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ORIGINAL PAPER

The bacterial diversity on steam vents from Paricutín and Sapichu volcanoes

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Abstract

Vapor steam vents are prevailing structures on geothermal sites in which local geochemical conditions allow the development of extremophilic microorganisms. We describe the structure of the prokaryotic community able to grow on the walls and rocks of such microecosystems in two terrestrial Mexican volcanoes: Paricutín (PI and PII samples) and its satellite Sapichu (S sample). The investigated samples showed similar diversity indices, with few dominant OTUs (abundance > 1%): 21, 16 and 23, respectively for PI, PII and S. However, each steam vent showed a particular community profile: PI was dominated by photosynthetic bacteria (*Cyanobacteria* and *Chloroflexia* class), PII by *Actinobacteria* and *Proteobacteria*, and S by *Ktedonobacteria* class, *Acidobacteria* and *Cyanobacteria* phyla. Concerning the predicted metabolic potential, we found a dominance of cellular pathways, especially the ones for energy generation with metabolisms for sulfur respiration, nitrogen fixation, methanogenesis, carbon fixation, photosynthesis, and metals, among others. We suggest a different maturity stage for the three studied fumaroles, from the youngest (PI) to the oldest (S and PII), also influenced by the temperature and other geochemical parameters. Furthermore, four anaerobic strains were isolated, belonging to Clostridia class (*Clostridium sphenoides*, *C. swellfunanium* and *Anaerocolumna cellulosilytica*) and to Bacilli class (*Paenibacillus azoreducens*).

Keywords Extreme environment · Volcanic fumaroles · Anaerobic bacteria · Microbial biodiversity · Predictive metagenomics profiling

Introduction

Fumaroles, also called steam vents, are commonly found at geothermal systems. They occur when steam and volcanic gases escape through the Earth's crust as a result of magma

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degassing and/or geothermal heating of groundwater at a shallow depth (Costello et al. 2009; Benson et al. 2011). These environments usually have high metal contents that, combined with the changing temperatures (60–95 °C) of the steam and gases, allow the formation of mineral crusts along the cooler walls, as well as the development of microorganisms on these mineral precipitates. Due to the extreme conditions of these environments, they are of special interest for astrobiology (Hedlund et al. 2014), microbial ecology (Benson et al. 2011; Cuecas et al. 2014), physiology (Barton 2005) and biotechnology (Bhowmick et al. 2009). The microbial diversity of these geothermal sites is, in general, strongly influenced by local geochemical conditions (Crossey et al. 2016).

The microbial communities of hot springs have been extensively studied in sites such as the Yellowstone National Park, in USA (e.g., Inskeep et al. 2013). However, most of these studies have focused on thermally heated soils surrounding vents (Costello et al. 2009; Cuecas et al. 2014; Stott et al. 2008; Tomova et al. 2010; Huang et al. 2011), with few studies exploring the microbial diversity associated with fumaroles themselves (Mayhew et al. 2007; Benson et al. 2011; Wall et al. 2015). Besides, works targeting the microbial communities inhabiting on the early stages of volcanic structures, which are the pioneer organisms on these environments, are even less common (Kelly et al. 2014). For instance, Kelly et al. (2011, 2014) studied the diversity of these colonists on crystalline volcanic rocks collected on Torfajökull Volcano and Fimmvörðuháls Lava Flow, both in Iceland. Clone libraries for these samples showed a predominance of *Proteobacteria* phylum (60–97%) in younger rocks (3–5 months), and of *Acidobacteria* (19–42%), *Actinobacteria* (6–16%), *Bacteroidetes* (6–11%), *Verrucomicrobia* (9–20%), *Firmicutes* (7%), and *Proteobacteria* (9–27%) phyla in the oldest ones (approx. 1750 years). Similar results were observed by Cockell et al. (2009), on basaltic glass and crystalline basalt collected near Hekla Volcano, also in Iceland. In a broader study, Wall et al. (2015) observed that, on deposits and steam waters of fumaroles collected from Hawaii's Volcanoes National Park, the dominant phyla were *Cyanobacteria* (37%), *Chloroflexi* (14%), *Proteobacteria* (10%), *Bacteroidetes* (4%), *Firmicutes* (4%), and *Deinococcus-Thermus* (3%), with a 25% of unclassified bacteria. These results show that, although a certain number of groups of bacteria are characteristic of these environments, their population profiles are very different, probably dependent on physical characteristics of the fumarole (pH, mineralogy, porosity, etc.) and its aging.

The central region of Mexico is crossed by a large number of geothermal sites and volcanoes (maars, cinder and lava cones) forming the Trans-Mexican Volcanic Belt (TMVB). Around 1000 Late Quaternary eruptive events of the TMVB were registered in the northern part of Michoacán state and in the southern part of Guanajuato state, forming the Michoacán–Guanajuato Volcanic Field (MGVF). The MGVF has an extension of around 40,000 km^2 and is characterized by over 900 cinder cones and scattered shield volcanoes, stratovolcanoes, lava domes, and maars (Hasenaka and Carmichael 1985). The Paricutín is the most recently active monogenetic cinder cone in the MGVF. Its activity period (1943–1952) has been well documented (Gardine et al. 2011) as well as its mineralogical composition (Cebriá et al. 2011).

These environments are almost unexplored from the microbial ecology perspective. For instance, we studied the bacterial diversity of the Los Azufres geothermal site, also located on the MGVF (Brito et al. 2014), and, recently, we published a preliminary study describing the bacterial diversity of six soils located in the Paricutín volcano (Medrano-Santillana et al. 2017). Both studies were performed using "classical" molecular approaches: terminal restriction fragment length polymorphism (TRFLP) combined with clone libraries.

In the present study, we focus on the very thin biomats over mineral crusts and make a description of the microbial diversity in three different samples. By applying next generation sequencing (NGS) approaches, we improve the sensitivity and the accuracy for the detection of microbial taxa, especially for the low-abundant species (Oberauner et al. 2013; Hong et al. 2015). This is the first study considering the application of high-throughput sequencing (HTS) methods for studying the bacterial diversity of Mexican steam vents. We also describe here anaerobic bacteria strains isolated from the same habitat. With this analysis, we intend to check if we can identify a typical bacterial community profile for terrestrial volcanic fumaroles, by comparing our results with the few previous ones for other similar sites, as well as contribute to the knowledge about the bacterial colonization process on these ephemeral environments.

Materials and methods

Sampling site and sample collection

The Paricutín volcano (Fig. 1) is located in Nuevo San Juan Parangaricutiro village, a *purépecha* village in the state of Michoacán (Mexico). It has a satellite, called Sapichu (first panel in Fig. 2a). They are surrounded by a wide area of solidified lava and ash, at nearly 2808 m.a.s.l. On April 2014, two fumaroles in Paricutín's cone and one in Sapichu's cone, showing steam deposits, were sampled. The inner walls of the vents were covered by colored evaporites, probably composed of iron oxides (Fig. 2b–d). Samples were collected from hot stones located inside the vents (10–20 cm deep) with a sterile metal spoon to scrape the superficial

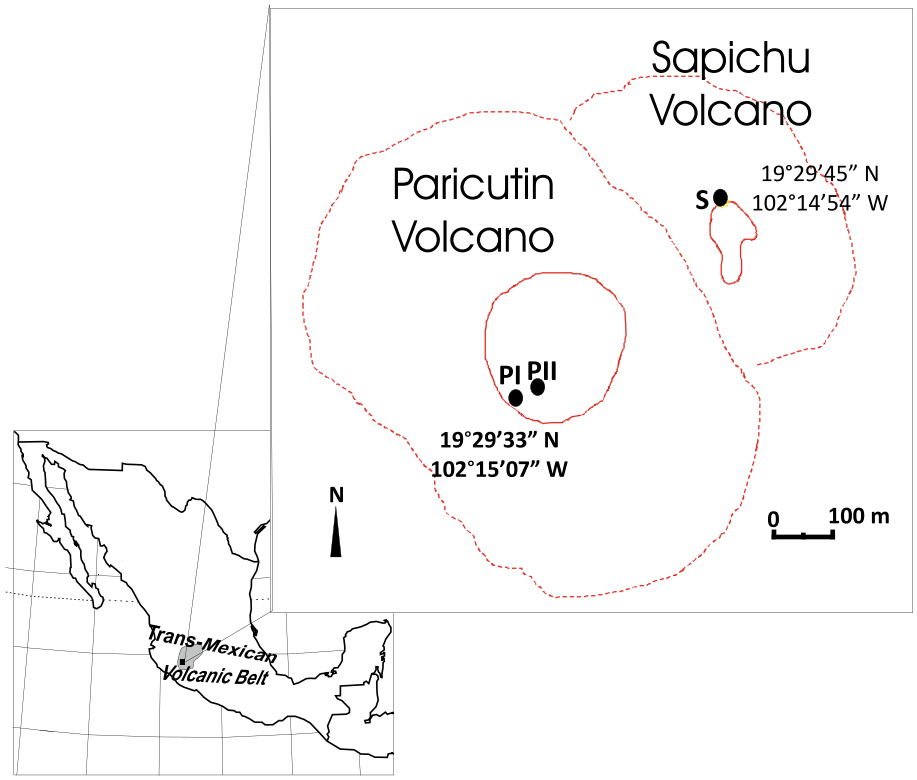

Fig. 1 Mexican map highlighting the Trans-Mexican volcanic belt and the sampling site. The sampling points (PI, PII and S) are marked by the black solid circles

layer of the rocks (less than 2 mm thick; Fig. 2e, f). All the samples showed the development of very thin embedded biomats.

The Sapichu sample was designed S while, for Paricutín Volcano, the PI sample was taken from a vent at the top of crater border and the PII sample from a vent located inside the crater, about 3 m far from the last.

The samples for microbial isolation were incubated, about 6 h after collection, under an improvised sterile system (using a Bunsen burner). These and the samples for DNA extraction were transported from the point of collection to the laboratory inside a styrofoam box (about 1 day trip). Before DNA extraction, the samples were frozen (− 20° C).

Microbial diversity

Total DNA was extracted with PowerSoil® DNA Isolation Kit (MoBio Laboratories) according to the manufacturer recommended procedure. Sequencing was performed at Langebio-CINVESTAV Genomic Services Laboratory with MiSeq® platform. The V3–V4 region was amplified using the primers 357F 5′-barcode-CTC CTA CGG GAG GCA GCA G-3′ (Turner et al. 1999) and CD(R) 5′-barcode-CTT GTG CGG GCC CCC GTC AAT TC-3′ (Rudi et al. 1997).

A total of 1,544,861 raw sequences were obtained: 610,343 for PI, 455,747 for PII and 478,771 for S. The quality of the reads was evaluated with fastqc v.0.11.5 software (Bioinformatics 2016), and only R1 paired-end fragment was approved for quality and used in the present analysis. The reads were processed using USEARCH 11.0 package (Edgar 2010). They were first filtered for eliminating small sequences and for trimming (at 275 bp) all the remaining (93.1%) reads to guarantee an acceptable quality level. Then the pool of reads was submitted to a de-replication algorithm, resulting in 544, 898 unique sequences (92.3% being singletons). Reads with less than 5 occurrences were removed. The remaining sequences (12,151) were clustered by similarity (in a 97% identity basis, to take into account errors due to PCR and sequencing, paralogs, etc.) and filtered for chimeras, resulting in 1,139 Operational Taxonomic Units (OTUs). The obtained OTUs were then associated with the complete pool of sequences and checked for a preliminary taxonomy. The RDP 16S rRNA (training set v16) was used as reference database. The raw sequences of the present project were deposited in the Sequence Read Archive database of NCBI and assigned accession numbers SAMN09522127 (PI), SAMN09522128 (PII) and SAMN09522129 (S).

Predictive analysis of microbial community functional gene structure

We applied Tax4Fun2 tools (Wemheuer 2018) for predicting the functional genes structure and metabolic pathways of the microbial community 16S rRNA sequences (OTUs) found in the samples from Paricutín and Sapichu. This

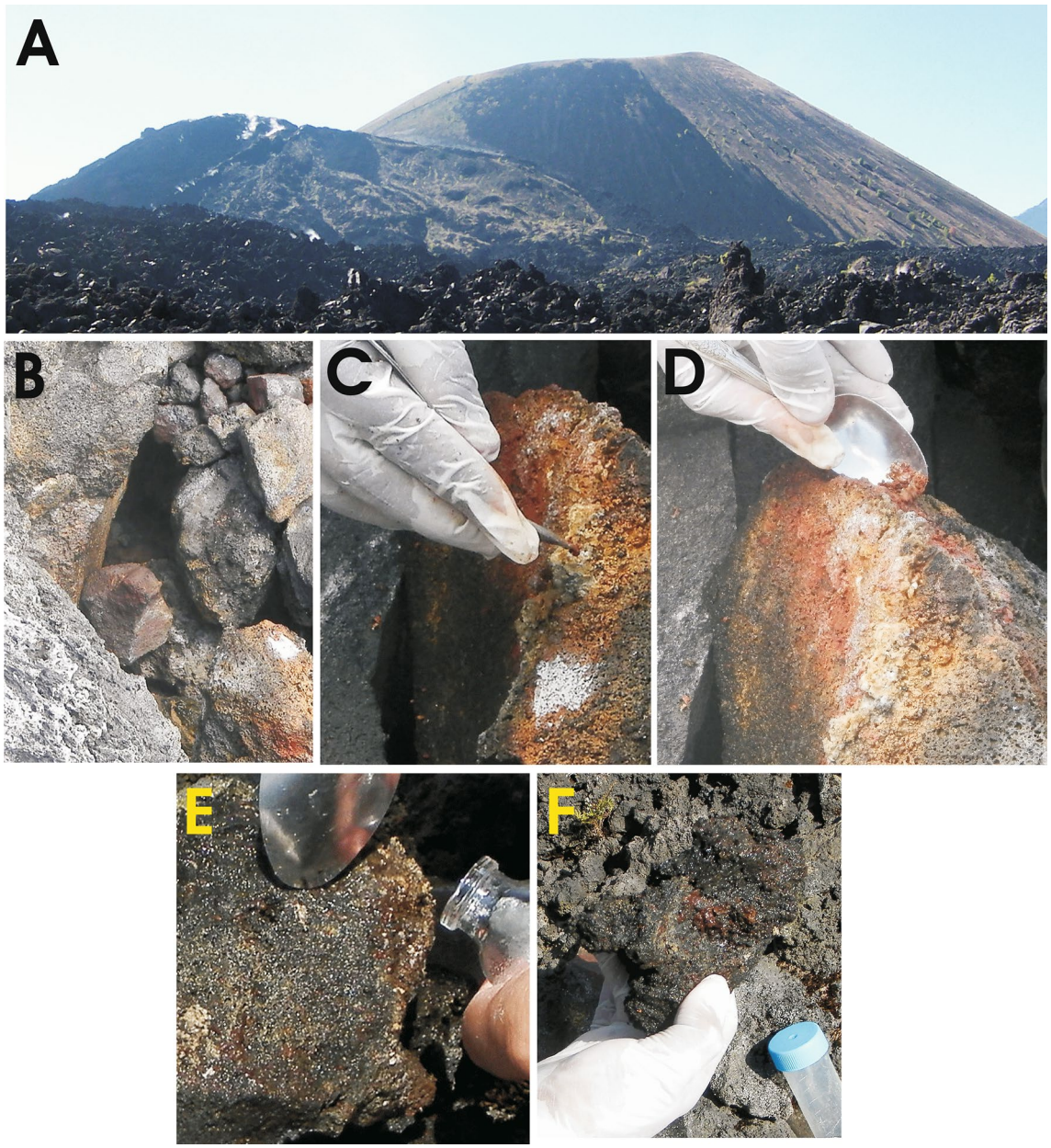

Fig. 2 **a** Sapichu (foreground volcano) and Paricutín (background volcano), with solidified lava in the first plane; **b–d** details of sample points and collection procedure; and **e**, **f** view of thin surface biomats

software uses NCBI BLAST+ (U.S. National Library of Medicine) for OTUs labelling. A pre-computed association matrix based on KEGG (Kyoto Encyclopedia of Genes and Genomes) organism functional profiles was used for association, followed by the abundance calculation. The identified KEGG Ortholog functional genes (KO's) were then automatically associated with empirical metabolic pathways. Besides, KO's lists were also screened by hand for important enzymes for biogeochemical cycles, especially the ones associated to metals and sulfate reduction metabolisms. The metabolic potential was predicted (in silico) by determining the abundance of specific genes.

Enrichments and isolation procedures

The incubations were conducted in 60-mL glass bottles sealed with gastight blue butyl rubber septa, containing 5 g of samples and 40 mL of mineral medium (MM, Fude et al. 1994). The composition of this MM was (per liter): 0.18 g $MgSO_4.7H_2O$; 0.045 g $C_3H_5O_7Fe_3$; 0.022 g K_2HPO_4

and 0.9 g tryptone; pH was adjusted to 6.5–7.0 with HCl. After autoclaving (121 °C during 20 min), MM was cooled under N_2 gas stream. Sterile anoxic solutions of (final concentrations) $FeSO_4$ [10 mM], $C_3H_5O_3Na$ [5 mM], Na_2CO_3 [30 mM], Na_2SO_3 [10 mM], 1 mL L^{-1} of Se-W (Widdel and Bak 1992), trace elements and vitamins were added to the MM (Overmann et al. 1992). The trace elements solution contained (per liter): 2.5 g EDTA, 10 mg $ZnSO_4.7H_2O$, 1.5 g $MnSO_4.H_2O$, 5.0 g $FeSO_4.7H_2O$, 390 mg $CuSO_4.5H_2O$, 240 mg $CoNO_3.6H_2O$, and 177 mg $Na_2B_4O_7.10H_2O$. The vitamin solution contained (per liter): 0.2 mg biotin, 10 mg acid nicotinic, 50 mg thiamine, 50 mg riboflavin, and 50 mg inositol. The Se-W solution contained (per liter): 4.4 g NaOH, 6 mg $Na_2SeO_3.5H_2O$, and 4 mg Na_2WO_4.

A pre-inoculation and two subsequent enrichments were done, in time intervals of 30 days, at room temperature and taking 10% of inoculum into fresh medium each time. After that, isolation was performed in the same medium supplemented with agar (1.6%) and using the roll tube method (Hungate 1969). The inoculum was serially tenfold diluted in roll tubes and single colonies were picked, under N_2 atmosphere. The purity of strains was checked by microscopy. Cells with similar morphology were obtained after two or three roll tube dilution series.

Identification of isolates

Identification of isolates was performed by sequencing the 16S rRNA gene. The procedure of Cortés-Palomec et al. 2008 was adapted for extraction of total DNA from isolates. Briefly, the pellets were washed with 1 mL TGE solution (25 mM Tris–HCl at pH 8; 50 mM Glucose; 10 mM EDTA) and lysed by freezing (20 min at − 20 °C); re-suspended on 160 μL CTAB buffer (Tris–HCl, 0.1 M, pH 8; cetyltrimethyl ammonium bromide, 50 mM; NaCl, 1.4 M; EDTA 20 mM, pH 8; PVP-40 [polyvinylpyrrolidone], 1 mM; ascorbic acid, 28 mM; diethyldithio-carbamic acid sodium salt, 22 mM; with the addition of β-mercaptoethanol, 100 µL/10 mL, just before its use) and 3.2 µL of β-mercaptoethanol and briefly vortexed. After, the suspension received 20 µL of *La Porta* solution (Tris–HCl, 0.1 M, pH 8; EDTA, 50 mM, pH 8; NaCl 50 mM; sterilized and with the addition 100 µL/10 mL β-mercaptoethanol just before its use), and 100 µL of sodium dodecyl sulfate (SDS, 20% p/v), vortexed after each addition, and incubated at 65 °C for 30 min. Then, 180 µL of potassium acetate, 5 M, was added, the suspension was vortexed once more and centrifuged (12,000*g*, 30 min). After the cellular lysis, a deproteinization with phenol–chloroform-alcohol isoamilic and DNA precipitation (sodium acetate, 3 M, pH 5.2; 500 µL isopropyl alcohol; 500 µL ethyl alcohol 70%) were performed (Brito et al. 2013).

PCR was performed in a C1000 Touch® thermal cycler (BIO-RAD), for 16S rDNA amplification, using primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′; Lane 1991) and 1513R (5′-GGT TAC CTT GTT ACG ACT T-3′; Satchanska and Selenska-Pobell 2005). The PCR mix (50 µL) was prepared with: PCR buffer 1X; 2 µM $MgCl_2$; 0.5 µM dNTP (dATP, dCTP, dGTP, and dTTP); 0.5 µM of each primer; 0.25 U/µL of Taq polymerase (Fermentas) and 0.1–1 µL of DNA. The reactions were cycled with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 52 °C for 45 s, extension at 72 °C for 1 min, and then a final extension step at 72 °C for 10 min. The PCR products were purified using Ilustra GFX PCR DNA purification kit (Amersham), following manufacturer's instructions. The PCR products were Sanger sequenced by Langebio-CINVESTAV Genomic Services Laboratory. The sequences were compared to those in the database of the National Center for Biotechnology Information (NCBI) website using the BLAST method. The 16S rRNA sequences of isolates and the closest references were aligned with the MAFFT program [Multiple Alignment using Fast Fourier Transforming; Katoh and Standley 2013]. The phylogenetic tree was constructed with the MEGA 6 (Tamura et al. 2013) software using the maximum likelihood method. The confidence of the phylogenetic tree was assessed by bootstrap using 1000 re-samplings. The sequences determined in this study have been submitted to GenBank database and assigned accession numbers MH553047—MH553050.

Results and discussion

Despite the ubiquity of geothermal systems around the world, there are only few studies exploring the bacterial diversity on steams of terrestrial volcanoes. Most of these studies have been done by applying TRFLP or DGGE techniques, frequently accompanied by clone libraries analyses (Mayhew et al. 2007; Costello et al. 2009; Benson et al. 2011; Wall et al. 2015). In the present work, we applied, for the first time to the Paricutín–Sapichu volcano system, a HTS approach to describe in detail the bacterial community profile and the predicted metabolic potential of three steam vents. Unlike a preliminary study (Medrano-Santillana et al. 2017) for this site, here we sampled the walls of the steam vents instead of the soil around them.

A chemical and physicochemical characterization of some Paricutín's fumaroles was presented in Medrano-Santillana et al. (2017). The measured temperatures ranged from 62 to 80 °C, while the pH was found to be from slightly acid to neutral (5.3–7.9). Samples showed high contents of Fe (20–34, 546 mg kg^{-1}) and S (94–256 mg kg^{-1}). For other metals and metalloids, the concentrations were typical of volcanic environments (Delfosse et al. 2003; Amaral et al. 2006; Tomova et al. 2010), the highest observed

values being the ones for As (0.03–4.40 mg kg^{-1}) and Pb (7.3–51.5 mg kg^{-1}). The steams characterized in the study by Medrano-Santillana et al. (2017), collected in 2010, are not the same as the ones in the present work (sampled in 2014).

Due to the hard access conditions of the site, we could not measure the composition of the mineral crust on the walls of fumaroles nor the steam-gas mixture. According to Krauskopf (1948), at the eruptive period of Paricutín, the emission of sulfur gases was prominent.

It is worth to note that, even with the great potential of HTS methods for detecting rare bacterial communities, such approaches still present the traditional biases with respect to DNA extraction and PCR amplification. Those are challenging in samples such as volcanic mud, volcanic glass and steam deposits, which are microbial environments that have both very low biomass and abundance, mainly due to high metal richness.

Diversity of microbial communities

A total of 12,151 reads with at least 5 cases in the pool resulted in 1,139 OTUs (about 700 in each sample). Similar diversity indices were found for the three samples, revealing an intermediary evenness (J index between 0.5 and 0.6), which resulted in a moderate estimate for the diversity (according to Shannon–Wiener entropy and Simpson indices). These calculations are presented in Table 1. The diversity we found is consistent with previous measurements for steam vents and volcanic rocks in the literature (e.g., Kelly et al. 2010, 2011) based on clone library data. This diversity, not so low as could be expected for an extreme environment, suggests that we are dealing with a dynamic community. Continually changing conditions may produce intermediate levels of disturbance and benefit a relative increase in diversity (e.g., Connell 1978).

Few OTUs (60) represented above 1% of the total community each (Tables 2, 3, 4). Some of these dominant populations were related to genera or species isolated from geothermal sites, such as those found in hot springs (OTUs 3, 12, 18, 23, 25, 28, 40, 43 and 49) or active fumaroles (OTU 38). Most are known to be either thermophilic (bacteria related to OTUs 2, 3, 5, 10, 12,14, 23, 25, 34, 40, 43, 45, 49 and 82) and/or spore-forming bacteria (OTUs 2, 5, 10, 28, 34, 38, 45 and 82). Others are related to bacteria known to be metal resistant (OTU 2) or alkaliphilic (OTUs 23, 36 and 67). Finally, OTUs 3 and 18 are related to bacteria able to grow on biomats with photosynthetic metabolism. The distribution of OTUs in the three samples, according to their phyla, is shown in Fig. 3.

Table 1 The diversity and related indices

	PI	PII	S	Total
Nr. of reads	630,581	467,624	495,848	1,594,053
Nr. of seqs. mapped to the OTUs	443,297	359,803	366,718	1,169,818
Nr. of OTUs (based on RDP)	721	696	714	1,139
Shannon–Wiener ($H^{'}$)	3.59	3.34	3.96	
$e^{H'}$	36.28	28.50	52.59	
Evenness (J)	0.546	0.512	0.603	
Simpson's index	0.918	0.905	0.957	

Although similar in richness, each steam vent showed a particular community profile different from the others (Fig. 4). Some 299 OTUs (26.3%) were omnipresent in the 3 samples (see Online Resource 1), but only 3 of them were dominant (more than 1% of relative abundance) in more than one sample (OTUs 1, 3 and 38), while no OTU represented more than 1% in all the samples. By comparing the samples two by two, one can see that S and PI samples are the least close ones, with only 31 OTUs in common. On the other hand, S sample shares 185 OTUs with PII, and PI has 178 OTUs in common with PII. A similar pattern of a distinct profile for each sample site has been observed before, from library clone analysis, for soil samples of the same volcanic complex (Medrano-Santillana et al. 2017), and by other authors for marine volcanic fumaroles (Kelly et al. 2011) and volcanic rocks (Crossey et al. 2016). These results support the idea that, in these extreme environments, the biodiversity of pioneering microorganisms responsible for the colonization of the site may largely vary even on small spatial scales, as suggested by Crossey et al. (2016). They suggest that probably this colonization is much dependent on geobiological processes that are influenced by deep subsurface geologic mechanisms.

The temperature may also be a decisive parameter for determining the community profile. Considering the distribution of thermophilic genera in the main bacterial populations of Tables 2, 3, 4, in S they dominate (68%), PI shows an intermediate fraction (58%) and PII is mesophilic dominated (only 27% of thermophiles). This is consistent with the location of the fumaroles: Sapichu is noticeably more active than Paricutín.

Members of the phyla *Cyanobacteria*, *Firmicutes* (class *Clostridia*) and *Chloroflexi* (class *Chloroflexia*), which were present and relatively dominant in all the samples, are commonly reported in hot springs (Miller et al. 2009), but they are not usually dominant in the fumaroles (Costello et al. 2009) and steam vents (Benson et al. 2011). In hot springs, anoxygenic phototrophs (Chloroflexus-like bacteria) compete with oxygenic phototrophs (Cyanobacteria-like) for inorganic carbon (Wang et al. 2013), nutrients and physical space (Miller et al. 2009) in lighted conditions. It is also possible that this competition occurs on

Table 2 The main bacterial populations (OTUs from Miseq® sequencing) found for PI sample according to their relative richness (only for abundance in the sample higher than 1%)

OTU	Number of reads	% by sample	% of total	Class[a]	Genus[b]
OTU_1	99656	22.48	10.35	Cyanobacteria (1.00)	–
OTU_3	62993	14.21	5.72	Chloroflexia (0.77)	*Roseiflexus* (0.45)
OTU_{20}	17153	3.87	1.47	Cytophagia (0.73)	*Flectobacillus* (0.08)
OTU_{15}	16683	3.76	1.43	Chloroflexia (0.28)	*Oscillochloris* (0.01)
OTU_{12}	15306	3.45	1.44	Deinococci (1.00)	*Meiothermus* (1.00)
OTU_{13}	14779	3.33	1.34	Cyanobacteria (1.00)	–
OTU_{568}	12973	2.93	1.19	Cyanobacteria (1.00)	–
OTU_{41}	10587	2.39	0.98	Acidobacteria (0.02)	–
OTU_{18}	10469	2.36	1.00	Chloroflexia (1.00)	*Chloroflexus* (1.00
OTU_{17}	10277	2.32	0.94	Cytophagia (0.96)	*Ohtaekwangia* (0.69)
OTU_{26}	8062	1.82	0.75	Acidobacteria (0.94)	–
OTU_{23}	7826	1.77	0.72	Alphaproteobacteria (1.00)	*Elioraea* (1.00)
OTU_{43}	7675	1.73	0.78	Deinococci (1.00)	*Meiothermus* (1.00)
OTU_{32}	7594	1.71	0.67	Clostridia (0.13)	*Saccharofermentans* (< 0.01)
OTU_{30}	7444	1.68	0.65	Alphaproteobacteria (0.96)	*Stella* (0.22)
OTU_{57}	5906	1.33	0.55	Acidobacteria (1.00)	–
OTU_{40}	5856	1.32	0.51	Betaproteobacteria (0.97)	*Tepidimonas* (0.26)
OTU_{25}	5598	1.26	0.71	Deinococci (1.00)	*Meiothermus* (1.00)
OTU_{44}	5546	1.25	0.48	Acidobacteria (1.00)	–
OTU_{46}	5405	1.22	0.46	Acidobacteria (1.00)	*Aridibacter* (0.64)
OTU_{42}	5098	1.15	0.44	Cyanobacteria (1.00)	–

[a]Numbers in parenthesis show the probability to belong to the class

[b]Numbers in parenthesis show the probability to belong to the genus

Table 3 The main bacterial populations (OTUs from Miseq® sequencing) found for PII sample according to their relative richness (only for abundance in the sample higher than 1%)

OTU	Number of reads	% by sample	% of total	Class[a]	Genus[b]
OTU_2	90374	25.12	7.78	Actinobacteria (1.00)	*Microbacterium* (0.92)
OTU_4	44225	12.29	3.81	Betaproteobacteria (1.00)	*Methylovorus* (0.06)
OTU_{10}	24479	6.80	2.11	Clostridia (0.92)	*Symbiobacterium* (0.68)
OTU_1	21228	5.90	10.35	Cyanobacteria (1.00)	–
OTU_8	19671	5.47	1.69	Betaproteobacteria (1.00)	*Burkholderia* (0.98)
OTU_{14}	12747	3.54	1.10	Alphaproteobacteria (1.00)	*Rhizobium* (0.91)
OTU_{19}	9477	2.63	0.82	Actinobacteria (1.00)	*Rhodococcus* (1.00)
OTU_{21}	9011	2.50	0.78	Cytophagia (0.95)	*Hymenobacter* (0.27)
OTU_{77}	7806	2.17	0.67	Actinobacteria (1.00)	*Curtobacterium* (0.54)
OTU_{24}	7700	2.14	0.66	Sphingobacteriia (1.00)	*Sediminibacterium* (1.00)
OTU_{27}	7676	2.13	0.66	Sphingobacteriia (0.98)	*Niastella* (0.22)
OTU_{31}	7484	2.08	0.65	Cytophagia (0.99)	*Siphonobacter* (0.16)
OTU_{67}	5589	1.55	0.48	Bacilli (1.00)	*Paenibacillus* (0.93)
OTU_{48}	4986	1.39	0.43	Actinobacteria (1.00)	*Crossiella* (0.19)
OTU_{38}	3758	1.04	0.98	Bacilli (1.00)	*Bacillus* (1.00)
OTU_3	3757	1.04	5.72	Chloroflexia (0.77)	*Roseiflexus* (0.45)

[a]Numbers in parenthesis show the probability to belong to the class

[b]Numbers in parenthesis show the probability to belong to the genus

Table 4 The main bacterial populations (OTUs from Miseq® sequencing) found for S sample according to their relative richness (only for abundance in the sample higher than 1%)

OTU	Number of reads	% by sample	% of total	Class[a]	Genus[b]
OTU_5	41138	11.22	3.53	Ktedonobacteria (0.07)	*Thermosporothrix* (< 0.01)
OTU_6	39908	10.88	3.42	Acidobacteria (1.00)	–
OTU_9	23886	6.51	2.05	Chthonomonadetes (1.00)	*Chthonomonas* (1.00)
OTU_7	21219	5.79	1.82	Acidobacteria (1.00)	–
OTU_{11}	17541	4.78	1.51	Cyanobacteria (1.00)	–
OTU_{22}	16101	4.39	1.38	Gammaproteobacteria (0.97)	*Rhodanobacter* (0.21)
OTU_{16}	12821	3.50	1.10	Cyanobacteria (1.00)	–
OTU_{28}	9301	2.54	0.80	Ktedonobacteria (0.13)	*Thermogemmatispora* (< 0.01)
OTU_{29}	8460	2.31	0.73	Deltaproteobacteria (0.92)	*Geobacter* (0.03)
OTU_{50}	8216	2.24	0.71	Cyanobacteria (0.98)	–
OTU_{38}	7618	2.08	0.98	Bacilli (1.00)	*Bacillus* (1.00)
OTU_{33}	7162	1.95	0.62	Anaerolineae (0.03)	*Longilinea* (< 0.01)
OTU_{36}	7111	1.94	0.61	Bacilli (0.30)	*Paenibacillus* (< 0.01)
OTU_{34}	6575	1.79	0.56	Ktedonobacteria (0.09)	*Thermosporothrix* (< 0.01)
OTU_{39}	6497	1.77	0.56	Acidobacteria Gp4 (1.00)	–
OTU_{35}	6464	1.76	0.55	Acidobacteria Gp3 (1.00)	–
OTU_{45}	5968	1.63	0.51	Ktedonobacteria (0.62)	*Thermosporothrix* (0.15)
OTU_{313}	4584	1.25	0.39	Cyanobacteria GpI (0.98)	–
OTU_{49}	4441	1.21	0.38	Deinococci (1.00)	*Meiothermus* (1.00)
OTU_{52}	4415	1.20	0.38	Cyanobacteria (1.00)	–
OTU_{82}	4133	1.13	0.35	Ktedonobacteria (0.84)	*Thermosporothrix* (0.34)
OTU_{54}	3951	1.08	0.46	Cyanobacteria (1.00)	–
OTU_{37}	3765	1.03	0.49	Nitrospira (1.00)	*Nitrospira* (1.00)

[a]Numbers in parenthesis show the probability to belong to the class

[b]Numbers in parenthesis show the probability to belong to the genus

the microecosystems of steam deposits on volcanic vents like PI. In addition, Wall et al. (2015) found that these groups dominate in light-exposed steam vents, while in cave-like vents or vents with minimal sun exposure, they are minority or inexistent. They also found that *Cyanobacteria* phylum dominate lower temperature vents, while the higher temperature vents tend to have more *Cloroflexi*. It is worth reinforcing that our samples were not taken from mature mats, but from the superficial layer of the stones internal to the steams, where the microorganisms are forming a thin layer over the mineral residues and receive only indirect light.

While PI appeared dominated by primary producers, PII sample, on the other hand, was dominated by heterotrophic bacteria affiliated within the *Actinobacteria* and the *Proteobacteria*. The presence of bacteria related to these phyla is also reported in other thermal vents (Costello et al. 2009; Benson et al. 2011; Wall et al. 2015). The most abundant OTU is related to *Microbacterium*, one genus of *Actinobacteria* with species highly resistant and able to transform metals (Pattanapipitpaisal et al. 2001; Gómez-Ramírez et al. 2015; Fierros-Romero et al. 2016, 2017).

Finally, Sapichu S sample was dominated by OTUs related to *Chloroflexi* (class *Ktedonobacteria*), *Acidobacteria* and *Cyanobacteria* phyla. The *Acidobacteria* also dominate extremely dry non-fumarole and warm-fumarole soil of Socompa Volcano (Costello et al. 2009), while on deposits and steam waters from four Hawaii's volcanoes they were minor (Wall et al. 2015). Similar to other classes of phylum *Chloroflexi* (Cavaletti et al. 2006), the *Ktedonobacteria* includes thermophilic species, aerobic heterotrophs, branched mycelium-forming and spore-forming bacteria (Yabe et al. 2010).

Predictive metabolic potential of microbial communities

In Fig. 5, we show the distribution of metabolic pathways, found using Tax4Fun2, from the first to the third levels highlighting the energy metabolism routes. Predicted pathways were dominated by cellular metabolism (46.3% of the pathways and about 51% of the functional genes) and environmental information processing (8.2% of the pathways and about 26% of the functional genes). Among the cellular

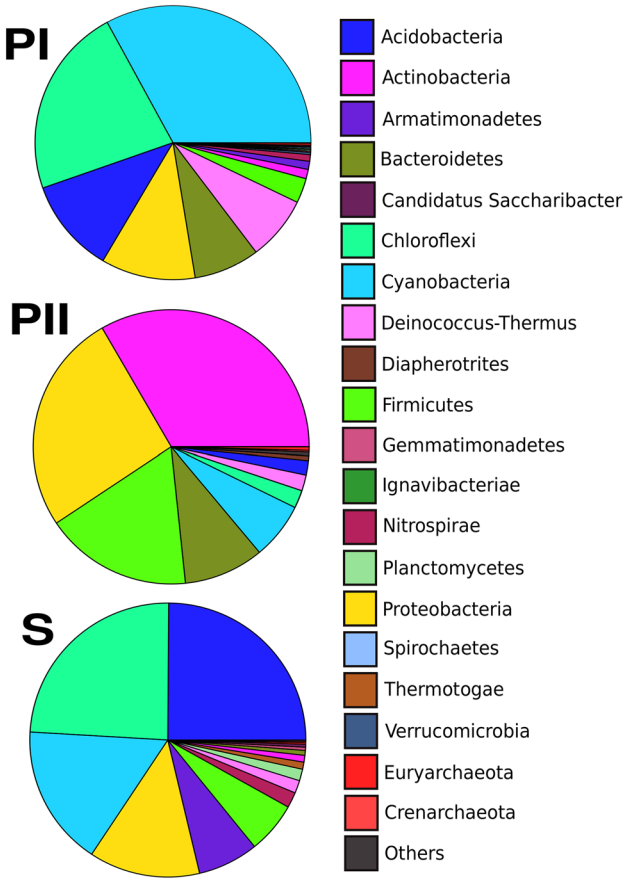

Fig. 3 Relative abundance of prokaryotic phyla in the three samples (PI, PII and S). OTUs were grouped at 97% sequence similarity using USEARCH package

metabolism pathways, stands out (in level 2) the metabolism for generating energy, with metabolisms for sulfur respiration, nitrogen fixation, carbon fixation and photosynthesis, among others. These mechanisms are of special interest for understanding this kind of extreme environments.

Nevertheless, this automatic prediction of metabolic pathways is very incomplete for such mechanisms because the KEGG database is feeded mainly by the most studied organisms, especially the human being. Thus, we searched directly in the KO's list for metabolic pathways of interest. As an example of the results, KO's for specific metal metabolisms can be found in Fig. 6 (see similar figures for other categories of pathways in Online Resources). We also found specific mechanisms for nitrogen and methane, this last one associated to the archaeal organisms in the pool. The grouping of KEGG functionals in metabolic pathways associated to the categories quoted above is shown in Table 5. One can note that, in the three samples, sulfur metabolism is dominant, although carbon- fixation and metabolism of metals are also relevant. It is well known that the elemental sulfur may come from reactions between mantle-derived hydrogen sulfide and sulfur dioxide gas emanating from volcanic fumaroles. We detected KO's related to assimilatory sulfate reduction, dissimilatory sulfate reduction and oxidation. From this we can infer that the reduction of elemental sulfur to hydrogen sulfide, utilizing both H_2 and organic compounds as the electron donors occurs. In addition, the anoxygenic photosynthesis was detected, which can be related to the production of sulfate that can further be used as an electron acceptor in the remineralization of organic carbon by sulfate-reducing bacteria.

Concerning the distinct metabolic potential among the three samples, we found that PI presented the highest potential, with larger fractions of KO's for different metals (Co, Mg, Hg, Se, Cr, Zn, Cu and Au), N, S and carbon cycles. S presented an intermediate potential, with high contents of KO's for N, S, carbon cycles and some metals (Hg, Se, Cr and Zn). Finally, PII only showed relatively high fractions of KO's for Calvin cycle and the metals: Cu, Au, As, Mn and Ni.

Bacterial isolation

In a previous study (Medrano-Santillana et al. 2017), we attempted to isolate microorganisms from Paricutín soil samples surrounding the fumaroles without success. Based on diversity data obtained by cloning and sequencing approaches, a dominance of thermophilic anaerobic spore-forming bacteria could be described. In addition, a significant abundance of OTUs related to *Massilia*, *Paenibacillus* and *Desulfonatrum* genera was observed. We verified that bacterial populations able to use sulfate, sulfur, Fe(III), nitrate and Mn(IV) as electron acceptors dominate.

In the present study, we also aimed to prospect anaerobic bacteria from the Paricutín volcanic fumaroles. Several culture media were tested but only those supplemented with yeast extract (1 g L^{-1}) allowed the development of colonies on solid media. Four anaerobic strains could be isolated: three belonging to Clostridia class and 1 to Bacilli class (see Online Resource 3), all of them were spore-forming microorganisms. The three *Clostridia* isolates were related to *Clostridium sphenoides* (VPTFN03, 99% similarity), to *Clostridium swellfunanium* (VPTFN01, 97% similarity) and to *Anaerocolumna cellulosilytica* (VPTFN02, 94% similarity). The other isolate, VPTFN04, also belongs to *Firmicutes* phylum, closely related to *Paenibacillus azoreducens* (99% similarity).

Although a specific medium for sulfate reducers was used, all the obtained isolates were typically fermentative. Nevertheless, the strains VPTFN03 and VPTFN04 produced a characteristic black precipitate when grown with Fe_2SO_4 under anaerobic conditions. The type strain of *C. swellfunanium* S11-3-10 (the closest related VPTFN01) was isolated in 2014 from the Luzhou Chinese liquor (Liu et al. 2014). It is capable of surviving on the presence of ethanol (7% v/v)

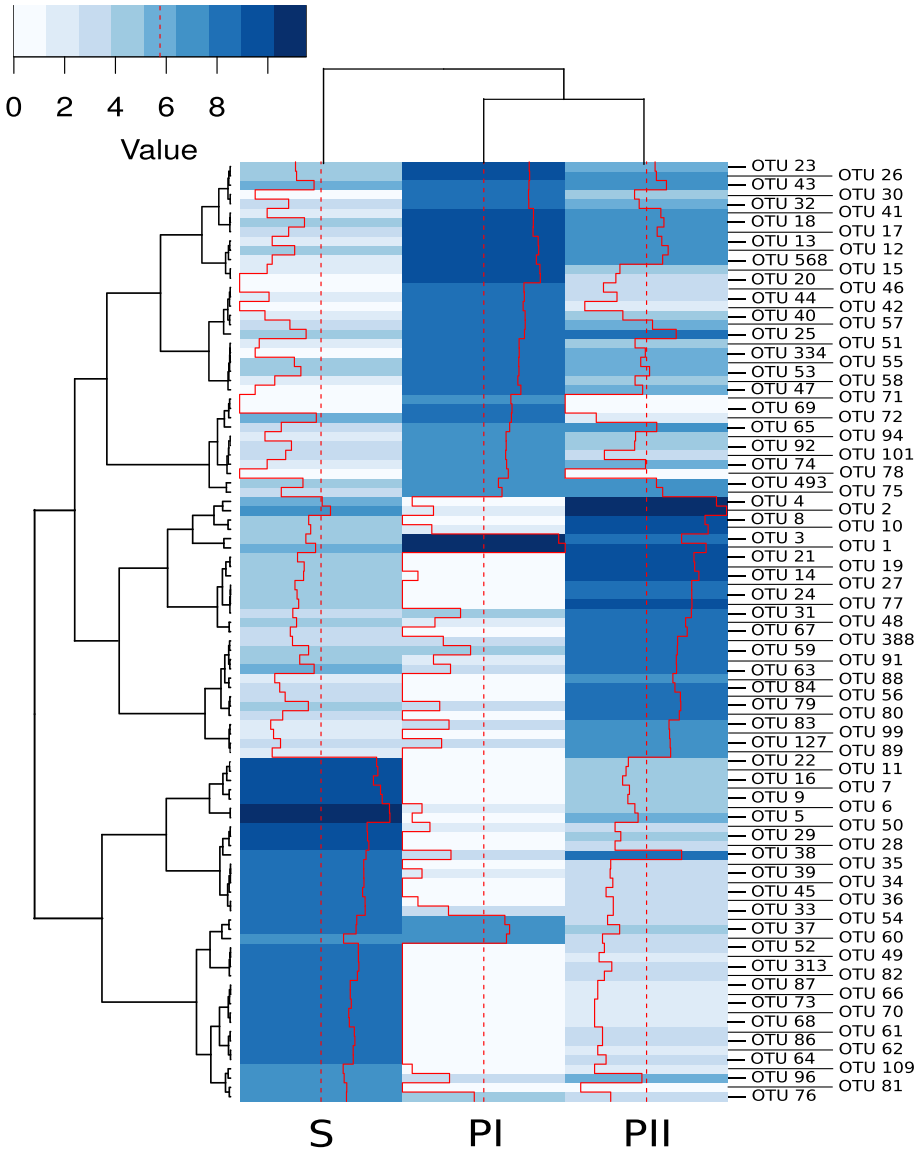

Fig. 4 Distribution of the top 101 most abundant (more than 0.1%) OTUs among the three samples (S, PI and PII). The color intensity marks the abundances according to the scale indicated on the top left side of the figure. The red trace represents the distribution of OTUs in each sample in logarithmic scale

and presents a peritrichous flagellum (Liu et al. 2014). The closest relative to VPTFN02, *Anaerocolumna cellulosilytica,* is a strictly anaerobic bacterium which was isolated from a methanogenic reactor, recently proposed as a new genus in the Clostridiales order (Ueki et al. 2016). The strain *P. azoreducens* CM1, the closest relative to VPTFN04, was isolated from a wastewater treatment plant, and is a facultative anaerobe, capable of degrading several textile dyes (Meehan et al. 2001).

Although we were successful in isolating these four strains, and also in their phylogenetic identification by 16S rRNA gene sequencing, we were not able to maintain the isolates in culture media. As time passed by, neither viable cells nor spores could be observed on the culture media despite all exhaustive attempts to rescue the strains from cultures preserved at − 20 °C or at 4 °C. Similar problems were reported by Rosa et al. (1975) for *Sulfolobus acidocaldarius*, an acidophile strain, also irreversibly lost in the first trial. The maintenance of viable cells was only achieved at sub-optimal temperatures, with hydrogen concentrations close to the expected concentration inside the cells. The authors argue that acidophilic microorganisms such as *Sulfolobus acidocaldarius* probably have specific metabolic mechanisms used for maintaining the pH gradient across the cell membrane. Since the growth of isolates on liquid cultures is an essential requirement for its maintenance as stock, to continue this study we need to re-isolate these strains with a strict control of microenvironmental conditions.

Comparison of diversity applying HTS and the clone libraries

The comparison of the diversity obtained in this paper, by HTS, and the diversity obtained in the soil surrounding

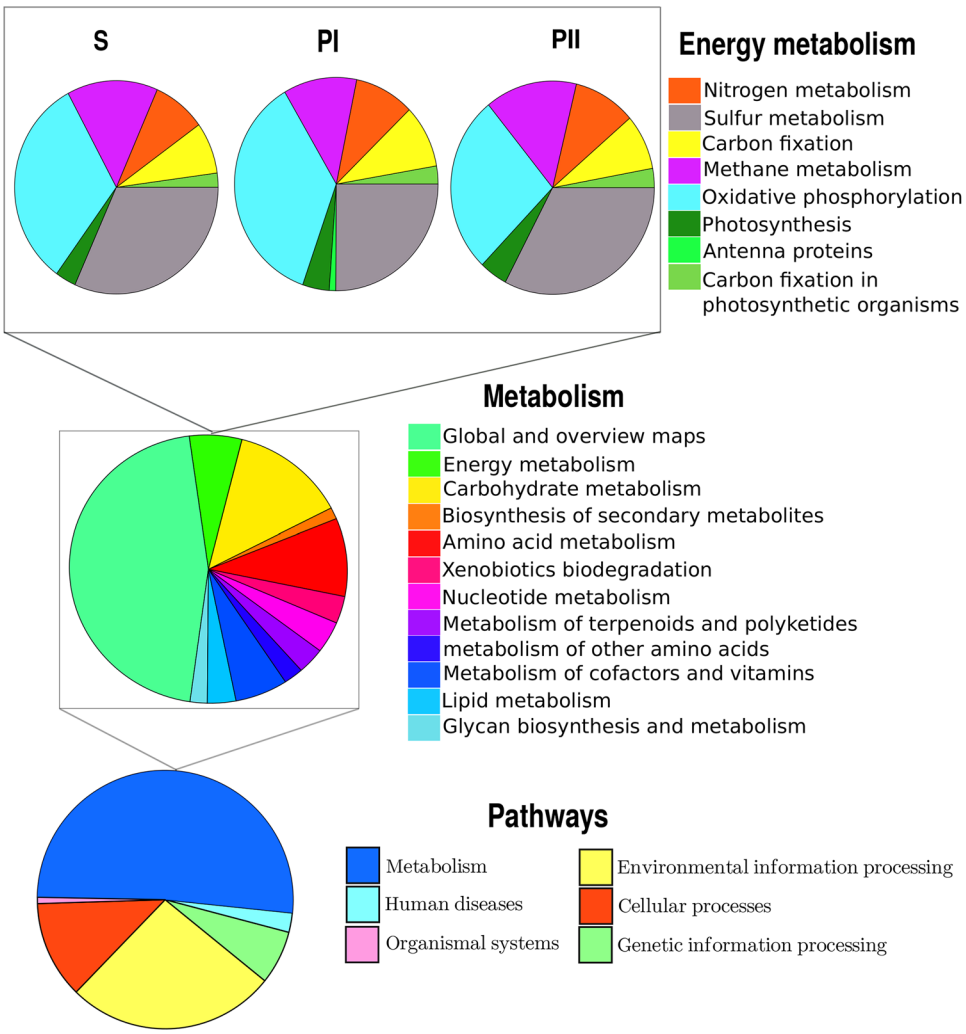

Fig. 5 Predicted metabolic pathways separated according to categories, from the first to the third levels, highlighting the energy metabolism routes, from Tax4Fun2 analysis

other fumaroles from the Paricutín volcano by clone libraries (Medrano-Santillana et al. 2017), highlights 15 genera in common (50% of the ones in that work). These include six genera (*Meiothermus, Paenibacillus, Methylobacterium, Thermoanaerobacter, Rubrobacter, and Ktedonobacter*) relatively abundant according to the present work, and nine rare populations (less than 0.1% of relative abundance).

Conclusions

The autotrophs such as *Cyanobacteria* phylum and *Chloroflexia* class are typical pioneering microorganisms, while among the heterotrophs the colonizers are usually the ones with spore-forming capability (Medrano-Santillana et al. 2017). Spore-forming microorganisms may arrive to the fumarole both from exogenic (by the wind or rain) and/or endogenic (fluid mixing dragged through conduits by mantle-derived volatiles) sources. By applying two distinct techniques, we could observe both groups in steam vents of Paricutín–Sapichu volcano system. Those results suggest that our samples have different maturity stages: PI is possibly the youngest fumarole, with an intermediate temperature, dominated by phototrophs (containing a fraction of only about 10% of spore-forming organisms) and presenting the highest predicted metabolic potential, while PII (probably the warmest) and S (probably the hottest) may represent different possibilities for more evolved fumaroles. Another remarkable point is that all bacterial classes in PI are also present in PII and/or S, but not the opposite. In a general context, the bacterial community profile of PI is very similar to the one found for fumaroles in Hawaii by Wall et al. (2015). Another possible explanation for the fact that PI is dominated by phototrophs is related to the position of the fumarole: it was located at the top of the Paricutín's cone rim, probably receiving more sunlight than the others. PII is dominated by heterotrophs (with about 30% of populations being spore-forming) and shows the lowest fraction of KO's related to the pathways considered in this study and a slightly

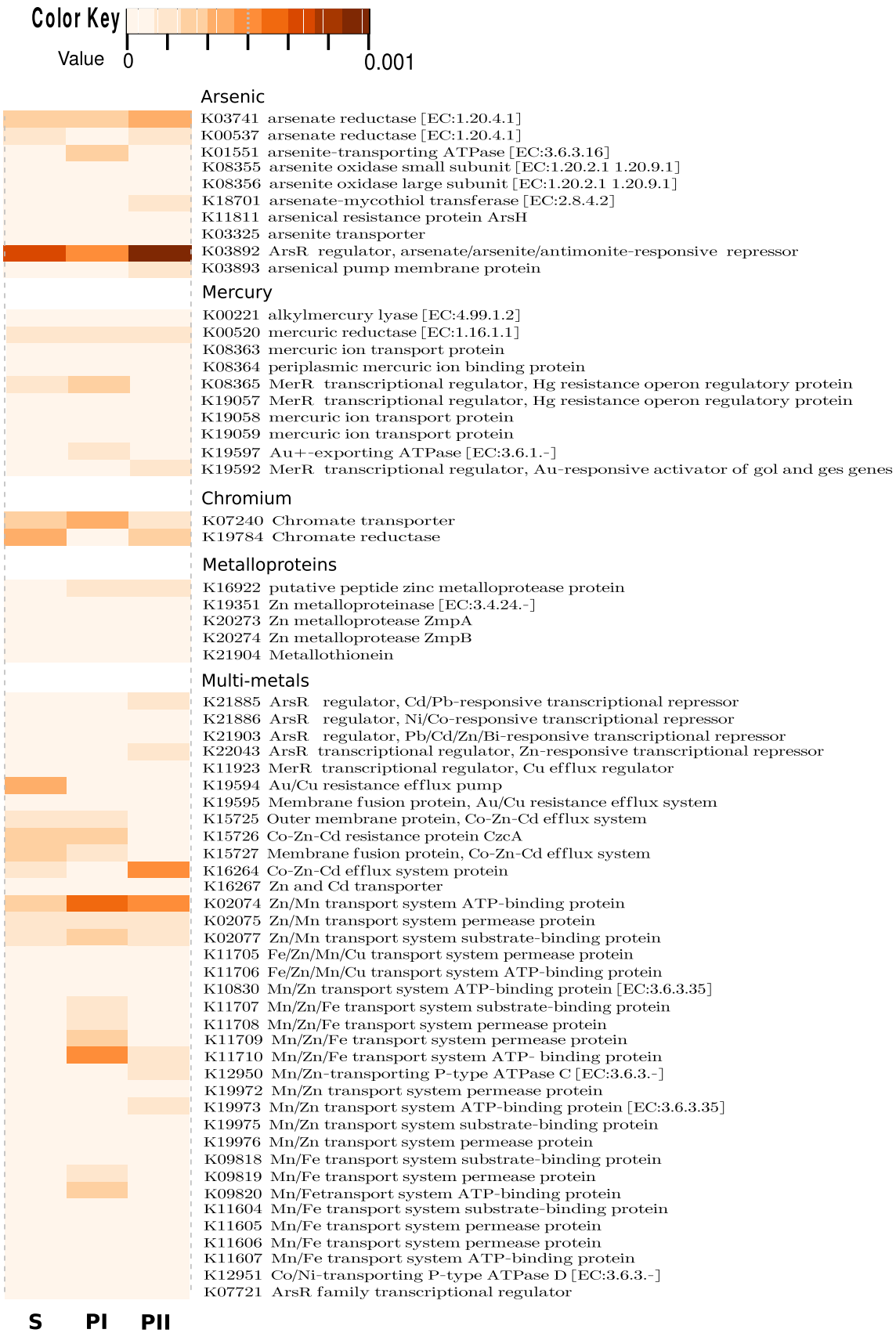

Fig. 6 Heatmap showing the predicted abundance of KO's related to metal pathways. The color intensity marks the abundances according to the scale indicated on the top left side of the figure

Table 5 KEGG pathways for energy metabolism and metals, separated in the different categories and for the three distinct samples (relative abundances)

	KEGG energy metabolism pathways	S	PI	PII	Number of EC's	Number of KO's
C-fixation	Reductive pentose phosphate (Calvin) cycle	5.85	6.30	7.75	10	15
	Reductive citric acid (Arnon–Buchanan) cycle	8.92	8.40	8.68	18	36
	Reductive acetyl-CoA (Wood–Ljungdahl) pathway	1.56	1.66	1.01	8	9
	3-Hydroxypropionate bicycle	6.47	7.31	4.04	14	18
	Hydroxypropionate-hydroxybutyrate cycle	5.46	5.28	5.01	16	15
	Dicarboxylate-hydroxybutyrate cycle	0.41	0.19	0.18	1	2
Photosynthesis	F-type H+ -transporting ATPase	3.45	2.85	5.19	1	8
	Cytochrome b6-f	0.57	0.39	0.02	1	8
	Photosystem I	0.00	0.30	0.00	0	12
	Photosystem II	0.00	0.65	0.00	1	24
	Photosynthetic electron transport	0.14	0.20	0.00	1	4
CH_4-metabolism	Methanogenesis ($CO_2 \rightarrow CH_4$) by methanogenic archaea	0.69	0.33	0.34	7	14
	Methanogenesis (acetate → methane)	3.60	2.68	4.23	4	6
	Methanogenesis (methylamine/dimethylamine/trimethylamine → methane) by methanogenic archaea	0.24	0.01	0.09	1	4
	Other KO's related to methane metabolism	0.33	1.14	0.36	3	8
	formaldehyde assimilation, the serine pathway	4.14	4.37	4.25	9	10
N-metabolism	Anammox	0.09	0.36	0.14	2	2
	Nitrification	0.48	0.71	0.04	3	6
	Nitrogen fixation	0.41	0.21	0.23	1	4
	Denitrification	1.28	1.00	0.34	6	10
	Assimilatory nitrate reduction	0.82	0.90	0.75	3	3
	Dissimilatory nitrate reduction	2.11	1.58	1.83	4	9
S-respiration	Assimilatory sulfate reduction	2.43	2.73	0.97	3	6
	Dissimilatory sulfate reduction and oxidation	0.29	0.91	0.12	3	5
	SOX system	2.03	2.60	0.20	0	6
	Other KO's related to sulfur metabolism	15.80	10.32	14.98	23	37
Metals	Arsenic	3.70	3.06	5.78	4	10
	Mercury	1.07	1.06	0.62	3	10
	Chromium	5.84	9.82	11.01	0	2
	Metalloproteins	0.19	0.55	0.48	1	5
	Multi-metals	8.90	9.14	9.49	2	36
	Cobalt	2.81	3.15	2.54	12	15
	Copper	0.06	0.34	0.47	1	18
	Magnesium	2.18	5.15	2.83	4	10
	Manganese	0.57	0.47	0.81	0	5
	Nickel	1.00	0.47	2.03	1	6
	Selenium	1.80	1.26	1.00	4	10
	Zinc	4.32	2.13	2.20	5	12

The numbers of E.C. (Enzyme Commission) and K.O. (KEGG Orthologs) found in each category are also shown

lower diversity. Finally, S is dominated by spore-forming microorganisms (more than 40%), presents an intermediate metabolic potential and a slightly higher diversity. As pointed above, the particular profile of each fumarole may also depend on geobiological processes, such as the gaseous and solid phase composition and, especially, the different temperature gradients of the fumaroles, which may depend, for instance, of how deep the sample was collected. Nevertheless, more studies are needed to understand what drives the development and evolution of different bacterial communities among the distinct steam vents. Objectively, it is important to follow the evolution of the community profiles of the fumaroles and to relate them to the variations of physicochemical parameters, such as

temperature, pH, exposure to light, composition of the mineral crust and the steam-gas mixture. This is not trivial since the fumaroles are usually ephemeral systems: they do not live long while new ones continually appear. Besides, the difficulty to access a volcano, even if it is a terrestrial volcano, increases the challenge to decipher the microbial community structure in fumaroles.

In summary, the steam vents of Paricutín–Sapichu volcano system revealed a moderate diversity of bacterial populations, with a distinct community fingerprint for each fumer but a similar predicted metabolic potential. The communities are composed by bacterial populations probably adapted to specific local conditions, such as high temperatures, indirect solar radiation and different electron acceptors/donors, which may vary from one fumarole to the other. Most of the detected populations were similar to those observed on other geothermal sites, which show some thermophilic capability, spore-forming, and with ability to grow on biomats. Some populations were similar to those described as metal resistant, halotolerants, alkaliphilics or acidophilics. The predictive analysis for metabolic potential revealed a dominance of cellular metabolic pathways, especially the ones for energy generation with metabolisms for sulfur, nitrogen, methane, carbon, photosynthesis and metals. This study also shows evidence of the difficulty to maintain pure cultures from these ecological niches. Despite all these complications, it is very important to continue the microbial research of extreme environments, such as fumarolic volcano vents, seeking insights about the first microbial colonization of this kind of ecosystems. For further studies, we intend to verify the temporal microbial colonization using more vents as study models and following the largest number of geochemical parameters as possible.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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