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Microbial diversity in Tunisian geothermal springs as detected by molecular and culture-based approaches

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Abstract Prokaryotic diversities of 12 geothermal hot springs located in Northern, Central and Southern Tunisia were investigated by culture-based and molecular approaches. Enrichment cultures for both aerobic and anaerobic microorganisms were successfully obtained at temperatures ranging from 50 to 75°C. Fourteen strains including four novel species were cultivated and assigned to the phyla *Firmicutes* (9), *Thermotogae* (2), *Betaproteobacteria* (1), *Synergistetes* (1) and *Bacteroidetes* (1). Archaeal or universal oligonucleotide primer sets were used to generate 16S rRNA gene libraries. Representative groups included *Proteobacteria*, *Firmicutes*, *Deinococcus-Thermus*, *Thermotogae*, *Synergistetes*, *Bacteroidetes*, *Aquificae*, *Chloroflexi*, candidate division OP9 in addition to other yet unclassified strains. The archaeal library showed a low diversity of clone sequences belonging to the phyla *Euryarchaeota* and *Crenarchaeota*. Furthermore, we confirmed the occurrence of sulfate reducers and methanogens by amplification and sequencing of dissimilatory sulfite reductase (*dsrAB*) and methyl coenzyme M reductase α-subunit (*mcrA*) genes. Altogether, we discuss the diverse prokaryotic communities arising from the 12 geothermal hot springs studied and relate these findings to the physico-chemical features of the hot springs.

Keywords Archaea · Biodiversity · Ecology · Phylogeny · Physiology of thermophiles · Thermophile ecology · Systematics

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Introduction

Many terrestrial hot springs exist on Earth. The microbial communities comprising these hot springs are of considerable interest since they are analogs for primitive Earth (Pentecost 1996; Cavicchioli 2002), and are a source of thermostable enzymes (Demirjian et al. 2001; Schiraldi and De Rosa 2002). The terrestrial hot springs located in Iceland, New Zealand, Japan, the United States (National Park of Yellowstone), Italy (area of Naples), and Russia (Peninsula of Kamchatka) are the most extensively studied. In spite of active investigations, we know of only few factors that affect the diversity and the composition of the microbial communities in hot springs. Previous studies have shown that microbial community composition is influenced by physical variables in addition to chemical and biological factors such as salinity, pH, temperature and availability in nutrients among others (Lamberti and Resh 1983; Nold and Zwart 1998; Arnon et al. 2005; Fierer and Jackson 2006). It was also shown that the metabolic

processes of the native prokaryotic communities are closely associated with their environment.

The borders of Tunisia span the Sahara domain from the South, which is a part of the old African continent, to the younger alpine domain in the North. These two domains meet at the South Atlasic accident marked by the faults of Negrine-Kebili and Gafsa-Medenine. As a result three thermal regions exist in North Western, Central/Eastern and Western Tunisia (Jellouli 2002). Previous findings suggest that the thermal water of Tunisia is of meteoric origin. In these regions, water infiltrates by gravity deep into rock layers where it is heated and supplies the hot springs (Zouari and Mamou 1992; Bouri et al. 2008).

Hot springs are abundant in North Africa, and particularly in Tunisia where they are used for baths (Hammam). Although these have been well characterized in terms of their geological and geochemical properties, Tunisian hot springs have not been studied for their microbial communities. Such studies are required not only to understand the microbial communities that are native to the hot springs, but also to further identify novel taxa. Herein, we describe the microbial diversity, as determined by culture-based and molecular approaches, for 12 hot springs located in the three Tunisian thermal regions.

Materials and methods

Locality description

All samples used were individually collected from each Tunisian hot spring and marked. Major tectonic events have subdivided the area into a number of individual entities that are rather independent and characterized by their specific features and hydrogeothermal potentials. For this study, we concentrated on several springs that were located in three different thermal regions with increasing geological complication from the southeast to the northeast (Fig. 1).

In view of their meteoric origin, the Tunisian springs are dependent on the geology of the site and the nature of the crossed rocks.

Sample collection and isolation procedure

Sediment and water samples (43–72°C) were collected from geothermal hot springs located in Northeast, Northwest and South Tunisia during spring 2006, autumn 2006 and summer 2007 (Fig. 1). Samples collected for molecular analyses were stored at −80°C.

Samples for cultures were stored in the dark at 4°C until use. For anaerobic studies, the samples were reduced by adding 0.1 g/L $Na_2S{\cdot}9H_2O$.

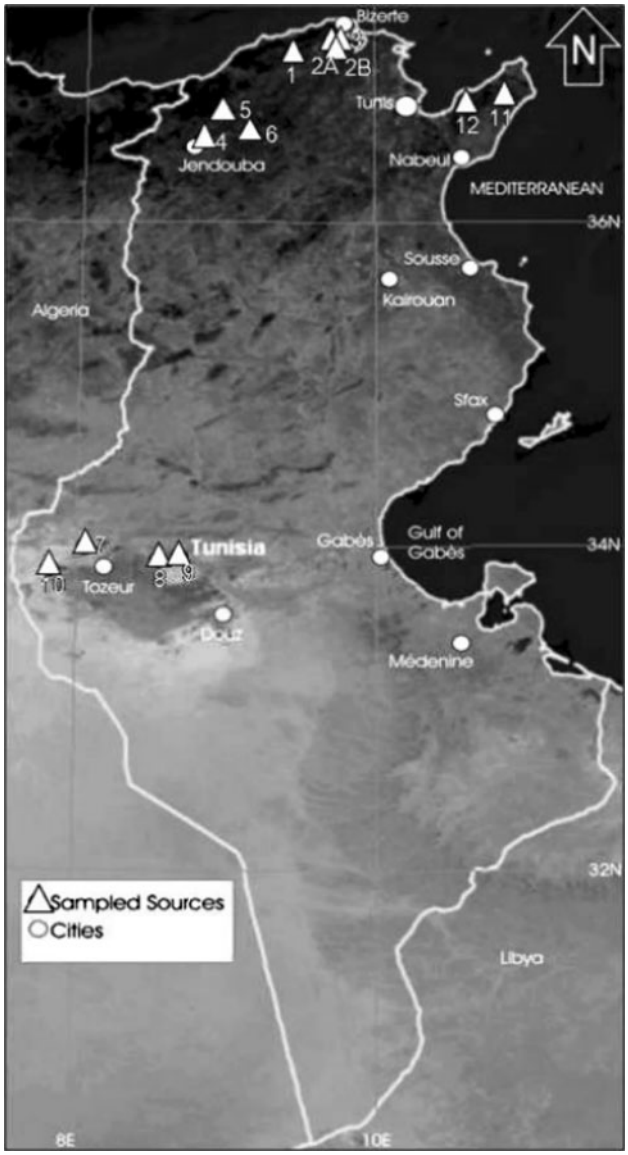

Fig. 1 Distribution map of the studied springs of Tunisia (modified from World wind: 9.58487E_33.79703N)

Enrichment, isolation and cultivation of aerobic strains were performed in medium 162 (Degryse et al. 1978) according to the modifications of Alfredsson et al. (1988) and by addition of 20 mL of phosphate buffer (containing per liter: KH_2PO_4: 5.44 g; $Na_2HPO_4{\cdot}2H_2O$: 21.4 g). For anaerobic cultures, we used Ravot medium (Ravot et al. 1995) and Widdel medium (Widdel and Pfennig 1981), two media suitable to grow fermentative prokaryotes (notably *Thermotogae*) and sulfate reducers.

The enrichment–isolation procedure was as follows: after incubation at different temperatures, positive enrichment cultures were subject to repeated dilutions for extinction series (Baross 1995) at the specified temperature and then spread on agar plates. Colonies were picked and purified by repeated streaking on plates.

Temperature, electrical conductivity and pH were measured with a portable thermometer Temp JKT (OAKTON Instruments, USA) and a portable pH meter (pH 320, WTW, France) at the sampling sites. Salinity was measured with a portable refractometer FG-211 (ARCADIA, Germany).

DNA extraction and 16S rRNA gene amplification

Genomic DNA was extracted from 10 mL environmental or enrichment culture samples with a FastDNA® SPIN Kit for soil (MP Biomedicals) according to the manufacturer's instructions. To avoid contamination, all manipulations were carried out in a PCR cabinet (Biocap™RNA/DNA, erlab®), using Biopur® 1.5 mL Safe-Lock micro-test tubes (Eppendorf™), RNase/DNase free water (MP Biomedicals™) and UV-treated plasticware and pipettes. The DNA extraction from isolates was according to Birnboim and Doly (1979). The 16S rRNA gene sequences were selectively amplified using the following primers: 8F and 1492R (Teske et al. 2002), the bacterial primers W02 and W18 (Godon et al. 1997), the archaeal PCR primers arc 21F-arc 958R (DeLong 1992), A8F-A1492R (Casamayor et al. 2002) (in the first round) and then arc915-arc344 (Eden et al. 1991) (in the second round if no significant band was observed on the gel after the first round). PCR cycles were as follows: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 1.5 min at the corresponding annealing temperature (58°C for arc 21F-arc 958R, 60°C for W02-W18, 53°C for 8F-1492R, 51°C for A8F-A1492R, and 57°C for arc915-arc344) and 72°C for 2 min, and a final extension step at 72°C for 6 min.

16S rRNA gene cloning and sequencing

The PCR products of 16S rRNA genes from the environmental samples were cloned into pGEM-T vector (Promega, Charbonnières, France). These led to the construction of libraries and sequences for single strands, above 700 bp long. Cloned sequences were aligned and compared to known sequences using NCBI Blast search program (Altschul et al. 1990).

Gene amplification

Methanogen-specific *mcrA* fragments were amplified with MCR (Springer et al. 1995; Lueders et al. 2001) and ME (Hales et al. 1996) primer sets which allow for the amplification of a region of ~503 and ~760 bp of the *mcrA* gene. The PCR products were then cloned into pGEM-T vector.

A portion of the *dsrAB* gene (1.9 kb) was amplified using the DSR primer sets DSR1F and DSR4R (Klein et al. 2001). PCR cycles were as follows: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 50°C (for MCR and ME) and 54°C (for DSR) for 1.5 min and 72°C for 2 min, and a final extension step at 72°C for 6 min.

The PCR products were separated by electrophoresis and bands showing the expected size were excised and purified (QIAquick Gel Extraction kit, Qiagen, Hilden, Germany). The products were then cloned into pCR2.1 (TOPO TA cloning kit, Invitrogen, Cergy Pontoise, France).

Phylogenetic reconstruction

The 16S rRNA gene sequences were first assessed for chimeras using the CHIMERA-CHECK online analysis program of the RDP-II database (Cole et al. 2003), and for homologies to known sequences in GenBank at nucleotide and amino acid level using Blast analysis (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al. 1990). The *dsrAB* sequences were translated into amino acids using the BioEdit 7.0.5.3 program (Hall 1999) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with the Clustal W program (Thompson et al. 1994). PHYLO-WIN with the neighbor-joining method (Saitou and Nei 1987) and PAM distance (Dayhoff et al. 1978) were then used for phylogenetic tree construction. The robustness of inferred topologies was tested by bootstrap resampling (500 replicates).

Statistical analysis

Principal component analysis (PCA) for physico-chemical data was calculated with StatBox 6.7 (GrimmerSoft). Chi-square test was calculated using the Excel statistical task.

Accession numbers

The sequences were deposited in the EMBL Nucleotide Sequence Database as follows: 16S rRNA genes (FN666100 to FN666231 and FN666239 to FN666246), *dsrAB* genes (FN666232 to FN666236) and *mcrA* genes (FN666237 and FN666238).

Results and discussion

Hot springs and samples

Twelve hot springs located in the three thermal regions of Tunisia were investigated by collection of 13 samples of sediments and water. Spring 2A is a stream that discharges into spring 2B, even though they are considered as separate springs due to their different temperature, pH and salinity. These two springs are located in the same 20-m wide

region as springs 1 and 3; therefore, they have the same name (Hammem Atrous). While the majority of Tunisian hot springs are in use, these two springs are not in use. Their temperature range, pH and salinity in addition to their chemical composition (data provided by the Office of the Tunisian Balneology) determined at the sample site are reported in Table 1.

Generally, the pH of spring waters is neutral, but two of the springs tested were slightly alkaline (spring 2B at pH 8.44 and spring 10 at pH 8). The salinity ranged from 3 g/L to approximately 20 g/L NaCl, with the most saline springs located in the North and South (springs 1, 2, 3, 4, 11 and 12). Temperatures ranged from 37.5°C (spring 2B) to 73°C (spring 5). Generally, springs with the highest temperatures were the least saline (springs 5, 6, 7, 8 and 9). Spring 5 (Ain Essalhine), which has the highest recorded temperature in Tunisia, is located in a complex geological area (Jellouli 2002). In this spring, we noted the presence of hydrogen sulfide (H_2S) and gas bubbles of unknown composition.

Isolation and enrichment of thermophilic prokaryotes

Following incubation at temperatures ranging from 50 to 75°C, we obtained enrichment cultures for springs 2B, 5, 6, 8, 10, 11 and 12 on media 162 or Ravot (Table 2). No growth was observed at temperatures above 75°C (using all media) or on the Widdel medium designed for sulfate reducers. Fourteen strains were isolated as pure cultures and their 16S rRNA genes were amplified, sequenced and compared to known sequences (Table 2). Strains affiliated to the genus *Anoxybacillus* (Bacillaceae) were isolated from springs 2B, 5, 10 and 12. Specifically, isolate AE3 from spring 5 showed 99% of similarity with *Anoxybacillus flavithermus*. Strain AE5 from spring 10 showed 100% identity with *Anoxybacillus pushchinoensis*. Also, isolate AE2 from spring 2B showed 99% of similarity with '*Anoxybacillus kamchatkensis* subsp. *asaccharedens*', and isolate AE7 from spring 10 showed 99% identity with *A. kamchatkensis*. On the other hand, isolates A06 and H07 from springs 10 and 12 appeared to be novel species as '*Anoxybacillus tunisensis*' (Sayeh et al., submitted). Furthermore, isolates showing 98–100% identity with *Tepidibacter formicigenes* (J2B), *Bacillus licheniformis* (AE6), *Hydrogenophilus thermoluteolus* (AE9), *Geobacillus lituanicus* (AE8) and *Thermonema rossianum* were obtained from springs 2B, 5, 6, 10, and 11, respectively. While three isolates, R8 from spring 8, R101 from spring 10 and G60 from spring 11, showed some similarity to previously described isolates (Stackebrandt and Ebers 2006), they in fact appeared to be novel species. They will be described as '*Fervidobacterium geothermalis*' (*Thermotogales*), '*Thermoanaerovibrio tozeurensis*' (*Synergistetes*) and '*Thermosipho korbousensis*' (*Thermotogales*) (Sayeh et al., submitted).

Prokaryotic diversity

Often, the comparison of the 16S rRNA gene sequences obtained from the springs to public databases resulted in a

Table 1 Physico-chemical data of Tunisian hot springs

No.	Sample	Location	pH	*T* (°C)	*S* (g/L)	*C* (mS/cm)	Ca[a]	Mg[a]	Na[a]	K[a]	Cl[a]	SO_4^a	HCO_3^a
1	Hammem Atrous (Ichkeul)	ND	6.27	48.5	11	3.63	ND	ND	ND	ND	ND	ND	ND
2A	Hammem Atrous (Ichkeul)	ND	6.21	51.5	13	3.78	ND	ND	ND	ND	ND	ND	ND
2B	Hammem Atrous (Ichkeul)	ND	8.44	37.5	18–20	5.42	ND	ND	ND	ND	ND	ND	ND
3	Hammem Atrous (Ichkeul)	ND	6.26	49	11	3.74	800	486	3020	78	5484.7	2047.1	439.2
4	Hammem Sayala	ND	6.9	43.2	13	6.63	280	121.6	4300	28	7455	903	355
5	Ain Essalhine	40.83175′N/6.39935′E/ altitude 212F	6.49	73	1	6.64	80	24.32	215	7	355	70	244
6	Ain Essalhine (spring called Kibrite)	ND	6.67	66.2	1	7.77	ND	ND	ND	ND	ND	ND	ND
7	Drilling of Nefta	33.52844′N/7.52842′E/ altitude 285F	6.68	70.1	4	6.58	520	31.61	500	92.5	603.5	1200.5	170.8
8	Hammem Mahassen	34.0083′N/8.1456′E/ altitude 328F	6.48	71.5	3	6.32	376.7	43.12	347.01	75.12	485.3	991.8	207.4
9	Hammem Bouhlel	34.00939′N/8.15729′E/ altitude 240F	7.73	60.4	3	6.65	391.52	40.91	323.11	77.86	472.84	981.26	219.4
10	El Hamma of Tozeur[b]	34.00314′N/8.10432′E/ altitude 350F	8	51.6	5	7.73	372	138.62	648.6	23.4	1001.1	1339.2	140.3
11	Ain Atrous	40.7690′N/6.39935′E/ altitude 4F	6.7	58.6	11	2.96	800	24.7	2935.2	89.7	4970	2110.1	536.8
12	Ain Sbia	ND	7.2	50	11	ND	780	182.4	3088.9	93.2	5266	1364	512.4

S salinity, *C* electrical conductivity, *F* feet

[a] Mineral composition (mg/L) of water springs is supplied by the Tunisian Office of Balneology (Office du thermalisme)

[b] The water of the drilling feeds Hammam El Borma

Table 2 Thermophilic bacteria isolated from Tunisian hot springs in this study

Sample	Enrichment and isolation media	*T* (°C) of isolation[a]	Strain	Most closely related isolate	Sequence identity (%)[b]
2B	162	65	AE2	*Anoxybacillus kamchatkensis* subsp. '*asaccharedens*'	99
	Ravot	50	J2B	*Tepidibacter formicigenes*	99
5	162	70	AE3	*Anoxybacillus flavithermus*	99
	162	65	AE6	*Bacillus licheniformis*	100
6	162	65	AE9	*Hydrogenophilus thermoluteolus*	99
8	Ravot	70	R8[c]	*Fervidobacterium pennivorans*	98
10	162	65	AE5	*Anoxybacillus pushchinoensis*	98
10	162	60	AE7	*Anoxybacillus kamchatkensis*	99
10	162	50	AE8	*Geobacillus lituanicus*	99
10	162	65	A06[c]	*Anoxybacillus flavithermus*	99
10	Ravot	60	R101[c]	*Thermoanaerovibrio acidaminovorans*	97
11	162	65	G11	*Thermonema rossianum*	99
11	Ravot	65	G60[c]	*Thermosipho japonicus*	98
12	162	65	H07[c]	*Anoxybacillus flavithermus*	99

[a] Temperature of positive enrichment culture and isolation

[b] The sequence identity was based on Blast analysis

[c] The temperature of isolation of these strains corresponds to the optimum temperature for growth

low similarity value. 75% of the sequences were below the threshold value (98.7% 16S rRNA gene sequences similarity) generally admitted for the separation of species (Stackebrandt and Ebers 2006), and 31% fell below the genus threshold (95%) (Ludwig et al. 1998). This finding may indicate that there are numerous unknown microorganisms in these springs.

Bacteria

Fifty clones were sequenced for each sample and were compared to known sequences or isolates (Table S1). Our phylogenetic analysis placed these clones in nine major Bacteria phyla (Fig. 2). These were as follows: *Firmicutes* (97 clones) including *Clostridium*-like sequences and *Bacillus*-like sequences; *Proteobacteria* [*Alphaproteobacteria* (44 clones), *Betaproteobacteria* (2 clones), *Deltaproteobacteria* (36 clones), *Gammaproteobacteria* (47 clones)], *Deinococcus-Thermus* (6 clones), *Nitrospirae* (3 clones), *Thermotogae* (5 clones), *Synergistetes* (4 clones), *Aquificae* (8 clones), *Bacteroidetes* (5 clones), *Chloroflexi* (green non-sulfur bacteria) (1 clone) and the environmental candidate division OP9 (2 clones). In addition, 12 clones were closely related to unclassified Bacteria.

The 16S rRNA diversity is shown in Fig. 2. Libraries constructed from DNA extracts from spring 5 (Ain Essalhine) and amplified with the different primer pairs showed some unique sequences, but for the most part the phylogenetic groups are known (data not shown). Therefore, a combination of results obtained using different primer sets were used to characterize this spring. For spring 8 (Hammem Mahassen), the amplification of environmental 16S rRNA was unsuccessful. In order to avoid contamination, we did not perform nested PCR for the amplification of bacterial 16S rRNA.

(i) *Proteobacteria*. *Proteobacteria* were largely represented in all clone libraries constructed from the different springs. Diversity among this taxon varied at the subdivision level from spring to spring.

(a) *Alphaproteobacteria*. Alphaproteobacterial-related sequences were present in five of the hot springs (1, 2A, 2B, 10 and 12), and were particularly numerous in spring 1 (91%). Sequences derived from spring 1 (48.5°C, pH 6.27 and 1.1% of NaCl) were closely related (97–99% identity) to marine clones and isolates. P2RS50 was affiliated to the deep sea strain P24, *Oceanibaculum indicum*, a moderately halophilic and mesophilic bacterium where growth is observed at salinities of 0–9% and at temperatures of 10–42°C (Lai et al. 2009). Clones P2RS76 and P2RS60 were related to *Parvibaculum lavamentivorans*, an aerobic heterotrophic mesophilic bacterium (Schleheck et al. 2004), and to the marine isolate JP57, respectively. *Alphaproteobacteria* were less abundant in all other clone libraries yet were correlated to clones originating from soil samples. In fact, this genus represented only 18% of the bacterial sequences from spring 2A and showed 99% 16S rRNA sequence similarity to *Rhizobiales* bacterium clone RDX 10, which is from contaminated soil (Roh et al.

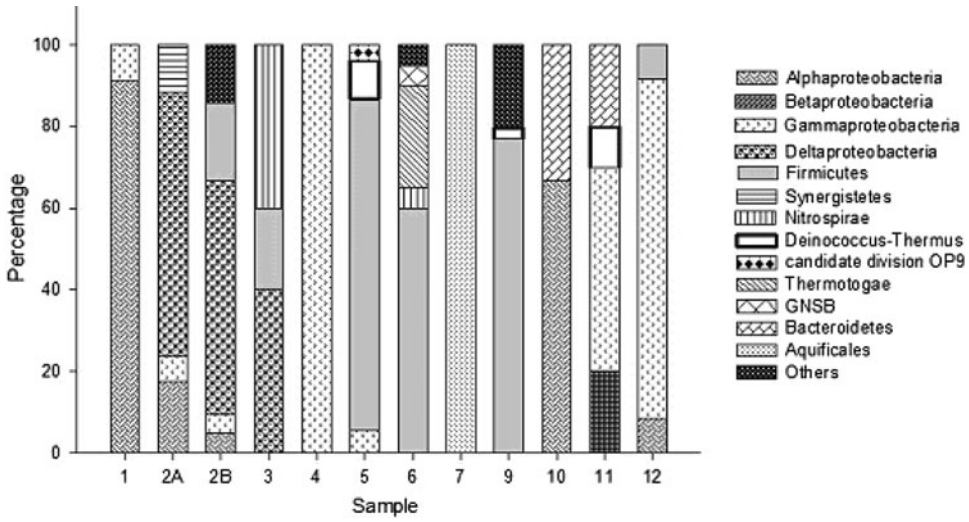

Fig. 2 Phylum level grouping of bacterial 16S rRNA gene sequences obtained from springs

2009). Springs 2B, 10 and 12 contained only one to six sequences of *Alphaproteobacteria* with an identity of 92–94%.

(b) *Betaproteobacteria*. *Betaproteobacteria* were retrieved only in spring 11, with two clones related to clone 390 from Altamira Cave (91% similarity).

(c) *Deltaproteobacteria*. Members of this group were dominant in springs 2A and 2B. Eighteen clones from spring 2A and 10 clones from spring 2B were 96–97% similar to *Desulfopila aestuarii*, an anaerobic sulfate-reducing bacterium isolated from estuarine sediment in Japan. For optimal growth, *D. aestuarii* requires a pH of 6.3–8.5 (optimum growth 7.5–7.6), temperatures ranging from 10 to 40°C (optimum 35°C), and NaCl concentrations from 0 to 5% (w/v) (optimum 1%; Suzuki et al. 2007). Growth parameters for *D. aestuarii* are consistent with the physico-chemical conditions of springs 2A and 2B (Table 1) located in the Ichkeul area where elevated concentrations of sulfate have been reported (Jellouli 2002). In addition, we also found 3 clones that are affiliated to environmental *Deltaproteobacteria* in springs 2A, 2B and 3. Clone P10B3RS3 (spring 3) was related to Dan_Bac69, an uncultured clone from oil production water whose closest isolate is *Desulfoglaeba alkanexedens* (DQ303457; 92% similarity), a mesophilic sulfate-reducing alkane-oxidizing bacterium with optimum growth at 31–37°C and a pH of 6.5–7.2, respectively. This organism does not require NaCl for growth, but salt concentrations as high as 55 g/L are tolerated (Davidova et al. 2006).

(d) *Gammaproteobacteria*. Sequences affiliated to this taxon were dominant in clone libraries from springs 4 (100%), 11 (50%) and 12 (greater than 90%). *Gammaproteobacteria* were also present at lower quantities (less than 9%) in springs 1, 2A, 2B and 5, and were absent in the libraries from springs 3, 6, 7, 9 and 10. Most gammaproteobacterial sequences were *Pseudomonas*-related sequences where 20 clones from spring 4 were affiliated to *Pseudomonas stutzeri*. Clone P1B1G34 (4 clones) from spring 11 was similar to isolate *Thiofaba tepidiphila* (95% 16S rRNA gene sequence similarity). This is an obligate chemolithoautotrophic sulfur-oxidizing bacterium isolated from hot springs with optimum growth conditions at 45°C, pH 6.5 and absence of NaCl (Mori and Suzuki 2008).

(ii) *Firmicutes*. This phylum was highly represented in the clone libraries from springs 5, 6 and 9 (81, 60 and 76% of the bacterial sequences, respectively). These springs share similar conditions including elevated temperatures (between 60.4 and 73°C), near neutral pH and low salinity (between 0.1 and 0.3%). Retrieved sequences included *Clostridium*- and *Bacillus*-like sequences, primarily those related to hydrothermal sediment clones and isolates. The bacterial clone library from spring 5 showed sequences of *Clostridiales* affiliated to the sulfate reducer *Desulfosporosinus*, the *Desulfosporosinus meridiei* strain S10 isolated from a shallow aquifer polluted with gasoline and also the *Desulfosporosinus auripigmenti* strain OREX-4 collected from surface sediments of the Upper Mystic Lake (Newman et al. 1997). The latter strain is shown to have optimal growth at a temperature of 25–30°C; however, this is lower than the temperature we recorded for spring 5 (73°C). Sequence P2RS19 from spring 5 was closely related (96% of similarity) to the sequence of *Caldicellosiruptor kristjanssonii* (I77R1B), an anaerobic cellulolytic, neutrophilic (pH 5.8–8.0), thermophilic (optimum at 78°C) bacterium from an Icelandic hot spring (Bredholt et al. 1999). The physico-chemical conditions found in spring 5 (73°C, pH 6.49) were satisfactory for the growth of this bacterium. Sequences from spring 5 were closely related to sequences from anoxic rice paddy soils, where P2RS3 (10 clones) were related to clone BCV51, while P14b5RS45 (4 clones) were related to strain XB90. The isolate BSV51 and the clone XB90 belonged to

Clostridiales cluster XIVa. Four other sequences from spring 5 were affiliated with two clones from rice field soil. The second highest proportion of *Firmicutes* was found in spring 9 where the majority of sequences (20 clones) were affiliated to clone D6-41 (93% similarity) from aerated pig manure slurry, and 12 other clones affiliated to sequences from urban aerosol, showing 97% similarity with the isolate *Bacillus niacini*. Clone P9B2BRS14 from spring 2B was related to *Halobacillus* sp. SR5-3 (96% similarity). This organism is a moderate halophile, growing in NaCl concentrations of 10% and up to 30% (Namwong et al. 2006). There were no sequences from springs 2A, 4, 7, 10 and 11 that were affiliated with the phylum *Firmicutes*.

(iii) *Deinococcus-Thermus*. This group was found only in springs 5, 9 and 11 clone libraries. The majority of sequences were related to *Thermus thermophilus* and *Meiothermus timidis*. Such organisms are thermophilic, aerobic and chemo-organotrophs that are frequently isolated from hot springs (Kanokratana et al. 2004).

(iv) *Aquificae*. All members of *Aquificales* are aerobic, generally utilizing H_2 or S as electron donors and O_2 or NO_3^- as terminal electron acceptors (Madigan and Martinko, 2006). Spring 7 contained members from this group which were affiliated to *Hydrogenobacter subterraneus*, an extremely thermophilic (optimal temperature 78°C), strictly aerobic and heterotrophic bacterium from subsurface geothermal water. This is the sole taxon found in the bacterial clone library of spring 7. As this is found in the hyperthermophilic smokers (Purcell et al. 2006), it suggests that H_2 could be a main energy source in hot spring 5 (Spear et al. 2005).

(v) *Nitrospirae*. Only two clones from spring 3 and one from spring 6 were affiliated with this group. P3RS26 showed similarity to three clones. These included clone D14, which was obtained from fuel cell enriched with artificial wastewater, the sulfate-reducing bacteria *Thermodesulfovibrio islandicus* (92% similarity), and *Thermodesulfovibrio hydrogeniphilus*, which was previously isolated from the same Tunisian spring (91% similarity) (Haouari et al. 2008).

(vi) *Bacteroidetes*. Two clones from spring 11 and three clones from spring 10 were affiliated to the phylum *Bacteroidetes*. Clone P7b10RS1 showed 94% 16S rRNA sequence similarity with the sequence of *Rhodothermus marinus*, a submarine thermophilic bacterium (Alfredsson et al. 1988).

(vii) *Thermotogales*. Five clones from spring 6 were closely related to an uncultured *Thermotogales* (clone B17_otu14) bacterium obtained from an oil field.

(viii) *Green non-sulfur bacteria*. Clone P3RS44 from spring 6 was strongly related (98% similarity) to a green, non-sulfur bacterium from the Yellowstone National Park: OpB11.

(ix) *Candidate division OP9*. Sequence P14b5RS47 (2 clones) from spring 5 was affiliated with the clone OPB47 (94% similarity) from the Obsidian Pool (Yellowstone National Park).

(x) *Synergistetes*. Four clones (P3RS67) from spring 2A were highly homologous to an uncultured *Synergistes* sp., clone D004011B05 from the Alaskan mesothermic petroleum reservoir. *Synergistetes* have been found in an assortment of anaerobic habitats. Despite the various habitats, the isolates show striking physiological resemblances in that they are all anaerobic, neutrophilic, Gram negative rods that ferment amino acids (Hugenholtz et al. 2009).

Statistical analysis

Our findings showed that the microbial diversity varied tremendously among the springs, regardless of their close proximity (e.g., sources 1, 2B and 3 are found within a 20 m zone) or geological zone (sources 1, 2B, 3, 4 and 5 belong to the same geological region). To assess the relationship between physico-chemical parameters and microbial diversity found in the Tunisian thermal springs, we performed a statistical analysis. A first PCA of the main physico-chemical data divided the springs into five groups visible on F1–F2 (97% of the whole variance). The groups were differentiated by their temperatures and salinities, which were inversely correlated to the first axis, and secondarily by their pH that is shown on the second axis (Fig. 3). The second PCA included all available physico-chemical variables. In this case, fewer samples showed that salinity positively correlated to the ionic charge of water and negatively correlated with temperature.

We then studied the distribution of taxonomic groups versus water characteristics.

A Chi-square test of independence showed that the composition of microbial communities was dependent on the physico-chemical characteristics of water. Figure 2 shows the composition of bacteria in the five groups determined by PCA (Table 3). The data indicated that at high temperatures (60°C) sequences related to the *Firmicutes* phyla are highly represented (group 1 and group 5). At lower temperatures (particularly groups 2, 3 and 4), sequences belonging to the *Proteobacteria* phylum are largely represented in the clone libraries. Group 2 largely contained the *Gammaproteobacteria* subdivision, where the genus *Pseudomonas* was most represented. Group 4 represented only spring 2B (the coldest spring in this study) that was dominated by *Alphaproteobacteria* and showed a large amount of *Deltaproteobacteria*. We also noted the presence of *Firmicutes*-related sequences which are found in mesophilic habitats. Group 3 contained *Deltaproteobacteria*. Most representatives of this subdivision are

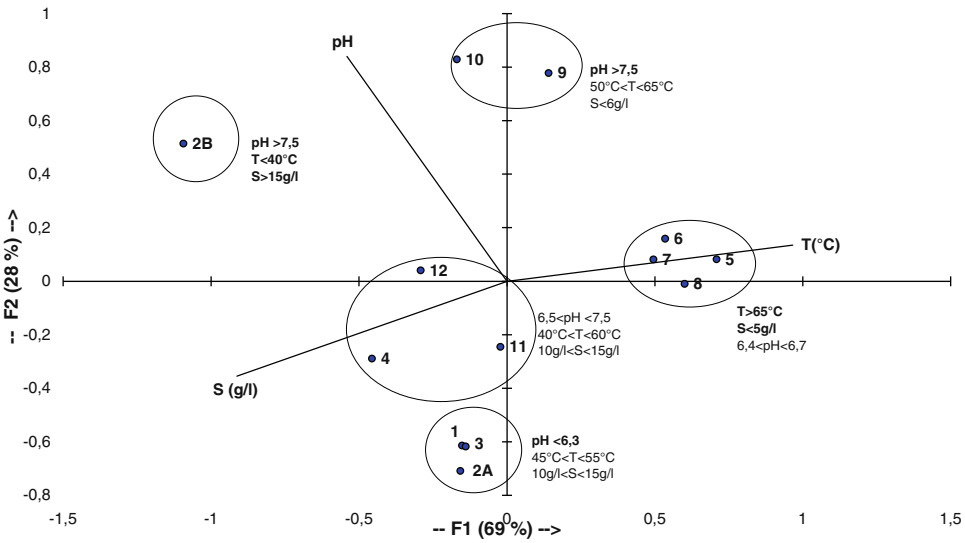

Fig. 3 Statistical analysis of physico-chemical data of Tunisian hot springs

Table 3 Characteristics of the different groups of Tunisian hot springs

Group	Springs	T (°C)	pH	Salinity (g/L)
1	5, 6, 7 and 8	>65	$6.4 < pH < 6.7$	<5
2	11, 12 and 4	$40 < T < 60$	$6.5 < pH < 7.5$	$10 <$ salinity < 15
3	1, 2A and 3	$45 < T < 55$	<6.3	$10 <$ salinity < 15
4	2B	<40	>7.5	>15
5	9 and 10	$50 < T < 65$	>7.5	<6

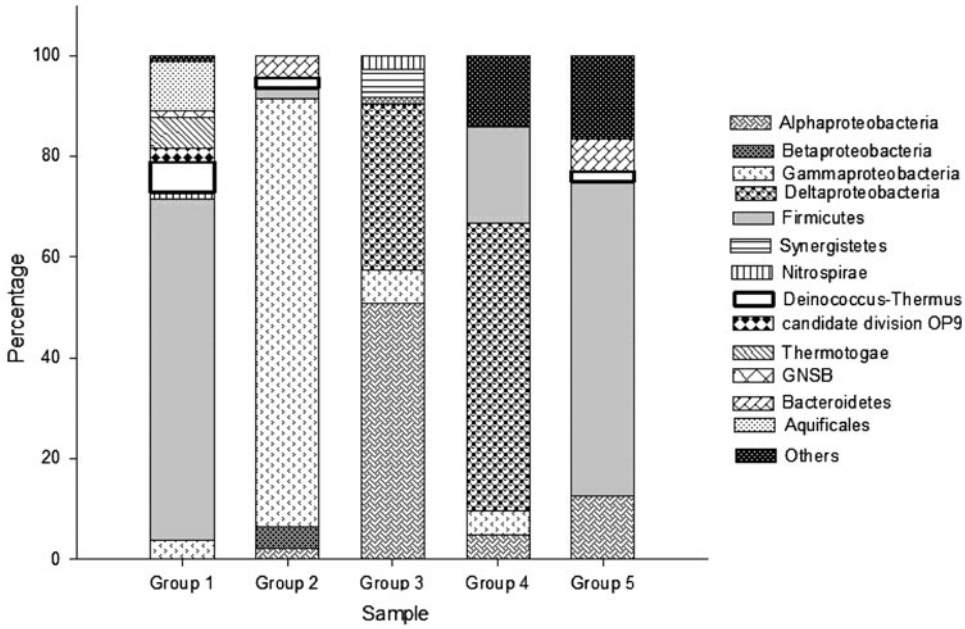

Fig. 4 Phylum level grouping of bacterial 16S rDNAs obtained per group of springs based on PCA results (Distribution in the main taxonomic groups: *Alpha-*, *Delta-*, *Gammaproteobacteria*, *Firmicutes* and others depend on physico-chemical characteristics/Chi-square test, $p = 2.6 \times 10^{-71}$, very highly significant)

sulfate reducers. We found that source 3 (group 3) was high in sulfates. Furthermore, springs belonging to group 3 were slightly acid, yet sequences affiliated to *Sulfobacteriales* or *Acidobacteriales* were not present in their archaeal and bacterial clone libraries. These findings are contrary to other acid sources, such as the Champagne pool (Iceland) and Bor Khlueng (Thailand), where sequences belonging to these orders were abundant (Hetzer et al. 2007; Kanokratana et al. 2004). On the other hand, sequences belonging to clone AK8 from the hot acid spring Bor Khlueng were largely represented in the archaeal clone library from group 3 (Fig. 4).

Diversity of *Archaea*. The analysis of the archaeal clone libraries obtained from Tunisian hot springs (Table S2) showed clones belonging to two major phyla: *Crenarchaeota* and *Euryarchaeota*. The crenarchaea were highly represented (274 *Crenarchaeota* clones as compared to 88 *Euryarchaeota*) and distributed among the hot springs. Nearly all archaeal sequences were associated to uncultured *Archaea*, except for clone P8a8RS5 that affiliated to the haloarchaea *Haloferax larsenii* strain ZJ203 with 94% similarity. According to Blast searches, other sequences are related to environmental clones. Some of these are also correlated to cultivated species including: P6a4RS35 (*Methanosaeta thermophila* PT), P6a4RS43 (*Methanospirillum hungatei*), P10a5RS68 (*Archaeoglobus fulgidus* VC-16), P14A5RS89 (*Thermoproteus neutrophilus*), P8a8RS43 (*Natronomonas pharaonis*), P1A2RS28 (*Halococcus* sp. HPA-87) and P8a8RS37 (*Halorhabdus tiamatea* SARL4B).

(i) *Phylum Crenarchaeota*. The spring 3 clone library revealed the presence of a single archaeal clone with 95% similarity to clone AK8, which was obtained from the Bor Khlueng hot spring in Thailand. This clone was also found in spring 4 (7 clones with 98% similarity sequence). The presence of this clone in all three springs is of interest since each spring has dissimilar physico-chemical characteristics. Regardless, it suggests that clone AK8 could be ubiquitous to terrestrial environments.

Clone P4E1RS1 (from spring 1) was highly related to the crenarchaeal clone ERB-A-A2, which was obtained from hot mineral and acidic soil (Soo et al. 2009). Unlike ERB-A-A2, the P4E1RS1 clone was found in a source with a neutral pH and a temperature of 48°C. Sequences P4E1RS6 (16 clones) and P4E1RS32 (12 clones) from spring 1 were related to clone Elev_16S_arch_1892 and clone Elev_16S_988, respectively, both from soils influenced by elevated atmospheric CO_2. This finding may be explained by the fact that Jebel Ichkeul is a massive carbonate reservoir (Jellouli 2002) with a high rate of atmospheric CO_2 emitted. Sequence P1A2RS38 from spring 2A was identical to clone HTA-G6. While HTA-G6 was obtained from a freshwater reservoir whose temperature is at 14°C and pH at 7 (Stein et al. 2002), P1A2RS38 grew at 51.5°C and at a pH of 6.21. Clone P6a4RS7 from spring 4 (temperature 43.2°C) showed 98% similarity to an uncultured *Crenarchaeota* clone: r-MDS-E06 from mesophilic digested sludge (37°C). P10a5RS61 from spring 5 was related to an uncultured *Crenarchaeota* (clone: Haud LA42). This clone is from geothermal subsurface waters within the Miscellaneous Crenarchaeotic Group (MCG), which is dominant in the upper mat of a hot water stream in the Hishikari gold mine (Japan) (Nunoura et al. 2005). Other crenarchaeotic sequences were affiliated to submarine hydrothermal clones within the MCG group that have also been found in other sedimentary, aquatic and terrestrial environments (Teske et al. 2002). Clone P14A5RS55 was affiliated to an uncultured *Crenarchaeota* clone OPPD030 from the Yellowstone geothermal system (Obsidian Pool). Clone P9a2BRS78 from spring 2B was related to clone HMMVCen-19 that was obtained from the Haakon Mosby mud volcano. HMMVCen-19 belongs to the Marine Benthic group which has often been found at methane seeps, but is not restricted to seeps (Lösekann et al. 2007). It is possible that spring 2B originates at great depth. Two clones of *Thermoproteales* were obtained from spring 5. One of these included P14A5RS89, which showed 94% similarity with clone Hverd033N obtained from an Icelandic hot spring.

(ii) *Phylum Euryarchaeota*. Spring 8 (Hammem Mahassen; $T = 71.5$°C, pH 6.48, NaCl 0.3%) is characterized by an abundance of *Euryarchaeota* represented by the class of *Halobacteriales*, which exists in high saline environments. Clone P8a8RS5 is far related (94%) to *Haloferax larsenii* isolated from a solar saltern, but this genus also includes species that are capable of long-term survival at low (4%) salt concentrations (Elshahed et al. 2004). A total of 6 clones represented by clone P8a8RS30 were similar (96%) to clone ss007 found in dry saline soils (Walsh et al. 2005). Another 16S rRNA sequence was *Halorhabdus tiamatea* (SARL4B strain, 94%), an extremely anaerobic halophilic archaeon with a NaCl growth range between 10 and 30% (w/v), optimal at 27% (w/v) NaCl (Antunes et al. 2008). The archaeal clone P8a8RS47 showed 98% similarity with the haloarchaeon clone from estuarine sediments 9AH, which is capable of growth at 3.5% NaCl (w/v). It has been demonstrated that some haloarchaea are capable of growth at salinities near that of seawater, even if they prefer higher salinities (Purdy et al. 2004). Our findings parallel this hypothesis and further showed that these halophilic microorganisms are likely to exist in low-salinity environments (0.3%). We also found in spring 2A (NaCl 1.3%) seven *Halobacteriales* clones. The representative sequence of the seven *Halobacteriales* clones was P1A2RS28, which is comparable to clone Fav1-Arch31 and showed 95% similarity with the *Halococcus* sp., HPA-87. Previous studies showed that *Halococcus*-related sequences were recovered from a sulfur spring at <1% NaCl (Elshahed et al. 2004). Clone Fav1-Arch31 was found in corals and was responsible for anaerobic denitrification (Siboni et al. 2008). Other sequences of *Halobacteriales* with similarities (less than 95%) to known clones were also found in different springs. These sequences have been recorded in spring 8, which contained the highest proportion of halophilic *Euryarchaeota*, and also in springs 2A, 2B and 9. Sequences comparable to clone haloarchaea TX4CA found in saline-alkaline soil were present in springs 8, 2A and 2B, yet only spring 2B was alkaline. *Halalkalicoccus jeotgali* is the closest isolate. Other sequences similar to haloarchaea that can survive at

low salinities were also represented in the archaeal clone libraries such as clone ZAR23 from the low-salt, sulfide- and sulfur-rich springs 2A and 2B (salinity between 0.7 and 1%) (Elshahed et al. 2004).

In addition, low proportions of other lineages of the *Euryarchaeota* are represented in the Tunisian hot springs. For example, sample 4 contained ten clones represented by P6a4RS35 that were affiliated (97%) to clone TP123 obtained from phenol-degrading methanogenic consortia whereby the thermophilic methanogenic archaeon *Methanosaeta thermophila* PT being the nearest cultivated representative. *Methanosaeta* species are the most prevalent methanogenic archaea found in a wide variety of communities. One clone, P6a4RS43, which belongs to the family *Methanomicrobiales*, was found in sample 4. Clone P10a5RS68 from spring 5 (4 clones) was 91% similar to clone PW45.1 obtained from a petroleum reservoir and the isolate *Archaeoglobus fulgidus* (VC-16), a hyperthermophilic sulfate-reducing archaeon within the family of *Archaeoglobales*. Spring 10 contained *Euryarchaeota* (27 clones) whose representative, P6a10RS93, affiliated (97%) to clone SAGM-J from deep South African gold mine waters (Takai et al. 2001).

Study of functional genes

The search for functional genes for sulfate-reducing microorganisms and methanogens was performed on samples containing sequences affiliated to the 16S rRNA clone libraries or with high sulfate content. The hot springs corresponding to these criteria were springs 2A, 2B, 4, 5 and 6. Only the PCR amplification for *dsrAB* genes and *mcrA* from the Ain Essalhine hot spring (spring 5) was successful. PCR results for *dsrAB* revealed two clones related to the genus *Desulfotomaculum* (*Clostridiales*). Two other clones were related to the genus *Desulfacinum* (*Deltaproteobacteria*). These clones were not represented in the 16S rRNA libraries; in addition, sequences for sulfate-reducing bacteria were not revealed in the functional gene library. Only *Archaeoglobus*-related sequences were

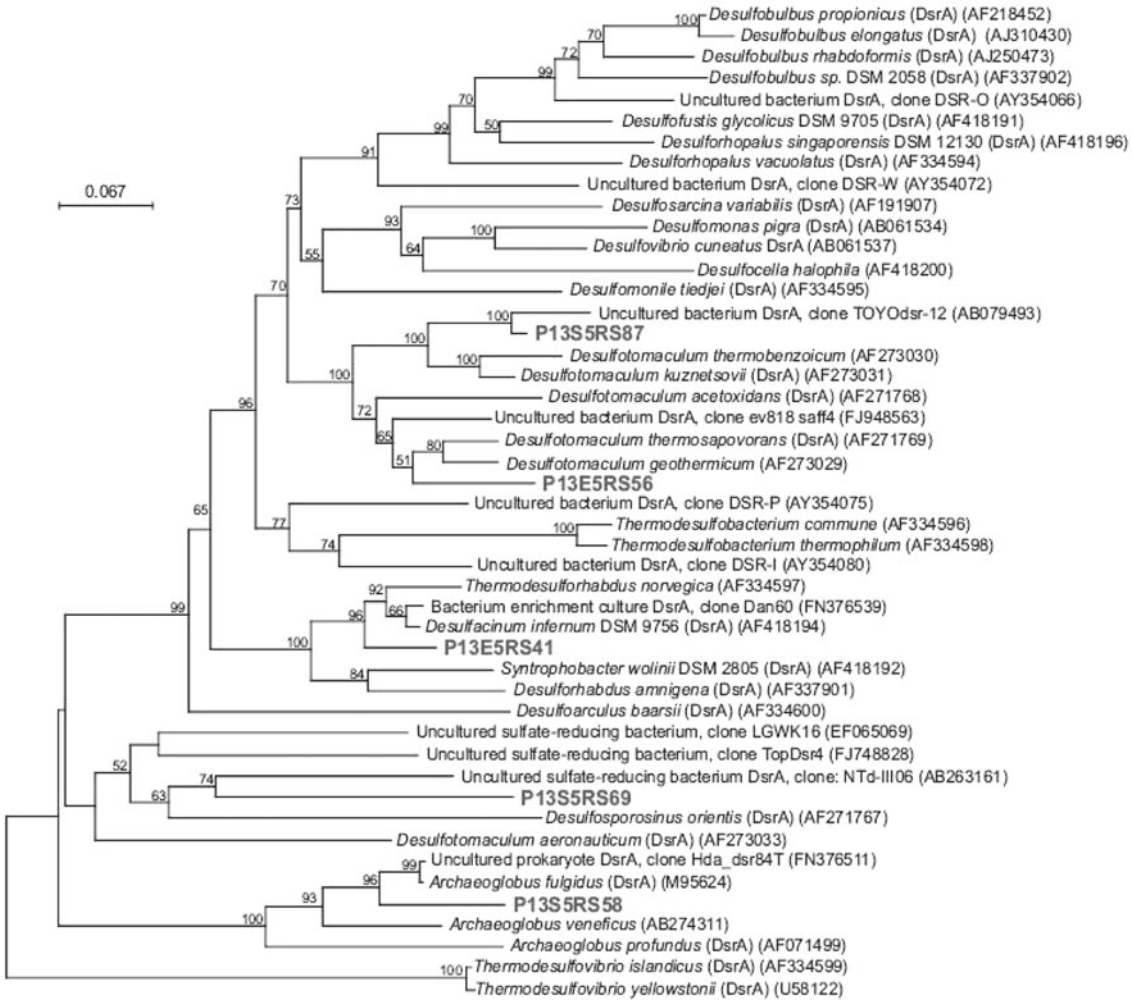

Fig. 5 Neighbor-joining tree based on the translated partial-length DsrA amino acid sequences from spring 5

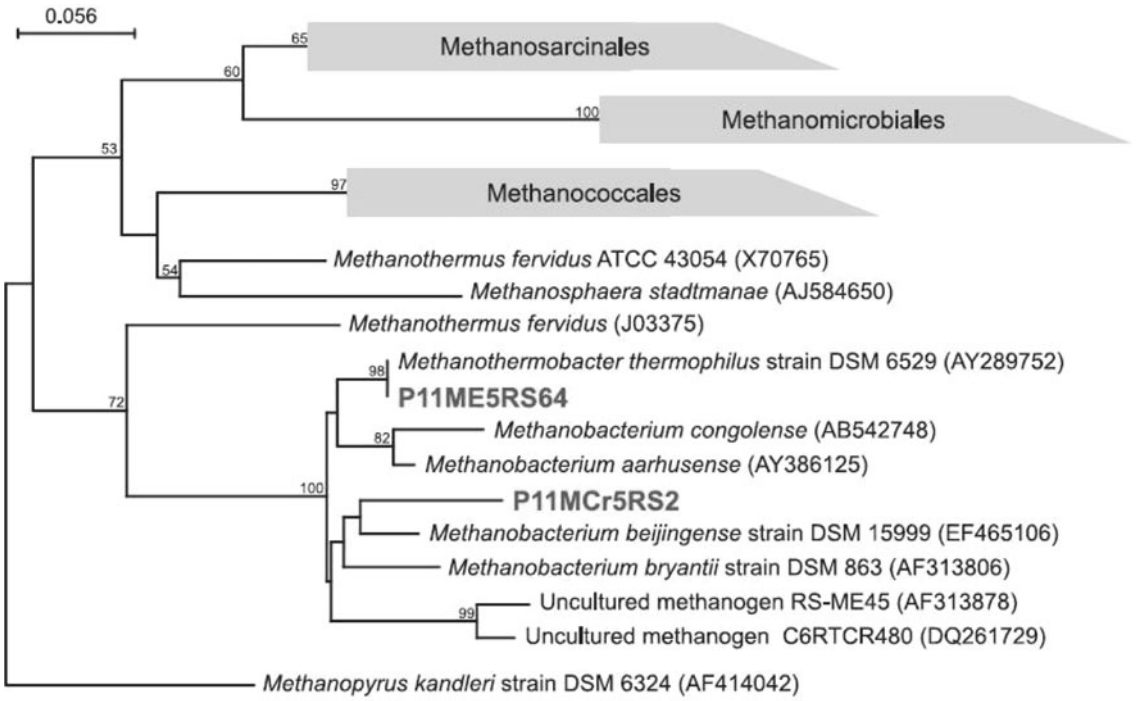

Fig. 6 Phylogenetic dendrogram indicating the relationship among translated mcrA amino acid sequences from spring 5 (Ain Essalhine), with known methanogens sequences in GenBank

found in the two libraries (16S rRNA and functional genes: Fig. 5).

The amplification of *mcrA* gene was successful for spring 5 and showed two clones (P11ME5RS64 and P11MCr5RS2) related to *Methanothermobacter thermophilus* and *Methanobacterium beijingense*, respectively (Fig. 6).

Altogether this study reveals the diversity and distribution of microorganisms in clone libraries, but is not a quantitative study. Despite the occurrence of *Thermotogales* in Tunisian hot springs, there were no *Thermotogales*-related 16S rRNA sequences detected in the clones libraries constructed from the same samples used for their enrichment cultures (springs 8 and 11). These findings may be due to either missed groupings following PCR amplification as a cause of primers used (Marchesi et al. 1998) or that *Thermotogales* are not a dominant component of the terrestrial hot springs but are instead fast-growing bacteria particularly adapted to the selective growth conditions used in this work.

Previous studies showed that evaluating the tolerance of isolates to in situ settings (with respect to temperature, salinity, acidity) can establish whether they are indigenous to their environments (Magot 2005). In the case for bacterial and archaeal phylotypes identified by PCR amplification, their autochthonous origin is suggested by their phylogenetic affiliation (Pham et al. 2009).

The presence of *Deltaproteobacteria* in habitats with low sulfate content could be explained by the fact that these microorganisms play an important role in the fermentation and anaerobic oxidation of organic compounds (Muyzer and Stams 2008). In spring 5, sequences are closely affiliated with *Clostridiales* detected in rice soil, which is one of the major sources of atmospheric methane (Hengstmann et al. 1999; Chin et al. 1999). This suggested that source 5 contains methane. We confirmed the presence of methanogens by amplification of *mcrA* gene. Further, there were no sequences of methanogenic archaea found in the archaeal clone library from this sample, despite the use of additional primer pairs. While the primers used are universal (and therefore not highly specific), the number of clones sequenced (100 clones for *Archaea*) remains low when compared to that required to exhaustively identify the entire diversity. Indeed, the number of clones analyzed in this study is minor as compared to the number of individual unidentified microbes likely to be present (Curtis and Sloan 2005). While the aim of our study was to detect and classify the dominant microbial communities present in Tunisian hot springs, we acknowledge that the approaches used, and available for use, do not permit identification of rare taxa.

The presence of soil-based clones in Tunisian springs may be explained by contamination of soil microorganisms or leaching of soil into the water sources. This is the case for source 1 (Hammem Atrous), where the source contacts

soil. Indeed, over 65% of the clones analyzed from source 1 are strongly related to sequences from soil samples. Other sources (particularly 2A, 2B, 6, 8, 9, 11 and 12) had fewer soil-based clones. Furthermore, our findings for the abundance of *Crenarchaeota* in the clone libraries as compared to the overall *Euryarchaeota* are likely explained by low-saline concentrations, and are in accordance with previous work (Jiang et al. 2007).

Conclusion

Herein, we used a combination of cultivation and molecular techniques to identify the microbial diversity of thermal springs in Tunisia. Using these multidisciplinary approaches, we found that depending on the technique used, clone libraries mildly differed. These findings are in agreement with other studies showing that the diversity in a library could not be exhausted (Spear et al. 2005; Curtis and Sloan 2005). To confidently portray the microbial diversities in the springs, we choose to show only the most abundant rRNA genes. The study of functional genes further complemented our findings, and highlighted the presence of microorganisms which did not appear in the clone library but whose presence was suspected due to physico-chemical data. Using both cloning and sequencing techniques, in addition to cultivation, we could study the prokaryotic diversity even though the majority remains uncultured (99%) (Amann et al. 1995). Comparison of these clones to their closest isolates allowed us to hypothesize their role in their respective habitats. This becomes more difficult when the closest relatives are only environmental clones or when the majority of clones show less than 95% sequence similarity, thereby making it difficult to affiliate them to a genus or species. Therefore, our study permitted the isolation and characterization of the strains present in the 12 thermal Tunisian springs, led to the identification of some novel strains and we determined their function through the study of functional genes.

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