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Diversity of Both the Cultivable Protease-Producing Bacteria and Their Extracellular Proteases in the Sediments of the South China Sea

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Abstract Protease-producing bacteria are known to play an important role in degrading sedimentary particular organic nitrogen, and yet, their diversity and extracellular proteases remain largely unknown. In this paper, the diversity of the cultivable protease-producing bacteria and their extracellular proteases in the sediments of the South China Sea was investigated. The richness of the cultivable protease-producing bacteria reached 10^6 cells/g in all sediment samples. Analysis of the 16S rRNA gene sequences revealed that the predominant cultivated protease-producing bacteria are *Gammaproteobacteria* affiliated with the genera *Pseudoalteromonas*, *Alteromonas*, *Marinobacter*, *Idiomarina*, *Halomonas*, *Vibrio*, *Shewanella*, *Pseudomonas*, and *Rheinheimera*, with *Alteromonas* (34.6%) and *Pseudoalteromonas* (28.2%) as the predominant groups. Inhibitor analysis showed that nearly all the extracellular proteases from the bacteria are serine proteases or metalloproteases. Moreover, these proteases have different hydrolytic ability to different proteins, reflecting they may belong to different kinds of serine proteases or metalloproteases. To our knowledge, this study represents the first report of the diversity of bacterial proteases in deep-sea sediments.

Co-first author, Ming-Yang Zhou and Xiu-Lan Chen had equal contribution to this work.

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Introduction

Abundant particulate organic material from marine creatures deposits to the sea floor and has an important influence on the chemical composition of deep-sea sediment and on global biogeochemical cycles. The sinking of particulate organic material to deep-sea sediment is influenced by many factors, such as water column production, season, location, and planktons [4, 11]. It was estimated that the total input of particulate organic nitrogen (PON) to the deep-sea sediment is 24–80 μmol m^{-2} day^{-1} in different area and time [3]. The PON is first decomposed into dissolved organic nitrogen (DON) and then is ammonified, nitrified, and denitrified, which is mainly performed by bacteria and their enzymes [3, 12]. Bacterial enzymatic activity in deep sea is generally considered as the initial and rate-limiting step in carbon oxidation and nitrogen recycling [20]. Ectoproteolytic activity in deep sea has been studied in some area using a fluorogenic substrate analog [2, 11, 20]. However, diversity of the bacteria and their enzymes participating in the degradation of sedimentary PON and DON are relatively unknown. Since proteins are the important component of sedimentary PON and DON, protease-producing bacteria should be the important decomposers on them.

Some bacteria from deep-sea sediments, such as *Pseudoalteromonas* [6, 7, 21], *Pseudomonas* [23], and *Alkalimonas collagenimarina* [16], have been demonstrated to be protease-producing bacteria and some proteases form them have been studied. However, there have been only a few studies on the diversity of sedimentary protease-producing bacteria. By far, the best investigation on the diversity of protease-producing bacteria was performed by Olivera et al [17], who screened 14 protease-producing strains belonging to the genera *Pseudoalteromonas*, *Shewanella*, *Colwellia*, *Planococcus*, and the family *Flavobacteriaceae* from sub-Antarctic sediments. Up to date, no investigation on the diversity of the bacterial proteases in sediments has been reported.

The South China Sea is a marginal sea of China. It extends from the Strait of Malacca in the southwest to the Strait of Taiwan in the northeast and covers an area around 3,500,000 km². The South China Sea has a remarkable amount of biological diversity, including over 30% of the world's coral reefs and many valuable fisheries. However, microbiological study, especially on the functionality, has seldom been carried out in this area. In this paper, the sediments of eight stations in the South China Sea were sampled, representing several distinct geographical characteristics and biogeochemical backgrounds. By cultivation and screening, 78 protease-producing strains were isolated from these sediments and their diversity was studied by a phylogenetic analysis of their 16S rRNA gene sequences. Moreover, the diversity of the proteases secreted by these strains was investigated by using different substrates and inhibitors.

Methods

Sampling and the Geochemical Characteristics of Samples

Sediment samples of the South China Sea were collected using a 0.1-m^2 stainless steel Gray O'Hara box corer or a deep-sea sediment grab sampler during the South China Sea Open Cruise of R/V Shiyan 3 in the August of 2007 (Fig. 1). Only undisturbed samples were used to ensure the integrity of the surface sediment structures. Replicate surface sediment subcore samples down to 5 cm depth for microbiological analysis were taken aseptically with sterile 60-ml syringes (luer end removed) and stored in airtight sterile plastic bags at 4°C. Sediment samples for environmental analysis were taken with the same manner and stored in airtight sterile plastic bags in a −20°C freezer during the cruise and −80°C after returning to the laboratory. Surface sediment temperature and pH were measured *in situ*. Sediment organic carbon (OrgC) and nitrogen (OrgN) contents were measured in the laboratory with a PE 2400 Series II CHNS/O analyzer (Perkin Elmer, USA).

Cultivation and Screening of Protease-Producing Bacteria

One gram (wet weight) sediment of every sample was serially tenfold diluted to 10^{-6} dilution with sterile artificial sea water. Aliquots of 100 µl diluted deep-sea sediment samples (10^{-2}–10^{-6} dilution) were spread on screening plates with a medium composed of 0.2% yeast extract, 0.3% casein, 0.5% gelatin, 1.5% agar powder, and

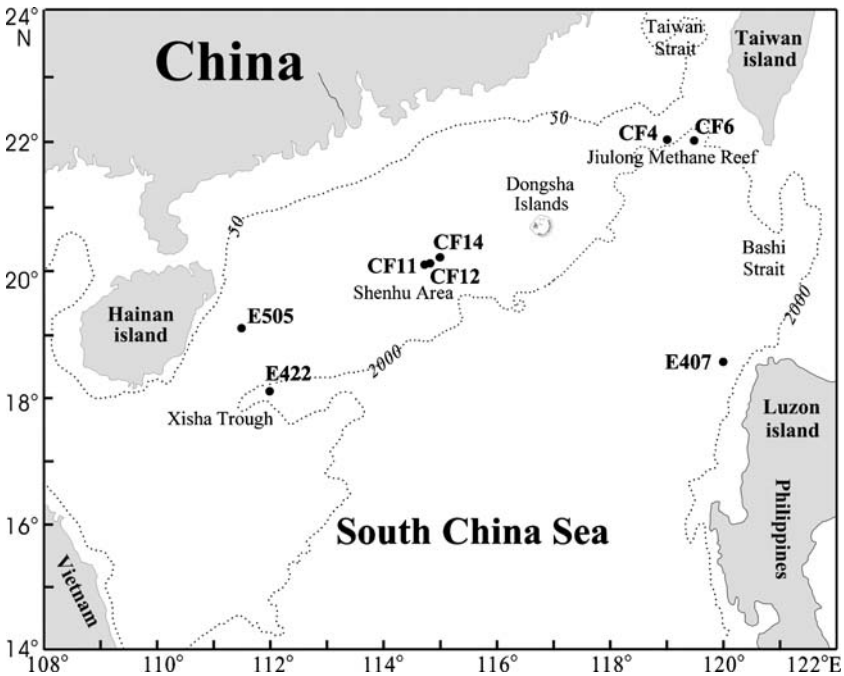


Figure 1 Geographic location of sampled sediment stations in the South China Sea

artificial seawater (pH7.0). The plates were then incubated at 15°C for a proper time to form detectable colonies with clear hydrolytic zone. Morphologically different colonies with or without hydrolytic zone were selected and further purified by repeatedly streaking on the same medium. The purified strains were stored in 15% glycerol at −80°C for use.

Amplification of 16S rRNA Genes and Phylogenetic Analysis

Genomic DNA from isolates was extracted by using BIOTEKE genomic DNA purification kit. The 16S rRNA genes were amplified from genomic DNA by polymerase chain reaction with two primers (1492r: 5′-GGTTACCTTGTTACGACTT-3′, 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′) [1]. These genes were ligated into pGEM-T cloning vectors (Promega) and sequenced by Biosune Inc. (Shanghai, China). Sequence alignment was performed using CLUSTAL X (v 1.83). Isolates with two or more than two different bases in their 16S rRNA gene sequences were taken as different strains. Neighbor-joining trees were constructed using MEGA version 3.1 [15] with neighbor-joining method and Kimura two-parameter model.

Analysis of the Hydrolysis Ability of Proteases to Casein, Elastin, and Gelatin

Plates were prepared with three media: basic medium (0.2% yeast extract, 0.5% gelatin, 1.5% agar powder, and artificial seawater, pH8.0) with 0.5% (*w*/*w*) casein, 0.5% (*w*/*w*) gelatin, and 0.5% (*w*/*w*) elastin powder, respectively. The strains which produced hydrolytic zone on the screening plates were streaked on three kind plates, respectively, and incubated at 15°C for 4 days. Then, for each strain, the diameter of its colony and the diameter of the hydrolytic zone it produced were measured, and a ratio of the hydrolytic zone diameter to the colony diameter (hydrolytic zone/colony, H/C) was calculated.

Analysis of the Inhibitory Effect of Protease Inhibitors on Protease Activity

The protease-producing strains were grown in the liquid screening medium at 15°C, 200 rpm for 4 days. The culture was centrifuged at 12,000×*g*, 4°C for 12 min. The protease activity of the supernatant was measured as previously described [5]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μg tyrosine per minute. The supernatant diluted appropriately with 50 mM Tris-HCl (pH8.0) was pre-incubated with 1.0 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 1.0 mM 1,10-phenanthroline (OP; Sigma), 0.1 mM E-64 (Merk), and 0.1 mM pepstatin A (Merk) at 20°C for 20 min, respectively. After incubation, the protease activity of every sample was measured. The activity of a sample without any inhibitor was taken as 100%, and the relative activity (%) of samples was calculated. The inhibition ratio was taken as the result of control activity minus the relative activity of a sample.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences resulting from this study were deposited in GenBank under the accession numbers FJ169962–FJ170039.

Results

Station Description and Sample Characteristics

The sampling stations with variable water depth (154–2,456 m) were located in different areas of the South China Sea (Fig. 1; Table 1). The Xisha Trough (station E422) and the Jiulong methane reef area off the Southwest Taiwan Island (stations CF4 and CF6) were previously identified as gas hydrate prospective areas [5, 10]. The existence of gas hydrates in the Shenhu area (stations CF11, CF12, and CF14) southwest of the Dongsha Islands was inferred from

Table 1 Characteristics of the sampling stations

Station	Location (E, N)	Depth (m)	Temperature (°C)	pH	OrgC (%)	OrgN (%)	C/N
E505	111°29.029′, 18°59.897′	154	17.3	6.94	0.47	0.06	7.83
CF11	114°34.477′, 19°43.341′	1,050	5.2	5.9	1.32	0.16	8.25
CF12	114°44.956′, 19°44.946′	1,153	8.5	6.93	1.32	0.16	8.25
CF14	115°12.971′, 19°54.256′	1,220	3.6	6.94	1.32	0.15	8.80
CF4	118°57.042′, 22°0.545′	1,632	2.8	6.97	0.65	0.07	9.29
E407	112°0.017′, 18°29.810′	1,800	4.7	6.97	0.67	0.06	11.17
CF6	119°30.060′, 22°0.316′	2,441	5.5	6.90	0.63	0.08	7.88
E422	112°0.793′, 18°0.341′	2,456	3.8	7.09	0.94	0.11	8.55

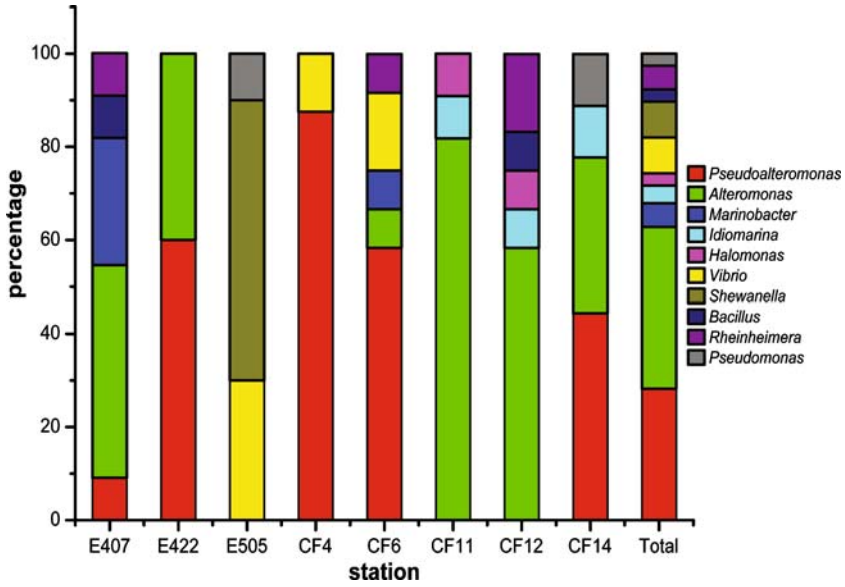


Figure 2 Relative percentage abundances of the phylotypic groups of cultivable protease-producing bacteria recovered from eight sample stations in the South China Sea

the evidence of seep carbonate nodules and was verified by direct sampling during a recent cruise [8].

All sediments displayed nearly neutral pH, with the only exception of station CF11. The content of OrgC and OrgN in the sediments was in the range of 0.47–1.32% (OrgC) and 0.06–0.16% (OrgN), with the highest values in the sediments from the gas hydrate-bearing stations CF11, CF12, and CF14, and the lowest values in the sediment from the shallow-water station E505 (Table 1). The highest C/N ratio was observed in station E407 (11.17) and the lowest in station E505 (7.83).

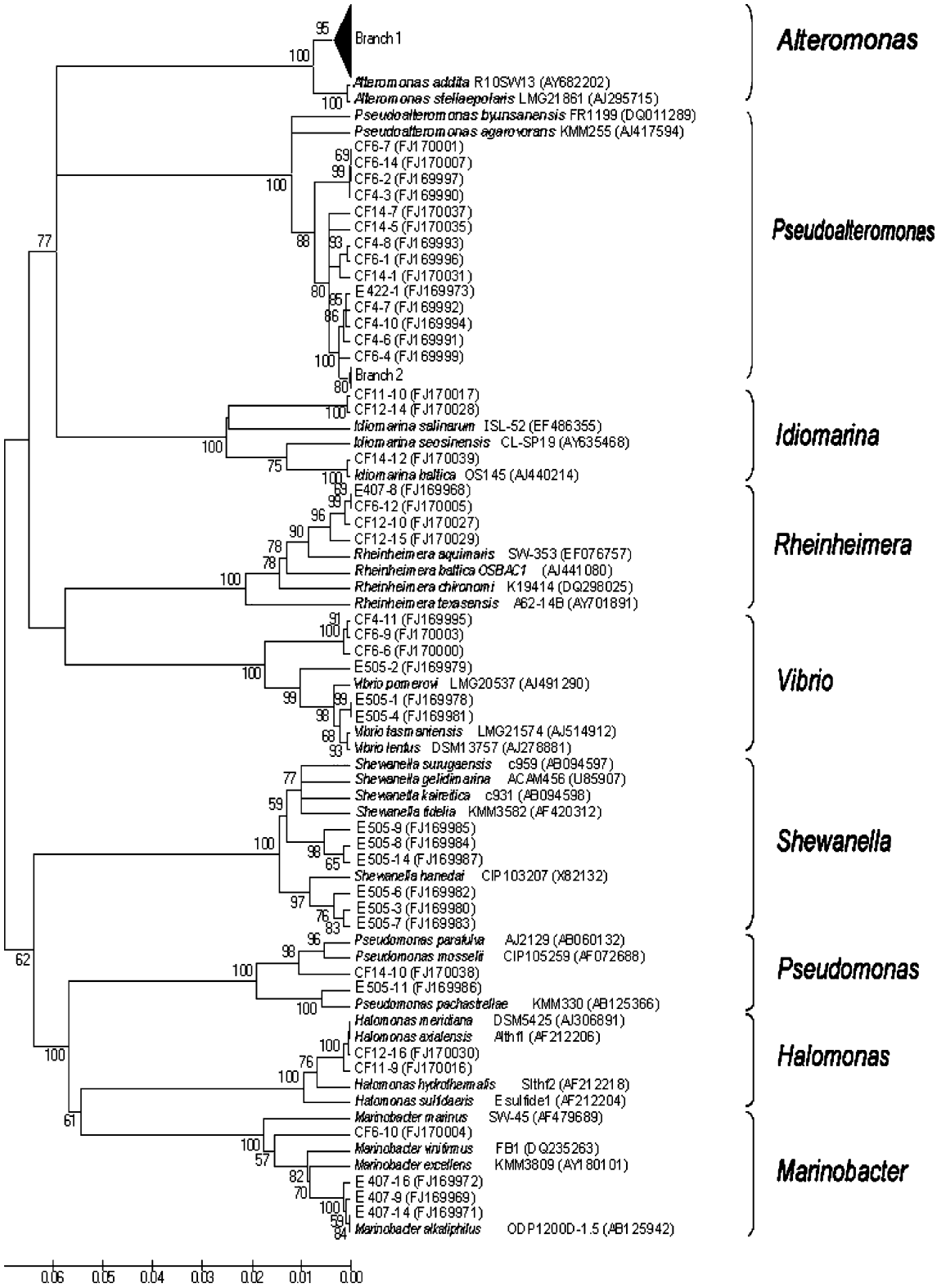
Screening of Protease-Producing Bacteria from Sediments

After cultivation on screening plates, a lot of colonies appeared on the plates with 10^{-2}–10^{-4} diluted samples (Suppl. Fig. S1). Quantitative statistic by manual count showed that the abundance of cultivated bacteria reached 10^6 cells/g in all sediment samples, and more than 90% colonies produced obvious hydrolytic zone in all samples. The difference of particulate organic material content and C/N ratio among the stations could not lead to obvious difference in the riches of cultivable protease-producing bacteria. One hundred two colonies were purified for diversity analysis.

Diversity of Protease-Producing Bacteria in the Sediments

Nearly complete 16S rRNA genes of the 102 isolates were amplified and sequenced. Isolates with the same (or only one base difference) 16S rRNA gene sequence were considered as the same strain, and consequently, 78 different strains were got. Based on the sequences of their 16S rRNA genes, the phylogenetic affiliation of these strains was analyzed. Except that one strain (E407-10) was a gram-positive bacterium belonging to genus *Bacillus*, the strains were mainly affiliated with the class *Gammaproteobacteria* and grouped in the genera *Pseudoalteromonas*, *Alteromonas*, *Marinobacter*, *Idiomarina*, *Halomonas*, *Vibrio*, *Shewanella*, *Pseudomonas*, and *Rheinheimera*. *Alteromonas* (34.6%) and *Pseudoalteromonas* (28.2%) were the predominant groups. The other genera comprised between 2.6% and 7.7% of all strains. However, the result also showed that the predominant bacteria in the sediments of different sites and depth were different. In the shallowest sediment sample (E505), the predominant protease-producing bacteria were *Shewanella* (60%) and *Vibrio*

Figure 3 Neighbor-joining phylogenetic tree of the protease-producing *Gammaproteobacteria* recovered from eight sampled sediment stations in the South China Sea based on the 16S rDNA sequences. Branch 1 included *Alteromonas* strains of 407-1 (FJ169962), 407-3 (FJ169964), 407-4 (FJ169965), 407-5 (FJ169966), 407-6 (FJ169967), 422-2 (FJ169974), 422-4 (FJ169976), CF6-3 (FJ169998), CF11-1 (FJ170008), CF11-2 (FJ170009), CF11-3 (FJ170010), CF11-4 (FJ170011), CF11-5 (FJ170012), CF11-6 (FJ170013), CF11-7 (FJ170014), CF11-8 (FJ170015), CF11-11 (FJ170018), CF12-1 (FJ170019), CF12-2 (FJ170020), CF12-3 (FJ170021), CF12-4 (FJ170022), CF12-5 (FJ170023), CF12-6 (FJ170024), CF12-7 (FJ170025), CF14-2 (FJ170032), CF14-3 (FJ170033), CF14-4 (FJ170034), *Alteromonas macleodii* LMG24082 (AM885870), and *Alteromonas marina* SW-47 (AF529060). The neighbor-joining phylogenetic tree of strains in branch 1 based on the 16S rDNA sequences was shown in supplementary Figure S2. Branch 2 included *Pseudoalteromonas* strains of CF6-8 (FJ170002), 407-2 (FJ169963), CF14-6 (FJ170036), 422-3 (FJ169975), CF6-13 (FJ170006), CF4-2 (FJ169989), 422-5 (FJ169977), and CF4-1 (FJ169988). The neighbor-joining phylogenetic tree of strains in branch 2 based on the 16S rDNA sequences was shown in supplementary Figure S3


Branch 1
Alteromonas addita R10SW13 (AY682202)
Alteromonas stellaepolaris LMG21861 (AJ295715)
Alteromonas
Pseudoalteromonas byunsanensis FR1199 (DQ011289)
Pseudoalteromonas agarovorans KMM255 (AJ417594)
CF6-7 (FJ170001)
CF6-14 (FJ170007)
CF6-2 (FJ169997)
CF4-3 (FJ169990)
CF14-7 (FJ170037)
CF14-5 (FJ170035)
CF4-8 (FJ169993)
CF6-1 (FJ169996)
CF14-1 (FJ170031)
E422-1 (FJ169973)
CF4-7 (FJ169992)
CF4-10 (FJ169994)
CF4-6 (FJ169991)
CF6-4 (FJ169999)
Pseudoalteromonas
Branch 2
CF11-10 (FJ170017)
CF12-14 (FJ170028)
Idiomarina salinarum ISL-52 (EF486355)
Idiomarina seosinensis CL-SP19 (AY635468)
CF14-12 (FJ170039)
Idiomarina baltica OS145 (AJ440214)
Idiomarina
E407-8 (FJ169968)
CF6-12 (FJ170005)
CF12-10 (FJ170027)
CF12-15 (FJ170029)
Rheinheimera aquimaris SW-353 (EF076757)
Rheinheimera baltica OSBAC1 (AJ441080)
Rheinheimera chironomi K19414 (DQ298025)
Rheinheimera texasensis A62-14B (AY701891)
Rheinheimera
CF4-11 (FJ169995)
CF6-9 (FJ170003)
CF6-6 (FJ170000)
E505-2 (FJ169979)
Vibrio pomeroyi LMG20537 (AJ491290)
E505-1 (FJ169978)
E505-4 (FJ169981)
Vibrio tasmaniensis LMG21574 (AJ514912)
Vibrio tentus DSM13757 (AJ278881)
Vibrio
Shewanella suzugaensis c959 (AB094597)
Shewanella gelidimarina ACAM456 (U85907)
Shewanella kaireitica c931 (AB094598)
Shewanella fidelia KMM3582 (AF420312)
E505-9 (FJ169985)
E505-8 (FJ169984)
E505-14 (FJ169987)
Shewanella hanedai CIP103207 (X82132)
E505-6 (FJ169982)
E505-3 (FJ169980)
E505-7 (FJ169983)
Shewanella
Pseudomonas paralutea AJ2129 (AB060132)
Pseudomonas mosselii CIP105259 (AF072688)
CF14-10 (FJ170038)
E505-11 (FJ169986)
Pseudomonas pachastrellae KMM330 (AB125366)
Pseudomonas
Halomonas meridiana DSM5425 (AJ306891)
Halomonas axialensis Alth1 (AF212206)
CF12-16 (FJ170030)
CF11-9 (FJ170016)
Halomonas hydrothermalis SIthf2 (AF212218)
Halomonas sulfidaeris E sulfide1 (AF212204)
Halomonas
Marinobacter marinus SW-45 (AF479689)
CF6-10 (FJ170004)
Marinobacter vinifirmus FB1 (DQ235263)
Marinobacter excellens KMM3809 (AY180101)
E407-16 (FJ169972)
E407-9 (FJ169969)
E407-14 (FJ169971)
Marinobacter alkaliphilus ODP1200D-1.5 (AB125942)
Marinobacter
0.06 0.05 0.04 0.03 0.02 0.01 0.00

(30%). In contrast, the predominant protease-producing bacteria in the sediment sample deeper than 1,000 m were *Pseudoalteromonas* and *Alteromonas* (Fig. 2).

A distance-based neighbor-joining tree was constructed with the *Gammaproteobacteria*-related sequences from this study and reference sequences from the GenBank database (Fig. 3). The closest neighbors of most of the strains still lack taxonomic standing or belong to uncultured bacterium clones and in most case from marine sources. Isolates related to *Alteromonas* were the most frequently recovered isolates (recovered from six sediments) and formed the largest group in term of abundance (27 of 78 isolates; branch 1 in Fig. 3, Fig. S2). Twenty-two *Pseudoalteromonas* strains recovered from five sediments were closely related and formed a sister group of *Pseudoalteromonas byunsanensis* FR1199 and *Pseudoalteromonas agarovorans* KMM255. The phylogenetic relationships of the other strains to their closely related species were also shown in Fig. 3. Several isolates, such as CF12-14, E505-2, E505-8, and E407-8, exhibited a distant relationship with any of the previously identified species. They may represent potentially new species, which merit further study.

Diversity of Bacterial Extracellular Proteases in the Sediments

The diversity of the bacterial extracellular proteases in the sediments was investigated with protease inhibitors (Table 2). PMSF (serine protease inhibitor), OP (metalloprotease inhibitor), E-64 (cysteine protease inhibitor), and pepstatin A (aspartic protease inhibitor) were used to inhibit the activities of the proteases secreted by the screened strains for identification of these proteases. Among the 78 strains, 16 of them did not produce enough proteases for inhibition analysis. PMSF inhibited the activities of the proteases from all the detected 62 strains by 23–100%, showing all the strains produce serine proteases in different proportion. Among them, the activities of the proteases from 13 strains were inhibited by PMSF by more than 90%, which indicated that these strains mainly or only produce serine proteases. Of all 62 strains, OP inhibited the activities of the proteases from 47 strains by 20–84% and had a little or no inhibitory effect on the others, showing a majority of the screened strains produce metalloproteases. Moreover, the activities of the proteases from most of the screened strains were inhibited by both PMSF and OP, indicating these strains simultaneously produce serine proteases and metalloproteases. E-64 and pepstatin A only had less than 10% or no inhibitory effect on the activities of all proteases, showing that these strains hardly produce cysteine or aspartic protease. Therefore, nearly all the extracellular proteases from the bacteria in the sediment samples are serine proteases or metalloproteases.

In addition, the bacterial protease diversity in the sediments was also investigated by detecting the hydrolysis ability of the proteases to different proteins casein, gelatin, and elastin, which was analyzed by measuring the H/C ratio on plates. Of the 78 strains, only 68 obviously showed a hydrolytic zone, which were shown in Table 2. Since these strains were screened with casein- and gelatin-containing medium, all the extracellular proteases from these strains could hydrolyze casein and gelatin with obvious hydrolytic zone except for the protease from strain CF12-7 which did not hydrolyze gelatin. However, these proteases displayed very different hydrolysis abilities to casein and gelatin. The extracellular proteases from strains E407-5, CF12-2, CF12-3, and CF12-7 of *Alteromonas* and strains CF4-3, CF6-7, and CF6-14 of *Pseudoalteromonas* had high caseinolytic activity with H/C ratio more than 6, and the extracellular proteases from strains CF4-3, CF6-2, CF6-7, and CF6-14 of *Pseudoalteromonas* and strain E505-3 of *Shewanella* had high gelatinolytic activity with H/C ratio more than 6. In contrast, only 47 strains could produce obvious hydrolysis zone around a single colony on elastin plates. The extracellular proteases from strain CF6-2 and CF6-14 of *Pseudoalteromonas* had high elastinolytic activity with H/C ratio more than 6. A lot of strains produced proteases with high activity to all three proteins. The good examples were strains CF6-2, CF6-14, E505-3, CF12-10, and E407-8. These differences in substrate specificity reflected that the proteases produced by these strains may belong to different kinds of serine proteases or metalloproteases.

Discussion

In general, cultivability (cultivable population vs. total cell population) of the deep subseafloor microbial community ranges from 0.1% to 0.001% [9, 13, 19] and the cultivable populations from deep marine sediments number between 10^4 and 10^7 cells cm^{-3} [14]. The richness of the cultivable protease-producing bacteria in eight sediments of the South China Sea studied in this paper was ~10^6 cells/g in all sediments, which is in the average range of previous demonstrations. This result indicates the existence of a sizable population of protease-producing bacteria in the sediments. Huston and Deming reported that total and actively respiring bacteria in sediment have strong positive relationships to POC and PON content, but weaker relationships to C/N ratios [11]. Although there are some differences in the OrgC and OrgN content and C/N ratio among the stations, these differences can not result in an obvious difference in the riches of cultivable protease-producing bacteria.

Gammaproteobacteria are an important population which is frequently encountered in the analysis of sedi-

Table 2 Diversity analysis of the extracellular proteases produced by the screened strains

Genera	Strains	H/C ratio[a]			Inhibition Ratio[c] (%)			
		Casein	Gelatin	Elastin	PMSF (1mM)	OP (1mM)	E64 (0.1mM)	P-A (0.1mM)
Alteromonas	CF12-2	6.81	2	1.8	48.27	59.3	−0.21	−1.44
	CF12-3	6.61	4	Thin[b]	45.52	61.11	0.36	0.6
	E407-5	6.13	3	Thin	68.7	55.48	0.08	1.53
	CF12-7	6	3.21	0	38.79	62.87	−3.26	3.07
	CF11-7	5.94	4.1	Thin	70.94	53.17	6.53	−1.18
	CF11-3	5.83	5.67	1.29	80.09	32.72	−6.13	−5.98
	E422-2	5.63	2.75	1.2	64.78	29.44	−1.95	2.16
	E407-1	5.47	4	1.46	75.81	45.39	2.36	−0.2
	CF11-4	5.22	2	1.4	53.49	60.11	−2.9	2.66
	CF11-11	5.18	4.75	1.62	79.28	35.54	2.71	0.69
	CF11-1	5.17	4.42	1.88	41.33	58.78	1.82	−3.13
	E407-6	5	4.08	1.99	61.34	54.61	−5.76	−5.98
	CF11-6	5	3.33	2.17	83.32	19.4	−3.97	−2.13
	CF14-2	5	2.67	1.4	73.82	50.58	3.07	9.27
	CF14-4	5	5.17	1.25	93.02	14.49	4.76	−0.03
	CF12-1	4.75	3.94	1.43	46.56	63.12	−1.44	−2.89
	CF14-3	4.75	3.81	1.54	68.21	48.04	−9.42	−3.05
	E407-4	4.72	4.38	Thin	66.24	57.49	−6.63	−4.17
	CF11-8	4.67	3.67	2	43.75	69.34	1.05	−7.57
	CF12-5	4.57	4.58	1.25	28.94	65.18	2.43	−2.06
	CF6-3	4.25	4.5	1.33	71.78	45.85	3.81	0.53
	E407-3	4.15	3.04	1.61	67.54	59.79	5.4	5.75
	E422-4	4.04	2.89	Thin	69.95	44.69	1.6	7.47
	CF12-4	3.91	3	1.75	88.19	20.55	−2.23	−8.23
	CF11-2	1.45	1.83	Thin	94.29	1.66	−4.76	−4.7
	CF11-5	1.3	2.22	1.6	91.7	20.28	1.62	3.61
Idiomarina	CF11-10	2.08	2.67	Thin	–	–	–	–
	CF14-12	1.8	0	Thin	–	–	–	–
	CF12-14	1.39	3.06	Thin	–	–	–	–
Pseudoalteromonas	CF4-3	7.83	6.8	0	92.69	35.17	−0.17	−3.17
	CF6-14	6.67	8.15	6.71	90.91	5.06	−1.64	−3.67
	CF6-7	6.46	6.67	0	94.91	18.83	−3.67	−0.2
	CF6-2	5.89	6.5	9	93.92	15.53	3.54	−0.1
	E422-5	4.13	3.39	1.79	54.23	48.87	1.91	8.32
	E422-3	3.8	3.67	1.83	34.12	56.11	−1.72	1.7
	CF14-7	3.8	2.3	0	39.81	49.93	3.85	8.31
	CF6-8	3.69	3.91	Thin	54.68	31.41	3.26	2.89
	CF14-1	3.57	2.63	Thin	43.63	62.97	8.59	5.02
	E407-2	3.5	3.06	1.3	54.04	46.1	40.77	1.3
	CF6-4	3.5	3.29	1.53	50.23	23.58	−0.63	−2.38
	CF14-6	3.33	3	1.65	50.09	58.81	6.03	4.52
	E422-1	3.21	1.9	1.38	44.93	49	−7.3	−4.88
	CF4-1	3.21	3	1.58	43.71	58.33	−0.22	−1.09
	CF4-6	3	3.35	1.58	48.33	80.53	0.79	0.95
	CF4-2	2.98	3.69	1.38	47.44	54.04	3.73	4.77
	CF4-10	2.83	3.39	1.78	40.63	51.14	4.82	4.04
	CF6-13	2.81	4.6	1.56	69.69	36.01	5.97	3.4

Table 2 (continued)

Genera	Strains	H/C ratio[a]			Inhibition Ratio[c] (%)			
		Casein	Gelatin	Elastin	PMSF (1mM)	OP (1mM)	E64 (0.1mM)	P-A (0.1mM)
	CF14-5	2.81	1.46	Thin	37.4	66.78	1.69	−9.24
	CF4-7	2.71	3.88	1.8	41.04	57.39	−4.16	0.27
	CF6-1	2.61	2.5	1.52	43.86	52.31	7.46	9.26
	CF4-8	2.57	2.69	1.42	47.02	66.06	−3.36	4.57
Shewanella	E505-3	3.8	7.75	3.11	84.2	20.97	−2.33	3.49
	E505-6	3.54	4.33	1.75	81.75	10.6	4.42	1.9
	E505-8	3.25	3.36	Thin	91.8	3.92	−1.28	1.02
	E505-7	2.77	4.5	1.75	63.21	24.2	3.68	2.28
	E505-9	2.5	3.5	Thin	77.98	26.77	−1.89	1.56
	E505-14	2.22	3.23	1.64	53.64	2.52	6.64	−1.82
Vibrio	E505-2	4.15	5.33	0	–	–	–	–
	E505-1	1.63	2.73	1.53	58.55	52.5	10.62	7.76
	CF4-11	1.62	3.33	0	99	−8.84	−1.48	−3.19
	CF6-6	1.62	2.5	0	99.3	−9.18	−4.32	−5.99
	E505-4	1.5	1.8	0	23.44	83.87	10.07	1.49
	CF6-9	1.45	2.16	1.36	103.41	−0.3	2.18	0.34
Rheinheimera	CF12-15	3.5	3.75	2.25	94.5	6.98	−0.84	−0.56
	CF6-12	3.17	3.64	2.45	–	–	–	–
	CF12-10	3.15	3.5	3.25	100.07	5.51	−0.05	−0.95
	E407-8	2.13	5.33	3.33	86.82	−3.51	−3.42	−3.63
Bacillus	CF12-9	1.67	2.06	3.72	–	–	–	–

PMSF phenylmethylsulfonyl fluoride, *OP* 1,10-phenanthroline, *P-A* pepstatin A

[a] H/C ratio is the ratio of the hydrolytic zone diameter to the colony diameter of a colony on the plate

[b] Thin represents a slight hydrolytic zone formed by the strain

[c] The activity of a sample without any inhibitor was taken as control (100%). The inhibition ratio was taken as the result of control activity minus the relative activity of a sample with an inhibitor

mentary bacterial diversity [12, 18, 22]. An analysis of the diversity of protease-producing bacteria in sub-Antarctic sediments shows that *Gammaproteobacteria* are also the main protease-producing bacteria. Of 19 cultivable strains, 17 strains are *Gammaproteobacteria*, which are affiliated with the genera *Pseudoalteromonas*, *Shewanella*, *Colwellia*, and *Planococcus* [17]. Our result in this paper showed that of 78 protease-producing strains recovered from the sediments of the South China Sea, 77 strains are *Gammaproteobacteria*, affiliated with the genera *Pseudoalteromonas*, *Alteromonas*, *Marinobacter*, *Idiomarina*, *Halomonas*, *Vibrio*, *Shewanella*, *Pseudomonas*, and *Rheinheimera*. Among them, *Alteromonas* and *Pseudoalteromonas* are predominant, accounting for 34.6% and 28.2% of the total strains, respectively. Although only a small portion of the cultivable protease-producing bacteria was selected to study the diversity, differences in the diversity of protease-producing bacteria between stations still could be observed (Fig. 2). However, no obvious relationship between the bacterial diversity and the OrgC and OrgN content or C/N ratio among the sites was observed. The predominant bacteria in the sediments of different sites and depths are different. In the shallowest sediment sample (E505), the predominant protease-producing bacteria are *Shewanella* (60%) and *Vibrio* (30%). In contrast, the predominant protease-producing bacteria in the sediment sample deeper than 1,000 m are *Pseudoalteromonas* and *Alteromonas*.

Ectoproteolytic activity in deep sea has been studied in some area [2, 11, 20]. Protease activity in sediments correlates positively with the abundance of both total and actively respiring bacteria, which play an important role in the nitrogen depletion of sinking particular organic material [11]. The total proteolysis activity in the sediments in this study was not measured. However, the proteases-producing ability of the selected strains was studied. Overall, the *Idiomarina* strains and the *Bacillus* strain have relatively low protease-producing ability, while the strains of the other genera have relatively high protease-producing ability at the experimental culture condition. The study of Xiong et al. also shows that *Pseudoalteromonas* strains are the

bacteria with high protease-producing ability in deep-sea sediments [21]. Although ectoproteolytic activity in deep sea has been demonstrated, there has been no report on ectoprotease diversity in deep sea. Our results showed that nearly all the extracellular proteases from the sedimentary bacteria are serine proteases or metalloproteases. Moreover, these proteases have different hydrolytic ability to different proteins, reflecting they belong to different kinds of serine proteases or metalloproteases. Studying these proteases would be significant not only for clarifying the degradation of sedimentary PON and DON but also for finding new type proteases for a future technological application. Our results in this study on the diversity of bacterial proteases in sediments are preliminary. Detailed identification of these proteases is being studied using molecular biotechnology and biochemical technology.

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References

1. Annette SE, Megan LP, Libby AS, Sarah Q, Philip CB (2004) Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "*Epsilonproteobacteria*". FEMS Microbiol Ecol 51:31–53
2. Bianchi A, Calafat A, Wit RD, Garcin J, Tholosan O, Cacho I, Canals M, Fabrés J, Grout H, Masqué P, Sanchez-Cabeza J-A, Sempéré R (2003) Microbial activity at the deep water sediment boundary layer in two highly productive systems in the Western Mediterranean: the Almeria–Oran front and the Malaga upwelling. Oceanol Acta 25:315–324
3. Brunnegard J, Grandel S, Stahl H, Tengberg A, Hall POJ (2004) Nitrogen cycling in deep-sea sediments of the Porcupine Abyssal Plain, NE Atlantic. Prog Oceanogr 63:159–181
4. Buesseler KO, Trull TW, Steinberg DK, Silver MW, Siegel DA, Saitoh S-I, Lamborg CH, Lam PJ, Karl DM, Jiao NZ, Honda MC, Elskens M, Dehairs F, Browng SL, Boyd PW, Bishop JKB, Bidigare RR (2008) VERTIGO (VERtical Transport In the Global Ocean): a study of particle sources and flux attenuation in the North Pacific. Deep-Sea Res II 55:1522–1539
5. Chen DF, Huang YY, Yuan XL, Cathles LMIII (2005) Seep carbonates and preserved methane oxidizing archaea and sulfate reducing bacteria fossils suggest recent gas venting on the seafloor in the Northeastern South China Sea. Mar Petrol Geol 22:613–621
6. Chen X-L, Zhang Y-Z, Gao P-J, Luan X-W (2003) Two different proteases produced by a deep-sea psychrotrophic strain *Pseudoalteromonas* sp. SM9913. Mar Biol 143:989–993
7. Chen X-L, Xie B-B, Lu J-T, He H-L, Zhang Y-Z (2007) A novel type of subtilase from the psychrophilic bacterium *Pseudoalteromonas* sp. SM9913: catalytic and structural properties of deseasin MCP-01. Microbiol-SGM 153:2116–2125
8. Chen Z, Yan W, Chen MH, Wang SH, Lu J, Zhang F, Xiang R, Xiao SB, Yan B, Gu SC (2006) Discovery of seep carbonate nodules as new evidence for gas venting on the northern continental slope of South China Sea. Chin Sci Bull 51:1228–1237
9. D'Hondt S, Jørgensen BB, Miller DJ, Batzke A, Blake R, Cragg BA, Cypionka H, Dickens GR, Ferdelman T, Hinrichs K-U, Holm NG, Mitterer R, Spivack A, Wang G, Bekins B, Engelen B, Ford K, Gettemy G, Rutherford SD, Sass H, Skilbeck CG, Ailleo IW, Guerin G, House CH, Inagaki F, Meister P, Naehr T, Niitsuma S, Parkes RJ, Schippers A, Smith DC, Teske A, Wiegel J, Padilla CN, Acosta JLS (2004) Distribution of microbial activities in deep subseafloor sediments. Science 306:2216–2221
10. Han XQ, Suess E, Huang YY, Wu NY, Bohrmann G, Su X, Eisenhauer A, Rehder G, Fang YX (2008) Jiulong methane reef: Microbial mediation of seep carbonates in the South China Sea. Mar Geol 249:243–256
11. Huston AL, Deming JW (2002) Relationships between microbial extracellular enzymatic activity and suspended and sinking particulate organic matter: seasonal transformations in the North Water. Deep-Sea Res II 49:5211–5225
12. Hunter EM, Mills HJ, Kostka JE (2006) Microbial community diversity associated with carbon and nitrogen cycling in permeable shelf sediments. Appl Environ Microbiol 72:5689–5701
13. Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki K, Nealson K, Horikoshi K (2003) Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk. Appl Environ Microbiol 69:7224–7235
14. Kobayashi T, Koide O, Mori K, Shimamura S, Matsuura T, Miura T, Takaki Y, Morono Y, Nunoura T, Imachi H, Inagaki F, Takai K, Horikoshi K (2008) Phylogenetic and enzymatic diversity of deep subseafloor aerobic microorganisms in organics- and methane-rich sediments off Shimokita Peninsula. Extremophiles 12:519–527
15. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163
16. Kurata A, Miyazaki M, Kobayashi T, Nogi Y, Horikoshi K (2007) *Alkalimonas collagenimarina* sp. nov., a psychrotolerant, obligate alkaliphile isolated from deep-sea sediment. Int J Syst Evol Microbiol 57:1549–1553
17. Olivera NL, Sequeiros C, Nievas ML (2007) Diversity and enzyme properties of protease-producing bacteria isolated from sub-Antarctic sediments of Isla de Los Estados, Argentina. Extremophiles 11:517–526
18. Polymenakou PN, Bertilsson S, Tselepides A, Stephanou EG (2005) Bacterial community composition in different sediments from the eastern Mediterranean Sea: a comparison of four 16S ribosomal DNA clone libraries. Microbioal Ecol 50:447–462
19. Süß J, Engelen B, Cypionka H, Sass H (2004) Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. FEMS Microbiol Ecol 51:109–121
20. Talbot V, Bianchi M (1997) Bacterial proteolytic activity in sediments of the Subantarctic Indian Ocean Sector. Deep-Sea Res II 44:1069–1084
21. Xiong H, Song L, Xu Y, Tsoi M-Y, Dobretsov S, Qian P-Y (2007) Characterization of proteolytic bacteria from the Aleutian deep-sea and their proteases. J Ind Microbiol Biotechnol 34:63–71
22. Xu H, Wu M, Wang X, Yang J, Wang C (2008) Bacterial diversity in deep-sea sediment from northeastern Pacific Ocean. Acta Ecol Sinica 28:479–485
23. Zeng R, Zhang R, Zhao J, Lin N (2003) Cold-active serine alkaline protease from the psychrophilic bacterium *Pseudomonas* strain DY-A: enzyme purification and characterization. Extremophiles 7(4):335–337