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Biological characterization of two marine *Bdellovibrio*-and-like organisms isolated from Daya bay of Shenzhen, China and their application in the elimination of *Vibrio parahaemolyticus* in oyster

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ABSTRACT

Bdellovibrio-and-like organisms (BALOs) are a group of highly motile delta-proteobacteria that prey on other gram-negative bacteria. However, nothing is known of the application potential of marine BALOs in safeguarding seafood safety. Here, biological characterization of two marine BALOs strains and their application in the elimination of *Vibrio parahaemolyticus* in oyster (*Crassostrea ariakensis*) at the laboratory scale were investigated.

BALOs strains BDH12 and BDHSH06 were isolated from sediment of Daya bay in Shenzhen of China, with *Shewanella putrefaciens* strain 12 and *V. parahaemolyticus* strain SH06 as preys, respectively, when using double layer agar technique. They were identified as BALOs morphologically by transmission electron microscopy, while partial 16S rDNA sequencing analysis revealed that they showed no close relationships with members of the known genera *Bdellovibrio*, *Bacteriolyticum*, *Bacteriovorax*, or *Peredibacter*.

Biological characterizations revealed that both strains had the optimal pH, salinity and temperature at 7.2, 3% and 30 °C, correspondingly. They could not utilize autoclaved, dead cells as hosts. Prey range analysis revealed that individually, BDH12 and BDHSH06 lysed 82.5% (47 strains) and 84.2% (48 strains) of the total 57 preys tested respectively. In combination, they lysed 98.2% (56 of 57) strains. All strains of *V. parahaemolyticus*, *Vibrio cholerae* and *Vibrio alginolyticus* tested could be lysed by both strains.

A 7-day laboratory-scale *V. parahaemolyticus* elimination experiment in oyster showed that in the control, the cell counts of total vibrios and *V. parahaemolyticus* strain Vp plus in water and in oyster intestines were on the rise, whereas in the BALOs treated groups, their numbers were down from 8.09 ± 0.05 log CFU/ml and 8.02 ± 0.04 log CFU/ml to 2.39 ± 0.01 log CFU/ml and 2.33 ± 0.01 log CFU/ml, respectively. The same patterns could also be observed in oyster intestines. Results of this study indicate the feasibility of using BALOs to biologically control or even eliminate *V. parahaemolyticus* in seafood oyster.

1. Introduction

Vibrio parahaemolyticus is a human pathogen that is widely distributed in the marine environments. It is frequently isolated from a variety of raw seafoods, particularly shellfish (Su and Liu, 2007) and could cause food poisoning when not properly prepared before consumption. In 1998, the largest *V. parahaemolyticus* outbreak reported to date in the USA involving 416 cases was linked to consumption of raw oysters (DePaola et al., 2000). In 2006, an outbreak of *V. parahaemolyticus* infections resulted in 177 cases and was again linked to the consumption of contaminated raw shellfish including oysters (Balter et al., 2006). Outbreaks of *V. parahaemolyticus* infection have heightened concerns about the safety of raw oyster consumption.

To control *V. parahaemolyticus* infections, the emergence of innovative FDA proposed post-harvest treatment of shellfish using interventions such as Individual Quick Freezing (IQF), introduced in 1989, Heat–Cool Pasteurization (HCP), introduced in 1995, and High Hydrostatic Pressure (HHP), introduced in 1999. These technologies, which are currently commercially available, have made it possible to bring raw and safer products to consumers (Meujo et al., 2010). But unfortunately, oysters cannot survive any of the above technologies (Mississippi Department of Marine Resources, USA, 2006) and could not satisfy those consumers who praise for live oysters.

Bdellovibrio-and-like organisms (BALOs), a less well-studied group of bacteria, are small predatory parasitic bacteria with a unique ability to penetrate, reproduce in and subsequently kill other gram-negative bacteria. BALOs are currently being considered as potential microbial control or therapeutic agents (Sockett and Lambert, 2004). Until now, as

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live protective cultures to be applied in food production system, BALOs have only been explored to a rather limited extent (Fratamico and Cooke, 1996). Nothing is known of their application in elimination of *V. parahaemolyticus* in oyster.

To explore the practical application of BALOs in seafood safety, we first biologically characterized two marine BALOs isolated from Daya Bay of Shenzhen, China, and then examined their abilities to eliminate *V. parahaemolyticus* in oysters at the laboratory scale.

2. Materials and methods

2.1. Prey strain and cultivation

Shewanella putrefaciens strain 12 and *V. parahaemolyticus* strain SH06 were used as prey strains, which were isolated and identified by 16S rDNA sequencing in our laboratory. *V. parahaemolyticus* strain SH06 contains no TDH (thermostable direct haemolysin) and no TRH (TDH-related haemolysin) gene. Fresh cultures of prey bacteria for BALOs isolation were prepared as follows: prey bacteria were cultured with shaking at 160 rpm include Marine broth 2216E (pH 7.6). After incubation at 30 °C for 12–16 h (late exponential phase, 1×10^8 CFU/ml), prey cells were pelleted at 5000 × *g* for 20 min at 4 °C. The resulting pellet was re-suspended and adjusted to 5×10^9 CFU/ml with 2216E broth after optical density measurement (1×10^8 CFU/ml gave ca. 0.1 $OD_{600\ nm}$), and then stored at 4 °C.

2.2. Isolation of BALOs

In the experiment 10 g sediment from Daya Bay of Shenzhen, China was aseptically added to 90 ml dilute nutrient broth (DNB) (Stolp and Starr, 1963), and subsequently mixed with 10 ml of *S. putrefaciens* strain 12 or *V. parahaemolyticus* strain SH06 cultures. The mixture was incubated at 30 °C at 250 rpm for 24 h, followed by centrifugation at 5000 × *g* for 20 min at 4 °C. The resulting supernatant was passed three times through a 0.45-μm-pore-size membrane (Millipore Corp., USA) to remove any potential unsusceptible bacteria, prey residuals and debris. The filtrate was subjected to centrifugation at 27,000 × *g* for 20 min at 4 °C to concentrate BALOs and eliminate potential bacteriophages in the supernatant. The BALOs-containing pellet was re-suspended with 1 ml DNB and diluted to 10^{-4} dilution with DNB in a series of 10-fold dilution. These dilutions (10^0–10^{-4}) were then checked for viable BALOs with the standard double layer agar plating technique (Stolp and Starr, 1963). Plates were left undisturbed to solidify and incubated at 30 °C for 4 to 6 days for the presence of BALOs plaques. Plaques visible after 2 to 3 days and with further expansion for several days were considered as potential BALOs. For purification, a plug containing one plaque and the surrounding preys was lifted from the plate and subjected to double layer agar plating technique, until lytic plaques on an agar plate were uniform in size.

2.3. Preparation of attack-phase BALO predators

To prepare attack-phase BALOs, a BALO plague was picked with a sterile inoculation loop from a freshly grown double-layer agar plate and inoculated into an Erlenmeyer flask that contained 50 ml DNB medium and 0.5 ml of one of the two freshly prepared prey strains. Incubation was performed at 30 °C with shaking at 200 rpm for 24 h. Then, 5 ml of this culture was used for the second-round inoculation for 24 h. Then, prey cells were removed by centrifugation at 5000 × *g* for 20 min at 4 °C, and the resulting supernatant was passed twice through a 0.45-μm-pore-size membrane filter to remove the prey cell residues. Filtrate with attack-phase BALOs was concentrated with centrifugation at 27,000 × *g* for 20 min at 4 °C, and the resulting pellet was then re-suspended in 1 ml sterile phosphate buffered saline (PBS: 10 mM $NaH_2PO_4\times2H_2O$, 10 mM $Na_2HPO_4\times2H_2O$, 150 mM NaCl, pH 7.2), stored at 4 °C. The BALOs concentration was adjusted to 1×10^{10} PFU/ml with PBS following the optical density measurement (1×10^9 PFU/ml gave $OD_{570\ nm}$ ca. 0.13) before use.

2.4. BALOs identification

We used two methods to identify BALOs: transmission electron microscopy and PCR-based sequencing analysis of 16S rDNA.

2.4.1. Transmission electron microscopy

To examine the morphology of attack-phase BALOs, a small drop of freshly prepared predator suspension was placed on a Formvar carbon-coated 400-mesh copper grid for 2 min at room temperature. The grid was then lifted gently with a pair of forceps, and the excess liquid was removed. Samples were counterstained with a 1% (wt/vol) phosphotungstic acid solution (pH 6.4) for 15 min and examined with a Model ZEX100CX II transmission electron microscope (Jeol, Akishima, Japan) at an accelerating voltage of 100 kV (Koval and Hynes, 1991). In the free attack-phase, BALOs are small (0.25 to 0.40 μm in width, 1 to 2 μm in length) vibrioid to rod-shaped bacteria that possesses one polar flagellum; cells with such features under the microscope were considered as BALOs candidates. Further identification was based on 16S rDNA sequencing analysis.

2.4.2. Phylogenetic analysis of partial 16S rDNAs

Genomic DNA of attack-phase BALOs was extracted with Instagene Matrix Kit (Bio-Rad Laboratories, USA), basically according to the manufacturer's instructions. DNA was re-suspended in 1 × TE (10 mM Tris-HCl, 100 mM EDTA, pH 8.0) buffer and stored at −20 °C. DNA concentration was assayed at 260 nm and 280 nm using a Model MPS-2000 Shimadzu spectrophotometer and diluted with 1× TE for PCR.

To avoid potential amplification from bacterial prey DNA, partial 16S rDNA PCR was performed with a universal bacterial forward primer 63 F (5′-cag gcc taa cac atg caa gtc-3′, positions 63–83 of 16S, *Escherichia coli* numbering) and a *Bdellovibrio*-specific reverse 842R [5′-cga(t) cac tga agg ggt caa-3′, positions 842–824 of 16S, *Escherichia coli* numbering]. These primers were designed for almost all the *Bdellovibrios* based on the *Bdellovibrio* 16S rRNA sequences, which were synthesized by Invitrogene Co., Ltd. (Guangzhou, China). PCR was performed according to Cai et al. (2008), in a total reaction volume of 50 μl. Amplification was performed on a thermal cycler (PCR Express, Hybaid, Middlesex, UK), with an initial cycle of denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 45 s and extension at 72 °C for 1 min., and a final extension step at 72 °C for 10 min. Successful amplifications were confirmed by electrophoresis of 5 μl PCR products on a 1% agarose gel. The products were purified with a PCR purification kit (Takara, Dalian, China). PCR direct sequencing was performed as in Thompson et al. (1992).

The 16S rRNA gene sequences (ca. 800 bp) obtained were then deposited in Genbank (accession number of BDH12, EF011103; and BDHSH06, EF094471). BLAST-N searches were used to identify the species most related to strains BDHSH06 and BDH12, respectively. A phylogenetic tree was constructed using the Neighbour-Joining method of Saitou and Nei (1987) with MEGA software with a bootstrap analysis of 1000 replicates (Felsenstein, 1985).

2.5. Characterization of lytic ability of BALOs strains BDHSH06 and BDH12

Table 1 lists 57 bacterial strains used as prey as well as their origins. Lytic abilities of BDH12 and BDHSH06 on these preys were assessed by their abilities to form clear lytic halos on a lawn of preys with double layer agar plating technique: 1 ml of fresh prey cells was added to 0.1 ml of the fresh attack-phase BDH12 or BDHSH06 and 3.5 ml of top agar as mentioned above. They were vortexed to mix and poured over the pre-warmed bottom agar. Plates were incubated for 4