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ISOLATION OF FOUR NEW STRAINS OF *CHLAMYDOMONAS REINHARDTII* (CHLOROPHYTA) FROM SOIL SAMPLES[1]

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ABSTRACT

We describe a method for the efficient extraction of flagellate phototrophs from soil samples. Soils of various types from a wide range of natural environments were sampled for Chlamydomonas reinhardtii *Dangeard. Test matings of candidate unicellular green algae were used to identify 19 heterothallic isolates that were interfertile with lab strains. Four of these isolates were genetically distinct from previously isolated strains and from each other, as indicated by the distribution of two transposons on restriction fragments of their genomic DNA and by allozyme variation at six of nine loci.*

Key index words: Chlamydomonas reinhardtii; *Chlorophyta; isolation techniques; new strains; soil algae*

Chlamydomonas reinhardtii Dangeard is a model system well suited to molecular, genetic, cytological, and evolutionary research (VanWinkle-Swift 1992). Many of these investigations require intraspecific variation, and the availability so far of only five distinct wild-type isolates has limited potential research. For several decades, only spores derived from a single zygote were available. Strains CC-1009 and CC-1010 (strain numbers are those used by the *Chlamydomonas* Genetics Center at Duke University to refer to strains in their collection) were collected in 1945 by G. M. Smith in Amherst, MA. CC-1373 was also isolated from Massachusetts by Smith and, although designated by Hoshaw and Ettl (1966) as *C. smithii,* is interfertile with *C. reinhardtii.* More recently, CC-2290 was derived from a soil sample from Minnesota (Gross et al. 1988); CC-1952 was extracted from the same sample and appears to be genetically equivalent. Spanier et al. (1992) isolated CC-2342, CC-2343, and CC-2344 from soils in Pittsburgh, PA, Melbourne, FL, and Malverne, PA, respectively.

We undertook a search for new strains of *C. reinhardtii* in order to add to the genetic diversity available for experimental study. Because little is known about the ecology of *C. reinhardtii,* we sampled many different soil types. A new technique was developed to assay soil efficiently for *C. reinhardtii.* It was hoped that widespread sampling and methodical soil analysis, in addition to producing new strains, would yield some information on the environmental distribution of the alga. We describe here the methods that permitted the isolation and preliminary characterization of four new strains of *C. reinhardtii.*

Laboratory wild-type strains CC-1010, CC-1373, CC-1952, CC-2342, CC-2343, and CC-2344 were provided by the *Chlamydomonas* Genetics Center at Duke University, Durham, NC. All lab strains and flagellates isolated from soil samples were maintained under axenic conditions on Bold's minimal medium (Harris 1989).

Twenty-two localities were sampled, mostly in the region of Montréal, Québec; localities in Ontario and the midwestern United States were also sampled as opportunities arose. Within these 22 localities, 352 samples were taken, spanning five different soil habitats (see Table 1). Each sample consisted of a sandwich-bagful of soil (approximately 150 mL) taken randomly from several spots within a given habitat area.

Approximately 150 mL of distilled water was added to each soil sample in a glass jar, and the mixture was left for 24–48 h under fluorescent lights. A 20-mL aliquot of the culture was centrifuged for 5 min at approximately 2000 × *g.* The supernatant was discarded, and motile phototrophs were isolated from 500 μL of the resuspended pellet of cells using the following device. A 9-in. Pasteur pipette was plugged with cotton at the wide end and autoclaved. A rubber bulb was used to draw a freshly autoclaved solution of 0.15% agarose in Bold's medium into the pipette to just under the cotton plug, under sterile conditions. The tip of the pipette was then sealed by flaming. The resulting device was a glass tube filled with sterile agarose, plugged at one end with cotton, and sealed at the tip.

To extract potential *Chlamydomonas* from a sample, the cotton plug was removed from a pipette, 500 μL of soil extract was introduced with a micropipette, and the top opening was plugged with sterile agar. The sample was thus trapped at the end of the agarose gel inside the pipette. The pipette was then covered with aluminum foil, with the exception of 5 cm at the tip, which was left exposed. The wrapped pipette was then placed under bright light, illuminating the uncovered tip. After 24 h, the illuminated end was broken off and a plate of Bold's agar was spread with the agarose from the broken tip. This inoculated plate was placed under illumination to cultivate colonies of phototactic microorganisms.

When green colonies resembling those of *Chlamydomonas* were observed, two were picked from each plate and spread on new plates. When they had achieved sufficient growth, a loopful of each culture was suspended in "M-N," a nitrogen-free liquid medium that induces gametogenesis in *Chlamydomonas* (Harris 1989). Aliquots were mixed with gametes of CC-1010 (mating type plus, or mt+) and CC-1952 (mt−) and cultured alone in separate wells of a multiwell plate. The following day, the mixtures were examined for the floating mats of zygospores characteristic of a mating reaction (Harris 1989). Isolates that mated with CC-1010 or CC-1952, but that did not mate when cultured separately, were identified as *C. reinhardtii.*

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TABLE 1. *Habitat types samples for Chlamydomonas reinhardtii. A, number of samples taken; B, number yielding flagellate phototrophs; C, number yielding* C. reinhardtii.

Soil type	A	B	C
Banks of lakes, ponds, streams; marshes, bogs	100	52	0
Forest floors	47	18	0
Exposed soil in meadows, forest clearings	105	39	0
Ditches, sides of roads	35	11	0
Rocky areas	3	1	0
Arable fields, vegetable gardens, cow pastures	62	40	19
Total	352	161	19

Four isolates, CC-2935 (mt−), CC-2936 (mt+), CC-2937 (mt+), and CC-2938 (mt−), were chosen for further study. Total genomic DNA was extracted by a modification of the method of Weeks et al. (1986). Cells were pelleted by centrifugation of 100 mL culture at 6000 × *g* for 5 min, resuspended in 1.6 mL deionized water, and disrupted by adding 3.4 mL of lysis buffer (2% SDS, 0.4 M NaCl, 0.04 M EDTA, 0.1 M Tris-HCl) and incubating at 50° C for 15 min. The cell lysate was extracted twice with an equal volume of 25:24:1 phenol : chloroform : isoamyl alcohol, with centrifugation at 12,000 × *g* for 10 min each time. DNA was ethanol-precipitated, resuspended in 100 μL deionized water, digested with *Bam*HI, and size-fractionated overnight on agarose gels. The DNA was vacuum-transferred to positively charged nylon membranes (Amersham or ICN) and hybridized overnight with fluorescein-dUTP-labeled probes for internal fragments of TOC1 (Day et al. 1988) and Gulliver (Ferris 1989), two transposons recently cloned from the nuclear genomes of lab strains of *C. reinhardtii.* ECL (Amersham) reagents and protocols were used for probe labeling, hybridization, and detection, except that the stringency washes were for 45 min each, rather than 15 min. The chemiluminescent probes were detected by exposure to X-ray films (Amersham or Fuji) for 10–20 min.

For allozyme electrophoresis, 250-mL cultures of the four new strains and previous isolates were pelleted by centrifugation at 6000 × *g* for 5 min. The pellets were resuspended in 100 μL distilled water in 1.5-mL microfuge tubes. The samples were frozen at −20° C and thawed twice in order to lyse the cells and then stored at −80° C. The samples were thawed and centrifuged, and the supernatants were submitted to horizontal starch gel electrophoresis, using gels of 12% hydrolyzed starch (Murphy et al. 1990). Loci were numbered and alleles were lettered beginning with the most anodally migrating bands.

Of the 352 samples taken, only those from cultivated fields on the outskirts of Farnham, Québec, yielded *C. reinhardtii* (Table 1). Thirty-seven of the 52 samples taken haphazardly within these fields on 23 June 1993 contained flagellate phototrophs. Two colonies from each sample were cultivated. Of these 74 colonies, 27 produced gametes that mated with CC-1010 or CC-1952. Four of these were chosen for genetic comparison with lab strains. Both TOC1 and Gulliver were more abundant in the four new isolates than in any of the lab strains (Fig. 1). Because few or none of the *Bam*HI fragments hybridizing to TOC1 or Gulliver were shared between the new isolates and the lab strains, the possibility that soil samples were inadvertently contaminated with our lab lines can be ruled out.

While the four Farnham isolates shared a high copy number of both transposons, they did not appear to be particularly closely related to each other, because repeated transposition of both TOC1 and Gulliver has occurred since their divergence. Ferris (1989) found that several derivatives of CC-1009

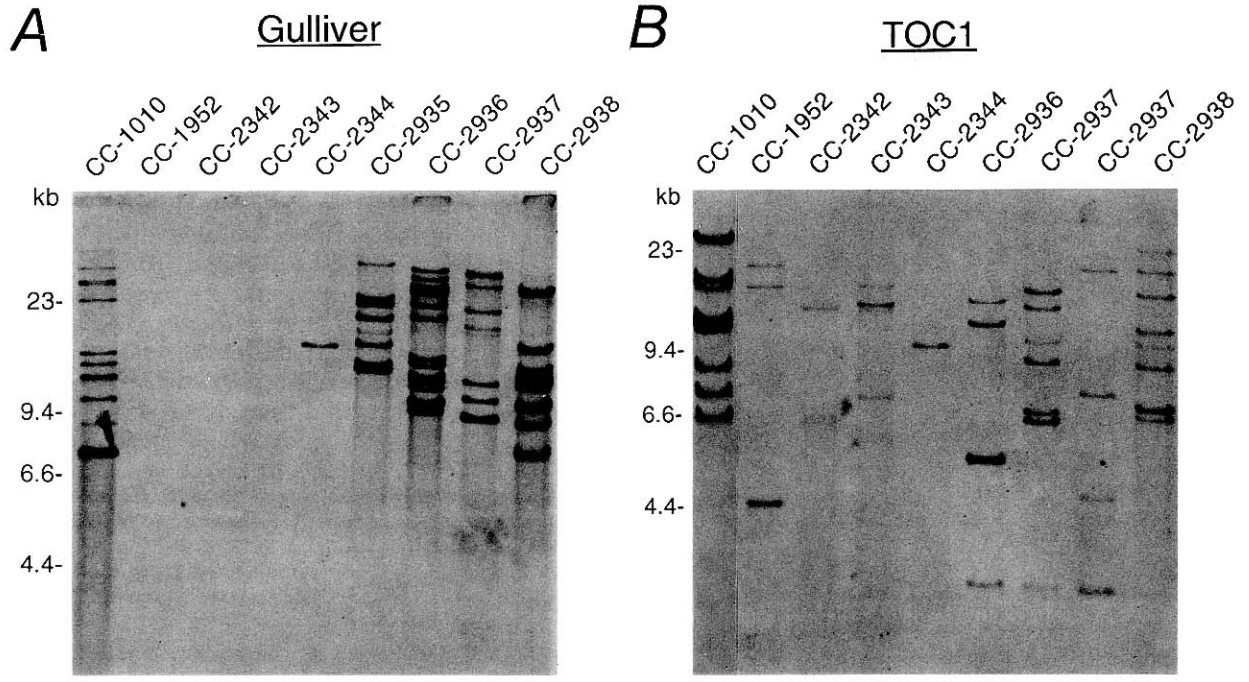

FIG. 1. Hybridization to *Bam*HI-digested genomic DNA from lab strains and new isolates of *Chlamydomonas reinhardtii,* vacuum-blotted to positively charged nylon membranes. Positions of size markers are indicated on the left of each blot. A) Hybridization with a labeled internal *Eco*RI-*Bam*HI fragment of Gulliver. B) Hybridization with an internal *Bam*HI-*Pst*I fragment of TOC1.

TABLE 2. *Electrophoretic phenotypes of strains at polymorphic loci. Three gel and tray buffer systems were used to examine the following nine presumptive loci: Tris citrate, pH 8.0, was used for cytosol aminopeptidase (CAP; EC 3.4.11.1) and phosphogluconate dehydrogenase (PGDH; EC 1.1.1.44, two loci); amine citrate, pH 6.1, was used for malate dehydrogenase (MDH; EC 1.1.1.37, two loci); and lithium borate–Tris citrate, pH 8.4, was used for dihydrolipoamine dehydrogenase (DDH; EC 1.8.1.4), glucose dehydrogenase (GCDH; EC 1.1.1.118), superoxide dismutase (SOD: EC 1.15.1.1), and xanthine dehydrogenase (XDH; EC 1.1.1.204). MDH-1, GCDH, and SOD were monomorphic.*

	Strain number								
Locus	CC-1010	CC-1952	CC-2342	CC-2343	CC-2344	CC-2935	CC-2936	CC-2937	CC-2938
CAP	A	A	B	A	A	A	A	A	A
DDH	B	B	B	A	B	A	B	A	B
MDH-2	B	B	B	B	B	B	B	B	A
PGDH-1	B	B	B	B	A	B	B	B	B
PGDH-2	B	B	B	B	A	B	B	B	B
XDH	B	B	B	B	A	B	B	B	B

and CC-1010 differed in the presence or absence of specific copies of Gulliver, but this variation was much less striking than that evident in Figure 1A. As a further indication of their genetic distinction, Farnham isolates CC-2936 and CC-2938 each contained a bright band on the background of genomic *Bam*HI fragments, indicative of a highly repeated sequence, which was absent from any other strain we examined (Fig. 2). Genetic variation among the lab strains and new isolates was also evident at six of nine allozyme loci, although our data cannot address the possibility that multiple alleles were segregating within a population of interfertile lineages at Farnham (Table 2). Strain CC-2342 was characterized by a unique CAP allele. Strain CC-2344 had unique alleles at PGDH-1, PGDH-2, and XDH.

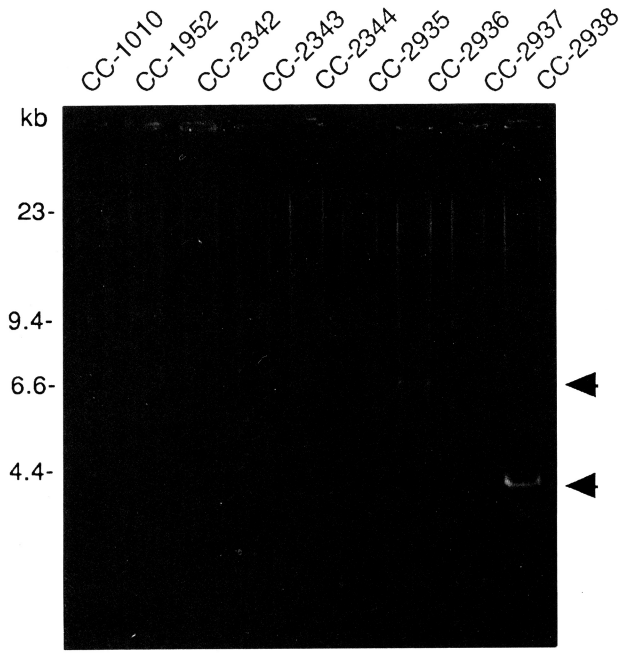

FIG. 2. Ethidium bromide fluorescence staining of *Bam*HI-digested genomic DNA from lab strains and new isolates of *Chlamydomonas reinhardtii*, size-fractionated by electrophoresis in 0.65% agarose. Positions of size markers are indicated on the left. Arrowheads indicate unique bands of repeated sequences in the DNA of isolates CC-2936 and CC-2938.

Strain CC-2938 was characterized by a unique allele at malic dehydrogenase-2 and strains CC-2343, CC-2935, and CC-2937 shared a DDH allele. All nine samples were monomorphic at GCDH, SOD, and malic dehydrogenase-1.

Using the agarose pipettes, which can also be used to clean contaminated *Chlamydomonas* cultures, a variety of flagellate phototrophs was extracted from soil samples. Many morphologically varied green colonies appeared on plates spread with the pipette tip contents but failed to mate with lab *C. reinhardtii*. Other *Chlamydomonas* species may have constituted a portion of these flagellate phototrophs, which are apparently ubiquitous in soils and were found in almost half the samples collected (Table 1). *Chlamydomonas reinhardtii*, however, was isolated only from Farnham. At the time of collection, the fields were newly planted, with little vegetation, and there had recently been a heavy rainfall. A second collection at this locality was made on 27 July 1993. Only one sample yielded *C. reinhardtii*, perhaps because the soil was dry and hardened by hot dry weather and a maize crop had grown up.

The soil samples in which *C. reinhardtii* was found were from well-fertilized arable fields recently disturbed by plowing, whereas the other soil habitats sampled included marshes, mud trails, water runoffs, sandpits, forest floors, and meadows (see Table 1). The absence of *C. reinhardtii* from any habitat but cultivated fields suggests a preference for disturbed, nutrient-rich environments. The adaptability of *C. reinhardtii* to lab conditions, with rapid growth in unoccupied, nutrient-rich environments, is also consistent with a "weed" ecology.

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