

DIVERSITY OF PROKARYOTES AT A SHALLOW SUBMARINE VENT OF PANAREA ISLAND (ITALY) BY HIGH-THROUGHPUT SEQUENCING

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ABSTRACT. To determine microbial community composition and possible key microbial processes in the shallow-sea hydrothermal vent system off Panarea Island (Italy), we examined bacterial and archaeal communities of sediment and fluid samples from a hot vent by 16S rDNA Illumina sequencing technique. Both high abundant (>1% of total sequences), low abundant (from 0.1 to < 1%) and rare (< 0.1%) phylogenetic groups were responsible for the distinct prokaryotic communities characterizing the heated sediment and fluid. The bacterial and archaeal communities from sediment were dominated by sequences affiliated with *Rhodovulum* genus (*Alphaproteobacteria*), including phototrophic ferrous-iron-oxidizing purple bacteria, *Thiohalospira* and *Thiomicrospira* (*Gammaproteobacteria*), typically involved in the sulphur cycle, and *Methanococcus* (*Euryarchaeota*). Fluid communities were dominated by anoxygenic phototrophic members of *Chlorobium*, followed by *Thiomicrospira* (*Gammaproteobacteria*), *Sulfurimonas*, *Arcobacter* and *Sulfurospirillum* (*Epsilonproteobacteria*), and *Methanosarcina* (*Euryarchaeota*). Obtained sequences were affiliated with prokaryotes taking a key part in the carbon, iron and sulphur cycling at the shallow hydrothermal system off Panarea Island. Despite the huge sequencing efforts, a great number of *Bacteria* and *Archaea* still remains unaffiliated at genus level, indicating that Black Point vent represents a hotspot of prokaryotic diversity.

1. Introduction

The diversity of marine microorganisms is much larger than previously known by culture-dependent methods and has been defined as impressive. Conventional molecular, culture-independent methods have greatly increased our understanding of the microbial communities inhabiting the marine environment (Sogin *et al.* 2006).

To analyze the real microbial diversity, powerful sequencing technologies have recently been developed and have been used to study the microbial richness in marine samples (Venter *et al.* 2004). The Illumina sequencing technique, allowing to increase the number of operational taxonomic units (OTUs) of an order of magnitude (millions), has initiated a new era in the study of microbial diversity by offering for the first time the opportunity of revealing simultaneously a large number of individuals and their identity (Bartram *et al.* 2011).

The submarine thermal systems are considered extreme environments because they are characterized by physico-chemical and nutritional conditions prohibitive for most organisms.

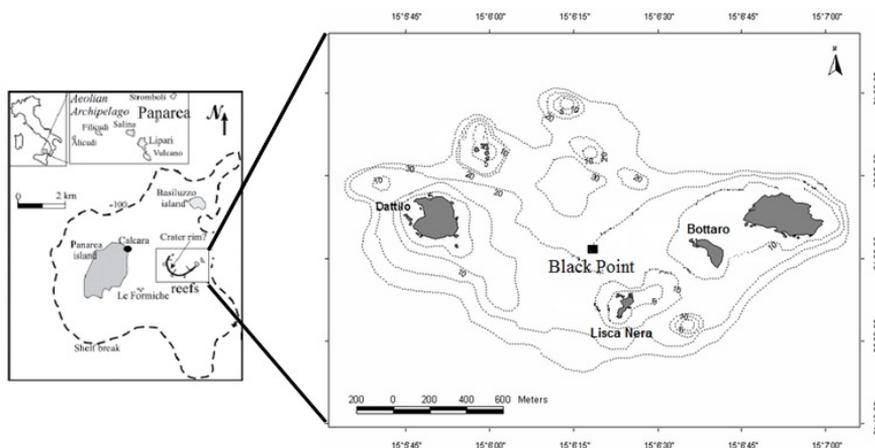


FIGURE 1. Map of Panarea and surrounding islets. Black Point: sampling site.

They represent investigation areas particularly suitable for studying the interactions between the geosphere and the biosphere. Biological communities living in the immediate vicinity of the fluid and gases emissions are greatly affected by them. The direct effect of vents on the biota is to exclude the majority of eukaryotes that are less tolerant to the strong chemical and physical gradients than prokaryotes.

Several studies carried out at the shallow hydrothermal vents of the Eolian Islands (Italy) using conventional molecular techniques, such as Fluorescent In Situ Hybridization, the Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) have led to the conclusion that, like the vents near Vulcano Island, even those off Panarea Island host populations of *Bacteria* and *Archaea* not yet known (Maugeri *et al.* 2010a). Genomic investigations on microbial community of thermal vents of Panarea Island have been carried out only in the last decade (Manini *et al.* 2008; Maugeri *et al.* 2010b, 2013) and indicated that many questions about the microbial structure and composition remained unresolved, needing a more powerful tool.

In this paper the new molecular tool of next-generation sequencing Illumina Solexa was applied to investigate the prokaryotic diversity from sediment and fluid samples collected from a shallow submarine vent of Panarea Island, Eolian Islands (Italy).

2. Materials and methods

Study site and sample collection. The study site was located inside the area delimited by Dattilo, Bottaro, Lisca Nera islets, off the eastern coast of Panarea Island, which is affected by a widespread exhalative and hydrothermal activity at the sea floor (Figure 1). The site, named Black Point (38°38'23"N, 15°06'28"E) due to the presence of black sulphide and manganese incrustations, is a submarine crater lying at a depth of about 23 m.

In August 2009 fluid and surface sediment samples were collected by SCUBA divers in the immediate vicinity of the emissions (< 5 cm) by using sterile bottles and sterile polycarbonate tubes (\varnothing 5.5 cm), respectively.

Physico-chemical analyses were performed by Dr. Italiano and his staff, according to the procedures in use at the Geochemical Laboratory of the *Istituto Nazionale di Geofisica e Vulcanologia* (Palermo, Italy). The main parameters and gas composition of the fluid are reported in Table 1. Samples were immediately frozen and maintained at -20°C until DNA extraction.

TABLE 1. Characteristics of the fluid sample collected from Black Point vent.

Physical and chemical properties			Gas composition (%)						
T $^{\circ}\text{C}$	pH value	Cond (mS/cm)	O $_2$	H $_2$	N $_2$	CO	CH $_4$	CO $_2$	H $_2\text{S}$
74	3.33	66.0	0.04	0.01	0.44	0.01	0.09	97.90	0.40

DNA extraction. The genomic DNA was directly extracted from fluid (1 l) and sediment sample (250 mg) by using the PowerSoil $^{\circledR}$ DNA Isolation Kit (MO-BIO Laboratories, Inc. USA) according to the manufacturer's instructions. Quality and concentration of DNA were checked by UV/Vis spectroscopy (NanoDrop ND-1000, Peqlab, Erlangen, Germany). DNA was used as template for high-throughput sequencing.

V3 region sequencing and trimming. The V3 region of genes coding for 16S rRNA was amplified using the universal primers U341f (5'-CCTACGGGRSGCAGCAG-3') (Hansen *et al.* 1998) and U529r (5'-ACCGCGGCKGCTGGC-3') (DasSarma, Fleischmann, and Rodriguez-Valera 1995). Solexa Illumina platform was used to generate V3 amplicon reads.

Read pairs were merged using the program STICH with default parameters.¹ From the merged reads, primers were clipped off by including a quality control step using the "Pipeline Initial Process" function of the RDP Pyrosequencing Pipeline (Cole *et al.* 2009). Hereby, deviating from the standard parameters a minimum sequence length of 110 bp was chosen. Chimeric sequences were excluded by using USEARCH.²

Analysis of prokaryotic communities. Diversity analyses were conducted using the software program Mothur (Schloss *et al.* 2009).³ The nonparametric Abundance Coverage Estimator (ACE), the Chao1 richness estimator and the Shannon diversity index (H') (Shannon and Weaver 1997) were computed for all recovered sequences from each sample. Diversity estimators were calculated at 97% similarity level.

To generate taxonomic profiles sequences were assigned to taxonomic groups by using the naïve Bayesian classifier v.2.1 (Wang *et al.* 2007) from the Ribosomal Database Project (RDP) with a bootstrap cut-off of 50%.

¹URL: <https://github.com/audy/stich>

²URL: <http://www.drive5.com/usearch/>

³URL: http://www.mothur.org/wiki/Download_mothur

3. Results

Analyses of prokaryotic communities. The Illumina-based analysis produced 3,611,014 sequences and after quality processing, removing chimeras, and a cut-off of 50%, 2,622,889 high quality sequences resulted from sediment and fluid samples (Table 2).

TABLE 2. Amplicon reads obtained by Illumina sequencing, prokaryotic diversity, as demonstrated by Shannon index (H'), richness estimators (Chao1 and ACE) and percentage of taxonomic coverage in sediment and fluid samples collected from Black Point vent (abbreviations: TC, taxonomic coverage; OTU, operational taxonomic unit).

	SAMPLE	
	Sediment	Fluid
Number of reads	1,762,460	1,848,554
Number of high quality reads	1,238,203	1,384,686
Unique reads	295,078	113,981
OTUs at 97% genetic similarity	25,981	19,360
Shannon (H')	9.25	8.81
Chao1 (TC)	48,843 (53.19%)	26,993 (71.72%)
ACE (TC)	63,761 (40.75%)	26,311 (73.58%)
Number of sequences affiliated with <i>Bacteria</i>	1,235,678	1,374,762
Number of sequences affiliated with <i>Archaea</i>	1,169	823

The highest number of *unique* sequences (i.e. present only in one of the two samples), constituting the 23.83% of all high quality sequences, was observed in sediment sample.

The highest value of Shannon index (9.25) was observed in the sediment (Table 2). OTUs, identified at the 97% genetic similarity, and synthetic richness estimators (Chao1 and ACE) were higher in sediment than in fluid.

Taxonomic coverage (TC) was from 40.75 to 73.58%, indicating that the prokaryotic richness was not entirely covered by the sequencing effort.

Phylogenetic analysis of both samples assigned 2,610,440 sequences to *Bacteria* (99.52% of all sequences with high quality) and 1,992 sequences to *Archaea* (0.07% of all sequences with high quality), whereas 10,457 sequences remained unclassified at domain level. The highest number of bacterial sequences was observed in the fluid sample, while number of archaeal sequences was highest in the sediment.

Bacterial phylotypes. Bacterial sequences were identified and grouped at phylum, class (limited to *Proteobacteria*) and genus level. The 10.90% and 3.90% of bacterial sequences remained unclassified at phylum level, in sediment and in fluid samples, respectively.

The number of classified phylogenetic groups were 33 in sediment and 28 in fluid, the relative abundance of each phylum or class is reported in Table 3. Based on the percentage of sequences assigned to *Bacteria*, retrieved phylogenetic groups have been distinguished into three groups: high abundant (HAP) (abundance > 1%), low abundant (LAP) (abundance ranging from 0.1 to < 1%) and rare (RP) (abundance < 0.1%) phylogenetic groups.

Most of bacterial sequences were affiliated with nine groups, five of them (*Alfa-Gamma* and *Epsilon-proteobacteria*, *Actinobacteria* and *Bacteroidetes*) were common to fluid and sediment samples (Table 3, highlighted in bold). *Deltaproteobacteria*, *Acidobacteria*,

TABLE 3. High abundant (> 1% of total classified sequences) (HAP), low abundant (LAP) (abundance ranging from 0.1 to < 1%) and rare (RP) (abundance < 0.1%) bacterial phylogenetic groups from sediment and fluid samples distributed in phyla (including classes of *Proteobacteria*). HAP common to sediment and fluid are represented in bold. The *unique* phyla (present in only one sample) are indicated with an asterisk (*).

	Phylogenetic group in sediment	Relative abundance (%)	Phylogenetic group in fluid	Relative abundance (%)
HAP	<i>Alphaproteobacteria</i>	38.402	<i>Chlorobi</i>	28.198
	Gammaproteobacteria	34.169	<i>Betaproteobacteria</i>	26.110
	<i>Actinobacteria</i>	9.752	Alphaproteobacteria	10.309
	<i>Deltaproteobacteria</i>	4.028	Actinobacteria	8.957
	Bacteroidetes	3.540	<i>Firmicutes</i>	8.025
	<i>Acidobacteria</i>	2.177	Gammaproteobacteria	6.895
	<i>Verrucomicrobia</i>	2.056	Epsilonproteobacteria	5.153
	Epsilonproteobacteria	1.805	Bacteroidetes	3.919
	<i>Cyanobacteria</i>	1.710	<i>Fusobacteria</i>	1.219
	LAP	<i>Deinococcus-Thermus</i>	0.570	<i>Deltaproteobacteria</i>
<i>Planctomycetes</i>		0.410	<i>Acidobacteria</i>	0.336
<i>Firmicutes</i>		0.401	<i>Cyanobacteria</i>	0.169
TM7		0.317		
<i>Deferribacteres</i>		0.116		
RP	<i>Betaproteobacteria</i>	0.090	TM7	0.099
	<i>Chlamydiae</i>	0.084	<i>Deinococcus-Thermus</i>	0.095
	<i>Aquificae</i>	0.076	<i>Aquificae</i>	0.031
	<i>Chlorobi</i>	0.074	<i>Verrucomicrobia</i>	0.028
	OD1	0.070	<i>Spirochaetes</i>	0.020
	<i>Lentisphaerae</i>	0.058	<i>Planctomycetes</i>	0.012
	<i>Chloroflexi</i>	0.031	OD1	0.010
	<i>Fusobacteria</i>	0.022	<i>Chlamydiae</i>	0.009
	WS3*	0.014	<i>Deferribacteres</i>	0.007
	<i>Spirochaetes</i>	0.010	SR1	0.006
	<i>Nitrospira</i>	0.007	<i>Chloroflexi</i>	0.004
	SR1	0.004	<i>Thermodesulfobacteria</i>	0.004
	<i>Thermotogae</i>	0.003	<i>Nitrospira</i>	0.003
	<i>Tenericutes*</i>	0.001	<i>Lentisphaerae</i>	0.002
	BRC1*	< 0.001	<i>Synergistetes*</i>	0.001
	<i>Fibrobacteres*</i>	< 0.001	<i>Thermotogae</i>	0.001
	<i>Gemmatimonadetes*</i>	< 0.001		
	OP11*	< 0.001		
	<i>Thermodesulfobacteria</i>	< 0.001		

Verrucomicrobia and *Cyanobacteria* were the high abundant groups retrieved only in sediment, while *Chlorobi*, *Betaproteobacteria*, *Firmicutes* and *Fusobacteria* were the high abundant groups only in fluid.

Rare phylogenetic groups were more numerous in sediment (19) than in fluid (16). Among RP, six were *unique* in sediment, only one was *unique* in fluid.

Archaeal phylotypes. The 28.22% and 30.86% of archaeal reads remained unclassified at phylum level in sediment and in fluid samples, respectively.

Euryarchaeota was the dominant group in both samples, contributing for more than 90% of classified archaeal sequences. *Crenarchaeota* were more abundant in fluid than in sediment. Sequences assigned to *Korarchaeota* reached 0.18% only in fluid sample.

Prokaryotic genera from sediment and fluid. A total of 763 bacterial genera were recorded, 389 in sediment, 122 in fluid, and 252 genera common to both samples.

The bacterial community from sediment was dominated by sequences affiliated with *Rhodovulum* genus (*Alphaproteobacteria*), including phototrophic ferrous-iron-oxidizing purple bacteria, and with *Thiohalospira* and *Thiomicrospira* genera (*Gammaproteobacteria*), typically involved in the sulphur cycle. Other abundant genera contained several members of *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia* and *Bacteroidetes*.

The fluid bacterial community was dominated by sequences affiliated with anoxygenic phototrophic members of *Chlorobium*, together with members of *Proteobacteria* drawn in the sulphur cycle, such as *Thiomicrospira* (*Gammaproteobacteria*), *Sulfurimonas*, *Arcobacter* and *Sulfurospirillum* (*Epsilonproteobacteria*). Other bacterial genera abundant in fluid were affiliated with *Betaproteobacteria*, *Actinobacteria*, and *Firmicutes*.

The number of archaeal genera was higher in fluid (11) than in sediment (6). The euryarchaeotal genera *Methanosarcina*, *Halomicrobium*, *Halobiforma*, *Halobacterium* and *Natronomonas* dominated the archaeal community in fluid, whereas the genus *Methanococcus* in that of heated sediment. Among *Crenarchaeota*, *Staphylothermus* and *Thermocladium* genera were retrieved in fluid and *Thermodiscus* in sediment.

4. Discussion

The hydrothermal system off Panarea Island received a great scientific interest since November 2002 when a sudden and massive increase in gas emission has been observed near the islet of Bottaro off the eastern coast of Panarea (Gugliandolo, Italiano, and Maugeri 2006; Maugeri *et al.* 2010b). After the crisis, the investigations have been intensified mainly at Black Point site, which maintained typical characteristics of hydrothermal fluids (Hamel 2010). Shallow hydrothermal vents of Black Point site are characterized by emission of gases, consisting mainly of CO₂ and H₂S. The fluid of the examined vent was characterized by low pH and high temperature.

To gain a better understanding of diversity of *Bacteria* and *Archaea* associated with fluid and heated sediment at Black Point site, the Illumina (Solexa) sequencing technique was used. This tool enabled us to detect and enumerate also microorganisms occurring at very low relative abundance (lower than 0.1%).

As resolved by the number of high quality sequences, 99.52% of them was assigned to *Bacteria* and 0.07% to *Archaea*, indicating that *Archaea* represent a minor component of prokaryotic community at the hydrothermal vent system of Black Point. These findings confirmed previous data from the same area, as well as from different shallow hydrothermal systems (Hirayama *et al.* 2007; Sievert, Kuever, and Muyzer 2000).

Although the extraordinary number of molecular signatures obtained by sequencing efforts, the synthetic estimators (ACE and Chao1) indicated that the richness was not entirely covered for the prokaryotic community.

The degree of diversity, predicted by the H' index, higher in sediment than in fluid, was confirmed by further taxonomic analysis in the recovering of 33 bacterial phylogenetic lineages (phyla and classes) in sediment and of 28 in fluid, grouping a huge number (763) of genera.

All reads referred to *Proteobacteria* dominated both in sediment and in fluid samples reaching a total percentage of 70.44 and of 47.11% of bacterial sequences, respectively. The proteobacterial classes were differently represented in fluid and sediment samples, since

Alphaproteobacteria, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* dominated the sediment community, whereas *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria* were more abundant in fluid.

The co-presence of chemosynthetic and photosynthetic microorganisms represents a distinctive trait of this site, as well as of other thermal shallow systems in different geographical locations (Tarasov *et al.* 2005).

Differently from non-heated marine environments, *Epsilonproteobacteria* are typical members of hydrothermal sites, both at deep- and shallow-sea vent locations, where they are numerically abundant bacteria and play a key role in catalyzing the elemental sulfur reduction and oxidation. The most abundant sequences within *Epsilonproteobacteria* were here related to sulfur-oxidizing *Sulfurimonas*, *Arcobacter* and *Sulfurospirillum*.

Dominant *Gammaproteobacteria* were related to the sulphide-oxidizing *Thiohalospira* and *Thiomicrospira* genera. *Thiomicrospira* is one of the most abundant culturable, sulfur-oxidizers at shallow vents.

Phototrophic *Bacteria* included members of *Alphaproteobacteria*, *Chlorobi* and *Cyanobacteria*.

The fluid bacterial community was dominated by sequences affiliated with anoxygenic phototrophic members of *Chlorobium*, whereas the genus *Rhodovulum* (*Alphaproteobacteria*), including phototrophic ferrous-iron-oxidizing purple bacteria, was predominant in sediment.

In addition to dominant anoxygenic phototrophs, bacterial methanotrophs (*Verrucomicrobia*) and archaeal methanogens (*Methanosarcina* and *Methanococcus*) were abundant, playing a relevant role in the carbon cycle.

In this study, for the first time, a great variety of bacterial and archaeal genera, occurring not only as dominant but also at very low abundance at shallow hydrothermal vents of Panarea, was resolved by using the new high-throughput sequencing Illumina technique.

Panarea vent system represents an unusual ecosystem, where coastal prokaryotic communities cohabit with those living at extreme locations. Despite the huge sequencing efforts, a great number of *Bacteria* and *Archaea* still remains unaffiliated at genus level, indicating that Black Point vent represents a hotspot of prokaryotic diversity until now unknown.

Acknowledgments

We would like to thank Dr. Franco Italiano at the *Istituto Nazionale di Geofisica e Vulcanologia* (Palermo, Italy) for help with the sample collection and for supplying the geochemical data. We are indebted to Professor Jörg Overmann at the *Leibniz-Institute DSMZ German Collection of Microorganisms and Cell Cultures* (Braunschweig GmbH, Germany) for valuable scientific and technical support.

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