatom *Cylindrotheca fusiformis* for a number of years (Darley and Volcani 1969; Sullivan and Volcani 1973; Borowitzka and Volcani 1977; Okita and Volcani 1978, 1980; Aline et al. 1984; Reeves and Volcani 1985). Extending our work by adopting a molecular biological approach has required developing procedures for isolating and manipulating nucleic acids from *C. fusiformis*. To our surprise, upon extraction of DNA from this organism, several pairs of lower molecular weight bands were observed in addition to high molecular weight genomic DNA, by agarose gel electrophoresis. In this paper, we identify these as two small circular DNA plasmids, called pCf1 and pCf2. We describe the cloning and characterization of the plasmids, and show that they are similar to each other and hybridize to chloroplast and nuclear DNA.

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Materials and methods

Enzymes and reagents. Restriction endonucleases, T4 DNA ligase, *Escherichia coli* DNA polymerase I, and *E. coli* DNA polymerase I large fragment (Klenow) were from Pharmacia Molecular Biologicals (PMB), Piscataway, NJ or Bethesda Research Laboratories (BRL), Gaithersburg, Md. The 1 kb ladder DNA size marker, lambda phage DNA, and Elutip columns were from BRL, and the 10 bp *Xho* I linker, unlabeled nucleotides, and O-ligolabeling (random priming) kit were from PMB or BRL. NucTrap columns were from Stratagene, and radioactively labeled nucleotides and Hybond nylon membrane were from Amersham, Arlington Heights, Ill. We obtained Hoechst dye 33258 from Calbiochem-Behring, La Jolla, Calif., the Prep-A-Gene DNA purification kit from BioRad, Richmond, Calif., protease Type XIV, proteinase K (P–0390), RNase A, RNase T1, DNase I, and agarase from Sigma Chemical Co., St. Louis, Mo., and Bacto-Tryptone from Difco Laboratories, Detroit, Mich. All other reagents and chemicals were obtained from standard chemical sources. Enzymes were used under conditions recommended by the supplier or other published sources (Maniatis et al. 1982).

Culture conditions and organelle isolation procedures. Maintenance and mass culturing of *C. fusiformis* were as previously described (Darley and Volcani 1969; Paul and Volcani 1976) except that greater yields of plasmids were obtained by adding 1 g/l Bacto-Tryptone to the artificial sea water medium and allowing cells to grow to stationary phase (approximately 4.5×10^6 cells per milliliter culture). Cells were harvested using an IEC PR–6000 centrifuge at 3200 rpm and 4° C for 15 min. Yield was approximately 1.2 g wet weight (0.23 g dry weight) of cells per liter of culture.

DNA extraction and purification. Two methods were used to extract total DNA from *C. fusiformis* cells. The first method was derived predominantly from Ullrich et al. (1980), and Specht et al. (1982), and involved lyophilization of cells followed by grinding and gentle lysis at room temperature in a toluene/SDS solution. DNA extracted in this manner was purged of RNA and protein by RNase A and protease Type XIV treatments (Bowman and Dyer 1982). The yield was approximately 70 µg DNA/g wet weight of cells. The second method was modified from a procedure used to isolate DNA from cells of *Chlamydomonas reinhardtii* (Method I, p 256, Rochaix et al. 1988). After harvesting, diatom cells were washed once with a 3.5% NaCl solution, and the cell pellet was quick-frozen in a dry ice-isopropanol bath and stored at −80° C. Cells were thawed to room temperature, and resuspended in 4.5 ml (per 1–2 g wet weight cells) 20 mM TRIS–HCl, pH 8.0, 100 mM NaCl, 50 mm Na_2EDTA. Then 250 µl of 20% SDS was added and mixed, followed by 250 µl of 10 mg/ml proteinase K, and the mixture was placed at 50° C. After 45 min, an additional 250 µl of proteinase K solution was added, and this was repeated after an additional 45 min. After a further 45 min of proteinase K digestion the solution was extracted twice with 8 ml water-saturated phenol, layers were separated by centrifuging at 16500 g, and 20° C, for 10 min in a Sorvall HB–4 rotor. Nucleic acids were precipitated upon the addition of 0.1 vol. 3 M sodium acetate, (pH 5.2) and 2 vol. ethanol, pelleted (Sorvall HB–4 rotor, 15 min, 20° C, 16500 *g*), washed twice with 70% ethanol (same centrifugation conditions), vacuum dried, and resuspended in 1 ml water. After RNase treatment, the average yield of DNA was 200 µg/g wet weight cells. Both methods of DNA extraction were used to produce data for this paper, however, the SDS-proteinase K method has become our preferred choice because of its simplicity, speed, and higher DNA yield.

Nucleic acids obtained by both methods were separated into components on gradients consisting of a 50% (w/w) CsCl solution containing up to 100 µg/ml nucleic acids and 10 µg/ml Hoechst 33258 dye (Muller and Gautier 1975; Manuelidis 1977). Centrifugation was in either a Beckman Ti60 rotor at $110000 \times g$ (42000 rpm), and 18° C, for 72 h, or a Beckman VTi65 rotor at $267000 \times g$ (55000 rpm), and 18° C, for 18 h. Longer centrifugation times or recentrifugation of isolated bands resulted in better resolution of the components on the gradients. DNA bands were removed from the gradients with an 18 gauge needle and syringe; dye was removed by repeated extraction with water-saturated *n*-butanol, and CsCl removed by dialysis at 4° C for 24 h against several changes of TE buffer (10 mM TRIS–HCl, pH 8.0, 1 mM Na_2EDTA).

Mitochondria were isolated according to Paul et al. (1975), and SDS-proteinase K treatment was used to extract mitochondrial DNA (M. Hildebrand et al., unpublished data).

Plasmid DNAs were isolated from *E. coli* using the method of Holmes and Quigley (1981).

Electron microscopic analysis of plasmid DNA. Diatom plasmid DNA molecules were extracted from low gelling temperature agarose gels (1.2%), as described by Wieslander (1979). Spreading of grids and shadow casting were performed as described by Reymond et al. (1980). Grids were examined with a Zeiss EM9S–2 transmission electron microscope.

Construction of cloning vectors. Plasmid pUC18X (constructed by Dr. Gail Baughman) was constructed by linearizing pUC18 (Yanisch-Perron et al. 1985) with *Hin*dIII, filling in the ends using a mixture of deoxynucleotides and the Klenow fragment of DNA polymerase I, and ligating a 10 bp *Xho*I linker onto these blunt ends. This construct retained β-galactosidase activity. Plasmid pKan18 was constructed as follows. Plasmid pUC18 was linearized by partial *Hae*III digestion and the ends were made blunt with the 3′ to 5′ exonuclease activity of Klenow fragment. A *Sal*I-*Hin*dIII restriction fragment containing the kanamycin resistance gene from pKC7 (Rao and Rogers 1979) was gel purified and the ends filled in as above. The resulting blunt-end fragment was ligated to *Hae*III digested, blunt-ended pUC18. Transformants containing this construction exhibited β-galactosidase activity and both ampicillin and kana-

mycin resistance. The *Sma*I site was removed from the inserted fragment by linearizing the construct by partial digestion with *Ava*I, and filling in the ends as described above. After recircularization of the plasmid, transformants were selected that retained β-galactosidase activity, and ampicillin and kanamycin resistance. pKan18 was selected from transformants that had lost the non-polylinker *Sma*I site, but retained the polylinker *Sma*I site.

Cloning of pCf1 and pCf2. Plasmid pCf1 was isolated from an agarose gel, digested with *Xho*I, then passed through an Elutip column (BRL). The purified DNA was ligated to *Xho*I – digested pUC18X. The resulting construct was designated pJDJ1 (Fig. 3). Gel-purified plasmid pCf2 was digested with *Bgl*II, and ligated to *Bgl*II – linearized pKan18. Two constructs were obtained, containing pCf2 in opposite orientations, pJDJ4 (Fig. 3), and pJDJ8 (not shown).

Restriction mapping. Initial restriction mapping was performed on gel-purified native plasmids and detailed maps were derived from the cloned material using double and reciprocal digestion techniques. Identical restriction endonucleases were used for both plasmids.

DNA hybridization. Standard procedures were used for agarose gel electrophoresis (Dretzen et al. 1981) and DNA transfer (Southern 1975) to nylon membranes. Inserts from pJDJ1 and pJDJ4, corresponding to pCf1 and pCf2, respectively, were isolated after electrophoresis by electroelution or by using Prep-A-Gene. Hybridization probes were synthesized from these inserts by the random priming technique (Feinberg and Vogelstein 1983, 1984), using a kit from BRL.

Hybridizations were performed at 42° C for 18–36 h in either 5 × SSPE (1 × SSPE consists of 180 mM NaCl, 10 mM $NaPO_4$, pH 7.7, 1 mM Na_2EDTA), 25% deionized formamide, 0.5% nonfat milk powder, and 0.1% SDS, or in 6 × NET (1 × NET consists of 150 mM NaCl, 15 mM TRIS–HCl, pH 8.0, 1 mM Na_2EDTA), 25% deionized formamide, 5 × Denhardt's solution (Denhardt 1966), 0.1% SDS, and 200 μg/ml degraded herring sperm DNA (Sigma D–3159). Washes were performed in either 2 × or 0.5 × SSC (1 × SSC consists of 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS at the temperatures indicated in the text.

Purification of upper band DNA free of plasmids. Undigested upper band DNA was electrophoresed on a 0.5% agarose gel. Gel slices containing high molecular weight DNA free of plasmids were isolated and treated with agarase (Sambrook et al. 1989). After overnight treatment, 210 μl samples were passed in 70 μl aliquots over a NucTrap column to remove residual oligosaccharides. Samples were then concentrated by ethanol precipitation.

Results

Isolation of DNA and identification of plasmids

Total DNA was extracted from 7–8 day cultures of *C. fusiformis,* and subjected to CsCl-Hoechst 33258 dye gradient centrifugation. The DNA separated into two bands in the gradients. The upper band of the gradient hybridized to chloroplast-specific (*rbcL* and *psbA*) and mitochondrial-specific (*cox2*) probes and to a 42 kb circular DNA from purified mitochondria (M. Hildebrand et al., unpublished results). These results suggest that the upper band contains both chloroplast and mitochondrial DNA. The lower band of the gradient hybridized to a nuclear-specific (*fcp1*) probe, suggesting that it contains nuclear DNA (M. Hildebrand et al., unpublished results). No contamination of the upper band by the lower band was detectable by hybridization analysis (M. Hildebrand et al., unpublished results). Upon long autoradiographic exposure very slight amounts of contamination of the lower band with the upper band were detectable. Thus, the upper band contains pure chloroplast and mitochondrial DNA and the lower band contains mostly nuclear DNA, with very slight contamination by chloroplast and mitochondrial DNA.

Samples of the upper and lower bands and total DNA were electrophoresed on an agarose gel (Fig. 1). Especially apparent in the upper band gradient samples (Fig. 1, lane 4) were three pairs of lower molecular weight bands, distinct from high molecular weight DNA. These bands were less apparent in the total DNA sample (lane 2), and barely visible in the lower band sample (lane 3). The most slowly migrating forms of these bands were extracted from agarose gels, and were shown by electron microscopy to consist of circular DNA molecules (Fig. 2). Contour lenghts were 1.4 and 1.3 μm (4.4 k and 4.1 kb, respectively). This corresponds in size to the intermediately migrating set of two bands on agarose gels (Fig. 1, lane 4, middle pair of arrows), which we assume are linear molecules. Supercoiled forms were also present (Fig. 1, lane 4, lowermost arrows). These plasmids (extrachromosomal genetic elements) were designated pCf1 (higher molecular weight) and pCf2 (lower molecular weight).

Cloning and restriction mapping of the plasmids

Two restriction endonucleases, *Xho*I and *Bgl*II, were found to digest pCf1 and pCf2, respectively, at a single site (Fig. 1, lanes 6 and 5). After isolation from agarose gels and linearization, the plasmids were cloned using pUC-derived vectors (see Materials and methods for details). The clones (Fig. 3) were designated pJDJ1 (pCf1 insert) and pJDJ4 (pCf2 insert). The identity of the cloned material with native plasmid was confirmed by hybridization analysis [Fig. 5B, lane 3 (pCf1), and 5C, lane 3 (pCf2)].

Initial data for restriction mapping were obtained using the upper band fractions from CsCl-Hoechst 33258 gradients. These were augmented with data from digests

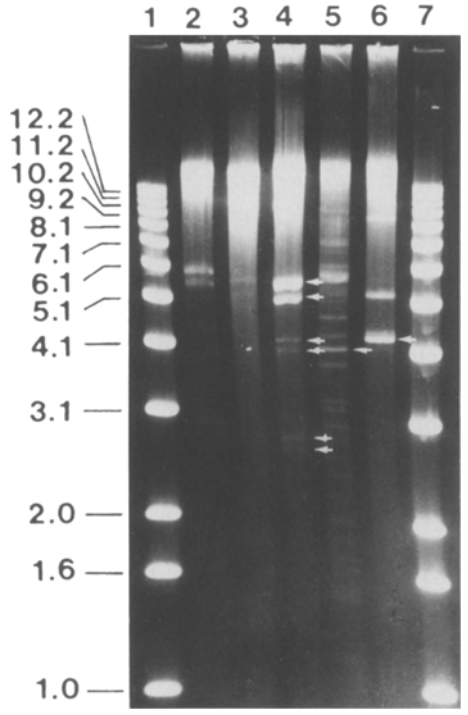

Fig. 1. Agarose gel electrophoretic separation of isolated diatom DNA. Photograph of an ethidium bromide-stained 1.2% agarose gel. Lanes 1 and 7, 1 kb ladder (BRL) molecular weight markers (sizes in kb are indicated to the left of the gel photograph); lane 2, total DNA extracted from *Cylindrotheca fusiformis;* lane 3, a sample of the lower band resulting from CsCl-Hoechst 33258 gradient separation of total DNA; lane 4, a sample of the upper band from the same gradient; lane 5, *Bgl*II digest of an upper band sample; and lane 6, *Xho*I digest of an upper band sample. *Arrows* adjacent to lane 4 mark the positions of open circular (slowest migrating), linear (intermediate migration), and supercoiled (fastest migrating) forms of pCf1 (uppermost of each pair) and pCf2 (lowermost of each pair). The *arrow* adjacent to lane 5 marks the position of linearized pCf2. The *arrow* adjacent to lane 6 marks the position of linearized pCf1

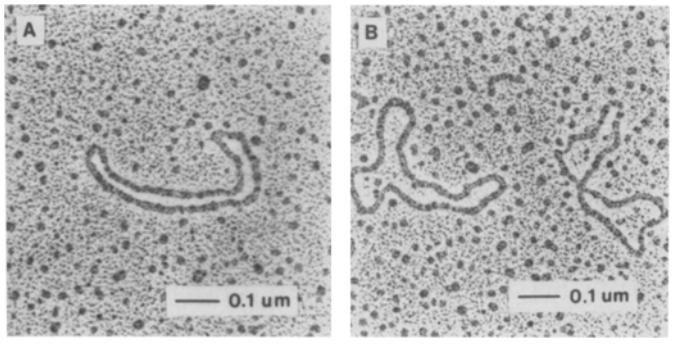

Fig. 2A–B. Electron micrographs of diatom plasmids. The open circular forms of the plasmids were isolated from an agarose gel and spread for electron microscopy. **A** pCf1. **B** pCf2. Magnification 35000×

of plasmid bands that had been isolated from agarose gels. Confirmation of the initial data and more detailed mapping was performed using the cloned material. The completed restriction maps are shown in Fig. 3. The size

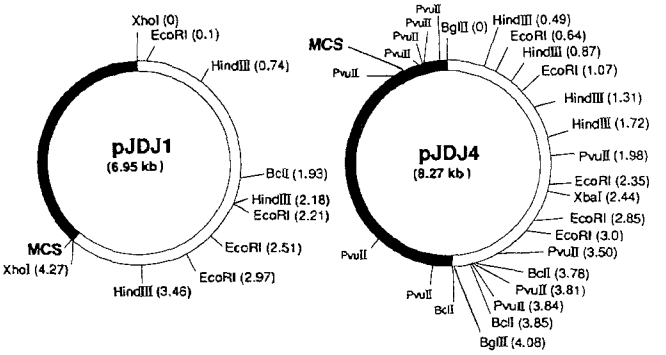

Fig. 3. Restriction maps of chimeric plasmids pJDJ1 and pJDJ4. Sizes in kilobase pairs (kbp) are in *parentheses* in the *center of the circles. Numbers in parentheses* adjacent to restriction endonuclease names indicate the distance in kilobase pairs relative to the unique *Xho*I site in pCf1 and the unique *Bgl*II site in pCf2. The *dark* portion of each map corresponds to the pUC-derived vector portion of the constructs. MCS, Multiple Cloning Site

of the plasmids as determined by restriction mapping was estimated as 4.27 kb for pCf1 and 4.08 kb for pCf2, in good agreement with the data obtained by electron microscopy.

Hybridization between the two plasmids

To determine whether the two plasmids are similar, blot hybridization experiments were performed (Fig. 4). pJDJ1 and pJDJ4 were digested to produce linearized insert (lane 1, band 1, and lane 3, band 1, respectively) and subfragments (lanes 2 and 4; see figure legend for restriction endonucleases used). The blots were hybridized under identical conditions, but washing stringency was varied by altering the temperature and/or the concentration of SSC. At 45° C and 2 × SSC, hybridization is observed between the two plasmids (Fig. 4A, lane 3, band 1 and 4B, lane 1, band 1). In Fig. 4A, lane 4, pCf1 hybridizes most strongly to a 410 bp *Hin*dIII fragment (band b), less strongly to a 630 bp *Hin*dIII-*Eco*Rl fragment (band a), and least strongly to a 240 bp *Hin*dIII-*Eco*Rl fragment (band c) in pCf2. The three hybridizing fragments are adjacent to each other on the pCf2 restriction map (Fig. 4A, lower). As washing temperature is increased the intensity of hybridization decreases differentially with regard to fragment. At 65° C in 0.5 × SSC (Fig. 4A, upper, lanes 3 and 4) virtually no hybridization is seen between the plasmids (the faint hybridization that is visible is due to slight contamination of the probe with vector sequences). Using pCf2 as a probe (Fig. 4B, upper), we observed three hybridizing fragments of decreasing intensity in pCf1; a 810 bp *Hin*dIII-*Xho*I fragment (band d), a 490 bp *Hin*dIII-*Eco*Rl fragment (band f), and a 640 bp *Hin*dIII-*Eco*Rl fragment (band e). These three fragments lie in the same region of pCf1 (Fig. 4B, lower); a smaller *Xho*I-*Eco*Rl fragment in this region either does not hybridize or was electrophoresed off the gel before blotting. These results show there are regions of similarity, though not identity, between the two plasmids.

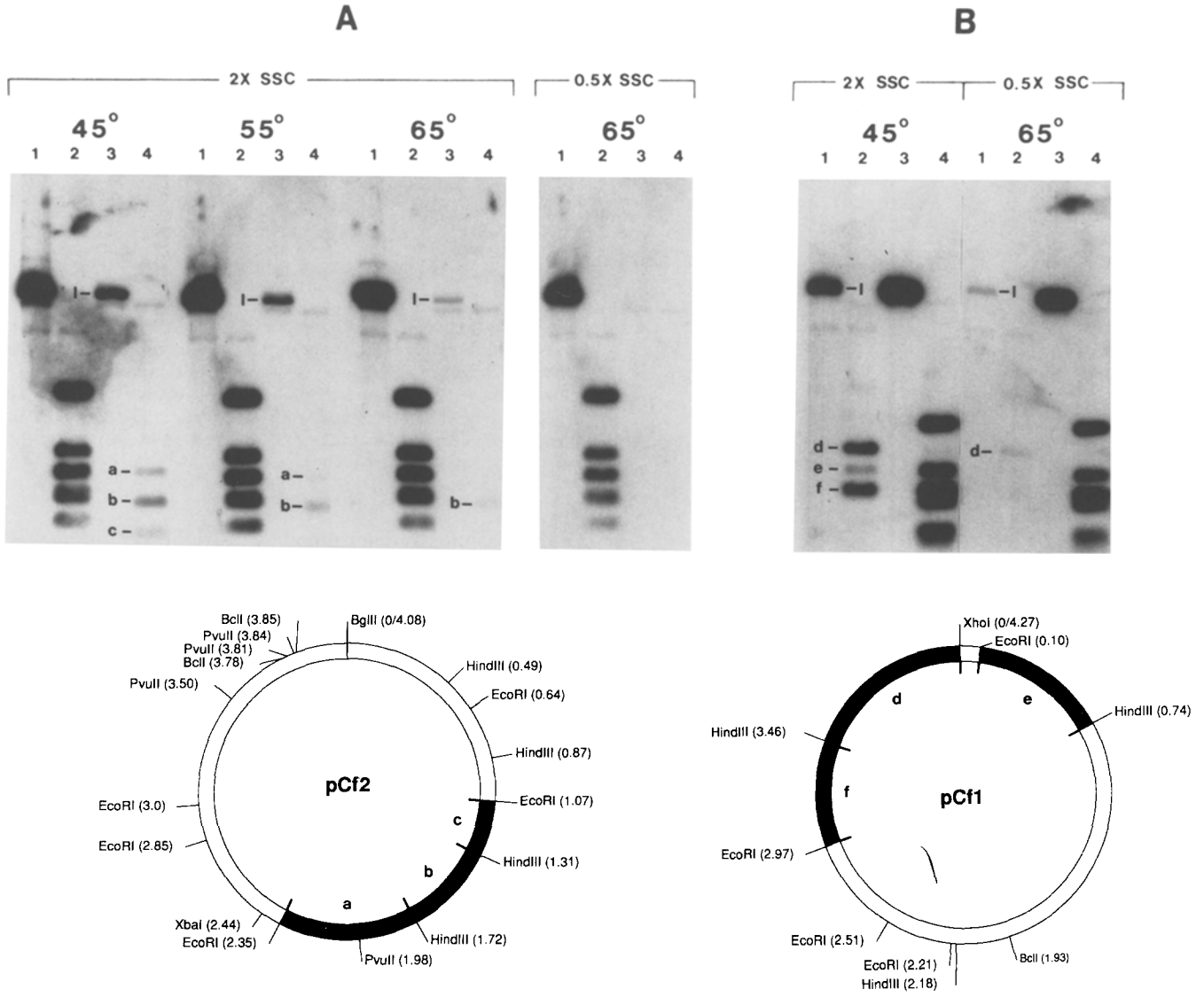

Fig. 4A–B. Similarity of pCf1 and pCf2. *Upper panel* Labeled pCf1 (**A**) and pCf2 (**B**) were used as hybridization probes against blots containing: lane 1, *Xho*I digested (linearized pCf1 insert) pJDJ1; lane 2, *Xho*I + *Hin*dIII + *Eco*RI digested pJDJ1; lane 3, *Bgl*II digested (linearized pCf2 insert) pJDJ4; lane 4, *Bgl*II + *Hin*dIII + *Eco*RI digested pJDJ4. *Letter I* denotes the position of linearized pCf1 (lane 1) and pCf2 (lane 3). *Letters a-c* and *d-f* denote subfragments of pCf1 and pCf2, respectively, that hybridize to the other plasmid. Hybridization wash temperature is indicated above each set of four lanes, and the concentration of SSC used in the washes is indicated in *brackets*. *Lower panel* Maps of pCf2 and pCf1 indicating the position of fragments a-c and d-f which hybridize to the other plasmid. Restriction site position nomenclature is as in Fig. 3

Hybridization between the plasmids and mitochondrial, nuclear, and chloroplast DNA

Hybridization experiments were performed to determine if similarity exists between the plasmids and mitochondrial, nuclear, or chloroplast DNA. Wash conditions were those under which the two plasmids do not hybridize with each other (65° C, 0.5 × SSC, 0.1% SDS). This allows discrimination between those bands specific for pCf1 or pCf2.

To test whether the plasmids hybridize to mitochondrial DNA, DNA was isolated from purified mitochondria. In addition to high molecular weight mitochondrial DNA, this preparation contains other lower molecular weight cellular DNA as a contaminant (M. Hildebrand et al., unpublished results). A blot of this DNA was probed separately with high molecular weight mitochondrial DNA, and cloned fragments representing pCf1 and pCf2 (Fig. 5A). The high molecular weight mitochondrial DNA hybridizes to itself, and the plasmids hybridize to the plasmid DNA contaminating the mitochondrial preparation but not to mitochondrial DNA.

The cloned plasmids were probed to blots of upper and lower band DNA (Fig. 5B and C). Nuclear DNA (lower band) is purified free of plasmids, but in this experiment, upper band DNA contains the native plasmids. Bands arising from plasmid restriction fragments are marked with asterisks. Since pCf1 and pCf2 do not hybridize with mitochondrial DNA (Fig. 5A), hybridizing fragments of upper band DNA, distinct from the

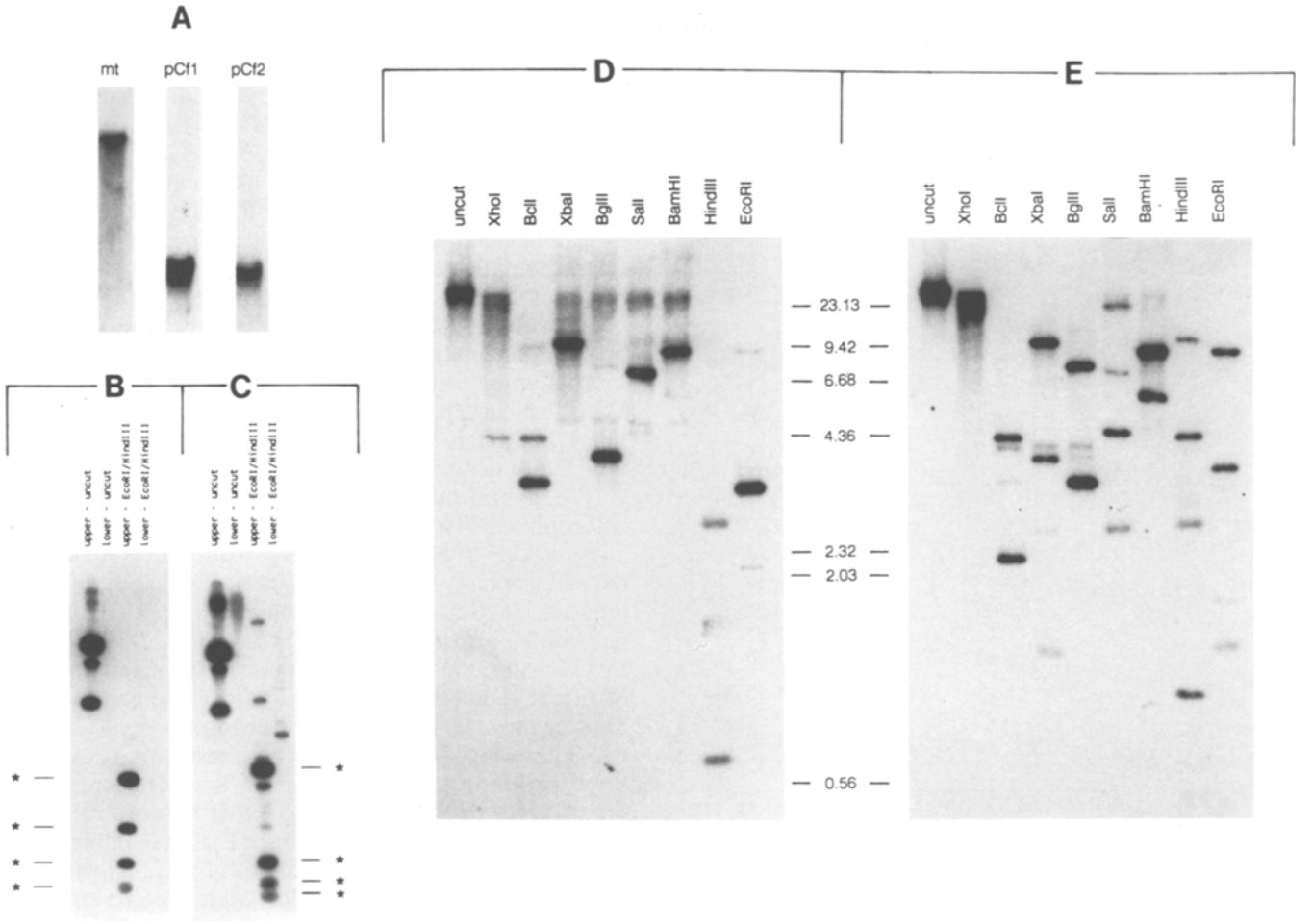

Fig. 5A–E. Hybridization of cloned pCf1 and pCf2 to mitochondrial, chloroplast, and nuclear DNA. **A** Hybridization of mitochondrial DNA (mt), pCf1, and pCf2 to a blot of DNA isolated from purified mitochondria. We have determined that the mitochondrial preparation contains other cellular DNA as a contaminant (M. Hildebrand et al., unpublished data), giving rise to the hybridization of pCf1 and pCf2. **B** and **C** Hybridization of pCf1 (**B**) and pCf2 (**C**) to blots of upper and lower band DNA. The DNA was either uncut or digested with *Eco*RI and *Hin*dIII. Bands marked with *asterisks* are derived from the plasmids. Other bands are from genomic chloroplast or nuclear DNA. **D** and **E** Hybridization of pCf1 (**D**) and pCf2 (**E**) to upper band DNA free of plasmids. Molecular weight markers in kb are shown between the two blots

native plasmid fragments, come from the chloroplast genome.

In the uncut upper band sample (Fig. 5B and C), cloned pCf1 and pCf2 hybridize intensely to the open circular, linear, and supercoiled forms of the native plasmids. They also hybridize to chloroplast DNA, as evidenced by hybridization to high molecular weight (genomic) chloroplast DNA above the three plasmid bands. pCf2 hybridizes to several restriction fragments of chloroplast DNA (Fig. 5C), but pCf1 does not hybridize to fragments other than those of the same molecular weight as plasmid-derived fragments (Fig. 5B). In addition, pCf2 hybridizes to lower band (nuclear) DNA (Fig. 5C), and pCf1 does not (Fig. 5B). This hybridization is not due to contamination of lower band with upper band DNA, since the restriction fragments hybridizing to pCf2 differ in molecular weight from those in the upper band sample (Fig. 5C). Additionally, if contamination were occurring, one would expect pCf1 also to hybridize to the lower band, which it does not (Fig. 5B).

To clarify further the nature of the hybridizing fragments in the chloroplast genome, we gel-purified high molecular weight upper band DNA free of the plasmids. The faintest bands visible in these hybridizations (Fig. 5D and E) have an identical molecular weight to bands expected from the native plasmids. Apparently, there is slight contamination with plasmids, but these fragments can be distinguished on the basis of hybridization intensity. In panel D, cloned pCf1 hybridizes to a single band in all digests but *Hin*dIII. In panel E, pCf2 hybridizes to either two or four fragments in the various digests. These results clearly show that pCf1 and pCf2 both hybridize to chloroplast DNA. Table 1 summarizes which chloroplast DNA restriction fragments hybridize. A summary of the hybridization experiments is presented in Table 2.

Discussion

Plasmid pCf1 is more abundant than pCf2 (Fig. 1, lane 4). By visual inspection of digests in which plasmids are

Table 1. Restriction fragments of upper band DNA hybridizing to pCf1 and pCf2

Restriction enzyme	pCf1		pCf2	
	Fragment number	Size (kb)	Fragment number	Size (kb)
BclI	19	3.28	16	4.24
			26	2.17
XbaI	8	8.46	8	8.46
			17	3.67
			25	2.38
			31	1.27
BglII	14	3.72	8	6.82
			15	3.21
SalI	10	6.50	5	13.60
			10	6.50
			11	4.34
			14	2.61
BamHI	7	7.97	7	7.97
			9	5.36
HindIII	23	2.52	2	8.77
	40	0.61	13	4.25
			23	2.52
			36	1.00
EcoRI	22	3.08	6	7.58
			19	3.38
			31	1.62
			34	1.42

Table 2. Summary of pCf1 and pCf2 hybridization data

	pCf1	pCf2	Nuclear DNA	Chloroplast DNA	Mitochondrial DNA
pCf1	++	+	−	++	−
pCf2	+	++	++	++	−

++, Hybridizes under high stringency conditions; +, hybridizes under low stringency conditions; −, does not hybridize

linearized, we estimate that the plasmids comprise 5% (pCf2) to 10% (pCf1) of the total amount of upper band DNA. Although not a rigorous determination of copy number, this suggests that the plasmids are abundant. In some isolations we were able to obtain open circular, linear, and supercoiled forms of the plasmids (Fig. 1, lane 4). It is unclear what the predominant form is in the cell. Their banding position in CsCl-Hoechst 33258 gradients indicates that the plasmids are both AT-rich (Muller and Gautier 1975; Manuelidis 1977).

The hybridization analyses (Figs. 4 and 5) yielded several interesting results. First, pCf1 and pCf2 share similar, but not identical sequences, which are located in specific regions of the plasmids (Fig. 4, lower). This has been substantiated by nucleotide sequence analysis of pCf1 and pCf2 (Hildebrand et al. 1991b, and unpublished data). The sequencing results bear upon the interpretation of the results of hybridization of pCf1 and pCf2 to nuclear, chloroplast, and mitochondrial DNA, and so merit some discussion. In the regions of hybridization between the two plasmids, there is a stretch of 650 bp with 77% nucleotide similarity, including three stretches of exact identity 19 nucleotides or greater in length (the largest being 25). These sequences hybridize under the lowest stringency conditions (45° C, 2 × SSC, 0.1% SDS) but not under the highest (65° C, 0.5 × SSC, 0.1% SDS). The hybridization experiments using pCf1 and pCf2 against mitochondrial, and upper and lower band DNA, were done under the highest stringency conditions. From this information we can conclude that a positive signal indicates that a significant portion of the plasmid sequences are found in the hybridizing DNA, and that hybridization is not just due to fortuitously similar sequences.

Under high stringency conditions, neither plasmid hybridizes to high molecular weight mitochondrial DNA, although both hybridize to contaminating plasmid DNA in the mitochondrial preparation (Fig. 5A).

The hybridization of pCf1 to a single fragment of chloroplast DNA in all digests but *Hin*dIII suggests that a single copy of pCf1-hybridizing sequences is present in the chloroplast genome (Fig. 5D). This contains one internal *Hin*dIII site. The genomic sequences and plasmids differ with regard to the *Xho*I, *Bc*II, *Hin*dIII, and *Eco*RI restriction sites. An estimate of the maximum size of the pCf1-hybridizing sequences can be made on the basis of the smallest single hybridizing fragment. This is 3.08 kb, in the *Eco*RI digest. These results indicate that the hybridizing genomic sequences are not identical to the plasmid. pCf2 hybridizes to either two or four fragments of chloroplast DNA (Fig. 5E). The simplest explanation for this is that two copies of pCf2-hybridizing sequences are present in the chloroplast genome and that both copies have the same restriction sites. Thus, they appear to be similar to each other. These sequences contain internal *Xba*I *Sal*I, *Hin*dIII, and *Eco*RI sites. No *Sal*I site is found in the native plasmid, and other differences in expected restriction fragment size are observed. As with pCf1, this indicates that an identical copy of pCf2 is not present in the genome.

Four fragments hybridize to both pCf1 and pCf2 (Table 1). This indicates that the pCf1-hybridizing and one copy of the pCf2-hybridizing sequences are located close to each other in the chloroplast genome. Since these hybridizations were done under high stringency conditions, other chloroplast DNA fragments that may hybridize less strongly were not included in this analysis.

Plasmids hybridizing with chloroplast DNA have been described in other organisms. A heterogeneously sized class of circular DNAs (2.5–27 kb) were found in bleached mutants of *Euglena gracilis* (Heizmann et al. 1982). Some of these hybridize with chloroplast DNA. A 14.1 kb circular plasmid is present in *Acetabularia cliftonii* chloroplasts (Ebert et al. 1985). Results suggest that the entire sequence of this plasmid is present in the chloroplast genome (Ebert et al. 1985). In *Chlamydomonas moewusii* a 5.9 kb linear DNA molecule hybridizes with chloroplast DNA (Turmel et al. 1986). The presence of restriction fragments of the same sizes in the plasmid and chloroplast DNA suggests that the entire plasmid sequence is also found in the chloroplast genome (Turmel et al. 1986). The results in *A. cliftonii* and *C. moewusii* are

in contrast to ours, since the genomic fragments that hybridize to pCf1 and pCf2 are different from those in the native plasmid.

In addition to hybridizing with chloroplast DNA, pCf2 hybridizes with nuclear DNA (Fig. 5C). Interorganellar sequence transfer between higher plant chloroplast, mitochondrial, and nuclear DNA has been documented (Stern and Lonsdale 1982; Timmis and Scott 1982; Whisson and Scott 1985; Schuster and Brennicke 1987). Sequence similarity between plasmids, organellar, and nuclear DNA has also been shown in the maize mitochondrial S-1 and S-2 plasmids (Kemble et al. 1983). To our knowledge, our results are the first example of hybridization between plasmids, chloroplast DNA, and nuclear DNA.

In this paper, we have not addressed the question of where the plasmids are located in the cell. They are enriched in a purified chloroplast preparation (not shown), and our current working hypothesis is that they are located in the chloroplast. More experiments are needed to confirm this. We have also completed the nucleotide sequence determination of the plasmids (Hildebrand et al. 1991b, and unpublished data). More complete characterization of the plasmid-hybridizing sequences in the genome will help clarify the function of the plasmids, and how they have interacted with the chloroplast and nuclear genomes of *C. fusiformis*.

As reported (Hildebrand et al. 1991a), pCf1 and pCf2 also hybridize with plasmids and upper band DNA of other plasmid-containing diatoms. These results suggest that features of the plasmids are found in several diatom species. A more detailed analysis of pCf1 and pCf2 may help elucidate the role that plasmids play in diatoms.

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