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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Hyperthermophile diversity microbes in the Calientes geothermal field, Tacna, Peru

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Abstract

Hyperthermophile microorganisms have been discovered worldwide, and several studies regarding biodiversity and the potential biotechnological applications have been reported. In this work, we describe for the first time the diversity of hyperthermophile communities in the Calientes Geothermal Field (CGF) located 4400 m above sea level in Tacna Region, Perú. Three hot springs were monitored and showed a temperature around 84 to 88 °C, for the microbiome analyzed was taken by sampling of sediment and water (pH 7.3–7.6). The hyperthermophile diversity was determined by PCR, DGGE, and DNA sequencing. The sediments analyzed showed a greater diversity than water samples. Sediments showed a more abundant population of bacteria than archaea, with the presence of at least 9 and 5 phylotypes, respectively. Most interestingly, in some taxa of bacteria (Bacillus) and archaea (Haloarcula and Halalkalicoccus), any of operational taxonomic units (OTUs) have not been observed before in hyperthermophile environments. Our results provide insight in the hyperthermophile diversity and reveal the possibility to develop new biotechnological applications based on the kind of environments.

Keywords Hyperthermophiles · Bacteria · Archaea · Geothermal field · DGGE

Introduction

The earth hosts various unique, unknown and extreme niches, among all of them, that have hyperthermophile environments, these hot spots could be found in the deep of the sea [1], and many terrestrial geothermal systems, including along tectonic boundaries, spreading centers, or "hot spots," where magma bodies may reach within a few kilometers of the Earth's surface [2–6]. The reports of these thermic habitats are terrestrial geothermal fields caused mainly by volcanism [7–11]. Some examples are known, such as geothermal fields reported from the Devonian Drummond Basin (Australia), the Devonian Rhynie cherts (Scotland), and the Yellowstone-style geothermal landscapes of Patagonia, Argentina ([12]).

These geothermal fields are also found in the Peruvian Andes. Here, geothermal activity is usually manifested on the surface as hot springs, geysers, and fumaroles. Water in these pools is often recycled through the geysers and back to the pool. Most of the hot springs in the Peruvian Andes discharge alkaline water; however, pH can vary between 1 and 9 [13]. The typical discharge of these springs is 5 L s^{-1}, but discharges of up to 60 L s^{-1} have been measured in the CGF [13].

The hot springs's compositions have been monitored by geologic drivers (climate, tectonics, heat input) that a role as distal factors, such as water-rock interaction, hardness of water, conductivity, microbial metabolism, mixing, and other physicochemical characteristics could be important for the study of the environments [4]. The physicochemical characteristics in the hot springs occur in soils rich in iron and calcium salts, which generate yellow and whitish colorations.

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The chemical conditions in these sites facilitate the growth of organisms that can oxidize hydrogen, sulfur, hydrogen sulfide, and thiosulfate [14]; reduce sulfur [15], nitrates, or nitrites [16]; or reduce iron in a photosynthesis-dependent way [17]. Therefore, microorganisms dominate the biota associated with these sites, their associated high temperatures, and unique characteristics [14]. Hyperthermophile microorganisms can be found living in geothermal fields, which are fairly accessible and therefore constitute one of the most studied habitats in terms of hyperthermophile diversity [2, 8, 18].

For these environments, a variety of classification have been used to define the microorganisms that live in that kind of temperature habitat, the most common classification have been used on optimal growth temperature, psychrophiles growth temperature of ≤20 °C [19], mesophiles having a 20 to 45 °C [20], thermophiles having an optimal growth temperature ranging from 45 to 80 °C [21], and hyperthermophiles having an optimal growth temperature of ≥80 °C [21]. For the thermophiles and hyperthermophiles, the clades most commonly studied are the bacterial and archaeal phylum [17], based on experience focused on study on the microbial communities by culture-based approaches initially used to study microbial diversity [21]. Microbial exploration due to the development of molecular biological techniques has greatly improved in the recent decades. Advances in cultivation-independent methods for examining uncultured microbes, including single-cell genomics and deep sequencing of environmental samples, have begun yielding complete or near-complete genomes from many novel lineages [21].

Additional terms are commonly used to describe polyextremophiles, such as thermoacidophiles, capable of growth at high temperature and low pH, halophilic thermoalkaliphiles, capable of growth at high temperature, high salt, and high pH, and thermophilic piezophiles, capable of growth under high temperature and pressure [6, 22–24]. A technique culture-independent that has been described as denaturing gradient gel electrophoresis (DGGE) analysis of 16 S rRNA gene segments has been used to profile complex microbial communities and to infer the phylogenetic affiliation of the community members [25].

Since the application of the Taq polymerase from the hyperthermophile *Thermus aquaticus* [26], the biological application for the microorganisms on the hot springs environments has made a paradigm shift in the industry. The potential biotechnological of the thermophiles and hyperthermophiles or their enzyme applications are a variety of advantages as bioremediation [27], the production of biomolecules [28], production of biofuels [29], biomining [30], in agriculture, biosurfactants could substitute chemical surfactants as adjutants in herbicide and pesticide formulations [31], or a microorganism for a biomarker of the wasted o extreme environment [32]. This application of the thermophiles or their enzymes is not surprising. They have a remarkable capacity to work in environmental fluctuations such as pH, temperature, and other possibilities [33].

Here, we analyzed for the first time the microbial diversity in the Calientes Geothermal Field (CGF) of the Peruvian Andes, the highest-known geothermal field. We collected water and sediment samples from three hot springs in the CGF, and the abundance and diversity of bacteria and archaea were determined. These data allowed us to describe, for the first time, the hyperthermophile diversity present in the CGF and opened the possibility to develop novel biotechnological applications based on the bioresources present in the unique hyperthermophilic microorganisms inhabiting this environment.

Materials and methods

Sample collection

The CGF is located in the Cordillera Occidental in Candarave Province, Tacna Region, Peru (17° 15'30" S, 70° 9' W) at 4400 m above sea level (Fig. 1 a, b), as they represent the most likely places to identify hyperthermophiles. A total of four water (W) samples (3 L) were collected, one from each hot spring at a depth of 1 m (samples G1 (W), G2 (W), and G3 (W)). Also, a water sample G2S (W) was collected from the surface of G2. Each sample was filtered on-site using sterile filter units (0.2 μm, 25 mm diameter, Millipore Sterivex^{TM}, Darmstadt, Germany). Sediments on the filters were retained, and filtered water was discarded. Pool temperature and pH were also measured. A total of five sediment (S) samples were collected using sterile spatulas, one from each pool at a depth of less than 10 cm (samples G1 (S), G2 (S), and G3 (S)). Two additional sediment samples were taken from G1 and G2 at a depth greater than 10 cm (G1T (S) and G2T (S)). The collected sediment was placed in sterilized 50-mL Falcon tubes. Filters and sediment samples were stored at room temperature until DNA extraction.

Physicochemical analysis of the CGF water

The composition and content of chemical elements in the water of three geysers G1, G2, and G3 were evaluated. For this, 1 L of water was collected from the surface of each geyser in a glass flask, and the pH was brought to 2 with nitric acid (1 N) and hermetically covered. The three samples were sent to a private laboratory for physicochemical analysis of total solids, total hardness, conductivity, and the composition of the trace elements (EPA 200), and anions (EPA 300).

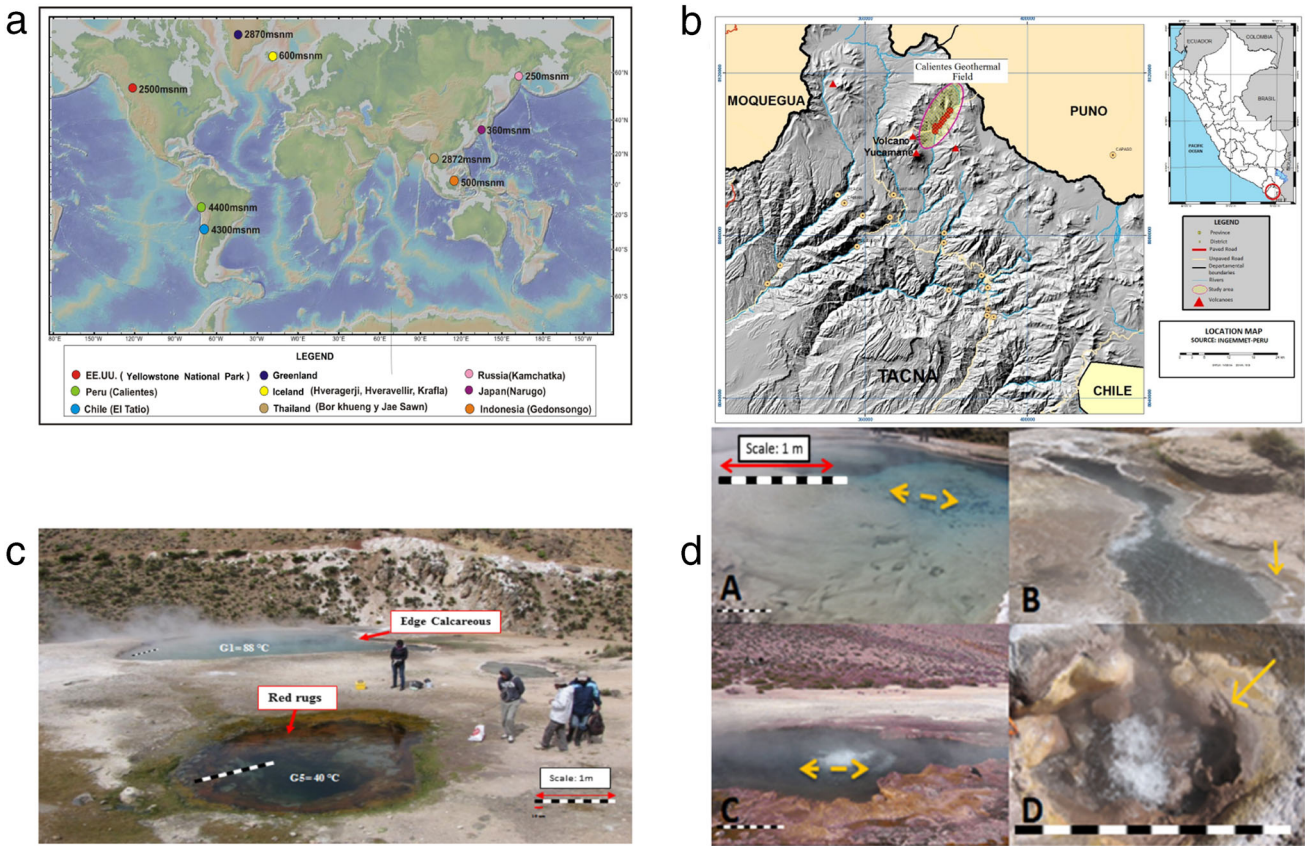

Fig. 1 The Calientes Geothermal Field (CGF) geographical characteristics. a Geographical world location of CGF and comparison with other GF. **b** Location of the CGF in Peru, South America. **c** The arrows indicate the location where water and sediment samples were taken (Source: INGEMMET-Peru). Panoramic view of hot springs in the CGF. Biofilms are visible in pool G5; calciferous borders are visible in pool G1. The bar approximates 1 m. **d** Sampling locations in the CGF. (A) Hot spring 1 (G1), arrows indicate where water sample (G1 (W)) was collected; (B) G1, arrow indicates where sediment sample (G1 (S)) was collected; (C) G2, arrows indicate where water samples (G2 (W) and G2S (W)) were obtained; (D) G3, arrow indicates the calciferous sediment. Scale bars represents 1 m of length

Genomic DNA extraction and amplification of 16 S rRNA

Genomic DNA was extracted from sediment (600 mg) or approximately 1 cm^2 of the filter from each sample using the MOBIO's Power Soil DNA isolation kit (QIAGEN, California, USA.) according to the manufacturer's instructions. Amplification of the 16 S rRNA gene from Bacteria and Archaea was performed by nested PCR. For archaea analysis, the first PCR included the primers Ar4F and Un1492 and produced fragments of 1500 bp (Table 1). These were used as templates for the nested PCR with the primers Ar3F (positions 7–26) and Ar9R (positions 906–927) [34], which generated an 880 bp fragment. For bacterial analysis, primers Eub9-27F and Eub1542R were used for the first PCR [35] (Table 1). PCR (25 μL final volume) conditions were as follows: 2 mM $MgCl_2$ (Roche, Switzerland), 200 μM dNTPs (Promega, Wisconsin, USA), 1 pmol of each oligonucleotide, 2.5 U Gotaq ((Promega, Wisconsin, USA), and 10–100 ng template DNA. DNA was denatured for 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 78 s. Fragments were further used for denaturing gradient gel eletrophoresis (DGGE).

Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed according to [41]. PCR products from bacterial 16 S rRNA were generated with primers 341F-GC and 534R (Table 1) [39]. For archaea, primers 344F-GC [40] and 915R [40] (Table 1) were used. Primer 344F-GC contains a 5' 40-nucleotide GC clamp that provides stability during DGGE [39]. PCR products were loaded on 7.5% polyacrylamide gels (MERCK, USA) containing a denaturing linear gradient from 30 to 60% for bacteria and 20 to 70% for Archaea according to Green et al. (2017) [25]). Urea (7 M) and 40% formamide were defined as 100%. Separations were achieved at 60 °C and 200 V for 6 h (BioRad D

Table 1 List of primers used in this study

Name	Domain	Position	Sequence (5'-3')
Ar4F[a]	Archaea	8–25	TCY GGT TGA TCC TGC CRG
Un1492R[b]	Universal	1492–1510	GGT TAC CTT GTT ACG ACT T
Ar3F[c]	Archaea	7–26	TTC CGG TTG ATC CTG CCG GA
Ar9R[d]	Archaea	906–927	CCC GCC AAT TCC TTT AAG TTT C
Ar344 F[e]	Archaea	344–363	TCG CGC CTG CTG CTC CCC GT
Ar915R[f]	Archaea	915–934	GTG CTC CCC CGC CAA TTC CT
27F[g]	Bacteria	8–27	AGA GTT TGA TCC TGG CTC AG
1542R[h]	Bacteria	1542–1525	AGA AAG GAG GTG ATC CAG CC
341 F-GC(P3)[i]	Bacteria	111–130	GGA ATC TTC CAC AAT GGG CG
534 R(P2)[j]	Bacteria	361–380	TTC CCC ACG CGT TAC TCA CC
344F-GC[k]	Archaea	111–130	ACG GGG CGC AGC AGG CGC GA
915R[l]	Archaea	361–380	GTG CTC CCC CGC CAA TTC CT
G+C Clamp			CGC CCG CCG CGC CCC GCG CCC
			GTC CCG CCG CCC CCG CCC C

[a, b, c, d], [34]; [e], [36]; [f], [37]; [g, h], [38]; [i, j], [39]; [k, l], [40]

Gene, California, Estados Unidos). Gels were developed with SYBR Gold (Invitrogen; final concentration 2.5×). Bands were observed by UV translumination (Vilber Lourmat, Germany).

Re-PCR of DGGE bands

The brightest DGGE bands (Fig. 2 a, b) were cut out of the gel with a sterile scalpel and placed in Eppendorf tubes containing 100 μL of distilled sterile water (MERCK, USA). Samples were incubated at 37 °C for 1 h and 4 °C for 24 h. Recovered DNA (5 μL) from each band was amplified by PCR in 25 μL. The primers used for re-PCR had the same sequence as the original amplification but lacked the GC clamp.

Sequencing of DGGE bands

DNA samples were sent to Macrogen Inc. (Seoul, South Korea) for sequencing in an ABI PRISM 3700 (Applied Biosystems). The forward and reverse primers were 341F and 907R for bacteria and 344F and 915R for archaea.

Denaturing gradient gel electrophoresis (DGGE)

To analyze the diversity of microbes in the samples, a matrix of the distribution of DNA bands was prepared in a way to clarify the presence or absence of different microbial species. This relationship was represented graphically by cluster analysis (cluster-WPGMA), based on the similarity percentage between samples (Multi-Variate Statistical Package, version 3.12d; Kovach Computing Services, Wales, UK).

Sequence analysis

A total of 16 S rRNA sequences were compared with available sequences in the database of the National Center for Biotechnology Information (NCBI), using BlastN. The cladograms were made in MEGA X [42].

Results

Ambiental and physical chemistry characteristics of the CGF

There were more than 87 geothermal features in the CGF, whose water temperature ranged around 37 and 88 °C and pH almost neutral (7). Vents with temperatures close to 40 °C contained layers of red, green, and gray biofilms, some of which were exposed to the air (Fig. 1c). The three hot springs chosen for this study had the highest water temperatures (G1 = 88 °C; G2 = 88 °C; G3 = 84 °C), and pH was close to neutral (G1 = 7.3; G2 = 7.14 and G3 = 7.6). Coherent to most thermal water found in the CGF, these pools had a distinct white-yellow coloration and a hard bottom, which is characteristic of carbonate and other mineral precipitates (Fig. 1 c and d; [13]).

The composition chemical of water showed certain similarities among the total solids, sulfates, and bicarbonates concentration in G1 and G2, with a similarity above 99% in all chemical characteristics (Tables 2 and 3). While trace element composition showed a more difference between G1 and G2, aluminum, calcium, magnesium, and manganese with similarities around 23.4%, 79.3%, 76.82%, and 35.4% within G2 have detected more concentration than G1 in these trace

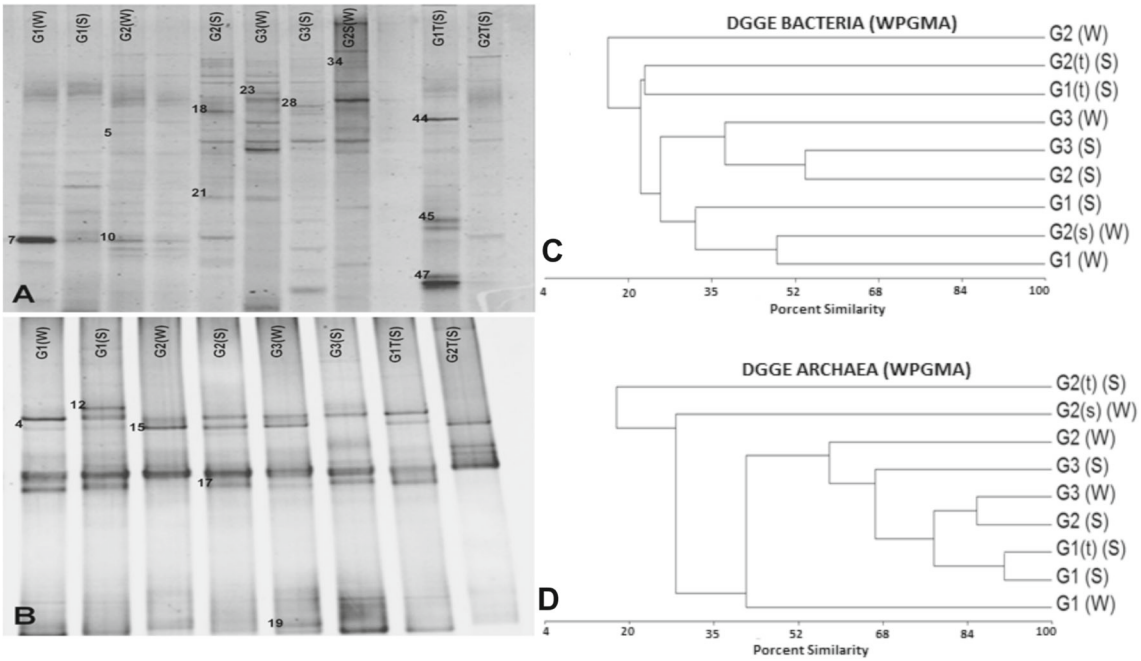

Fig. 2 DGGE diversity profiles. A PCR-DGGE analysis of the predominant bacterial/archaeal communities of water and sediment samples obtained from the CGF. Total DNA was extracted from samples, and the 16 S rRNA gene was amplified by nested PCR using primers specific for bacteria (A) or archaea (B). Each labeled well represents a separate sample with a denaturing linear gradient from 30 to 60% for bacteria and 20 to 70% for archaea. The bands had been sequenced and compared in the NCBI database, the best results are shown in Table 4. **B** Numbered bands were submitted for sequencing. UPGMA (unweighted pair-group method using arithmetic averages) dendrogram generated from bacterial denaturing gradient gel electrophoresis (DGGE) profiles. Samples are from water (W), sediment (S), **C** bacteria, and **D** archea. G1, G2, and G3 are the hot springs used for sample collection. (W), water sample collected at 1 m of depth; (s) (W), water sample collected at the surface; (S), sediment sample collected at less than 10 cm of depth; (t) (S), sediment sample collected at more than 10 cm of depth

elements. However, G3 revealed the most variations in its chemical parameters about G1 and G2, within more bicarbonate over 188%, but a total solid lesser of the other two points (<50%). The trace element has been different in all of the components only the calcium reported moderate similarity with G2 (~95.9) (Table 3).

Composition and diversity of microbial communities in CGF

To analyze the bacterial and archaeal diversity of the hot springs, DGGE was used to visualize several PCR-amplified 16 S rRNA gene fragments. A 30–60% urea-formamide denaturing gradient gel was used to separate the bacterial PCR products obtained from primers 341F-GC and 534R (Table 1; Fig. 2A), and a 20–70% gel was used to separate the archaeal PCR products obtained from primers 344F-GC and 915R (Fig. 2B). Next, cluster analysis (WPGMA) of DGGE bands was used to look for similarities in bacterial and archaeal composition between samples and hot springs. Bacterial samples (Fig. 2C) showed high diversity. Indeed, the most similar clusters, samples G2 (S) and G3 (S), had less than 60% similarity. Analysis of archaeal samples (Fig. 2D) revealed both diversity and similarity. The highest percentage

Table 2 General characteristics of the three hot springs in the CGF selected for biodiversity analysis

Hot springs number	Activity	Parameters			Visual description
		Temperature (°C)	pH	Diameter (m)	
G1	+++	88	7.3	10	Whitish calcareous sediment
G2	++	88	7.4	4.5	Stony and gray
G3	++	84	7.6	0.89	Chalky whitish beige

The air temperature was 11 °C when the samples were collected
++, active
+++, very active

Table 3 Physico-chemical analysis of the CGF water of the three hot springs in the CGF selected for biodiversity analysis

Elements	Geysers (mg L^{-1})		
	G1	G2	G3
Aluminum	0.128	0.030	<0.001
Arsenic	12.1338	12.0930	7.3290
Cadmium	<0.0002	<0.0002	<0.0002
Calcium	37.260	46.998	49.421
Cobalt	<0.00007	<0.00007	<0.00007
Copper	<0.001	<0.001	<0.001
Chromium	<0.002	<0.002	<0.002
Phosphorus	<0.2	<0.2	<0.2
Iron	0.023	<0.001	0.046
Lithium	8.2425	8.9223	5.5001
Magnesium	0.285	0.371	4.275
Manganese	0.0540	0.1414	0.6112
Mercury	0.00270	0.00164	0.00081
Molybdenum	0.06133	0.06397	0.04687
Potassium	85.6	86.4	50.1
Selenium	<0.002	<0.002	<0.002
Sodium	1426.07	1510.39	856.95
Zinc	0.0055	0.0030	0.0016
Sulfate	85.80	85.12	91.41
Total Solids	3920	3950	1930
Bicarbonates[a]	88.6	99.8	188.2
Total hardness[a]	96.2	124.7	145.5
Conductivity[b]	6970.00	7020.00	4440.00

[a], unity (mg HCO3/L); [b], unity (µS/cm)

of similarity was between G1 (S) and G1 (t) (S) (90%), followed by G2 (S) and G3 (W) (85%), and by the G3 group, which showed an equal similarity among them (65%). All other samples exhibited high diversity, with less than 60% similarity.

Phylogenetic diversity of bacteria and archaea in CGF

To identify the phylotypes present in the CGF, 16 S rRNA sequences obtained from the brightest DGGE bands were compared with the NCBI database using BLASTN. The threshold for similarity was set at greater than or equal to 97%, which excluded 80.4% and 79.2% of bacterial and archaeal sequence results, respectively. Sequence analysis revealed 124 operational taxonomic units (OTUs) in sediment for bacteria and 54 OTUs for archaea. The analysis of bacterial sequences revealed the presence of the phylotypes Firmicutes, Deinococci, Gammaproteobacteria, and Chloroflexi (Fig. 3, Table 4). The blast hits obtained (except for Enterobacteriales) were associated with high salinity environments; however, the bands 21, 28, and 34 are high affinity with thermophilic microorganisms like *Meiothermus* spp. and *Fervidobacterium* spp. associated with geothermal environment [43, 44], and the bands 7, 10, 23, and 44 within a relationship with the clade of *Bacillus* spp. included *Bacillus simplex* and *Bacillus muralis* [45, 46]. However, bands 5, 18, 21, and 23 are associated with Exiguobacterium species; this clade is not reported to live above 80 °C, although relationship in a hot spring or thermophilic application [47, 48], and *Chloroflexus* spp. are able to live until 70 °C [49, 50] This suggests the potential formation of microenvironments in the niches and/or the potential presence of more thermophilic variants of these species. For the archaea domain, the sequences belonged to the phylotype Halobacteria (Fig. 4, Table 4). Sequences from Band4 and Band15 were related to *Haloarcula* spp., a species that has not been reported to grow at high temperatures [51]. Meanwhile, Band17 is closed *Halalkalicoccus* spp., a genus commonly grown in mesophilic environments; these have been more associated with subterranean water environments with high salinity (>6%) [52–54]. Band19 was related to *Haloarcula* spp., another halophilic archaeon, also not reported to grow at high temperatures, and more "alkaliphilic" than *Haloarcula* [23]; however, Band12 and Band19 are associated with a high similarity (>97%) to uncultured archaeon.

Discussion

Geothermal fields are excellent sites for the study of hyperthermophile microorganisms, which are of great interest to biotechnology. Here, we characterized for the first time the bacterial and archaeal diversity of the CGF, the highest-elevation geothermal field in the world (Fig. 1). Although other CGF has been reported around the world, as Lardello, Italy (160 to 860 m a.s.l.) [55]; Rotokawa, New Zealand (500 m a.s.l) [56]; Hengill, Iceland (800 m a.s.l.) [57]; Yellowstone park, USA (2.500 m a.s.l) [58]; and GCF in Latin America as Copahue, Argentina (1600 m a.s.l) [24], Tatio, Chile (4.200) [22], in this study showed an environment diversity assay with an elevation around 4.400 m a.s.l, this could give an advantage and restriction in this environment that will be presented in discussion.

About the physicochemical, the pH in this CGF is moderately alkaline in the three hot springs around 7.3 to 7.6, against other CGF with evidence of chemotroph metabolism (Fig. 1b), like Copahue or Yellowstone with a pH around 2 to 5 [24, 58], but similar pH to hot spring with the presence of high concentration of microorganism photosynthetic [59]. This could be explaining to the dessert that it is more heavy metal-free and that these alkalize the water [60], this could be supported due to the fact that Tatio hot spring in Chile has been reported similar pH (Table 3) [22]. The conductivity

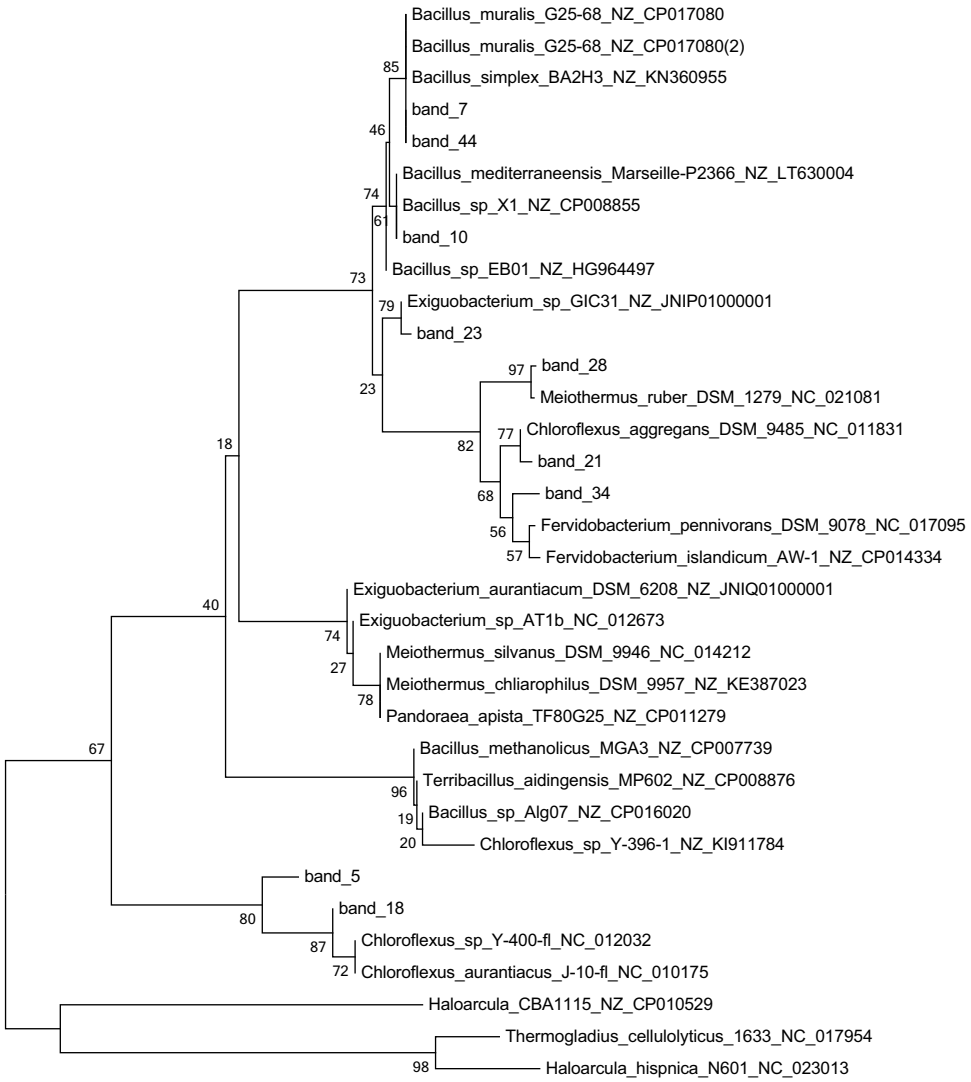

Fig. 3 Phylogenetic tree of bacterial diversity in the CGF based on partial 16 S rRNA gene sequences. The brightest bands obtained from the DGGE were cut, re-amplified, and sequenced. Sequences were compared with BLASTN to the NCBI database. Sequences from this study are in red, indicating the corresponding DGGE band number. Reference sequences were chosen to represent the greatest diversity of bacteria. Scale bar at the bottom left represents 0.2 (20%) nucleotide sequence difference

is similar to that reported for other authors with present chemotrophic microorganisms, and the temperature consistency among the three hot springs was studied (Tabla 2), which is a similar temperature for other CGF [24].

Microbial diversity in the CGF was abundant and varied among samples and collection sites. The WPGMA cluster analysis of Bacteria in sediment and water samples did not show a clear cluster (Fig. 2), indicating high diversity in this environment. These results confirm that each testing site and type of sample presents different physical and chemical conditions that resulted in adaptation of the microbes present in those sites (Tables 2 and 3) [14–17, 61]. Abiotic factors such as the ion cocktail (nutrients) of each site, as well as the temperature that determines the microbial communities, were observed in a particular location [62, 63]. Although, samples such as G2 (S) and G3 (S) showed reasonably high similarity. Differences in the environment surrounding geothermal fields may also influence microbial diversity. For example, the Yellowstone National Park has more abundant flora and fauna than the CGF or any hot spring in a desertic zone, and animals such as birds and mammals may facilitate the spread of bacteria among thermal features [8, 14, 64, 65].

PCR-DGGE finger printer in bacterial and archaea showed different taxas in the environment samples in both phylum (Fig. 2) and have no representative difference in the sediments in bacteria (Fig. 2b); however, the archaea revealed a more significative distribution according to the CGFs location (Fig. 1 c and d). Furthermore, the abundance in the CGF samples was not considered because this intensity in the bands may have been obtained by an amplification artifact [66].

DGGE and sequence analysis revealed a higher diversity of bacteria than archaea. Additionally, 80.4% of bacteria and 79.2% of archaea sequences had a BLASTN similarity of less than 97%, constituting a variety of unknown microorganisms that could serve as a platform for new studies and

Table 4 BLASTN phylogenetic analysis of bacterial and archaeal 16 S rRNA gene sequences obtained from DGGE

Sequence	Hot springs number	Accession	Best match	% Identity
Bacteria				
Band05	G2 (W)	NZ_CP007739	Bacilllus methanolicus strain MGA3	100.0
Band07	G1 (W)	KY615350	Bacillus simplex strain E204	100.0
Band10	G2 (W)	NR_144741	Bacillus mediterraneensis strain Marseille-P2366	100.0
Band18	G2 (S)	NR_074263	Chloroflexus aurantiacus strain J-10-fl	97.7
Band21	G2 (S)	NR_074226	Chloroflexus aggregans strain DSM 9485	97.8
Band23	G3 (W)	NZ_JNIP01000001	Exiguobacterium sp. E11_27	98.3
Band28	G3(S)	NR_145943	Meiothermus roseus strain YIM 71031	100.0
Band34	G2S (W)	MK299259	Uncultured Chthonomonadales bacterium	99.0
Band44	G1T (S)	AY795693	Bacterium Schreyahn_K9.Sali	98.4
Band45	G1T (S)	EU876657	Uncultured proteobacterium clone DB2	99.3
Band47	G1T (S)	EU919218	Uncultured Raoultella sp. clone QRSYY3	99.4
Archeae				
Band04	G1 (W)	JX188260	Haloarcula sp. HMC-3	99.6
Band12	G1 (S)	HM234400	Uncultured archaeon clone A3-14	97.2
Band15	G2 (W)	AB074561	Halobacterium sp. NCIMB 714	99.8
Band17	G2 (S)	NC_014297	Uncultured Halalkalicoccus sp	99.4
Band19	G3 (W)	HQ425124	archaeon BC32	97.7

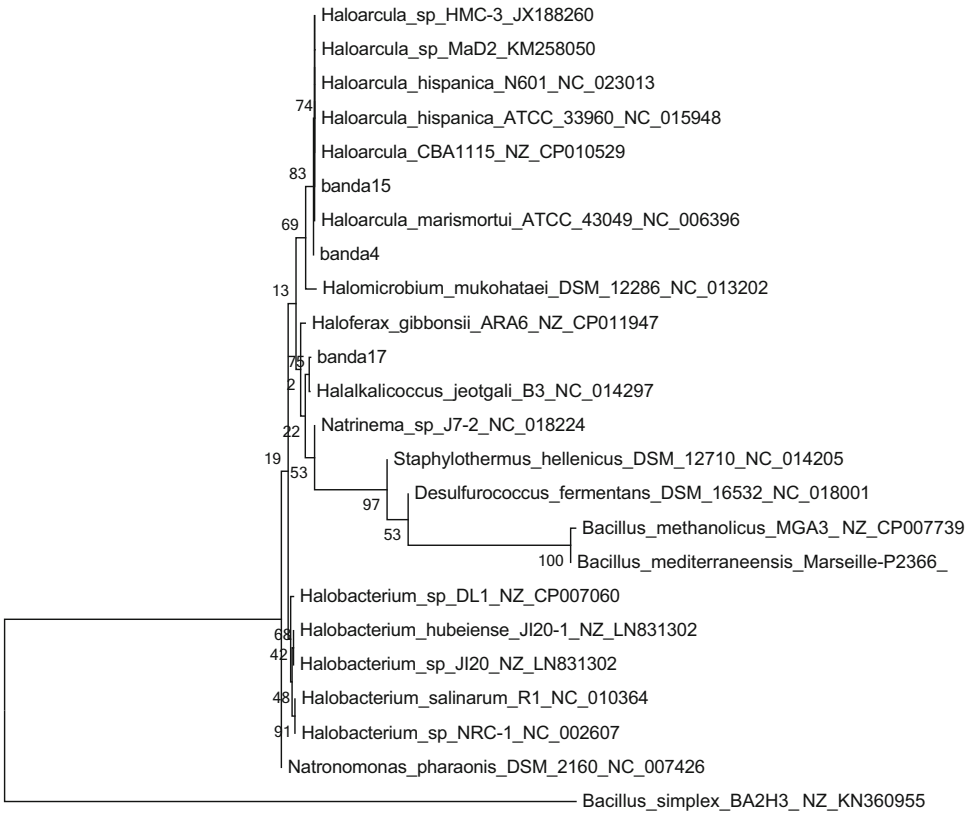

Fig. 4 Phylogenetic tree of archaeal diversity in the CGF based on partial 16 S rRNA gene sequences. Sequences were compared with BLASTN to the NCBI database. Sequences from this study are in red, indicating the corresponding DGGE band number. Reference sequences were chosen to represent the greatest diversity of archaea. Scale bar at the bottom left represents 0.2 (20%) nucleotide sequence difference

hyperthermophile research. This match was similar to other experiences from other hot springs reported in the literature [18, 67, 68].

Here, we demonstrated for the first time the presence of different taxa of hyperthermophiles (Table 3) in the GCF. Also, we found a new clade of hyperthermophile bacteria represented by bands 5, 18, 45, 47, and 48 (Fig. 3) and observed new branches (bands 28 and 21), which are not related to known sequences in GenBank; in this case, the bands associated with uncultured microorganism and proteobacteria are commonly associated with mesophilic and pathogenic but was reported to geothermal environments, which suggest a new clade with environment enzyme like as metal resistance associate with antibiotic resistance capacity [46, 51, 69]. It is possible that these new hyperthermophile bacterial species may be endemic to the CGF, indicating a high level of endemism for bacteria in this site [70–72]. Likewise, we identified a new clade of Archaea in bands 12 and 19 (Fig. 4) Several taxa reported in this study, such as *Bacillus* spp., Gammaproteobacteria, *Chloroflexus aggregans*, Meiothermus, and Archaeas have been previously reported in this type of environment [73]. However, we also determined the presence of some taxa that have not been described in thermal springs before. These include uncultured *Exiguobacterium* spp., *Halobacterium* spp., and *Halalkalicoccus* spp. These taxa may have adapted physiologically and metabolically to take advantage of the thermal spring environment [17, 74–76].

The biodiversity analysis of the three evaluated geysers shows interesting results. It was determined that there is a similarity between the prokaryotic members identified in the geysers (G1 and G2), between bacterial members (*Bacillus muralis*, *B. simplex*, *Bacillus mediterraniensis*, and *Chloroflexus*) and archaea (*Halobacterium* sp, *Halalkalicoccus* sp., and band 12, which would be related by its proximity to halophilic members); on the other hand, the diversity analysis of the geyser (G3) shows different members of the bacteria domain such as Exiguobacterium and *Meiothermus ruber*, as well as in archaea the appearance of a band (19) distant from the halophilic group. These results added to the fact that there are similarities in the temperature and pH data (Table 2), and concentrations of certain metals (Table 3) would lead us to suppose that geysers (G1 and G2) tend to have a common origin and G3 a different origin.

In summary, the three sites tested had similar pH and water temperature, but each one of them had unique microbial diversity. Entire new clades of archaea and bacteria were identified, while other taxa of archaea and bacteria have uniquely diversified to live in these springs, which showed a high degree of microbial endemism. These results, the first of their kind in the CGF, will set the stage for continued research into the metabolic strategies, use, and identification of hyperthermophiles. This study is also important on biotechnological applications from the singular metabolism at a high temperature, which would have industrial benefits and improve the circular economy.

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Declarations

Conflict of interest The authors declare no competing interests.

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