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ORIGINAL PAPER

Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough

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Abstract The Southern Okinawa Trough is an area of focused sedimentation due to particulate matter export from the shelf of the East China Sea and the island of Taiwan. In order to understand the geomicrobiological characteristics of this unique sedimentary environment, bacterial cultivations were carried out for an 8.61 m CASQ core sediment sample. A total of 98 heterotrophic bacterial isolates were characterized based on 16S rRNA gene phylogenetic analysis. These isolates can be grouped into four bacterial divisions, including 13 genera and more than 20 species. Bacteria of the *γ-Proteobacteria* lineage, especially those from the *Halomonas* (27 isolates) and *Psychrobacter* (20 isolates) groups, dominate in the culturable bacteria assemblage. They also have the broadest distribution along the depth of the sediment. More than 72.4% of the isolates showed extracellular hydrolytic enzyme activities, such as amylases, proteases, lipases and Dnases, and nearly 59.2% were cold-adapted exoenzyme-producers. Several *Halomonas* strains show almost all the tested hydrolases activities. The wide distribution of exoenzyme activities in the isolates may indicate their important ecological role of element biogeochemical cycling in the studied deep-sea sedimentary environment.

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Introduction

The Southern Okinawa Trough is an active marginal backarc basin at a nascent stage of evolution, where seamounts, hydrothermal vents and chimneys have been identified (Glasby and Notsu 2003; Hsu et al. 2003). This is also an area of focused sedimentation along the path of the Kuroshio Current (Wei et al. 2005; Jeng and Huh 2006), which is the biggest western boundary current of the Pacific Ocean. Due to its high speed, great depth and width, the Kuroshio Current transports a huge amount of momentum, materials, heat and moisture from the tropical western Pacific warm pool to the northern mid-latitudes, impacting significantly on the fishery and climate regime of the west Pacific Ocean and the bordering East Asia continent (Nakata and Hidaka 2003). Complicated interactions of hydrological, geological, chemical and biological processes at the water-sediment and land-ocean interfaces make the Southern Okinawa Trough area a focused study site in global change and marine environmental research (Wei 2005).

Evidences indicate that the Southern Okinawa Trough is an important site for particulate organic matter (POC) export from the island of Taiwan and the shelf of the East China Sea (Kao et al. 2003), which may stimulate the metabolic activity of sedimentary heterotrophic microorganisms. Extracellular enzymes produced by sediment bacteria play important roles in deposited and buried organic matter decomposition, nutrient recycling, and earth

element transformation and mobilization. Psychrophilic enzymes produced by deep-sea cold-adapted bacteria display a high catalytic efficiency, not only important for in situ biogeochemical processes, but also for their potentials in biotechnology and industry applications (Gerday et al. 2000; Demirjian 2001; van den Burg 2003). However, very little effort has been made to understand the microbial ecophysiology in the unique Southern Okinawa Trough sedimentary environment (Jean et al. 2005).

The current article studied the diversity of culturable heterotrophic bacteria and their extracellular hydrolytic enzymes, especially at low temperature, in the deep-sea sediments of the Southern Okinawa Trough.

Materials and methods

Sediment core collection and description

Deep-sea sediment cores were collected during Leg 2 of the Chinese–French joint MD147/MARCO POLO 1/IMAGES XII cruise of R/V Marion Dufresne in the tropical and sub-tropical western Pacific during May and June of 2005. An 8.61 m sediment CASQ core MD05-2907 (24°47.19′N, 122°29.30′E) was retrieved from the seafloor of the Southern Okinawa Trough at a water depth of 1,245 m (Fig. 1). The CASQ core sampler has a 25 × 25 cm^2 section area. On board, the box core was open from the side and sediment samples for microbial study were taken aseptically at depths 0.3, 2, 4, 6, and 8.6 mbsf (meters below the seafloor) and stored at 4°C in air-tight sterile plastic bags. The topmost 30 cm sediment was disturbed inside the core sampler, so sediment at depth 30 cm was collected instead of the very top sediment. The choice of this "surface" layer also avoided microbial contamination from the overlaying seawater. The samples, from the surface layer to the deepest layer, were named K0, K2, K4, K6, and K8 for further references.

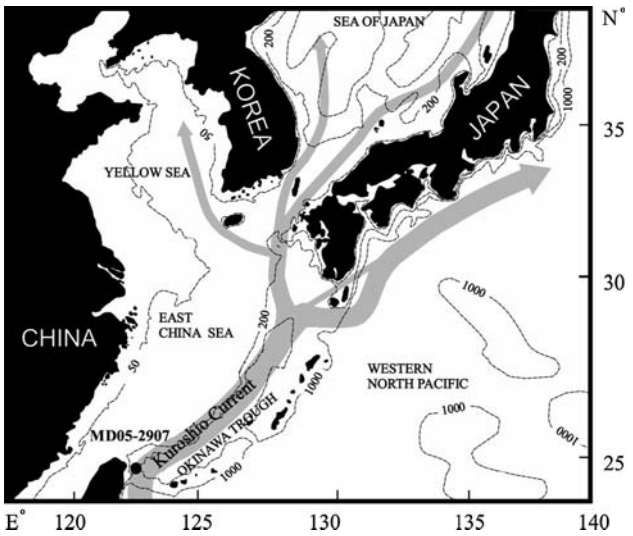

Fig. 1 Sampling site of core MD05-2907 at the Southern Okinawa Trough

Heterotrophic bacteria isolation

For heterotrophic bacteria cultivation, one gram of 4°C stored sediment samples from each depth was serially diluted in sterilized artificial seawater and plated in triplicates onto 2216 marine agar (Difco formula) or low nutrient 2216 marine agar (with only 10% original yeast extract and peptone concentrations) plates and cultivated at 28 or 4°C in the dark, respectively. After growing for several days to a few weeks, colonies were randomly picked and re-streaked 2–3 times to ensure purity of the isolates. A total of 98 strains were isolated. For clarity, each isolate was given a unique identifier, such as K0-28L-001, in which the first letter number combination stands for sediment layer, the second number letter combination designates cultivation condition (in this case the culture was grown at 28°C and on low nutrient 2216 marine agar medium, if the letter "L" is missing, then regular 2216 marine agar medium was used) and the third designated the serial number of the isolate.

The determination of facultative anaerobes of the isolates was carried out by stab culture method with 2216 marine agar medium. After incubation for a few days, all of the 98 isolates grew positively at the bottom of the test tube, indicating that they were all facultative anaerobic bacteria.

Phylogenies of the bacterial isolates

The phylogenies of the bacterial isolates were determined by 16S rRNA gene (16S rDNA) sequence analysis. A simple boiling method was used for rapid bacterial genomic DNA extraction (De Medici et al. 2003). For 16S rDNA amplification, primers 27F and 926R were used (Dang et al. 2006). PCR products were examined by electrophoresis on 1% agarose gels. Primer 27F was also used for DNA sequencing using an ABI 3770 automatic sequencer (Applied BioSystems, USA) with purified PCR products as templates. To simplify the molecular taxonomy analysis of the isolates, sequences with 98% or higher similarity were assumed to be potentially from a single species. The grouping of similar sequences was carried out using the DOTUR program (Schloss and Handelsman 2005).

Bioinformatic determination of the sequence affiliations followed the standard methods (Dang and Lovell 2000). For each sequence, a query was made by the online BLAST

program to the NCBI GenBank database for an initial determination of the nearest neighbor sequences (Altschul et al. 1997). Sequences were aligned using the CLUSTAL_X program (version 1.8) (Thompson et al. 1997), and the fragments (>730 bp) covering at least the V1 to V4 hypervariable regions of bacterial 16S rDNA were used for phylogenetic analysis. Phylogenetic trees were constructed with programs of the PHYLIP package (version 3.65) (Felsenstein 1989). Program DNADIST was used for distance matrix calculation and phylogenetic trees were constructed from evolutionary distances by the neighbor-joining method (Saitou and Nei 1987) implemented through the program NEIGHBOR.

The 16S rDNA sequences determined have been deposited in the NCBI GenBank database under accession number DQ356954 to DQ357051.

Screening of extracellular hydrolytic enzymes

The bacterial extracellular amylases, acidic (pH 5.0) and neutral (pH 7.0) proteases, lipases and chitinases were screened using 2216 marine agar plates supplemented with 0.5% (w/v) soluble starch (Sangon, China) (Sánchez-Porro et al. 2003), 2% (w/v) sterile skim milk (Oxoid, UK) (Zhang and Austin 2000), 1% (v/v) Tween 80 (polyoxyethylene sorbitan mono-oleate) (Sigma, USA) and 1% (w/v) arabic gum powder (Sigma, USA) (Moreno and Landgraf 1998), and 1% (w/v) arabic gum powder and 13–14% (wet weight) colloidal chitin prepared by the method of Gómez Ramírez et al. (2004) and Rojas-Avelizapa et al. (1999), respectively. The extracellular alkaline proteases were screened by LB agar plates adjusted to pH 10.0 and 3% NaCl salinity. The extracellular Dnases were screened using Dnase test agar (Haibo, China) plates adjusted to 3% NaCl salinity (West and Colwell 1984). For the detection of amylase-producers, the plates were flooded with 0.3% I_2—0.6% KI solution. For the detection of Dnase-producers, the plates were flooded with 1 M HCl solution. For all the tested enzymes, a clear or dim halo around a colony after incubation at 28 or 4°C for several days to a few weeks indicated a positive exoenzyme-producing isolate.

Results

Phylogenies of the bacterial isolates

Most of the 16S rDNA sequences of our isolates had quite high sequence identity (usually >98%) to the nearest neighboring GenBank sequences, usually determined from culturable bacterial strains. Several isolates, such as K2-28-058, K0-28L-010, K6-28L-034, K2-28-011 and the closely related strains, have only moderate 16S rDNA sequence similarity (94–97%) to their GenBank best match sequences from taxonomically well determined bacteria species, indicating that they may be new species or even new genus. The strain K2-28-058 was eventually identified as a new species in a new bacterial genus, *Wangia profunda* (Qin et al. 2007). Most of the 16S rDNA GenBank nearest neighboring sequences of our isolates were originally obtained from deep-sea sediment environments, consistent to the in situ environmental characteristics of our isolates. The Southern Okinawa Trough deep-sea sediments harbored typical deep-sea sedimentary bacteria and certain novel bacterial microorganisms.

Based on the phylogeny of the nearest neighboring GenBank sequences, four bacterial groups at division or phylum level could be recognized in our isolates, including *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* of the α- and γ-*Proteobacteria* subdivisions. The isolates are quite diverse, a total of 14 different genera can be identified, including *Alcanivorax*, *Bacillus*, *Cobetia*, *Halomonas*, *Methylarcula*, *Micrococcus*, *Myroides*, *Paracoccus*, *Planococcus*, *Pseudomonas*, *Psychrobacter*, *Sporosarcina*, *Sufflavibacter* and *Wangia* (Table 1). Molecular systematic analysis of the 16S rDNA sequences of our bacterial isolates further confirmed most of their phylogenetic affiliations inferred from the nearest neighbor sequences, and more than 20 bacterial species may be identified (Fig. 2). However, based on our phylogenetic analysis, the genus *Sufflavibacter* should be a synonym of the genus *Wangia* (Qin et al. 2007; Kwon et al. 2007). Thus, our isolates belong to 13 different bacterial genera described above, excluding *Sufflavibacter*. The moderately halophilic marine *Halomonas* isolates (7 species) showed the highest inter-species diversity. They are also the most abundant bacterial group (27 isolates) of our isolates, followed by the *Psychrobacter* (20 isolates) and *Pseudomonas* (12 isolates) species.

The use of low nutrient media and in situ temperature (4°C) for initial incubation benefited us for isolating diverse microbial strains. Several strains, including 1 *Alcanivorax* isolate, 7 *Halomonas* isolates, 8 *Pseudomonas* isolates and 2 *Wangia* isolates, were only isolated from low nutrient agar plates (Table 1), and a few other strains, including 2 *Halomonas* isolates, 1 *Myroides* isolate and 1 *Sporosarcina* isolate, were only isolated from in situ temperature (4°C) cultivations. All the strains collected from the deepest sediment sample (K8, at 8.6 mbsf) were isolated from low nutrient media, in situ temperature incubation, or the combination of these two conditions.

Screening of extracellular hydrolytic enzymes

Of the 98 isolates screened, more than 72.4% showed extracellular hydrolytic enzyme activities, and nearly

Table 1 Potential phylogenetic affiliations of the cultivated heterotrophic bacteria isolated from the Southern Okinawa Trough deep-sea sediment

Nearest neighbor of partial 16S rDNA sequence	GenBank accession number	Isolates number in our strain collection	Similarity (%)
α-Proteobacteria			
Paracoccus sp. JL1148	DQ985067	K0-28L-028, K4-28-038, K4-28L-025, K8-4L-015	100
Bacterium WP3ISO7	DQ985868	K0-28-006, K0-28L-002, K0-28L-012	99
γ-Proteobacteria			
Cobetia sp. MACL02	EF198244	K0-28-003, K0-28-008, K0-28L-003, K0-28L-013, K0-4-001, K0-4-008, K0-4-014, K0-4L-001, K0-4L-002, K0-4L-003, K4-4L-006, K4-4L-007	99–100
Halomonas sp. BSi20362	EF673259	K0-4-006, K0-4-010	98
Halomonas sp. Y2	EF205533	K0-28-001, K0-4-004, K6-28-041	99
Halomonas sp. Sa5-2XX	AB305245	K0-28L-004	99
Halomonas sp. HI10	EF554891	K0-28L-011, K6-28L-030, K6-28L-034	99–100
Halomonas sp. Splume4.1864c	AF212216	K0-28L-040, K4-28-020, K4-28L-022, K4-4-025, K4-4L-005, K6-4-035, K8-4L-014	99–100
Halomonas sp. Y19	EF177668	K0-28L-010	100
Halomonas sp. IW2-2	AB305253	K2-28-012	99
Halomonas sp. MBIC2031	AB025599	K2-28-059	100
Halomonas hydrothermalis	AF212218	K4-28L-015, K4-28L-028	99
Uncultured organism clone ctg_NISA323	DQ396152	K4-28-015, K2-28-055, K2-28L-053, K2-28L-057, K4-28-034, K8-28L-038	100
Uncultured bacterium clone S-14	EF118004	K0-28-004	99
Alcanivorax sp. EPR 10	AY394868	K2-28L-051	100
Pseudomonas sp. QZ1	EF542804	K0-28-007, K0-4-017, K0-4-018	99–100
Pseudomonas sp. GW12	EF550162	K6-28-040	99
Pseudomonas stutzeri	AJ312176	K6-28L-029, K6-28L-031, K6-28L-032, K6-28L-033, K6-28L-035, K6-4L-018, K8-28L-039	99–100
Uncultured bacteria clone W26	AY770966	K0-28L-041	99
Psychrobacter sp. "A1 isolate-4"	EF474164	K0-4L-004, K2-28-054, K2-28-057, K2-28L-043, K2-4L-010, K4-28-036, K4-4L-008, K8-28L-037, K8-4L-016	99–100
Uncultured organism clone ctg_CGOF227	DQ395702	K2-4L-009, K2-4L-011, K4-4-028, K4-28-031, K4-28L-019, K4-28L-020, K4-28L-024, K4-28L-027, K4-4-020, K8-4-041, K8-4L-013	99–100
Bacteroidetes			
Flavobacterium sp. V4.MO.31	AJ244697	K2-28-052, K2-28-053, K2-28-058, K2-28L-052	99–100
Sufflavibacter litoralis IMCC 1001	DQ868538	K2-28L-055, K2-28L-058	100
Myroides odoratimimus	AJ854059	K4-4-032	99
Firmicutes			
Bacterium JL-74	AY745842	K0-28-002	99
Bacillus sp. 122004	EF522807	K2-28-009	99
Bacillus sp. CNJ817 PL04	DQ448789	K2-28-010	99
Planococcus rifitiensis	AJ493659	K4-28-026, K4-28L-016	99
Sporosarcina sp. Tibet-S2a1	DQ108400	K2-4-053	99
Sporosarcina sp. SK 55	DQ333897	K0-4-012, K2-28-011, K2-4-037, K2-4-051	98–99
Actinobacteria			
Micrococcus sp. UFLA 11-LS	EF194088	K0-28-005	99

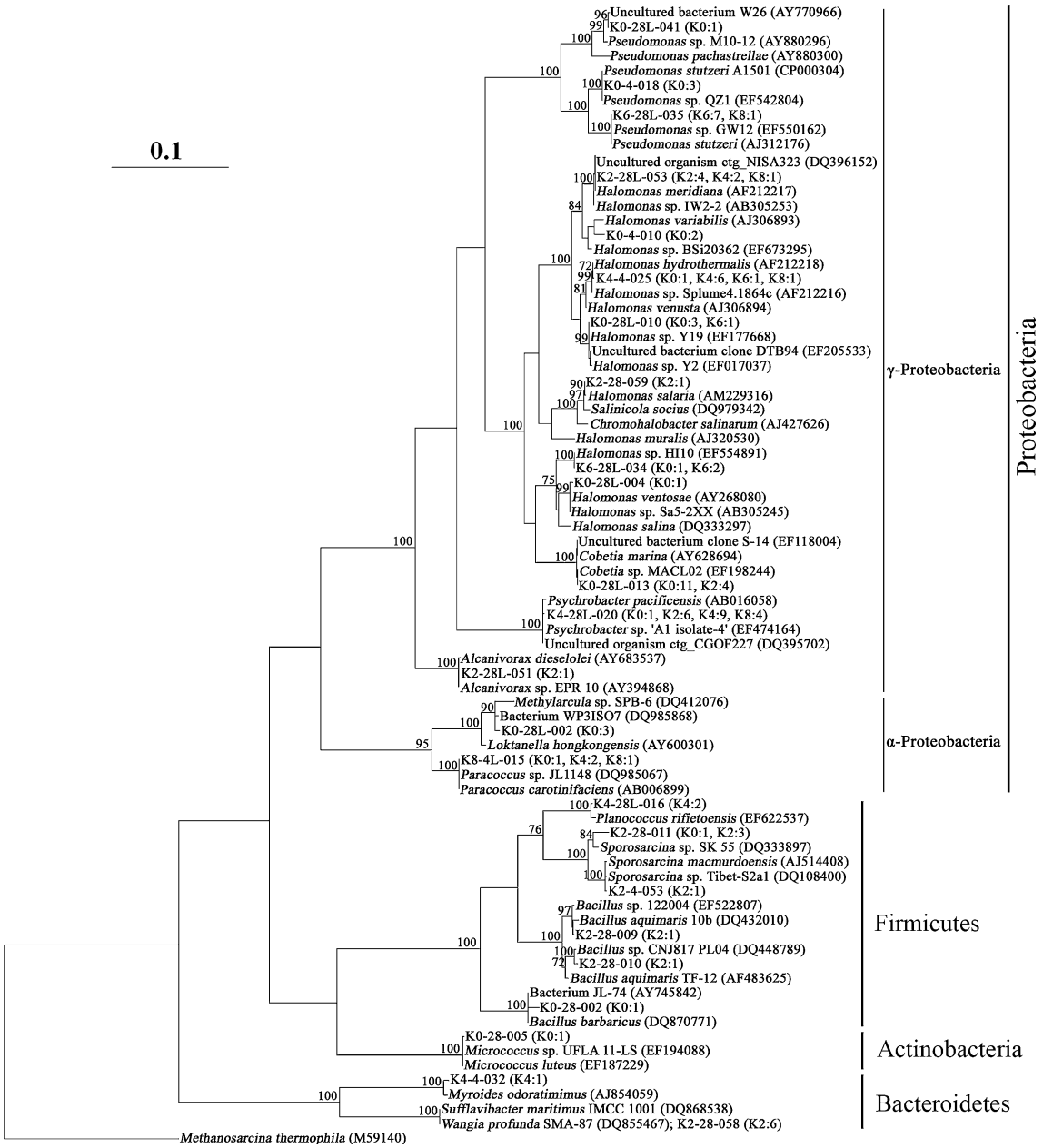

Fig. 2 Phylogenetic tree constructed based on partial 16S rDNA sequences using the neighbor-joining method for the bacterial isolates recovered from the Southern Okinawa Trough deep-sea sediments. The tree branch distances represent nucleotide substitution rate, and the scale bar represents the expected number of changes per homologous nucleotide position. Bootstrap values greater than 70% of 100 resamplings are shown near nodes

59.2% were cold-adapted exoenzyme-producers. There were 35, 30, 32, and 23 isolates producing extracellular amylases, proteases, lipases and DNases, respectively.

The strains producing amylase activity were the most diverse and abundant functional group of our isolates. Thirty-four isolates belonging to 8 different genera produced extracellular amylases at 28°C, and 19 of these isolates and 1 other isolate belonging to 5 different genera also produced amylases at 4°C (Table 2).

Ten isolates at 28°C and 11 isolates at 4°C produced acidic proteases, 8 isolates at 28°C and 13 isolates at 4°C produced neutral proteases, and 7 isolates at 28°C and 7 isolates at 4°C produced alkaline proteases. A few strains, including 3 *Halomonas* isolates, 2 *Bacillus* isolates, 1 *Myroides* isolate, 1 *Planococcus* isolate and 1 *Sporosarcina* isolate, produced proteases in all the pH conditions tested. Five strains belonging to *Cobetia*, *Halomonas*, *Pseudomonas* or *Psychrobacter* produced acidic proteases

Table 2 Screening result of the extracellular enzyme-producing bacteria from the southern Okinawa Trough deep-sea sediments

Bacterial affiliation	Amylase		Protease						Lipase		DNase	
			Acidic		Neutral		Alkaline					
	28°C	4°C	28°C	4°C	28°C	4°C	28°C	4°C	28°C	4°C	28°C	4°C
Paracoccus												
Methylarcula	1									1		
Alcanivorax									1		1	
Cobetia	5			2					2			
Halomonas	7	5	3	4	2	2	2	2	2	4	3	4
Pseudomonas	10	9		1				1	8	1		1
Psychrobacter	2		1	3	1	8		2		13		3
Wangia	5	3	1								4	
Myroides			1	1	1	1	1				1	1
Bacillus	2	1	2		3		2				2	2
Planococcus			1			1	1	2			1	2
Sporosarcina	2	2	1		1	1	1				2	2
Micrococcus										1		
Total isolates	34	20	10	11	8	13	7	7	13	20	14	15

only in low temperature cultivations (4°C). Four isolates belonging to *Planococcus* or *Psychrobacter* produced neutral proteases only in low temperature cultivations. Three strains belonging to *Pseudomonas* or *Psychrobacter* produced alkaline proteases only in low temperature cultivations. Twelve isolates belonging to *Psychrobacter* produced proteases only in low temperature cultivations in all the pH conditions tested.

Thirteen isolates produced extracellular lipases at 28°C and 20 isolates at 4°C, including one *Pseudomonas* isolate with lipase activity at both cultivation temperatures. Several isolates, including 4 *Halomonas* isolates, 1 *Methylarcula* isolate, 1 *Micrococcus* isolate and 12 *Psychrobacter* isolates, produced extracellular lipases only at 4°C.

Fourteen isolates produced extracellular Dnases at 28°C and 15 isolates at 4°C, including 2 *Bacillus* isolates, 1 *Myroides* isolate, 1 *Planococcus* isolate and 2 *Sporosarcina* isolates with the DNase activity at both temperatures. Seven isolates related to *Halomonas*, *Pseudomonas* or *Psychrobacter* showed the DNase activity only at 4°C.

The screening of extracellular chitinase-producing bacteria showed that none of our isolates actually had the extracellular chitinase activity.

Our *Halomonas* sp. Splume4.1864c-like strains (Table 1) showed all the detectable extracellular hydrolytic enzymes activities at both 28°C and 4°C, excluding the extracellular chitinase activity. The *Psychrobacter pacificensis*-like isolates (Fig. 2) also showed a broad spectrum of the extracellular hydrolytic enzymes activity, but were mainly active in the low temperature cultivation conditions. A few isolates related to *Paracoccus* sp. JL1148 (4 isolates), *Halomonas hydrothermalis* (2 isolates), *Halomonas* sp. BSi20362 (2 isolates) and *Halomonas* sp. Y19 (1 isolate) didn't show any of the extracellular hydrolytic activities tested.

Discussion

Deep-sea sediments constitute the largest compartment of the global biosphere. It is also the largest relatively unexplored habitat on earth (Whitman et al. 1998; D'Hondt et al. 2002; Parkes et al. 2005; Schippers et al. 2005). With the advance of molecular approaches (Amann et al. 1995), diverse bacterial and archaeal genotypes, even at phylum level, have been discovered (Schloss and Handelsman 2004; Schleper et al. 2005). However, isolation is still a necessary approach to obtain novel microbes and physiological characteristics for understanding their ecophysiological and environmental functions, and for their application potentials (Vandamme et al. 1996; Palleroni 1997; Sfanos et al. 2005).

The deep-sea sediments of the studied Southern Okinawa Trough may provide an extreme environment due to its permanent low temperature (~5°C) (Mottl 2005). To enrich a broad diversity of deep-sea bacteria, particularly those with dominant environmental relevance, we used several combinations of culture media and cultivation conditions. It turned out that low nutrient condition, in situ temperature incubation and the combination of these two may be necessary for the isolation of certain deep-sea indigenous sediment bacteria. Several isolates, such as strain K2-28-058, were found to have the potential being novel bacterial species (Qin et al. 2007).

To date, bacteria isolated from deep-sea environments predominantly fall within the *γ-Proteobacteria* subdivision, and mainly within the genera *Shewanella*, *Mortiella*, *Colwellia*, *Photobacterium*, *Psychrobacter* or *Pseudomonas* (Kato et al. 1996; DeLong et al. 1997; Sü*β* et al. 2004; Wang et al. 2004), though *α-Proteobacteria* may be abundant in certain unique environments (Sü*β* et al. 2004). In the Southern Okinawa Trough deep-sea sediments, the most dominant culturable heterotrophic bacteria are marine *Halomonas* and *Psychrobacter*, and for certain specific sediment layers, *Cobetia*, *Pseudomonas* or *Wangia* species might dominate. Some *Halomonas* and *Psychrobacter* strains were also isolated previously from the nearby tropical West Pacific Warm Pool deep-sea sediments (Wang et al. 2004). The predominant culturable heterotrophic bacteria from the coastal subseafloor sediments collected from the southwestern Okhotsk Sea of the northwestern Pacific Ocean are *Halomonas*, *Psychrobacter* and *Sulfitobacter* (Inagaki et al. 2003). The consistent recovery of *Halomonas* and *Psychrobacter* bacteria indicates that they are ubiquitous in marine sediments, at least in the west Pacific. The distinct distribution of the other bacterial groups might indicate that their distributions could be restricted by certain environmental conditions.

Most of the oceanic sedimentary mineralization occurs over the continental margin, one of the most important boundaries on Earth (Walsh 1991), where bacteria are the major players for the organic matter mineralization process. Besides to be low-nutrient- and cold-adaptive, our Southern Okinawa Trough deep-sea sediment strains represent diverse ecophysiology in culture, and might play various ecological and geomicrobiological roles in situ. The production and secretion of extracellular hydrolytic enzymes in deep-sea sedimentary environment may have important biogeochemical implications, especially in organic biopolymer compounds degradation, nutrients recycling and bio- or geo-elements mobilization. Microbial extracellular hydrolytic enzymes are the major biological mechanism for the decomposition of sedimentary particular organic carbon and nitrogen. Besides, microbial degradation of extracellular DNA in deep-sea ecosystem may provide another suitable C and N source for sediment prokaryote metabolism (Dell'Anno and Danovaro 2005). Our study showed that diverse and abundant bacterial isolates could secrete at least one of the extracellular enzymes screened, indicating that the in situ microbiota might have developed the genetic and physiological adaptivity for utilizing the high content of particulate organic matters in the Southern Okinawa Trough deep-sea sediments via exoenzyme production. Some strains even harbored all the extracellular hydrolytic enzymes screened, except for the chitinase. The major source of chitin in the deep-sea sediments may be dead bodies and detritus of marine planktonic crustaceans exported from the water column. The lack of extracellular chitinase activity and the prevalence of the other extracellular hydrolytic enzymes activities of our bacterial isolates indicate that the terrestrial export of the particulate organic matters may be the major source of the biopolymers buried in the studied deep-sea sediments. Diverse and abundant bacterial producers of extracellular hydrolytic enzymes, including chitinases, have been isolated from the deep subseafloor organics- and methane-rich sediments off Shimokita Peninsula (Kobayashi et al. 2008). The microbial ecophysiology may present a good bioindicator of the terrestrial impact on the marine benthic microbial ecosystem in the Southern Okinawa Trough deep-sea environment. The diverse extracellular enzymes detected in the current study might also provide a resource for novel biocatalysts discovery and application, especially for low-temperature conditions.

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