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Newly isolated bacterium *Tenacibaculum* sp. strain Pbs-1 from diseased pearl oysters is associated with black-spot shell disease

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ABSTRACT

In recent years, black-spot shell disease has appeared on the shells of Akoya pearl oysters, *Pinctada fucata*, causing serious problems in cultured pearl production. This disease was believed to be caused by boring of *Polydora ciliata* into calcareous substrate, and a control measure against *P. ciliata* was developed. As a result, boring activity of *P. ciliate* decreased, but black-spot shell disease remains a serious problem. We detected the genus *Tenacibaculum* specifically from the shells of diseased oysters using PCR-DGGE, and isolated *Tenacibaculum* sp. strain Pbs-1 from the diseased oyster shells. Analyses of 16S rRNA gene sequence homology and its biochemical and morphological characteristics suggested that strain Pbs-1 was a new species of *Tenacibaculum*. We conducted four infectivity experiments by different infection methods: smear, injection, immersion, or a combination of wound and immersion. The highest infectivity (100%) in the shell was observed in the test group by the smear method, which was accompanied by severe clinical signs that included a focal blackish discoloration of the shell. The results of the infectivity experiments indicated that the newly isolated *Tenacibaculum* sp. strain Pbs-1 is one of the putative causative agents of black-spot shell disease in Akoya pearl oysters.

1. Introduction

The cultured pearl industry in Japan was established by Kokichi MIKIMOTO in 1893, and currently, aquaculture is carried out in Mie Prefecture, Ehime Prefecture and Nagasaki Prefecture (Nagai, 2013). In the past, the cultured pearl industry was an important contributor to the regional economy and pearls were one of the country's valuable exports. However, since the 1990s, the production of cultured pearls has decreased significantly and remains low because of the high mortality rate among pearl oysters caused by deterioration of the environment and disease. In recent years, a black-spot shell disease (Fig. 1) has appeared on the shells of the Akoya pearl oyster, *Pinctada fucata*, causing serious problems in cultured pearl production, which was characterized by its major symptom, a focal blackish discoloration of the shell. This symptom was first reported in the 1950s and subsequently increased year by year (Mizumoto, 1964). Black-spot shell disease not only affects the quality of the pearls but also threatens the survival of the organism. Mizumoto (1964) described many small holes made by the boring *Polydora ciliata* on the shell surface foci of diseased pearl oysters. Polydorids (Polychaeta, Spionidae) commonly possess the same boring activity. Habitat types and boring activity of 32 species of polydorids have been studied by Sato-Okoshi (1999), who described that the boring mechanism is the same among the polydorid species and the existence of concentric-edged holes or worm-eaten structure in calcareous substrata provides evidence of polydorid infestation. Research into the ecology of *P. ciliata* was carried out, and a parasitic control measure such as NaCl-supplemented sea water bathing was developed (Mizumoto, 1964, 1966). In response to this control measure, boring activity by *P. ciliata* decreased; however, in recent years, the impact of black-spot shell disease has once again increased in severity.

Various species of pathogenic bacteria have been reported from cultured fish and shellfish: *Edwardsiella tarda* (Nakatsugawa, 1983), *Flexibacter maritimus* (Wakabayashi et al., 1986), *Tenacibaculum maritimum* (Avendaño-Herrera et al., 2006a), *Lactococcus garvieae* (Meyburgh et al., 2017), *Mycobacterium* sp. (Jacobs et al., 2009), *Pseudomonas plecoglossicida* (Zhang et al., 2014), *Streptococcus iniae* (Agnew and Barnes, 2007), *Vibrio alginolyticus* (Rameshkumar et al., 2017), and some other minor or unknown species. In Japan, around 200 million US dollars (including the cost of medication) are lost every year

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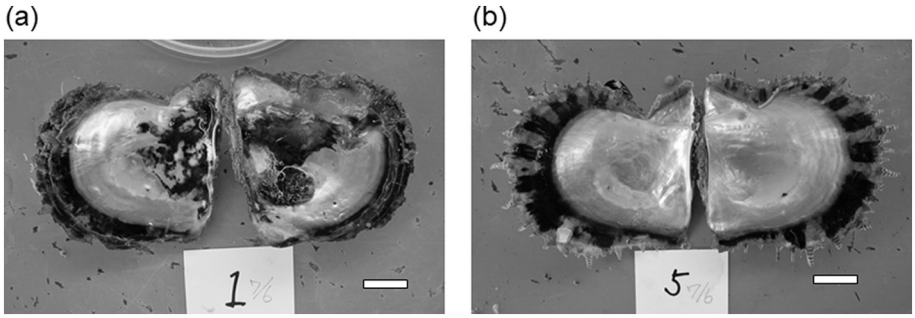

Fig. 1. Photographs of the shell of *Pinctada fucata*.
(a) A black-spot shell diseased individual; (b) A healthy individual. The bar in the photographs indicates 2 cm.

as a result of bacterial infections in the mariculture industry (Kusuda and Kawai, 1998). However, to the best of our knowledge, there are no reports about the bacterial infection of pearl oysters, although, in Western Australia, the early literature contained a report about *Vibrio* bacterial infections of pearl oyster *Pinctada maxima* (Pass et al., 1987).

In recent years, culture-independent molecular methods based on the 16S rRNA gene sequence have become widely used for the detection of environmental microorganisms (Ranjard et al., 2000). Among them, the denaturing gradient gel electrophoresis (DGGE) technique allows for the simultaneous analysis of multiple samples and comparison of microbial communities based on temporal and spatial differences (Muyzer et al., 1993). Furthermore, PCR-DGGE has the advantage that data comparing major microbial communities can be observed immediately after electrophoresis (Hanning and Ricke, 2011).

In this study, we first detected bacteria in the shell of diseased Akoya pearl oysters using PCR-DGGE, then we isolated and characterized the bacteria. Furthermore, we reproduced the disease by infecting the isolated strains into healthy individuals.

2. Materials and methods

2.1. Collection of pearl oysters

Pearl oysters *Pinctada fucata martensii* (Akoya) were collected from the pearl oyster farm in Ago Bay in Shima City, Mie Prefecture, Japan, and Shibata Bay in Uwajima City, Ehime Prefecture, Japan, and Van Phong Bay in Van Thanh, Viet Nam. Sampling was conducted once a month between June 2015 and July 2016. Three individual diseased oysters (Fig. 1) and healthy oysters were collected in one sampling. After excision from the shells, the oysters were transferred into 1.5 ml sterile centrifuge tubes and stored at −30 °C prior to DNA extraction.

2.2. DNA extraction

Total DNA was extracted from the samples using a vigorous bead-beating DNA extraction kit (UltraClean Soil DNA Isolation Kit; Mo Bio Laboratories, CA, USA) following the manufacturer's instructions. The genomic DNA was then quantified with a spectrophotometer (Nanodrop 1000 Spectrophotometer; Thermo Fisher Scientific K.K., Kanagawa, Japan) and stored at −30 °C for later use.

2.3. 16S rRNA gene amplification, DGGE analysis and sequencing

PCR amplification of the 16S rRNA gene of bacteria was performed in a 60 μl volume containing approximately 5 ng of template DNA, 1 × Ex Taq buffer, 200 μM dNTP mix, 0.25 μM of each primer and 0.5 U Ex Taq HS (TaqDNA polymerase; Takara Bio Inc., Shiga, Japan). The universal primers GC-341f and 518r (Muyzer et al., 1993) were used under the following thermal cycle conditions: initial denaturation at 94 °C for 3 min; 20 touchdown cycles of denaturation (at 98 °C for 10 s), annealing (at 65 °C for 30 s, decreasing by 0.5 °C each cycle) and extension (at 72 °C for 30 s); 15 standard cycles of denaturation (at 8 °C for 10 s), annealing (at 55 °C for 30 s) and extension (at 72 °C for 30 s), and a final extension at 72 °C for 3 min. Successful DNA amplification was verified by electrophoresis in a 2% agarose gel in 0.5 × TAE buffer diluted from 50 × TAE buffer (2 M Tris base, 1 M glacial acetic acid and 50 mM EDTA) with a 100 bp Ladder marker (Nippon Gene, Tokyo, Japan).

The amplified 16S rRNA gene fragments were analyzed by DGGE with a Dcode Population Base System (Bio-Rad, Laboratories, CA, USA). PCR products were separated on an 8% acrylamide gel with a linear gradient of the denaturants urea and formamide increasing from 25% at the top of the gel to 60% at the bottom. Electrophoresis was performed at 70 V and 60 °C for 14 h in 0.5 × TAE buffer, with a DNA marker on either side of the samples. The gel was stained with ethidium bromide and documented using a UV-transilluminator (MultiDoc-It TM Digital Imaging System; UVP, CA, USA) and a C-5060 Wide Zoom imaging system (Olympus, Tokyo, Japan).

The predominant bands were stabbed with a sterile pipette tip and placed into 1.5 ml tubes with 100 μl of sterile water. DNA was eluted from the gel pieces by overnight incubation at 4 °C, and the eluates were used as template DNA and reamplified as described above. The amplified products were purified using a QIAquick PCR purification kit (Qiagen K. K., Tokyo, Japan), followed by direct sequencing with the reverse primer 518r using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Sequence similarities between the isolates and sequences available in public databases were then searched using the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov).

2.4. Isolation and characterization of the pathogenic bacterium

To isolate the pathogenic bacterium, 1 g (wet weight) of shell pieces from diseased Akoya pearl oysters was transferred to a conical beaker containing 100 ml of modified AO medium (5% tryptone, 0.5% yeast extract, 0.2% beef extract, 0.2% sodium chloride) with 70% sea water (Pazos et al., 1996), and incubated at 25 °C on a rotary shaker at 140 rpm (Bioshaker BR-180LF; Taitec Corp., Saitama, Japan) for 3 days. A loop-full of the culture medium containing the cultured bacteria was spread onto AO agar plates (1.5% *w/v*). The pathogenic bacterium was isolated as a single colony.

The morphology and motility of the bacteria were first observed by phase-contrast microscopy (Olympus BX-51, Tokyo, Japan). Genomic DNA was extracted from a colony of isolates, and the 16S rRNA gene was amplified by PCR using the eubacterial universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG -3′) and 1525r (5′- AAAGGAGGTGATCC AGCC -3′). The amplified DNA was purified and sequenced as described above using the primers, r1L, r2L, r3L, r4L, f3L and 926f (Tanaka et al., 2010). Sequence similarities between the isolates and sequences available in public databases were then searched using the BLAST program on the NCBI website. Biochemical characterization was