CHAPTER 2. AN ULTRASTRUCTURAL STUDY OF VEGETATIVE CELLS OF *POLYKARYON PYRENOIDOSUM*

Introduction

In this paper ultrastructural observations made on vegetative cells of the chromophyte alga *Polykaryon pyrenoidosum* (Trenkwalder) Bailey *et* Misner (strain SAG 31.83) are reported. The alga was originally described as *Botrydiopsis pyrenoidosa* and placed in the Xanthophyceae (Trenkwalder, 1975; Ettl, 1978). However, analyses of nuclear-encoded small subunit ribosomal RNA (18S rRNA) gene sequences indicate that the alga is not closely related to *Botrydiopsis intercedens* Vischer *et* Pascher (GenBank acc. no. U41647) and probably does not belong in the Xanthophyceae (Bailey & Misner, submitted). Results of the latter study imply that *P. pyrenoidosum* cannot confidently be placed in any known class of chromophyte algae so the monotypic genus *Polykaryon* was established and placed in its own family (Polykaryaceae) and order (Polykaryales) within the Heterokontophyta (Bailey & Misner, submitted).

In an attempt to further ascertain the phylogenetic and taxonomic affinities of *P. pyrenoidosum* the ultrastructure of vegetative cells of the species were examined. This study revealed that the fine structure of *P. pyrenoidosum* cells is similar to that observed in other heterokont algal species. *Polykaryon pyrenoidosum* is, however, characterized by a combination of features that are useful for identification. For example, cells possess an extracellular fringe, most cells are multinucleate, and nascent flagellar hairs (mastigonemes) are often present in the endomembrane system of coccoid vegetative cells.

Materials and Methods

Polykaryon pyrenoidosum (strain SAG 31.83) was obtained from the Sammlung von Algenkulturen Göttingen, Germany (www.epsag.unigoettingen.de/html/sagintro .html). Cultures were grown in 1 L of DYIV medium (Andersen *et al.,* 1997) under a 12:12 light:dark cycle at 18^{o}C and harvested by centrifugation (1300 x g). Cells were resuspended and fixed at 0ºC with 3 mL of 2.6% glutaraldehyde in 0.66 M cacodylate buffer (pH 7.0). After 20 seconds 1 mL of 4% osmium tetroxide in 0.66 M cacodylate buffer was added and cells were fixed for 1 hr at room temperature. Cells were then rinsed with deionized water (DI) and dehydrated in 50 and 70 % ethanol solutions (15 min. each). Cells were left in 1% uranyl acetate in 70% ETOH at 4 ºC. Dehydration was continued the following day to 100% EtOH followed by two changes of propylene oxide 15 min. each. Cells were infiltrated with 1:1 100% propylene oxide and 100% Spurr's epoxy resin (Spurr, 1969) for 1 hr with constant rotation. The propylene oxide:Spurr's was then replaced with 100% Spurr's epoxy resin and left on a rotator overnight. Following overnight infiltration cells were transferred into BEEM capsules containing fresh 100% Spurr's epoxy resin, left for 1 hr at room temperature, and then polymerized at 70^{o}C for eight hours.

Thin sections (90-100 nm) were cut on a Reichert-Jung Ultracut E ultramicrotome, flattened with chloroform fumes and collected on formvar coated 200 mesh ultrahigh transmission copper grids. Grids were post-stained with 2% uranyl acetate (in 50% EtOH) for 15 min., then rinsed with DI water, and then stained with Reynolds' lead citrate (Reynolds, 1963). Sections were examined using a Philips EM 400 TEM at an accelerating voltage of 80kV and micrographs were taken using Kodak EM 4489 film.

Negatives were scanned with a Microtek Scanmaker 4 and digitized images were processed using Adobe Photoshop, v. 7.0.

Cells from two separate subcultures of *P. pyrenoidosum* were prepared as described above.

Results

Cell diameters for cultured *P. pyrenoidosum* cells ranged from 7.5 – 50 μm and the mode and mean diameters for 334 cells were 12.5 μm and 17 μm, respectively (Bailey & Misner, submitted). Cells possessed an entire cell wall ranging in thickness from 80-740 nm, depending upon the cell, with larger cells typically having thicker cell walls (Figs 20-25, 28, 30, 31). Most fixed cells possessed fine fibril-like extensions of the outer cell wall that has been termed 'fringe' (Figs 20-22, 25, 28-31). The micrograph shown in Fig. 29 suggests that this fringe may be an elaboration of the cell wall because the fringe and cell wall are identically stained. There is, however, a darker staining layer of material between the fringe and inner cell wall (Fig. 29, see also Figs 21, 30). In higher magnification images the continuity of the fringe and cell wall is not apparent and it is possible that the cells' fringe may be mucilaginous in nature (Figs 20-22, 25, 28, 30, 31). A few cells examined possessed electron dense inclusions at the cell periphery, just beneath the plasmalemma (Figs 20, 24, 25). Cells typically contained many (Figs 20-26, 32, 36, 40, 43) membrane bound vesicles and some of these vesicles appeared to be devoid of contents except for tiny electron opaque granules (e.g., Figs 20-26, 32, 36, 40, 43). It is likely that some vesicles are contractile vacuoles whereas others may contain, or have contained, photosynthate (e.g., putative lipid droplet in Fig. 22). Other,

sometimes numerous membrane bound structures were also apparent that contain more darkly staining material (Figs 20, 21, 26). Closer examination revealed that these structures are bounded by a double membrane and are therefore organellar in origin (Fig. 27). These are interpreted as poorly fixed or senescent mitochondria.

Single nuclear profiles were observed in some sections (Figs 20, 22, 32, 35-37), but other cells possessed numerous nuclei (Figs 23-25) that are spherical (Fig. 23) or elongate (Fig. 32). Nuclei are chromatin rich and may contain a well-developed nucleolus.

Each cell contained two or more plastids that appear elongate or lenticular in longitudinal section (Figs 20-25). Pyrenoids were observed in some sections and formed one or more semi-immersed bulges between the outer thylakoids of the plastid and the plastid membranes (Figs 22, 28). Traversing thylakoids were not observed within pyrenoids and chloroplasts of vegetative cells did not contain an eyespot (or stigma). *P. pyrenoidosum* plastids are surrounded by four unit membranes (Figs 30, 31) and a chloroplast endoplasmic reticulum (CER) is present: the confluence of the outer nuclear and outer chloroplast membranes is evident in Fig. 32. Girdle lamellae encircle the plastid beneath plastid membrane (Figs 21, 22, 30, 31), thylakoids traverse the plastid in tightly adherent groups of three (or four)(Fig. 33), and plastids bearing one to many plastoglobuli were commonly observed (Figs 28, 34).

One to several mitochondrial profiles with tubular cristae were observed in sections of each cell (Figs 20, 21, 34, 36). Cells contained a single Golgi body (dictyosome) with 4 – 10 cisternae that is positioned near a nucleus (Figs 32, 35-37). The *cis*- (forming) cisternae of the Golgi are proximal to the nucleus whereas the *trans*- face

is distal to the nucleus (Figs 32, 35, 36). Endomembranous vesicles derived from the *trans*- face of active Golgi bodies are shown in Figs 35 and 37.

One or two centrioles were occasionally viewed in *P. pyrenoidosum* vegetative cells (Figs 37-39), and a structure closely resembling a connecting fiber (or connecting band) was observed (Figs 38, 39).

The most distinctive subcellular feature, consistently observed in cells was the presence of membrane bound vacuoles containing hollow tubules (Figs33, 40-44). The diameters of 50 cross-sectioned tubules were measured and ranged from 12.7 – 20 nm with a mean diameter of 16 nm. Vacuoles containing tubules were found in the cytoplasm (Figs 41-44) or within membranous sacs formed by the outer membrane of the CER (Fig. 33, 44) and outer membrane of the nucleus (Fig. 40). In all cases the tubules, despite their juxtaposition to other organelles, are located within the cells' endomembrane system. Within vacuoles tubules are sometimes viewed in longitudinal and cross-section (Figs 40, 42) indicating that 1) 'bundles' of tubules are present that are oriented in different directions, or 2) the tubules are folded or undulate within the vesicle. Longitudinally or obliquely sectioned tubules appear to be inside the chloroplast shown in Fig. 40. However, this section is interpreted as a longitudinal or oblique section through a 'tubule pouch' formed within or near the concave face of a 'curved' (i.e., sickle-shaped) chloroplast (c.f. Figs 33 and 44): a schematic of the section seen in Fig. 44 is illustrated in Fig. 45. There is than no reason to suspect that tubules within the cell are found outside the endomembrane system. These tubules are putatively identified as nascent flagellar hairs (FHs).

Discussion

The organization of *P. pyrenoidosum* cells is similar to other heterokont algae (see Hibberd, 1976, 1990; Kristiansen 1990; Moestrup & Andersen, 1991; Wujek, 1996). The presence of a CER and chloroplasts with girdle lamellae and adherent thylakoids in groups of three, and mitochondria with tubular cristae are features shared by most photosynthetic heterokonts (Hibberd & Leedale, 1971; Belcher & Swale, 1972a, b, 1976; Belcher, 1974; Hibberd, 1977; Heywood, 1980; Andersen, 1982; Preisig &Wilhelm, 1989; Andersen *et al.,* 1993; Bailey *et al.,* 1998; Boddi *et al.,* 1999; Gulliou *et al.,* 1999; O'Kelly & Wujek, 2001). Also, as in many other heterokonts, the single Golgi body of *P. pyrenoidosum* lies close to the nucleus and the cisternae are curved in a concave manner with respect to the nucleus (Hibberd, 1971, 1976; Belcher & Swale, 1972a; Preisig & Hibberd 1983; Thomsen, 1988; Andersen, 1989; Kristiansen, 1990; Wujek, 1996; Andersen *et al.*, 2002). Thus, ultrastructural and molecular evidence indicate that *P. pyrenoidosum* is a member of the Heterokontophyta (Bailey & Misner submitted).

Coccoid vegetative cells of *Polykaryon pyrenoidosum* fixed for TEM from two different subcultures exhibited three noteworthy features. First, the cell walls of all cells examined possessed a fibrillar layer termed 'fringe'. The composition of the 'fringe' has not been confirmed and may be composed of cell wall material (e.g., cellulose and pectin), mucopolysaccharides, peptidoglycans and/or other compounds. Despite its unknown composition, the 'fringe' is a consistent characteristic of the alga that seems useful for purposes of identification.

Second, most *P. pyrenoidosum* vegetative cells are multinucleate and the light (Bailey & Misner, submitted) and TEM observations allow us to conclude that the

number of nuclei within a cell is correlated with cell size, with larger, presumably older cells having more nuclei than smaller, younger cells.

Third, many *P. pyrenoidosa* cells examined possessed one or more membrane-bound vacuoles containing hollow tubules (Figs 29, 36-40). In two cells examined this structure occupied approximately one-third of the cells' total volume (not shown). Thin sections through some vesicles in which both longitudinal and cross sectional views of the tubules are apparent suggest that, at least in some instances, the tubules may be folded within the delimiting membranous sac (Figs 36, 38). Alternatively, these observations may be explained if one assumes the vesicle contains two 'bundles' of tubules that are oriented perpendicularly with respect to one another. Homologous structures that are similar in cross sectional diameter have been observed in many other heterokont species (Bouck 1969, 1971,1972; Heywood 1972, 1973, 1980; Clarke & Pennick ,1975; Andersen, 1989; Pipes *et al*., 1981). As in *P. pyrenoidosum* these tubules are always found intracellularly within the endomembrane system and have been observed in the cytosol or associated with chloroplasts, mitochondria or nuclei (Leedale *et al*., 1970; Belcher & Swale, 1972a, b, 1976; Moestrup, 1982; Andersen *et al*., 1993; Guillou *et al*., 1999). The overwhelming consensus is that the tubules probably are incompletely formed (immature) FHs. Although there were not clear observations of terminal filaments or a base these 16 nm diameter tubules are putatively identified as nascent flagellar hairs (FHs) based upon previous studies of heterokont algae (Bouck 1969, 1971, 1972; Heywood 1972, 1973). A non-exhaustive survey of previously published data for 12 other species of heterokont algae revealed FH diameters ranging from 15-26 nm (Bouck, 1969; Hibberd, 1970, 1971, 1980; Heywood, 1972, 1973; Chen & Haines 1975; Mignot,

1976; Andersen *et al*., 1993; Boddi *et al*., 1999; Guillou *et al.*, 1999). These and other data lead Andersen *et al.* (1991) to concisely define tubular flagellar hairs as "Filamentous appendages consisting of at least a hollow shaft (> 15 nm diam.) often with one or more terminal filaments." The diameters of vesicle bound tubules in *P. pyrenoidosum* measured *ca*. 16 nm; given their appearance and size it is concluded that they are FHs.

Trenkwalder (1975) reported that *P. pyrenoidosum* (as *Botrydiopsis pyrenoidosa*) reproduces by means of autospores or zoospores and the zoospores are more-or-less amoeboid or pear shaped (12-15 μm long x 6-7 μm wide). Despite numerous hours of observation, no autospores or zoospores have been identified in the *P. pyrenoidosum* cultures. It is suspect that autospores are formed as the multinucleate cytosol of the mother cell is partitioned into individual walled daughter cells but have no direct evidence for such a process. Trenkwalder (1975) observed zoospores possessing only one long, anteriorly directed flagellum visible in the light microscope. The TEM observations of vegetative cells indicate that they contain two basal bodies (or centrioles: Figs 37-39). Thus, it is possible that swimming cells possess a second short (mature) flagellum that is non-emergent or too small to see at the light microscopic level. Alternatively, the zoospores could indeed be uniflagellate in which case the basal body usually giving rise to the mature flagellum is barren. In any event, all the observations raise an interesting question: If *P. pyrenoidosum* no longer produces swimming cells in culture, why are FHs prolifically constructed within the endomembrane system of coccoid vegetative cells?

The taxonomic position of *Polykaryon pyrenoidosum*

Sequence analyses of nuclear-encoded small subunit ribosomal RNA (18S rRNA) genes indicate the *P. pyrenoidosum* belongs in the Heterokontophyta, but the class (if any) to which the species should be assigned is unclear (Bailey & Misner, submitted). The gene encoding the large subunit of RuBisCO (*rbc*L) has been extensively used to infer phylogenetic relationships among heterokont algae (Daugbjerg & Andersen, 1997a, b; Bailey *et al.,* 1998, Daugbjerg & Guillou 2001; Kawai et al., 2003). However, initial attempts to determine the *rbc*L sequence for *P. pyrenoidosum* using 'standard' primers (Daugbjerg & Andersen, 1997a, b) have been met with mixed success: data are unavailable for the first (5') half of the gene. The results of the ultrastructural study, however, provide convincing evidence for excluding *P. pyrenoidosum* from several classes of heterokont algae.

Polykaryon pyrenoidosum is coccoid and therefore can be excluded from the Raphidophyceae whose members are wall-less flagellates (Heywood & Leedale, 2000). Morphological and reproductive features, i.e., absence of multicellularity, plasmodesmata, and sporangia, preclude placing the species in the Phaeophyceae (Clayton, 1989; 1990) and the absence of a siliceous frustule excludes *P. pyrenoidosum* from the Bacillariophyceae (Graham & Wilcox, 2000). The absence of an external siliceous skeleton (as in silicoflagellates) and/or tentacles supported by microtubular triads (as in pedinellids) suggest that the species is not a member of the Dictyochophyceae (Moestrup 1995; Daugbjerg 1996; Moestrup & O'Kelly 2000). In fact, the absence of silica deposition vesicles implies that *P. pyrenoidosum* does not belong in any heterokont algal class whose members regularly produce siliceous walls,

cysts or scales. The marine filamentous alga *Schizocladia ischiensis* Henry, Okuda *et* Kawai (Schizocladiophyceae) is unlikely to be a close relative of *Polykaryon* and produces swimming cells with eyespots, which *Polykaryon* does not (Trenkwalder 1975; Kawai *et al.,* 2003). The classes Chrysomerophyceae (O'Kelly, 1989; Cavalier-Smith *et al*., 1995), Pelagophyceae (Andersen *et al*., 1993), and Pinguiophyceae (Andersen *et al*., 2002; Kawachi *et al*., 2002) seem unlikely taxa in which to classify *P. pyrenoidosum* because the latter contain estuarine or marine species whereas *P. pyrenoidosum* is known only from freshwater habitats. Nevertheless, it must be remembered that several heterokont algal classes contain both freshwater and marine representatives (e.g., Bacillariophyceae, Eustigmatophyceae, and Raphidophyceae) so this criterion should be applied with reservation.

Potential relationships between *Polykaryon* and the remaining classes of heterokont algae (Chrysophyceae, Eustigmatophyceae, Phaeothamniophyceae, Synurophyceae and Xanthophyceae) are equally obscure. *Polykaryon* is multinucleate and possesses many chloroplasts and these features are uncommon among the Chrysophyceae *sensu stricto* (Preisig & Andersen, 2000). Furthermore, the Chrysophyceae and its sister class, the Synurophyceae, are capable of producing siliceous stomatocysts, which are unknown in *Polykaryon*. *Polykaryon* also lacks bilaterally symmetrical siliceous scales characteristic of synurophytes (Andersen, 1987; Andersen & Preisig, 2000). The complement of photosynthetic pigments in *P. pyrenoidosum* chloroplasts is unknown, which is unfortunate because pigment profiles are useful phylogenetic markers within heterokonts (Jeffrey 1989; Bjørnland & Liaaen-Jensen 1989). For example, pigment analysis might exclude *P. pyrenoidosum* from the

Eustigmatophyceae which lack chlorophyll *c* or the Xanthophyceae in which fucoxanthin is absent (Hibberd, 1990). Most eustigmatophytes surveyed at the ultrastructural level possess so-called lamellate vesicles (or lamellate storage vesicles: Hibberd & Leedale,1972; Maruyama *et al.*, 1986; Santos 1996; Schnepf *et al.,* 1995/96) that are absent in *P. pyrenoidosum*. Furthermore, encircling girdle lamellae are lacking in eustigmatophyte chloroplasts (Santos 1996); *P. pyrenoidosum* possesses girdle lamellae. According to Trenkwalder (1975) *P. pyrenoidosum* zoospores lack a stigma (eyespot), which is a prominent characteristic of most, but not all (Schnepf *et al*., 1995/96), eustigmatophyte swimming cells. For these three reasons it seems that *P. pyrenoidosum* is a highly unlikely candidate for inclusion in the Eustigmatophyceae. Some *P. pyrenoidosum* cells possessed electron dense vesicles at the cell periphery similar to those observed in *Phaeobotrys, Phaeoschizochlamys, Phaeothamnion, Stichogloea* and *Tetrasporopsis* (Phaeothamniophyceae) (Figs. 24, 25: Dop &Van Oers, 1979; Entwisle & Andersen, 1990; Bailey *et al.,* 1998). It is unknown if homologous vesicles are found in other taxa placed in the Phaeothamniophyceae (Bailey *et al*., 1998). It is possible that the electron dense vesicles are involved in production of extracellular components of cell walls or mucilaginous sheaths (i.e., in *Phaeoschizochlamys* and *Stichogloea*) and the fringe possessed by *P. pyrenoidosum* (Figs 20-22, 28, 29).

As in *Polykaryon* several well-known xanthophyte genera have cell walls, multiple nuclei and many plastids including *Asterosiphon, Botrydium* and *Vaucheria* (Ettl, 1978; Reith 1980), although the latter three taxa are not coccoid in nature. It is possible that the multinucleate condition evolved independently in these taxa and is therefore phylogenetically uninformative. Nevertheless, *vegetatively* multinucleate cells

are apparently rare among heterokont algae; multinucleate cells or structures in heterokonts are otherwise almost always associated with reproduction (i.e., the production of swimming cells, spermatozoids, autospores, etc.).

In summary, fourteen classes of heterokont algae are recognized and this data provide a reasonable basis for excluding *P. pyrenoidosum* from eleven of these taxa. Thus, three possibilities remain: Ultrastructural and other features imply that *P. pyrenoidosum* is most similar to species placed in the Phaeothamniophyceae and Xanthophyceae, although the data do not rule out, however unlikely, that the species is an unusual member of the Chrysophyceae. If other data do not support any of these hypotheses then erecting a new taxon at the rank of class that includes *P. pyrenoidosum* seems justifiable.