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NOTE

Rickettsiaceae-like Microorganisms in the Gill and Digestive Gland of the Hard Clam, *Meretrix lusoria* Röding

Intracellular prokaryotic organisms from marine bivalves were first reported by J. C. Harshbarger, S. C. Chang, and S. V. Otto (*Science* **196**, 666–668, 1977). Rickettsia-like parasites have been detected by light and electron microscopy in the tissues of a number of marine bivalves (see review in C. R. Fries and D. M. Grant, *J. Invertebr. Pathol.* 57, 166–171, 1991). Although most of the Rickettsiales are intracellular symbiotic prokaryotes and usually exhibit no host response, some of them have been suggested to lead to increased mortality in the infected animals (R. A. Elston, *J. Fish Dis.* **9**, 69–71, 1986; G. Gulka and P. W. Chang, *J. Fish Dis.* 8, 319–323, 1984; G. Le Gall, D. Chagot, and M. H. Grizel, *Dis. Aquat. Org.* **4**, 229–232, 1988).

The purpose of this paper is to document the discovery of a rickettsia-like microorganism in the epithelial cells of gill and digestive gland of the hard clam, *Meretrix lusoria* Röding, collected in Taiwan. This is the first paper to describe the morphological characteristics of rickettsia-like organisms in infected *M. lusoria*. The organisms reported in the present study show no significant host response and was not lethal to the host.

Hard clams, *M. lusoria*, were obtained monthly from a farm in Changua, Taiwan, from December 1992 through April 1993. The clams were sacrificed and a portion of the clam's tissue was fixed in 10% buffered formalin, embedded in Paraplast, sectioned, and stained with Mayer's hematoxylin and eosin, Feulgen, Acridine Orange, Gram's, or Price's Giemsa stains, respectively, for light microscopy. The remaining tissue was fixed in cold 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in alcohol, embedded in Spurr's resin, and sectioned for electron microscopy.

Upon histopathological examination, most specimens of *M. lusoria* had basophilic intracellular inclusions in the epithelial cells of the gill filaments and digestive glands (Fig. 1). Heavy infections were found

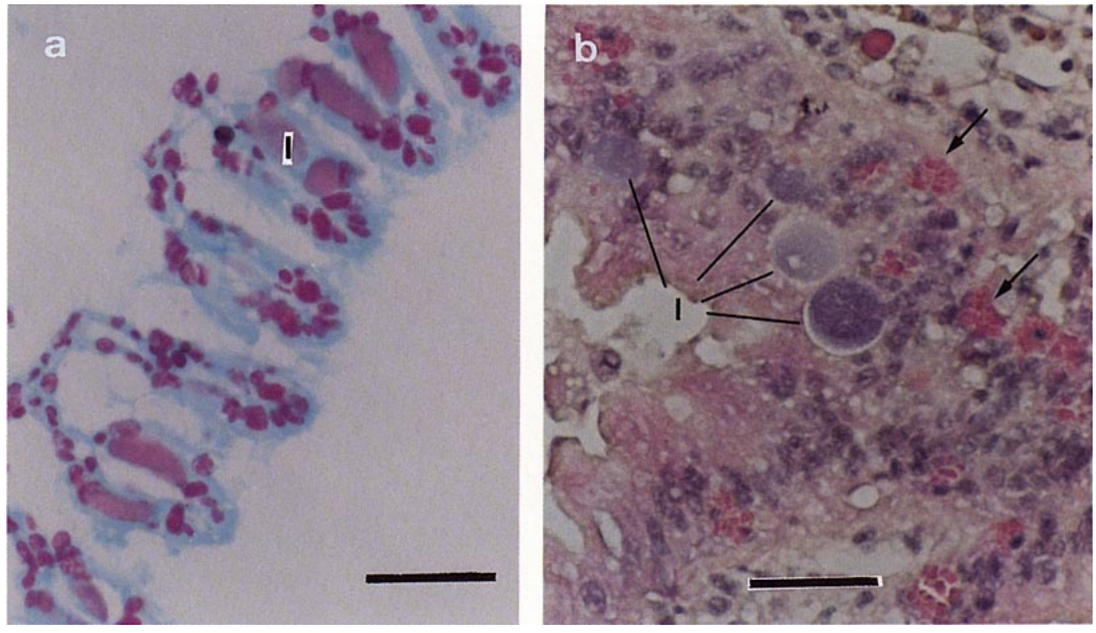

FIG. 1. (a) Rickettsiales-like inclusions in the gill tissue of *M. lusoria*. Feulgen's stain. I, Feulgen-positive inclusion. (b) Rickettsiales-like inclusions in the epithelium of digestive gland of *M. lusoria*. Numerous eosinophilic granulocytes were noticed (arrows). Mayer's hematoxylin and eosin stain. I, basophilic inclusions. Bar = 100 μm.

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in the gill tissue. These inclusions usually were bean- or rod-shaped in the gill and spherical in the digestive gland. The inclusions in the gill, as measured in sectioned material, were about 90 by 40 μm and about 22 μm in diameter in the digestive gland. Numerous deep-stained particles were found to be confined within an inclusion. The inclusions were Feulgen-positive, Gram-negative, fluoresced green when stained with Acridine Orange at pH 3.8, and were also DNase digestible.

Semithin resin sections of approximately 1 μm stained with methylene blue presented numerous Rickettsiales-like organisms in a membrane-bound cytoplasmic inclusion within the epithelial cells of the digestive gland tubule. Electron microscopy of gill tissue revealed the microorganisms, bounded by a plasma membrane and a thin, rippled cell wall, typical of Rickettsiaceae (Fig. 2a). They occurred in membrane-bound vacuoles in the cytoplasm of the ciliated epithelial cells, forming inclusions that contained variable numbers of organisms and fibril materials. The organisms were usually observed as pleomorphic, coccoid, or rod-shaped (Fig. 2a). From electron micrographs, these individual organisms measured approximately 1.0–4.0

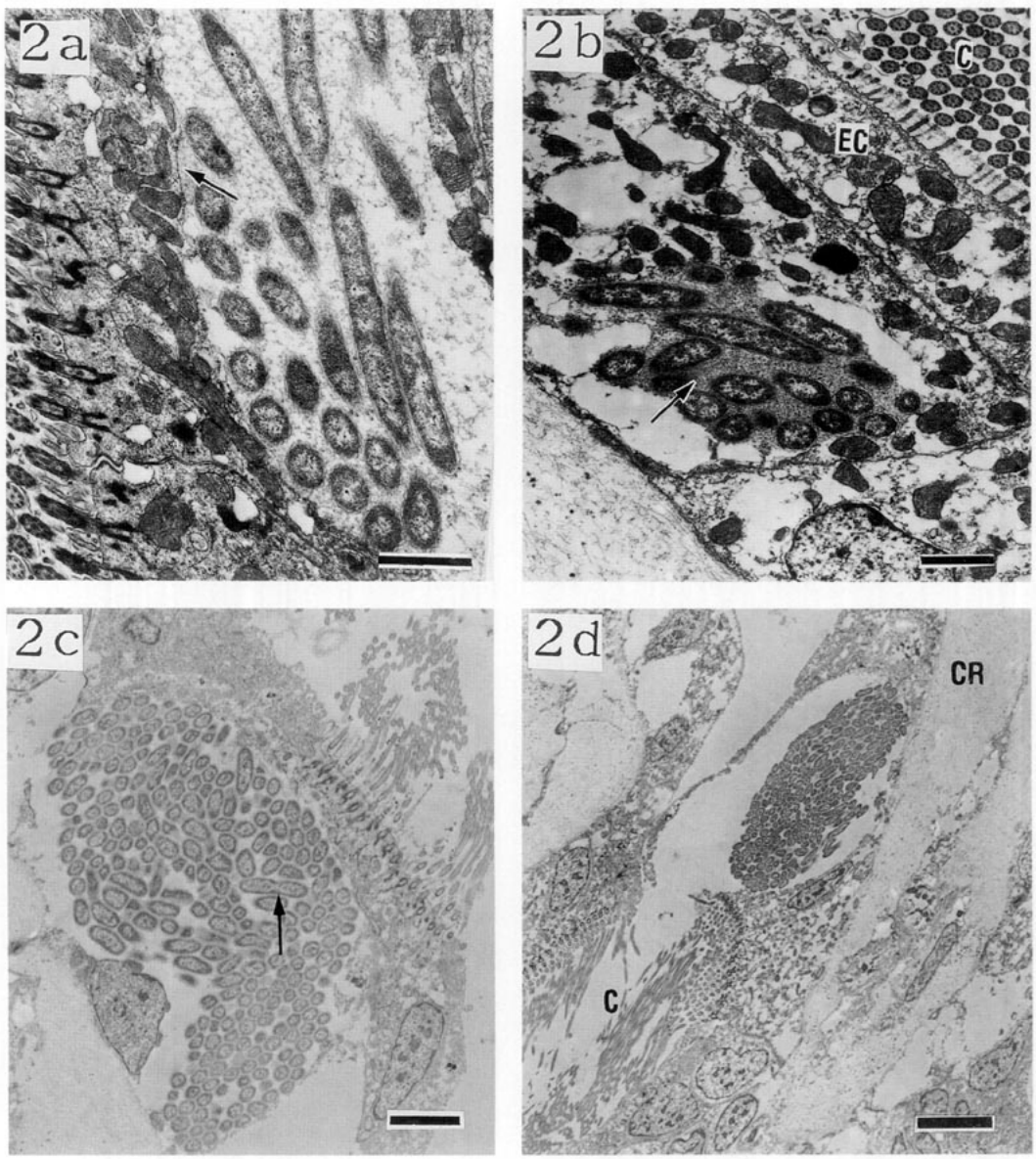

FIG. 2. Transmission electron micrographs of Rickettsiaceae-like inclusion in the gill of *M. lusoria*. (a) The inclusion was separated from the host cytoplasm by a membrane (arrow) and contained numerous Rickettsiaceae-like organisms and fibril materials. (b) Rickettsiaceae-like organisms surrounded by dense fibril materials (arrow). Note the adjacent cell with numerous vacuoles in the cytoplasm. (c) Binary fusion is shown (arrow). (d) A ruptured gill epithelial cell containing numerous Rickettsiaceae-like organisms. C, cilia; CR, chitinous rod; EC, epithelial cell. Bar in a, b = 1 μm; in c = 2 μm; in d = 3 μm.

μm on the long axis by 0.4 ± 0.05 μm along the short axis (ranged from 0.3–0.5 μm). In some cases, prokaryocytes surrounded by dense fibril materials were observed (Fig. 2b) and were considered to be at the early stage of proliferation of the microorganism. Electron micrographs of digestive gland, however, showed a different type of microorganism in the diverticular epithelial cells. The bacterial cells were coccoid or short rod in shape, measuring approximately 180–350 nm in diameter and up to 950 nm in length (Fig. 3). Both types of the microorganisms were filled with ribosomes at the periphery and the inner part was free (or nearly free) of ribosomes and contained threadlike materials of varying diameter that were considered to be the DNA (Fig. 2a). From the above characterization, those microorganisms were suggested to be Rickettsiaceae-like organisms.

Cytopathological effects in infected host cells may result in the complete destruction of the cytoplasm. The adjacent epithelial cells retained an abnormal appearance and vacuolization was observed (Fig. 2b). Large inclusions within hypertrophied ciliated cells were generally observed (Fig. 2c), and ruptured cells associated with an inclusion of the prokaryocyte were also seen (Fig. 2d). The expansion of the microcolony resulted in the compression of the cytoplasmic organelles against the cell membrane (Figs. 2c, 2d).

Rickettsiae generally are obligate intracellular parasites found in a number of species of marine bivalves, about which little is known on the classification and even less is understood about their transmission in the marine environment. Most rickettsiae have ribosomes and DNA filaments interspersed throughout the cytoplasm; however, some have a central area containing

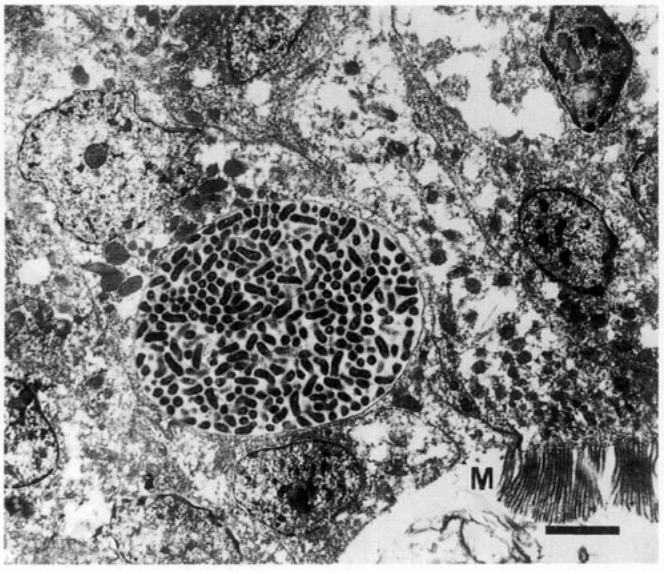

FIG. 3. Transmission electron micrograph of Rickettsiaceae-like inclusion in the epithelium of digestive gland of *M. lusoria*. M, microvilli. Bar = 2 μm.

mainly filaments and are found in several marine bivalves including: *Crassostrea gigas* (M. Comps, G. Tige, J. L. Duthoit, and H. Grizel, *Halitois* 8, 317–321, 1979), *Placopecten magellanicus* (G. Gulka, P. W. Chang, and K. A. Marti, *J. Fish Dis.* 6, 355–364, 1983), *Pecten maximus* (G. Le Gall, D. Chagot, and M. H. Grizel, *Dis. Aquat. Org.* 4, 229–232, 1988), and *Tridacna crocea* (C. L. Goggin and R. J. G. Lester, *J. Invertebr. Pathol.* 56, 135–138, 1990). On the basis of size and morphology, the microorganism found in the gill epithelial cells in this paper is similar to the rickettsia-like organism infecting the sea scallop, *P. magellanicus*. Both of them had a high rate of occurrence, up to 100% (G. Gulka and P. W. Chang, *J. Fish Dis.* 8, 309–318, 1984).

Aside from the fact that an endospore-like body was not present, the morphological characteristics of those prokaryocytes found in this study are considered to be very similar to those of the genus *Coxiella* in the tribe Rickettsieae of the family Rickettsiaceae (E. Weiss and J. M. Moulder, "Bergey's Manual of Systematic Bacteriology," Vol. 1, pp. 687–739, 1984).

In addition to the common appearance of microorganism, phage-containing rickettsiae in a membrane-bound cytoplasmic inclusion were also present within diverticular epithelial cells (Fig. 4a). These phage-infected microorganisms were more pleomorphic and larger than those of uninfected microorganisms, with a size of 0.3–1.0 μm in diameter and up to 1.7 μm in length. The diameter of the phages, as observed in thin sections, were approximately 41 ± 1 nm (n = 50) (Fig. 4b).

Similarly, intracytoplasmic prokaryocytes with phage were also observed in the digestive gland of the marine bivalves *Mercenaria mercenaria* and *Tellina tenuis* (J. C. Harshbarger, S. C. Chang, and S. V. Otto, *Science* 196, 666–668, 1977; J. S. Buchanan, *J. Fish Dis.* 1, 27–43, 1978). In comparison, the diameter of phage reported in the present study is smaller than those reported previously (50 and 66–68 nm).

During the past decades, high mortalities of the cultured hard clam, *M. lusoria*, occurred during the summer in Taiwan. The main causes for the mass mortalities are still unknown, although water pollution was suggested to be the major factor (W.-Y. Tseng, *Bull. Tai. Fish. Res. Inst.* 26, 1–35, 1976). Several pathogen-like viruses or bacteria have also been isolated from the hard clam and some of them were suggested to be possible causes for the mortalities (M.-K. Yang, C.-F. Lo, R. F. Huber, and G.-H. Kou, *COA Fish. Ser.* 10, 56–63, 1977; C.-F. Lo, Y.-W. Hong, S.-Y. Huang, and C.-H. Wang, *Fish Pathol.* 23, 147–154, 1988).

Since rickettsia-like infections associated with mortalities of bivalve mollusk have previously been reported (G. Gulka, P. W. Chang, and K. A. Marti, *J. Fish Dis.* 6, 355–364, 1983), and indirect lethal effects of the microorganisms have also been suggested by G.

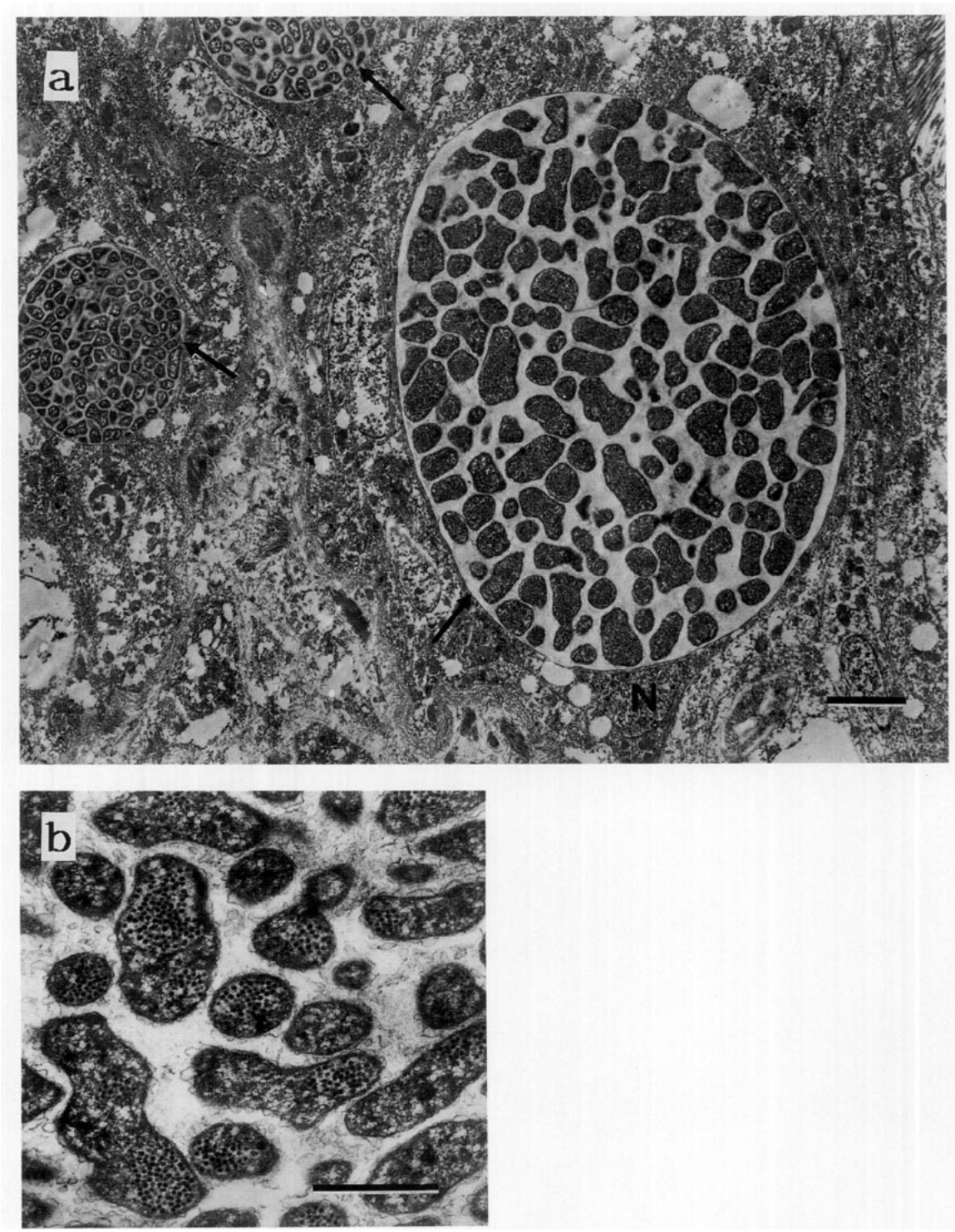

FIG. 4. (a) Three typical membrane-bound (arrows) intracytoplasmic inclusions in the absorptive epithelium of digestive gland of *M. lusoria*. Two smaller microcolonies containing Rickettsiaceae-like organisms that are phage free and one larger microcolony containing Rickettsiaceae-like organisms associated with phage are shown. N, nucleus. Bar = 2 μm. (b) Higher magnification of Rickettsiaceae-like organisms in the absorptive cell of digestive gland, showing hexagonal phage particles in the cytoplasm. These particles have a diameter of 41 ± 1 nm. Bar = 1 μm.

Gulka and P. W. Chang (*J. Fish Dis.* 8, 309–318, 1984), factors causing the mass mortality of *M. lusoria* in the summer in Taiwan should be reexamined. According to the observations of Gulka and Chang (*J. Fish Dis.* 8, 309–318, 1984), prokaryocytes released after the rupture of epithelial cells may infect nearby (and distant) animals via seawater transport. The present study shows that the same infection mechanism by the Rickettsiaceae-like organisms may occur in *M. lusoria* in Taiwan. An investigation of serological characteristics for these microorganisms is in progress and the results will be published elsewhere.

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