

Characteristics of the cultivable bacteria from sediments associated with two deep-sea hydrothermal vents in Okinawa Trough

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Abstract In this study, different culture-dependent methods were used to examine the cultivable heterotrophic bacteria in the sediments associated with two deep-sea hydrothermal vents (named HV1 and HV2) located at Iheya Ridge and Iheya North in Okinawa Trough. The two vents differed in morphology, with HV1 exhibiting diffuse flows while HV2 being a black smoker with a chimney-like structure. A total of 213 isolates were identified by near full-length 16S rRNA gene sequence analysis. Of these isolates, 128 were from HV1 and 85 were from HV2. The bacterial community structures were, in large parts, similar between HV1 and HV2. Nevertheless, differences between HV1 and HV2 were observed in one phylum, one class, 4 orders, 10 families, and 20 genera. Bioactivity analysis revealed that 25 isolates belonging to 9 different genera exhibited extracellular protease activities, 21 isolates from 11 genera exhibited extracellular lipase activities, and 13 isolates of 8 genera displayed antimicrobial activities. This is the first observation of a large population of bacteria with extracellular bioactivities existing in deep-sea hydrothermal vents. Taken together, the results of this study provide

new insights into the characteristics of the cultivable heterotrophic bacteria in deep-sea hydrothermal ecosystems.

Keywords Deep-sea hydrothermal vent · Sediment · Cultivable bacteria · Lipase · Protease · Antimicrobial

Introduction

Since their first discovery in 1977, hydrothermal vents have drawn a great deal of attention. Nowadays it is known that hydrothermal vents possess unique geological structures, complex physicochemical characteristics, and diverse biotic communities (Anderson et al. 2014; Kiliyas et al. 2013; Nakamura et al. 2013). The vent biome contains macrofauna and microorganisms, the former including tube worms, mussels, shrimps, crabs, barnacle, and sponges (Buckeridge et al. 2013; Edwards and Nelson 1991; Kim et al. 2013; Nishijima et al. 2010; Plouviez et al. 2013; Watsuji et al. 2010), and the latter including various bacteria and archaea (Polz and Cavanaugh 1995; Takai et al. 2001, 2004; Zhao et al. 2014). Microorganisms play important roles in deep-sea hydrothermal ecosystems. In the absence of photosynthesis, the food chain in the vent system relies on chemoautotrophic prokaryotes, which produce complex organic molecules with energy obtained by oxidizing the inorganic compounds available in the environments. Studies have shown that some chemoautotrophic microorganisms, such as *Thioreductor*, *Sulfurovum*, and *Sulfurimonas*, can use reduced sulfur compounds, Fe(II), Mn(II), H₂, and CH₄ as electron donors to obtain energy and fix carbon, thus providing a basis for hydrothermal vent ecosystems (Biddle et al. 2012; Inagaki et al. 2004; Kanaparthi et al. 2013; Mino et al. 2014; Nakagawa et al. 2005a). In addition, heterotrophic bacteria

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were also rich in hydrothermal systems (Rajasabapathy et al. 2014), and they play important roles in carbon and nitrogen cycling by decomposing particulate organic materials (Zhou et al. 2009a, b).

To date, a number of studies on the microbial communities in deep-sea hydrothermal vents have been reported, mainly focusing on habitats inside or outside of the chimney structure (Takai et al. 2001), on the outer surface of deposits around the vents, typically as mats (Moyer et al. 1995), on and within macrofauna as symbionts (Watsuji et al. 2010), and within the plume of hydrothermal fluid in the overlying seawater (Takai et al. 2004). In most documented reports, the microbial diversity is investigated by culture-independent methods, because the majority of microbes in the vent systems are difficult to cultivate (Amann et al. 1995; Inagaki et al. 2006; Zhou et al. 2009a, b). Nevertheless, culture-dependent method is important, as it is essential for the discovery of new species, novel drug, and natural products with industrial application potential (Guan et al. 2011; Thornburg et al. 2010). Moreover, culture-dependent methods help us understand the complex process that occurs at hydrothermal vents by providing us with model organisms to test the hypothesis, often raised by culture-independent techniques and that thus both methods need to be interrelated for a more comprehensive understanding of the system. Recent studies indicate that cultivable bacteria in shallow water hydrothermal vents are abundant and diverse, and that some of these bacteria possess strong enzyme-producing capacities (Mohandass et al. 2012; Raghukumar et al. 2008; Rajasabapathy et al. 2014). However, similar bioactivity studies of microbes from deep-sea hydrothermal vents are scarce.

Investigations on the microbial community in the hydrothermal vents in the vicinity of Okinawa Trough have been reported (Nakagawa et al. 2005b, c; Takai et al. 2003, 2004). In these reports, systematic survey with culture-dependent method was used only for microbes associated with hydrothermal plume, chimney structures, and vent fluids (Nakagawa et al. 2005b, c). In addition, most of these studies target on autotrophic bacteria, while the heterotrophic bacteria in these vents are largely overlooked. Therefore, the focus of our present study is heterotrophic bacteria. We aimed to investigate the characteristics of the cultivable heterotrophic bacteria in the sediments associated with two deep-sea hydrothermal vents in Iheya North and Iheya Ridge of Okinawa Trough, which exhibited different geochemical features. Moreover, since the current knowledge on the bioactive compounds secreted by deep-sea hydrothermal microorganisms is very limited, we also aimed to investigate the potential of the identified bacteria to produce extracellular protease, lipase, and antimicrobial molecules.

Materials and methods

Sample collection and processing

The samples used in this study were obtained by means of the scientific research vessel KEXUE during the cruise of April 2014. The cruise was part of the research project “Western Pacific Ocean System: Structure, Dynamics, and Consequences—Deep Ocean Environments and Ecosystems” supported by Chinese Academy of Sciences (Tollefson 2014). The sediments approximately 40 meters from Hydrothermal vent 1 (HV1) and Hydrothermal vent 2 (HV2) located in Iheya Ridge and Iheya North respectively, of Okinawa Trough were collected by push-cores on the Remotely Operated Vehicle (ROV) equipped on the KEXUE vessel. The temperature and dissolved oxygen concentration of the sample sites were detected by a conductivity-temperature-depth sampler and a dissolved oxygen sensor, respectively. Undisturbed portions of the samples were immediately processed on board by serial dilutions in sterilized seawater and plating on different solid mediums (see below) in an aseptic environment. The remaining samples were stored at 4 and -80°C .

Bacterial isolation

Three different media were used for bacterial isolation, namely Marine Agar 2216 (Kobayashi et al. 2008), modified R2A (Yin et al. 2013), and Leibovitz's L-15 Medium (Thermo Scientific HyClone, Beijing, China) (Futai et al. 2006). Marine 2216 is a common medium for isolation of marine heterotrophic bacteria. However, compared to marine environments, the nutrients of 2216E medium are rich. To obtain more diverse heterotrophic bacteria, modified R2A and Leibovitz's L-15 were also used. Modified R2A medium prepared with seawater is less nutritious than marine 2216 and widely used for isolation of marine bacteria. Leibovitz's L-15 Medium, purchased from Thermo Scientific HyClone, Beijing, China, is a kind of synthetic medium. It is the least nutritious medium and contains inorganic salts, a small amount of amino acids, and vitamins. It is used to isolate bacteria that demand little nutrient. The plates were incubated at the temperatures of 4, 16, 28, and 40°C under aerobic condition for 3 days to 1 month. The bacterial isolates emerged on the plates were first screened on the basis of colony characteristics including size, shape, margin, color, and opacity (Valiente Moro et al. 2013). One colony per type was selected per plate and re-cultured for further purification. The purified isolates were resuspended in medium containing 15 % (v/v) glycerol and stored at -80°C .

Bacterial identification and phylogenetic analysis

Genomic DNA was prepared from the bacteria with TIANamp bacteria DNA kit (TIANGen, Beijing, China). The nearly complete 16S rRNA genes (~1400 bp) of the bacteria were amplified using primers 27F and 1492R (Kobayashi et al. 2008) with Ex Taq DNA polymerase (Takara, Dalian). The PCR products were purified and sequenced. The 16S rRNA gene sequence similarities were searched using the BLASTn program in the National Center for Biotechnology Information database, the RDP Classifier (Wang et al. 2007), and the EzTaxon server 2.1 (Kim et al. 2012). Evolutionary distances were calculated according to the two-parameter model of Kimura (Kimura 1980). Phylogenetic dendrograms were constructed with maximum likelihood method (Rogers 1997), and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 4.0 software (Tamura et al. 2007).

Geochemical analysis

Chemical composition of the samples was analyzed by the Research Center of Analysis and Measurement (RCAM), Institute of Oceanology, Chinese Academy of Sciences. Major and trace elements were analyzed by an inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Perkin Elmer, USA) using a strong acid digestion method (Lee et al. 2006). The concentrations of total organic carbon (TOC) and total nitrogen (TN) were determined using a PE 2400 Series II CHNS/O analyzer (Perkin Elmer, USA).

Screening for extracellular protease, lipase, and antibacterial activity

For protease activity assay, the bacteria were plated on Marine 2216 medium supplemented with 1 % (w/v) skim milk. The plates were incubated as described above (section “Bacterial isolation”). The diameter of the colony (C) and the diameter of the hydrolytic zone (Z) around the colony were recorded. The ratio of Z/C was calculated and used as an indicator of enzymatic activity. For lipase activity assay, the bacteria were plated on Marine 2216 medium supplemented with 1 % olive oil, 1 % Tween-80, and 1.6 ‰ Bromocresol purple. Lipid was decomposed to fatty acid, which makes Bromocresol purple changing from purple to yellow. For each isolate, the Z/C ratio was determined by the method above. For antimicrobial activity assay, the bacterial cultures were spotted on a sterile filter-paper disk placed on a plate spread with the Gram-positive bacterium *Micrococcus luteus* (purchased from CGMCC, Beijing, China) or the Gram-negative bacterium

Escherichia coli DH5 α (purchased from Tiangen, Beijing, China). The Z/C value was determined as above and used as an indicator of inhibition.

Nucleotide sequence

The 16S rRNA gene sequences of the isolates examined in this study have been deposited in GenBank databases under the accession numbers KM978994-KM979121 (for HV1 isolates) and KM979122-KM979206 (for HV2 isolates).

Results

Sampling sites

Sediment samples were collected from two deep-sea hydrothermal vent fields in Iheya Ridge and Iheya North in Okinawa Trough (Fig. 1). The two vents exhibited different morphologies. Hydrothermal vent 1 (HV1) had no obvious chimney structure but had diffuse flows, while Hydrothermal vent 2 (HV2) was a typical black smoker with a chimney-like structure by the ROV observation at the time of sampling. Samples of HV1 were brown poly-metallic ooze, while samples of HV2 were black sulfides. The characteristics of the samples are indicated in Table 1, which shows that there existed apparent differences in physicochemical features between the fields of HV1 and HV2 in terms of the contents of total organic carbon (TOC), total nitrogen (TN), and metal irons. The TOC and

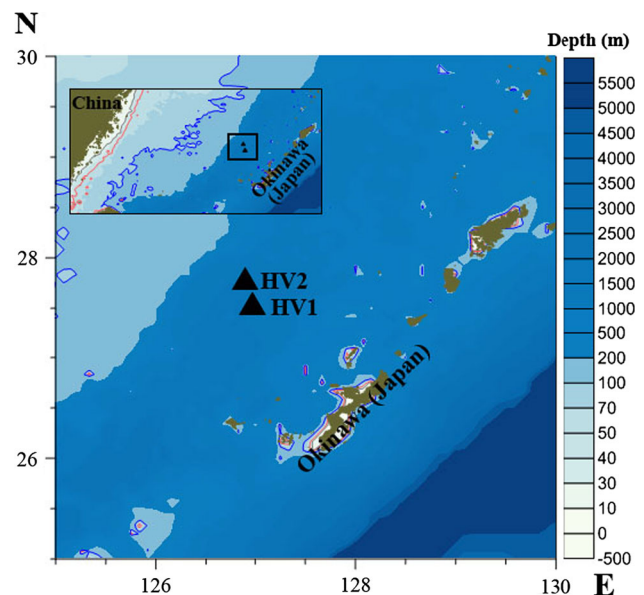


Fig. 1 Sampling locations in Okinawa Trough. The locations of hydrothermal vent 1 (HV1) and hydrothermal vent 2 (HV2) are indicated by triangles

Table 1 Characteristics of the samples used in this study

| Characteristic | HV1 | HV2 |
|-----------------------|-----------------|-----------------|
| Location | 126.97°E27.55°N | 126.90°E27.79°N |
| Depth (m) | 1388 | 995 |
| Temperature (°C) | 4 | 4 |
| Dissolved oxygen (μM) | 315 | 300 |
| TOC ^a (%) | 0.13 | 0.65 |
| TN ^b (%) | 0.01 | 0.10 |
| Metal (mg/kg) | | |
| Cu | 62.9 | 45,646 |
| Fe | 62,963 | 126,066 |
| Mg | 25,740 | 10,585 |
| Mn | 1753 | 588.4 |
| Pb | 32.9 | 11,352 |
| Zn | 133.1 | 140,836 |
| Ba | 162.4 | 2399 |
| Ca | 68,250 | 6413 |

^a TOC total organic carbon

^b TN total nitrogen

TN in HV2 were 5.0 and 10.0 times respectively, more than those in HV1. Of the metal ions that had been identified, Cu, Pb, and Zn were 725.7, 345.0, and 1058.1 times more abundant in HV2 than in HV1, while Fe and Ba were 2–14 times higher in HV2 than in HV1. In contrast, the contents of Ca, Mg, and Mn in HV1 were 10.6, 2.4, and 3.0 times, respectively, more than those in HV2 (Table 1). The temperature and dissolved oxygen concentrations of the two sites were similar (Table 1).

Structure of the cultivable bacterial community in HV1

Of the isolates obtained from HV1 (Fig. 2), 128 were identified by near full-length 16S rRNA gene sequence analysis. The results showed that the 128 isolates were classified into 4 phyla, 5 classes, 11 orders, 19 families, 26 genera, and 68 species (Fig. 3). The species classification was according to a 97 % identity threshold (Guan et al. 2011). At the phylum level, *Proteobacteria* (87 isolates) was the dominant group, which was followed in abundance by *Firmicutes* (37 isolates), *Bacteroidetes* (2 isolates), and *Actinobacteria* (2 isolates) (Fig. 4a). Except for the *Proteobacteria* phylum, which contains two classes, i.e. *Gammaproteobacteria* (80 isolates) and *Alphaproteobacteria* (7 isolates), the other three phyla each contains only one class, i.e. *Bacilli* (37 isolates), *Actinobacteria* (2 isolates), and *Flavobacteria* (2 isolates) (Fig. 4b). At the order level, *Alteromonadales* (46 isolates) was the largest group, which was followed in size by *Bacillales* (37 isolates),

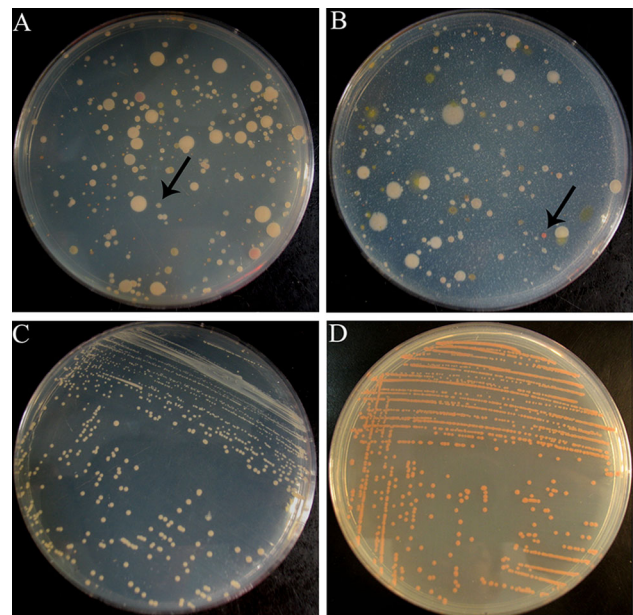


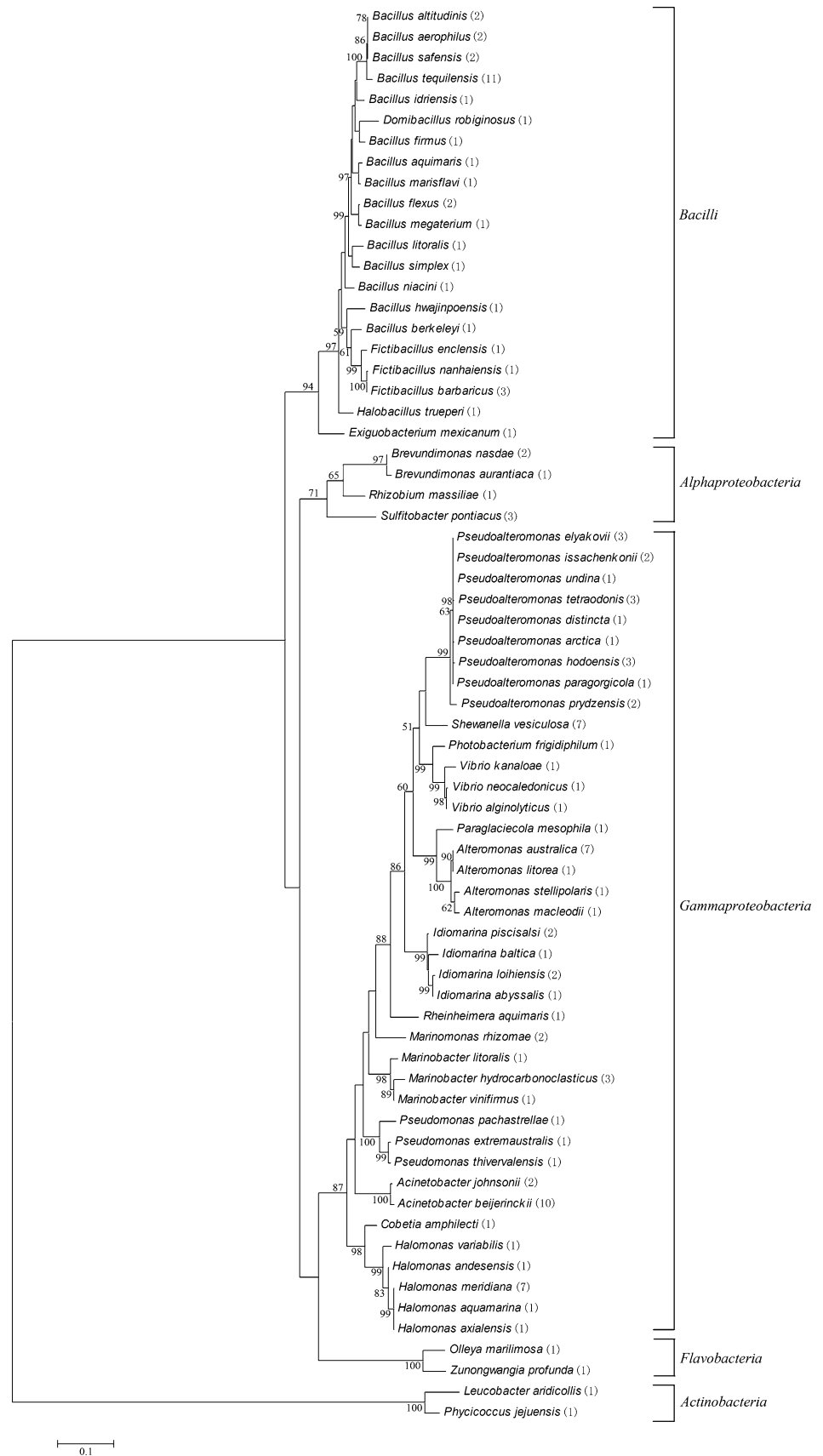
Fig. 2 Morphologies of representative isolates. **a** bacterial colonies formed in Marine Agar 2216 medium. **b** bacterial colonies formed in agar R2A medium. **c**, **d** arrow-indicated colonies in **a** and **b** were streaked in Marine Agar 2216 medium and agar R2A medium, respectively

Pseudomonadales (15 isolates), and *Oceanospirillales* (14 isolates) (Fig. 4c). The other 7 orders each contains less than four isolates. The largest family was *Bacillaceae 1* (35 isolates), which was followed by *Pseudoalteromonadaceae* (17 isolates), *Alteromonadaceae* (16 isolates), *Halomonadaceae* (12 isolates), *Moraxellaceae* (12 isolates), and *Alteromonadaceae* (6 isolates) (Fig. 4d). For the remaining 13 families, each contains less than five isolates. *Bacillus* (29 isolates) was the dominant genus and comprised of the largest amount of species (15 in number). Next to *Bacillus* in species diversity were *Pseudoalteromonas*, *Halomonas*, *Alteromonas*, and *Idiomarina*, which contain 9, 5, 4, and 4 species respectively.

Structure of the cultivable bacterial community in HV2

A total of 85 isolates from HV2 were identified by near full-length 16S rRNA gene sequence analysis. The 85 isolates were classified into 3 phyla, 4 classes, 9 orders, 15 families, 20 genera, and 50 species (Fig. 5). The three phyla were *Proteobacteria* (68 isolates), *Firmicutes* (16 isolates), and *Bacteroidetes* (1 isolates) (Fig. 4a). The *Proteobacteria* phylum contains two classes, i.e. *Gammaproteobacteria* (66 isolates) and *Alphaproteobacteria* (2 isolates), while the other two phyla each contains only one class, i.e. *Bacilli* (16 isolates) and *Flavobacteria* (1 isolates) (Fig. 4b). At the order level, *Alteromonadales*

Fig. 3 Phylogenetic tree of the isolates from HV1 based on 16S rRNA gene sequences (1323 bp). The phylogenetic tree was constructed using maximum likelihood method. The numbers on the branches indicate bootstrap values based on 1000 replications. The numbers in parentheses indicate strain numbers. *Scale bar* represents number of changes per nucleotide position



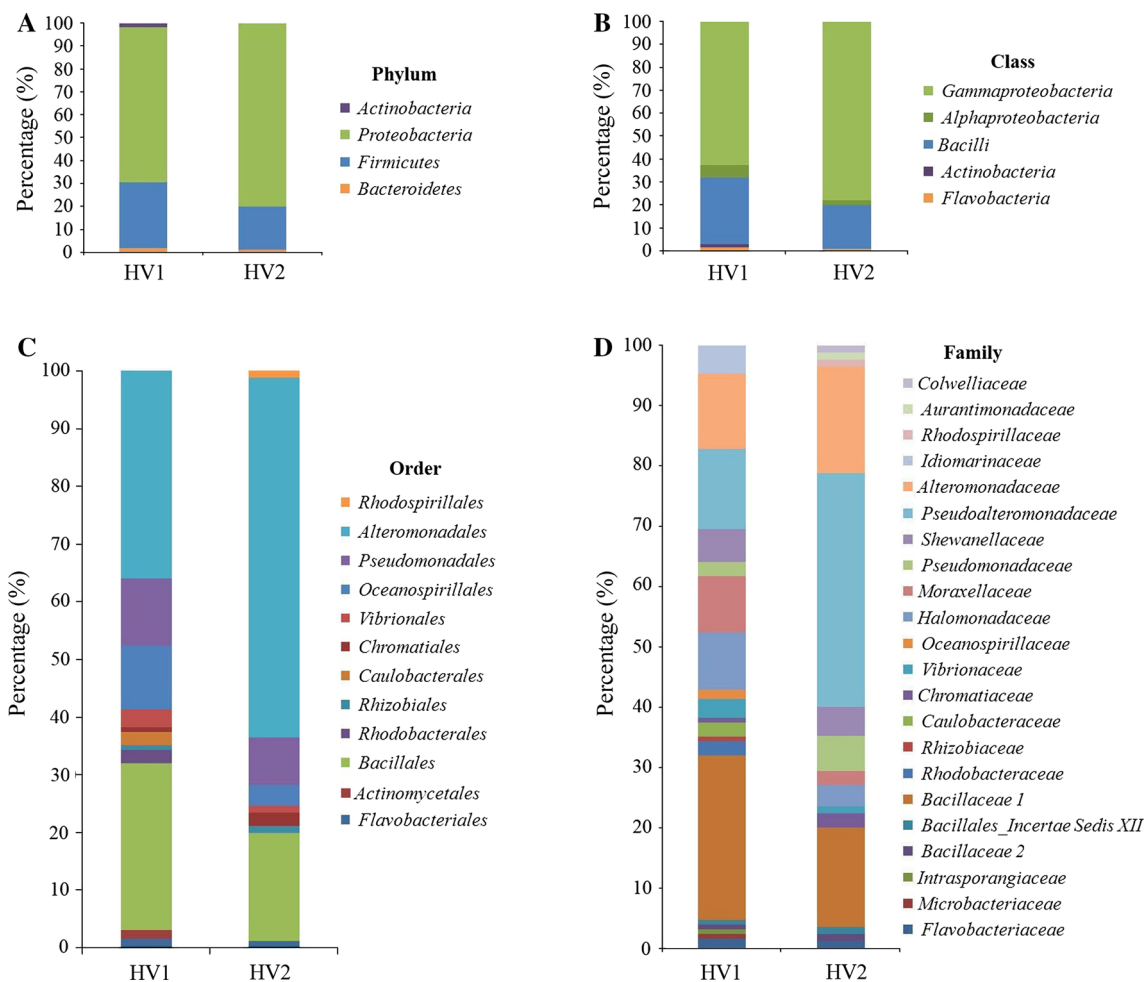


Fig. 4 Diversity and distribution of HV1 and HV2 isolates. The isolates were classified at the levels of phylum (a), class (b), order (c), and family (d). Each color represents the percentage of the taxon in the total isolates. (Color figure online)

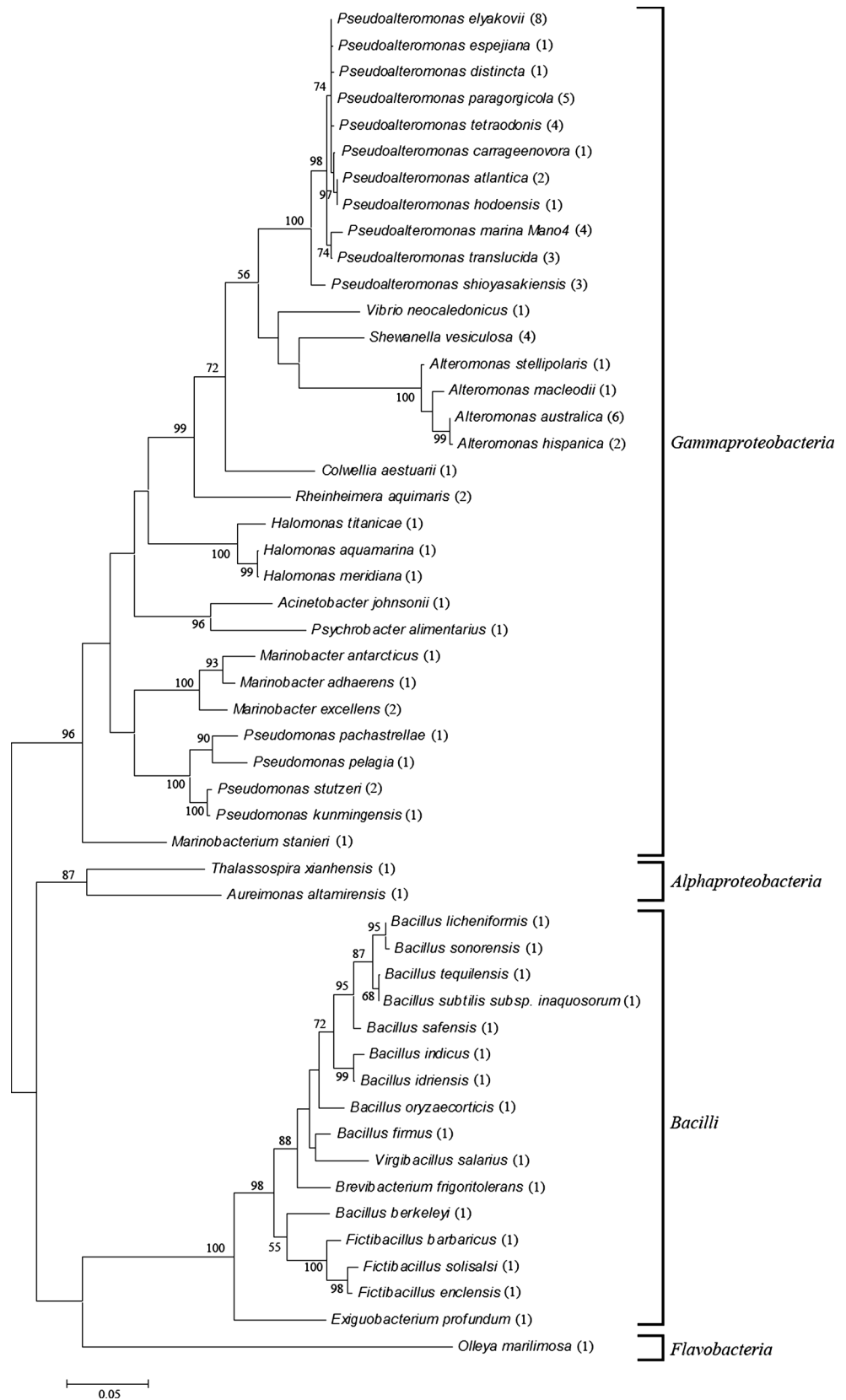
(53 isolates) was the largest group, which was followed in size by *Bacillales* (16 isolates) and *Pseudomonadales* (7 isolates) (Fig. 4c). For the other 6 orders, each has less than four isolates. The dominant families were *Pseudoalteromonadaceae* (33 isolates), *Alteromonadaceae* (15 isolates), and *Bacillaceae 1* (14 isolates) (Fig. 4d). The other 12 families each contains less than five isolates. The most populous genus was *Pseudoalteromonas* (33 isolates), which contains 11 species. Next to *Pseudoalteromonas* in species diversity were *Bacillus*, *Pseudomonas*, and *Alteromonas*, which contain 10, 4, and 4 species respectively.

Comparison of the bacterial communities in HV1 and HV2

Of the four phyla identified in this study, *Actinobacteria* was found only in HV1, while the other three phyla (*Proteobacteria*, *Firmicutes*, and *Bacteroidetes*) occurred in both HV1 and HV2 (Fig. 6a). At the class level,

Gammaproteobacteria, *Alphaproteobacteria*, *Bacilli*, and *Flavobacteria* were shared by HV1 and HV2, whereas *Actinobacteria* occurred only in HV1 (Fig. 6b). Eight orders, i.e. *Flavobacteriales*, *Bacillales*, *Rhizobiales*, *Chromatiales*, *Vibrionales*, *Oceanospirillales*, *Pseudomonadales*, and *Alteromonadales*, occurred in both HV1 and HV2, while *Rhodospirillales* was isolated only from HV2; the other three orders, i.e. *Rhodobacterales*, *Caulobacterales*, and *Actinomycetales* were isolated only from HV1 (Fig. 6c). At the family level, *Rhodobacteraceae*, *Rhizobiaceae*, *Caulobacteraceae*, *Microbacteriaceae*, *Intrasporangiaceae*, *Oceanospirillaceae*, and *Idiomarinaceae* occurred only in HV1, and *Rhodospirillaceae*, *Aurantimonadaceae*, and *Colwelliaceae* occurred only in HV2. The other 12 families were common to HV1 and HV2 (Fig. 6d). Of the 26 genera from HV1 and the 20 genera from HV2, 13 were shared by both sites (Fig. 6e). These 13 genera were *Pseudoalteromonas*, *Marinobacter*, *Alteromonas*, *Halomona*, *Bacillus*, *Olleya*, *Exiguobacterium*,

Fig. 5 Phylogenetic tree of the isolates from HV2 based on 16S rRNA gene sequences (1267 bp). The phylogenetic tree was constructed using maximum likelihood method. The numbers on the branches indicate bootstrap values based on 1000 replications. The numbers in parentheses indicate strain numbers. *Scale bar* represents number of changes per nucleotide position



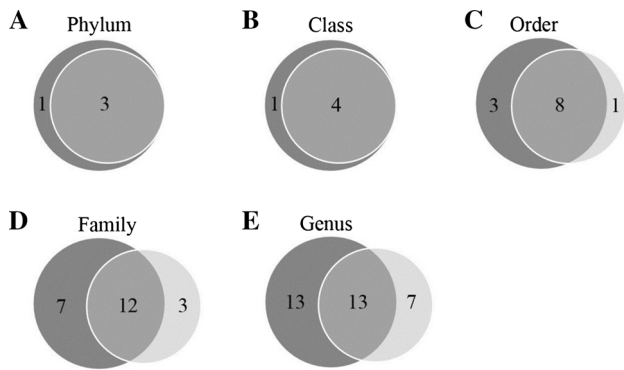


Fig. 6 Venn diagrams showing isolate diversity and distribution in HV1 and HV2. Isolates were classified at the phylum (a), class (b), order (c), family (d), and genus (e) levels, and their distributions in HV1 (deep grey) and HV2 (light grey) are indicated by numbers

Rheinheimera, *Vibrio*, *Acinetobacter*, *Pseudomonas*, *Shewanella*, and *Fictibacillus*. For the other genera, *Zunongwangia*, *Halobacillus*, *Leucobacter*, *Domibacillus*, *Sulfitobacter*, *Rhizobium*, *Brevundimonas*, *Photobacterium*, *Marinomonas*, *Cobetia*, *Paraglaciecola*, *Idiomarina*, and *Phycococcus* were only found in HV1, while *Virgibacillus*, *Brevibacterium*, *Thalassospira*, *Aureimonas*, *Psychrobacter*, *Colwellia*, and *Marinobacterium* were found only in HV2. In summary, the 213 isolates obtained from HV1 and HV2 belonged to 4 phyla, 5 classes, 12 orders, 22 families, 33 genera, and 96 species.

Screening for isolates with extracellular activities

Isolates that exhibited extracellular protease activity

Ninety-six isolates representing the 96 different species of HV1 and HV2 were examined for the potential to produce extracellular protease/lipase or antimicrobial activity. Forty-four isolates (45.8 %) were found to exhibit at least one type of activity, but no isolates exhibited all three types of activities (Table 2). Representative isolates with protease, lipase, and antimicrobial activities are shown in Fig. 7. Extracellular protease activity was identified in 25 isolates of 9 different genera (Fig. 8). Of these isolates, 13 are of the *Bacillus* genus, two, three, and two belong to the genera of *Exiguobacterium*, *Fictibacillus*, and *Alteromonas* respectively, while the remaining five isolates belong to the genera of *Brevibacterium*, *Rheinheimera*, *Marinomonas*, *Halomonas*, and *Pseudoalteromonas* respectively (Fig. 8). Relatively high levels of activity ($Z/C \geq 3$) were displayed by 10 isolates, i.e. *Fictibacillus barbaricus*, *Fictibacillus solisalsi*, *Bacillus tequilensis*, *Bacillus safensis*, *Bacillus megaterium*, *Bacillus aerophilus*, *Bacillus firmus*, *Bacillus altitudinis*, *Bacillus subtilis* subsp. *Inaquosorum*, and *Rheinheimera aquimaris* (Table 2).

Isolates that exhibited extracellular lipase activity

In the screening of extracellular lipase activity, 21 isolates were found to be positive. Seven of the isolates belong to the genus of *Pseudoalteromonas*, and the other 14 isolates belong to the genera of *Exiguobacterium*, *Domibacillus*, *Brevibacterium*, *Brevundimonas*, *Halomonas*, *Acinetobacter*, *Shewanella*, *Colwellia*, *Paraglaciecola*, and *Fictibacillus* (Fig. 8). Nine isolates of the genera *Exiguobacterium*, *Domibacillus*, *Halomonas*, *Acinetobacter*, *Pseudoalteromonas*, and *Paraglaciecola* showed high levels of activity ($Z/C \geq 3$) (Table 2).

Isolates that exhibited antimicrobial activity

When the Gram-positive bacterium *M. luteus* was used as a target strain, 12 isolates were found to be able to inhibit *M. luteus* growth to different extents. Six of the 12 isolates belong to the genus *Bacillus*, and the other six isolates belong to the genera of *Virgibacillus*, *Rheinheimera*, *Marinomonas*, *Pseudoalteromonas*, *Alteromonas*, and *Halomonas* (Fig. 8). Four of these isolates, i.e. *Bacillus tequilensis*, *Bacillus safensis*, *Bacillus aerophilus*, and *Bacillus altitudinis*, exhibited relatively strong inhibitory effects ($Z/C \geq 2$) (Table 2). When the Gram-negative bacterium *E. coli* was used as a target strain, only one isolate, *Vibrio neocaledonicus*, showed apparent inhibitory activity (Table 2).

Isolates that exhibited two types of extracellular activity

There were 15 isolates showing two types of extracellular activity. Specifically, four isolates from the genera of *Exiguobacterium*, *Fictibacillus*, and *Brevibacterium* exhibited both protease and lipase activities; 11 isolates from the genera of *Bacillus*, *Halomonas*, *Pseudoalteromonas*, *Alteromonas*, *Marinomonas*, and *Rheinheimera* exhibited protease and antibacterial activities (Table 2). No isolates showing both lipase and antibacterial activities were found.

Discussion

In this study, we examined the cultivable heterotrophic bacterial communities in sediments associated with two deep-sea hydrothermal vents, HV1 and HV2, in Iheya Ridge and Iheya North respectively, via culture-dependent methods. A total of 213 isolates were obtained from the two vent fields, which were classified into 96 species. This species ratio (96/213) or operational taxonomic units (OTU) in all isolates was similar to that (95/221) observed in a shallow water hydrothermal vent in Espalamaca (Rajabapathy et al. 2014). For both HV1 and HV2,

Table 2 Characteristics of isolates with extracellular protease activity, lipase activity, and antimicrobial activity

| Serial number | Nearest type strain | % Identity | Lipase | Protease | Antibacterial | |
|---------------|--|------------|--------|----------|--------------------------------|------------------------------|
| | | | | | Inhibition of <i>M. luteus</i> | Inhibition of <i>E. coli</i> |
| 1 | <i>Exiguobacterium mexicanum</i> | 99.56 | +++ | ++ | – | – |
| 2 | <i>Exiguobacterium profundum</i> | 99.86 | ++ | + | – | – |
| 3 | <i>Fictibacillus barbaricus</i> | 99.64 | – | +++ | – | – |
| 4 | <i>Fictibacillus enclensis</i> | 99.36 | + | ++ | – | – |
| 5 | <i>Fictibacillus solisalsi</i> | 99.86 | – | +++ | – | – |
| 6 | <i>Virgibacillus salarius</i> | 99.79 | – | – | + | – |
| 7 | <i>Domibacillus robiginosus</i> | 98.59 | +++ | – | – | – |
| 8 | <i>Brevibacterium frigoritolerans</i> | 100 | ++ | ++ | – | – |
| 9 | <i>Bacillus tequilensis</i> | 99.93 | – | +++ | ++ | – |
| 10 | <i>Bacillus berkeleyi</i> | 99.00 | – | + | – | – |
| 11 | <i>Bacillus safensis</i> | 100 | – | +++ | ++ | – |
| 12 | <i>Bacillus megaterium</i> | 97.86 | – | +++ | – | – |
| 13 | <i>Bacillus flexus</i> | 100 | – | + | + | – |
| 14 | <i>Bacillus idriensis</i> | 98.96 | – | ++ | – | – |
| 15 | <i>Bacillus aerophilus</i> | 99.86 | – | +++ | ++ | – |
| 16 | <i>Bacillus firmus</i> | 99.42 | – | +++ | – | – |
| 17 | <i>Bacillus aquimaris</i> | 99.28 | – | + | – | – |
| 18 | <i>Bacillus altitudinis</i> | 100 | – | +++ | ++ | – |
| 19 | <i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> | 99.93 | – | +++ | – | – |
| 20 | <i>Bacillus sonorensis</i> | 99.29 | – | + | – | – |
| 21 | <i>Bacillus indicus</i> | 99.93 | – | ++ | + | – |
| 22 | <i>Brevundimonas nasdae</i> | 99.69 | ++ | – | – | – |
| 23 | <i>Rheinheimera aquimaris</i> | 100 | – | +++ | + | – |
| 24 | <i>Vibrio neocaledonicus</i> | 99.58 | – | – | – | + |
| 25 | <i>Marinomonas rhizomae</i> | 98.93 | – | + | + | – |
| 26 | <i>Halomonas meridiana</i> | 99.93 | +++ | – | – | – |
| 27 | <i>Halomonas axialensis</i> | 99.93 | +++ | – | – | – |
| 28 | <i>Halomonas andesensis</i> | 97.85 | – | + | + | – |
| 29 | <i>Halomonas titanicae</i> | 98.96 | +++ | – | – | – |
| 30 | <i>Acinetobacter johnsonii</i> | 99.36 | +++ | – | – | – |
| 31 | <i>Acinetobacter beijerinckii</i> | 99.76 | +++ | – | – | – |
| 32 | <i>Shewanella vesiculosa</i> | 100 | + | – | – | – |
| 33 | <i>Pseudoalteromonas elyakovii</i> | 99.78 | ++ | – | – | – |
| 34 | <i>Pseudoalteromonas tetraodonis</i> | 99.92 | ++ | – | – | – |
| 35 | <i>Pseudoalteromonas distincta</i> | 99.71 | ++ | – | – | – |
| 36 | <i>Pseudoalteromonas paragorgicola</i> | 99.78 | ++ | – | – | – |
| 37 | <i>Pseudoalteromonas shioyasakiensis</i> | 99.93 | ++ | – | – | – |
| 38 | <i>Pseudoalteromonas translucida</i> | 99.78 | ++ | – | – | – |
| 39 | <i>Pseudoalteromonas atlantica</i> | 99.93 | – | ++ | + | – |
| 40 | <i>Pseudoalteromonas espejiana</i> | 99.71 | +++ | – | – | – |
| 41 | <i>Paraglaciecola mesophila</i> | 99.93 | +++ | – | – | – |
| 42 | <i>Alteromonas litorea</i> | 98.80 | – | ++ | + | – |
| 43 | <i>Alteromonas macleodii</i> | 99.42 | – | + | – | – |
| 44 | <i>Colwellia aestuarii</i> | 99.69 | ++ | – | – | – |

+, $Z/C \leq 2$; ++, $2 < Z/C < 3$; +++, $Z/C \geq 3$; – negative. Z, zone diameter; C, colony diameter

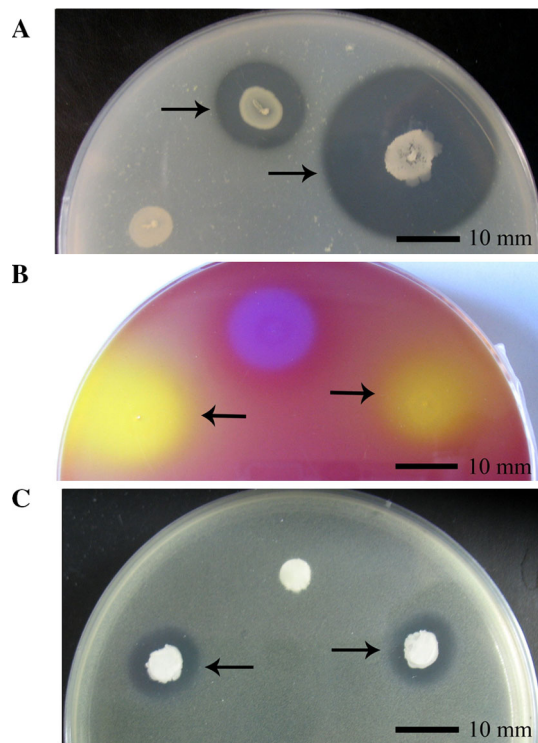


Fig. 7 Representative isolates with extracellular protease activity (a), lipase activity (b), and antibacterial activity (c). a Cultures of three bacteria were spotted on a plate containing skim milk. b Cultures of three bacteria were spotted on a plate containing olive oil, Tween-80, and Bromocresol purple. c Cultures of three bacteria were spotted onto filter discs placed on a plate spread with *Micrococcus luteus*. For all panels, arrows indicate strains with extracellular bioactivity. (Color figure online)

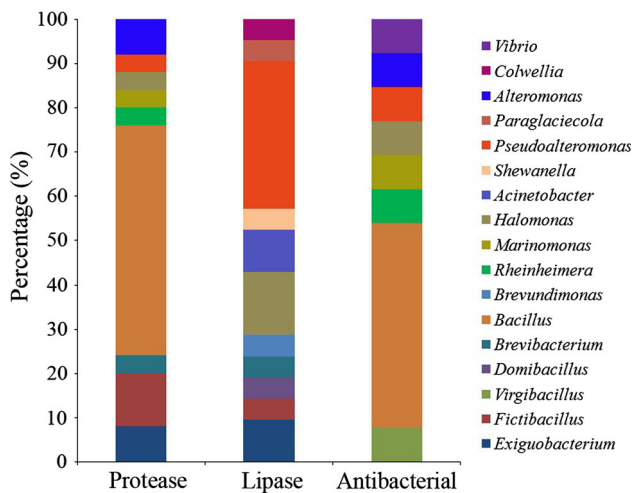


Fig. 8 Diversity of the bacterial isolates with extracellular protease activity, lipase activity, and antibacterial activity. The diversity is presented at the genus level

Proteobacteria and *Gammaproteobacteria* were most abundant at the phylum and class level respectively. *Gammaproteobacteria* appears to be a common inhabitant

of hydrothermal vents and has been reported to exist in different vent systems. For example, Perez-Rodriguez et al. (2013) found a large amount of *Gammaproteobacteria* in cooling and less reducing vent fields, and Rajasabapathy et al. identified abundant members of *Gammaproteobacteria* in a shallow hydrothermal field (Rajasabapathy et al. 2014). Previous reports showed that some *Pseudoalteromonas* and *Marinobacter*, both belonging to *Gammaproteobacteria*, may take part in the Mn-oxidizing process in hydrothermal vents (Mason et al. 2009; Orcutt et al. 2011), and that *Marinobacter* is thought to participate in Fe oxidation (Edwards et al. 2003; Kaye et al. 2011). In our study, *Pseudoalteromonas* was found to be the dominant genus in both HV1 and HV2, and *Marinobacter* also occurred in HV1 and HV2. Considering the possible role of *Pseudoalteromonas* and *Marinobacter* in metal processing, these observations are consistent with the fact that the contents of Mn and Fe were relatively high in the sampling sites of HV1 and HV2. In addition to *Pseudoalteromonas* and *Marinobacter*, seven other genera, i.e. *Alteromonas*, *Halomona*, *Vibrio*, *Acinetobacter*, *Shewanella*, *Pseudomonas*, and *Rheinheimera*, of the *Gammaproteobacteria* phylum were also isolated from both HV1 and HV2. Of these 7 genera, the first 6 have been detected in hydrothermal fields around the world (Kaye et al. 2011; Martins et al. 2013; Pena et al. 2012; Raguenees et al. 2003; Rajasabapathy et al. 2014), while the last one was identified in vent environments for the first time. The roles of these bacteria in hydrothermal vent ecosystems remain to be elucidated. It should be noted that *V. alginolyticus*, which is an aquaculture pathogen to fish and invertebrate marine animals, was isolated from HV1. It will be interesting for future studies to investigate whether it is pathogenic to vent fauna.

Firmicutes and *Bacilli* were the second largest phylum and class respectively, in both HV1 and HV2. Within the *Firmicutes* phylum, three genera, i.e. *Bacillus*, *Exiguobacterium*, and *Fictibacillus*, were shared by HV1 and HV2. In the past, reports on *Firmicutes* found in hydrothermal vents were rare. However, recent studies indicate that this bacterial group, which can tolerate a wide range of temperatures, exists in varying phenotypes in different hydrothermal environments (Cao et al. 2014; Price et al. 2013). In a shallow water hydrothermal vent at D. João de Castro Seamount (DJCS), researchers isolated 22 strains, 15 of which were within the genus *Bacillus*, and the growth of these bacteria was promoted by Fe and Mn (Mohandass et al. 2012). Another report showed that diverse spore-forming *Bacillus* spp. from Guaymas Basin hydrothermal sediments are involved in copper-mediated Mn(II) oxidation and precipitation (Dick et al. 2006). Given these findings and the observation that the sediments of HV1 and HV2 were rich in metal ions, it is possible that the abundant *Bacillus* found in our study may play a role in metal oxidation.

Of the phyla shared by HV1 and HV2, *Bacteroidetes* was the smallest one. The percentages of *Bacteroidetes* isolates in the two fields were much less than that reported for a shallow hydrothermal vent field in Espalamaca (Rajasabapathy et al. 2014). This is probably due to the difference in the geochemical features of these vent systems. The shallow water hydrothermal vent in Espalamaca is exposed to the sunlight, and consequently both photosynthesis and chemosynthesis exist, which leads to production of more abundant organic matters relative to deep-sea hydrothermal vents. Since members of *Bacteroidetes* are known to play an important role in degradation of organic compounds (Gomez-Pereira et al. 2012), its scarcity in HV1 and HV2 may reflect a harshness of the environments in these two vent niches.

In addition to similarities, we also observed dissimilarities between HV1 and HV2 in terms of bacterial diversity. HV1 and HV2 differed in one phyla, one class, four orders, ten families, and 20 genera. It is likely that the differences in the geological, physical, and chemical characteristics of the vent systems, which are known to select and shape the population structures of the ecosystem (Klochko et al. 2012; Lai et al. 2014), may account for the difference in the bacterial communities observed in HV1 and HV2. Huston and Deming (2002) reported that the amounts of organic carbon and organic nitrogen in water would affect heterotrophic bacterial communities. In our study, we found that the contents of TOC and TN were significantly different between HV1 and HV2, which may contribute at least in part to the difference in the heterotrophic bacterial communities between the two sites.

To our knowledge, no systematic studies on the production of extracellular bioactive compounds by deep-sea hydrothermal microorganisms have been documented. One report by Mohandass et al. showed that bacteria from a shallow water hydrothermal vent in D. João de Castro Seamount (DJCS), Azores, Portugal were able to produce degradative enzymes like amylase (Mohandass et al. 2012). In two other reports, *Pseudoalteromonas* from the sediments of South China Sea and *Bacillus* spp. from the coastal sediments of King George Island produced extracellular proteases (Zhou et al. 2009a, b; Zhou et al. 2013). In our study, we identified diverse protease-producing isolates from HV1 and HV2, most of which belonging to the *Bacillus* genus. We also detected a large number of lipase-producing bacteria, two of which, i.e. *Domibacillus* and *Paraglaciecola*, were found for the first time to secrete lipase. Hydrothermal vents are considered the next hot spots for novel drug discovery, especially antibacterial drugs (Andrianasolo et al. 2009, 2012; Tasiemski et al. 2014; Taylor 2013; Thornburg et al. 2010). In our study, antibacterial properties were detected in 13 isolates, and that relatively strong inhibitory effects on Gram-positive bacteria were observed with isolates of the

Bacillus genus. These results are comparable to the previous reports that antimicrobial activities were observed with *Bacillus* from widely different sources such as rhizosphere plants and gastrointestinal tract (Mouloud et al. 2013; Thankappan et al. 2014).

In conclusion, the results of this study demonstrate that diverse cultivable bacteria exist in sediments associated with two deep-sea hydrothermal vents, HV1 and HV2, in Okinawa Trough, and that although the bacterial community structures in HV1 and HV2 are largely similar, differences between these two sites do exist, possibly as a result of specific adaptation to the local environmental selection. In addition, we also provide the first evidence that a large population of bacteria with extracellular bioactivities flourishing in deep-sea hydrothermal vents, which suggests a potential for industrial/medical application and provides a basis for future studies of the biological roles of microorganisms in hydrothermal ecosystems.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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