Culturable bacterial phylogeny from a shallow water hydrothermal vent of Espalamaca (Faial, Azores) reveals a variety of novel taxa

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Phylogenetic diversity of the 16S rRNA gene associated with the domain bacteria was examined at the level of operational taxonomic units (OTUs) using the rarefaction analysis from a newly identified shallow water hydrothermal vent, Espalamaca in the Azorean Island Faial (Horta), North Atlantic Ocean. Diluted sea water nutrient agar amended with metals manganese, lead, iron and element sulphur, could help in retrieving highest OTUs (95) from the vent and 39 OTUs from nonvent. Molecular tools implemented on bacterial census indicated the occupancy of γ-Proteobacteria by 55.78%, α-Proteobacteria by 21.05% and 12.63% of Bacteroidetes in the total population. Occurrence of novel species maximized with α-Proteobacteria (11/20) followed by Bacteroidetes (5/12) signified the necessity of studying these groups to strengthen the biodiversity database. Shannon index (H') and the Chao I richness estimator illustrated a strong bacterial community in the venting area. The current study confers many bacterial genera which were not reported earlier in any of the shallow water vents and adds 33 new taxa to the database.

Keywords: Bacterial phylogeny, metals, novel taxa, rarefaction analysis, shallow vent.

THE hydrothermal vent ecosystem is known for its higher temperature with various gases, elements and metals. Biological productivity at the deep sea hydrothermal vents (> 200 m) is not maintained by photosynthetic products, but by the chemosynthesis of organic matter by vent microbes, using energy from chemical oxidation to produce organic matter from CO_2 and mineral nutrients[1,2]. Geochemically reactive shallow water hydrothermal vents (< 200 m) are exposed to sunlight and their biological production is maintained by photosynthesis as well as chemosynthesis. Shallow hydrothermal vents offer a variety of habitats to metabolically diverse microbes. Though cultivation-based methods alone cannot explore the entire microbial community, they do elaborate their metabolic activities in biogeochemical cycles which can be applied in environmental biotechnology.

In general, hydrothermal vent environment represents highly productive ecosystems; the important primary producers in vent food webs are the bacteria that oxidize sulphur, methane, hydrogen and iron[3,4]. Thus hydrothermal vent researchers have focused on the isolation of specialists like thermophilic and chemosynthetic microbes[5,6]. But the roles of heterotrophic bacteria which are adapted to metal-rich environments have rarely been addressed[7,8]. Further, various elements present in the shallow hydrothermal vents are oxidized and utilized by heterotrophic bacterial groups as electron acceptors[9], although they do not depend only on these for their growth.

Knowledge of hydrothermal vent bacteria may offer significant information because they react quickly to changes in the concentration and availability of metals within their environment. Little is known about how microorganisms from marine hydrothermal environments interact with metals, but their interactions are generally described in one of three ways: the metals are toxic and elicit a response; they are oxidized or reduced to conserve energy in dissimilatory reactions; or they are taken up and utilized in assimilatory reactions[10]. Previous studies demonstrated that heterotrophic bacteria not only function as decomposers, but also channel-dissolved organic and inorganic nutrients into higher trophic levels through the microbial food-web, thus supporting in the cycling of bio-elements[11,12]. Since heterotrophic bacteria are highly abundant in the ocean and play a significant role in the biogeochemical cycle of carbon, nitrogen and sulphur[13,14], it is necessary to study their diversity and adaptations to various elements and metals in the hydrothermal vent ecosystem.

A new shallow hydrothermal vent field was discovered during 2010 at a depth of 30 m, which is located close to the Faial Island, just outside the Espalamaca (38°33′N; 28°39′W). Research on various aspects to understand this shallow water hydrothermal vent is underway. This study focused on getting information on four main issues from the study area. First, is to know the culture-dependent

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bacterial phylogeny; second, to understand the community variation of bacterial diversity between the vent and nonvent; third, to know what made the bacterial species survive in their respective ecosystem and fourth, to estimate the new/novel bacterial species this area has and to add to the existing bacterial data bank.

Materials and methods

Geological setting

The Azores is an archipelago of nine islands situated in the North Atlantic. The islands spread across an extent of 617 km and are aligned along major tectonic lineaments generally trending WNW–ESE. All islands rise from volcanic edifices sitting on a rugged elevation roughly delineated by the 2000 m depth contour and named the Azores Plateau[15]. Faial and Pico are two islands located in the central group of the Portuguese archipelago of the Azores (Northeast Atlantic). Both islands are estimated to have emerged during the Pleistocene (800 and 270 ky BP respectively) and are located east of the Mid-Atlantic Ridge. A 5 km wide shelf unites both islands creating a unique shallow water structure in an archipelago where seafloor elsewhere between islands typically exceeds depths of 1000 m (refs 16 and 17).

A passage which is 6 km wide in its narrowest section currently separates Faial and Pico. Large expanses of this inter-island shelf are shallower than 100 m and a sill straddling between the Espalamaca head land (Faial Island) to Madalena (Pico Island) bears a maximum depth of 63 m (ref. 18).

In Faial Island, the Espalamaca degasification low temperature 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. The area has been surveyed in detail, during summer 2010, with a multibeam echosounder. 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 concentration of methane, no sulphur, temperature as high as 35°C and pH value of 5.7 (Colaço, pers. commun.). This hydrothermal field is also integrated in a larger protected area designated Baixa do Sul (Canal Faial-Pico), recently classified and integrated the Faial Island Natural Park.

Sampling area and description

Surface and bottom water samples were collected from venting and nonventing areas at Espalamaca (Figure 1). Sediment samples were collected from the vent and nonvent areas. In the vent, sediments were collected from the bubbling area where crevice was present (VSD) and from the bubbling area where crevice was absent (VSG). Figure 2 indicates the vent sediment sampling site. Samples were collected by scuba diving during October 2010 under Indo-Portugal bilateral programme and in August 2012, the samples were collected by the Portuguese counterpart and sent to India for analysis. All the samples were transported with ice packs and the analyses were carried out at CSIR-National Institute of Oceanography, Goa, India.

Enumeration and isolation of culturable heterotrophic bacteria

One hundred micro litres of serially diluted water and sediment samples were spread-plated on the nutrient agar (M001, HiMedia) prepared in 50% sea water (SWNA). pH of the medium was maintained at 5.7 for vent bottom water and 8.2 for sediment samples. All the plates were incubated at 30 ± 2°C up to 72 h and final counts of colonies were made. Morphologically different bacterial isolates were quadrant streaked several times to obtain pure cultures.

Enumeration and isolation of metals/element-resistant bacteria

To enumerate the bacteria resistant to manganese and lead, 100 µl of serially diluted sea water and sediment samples were spread-plated on diluted SWNA (quarter strength of nutrient broth in 50% sea water and 1.8% agar) amended with 1 mM $MnCl_2$ and 1 mM $Pb(NO_3)_2$ respectively. Heterotrophic iron bacteria were isolated using 1 mM ferrous iron/0.02% (w/v) yeast extract prepared in 50% sea water (modified slightly from Johnson *et al.*[19]). Heterotrophic thiosulphate bacteria were isolated using the method provided by Pandey *et al.*[20]. Briefly, the samples were spread-plated in media containing

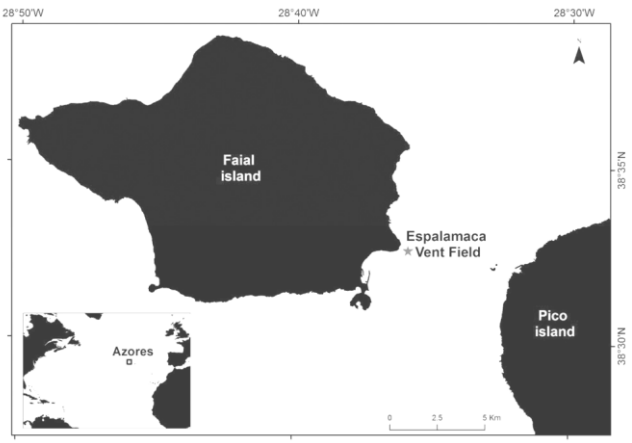

Figure 1. Sampling site of the shallow water hydrothermal vent of Espalamaca in the Azores Islands, Portugal. Asterisk indicates the sampling site located between Faial and Pico Islands.

(l^{-1}) yeast extract 2.0 g, bacteriological agar 18 g, sodium thiosulphate 5 g prepared in 50% sea water. All the plates were incubated in dark condition at 30 ± 2°C up to 72 h and bacterial colonies were counted. Morphologically different bacterial isolates from each medium plate amended with various elements were quadrant streaked several times on the same medium to obtain pure cultures. Pure bacterial cultures thus obtained were stored at 4°C for short-term storage and at –80°C with 30% glycerol for long-term storage. Further, methanotrophic populations were also assessed using NMS media[21].

16S rRNA gene sequencing and phylogenetic analysis

Bacterial cells grown overnight on the respective liquid media were centrifuged at 8000 rpm for 10 min. Genomic DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. For 16S rRNA gene amplification, eubacterial primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) were used[22]. PCR amplification was performed in 50 µl reaction volume containing 5 µl of 10X reaction buffer, 5 µl of 15 mM $MgCl_2$, 4 µl of 2.5 mM dNTP, 2 µl of each primer (10 pmol μl^{-1}), 1 µl of template (25–50 ng), and 0.5 µl of *Taq* DNA polymerase (5 U μl^{-1}) and made up with sterile double-distilled water. PCR profile consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 60 sec, 53°C for 60 sec, 72°C for 90 sec and a final extension of 7 min at 72°C. PCR products (~ 1500 bp) were examined by 1% agarose gel electrophoresis with TAE buffer. The PCR products were gel-purified using a Gel Extraction Kit or purified with PCR cleanup kit (Sigma) according to the manufacturer's instructions.

The purified PCR products were sequenced on automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) with the bacterial primers 27F, 518F and 1492R. The sequences thus obtained were combined to get nucleotide sequences of 16S rRNA gene using DNAbaser software (version 3.5.3). The PINTAIL 1.0 program[23] was used for chimera checking and no differences were detected from our sequences. The acquired nearly complete sequences were subjected to BLASTn on the National Center for Biotechnology Information (NCBI) and EzTaxon 2.1 server[24] to identify sequences with the highest similarity. 16S rDNA sequence similarity levels of ≥ 99% were considered as the same species, whereas phylotypes clustered in a particular genus with < 99% sequence similarity were considered as potential novel species. Multiple and pairwise sequence alignment was performed using Clustal X[25]. Neighbour-joining[26], maximum parsimony[27] and maximum likelihood[28] analysis were performed to reconstruct phylogenetic trees using MEGA 5 (ref. 29). The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1000 replications.

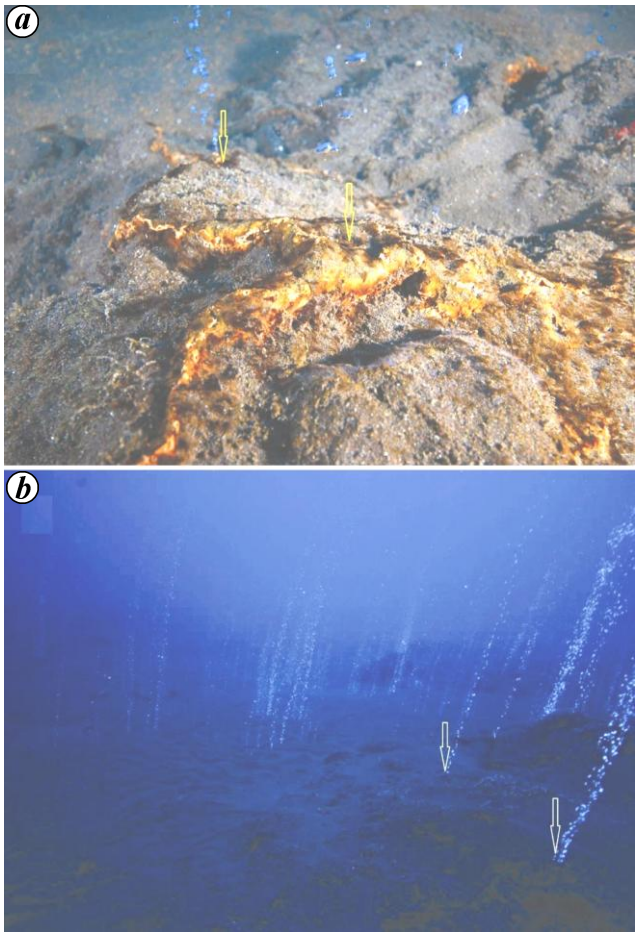

Figure 2. Espalamaca hydrothermal vent site. Yellow arrow indicates the presence of crevice from which bubbles are coming out (***a***) and white arrow indicates bubbles coming out without any crevice (***b***).

Accession number for bacterial 16S rRNA gene sequences

The sequences obtained from this study were submitted to GenBank with accession numbers from KC534142 to KC534459.

Statistical analysis

Rarefaction analysis was performed by plotting the number of phylotypes/OTUs observed against the total number of isolates using EcoSim700 (ref. 30) to estimate the representation of phylotypes. Good's coverage of bacterial isolates was calculated using the formula $C = [1–(n1/N)]*100$, where C is the homologous coverage, $n1$ is the number of OTUs appearing only once and N is the total number of isolates observed. Shannon and Chao I indices were calculated using on-line program (http://fastgroup.sdsu.edu/cal_tools.htm).

Table 1. Abundance of culturable bacteria on various isolation media from shallow water hydrothermal vent and nonvent sites at Espalamaca

	Venting site				Nonvent site		
	Surface water*	Bottom water*	Crevice sediment**	Non-crevice sediment**	Surface water*	Bottom water*	South sediment**
HB	1.22×10^5	7.62×10^4	8.27×10^5	1.54×10^5	2.38×10^4	2.65×10^4	3.70×10^4
MnB	9.00×10^4	1.02×10^4	4.73×10^5	1.15×10^5	5.55×10^4	4.80×10^4	3.06×10^4
PbB	8.25×10^4	8.80×10^3	5.70×10^5	7.43×10^4	2.45×10^4	1.85×10^4	1.00×10^4
TsB	3.65×10^4	2.40×10^4	3.70×10^5	6.00×10^4	1.30×10^4	8.50×10^3	2.20×10^4
FeB	ND	ND	2.71×10^5	5.33×10^5	ND	ND	2.70×10^4
MeB	6.70×10^3	1.22×10^3	1.97×10^5	1.74×10^5	5.10×10^3	2.40×10^3	7.03×10^3
Proportions to HB (%)							
MnB/HB	73.77	13.38	57.19	74.67	233.1	181.1	82.07
PbB/HB	67.62	11.54	68.92	48.24	102.9	69.81	27.02
TsB/HB	29.91	31.49	44.74	38.96	54.62	32.07	59.45
FeB/HB	ND	ND	32.76	346.1	ND	ND	72.97

HB, Heterotrophic bacteria on sea-water nutrient agar; MnB, Manganese-tolerant bacteria on 25% nutrient broth with 1 mM $MnCl_2$; PbB, Lead-tolerant bacteria on 25% nutrient broth with 1 mM $Pb(NO_3)_2$; TsB, Thiosulphate-tolerant bacteria on 25% nutrient broth with 0.5% $Na_2S_2O_3$; MeB, Methanotrophs on NMS media; FeB, Iron-tolerant bacteria on 0.02% yeast extract in sea water with 1 mM $FeSO_4$; ND, Not detectable; *CFU ml^{-1}; **CFU g^{-1}.

Results

Comparison of metal-tolerant bacterial retrieval with heterotrophs

Total heterotrophic bacterial populations (HB) which appeared on SWNA were one order higher in venting sites compared to the nonvent area. While implementing various elements into the diluted nutrient media, the bacterial populations and their ratios were varied for each sample. However, bacterial populations observed in the vent were invariably higher than the nonventing samples (Table 1). The Mn-tolerant bacterial colonies (MnB) retrieved from the nonvent water samples were double the counts of heterotrophic bacteria (5.55×10^4 and 2.38×10^4 CFU ml^{-1}). Pb-resistant bacteria (PbB) from nonvent surface water (2.45×10^4 CFU ml^{-1}) were almost equal to HB (2.38×10^4 CFU ml^{-1}). In the vents, especially in bottom waters, the MnB was lowered by the heterotrophic counts. The retrieval rates of MnB and PbB to HB exhibited a ratio of 13.38% and 11.54% respectively. Whereas the Fe-resistant bacteria (FeB) in the VSG sediment topped the heterotrophic counts. The ratio of FeB to HB in VSG was 346.1%, considered to be the highest bacterial retrievability (5.33×10^5 CFU g^{-1}) in VSG than any other element implemented. In case of thiosulphate, the ratio of thiosulphate bacteria (TsB) to HB was found to be 30–60% in all the samples. Methanotrophs were retrieved one order less compared to other groups, except in vent sediments (10^5 CFU g^{-1}; Table 1).

Addition of metals in the isolation media will attract the metal-resistant bacteria since the hydrothermal vent regions are rich in various metals and elements. At the same time growth of the bacteria should not be hampered by low or high concentrations of metals used in the medium. Hence, we have chosen 1 mM concentration for two reasons. One is based on studies conducted by Fernandes *et al.*[31] on Mn oxidizing bacteria, which revealed that 1 mM concentration could retrieve maximum number of organism; increasing beyond this concentration leads to lesser retrieval rates. Second to salvage potential metal-tolerant bacteria this could be used further for removal of heavy metals. Later, our own studies on the above metals showed that vent organisms could grow at 5 mM of Mn (tested up to 50 mM) and 2 mM of Pb (tested up 10 mM; data not shown).

16S rRNA gene-based diversity

A total of 318 bacterial colonies which appeared on various isolation media were selected for 16S rRNA gene sequencing analysis. Highly diversified bacterial phylotypes spanned nearly 30 families and 6 phyla, Actinobacteria, Bacteroidetes, Firmicutes, α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria. γ-Proteobacteria dominated with 68.7% (152/221) in the vent and 62.8% (61/97) in the nonvent; α-Proteobacteria, 16.7% (37/221) in the vent isolates and 29.9% (29/97) in the nonvent; Firmicutes 3.2% (7/221) in the vent isolates and 4.1% (4/97) in the nonvent; β-Proteobacteria 0.45% (1/221) in the vent and 3.1% (3/97) in the nonvent. Bacteroidetes (10%) and Actinobacteria (0.9%) were only retrieved from vent samples. Details of the phylotypes and novel taxa obtained are given in Figure 3.

Diversity of vent bacteria

A total of 113 phylotypes were obtained from 318 sequences in which 95 phylotypes were from the vent.

γ-Proteobacteria was found to be the dominant phyla with its members like *Alcanivorax*, *Amphritea*, *Halomonas*, *Marinobacter*, *Pseudoalteromonas*, *Vibrio*, etc. and covered 53 OTUs belonging to 16 genera. *Vibrio*, established with 13 different species (Figure 4), was found to be the dominant genus and all the *Vibrio* species were retrieved from the vent sediments. Among the 53 OTUs, 10 were expected to be novel taxa since their identities with the type strain sequences were lower than 99%. Further, the phylogentic relationship executed with neighbouring sequences expressed distinct variations which were clearly noticeable in the phylogenetic tree (Figure 5 *a* and supplementary figures, S1 *a*, S2 *a*, online). This may be confirmed with polyphasic taxonomic approaches. The second major phylum of this study was found to be α-Proteobacteria, covering 20 OTUs belonging to 12 genera. The major contributors were *Erythrobacter*, *Hyphomonas*, *Sulfitobacter* and *Thalassospira* each with 3 species. Further, 55% of the α-Proteobacteria obtained from venting area was found to be novel species and its evolutionary relationships with the closest matches are represented in Figure 5 *b* and supplementary figures S1 *b*, S2 *b* (see online). The phyla Bacteroidetes contributed 12 phylotypes; *Aequorivita*, *Arenibacter* and *Maribacter* contributed two species each and the remaining genera were found to contain only one each. An interesting result of this study was that Bacteroidetes group was only retrieved from the venting site. In addition, around 42% of Bacteroidetes comprised of potential novel species and their phylogenetic relationships with closely related taxa are presented in Figure 5 *c* and supplementary figures S1 *c*, S2 *c* (see online). The 16S rRNA genes from Actinobacteria, Firmicutes and β-Proteobacteria were less prominent sequences observed in the venting area with 7, 1 and 2 OTUs respectively.

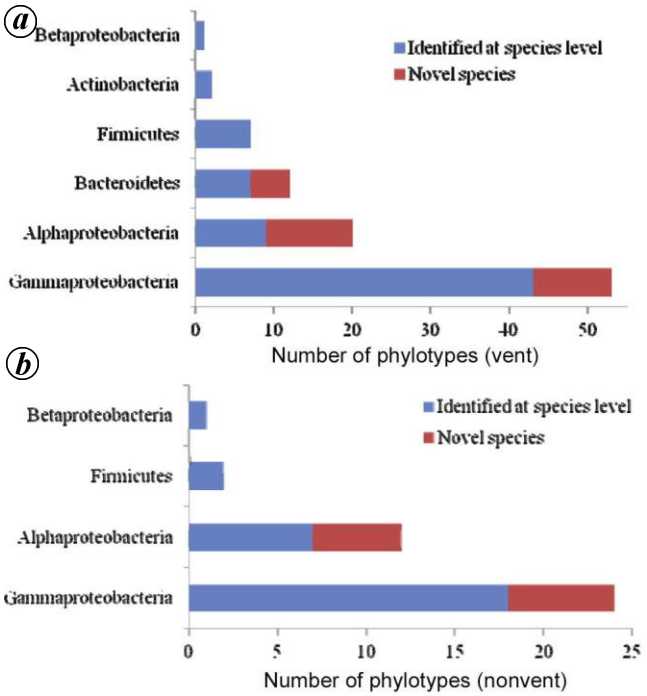

Figure 3. Distribution of bacterial phylotypes (given in numbers) over various phyla from (***a***) vent and (***b***) nonvent samples. The set labelled with 'identified at species level' contains phylotypes with ≥ 99% 16S rRNA sequence similarity. The set 'novel species' represents phylotypes that belong to novel species considering the threshold level of ≤ 99% 16S rRNA gene sequence similarity.

Diversity of nonvent bacteria

Phylogenetic analysis of nonvent bacteria revealed that the 16S rRNA gene clusters differ greatly compared to the nearby shallow vents. A total of 97 bacterial colonies tested from the nonvent area resulted in 39 phylotypes affiliated to four phyla, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and Firmicutes. As in the venting area, γ-Proteobacteria was found to be the dominant phyla in the nonventing area. It contributed 24 phylotypes belonging to ten genera. *Pseudoalteromonas* was found to be the dominant genus containing seven bacterial species, followed by *Marinobacter* with three species (Figure 4). Around 25% of the nonvent γ-Proteobacteria belongs to novel taxa. The second most abundant phylogenetic class in the nonvent bacteria was found to be α-Proteobacteria with 12 phylotypes belonging to 10 genera. As in the venting area *Hyphomonas* dominated (three species) followed by *Erythrobacter* (two species). Nearly 42% of α-Proteobacteria taxa was accounted to be novel, including one potential new genus (Figure 5 *d* and supplementary figures S1 *d*, S2 *d* (see online)). On the other hand, only two clusters appeared in *Firmicutes* affiliated to *Bacillus* and *Brevibacillus*, whereas the phylum β-Proteobacteria contained only one cluster affiliated to *Limnobacter* sp.

Comparative analysis of vent and nonvent bacterial diversity

Overall 221 bacterial colonies from vent samples and 97 bacterial colonies from nonvent samples were selected for the culturable diversity analysis. The number of vent bacterial isolates was nearly double compared to nonvent. This is due to an extra sediment sample from the venting site (details are given in materials and methods section). Another reason is comparatively more distinct colonies appeared in the vent than in the nonvent area. Gene sequence results indicated 113 OTUs (95 OTUs from venting area and 39 OTUs from nonventing area). Though the number of OTUs varied, the rarefaction curve clearly indicated that the venting area was richer with more number of species than the nonvent area. There were 21 phylotypes belonging to 13 genera observed to be common in both the areas, i.e. *Alcanivorax*, *Amphritea*, *Brevibacillus*, *Citreicella*, *Erythrobacter*, *Halomonas*,

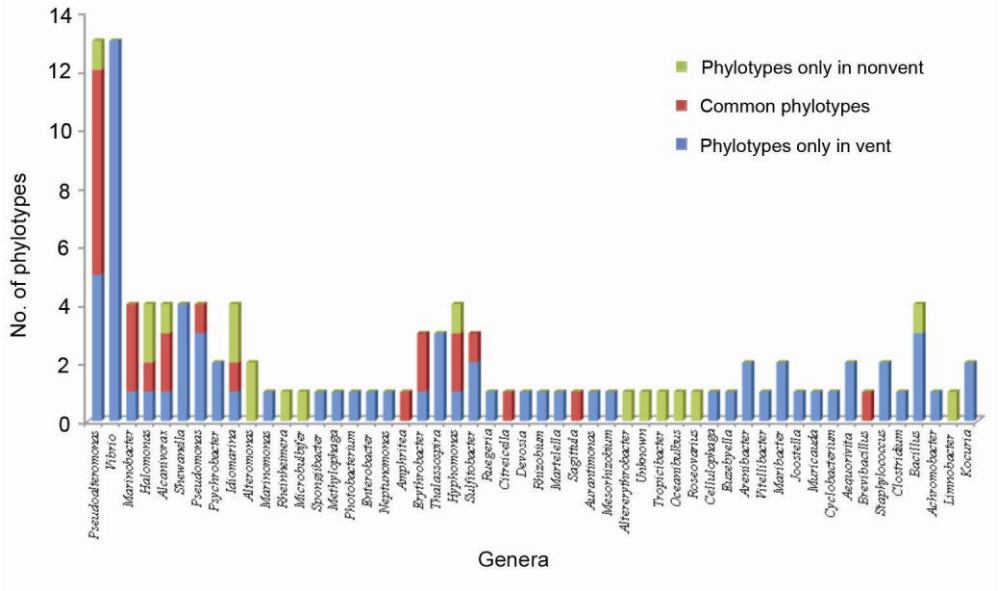

Figure 4. Number of vent, nonvent and common phylotypes retrieved from each genus.

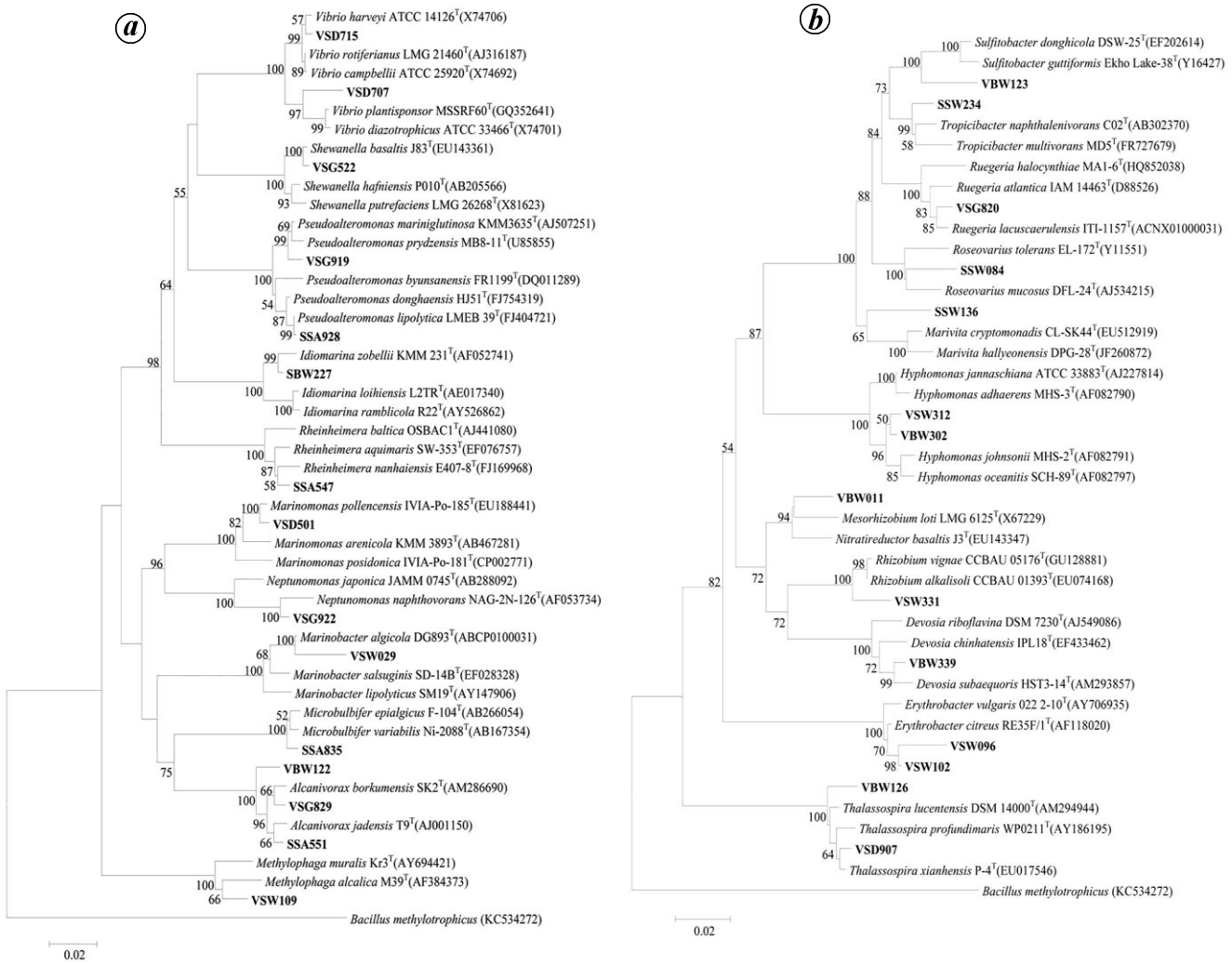

Figure 5. (*Contd*)

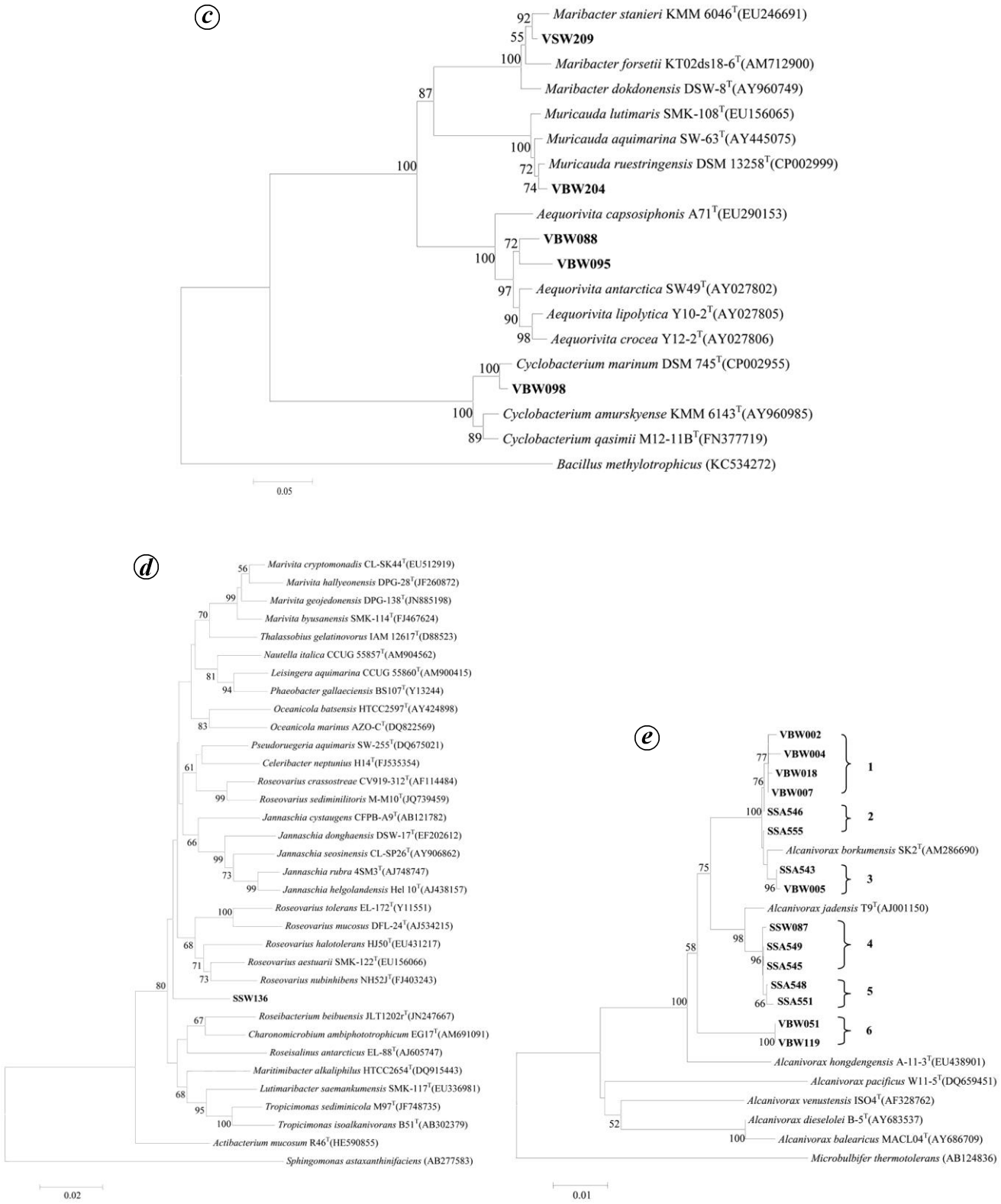

Figure 5. Phylogenetic relationships of representative novel taxa obtained from vent and nonvent regions of Espalamaca based on neighbour-joining analysis of 16S rRNA gene sequences data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap percentages of > 50 are shown on the nodes. The evolutionary distances were computed using the Maximum Composite Likelihood method. *Bacillus methylotrophicus* (KC534272) was used to assign an out-group for (***a***)–(***d***). *Microbulbifer thermotolerans* (AB124836) was used as out-group for (***e***). ***a***–***c***, Phylogenetic relationships of novel species obtained from (***a***) γ-Proteobacteria; (***b***) α-Proteobacteria and (***c***) Bacteroidetes. (***d***) Phylogenetic tree representing SSW136 as novel genus. (***e***) Phylogenetic variations of *Alcanivorax* isolates. Numbers from 1 to 6 indicate six possible groups of novel strains belong to the genus *Alcanivorax*.

Table 2. Distribution of bacteria, number of phylotypes obtained, their diversity indices and coverage of operational taxonomic units from vent and nonvent

	No. of bacterial isolates	No. of phylotypes	Shannon index (H')	Good's coverage (C)	Chao I
Vent	221	95	4.183	74.66	233.61
Nonvent	97	39	3.305	77.31	161.40

Hyphomonas, Marinobacter, Pseudidiomarina, Pseudoalteromonas, *Pseudomonas*, *Sagittula* and *Sulfitobacter*. *Pseudoalteromonas* and *Vibrio* were found to be major genera in this study. All *Vibrio* species exclusively came from the vent samples, whereas *Pseudoalteromonas* was retrieved in both the areas. Even though γ-Proteobacteria followed by α-Proteobacteria were supposed to be the dominant phylogenetic classes in the analysed area, Bacteroidetes also contributed significantly in the venting area with 12 different phylotypes. Actinobacteria was the least prominent phylum observed from the vent samples with two species. In addition Firmicutes and β-Proteobacteria were common in both the areas.

A total of 33 novel taxa were recovered during this study. This number may slightly increase when we perform DNA–DNA hybridization for a few other strains, although their 16S rRNA gene sequence similarity was found to be ≥ 99%. For example, we came across three phylotypes of novel *Alcanivorax*; however, when we constructed a phylogenetic tree for the *Alcanivorax* isolates we were able to get six different clusters (Figure 5 *e* and supplementary figures S1 *e*, S2 *e* (see online)).

Statistical analysis

The Shannon index (H') for venting area was observed to be higher (4.183) than the nonvent ones (3.305). At the same time Chao I richness estimator gave a stronger richness in the venting area (233.61) than in the nonvent area (161.9). Coverage values were almost equal in both the sampling areas (74.66 in vent and 77.31 in nonvent; Table 2). These results strongly indicate that the venting area is diversified with a variety of bacteria, whereas in the nonvent area even though it is located close to the vent, bacterial diversity is comparatively low.

Rarefaction analysis conducted with various phylotypes retrieved from the SWNA and metal-amended media revealed high richness in bacterial species from the vent. Only in thiosulphate media the species richness in nonvent was comparable with vent taxa, whereas all other media yielded a higher number of phylotypes in the vent samples (Figure 6). On the other hand, low retrieval of bacterial isolates was observed in Fe amended medium. In fact, the water samples did not give any bacterial colony when 1 mM Fe was added. Hence we did not study rarefaction analysis for Fe medium.

Discussion

Bacterial association with hydrothermal systems is generally described in one of four ways based on their habitat. First, microbes in the crevices below the surface; second, microbes on the outer surface of sulphide deposits; third, microbes associated with invertebrates and fourth, microbes within the plume of hydrothermal fluid in the overlying sea water. Except the first, the remaining habitat microbes are generally mesophilic aerobic bacteria and some of them can oxidize Mn and Fe for their respiration[3,10]. Hence the exploration of shallow water hydrothermal bacterial communities provides interesting insights into bacterial interactions with hydrothermal vent fluids and adaptations towards various elements.

The main drawback of using basic/normal nutrient media was that it could yield only a moderate level of bacterial diversity. In the present study we have amended Mn, Fe, Pb and thiosulphate in the diluted nutrient media to overcome this limitation. Use of diluted nutrient media may give way to oligophilic bacteria[32]. However, studies like this usually recommended low nutrient and high supply of metals (substrates) so that the bacteria could attack the metals in a better way. On the other hand, we have used the full strength nutrient media without metals that takes care of vast groups of heterotrophic bacteria. The results were obviously interesting because we could retrieve 95 OTUs from vent and 39 OTUs from nonvent area. Innovative culturing methods like preparation of isolation media with the sea water recreates physical and chemical conditions found in the ocean similar to the organically rich media[33]. Different combination of metals in the regular media prepared in sea water helped to get maximum colonies with distinct morphology.

The mean values of heterotrophic bacterial numbers in water and sediment samples of hydrothermal vent off the Island of Vulcano (Eolian Islands, Italy), were 0.22×10^4 CFU ml^{-1} and 14×10^4 CFU g^{-1} respectively[34], whereas in the present study we could retrieve 9.9×10^4 CFU ml^{-1} and 49×10^4 CFU g^{-1} respectively. This indicates that hydrothermal fluids of Espalamaca have a rich and diverse bacterial load. This higher bacterial abundance may be due to the Azorean vent fluids which have much higher trace elements and gas concentrations than the ambient Atlantic sea water to raise high bacterial numbers[35].

The 16S rRNA gene sequence data of the Espalamaca bacterial isolates were compared with the public database

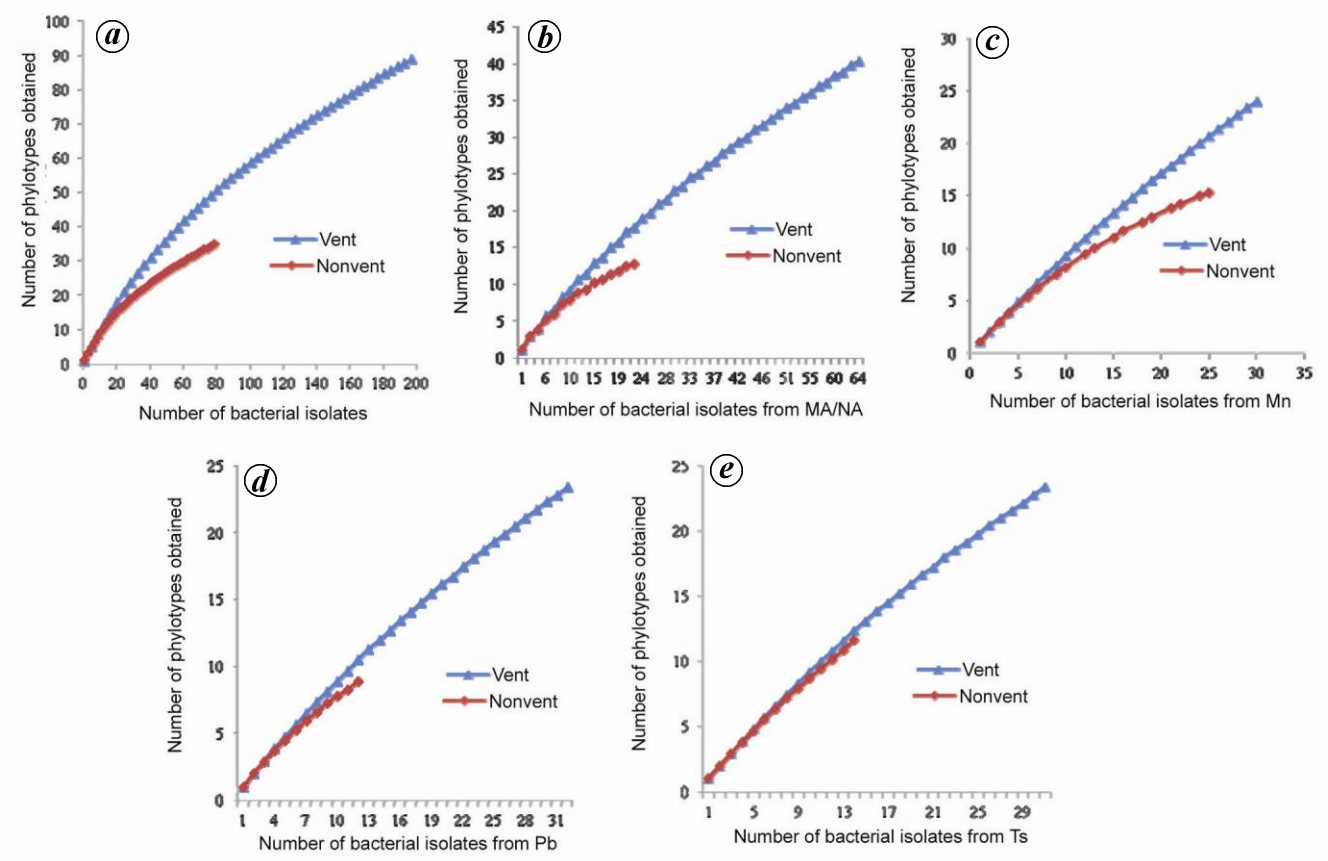

Figure 6. Rarefaction curves of operational taxonomic units (OTUs) based on 16S rRNA gene. Bacterial isolates were grouped into OTUs based at a level of sequence similarity of ≥ 99%. (***a***) Overall OTUs from vent and nonvent samples. ***b***–***e***, OTUs from the isolation media: (***b***) Seawater nutrient agar (SWNA), (***c***) SWNA with 1 mM $MnCl_2$, (***d***) SWNA with 1 mM $Pb(NO_3)_2$ and (***e***) SWNA with 0.5% thiosulphate.

NCBI GenBank and those with a similarity level of ≥ 99% sequence identity were assigned as same species. The standard value of DNA–DNA relatedness for defining a species is less than 70% (ref. 36), which was achievable with ≤ 97% of 16S rRNA gene homology[37]. Although most of our sequence similarity level was ≥ 97%, some reports represented ≥ 97.5% and ≥ 98% of sequence identity to assign similar species[38,39]. However, recent studies authenticated that 16S rRNA sequence similarity level of ≥ 99% can cover DNA–DNA hybridization values of ≥ 70% and less than 99% match was considered as a novel species[40]. Hence, we opted to use cut-off level of 99% for assigning novel taxa. More than half of the vent and nearly 41% of nonvent α-Proteobacteria possessed potential novel taxa respectively. Though γ-Proteobacteria was found to be a major phylum, comparatively it yielded less number of novel taxa, i.e. 19% and 25% from vent and nonvent respectively. Bacteroidetes, the phylum found to thrive only in the venting area (Espalamaca), also held 42% of novel bacteria. Even though the 16S rRNA gene sequence similarity exceeds 99% and the closest relatives are found to be very few, several studies reported various novel taxa by performing DNA–DNA hybridization with the closest type strains[41,42]. From this point of view we have pooled 15 *Alcanivorax* isolates (from this study) to check their phylogenetic relationships with their neighbour species. All of them have close relations with only two type strains *A. borkumensis* and *A. jadensis*. From the phylogenetic tree it is evident that there are six different clusters (Figure 5 *e* and supplementary figures S1 *e*, S2 *e* (see online)), whereas only three phylotypes were observed to contain less than 99% sequence similarity (Figure 5 *a* and supplementary figures S1 *a*, S2 *a* (see online)). This may be confirmed by DNA–DNA relatedness.

We conducted a simple analysis to explain the effect of more constrained and relaxed cut-off points with 98% and 99.5% similarity respectively. This analysis had shown a total of 13 novel taxa when we used constrained cut-off level, whereas the relaxed cut-off level yielded 60 novel taxa overall. Even though various cut-off rates indicate distinct number of novel species from the study site, it is concluded that minimum 13 strains should be novel. However, here we report 33 novel taxa from our study area using a cut-off value of 99%.

The 16S rRNA gene sequence data of this study concluded the abundance of six different phylogenetic classes from the venting area and four from nonvent area.

γ-Proteobacteria was found to be the dominant phylum in both the vent (69%) and nonvent (63%) regions, but it was diversified with distinct species depending on the area. Phylogenetic analysis of DGGE from the shallow hydrothermal vent of Southern Tyrrhenian Sea by López-García *et al.*[43] and Maugeri *et al.*[44] in the Rainbow hydrothermal sediment at MAR libraries also supports the dominance of γ-Proteobacteria in the vents. Podgorsek *et al.*[45] reported the abundance of α- and γ-Proteobacteria by culture-dependent approach from low-temperature hydrothermal fluids of the North Fiji Basin. Studies from the shallow submarine hydrothermal vent in Taketomi Island, Japan were also supportive of γ-Proteobacteria dominancy in the vents[38]. Cho and Giovannoni[46] reported that members of γ-Proteobacteria have been found not only from the hydrothermal vent systems but also in most of the oceans.

Bacteroidetes thrive in a variety of marine ecosystems, including hydrothermal vents[9,47]. Members of this group are prevalent in marine ecosystems where they play a key role in degradation of organic matter[48]. In this study we could obtain 12 phylotypes, 11 of them belonging to Flavobacteria and 1 reported to be Sphingobacteria. This result suggested that the abundance of Bacteroidetes depends on the amount of organic matter generated and its absence in the nonvent area could be due to less generation of organic matter compared to venting area of Espalamaca. This was supported by Kirchman[49], who mentioned that representatives of the class Flavobacteria may play a significant role in the degradation of complex organic matter. Current data from the Espalamaca indicate that 42% of Bacteroidetes belongs to novel taxa and its role needs to be identified. Phylum Bacteroidetes is not only present in the warm water vents, but also exists in the cold ecosystem like Antarctica with large number of novel taxa[40].

Members of Actinobacteria form a relatively small fraction at hydrothermal vents compared to non-thermal environments[50]. However, in this study we came across two OTUs of Actinobacteria from the vents and none from the nonvent regions. Members of ε-Proteobacteria, distinguished as characteristic of deep and shallow hydrothermal systems[50,51], were not observed in this study. Although culture dependent bacterial diversity approach did not show the culturability of ε-Proteobacteria, culture independent molecular analysis revealed the abundance of such groups in hydrothermal vent systems[38]. On the other hand, 77% of bacterial isolates were reported to be Firmicutes in the shallow water hydrothermal vent of DJCS Azores[8]; however, in the present study we could retrieve only 7 OTUs out of 113. This may be due to inconsistency in the concentration of elements and gases present over the study area.

Out of the total 113 OTUs, 21 were observed to be common in vent and nonvent areas. In the remaining 92 OTUs, 74 belong to vent and 18 to nonvent areas. The number of phyla obtained from this study area is high and only one-third of the genera were reported earlier from various shallow water hydrothermal vents, including *Alteromonas*, *Aurantimonas*, *Bacillus*, *Erythrobacter*, *Flexibacter*, *Halomonas*, *Hyphomonas*, *Idiomarina*, *Marinobacter*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Staphylococcus*, *Sulfitobacter*, *Thalassospira* and *Vibrio*[8,9,34,52,53]. Each of the studies explored different kinds of bacterial communities with some common species. But in the present study, most of the bacteria were not reported earlier and are being reported for the first time in shallow water vents; at the same time these groups were reported in various marine environments. Some of the bacteria which were reported in other shallow vents, like *Alcaligenes*, *Brevibacterium*, *Colwellia*, *Halobacillus*, *Halothiobacillus*, *Micrococcus*, *Rhodomicrobium*, *Sulfurivirga*, *Thioalkalivibrio*, *Thiomicrospira*, *Xanthomonas*[8,38,54,55] were not found in the Espalamaca vent. This might be due to the bacterial preferences in accordance with their habitat choice or on the physico-chemical and nutritional requirements.

Members of *Vibrio* and *Pseudoalteromonas* were observed to be predominant in this study. This was coincident with the study of Hirayama *et al.*[38], where aerobic heterotrophs isolated from hydrothermal system of Taketomi Island, Japan were associated with *Pseudoalteromonas* and *Vibrio*. Though a variety of *Vibrio* species were reported from various marine environments, the present study concludes that the abundance of *Vibrio* occurred only in the venting area, especially from the vent sediments, whereas presence of *Pseudoalteromonas* has been recorded in both sea water and sediment samples. The role of heterotrophic sulphur oxidizers belonging to the genera *Acinetobacter*, *Pseudomonas* and *Vibrio* has been described previously in hydrothermal vent[56]. Organisms reported for Mn and Fe oxidization by Sudek *et al.*[52] from Vailulu'u Seamount were comparable to our study because most of their genera were found to be common, e.g. *Alteromonas*, *Aurantimonas*, *Halomonas*, *Hyphomonas*, *Idiomarina*, *Marinobacter*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Thalassospira* and *Vibrio*. However, the vent at Espalamaca could harbour more number of phylotypes than the Vailulu'u Seamount. Since most of the isolates were obtained through the metal-amended media, we could expect better survival over metals/elements. Some of the isolates like *Halomonas* and *Marinobacter* localized with hydrothermal vent, deep-sea and sub-seafloor have been already reported for their involvement in metal cycling[57].

Our previous study on cultured bacterial diversity conducted at DJCS, another shallow water hydrothermal vent located in the Azorean Island, explored 10 different RFLP patterns belonging to *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Halomonas*, *Micrococcus*, *Pseudoalteromonas* and *Staphylococcus*[8]. But in Espalamaca we could obtain only the former four genera and not the latter three. In this study, however, we could retrieve more number of

bacterial isolates to accomplish the maximum culturable diversity compared to DJCS. Bacteroidetes and α-Proteobacteria members were not observed in the DJCS site, but Actinobacteria, Firmicutes, β-Proteobacteria and γ-Proteobacteria members were common between DJCS and Espalamaca. However, DJCS is rich in Gram-positive spore-producers, whereas Espalamaca is rich with Gram-negative non-spore producers. This shows that the bacterial populations and diversity vary from one vent to another; at the same time some similarities also can be seen.

Conclusions

Results obtained from the diversity of culturable bacteria analysed from the Azorean shallow water vent Espalamaca region indicate a unique bacterial phylogeny. In general, the bacterial genera which are normally reported from other shallow water vents were minimized, but these vents were overloaded with many uncommon bacteria. Espalamaca has a slightly higher population than the hydrothermal vent off the Island of Vulcano (Eolian Islands, Italy). Bacteriological data on culture-dependent organisms from this study bring many new phylotypes into the existing bacteriological database. Espalamaca diversified with 55% of the new taxa into α-Proteobacteria and 19% to γ-Proteobacteria. The phylum Bacteroidetes appeared only in the vent samples and was able to bring 42% of novel phylotypes from this ecosystem. Metal-amended media helped isolate maximum number of bacteria for phylogenetic analysis. The rarefaction and Shannon index clearly indicated that the venting area was always richer with more species than the nonvent area. This area seems a highly potent and promising site for many new taxa which may have various interesting applications in bioremediation, especially in metals.

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