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Full Paper

Physiological characterization of aerobic culturable bacteria in the intestine of the sea cucumber *Apostichopus japonicus*

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Various aerobic culturable bacteria (1,133 isolates) were isolated from the gut of *Apostichopus japonicus* (black adult, green adult, black small, green small, black juvenile, and green juvenile sea cucumbers) and from the sea sediment and the seawater using different culture conditions and without enrichment culture. By molecular analysis of partial 16S rRNA gene sequences of 231 isolates, they were tentatively affiliated with 53 described species in the phyla Firmicutes (42 species), Proteobacteria (9 species) and Actinobacteria (2 species). Eighteen species were often found among the intestines and the sea sediment. High diversity was observed in the genus *Bacillus* (20 species), *Oceanobacillus* and *Virgibaillus* but there were no isolates affiliated to members of the genus *Vibrio,* well-known sea pathogens. There were no clear differences in the bacterial communities among the hosts varied in size and color. Most isolates showed various polysaccharide degradation activities, suggesting their possible contributions in the digestion of organic matters in the gut.

Key Words——aerobic culturable bacteria; *Apostichopus japonicus*; diversity; Omura bay; polysaccharide degradation; sea cucumber; 16S rRNA gene

Introduction

Sea cucumbers are found in various sea environments like sea floors from the deep sea to intertidal areas (Foster and Hodgson, 1995; Uthicje et al., 2009). Sea cucumbers belong to the phylum Echinodermata and they mainly feed on detritus containing organic matter, microalgae, and bacteria (Massin, 1982; Moriarty, 1982; Yingst, 1976).

Gut microorganisms are important for digestion of diets, but the relationships between host sea cucumbers and their gut bacteria and bacterial functions are not still clear. Studies on bacteria associated with sea cucumbers were reported only for *Holothuria atra* and *Molpadia musculus* (Amaro et al., 2009; Ward-Rainey et al., 1996).

Ward-Rainey et al. reported preliminary research on aerobic bacterial flora of *Holothuria atra*. In their research, only 23 isolates were characterized by 16S rRNA gene sequence analysis (the first 300 nucleotides) and they were affiliated to the genera *Vibrio* and *Bacillus*. It was reported that the bacterial community of an abyssal holothurian, *Molpadia musculus* was analyzed using non-culturing methods (Amaro et al.,

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2009). Amaro et al. found that the gut bacterial composition was similar to that of the organic matter-rich sediments and members of the phylum Bacteroidetes dominated in the bacterial community (Amaro et al., 2009). They recently found that a substantial number of bacterial OTUs (Operational Taxonomic Unit) were associated uniquely with the gut contents and suggested the possibility of wide and highly diversified interactions between prokaryotes and deep-sea holothurians (Amaro et al., 2012). Recently Enomoto et al. reported that Gammaproteobacteria members containing *Vibrio* spp. were isolated as culturable bacteria from the intestine of *Apostichopus japonicus* (Enomoto et al., 2012). Using molecular techniques, they also found that Proteobacteria members were main metabolically active microbial populations in the intestine of *Apostichopus japonicus*.

In this paper, we have isolated many varied aerobic culturable bacteria associated with *Apostichopus japonicus* using different culture conditions and investigated their diversity and physiological characters including the tests for polysaccharide degradation ability of the isolates to understand the digestive symbiosis in sea cucumbers.

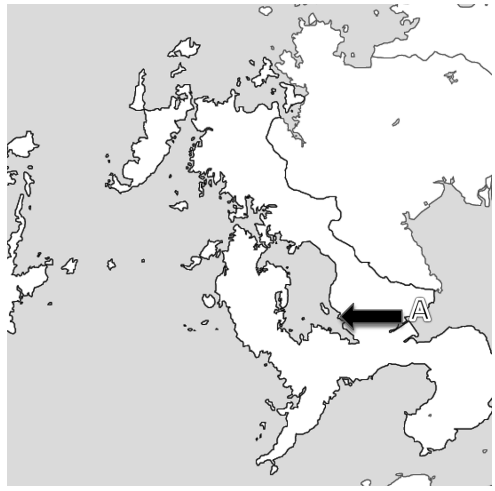

Fig. 1. Map of sampling site and environmental conditions. Sampling point: 32°53′N, 129°57′E (near Kushima, Omura Bay, Nagasaki, Japan), Sampling data: 2011.1.28, Surface water temperature: 7.4°C, Salinity of surface water 2.85%, Water depth of samples: 4 m, Temperature: 7.1°C, Salinity: 2.93%. Reference data—Open sea surface water temperature: 13–15°C (Data from Japan Meteorological Agency).

Materials and Methods

Sample collection. Six kinds of *Apostichopus japonicus* samples (black adult, green adult, black small, green small, black juvenile, and green juvenile sea cucumbers), the sea sediment and the seawater were collected at the coastal waters of Kushima, Omura, Nagasaki, Japan, on January 28, 2011 (Fig. 1). The genetic relationship between black and green types in *Apostichopus japonicus* was examined using 11 microsatellite markers and it was concluded that sympatric black and green types belonged to the same population (Kanno et al., 2006). The surface water temperature was 7.4°C and the salinity of surface water was 2.85% at the sampling point. Samples were collected at a water depth of 4 m. The temperature and the salinity of the water depth of 4 m were 7.1°C and 2.93%, respectively. The surface water temperature and 50 m depth water temperature of the open sea near the Nagasaki area were 13–15°C. (These data were obtained from Japan Meteorological Agency.)

The samples were immediately transferred and aseptically dissected in our laboratory. The whole intestine was excised from the animal body aseptically using sterilized instruments. The weights of whole intestines of *Apostichopus japonicus* samples (black adult, green adult, black small, green small, black juvenile, and green juvenile sea cucumbers) were 54.0 g, 39.4 g 5.5 g, 5.5 g, 1.4 g and 1.7 g, respectively. To isolate bacteria from both the intestinal wall and contents, the intact guts were crushed and mixed and the same weight of 3.5% saline solution was added to the mixtures. The gut suspensions thus obtained were used for isolation of bacteria. The same weight of 3.5% saline solution was added to the sediment sample and the mixture was used for isolation of bacteria. The seawater sample was used directly for isolation of bacteria. Fifty microliters of each sample was spread on a plate and the plates were aerobically incubated at 30°C for 2 weeks.

Growth media. Luria-Bertani medium (LB) and Horikoshi medium were used with slight modifications. Polysaccharides such as carboxymethyl cellulose sodium salt (CMC) (Wako Pure Chemicals, Osaka, Japan), xylan (Sigma), sodium alginate (Wako Pure Chemicals) and soluble starch (Nacalai Tesque, Kyoto, Japan) were added to Horikoshi medium as carbon sources (final concentration 1%).

LB solid medium (pH 7) contained 1% tryptone (Difco), 0.5% yeast extract (Difco), 3.5% NaCl, and 1.5% agar (Wako Pure Chemicals). Horikoshi solid medium

(pH 7) contained 1% polysaccharide, 0.5% peptone (BD), 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 3.5% NaCl, and 2% agar. Sodium alginate solid medium contained 2.5% agar. For 10% NaCl media, the NaCl concentration of growth media was 10% instead of 3.5%.

For alkaline agar plates, Na_2CO_3 (autoclaved separately) was added to neutral agar medium (final pH: pH 10.3–10.5). The Na_2CO_3 concentration of the alkaline plate was 1%.

Isolation of bacteria. In order to isolate various bacteria, the gut suspension was directly plated on agar plates without enrichment culture. High salt concentration or high pH were used for isolation conditions to isolate various bacteria because marine water is semi-alkaline and contains 3.5% NaCl. Seventeen different media were prepared by combination of pH, NaCl concentration and carbon source (Table S1). The plates were incubated at 30°C aerobically for 2 weeks to obtain slowly growing bacteria. Bacteria were isolated from each plate, purified and stored in slants for further analysis.

Physiological and biochemical characteristics of isolates. Polysaccharide degradation activities were detected by plate methods using starch (S), CMC, alginate (AL), xylan (XL) or agar as the substrate. The following plates were prepared for detection of enzyme activities.

1. Neutral agar plates

1–1. Amylase detection: Horikoshi agar medium containing 1% potato starch instead of soluble starch was used for amylase detection. The amylase-producing colony showed a turbid halo around the colony.

1–2. Cellulase detection: Basic neutral agar medium for cellulase detection contained 0.1% CMC, 3.7% marine broth, 0.6% $MgCl_2 \cdot 6H_2O$, 1.5% agar, 1.6% NaCl, 0.0015% congo-red, adjust pH to 7.0 with 1 M NaOH. A clear zone around a colony suggested cellulase activity.

1–3. Alginate lyase detection: The basic neutral agar medium for alginate lyase detection contained 1% sodium alginate, 3% NaCl, 0.07% KCl, 0.26% $MgSO_4$, 0.5% $MgCl_2$, 0.1% $CaSO_4$, 0.5% peptone, 0.01% ferric phosphate, 0.1% yeast extract, 2% agar; adjusted to pH 7.0 with 1 M NaOH. After 2 weeks' incubation at 30°C, 70% ethanol was added to the plates. A clear zone around the colony indicated the presence of alginate lyase.

1–4. Xylanase detection: Horikoshi agar medium containing 1% xylan was used for xylanase detection. The xylanase-producing colony showed a clear zone around the colony.

1–5. Agarase detection: Horikoshi agar medium without polysaccharide was used for agarase detection. The agarase-producing colony showed a dent around the colony.

2. Alkaline agar plates

For alkaline agar plates, Na_2CO_3 (autoclaved separately) was added to neutral agar medium (final pH: pH 10.3–10.5). The Na_2CO_3 concentration of the alkaline plate was 1%.

All isolates were tested for salt tolerance: 0%, 3.5%, 10%, 15%, 20%, and 25% NaCl (w/v); pH tolerance (pH 7 and pH 10); and effect of oxygen. Growth ability under various conditions of salinity or pH was measured at 30°C for 2 weeks. The isolates were divided into two groups by effect of pH on growth, neutrophilic bacteria (NE) that grew only at pH 7, and alkaliphilic bacteria (ALK) that grew both at pH 7 and pH 10.

Anaerobic growth was examined using a gaspak (COSMO BIO) at 30°C for 2 weeks, and then the growth condition was changed to the aerobic condition at 30°C for 2 weeks. The isolates were assigned to three groups, facultative anaerobic bacteria (FA), anaerobic tolerant bacteria (AT) and aerobic bacteria (A). Facultative anaerobic bacteria form colonies under both aerobic and anaerobic cultivation. Anaerobic tolerant bacteria do not form colonies under anaerobic condition for 2 weeks but form colony under aerobic cultivation after anaerobic cultivation. Aerobic bacteria do not form colonies under anaerobic conditions for 2 weeks and also do not form colony under aerobic cultivation after anaerobic cultivation.

Molecular identification of the isolates. Partial analysis of the 16S ribosomal RNA (rRNA) gene of the isolates was carried out. The 16S rRNA gene was amplified using bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) and the purified PCR product was sequenced with the dideoxynucleotide chain-termination method using a 3,130 or 3,730 DNA sequencer (Applied Biosystems). Primers 27F, 520R (5′-ACCGCGGCTGCTGGC-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) were used in gene sequencing reactions. Sequences of the partial 16S rRNA genes were assembled and edited using Sequencher (version 4.10.1 demo, Gene Codes Corporation) and MacVector (version 10.0.2). Nucleotide sequences of the partial 16S rRNA genes

have been submitted to GenBank/EMBL/DDBJ databases under accession numbers AB734817 through AB735047 (Table S2).

The partial 16S rRNA gene sequences were compared with other sequences in DDBJ database using the BLAST program and compared with type strain sequences in the Ribosomal database project (RDP). When an isolate showed ≥97% identity with a certain type strain, the isolate was assigned to the species. When an isolate showed <97% identity with all type strain sequences, the isolate was assigned to a tentative species.

Pairwise similarity values were calculated by using the Sørensen similarity index: $S=2ab/(a+b)$, where a and b are the number of species in any two categories and ab is the number of common species (Wolda, 1981). A similarity value of 1 indicates that species compositions are identical and a similarity value of 0 indicates that no species are shared.

Results

Isolation of bacteria

In order to isolate various bacteria, the gut suspensions from samples were directly plated on agar plates without enrichment culture and 17 isolation media were used (Table S1). The number of colony forming units (cfu)/g of gut suspension in sea cucumbers was 1.3×10^4 cfu/g to 2.7×10^4 cfu/g in Horikoshi medium (pH 7). The number of cfu/g of the sea sediment was 5.5×10^4 cfu/g in Horikoshi medium (pH 7) but the number of cfu/g of the seawater was 8.6×10^1 cfu/g in Horikoshi medium (pH 7). The viable counts of the sea sediment were similar to those of the gut suspensions, but there were very low viable counts in the seawater sample.

A total of 1,133 isolates were purified and analyzed regarding physiological characteristics. Among them, 231 isolates were analyzed phylogenetically using partial 16S rRNA gene sequences (Table S2).

Phylogenetic analysis of bacterial isolates

The partial 16S rRNA gene sequences were compared with type strain sequences in a database (Table 1, Table S2). Based on analysis of partial 16S rRNA gene sequences, 231 isolates from various samples were classified into 53 species with the criterion of 97% sequence identity with type strain species. The 53 species affiliated to the phyla Firmicutes (42 species), Proteobacteria (9 species), and Actinobacteria (2 species). Twelve genera of the phylum Firmicutes belonged to the families Bacillaceae 1 (*Bacillus*), Bacillaceae 2 (*Oceanobacillus, Virgibacillus, Gracilibacillus* and *Halobacillus*) and Planococcaceae (Lysinibacillus, *Planococcus* and *Sporosarcina*). The species of the genus *Bacillus* were mainly *Bacillus aryabhattai, Bacillus clausii, Bacillus hunanensis*, *Bacillus licheniformis, Bacillus marisflavi* and *Bacillus oshimensis.* The species of the genus *Oceanobacillus* were mainly *Oceanobacillus oncorhynchi* subsp*. incaldanensis* and *Oceanobacillus kimchii.* The species of the genus *Virgibacillus* were mainly *Virgibacillus dokdonensis* and *Virgibacillus halodenitrificans.*

The species of the phylum Proteobacteria mainly belonged to the genera *Pseudomonas*, *Psychrobacter, Halomonas* and *Pseudoalteromonas.* There were no isolates affiliated to members of the genus *Vibrio*. The species of the phylum Actinobacteria belonged to the genera *Nocardiopsis*, *Streptomyces* and *Williamsia.* The closest relatives of these isolates were observed in various locations including coastal environments, sea animals, and soil. A few strains were isolated from the seawater sample in this research and they belonged with genera *Psychrobacter*, *Pseudomonas* and *Williamsia* (actinobacteria).

Table 2 shows pairwise comparisons of species compositions between different size groups of the sea cucumber and between the sea cucumber and sea sediment as expressed by the Sørensen index. The highest value was observed between the adult and the small sea cucumber groups. Values of similarity index for the sea sediment increased as the body size of sea cucumber increased. The similarity index for comparison between the black and green groups was 0.568 and there was no clear difference between them.

Twenty-seven isolates (in Table S2) showed less than 97% identities with any type strain sequences, suggesting that these isolates were new species or new genera. Among the 27 isolates, we found 6 tentative species defined with ≥97% sequence identity (607–613 bp of partial 16S rRNA gene sequence). It is worth noting that almost all (26 out of 27) isolates were obtained from alkaline agar plates (pH 10.3–10.5) and 23 isolates were found on the plates containing 10% NaCl. Four tentative species (tentative species 1: isolates U0063, U0071, U0112, U0179, U0195, U0204, U0211, U0241, U0281, U378; tentative species 2: isolate U0217; tentative species 3: isolate U0557; tenta-

Table1. Phylogenetic affiliation for isolates (231 strains) from various specimens.

Phylum/class/family	Genus	Species /tentative species	Specimens								Number of specimens
			①	③	⑤	⑥	⑦	⑧	⑨	⑩	
phylum Firmicutes											
family Bacillaceae 1	*Bacillus* (20)	*Bacillus aerophilus/Bacillus altitudinis/Bacillus stratosphericus*[*]	+	+	+	+	+	+		+	7
		Bacillus amyloliquefaciens[*]	+	+						+	3
		Bacillus aquimaris	+			+					2
		Bacillus aryabhattai[*]		+	+	+	+	+		+	6
		Bacillus cereus						+			1
		Bacillus clarkii/Bacillus polygoni	+		+						2
		Bacillus clausii[*]	+	+	+	+	+	+		+	7
		Bacillus farraginis						+			1
		Bacillus firmus				+				+	2
		Bacillus gibsonii	+								1
		Bacillus horikoshii						+			1
		Bacillus hunanensis[*]	+	+	+	+	+	+		+	7
		Bacillus hunanensis/Bacillus lehensis		+	+						2
		Bacillus krulwichiae								+	1
		Bacillus licheniformis[*]	+	+	+	+	+	+		+	7
		Bacillus marisflavi[*]		+		+	+	+		+	5
		Bacillus methylotrophicus				+					1
		Bacillus okhensis/Bacillus wakoensis		+							1
		Bacillus okhensis/Bacillus krulwichiae					+				1
		Bacillus oshimensis[*]		+		+	+				3
		Bacillus polygoni						+			1
		Bacillus pseudofirmus		+	+						2
		Bacillus pumilus		+							1
		Bacillus pumilus/Bacillus safensis[*]		+		+				+	3
		Bacillus subtilis				+	+				2
		Bacillus vietnamensis			+						1
family Bacillaceae 2	*Filobacillus* (1)	*Filobacillus milensis*								+	1
family Bacillaceae 2	*Geomicrobium* (1)	*Geomicrobium halophilum*[*]	+	+		+	+	+		+	6
family Bacillaceae 2	*Gracilibacillus* (3)	*Gracilibacillus dipsosauri*	+								1
		Gracilibacillus halotolerans					+				1
		Gracilibacillus saliphilus	+								1
family Bacillaceae 2	*Halobacillus* (2)	*Halobacillus kuroshimensis*[*]	+	+	+						3
		Halobacillus trueperi				+					1
		Halobacillus yeomjeoni/Halobacillus trueperi/Halobacillus litoralis		+							1
family Bacillaceae 2	*Halolactibacillus* (1)	*Halolactibacillus alkaliphilus*			+						1
family Bacillaceae 2	*Oceanobacillus* (6)	*Oceanobacillus chironomi*					+				1
		Oceanobacillus oncorhynchi subsp. incaldanensis[*]		+	+		+	+		+	5
		Oceanobacillus kimchii[*]		+				+		+	3
		Oceanobacillus picturae		+						+	2
		Oceanobacillus profundus								+	1
		Oceanobacillus sojae				+					1
family Bacillaceae 2	*Salsuginibacillus* (1)	*Salsuginibacillus kocurii*	+				+				2
family Bacillaceae 2	*Virgibacillus* (4)	*Virgibacillus dokdonensis*[*]	+	+	+	+	+	+		+	7
		Virgibacillus chiguensis			+						1
		Virgibacillus halodenitrificans[*]	+	+	+	+	+	+			6
		Virgibacillus marismortui[*]		+	+	+					3
		Virgibacillus marismortui/Virgibacillus salarius[*]	+	+	+	+	+	+			6

Table1. Continued.

Phylum/class/family	Genus	Species /tentative species	Specimens								Number of specimens
			①	③	⑤	⑥	⑦	⑧	⑨	⑩	
family Planococcaceae	*Lysinibacillus* (1)	*Lysinibacillus fusiformis*					+	+			2
family Planococcaceae	*Planococcus* (1)	*Planococcus maritimus*								+	1
family Planococcaceae	*Sporosarcina* (1)	*Sporosarcina saromensis*		+							1
phylum Proteobacteria											
class alpha	*Pseudovibrio* (1)	*Pseudovibrio japonicus*						+			1
class gamma	*Ferrimonas* (1)	*Ferrimonas senticii*					+				1
class gamma	*Halomonas* (1)	*Halomonas meridiana*								+	1
class gamma	*Pseudomonas* (3)	*Pseudomonas cedrina* subsp. *fulgida*				+	+				2
		*Pseudomonas gessardii**		+					+	+	3
		Pseudomonas libaniensis								+	1
class gamma	*Pseudoalteromonas* (1)	*Pseudoalteromonas tetraodonis*						+			1
class gamma	*Psychrobacter* (2)	*Psychrobacter celer*							+		1
		Psychrobacter nivimaris							+		1
phylum Actinobacteria											
family Nocardiopsaceae	*Nocardiopsis* (1)	*Nocardiopsis lucentensis*		+							1
family Streptomycetaceae	*Streptomyces*	*Streptomyces gougerotii*/*Streptomyces rutgersensis*						+			1
family Williamsiaceae	*Williamsia* (1)	*Williamsia serinedens*							+		1
		Number of nearest type strain species	16	26	17	20	20	20	4	21	

①–⑧ indicated samples from black adult ①, green adult ③, black small ⑤, green small ⑥, black juvenile ⑦, and green juvenile ⑧ sea cucumbers, respectively. ⑨ and ⑩ indicated samples from seawater ⑨ and sea sediment ⑩, respectively. +indicated presence of the species. ( ) indicated number of the speies. * indicated the species found in more than 3 samples. Display of more than one species in the column of species indicated the same identity in the comparison range.

Table 2. Similarity indices for the different size groups of sea cucumbers (adult, small and juvenile) and the sea sediment.

Samples	Adult	Small	Juvenile	Sediment
Adult				
Small	0.633			
Juvenile	0.467	0.500		
Sediment	0.528	0.449	0.408	

tive species 4: U1120) were found only in the intestine of sea cucumbers and 2 tentative species (tentative species 5: isolates U0034, U0038, U0062, U0094, U0137, U0147, U0167, U0205, U0326, U0377; tentative species 6: isolates U0087, U0100, U0142, U0320) were found in both the intestine and the sea sediment.

Polysaccharide degradation ability of isolates

The 231 isolates from various samples showed various polysaccharides degradation ability and degraded one or more substrates (S, CMC, AL or XL). Twenty-seven, 14 and 14 species from the intestines showed amylase activity, cellulase activity and xylanase activity, respectively (Fig. 2, Table S2). There were no iso-

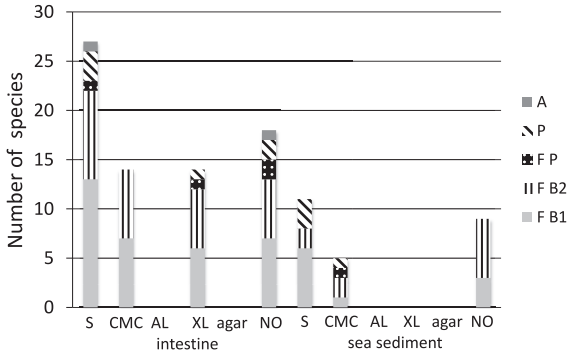

Fig. 2. Polysaccharide-degradation ability of isolates.

S, CMC, AL, XL, agar and NO indicate starch degradation activity, CMC degradation activity, alginate degradation activity, xylan degradation activity, agar degradation activity and no degradation activity of polysaccharides mentioned in this study, respectively. Gray box (FB1): the phylum Firmicutes, the family Bacillaceae 1, Vertical stripes (FB2): the phylum Firmicutes, the family Bacillaceae 2, Dotted box (FP): the phylum Firmicutes, the family Planococcaceae, Diagonal stripes (P): the phylum Proteobacteria, Black box (A): the phylum Actinobacteria. Fig. 2 was summarized from Table S2.

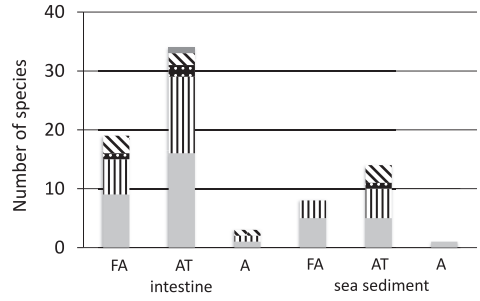

Fig. 3. Effect of oxygen on the growth of isolates.

Facultative anaerobic bacteria (FA) form colonies under both aerobic and anaerobic cultivation. Anaerobic-tolerant bacteria (AT) do not form colonies under anaerobic conditions for 2 weeks but form colonies under aerobic cultivation after anaerobic cultivation. Aerobic bacteria (A) do not form colonies under anaerobic conditions for 2 weeks and also do not form colonies under aerobic cultivation after anaerobic cultivation. Gray box (FB1): the phylum Firmicutes, the family Bacillaceae 1, Vertical stripes (FB2): the phylum Firmicutes, the family Bacillaceae 2, Dotted box (FP): the phylum Firmicutes, the family Planococcaceae, Diagonal stripes (P): the phylum Proteobacteria, Black box (A): the phylum Actinobacteria. Fig. 3 was summarized from Table S2.

lates showing alginate or agar degradation activities. On the other hand, 18 species from the intestines had no activity to degrade these polysaccharides. Most of the species showing various polysaccharide-degradation activities belonged to the families Bacillaceae 1 and 2 (Fig. 2). The bacterial diversity of polysaccharide-degrading isolates was almost identical among samples from the 6 kinds of sea cucumbers and the sea sediment except for xylan degradation activity. The species showing xylan degradation were detected in the intestines but few in the sediment (Fig. 2).

Amylase-producing isolates were mainly affiliated with the genus *Bacillus*, namely *Bacillus amyloliquefaciens, Bacillus aryabhattai, Bacillus clausii*, *Bacillus hunanensis*, *Bacillus licheniformis*, *Bacillus oshimensis* and *Bacillus subtilis*. The majority of cellulase-positive isolates were affiliated to *Virgibacillus dokdonensis*, *Bacillus hunanensis* and *Bacillus oshmensis*. The xylanase-positive isolates were mainly *Bacillus stratosphericus*/*Bacillus aerophilus*/*Bacillus altitudinis* group, *Bacillus pumilus*/*Bacillus safensis* group, *Bacillus subtilis*. The isolates of *Geomicrobium halophilum, Virgibacillus halodenitrificans* and *Virgibacillus marismortui* showed no polysaccharide-degradation ability.

Physiological characteristics of the isolates

Figure 3 and Table S2 shows the effect of anaerobic conditions for growth of the isolates. The 231 isolates from various samples were divided into three groups, facultative anaerobic bacteria (FA), anaerobic tolerant bacteria (AT) and aerobic bacteria (A). Diversity of FA, AT and A groups was similar between the intestines and the sea sediment and most of the isolates belonged to the families Bacillaceae 1 and 2 (Fig. 3). Facultative anaerobic isolates were mainly affiliated with *Virgibacillus dokdonensis*, *Bacillus licheniformis*, *Bacillus aerophilus*/*Bacillus altitudinis*/*Bacillus stratosphericus* group and *Oceanobacillus oncorhynchi* subsp*. incaldanensis*.

Anaerobic-tolerant isolates were mainly affiliated with *Bacillus clausii*, *Bacillus hunanensis*, *Bacillus oshimensis*, *Bacillus marisflavi*, *Geomicrobium halophilum*, *Virgibacillus halodenitrificans, Oceanobacillus kimuchii* and *Pseudomonas gesardii*. Aerobic isolates were affiliated with the genera *Bacillus, Halobacillus* and *Pseudomonas*.

Salinity tolerance of the isolates was examined (Table S2). Eleven isolates were halophilic (≧25% NaCl conc.) and belonged to the genera *Halobacillus, Virgibacillus* and *Oceanobacillus*. Most of isolates showing 20−25% NaCl tolerance belonged to the family Bacillaceae 2, such as the genera *Halobacillus, Virgibacillus* and *Oceanobacillus*. On the other hand, most isolates showing 10−15% NaCl tolerance belonged to the genera *Bacillus, Geomicrobium* and *Pseudomonas.* It

appears that the strains isolated from 3.5% NaCl plates showed 10−15 % salinity tolerance and the strains isolated from 10% NaCl plates showed 15−20% salinity tolerance (Table S2). The salinity tolerance of the isolates was similar among samples from the intestines and the sea sediment.

All isolates (231 strains) were examined for growth responses to pH shift (pH 7→pH 10 or pH 10→pH 7) (Table S2). All alkaliphilic strains isolated from alkali medium were able to grow at pH 7, and more than half of the isolates from pH 7 were able to grow at pH 10. All neutrophiles were mainly affiliated with the family Bacillaceae 1, such as *Bacillus amyloliquefaciens, Bacillus aryabhattai* and *Bacillus subtilis.* The isolates belonging to the family Bacillaceae 2 and more than half of the isolates belonging to the family Bacillaceae 1 were alkaliphiles.

Discussion

In this report, we isolated various aerobic culturable bacteria from the guts of *Apostichopus japonicus*. Analysis of partial 16S rRNA gene sequences of 231 isolates indicated that they were classified into 53 species in the families Bacillaceae 1 and 2 of the phylum Firmicutes, the class Gammaproteobacteria and the phylum Actinobacteria. High diversity was observed in the genus *Bacillus* (20 species), *Oceanobacillus* (6 species) and *Virgibaillus* (4 species). The isolated species were often observed in sea environments, sea animals and the Far East area. Most isolates showed salt-tolerance and alkaliphilic propertieis, suggesting that these isolates were derived from sea environment.

Microbial diversity was almost identical among the samples of adult, small, juvenile sea cucumbers and also among the samples of black and green sea cucumbers. Moreover, a substantial number of bacterial species were found to be common between the holothurians' gut and the sea sediment. In contrast to our culture-dependent method, Amaro et al. performed culture-independent methods and reported that the gut bacterial composition of the abyssal holothurian *Molpadia musculus* was similar to that of the organic matter-rich sediments (Amaro et al., 2009). Recently, they also found that ca. 82% of total bacterial OTUs were common between the gut contents and the surrounding sediments (Amaro et al., 2012).

Surprisingly, there were no isolates affiliated to members of the genus *Vibrio* among various samples of *Apostichopus japonicus*, the sea sediment or the seawater collected in this research. On the other hand, Enomoto et al. reported that Gammaproteobacteria members including *Vibrio* spp. were isolated as culturable bacteria from the intestine of *Apostichopus japonicus* (Enomoto et al., 2012). It was reported that the frequency and level of *Vibrio* species were much lower during winter than summer months (Chowdhury et al., 1990; Colwell, 1979). *Vibrio* species are well known pathogens for sea animals (Austin, 2010). The seawater temperature of the open sea near Nagasaki area was ca.15˚C in Jan. 2011 (Data from Japan Meteorological Agency). But Omura Bay is an inland bay and the seawater temperature was less than 10˚C in winter, ca. 5 degree lower than the open seawater temperature near Nagasaki. Omura Bay has been known as a production area of sea cucumbers since the Edo Era. Probably this low temperature in winter contributes to the production of healthy sea cucumbers in Omura Bay.

Detritus is a source of nutrient for detritus feeders and bacteria are the main decomposers that degrade these materials (Hagen et al., 2012). Therefore, we analyzed polysaccharide degradation of the isolates. Most isolates showed starch, CMC or xylan degradation abilities but few isolates were able to degrade alginate or agar. On the other hand, most isolates were facultative anaerobic bacteria or anaerobic-tolerant bacteria, indicating that most isolates were alive in the intestine of the sea cucumber. Although there has never been convincing evidence for intestinal environments of sea cucumbers, it is highly probable that oxygen will enter the intestine of the sea cucumber from the mouth with the detritus food and also some amount can penetrate from the body tissues. Our results suggested that the aerobic culturable isolates in this study potentially contribute to digest detritus and supply fermentation products (minor components and vitamins) to their host sea cucumber, although it is yet unclear whether aerobic isolates obtained in this study are permanent residents in the intestines or not.

High salt concentration or high pH were used for isolation conditions to isolate various bacteria because marine water is semi-alkaline and contains 3.5% NaCl. Most of the isolates showing 20−25% NaCl tolerance belonged to the family Bacillaceae 2, such as the genera *Halobacillus, Virgibacillus* and *Oceanobacillus*. Isolates belonging to the family Bacillaceae 2 and more than half of isolates belonging to the family Bac-

illaceae 1 were alkaliphiles. On the other hand, 27 isolates (Table S2) showed less than 97% identities with any type strain sequence. These isolates were classified into 6 groups with ≥97% sequence identity (607–613 bp of partial 16S rRNA gene sequence). Most of them were alkaliphiles obtained from plates at pH 10 and 10% NaCl. These results suggested that the intestines of holothurians are resources for new species.

Many isolates classified into the species mentioned below were reported to have denitrification ability: *Virgibacillus halodenitrificans* (Denariaz et al., 1989), *Oceanobacillus oncorhynchi* subsp*. incaldanensis* (Raats and Halpern, 2007), *Pseudomonas gessardii* (Verhille et al., 1999), *Pseudovibrio japonicus* (Hosoya and Yokota, 2007), *Gracilibacillus dipsosauri* (Lawson et al., 1996) and *Oceanobacillus chironomi* (Raats and Halpern, 2007). Probably these isolates play an important role to denitrify nitrate derived from human sewage from cities surrounding Omura Bay but the role in the intestine was not clear.

Although culture-dependent approaches limit our ability to quantify prokaryotic diversity in the holothurian gut, we believe that physiological examinations of isolated bacteria contribute to a further understanding of invertebrate-microbe interactions in combination with metagenomic approaches.

Supplementary Materials

Table S1. The number of the isolates obtained by different culture conditions.

Table S2. Diversity and physiological characteristics of isolates.

Supplementary tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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References

Amaro, T., Luna, G. M., Danovaro, R., Billett, D. S. M., and Cunha, M. R. (2012) High prokaryotic biodiversity associated with gut contents of the holothurian *Molpadia musculus* from the Nazaré Canyon (NE Atlantic). *Deep-Sea Res. I*, **63**, 82–90.

Amaro, T., Witte, H., Herndl, G. J., Cunha, M. R., and Billett, D. S. M. (2009) Deep-sea bacterial communities in sediments and guts of deposit-feeding holothurians in Portuguese canyons (NE Atlantic). *Deep-Sea Res. I*, **56**, 1834–1843.

Austin, B. (2010) Vibrios as causal agents of zoonoses. *Vet. Microbiol.*, **140**, 310–317.

Chowdhury, M. A. R., Yamahaka, H., Miyoshi, S., and Shinoda, S. (1990) Ecology and seasonal distribution of *Vibrio parahaemolyticus* in aquatic environment of a temperate region. *FEMS Microbiol. Ecol.,* **74**, 1–10.

Colwell, R. R. (1979) Human pathogens in the aquatic environment. *In* Aquatic Microbial Ecology, ed. by Colwell, R. R. and Foster, J., University of Maryland Sea Grant, College Park, pp. 337–344.

Denariaz, G., Payne, W. J., and Gall, J. L. (1989) A halophilic denitrifier, *Bacillus halodenitrificans* sp. nov. *Int. J. Syst. Bacteriol.,* **39**, 145–151.

Enomoto, M., Nakagawa, S., and Sawabe, T. (2012) Microbial communities associated with holothurians: Presence of unique bacteria in the coelomic fluid. *Microbes Environ.,* DOI: 10.1264/jsme2.ME12020.

Foster, G. G. and Hodgson, A. N. (1995) Annual reproductive cycles of three sympatric species of intertidal holothurians (Echinodermata) from the coast of the Eastern Cape Province of South Africa. *Invertebr. Reprod. Dev.,* **27**, 49–59.

Hagen, E. M., McCluney, K. E., Wyant, K. A., Soykan, C. U., Keller, A. C., Luttermoser, K. C., Holmes, E. J., Moore, J. C., and Sabo, J. L. (2012) A meta-analysis of the effects of detritus on primary producers and consumers in marine, freshwater, and terrestrial ecosystems. *Oikos*, DOI: 10.1111/j.1600-0706.2011.19666. x.

Hosoya, S. and Yokota, A. (2007) *Pseudovibrio japonicus* sp. nov., isolated from coastal seawater in Japan. *Int. J. Syst. Evol. Microbiol.,* **57**, 1952–1955.

Kanno, M., Suyama, Y., Li, Q., and Kijima, A. (2006) Microsatellite analysis of Japanese sea cucumber, *Stichopus (Apostichopus) japonicus*, supports reproductive isolation in color variants. *Mar. Biotechnol.*, **8**, 672–685.

Lawson, P. A., Deutch, C. E., and Collins, M. D. (1996) Phylogenetic characterization of a novel salt-tolerant *Bacillus* species: Description of *Bacillus dipsosauri* sp. nov. *J. Appl. Bacteriol.,* **81**, 109–112.

Massin, C. (1982) Food and feeding mechanisms, Holothuroidea. *In* Echinoderm Nutrition, ed. by Jangoux, M. and Lawrence, J. M., Balkema, Rotterdam, pp. 43–55.

Moriarty, D. J. W. (1982) Feeding of *Holothuria atra* and *Stichopus chloronotus* on bacteria, organic carbon and organic nitrogen in sediments of the Great Barrier Reef. *Aust. J. Mar. Freshwater Res.,* **33**, 255–263.

Raats, D. and Halpern, M. (2007) *Oceanobacillus chironomi* sp. nov., a halotolerant and facultatively alkaliphilic species

isolated from a chironomid egg mass. *Int. J. Syst. Evol. Microbiol.,* **57**, 255–259.

Uthicje, S., Schaffelke, B., and Byrne, M. (2009) A boom and bust phylum? Ecological and evolutionary consequences of density variations in echinoderms. *Ecol. Monogr.*, **79**, 3–24.

Verhille, S., Baida, N., Dabboussi, F., Hamze, M., Izard, D., and Leclerc, H. (1999) *Pseudomonas gessardii* sp. nov. and *Pseudomonas migulae* sp. nov.*,* two new species isolated from natural mineral waters. *Int. J. Syst. Evol. Microbiol.,* **49**, 1559–1572.

Ward-Rainey, N., Rainey, F. A., and Stackebrandt, E. (1996) A study of the bacterial flora associated with *Holothuria atra*. *J. Exp. Mar. Biol. Ecol*., **203**, 11–26.

Wolda, H. (1981) Similarity indices, sample size and diversity. *Oecologia (Berl)*, **50**, 296–302.

Yingst, J. Y. (1976) The utilisation of organic matter in shallow marine sediments by an epibenthic deposit-feeding Holothurian. *J. Exp. Mar. Biol. Ecol.*, **23**, 55–69.