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Comparative Physiology, Biochemistry, and the Taxonomy of *Chlorella* (*Chlorophyceae*)[1]

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Abstract: According to biochemical and physiological characters, 77 strains of *Chlorella* were assigned to 12 taxa. The characters used are presence or absence of hydrogenase, formation of secondary carotenoids under nitrogen-deficient conditions, liquefaction of gelatin, products of glucose fermentation, ability to reduce nitrate, thiamine requirement, acid tolerance, salt tolerance, thermophily, and base composition of DNA (GC content). On the other hand, certain nutritional characters, i. e., utilization of organic carbon and nitrogen compounds, were found to be more or less strain-specific, highly variable, and therefore unsuitable for taxonomy.

For over 50 years strains of *Chlorella* (*Chlorophyceae, Chlorococcales*) have served as model organisms in plant physiological and biochemical research. The taxonomy of these very common algae, however, has remained in a state of chaos. For lack of conspicuous morphological characters, classical taxonomy was unable to define the *Chlorella* species.

In the course of time there have been several attempts to apply nutritional characters in *Chlorella* taxonomy. These, however, have failed or have led to the description of a multitude of ill-defined taxa. For example, Shihira & Krauss (1965) have created no less than 30 taxa for 41 strains studied. On the other hand, we have found that properties like the utilization of organic nitrogen or carbon sources are more or less strain-specific, highly variable, and therefore unsuitable for taxonomy in *Chlorella* (Kessler & Czygan 1970, Kessler 1972a).

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During the past 14 years we were able to show that a sound taxonomy for *Chlorella* can be based on certain biochemical and physiological characters (KESSLER & SOEDER 1962, KESSLER et al. 1963, KESSLER 1967a, 1976). In this work, 77 strains were assigned to 12 taxa. FOTT & NOVÁKOVÁ (1969) have found that these taxa are also morphologically different, can be distinguished by careful microscopy, and therefore are in agreement with the classical species concept.

Differential Characters from Biochemistry and Physiology

The starting point of our work was the observation that some strains of *Chlorella* contain the hydrogen-activating enzyme hydrogenase, whereas others are unable to metabolize molecular hydrogen. This result suggested the possibility to use the presence or absence of hydrogenase as a biochemical character for the separation of *Chlorella* species. Hydrogenase is a rather remarkable enzyme (KESSLER 1974a). It catalyzes reactions involving an uptake or a production of hydrogen (Table 1). Reactions of this type must have been very important for the early anaerobic organisms on earth. Due to its high sensitivity against oxygen, however, the enzyme is usually inactive in present aerobic algae. Thus hydrogenase seems to be something like a "living fossil" among the enzymes. We therefore regard it as a primitive, phylogenetically significant, and for that reason exceptionally important taxonomic character.

Another striking difference between different strains of *Chlorella*, which proved to be valuable for taxonomy, is the pigmentation under nitrogen-deficient conditions. Most nitrogen-deficient algae become pale, due to a loss of chlorophylls and carotenoids, but some assume an intense orange colour. This is caused by the formation of so-called "secondary carotenoids", which are chemically different from the normal "primary" carotenoids.

Other biochemical characters of species-specific and taxonomical significance in the genus *Chlorella* are the ability or inability to liquefy gelatin (SOEDER 1963), the products of fermentation formed from glucose under anaerobic conditions (VINAYAKUMAR & KESSLER 1975), the ability or inability to reduce nitrate, and a requirement for thiamine (KESSLER & ZWEIER 1971).

In addition to these biochemical characteristics, some physiological tolerances are also species-specific and taxonomically significant. These are acid tolerance, salt tolerance, and thermophily (KESSLER 1965, 1972b, 1974b). It was surprising to find that, for example, the strains of one *Chlorella* species are able to grow at a pH as low as 2.0, but the most sensitive species will tolerate only acidities at or above pH 6.0. A similar situation was found with salt tolerance. Whereas some species are able to grow in the presence of 4 or 5% sodium chloride, the most sensitive one will not even tolerate 1% NaCl. Most strains of *Chlorella* will grow only at temperatures up to 30 or 32 °C, but the members of one taxon are thermophilic and able to grow at 38 to 42 °C.

Table 1. Reactions catalyzed by the hydrogen-activating enzyme hydrogenase

$$H_2 \rightleftharpoons 2\,H^+ + 2\,e^-$$
$$R + H_2 \longrightarrow RH_2$$
$$CO_2 + 2\,H_2 \xrightarrow{\text{light}} (CH_2O) + H_2O$$
$$O_2 + 2\,H_2 \longrightarrow 2\,H_2O$$
$$NO_2^- + 3\,H_2 \longrightarrow NH_3 + H_2O + OH^-$$
$$RH_2 \longrightarrow R + H_2$$

Not unexpectedly, the base composition of DNA, i. e., the guanine + cytosine content or "GC-content", was found to be the most important and most sensitive character for the separation and definition of *Chlorella* taxa (HELLMANN & KESSLER 1974a).

Grouping of Strains into Species

According to these results we could clearly separate and define 12 *Chlorella* taxa (Table 2). Each taxon is biochemically and, according to the work of FOTT & NOVÁKOVÁ (1969), also morphologically uniform, with very little variation among the different strains. Table 3 shows that, for example, the GC content of the DNA of 14 strains of *Chl. protothecoides* is between 61.5 and 62.0%. The acid limit of growth is at pH 3.5 to 4.0, and all the strains are able to grow in the presence of 3 to 4% NaCl. Table 4 gives the data for three morphologically different varieties of *Chl. fusca*. It is evident that there are clear-cut differences in the base composition of DNA. Initially, we assigned 19 strains to *Chl. vulgaris* (Table 5). The investigation of their DNA, however, revealed distinctly different values for two strains. Most likely they belong to a different, so far undescribed species. This is also supported by their much smaller salt tolerance.

The work of FOTT & NOVÁKOVÁ (1969) has shown that, with two exceptions, all of our 12 taxa are morphologically different. The two exceptions are *Chl. vulgaris* and *Chl. sorokiniana*, which was also designated as *Chl. vulgaris f. tertia* by FOTT & NOVÁKOVÁ (1969). These

Table 2. Biochemical and physiological characters of 12 *Chlorella* taxa (77 strains).
Hydrogenase, secondary carotenoids, liquefaction of gelatin, acid tolerance (pH limit), salt tolerance (% NaCl), thermophily, lactic acid fermentation, nitrate reduction, thiamine (B_1) requirement, base composition of DNA (% GC), and number of strains studied

Species	hydr.	s. car.	gel. liqu.	pH	NaCl %	therm.	lact. ferm.	NO_3 red.	B_1 requ.	DNA % GC	strains
Chl. fusca var. vacuolata SHIHIRA et KRAUSS	+	+	+	3.5	3	—	+	+	—	50	10
Chl. fusca var. fusca SHIHIRA et KRAUSS	+	+	+	4.0	2	—	+	+	—	55	1
Chl. fusca var. rubescens (DANGEARD) KESSLER et al.	+	+	—	4.5	3	—	+	+	—	58	1
Chl. homosphaera SKUJA	+	+	—	6.0	0	—	—	+	—	69	1
Chl. zofingiensis DÖNZ	—	+	—	5.0	1	—	+	+	—	49	3
Chl. minutissima FOTT et NOVÁKOVÁ	—	—	—	5.5	1	—	—	+	—	43	1
Chl. saccharophila (KRÜGER) MIGULA	—	—	—	2.0	4	—	—	+	—	50	6
Chl. luteoviridis CHODAT	—	—	—	3.0	5	—	—	+	—	44	6
Chl. kessleri FOTT et NOVÁKOVÁ	+	—	—	3.0	2	—	—	+	—	56	7
Chl. sorokiniana SHIHIRA et KRAUSS (= *Chl. vulgaris f. tertia* FOTT et NOVÁKOVÁ)	+	—	—	4.0	2	+	+	+	—	65	10
Chl. vulgaris BEIJERINCK	—	—	—	4.0	3	—	+	+	—	62	17
Chl. protothecoides KRÜGER	—	—	—	4.0	4	—	+	—	+	62	14

Table 3. Base composition of DNA (% GC), acid limit of growth (pH), and salt tolerance (% NaCl) of 14 strains of *Chlorella protothecoides*

Strain	DNA % GC	pH	NaCl %
211-7a	62.0	4.0	4
211-7b	62.0	3.5	4
211-7c	62.0	3.5	4
211-7d	61.5	3.5	4
211-8d	62.0	3.5	4
211-10a	62.0	3.5	4
211-10b	61.5	4.0	4
211-10c	61.5	4.0	4
211-10d	61.9	4.0	4
211-10e	61.5	4.0	4
211-11a	61.8	4.0	3
211-11i	61.6	4.0	3
211-13	61.6	4.0	4
211-17	61.6	4.0	4

Table 4. Base composition of DNA, acid limit of growth, and salt tolerance of three varieties of *Chlorella fusca*

	Strain	DNA % GC	pH	NaCl %
var. vacuolata	211-8a	50.3	3.5	3
	211-8b	50.3	3.5	3
	211-8c	50.3	3.5	3
	211-8e	50.2	3.5	3
	211-8g	50.3	3.5	3
	211-8h	50.3	3.5	3
	211-11m	50.3	3.5	3
	211-11n	50.3	3.5	3
	211-15	50.5	3.5	3
	C-1.1.10	50.3	3.5	3
var. fusca	343	55.3	4.0	2
var. rubescens	232/1	57.8	4.5	3

two taxa seem to be morphologically identical. But they are strikingly different in two important physiological characters: *Chl. sorokiniana*, in contrast to *Chl. vulgaris*, contains the enzyme hydrogenase and, in addition, is thermophilic. Thus *Chl. sorokiniana* has two properties which can be regarded as primitive. Furthermore, there is a distinct difference between the two taxa in the GC content of their DNA (Table 2). However, there is no doubt that *Chl. sorokiniana* is rather closely related to *Chl. vulgaris.* It seems reasonable to speculate that *Chl. vulgaris* might have evolved from *Chl. sorokiniana* by loss of the "primitive" characters hydrogenase and thermophily. Another step, i. e., loss of the abilities to reduce nitrate and to synthesize thiamine,

Table 5. Base composition of DNA, acid limit of growth, and salt tolerance of 17 strains of *Chlorella vulgaris* and 2 related strains

Strain	DNA % GC	pH	NaCl %
211-1e	62.0	4.0	3
211-8l	62.3	4.0	4
211-8m	62.5	4.0	3
211-11b	62.3	4.0	3
211-11c	62.0	4.0	3
211-11f	62.0	4.0	3
211-11j	62.3	4.0	3
211-11l	62.5	4.5	3
211-11p	62.5	4.5	3
211-11q	62.5	4.5	4
211-11s	62.3	4.0	4
211-11t	62.3	4.0	3
211-12	61.8	4.0	4
211-19	63.0	4.0	3
211-21	62.5	4.0	3
395	62.3	4.0	4
396	63.3	4.0	4
211-11r	57.7	4.5	1
211-30	57.2	4.5	0

might have led from *Chl. vulgaris* to *Chl. protothecoides*, which is otherwise very similar to *Chl. vulgaris*, even in the base composition of its DNA. The three species discussed can be combined into an informal "*Chl. vulgaris*-group".

Apart from the obvious relatedness of the three varieties of *Chl. fusca* (resp. the "*Chl. fusca*-group") and the members of the "*Chl. vulgaris*-group" mentioned before, the remaining species demonstrate no evident similarities among themselves or towards these groups. This leads to the important, basic problem of how to define and to delimitate a species and, in addition, a genus of these morphologically simple algae.

Of special interest in this connexion is *Chl. sorokiniana.* This taxon originally seemed to comprise 15 thermophilic strains which have identical biochemical and physiological properties. The study of the base composition of their DNA, however, revealed the existence of three groups of strains with different GC values (Table 6). The main group of 10 strains has 64.4 to 65.7% GC. It should be noted that they are from widely different geographic locations, i. e., from North America, Japan, Israel, and Europe. Two additional strains are distinctly dif-

Table 6. Base composition of DNA, upper temperature limit of growth, and geographic origin of *Chlorella sorokiniana* (= *Chl. vulgaris f. tertia*) and related groups of thermophilic strains

Strain	DNA % GC	Temp. limit	Origin
211-8k	64.9	42°	U.S.A.
211-11d	64.4	40°	U.S.A.
211-11k	65.0	38°	U.S.A.
211-32	64.4	42°	Japan
211-33	65.0	42°	Japan
211-34	65.0	40°	Japan
C-1.1.8	65.0	38°	Europe
C-1.6.7	64.4	40°	Europe
SLESS 1	64.5	38°	Israel
SLESS 2	65.7	38°	Israel
211-31	68.8	38°	Europe
1-9-30	68.2	42°	U.S.A.
211-40a	78.6	38°	(Zoochlorella)
211-40c	78.6	38°	(Zoochlorella)
Prag A 14	77.8	38°	Europe

ferent, with 68.2 to 68.8% GC. The final group of three strains is very much different, having GC values of 77.8 to 78.6%. This group includes two zoochlorellas isolated from the fresh-water sponge *Spongilla*. These results raise the question whether we can consider the three groups of strains as members of one species. In view of the very small variation of the GC values within all other taxa, this seems rather unlikely, especially for the third group which is so much different. One might perhaps regard these groups of strains, which only differ in the base composition of their DNA, as a species developing into several new taxa. Otherwise, they might represent a group of morphologically, physiologically, and biochemically similar, but not really related taxa from thermophilic environments.

Another interesting problem is posed by two strains which have properties seemingly intermediate between those of *Chl. saccharo-*

phila and *Chl. vulgaris* (Table 7). Strains 211-1a and C-1.3.1 were originally assigned to *Chl. saccharophila*, mainly because of their characteristic extreme acid tolerance. The study of their fermentation, however, revealed a production of lactic acid as it is typical for *Chl. vulgaris*. The GC content of their DNA is intermediate between those of *Chl. saccharophila* and *Chl. vulgaris*. In addition, SOEDER (1963) has found that 211-1a agrees with *Chl. vulgaris* also in the reaction of its cell wall with ruthenium red. Concerning the shape of its cells, it again assumes a position intermediate between the ellipsoidal cells of *Chl. saccharophila*

Table 7. Comparison of strains 211-1a and C-1.3.1 with *Chlorella saccharophila* and *Chl. vulgaris*

Species	Limit at pH	Lactic acid fermentation	DNA % GC
Chl. saccharophila	2.0	—	50
211-1a, C-1.3.1	2.0	+	57
Chl. vulgaris	4.0	+	62

and the spherical cells of *Chl. vulgaris*. It is tempting to speculate that these two strains might be hybrids of *Chl. vulgaris* and *Chl. saccharophila*. This, of course, would have considerable consequences concerning the existence of sexuality in *Chlorella*. On the other hand, these intermediate strains may represent remnant "stepping stones" in the phylogeny from one to the other species.

Chlorella, a Natural Genus?

Finally, we have to consider the problem of the nature and reality of the genus *Chlorella* itself. The 12 *Chlorella* taxa show a remarkable diversity of biochemical and physiological properties. This becomes the more evident if we compare the situation within the genus *Chlorella* with that of two morphologically better defined genera, i. e., *Ankistrodesmus* and *Scenedesmus*. In all strains of these genera we found hydrogenase, liquefaction of gelatin, and the ability to synthesize secondary carotenoids (KESSLER & CZYGAN 1967). On the other hand, there are species-specific, but comparatively small, differences in acid tolerance and in the base composition of DNA (KESSLER 1967b, HELLMANN & KESSLER 1974b). This latter character again is the most interesting one (Table 8). Within the genus *Scenedesmus*, we find GC values from 52 to 62%. For *Ankistrodesmus*, including *Raphidium*, 63 to 70% GC are typical. Within the genus *Chlorella*, however, the GC values cover the amazingly wide range from 43 to 79%. These data raise the question whether we can regard *Chlorella* as a natural genus which combines a number of truly related species. According to our results it might rather represent an assembly of morphologically similar species of polyphyletic origin. Experiments on DNA hybridization are now in progress which, we hope, will give more information concerning this problem.

Table 8. Base composition of DNA and acid limit of growth within the genera *Chlorella*, *Ankistrodesmus*, and *Scenedesmus*

Genus	DNA % GC	Limit at pH
Chlorella	43–79	2.0–6.0
Ankistrodesmus	63–70	3.5–5.5
Scenedesmus	52–62	3.5–5.5

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