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NOTE

Rare occurrence of heart lesions in Pacific oysters *Crassostrea gigas* caused by an unknown bacterial infection

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ABSTRACT: On rare occasions, small cream-coloured cysts have been observed in the heart and pericardial cavity of Pacific oysters *Crassostrea gigas* from British Columbia, Canada. Histopathology revealed the presence of large colonies of bacteria (up to 800 µm in diameter) causing significant host response and hypertrophy of the heart epithelium. The causative bacteria were characterized as follows: Gram-negative, coccoid to small rod-shaped, typically <1.5 µm in size, cell walls highly endowed with surface fimbriae and division via binary fission. Although these bacteria shared some morphological characteristics with the order Rickettsiales, they did not require an intracellular existence for multiplication. Unfortunately, a cultured isolate was not available, and a retrospective attempt to further characterize the bacteria using DNA sequence analysis of a fragment from the 16S rDNA region proved to be uninformative.

KEY WORDS: Pacific oysters · Lesions · Bacteria

INTRODUCTION

In the fall of 2003, small cream-coloured cysts (up to 1 mm in diameter) were observed in the heart and pericardial cavity of adult Pacific oysters *Crassostrea gigas* from 2 locations in British Columbia (BC), Canada. These macroscopic lesions were recorded in approximately 3% of the oysters examined from both Ladysmith Harbour and Lemmens Inlet (1 of 30 and 3 of 120, respectively). Examination of tissue samples via histology and electron microscopy revealed that these cysts were being caused by large colonies of coccoid to short rod-shaped bacteria. This paper represents the first report of macroscopic lesions in the heart of *C. gigas* and describes the pathology and the morphological characteristics of the bacteria involved with the infection.

MATERIALS AND METHODS

Histopathology

Transverse cross sections including heart tissue from 120 oysters were preserved in Davidson's solution (Howard et al. 2004) and processed using routine histology techniques for paraffin embedding. Tissue sections were cut at 5 µm thickness and stained with haematoxylin and eosin (H&E). Additional tissue sections from the 4 oysters with lesions were stained using Giemsa and Gram stains

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(Howard et al. 2004). Histology slides were examined and photographed via light microscopy (100× to 1000× magnification).

Transmission electron microscopy (TEM)

Heart tissue lesions from 2 oysters were cut into small pieces (2 to 3 mm cubes), preserved in 2.5% gluteraldehyde in Sorenson's phosphate buffer (pH 7.2) and stored at 4°C for 12 to 25 d. Tissues were rinsed 3× with the same buffer, post fixed for 2 h in 1% osmium tetroxide, rinsed another 3× with buffer, dehydrated through an ethanol series and acetone followed by infiltration and embedding in epoxy resin (Eponate 12 kit Marivac). Thin sections were mounted on grids, stained with uranyl acetate and lead citrate and examined using a Zeiss EM 10 transmission electron microscope.

Polymerase chain reaction (PCR) and sequencing

Small pieces of heart tissue containing lesions were preserved and stored in 95% ethanol for approximately 12 yr. Recently, DNA from 1 sample was extracted using a DNeasy tissue Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA concentration and purity was measured using a Nanodrop spectrophotometer (166 ng μl^{-1}). A PCR assay was conducted using a previously published primer pair (968f and 1401r) designed to amplify bacterial 16S ribosomal DNA (Nübel et al. 1996). The PCR reaction was performed using HotStar Taq (Qiagen) with 1 µl of undiluted DNA as template in a 25 µl reaction volume with final concentrations of the following: 1× buffer, 5× Q-Solution, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM forward and reverse primer and 0.05 U μl^{-1} of Taq polymerase. The thermal cycling conditions were 5 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; followed by a final cycle of 10 min at 72°C. The PCR product was visualized on 1.5% agarose gels stained with SYBR Safe (Thermofisher) and then purified for sequencing using EXOSap-IT (USB Corporation). The sequencing reaction was carried out using Big Dye Terminator v3.1 (Thermofisher) with 1 µl of purified PCR product as template, according to the manufacturer's protocol; the products were then purified using the DyeEX 2.0 Spin Kit (Qiagen) and run on an ABI 3130xl genetic analyzer.

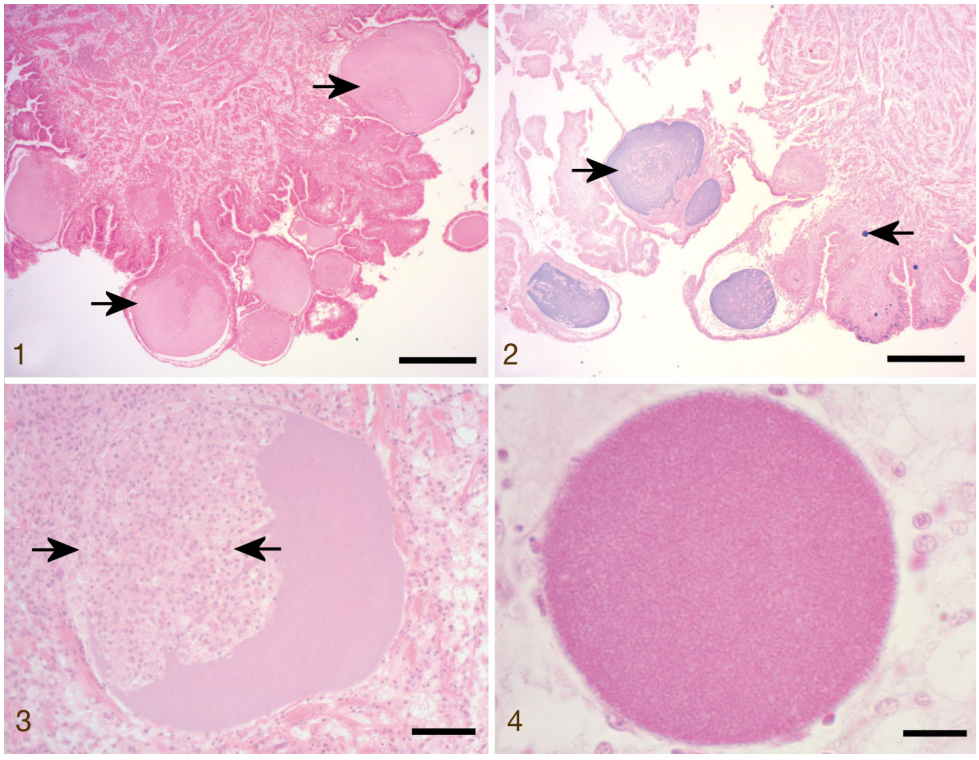
RESULTS

Histopathology

The bacterial colonies occurred primarily in the heart, but in 2 of 4 specimens were also observed to a lesser degree in the connective tissue of the mantle, gills, labial palps and digestive gland. The colonies consisted of bacteria that were weakly basophilic with H&E (Figs. 1 & 3), strongly basophilic with Giemsa stain (Fig. 2) and Gram negative (Fig. 4). The size of the colonies ranged from 10 to 800 µm in diameter and caused hypertrophy of the heart epithelium (Fig. 1). Smaller colonies (<200 µm diameter) were usually spherical and appeared to be contained by the host (Fig. 4). Larger colonies were often irregularly shaped and associated with host responses consisting of haemocyte infiltration (Fig. 3).

TEM

The bacterial colonies were usually contained within a cyst wall (Figs. 5–7), which consisted of amorphous material and sometimes incorporated remnants of degenerating cells and elongated muscle fibres (Fig. 5). In addition to numerous bacteria, the cysts contained haemocytes, which were often located along the internal surface of the cyst wall (Figs. 5–7). Some of these haemocytes were necrotic, but others contained necrotic-looking bacterial cells within phagosomes (Fig. 5). Phagocytosis by host haemocytes was commonly observed (Figs. 5 & 6). However, the colonies did not appear to have an intracellular habitat, and bacteria were observed to multiply within the cyst matrix (Figs. 9–11). Some cysts also contained disrupted striated heart muscle fibres, usually with closely associated bacteria (Figs. 7, 8 & 11). The bacteria were predominantly coccoid and short rod-shaped; however, a few pleomorphic specimens were also observed (Figs. 5–11). Individual bacteria measured from 0.32 to 0.65 µm in diameter and up to 1.75 µm in length (n = 78), and division by binary fission was commonly observed (Figs. 9–11). All bacteria were highly endowed with surface fimbriae (filaments or pili) (Figs. 8–11). The interior of most bacteria was formed by granular electron-dense material which often had a mottled appearance, the density of which increased substantially near the outer membrane. An electron-dense cap was also observed in some specimens (Figs. 9 & 11).



Figs. 1 to 4. Histological tissue sections of *Crassostrea gigas* heart tissue showing lesions caused by large bacterial colonies. Fig. 1. Low magnification showing multiple large colonies of bacteria (arrows) causing hypertrophy of the heart epithelium (H&E stain, scale bar = 500 µm). Fig. 2. Low magnification showing multiple colonies of bacteria (arrows) ranging from 10 to 800 µm in diameter (Giemsa stain, scale bar = 500 µm). Fig. 3. Close up of a ruptured bacterial colony which has elicited host response (arrows) consisting of abundant haemocyte infiltration (H&E stain, scale bar = 50 µm). Fig. 4. High magnification showing a single discrete bacterial colony which appears to be contained by the host (Gram stain, scale bar = 10 µm)

Sequence analysis

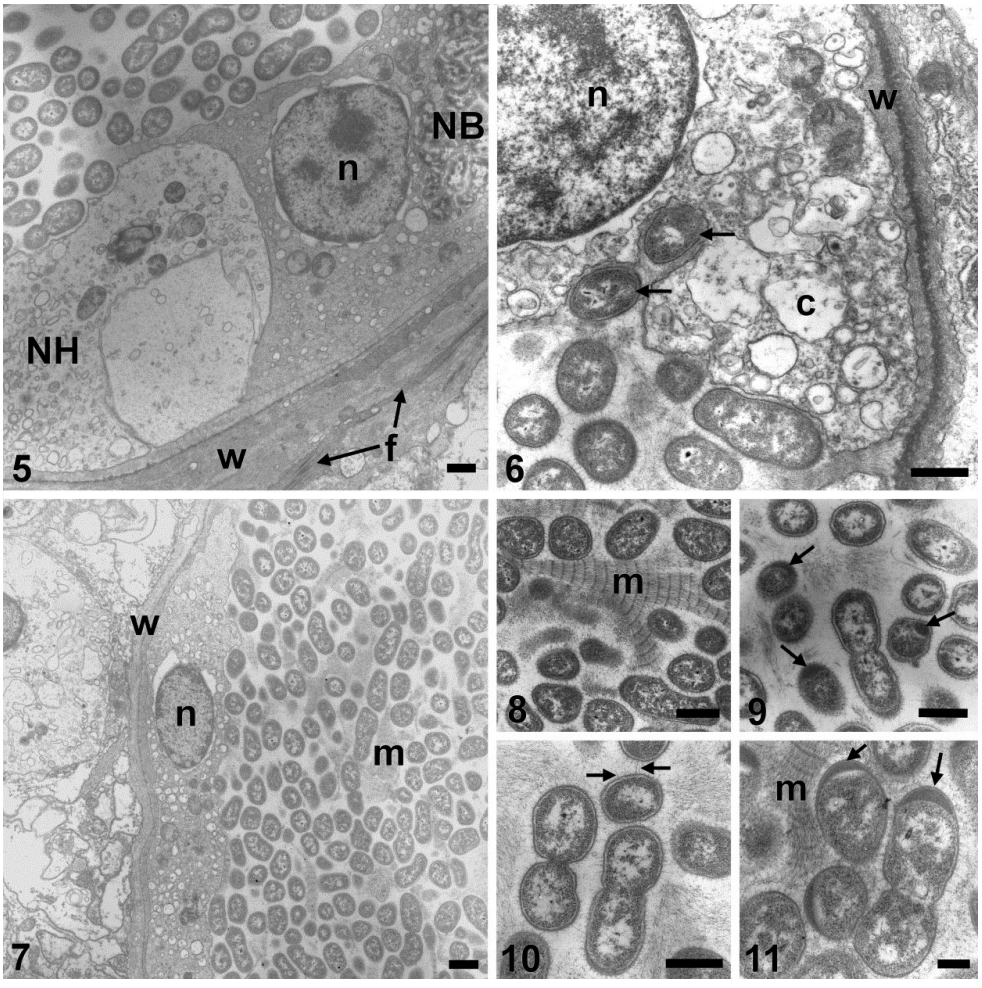
A 403 bp sequence fragment from the 16S rDNA region was obtained from a single sample (GenBank accession no. KY799077) with BLAST results identifying most closely with uncultured bacteria from multiple origins (the top 50 results showing 93 to 95% similarity over the entire fragment).

DISCUSSION

The histopathology and morphological characteristics of the bacteria observed between specimens from both locations were identical, suggesting that a common agent was responsible for the lesions. Examination by electron microscopy revealed that the bacteria possessed abundant fimbriae, a common feature among other Gram-negative pathogenic bacteria (Cheville 1994). Some of the bacteria appeared to be closely associated with heart muscle fragments within the cysts, presumably the result of rupture and dissociation of the heart muscle by large bacterial colonies.

Although the characteristics of the bacteria reported in this study somewhat resemble those described for the order Rickettsiales (Weiss & Moulder 1984, Cheville 1994), they appeared to lack the obligate intracellular tropism which is a defining characteristic of this group. Rickettsiales-like prokaryote organisms (commonly referred to as RLP or RLO) have been reported from >25 species of bivalve molluscs over a wide geographic distribution (for example, see Harshbarger et al. 1977, Elston & Peacock 1984, Elston 1986, Fries & Grant 1991, Sun & Wu 2004, Bower 2010). These RLP infections typically occur in the epithelial cells of the gills or digestive gland and in most instances are considered to be benign; only in rare cases have they been implicated with disease and mortality among bivalves.

Unfortunately, little is known about the biochemical or growth characteristics of the oyster heart bacteria because isolation and culture was not attempted at the time of necropsy in 2004 and because subsequent occurrence of heart lesions in BC oysters has been extremely rare (<0.5%). Also, the attempt to gain more insight into the identity



Figs. 5 to 11. Transmission electron micrographs. Fig. 5. Two haemocytes, one of which appears necrotic (NH) located on the interior of the cyst wall (w) that incorporated cell remnants and elongated muscle fibres (f). The healthy looking haemocyte contains many necrotic-looking bacteria (NB) within a phagosome adjacent to its nucleus (n). Scale bar = 0.5 µm. Fig. 6. Two bacteria (arrows) being phagocytized by a host haemocyte sectioned through its nucleus (n) and cytoplasmic vacuoles (c). The haemocyte is adjacent to the cyst wall (w). Scale bar = 0.5 µm. Fig. 7. An elongate haemocyte (n) containing a few phagocytized bacteria is stretched along the interior surface of the cyst wall (w); numerous bacteria and heart muscle (m) fragments are also present within the cyst. Scale bar = 1.0 µm. Fig. 8. Numerous bacteria closely associated with striated muscle (m) of the heart. Scale bar = 0.5 µm. Fig. 9. Three bacteria with electron-dense caps (arrows) surrounding an example of a bacteria undergoing binary fission. Scale bar = 0.5 µm. Fig. 10. Abundant fimbriae (arrows) protruding from the outer surface of the bacteria cell walls. The 2 bacteria in the central portion of the image are in the process of binary fission: (right) in early stage division showing invagination of the cell wall, and (left) exhibiting late-stage division and about to pinch off to form separate daughter cells. Fig. 11. Several bacteria with electron-dense caps (arrows), one of which is in the early stages of cell division. Fragments of heart muscle (m) located near the bacteria. Scale bar = 0.25 µm

of the bacteria via sequence analysis of a 16S rDNA fragment proved to be uninformative. Considering the complex nature of bacterial taxonomy and the lack of conclusive evidence supporting specific taxonomic affinities to the Rickettsiales, it was decided at this time to refer to the causative microorganisms simply as unknown bacteria. Nonetheless, this report documents new findings concerning lesions in the heart of Pacific oysters that are caused by a bacterial infection. Although these lesions are a rather sporadic and rare phenomenon in BC, further research and identification of these bacteria is warranted given that *C. gigas* is an economically important species that is cultivated in many parts of the world.

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