ACTA
PROTOZOOLOGICA

Bacterial Endocytobionts within Endosymbiotic Ciliates in *Dreissena polymorpha* (Lamellibranchia: Mollusca)

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Summary. This paper documents a multi-level microcosm system: *Dreissena*-ciliates-bacteria-viruses. In the first comprehensive investigation of endocytobionts present in the cytoplasm of endosymbiotic ciliates from mussels, bacteria are described from *Conchophthirus acuminatus* and an undescribed *Ophryoglena* sp. species which are, respectively, commensal and parasitic in the freshwater bivalve *Dreissena polymorpha*. Light microscopy, electron microscopy, Feulgen staining, and *in situ* observation indicated that in some populations of *C. acuminatus* practically all individuals were infected with cytoplasmic bacteria. Some of these bacteria were in the α-subgroup of proteobacteria. *In situ* hybridization indicated that some other eubacteria with a very similar morphology were also present in the cytoplasm of *C. acuminatus*. Using *in situ* hybridization with appropriate oligonucleotide probes, a large amount of bacteria, most of which were also in the α-subgroup, were observed in the cytoplasm of each specimen of *Ophryoglena* sp. examined. Some bacteria with virus particles were also observed in a population of the *Ophryoglena* sp. The bacteria in *C. acuminatus* were not likely the same as in the *Ophryoglena* sp. The presence of α-subgroup proteobacteria in the cytoplasm of both endosymbiotic ciliates, in conjunction with previous reports of these bacteria in free-living species, indicates that they are widely established endocytobionts in these protists.

Key words: bacteria, *Conchophthirus acuminatus*, *Dreissena polymorpha*, *in situ* hybridization, *Ophryoglena* sp.

INTRODUCTION

The freshwater macrofouling bivalve *Dreissena polymorpha*, commonly known as the zebra or wandering mussel, has been continually spreading throughout Eastern and Western Europe since the beginning of the 19th century. Although considerable research has been carried out to understand the ecological interrelationships of this bivalve with other aquatic organisms, relatively little effort has been made to investigate the

diversity, distribution, and significance of endosymbiotic organisms present within these mussels. This gap of information is currently being addressed as a project of the International Research Consortium on Molluscan Symbionts (IRCOMS) (Molloy *et al.* 2001, Molloy 2002). IRCOMS investigations have documented that ciliates are the most common endosymbionts of *D. polymorpha*. Five host-specific ciliates (*Conchophthirus acuminatus*, *C. klimentinus*, *Hypocomagalma dreissenae*, *Sphenophrya dreissenae*, and *S. naumiana*) are known from the mantle cavity of this mussel and at least one undescribed *Ophryoglena* sp. from its digestive gland. The nature of the symbiotic relationships of these

species with their dreissenid hosts is typically not well defined, but appears to range from commensalism, e.g., *C. acuminatus,* to parasitism, e.g., *Ophryoglena* sp. (Molloy *et al.* 1997, Laruelle *et al.* 1999).

Intracellular bacteria have been observed in the cytoplasm, nuclei, and perinuclear space of a number of free living ciliate species, but their phylogenetic positions have rarely been determined (Görtz 1983, 1998; Fokin 1993; Fokin and Karpov 1995; Fokin *et al.* 1996, 2000). In this IRCOMS investigation, we report bacterial endocytobionts within endosymbiotic ciliates, in particular, within two species from *D. polymorpha*: the commensal *C. acuminatus* (Scuticociliatida, Conchophthiridae) (Fig. 1) and the undescribed parasitic *Ophryoglena* sp. (Hymenostomatida, Ophryoglenidae) (Fig. 2).

MATERIALS AND METHODS

Organisms: *Dreissena polymorpha* populations from the following five locations were dissected to collect endosymbiotic ciliates: lake Bodensee at Konstanz and a lake near Karlsruhe in Germany, from the Volkerak and Haringvliet waterbodies in the Netherlands, and from the Ivankovskoye reservoir in Russia. *C. acuminatus* was obtained from all locations, and the *Ophryoglena* sp. from all except the Karlsruhe lake. Although the majority ciliate populations examined contained bacteria in their cytoplasm, the research efforts outlined below focused primarily on specimens of *C. acuminatus* from Karlsruhe and Volkerak and on the *Ophryoglena* sp. from Bodensee, Volkerak, and Haringvliet.

Microscopy: live ciliates were temporarily immobilized following the methods of Skovorodkin (1990). Photomicrographs were taken with DIC and fluorescence optics (Axioskop, Zeiss, Germany and Zeiss confocal laser scanning microscope, LSM 410).

Staining: Feulgen procedure was done according to previous work (Fokin 1989). For *in situ*- hybridization, cells were fixed with 4% formaldehyde (w/v, freshly prepared from paraformaldehyde) in phosphate buffered saline solution (PBS) pH 7.2, for 2 h and washed with PBS. Cells were incubated with oligonucleotide probes in hybridization buffer (Fokin *et al.* 1996). A eubacterial specific probe (5'-GCTGCCTCCCGTAGGAGT-3'), labeled with fluorescein (FITC) and an α-subgroup of proteobacteria specific probe (5'-GCGTTCGCTCTGAGCCAG-5'), labeled with tetramethylrhodamine (TRITC) (Amann *et al.* 1990, 1991) were used.

Specimens were examined using a Zeiss LSM 410 with a plan neofluar 100x oil immersion objective. For detection of FITC and TRITC, an argon-ion laser (488 nm) and a helium-neon laser (543 nm) with appropriate emission filters, BP 510-525 nm, and 575-640 nm, respectively, were used. Color pictures were taken by a Polaroid recorder on a Zeiss LSM 410.

Electron microscopy: immediately after collection, during zebra mussel dissection, the ciliates were immersed in 2% glutaraldehyde (Grade I, Sigma Chemical Co.) in 0.025 M sodium cacodylate buffer (pH 7.4) for 90 min at 4°C. After centrifugation at 200 G, the cell

pellets were rinsed in buffer solution (0.05 M) and post-fixed with 1% osmium tetroxide (Sigma) buffered with sodium cacodylate. To facilitate manipulations, the cells after fixation were first embedded in agar. Following their dehydration through graded alcohol, the ciliates were embedded in Epon-Araldite (Sigma). Ultra-thin sections (60-80 nm), cut with a diamond knife on a LKB Ultratom V ultramicrotome, were placed on copper grids and stained with uranyl acetate and lead citrate. Ultrastructural observations were made using a Jeol CX100 (80KV) transmission electron microscope.

RESULTS

Bacteria in the cytoplasm of *Conchophthirus acuminatus* (Figs 1, 11)

Prevalence of infection was high, and in some *C. acuminatus* populations practically all individuals were infected. Using DIC and phase-contrast microscopy, relatively large bacteria (2.0-6.0 x 0.5-0.7 µm) were detected in four of the five *C. acuminatus* populations investigated, with infection intensities ranging from about 5 to 70 of these large bacteria per ciliate. Whereas bacteria were occasionally visible in live intact cells, they were more easily seen in squashed preparations. The best methods of detection, however, were Feulgen-staining and the *in situ* hybridization.

According to the DIC pictures of squashed living cells, the bacteria appeared to be well distributed throughout the cytoplasm. From light microscopic observations, it seemed that at least some bacteria were located in individual vacuoles. Electron microscopic images, however, did occasionally reveal membranes surrounding some bacteria, but whether these were truly endocytobiotic vacuoles was unclear. A clear zone frequently separated the bacteria from host cytoplasmic structures (Figs 3-5). Bacteria were sometimes observed close to the cisternae of endoplasmic reticulum without ribosomes (Fig. 5). No clear nucleoid structures were found inside of the bacteria. In rare cases, a portion of the bacterial "cytoplasm" appeared to have a different density (Fig. 5). The cell wall structure suggests that all bacteria could be Gram-negative (Figs 3, 4).

Using an *in situ* hybridization procedure with two oligonucleotide probes, at least two types of bacteria were detected in the cytoplasm: an α-subgroup of proteobacteria and one more type of eubacteria (Figs 12, 15). Since all bacteria had similar dimensions and shapes, we were not able to discriminate among them morphologically, but the two types always were located in
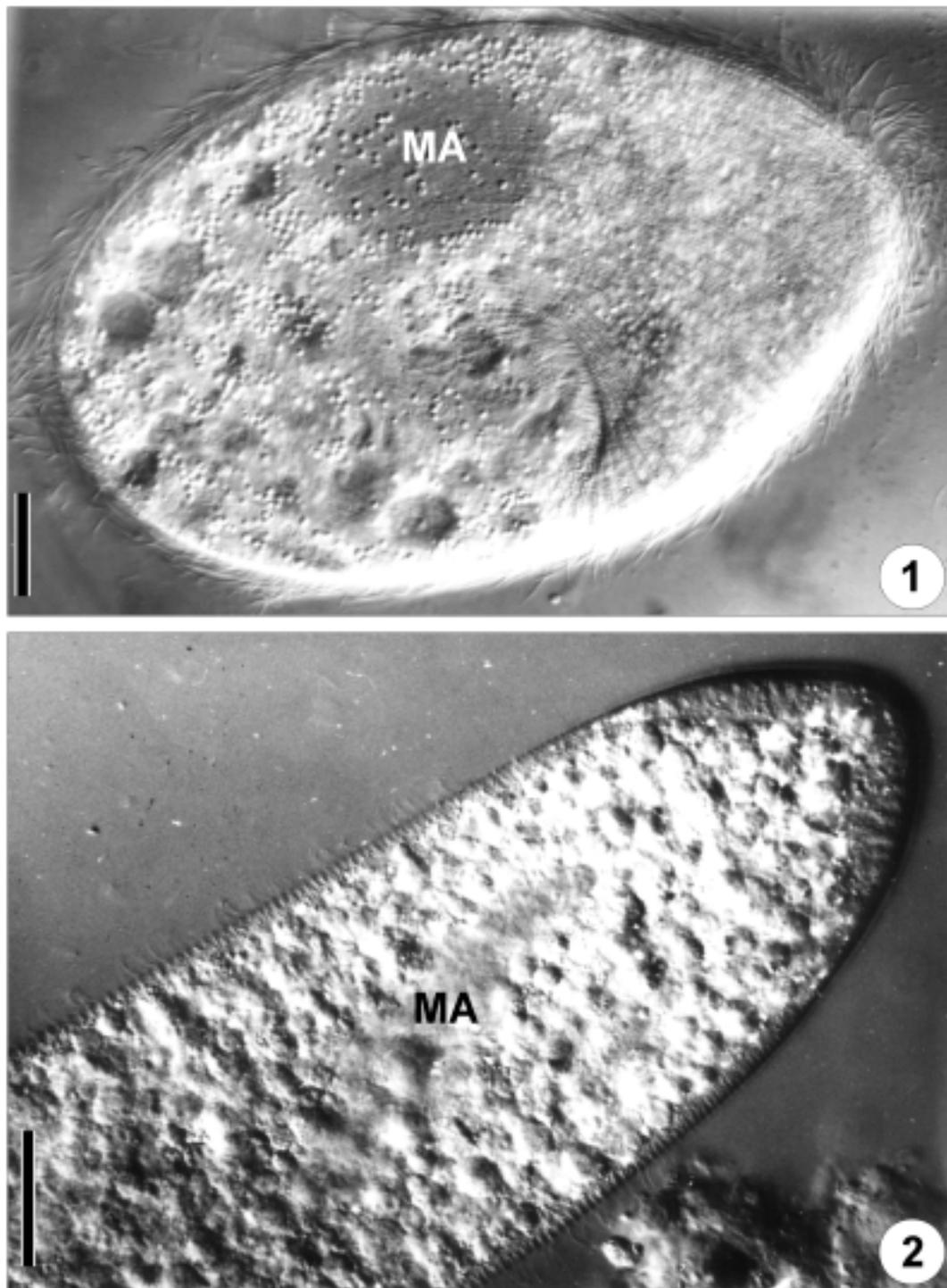
Figs 1-2. Ciliates investigated. **1** - *Conchophthirus acuminatus*, an endocommensal from the mantle cavity of *Dreissena polymorpha*; and **2** - *Ophryoglena* sp., a parasite of the digestive gland of *D. polymorpha*. Living cells, DIC contrast, MA - macronucleus. Scale bars 10 µm (1); 20 µm (2).
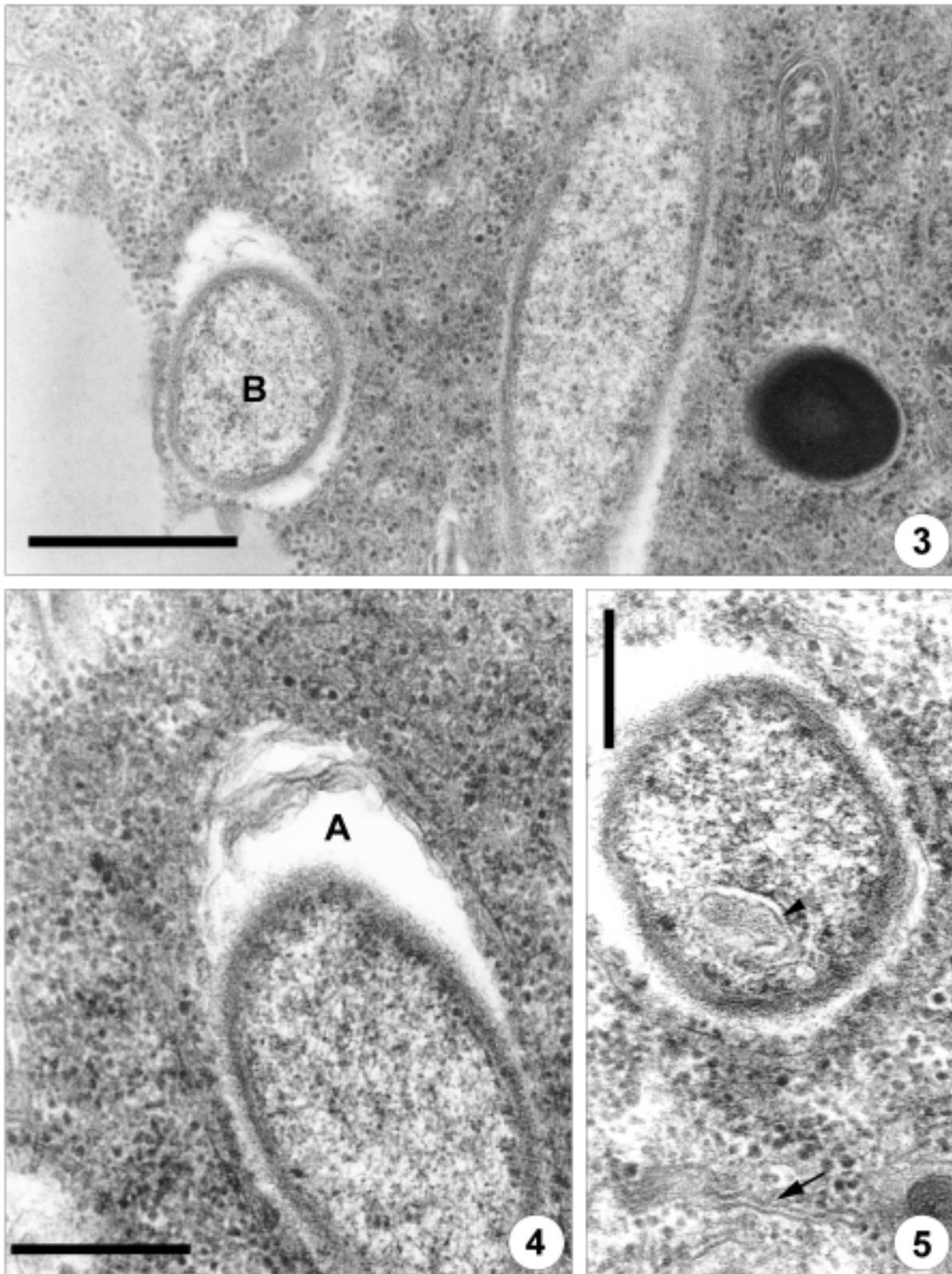
Figs 3-5. Electron micrographs of bacteria in the cytoplasm of *Conchophthirus acuminatus*. A - clear space separates the bacteria from host cytoplasmic structures; membranes surrounding some bacteria may be associated with individual endocytobiotic vacuoles. B - bacteria, arrowhead - region of bacterial cytoplasm with different density, large arrow - cisternae of endoplasmic reticulum. Scale bar 0.5 μm.
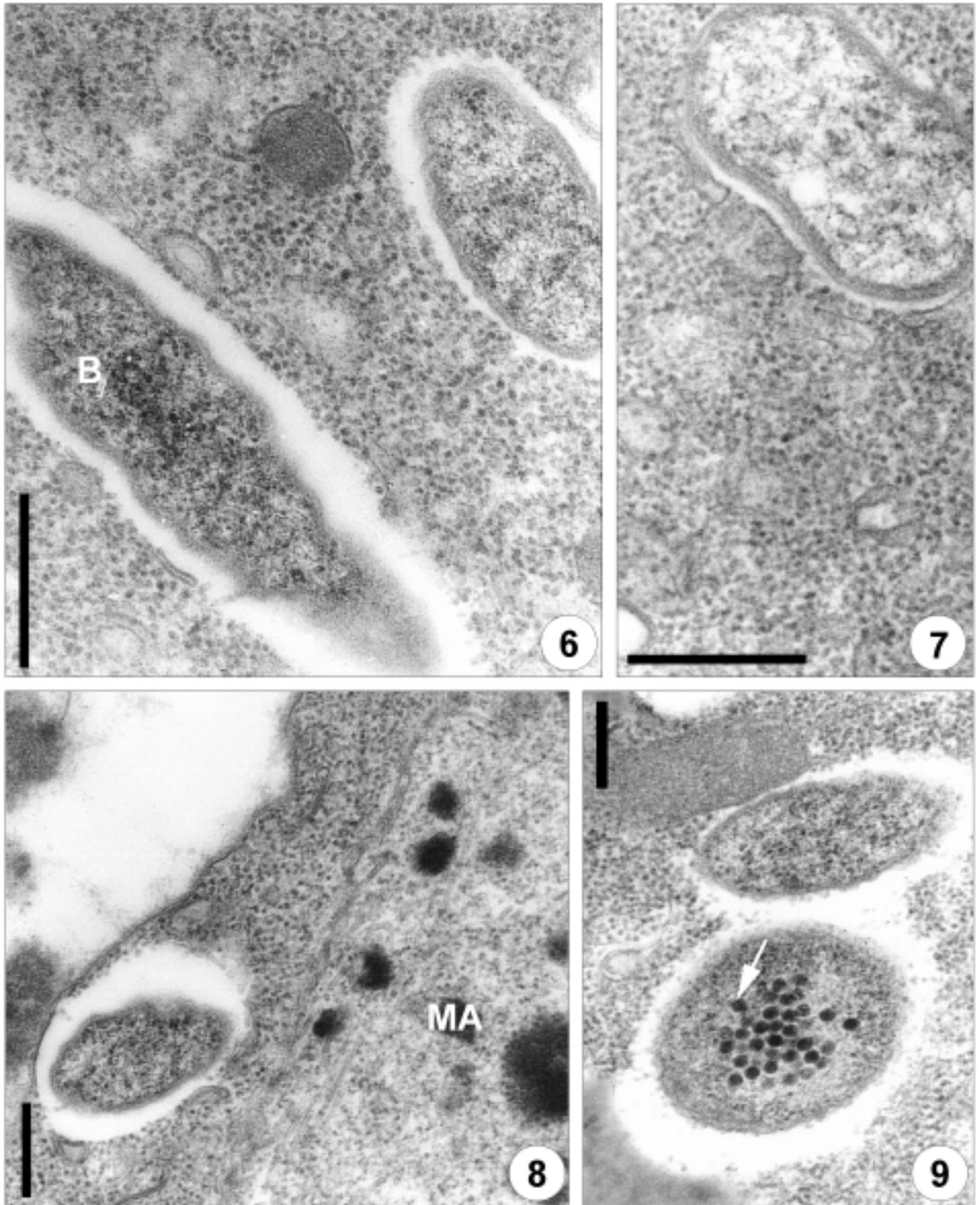
Figs 6-9. Electron micrographs of bacteria in the cytoplasm of *Ophryoglena* sp. **6, 8, 9** - no host membrane surrounding the bacteria was observed; instead, clear space separated the bacteria from host cytoplasmic structures. **7** - another type of bacterium was found, apparently, enclosed in an individual host vacuole; **9** - virus particles were observed in some of the bacteria in one of *Ophryoglena* population. B - bacteria, arrow - virus particles, MA - macronucleus. Scale bar 0. 5 μm.
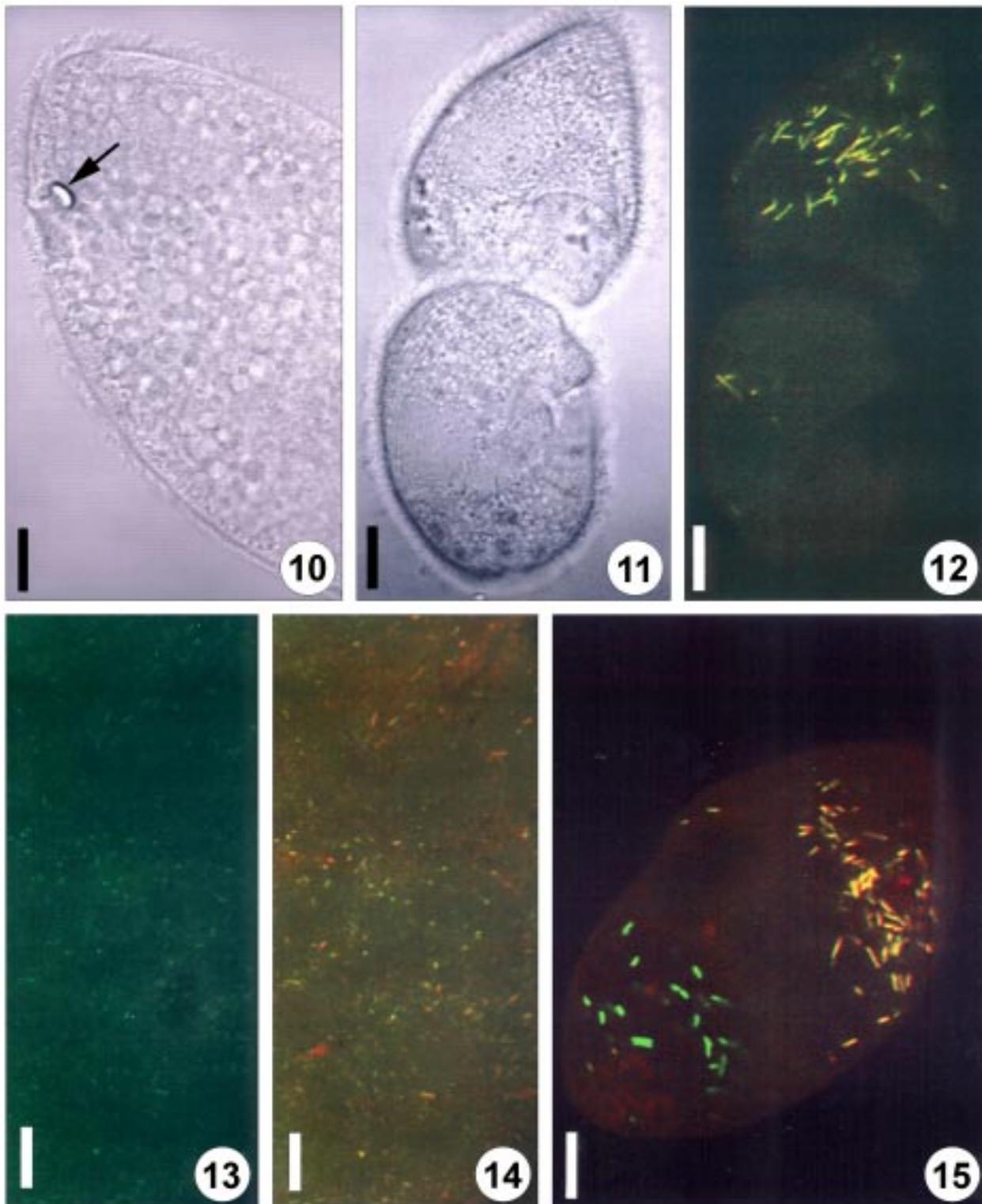
Figs 10-15. *In situ* hybridization. **10** - *Ophryoglena* sp. cell - the organelle of Lieberkühn (arrow). **11** - *Conchophthirus acuminatus* in division process; **12** - the same dividing cells labeled with eubacteria- and α-subgroup-specific probes. Bacteria in the cytoplasm (double labeling yellow). **13** - bacteria in the cytoplasm of *Ophryoglena* sp., the same cell as presented on fig.10. A labeling with eubacteria-specific probe (green); **14** - the same region with double labeling. Main bacteria are labeled as α-subgroup bacteria (orange) and some are labeled just as eubacteria (green). **15** - two different types of bacteria in the cytoplasm of *C. acuminatus*. Double labeling: populations of α-subgroup bacteria (orange) and some other eubacteria (green). The populations do not mix in the cytoplasm. Figs 10, 11 - DIC; Figs 12-15 - fluorescence microscopy. Scale bars 10 μm (10-12, 15); 6 μm (13, 14).

separate parts of the cell (Fig. 15). The α-subgroup was the most frequent type detected, and the *C. acuminatus* in some mussels appeared to have only this type (Fig. 12). Distribution of the bacteria during ciliate division appeared to be arbitrary (Figs 11, 12), with some progeny possibly receiving no bacteria. Experimental infection of aposymbiotic *C. acuminatus* with bacteria was not attempted.

Bacteria in the cytoplasm of *Ophryoglena* sp. (Figs 2, 10)

In living *Ophryoglena* it was impossible to detect any endocytobionts because of the opacity of the cytoplasm (Fig. 2), but bacteria were visible in squashed preparations of live specimens. *In situ* observations indicated that at least two types of bacteria (usually totaling more than 100 individuals) were present in each ciliate. Electron microscopy revealed that one type was rod-shaped, 1.5-2.0 x 0.3-0.5 µm. No host membrane was observed surrounding this type, but a clear space separated individual bacterial cells from host cytoplasmic structures (Figs 6, 8, 9). The second type of bacterium was short and rod-like, measured about 1.0 x 0.4 µm, and appeared enclosed in individual host vacuoles (Fig. 7). The two-membrane cell wall structure typical of Gram-negative bacteria was present in both types (Figs 6, 7). Both types of bacteria were generally located everywhere in the cytoplasm, sometimes even close to the nuclei (Fig. 8). The inner structure of the short bacteria was distinctive, differing from that of the larger rod-shaped type as well as the bacteria in *C. acuminatus* (Figs 3, 6, 7). In one population of *Ophryoglena* from the Netherlands, virus particles were observed in bacteria which morphologically resembled the larger rod-shaped type (Fig. 9).

Using the *in situ* hybridization procedure with two oligonucleotide probes, α-subgroup proteobacteria were identified in the cytoplasm of the *Ophryoglena* sp., along with a smaller number of other eubacteria (Figs 13, 14). From the size of the bacteria observed, it appeared that the large rod-shaped bacteria belonged to the α-subgroup and that the short ones were a mixture of both α-subgroup proteobacteria and other eubacteria (Figs 13, 14).

DISCUSSION

This is the first comprehensive investigation of bacteria present in the cytoplasm of endosymbiotic ciliates from mussels. Previous investigations of bacterial endocytobionts in ciliates have focused mostly on free-living host species. It is also the first detailed investigation of the cytoplasm of either *C. acuminatus* or the undescribed *Ophryoglena* sp.

Conchophthirus acuminatus

Conchophthirus acuminatus is an obligate commensal of the mantle cavity, and *Dreissena* spp. are its only known hosts (Molloy *et al.* 1997, Laruelle *et al.* 1999). Intensity of infection can be over several thousand ciliates per mussel (Burlakova *et al.* 1998, Karatayev *et al.* 2000), and the species is widely distributed in European *Dreissena* populations (Molloy *et al.* 1997).

Four from five *C. acuminatus* populations we sampled in this study were infected, but these bacteria are not present in a number European *C. acuminatus* populations (authors, unpublished data). However, we observed a high prevalence of bacterial infection in these four *C. acuminatus* populations, suggesting that where present, infection is usually of high intensity. The range in infection intensity recorded (i.e., 5 to 70 bacteria per host) was understandable since we observed that bacteria did not appear to be equally distributed between ciliate progeny during *C. acuminatus* division (Figs 11, 12).

Intracellular bacteria are known to be site specific, e.g., residing in the cytoplasm, in the micro- or macronuclei, or in the perinuclear space (Heckmann and Görtz 1991, Fokin 1993, Fokin and Karpov 1995). Some bacterial endocytobionts cannot live together in the same region of the host cell (Fokin 1993). Using *in situ* hybridization, we recorded two different types of bacteria in *C. acuminatus* and noted their presence in separate regions of the host cytoplasm (Fig. 15). In addition to a possible inhibitory (i.e., antibiotic) effect of one type of bacterium on another, we suggest that the lack of a uniform bacterial distribution might be due in part to a heterogeneous distribution of key host metabolites or nutrients. The ability of these bacteria to be cultivated outside of their host was not tested. Because of the relative ease of collection of infected *C. acuminatus*, this ciliate could be a very valuable organism for studying the dynamics of niche preferences among intracellular bacteria.

Ophryoglena sp.

Although ophryoglenine ciliates have been extensively studied (Canella 1976, Puytorac *et al.* 1983, Lynn *et al.* 1991, Kuhlmann 1993), their endocytobionts have never been investigated. Moreover, the *Ophryoglena*

sp. from the zebra mussel digestive gland is only currently being described as a new species (authors, in preparation) and has received very limited research attention. Since this *Ophryoglena* sp. is parasitic, the role of its cytoplasmic bacteria may be more specialized than in free living ciliates. Evidence of this is that every *Ophryoglena* specimen examined in this study was infected by similar types of bacteria. A continual problem in investigating the majority of intracellular bacteria within ciliates has been the inability to cultivate them outside of their host. Preliminary tests indicate that this is true for the bacteria of *Ophryoglena* as well. This suggests that the majority of these bacteria found in ciliates likely are obligate symbionts.

Overview

Our observations of α-subgroup proteobacteria in two endosymbiotic ciliate species, in conjunction with reports of these bacteria from a number of free-living ciliate species (Görtz 1998, Brigge *et al.* 1999, Fokin *et al.* 2000, Fokin unpublished data), indicate that they are widely established endocytobionts in these protists.

This paper documents a multi-level microcosm system: *Dreissena*-ciliates-bacteria-viruses. The presence of different bacteria inside ciliate cells has been well documented (Heckmann and Görtz 1991, Fokin 1993, Brigge *et al.* 1999), but never as part of such a multi-level microcosm. Such a system could be a useful one for microbiological and ecological investigations. The cytoplasm is an initial environment for establishment of any endocytobiont, and thus, among the cytoplasmic bacteria both phylogenically old and young forms can be found. Thus, investigations of cytoplasmic bacteria or cytoplasmic stages of endonucleobionts could, therefore, potentially have significant impacts on theories of ecology and evolution of symbiotic microbes (Fokin *et al.* 2000).

The relationship between *Dreissena*'s endosymbiotic ciliates and their endocytobiotic bacteria had previously never been studied. It is likely that future investigations of ciliates from other *Dreissena* populations will reveal even a wider diversity of intracellular bacteria than reported herein.

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