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Polynucleobacter: Symbiotic bacteria in ciliates compensate for a genetic disorder in glycogenolysis

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Abstract

Ciliates of a group of closely related species of the genus *Euplotes* harbor *Polynucleobacter* bacteria, and apparently need them in order to successfully reproduce. In the present paper we investigate the possible metabolic correlation between the two partners in this symbiosis. We examine and compare behavioral, ultrastructural and cytochemical analysis specimens of *E. harpa*, with and without their symbionts. The results show that aposymbiotic cells do not undergo a sharp blockage of their cell cycle: they are able to start and go on with DNA replication and, in some cases, nuclear division; in a few cases they can even start cytoplasmic division. Electron microscopical observation reveals that these cells are no longer able to perform glycogenolysis. A similar deficiency was observed in aposymbiotic *E. aediculatus*. In all likelihood, this failure deprives them of an important energy source useful for the accomplishment of the energetically costly fission process. These results are in agreement with the assumption that the bacteria compensate for their hosts' metabolic disorder and prove that this disorder concerns glycogen metabolism. The fact that *Polynucleobacter*-like bacteria are present in every organism of several different, closely correlated, *Euplotes* species and are vertically transmitted from generation to generation, indicates that we are dealing with a hereditary disorder, possibly an enzymatic deficiency, as happens in the human genetic diseases referred to as glycogenosis, and that the bacteria make up for this deficiency.

Keywords: *Euplotes*, glycogen, *Polynucleobacter*, prokaryotic-eukaryotic interactions

1. Introduction

Permanent symbiosis, with all cells of all populations being infected, is known in a limited number of *Euplotes* species. Most of these ciliates appear to depend on their symbionts. The best known example of these bacterial symbioses is the association between *E. aediculatus* and *Polynucleobacter necessarius* (Heckmann, 1975; Fujishima and Heckmann, 1984). Previous experiments have proved that the elimination of the symbiont from the host through antibiotics or other means, impairs the reproductive cycle of the ciliate, which eventually dies. On the other hand, re-infection of aposymbiotic cells results in the rescue of host cells and cultures (Fujishima and Heckmann, 1984). Similar bacteria (easily recognizable due to the presence of nucleoids) were found in other fresh water *Euplotes* species, all sharing with *E. aediculatus* the 9 type I fronto-ventral cirral pattern (*E. patella* type). This observation suggests that the species in this group suffer from a common deficiency that arose in a common ancestor and is compensated by the symbiont (Heckmann et al., 1983). A bacterium, with the same morphology and a high similarity value (98.4%) with *P. necessarius* in 16S rRNA gene sequence, was also reported in *E. harpa,* a species with a cirral pattern 10, living in a brackish environment. Antibiotic experiments showed that eliminating the bacteria impairs the reproductive cycle in *E. harpa,* as in the fresh water species mentioned above (Vannini et al., 2003; Vannini et al., 2005). This finding indicates that the presence of *Polynucleobacter*-like bacteria might represent a plesiomorphic character for a broader group of *Euplotes* species. On the other hand, in spite of morphological and ecological differences, *E. harpa* and *E. aediculatus* cluster together in phylogenetic trees inferred from 18S rRNA gene sequences (Petroni et al., 2002).

Furthermore, free living bacteria and environmental

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clones from a broad spectrum of fresh water habitats, with a high 16S rRNA gene sequence similarity with *P. necessarius,* were isolated. Nevertheless, information about the real function of symbiotic *Polynucleobacter* is lacking. Exactly what metabolic deficiency do they compensate for? What stage of the extremely complex reproductive process of the ciliate do they interfere with?

In this paper, a comparative behavioral, ultrastructural and cytochemical analysis is performed on *E. harpa* and *E. aediculatus* with and without their symbionts, to point out possible variations ascribable to the symbiont deprivation. The results show that the ciliates are no longer able to perform glycogenolysis without the bacteria.

2. Materials and Methods

Source of the samples and culture conditions

Three strains of *Euplotes harpa,* obtained from samples collected in brackish environments in different geographical regions, were used for preliminary tests: FSP1.4 (pond close to the estuary of the Serchio River, Pisa, Italy), BOD2 (Baltic Sea, Boderne, Denmark), FC1 (estuary of the Chidro River, Taranto, Italy).

Cells from strains FC1 and BOD2 were previously shown to harbor *Polynucleobacter*-like bacteria and a still unknown secondary bacterial symbiont, while FSP1.4 cells harbor only *Polynucleobacter*-like bacteria (Vannini et al., 2005). To exclude any possible interference of the secondary symbiont, more targeted *in vivo* experiments, cytochemical techniques and Feulgen staining procedure were performed on *E. harpa* strain FSP1.4. Stock STIR1 of *E. aediculatus* (Stirone River, Italy) was also used for preliminary experiments. Cells of *E. harpa* and of *E. charon* (strain LIV31) were maintained in artificial seawater (salinity of 5‰ and 33‰, respectively) and at 20°C. Cultures of *Dunaliella tertiolecta*, grown in the same conditions were used as food. Cells belonging to the freshwater species *E. aediculatus* (strain STIR1) were maintained in a medium consisting of spring water enriched with boiled rice grains at 20°C.

Antibiotic treatments

Preliminary antibiotic treatments were performed with 1,000 u/ml of Penicillin-G for five days, on *E. harpa* (strains FSP1.4, BOD2, FC1) and *E. aediculatus* (strain STIR1) according to the protocol described elsewhere, whose efficiency in eliminating *Polynucleobacter* from the host cytoplasm without any apparent damage to the ciliate cells was verified by observations on cellular behavior, TEM analysis, and FISH experiments for both species (Vannini et al., 2003; Vannini et al., 2005). The same treatments were also performed on specimens belonging to the species *E. charon* (strain LIV31), which doesn't harbor any prokaryotic symbiont. Then *in vivo* experiments were performed on *E. harpa* FSP1.4 cells. Each experimental group consisted of 21 isolated cells. After five days of exposure to the antibiotic, clones resulting from isolated specimens were transferred to the following media without any antibiotic addition: 1) their growth medium without the addition of food organisms; 2) their growth medium with food and 3) their growth medium with food and 0.1 M glucose. In all cases, the same number of isolated cells, taken as a control, was maintained for five days in culture medium without any antibiotic addition, and then treated in the same way. The growth rate of *E. harpa* (strain FSP1.4) was estimated by counting the number of *E. harpa* cells in each treated and control clone every 24 hours from the beginning of the experiment, for a total period of 10 days. The number of cellular divisions was inferred by calculating the base-2 logarithm of the ciliate number. The Kruskal-Wallis non-parametric test was used to assess the significance of possible differences occurring between the experimental populations.

In situ *hybridization*

In order to test the presence of *Polynucleobacter* symbionts, fluorescence *in situ* hybridization on ciliate cells was performed at the end of the antibiotic experiments (ten days after beginning the treatment) according to the protocol described by Vannini et al., 2005. We used as a probe Poly_862 (5'-GGCTGACTTCACGCGTTA-3'), targeting *Polynucleobacter*-like bacteria (Vannini et al., 2005).

Electron microscopy

For transmission electron microscopy (TEM) cells were fixed and processed as previously described (Vannini et al., 2005). The sections for TEM observation were contrasted either with uranyl acetate and lead citrate or with the Thiéry method which is specific for polysaccharidic substances (Thiéry, 1967).

Cytochemistry

Glucose-6-phosphatase activity was revealed cytochemically by the formation of an electron-dense lead precipitate. Control and treated samples were fixed with glutaraldehyde (2% for 5 min) on the 5th day, then they were pre-incubated in a tris-maleate buffer 0.2 M pH 6.5 for 10 min, incubated in the same buffer containing 4 nM glucose-6-phosphate and lead nitrate 3 mM for 1 h. As a control, some cells were incubated in the same medium not containing glucose-6-phosphate. Then, the cells were rinsed in the same buffer and postfixed in OsO_4 for 30 min, dehydrated and embedded as for routine electron

microscopy. The sections were stained only with uranyl acetate.

Feulgen staining procedure

The antibiotic treatment was performed as reported above on the strain FSP1.4 of *E. harpa*. After 5 days, both treated and control specimens were divided into two groups. The first one, consisting of 329 control and 355 treated cells respectively was directly concentrated with a micropipette on cover slides, fixed in Sanfelice and processed for Feulgen procedure; the second group, 144 control and 129 treated cells, was transferred to a new medium in the presence of food and processed for Feulgen procedure after 36h. All the samples were observed with the light microscope to verify and quantify the presence and the progression of the reorganization bands in the macronucleus.

3. Results

The elimination of bacteria inhibits glycogenolisis

In the preliminary antibiotic experiments performed in the presence of food we verified that the specimens of *E. charon* were not affected by the treatment: differences were never observed between control and treated specimens in behavior and fission rate. On the contrary, the three *E. harpa* strains, as well as *E. aediculatus*, transferred to the culture medium with food after the antibiotic treatment, were not able to perform divisions starting the second day of the treatment.

In any case, the treated cells did not immediately die; they fed and moved like the control cells at least until the 10th day from the beginning of the experiment. Only a few days later did their movements become slow, and eventually they died. TEM observation on thin sections revealed that the only ultrastructural difference between treated and control cells concerned the amount of polysaccharidic reserve substances that, in *Euplotes*, are stored as glycogen in the typical form of rosettes (like in the human liver). Indeed, an extremely high amount of glycogen rosettes was observed in the cytoplasm of aposymbiotic fed *E. harpa* and *E. aediculatus,* compared to the bacteria-bearing specimens (Figs. 1a, b).

It is known that the amount of glycogen particles in a cell varies according to nutritional conditions and energy needs. To exclude the possibility that the higher glycogen amount in treated cells might be the direct consequence of a minor energy request due to fission blockage, a series of experiments without the addition of food organisms was performed on *E. harpa* FSP1.4 (see Methods). In this condition, aposymbiotic cells ceased to divide on the 2nd day, as in the preliminary experiments described earlier, but the fission rate of the control cells decreased dramatically starting the 5th day of the experiment. The maximum number of divisions reached by control ciliates was of 3.1 (mean value on the 10th day), while the same value for aposymbiotic cells was 0.1. As expected (Rosati et al., 1981), at the end of the experiment, the cytoplasm of the control starved cells contained significantly less glycogen particles than the cytoplasm of the well-fed cells. On the contrary, the cytoplasm of treated cells was still full of glycogen particles.

The addition of glucose to the culture medium with food did not restore the fission capacity in the treated cells and did not modify the fission rate in the control cells. Maximum numbers of divisions reached by the aposymbiotic experimental populations with and without added glucose, were 1.3 and 1.5, respectively. Maximum division numbers reached by the control populations with and without glucose were 6.9 and 7.1, respectively. Statistical analysis revealed no highly significant difference between the two control groups ($p=0.9$), nor between the two aposymbiotic groups ($p=2.5\times10^{-4}$). The same kind of analysis underlined highly significant statistical differences between the control and treated populations ($p<10^{-5}$) (Fig. 2).

Electron microscopy revealed that in the cytoplasm of aposymbiotic ciliates, maintained in the presence of glucose, the amount of glycogen actually increased (Fig. 1c). Thus *E. harpa* appear to be able to perform glycogenosynthesis but not glycogenolysis once deprived of *Polynucleobacter*-like bacteria. It is worth noting, however, that at least one treated cell carried out a cellular division on the 9th day of the experiment. The total absence of bacterial symbiont in both daughter cells was verified by *in situ* hybridization.

Aposymbiotic cells are able to start binary fission

The analysis of *E. harpa* FSP1.4 specimens stained with Feulgen procedures showed that both treated and control cells were able to start the fission process. The results obtained are summarized in Table 1. In the absence of food organisms, the percentages of the different steps of the reorganization band progression, indicative of ongoing DNA synthesis, were very similar in treated and control cells. Under feeding conditions, i.e., in conditions stimulating division, control cells were able to go ahead and begin a new cycle (as demonstrated by the high percentage of dividers and cells without bands) but, although it appeared that aposymbiotic cells were also able to start and carry on macronuclear DNA replication, only in a few cases did they complete macronuclear replication (0.78%), occasionally followed by an anomalous division; only in very few cells was the fission process regularly accomplished (1.55%). Apparently micronucleus divided regularly in any case (Figs. 3a–c).

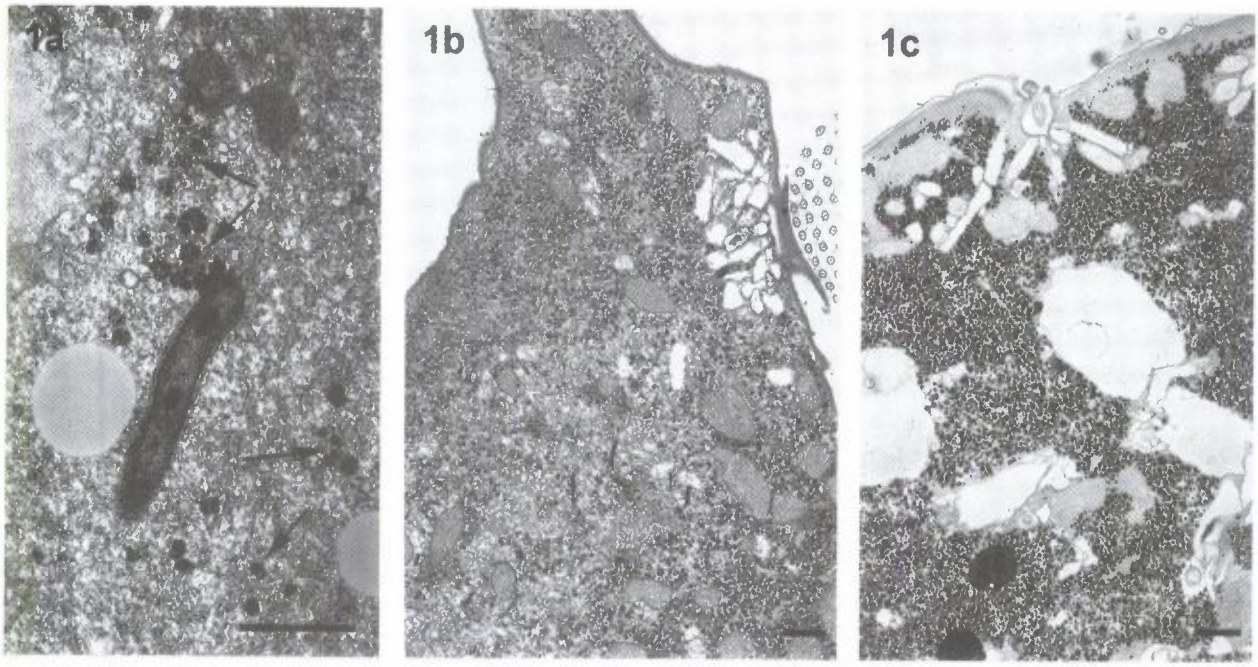

Figure 1. Transmission electron micrographs of *Euplotes harpa* cytoplasmic portions. a) Control fed cell. Scattered glycogen rosettes are present. A *Polynucleobacter* bacterium is also visible. b) Treated cell. Glycogen rosettes are more numerous throughout the cytoplasm. Bacteria are not present. c) Cell treated and transferred to a medium containing 0.1 M glucose. The section was contrasted with the Thiéry method for polysaccharidic substances. The number of glycogen rosettes is extremely high. The cytoplasm vacuolization is indicative of stress conditions. Arrows point to glycogen rosettes. Bars = 1 μm.

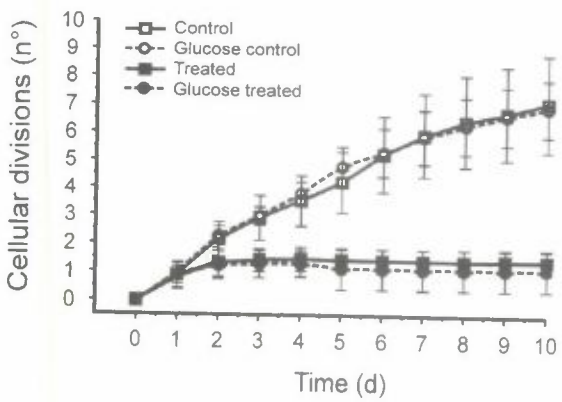

Figure 2. Number of cellular divisions in symbiotic and aposymbiotic *Euplotes harpa* FSP1.4, in normal culture medium and with the addition of 0.1 M glucose. Differences between control and treated populations are highly significant ($p<10^{-5}$).

Table 1. The cells are grouped according to the position of the DNA reorganization bands in macronucleus evidenced by Feulgen protocol.

	Starved		Fed	
	Treated %	Control %	Treated %	Control %
Without bands	57.75	57.75	37.21	43.06
Initial	23.10	22.80	29.45	31.94
Intermediate	9.86	13.07	17.83	8.33
Advanced	9.29	6.38	13.18	7.64
Joined at the end of replication	0.00	0.00	0.78	0.00
Dividers	0.00	0.00	1.55	9.03

Glucose-6-phosphatase activity in control and antibiotic treated cells

In control cells the reaction product, indicative of the presence of the active enzyme, was only localized at the cell membrane level (Fig. 4a) while in treated cells, the mitochondrial cristae were also selectively stained (Fig. 4b). Moreover, many lipid droplets appeared partially degraded and surrounded by mitochondria (Fig. 4b). The reaction product was never found in control specimens in which the substrate was omitted.

4. Discussion

The scenario we obtained after symbiont deprivation in different strains of *E. harpa* and a strain of *E. aediculatus* perfectly corresponds to that obtained in previous studies on *E. aediculatus* (Heckmann et al., 1983; Vannini et al., 2005) and *E. harpa* (Vannini et al., 2005). Thus, corresponding results have been obtained in different

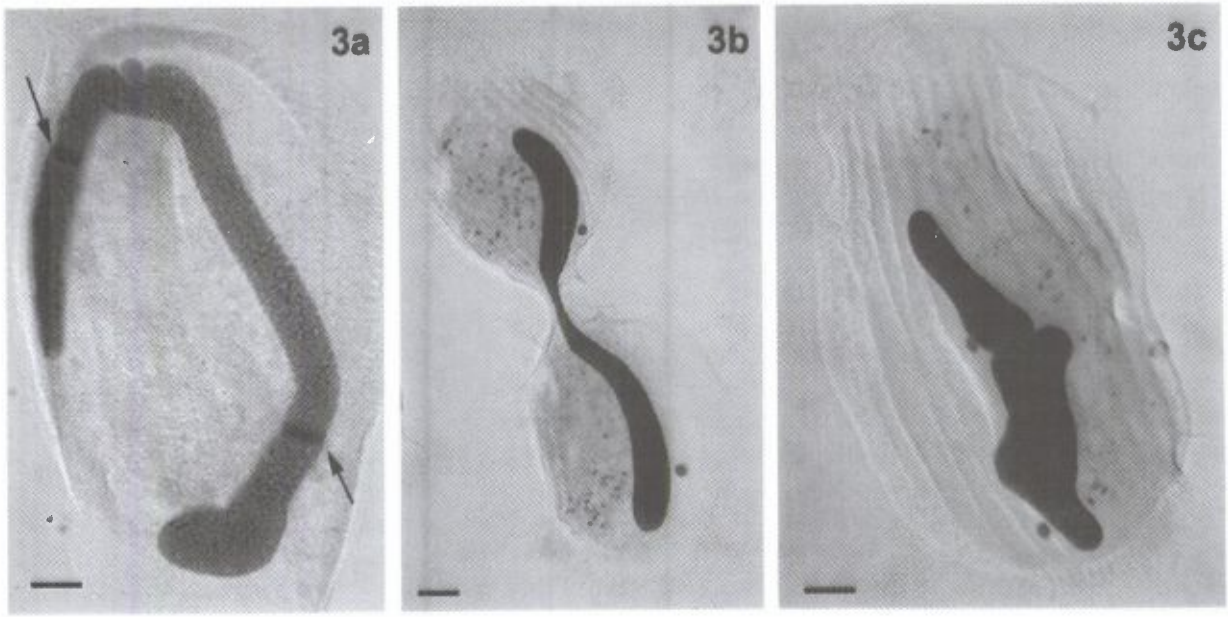

Figure 3. *Euplotes harpa* cells stained by Feulgen procedure. a) A control cell with the macronuclear reorganization band (arrows) at an intermediate level. b) Dividing control cell. c) The anomalous macronuclear division in a treated cell. Bars = 10 μm.

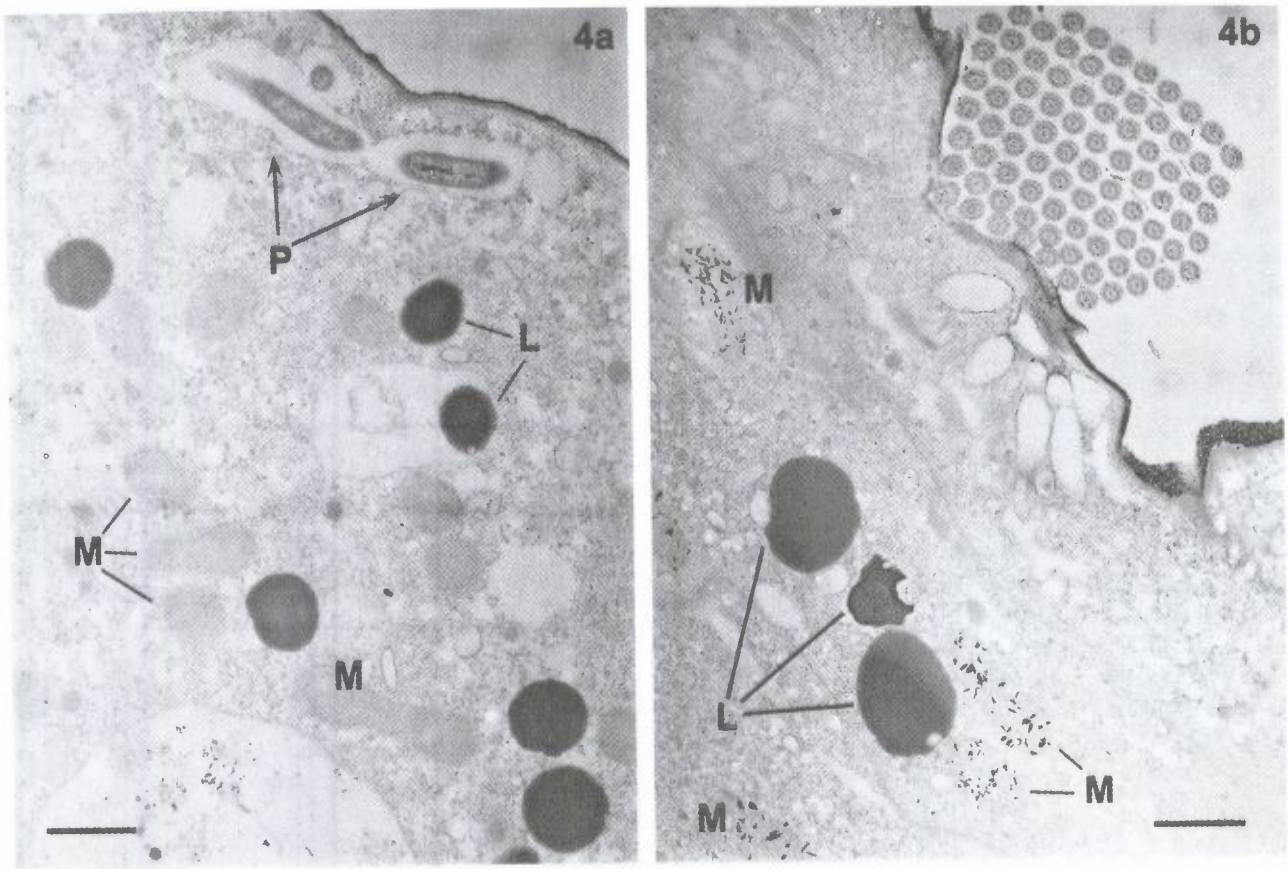

Figure 4. Localization of glucose-6-phosphatase. a) Control cell. Lead precipitate is present only at the cell membrane level. *Polynucleobacter* bacteria are present. b) Treated cell. Lead precipitate is also localized on mitochondrial crstae. Note the partially degraded lipid droplets. L = lipid droplets; M = mitochondria; P = *Polynucleobacter*. Bars = 1 μm.

species, at different times, by different researchers. Moreover, since *E. charon* is a species which doesn't harbor any prokaryotic symbionts, and is never affected at all by the treatments, a direct effect of Penicillin-G on ciliate cells of *Euplotes* genus may be reasonably excluded at least in our experimental conditions. This data, on the whole, definitely proves that *Polynucleobacter*-like bacteria really do interfere with the reproductive process of the different species they inhabit.

Our results are also in agreement with the assumption that the bacteria compensate for a metabolic disorder in their hosts and for the first time prove that this disorder concerns glycogen metabolism. Indeed electron microscopical observation of aposymbiotic *E. harpa* and *E. aediculatus* showed that they are not able to metabolize their polysaccharidic reserve substances. The fact that *Polynucleobacter*-like bacteria are present in every organism of several different, phylogenetically correlated, *Euplotes* species, are vital for them and are vertically transmitted from generation to generation, indicates that we are dealing with a hereditary disorder, possibly an enzymatic deficiency.

The first visible step in the reproductive process of *Euplotes* is the appearance of reorganization bands at both ends of the typically C shaped macronucleus. These bands, which then move toward the center of the macronucleus, fuse and finally disappear, indicate the ongoing DNA synthesis and reorganization. Absence or paucity of food is supposed to block the S phase of the cell cycle in ciliates (Hausmann et al., 2003). In line with this assumption, most starved *E. harpa* appear without or with only initial reorganization bands in their macronucleus, independently from the presence or the absence of the symbionts. The observation of fed specimens, however, demonstrates that aposymbiotic cells are able to start and go on with DNA replication, in some cases nuclear division and very rarely even cytoplasmic division; the higher total percentage of reorganization bands observed in treated specimens is compensated by the number of dividers observed in control cells all able to complete the whole process. It is very likely that failure in performing glycogenolysis deprives aposymbiotic organisms of an important energy source, thus preventing the accomplishment of the whole reproductive process. Indeed, the reproductive process in ciliates has a high energy cost given that, besides the replication of the nuclei and of the other internal cellular organelles, it involves the doubling and reorganization of the specialized cortical structures.

Polysaccharidic reserve substances in ciliates are present in different forms. The most common are glycogen and other similar carbohydrates commonly referred to with the general term paraglycogen, introduced by Bütschly (1889). They show different features when examined at the electron microscope level. Paraglycogen usually appears in the form of granules of variable density and size ranging from 0.1 to 15 μm. Glycogen is present either as single spherical particles 15–30 nm in diameter (as in human muscle) or in the form of rosettes as in the human liver. The latter is less frequent in ciliates and, within the Euplotidae family, is a typical trait of all the species of the genus *Euplotes* (Verni and Rosati, 1980). The different forms of polysaccharidic reserve substances react differently to cytochemical treatments (Verni and Rosati, 1980) and, presumably, different enzymatic batteries are involved in their synthesis and degradation. Interestingly, a comparative study on different *Euplotes* species revealed that glycogen rosettes at least in *E. harpa*, *E. aediculatus* and *E. eurystomus* (another *Polynucleobacter*-like bacteria-bearing species) are, on the average, larger than in the other *Euplotes* species (unpublished data). This might be the result of a somewhat difficult glycogenolysis, even in the presence of their symbionts.

The similarity of *Euplotes* and human liver glycogen led us to verify in both treated and control cells the activity of Glucose-6-phosphatase (G6Pase), an enzyme found mainly in the liver and the kidneys, which plays the important role of providing glucose during starvation by glycogenolysis or gluconeogenesis. The latter process, i.e., the generation of glucose from non-polysaccharidic organic molecules, occurs during periods of starvation or intense exercise and is highly exergonic. G6Pase is a membrane bound enzyme; it is associated in mammals with endoplasmic reticulum membranes (Van Schaftingen and Gerin, 2002). We localized active G6Pase on the cell membrane of both control and treated specimens but only in aposymbiotic specimens was the enzyme also present at the mitochondrial cristae level. This presence, together with the evident degradation of lipid droplets, suggests that aposymbiotic cells perform gluconeogenesis through the glyoxylate cycle even in the presence of food, as an emergency energy source. The glyoxylate cycle replenishes intermediates of the Krebs cycle and conserves carbon that would otherwise be oxidized and lost to biosynthetic pathways, with the final result of a net conversion of fats to carbohydrates. It has been found in many forms of life and is widely distributed among bacteria, fungi and higher plants. It has also been reported in autotroph (Ono et al., 2003) as well as eterotroph (De Duve and Badhuin, 1966) protists.

The occurrence of G6Pase at the cell membrane level has already been reported in some protists such as *Ochromonas* (Patni and Aaronson, 1974) and the ciliate *Paramecium* (Gu et al., 2002). It has never been reported in the internal mitochondrial membranes.

A number of human diseases, due to the abnormal metabolism of glycogen, are known and referred to as glycogenosis. Most of them are characterized by the accumulation of glycogen in tissues. They are recognized, depending on the enzyme deficiency involved, all of autosomal recessive inheritance, but with a different gene for each enzyme deficiency. The presence of active G6Pase

indicates that in the *Euplotes* "glycogenosis" a deficiency of this enzyme is not involved. Other important metabolic factors in glycogenolysis, however, would seem to be.

Eukaryotic cells, as genetic entities, most often involve several physically associated genomes that direct the metabolic cell equilibrium. Thus, in the coleopteran insects of the genus *Sitophilus*, in addition to the nucleus and the mitochondrial genomes, two other intracellular bacterial genomes are also present. They coexist with the eukaryotic host cell genomes, and intervene in the physiology and reproduction of the host (Heddi et al., 2001). This is also the case of the parasitic wasp *Asobara tabida* in which a direct relationship between oocyte production and the symbiotic bacterium *Wolbachia* density has been demonstrated. Indeed the removal of *Wolbachia* by means of antibiotic treatment specifically inhibits oogenesis (Dedeine et al., 2001). There are indications of similar genomic cooperation even in ciliates. It has been reported that symbionts provide *Paramecium octaurelia* with folic acid and biopterin needed for their growth (Soldo et al., 1982), while the infection with the bacterium *Holospora obtusa* may confer heat-shock resistence to *Paramecium caudatum* by enhancing the gene expression (Hori and Fujishima, 2003). The species-specific symbiont *Devosia euplotis* is necessary for food digestion in *Euplotes magnicirratus* (Vannini et al., 2004). The consortium between *Euplotes* species and *Polynucleobacter* bacteria is a further demonstration of the importance of these genome associations in solving life problems. Bacteria of the *Polynucleobacte*r group, comprehensive of free living and obligate symbiotic forms strictly phylogenetically correlated (Hahn, 2003) to each other, might also represent a good model for studies on symbiosis establishment and evolution.

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