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Mironov Timofey Ivanovich

NEW MODEL SYMBIOTIC SYSTEM CILLIATE *PARAMECIUM MULTIMICRONUCLEATUM/ CA.* TRICHORICKETTSIA MOBILIS.

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Supervisor:
Associate Professor, Ph.D.
Sabaneyeva E. V.

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Abbreviations list

CLSM— confocal laser scanning microscope;

EB — elementary bodies;

FISH— fluorescence in situ hybridization;

FLIP7— fly larvae immune peptide;

HLB— holospora-like bacteria;

Ma— somatic macronucleus;

Mi— generative micronucleus;

MIC— minimal inhibitory concentration;

PHA— polyhydroxyalcanoate;

RB — reticular body;

RER — rough endoplasmic reticulum;

RLB— ickettsia-like bacteria;

RLO— rickettsia-like organisms;

SFG— spotted fever group;

TEM— transmission electron microscopy;

TG— typhus group;

1. Introduction

Relevance of the study

Symbiosis is a widespread phenomenon of prolonged living together of non-related organisms. At present, a great number of symbiotic associations involving practically all groups of living organisms has been described. In the modern sense of the term, symbiosis refers to the coexistence of organisms belonging to different species regardless of whether they have a positive or negative effect on each other, i.e., in the meaning originally proposed by Anton de Bary (de Bary, 1879, cit. ex Daida et al., 1996). Among the biodiversity of symbiotic associations, endosymbioses of protists occupy special position. Their frequent occurrence in nature is connected, first of all, with the ability of many protists to feed by phagocytosis (Schweikert et al., 2013). Ingested microorganisms can avoid lysosomal degradation in the host cell and become endosymbionts.

Symbioses, in which a host cell is a protist, are of great interest to the researchers in the field of microbial ecology and in terms of evolutionary biology. Recently, the holobiont concept proposed by Lynn Margulis (Margulis, 1991), according to which an organism with its inherited inhabitant is considered as a whole entity, has been widely discussed in the literature. In modern interpretation of this concept, a holobiont possesses a hologenome, i.e., a summarized genome of a microorganism and all associated microorganisms. The holobiont is prone to natural selection and undergoes evolutionary changes (Zilber-Rosenberg, Rosenberg, 2008; Bordenstein, Theis, 2015; Theis et al., 2016). Investigations of symbiotic systems, in which a unicellular eukaryote is a host microorganism, i.e., the interactions between the partners take place at the cellular level of organization, can provide a good basis for further elaboration of the holobiont concept. Obviously, in the studies of such models an important factor is their stability, that is resistance to various different external factors, including antibiotics and antimicrobial peptide complexes.

Application of the holobiont concept to the studies of sybioses in protists can also lead to a new approach in the taxonomy of these organisms, figuratively coined “next generation taxonomy”, in which the endosymbionts of the protist and the host features associated with them are regarded as important taxonomic features (Serra et al., 2020).

Another important aspect of the studies of endosymbiosis in protists is revealing fine mechanisms of the partner interactions at the cellular level of organization. Analysis of these mechanisms can elucidate some poorly studied functions of the eukaryotic cell. Besides that, lately, the studies of symbiotic systems in protists gained special relevance due to a growing number of publications on findings of microorganisms identical or related to intracellular pathogens of humans and animals in different protists (Molmeret et al., 2005; Ferrantini et al., 2009; Schrallhammer et al.,

2013). Thus, *Acanthamoeba* appeared to be able to carry in its cytoplasm dangerous causative agents of pneumonia in humans - *Legionella* or *Mycobacterium*, while ciliates often host rickettsia-like bacteria (RLB) (Ferrantini et al., 2009; Schrallhammer et al., 2013). On these grounds, it has been suggested that protists can serve as a natural reservoir for microorganisms potentially pathogenic for humans (Molmeret et al., 2005; Fokin et al., 2014). Therefore, the search for new symbiotic systems formed by protists and other microorganisms and their investigation has not only purely theoretical, but practical implication as well.

Goal and objectives of the study

The goal of the study was to describe a new model symbiotic system formed by the ciliate *Paramecium multimicronucleatum* and the motile intranuclear bacterium and to assess the stability of this system under exposure to antibiotics and antimicrobial peptides.

The objectives of the study were

1. To characterize morphology and ultrastructure of the motile endosymbiont inhabiting the macronucleus of the ciliate *Paramecium multimicronucleatum.*
2. To determine the phylogenetic relationships and taxonomic position of the motile intranuclear symbiont of *P. multimicronucleatum*.
3. To assess stability of this symbiotic system formed by the ciliate *Paramecium multimicronucleatum* and the motile intranuclear bacterium exposed to antibiotics with different mode of action
4. To assess resistance/susceptibility of the symbiotic system to the antimicrobial peptide complex FLIP7 and its chromatographic fractions.

Principal findings to be considered

1. A new species of bacteria belonging to family *Rickettsiaceae* (*Rickettsiales*), *Ca*. Trichorickettsia mobilis, was described
2. Some representatives of *Rickettsiaceae* family can possess a well-developed flagellar apparatus
3. The symbiotic system P. multimicronucleatum/*Ca*. T. mobilis is resistant to administration of antibiotics and antimicrobial peptide complex FLIP7, stability of the symbiotic system being associated with the production of bacterial persisting forms
4. Due to its stability the symbiotic system P. multimicronucleatum/*Ca*. T. mobilis represents a convenient model for further elaboration of the holobiont concept.

Scientific novelty of the study

For the first time a new species of intranuclear symbiotic bacteria, *Ca*. Trichorickettsia mobilis, inhabiting the macronucleus of the ciliate *Paramecium multimicronucleatum*, was described using morphological and molecular techniques. This species was found to belong to the family *Rickettsiaceae* (*Rickettsiales*). For the first time the presence of flagella was demonstrated in a representative of *Rickettsiaceae.* For the first time the symbiotic system *P. multimicronucleatum*/ *Ca*. T. mobilis was shown to be highly resistant to a number of antibiotics, including those used for treating rickettsioses in humans. Also, for the first time, high resistance of the symbiotic system *P. multimicronucleatum*/*Ca*. T. mobilis to the antimicrobial peptide complex FLIP7 and its fractions was demonstrated. The symbiotic system *P. multimicronucleatum*/*Ca*. T. mobilis was first proposed to be regarded as a model to further develop the holobiont concept.

Theoretical and practical implications of the study

The revealed morphological peculiarities of the species *Ca*. Trichorickettsia mobilis (the presence of the flagellar apparatus) call for amendment of the diagnosis not only of the family *Rickettsiaceae*, but of the whole order *Rickettsiales*. Besides, the question arises concerning the need to reconsider possible paths of evolution of the bacteria belonging to this order. The data providing evidence for the high stability of the symbiotic system *P. multimicronucleatum*/*Ca*. T. mobilis obtained in the study make it possible to consider this symbiotic system as a convenient model for further development of the holobiont concept.

Since the work with pathogenic microorganisms is limited by substantial technical and organizational requirements, further studies of this symbiotic system have important practical implications, because close relationship of *Ca*. T. mobilis and rickettsiae pathogenic to humans makes it possible to use this symbiotic system as a model for studying fine mechanisms of rickettsia-eukaryotic cell interaction.

The results of the work can be used in the lecture courses on cell biology, symbiosis and microbial ecology for students in biology at Higher Educational Institutions.

Personal contribution of the author

The results presented in the thesis were obtained by the author personally. Sequencing and phylogenetic analysis were carried out in collaboration with the colleagues from the University of Pisa (Italy) (Vannini,…, Mironov, et al., 2014). Antimicrobial peptide complex FLIP7 and its

chromatographic fractions were obtained by Andrey Yurievich Yakovlev (Laboratory of Biopharmacology and Insect Immunology, SPbU). The results of the work were discussed and published together with the co-authors and the scientific supervisor.

Approbation of the results of the study

The results of the study were presented at the scientific conferences: 17th International Puschino Conference for Young Scientists (Puschino, 2013), VI All-Russian Congress of young scientists in biology “Symbiosis-Russia” (Irkutsk, 2013), 17th All-Russia Congress “Structure and Functions of the Cell Nucleus” (Saint-Petersburg, 2014), “7th Youth school-conference on Molecular and Cell Biology” Institute of Cytology, RAS (Saint-Petersburg, 2020).

Publications

10 contributions based on the materials of the thesis were published: 3 scientific articles in journals indexed by WoS/Scopus, all of them ranking Q1 (SJR), and 7 publications in the Materials of International and All-Russia Conferences.

Scientific articles in journals:

1) Vannini C., Boscaro V., Ferrantini F., Benken K., **Mironov T**., Schweikert M., Görtz H., Fokin S., Sabaneyeva E., Petroni G., 2014. Flagellar movement in two bacteria of the family *Rickettsiaceae*: a re-evaluation of motility in an evolutionary perspective. PLoS One. 9(2), e87718.

2) **Mironov T**., Sabaneyeva E., 2020. A robust symbiotic relationship between the ciliate *Paramecium multimicronucleatum* and the bacterium *Ca*. Trichorickettsia mobilis. Frontiers in Microbiology.11, 603335.

3) **Mironov T**., Yakovlev A., Sabaneyeva E., 2022. Together forever: Inseparable partners of the symbiotic system *Paramecium multimicronucleatum*/*Ca*. Trichorickettsia mobilis. Symbiosis. 1-12.

Abstracts of the presentations:

1) **Mironov T.**, Yashchenko V., Benken K., Fokin S., Schweikert M., Sabaneeva E., 2013. A new three-component symbiotic system (protist-bacteria-virus) in Paramecium multimicronucleatum ciliate cell. VI Russian Congress of young biologists with international participation "Symbiosis-Russia 2013", Russia, Irkutsk. 98-99. (in Russian)

2)**Mironov T.**, Benken K., Sabaneeva E., 2014. Violation of the spatial organization of the macronucleus of the ciliate Paramecium multimicronucleatum infected with the motile endonucleobiont Trichorickettsia mobilis, Russia. Cytology. 56, 672. (in Russian)

3) **Mironov T.**, Yakovlev A., Chernysh S., Sabaneeva E., 2020. Stability of the symbiotic system of the ciliate Paramecium multimicronucleatum/Ca bacterium. Trichorickettsia mobilis when exposed to antibacterial agents. Genes and Cells. 15(3), 193-194.(in Russian)

4) **Mironov T**., Benken K., Boscaro V., Fokin S., Schweikert M., Sabaneyeva E., 2013. Rickettsia-like motile intranuclear endobionts of the ciliate *Paramecium multimicronucleatum*. The 17th international pushchino school conference of young scientists «Biology – the science of the XXI century», Russia, Pushchino. 57-58.

5)**Mironov T**., Yashenko V., Benken K., Boscaro V., Fokin S., Schweikert M., Sabaneyeva E., 2013. A triple symbiotic system in *Paramecium multimicronucleatum.* Ciliates as model system to study genome evolution, mechanisms and non-Mendelian inheritance and environmental adaptation, Estonia, Tallin. 61.

6) Sabaneyeva E., **Mironov T**., Szkoli F., Castelli M., Pasqualetti C., Kaltz O., Petroni G., 2014. Sensitivity to antibiotic in *Trichorikettsia mobilis.* Frontiers in ciliate genome evolution adaptation, and symbiosis, Italy, Piza. 10-11.

7) Sabaneyeva E., **Mironov T**., Vannini C., Boscaro V., Ferrantini F., Benken K., Schweikert M., Goertz H., Fokin S., Petroni G., 2015. *Paramecium* and its motole endosymbionts: for better and for worse. VII European congress of protistology, Spain. 189.

2. Literature review

2.1 Symbiosis and classification of symbioses

Symbiosis is one of the most contradictory and widely discussed phenomena. The concept of symbiosis was proposed as early as in the XIX century by Anton de Bary (1831-1888) as a general term to designate coexistence of dissimilar organisms without specifying the character of relationship between the partners in the system – mutualism, commensalism or parasitism (after Oulhen et al., 2016).

At present, the term symbiosis is used in this, the broadest sense of the word and reflects the insufficient study of the relationship between the host and the endosymbiont as well as the possibility of transition from one type of relationship to another. For example, in symbiotic association between the ciliate *Paramecium biaurelia* and the bacterium *Holospora caryophila* at the stage of logarithmic growth of the culture the relationship between the partners is mutualistic, while in the stationary phase of ciliate growth bacteria switch to parasitism, bacteria of different strains of the same species being able to produce different effects on the host cell **(**Bella et al., 2016). Moreover, although bacteria belonging to the genus *Holospora* are energy parasites (they consume host's ATP) (Görtz, Schmidt, 2005), under some conditions, such as osmotic stress (Duncan et al., 2010) or temperature changes (Fujishima et al., 2005), their presence promotes viability of the host ciliate. Besides that, stability of the symbiotic system depends on external factors to a great extent. Thus, during cultivation of the ciliate *P. bursaria* carrying symbiotic *Chlorella* in its cytoplasm in the dark, the symbionts are gradually digested in the host cell food vacuoles (Kodama, Fujishima, 2014).

Since it is often not possible to determine the type of relationship between the host and the symbiont, the most popular is classification distinguishing symbionts by their localization in the host. This classification enables sorting still poorly studied symbiotic associations into one of the three groups (Nardon, Charles, 2001): ectosymbionts, endosymbionts and endocytobionts.

I. Ectosymbiosis. Both partners of the symbiotic system interact only by their surfaces. The smaller organism is called the ectosymbiont. It never invades the host. This type of symbiosis occurs between multicellular organisms, e.g., between the colonies of annelids *Branchiobdellidans* and fresh water crustaceans (Williams et al, 2013), as well as between the unicellular organisms, like in the case of association between the ciliate *Paramecium primaurelia* and the bacterium *Ca.* Deianiraea vastatrix (Castelli et al, 2019).

II. Endosymbiosis. The inner space of the host serves as an environment for the symbiont, which is called "endosymbiont". Such an interaction usually does not lead to penetration of the symbiont into the host cells. Most often the endosymbiotic associations are found in the digestive tract, internal body

cavities, and sometimes in the inner organs (Taylor et al, 2007; Donaldson et al, 2017). The classical example of such symbiosis is the association of wood-feeding termites with flagellates belonging to the orders Hypermastigida and Oxymonadida (Stingl et al., 2005).
III. Endocytobiosis. Most part of its life cycle the symbiont is localized inside the host cell, either in a vesicle formed by the host cell membrane (vacuole) or just freely lie "naked" in the cytosol. Vacuoles of the host occupied by symbionts are called symbiontosomes. In Russian literature the term "symbiontophorous vacuole" is traditionally used. In multicellular organisms the cells containing endocytobionts are called bacteriocytes (Szklarzewicz, Michalik, 2017) or mycetocytes (Douglas, 1989). For example, the symbiotic bacteria of aphids, *Buchnera aphidiclola*reside in bacteriocytes (Braendle et al., 2003). Such cells often form special structures, bacteriomes.

Symbioses occur in all biocenoses, and representatives of all kingdoms of living organisms can form symbiotic associations.

2.2 History of the studies of symbiosis

Ideas of Anton de Bary were further developed in the studies by Andrei Sergeevich Famintsin (1835-1918), who had worked for some time in Frieburg under the supervision of de Bary. In his work Famintsin suggested the symbiotic origin of chloroplasts (Famintsin, 1912).

A significant contribution to the studies of symbiotic systems was made by Russian scientist Konstantin Sergeevich Merezhkovsky (1855-1921). His studies were aimed at investigation of the ways of speciation and elaboration of systematics of living organisms. According to Merezhkovsky, symbiosis is the central mechanism of formation of large phylogenetic groups (Merezhkovsky, 1909). According to his concept, all life forms were formed from two independent lines – a mycoplasm and an amoeboplasm. The mycoplasm appeared earlier and gave origin to cyanobacteria and plastids. The amoeboplasm appeared later, when autotrophs have already formed the substrate for feeding heterotrophs. In modern understanding, the mycoplasm corresponds to prokaryotes, and amoeboplasm – to eukaryotes. At the same time all modern species diversity is a result of symbiosis of a Monera (a mesophyll heterotroph lacking nucleus) with bacteria. The second round of symbiosis, according to Merezhkovsky, is connected with invasion of phototrophs into the cells of some nucleus possessing organisms, which led to the appearance of plants. As for fungi, Merezhkovsky joined them with bacteria in a "mycoid kingdom" basing on similarity of their physiology and structure: extreme viability in unfavorable conditions and the presence of high content of nitrogen in their cell wall (Fig. 1). The presence of the nucleus Merezhkovsky considered a secondary feature (Merezhkovsky, 1909; Kowallik, Martin, 2021). The works by Merezhkovsky formed the grounds for the theory of symbiogenesis. As early as in 11 years after the work of Merezhkovsky, another Russian investigator,

Boris Mikhailovich Kozo-Polyansky (1890-1957) formulated the symbiogenetic theory, according to which green photosynthetic subunits, originated as Cyanophyceae were ingested by larger cells and could form symbiotic relationships, which led to appearance of chloroplasts and mitochondria.

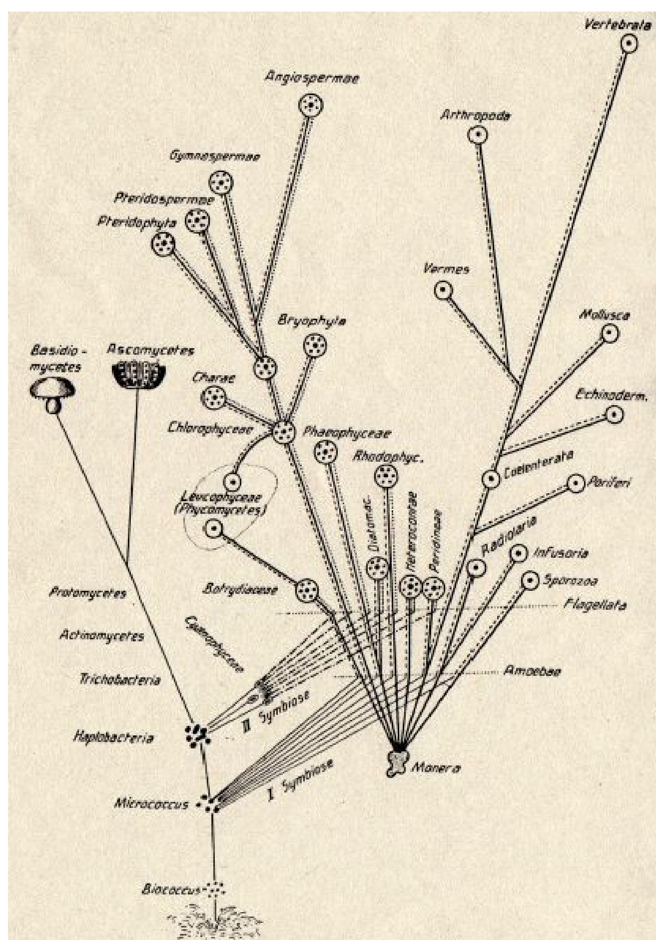


Fig. 1. Origin of eukaryotes according to K.S. Merezhkovsky (From: Merezhkovsky, 1909).

B.M. Kozo-Polyansky also stated, that symbiosis and evolution together led to rapid changes in the organisms, such as the appearance of plastids during just a few generations, and not during many generations as believed by many evolutionists (Kozo-Polyansky, 1924; Fet, 2021).

The ideas of symbogenesis were actively developed by the American researcher Lynn Margulis (1938-2011). Her work was aimed at investigation and systematization of the known symbiotic systems to understand the causes of their origin and their role in evolution. In her studies L. Margulis emphasized that the Earth Biosphere abounds in symbiotic associations of different organisms. In some cases, the symbiotic association will possess the features n ot characteristic of the partners taken separately. For instance, mycoriza between the desert plants and fungi. However, mechanisms causing coming together of the two organisms and interchange of the products of metabolism and genetic material remain unclear (Margulis, 1976).

In 1967 L. Margulis (Sagan) proposed a symbiogenetic theory of the origin of eukaryotes and organelles. According to this theory, the first eukaryotes were formed as a result of phagocytosis of aerobic phototrophs by heterotrophic prokaryotes. Importantly, according to Margulis, the eukaryotes appeared many times in evolution. Further symbiosis of eukaryotes with spirochaets led to the appearance of eukaryotic flagellum, and symbiosis with cyanobacteria resulted in the appearance of plants (Fig. 2) (Sagan, 1967).

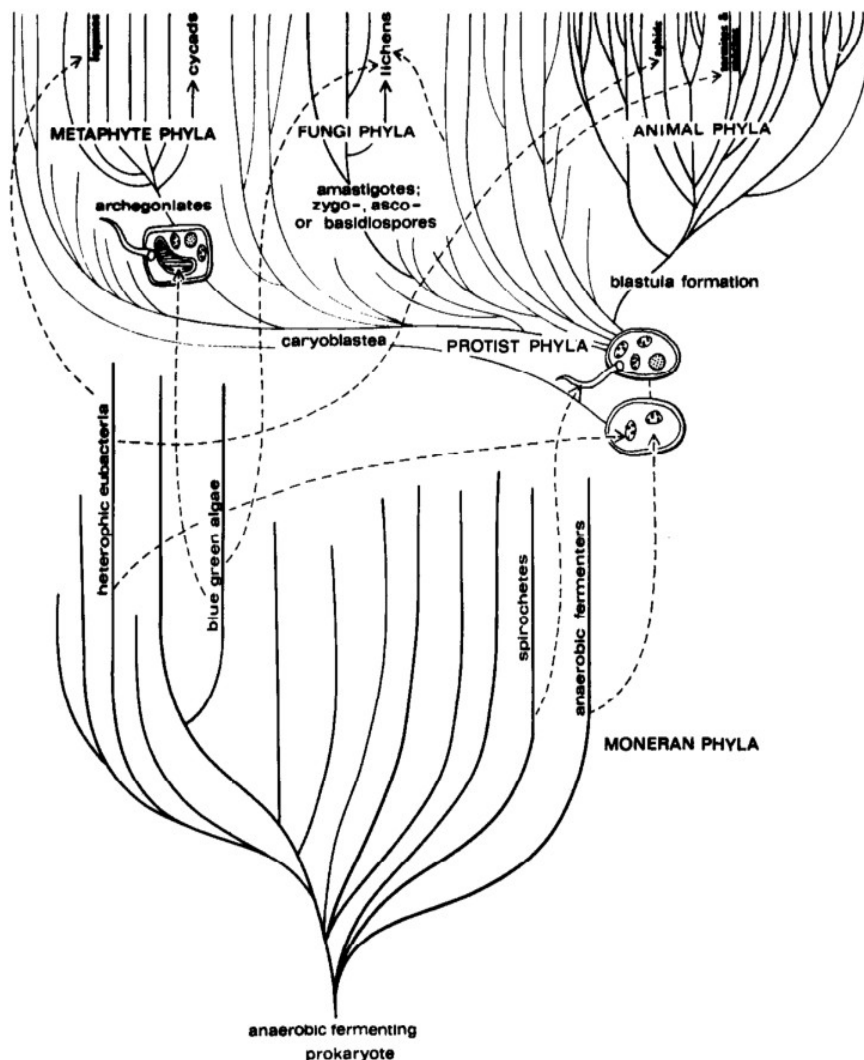


Fig. 2. Origin of eukaryotes according to L. Margulis (From: Margulis, 1976).

It should be noted that not all hypotheses of L. Margulis proved to be true. Thus, molecular methods failed to support the origin of the protist flagella from spirochaetes. Her another important statement concerning the multiple origin of eukaryotes has not been supported either. To prove this hypothesis, it was necessary to find organisms with some intermediate variants of symbiosis: eukaryotes with a bacterial flagellum, eukaryotes which have never possessed mitochondria, i.e., primarily anaerobic. However, by the present time, no such organisms have been found, which,

according to L. Margulis might be connected with their inaccessibility. Nevertheless, most investigators are inclined to think that modern eukaryotes had one common ancestor, LECA (last eukaryotic common ancestor) (Lane, 2011). Most likely, this ancestor appeared as a result of fusion of an anaerobic archaean and an alphaproteobacteria. This suggestion was justified when a novel large phylogenetic group Asgardarchaea comprising Lokiarchaea, Torarchaea, Odinarchaea and Cheimdallarchaea was found in deep geothermal vents. Basing on molecular data obtained by DNA analysis of Asgardarchaea, it was shown that they are close to eukaryotes, Cheimdallarchaea being the closest relatives (Zaremba-Niedzwiedzka et al, 2017). Since ribosomes, cytoskeleton and transport proteins in these archaeans are similar to those of eukaryotes, it was suggested that they are capable of phagocytosis and vesicular transport (Zaremba-Niedzwiedzka et al, 2017). However, observations of living cells in laboratory culture failed to reveal phagocytosis and vesicular traffic, but demonstrated the ability of Asgardarchaea to form various protrusions (Imachi et al., 2020). Thus, living cell observations called into question the phagocytic theory of the evolution of eukaryotes, but at the same time evidenced for the hypothesis of Kunin and his colleagues on the origin of eukaryotes by fusion of the archaean's protrusions around bacteria followed by isolation of the symbionts and their transformation into mitochondria (Yutin et al, 2009). This hypothesis was further developed by Baum and Baum, who proposed an explanation of the origin of other organelles of the eukaryotic cells, such as the nucleus, nuclear pore complexes, ER and Golgi complex basing on the fusion of the archaean's protrusions (Fig. 3) (Baum, Baum, 2014).

Extensive studies of symbiotic systems and recognition of their importance in ecology and evolution has led to further elaboration of a holobiont concept first proposed by Lynn Margulis (1991). In the original meaning, a holobiont is a host with its inherited endosymbiont. According to the modern understanding, any organism together with its microbiota should be regarded as a single entity possessing a hologenome, the summarized genome of the host and its microbiota (Zilber-Rosenberg, Rosenberg, 2008; Bordenstein, Theis, 2015; Theis et al., 2016). The holobiont is prone to natural selection and can be considered as an evolutionary unit (Rosenberg, Zilber-Rosenberg, 2018). The holobiont concept, though eagerly accepted by many scholars (Bosch, Miller, 2016; O'Malley, 2017; Simon et al., 2019), has been criticized on the part of the adepts of the ecological approach to the studies of symbiotic systems (Moran, Sloan, 2015; Douglas, Werren, 2016; Foster et al., 2017) and hotly debated (Morris, 2018; Baedke et al., 2020).

The main points of criticism concerned restrictions of the holobiont concept with regard to host fidelity and the way of symbiont transmission (Douglas, Werren, 2016). Indeed, it seems that the most important limitation of the holobiont concept is the stability of symbiotic relationship, since the system prone to easy changes of the partners could be hardly considered a single entity. Despite the continuous debate on the importance of selection at the holobiont level, at present, the holobiont concept is

considered a highly promising approach in evolutionary thinking (Morris 2018; Baedke et al. 2020). Accumulating data on microbial ecology and, in particular, on symbiotic associations formed by unicellular eukaryotic microorganisms and various bacteria could provide a comprehensive range of relatively simple model holobionts to be used in further research.

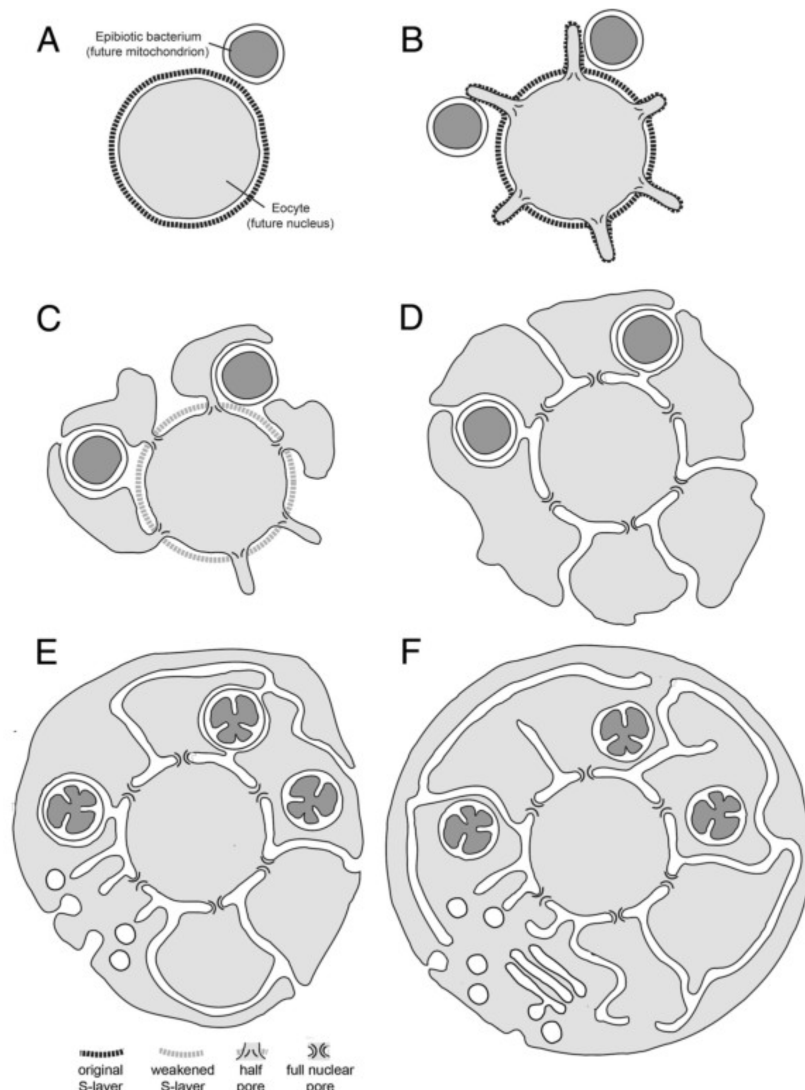


Fig. 3. Formation of a eukaryotic cell according to Baum and Baum (From: Baum, Baum, 2014).

2.3 Symbionts of protists

Heterotrophic way of feeding, which is intrinsic to many protists, increases probability of endosymbiont acquisition. Since in protists the cellular and the organismal level of organization coincide, endocytobionts of protists at the same time are the endosymbionts, and further we will use the latter term. Diversity of microorganisms inhabiting the protist cells is very high, and it encompasses both, pro- and eukaryotic organisms which are in principle capable of forming symbiotic associations(Reisser, 1985). The nature of the relationships between the protist and its

endosymbiont varies in a vast range from mutualism to parasitism. For example, in most cases the symbiosis between a protist with green algae is mutually beneficial (Foster, Zehr, 2019), while the endosymbionts from the orders *Rickettsiales* and *Holosporales* are considered as parasites (Floriano et al 2018; George et al, 2020; Husnik et al., 2021). The endosymbionts can occupy various cell compartments: cytosol, cell cortex, nucleus, perinuclear space, ER (Ossipov et al., 1996). Cytoplasmic endosymbionts can be localized directly in the host cell cytosol or can be enclosed by the host cell membrane and reside in a special vacuole, sometimes called symbiontophorous vacuole (Ossipov et al., 1996). The endosymbionts localized in the cytosol are protected from the host cell lysosome attack since there is no membrane which could fuse with the lysosome membrane (Fok, Allen 1998). The more so, intranuclear endosymbionts cannot be affected by the lysosomal hydrolases. As for the endosymbionts residing in the vacuoles of the host cell, the mechanisms preventing the fusion of lysosomes with the membrane of the symbiontophorous vacuole have not been studied yet. Basing on the analysis of a great amount of data, existence of two evolutionary strategies of interaction between an endosymbiont and its host-protist has been proposed. The first one is that microorganisms that invaded the protist are no longer recognized as alien objects by the host and in the more evolutionary advanced systems become additional cell organelles. The second strategy is that endosymbionts, to avoid the attack by the host's hydrolytic enzymes, occupy some safe compartment of the host cell, e.g., the nucleus **(**Ossipov et al., 1996).

The absence of host cell membranes enclosing the cytoplasmic bacteria may be indicative of close symbiotic interactions of the two organisms. Another evidence for the long-standing relationship between the host and its endosymbiont is its inability to grow independently from the host cell in the culture medium (Eschbach et al., 2009).

2.4 Endosymbionts of amoebae

Most free-living amoebae are heterotrophs, bacterial colonies, unicellular algae and fungi of ponds and lakes being their main source of energy (Leidy, 1878). Amoebae consume a great number of microorganisms, reducing their numbers by a factor of 1000 (Habte, Alexandr, 1978), therefore, they are rightfully considered to be effective natural regulators of the population of aquatic microorganisms. However, some free-living bacteria, fungi and algae can avoid digestion in the amoeba phagosomes and finally colonize the protist. Endosymbionts can survive and propagate inside the protist for a long time. Biodiversity of the endosymbionts of free-living amoeba (*Acanthameba sp*., *Hartmannella sp*.) is represented by the following groups: *Proteobacteria* (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria); *Actinobacteria; Firmicuies; Chlamydiae*;

Bacteroidetes; Ascomycota; *Chlorella*(Smirnov et al., 1995; Ossipov et al., 1997; Horn, Wagner, 2004).

Depending on the systematic group of the endosymbiont, their number in the host cell can vary significantly (Horn, Wagner, 2004). Thus, the number of endosymbiotic Alfa- and Betaproteobacteria per one protist usually is not too high and can range from 5 to 100 cells (Horn et al., 2002; Horn, Wagner, 2004), while bacteria from the phylum *Bacteroidetes* can completely fill not only the trophozoite, but also the cyst of the amoeba (Horn et al., 2001). Interestingly, the endosymbionts occupy different compartments of the *Acanthamoeba* cell: Alphaproteobacteria predominantly occupy the host cell cytosol, while the representatives of the phylum *Bacteroidetes* reside mostly in the host vacuoles (Horn et al., 2001; Horn et al., 2002). This fact evidences for the independent evolutionary adaptations to one and the same host in different phyla of bacterial endosymbionts (Horn, Wagner, 2004).

*Acanthamoeba,*besides being an opportunistic pathogen of humans itself, has been shown to play an important role in dissemination of bacterial diseases of humans (Heinz, et al., 2007; Król-Turmińska, Olender, 2017). That is why this amoeba, a vector of human pathogenic microorganisms, is often figuratively referred to as the “Trojan horse” (Horn, 2004). Contrary to rapidly changing environmental conditions, the amoeba provides the bacteria with a habitat with more stable conditions. Constantly maintained microaerobic environmental conditions inside the host cell allow endosymbiotic actinobacteria *Mobilincus curtisii*to survive unfavorable environmental conditions (Tomov, 1999). The endosymbiont is not only maintained inside the host, but actively propagates. This bacterium cannot survive in aerobic environment, therefore, when exposed to unfavorable conditions without their host, the endosymbionts lose the ability to reproduce and die. However, if *M. curtisii* gets inside the human organism, the pathogenic properties of this bacterium can manifest themselves, causing dysfunction of the urogenital system (Onderdonk et al, 2016).

Association between the facultative bacterial pathogen *Legionella pneumophila* and its host *Acanthamoeba sp.* is the most studied one. Once in the human body, *L. pneumophila* can cause pneumonia, however, the patient infected with *Legionella,* is not contagious. The amoebae can inhabit damp premises, vent holes, pipelines, bathrooms and hospital kitchens (Kwalk et al., 1998, Yoshida et al., 2018). *L. pneumophila* can infect humans by different ways, including the one using *Acanthamoeba sp.* as a vector, since many representatives of this genus can invade immunocompromised humans(Marciano-Cabral et al., 2003). If the infection is mediated by the amoeba, the patient is not contagious. An alternative option for *L. pneumophila* to enter the human respiratory system directly is related to the ability of the bacteria to lyse the host cell and spread by airborne droplets (Schuster, Visvesvara, 2004). Despite the bacterial ability to grow independently from the host, forming biofilms, propagation inside the host cell ensures better dissemination of *L.*

pneumophila (Horn, Wagner, 2004). Close and prolonged symbiosis between amoeba and bacterium led to coevolution of the partners, which is supported, in particular, by the horizontal gene transfer revealed with DNA analysis. In many of the studied symbiotic associations, eukaryotic genes have been found in the genome of *Legionella* (Gomez-Valero, Buchrieser, 2019).

Another pathogenic representative of the amoeba endosymbionts is *Mycobacterium avium*. This bacterium can survive inside the protist and even live between the layers of the host cyst wall. If it gets inside the human being, it can infect alveolar macrophages, which results in mycobacteriosis of the lung tissue (Steinert et al., 1998; Busatto et al., 2019). The bacterium cannot significantly increase its numbers inside the amoeba. This seems to be related either to the low division rate of *M. avium*, which is characteristic of all representatives of this genus, or to the low specificity of symbiotic interactions (this bacterium can live outside the host amoeba and, contrary to *Legionella pneumophila*, can infect humans directly, bypassing the protist (Yoshida et al., 2018).

Bacteria of the order *Chlamydiales* constitute a completely different group of the obligate endosymbionts of amoeba. These bacteria are known due to their pathogenicity to humans. For example, *Chlamydia pneumoniae* is a causative agent of 10% pneumonia, and it causes a great number of chronic diseases, such as asthma and cardio-vascular diseases (Horn, Wagner, 2004). This group of endosymbionts is characterized by a peculiar life cycle with two morphologically different forms. One of them is called a reticular body (RB). This bacterial form is metabolically active and is able to divide. RB can form inactive elementary bodies (EB). These are incapable of division, but can survive outside the host protist, reactivate and become RB, and, in fact serve as an infectious bacterial form (Greub, 2018).

2.5 Endosymbionts of flagellates

Other groups of Protista are also rich in endosymbionts. Thus, by the end of the last century not less than 69 flagellate species harboring endosymbiotic microorganisms have been found and investigated, among them 8 hosted endonucleobionts and 9 with endosymbionts in the perinuclear space (Ossipov et al., 1996). Flagellates can form endosymbiotic associations with various cyanobacteria, and many of them form special structures for its endosymbiont. For example, the flagellate *Citharestis apsteinii* forms on its surface special chambers with transparent walls and a narrow aperture, so that the endosymbionts could get inside (Ossipov et al., 1996).

All discovered endosymbionts of the flagellates have a Gram-negative morphotype, and the majority of them inhabits the host cytoplasm. Cytoplasmic endosymbionts can reside in a symbiontophorous vacuole or directly in the cytosol of the protist. On the contrary, endonucleobionts never occur in vacuoles. Interestingly, the endosymbionts residing in the perinuclear space are never

observed in rough endoplasmic reticulum (RER), although these organelles are a uniform compartment by its origin (Ossipov et al., 1996). An only exception to this rule has been found in a chrysomonad *Paraphysomonas vestita,* in which bacteria colonized both the perinuclear space and the RER (Tanichev, Karpov,1992; Ossipov et al., 1996).

All representatives of *Glaucophyceae* bear non-differentiated cyanobacteria in their cytoplasm. Due to the presence of the cell wall, pigment composition and low dependance on the host cell genome, these endosymbionts can be easily distinguished from the chloroplasts (Ossipov et al., 1996).

2.6 Endosymbionts of ciliates

Diversity of microorganisms inhabiting ciliates encompasses nearly all pro- and eukaryotic organisms, that can become symbionts: bacteria (Goertz, 2010; Sabaneyeva et al., 2018; Fokin et al., 2019), archaea (Bruggen et al., 1985; Wrede et al., 2012), unicellular algae (Kodama, Fujishima, 2005; Simek et al., 2016; Flemming et al., 2020), yeasts (Goertz, 1982; Summerer et al., 2007), microsporidia (Fokin et al., 2008; Yakovleva et al., 2020). The ciliate cell as an environment for diverse symbiotic microorganisms is characterized by a number of morphological peculiarities: stability of the internal environment, high reproduction activity, and the presence of various large compartments in which the endosymbiont can exist (Goertz, 2001). All representatives of ciliates are characterized by the presence of heteromorphous nuclear apparatus, i.e., the simultaneous presence of two nuclei, the somatic macronucleus (Ma) and the generative micronucleus (Mi), in their cytoplasm (Ossipov, 1981). The somatic nucleus is highly amplyploid (part of its genome is amplified), and its chromatin is decondensed. Ma is usually very large, and can have different shape. A positive correlation between the ciliate size and the size of its Ma has been observed (Ossipov, 1981). Diploid generative nucleus, Mi, is characterized be highly condensed chromatin, and the number of Mi per ciliate cell can reach several dozens. Normally, its size is small and varies from a few tenths of micron to 10 µm. In both nuclei the nuclear envelope is preserved in the course of division, which ensures maintenance of the endosymbionts in the nucleus, however, during sexual process (conjugation) the endosymbionts are often eliminated from the Ma. Infection of Mi makes the ciliate incapable of the sexual process (Ossipov, 1981).

As a rule, the endosymbionts colonize some specific compartment of the host cell, which is practically inaccessible for the bacteria non-adapted to symbiosis. For instance, food bacteria *Enterobacter sp.* can get inside the Ma together with the infectious forms of *Holospora obtusa*, but they cannot be maintained in the ciliate and are lost very soon (Fokin, Skovorodkin, 1991).

Affinity of the endosymbiont to a certain compartment may evidence for evolutionary advanced symbiotic relationship between the ciliate and its inhabitant (Goertz, Fokin, 2009).

Variations in the location of the bacterial endosymbiont is a very unusual event. Getting into another compartment of the ciliate may be only occasional and transitory, i.e., the endosymbiont is cleared from the host cell in several generations (Fokin, Skovorodkin, 1991). At the same time, alongside with the endosymbionts with strict host specificity to a certain compartment (one of the nuclei or the cytoplasm), there are endosymbionts lacking compartment specificity, which occur more rarely.

Let us consider in detail endosymbionts inhabiting the cytoplasm and nucleus of ciliates.

2.6.1 Cytoplasmic endosymbionts of ciliates

One of the most studied endosymbionts residing in the cytoplasm of ciliates are representatives of the genus *Caedibacter,* which manifest Gram-negative morphotype and belong to *Gammaproteobacteria*(Beier et al., 2002). Depending on the species (*C. taeniospiralis, C. pseudomutans, C. paraconjugatus*), the size of the endosymbiont can vary within the range of 0.4-1.9 µm (width) and 2-4 µm (length). Bacteria of the genus *Caedibacter* can colonize the ciliates of *P. aurelia* species complex (Goertz, Fokin, 2009). It should be noted that basing on molecular data, the species *C. caryophilus* inhabiting the Ma of *P. caudatum* and*C. varicaedens* from cytoplasm of *P. biaurelia* formerly assigned to the same genus, have been recently renamed and attributed to the genus *Caedimonas*belonging to *Alphaproteobacteria*(Schrallhammer et al., 2018).

All bacteria belonging to the genera *Caedibacter* and *Caedimonas* are non-motile. Their main peculiarity is the ability to produce the so-called R-body (refractile body), which got its name due to its refractive capacity, making them easily discernable with phase contrast light microscopy (Schrallhammer et al., 2018). These R-bodies turned out to be the cause of the so-called killer-effect, described by Sonneborn as early as in 1943 (Sonneborn, 1943). The killer-effect is based on the ability of the infected ciliates to release R-bodies in the medium. Once in the non-infected ciliate, the R-body is activated and causes death of the target cell (Pond et al., 1989).

R-bodies are rolled proteinaceous ribbons of 0.8 µm wide and more than 20 µm long. Viral capsids often occur at the end of this ribbon. It has been shown that part of the proteins of R-bodies are coded by the bacteriophage genome (Pond et al., 1989). The ribbons are tightly packed in the bacterial cell and remain rolled until they get activated (Kusch, Goertz, 2005). Once ingested and taken up in the food vacuole by a *Caedibacter*-free paramecium, this ribbon unrolls (activation of R-body) due to decrease in the pH level (values lower than 6.5) caused by the fusion of the food vacuole with acidosomes (Schrallhammer et al., 2012; Koehler et al., 2019). Activation of R-body leads to drastic changes in the target ciliate: formation of a large aboral lump, apparently hampering normal feeding of the ciliate; motility problems, visualized as rotation; formation of giant vacuoles, a sharp decline in cell response, eventually causing the death of the target cell κ (Pond et al., 1989; Koehler et al., 2019).

Experiments with recombinant *Escherichia coli* producing R-bodies supported the idea that the uncoiling ribbon of the R-body serves only as a releasing factor of some unknown toxin, since R-body bearing *E. coli* did not cause death of susceptible ciliate strain when ingested (Schrallhammer et al., 2012). Not all bacteria in the population have R-bodies, the percent of R-body carrying bacteria varies from 1% to 35%, rarely 50%, depending on the endosymbiont species and abundance of nutrition (Preer et al., 1974).

Among endosymbionts of ciliates, *Caedibacter* and *Caedimonas* are not the only bacterial genera possessing killer-effect. Bacteria of the genus *Lyticum* can colonize the cytoplasm of the same ciliate species as *Caedibacter* (*P. biaurelia, P. tetraurelia, P. octaurelia*). According to observations made by Preer, *Lyticum* has a toxin capable of lysis of the non-infected ciliate in 30 min at room temperature (Preer et al., 1974). However, the mechanism of toxicity is absolutely unclear. *Lyticum* is a rather large cell: width, 0.6-0.8 µm, length, 3-5 µm. These endosymbionts have numerous flagella all over the cell surface, however, they are incapable of active movement (Preer et al., 1974; Boscaro et al, 2013).

Archaeans are other comparatively well studied cytoplasmic endosymbionts of ciliates. Many of them are able to decompose organic matter under anaerobic conditions releasing methane (Fenchel, Finlay, 1991). Typical habitats for methanogenic archaeans are the digestive tract of vertebrates (Patra et al, 2017), anaerobic swamp cenoses (Boone, 2015) and bottom marine sediments (Milkov, 2004). *Methanobacterium formicicum* belongs to methanogenic archaeans capable of colonizing marine ciliates inhabiting anaerobic habitats. This endosymbiont is 2-7 µm long with a diameter of 0.4 µm, lacking flagella. Sometimes the endosymbionts form chains up to 6 cells long (Bruggen et al., 1985). It should be noted that *M. formicicum* has been found in the gut of most farm animals (Chellapandi et al., 2018). Methanogenic endosymbionts have been found in many ciliates: *Metopus striatus* (Bruggen et al., 1985), *Metopus palaeformis* (Embley, Finlay, 1994) и*Plagiopyla frontata* (Fenchel, Finlay, 1991). Methanogens help ciliates to survive in the absence of oxygen, providing some metabolic benefits, also (Fenchel, Finlay, 1991). Anaerobic ciliates lack mitochondria in their cytoplasm, which, apparently, have been lost in the course of evolution in anaerobic environment, and, instead, possess hydrogenosomes, producing carbon dioxide, acetate and hydrogen from pyruvate (Muller, 1972; Bruggen et al., 1985). In *Plagiopyla frontata*, there are about 5000 hydrogenosomes and 3500 endosymbiotic methanogens per cell (Fenchel, Finlay, 1991). Studies of the division cycle of methanogenic endosymbionts revealed that their division is directly connected to the division of the host cell. Hydrogenosomes divide mostly at the start of cell division, while methanogens synchronously double their numbers at the last stage of ciliate division, i.e., bacterial division is under control of the host cell division cycle (Fenchel, Finlay, 1991).

The ongoing recent search of novel endosymbiotic systems in ciliates resulted in discovery of many previously unknown prokaryotic endosymbionts inhabiting cytoplasm of different ciliates: *Ca.* Fokinia solitarium in *Parameciumspp.* (Szokoli et al., 2016), *Ca*. Bealeia paramacronuclealis in *P. biaurelia* (Szokoli et al, 2016), *Ca.* Megaira venefica in *P. bursaria*, *P. nephridiatum*, *P. putrinum* (Lanzoni et al., 2019), *Ca.* Mystax nordicus in P. *nephridiatum* (Korotaev et al., 2020), *Ca*. Pinguicoccus supinus in *Euplotes vanleeuwenhoeki* (Serra et al, 2020), to name a few.

Other equally interesting cytoplasmic endosymbionts of ciliates belong to the group of phototrophic eukaryotic organisms. The most studied is the symbiotic association of the ciliate *P. bursaria* and the green algae *Chlorella* (Kodama, Fujishima, 2009; Esteban et al., 2010). If the ciliates are maintained under light exposure, the algae located in the host cell cytoplasm is capable of photosynthesis. It should be noted that *Chlorella* obtains the carbon dioxide, which is necessary for photosynthesis, not only from the cultivation medium, but also from the host cell. Sugars and oxygen produced in the course of photosynthesis are released into the cytoplasm of the ciliate. In the dark the number of the endosymbionts is drastically reduced, since the *Chlorella* containing vacuoles fuse with the lysosomes, and their contents are digested. *P. bursaria* feeds not only at the expense of its endosymbionts, but is able to ingest and digest bacteria from the medium, as all ciliates, that is why it can successfully survive in the dark as well (Kodama, Fujishima, 2009; Esteban et al., 2010).

Viruses capable of infecting *Chlorella* have been revealed in many *P. bursaria* strains. Viruses do not invade the ciliate cell, but stay on its surface in the ciliary pockets in the area of the buccal cavity (Yamada et al., 2006). If the ciliate dies, *Chlorella* are released into the medium where they get infected by the viruses. In 24-48 h after the infection *Chlorella* dies, releasing viral capsids into the environment, where they can again associate with the surface of *P. bursaria* (Yashchenko et al., 2012). By using this strategy, after the death of each ciliate about 300000 viral particles are released, ensuring their high concentration in the medium (Van Etten et al., 1991).

2.6.2 Intranuclear endosymbionts of ciliates

Bacteria of the genus *Holospora* (*Holosporales)*

Endosymbionts of the genus *Holospora* are the most studied of all endonucleobionts of ciliates. They occur only in the ciliates of the genus Paramecium, which includes about 20 species. At present, more than 10 Holospora species are known; all of them belong to Alfaproteobacteria and do not have killer-effect. Holospora can colonize Ma or Mi of paramecia, filling it completely (Fokin, Goertz, 2009). This intranuclear endosymbiont is characterized by a two-phase life cycle. The physiologically active reproductive form of the bacterium is small (about 2-3 µm long and less than 1 µm wide) and is

incapable of infection. The infectious form is bigger (up to 25 μm, length and 1 μm, width) and can infect a new host if it gets inside. Neither of these forms can survive outside the host cell even in the balanced medium, i.e., the relationship is obligatory for the bacterium. However, the infectious forms of many *Holospora* species maintain their infectious capacity if they are cryoconserved for 6 months at -85 (Fujishima et al., 1991; Milot, Kaltz, 1996; Kawai, Fujishima, 1996, Fujishima et al., 2012). The endosymbionts of the*Holospora*genus are not only species-specific, but also nucleus-specific, residing either only in Ma, or in Mi (Fokin,1993; Fokin, Goertz, 2009). Thus, *H. undulata* and *H. obtusa* occur in *P. caudatum*, the first one in Mi, and the second one – in Ma, while *H. acuminata* and *H. curviuscula* are endosymbionts of *P. bursaria*, occupying Mi and Ma, correspondingly.

H. obtusa is a good model object for studying host-endosymbiont relationship, since, as all representatives of this genus it is highly infectious. The infectious form has to be phagocytosed by a ciliate to infect a new host cell. After getting into the buccal cavity of a ciliate, it gets into the food vacuole of the host cell. The infectious form manages to avoid the lysosome attack of the host cell due to its ability to escape from the food vacuole in response to acidification of the phagosome contents caused by its fusion with acidosomes (Fokin, Goertz, 2009). Then the infectious form becomes surrounded by flattened vesicles carrying ribosomes, which form a secondary transport vesicle. The bacterium can get inside Ma in the course of the so-called “sluicing” mechanism, i.e., fusion of the membranes surrounding the bacterium with Ma envelope (Ossipov, 1981; Goertz, 1989). In the Ma the infectious form starts dividing and producing reproductive forms, which later differentiate into the infectious forms. After division of the infected ciliate the infectious forms get into the residual body and later can be released via the cytoproct. Reproductive forms remain in the somatic nucleus, divide and can later differentiate into infectious forms capable of infecting new hosts (Goertz, 2001).

Phylogenetic analysis of the SSU rRNA gene of the endosymbionts belonging to the genus *Holospora* demonstrated that phylogenetically they are not too far from Rickettsiales, and at present they are regarded as a family *Holosporaceae* in the order *Holosporales*. Their relatedness to *Rickettsiales* or to *Rhodospirillales* is still debated (Castelliet al., 2019; Schrallhammer, Potekhin, 2020).

Holospora-like bacteria

In the last decade a group of intranuclear Holospora-like bacteria (HLB) has been proposed; this group includes *Ca*. Gorzia infectiva (Boscaro et al., 2013), *Ca*. Gorzia schahrazadis from the Ma of *Paramecium multimicronucleatum* (Serra et al., 2016), *Ca.* Gorzia yakutika inhabiting Ma of *P. putrinum* (Belyavskaya et al., 2020), *Ca*. Hafkinia simulans from the Ma of Frontonia salmastra (Fokin et al., 2019) and *Ca*. Preeria caryophila, previously known as *Holospora caryophila* (Potekhin et al.,

2018), which occurs in Ma of several representatives of *P. aurelia* species complex. They are similar to *Holospora* in the presence of two morphological forms in their life cycle, the presence of an infectious tip in the infectious form and in strictly intranuclear localization. At the same time, there are several features distinguishing them from *Holospora*: they lack strict host species specificity, but they are exclusively macronuclear endosymbionts and never occur in Mi. Interestingly, contrary to *Holospora*, HLB can be occasionally observed in the cytoplasm of the host cell, which might be an adaptation of the endosymbiont to conjugation and autogamy of the host cell permitting it to invade the newly formed macronuclear anlagen.

Other intranuclear endosymbionts

A very special case is *Ca*. Paraholospora nucleivisitans, which is only distantly related to *Holospora* and differs significantly from the latter in its biology. The most important difference is the absence of an infectious form, typical for bacteria of the genus *Holospora* (Eschbach et al., 2009) and thus, *Ca*. P. nucleivisitans is not infective. Therefore, at present this bacterium is not considered an HLB (Schrallhammer, Potekhin, 2020). This endosymbiont has been found in a tropical ciliate *P. sexaurelia* and it can colonize both, the cytoplasm and the Ma of the host cell (Eschbach et al., 2009), most often occurring either in the ciliate cytoplasm, or in its Ma. In the last case, only a few if any bacteria are left in the cytoplasm. The bacterium is 0.4-0.8 µm across and up to 25 µm long and is slightly curved. Like most endosymbionts of ciliates, it has a Gram-negative morphotype and is susceptible to kanamycin treatment (Eschbach et al., 2009). Contrary to *Caedibacter*, *P. nucleivisitans* has no killer-effect. *P. sexaurelia* undergoes autogamy, which results in the formation of a new Ma, but this process does not harm its endosymbiont. Contrary to many other bacteria which are sometimes eliminated from Ma in the course of autogamy, *Paraholospora* migrates into Ma before the start of the autogamy, and is released from it into the cytoplasm after the formation of the new Ma (Eschbach et al., 2009).

Another inhabitant of *P*. *caudatum* Ma is the bacterium *Nonospora macronucleata.* This endosymbiont, like most bacterial endosymbionts, has a Gram-negative morphotype. Most often these bacteria are revealed as small rods about 1 µm long and 0.2-0.3 µm across. Besides this form, long chains of dividing forms up to 10 µm long occur in the ciliate Ma, and, infrequently, bacteria about 5 µm long. The endonucleobiont does not have any flagella and an infectious form (Fokin et al., 1987). These bacteria are mainly localized in the central part of Ma, which is devoid of the condensed chromatin. The endosymbiont is preserved in the nucleus during conjugation. The infected cells do not demonstrate any killer effect, and when homogenized, the released bacteria are only slightly infectious (Fokin et al., 1987).

A unique case is a motile bacterium inhabiting Ma of the ciliate *P. multimicronucleatum*, described in the strains isolated from populations collected in USA and Moldova (Vishnyakov, Rodionova, 1999). This endosymbiont can move inside the Ma due to a great number of flagella, which can be up to 10 µm long and 20 nm across (Vishnyakov, Rodionova, 1999). The endosymbiont has two morphological forms – a rod-like form (length, 2-2.5 µm, width, 0.4-0.6 µm) and an ovoid form (length,1.3-1.8 µm, width, 0.4-0.6 µm). Inside Ma, they move at the speed of 10-15 µm/sec, and in the medium their speed encreaases up to 20 µm/sec. Sometimes nonmotile forms of this bacterium occur, which are about 2-2.5 µm long and 1.5-2 µm across. Addition of ampicillin in the medium leads to formation of septate forms and loss of the endosymbiont motility; sometimes filamentous forms up to 75-80 µm have been observed. The homogenate obtained from the infected ciliates is not infectious, however, experimental infection by microinjection of bacteria into Ma has been possible (Vishnyakov, Rodionova, 1999). Fluorescence in situ hybridization with the oligonucleotide probes specific to fragments of the 16S rRNA gene has shown that the intranuclear motile endosymbiont of the ciliate *P. multimicronucleatum* belongs to *Eubacteria*, but is not related to *Holospora* (Vishnyakov, Rodionova, 1999).

2.7 Rickettsia and Rickettsia-like bacteria

According to Bergey's Manual of Systematic Bacteriology, representatives of the order *Rickettsiales* belong to the class Alphaproteobacteria, have a Gram-negative morphotype and can propagate exclusively within eukaryotic cells (Dumler et al., 2015). Due to the intracellular life style of rickettsia the metabolic cooperation between these bacteria and the host is extremely strong. The life cycle of the majority of *Rickettsiales* can be divided in two stages. The bacterium spends most part of its life inside the host cell, where it replicates. During the extracellular stage, the bacteria have been believed to search for a new host cell and to be incapable of fission. However, recently an ectosymbiont of the ciliate *Paramecium primaurelia* has been found, which, according to phylogenetic analysis, belongs to *Rickettsiales* (Castelli et al., 2019). It has been shown that this bacterium, called *Ca.* Deianiraea vastatrix, remains associated with the surface of the ciliate and can divide outside the host cell. The bacterium has never been observed inside the host cell. Such surprising features of this bacterium, which have never been observed in representatives of *Rickettsiales* before, compelled the scholars to make significant adjustments in the description of the whole order and to distinguish within it the family *Ca.* Deianiraceae (Castelli et al, 2019).

The order *Rickettsiales* includes bacteria capable of causing severe human diseases, like *Rickettsia rickettsii*(the causative agent of Rocky Mountains spotted fever) и*Rickettsia prowazekii* (the causative agent of epidemic typhus) (Walker, Ismail, 2008). For a long time, the interest in this group

was focused on phylogeny and evolution of a small group of pathogenic bacteria belonging to the genus *Rickettsia.* Relatedness of mitochondria to rickettsia revealed later caused a surge of interest in the study of the phylogeny and evolution of these bacteria (Anderson et al., 1998). Subsequently, bacteria of the order *Rickettsiales* not belonging to the genus *Rickettsia* were named Rickettsia-like organisms (RLO). Most part of RLO are not connected with the blood-sucking arthropods parasitizing mammals, but colonize a wide range of aquatic organisms including protists (Schrallhammer et al., 2013; Castelli et al., 2016; Castelli et al., 2019).

At present, the order *Rickettsiales* comprises two well-studied families, *Rickettsiaceae* and *Anaplasmaceae*, and two recently described families *Ca*. Midichloriaceae (Montagna et al., 2013) and *Ca*. Deianiraeaceae (Castelli et al, 2019). The family *Holosporaceae,* which until recently has been considered as belonging to the order *Rickettsiales,* lately has been removed from *Rickettsiales* and is regarded as a sister order *Holosporales* (Potekhin, Schrallhammer, 2021). Since representatives of *Anaplasmaceae* infect only Metazoans, and have not been found in protists (Castelli et al., 2016; Castelli et al., 2019), we will not consider it here and will confine this review to a more detailed description of some representatives of the two other families, *Ca.* Midichloriaceae и*Rickettsiaceae.*

2.7.1 Family *Ca*. Midichloriaceae

The family *Ca*. Midichloriaceae has been recently distinguished as a separate family within the order *Rickettsiales.* The members of this family can colonize a very wide range of multicellular organisms: Placozoa (Driscoll et al., 2013), Cnidaria (Fraune et al., 2007), ticks (Epis et al., 2008), lice (Matsuura et al., 2012), bugs (Richard et al., 2008), gadflies (Hornok et al., 2008), as well as fishes (Cafiso et al., 2015) and mammals (Bazzocchi et al., 2013), including humans (Mediannikov et al., 2004). The representatives of the family *Ca*. Midichloriaceae have been also found in amoeba (Fritsche et al., 1999) and in ciliates (Boscaro et al., 2013; Szokoli et al., 2016). The first described member of this family, which got a formal description, was *Ca*. Midichloria mitochondrii found in the tissues of the tick *Ixodes ricinus,* in salivary glands and, even more, in oocytes. The species got its name due to its ability to infect mitochondria of the host cells (Sassera et al., 2006). The influence of this bacterium on mitochondria of the host cell still remains unclear. However, according to the newest data, obtained by means of mathematic modelling, the bacterium does not cause any adverse effect on the host mitochondria (Comandatore et al., 2021). Interestingly, in most cases the bacterial fission occurs outside the host mitochondria, and the probability of finding the endosymbiont inside mitochondria increases with the maturation of the tick oocyte (Comandatore et al., 2021). It should be noted, that contrary to the longstanding view prevailing in microbiology that all members of the order

Rickettsiales are devoid of flagella, the genome of this species has been found to contain 26 genes coding for different parts of the bacterial flagellar apparatus (Sassera et al., 2011).

Since in the course of investigations of the "red spot disease" in the rainbow trout a bacterium phylogenetically close to *Ca*. M. Mitochondrii has been found, and the disease was often concomitant with simultaneous infestation by a parasitic ciliate *Ichthyophthirius multifiliis,* it was suggested that the ciliate could serve as a vector to transfer the bacterium from one host to another (Zaila et al., 2017). The experiments demonstrated that a ciliate can be infected by this bacterium transferred from the infested fish at least transiently, and can transfer the infection to its progeny (Pasqualetti et al., 2021). The study of this disease is both of scientific and of practical interest, since it affects a significant part of trout in artificial reservoirs and tanks, and the high density of fishes increases significantly the spread of the disease (Howell et al., 2019).

The members of *Ca*. Midichloriaceae family hosted by protists are *Ca*. Bandiella (Senra et al., 2016), Ca. Jidaibacter acanthamoebae (Fritsche et al., 1999), *Ca*. Anadelfobacter (Vannini et al., 2010), *Ca*. Cyrtobacter (Vannini et al., 2010; Boscaro et al., 2013a), род*Lyticum* (Boscaro et al., 2013b), *Ca*. Defluviella procrastinata (Boscaro et al., 2013), *Ca*. Fokinia (Szokoli et al., 2016). Interestingly, of all aforementioned bacteria, the genus *Ca*. Bandiella holds a special position, because besides ciliates and Euglenozoa it can infect various metazoans, including sponges Longford et al., 2007), placozoans (Driscoll et al., 2013), and cnidarians (Fraune, Bosch, 2007). The only formally described species of this genus is *Ca*. Bandiella woodruffi, found in the cytoplasm of the ciliate *Euplotes woodruffi* (Senra et al., 2016). *Ca.* Jidaibacter acanthamoebae is an endosymbiont of the amoeba *Acanthamoeba keratitis,* which causes corneal keratitis in humans (Fritsche et al., 1999). Bacterial genome sequencing revealed 35 genes coding for the flagellar proteins in this bacterium, however, the flagella are absent. Since the genome in one of the strains appeared to be large enough (2.4 Mbp) and had many genes common with free-living Alfaproteobacteria, some researchers suggested that this species could be an intermediate link between the free-living and symbiotic Alfaproteobacteria (Schultz et al., 2016). The species of the genera *Ca*. Anadelfobacter and *Ca*. Cyrtobacter are associated with the species of the genus *Euplotes*, while the genera *Lyticum*, *Ca*. Defluviella и*Ca*. Fokinia are found in different *Paramecium* species. *Lyticum flagellatum* and *Lyticum sinuosum*, known since the 1970-s, have been recently redescribed using molecular techniques (Boscaro et al., 2013). It is noteworthy, that all representatives of the family *Ca*. Midichloriaceae are cytoplasmic endosymbionts and they never occur in the host nucleus.

The presence of such a wide host range in these bacteria allowed researchers to suggest the ability of bacteria of the family *Ca*. Midichloriaceae to be transmitted horizontally between different phylogenetic groups of living organisms. This hypothesis has not been proved for the members of *Ca*. Midichloriaceae as yet, however, there are literature data arguing in favor of this ability in a

representative of the family *Rickettsiaceae* close to midichloria (Schrallhammer et al., 2013, Modeo et. al., 2020).

2.7.2 Family *Rickettsiaceae*

For a long time, the family *Rickettsiaceae* comprised only the genera *Rickettsia* and *Orientia,* as well as the recently described genus *Ca*. Megaira (Schrallhammer et. al, 2013). The genera *Rickettsia* and *Orientia* are mostly represented by pathogens of mammals, including humans. Once in the host organism, the bacteria colonize and damage the host cells, which may cause different pathologies in the host in general. Horizontal transfer of pathogenic rickettsia occurs due to the bites of blood-sucking arthropods, such as ticks, lice and fleas. Representatives of these genera are causative agents of severe infectious diseases of humans, like Rocky Mountain Spotted Fever (*R. rickettsii*), epidemic typhus (*R. prowazekii*), Mediterranean spotted fever (*R. typhi*), tsutsugamushi fever (*O. tsutsugamushi*), etc. (Gillespie et al., 2007). At present, much is known about the natural sources, vectors and pathologies caused by rickettsia. However, unfortunately, other representatives of this family, which are non-pathogenic to humans, are poorly studied. At the same time, studying other genera belonging to *Rickettsiaceae* family could give a better insight into the mechanisms of interaction of rickettsia with eukaryotic cells.

Representatives of *Rickttsiaceae*, which are non-pathogenic for humans, have been found in the cells of multicellular organisms, such as arthropods (Gottlieb et al., 2012), cnidarians (Fraune, Bosch, 2007), leeches (Kikuchi Y., et al., 2002), in plant cells (Davis et al., 1998) and in protists (Vannini et al., 2004).

One of the first RLB forming symbioses with ciliates was *Ca*. Cryptoprodotis polytropus, found in the cytoplasm of the ciliate *Pseudomicrothorax dubius* (Ferrantini et al., 2009). This bacterium is not surrounded by host membrane, it lives and divides in the cytoplasm and is non-motile. The peculiarity of this symbiotic system is incomplete infection - only 90% of *P. dubius* are infected with it. In the author's opinion, partial infection of ciliates indicates that this bacterium is not essential for the protist and represents a highly infectious parasite (Ferrantini et al., 2009).

The genus *Ca*. Megaira has been described and included in Rickettsiales family only recently, in 2005. The first representative of this genus described in ciliates was *Diophrys oligothrix* (Vannini et al., 2005). Later representatives of this genus were found in other ciliate species, such as *Euplotes octocarinatus*, *Paramecium caudatum*, *P. primaurelia*, *P. bursaria*, *Spirostomum minus* (Schrallhammer et al, 2013; Lanzoni et al., 2019), and in the vampyrellid amoeba *Hyalodiscus flabellus* (Hess, 2017). Also, bacteria *Ca*. Megaira can form symbiotic associations with multicellular organisms, such as cnidarians (Fraune, Bosch, 2007), green algae (Hollants et al, 2013), ascidians

(Dishaw et al., 2014), fishes (Miyake et al., 2016) and annelids (Murakami et al., 2017). At present, the genus *Ca*. Megaira comprises two endosymbiont species, *Ca*. Megaira polyxenophila (Schrallhammer et al, 2013) and *Ca*. Megaira venefica (Lanzoni et al., 2019). The first one is found depending on the host species, both, in the cytoplasm (*P. primaurelia*) and in the macronucleus (*P. caudatum*), while the latter resides exclusively in the host cytoplasm (*P. bursaria*, *P. nephridiatum*, *P. putrinum*). Differences in in localization of *Ca*. M. polyxenophila can be related to the autogamy in *P. aurelia*, which makes impossible stable infection of Ma (Potekhin et al., 2018).

The bacterial size differs significantly even within one species. The length of *Ca*. M. polyxenophila inhabiting the cytoplasm of one *P. primaurelia* strain is three times the length of bacteria of the same species from other strains (Lanzoni et al., 2019). It should be mentioned, that the size variability within one species is generally typical for rickettsia, e.g., the length of *R. prowazekii* can vary from 4 to 10 µm (Anderson et al., 1965).

2.8 Modern lines of research on symbioses between protists and other microorganisms

Modern studies of symbiotic systems formed by protists and other microorganisms concern three main aspects of the problem of symbiosis: ecological, evolutionary and cytological (cellular biological). In other words, they are aimed at revealing the role of symbiotic systems in evolution, at investigation of ecological consequences of symbiotic association formation and at elucidating fine mechanisms of the partner interactions within a symbiotic system.

2.8.1 Evolutionary aspect of the problem of symbiosis between the protists and other microorganisms

Interaction of the organisms can serve as a powerful factor of mutual evolution of host and its symbiont, leading to formation of new taxonomic groups (López-García et al, 2017). In many ecosystems, symbioses are the basis for its functioning and evolutionary processes. Symbioses permit both, the host and its symbiont colonize new niches inaccessible for them separately. In some cases, the interconnection between the host and the symbiont becomes so close, that they can be considered as a single evolutionary unit, i.e., an archaeon and a bacterium forming a eukaryotic cell (Fisher et al., 2017).

The evolutionary process is connected with the deepness of cooperation between the symbiont and the host and depends on the benefits they get from interacting with each other. The symbiont-host interaction is realized at the morphological, physiological and genetic levels. The more metabolic and ecological links there are between the partners in the symbiotic association, the more dependent on each other are the partners (Estrela et al., 2016).

Symbiosis results in reduction of the bacterial genome. For example, bacteria *Desulfovibrio* occur both, as free-living forms and in symbiotic association with the protist *Trichonympha collaris*. The genome size of the ectosymbionts is 1.4-1.6 Mbp, while that of free-living forms is 2.9-3.7 Mbp (Takeuchi et al., 2020).

The main difficulty in the studies of the genome reduction processes is inability of most of the endosymbionts to grow outside the host cell. One of the few symbiotic associations permitting the investigators to assess the genome reduction of the endosymbiont caused by its shift to the symbiotic relationship is the association between the ciliate *Euplotes* and the bacterium *Polynucleobacter.* This symbiotic system has been known for a long time and has attracted attention of the researchers primarily due to the obligatory relationship between the partners (Heckmann, 1975). Loss of the endosymbiont leads to the stop of division and death of the ciliate. It is believed to be connected with the disorders in the carbohydrate metabolism in the ciliate, which is manifested in accumulation of glycogen granules in the cytoplasm (Vannini et al., 2007). The free-living form of *Polynucleobacter* found recently permitted the investigators to assess the genome reduction of this bacterium in the course of obligatory symbiosis. On average, the genome size of the free-living *Polynucleobacter* is more than 2000 Kbp, while the genome of the endosymbiotic form is about 1800 Kbp. The genome reduction is concomitant with the reduction of protein coding genes (Boscaro et al., 2017). Interestingly, the rate of genome reduction in many bacterial symbionts correlates with the frequency of mutations (Bourguignon et al., 2020).

Although the tendency of the protist symbiont genome to reduction is considered to be an established fact, it is unclear which processes cause genome reduction, how the reduction tells on genome structure and if there are any differences in the genome reduction in protists and in multicellular organisms. The loss of function of a particular gene in a symbiont is easy enough to explain: in the course of symbiotic interaction the symbiont loses the need to maintain some part of phenotypic features, which leads to attenuation of the selection pressure on these features. As a result, mutations in these genes are retained which leads to disfunction of the products of this gene (Boscaro et al., 2017). For example, the flagellar apparatus in symbiotic bacteria is often lost in the course of symbiosis (Husnik et al., 2021). Much more unexpected is the reduction of genes encoding the components of the energy metabolism of the symbiont, in particular, ATP/ADP translocase. According to a number of investigators, it is connected with the ability of the symbiont to obtain ATP directly from the host cell (Moran, Bennett; 2014; Mehari et al., 2016; George et al., 2020).

The sequence of the gene that has lost its functions can be retained in the symbiont genome for a long time. As a result, the symbiont genome accumulates different non-functional gene residues, which may lead to extinction of small closed populations. This mechanism, called Muller's ratchet,

plays an important role in evolution. Its emergence is facilitated by disorders in DNA recombination and reparation of the symbiont (Naito, Pawlowska, 2016).

In the course of long-term symbiosis some prokaryotes formed a group of “professional” symbionts. These organisms are adapted to symbiotic relationship and in most cases, they are able to form obligatory symbiotic associations and can infect a wide host range. “Professional” symbionts comprise the following prokaryotes: representatives of the orders *Rickettsiales* (Floriano et al., 2018) and *Holosporales* (George et al., 2020), *Cyanobacteria* (Hilton et al., 2013; Nakayama, Inagaki, 2017; Nowack et al., 2018), *Polynucleobacter* (Boscaro et al., 2017), *Kinetoplastibacterium* (Harmer et al., 2018), *Arcobacter sp.* (Hamann et al., 2016), *Desulfarcum epimagneticum* (Monteil et al., 2019), *Methanobrevibacter, Methanocorpusculum* (Lind et al., 2018), *Methanobacterium* (Beinart et al., 2018). The genome of the “professional” symbionts is compact and well-ordered, it contains a small number of non-coding sequences and mobile elements. However, their genome retains a large amount of recombinant DNA and reparation systems (George et al., 2020). Some of the “professional” symbionts are infectious, e.g., representatives of the families *Holosporaceae* (Ossipov, Podlipaev, 1977) and *Chlamydiaceae* (Taylor-Brown et al., 2015). Contrary to animals, protists have a higher propagation rate due to the absence of an embryonic and postembryonic stages, which leads to a quicker endosymbiont genome reduction (Mira, Moran, 2002).

According to many authors, it was from the “professional” symbionts of protists that permanent organelles such as mitochondria, plastids and chromatophores ofthe protist *Paulinellachromatophora* were formed (Husnik et al., 2021). Plastids are organelles performing photosynthesis in the plant cells. All plastids found in eukaryotes are subdivided in 3 groups: primary, secondary and tertiary. The primary plastids have been found in most of algae and in high plants, while the secondary and the tertiary plastids enclosed in three or more membranes occur in different planktonic organisms, such as diatoms or dinoflagellates (Chan et al., 2010).

Primary plastids originated about 1.5 billion years ago (Hedges, 2004). They appeared as a result of ingestion by a heterotrophic protist of a cyanobacterium that managed to survive in the phagosome (Margulis, 1970; Kutschera, Niklas, 2005). Later, the endosymbiont surrounded by two membranes (the membrane of the food vacuole and its own plasma membrane) lost some part of the genome and acquired vertical transmission ability. In the course of this process, an ancestral branch of eukaryotes that gave origin to Plantae Kingdom which includes algae and higher plants could be formed(Rodriguez-Expeleta et al., 2005; Chan, Bhattacharya, 2010). Due to molecular phylogeny techniques, it has been found that the plastids of plants originated from cyanobacteria as a result of partial gene transfer from the endosymbiont into the host cell nucleus (Bhattacharya et al., 2004; Rodriguez-Ezpeleta et al., 2005; Weber et al., 2006).

While symbiosis with cyanobacteria explains the origin of primary plastids in plants, many phototrophic protists, as a rule, possess secondary plastids, which makes their studies much more difficult. An exclusion of this rule is the amoeba *Paulinella chromatophora*, carrying in its cytoplasm chromatophores, organelles functioning like plastids, but originating in evolution much later, than plastids. Molecular analysis of *P. chromatophora* 18S rRNA permitted the researchers to date the appearance of chromatophore in the range from 90 to 140 million years ago (Delaye et al., 2016). The chromatophore is U-shaped. It originated from an alfacyanobacterium of the genus *Synechococcus* (Martin et al., 2005). Interestingly, the closest relative of *P. chromatophora*, *P. ovalis*, is heterotrophic, lacks plastids, and feeds mainly on cyanobacteria (Lhee et al., 2019). Therefore, independent origin of primary plastids in *P. chromatophora*may serve as a good example of organelle formation due to phagotrophic way of feeding.

Unlike plants and *P. chromatophora*, which have primary plastids, appearance of secondary plastids in other phototrophic eukaryotes happened as a result of secondary symbiosis. The secondary plastids are enclosed in three or four membranes retained after ingestion of a plastid-carrying eukaryote by a heterotrophic eukaryote. There are different points of view concerning the origin of secondary plastids in eukaryotes. Molecular data evidence that the secondary endosymbiosis with green algae occurred twice in evolution, which resulted in formation of two groups – euglenids and chlorarachniophyte algae (Archibald, 2015). As for the ingestion of a red alga by a eukaryote, the views of the researchers differ. According to Chromalveolata hypothesis, this event happened only once in evolution (Cavalier-Smith, 1999), some organisms in this group (i.e., ciliates and oomycetes) later losing their plastids. Another viewpoint is that secondary plastids in some groups might have evolved as a result of tertiary symbiosis, the phenomenon occurring in some dinoflagellates (Archibald, 2015).

A distinctive feature of organelles is the transfer of some part of the genetic information to the host, which results in the loss of independence by the organelle and its dependence on the protein-synthesizing apparatus of the host cell. At the same time, many symbionts are still in the initial stages of integration with the protist and therefore retain partial independence. For example, *Ca*. Kinetoplastibacterium crithidii colonizing the trypanosomatid *Angomonas deanei* perform minor protein import from the host cell (Morales et al., 2016) and have synchronized their division cycle with the host cell cycle, which permits the endosymbionts to be distributed between the daughter host cells (Catta-Preta et al., 2015), blurring the line between the concepts of endosymbiont and organelle. Many diatoms can form symbiotic associations with nitrogen-fixing cyanobacteria. Thus, so-called spheroid bodies, which are in fact endosymbiotic cyanobacteria surrounded by host cell membranes have been described in freshwater diatoms of the genera *Rhopalodia*и*Epithemia* (Nowack, Weber, 2018). Phylogenetic analysis of 16S and 18S rDNA demonstrated that both, the host and the endosymbiont

are monophyletic, i.e., apparently, all spheroid bodies appeared as a result of a single event, which happened about 12 million years ago and led to the formation of symbiotic association (Nakayama et al., 2011). A limited number of spheroid bodies (2-8 per host cell depending on its size), vertical transmission and inability to survive outside the host cell evidence for their significant integration into the host cell (Nowack, Weber, 2018). Genome analysis of *Rhopalodia* and *Epithemia* spheroid bodies demonstrated that their genomes are markedly reduced compared to the genomes of closely related free-living cyanobacteria *Cyanothece* spp. (2.29 against 4.68-5.36 Mbp, respectively). According to a number of scholars, in the course of evolution nitrogen-fixating endosymbiotic cyanobacteria of diatoms might give origin to a new organelle, a nitroplast (Nowack, Weber, 2018).

2.8.2 Importance of symbiotic associations between protists and other microorganisms for their ecology

Due to the symbioses a protist host acquires new features, increases its ecological plasticity which finally permits it to colonize new ecological niches. Thus, symbiotic associations are directly involved in the structure of the ecosystem and links within it. Although many protists are able to ingest various microorganisms, they possess some selectivity with respect to food particles (Montagnes et al., 2008). Besides that, there should be a certain concentration of food particles in the environment to maintain the population (Weisse, 2002). The absence or low concentration of the preferred food particles would lessen the protist fission rate and slow down their dissemination in an ecosystem. At the same time the presence of endosymbiotic microorganisms in the protist cell will provide an extra source of nutrition, both, in the form of dividing endosymbionts which can be digested if necessary, and in the form of the products of their metabolism (Gast et al, 2009; Yuuki, Fujishima, 2014). A classic example of such relationship is the symbiotic system *P. bursaria*-endosymbiotic *Chlorella*mentioned above. Besides trophic benefits, the protist involved in the symbiotic association, can acquire from the symbiont extra advantages compared to its nutritional competitors or higher viability upon changes of the environmental conditions. For instance, bacteria of the genus *Caedibacter*, infecting the cytoplasm of *Paramecium*, release R-bodies in the medium, causing the death of not infected ciliates (Schrallhammer et al, 2018). Adverse effect on the endosymbiont-free ciliates gives competitive advantages to the endosymbiont carrying congeners. The number of the infected specimens in the population increases together with the number of bacterial endosymbionts. In the ecosystems with a greater number of organisms susceptible to R-bodies, the infected ciliates have a great competitive advantage (Kusch, Goertz, 2005). On the contrary, in places rich in nematodes, rotifers and other organisms resistant to the toxin the infection is disadvantageous for the ciliates, since

the endosymbionts use host cell resources for their purposes becoming a burden (Landis, 1981; Koehler et al., 2019).

Other endosymbionts of paramecia, bacteria *Holospora obtusa*, can increase ecological plasticity of the host by promoting synthesis of the heat shock proteins in the host, which allows the latter to survive at the increased temperature up to 35 and maintain their endosymbionts (Hori et al., 2008). Finally, the endosymbionts can both, change the environment in close proximity to the protist, and to change the protist host itself, permitting it to survive in the aggressive conditions of the environment. Most often colonization of an unfavorable environment due to symbioses occurs in anaerobic and microaerophilic conditions.

Many protists are able to survive in microaerophilic and even in anaerobic conditions, such as sediments, standing water and animal digestive tract. There is a certain amount of O_2 in such habitats, however, due to high concentration of H_2S the oxygen respiration is blocked completely. The protists inhabiting anaerobic environments, lose enzymes necessary for oxidative phosphorylation. The representatives of the free-living anaerobic ciliates *PlagiopylaиSonderia* were first described by Fenchel and colleagues in 1977 (Fenchel et al, 1977). At present a great number of anaerobic ciliates from different genera including *Geleia* (Edgcomb et al., 2011), *Metopus* (Fenchel, Omar et al, 2017), *Caenomorpha* (Li et al, 2017 is known. The members of these genera were isolated mostly from the samples collected from the marine sediments rich in sulfides. These ciliates lack mitochondria and the enzyme cytochrome oxidase. The ciliates lacking mitochondria use hydrogenosomes for oxidative phosphorylation in the absence of oxygen (Finlay, Fenchel, 1989). The main way of feeding of anaerobic ciliates is phagocytosis of bacteria and archea, inhabiting the marine sediments rich in sulfides. As a result of digestion of prokaryotes, the protists acquire organic matter to be further oxidized in hydrogenosomes. ATP is synthesized in the hydrogenosomes, and the byproducts of this process are molecular hydrogen, carbon dioxide and acidic acid (Barbara et al, 2010).

Most often anaerobic ciliates form symbiotic associations with methanogenic archaea (Fenchel, Finlay, 1991). Ciliate metabolites produced by hydrogenosomes can be advantageous for formation of the symbiotic system in anaerobic conditions. Symbiotic methanogens can use hydrogen produced by hydrogenosomes of the host cell for reduction of CO_2 with the following generation of energy and methane as a by-product (Joens et al., 1987). The ciliates definitely benefit from such symbiotic association, since under inhibition of methanogenesis their growth substantially slows down (Finlay, Fenchel, 1992).

Due to the inherent property of coenzyme F420 in methanogens to fluoresce in UV light, the search and study of methanogens is greatly simplified (Joens et al., 1987). Methanogens have been found in many free-living anaerobic ciliates: *Metopus contortus*, *M. striatus*, *M. palaeformis*, *Trimyema sp*. Sometimes the host cell is co-inhabited by several species of methanogens

simultaneously. It has been shown that such symbioses could have been formed repeatedly, independently of each other and under the influence of various factors (Embley et al., 2004). Not only the methanogens can colonize anaerobic ciliates, but also representatives of *Desulfobacteriaceae*. These Proteobacteria, as well as methanogens, can use hydrogen produced by host cell hydrogenosomes as a reducing agent. However, in the case of desulfobacteria they will reduce sulfur compounds to sulfides (Widdel, Bak, 1992). Interestingly, several types of symbionts that are not metabolically and phylogenetically similar can interact with a single ciliate cell. In the ciliate *Parduzcia orbis*, inhabiting microaerobic conditions of marine sediments rich in sulfur compounds, 4 ectosymbionts related to the metabolic processes of the host cell have been found simultaneously. The ectosymbionts of *P. orbis* are localized in cavities close to the oral cavity. 16S rRNA sequence analysis revealed two sulfatereducers belonging to the families *Desulfobulbaceae* and *Desulfobacteraceae*, a methanogenic archaean from the family *Methanobacteriales* and a methanotroph. In this symbiotic association the ciliate functions as an ecological niche for the symbionts involved both in a metabolic communication with the host cell and in interaction with each other. The ciliate in the described case, according to the authors, gets an opportunity to ingest and digest the symbionts if necessary; no other benefits of symbiosis with such a great number of symbionts have been established as yet (Edgcomb et al., 2011).

2.8.3 Fine mechanisms of interaction between the protists and their endosymbionts

The question about the mechanisms of interaction between the partners in the symbiotic system is another aspect of the problem of symbiosis, no less interesting, but much more difficult to investigate. The difficulties of such studies are connected, firstly, with the necessity to find methodical approaches and techniques not only for studying of each particular symbiotic system, but for different steps in its formation and existence. Many symbionts possess various systems of interaction with the host. These include, for instance, the secretory systems of the IVth and the VIth types permitting the symbionts to release effectors into the eukaryotic host cell as well as in the prokaryotic cells of competitor bacteria (Castelli et al., 2019; George et al., 2020). In contrast to the vast amount of data obtained during studies of fine mechanisms of interaction between pathogenic microorganisms and the cells of Metazoans (Cossart, Sansonetti, 2004; Shames et al., 2009; Stradal, Schelhaas, 2018), this aspect remains poorly studied as regards the endosymbionts of protists. To a greater extent, this issue is covered for systems, in which the cell of a protist (*Dictyostelium discoideum* or *Acanthamoeba castellanii*) infected with a human pathogen (*Mycobacterium* or *Legionella*) is used as a model for revealing key elements of the signaling pathways involved in the process or maintaining of infection (Solomon et al., 2003; Fajardo et al., 2004; Williams et al., 2006; Steinert, 2010; Mori et al., 2018).

Thus, in the experiments made on the slime mold *Dictyostelium*, it was shown that mutation in coronin, the protein regulating dynamics of actin cytoskeleton leads to intensive growth of the bacterium *Mycobacterium marinum* in the cell (Solomon et al., 2003). Using various mutated strains of *Dictyostelium* infected with *Legionella pneumophila*, it has been demonstrated that this bacterium actively modulates phosphoinositides of the host cell membrane to form a habitable compartment (Swart, Hilbi, 2020). It has been shown, that infection of *Acanthamoeba castellanii* with *Legionella* leads to the increased expression of the host genes coding for Rab and Arf GTPases throughout the infection period, while the increased expression of the Rho GTPase has been registered only during the first day after the infection, and the expression of the host genes related to carbon metabolism, energy production and cell cycle decreased significantly (Li et al., 2020).

As for the symbiotic systems formed by protists and the microorganisms that are not regarded as pathogenic, the information is scarce. The best studied are fine mechanisms of interaction between the ciliate *P. caudatum* and its macronuclear endosymbiont *H. obtusa* (Iwatani et al., 2005; Fujishima, 2009, Sabaneyeva et al., 2005; Sabaneyeva et al., 2009; Fujishima et al., 2012). It has been established that activation of the infectious form of *Holospora* happens within minutes after it is ingested in the phagosome as a result of slight acidification of a newly formed phagosome following its fusion with acidosomes carrying vacuolar ATPase in their membranes. The activated infectious form escapes from the food vacuole with the infectious tip forward (Iwatani et al., 2005). Inhibition of V-ATPase with kanamycin A blocks acidification of the contents of the phagosome, and holospora does not get into the nucleus Fujishima, Kawai, 1997).

A group of Japanese researchers managed to find out that an 89kDa protein, which is characteristic for the infectious forms and absent from the reproductive forms, plays an important role in the exit of the infectious form from the phagosome (Iwatani et al., 2005). Using monoclonal antibodies to this protein they demonstrated that the fusion of the phagosome containing the infectious form with acidosomes leads to relocation of the 89kDa protein (it moves from the infectious tip to the bacterial surface, remaining connected with the tip). Apparently, this protein corresponds to the fine fibrous material that mediates the connection of the infectious tip with the evaginating phagosome membrane, often described in electronographs (Iwatani et al., 2005; Fokin, Goertz, 2009; Fujishima et al., 2012). Basing on the TEM data, a number of investigators believes that the infectious form is released from the phagosome enclosed by the host cell membrane (Fokin, Goertz, 2009; Sabaneyeva et al., 2009), while others state that the bacterium ruptures the membrane of the food vacuole and gets into the host cytosol (Iwatani et al., 2005; Fujishima et al., 2012).

Despite this controversy, the involvement of the host actin in the process of infection is acknowledged by the both groups. Participation of the host cell actin in the release of the bacterial infectious form from the food vacuole has been demonstrated using two techniques – by

immunocytochemistry with specific antibodies against paramecium actin and by using a plasmid containing the actin gene and GFP gene (Sabaneyeva et al., 2009). Since the 89kDa protein has two actin binding domains (Iwatani et al., 2005), it has been suggested to promote interaction between the infectious form with the host cell microfilaments that ensure transport of bacteria to Ma. The 89kDa protein remains associated with the infectious form all the time while the bacterium is in the cytoplasm of the ciliate; when the bacterium enters the host nucleus, actin and the 89kDa protein remain outside (Iwatani et al., 2005; Fujishima et al., 2009). Interestingly, microtubules of the host also play a certain role in the infection, apparently, ensuring the correct orientation of the bacterium with respect to the nuclear envelope at the moment of its penetration into the nucleus (Sabaneyeva et al., 2005). Anyway, microtubule depolymerization in the presence of nocodazole prevents ingress of bacteria into the nucleus without affecting their ingestion in the food vacuole in the course of phagocytosis and their exit from the phagosome into the cytosol.

A number of key points still remain unresolved: whether the membrane is enclosing the bacterium after its egress from the phagosome; how the bacterium finds the specific nucleus and gets to it; how it penetrates the nuclear envelope and gets into the nucleus (Iwatani et al., 2005; Fujishima et al., 2009). According to the authors, some specialized lipopolysaccharides present on the surface of the infectious form and specific receptors on the ciliate nuclear envelope could be responsible for this process. This might explain nuclear specificity, host specificity and strain specificity of *Holospora* bacteria (Fujishima et al., 2012). Another protein found in the infectious form of *Holospora* and, obviously, playing an important but not clear role at the last steps of the process of infection and in maintaining of bacteria in the nucleus has a molecular weight of 63kDa (Abamo et al., 2008). After the bacterium gets into Ma, it secretes this protein in the host nucleus, the amount of the protein in the nucleus increasing during the first day of infection (Abamo et al., 2008). Experiments using specific inhibitors of protein synthesis in prokaryotes demonstrated that alongside with the 63kDa protein pre-existing in the infectious form, some part of the protein is synthesized anew ensuring the increase of its concentration in the nucleus before reproductive forms appear in the nucleus. At the start of differentiation of the reproductive forms into the infectious ones, the synthesis of this protein by bacteria was registered again. The role of the 63kDa protein remains unclear, however, since it has DNA-binding domains in its amino acid sequence, the authors suggest that by binding the host cell DNA this protein might prevent DNase activity and creates necessary conditions for the division of the infectious forms and production of reproductive forms (Abamo et al., 2008). This protein might also serve to block receptors or signaling pathways in the nucleus leading to changes in the expression of genes promoting maintenance of the endosymbiont in the nucleus. Besides that, it has been suggested that the 63kDa protein has an antiapoptotic activity, which allows holospora to propagate inside the nucleus (Fujishima et al., 2012). Such strategy, characterized by the presence of apoptosis blocking

mechanism has been registered in *Rickettsia rickettsii*, which is phylogenetically not too far from *Holospora* (Clifton et al., 1998). However, it should be noted that that apoptosis as such is unique to multicellular organisms, while protists have programmed apoptosis-like cell death, which has only a number of features in common with classical apoptosis (Kaczanowski et al., 2011). Therefore, apparently, one can only speak about possible blocking of programmed cell death similar to apoptosis by holospora.

Relatively well-studied are the relationships between the ciliates *Paramecium burasria* and the phototrophic *Chlorella spp*. This symbiotic association can be used as a model for studying fine mechanisms of the partner interactions. The cytoplasm of many*P. burasria* strains is filled with many alga cells, each chlorella residing in an individual perialgal vacuole different from the food vacuole. The egress of the *Chlorella* from the food vacuole and its transformation into an endosymbiont is fairly well understood.

As in the case of *Holospora*, *Chlorella*, upon ingestion into the phagosome, can avoid digestion with lysosomal enzymes and egress from the phagosome one at a time (Kodama, Fujishima, 2005). However, contrary to *Holospora* that egress from the phagosome immediately after its fusion with acidosomes, *Chlorella* does it much later, when the lysosome enzymes have already started to digest its contents. Some part of algae become damaged by enzymes and finally they are digested, while another part remains intact and manages to avoid digestion (Kodama, Fujishima, 2010).

Living green chlorella are enclosed by a perialgal vacuole and reside in the ciliate cell cortex between the trichocytes (Kodama, Fujishima, 2005; Kodama, Fujishima, 2010; Kodama et al., 2011). Interestingly, in case of substitution of chlorella by yeasts or polysterol spheres of comparable size (3μm and more), they were also able to egress from the food vacuoles (Kodama, Fujishima, 2012). At the same time, polysterol spheres with a diameter of 0.81 μm, Indian ink particles and food bacteria remained in the digestion vacuoles (Kodama, Fujishima, 2005). Localization of *Chlorella* in the cell cortex ensures its minimal contact with the lysosomal enzymes, however, the main role in the defense from the host lysosomes belongs to the perialgal vacuole (Kodama, Fujishima, 2009). Interestingly, acid phosphatase activity is registered in trichocysts when chlorellae get into the subcortical layer, which is not typical for *Chlorella*-free paramecia. When a chlorella enclosed in a perialgal vacuole appears in the vicinity of the host cell cortex, a part of the trichocysts was digested by the host cell enzymes leaving some space for a perialgal vacuole containing a chlorella. Destruction of trichocysts and vacating space for new endosymbionts has been also observed upon chlorella fission and reinfection (Kodama, Fujishima, 2011). Molecular mechanism causing the fusion of lysosomes with some trichocytes during the formation of the symbiotic system *P. bursaria/Chlorella* still remains unclear.

Japanese researchers used the protein translation inhibitor cycloheximide, the antibiotic chloramphenicol and the photosynthesis inhibitor dichlorphenyl urea to elucidate the relationship between the chlorella and the ciliate. They managed to show that during photosynthesis chlorella produces produces proteins necessary for maintaining the structure of their nuclei and chloroplasts. Disorders in translation led to the rapid lysis of organelles, disorders in the perialgal vacuole structure and digestion of the symbiont with lysosomal enzymes of the host cell (Kodama, Fujishima, 2011). Despite the abundance of the obtained data, many aspects of the partner relationships remain unclear, in particular, the mechanism of resistance of some part of chlorella to lysosomal degradation.

Fine mechanisms of interaction between protists and their endosymbionts in other symbiotic systems are even less studied. Thus, absolutely unknown are mechanisms of penetration into the host cell of bacteria devoid of specialized infectious form (e.g., *Lyticum*, *Ca*. Megaira, *Ca*. Fokinia), widespread occurrence of some of these species in nature (*Ca*. Megaira venefica) and the fact that they can be found in various host species suggesting the existence of an infectious agent and the ability of horizontal transfer, although they have not been registered as yet. Besides that, it is not clear, why the endosymbionts residing “naked” in the host cytosol do not undergo autophagy on the part of the host cell, and what the mechanisms for controlling the endosymbiont number. These questions could be answered by applying modern recently elaborated technical approaches to the studies of symbiotic systems in protists.

2.8.4 Methodical approaches to the studies of symbiotic systems in ciliates

For many years description of symbiotic systems with ciliates serving as hosts has been based exclusively on morphological characteristics of the partners obtained by means of light microscopy and electron microscopy. In the cases when the symbiotic system could be maintained in the laboratory conditions this characteristic could be complemented with some information about the biological peculiarities of the system (the presence or absence of the killer-effect, infectiousness of the endosymbiont, the way of the endosymbiont transmission in the sexual process) (Serra, Fokin, 2016).

At present, with the development of molecular methods of investigation the use of full cycle SSU rRNA gene analysis (Amann et al., 1995) is ever growing. The algorithm of the study includes total DNA isolation followed by amplification and sequencing of the 18S rRNA gene of the host and 16S rRNA gene of the endosymbiont (if it is a prokaryote). In case of difficulties in identification of the host using the sequence of the 18S rRNA gene, a fragment of the ribosomal gene cluster including both internal transcribed spacers, ITS1 and ITS2, and/or COXI gene sequence are used. Then on the basis of the endosymbiont 16S rRNA gene analysis specific probes are designed, which are labeled with fluorochromes and used in fluorescence in situ hybridization to prove that the analyzed sequence

originated from the endosymbiont, and not from a contaminating microorganism. In case of a positive signal, the obtained sequence is used to determine the position of the endosymbiont on the phylogenetic tree.

Novel actively developing approaches in the studies of symbiotic associations based on the host and the endosymbiont genome and transcriptome analysis offer new perspectives for the researchers. The whole genome of some representatives of the species *Paramecium* and their endosymbiotic bacteria *Holospora* has been sequenced, which makes further comparative analysis of various hosts and endosymbionts possible (Garushyants et al., 2018). At the same time the obtained DNA sequences of the ciliates and the endosymbionts may become the basis for a more in-depth transcriptome analysis. Comparing transcriptomes and gene sequences of the partners of symbiotic systems which account for establishment and maintenance of the symbiotic system, will help to clarify molecular mechanisms ensuring the interaction between the host and the endosymbiont (Schrallhammer, Potekhin, 2020).

Investigation of the impact of the endosymbiont on the metabolism of the symbiotic system and assessment of its role in the vital functions of the host, and, consequently, the importance of the symbiotic system on a particular ecosystem are highly problematic. A fruitful approach to the study of the endosymbiont influence on its host-protist is comparing host fitness of the endosymbiont-bearing strains and experimentally obtained aposymbiotic cell lines (Kusch et al., 2002; Dusi et al., 2014; Bella et al., 2016; Grosser et al., 2018; Pasqualetti et al., 2020). Aposymbiotic cell lines are obtained by administration of antibacterial agents to endosymbiont-bearing cells. Traditionally, bacteriocide antobiotics, such as streptomycin (Kusch et al., 2002; Dusi et al., 2014; Bella et al., 2016; Grosser et al., 2018) and ampicillin (Vyshnyakov, Rodionova, 1999) are used for this purpose. Comparison of the propagation rate of aposymbiotic and endosymbiont-bearing cell lines allows the researchers to evaluate the influence of the endosymbiont on its host and the rate of the host dependence on symbiotic relationships.

At present, several mechanisms of bacterial antibiotic resistance have been discovered, among them one may distinguish the primary mechanisms and the acquired ones. The primary antibiotic resistance is related to the physical and chemical peculiarities of bacterial cell structure and is not subjected to genetic modifications. Thus, primary antibiotic resistance is ensured by the presence of the outer membrane of Gram-negative bacteria, lipopolysaccharides and the ability of bacteria to form biofilms to protect themselves from the zooplankton and drugs (Monore, 2007; Mah, 2012). The acquired resistance is caused by the appearance of previously absent ways of protection, e.g., mutations leading to nonsusceptibility to the antibiotic, or by acquisiton of the resistance gene from a plasmid or bacteriophage. Also, mutations in the regulatory elements which launch synthesis of molecules increasing bacterial resistance to antibiotics, can be regarded as acquired resistance (Rhodes

et al., 2016). Mechanisms of bacterial antibiotic resistance include enzymatic inactivation of antibiotics, decrease of membrane permeability by channel regulation, activation of the efflux pumps, and modification of the antibiotic target (Schweizer, 2012), and L-form transition (Mercier, 2013). Besides, bacteria can combine these mechanisms to increase their resistance. For example, under antibiotic administration, *Pseudomonas aerogenes* decreases the outer membrane permeability and increases the number of the efflux pumps (Blair et al., 2015).

Representatives of the family *Rickettsiaceae* belong to obligate Gram-negative endosymbionts. Universal resistance of rickettsia to antibiotics is related to the efflux system (Rolain, Raoult, 2005). Rickettsioses do not respond to the treatment with β-lactam antibiotics, firstly, due to their ability to synthesize β-lactamase. Also, a penicillinase homologue has been found in *R. felis* (Ogata et al., 2005). The rickettsial resistance to antibiotics suppressing protein synthesis, such as erythromycin, is ensured by modifications of 23S rRNA. No emr gene coding for erythromycin resistance enzyme has been found in the rickettsial genome. However, it has been shown that frequent mutations in 23S rRNA gene can lead to appearance of the non-susceptible to erythromycin strain (Vester, Douthwaite, 2001). Resistance of rickettsia to aminoglycosides, such as gentamycin and streptomycin, is presumed to be caused by the enzyme aminoglycosidacetyltransferase (Rolain, Raoult, 2005). However, although the rickettsial resistance to aminoglycosides is well documented, its mechanism still remains unclear.

Antimicrobial complex FLIP7 (Fly Larvae Immune Peptides) elaborated in the laboratory of biopharmacology and immunology of SPbU (Chernysh et al. 2015; Chernysh et al. 2018)is of particular interest for the studies of stability of the symbiotic relationships. It is a complex composed of antibacterial peptides isolated from a blue blow fly *Calliphora vicina* (Diptera: Calliphoridae). Antibacterial peptides comprised in FLIP7 are defensins, cecropines, diptericins, proline-rich peptides and some unknown antibacterial agents (Chernysh et al. 2015; Chernysh et al. 2018). This complex is active both, against Gram-negative and Gram-positive bacteria, including *Staphylococcus aureus,Escherichia coli*, *Klebsiella pneumonia* and *Acinetobacter baumanni* (Chernysh et al. 2015). Due to its broad range of action and composition, presumably, administration of FLIP7 may be useful in the cases of bacterial antibiotic resistance and can be considered as an advanced means of obtaining aposymbiotic cell lines.

3. Material and methods

3.1 Cell Cultures

Four *Paramecium multimicronucleatum* strains infected with the motile intranuclear endosymbiont *Ca*. Trichorickettsia mobilis (LSA11-2, Büsnau, AB9-4,Kp154-4) and three control non-infected strains of the same species (MSA, CyP5-3, Esa 3-2)were used in the experiments (Table 1). The strain LSA11-2 originated from a sample taken in Lucca (Italy) in the summer 2011. The ciliates were cloned in a multi-well plastic plate in a wet chamber at room temperature in lettuce infusion inoculated with *Klebsiella (Enterobacter) aerogens.* Büsnau strain was kindly provided by Dr. M. Schweikert (Technical University of Stuttgart), the rest of the strains were made available by N. A. Lebedeva (RC CCM culture collection at the Core Facility Center for Cultivation of Microorganisms (Saint-Petersburg State University). The ciliates were maintained in tubes with the lettuce infusion inoculated with *Klebsiella aerogenes* at room temperature in the dark. Twice a week the cultures were fed by adding 1–2 mL of fresh bacterized medium.

Table 1. *P. multimicronucleatum* strains used in the experiments

Strain	Origin	Intranuclear motile endosymbiont	Years of isolation	Collector
LSA 11-2	Lucca, Italy	present	2011	Sabaneyeva E. V.
Büsnau	Stuttgart, Germany	present	1988	Goertz H.-D.
94/AB9-4	Boston, USA	present	1994	Scoblo I. I.
Kp154-4	Krasnoyarsk, Russia	present	2012	Potekhin A. A.
MSA	Cyprus, Greece	No	2016	Lebedeva N. A.
CyP5-3	Cyprus, Greece	No	2016	Lebedeva N. A.
ESa 3-2	Saarema, Estonia	No	2017	Lebedeva N. A.

3.2 Living cell observations

Living cell observations were performed using the cells immobilized with Skovorodkin's compression device (Skovorodkin, 1990) and a Leica 6000B microscope (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with a digital camera DFC500 in Nomarsky imaging mode.

3.3 Experimental infections

The contents of the three tubes with the infected strain LSA11-2 were concentrated by centrifugation at 800 rpm for 10 min. The supernatant was discarded, and 1 mL of the sediment was homogenized with a glass syringe for insulin injections. Periodically the homogenate was checked for the presence of the intact cells. Then the homogenate was added to a well containing 1 mL of non-infected cells (MSA strain), so that the volume ratio of the homogenate and symbiont-free cell suspension was 1:1. The cells were checked for infection in 20 min, 2 h, 1 day and in a week.

3.4 Killer-effect tests

These experiments were performed in plastic Petri dishes.10 infected living cells of the LSA11-2 strain were placed in a drop of fresh culture medium. Then 10 cells of the endosymbiont free MSA strain were added to the same drop. In 30, 90, and 120 min, the cells were checked using a stereomicroscope to assess the number of dead cells.

3.5 Feulgen staining of the whole cell mounts

The ciliates were fixed in Bouin solution in the glass 3-well plates, transferred to the slides, and the fixative was removed. Then the slides were dehydrated in a series of alcohol (70°-96°), and the cells were mounted in celloidin. After that the slides were gradually rehydrated (96°-70°-distilled water) and kept in 6N hydrochloric acid for 20 min. The slides were washed in distilled water and stained in Fucsin solution for 20 min. Then the slides were briefly rinsed in acidic ethanol (70°) and the cytoplasm of the cells was counterstained with Light Green in 70° ethanol. Then the slides were dehydrated in a series of ethanol and xylol and mounted in Canada balsam.

3.6 Transmission Electron Microscopy (TEM)

The cells were fixed in a mixture of 1.6% paraformaldehyde and 2.5% glutaraldehyde prepared with a phosphate buffer (0.1 M, pH 7.4) for 1.5 h at room temperature. Alternatively, 2.5% glutaraldehyde diluted with 0.1M cacodylate buffer (pH 7.4) was used as a fixative. The ratio of the fixative and the cell suspension volumes was 2:1. Then the cells were concentrated by centrifugation at 4000 rpm for 5 min and the supernatant was discarded. The cells were transferred to the Eppendorf

tubes and washed in the same buffer containing 12.5% sucrose and postfixed in 1.25-1.6% OsO4 (1 h at 4°C). Then the cells were washed in the same buffer, dehydrated in ethanol gradient followed by ethanol/acetone mixture (1:1) and 100% acetone. After each step the cells were concentrated by centrifugation. Before embedding the cells were soaked in the mixture ofacetone and resin (3:1, 1:1, 1:3). The material was embedded in Epoxy embedding medium (FlukaChemie AG, St. Gallen, Switzerland) prepared according to the manufacturer's protocol. For the final steps, the cells were kept for 2h in the resin (2 changes) at room temperature, then placed in a BioSan thermoblock (Latvia) at 37°C for 52h, 45°C for 24h and 60°C for 24h. The blocks were sectioned with a Leica EM UC6 Ultracut, and ultrathin sections were stained with aqueous 1% uranyl acetate followed by 1% lead citrate. All samples were examined with a JEM-1400 electron microscope (JEOL Ltd., Tokyo, Japan) or JEM-2100 (JEOL Ltd., Tokyo, Japan) in the Core Facility Center "Development of Cellular and Molecular Technologies" (Saint-Petersburg State University) at 90 kV.

3.7 Negative staining

For negative staining several LSA11-2 cells were briefly washed in distilled water. The cells were squashed in a tiny drop of water and the resulting suspension was placed on a Pioloform coated copper grid. Bacteria were allowed to precipitate for 2–3 min, then a drop of 1% uranyl acetate in distilled water was added for about 40 sec. The liquid was then absorbed with filter paper and the grid was air-dried for 24h. The samples were analyzed with a JEM-1400 electron microscope (JEOL Ltd., Tokyo, Japan) or JEM-2100 (JEOL Ltd., Tokyo, Japan) in the Core Facility Center "Development of Cellular and Molecular Technologies" (Saint-Petersburg State University).

3.8 Atomic Force Microscopy

The ciliates were briefly washed in the sterile lettuce medium, squashed in a small drop of medium on the cover slip and air dried. The images were obtained with a NTEGRA Aura (NT MDT, Russia) scanning probe microscope in the Core Facility Center for Microscopy and Microanalysis (SPbU).

3.9 DNA Extraction and Characterization of the SSU rRNA gene of the host and the endosymbiont

DNA extraction was performed using the protocol for mycelium DNA of NucleoSpinTM Plant DNA Extraction Kit (Macherey-Nagel GmbH and Co., Düren NRW, Germany). Amplification of the

host 18S rRNA gene from total DNA was performed with the 18S_F9 (Medlin et al., 1988) and 18S R1513Hypo primers (Petroni et al., 2002). PCR products were directly sequenced with the same primers. The 16S rRNA gene of the endosymbiont was amplified by a PCR employing universal eubacterial primers 16S_αF19a(Vannini et al., 2004)and 16S_R1522a(Vannini et al., 2014)or by a touchdown PCR reaction employing primers specifically designed by our Italian colleagues: RickFla_F69 5′-GTTAACTTAGGGCTTGCTC-3′, RickFla_F87 5′-CTCTAGGTTAATCAGTAGCAA-3′, RickBas_F166 5′-ATGCTAATGCCGTATATTCTC-3′, Rick_R1270 5′- TTTTAGGGATTTGCTCCACG-3′, Rick_R1455 5′-CCGTGGTTGGCTGCCT-3′ (Vannini, …Mironov et al., 2014). PCR products were directly sequenced using the same primers and TaKaRa ExTaq polymerase.

3.10 Fluorescence in situ Hybridization (FISH)

In order to confirm that the obtained sequence indeed belonged to the macronuclear symbiont of the paramecium, and was not an occasional contamination, fluorescence *in situ* hybridization was carried out using a specially designed oligonucleotide probe RickFla_430 (5′-TCTTCCCTGCTAAAAGAACTTT-3; Vannini, …, Mironov et al., 2014) conjugated to Cy3 fluorochrome. FISH with this probe was also carried out to identify the motile endosymbionts in the Busnau, AB9-4 and Kr154-4 strains and to specifically reveal the motile endosymbionts in the cells upon antibiotic and FLIP7 administration. Alongside with the species-specific probe, we used the nearly universal eubacterial probe Eub_338 (5′-GCTGCCTCCCGTAGGAGT 3′; Amann et al., 1990) labeled with FITC. In some experiments Alfaproteobacteria-specific probe Alf_1b (5′-CGTTCGYTCTGAGCCAG-3′; Manz et al., 1992) labeled with Cy3 was used.

The cells were fixed with cold 4% paraformaldehyde in 0.2M PBS in a microwell for 1 h and transferred to the Super Frost slides (Menzel-Gläser, Germany) for better cell adhesion and cell loss decrease during the following manipulations. The fixative was removed with a thin capillary and filter paper and 0.2M PBS was added for 5 min to remove the remaining fixative solution. Then the buffer was removed and replaced with 70% methanol for up to 1h. Changes of fixative and washes were controlled under a stereomicroscope. After a brief final wash in PBS, the slides were incubated for 30 min in hybridization buffer. Hybridization was carried out according to Manz and colleagues (Manz et al., 1992) in the hybridization buffer composed of 5M NaCl (18%), 1 M TrisHCl (2%), distilled water (50%), formamide (30%) and 10% SDS (0.001%) and containing the oligonucleotide probe (10 ng/µL) in a wet chamber in a BioSan thermoblock (Latvia) at 46℃ for 1.5 h. In order to remove the unbound probe hybridization was followed by two incubations in washing solution at 48℃, each for 30 min. The slides were mounted in polyvinyl alcohol Mowiol (Calbiochem, Germany) in 40% glycerol prepared

according to manufacturer's protocol and containing the antifading agent PPD and DAPI for counterstaining nuclei. The slides were analyzed with a Leica TCS SPE Confocal Laser Scanning Microscope (CLSM) in the Core Facility Centre for Microscopy and Microanalysis (SPbU). For fluorochrome excitation three lasers with the wavelengths 365, 488, 555 nm were used. The images were processed with ImageJ or Fiji open access software.

3.11 Phylogenetic analysis

Phylogenetic analysis was performed together with our colleagues from the University of Pisa (Italy) on 32 sequences. Sequence lengths were reduced to that of the shortest one, and the long inserts present only in the motile macronuclear symbiont of LSA11-2 strain were removed, which resulted in a 1338 character matrix. Maximum Likelihood (ML) and Bayesian Inference (BI) methods were employed, using the software PHYML (Guindon, Gascuel, 2003) and MrBayes (Huelsenbeck, Ronquist, 2001), respectively. The best substitution model was selected according to the AIC parameter calculated by jModelTest (Posada, 2008). Bootstrapping (1,000 pseudoreplicates) was applied to the ML analysis. Similarity values were calculated on the unmodified matrix. Bacteria from other families of Rickettsiales (*Midichloriaceae* and *Anaplasmataceae*) were taken as an outgroup.

3.12 Antibiotic Treatment

In order to assess stability of the symbiotic system and to obtain aposymbiotic cell lines the ciliates of the infected strains were treated with antibiotics characterized bydifferent mode of action (Table 2). Stock solutions of streptomycin (Sigma-Aldrich, St. Louis, United States), ampicillin (Sigma-Aldrich, St. Louis, United States) and tetracycline (Sigma-Aldrich, St. Louis, United States) were prepared in distilled water, stock solutions of chloramphenicol (Sigma-Aldrich, St. Louis, United States) — in ethanol. Fifty ciliates of each strain were placed in a microwell of a 24-microwell plate containing 0.5 mL of slightly bacterized lettuce infusion. In the first set of experiments, performed with all antibiotics, except tetracycline, an antibiotic was added to adjust the final concentration to 100, 250, 500, and 1,000 μg/mL. The microwell plates were kept in the dark at room temperature.
The host cells were checked for viability and the presence of the endosymbionts on the 3rd, 10th, and 15th day after the treatment. After the first check 3–4 drops of bacterized culture medium were added twice a week to feed the ciliates. Since tetracycline proved to be extremely harmful for the host in the preliminary tests, tetracycline was administered in concentrations 10-fold lower than in the experiments with the other three antibiotics — 10, 25, 50 and 100 μg/mL, and the results of this experiment were checked on the 3rd day. Besides that, we used an extra control endosymbiont free

strain (CyP5-3) in the experiments with tetracycline. Each experiment (each concentration used with each strain) was repeated three times. In another set of experiments, 1,000 µg/mL ampicillin was repeatedly (every 3rd day) administered to the microwell containing the same volume of the culture medium and the same number of Kr154-4 cells together with the bacterized culture medium. The ciliates were kept for 21 days.

Table 2. Antibiotics used in the experiments

Antibiotic	Class	Mechanism of action	Toxicity against eukaryotes	Reference
Streptomycin	Aminoglycoside	inhibits protein synthesis	low	Pagkalis et al., 2011
Ampicillin	β-lactam	inhibit bacterial cell wall synthesis	very low	Rafailidis et al., 2007
Chloramphenicol	Amphenicol	inhibits protein synthesis	high	Barnhill et al, 2012
Tetracycline	Tetracycline	inhibits protein synthesis	high against some protozoan	Connell et al., 2003

The cells were checked periodically for the host viability with a stereomicroscope and the presence of endosymbionts using Nomarsky optics. The culture was considered infected if five randomly chosen cells in a row manifested motile endosymbionts in their macronucleus. As sometimes it was impossible to obtain clear images of living bacteria due to their fast motility, for microphotography ciliates were fixed with 4% paraformaldehyde and observed with DIC. On the 5th (after the second ampicillin treatment) and on the 15th day of the experiment a part of the ciliates was fixed for TEM, and a part of the cells was fixed for FISH.

3.13 FLIP7 complex extraction and fractioning

FLIP7 (Fly Larvae Immune Peptides) extraction was performed using diapausing *Callifora vicina* larvae inoculated with Escherichia coli M17 strain (Microgen, Russia) to induce immune response as described elsewhere (Chernysh et al., 2015; Gordya et al., 2017). On the next day after immunization the hemolymph was collected, centrifuged to exclude cell fraction and fats, purified, and lyophilized. Prior to use, the lyophilized sample was dissolved in deionized water and sterilized by filtration through a membrane with a pore size of 0.22 µm (Milliex-GS; Merck Millipore, Billerica, MA, USA).

FLIP7 fractions were obtained using 10 mg of lyophilized FLIP7 with Shimadzu LC20 Prominence HPLC system equipped with Vydac C18 column (250 x 10 mm, 5 µm, Grace, USA),

equilibrated with 0.05% TFA. Substances were eluted with a linear gradient of acetonitrile (ACN) from 2 to 60% for 60 min on the 2.5 ml/min flow rate. Chromatographic fractions were automatically collected with 1 min intervals. The fractions' optical densities were registered by means of a UV detector at wavelength of 214 nm. The fractions were lyophilized for storage, and dissolved in deionized water prior to use. FLIP 7 and its fractions were kindly provided by Dr. A. Yakovlev (Laboratory of Insect Biopharmacology and Immunology, Biological Faculty, Saint-Petersburg State University).

3.14 Whole FLIP7 treatments

In preliminary experiments aimed to approximately determine the range of FLIP7 concentrations to be used in further experiments, only one of the endosymbiontcarrying strains, Kr154-4, and the endosymbiont-free CyP5-3 strain, which served as a control, were used. Ciliates were washed in the sterile lettuce infusion to get rid of food bacteria and were placed in wells of the 24-well plate containing 0.5 mL of the fresh sterile lettuce medium, 50 cells per well. The stock solution of FLIP7 was administered to the wells to adjust the final concentrations to 0.2, 2, 10 and 20 mg/mL. The effect was checked immediately after FLIP7 administration and the observations were continued throughout 1 h after the start of the experiment using a stereomicroscope MSP-1 (LOMO, Russia). In the trials with lower FLIP7 concentrations (20 and 100 µg/mL) the cells were checked for viability in 1h, 2h and on the 3d day after administration of FLIP7. Viability of the motile intranuclear endosymbionts was registered under a Leica B6000 microscope equipped with DIC and a digital camera DFC 500. The culture was considered infected if five randomly chosen cells in a row manifested motile endosymbionts in their macronucleus. Sometimes, as it was impossible to obtain clear images of living bacteria due to their fast motility, for microphotography ciliates were fixed with 4% paraformaldehyde and observed with DIC.

In the second set of experiments the FLIP7 concentration range was 20, 100, 200, and 400 µg/mL. Cells of the two Trichorickettsia-carrying strains, Kr154-4 and LSA11-2, and the control Esa3-2 strain were washed and placed in the wells, as described above. Each experiment (all four concentrations tested for the three strains) was repeated three times. The effect of FLIP7 was assessed in 1 h and on the 7th day after the start of the experiment. Each experiment was repeated three times. Besides living cell observations, fluorescence *in situ* hybridization using species specific probe, RickFla_430, was carried out to reveal the endosymbiont location in the host cell. In order to follow the changes in the distribution and fine structure of both, the host and the endosymbionts, the cells were fixed for TEM in a week after administration of the whole FLIP7 (100 µg/mL).

3.15 Assessment of FLIP7 HPLC fractions' effects

47 HPLC fractions of FLIP7 (4-50) were used in the experiment. 50 cells of the strain Kr154-4 were washed from the medium containing food bacteria and placed in the wells with 500 µL sterile medium, as in the previous experiments. The final concentration of all fractions usedcorresponded to 1.2 mg of whole FLIP per mL (hereinafter – "FLIP7 equivalents/mL"). The ciliates were checked for viability in 5 min after the start of the experiment with a stereomicroscope MSP-1 (LOMO, Russia). Most active fractions were selected, and these were tested in lower concentrations (120, 240 and 360 µg of FLIP7 eqvl./mL). The cells were checked in 1 h after the start of the experiment. In another set of experiments viability of the infected Kr154-4 ciliates and the control CyP5-3 cells was checked on the 7th day after administration of the most active fractions in the concentration of 120 µg of FLIP7 eqvl./mL.

4. Results

4.1 General characteristics of the symbiotic system *Paramecium multimicronucleatum*/*Ca.* Trichorickettsia mobilis.

4.1.1 Identification of the strain LSA11-2 ciliate species

The species of the ciliate host was determined basing on morphological and molecular data. Using Feulgen staining in modification by De Lamater and living cell observations performed with differential interference contrast it was shown that according to criteria proposed by Fokin (Fokin, 2010/2011), by their morphotype (in particular, the form and the size of the cells and the number and the size of the micronuclei) the ciliates of the LSA 11-2 strain belong to *Paramecium multimicronucleatum* (Fig. 4).Morphological data are in good agreement with molecular data obtained by analysis of the fragment of the 18S rRNA gene sequence (Vannini,…, Mironov et al., 2014). The fragment of 1710 b.p. demonstrated more than 78% identity with the published *P. multimicronucleatum* 18S rRNA gene sequences present in NCBI Blastn data base (Vannini,…, Mironov et al., 2014).

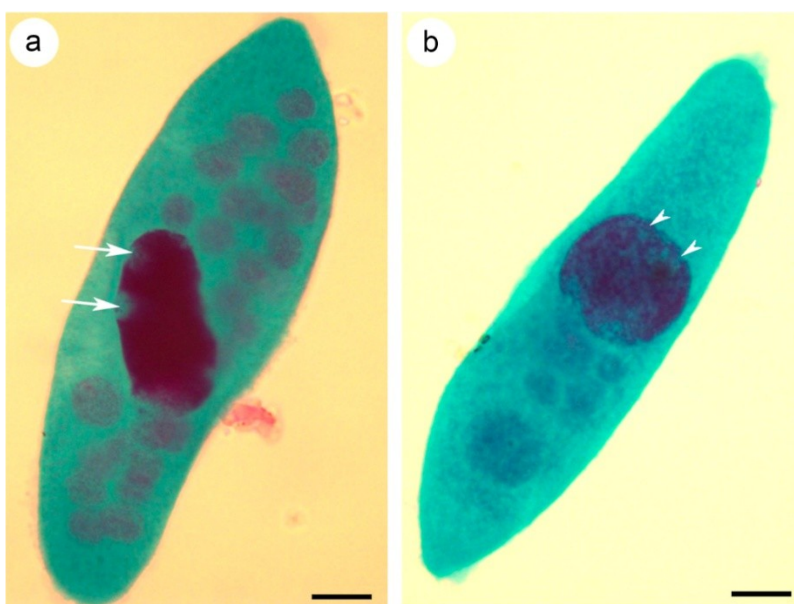


Fig. 4. *Paramecium multimicronucleatum* (strain LSA11-2). Feulgen staining in modification by De Lamater. a – white arrows point to lacunae in the ciliate Ma; b – white arrowheads mark the regions with lower chromatin condensation. Scale bar 10 µm.

4.1.2 Localization of the motile endosymbiont in the ciliates *P. multimicronucleatum* (strain LSA11-2)

Contrary to the normal intensive even staining of the macronucleus of paramecia free from the intranucler endosymbionts, in the Ma of the LSA11-2 ciliates stained by Feulgen technique one can clearly see either Feulgen-negative cavities (lacunae) or uneven staining with brighter focuses and less intensively stained background (Fig. 4). Also, lacunae in Ma were observed in the cells stained with DAPI (Fig. 5).

In the course of living cell observations of the LSA11-2 cells using Nomarsky contrast, also, cavities or lacunae with actively moving bacteria were easily detected (Fig. 6). Most part of bacteria were moving directly under the Ma nuclear envelope, axis-turning while propulsion. Upon collision with an obstacle (the border of the lacuna), the endosymbionts changed abruptly the direction of their movement. The number of bacteria within one lacuna varied; it was not possible to estimate their number due to their high motility, however, in some cases we managed to register numerous bacteria in one lacuna.

In squashed living ciliates, the bacteria were seen to keep moving within the vesicles containing fragments of the disrupted Ma for several minutes. After dissolving of the Ma fragments in the medium, the bacteria soon stopped moving and, possibly, died (Vannini, …, Mironov et al., 2014).

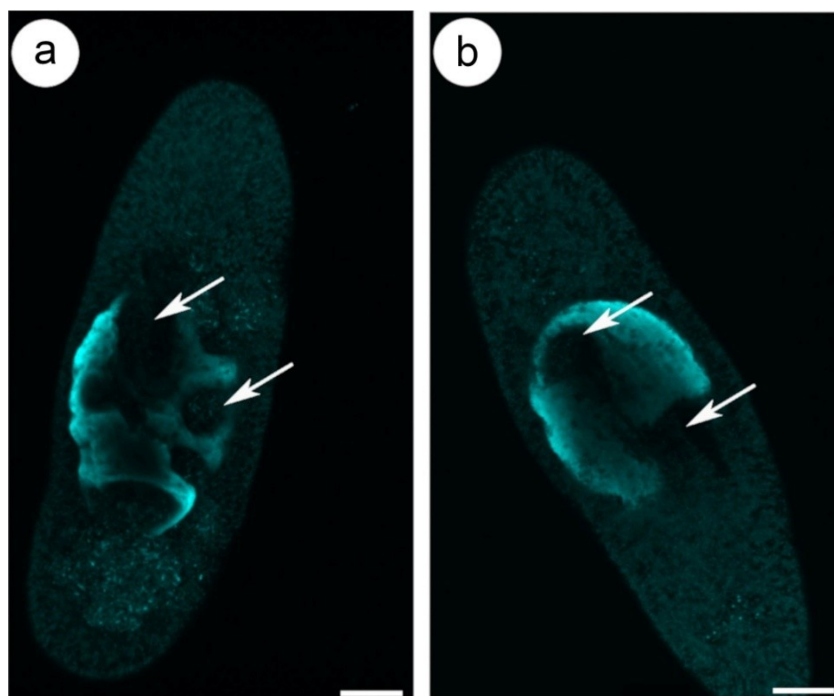


Fig. 5. The ciliate *P. multimicronucleatum* (strain LSA11-2), DAPI staining. Confocal laser scanning microscopy. White arrows point to lacunae in Ma. Scale bar 10 µm.

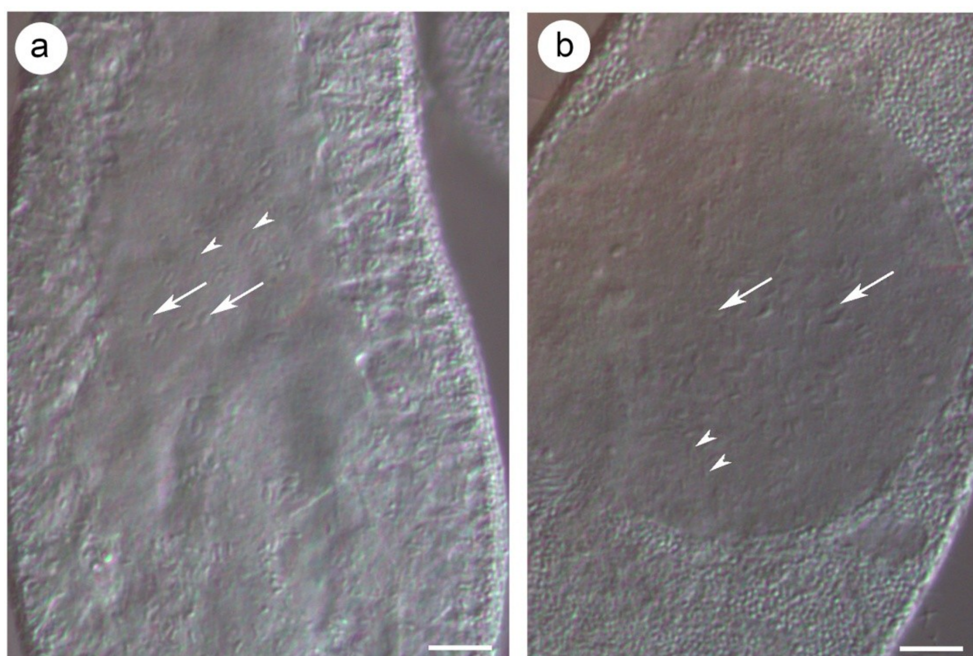


Fig. 6. Motile endosymbionts in the macronucleus of the ciliate *P. multimicronucleatum* (strain LSA11-2), Nomarsky contrast. Rod-like bacteria are marked with white arrowheads, white arrows point to ovoid forms. Scale bar 10 µm.

In TEM images, chromatin-free areas were distinct in the infected by motile endosymbionts Ma of the ciliates of LSA11-2 strain (Fig. 7a). In some cases, the electron density in these areas was uneven. Electron microscopy showed that the endosymbionts in Ma are not surrounded by any additional mmembranes, which could serve as a symbiontophorous vacuole, and the bacteria can be located not only in the chromatin-free lacunae on the macronuclear periphery, but also in the immediate vicinity to the chromatin bodies and nucleoli (Fig. 7b, c, d).

In living ciliates and in the cells fixed with paraformaldehyde, polymorphism of the bacterial population was quite conspicuous. Some bacteria were rod-like and actively moving, while others on the contrary, were ovoid and were less motile. However, various intermediate forms were also present (Fig. 6).

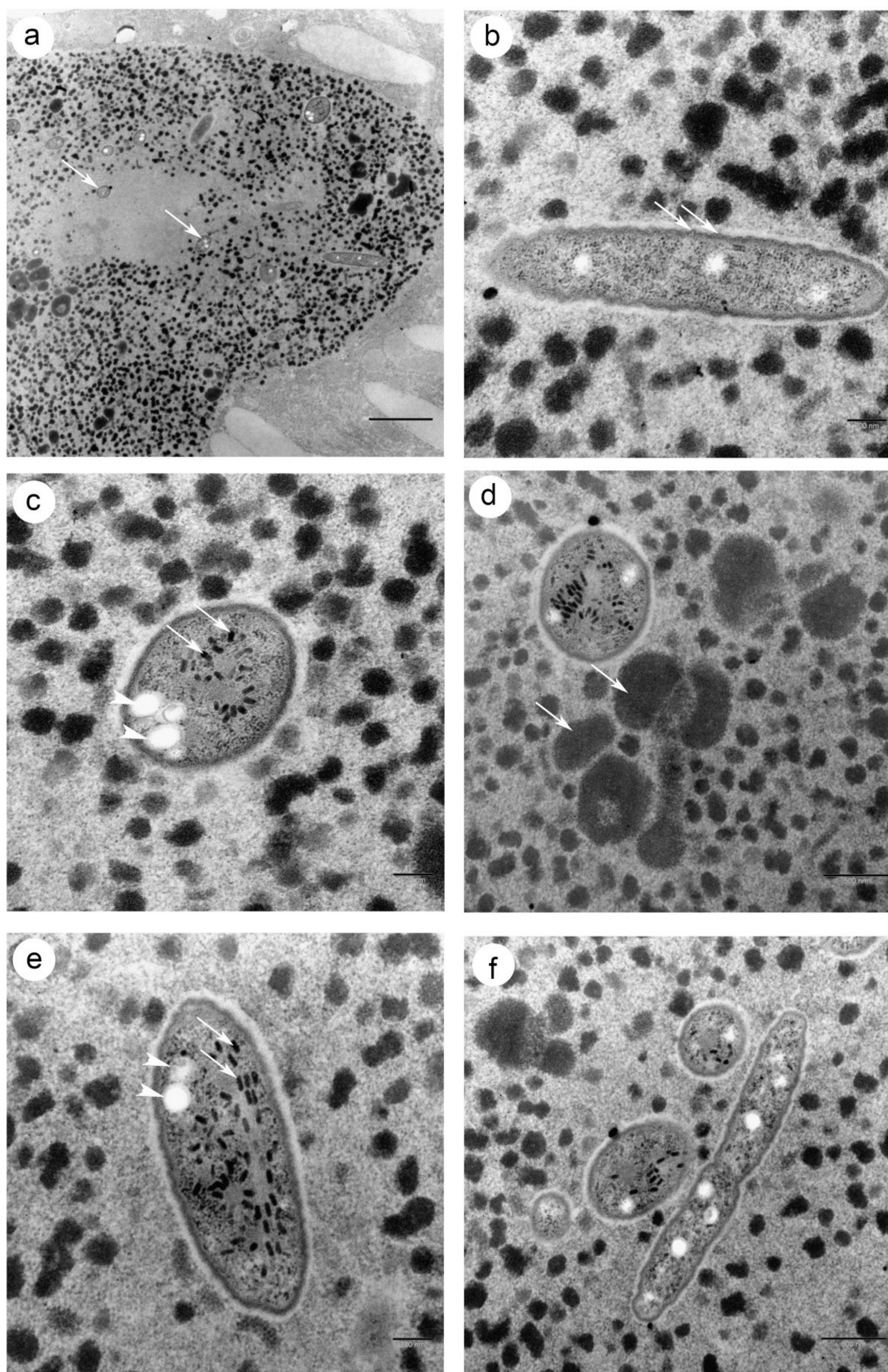


Fig. 7. Motile endosymbiont in the macronucleus of the ciliate *P. multimicronucleatum* (strain LSA11-2). Transmission electron microscopy. a – white arrows point to endonucleobionts; b – white arrows indicate the outer bacterial membrane; c and e – white arrowheads show the PHA granules in the cytoplasm of the endosymbiont; white arrows demonstrate the virus-like particles; d – nucleolus elements are marked with white arrows; f – white arrow points to the dividing endosymbiont in the host cell Ma. Scale bar a – 2 μm, b, c, e – 200 μm, d, f – 500 мкм.

4.1.3 Morphology and ultrastructure of the endosymbiot retrieved from the ciliate *P. multimicronucleatum* LSA11-2

Analysis of the fine sections showed that the bacterial cell wall structure corresponds to the Gram-negative morphotype (Fig. 7b). In TEM images, as well as in living cells, rod-like and ovoid forms of bacteria could be easily distinguished. Small electron dense granules, obviously, ribosomes, and rounded electron lucid inclusions were seen in the cytoplasm of both bacterial forms. Besides that, electron dense virus-like particles were found in the majority of the endosymbionts. In our sections the virus-like particles used to be located around some fine fibrous material resembling viroplasm, most often arranged regularly (Fig. 7c, e). Nucleoid elements were not observed. Although living cell observations of the actively moving endosymbionts suggested the presence of a well-developed locomotory apparatus in these bacteria, flagella were nearly invisible in fine sections (Vannini,…, Mironov et al., 2014). Occasionally, dividing forms occurred among endosymbiotic bacteria, which argues for proliferative capacities of the bacteria inside the host nucleus.

Since fine section analysis failed to reveal flagella, which could account for high motility of the endosymbionts, on the bacterial surface, we took two approaches enabling discrimination of appendages on the bacterial cell: negative staining using uranyl acetate and atomic force microscopy. Negative contrast staining of the endosymbionts from the Ma of the ciliate *P. multimicronucleatum* with uranyl acetate revealed numerous flagella on the rod-like bacteria, characteristic of the polytrichs (Fig. 8). The length of a flagellum could reach 10 µm, while its width – 10 nm. The size of a rod-like bacterium determined with TEM was 2-2.5 µm long and 0.6-0.8 µm wide (Vannini,…, Mironov et al., 2014).

Analysis made with atomic force microscopy also revealed two forms of the endosymbionts with different morphology, however, because of the greater thickness of the ovoid forms it was not possible to obtain their satisfactory images. The dimensions of the rod-like bacteria corresponded to the size estimated with negative staining, being 2-2.5, length, 0.6-0.8 -width. Assessing the surface of bacteria by means of atomic force microscopy also permitted us to reveal numerous flagella evenly distributed on the cell surface (Fig. 9), supporting our data obtained with negative staining technique.

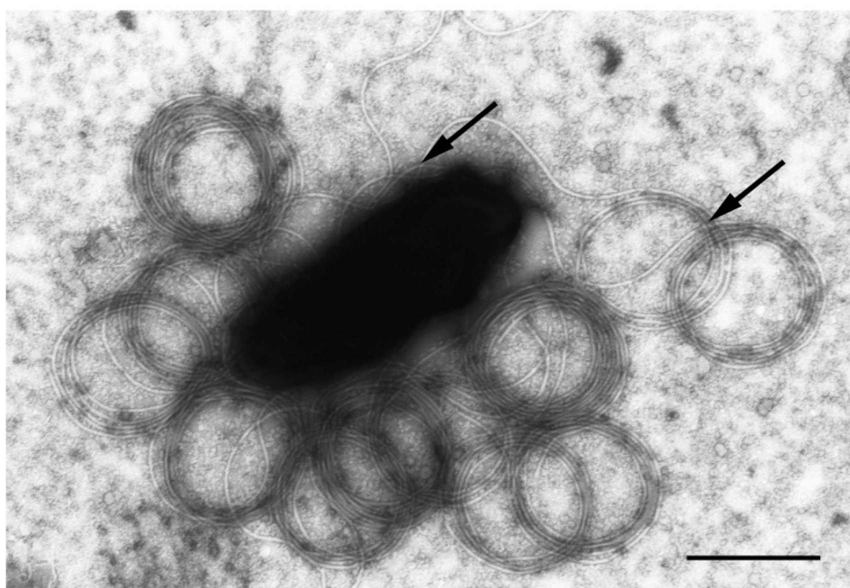


Fig. 8. Endosymbiotic bacterium isolated from the ciliate *P. multimicronucleatum,* strain LSA11-2. Negative staining, TEM. Arrows point to flagella. Scale bar, 1 μm. From Vannini,…, Mironov et al., 2014, with modifications.

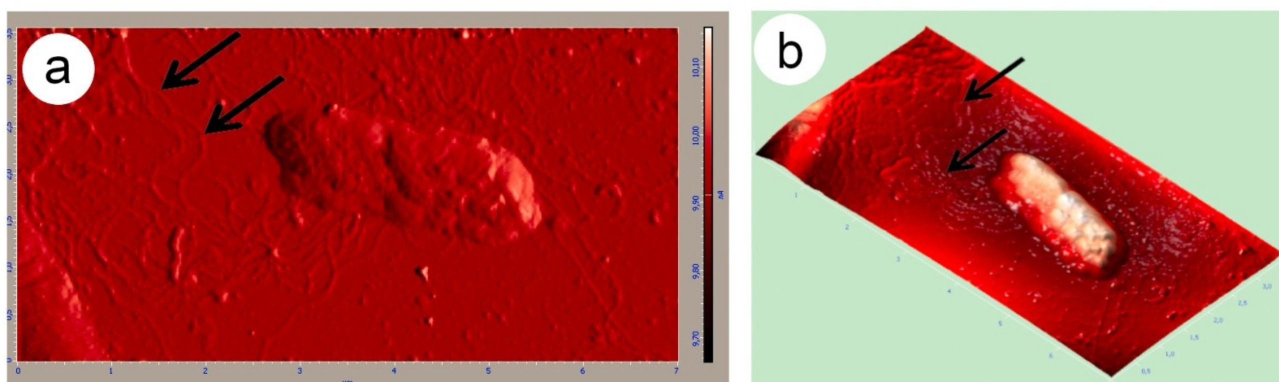


Fig. 9. Endosymbiotic bacterium isolated from the ciliate *P. multimicronucleatum*, strain LSA11-2. Atomic force microscopy; a – mag image, b – 3D model. Black arrows point to bacterial flagella.

4.1.4 Phylogenetic position and taxonomy of the intranuclear endosymbionts of the ciliate *P. multimicronucleatum*, strain LSA11-2

Phylogenetic relationship of the motile intranuclear bacteria inhabiting strain LSA11-2 was established by analysis of the 16S rRNA gene fragment (Vannini,…Mironov et al., 2014). After PCR amplification using primers RickFla_F69 (5′-GTTAACTTAGGGCTTGCTC-3′), RickFla_F87 (5′-CTCTAGGTTAATCAGTAGCAA-3′), RickBas_F166 (5′-ATGCTAATGCCGTATATTCTC-3′), Rick_R1270 (5′-TTTTAGGGATTTGCTCCACG-3′), Rick_R1455 (5′-CCGTGGTTGGCTGCCT-3′) followed by sequencing using the same primers we obtained a gene fragment of 1433 b.p. which was

registered in the NCBI nucleotide data base (accession number HG315611) (Vannini,…,Mironov, et al., 2014). The search in NCBI BLASTn data base showed that the obtained sequenceFlu has 97,3% homology with the sequences of members of *Rickettsiaceae* family (Rickettsiales).

Fluorescence *in situ* hybridization with specially designed oligonucleotide probes TrichoRick_142 and RickFla_430 specific for the obtained sequence (Vannini,…,Mironov, et al., 2014) confirmed that this sequence belonged to the intranuclear endosymbiotic bacterium, and not to the food bacteria or contaminations (Fig. 10, 11). In the Ma of the strain LSA11-2 intensive fluorescence was observed both, in red (TrichoRick_142/RickFla_430-Cy3), and in green (Eub_338-Fluo3) channels. In the images obtained by merging two channels, colocalization of the two signals was observed, since intranuclear endosymbionts were recognized by the two probes simultaneously. At the same time, phagosomes demonstrated only green fluorescence, which resulted from hybridization of the eubacterial probe with food bacteria serving as a negative control. DAPI staining permitted us to determine the nuclear localization of the endosymbiont (Fig. 10d, 11d).

Having ascertained that the obtained sequence indeed belonged to the endosymbiont and is not a result of contamination, phylogenetic analysis using 32 sequences was performed in collaboration with our Italian colleagues. Sequences of two other families of *Rickettsiales* were taken as an outgroup. Analysis of these sequences showed that the motile intranuclear endosymbiont of *P. multimicronucleatum* strain LSA11-2 forms a sister branch both, to validly described rickettsia of arthropods including causative agents of severe human diseases (*Rickettsia rickettsia, R. prowazekii, R. typhi*) and to the organisms related to R. limonia, which have a wider host range, but which have not got a formal description (Fig. 12). According to modern view, a species should be considered a representative of the genus *Rickettsia* only in case its 16S rRNA gene sequence has at least 98.1% identity with the sequences of other representatives of this genus (Fournier, Raoult, 2009).

Thus, the motile intranuclear endosymbiont of the ciliate *P. multimicronucleatum* represents a new genus in the family *Rickettsiaceae*. According to the International Code of Nomenclature of Prokaryotes (ICNP), uncultivable bacteria characterized only basing on the DNA analysis are granted the *Candidatus* status. Due to the presence of a great number of flagella ensuring the motility of this bacterium in the host nucleus, we proposed for this endosymbiont the name *Ca*. Trichorickettsia mobilis (Vannini, ….Mironov et al., 2014).

It should be noted that another species of Rickettsia-like endosymbionts, having a broad host range including ciliates, *Ca*. Megairapolyxenophila, belongs to another clade (Fig. 12).

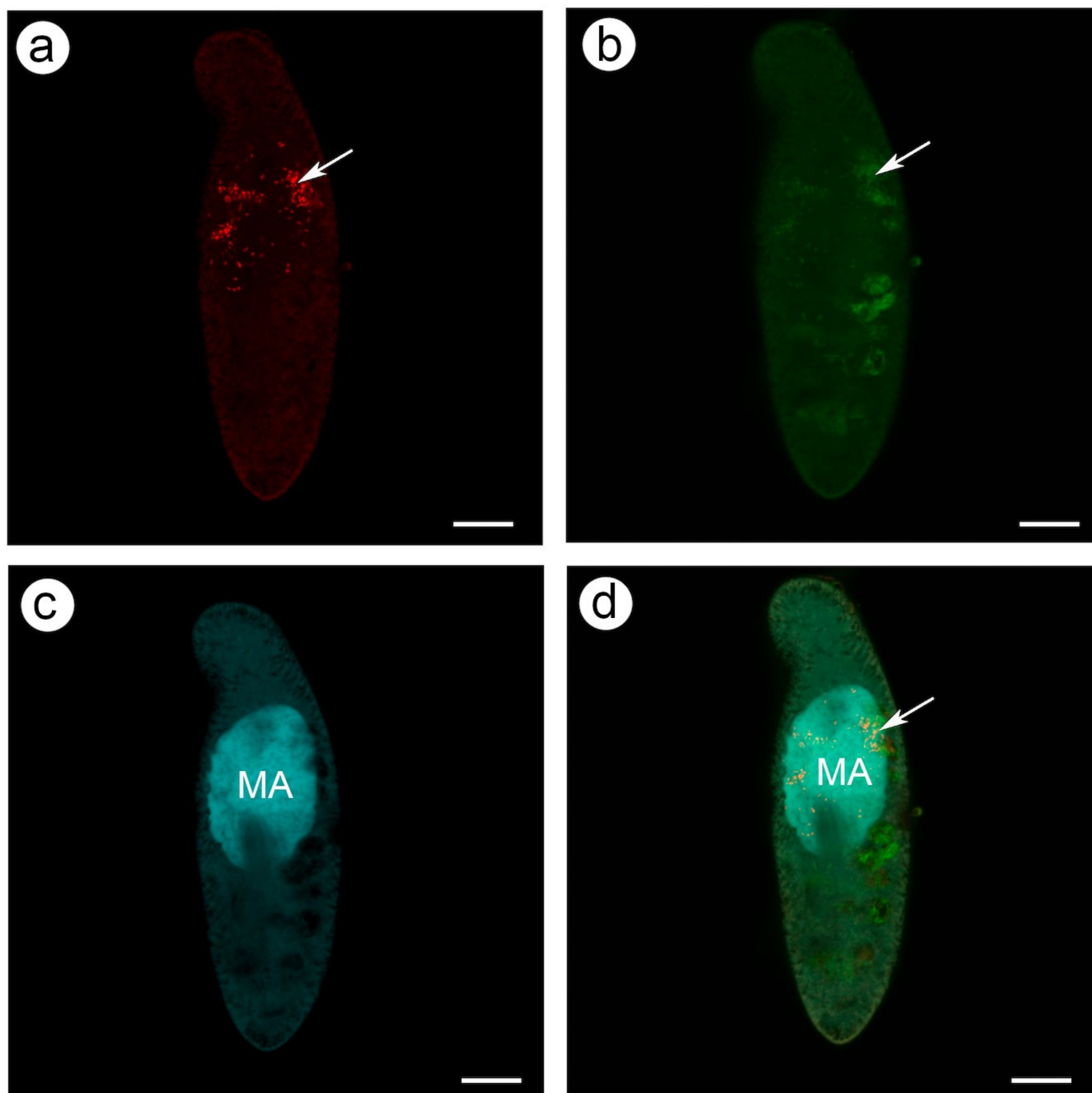


Fig. 10. Ciliate *Paramecium multimicronucleatum* (strain LSA11-2). Fluorescence in situ hybridization, confocal laser scanning microscopy. a - TrichoRick_142 probe (Cy3, red signal); b – eubacterial Eub_338 probe (Fluo3, green signal); c – ciliate macronucleus revealed with DAPI (cyan); d – merge (image obtained in three channels merged). Endosymbiotic bacterium is revealed with both, species specific and eubacterial probes, which results in yellow signal. Scale bar, 10 µm.

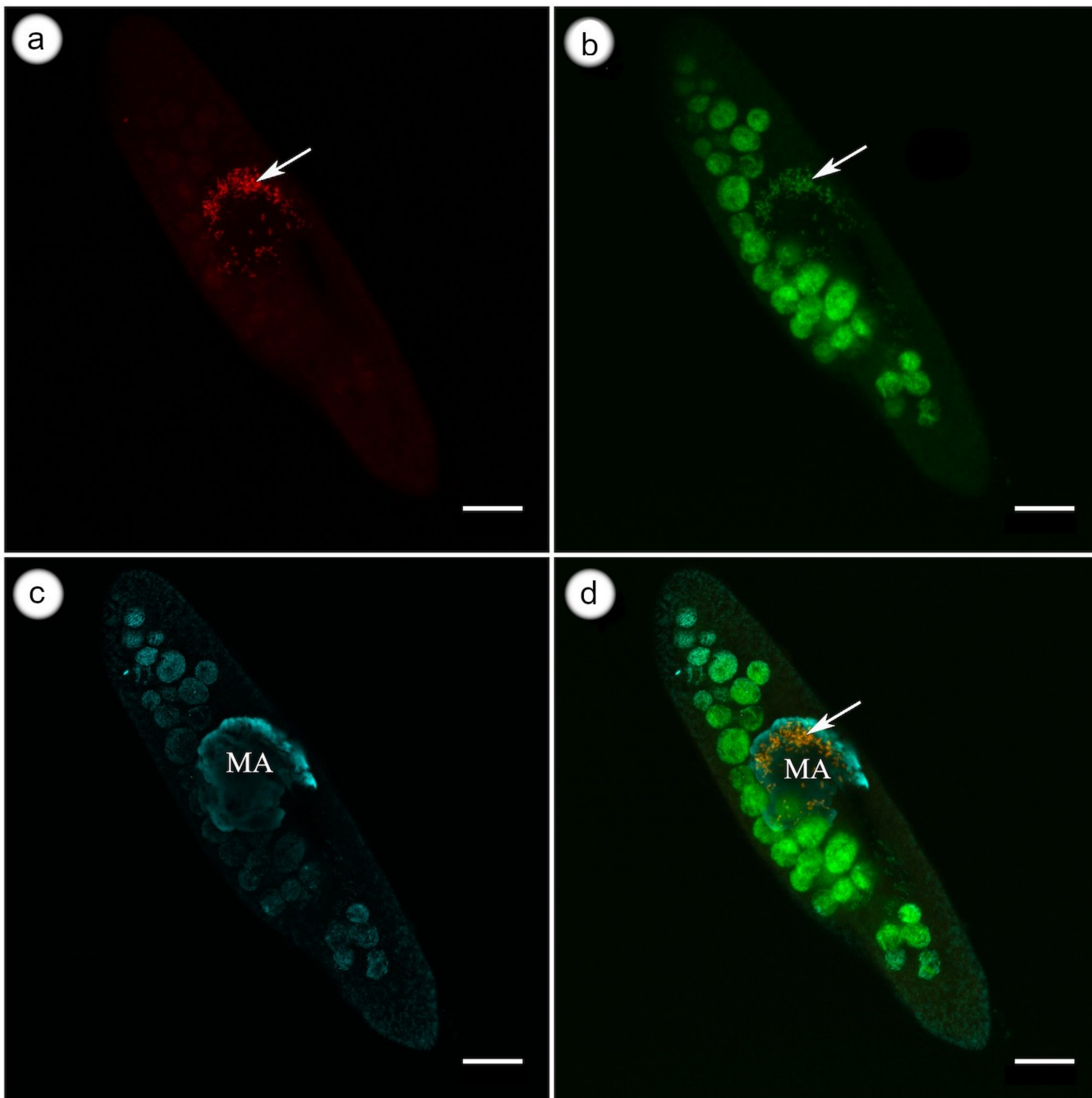


Fig. 11. Ciliate *Paramecium multimicronucleatum* (strain LSA11-2). Fluorescence in situ hybridization, confocal laser scanning microscopy. a – species specific probe RickFla_430 (Cy3, red signal); b – eubacterial Eub_338 probe (Fluo3, green signal); c – ciliate macronucleus revealed with DAPI (cyan); d – merge (image obtained in three channels merged). Endosymbiotic bacterium is revealed with both, species specific and eubacterial probes, which results in yellow signal. Scale bar, 10 μm.

4.1.5 Biological properties of the ciliates of the LSA11-2 strain and their endosymbiont *Ca*. Trichorickettsia mobilis

In none of the experiments aimed at revealing the killer-effect, have we registered the naïve cell death at any time. Thus, paramecia infected with motile intranuclear endosymbionts do not possess any killer capacity towards non-infected cells.

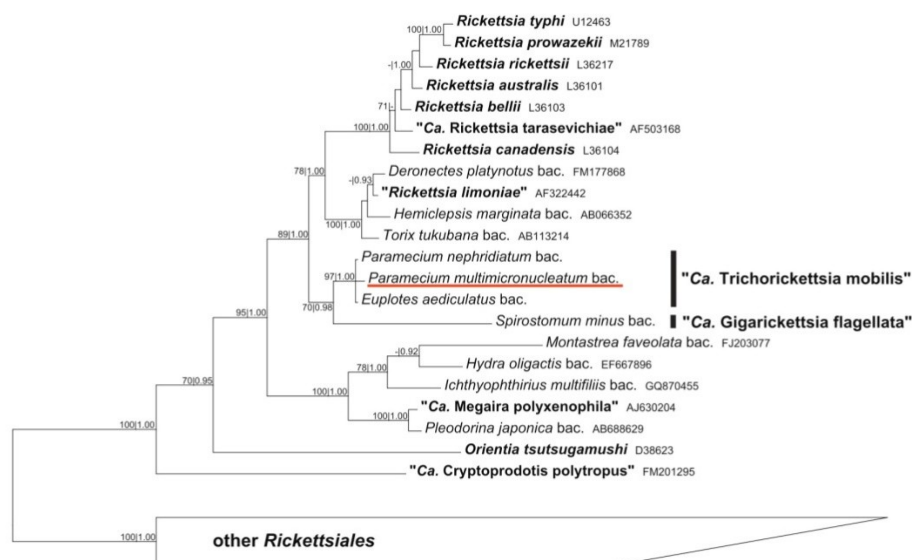


Fig. 12. Bayesian phylogenetic tree of bacteria belonging to Rickettsiaceae family. The tree was built employing GTR+I+G model. Numbers associated to nodes represent Maximum Likelihood bootstraps and Posterior Probabilities. Values below 70|0.85 are omitted. Taxa that received a formal or a provisional name are in bold. Bac – bacterium associated with. From Vannini, ….Mironov et al., 2014, with modifications.

None of experimental infections of endosymbiont-free *P. multimicronucleatum* strains with the homogenate made of the infected ciliates was successful, which evidences for the absence of infectious capacity of the bacterium *Ca*. Trichorickettsia mobilis under experimental isolation of the endosymbionts from the host cell.

4.2 Affiliation of macronuclear endosymbionts from the *P. multimicronucleatum* strains Busnau, Кр154-4 and AB9-4 to the species *Ca*. Trichorickettsia mobilis

Two more strains of *P. multimicronucleatum* harbouring motile endosymbionts in Ma revealed with Nomarsky optics (Kr154-4 and AB9-4) were maintained in RC CCM ciliate culture collection of the Core Facility Centre for Cultivation of Microorganisms of SPbU. *P. multimicronucleatum* Busnau strain from the collection of Technical University of Stuttgart also contained motile bacteria in Ma.

Fluorescence *in situ* hybridization with the species-specific oligonucleotide probe RickFla_430 designed for LSA11-2 strain confirmed that these three species belonged to the newly described species *Ca*. Trichorickettsia mobilis (Fig. 13), which enabled further studies of the stability of this endosymbiotic system using all four strains.

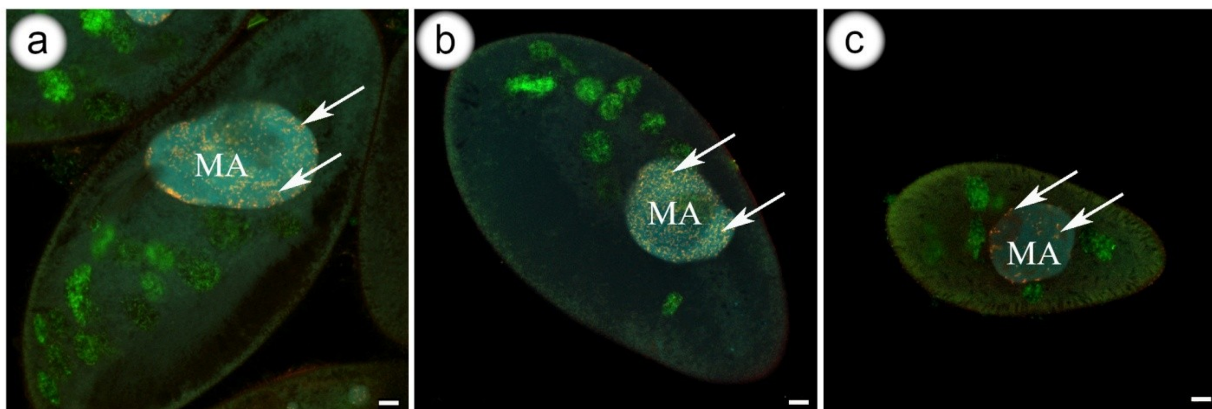


Fig. 13. Bacteria *Ca*. T. mobilis in different strains of *P. multimicronucleatum.* Fluorescence *in situ* hybridization using RickFla_430 probe conjugated to Cy3 (red signal) and the eubacterial Eub_338 probe conjugated with Fluo3 (green signal), the nuclei counterstained with DAPI. A merge image (three channels merged) is shown for each strain. White arrows point to the symbiotic bacteria in Ma. a – Kr154-4; b – AB9-4; c – Büsnau. Scale bar, 10 µm. From Mironov, Sabaneyeva, 2020, with modifications.

4.3 Effect of antimicrobial agentsadministration on viability of the partners of the symbiotic system *P. multimicronucleatum* / *Ca.* Trichorickettsia mobilis

4.3.1 Effect of antibiotic administration on viability of the partners of the symbiotic system *P. multimicronucleatum* / *Ca.* Trichorickettsia mobilis

Prior to the experiments aimed at assessment of the influence of antibiotics on the viability of the endosymbiont and the host ciliate, each strain was checked for the presence of the endosymbionts using Nomarsky contrast or FISH experiments with the species-specific probe RickFla_430 (Vannini et al., 2014). All strains (LSA 11-2, Busnau, Кр154-4 иAB9-4), except the control ones (MSA и CyP5-3), demonstrated 100% prevalence of infection with *Ca*. T. mobilis; all endosymbiotic bacteria were located exclusively inside the macronucleus, were motile and were never found in the cytoplasm. Results of experimental treatments with streptomycin, ampicillin and chloramphenicol of the four

strains infected with *Ca*. T. mobilis and the control trichorickettsia-free strain MSA are summarized in a table (Table 3).

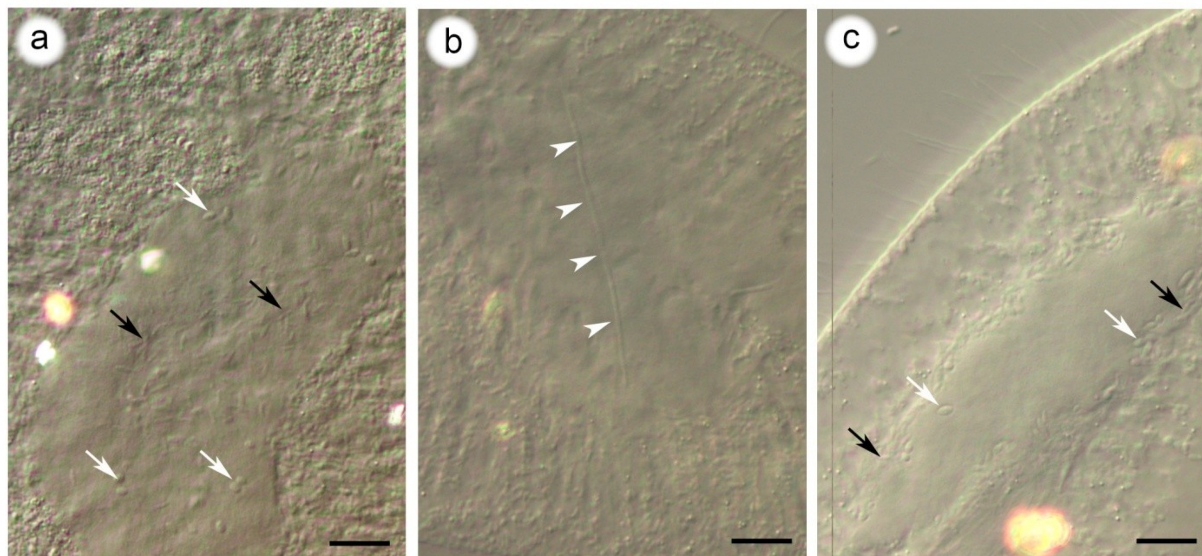
Table 3. The effect of streptomycin, ampicillin and chloramphenicol on *Paramecium multimicronucleatum*/*Ca*. Trichorickettsia mobilis symbiotic system.

antibiotic	concentration	100 µg/mL			250 µg/mL			500 µg/mL			1000 µg/mL		
	day strain	3	10	15	3	10	15	3	10	15	3	10	15
Streptomycin	LSA11-2	+b	+b	+b	+b	+b	+b	+b	+b	+b	-	-	-
	Büsnau	+b	+b	+b	+b	+b	+b	+b	+b	+b	-	-	-
	Кр154-4	+b	+b	+b	+b	+b	+b	+b	+b	+b	+b	+b	+b
	AB9-4	+b	+b	+b	+b	+b	+b	+b	+b	+b	+b	+b	+b
	MSA (contr)	+	+	+	+	+	+	+	+	+	+	+	+
Ampicillin	LSA11-2	+bf	+bf	+bf	+bf	+bfo	+bf	+bf	+bfo	+bf	+bfo	+bfo	+bfo
	Büsnau	+bf	+bfo	+bf	+bf	+bfo	+bf	+bf	+ bfo	+bf	+bfo	+bfo	+bfo
	Кр154-4	+b	+b	+b	+bf	+bf	+bf	+bf	+bf	+bf	+bfo	+bfo	+bfo
	AB9-4	+b	+b	+b	+ bf	+ bf	+ bf	+ bf	+ bf	+ bf	+ bfo	+ bfo	+ bfo
	MSA (контр)	+	+	+	+	+	+	+	+	+	+	+	+
Chloramphenicole	LSA11-2	-	-	-	-	-	-	-	-	-	-	-	-
	Büsnau	-	-	-	-	-	-	-	-	-	-	-	-
	Кр154-4	+b	+b	+b	+b	+b	+b	+b	+bfo	+bfo	-	-	-
	AB9-4	+b	+b	+b	+b	+b	+b	+b	+bo	+b	-	-	-
	MSA (контр)	+	+	+	+	+	+	+	+	+	-	-	-

«+» – ciliates alive, «-» – 100% ciliates dead, «b» – motile bacteria observed in Ma of ciliates, «o» – ovoid forms of bacteria present, «f» – filamentous forms present.

As seen from the table, streptomycin at all concentrations used affected neither viability of the host cells, nor motility or nuclear location of their endosymbionts. The only exception was the highest concentration (1000 µg/mL) used with LSA11-2 and Büsnau strains, which resulted in the host cell death in both strains as early as by the 3rd day after the start of the experiment. Administration of ampicillin did not affect viability of the host or its endosymbiont, but led to the changes of the

morphotype of the latter. Alongside with the rod-like motile bacteria non-motile ovoid and/or filamentous forms of trichorickettsia could be observed (Fig. 14a, b). Filamentous forms of bacteria could reach more than 50 µm long and occurred in LSA11-2 and Büsnau strain in all ampicillin concentrations. In AB9-4 and Kr154-4 strains, the filamentous forms were observed starting with 250



µg/mL concentration and ovoid forms were registered only under the highest concentration, 1000 µg/mL (Fig. 14a, b).

Fig. 14. *Ca*. Trichorickettsia mobilis in the macronucleus of *Paramecium multimicronucleatum* after antibiotic administration. Cells fixed with paraformaldehyde, Nomarsky contrast. a –LSA11-2, 10th day after ampicillin administration (1000 µg/ml); b - LSA11-2, 3rd day after ampicillin administration (500 µg/ml); c – Kr154-4, 10th day after chloramphenicol administration (500 µg/ml). Short rod-like cells are marked with black arrows, ovoid forms – with white arrows. Arrowheads point to filamentous forms of bacteria. Scale bar, 10 µm. From Mironov, Sabaneyeva, 2020, with modifications.

Interestingly, treatment with ampicillin caused the exit of trichorickettsia from the Ma into the host cytoplasm. In FISH experiments using Alf1b probe specific to Alfaproteobacteria (Fig. 15) and the species-specific probe RickFla_430 (Fig. 16) the positive signal was registered both, in Ma and beyond it. Noteworthy, the signal occurred outside the food vacuoles, the contents of which were revealed with the nearly universal eubacterial probe Eub_338 and were not associated with the species-specific probe RickFla_430 (Fig. 16).

Formation of ovoid and filamentous trichorickettsia was also observed in the ciliates of AB9-4 and Kp154-4 strains under chloramphenicol administration at 500 µg/ml (Table 3, Fig. 14c). It should be noted that the effect of chloramphenicol differed significantly in various trichorickettsia-infected strains. Contrary to the effect of ampicillin, administration of chloramphenicol to the ciliates of LSA11-2 and Büsnau strains in any concentration led to the cell death. At the same time the ciliates of

AB9-4 and Kp154-4 strains survived and maintained motile endosymbionts in all chloramphenicol concentrations, except for the highest, 1000 μg/ml, which was lethal both for all infected ciliates and for the control non-infected strain MSA. This concentration caused the host cell death as early as in several hours after the start of the experiment (Table 3).

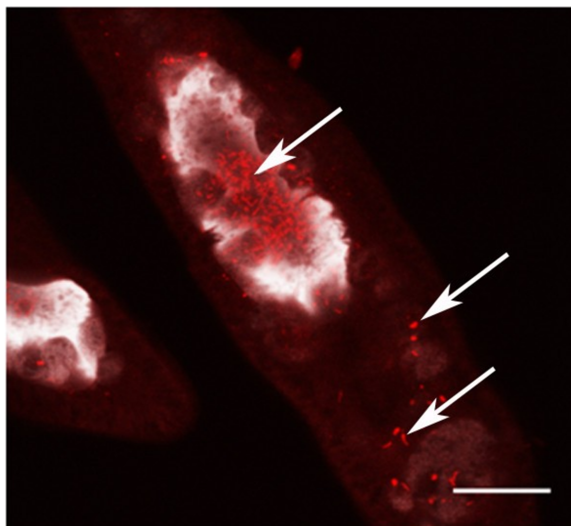


Fig. 15. *P. multimicronucleatum* (strain LSA11-2), 3d day after the ampicillin treatment. FISH using the Alfaproteobacteria-specific probe Alf1b (Cy3, red signal). Arrows point to trichorickettsia inside the macronucleus (counterstained with DAPI, white signal) and in the cytoplasm. Scale bar, 20 μm. From Mironov, Sabaneyeva, 2020, with modifications.

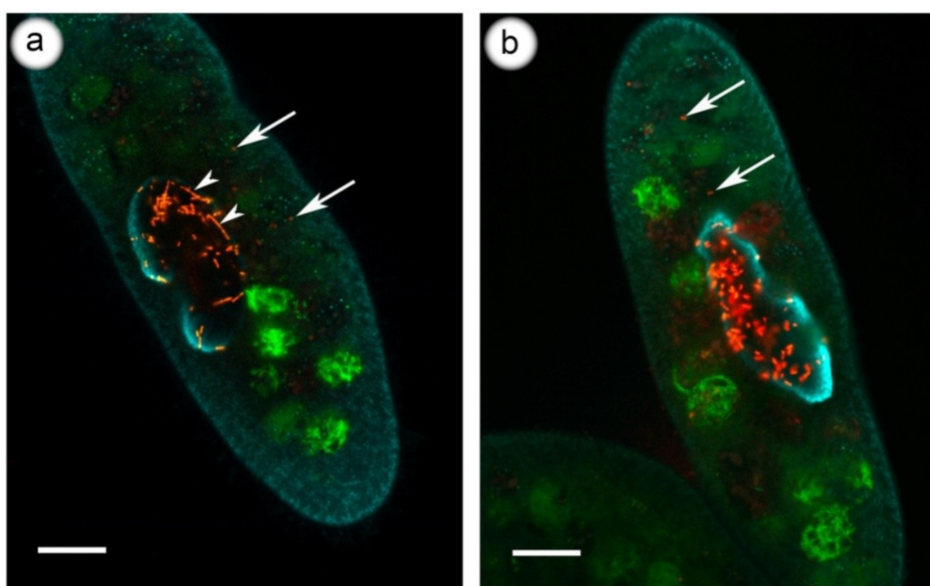


Fig. 16. *Paramecium multimicronucleatum* (strain Kr154-4) infected with the intranuclear Ca. Trichorickettsia mobilis on the 15th day of the repeated ampicillin administration (1000 μg/ml). Fluorescence in situ hybridization with the species-specific probe RickFla_430 (Cy3, red signal) and the eubacterial probe Eub_338 (Fluo3, green signal), macronucleus slightly counterstained with DAPI (cyan) to see the nuclear contour. Confocal laser scanning microscopy. a – Filamentous forms (white

arrowheads) are seen in the macronucleus alongside with the short rod-shaped bacteria; b – some trichorickettsia (arrow) can be seen outside the host macronucleus; the bacterium marked with a two-headed arrow resides in the vacuole devoid of food bacteria. Scale bar, 10 µm. From Mironov, Sabaneyeva, 2020, with modifications.

Since Büsnau strain was lost in the course of time, the effect of tetracycline treatment was assessed using the three remaining strains — LSA11-2, Kr154-4, and AB9-4. These three strains and the non-infected control strains MSA and CyP5-3 proved to be highly susceptible to tetracycline, which had to be used in the concentrations which were10-fold less, than those used with the three other antibiotics tested. Only under the lowest tetracycline concentration, 10 µg/mL, paramecia of all strains survived the treatment (Table 4).

Table 4. Effect of tetracycline on infected with trichorickettsia and non-infected strains of the ciliate *P. multimicronucleatum.*

Strain/concentration	10 µg/ml	25µg/ml	50 µg/ml	100 µg/ml
LSA11-2	+b	-	-	-
Kp154-4	+b	-	-	-
AB9-4	+b	-	-	-
MSA (control)	+	-	-	-
CYP5-3 (control)	+	-	-	-

«+» – ciliates alive, «-» – 100% ciliates dead, «b» – motile bacteria observed in Ma of ciliates.

All surviving infected with trichorickettsia ciliates maintained their motile endosymbionts in Ma. In none of the experiments did we manage to obtain aposymbiotic cell lines (trichorickettsia cleared from the host cells).

4.3.2 Fine structure and localization of trichorickettsia after ampicillin treatment

In the intact ciliates, the outer and the plasma membranes of *Ca*. T. mobilis were usually rather closely apposed, so that the periplasmic space is hardly discernable, regardless of the way of fixation (Fig. 17).

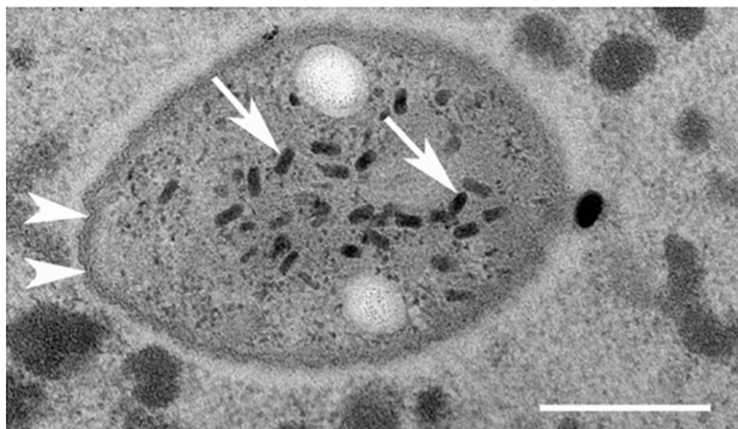


Fig. 17. Endosymbionts *Ca*. T. mobilis in the macronucleus of *P. multimicronucleatum* (strain Kr154-4) in the absence of antibiotic treatments. Transmission electron microscopy. White arrowheads point to the outer membrane tightly adjacent to the plasma membranes; virus-like particles in the bacterial cells are marked with white arrows. Scale bar, 200 nm. From Mironov, Sabaneyeva, 2020, with modifications.

In the macronucleus of the ciliates treated with ampicillin, in the same cross-section, alongside with the bacteria possessing tightly adjacent outer and plasma membranes, some part of the endosymbionts demonstrated enlarged periplasmic space, suggesting disorders in the bacterial cell wall (Fig. 18a-e). Independently of ampicillin concentration and duration of the treatment, the infection in Ma was preserved, bacterial cells with the enlarged periplasmic space being registered on the 5th day after the start of the experiment, as well as on the 15th day. Some endosymbionts located in the macronucleus were surrounded by a thin layer of fine fibrous material with electron density similar to that of the interchromatin compartment, but differing from the latter by regular arrangement (Fig. 18f). Interestingly, in bacteria enclosed in fine fibrous material the virus-like particles showed signs of disassembly, their outlines being less sharp, however, no perceptible detachment of the outer membrane of the bacterial cell which could evidence for the cell wall disorders was noted. Since all our TEM images represented cross or oblique sections of the bacteria, it was not possible to distinguish filamentous forms of *Ca.* T. mobilis from the rod-shaped forms in the host Ma with certainty.

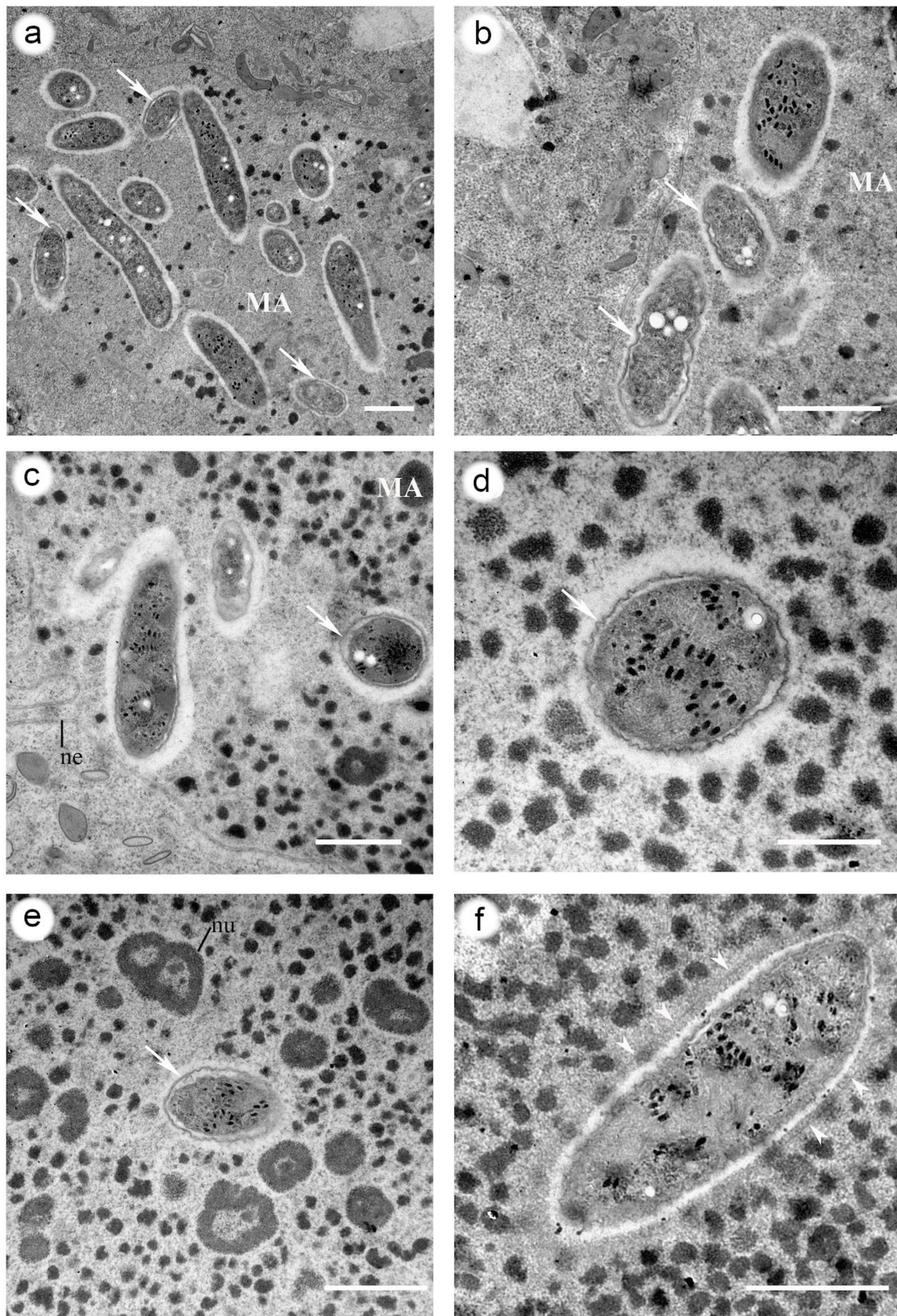


Fig. 18. *Ca*. T. mobilis in the macronucleus of *P. multimicronucleatum* (strain Kr154-4) after repeated ampicillin treatments. Transmission electron microscopy. a, b, f – 5th day, c, e – 15th day after the start of the experiment; a-e – bacterial cells with the detached outer membrane are marked with arrows; f – a bacterium surrounded by a layer of fine fibrous material (arrowheads). MA, macronucleus; ne, nuclear envelope; nu, nucleoli. Scale bar, a – e – 1 µm; f – 500 nm. From Mironov, Sabaneyeva, 2020, with modifications.

By means of electron microscopy we managed to confirm our observations of bacterial egress from the host macronucleus upon ampicillin treatment made with FISH. The bacterial forms released from the host macronucleus only occasionally lay “naked” in the host cytoplasm (Fig. 19a), sometimes, with a fine horseshoe-like cistern resembling a phagophore found in their vicinity. However, most of the endosymbiotic bacteria residing in the host cytoplasm after ampicillin treatment were located inside the vacuoles bounded by a single membrane (Fig. 19b-e). In some cases, lysosomes were found in the close proximity to the endosymbiont containing vacuole and seemed to fuse with it (Fig. 19b). “Naked” *Ca*. T. mobilis and most of the endosymbionts enclosed in the cytoplasmic vacuoles looked very much the same as those located inside the macronucleus, however, we frequently observed patterns suggesting the exit of virus-like particles from the bacterial cell upon its getting into the host vacuole (Fig. 19c, d). The contents of these vacuoles were of medium electron density, comparable with the contents of lysosomes. Noteworthy, the endosymbionts inside these vacuoles were always surrounded by an electron lucid halo, presumably resulting from the numerous poorly preserved bacterial flagella. In rare cases the virus-like particles underwent disassembly, while the endosymbiont manifested blebbing of its surface (Fig. 19d). The general appearance of the endosymbiont containing vacuoles (Fig. 19a-e), differed significantly from the newly formed phagosomes with the food bacteria (Fig. 19f). The former contained exclusively *Ca*. T. mobilis, which were easily recognizable by the presence of the virus-like particles (Fig. 19a-e), and never included any food bacteria. On the contrary, the phagosomes enclosed only the food bacteria, the space between them being always electron lucid (Fig. 19f), which distinguished them from the vacuoles containing the endosymbionts.

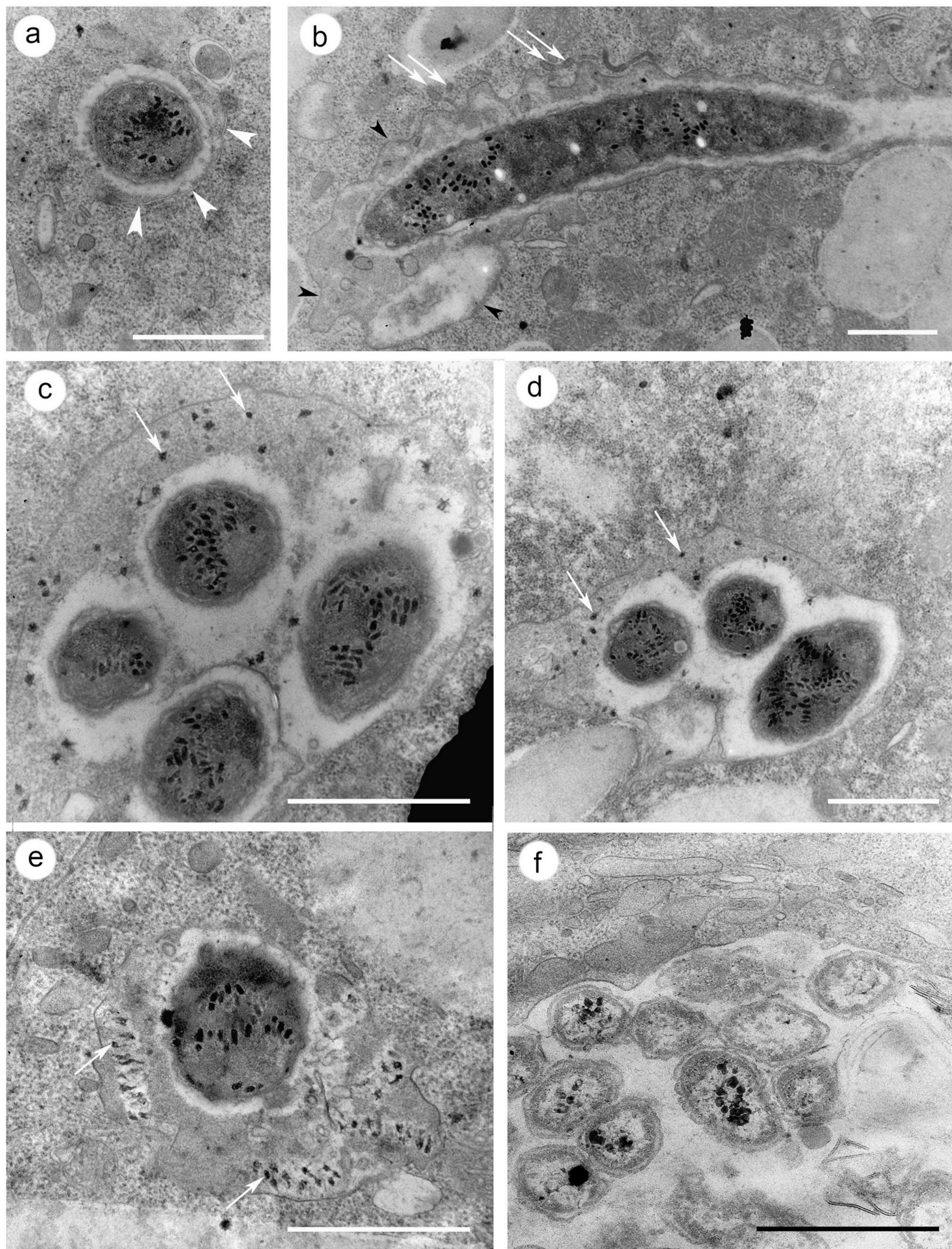


Fig. 19. *P. multimicronucleatum* (strain Kr154-4) infected with trichorickettsia on the 5th day after repeated ampicillin treatment. Transmission electron microscopy; a–d –*Ca*. Trichorickettsia mobilis in the host cytoplasm; a – the "naked" bacterium lying free in the cytoplasm (cross section); a horseshoe-like cisterna resembling a phagophore is shown with white arrowheads; b –oblique section of the filamentous form enclosed in a vacuole (black arrowheads point to the vacuole membrane, white arrows indicate the sites of its fusion with lysosomes); c, d, e – cross sections of bacteria inside the vacuoles in the host cytoplasm (arrows point to the virus-like particles outside the bacteria); f – food bacteria *Klebsiella aerogenes* in a food vacuole. Scale bar, 1 µm. From Mironov, Sabaneyeva, 2020, with modifications.

4.3.3 The effect of the antimicrobial peptide complex FLIP7 on *Paramecium multimicronucleatum*/Ca. Trichorickettsia mobilis symbiotic system

In order to assess the range of FLIP7 concentrations to be used in further experiments, preliminary experiments were performed with high (0.2, 2, 10 and 20 mg/mL) and low (20 и 100μg /mL) concentrations of FLIP7; the experiment was repeated only once for each concentration. First, the cells of the infected Kr154-4 and the endosymbiont free CyP5-3 strains were treated with high FLIP7 concentrations. Administration of FLIP7 at high concentrations caused death of ciliates, both, of the endosymbiont bearing and of the control ciliates. The highest concentration of FLIP7 (20 mg/mL) caused death of all ciliates as early as in 1 min after the start of the experiment, while in the lowest concentration (0.2 mg/mL) all ciliates died in 20 min. The control cells demonstrated similar timing data for all concentrations. With lower FLIP7 concentrations (20 и 100μg /mL) ciliates of both strains survived the short-term (1 h) treatment, cell death was registered only for the control CyP5-3 strain on the 3d day after administration of FLIP7 100μg /mL). All infected ciliates maintained motile endosymbionts in Ma.

In further experiments, FLIP7 was used at concentrations of 20, 100, 200 and 400 μg/mL. Viability of two infected strains, Kr154-4 and LSA11-2, and the control Esa2-3 strain was tested in three independent experiments performed at different days. The effects of the short-term (1h) and long-term (7 days) treatments with lower FLIP7 concentrations on the viability of the ciliates and the ability of the infected cells to maintain the endosymbiotic bacteria in their macronucleus are summarized in Table 5. At 20 μg/mL, FLIP7 did not affect ciliate viability in the experimental and in the control, Esa2-3, strains. The same was true for the short-term treatment (1h) with 100 μg/mL FLIP7, however in 7 days after the start of the experiment the ciliates of the control strain died, while the trichorickettsia-infected cells survived. The control strain and one of the infected strains (Kr154-4) proved to be more susceptible to FLIP7 used at 200 μg/mL and died as early as in 1h after the beginning of the experiment, while the other Trichorickettsia-bearing strain, LSA11-2, appeared to be more resistant and remained alive even on the 7th day of the experiment. Thus, in the further experiments only the more susceptible strain Kr154-4 was used. The highest concentration of FLIP7 administered in these experiments (400 μg/mL) proved to be deleterious for both, Trichorickettsia-bearing and Trichorickettsia-free strains. All surviving ciliates of the LSA11-2 and Kr154-4 strains maintained motile endonucleobionts in their Ma.

Table 5. The effect of antimicrobial peptide complex FLIP7 on the symbiotic system P. multimicronucleatum/*Ca*. T. mobilis on the 1st and the 7th day after administration.

Strain	FLIP7 concentration							
	20µg/mL		100 µg/mL		200 µg/mL		400 µg/mL	
	1h	7d	1h	7d	1h	7d	1h	7d
ESa3-2 (control)	+	+	+	-	-	-	-	-
Kp155-4	+b	+b	+b	+b	-	-	-	-
LSA11-2	+b	+b	+b	+b	+b	+b	-	-

«+» – ciliates alive, «-» – 100% ciliates dead, «b» – motile bacteria observed in Ma of ciliates. From: Mironov et al, 2022, with modifications.

4.3.4 Localization and ultrastructure of bacteria *Ca*. Trichorickettsia mobilis after administration of antimicrobial peptide complex FLIP7 to the host ciliates

As demonstrated by living cell observations and fluorescence in situ hybridization (FISH) with the species-specific oligonucleotide probe RickFla_430, bacteria *Ca*. T. mobilis were detected in Ma of all surviving ciliates of the LSA11-2 and Kr154-4 strains (Fig. 20, Fig. 21). Interestingly, on the 7th day after FLIP7 administration at 100 µg/mL ovoid forms of Trichorickettsia wereobserved in Ma of host ciliates (Fig. 20a).

In FISH experiments performed using the species-specific strain RickFla_430, although most part of Trichorickettsia were still localized in the macronucleus of the host cell, single bacteria surrounded by DAPI-positive chromatin were present in the host cytoplasm (Fig. 21).

By means of TEM, ovoid forms of Trichorickettsia residing in the Ma of the infected ciliates treated with FLIP7 at 100 µg/mL (Fig. 22a, b). Their appearance resembled that of the bacteria found in non-treated paramecia (Fig. 22c); these forms are characterized by the presence of virus-like electron-dense particles and electron-lucid areas, apparently, corresponding to polyhydroxyalkanoate (PHA) inclusions. In the cytoplasm of Trichorickettsia-bearing ciliates subjected to FLIP7 treatment large vacuoles packed with elongated bacterial forms much smaller in diameter than the ovoid forms and overloaded with PHA inclusions were registered (Fig. 22a, b). Importantly, we did not manage to find any virus-like particles in Trichorickettsia located in the vacuoles in the host cytoplasm. Interestingly, a few smaller forms with PHA inclusions were noted outside the host cell in the vicinity of the ciliate cortex, suggesting that these forms could be released from the host cell (Fig. 22d).

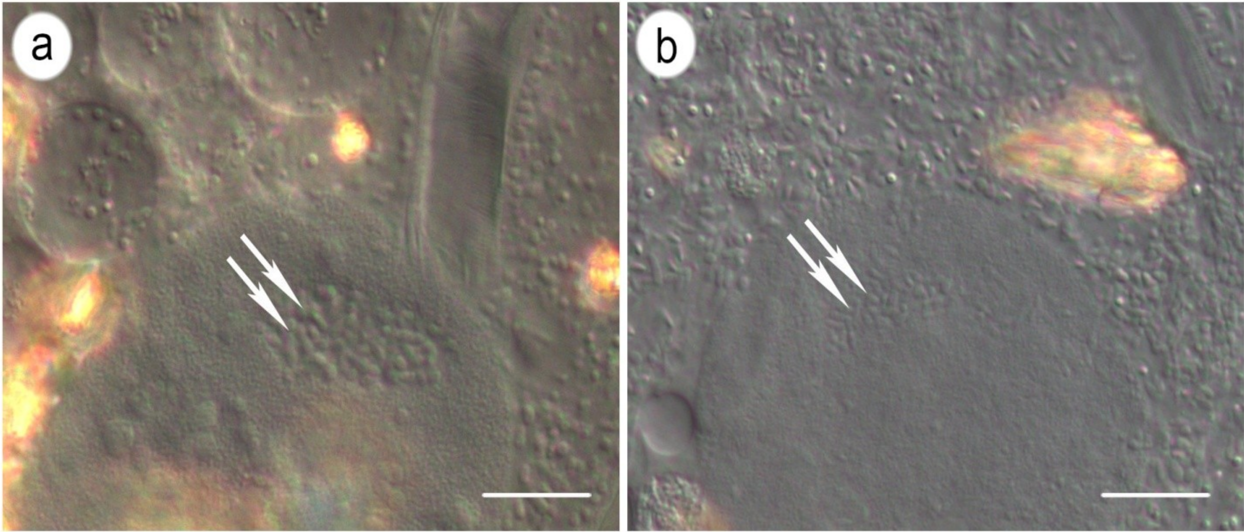


Fig. 20. Living ciliate *P. multimicronucleatum* (strain Kr154-4) infected with *Ca*. T. mobilis. a – 7 days after FLIP7 administration (100 μg/mL), b – no treatment. Arrows point to the endosymbionts in the host cell macronucleus. Scale bar 10 μm. From: Mironov et al., 2022, with modifications.

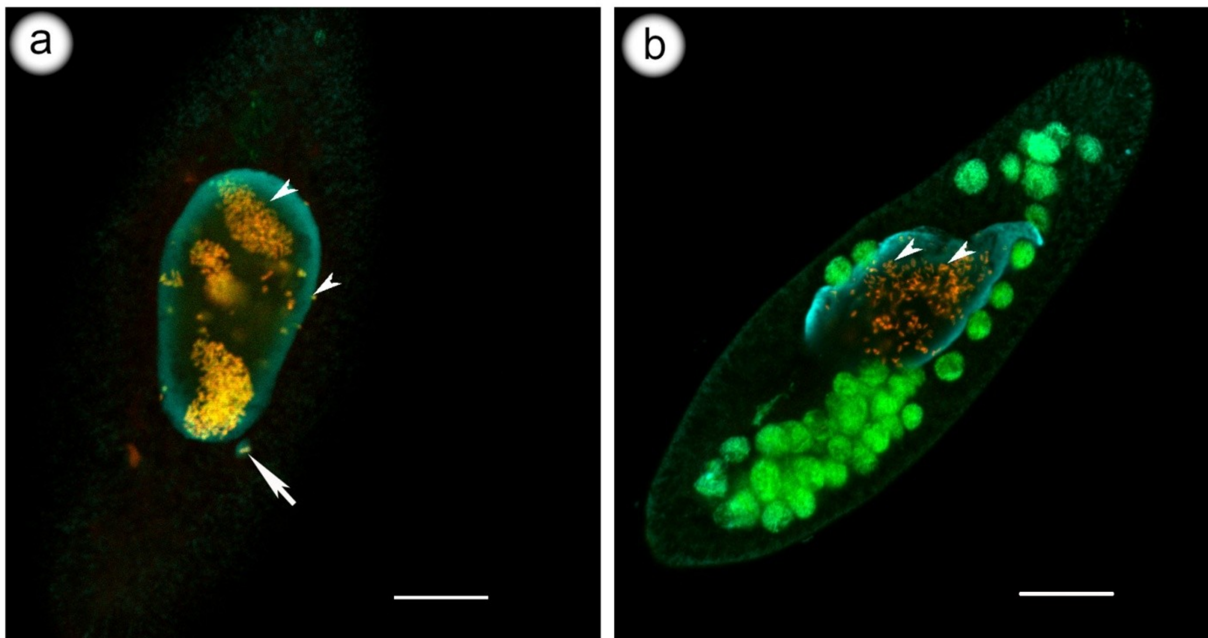


Fig. 21. Ciliate *P. multimicronucleatum* infected with *Ca*. T. mobilis. Fluorescence in situ hybridization with the species-specifc RickFla_430 probe (red signal) and the nearly universal probe for eubacteria Eub338 (green signal), macronucleus counterstained with DAPI. Confocal laser scanning microscopy. a − 7 days after FLIP7 administration, b – untreated cell. Trichorickettsia are orange-yellow due to overlay of the two channels (white arrowheads). Arrow points to the bacterium located beyond Ma boundaries. Scale bar 20 μm. From: Mironov et al., 2022, with modifications.

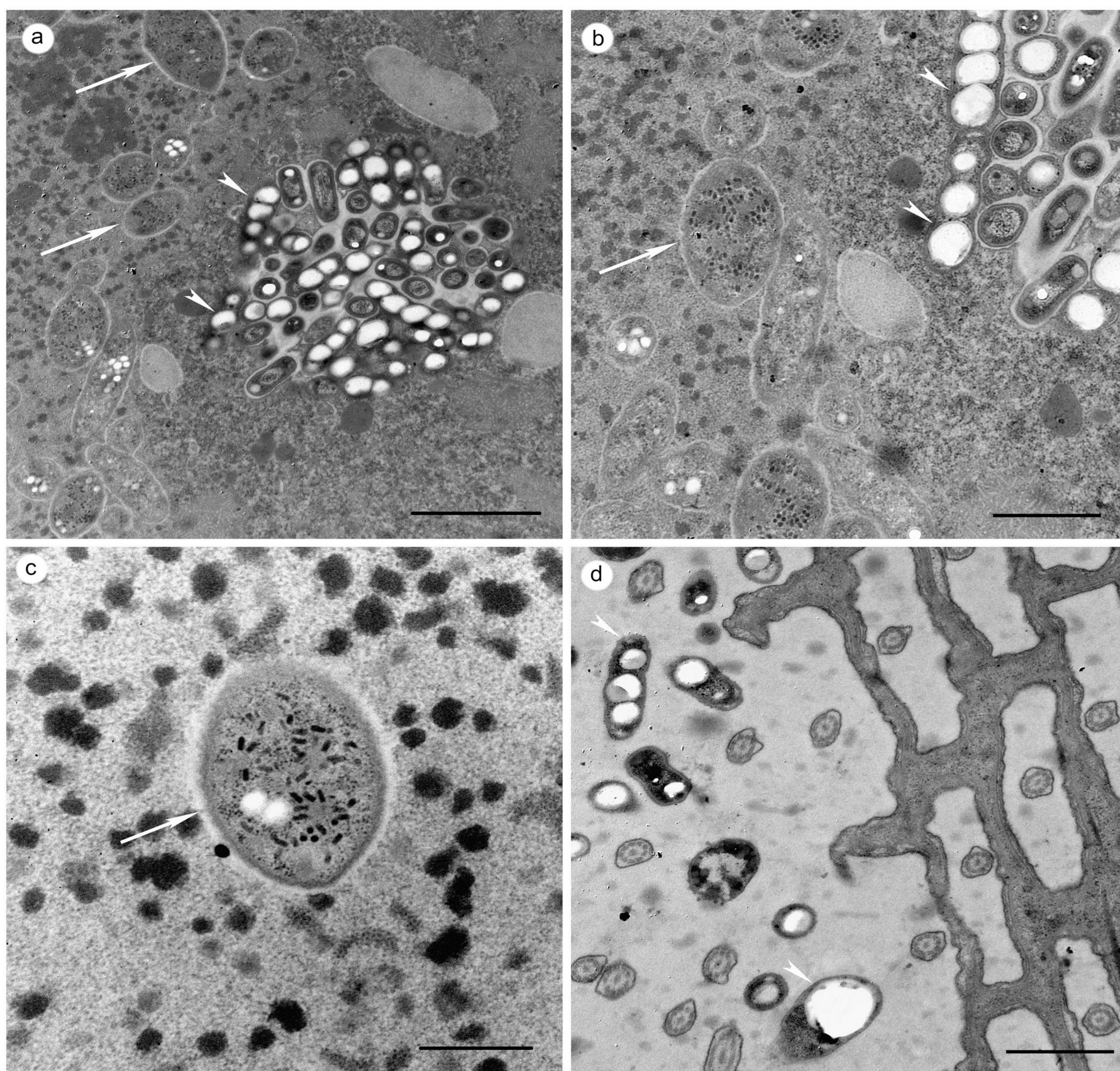


Fig. 22. Fine structure of the two forms of Trichorickettsia in *P. multimicronucleatum*, transmission electron microscopy. a, b, d – after FLIP7 administration, c – control. Arrows point to the forms with virus-like particles, arrowheads – to the smaller forms with PHA granules. Scale bar a – 2 μm, b, d – 1 μm, c – 0.5 μm. From: Mironovetal., 2022, with modifications.

4.3.5 The effect of FLIP7 HPLC fractions

In order to assess the effectivity of 47 different HPLC fractions of FLIP7, we chose the strain Kr154-4 infected with *Ca*. T. mobilis as the most susceptible to antimicrobial peptide complex FLIP7. The final concentration of each tested fraction was 1.2 mg of FLIP7 eqvl./mL. The activity of the fractions against the ciliates *P. multimicronucleatum*is shown in Fig. 23. In 5 min after the treatment all

ciliates died in the presence of fractions 12, 16-19, 23, 26-39. In the case of three fractions (7, 15 and 20) the ciliates appeared swollen in 20 min after the start of the experiment and died in 30 min; these fractions were considered "sublethal". In ciliates treated with fractions 6, 14, 24 and 25, we did not observe any external changes, however, the cells also died in 30 min after start of the experiment, and they were also considered "sublethal". In the rest of the tested fractions the ciliates stayed viable, motile endosymbionts being present in the macronucleus.

Since the goal of the further study was not only to compare the viability of Trichorickettsia-bearing and Trichorickettsia-free cell lines and the ability of the former to maintain their endosymbionts in various FLIP7 fractions, but also to detect the fraction which would remove Trichorickettsia from the host cells, so as to obtain an aposymbiotic cell line, "lethal" (12, 17, 23, 26, 30, 31, 33, 36) and "sublethal" (7, 15, 20) fractions were selected for further experiments and used at lower concentrations (120, 240 and 360 μg of FLIP7 eqvl./mL).

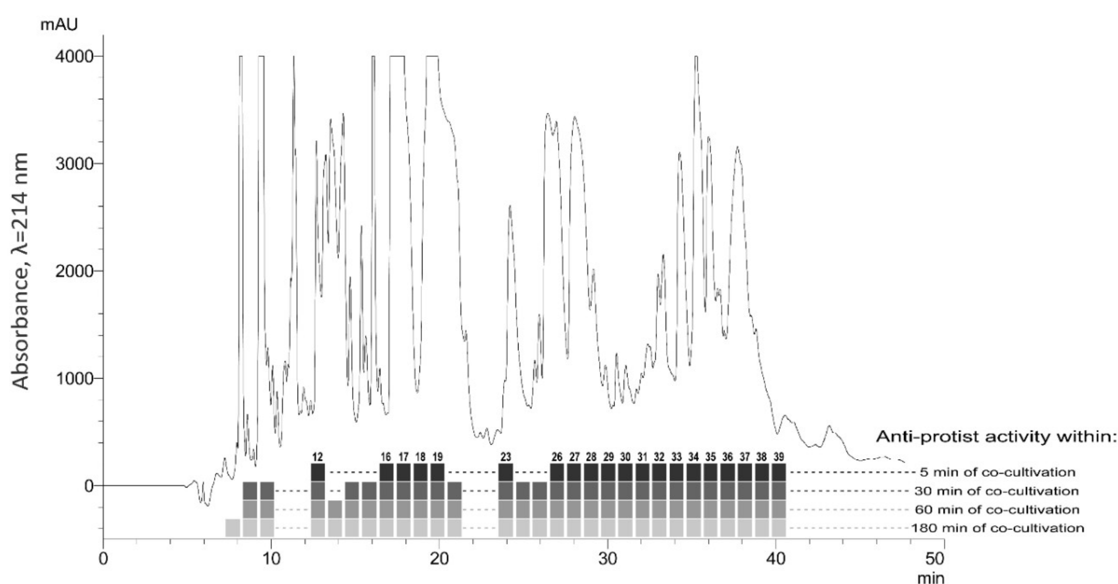


Fig. 23. Activity of chromatographic fractions of the antimicrobial peptide complex FLIP7 (1.2 mg FLIP7 eqvl./mL) tested on Trichorickettsia-bearing *P. multimicronucleatum.* The activity of each fraction (ciliate death time) was mapped on the chromatographic profile (histograms in the areas of the corresponding fractions). From: Mironov et al, 2022, with modifications.

The results of these treatments are shown in Table 6. Both, the endosymbiont harboring Kr154-4 cells and the control Cyp5-3 cells survived in all fractions used at a concentration of 120 μg of FLIP7 eqvl./mL. A twofold increase of concentration (240 μg of eqvl. /mL of FLIP7) led to the death of the Trichorickettsia-free control ciliates in the fractions 20 and 33 in 1h after the treatment, while in all other fractions the ciliates survived. At the highest concentration (360 μg of FLIP7 eqvl. /mL), fractions 20, 26, 33 and 36 caused death of the control non-infected ciliates, and only the

presence in the medium of the fraction 33 caused death of the infected Kr154-4 strain. In all concentrations of all FLIP7 fractions tested, motile endosymbiotic bacteria were always registered in the surviving ciliates of the Kr154-4 strain.

Table 6. Survival of Trichorickettsia-bearing and Trichorickettsia-free strains of ciliates *P. multimicronucleatum* under FLIP7 fractions.

Fraction N	**Strain**	**Fraction concentration per whole FLIP7**		
		120 мкг/мл	240 мкг/мл	360 мкг/мл
7	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
12	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
15	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
17	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
20	CyP5-3(control)	+	-	-
	T. - bearing	+	+	+
23	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
26	CyP5-3(control)	+	+	-
	Кр154-4	+	+	+
30	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
31	CyP5-3(control)	+	+	+
	Кр154-4	+	+	+
33	CyP5-3(control)	+	-	-
	Кр154-4	+	+	-
36	CyP5-3(control)	+	+	-
	Кр154-4	+	+	+

«+» –ciliates survive within 1 h after fraction administration, «-» –ciliates die within 1 h. From: Mironov et al., 2022, with modifications.

In the long-term experiment Trichorickettsia-bearing Kr154-4 strain and the endosymbiont-free strain CyP5-3 were treated with the same FLIP7 fractions used at the lowest concentration (120 µg), the viability of the cells was assessed on the 7th day after the start of the experiment. Viability of Trichorickettsia-bearing and the endosymbiont-free cells differed drastically. The endosymbiont-free control cells (CyP5-3 strain) died in all fractions tested, while the ciliates of the endosymbiont harboring strain Kr154-4 died only in the 7th, 17th and the 33d fraction. Surprisingly, in the 12th and the 30th fractions the host cell number seemed to increase (about 68-70 cells compared to 50 taken in the experiment). Correspondingly, about one third of the cells divided at least once during the experiment, suggesting that these fractions do not affect the cell division. As in the previous experiments, all surviving cells of the Kr154-4 strain continued to maintain motile bacteria in their macronucleus.

5. Discussion

5.1 Intranuclear localization of motile endosymbionts of the ciliate *P. multimicronucleatum*

Analysis of the sequence encoding 16S rRNA of the endosymbiont demonstrated a high rate of homology (97.3% identity) to the sequences of other representatives of the family *Rickettsiaceae* (order *Rickettsiales*). Bacteria of the order Rickettsiales have a very wide host range (Merhej, Raoul, 2011). Some of them are human pathogens, others are endosymbionts of arthropods, and lately, a growing number of representatives of this group has been found in protists (Vannini, 2010; Castelli et al., 2019). Nuclear localization is typical for a number of representatives of the family *Rickettsiaceae*, e.g., rickettsia from the Spotted Fever Group (SFG) inhabit exclusively the nucleus of a eukaryotic cell (Yu, Walker, 2005). Moreover, Ogata and colleagues (Ogata et al, 2006) found that *Rickettsia bellii*, belonging to the basal group of rickettsia, which separated before divergence of the two groups, Spotted Fever Group (SFG) and Typhus Group (TG), is also capable of invading the host nucleus and propagate inside it. Obviously, the nucleus is a cell compartment safely protected from the attack of lysosomal hydrolases, which provides endonucleobionts with the medium rich in nutrients; besides, intranuclear location is optimal for an endosymbiont as regards to possibility of controlling host gene expression (Осипов, 1981; Bierne, Cossart, 2012).

Other endonucleobionts of paramecia, representatives of the genera *Holospora* and *Caedimonas*,are assigned at present to the order *Holosporales.* Relatonship of *Holosporales* and *Rickettsiales* is still debated (Castelli et al., 2018). Phylogenetic position of the intranuclear endosymbiont *Nanospora macronucleata*, previously described in *Paramecium caudatum*, still remains uncertain.

5.2 Flagella in bacteria of the order *Rickettsiales*

The data obtained with atomic force microscopy and negative contrast unequivocally demonstrated the presence of numerous flagella on the surface of the intranuclear endosymbiont of *P. multimicronucleatum.* This is not surprising, since vigorous motility of bacteria in the nucleus perceivable in the host nucleus during living cell observations with Nomarsky contrast, *a priori* suggested their existence. At the same time, according to the description of the order Rickettsiales given in the Bergey's Manual of Systematic Bacteriology (Volume 2: The Proteobacteria), all members of this order lack flagella. However, studies of the endosymbiont inhabiting mitochondria of the tick *Ixodes ricinus*, *Midichlora mitochondrii*, have revealed 26 genes coding for the flagellum proteins, though no flagella have been found using transmission electron microscopy (Mariconti, 2012).

Besides, the genus *Lyticum* from the cytoplasm of paramecia, the representatives of which bear non-functional flagella, has been redescribed (Boscaro et al., 2013).Basing on the 16S rRNA gene analysis, the genus *Lyticum* was assigned to a new family, *Midichloriaceae* (order*Rickettsiales*). These data provided the basis for the suggestion that the ancestors of bacteria from the order *Rickettsiales* once had possessed functional flagella, which were lost in the course of evolution (Sassera et al., 2011, Mariconti et al., 2012; Boscaro et al., 2013, Castelli et al., 2019). The fact that we managed to find a representative of the order *Rickettsiales* with actively functioning flagella supports this suggestion with high probability and evidences for the presence in some representatives of this order not only of genes coding for the flagella proteins, but of actively functioning flagella as well. At the same time, we cannot exclude the possibility of the secondary origin of flagella and motility in the intranuclear symbionts of *P. multimicronucleatum* as a result of horizontal gene transfer, e.g., from some other bacterium capable of forming symbioses with ciliates. Cases of horizontal gene transfer between bacteria inhabiting one and the same protist have been registered in literature (Ogata et al., 2006). Electron-dense particles resembling viral capsids, which could take part in gene exchange, were found in the cytoplasm of *Ca*. T. mobilis. In principle, independent appearance of flagella in the representatives of one and the same order is possible, but at the present time this hypothesis is based only on assumptions (Vannini,…Mironov, et al., 2014). The final choice of a particular hypothesis will be feasible only in case of full genome characteristics of the endosymbiont. However, our data argue against the belief that all *Rickettsiales* lack flagella and require reconsidering the description of the order *Rickettsiales.*

5.3 Phylogenetic position and taxonomy of the motile intranuclear endosymbionts

The obtained sequence of the 16S rRNA gene of the motile intranuclear endosymbiont had only 97.3% identity with the representatives of the family *Rickettsiaceae* (order *Rickettsiales*) (Vannini,…Mironov, et al., 2014). Following the guidelines given by the prominent specialists in the field of studying *Rickettsiales* (Fournier, Raoult, 2009), according to which a bacterial species can be included in the genus Rickettsia only if its 16S rRNA gene sequence shows a similarity value of 98.1% or higher with respect to other members of the genus, the motile intranuclear endosymbiont of the ciliate P. multimicronucleatum belongs to a new genus in the family *Rickettsiaceae*. In the course of studies performed by our Italian colleagues, bacteria with nearly identical sequences were found in Ma of another strain of *P. multimicronucleatum,* and in the cytoplasm of the ciliates *P. nephridiatum* and *Euplotes aediculatus*(Vannini,…Mironov, et al., 2014). Since this bacterium is incultivable outside the host cell, it should be granted the “Candidatus” status. To emphasis the phylogenetic relationship of

this bacterium to rickettsia and its peculiar feature – the presence of numerous flagella, the proposed name for this bacterium was "*Candidatus* Trichorickettsia mobilis" (Vannini, …, Mironov et al., 2014).

Diagnosis of "*Candidatus* Trichorickettsia mobilis". *Trichorickettsia mobilis* (Tric.ho.ric.ket'tsi.a mo'bi.lis; Gr. masc. n. thrix, hair, N.L. fem. n. *Rickettsia*, from the name of a related genus, N.L. fem. n. *Trichorickettsia*, hairy *Rickettsia*; L. adj. mobilis, motile). Rod-shaped bacterium, up to 2.6 mm in length and 1.3 mm in width. Macronuclear or cytoplasmic symbiont of protist ciliates of the genera *Paramecium* and *Euplotes*. Displaying flagella and swimming behavior inside the host cell of *P. multimicronucleatum*. Electron-dense cytoplasm, frequently hosting particles resembling viral capsids with various shapes arranged in regular fashions. Belonging to the family *Rickettsiaceae* in the order *Rickettsiales*. Basis of assignment: 16S rRNA gene sequence (accession number: HG315612) and positive match with the specific FISH oligonucleotide probe TrichoRick_142 (5'-GTTTCCAAATGTTATTCCATAC-3'). Uncultured thus far (Vannini, …, Mironov et al., 2014).

5.4 Occurrence of the species *Ca.* Trichorickettsia mobilis

Analysis of *P.multimicronucleatum* strains from RC CCM culture collection of the Core Facility Centre for Cultivation of Microorganisms of SPbU by means of fluorescence in situ hybridization with the species-specific probe revealed two more strains (AB9-4 иKp154-4) of this ciliate species harbouring bacteria *Ca*. T. mobilis in the Ma. Morphology and the fine structure of the bacterial endosymbiont of the strain AB9-4 were described by Vishnyakov and Rodionova as early as in 1999 (Vishnyakov, Rodionova, 1999), however, their phylogenetic position still remained obscure. This strain was isolated from nature in Boston (USA) in 1994 and has not lost its endosymbionts during more than 25 year existence in the laboratory culture, which evidences for the high stability of the symbiotic system. The strain Kr154-4 originated from a population isolated in Krasnoyarsk, while the strain Büsnau, kindly provided by Dr. M. Schweikert, was retrieved from a waterbody in Germany. *Ca*. T. mobilis, inhabiting the cytoplasm of the of *P. nephridiatum* and *Euplotes aediculatus*, was found in the strains isolated in Italy and in India, correspondingly, and trichorickettsia, described later in the cytoplasm of *P. calkinsi* originated from the population isolated from the wastewater stream on Cyprus (Sabaneyeva et al., 2018). Thus, the bacterium *Ca*. T. mobilis is widespread among the ciliates of the Northern hemisphere, occurs in different ciliate hosts and can reside both, in the nucleus and in the cytoplasm of the host cell. The absence of host specificity suggests the existence of horizontal transfer of the endosymbiont between different hosts, alongside with vertical transmission.

5.5 The importance of studying “Non-model *Rickettsiales*”

Symbiotic systems including protists as hosts may become a valuable source of information about diversity and evolution of bacteria belonging to the order *Rickettsiales*. Our results suggest that certain peculiarities of organization in bacteria ancestral to the order *Rickettsiales* (e.g., the presence of flagella) could have been preserved in the symbionts of protists (Blanc et al., 2007), which might have been the original hosts of *Rickettsiales* bacteria. Besides that, it should be noted that all most known representatives of the family *Rickettsiaceae* have been found in the terrestrial organisms, while *Ca*. T. mobilis inhabits freshwater and sea organisms, i.e., in absolutely different conditions. Lately, an increasing number of *Rickettsiales* bacteria, often referred to as “non-model Rickettsiales” is being found in sea (Sunagawa et al., 2009; Vannini et al., 2005) and freshwater (Ferrantini et al., 2009; Schrallhammer et al., 2013; Fraune, Bosch, 2007; Kawafune et al., 2012; Castelli, 2016) organisms. The studies of the representatives of this group can elucidate the evolutionary pathways of *Rickettsiales* bacteria. Since the bacterium *Ca*. T. mobilis is closely related to pathogenic rickettsia, causative agents of severe human diseases, the symbiotic system *P. multimicronucleatum*/*Ca.* T. mobilis can serve as a model for studying fine mechanisms of the host-endosymbiont interactions.

5.6 Susceptibility of the symbiotic system *P. multimicronucleatum/Ca.* T. mobilis to antibiotic treatment

Symbiotic system *Paramecium multimicronucleatum*/*Ca*. Trichorickettsia mobilis demonstrated high stability under antibiotic treatments. Streptomycin, the antibiotic of the aminoglycoside group with a wide range of action, which is often used for obtaining aposymbiotic cell lines in ciliates (Grosser et al., 2018), proved to be absolutely ineffective. These results are in good agreement with the data obtained in the course of treating human rickettsioses with aminoglycosides (Rolain, Raoult, 2005). Also, *Ca*. T. mobilis demonstrated resistance to ampicillin, one of the penicillin group of antibiotics. This is not very surprising taking into account resistance of pathogenic rickettsia to the majority of beta-lactam antibiotics (Rolain et al., 1998). Contrary to our expectations, we did not manage to obtain aposymbiotic cell lines with antibiotics used in clinical practice for the treatment of rickettsioses, either (chloramphenicol, tetracycline). Exposure to high concentrations of chloramphenicol turned out to beharmful for ciliates, which is in accordance with the data on high toxicity of this drug (Shukla et al., 2011). Presumably, toxic effect of chloramphenicol is related to disorders in protein synthesis in the host mitochondria (Barnhill et al., 2012).Our attempt to obtain endosymbiont-free cells after tetracycline administration failed apparently due to high susceptibility of the host cells to tetracycline. The spectrum of activity of this group of antibiotics is known to

encompass some parasitic protists, such as *Plasmodium falciparum*, *Giardia lamblia*, *Trichomonas vaginalis* and some others (Chopra et al., 2001). Although molecular mechanism of the antiparasitic effect of tetracyclines in protists remains unknown, it is believed that it could be caused by the inhibition of the protein synthesis in their mitochondria (Chopra et al., 2001). Taking into account our data obtained with tetracycline administration, one may suggest that *P. multimicronucleatum* falls in the list of eukaryotes susceptible to tetracycline treatment as well. Possibly, tetracycline, like chloramphenicol, affects protein synthesis in the mitochondria of this species. Surprisingly, representatives of the same genus, *P. primaurelia* and *P. pentaurelia*, seem not to be susceptible to tetracycline, as aposymbiotic cell lines have been obtained with higher concentrations of this antibiotic while clearing the ciliates from *Ca*. Megaira polyxenophila (Pasqualetti et al., 2020).

In general, antimicrobial susceptibility testing in obligate intracellular bacteria is a challenging task, since it is limited by a range of the applicable tests (Vanrompay et al., 2017). One of a few assessed parameters applicable for uncultivable bacterial strains is the minimal inhibitory concentration (MIC value). Moreover, estimation of the final effective antibiotic concentration still remains elusive, as the concentration inside the host can differ from that in the culture medium (Wisseman, 1982). Besides that, the half-life of the particular antibiotic should differ depending on peculiarities of the host cell metabolism. Therefore, for the same intracellular bacterium, e.g., *Chlamydia*, MICs may differ in different cell lines (Suchland et al., 2003). In our study, we observed the differences of the ciliate strains (LSA11-2 and Büsnau *versus* Kr154-4 and AB9-4) in their susceptibility to administration of chloramphenicol and the highest concentration of streptomycin. The reason for higher vulnerability of the two strains (LSA11-2 and Büsnau) remains unclear. We suggest that the differences in strain susceptibility to antibiotics might be connected either with the strain differences at the level of gene transcription and protein modifications, or, alternatively, it could be another example of intraspecies variability at the genome level, like the one observed in *Paramecium calkinsi* infected with cytoplasmic *Ca*. T. mobilis (Sabaneyeva et al., 2018).

Overall, the antibiotic resistance pattern of *Ca*. T. mobilis seems to be very similar to that of pathogenic rickettsia, however, we did not manage to expel the endosymbionts from the host cells by administering antibiotics used as a first-line treatment of rickettsioses. All our experiments led to two possible outcomes: either the host survived together with its endosymbiont, or the host died. Apparently, in the latter case the death of the host was accompanied by the death of the endosymbiotic bacteria. There could be two possible explanations of these data. On the one hand, it is tempting to suggest that the host might have become dependent on its endosymbiont, and the death of the ciliate might be caused by the death of its endosymbionts upon administration of tetracycline or a high dosage of chloramphenicol. On the other hand, we cannot exclude another option – that tetracycline and high concentration of chloramphenicol may be harmful for the host itself, possibly, by affecting its

mitochondria.Be that as it may, so far, it seems impossible to separate the partners of the symbiotic system *P. multimicronucleatum/Ca*. Trichorickettsia mobilis, which can be regarded as an ideal model holobiont in both, original and modern, senses of this term, as it not only represents a host with an inherited endosymbiont, but appears as a real single entity demonstrating significant stability under antibiotic pressure.

5.7 Release of the Intranuclear Bacteria into the Host Cytoplasm

A thin layer of a fibrous material surrounding some of the bacterial cells in the host nucleus looks very much like the one seen around *Holospora obtusa* in the Ma of the nonspecific host *P. multimicronucleatum* under experimental infection under nocodazole treatment before the bacteria are expelled from the macronucleus (Fokin et al., 2005). This layer has been proposed to play a role in the extrusion of bacteria from the Ma, however, it is not clear whether this is a feature typical only for *P. multimicronucleatum*, or it is a more general cellular mechanism for extrusion of alien material from the nucleus. Interestingly, a similar layer was registered around the infectious forms of two other endonucleobionts residing in the macronucleus of two other species of paramecia - *Holospora curvata* from *P. calkinsi* and *Holospora bacillata* from *P. woodruffi* (Fokin, Sabaneyeva, 1997). In this case, the fibrillar layer has been also believed to be connected with the release of the endosymbionts from the host macronucleus. Thus, formation of such layer seems to be typical at least for *Paramecium* species. The composition of this layer remains enigmatic.

5.8 Formation of Persister Cells upon Treatment with Ampicillin and Chloramphenicol

Upon ampicillin treatment, we observed transition of motile intranuclear short rod-shaped bacteria to filamentous and ovoid forms in all tested strains. These results are in good agreement with the data of Vishnyakov and Rodionova (1999) on the morpphotype changes in a motile intranuclear endosymbiont inhabiting the macronucleus of *P. multimicronucleatum.*

Since beta-lactam antibiotics inhibit the cell wall synthesis, formation of filamentous (or septate, as they were called by Vishnyakov and Rodionova) and ovoid bacterial cells must have been caused by disorders in the bacterial cell wall. Extension of the periplasmic space seen in some bacteria with TEM supports this idea, suggesting that ampicillin treatment induces formation of the cell wall deficient forms in *Ca*. T. mobilis. Noteworthy, formation of spheroplasts (bacteria lacking the cell wall) upon G-penicillin treatment has been reported in *R. prowazekii* (Wissemann et al., 1982), a pathogenic rickettsia, which can be sustained in the organism for a long time and can cause relapse of the infection (Raoult, Roux, 1999; Weissmann, 2005; Sekeyová et al., 2019).

The recurrent diseases are generally believed to be caused not by reinfection, but by populations of persister cells, i.e., quiescent forms of bacteria (Fauvart et al., 2011; Brauner et al., 2016; Van den Bergh et al., 2016; Levin-Reisman et al., 2017; Trastoy et al., 2018; Mickiewicz et al., 2019). Most often persisters are represented by the cell wall deficient forms (L-forms), which are believed to cause relapse of the disease long time after the patients' recovery due to antibiotic administration (Onwuamaegbu et al., 2005; Mickiewicz et al., 2019). Administration of antibiotics affecting cell wall synthesis to bacteria can induce transition to L-form state (Mercier et al., 2014; Errington et al., 2016). L-forms, or so called spheroplasts, have been reported periodically in a wide range of bacteria, from *Mycoplasma tuberculosis* (Slavchev et al., 2016) to uropathogenic *Escherichia coli* (Mickiewicz et al., 2019).

Although the mechanism of *R.prowazekii* latency has not been definitely established (Sekeyová et al., 2019), possibly, it is persisting spheroplasts that cause recrudescence. Interestingly, recurrence of other rickettsia-borne diseases, such as Mediterranean spotted fever and murine typhus, has been registered even after treatment with chloramphenicol (Shaked et al., 1989). Disease relapses and persistence of the closely related to rickettsia species *Orientia tsutsugamushi* (scrub typhus rickettsia) in tissues of recovered patients have been reported after both, chloramphenicol and doxycycline therapy (Kelly et al., 2017). Our data evidencing for the transition of *Ca*. T. mobilis into ovoid forms upon exposure to sublethal concentrations of chloramphenicol in the strains Kr154-4 and AB9-4 seem to be in line with clinical observations on rickettsioses. It is very likely, that the ovoid forms of *Ca*. T. mobilis correspond to spheroplasts of *R. prowazekii* and should be considered as persisters, which we believe to be responsible for stability of the symbiotic system *P. mutimicronucleatum*/*Ca*. T. mobilis. The faint signal produced in FISH experiments by the endosymbionts located in the host cytoplasm (Fig. 16) may be caused by a low level of ribosome synthesis in the dormant persister cells, induced by antibiotic treatment.

Interestingly, *Ca*. T. mobilis seems to combine features characteristic of two groups of *Rickettsia*: intranuclear localization, which has been registered for the spotted fever group (SFG) rickettsia, and spheroplast transition, so far proposed only for *Rickettsia prowazekii*, belonging to the typhus group (TG).

5.9 The nature of the endosymbiont containing vacuolesin ampicillin treated cells

Exposure of the symbiotic system to ampicillin leads to the release of some endosymbionts into the host cytoplasm. Our detection of a phagophore-like structure closing on a "naked" endosymbiont in the host (Fig. 19a) and of lysosomes fusing with the membrane of the endosymbiont containing vacuole (Fig. 19b) suggests an autophagy-related process. The vacuoles in the host cytoplasm

containing endosymbionts might be autophagolysosomes, since they possess one membrane and the electron density of their contents seems to be similar to that of the lysosomes. It is not clear whether the released endosymbionts survive. The presence of virus-like particles outside the bacterial cells may be considered as a sign of bacterial degradation, however, only in rare cases did we observe blebbing (formation of bubbles) of the bacterial membrane (Fig. 19e), reminding of the images interpreted as L-form proliferation (Errington, 2012). However, most of the bacteria seemed to remain intact, which is in good agreement with the recent finding that *Ca*. T. mobilis can survive in the planarian enterocytes for 7 days (Modeo et al., 2020).

We suppose that egress of bacteria *Ca*. T. mobilis from the Ma into the host cytoplasm could trigger autophagy, a process used to clear cells from alien microorganisms. Although autophagy, in particular, xenophagy, is a mechanism used by a cell to control pathogenic microorganisms, many intracellular pathogens manage to develop means of escape from the host autophagosomes or to hijack this dangerous compartment for their survival and propagation (Siqueira et al., 2018; Khandia et al., 2019). Some of them, e.g., *Shigella flexneri* or *Salmonella typhimurium*, inhibit the host autophagy machinery to prevent xenophagy, while others, like *Ehrlichia chaffeensis*, induce autophagy to obtain host cytosolic nutrients without running the risk of autophagic clearance (Jiao, Sun, 2019). Thus, a representative of the sister to Rickettsiaceae family *Anaplasmataceae*,*Anaplasma phagocytophilum*, subverts early autophagosomes of the host cell to facilitate bacterial proliferation (Niu et al., 2008). Other bacteria, such as *Porphyromonas gingivalis* or *Brucella* adapt to survival in the autophagosome by preventing its fusion with the host lysosomes (Khandia et al., 2019), whereas *Coxiella brunetti* is known to recruit autophagy related proteins to its replicative vacuole, and inhibition of autophagy leads to impairmentof replication in *Coxiella* (Pareja et al., 2017). Keeping this in mind, we cannot exclude the possibility that *Ca*. T. mobilis, likewise, might be able to manipulate the host cell autophagy to ensure its survival. At the same time, *Ca*. T. mobilis might use autophagy as an extra host sparing way for egress from the host cell, like *Brucella*, *Mycobacterium marinum* and, possibly, *Francisella* (Freidrich et al., 2012).

5.10 Activity of the antimicrobial peptide complex FLIP7 on the stability of symbiotic system *Paramecium multimicronucleatum*/*Ca*. Trichorickettsia mobilis

At present, antimicrobial peptides are suggested to be very promising agents against a wide range of Gram-negative and Gram-positive bacteria including antibiotic-resistant strains (Chernysh et al., 2015). Also, antimicrobial peptides have been shown to be active against some fungi, protists and viruses (Boulanger et al. 2006; Huan et al. 2020; El-Dirany et al. 2021). Bacteria of the order Rickettsiales are characterized by a wide host range, *Wolbachia*, a representative of the family

Anaplasmataceae, parasitizing insects (Dumler and Walker 2005). In this regard, one could expect that antimicrobial peptides produced by insects might possess a certain activity against rickettsia, which are known to be resistant to many antibiotics (Rolain et al. 1998; Rolain 2007). Nevertheless, in our experiments, we failed to obtain aposymbiotic (endosymbiont-free) cell lines by administration of the antimicrobial peptide complex FLIP7 or its fractions to the *P. multimicronucleatum* strains bearing Trichorickettsia in the macronucleus. The results of treatments Trichorickettsia-harboring strains with FLIP7 and its HPLC fractions conform to our results obtained in the experiments using antibiotic treatments (Mironov, Sabaneyeva, 2020) and, in a way, resembles the “all-or-none” principle of physiology. Either no effect is registered, the host cells looking healthy and keeping proliferating endosymbionts in their Ma, or the ciliates die, presumably, together with their endosymbionts. This feature of Trichorickettsia is in good agreement with our data on resistance of Trichorickettsia to antibiotic treatments and the well-known resistance to antibiotic treatments demonstrated by pathogenic bacteria from the family *Rickettsiaceae* causing severe human diseases (Rolain et al. 1998; Rolain 2007).

High concentrations of FLIP7 proved to be harmful to ciliates, arguing for the anti-protist activity of this antimicrobial complex (Mironov et al., 2022). Surprisingly, the strains harboring Trichorickettsia demonstrated higher viability after administration of FLIP7 or its HPLC fractions than the control strain, which was especially evident in the experiments using fractions 20, 26 and 36 (Mironov et al., 2022). All control non-infected ciliate strains appeared to be more susceptible to the antimicrobial peptide complex and its fractions than the Trichorickettsia-bearing strains. Although it would be too early to irrevocably state that it is the presence of the endosymbiotic bacteria in the macronucleus of *P. multimicronucleatum* that makes the strains resistant against antimicrobial peptides, this possibility should be taken into consideration. Since we have failed to obtain any aposymbiotic cell lines, it is not feasible to find an unequivocal answer to the question whether Trichorickettsia bacteria confer special features to their host, or resistance to antibiotics and antibacterial peptides is just an intrinsic character of particular ciliate strains independent on the presence of the endosymbionts.

In our experiments we used HPLC fractions of FLIP7 eluted basing on their hydrophobic properties. Some of the fractions demonstrated high rate of cytotoxicity against ciliate strains. Previously, it has been shown that these fractions contain antimicrobial peptides of *Calliphora vicina* (Chernysh et al. 2018; Gordya et al. 2018), implying that FLIP7 activity against ciliates is due to antimicrobial peptides. However, in some of the fractions (from 7 to 20) demonstrating anti-ciliate activity antimicrobial peptides have not been registered, which might mean that these fractions comprise some compounds with specific anti-protist properties.

In the short-term experiments, only the 33[d] fraction appeared to be effective against Trichorickettsia-carrying ciliates. This fraction is known to contain a cecropin with MW 4156 Da (Gordya et al. 2018). Insect cecropins are linear amphipathic alpha-helical peptides particularly effective against Gram-negative bacteria (Chernysh et al. 2018) as well as against some protists (Boulanger et al. 2006; El-Dirany et al. 2021). Possibly, it is the presence of cecropins that account for the anti-ciliate activity of this fraction.

After FLIP7 treatment of Trichorickettsia-carrying *P. multimicronucleatum* we observed formation of two distinct populations of the endosymbionts: the nuclear ovoid forms containing virus-like particles and the cytoplasmic elongated slimmer forms enriched in polyhydroxyalcanoate (PHA) inclusions (Mironov et al., 2022). Thus, the stress caused by administration of the antimicrobial peptide complex FLIP7 causes release of the bacteria from the nucleus into the cytoplasm similarly to the effect observed after some antibiotic treatments (Mironov, Sabaneyeva, 2020). Although the smaller forms were registered within vacuoles in the host cytoplasm, they resembled "naked" (host membrane-free) Trichorickettsia inhabiting the cytoplasm of *P. calkinsi* (Sabaneyeva et al. 2018), the main difference being the presence of flagella in the latter case. Trichorickettsia localized in the vacuoles in the host cytoplasm and those registered outside of the host cell were rich in PHA inclusions, the presence of which has been already recorded in cytoplasmic Trichorickettsia inhabiting *P. calkinsi* (Sabaneyeva et al. 2018). PHA granules are known to be synthesized by bacteria in unbalanced growth conditions; they serve as a source of energy and carbon supply and facilitate bacterial survival under stressful conditions (Kadouri et al. 2005; Tian et al. 2005; Pötter and Steinbüchel 2006). Interestingly, some lipid modifications play an important role in mechanisms of resistance to antimicrobial peptides in Gram-negative bacteria (Moravej et al. 2018). PHA are mostly typical for free-living bacteria, however, there are some cases of their presence in the symbiotic species, *Rhizobia* and *Burkholderia*, as well (Lakshman and Shamala 2003; Kim et al. 2013). The ability of *Burkholderia* to produce PHA is believed to play an important role in sustaining its infectious levels and, moreover, to affect the host fitness (Kim et al. 2013). Last, but not least, it has been proposed, that PHA granules are produced in environmentally acquired endosymbionts, and not in vertically transmitted bacteria (Kim et al. 2013). It seems, that Trichorickettsia exploits the same strategy. On the one hand, synthesis of PHA granules by trichorickettsia could be concomitant with the development of small forms, which can be later released from the host cell to ensure their survival in the environment and, possibly, their horizontal transmission. It should be noted, that since *Ca*. T. mobilis was revealed in the strains of different geographic origin (Europe, United States of America and Siberia), theoretically, it could possess high infectious capacity. Nevertheless, the way of its transmission still remains unclear (Modeo et al., 2020). On the other hand, it is the synthesis of PHA by symbiotic Trichorickettsia that might account for the higher fitness of the Trichorickettsia-carrying

ciliate strains compared to Trichorickettsia-free ones. The mode of the anti-protist action of antimicrobial peptides is believed to be based firstly on membrane permeabilization (Torrent et al., 2012), and the presence of extra lipid supply could be an important factor ensuring resistance of ciliates to the membrane-tropic chemical compounds.

Thus, we showed that the symbiotic system *Paramecium multimicronucleatum*/*Ca*. Trichorickettsia mobilis is very stable and its partners are inseparable under conventional antibiotic treatments or administration of antimicrobial peptide complex FLIP7. This peculiarity makes the system a good candidate for a model holobiont, which can be used in further elaboration of the holobiont concept. Stability of the system is most probably ensured by the ability of the endosymbiont to produce dormant persisters in the presence of ampicillin and chloramphenicol, a feature uniting them with pathogenic *R. prowazekii.* Ovoid and filamentous forms of *Ca*. T. mobilis might be subsequently investigated to elucidate the issue of bacterial persistence. Moreover, we propose that autophagy plays a role in the survival of *Ca*. T. mobilis released from the host macronucleus into the cytoplasm. Analysis of the host cell molecular mechanisms affected by the endosymbiont under administration of certain antibiotics and antimicrobial peptides might enrich our knowledge of bacterial-host crosstalk in the symbiotic system under destabilizing conditions.

Conclusions

1. The motile endonuclear symbiont of the ciliate *Paramecium multimicronucleatum* possesses flagella and carries virus-like particles.
2. The motile macronuclear endosymbiont of the ciliate *P. multimicronucleatum* belongs to the family *Rickettsiaceae* (order *Rickettsiales*) and represents a new species, *Ca*. Trichorickettsia mobilis.
3. The symbiotic system *P. multimicronucleatum*/*Ca.* T. mobilis is stable under administration of antibiotics with different mode of action, including those used for treatment of rickettsioses.
4. The symbiotic system P. multimicronucleatum/*Ca*. T. mobilis is resistant to the effect of antimicrobial peptide complex FLIP7 and its chromatographic fractions.
5. Upon administration of some antibiotics and antimicrobial peptides complex FLIP7 the intranuclear endosymbiont *Ca*. T. mobilis is able to produce persisting forms released into the host cytoplasm.

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