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NOTE

Phage particles infecting branchial Rickettsiales-like organisms in banded carpet shell *Polititapes virgineus* (Bivalvia) from Galicia (NW Spain)

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ABSTRACT: Basophilic intracellular prokaryotic-like colonies were observed in the gills of banded carpet shell *Polititapes virgineus* (= *Tapes rhomboides*) (Linnaeus, 1767) from a natural bed in Galicia (NW Spain). Light microscope observations suggested the presence of 2 types of colonies, but transmission electron microscopy revealed that these were the same Rickettsiales-like colonies, one infected and the other uninfected by phage particles. This is the first report of the presence of phage particles in Rickettsiales-like organisms in the gills of *P. virgineus*.

KEY WORDS: Virus · Prokaryotic organisms · Ultrastructural analysis

INTRODUCTION

Intracellular prokaryotic organisms such as Rickettsiae, Chlamydiae and Mycoplasma were reported for the first time in marine bivalves by Harshbarger et al. (1977). Rickettsial and chlamydial organisms are prokaryotic organisms, generally intracellular obligate parasites and Gram-negative, belonging to the orders Rickettsiales and Chlamydiales, respectively. Rickettsiae or rickettsia-like organisms have been most commonly reported in bivalves (Fryer & Lannan 1994, Renault & Cochennec 1994, Wen et al. 1994, Villalba et al. 1999, Wu & Pan 2000, Molloy et al. 2001, Hine & Diggles 2002, Sun & Wu 2004). Branchial rickettsia-like infections have been associated with mortalities in bivalve populations (Gulka & Wen Chang 1985, Le Gall et al. 1988, Norton et al. 1993, Villalba et al. 1999, Wu & Pan 2000, Sun & Wu 2004).

During a histological survey in a natural bed of the banded carpet shell *Polititapes virgineus* (=*Tapes rhomboides*) from Galicia (NW Spain) to search for the cause of a significant recent population decline, we found a high prevalence and density of branchial intracellular prokaryotic organisms, some of which were infected by phage particles. Phages have previously been described hyperparasitizing prokaryotic organisms in cells of some bivalve species (Harshbarger et al. 1977, Buchanan 1978, Meyers 1979, Johnson & Le Pennec 1995, Wu & Pan 2000, Molloy et al. 2001, Sun & Wu 2004).

The purpose of the present study was to investigate the basophilic intracellular prokaryotic-like colonies observed in the gills of *Polititapes virgineus* in Galicia.

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MATERIALS AND METHODS

A sample of 30 clams (45.6 ± 2.7 mm long, 21.30 ± 5.33 g mass) was collected for a histological survey in June 2010. Gills were processed by histological techniques and stained with Harris' hematoxylin and eosin (H&E) for analysis by light microscopy. The infection intensity of prokaryotic colonies in each clam was calculated from the number of colonies in the gill measured at 1000× magnification in 10 fields (equivalent area: 0.38 mm^2). Thirty colonies of each type were measured using the imaging software Cell* (Olympus). To further characterize prokaryotic colonies the following additional staining techniques were used: Gram's Brown and Hopps method, the Pinkerton method for rickettsial organisms, the Ziehl-Neelsen method for acid-fast bacteria and Feulgen methods for DNA. Gills embedded in paraffin blocks and fresh tissue were processed for analysis with a transmission electron microscope (TEM).

RESULTS AND DISCUSSION

Light microscope observations of H&E-stained tissues revealed the presence of 2 kinds of branchial intracellular inclusion colonies in all individuals sampled. The most abundant type of colony (Type I) had a granular appearance (14.87 ± 4.34 µm length; mean ± SD, n = 30) whereas the other (Type II) (15.53 ± 4.04 µm length, n = 30) was scarce and had a granular and bacillary appearance, with larger microorganisms inside and stronger basophilia (Fig. 1). The Type I colony is similar to those described by Villalba et al. (1999) in the same bivalve species. The mean intensity of Type I colonies was higher (47 ± 24 colonies per area [0.38 mm^2]; n = 15) than the mean intensity of Type II colonies (2 ± 1 colonies per area [0.38 mm^2]; n = 15). Both types of colony were Gram-negative, acid-fast negative and Feulgen positive (with stronger reaction in Type II), and they took on a red colour typical of rickettsial colonies analyzed using the Pinkerton methods; similar results have been reported by several authors (Wen et al. 1994, Gardner et al. 1995, Wu & Pan 2000).

TEM examination revealed 2 types of intracellular colony inside vacuoles of host cells. Type I colonies were densely packed, composed of pleomorphic prokaryotic organisms measuring up to 2.5 µm in length (Fig. 2A). They showed the typical prokaryotic nucleoid and ribosomes distributed randomly. A thin rippled wall and a plasma membrane (electro-dense) separated by a pale layer (electro-lucent) were observed (Fig. 2B). Some colonies exhibited 2 nucleoids and a transverse constriction, suggesting binary fission (Fig. 2B). Similar colonies have been observed by Fries & Grant (1991), Fries et al. (1991), Villalba et al. (1999), and Hine & Diggles (2002). Type II colonies were larger (measuring up to 3.5 µm) and infected by apparently icosahedral phage particles with paracrystalline disposition. Phage infection provokes the enlargement of the prokaryotic organism, causing breakage and liberation of virus particles into the cytoplasm of the host cell (Fig. 3). In addition, we occasionally observed prokaryotic organisms both

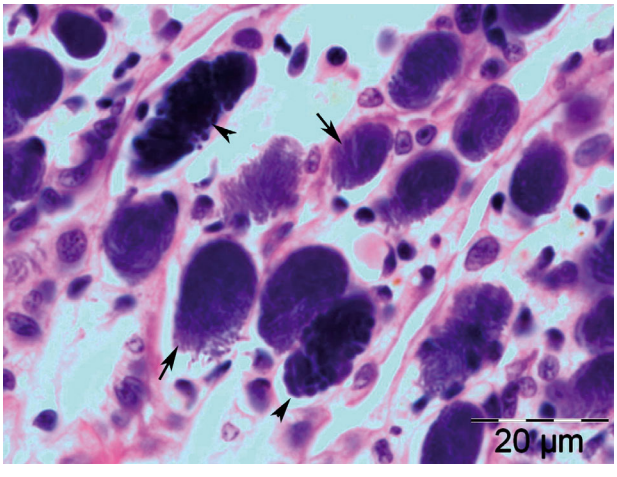

Fig. 1. Light micrograph of a heavily infected gill of *Polititapes virgineus* with 2 types of intracellular prokaryotic-like colonies: Type I (arrows) and Type II (arrowheads) (H&E staining). Scale bar = 20 µm

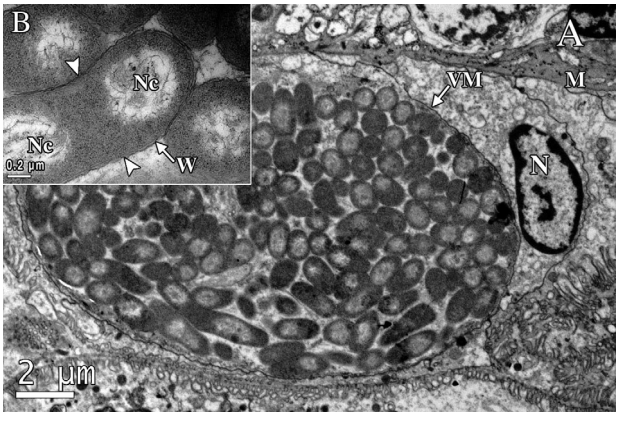

Fig. 2. (A) Ultrathin section of an intracellular colony of Rickettsiales-like organisms Type I in the gill of *Polititapes virginieus*. N: nucleus of cell host; M: membrane of cell host; VM: vacuole membrane. (B) Detail of a Rickettsiales-like organism with a transverse constriction, suggesting binary fission (arrowheads). Nc: nuleoid; W: wall. Scale bar = 0.2 µm

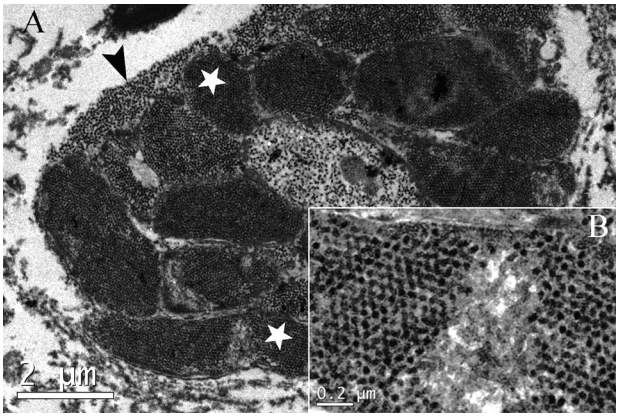

Fig. 3. (A) Ultrathin section of one Type II colony with prokaryotic organisms containing phage particles mostly occurring in paracrystalline arrays (stars) and free phage particles in the cytoplasm of the host cell (arrowhead). (B) Detail of phage particles

infected and uninfected by phages in the same colony.

Rickettsiae and Chlamydiae are commonly distinguished (using electron microscopy) by taking into account their morphology and division system. Chlamydial species are all coccoid and they have a complex developmental cycle (reticulate and elementary bodies), whereas rickettsial species are pleomorphic and replicate by binary fission (Fryer & Lannan 1994). Few intracellular prokaryotic organisms infecting aquatic animals have been sufficiently characterized for precise taxonomic placement (Fryer & Lannan 1994). In the case of marine invertebrates, the principal problem in the study of intracellular obligate parasites is the absence of cell lines. Molecular techniques can help to identify these organisms, as in the case of the identification of '*Candidatus* Xenohaliotis californiensis' in *Haliotis* spp. (Friedman et al. 2000).

The terms rickettsia-like and chlamydia-like were used in some reports to refer to intracellular inclusion bodies using only light microscope observations. Nevertheless, without ultrastructural data it is only possible to name these inclusions 'intracellular inclusion bodies' or 'intracellular prokaryotic-like inclusions'.

We consider the branchial intracellular prokaryotic colonies found in *Polititapes virgineus* to be organisms belonging to the order Rickettsiales, because we observed pleomorphic morphology and only division by binary fission, with an absence of reticulate and elementary bodies.

High prevalence and intensity suggest that these Rickettsiales-like organisms could be related to a mortality episode which caused a decline in the population. More studies are being conducted to study population dynamics and pathology in *Polititapes virgineus* beds.

Results of ultrastructural observations indicated that the Type II colony, observed by light microscope, corresponded to the same Rickettsiales-like organism infected by phage particles. This was concluded by the observation of 1 colony with both infected and uninfected Rickettsiales-like organisms.

Chlamydial and rickettsial organisms infected by phages have been reported in different bivalve tissues (Harshbarger et al. 1977, Buchanan 1978, Wen et al. 1994, Johnson & Le Pennec 1995, Comps & Tigé 1999, Wu & Pan 2000, Molloy et al. 2001, Sun & Wu 2004) but not in gills, as in the present study.

Different authors have suggested that the spreading of the pathogenic rickettsial and chlamydial organisms might be controlled by the phage infection; this can infect all the bacteria in a colony, causing obvious damage such as lysis of the host cells (Harshbarger et al. 1977, Comps & Tigé 1999, Molloy et al. 2001).

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