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EXPERIMENTAL ARTICLES

Obtaining and Characterization of *"Holospora curviuscula"* and *Holospora obtusa*, Bacterial Symbionts of the Macronuclei of *Paramecium bursaria* and *Paramecium caudatum*

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Abstract—The symbionts of the macronuclei of *Paramecium bursaria* and *P. caudatum, "Holospora curviuscula"* 02AZ16 and *H. obtusa* 88Ti, respectively, were obtained and investigated. The 16S rDNA nucleotide sequences of *"Holospora curviuscula"* were obtained for the first time. The differences in 16S rDNA (3.4%) suggest their classification within the genus *Holospora*. Molecular phylogenetic analysis of the symbionts revealed that these intranuclear symbionts of the ciliates belonged to the order *Rickettsiales*, forming within a compact cluster of related species.

Keywords: Holospora, intranuclear symbiosis, *Paramecium,* 16S rDNA, phylogenetic analysis.

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Bacteria of the genus *Holospora* are obligate intranuclear endobionts of ciliates (symbionts of the macronucleus MA and micronucleus MI). Intranuclear symbiosis is characterized by high species and nuclear specificity [1, 2]. Based on their morphological characteristics, as well as host and nucleus type specificity, nine *Holospora* species have been described: *H. obtusa*, a macronuclear symbiont; *H. elegans*, sequences of "*H. recta"* and *H. undulata*, micronuclear symbionts of *P. caudatum*; "*H. curviuscula*" and *"H. acuminata,"* macro- and micronuclear symbionts of *P. bursaria,* respectively; *H. caryophila*, a macronuclear symbiont of *P. aurelia;* and *"H. bacillata"* and *"H. curvata,"* macronuclear symbionts of *P. calkinsi* and *P. woodruffi*, respectively [3–10].

Members of the genus *Holospora* possess a complex life cycle, during which noninfectious, short (1.5–2.0 μm) reproductive forms (RF) arrive into the daughter nuclei after division of the host cell (vertical transfer of the symbiont); differentiate; and form long (5.0–20.0 μm) infectious forms (IF) unable to divide and possessing a hypertrophied periplasmic space [11, 12]. The IFs are released into the medium and subsequently penetrate into the digestive vacuole via the cytostome, travel to the cytoplasm without disrupting the vacuole, and arrive into the relevant nucleus (MA or MI) by a sluicing mechanism that does not disrupt the nucleus (horizontal transfer of the symbiont) [13].

Since intranuclear symbionts cannot be obtained in pure culture, their systematic position is still insufficiently studied. In 1991 Amann et al. carried out phylogenetic analysis of one species (*H. obtusa*) and demonstrated its relation to *Alphaproteobacteria* [14].

The goal of the present work was to obtain "*H. curviuscula*" and *H. obtusa*, macronuclear endosymbionts of *P. bursaria* and *P. caudatum*, and to characterize their taxonomic position using the results of 16S rRNA gene sequencing and comparative molecular phylogenetic analysis.

MATERIALS AND METHODS

The following environmental isolates of two species of the intranuclear symbionts were used in the present work: strain 02AZ16 from *Paramecium bursaria* isolated in the Astrakhan biosphere reserve and strain 88Ti from *P. caudatum* isolated in Tajikistan. Both isolates were obtained from the infected clones maintained in the CCCS (Culture Collection of Ciliates and their Symbionts) of the laboratory of Unicellular Caryology, St. Petersburg State University.

The macronuclei were isolated as follows [11]. The ciliate cells were lysed in a buffer containing 10 μM tris(hydroxymethyl)aminomethane (pH 7.9), 0.25 M sucrose, 3 μM $CaCl_2$, 8 μM $MgCl_2$, 0.1% spermidine, and 0.2% Nonidet P40. The nuclei were then concentrated in 1.6 M sucrose at 700 rpm for 10 min. The purified vegetative nuclei of the host cells were used for PCR on a Mastercycler DNA amplifier (Eppendorf). For amplification and sequencing, the primers 5'-AGAGTTTGATCCTGGCTCAG-3' (*E. coli* positions 2–21) and 5'-ACGGCTACCTTGTTAC-

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Comparative characterization of strains 02AZ16, 88Ti, and the type strains of *H. obtusa* and *H. elegans* [12]

Feature	*H. obtusa* [12]	*H. elegans* [12]	"*H. curviuscula*" 02AZ16	*H. obtusa* 88Ti
Gram reaction	Gram–	Gram–	Gram–	Gram–
IF size, μm	0.8–1.0 × 10–25	0.8–1.0 × 10–20	0.4–0.5 × 4.0–10.0	0.8–1.0 × 10–25
Morphology of infectious forms	Rods with rounded ends	Thin rods with tapered ends	Curved, with tapered ends	Rods with rounded ends
Host	*Paramecium caudatum*	*Paramecium caudatum*	*Paramecium bursaria*	*Paramecium caudatum*
Nucleus of symbiont localization	MA	MI	MA	MA

GACCT-3' (1507–1487), complementary to the "beginning" and "end" of 16S rDNA, were used.

PCR was carried out in 50 μl of the following mixture: 5 μl 10x buffer (Sibenzim, Russia), 200 μM of each dNTP, 0.5 μM of each primer, and 1.25 U *Taq* polymerase (Sibenzim, Russia). The hybridization conditions were as follows: a cycle for 4 min at 94°C; 35 cycles for 1 min at 94°C 1 min at 60°C, and 1 min at 72°C; and 1 cycle for 3 min at 72°C. The sequencing was carried out by Amber (St. Petersburg, Russia).

The 16S rDNA sequences thus obtained and the GenBank sequences (http://www.ncbi.nlm.nih.gov/Entrez) were used for phylogenetic analysis of the symbionts. The similarity between the isolates (%) was determined using the BLAST software package [15]. Phylogenetic analysis was carried out by constructing evolutionary trees with the Mega version 4.0 software package [16].

RESULTS AND DISCUSSION

Comparative characterization of the isolates 02AZ16 (Fig. 1) and 88Ti (Fig. 2) is presented in the table. According to all morphological criteria, characteristic life cycle, IF structure, host specificity (*P. bursaria* and *P. caudatum*) and nucleus type (MA), both isolates belonged to the genus *Holospora*. For species identification of the symbionts, 16S rDNA sequences of the isolates were obtained and comparative molecular phylogenetic analysis was carried out. The 16S rDNA sequences of strains 88Ti and 02AZ16 were deposited in GenBank (accession nos. JF713682 and JF713683, respectively).

Phylogenetic analysis of strains 02AZ16 and 88Ti revealed their position among rickettsia-like endosymbionts of the family *Holosporaceae* within the phylum *Alphaproteobacteria* (Fig. 3). Analysis of the 16S rRNA gene sequence of strain 88Ti and the GenBank sequence of the type strain *H. obtusa* X58198 showed high similarity of 99.7% (differences in 4 positions out of 1405). Thus, isolate 88Ti may be classified as *H. obtusa*. Importantly, the two investigated *H. obtusa* strains—namely, the strain described previously [14] and the one used in the present work—although isolated in distant (4000 km) sites, exhibited high similarity of their 16S rDNA sequences (99.7%). Differences in four positions—188, 191, 765, and 776 (numbered for *H. obtusa* X58198 from GenBank)—were found.

We also obtained the 16S rDNA sequence of another subject of the present work, isolate 02AZ16, the symbiont of *P. bursaria* macronucleus. Based on the morphology of its infectious forms, host specificity, and nucleus type specificity, this isolate was previously described as "*H. curviuscula*" [8]. The 16S rDNA sequence of "*H. curviuscula*" 02AZ16 obtained in the present work differed from the sequence of *H. obtusa* X58198 in 48 positions out of 1413 (96.6%), thus confirming that the isolates belonged to different species within the same genus.

The phylogenetic tree (Fig. 3) shows that members of the genus *Holospora* form a compact group of closely related species. Within this group, *"Candidatus* Paraholospora nucleivisitans," the recently described intracellular symbiont of *Paramecium sexaurelia*, exhibits the highest similarity to the genus *Holospora*. However, the results of phylogenetic analysis and its morphological characteristics (absence of a complex life cycle, occurrence mainly in the cytoplasm and occasionally in MA) suggested that it be classified within another genus [17].

The *Caedibacter* group, which comprises both the nuclear and cytoplasmic symbionts of paramecia, is a sister group. These bacteria were grouped within one genus due to their characteristic morphological feature, ability to form R bodies. The presence of such bodies in a symbiont makes the paramecium host the

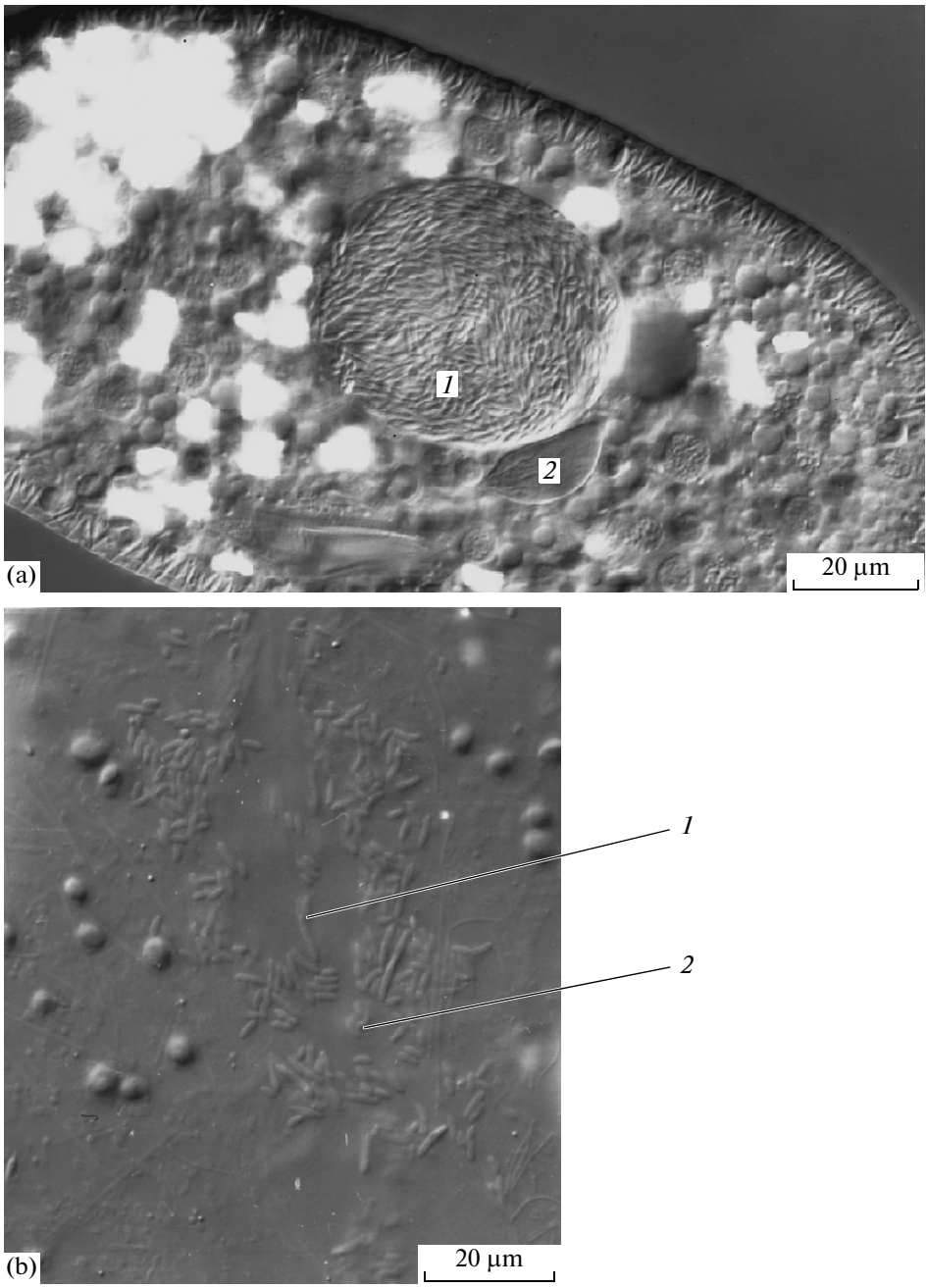

Fig. 1. "*H. curviuscula* 02AZ16 in the macronucleus of *P. bursaria*. Symbionts in the host nucleus: MA (*1*) and MI (*2*) (a) and in the crushed host cell: IF (*1*), RF (*2*) (b).

killer of symbiont-free paramecia. Phylogenetic analysis revealed, however, that *C. taeniospirales* localized in the cytoplasm belongs to the *Gammaproteobacteria*, while the intranuclear symbiont *C. caryophilus* belongs to the *Alphaproteobacteria* [18]. Thus, the species of this genus belonged to different phyla. This is a demonstration of the ambiguity of the morphological criteria for description of bacterial species.

Generally, the ciliates of the genus *Paramecium* carry various intranuclear bacterial obligate endosymbionts, which have not been cultured outside the host cells. Molecular phylogenetic analysis of the symbionts makes it possible to determine their taxonomic position and to trace their evolutionary relations. The family *Rickettsiaceae* and the progenitors of mitochondria have previously been shown to be sister groups [19]. Elucidation of the phylogenetic position of the known rickettsia-related symbionts and determination of the position of the previously unstudied isolates will outline the group of symbiotic bacteria

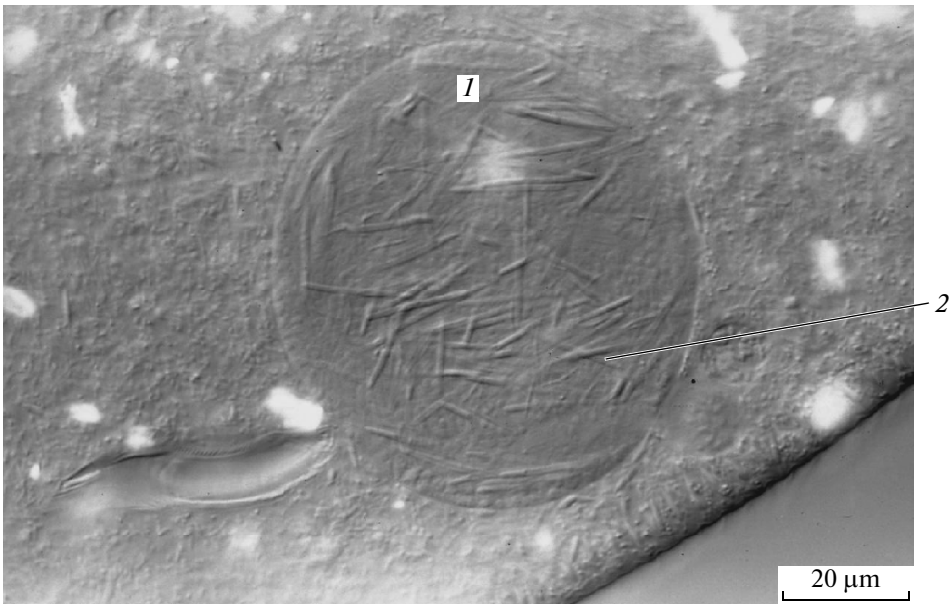

Fig. 2. *H. obtusa* in the macronucleus of *P. caudatum:* MA (*1*) and IF (*2*).

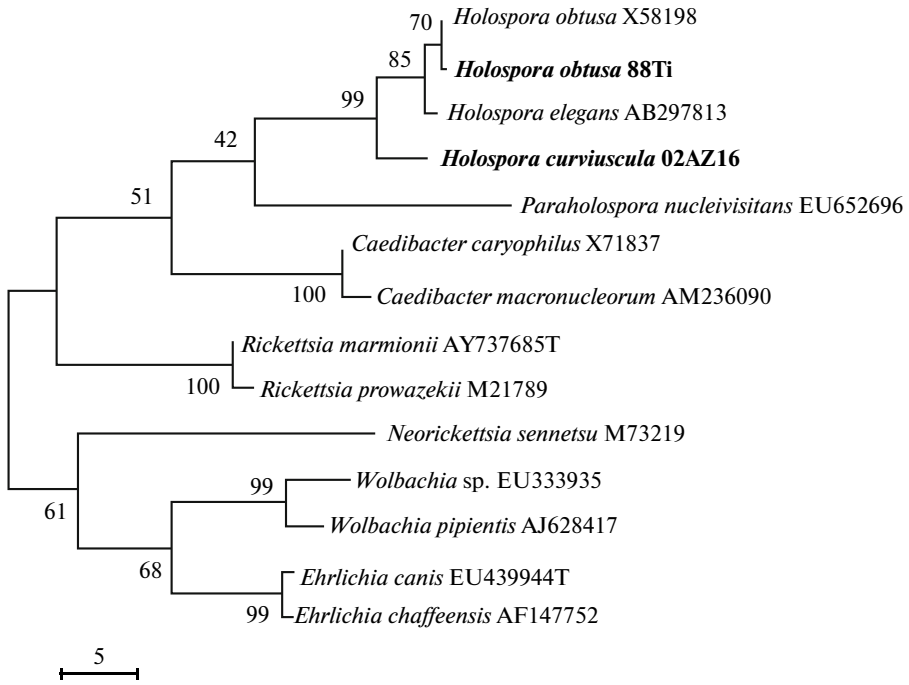

Fig. 3. Phylogenetic tree of the family *Rickettsiaceae* constructed based on 16S rDNA sequences [17].

important for our understanding of the evolution of the mitochondria and the eukaryotic nucleus. In general, determination of the taxonomic position of obligate endosymbionts and of the phylogenetic relations between the symbionts from different hosts, as well as comparison with the host phylogeny, is promising for analysis of the prerequisites of symbiosis and of its evolutionary consequences.

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