From Symbiosis to Eukaryotism
ENDOCYTOBIOLOGY VII
E. Wagner *et al.*, Eds.
University of Geneva, 1999, pp. 169-177

Motile intranuclear symbionts of ciliate *Paramecium multimicronucleatum*

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Abstract

A new species of intranuclear symbiotic bacteria was found in ciliate *Paramecium multimicronucleatum*, collected in two very remote areas: the USA and Moldova (former USSR). These symbionts inhabit macronuclei of *P. multimicronucleatum* and are able to move in caryoplasm. The bacteria have numerous flagella, which are arranged all around the surface of the symbiont cell. Their flagella do not have appendages. Lengths of some flagella are more than 10 μm, and their diameter is about 20 nm. The shape of the cells is variable: oval or rod-like. The symbionts have a dispersed or centrally located nucleoid. *In situ* hybridization with labeled group-specific oligonucleotides showed that the symbionts are Eubacteria, but a probe specific for *Holospora* (the most studied genus of ciliate intranuclear symbionts) did not show any positive signal. At present, infection of non-infected ciliates was successful only by using a microinjection method. The fine structure of these new symbionts was studied.

1. Introduction

Endosymbionts of ciliates are known since the end of the 19th century. At present, many cytoplasmic and intranuclear symbionts in ciliates have been found and studied [Ossipov & Ivakhnuk, 1972; Preer *et al.*, 1974; Goertz, 1986; Heckmann & Goertz, 1991]. However, all of them are immobile although some representatives of some species have flagella. Three years ago a new type of intranuclear symbiont was found in *Paramecium*

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multimicronucleatum collected in two very distant areas of Earth - the USA and Moldova (former USSR). The unique feature of these symbionts, in contrast to other symbionts in ciliates, is their ability to undergo very active locomotion over the whole volume of the nucleus. The present communication is the first description of these new symbionts.

2. Materials and methods

The subject of our investigation are new intranuclear symbionts which were found in the macronucleus of the ciliate *Paramecium multimicronucleatum*. The ciliates - hosts of these symbionts - were collected in a pond of the city of Boston, Massachusetts, USA (these ciliates were collected in 1994 by I.I. Skoblo), and in the river Dnestr near the city of Bender, Moldova, in 1996. Light optical observations were carried out with a Polyvar microscope (Carl Zeiss) using Nomarski differential interference contrast. For electron microscopy the ciliates (with symbionts) were fixed with 2.5 % glutaraldehyde, posttfixed with 1 % OsO_4, dehydrated with ethanol and embedded in Spurr. Thin sections were stained with lead citrate and uranyl acetate. For investigation of the symbiont flagellum apparatus the symbionts were removed from the nucleus with a micropipette and transferred to a drop of sterile culture medium on a formvar-coated copper grid. The cells were fixed with OsO_4 vapor and allowed to settle. The culture medium was removed with filter paper and cells were shadowed with platinum-carbon at an angle of 20 degrees. In an experiment with antibiotic treatment of infected paramecia we used ampicillin solution in culture medium at concentrations of 0.1, 0.5, 1.0, 10.0, 50.0 and 100.0 mg/ml. The nucleoids of the symbionts were detected after staining with DAPI at a concentration of 1 µg/ml. Before the staining procedure the symbionts were removed from the nucleus by micropipette and transferred to a drop of sterile culture medium with albumin (2-4 mg/ml). After that the drop was dried and the symbionts were fixed in ethanol and acetic acid (3:1) and stained with DAPI. The taxonomic position of symbionts was determined by using labeled oligonucleotides specific for Eubacteria and bacteria of the genus *Holospora* [Amann, 1996]. The infection of non-infected ciliates was carried out as described in Ossipov *et al.* [1976]. All microinjection procedures were done as described in Rautian *et al.* [1996].

3. Results and discussion

The present paper is the first description of a motile intranuclear bacterial symbiont of *Paramecium multimicronucleatum*. The symbionts inhabit exclusively macronuclei of the ciliate *P. multimicronucleatum* (fig. 1). Size and shape of the symbionts are variable. There are two main forms of symbionts: rod-shaped ones (length - about 2.0-2.5 µm, width -

0.4-0.6 μm) and short, oval morphs (length - 1.3-1.8 μm, width - 0.4-0.6 μm). Both forms move over the whole volume of the nucleus. The velocity of their locomotion is about 10-15 μm/s. However, it increases up to 20 μm/s when the nucleus begin to break down and flow out of the cell. Probably this is the result of a decrease in the viscosity of the environment. Sometimes symbiont cells can be observed which are much thicker (length - about 2.0-2.5 μm, width - about 1.5-2.0 μm). These bacteria are unable to move. When the ciliates starve or when antibiotics are added to culture medium, the amount of these immovable forms increases and they take on a new feature – some form septa (fig. 2). We

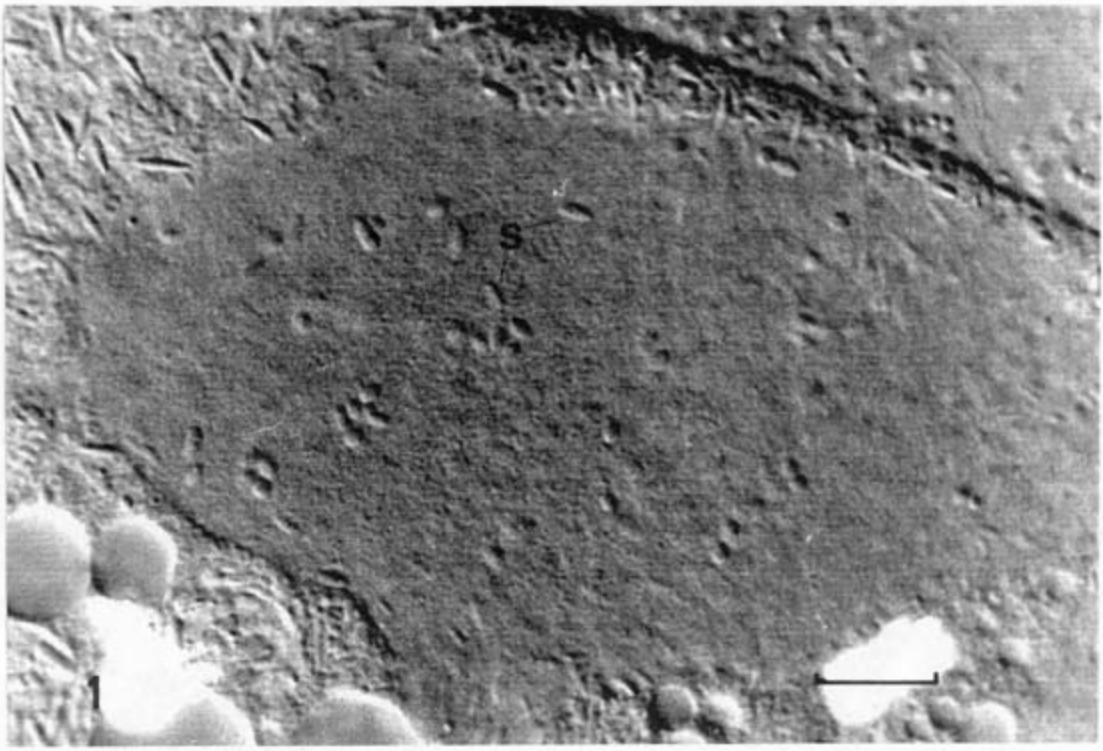


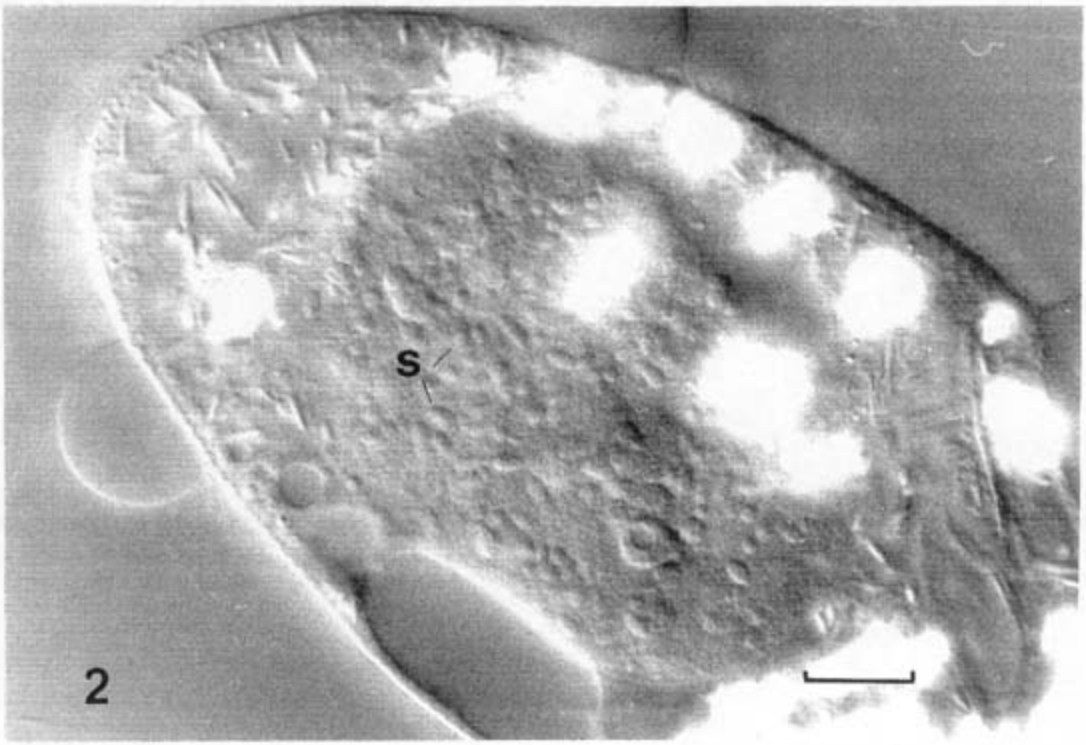
Fig. 1: Symbionts in the nucleus of P. multimicronucleatum. *s - symbionts. Bar - 10 μm.*

Fig. 2: Symbionts with septa in the nucleus of starving P. multimicronucleatum. *s - symbionts. Bar - 10 μm.*

Fig. 3: Flagellar apparatus of symbiont from ciliates collected in Moldava. *Bar - 1 μm.*

Fig. 4: Flagellar apparatus of symbiont from ciliates collected in the USA. *Bar - 1 μm.*

Fig. 5: Thin section of symbiont in nucleus of P. multimicronucleatum. *bw - bacterial wall, b - blebs of outer membrane, f - flagellum, lg - lipid granule, ii - irregular inclusions (perhaps the nucleoid). Bar - 0.4 μm.*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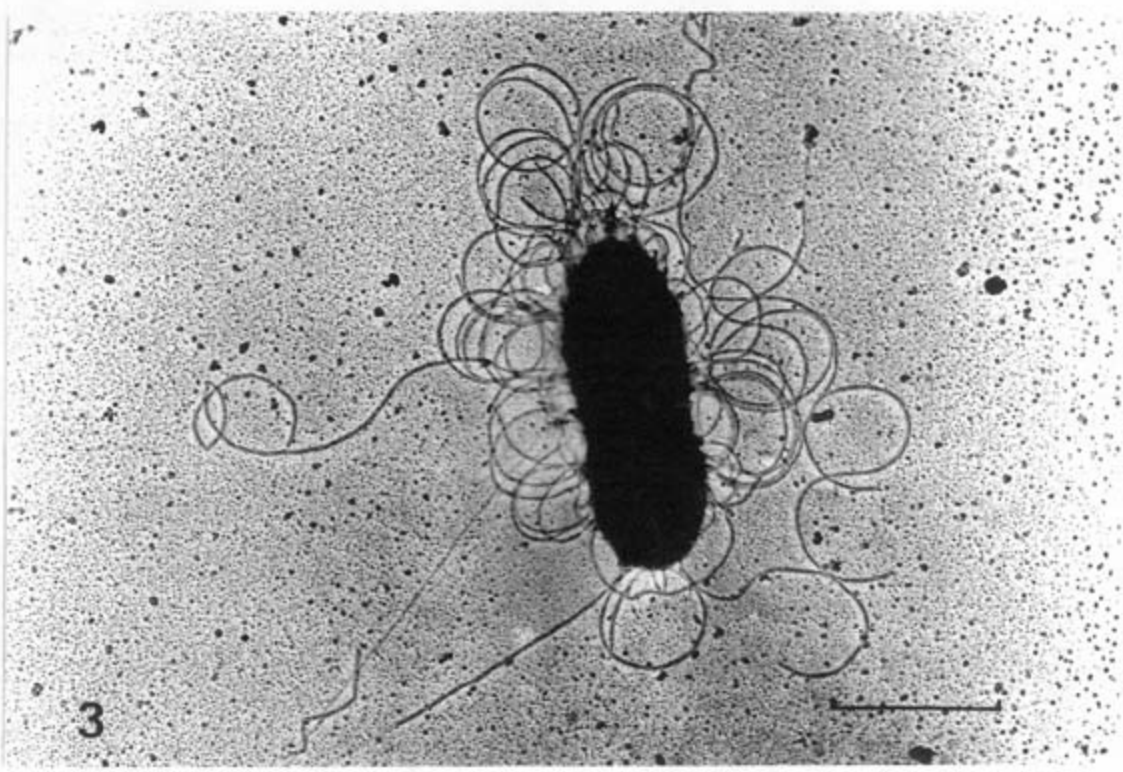
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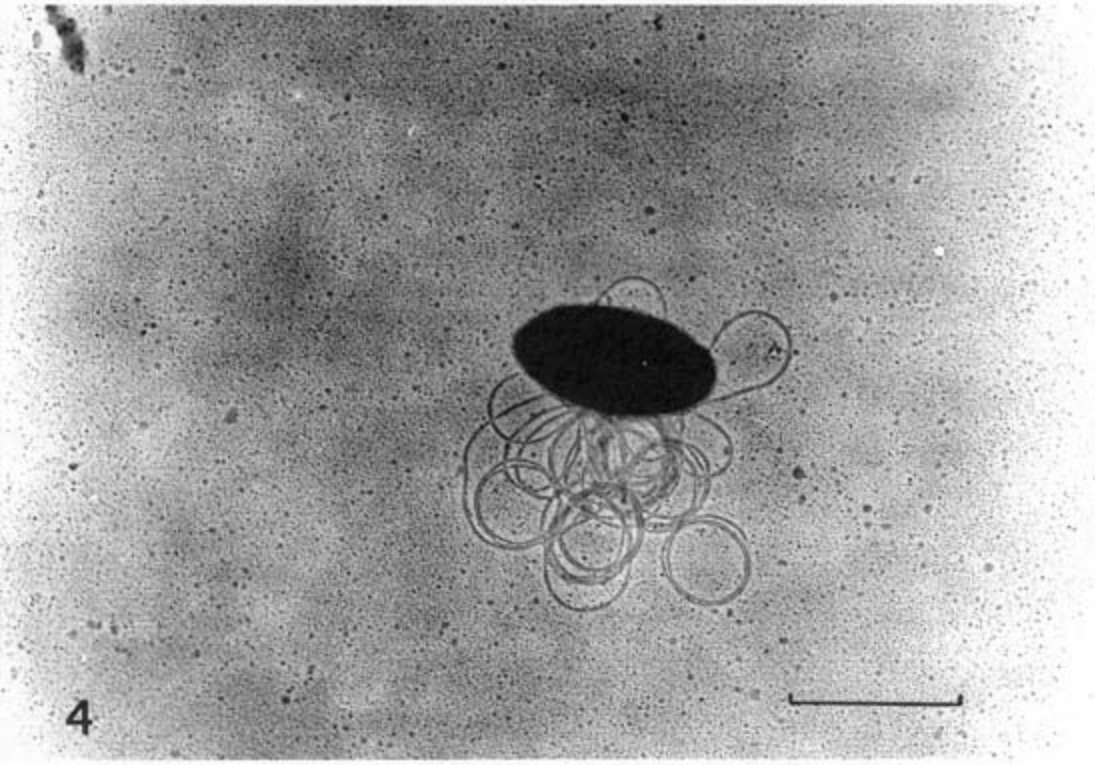

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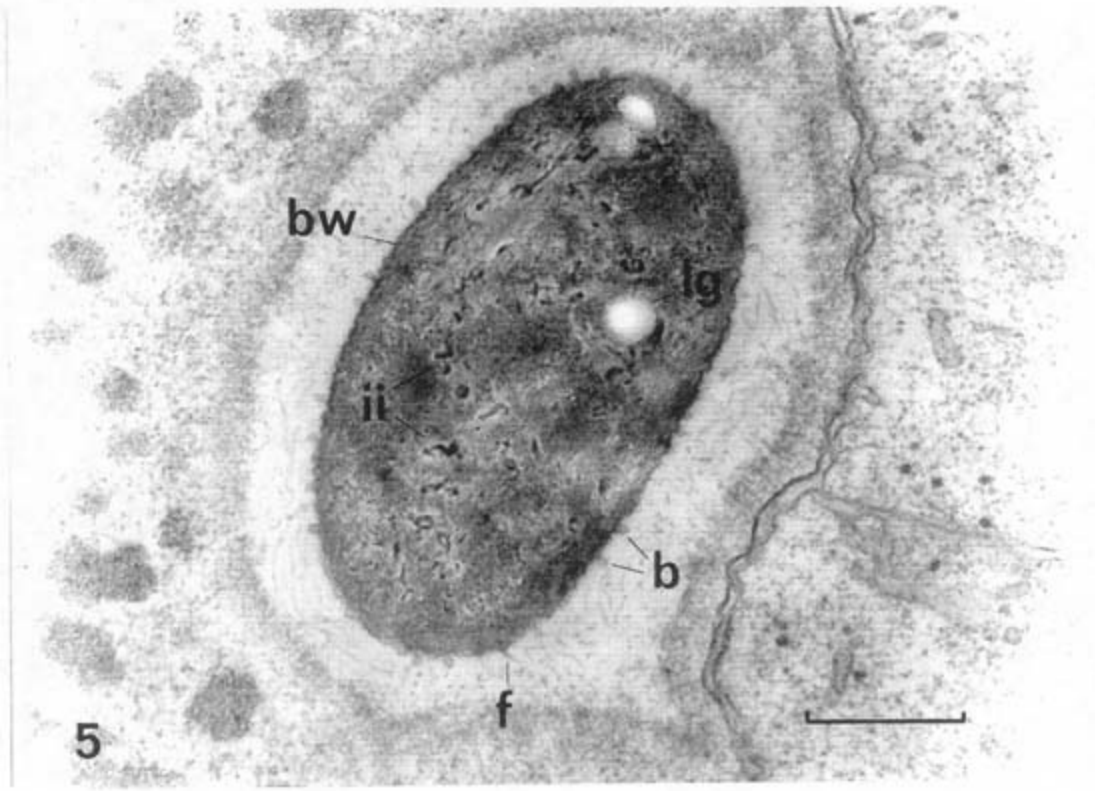

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observed that only oval-shaped symbionts are capable of fission, allthough we cannot exclude that the rod-shaped symbionts also are capable of division by fission.

Bacteria have numerous flagella arranged all around the surface of the symbiont cell inhabiting the ciliates collected in Moldova (fig. 3) and, probably, they have a local place of attachment on the cell surface of symbionts living in macronucleus of the ciliates collected in the USA (fig. 4). Maximum length of flagella is about 15 µm and their diameter is about 20 nm. Flagella do not have any pili or other appendages.

The fine structure of the symbionts of the ciliates collected in Moldova was investigated. The cell wall of the bacteria consists of two membranes with a thin layer of murein (fig. 5). Such organization of the cell wall is most readily visible in the symbionts which have a space between the internal and external membranes (fig. 6). This structure of the bacterial wall might suggest that the symbionts are gram-negative bacteria. The external membrane is covered with small membrane-bound vesicles over the whole surface. Sometimes among these structures one can detect the bases of the bacterial flagella (fig. 5). Flagella are particularly well-defined on the sections which passed close to the symbionts (fig. 7). Frequently the space around the symbiont is free of chromatin. At the boundary of the space the chromatin is more dense. Most probably this is a result of movement of the flagella (fig. 5).

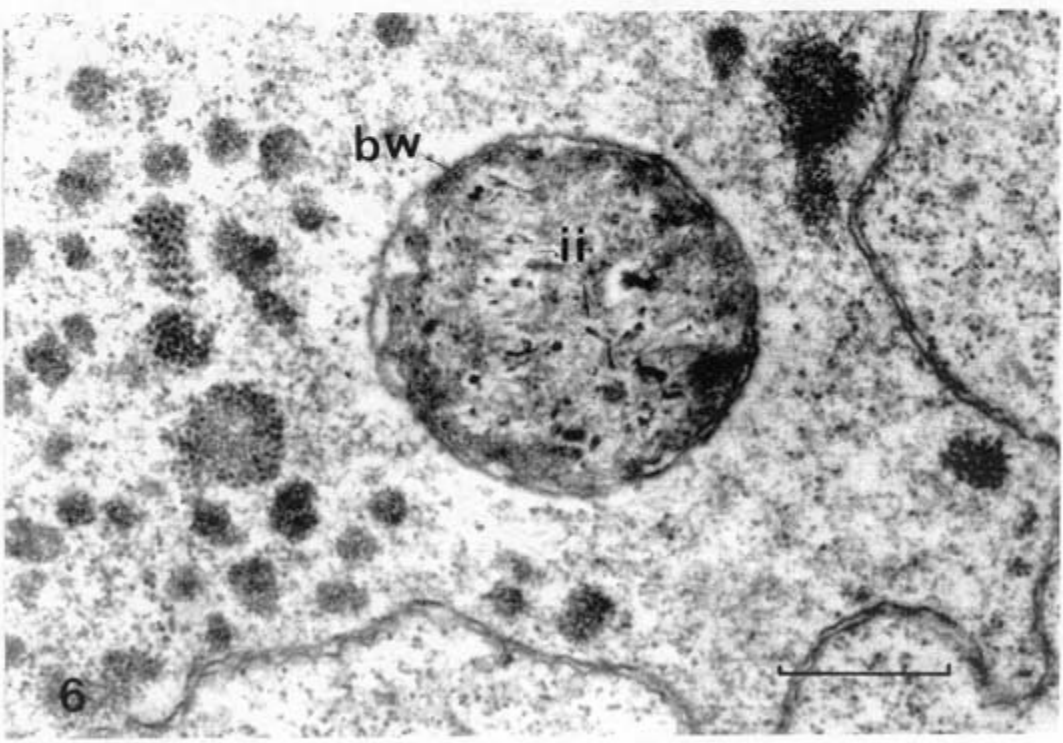

Fig. 6: Thin section of symbiont in nucleus of starving P. multimicronucleatum. *bw - bacterial wall, ii - irregular inclusions (perhaps the nucleoid). Bar - 0.4 µm.*

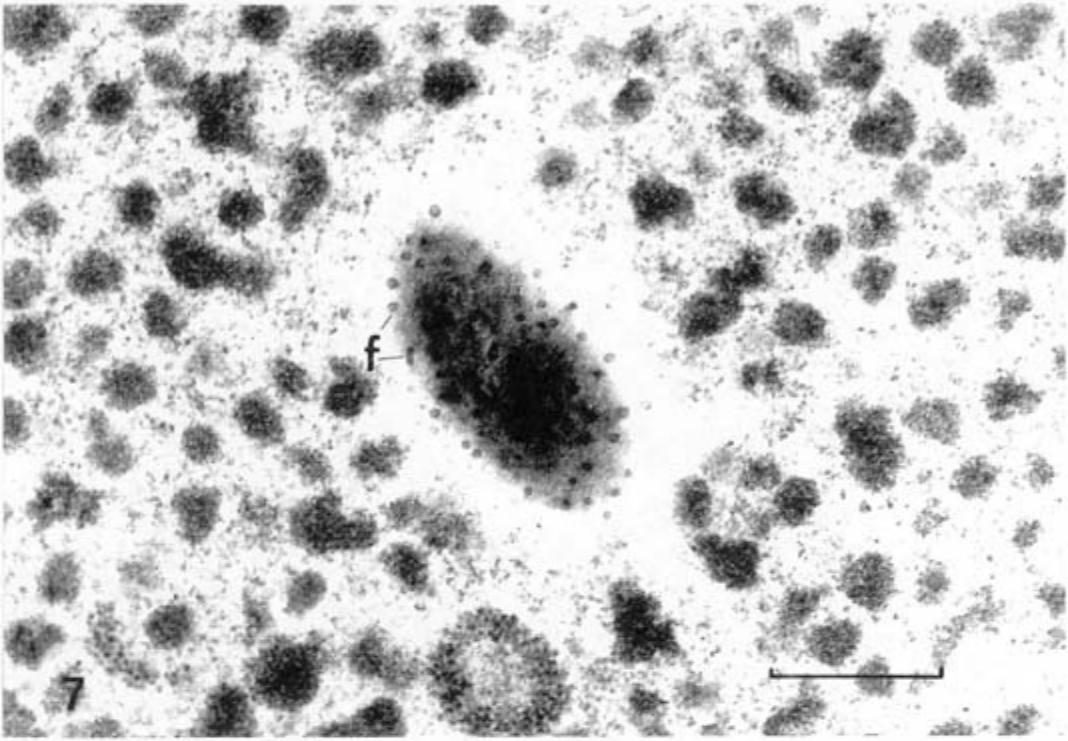

Fig. 7: Thin section of nucleus of P. multimicronucleatum beside symbiont. *f - flagellum. Bar - 0.4 µm.*

The cytoplasm of the bacteria exhibits two remarkable features: electron-transparent, oval granules and electron-dense, irregularly shaped inclusions (fig. 5). The oval granules look like polyhydroxybutyrate or polyphosphate granules or lipid droplets (Beveridge, 1989). However, the first mentioned granules are membrane-bound, whereas polyphosphate granules usually have a dark thin border. In our case we cannot find these structural features. So they might be considered to be lipid droplets.

The irregular inclusions might indicate a nucleoid which has this peculiar structure of packing. In some symbiont cells these inclusions can form a compact regular structure (fig. 6). In this case the inclusions are not scattered over the cytoplasm but occupy a strict region of the cell. Often these inclusions can be found in symbionts which have a cell wall with a space between internal and external membranes. Perhaps these symbionts are at the stage of destruction.

Most of the symbionts of oval and long shapes stained by DAPI show a uniform fluorescence. Only some of the cells have a single local field of fluorescence arranged in the center of the cell. We could not obtain preparations of symbiont with cross septa because they collapse very easily during preparation. So, for the present, we cannot confirm

electron-microscopic data by this method that the compact regular structure of irregul inclusions (fig. 6) is a nucleoid.

Ampicillin at a concentration of 0.5-10 mg/ml influences the symbionts in two ways. Sor symbionts stop their movement and acquire a form of large oval cells with cross septa (fi 2). On electron-microscopic sections they are the cells which have the space betwe external and internal membranes of the bacterial wall (fig. 6). Other symbionts stop the fission and become longer, sometimes up to 75-80 µm (fig. 8). Meanwhile they retain t ability to move inside the nucleus. Ampicillin at concentrations below 0.5 mg/ml does n influence the symbionts, whereas a higher concentration of it is lethal for the ciliates.

First experiments to determine a taxonomic position of these new symbionts b hybridization *in situ* with labeled oligonucleotides showed that they are Eubacteria but d not belong to the genus *Holospora*.

We investigated the infectivity of these symbionts in several experiments. At first we use the standard procedure for *Holospora* infection [Ossipov *et al.*, 1976]. The homogena from symbiont-bearing *P. multimicronucleatum* was added to the culture medium uninfected *P. multimicronucleatum*. In the case of *Holospora* many paramecia becon

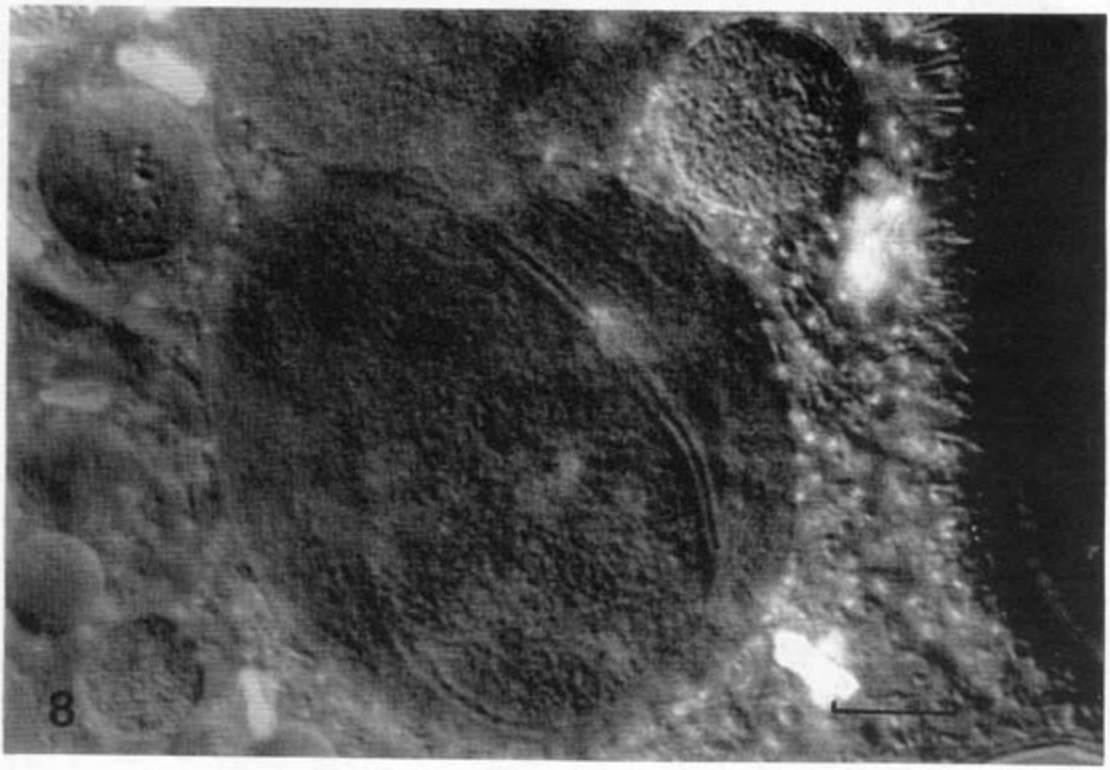

Fig. 8: Long form of symbiont in nucleus of P. multimicronucleatum after ampicilli treatment. *Bar - 10 µm.*

infected. However, in the experiments with these new symbionts, infected cells were never obtained. Obviously the symbionts collapsed in the culture medium because of the difference between the osmotic pressure in the nucleus and that outside of the ciliate cell. An increase of the osmotic pressure of the culture medium by adding of albumin (2-3 mg/ml) allowed one to conserve most of the cells when they came out of the nucleus.
We could obtain a few infected clones of *P. multimicronucleatum* using the method of direct microinjection from the nucleus of the infectious cell to the nucleus of the non-infectious ciliate. Ciliates of these clones are stable in keeping the symbionts over many months.

Acknowledgments
I am grateful to Prof. A.V. Pinevich for help in analysis of the electron-microscopic data and for his thoughts. The work is supported by the Russian Basic Research Foundation under the projects No. 96-04-48951 and No 98-04-49761.

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