Symbiosis, 26 (1999) 1–23
Balaban, Philadelphia/Rehovot

Review article

Epixenosomes: Symbionts of the Hypotrich Ciliate *Euplotidium itoi*

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Received October 20, 1997; Accepted May 9, 1998

Abstract

Peculiar episymbionts (epixenosomes) are located on the surface of the marine ciliate *Euplotidium itoi*. Reproduction, redistribution between the two offspring *Euplotidium* cells, and the developmental transformation (maturation) of epixenosomes are well coordinated with the ciliate's reproductive cycle. Not only does the ciliate strongly influence the epixenosomes, but the epixenosomes influence the hypotrich. During their reproductive stage epixenosomes have a bacteria-like morphology and divide like prokaryotes, but when mature they have both prokaryotic and eukaryotic traits. Although they lack a membrane-bounded nucleus, their DNA hybridizes with eukaryotic rDNA probes obtained from organisms belonging to three different kingdoms (Protista, Animalia, Plantae). Their DNA hybridizes with the gene encoding for β tubulin in another related ciliate (*Euplotes crassus*). They lack mitochondria and other membrane-bounded organelles, but display their own distinctive cell compartmentalization. Tubules, present in their cytoplasm, positively react with different antitubulin antibodies. The most prominent structure of mature epixenosomes is their unique sophisticated extrusive apparatus that shares features of both prokaryotes and eukaryotes. A 40 μm long tubular process terminates with a "head" mainly consisting of the epixenosome genetic material. The ejected tubes, detached from the ciliate host, are rapidly deployed into the surroundings. Ejection is triggered by the detection of external signals through membrane receptors followed by activation of an adenylate cyclase-cyclic AMP system. Neither the trigger nor the biological significance of ejection in the natural environment are known.

Keywords: epixenosomes, episymbiosis, *Euplotidium*, cell evolution, prokaryotic-eukaryotic border

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1. Introduction

The most fundamental division in the living world is between prokaryotic and eukaryotic organisms. However, even at this level it is not easy in many cases to determine the phylogenetic position of an organism. So, for example, according to some authors the group of bacteria named archaebacteria (the Archaea), separate from the rest of the true bacteria, and represent a third domain of life (for bibliography see Gray, 1996). This viewpoint, however, is not accepted by all phylogeneticists. The living world cannot be easily subdivided in categories with sharp boundaries and many features are needed to determine an organism. Even among organisms that have been clearly determined as prokaryotic or eukaryotic, some do not completely fit in either category. For example a bacterium, *Epulopiscium fishelsoni,* up to 600 μm long (Clements and Bullivant, 1991; Angert et al., 1993), reaches lengths typical of large protists. The most distinctive trait of prokaryotes is regarded the lack a true nucleus surrounded by a membrane and any other membrane-bounded organelles. Nevertheless the bacterium *Gemmata obscuriglobus* has a membrane bounded nucleoid (Fuerst and Webb, 1991) while membrane bounded inclusions are frequently found in cyanobacteria (for review see Jensen, 1993). An invagination of the plasmamembrane segregates a nucleoid by a double membrane in the prokaryote *Nitrosolobus multiformis* (Jensen, 1994). On the other hand, *Cyanidioschizon merolae,* an eukaryote belonging to the cyanidial algal group, divides amitotically like prokaryotes (Nagashima et al., 1992).

A more enigmatic example of "exception to the rules" is certainly represented by epixenosomes. Epixenosomes (from the ancient Greek = external alien bodies) are unique microbes living on the dorsal surface of the marine hypotrich ciliate *Euplotidium itoi.* They are still referred to by this generic term because although they have been studied extensively (Verni and Rosati, 1990; Rosati et al., 1993a; Rosati et al., 1996; Rosati et al., 1997; Rosati et al., 1998), their nature as prokaryotes or eukaryotes has not yet been determined. It appears premature to name them according the binomial nomenclature.

This article presents a comprehensive view of this epixenosome-ciliate association.

2. The Ciliate Host

Euplotidium itoi a marine, psammophilic hypotrich ciliate, was discovered near a shore of the sea of Japan by Ito (1958). Our specimens, collected from a rocky shore near Leghorn (Ligurian sea), augmented Ito's light microscopic descriptions with electron microscopic observation. Many large pools, more or less interconnected and linked to the open sea by channels, harbor *Euplotidium*

itoi. Some were easily found in pools whose bottom was covered by medium coarse sand but only when, due to the low tide, the pools were separated from each other. *E. itoi*, like the other species of the same genus (Tuffreau, 1985) have a restricted, well characterized ecological niche. Only in rare cases can they be found in abundance. Unlike many common ciliates, *Euplotidium* species are not well suited to be grown in the laboratory. Although successfully grown in the laboratory, the cultures never grew optimally (Giambelluca et al., 1995). The cell cycle in the laboratory, for example, was unusually long i.e., 48 h, compared to that of the related species *Euplotes crassus* that, under the same conditions, reproduces at a rate of 2 fission per day (Luporini and Bracchi, 1973). We maintained *E. itoi* at first in the original sea water and subsequently, when their reproductive cycle became regular, we transferred them to artificial sea water enriched with food (i.e. a chlorophyte flagellate *Dunaliella salina* and a diatom *Pheodactilum tricornutum*).

E. itoi is oval in shape, 60–90 µm long and 40–52 µm wide. On its ventral surface are twelve frontoventral cirri, six transversal cirri and one left marginal cirrus. The huge oral cavity is surrounded by a well developed series of membranelles and a reduced paroral membrane. A prominent peristomial plate is present. On the convex dorsal surface are five kineties of short cilia (dorsal bristles) (Fig. 1) Epixenosomes lie along both sides, and at the anterior end of the dorsal surface in a cortical band that appears as a sort of scarf located within well defined limits (Verni and Rosati, 1990). The ultrastructural description of *E. itoi* is given elsewhere (Lenzi and Rosati, 1993).

3. The Epixenosomes

Epixenosomes are present in two different forms. Form I spherical, 1 µm in diameter, bacteria-like in ultrastructure, are mainly located in the central region of the epixenosomal band. They are surrounded by two membranes enclosing a granular matrix in which only clear zones containing filamentous material can be distinguished (Fig. 2a). They divide like prokaryotes by direct binary fission (Fig. 2b). These form I epixenosomes transform into form II epixenosomes by gradually acquiring a more complex structure (Fig. 2c) according to a well defined pattern (Rosati et al., 1993a). Fully formed form II epixenosomes are larger and egg shaped (2–2.5 µm long and 1.2–1.3 µm wide) (Fig. 3a). They are unable to divide and have a far more complicated internal structure. Cytochemical studies, mainly based on enzyme localization, showed that a functional compartmentalization corresponds to the structural complexity (Rosati et al., 1996). The following compartments have been found:

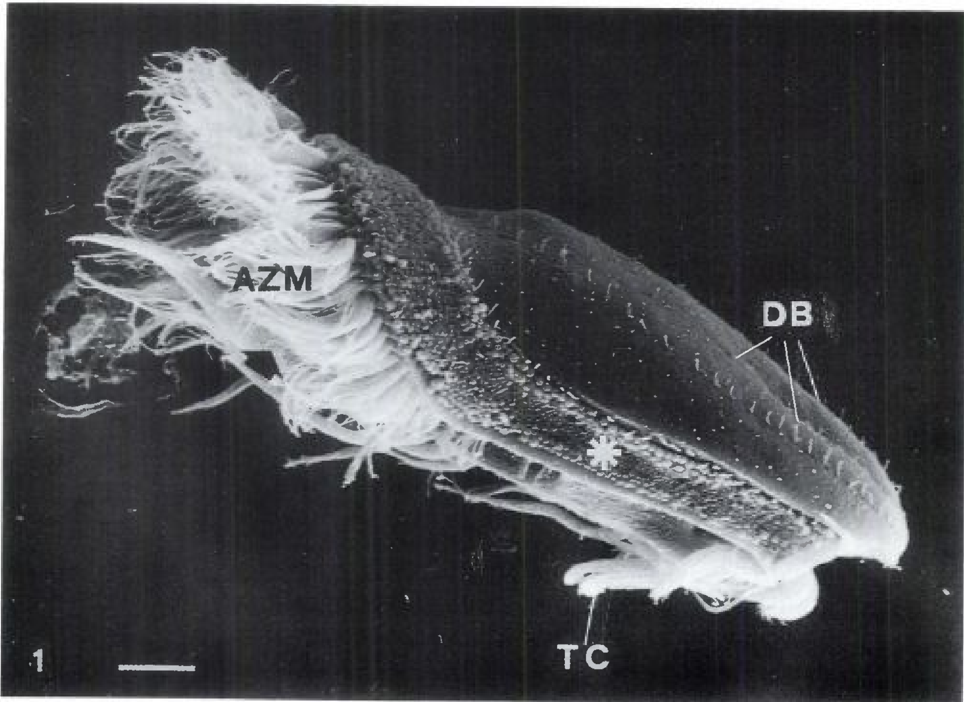

Figure 1. *Euplotidium itoi* as seen with the scanning electron microscope (from Rosati et al. 1993a), side view. Part of the adoral membranelles (AZM) and some transversal cirri (TC) are visible on the ventral surface; the dorsal surface bears dorsal bristles kineties (DB). Asterisks indicate the epixenosomal band. Bar = 10 μm.

1) a space (IM) between the two external, delimiting membranes in which active acid phosphatase has been demonstrated (Rosati et al., 1996). This enzyme is cytosolic in prokaryotes, but contained in plastids, mitochondria and lysosomes in eukaryotes. In epixenosomes form II it is enclosed in the only space delimited by true membranes.

2) An apical electrondense zone (referred to as dome-shaped zone = DZ, under the cell membranes in the upper region of the cell body) is not delimited by a membrane or a thin envelope. The dome portion of the epixenosomes contains DNA and basic proteins (Verni and Rosati, 1990; Rosati et al., 1996). Its ultrastructure resembles eukaryotic heterochromatin. By means of the *in situ* hybridization technique at the ultrastructural level (Rosati et al., 1995; Rosati et al., 1998) a specific labeling, indicating that hybridization took place, was obtained at the DZ level by using the following eukaryotic probes suitable for detecting rRNA genes: a fragment of the 18S rRNA gene from *Pisum sativum*;

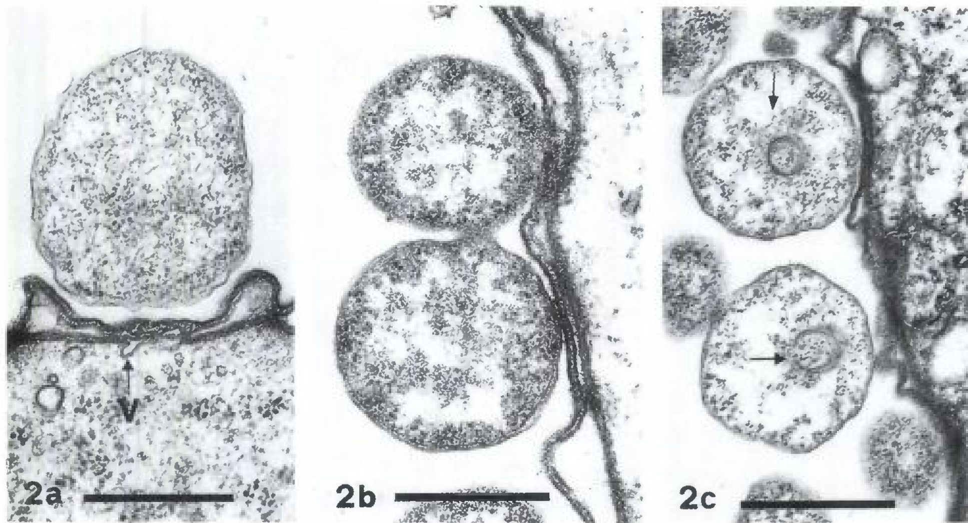

Figure 2. Form I epixenosomes a. resting stage (from Lenzi and Rosati, 1993), (V) = parasomal sac-like vesicle; b. the end of binary fission; c. initial stage during the transformation from form I to form II. Arrows indicate the forming extrusive apparatuses. Bars = 0.5 μm

5000 bp Eco RI fragment from the macronuclear gene of *Euplotes crassus* containing the 3' end of the 18S rRNA and the complete sequence of the 28S rRNA; and a PCR product corresponding to the 800-bp 5' end of 18S rRNA from *Bos taurus*. Successful *in situ* hybridization, was also obtained when the gene encoding for β tubulin in *Euplotes crassus* (provided by the C. Jahn's laboratory) was used as probe. No positive reaction was observed with three different prokaryotic probes: an oligonucleotide suitable for detecting *Escherichia coli* 16S rRNA; an oligonucleotide whose sequence was reported in the literature as complementary to all bacterial 16S rRNA sequenced (Amann et al., 1991); a PCR product corresponding to the 800 pb 5' end of 16S rRNA from *Bacillus subtilis*.

3) A roundish body (RB) with a rather constant size (200 nm in diameter) and a precise location just under the dome shaped zone, containing reserve polysaccharides and enzymes, in particular peroxidases (Fig. 3b) (Rosati et al., 1996) i.e., enzymes cytosolic in prokaryotes and confined in special cell compartments in eukaryotes. The RB is delimited by a thin layer, made particularly visible by staining procedures specific for glycosiyated substances (Rosati et al., 1996).

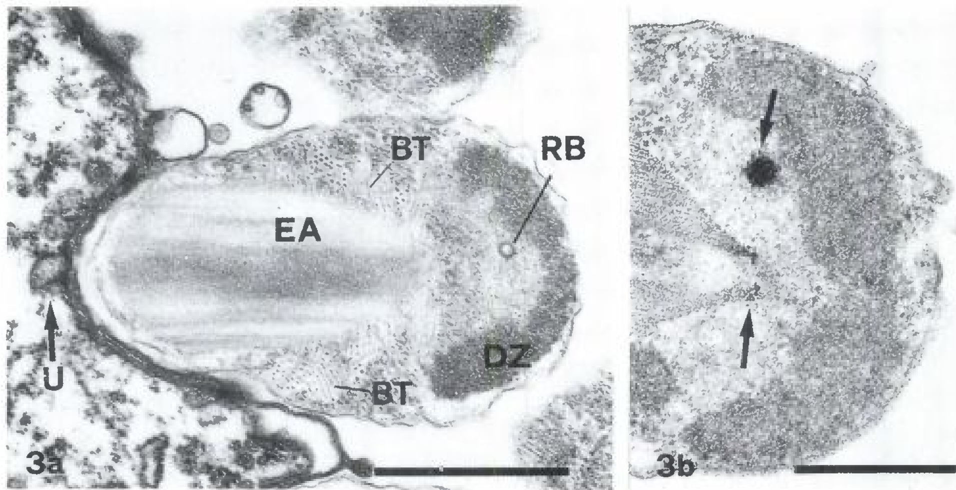

Figure 3. Fully formed form II epixenosomes a. longitudinal section of a specimen prepared according the standard procedure for electron microscopy; (BT) = basket tubules; (DZ) = dome shaped zone; (EA) = extrusive apparatus; (RB) = roundish body; (V) = parasomal sac-like vesicle. Bar = 1 μm; b. specimen treated for the localization of endogenous peroxidase (from Rosati et al., 1996). Arrows point to the reaction product revealing the presence of the enzyme. Bar = 0.5 μm.

4) An extrusive apparatus (EA) consisting of a ribbon with smooth surfaces in which lectin binding sites are present (unpublished results). The ribbon is coiled up around a granular central core (150 nm in diameter) which contains a bundle of fibrils (CF) (20 nm thick and 0.8 μm long) in its upper region (Verni and Rosati, 1990; Rosati et al., 1993a). Cytochemical analysis (Rosati et al., 1993a; Rosati et al., 1996) showed that the EA is immersed in a proteinaceous matrix whose nature is somehow different from the remaining cytoplasm. The whole structure, which has an almost oval shape well suited to that of the epixenosome, is separated from the cytoplasm and the central core by thin membranous layers along which active adenylate cyclase, i.e., a typically membrane bound enzyme in both prokaryotes and eukaryotes (Rosati et al., 1996). At its top, a "ring" of peroxidase is present (Fig. 3b) (Rosati et al., 1996).

5) A basket formed by bundles of regularly arranged tubules (BT) surrounding the extrusive apparatus (Rosati et al., 1993b). Some of these tubular bundles, particularly in the apical region where the EA forms a sort of a tip, appear to be directly associated to the free end of the EA layers (Fig. 3a). The values of the inner and outer diameters of BT (20–24 nm and 12–14 nm, respectively) fall into the range reported for tubulin microtubules (Margulis, 1993), moreover they

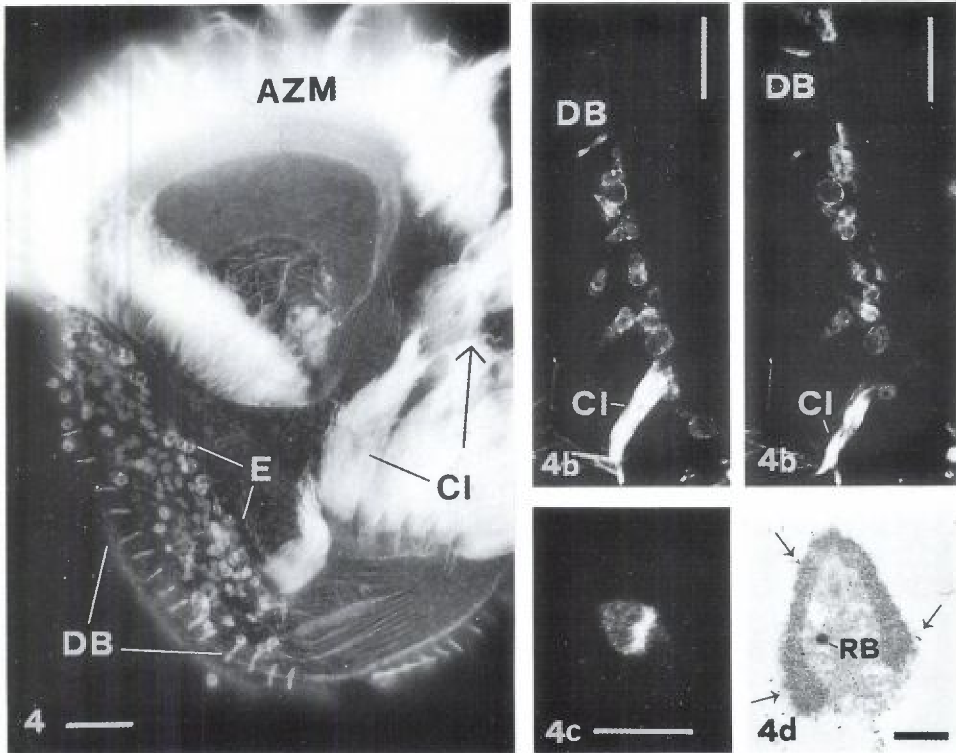

Figure 4. Immunocytochemistry with antitubulin antibodies (from Rosati et al., 1993b) a. micrograph of whole *Euplotidium*. Fluorescence is evident at the level of the ciliary structures and along a peripheral ring in epixenosomes (E). Bar = 10 μm; b. confocal micrographs of optical sections from the same portion of the epixenosomal band. Bar = 2 μm; c. the head of an ejected extrusive apparatus shows fluorescent labelling after exposition to an antitubulin antibody. Bar = 1 μm; d. section of the head at the electron microscope. The tubules associated with the head appear labelled by gold particles (arrows). Bar = 0.5 μm. (AZM) = adoral zone of membranelles; (CI) = cirri; (DB) = dorsal bristles; (RB) = roundish body.

are delimited by a wall made up of globular subunits. These structures are particularly well preserved by fixation in the presence of tannic acid, a procedure generally used for the maintenance of tubulin microtubules, and are sensible to nocodazole and cold temperature (4°C), two factors known to depolymerize cytoplasmic microtubules. Both cold and nocodazole depolymerize the cytoplasmic microtubular network in *Paramecium* (Torres and Delgado, 1989; Adoutte et al., 1991). Moreover BT show a positive

immunoreaction against different antitubulin antibodies. The pictures shown (Figs. 4a–d) were obtained following treatment with monoclonal anti *Paramecium* axonemal α-tubulin antibody (kindly provided by A. Fleury, Université Paris-Sud). A similar pattern of decoration at the ultrastructural level was obtained with a rabbit antitubulin antiserum (Sigma) containing both α and β antitubulin antibodies and a monoclonal antibody specific for α tubulin (Amersham) (Rosati et al., 1993b). Some positive results obtained in prokaryotes by use of fluorescent antitubulin antibodies are attributable to hsp65 (heat shock 65 kDa protein) (Munson et al., 1993; Bermudes et al., 1994). Although in the case of epixenosome positive results with antitubulin antibodies are strenghtened by those obtained with in situ hybridization (Rosati et al., 1998), only sequence analyses will definitely prove that the BT consist of tubulin.

4. The Association Between *Euplotidium itoi* and Epixenosomes

The association between *E. itoi* and epixenosomes is constant in nature. Indeed every *E. itoi* specimen analyzed soon after collection, in the course of several years, carried the typical band of epixenosomes. Recently, on one occasion, did we collect *E. itoi* and at the same time another species of the same genus, *E. arenarium*. Since Magagnini and Nobili (1964) described *E. arenarium* at the light microscope level the presence of epixenosomes was not detected. We prepared specimens for both scanning and transmission electron microscope (Verni and Rosati, 1990) and we saw that *E. arenarium* too, harbors epixenosomes on its dorsal surface, located exactly as in *E. itoi* (Fig. 5). This new finding confirms the hypothesis, already presented (Verni and Rosati, 1990) that epixenosomes are present on different *Euplotidium* species. Epixenosomes are probably present also on *Gastrocyrrus trichocystus*, species of a related genus, in which "trichocysts" have been described that share their dimension and well defined localization with them (Ito, 1958). This indicates that we are dealing with an ancient and well established relationship.

Epixenosomes are inserted, along the epixenosomal band, in matching depressions of the *Euplotidium* cortex which, like in most ciliates, consists of the external plasmamembrane, the alveolar system and layers of subcortical microtubules; intra-alveolar plates are typically present as in other euplotid ciliates (Hausmann and Kaiser, 1979; Kloetzel, 1991). As epixenosomes are present in two forms differing from each other in structure and size, there are also two types of cortical depressions (compare Figs. 2a and 3a). A fusion between the plasmamembrane of epixenosomes and that of their host has never been observed: the two organisms are always separated by an irregular, electron transparent space ranging between 25 and 75 nm. Roughly in the center of each

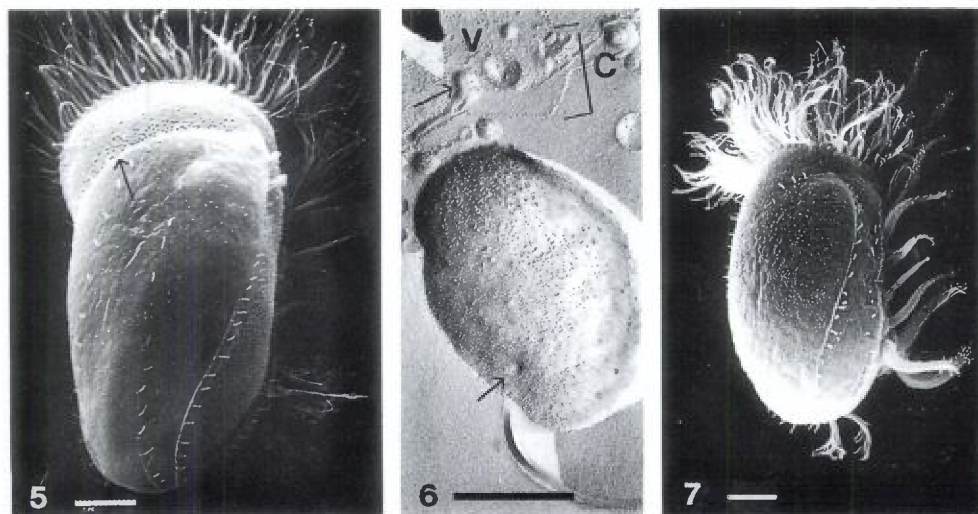

Figure 5. *Euplotidium* arenarium: dorsal view. This species too bears an epixenosomal band. Empty cortical depressions (arrow) are visible where symbionts have been lost during the preparation for electron microscopy. Bar = 10 µm.

Figure 6. Freeze-fractured epixenosome. In the region set toward the ciliate cortex (C) intramembraneous particles have a greater density; arrow points to a "specialized site". V = parasomal sac-like vesicle; VE = external vesicles (see text and Fig. 9). Bar = 0.5 µm.

Figure 7. A starved *Euplotidium itoi*. Epixenosomes are absent and with them disappeared the cortical depressions. Bar = 10 µm.

depression are one or two coated vesicles, often containing a fuzzy material, open to the exterior passing through the alveolar system and fusing with the plasmamembrane (Figs. 2a, 3a and 6). These vesicles are like "parasomal sacs", structures that in ciliates (*Euplotidium* included) are part of ciliate kinetids (Corliss, 1979). They are considered the sites of exocytosis and/or pinocytosis. As parasomal sac-like vesicles not associated with ciliary structures were observed only in the epixenosomal band, we hypothesize that these structures play a role in the relationship between the ciliate and the epixenosomes (Lenzi and Rosati, 1993). Intramembraneous particles detected by freeze-etching (Rosati et al., 1996) are particularly abundant in the epixenosomal region towards the ciliate cortex (Fig. 6) relating to the other regions of the cell membrane. Neither dense material adhering to the cytoplasmic side of the cortical membranes nor a high density of intramembrane particles, as reported

in the association between devescovinid flagellates and episimbiotic bacteria (Tamm, 1980) differentiate the cortex of the cortical band in *Euplotidium*. In *Staurojoenina*, a flagellate symbiont of dry-wood eating termites, epibiotic bacteria are associated with cytoplasmic protrusions each underlain by a single submembraneous microtubule (Dolan and Margulis, 1997). In *Euplotidium* the subcortical sheets of microtubules seem less organized where depressions in which epixenosomes are inserted are present (Lenzi and Rosati, 1993).

All our attempts to mechanically remove epixenosomes from *Euplotidium*, without damage to the ciliate, were unsuccessful. Neither centrifugation, sonication, electric shock, sharp variations in temperature, pH or ionic strength, sufficed. Monosaccharides known to play a role in the cell membrane binding capacity were also used. Each of the following substances was added to the sea water in the highest dose which had no appreciable effect on the ciliate: glucose, mannose, galactose, fucose, N-acetyl-galactosamine (GlcNAc) and N-acetyl-galactosamine (GalNAc). About 100 *Euplotidium* were treated in each experiment. Some specimens were isolated at intervals (15, 30, 60, and 120 min) and observed at the light microscope or prepared for electron microscopical observation to check the presence of epixenosomes. A partial removal of epixenosomes was attained with 0.5–1M GlcNAc or GalcNAc. This suggests the involvement of glycosidic material in maintaining the association. Nevertheless the space between *Euplotidium* and epixenosomes always appears electrontrans-parent, even when fixatives or staining procedures for glyco-silated substances were used.

Only by prolonged starvation or when protein synthesis was inhibited (by adding 20 µg/ml cycloheximide) epixenosomes were removed. Under these conditions Euplotidium cell division stopped as well. In both cases at first only form II epixenosomes were present, then they were gradually lost until they completely disappeared. Intriguingly whenever epixenosomes were removed the ciliate surface appeared empty and smooth and no cortical depression remained (Fig. 7). Yet the parasomal sac-like vesicles remained (Lenzi and Rosati, 1993). A certain percentage of the epixenosome-free *Euplotidium* population recovered its reproductive capacity once conditions were normalized. Strains lacking epixenosomes were obtained from these specimens. It appears that epixenosomes are not vital for the ciliate, at least in a non-competitive environment. To verify whether the association with *Euplotidium* is vital for epixenosomes is more difficult. Attempts to grow the episymbionts in culture media for different bacteria, failed; however this negative result may be due either to the indispensability of the association or to the peculiarity of the organism and its metabolism.

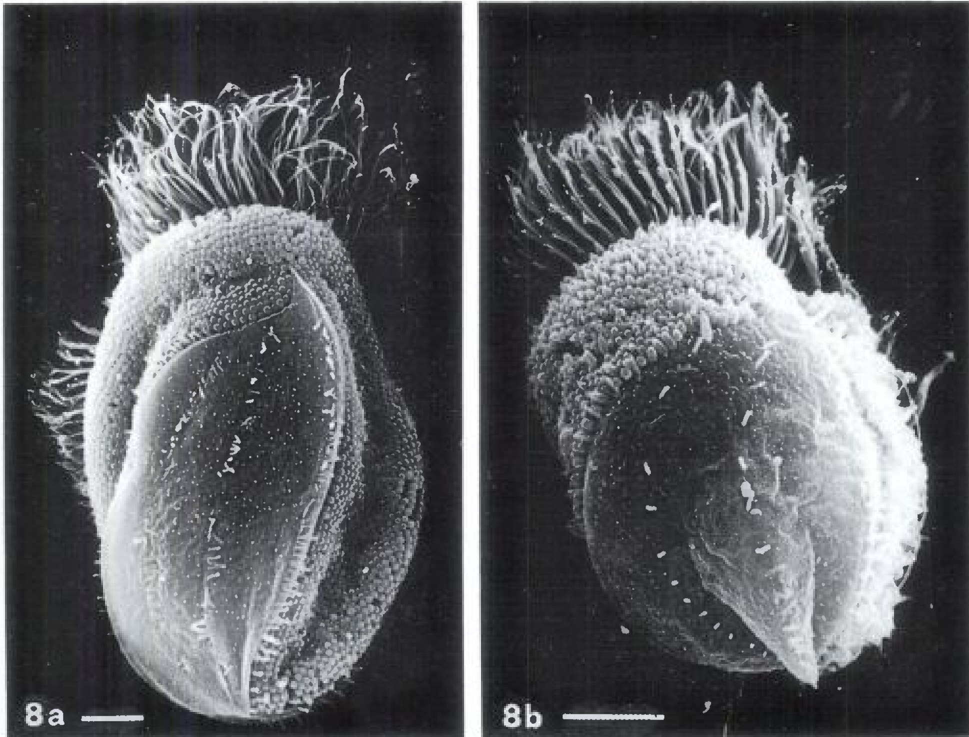

Figure 8. The behavior of the epixenosomal band during the ciliate host binary fission. a. the epixenosomal band widens and a central furrow appears, while morphogenetic events occurs in *E. itoi*. At this stage the dorsal bristles (DB) are separated in two distinct sets, both with more numerous cilia than during the non dividing stage (compare with Fig. 1). Bar = 10 μm; b. a just separated Euplotidium daughter cell (proter): the epixenosomal band is still wider than in the non dividing stage. Bar = 10 μm.

5. Behavior of Epixenosomes and the Epixenosomal Band during Euplotidium Binary Fission

At the end of the morphogenetic events following the binary fission of *E. itoi*, each offspring cell has a similar epixenosomal band. The analysis of the variations of the epixenosomal band and the behavior of epixenosomes during this process showed that the cortical region of *Euplotidium* corresponding to the epixenosomal band is a very specialized zone (Giambelluca and Rosati, 1996). Changes occur in this region according to a pattern that is well

coordinated with morphogenetic events (Fig. 8a): the number of dividing epixenosomes begins to increase and the cortical region in which they lie begins to widen soon after the appearance of the macronuclear replication band, i.e., during a very early stage of *E. itoi* morphogenesis in which the development of new ciliary structures is not yet evident at the cell surface. Then the gradual widening of the cortical band region, its increase in length due to the lateral shifting of the forming opisthe, the appearance of a central furrow full of form I epixenosomes most of which are dividing, and the recovery of the typical non-dividing features, proceed along precise steps together with *E. itoi* morphogenesis. Soon after separation both proter (Fig. 8b) and opisthe still have a wide band (20–25 μm versus 13–15 μm in non dividing stage). The central furrow, however, becomes rapidly less evident and as soon as the host cell completely resumes its non-dividing morphotype the epixenosomal band too returns to its typical width. Observations with the transmission electron microscope revealed that, while the cortical region of the epixenosomal band narrows and the total number of epixenosomes decreases (it is not clearly understood how) most epixenosomes have a morphology half-way between form I and form II. The correspondence between the increase and decrease in the number of epixenosomes and their transformation from form I to form II, with the widening and narrowing of the *Euplotidium* cortical region in which they lie, reveals that a close relationship exists between the two organisms. But perhaps more significant is the fact that when *E. itoi* devoid of epixenosomes (obtained for example by starvation) undergo binary fission their cortical region corresponding to the epixenosomal band does not widen (Giambelluca and Rosati, 1996).

6. Euplotidium and Epixenosomes Influence Each Other

These observations not only indicate that the ciliate cell strongly affects the epixenosomes, but also that the epixenosomes affect the ciliate at least to a certain extent. It is worth remembering here that the cortex of ciliates is a complex structure that cannot be imagined as an elastic surface that can be stretched to lodge a great number of epixenosomes during morphogenesis and relaxed when the number of epixenosomes decreases. Contribution of new plasmamembrane and alveolar material is needed for its widening; while for its narrowing the reabsorbtion of these materials has to take place. The fact that this phenomenon occurs at the level of the epixenosomal band only when epixenosomes are present is indicative of a direct influence of the symbionts. Moreover, not only the multiplication of epixenosomes but also their differentiation from form I to form II is correlated with the host cell cycle. The

case of the gram negative bacterium *Holospora obtusa* living in the macronucleus of the ciliate *Paramecium caudatum* (Görtz, 1988) is well known. This bacterium shows a developmental cycle with infectious and reproductive forms that differ from each other in terms of morphology and electrophoretic polypeptide pattern (Görtz et al., 1990, 1992). The reproductive short form is found predominantly in the macronucleus of vegetatively growing host cells and can multiply by binary fission. It ceases the growth and differentiates to the infectious form when the starved host stops dividing It has been suggested that the bacteria need host protein synthesis to maintain reproductive forms and that the host proteins may suppress the bacterial gene expressions for infectious form-specific proteins (Fujishima, 1992; Lehman et al., 1997). Perhaps it is possible to imagine a similar mechanism in the association between *Euplotidium* and epixenosomes: specific proteins coming into play during the different stages of the ciliate's morphogenesis could induce the multiplication and the differentiation of epixenosomes, while the stages of the epixenosome cycle could influence the behavior of the cortical band in which they are lodged. This implies that the two organisms in some way "communicate". It may be hypothesized that the parasomal-sac like vesicles are involved in this communication as exocytotic or pinocytotic sites.

By means of various fixation methods for thin sectioning electron microscopy, scanning electron microscopy and freeze etching (for technical details see: Verni and Rosati, 1990; Rosati et al., 1996) membrane-bounded vesicles (80–100 nm in diameter) have often been observed as emerging from (or fusing with) the cell membrane of epixenosomes and attached to or close to the cell membrane corresponding to the epixenosomal band region of *Euplotidium* (Figs. 6 and 9). On the basis of my very long experience in electron microscopy, an artifactual nature of these vesicles appears improbable. Moreover, freeze etching preparations revealed specialized sites on the epixenosomal surface (Fig. 6), like those considered as "pinocytotic sites" in other protists (Dubremetz and Entzeroth, 1993). It can be hypothesized that the vesicles are formed by evagination of the cell membrane of one organism and fuse with the cell membrane of the other. Whether the vesicles emerge from *Euplotidium* or from epixenosomes or from both organisms, cannot be detected on the basis of static images. In any case their involvement may be hypothesized as a direct passage of material between epixensomes and *Euplotidium*. Similar vesicles were sometimes found in the cytoplasm of the ciliate, just under the cortex (Fig. 9d) while in other cases they are in contact with the internal alveolar membrane. It is difficult to reconstruct the origin and the movements of these cytoplasmic vesicles as well as their relation with the vesicles at the plasmamembrane.

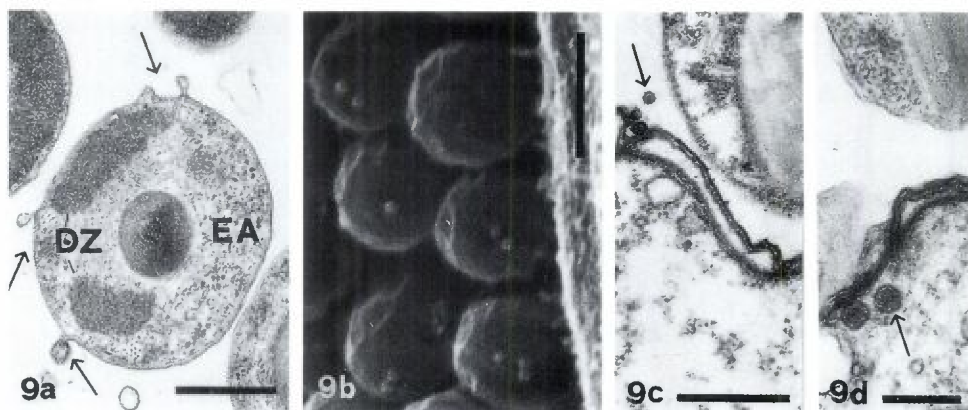

Figure 9. Membrane bounded, external vesicles (see also Fig. 6); a. cross section of the apical region of an epixenosome; arrows indicate the vesicles more or less fused with the epixenosomal external membranes. (DZ) = dome shaped zone; (EA)= extrusive apparatus. Bar = 0.5 μm; b. epixenosomes at the scanning electron microscope. Vesicles are visible on their surface. Bar = 1 μm; c. vesicles can be found also externally to the ciliate cortex (arrow). Bar = 0.5 μm; d. vesicles just under the ciliate cortex (arrow). Bar = 0.25 μm.

7. Entry of Epixenosomes and Foreign Bacteria by Invagination of the Cortex at the Epixenosomal Band

Invaginations of the whole cortex have been observed at the epixenosomal band (Rosati, 1994) in some *Euplotidium* specimens grown in the laboratory. Such invaginations were found only in specimens of a certain container, while they were apparently absent in specimens maintained in the same manner in other containers. Only once were cortical invaginations observed in *Euplotidium* just collected from the sea: in this case they were found in all the specimens (15) observed. These invaginations contain foreign bacteria, a few epixenosomes and a certain number of the above mentioned membrane bounded 80–100 nm vesicles (Figs. 10a and b). Subsequently, intracellular vacuoles are formed, delimited by the whole cortex with the plasmamembrane inward and the alveolar system toward the ciliate cytoplasm (Fig. 10c). No sign of digestion could be observed within these vacuoles. The alveolar system is gradually dissolved in small regions. At these sites, delimited by a single unit membrane (Fig. 10d), bacteria and epixenosomes can escape from the vacuole. Once in the *Euplotidium* cytoplasm bacteria are surrounded by rough endoplasmic reticulum membranes. In the cytoplasm of these invagination-forming *Euplotidium*, well preserved bacteria, able to divide, are often found. More rarely, epixenosomes in a resting

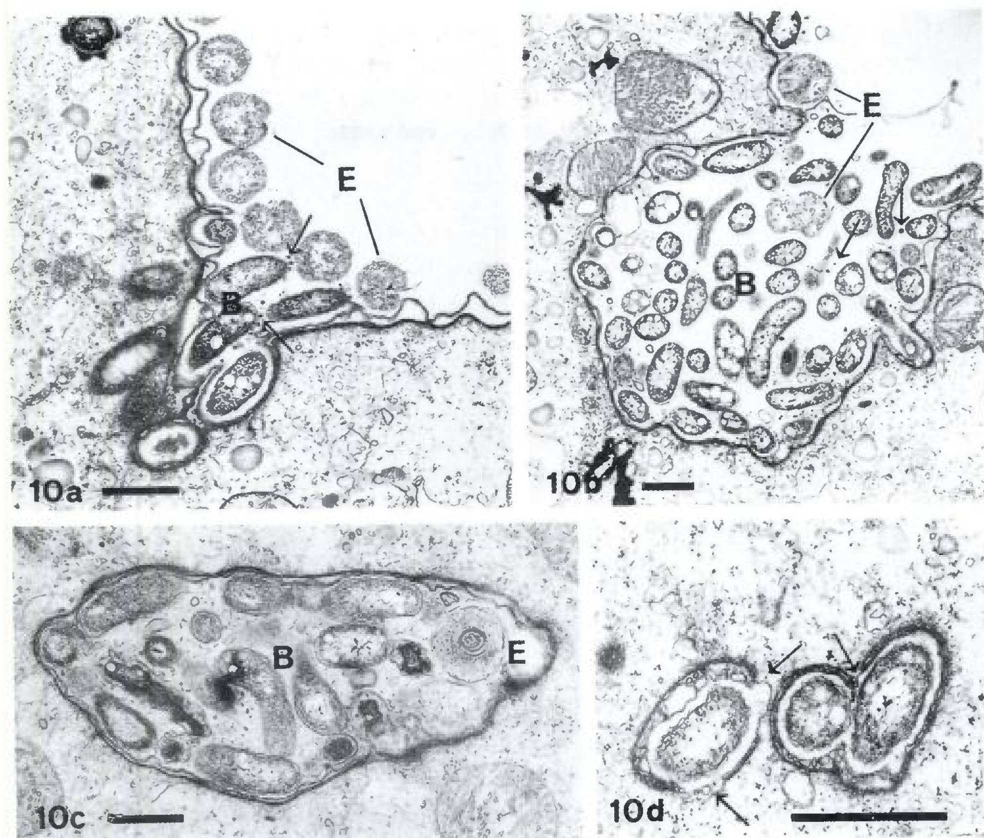

Figure 10. Entry of foreign bacteria and epixenosomes through cortical invaginations (from Rosati, 1994), (B) = foreign bacteria; (E) = epixenosomes, a. an early stage of the process, arrows point to the external vesicles;. Bar = 1 μm; b. a more advanced stage. Bar 1 μm; c. a fully formed vacuole, delimited by the whole inverted cortex. Bar = 1 μm; d. section interesting two branches of a cortical vacuole. Arrows mark zones delimited by a single membrane. Bar = 1 μm. (B) = foreign bacteria; (E) = epixenosomes.

or ejected state were observed inside the cytoplasm of these same *Euplotidium* specimens. Neither bacteria nor epixenosomes were detected in the cytoplasm of specimens that did not form invaginations. The alterations of mitochondrial features found in specimens with cortical vacuoles suggest an infectious nature of the foreign bacteria. Also in line with this hypothesis is the observation that the cortical invaginations were found in every case in specimens growing in the same container or living in the same natural pool (Rosati, 1994). The entry of pathogenic or symbiotic bacteria by means of cell surface invaginations has

been reported in many eukaryotic cells delimited by a simple unit membrane; but it is unusual in ciliates that are delimited by a complex cortex. Most cases of infections or establishment of symbiosis have been reported as occurring in ciliates through food vacuoles (for review see Fenchel, 1987; Görtz, 1988). Only one case has been described (Soldo et al., 1993) in which "xenosomes" enter through the cortex in a ciliate: in this case, however, penetration occurs by rapid dissolution and re-sealing of the cortical layers. The phenomenon here described cannot be compared to the well known case of *Kentrophorus fistolosum* (Raikov, 1971) a mouthless ciliate which feeds on bacteria living on its surface, taking them up by invaginations of the surface itself (which is apparently delimited by a single membrane) and digesting them inside the so formed food vacuoles.

The formation of cortical invaginations takes place only at the level of the epixenosomal band; once again the uniqueness of this cortical region is evident. Nevertheless cortical invagination remains obscure: what is the role, if any, of epixenosomes? Are some epixenosomes introduced into the cell because of sporadically associated bacteria? What happens to epixenosomes once they are inside the cell?

8. Ejection

The ejection process was reconstructed by observations of living organisms at the interferential contrast microscope and on the basis of more than 100 different micrographs obtained by transmission and scanning electron microscopes (Rosati et al., 1993a). Nothing is known about the factor(s) causing the ejection in the natural environment. In the laboratory a massive ejection can be obtained in different ways (osmotic shock, pressure, freezing-thawing) all of which cause, as a final step, the death of *E. itoi*. More simply some specimens were put in a very small amount of sea water on a microscope slide, covered with a cover slip and observed under the microscope. In this condition the ciliate stops swimming in a short time although it is still able to move its ciliature (cirri and membranelles). At this moment a massive ejection takes place. The ejection is thereafter followed by the detachment of the entire epixenosome body from the *Euplotidium*. The ejected tubes appear rigid and are rapidly dispersed around the ciliate host due to the currents generated by its still moving ciliary structures. Generally a few seconds later the ciliate cell disintegrates, probably because of the altered salinity of the water. The first step in the ejection is the opening of the cell membrane in the apical region of the epixenosomes. Then the EA begins to unroll from the inside by the oblique slipping of its layers one into the other. The forming tube slips into a perfectly fitting hole of the DZ and takes away this structure (Figs. 11a and b). At the

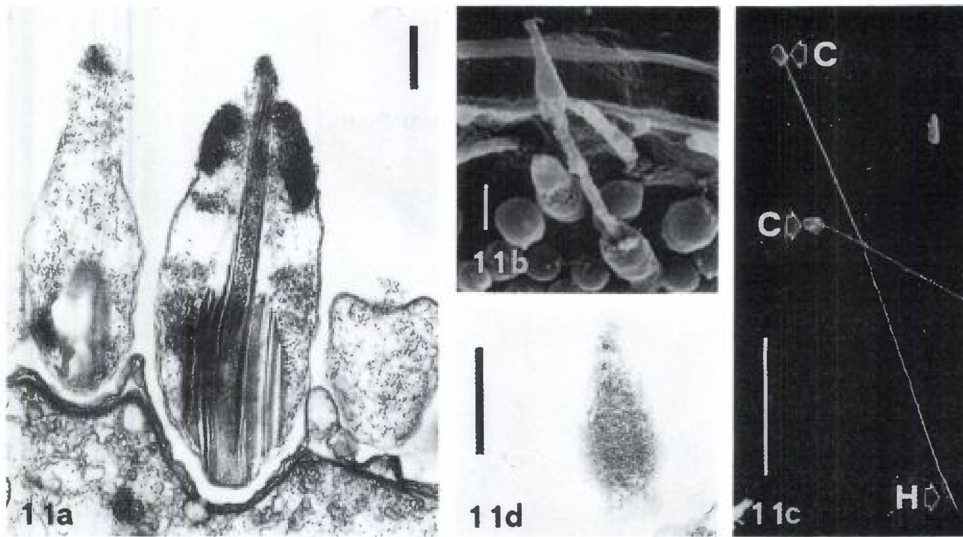

Figure 11. The ejection process a. a very early stage of the process (from Verni and Rosati, 1990). Bar = 0.5 μm; b. a slightly subsequent stage as seen at the scanning electron microscope;. Bar = 1 μm; c. the tube at the end of the process (from Verni and Rosati, 1990). (C) = cup, (H) = head. Bar = 10 μm; d. a superficial section of the head: it mainly consists of the DZ material. Bar = 1 μm.

end of the process the tube is 40 μm long and 150 nm in diameter (Fig. 11c); it emerges from a sort of "cup" consisting only of the epixenosomal external membranes and ends with a "head" consisting of the DZ (Fig. 11d), a portion of cytoplasm containing ribosomes and the RB (Fig. 4d). At its distal end the tube remains open from the onset of ejection and the apical fibrils of the core (CF) emerge slightly from it, thus forming the very tip of the structure. Some BT are often found associated with the tube and the head (Fig. 4d). As *in vivo* treatments with nocodazole or cold temperature (4°C) inhibit the ejecting capacity of epixenosomes, it may be supposed that BT, the only epixenosomal structure affected under these experimental conditions (Rosati et al., 1993b), play an important role in the ejecting process.

The EA appears to be a very efficient structure: when it is rolled up in the resting position its overall dimensions are such that it can be easily lodged inside the epixenosomes while, once ejected, it reaches a length at least 15 times that of the organism. Moreover the tubular shape thus formed, together with the ratio between length and diameter, make the tube much more resistant than the extended flat ribbon whatever its chemical nature may be. Besides, it is not necessary to imagine a chemical linking up among the coils:

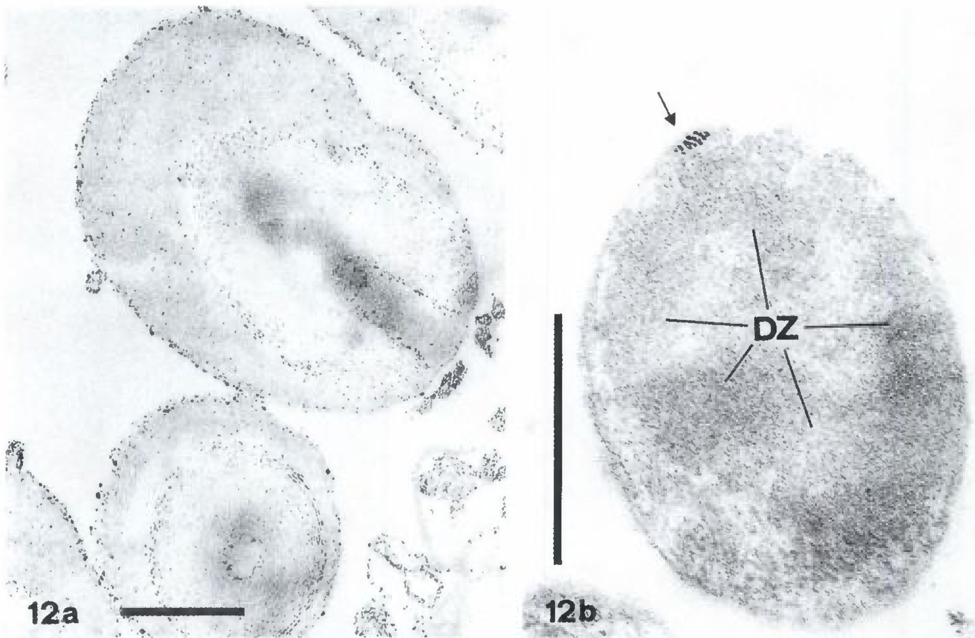

Figure 12. Enzyme localization a. as it is evident in both epixenosomes shown in the micrograph, cross and longitudinally sectioned respectively, the reaction product indicative for the presence of active adenylate cyclase, is mainly localized along the external membrane, at the periphery of the EA and along the border of the central core. Bar = 0.5 µm; b. oblique cross section interesting the dome-shaped zone (DZ) in a specimen prepared for detection of membrane receptors coming into play in the activation of the ejecting process: the localization, revealed by gold particles, is very precise (arrow) (from Rosati et al., 1997). Bar = 0.5 µm.

indeed the ribbon unrolls in an oblique pattern and the different coils partially overlap, thus ensuring the continuity of the tube.

The presence of active adenylate cyclase (AC) on the layers delimiting the EA (Fig. 12a) (Rosati et al., 1996), led us to explore the role of the AC-cAMP system and the possible involvement of membrane receptors in the activation of the ejecting process. The results obtained, by means of in vivo treatments and cytochemical procedures, demonstrated that the process is triggered by the detection of external signals through membrane receptors and the consequent activation of the AC-cAMP system as transduction mechanism. The membrane receptors coming into play have a precise localization at the top of the organism (Fig. 12b), just where the membrane interruption appears as a first step of the whole process (Rosati et al., 1997).

9. What's the Significance of the Ejection?

As described above, ejection causes the disruption of the epixenosome while throwing its genetic material far from the host. This observation led us to consider the possibility of a dispersive role of the extrusive process. As a speculation, considering that we observe the process only when the ciliate host is dying, it may be supposed that under these conditions *Euplotidium* produces the molecule(s) capable of activating the ejection of its symbionts. In this way the symbionts would have the possibility of finding another host. This would be a dispersive process recalling that of the polar filament in *Microsporidia* spores (Larsson, 1986). The reinfection of the epixenosome-free stocks of *E. itoi* produced in the laboratory was never obtained by maintaining a certain number of symbiont bearing and symbiont-free specimens in the same container, or rapidly transferring epixenosome-free specimens to a container in which the ejection had been provoked.

A physiological ejection may also occur to regulate the number of epixenosomes during the host cell cycle. Indeed, soon after the separation of the offspring cells at the end of the *Euplotidium* binary fission there are newly divided epixenosomes, starting their differentiation in form II epixenosomes, in the central region of the epixenosomal band, and old form II epixenosomes at the periphery. Then the number of the epixenosomes rapidly decreases, reaching the values typical of non dividing ciliates (Giambelluca and Rosati, 1996). We hypothesize that *Euplotidium* produces the substances which activate the ejection in this phase of the cell cycle as well, thus enabling the renewal of epixenosomes on its surface at each generation.

The ejection of epixenosomes may be significant in defense for the ciliate. In this case the epixenosomes would function as some extrusomes like the pigment granules of *Blepharisma japonicum* (Miyake et al., 1990) and the trichocysts of *Paramecium* whose discharge is a response against predator attacks (Harumoto and Miyake, 1991; Miyake and Harumoto, 1996). However, these are, for the moment, only speculations: till now we have not been able to single out possible *Euplotidium* predators among the species living in the same ecological niche.

10. Concluding Remarks

The results here reported, considered altogether, form an integrated picture and appear to point in the same direction. In particular the positive responses obtained by in situ hybridization against rRNA eukaryotic probes and a gene encoding for β tubulin seem to favor the hypothesis that epixenosomes are eukaryotes. Horizontal transfer events between kingdoms, deduced by sequence analysis, have been proposed in the literature (Sprague, 1991; Vaughn et al.,

1995). If the possibility of a transfer of the tubulin gene from *Euplotidium* to epixenosomes may be considered, it is not easy to imagine the horizontal transfer of rRNA genes. The nucleotide sequence of rRNA genes has been chosen and extensively used to study prokaryotic and eukaryotic evolution. The definitive identification of epixenosomes as prokaryotes or eukaryotic organisms, however, requires the analysis of their genome.

The most similar structures to the epixenosomal EA so far described are the R bodies of *Caedibacter teniospiralis*, the bacterium that confers the killer trait to *Paramecium aurelia* (Preer et al., 1974); that is a prokaryotic organism. The EA, however, is a far more complex structure as it is immersed in a proteinaceous matrix, separated and different from the cytoplasm, delimited by glycosilated layers in which enzymes are located and presents a differentiated central core containing the CF. There are also eukaryotic organelles consisting of coiled ribbons which unroll from the inside during ejection; for example the ejectisomes of Cryptomonadinae (Anderson, 1962). According to Preer et al. (1974) it is difficult to imagine whether the similarity between the prokaryotic R bodies and the eukaryotic ejectisomes is derived from an evolutionary convergence or a phylogenetic relationship. The presence of a similar structure in epixenosomes is an interesting point in considering the question.

Even the episymbiotic association between epixenosomes and the ciliate host, although not fully understood, certainly presents peculiarities. Of particular interest is the different behavior of the cortical region corresponding to the epixenosomal band whether the episymbionts are present or not. In ciliates specialized cortical regions exist for morphogenetic events during both binary fission and conjugation. This is particularly evident in hypotrich ciliates (Fleury, 1991; Kloetzel, 1991), in which differentiated ciliary structures have a discontinuous distribution characteristic for each species. Morphogenetic processes take place according well defined patterns to which phylogenetic value is awarded: they are physiological phenomena genetically "programmed" in particular phases of the life cycle of the ciliate cell. Our results indicate that differentiation in epixenosomal band of the cortical zone of *Euplotidium* may be induced by external elements. Its widening and narrowing during binary fission is related to the presence of epixenosomes. The formation of cortical invagination in the same cortical zone is probably due to the presence of invasive bacteria from the medium.

Acknowledgements

The author is indebted to all those who have been involved in this study during the years. Particular thanks are due to Franco Verni, Paola Lenzi and

Anita Giambelluca who shared with me the most part of the "adventure" and to Simone Gabrielli for his technical and photographic assistance.

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