

# Procaryotic infections in the mussel *Mytilus galloprovincialis* and in its parasite the turbellarian *Urastoma cyprinae*

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**ABSTRACT:** Mussels *Mytilus galloprovincialis* from the Thau lagoon (Mediterranean coast of France) were regularly sampled to determine the prevalence and intensity of parasitic infections. Microscopically, hepatopancreatic tubules of the mussel appeared infected by a rickettsia-like organism (RLO). Each RLO were surrounded by 2 unit membranes, and colonies composed of several bacteria were enclosed within a vacuolar membrane of the host cell. In addition, examination by transmission electron microscopy revealed that the RLO was infected by phage particles. Histological investigations of the turbellarian *Urastoma cyprinae* parasitizing the mussels have shown that this ectoparasite was also infected by 2 types of procaryotes, a chlamydia-like organism (CLO) and a mollicute-like organism (MLO). The CLO displayed characteristic developmental stages of the Chlamydiales and was secondarily infected by electron-dense particles presumed to be phage particles. The MLO exhibited some morphological characteristics of the mollicutes, in that the microorganisms were bounded by a single membrane sharing a trilaminar structure. Neither of these microorganisms have previously been reported in Platyhelminthes.

**KEY WORDS:** *Mytilus galloprovincialis* · Turbellarian *Urastoma cyprinae* · Rickettsiales · Chlamydiales · Mollicutes · Phage infection

## INTRODUCTION

Although no important diseases have affected the mussel industry in the Thau lagoon (French Mediterranean coast) during the past decade, a routine survey to check for growth and mortality is regularly performed. Two years of sampling *Mytilus galloprovincialis* has shown that this mollusc serves as the host for several endemic parasites, particularly *Mytilicola intestinalis* (Steuer 1902), *Marteilia maurini* (Comps et al. 1982) and *Urastoma cyprinae* (von Graff 1913). In addition, during the survey histological examinations revealed procaryotic infections within the digestive gland of both mussel specimens and in the digestive tract of *U. cyprinae*.

We report here the morphological features of the procaryotes found in the mussel *Mytilus galloprovin-*

*cialis* and in its ectoparasite, the turbellarian *Urastoma cyprinae*. More extensive field studies are currently underway to better identify these microorganisms.

## MATERIALS AND METHODS

Morphological characterization of the procaryotes was made from naturally infected mussels parasitized by the turbellarian, *Urastoma cyprinae*. For light microscopy studies, specimens were fixed in Davidson fixative (Shaw & Battle 1957) for 2 h. Sections were stained by the Mann-Dominici method (Gabe 1968). For transmission electron microscopy, tissues were fixed overnight at 4°C in 2.5 % glutaraldehyde buffered with 0.4 M sodium cacodylate (pH 7.2), postfixed at room temperature in 1 % OsO<sub>4</sub> and embedded in Epon resin. Sections were contrasted with saturated uranyl acetate in 50 % ethanol, followed by lead citrate (Reynolds 1963).

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## RESULTS

## Electron microscopy

## RLO-infection in the mussel

## Light microscopy

Extensive examinations of mussel digestive tracts revealed inclusion bodies (ib) within the cytoplasm of digestive cells (Fig. 1a). In semi-thin sections, ibs 4 to 10  $\mu\text{m}$  in diameter appeared granular and exhibited different degrees of basophilia.

Ibs consisted of membrane-bound microcolonies of microorganisms with a procaryotic morphology (Fig. 1b). Typically, only 1 ib was found within infected cells; however, multiple ibs were also detected in some cases (Fig. 1c). The microorganisms were rod-shaped, about 500 to 800 nm long and 400 nm in diameter, and surrounded by an envelope which consisted of 2 trilaminar membranes. Their cytoplasm was electron-dense at the periphery while the center was reticulate.

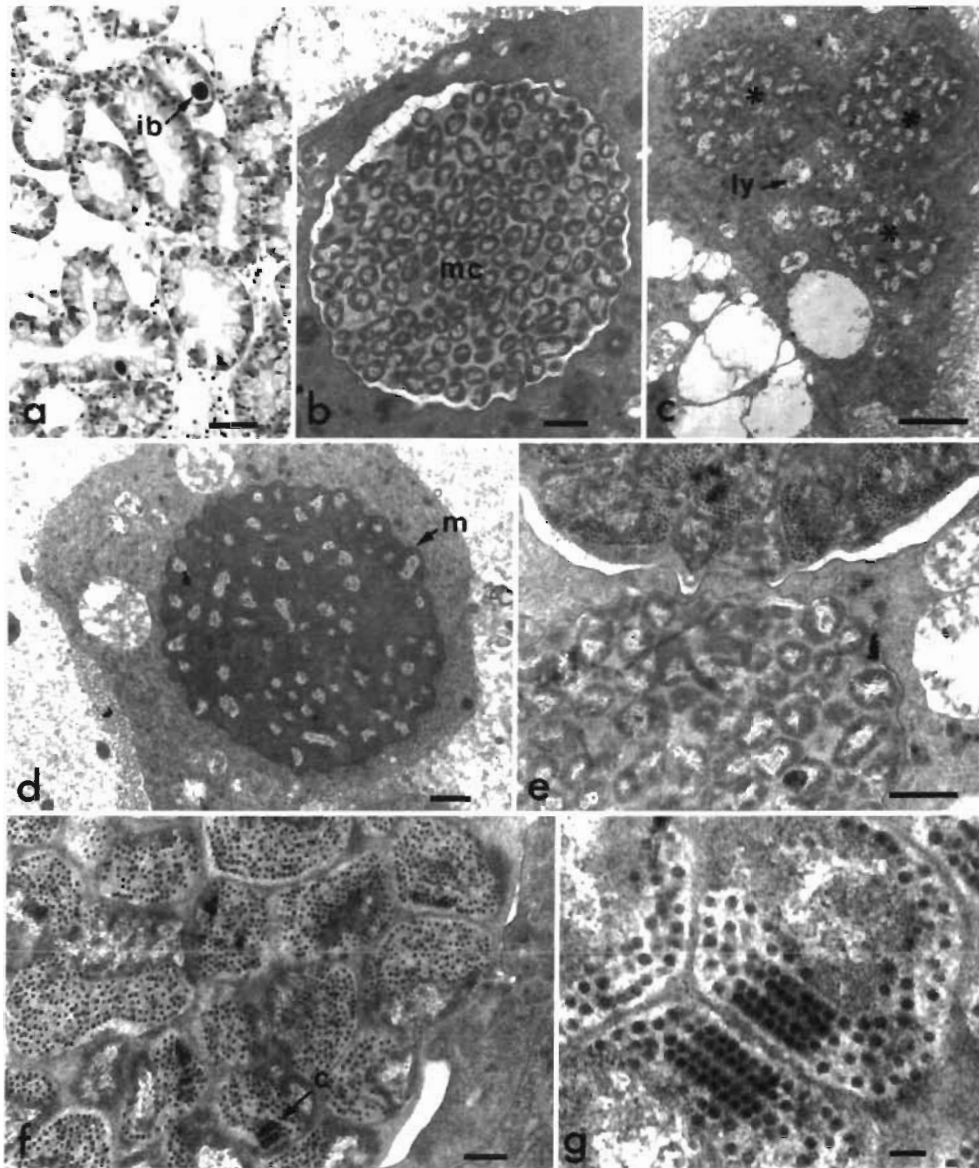


Fig. 1. Morphology of the rickettsia-like organism (RLO) infecting *Mytilus galloprovincialis*. (a) Section through the digestive diverticula displaying basophilic inclusion bodies (ib) localized within the tubule epithelium. Hematoxylin-eosin. Scale bar = 50  $\mu\text{m}$ . (b) Electron micrograph of an ib: RLOs exhibit a characteristic swollen outer membrane; the center of the cells is occupied by finely reticulated dense fibers. mc: microcolony. Scale bar = 500 nm. (c) Enlarged epithelial digestive cell containing 3 microcolonies of RLOs (\*); lysosomes (ly). TEM. Scale bar = 2  $\mu\text{m}$ . (d) In some parasitic vacuoles, the RLOs are found within electron-dense material; the membrane (m) of the vacuole appears to consist of a single thick layer; TEM. Scale bar = 500 nm. (e) Digestive cell containing microcolonies of phage-infected and uninfected RLOs; note the size difference of the 2 types of RLOs. TEM. Scale bar = 500 nm. (f) RLOs with severe phage infections; phage particles are dispersed throughout the cells or arranged in paracrystalline arrays (c). TEM. Scale bar = 200 nm. (g) Detail of paracrystalline arrays of phage particles. TEM. Scale bar = 100 nm.

Some bacteria were undergoing binary fission. In some ibs, the microorganisms were observed within finely granular and electron-dense material (Fig. 1d). Other ibs consisted of greatly distended microorganisms containing electron-dense phages (Fig. 1e). These particles were icosahedral and had a diameter of about 45 nm and appeared randomly scattered through the cytoplasm or formed paracrystalline arrays (Fig. 1f,g).

### Procaryotic infections in the turbellarian parasite of the mussel

#### Light microscopy

Sections of the turbellarian parasitizing the gills of the mussels displayed similar ibs localized among the sub-tegumentary glands. Larger in size (15 to 35  $\mu\text{m}$ ) than the ibs found in the mussels, these bodies were less basophilic (Fig. 2a).

#### Electron microscopy

In the worm *Urastoma cyprinae*, 2 types of microorganisms were observed within sub-tegumentary cells, a chlamydia-like organism (CLO) and a mycoplasma-like organism (MLO).

The CLO displayed the main developmental stages that occur during the life cycle of the Chlamydiales. The reticulate bodies were ovoid, 450 to 600 nm in size, and capable of dividing (Fig. 2b,c). As in the mussel, the bacteria were often tightly packed and included in dense material (Fig. 2d). The ibs exhibited central condensation of the fibrillar material (Fig. 2e). The elementary bodies were pleomorphic and measured about 200 to 250 nm in diameter (Fig. 2f). They were bounded by a complex envelope consisting of 3 electron-dense layers separated by 2 intermediate electron-luscent layers (Fig. 2g). The nucleoid exhibited an irregular profile and its high electron density contrasted with the surrounding cytoplasm. In some micro-colonies of reticulate bodies, several elementary bodies were found within a membrane-bound vacuole (Fig. 2h).

In highly parasitized worms, numerous reticulate bodies appeared to contain virus-like particles (Fig. 3a). These particles, about 50 nm in diameter, were scattered throughout the cytoplasm or formed paracrystalline arrays which occupied wholly or partly the reticulate bodies (Fig. 3b). Morphologically, the particles appeared spherical or hexagonal in outline and each consisted of a dense core surrounded by a unit membrane-like envelope. Earlier stages showed incomplete particles which consisted of electron-dense

material associated with the DNA fibrils. Maturing particles were also found dispersed throughout the parasitic vacuole, probably originating from lysed infected reticulate bodies (Fig. 3c).

Additionally, ultrastructurally in 1 CLO-infected *Urastoma cyprinae*, basophilic cells contained unusual polymorphic bodies exhibiting procaryotic structures. The more distinctive form was rod shaped, 150 to 400 nm long and 40 nm wide, while others were paraspherical or pear shaped (Fig. 4a). These particles were enclosed by a single membrane showing a trilaminar structure and their cytoplasm appeared finely granular and more or less electron-dense (Fig. 4b). Among the dense particles, ovoid bodies, 70 to 180 nm in size, displayed clear cytoplasm which contained structures analogous to ribosomes and DNA strands and median trilaminar elements (Fig. 4c).

### DISCUSSION

Since the first report of a rickettsia-like infection in the marine mollusc *Mya arenaria* (Harshbarger et al. 1977), numerous procaryotes related to the Rickettsiales, Chlamydiales and to the Mycoplasmas have been described from various marine invertebrates. During the past several years, reviews have contributed to the knowledge base about these organisms (Laukner 1983, Fries & Grant 1991, Chiou-Ming Wen et al. 1994). In the mussels, cases of infection by parasites belonging to these groups of organisms were reported in *Mytilus edulis* and *M. californianus* from the United States by Yevich & Barszcz (1980) and in *M. edulis* and *M. galloprovincialis* from Spain by Figueras & Montes (1988), Figueras et al. (1991) and Robledo et al. (1994). Additionally, a CLO was identified by Cajaraville & Angulo (1991) in *M. edulis* from the Basque coast. However, little information is presently available about such infections in marine worms since only 1, *Neorickettsia helminthoeca*, has been detected in the trematode *Nanophyetus salmincola* (Farrell et al. 1964). This rickettsia is a pathogen of canines (Millemann et al. 1964).

According to its morphological features and its replicative cycle by single binary fission, the procaryote found in the digestive cells of the mussel *Mytilus galloprovincialis* might be related to the order Rickettsiales. Based on its ability to produce membrane-bound cytoplasmic vacuoles in an invertebrate host, this microorganism could be considered closely related to the Wolbachiae. This is the first report of an RLO to be described in the mussel *M. galloprovincialis*; only CLOs have been previously reported in this species (Cajaraville & Angulo 1991, Robledo et al. 1994). Previously, Figueras et al. (1991) identified Rickettsiae in



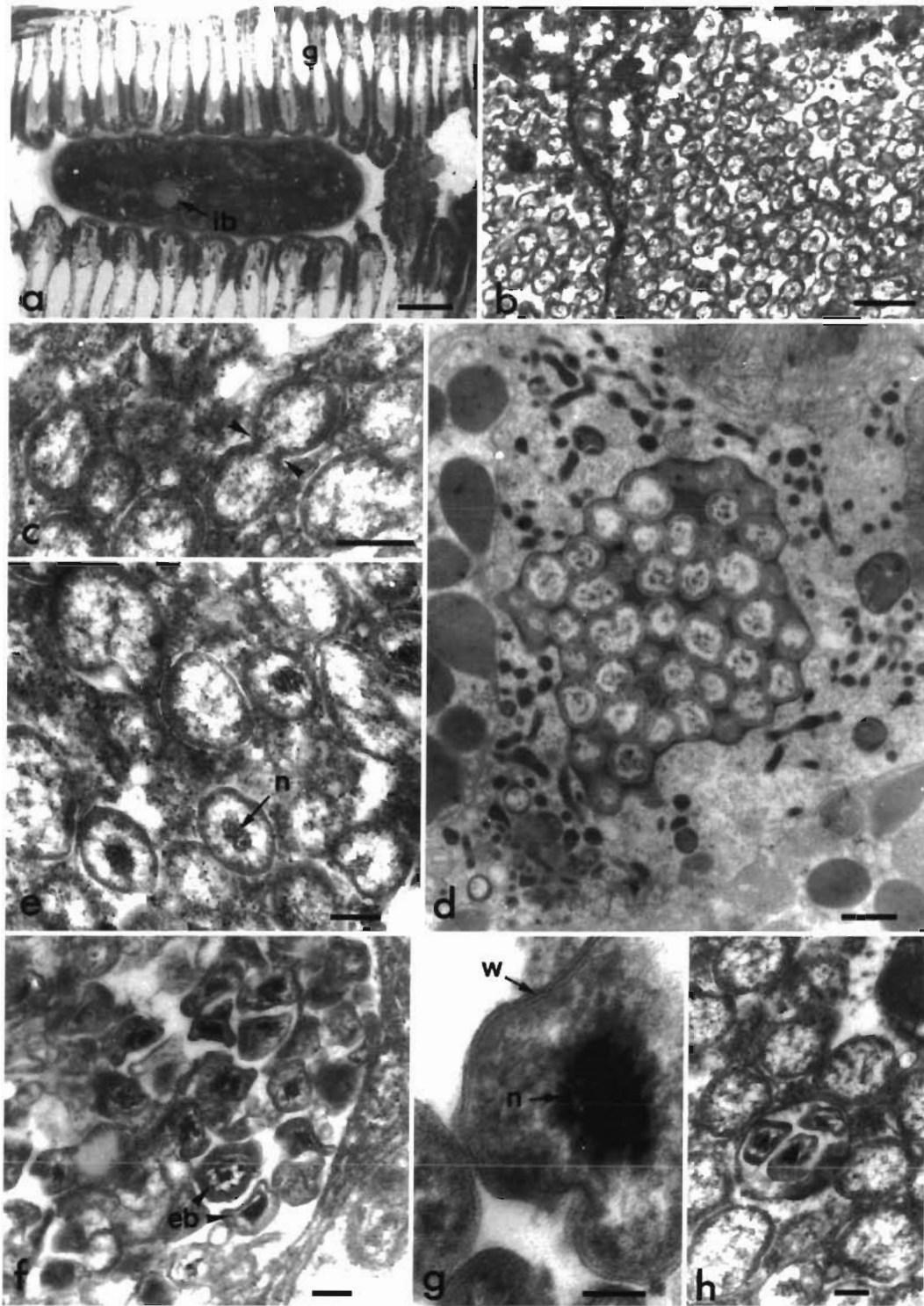


Fig. 2. Histology of the chlamydia-like organism (CLO) infection in *Urostoma cyprinae*. (a) Section showing the turbellarian localized in the gills (g) of the mussel; inclusion body (ib). Toluidine blue. Scale bar = 50  $\mu$ m. (b) CLO cytoplasmic inclusion found in the subtegumentary gland. TEM. Scale bar = 1  $\mu$ m. (c) Reticulate body undergoing fission (arrowheads). TEM. Scale bar = 500 nm. (d) Cytoplasmic inclusion containing paraspherical reticulate bodies within electron-dense material. TEM. Scale bar = 500 nm. (e) Ibs characterized by the presence of a central electron-dense nucleoid (n). TEM. Scale bar = 200 nm. (f) Microcolony of elementary bodies (eb). TEM. Scale bar = 200 nm. (g) Electron micrograph of an eb displaying the fine structure of the wall (w); nucleoid (n). Scale bar = 50 nm. (h) Microcolony of reticulate bodies containing eb within a membrane-bound vacuole. TEM. Scale bar = 200 nm

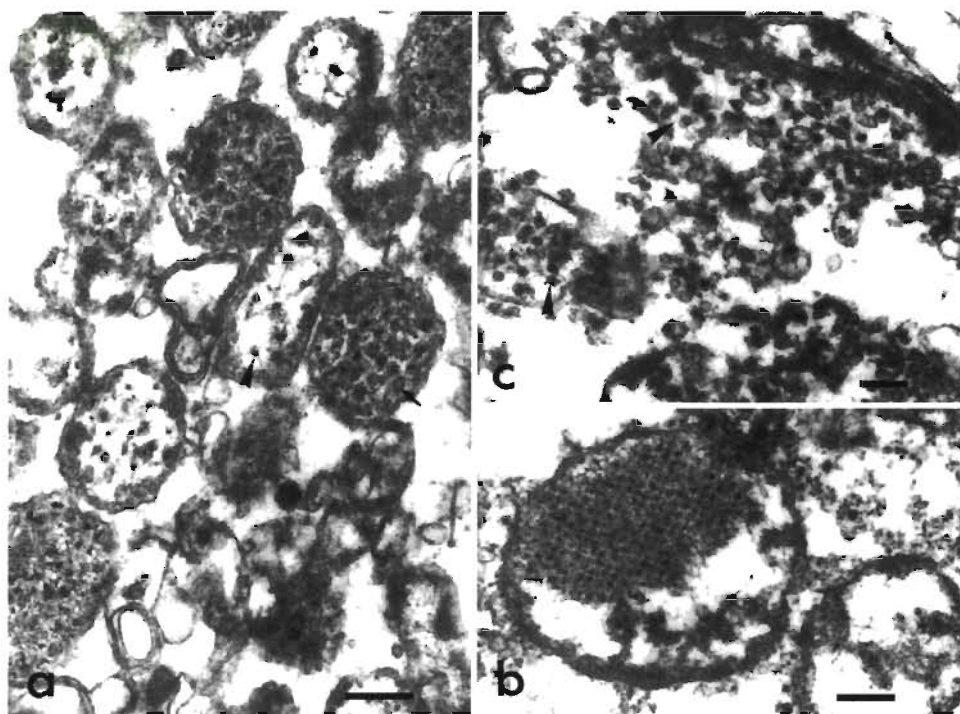


Fig. 3. Fine structure of phage-infected CLO. (a) Different stages of phage development are present in the same microcolony of procaryotes. First particles form among the DNA fibres in the central area of the CLO (arrowhead). Some CLOs are completely full of phage particles (arrow). TEM. Scale bar = 200 nm. (b) Enlarged CLO containing numerous particles in regular arrangement. TEM. Scale bar = 200 nm. (c) When the membrane of the microorganism is ruptured, phage particles (arrowheads) are scattered throughout the parasitic vacuole among cellular debris. TEM. Scale bar = 200 nm

this mollusc through light microscopic examinations, but did not illustrate the developmental mode of the procaryote. Although the RLO-infected mussel cells exhibited some cytopathic effects, including hypertrophy and abnormal vacuolization of the cytoplasm, no characteristic histological changes were seen in the neighbouring cells of the digestive tubules. However, some changes in the structure of the parasitic vacuole, such as an increase in the density of its content or the thickening of its membrane, could correspond to host reactions. Apparently, spreading of the pathogen is also controlled by the phage, which can infect all the bacteria contained within a vacuole. Similar phage infections of procaryotes were reported in other molluscs such as *Mercenaria mercenaria* (Harshbarger et al. 1977), *Tellina tenuis* (Buchanan 1978), and in other invertebrates including the snail *Bithynia siamensis goniomphalus* (Adam et al. 1994) and the scorpion *Buthus occitanus* (Morel et al. 1974).

The CLO found in *Urastoma cyprinae* is the first infectious agent described in this species. It exhibits some similarities with the Chlamydiales reported in marine invertebrates (Harshbarger et al. 1977, Comps & Deltreil 1979, Joly & Comps 1980, Cajaraville & Angulo 1991), but differs in the morphology of its ele-

mentary bodies. To date, none of these organisms have been classified in the order Chlamydiales and their relationship to their hosts is obscure. Concerning the parasite of *U. cyprinae*, although the sampled worms demonstrated 80% infection prevalence, we were unable to determine what effect, if any, this parasite has on its host. CLO development and its potential pathogenic effect on the worms may be seriously limited by the ability of the infecting phage to destroy this microorganism.

According to its morphological characteristics (pleomorphic procaryotic cell delimited only by a plasma membrane), the second intracellular microorganism found in *Urastoma cyprinae* appears closely related to the Mollicutes (Razin & Freundt 1983), but phenotypic characters alone were not sufficient to determine its specific placement. Few organisms belonging to this group have been reported in marine invertebrates. Harshbarger et al. (1977) first reported a mycoplasma-like organism within the gut cells of the American oyster *Crassostrea virginica*. Later, a new type of mycoplasma was described by Krol et al. (1991) in the hepatopancreatic cells of *Litopenaeus vannamei*.

The observation of new intracytoplasmic procaryotes in marine invertebrates confirms that these animals,

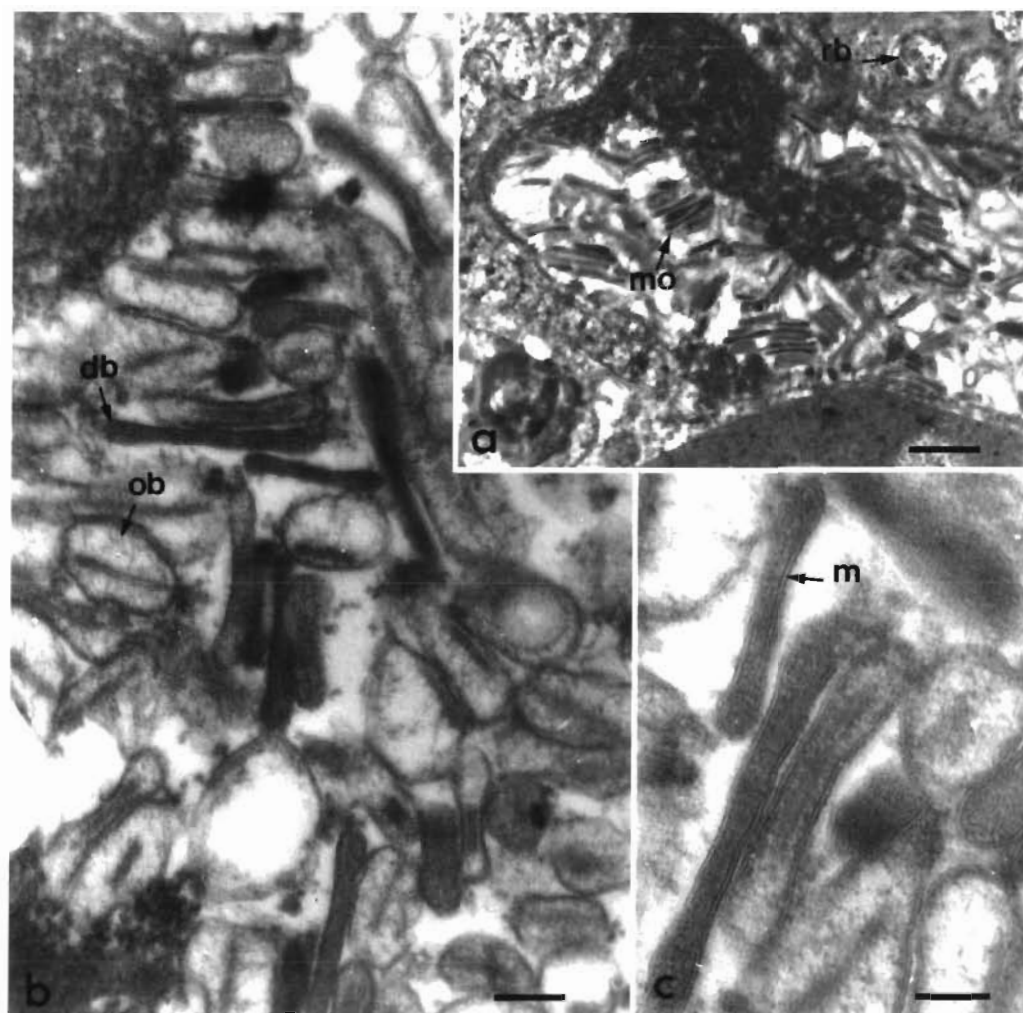


Fig. 4. Mycoplasma-like organism (MLO) from *Urastoma cyprinae*. (a) Electron micrograph of MLO-infected tissues (mo); reticulate body (rb) inside a CLO-infected cell. Scale bar = 500 nm. (b) Two types of bodies are found in the MLO-containing cells: dense bodies (db) and ovoid bodies (ob). TEM. Scale bar = 100 nm. (c) Higher magnification of dbs: finely granular, the cytoplasm is surrounded by a single membrane (m). TEM. Scale bar = 50 nm

particularly bivalves, are common hosts for these types of parasites. Generally, they cause mild cytopathic effects only in the infected cells. To date, no report of serious disease by these pathogens has been published, except in the scallop *Pecten maximus*. In this case, a rickettsia was suspected to have caused mortalities in scallop beds located in Brittany (France), but its role in the epizootic was not experimentally demonstrated (LeGall et al. 1988).

The pathogenicity and the transmission of these microorganisms remain in actual fact unknown and, until now, were only the subject of various hypotheses (Fries & Grant 1991, Renault & Cochenne 1994). It is obvious that more experimental data is needed to determine the exact relationship between these microorganisms and their hosts.

Purification of the CLO present in the Turbellarian *Urastoma cyprinae* is being conducted to further characterize this microorganism and to attempt experimental transmission.

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