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First evidence of bacterial endocytobionts in the lobose testate amoeba *Arcella* (Amoebozoa, Arcellinida) [1]

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Summary

While prokaryotic symbionts in ciliates are extensively searched, free-living amoeba species of no importance to human health have been widely neglected in symbiont studies. The present paper gives the first evidence of bacterial endocytobionts in *Arcella*, a lobose testate amoeba. Clonal cultures of different *Arcella* species were investigated for the presence of endocytobionts using 16S rRNA gene sequencing, fluorescent *in situ* hybridization (FISH) and transmission electron microscopy (TEM). A rich diversity of eubacterial sequences have been identified by amplification of partial 16S rRNA gene sequences either by direct isolation of DNA from the testacean cell or following cultivation of bacteria from individual *Arcella* cells on agar plates. FISH with different probes against various eubacterial targets demonstrated the presence of single bacterial cells scattered in the cytoplasm that were clearly different from those aggregated within the food vacuoles. α-Proteobacteria and Gram-positive bacteria were visualized in different host species kept in clonal cultures. Transmission electron microscopical surveys revealed single rod-shaped bacteria located in different parts of the cytoplasm. Bacteria from the same host clone appear to cover a wide phylogenetic range. Although some belong to the same taxa as symbiotic bacteria of other eukaryotic organisms, others are close relatives of human pathogens, suggesting the potential role of *Arcella* species as reservoires. This idea was supported by the presence of *Legionella sp.* in 3 different *Arcella* samples from natural environment. In symbiont-bearing *Arcella* clones lysis of host cell has not been detected, but cells in older cultures occasionally started cyst formation. This phenomenon might indicate that the endocytobionts detected so far are not harmful to the host cells, though their possible beneficial role is still to be proved.

Key words: Bacteria, symbiosis, endocytobionts, testate amoebae, *Arcella*

Introduction

Intracellular bacteria have long been known to occur in amoeboid organisms. However, most of the amoeboid hosts studied are pathogens, mostly obtained from culture collections (Declerck et al., 2005). There are only a few reports in the literature of endocytobionts of non-pathogenic amoeboid hosts (Michel et al., 2000). Most research on endocytobionts in free-living protozoa was performed

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on ciliates (Görtz, 2001; Fokin et al., 2003). Testate amoebae have not been examined so far in this respect.

The presence of prokaryotes in amoebae was reported several times in the second half of the nineteenth century. An important example from the early studies is that of Nägler (1910) who observed the invasion of an amoeba by bacteria. Thus, already Nägler recognized the presence of bacteria in amoebae as a phenomenon.

A wide range of phylogenetically different bacteria has been discovered in amoebae, mainly without a specific relationship with the host. This means that bacteria are not confined to a certain amoeboid taxon.

According to our present knowledge, testate amoebae are clonal, asexual organisms (Meisterfeld, 2001). Signs of autogamy have been documented only once, though convincingly, in an *Arcella* species (Mignot and Raikov, 1992). This process must be very rare and, therefore, testate amoebae display a high morphological variability. The extreme morphological polymorphism has often been encountered during faunistic analyses, especially in *Difflugia* species.

The aim of the present study, to reveal endocytobionts in testate amoebae, was stimulated by the idea that bacteria might contribute to the survival and genetic polymorphism of testate amoebae. As a research object, we chose the representatives of the genus *Arcella* Ehrenberg, 1830, one of the first testate amoeba genera described. The features making them an ideal research object are the flat, transparent organic test and the relatively large size. Some data are available on their culture conditions (Netzel, 1975). *Arcella* species can be easily collected in the nature and the identification of the morphospecies is less problematic than, for instance, in *Difflugia* species. The above considerations are supported by more than 70 papers in which *Arcella* species have been used in experiments (e.g., Moraczewsky, 1970; Netzel, 1975).

Initially, we tried to find out whether endocytobionts are or are not present in the *Arcella* species under investigation. The subsequent aims were to determine the phylogenetic affiliation of the detected bacteria, the prevalence of endocytobionts in different *Arcella* species, the duration of bacterial presence and the localization of bacteria within the host cell.

The presence of human pathogens in amoebae is an important issue in public health (Rowbotham, 1980). Therefore, we investigated the occurrence of *Legionella*, the genus including the causative agent of the Legionnaire´s disease, *L. pneumophila,* in both clonal cultures and environmental samples of *Arcella* spp. (Rowbotham, 1980).

Material and Methods

Different species of *Arcella* (Amoebozoa, Arcellinida) were subjected to a complex methodological approach in order to find and characterize endocytobionts. *Arcella* cells both from clonal cultures and directly from environmental samples were used.

Clonal cultures of several *Arcella* species were set up in order to investigate endocytobionts. Cells were transferred to sterile plates filled with mineral water (Danone Vitalinea) after several subsequent washings in the same medium. Cultures were fed on *Enterobacter aerogenes*. The clone of *Arcella rotundata* Playfair isolated from the Ipoly River, North Hungary in 2005 was involved in all FISH, TEM and molecular investigations. Other clones included in the FISH studies were of *A. polypora* Penard (isolated from the peatland near Ócsa in 2006) and *Arcella excavata* Cunningham (from the Danube River, 2007).

In situ visualization of endocytobionts was performed on both clonal cultures and environmental samples of different *Arcella* species by fluorescent *in situ* hybridization following a standard protocol (Amann et al., 1992). After 3-12 hours of fixation in paraformaldehyde solution (4° C), specimens of *Arcella spp.* were washed in 1xPBS solution, transferred to slides and dehydrated in an increasing ethanol series. Hybridization was carried out at 46° C. Formamide concentration of the hybridization buffer was determined according to the applied fluorescently labelled probes. Probes were targeted against large groups of bacteria presumed to occur as endocytobionts, such as Eubacteria (5' ALEXA 546 - GCT GCC TCC CGT AGG AGT – 3‘), α-Proteobacteria (5’ Rhodamine – GGT AAG GTT CTG CGC – 3’) and low GC content Gram-positive bacteria (5’ Rhodamine – GGA AGA GTC CCT ACT GCT G -3’). A Nikon Eclipse 80i or an Olympus BX61 epifluorescence microscope was used for observations.

Bacterial strains were isolated by spreading single *Arcella* cells on agar plates. Prior to spreading, the host cells were washed five times in sterile water in order to remove the extracellular bacteria. Two types of agar plates were designed to imitate the intracellular composition of the host cell, modified R2 (mR2) medium and general amoeba-containing (GAC) medium. The mR2 medium contained 0.5 g yeast extract, 0.5 g dextrane, 0.5 g casamino acids, 0.25 g casein hydrolysate, 0.25 g meat peptone, 0.25 g starch, 0.25 g tryptone, 0.25 g cellulose, 0.25 g trypcasin peptone, 0.3 g K_2HPO_4, 0.048 g $MgSO_4$ and 15 g agar in 1 l destilled

water (final pH 6,5). The GAC medium contained 40 ml of well-growing *Arcella* culture (approximately 50 cells), 0.4 g K_2HPO_4, 1 ml vitamin solution (DSMZ-603; http://www.dsmz.de/media/media.htm), 1 ml trace element solution SL-4 (DSMZ-14) and 15 g agar in 1 l destilled water (final pH 6.5). All media were autoclaved for 20 min at 121°C. Vitamin and trace element solutions were filtered (through a 0.2 µm pore membrane) and added after autoclaving. Plates were incubated at 28°C for 2 weeks aerobically. The isolated bacterial strains were maintained on the same slants they were isolated from, also at 28°C. The isolates were conserved by freezing right after isolation.

Genomic DNA from the isolated bacterial strains was extracted by Bacterial Genomic DNA Miniprep Kit (V-GENE) using 24 hour-old strains and following the manufacturer's description. The genomic DNA was detected by agarose gel electrophoresis and part of the 16S rRNA gene was amplified by PCR using the bacteria-specific Bac27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and Univ1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers. PCR reactions were done in 50 µl volumes of 1x Taq buffer (Fermentas) containing 1 µl extracted DNA, 0.2 of each primer, 2mM $MgCl_2$, 200 µM of each dNTPs and 1.25 U of Taq DNA Polymerase (Fermentas). The PCR program had an initial denaturation step at 98° C for 5 min, prior to addition of Taq DNA polymerase, and followed by 30 cycles of 94° C for 30 s, 52° C for 30 s, and 72°C for 1 min with a final extension cycle at 72°C for 10 min. After electrophoretic detection the PCR products were grouped by their ARDRA patterns using the enzymes Hin 6I and Alu I (Fermentas) (37°C, overnight incubation) (Massol-Deya et al., 1995). The group representatives were cleaned using the PCR-M Clean Up System Kit (Viogene), and sequenced. Sequencing reactions were carried out with an ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction v3.0 kit (Perkin Elmer) using the primer Bac519R: 5'-GWATTACCGCGGCKGCTG-3'. The partial 16S rRNA genes were sequenced with an ABI 310 Genetic Analyzer (approximately 400 bp).

The 16S rDNA partial sequences were identified by comparison with sequences available in the NCBI GenBank database (available on the web site www.ncbi.nlm.nih.gov) using BLAST program, megablast algorithm (Altschul et al., 1997).

The nucleotide sequences of the representative isolates were deposited in the EMBL nucleotide database. For accession numbers see Table 1.

Cells for transmission electron microscopy were

Table 1. List and phylogenetic affiliation of bacterial strains isolated from *Arcella rotundata* specimens planted individually on agar plates

Reference strains	Number of isolates	Medium	Closest relative	Matching base pairs	Sequence similarity	Phylogeny
ESZ 10t	2	R2M	*Sphingomonas* sp. KH406	421/422	99%	α-Proteobacteria
ESZ 2t	1	R2M	*Acidovorax* sp. 10407	463/468	98%	β-Proteobacteria
ESZ 22t	8	GAC	*Variovorax paradoxus* strain E4C	380/384	98%	β-Proteobacteria
ESZ 25t		R2M		770/770	100%	
ESZ 26t				748/748	100%	
ESZ 29t				742/742	100%	
ESZ 31t				790/790	100%	
ESZ 11t	2	R2M	*Paenibacillus graminis* RSA19	487/491	99%	Firmicutes
ESZ 27t	2	R2M	candidatus *Chryseobacterium massiliae*	473/477	99%	Bacteroidetes
ESZ 21t	1	R2M	Uncultured *Verrucomicrobia* bacterium clone AKYG980	496/514	96%	Verrucomicrobiales

fixed with 2% glutaraldehyde in 0.1M cacodylate buffer at room temperature for 1 hour, then at 4° C overnight, rinsed in cacodylate buffer, then postfixed in 0.5% osmium tetroxide. After dehydration in a graded series of ethanol the cells were transferred into propylenoxyde in order to remove ethanol, then embedded in Durcupan ACM epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The examinations were performed on a JEOL 100CX-II electron microscope.

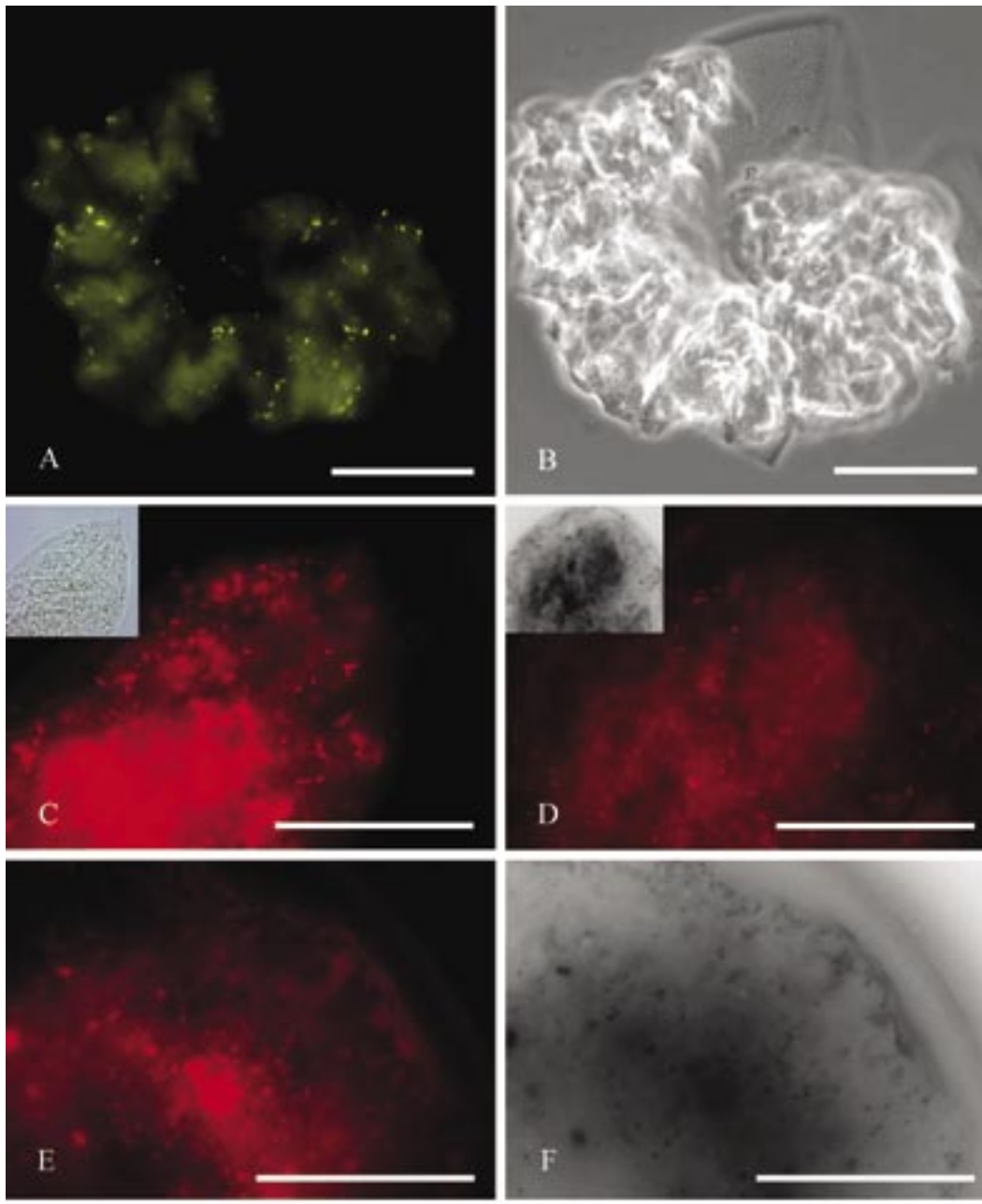

Fig. 1. Endocytobiont bacteria in *Arcella* species demonstrated by FISH. A – Bacteria in the cytoplasm of *Arcella rotundata* (probe against Eubacteria, label: ALEXA546); B – the same object in phase-contrast illumination indicating the rest of the *Arcella* shell; C – short rod-shaped bacteria (the same host species, α-Proteobacteria specific probe, label: rhodamine); D – coccoid bacteria (the same host species, low per cent GC content specific Gram-positive bacteria specific probe, label: rhodamine); E – coccoid bacteria in *Arcella polypora* (low per cent GC content specific Gram-positive bacteria specific probe, label: rhodamine); F – the same object with visible edge of the *Arcella* shell in black and white, inverted picture. Scale bars: 20 μm.

To detect bacterial pathogens in *Arcella* species we investigated both clonal cultures and cells from environmental samples for the presence of *Legionella spp. Arcella* cells were collected from environmental samples by a micropipette and washed five times in sterile water to clean the shell surface from bacteria. Following the above-mentioned first PCR, a nested one was carried out with *Legionella* specific primers (JFP: 5'-GAG GCA GCA GTG GGG AAT-3', JRP: 5'-CCC AGG CGG TCA ACT TAT-3', Cloud et al., 2000) with the temperature profile of 93°C initial denaturation for 3 min, than 38 cycles of 94° C for 45 s, 57° C for 45 s and 72° C for 45 s, with a final extension of 72° C for 10 min.

Results

In some *Arcella* species endocytobionts were explored by all the methods described above. The first *in situ* hybridization was performed on *Arcella rotundata* specimens from clonal culture using a universal Eubacteria specific primer. Short rod-shaped bacteria were scattered throughout the cytoplasm. This examination was repeated on the same host clone using the same probe two months later; the same signals were found again. Another two months later the same host species was investigated with different specific primers, against α- and β, γ-Proteobacteria, low GC content Gram-positive bacteria and Verrucomicrobiales. We detected specific signals in the case of α-Proteobacteria specific probe, showing short rod-shaped bacteria and low per cent GC content Gram-positive bacteria specific probe yielding coccoid prokaryotes. In both cases we observed bacteria individually scattered throughout the cytoplasm. For Verrucomicrobiales specific probe we obtained only aspecific signals without appraisable results. Applying β, γ-Proteobacteria specific probe we did not find any signals.

Further species from clonal cultures were also involved in the FISH studies. *Arcella excavata* was positive for the universal Eubacteria probe and *Arcella polypora*, for the low GC content Gram-positive bacteria probe, showing coccoid bacteria of the same size as in *A. rotundata* (Fig. 1.).

Arcella megastoma Wailes and *A. vulgaris* Ehrenberg from environmental samples were investigated with FISH using universal Eubacteria probe. In both cases weak, aspecific signals were observed.

From *Arcella rotundata* specimens 16 bacterial strains were isolated and subjected to phylogenetic analysis. The ARDRA grouping resulted in 6 phylotypes. Partial 16S rRNA gene sequences showed similarity to phylotypes of five phyla with 96-100% sequence similarity to the closest relatives (Table 1).

Most of the isolated bacteria (11 isolates) belonged to the group of Proteobacteria, namely to the genera of *Sphingomonas*, *Acidovorax* and *Variovorax*. Species of these taxonomic groups are mostly common environmental bacteria, often isolated from soil and rhizosphere. Eight isolates arising from different isolation events from *Arcella* hosts proved to be 100% identical to a specific strain of *Variovorax paradoxus*. Two isolates showed high sequence similarity to the type strain of *Paenibacillus graminis,* a low per cent GC Gram-positive bacterium, which is also a common environmental species of soil and plant roots (Berge et al., 2002); intrafungal strains have recently been detected within the genus (Bertaux et al., 2003). Another two isolates were identified as candidatus *Chryseobacterium massiliae*. Their closest relative was first detected as an amoeba-resisting bacterium from human nasal swabs by amoebal coculture (Greub et al., 2004). Our isolate showed 99% sequence similarity to that strains according to partial 16S rRNA sequence.

Only one isolate grouped to the Verrucomicrobiales. This strain showed only 96% sequence similarity to the known species, and may represent a new taxon. Though this isolate could grow well on the agar plates surrounded by other bacterial colonies, after isolation it could not survive the passaging procedure.

Transmission electronmicroscopy revealed short rod-shaped bacteria of 600-800 nm length in *A. rotundata* host (Fig. 2). Single bacteria without a surrounding vacuole were localized mostly in the upper cytoplasm. In some cases more bacterial cells appeared in close vicinity. A remarkable space could be observed between the endocytobiont and the host cytoplasm. The membrane of the bacteria was folded. Even possibly dividing forms could be detected. Occasionally nucleoid was visible in the middle part of several individuals. On account of the thin wall they seem to be Gram-negative bacteria. Besides short rods, some very clear globular bodies could be seen in the cytoplasm, in size about the half of the rods, showing an apparently thicker, frothy and irregular outline. These bodies were supposed to be Gram-positive bacteria owing to the thick wall-like outer border, but the central part of the assumed bacterium cell was invisible. Organelles of the *Arcella* host seem to be free from endocytobionts.

We were curious about the occurrence of pathogenic bacteria in the testacean host and, therefore, examined several clonal cultures and environmental samples of *Arcella* for the presence of *Legionella*. Genus specific primer against *Legionella spp.* yielded

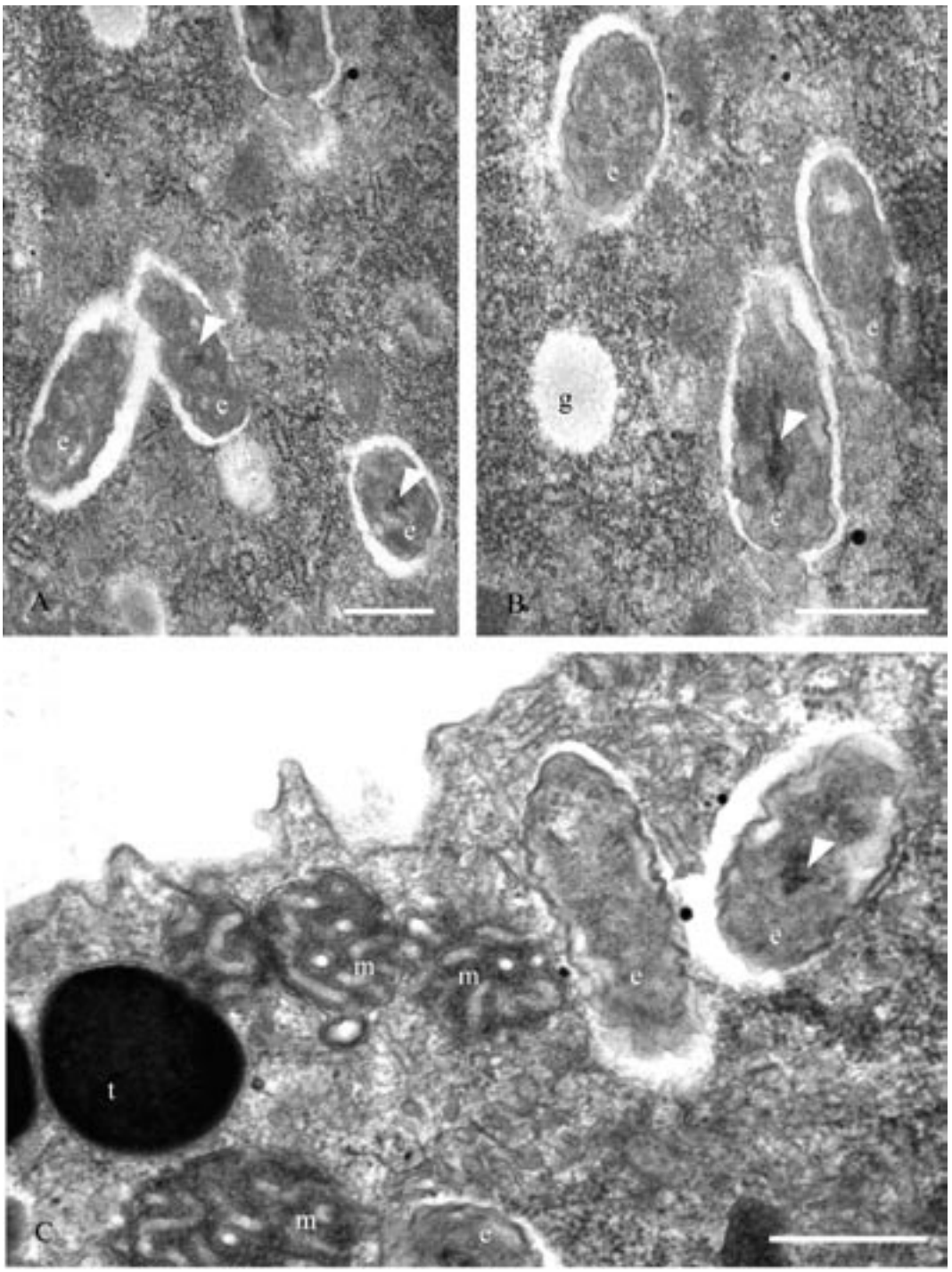

Fig. 2. Transmission electron micrographs of endocytobionts in *Arcella rotundata* from clonal culture. A – Short rod-shaped bacteria located in the cytoplasm; B – one possible Gram-positive coccus among three short rods; C – bacteria in the peripheral cytoplasm surrounded by numerous mitochondria; dark vesicles are thecagenous granules produced for shell construction for the *Arcella* daughter cell. Abbreviations: e – rod-shaped endocytobiont, t – thecagenous granule, m – mitochondrium, g – possible Gram-positive coccus; arrowhead: nucleoid. Scale bars: 500 nm.

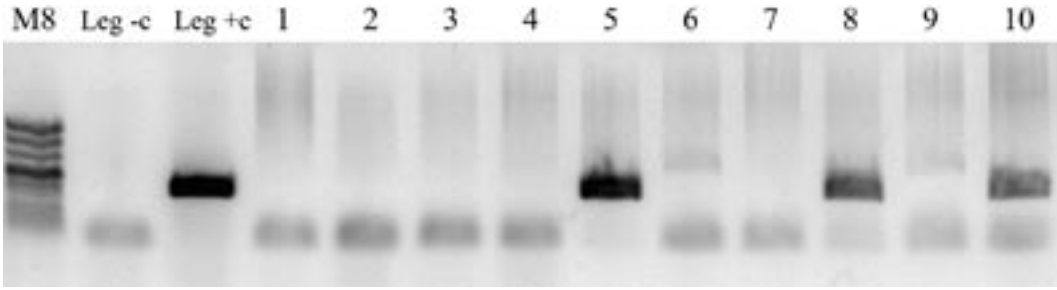

Fig. 3. Detection of *Legionella* sp. in *Arcella* spp. on agarose gel. Positive *Arcella* species are *A. discoides, A. megastoma, A. vulgaris*, positive control was *Legionella pneumophila* DNA. Abbreviations: M8 – molecular weight standard, Leg-c – negative control, Leg+c –positive control; 1 – *Arcella rotundata*, 2 – *A. polypora*, 3 – *A. discoides* clone 1, 4 – *A. hemisphaerica* clone 1, 5 – *A. discoides* clone 2, 6 – *A. excavata*, 7 – *A. formosa*, 8 – *A. megastoma*, 9 – *A. hemisphaerica* clone 2, 10 – *A. vulgaris*.

three positive results among the examined host species: *A. discoides* Ehrenberg, *A. megastoma* and *A. vulgaris* (Fig. 3.).

Discussion

Our investigation demonstrated for the first time the presence of endocytobiont bacteria in the cytoplasm of the lobose testate amoeba *Arcella*. While the overwhelming majority of previous studies focused on free-living amoebae with certain pathogenic affinities (*Acanthamoeba*, *Naegleria*, etc.), we chose a host organism which at no time displays an invasive attitude.

We have revealed the presence of endocytobiotic bacteria in the cytoplasm of different *Arcella* species by FISH and visualized rod-shaped α-Proteobacteria and coccoid Gram-positive bacteria, the latter in two species (*Arcella rotundata* and *A. polypora*). We encountered two different endocytobionts during FISH examinations of identical *Arcella rotundata* clonal culture at the same time, which is at variance with the predominating view (Horn and Wagner, 2004) that only one prokaryotic endocytobiont occurs in one eukaryotic host isolate. By agar plate culture we identified the presence of even more bacterial strains from *Arcella rotundata* host (Table 1). However, caution is needed when interpreting these findings, since the culture method is not selective for endocytobionts and thus we can expect a great portion of the strains to be temporary residents of environmental origin.

Nevertheless, the unknown Verrucomicrobia and the candidatus *Chryseobacterium massiliae* strains detected can be considered as possible endocytobionts, because of their symbiotic or endocytobiotic affinities (Greub et al., 2004). Besides, *Variovorax paradoxus* could be also an interesting member, maybe the conductor, of the amoeba colonizing bacteria association due to its ability to interfere with the communication of other bacteria through the utilization of acyl-homoserine lactones as the sole source of energy and nitrogen (Leadbetter and Greenberg, 2000). However, the lack of signals in the case of β-γ-Proteobacteria specific probes is remarkable. On the one hand, this result shows a great contrast with the 16S rRNA partial gene sequence studies, since most of the sequences obtained belong to the β-Proteobacteria (*Acidovorax* and *Variovorax* strains, see Table 1). On the other hand, the food organism *Enterobacter aerogenes* used to maintain clonal cultures is a member of γ-Proteobacteria. This controversal result, however, excludes the possibility that the strong signal by the general Eubacterium specific probe (Fig. 1, A) would sign *Enterobacter aerogenes*. Strikingly, this bacterium was also missing from the cultivation spectrum, although it could grow on the medium used in this study.

By means of transmission electron microscopy we found one rod-shaped, most likely Gram-negative bacterium and the presence of Gram-positive bacteria has not been proved yet. The latter result is fairly unusual, since the current view is that Gram-positive bacteria in general occur freely in the environment. However, we do not reject the possibility of a Gram-positive endocytobiont, since FISH experiments in two different host species yielded coccoid Gram-positive bacteria. Furthermore, two distinct isolates from *A. rotundata* host showed high sequence similarity to *Paenibacillus graminis*, a Gram-positive bacterium and otherwise a common environmental species. We cannot exclude that a coccoid stage of this species might occur in intracellular environment, but this idea has to be confirmed by further

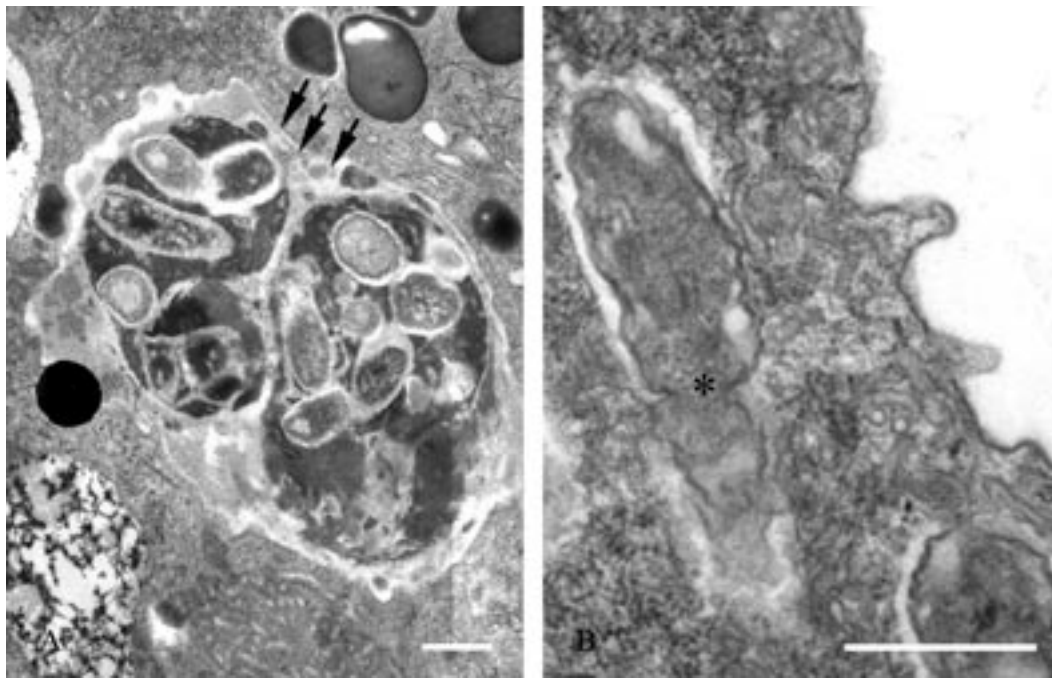

Fig. 4. Transmission electron micrograph of *Arcella rotundata* cytoplasm. A – Food vacuole full of bacteria and other food organisms; B – endocytobiont in division. Arrows show the food vacuole, * - dividing endocytobiont. Scale bars: 500 nm.

experiments. The recent finding on an intracellular *Paenibacillus* species in an ectomycorrhizal fungus *Laccaria bicolor* supports the above considerations (Bertaux et al., 2003).

Food vacuoles containing prokaryotes looked completely different, which made misinterpretation of food bacteria as endocytobionts unlikely.

The rod-shaped bacteria seemed similar to each other in appearence; moreover, some dividing specimens were detected (Fig. 4, A, B) sustaining the concept of their endocytobiotic nature.

The number of endocytobionts per host cells appeared to be high: in all the positive FISH experiments at least 100 bacterium specimens were estimated within one host cell. This result was consistent with the results of the analysis of transmission electron micrographs. The bacteria were not surrounded by a vacuolar membrane of the host, which indicated their proteobacterial nature (Amann et al., 1997) and agreed well with the FISH results about the possible occurrence of an α-Proteobacterium in the cytoplasm of *A. rotundata*.

It is still unclear whether the associations of bacteria and host encountered in our research were transient or permanent. In specimens from the same clonal culture of *A. rotundata* positive fluorescent signals were exhibited over a four-month time-span, but hether the same or different bacterial species were present on every occasion remains uncertain.

A remarkable difference was revealed in the distribution of *Legionella* infection among *Arcella* hosts: clonal host cultures were not infected, while 3 out of 6 environmental samples contained *Legionella* sp. Presence of the human pathogen *Legionella pneumophila* has not been confirmed yet. Sequencing of the targeted 16S rRNA gene region will reveal the affiliation of *Legionella* sp. in *Arcella* species. However, it is worth to point it out that none of the hosts kept in clonal cultures for weeks or months (*Arcella discoides* clone 1, *A. excavata*, *A. hemisphaerica* clone 1) or years (*A. polypora, A. rotundata*) contained this bacterium genus. All the host species harboring *Legionella* sp. (*A. discoides* strain 2, *A megastoma, A. vulgaris*) spent in the laboratory only a few hours or days prior to the amplification of the partial 16S rRNA gene sequence in order to avoid the infections in the laboratory. Consequently, we suppose that if this well known transient prokaryote disappears from the host after a short period, the resident bacteria in *A. rotundata* and *A. polypora* indicated by FISH are endosymbionts. On the other hand, our FISH experiments showed that that *Arcella* specimens freshly isolated from environmental samples exhibited very week, aspecific signals. Adjustment of experimental conditions, such as changing formamide concentration in FISH

hybridization buffer, increasing probe specificity and number of experiments, etc., might refine this result.

At present, we cannot estimate the role of the endocytobionts detected. Lysis of host cell has not been detected; instead cells in older cultures occasionally started cyst formation. Tis phenomenon might indicate that the endocytobionts revealed so far are not harmful for the host cells; nevertheless, their possible beneficial role is still to be proved.

Te combination of the molecular and morphological methods applied seems to be succesful for exploration of endocytobionts in *Arcella* species. In the future we plan to study the role of endocytobionts within the host by means of regular analyses of clonal *Arcella* cultures. We have to answer which bacteria are symbionts (*sensu lato*) and which of them utilize the *Arcella* host in a transient manner, as a reservoire. Te type of host - endocytobiont relationship must be studied in detail to decide whether a particular endocytobiont is beneficial, harmful or indifferent for the host. Te ultimate purpose of the *Arcella* - endocytobiont relationship research is to ascertain whether the endocytobionts are able to influence the viability and morphology of their testate amoeba hosts.

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