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CELL DIVISION IN THE GREEN MICROALGA MARVANIA GEMINATA: SEMI-EXOGENOUS AUTOSPOROGENESIS, ROLE OF COATED PIT-MICROTUBULE COMPLEXES, AND SYSTEMATIC SIGNIFICANCE

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SUMMARY

Cell division in the unicellular coccoid green alga *M. geminata* has been studied with transmission electron microscopy. Unlike the division cycle in other coccoid green algae, mitosis occurs not before the cell has expanded until about twice its original volume. Cell expansion involves the escape of part of the protoplast through a break in the cell wall. During mitosis the nuclear envelope remains intact. The spindle is not associated with centrioles, which seem to be absent throughout the cell cycle. The division site at the plasma membrane is marked by a short cortical microtubule which appears before division is initiated. The plasma membrane near the microtubule invaginates to form a coated pit-like structure. The latter continues to grow inward along with the short microtubule, and in effect constitutes the cleavage furrow. After furrowing, each daughter cell forms a wall layer at the new septum. Cells separate after the old, common wall layer is broken at the level of the septum. A model explaining cytokinetic events, in particular the furrowing mechanism, is presented. It is concluded that *M. geminata* may be considered as a member of a broadly conceived order Chlorococcales.

1. INTRODUCTION

Light microscopical features of the external morphology and the cellular organization of the plant body (including its reproductive structures) continue to play a dominant role in current text book presentations of the higher-level classification of green algae (example: ETTL 1980). For example, unicellular and colonial non-flagellated forms are generally assigned to the order Chlorococcales, yet few will doubt that the order thus conceived is artificial, heterogenous, and cannot be defined by sharp, unambiguous criteria (cf., KOMÁREK & FOTT 1983). Especially those coccoid green algae in which the products of cell division are held together due to the persistence of extra-cellular substances (mucilage, walls) which results in more or less coherent tissue- or filament-like complexes, illustrate the difficulty of establishing discrete morphological categories (taxa) in what appears to be a virtual continuum of increasing levels of morphological complexity among the green algae.

The question of how to distinguish between multicellular chlorococcalean forms and those with higher grades of organization like filaments and tissue-like

complexes has led BOLD and co-workers to focus their attention to the potential systematic value of the patterns of cell division that occur in coccoid green algae. This resulted in the recognition that two basic types of cell division exist, viz. 'desmoschisis', or vegetative cell division, and 'eleutheroschisis', or non-vegetative division. It was proposed that the order Chlorococcales would be restricted as to include algae that divide solely by eleutheroschisis, and that those known to be capable to divide by desmoschisis as well would have to be assigned to a separate order Chlorosarcinales (see BOLD & WYNNE 1985 for a recent summary, and SILVA 1982, TSCHERMAK-WOESS 1982, and ETTL & KOMÁREK 1982 for a critical discussion.)

The problematic taxonomic position of the so-called primitive Ulotrichales (which might be regarded as a natural link between Chlorococcales and truly filamentous green algae) has been discussed in various papers by HINDÁK (e.g., 1962, 1978, 1982). His concept of the Chlorococcales is based more on the inability to form filaments than on the occurrence of specific cell division patterns. The term 'filament' was obviously applied in a broad sense, as diad-forming green algae like *Elakatothrix* are considered to be filamentous in HINDÁK's view, and therefore are placed in the order Ulotrichales.

The spherical unicellular non-motile green microalga *Marvania geminata* Hindák 1976, presents a special case in point that illustrates the problems mentioned above concerning the taxonomic delimitation of the order Chlorococcales. The vegetative cell features of *Marvania* obviously suggest an affinity with chlorococcalean algae, yet its characteristic and only mode of cell division involving a budding-like mechanism (HINDÁK 1976; HEYNIG 1980; REYMOND *et al.* 1986) is not known to occur in any member of the Chlorococcales, nor in any other green alga, for that matter. The fact that under certain conditions linear (filament-like) cell complexes can be produced led HINDÁK (1976, 1978) to conclude that *M. geminata* should be classified, at least tentatively, in the order Ulotrichales.

It now seems beyond doubt that for the establishment of a more stable and 'natural' classification of green micro-algae, especially at intermediate and higher taxonomic levels, it is essential to de-emphasize gross morphological and reproductive features and to search for novel taxonomic markers, both structural and biochemical, at cellular and macromolecular levels. For this reason, a preliminary ultrastructural study of *M. geminata* was undertaken by REYMOND *et al.* (1986), who found that its seemingly exotic method of cell division is basically a special form of autosporogenesis. Our present report examines the ultrastructural aspects of cell division in *M. geminata* in more detail.

2. MATERIALS AND METHODS

A strain of *Marvania geminata* Hindák, (nr. 1978/21, isolated by Dr. F. Hindák, Bratislava, Czechoslovakia) was grown as a unialgal culture in aerated Woods Hole MBL medium following NICHOLS (1973), with TRIS being replaced by 2 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) at 16 °C

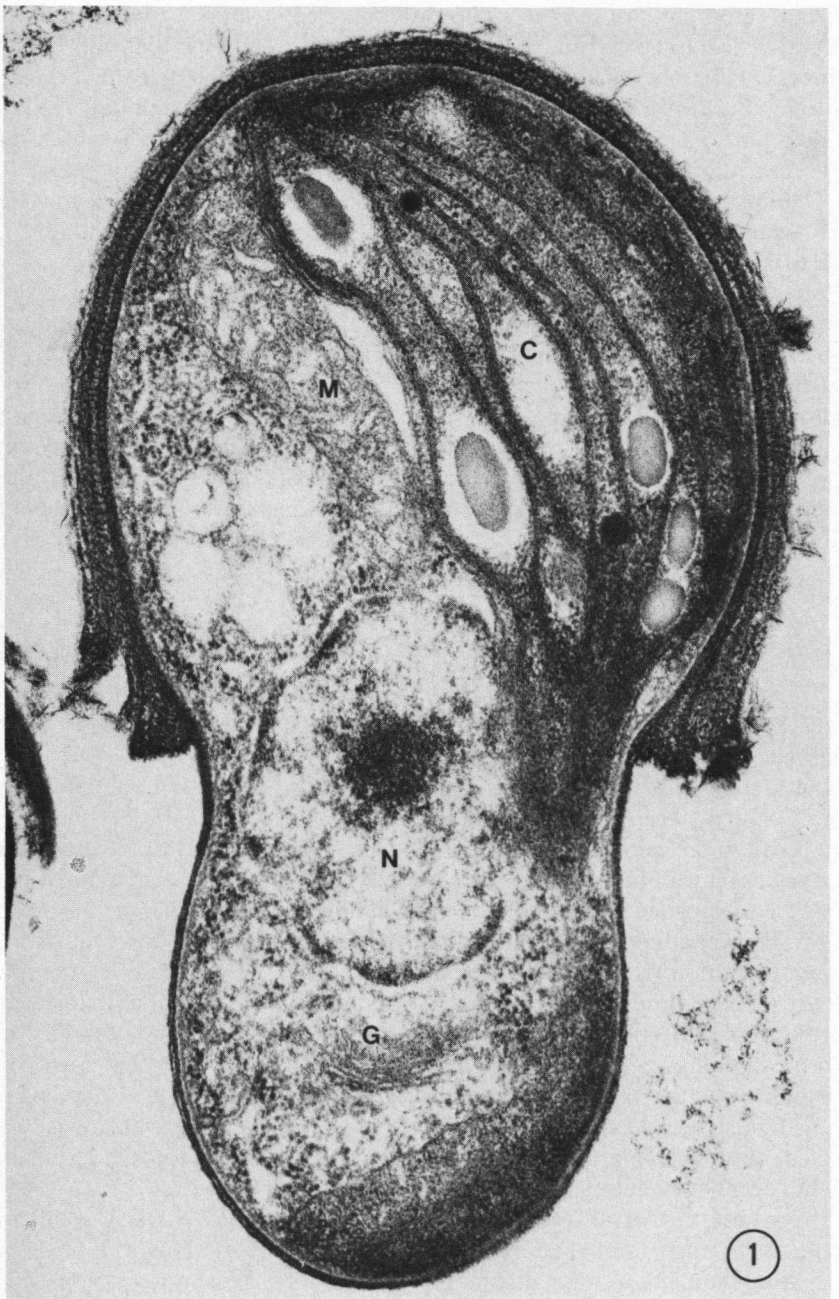


Fig. 1. *Marvania geminata.* Advanced stage of cell expansion prior to cell division, with old and new (inner) wall layers clearly visible. C, chloroplast; G, Golgi body; M, mitochondrion; N, nucleus. ×51000.

under a 16 h light: 8 h darkness photoperiod. Exponentially growing cultures were sampled for electron microscopy during the light period (the highest numbers of dividing cells being noted at approximately the sixth h of light) using the following protocols. (1) Centrifugation of 10 ml samples in a table centrifuge at 3500 rpm for 5 min, followed by resuspension in c. 4 ml fixative (1% glutaraldehyde in medium containing 0.1 M HEPES, pH 7.2) at room temperature for 30–50 min; centrifugation followed by embedding of the pelleted cells in warm 1.5% agar; repeated washing of the immobilized cells in medium containing 0.1 M HEPES; postfixation in 1% aqueous OsO_4 for 90 min at room temperature followed by a thorough wash in distilled water. (2) As in method (1), except that the primary fixative consisted of 0.2% glutaraldehyde in medium, and that embedding in agar took place after osmication. (3) As in method (2), except that the primary fixative consisted of 0.2% glutaraldehyde, 0.2% tannic acid (galloyl glucose, MW 1701.21, Polysciences) and 0.05% saponin (Polysciences) in medium. The specimens, cut in c. 8 mm^3 blocks, were dehydrated with acetone or acidified 2,4-dimethoxypropane, embedded in Spurr's resin, and sectioned using a Reichert Ultracut-E microtome and a diamond knife. Observations were made and photographs taken using Philips EM-300 (Leiden) and Zeiss EM-10 (Lausanne) electron microscopes.

3. RESULTS

The general ultrastructure of *M. geminata* has been the subject of a previous study (REYMOND *et al.* 1986), and will not be repeated in the present report which will focus on the details of cell division.

The first stage of each division cycle involves an increase in volume of the initially spherical cell until it has doubled in size. This cell expansion is effected by a break in the cell wall and a bulging of the protoplast out of the seemingly rigid encasing wall in a budding-like fashion (*fig. 1*). At the same time, a new thin wall is deposited all around the cell, including the bulging part of the protoplast. The expanding cytoplasm contains the nucleus (which shows no sign of an imminent mitosis yet) and the elongating parietal chloroplast. By the time the expanded cell has reached a length of about twice its original diameter, a more or less mirror-like symmetrical intracellular organization is established, with the initially single mitochondrion and Golgi-body having duplicated, and the nucleus having assumed a central position at what might be described as the 'isthmus' between the two semi-cells (*fig. 2*). After the chloroplast has cleaved, the nucleolus disappears, and irregular condensation of chromatin patches occurs, indicating that the nucleus is entering prophase.

At this first stage of mitosis a short plasma membrane (PM) associated microtubule (MT) appears at the level of the 'isthmus' (*fig. 4*). This MT is visible in no more than 5 or 6 consecutive transverse sections. Most observations seem to indicate the presence of one single cortical MT, but on one occasion a second MT was noted in similar position at the opposite side of the nucleus (micrograph not shown). As the nucleus passes through prometaphase during which a much

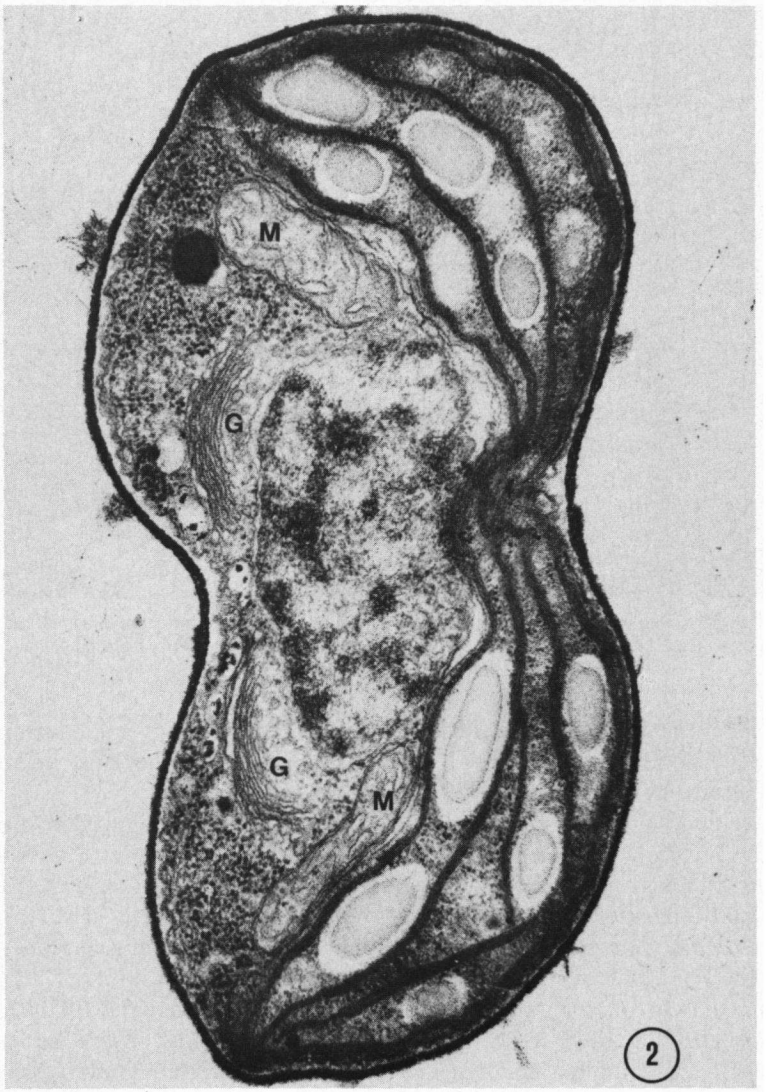
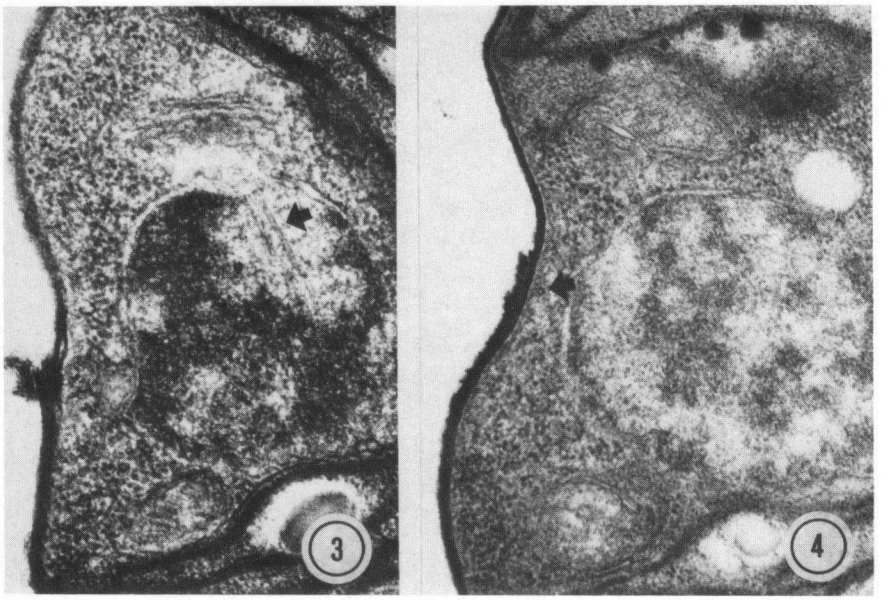


Fig. 2. *M. geminata*. Cell expansion is completed, and mitosis has been initiated (prophase). Mitochondria (M) and Golgi bodies (G) have duplicated, and chloroplast division is underway. × 52000.

reduced spindle apparatus is developed (*figs. 3, 5*) the PM subtending the cortical MT forms a minute indentation or pit with a diameter of less than 50 nm. The pit PM is associated with a densely staining abluminal coat (*figs. 6–8*), which is particularly well visible when a primary aldehyde fixative containing tannic acid is employed. This suggests that proteins are a major constituent of the coating material. There is some evidence (not shown) that the coated pit may be associated with two MTs instead of one MT.



Figs. 3, 4. *M. geminata*. Fig. 3. Prophase nucleus in which spindle microtubules are being formed (arrow). ×40000. Fig. 4. Cell at prophase in which a plasma membrane-associated microtubule (arrow) marks the presumptive division site. ×42000.

The mitotic apparatus is enclosed by an intact nuclear envelope during most of the division period, if not permanently. Centrioles were not observed during any stage of the cell cycle.

After completion of mitosis the now elongated PM invagination proceeds to grow inward. The coating material/MT complex persists at the front end of the invagination or furrow, at least until about half of the internuclear cytoplasm has been bisected. A just completed septum with an associated MT is shown in *fig. 9*. At no time have cytokinetic MTs been observed during cleavage, apart from the 1 or 2 MTs just described.

Immediately following completion of the septum (shown in *fig. 10*) the dictyosome in each daughter cell begins to release a steady flow of vesicles from its trans-side, containing amorphous electron dense material. These vesicles migrate towards the septum where the amorphous substance is discharged via exocytosis (*figs. 10–13*). This results initially in the development of a delicate single wall layer in the septum, but after some period of intense vesicle traffic from the dictyosomes to the septum PM a double-layered wall is formed. The transition from a single layer to a bi-layer has apparently been captured in the cell shown in *fig. 10* (arrow). Although wall formation activity is most evident at the transverse septum, it is certainly not restricted to this region alone since the new wall layers at the septum are continuous with layers deposited elsewhere. Due to the similar texture and staining properties, however, it is difficult to differentiate between old and new layers where they are closely appressed (*fig.*

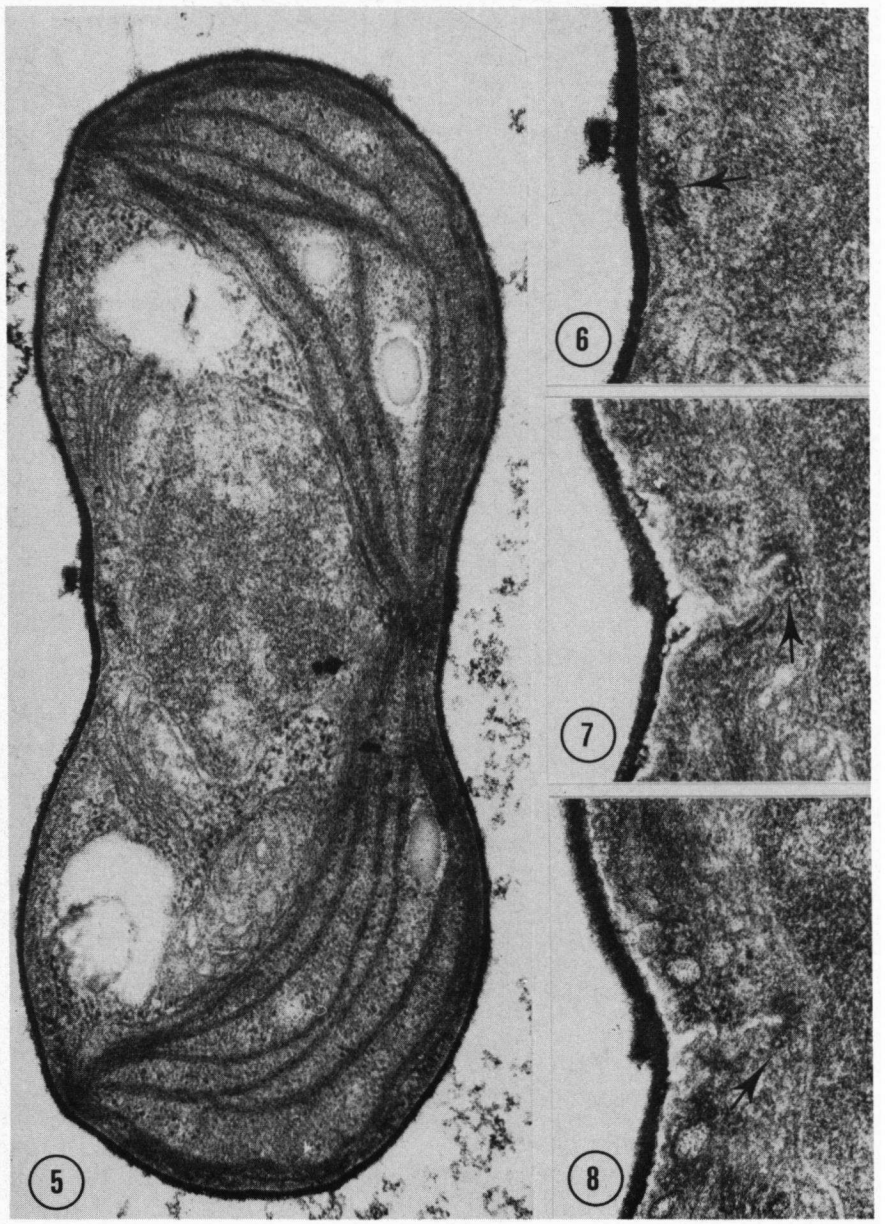
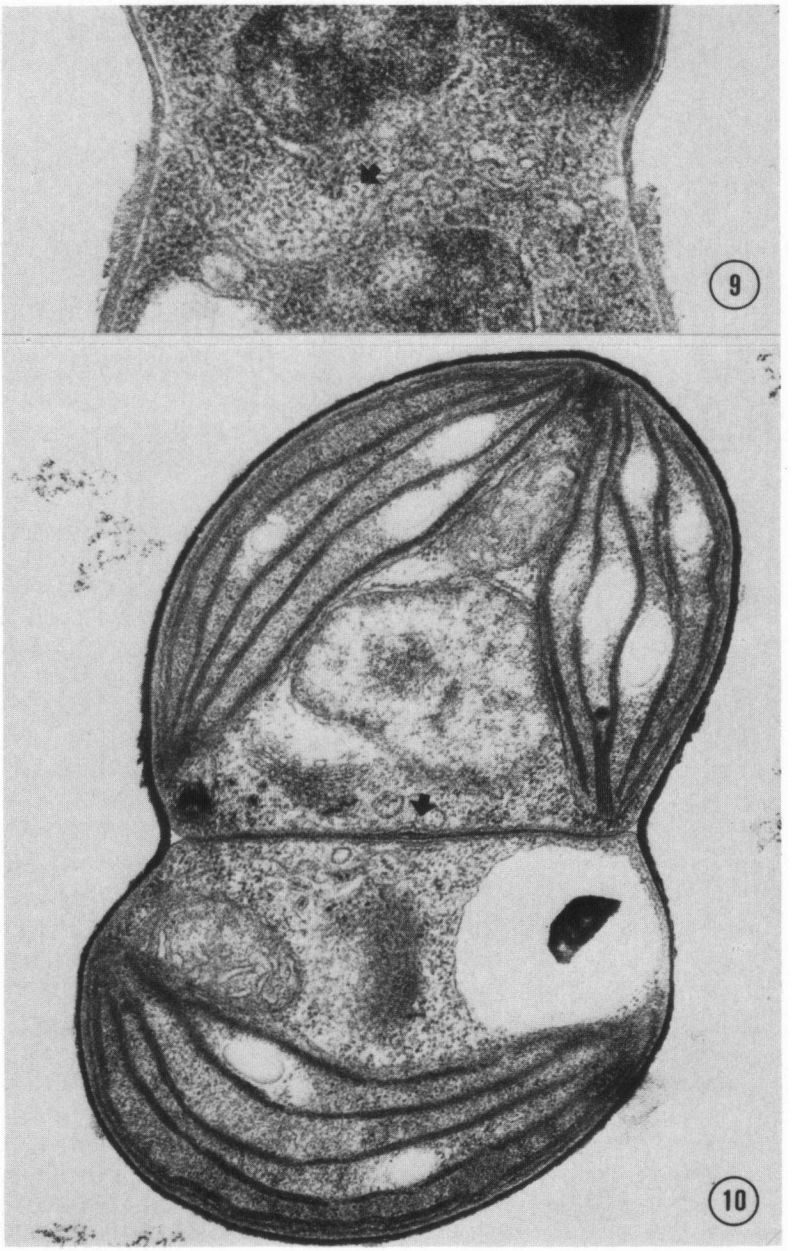


Fig. 5–8. *M. geminata.* Fig. 5. The mitotic nucleus (metaphase?) is surrounded by an intact nuclear envelope. Furrowing has started by the formation of a coated pit-like invagination of the plasma membrane, with one or two associated microtubules. ×47000. Fig. 6. Enlarged portion of fig. 5, showing coated pit (arrow) in more detail. ×80000. Figs. 7, 8. Non-adjacent serial sections of an advanced coated pit, with associated microtubule (arrows). ×60000.



Figs. 9, 10. *M. geminata*. Fig. 9. A just completed, delicate septum spans the internuclear cytoplasm after telophase. A septum-associated microtubule can be seen about halfway across the division plane (arrow). × 44000.

Fig. 10. Early stage of septum wall formation, with wall precursors being produced by the Golgi body of each cell. Part of the new wall has already become bi-layered (arrow). × 35000.

11, inset). As the two wall layers at the septum increase in thickness, the old outer cell wall that encloses the two daughter cells breaks at the level of the septum (*fig. 12–13*, arrows). This allows the cells to convert to a more spherical shape, and eventually to separate.

4. DISCUSSION

4.1. Morphological and taxonomic considerations

On the basis of the present observations on cell division in *M. geminata*, combined with information obtained by REYMOND et al. (1986), it is concluded that the division cycle of this alga is characterized by the following structural features: (1) pre-mitotic expansion of the cell and localized rupture of the cell wall ('budding'); (2) pre-mitotic duplication of the chloroplast, Golgi apparatus, and mitochondrion; (3) absence of centrioles during mitosis and the rest of the cell cycle; (4) marking of the division site at prophase by one or two short cortical MTs; (5) formation of a coated pit-like invagination of the plasma membrane at the site marked by cortical MTs at prometaphase; (6) following telophase, ingrowth of the now elongated coated pit-like PM invagination associated apically with the co-migrating 1 or 2 MTs, resulting in cell cleavage; (7) absence of cytokinetic MTs other than those mentioned; (8) deposition of two distinct wall layers by enhanced Golgi activity at the new septum that separates the two daughter cells; (9) rupture of the common outer wall layers at the level of the septum, followed by separation of the two cells.

The cytokinetic details noted in *M. geminata* and listed above are not particularly informative as to its taxonomic affinities among green algae. The over-all aspects of the 'budding-like' cell division appears to be unique although the pattern of cell wall deposition during cell expansion and following cleavage is essentially the same as the formation of walls in Chlorococcales during autosporogenesis (REYMOND *et al.* 1986). Assignment of *M. geminata* to the Chlorococcales does not seem to be contradicted by any of the other features listed. Nevertheless, it is obvious that the cell division mechnism of *M. geminata* represents a variation on the chlorococcalean pattern in at least two important respects.

First, the typical cell cycle in Chlorococcales is characterized by a prolonged period of cytokinetic inactivity and gradual cell expansion, followed by two or more cleavages occurring within the confines of the mother cell wall (KOMÁREK & FOTT 1983). Cell expansion occurs in the progeny after rupture or desintegration of the mother wall. However, in *M. geminata* an apparently reversed sequence is seen: after some period of nuclear inactivity, the beginning of a new division cycle is marked by expansion of the protoplast, causing rupture of the cell wall, with subsequent cytokinesis being delayed until the cell volume has been doubled. This seems to indicate that the cell cycle control mechanisms in *M. geminata* and in Chlorococcales are fundamentally different.

A second deviation of *M. geminata* from the usual chlorococcalean division pattern is the formation of only two cells per division cycle. (The formation of multiple daughter cells as reported by HINDÁK (1976) was not observed in

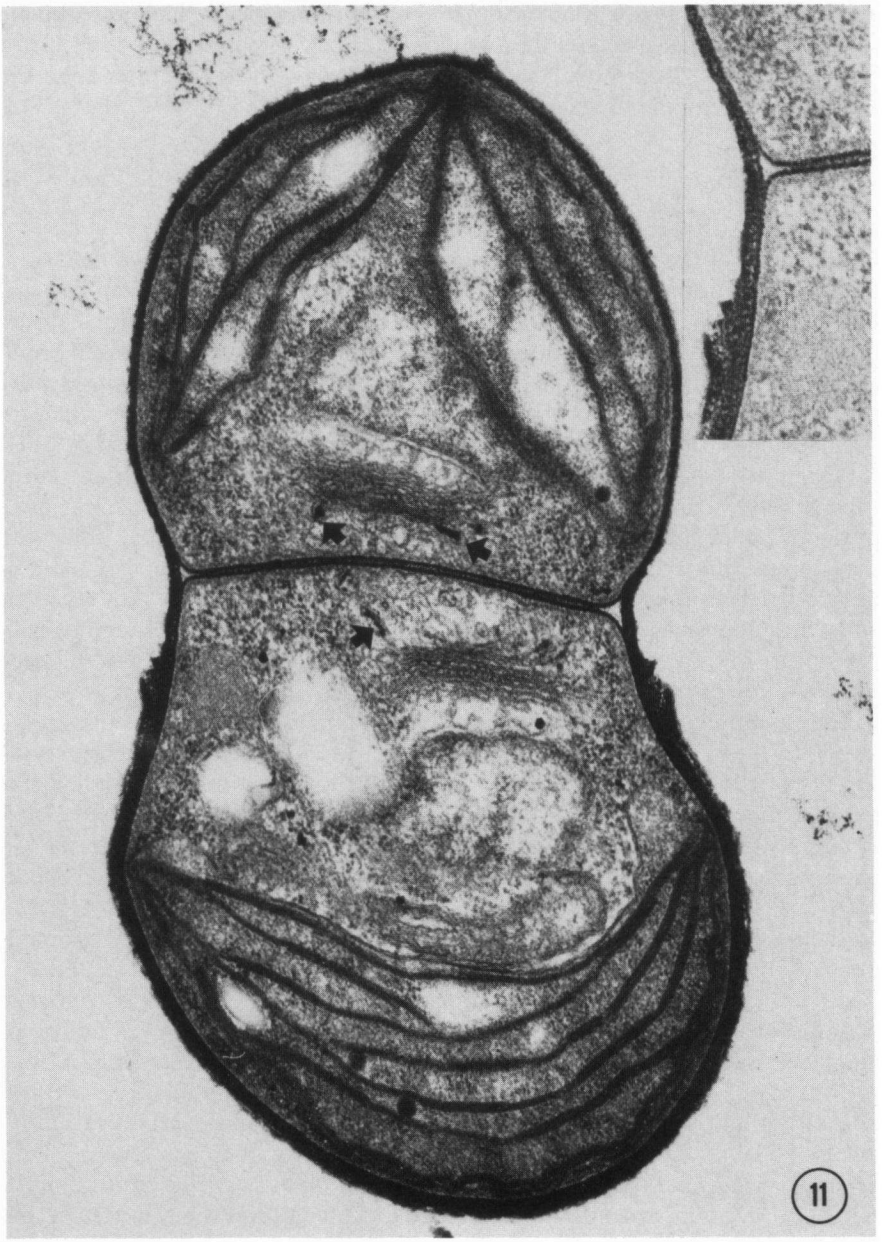
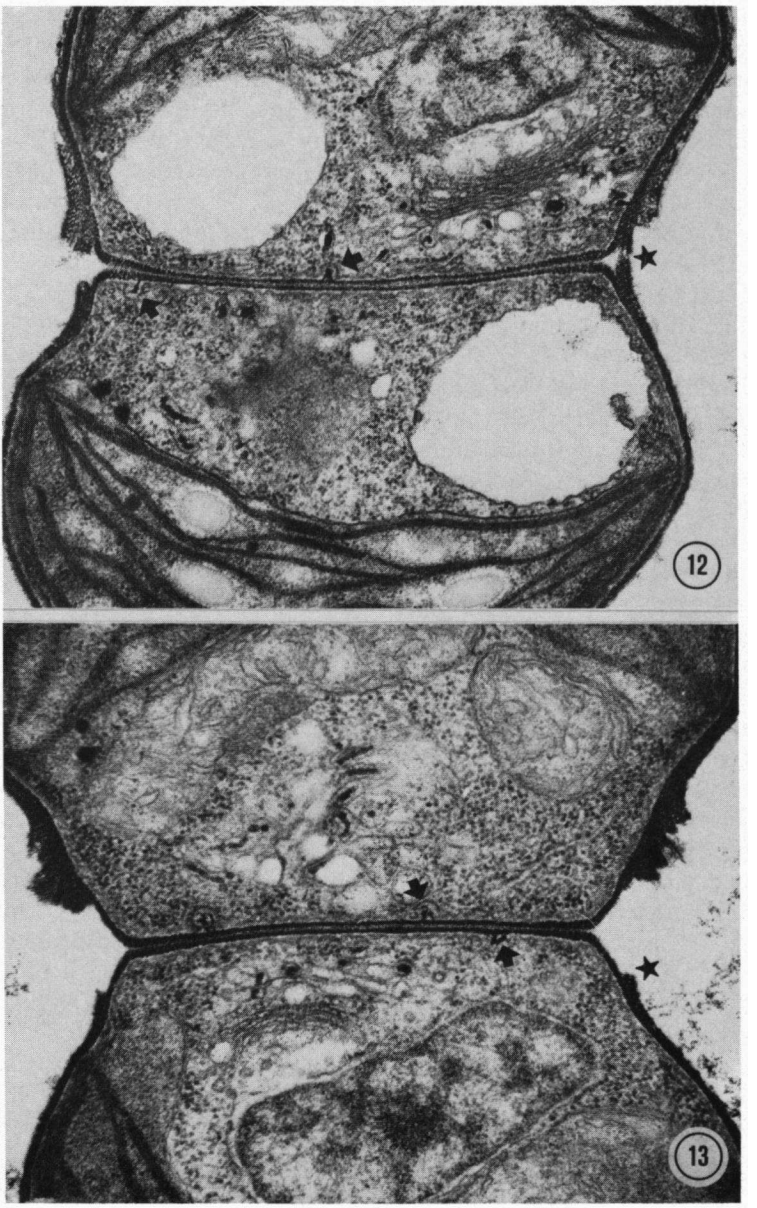


Fig. 11. *M. geminata*. More advanced stage of septum wall formation, with continued excretion of wall material via Golgi-derived vesicles (arrows). The wall is now bilayered throughout. × 39000. Inset: Enlarged portion of fig. 11, showing the junction of transverse and lateral walls. × 59000.



Figs. 12, 13. *M. geminata*. Golgi-derived vesicles apparently fuse with the septum plasma membrane, thereby discharging their electron dense contents (arrows). Old wall layers break at the level of the septum (asterisk in fig. 12), and their remnants remain visible for some time (asterisk in fig. 13). ×37000, ×45000.

our cultures). This deviation, however, may be more apparent than real. Cell division resulting in one pair of daughter cells typically occurs in pseudo-filamentous forms that appear to be more (*Cylindrocapsa*) or less (*Sphaeroplea, Microspora*) closely related to Chlorococcales on structural and ultrastructural grounds (SLUIMAN 1984, 1985). If this affinity with the Chlorococcales (which future EM studies may reveal to exist in other simple or loosely organized filaments as well, like *Elakatothrix, Radiofilum, Fusola, Fottea*) is taken as an argument for expanding the currently accepted morphology-based limits of the order, it is clear that *M. geminata* belongs to this more broadly conceived order Chlorococcales.

On the basis of all information now available (REYMOND *et al.* 1986, present paper), cell division of *M. geminata* appears to be more similar to the known division patterns of Chlorococcales than initial light microscopic observations (HINDÁK 1976) would suggest. Whereas the basic cell division type in Chlorococcales is the endogenous formation of multiple spores, the division pattern in *M. geminata* is probably best described as 'semi-exogenous formation of autospore diads'.

4.2. The role of coated pits and microtubules in cleavage

In *M. geminata*, the first visible sign of a determination of the cleavage site appears during prophase when a PM-associated MT is formed. The PM then invaginates and at the same time becomes associated on its cytoplasmic face with electron-dense coating material. The resulting structure is highly reminiscent of 'coated pits', i.e. PM specializations that have been implicated in the formation of coated vesicles (for references and discussion, see EMONS & TRAAS 1986). Although the coated pit in *M. geminata* does not give rise to a coated vesicle, a comparison with the development of coated pits/vesicles in other systems can be helpful to understand its role in the process of cell cleavage.

Coated pits arise by invagination of PM regions that are coated with proteins, the best characterized of which is clathrin. It is believed that the assembly of the cage-like lattice of clathrin from triskelions along the cytoplasmic surface of the phospholipid bilayer helps (or, induces) the latter to invaginate and to bud off as a cytoplasmic vesicle (DARNELL *et al.* 1986; HEUSER & EVANS 1980; KANASEKI & KADOTA 1969).

A similar mechanism may cause the PM in dividing cells of *M. geminata* to curve into a pit at a predetermined site. How this cleavage site is fixed is not clear, but the consistent presence of a MT before the pit has developed, and its close and persistent association with the coating material suggests that there is a microtubular involvement in the clustering of coat protein monomers at the cleavage site. According to the membrane motility model of coated vesicle formation (OCKLEFORD 1981), the protein monomers may be inserted in the PM at this specific site from the subtending cortical cytoplasm through some unknown microtuble/monomer interaction, or may be incorporated in more distant parts of the PM, and arrive at the pit site by lateral transport in the plane of the fluid membrane bilayer.

The fact that the coated pit is not pinched off to form a coated vesicle but rather persists as an ever-elongating narrow PM invagination may be explained by assuming that the process of coat assembly has stopped once a certain radius of pit curvature is reached, without interrupting the addition of new PM precursors to the invaginating membrane (cf. OCKLEFORD 1981). The observed co-migration of the apical PM coat and its associated MT into the cell again suggests that MTs play a role in maintaining the integrity of the coat material as a subcellular structure visible in the electron microscope. It is understood that coated vesicles are prevented from fusing with other membranes by the presence of the coating material. This implies that in *M. geminata* the apical coat has to be disassembled before the furrow reaches the PM at the opposite side of the cell. Although the actual process of coat disassembly could not be observed in the present study, the presence of a MT alongside a just completed septum (*fig. 9*) may be taken as indirect evidence that at this site the close PM/coat/MT-association might have ended.

Coated pit-like structures involved with plant cell division have not been described before, yet they are clearly visible in a few published electron micrographs of dividing coccoid green algae. These include *Tetracystis aeria* (fig. 11 in DEASON & O'KELLEY 1979), and *Friedmannia israelensis* (figs. 1–3 in DEASON *et al.* 1979). In some of these figures, also the pit-associated MT (MTs?) seems to be present. Lack of detailed information conerning the cytokinetic process in these organisms prevents a useful comparison to division events in *M. geminata.*

It is known that green plants form new transverse septum membranes during cellular division by localized incorporation of endomembrane elements (i.e., ER- or Golgi-derived vesicles), or by folding in of lateral PM (invagination) (SLUIMAN 1984). The latter process, which has been reported to be characteristic of many green algae and is often casually referred to as 'furrowing', has never been described in any detail; in particular, the precise mechanism of membrane growth in the furrow has received little attention. Only very recently, detailed observations on the cleavage mechanism in the green flagellate *Carteria* were published by DOMOZYÇH (1987). His conclusion that cell division is effected through ingression of existing PM into the cell was based on the lack of critical evidence that membrane is physically transferred from an endomembrane component (ER, Golgi apparatus) to the furrow PM. *M. geminata*, however, produces a furrow whose apical PM bears an intrinsic, and ultrastructurally recognizable marker in the form of a membrane-associated coat. The present study, therefore, presents the first positive evidence of the occurrence of lateral transport-dependent invagination of PM during cellular division in a green alga.

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REFERENCES

BOLD, H. C. & M. J. WYNNE (1985): *Introduction to the algae. Structure and reproduction.* Second edition. Prentice-Hall, Inc., Englewood Cliffs, N.J.

DARNELL, J., H. LODISH & D. BALTIMORE (1986): *Molecular cell biology.* Scientific American Books, W. H. Freeman, New York.

DEASON, T. R. & J. C. O'KELLY (1979): Mitosis and cleavage during zoosporogenesis in several coccoid green algae. *J. Phycol.* **15**: 371–378.

DEASON, T. R., P. E. RYALS, J. C. O'KELLY & K. W. BULLOCK (1979): Fine structure of mitosis and cleavage in Friedmannia israelensis. *J. Phycol.* **15**: 452–457.

DOMOZYCH, D. S. (1987): Cell division in Carteria crucifera (Chlorophyta): the role of the endomembrane system and phycoplast. *Protoplasma* **136**: 170–182.

EMONS, A. M. C. & J. A. TRAAS (1986): Coated pits and coated vesicles on the plasma membrane of plant cells. *Eur. J. Cell. Biol.* **41**: 57–64.

ETTL, H. (1980): *Grundriss der allgemeinen Algologie.* G. Fischer Verlag, Stuttgart.

– & J. KOMÁREK (1982): Was versteht man unter dem Begriff 'coccale Grünalgen'? (Systematische Bemerkungen zu den Grünalgen II). *Arch. Hydrobiol.* Suppl. 60 (*Algological Studies* **29**): 345–374.

HEYNIG, H. (1980): Einige Bemerkungen zu den Gattungen Marvania Hindák 1976 und Hortobagyella Hajdu 1975. *Arch. Protistenk.* **123**: 450–454.

HEUSER, J. & L. EVANS (1980): Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell. Biol.* **84**: 560–583.

HINDÁK, F. (1962): Beitrag zur Phylogenese und Systematik der Ulothrichales. *Biológia, Bratislava* **17**, 641–649.

– (1976): Marvania geminata gen. nov. et sp. nov., a new green alga. *Arch. Hydrobiol.* Suppl. 49 (*Algological Studies* **16**): 261–270.

– (1978): Survey of the genera of simple ulotrichacean algae (Chlorophyceae). *Proc. Crypt. Symp. SAS*: 69–79.

– (1982): Systematic position of some genera of green algae characterized by the formation of mucilaginous or pseudofilamentous colonies. *Preslia, Praha* **54**: 1–18.

KANASEKI, T. & K. KADOTA (1969): The 'vesicle in a basket'. A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain, with special reference to the mechanism of membrane movements. *J. Cell Biol.* **42**: 202–220.

KOMÁREK, J. & B. FOTT (1983): Chlorophyceae (Grünalgen), Ordnung: Chlorococcales. In: HUBER-PESTALOZZI, G.: *Das Phytoplankton des Süsswassers* **7** (1). E. Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obermiller), Stuttgart.

NICHOLS, H. W. (1973): Growth media – fresh water. In: STEIN, J. R. (ed.): *Handbook of Phycological Methods. Culture methods and growth measurements.* pp. 7–24. Cambridge University Press, Cambridge.

OCKLEFORD, C. D. (1981): Coated vesicles. In: HARRIS, J. R. (ed.): *Electron Microscopy of Proteins,* Vol. 1, pp. 255–299. Academic Press, London.

REYMOND, O. L., F. HINDÁK & H. J. SLUIMAN (1986): Morphologie cellulaire et cycle de développement chez l'algue verte Marvania geminata Hindák. *Arch. Sc. Genève* **39**: 243–255.

SILVA, P. C. (1982): Thallobionta. In: PARKER, S. P. (ed.): *Synopsis and classification of living organisms.* pp. 59–161. McGraw-Hill, New York.

SLUIMAN, H. J. (1984): A pathway of plasma membrane biogenesis bypassing the Golgi apparatus during cell division in the green alga Cylindrocapsa geminella. *J. Cell Sci.* **72**: 89–100.

— (1985): Mitosis and cell division in Cylindrocapsa geminella (Chlorophyceae). *J. Phycol.* **21**: 523–532.

TSCHERMAK-WOESS, E. (1982): Ueber die Abgrenzung der Chlorosarcinales von den Chlorococcales. *Pl. Syst. Evol.* **139**: 295–301.