Flagellated endosymbiotic bacteria in a marine
*Frontonia* sp. (Oligohymenophorea, Peniculida)

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**Summary**

*Frontonia* sp. was repeatedly found in sea water samples collected from a shore near Leghorn (Ligurian sea). Endosymbiotic rod shaped bacteria 5–6 µm long were found in the cytoplasm of each *Frontonia* sp. specimen examined at the fluorescent microscope following DAPI staining procedure. These bacteria, as revealed by electron microscopy, are contained in vacuoles (one or two symbionts are present in each vacuole) and are covered by numerous flagella which are in close contact with the vacuolar membrane. Flagellated symbiotic bacteria in ciliates have been seldom described. All the cases referred in the literature concern *Paramecium*, a genus that mainly consists of fresh-water species. This is the first record of flagellated bacteria living in a marine ciliate species belonging to a different taxon. The fact that these symbionts have been constantly observed in just collected *Frontonia* and are maintained in specimens grown in the lab for at least one month, suggests that we are not dealing with an occasional relationship. The abundance of cytoplasmic vesicles and glycogen particles, around and sometimes within the vacuoles, suggests a movement of metabolites between the host cytoplasm and the symbionts. The bacteria maintained their motility once outside the host cell.

**Key words:** ciliates, endosymbiosis, flagellated bacteria, *Frontonia*, symbiosis, ultrastructure.

**Introduction**

Bacterial symbionts have been observed in a variety of ciliate species. Many reports deal with occasional or not understood associations. Nevertheless a certain number of well established relationships between ciliates and bacteria have been described and studied in detail. Fresh-water species of a major group of the hypotrich *Euplotes* depend upon symbionts (Heckmann, 1975; Schmidt and Heckmann, 1980). The best known species is *Polynucleobacter necessarius* (Heckmann and Schmidt, 1987): it cannot be grown outside its host nor can *Euplotes* grow and multiply without the bacterium. Both organisms form an evolutionary entity. In other permanent associations, it has been demonstrated that the symbiotic bacteria, although not essential, provide true benefits to their ciliate host. Epixenosomes (Rosati, 1999), endosymbiotic bacteria related to *Verrucomicrobia* (Petroni et al., 2000), defend their host, namely ciliates of *Euplodiidium* genus, from predators (Rosati et al., 1999). Ectosymbiosis involving sulphate reducers bacteria are widespread in ciliates living in the anaerobic environment (Fenchel and Finlay, 1995), while sulfur-oxidizer bacteria can be found on ciliates living in habitats whose common denominator is the production of sulfide in close proximity to a source of oxygen. In some cases these bacteria are vital for the host (Bauer-Nebelsick et al., 1996).

As concern endosymbiosis, well known is the mutualistic association between ciliates adapted to an anaerobic life stile, particularly those possessing hydrogenosomes, and methanogenic bacteria. The symbionts use intracellular hydrogen as a substrate for methane formation: in this way they guarantee to their hosts the partial low pressure of hydrogen required for the functioning of hydrogenosomes (Fenchel and Finlay, 1995). It has been suggested a multiple acquisition and replacement of endosymbiotic methanogenic bacteria during their host adaptation to the various anaerobic ecological niches (van Hoek et al., 2000): this represents a further indication that we are dealing with an association truly favorable for both partners.

A number of different endosymbiotic bacteria confer killer traits upon their ciliate host. This trait has been reported in different species of *Paramecium* (for review see Preer et al., 1974), in *Parauronema acutum* (Soldo and Brickson, 1978) and in some *Euplotes* species (Heckmann et al., 1967; Nobili et al., 1976). In every case cells bearing these symbionts may kill cells of other strains (or even...
other species) that do not bear symbionts. While the symbionts that confer the killer trait to *Euplotes* have not been identified, those of *Paramecium* have been diffusely studied and included in different genera and species (for review see Quackembush, 1988). The most known are bacteria of *Caedibacter* genus, most of which are able to produce R bodies (refractile bodies). Very likely the presence of killer symbionts means a selective advantage in intraspecific competition for food.

In the present paper the constant association between flagellated bacteria and the marine ciliate *Frontonia* sp. is described for the first time.

### Material and Methods

Samples of sand and seawater were repeatedly collected during two years (1997–1998) from tidal pools in a rocky shore near Leghorn (Ligurian Sea). The samples were transferred in the lab and checked for the presence of *Frontonia*. Once identified, specimens of *Frontonia* were singly picked up with a micropipette and used in different ways: a) transferred in artificial sea water enriched with the green flagellate *Dunaliella salina* and the diatom *Pheodactilm tricornutum* to start cultures; b) fixed with 15% formaldehyde in distilled water added with 0.25M NaCl for DAPI staining procedure; c) processed for scanning electron microscopy; d) processed for transmission electron microscopy as described below. The latter three methods were successively applied to specimens picked up from cultures grown in the lab for at least one month.

### Electron microscopical techniques

For observation at the scanning electron microscope (SEM) specimens of *Frontonia* sp. were fixed with 2% OsO₄ in sea water, placed on coverslips coated with poly-L-lysine hydrobromide, dehydrated in ethanol and, after critical point drying, coated with gold and examined at a Jeol/JSM-5410.

For transmission electron microscopy (TEM) the specimens were fixed with 1:1 mixture of 5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) and 4% OsO₄ in distilled water. Then after ethanol dehydration, they were embedded in epon-araldite mixture. The sections were contrasted with uranyl acetate and lead citrate prior to examination in a SX 100 Jeol electron microscope. Some sections were picked up with mylar rings and treated according Thiéry procedure for polysaccharides (Thiéry, 1967).

### Results

A specimen of *Frontonia* sp. as it appears at SEM is shown in figure 1.

Observations by the fluorescence optical microscope following DAPI staining procedure were made either on specimens just collected from the sea at different times or on specimens of various cultures grown in the lab for at least one month. Hundreds of *Frontonia* sp. individuals were examined on the whole. In every case rod shaped bacteria 5–6 mm long were observed in the cytoplasm, always with an apparently similar density (Figs 2, 3). In some cases a few curved or spiral bacteria were also identified (Fig. 3).

Electron microscopical observation showed (Figs 4, 5) that the bacteria are delimited by two membranes, have a diameter of about 0.5 mm and a wrinkled surface from which numerous flagella emerge. The flagella, that do not have appendages, completely surround the cell. The bacteria are enclosed in cytoplasmic vacuoles bounded by smooth membranes, not regularly lined up by ribosomes along the host cytoplasm side. They are separated from the host by a wide space, the vacuolar space. In limited zones the bacterial and host membranes are however in close contact with each other (Figs 4, 5). One or two (Figs 6, 7) symbionts are present in the same vacuole. Probably, in many cases dividing individuals have been found (Fig. 8). The flagella extend from the bacterial surface into the vacuolar space, reaching the vacuolar membrane (Figs 4–7). In the host cytoplasm surrounding the vacuoles numerous membrane bounded vesicles of various size, some of which contain an amorphous material, are present (Figs 4–7).

Thiéry procedure, specific for polysaccharidic substances, revealed that also glycogen granules, in the form of small granules (b particles), are particularly abundant in these cytoplasmic regions (Fig. 9). Occasionally glycogen granules are present even in the vacuolar space (Fig. 10). In the same pictures cytoplasmic vesicles can be seen in close contact with the vacuole membrane. Their membranes, at variance with the vacuolar one, positively reacted to Thiéry procedure. Only rarely the curved bacteria could be identified on thin sections; anyway they too possess flagella (Fig. 11).

The endosymbiotic bacteria of *Frontonia* can be seen moving for a while under the interferential contrast microscope only once the host cell is broken (for example by a pressure on the coverslip or for an altered salinity). This is a strong argument that they are really flagellated.

### Discussion

Endosymbiotic flagellated bacteria were present in all the specimens of *Frontonia* sp. examined either soon after their collection (they were collected repeatedly, at irregular interval time, during two years) or after one month of permanence in the lab. The constancy of the finding, the observation that the flagellated bacteria are able to repro-
Figs 1–5. *Frontonia* sp. and its symbionts: 1 – ventral view of the ciliate at the scanning electron microscope (arrow points to the cytostome); 2, 3 – photographs printed down from colored slides of DAPI stained specimens (2 – the nuclear apparatus and rod shaped bacteria in the cytoplasm are evidenced, 3 – at higher magnification some curved bacteria (arrows) can be distinguished among the rod shaped ones); 4, 5 – different sections of rod shaped bacteria (arrows point to the flagella, asterisks indicate the bacterial and vacuolar membranes in close contact). CV – cytoplasmic vesicles, Ma – macronucleus, Mi – micronuclei, VM – vacuolar membrane, VS – vacuolar space. Scale bars: 1, 2 – 50 µm, 3 – 10 µm, 4, 5 – 1 µm.
Figs 6–11. Electron microscopy of the bacteria: 6, 7 – two symbionts are present in the same vacuole; 8 – a bacterium during binary fission; 9, 10 – sections stained according Thiéry procedure for polysaccharides (glycogen granules are visible in the cytoplasm surrounding the vacuole and inside the vacuole itself; arrows indicate cytoplasmic vesicles whose membrane positively reacted to the staining procedure.); 11 – a section of a curved bacterium. DV – digestive vacuole. Scale bars: 1 µm throughout.
duce within the host cytoplasm and that they are maintained in the stocks grown in an artificial environment, suggest that we are not dealing with an occasional relationship but with a well established association, very likely advantageous for both partners. The presence of cytoplasmic vesicles and glycogen granules in close association with the vacuolar membrane suggests movements of metabolites between the host cytoplasm and the symbionts (Bigliardi et al., 1989). So, whatever the nature of the association between the ciliate host and the flagellated bacteria, apparently this symbiosis involves a highly integrate system.

Ultrastructural studies on *Frontonia* species are scanty and limited to some features such as the cortex of *F. leucas* (Gil, 1981) and the trichocysts of *F. vesiculosa* (Yusa, 1965). So, although not reported in those studies, the presence of endosymbionts cannot be excluded. Some kind of bacteria were reported in *Frontonia* sp. by Fokin (1993). Anyway, considering also that *Frontonia* sp. specimens here studied were all collected along the same shore, further investigations are needed to consider this association constant and widespread in nature.

Endosymbiotic, flagellated bacteria have been described in a certain number of *Paramecium* species. In some cases these bacteria inhabit the macronucleus. Flagellated intranuclear symbionts, able to move in caryoplasm, have been found in *P. multimicronucleatum* collected in two very remote geographical areas (Vishnyakov and Rodionova, 1999). Cytoplasmic flagellated bacteria in ciliates were firstly described in *P. aurelia* as lambda and sigma particles (Beale et al., 1969). They are among the bacteria able to confer the killer trait of the “rapid lysis” type to their host and are now designated *Lyticum flagellatum* and *L. sinueum* respectively (Görtz, 1988). They do not produce R bodies. Cytoplasmic flagellated bacteria have been also reported in stocks of *P. caudatum* (Bass et al., 1987) and *P. woodruffi* (Fokin, 1989) and were named *Pseudolyticum multimicronucleatum* and *Ps. minutus* respectively; they both do not produce real R bodies but different structures, do not confer killer trait to their host and do not infect the endosymbiont-free cell lines. They were never observed to move either in the cytoplasm of the ciliate or outside the cell. Some motile infectious bacteria were reported in the cytoplasm of *P. caudatum* by Dieckmann (1981).

Here we describe for the first time flagellated bacteria in the cytoplasm of a ciliate species that do not belong to *Paramecium* genus and that, differently from most *Paramecium* species, is marine. These bacteria maintained their motility once outside the host cell. Most of them are rod shaped and, except for a major length, they are very similar to *L. flagellatum* in morphology and localization. The possibility that, like the latter symbionts, they confer the killer capacity to their host could not be tested, as symbiont-free *Frontonia* sp. were not available. The curved forms are rare: the difficulty to identify them in thin sections rendered it impossible to determine whether they represent a particular stage of the life cycle or they are a different species.

As far as we know none of the flagellated bacteria living in *Paramecium* species have been identified: they have been distinguished and classified on the basis of their morphological characteristics and their relationship with the host. An indication obtained by in situ hybridization with labeled group-specific oligonucleotides concerns the motile intranuclear symbionts of *P. multimicronucleatum* (Vishnyakov and Rodionova, 1999): they are Eubacteria, but a probe specific for *Holospora* (Amman, 1996), a well studied genus of infectious intranuclear symbionts in *Paramecium* (Görtz, 1988; Fokin et al., 1996), did not show any positive signal. It would be of a great interest to determine the possible phylogenetic relationships among the flagellated bacterial endosymbions found in various *Paramecium* species and in *Frontonia*.

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**References**


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