Chapter Ⅳ

Diversity and function of aerobic culturable bacteria in the intestine of deep-sea holothurian

4.1 Introduction

Deep-sea is one of unexplored regions even today. Holothurians (~1430 species) are found on various sea floors from deep sea floors to intertidal areas (Foster et al., 1995, Uthicke et al., 2009). Holothurians belong to phylum Echinodermata and their diet is detritus such as organic matter, microalgae, and bacteria (Massin, 1982, Moriarty, 1982, Yingst, 1976).

Gut bacteria play an important role in digestion of diets. Studies on bacteria associated with Holothurians were reported only for *Holothuria atra* (Ward-Rainey et al, 1996) and *Molpadia musculus* (Amaro et al., 2009, 2012). Ward-Rainey et al. reported partial aerobic bacterial flora of *Holothuria atra* (Ward-Rainey et al, 1996). In their report, only 23 isolates were characterized by 16S rRNA gene sequences analysis (the first 300 nucleotides) and they were affiliated to the genera *Vibrio* and *Bacillus*. On the other hands, Amaro et al. used non-culturing methods to analyze bacterial community of abyssal holothurian, *Molpadia musculus* (Amaro et al., 2009). Their results suggested that the gut bacterial composition was similar to that of the organic matter-rich sediments. Members of Cytophaga- Flavobacteria-Bacteroides (CFB) group dominated in the bacterial community (Amaro et al., 2009). Recently, they also found that ca. 82% of total bacterial OTUs (Operational Taxonomic Unit) were common between the gut contents and the surrounding sediments (Amaro et al., 2012). Enomoto et al. also reported recently that γ *-Proteobacteria* members were mainly isolated as culturable bacteria from the intestine of *Apostichopus japonicus* (Enomoto et al., 2012). Using the

molecular techniques, they also found that *Proteobacteria* members were main metabolically active microbial populations in the intestine of *Apostichopus japonicus*.

Gut microorganisms play an important role in digestion of diets, but the diversity and function of aerobic culturable bacteria in the intestine of the deep-sea holothurian are still unclear.

In this report, I isolated ninety-two aerobic culturable bacterial strains from each part of the intestine of the deep-sea holothurian collected at the southeast of Fukue Island, Nagasaki, Japan, water depth of 236 m and in November 2010. I found that the aerobic culturable isolates belonged to 45 nearest type strain species (below, referred to as species). The bacterial diversity was similar among three parts, i.e. anterior, mid and posterior parts of the intestine. Most isolates showed various polysaccharide degradation activities but few isolates showed alginate or agar degradation activities probably because there were no seaweeds in deep-sea. On the other hand, when I compared the functions and properties of several species in three parts, the posterior part was likely to be different from the anterior or mid parts. Maybe the posterior part was related to the digestion of polysaccharides or high salt environment.

4.2 Materials and Methords

Sample collection and dissection. Deep-sea holothurian specimen was collected at southeast of Fukue Island, Nagasaki, Japan (32°30'N, 129°09'E), at a water

depth of 236 m in November 21, 2010 (Fig.1). The temperature of seawater at the water depth of the sampling point was estimated to be ca.13-14 °C from data in Japan Meteorological Agency. The specimen was kept in icebox and aseptically dissected in our laboratory in November 24, 2010. Whole intestine was excised from the animal body aseptically using sterilized instruments. Fraction of intestine was carried out according to Shimizu et al (Shimizu et al., 1992). The intact intestine was divided into three parts, the anterior part (0.71g), the mid part (0.88g) and the posterior part (1.80g) (Fig. 2). To isolate bacteria from both intestinal wall and contents, 1 ml of saline was added to each part and each part was crushed and mixed enough. Each suspension thus obtained was used for isolation of bacteria and 50 μl of the each suspension was spread on plates.

Growth media. Luria-Bertani medium (LB) and Horikoshi medium were used basically. But NaCl concentration was 3.5% instead of 1%. Polysaccharides such as carboxymethyl cellulose sodium salt (CMC) (Wako pure chemicals, Osaka, Japan), xylan (Sigma), sodium alginate (Wako pure chemicals, Osaka, Japan) 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, 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. Sodium alginate solid medium contained 2.5% agar. For 10% NaCl media, 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: pH10.3-10.5). Na_2CO_3 concentration of alkaline plate was 1%.

Isolation of bacteria. Fifty μl of the gut suspensions 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 pH and contains 3.5% NaCl. Seventeen different media were prepared by combination of pH, NaCl concentration and carbon source (Table 1). The plates were incubated at 30 ℃ aerobically for two 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 CMC, xylan, alginate, starch or agar as 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. Amylase-producing colony showed turbid halo around a 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 1N NaOH. 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; adjust the pH to 7.0 with 1N NaOH. After two weeks' incubation at 30 °C, 70% ethanol was filled into 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. Xylanase-producing colony showed clear zone around a colony.

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

2. Alkaline agar plates

For alkaline agar plates, Na_2CO_3 (autoclaved separately) was added to neutral agar medium (final pH: pH10.3-10.5). Na_2CO_3 concentration of alkaline plate was 1%.

All isolates were tested for salt tolerance: 0%, 3.5%, 10%, 15%, 20%, 25% NaCl (w/v), pH tolerance (pH7 and pH10) and effect of oxygen.

Anaerobic growth was examined using gaspak (COSMO BIO) at 30 °C for two weeks, and then growth condition was changed to the aerobic condition at 30°C for two weeks. The isolates were assigned to three groups, facultative anaerobic bacteria (FA), anaerobic tolerant bacteria (AT) and aerobic bacteria (A). Facultative anaerobic bacteria form colony in both aerobic and anaerobic cultivation. Anaerobic tolerant bacteria do

not form colony in anaerobic condition for two weeks but form colony in aerobic cultivation after the anaerobic cultivation. Aerobic bacteria do not form colony in anaerobic condition for two weeks and also do not form colony in aerobic cultivation after the anaerobic cultivation. Growth ability at various conditions of salinity or pH was measured at 30 °C for two weeks. The isolates were divided into two groups by effect of pH on growth, neutrophilic bacteria (NE) that grew only at pH7, and alkaliphilic/alkali-tolerant bacteria (ALK) that grew both at pH 7 and pH10.

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'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') and the purified PCR product was sequenced with dideoxynucleotide chain-termination method using 3130 or 3730 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 AB741781 through AB741873 except AB741857 (Supplementary Table 1; see JJSE Web site).

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

Ribosomal database project (RDP). Each isolate was assigned to the nearest type strain species (Supplementary Table 1; see JJSE Web site). When isolate showed more than 97% identities with some type strain sequences, the isolate was assigned to the nearest type strain species (below, referred to as species). When isolate showed less than 97% identities with any type strain sequences, the isolate was assigned to the tentative nearest type strain species (below, referred to as tentative species).

4.3 Results

Isolation of bacteria

Fig.2 shows photographs of the deep-sea holothurian and its dissection (C), including anterior intestine (1), mid intestine (2), posterior intestine (3), Polian vesicle (4) and respiratory trees (5). But Cuvierian tubules were not detected in the specimen. The each intestine suspension was directly plated on agar plates and seventeen isolation media were used. Table 1 summarized number of the isolates obtained by different cultural conditions. The intact intestine was divided into three parts, the anterior part, the mid part and the posterior part. The each part was crushed and mixed enough and the suspensions thus obtained were used for isolation of bacteria. Number of colony forming units (cfu) per g of the anterior, mid and posterior parts were 1.4×10^4 cfu/g, 0.6×10^4 cfu/g and 0.58×10^4 cfu/g in LB medium, respectively. Similar cfu numbers were obtained in Horikoshi media (pH7), but lower cfu numbers in the alkaline Horikoshi media (ca. pH10.3).

Twenty-four, 33 and 35 isolates were obtained from the anterior, mid and posterior suspensions, respectively. In total, 92 isolates were purified and analyzed further.

Phylogenic analysis of bacterial isolates

The partial 16S rRNA gene sequences were done and compared with other sequences in DDBJ database using BLAST program and compared with the type strain sequences in Ribosomal database project (RDP). Table 2 summarized the species/the tentative species of the isolates as determined via BLAST (Supplementary Table 1; JJSE Web site). By partial 16S rRNA gene sequences of the isolates, the isolates belonged to 45 species. Fourteen species were detected in multiple locations of three parts, i.e. anterior, mid and posterior parts of the intestine. The isolates belonged to the phyla Firmicutes (33 species) and Proteobacteria (12 species) (Table 2). Among 33 species of the phylum Firmicutes, 21 species belonged to the family Bacillaceae 1, the genus *Bacillus*. Ten species belonged to the family Bacillaceae 2, the genera *Gracilibacillus*, *Halobacillus*, *Oceanobacillus*, *Thalassobacillus* and *Virgibacillus*. Twelve species of the phylum Proteobacteria belonged to the genera *Vibrio*, *Halomonas, Photobacterium, Pseudomonas* and *Marinobacter.* Among them, high diversity was found in the genera *Bacillus* and *Vibrio*. The closest relatives of these isolates were observed in various sea environments. The bacterial diversity was similar among three parts, i.e. anterior, mid and posterior parts of the intestine (Fig.3) and 14 species (indicated by star in Table 2) were detected in multiple parts of the intestine. But, the number of species belonged to

the family Bacillaceae 2 decreased in the posterior part of the intestine compared with those in the anterior or mid parts of the intestine (Fig.3).

Three isolates (isolate no. C214, C254 and C271) showed less than 97% identities with any type strain sequences. The isolates were assigned to the tentative species. (Supplementary Table 1; see JJSE Web site).

Polysaccharide degradation ability of isolates

Many isolates from the specimen showed polysaccharides degradation ability and degraded one or more substrates (S, CMC, AL and XL). Twenty-eight, 12, 3 and 12 species showed amylase activity, cellulase activity, alginate lyase activity and xylanase activity, respectively (Fig.4, Supplementary Table 1; see JJSE Web site). No agarase activity was observed in all isolates.

Amylase producing isolates were mainly affiliated with the genus *Bacillus* (15 tentative species). Twelve species such as *Bacillus horikoshii*, *B. hunanensis, B. licheniformis, B. megaterium, B. vietnamensis, Halobacillus kuroshimensis, H. trueperi, Photobacterium rosenbergii, Pseudomonas cedrina, P. libanensis, Vibrio pomeroyi,* and *V. rotiferianus* were found in multiple locations of three parts, i.e. anterior, mid and posterior parts of the intestine.

Most cellulase positive isolates belonged to the genera *Bacillus* (5 species) and *Vibrio* (4 species). Three species such as *Jeotgalibacillus campisalis, Vibrio pomeroyi* and *V. rotiferianus* were found in multiple locations. Only 3 isolates producing alginate-lyase were affiliated with *Gracilibacillus dipsosauri, Pseudomonas synxantha*

and *Vibrio agarivorans*. All isolates producing xylanase were most closely related to the genera *Bacillus* (9 species) and *Pseudomonas* (3 species). One species, *Pseudomonas libanensis* was found in multiple locations. Several species showed multiple polysaccharides degradation activities. On the other hand, 11 species had no polysaccharides degradation ability and 7 species of them in the anterior or mid parts of the intestine belonged to the family Bacillaceae 2 but the number of them decreased in the posterior part of the intestine (Fig.4). It was observed that in the posterior part, the number of xylan degrading species related to the family Bacilliaceae 1 increased and the number of starch degrading species related to the order γ -Proteobacteria also increased (Fig.4).

Physiological characteristics of the isolates

Fig.5 and Supplementary Table 1 (JJSE Web site) showed effect of anaerobic condition for growth of the isolates. The isolates were divided into three groups, facultative anaerobic bacteria (FA), anaerobic tolerant bacteria (AT) and aerobic bacteria (A). Twenty-five FA species were mainly affiliated with the phylum Proteobacteria and the family Bacillaceae 1. Six species such as *Bacillus licheniformis, Photobacterium rosenbergii, Pseudomonas cedrina, P. libanensis, Vibrio pomeroyi* and *V. rotiferianus* were found in multiple locations. Thirty AT species were mainly affiliated to the genera *Bacillus* (16 species), *Gracilibacillus, Halobacillus, Oceanobacillus* and *Virgibacillus*. Only one aerobic (A) species, *Halobacillus kuroshimensis* was found in multiple locations. The species belonging to the family

Bacillaceae 2 were mainly affiliated to AT group and species belonging to the family Bacillaceae 1 were mainly affiliated to FA or AT groups (Fig.5). The species belonging to the phylum Proteobacteria were mainly affiliated to FA group (Fig.5).

High salt concentration or high pH were used for isolation conditions to isolate various bacteria because marine water is semi-alkaline pH and contains 3.5% NaCl. Salinity tolerance of the isolates was examined (Fig.6, and Supplementary Table 1; JJSE Web site). The isolates most closely related to *Halobacillus kuroshimensis* and *Bacillus clausii* showed the highest salt tolerance (25 % NaCl) and some strains were able to grow in absent of NaCl. Twelve species were halophilic (20-25 % NaCl conc.) and mainly belonged to the family Bacillaceae 2, the genera *Halobacillus, Virgibacillus* and *Oceanobacillus*. Thirty-two species were moderate halophilic (10-15% NaCl) and 18 species belonged to the family Bacillaceae 1, the genus *Bacillus* and 9 species belonged to γ-proteobacteria such as the genera *Pseudomonas*, *Vibrio* and *Photobacterium*. Seven species were slight halophiles (3.5% NaCl) belonged to the genera *Vibrio*, *Photobacterium*, *Jeotgalibacillus* and *Bacillus*. It appears that the species belonging to the family Bacillaceae 2 are most salt-tolerant (more than 20% NaCl) and those belonging to the phylum Proteobacteria or the family Bacillaceae 1 were moderate halophilic (10-15% NaCl)(Fig.6). It was observed that number of species related to slight halophiles (3.5%) decreased in the posterior part compared with those in other parts.

All isolates were examined for growth responses to pH shift (pH7→pH10 or pH10→pH7). All alkaliphilic/alkali-tolerant strains isolated from alkali medium were

able to grow at pH 7, while half of species isolated from pH 7 were able to grow at pH 10. Nineteen species were the neutrophilic species (NE) growing only at pH7. Eleven species were affiliated with the genus *Bacillus*.

4.4 Discussion

In this report, I isolated aerobic culturable bacteria from each part of the gut of deep-sea holothurian using different culture conditions. The deep-sea holothurian was collected at southeast of Fukue Island, Nagasaki, Japan (32°30′ N, 129°09′ E), at a water depth of 236 m in November 21, 2010. Ninety-two aerobic culturable bacterial strains were isolated from each part of the intestine of deep-sea holothurian. By partial 16S rRNA gene sequences of the isolates, the isolates belonged to 45 species. The bacterial diversity was similar among three parts, i.e. anterior, mid and posterior parts of the intestine and 14 species were detected in multiple parts of the intestine. But, the number of species belonged to the family Bacillaceae 2 decreased in the posterior part of the intestine compared with those in the anterior or mid parts of the intestine. (I will discuss this later.)

As shown in Table2, the isolates belonged to the phyla Firmicutes (33 species) and Proteobacteria (12 species). Among 33 species of the phylum Firmicutes, 21 species belonged to the family Bacillaceae 1, the genus *Bacillus*. Recently, Enomoto et al. reported that Proteobacteria members were mainly isolated as culturable bacteria from the intestine of *Apostichopus japonicus* (Enomoto et al., 2012). These results suggested

that the sea environments such as deep sea or intertidal areas maybe affected diversity of aerobic culturable bacteria.

Detritus is organic materials and is used as a source of nutrient for detritus feeders (Hagen et al., 2012). Most important components of detritus are recalcitrant polysaccharides and bacteria are the main decomposers that degrade these materials. Therefore, I analyzed polysaccharide degradation of the isolates. I found that many isolates showed various polysaccharide degradation activities. High diversity was observed in starch degradation isolates, suggesting the large amount storage of starch in detritus, for example algae. But, there were few isolates showing alginate or agar degradation activities probably because deep-sea was not suitable area for seaweeds which contained a lot of alginate or agar. As mentioned in Fig.4, 11 species had no polysaccharides degradation ability and 7 species of them in the anterior or mid parts of the intestine belonged to the family Bacillaceae 2 but the number of them decreased in the posterior part of the intestine. It was observed that in the posterior part, the number of xylan degrading species related to the family Bacilliaceae 1 increased and the number of starch degrading species related to the order γ-Proteobacteria also increased (Fig.4). It was also observed that number of species related to slight halophiles (3.5%) decreased in the posterior part compared with those in other parts. These results suggested that the posterior part had a different role or environment compared with the anterior or mid parts, maybe the posterior part was involved in the digestion of polysaccharides.

I found that almost all isolates were facultative anaerobic bacteria or anaerobic

tolerant bacteria. It appears 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. These results suggested that the aerobic culturable isolates potentially contributed to digest detritus and supply metabolic products (minor components and vitamins) to their host sea cucumber.

Three isolates (isolate no. C214, C254 and C271) showed less than 96% identities with any type strain sequences and two of them were obtained from alkaline plates and 10% NaCl. These results suggested that the intestines of deep-sea holothurians were still new resource for new species.

The temperature of seawater at the water depth of the sampling point was estimated to be ca.13-14 °C in November 2010 from data of Japan Meteorological Agency. But, it was surprising that the full year temperature of the sampling point was estimated to be ca.12-15 °C throughout the year (from data in Japan Meteorological Agency). These results suggested that this deep-sea environment seemed a little low temperature but more suitable environment for deep-sea holothurian and their intestinal bacteria.

In this study, only one specimen, deep-sea holothurian was obtained and investigated. Therefore, future challenges remain regarding individual variations, morphological descriptions, haplotypes of host species and surrounding environments including sediments.

4.5 Summary

Ninety-two aerobic culturable bacterial strains were isolated from each part of the intestine of the deep-sea holothurian collected at 32°30' N, 129°09' E (southeast of Fukue Island, Nagasaki, Japan) and water depth of 236 m in November 2010. The temperature of seawater at the water depth of the sampling point was estimated to be ca.13-14 °C from data in Japan Meteorological Agency. By partial 16S rRNA gene sequences of the isolates, the isolates belonged to 45 nearest type strain species (below, referred to as species). High diversity was observed in the genera *Bacillus* (21 species) and *Vibrio* (6 species). The bacterial diversity was similar among three parts, i.e. anterior, mid and posterior parts of the intestine and 14 species were detected in multiple parts of the intestine. Most isolates showed various polysaccharide degradation activities but few isolates showed alginate or agar degradation activities probably because there were no seaweeds containing alginate or agar in this deep-sea. Comparing the functions and properties of several species in three parts, the posterior part was likely to be different from the anterior or mid parts.

Table1 Number of the isolates obtained by differrent culture conditions

pH	Medium	Sanility	part of the intestine			subtal
			anterior	mid	posterior	
pH 7	LB	3.5%	–	6	–	
	CMC	3.5%	1	3	–	
		10%	2	2	4	
	S	3.5%	2	3	1	
		10%	–	1	3	
	AL	3.5%	1	1	1	
		10%	4	2	7	
	XL	3.5%	2	3	1	
		10%	3	3	2	
subtotal			15	24	19	58
pH 10	CMC	3.5%	1	5	2	
		10%	–	–	2	
	S	3.5%	3	2	5	
		10%	–	–	–	
	AL	3.5%	2	–	1	
		10%	1	–	–	
	XL	3.5%	1	2	4	
		10%	1	–	2	
subtotal			9	9	16	34
Total			24	33	35	92

Table2 Phylogenetic affiliation for isolates (92 strains) from various parts of intestine

phylum/class/family	genus	species /tentive species	part of the intestine		
			anterior	mid	posterior
phylum Firmicutes					
family Bacillaceae 1	Bacillus (21)	*Bacillus aerophilus*			+
		Bacillus aerophilus/Bacillus altitudinis	+		
		Bacillus altitudinis/Bacillus stratosphericus	+		
		Bacillus aquimaris			+
		Bacillus aryabhattai		+	
		Bacillus aurantiacus			+
		Bacillus clarkii/Bacillus polygoni		+	
		Bacillus clausii		+	
		Bacillus flexus	+		
		Bacillus hemicellulosilyticus			+
		*Bacillus horikoshii**	+		+
		Bacillus horti		+	
		*Bacillus hunanensis**	+		+
		Bacillus hunanensis/Bacillus oshimensis			+
		Bacillus hwajinpoensis			+
		Bacillus lehensis		+	
		*Bacillus licheniformis**		+	+
		Bacillus marisflavi		+	
		*Bacillus megaterium**	+	+	
		Bacillus neizhouensis	+		
		*Bacillus oshimensis**	+		+
		Bacillus pseudalcaliphilus			+
		Bacillus pumilus		+	
		*Bacillus vietnamensis**	+		+
		Bacillus wakoensis			+
family Bacillaceae 2	*Gracilibacillus* (1)	*Gracilibacillus dipsosauri*	+		
	Halobacillus (2)	*Halobacillus kuroshimensis**	+	+	+
		*Halobacillus trueperi**		+	+
	Oceanobacillus (3)	*Oceanobacillus kimchii*		+	
		Oceanobacillus oncorhynchi	+		
		Oceanobacillus sojae	+		
	Thalassobacillus (1)	*Thalassobacillus devorans*	+		
	Virgibacillus (3)	*Virgibacillus dokdonensis*	+		
		*Virgibacillus halodenitrificans**		+	+
		Virgibacillus marismortui		+	
family Planococcaceae	*Jeotgalibacillus* (1)	*Jeotgalibacillus campisalis**	+		+
family Staphylococcaceae	*Staphylococcus* (1)	*Staphylococcus warneri*		+	
phylum Proteobacteria					
class gamma	*Halomonas* (1)	*Halomonas meridiana*			+
	Marinobacter (1)	*Marinobacter alkaliphilus*			+
	Photobacterium (2)	*Photobacterium lutimaris*	+		
		*Photobacterium rosenbergii**		+	+
	Pseudomonas (3)	*Pseudomonas cedrina**		+	+
		*Pseudomonas libanensis**	+	+	+
		Pseudomonas synxantha		+	
	Vibrio (5)	*Vibrio agarivorans*			+
		Vibrio harveyi		+	
		Vibrio mediterranei	+		
		*Vibrio pomeroyi**	+	+	
		*Vibrio rotiferianus**	+		+
	45 species				
	* indicated the species/tentive species found in multiple parts of the intestine				

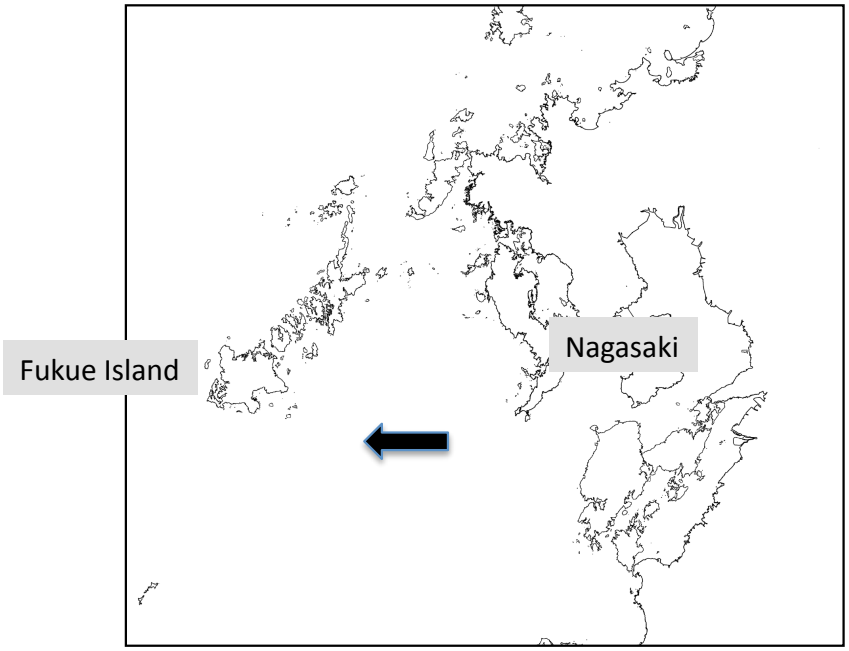

Fig.1 Map of sampling site (arrow) and environmental conditions.
Sampling point : 32°30′ N, 129°09′ E
(Southeast of Fukue Island, Nagasaki, Japan)
Water depth: 236 m
Sampling data: November 21, 2010

Temperature of water depth 200 m : ca.13-14 ℃
Sea surface temperature: ca. 21-22 ℃
（Data from Japan Meteorological Agency)

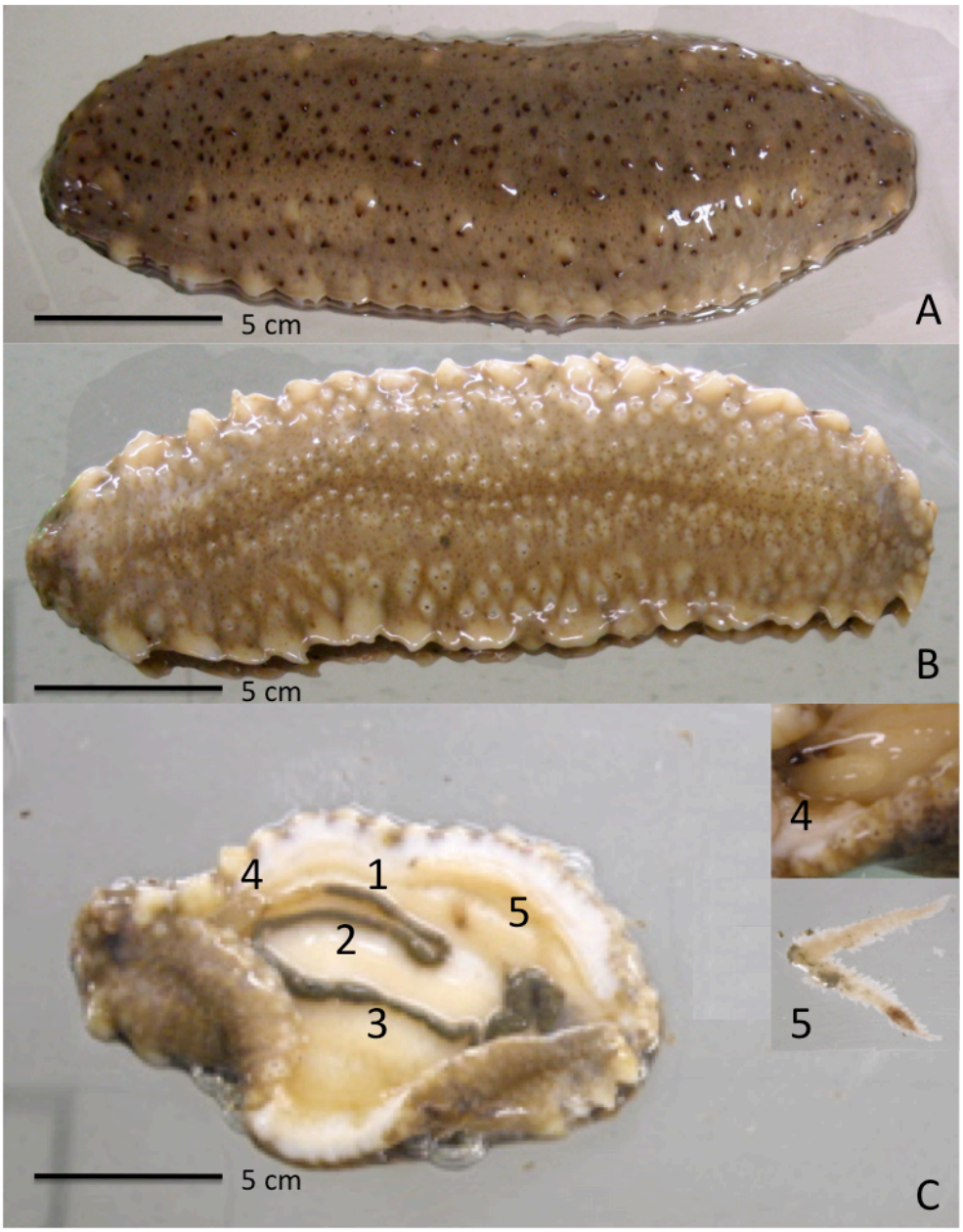

Fig. 2 . Photograph of the deep-sea holothurian (dorsal side A, ventral side face B) and it dissection (C), including anterior intestine (1), mid intestine (2), posterior intestine (3), Polian vesicle (4) and respiratory trees (5). Cuvierian tubules were not detected in the sample.

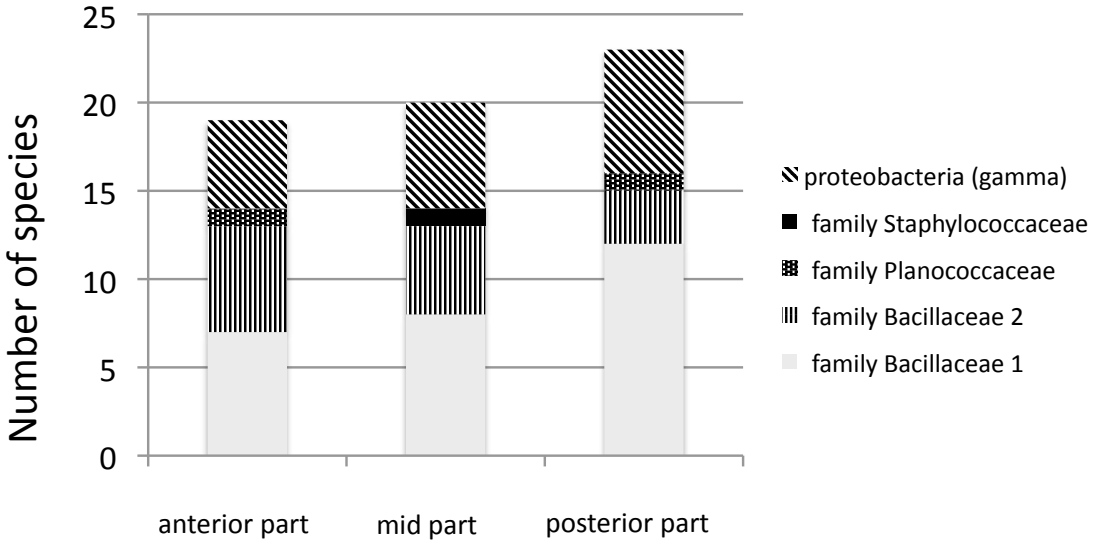

Fig.3 Number of the species of isolates in each part of the intestine
The species were divided into five groups: the family Bacillaceae 1 (gray box), the family Bacillaceae 2 (vertical stripes), the family Planococcaceae (dotted box), the family Staphylococcaceae (black box) and the order γ-Proteobacteria (diagonal stripes box). Fig.3 was summarized from Supplementary Tables 1 (JJSE web site).

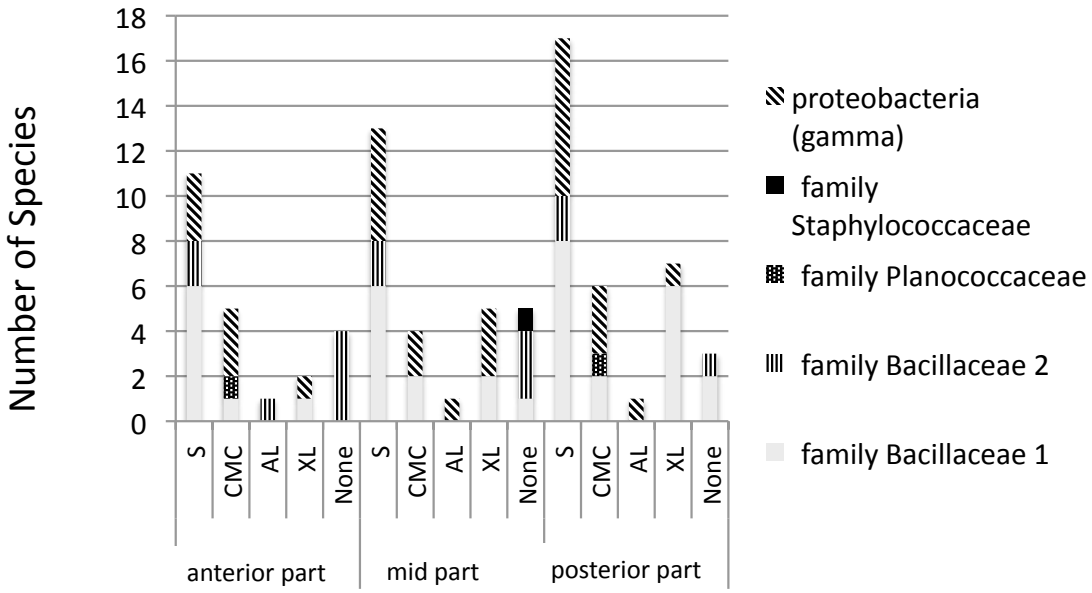

Fig.4 Number of the species of isolates degrading various polysaccharides
The species were divided into five groups shown in the legend of Fig.3. S, CMC, AL, XL and None indicated starch degradation activity (S), CMC degradation activity (CMC), alginate degradation activity (AL), xylan degradation activity (XL) and No degradation activity (None) of polysaccharides mentioned in this study, respectively. All isolates showed no agarase activity.

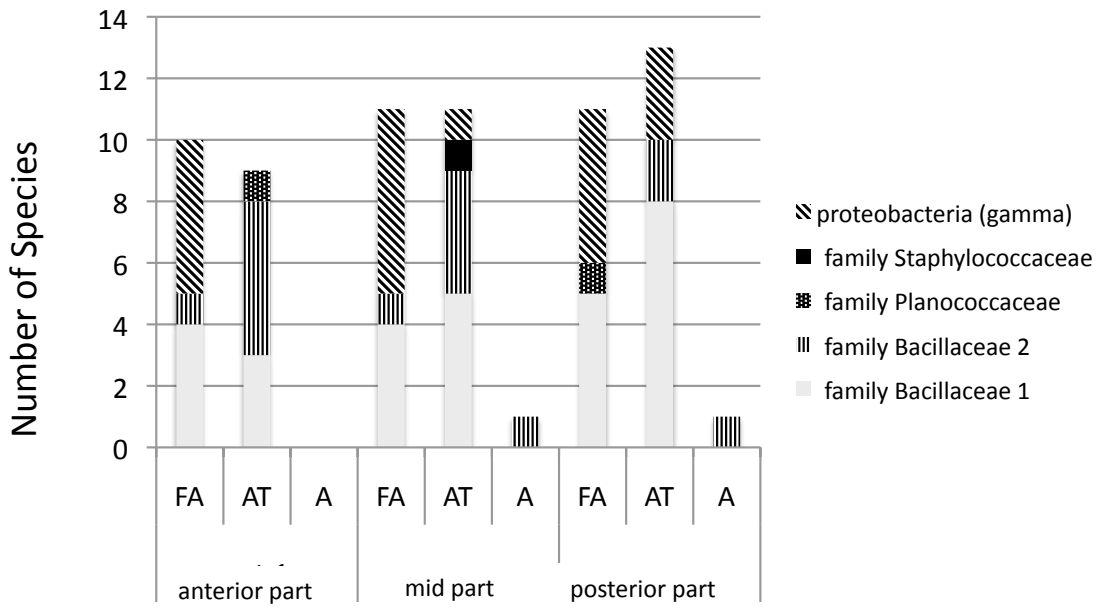

Fig.5 Number of the species of isolates classified by effect of oxygen on the growth
The species were divided into five groups as shown in Fig.3. Facultative anaerobic bacteria (FA) form colony in both aerobic and anaerobic cultivation. Anaerobic tolerant bacteria (AT) do not form colony in anaerobic condition for two weeks but form colony in aerobic cultivation after the anaerobic cultivation. Aerobic bacteria (A) do not form colony in anaerobic condition for two weeks and also do not form colony in aerobic cultivation after the anaerobic cultivation.

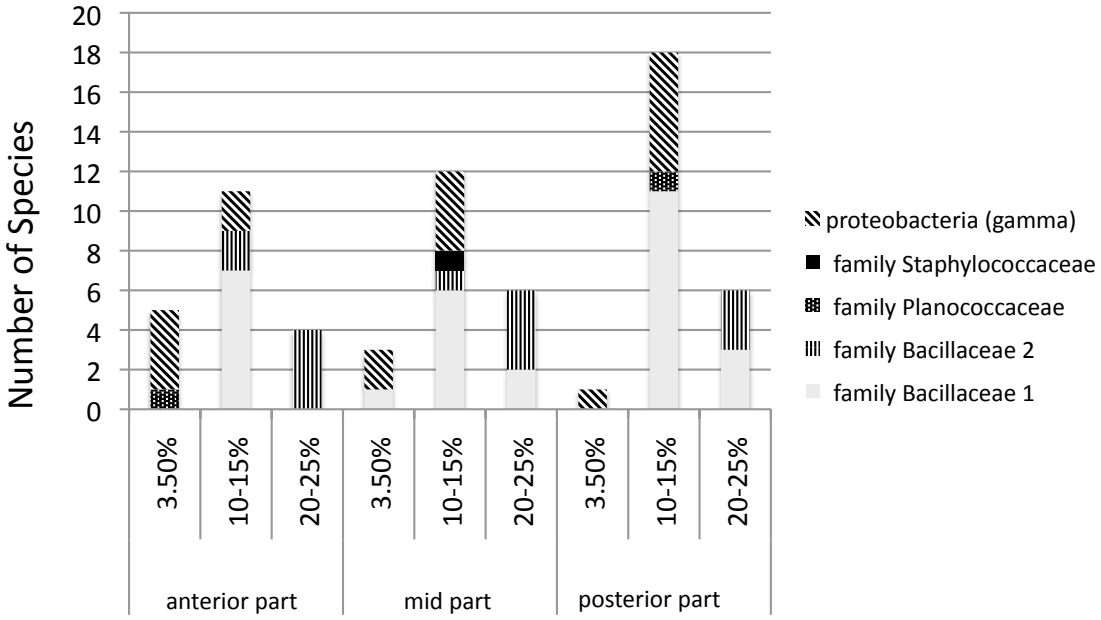

Fig. 6 Number of the species of isolates classified by effect of NaCl concentration on the growth
The species were divided into five groups as shown in Fig.3.

Supplementary Table 1

Isolate No.	Accession number of isolates	isolation medium	letters	part of the intestine	Accession nnumber of type strain	species/tentative species	Identities	Degrading activities on polysaccharids				Requiment of oxygen	Maximum NaCl concentration for growth	pH tolerance	phylum	family
								S	CMC	AL	XL					
C210	AB741781	LB(pH7,3.5%)	528	mid	EF114313	*Bacillus aryabhattai*	522/522 (100%)	+	+	−	−	FA	10	NE	firmicutes	family Bacillaceae 1
C212	AB741782	LB(pH7,3.5%)	818	mid	AF483624	*Bacillus marisflavi*	818/818 (100%)	−	−	−	+	AT	20	NE	firmicutes	family Bacillaceae 1
C214	AB741783	LB(pH7,3.5%)	623	mid	AJ009793	*Virgibacillus marismortui*	555/577 (96%)	−	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 2
C219	AB741784	LB(pH7,3.5%)	825	mid	AJ310149	*Halobacillus trueperi*	816/825 (98%)	+	−	−	−	AT	20	ALK	firmicutes	family Bacillaceae 2
C220	AB741785	LB(pH7,3.5%)	819	mid	L37603	*Staphylococcus warneri*	819/819 (100%)	−	−	−	−	AT	15	NE	firmicutes	family Staphylococcaceae
C221	AB741786	LB(pH7,3.5%)	509	mid	AJ491290	*Vibrio pomeroyi*	503/509 (98%)	+	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C234	AB741787	CMC(pH10,10%)	817	posterior	HM054473	*Bacillus hunanensis*	816/817 (99%)	+	−	−	−	AT	20	ALK	firmicutes	family Bacillaceae 1
C235	AB741788	CMC(pH10,10%)	813	posterior	AB125942	*Marinobacter alkaliphilus*	807/813 (99%)	+	−	−	−	AT	15	ALK	proteobacteria	gamma
C236	AB741789	CMC(pH10,3.5%)	594	anterior	AY190535	*Jeotgalibacillus campisalis*	590/594 (99%)	−	+	−	−	AT	3.5	ALK	firmicutes	family Planococcaceae
C240	AB741790	CMC(pH10,3.5%)	499	mid	X76440	*Bacillus clausii*	497/499 (99%)	+	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 1
C241	AB741791	CMC(pH10,3.5%)	589	mid	D87035	*Bacillus horti*	585/589 (99%), Gaps = 1/589 (0%)	+	−	−	−	AT	3.5	ALK	firmicutes	family Bacillaceae 1
C242	AB741792	CMC(pH10,3.5%)	540	mid	AJ842344	*Photobacterium rosenbergii*	537/540 (99%)	+	−	−	−	FA	3.5	ALK	proteobacteria	gamma
C243	AB741793	CMC(pH10,3.5%)	797	mid	D87035	*Bacillus horti*	791/797 (99%), Gaps = 2/797 (0%)	+	−	−	−	AT	3.5	ALK	firmicutes	family Bacillaceae 1
C245	AB741794	CMC(pH10,3.5%)	812	mid	GU784860	*Oceanobacillus kimchii*	807/812 (99%)	−	−	−	−	FA	20	ALK	firmicutes	family Bacillaceae 2
C246	AB741795	CMC(pH10,3.5%)	574	posterior	AB188090	*Bacillus oshimensis*	571/574 (99%)	−	−	−	−	FA	15	ALK	firmicutes	family Bacillaceae 1
C247	AB741796	CMC(pH10,3.5%)	427	posterior	AJ492830	*Pseudomonas cedrina*	425/427 (99%)	+	−	−	−	FA	15	ALK	proteobacteria	gamma
C254	AB741797	AL(pH10,10%)	453	anterior	AJ717299	*Thalassobacillus devorans*	437/453 (96%)	−	−	−	−	AT	20	ALK	firmicutes	family Bacillaceae 2
C258	AB741798	AL(pH10,3.5%)	621	anterior	EU925618	*Bacillus neizhouensis*	609/616 (98%)	−	−	−	+	FA	15	ALK	firmicutes	family Bacillaceae 1
C259	AB741799	AL(pH10,3.5%)	715	anterior	AJ316187	*Vibrio rotiferianus*	691/699 (98%)	−	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C263	AB741800	AL(pH10,3.5%)	524	posterior	AF057645	*Pseudomonas libanensis*	523/524 (99%)	+	+	−	−	AT	10	ALK	proteobacteria	gamma
C265	AB741801	XL(pH10,10%)	535	anterior	AB188090	*Bacillus oshimensis*	530/535 (99%)	+	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 1
C270	AB741802	XL(pH10,10%)	565	posterior	HM054473	*Bacillus hunanensis*	565/565 (100%)	+	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 1
C271	AB741803	XL(pH10,10%)	764	posterior	AJ605773	*Bacillus aurantiacus*	732/759 (96%), Gaps = 6/759 (0%)	+	−	−	+	AT	20	ALK	firmicutes	family Bacillaceae 1
C277	AB741804	XL(pH10,3.5%)	677	anterior	AB043865	*Bacillus horikoshii*	675/677 (99%), Gaps = 1/677 (0%)	+	−	−	−	AT	10	ALK	firmicutes	family Bacillaceae 1
C280	AB741805	XL(pH10,3.5%)	544	mid	D84025	*Pseudomonas synxantha*	540/544 (99%)	+	−	+	+	FA	10	ALK	proteobacteria	gamma
C281	AB741806	XL(pH10,3.5%)	475	mid	X76444/AB292819	*Bacillus clarkii/Bacillus polygoni*	474/475 (99%)	+	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 1
C285	AB741807	XL(pH10,3.5%)	581	posterior	AY190535	*Jeotgalibacillus campisalis*	581/583 (99%), Gaps = 1/583 (0%)	−	+	−	−	FA	10	ALK	firmicutes	family Planococcaceae
C287	AB741808	XL(pH10,3.5%)	637	posterior	HM054473/AB188090	*Bacillus hunanensis/Bacillus oshimensis*	634/637 (99%)	+	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 1
C288	AB741809	XL(pH10,3.5%)	772	posterior	X76449	*Bacillus pseudalcaliphilus*	743/760 (97%)	+	−	−	+	FA	10	ALK	firmicutes	family Bacillaceae 1
C291	AB741810	XL(pH10,3.5%)	521	posterior	AB043851	*Bacillus wakoensis*	521/521 (100%)	−	−	−	+	FA	15	ALK	firmicutes	family Bacillaceae 1
C295	AB741811	S(pH10,3.5%)	515	anterior	DQ534014	*Photobacterium lutimaris*	512/515 (99%)	+	−	−	−	FA	3.5	ALK	proteobacteria	gamma
C298	AB741812	S(pH10,3.5%)	816	anterior	HM054473	*Bacillus hunanensis*	815/816 (99%)	+	−	−	−	FA	15	ALK	firmicutes	family Bacillaceae 1
C302	AB741813	S(pH10,3.5%)	411	anterior	X74710	*Vibrio mediterranei*	407/411 (99%)	−	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C304	AB741814	S(pH10,3.5%)	540	mid	AJ842344	*Photobacterium rosenbergii*	535/540 (99%)	+	−	−	−	FA	10	ALK	proteobacteria	gamma
C305	AB741815	S(pH10,3.5%)	683	mid	X76440	*Bacillus clausii*	673/677 (99%)	+	−	−	−	FA	10	ALK	firmicutes	family Bacillaceae 1
C307	AB741816	S(pH10,3.5%)	550	posterior	AB043846	*Bacillus hemicellulosilyticus*	547/550 (99%)	+	+	−	+	AT	10	ALK	firmicutes	family Bacillaceae 1
C309	AB741817	S(pH10,3.5%)	565	posterior	AJ842344	*Photobacterium rosenbergii*	560/565 (99%)	−	+	−	−	FA	10	ALK	proteobacteria	gamma
C310	AB741818	S(pH10,3.5%)	623	posterior	AF057645	*Pseudomonas libanensis*	619/623 (99%)	+	−	−	−	AT	15	ALK	proteobacteria	gamma
C312	AB741819	S(pH10,3.5%)	516	posterior	AJ310647	*Vibrio agarivorans*	505/516 (97%)	+	−	+	−	FA	10	ALK	proteobacteria	gamma
C313	AB741820	S(pH10,3.5%)	388	posterior	AB043865	*Bacillus horikoshii*	388/388 (100%)	+	−	−	−	FA	10	ALK	firmicutes	family Bacillaceae 1
C317	AB741821	CMC(pH7,10%)	580	anterior	AB099708	*Bacillus vietnamensis*	579/580 (99%)	+	−	−	−	FA	10	NE	firmicutes	family Bacillaceae 1
C318	AB741822	CMC(pH7,10%)	780	anterior	AJ831844/AJ831842	*Bacillus aerophilus/Bacillus altitudinis*	780/780 (100%)	−	−	−	−	AT	10	NE	firmicutes	family Bacillaceae 1
C324	AB741823	CMC(pH7,10%)	569	mid	AY543169	*Virgibacillus halodenitrificans*	568/569 (99%)	−	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 2
C327	AB741824	CMC(pH7,10%)	811	mid	AB195680	*Halobacillus kuroshimensis*	808/811 (99%), Gaps = 2/811 (0%)	+	−	−	−	AT	25	NE	firmicutes	family Bacillaceae 2
C333	AB741825	CMC(pH7,10%)	679	posterior	AY543169	*Virgibacillus halodenitrificans*	675/676 (99%)	−	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 2
C334	AB741826	CMC(pH7,10%)	586	posterior	CP000002	*Bacillus licheniformis*	580/580 (100%)	+	−	−	−	FA	15	NE	firmicutes	family Bacillaceae 1
C335	AB741827	CMC(pH7,10%)	808	posterior	AJ310149	*Halobacillus trueperi*	806/808 (99%)	+	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 2
C336	AB741828	CMC(pH7,10%)	740	posterior	AF483625	*Bacillus aquimaris*	735/740 (99%)	+	−	−	−	AT	15	NE	firmicutes	family Bacillaceae 1
C339	AB741829	AL(pH7,10%)	589	anterior	AJ640134	*Oceanobacillus oncorhynchi*	577/589 (97%)	−	−	−	−	AT	20	ALK	firmicutes	family Bacillaceae 2
C341	AB741830	AL(pH7,10%)	558	anterior	AJ831842/AJ831841	*Bacillus altitudinis/Bacillus stratosphericus*	558/558 (100%)	−	−	−	−	AT	10	NE	firmicutes	family Bacillaceae 1

C344	AB741831	AL(pH7,10%)	768	anterior	AJ831844/AJ831842	*Bacillus aerophilus/Bacillus altitudinis*	768/768 (100%)	−	−	−	−	AT	10	NE	firmicutes	family Bacillaceae 1
C347	AB741832	AL(pH7,10%)	704	anterior	AY822043	*Virgibacillus dokdonensis*	691/704 (98%)	−	−	−	−	AT	10	NE	firmicutes	family Bacillaceae 2
C350	AB741833	AL(pH7,10%)	823	mid	AB195680	*Halobacillus kuroshimensis*	816/820 (99%), Gaps = 3/820 (0%)	+	−	−	−	A	20	NE	firmicutes	family Bacillaceae 2
C355	AB741834	AL(pH7,10%)	684	mid	AB195680	*Halobacillus kuroshimensis*	682/685 (99%), Gaps = 2/685 (0%)	+	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 2
C358	AB741835	AL(pH7,10%)	753	posterior	AF541966	*Bacillus hwajinpoensis*	746/749 (99%), Gaps = 1/749 (0%)	−	−	−	−	AT	15	NE	firmicutes	family Bacillaceae 1
C359	AB741836	AL(pH7,10%)	528	posterior	CP000002	*Bacillus licheniformis*	528/528 (100%)	+	−	−	−	AT	10	NE	firmicutes	family Bacillaceae 1
C360	AB741837	AL(pH7,10%)	557	posterior	AB099708	*Bacillus vietnamensis*	557/557 (100%)	+	−	−	−	AT	10	NE	firmicutes	family Bacillaceae 1
C361	AB741838	AL(pH7,10%)	665	posterior	AY543169	*Virgibacillus halodenitrificans*	663/665 (99%)	−	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 2
C362	AB741839	AL(pH7,10%)	813	posterior	AB195680	*Halobacillus kuroshimensis*	810/814 (99%), Gaps = 2/814 (0%)	+	−	−	−	A	25	NE	firmicutes	family Bacillaceae 2
C365	AB741840	AL(pH7,10%)	772	posterior	AF483625	*Bacillus aquimaris*	760/772 (98%)	+	−	−	−	AT	15	NE	firmicutes	family Bacillaceae 1
C367	AB741841	AL(pH7,10%)	520	posterior	AF541966	*Bacillus hwajinpoensis*	515/520 (99%)	−	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 1
C370	AB741842	XL(pH7,10%)	420	anterior	AB101591	*Gracilibacillus dipsosauri*	420/420 (100%)	+	−	+	−	AT	15	ALK	firmicutes	family Bacillaceae 2
C375	AB741843	XL(pH7,10%)	598	anterior	AB473561	*Oceanobacillus sojae*	596/598 (99%)	−	−	−	−	AT	20	ALK	firmicutes	family Bacillaceae 2
C377	AB741844	XL(pH7,10%)	760	anterior	AB195680	*Halobacillus kuroshimensis*	758/761 (99%), Gaps = 2/761 (0%)	+	−	−	−	FA	25	ALK	firmicutes	family Bacillaceae 2
C379	AB741845	XL(pH7,10%)	533	mid	CP000002	*Bacillus licheniformis*	531/532 (99%)	+	−	−	−	FA	15	ALK	firmicutes	family Bacillaceae 1
C380	AB741846	XL(pH7,10%)	535	mid	X76440	*Bacillus clausii*	534/535 (99%)	−	−	−	−	AT	25	NE	firmicutes	family Bacillaceae 1
C381	AB741847	XL(pH7,10%)	836	mid	AB195680	*Halobacillus kuroshimensis*	831/837 (99%), Gaps = 2/837 (0%)	+	−	−	−	A	20	ALK	firmicutes	family Bacillaceae 2
C392	AB741848	XL(pH7,10%)	655	posterior	CP000002	*Bacillus licheniformis*	653/655 (99%)	+	−	−	+	FA	15	NE	firmicutes	family Bacillaceae 1
C397	AB741849	XL(pH7,10%)	568	posterior	AJ842344	*Photobacterium rosenbergii*	565/569 (99%), Gaps = 1/569 (0%)	+	−	−	−	FA	15	NE	proteobacteria	gamma
C416	AB741850	S(pH7,10%)	711	mid	CP000002	*Bacillus licheniformis*	710/711 (99%)	+	−	−	−	FA	15	NE	firmicutes	family Bacillaceae 1
C417	AB741851	S(pH7,10%)	672	posterior	CP000002	*Bacillus licheniformis*	671/672 (99%)	+	+	−	−	FA	15	NE	firmicutes	family Bacillaceae 1
C431	AB741852	S(pH7,10%)	777	posterior	AY543169	*Virgibacillus halodenitrificans*	777/777 (100%)	−	−	−	−	AT	20	NE	firmicutes	family Bacillaceae 2
C435	AB741853	CMC(pH7,3.5%)	706	anterior	AJ491290	*Vibrio pomeroyi*	699/706 (99%), Gaps = 1/706 (0%)	+	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C442	AB741854	CMC(pH7,3.5%)	531	mid	AJ491290	*Vibrio pomeroyi*	525/531 (98%)	+	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C444	AB741855	CMC(pH7,3.5%)	421	mid	D16273	*Bacillus megaterium*	417/421 (99%)	+	+	−	−	AT	10	NE	firmicutes	family Bacillaceae 1
C446	AB741856	CMC(pH7,3.5%)	568	mid	AY876289	*Bacillus pumilus*	568/569 (99%)	−	−	−	+	FA	10	NE	firmicutes	family Bacillaceae 1
C460	AB741858	AL(pH7,3.5%)	477	anterior	AJ491290	*Vibrio pomeroyi*	471/477 (98%)	+	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C463	AB741859	AL(pH7,3.5%)	462	mid	AF057645	*Pseudomonas libanensis*	461/462 (99%)	+	−	−	−	AT	10	ALK	proteobacteria	gamma
C466	AB741860	AL(pH7,3.5%)	664	posterior	AJ316187	*Vibrio rotiferianus*	662/664 (99%)	+	+	−	−	FA	3.5	ALK	proteobacteria	gamma
C471	AB741861	XL(pH7,3.5%)	544	anterior	AF057645	*Pseudomonas libanensis*	542/544 (99%)	−	−	−	+	FA	15	NE	proteobacteria	gamma
C475	AB741862	XL(pH7,3.5%)	655	anterior	AJ316187	*Vibrio rotiferianus*	654/655 (99%)	+	+	−	−	FA	15	ALK	proteobacteria	gamma
C483	AB741863	XL(pH7,3.5%)	673	mid	AF057645	*Pseudomonas libanensis*	671/673 (99%)	+	−	−	+	AT	15	ALK	proteobacteria	gamma
C484	AB741864	XL(pH7,3.5%)	542	mid	AJ492830	*Pseudomonas cedrina*	540/542 (99%)	+	−	−	−	FA	15	ALK	proteobacteria	gamma
C487	AB741865	XL(pH7,3.5%)	535	mid	AY750575	*Vibrio harveyi*	529/535 (98%)	−	+	−	−	FA	15	ALK	proteobacteria	gamma
C494	AB741866	XL(pH7,3.5%)	761	posterior	AJ831844	*Bacillus aerophilus*	761/761 (100%)	−	−	−	+	AT	15	NE	firmicutes	family Bacillaceae 1
C503	AB741867	S(pH7,3.5%)	749	anterior	D16273	*Bacillus megaterium*	747/749 (99%)	+	−	−	−	FA	10	NE	firmicutes	family Bacillaceae 1
C506	AB741868	S(pH7,3.5%)	540	anterior	AB021185	*Bacillus flexus*	538/540 (99%)	+	+	−	−	AT	15	NE	firmicutes	family Bacillaceae 1
C512	AB741869	S(pH7,3.5%)	556	mid	AF057645	*Pseudomonas libanensis*	554/556 (99%)	+	−	−	+	FA	10	NE	proteobacteria	gamma
C520	AB741870	S(pH7,3.5%)	563	mid	AJ492830	*Pseudomonas cedrina*	561/564 (99%), Gaps = 1/564 (0%)	−	−	−	+	FA	15	NE	proteobacteria	gamma
C521	AB741871	S(pH7,3.5%)	669	mid	AY793550	*Bacillus lehensis*	668/669 (99%)	+	−	−	−	AT	15	ALK	firmicutes	family Bacillaceae 1
C528	AB741872	S(pH7,3.5%)	578	posterior	AF057645	*Pseudomonas libanensis*	576/578 (99%)	−	+	−	+	FA	10	NE	proteobacteria	gamma
C538	AB741873	S(pH7,10%)	746	posterior	AJ306891	*Halomonas meridiana*	746/746 (100%)	+	−	−	−	AT	15	ALK	proteobacteria	gamma

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/alkali-tolerant bacteria . Display of more than one species in the column of nearest type strain species indicated the same idenntity in the comparison range.