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Taxonomic identification and ultrastructural characterization of a Chilean strain of *Dunaliella*

Identificación taxonómica y caracterización ultraestructural de una cepa chilena de *Dunaliella*

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ABSTRACT

The ultrastructure of a Chilean strain of *Dunaliella salina.* (Chlorophyceae), a halophilic unicellular green alga, is described. Previous ultrastructural observations for this species are confirmed and extended. The occurrence of *Dunaliella salina* (Dunal) Teodoresco is reported for the first time for Chile.

Key words: Chlorophyceae, *Dunaliella salina*, ultrastructure, Chilean strain.

RESUMEN

Se describe la ultraestructura de una cepa chilena de *Dunaliella salina* (Chlorophyceae), una alga verde unicelular halotolerante. Observaciones previas sobre la ultraestructura de la especie fueron confirmadas y extendidas. La presencia de *Dunaliella salina* (Dunal) Teodoresco es informada por primera vez para Chile.

Palabras claves: Chlorophyceae, *Dunaliella salina*, ultraestructura, cepa chilena.

INTRODUCCION

The genus *Dunaliella*, family *Polyblepharidaceae*, order *Volvocales*, class *Chlorophyceae*, includes a variety of ill defined species (ca. 30 species *sensu* Massyuk 1973 and Melkonian and Preising, 1984). Distinguishing features of *Dunaliella* are the following: (1) The cells lack a cell wall, but have a surface coat. The cell is highly responsive to osmotic changes permitting rapid changes in cell shape. (2) Asexual flagellated cells may produce thick walled cysts, which allow survival under environmental stress. (3) Natural habitats which include brine lakes, small pools and salt water ditches near the sea contain a wide range of salt concentrations (Massyuk 1973, Ben-Amotz, & Avron 1980, Borowitzka & Borowitzka 1988). High light intensity, temperature and salt concentrations cause *Dunaliella salina* to accumulate carotenoids (Lerche 1937, Borowitzka 1981 and Borowitzka 1988). Two halophilic species, *Dunaliella salina* (Dunal) Teodoresco and *D. bardawil* (Ben-Amotz & Avron 1983) a nomen nudum and actually considered a strain of *D. salina* Teodoresco, have the ability to accumulate very large amounts, sometimes more than 10% of the algal dry weight is beta-carotene, both in nature or under laboratory conditions (Ben-Amotz *et al.* 1988). The ability to grow at high salinity, with the concomitant production of high levels of beta-carotene and glycerol, has made these flagellates very suitable for commercial exploitation (Borowitzka *et al.* 1984).

The taxonomy of the genus is still uncertain, but it is rich in species, subspecies, ecotypes and isolates (Massyuk 1973, Ginsburg 1987). Major studies contributing to the taxonomy of the genus *Dunaliella* include those by Teodoresco (1905), Lerche (1973), Butcher (1959), Massyuk (1973, in Ukranian), and Melkonian & Preising (1984). Physiological and biochemical studies are far more prevalent than ultrastructural studies. The general ultrastructure of *D. salina* has been

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previously investigated by Trezzi *et al.* (1964), Vladimirova (1978), and Melkonian & Preising (1984). Other contributions give only partial information (Tomasello *et al.* 1980, Trezi *et al.* 1965). Table 1 list the species of *Dunaliella* studied with TEM.

TABLE 1

Species of *Dunaliella* for which fine structural details are known.
Especies de *Dunaliella* para las cuales se conocen detalles estructurales finos.

Dunaliella salina:	(1)	Trezzi *et al.*,1964 Trezzi *et al.*,1965
	(2)	Peterfi & Manton, 1968
	(3)	Werz & Kellner, 1970
	(4)	Vladimirova, 1978
	(5)	Tomasello *et al.*,1980
	(6)	Melkonian & Preising,1984
Dunaliella bardawil:	(1)	Ben-Amotz *et al.*,1982
Dunaliella viridis:	(1)	Anghel *et al.*,1980
Dunaliella primolecta:	(1)	Hyams & Chasey,1974
	(2)	Eyden,1975
Dunaliella tertiolecta:	(1)	Oliveira *et al.*, 1980
	(2)	Hoshaw & Maluf, 1981
Dunaliella bioculata:	(1)	Marano, 1976
	(2)	Chardard, 1987
Dunaliella lateralis:	(1)	Watanabe & Floyd, 1989

A review of the literature concerning the distribution of species of *Dunaliella* confirms that the genus has not been reported for Chile and that only *D. viridis* has been reported from other countries of South America (Massyuk 1973, Ginsburg 1987). Williams (1981) points out that South America has many large salt lakes in a variety of regions but, unfortunately, little biological work has been carried out in them and the literature is sparse and scattered.

To determine the feasibility of industrial biomas and/or secondary metabolite production, a research program under the sponsorship of the United Nations Development Program (UNDP) and the Chilean goverment, with the participation of research teams of three Chilean universities and one fishing factory, has been undertaken. One component of this program included a systematic study of *D. salina.* This paper represents the first contribution on the taxonomy and ultrastructure of a Chilean species of *Dunaliella.*

MATERIALS AND METHODS

Water samples containing a red bloom of *Dunaliella* sp. were obtained during October 1987 from a seaside pool "Sector La Rinconada" (Lat. 23°26'40", 70°30' 50"W), situated at 30 km north of the city of Antofagasta. Unialgal, clonal, but not axenic cultures were obtained and grown in Erdschreiber (ES) seawater medium, illuminated with cool-white fluorescent tubes at an irradiance of 30-200 $\mu Em^{-2} s^{-1}$ on a 12:12 light: dark photoregime, 18-20°C and without aeration. In order to compare morphological and ultrastructural characteristic and to identify the Chilean strain, a cultured strain of *Dunaliella salina* (LB 200) was obtained from the Culture Collection of Algae at the University of Texas (Starr & Zeikus 1987). Flagellated cells from 8- to 10-day-old cultures (active cells) and from stationary phases (25-30 days) were examined with light and transmission electron microscopy.

The following conditions and species were used.

No 1 *Dunaliella salina* (UTEX LB 200). Growth medium Erdschreiber + 100 g Na Cl/l (stationary phase).
No 2 *Dunaliella* sp. Antofagasta (Chile). Growth medium Erdschreiber + 100 g NaCl/l (stationary phase).
No 3 *Dunaliella salina* (UTEX LB 200). Growth medium Erdschreiber + 100 g NaCl/l (active cells).
No 4 *Dunaliella* sp. Antofagasta). Growth medium Erdschreiber + 100 g NaCl/l (active cells).

Light microscopic observations were carried out with a Zeiss microscope fitted with phase contrast and interference optics. For transmission electron microscopy, cells from conditions 1-4 were fixed in a combination of 8% glutaraldehyde in 10% NaCl, 16% aqueous paraformaldehyde, and 4% aqueous Os04. Thus, the final con-

centrations in the mixture were 2% glutaraldehyde, 0.8% paraformaldehyde and 0.2% Os04 in 5% NaCl. The cells were rinsed four times with distilled water, "en bloc" stained in 0.5% aqueous uranyl acetate, dehydrated in acetone, and embedded in Spurr's resin (Spurr 1969). Sections were cut with a diamond knife, post-stained in KMn04 and lead citrate and observed with a Zeiss EM-10 CA TEM operated at either 60 or 80 KV.

In a second sample of cells from #4, the initial fixative consisted of 8% aqueous glutaraldehyde and 4% Os04, combined to yield final concentrations of 2% glutaraldehyde and 1% Os04. These cells were rinsed with distilled water, post-fixed in 1% aqueous Os04, and then treated as above.

RESULTS AND DISCUSSION

The Chilean strain of *Dunaliella* is ellipsoidal to cylindrical (variable under culture conditions) with two apical flagella as long as the cell. The posterior of the cell is occupied by a single cup-shaped chloroplast, with a single large pyrenoid. The nucleus is located in the middle of the cell. The cell size varies between 10-19 μm in length and 8-21 μm in width. Both in the natural environment and under laboratory culture conditions the strains vary in color from yellow-green to deep red. This combination of features is characteristic of *Dunaliella salina* (Dunal) Teodoresco.

Figure 1 is a general diagram of a cell of *D. salina.* This alga has been isolated from salt marshes, pools, and salt lakes from many parts of the world (Hammer 1981). However, to the author's knowledge, it has not been reported from Chile.

In the natural environment and under laboratory culture conditions the strain varied from yellow-green to deep red colour.

The above features have allowed us to identify of the strain with the species *D. salina* (Dunal) Teodoresco.

Ultrastructural Features

The cells are wall-less but thet do have a surface coat (Fig. 2). In most of our preparations the external surface shows a layer of amorphous material of variable thickness and distribution. This "fuzzy" material, possibly glycoprotein (Chardard, 1987), has been observed in other species, *i.e., D. tertiolecta* (Oliveira *et al.* 1980), *D. primolecta* (Eyden 1975), *D. bioculata* (Chardard 1987) and *D. salina* (Melkonian & Preising 1984). This material is probably a generic characteristic of *Dunaliella* (Melkonian & Preising 1984, Watanabe & Floyd 1989). Even though *Dunaliella* lacks a cell wall, the presence of a surface coat is postulated to be a mechanism to withstand a wide range of osmotic conditions (Oliveira *et al.* 1980).

The single, large and cup-shaped chloroplast fills about half the cell volume, with the pyrenoid centrally located, surrounded by starch granules, and penetrated by multiple pairs of thylakoids. The pairs of thylakoids extend only part way into the pyrenoid (Fig. 3). The penetration of the thylakoid membranes seems to be a consistent feature of biflagellate algae whose basal bodies are in the clockwise absolute orientation (Watanabe & Floyd 1989). Chloroplast lobes extend anteriorly almost completely enclosing the central region of cytoplasmic matrix and nucleus. Cells in the stationary phase contain numerous large starch granules. Fig 4 shows small droplets located in the interthylakoid space of the chloroplast. Ben-Amotz *et al.* (1987) have called these accumulations beta-carotene globules.

The nucleus contains a single prominent nucleolus (see Fig. 1). Actively growing cells contain numerous vacuoles with thick membranes and electron-translucent contents. In contrast, stationary-phase cells possess slightly smaller vacuoles. In either case the vacuoles are located centrally near the nucleus.

Golgi bodies are found intercalated between the anterior end of the nucleus and the basal bodies (Fig. 2). This consistent disposition is also considered to be a generic characteristic (Melkonian and Prei-

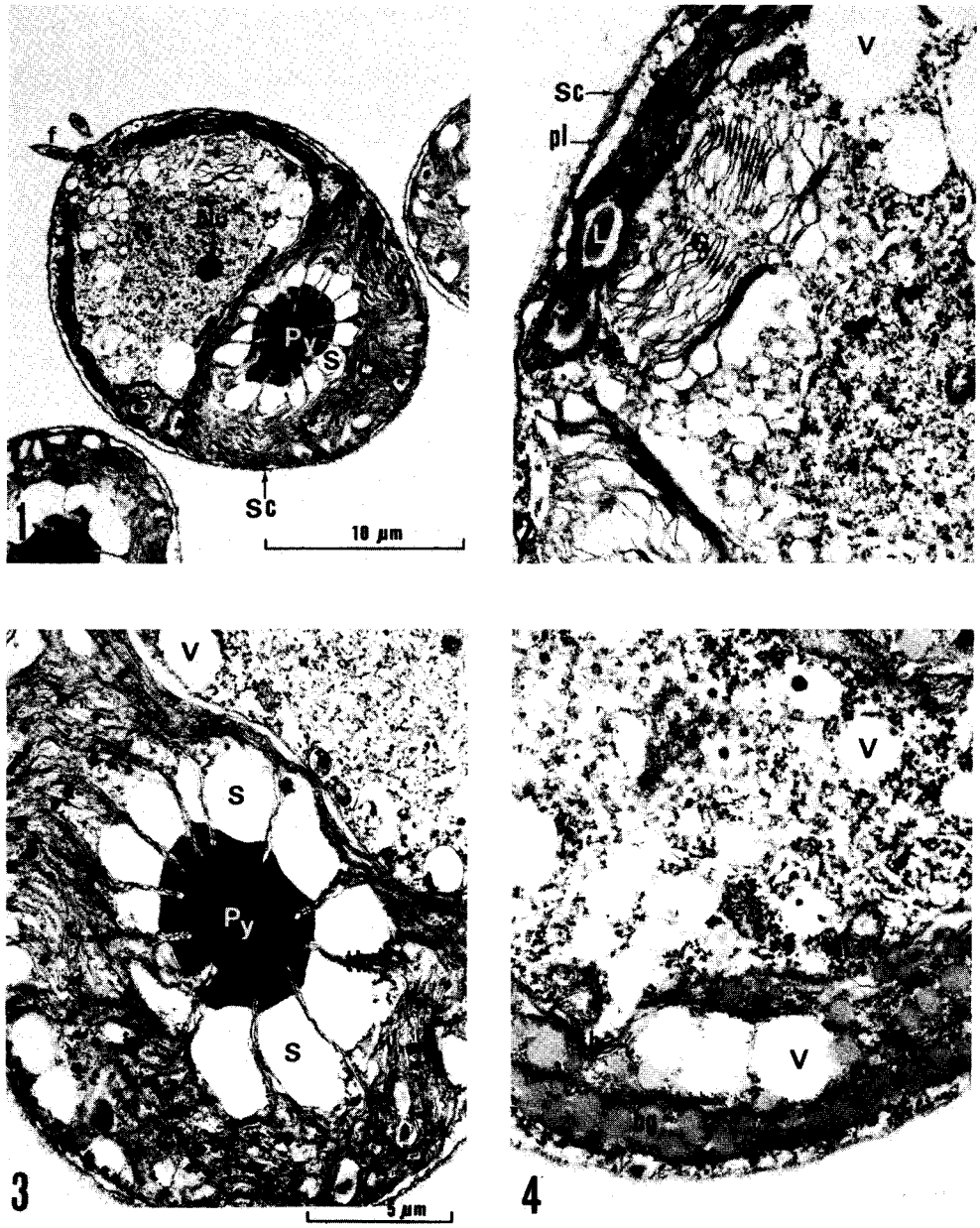

Fig. 1-4: Dunaliella salina (strain CONC-101) vegetative cell general features. Scale bars = 10 µm (Fig. 1); 5 µm (Figs. 2-4). Fig. 1. Longitudinal section showing flagella, nucleous, nucleolus, chloroplast, pyrenoid, starch grains, vacuoles. Fig. 2. Section through the Golgi body, see also the plasmalemma and the surface coat. Fig. 3. Section through the pyrenoid showing the traversing pairs of thylakoids. Fig. 4. Section showing the beta-carotene accumulation (beta-carotene globules).

Dunaliella salina (strain CONC-101), caracteres generales de una célula vegetativa. Escala 10 µm (Fig. 1); 5 µm (Figs. 2-4) Fig. 1. Sección longitudinal mostrando los flagelos, nucleo, nucleolo, cloroplasto, pirenoide, gránulos de almidón, vacuolas. Fig. 2. Sección longitudinal a través del aparato Golgi, también se observa claramente la membrana celular (plasmalemma) y la cubierta superficial. Fig. 3. Sección a través del pirenoide mostrando los pares de tilacoide penetrando en él. Fig. 4. Sección mostrando la acumulación de beta-caroteno (glóbulos de beta-caroteno).

Note: Abbreviations used in figures. bg = beta-carotene globules; chl = chloroplast; f ∴ flagella; G = Golgi body; L = lipid body; N = nucleus; Nu = nucleolus; pl = plasmalemma; Py = pyrenoid; S = starch grain; Sc = surface coat; th = thylacoids; V = vacuole.

Abreviaciones usadas en las figuras: bg = glóbulos de beta-caroteno; chl = cloroplasto; f = flagelos; G = aparato Golgi; L = corpúsculo de lípido; N = núcleo; Nu = nucleolo; pl = plasmalema; Py = pirenoide; S = gránulo de almidón; Sc = cubierta superficial; th = tilacoides; V = vacuolas.

sing 1984). The endoplasmic reticulum is typically in close proximity to the plasmalemma over most of the cell. The mitochondria are consistently near the basal bodies.

The features of the flagellar apparatus of each strain fit the description given for *D. lateralis* by Watanabe and Floyd (1989). Two flagella of equal length are present. The typical transition region pattern described for the Chlorophyceae (Melkonian & Preising 1984) connects the flagella to the basal bodies. The basal bodies are linked by a prominent distal connecting fiber which is striated and connected to basal body triplets. The basal bodies are also connected proximally by non-striated electron-dense material which extends from proximal sheaths. Two proximal fibers are present and connected laterally to the basal bodies.

The flagellar rootlet system is 4-2-4-2 and the basal bodies are offset in the clockwise absolute orientation without overlap. Also, two accessory basal bodies are located near the two-membered rootlets. A SMAC (striated microtubule-associated component) was not observed in any of the isolates. As in *D. lateralis* the rhizoplast appears to be non-striated, unbranched, and extends from the proximal sheaths on the basal body to mitochondria.

Taxonomy of Dunaliella salina

Butcher (1959) comments that either *D. salina* is a very variable species or there are several distinct species included under the same name. The criteria applied by Butcher were only morphological. Ginsburg (1987) suggests that the major problem facing the taxonomist with regard to *Dunaliella* is the use of only morphological criteria. Consequently, the application of physiological and biochemical criteria might be more useful to the taxonomist than the usual morphological ones. However, even this approach appears to be disputable in the light of the existing data. For example, Ben-Amotz & Avron (1982) separate *D. bardawil* from *D. salina* on the basis of biochemical features, *i.e.* globules of beta-carotene are present in *D. bardawil,* and absent from *D. salina* (probably *D. salina* of Ben-Amotz and Avron 1982 is *D. parva*). Borowitzka and Borowitzka (1988) reported beta-carotene globules in *D. salina.* Likewise the glycerol accumulating *D. bardawil* may be identical to one of the beta-carotene-accumulating variants of *D. salina* described by Drokova (1961) and Massyuk (1961). It must be pointed out that *D. bardawil* Ben-Amotz and Avron should be considered and illegitimate name because it lacks an original Latin description and, according to Borowitzka & Borowitzka (1987), is actually a strain of *D. salina* (Dunal) Teodoresco. Borowitzka & Borowitzka (1988) also state that "many of the strains in the UTEX culture collection are misnamed". The UTEX-200 strain (= CCAP 19/3) investigated by Melkonian & Preising (1984) as *D. salina* probably belong to *D. viridis.* These authors found eyespots in the UTEX strain, which were "only rarely seen in our material and consisted of a single layer of lipid globules located in an anterior lobe of the chloroplast" (Melkonian & Preising 1984). The presence or absence of eyespots is a distinguishing feature between *D. salina* and *D. viridis.* Our observations on the strain UTEX-200 have not demonstrated neither the presence of the eyespots nor the development of beta-caroteno globules. It remains green at high salt concentration (personal observations). Other ultrastructural features of UTEX-200 strain and the Chilean strain of *D. salina* are similar.

The boundaries of species of *Dunaliella* are vague as a result of the considerable difficulty in integrating morphological and physiological data. It appears that the *D. salina* complex (*D. salina, D. pseudosalina, D. bardawil* and *D. parva*) corresponds to the two species originally described by Teodoresco (1905), *D. salina* (Dunal) Teodoresco and *D. viridis.* Teodoresco. This is in agreement with Butcher (1959), Bass-Becking (1931) and Johnson *et al.* (1958). All are species extremely salt-tolerant and some of them remain green at all salt concentrations.

All the morphological and ultrastructural features are similar to those given for

D. salina (Dunal) Teodoresco. However, recent chemical data show the Chilean cells to have a higher concentration of alpha-carotene than beta-carotene. If this characteristic is constant under natural and laboratory conditions, our isolate could be treated as a dictinct physiological race if not as a new species, as suggested by Borowitzka (personal communication).

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