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Possible new intranuclear symbionts of *Paramecium caudatum*

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Summary

Intranuclear bacteria inhabiting macronucleus of *Paramecium caudatum* isolated from three populations from North America and Eastern Europe were studied. Interference contrast and electron microscopy was used to describe symbiont morphology. Symbiotic bacteria from such distant populations are rather similar. They are rod-shaped with 1–2 μm length and 0.3–0.4 μm diameter; cell wall is typical for gram-negative bacteria; membrane vesicles on the surface of some bacteria indicate the secretion of some periplasm material into the nucleus. According to the *in situ* hybridization with group-specific probes, these symbionts belong to a-Proteobacteria subdivision. The proposal of α-Proteobacteria advantages for intracellular and intranuclear life are discussed.

Key words: intranuclear symbiosis, *Paramecium*, α-Proteobacteria, macronucleus

Introduction

The first description of symbiotic bacteria in ciliates was made more than a century ago (Hafkine, 1890). Since then a lot of various kinds of symbionts have been found (Ball 1969; Ossipov et al., 1997; Görtz and Brigge, 1998). Most of them are uncultivable, so the determination of their systematic relationship faced problems and, usually, systematic position of these bacteria is unclear. After development of the macrosystem of Prokaryote based on rRNA sequence comparison (Woese, 1987) and powerful express method for the attribution of the given bacteria to a particular group had been proposed (Stahl and Amann, 1991; Amann et al., 1995). As a result of an accumulation of thousands of RNA gene sequences from different bacteria specific short sequences – oligonucleotide "signatures" – were discovered. They are identical for representatives of particular groups and are absent in rRNAs of other bacteria. Up to now dozens of "signatures" specific for groups of different rank – main domains, divisions and subdivisions, genera etc. – have been found. Using *in situ* hybridization with the set of labelled oligonucleotides it is possible to dtermine systematic position of bacteria. This approach is especially useful for uncultivable bacteria, e.g. intracellular symbionts.

The present work is devoted to the description of new intranuclear symbionts found in *P. caudatum*. The ciliates were collected in the natural populations on different continents separated by thousands of kilometres. We used light and electron microscopy and some physiological tests for symbiont description and FISH for their systematic attribution.

Material and Methods

P. caudatum strains were collected on two different continents: North America (94AB 4–1, 94AB 4–3, 94AB 4–6, 94AB 4–7, 94AB 4–12, 94AB 4–15, 94AB 4–16, and 94AB 8–3, 94AB 8–4 are from two different ponds of the city of Boston, Massachusetts, USA, 1994), and Eastern Europe (97KM 3–23 and 97KM 3–24 in the lake in the Kaliningrad region, near the village Morskoye, 1997). All these clones are maintained in the Culture Collection of the free-living unicellular organisms at the Laboratory of Protozoan Karyology of Biological Research Institute of St.Petersburg University.

The ciliates were cultivated at room temperature on standard lettuce buffered medium inoculated with *Klebsiella aerogenes* as a prey organism.

Light-microscopic observations were carried out with a microscope Polyvar (Reichert-Jung, Austria), using differential interference contrast. For electron microscopy, the ciliates (with symbionts) were fixed with 2,5 % glutaraldehyde and postfixed 1 % solution OsO_4 in 0.1 M phosphate buffer, pH 7.4.

Attribution of symbionts to a particular group of Prokaryotes has been done using fluorescein- and rhodamin-labeled oligonucleotide "signatures" for Eubacteria, a-, b-, g-Proteobacteria and for genus Holospora (Amann et al., 1995). The whole paramecia cells with symbionts were fixed with fresh 4% paraformaldehyde in PBS, pH 7.2, and hybridized according to the protocol published by Amann and coworkers (Amann et al., 1991).

Results

With the light microscope**,** a long-standing monitoring of ciliate clones from the laboratory collection of unicellular organisms has allowed to find in the macronuclei (Ma) of *P. caudatum* from several clones (97KM 3–23, 97KM 3–24, 94AB 4–1, 94AB 4–3, 94AB 4–6, 94AB 4–7, 94AB 4–12, 94AB 4–15, 94AB 4–16, 94AB 8–3, 94AB 8–4) very small bacteria. They could be seen in Ma while micronucleus and cytoplasm of infected cells were free of symbionts (Figs 1 a, b). The appearance of micro- and macronuclei did not differ from the typical morphology of uninfected paramecia.

In the Ma of all strains studied the symbionts could be distributed more or less uniformly throughout caryoplasm, or organized in the dense groups of the various size and shape. Sometimes such groups occupied up to 1/4–1/2 of Ma volume.

The symbiotic bacteria have rod-like shape (length about 1 and diameter about 0.3 mm). Sometimes their length could range up to 4–5 microns. Some of such cells possessed a transverse fission which may be an indication of division process.

Ciliates of strains 97KM 3–23 and 97KM 3–24, unlike the American strains, in addition to symbionts of typical shape and size also had elongated and curved ones up to extreme variants – open-ended rings or spirals. At the same clones some of the symbionts, especially elongated ones, had refractive spherical structures.

For electron-microscopic study one clone from each group (97KM 3–24, 94AB 4–7 and 94 AB 8–3) was used. Symbiotic bacteria of ciliate clones 94AB 4–7 and 94AB 8–3 had similar morphology (Figs 2, 4). The cells were rod-shaped with the length varied from 1 up to 5 microns, and the diameter has 0.2–0.3 microns. The elongated cells, as a rule, were at different stages of division. Symbiont cell wall had a slightly wavy outline. Its structure is characteristic for gram-negative bacteria: there is a thin electron-transparent peptidoglycan layer between outer and plasma membranes. There are no appendages outside the bacterial wall. The cytoplasm has heterogeneous contents. There are electron-dense strands of irregular shape. Similar structures were earlier found in motile intranuclear symbionts of *P. multimicronucleatum* which were interpreted as nucleoid elements (Vishnyakov and Rodionova, 1999).

The ultrastructure of infected Ma, besides the presence of symbionts, did not differ from the uninfected Paramecium Ma: small chromatin bodies and nucleoli had typical size and distribution. The majority of bacteria of all clones had no direct contact with chromatin elements and were surrounded by electron-transparent area.

The symbiont cells in ciliate clone 97KM 3–24 had the characteristics described above for clones 94AB 4–7 and 94AB 8–3 (Figs 3, 5). However they had some differences. There were homogeneous spherical structures of moderate electron density in the cytoplasm of some bacteria (Fig. 5). These structures had the diameter about 80–100 nm. Probably these elements correspond to refractive structures, which occurred in some cells and were visible at the level of light microscope.

On the surface of the cells small membrane vesicles (blebbs) are recognized (Fig. 3).

The cytoplasm of elongated and curved cells was less electron-dense and more homogeneous (Fig. 5). In the same cells in the cytoplasm in close proximity to the plasma membrane electron-lucent area appeared (Fig. 5). In some bacteria both inside and outside the cell the vesicles of various size could be seen. Finally, the separate fragments, as well as the vesicles, were found in the area between normal symbionts. It is possible that the described structures appeared as the result of cell destruction (Fig. 5).

For determination of symbiont systematic position in macrosystem of Prokaryota the symbionts of ciliate strains, 97KM 3–24, 94AB 4–7 and 94AB 8–3, were used. All these bacteria had a positive signal with fluorescent probes specific for Eubacteria (positive control) and a-Proteobacteria (Fig. 1b). However, the signal was absent with probes for b-Proteobacteria and g-Proteobacteria (negative control).

The sequence used as a probe for a-Proteobacteria, can be found in 16S RNA of all a-Proteobacteria, and also a few representatives of s-Proteobacteria and spirochaetes (Stahl and Amann, 1991; Amann et al., 1995).

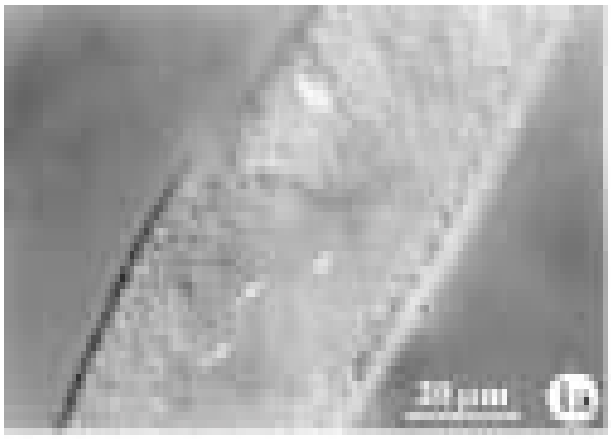
Fig. 1. a – Part of *P. caudatum* cell infected with symbiotic bacteria, strain 94 AB4–7 (arrows – groups of symbiotic bacteria in Ma); ➢ **b** – the same *Paramecium* cell: symbiotic bacteria visualised with the oligonucleotide probe specific for a–Proteobacteria.

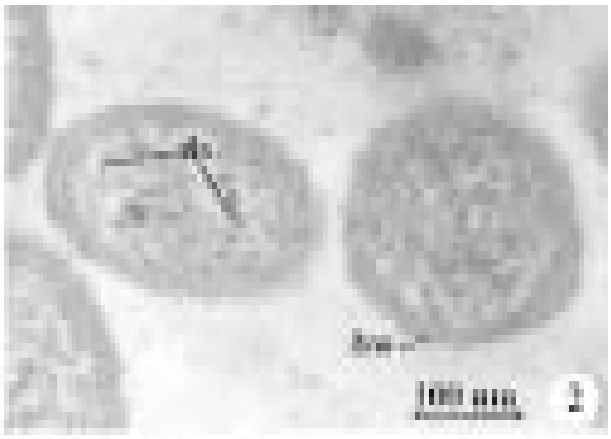
Fig. 2. Ultrastructure of symbiotic bacterium of the strain 94AB 8–3; bw – bacteria wall; ds – electron dense strands.

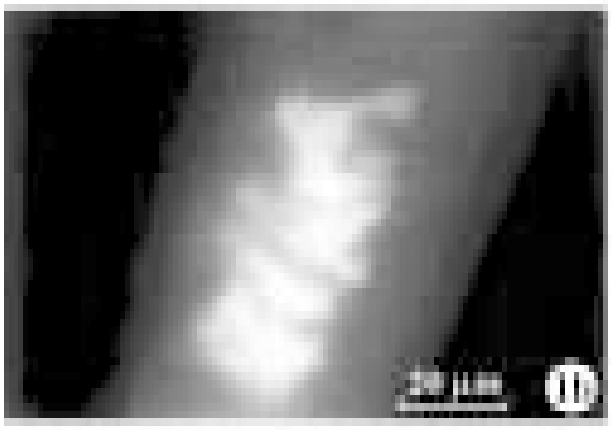
Fig. 3. Ultrastructure of symbiotic bacterium of the strain 97KM 3–24; mv – membrane vesicles, for other explanations see Fig. 2.

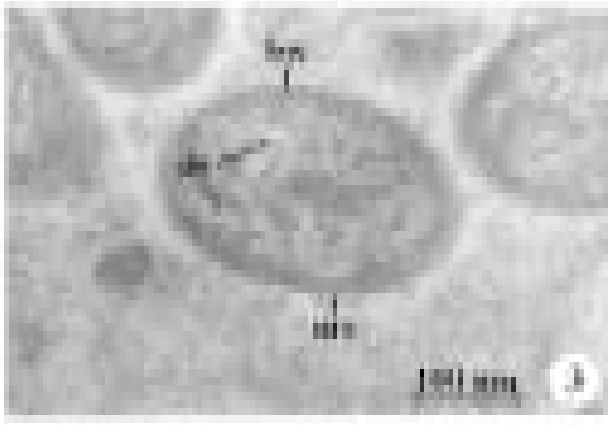
Fig. 4. Ultrastructure of the Ma *P. caudatum* infected with symbiotic bacteria, strain 94 AB 8–3; cb – chromatine body; n – nucleolus; sb – symbiotic bacterium.

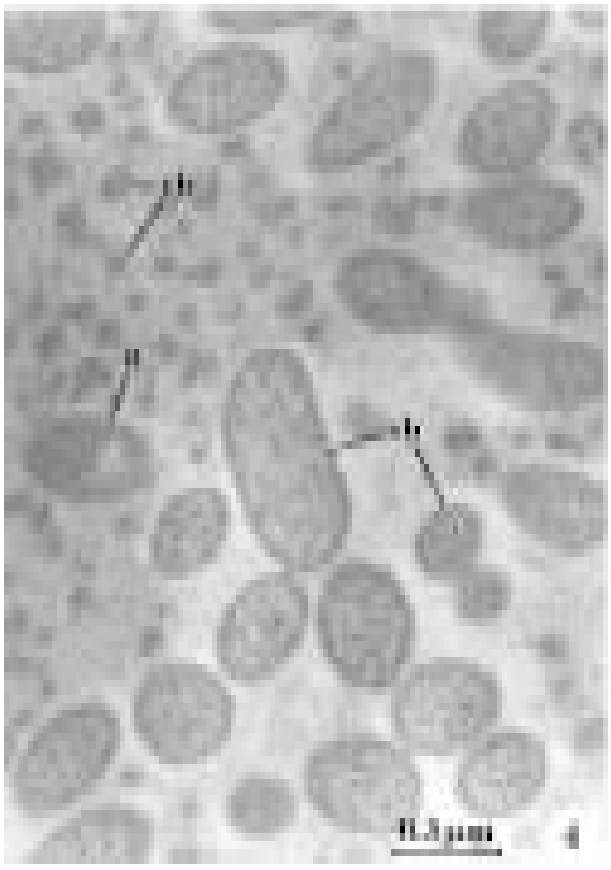
Fig. 5. Ultrastructure of the Ma *P. caudatum* infected with symbiotic bacteria, strain 97 KM 3–24; ip – invagination of plasm membrane; ss – spherical structure, for other explanations see Figs 3 and 4.

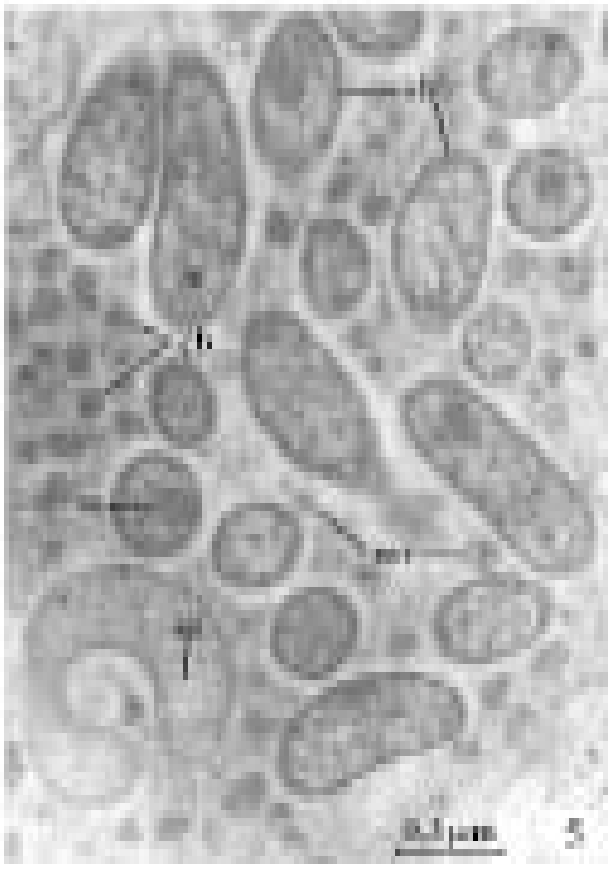












Discussion

Intranuclear symbionts isolated in two very distant natural populations, in North America and Eastern Europe, were shown to have many common features. Symbionts from both locations are rod-shaped gram-negative bacteria with similar size; they occupy the Ma and often form dense aggregations; inside the aggregation chromatin bodies are less dense and nucleoli are absent, while outside the group of symbionts the structure of Ma doesn't differ from that of the uninfected Ma. As it was shown by *in situ* hybridization with group-specific oligonucleotides, both symbionts belong to a-Proteobacteria. All these properties are nearly the same as those described previously for *Nonospora macronucleata* (Fokin et al., 1987) and ms-2 (Görtz, 1980) According to our data, *N. macronucleata* is also a representative of a-Proteobacteria (Vishnyakov and Rautian, 1999). Nevertheless, there are some differences between symbionts from two populations as well as between both of them and *N. macronucleata*. Some bacteria cells from 97KM3–23 and 97KM3–24 possess refractive structure. The proportion of the cells with this structure could change during the life span of the host strain. Thus, this may be a reaction of the symbionts to various external and/or internal factors. As it was seen with electron microscope, these structures don't have ultrastructure features characteristic for so-called R-bodies of *Caedibacter* (Preer et al.,1966; Pond et al., 1989). There is some correlation between the presence of these structures and different variants of the cell shape and obvious various stages of symbiont destruction: they were more frequent in elongated cells, which were more often subject to lysis. Alternative interpretation of these findings is also possible. The membrane vesicles which were seen on the surface of symbiotic bacteria with that electron dense body, might be a morphological manifestation of secretion process. This was shown for many gram-negative bacteria (Beveridge, 1999). This interpretation is attractive because such secretion might be a form of host-symbiont communication; obviously, more information is necessary to support his presumption. Anyway, the presence of refractive structures inside the cells as well as membrane vesicles and fragmented (distracted?) bacterial cells in infected nuclei were never observed in American populations. Comparison with *N. macronucleata* also reveals some differences. For example, *N. macronucleata* forms chains (Fokin et al., 1987), while we never observed them in symbionts studied here.

To sum up, at the present state it is impossible to say, whether *N. macronucleata* is a worldwide distributed variable symbiont or whether there are several different bacteria with more or less similar morphology caused by the same host intranuclear habitat.

An attribution of both symbionts to a-Proteobacteria raised up another interesting aspect of intranuclear symbiosis. At present the systematic position of several intranuclear bacterial symbionts is known. For *Caedibacter caryophila* and *Holospora obtusa* the complete sequencing of 16S and 23S rRNAs had been done (Amann et al.,1991; Springer et al., 1993) and they were found to be members of a-Proteobacteria. Using the same approach as we applied here, another 6 *Holospora* species, namely *H. undulata*, *H. elegance*, *H. acuminata*, *H. recta*, *H. curviuscula* and *H. caryophila* (Fokin et al., 1996; Vishnyakov and Rautian, 1999) were attributed to a-Proteobacteria. Another recently described bacteria inhabiting the Ma of *P. multimicronucleatum* (Vishnyakov and Rodionova, 1999) was also found to be a-Proteobacteria. Finally, *N. macronucleata* and symbionts described in this study also belong to this subdivision. It should be stressed that there is no any other intranuclear symbiont of Ciliates with known systematic position which would be a representative of another bacterial group. Whether it is a coincidence or representatives of this group have properties promoting formation of intranuclear relationships, further research can show.

a-Proteobacteria include a lot of intracellular bacteria, either symbionts or pathogens, inhabiting plant or animal cells. Also mitochondria, according to 16S rRNA sequencing and properties of many other genes, have a common phylogenetic root with other a-Proteobacteria (Woese, 1987; Lang et al., 1999). Recently very similar gene complexes that are necessary for penetration and establishment of relationships with eukaryotic cell were identified in plant symbiont *Rhizobium meliloti* and in animal intracellular pathogen *Brucella abortus* (Sola-Landa, et al., 1998; LeVier et al., 2000). Numerous intracellular bacteria are well known among all divisions of Proteobacteria. For well studied Enterobacteria (belonging to g-Proteobacteria) it was shown that the ability to penetrate host cell and thus become intracellular pathogen depends on a set of genes which are incorporated into the genome as a single block. These blocks were named "pathogenicity islands" and, because of different from the rest of the genome G+C content, they are believed to have been gained by lateral transfer not long ago (Hensel, 2000). In contrast to Enterobacteriacea, intracellular a-Proteobacteria have the same G+C content in "symbiotic genes" and in the other parts of genome. This allowed Moreno (1998) to proposed, that, perhaps, in a-Proteobacteria since the origination of this subdivision (that is to say, long ago), the general secretary pathway III was used to establish symbiotic relations with eukaryotic cells. As an ancient system, this may have evolved in different ways and, probably, lead to establishment of intranuclear symbiosis. Accumulation of new data on intranuclear symbionts and their genes, involved in the control of symbiotic relations, will show whether these speculations are close to reality.

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