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ENDOBACTERIA IN THE DIATOM *PINNULARIA* (BACILLARIOPHYCEAE). I. "SCATTERED ct-NUCLEOIDS" EXPLAINED: DAPI–DNA COMPLEXES STEM FROM EXOPLASTIDIAL BACTERIA BORING INTO THE CHLOROPLASTS[1,2]

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A local strain of the pennate diatom *Pinnularia* cf. "*nobilis*" was investigated using cytochemistry and fluorescence and EM techniques. The regular perforation of the chloroplasts of *P. "nobilis"* and the lack of a typical diatom pyrenoid were confirmed at the ultrastructural level. Cavities and channels in the complex secondary plastid were found to harbor symbiotic bacteria, and their DNA elicited DAPI fluorescence. Wheat germ agglutinin, labeling bacteria walls, elicited a similar fluorescence pattern. Previous speculation that the apochlorotic DNA-positive dots in the plastids of several *Pinnularia* species are "scattered ct-nucleoids" is thus refuted. Bacteria were rod shaped and gram negative. They resided in the lumen of the endoplasmic reticulum (ER) during host interphase confined to the specialized ER compartment housing the secondary plastid, that is, to the space between the third and the fourth membrane profile, encircling the chloroplast. TEM images of chemically and cryofixed cells revealed that cavities resulted from the interaction of bacteria with the plastid according to the following sequence, alignment, attachment, deformation, and disintegration. This occurred without visible injury to the primary chloroplast envelope or the relict cell membrane of the reduced ancestral red alga that surrounds the chloroplast. The patterned arrangement of bacteria suggests recognition sites on the vestigial cell membrane, thought to interact with surface groups on the bacteria. The intimate association between bacteria and secondary plastid inside the common specialized ER cisterna suggests they form a functional unit. Comparison of thylakoid profiles, disrupted by bacteria in *Pinnularia*, with those disrupted by the pyrenoid in other pennate diatoms (e.g. *Trachyneis*) revealed a significant ultrastructural resemblance. No aposymbiotic *Pinnularia* cells were found at the sampling site.

Key index words: **chloroplasts; diatoms; DNA; endobacteria; endoplasmic reticulum; *Pinnularia*; pyrenoids; RUBISCO; symbiosis; *Trachyneis***

Abbreviations: **DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; ER, endoplasmic reticulum; FM, fluorescence microscope; FS, freeze substitution; HPF, high pressure freezing; rCM, relict cell membrane of the reduced red alga; WGA, wheat germ agglutinin.**

Diatoms are important aquatic primary producers, displaying a great diversity in chloroplast morphology, which caught the interest of many early phycologists (Geitler 1932). Geitler's focus on a survey of freshwater diatoms revealed that most pennates possess band- or platelike chloroplasts with smooth or lobed edges and contain either a single large or numerous small pyrenoids (Geitler 1932, 1937b). The complete lack of a conventional diatom pyrenoid in *Pinnularia "nobilis"* was thus unexpected (Geitler 1937a). The other striking feature of the *Pinnularia* plastid discernible in bright-field LM was its "sieve-like perforations" with grooves ranging from 0.5 μm up to more than 1 μm in diameter, occasionally clustered, but mainly evenly scattered over the chloroplast area and "at a superficial glance appearing as small granules" (Geitler 1937a, Fig. 1, a–d). Geitler emphasized that such a chloroplast was unknown in other genera and that the grooves, accentuated in his drawings as trans-plastidial channels in profile (Geitler 1937a, Fig. 1e), could be of physiological significance. His observations were affirmed with LM techniques by Gschöpf (1952) and Tschermak-Woess (1953), who found perforated plastids devoid of pyrenoids in several large *Pinnularia* species.

In a reexamination of *P.* cf. *"nobilis"* and a number of other *Pinnularia* species with modern LM optics more than half a century later, Mayama and Shihira-Ishikawa (1994a,b,c, 1996) argued that Geitler's interpretation was fallacious, because their LM differential interference contrast (DIC) images were deciphered as granules instead of chloroplast invaginations, which, in addition, stained with the nuclear dye DAPI. They described these "DAPI spots" to be "globular chloroplast (=ct) nucleoids, clearly embedded within the chloroplast itself," which would have been an exciting novelty for diatoms, previously shown to have their ct-DNA, like other chromophytes, visible as a peripheral ring when stained with DAPI (Coleman 1985). Consequently, they subdivided the genus *Pinnularia* on the basis of absence or presence of "scattered ct-nucleoids" (in addition to the peripheral ring) into more "primitive" and more "advanced" forms (Mayama and Shihira-Ishikawa 1994c,1996). Their argument that lack of fluorescence after DNAse treatment would confirm that they were *ct*-nucleoids is unconvincing, however, and their own clear optical sections generated with

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a confocal laser scanning microscope (Mayama and Shihira-Ishikawa 1994a, Figs. 7f and 8, c and d) show that they failed to notice also rod-shaped blue-light–emitting structures, some of which were not flush with the plastid surface. Nevertheless, their hypothesis was highlighted and generalized in a review article on diatom chloroplasts (Mann 1996).

A TEM image showing that chloroplast invaginations are real in *Pinnularia* and are caused in a local strain (U-strain) by exoplastidial bacteria located within the endoplasmic reticulum (ER) lumen (Schmid 1994, Fig. 14) was probably overlooked, because space limitations restricted an explanation to merely mention "bacteria" in the figure legend. A short report was recently issued as an abstract (Schmid 2000), and the lack of a classic diatom pyrenoid was reaffirmed using modern pyrenoid stains (Schmid 2001). Pyrenoid dyes stained apochlorotic dots in *Pinnularia*, particularly clearly when they occurred in clusters (Schmid 2001, Fig. 39), delineating a pattern comparable with that of DNA–DAPI complexes. A first screening with polyclonal antibodies directed against the large subunit of RUBISCO revealed a similar pattern (Schmid 2003). Hence, a search for the phylogenetic position of the bacteria and for their metabolic functions is in progress. Results presented here may stimulate the search for similar complex associations in other chromophytes, especially because the origin of RUBISCO in this group has become subject of great interest (Kowallik 1992, Jenks and Gibbs 2000). The "complex (= secondary) plastid" (Sitte 1993, Maier et al. 1996) of chromophytes that evolved from the reduction of a eukaryote after secondary endocytobiosis (Gibbs 1990, Sitte 1993, Melkonian 1996, Medlin et al. 1997, McFadden 2001) was convincingly defined for diatoms by Kowallik et al. (1995) as a "streamlined red alga." The information on this *Pinnularia* is split into two parts, the present article describing the relationship between the endobacteria and the host diatom cell during interphase and Schmid (2003, this issue) illustrating stages of bacterial translocation to the cell center concomitant with the host cell cycle, and the scars left in the chloroplasts, before plastid rearrangement and division.

A corollary of the work on this chimeric diatom/bacteria system is the compelling evidence that the resolution of a confocal laser scanning microscope, which, in combination with DNA-complexing fluorochromes, is now widely used as a diagnostic tool for localization of extranuclear DNA, is *not* sufficient to replace TEM images of ultrathin sections.

This *Pinnularia* work is a tribute to the late Professor Dr. L. Geitler (1899–1990) and a reminder of his critical and visionary observations (Tschermak-Woess and Weber 1989, Schmid 1991).

MATERIALS AND METHODS

Pinnularia cf. "*nobilis*" (U-strain) was collected in autumn 1991 from a shallow ditch in a mire at the base of the Untersberg, in the limestone area of Salzburg, and identified by Dr. K. Krammer as *P. mesogongyla* Ehrenberg, previously assigned by Cleve, Hustedt, and others to *P. nobilis* Ehrenberg (which is, however, according to Krammer, a fossil taxon; *P. mesogongyla* is not related to *P. gibba* var. *mesogongyla*: see refs. and discussion in Krammer 2000). The size range of the U-strain *Pinnularia* slightly exceeds that given for *P. mesogongyla* in Krammer (2000). U-strain cells are 200 to 290 μm long and 33 to 40 μm wide. The valve outline is not stable, the larger cells exhibit a polar transapical widening that is lost below 270 μm, the central swelling is lost below 220 μm. Complexity of the raphe fissure and width of internal opening of alveoli varied with the cell size.

Pinnularia cells were roughly cleaned and concentrated by filtering through nets of decreasing mesh sizes and have been kept since then as a natural batch culture, together with the fine silt/detritus required for normal growth of this species, in 250-mL glass dishes, at 22 ± 1° C and a natural light regime. Once a week, medium was decanted, the algal–silt mix rinsed twice with deionized water, and then again provided with sterile filtered water from the habitat, supplemented with 1 $mL \cdot L^{-1}$ of silicate and 1 $mL \cdot L^{-1}$ of vitamin B_{12} stock solution (1500 mg $Na_2SiO_3.9H_2O$ and 1 mg vitamin B_{12}, each in 100 mL double distilled water). Since 1994, cells from batch culture and the natural habitat were monitored for presence of endobacteria with DAPI–DNA fluorescence.

Cell-concentration for LM assays and TEM fixes was by means of repeated washes of aliquots in sterile filtered medium, taking advantage of the higher specific gravity of the diatoms relative to the silt, which allowed centrifugation or filtering at any stage of processing to be avoided. Single cells were transferred with a mouth pipette under a dissecting microscope. The mouth pipette was constructed from two pieces of a sterile infusion tube coupled via a Luer lock system to a sterile bacterial dense disposable filter holder (Schleicher & Schuell, Dassel, Germany, Red-Ring, 0.2 μm pore size). One end was fitted with a mouth piece and the other with a glass pipette pulled out over a small flame to the appropriate bore required for the diameter of the cell to be transferred.

LM and fluorescence microscopy (FM). For vital staining of DNA, 10 μL of an aqueous solution (1 $\mu g \cdot mL^{-1}$) of DAPI (Polysciences, Warrington, PA, USA) was added to 1-mL diatom samples immediately before preparation of slides for observation. For DNA localization with DAPI in isolated plastids, diatoms were fixed and prepared as described in Schmid (1989) or in Edgar and Zavortink (1983).

Assay for bacterial peptidoglycan walls (N-acetyl-glucosamine residues). Fixed and permeabilized cells were briefly reduced with 1 $mg \cdot mL^{-1}$ Na-borohydride (Sigma Aldrich, Vienna, Austria) in PBS, rinsed three times in PBS, and blocked in PBS containing 1% BSA (Serva, Heidelberg, Germany). Cells were either dehydrated in a graded ethanol series in 10% steps from 10% to 100%, embedded in JB-4 (Polysciences, Warrington, PA, USA), and sectioned before incubation or transferred as whole cells to 0.5-mL centrifuge tubes containing wheat germ agglutinin (WGA) tagged with Texas red (Molecular Probes, Leiden, The Netherlands) in the dilution recommended by the manufacturer. After cross-reaction, sections and cells were rinsed in PBS and mounted in 50% glycerol in PBS containing 1% *p*-phenylenediamine as an antifading substance. Observation and documentation was with a Reichert Polyvar (Reichert, Vienna, Austria) using DIC optics and the epifluorescence attachment with the filter combinations U-1 (exc. 330-80, DM 420, barr. 418 nm) for DAPI and G-1 (510-46, 580, 590 nm) for WGA Texas red. A Yashica 108 Multiprogramme camera coupled to the Polyvar photoautomatic device (Reichert, Vienna, Austria) was used for recording on a Kodak T Max 100 or 400 ASA film (Kodak Ltd., London, UK).

EM and TEM. Results are based on 40 sectioned cells from four chemical and two cryofixations. All contained symbiotic bacteria.

Chemical fixations: Pinnularia (U-strain) cells were fixed in the autumn of 1991 and in January 1992 in four different modifications using buffered glutaraldehyde at a final concentration of 2.5% and 0.2% buffered OsO_4 (both from Serva), with or with-

out addition of 0.5% potassium ferricyanide (Merck, Darmstadt, Germany). Either a conventional fixative with prefixation in aldehyde, three rinses in half-strength buffer, and postfixation in osmium was used or both fixatives were applied simultaneously. Tannic acid was also added as additional mordant and to enhance contrast. After *en bloc* stabilization in 1% aqueous uranyl acetate (Serva) overnight at 4° C, diatoms were dehydrated in a graded acetone series in 10% steps from 10% to 100%, followed by anhydrous acetone (molecular sieve) and flat embedded in Spurr's resin (Serva). Preselected diatoms were mounted in the desired orientation onto Spurr blocks and cut with a diamond knife on a Reichert Ultracut (Reichert, Vienna, Austria). Sections were stained in the conventional way with uranyl acetate and Reynold's lead citrate or staining was omitted when K-ferricyanide had been added to the fixative.

Cryofixations: High pressure freezing (HPF) of *Pinnularia* cells was performed in cooperation with Prof. M. Müller, ETH-Zürich (December 1998) using a Bal-Tec HPM 010 (Balzers Union, Balzers, Liechtenstein) at 2100 bar (Müller and Moor 1984, Studer et al. 1989). Frozen cells were transferred in liquid nitrogen (LN_2) to Salzburg and stored until freeze substitution. Rapid freezing (RF; January 1999) allowed specimens to attach to Thermanox strips, which were plunged frozen in liquid propane and transferred to LN_2, following standard procedures. Freeze substitution (FS) was carried out in a Reichert-Jung CS-Auto (Reichert-Jung Ag, Vienna, Austria) (December 1998 and January 1999) and followed the time and temperature protocols of Müller et al. (1980) and Steinbrecht and Müller (1987).

After warming to 4° C, strips and tubes containing diatoms were rinsed in acetone, transferred to a cold mix of acetone/methanol containing 1% uranyl acetate, left overnight at 4° C (modified after Lancelle et al. 1986), rinsed once in acetone/methanol and twice in acetone, and gradually infiltrated with the low viscosity Spurr mixture at room temperature. Polymerization was as advised (Spurr 1969). Sections of cryofixed cells were stained at room temperature for 30 min in aqueous 1% $KMnO_4$ and poststained for 45 min in 2% uranyl acetate dissolved in 30% methanol, followed by 2 to 5 min in Pb-citrate (modified after Hess 1990) or stained with a modified Thiéry method (Weber 1989).

The marine *Trachyneis aspera* (Ehr.) Cleve, a pyrenoid-containing pennate diatom, was collected in 1998 from the Red Sea at Sharm el Sheikh and chemically fixed. All specimens were examined with a transmission electron microscope (model 400, Philips, Eindhoven, Netherlands) and documented on Agfa Scientia or Kodak (Washington, PA, USA) electron microscope film Estar thick base 4489 (Agfa-GeVaert, Belgium).

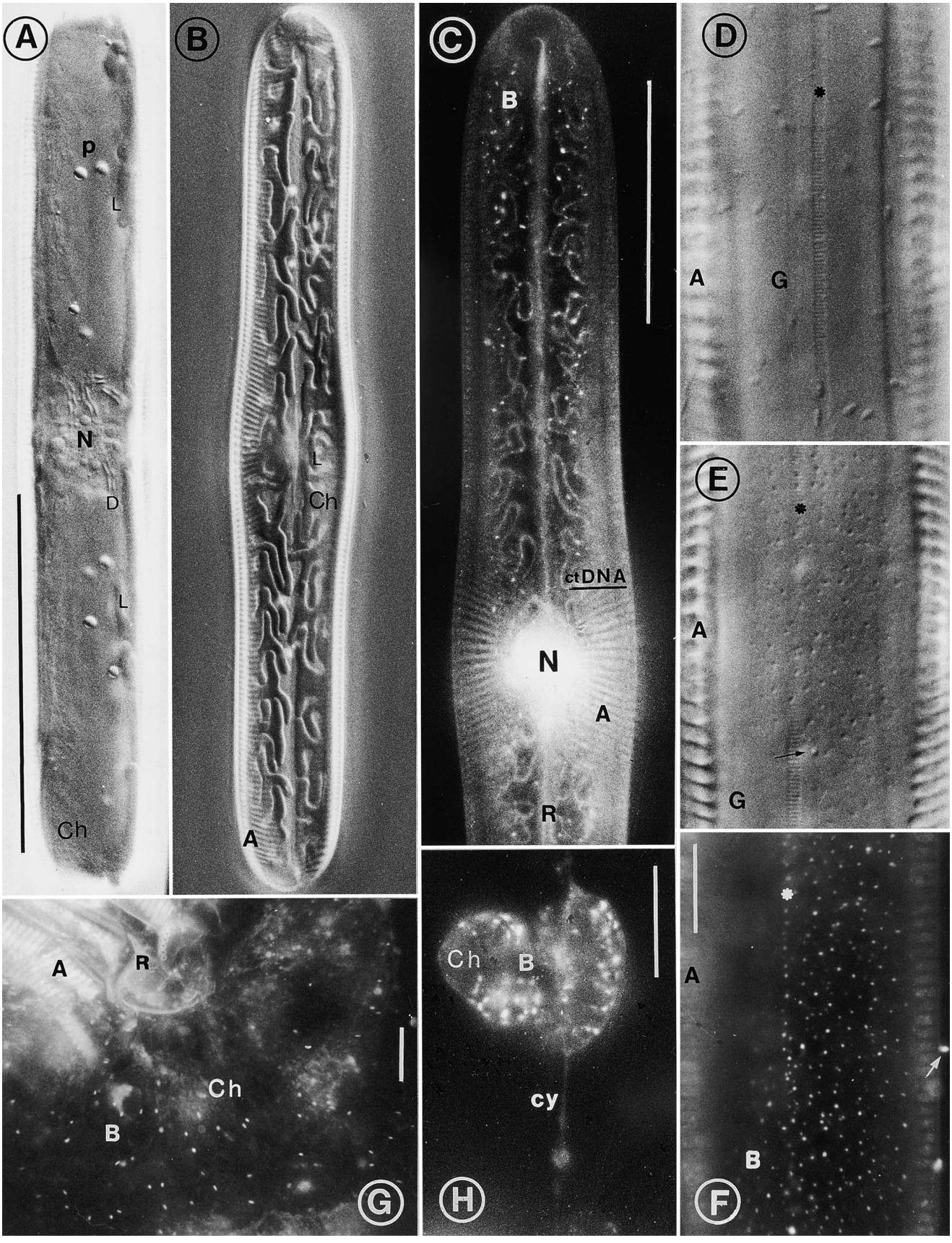
RESULTS

Cell organization of the living U-strain cells (LM-DIC and FM). The two golden-yellow platelike chloroplasts were embedded in the cortical cytoplasm, lying against the girdle sides during interphase. They reached without interruption from pole to pole, below the alveoli of the valve, and were highly lobed at their edges (Fig. 1). The chloroplast margin gave bright fluorescence with DAPI, indicating peripheral ct-DNA (Fig. 1C). Comparison of images of endobacteria with those of exobacteria by focusing on different optical planes showed their size difference (Fig. 1, D and E). The apochlorotic dots (endobacteria) appeared as invaginations in DIC optics when compared with spherical organelles lying close by (Fig. 1E). No autofluorescence originated from these spots when excited with UV, blue, or green light (not shown), whereas vital staining with the DNA-specific fluorochromes acridine orange (not shown) and DAPI was positive (Fig. 1, C, F–H), in both cases showing the DNA-dye–specific emission spectra. Fluorescent particles were often seen to be arranged in a row below, and aligned with, the series of slits in the girdle band of the epitheca (Fig. 1, D–F), probably resulting from a specific local adhesion of the cytoplasm along this part of the cell wall. Pressure, gently applied during observation, squeezed the plastids and cytoplasm out of the cell, revealing that particles were rod shaped and not restricted to the (then denatured) plastids (Fig. 1, G and H). No aposymbiotic *Pinnularia* cells have been found at the sampling site.

Fixed cells of Pinnularia *(LM-DIC, FM).* When fixed in ethanol:acetic acid (Geitler's main fixative; Schmid 1989), the plastid appeared sieve-like perforated (Fig. 2, A and B). DIC images of isolated plastids revealed small "dots" within chloroplast cavities (Fig. 2A). DAPI staining displayed them as DNA-positive dot- or rod-shaped particles clearly inside "holes" in the plastids (Fig. 2B). The lectin WGA bound to bacteria in whole cells (not shown) as well as in sections (Fig. 2C), indicating a recognition of *N*-acetyl-glucosamine acid residues (Kleinig and Sitte 1992, Madigan et al. 2000).

Host ER -membrane system. During interphase the central nucleus was pulled out into numerous tentacles, distally thinning out into ER cisternae, along which Golgi bodies were paired (Figs. 3, A–C, and 8). This is an ideal compromise, allowing the nuclear envelope (= perinuclear cisterna) to bear a dense pattern of nuclear pores (Fig. 3, A, C and inset, and E) and simultaneously bud vesicles from its outer membrane toward the *cis*-face of the dictyosomes (Fig. 3, A–C) (Schmid 1994, Fig. 15). These specialized domains of the host membrane system (perinuclear cisterna and regions subjacent to the dictyosomes) lacked ribosomes, although their outer membrane was continuous with the

FIG. 1. LM-DIC and DAPI-DNA fluorescence images of live *Pinnularia* cells. (A–C) Surveys of the platelike chloroplasts (Ch) with lobed margins (L) in DIC optics in girdle (A) and valve view (B) and in the FM (C, valve view) with the chloroplast-DNA (ct-DNA) delineating the lobes. Paired dictyosomes (D) surround the nucleus (N); polyphosphate granules (P) are in the vacuole; alveolate chambers in the valve (A) are visible as transapical striation. DAPI emission in (C) also highlight nuclear (N) and bacterial (B) DNA, the latter visible also outside the contours of the chloroplast. Valve structures (R, raphe; A) are visible due to reflexion phenomena. (D and E) Two focal planes in DIC optics of the central girdle band region (G) show the difference between exobacteria (D) and endobacteria (E). Note the different location of the "shadow" on a lipid droplet in the cytoplasm (arrow in E) and on the plastid dots (E), indicating convex and concave structures. The DAPI-DNA image in F corresponds to E; white arrow point to exobacteria. Valve chambers (A) from the side; asterisks mark a row of slits (D–F) in the girdle band and apparent alignment of bacteria (F). (G and H) Details of a squashed DAPI-stained cell. Cytoplasm and chloroplast (Ch) with rod-shaped bacteria (B) extruded from the polar region of the broken cell wall (A, R). A cytoplasmic droplet (H) connected to the main cell with a thin strand (cy) contains chloroplast fragments (Ch) and bacteria (B). Scale bars, 100 μm (A and B), 50 μm (C), 10 μm (D–H).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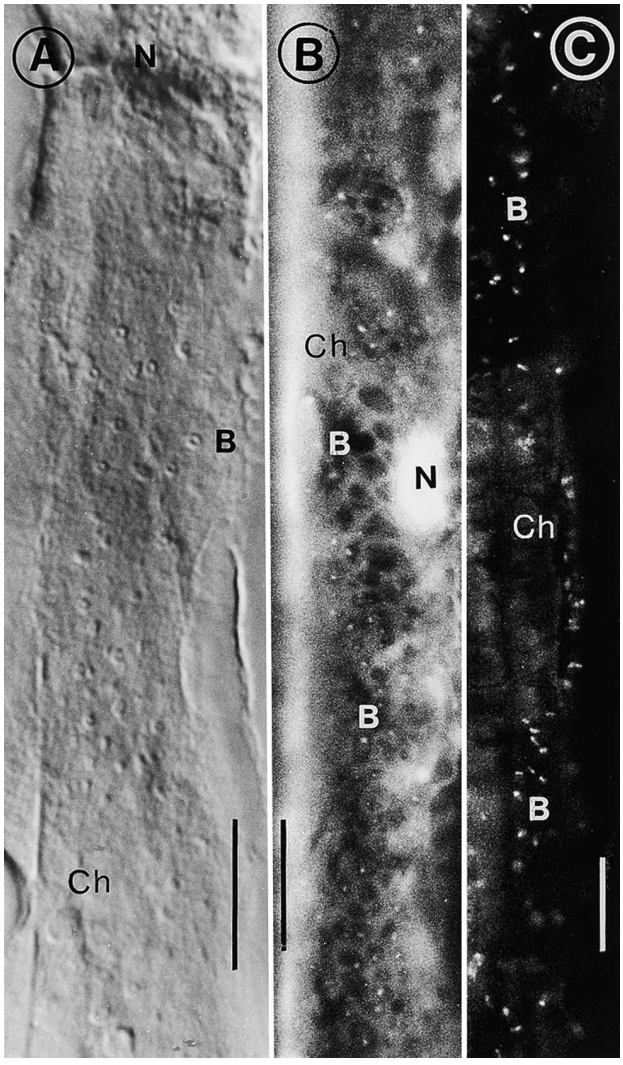


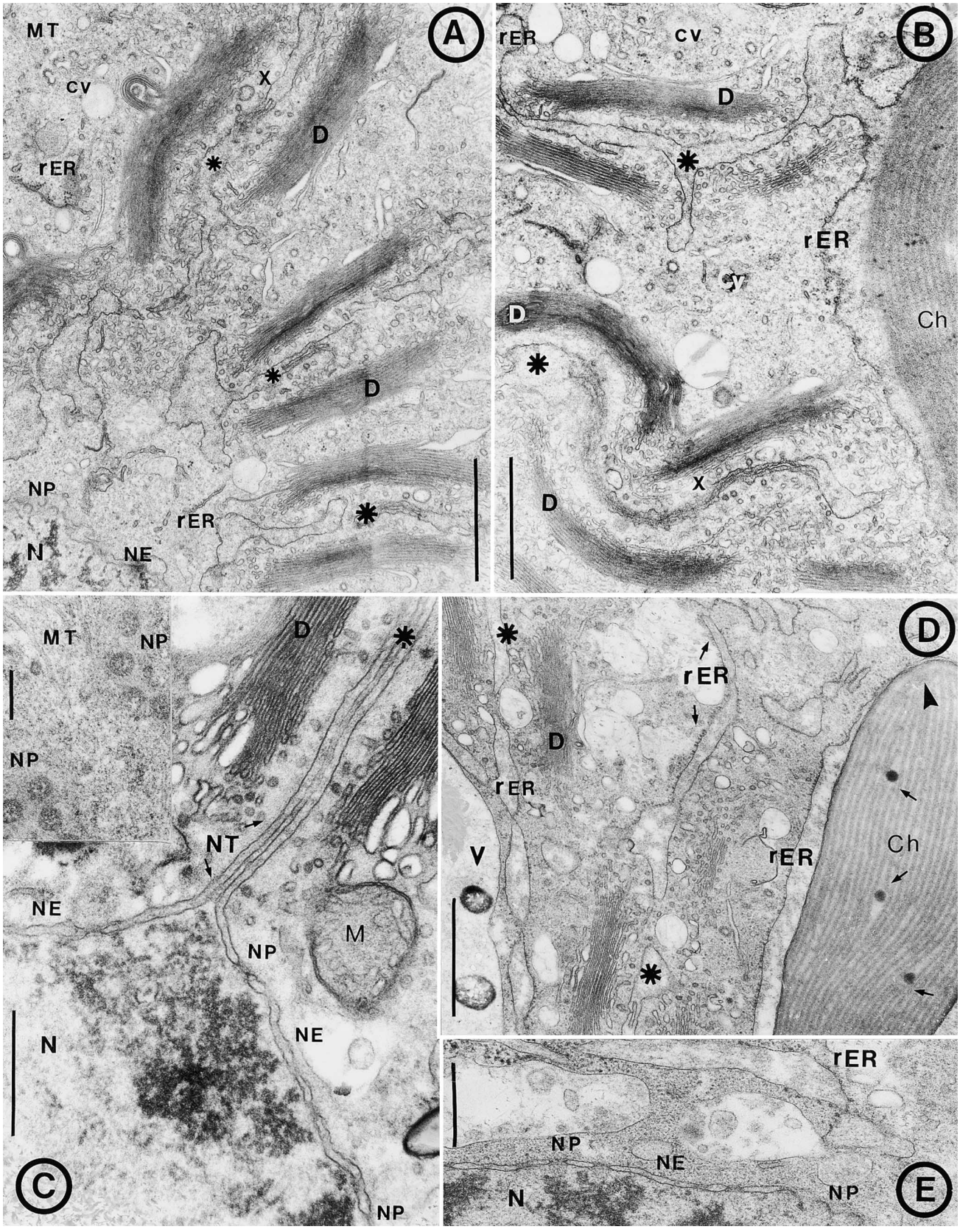
FIG. 2. LM and FM images of bacteria in fixed *Pinnularia* cells. (A and B) Isolated chloroplasts (Ch) in DIC optics show small granules (B) inside holes in the plastid that correspond to DAPI–DNA complexes; N, nuclear region. (C) Glancing thick section of a lectin (WGA Texas red) labeled plastid (Ch) indicating bacterial walls (B). Scale bars, 10 μm.

rough ER system of the diatom cell and the rough ER membrane surrounding the secondary plastid (Fig. 3).

Complex chloroplast. Exceptional among diatoms, the *Pinnularia* chloroplast was devoid of any trace of a typical pyrenoid in TEM profiles, despite having been sectioned in all three possible planes. Thylakoids were densely stacked into lamellae that left little space for chloroplast stroma (Figs. 4, A and B, and 5, A–D). During interphase, lamellae were continuous over long distances and in longitudinal section lay almost parallel to each other and to the chloroplast surface (Figs. 3, B and D, 4, A and B, and 5, A–D). Their ends were alternately interdigitated between others. In addition, single thylakoids traversed the matrix and joined adjacent lamellae (Fig. 4, A and B). Lipid droplets (plastoglobules) were closely packed in the stroma between intact lamellae, and single lamellae were seen to terminate also at single osmiophilic granula (Figs. 3D and 4B), but several stacks were simultaneously interrupted by bacteria (see below). Plastid edges displayed thylakoid free regions containing fibrillar material (Fig. 3D) (Schmid 2003, Fig. 3, a and d), known from other diatoms to contain plastid-DNA and supporting the results with DAPI staining (Fig. 1C).

As in other diatoms (e.g. Maier et al. 1996), the chloroplasts were surrounded by four membranes altogether, the two innermost invariably parallel, constituting the primary chloroplast envelope, which in turn was enveloped by a membrane homologous to the cell membrane of the engulfed red alga (e.g. Maier et al. 1996) (Fig. 4, C–G). Ideal cross-sections, particularly of prophase plastids (Fig. 4D), showed fibrillar connections between this vestigial red-algal cell membrane (rCM) and the chloroplast envelope, resembling a "fibrous lamina" found in several diatoms to be associated with the cell membrane of the host (Schmid 1994, Schmid et al. 1996). The cytoplasmic space between the chloroplast envelope and the rCM together with its "periplastid reticulum" (Fig. 4D) (Gibbs 1981) appeared only rudimentary in this *Pinnularia* and was restricted to the inner chloroplast surface, facing the vacuole. During interphase it was confined to the regions of the diatom where bacteria invaginate the plastid (Figs. 5, A and D, 6, A and D, and 8). Finally, the complex chloroplast was completely surrounded by a single ER membrane, studded with ribosomes arranged as polysomes (Figs. 3, B and D, 4, B–G, 5, A–E, 6, A and C–F, and 8). At the outer plastid surface, this ER membrane was contiguous with the rCM but at a variable spacing at its inner surface, where the intracisternal lumen was over large areas inflated, irrespective of whether cells were con-

FIG. 3. TEM profiles of the *Pinnularia* host membrane system (all chemically fixed). (A) Survey from nucleus (N) in the lower left corner to the periphery. NP, nuclear pores; NE, ribosome-free nuclear envelope vs. ribosome-containing ER (rER). Dictyosomes (D) paired along specialized domains of ER cisternae with duplicated membranes lacking ribosomes (asterisks, X), coated vesicles (cv) at their *trans* face. (B) Peripheral region with secondary chloroplast (Ch) inside a special ER compartment (rER), topologically continuous with the ribosome free ER domain subjacent to dictyosomes (asterisks); X marks transition vesicles. (C) Detail of nucleus (N) with nuclear pores (NP), the ribosome-free nuclear envelope (NE) extending into a tentacle (NT) lacking ribosomes (asterisk) subjacent to dictyosomes (D). (Inset) Glancing section of nuclear envelope with nuclear pores (NP) and associated microtubules (MT) but no ribosomes. (D) Survey from vacuole (V, left) to chloroplast (Ch, right), demonstrating continuity of ER membranes with (rER) and without ribosomes (asterisks). Arrows in the chloroplast point to ends of single thylakoid stacks, arrowhead to ct-DNA. Note inflated space of the plastid containing rER at the side facing the vacuole in B and D. (E) Detail of nuclear region (N). Note lack of ribosomes at the nuclear envelope (NE) as compared with the rER. Scale bars, 1 μm (A, B, D), 0.5 μm (C, E), 0.2 μm (inset of C).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ventionally fixed (Figs. 3, B and D, 4G, 5, 6, and 8) or cryoimmobilized (Fig. 7A) (Schmid 2003, Fig. 4a). At the plastid edge, isolated lobes were found inside a common swollen ER cisterna (cf. Fig. 1, B and C with Fig. 4G), that is, the secondary plastid is enclosed within the lumen of a specialized rough ER cisterna.

Size and ultrastructure of the bacteria. Endobacteria were ultrastructurally different from the exobacteria sticking in the exocellular mucous capsule (not shown) and were smaller (0.3–0.4 μm wide and 0.8–1.0 μm long during the interphase) than exobacteria (cf. Fig. 1D and E). TEM images suggest endobacteria were gram negative, because of a clearly visible extra wall with the typical trilamellate image separated from the plasma membrane by a periplasmic space (Figs. 5E and 6). A fuzzy coat external to this wall displayed a periodically spaced substructure in ideal cross-sections and glancing sections, which appeared to run in helical gyres around the cell body (Figs. 5A and 6). When prepared by HPF-FS (Fig. 7A), the bacterial image resembled that of *Escherichia coli* prepared in a similar way (Studer et al. 1989). The interior was filled with a fibrous network and ribosomes, which were smaller than those attached to the host ER (Fig. 6E), and both were only ill defined in unstained sections. Circular membrane profiles were local invaginations of the cell membrane (Figs. 5F and 6E). They occurred more frequently in bacteria that were on their way to the central cytoplasm in pre-prophase (Schmid 2003).

Intracellular location of bacteria. This was dependent on the cell cycle of the host (Schmid 2003) and in the U-strain *Pinnularia* restricted to the lumina of the ER cisternae (Figs. 5, 6, 7A, and 8). During interphase endobacteria were exclusively found in the inflated space between the vestigial cell membrane of the reduced endocytobiont and the ER membrane, where they appeared to bore either singly in more or less regular intervals into the chloroplast or were present in clusters (Figs. 5A, 7A, and 8). The distribution of bacteria profiles in plastid-glancing sections (Fig. 5F) agreed with FM patterns of DAPI, WGA, and large subunit of RUBISCO (Schmid 2003) complexes. Bacteria were never found to occupy the space of the nuclear envelope. They did not even invade the nuclear tentacles, which were free of nuclear pores and thus free also of mechanical barriers (Fig. 3, A–D, and summarized in Fig. 8). No dividing bacteria have been detected in the vicinity of the plastids.

Interaction with secondary plastids. The bacteria, cohabiting the same intracisternal compartment of the diatom ER system as the secondary plastid, were not seen to align themselves with these ER membranes during interphase. Contact between the luminal face of the ER and bacteria was detected only at bacterial poles, via filamentous aggregates (Figs. 5, B–E, 6, A and D–F, and 8). Periodically spaced structural links were also visible between the fibrous coat of the bacteria and the exterior side of the rCM (Figs. 5A and 6), to which bacteria had a much greater affinity than to the ER membrane (Figs. 5, 6, 7A, and 8). TEM profiles suggested that bacteria immediately align very tightly with the rCM, as soon as they come near it, regardless of whether the point of contact was a pole (Figs. 5, A–D, 6, A and D–G, 7A, and 8) or a lateral wall region (Figs. 5, C and E, 6, A, C, and E, and 8). At the area of attachment the rCM and chloroplast envelope were concavely deformed, as were the thylakoids initially (Figs. 5A, 7A, 8, and 9). TEM images revealed that where the bacterium bores into the plastid, concave (pigmented) thylakoids progressively disintegrate (they are pierced; Figs. 5, A–D, 6, 7A, and 9) without disruption of the rCM or the chloroplast envelope, which appeared locally almost fused face to face (Figs. 5E, 6, B and C, 7A, 8, and 9) and were occasionally left over as membrane outfolds (Figs. 5A and 6, A and B). This provided conclusive evidence that bacteria only invaginate the chloroplasts but do not actually invade them. Some images showed the nearby thylakoids oriented toward the bacterium (Fig. 7A). Local disintegration of pigmented thylakoids was visible by LM as apochlorotic spots.

DISCUSSION

Ultrastructure of Pinnularia *and its chloroplasts after different fixations.* Micrographs displaying aspects of the cytoplasmic ultrastructure of a typical *Pinnularia* cell have been previously published by Drum et al. (1966, *P. "nobilis"*), Pickett-Heaps et al. (1978, 1979, *P. viridis*, *P. maior*), Schmid (1994), and Schmid et al. (1996, *P.* cf. *"nobilis"*, *P. maior*). Plunge freezing resulted in 99% trash because the *Pinnularia* cells are too large and too vacuolated, and the cost of HPF is too great for screening. Moreover, there were problems with insufficient contrast in the combination with Spurr embedding and other deleterious effects (Lancelle et al. 1986, Studer et al. 1989, Hess 1990). Thus the method of choice for this large diatom was chemical fixation, despite a number of unavoidable artifacts, such as the "negative image" of thylakoids (as in cryoimmobilized cells) when simultaneously fixed using tannic acid. On the other hand, plastid ultrastructure is almost as well preserved as after cryofixation, delineating densely stacked lamellae, which leave little space for a chloroplast stroma. In light of the information gained from TEM studies of chloroplasts from the green and chromophyte algae, it is difficult to imagine that this matrix, which is also traversed by thylakoids joining neighboring lamellae, should contain the total amount of RUBISCO in a dispersed form, as in higher plants. This question is still unresolved and of special interest because molecular studies revealed the proteobacterial origin of the RUBISCO in the red algal lineage (Kowallik 1992, 1999, Jenks and Gibbs 2000).

As in other diatoms, single lamellae terminate among intact lamellae, which seems one of the prerequisites for maintaining the internal stability of a chloroplast more than 200 μm long. However, at the sites where bacteria exert pressure perpendicular to the thylakoids, whole stacks of lamellae, often 10 to 15, are pierced simultaneously, which locally weakens the mechanical strength of their membranes (and its mem-

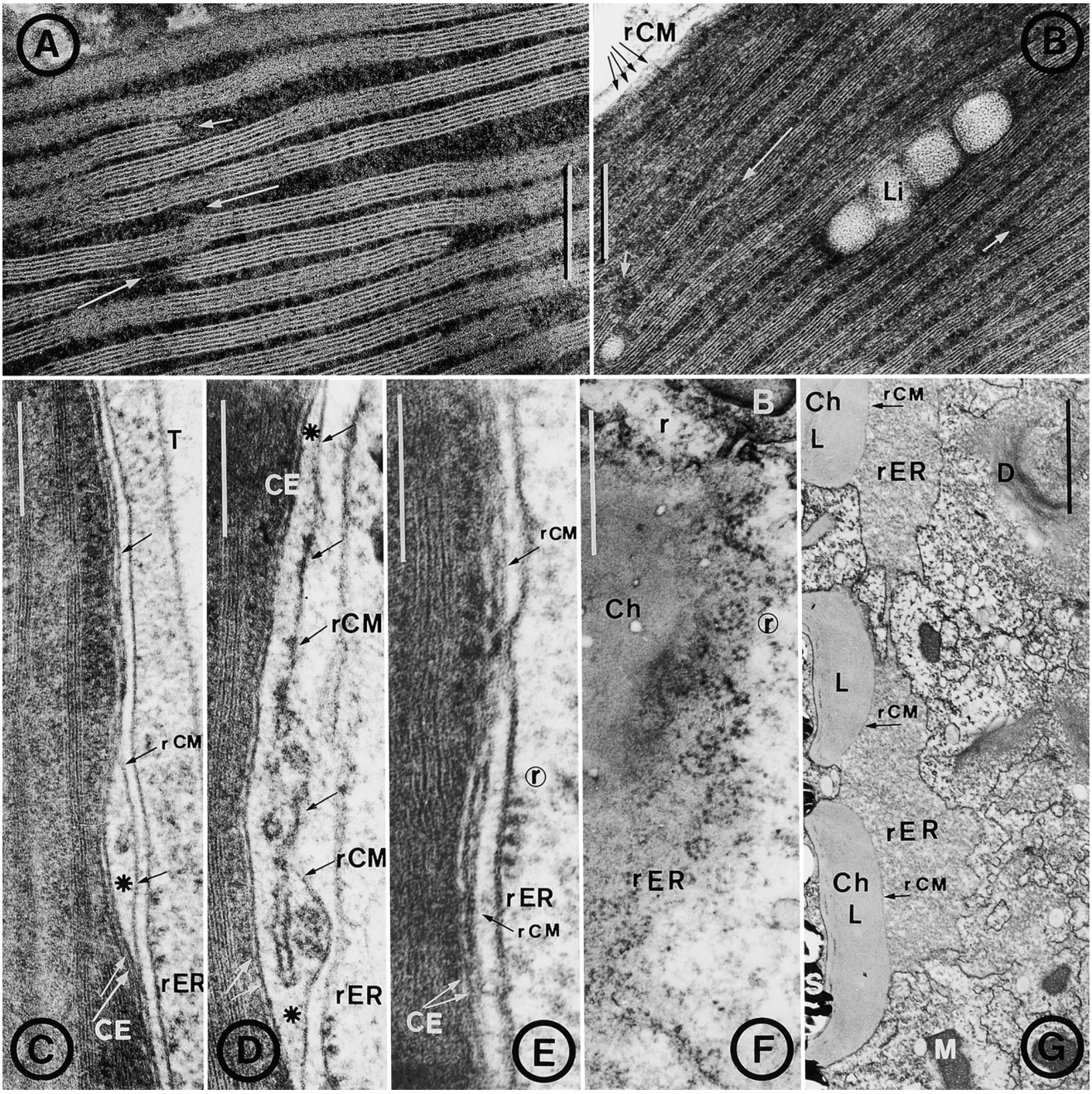
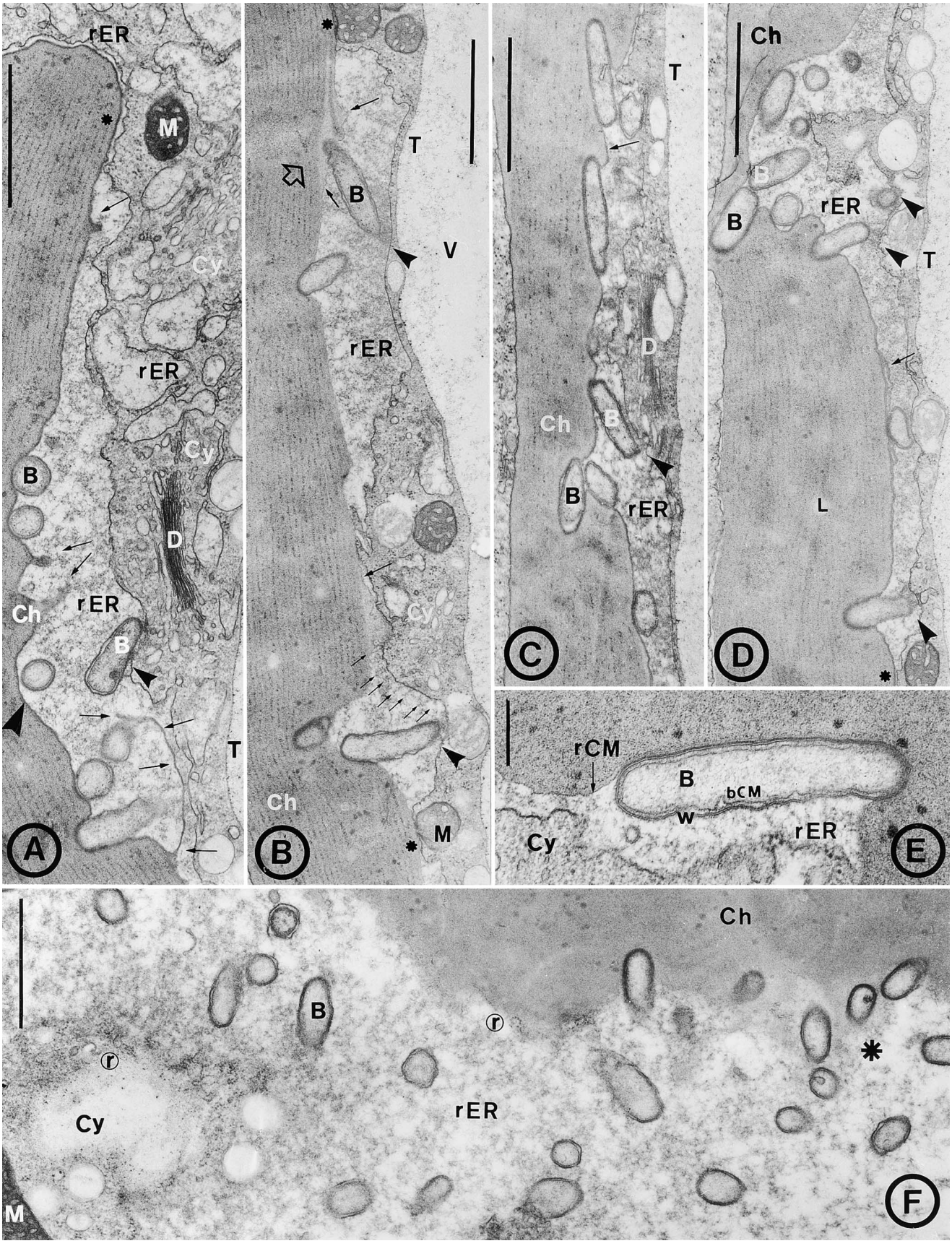


FIG. 4. TEM profiles of the complex chloroplast of *Pinnularia.* (A and B) Comparison of details of the chloroplast interior of a cryofixed (A) and a chemically fixed cell (B); single lamellae end (short white arrow) in the matrix or at lipid droplets (Li) and single thylakoids join stacks (long arrows). Black arrows point to a periodic pattern of fibrils in the relict cell membrane of the macroendosymbiont (rCM). rER membrane of the host is external to it. (C–G) Details of the membrane profiles surrounding the plastid: (C–E) prophase and (F and G) interphase. CE and white arrows point to the primary chloroplast envelope; rCM and black arrows indicate the relict cell membrane of the secondary plastid coated with a "fibrous lamina" (asterisks) on its cytoplasmic face and membrane infolds (="periplastid reticulum"). rER is the boundary membrane of the ER compartment containing secondary plastid (Ch) and bacteria (B) and is beset with groups of ribosomes (r) seen as polysomes in glancing sections (F). (G) Survey image of a cross-section through the chloroplast margin shows separated lobes (Ch-L) inside a continuous rER compartment. D, dictyosomes; M, mitochondrium; T, tonoplast). (A) Cryofixation (rapid frozen-freeze substituted), (B–G) chemical fixations. Scale bars, 0.2 μm (A–E), 0.5 μm (F), 2.0 μm (G).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brane skeleton) and thus the integrity of the whole chloroplast as soon as the tight connection between bacteria and plastid is cleaved. The small holes drilled by the bacteria into the pigmented thylakoids become larger after chemical fixations, explaining Geitler's (1937a) description of a sieve-like perforated chloroplast in *P. "nobilis."* Glancing sections cut parallel or obliquely to lamellae of the interphase chloroplast did not display any extraordinary feature in TEM, apart from the bacteria profiles within smooth even lamellae. The dramatic event apparently taking place at the diatom's pre-prophase therefore deserves particular attention (Schmid 2003).

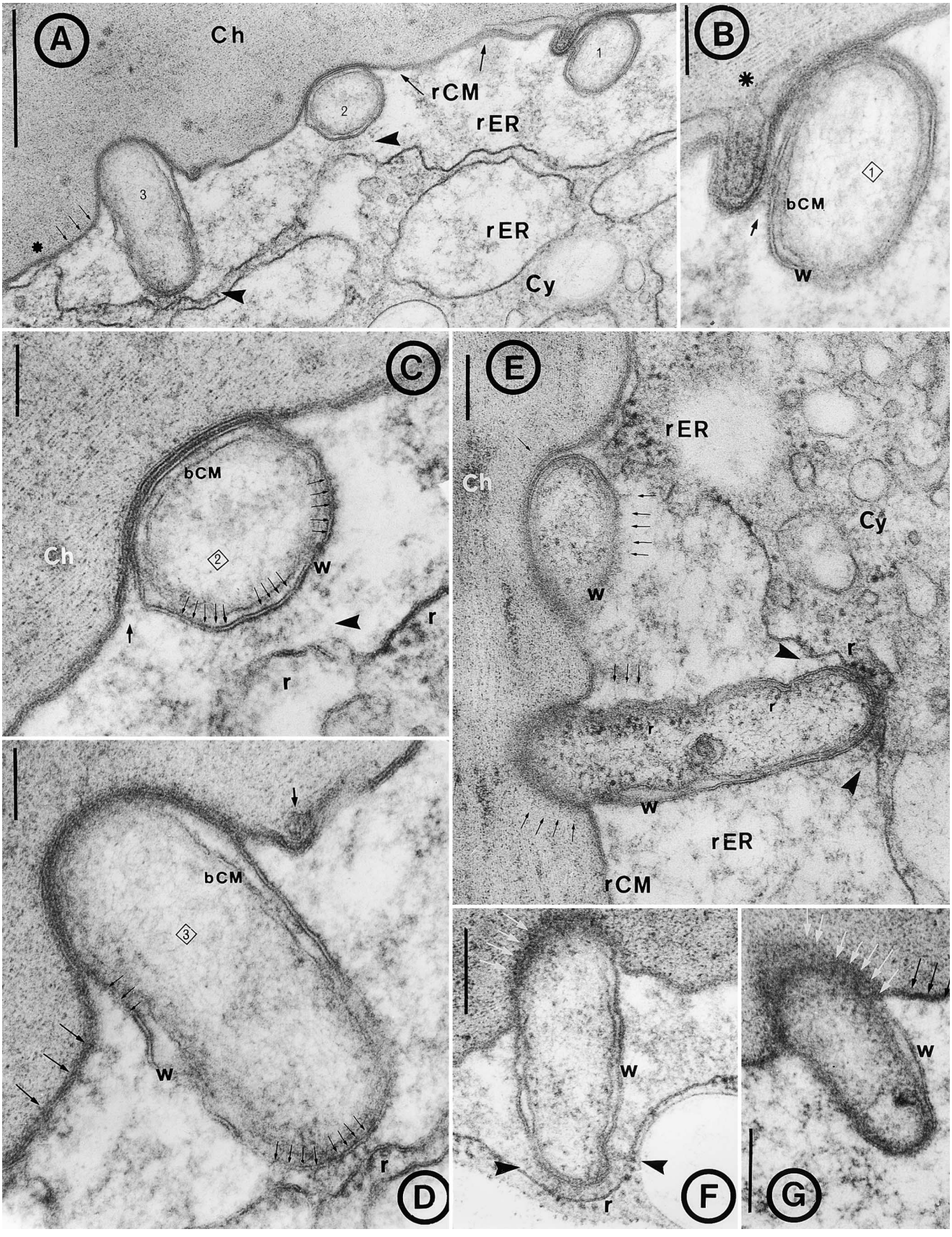
In conventional diatom chloroplasts the only structure that is seen to interrupt thylakoids in a similar way is the pyrenoid (Drum et al. 1966, Schmid 2001). For direct comparison, Figure 7B shows a detail of the chloroplast of the marine pennate diatom *Trachyneis aspera*, which contains a bar-shaped, embedded, pyrenoid running almost the length of the plastid and has at the side facing the vacuole, a "stalk region," directed toward the plastid envelope. Here the thylakoids are interrupted as in *Pinnularia*.

Plastid DNA. Unlike green algae and higher plants, in which chloroplast DNA occurs in distinct nucleoids throughout the chloroplast matrix and/or around pyrenoids (Coleman 1985, van den Hoek et al. 1995), matching the current theory of a direct cyanobacterial origin of chloroplasts in the green line chloroplasts in chromophytes are, as revealed from ultrastructural and molecular studies, derivatives of a secondary endocytobiosis (Gibbs 1990, Kowallik 1992, Sitte 1993, Melkonian 1996, Medlin et al. 1997). Concurrent with the uptake of the red algal plastid progenitor into the host and the loss of phycobilisomes (Kowallik et al. 1995), a transposition of thylakoids, pyrenoids, and plastid-DNA must have taken place. The DNA was eventually dislodged into a circular array around the chloroplast edges (Dodge 1973, Coleman 1985), and only few exceptions are reported (van den Hoek et al. 1995). It cannot be excluded that diatom plastids may show exceptions too, but this is unlikely in *Pinnularia*, where spots stained by pyrenoid dyes (Schmid 2001) are similar to those recognized by the lectin WGA (indicating bacterial walls) and not only display a pattern comparable with the scattered DAPI spots but correlate with bacteria profiles in TEM chloroplast-glancing sections. The unequivocal evidence for the bacterial origin of DNA–DAPI fluorescence disproves previous speculation that they were scattered globular ct-nucleoids (Mayama and Shihira-Ishikawa 1994a,b,c, 1996, Mann 1996) and at the same time shows the importance of not overestimating the otherwise exciting resolution and advances of a confocal laser scanning microscope. As often in cell biology, and currently ignored far too much despite an increasing demand to correlate molecular data with ultrastructure, organization and intimate localization of subcellular structures can be resolved only with the electron microscope (Schmid 1989).

Topological affiliation of bacteria with the intracisternal space. These bacteria live inside the ER; thus any speculation that their outer cover, resembling a biomembrane in profile, could be a derivation of the host's plasmalemma can be excluded. This additional wall is typical of gram-negative bacteria (Kleinig and Sitte 1992, Madigan et al. 2000). The U-strain *Pinnularia* is not the only organism harboring bacteria within the cisternae of the rough ER. Rod-shaped bacteria were found in *Mallomonas papillosa* (Belcher 1969) inside swollen rough ER cisternae branching off the inflated regions of the ER compartment housing the secondary plastid. No micrograph was supplied, showing a closer association of bacteria with the plastids, and no further comment on this symbiosis was made. A similar scenario was published for *Ochromonas monicis* (Doddema and van der Veer 1983) in which bacteria were suspected to provide vitamins.

Although the ER membrane is continuous from the rough ER membrane surrounding the secondary plastid through the outer membrane of the nuclear envelope, bacteria in the U-strain *Pinnularia* never invade the latter. Instead, during interphase, they remain restricted to the specialized ER compartment that contains the secondary plastid and do not even migrate into cytoplasmic parts of the rough ER. This implies a type of barrier that is active only during interphase and controlled by a mechanism that is synchronous with the cell cycle. In addition, it reflects perhaps functional separation into regional subdomains, as previously reported for a variety of cells (Denecke 1996), and supported in *Pinnularia* by the lack of ribosomes on the

FIG. 5. TEM images of longitudinal sections through *Pinnularia* cells showing surveys of chloroplast–bacteria associations (all chemically fixed); A–D are cut perpendicular to thylakoid stacks, all the same orientation with the vacuole (V; T, tonoplast) to the right side, and E and F are glancing sections. (A–D) Intraluminal space of the rER compartment inflated at the side facing the vacuole but not in the polar region of the plastid (A) or subjacent to mitochondria (asterisks in A–D). Black arrows in A–E indicate the rCM. Note large outfold of the rCM in the lower part of A and smaller ones poleward and in the middle region of D (black arrows). A–E show the affinity of bacteria (B) to the rCM, either with their poles (A–D) or laterally (C, E) and single or in clusters; (D) the plastid is almost perforated. Large arrowhead in A indicates a fuzzy halo around a bacterium; small arrowheads point to fibrillar connections between bacteria walls and the rER membrane (A–D), which show a distinct periodicity (small arrows in B). Adhesion of bacteria with one pole to the plastid, whereas the other is still in contact with the rER membrane is seen in B–D but best in B (large open arrow). (E) Detail of attached bacterium aligned with its lateral wall (W) with the rCM (arrow). bCM, bacterial cell membrane; Cy, host cytoplasm. (F) Distribution of bacteria in the plastid (Ch), upper right, and the inflated lumen of the rER compartment (rER). Host cytoplasm (Cy, M) in the lower left; r inside a circle indicates polysomes; asterisk marks bacteria with infolds of their cell membrane. Scale bars, 1 μm (A–D, F), 0.2 μm (E).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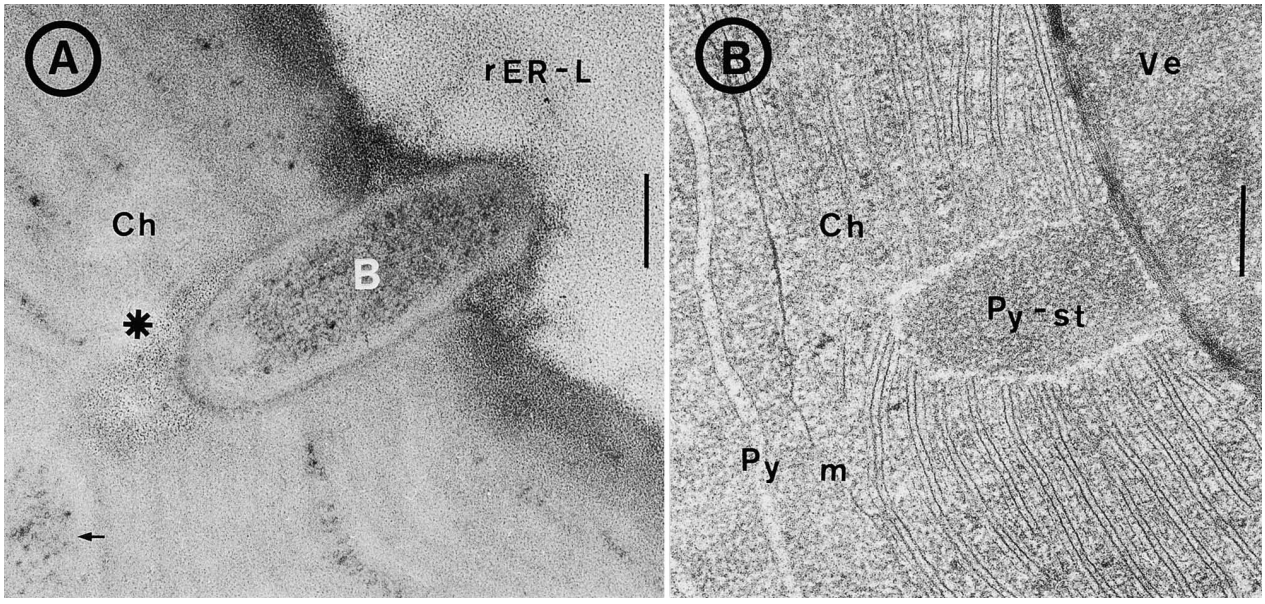

FIG. 7. TEM images of cross-sections through the chloroplasts of a cryofixed (HPF-FS) *Pinnularia* (A) and a chemically fixed *Trachyneis aspera* (B). Note resemblance in the appearance of the interrupted thylakoids caused by the bacterium in *Pinnularia* and by the pyrenoid stalk (py-st) in *Trachyneis*. Disintegrated thylakoids (asterisk) subjacent to the bacterium in A and also between pyrenoid stalk and membrane bound pyrenoid (m, py) in *Trachyneis*. Arrow in A indicates chloroplast matrix. rER-L, Er-lumen; Ve in B marks a cytoplasmic vesicle attached to the noninflated rER membrane. Scale bars, 0.2 μm.

nuclear envelope and on the ER region budding off vesicles to the dictyosomes and with findings in other protists, in which bacteria are restricted to distensions of the nuclear envelope, as they are in the chrysophytes *Paraphysomonas* (Preisig and Hibberd 1984) and *Chrysosphaerella* (Wujek 1984) or in a *Paramecium* (Jenkins 1970). Thus, from what is now generally known about the functional specializations of the ER and from what is visible from TEM images, statements that complex diatom (and other chromophyte) plastids would "usually lie within the perinuclear cisterna" (Cavalier-Smith 2000) appear as unrealistic simplifications.

The intracisternal space of the rough ER is a unique aqueous environment, transiently containing the proteins that follow different secretory pathways and sugars for their core glycosylation, and it is also the reservoir for luminal resident proteins (Kleinig and Sitte 1992). Altogether this makes it a nutritional paradise for endobacteria. However, the ER lumen is also reported to store defense related proteins, synthesized during interaction of cells with pathogenic microorganisms (Denecke 1996). Remarkably, bacteria thriving in the lumen of the rough ER in *Pinnularia* apparently do not elicit a visible defense response of the host apart from retarding the rate of division (Schmid 2003) and in turn do not seem to disturb normal function of this host ER, such as protein synthesis or morphogenetic blueprint function during cell wall formation (Schmid 1994, 2003, Schmid et al. 1996). This points to a great immunological compatibility between host and bacteria, which is not likely to be transient (i.e. facultative).

FIG. 6. TEM images of longitudinal sections through *Pinnularia* cells showing details of bacteria–plastid interactions (all chemically fixed). (A) Alignment of three bacteria (1, 2, 3) with the rCM (arrows) and their details in (B–D). Asterisk marks border of inflated rER space; small arrows point to fibrillar extensions from the rCM, arrowheads to fibrillar connections between bacteria and the rER membrane. (B) Bacterium (1) attached to an outfold of the macroendosymbiont's surface (arrow); asterisk indicates a possible twist of plastid membranes. Note bacterial cell membrane (bCM) and bacterial wall (W). (C) Bold arrow points to fibrillar connection between bacterium (2) and rCM; arrowhead marks fibrils between bacterium and rER; light arrows mark fibrils extending from the bacterial surface. (D) Long arrows point to fibrillar extensions of the rCM; short bold arrow indicates membrane infolds of the rCM. Light short arrows indicate fibrils extending from the bacterial surface (3): in the vicinity of the rER membrane they have a similar spacing as the ribosomes (r) at its cytoplasmic face. (E) Profiles of bacteria attached with a pole and the broad side to the rCM, while still in contact with the rER via fibrils (arrowheads). Small arrows indicate fibrils radiating from the bacterial surface into the lumen of the ER compartment and through the rCM into the chloroplast. Note difference in size of the ribosomes in the host cytoplasm (r, Cy) and bacteria (small r). (F and G) Bacteria showing surface structures in helical gyres (white arrows); black arrows indicate fibrils extending from the rCM; arrowheads mark region of fibrillar connection between bacterium and rER membrane. Scale bars, 0.2 μm (A, E–G), 0.1 μm (B–D).

The rCM. The U-strain *Pinnularia* is also a perfect candidate to support the need to discriminate between the two outer membrane profiles that form additional eukaryotic envelopes around the primary chloroplast envelope, that is, the ER membrane belonging to the host membrane system, and the vestigial cell membrane of the engulfed and reduced plastid containing endocytobiont (Gibbs 1981, 1990, Maier et al. 1996). This membrane was previously termed the "periplastid membrane" by Cavalier-Smith (1989) to denote its different origin, chemistry, and function. However, information gained since then from the results of molecular studies that unequivocally identified the secondary plastid of diatoms as a "streamlined red alga" (Kowallik et al. 1995) allows a more precise definition—rCM. A derivation of the rCM from the host plasmalemma that was originally involved in phagocytosis of the plastid progenitor can be excluded, first on the basis that the rCM is totally enveloped by a single ER membrane and second because in diatoms, the space between the rCM and the primary chloroplast envelope also shows cytoplasm containing a membranous reticulum at defined sites (Drum et al. 1966, Crawford 1973), as in *Pinnularia*, continuous with (and derived from?) the rCM (Gibbs 1981). In both cases, the "rules of compartmentation" (Sitte 1981, Schnepf 1984) would be grossly violated if the rCM were a derivative of the host plasmalemma. For the same reason (i.e. the presence of cytoplasm between the chloroplast envelope and the rCM), the possibility can be excluded that the third membrane might be, as is still sometimes suggested, just the ribosome free other face of a flat ER cisterna of the host, which would surround the chloroplast as a "fold," because this would imply that this cytoplasm would be of host origin. If, however, the third membrane is taken as the vestigial cell membrane of the reduced endocytobiont, then this cytoplasm is of red algal origin, as indicated clearly from TEM images and molecular analyses of *Cryptophyceae* (Gibbs 1981, Sitte 1993, Maier et al. 1996). The difficulties envisioned in this case for a plastid protein transport through the former cell membrane (discussed in Melkonian 1996) could be easily circumvented by vesicular traffic (i.e. endocytosis and transport via the periplastid reticulum into the cytoplasm housing the primary chloroplast; Gibbs 1981) and could coincide with a cell and thus plastid cycle dependent fluctuation in the volume of this cytoplasm (Schmid 2003).

Moreover, the affiliation behavior of the bacteria favors the view of the rCM being the reduced retained plasmalemma of the endocytobiont. If it were just part of an ER fold, then the membranes of this ER cisterna would exceed the membranes of the nuclear envelope in polarization, because bacteria living inside the perinuclear space (Preisig and Hibberd 1984, Wujek 1984) were equidistant to both the inner and the outer membrane. In contrast, bacteria in *Pinnularia* have a much greater affinity to the rCM than to the ER membrane. As is obvious from TEM images, this association is a very tight attachment, although transiently cleaved during the cell division events, but reestablished in post-telophase (Schmid 2003). EM profiles of bacteria aligned with the rCM in *Pinnularia* show a compelling resemblance to EM profiles of *Mixotricha paradoxa* (Cleveland and Grimstone 1964) and similar flagellates (Tamm 1978), where exosymbiotic bacteria are intimately associated with the host cell surface, fitting into distinct pits (Cleveland and Grimstone 1964, Margulis 1993). Considering that the rCM is the retained, though reorganized, cell membrane of the red alga, engulfed by the diatom progenitor, it may not be too absurd to envisage this alliance to be of a similar construction that allows coordinated signal transduction and easier translocation of molecules between the partners. Intriguingly, in both cases there is a high degree of intimacy between membrane and bacteria, and in both cases the alignment of bacteria with the membrane, rCM, or flagellate-cell membrane occurs in a distinct pattern. In contrast to *Mixotricha* and others, however, where division stages are found among the adherent bacteria (Cleveland and Grimstone 1964), in *Pinnularia* no dividing bacteria were seen as long as they are in the vicinity of the chloroplast.

Bacteria and complex plastids: "zipper mechanism" (=alignment, adhesion, deformation). Although the concept of the invagination mechanism seems rather obvious (see below) and intracellular long distance transport is under the influence of the host (Schmid 2003), the mechanisms by which endobacteria are attracted to the plastids are difficult to interpret. No bacterial flagellum that could assist in propulsion has been observed in interphase. Some micrographs suggest the presence of fine fibrils between the external face of the bacteria and the internal face of the rough ER, but their origin and thus their orientation is undetermined. Although fibrils were often seen in a spacing similar to that of the ribosomes attached to the ER, speculation that they were synthesized by the host ER and connected, perhaps as a specific type of marker, to a bacterial pole is not yet supported.

Nothing is known about regulatory mechanisms that ensure the equal distribution of bacteria, as visually assessed from LM and FM patterns and TEM profiles. Morphological evidence suggests that alignment of bacteria with the rCM is receptor mediated via linking structures on their extraplasmatic faces. Bacterial surface groups, perhaps correlating with fibrils, distinctly spaced along helical gyres on their external surface could interact with bacteria recognition sites on the vestigial red algal cell membrane organized in a pattern that would define the area of physical contact between bacteria and rCM (Schmid 2003). Although not yet detected, clusters of bacteria lysing large grooves into the plastid could indicate a local disturbance of such a pattern, the nature of which is still unknown. Sharon and Lis (1989) proposed that most bacterial host recognition (and phagocytosis) is mediated via lectins. The ability to visualize a "fibrous lamina" on the cytoplasmic side of the rCM over larger distances is limited by constraints of the TEM technique.

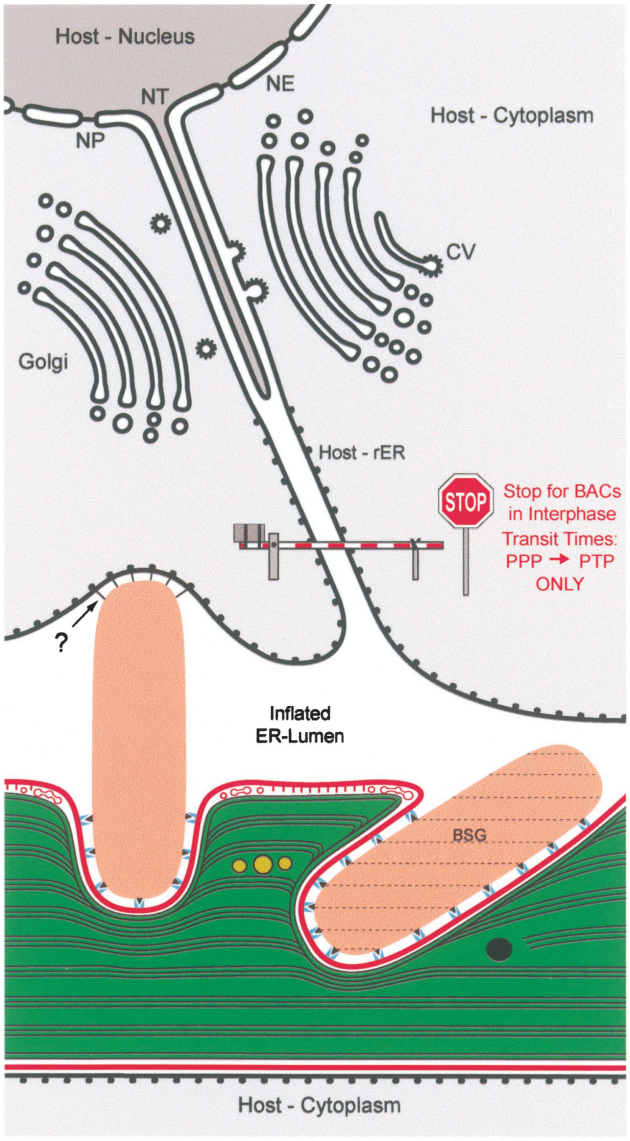

FIG. 8. Schematic representation of the ultrastructural relationship between host, secondary chloroplast, and endobacteria during the interphase of the U-strain *Pinnularia*. Translocation of bacteria (BACs) restricted to the period between pre-prophase (PPP) and post-telophase (PTP) of the host.

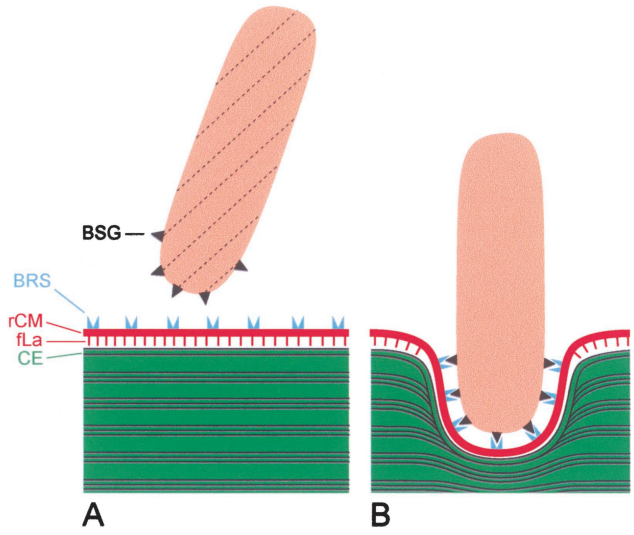

FIG. 9. Schematic drawing of the (A) alignment, (B) adhesion, and deformation ("zipper mechanism") during the interaction of bacteria and secondary chloroplast in the U-strain *Pinnularia* and the disintegration of thylakoids.

Hence, such images do not allow determination as to whether the rCM is totally coated with a fibrous lamina or whether it forms a mosaic distribution conforming with the patterned bacterial adhesion. At present, this issue about the regularity of the porous pattern is as enigmatic as it was six decades ago (Geitler 1937a).

The compiled TEM images of plastid bacterial associations in *Pinnularia* strongly suggest that progressive adhesion of the bacterial surface to the rCM is the key mechanism that creates invaginations in the chloroplasts and simultaneously pulls the bacterium into it. Concomitant with a successful extraplasmatic recognition and adhesion, a local reorientation of the internal membrane skeletons would be essential to bring about invagination. This supportive framework would be expected to contain proteins with tensile or shear resisting properties. Initially, this process is very similar to a "zipper mechanism" proposed by Reisser (1990) to explain phagocytosis, although in the case of the plastid bacterial relation in *Pinnularia*, no actual ingestion follows.

Disintegration of thylakoids but not of the primary and secondary envelopes. Bacterial attachment to, and deformation of, the rCM and chloroplast envelope exerts pressure on the thylakoids perpendicular to their surfaces. Because of their anchorage in the matrix, tensional shear stress results in a concave deformation and then disintegration of the nearby thylakoids and stroma into a cylinder of granular material extending from the bacterium. This indicates that thylakoid membranes are not only functionally different from the enveloping membranes, but also in their physicoelastic properties. Because no visible lysis of the rCM or chloroplast envelope appears but membrane is occasionally left over as outfolds, it is concluded

that the bacterial action has very specific, though unknown, transmembrane effects. Comparison with *Trachyneis* suggests that bacteria are involved in a structural (and functional?) dialogue with thylakoids, known hitherto only from pyrenoids. Pyrenoids as well as bacteria cause regions devoid of pigmented thylakoids and stand out in LM as apochlorotic sites (for pyrenoids see Mann 1996, Figs. 5–10). The morphological integrity of the rCM and the primary chloroplast envelope reflects perhaps their ancestral functions and properties as "vestigial cell membranes." Moreover, both the rCM and the chloroplast envelope (together with their membrane skeletons) are locally deformed and stretched during invagination, which could activate transmembrane channels and elicit a cascade of signals, eventually leading to the observable ultrastructural changes in the chloroplast interior. Some TEM profiles showed bacteria inside invaginations into the chloroplast, enveloping plastid membranes deeply concave, but the thylakoids oriented toward the bacterium (i.e. convex) and an amorphous area above the bacterium. Such images strongly suggest a sophisticated *trans*-membrane communication between bacteria and thylakoids, and perhaps the best way to explain such snapshots is to assume that bacteria were "pulling out" at the time of fixation. Bacteria indeed leave the plastids before the host prophase, that is, before the chloroplast moves to the valves, creating "scars" in the thylakoids (Schmid 2003). Because the rCM and the primary chloroplast envelope have not been seen to become perforated by single bacteria in the U-strain *Pinnularia*, transplastidial channels (not shown) are assumed to be created either by clustered bacteria or during expansion of the lobed edge of the plastid, when adjacent lobes make contact laterally and fuse. Fusion of lobes is preceded by expansion of the ER cisterna, and bacteria, present within this continuous sac, could get trapped, surrounded by the expanding secondary chloroplast.

Endobacteria and the Pinnularia *habitat.* The presence of bacteria inside the U-strain *Pinnularia* since 1991 in batch culture, as well as in the natural habitat, and their morphological difference from those attached to the cell surface indicates that this is neither a pathogenic nor a facultative scenario but a stable symbiosis, in which a not yet specified capability of the bacteria is exploited by the host, which provides in exchange a constant environment within a distinct subcellular compartment. Bacteria in turn adapt their generation cycle to that of the living shelter (Schmid 2003). Certain *Pinnularia* species are apparently associated with bacteria. The DNA-DAPI fluorescence of several *Pinnularia* species from Japan, though interpreted as showing ct-nucleoids (Mayama and Shihira-Ishikawa, 1994a,b,c, 1996), demonstrates that those taxa probably house bacteria too. Pickett-Heaps et al. (1978) report in passing that "numerous endobacteria are apparent" in both *Pinnularia* species they investigated from Colorado (USA), *P. maior* and *P. viridis*, but they did not comment further on their intracellular location. In Salzburg, bacteria profiles have been seen in TEM images of two other local large, *Pinnularia* strains, though intriguingly with species specific variations (unpublished data). Thus it is probably safe to assume that the *Pinnularia* species investigated by Geitler (1937a, *P.* cf. *nobilis*), Gschöpf (1952, *P. viridis*), and Tschermak-Woess (1953, *P. maior*), and others also may have had bacterial symbionts.

The endobacteria of the U-strain *Pinnularia* were found to be exclusively extraplasmatic with respect to the host cytoplasm and with respect to the streamlined endocytobiont. They exist in the ER lumen, the "no man's land" between the host and the secondary plastid. It is interesting that the few reported examples of ER-residing bacteria stem only from chromophyte algae, including this *Pinnularia* and some photosynthesizing flagellates (see above), all complex organisms created by secondary endocytobiosis, fueling the speculation that bacteria could have been exosymbionts of the engulfed plastid ancestor ("primary host") in *Pinnularia.* This chimeric association might thus be interpreted as an ancestral constellation whereby bacteria may have played a role as "couriers" between the two different cytoplasmic territories and eventually became intrinsic also to the "secondary host" in an as yet undefined function.

On the other hand, the *Pinnularia*–bacteria association could be the result of a novel adaptive symbiosis, which allowed the complex organisms to adapt to, and now flourish in, their typical oligotrophic dystrophic habitats (as described to be specific for most of this genus; Krammer 1992), an otherwise perhaps adverse ecological niche. In this case too bacteria would become indispensable to the host and thus indirectly influence also the distribution and diversity of a species. The U-strain *Pinnularia* is the first pennate diatom encountered in our laboratory to be apparently dependent on a layer of natural substrate for healthy growth over longer periods. That the reason for this would be the need for a "sunscreen," allowing the cells to find optimal light conditions via photophobic responses (Wenderoth 1980), can be excluded on the basis of the observation that the downward migration of the circadian rhythm occurs during the dark (!) phase, a behavior found to be typical also for *P. viridis* living in a similar habitat (Harper 1976). Hence, it is reasonable to assume that this vertical movement is routed along a chemical gradient, perhaps as a response to the bacterium's requirements, although this has yet to be proven.

That the experimental phase of establishing bacterial–algal–host chimeras did not cease in the past but actually still progresses may be also displayed with bacteria living and dividing in the plastid stroma in a dinoflagellate (*Woloszynskia pascheri*, Wilcox 1986) and perhaps even inside a cyanobacterium (*Pleurocapsa minor*, Wujek 1979). Such examples not only substantiate Mereschkowsky's (1910) revolutionary visions of "symbiogenesis as the driving mechanism for evolution," they are also consistent with modern endosymbiotic theories based on molecular genetic analyses

(Kowallik 1999, McFadden 2001) and simultaneously allow these theories to be tested. The TEM survey of our *Pinnularia*–bacteria system revealed that it may be ideally suited to act as a model system for exploring homology and evolutionary history of the RUBISCO in chromophyte algae, predicted, on the basis of molecular data, to be of proteobacterial origin (Kowallik et al. 1995, Jenks and Gibbs 2000).

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Belcher, J. H. 1969. Some remarks on *Mallomonas papillosa* Harris and Bradley and *M. calceolus* Bradley. *Nova Hedw.* 18:257–70.

Cavalier-Smith, T. 1989. The kingdom chromista. *In* Green, J. C., Leadbeater, B. S. C. & Diver, W. I. [Eds.] *The Chromophyte Algae: Problems and Perspectives*. Systematics Association Special Vol. 38. Clarendon Press, Oxford, pp. 381–407.

Cavalier-Smith, T. 2000. Membrane heredity and early chloroplast evolution. *Trends Plant Sci.* 5:174–82.

Cleveland, L. R. & Grimstone, A. V. 1964. The fine structure of the flagellate *Mixotricha paradoxa* and its associated micro-organisms. *Proc. R. Soc. Lond. B* 159:668–86.

Coleman, A. W. 1985. Diversity of plastid DNA configuration among classes of eucaryote algae. *J. Phycol.* 21:1–16.

Crawford, R. M. 1973. The protoplasmic ultrastructure of the vegetative cell of *Melosira varians* C. A. Agardh. *J. Phycol.* 9:50–61.

Denecke, J. 1996. Mechanisms controlling function, identity and integrity of the plant endoplasmic reticulum. *In* Smallwood, M., Knox, J. P. & Bowles, D.J. [Eds.] *Membranes: Specialized Functions in Plants*. Bios Scientific Publishers Ltd., Oxford, pp. 367–82.

Doddema, H. J. & van der Veer, J. 1983. *Ochromonas monicis* sp. nov., a particle feeder with bacterial endosymbionts. *Cryptog. Algol.* 4: 89–97.

Dodge, J. D. 1973. *The Fine Structure of Algal Cells*. Academic Press, London, 261 pp.

Drum, R. W., Pankratz, S. & Stoermer, E. F. 1966. Electron microscopy of diatom cells. *In* Helmcke, J. G. & Krieger, W. [Eds.] *Diatomeenschalen im elektronenmikroskopischen Bild*. Cramer, Lehre, Germany, Teil VI, plates 514–613.

Edgar, L. A. & Zavortink, M. 1983. The mechanism of diatom locomotion. II. Identification of actin. *Proc. R. Soc. Lond. B* 218:345–8.

Geitler, L. 1932. Der Formwechsel der pennaten Diatomeen. *Arch. Protistenk.* 76:1–226.

Geitler, L. 1937a. Chromatophor, Chondriosomen, Plasmabewegung und Kernbau von *Pinnularia nobilis* und einigen anderen Diatomeen nach Lebendbeobachtungen. *Protoplasma* 27:534–43.

Geitler, L. 1937b. Der Chromatophorenbau der Diatomeen *Gyrosigma attenuatum* und *Nitzschia sigmoidea*. *Beih. Bot. Centralbl.* 57:425–31.

Gibbs, S. P. 1981. The chloroplast endoplasmic reticulum: structure, function and evolutionary significance. *Int. Rev. Cytol.* 72: 49–99.

Gibbs, S. P. 1990. The evolution of algal chloroplasts. *In* Wiessner, W., Robinson, D. G. & Starr, R. C. [Eds.] *Experimental Phycology: Cell Walls and Surfaces, Reproduction and Photosynthesis*. Springer-Verlag, Berlin, Heidelberg, pp. 145–57.

Gschöpf, O. 1952. Das Problem der Doppelplättchen und ihnen homologer Gebilde bei den Diatomeen. *Öst. Bot. Z.* 99:1–36.

Harper, M. A. 1976. Migration rhythm of the benthic diatom *Pinnularia viridis* on pond silt. *N. Z. J. Mar. Freshw. Res.* 10:381–4.

Hess, M. W. 1990. Spurr resin affects the ultrastructure of freeze-fixed/freeze-substituted pollen grains. *Ultramicroscopy* 34:219–21.

Jenkins, R. A. 1970. The fine structure of a nuclear envelope associated endosymbiont of *Paramecium*. *J. Gen.Microbiol.* 61:355–9.

Jenks, A. & Gibbs, S. P. 2000. Immunolocalization and distribution of form II RUBISCO in the pyrenoid and chloroplast stroma of *Amphidinium carterae* and form I RUBISCO in the symbiont-derived plastids of *Peridinium foliaceum* (Dinophyceae). *J. Phycol.* 36:127–38.

Kleinig, H. & Sitte, P. 1992. *Zellbiologie*, 3rd ed. Gustav Fischer, Stuttgart, Jena, New York, 591 pp.

Kowallik, K. V. 1992. Origin and evolution of plastids from chlorophyll a+c-containing algae: suggested ancestral relationship to red algal plastids. *In* Lewin, R. A. [Ed.] *Origins of Plastids*. Chapman and Hall, New York, London, pp. 223–63.

Kowallik, K. V. 1999. Endosymbiose, ein Motor der Evolution. *Biol. Heute* 1:1–5.

Kowallik, K. V., Stoebe, B., Schaffran, I., Kroth-Pancic, P. & Freier, U. 1995. The chloroplast genome of a chlorophyll a+c-containing alga, *Odontella sinensis*. *Plant Mol. Biol. Rep.* 13:336–42.

Krammer, K. 1992. *Pinnularia. Eine Monographie der europäischen Taxa*. Bibliotheca Diatomologica Bd. 26, J. Cramer, Berlin, Rúgell, Liechtenstein, 353 pp.

Krammer, K. 2000. *The genus* Pinnularia. *In* Lange-Bertalot, H. [Ed.] *Diatoms of Europe*. Vol. 1. A. R. G. Ganter Verlag KG, 703 pp.

Lancelle, S. A., Callaham, D. A. & Hepler, P. K. 1986. A method for rapid freeze fixation of plant cells. *Protoplasma* 131:153–65.

Madigan, M. T., Martinko, J. M. & Parker, J. 2000. *Biology of Microorganisms*, 9th ed. Prentice Hall International Editions, London, UK, 1175 pp.

Maier, U. G., Hofmann, C. J. B. & Sitte, P. 1996. Die Evolution von Zellen. *Naturwissenschaften* 83:103–12.

Mann, D. G. 1996. Chloroplast morphology, movements and inheritance in diatoms. *In* Chaudhary, B. R. & Agrawal, S. B. [Eds.] *Cytology, Genetics and Molecular Biology of Algae*. SPP Academic Publishing, Amsterdam, The Netherlands, pp. 249–74.

Margulis, L. 1993. *Symbiosis in Cell Evolution. Microbial Communities in the Archean and Proterozoic Eons*, 2nd ed. W.H. Freeman & Company, San Francisco, 452 pp.

Mayama, S. & Shihira-Ishikawa, I. 1994a. Putative nucleoids scattered in chloroplast of *Pinnularia nobilis* (Bacillariophyceae). *Jpn. J. Phycol.* 42:437–41.

Mayama, S. & Shihira-Ishikawa, I. 1994b. Observations on the scattered chloroplast nucleoids in three *Pinnularia* species, *P. major*, *P. viridis*, and *P. viridiformis*. Abstracts, 5th Intern. Phycol. Congr. Chinese Academy Science, Qingdao, China, p. 27.

Mayama, S. & Shihira-Ishikawa, I. 1994c. Distribution types of chloroplast nucleoids in diatoms. Abstracts of the 13th Int. Diatom Symposium, Jean Gilder Congress, Naples, Italy 1994, p. 45.

Mayama, S. & Shihira-Ishikawa, I. 1996. Configurations of chloroplast nucleoids in *Pinnularia* (Bacillariophyceae). Abstracts of the 14th Int. Diatom Symposium, Tokyo 1996, p. 76.

McFadden, G. I. 2001. Primary and secondary endosymbiosis and the origin of plastids. *J. Phycol.* 37:951–9.

Medlin, L. K., Kooistra, W. H. C. F., Potter, D., Saunders, G. W. & Andersen, R. A. 1997. Phylogenetic relationships of the "golden algae" (haptophytes, heterokont chromophytes) and their plastids. *Plant Syst. Evol.* 11(Suppl.):187–219.

Melkonian, M. 1996. Phylogeny of photosynthetic protists and their plastids. *Verh. Dtsch. Zool. Ges.* 89:71–96.

Mereschkowsky, C. 1910. Theorie der zwei Plasmaarten als Grundlage der Symbiogenesis, einer neuen Lehre von der Entstehung der Organismen. *Biologisch. Centralb.* 30:278–303.

Müller, M. & Moor, H. 1984. Cryofixation of thick specimens by high pressure freezing. *In* Revel, J.-P., Barnard, T. & Haggis, G.-H. [Eds.] *The Science of Biological Specimen Preparation*. SEM Inc., AMF O'Hare, Chicago, pp. 131–8.

Müller, M., Marti, T. & Kriz, S. 1980. Improved structural preservation by freeze substitution. *In* Brederoo P. & de Priester W. [Eds.] *Electron Microscopy 1980*, Vol. II. Proc. 7th Europ. Congr. Electron Microsc., Leiden, pp. 720–21.

Pickett-Heaps, J. D., Tippit, D. H. & Andreozzi, J. A. 1978. Cell division in the pennate diatom *Pinnularia*. I. Early stages in mitosis. *Biol. Cell.* 33:71–8.

Pickett-Heaps, J. D., Tippit, D. H. & Andreozzi, J. A. 1979. Cell divi-

sion in the pennate diatom *Pinnularia*. IV. Valve morphogenesis. *Biol. Cell.* 35:199–206.

Preisig, H.-R. & Hibberd, D. J. 1984. Virus-like particles and endophytic bacteria in *Paraphysomonas* and *Chromophysomonas* (Chrysophyceae). *Nord. J. Bot.* 4:279–85.

Reisser, W. 1990. Participation of algal cell wall surface structures in the formation of host-symbiont-interface of endocytobiotic systems. *In* Wiessner, W., Robinson, D. G. & Starr, R. C. [Eds.] *Experimental Phycology: Cell Walls and Surfaces, Reproduction and Photosynthesis.* Springer-Berlin, Heidelberg, pp.55–68.

Schmid, A. M. 1989. Geitler's "Plattenband" in the diatom *Synedra* cf. *ulna* in the light of TEM investigations. *Pl. Syst. Evol.* 164: 239–52.

Schmid, A. M. 1991. A life for cell biology—a life for diatoms. Obituary: Professor Dr. Lothar Geitler (May 18, 1899—May 1, 1990). *Diatom Res.* 6:181–93.

Schmid, A. M. 1994. Aspects of morphogenesis and function of diatom cell walls with implications for taxonomy. *Protoplasma* 181: 43–60.

Schmid, A. M. 2000. "Scattered cp-nucleoids" in diatoms explained: bacteria—inside the endoplasmic reticulum—pierce the plastids of *Pinnularia nobilis*. *J. Phycol.* 36:61–2.

Schmid, A. M. 2001. Value of pyrenoids in the systematics of the diatoms: their morphology and ultrastructure. *In* Economou-Amilli, A. [Ed.] *Proceedings of the 16th International Diatom Symposium.* University of Athens Press, Athens, 2000, pp. 1–32.

Schmid, A. M. 2003. Endobacteria in the diatom *Pinnularia* (Bacillariophyceae). II. Host cell cycle-dependent translocation and transient chloroplast scars. *J. Phycol.* 39:139–153.

Schmid, A. M., Eberwein, R. & Hesse, M. 1996. Pattern morphogenesis in cell walls of diatoms and pollen grains: a comparison. *Protoplasma* 193:144–73.

Schnepf, E. 1984. The cytological viewpoint of functional compartmentation. *In* Wiessner W., Robinson D. & Starr R. C. [Eds.] *Compartments in Algal Cells and their Interaction*. Springer Berlin, Heidelberg, pp.1–10.

Sharon, N. & Lis, H. 1989. Lectins as cell recognition molecules. *Science* 246:227–46.

Sitte, P. 1981. Die Endosymbionten Hypothese - eine kritische Betrachtung zur Zell-Evolution. *Nova Acta Leopoldina NF56* 251:41–58.

Sitte, P. 1993. Symbiogenetic evolution of complex cells and complex plastids. *Eur. J. Protistol.* 29:131–43.

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31–43.

Steinbrecht, R. A. & Müller, M. 1987. Freeze substitution and freeze drying. *In* Steinbrecht R. A. & Zierold K. [Eds.] *Cryotechniques in Biological Electron Microscopy.* Springer, Berlin, Heidelberg, pp.149–72.

Studer, D., Michel, M. & Müller, M. 1989. High pressure freezing comes of age. *Scann. Microsc.* 3(Suppl.):253–69.

Tamm, S. L. 1978. Membrane specialisations associated with the attachment of prokaryotes to a eucaryotic cell. *J. Cell Biol.* 79:216a.

Tschermak-Woess, E. 1953. Über auffallende Strukturen in den Pyrenoiden einiger Naviculoideen. *Österr. Bot. Z.* 100:160–78.

Tschermak-Woess, E. & Weber, A. 1989. Lothar Geitler on the occasion of his 90th birthday on May 18th 1989. [Foreword of the editors & list of publications of Lothar Geitler since 1979.] *Pl. Syst. Evol.* 164: No.1–4, 165: No.1–2.

van den Hoek, C., Mann, D. G. & Jahns, H. M. 1995. *Algae*: *An Introduction to Phycology.* Cambridge University Press, Cambridge, 623 pp.

Weber, M. 1989. Thiéry test in combination with a uranyl acetate-lead citrate staining. *Stain Technol.* 64:147–9.

Wenderoth, K. 1980. Photokinese und photophobische Reaktionen der Kieselalge *Navicula peregrina*. Film no. C-1388, IWF, Institut f. wissenschaftl. Film, Göttingen.

Wilcox, L. W. 1986. Prokaryotic endosymbionts in the chloroplast stroma of the dinoflagellate *Woloszynskia pascheri*. *Protoplasma* 135:71–9.

Wujek, D. E. 1979. Intracellular bacteria in the blue-green alga *Pleurocapsa minor*. *Trans. Am. Micros. Soc.* 98:143–5.

Wujek, D. E. 1984. Bacteria in the perinuclear cisterna of the chrysophyte *Chrysosphaerella*. *Trans. Kansas Acad. Sci.* 87:32–5.