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Identification and classification of the principal microflora of the sea pineapple *Halocynthia roretzi* using MALDI biotyping and 16S rRNA analysis

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ABSTRACT: *Halocynthia roretzi* (phylum Cordata), also called 'sea pineapples', live in shallow coastal waters and typically feed on plankton and detritus that they filter from seawater. It has been reported that symbiotic microflora associated with *H. roretzi* act as protective agents that strengthen its immune system or control energy metabolism. This study analyzed the culturable microflora from the coelomic fluid of the sea pineapple using MALDI-biotyping and 16S rRNA sequencing, combining a recent technology with the conventional method of bacteria identification. The MALDI-biotyper enabled the classification of the symbiotic microflora into 5 groups based on the specific patterns of their mass spectrum. The 16S rRNA sequencing was then used to establish the identity of the dominant bacteria in 4 groups, later revealed as 2 groups of *Vibrio* spp., *Shewanella* spp. and *Bacillus* spp. MALDI-biotyping was applied for the identification of microorganisms directly from cultured agar, and, coupled with numerical taxonomic analyses, we determined the major microflora associated with *H. roretzi.*

KEY WORDS: *Halocynthia roretzi* · Microflora · MALDI-biotyper · 16S rRNA

INTRODUCTION

The sea pineapple *Halocynthia roretzi* belongs to the phylum Chordata, which is composed of sac-like marine invertebrate filter feeders, and is a popular edible ocean resource in Far East Asia (especially Korea and northern Japan). There has been considerable debate regarding the evolutionary origins of the chordates and of vertebrates, and also on the relationships between the first chordates and invertebrate deuterostomes (Dehal et al. 2002). Sea pineapples are marine animals that have characteristics falling between invertebrates (lacking backbone) and vertebrates (larval stage), thus they can potentially provide new clues about the origins of key vertebrate systems and structures as they possess a vertebrate-like gut, nervous system and immune system (Chiba et al. 2004). Sea pineapples have also been found to have bacterial symbionts similar to other marine invertebrates. They offer a more nutrient-rich environment than seawater and sediments, thus providing nourishment and a safe habitat for their symbionts.

Marine bacteria–invertebrate associations have been reported in marine annelids, e.g. in *Riftia pachyptila* (Cavanaugh et al. 1981, Bright & Sorgo 2003), *Olavius algarvensis* (Dubilier et al. 2001, Ruehland et al. 2008), sponges (Vacelet & Donadey 1977, Friedrich et al. 1999), molluscs (Kimbell & McFall-Ngai 2003, McFall-Ngai et al. 2010), clams (Fisher 1990, Newton et al.

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2007, Southward 2009) and mussels (Distel & Cavanaugh 1994, Duperron et al. 2009). The diversity of these associations is very broad, and there are still many questions about the origin and the exact relationship between the host and its symbionts.

This study analyzed the culturable aerobic bacteria from the coelomic fluid of sea pineapple using a MALDI-TOF MS (matrix-associated laser desorption ionization-time of flight mass spectrometry; Bruker Daltonics) biotyper (MALDI-biotyper) combined with 16S rRNA sequencing, thus pairing a new method of identifying bacteria with a classical method. The MALDI-biotyper was developed recently as a new method for rapid identification of bacteria and yeasts (De Bruyne et al. 2011) based on measures of abundant protein, making it an ideal method for measuring non-purified extracts and intact bacterial cells. The MALDI-biotyper also has the benefits of faster analysis time and cost effectiveness (Dhiman et al. 2011), making it an ideal candidate for bacteria identification. 16S rRNA sequencing is a widely used method for identifying bacterial isolates and examining phylogenic relationships between bacteria (Weisburg et al. 1991).

MATERIALS AND METHODS

Coelomic fluid collection and culture of bacteria

Coelomic fluid was collected from the stomach and intestines of sea pineapple following the method described in Cha et al. (2011) with some modification. Briefly, 10 sea pineapples were transferred to the laboratory on ice from Tongyoung, South Korea and the coelomic fluid was immediately collected from below the tunic matrix using 3 ml syringes without damaging the internal organs and avoiding contamination with seawater. The collected sample was diluted 10× with phosphate buffered saline (PBS, 3 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4, pH 7.4). The diluted coelomic fluids were then cultured in tryptone soya agar (TSA; Oxoid) and seawater agar (seawater, 750 ml; tap water, 250 ml; agar, 20 g; peptone, 10 g; beef extract, 10 g) at 25°C for 48 h. *Vibrio alginolyticus* ATCC 17749 and *V. harveyi* ATCC 14126 were used as reference strains.

MALDI-biotyper analysis

Protein extraction from the bacterial isolates was performed using formic acid as described by Kurokawa et al. (2013) with some modifications. Individual colonies were loaded into the MSP 96 target polished steel plate (Bruker Daltonik). After the samples dried, 1 µl matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was added onto the samples and allowed to crystallize with the samples.

Mass spectra were analyzed using a Microflex LT mass spectrometer (Bruker Daltonik) under the control of flexControl software (v.3.0, Bruker Daltonik). Positive ions were extruded with an accelerating voltage of 20 kV, and spectra were analyzed within a mass/charge (m/z) ratio of 2000 to 20 000 in the positive linear mode. Each spot was measured 3 times using a perfect flex control method (MBT_FC.par) and a perfect auto executes method (MBT_autox.axe). A single standard spectrum was generated from 20 spectra selected from 24 raw spectra with flexAnalysis 3.3 (Bruker Daltonik) and used for MSP creation. Logarithmic scores of 0 to 3.0 were assigned by the MALDI-Biotyper according to spectra peak matching patterns as follows: scores of 0 to 1.699 indicated no reliable identification; scores of 1.700 to 1.999 indicated probable genus identification; scores of 2.000 to 2.299 indicated secure genus identification and probable species identification; and scores of 2.300 to 3.000 indicated highly probable species identification.

Phylogenetic relationships based on 16S rRNA

The phylogenetic relationships between isolates were compared using the 16S rRNA gene as previously described (Chun & Goodfellow 1995). Bacterial genomic DNA was extracted using an AccuPrep® Genomic DNA extraction kit (Bioneer) according to the manufacturer's instructions. PCR was performed using a 20 µl reaction mixture containing 1 µl template DNA, 0.05 µM of universal primer (F27, R1494, Neilan et al. 1997, Weisburg et al. 1991), and the AccuPower PCR® premix (Bioneer). Amplification was performed using a C-1000TM thermo cycler (Bio-Rad). The PCR conditions consisted of an initial denaturation cycle of 96°C for 2 min; 30 cycles of denaturation step at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s; and a final extension step at 72°C for 5 min. The amplified PCR products were cloned into the pJET1.2 cloning vector (Fermentas) and the nucleotide sequences were determined by SolGent (Daejun, South Korea) using an ABI 3130xl Genetic analyzer (Life Technologies). A phylogenetic tree was constructed using the neigh-

bor-joining algorithm with the molecular evolutionary genetics analysis (MEGA) v.4.0 (Tamura et al. 2007) and 1000 replicates for bootstrap analysis.

RESULTS AND DISCUSSION

A total of 200 colonies were selected from the bacteria cultured from the coelomic fluid of sea pineapple and grown on TSA and sea water agar. After initial screening, a total of 30 dominant colonies were selected based on the MSP dendrogram. The MALDI-biotyper was able to classify the dominant isolates and 2 reference strains into 5 groups (referred to as M groups) based on the specific patterns of their mass spectrum (Fig. 1), including *Vibrio* spp. (*V. fortis*, *V. harbeyi*, *V. chagasii*, *V. alginolyticus* and *V. gigantis*) in M Group 4, and *Bacillus* spp. (*B. megaterium*, *B amyloiquefaciens*, *B. pumilus*) in M Group 3; however, complete identification of over half of the strains (19/32) could not be achieved using the MALDI-biotyper. Of these latter strains (marked as 'no reliable identification'), most belonged to the M Groups 1, 2 and 5, which may have been due to the unavailability of an appropriate database to allow proper identification. From the 16S rRNA sequencing, the identity of the dominant bacteria in the 4 groups (referred to as R groups) were identified as 2 different *Vibrio* spp. in R Group I and II, *Shwanella* spp. in R Group III and *Bacillus* spp. in R Group IV (Fig. 2). Although, colony numbers 14, 3 and 2 were not identified by MALDI-biotyper, 16S rRNA sequencing showed that they matched to *Vibrio* spp., *Bacillus* spp. and *Shewanella* spp., respectively (Table 1). These results suggest that *Vibrio* is the main genus of the bacterial community in sea pineapples, followed by *Bacillus* and *Shewanella*.

Members of the genus *Vibrio* are common marine bacteria which have been isolated from diverse marine niches, e.g. some of them are associated with toxic shellfish poisoning (Eastaugh & Shepherd 1989). However, horizontal transmissionof symbionts, such as those exhibited in the squid *Euprymna scolopes,* could have a very specific symbiont selection. Juvenile squid, through a mechanism called ciliary mucus currents, produces a mucus stream that expels or inhibits the colonization by nonsymbiotic bacteria while at the same time allowing the necessary bacteria to colonize. In this case, *V. fischeri* present in the seawater are trapped in the mucus and are allowed to eventually colonize the crypts or light organ (Ruby 1999, McFall-Ngai & Ruby 2000). Recently, some *Vibrio* species (*V. alinolyticus* and *V. harveyi*) commonly associated with bivalves have been observed to be influenced by environmental parameters such as salinity and temperature (Arias et al. 1999, Pujalte et al. 1999).

Bacillus spp. isolated from marine invertebrates including oyster, abalone and shrimp (Hernández-

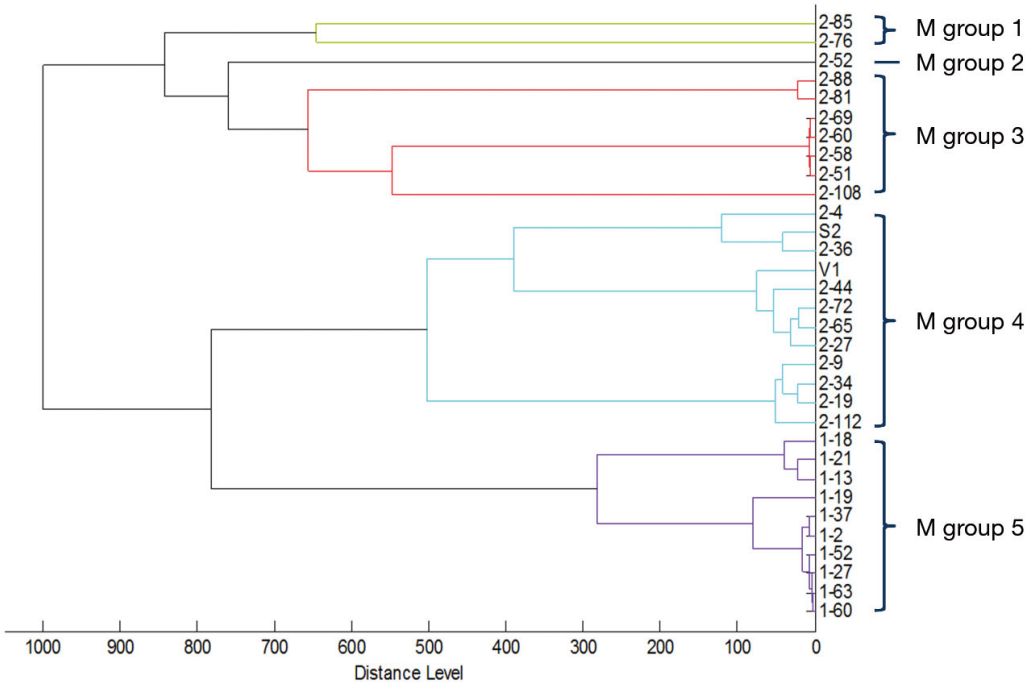

Fig. 1. Dendrogram analysis showing taxonomical clustering of 32 bacterial isolates from the MALDI-biotyper analysis (designated M groups). Distance level shows the phylogenic distance among strains

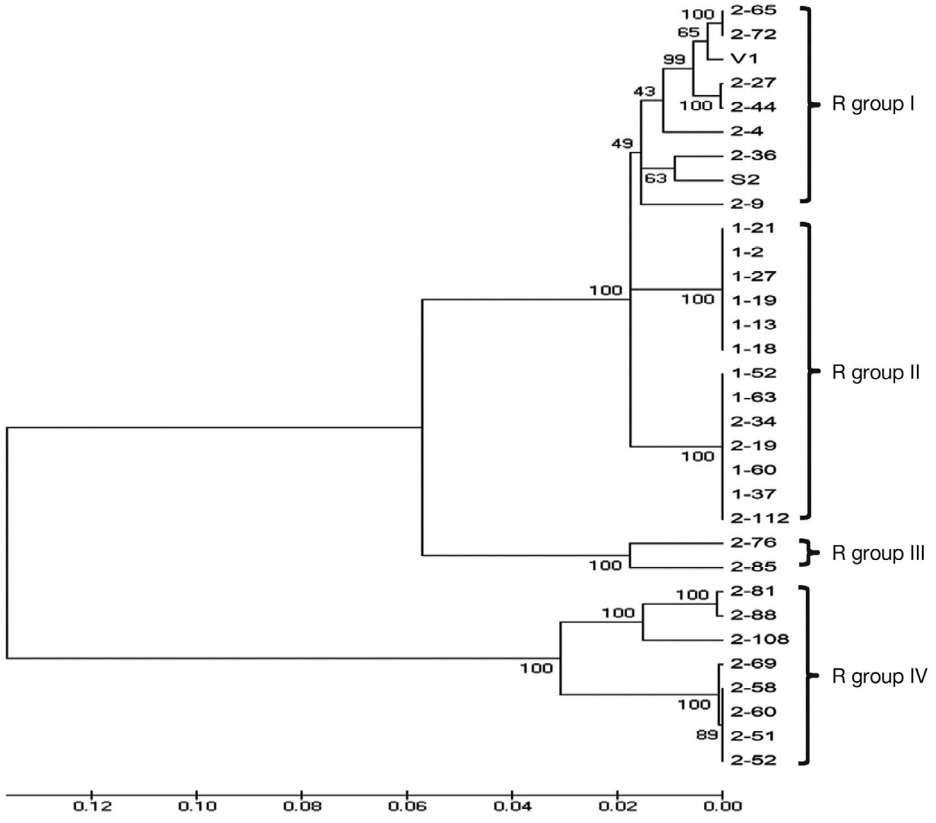

Fig. 2. Phylogenetic relationships of 32 bacterial isolates based on 16S rRNA gene sequences (designated R groups). The tree was calculated using the maximum-likelihood method with 1000 bootstrap replications. Scale bar indicates 0.12 substitutions per nucleotide position

Zárate & Olmos-Soto 2006, Ziaei-Nejad et al. 2006) presented valuable information in the aspect of biological purposes. For example, *Bacillus* used as a probiotic in shrimp were able to colonize both the culture water and the shrimp digestive tract, leading to increased shrimp survival by replacing *Vibrio* spp. in the gut of the shrimp (Rengpipat et al. 1998) and enhancing cellular and humoral responses (Rengpipat et al. 2000).

The genus *Shewanella* is widely distributed in nature, especially in marine environments (Bowman et al. 1997, Bozal et al. 2002). They are classified as either psychrophilic or halophilic, and can produce large amounts of eicosapentanoic acid (EPA), which is a critical component of the glycolipids and phospholipids that are known to induce larval settlement in sea urchins (Kitamura et al. 1993) and barnacles (Vogan et al.2003).

Taking the above into consideration, we conclude that the sea pineapple may have been colonized with bacterial symbionts—*Vibrio* spp., *Bacillus* spp., and *Shewanella* spp.—similar to squids, bivalve, shrimp and other marine organisms. The diversity of these microflora is broad and many questions still exist regarding the relationship between the host and symbiont and its origin. The results presented in this study suggest that the symbiotic bacteria of sea pineapple may affect its settlement or growth in various marine environments, although this aspect requires further study. The vast array of marine invertebrate symbioses remains largely unexplored, but the recent development of new molecular technologies, such as those employed in this study, proves promising (particularly for phylogenetic analysis and identification of novel culturable bacteria). In addition, our work further establishes the potential of MALDI-biotyper as a rapid and convenient method for the taxonomic classification of bacterial species.

Using the MALDI-biotyper and 16S rRNA sequencing, we were able to identify the major symbiotic microflora in the coelomic fluid of sea pineapples. Furthermore, the results obtained will help to further the understanding of the phylogenic and evolutionary aspects of the relationship between hosts and symbionts—particularly in marine organisms.

Table 1. The main bacterial species colonizing sea pineapple *Halocynthia roretzi* identified using MALDI-biotyper and 16S rRNA sequencing

No.	Strain	MALDI-biotyper	M group	16S rRNA (Accession no.)	R group
1	1-2	No reliable identification	5	*Vibrio* sp. V759 (DQ146990.1)	II
2	1-13	No reliable identification		*Vibrio* sp. V759 (DQ146990.1)	
3	1-18	No reliable identification		*Vibrio* sp. V759 (DQ146990.1)	
4	1-19	No reliable identification		*Vibrio* sp. V759 (DQ146990.1)	
5	1-21	No reliable identification		*Vibrio* sp. V759 (DQ146990.1)	
6	1-27	No reliable identification		*Vibrio* sp. V759 (DQ146990.1)	
7	1-37	No reliable identification		*Vibrio* sp. A9m (AB472064.1)	
8	1-52	No reliable identification		*Vibrio* sp. A9m (AB472064.1)	
9	1-60	No reliable identification		*Vibrio* sp. A9m (AB472064.1)	
10	1-63	No reliable identification		*Vibrio* sp. A9m (AB472064.1)	
11	2-19	No reliable identification	4	*Vibrio* sp. A9m (AB472064.1)	
12	2-34	No reliable identification		*Vibrio* sp. A9m (AB472064.1)	
13	2-112	No reliable identification		*Vibrio* sp. A9m (AB472064.1)	
14	2-4	*V. fortis*		*Vibrio* sp. BWDY-52 (DQ328953.1)	I
15	2-9	No reliable identification		*V. shilonii* strain SW-2 (AY911395.1)	
16	2-27	*V. harveyi*		*V. harveyi* AM11 (AB512470.1)	
17	2-36	*V. chagasii*		*Vibrio* sp. V140 (DQ146978.1)	
18	2-44	*V. harveyi*		*Vibrio* sp. V639 (DQ146989.1)	
19	2-65	*V. harveyi*		*V. harveyi* strain SW-3 (AY911396.1)	
20	2-72	*V. harveyi*		*V. harveyi* strain SW-3 (AY911396.1)	
21	V1 (ref)	*V. alginolyticus*		*V. alginolyticus* strain BPRIST053	
22	V2 (ref)	*V. harveyi*		*Vibrio* sp. BWDY-62	
23	2-51	*B. megaterium*	3	*B. megaterium* strain HDDMG02 (EU723818.1)	IV
24	2-58	*B. megaterium*		*B. megaterium* strain Jz11 (JF833087.1)	
25	2-60	*B. megaterium*		*B. megaterium* strain Jz11 (JF833087.1)	
26	2-69	No reliable identification		*B. megaterium* strain LAMA 262 (HM104232.1)	
27	2-81	No reliable identification		*B. polyfermenticus* strain bA8 (JF772465.1)	
28	2-88	*B. amyloliquefaciens*		*B. subtilis* strain ET (HQ266669.1)	
29	2-108	*B. pumilus*		*B. pumilus* strain BPT-18 (EF523475.1)	
30	2-52	No reliable identification	2	*B.megaterium* strain SB 3112 (GU191918.1)	
31	2-76	No reliable identification	1	*Shewanella* sp. NF1-13 (FJ196010.1)	III
32	2-85	No reliable identification		*Shewanella* sp. CGB9 (GU070668.1)	

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