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Full Paper

Diversity and function of aerobic culturable bacteria in the intestine of the sea cucumber *Holothuria leucospilota*

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Sea cucumbers play an important role in nutrient cycling of marine ecosystems by consuming sediments and moving sand, thus occupying a similar niche to earthworms in terrestrial ecosystems. However, our understanding of microbial diversity and functions associated with sea cucumbers is meager. Here, we isolated 141 bacterial strains under aerobic conditions using various media from the intestine of *Holothuria leucospilota*, a common sea cucumber in Japanese warm waters. By partial 16S rRNA gene sequences of the isolates, the isolates were tentatively affiliated with 55 described species. Among them, 23 species were common between 2 individuals of *H. leucospilota*. High diversity was observed in the genera *Bacillus* and *Vibrio*, which are often found in marine sediments, marine animals and other various environments. Most isolates showed various polysaccharide degradation activities and were able to grow under or were tolerant of anaerobic condition. We suggest that these aerobically isolated bacteria can play a role in digestion of detritus in aerobic and/or anaerobic regions of the intestine.

Key Words——aerobic culturable bacteria; diversity; function; *Holothuria leucospilota*; 16S rRNA gene; sea cucumber

Introduction

Holothurians (~1,430 species), or sea cucumbers are found on various sea floors from deep sea to intertidal areas (Foster and Hodgson, 1995; Kerr and Kim, 2001; Uthicke et al., 2009). Holothurians belong to the class Holothuroidae and their main diet is detritus such as organic matter, microalgae, and bacteria (Massin, 1982; Moriarty, 1982; Yingst, 1976), although there has never been a common understanding of how holothurians fulfill their dietary and energy requirements.

Gut bacteria potentially play an important role in digestion of diets. However, there have been only a few reports on the microbiota in the digestive tract of sea cucumbers (Amaro et al., 2009, 2012; Ward-Rainey et al., 1996). Ward-Rainey et al. reported aerobic bacterial microbiota of *Holothuria atra*. In their report, only 23 isolates were identified by 16S rRNA gene sequences analysis and they were affiliated to the genera *Vibrio* and *Bacillus*. Amaro et al. used non-culturing methods to analyze the bacterial community of an abyssal holothurian, *Molpadia musculus*. Their results suggested that the gut bacterial composition was similar to that of the organic matter-rich sediments. Members of the phylum Bacteroidetes dominated in the bacterial community (Amaro et al., 2009). Recently Amaro et al. also

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reported the occurrence of wide and highly diversified interactions between prokaryotes and deep-sea holothurians (Amaro et al., 2012). Enomoto et al. reported that Gammaproteobacteria members were mainly isolated as culturable bacteria from the intestine of the Japanese spiky sea cucumber *Apostichopus japonicus* (Enomoto et al., 2012). Using molecular techniques, they also found that Proteobacteria members were the main metabolically active microbial populations in the intestine of *A. japonicus*.

In this report, we investigated the biological diversity and function of bacteria in the intestine of *Holothuria leucospilota,* a common sea cucumber in Japanese warm waters. We isolated 141 bacterial strains under aerobic conditions using media differing in salt concentration, pH, and carbon sources, and tested tolerance to anoxic conditions and heat treatment. Our data provide an insight into the symbiosis between the gut bacteria and the holothurian sea cucumbers.

Materials and Methods

Sample collection. *H. leucospilota* is a large, black sea cucumber species which is found throughout the tropical and subtropical Indo-Pacific region and it is a common sea cucumber in the shallow waters of western Japan (Drumm and Loneragan, 2005; Matsuno and Ishida, 1961; Sloan, 1979). *H. leucospilota* (2 specimens) were collected from the coastal waters of Koe-cho, Nagasaki, Japan on April 24, 2009. Both samples were immediately transferred to our laboratory and the entire intestine was removed with sterile instruments. To isolate bacteria from both the intestinal wall and contents, the intact gut was crushed and mixed sufficiently and the gut suspension thus obtained was used for isolation of bacteria.

Growth media. Luria-Bertani medium (LB) and Horikoshi medium were used with slight modifications. LB solid medium (pH 7) contained 1% tryptone (Difco), 0.5% yeast extract (Difco), 3.5% NaCl, and 1.5% agar (Wako Pure Chemicals, Osaka, Japan). 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. As polysaccharides, 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. Horikoshi medium with sodium alginate as a polysaccharide contained 2.5% agar.

High salt concentration or high pH were used for isolation conditions to isolate various bacteria because marine water has a semi-alkaline pH and contains 3.5% NaCl. In total 13 different media were prepared by combination of pH (7 or ca. 10), NaCl concentration (3.5 or 10%) and polysaccharides (CMC, xylan, sodi-

Table 1. The number of the isolates obtained by different cultural conditions and their physiological characteristics.

pH	Medium	Salinity	Specimen		Subtotal	Degrading activities on polysaccharides					Requirement of oxygen			Maximum NaCl concentration for growth					pH tolerance	
			①	②		S	CMC	AL	XL	Agar	FA	AT	A	3.5%	10%	15%	20%	25%	NE	ALK
pH 7	LB	3.5%	11	10		18	–	9	1	2	18	2	1	11	9	–	1	–	12	9
	CMC	3.5%	10	7		10	–	4	–	–	12	2	3	4	11	2	–	–	3	14
	S	10%	2	3		1	–	–	–	–	2	2	1	–	1	2	1	1	–	5
	AL	10%	7	1		4	–	–	–	–	2	3	3	–	3	2	3	–	5	3
	XL	3.5%	16	14		15	–	8	5	1	18	8	4	13	13	3	1	–	11	19
		10%	5	5		5	–	2	1	–	–	8	2	–	3	5	–	2	7	3
	Subtotal		51	40	91	53	0	23	7	3	52	25	14	28	40	14	6	3	38	53
pH 10	CMC	3.5%	8	5		5	10	1	–	1	2	9	2	5	5	1	1	1	–	13
	S	3.5%	4	5		3	5	4	–	–	4	5	–	3	6	–	–	–	–	9
		10%	1	3		2	2	–	–	–	–	4	–	–	–	–	4	–	–	4
	AL	3.5%	6	6		8	10	6	1	1	–	10	2	5	6	1	–	–	–	12
		10%	4	0		2	2	3	–	–	–	2	2	–	–	4	–	–	–	4
	XL	3.5%	2	2		3	2	–	–	–	–	4	–	–	4	–	–	–	–	4
		10%	1	3		2	4	–	–	–	–	3	1	–	4	–	–	–	–	4
	Subtotal		26	24	50	25	35	14	1	2	6	37	7	13	25	6	5	1	0	50
	Total		77	64	141	78	35	37	8	5	58	62	21	41	65	20	11	4	38	103

Abbreviations: S, starch; CMC, carboxymethyl cellulose sodium salt; AL, alginate; XL, xylan; FA, facultative anaerobic bacteria; AT, anaerobic tolerant bacteria; A, aerobic bacteria; NE, neutrophilic bacteria; ALK, alkaliphilic bacteria.

um alginate or starch) (Table 1). To adjust pH for alkaline conditions, 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. The gut suspension (50 μl) was directly plated on agar plates. The plates were incubated at 30°C aerobically for 2 weeks to obtain slowly growing bacteria. Bacteria with different morphological colonies (e.g. colony size, shape and color) 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 CMC, xylan, alginate, starch or agar as the substrate. The following plates were prepared for detection of enzyme activities. NaCl concentration (3.5 or 10%) and pH (7 or 10.3–10.5) were adjusted to the same conditions in which each isolate originated.

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.

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

3. Cellulase detection: The agar medium for cellulase detection contained 0.1% CMC, 3.7% marine broth, 0.6% $MgCl_2 \cdot 6H_2O$, 1.5% agar and 0.0015% congo-red. A clear zone around the colony suggested cellulase activity.

4. Alginate lyase detection: The agar medium for alginate lyase detection contained 1% sodium alginate, 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 and 2% agar. 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.

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 at 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 at 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 a colony in both aerobic and anaerobic cultivation. Anaerobic tolerant bacteria do not form a colony in anaerobic conditions for 2 weeks but form a colony in aerobic cultivation after the anaerobic cultivation. Aerobic bacteria do not form a colony in anaerobic conditions for 2 weeks and also do not form a colony in aerobic cultivation after the anaerobic cultivation.

For thermal tolerance, all isolates were incubated on agar plates at 50°C for 72 h aerobically, and then the incubation condition was shifted down to 30°C for 2 weeks. Isolates that were able to form a colony on agar plates at 50°C for 72 h aerobically, were assigned as thermophiles (TO). Isolates that were not able to form a colony on agar plates at 50°C for 72 h aerobically but were able to form a colony at 30°C incubation after the 50°C incubation, were assigned as thermotolerant bacteria (TT). Isolates that were not able to form a colony on agar plates at 50°C for 72 h aerobically and also were not able to form a colony at 30°C incubation after the 50°C incubation, were assigned as non-thermal bacteria (N).

Molecular identification of the isolates. Partial analysis of 16S ribosomal RNA (rRNA) gene of the isolates was carried out. The 16S rRNA gene was amplified using bacterial primers 27F (5′-AGAGTTTGATCCTGGCT CAG-3′) and 1492R (5′-GGTTACCTT GTTACGACTT-3′) and the purified PCR product was sequenced with the dideoxynucleotide chain-termination method using a 3130 or 3730 DNA sequencer (Applied Biosystems). Primers 27F, 520R (5′-ACCGCGGCT GCTGGC-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 AB719059 through AB719199 (Table S1; see JGAM Web site).

The partial 16S rRNA gene sequences were compared with other sequences in the DDBJ database using the BLAST program and compared with type strain

Table 2. Phylogenetic affiliation for isolates from *Holothuria leucospilota* specimen ①.

Phylum/Class	Genus/Clade	Number of species	Species/tentative species	Number of isolates
Firmicutes	*Bacillus*	14	*aerophilus/stratosphericus*	1
			aquimaris	1
			clarkii	1
			*clausii**	1
			*horneckiae**	2
			*hunanensis**	1
			*lehensis**	11
			*marisflavi**	3
			megaterium	1
			murimartini	1
			*oshimensis**	1
			*patagoniensis**	2
			*plakortidis**	1
			pumilus	1
			*stratosphericus**	1
	Geomicrobium	1	*halophilum*	**3(2)**
	Gracilibacillus	2	*dipsosauri*	2
			ureilyticus	**2(1)**
	Halobacillus	2	*salinus*	1
			*trueperi**	1
	Oceanobacillus	2	*iheyensis*	1
			*profundus**	1
	Staphylococcus	1	*haemolyticus**	1
	Virgibacillus	1	*dokdonensis**	4
	Subtotal	23		45
Proteobacteria				
Alphaproteobacteria	*Ruegeria*	1	*lacuscaerulensis*	2
Gammaproteobacteria	*Halomonas*	1	*denitrificans*	1
	Photobacterium	1	*rosenbergii**	1
	Pseudoalteromonas	3	*tetraodonis*	1
			mariniglutinosa	1
			prydzensis	1
	Vibrio	13		
	harveyi clade	7)	*alginolyticus**	3
	harveyi clade		*alginolyticus/harvey/communis*	2
	harveyi clade		*azureus**	1
	harveyi clade		*communis**	3
	harveyi clade		*harveyi**	5
	harveyi clade		*owensii**	1
	harveyi clade		*rotiferianus**	1
	harveyi clade		*natriegens**	1
	orientalis clade	1)	*brasiliensis*	1
	halioticoli clade	1)	*ezurae*	1
	splendidus clade	3)	*gigantis**	1
	splendidus clade		*tasmaniensis*	1
	splendidus clade		*gallaecicus*	**1(1)**
		1)	*mediterranei**	1
	Subtotal	19		30
Actinobacteria	*Paraoerskovia*	1	*marina*	1
	Nocardiopsis	1	*salina*	1
	Subtotal	2		2
	Total	44		77

The boldface with brackets means the low identities (less than 97%).* indicates the species found in two specimens. The display of more than one species in the column of species indicates the isolates showed the same identity with more than one type strain species.

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 any type strain sequences, the isolate was assigned to the tentative species.

Results

Isolation of bacteria

The gut suspensions from two specimens were directly plated on agar plates and 13 isolation media were used. Table 1 summarizes a number of the isolates obtained under different cultural conditions. The total weights of intestine and intestinal contents were 24.3 g and 26.8 g for specimen ① and ②, respectively. The number of colony forming units (cfu) per g of gut suspension in specimen ① and ② were 3.1×10^4 cfu/g and 2.2×10^4 cfu/g in LB medium, respectively. Total cfu of gut suspension in specimen ① and ② were 7.5×10^5 cfu and 5.9×10^5 cfu in LB medium, respectively. Numbers of cfu varied in the range of 1.4×10^4 to 3.2×10^4 cfu/g in Horikoshi media (pH 7, 3.5% NaCl) and 1.6×10^2 to 1.2×10^3 cfu/g in the alkaline Horikoshi media (ca. pH 10.3, 3.5% NaCl). We obtained 77 isolates from specimen ① and 64 isolates from specimen ②. In total, 141 isolates were purified and analyzed further.

Phylogenic analysis of bacterial isolates

The partial 16S rRNA gene sequences of 141 isolates were determined and compared with the type strain sequences. Table 2 and Table 3 summarize the species/the tentative species of the isolates as determined via BLAST (Table S1; JGAM Web site). The isolates were tentatively affiliated with 55 described species in the phyla Firmicutes, Proteobacteria, and Actinobacteria (Tables 2 and 3). The isolates from specimen ① and ② were affiliated to 44 and 34 species, respectively. High diversity was observed in the genera *Bacillus* and *Vibrio* among all isolates of specimen ① and ②. In total, 23 species of isolates were found in both specimens; 9 species belonged with the genus *Bacillus* and another 9 species belonged with the genus *Vibrio*.

The isolates affiliated to *Bacillus lehensis* were frequently observed in specimen ① (11 isolates) and ② (9 isolates). These isolates originating from alkaline plates varied with polysaccharides and/or NaCl concentration. Among 20 isolates, 19 isolates showed more than 99% sequence identity with the type strain of *B. lehensis* and 14 isolates shared 100% sequence identity with each other. The isolates affiliated to *Vibrio harveyi* were another major group. We obtained 11 isolates affiliated to *V. harveyi* from specimen ① and ②. All these isolates showed more than 99% sequence identity with each other.

We also found that 7 isolates (isolate no. C079, C088, C093, C116, C117, C125 and C140) showed less than 97% identities with any type strain sequences. These isolates were assigned to the tentative species (strains marked with an asterisk in Table S1; see JGAM Web site). Among the 7 isolates, we found 4 tentative species defined with ≥97% sequence identity. The isolates classified into the same tentative species shared nearly 100% sequence identity with each other. The 4 tentative species shared 89–96% sequence identity with described species. It is worth noting that almost all (6 out of 7) isolates were obtained from alkaline agar plates (pH 10.3–10.5).

Polysaccharide degradation ability of isolates

Many isolates from both samples showed polysaccharide degradation ability and degraded one or more substrates (S, CMC, AL, XL or Agar). Among the 55 isolated species, 29, 12, 24, 5, and 4 species showed activities of amylase, cellulase, alginate lyase, xylanase and agarase, respectively (Fig.1, Table S1; see JGAM Web site).

Amylase-producing isolates were mainly affiliated with the genera *Vibrio* (12 species) and *Bacillus* (9 species). Most cellulase-producing isolates were affiliated to the genus *Bacillus* (8 species). Half of the isolates producing alginate-lyase were affiliated with the genus *Vibrio* (12 species), especially the harveyi and splendidus clades. Several isolates producing alginate-lyase were affiliated with the genus *Bacillus* (7 species). All isolates producing xylanase were most closely related to the genera *Bacillus* (4 species) and *Gracilibacillus*. A few agarase-producing isolates were closely related to the genera *Vibrio, Photobacterium* and *Shewanella*.

There were 5 species showing both amylase and cellulase activities, among them 4 species were affiliated with the genus *Bacillus.* We found 13 species showed both amylase and alginate lyase activities and most were affiliated with the genus *Vibrio.* On the other hand, 21 species had no polysaccharide-degradation

Table 3. Phylogenetic affiliation for isolates (64 strains) from *Holothuria leucospilota* specimen ②.

Phylum/Class	Genus/Clade	Number of species		Species/tentative species	Number of isolates
Firmicutes	*Bacillus*	14		*altitudinis*	1
				*clausii**	3
				gibsonii	2
				*horneckiae**	3
				*hunanensis**	**2(1)**
				hwajinpoensis	1
				*lehensis**	9
				*marisflavi**	1
				*oshimensis**	1
				*patagoniensis**	**2(1)**
				*plakortidis**	1
				polygoni	1
				vietnamensis	1
				*stratosphericus**	1
	Halobacillus	1		*trueperi**	1
	Oceanobacillus	1		*profundus**	1
	Sporosarcina	1		*ureae*	**1(1)**
	Staphylococcus	2		*haemolyticus**	1
				warneri	1
	Virgibacillus	1		*dokdonensis**	1
	Subtotal	20			35
Proteobacteria					
Gammaproteobacteria	*Photobacterium*	1		*rosenbergii**	2
	Shewanella	1		*gaetbuli*	1
	Vibrio	11			
	harveyi clade		8)	*alginolyticus**	2
	harveyi clade			*alginolyticus*/*harveyi*/*communis*	1
	harveyi clade			*azureus**	1
	harveyi clade			*communis**	2
	harveyi clade			*harveyi**	6
	harveyi clade			*natriegens**	1
	harveyi clade			*natriegens*/*alginolyticus*	1
	harveyi clade			*owensii**	2
	harveyi clade			*parahaemolyticus*	1
	harveyi clade			*rotiferianus**	2
	splendidus clade		1)	*gigantis*/*crassostreae*	1
				pomeroyi/*gigantis*	1
				*gigantis**	1
			1)	*mediterranei**	2
			1)	*neptunius*	1
	Subtotal	13			28
Actinobacteria	*Micrococcus*	1		*luteus*	1
	Subtotal	1			1
	Total	34			64

The boldface with brackets means the low identities (less than 97%). * indicates the species found in two specimens. The display of more than one species in the column of species indicates isolates showed the same identity with more than one type strain species.

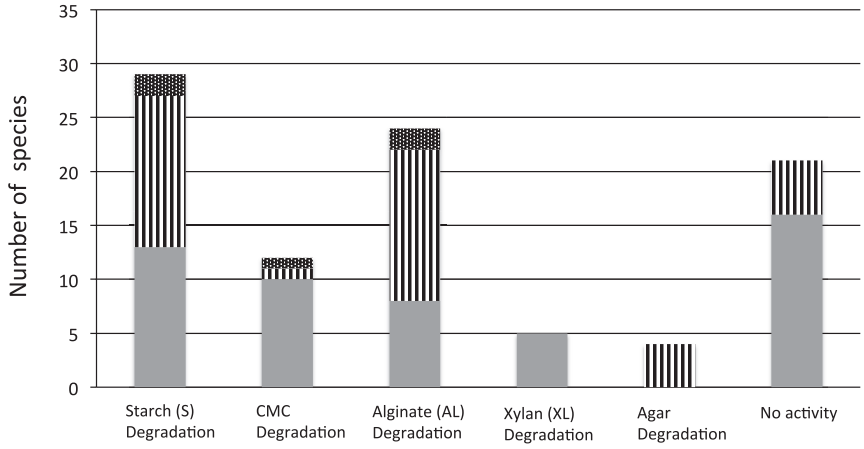

Fig. 1. Number of the species of isolates degrading various polysaccharides.

The species were divided into three groups: the phylum Firmicutes (black box), the phylum Proteobacteria (vertically striped box) and the phylum Actinobacteria (dotted box). Figure 1 summarizes Table S1 (see JGAM Web site).

abilities.

Physiological characteristics of the isolates

Figure 2 and Table S1 (JGAM Web site) show the effect of anaerobic conditions on the growth of the isolates. The isolates were divided into three groups, facultative anaerobic bacteria (FA), anaerobic tolerant bacteria (AT) and aerobic bacteria (A). The members of FA (23 species) were mainly affiliated with the phylum Proteobacteria and most of them belonged to the harveyi clade of the genus *Vibrio*. The members of AT (24 species) were affiliated to the genus *Bacillus* in the order Bacillales, the phylum Firmicutes.

The anaerobic cultivation for 2 weeks revealed that 17 species were aerobic bacteria. It appears that the average time for detritus to stay in the intestine of holothurian is several days. Therefore, all aerobic isolates (A species) were subject to anaerobic cultivation for 2 days. The aerobic strains did not form colonies in anaerobic conditions for 2 days, but they were able to form colonies in aerobic cultivation after the 2 days of anaerobic cultivation.

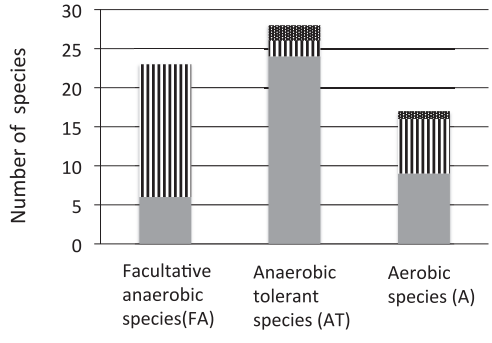

Fig. 2. Number of the species of isolates classified by effect of oxygen on growth.

The species were divided into three groups: the phylum Firmicutes (black box), the phylum Proteobacteria (vertically striped box) and the phylum Actinobacteria (dotted box). Figure 2 summarizes Table S1 (see JGAM Web site).

Salinity tolerance of the isolates was examined (Fig. 3, and Table S1; JGAM Web site). The isolates affiliated to *Halobacillus trueperi*, *Geomicrobium halophilum* and *Bacillus hwajinpoensis* showed the highest

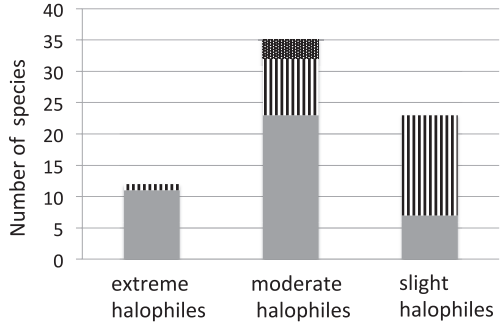

Fig. 3. Number of the species of isolates classified by effect of NaCl concentration on growth.

The species were divided into three groups: the phylum Firmicutes (black box), the phylum Proteobacteria (vertically striped box) and the phylum Actinobacteria (dotted box). Slight halophiles (SH): maximum salinity for growth is 3.5% NaCl, moderate halophiles (MH): maximum salinity for growth is 10–15% NaCl, extreme halophiles (EH): maximum salinity for growth is more than 20% NaCl. Figure 3 summarizes from Table S1 (see JGAM Web site).

tolerance (25% NaCl) and some strains were able to grow in the absence of NaCl. We found 12 species were halophilic (20–25% NaCl conc.) and among them, 11 species were affiliated to the genera *Bacillus, Halobacillus, Virgibacillus* and *Geomicrobium* in the phylum Firmicutes. We found 35 species were in the moderate halophilic (10–15% NaCl) group, mainly consisting of *Bacillus* (16 specise) and *Vibrio* (7 species). Slight halophiles (3.5% NaCl) were 23 species, which were affiliated to the genera *Vibrio*, *Photobacterium*, *Ruegeria*, *Pseudoalteromonas, Shewanella* and *Bacillus*. It appears that the species belonging to the phylum Firmicutes are more salt-tolerant than those belonging to the phylum Proteobacteria (Fig. 3).

All isolates were examined for growth responses to pH shift (pH 7→pH 10 or pH 10→pH 7). All alkaliphilic strains isolated from alkali medium were able to grow at pH 7, while only half of the strains isolated from pH 7 were able to grow at pH 10. Neutrophilic species (NE), defined as isolates growing only at pH 7 were 26 species, including 10 species affiliated with the genus *Vibrio*. Members of the alkaliphiles were 38 species affiliated with the phyla Firmicutes, Proteobacteria and Actinobacteria. The main genera of alkaliphiles were *Bacillus* and *Vibrio*.

Discussion

In this report, we isolated various aerobic bacteria from the gut of *H. leucospilota* using different culture conditions. By molecular identification using 16S rRNA gene sequences, the majority of isolates were affiliated to the phyla Firmicutes and Proteobacteria. High diversity of the species was observed in the genera *Bacillus* and *Vibrio*, which are often found in marine sediments, marine animals and other various environments. In total, 23 species of isolates were common in 2 individuals of *H. leucospilota* and 9 species belonged to the genus *Bacillus* while another 9 species belonged with the genus *Vibrio* (Tables 2 and 3). These results suggested that the intestine of holothurians was one of the suitable habitats for these bacteria.

Detritus is composed of organic materials, which are the nutrient source for detritus feeders (Hagen et al., 2012). The majority comprise recalcitrant polysaccharides, which in many cases can be degraded only by microorganisms. Therefore, we analyzed the polysaccharide degradation of the isolates. We found that many isolates showed various polysaccharide degradation activities. High diversity was observed in starch degradation isolates, suggesting the stock of starch in detritus, for example algae. The facultative anaerobic isolates were mainly affiliated to the genus *Vibrio* and they degraded starch, alginate and agar. The anaerobic-tolerant isolates were mainly affiliated to the genus *Bacillus* and they degraded starch, CMC and xylan.

We found that the isolates were divided into facultative anaerobic, anaerobic tolerant and aerobic bacteria by means of 2-week anaerobic cultivation. We also examined the effect of 2-day anaerobic conditions simulating the digestive process of holothurian and we found that the isolates classified into aerobic bacteria were able to form colonies in aerobic cultivation after the 2-day anaerobic conditions. These results suggested that the aerobic isolates were potentially tolerant for anaerobic conditions in the intestine of holothurians. On the other hand, all 62 anaerobic tolerant (AT) isolates were examined for heat tolerance because most of the AT isolates belonged to the phylum Firmicutes and had the ability to form spores. More than 60% of AT isolates lacked tolerance, suggesting that spore formation was not always the reason for anaerobic tolerance.

Oxygen will enter from the mouth with the detritus food and also some amount can penetrate from the body tissues. Some regions in the intestine can contain more or less oxygen, and these aerobic bacteria can play a role in the gut symbiotic system. Our results suggested that in the intestine, the majority of isolates could provide degrading enzymes and/or metabolites (fermentation products) useful for their host.

Recently, Amaro et al. also found that ca. 82% of total bacterial OTUs (Operational Taxonomic Units) were common between the gut contents and the surrounding sediments (Amaro et al., 2012). Figure 4 shows one model for the facultative symbiotic association among the host holothurian, the aerobic bacteria and the bacteria unique to the intestine of the holothurian. In marine ecosystems, aerobic bacterial degradation of detritus occurs in the seabed and the holothurian ingests the detritus with the aerobic bacteria. As mentioned above, the aerobic bacteria and the bacteria unique to the intestine produce metabolites useful for their host. Several days later, the detritus with the aerobic bacteria is excreted to the seabed again. The present study suggests the facultative symbiotic association among the host holothurian, the aerobic bacteria and the bacteria unique to the intestine.

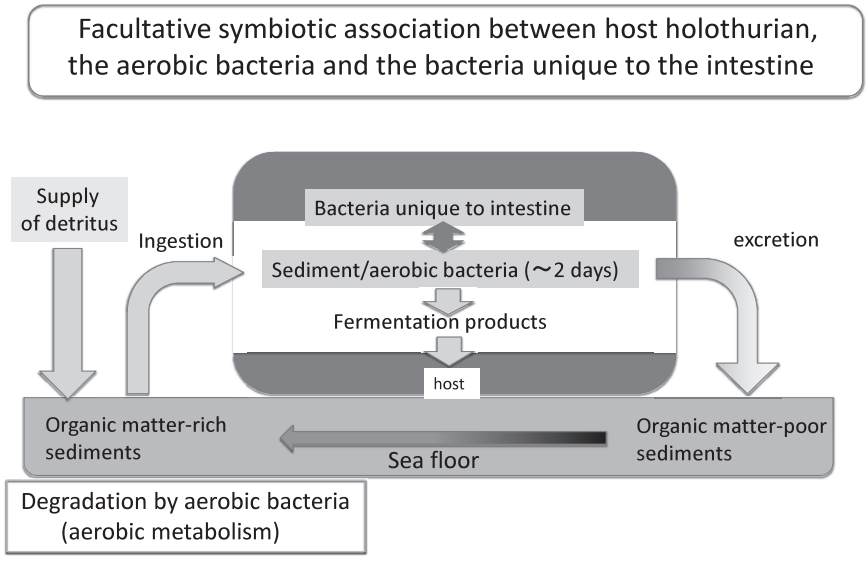

Fig. 4. Model for the facultative symbiotic association between host holothurian, aerobic bacteria and bacteria unique to the intestine of *H. leucospilota.*

We found 7 isolates showing less than 96% identities with any type strain sequences and 6 of these isolates were obtained from alkaline plates and 10% NaCl. These results suggested that the intestines of holothurians were new resources for new species.

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Supplementary Materials

Table S1. Diversity and physiological characteristics of isolates.

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

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