

Chapter II

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

2.1 Introduction

Holothurians (~1430 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 Holothuroidea 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 energetic 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 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 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 the molecular techniques, they

also found that Proteobacteria members were main metabolically active microbial populations in the intestine of *A. japonicus*.

In this report, I investigated the biological diversity and function of bacteria in the intestine of *Holothuria leucospilota*, a common sea cucumber in Japanese warm waters. I 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.

2.2 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 shallow waters of western Japan (Drumm and Loneragan, 2005; Matsuno and Ishida, 1961; Sloan, 1979). *H. leucospilota* (2 specimens) were collected at coastal waters of Ko-e cho, Nagasaki, Japan in April 24, 2009. Both samples were immediately transferred to our laboratory and the entire intestine was removed with sterile instruments. Whole intestine was excised from the animal body aseptically using sterilized instruments. To isolate bacteria from both intestinal wall and contents, the intact gut was crushed and mixed enough 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% KH₂PO₄, 0.02% MgSO₄•7H₂O, 3.5% NaCl, and 2% agar. As polysaccharides, carboxymethyl cellulose sodium salt (CMC) (Wako pure chemicals, Osaka, Japan) 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 is 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, sodium alginate or starch) (Table 1). To adjust pH for alkaline condition, Na₂CO₃ (autoclaved separately) was added to neutral agar medium (final pH: pH10.3-10.5). Na₂CO₃ concentration of 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 two weeks to obtain slowly growing bacteria. Bacteria with different morphological colony (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 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 condition in which each isolate originated.

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.

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

3. Cellulase detection: The agar medium for cellulase detection contained 0.1% CMC, 3.7% marine broth, 0.6% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.5% agar and 0.0015% congo-red. Clear zone around a 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 two weeks' incubation at 30°C, 70% ethanol was filled into 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%, 25% NaCl (w/v), pH tolerance (pH7 and pH10) and effect of oxygen. 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 bacteria (ALK) that grew both at pH 7 and pH10.

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.

For thermal tolerance, all isolates were incubated on agar plates at 50°C for 72h aerobically, and then incubation condition was shifted down to 30°C for two weeks. Isolates that were able to form colony on agar plates at 50°C for 72h aerobically, were assigned as thermophiles (TO). Isolates that were not able to form colony on agar plates at 50°C for 72h aerobically but were able to form colony at 30°C incubation after the 50°C incubation, were assigned as thermo-tolerant bacteria (TT). Isolates that were not able to form colony on agar plates at 50°C for 72h aerobically and also were not able to form 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'-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 AB719059 through AB719199 (Supplementary Table 1; see JGAM 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). When an isolate showed $\geq 97\%$ 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.

2.3 Results

Isolation of bacteria

The gut suspensions from two specimens were directly plated on agar plates and 13 isolation media were used. Table 1 summarized 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. 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 (pH7, 3.5%NaCl) and 1.6×10^2 to 1.2×10^3 cfu/g in the alkaline Horikoshi media (ca. pH10.3, 3.5%NaCl). I obtained 77 isolates from the specimen ① and 64 isolates from the 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 summarized the species/the tentative species of the isolates as determined via BLAST (Supplementary Table 1 ; JGAM Web site). The isolates were tentatively affiliated with 55 described species in the phyla Firmicutes, Proteobacteria, and Actinobacteria (Table 2 and 3). The isolates from the 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, the 23 species of isolates were found in both specimens and 9 species belonged with the genus *Bacillus* and other 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 originated 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. I obtained 11 isolates affiliated to *V. harveyi* from specimen ① and ②. All these isolates showed more than 99% sequence identity with each other.

I 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 asterisk in Supplementary Table 1; see JGAM Web site). Among the 7 isolates, I found 4 tentative species defined with $\geq 97\%$ 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 polysaccharides 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, Supplementary Table 1; see JGAM Web site).

Amylase-producing isolates were mainly affiliated with the genera *Vibrio* (12 species) and *Bacillus* (9 species). Most cellulase positive 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*. I 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 abilities.

Physiological characteristics of the isolates

Fig. 2 and Supplementary Table 1 (JGAM Web site) show the effect of anaerobic condition on 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 colony in anaerobic condition for 2 days, but they were able to form colony in aerobic cultivation after the 2 days anaerobic cultivation.

Salinity tolerance of the isolates was examined (Fig. 3, and Supplementary Table 1; JGAM Web site). The isolates affiliated to *Halobacillus trueperi*, *Geomicrobium halophilum* and *Bacillus hwajinpoensis* showed the highest tolerance (25 % NaCl) and some strains were able to grow in absent of NaCl. I 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. I found 35 species were 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 (pH7→pH10 or pH10→pH7). All alkaliphilic strains isolated from alkali medium were able to grow at pH 7, while only half of strains isolated from pH 7 were able to grow at pH 10. Neutrophilic species (NE) defined as isolates growing only at pH7 were 26 species,

including 10 species affiliated with the genus *Vibrio*. Members of alkaliphiles were 38 species affiliated with the phyla Firmicutes, Proteobacteria and Actinobacteria. The main genera of alkaliphiles were *Bacillus* and *Vibrio*.

2.4 Discussion

In this report, I 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* that were 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 to the genus *Vibrio* (Table 2 and Table 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 majorities comprise recalcitrant polysaccharides, which in many cases can be degraded only by microorganisms. 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 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.

I found that the isolates were divided into facultative anaerobic, anaerobic tolerant and aerobic bacteria by means of 2-week anaerobic cultivation. I also examined the effect of 2-day anaerobic condition simulating the digestive process of holothurian and I found that the isolates classified into aerobic bacteria were able to form colonies in aerobic cultivation after 2-day anaerobic condition. These results suggested that the aerobic isolates were potentially tolerant for anaerobic condition in the intestine of holothurians. On the other hand, all 62 anaerobic tolerant (AT) isolates were examined for heat tolerance because most of AT isolates belonged to the phylum Firmicutes and had ability to form spores. More than 60 % of AT isolates showed non-heat 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. My 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 Unit) were common between the gut contents and the surrounding sediments (Amaro et al., 2012). Fig. 4 shows one model for the facultative symbiotic association among host holothurian, the aerobic bacteria and the bacteria unique to the intestine of holothurian. In marine ecosystem, aerobic bacterial

degradation of detritus occurs in the seabed and the holothurian ingested 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 host holothurian, the aerobic bacteria and the bacteria unique to the intestine.

I 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.

2.5 Summary

Sea cucumbers play an important role in nutrient cycling of marine ecosystems by consuming sediments and moving sand, thus occupying a similar niche of earthworms in terrestrial ecosystems. However, our understanding of microbial diversity and functions associated with sea cucumbers is meager. Here, I isolated 141 bacterial strains in aerobic condition 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* that were often found in marine sediments, marine animals and other various environments. Most

isolates showed various polysaccharide degradation activities and were able to grow or were tolerant under anaerobic condition. I suggest that these aerobically isolated bacteria can play a role in digestion of detritus in aerobic and/or anaerobic regions of the intestine.

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.

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

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

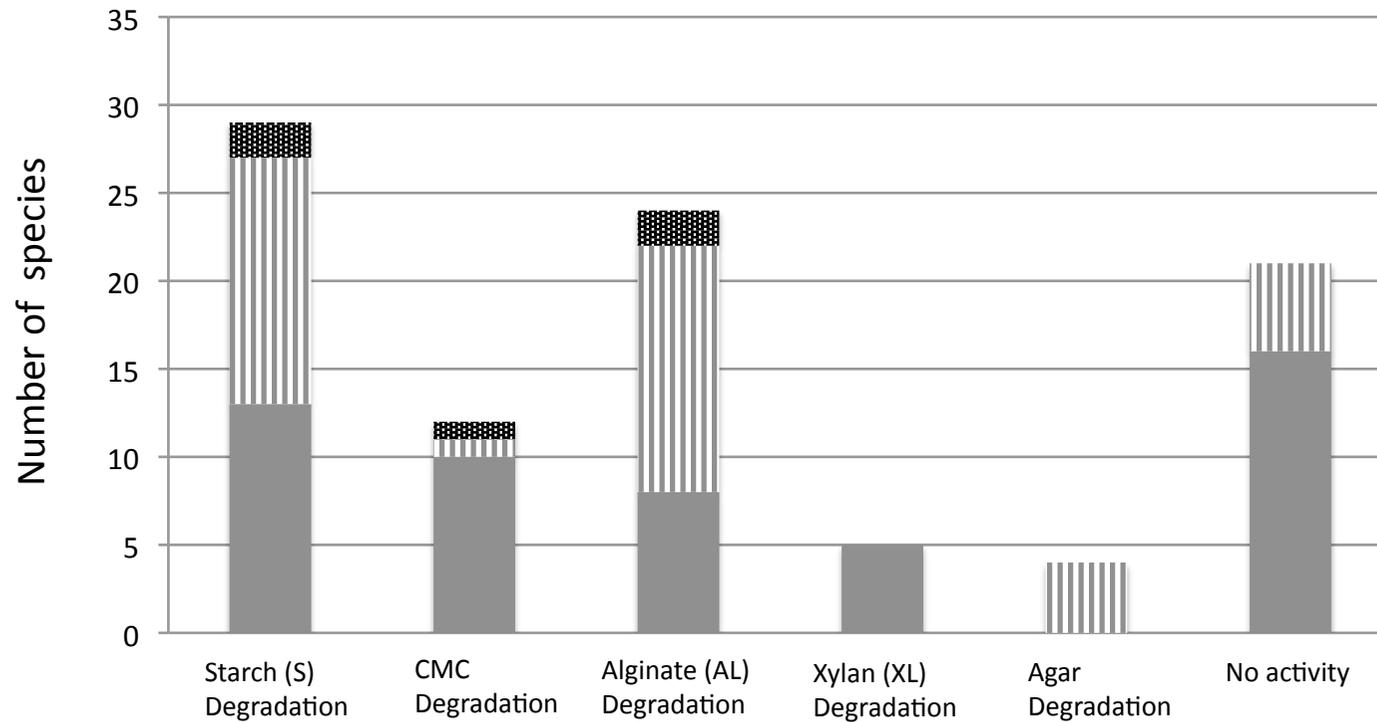


Fig.1 Number of the species of isolates degrading various polysaccharides
The species were divided into three groups: the phylum Firmicutes (black box), the phylum Proteobacteria (vertical stripes box) and the phylum Actinobacteria (dotted box). Fig.1 was summarized from Supplementary Table 1.

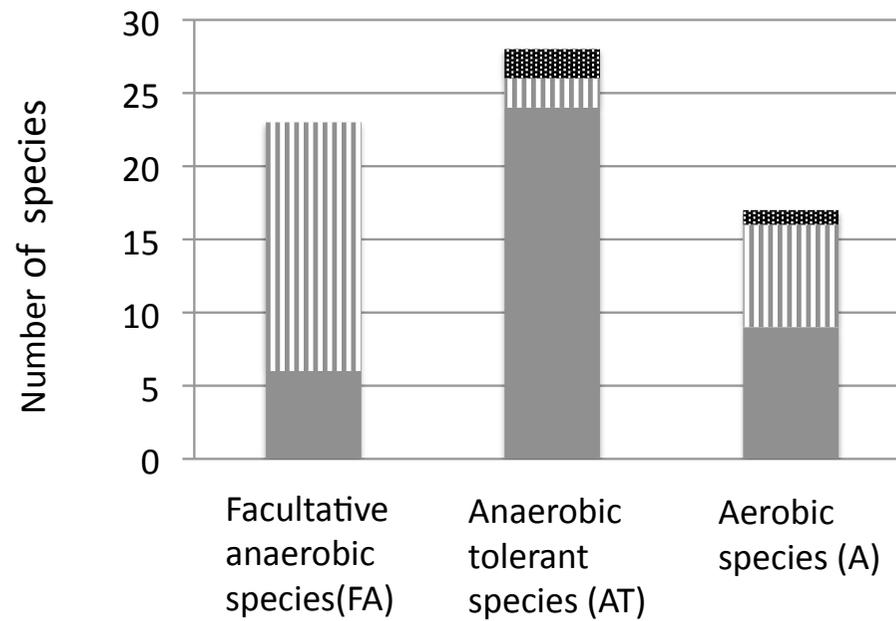


Fig.2 Number of the species of isolates classified by effect of oxygen on the growth
The species were divided into three groups: the phylum Firmicutes (black box), the phylum Proteobacteria (vertical stripes box) and the phylum Actinobacteria (dotted box). Fig.2 was summarized from Supplementary Table 1.

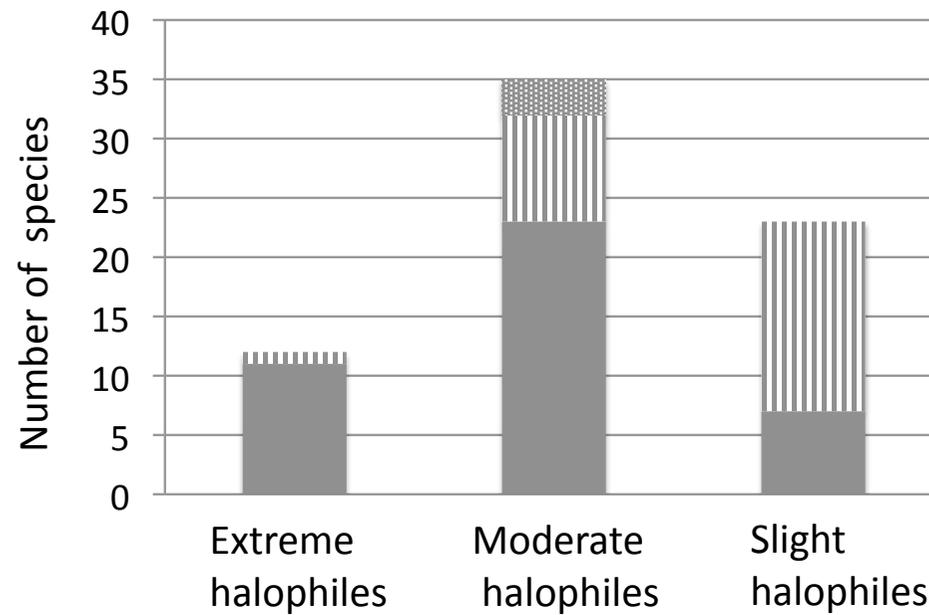


Fig. 3 Number of the species of isolates classified by effect of NaCl concentration on the growth. The species were divided into three groups: the phylum Firmicutes (black box), the phylum Proteobacteria (vertical stripes box) and the phylum Actinobacteria (dotted box). Slight halophiles (SH): maximum concentration for growth is 3.5 % NaCl, moderate halophiles (MH): maximum concentration for growth is 10-15 % NaCl, extreme halophiles (EH): maximum concentration for growth is more than 20 % NaCl. Fig.3 was summarized from Supplementary Table 1.

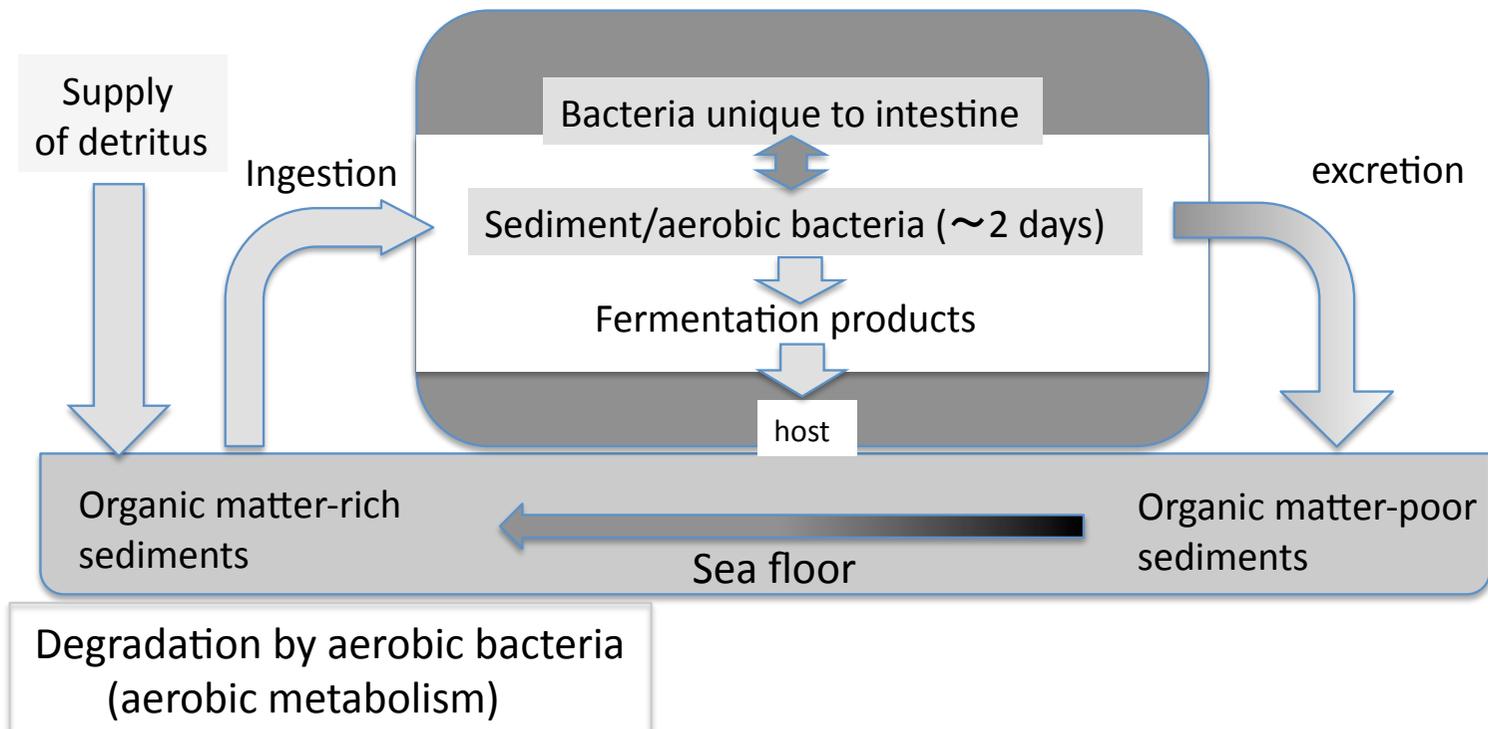


Fig.4 Model for the facultative symbiotic association between host holothurian, aerobic bacteria and the bacteria unique to the intestine of *H. leucospilota*.

| Supplementary Table 1 | | | | | | | | | | | | | | | | |
|-----------------------|---------------|------------------|----------|--|---------------------------------|----------------------------------|----------------------|-----|----|----|--------------------------|--|----------------------|-----------------|--------|----------------|
| Isolate No. | Accession No. | Medium | Specimen | species / tentative species | Accession No. of type strain | Identities | Degrading activities | | | | Requirement of oxygen | Maximum NaCl concentration for growth | Thermal tolerance | pH tolerance | phylum | |
| | | | | | | | S | CMC | AL | XL | | | | | | Agar |
| C001 | AB719059 | LB(pH7, 3.5%) | ① | <i>Vibrio alginolyticus</i> | X56576 | 594/598 (99%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C002 | AB719060 | LB(pH7, 3.5%) | ① | <i>Vibrio tasmaniensis</i> | AJ316192 | 767/772 (99%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C003 | AB719061 | LB(pH7, 3.5%) | ① | <i>Bacillus horneckiae</i> | EU861362 | 628/628 (100%) | - | - | - | - | - | A | 3.5 | TT | ALK | Firmicutes |
| C004 | AB719062 | LB(pH7, 3.5%) | ① | <i>Vibrio natriegens</i> | X74714 | 775/778 (99%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C005 | AB719063 | LB(pH7, 3.5%) | ① | <i>Bacillus horneckiae</i> | EU861362 | 609/609 (100%) | - | - | - | - | - | FA | 3.5 | TT | ALK | Firmicutes |
| C006 | AB719064 | LB(pH7, 3.5%) | ① | <i>Vibrio brasiliensis</i> | AJ316172 | 586/599 (97%) | + | - | - | - | + | FA | 3.5 | N | ALK | Proteobacteria |
| C007 | AB719065 | LB(pH7, 3.5%) | ① | <i>Bacillus stratosphericus</i> | AJ831841 | 550/552 (99%) | - | - | - | + | - | FA | 20 | TO | ALK | Firmicutes |
| C008 | AB719066 | LB(pH7, 3.5%) | ② | <i>Vibrio harveyi</i> | AY750575 | 753/760 (99%) | + | - | - | - | - | FA | 10 | N | NE | Proteobacteria |
| C009 | AB719067 | LB(pH7, 3.5%) | ② | <i>Vibrio pomeroyi/ Vibrio gigantis</i> | AJ491290/ EF094888 | 787/793 (99%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C011 | AB719068 | LB(pH7, 3.5%) | ② | <i>Photobacterium rosenbergii</i> | AJ842344 | 639/643 (99%) | + | - | - | - | + | FA | 3.5 | N | NE | Proteobacteria |
| C012 | AB719069 | LB(pH7, 3.5%) | ① | <i>Vibrio harveyi</i> | AY750575 | 563/569 (98%) | + | - | - | - | - | FA | 10 | N | NE | Proteobacteria |
| C013 | AB719070 | LB(pH7, 3.5%) | ① | <i>Vibrio rotiferianus</i> | AJ316187 | 791/795 (99%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C014 | AB719071 | LB(pH7, 3.5%) | ① | <i>Vibrio harveyi</i> | AY750575 | 515/521 (98%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C015 | AB719072 | LB(pH7, 3.5%) | ① | <i>Vibrio harveyi</i> | AY750575 | 580/586 (98%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C016 | AB719073 | LB(pH7, 3.5%) | ② | <i>Vibrio rotiferianus</i> | AJ316187 | 788/793 (99%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C017 | AB719074 | LB(pH7, 3.5%) | ② | <i>Vibrio harveyi</i> | AY750575 | 546/552 (98%) | + | - | - | - | - | FA | 10 | N | NE | Proteobacteria |
| C018 | AB719075 | LB(pH7, 3.5%) | ② | <i>Vibrio alginolyticus</i> | X56576 | 760/768 (98%), Gaps = 3/768 (0%) | + | - | + | - | - | FA | 10 | N | NE | Proteobacteria |
| C019 | AB719076 | LB(pH7, 3.5%) | ② | <i>Micrococcus luteus</i> | AJ536198 | 634/646 (98%) | + | - | - | - | - | AT | 10 | N | ALK | Actinobacteria |
| C021 | AB719077 | LB(pH7, 3.5%) | ② | <i>Vibrio neptunius</i> | AJ316171 | 823/837 (98%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C022 | AB719078 | LB(pH7, 3.5%) | ② | <i>Bacillus clausii</i> | X76440 | 589/590 (99%) | + | - | + | - | - | AT | 10 | TO | ALK | Firmicutes |
| C023 | AB719079 | LB(pH7, 3.5%) | ② | <i>Vibrio gigantis</i> | EF094888 | 792/793 (99%) | + | - | + | - | - | FA | 3.5 | N | NE | Proteobacteria |
| C024 | AB719080 | CMC(pH7, 3.5%) | ① | <i>Vibrio harveyi</i> | AY750575 | 642/648 (99%) | + | - | + | - | - | FA | 10 | N | ALK | Proteobacteria |
| C025 | AB719081 | CMC(pH7, 3.5%) | ① | <i>Ruegeria lacuscaerulensis</i> | U77644 | 734/753 (97%) | - | - | - | - | - | A | 3.5 | N | NE | Proteobacteria |
| C026 | AB719082 | CMC(pH7, 3.5%) | ① | <i>Vibrio ezuriae</i> | AY426980 | 755/760 (99%) | - | - | + | - | - | A | 3.5 | N | NE | Proteobacteria |
| C027 | AB719083 | CMC(pH7, 3.5%) | ② | <i>Vibrio harveyi</i> | AY750575 | 610/616 (99%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C028 | AB719084 | CMC(pH7, 3.5%) | ② | <i>Vibrio alginolyticus/ Vibrio harveyi/ Vibrio communis</i> | X56576/ AY750575/ GU078672 | 497/497 (100%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C030 | AB719085 | CMC(pH7, 3.5%) | ① | <i>Vibrio azureus</i> | AB428897 | 728/736 (98%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C031 | AB719086 | CMC(pH7, 3.5%) | ① | <i>Pseudoalteromonas prydzensis</i> | U85855 | 758/772 (98%) | - | - | - | - | - | A | 10 | N | NE | Proteobacteria |
| C032 | AB719087 | CMC(pH7, 3.5%) | ① | <i>Virgibacillus dokdonensis</i> | AY822043 | 804/805 (99%) | - | - | - | - | - | FA | 15 | TT | ALK | Firmicutes |
| C034 | AB719088 | CMC(pH7, 3.5%) | ① | <i>Virgibacillus dokdonensis</i> | AY822043 | 819/820 (99%) | - | - | - | - | - | AT | 15 | TT | ALK | Firmicutes |
| C036 | AB719089 | CMC(pH7, 3.5%) | ① | <i>Bacillus clausii</i> | X76440 | 383/390 (98%) | - | - | - | - | - | AT | 10 | TO | ALK | Firmicutes |
| C037 | AB719090 | CMC(pH7, 3.5%) | ① | <i>Vibrio communis</i> | GU078672 | 768/771 (99%) | + | - | + | - | - | FA | 3.5 | N | ALK | Proteobacteria |
| C038 | AB719091 | CMC(pH7, 3.5%) | ① | <i>Virgibacillus dokdonensis</i> | AY822043 | 592/604 (98%) | - | - | - | - | - | FA | 10 | TT | ALK | Firmicutes |
| C039 | AB719092 | CMC(pH7, 3.5%) | ② | <i>Vibrio communis</i> | GU078672 | 764/767 (99%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C040 | AB719093 | CMC(pH7, 3.5%) | ② | <i>Vibrio harveyi</i> | AY750575 | 778/785 (99%) | + | - | + | - | - | FA | 3.5 | N | ALK | Proteobacteria |
| C041 | AB719094 | CMC(pH7, 3.5%) | ② | <i>Vibrio alginolyticus</i> | X56576 | 608/608 (100%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C042 | AB719095 | CMC(pH7, 3.5%) | ② | <i>Vibrio owensii</i> | GU018180 | 758/761 (99%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C043 | AB719096 | CMC(pH7, 3.5%) | ② | <i>Vibrio harveyi</i> | AY750575 | 598/603 (99%) | + | - | - | - | - | FA | 10 | N | ALK | Proteobacteria |
| C044 | AB719097 | CMC(pH10, 3.5 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 590/591 (99%) | + | + | - | - | - | AT | 10 | TT | ALK | Firmicutes |
| C045 | AB719098 | CMC(pH10, 3.5 %) | ① | <i>Oceanobacillus theyensis</i> | AB010863 | 831/846 (98%), Gaps = 1/846 (0%) | - | + | - | - | - | AT | 20 | TT | ALK | Firmicutes |
| C046 | AB719099 | CMC(pH10, 3.5 %) | ② | <i>Bacillus lehensis</i> | AY793550 | 780/783 (99%) | + | + | - | - | - | AT | 10 | N | ALK | Firmicutes |
| C047 | AB719100 | CMC(pH10, 3.5 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 805/808 (99%) | + | + | - | - | - | AT | 10 | TT | ALK | Firmicutes |
| C048 | AB719101 | CMC(pH10, 3.5 %) | ① | <i>Bacillus murimartini</i> | AJ316316 | 820/826 (99%) | - | - | - | - | - | A | 3.5 | N | ALK | Firmicutes |
| C049 | AB719102 | CMC(pH10, 3.5 %) | ① | <i>Bacillus patagoniensis</i> | AY258614 | 833/838 (99%) | - | + | - | - | - | AT | 3.5 | TT | ALK | Firmicutes |
| C050 | AB719103 | CMC(pH10, 3.5 %) | ① | <i>Geomicrobium halophilum</i> | AB449106 | 457/467 (97%) | - | - | - | - | - | AT | 25 | TT | ALK | Firmicutes |

| | | | | | | | | | | | | |
|------|----------|-----------------|---|--|----------------------------|-----------------------------------|-----------|----|-----|----|-----|----------------|
| C051 | AB719104 | CMC(pH10,3.5 %) | ① | <i>Gracilbacillus ureilyticus</i> | EU709020 | 771/791 (97%) | + - - - - | AT | 10 | TT | ALK | Firmicutes |
| C052 | AB719105 | CMC(pH10,3.5 %) | ① | <i>Bacillus patagoniensis</i> | AY258614 | 816/820 (99%) | - + - - - | AT | 10 | N | ALK | Firmicutes |
| C053 | AB719106 | CMC(pH10,3.5 %) | ② | <i>Bacillus lehensis</i> | AY793550 | 677/678 (99%) | + + - - - | AT | 3.5 | N | ALK | Firmicutes |
| C054 | AB719107 | CMC(pH10,3.5 %) | ② | <i>Vibrio mediterranei</i> | X74710 | 776/779 (99%) | - + - - - | FA | 3.5 | N | ALK | Proteobacteria |
| C055 | AB719108 | CMC(pH10,3.5 %) | ② | <i>Vibrio mediterranei</i> | X74710 | 757/771 (98%) | - + + - + | FA | 3.5 | N | ALK | Proteobacteria |
| C056 | AB719109 | CMC(pH10,3.5 %) | ② | <i>Bacillus gibsonii</i> | X76446 | 797/801 (99%) | - + - - - | A | 15 | N | ALK | Firmicutes |
| C059 | AB719110 | S(pH7,10 %) | ① | <i>Staphylococcus haemolyticus</i> | X66100 | 761/763 (99%) | - - - - - | FA | 20 | N | ALK | Firmicutes |
| C061 | AB719111 | S(pH7,10 %) | ① | <i>Halobacillus trueperi</i> | AJ310149 | 841/844 (99%) | - - - - - | AT | 20 | N | ALK | Firmicutes |
| C064 | AB719112 | S(pH7,10 %) | ② | <i>Staphylococcus warneri</i> | L37603 | 829/829 (100%) | - - - - - | FA | 15 | N | ALK | Firmicutes |
| C065 | AB719113 | S(pH7,10 %) | ② | <i>Bacillus vietnamensis</i> | AB099708 | 578/578 (100%) | + - - - - | AT | 15 | N | ALK | Firmicutes |
| C066 | AB719114 | S(pH7,10 %) | ② | <i>Vibrio owensii</i> | GU018180 | 569/573 (99%) | - - - - - | A | 10 | N | ALK | Proteobacteria |
| C077 | AB719115 | S(pH10,3.5 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 520/524 (99%) | + + - - - | AT | 10 | TT | ALK | Firmicutes |
| C078 | AB719116 | S(pH10,3.5 %) | ① | <i>Photobacterium rosenbergii</i> | AJ842344 | 764/767 (99%) | - - + - - | FA | 3.5 | N | ALK | Proteobacteria |
| C079 | AB719117 | S(pH10,3.5 %) | ① | <i>Vibrio gallaecicus</i> | EU541605 | 734/759 (96%)* | - - + - - | FA | 3.5 | N | ALK | Proteobacteria |
| C080 | AB719118 | S(pH10,3.5 %) | ② | <i>Bacillus patagoniensis</i> | AY258614 | 518/521 (99%) | - + + - - | AT | 10 | N | ALK | Firmicutes |
| C082 | AB719119 | S(pH10,3.5 %) | ② | <i>Photobacterium rosenbergii</i> | AJ842344 | 445/449 (99%) | - - + - - | FA | 3.5 | N | ALK | Proteobacteria |
| C083 | AB719120 | S(pH10,3.5 %) | ① | <i>Bacillus oshimensis</i> | AB188090 | 419/423 (99%) | - + - - - | AT | 10 | N | ALK | Firmicutes |
| C086 | AB719121 | S(pH10,3.5 %) | ② | <i>Bacillus lehensis</i> | AY793550 | 551/555 (99%) | - + - - - | AT | 10 | N | ALK | Firmicutes |
| C087 | AB719122 | S(pH10,3.5 %) | ② | <i>Bacillus clausii</i> | X76440 | 441/449 (98%) | + - - - - | FA | 10 | TO | ALK | Firmicutes |
| C088 | AB719123 | S(pH10,3.5 %) | ② | <i>Bacillus hunanensis</i> | HM054473 | 793/819 (96%), Gaps = 2/819 (0%)* | + + - - - | AT | 10 | N | ALK | Firmicutes |
| C090 | AB719124 | S(pH10,10 %) | ② | <i>Bacillus lehensis</i> | AY793550 | 438/443 (98%), Gaps = 1/443 (0%)* | + - - - - | AT | 20 | N | ALK | Firmicutes |
| C091 | AB719125 | S(pH10,10 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 522/525 (99%) | - + - - - | AT | 20 | N | ALK | Firmicutes |
| C092 | AB719126 | S(pH10,10 %) | ② | <i>Bacillus oshimensis</i> | AB188090 | 668/668 (100%) | + - - - - | AT | 20 | N | ALK | Firmicutes |
| C093 | AB719127 | S(pH10,10 %) | ② | <i>Sporosarcina ureae</i> | AF202057 | 467/521 (89%), Gaps = 1/521 (0%)* | - + - - - | AT | 20 | TT | ALK | Firmicutes |
| C095 | AB719128 | AL(pH7, 10%) | ① | <i>Bacillus marisflavi</i> | AF483624 | 818/818 (100%) | - - - - - | A | 15 | N | ALK | Firmicutes |
| C096 | AB719129 | AL(pH7, 10%) | ① | <i>Vibrio alginolyticus/ Vibrio harveyi/ Vibrio communis</i> | X56576/ AY750575/ GU078672 | 513/513 (100%) | + - - - - | AT | 10 | N | ALK | Proteobacteria |
| C097 | AB719130 | AL(pH7, 10%) | ① | <i>Bacillus aquimaris</i> | AF483625 | 652/659 (98%) | + - - - - | A | 10 | N | NE | Firmicutes |
| C098 | AB719131 | AL(pH7, 10%) | ① | <i>Pseudoalteromonas tetraodonis</i> | AF214730 | 770/770 (100%) | + - - - - | A | 10 | N | NE | Proteobacteria |
| C099 | AB719132 | AL(pH7, 10%) | ② | <i>Staphylococcus haemolyticus</i> | X66100 | 596/597 (99%) | - - - - - | FA | 15 | N | NE | Firmicutes |
| C101 | AB719133 | AL(pH7, 10%) | ① | <i>Virgibacillus dokdonensis</i> | AY822043 | 678/680 (99%) | + - - - - | FA | 20 | TT | ALK | Firmicutes |
| C102 | AB719134 | AL(pH7, 10%) | ① | <i>Halomonas denitrificans</i> | AM229317 | 789/806 (98%) | - - - - - | AT | 20 | N | NE | Proteobacteria |
| C103 | AB719135 | AL(pH7, 10%) | ① | <i>Halobacillus salinus</i> | AF500003 | 834/836 (99%) | - - - - - | AT | 20 | N | NE | Firmicutes |
| C104 | AB719136 | AL(pH10,3.5 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 540/543 (99%) | + + - - - | AT | 10 | TT | ALK | Firmicutes |
| C105 | AB719137 | AL(pH10,3.5 %) | ① | <i>Vibrio mediterranei</i> | X74710 | 712/729 (97%) | - + + - + | A | 3.5 | N | ALK | Proteobacteria |
| C108 | AB719138 | AL(pH10,3.5 %) | ② | <i>Bacillus lehensis</i> | AY793550 | 833/836 (99%) | + + - - - | AT | 10 | N | ALK | Firmicutes |
| C109 | AB719139 | AL(pH10,3.5 %) | ② | <i>Bacillus plakortidis</i> | AJ880003 | 781/795 (98%), Gaps = 1/795 (0%)* | - - + - - | AT | 10 | N | ALK | Firmicutes |
| C110 | AB719140 | AL(pH10,3.5 %) | ② | <i>Bacillus horneckiae</i> | EU861362 | 657/657 (100%) | - + + - - | AT | 3.5 | N | ALK | Firmicutes |
| C111 | AB719141 | AL(pH10,3.5 %) | ② | <i>Bacillus clausii</i> | X76440 | 489/490 (99%) | + + - - - | AT | 3.5 | TO | ALK | Firmicutes |
| C112 | AB719142 | AL(pH10,3.5 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 740/743 (99%) | + + - - - | AT | 10 | TT | ALK | Firmicutes |
| C113 | AB719143 | AL(pH10,3.5 %) | ① | <i>Bacillus hunanensis</i> | HM054473 | 816/817 (99%) | - + + - - | A | 3.5 | N | ALK | Firmicutes |
| C115 | AB719144 | AL(pH10,3.5 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 551/556 (99%) | + + + - - | AT | 10 | N | ALK | Firmicutes |
| C116 | AB719145 | AL(pH10,3.5 %) | ① | <i>Geomicrobium halophilum</i> | AB449106 | 747/823 (90%), Gaps = 2/823 (0%)* | + - + - - | AT | 15 | N | ALK | Firmicutes |

| | | | | | | | | | | | | |
|------|----------|----------------|---|--|----------------------------|------------------------------------|-----------|----|-----|----|-----|----------------|
| C117 | AB719146 | AL(pH10,3.5 %) | ② | <i>Bacillus patagoniensis</i> | AY258614 | 414/437 (94%) * | + + - + - | AT | 3.5 | N | ALK | Firmicutes |
| C119 | AB719147 | AL(pH10,3.5 %) | ② | <i>Bacillus lehensis</i> | AY793550 | 817/821 (99%), Gaps = 1/821 (0%) | + + - - - | AT | 10 | N | ALK | Firmicutes |
| C122 | AB719148 | AL(pH10,10 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 819/827 (99%) | - - + - - | AT | 15 | N | ALK | Firmicutes |
| C123 | AB719149 | AL(pH10,10 %) | ① | <i>Bacillus lehensis</i> | AY793550 | 540/544 (99%) | + + - - - | A | 15 | N | ALK | Firmicutes |
| C124 | AB719150 | AL(pH10,10 %) | ① | <i>Nocardiopsis salina</i> | AY373031 | 781/793 (98%) | + + + - - | A | 15 | N | ALK | Actinobacteria |
| C125 | AB719151 | AL(pH10,10 %) | ① | <i>Geomicrobium halophilum</i> | AB449106 | 717/788 (90%), Gaps = 1/788 (0%) * | - - + - - | AT | 15 | N | ALK | Firmicutes |
| C126 | AB719152 | XL(pH7, 3.5%) | ① | <i>Bacillus aerophilus/ Bacillus stratosphericus</i> | AJ831844/ AJ831841 | 829/829 (100%) | - - - + - | AT | 10 | TO | ALK | Firmicutes |
| C127 | AB719153 | XL(pH7, 3.5%) | ① | <i>Vibrio alginolyticus</i> | X56576 | 601/601 (100%) | + - - - - | FA | 10 | N | ALK | Proteobacteria |
| C128 | AB719154 | XL(pH7, 3.5%) | ① | <i>Ruegeria lacuscaerulensis</i> | U77644 | 734/753 (97%) | - - - - - | AT | 3.5 | N | NE | Proteobacteria |
| C129 | AB719155 | XL(pH7, 3.5%) | ① | <i>Vibrio gigantis</i> | EF094888 | 828/831 (99%), Gaps = 2/831 (0%) | - - + - - | FA | 3.5 | N | NE | Proteobacteria |
| C130 | AB719156 | XL(pH7, 3.5%) | ① | <i>Bacillus plakortidis</i> | AJ880003 | 592/593 (99%) | - - - - - | AT | 10 | TT | ALK | Firmicutes |
| C131 | AB719157 | XL(pH7, 3.5%) | ② | <i>Bacillus stratosphericus</i> | AJ831841 | 454/462 (98%) | - - - + - | AT | 10 | TO | ALK | Firmicutes |
| C132 | AB719158 | XL(pH7, 3.5%) | ② | <i>Vibrio parahaemolyticus</i> | AF388386 | 816/821 (99%), Gaps = 2/821 (0%) | + - - - - | FA | 10 | N | ALK | Proteobacteria |
| C134 | AB719159 | XL(pH7, 3.5%) | ② | <i>Bacillus gibsonii</i> | X76446 | 809/813 (99%) | - - - - - | A | 3.5 | TT | ALK | Firmicutes |
| C135 | AB719160 | XL(pH7, 3.5%) | ② | <i>Shewanella gaetbuli</i> | AY190533 | 787/791 (99%) | - - + - + | FA | 3.5 | N | NE | Proteobacteria |
| C136 | AB719161 | XL(pH7, 3.5%) | ② | <i>Vibrio gigantis/ Vibrio crassostreae</i> | EF094888/ EF094887 | 817/820 (99%) | + - + - - | FA | 3.5 | N | NE | Proteobacteria |
| C137 | AB719162 | XL(pH7, 3.5%) | ② | <i>Bacillus altitudinis</i> | AJ831842 | 776/776 (100%) | - - - + - | AT | 15 | TO | ALK | Firmicutes |
| C138 | AB719163 | XL(pH7, 3.5%) | ② | <i>Vibrio harveyi</i> | AY750575 | 553/558 (99%) | + - - - - | FA | 15 | N | ALK | Proteobacteria |
| C139 | AB719164 | XL(pH7, 3.5%) | ① | <i>Pseudalteromonas mariniglutinosa</i> | AJ507251 | 601/604 (99%) | - - - - - | FA | 3.5 | N | NE | Proteobacteria |
| C140 | AB719165 | XL(pH7, 3.5%) | ① | <i>Gracilibacillus urelyticus</i> | EU709020 | 529/546 (96%) * | - - - - - | AT | 10 | TT | ALK | Firmicutes |
| C141 | AB719166 | XL(pH7, 3.5%) | ① | <i>Vibrio owensii</i> | GU018180 | 504/507 (99%) | + - - - - | FA | 10 | N | ALK | Proteobacteria |
| C142 | AB719167 | XL(pH7, 3.5%) | ① | <i>Gracilibacillus diposauri</i> | AB101591 | 606/606 (100%) | + - - + - | A | 15 | TT | NE | Firmicutes |
| C143 | AB719168 | XL(pH7, 3.5%) | ① | <i>Vibrio communis</i> | GU078672 | 763/767 (99%) | + - + - - | FA | 3.5 | N | NE | Proteobacteria |
| C145 | AB719169 | XL(pH7, 3.5%) | ① | <i>Bacillus pumilus</i> | AY876289 | 589/589 (100%) | - - - + - | AT | 10 | TO | ALK | Firmicutes |
| C146 | AB719170 | XL(pH7, 3.5%) | ① | <i>Vibrio harveyi</i> | AY750575 | 817/824 (99%), Gaps = 1/824 (0%) | + - + - - | FA | 10 | N | ALK | Proteobacteria |
| C148 | AB719171 | XL(pH7, 3.5%) | ① | <i>Vibrio alginolyticus</i> | X56576 | 639/639 (100%) | + - - - - | FA | 10 | N | ALK | Proteobacteria |
| C150 | AB719172 | XL(pH7, 3.5%) | ① | <i>Vibrio communis</i> | GU078672 | 798/803 (99%) | + - + - - | FA | 3.5 | N | NE | Proteobacteria |
| C151 | AB719173 | XL(pH7, 3.5%) | ① | <i>Vibrio alginolyticus/ Vibrio harveyi/ Vibrio communis</i> | X56576/ AY750575/ GU078672 | 550/551 (99%) | + - - - - | FA | 10 | N | ALK | Proteobacteria |
| C152 | AB719174 | XL(pH7, 3.5%) | ① | <i>Oceanobacillus profundus</i> | DQ386635 | 433/433 (100%) | - - - - - | AT | 10 | TT | ALK | Firmicutes |
| C154 | AB719175 | XL(pH7, 3.5%) | ② | <i>Vibrio rotiferianus</i> | AJ316187 | 812/818 (99%) | + - + - - | FA | 3.5 | N | NE | Proteobacteria |
| C155 | AB719176 | XL(pH7, 3.5%) | ② | <i>Bacillus horneckiae</i> | EU861362 | 589/589 (100%) | - - - - - | A | 3.5 | TT | ALK | Firmicutes |
| C156 | AB719177 | XL(pH7, 3.5%) | ② | <i>Bacillus horneckiae</i> | EU861362 | 606/606 (100%) | - - - - - | A | 3.5 | N | ALK | Firmicutes |
| C157 | AB719178 | XL(pH7, 3.5%) | ② | <i>Vibrio azureus</i> | AB428897 | 717/725 (98%) | - - + - - | FA | 3.5 | N | NE | Proteobacteria |
| C159 | AB719179 | XL(pH7, 3.5%) | ② | <i>Vibrio natriegens/ Vibrio alginolyticus</i> | X74714/ X56576 | 646/646 (100%) | + - - - - | FA | 10 | N | ALK | Proteobacteria |
| C160 | AB719180 | XL(pH7, 3.5%) | ② | <i>Virgibacillus dokdonensis</i> | AY822043 | 522/524 (99%) | + - - - - | FA | 20 | TT | ALK | Firmicutes |
| C161 | AB719181 | XL(pH7, 3.5%) | ② | <i>Vibrio communis</i> | GU078672 | 800/804 (99%) | + - - - - | FA | 3.5 | N | NE | Proteobacteria |
| C164 | AB719182 | XL(pH7, 10%) | ① | <i>Bacillus marisflavi</i> | AF483624 | 816/818 (99%) | - - - - - | AT | 15 | N | ALK | Firmicutes |
| C165 | AB719183 | XL(pH7, 10%) | ① | <i>Bacillus megaterium</i> | D16273 | 807/809 (99%) | + - - - - | A | 10 | N | NE | Firmicutes |
| C170 | AB719184 | XL(pH7, 10%) | ② | <i>Vibrio natriegens</i> | X74714 | 700/700 (100%) | + - - - - | A | 10 | N | ALK | Proteobacteria |
| C171 | AB719185 | XL(pH7, 10%) | ② | <i>Bacillus marisflavi</i> | AF483624 | 599/602 (99%) | + - + - - | AT | 15 | N | NE | Firmicutes |
| C174 | AB719186 | XL(pH7, 10%) | ② | <i>Halobacillus trueperi</i> | AJ310149 | 808/811 (99%) | - - - - - | AT | 25 | N | NE | Firmicutes |
| C175 | AB719187 | XL(pH7, 10%) | ② | <i>Oceanobacillus profundus</i> | DQ386635 | 543/543 (100%) | - - - - - | AT | 15 | TT | ALK | Firmicutes |
| C176 | AB719188 | XL(pH7, 10%) | ① | <i>Bacillus marisflavi</i> | AF483624 | 444/445 (99%) | + - - - - | AT | 15 | TT | NE | Firmicutes |
| C177 | AB719189 | XL(pH7, 10%) | ① | <i>Paraerskovia marina</i> | AB445007 | 709/709 (100%) | - - + - - | AT | 10 | N | NE | Actinobacteria |
| C180 | AB719190 | XL(pH7, 10%) | ① | <i>Gracilibacillus diposauri</i> | AB101591 | 827/827 (100%) | + - - + - | AT | 15 | TT | NE | Firmicutes |

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|---|----------|---------------|---|-------------------------------|----------|----------------|---|---|---|---|---|----|----|----|-----|------------|
| C184 | AB719191 | XL(pH7, 10%) | ② | <i>Bacillus hwajirpoensis</i> | AF541966 | 523/523 (100%) | - | - | - | - | - | AT | 25 | N | NE | Firmicutes |
| C187 | AB719192 | XL(pH10,3.5%) | ① | <i>Bacillus clarkii</i> | X76444 | 566/567 (99%) | - | - | - | - | - | AT | 10 | TT | ALK | Firmicutes |
| C188 | AB719193 | XL(pH10,3.5%) | ② | <i>Bacillus humanensis</i> | HM054473 | 816/817 (99%) | + | - | - | - | - | AT | 10 | TT | ALK | Firmicutes |
| C189 | AB719194 | XL(pH10,3.5%) | ① | <i>Bacillus lehensis</i> | AY793550 | 817/820 (99%) | + | + | - | - | - | AT | 10 | TT | ALK | Firmicutes |
| C190 | AB719195 | XL(pH10,3.5%) | ② | <i>Bacillus lehensis</i> | AY793550 | 659/666 (98%) | + | + | - | - | - | AT | 10 | TT | ALK | Firmicutes |
| C192 | AB719196 | XL(pH10,10%) | ② | <i>Bacillus lehensis</i> | AY793550 | 822/830 (99%) | - | + | - | - | - | AT | 10 | N | ALK | Firmicutes |
| C196 | AB719197 | XL(pH10,10%) | ② | <i>Bacillus polygoni</i> | AB292819 | 798/798 (100%) | - | + | - | - | - | AT | 10 | N | ALK | Firmicutes |
| C197 | AB719198 | XL(pH10,10%) | ① | <i>Bacillus lehensis</i> | AY793550 | 523/526 (99%) | + | + | - | - | - | AT | 10 | N | ALK | Firmicutes |
| C201 | AB719199 | XL(pH10,10%) | ② | <i>Bacillus lehensis</i> | AY793550 | 548/551 (99%) | + | + | - | - | - | A | 10 | N | ALK | Firmicutes |
| Abbreviations: S, starch; CMC, carboxymethyl cellulose sodium salt; AL, alginate; XL, xylan; FA, facultative anaerobic bacteria; AT, anaerobic tolerant bacteria; A, aerobic bacteria; TO, thermophiles; TT, thermo-tolerant bacteria; N, non-thermal bacteria; NE, neutrophilic bacteria; ALK, alkaliphilic bacteria. Display of more than one species in the column of nearest type strain species indicated the same identity in the comparison range. *: means the new species or genus | | | | | | | | | | | | | | | | |