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Short Communication

The hyperparasite of the rickettsiales-like prokaryote, *Candidatus* Xenohaliotis californiensis has morphological characteristics of a *Siphoviridae* (*Caudovirales*)

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ABSTRACT

Transmission electron microscopy analysis (TEM) of the rickettsiales-like prokaryote, *Candidatus* Xenohaliotis californiensis (*C*Xc), pathogen of *Haliotis* spp. from the West Coast of North America, were found to be infected by a bacteriophage hyperparasite previously described in red abalone from California. The hyperparasite has an icosahedrical-like capsid with a narrow long flexible tail, this morphological characteristic tentatively place this virus in the Family *Siphoviridae* from the order *Caudovirales*. TEM images also showed the bacteriophage in different stages of assembly in the cytoplasm of *C*Xc, demonstrating its lytic cycle.

1. Introduction

Candidatus Xenohaliotis californiensis (*C*Xc) is a rickettsiales-like prokaryote that is commonly found in intracellular membrane bound vacuoles (MBVs) in the digestive tract of *Haliotis* spp. from the western coast of North America (Friedman et al., 2000; Cáceres-Martínez et al., 2011). This intracellular bacteria is considered the etiological agent of Withering Syndrome (WS) a chronic disease of abalone (Gardner et al., 1995; Friedman et al., 2000). The MBVs of the parasite can be detected by conventional histology of infected tissues. These stain violet to light purple with hematoxylin and eosin (H & E) and show a smooth appearance. In recent studies this pathogen was found to be affected by a bacteriophage hyperparasite in cultured red abalone *Haliotis rufescens* and wild black abalone *Haliotis cracherodii* in California, USA (Friedman and Crosson, 2012; Friedman et al., 2014). Bacteriophage infected MBVs (iMBVs) are characterized by a deep purple to navy blue staining pattern with H & E and have a rough granular appearance. To date there is no data on the identity and inherent characteristics of this bacteriophage. Furthermore, it is unknown if this bacteriophage is found in other species of abalone and in different localities. A TEM analysis of red abalone *H. rufescens* and yellow abalone *Haliotis corrugata* from Baja California, Mexico, revealed the morphological characteristics of these viruses and different stages of assembly in the cytoplasm of *C*Xc.

2. Materials and methods

Tissue samples were obtained from three red abalone *H. rufescens* with the external signs of WS (see Gardner et al., 1995) from an aquaculture facility. The posterior esophagus (PE) was excised aseptically and washed thoroughly with filtered (0.2 μm) seawater (FSW) to remove all exogenous bacteria. The PE was macerated adding 10 ml of FSW to facilitate homogenization. The macerated PE from was passed though progressively smaller filters and precipitated following the methodology described by Cruz-Flores et al. (2015). A similar sample was obtained from two wild yellow abalone. The PE of both organisms was removed aseptically and washed thoroughly with FSW, but in this case 1–2 mm^2 pieces of the PE were carefully cutout from the two samples. In both cases, the precipitate and the infected tissue were fixed in 3% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4 for 4 h at 4 °C, washed once in 0.1 M sodium cacodylate for 12 h at 4°, post-fixed in 1% OsO_4, and stored at 4 °C in 0.1 M sodium cacodylate. Fixed samples were processed for TEM in the Microbiology Department of the Centro de Investigación Científica y de Educación Superior de Ensenada. Parallel 5 mm^2 pieces of the PE were process by routing histology and stained with H & E from each abalone as described by

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Cáceres-Martínez et al. (2011) to confirm the presence of the iMBVs.

3. Results

Histological analysis of parallel tissue sections from both species of abalone revealed the presence of the MBVs (Fig. 1A) and the iMBVs (Fig. 1A and B). In the MBVs it was not possible to observe individual bacteria by light microscopy, while in the iMBVs large pleomorphic bacteria could be easily observed. These large pleomorphic bacteria are expelled into the lumen of the PE when the iMBVs and the host cell rupture (Fig. 1B).

TEM examination of the uninfected bacteria in this study showed the typical size and shape of *C*Xc (round to oval and a mean width of 0.4 μm by a mean length of 1.7 μm, $N = 30$) as has been previously described by Friedman et al. (2000) and Cáceres-Martínez et al. (2011) TEM results showed that infected bacteria varied in greatly in size and shape and had a width range of 1.0–4.5 μm ($N = 30$) and a length range of 0.5–3.0 μm ($N = 30$) (Fig. 2). These bacteria contained icosahedral-like phage capsids, the majority of them with small electron dense granules that had a dark core while others were interpreted as proheads with a clear core (Fig. 2B). The size of capsids with electron dense granules ranged from 50–60 nm ($N = 30$) and were larger than the proheads which had sizes that ranged from 25–40 nm ($N = 30$). Some of phages with electron dense capsids displayed a long uniform and flexible tail attached to the vertex of the capsid. The tail measured 35–75 nm (from vertex to end of tail) ($N = 21$) and had a width of 10–12.5 nm ($N = 21$) (Fig. 2D–H). The total length (capsid to end of tail) of the apparently complete phages ranged from 80 to 110 nm. In some areas where apparently complete phages were observed the nearest tri-laminar cell wall of the bacteria appeared degraded (Fig. 2C). TEM images of the infected yellow abalone tissues also showed *C*Xc containing similar bacteriophage hyperparasites confirming this hyperparasitism in yellow abalone.

4. Discussion

According to Ackermann (2007) and Ackermann and Prangishvili (2012) bacteriophages are classified into one order and 13 families. Over 6300 phages have been examined by the electron microscope since 1959. At least 4950 phages (96%) are tailed. They constitute the order *Caudovirales* that contains three families. *Siphoviridae* or phages with long, noncontractile tails that are by far the largest family (over 3600 or 57.3%). The other families are the *Myoviridae* and *Podoviridae*. Bacteriophages occur in over 140 bacterial and archaeal genera. The presence of a tail of the bacteriophage in this study undoubtedly demonstrates that this phage belongs to the order *Caudovirales*. The uniformly thin long tail without narrowing near the attachment to the vertex of the capsid and the absence of large terminal fixation structures as well as the flexible appearance shows evidence that the bacteriophage in *C*Xc belongs to the family *Siphoviridae* (Ackermann, 2003; Ackermann and Prangishvili, 2012). Bacteriophages from the family *Myoviridae* also have tails but these can be differentiated from the family *Siphoviridae* due to their rigid and relatively thick tail (16–20 nm) (Murphy et al., 1995). Additionally the placement of this virus in the order *Caudovirales* suggests this as a double stranded DNA virus. More detailed ultra-structure observations such as negatively stained images and DNA analysis according to the International Committee on the Taxonomy of Viruses are needed to determine the precise taxonomic placement of this bacteriophage. In this study the appearance of the membrane and cytoplasm as well as the phage morphology within host cells suggest that viral particles without tail are in different phases of assembling: the proheads are in an initial phase, the capsids with the electron dense core without a tail are in a intermediate phase, while those that contain a capsid with an electron dense core and a tail are in the final phase of assembly when bacterial cell lyses could occur (Penso, 1955; Voyles, 2002). This is in accordance with the normal progression of the lytic cycle by a virulent bacteriophage. Further evidence of the lytic phase of the bacteriophage was observed through histological sections which showed the rupture of the iMBVs and the released of large pleomorphic bacteria to the lumen of the PE.

Some of the earliest observation of bacteriophages in a rickettsiales-like organisms form marine mollusks were made by Buchanan (1978) in the clam *Tellina tenuis*. Since then bacteriophage have been detected in other rickettsiales-like organisms in other marine mollusks such as the orient clam *Meretrix lusoria* (Wen et al., 1994) the mediterranean mussel *Mytilus galloprovincialis* (Comps and Tigel, 1999), the suminoe oyster *Crassostrea ariakensis* (Sun and Wu, 2004), red abalone (Friedman and Crosson, 2012) and black abalone (Friedman et al., 2014). Phages infecting rickettsiales-like organisms have been observed in the scorpion *Buthus occitanus* (Morel et al., 1974) and in the intracellular salmon pathogen *Piscirickettsia salmonis* (Yuksel et al., 2001), that was once considered in the order Rickettsiales. According to Ackermann (2003), in Rickettsias and Clamidias, four bacteriophages have been detected, one *Podoviridae*, two Polyhedral, filamentous, and pleomorphic phages and one *Siphoviridae*. These morphological obser-

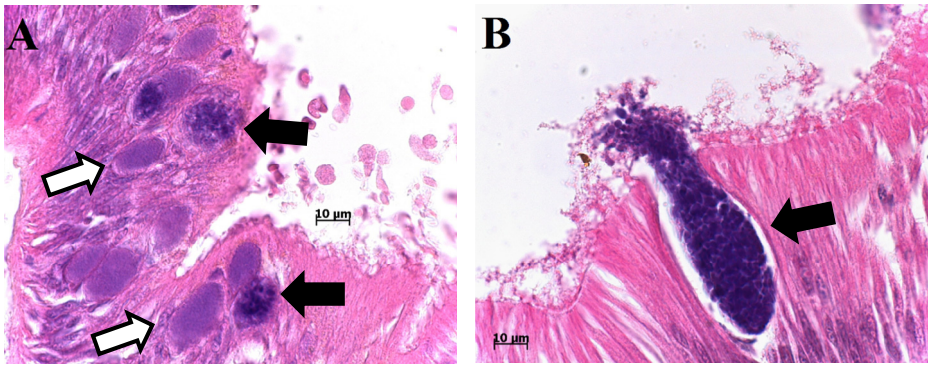

Fig. 1. *Candidatus* Xenohaliotis californiensis (*C*Xc) infecting the gastrointestinal epithelia of abalone. Gastrointestinal epithelia of abalone infected by membrane bound vacuoles (MBVs) formed by *Candidatus* Xenohaliotis californiensis and bacteriophage infected membrane bound vacuoles (iMBVs). (A) Presence of MBVs (white arrows) and iMBVs (arrows blacks) in the posterior esophagus, note the clear difference in morphology and staining pattern between the two inclusions. (B) High magnification of the rupture process of an iMBV and the host cell were the infected bacteria are released into the lumen. Hematoxylin and eosin.