Bacterial diversity in the intestine of sea cucumber *Stichopus japonicus*

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Abstract

The intestinal bacterial diversity of *Stichopus japonicus* was investigated using 16S ribosomal RNA gene (rDNA) clone library and Polymerase Chain Reaction/Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The clone library yielded a total of 188 clones, and these were sequenced and classified into 106 operational taxonomic units (OTUs) with sequence similarity ranging from 88 to 100%. The coverage of the library was 77.4%, with approximately 88.7% of the sequences affiliated to *Proteobacteria. Gammaproteobacteria* and *Vibrio* sp. were the predominant groups in the intestine of *S. japonicus*. Some bacteria such as *Legionella* sp., *Brachybacterium* sp., *Streptomyces* sp., *Propionigenium* sp. and *Psychrobacter* sp were first identified in the intestine of sea cucumber.

Keywords: Intestinal bacterial diversity, 16S rDNA, PCR-DGGE, Sequencing, *Stichopus japonicus*

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Introduction

Sea cucumber *Stichopus japonicus* is one of the most important holothurian species in coastal fisheries. The aquaculture of sea cucumber has rapidly developed in many Asian countries in recent decades (Conand, 2004). However, several factors increase the chance of infection for sea cucumber, such as high density breeding, germplasm degradation and water pollution. Intestinal microbiota is known to play a crucial role on the health status of aquatic animals (Liu *et al*., 2011). Microbes can influence the function of aquatic animals in various ways, including nutritional status, development and maintenance of the immune system, and defense against pathogens (Tannock, 1999). However, there have been only a few reports on the microbiota in the digestive tract of sea cucumbers.

Ward-Rainey *et al.* (1996) reported aerobic bacterial microbiota of *Holothuria atra*. Amaro *et al*. (2009, 2012) studied the bacterial community of an abyssal holothurian, *Molpadia musculus*, by using non-culturing methods. Recently, Enomoto *et al.* (2012) reported the culturable bacteria from the intestine of the Japanese spiky sea cucumber *Apostichopus japonicus.* Zhang *et al*. (2012, 2013) have isolated many varied aerobic culturable bacteria associated with *Holothuria leucospilota* and *A. japonicus*. However, neither culturable nor uncultrable bacterial microbial communities in the intestine of sea cucumber *S. japonicus* are fully understood.

In this paper, 16S rDNA clone library analysis approaches and PCR-DGGE fingerprinting of the 16S rDNA V3 regions were used to study the bacterial diversity in the intestine of *S. japonicus*. Our data provide an insight into the symbiosis between the intestinal bacteria and the holothurian sea cucumbers.

Materials and methods

Sampling and collecting of intestinal bacteria

Sea cucumber *S. japonicus* was captured from the coast of Dalian (Liaoning, China) during June 2011 to June 2012. Twenty healthy individuals were sampled each month. These sea cucumbers had an average weight of approximately 200 g and were about 3 years old. The body surface of these animals were sterilized for 30 s with 75% ethanol and then washed with sterilized distilled water. They were dissected and the all intestinal tracts were aseptically removed from the abdominal cavity and rinsed with sterilized distilled water on a super-clean bench. The intestinal tracts of 20 healthy individuals were sampled each month and used for each experiment. Six grams (wet weight) of the intestinal mixture was homogenized in a tissue grinder for 5 min followed by vigorous vortexing in 9 mL of sterile PBS buffer, and then centrifuged at 700×*g* for 5 min. The supernatant was transferred into a sterile tube and centrifuged at 10000×*g* for 1 min at 4°C. The supernatant was discarded and the remaining precipitate was stored at

−80°C until further processing.

DNA extraction and construction of 16S rDNA clone library

Total genomic DNA was isolated from the precipitated material using a bacterial genomic DNA Extraction Kit (TaKaRa Corporation Ltd., Dalian, China) and stored at −20°C until use.

To analyze the intestinal bacterial diversity of *S. japonicus*, nearly full-length 16S rDNA products were amplified with the universal primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGTTACCTTGTTACGACT T-3′). 16S rDNA clone library was constructed (Supplemental Materials). The V3 region of the 16S rRNA genes was amplified with Eubacteria-specific primers 341f (5’ -CCTACGGGAGGCAGCAG-3′) and 534r (5′-ATTACCGCGGCTGCTGG-3′). A GC-clamp (CGCCCGCCGCGCGCGGCGGGCG GGGCG GGGGCACGGGGGG) was applied to the 5′ end of the forward primer to increase the sensitivity of the DGGE analysis. The amplification condition was according to Liu *et al*. (2011).

PCR-DGGE analysis

The PCR-amplified 16S rRNA gene products (generated with 341f-GC/534r primer pair) were analyzed by DGGE performed with the Bio-Rad Dcode^{TM} mutation detection system (Bio-Rad, Hercules, CA, USA) according to Yang *et al*. (2007). Principle component analysis, based on the densities and migration of the bands, was performed using Quantity One (Version 4.5). The output data combined with MVSP V3.13 analysis was used to calculate the Shannon-Wiener index (H′) that was compared to the flora diversity of the samples.

Purified PCR products from the eluted DGGE were ligated into PMD19-T vector (TaKaRa Corporation Ltd., Dalian, China), and the ligation products were transformed into *Escherichia coli* JM 109 cells (TaKaRa Corporation Ltd., Dalian, China) according to the manufacturer’s instructions.

For each clone library generated from the eluted DGGE bands, positive clones that contained the inserts were chosen and cultured in 1 ml sterile SOC liquid media containing 100 μg/mL of Ampicillin. After incubating at 37°C for 3h, 1 μL of the liquid was used as a DNA template for PCR amplification with the RV-Mf and M13-47r primers. Samples that yielded the correct sizes of the inserted DNA fragments were subjected to DNA sequencing.

Results

As shown in the supplemental Table 1, the 106 operational taxonomic units (OTUs) included were found and used for subsequent phylogenetic analysis. The nearest relatives at the phylotype level were obtained using BLAST. The coverage of the library was approximately 77.4%.

Table 1: Closest relative as determined by a blast search, similarity to this relative and accession number for the major bands from the 16S rDNA V3 DGGE gels.

Band no.	Closest relative (obtained from BLAST search)	Identity (%)	Accession no.
1	*Vibrio gallaecicus* strain Col 42	98	GU194171.1
2	Uncultured *Shewanella* sp. isolate DGGE gel band B10	96	EU437395.1
3	Uncultured *Vibrio* sp. clone H8	99	GU211025.1
4	*Leisingera* sp. 34	100	JN797827.1
5	Uncultured *Alistipes* sp. clone EMP_U8	96	EU794272.1
6	Uncultured *Propionigenium* sp., clone: L-09-15	100	AB550455.1
7	Uncultured *Propionigenium* sp., clone: S-40-28	100	AB550454.1
8	Uncultured *Arcobacter* sp. clone ATB-KS-14134	99	JQ845788.1
9	*Vibrio ichthyoenteri* strain SF11070701B	100	JQ904784.1
10	*Vibrio* sp. GE170_2012_SAC	99	JX047592.1
11	*Pseudomonas* sp. An30H-SC-S	98	AB267465.1
12	Uncultured *Arcobacter* sp. clone ATB-KS-14134	99	JQ845788.1
13	*Pseudoalteromonas* sp. W19	97	DQ521087.1
14	Uncultured *Rhodobacteraceae* bacterium clone: No. 25	100	AB695103.1
15	*Vibrio* sp. Vb199 partial 16S rRNA gene, isolate Vb199	100	HE818177.1
16	*Ferrimonas* balearica strain KJ-W41	97	JQ799133.1

Eight major phylogenetic lineage was identified, including Gammaproteobacteria (76.4%), Alphapproteobacteria (6.6%), Firmicute (6.6%), Epsilonproteobacteria (5.7%), Actinobacteria (1.9%), Deltaproteobacteria (0.9%), Fusobacteria (0.9%) and Verrucomicrobia (0.9%). Approximately 88.7% sequences were affiliated with Proteobacteria, and Gammaproteobacteria was the predominant group.

Only four OTUs, such as *Fangia*, *Caedibacter*, *Holosporaceae* and *Candidatus Odyssella* could be identified at the family level. Seven OTUs including Gammaproteobacteria Epsilonproteobacteria, Epsilonproteobacteria, Epsilonproteobacteria, Alphaproteobacteria, Alphaproteobacteria and Deltaproteobacteria could be identified at the class level, while only one OUT (Verrucomicrobia) could be identified at the phylum level.

Our results showed that the bacteria represented by the library contained a diverse range of species. Among the different species identified, *Vibrio* sp. was the most abundant bacteria in the intestine of *S. japonicus*, followed by uncultured bacterium and *Pseudomonas* sp. (Fig. 1A). The V3 regions of the amplified 16S rDNA sequences were subjected to PCR-DGGE (Fig. 1B). The Shannon-Wiener index (H′) of the DGGE analysis was 2.18. In order to identify the bacterial species, 16 major bands in the DGGE profiles were eluted from the polyacrylamide gels and cloned, and three positive clones were sequenced for each band.

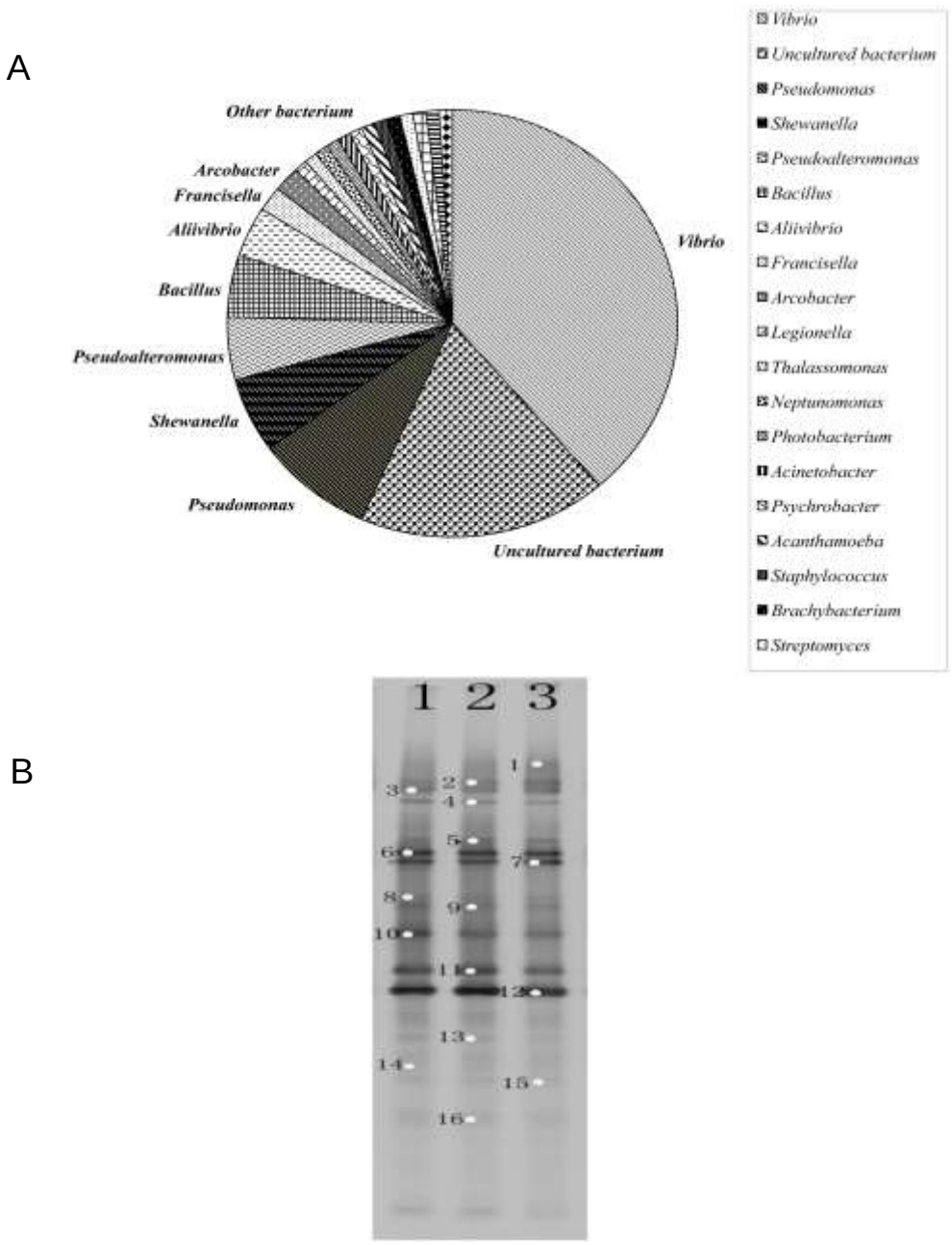

Figure 1: Distribution of the predominant genus in the intestinal microbial diversity of sea cucumber *Stichopus japonicus* by clone library analyses (A) and denaturing gradient gel electrophoresis (DGGE) analysis (B) (lane1, lane2, and lane3 are the same samples).

On comparison with other sequences available in the NCBI database, these sequences exhibited high similarity to the 16S rRNA gene sequences of several bacterial strains with similarities higher than or equal to 96% (Table 1). Overall, the results indicated that *Vibrio* sp., *Pseudomonas* sp., *Shewanella* sp., *Leisingera* sp., *Alistipes* sp., *Propionigenium* sp., *Arcobacter* sp., *Pseudoalteromonas* sp., *Ferrimonas* sp. and *Rhodobacteraceae* are the major groups of bacteria in the intestine of *S. japonicus*. Although DGGE profiles

and sequence analyses revealed different bacterial compositions, both methods identified *Vibrio* sp. as the predominant bacteria in the intestine of *S. japonicus.*

Discussion

Many molecular biology approaches have been used to study the diversity of microbial communities (Liu *et al*., 2010). As conventional culture-based techniques do not present a correct picture of the bacterial diversity in the intestine (Liu *et al*., 2011), we resorted to molecular methods by amplifying 16S rRNA genes form template derived from total DNA isolated from whole intestine samples and then analyzed the resulting sequences by PCR–DGGE and 16S rDNA clone library approaches to investigate the intestinal bacterial diversity of *S. japonicus.*

In our study, Gammaproteobacteria was the predomainat group (supplemental Table 1). This observation is consistent with the main populations of culturable bacteria in the intestine of *A. japonicus* (Enomoto *et al.,* 2012). Whereas, by molecular analysis of partial 16S rRNA gene sequences of 231 isolates from *A. japonicus*, species in the phylum Firmicutes were the predominant group (Zhang *et al*., 2013). Moreover, the members of phylum Bacteroidetes dominated the bacterial community of abyssal holothurian *M. musculus*, which was similar to that of the organic matter-rich sediments (Amaro *et al.,* 2009). These results suggest that bacterial diversity in the intestine of sea cucumber is varied in different sea areas.

Eighteen OTUs obtained from 16S rDNA clone library showed less than 97% sequence similarity to the existing 16S rRNA gene sequences via BLAST search of NCBI database, suggesting these bacterial species have not been characterized yet. Stackebrandt and Goebel (1994) indicated that the prokaryote in which 16S rDNA sequence differed by more than 3% from that of all other organisms of one group might be regarded as a new species and genus, respectively.

Although DGGE profiles and sequence analyses revealed different bacterial compositions, both methods identified *Vibrio* sp. as the predominant bacteria in the intestine of *S. japonicus.* These results were consistent with that of previous studies. Ward-Rainey *et al*. (1996) reported, that from 43 isolates of aerobic bacterial microbiota in *H. atra*, 24 were affiliated to the genera *Vibrio* and neighboring taxa. High diversity was observed in the genera *Bacillus* and *Vibrio* in the intestine of *H. leucospilota*, a common sea cucumber in Japanese warm waters (Zhang *et al*., 2012). However, as for culturable bacteria associated with *A. japonicus*, the genus *Bacillus*, *Oceanobacillus* and *Virgibaillus* dominated, but there were no isolates affiliated to members of the genus *Vibrio* (Zhang *et al*., 2013).

Our results indicated that *Vibrio* sp. and *Pseudomonas* sp. were the major groups of bacteria in the intestine of *S. japonicus*, which are the most

commonly reported genera of gut microflora in aquatic invertebrates (Harris, 1993). Among the OTUs identified as *Vibrio* sp., 12 OTUs belong to *Vibrio splendidus,* which is a common coastal planktonic bacterium (Nealson *et al*., 1993), and is usually predominant in the culturable bacterial communities (Urakawa *et al.,* 1999). Furthermore, some bacteria have not been characterized previously from the intestine of sea cucumber, such as *Legionella* sp., *Brachybacterium* sp., *Streptomyces* sp., *Propionigenium* sp. and *Psychrobacter* sp.

In conclusion, the intestinal bacterial diversity of *S. japonicus* was examined by 16S rDNA clone library and PCR-DGGE analyses. Gammaproteobacteria and *Vibrio* sp. are the predominant groups in the intestine of *S. japonicus*. Some bacteria such as *Legionella* sp., *Brachybacterium* sp., *Streptomyces* sp., *Propionigenium* sp. and *Psychrobacter* sp were first identified in the intestine of sea cucumber.

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