Protistology

In shadow of *Holospora* – The continuous quest for new Holosporaceae members

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Summary

Bacteria from the family Holosporaceae are known as obligate endosymbionts of eukaryotes, mostly protists. In the last decade the knowledge about Holosporaceae significantly expanded, new members of the family were found in various hosts and reported from different environments. The studies of *Holospora*, the type genus of the family, have been initiated in the 1970-ies in the Soviet Union by the research groups headed by D. Ossipov and B. Gromov. In this review, we aimed to describe current diversity of Holosporaceae, showing the past, the present and possible future directions of research on this specific and fascinating group of bacteria.

Key words: ciliates, infection, intracellular bacteria, *Paramecium*, protists, symbiosis

Introduction

Symbiosis is one of the most global biological phenomena. Studies of symbiosis now echo in all biological disciplines, from microbiology and zoology to physiology and evolution. This renaissance of symbiosis research became possible due to molecular approaches to study complex multi-organismal associations. Several decades ago, it was much more difficult to dissect symbiotic systems and sometimes even to figure out which organisms live together in the intimate interactions. Boris Gromov, Professor of Saint Petersburg State University, a prominent microbiologist of his time, had very broad scientific interests, one of which was symbiosis. Together with colleagues he started several lines of research of diverse symbiotic systems: they isolated and described algal parasites currently known as aphelids and chytridiomycetes (Gromov, 1976; Karpov et al., 2014), approached triple associations between eukaryotic hosts, microalgae and their viruses, and also studied peculiar system of intranuclear symbiosis between *Holospora* bacteria and *Paramecium*. In this review, we will try to show the state-of-the-art of the knowledge on Holosporaceae (Alphaproteobacteria), the family which first representatives were redescribed and recognized as valid species by B. Gromov.

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Professor Boris Gromov' studies of Holospora: impact and insights

Bacterial symbionts of protists represent one of the emerging peculiar ecological groups of bacteria. The first proper descriptions of bacteria inhabiting nuclei of the ciliate *Paramecium* were fulfilled by Wladimir Hafkine at the end of the XIX century (Hafkine, 1890), but then for many years symbionts of protists remained neglected. Microbiologists traditionally critically assessed non-cultivated bacteria, as there were no approaches to characterize such objects according to the standard rules on pure cultures, and only observations were not considered sufficient. Thus, mostly symbionts of protists were occasionally described by zoologists, and such descriptions were sometimes episodic and incomplete (Görtz and Fokin, 2009). It was even difficult to prove that the "particles" observed within the cells of protists were bacteria. However, as the ciliate *Paramecium* became a model protozoan deeply studied by the American geneticist Tracy Sonneborn and his disciples, the information on "particles" present in paramecia has been accumulating (Jurand and Preer, 1968; Beale et al.,1969; Gibson, 1974). Such particles, as cytoplasmic factors, were named with letters of the Greek alphabet, although numerous evidences suggested that they should be regarded as bacteria: they contained DNA, RNA and enzymes, were sensitive to antibiotics, had components of bacterial cell walls and appeared to be Gram-negative. In the middle of the 1970-ies, when methods of DNA analysis such as nucleic acids hybridization and DNA renaturation kinetics started to develop, it was finally accepted that these particles were indeed symbiotic bacteria (Preer et al., 1974). However, qualified microbiological expertise was still lacking, and it was not clear how to incorporate uncultivable bacteria, in particular intracellular symbionts of eukaryotes, in bacterial systematics and taxonomy.

In 1970, Dmitry Ossipov, a protistologist from Leningrad State University (USSR), collected some cells containing impressive symbionts in the micronuclei from a natural population of *Paramecium caudatum*. These symbionts were negatively stained by Gram. Ossipov and Ivakhnyuk (1972) were the first to show using light microscopy that two different forms of symbionts, later called infectious form and reproductive form, succeeded each other during infection development. They also demonstrated that symbionts were highly infectious, and that the infection cycle could be accomplished in a certain chain of events. Later Ossipov (1973) found that these new "particles" were highly specific for the ciliate host species. According to the tradition of those times, the intranuclear symbionts were named "omega-particles", although the authors were convinced that they had described bacteria (Ossipov and Ivakhnyuk, 1972). Moreover, they supposed that those symbionts had been observed by different authors several times before, giving priority to the description of the so-called "*Holospora undulata*" made by Hafkine (Hafkine, 1890).

Ossipov joined his efforts with B. Gromov (Fig. 1) to describe omega-particles and iota-particles found later in *P. caudatum*' macronucleus (also described by Hafkine as "*Holospora obtusa*") in a microbiologically correct way. Gromov and Ossipov worked together thoroughly studying these symbiotic systems for several years, and in 1981 they published a scientifically correct description of *Holospora undulata* and *H. obtusa*, mentioning that "these symbionts have now been examined better than some named bacteria which have definite positions in bacterial classification" (Gromov and Ossipov, 1981). They carefully referred to the descriptions made by Hafkine as the types, and amended them with their own data. It was a milestone work, as it was one of the first valid descriptions of intracellular symbionts of protists. In parallel, similar work aiming to properly describe endosymbionts of *Paramecium* and to confer correct names on them was done by John Preer and his group (Preer et al., 1974; Preer and Preer, 1982). Next years showed that Gromov and Ossipov had the honor to describe the main model protozoan symbiont, as *Holospora* obviously remains the best-studied symbiont of ciliates.

Biology of *Holospora* has been described in all details in several reviews (Görtz, 1986; Görtz, 2006; Fokin and Görtz, 2009; Schrallhammer and Potekhin, 2020). Avoiding redundancy with these publications, we will summarize here the main contributions made by B. Gromov. His excellent work on morphology and ultrastructure of omega (Ossipov et al., 1973) and iota (Gromov et al., 1976) particles (*H. undulata* and *H. obtusa*, respectively) and on ultrastructure of infected nuclei followed the initial observation that morphology of intranuclear symbionts was changing during infection. Transmission electron microscopy not only allowed to discern morphology of both spindle-shaped (RFs) and spiral-shaped (IFs) bacteria, but also provided the first insight in physiology of infection, e.g., the electron-lucid space on one end of the infectious spiral form, later called "infection

Fig. 1. Prof. Boris Gromov and his wife and co-worker Kira Mamkaeva in the laboratory, photographed by Prof. Dmitry Ossipov, 1999 (courtesy of D.V. Ossipov).

tip", was detected. The infection tip, also known as recognition tip, is an important part of the IF responsible for escape from the phagosome and for nuclear membrane penetration; probably, it functions as an effector-secretion zone during cross-talk between the bacterium and the host (Schrallhammer and Potekhin, 2020). It was shown that symbionts significantly influenced the chromatin in the infected micronucleus (Ossipov et al., 1973), while macronuclear chromatin upon infection looked intact (Gromov et al., 1976). Intranuclear bacterial symbionts were considered as a potential tool to study the structure and functions of the dimorphic nuclear apparatus of ciliates (Ossipov et al., 1976; Ossipov, 1981).

Gromov and coauthors coined the term "spore" for the infectious form of *Holospora* (Gromov et al., 1974). This term was suggested by Hafkine and even was reflected in the name of the symbiont (Hafkine, 1890), but as *Holospora* "spores" are quite different from traditional bacterial endospores, the term had to be re-introduced and explained. The spore of *Holospora* is responsible for distribution and survival of bacteria in the environment and for the infection of new paramecia. Studying spores of iota-particles, Gromov's group demonstrated that they were sensitive to changes in pH. The authors used hydrochloric acid to provoke response of the infectious form. Such drastic change of pH resulted in egress of the contents from the middle part of the spore envelope (Gromov et al., 1974). Later it was shown that *Holospora* are in fact sensitive to

acidification of the digestive vacuole (Fujishima et al., 1997). However, pH changes much more smoothly in *Paramecium* phagosomes, and the "bacterial body" remains inside the spore cell wall. Instead, acidification triggers secretion of proteins via infection tip, helping bacteria to escape from the vacuole and to start their trip through the cytoplasm to the nucleus (Kawai and Fujishima, 2000).

At that time, there were no other ways to characterize a bacterium and attribute it to a certain group than observations on its morphology and ultrastructure. Alpha-particles from the macronucleus of *Paramecium biaurelia* described by Preer (1969) were suggested to belong to Myxobacteria. Gromov, however, supposed that according to morphology, alpha-particles do belong to the genus *Holospora* but not to the genus *Cytophaga* as suggested by the American colleagues (Preer et al., 1974). In fact, later alpha-particles were indeed described as *Holospora caryophila* (Preer and Preer, 1982), and recently redescribed as *Preeria caryophila*, one of *Holospora*-like bacteria (Potekhin et al., 2018). Judging by iota, omega and alpha particles ultrastructure and life cycle, Gromov supposed that more likely those symbionts were related to *Rickettsia* (Gromov et al., 1976). Indeed, in life cycles of *Holospora* and some rickettsia-like bacteria small forms propagate inside the host cell and differentiate into bigger cells with non-typical morphology, which do not divide and function as transmission agents (Gromov, 1978). This idea was proven to be absolutely right later, when molecular phylogenetics appeared, and *Holospora* even provided its name to the order Holosporales, which is a close evolutionary relative of the order Rickettsiales (Szokoli et al., 2016).

Gromov summarized the current knowledge of his time about symbionts of protists and arthropods in the review (Gromov, 1978) where he discussed also the difference between uncultivable symbionts and cell organelles, and evolutionary aspects of such symbioses. He was limited in technological solutions to assess these problems experimentally, but he had a clear understanding of the perspectives of research in this field. The further development of science confirmed that many of his estimates and expectations were correct.

Diversity of Holosporaceae

Forty years passed since the description of *Holospora* had been published. In this period of
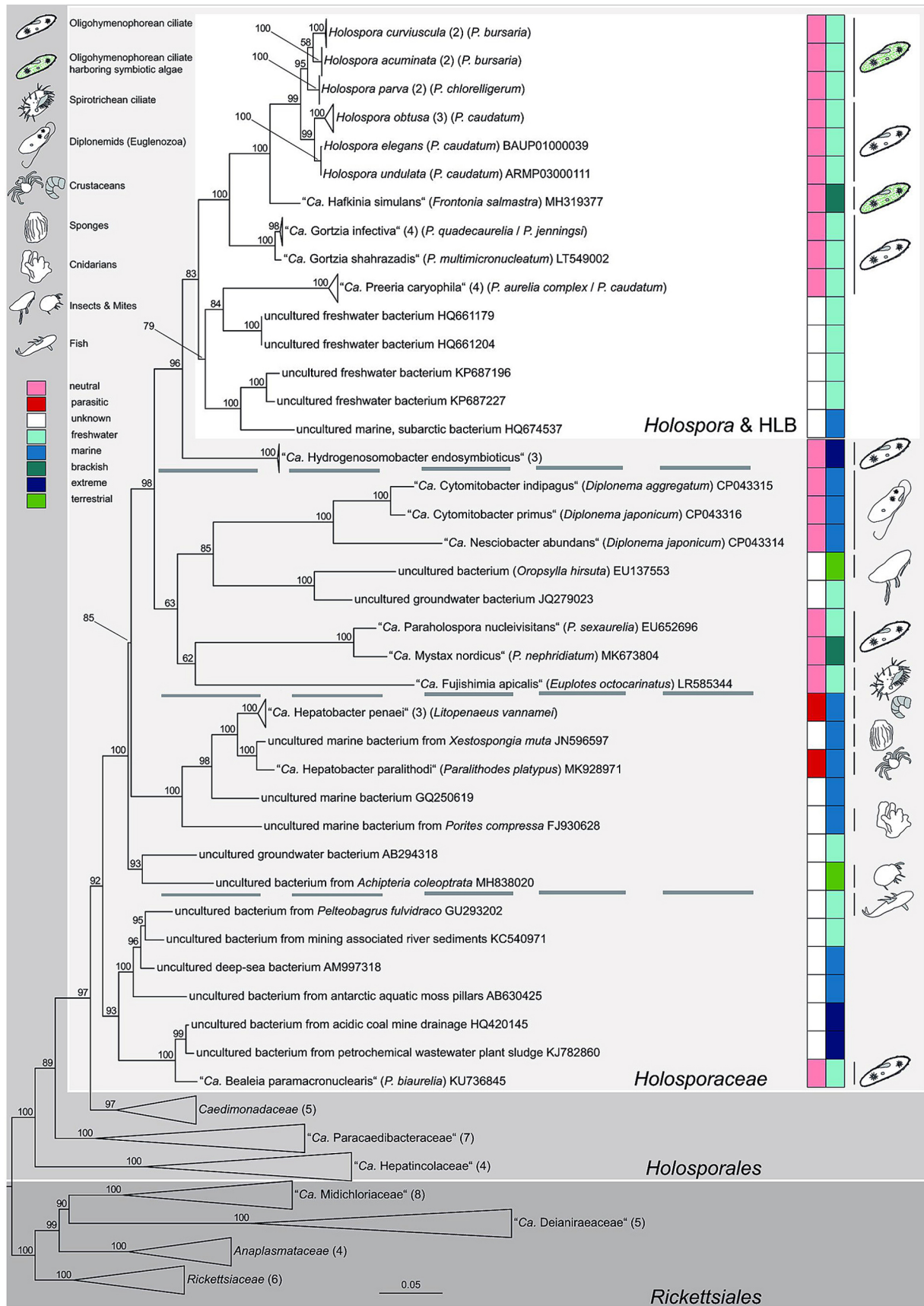
time, microbiology made a huge step forward due to emergence of molecular approaches for characterization of prokaryotes. This revolution significantly affected uncultivable microorganisms, as now they can be correctly characterized and classified according to their DNA sequences (Yarza et al., 2008; Murray et al., 2020). Numerous environmental bacteria are unable to grow under laboratory conditions and, thus, it is impossible to obtain them in microbiologically pure culture. Nevertheless, now they can be characterized by "full rRNA cycle" (Amann et al., 1991), allowing to define their systematic affiliation and taxonomic rank. In the last decade, the number of properly described *Paramecium* endosymbionts increased almost threefold. Mostly symbionts of *Paramecium* belong to two sister orders within Alphaproteobacteria, namely Rickettsiales and Holosporales, although there is a debate on the phylogenetic position and rank of the latter (Szokoli et al., 2016; Muñoz-Gómez et al., 2019). According to the phylogenetic system proposed by Szokoli and colleagues, the order Holosporales comprises four families: Holosporaceae, Caedimonadaceae, Paracaedibacteraceae, and Hepatincolaceae. Here we will review current diversity of the family Holosporaceae, which includes only endosymbiotic bacteria of protozoan and metazoan hosts.

Currently there are four main clusters on the Holosporaceae molecular phylogenetic tree inferred from the 16S rRNA gene sequences (Fig. 2). The type genus of the order Holosporales and of the family Holosporaceae is *Holospora*. This genus includes several species of highly infectious bacteria (Fig. 3, A-D.) that are very selective for the *Paramecium* host species and for the compartment, either micronucleus or macronucleus of the ciliate (Schrallhammer and Potekhin, 2020). *Holospora* phylogeny suggests co-evolution of *Holospora* with *Paramecium*, as separate branches are formed by species infecting different hosts, *P. caudatum, P. chlorelligerum* and *P. bursaria*. In general, there are different views on the number of *Holospora* species. Several species, described in pre-molecular era, such as *H. bacillata* and *H. curvata* (Fokin and Görtz, 2009), have not been found again yet and, thus, need to be confirmed. *Holospora undulata* and *H. elegans* (third species described by Hafkine) colonizing micronucleus of *P. caudatum* are, probably, two different morphological variants of the same species, as there are almost no differences in their genomes (Garushyants et al., 2018). It seems likely that *H. recta*, one more micronuclear symbiont of *P. caudatum*, is the third morphotype of the same species, as straight elongated infectious forms — diagnostic feature of *H. recta* — are relatively frequently observed among *H. undulata* spores as well (Skoblo et al., 1996; N. Lebedeva, pers. comm.). There are three genera closely related to *Holospora* that include also infectious bacteria inhabiting the nuclei of ciliates. Those are: "*Candidatus* Gortzia" (Boscaro et al., 2013) and "*Candidatus* Preeria" (Potekhin et al., 2018) colonizing different *Paramecium* species, and "*Candidatus* Hafkinia" (from here on bacterial taxa will be referred to without the "*Candidatus*" prefix) residing in the other oligohymenophorean ciliate, *Frontonia salmastra* (Fokin et al., 2019). These symbionts cluster on neighboring branches together with *Holospora* (Fig. 2) and are generally termed *Holospora*-like bacteria (HLB). All of them are ultrastructurally similar to *Holospora* and characterized by biphasic life cycle. HLB, except *Hafkinia*, reside preferentially but not exclusively in macronuclei of their hosts, being able to roam as *Preeria caryophila* (Potekhin et al., 2018) and even persist as *Gortzia shahrazadis* (Serra et al., 2016) also in cytoplasm. *Preeria* (Fig. 3, E) seems to be more diverged from *Holospora* and other HLB, and it groups closely with several anonymous freshwater and marine bacteria known only by their 16S rRNA gene sequences obtained from metagenomic studies (Fig. 2). Since the pervasive review on *Holospora* and HLB was recently published (Schrallhammer and Potekhin, 2020), we will not discuss here in more detail biology of these representatives of Holosporaceae.

One more distantly related genus first diverging in the large clade of HLB is *Hydrogenosomobacter*.

Fig. 2. 16S rRNA gene phylogenetic reconstruction of the order Holosporales focusing on taxonomic and ecological diversity within Holosporaceae family. Maximum likelihood tree was calculated with IQ-TREE based on 93 16S rRNA gene sequences. 1,320 characters were considered. TVMe+R5 was the applied best fit evolutionary model, and Ultrafast Bootstrap support values of IQ-TREE are reported if higher than 50 %. Numbers of utilized sequences within collapsed groups are indicated by figures in brackets. Four clades of Holosporaceae are outlined with dashed lines. HLB stands for *Holospora*-like bacteria, „Ca." stands for *Candidatus*. Scale bar: 0.05 sequence divergence.

Oligohymenophorean ciliate

Oligohymenophorean ciliate harboring symbiotic algae

Spirotrichean ciliate

Diplonemids (Euglenozoa)

Crustaceans

Sponges

Cnidarians

Insects & Mites

Fish

neutral
parasitic
unknown
freshwater
marine
brackish
extreme
terrestrial

Holospora curviuscula (2) (*P. bursaria*)
Holospora acuminata (2) (*P. bursaria*)
Holospora parva (2) (*P. chlorelligerum*)
Holospora obtusa (3) (*P. caudatum*)
Holospora elegans (*P. caudatum*) BAUP01000039
Holospora undulata (*P. caudatum*) ARMP03000111
"*Ca.* Hafkinia simulans" (*Frontonia salmastra*) MH319377
"*Ca.* Gortzia infectiva" (4) (*P. quadecaurelia* / *P. jenningsi*)
"*Ca.* Gortzia shahrazadis" (*P. multimicronucleatum*) LT549002
"*Ca.* Preeria caryophila" (4) (*P. aurelia complex* / *P. caudatum*)
uncultured freshwater bacterium HQ661179
uncultured freshwater bacterium HQ661204
uncultured freshwater bacterium KP687196
uncultured freshwater bacterium KP687227
uncultured marine, subarctic bacterium HQ674537

Holospora & HLB

"*Ca.* Hydrogenosomobacter endosymbioticus" (3)
"*Ca.* Cytomitobacter indipagus" (*Diplonema aggregatum*) CP043315
"*Ca.* Cytomitobacter primus" (*Diplonema japonicum*) CP043316
"*Ca.* Nesciobacter abundans" (*Diplonema japonicum*) CP043314
uncultured bacterium (*Oropsylla hirsuta*) EU137553
uncultured groundwater bacterium JQ279023
"*Ca.* Paraholospora nucleivisitans" (*P. sexaurelia*) EU652696
"*Ca.* Mystax nordicus" (*P. nephridiatum*) MK673804
"*Ca.* Fujishimia apicalis" (*Euplotes octocarinatus*) LR585344
"*Ca.* Hepatobacter penaei" (3) (*Litopenaeus vannamei*)
uncultured marine bacterium from *Xestospongia muta* JN596597
"*Ca.* Hepatobacter paralithodi" (*Paralithodes platypus*) MK928971
uncultured marine bacterium GQ250619
uncultured marine bacterium from *Porites compressa* FJ930628
uncultured groundwater bacterium AB294318
uncultured bacterium from *Achipteria coleoptrata* MH838020
uncultured bacterium from *Pelteobagrus fulvidraco* GU293202
uncultured bacterium from mining associated river sediments KC540971
uncultured deep-sea bacterium AM997318
uncultured bacterium from antarctic aquatic moss pillars AB630425
uncultured bacterium from acidic coal mine drainage HQ420145
uncultured bacterium from petrochemical wastewater plant sludge KJ782860
"*Ca.* Bealeia paramacronuclearis" (*P. biaurelia*) KU736845

Holosporaceae

Caedimonadaceae (5)
"*Ca.* Paracaedibacteraceae" (7)
"*Ca.* Hepatincolaceae" (4)

Holosporales

"*Ca.* Midichloriaceae" (8)
"*Ca.* Deianiraeaceae" (5)
Anaplasmataceae (4)
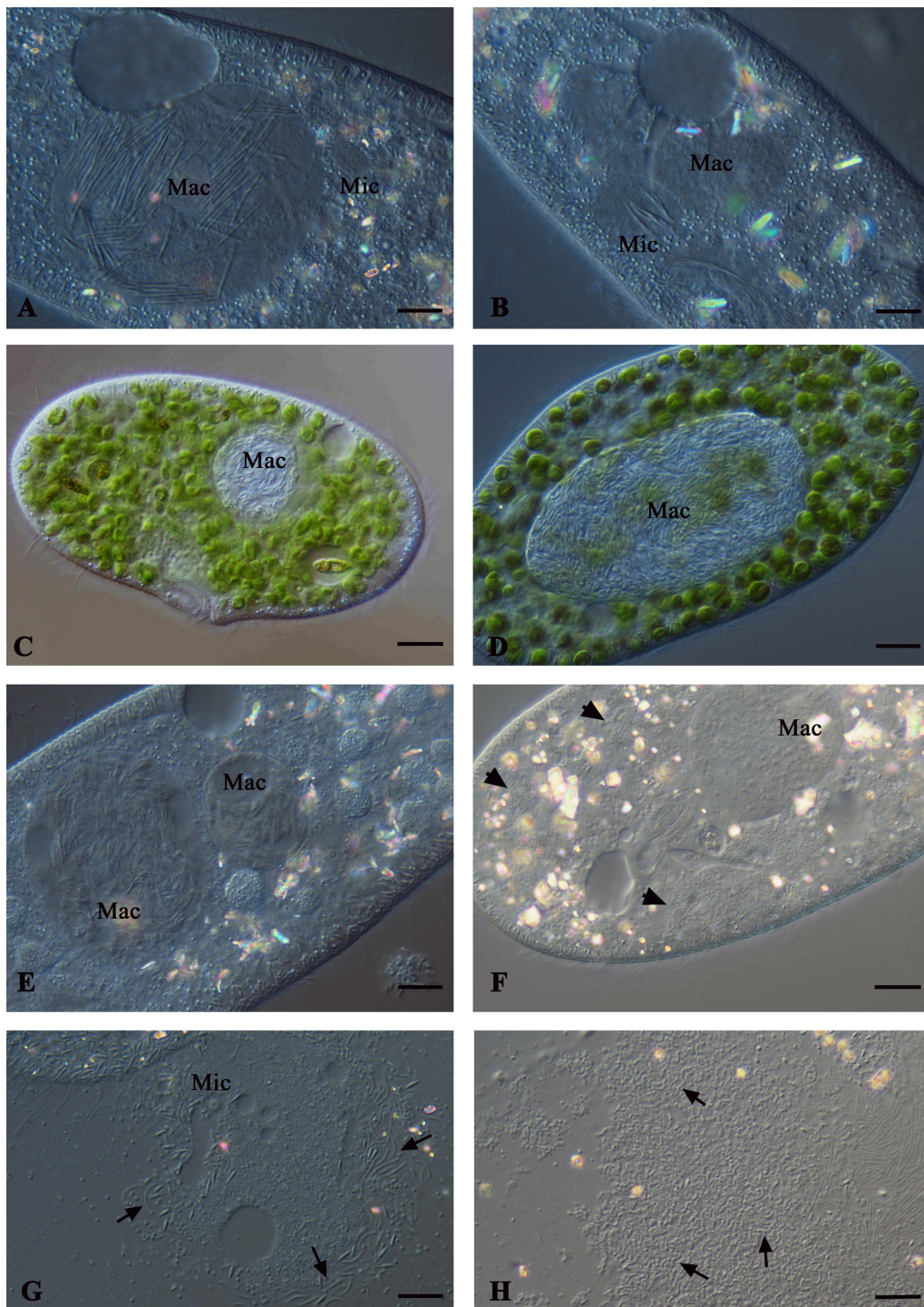*Rickettsiaceae* (6)

0.05

Rickettsiales

Fig. 3. Different *Paramecium* symbionts from the family Holosporaceae. Both infectious forms and reproductive forms are present in *P. caudatum* in the macronucleus infected by *Holospora obtusa* (A) and in the micronucleus infected by *Holospora undulata* (B); *Holospora parva* residing in macronucleus of *P. chlorelligerum* (C); *P. bursaria* macronucleus hyperinfected by *Holospora curviuscula* (D); *Preeria caryophila* in the splitted macronucleus of *P. caudatum* (E); aggregates of *Mystax nordicus* (black *arrowheads*) in the cytoplasm of *P. nephridiatum* (F); *Paraholospora nucleivisitans* (G) and *M. nordicus* (H) released from ruptured host cells, black *arrows* point on bacteria with characteristic appearance. DIC microscopy. *Abbreviations*: Mac – macronucleus, Mic – micronucleus. Scale bars: 10 μm.

Its only known species, *H. endosymbioticus*, is one of the partners in a complex symbiotic association (Takeshita et al., 2019). Its host, an unknown anaerobic scuticociliate (Oligohymenophorea), which closest relative by molecular data is another anaerobic species, *Cyclidium porcatum*, lives in stable association with two cytoplasmic endosymbionts. One of them is methanogenic archaeon *Methanoregula* sp., and the other is *H. endosymbioticus*. Symbionts do not interact with each other sharing host's cytoplasm, but both tend to be surrounded by host' hydrogenosomes. Archaea and bacteria in this system are more likely to be competitors than mutualistic partners, as the ratio between them in each ciliate can be different. Both *Methanoregula* and *Hydrogenosomobacter* were found in physical association with host hydrogenosomes. While methanogenic archaea are usual symbionts of anaerobic ciliates helping them to utilize hydrogen appearing in the course of energy production (Emblay and Finlay, 1993-1994; Borrel et al., 2020), occurrence of a bacterial symbiont in the same system is not that common. The reason for that might be either the need for direct acquisition of some substances produced by hydrogenosome, or even using bacteria as a certain backup for hydrogen utilization. In any case, *Hydrogenosomobacter* is an exciting example of an anaerobic member of the evolutionary group formed by aerobic bacteria, showing that metabolic adaptation in symbiosis may be really transforming. Despite belonging to the group, which almost exclusively consists of intranuclear symbionts of ciliates, *H. endosymbioticus* was not detected in the host nucleus. Interestingly, *Cyclidium porcatum* was also reported to host two different prokaryotic endosymbionts (Esteban et al., 1993), which might mean that *Hydrogenosomobacter* is able to colonize different, though related, ciliate species, or, at least, that there can be more such cooperations between archaea and bacteria in anaerobic protists.

The next clade of the Holosporaceae phylogenetic tree (Fig. 2) includes a rather interesting mixture of microorganisms. It is further subdivided into two clusters. One of them is formed exclusively by endosymbionts of ciliates. The other unites three endosymbionts of *Diplonema* (Euglenozoa), and two sequences of uncultured bacteria. One of the latter belongs to some bacterium from groundwater. The other was detected in the bacterial community of a flea *Oropsylla hirsuta* collected from the black-tailed prairie dog *Cynomys ludovicianus* (Jones et al., 2008); the bacterium itself remained unknown.

The first described representative of this Holosporaceae clade was *Paraholospora nucleivisitans* (Eschbach et al., 2009), the nuclear / cytoplasmic symbiont of *Paramecium sexaurelia*, one of the sibling species of the *P. aurelia* complex. It was found in two different *P. sexaurelia* strains, and both of them probably originated from tropics. Currently, several *Paraholospora nucleivisitans* isolates are maintained in the RC CCM collection of Saint Petersburg State University (Russia), and all of them are also hosted by *P. sexaurelia* (A. Potekhin, N. Lebedeva, unpublished). Thus, it seems that *Paraholospora nucleivisitans* may have a rather narrow host range. These bacteria are up to 25 μm long, and have very peculiar shape, bended and curved (Fig. 3, G). The authors describing *Paraholospora* reported that while symbionts mostly resided in host cytoplasm, still in 10% of cells of the initial strains and further of the subcloned cell lines they were observed in the macronuclei, and very rarely in both compartments together (Eschbach et al., 2009). Such shuttling between cytoplasm and macronucleus is a very special feature of this symbiont, the only other holosporaceaen known to be able to propagate in both compartments is *Gortzia shahrazadis* (Serra et al., 2016). At the same time, neither presence of specific stages in the life cycle nor ability to infect other paramecia was characteristic for *Paraholospora*. At the time of the description, its closest phylogenetic relative was *Holospora*, which got reflected in the name of this symbiont. Now it looks misleading, as *Paraholospora* is indeed far-divergent from *Holospora* and does not belong to the group of HLB. The symbiotic association between *Paramecium* and *Paraholospora* can be stable for years, and bacteria do not show considerable fluctuations in prevalence or abundance in the host culture (Eschbach et al., 2009; A. Potekhin, E. Pen'kova, unpublished)

Currently, the closest relative of *Paraholospora* is *Mystax nordicus*, a cytoplasmic symbiont of *Paramecium nephridiatum* (Korotaev et al., 2020). This bacterium got its name due to its moustache-reminding shape, and it really has a remarkable appearance (Fig. 3, H), which allowed to re-describe it according to observations and initial description made in the 1980-ies (Fokin, 1989). In one of the studied combinations, *Mystax* shared the host cytoplasm with another bacterial symbiont, *Megaira venefica*. Symbionts were never observed inside the macronucleus; instead, they were aggregating in big clusters (Fig. 3, F) close to mitochondria. The load

of symbionts varied from single bacterial cells per *Paramecium* to hyperinfection with numerous symbionts literally sticking out of the ciliate cortex (Korotaev et al., 2020). Neither both transmission electron microscopy and atomic force microscopy analyses showed the presence of an infection tip as in *Holospora*, nor different morphological forms were observed for *M. nordicus*, thus rejecting the possibility of a specific infection cycle similar to that of *Holospora* and HLB. The prevalence of the symbionts in the culture tended to reduce with time, but the symbiont-bearing strain has been maintained in RC CCM collection of Saint Petersburg State University already for four years (E. Sabaneyeva, N. Lebedeva, pers. comm.).

Not only *Paramecium* and its closest relative *Frontonia* may host Holosporaceae members among ciliates. Recently, one more representative of the family was documented during extensive screening for bacterial symbionts in different *Euplotes* (Spirotrichea) species (Boscaro et al., 2019). The cytoplasmic symbiont of freshwater species *E. octocarinatus* named *Fujishimia apicalis* in honor of Masahiro Fujishima, one of the pioneers in the *Holospora* studies, was among several novel genera and species of endosymbionts discovered in that work. If not predicted first from metagenomic data, it might have been easily missed, as it shared the host's cytoplasm together with *Polynucleobacter*, an essential symbiont of *E. octocarinatus*, and four other bacterial symbionts from different families of Rickettsiales and Holosporales (Boscaro et al., 2019). *Fujishimia apicalis* was found only in one population of *E. octocarinatus*, and it was present not in all cells of that population, thus putting in question stable vertical transmission. Symbionts had coccoid shape non-typical for Holosporaceae and were really tiny (about 0.5 µm). They were neither detected in the macronucleus, nor distributed throughout the cytoplasm, but were localized in groups at the margin of host cells, often in an anterior position. There are no data about *F. apicalis* ultrastructure, its ability for horizontal transmission, effects on host and any other biological peculiarities.

A very interesting set of Holosporaceae endosymbionts was detected and characterized in marine diplonemids *Diplonema japonicum* and *D. aggregatum* (Euglenozoa). All trophic cells of the strains of both species appeared to contain numerous short (0.9−1.4 µm) rod-shaped bacterial cells scattered throughout the cytoplasm beneath the cell surface. Symbionts were also present though less numerous in swimming host cells. The new genus

Cytomitobacter, accommodating *C. primus* from *D. japonicum* and *C. indipagum* from *D. aggregatum*, was established according to the results of full rRNA cycle characterization of the symbionts (Tashyreva et al., 2018). In further study, one more symbiont was discovered in the same strain of *D. japonicum*. It coexisted in the host's cytoplasm with *C. primus* and was even more abundant. Despite close relation with *Cytomitobacter*, it was divergent enough to assign it to another genus, *Nesciobacter* (George et al., 2020). All three endosymbionts were purely cytoplasmic, never observed in the nucleus or externally of the hosts. The morphology of the endosymbionts remained unaltered throughout the host cell cycle, with no signs of differentiation into specific forms. Bacteria were host-specific and did not show ability to infect symbiont-free diplonemid species under laboratory conditions. Symbionts did not affect their hosts negatively as the association was very stable, and *Diplonema* fitness was not impeded. Interestingly, the authors did not manage to eliminate symbionts with antibiotics (Tashyreva et al., 2018), which can be explained either by resistance of these bacteria to the treatment, or by the necessity of the symbionts to their host. Symbionts preferred to be localized adjacent to the mitochondrion, and occasionally were seen wrapped by the organelle or even inside of it (Tashyreva et al., 2018), which is an extremely rare case of intracellular localization for the symbionts (Stavru et al., 2020).

Bealeia paramacronuclearis, a symbiont found in two *Paramecium biaurelia* strains isolated in the USA (Szokoli et al., 2016), up to now remains the only "taken alive" and properly characterized bacterium in the third clade of Holosporaceae phylogeny (Fig. 2). These small rod-shaped endosymbionts were located in close proximity to the *Paramecium* macronucleus but not inside of it, and could sometimes be found in deep folds of the nuclear envelope. *Bealeia* often formed clusters of several cells laying in parallel orientation. No morphological varieties or ability to infect other *Paramecium* strains were observed for *B. paramacronuclearis*. It is worth noting that *Bealeia* were not sole in one of their host strains, but sharing the cytoplasm with other symbionts identified as *Fokinia cryptica* from Midichloriaceae family of the order Rickettsiales (Szokoli et al., 2016). The second symbiont resided in symbiontophorous vacuoles localized closer to *Paramecium* cortex.

The clade containing *Bealeia* includes also several uncultured bacteria which 16S rRNA gene sequences were retrieved from wastewaters such as acidic mine drainage (Brown et al., 2011), from

deep-sea surface sediments (Schauer et al., 2010), and from Antarctic aquatic "moss pillars" microbial communities (Nakai et al., 2012). Interestingly, one uncultured bacterium, which 16S rRNA gene sequences also drop inside this group, derived from stomach microbiome of the yellow catfish *Pelteobagrus fulvidraco* (Wu et al., 2012). Nothing is known about the biology of these bacteria.

The last, fourth clade of Holosporaceae molecular phylogenetic tree includes several 16S rRNA gene sequences of uncultivated aquatic groundwater and marine bacteria and, interestingly, several symbionts of metazoan hosts (Fig. 2). The most prominent are two species of *Hepatobacter*, pathogenic bacteria parasitizing on crustaceans. Both species cause necrotizing hepatopancreatitis in their hosts, *H. penaei* in the Pacific white shrimp P*enaeus vannamei* (Nunan et al., 2013), and *H. paralithodi* in the blue king crab *Paralithodes platypus* (Ryazanova et al., 2020). Both *Hepatobacter* species cannot be cultivated on cell-free media, and both colonize only epithelial cells of the hepatopancreas of their host. However, two species seem to be rather different from each other. The shrimp pathogen *H. penaei* has two forms, a tiny rod-shaped and a longer helical form, which is motile due to presence of several flagella (Frelier et al., 1992; Nunan et al., 2013). Motility, probably, helps the bacterium to move in its environment, either water or digestive system of a shrimp where it has to reach the hepatopancreas. The crab pathogen *H. paralithodi* is also differentiated into two main morphotypes, spherical or rod-like intermediate bodies dividing in parasitophorous vacuoles in the infected epithelial host cells, and small dense spore-like elementary bodies considered as infectious stage (Ryazanova et al., 2020). None of these two forms is motile or has flagella. While *H. penaei* reside as solitary cells or as aggregates directly in cytoplasm of the infected host cells, *H. paralithodi* can be enclosed in parasitophorous vacuoles. Bacteria of both species were never observed in the nucleus of the host cells. Epitheliocytes infected by *Hepatobacter* rupture at the late stages of the lethal infection, and bacteria appear in the lumen of hepatopancreatic tubules. Actually, similarity between 16S rRNA gene sequences of *H. penaei* and *H. paralithodi* was 94.1−94.7% (Ryazanova et al., 2020), which is considered as threshold to discriminate bacterial genera (Yarza et al., 2008); so, whether *H. penaei* and *H. paralithodi* indeed belong to the same genus or not, they are quite diverged from each other.

16S rRNA gene sequences coming from meta-genomic studies on several other metazoans fall into the same clade. Recently, a holosporaceaen symbiont was detected molecularly in the moss mite *Achipteria coleoptrata* (Konecka and Olszanowski, 2019). The authors only obtained its 16S rRNA gene sequence and did not provide any insight on where bacteria were localized; they even suppose it might be an intracellular endobiont of some protozoan symbionts of the mite. In any case, this bacterium likely was numerous in the host mite, as its sequence was obtained directly in PCR screening for some Anaplasmataceae endosymbionts like *Wolbachia*, but not in total metagenomic analysis of mite microbiome. The Polish researchers also performed a large-scale phylogenetic search (Konecka and Olszanowski, 2019) and found two other 16S rRNA gene sequences of holosporacean origin in the data of microbiome studies of two marine organisms, a giant barrel sponge *Xestospongia muta* (Montalvo and Hill, 2011) and Hawaiian corals *Porites compressa* and *P. lobata*. These bacteria group together with *Hepatobacter*, forming a "marine" cluster in the Holosporaceae molecular phylogenetic tree (Fig. 2).

State-of-the-art and future perspectives of Holosporaceae studies

The family Holosporaceae includes many different bacteria that at a first glance do not have much in common. The main feature of Holosporaceae is that all its known members are obligate symbionts that cannot survive outside the eukaryotic host. Although the hosts are mostly unicellular organisms, some representatives of Holosporaceae colonize metazoan animals (but in some cases, it cannot be excluded that they also inhabit protists associated with metazoan hosts). The character of interactions with the host in many cases is unknown, and for several better-explored systems, it ranges from commensalism to mild parasitism, rarely deleterious effects on host may develop as in associations between crustaceans and *Hepatobacter* (Nunan et al., 2013; Ryazanova et al., 2020). Holosporaceae are known from different environments, infecting freshwater and marine hosts, aerobic and anaerobic protists, just a few were detected in connection with terrestrial arthropods; their 16S rRNA gene sequences were detected in metagenomic studies of Antarctic microbial communities, wastewater plant sludges and acidic coalmine drainages. Interestingly,

symbionts of diverse marine organisms cluster in one subclade in the molecular phylogeny of the family, which is tempting to explain by the possibility of cross-infections of different hosts. On the other hand, rather strict host specificity is a remarkable feature for the best-studied representatives of the family, *Holospora* and HLB. Several species have complex biphasic life cycles with altering morphologically different forms. These representatives (*Holospora*, HLB, *Hepatobacter*) are infectious bacteria, and one of the forms is responsible for horizontal transmission of the symbionts similarly to some rickettsia-like bacteria such as *Rickettsiella*, the pathogen of arthropods (Cordaux et al., 2007). Gromov at the dawn of Holosporaceae studies compared life cycles of *Holospora* and *Rickettsiella* (Gromov, 1978). This reminiscence was independently noticed by Ryazanova and colleagues describing *Hepatobacter paralithodi* infectious cycle in crab (Ryazanova et al., 2020). Thus, parallels between *Rickettsiella* and infectious Holosporaceae arose repeatedly. At the same time, many species are not infectious, or at least infection ability was not present under laboratory conditions. Such bacteria do not show morphological variations and seem to lack specific pre-adaptations for invasion of the hosts. The infectivity and ability for horizontal transmission of these bacteria will be probably unveiled by future genomic and transcriptomic studies.

One more distinguishing feature of many representatives of the family is a common coexistence with other symbionts in the host cell. Indeed, *Hydrogenosomobacter* shares host ciliate cytoplasm with methanogenic archaea (Takeshita et al., 2019), *Mystax* was found in paramecia hosting also *Megaira* (Korotaev et al., 2020), *Bealeia* neighbored *Fokinia* in *Paramecium* cytoplasm (Szokoli et al., 2016), and *Fujishimia* was one of the several symbionts all together colonizing *Euplotes* (Boscaro et al., 2019). There were several reports about co-occurrence of *Holospora* and HLB with other symbionts, either cytoplasmic or sharing the macronucleus with them (see: Schrallhammer and Potekhin, 2020). *Cytomitobacter* and *Nesciobacter* symbionts even stay in more or less constant ratio inside *Diplonema japonicum* cells throughout trophic phase of the host's life cycle, which puts in question possible partitioning of some unknown functions (George et al., 2020). It is unknown whether symbionts compete for the host cell resources or coexist peacefully in such polybacterial infections, providing one possible direction of further research.

Now biology is rapidly moving to genomic and postgenomic era. Methods of next generation sequencing allow to obtain complete metagenomes of complex symbiotic associations, without spending efforts to purify and sequence genomes of the partners separately, which sometimes was anyway not possible. Mining genomes provides clues to the nature of symbiotic relations, either parasitic or mutualistic, to metabolic peculiarities of the partners, and to the evolution of symbiosis. The genome studies are then followed by transcriptome analyses that open wide perspectives to understand the interactions between the partners when the symbiotic system is formed and established, the mechanisms of symbiosis regulation and symbiont's maintenance. Obviously, the symbioses in which Holosporaceae members are involved will be also dissected by genomic and transcriptomic approaches in forthcoming studies. Currently, just seven genomes of holosporaceans from two clades have already been sequenced and published. These are the genomes of four *Holospora* species (Dohra et al., 2013, 2014; Garushyants et al., 2018), namely *H. obtusa, H. undulata* and *H. elegans* (interestingly, exactly those three species had been described by Wladimir Hafkine) from *Paramecium caudatum* and *H. curviuscula* from *P. bursaria*; and the genomes of two *Cytomitobacter* species and *Nesciobacter abundans* from two species of diplonemids (George et al., 2020). Detailed comparative analysis of these genomes is not among objectives of this review, so we will just mention several most important findings shedding light on the biology of the studied symbiotic systems.

All known Holosporaceae up to date are obligate symbionts and cannot grow on any laboratory medium. For many years, this feature has been evoking parallels between obligate bacterial symbionts and cell organelles such as mitochondria and plastids, and suggesting the strong dependence of symbionts on the host cell (Gromov, 1978; Wernegreen, 2017; Wein et al., 2019). Indeed, almost all known obligate symbionts which genomes are sequenced show significant metabolic reduction accompanied by more or less severe genome shrinking (Klasson and Andersson, 2004; Husnik et al., 2013; Moran and Bennett, 2014). *Holospora* and diplonemids' symbionts are not an exclusion of this evolutionary rule. The genomes of the three latter are among the smallest genomes of protist symbionts being only 615−628 kb (George et al., 2020), which is the same size range as, for example, the genomes

of *Buchnera aphidicola*, obligate mutualistic symbionts of aphids that are considered as "semiorganelles" (Shigenobu et al., 2000). *Holospora* genomes range from 1.27 to 1.72 Mb and encode approximately three times more proteins than genomes of diplonemid symbionts (Garushyants et al., 2018; George et al., 2020). However, unlike *Cytomitobacter* and *Nesciobacter, Holospora* are highly infectious bacteria with complex life cycle, so they need to encode many proteins unnecessary to vertically transmitted morphologically invariant symbionts. Such difference in genome size is also partly explained by numerous mobile elements, repeats and phage genomes contained in *Holospora* genomes (Garushyants et al., 2018), while diplonemid symbionts have very compact genomes with densely packed genes, almost devoid of pseudogenes and mobile elements (George et al., 2020). Functionally, the genomes of *Holospora, Cytomitobacter* and *Nesciobacter* furnish rather similar metabolic abilities to these, at a first glance so different, symbionts. Neither *Holospora* nor diplonemid symbionts provide nutrients, amino acids or some inevitable metabolites to their hosts, thus not being physiologically mutualistic. Instead, these bacteria strongly depend on a host for amino acids, nucleotides and vitamins. Moreover, both *Holospora* and diplonemid symbionts are deficient for glycolysis, Krebs cycle and pentose phosphate pathway, they do not have electron transport proteins and lack functional ATP-synthase complex retaining only its part, which is probably used as an ATP-driven proton pump (George et al., 2020). Inability to obtain ATP utilizing carbohydrates is, probably, compensated by adaptation to pure "energetic parasitism", when symbionts encode nucleotide translocases and nucleotide reductases, allowing to steal nucleotides from the host and use them as energy source (Garushyants et al., 2018). Such life strategy explains preferential localization of many Holosporaceae symbionts in close proximity to mitochondria or even in direct contact with these organelles (Fokin, 2004; Tashyreva et al., 2018; Korotaev et al., 2020). It seems quite likely that other Holosporaceae are also "energy parasites". However, symbioses involving them are evolutionary well adjusted, since negative effects on the host are not commonly observed, and even being parasites, these bacteria cannot be referred to as pathogens.

Unexpectedly, *Holospora* lack type III and type IV protein secretion systems used by many intracellular pathogens during entry and persistence in the host cell (Garushyants et al., 2018), and also they miss type VI secretion system commonly used in interactions between prokaryotes and sometimes exploited for intracellular maintenance of endobionts (Hernandez et al., 2020). Diplonemid symbionts possess type II and type IV secretion systems components that may act as a "hybrid" secretion system to deliver effector proteins to the host cell, and also partial type VI secretion system, which may be used in interaction between symbionts inside the host (George et al., 2020).

Finally, new findings of bacteria belonging to Holosporaceae family will definitely continue to occur. Several dozens of different symbionts of ciliates were described in pre-molecular period without possibility to classify them correctly (Görtz and Fokin, 2009). In the last years, several such symbiotic systems "from the past" were collected from nature again, and symbionts were identified according to the existing rules of bacterial systematics; the story of *Mystax nordicus* is one good example (Korotaev et al., 2020). The sequence of the 16S rRNA gene is the only witness of existence for many uncultivable bacteria, and there are also anonymous Holosporaceae members from very different ecological niches among them. We believe that many more symbionts belonging to this family still remain completely unknown. Continuous screening of *Paramecium* symbionts revealed at least three new genera belonging to Holosporaceae in the last two years (A. Potekhin, N. Lebedeva, unpublished data). Recently characterized cytoplasmic symbionts of *Paramecium polycaryum* (Castelli et al., accepted) belong to such novel genus, which got the name *Gromoviella* in honor and memory of Prof. Boris Gromov, one of the founders of Holosporaceae studies.

Acknowledgements

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