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Cell wall development, microfibril and pyrenoid structure in type strains of *Chlorella vulgaris, C. kessleri, C. sorokiniana* compared with *C. luteoviridis* (Trebouxiophyceae, Chlorophyta)

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With 14 figures in the text

Abstract: Ultrastructural examination of three glucosamine-type Chlorella species (C. vulgaris var. vulgaris, C. kessleri, C. sorokiniana), forming a related group in phylogenetic trees inferred from 18S rRNA gene sequences (FRIEDL 1995, HUSS et al. 1999), revealed a similar cell ultrastructure but some differences in early and later stages of the cell wall development. All species mentioned above contain the monosaccharide glucosamine as the main constituent of the rigid cell wall (TAKEDA 1991, 1993a, 1993b). A thin electrondense layer is the first visible structure covering the young daughter protoplasts of C. vulgaris and C. sorokiniana. Layered microfibrils can be observed in cross-sections of adult cell walls. Remnants of the broken maternal cell walls (MCW) persist in a culture medium. In C. kessleri the initial electrondense layer was not found. The cell wall is hardly visible, no microfibrillar structure was detected. No MCW remnants were found in the medium. Negatively stained microfibrils of all the three species obtained by 1N NaOH and 2M TFAA treatment are straight or slightly bent. The pyrenoid is transversed by two thylakoids. The rigid cell wall of C. luteoviridis is composed of glucose and mannose (TAKEDA 1991, 1993a, 1993b). However some ultrastructural features of C. luteoviridis resemble that of glucosamine-type chlorellas (the thin electrondense layer covering the young daughter protoplasts, microfibrillar structure of the adult cell wall visible on cross-sections, MCW remnants persisting in a medium). Microfibrils do not form a net, they are kinked and flexuous. C. luteoviridis differs from glucosamine-type species in the pyrenoid structure (the pyrenoid is bisected by four or two thylakoids). Thickness of microfibrils in all studied species is about 5 nm.

Key words: Trebouxiophyceae, Chlorophyta, Chlorella vulgaris, C. kessleri, C. sorokiniana, C. luteoviridis, rigid cell wall, microfibrils, pyrenoid structure.

Introduction

A taxonomy of the genus *Chlorella* BEIJERINCK based on light microscopy observations was published by FOTT & NOVÁKOVÁ (1969). In this monograph the

authors established the nomenclatoric types (iconotypes and type cultures) and documented the phenoplasticity of eight species and four varieties. After numerous chemotaxonomical investigations of Kessler and his co-workers (Kessler 1992, KESSLER & HUSS 1992) it became evident that the genus Chlorella represents taxonomically heterogeneous assembly of simple unicells. KALINA & PUNČOCHÁŘOVÁ (1987) and ANDREYEVA (1998) restricted the list of Chlorella species only to those, which do not contain the algenan layer in their cell walls. A new insight into the taxonomy of coccoid green algae was achieved by employment of 18S rRNA gene sequence data. Taxa of Chlorella were dispersed over two classes: the Chlorophyceae and the Trebouxiophyceae (FRIEDL 1995, HUSS et al. 1999). The class Trebouxiophyceae has been established by FRIEDL (1995). HANAGATA & CHIHARA (1997) proposed that only the species of Chlorella vulgaris-group should be kept in the genus Chlorella (Trebouxiophyceae). The existence of a distinct group containing C. vulgaris BEIJERINCK, C. lobophora ANDREYEVA, C. sorokiniana SHIHIRA et KRAUSS and C. kessleri FOTT et NOVÁková has been also resolved in the 18S rRNA phylogeny (Huss et al. 1999). TAKEDA (1991, 1993a, 1993b) proposed the monosaccharide composition of rigid cell walls as a taxonomical marker. He found that the rigid cell walls of C. vulgaris, C. sorokiniana and C. kessleri consisted of glucosamine, while other Chlorella species (concerning C. luteoviridis) possessed glucose and mannose as a main constituent of the rigid cell wall. KAPAUN & REISSER (1995) studied electronmicroscopic preparations of rigid cell walls of symbiotic Chlorella Pbi strain, which belongs to the Chlorella vulgaris /sorokiniana cluster (Huss et al. 1999, Reisser & WIDOWSKI 1992). The rigid cell wall of this strain is composed of the monosaccharide glucosamine (KAPAUN et al. 1992). In both freeze-etched and negatively stained samples KAPAUN & REISSER (1995) revealed microfibrils of diameter about 5 nm. They investigated the rigid cell wall by sugar analysis, infra red spectroscopy, lectin binding, enzymatic degradation, X-ray diffraction and concluded that it was composed of the polysaccharide glycosaminoglycan, which can be regarded as a chitin-like glycan. The authors assumed the same nature of microfibrils in related species with glucosamine rigid cell wall (C. kessleri, C. sorokiniana and C. vulgaris). Another character indicating a closer evolutionary relationship of species with glucosamine cell walls is the identical pyrenoid structure. The thylakoid that penetrates into the pyrenoid matrix, is uniformly double-layered (IKEDA & TAKEDA 1995).

The purpose of this study is to compare an early stage of the cell wall development, the structure of extracted microfibrils and the pyrenoid morphology in type strains of three glucosamine-type species of *Chlorella: C. vulgaris* var. *vulgaris, C. kessleri* and *C. sorokiniana.* Ultrastructure features mentioned above are compared with *C. luteoviridis*, which shows certain resemblance to glucosamine-type species. The microfibrillar structure of a *Chlorella*-like species with glucose and mannose as a main constituent of the rigid cell wall has not been studied yet.

Material and methods

Chlorella type strains were obtained from the Culture Collection of Algae at the Department of Botany, Faculty of Natural Science, Charles University in Prague (CAUP): *C. vulgaris* var. *vulgaris* strain H1955 (identical with SAG 211-11b); *C. kessleri* strain H1901 (identical with SAG 211-11 g); *C. sorokiniana* strain H1957 (identical with SAG 211-8k) and *C. luteoviridis* strain H1906 (identical with SAG 211-2a). *Chlorella* cultures were grown in 60 ml glass tubes filled with BOLD's Basal Medium (STEIN 1975), aerated with filtered air under continuos illumination of light intensity 100 mW cm⁻². Cells were cultivated at 25 °C for 6 days.

Preparation of the ultrathin sections. Actively growing cells were harvested by centrifugation, fixed with 2% (w/v) glutaraldehyde in 0.05 M phosphate buffer (pH 6.9) at room temperature overnight, and postfixed with 1% OsO₄ in 0.05 M phosphate buffer for 2 hours at 12 °C and with 1% uranyl acetate in methanol overnight. Cells were dehydrated in graded concentration of ethanol, than transferred into butanol and embedded in SPURR's low viscosity epoxy medium (SPURR 1969). Sections were cut with a diamond knife by an Ultracut microtome and collected on a Formvar-coated copper grid. Sections were poststained with uranyl acetate and bismuth oxynitrate.

Preparation of the cell walls. Algal cells (ca. 1 ml of packed cell volume) were mechanically broken by vortexing (on a Griffin & George Ltd. homogenizer) in a plastic 7 ml tube in the presence of glass beads (0.5 mm diameter, Sigma). Cell walls were gathered by successive centrifugation (1500 r.p.m. for 5 min. – sediment was under interest, cell organelles and diluted material were eliminated; 800 r.p.m. for 1 min. – supernatant was under interest, unbroken cells and cell conglomerates were eliminated; 4200 r.p.m. – broken cell walls were harvested). Cell walls were washed with double destilled water by repeated centrifugation.

Extraction of the cell walls. Cell wall preparations were extracted according to KAPAUN & REISSER (1995) by alkaline treatment (1 N NaOH, 20 min, 100 °C) and, after washing with water, with 2 M trifluoroacetic acid (TFAA, 2 h, 110 °C). Rigid cell wall material was diluted and dropped onto Formvar-coated copper grids. Negative staining was achieved by 2% uranyl acetate, after 5 min the liquid was carefully removed. All samples were examined with a transmission electron microscope Philips 300.

Results

Cell wall

The cell walls of the tree species (C. vulgaris var. vulgaris, C. sorokiniana and C. luteoviridis) differ in its development, in electron density after contrasting solu-



tion application, and in the ability to persist in the medium after autospore deliberation. A thin electrondense layer as the first visible structure of the newly formed cell wall was observed in autosporangia of *C. vulgaris* var. *vulgaris*, *C. sorokiniana* and *C. luteoviridis* (Figs 2, 6, 8). The cell wall thickness depends on the growth rate. In the intensively growing culture, the cell walls remain thinner. Layered microfibrils are visible on a cross-section of adult cell walls in all species named above (Figs 1, 4, 7). A cell wall structure of *C. kessleri* is hardly visible not only in young autospores but as well in adult cells, because of low contrast. Contrast of the cell wall was not increased even after additional staining with 2% potassium permanganate solution. No microfibrillar structure was detected even in a maternal cell wall (MCW) of the autosporangium (Figs 5, 6). MCW remnants persist in the culture medium of *C. vulgaris* var. *vulgaris*, *C. sorokiniana* and *C. luteoviridis* (Figs 2, 4, 7).

Pyrenoid

Pyrenoids of all studied species show a very uniform structure. The pyrenoid is surrounded by a starch sheath of two large plates. Pyrenoids in glucosamine-type species are bisected always by two thylakoids (Figs 1, 3, 5). Pyrenoid bisected by four thylakoids has never been observed in *C. vulgaris, C. sorokiniana* and *C. kessleri*. In *C. luteoviridis* 211-2a the pyrenoid is transversed by either four (Fig. 7) or two (Fig. 8) thylakoids. Pyrenoid bisected by four thylakoids has appeared more frequently (about 80% of cells, where the thylakoids were clearly visible), however the independent thylakoids were difficult to follow in same parts of pyrenoid. A starch sheath bisected by another group of thylakoids, except those passing through the pyrenoid, was occassionally observed.

Microfibrils

Glucosamine-type group *C. vulgaris* var. *vulgaris*. Extracted cell walls appear to be reticulate with minimum of a positively stained interfibrilar material. Relatively long straight microfibrils are visible to protrude from the extracted cell wall, especially where the wall has ruptured (Fig. 9). Detailed study of negatively

Figs 1-6. 1. Chlorella vulgaris var. vulgaris – vegetative cell, developed cell wall with microfibrillar structure, two thylakoids bisect the pyrenoid. 2. C. vulgaris var. vulgaris – sporangium, note the thin electrodense layer as a first visible structure of the cell wall (arrowhead). 3. Chlorella sorokiniana – vegetative cell, pyrenoid is bisected by two thylakoids. 4. C. sorokiniana – microfibrillar maternal cell wall (MCW) surrounding the cell walls of the autospores. 5. Chlorella kessleri – vegetative cell with parietal chloroplast, note the hardly visible cell wall, the pyrenoid is bisected by two thylakoids. 6. C. kessleri – sporangium, newly forming autospores are not divided by a thin electrondense layer. [Bar = $0.5 \, \text{um.}$]



Figs 7-10. 7. Chlorella luteoviridis – vegetative cell, MCW remnants persist in the medium, the pyrenoid is bisected by four thylakoids. 8. C. luteoviridis – vegetative cell, two thylakoids bisect the pyrenoid. Figs 9-10. Extracted cell walls. 9. C. vulgaris var. vulgaris, reticulate structure of an empty cell wall, note long protruding microfibrils (arrowhead). 10. C. luteoviridis – empty cell walls with a fuzzy surface and a large amount of a positively stained interfibrilar material. [Bar = $0.5 \mu m$.]

Figs 11-14. Negatively stained cell wall microfibrils extracted by 1N NaOH and 2M TFAA. 11. C. vulgaris var. vulgaris, note the kinked microfibril (arrowhead). 12. C. kessleri. 13. C. sorokiniana. 14. C. luteoviridis, flexuous microfibril-like structures embedded in interfibrillar material. [Bar = $0.1 \,\mu$ m.]



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stained microfibrils (Fig. 11) revealed the thickness from 4 to 6 nm. Some of the microfibrils are bent (arrow), this is probably an artefact due to acid hydrolysis or drying on the grid.

C. kessleri. Extracted cell walls of *C. kessleri* (not shown) strongly resemble that ones of *C. vulgaris*. Negatively stained microfibrils appear straight, but shorter and more randomly oriented compared with *C. vulgaris* (Fig. 12). They form a net of higher density. Occasionally two or three microfibrils form a bundle. A small amount of additional resistant material surrounds the microfibrils. The thickness of MFs vary from 3 to 5 nm.

C. sorokiniana. Extracted cell walls of *C. sorokiniana* (not shown) resemble that ones of *C. vulgaris* and *C. kessleri*. Microfibrils are 3 to 5 nm thick (Fig. 13), which is comparable with *C. kessleri* MFs. Microfibrils are almost straight and they show an interwoven random orientation. A bundle of two MFs is only seldom formed. The length of MFs is very difficult to follow.

Glucosomannan-type group *C. luteoviridis.* The appearance of extracted cell walls of *C. luteoviridis* completely differs from the glucosamine species. The surface of empty walls is fuzzy, no reticulate structure is visible, a large amount of a positively stained interfibrillar material is present (Fig. 10). This material (probably of polysaccharidal nature) is able to resist 1 N NaOH and 2M TFAA extractions. Higher magnification shows microfibril-like structures embedded in interfibrillar material, creating protrusions of the surface (Fig. 14). "Microfibrils" do not form a net, they are bent and flexuous. Occasionally helices of two or more are visible. Their thickness is from 4 to 6 nm. The length cannot be measured since only occasionally is a fibrillar end detectable.

Discussion

Some ultrastructural features of the glucan-type species *C. luteoviridis* resemble that of glucosamine-type chlorellas. They are: 1) the thin electrondense layer as the first visible structure of the cell wall, 2) layered and microfibrillar structure visible on cross-sections of the adult cell wall and 3) the cell wall remnants persisting in the medium. On the other hand the close relationship of *C. luteoviridis* with *C. saccharophila* is supported by 18S rRNA phylogeny, while glucosamine-type chlorellas form a distinct group (Huss et al. 1999).

The ability to persist in the medium was ascribed to the presence of an acetolysis resistant biopolymer (algenan) in MCW (ATKINSON et al. 1972, CORRE et al. 1996). MCW can accumulate in the medium also in strains, which do not produce algenans. The resistence to acetolysis may be a result of the presence of additional sugars in the cell wall (BURCZYK et al. 1995). This is probably the case

of C. vulgaris var. vulgaris and C. sorokiniana. It is speculative whether the resistance might be caused by chitin-like glycans.

According to our investigations *C. luteoviridis* (strain 211-2a) shows not the same but very similar ultrastructural features in the pyrenoid structure as do the glucosamine-type species. IKEDA & TAKEDA (1995) studied the pyrenoid structure in a different strain (211-2b) of *C. luteoviridis*. They have found the thylakoids connected with the surface of the pyrenoid but not penetrating it. Tubelike structures surrounded by numerous pyrenoglobuli penetrated the pyrenoid matrix. Neither tubelike structures nor pyrenoglobuli were visible in our micrographs. The rigid cell wall of *C. luteoviridis* 211-2a is composed mostly of glucose and of about 9% mannose (TAKEDA 1991).

The appearance of extracted cell walls of *C. luteoviridis* differs considerably from the glucosamine-type species (compare Figs 9 and 10). Microfibril-like structures are embedded in interfibrillar material and their appearance strongly contrasts with sharply delimited MFs of the glucosamine-type species. Microfibrillar structure probably reflects the sugar composition of the rigid cell wall. The microfibril polysaccharide may be mannoglucan. However other possibilities that both glucan and mannan participated to form microfibrils can not be excluded (TAKEDA pers. comm.).

The thickness of microfibrils of *C. kessleri*, *C. sorokiniana*, *C. vulgaris* var. *vulgaris* and *C. luteoviridis* is about 5 nm which is in congruence with the results of KAPAUN & REISSER (1995). The thickness of microfibrils can vary considerably even in one single cell wall. The measurement of microfibril's dimension in negative stained preparations is not very precise especially for thin MFs. It is sometimes difficult to estimate whether certain imprint represents one or two MFs. Neither dimensions nor appearance of MFs can be used to determine whether they are composed of cellulose, chitin or other polysaccharides.

The comparison of the species belonging to the *Chlorella vulgaris*-group suggests the congruence of molecular data with the conclusions based on ultra-structural and chemotaxonomical features.

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