Bacterial diversity at a shallow-water hydrothermal vent (Espalamaca) in Azores Island

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A low-temperature shallow-water hydrothermal vent field was discovered during the summer of 2010 in the Faial-Pico channel off the Espalamaca headland, Faial Island, Azores, Portugal, NE Atlantic. The present study analyses bacterial communities present in shallow-water hydrothermal vent of Espalamaca using SSU rRNA-based clone library approach. Clones of shallow vent sediment sample revealed the dominance of Proteobacteria (including α, γ, ε, δ, ζ subdivisions) and Bacteroidetes with 36% and 28% of the whole community respectively. The dominance of γ-Proteobacteria is the unique characteristic of this shallow vent and it coincides with the South Tonga Arc and Bahía Concepción (Pacific Ocean), whereas ε-Proteobacteria groups were reported to be high in the majority of the hydrothermal vents. Though the sampling sites of the venting and non-venting regions of Espalamaca were only 500 m apart, high variation (>80%) of phylotypes was found between the regions.

Keywords: Bacterial diversity, clone library, hydrothermal vent, phylogeny, shallow water.

MARINE hydrothermal vents are known for their characteristic habitat since the fluids emitted from the vents are distinguished by high temperatures as well as high concentration of CO_2, H_2S and heavy metals. The vents occurring at a depth of below 200 m and 0–200 m are defined as deep-sea and shallow hydrothermal vents respectively. Primary productions in deep-sea hydrothermal vent ecosystems are mainly based on chemolithoautotrophy[1,2], whereas shallow-water hydrothermal vent ecosystems are largely influenced by photosynthesis[3]. Shallow-water hydrothermal systems can be described as high-energy environments, where microbial metabolism is fuelled by light and chemical energy sources[4]. Hence, the microbial communities inhabiting shallow hydrothermal vents would be different from their deep-sea counterparts, with a dominance of photosynthetic microbial lineages. Though shallow submarine hydrothermal vents are less understood habitats in the ocean, they have been the focus of research during the past three decades[5].

Low-temperature hydrothermal vents, which form by the mixing of high-temperature hydrothermal fluids and sea water, are omnipresent in recent submarine hydrothermal systems[6]. Microorganisms are involved in the transformation of inorganic compounds released from hydrothermal vent emissions; hence they are at the basis of the hydrothermal system food web[7]. Understanding the microorganisms present in shallow-water hydrothermal vents is essential to interpret how they influence biogeochemical cycles. So far culture-independent molecular technique using 16S rRNA gene has been successfully studied for investigating the microbial communities in various hydrothermal systems[8–11].

Nine shallow-water hydrothermal vent fields have been identified so far within the Azores Archipelago, Portugal. When compared to deep-sea counterparts, shallow hydrothermal vent regions in the Azorean Islands, Portugal, are not explored in the aspect of diversity and ecology[12]. The proximity of the Azorean Islands (Portugal) provides accessible sampling sites for studying microorganisms inhabiting the hydrothermal vent ecosystems. D. Joao de Castro seamount (DJCS, located between the islands of Terceira and Sao Miguel) and Espalamaca (located in Faial–Pico channel off the Espalamaca headland) are well documented with regard to culture-dependent microbes[13–15] from the Azores Island. More recently, molecular diversity of culture-dependent bacteria was investigated using 16S rRNA gene sequencing from shallow vents in Espalamaca[15]. The results indicated that culturable fractions of Proteobacteria were dominant followed by Bacteroidetes, Firmicutes and Actinobacteria. Many of them were novel bacteria with no previous records of their existence in such environments. However, there are no reports on culture-independent bacteria based on microbial diversity in shallow-water hydrothermal vents in the Azores.

In this study, we explore the culture-independent microbial community structure from the shallow-water hydrothermal vent of Espalamaca using the 16S rRNA

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gene sequence clone library. The clone library data were also compared with few similar shallow-water hydrothermal vents to reveal the differences in microbial community pattern. Further, culture-independent community structure from shallow vent region was compared with the non-vent region of Espalamaca.

Materials and methods

Site description and sample details

Surface and bottom water as well as sediment samples were collected from the shallow hydrothermal vent (36 m depth) and non-vent (38 m depth) sites in Espalamaca (38°33′N; 28°39′W) during April 2014 by scuba divers (Figure 1). Sediment samples were collected using sterile polycarbonate tubes and water samples using Niskin samplers (2.5 l capacity). Samples were immediately brought to the laboratory at the University of Azores, Portugal.

Genomic DNA extraction

Sea-water samples (2.5 l) were filtered using 0.2 µm cellulose nitrate filters (Sartorius, France). Genomic DNA was extracted using EZNA Water DNA kit (D5525, Omega) following the manufacturer's instructions. Genomic DNA from the sediment samples was extracted using two steps. The first step was based on the method of Luna *et al.*[16] with phenol–chloroform method. In the second step, the DNA samples were purified following the EZNA soil DNA kit protocol (D5625, Omega). The presence of genomic DNA was confirmed using 1.0% agarose gel electrophoresis with 0.5× Tris borate EDTA (TBE) buffer.

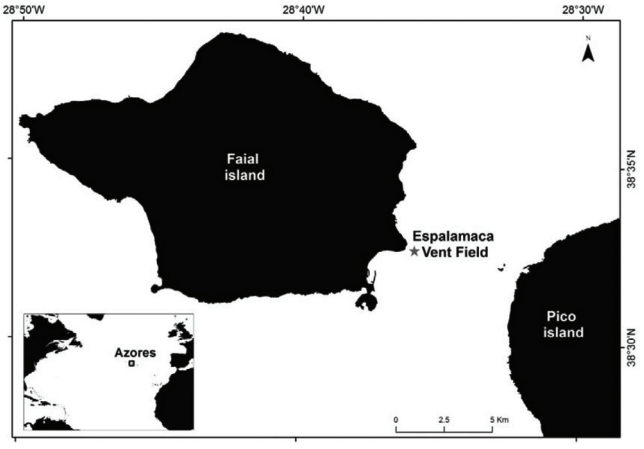

Figure 1. Sampling location at Espalamaca shallow-water hydrothermal vent, Azores, Portugal. Asterisk indicates the sampling site[15].

PCR amplification of 16S rRNA gene and clone library construction

Bacterial 16S rRNA genes were amplified using eubacterial primer sets 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′)[17]. The polymerase chain reaction (PCR) mixture (50 µl final volume) contained template DNA (≈100 ng), 10× PCR buffer, 40 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 20 pmol of each primer and 1 U *Taq* DNA polymerase (Ambion, Life Technologies). Amplification profile consisted of an initial denaturation step (94°C for 5 min) followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. Final extension was kept at 72°C for 10 min. PCR products were examined using 1% agarose gel electrophoresis with TBE buffer.

PCR products were purified on a 1% (w/v) agarose gel and extracted with a gel-extraction kit (Promega) according to the manufacturer's instructions. Purified PCR products were cloned into pJET1.2 cloning vector (Thermo Scientific) and transformed into competent *Escherichia coli* DH5α cells. The transformed clones were grown in a 1 ml Luria–Bertani broth (Merck) for 1 h at 37°C with shaking (225 rpm). Various volumes (50, 100 and 200 µl) from the above broth were spread-plated onto Luria–Bertani agar plates containing ampicillin (50 µg/ml) and incubated at 37°C for 16 h to obtain individual bacterial colonies.

DNA sequencing and phylogenetic analysis

Plasmids were isolated and purified from randomly selected clones using Plasmid MiniPrep kit (Invitrogen) following the manufacturer's instructions. The purified plasmids were directly sequenced using an automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). The partial sequences obtained were trimmed using DNA Baser software, version 3.0 and vectors were removed using NCBI on-line program VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Chimeric sequences were removed using an online tool DECIPHER[18]. Non-chimeric sequences were submitted to BLAST search program at NCBI to find the closest neighbour sequences in GenBank and for phylogenetic analysis. To generate taxonomic profiles, the assembled sequences were assigned to taxonomic groups using RDP (Ribosomal Database Project) classifier. Sequences were aligned using ClustalW sequence alignment program[19] and neighbor-joining phylogenetic trees[20] were constructed using MEGA 5.2 software[21] with bootstrap values based on 1000 replications[22]. The sequences were submitted to GenBank with accession numbers KP303396–KP303589.

Shannon and Simpson diversity indices were calculated using an on-line program (http://www.changbioscience.

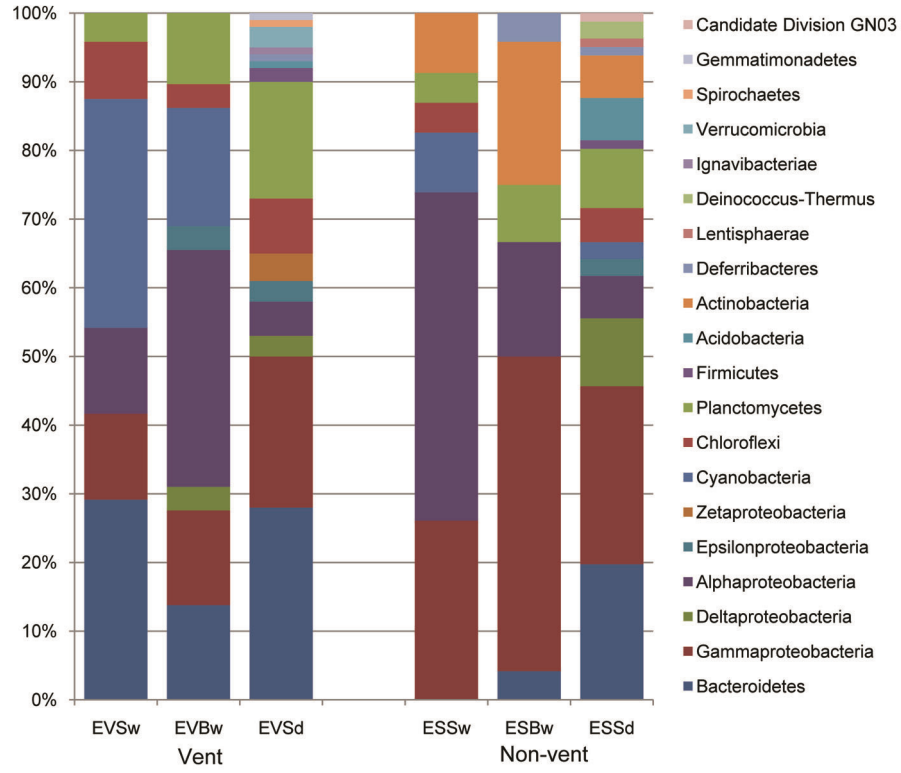

Figure 2. Relative abundance of bacterial phylogenetic groups in sediments and water samples of shallow hydrothermal vent (EVSw, EVBw and EVSd) and non-vent (ESSw, ESBw and ESSd) at Espalamaca.

com/genetics/shannon.html). Rarefaction analysis was performed by plotting the number of phylotypes against the total number of clones using EcoSim700 (ref. 23). Good's coverage was calculated using the formula $C = [(1 - (n1/N)] * 100$, where C is the homologous coverage, $n1$ the number of phylotypes appearing only once and N is the total number of clones.

Results

Bacterial diversity from hydrothermal vent region

After chimera removal, we analysed 100 clones from sediments (EVSd), 29 clones from bottom water (EVBw) and 24 clones from surface water (EVSw). Highly diverse bacterial 16S rRNA gene community structures were found from the shallow hydrothermal vent region in Espalamaca. The sequences were affiliated with Acidobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deferribacteres, Firmicutes, Gemmatimonadetes, Ignavibacteriae, Planctomycetes, Proteobacteria (includes α, γ, ε, δ, ζ subdivisions), Spirochaetes and Verrucomicrobia. Figure 2 summarizes relative proportions of the different groups in each clone library.

Proteobacteria: This phylum Proteobacteria was found to be dominant with 59 clones (38 phylotypes) in the hydrothermal vent region, which includes α, γ, ε, δ, ζ subdivisions. Among them, half of the bacterial clones were affiliated to γ-Proteobacteria, representing 12.5%, 13.8% and 22.0% of clones in the EVSw, EVBw and EVSd libraries respectively. The genus *Colwellia* was abundant in the sediment samples with seven clones. *Marinobacterium* was the only common genus between EVBw and EVSd libraries; all other phylotypes were sample-specific.

Eighteen clones (12 phylotypes) were affiliated with α-Proteobacteria, representing 12.5%, 34.5% and 5.0% of clones in the EVSw, EVBw and EVSd libraries respectively. Highly diverse α-Proteobacteria groups were found in EVBw when compared to the other two libraries and other subdivisions of Proteobacteria. Eight out of 18 clones belonged to SAR11 clusters. Interestingly, all the clones were observed only in sea-water libraries and not in the vent sediments.

Bacterial sequences belong to ε, δ and ζ subdivisions of Proteobacteria were also observed in the study area. Four clones (three phylotypes) were affiliated with δ-Proteobacteria and belonged to the families Syntrophobacteraceae (one clone) and Geobacteraceae (three clones). Four clones (two phylotypes) were affiliated to ε-Proteobacteria and grouped into the family Campylobacteraceae. Only one clone each of δ-Proteobacteria and ε-Proteobacteria was observed in

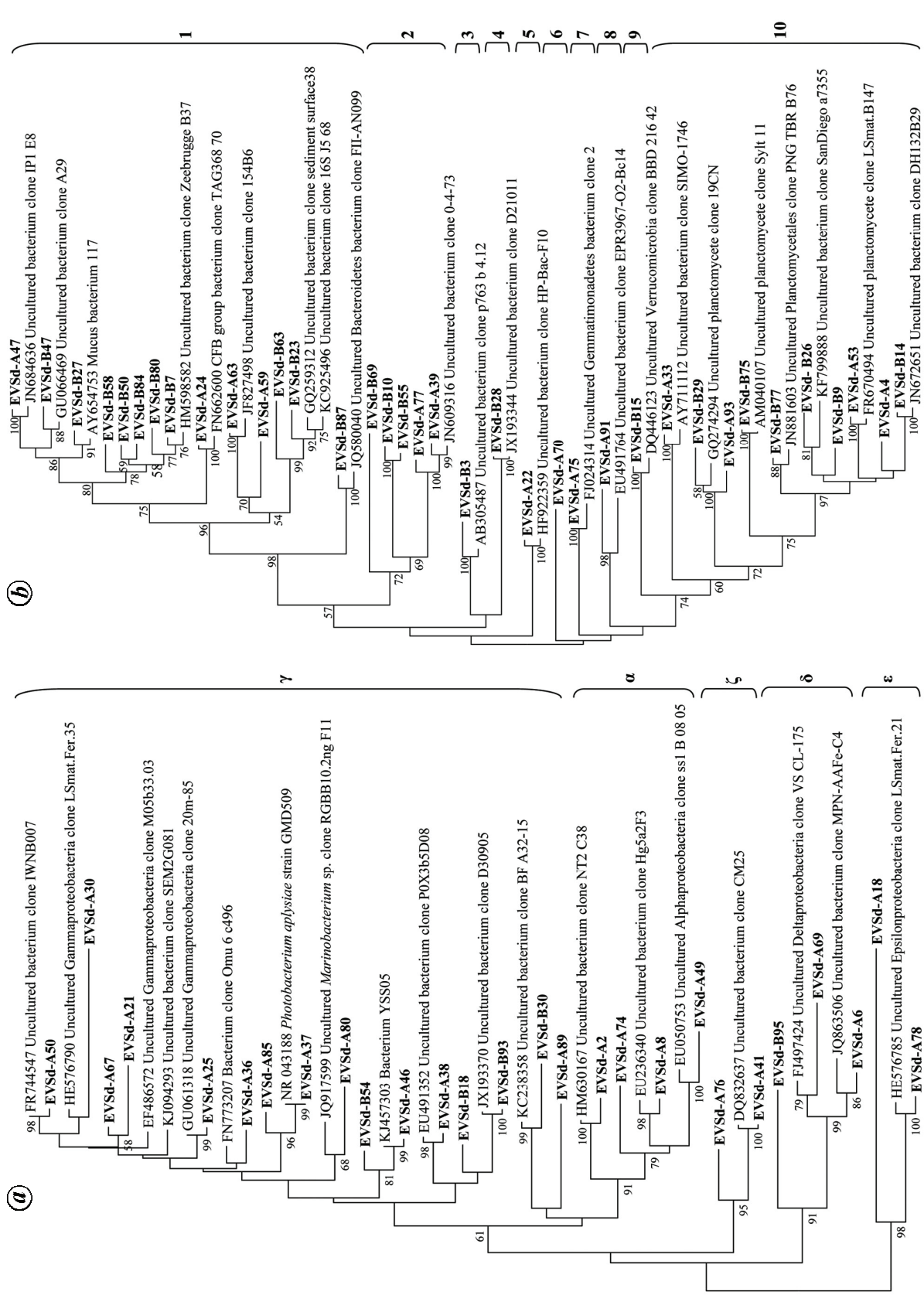

Figure 3. Neighbour-joining tree showing the evolutionary relationship of phylotypes based on 16S rRNA gene sequences from hydrothermal vent EVSd library: (*a*) Proteobacteria and (*b*) other groups. Bootstrap analysis was performed with 1000 replications and values above 50% are indicated at the nodes. 1, Bacteroidetes; 2, Chloroflexi; 3, Ignavibacteriae; 4, Acidobacteria; 5, Firmicutes; 6, Deferribacteres; 7, Gemmatimonadetes; 8, Spirochaetes; 9, Verrucomicrobia; 10, Planctomycetes.

EVBw; the remaining clones were solely observed in EVSd. Four clones (two phylotypes) belonging to the family Mariprofundaceae of ζ-Proteobacteria were observed only in the sediment library (EVSd). Figure 3 *a* shows detailed phylogenetic position and evolutionary relationships of clone sequences belonging to Proteobacteria.

Bacteroidetes: This was the second dominant phylum observed in the present study with 39 clones (17 phylotypes). The clones were closely related to the families Cryomorphaceae, Flammeovirgaceae, Flavobacteriaceae, Porphyromonadaceae and one unidentified group. Bacteroidetes clones were consistent with all the three libraries, representing 29.2%, 13.8% and 28.0% of clones in the EVSw, EVBw and EVSd libraries respectively. Four phylotypes from EVBw and three phylotypes from EVSw libraries was obtained and none of them was common. Whereas 15 phylotypes were found in EVSd library in which one phylotype was observed to be common with EVSw and four phylotypes were common with EVBw.

Planctomycetes: Twenty-one clones (11 phylotypes) belonged to the phylum Planctomycetes and accounted for 4.2%, 10.3% and 17.0% in the EVSw, EVBw and EVSd libraries respectively. Majority of the clones belonged to the family Planctomycetaceae and a few belonged to the family *Candidatus* Brocadiaceae. The sequences closely related with the genus *Blastopirellula* were common in all the three libraries. On the other hand, sequences closely related to the genus *Pirellula* were found to be common in the EVSd and EVBw libraries. The remaining phylotypes were observed only from the sediments (EVSd).

Cyanobacteria and chloroflexi: A total of 13 clones (five phylotypes) were affiliated to the phylum Cyanobacteria; interestingly, they were retrieved only from the sea-water samples. Cyanobacteria accounted for 33.3% and 17.2% in the EVSw and EVBw libraries respectively. They were observed to be predominant (33.3%) in the surface sea water when compared to Bacteroidetes (29.2%) and Proteobacteria (25.0%). All the five phylotypes observed in this group belonged to four different families, namely Bacillariophyta, Cryptomonadaceae, Chlorarachniophyceae and Family II. There were 11 clones belonging to the phylum Chloroflexi in which six phylotypes were observed. These accounted for 8.3%, 3.4% and 8.0% clones in the EVSw, EVBw and EVSd libraries respectively. These six phylotypes represented three different families, i.e. Anaerolineaceae, Ardenticatenaceae and Caldilineaceae.

Firmicutes, *Verrucomicrobia*, *Acidobacteria and other minor groups*: Three clones (one phylotype) belonged to the phylum Verrucomicrobia and three clones (one phylotype) were affiliated with the phylum Firmicutes. Acidobacteria, Deferribacteres, Spirochaetes, Gemmatimonadetes and Ignavibacteriae were some of the other phyla observed in this venting region with one clone each. It is noteworthy to mention here that all of these minor groups were observed only in the sediment library (EVSd). Figure 3 *b* shows phylogenetic position and evolutionary relationships of clone sequences belonging to phylogenetic groups other than Proteobacteria.

Bacterial diversity from non-vent region

We analysed 128 clones from three clone libraries of the non-vent samples (81 clones from sediments, 24 clones from bottom water and 23 clones from surface sea water). Similar to the venting site, high bacterial diversity was observed in the non-vent region as well. The sequences were affiliated to the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Candidate Division GN03, Chloroflexi, Cyanobacteria, Deferribacteres, Deinococcus–Thermus, Firmicutes, Lentisphaerae, Planctomycetes and Proteobacteria (includes α, γ, ε, δ subdivisions). Figure 2 summarizes the relative proportions of these groups in each clone library.

Proteobacteria: This phylum was predominant in the non-vent region with 68 clones (41 phylotypes), which includes α, γ, ε, δ subdivisions. Among them, more than 50% of the bacterial clones (22 phylotypes) were affiliated with γ-Proteobacteria, representing 26.0%, 45.8% and 25.9% in the ESSw, ESBw and ESSd libraries respectively. Alteromonadaceae, Halomonadaceae, Oceanospirillaceae, Pseudoalteromonadaceae, Hahellaceae, Colwelliaceae, Ectothiorhodospiraceae, Vibrionaceae, Piscirickettsiaceae, Methylococcaceae and some unidentified families were observed from the non-vent γ-Proteobacteria clones.

Twenty clones (10 phylotypes) were affiliated with α-Proteobacteria, contributing 47.8%, 16.7% and 6.2% in the ESSw, ESBw and ESSd libraries respectively. The predominant phyla observed form the surface sea-water samples were α-Proteobacteria followed by γ-Proteobacteria. Majority of the clones (eight each) belonged to the family Rhodobacteraceae and SAR11 groups. Hyphomonadaceae, Methylocystaceae and Rhodospirillaceae were some other families observed in this region. Similar to the vent region, SAR11 groups were observed only from the water samples in the non-vent region.

Eight clones (eight phylotypes) were affiliated with the subdivision of δ-Proteobacteria. Interestingly, all of them were retrieved from sediment samples contributing 8.6% clones to the ESSd library. These clones were affiliated with the families Syntrophobacteraceae, Cystobacterineae,

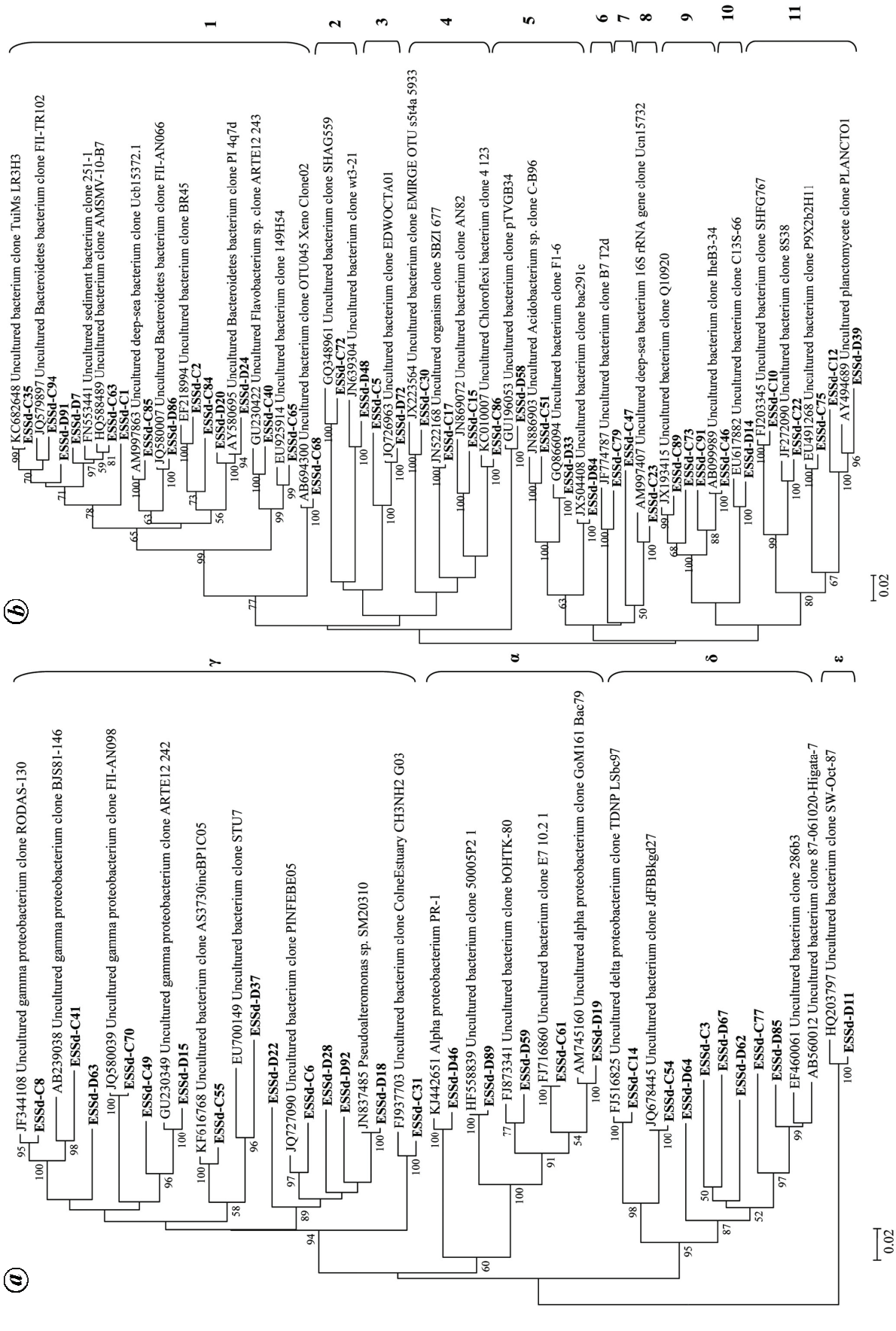

Figure 4. Neighbour-joining tree showing the evolutionary relationship of phylotypes based on 16S rRNA gene sequences from non-vent ESSd library: (***a***) Proteobacteria and (***b***) other groups. Bootstrap analysis was performed with 1000 replications and values above 50% are indicated at the nodes. 1, Bacteroidetes; 2, Deinococcus-Thermus; 3, Cyanobacteria; 4, Chloroflexi; 5, Acidobacteria; 6, Deferribacteres; 7, Candidate Division GN03; 8, Firmicutes; 9, Actinobacteria; 10, Lentisphaerae; 11, Planctomycetes.

Table 1. Bacterial community from the shallow-water hydrothermal vent Espalamaca compared with other shallow-water hydrothermal vent regions (in percentage)

Taxonomic affiliation	EVSd, Espalamaca	Loihi Seamount	Photic zone, South Tonga Arc	MV1, Milos	MV2, Milos	Juan de Fuca Ridge	Bahía Concepción
α-Proteobacteria	5.0	–	8.0	–	–	–	4.0
β-Proteobacteria	–	–	–	–	–	3.7	–
ε-Proteobacteria	3.0	60.5	17.0	60.0	59.0	52.4	7.0
γ-Proteobacteria	22.0	33.4	33.0	7.0	20.0	17.1	21.0
δ-Proteobacteria	2.0	2.1	8.0	5.0	6.0	9.8	10.0
ζ-Proteobacteria	4.0	–	–	–	–	–	–
Bacteroidetes	28.0	–	11.0	20.0	6.0	3.7	17.0
Cyanobacteria	–	–	0.4	2.0	–	–	14.0
Planctomycetes	17.0	–	9.0	–	3.0	–	–
Chloroflexi	9.0	–	4.9	–	–	–	10.0
Acidobacteria	1.0	–	3.0	–	–	–	–
Actinobacetria	–	–	3.4	–	3.0	–	4.0
Firmicutes	2.0	–	0.7	–	–	–	10.0
Verrucomicrobia	3.0	–	–	–	–	–	–
Deferribacteres	1.0	–	0.4	–	–	–	–
Spirochaetes	1.0	–	1.1	–	–	–	3.0
Ignavibacteriae	1.0	–	–	2.0	3.0	–	–
Deinococcus–Thermus	–	–	–	–	–	6.1	–
Gemmatimonadetes	1.0	–	–	–	–	–	–
Others	–	2.1	–	–	–	7.3	–
Unknown	–		–	4.0	–	–	–
Reference	Present study	9	33 and Murdock (pers. commun)	24	24	5	32

Desulfobulbaceae, Desulfarculaceae, Desulfobacteraceae and Campylobacteraceae. Two clones (one phylotype) belonged to the phylum ε-Proteobacteria and both were observed in the sediment library (ESSd). Figure 4 *a* shows phylogenetic relationships of clone sequences belonging to Proteobacteria.

Bacteroidetes: Seventeen clones consisting of 15 phylotypes belonged to this phylum. Only one clone represented ESBw while the remaining were found in the ESSd library, contributing 4.2% and 19.8% respectively. Majority of the clones were grouped into the family Flavobacteriaceae followed by Marinilabiliaceae, Cryomorphaceae, Saprospiraceae, Sphingobacteriaceae, Rhodothermaceae and Flammeovirgaceae.

Actinobacteria: A total of 12 clones (six phylotypes) were affiliated to this phylum, accounting for 8.7%, 20.8% and 6.2% clones in the ESSw, ESBw and ESSd libraries respectively. Five clones represented the family Acidimicrobineae, four clones represented the family Actinomycetales and one clone each was affiliated to Iamiaceae and Euzebyales. Actinobacteria was found to be the second dominant phylum in the ESBw library. Interestingly, Actinobacteria groups were not recovered from the venting region of Espalamaca.

Planctomycetes, *Chloroflexi and Cyanobacteria*: Ten clones (six phylotypes) were affiliated with Planctomycetes, and accounted for 4.3%, 8.3% and 8.6% in the ESSw, ESBw and ESSd libraries respectively. These clones were closely affiliated to the families Planctomycetaceae, Phycisphaeraceae and '*Candidatus* Brocadiaceae'. A total of five clones (four phylotypes) belonged to the phylum Chloroflexi, representing 4.3% and 4.9% of the in the ESSw and ESSd libraries respectively. Two clones each were affiliated with the family Leptolinea and the remaining one was unidentified. Four clones (three phylotypes) belonged to the phylum Cyanobacteria, representing 8.7% and 2.5% in the ESSw and ESSd libraries respectively.

Acidobacteria, *Firmicutes and other minor groups*: A total of five clones (three phylotypes) were affiliated to Acidobacteria. Acidobacterial clones were only recovered from the ESSd library and belonged to the groups Gp4, Gp21 and Gp22. Both the clones originated from the ESSd library. Two clones (two phylotypes) were affiliated to Deinococcus–Thermus, two clones (one phylotype) to Deferribacteres and one clone each belonged to the phyla Lentisphaerae, Firmicutes and Candidate Division GN03. Figure 4 *b* shows evolutionary relationships of phylotypes from clone sequences.

Discussion

In Faial Island, a low-temperature Espalamaca hydrothermal field has been discovered in the Faial–Pico channel off the Espalamaca headland (Faial Island, Azores, NE Atlantic). The main venting area, named Espalamaca vent field, extends for a few tens of metres at

approximately 35 m depth. Gas emissions can be observed venting out of the sediment, as well as through cracked hard ground. Preliminary analyses of the gaseous discharges from the vents suggest that they are mainly composed of CO_2, with low concentrations of methane, temperature as high as 35°C and pH of 5.7 (Ana Colaço, pers. commun.). CO_2 is the primary gas found in most of the shallow-water hydrothermal vents described previously[9,24]. This hydrothermal field is also integrated into a larger protected area designated Baixa do Sul (Canal Faial–Pico), recently classified and included under the Faial Island Natural Park.

Studies on microbial communities inhabiting the deep-sea hydrothermal venting regions of Atlantic Ocean have been made along the Lost City[25], Rainbow[26], Ashadze[27], Logatchev[28] and Lucky Strike vent fields[29]. However, microbial explorations from the shallow hydrothermal vent counterparts are still poorly explored in the Atlantic Ocean. The present study provides novel insight into the bacterial communities thriving in the shallow-water hydrothermal vent of Espalamaca, and how they vary from other shallow hydrothermal vent fields.

Genomic information of unculturable microbes from metagenomic clone libraries can help in understanding their physiology and role in the ecosystem[30]. Most of the 16S rRNA gene sequences obtained from the clone libraries were closely related with the uncultured neighbours in the GenBank database. At the same time, we identified the sequences through RDP database to reveal closely related organisms. The on-line tool DECIPHER helped to find out around nine chimeric sequences from the clone libraries which were omitted from further analysis.

Bacterial community structure obtained from the Espalamaca hydrothermal vent sediments was compared with other shallow-water hydrothermal vent regions (rich in CO_2 emission) reported from various oceans to find out the similarities and variations between them (Table 1). Some shallow-water hydrothermal vents like Taketomi Island[31] and Punta Mita[32] were not included in this comparison since they reported high concentration of methane. However, we have included microbial community studies from South Tonca Arc[33] and Juan de Fuca Ridge[5], where there were no reports of high gas concentration. The comparative study revealed that the phylum Proteobacteria (especially ε-, γ- and δ-Proteobacteria) was consistently present in every hydrothermal vent field. It is not surprising because this phylum is the most dominant and diverse group of the microbial assemblage[34]. However, Proteobacteria subdivisions and their proportions varied from one region to another. Bacteroidetes was observed in almost all the venting regions, except Loihi Seamount (Table 1).

Generally, ε-Proteobacteria groups predominant in the shallow hydrothermal vents of Loihi Seamount[9], Milos Island[24] and Juan de Fuca Ridge[5]. It is noteworthy to mention that the ε-Proteobacteria group has also been reported to represent a major part of microbial communities in deep-sea hydrothermal vents as well[8,35]. Results from previous studies revealed that ε-Proteobacteria accounted for a significant part of the domain bacteria, between 40% and 80% from the 16S rRNA clone libraries. However, microbial communities in the present study region revealed that the composition of ε-Proteobacteria was only 3% in total, which is a contrast to other hydrothermal vents. It is worth mentioning here that the ε-Proteobacteria clone sequences were affiliated to the order Campylobacteriales, which is generally mesophilic and microaerophilic in nature[36]. Further, one of the phylotypes (clones EVBw-F45 and EVSd-A78) belonging to the phylum ε-Proteobacteria was closely related with the GenBank sequence HE576785; interestingly, it was reported from a microbial mat in Lucky Strike hydrothermal vent field (Mid-Atlantic Ridge). In addition, clones EVBw-F45 and EVSd-A78 were closely related with the genus *Arcobacter* in RDP database, which is reported to be the producer of elemental sulphur in filamentous form from the oxidation of sulphide[37]. This reveals that even though mesophilic ε-Proteobacteria groups are present in low level in the study area, they are involved in sulphur oxidation process.

γ-Proteobacteria groups were predominant in the shallow hydrothermal vent of Espalamaca, similar to those in the South Tonga Arc[33] and Bahía Concepción[32] in the Pacific Ocean. α-Proteobacteria and γ-Proteobacteria are known to form large clusters in all the marine environments[38,39]. Bacteroidetes and γ-Proteobacteria together contributed 50% in the present study area followed by South Tonga Arc with 44% and Bahía Concepción with 38%. Other bacterial groups observed in this study also followed a similar pattern to the South Tonga Arc vents. For instance, domination of Bacteroidetes and γ-Proteobacteria followed by other Proteobacteria groups, Planctomycetes, Chloroflexi and even minor group signatures like Acidobacteria, Verrucomicrobia and Deferribacteres. On the other hand, large variations in bacterial communities were observed between the present study region and other shallow hydrothermal vent fields (Table 1). The present study revealed more than 15 phylogenetic groups, whereas in other venting regions it was reported to be less (Table 1).

In Milos and Juan de Fuca Ridge, the number of phylogenetic groups was around seven and in Loihi Seamount, it was reported to be three, excluding few unknown groups. This may be due to lower number of sequenced clones. Whereas in South Tonga Arc vents, a larger number of clones have been sequenced; hence the number of phylogenetic groups was more than 10. Even though clones sequenced from EVSd library ($n = 100$) were less than the photic zone vent of South Tonga Arc, we have identified more phylogenetic groups. Apart from these clone libraries (compared in Table 1), recently advanced technologies like Illumina sequencing are a

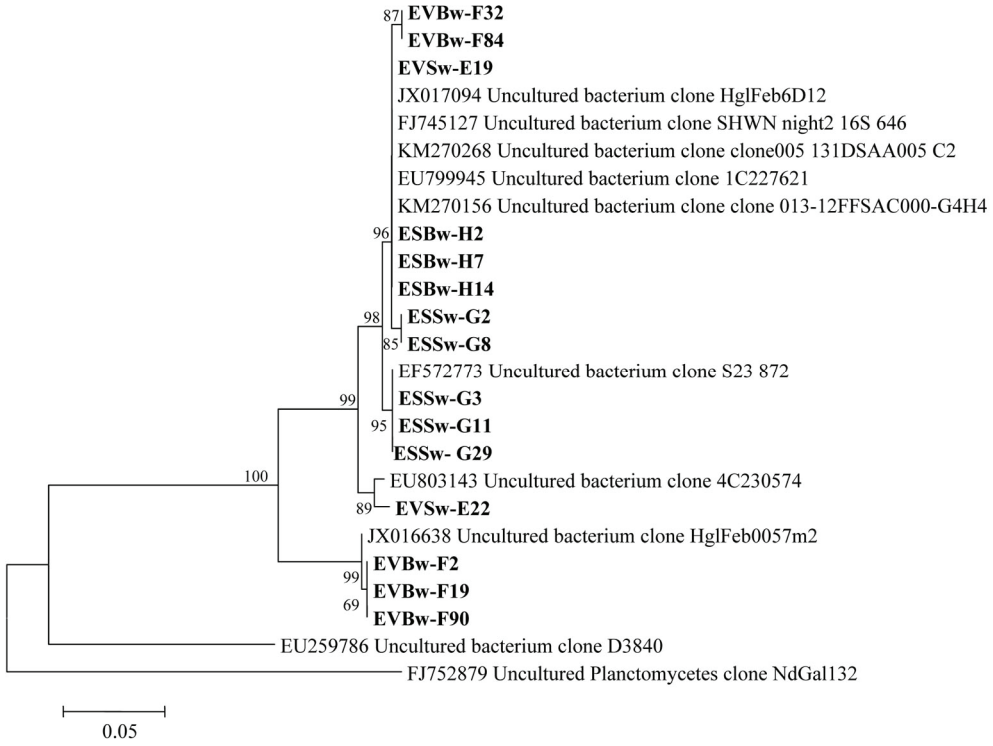

Figure 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of SAR11 phylotypes from Espalamaca waters. Bootstrap analysis was performed with 1000 replications and values above 50% are indicated at the nodes.

promising approach for studying microbial community structure. For instance, Lentini *et al.*[7] analysed prokaryotic communities from a shallow hydrothermal site in Eolian Islands, Italy, using Illumina sequencing technology, which resulted in about 35 phylogenetic groups.

Bacteria of the SAR11 groups often dominate marine microbial communities in both the surface and deep waters of the ocean and potentially mediate a large portion of the dissolved organic matter flux[40]. Though they are not in large proportion, we have observed a considerable number of SAR11 clones from the Espalamaca waters (seven clones from venting regions and eight from the non-venting region), accounting for 8.3%, 17.2%, 21.7% and 12.5% from the EVSw, EVBw, ESSw and ESBw libraries respectively. Figure 5 shows phylogenetic positions of each clone belonging to SAR11 groups obtained from this study. Clones belonging to SAR11 clade were not observed in sediments of both vent and non-vent regions.

The presence of Cyanobacteria and Chloroflexi groups confirms that these photosynthetic bacteria are involved in primary production in the shallow-water hydrothermal vents. Few of the clones (EVSd-B10, EVSd-B55, EVSd-B89 and EVSd-B92) belonging to Chloroflexi were closely related (96%, 91%, 91% and 97% sequence homology respectively) with a sequence in NCBI database reported from iron-oxide sediments in Volcano 1, Tonga Arc hydrothermal vent (GenBank accession number FJ905709).

A considerable number of clones ($n = 21$) was observed from the vent libraries belonging to the phylum Planctomycetes. They were affiliated with two orders, namely Planctomycetales and *Candidatus* Brocadiales, and closely related with the genera Pirellula, Blastopirellula, Rhodopirellula and '*Candidatus* Anammoxoglobus'. Interestingly, these groups are reported from the studies conducted by Storesund and Øvreås[41] on the diversity of Planctomycetes in low-temperature hydrothermal venting (iron-hydroxide deposits) at the Mohns Ridge, a part of the Arctic Mid Ocean Ridge. Members of Planctomycetes are reported to be involved in carbohydrate fermentation and sulphur reduction[42]. In addition, they are the only known organisms able to perform anaerobic ammonium oxidation (anammox), which could be a significant process in these ecosystems[43,44].

Members of Actinobacteria appear as a small fraction in the hydrothermal vents when compared to non-thermal environments[45]. In this study, we did not observe Actinobacteria members in the venting region, while Actinobacteria was found to be the second dominant phylum in the ESBw library of the non-vent region.

Shannon diversity index (H') for the venting region was found to be 2.28, 3.06 and 4.03 in the EVSw, EVBw and EVSd libraries respectively, whereas in the non-vent

region it was 2.40, 2.57 and 4.16 in the ESSw, ESBw and ESSd libraries respectively. Rarefaction curve analysis of vent and non-vent sediment libraries indicated that the non-vent region showed more phylotypes with fewer clones than the venting site (Figure 6). Overall, a total of 84 phylotypes each were obtained from both the sites and majority of them (84.5%) were unique to the ecosystem. Only a few phylotypes (15.5% of total) were observed to be common between the vent and non-vent libraries. This massive community variation (within 500 m distance) in clone libraries showed that microbes inhabiting the venting area are entirely different from the reference area which may be involved in metal or elemental transformation and oxidation pathways.

Culture-dependent analysis from the shallow hydrothermal vent of Espalamaca[15] revealed that the presence of γ-Proteobacteria (68.7%), α-Proteobacteria with (16.7%), Bacteroidetes (10%), Firmicutes (3.2%), Actinobacteria (0.9%) and β-Proteobacteria (0.45%). Former four phylogenetic groups were also obtained in culture-independent clone library analysis, whereas minor groups like Actinobacteria and β-Proteobacteria were not observed in vent clone libraries. However, many uncultured clones recovered from the same venting regions were affiliated to more than 10 phyla. Phylogenetic groups of Proteobacteria (ε, δ, ζ subdivisions), Acidobacteria, Chloroflexi, Cyanobacteria, Deferribacteres, Gemmatimonadetes, Ignavibacteriae, Planctomycetes, Spirochaetes and Verrucomicrobia obtained from non-culturable diversity were not detected in culture-dependent analysis. This indicated the importance of culture-independent sequence analysis for assessing the entire bacterial community in a particular ecosystem. *Roseovarius*, *Photobacterium*, *Vibrio* and *Pseudoalteromonas* are some of the common genera observed in both culture-dependent and culture-independent analysis. The present study analyses culture-independent microbial communities in the shallow hydrothermal vent area of the Azores Islands.

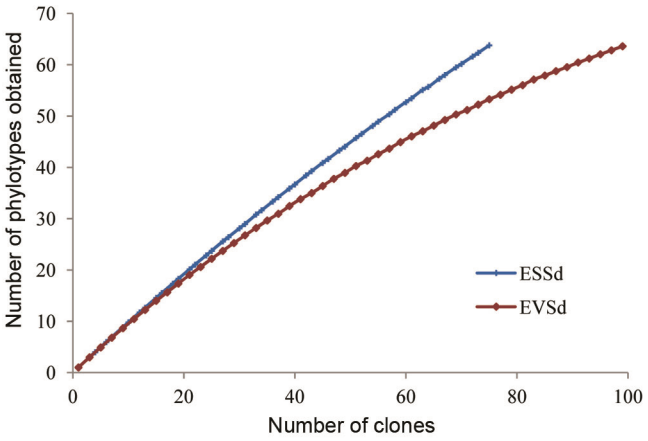

Figure 6. Rarefaction curve of 16S rRNA gene libraries from vent (EVSd) and non-vent (ESSd) sediments.

We explored the presence of functional genes from the sediment DNA sample using available primer sets (data not shown). Interestingly, we could see PCR amplification bands for the genes of methanol dehydrogenase (*mxaF*), carbonic anhydrase and *soxB*. Carbonic anhydrases is a metalloenzyme catalysing the reversible hydration of carbon dioxide to bicarbonate, and plays important roles in global carbon cycle[46]. Methanol dehydrogenase is highly conserved among distantly related methylotrophic species in α-, β- and γ-Proteobacteria[47]. The presence of *soxB* gene shows that there are active sulphur-oxidizing microbial communities in shallow hydrothermal vent. This additional information revealed the importance of shallow hydrothermal vent bacteria which are involved in global biogeochemical cycles.

Conclusion

The Espalamaca hydrothermal vent region harboured distinct bacterial communities when compared with a non-venting region located 500 m south of the vent site. Merely 15.5% of the phylotypes were observed to be common between the vent and non-vent libraries. Combination of culture-independent clone libraries and culture-dependent diversity analysis provided an overview of microbial communities in the shallow hydrothermal vent in Espalamaca. Groups of ε-Proteobacetria were reported to be dominant in various hydrothermal vent systems. However, the present study area exposed to a high abundance of γ-Proteobacetria and Bacteroidetes groups, similar to the hydrothermal vent of South Tonga Arc and Bahía Concepción in the Pacific Ocean. The presence of methanol dehydrogenase, carbonic anhydrase and sulphur-oxidizing genes in the study area favour further studies on functional gene diversity aspect which could reveal microbial metabolic pathways in the venting area. This forms first report on culture independent microbial diversity from shallow hydrothermal vents in Azores Island.

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