Chapter V

General discussion

General discussion

Benthic decomposing bacteria play a key role in material cycling on the earth and are rich in sedimentary detritus. Deposit-feeding sea cucumbers are the major bacteria-related detritus consumers and play an important role in improvement of sediment quality and nutrient cycles. The main aim of this study was to investigate the biological diversity, the physiological and biochemical characteristics of intestinal bacteria of sea cucumbers. In this study different species of sea cucumbers e.g. *H. leucospilota*, *Stichopus japonicus* and deep-sea holothurians that were collected form different places, were used to compile basic information of the bacterial flora associated with sea cucumbers.

In chapter Ⅱ, totally 141 aerobic culturable isolates were isolated from the intestine of sea cucumber *H. leucospilota* (2 specimens) collected at coastal waters of Ko-e cho, Nagasaki. The result of analysis of 16S ribosomal RNA (rRNA) gene showed that these isolates were tentatively affiliated with 55 described species which were divided into three groups: the phylum Firmicutes, the phylum Protobacteria and the phylum Actinobacteria. High diversity was observed in genera *Bacillus* and *Vibrio* that widely distributed in marine environments, e.g. marine sediments and marine animals. Twenty-three isolates were found in both specimens and 9 species belonged with the genus *Bacillus* and other 9 species with the genus *Vibrio*. These results suggested that the intestine of holothurians was one of the suitable habitats for these bacteria. Many

isolates from both samples showed degradation ability of one or multiple polysaccharides. Among the 55 species, 29, 12, 24, 5, and 4 species showed degradation ability of potato starch (S), carboxymethyl cellulose sodium salt (CMC), sodium alginate (AL), xylan (XL) and agar, respectively.

On the other side, most isolates were facultative anaerobic or anaerobic tolerant bacteria. The facultative anaerobic (FA) isolates were mainly affiliated to the genus *Vibrio* and they degraded starch, alginate and agar. The anaerobic tolerant (AT) isolates were mainly affiliated to the genus *Bacillus* and they degraded starch, CMC and xylan. Anaerobic growth experiment (2 days) showed that all the aerobic bacteria (A) could form colonies in aerobic cultivation after 2-day anaerobic condition.

Although there has never been convincing evidence for intestinal environments of sea cucumbers, it is highly probable that oxygen will enter from mouth with the detritus food and also some amount can penetrate from the body tissues. Some regions in the intestine can contain more of less oxygen, and these aerobic bacteria can play a role in the gut symbiotic system. I proposed that a facultative symbiotic association existed among host *H.leucospilota*, the aerobic bacteria and the bacteria unique to the intestine. In intestine, the majority of isolates could provide degrading enzymes and /or metabolites (fermentation products) useful for their host.

In chapter Ⅲ, various aerobic culturable bacteria (1131 isolates) were isolated from intestines of the sea cucumber *Apostichopus japonicus* individuals with different body size and body colour and environment samples (sea water and sediment). By molecular analysis of partial 16S rRNA gene sequencess of 231 isolates, they were

tentatively affiliated with 53 described species in the phyla Firmicutes (42 species), Proteobacteria (9 species) and Actinobacteria (2 species). High diversity was observed in the genus *Bacillus* (20 species), *Oceanobacillus* (6 species) and *Virgibaillus* (4 species), but there were no isolates affiliated to members of the genus *Vibrio*, well-known sea pathogens. Microbal diversity was almost similar among the sea cucumber samples with different body size and body colour. Twenty-seven, 14 and 14 species from the intestines showed amylase activity, cellulase activity and xylanase activity, respectively. No isolates showing alginate or agar degradation activities were detected. Most of the species showing various polysaccharides degradation activities belonged to the families Bacillaceae 1 and 2. The bacterial diversity of polysaccharide degrading isolates was almost similar among samples from the 6 kinds of sea cucumbers and the sea sediment except xylan degradation activity. Diversity of FA, AT and A groups was similar between the intestines and the sea sediment and most of the isolates belonged to the families Bacillaceae 1 and 2. These results indicated that most isolates were alive in the intestine of the sea cucumbers. It is proposed that facultative symbiotic relation also existed among and *A. japonicus* intestinal bacteria.

Surprisingly, no isolates affiliated to members of the genus *Vibrio* were detected. It was demonstrated that the frequency and level of *Vibrio* species were much lower during winter than summer months (Chowdhury et al., 1990; Colwell, 1979). The seawater temperature of the open sea near Nagasaki area was ca.15℃ in Jan. 2011 (Data from Japan Meteorological Agency). In contrast, Omura bay where the samples were collected was inland bay and the seawater temperature was less than 10℃ in

winter, ca.5 degree lower than the open seawater temperature near Nagasaki. The sampling was carried out in winter, and it is possible that low temperature of sea water affected the microbial community in the intestine of the sea cucumber.

In chapter Ⅳ, in total, 92 isolates (45 species) were purified from different parts of deep-sea holothurians. Analysis of partial 16S rRNA gene sequences of the isolates showed the isolates belonged to the phyla Fimicutes (33 species) and Proteobacteria (12 species). High diversity was observed in the genera *Bacillus* (21 species) and *Vibrio* (6 species). Nineteen species, 19 species and 23 species were obtained from the anterior, mid and posterior part of intestine, respectively. Fourteen species were detected in multiple parts of intestine. On the other hand, halobacteria, FA bacteria, AT bacteria and alkaliphilic/alkali-tolerant bacteria were detected and mainly belonged with phylum Firmicutes, family Bacillaceae.

Many isolates showed polysaccharides degradation ability and degraded one or more substrates (S, CMC, AL and XL). These isolates were mainly belonged with the genus *Bacillus*. Twelve *Bacillus* species with starch degradation ability were found in multiple locations of three parts of the intestine. It was observed that in the posterior part, the number of xylan degrading species belonging to the family Bacilliaceae 1 increased and the number of starch degrading species belonging to the class Gammaproteobacteria also increased. This result indicated that the posterior part of the intestine might had different intestinal environment or functions comparing the anterior or mid parts.

In conclusion, various aerobic culturable bacteria belonged with the phyla Firmicutes, Protobacteria and Actinobacteria were isolated and analyzed (Table 1). It was clearly demonstrated that high bacterial diversity was observed in the intestine of sea cucumbers. These study results indicated that the facultative symbiotic relationship existed between the host sea cucumber and intestinal bacteria. As a host, sea cucumbers supply habitat and food source to intestinal bacteria. On the other hand, aerobic bacteria might also promote the forming of anaerobic gut environment by consuming the dissolved oxygen in the intestine of sea cucumbers. Metabolites (e.g. vitamin and organic acids) which are beneficial for host sea cucumbers, might be provided by intestinal bacteria via decomposition of polysaccharides.

Based on the above results, it is proposed that sea cucumber is favorable model organism for studying on the cooperative interaction among bacteria and hosts (Fig. 4, in chapter Ⅱ). The bacteria detected in this study had also been reported in other marine animals and marine environments. So this model may be also applicable to other marine deposit-feeding benthos, such as clamworms and amphipods (Fig. 1) (Nascimento et al, 2011).

By partial 16S rRNA gene sequence analysis, a number of isolates isolated from each sea cucumber sample showed less than 97% identities with any type strain sequences. This result suggested that the intestines of sea cucumbers were resources for new species.

As been well known, only 0.1% of the total population of bacteria from any natural environment is culturable (Aslam et al., 2010). Figure 2 shows the probable

relationship of unculturable microbes and culturable microbes in marine animals, such as sea cucumbers. Various bacteria inhabit in the intestine of sea animals. A large number of the unculturable microbes have strict environment selection (the obligate symbionts to one special environment), while a few culturable microbes can habit in various environments (shuttle microbes).

There is growing evidence that horizontal gene transter is a very important mechanism in genome evolution, particularly among prokaryotes (Lawrence, 1999). Bolotin demonstrated that horizontal gene transfer happened among the lactic bacterium *Streptococcus thermophilus* and *Lactococcus lactis* sub. *lactis* and resulted in decreasing pathogenic potential of *Streptococcus thermophilus*. (Bolotin et al, 2004). Recent research on Japanese gut microbes indicated that gut microbes seemed to gain genes for glycolytic enzymes (e.g. porphyranase and agarase) from marine bacteria associated with seaweeds by horizontal gene transfer (Hehemann et al., 2010). So, as shuttle microbes, the culturable microbes potentially play an important role in transporting genetic information to various environments.

In this study, I investigated the bacterial diversity and physiological characters including polysaccharide degradation ability of the isolates to understand the digestive symbiosis in sea cucumbers. In the future, a combination of culture-based approaches and metagenomic approaches should make it possible to analyze the symbiosis between bacteria and sea animals in more detail and to utilize the microbial resources for industrial applications.

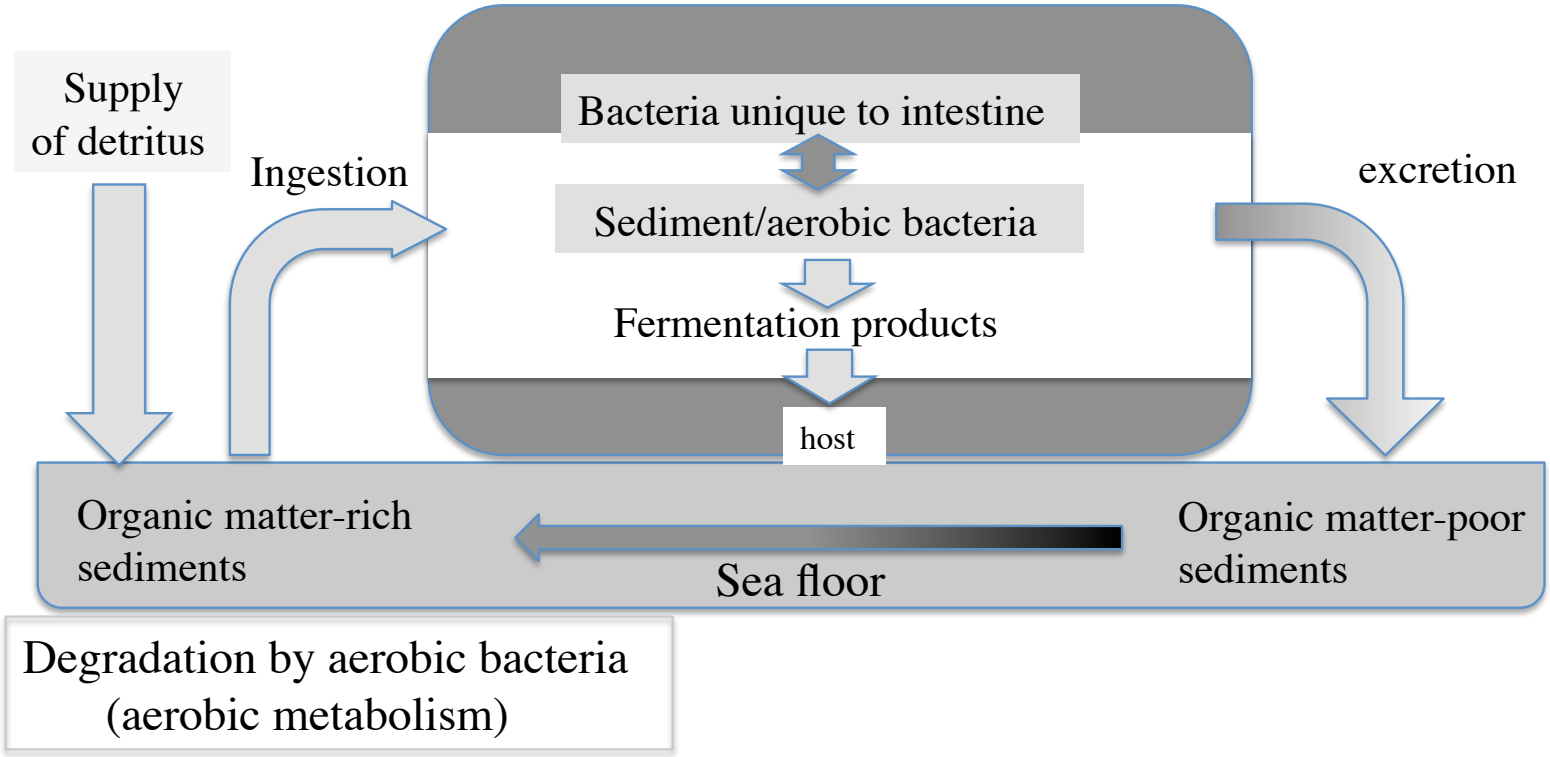

Fig. 1 Model for the facultative symbiotic association among host deposit-feeding benthos, aerobic bacteria and the bacteria unique to the intestine of host. For example, sea cucumbers, clamworms and amphipods.

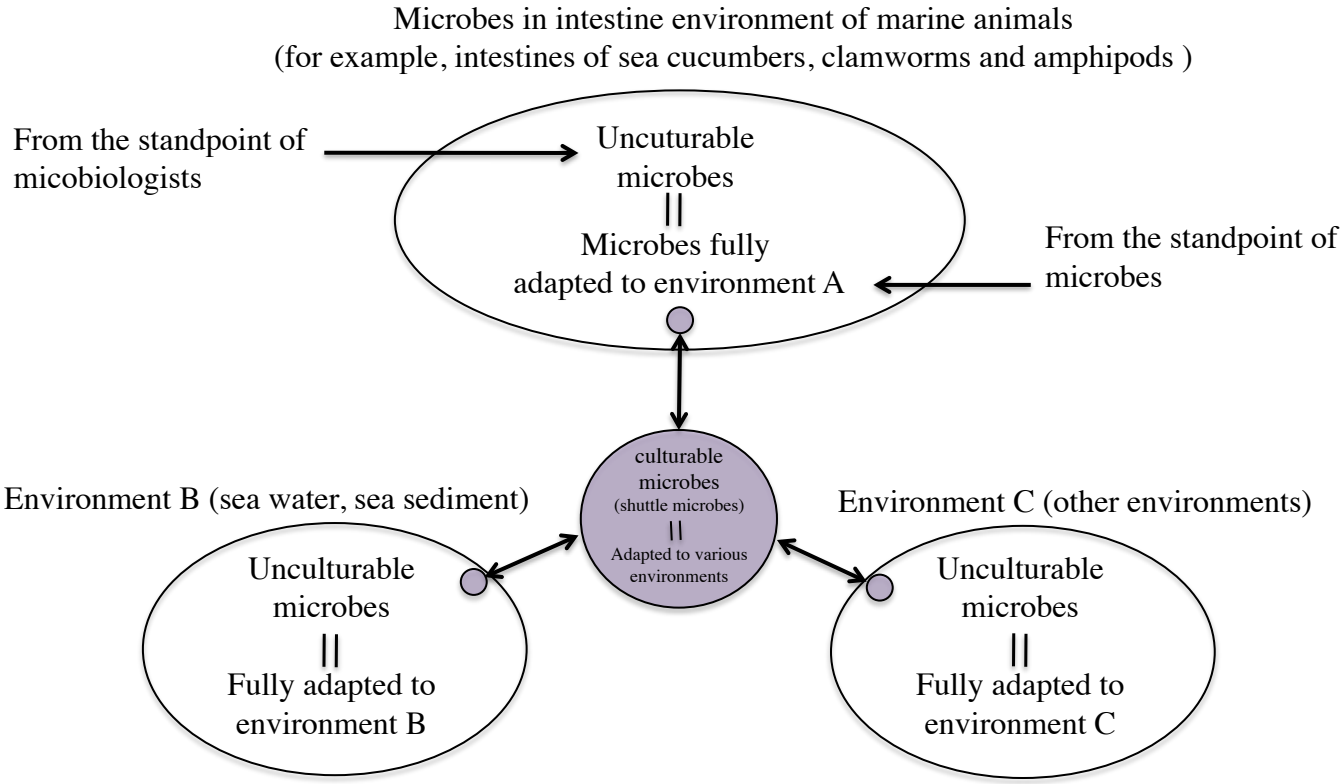

Fig. 2 Roles of Aerobic Culturable Microbes.
The aerobic culturable bacteria isolated from intestines of deposit-feeding animals might have three main function. ① Enhancing the forming of anaerobic intestinal environment by consuming dissolved oxygen in intestinal. ② Supplying metabolite (e.g. organic acids, vitamin and minor components) to host sea cucumbers. ③ Connecting intestinal environment to various environments, as shuttle bacteria (e.g. horizontal gene transfer).

Table1 Phylogenetic affiliation for isolates (464 strains) from various specimens

phylum/class/family	genus	species /tentative species		specimens					reference
				Holothuria leucospilota	*Apostichopus japonicus*	sediment	sea water	deep-sea holothurian	
phylum Firmicutes									
family Bacillaceae 1	*Bacillus* (34)	*Bacillus*	*aerophilus*					+	Shivaji et al., 2006
		Bacillus	*aerophilus/altitudinis*					+	Shivaji et al., 2006
		Bacillus	*aerophilus/altitudinis/stratosphericus*		+	+			Shivaji et al., 2006
		Bacillus	*aerophilus/stratosphericus*	+					Shivaji et al., 2006
		Bacillus	*altitudinis/stratosphericus*					+	Shivaji et al., 2006
		Bacillus	*altitudinis*	+					Shivaji et al., 2006
		Bacillus	*amyloliquefaciens*		+	+			Priest et al., 1987
		Bacillus	*aquimaris*	+	+	+		+	Yoon et al., 2003b
		Bacillus	*aryabhattai*		+	+		+	Shivaji et al., 2009
		Bacillus	*aurantiacus*					+	Borsodi et al., 2008
		Bacillus	*cereus*		+	+			** pp. 53, 65-67, 81, 92, 95
		Bacillus	*clarkii*	+					Nielsen et al., 1995
		Bacillus	*clarkii/polygoni*		+	+		+	Nielsen et al., 1995; Aino et al., 2008
		Bacillus	*clausii*	+	+	+		+	Nielsen et al., 1995
		Bacillus	*farraginis*		+	+			Scheldeman 2004
		Bacillus	*firmus*		+	+			** pp. 74, 99
		Bacillus	*flexus*					+	** pp. 74, 100
		Bacillus	*gibsonii*	+	+	+			Nielsen et al., 1995
		Bacillus	*hemicellulosilyticus*					+	Nogi et al., 2005
		Bacillus	*horikoshii*		+	+		+	Nielsen et al., 1995
		Bacillus	*horneckiae*	+					Vaishampayan et al., 2009
		Bacillus	*horti*					+	Yumoto et al., 1998
		Bacillus	*hunanensis*	+	+	+		+	Chen et al., 2011
		Bacillus	*hunanensis/lehensis*		+	+			Chen et al., 2011; Ghosh et al., 2007
		Bacillus	*hunanensis/oshimensis*					+	Chen et al., 2011; Yumoto et al., 2005
		Bacillus	*hwajinpoensis*	+				+	Yoon et al., 2004b
		Bacillus	*krulwichiae*		+	+			Yumoto et al., 2003
		Bacillus	*lehensis*	+				+	Ghosh et al., 2007
		Bacillus	*licheniformis*		+	+		+	Palmisano et al., 2001
		Bacillus	*marisflavi*	+	+	+		+	Yoon et al., 2003b
		Bacillus	*megaterium*	+				+	** pp. 75, 109
		Bacillus	*methylotrophicus*		+	+			Madhaiyan et al., 2010
		Bacillus	*murimartini*	+					Borchert et al., 2007
		Bacillus	*neizhouensis*					+	Chen et al., 2009
		Bacillus	*okhensis/krulwichiae*		+	+			Nowlan et al., 2006; Yumoto et al., 2003
		Bacillus	*okhensis/wakoensis*		+	+			Nowlan et al., 2006; Nogi et al., 2005
		Bacillus	*oshimensis*	+	+	+		+	Yumoto et al., 2005
		Bacillus	*patagoniensis*	+					Olivera et al., 2005
		Bacillus	*plakortidis*	+					Borchert et al., 2007
		Bacillus	*polygoni*	+	+	+			Aino et al., 2008
		Bacillus	*pseudalcaliphilus*					+	Nielsen et al., 1995
		Bacillus	*pseudofirmus*		+	+			Nielsen et al., 1995
		Bacillus	*pumilus*	+	+	+		+	Parvathi et al., 2007
		Bacillus	*pumilus/safensis*		+	+			Parvathi et al., 2007; Satomi et al., 2006
		Bacillus	*stratosphericus*	+					Shivaji et al., 2006
		Bacillus	*subtilis*		+				Palmisano et al., 2001
		Bacillus	*vietnamensis*	+	+			+	Noguchi et al., 2004
		Bacillus	*wakoensis*					+	Nogi et al., 2005
				20 species	26 species	18 species		25 species	

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phylum/class/family	genus	species /tentative species		specimens					reference
				Holothuria leucospilota	*Apostichopus japonicus*	sediment	sea water	deep-sea holothurian	
phylum Firmicutes									
family Bacillaceae 2	*Filobacillus* (1)	*Filobacillus*	*milensis*		+	+			Schlesner et al., 2001
family Bacillaceae 2	*Geomicrobium* (1)	*Geomicrobium*	*halophilum*	+	+	+			Echigo et al., 2010
family Bacillaceae 2	*Gracilibacillus* (4)	*Gracilibacillus*	*dipsosauri*	+	+			+	Waino et al., 1999
		Gracilibacillus	*halotolerans*		+				Waino et al., 1999
		Gracilibacillus	*ureilyticus*	+					Huo et al., 2010
		Gracilibacillus	*saliphilus*		+				Tang et al., 2009
family Bacillaceae 2	*Halobacillus* (3)	*Halobacillus*	*kuroshimensis*		+			+	Hua et al., 2007
		Halobacillus	*salinus*	+					Yoon et al., 2003a
		Halobacillus	*trueperi*	+	+			+	Spring et al., 1996
		Halobacillus	*yeomjeoni/trueperi/litoralis*		+				Yoon et al., 2005b; Spring et al., 1996
family Bacillaceae 2	*Halolactibacillus* (1)	*Halolactibacillus*	*alkaliphilus*		+				Cao et al., 2008
family Bacillaceae 2	*Oceanobacillus* (8)	*Oceanobacillus*	*chironomi*		+				Raats et al., 2007
			iheyensis	+					Lu et al., 2001
			kimchii		+	+		+	Whon et al., 2010
			oncorhynchi					+	Yumoto et al., 2005; Romano et al., 2006
			oncorhynchi subsp. incaldanensis		+	+			Romano et al., 2006
			picturae		+	+			Heyrman et al., 2003; Lee et al., 2006
			profundus	+	+	+			Kin et al., 2007
			sojae		+			+	Tominaga et al., 2009
family Bacillaceae 2	*Salsuginibacillus* (1)	*Salsuginibacillus*	*kocurii*		+				Carrasco et al., 2007
	Thalassobacillus (1)	*Thalassobacillus*	*devorans*					+	Garcia et al., 2005
family Bacillaceae 2	*Virgibacillus* (4)	*Virgibacillus*	*chiguensis*		+				Wang et al., 2008
		Virgibacillus	*dokdonensis*	+	+	+		+	Yoon et al., 2005a
		Virgibacillus	*halodenitrificans*		+			+	Denariaz et al., 1989; Yoon et al., 2004c
		Virgibacillus	*marismortui*		+			+	Arahal et al., 1999
		Virgibacillus	*marismortui/salarius*		+				Arahal et al., 1999; Hua et al., 2008
				8 species	21 species	7 species		10 species	
family Planococcaceae	*Jeotgalibacillus* (1)	*Jeotgalibacillus*	*campisalis*					+	Yoon et al., 2010
family Planococcaceae	*Lysinibacillus* (1)	*Lysinibacillus*	*fusiformis*		+				Priest et al., 1988; Ahmed et al., 2007
family Planococcaceae	*Planococcus* (1)	*Planococcus*	*maritimus*		+	+			Yoon et al., 2003c
family Planococcaceae	*Sporosarcina* (2)	*Sporosarcina*	*ureae*	+					Kampfer et al., 2010; Reddy 2003
		Sporosarcina	*saromensis*		+				An et al., 2007
family Staphylococcus	*Staphylococcus* (2)	*Staphylococcus*	*haemolyticus*	+					** pp. 394, 408
		Staphylococcus	*warneri*	+				+	** pp. 395, 419
				3 species	3 species	1 species		2 species	

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phylum Proteobacteria									
class alpha	*Pseudovibrio* (1)	*Pseudovibrio*	*japonicus*		+				Hosoya et al., 2007
	Ruegeria (1)	*Ruegeria*	*lacuscaerulensis*	+					Petursdottir et al., 1997
class gamma	*Ferrimonas* (1)	*Ferrimonas*	*senticii*		+				Campbell et al., 2007
class gamma	*Halomonas* (2)	*Halomonas*	*denitrificans*	+					Kim et al., 2007
		Halomonas	*meridiana*		+	+		+	Kaye et al., 2004
class gamma	*Marinobacter* (1)	*Marinobacter*	*alkaliphilus*					+	Takai et al., 2005
class gamma	*Photobacterium* (2)	*Photobacterium*	*lutimaris*					+	Jung et al., 2007
		Photobacterium	*rosenbergii*	+				+	Thompson et al., 2005
class gamma	*Pseudoalteromonas* (3)	*Pseudoalteromonas*	*mariniglutinosa*	+					Romanenko et al., 2003
		Pseudoalteromonas	*prydzensis*	+					Browman 1998
		Pseudoalteromonas	*tetraodonis*	+	+				Ivanova et al., 2001; Simidu et al., 1990
class gamma	*Pseudomonas* (5)	*Pseudomonas*	*cedrina*					+	
		Pseudomonas	*cedrina subsp. fulgida*		+				Dabboussi et al., 1999; Behrendt et al., 2009
		Pseudomonas	*gessardii*		+	+	+		Verhille et al., 1999
		Pseudomonas	*libaniensis*		+	+		+	Dabboussi et al., 1999
		Pseudomonas	*synxantha*					+	* pp. 373.
class gamma	*Psychrobacter* (2)	*Psychrobacter*	*celer*		+		+		Yoon et al., 2005c
		Psychrobacter	*nivimaris*		+		+		Heuchert et al., 2004
class gamma	*Shewanella* (1)	*Shewanella*	*gaetbuli*	+					Yoon et al., 2004a
class gamma	*Vibrio* (17)	*Vibrio*	*agarivorans*					+	Macian et al., 2001
		Vibrio	*alginolyticus*	+					Molitoris et al., 1985
		Vibrio	*alginolyticus/harvey/communis*	+					* pp. 521, 528-530; Chimetto et al., 2011
		Vibrio	*azureus*	+					Yoshizawa et al., 2009
		Vibrio	*brasiliensis*	+					Thompson et al., 2003
		Vibrio	*communis*	+					Chimetto et al., 2011
		Vibrio	*ezurae*	+					Sawabe et al., 2004
		Vibrio	*gallaecicus*	+					Beaz et al., 2009
		Vibrio	*gigantis*	+					Le Roux et al., 2005
		Vibrio	*gigantis/crassostreae*	+					Le Roux et al., 2005; Faury et al., 2004
		Vibrio	*harveyi*	+				+	* pp. 521, 528-529.
		Vibrio	*mediterranei*	+				+	Pujalte et al., 1986
		Vibrio	*natriegens*	+					* pp. 521, 537.
		Vibrio	*natriegens/alginolyticus*	+					* pp. 521, 530, 537.
		Vibrio	*neptunius*	+					Thompson et al., 2003
		Vibrio	*owensii*	+					Cano-Gomez et al., 2010
		Vibrio	*parahaemolyticus*	+					Molitoris et al., 1985
		Vibrio	*pomeroyi*					+	Thompson et al., 2003a
		Vibrio	*pomeroyi/gigantis*	+					Thompson et al., 2003a ; Le Roux et al., 2005
		Vibrio	*rotiferianus*	+				+	Gomez et al., 2002
		Vibrio	*tasmaniensis*	+					Thompson et al., 2003b
				23 species	9 species	3 species	3 species	14 species	

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phylum/class/family	genus	species /tentative species		specimens					reference
				Holothuria leucospilota	*Apostichopus japonicus*	sediment	sea water	deep-sea holothurian	
phylum Actinobacteria									
family Cellulomonadaceae	*Paraoerskovia* (1)	*Paraoerskovia*	*marina*	+					Khan et al., 2009
family Micrococcaceae	*Micrococcus* (1)	*Micrococcus*	*luteus*	+					Wieser et al., 2002
family Nocardiopsaceae	*Nocardiopsis* (2)	*Nocardiopsis*	*lucentensis*		+				Yassin et al., 1993
		Nocardiopsis	*salina*	+					Li et al., 2004
family Streptomycetaceae	*Streptomyces* (1)	*Streptomyces*	*gougerotii/rutgersensis*		+				Shirling et al., 1969; Shirling et al., 1968
family Williamsiaceae	*Williamsia* (1)	*Williamsia*	*serinedens*		+		+		Yassin et al., 2007
				3 species	4 species		1 species		
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