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Bacterial genetic diversity on settlement substrates during mass mortality of larvae of nona-porous abalone (*Haliotis diversicolor supertexta*)

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ABSTRACT

Settlement substrate samples for nona-porous abalone seed attachment from diseased and nondiseased abalone hatcheries during mass mortality occurrence were collected, the 16S rRNA gene clone libraries of the attachments were constructed using primers universal for the domain Bacteria. In total, 83 clones randomly selected were screened by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and clones with unique RFLP patterns were sequenced. Phylogenetic analysis revealed bacterial components different from previous studies in such environments. Apart from sequences originating from chloroplast of eukaryotic diatom (occupying 29.33%), all the cloned sequences fell into five lineages of the bacterial domains, the α-Proteobacteria, γ-Proteobacteria, Flavobacteria, Acidobacteria and an uncultured candidate division TM7. The α-Proteobacteria was predominant (56%) and was mainly comprised of *Sphingomonadales*, *Rhodobacterales*, and *Rhizobiales* affiliated clones. Other groups appeared just as minor components. Most of our sequences were closely related to sequences from attached bacteria, or sequences retrieved from extreme environments or specific environments, indicating that these bacteria might play an important function in bacterial attachment or in extreme environments. The community structure and the clone abundance in different groups of the two clone libraries were different, further research focusing on these different bacteria will help to reveal the potential pathogens of the abalones' mass mortality.

1. Introduction

Abalone is an economically important gastropod with high market values. Remarkable progress has been made since 1990s in abalone cultivation along the Chinese coastal areas. However, abnormal mortality has been threatening sustainable development of the abalone industry since its inception. Mass mortalities occurring in juveniles and seeds have been observed in many commercial farms since the early 1990s. Mortality usually occurred in the post-settlement nursery phase when spat remain on settlement plates, characterized by cessation of dietary intake and falling from the substratum. Most abalone hatcheries in Chinese coast suffered severe loss of abalone seeds, either larvae or juveniles (Zhang et al., 2004).

The causations are suspected to be the poor water quality, the lack of food caused by diatoms, virus and bacterial infection, etc. (Xu et al., 2006). However, the measures of improving the water quality and food quality could not prevent the abalone seeds from falling off (Xu et al., 2006), and virus infection is not confirmed (Song et al., 2003). *Vibrio alginolyticus* and other *Vibrio* spp. were the most frequently reported bacteria as dominant members in the abalone farming ponds during mass mortalities, and thus were suggested to be a main cause of this mass mortality (Wang et al., 2005; Cai et al., 2006; Wang et al., 2006), while in other cases, *Vibrio alginolyticus* were not the dominant members (Yang et al., 2003) and most of them were non-pathogenic (Li et al., 2003). At present, the exact cause of this large-scale mortality remains unknown. This is partly due to the incomplete understanding of the microbial composition in the abalone farming environments, especially on the abalones' attachment substrates. Most of the currently used methods for bacteria identification were mainly based on traditional cultivation and/or microscopy observation. It is generally known that the cultivated species are not always in accordance with naturally occurring species in environments and only small minorities of microbes grow and form colonies on agar plates. Many viable but nonculturable microbes are undetected during the selective isolation and cultivation processes, leading to the misunderstanding of the composition of environmental microbes (Rappe and Giovannoni, 2003; Schloss and Handelsman, 2004).

In contrast, the application of molecular biology techniques, especially the 16S rRNA gene (rDNA) amplification and sequence analysis, allows us to identify microorganisms directly from the environmental samples without the need of prior cultivation and isolation, and enables us to reveal in situ microbial diversity (Amann et al., 1995; Hugenholtz et al., 1998). Here we report the first 16S rDNA-based investigations for the bacterial genetic diversity on the

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attachment substrates of larvae of nona-porous abalones (*Haliotis diversicolor supertexta*) and revealed bacterial components different from previous studies in such environments. Furthermore, we compare the bacterial community structure on the attachments between the diseased and nondiseased abalone hatcheries, hoping that the information present here will assist in screening potential pathogens of abalone's disease.

2. Materials and methods

2.1. Description of the nona-porous abalone hatcheries

Samples were collected from two nona-porous abalone (*Haliotis diversicolor supertexta*) hatcheries, Dongshan abalone farm and Aquatic Test Base of Jimei University, located in southern Chinese coast of Fujian province. Dongshan abalone farm is a commercial farm specialized in culturing and selling live abalone. This farm suffered severely from frequently occurring mass morality of nona-porous abalone seeds since 1999. In November 2006, mass mortality of the nona-porous abalone occurred again and about 80% of the abalone seeds, either larvae or juveniles, died. Jimei Aquatic Test Base is mainly used for students' practice and teachers' scientific research. Mass mortality never occurred in Jimei abalone hatchery.

2.2. Sample collection

Samples were collected in November, 2006, when mass mortality was occurring in Dongshan abalone hatchery. The nona-porous abalones were 50 days old in Dongshan abalone hatchery, and 25 days old in Jimei abalone hatchery, respectively. Transparent plastic films (polythene with 0.2–0.3 mm thickness) precoated with diatoms were used for abalone larvae settlement. The plastic films were fished out from the abalone ponds and about 5 cm×5 cm plastic films were cut down with sterile surgical scissors. Each plastic film was then placed in a 5-ml sterile disposable centrifuge tube, and the tubes were immediately frozen in a small liquid nitrogen tank. The tank was transported to the laboratory and the samples were stored in a freezer at −20 °C before DNA extraction. The samples from Dongshan were designated as F4, and the samples from Jimei were designated as F0.

2.3. DNA extraction, PCR amplification and clone library construction

Plastic film samples were cut into small pieces and were incubated in 1.5 ml of lysis solution (45 mM glucose, 23 mM Tris [pH 8.0], 59 mM EDTA) containing lysozyme at a concentration of 0.5 mg/ml. After incubation at 37 °C for 2 h, lysates were transferred to another sterile centrifuge tube and the Plastic film species were rinsed again with 0.5 ml of lysis buffer. The lysates were pooled and cells were further lysed by the addition of 1/100 volume proteinase K (0.5 mg/ml) and 1/10 volume sodium dodecyl sulfate (SDS, 1%) for 30 min at a temperature of 55 °C. The cell lysates were extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1) and once with chloroform–isoamyl alcohol (24:1) before nucleic acids were precipitated with 1 volume of isopropanol and 0.4 volume of 7.5 M ammonium acetate at room temperature. Nucleic acids were recovered by centrifugation, washed once with 70% ethanol, and resuspended in TE. The primers used for amplification of bacterial 16S rDNA were 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTACCTTGTTACGACTT-3′). The amplification reaction mixture consisted of 0.5 μM of each primer, 200 μM dNTPs, 5 μl 10×PCR buffer, 1 unit Taq DNA polymerase (TaKaRa Biotechnology Co., Dalian, China) and 2 μl of DNA solution, with sterilized MilliQ water added to the total volume of 50 μl.

The amplification conditions were comprised of steps at 94 °C for 5 min, and 26 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension for 10 min at 72 °C to facilitate the TA cloning. The amplified products were gel-purified and ligated into the pMD18-T vector (TaKaRa Biotechnology Co., Dalian, China) and then transformed into competent cells of *E. coli* DH5α. The ampicillin-resistant clones were randomly picked and screened for inserts by performing colony PCR with M13 primers for the vector (Invitrogen, Shanghai, China).

2.4. PCR-RFLP screening and statistical analysis of the clone libraries

A total of 83 clones (44 from Dongshan sample and 39 from Jimei sample) with inserts of expected length were selected for subsequent RFLP analysis. A 1:10 dilution of the colony PCR products amplified by the M13 primers was reamplified using the primers 27F and 1492R with the same PCR program as described above. Two aliquots of 8–10 μl PCR products were separately digested by restriction endonucleases *Hha* I and *Afa* I (TaKaRa Co., Dalian, China). Restriction fragments were visualized on a 2.0% agarose gel and different clones could be discriminated according to their RFLP patterns. Phylotype diversity in the clone libraries obtained through PCR-RFLP analysis was subjected to statistical analysis. The following indices were calculated: (1) Taxa, the total numbers of RFLP pattern in each library; (2) Individuals, the total clone numbers examined; (3) Coverage (Mullins et al., 1995), which is derived from the equation Coverage = 1 − (*N*/Individuals), where *N* is the number of clones that occurred only once; and (4) Diversity indices (Dominance, Evenness, Shannon, Simpson), calculated using the statistical program PAST (http://folk.uio.no/ohammer/past).

2.5. DNA sequencing and phylogenetic analysis

Representative clones showing unique RFLP patterns were selected for sequencing, and the sequencing was carried out on an ABI model 377 automated DNA sequence analyzer (Applied Biosystems, Perkin-Elmer) using sequencing primer 27F. All the nucleotide sequences were checked for putative chimeras by the RDP CHIMERA_CHECK (Maidak et al., 2001) and compared to known 16S rDNA sequences in the database by using the BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were performed using the neighbor-joining algorithm by the software ClustalX (Thompson et al., 1997) and phylogenetic tree of each group was constructed by the software MEGA3 (Kumar et al., 2004). Bootstrap values were obtained with 100 resamplings.

2.6. Nucleotide sequence accession number

Clone sequences have been deposited in GenBank under the accession numbers EU367113 to EU367148.

3. Results

3.1. PCR screening, pattern frequency and statistical analysis of the two bacterial 16S rDNA clone libraries

After screening for inserts by colony PCR with M13 primers, a total of 83 clones were subjected to RFLP analysis in which 39 clones were in the Jimei library and 44 were in the Dongshan library. Restriction analysis indicated that Jimei clone library contained 16 RFLP patterns, and Dongshan contained 24. Although the number of clones for RFLP analysis in Jimei (39 clones) was smaller than that of Dongshan (44 clones), the coverage value of 74% in Jimei was higher than the 68% in Dongshan, indicating that the sampling size in Jimei was more sufficient than that in Dongshan and further sampling would reveal more unique clones in Dongshan library.

Details on the frequency of RFLP patterns for different clones are shown in Fig. 1. The most abundant RFLP pattern in Jimei corresponds to 15 clones (38.5% in total clones) compared to seven clones in