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Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*

I. *Chlorella sorokiniana*

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Abstract. Two different DNA hybridization methods were used to study the genetic relationships among strains of *Chlorella sorokiniana*. Closely related strains could be easily detected by comparison of DNA renaturation rates as determined spectrophotometrically, whereas the degree of relationship of more remotely related strains could be determined by means of reassociation of radioactively labelled DNA fragments with high-molecular-weight DNA bound to nitrocellulose filters. Strains assigned to *C. sorokiniana* proved to be a rather heterogeneous group with only 3 out of 17 strains being closely related to the type strain *C. sorokiniana* 211-8k^T. The description of new species or subspecific taxa on the basis of nucleotide sequence similarities, however, does not seem justified before more detailed knowledge of genome organization in *Chlorella* is available.

Key words: *Chlorella sorokiniana* – Taxonomy – DNA/DNA reassociation – DNA homology

Unicellular green algae of the genus *Chlorella* are widely used in basic research on, for example, photosynthesis and nitrate reduction, and strains of *Chlorella* have more recently attained importance in the field of biotechnology. Because of the variety of biochemical and physiological properties of *Chlorella* strains it is necessary to have a detailed knowledge of what is characteristic for different groups or taxa, that is, to have a clearcut taxonomy for the sake of reproducibility of research results, for identifying new isolates, and for choosing the most appropriate strains for special demands in biotechnology. The uniformity of the small, spherical cells and the lack of morphological features discernible in the light microscope, however, have in the past made it almost impossible to identify strains and to assign them unequivocally to certain species. In the last 25 years Kessler and coworkers have developed a taxonomical system based upon ten biochemical and physiological characters, which permits a division of *Chlorella* strains into 14 well characterized taxa (for reviews see Kessler 1982, 1984). The application of methods elucidating DNA similarities, such as the determination of DNA base composition (Hellmann and Kessler 1974) and DNA hybridization (Kerfin and Kessler 1978), however, indicated that some of the taxa might be quite heterogeneous on a genetic level. The first attempt of Kerfin and Kessler (1978) to apply DNA hybridization to the taxonomy of *Chlorella* in the same way as for prokaryotes (Schleifer and Stackebrandt 1983) was carried out with only two reference strains, namely *C. fusca* var. *vacuolata* 211-8b^T and *C. vulgaris* 211-8m. Consequently, the relationships within and between the other species remained unknown. In particular *C. sorokiniana*, which is characterized by the possession of hydrogenase (Kessler 1967) and its ability to grow at 38° C (Kessler 1972) proved to be heterogeneous in DNA base composition (55.0–78.6% G + C; Hellmann and Kessler 1974) as well as in some biochemical and physiological characters such as hydrolysis of starch (Kessler 1978) and salt tolerance (Kessler 1974). Extensive DNA hybridization studies were therefore applied to show whether or not *C. sorokiniana* is composed of several only distantly related groups of organisms similar in morphology and physiology and sharing high-temperature habitats.

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Abbreviations. 1 × SSC, standard saline citrate (0.15 M NaCl + 0.015 M trisodium citrate, pH 7.0); Tris, tris-(hydroxymethyl)-aminomethane; EDTA, ethylene diamine tetraacetate; CTAB, N-cetyl-N,N,N-trimethyl ammonium bromide; TEACl, tetraethyl ammonium chloride

Materials and methods

Strains and culture conditions

In addition to the 16 strains of *Chlorella sorokiniana* described by Kessler (1982) one more strain was included in this study, namely *C. sorokiniana* 211-40b, an endosymbiont of *Spongilla fluviatilis* (Schlösser 1982) (Table 1). The algae were grown in the medium of Kessler and Czygan (1970) with additional 0.02 mg each of $CuSO_4 \cdot 5\,H_2O$, $NiSO_4 \cdot 6\,H_2O$, and $CoSO_4 \cdot 7\,H_2O$ under the conditions described by Hellmann and Kessler (1974). The growth medium of strain 211-18 was supplemented with 40 mg/l thiamine dichloride (Kessler and Zweier 1971).

DNA extraction and purification

The algae were harvested by centrifugation, washed in Tris-EDTA-buffer (0.05 M Tris, 0.03 M EDTA, pH 7.0) and homogenized with glass beads as described by Kerfin and Kessler (1978). The cell extract was separated from the glass beads by washing with buffer and suction through a coarse sintered filter. DNA was purified by a modified method after Marmur (1961) using CTAB, RNAse A (Serva) and pronase K (Merck) for the removal of polysaccharides (Darby et al. 1970), RNA and proteins, respectively.

Table 1. The strains studied and their DNA base compositions

Species	Strain	Origin[a]	G + C content (mol%)[b]
Chlorella sorokiniana Shihira and Krauss	211-18	Göttingen	51.3
	211-11d	Cambridge	61.5
	C-1.1.8	Bethesda	62.2
	C-1.6.7	Bethesda	62.8
	211-8k^T	Göttingen	63.1
	211-32	Göttingen	63.9
	211-33	Göttingen	64.0
	Sless 2	B. Sless, Israel	64.8
	1-9-30	C. Sorokin, USA	64.9
	211-34	Göttingen	65.5
	Sless 1	B. Sless, Israel	65.7
	211-11k	Göttingen	66.6
	211-31	Göttingen	68.3
	211-40b[c]	Göttingen	73.5
	Prag A14	M. Baslerová, CSSR	73.7
	211-40c[c]	Göttingen	74.9
	211-40a[c]	Göttingen	75.2
C. vulgaris Beijerinck	211-11b^T	Göttingen	61.1
C. protothecoides Krüger	211- 7a^T	Göttingen	57.6
C. saccharophila (Krüger) Migula	211- 9a^T	Göttingen	51.9

[a] Göttingen, Algensammlung des Pflanzenphysiologischen Instituts der Universität Göttingen, FRG; Cambridge, Culture Centre of Algae and Protozoa, Cambridge, UK; Bethesda, culture collection at Bethesda, Maryland, USA. For additional information see Schlösser (1982) and Kessler (1972)
[b] *Escherichia coli* B (G + C content: 52.0 mol%; Gillis et al. 1970) was used as reference
[c] Endosymbiont of *Spongilla fluviatilis*
[T] Type strain

Labelling of DNA

DNA was labelled by means of the nick translation procedure (Rigby et al. 1982) with deoxy-(1′,2′,5′-^{3}H)-cytidine-triphosphate (Amersham) according to the specifications given by the nick translation kit (Bethesda Research Laboratories). Labelled DNA was separated from free nucleotides on an "Elutip-d" mini column (Schleicher and Schüll).

DNA base composition and DNA hybridization

Melting profiles of DNA preparations in 0.1 × SSC were determined at 260 nm with a Gilford 2600 spectrophotometer equipped with a thermoprogrammer. The guanine + cytosine content was calculated from the melting points (T_M-values) according to Owen et al. (1969). DNA of *Escherichia coli* B DSM 2840 was used as reference.

DNA/DNA hybridization on membrane filters was accomplished as described by Meyer and Schleifer (1978) in 3 × SSC at 60°C for 75 h under optimal conditions (25°C below T_M). The melting point of DNA was lowered by the additon of formamide (Blüthmann et al. 1973). Renaturation rates of DNA in solutions containing 50 µg/ml DNA in 2 × SSC/1 M TEACl, adjusted to pH 7.0 with tetraethyl ammonium hydroxide, were determined by the method of Huss et al. (1983) and degrees of binding (%D) calculated according to DeLey et al. (1970).

Table 2. Homology group I of *Chlorella sorokiniana*[a]

Strain	D value (%) with					
	C-1.6.7	211-32	211-33	Sless 1	Sless 2	1-9-30
211-8k^T	93	93	98	52	54	47
C-1.6.7		95	87	–	53	47
211-32			98	–	48	45
211-33				–	56	53
Sless 1					103	60
Sless 2						52

[a] Degrees of binding (%D) were determined by using the renaturation rate method
– = not determined

Results

Table 1 shows the G + C contents of all strains investigated. Differences to the data of Hellmann and Kessler (1974) mainly are due to different equations used for the calculation of the G + C contents.

Within *Chlorella sorokiniana*, at least three groups of strains can be distinguished. The first group is represented by only one strain, 211-18 (51.3 mol%). The second group contains the bulk of the strains, with DNA base compositions distributed between 61.5 and 68.3 mol%, while the third group comprises the three strains isolated as endosymbionts from *Spongilla fluviatilis* as well as strain Prag A 14 (73.5–75.2 mol%).

Closely related strains within these groups were detected by using the renaturation rate method for DNA hybridizaton (Huss et al. 1983). This method, originally developed for prokaryotes, has been modified to overcome two problems which have not been considered in the former study, namely the larger genome in comparison with prokaryotes and the high G + C content of the *C. sorokiniana* DNA. As far as we know, there is no report about the genome size of *Chlorella*, but rough estimations from the renaturation rates (Gillis et al. 1970) indicate a molceular weight of at least $1-2 \times 10^{10}$ daltons (the molecular weight of nuclear DNA from *Chlamydomonas reinhardii* is reported to be 6×10^{10} daltons; Howell and Walker 1976). This leads to very low renaturation rates which cannot be accurately determined. We therefore raised the DNA concentration to 50 µg/ml in spite of the higher unspecific binding to be expected (Huss et al. 1983). The high G + C content of *C. sorokiniana*, on the other hand, does not allow the melting of DNA samples in 2 × SSC buffer in the thermocuvettes of the spectrophotometer. We therefore lowered the melting temperature by addition of 1 M TEACl (Melchior and von Hippel 1973). This reduces the melting point by about 15°C. Moreover, TEACl has the additional advantage of enhancing DNA renaturation rates (Chang et al. 1974; Orosz and Wetmur 1977).

The DNA homology values obtained with the renaturation rate method can be arranged in three different homology groups (Tables 2–4). The first homology group (Table 2) contains three strains (C-1.6.7, 211-32, 211-33) which are highly related (87–98% D) as well as three moderately related strains (Sless 1, Sless 2, 1-9-30) with DNA homologies ranging from 47–54% D as compared with the type strain 211-8k^T. Strains Sless 1 and Sless 2, both isolated from a waste water pond, do not differ in their biochemical

and physiological properties (Kessler 1982). The high degree of binding of their DNAs (103%) indicate that both strains may be identical.

In the second homology group (Table 3), strains 211-11k and 211-31 are highly related (97% D) while we found about 60% binding between them and strain 211-34. Strains living in endosymbiosis with *Spongilla fluviatilis,* constitute the third homology group (93–102% D) (Table 4).

Homology values found between representatives of the three homology groups lie within the range of background values of this method, which may reach up to 40% D using the described conditions (data not shown). For a better resolution of DNA relationships for members of the three homology groups we used the membrane filter technique (Denhardt 1966). Results are shown in Table 5.

Table 3. Homology group II of *Chlorella sorokiniana*[a]

Strain	D value (%) with		
	211-31	211-34	211-8k^T
211-11k	97	64	36
211-31		62	33
211-34			26

[a] Degrees of binding (%D) were determined by using the renaturation rate method

Table 4. Homology group III of *Chlorella sorokiniana*[a]

Strain	D value (%) with		
	211-40b	211-40c	211-8k^T
211-40a	102	93	18
211-40b		99	–
211-40c			24

[a] Degrees of binding (%D) were determined by using the renaturation rate method
– = not determined

Using this method, corresponding homology groups were formed, while homology values of most of the remaining strains lie between 20 and 30%. It is only strain 211-18 which, like *Chlorella* species other than *C. sorokiniana,* exhibits a distinctly lower degree of binding (about 10% D).

Data from Tables 2 to 5 were combined to give an average linkage dendrogram illustrating DNA relationships within *C. sorokiniana* (Fig. 1).

Discussion

The determination of DNA homologies by comparing initial renaturation rates (DeLey et al. 1970) is a rapid and accurate method for investigating close phylogenetic relationships among bacteria (Huss et al. 1983). Nevertheless, this method has only rarely been applied to eukaryotes (Martini and Phaff 1973; Jahnke and Bahnweg, in press). The reason for this is mainly the more complex genome of eukaryotes resulting in very low renaturation rates which cannot be accurately determined. The relatively small complexity of the *Chlorella* genome, which is about 10 times that of *Escherichia coli,* does, however, permit the use of this method for taxonomic investigations within this genus. The problem of low renaturation rates was reduced by employing higher DNA concentrations (50 μg/ml) and by the addition of 1 M TEACl to the reaction mixture. TEACl not only lowers the melting temperature of the DNA, necessary for complete denaturation under the conditions used, but also enhances renaturation rates (Melchior and von Hippel 1973; Orosz and Wetmur 1977; Chang et al. 1974). The acceleration, however, did not exceed a factor of 2–3 and could not prevent a lower reproducibility of homology values (±7% D mean standard deviation as compared to ±2.4% D with prokaryotes). Another disadvantage is the low resolution of this method. Unspecific binding, as determined by reassociation of distantly related strains, goes up to about 40% D. DNA similarities in the range of 0–40% D have therefore to be determined by means of the membrane filter technique. Above 40% D, values obtained by both methods agree rather well, being slightly higher when determined optically. For a rapid recognition of closely related strains, the optical

Table 5. DNA-DNA hybridization of *Chlorella sorokiniana, C. vulgaris* 211-11b^T, *C. saccharophila* 211-9a^T, and *C. protothecoides* 211-7a^T

Source of filter-bound DNA	D value (%) with (^{3}H)-labelled DNA[a] from										
	211-8k^T	C-1.6.7	Sless 2	1-9-30	211-11k	211-34	211-40a	C-1.1.8	211-11d	Prag A 14	211-18
211-8k^T	100	99	31	35	20	37	15	19	23	16	8
C-1.6.7	86	100	43	42	16	31	12	16	20	15	8
Sless 2	33	42	100	33	14	36	16	27	25	21	9
1-9-30	36	45	38	100	21	39	14	29	28	22	10
211-11k	16	21	13	12	100	63	11	27	21	13	7
211-34	31	22	17	13	51	100	17	30	14	20	5
211-40a	30	29	20	19	24	29	100	30	27	36	8
C-1.1.8	16	16	8	9	15	24	7	100	13	12	5
211-11d	25	26	15	16	26	33	15	30	100	21	13
Prag A 14	23	25	14	19	21	33	27	31	27	100	10
211-18	10	11	8	7	10	17	6	15	13	9	100
211-11b^T	11	11	–	–	–	–	–	–	–	–	–
211-9a^T	3	–	–	–	–	–	–	–	–	–	–
211-7a^T	9	7	7	7	–	6	7	–	–	–	7

[a] The optimal renaturation temperature (T_M-25° C) was related in each case to the melting temperature of the (^{3}H)-labelled strain
– = not determined

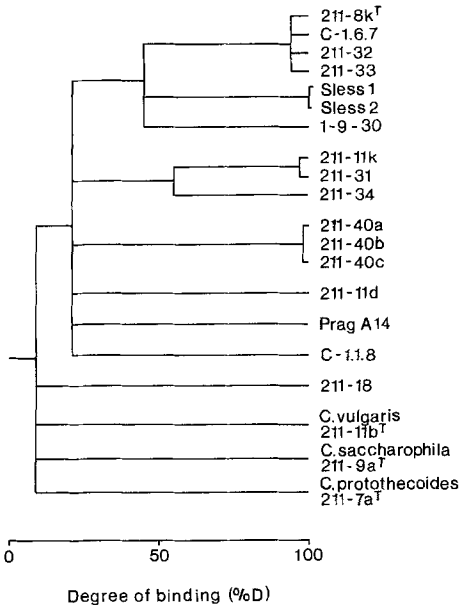

Fig. 1. Average linkage dendrogram of *Chlorella sorokiniana* based on DNA-DNA hybridization studies (see Tables 2–5)

method remains a simple routine method even when applied to eukaryotic organisms with relatively small genomes like *Chlorella*.

It is somewhat surprising that DNA hybridization between many *C. sorokiniana* strains is rather low (Fig. 1), in spite of the fact that all strains are characterized by the possession of hydrogenase and thermophily, by the absence of production of secondary carotenoids and of exoproteases as required for the liquefaction of gelatin, and by the abilities to ferment glucose to lactate and to reduce nitrate; in addition, there is no requirement of thiamine for growth (Kessler 1982). Some heterogeneity, however, is found in characteristics like production of exoamylases for starch hydrolysis, salt tolerance (limit at 1–3% NaCl) and acid tolerance (limit at pH 4.0–5.0), and especially in the G + C contents of the DNA (61.5–75.2 mol%; Table 1).

Strain 211-18, with the comparatively low G + C content of 51.3 mol%, not only shows very low DNA reassociation with *C. sorokiniana* (about 10% D) but also proved to be not thermophilic (Kessler, unpublished observation); it should therefore be excluded from this species.

The strains living in endosymbiosis with *Spongilla fluviatilis* (211-40a, 211-40b, 211-40c) which are closely related (93–102% D), as well as strain Prag A 14, are separated by remarkably high G + C contents (73–75 mol%), about 10 mol% higher as compared to the other strains. According to DeLey (1969), two bacteria, 10 mol% G + C apart, can theoretically share at most 10–30% of their nucleotide sequences. The observed homology of about 20% between the different G + C groups is thus rather high. This could be due to a number of homologous genes in common with additional repetitive sequences quite different in their G + C contents, or to the presence of multiple repeated conservative DNA sections such as rRNA and tRNA cistrons, as observed e.g. in *Neurospora crassa* by Brooks and Huang (1972), within an otherwise more heterogeneous genome.

It should be pointed out in this context that we used whole cell DNA without separation of chloroplast DNA (cp DNA), mitochondrial DNA (mt DNA), and repetitive sequences. The fraction of what is presumably cp DNA, which has a G + C content of 30–40 mol% in all strains of *Chlorella* investigated, varies between 1 and 10% in our preparations as estimated from the biphasic melting profiles. The proportion of mt DNA is assumed to be negligible. Since *Chlorella* cells have only one chloroplast, cp DNA should not markedly influence the renaturation rate of the chromosomal DNA. On the other hand, the presence of an appreciable amount of repetitive sequences would prevent the desired overall comparison of the whole genome, since initial reassociation would largely occur with those redundant sequences. The question of presence and amount of repetitive sequences in *Chlorella* is now under investigation. We believe, however, that our hybridization values are significant in a phylogenetic sense because strains of physiologically and biochemically more heterogeneous *Chlorella* species like *C. sorokiniana* (Fig. 1) and *C. saccharophila* partly show rather low DNA homologies, whereas only high values (90–100% D) were obtained within homogeneous species like *C. fusca* var. *vacuolata* and *C. kessleri* (results to be published).

Nucleotide sequence comparisons within eukaryotes have so far mostly been done in yeasts and higher fungi. Some authors have tried to correlate hybridization data with the delimitation of species (Price et al. 1978; Jahnke and Bahnweg, in press). They stated that well-defined species usually share at least 70–85% similar DNA sequences. We do not want to apply this boundary to unicellular green algae like *Chlorella* in the present state of research, without first acquiring a more detailed knowledge of genome organization in *Chlorella*, and in particular much more extensive hybridization data.

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