

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

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Running title: Bacterial diversity from shallow hydrothermal vent Azores

Abstract

A low-temperature shallow-water hydrothermal vent field has been discovered during summer 2010 in the Faial-Pico channel off the Espalamaca headland (Faial Island, Azores, NE Atlantic). Present investigation focused to analyze 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* (includes 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 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 of only 500 m difference, high variations (>80%) of phylotypes have been found between venting and non-venting region.

Keywords: Hydrothermal vent; Espalamaca; Clone library; SSU rRNA; phylogeny

Introduction

Marine hydrothermal vents are known for its characteristic habitat since the fluids emitted from the vents are distinguished by high temperatures, high concentrations of CO₂, H₂S 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 photosynthesis³. Shallow-water hydrothermal systems can be described as high energy environments, where microbial metabolism is fuelled by light and as well as by chemical energy sources⁴. Hence, the microbial communities inhabiting in the shallow hydrothermal vents would be different from those deep-sea counterparts with a dominance of photosynthetic microbial lineages. However, shallow submarine hydrothermal vents are less understood habitats in the ocean but have been intensively focused for research in past 3 decades⁵.

Low-temperature hydrothermal vents, which form by the mixing of high-temperature hydrothermal fluids and seawater, are omnipresent in recent submarine hydrothermal systems⁶. 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 web⁷. Understanding the microorganisms present in shallow water hydrothermal vents is essential to comprehend how microorganisms 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^{8,9,10,11}.

There are nine shallow water hydrothermal vent fields have been identified so far within the Azores Archipelago. When compared to deep-sea counterparts, shallow hydrothermal vent regions in the Azorean Islands are not explored in the aspect of diversity and ecology¹². The proximity of the Azorean Islands (Portugal) provides accessible sampling sites for studying microorganisms

inhabiting in 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 in the aspect of culture dependent microbes^{13,14,15} from the Azores Island. More recently molecular diversity of culture dependent bacteria was investigated using 16S rRNA gene sequencing from shallow vents in Espalamaca¹⁵. The results indicated that culturable fractions of *Proteobacteria* were dominant followed by *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. Many of those were novel bacteria and they were not having previous records for their existence in such environments. However, there is no report on culture independent based approach on microbial diversity in shallow water hydrothermal vents in the Azores.

In this investigation, we attempted to explore the culture independent microbial community structure from the shallow water hydrothermal vent of Espalamaca based on 16S rRNA gene sequence clone library approach. In addition, the clone library data was compared with few other shallow water hydrothermal vents of similar type to reveal the differences of 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 and sediment samples were collected from the shallow hydrothermal vent (36 m depth) and non-vent sites (38 m depth) in Espalamaca (38°33'N; 28°39'W) during April 2014 by scuba divers (Fig. 1). Sediment samples were collected using sterile polycarbonate tubes and water samples were collected using Niskin samplers (2.5 L capacity). Samples were immediately brought to the laboratory, University of Azores.

Genomic DNA Extraction

Seawater samples (2.5 L) were filtered using 0.2 µm cellulose nitrate filters (Sartorius). Genomic DNA was extracted from the filtered papers using E.Z.N.A water DNA kit (D5525, Omega) following the manufacturer's instructions. Genomic DNA from the sediment samples was extracted with 2 steps. The first step based on the method by Luna et al.¹⁶ with phenol-chloroform. In the second step, the above DNA samples were purified following the E.Z.N.A. Soil DNA kit protocol (D5625, Omega). The presence of genomic DNA was confirmed with 1.0 % agarose gel electrophoresis with 0.5X Tris Borate EDTA buffer (TBE) buffer.

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), 10x PCR buffer, 40 mM deoxynucleoside triphosphates, 2.5 mM MgCl₂, 20 pmol 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 by 1.0 % 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 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 1.0 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). Obtained partial sequences were trimmed using DNA baser software version 3.0 and vectors were removed using NCBI online program VecScreen (<http://www.ncbi.nlm.nih.gov/tools/vecsreen/>). Chimeric sequences were removed using an online tool Decipher¹⁸. Nonchimeric sequences were submitted to BLAST search program in 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 by using RDP (Ribosomal Database Project) classifier. Sequences were aligned using Clustal W sequence alignment program¹⁹ and neighbour-joining phylogenetic trees²⁰ were constructed using MEGA 5.2 software²¹ with bootstrap values based on 1,000 replications²². The sequences were submitted to GenBank with accession numbers KP303396 – KP303589.

Shannon and Simpson diversity indices were calculated using online program (<http://www.changbioscience.com/genetics/shannon.html>). Rarefaction analysis was performed by plotting the number of phylotypes against the total number of clones using EcoSim700²³. Good's coverage was calculated using the formula $C = [(1-(n1/N)]*100$ where C is the homologous coverage, n1 is 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 analyzed 100 clones from sediments (EVSd), 29 clones from bottom water (EVBw) and 24 clones from surface water (EVSsw). 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*. Relative proportions of the different groups in each clone library were summarized in Figure 2.

Proteobacteria: The phylum *Proteobacteria* was found to be dominant with 59 clones (38 phylotypes) in the hydrothermal vent region which includes α , γ , ϵ , δ and 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 *Cohwellia* was abundant in the sediment samples with 7 clones. *Marinobacterium* was the only common genus between EVBw and EVSd libraries; otherwise, 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 other two libraries and other subdivisions of *Proteobacteria*. Eight out of 18 clones were belonged to SAR11 clusters, interestingly all the clones were observed only in seawater libraries, 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 (3 phylotypes) were affiliated with δ -*Proteobacteria* belong to the families *Syntrophobacteraceae* (1 clone) and *Geobacteraceae* (3 clones). Four clones (2 phylotypes) were affiliated to ϵ -*Proteobacteria* and grouped into the family *Campylobacteraceae*. Only one clone each of δ -*Proteobacteria* and ϵ -*Proteobacteria* were observed in EVBw, remaining clones are solely observed in EVSd. Four clones (2 phylotypes) belong to the family *Mariprofundaceae* of ζ -*Proteobacteria* were observed only in the sediment library (EVSd). Detailed phylogenetic position and evolutionary relationships of clone sequences belong to *Proteobacteria* are given in Figure 3a.

Bacteroidetes: *Bacteroidetes* was the second dominant phyla observed in this 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 3 libraries, representing 29.2 %, 13.8 % and 28.0 % of clones in the EVSw, EVBw and EVSd libraries respectively. Four phylotypes from EVBw and 3 phylotypes from EVSw libraries were obtained and none of them were common. Whereas 15 phylotypes were found in EVSd library in which one phylotype was observed to be common with EVSw and 4 phylotypes were common with EVBw.

Planctomycetes: Twenty-one clones (11 phylotypes) were belonged to the phylum *Planctomycetes*, accounted for 4.2 %, 10.3 % and 17.0 % in the EVSw, EVBw and EVSd libraries respectively. Majority of the clones were belonged to the family *Planctomycetaceae* and few of the clones were belonging to the family “*Candidatus Brocadiaceae*”. The sequences closely related with the genus *Blastopirellula* were common in all the 3 libraries. On the other hand, sequences closely related to the genus *Pirellula* was found to be common in EVSd and EVBw libraries. Remaining phylotypes were observed only from the sediments (EVSd).

Cyanobacteria and *Chloroflexi*: A total of 13 clones (5 phylotypes) were affiliated to the phylum *Cyanobacteria*, interestingly they retrieved only from the seawater samples. *Cyanobacteria* accounted for 33.3 % and 17.2 % in the EVSw and EVBw libraries respectively. *Cyanobacteria* were observed to be predominant (33.3 %) in the surface seawater when compared to *Bacteroidetes* (29.2 %) and *Proteobacteria* (25.0 %). All the 5 phylotypes observed in this group were belonged to 4 different families namely, *Bacillariophyta*, *Cryptomonadaceae*, *Chlorarachniophyceae* and Family II. There were 11 clones belong to the phylum *Chloroflexi* in which 6 phylotypes were observed. These clones accounted for 8.3 %, 3.4 % and 8.0 % clones in the EVSw, EVBw and EVSd libraries

respectively. These 6 phylotypes represent 3 different families, *Anaerolineaceae*, *Ardenticatenaceae* and *Caldilineaceae*.

Firmicutes, *Verrucomicrobia*, *Acidobacteria* and other minor groups: Three clones (1 phylotype) were belonging to the phylum *Verrucomicrobia* and 2 clones (1 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). Phylogenetic position and evolutionary relationships of clone sequences belong to the phylogenetic groups other than *Proteobacteria* are given in Figure 3b.

Bacterial diversity from non-vent region

We analyzed 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 seawater). As like the venting site, high bacterial diversity was observed in the non-vent region also. 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). Relative proportions of these groups in each clone library were summarized in Figure 2.

Proteobacteria: The phylum *Proteobacteria* was predominant in the non-vent region with 68 clones (41 phylotypes) which includes α , γ , ϵ and δ and 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 % of clones 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 % of clones in the ESSw, ESBw and ESSd libraries respectively. The predominant phyla observed from the surface seawater samples was α -*Proteobacteria* followed by γ -*Proteobacteria*. Majority of the clones (8 clones each) were belonged to the family *Rhodobacteraceae* and SAR11 groups. *Hyphomonadaceae*, *Methylocystaceae* and *Rhodospirillaceae* were some other families observed from this region. Similar to the vent region, SAR 11 groups were observed only from the water samples in the non-vent samples also.

Eight clones (8 phylotypes) were represented in 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*, *Desulfobulbaceae*, *Desulfarculaceae*, *Desulfobacteraceae* and *Campylobacteraceae*. Two clones (1 phylotype) were belonging to the phylum ϵ -*Proteobacteria* and both the clones were observed in sediment library (ESSd). Phylogenetic relationships of clone sequences belong to *Proteobacteria* are given in Figure 4a.

Bacteroidetes: Seventeen clones consisting of 15 phylotypes belong to the phylum *Bacteroidetes*. Only one clone represents ESBw and remaining clones 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 (6 phylotypes) were affiliated to the phylum *Actinobacteria*, accounting for 8.7 %, 20.8 % and 6.2 % clones in the ESSw, ESBw and ESSd

libraries respectively. Five clones were represented the family *Acidimicrobinae*, four clones represented the family *Actinomycetales* and one clone each was affiliated to *Iamiaceae* and *Euzebyales*. *Actinobacteria* was found to be the second dominant in the ESBw library. Interestingly, *Actinobacteria* groups were not recovered from the venting region of Espalamaca.

Planctomycetes, *Chloroflexi* and *Cyanobacteria*: Ten clones (6 phylotypes) were affiliated with *Planctomycetes*, accounted for 4.3 %, 8.3 % and 8.6 % respectively in the ESSw, ESBw and ESSd libraries. These clones were closely affiliated to the families *Planctomycetaceae*, *Phycisphaeraceae* and “*Candidatus* Brocadiaceae”. A total of 5 clones (4 phylotypes) were belonging to the phylum *Chloroflexi*, representing 4.3 and 4.9 % of the clones in the ESSw and ESSd libraries respectively. Two clones each were affiliated with the family *Leptolinea* and the remaining one was unidentified. Four clones (3 phylotypes) were belonged to the phylum *Cyanobacteria*, representing 8.7 and 2.5 % respectively in the ESSw and ESSd libraries.

Acidobacteria, *Firmicutes* and other minor groups: A total of 5 clones were affiliated to *Acidobacteria* with 3 phylotypes. Acidobacterial clones were only recovered from the ESSd library and belong to the groups Gp4, Gp21 and Gp22. Both the clones were originated from ESSd library. Two clones (2 phylotypes) were affiliated to *Deinococcus-Thermus*, two clones (1 phylotype) were belonging to *Deferribacteres* and one clone each belongs to the phyla *Lentisphaerae*, *Firmicutes* and Candidate Division GN03. Evolutionary relationships of phylotypes from clone sequences are given in Figure 4b.

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 meters 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 CO₂, with low concentrations of methane, temperature as high as 35 °C and pH values of 5.7 (Colaço pers. comm.). CO₂ 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 the Faial Island Natural Park.

Studies on microbial communities inhabiting in the deep-sea hydrothermal venting regions of Atlantic Ocean have been attempted along the Lost City²⁵, Rainbow²⁶, Ashadze²⁷, Logatchev²⁸ and Lucky Strike vent fields²⁹. However, microbial explorations from the shallow hydrothermal vent counter parts are still poorly explored in the Atlantic Ocean. Present investigation provides a new insight into the bacterial communities thriving in the shallow water hydrothermal vent of Espalamaca (Azores, North Atlantic) and how the microbial communities vary from other shallow hydrothermal vent fields.

Genomic information of unculturable microbes from metagenomic clone libraries can help in understanding their physiology and their role in the ecosystem³⁰. 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 online tool Decipher helped to find out around 9 chimeric sequences from the clone libraries which were omitted for further analysis.

Bacterial community structure obtained from the Espalamaca hydrothermal vent sediments was compared with the other shallow water hydrothermal vent regions (rich with CO₂ emission) reported from various Oceans (Table 1) to find out the similarities and variations between them. Some of the other shallow water hydrothermal vents like Taketomi Island³¹ and Punta Mita³² are not

included in this comparison since they reported the high concentration of methane. However, we have included microbial community studies from South Tonga Arc³³ and Juan de Fuca Ridge⁵ where there were no reports of gas concentrations. The comparative study revealed that the phylum *Proteobacteria* (especially ϵ -, γ - and δ -*Proteobacteria*) was consistently present in every hydrothermal vent fields. It is not surprising because the phylum *Proteobacteria* is the most dominant and diverse group of the microbial assemblage³⁴. However, *Proteobacteria* subdivisions and their proportions varied from one region to another. Apart from that, the presence of *Bacteroidetes* was observed almost in all the venting regions except in Loihi Seamount (Table 1).

Generally, ϵ -*Proteobacteria* groups were predominant in the shallow hydrothermal vents of Loihi Seamount⁹, Milos Island²⁴ and Juan de Fuca Ridge⁵. It is noteworthy to cite that the ϵ -*Proteobacteria* group has also been reported to represent a major part of microbial communities at deep-sea hydrothermal vents as well^{8,35}. Results from the previous investigation revealed that ϵ -*Proteobacteria* accounted significant part in 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 are generally mesophilic and microaerophilic in nature³⁶. Further, one of the phylotypes (clones EVBw-F45 and EVSd-A78) belong 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 to that, clones EVBw-F45 and EVSd-A78 are closely related with the genus *Arcobacter* in RDP database which are reported to be the producers of elemental sulphur in filamentous form from the oxidation of sulphide³⁷. This information 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 which is coinciding with the South Tonga Arc³³ and Bahía Concepción³² from the Pacific Ocean. *α-Proteobacteria* and *γ-Proteobacteria* are known to form large clusters in all of 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 from this study were also in 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 side, huge variations of bacterial communities were observed between our results and other shallow hydrothermal vent fields indicated (Table 1). The present investigation revealed more than 15 phylogenetic groups, whereas in other venting regions it was reported less (Table 1).

In Milos and Juan de Fuca Ridge, the numbers of phylogenetic groups were around 7 and in Loihi Seamount, the numbers of phylogenetic groups were reported to be 3 excluding few unknown groups. This may be due to lower number of sequenced clones. Whereas, in South Tonga Arc vents, they sequenced larger number of clones 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 detected more phylogenetic groups. Apart from these clone libraries (compared in table 1), recently advanced technologies like Illumina sequencing are a promising approach for studying microbial community structure. For instance, Lentini et al⁷ analyzed prokaryotic communities from a shallow hydrothermal site in Eolian Islands (Italy), elucidated by Illumina sequencing technology, which resulted about 35 phylogenetic groups.

Bacteria of the SAR11 groups often dominate the marine microbial communities in both the surface and deep waters of the ocean and potentially mediate a large portion of the dissolved organic

matter flux⁴⁰. Though they are not in large proportion, we have observed a considerable number of SAR11 clones from the Espalamaca waters (7 clones from venting regions and 8 clones from the non-venting region), accounting for 8.3 %, 17.2 %, 21.7 % and 12.5 % of clones from EVSw, EVBw, ESSw and ESBw libraries respectively. The phylogenetic positions of each clones belonging to SAR11 groups obtained from this study are given in Figure 5. Clones belong to SAR11 clade was 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 (96.0, 91.0, 91.0 and 97.0 % 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 2 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 Øvreås⁴¹ on the diversity of the phylum *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 reduction⁴². 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 in a small fraction at hydrothermal vents when compared to non-thermal environments⁴⁵. In this investigation, we did not come across with *Actinobacteria*

members in the venting region while *Actinobacteria* was found to be the second dominant phyla in ESBw library of the non-vent region.

Shannon diversity index (H') for venting region was found to be 2.28, 3.06 and 4.03 respectively in the EVSw, EVBw and EVSd libraries whereas in the non-vent region it was 2.40, 2.57 and 4.16 respectively in the ESSw, ESBw and ESSd libraries. 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 (Fig. 6). Overall, a total of 84 phylotypes each were obtained from both the sites and the 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 in the venting area are entirely different from reference area which may be involved in metal or elemental transformation and oxidation pathways.

Culture dependent analysis from the shallow hydrothermal vent of Espalamaca¹⁵ 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 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 represents the first report on culture independent microbial community analysis in the shallow hydrothermal vent in the area of the Azores Islands.

We attempted to find out 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 (*mxhF*), carbonic anhydrase and *soxB*. Carbonic anhydrases is a metalloenzyme catalyzing the reversible hydration of carbon dioxide to bicarbonate and play important roles in global carbon cycle⁴⁶. 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 to 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 diversity¹⁵ analysis revealed an overview of microbial communities in the shallow hydrothermal vent in Espalamaca. Groups of ϵ -*Proteobacteria* were reported to be dominant in various hydrothermal vent systems; however, the present area exposed to a high abundance of the γ -*Proteobacteria* and *Bacteroidetes* groups which is coinciding with the hydrothermal vent of South Tonga Arc and Bahía Concepción in the Pacific Oceans. The presence of methanol dehydrogenase, carbonic anhydrase and sulphur oxidizing genes in the study area hinted us for further investigation on functional gene diversity aspect which could reveal microbial metabolic pathways in venting area. This forms first report on culture independent microbial diversity from shallow hydrothermal vents in Azores Island.

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Table 1. Bacterial community from the shallow water hydrothermal vent Espalamaca (our study) 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
<i>α-Proteobacteria</i>	5.0	-	8.0	-	-	-	4.0
<i>β-Proteobacteria</i>	-	-	-	-	-	3.7	-
<i>ε-Proteobacteria</i>	3.0	60.5	17.0	60.0	59.0	52.4	7.0
<i>γ-Proteobacteria</i>	22.0	33.4	33.0	7.0	20.0	17.1	21.0
<i>δ-Proteobacteria</i>	2.0	2.1	8.0	5.0	6.0	9.8	10.0
<i>ζ-Proteobacteria</i>	4.0	-	-	-	-	-	-
<i>Bacteroidetes</i>	28.0	-	11.0	20.0	6.0	3.7	17.0
<i>Cyanobacteria</i>	-	-	0.4	2.0	-	-	14.0
<i>Planctomycetes</i>	17.0	-	9.0	-	3.0	-	-
<i>Chloroflexi</i>	9.0	-	4.9	-	-	-	10.0
<i>Acidobacteria</i>	1.0	-	3.0	-	-	-	-
<i>Actinobacteria</i>	-	-	3.4	-	3.0	-	4.0
<i>Firmicutes</i>	2.0	-	0.7	-	-	-	10.0
<i>Verrucomicrobia</i>	3.0	-	-	-	-	-	-
<i>Deferribacteres</i>	1.0	-	0.4	-	-	-	-
<i>Spirochaetes</i>	1.0	-	1.1	-	-	-	3.0

<i>Ignavibacteriae</i>	1.0	-	-	2.0	3.0	-	-
<i>Deinococcus-</i>	-	-	-	-	-	6.1	-
<i>Thermus</i>							
<i>Gemmatimonadetes</i>	1.0	-	-	-	-	-	-
Others	-	2.1	-	-	-	7.3	-
Unknown	-		-	4.0	-	-	-
Reference	Present study	9	33 & Murdock (pers. com)	24	24	5	32

Legends to Figures

Fig. 1. Sampling location at Espalamaca shallow-water hydrothermal vent (Azores, Portugal).

The asterisk indicates the sampling site¹⁵

Fig. 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, Azores

Fig. 3. Neighbour-joining tree showing the evolutionary relationship of phylotypes based on 16S rRNA gene sequences from hydrothermal vent EVSd library (a, *Proteobacteria*; b, other groups). Bootstrap analysis was performed with 1,000 replications and the 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*

Fig. 4. Neighbour-joining tree showing the evolutionary relationship of phylotypes based on 16S rRNA gene sequences from non-vent ESSd library (a, *Proteobacteria*; b, other groups). Bootstrap analysis was performed with 1,000 replications and the 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*

Fig. 5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of SAR11 phylotypes from Espalamaca waters. Bootstrap analysis was performed with 1,000 replications and the values above 50 % are indicated at the nodes.

Fig. 6. Rarefaction curve of the 16S rRNA gene libraries from vent (EVSD) and non-vent (ESSd) sediments

Figure 1.

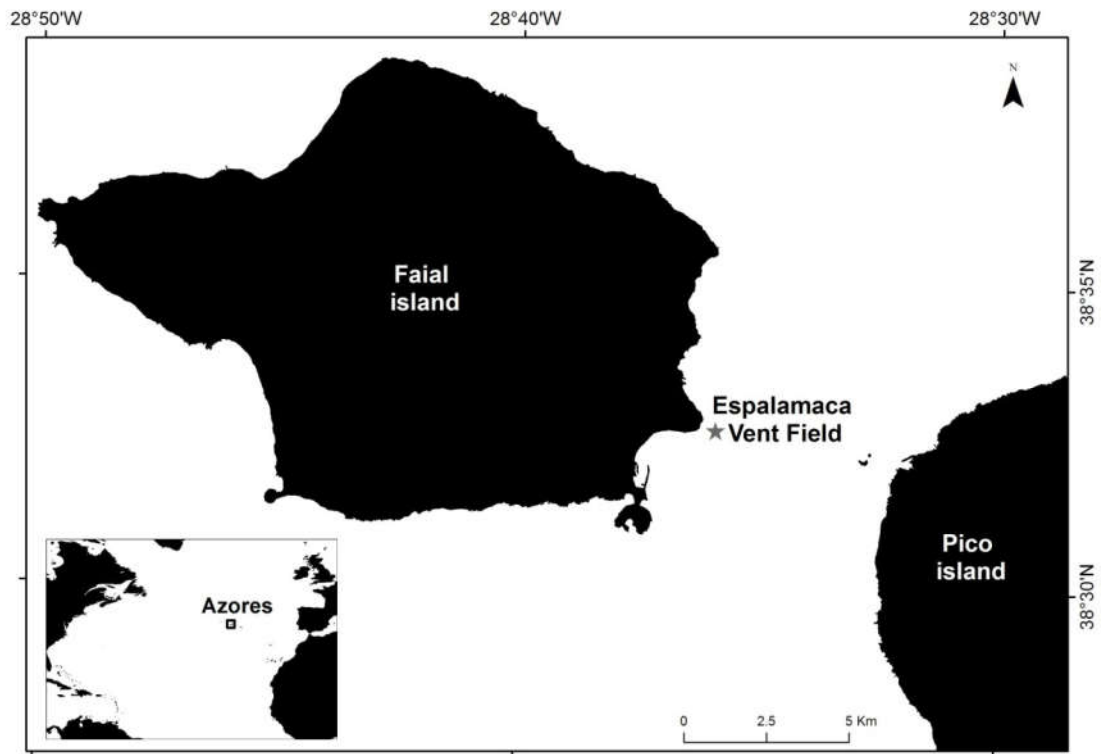


Figure 2.

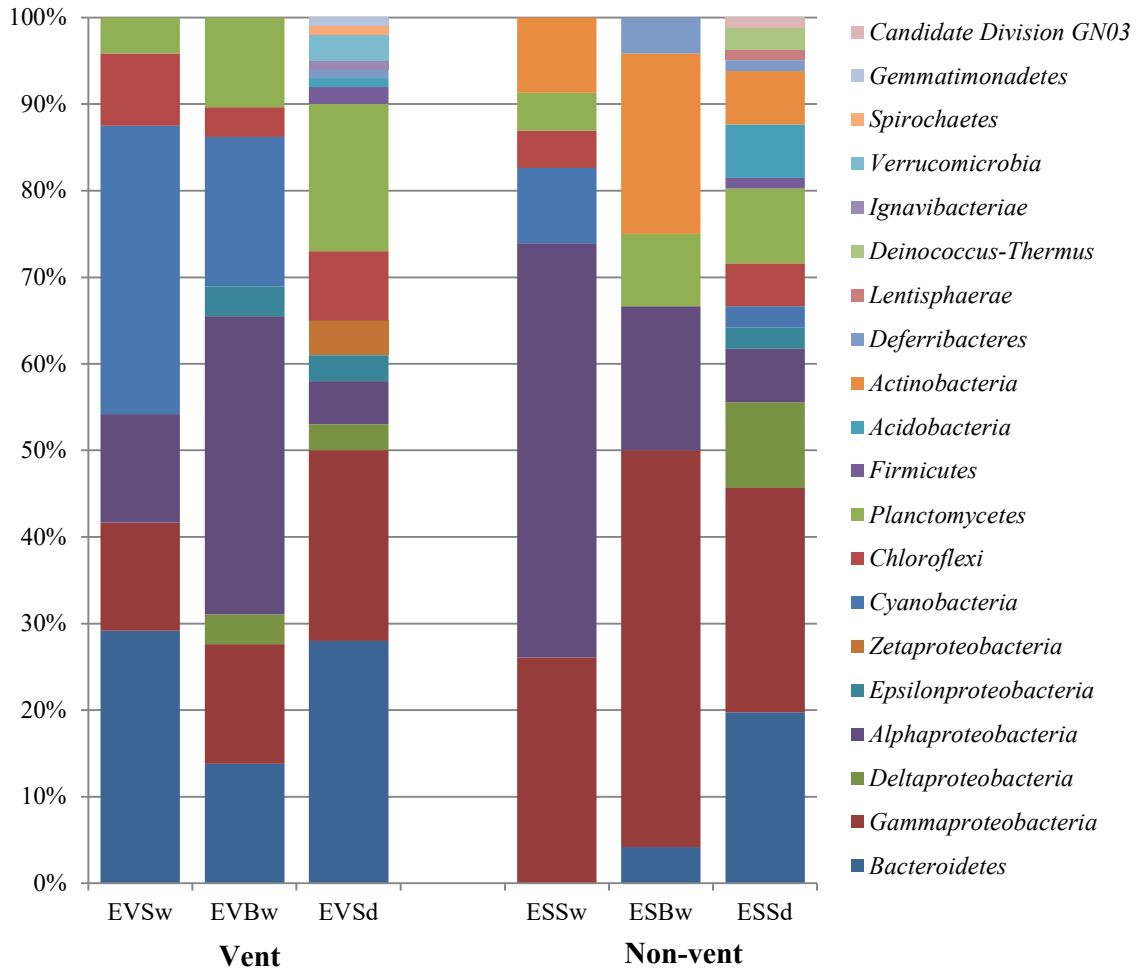


Figure 3a.

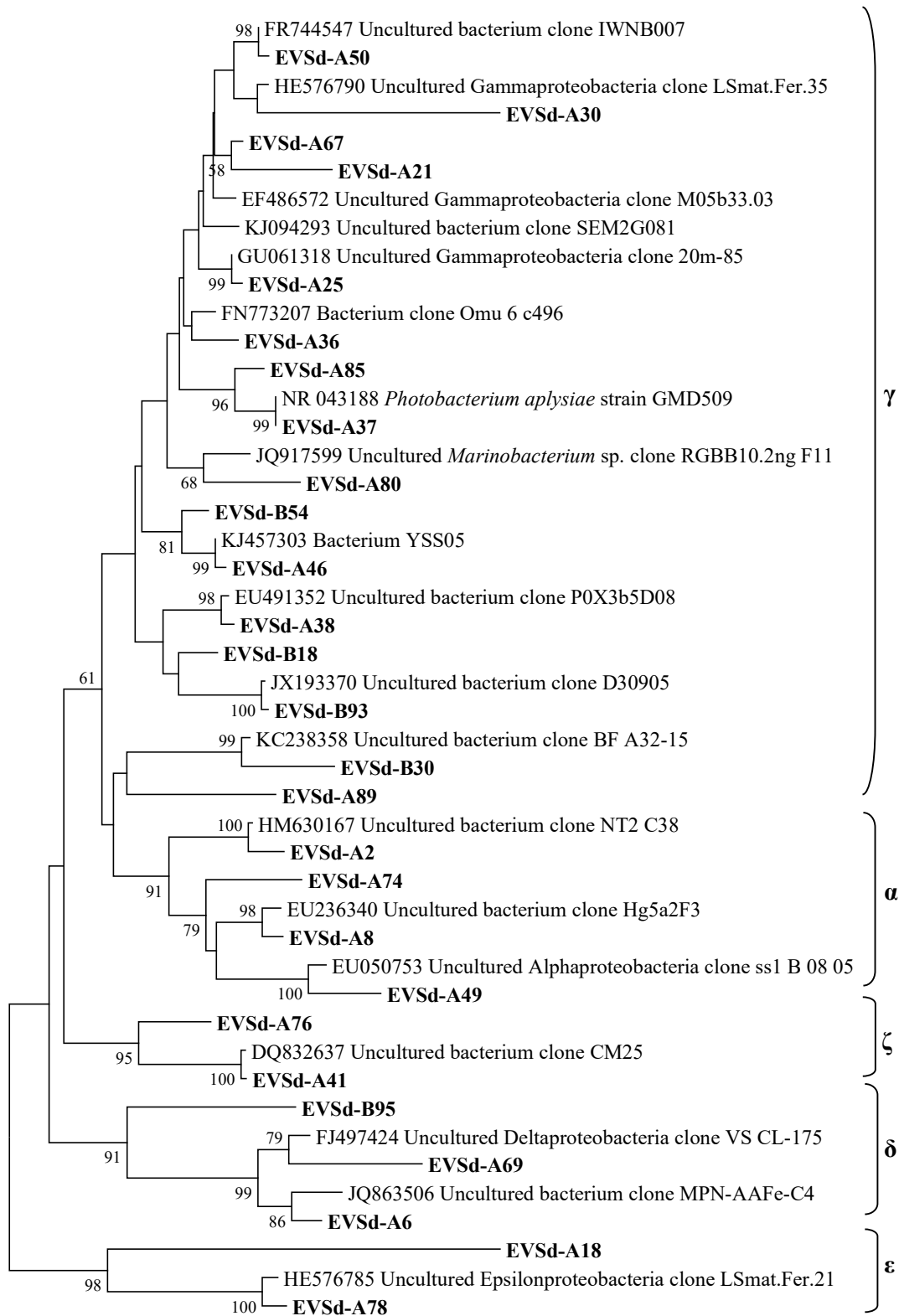


Figure 3b.

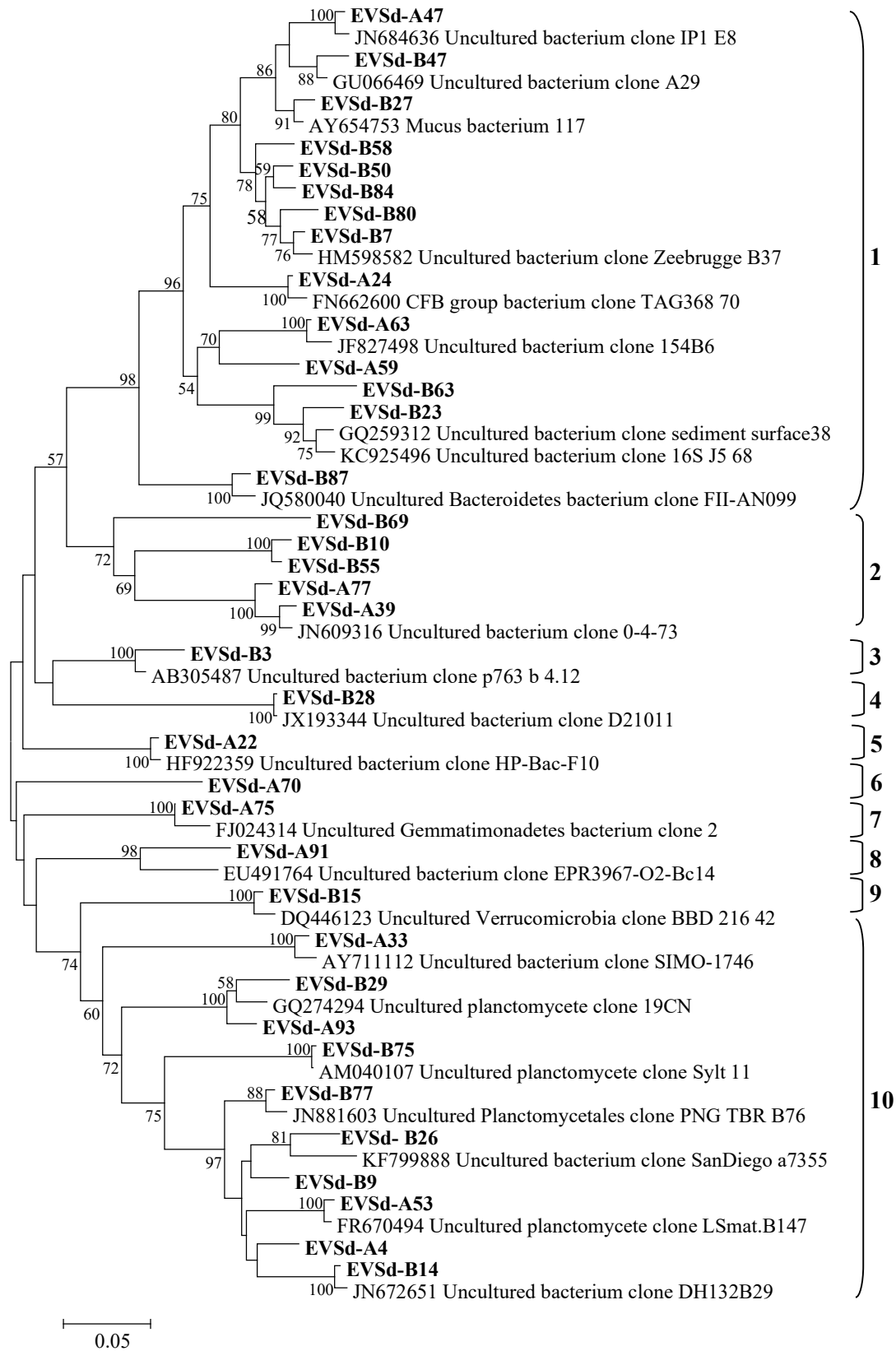


Figure 4a.

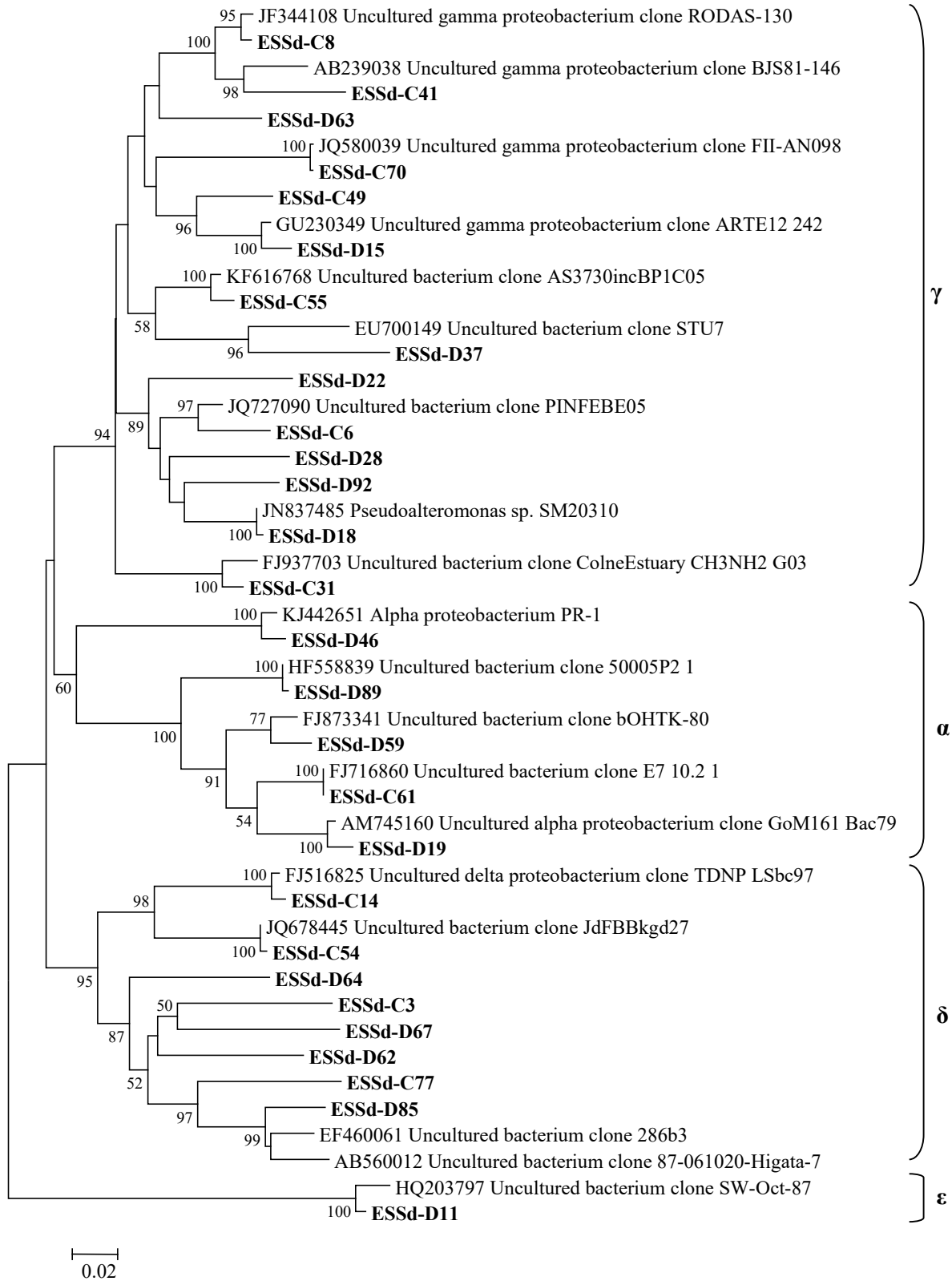


Figure 4b.

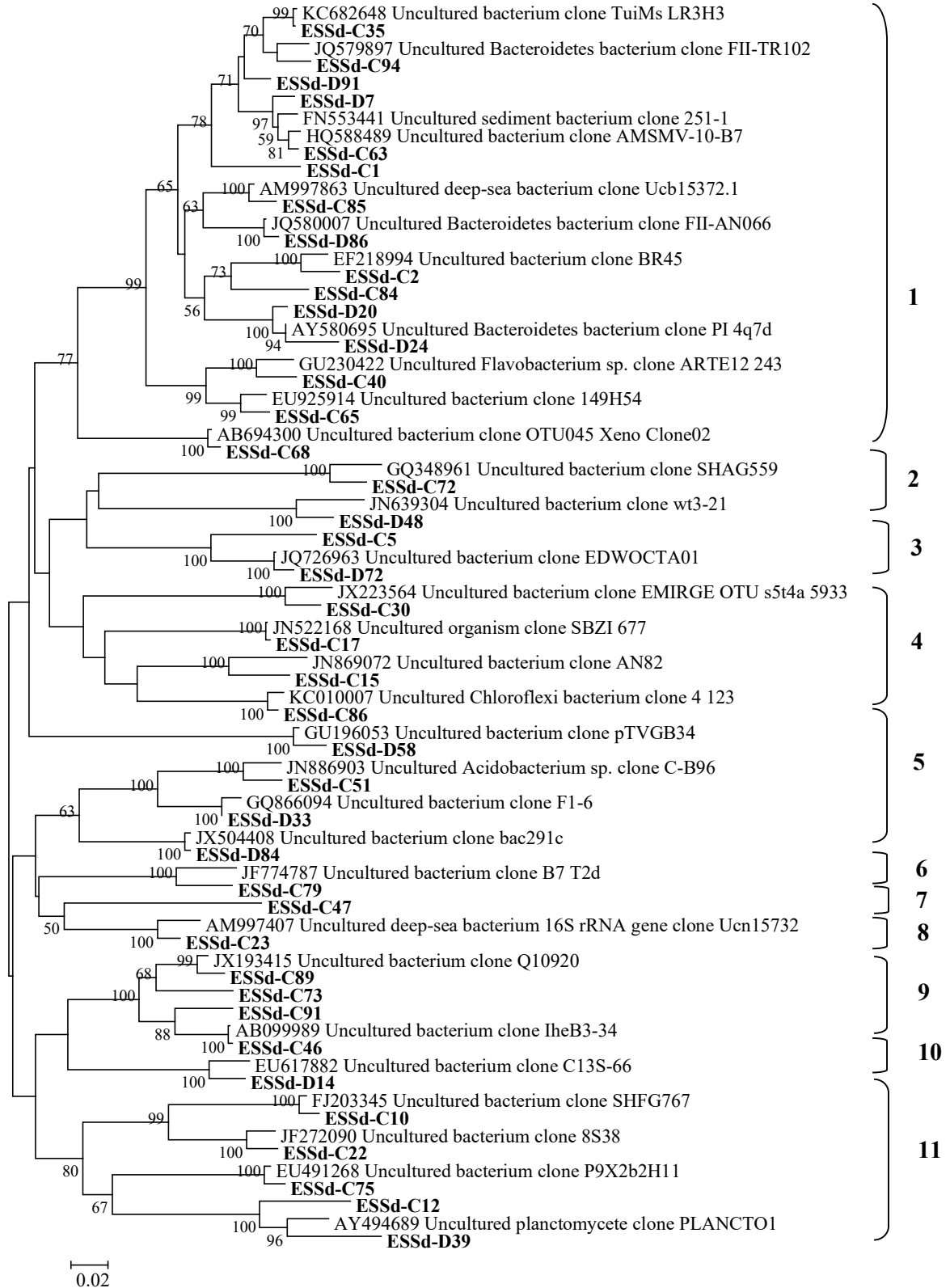


Figure 5.

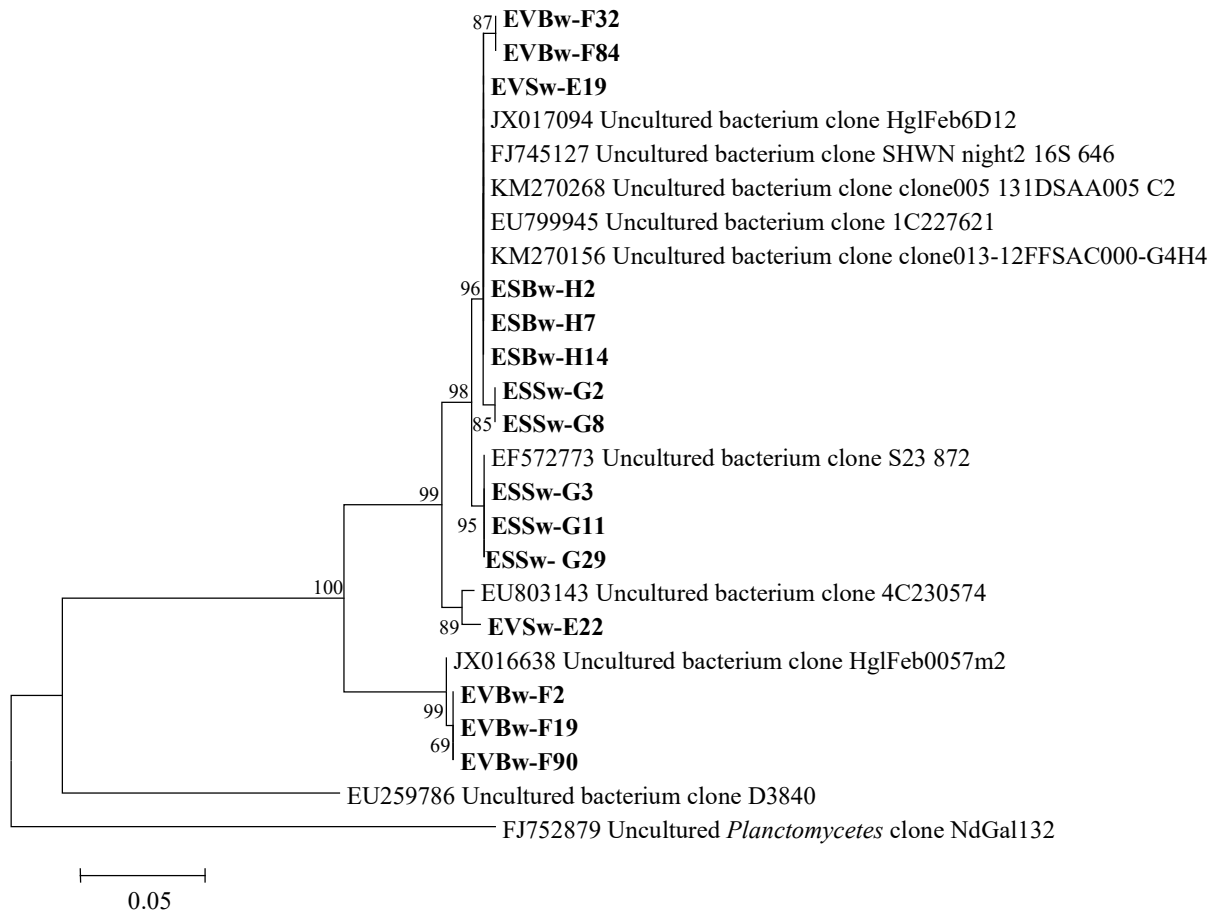


Figure 6.

