

Family-level diversity of extracellular proteases of sedimentary bacteria from the South China Sea

Jinyu Yang^{1,4}, Yangyang Feng¹, Xiulan Chen¹, Binbin Xie¹, Yuzhong Zhang^{1,2,3}, Mei Shi¹, Xiyang Zhang^{1*}

¹ State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Qingdao 266237, China

² Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao 266237, China

³ College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

⁴ Institute of Agro-Food Science and Technology, Shandong Academy of Agricultural Sciences/Key Laboratory of Agro-Products Processing Technology of Shandong Province/Key Laboratory of Novel Food Resources Processing, Ministry of Agriculture and Rural Affairs, Jinan 250100, China

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Abstract

Protease-producing bacteria and their extracellular proteases are key players in degrading organic nitrogen to drive marine nitrogen cycling and yet knowledge on both of them is still very limited. This study screened protease-producing bacteria from the South China Sea sediments and analyzed the diversity of their extracellular proteases at the family level through N-terminal amino acid sequencing. Results of the 16S rRNA gene sequence analysis showed that all screened protease-producing bacteria belonged to the class Gammaproteobacteria and most of them were affiliated with different genera within the orders Alteromonadales and Vibrionales. The N-terminal amino acid sequence analysis for fourteen extracellular proteases from fourteen screened bacterial strains revealed that all these proteases belonged to the M4 family of metalloproteases or the S8 family of serine proteases. This study presents new details on taxa of marine sedimentary protease-producing bacteria and types of their extracellular proteases, which will help to comprehensively understand the process and mechanism of the microbial enzymatic degradation of marine sedimentary organic nitrogen.

Key words: protease-producing bacteria, diversity, extracellular proteases, protease families, N-terminal amino acid sequencing, South China Sea

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1 Introduction

Microbial degradation of organic matter is a crucial process for the element and nutrient cycling in marine environments. Microbial extracellular enzymes play pivotal roles in this process by effectively catalyzing the decomposition of high molecular weight organic matter into smaller and assimilable units and thereby initiating the mineralization of organic matter (Arnosti, 2011; Arnosti et al., 2014; Azam and Malfatti, 2007; Boetius and Lochte, 1994; Fabiano and Danovaro, 1998; Patel et al., 2001; Poremba, 1995; Talbot and Bianchi, 1997; Zimmerman et al., 2013). Proteins constitute large portions of marine organic matters (Bridoux et al., 2015; Lloyd et al., 2013; Moore et al., 2014; Nelson and Baldock, 2005), protease-producing bacteria and the proteases they secrete are therefore usually considered to act as important players in the microbial degradation process of marine organic nitrogen, especially high molecular weight organic nitrogen (Chen et al., 2009; Herbert, 1999; Qin et al., 2010; Zhao et al., 2008, 2012; Zhou et al., 2009). Knowing the taxa of pro-

tease-producing bacteria and the types of the extracellular proteases they produce is a major requisite for the full and accurate understanding of the process and its ecological significance (Arnosti, 2011; Chen et al., 2009; Jørgensen and Boetius, 2007; Lloyd et al., 2013; Tsuboi et al., 2014). However, to date, there are only a few studies addressing the issue and knowledge on the above two aspects in most sea areas is still lacking.

Investigation of the diversity of marine protease-producing bacteria began with a work on the sub-Antarctic coastal areas (Isla de Los Estados, Argentina) in 2007, in which nineteen protease-producing bacteria were isolated from sub-Antarctic marine sediments and were found to belong to the genera *Pseudoalteromonas*, *Shewanella*, *Colwellia* and *Planococcus* and the family Flavobacteriaceae, with *Pseudoalteromonas* and *Shewanella* being the predominant ones (Olivera et al., 2007). Later, the diversities of the cultivable protease-producing bacteria in the sediments from several other marine areas have been successively reported, showing that the sedimentary protease-producing bac-

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*Corresponding author, E-mail: zhangxiyang@sdu.edu.cn

teria communities and the predominant groups of them were quite different among different sea areas. For examples, seventy-eight protease-producing bacterial strains screened from sediments of the tropical South China Sea were mainly affiliated with the genera *Pseudoalteromonas*, *Alteromonas*, *Marinobacter*, *Idiomarina*, *Halomonas*, *Vibrio*, *Shewanella*, *Pseudomonas* and *Rheinheimera* within the class Gammaproteobacteria and dominated by *Alteromonas* and *Pseudoalteromonas* (Zhou et al., 2009); while one hundred and twenty-four protease-producing strains screened from sediments of a temperate bay (Laizhou Bay, China) were mainly affiliated with the genera *Bacillus*, *Jeotgalibacillus*, *Halobacillus*, *Planococcus*, *Oceanobacillus*, *Pseudoalteromonas*, *Photobacterium*, *Halomonas*, *Rheinheimera*, *Alcanivorax*, *Celeribacter*, *Sulfobacter*, *Marinobacter* and *Ruegeria* within the phyla Firmicutes and Proteobacteria with *Bacillus*, *Pseudoalteromonas* and *Photobacterium* being the preponderant ones (Li et al., 2017).

In addition to the diversity of protease-producing bacteria, the diversity of the extracellular proteases produced by bacteria from marine sediments was also revealed through inhibitor analysis. Surprisingly, although the structural compositions of the sedimentary protease-producing bacteria communities and the predominant groups varied considerably in different sea areas, the principle types of the extracellular proteases of sedimentary protease-producing bacteria from different sea areas are consistent: being serine proteases and metalloproteases (Li et al., 2017; Zhang et al., 2015; Zhou et al., 2009, 2013). Proteases are divided into nine types based on their catalytic types, and proteases in each type are further classified into clans and then families. A family is sets of homologous proteases with a significant similarity in amino acid sequence and proteases are now divided into two hundred and sixty-seven families (Rawlings et al., 2018). Although the extracellular proteases from sedimentary bacteria have been shown to be serine proteases and metalloproteases, there are hitherto no reports investigating the diversity, at a more fine level (e.g., at the family level), of extracellular proteases from sedimentary protease-producing bacteria. Thus, it is unknown

what kinds of serine proteases and metalloproteases are secreted by sedimentary bacteria. In this study, we investigated the family-level diversity of the extracellular proteases from marine sedimentary bacteria. Protease-producing bacteria were screened from sediment samples of the South China Sea, their extracellular proteases were further purified and the N-terminal sequences of the purified extracellular proteases were determined to reveal the family-level diversity of marine sedimentary bacterial proteases.

2 Materials and methods

2.1 Isolation of protease-producing bacterial strains from the South China Sea sediments

Sediment samples were collected from seventeen stations of the South China Sea during August to September, 2008. These stations are distributed in different regions of the northern part of the South China Sea and in different water depths (35–1 320 m) (Fig. 1). The sediment samples were stored at 4°C before their use as sources to isolate bacteria. Screening of protease-producing bacteria from these sediment samples were performed with the conventional dilution-plate technique using a selective medium containing 0.2% yeast extract, 0.3% casein, 0.5% gelatin, 1.5% agar and artificial sea water (prepared with Sigma sea salts) (pH 8.0) at 15°C, as described in Zhou et al. (2009). Colonies showing hydrolysis zone on the selective plates were picked up and purified by repeated streaking on the selective medium. Strains purified were cultured in TYS broth (0.5% tryptone, 0.1% yeast extract and artificial seawater) at 15°C and stored at –80°C in TYS broth containing 15% (v/v) glycerol.

2.2 Identification of protease-producing bacterial strains through 16S rRNA gene sequencing

For phylogenetic identification of these protease-producing bacterial strains screened, chromosomal DNAs used as the templates were extracted with a bacterial genomic DNA isolation kit (BioTeke, China) and their 16S rRNA genes were PCR-amplified

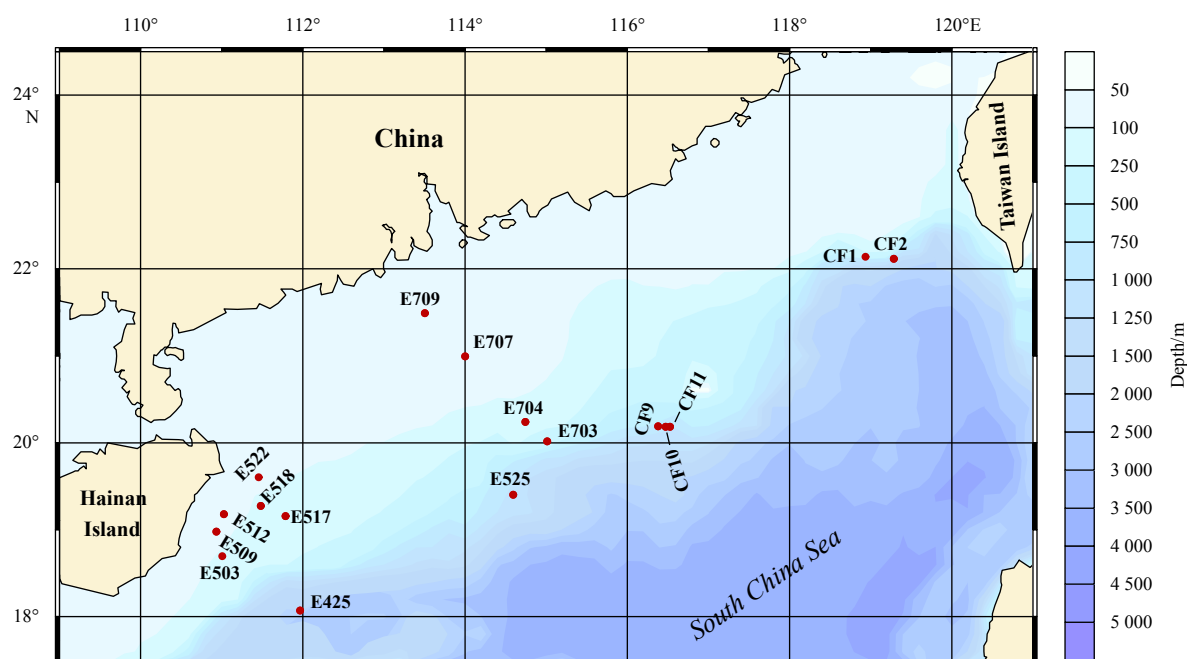


Fig. 1. Geographic location of sampling stations in the South China Sea.

using the primers 27F and 1492R. The 16S rRNA gene sequences were sequenced using an ABI 3730 automatic DNA sequencer. The obtained 16S rRNA gene sequences were subjected to BLAST searches against the GenBank database to determine the phylogenetic affiliation to different bacterial genera of strains screened. Sequence alignment and phylogenetic analysis were conducted using MEGA Version 5 (Tamura et al., 2011). Phylogenetic trees were generated using the Neighbour-Joining method (Saitou and Nei, 1987) with evolutionary distances being calculated according to the Kimura two-parameter model (Kimura, 1980) and bootstrap analysis being based on 1 000 replicates.

2.3 Hydrolysis ability towards casein and gelatin of extracellular proteases

The screened protease-producing bacterial strains were, respectively, streaked onto two solid media (containing 0.5% casein or 0.5% gelatin, 0.2% yeast extract, 1.5% agar and artificial sea water) and incubated at 15°C for 3–10 d. The hydrolytic zone diameter and colony diameter of every strain on each of the two solid media were, respectively, measured. The ratio of hydrolytic zone diameter to colony diameter (H/C) was then calculated to reflect the hydrolysis ability of the extracellular proteases of each strain towards casein or gelatin. The average H/C value of three colonies of each strain was taken.

2.4 Protease activity assay

Protease-producing strains were cultivated in medium containing 0.2% yeast extract, 0.5% gelatin, 0.5% casein and artificial sea water at 15°C at the speed of 200 r/min for 3 d, then the fermentation broth containing the extracellular proteases was collected by centrifugation. Protease activity was determined as described previously (Chen et al., 2003). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine from casein per minute.

2.5 Purification of extracellular proteases

Protease-producing strains were cultured in the fermentation medium containing 0.5% casein, 0.5% gelatin, 0.2% yeast extract and artificial seawater at 15°C until the protease activity reached maximum. The fermentation broth of each strain was centrifuged (10 000× g, 10 min) at 4°C and the supernatant was precipitated by adding ammonium sulfate at an appropriate concentration. The resultant precipitate was gathered by centrifugation (10 000× g, 10 min) and dissolved in 50 mmol/L Tris-HCl (pH 8.5). The solution obtained was dialyzed against 50 mmol/L Tris-HCl (pH 8.0) to remove excess ammonium sulfate. The dialyzed solution was then loaded onto a DEAE-Sepharose Fast Flow column (Amersham Biosciences, USA) pre-equilibrated with 50 mmol/L Tris-HCl (pH 8.0) and was subsequently eluted with a linear gradient of NaCl from 0 to 1.0 mol/L. Fractions with protease activity were collected and subjected to the following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.6 SDS-PAGE analysis for extracellular proteases

SDS-PAGE was performed onto 12.5% running gels for the purity examination and molecular weight determination of the target proteases in the active fractions. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 to visualize protein bands.

2.7 Zymography analysis for extracellular proteases

Gelatin zymography analysis for the extracellular proteases

partially purified was performed as described previously (He et al., 2012) with slight modification. In the analysis, proteases were firstly separated on SDS-polyacrylamide gels containing 0.2% gelatin. The gels were subsequently immersed in the Triton X-100 solution (2.5%, v/v) for 45 min to remove SDS and further soaked into 50 mmol/L Tris-HCl buffer at 37°C for 2 h to allow proteases to perform their activities to gelatin. The gels were finally stained with Coomassie brilliant blue R-250 and de-stained. The clear and white bands on the gels against the blue background represented protease bands.

2.8 N-terminal amino acid sequence determination

After the protease sample was separated in SDS-polyacrylamide gels by electrophoresis, the sample was transferred to the Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad) at a constant current of 200 mA in a CAPS buffer system for 2 h. Then the membrane was stained by Coomassie brilliant blue G-250 and de-stained. The N-terminal amino acid sequences of target proteases were determined using the Edman degradation method on a PROCISE 491 protein sequencer (Applied Biosystems, USA) at Peking University (China).

2.9 Assignment of proteases to different families

The N-terminal amino acid sequences obtained were BLAST searched against the GenBank non-redundant protein database to find homologues of the target proteases. The nearest homologue to each target protease found was searched for conserved domains in its full-length amino acid sequence through NCBI CD-Search service (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to identify the protease family it belongs to and the target protease was then assigned to the same family. Sequence alignment was performed with ClustalW (Chenna et al., 2003) and edited with ESPript (Robert and Gouet, 2014).

2.10 Nucleotide sequence accession numbers

The 16S rRNA gene sequences for the strains screened in this study were deposited in GenBank under the accession numbers from MF975547 to MF975636.

3 Results

3.1 Diversity of the protease-producing bacteria screened from the South China Sea sediments

A total of ninety protease-producing bacterial strains were screened from sediments samples collected from seventeen stations of the South China Sea (Fig. 1). Taxonomic belongingness to different bacterial genera of these new isolates was subsequently determined by comparatively analyzing their nearly complete 16S rRNA gene sequences. The results showed that all strains screened belonged to nine genera in the class Gammaproteobacteria of the phylum Proteobacteria, i.e., *Pseudoalteromonas*, *Photobacterium*, *Vibrio*, *Shewanella*, *Alteromonas*, *Idiomarina*, *Pseudomonas*, *Oceanospirillum* and *Psychrobacter*. On the whole, *Pseudoalteromonas* (44.4%), *Photobacterium* (20.0%), *Vibrio* (12.2%), *Shewanella* (10.0%) and *Alteromonas* (6.6%), were the preponderant groups, while *Idiomarina* (2.2%), *Pseudomonas* (2.2%), *Oceanospirillum* (1.1%) and *Psychrobacter* (1.1%), all containing only one or two strains, occupied very few proportions of the total bacteria screened. Meanwhile, *Pseudoalteromonas* (forty strains) was found to be present in fourteen sediments and to be dominant in nine samples (CF9, CF10, CF11, E425, E503, E512, E704, E707 and E709) (Fig. 2), hav-

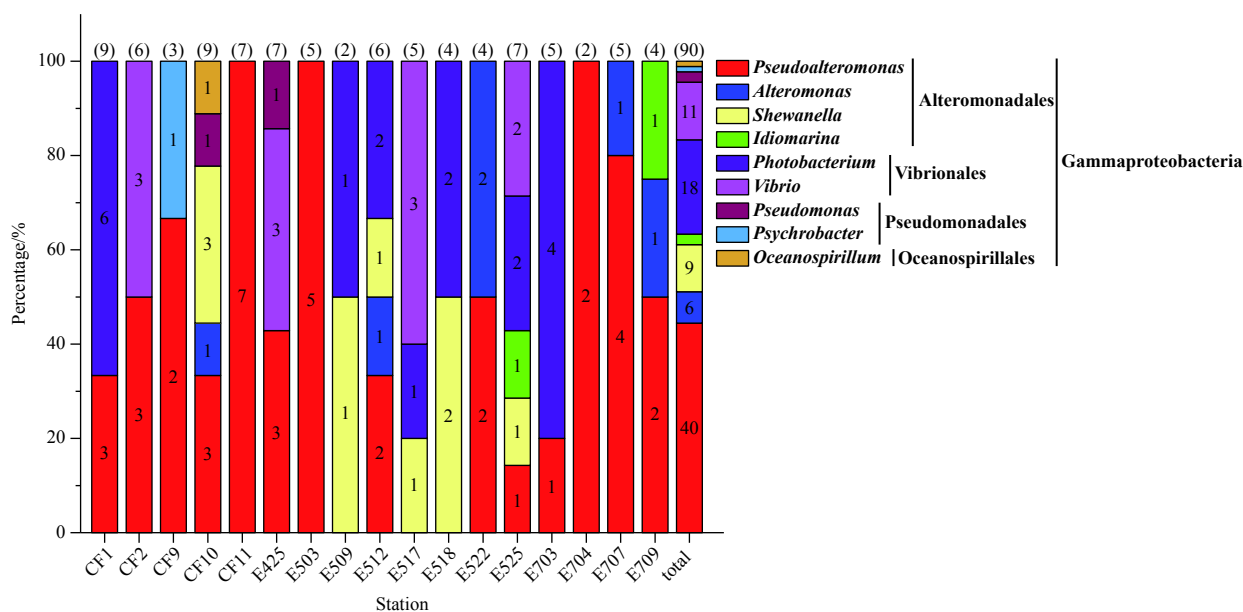


Fig. 2. Relative percentage abundance of the phylotypic groups of cultivable protease-producing bacteria isolated from seventeen stations in the South China Sea. The total number of strains screened from each station is shown in the parenthesis above each column. Strain numbers per genus are shown in the centers of sections of the columns.

ing a much wider distribution in different stations and greater quantity than other groups and hence representing the most frequently recovered and abundant group of the cultivable protease-producing bacteria in the South China Sea sediments studied. In addition, protease-producing bacteria from CF10 and E525 samples belonged to five genera, in a striking contrast to those from CF11, E503, E704 samples all belonging to only one genus (*Pseudoalteromonas*), constituting the two most diverse communities of the cultivable protease-producing bacteria among the seventeen stations.

Phylogenetic relationships of all the protease-producing strains to different genera were depicted in the neighbor-joining tree constructed based on 16S rRNA gene sequences (Fig. 3). Some *Pseudoalteromonas* and *Photobacterium* strains showing closely phylogenetic relationships to each other clustered into two branches in Fig. 3: Branch 1 (Fig. 4a) consisting of thirty-seven *Pseudoalteromonas* strains, all being closely related to *Pseudoalteromonas shioyasakiensis* (AB720724) isolated from a Pacific Ocean sediment (Matsuyama et al., 2014) and Branch 2 (Fig. 4b) consisting of seven *Photobacterium* strains, all being closely related to *Photobacterium lutimaris* (DQ534014) isolated from a tidal flat sediment in Korea (Jung et al., 2007).

3.2 Diversity of the bacterial extracellular proteases based on their hydrolytic ability towards gelatin and casein

The extracellular proteases diversity of the protease-producing bacteria screened from the South China Sea sediments was firstly preliminarily characterized by measuring the H/C ratios of colonies on the plates containing gelatin or casein. Judged from each strain's H/C ratios on plates containing casein or gelatin (Table 1), extracellular proteases from eighty-one strains (90% of the total strains) could hydrolyze casein, those from fifty-one strains (56.7% of the total strains) could hydrolyze gelatin and those from forty-six strains (51.1% of the total strains) could hydrolyze both the substrates. In particular, the extracellular proteases from five *Photobacterium* strains (CF1-1, CF1-3, CF1-8,

CF1-9 and E512-5) show very high gelatinolytic activity with the H/C ratios more than 5 and those from three *Pseudoalteromonas* strains (CF2-12, E512-3 and E707-8), three *Alteromonas* strains (E522-9, E522-12 and E709-2), five *Photobacterium* strains (CF1-5, E525-10, E703-4, E703-12 and E703-13), three *Shewanella* strains (CF10-3, E518-1 and E525-6), five *Vibrio* strains (CF2-6, CF2-9, E517-4, E517-9 and E525-9) and one *Pseudomonas* strain (CF10-6) showed relatively high gelatinolytic activity with the H/C ratios more than 3. In the meantime, extracellular proteases from one *Alteromonas* strain (E709-2) and one *Photobacterium* strain (E703-4) showed very high caseinolytic activity with the H/C ratios more than 5, and those from twelve *Pseudoalteromonas* strains (CF1-10, CF2-5, CF2-13, CF9-2, E503-8, E512-3, E704-4, E704-6, E707-6, E707-8, E707-9 and E709-10), one *Alteromonas* strain (E522-12), one *Photobacterium* strain (E512-5) and one *Oceanospirillum* strain (CF10-4), three *Shewanella* strains (CF10-5, E518-1 and E518-2) and nine *Vibrio* strains (CF2-6, CF2-8, CF2-9, E425-4, E425-5, E517-4, E517-7, E517-9 and E525-9) showed relatively high caseinolytic activity with the H/C ratios more than 3. Moreover, the extracellular proteases from twelve strains (including strains E512-3 and E707-8 of *Pseudoalteromonas*, E518-1 of *Shewanella*, E522-12 and E709-2 of *Alteromonas*, E512-5 and E703-4 of *Photobacterium* and CF2-6, CF2-9, E517-4, E517-9 and E525-9 of *Vibrio*) showed relatively high activity towards both gelatin and casein because their H/C ratios on the two kinds of plates containing gelatin or casein were both greater than 3 (Table 1). Altogether, differences in the hydrolysis ability towards gelatin and casein of the extracellular proteases from the screened strains reflect the difference in kinds or in substrate specificity of these proteases and their diversity and complexity.

3.3 Family-level diversity of the bacterial extracellular proteases analyzed by N-terminal amino acid sequencing

To identify their types at the family level, the extracellular proteases secreted by the protease-producing bacteria screened from the South China Sea sediments were purified and their N-

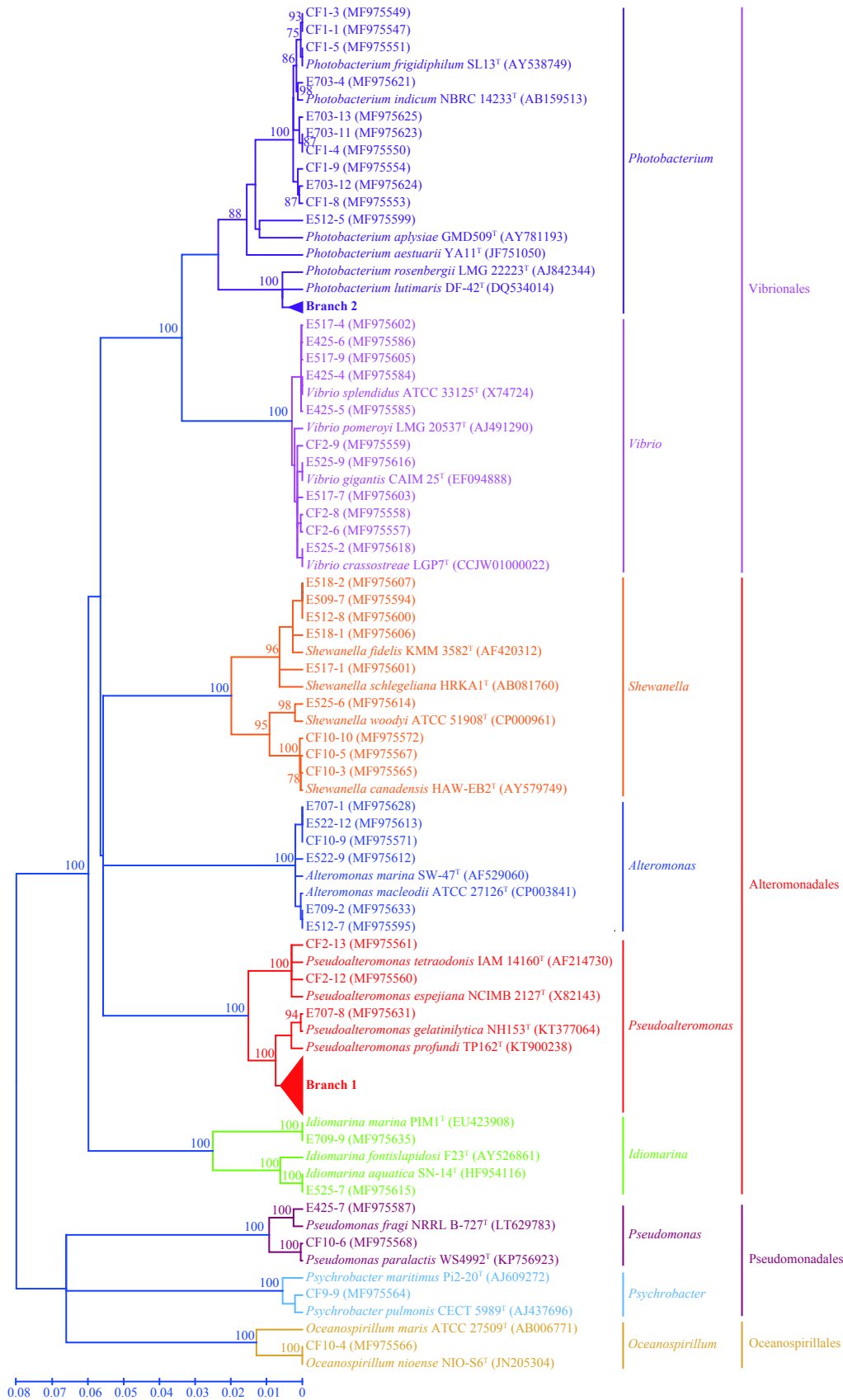


Fig. 3. Neighbor-joining tree of the protease-producing bacteria isolated from seventeen stations in the South China Sea based on their 16S rRNA gene sequences. Branch 1 contains 37 *Pseudoalteromonas* strains closely related to *Pseudoalteromonas shioyasakiensis* (AB270724); and Branch 2 contains 7 *Photobacterium* strains closely related to *Photobacterium lutimaris* (DQ534014).

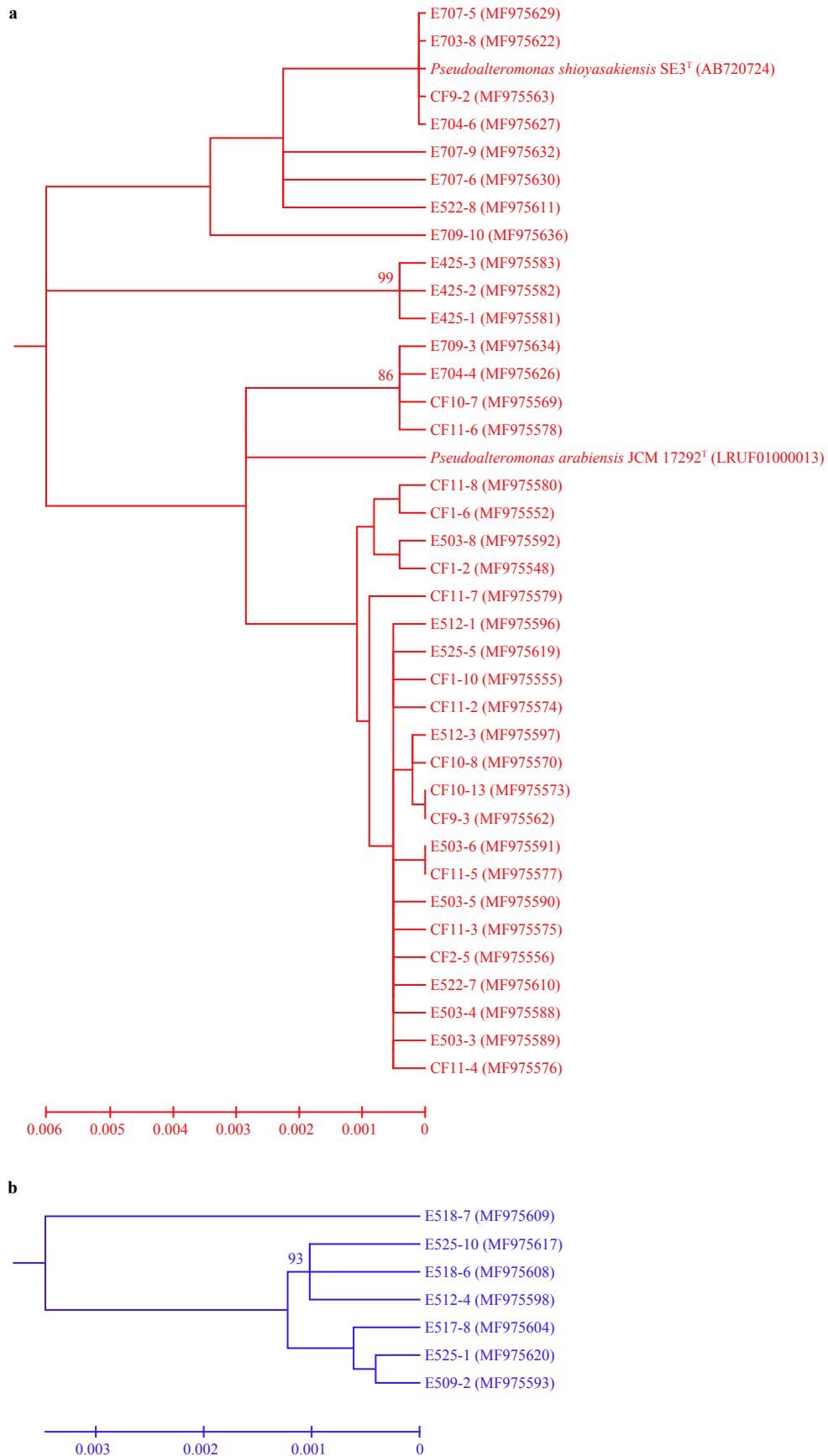


Fig. 4. The Neighbor-joining phylogenetic tree of the strains in Branch 1 (a) and Branch 2 (b) in Fig. 3 based on the 16S rRNA gene sequences.

Table 1. The H/C ratios of the strains on the plates containing gelatin or casein

Genera	Strain	H/C ratio ¹⁾		Genera	Strain	H/C ratio	
		Gelatin	Casein			Gelatin	Casein
<i>Pseudoalteromonas</i>	CF1-2	thin ²⁾	2.46	<i>Photobacterium</i>	CF1-1	7	2.83
	CF1-6	thin	2.41		CF1-3	5.65	2.18
	CF1-10	thin	3.27		CF1-4	thin	1.4
	CF2-5	2.85	3.18		CF1-5	3.65	2.7
	CF2-12	3.25	2.98		CF1-8	6.82	2.83
	CF2-13	thin	3.03		CF1-9	5.83	2.9
	CF9-2	thin	4		E509-2	thin	2.83
	CF9-3	thin	2.63		E512-4	1.3	thin
	CF10-7	thin	2.6		E512-5	5.25	3.89
	CF10-8	thin	1.79		E517-8	thin	thin
	CF10-13	thin	2.45		E518-6	1.3	thin
	CF11-2	1.36	2.18		E518-7	2	thin
	CF11-3	1.4	2.01		E525-1	thin	2.58
	CF11-4	1.4	1.5	E525-10	3.49	thin	
	CF11-5	thin	1.69	E703-4	5	5.9	
	CF11-6	1.3	2.3	E703-11	1.45	1.37	
	CF11-7	thin	2.43	E703-12	4.22	2	
	CF11-8	thin	2.17	E703-13	3.62	2.27	
	E425-1	thin	2.6	<i>Shewanella</i>	CF10-3	3.55	1.8
	E425-2	1.3	2.24		CF10-5	thin	3.78
	E425-3	thin	2.73		CF10-10	thin	2.43
	E503-3	thin	1.7		E509-7	2.67	2.7
	E503-4	thin	2.27		E512-8	thin	1.43
	E503-5	thin	2.04		E517-1	thin	2
	E503-6	thin	thin		E518-1	3.2	4.5
	E503-8	thin	3.25		E518-2	thin	3.5
	E512-1	1.98	2.12		E525-6	4.17	2.44
	E512-3	3.2	4		<i>Vibrio</i>	CF2-6	4.33
	E522-7	2.11	2.8	CF2-8		thin	3.51
	E522-8	thin	2.85	CF2-9		3.7	3.5
	E525-5	thin	thin	E425-4		2.51	3.85
	E703-8	thin	2.97	E425-5		3	3.25
	E704-4	thin	4	E425-6		2.67	3
E704-6	2.2	4.5	E517-4	3.2		5	
E707-5	3	2	E517-7	2		3.3	
E707-6	2.4	3.5	E517-9	3.1		4.7	
E707-8	3.9	4.4	E525-2	1.66		2.17	
E707-9	2.9	3.3	E525-9	3.78	3.3		
E709-3	1.2	3	<i>Idiomarina</i>	E525-7	thin	3	
E709-10	2.8	5		E709-9	thin	2	
<i>Alteromonas</i>	CF10-9	thin	2.5	<i>Pseudomonas</i>	CF10-6	3.4	thin
	E512-7	thin	thin		E425-7	1.52	2.76
	E522-9	4	2.75	<i>Oceanospirillum</i>	CF10-4	thin	3.2
	E522-12	4.33	3.22		<i>Psychrobacter</i>	CF9-9	thin
	E707-1	2.3	2				
E709-2	3.3	6					

Note: ¹⁾ H/C ratio is the ratio of the hydrolytic zone diameter to the colony diameter of a colony on the plate. The data were the average H/C values of three colonies of each strain. ²⁾ Thin represents a very slight hydrolytic zone formed by the strain.

terminal amino acid sequences were determined. When cultivated in the fermentation medium, the amount of extracellular protease secreted by each strain was determined by protease activity assay. Finally, we found that the amount of secreted extracellular proteases of only fourteen strains affiliated with the genera *Pseudoalteromonas* (eight), *Photobacterium* (two), *Vibrio* (three) and *Idiomarina* (one) was enough to be purified for the

subsequent N-terminal amino acid sequencing. Then, the extracellular proteases of these strains were purified by ion exchange chromatography. The different fractions from ion exchange chromatography were analyzed by protease activity assay and the fractions with protease activity were collected for zymography analysis to confirm the gelatin hydrolysis activity (Fig. 5). Then the N-terminal amino acid sequences of the purified proteases

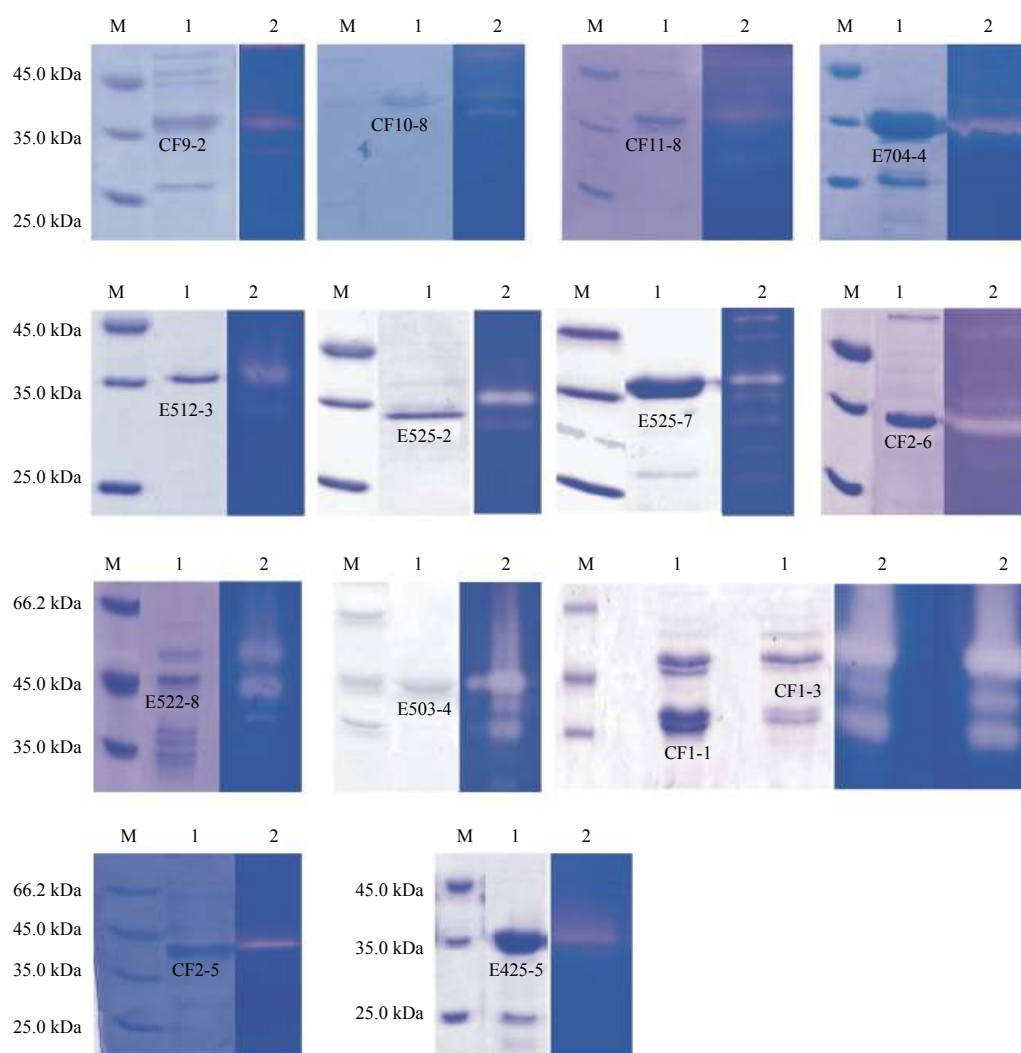


Fig. 5. The zymography analysis of extracellular proteases. M: molecular weight marker proteins; Lane 1: SDS-PAGE analysis of extracellular proteases after DEAE chromatography; and Lane 2: extracellular proteases activities to gelatin shown on gels.

were determined and BLAST searched. According to the results of the blast searching of the N-terminal amino acid sequences and the conserved domains searching of their homologues, all the sequenced extracellular proteases of the fourteen strains could be assigned to the M4 family of metalloproteases or the S8 family of serine proteases (Table 2), which was in good consistency with previous observations that metalloproteases and/or serine proteases were the principle extracellular proteases of the sedimentary protease-producing bacteria (Li et al., 2017; Zhang et al., 2015; Zhou et al., 2009). The alignments between the 14 N-terminal amino acid sequences and the previously experimental validated proteases in the same family were shown in Fig. 6. More specifically, thirteen extracellular proteases (of the total fourteen proteases to be N-terminally determined) from thirteen strains including seven *Pseudoalteromonas* strains (CF9-2, CF10-8, CF11-8, E704-4, E503-4, E522-8 and E512-3), two *Photobacterium* strains (CF1-1 and CF1-3), three *Vibrio* strains (CF2-6, E525-2 and E425-5) and one *Idiomarina* strain (E525-7) belonged to the M4 family and one protease from the *Pseudoalteromonas* strain CF2-5 belonged to the S8 family (Table 2), which indicated that the M4 and S8 proteases are probably the main types of the extracellular proteases of the screened strains.

4 Discussion

The major groups of the cultivable protease-producing bacteria recovered from the South China Sea in this study, including *Pseudoalteromonas*, *Photobacterium*, *Vibrio*, *Shewanella* and *Alteromonas*, all are members of the orders Alteromonadales and Vibrionales of the class Gammaproteobacteria. These groups (or at least some of them) and some other groups in the two orders were also found prevalent and predominant in the cultivable protease-producing bacteria communities from other sediment samples (Li et al., 2017; Olivera et al., 2007; Zhang et al., 2015; Zhou et al., 2009), indicating that Gammaproteobacteria belonging to the orders Alteromonadales and Vibrionales seem to be the major groups of the cultivable protease-producing bacteria in marine sedimentary environments. Intriguingly, members of the orders Alteromonadales and Vibrionales were also reported to be prolific producers of antagonistic materials (Long and Azam, 2001), and hence the extracellular proteases they secrete maybe represent one type of the inhibitory agents to other strains in view of the fact that some bacterial proteases, such as staphylolysin from *Pseudomonas aeruginosa* (Kessler et al., 1993) and lysostaphin from *Staphylococcus simulansare* (Schindler and Schuardt, 1964), have been reported to have lytic activities to-

Table 2. N-terminal sequences of and families of the extracellular proteases produced by strains screened from the South China Sea sediments

Strain	Genera	AMW/kDa	N-terminal sequences	Protease families
CF9-2	<i>Pseudoalteromonas</i>	35.0	ASATGPGANLKTGKY	M4
CF10-8	<i>Pseudoalteromonas</i>	35.0	ASATGPGANLKTGKY	M4
CF11-8	<i>Pseudoalteromonas</i>	35.0	ASATGPGANLKTGKY	M4
E704-4	<i>Pseudoalteromonas</i>	35.0	ASATGPGANLKTGKY	M4
E503-4	<i>Pseudoalteromonas</i>	45.0	ASATGPGGNLKTGKY	M4
E522-8	<i>Pseudoalteromonas</i>	45.0	ASATGPGGNLKTGKY	M4
CF1-1	<i>Photobacterium</i>	35.0	ASATGPGGNLKTGKY	M4
CF1-3	<i>Photobacterium</i>	35.0	ASATGPGGNLKTGKY	M4
E512-3	<i>Pseudoalteromonas</i>	35.0	AKALGTGPGGNIKTT	M4
CF2-6	<i>Vibrio</i>	35.0	AKASGTGPGGNIKTT	M4
E525-2	<i>Vibrio</i>	35.0	AKASGTGPGGNIKTT	M4
E425-5	<i>Vibrio</i>	35.0	AKSSGTGPGGNLKT	M4
E525-7	<i>Idiomarina</i>	35.0	AKALGTGPGGNLKT	M4
CF2-5	<i>Pseudoalteromonas</i>	40.0	SATNDPPFDLLGLN	S8

Note: AMW represents apparent molecular weight.

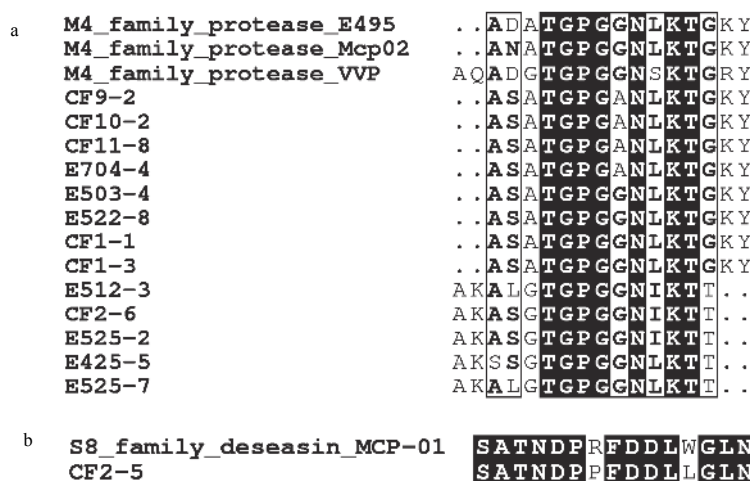


Fig. 6. Sequence alignments between the extracellular proteases and the previously experimental validated proteases from the corresponding families. a. Alignment of M4 family proteases. The accession numbers of the reference proteases are as followed: E495 (ACI28452), Mcp02 (ABL06977) and VVP (AAC44789). b. Alignment of S8 family proteases. The accession number of the reference protease MCP-01 is ABD14413. Black boxes with white characters indicate residue identity; bold characters residue similarity; and black-framed characters similarities between groups of residues.

ward cells of certain bacterial species.

Metalloproteases and serine proteases are both greatly diverse, including seventy-five and fifty-three families, respectively, to date, and each family contains one or more different proteases (<https://www.ebi.ac.uk/merops/>). Although it has been found that marine sedimentary bacteria mainly secrete metalloproteases and serine proteases (Li et al., 2017; Zhang et al., 2015; Zhou et al., 2009, 2013), the diversity of extracellular proteases from marine sedimentary bacteria at family level or even more fine level has not yet been revealed. In this study, through N-terminal amino acid sequencing, extracellular proteases of fourteen sedimentary strains screened were found belonging to the M4 family of metalloproteases or the S8 family of serine proteases. Most proteases of these two families are secreted enzymes able to degrade extracellular proteins and peptides to support the bacterial growth (Rawlings et al., 2018), meaning that they may act as key forces in decomposing nitrogenous organic matters to drive the nitrogen cycling in marine sedimentary environments. Moreover, a number of M4 and S8 proteases from marine sedi-

mentary bacteria have been identified and characterized, such as the M4 metalloproteases MCP-02 and HSPA, and the S8 serine proteases myroicolsin, deseasin MCP-01, MCP-03, P57, Sep1 and a *Planomicrobium* sp. 547 protease, which display particular structural and catalytic properties to efficiently decompose proteinaceous substrates (Chen et al., 2003, 2007; Gao et al., 2010; Geng et al., 2016; Li et al., 2016; Ran et al., 2013, 2014; Yan et al., 2009; Yang et al., 2011, 2013; Zhao et al., 2008), exemplifying the wide distribution of the M4 and S8 proteases in marine sedimentary bacteria and meanwhile highlighting their ecological roles in marine nitrogenous organic matters degradation. In addition, by applying the metaproteomic technique, deseasin MCP-01 (the S8 family) and metalloprotease MCP-02 (the M4 family) were directly detected in marine particulate organic matter (POM) samples obtained by filtering large volumes of oligotrophic seawater (≥ 600 L) collected at different depths (300 and 500 m) of the Sargasso Sea (Bridoux et al., 2015), further demonstrating the prevalence of the M4 and S8 family proteases in marine environments and also supporting our findings in this study that the

M4 metalloproteases and S8 serine proteases are the main types of the extracellular proteases of marine sedimentary strains.

To sum up, this study analyzed the diversity of cultivable protease-producing sedimentary bacteria from the South China Sea based on the 16S rRNA gene sequences analysis and their extracellular proteases at the family level through N-terminal amino acid sequencing. It was found that the protease-producing bacterial strains screened were mostly affiliated with different genera belonging to the orders Alteromonadales and Vibrionales of the class Gammaproteobacteria and their extracellular proteases mainly belonged to the M4 family of metalloproteases and the S8 family of serine proteases. These findings provide new details on taxa of cultivable protease-producing sedimentary bacteria and types of their extracellular proteases, which broaden our understanding to protease-producing bacteria and their extracellular proteases in marine environments and are helpful in accurately and completely elucidating the mechanism underlying sedimentary organic nitrogen degradation. In addition, it is worth mentioning that, it still remains a great technological challenge to purify all the bacterial extracellular proteases for N-terminal amino acid sequencing because the amount of some extracellular proteases was too small in our cultural condition to be purified. Therefore, new techniques are urgently required to more effectively and accurately reveal the diversity of marine bacterial extracellular proteases.

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