Annals of Microbiology (2018) 68:915–929
https://doi.org/10.1007/s13213-018-1401-8

ORIGINAL ARTICLE

Analysis of the diversity of aerobic, thermophilic endospore-forming bacteria in two Algerian hot springs using cultural and non-cultural methods

Mohamed Amine Gomri[1] · Tedj El Moulouk Khaldi[2] · Karima Kharroub[1]

Received: 7 June 2018 / Accepted: 1 November 2018 / Published online: 9 November 2018

Abstract

Terrestrial hot environments are important resources for isolation of thermophilic microorganisms. Few studies have been made on microbial diversity of Algerian geothermal sites. This paper reports the diversity of thermophilic, aerobic endospore-forming bacteria from water and sediment samples taken from Hammam Ouled Ali and Hammam Debagh, two hot springs with a wide range of temperatures and a very rich mineral composition, located in the region of Guelma, north-east of Algeria using culture-dependent and culture-independent approaches Sequences of the V4 region of the 16S rRNA gene from environmental DNA extracted from sediment samples were analyzed and a set of isolates from water and sediment have been characterized by phenotypic and molecular methods. Phylogenetic surveys using environmental DNA sequences indicated that three families dominated the two hot springs: *Planococcaceae*, *Bacillaceae*, and *Paenibacillaceae*. Phenotypic characterization revealed the morphological, biochemical, and physiological properties of these microorganisms, all of which exhibited a range of common extracellular enzymatic activities. Amplified ribosomal DNA restriction analysis (ARDRA) was used to cluster isolates into different phylotypic groups and phylogenetic analysis of 16S rRNA gene sequences of selected isolates showed that all were closely related to four genera of thermophilic Bacilli: *Bacillus*, *Anoxybacillus*, *Geobacillus*, and *Brevibacillus*. Our results provide important insights into the microbial ecology of Guelma hot springs. They showed that the phylogenetic diversity of bacterial communities within the two studied hot springs was mostly aerobic, with the presence of taxonomic groups of great biotechnological interest. Bioprospection of thermozymes and other biomolecules within these communities will probably provide a data basis for their industrial exploitation.

Keywords Thermophilic · Bacilli · Endospore-forming · Microbial diversity · Hot spring · Algeria

Introduction

Thermophilic and hyperthermophilic microorganisms inhabiting hot aqueous environments such as terrestrial hot springs have unique molecular adaptation capacities and can be a significant source of bioactive molecules (Gomes et al. 2016).

The phylum *Firmicutes* consists of at least 26 families and 223 genera. Some members of the *Firmicutes* are thermophiles and/or halophiles (Schleifer 2009).

Inside this phylum, thermophilic endospore-forming bacteria constitute an important taxonomic group with widely cited potential biotechnological applications (Raddadi et al. 2015). Many of their bioactive compounds such as antimicrobial agents, compatible solutes, and biopolymers can find their way into industrial, pharmaceutical, and medical use, but their thermozymes, especially hydrolases, are maybe the most exploited and studied resource (Charlesworth and Burns

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13213-018-1401-8) contains supplementary material, which is available to authorized users.

✉ Mohamed Amine Gomri
gomrima@umc.edu.dz

Tedj El Moulouk Khaldi
moulouk.khaldi@umc.edu.dz

[1] Equipe Métabolites des Extrêmophiles (METEX), Laboratoire de Recherche Biotechnologie et Qualité des Aliments (BIOQUAL), Institut de la Nutrition, de l'Alimentation et des Technologies Agro Alimentaires (INATAA), Université Frères Mentouri Constantine 1 (UFMC1), Route de Ain El Bey, 25000 Constantine, Algeria

[2] Laboratoire Alimentation, Nutrition et Santé (ALNUTS), Institut de la Nutrition, de l'Alimentation et des Technologies Agro Alimentaires (INATAA), Université Frères Mentouri Constantine 1 (UFMC1), Route de Ain El Bey, 25000 Constantine, Algeria

2016; Kambourova et al. 2016; Dumorne et al. 2017). For example, thermostable amylases, cellulases, and proteases of great industrial interest have been isolated from thermophilic strains of the *Firmicutes* (Elleuche et al. 2015; Contesini et al. 2017; Zhang et al. 2018). Indeed, there is an increasing focus on the utilization of renewable sources to satisfy the exponentially growing industrial needs. Microorganisms living in extreme habitats are an ideal source for polymer degraders, which allow to perform biotransformation reactions at non-conventional conditions under which many proteins are completely denatured (Antranikian 2007).

Algeria contains numerous and largely unstudied extreme environments. For example, over than 240 geothermal springs have been recorded in Northern Algeria but the data on their biodiversity is very limited. These springs might be considered as naturally well protected, renewable, and quasi-infinite resources (Fekraoui and Kedaid 2005; Amarouche-Yala et al. 2014), but can, on the other hand, be particularly vulnerable to microbial contamination due to human activities and their biodiversity could be significantly affected (Field 1999).

In this work, we investigate the diversity of thermophilic, aerobic endospore-forming bacteria of Hammam Ouled Ali and Hammam Debagh, two hot springs located in the region of Guelma, North-East Algeria, using culture-dependent and culture-independent methods.

Materials and methods

Studied sites, sampling, and isolation procedure

Environmental samples were collected from four different sites of two hot springs: Ouled Ali (36°34′ N; 7°23′ E) and Debagh (36° 27′ N; 7°16′ E) located in the Department of Guelma, North-East Algeria (Fig. 1). These springs are used for their therapeutic properties as baths and spas and are found to be near travertine deposits with a very rich mineral salt composition. Hammam Debagh is the hottest spring in Algeria, with temperatures rising up to 98 °C.

Water and sediment samples were used for isolation of thermophilic bacteria from each site. Four to eight sediment samples of 20 g from each site were used for the extraction of environmental DNA. Temperature and pH values of the sampling sites were measured in situ, and pH was rechecked at the laboratory. Samples were collected in sterile containers and were stored at 4 °C until further use.

Environmental DNA extraction and sequencing

Metagenomic DNA of 23 sediment samples was extracted using a previously described protocol (Miller et al. 1999). The quantity and quality of the genomic DNA was measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Extracted metagenomic DNA was purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific).

16S rRNA gene V4 variable region PCR primers (515/806 with forward primer barcode) were used in a 30-cycle PCR (5 cycles used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. After amplification, PCR products were checked in 2% (*w*/*v*) agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads, and the pooled and purified PCR product was used to prepare a DNA library using the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MRDNA (Shallowater, TX, USA) on an Illumina MiSeq following the manufacturer's guidelines.

Diversity survey of aerobic endospore-forming bacteria in environmental DNA

Sequence data were processed using the MRDNA analysis pipeline (MR DNA, Shallowater, TX, USA). Sequences were

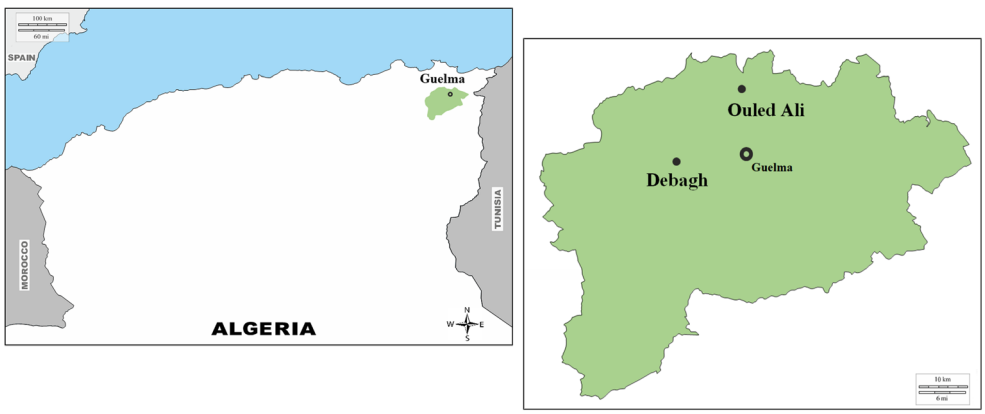

Fig. 1 Map of Algeria depicting sampling locations

joined and depleted of barcodes. Sequences < 150 bp and sequences with ambiguous base calls were removed. Sequences were denoised, operational taxonomic units (OTUs) generated, and chimeras removed using UCHIME (Edgar et al. 2011). OTUs were defined by clustering at 3% divergence (97% similarity) by CD-HIT (Li and Godzik 2006). Final OTUs were taxonomically classified using BLASTn against a curated database derived from Greengenes, RDPII, and NCBI (DeSantis et al. 2006; Cole et al. 2014; Benson et al. 2018).

Availability of data

The sequence data sets were submitted to NCBI Sequence Read Archive (SRA) under the accession number (SRP153378) for public access.

Isolation of bacteria

For the isolation of aerobic thermophilic bacteria, 1 g aliquots of sediment was added to enrichment broth (0.4% peptone, 0.2% yeast extract, 0.2% NaCl *w/v*) and incubated at 55 °C for 24 h. Of water samples or enrichment broths, 0.1 mL volumes were then sprayed onto five different growth media: M1, *Thermus* medium agar (Atlas 2005); M2, Medium 256 agar (Atlas 2005); M3, Nutrient agar (Sigma); M4, Plate Counting Agar (Sigma); M5, *Actinomycetes* Agar (Difco), pH adjusted to 7.2 ± 0.2. Plates were incubated at 55 °C for 72 h.

Further purification of isolates was done on *Thermus* agar plates. The isolated colonies were stored at 4 °C.

Phenotypic characterization

The selected isolates were characterized using 103 phenotypic tests based on minimal standards for describing new taxa of aerobic, endospore-forming bacteria and criteria described by the Bergey's Manual of Systematic Bacteriology (Logan et al. 2009; De Vos et al. 2011).

Colonies morphology was studied by inoculating the cultures on M1 plates and incubating at 55 °C for 24 h. Cell morphology was observed by light microscopy after Gram staining. The presence of endospores was investigated using the Schaeffer-Fulton technique (Bartholomew and Mittwer 1950).

The physiological and biochemical characteristics of each isolate were determined using standard procedures, following 24 to 48 h incubation at optimal growth temperatures, in triplicate. Temperature, pH, and NaCl requirements were studied by inoculating the cultures on M1 agar/broth and incubating at a range of temperatures (10–80 °C, with an interval of 5 °C), pH levels (5.0–10.0, with an interval of pH 0.5 unit), and NaCl concentrations (0.5, 1.0, 2.0, 3.0, 3.5, 4.0, 5.0, 7.5, 10.0, 12.5, and 15% *w/v*) to record the minimum, optimum, and maximum temperature-, pH-, and salinity-dependent growth of all isolates. Catalase activity was tested with 10% (*v/v*) H_2O_2, oxidase production was checked using Oxidase Strips (Sigma), and β-galactosidase production was checked using Ortho-Nitrophenyl-β-Galactoside (ONPG) disks (Fluka). Carbon substrate utilization was evaluated using 1% (*w/v*) sugars: (D(+)-glucose, D(+)-fructose, D(+)-galactose, D(+)-mannose, D(+)-saccharose, D(+)-maltose, D(+)-lactose, and dextrin), 0.1% (*w/v*) amino acids (L-tyrosine, L-glycine, L-glutamic acid, L-threonine, and L-lysine), 0.1% (*w/v*) organic acids (lactate, acetate, citrate, propionate, and succinate), 0.1% (*w/v*) alcohols (mannitol, glycerol, ethanol, and methanol), and 1% (*w/v*) polysaccharides (starch, pectin) on agar plates containing 0.05% (*w/v*) NH_4Cl and 0.005% (*w/v*) Na_2HPO_4. Acid production from growth on sugars (1%, *w/v*) was recorded on nutrient broth containing 0.003% of phenol red as a pH indicator. Other standard biochemical tests carried out included the following: Methyl Red (MR) and Voges Proskauer (VP) reactions, formation of indole, urease production, mannitol motility, and triple sugar iron tests (Tindall et al. 2007).

Screening for extracellular hydrolase production

The isolates were tested for extracellular enzymatic activities by growth on solid media. Amylolytic activity was tested on a growth medium containing 1% (*w/v*) of starch (Gordon et al. 1973). Caseinolytic and gelatinolytic activities were tested on growth media containing 1% (*w/v*) of casein and 0.4% (*w/v*) of gelatin, respectively (Frazier 1926; Priest et al. 1988). Cellulolytic activities were investigated using a growth media containing 0.5% (*w/v*) of carboxymethyl cellulose (CMC) (Bragger et al. 1989). Pectinolytic activities were determined using 1% (*w/v*) pectin (Soares et al. 1999), and extracellular lipases were screened in growth media containing 1% (*v/v*) of Tween 20 or Tween 80 (Khyami-Horani 1996). All cultures were incubated at optimal growth temperature for 48 to 72 h.

Phenogram construction

A dendrogram of the phenotypic characteristics of the isolates was constructed using NTSYS-pc ver. 2.02i (Exeter Software, Setauket, NY). A binary 0/1 matrix, based on the absence or presence of a phenotypic feature, was assembled. Simple matching (SM) coefficients were calculated, and a dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) (Gower 1971).

DNA extraction, 16S rRNA gene amplification

All strains were grown aerobically on M1 medium (pH 7.2) at 55 °C for 24 h. Genomic DNA was extracted using a modified protocol as described previously (Miller et al. 1999). The quantity and quality of the genomic DNA was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific).

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal bacterial primers E9F (GAGTTTGATCCTGGCTCA) (Farrelly et al. 1995) and U1510R (GGTTACCTTGTTACGACTT) (Reysenbach and Pace 1995).

A typical PCR contained the following (final concentration): 1× DreamTaq Buffer, 1% (*v*/*v*) bovine serum albumin, 1.25 U DreamTaq Polymerase (Thermo Scientific), 1 μM (each) primer, 200 μM of each deoxynucleoside triphosphate, and 10 to 100 ng template DNA in a 50-mL reaction volume. PCR conditions were as follows: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 85 s; and a final incubation at 72 °C for 5 min. PCR products were electrophoresed and visualized on a 1% (*w*/*v*) agarose gel.

Amplified ribosomal DNA restriction analysis and sequencing

Restriction digestion amplified ribosomal DNA restriction analysis (ARDRA) profiles on agarose gels, generated by AluI and HaeDIII fast digests (FD) (Thermo Fisher Scientific), were analyzed to avoid sequencing redundant clones.

The reaction mixture containing 3.33 μl amplified 16S rRNA gene product, 0.67 μl Tango buffer (10×) for AluI or 0.67 μl FD buffer (10×) for HaeIII FD, 0.33 μl AluI or 0.33 HaeIII FD (10 U/μl), and 5.67 μl deionized water was incubated at 37 °C for 3 h (AluI) or 5 min (HaeIII FD). AluI was inactivated by heating at 65 °C for 20 min. Restriction digest products were loaded into 2.5% (*w*/*v*) agarose gels, electrophoresed at 90 V for 90 min. Restriction fragment size was estimated by inclusion of a 100-bp DNA ladder (Thermo Fisher Scientific). The gel was visualized using a Bio-Rad Imager Gel Doc XR System, and the restriction patterns of each sample were compared. Fragments smaller than 100 bp were not included in this analysis. The NTYSIS-pc package (version 2.02i, Exeter Software, Setauket, NY) was used to score similarity and clustering analysis using the binary data. Dice coefficients were used to calculate the similarity among the isolates and dendrograms were constructed using the UPGMA method (Nei and Li 1979).

Amplicons of the selected isolates from the ARDRA were then purified with the NucleoSpin Gel and PCR Clean-up (Thermo Fisher Scientific). E9F and U1510R primers were used for capillary sequencing at the Central DNA Sequencing Facility, University of Stellenbosch.

Phylogenetic analysis

Identities with 16S rRNA sequences of described taxa were investigated using the nBLAST tool against the EzBioCloud Database of cultured organisms (Yoon et al. 2017). Multiple sequence alignments were performed with MEGA 7 software using the ClustalW algorithm (Thompson et al. 1994). 16S rRNA gene-based phylogenetic trees were constructed, based on neighbor-joining (Saitou and Nei 1986) and maximum composite likelihood models (Tamura et al. 2004) with 1000 bootstrap replications (Felsenstein 1985) using MEGA 7 program package (Kumar et al. 2016). The 16S rRNA gene sequence of *Sulfobacillus acidophilus* DSM 10332^T was used as outgroup.

Nucleotide accession numbers

Nucleotide sequences have been deposited in the NCBI database under accession numbers MF136820 to MF136840.

Results

Sampling sites

The samples for this study were collected from four different sites at two hot pools: Debagh1 (Db1), Debagh2 (Db2), Debagh3 (Db3), and Ouled Ali (OA). Temperature and pH values for each site are shown in Table 1. Chemical analyses of the spring waters presented by different studies showed some common parameters between the two hot springs: high temperature, slightly acid to neutral pH, high concentrations of silica, major concentrations of sodium chloride with large content of calcium sulfate, gas emissions of carbon dioxide and nitrogen at the thermal vents (Saibi 2009; Bahri et al. 2011; Amarouche-Yala et al. 2015).

Aerobic endospore-forming bacteria communities in hot springs sediments

We performed a MiSeq sequencing of the V4 region of 16S rRNA amplicons from metagenomic DNA extracted from sediment samples from the four sites. Filtration of the data generated 2,238,462 good sequence reads from the 23 pooled samples. Db1 samples had the most important reads (887,026), followed by Db2 (511,956), OA (479,579), and Db3 (359,901). About 193,318 good reads were classified under archaea, while 2,002,967 good reads were classified under bacteria. For the bacterial diversity, the result showed 30 phyla (see supplementary). *Firmicutes* abundance among other bacterial phyla is shown in Fig. 2.

Prevalence of *Firmicutes* within the total bacterial community in each of the four sites was significantly different. It was very low in Db2 and OA (1.66 and 6.13%, respectively) in comparison with Db1 (12.86%) and Db3 (17.92%) pools. In general, *Firmicutes* were relatively a minor taxonomic group in the four sites. Dominant phyla were different in Db1 (*Deinococcus-Thermus*), Db2 (*Aquificae*), Db3 and OA (*Chloroflexi*).

When studying *Firmicutes* diversity present in each site at the family taxonomic level, we notice that aerobic endospore-

Table 1 Localization and physicochemical data of the sampling sites

Parameters	Sampling sites			
	Ouled Ali (OA)	Debagh 1 (Db1)	Debagh 2 (Db2)	Debagh 3 (Db3)
Localization	(36°34′ N; 7°23′ E)	(36° 27′ N; 7°16′ E)		
Altitude (masl)	270	350		
Temperature (°C)	54 ± 1.0	91 ± 0.5	95 ± 0.5	56 ± 1.0
pH	7.0 ± 0.05	6.6 ± 0.02	6.8 ± 0.08	6.8 ± 0.1

formers are dominant in the four sites (Fig. 3). In fact, the presence of three major families of aerobic thermophilic endospore-forming bacilli is observed: *Planococcaceae*, *Bacillaceae*, and the *Paenibacillaceae* which were the *Firmicutes* dominant family in Db1 (61.61%) and Db3 (59.08%). *Clostridiaceae* and *Thermoanaerobacteraceae* were the most noticeable anaerobic endospore-forming families present in the sediment samples.

The major thermophilic aerobic endospore-forming genera of *Firmicutes* phylum present within the bacterial communities of the four sites were *Paenibacillus*, *Psychrobacillus*, and *Sporosarcina* with at least 1% of the total sequences (see supplementary). Genera such as *Bacillus*, *Geobacillus*, *Anoxybacillus*, and *Brevibacillus* were minor (less than 1%) in both hot springs.

Isolation of thermophilic bacteria

One hundred seven aerobic, thermophilic, chemoheterotrophic bacterial isolates were obtained from the cultivation of the different samples collected from the four sites. Literature indicates that the temperature used for the isolation procedure (55 °C) allows to identify a wide range of thermophilic heterotrophic aerobic bacterial taxa from both hot and mild environments (Trujillo 2001; Logan et al. 2009; Battista and Rainey 2015; De Vos 2015; De Vos et al. 2015). The selection and purification of these isolates were based on the morphological variability of their colonies and their microscopic aspect. The Ouled Ali site samples provided 49 isolates whereas 58 isolates came from Debagh three sites. Sediment samples from both hot springs yielded 63 isolates while water samples provided 44 isolates. Thirty-five isolates were collected from growth medium M1, 25 from M2, 33 from M3, 11 from M4, and 3 isolates from M5.

Phenotypic characterization and extracellular hydrolase production

Phenotypic characterization and screening for extracellular enzymatic activities was performed for 20 isolates selected by ARDRA analysis (Table 2). Colonies were smooth or moist, of circular shape, cream or transparent and generally non-pigmented after incubation at 55 °C for 48 h. Cells were typically Gram-positive, rod-shaped, and single or in chains. Based on microscopic observation, ellipsoidal endospores were located in either a terminal or subterminal position.

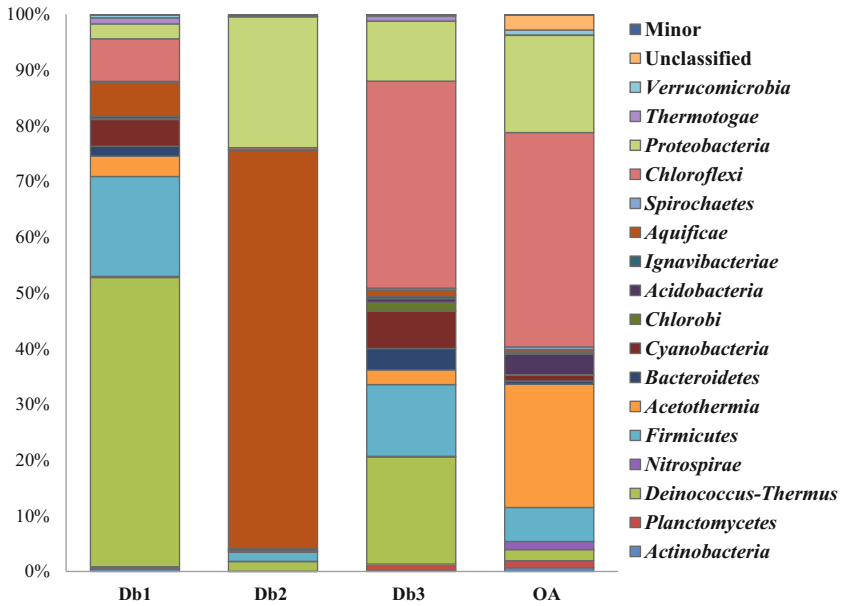

Fig. 2 Percentages of the major phyla in each site (the sequence percentage is above 0.5% in at least one site)

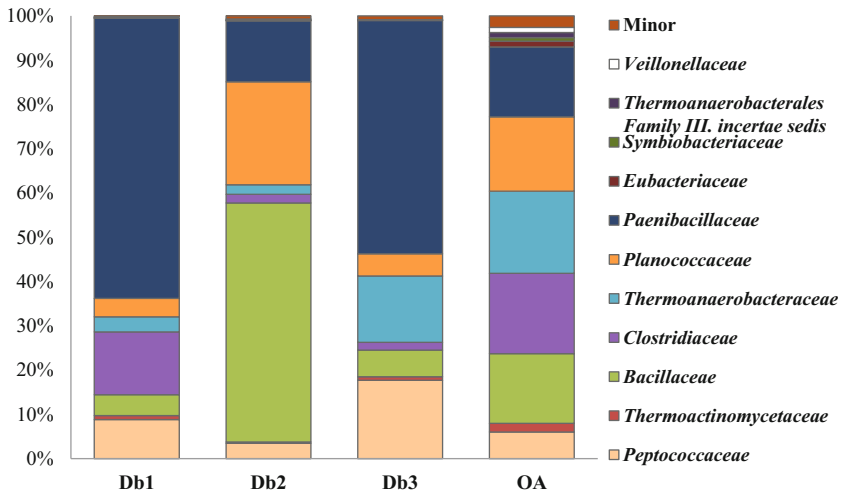

Fig. 3 Percentages of the major families within the *Firmicutes* phylum in each site (the sequence percentage is above 0.5% in at least one site)

Strains were moderately thermophilic and neutrophilic, with growth ranges between 30 and 75 °C and optimum growth between 50 and 60 °C, and at pH values from 5.0 to 9.0 with optimum growth between pH 6.5 and 7.5. All the strains were able to grow at 0% (*w*/*v*) NaCl and tolerated salt concentrations between 1 and 10% (*w*/*v*). Optimum growth occurred at 3% (*w*/*v*) for the most halotolerant strain.

Isolates were aerobic and were able to produce catalase and/or oxidase. The majority of the strains were not able to produce β-galactosidase. Glucose was used as carbon source by all strains while the utilization of other substrates was variable. Fermentation of sugars, RM, and VP reactions were negative in most cases, indicating the presence of aerobic metabolic pathways. Indole and urease tests were negative. Mannitol-mobility and triple sugar iron test results were variable (Table 2).

All of the 21 strains showed positive results for at least one of the seven extracellular hydrolytic activities tested. Amylases and proteases were the most commonly produced extracellular enzymes while lipolytic and cellulolytic activities were the least expressed enzymatic activities (Table 2).

The phenotype-based UPGMA dendrogram of selected strains is shown in supplementary. Strains showed no particular pattern related to the physicochemical properties of their isolation sites. Clusters did not reflect any specific phenotypic differences.

Amplified ribosomal DNA restriction analysis

ARDRA analysis was conducted in order to eliminate redundant clones from the set of isolates. Analysis profiles, using AluI and HaeIII of 45 isolates, were combined and used to construct a dendrogram using UPGMA cluster analysis based upon the similarity index calculated using Dice coefficient (Fig. 4). In total, 23 different bands, ranging in size from 100 to 1000 bp, were observed. AluI and HaeIII are the most frequently used enzymes for ARDRA of members of the genus *Bacillus*, giving the highest number of differentiating bands (Blanc et al. 1997; McMullan et al. 2004; Kuisiene et al. 2007). We noted that restriction digestion with AluI was more discriminative than HaeIII FD. Isolates could be assigned to 28 distinct clusters, divided into five groups at similarity levels of 70% (group V), 84% (groups III and IV), 87% (group II), and 92% (group I). Results were compared with phenotypic characters to select representatives of each pattern group for further molecular characterization.

Molecular characterization and phylogenetic analysis

Twenty-one organisms were selected for phylogenetic analysis: 8 originated from Debagh hot spring and 13 from Ouled Ali. 16S rRNA sequences were compared to the EzBioCloud version 2018-05 using the nBLAST tool, yielding sequence identities ranging from 98.69 to 100% between the selected strains and type strains (see supplementary). Isolates were affiliated with the following species: *Anoxybacillus gonensis* (Belduz et al. 2003), *Anoxybacillus flavithermus* (Pikuta et al. 2000; Dai et al. 2011), *Anoxybacillus thermarum* (Poli et al. 2009), *Bacillus paralicheniformis* (Dunlap et al. 2015), *Bacillus licheniformis* (Weigmann 1898), *Geobacillus thermoleovorans* (Nazina et al. 2001), and *Brevibacillus thermoruber* (Manachini et al. 1985; Shida et al. 1996).

The phylogenetic analysis is shown in Fig. 5. Strains affiliated to the *Bacillaceae* (Logan and Vos 2015b) and the *Paenibacillaceae* families (De Vos et al. 2015) were mostly related to four genera: *Bacillus*, *Anoxybacillus*, *Geobacillus*, and *Brevibacillus* with a predominance of *Bacillus* (10 strains) and *Anoxybacillus* (9 strains).

Discussion

In this work, the diversity of thermophilic endospore-forming bacteria in two Algerian Hot Springs with variable water

Table 2 Phenotypic characterization of the strains

Characteristic	Db101	Db114	Db120	Db134	Db159	Db27	Db32	Db59	OA105	OA107	OA113
Colonies aspect	Smooth	Smooth	Smooth	Smooth	Smooth	Moist	Moist	Moist	Moist	Moist	Smooth
Colonies pigmentation	Cream	Cream	Cream	Cream	Cream	Transparent	Transparent	Transparent	Transparent	Transparent	Cream
Gram	+	+	+	+	+	+	+	+	+	+	+
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Endospores	Subterminal	Terminal	Terminal	NO	Terminal	Subterminal	Subterminal	NO	Subterminal	Subterminal	NO
Motility	–	–	–	–	–	–	–	–	–	–	–
T° range (°C)	[35–65]	[35–65]	[35–65]	[35–65]	[30–70]	[25–60]	[30–60]	[25–70]	[35–70]	[25–70]	[25–70]
Optimum T° (°C)	60	55	55	50	50	50	50	55	55	50	50
pH range	[6.5–8.0]	[6.5–8.5]	[6.5–8.0]	[6.5–8.0]	[6.5–8.0]	[6.5–8.5]	[6.5–8.5]	[6.5–8.0]	[6.5–8.5]	[6.5–8.5]	[6.5–8.5]
Optimum pH	7.5	7.0	7.5	7.0	7.5	7.0	7.0	7.5	7.0	7.0	6.5
NaCl %	[0–3]	[0–3]	[0–3]	[0–3]	[0–3]	[0–10]	[0–10]	[0–5]	[0–2]	[0–3]	[0–3]
Optimum NaCl %	0	1	0.5	2	2	2	3	0.5	0	0.5	1
Catalase	+	+	–	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	–	+	+	+	+	+	+
β-Galactosidase	–	–	–	–	–	–	–	+	–	–	+
Production of:											
Indole	–	–	–	–	–	–	–	–	–	–	–
Gaz	–	–	–	–	–	–	–	–	–	–	+
H_2S	–	–	–	–	–	–	–	–	–	–	–
MR	–	–	–	–	–	+	–	–	–	–	–
VP	–	–	–	–	–	–	–	–	–	–	+
Acid from:											
D(+)-Glucose	–	–	+	+	–	–	–	–	–	–	+
D(+)-Fructose	–	–	–	+	+	–	–	+	–	–	+
D(+)-Galactose	–	–	–	–	–	–	–	–	–	–	–
D(+)-Lactose	–	–	–	+	+	–	–	–	–	–	+
D(+)-Saccharose	–	–	–	–	+	–	–	–	–	–	+
D(+)-Maltose	–	–	–	–	–	–	–	–	–	–	–
Dextrin	–	–	–	–	+	–	–	+	–	–	+
D(+)-Melibiose	–	–	–	–	+	–	–	–	–	–	–
Mannitol	–	–	–	–	+	–	–	–	–	–	+
D(+)-Mannose	–	–	–	–	–	–	–	–	–	–	–
Hydrolysis of:											
Cellulose	–	–	–	+	–	–	–	–	–	–	–
Starch	+	–	–	+	+	+	–	+	–	+	+
Pectin	–	–	+	–	–	–	–	–	–	–	–
Tween 20	–	–	–	–	–	–	–	–	–	–	–
Tween 80	–	–	–	–	–	–	–	–	+	–	–
Casein	–	+	+	+	+	+	+	–	+	+	+
Gelatin	+	–	–	–	–	+	–	+	–	–	–
Urea	–	–	–	–	–	–	–	–	–	–	–
Utilization of:											
D(+)-Glucose	+	+	+	+	+	+	+	+	+	+	+
D(+)-Fructose	–	+	+	+	+	+	+	–	–	+	+
Mannitol	–	+	–	+	–	+	–	–	–	–	–

Table 2 (continued)

D(+)-Melibiose	–	–	–	–	–	–	–	–	–	–	–
D(+)-Mannose	–	–	–	–	+	+	–	–	–	–	–
D(+)-Galactose	–	–	–	+	+	+	+	+	+	–	–
D(+)-Saccharose	–	–	+	+	+	+	+	–	+	+	–
D(+)-Maltose	–	–	–	+	–	–	–	–	–	–	–
Dextrin	–	–	–	–	–	–	–	–	–	–	+
D(+)-Lactose	–	+	+	–	+	+	+	–	+	+	+
L-Tyrosine	–	–	–	–	–	–	–	–	–	–	–
L-Glutamic acid	–	–	–	–	–	+	–	–	–	–	+
L-Glycine	–	–	–	–	–	–	–	–	–	–	–
L-Threonine	–	–	–	–	–	–	–	–	–	–	–
L-Lysine	–	+	–	–	–	+	–	–	–	–	–
Lactate	–	–	–	–	–	–	–	–	–	–	–
Acetate	–	–	–	–	–	–	–	–	–	–	–
Propionate	–	–	–	–	–	–	–	–	–	–	–
Succinate	–	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–	–	–	–	–	–	–	–
Ethanol	–	–	–	–	–	–	–	–	–	–	–
Methanol	–	–	–	–	–	–	–	–	–	–	–
Citrate	–	–	–	–	–	–	–	–	–	–	–
Pectin	–	–	–	–	–	+	–	–	–	–	+
Starch	–	–	+	+	–	–	–	+	–	–	+

Characteristic	OA117	OA123	OA126	OA129	OA130	OA140	OA21	OA23	OA28	OA30
Colonies aspect	Smooth	Smooth	Moist	Smooth	Smooth	Smooth	Smooth	Smooth	Moist	Smooth
Colonies pigmentation	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Yellow	Transparent	Cream
Gram	+	+	+	+	+	+	+	+	+	+
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Endospores	Terminal	Terminal	NO	Subterminal	Terminal	Terminal	Terminal	Terminal	Subterminal	Subterminal
Motility	–	–	–	–	–	–	–	–	–	+
T° range (°C)	[30–70]	[35–65]	[30–70]	[25–70]	[30–70]	[35–60]	[30–60]	[45–65]	[35–65]	[30–75]
Optimum T° (°C)	60	55	55	55	55	60	60	55	50	55
pH range	[6.5–8.0]	[6.5–8.0]	[6.5–8.5]	[6.5–8.5]	[5.0–9.0]	[6.5–8.0]	[6.5–8.0]	[5.5–8.0]	[5.5–8.0]	[6.5–8.5]
Optimum pH	7.0	7.0	7.0	6.5	7.0	7.0	7.5	7.5	7.0	7.0
NaCl %	[0–3]	[0–5]	[0–3]	[0–1]	[0–10]	[0–3]	[0–3]	[0–3]	[0–3]	[0–3.5]
Optimum NaCl %	1	2	1	0	0	2	1	1	0.5	1
Catalase	+	+	+	+	+	–	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
β-Galactosidase	–	–	–	–	–	–	–	–	–	–
Production of:										
Indole	–	–	–	–	–	–	–	–	–	–

Table 2 (continued)

Gaz	−	−	−	−	+	−	−	−	−	−
H_2S	−	−	−	−	−	−	−	−	−	−
MR	+	−	+	−	+	−	−	−	−	−
VP	−	−	−	−	−	−	+	−	−	−
Acid from:										
D(+)-Glucose	−	+	−	+	+	+	+	+	−	−
D(+)-Fructose	−	+	−	+	+	+	+	+	−	−
D(+)-Galactose	−	−	−	−	−	−	−	−	−	−
D(+)-Lactose	−	+	−	−	+	−	+	−	−	−
D(+)-Saccharose	−	+	−	−	+	−	−	−	−	−
D(+)-Maltose	−	−	−	−	−	−	−	−	−	−
Dextrin	−	−	−	−	+	+	−	−	−	−
D(+)-Melibiose	−	−	−	−	+	−	−	−	−	−
Mannitol	−	+	−	−	−	−	−	−	−	−
D(+)-Mannose	−	−	−	−	−	−	−	−	−	−
Hydrolysis of:										
Cellulose	−	−	+	−	−	−	−	−	−	−
Starch	+	−	+	+	+	+	−	−	−	+
Pectin	+	+	−	−	−	+	+	−	+	−
Tween 20	−	−	−	−	−	−	−	−	−	−
Tween 80	−	−	−	−	−	−	−	−	−	−
Casein	−	+	+	+	+	−	+	−	+	+
Gelatin	+	−	−	−	+	+	+	+	−	+
Urea	−	−	−	−	−	−	−	−	−	+
Utilization of:										
D(+)-Glucose	+	+	+	+	+	+	+	+	+	+
D(+)-Fructose	+	+	+	+	+	+	+	−	+	+
Mannitol	−	+	−	−	−	+	−	+	−	+
D(+)-Melibiose	−	+	−	+	−	−	+	−	−	−
D(+)-Mannose	−	+	−	−	−	−	+	−	−	−
D(+)-Galactose	+	+	−	+	−	+	+	−	−	+
D(+)-Saccharose	+	−	−	−	+	+	+	−	−	+
D(+)-Maltose	−	−	−	−	+	−	−	−	−	+
Dextrin	−	−	−	−	+	−	−	−	−	+
D(+)-Lactose	+	−	−	−	+	+	+	−	+	−
L-Tyrosine	−	−	−	−	−	−	−	−	−	−
L-Glutamic acid	+	−	+	−	−	+	−	+	−	+

Table 2 (continued)

L-Glycine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Threonine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Lysine	+	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+
Lactate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Acetate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Propionate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Succinate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	+	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+
Ethanol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Methanol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Citrate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pectin	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
Starch	+	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+

NO not observed, + positive result, − negative result

temperatures has been studied with culture-dependent and culture-independent approaches.

The prevalence of the *Firmicutes* among the bacterial communities of the two hot springs by MiSeq sequencing of the V4 region of 16S rRNA gene extracted from sediment samples was investigated. The results showed that this phylum was minor in the four sites. Indeed, bacterial communities in sites Db1 (91 °C) and Db2 (95 °C) were dominated by two other aerobic chemoheterotrophic and more thermophilic phyla: *Deinococcus-Thermus* and *Aquificae*, respectively (Reysenbach et al. 2001; Battista 2016), while phototrophic thermophilic phyla were the most abundant when temperatures were under 80 °C in Db 3 (75 °C) and OA (54 °C), where *Chloroflexi* was the most prevalent phylum (Castenholz 2001). We also found that the prevalence of *Firmicutes* was more important when the temperature of the pools increased: from 6.13% in OA (54 °C) to 12.87% in Db3 (75 °C), to finally reach its highest percentage (17.92%) in Db1 (91 °C). On the other hand, it seems that the limit temperature for the growth of the members of this group was below the temperature of Db2 pool (95 °C) where the prevalence of representative OTUs dropped to 1.66%.

Results of microbial diversity studies for similar environments using the same metagenomic approach were variable. In the Manikaran hot springs (India), *Firmicutes* (28 to 84%), *Aquificae* (2 to 64%), and *Deinococcus-Thermus* (1 to 18%) were the dominant phyla (Bhatia et al. 2015). In Jakrem hot springs (India), *Firmicutes* (61%), *Chloroflexi* (21%), and *Cyanobacteria* (13%) were the most abundant (Panda et al. 2015). *Firmicutes* represented 7.5% of the microbial diversity in Eryuan hot spring (China) (Menzel et al. 2015) and between 6 and 21% of the total microbial diversity in Ma'in hot spring (Jordan) (Hussein et al. 2017). In Murtazaabad hot springs (Pakistan), *Chloroflexi* and *Firmicutes* represented 29 and 10% of the bacterial diversity, respectively (Amin et al. 2017).

The diversity of *Firmicutes* within the four sites was mainly aerobic. Three families of aerobic endospore-forming *Firmicutes* were dominant, especially in Debagh sites: *Paenibacillaceae* (De Vos et al. 2015), *Bacillaceae* (Logan and Vos 2015a), and *Planococcaceae* (Shivaji et al. 2014). *Paenibacillus* (Ash et al. 1993), *Psychrobacillus* (Krishnamurthi et al. 2010), and *Sporosarcina* (Yoon et al. 2001) were, respectively, the most abundant genera of each family. In OA, where the temperature was the lowest among all the studied points, the distribution between aerobic and anaerobic families was more equal with more diversity than in Debagh sites. This is confirmed by the fact that more variability was found in the strains isolated from OA than in Db. Higher temperatures tend to limit the diversity of the environments (Gómez and Parro 2012). The limited depth of the studied pools (between 50 cm and 1 m) might be favorable to the presence of aerobic thermophiles since it is known that dissolved oxygen quantity is more important in surface water (Hayashi and Rosenberry 2002).

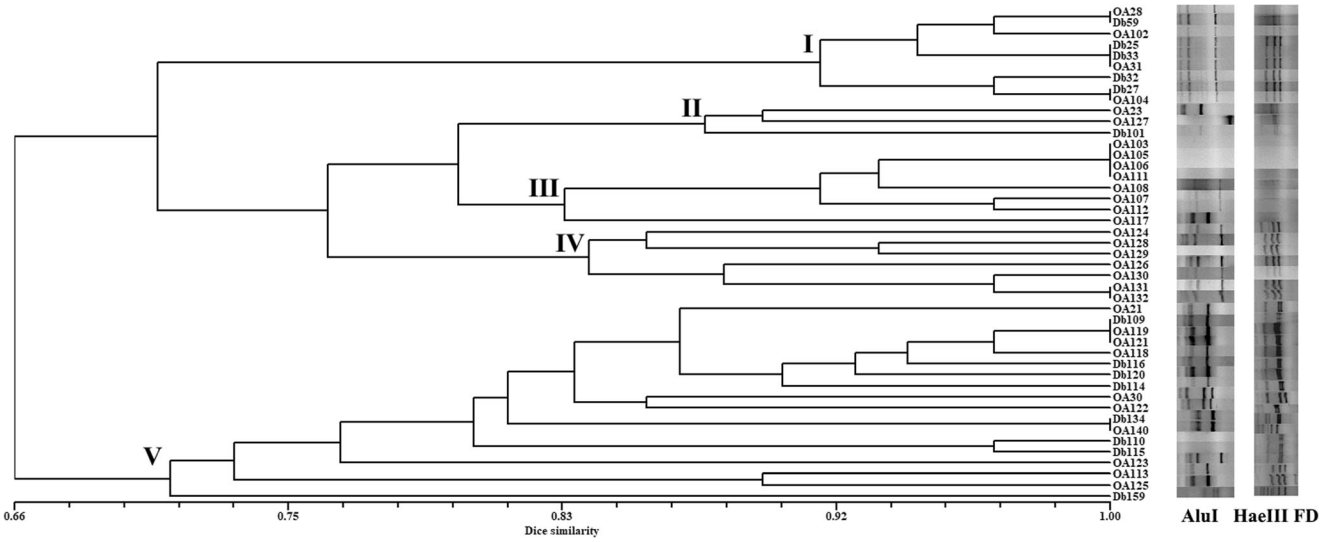

Fig. 4 Differences in restriction patterns. Dendrogram of 45 isolates ARDRA distances obtained by digestion with AluI + HaeIII FD. Isolates are regrouped under seven different pattern groups: I, II, III, IV, and V. The dendrogram was constructed using the UPGMA algorithm and Dice coefficients

Ninety-three isolates have been cultured on non-specific media (M1, M2, and M3). Most aerobic endospore-formers are chemoorganotrophs with aerobic metabolism and, despite their very wide diversity, will grow well on routine media such as nutrient agar or trypticase soy agar (Logan and Allan 2008).

From an initial set of 107 isolates, 13 strains from Ouled Ali and 8 strains from Debagh were selected for molecular characterization. Ninety percent of these strains belonged to the *Bacillus* and *Anoxybacillus* genera, with no significative distribution differences among the two hot springs of the study, in addition to two strains affiliated to *Geobacillus* and *Brevibacillus* isolated from Debagh and Ouled Ali hot springs, respectively. Reports have been made about the presence of representatives of these genera in hot springs located in geographically different sites: for example in Armenia (Panosyan 2017), Bulgaria (Derekova et al. 2008), China (Song et al. 2013), Morocco (Aanniz et al. 2015), Tunisia (Sayeh et al. 2010), Turkey (Adiguzel et al. 2009), and Russia (Foti et al. 2008). Isolates had typical phenotypic characters described for *Bacillales* members, they were commonly Gram-positive,

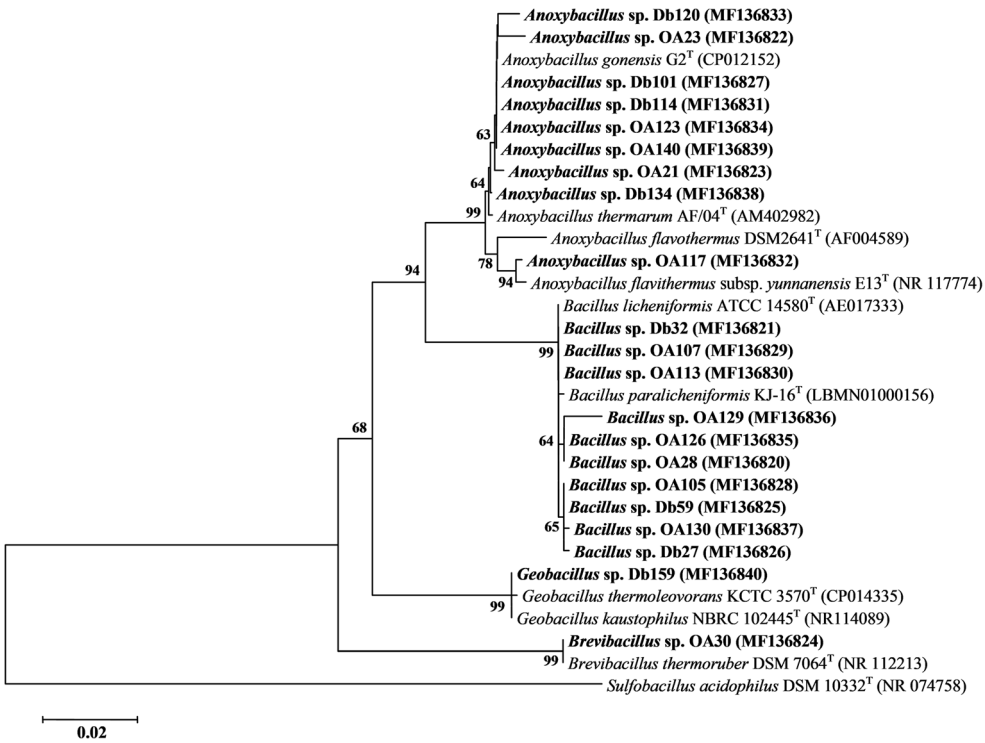

Fig. 5 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains of this study (indicated in bold letters) and strains of related genera of the family *Bacillaceae*. The strains and their corresponding Genbank accession numbers are shown following the organism name and indicated in parentheses. The phylogenetic tree was made using the neighbor-joining method with maximum composite likelihood model implemented in MEGA 7. The tree includes the 16S rRNA gene sequence of *Sulfobacillus acidophilus* DSM 10332^T as outgroup. Bootstrap consensus trees were inferred from 1000 replicates, only bootstrap values > 60% are indicated. The scale bar represents 0.02 nucleotide changes per position

rod-shaped, chemoorganotrophic, aerobic, and moderate thermophiles (De Vos et al. 2011; Logan and Vos 2015b). Identity between the strains of the study and type strains of databanks was between 98 and 100%. These type strains were isolated from different hot and mild environments. Thermophilic aerobic endospore-forming bacteria are widely distributed in water, soil, and many other environments and it is not uncommon to isolate them from mesophilic environments (Logan and Halket 2011; Müller et al. 2014).

All of the selected strains showed at least one extracellular enzyme activity among the tested activities. Amylases and caseinases production was numerically the most important and further studies of these enzymes might be interesting. For example, the proteasic activity of one of the strains isolated in this study, *Brevibacillus* sp. OA30, was investigated and an acid protease with remarkable properties was characterized (Gomri et al. 2018). Thermophilic aerobic endospore-forming bacteria are well known to be a good source of thermoenzymes, including xylanases, proteases, amylases, peroxidases, glucose isomerases, lipases, and DNA restriction enzymes (Maurer 2004; Schallmey et al. 2004; Satyanarayana et al. 2013; Panosyan 2017).

A dendrogram was constructed to discriminate the 21 isolates based on their phenotypic characters. It seems that the clusterization of the OTUs was generally not influenced by the strains origin. The physiology of the endospore-forming bacteria is influenced by complex multifactorial conditions of their environments and temperature might not be the decisive parameter in the variation of the phenotypic features of these microorganisms (Mandic-Mulec et al. 2016; Gauvry et al. 2017). A natural environment provides both sufficient nutrients and favorable physical and chemical conditions of temperature, pH, availability of water, redox, etc. (Carlin 2011).

ARDRA might be an interesting tool for the screening and the identification of new strains of heterotrophic thermophilic endospore-forming bacteria. Application of this technique in studies interested in the genetic variability of bacilli species revealed its ability to expose the exact lineage of the species rapidly (Kumar et al. 2014; Tiwari and Thakur 2014; Jain et al. 2017). The diversity rate of these groups can be very low in hot pool environments and ARDRA might help eliminate redundant clones in the prospecting process (Blanc et al. 1997; Brock 2012).

When comparing results from culture-dependent and culture-independent methods, we observed that the genera to which our isolated strains belonged (*Anoxybacillus*, *Bacillus*, *Geobacillus*, and *Brevibacillus*) had a very low distribution among the four sites. Similar observations were made on other hot springs in Malaysia (Chan et al. 2015), Tunisie (Sayeh et al. 2010), India (Kikani et al. 2015), and Bulgaria (Derekova et al. 2008) where these genera were the most frequently isolated while there abundance in environmental DNA was very low. It is important to mention that in culture-independent studies, the abundance of taxonomic groups of endospore-forming organisms belonging to the *Firmicutes* risk to be underestimated. The cell lysis of such organisms is often difficult, and so subsequent DNA purification and/or PCR-based applications may also be biased (De Vos 2011; Urbieta et al. 2015).

16S rRNA gene-based methods provide good phylogenetic information to the genus level, but, in themselves, give little information on function. Importantly, traditional approaches that group isolates on the basis of common metabolic properties may be limited in terms of phylogenetic insights, but provide clues to environmental factors favoring and selecting for particular groups and can be strong indicators of potential ecosystem function. A combined approach might allow to establish more clearly the links between diversity, community structure, physiological diversity, and ecosystem function, rather than merely characterizing the presence, absence, and identity of strains present (Mandic-Mulec and Prosser 2011).

Conclusion

This work is one of the few published studies describing the diversity of thermophilic bacteria of hot springs in Algeria. The sequencing of the V4 region of the 16S rRNA gene from environmental DNA extracted from sediment samples showed that *Firmicutes* were present as a minor phylum in the four sites with aerobic endospore-forming members belonging to *Planococcaceae*, *Bacillaceae*, and *Paenibacillaceae* families as major representatives.

Twenty-one thermophilic endospore-forming bacteria strains were isolated from Debagh and Ouled Ali hot springs and selected for phenotypic and genotypic characterization. Phylogenetic analysis based on the 16S rRNA sequences revealed that these strains were affiliated with genera *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Geobacillus* distributed between the two hot springs. All of the 21 isolates had at least one extracellular hydrolytic activity. Classical culture-based methods remain important for understanding the molecular adaptations of microbial guilds, particularly those isolated from extreme habitats with potential applications. One of the perspectives of this work is the use of Next-Generation Sequencing methods to explore the studied hot springs with a focus on bioprospection of thermozymes and other biomolecules of great biotechnological potential.

Acknowledgements Don Cowan and Thulani Makhalanyane, Centre for Microbial Ecology and Genomics, University of Pretoria, are gratefully acknowledged for their support in phylogenetics training. We also wish to thank Djamel Eddine Mekhancha, Laboratoire ALNUTS, Université Frères Mentouri Constantine 1 for his support during sampling, isolation, and phenotypic study.

Funding This study received financial support from the following organizations: The Algerian Ministry of Higher Education and Scientific Research and The Genomics Research Institute and the University of Pretoria, South Africa.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent N/A

References

Aanniz T et al (2015) Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. Braz J Microbiol 46:443–453. https://doi.org/10.1590/s1517-838246220140219

Adiguzel A, Ozkan H, Baris O, Inan K, Gulluce M, Sahin F (2009) Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. J Microbiol Methods 79:321–328. https://doi.org/10.1016/j.mimet.2009.09.026

Amarouche-Yala S, Benouadah A, Bentabet AEO, López-García P (2014) Morphological and phylogenetic diversity of thermophilic cyanobacteria in Algerian hot springs. Extremophiles 18:1035–1047. https://doi.org/10.1007/s12403-014-0130-x

Amarouche-Yala S, Benouadah A, el Ouahab BA, Moulla AS, Ouarezki SA, Azbouche A (2015) Physicochemical, bacteriological, and radiochemical characterization of some Algerian thermal spring waters. Water Qual Expo Health 7:233–249. https://doi.org/10.1007/s12403-014-0130-x

Amin A et al (2017) Diversity and distribution of thermophilic bacteria in hot springs of Pakistan. Microb Ecol 74:116–127. https://doi.org/10.1007/s00248-017-0930-1

Antranikian G (2007) Industrial relevance of thermophiles and their enzymes. In: Thermophiles: biology and technology at high temperatures, pp 113–186. https://doi.org/10.1201/9781420008852.ch8

Ash C, Priest F, Collins M (1993) Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie Van Leeuwenhoek 64:253. https://doi.org/10.1007/BF00873085

Atlas RM (2005) Handbook of media for environmental microbiology. CRC Press, Boca Raton. https://doi.org/10.1201/9781420037487

Bahri F, Saibi H, Cherchali M-E-H (2011) Characterization, classification, and determination of drinkability of some Algerian thermal waters. Arab J Geosci 4:207–219. https://doi.org/10.1007/s12517-010-0127-0

Bartholomew JW, Mittwer T (1950) A simplified bacterial spore stain. Stain Technol 25:153–156. https://doi.org/10.3109/10520295009110979

Battista JR (2016) *Deinococcus–Thermus* group. In: Encyclopedia of life sciences. Wiley. https://doi.org/10.1002/9780470015902.a0021151

Battista JR, Rainey FA (2015) Deinococcus. In: Whitman WB et al (eds) Bergey's manual of systematics of archaea and bacteria. Wiley. https://doi.org/10.1002/9781118960608.gbm00475

Belduz AO, Dulger S, Demirbag Z (2003) *Anoxybacillus gonensis* sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium. Int J Syst Evol Microbiol 53:1315–1320. https://doi.org/10.1099/ijs.0.02473-0

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Ostell J, Pruitt KD, Sayers EW (2018) GenBank. Nucleic Acids Res 46:D41–D47. https://doi.org/10.1099/00207713-47-4-1246

Bhatia S, Batra N, Pathak A, Green SJ, Joshi A, Chauhan A (2015) Metagenomic evaluation of bacterial and archaeal diversity in the geothermal hot springs of Manikaran, India. Genome Announc 3: e01544–e01514. https://doi.org/10.1128/genomea.01544-14

Blanc M, Marilley L, Beffa T, Aragno M (1997) Rapid identification of heterotrophic, thermophilic, spore-forming bacteria isolated from hot composts. Int J Syst Evol Microbiol 47:1246–1248. https://doi.org/10.1099/00207713-47-4-1246

Bragger J, Daniel RM, Coolbear T, Morgan HW (1989) Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. Appl Microbiol Biotechnol 31:556–561. https://doi.org/10.1007/BF00270794

Brock TD (2012) Thermophilic microorganisms and life at high temperatures. Springer Science & Business Media. https://doi.org/10.1007/978-1-4612-6284-8

Carlin F (2011) Origin of bacterial spores contaminating foods. Food Microbiol 28:177–182. https://doi.org/10.1016/j.fm.2010.07.008

Castenholz R (2001) Class I. "*Chloroflexi*" Bergey's manual of systematic bacteriology 1:427. https://doi.org/10.1002/9781118960608.cbm00016

Chan CS, Chan K-G, Tay Y-L, Chua Y-H, Goh KM (2015) Diversity of thermophiles in a Malaysian hot spring determined using 16S rRNA and shotgun metagenome sequencing. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.00177

Charlesworth J, Burns BP (2016) Extremophilic adaptations and biotechnological applications in diverse environments. AIMS Microbiol 2: 251–261. https://doi.org/10.3934/microbiol.2016.3.251

Cole JR et al (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res 42:D633–D642. https://doi.org/10.1093/nar/gkt1244

Contesini FJ, Melo RR, Sato HH (2017) An overview of *Bacillus* proteases: from production to application. Crit Rev Biotechnol 1–14. https://doi.org/10.1080/07388551.2017.1354354

Dai J, Liu Y, Lei Y, Gao Y, Han F, Xiao Y, Peng H (2011) A new subspecies of *Anoxybacillus flavithermus* ssp. *yunnanensis* ssp. nov. with very high ethanol tolerance. FEMS Microbiol Lett 320: 72–78. https://doi.org/10.1111/j.1574-6968.2011.02294.x

De Vos P (2011) Studying the bacterial diversity of the soil by culture-independent approaches. In: Endospore-forming soil bacteria. Springer, pp 61–72. https://doi.org/10.1007/978-3-642-19577-8_3

De Vos P (2015) Bacillales. In: Whitman WB et al (eds) Bergey's manual of systematics of archaea and Bacteria. Wiley. https://doi.org/10.1002/9781118960608.obm00057

De Vos P et al (2011) Phylum BXIII. *Firmicutes*. In: Boone DR, Castenholz RW, Garrity GM (eds) Bergey's manual® of systematic bacteriology, vol 3. Springer Science & Business Media, New York, pp 625–637. https://doi.org/10.1007/978-0-387-21609-6_29

De Vos P, Ludwig W, Schleifer K-H, Whitman WB (2015) "Paenibacillaceae" fam. nov. In: Whitman WB et al (eds) Bergey's manual of systematics of archaea and bacteria. Wiley. https://doi.org/10.1002/9781118960608.fbm00114

Derekova A, Mandeva R, Kambourova M (2008) Phylogenetic diversity of thermophilic carbohydrate degrading bacilli from Bulgarian hot springs. World J Microbiol Biotechnol 24:1697–1702. https://doi.org/10.1007/s11274-008-9663-0

DeSantis TZ et al (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72:5069–5072. https://doi.org/10.1128/aem.03006-05

Dumorne K, Córdova DC, Astorga-Eló M, Renganathan P (2017) Extremozymes: a potential source for industrial applications. J Microbiol Biotechnol 27:649–659. https://doi.org/10.4014/jmb.1611.11006

Dunlap CA, Kwon S-W, Rooney AP, Kim S-J (2015) *Bacillus paralicheniformis* sp. nov., isolated from fermented soybean paste. Int J Syst Evol Microbiol 65:3487–3492. https://doi.org/10.1099/ijsem.0.000441

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200. https://doi.org/10.1093/bioinformatics/btr381

Elleuche S, Schäfers C, Blank S, Schröder C, Antranikian G (2015) Exploration of extremophiles for high temperature biotechnological processes. Curr Opin Microbiol 25:113–119. https://doi.org/10.1016/j.mib.2015.05.011

Farrelly V, Rainey FA, Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol 61: 2798–2801. https://doi.org/10.1007/bf01919508

Fekraoui A, Kedaid F-Z (2005) Geothermal resources and uses in Algeria: a country update report. In: Proceedings

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 783–791. https://doi.org/10.2307/2408678

Field MS (1999) Karst hydrogeology and human activities: impacts, consequences, and implications. Environ Eng Geosci V:487–489. https://doi.org/10.2113/gseegeosci.V.4.487

Foti MJ, Sorokin DY, Zacharova EE, Pimenov NV, Kuenen JG, Muyzer G (2008) Bacterial diversity and activity along a salinity gradient in soda lakes of the Kulunda Steppe (Altai, Russia). Extremophiles 12: 133–145. https://doi.org/10.1007/s00792-007-0117-7

Frazier WC (1926) A method for the detection of changes in gelatin due to bacteria. J Infect Dis 302–309. https://doi.org/10.1093/infdis/39.4.302

Gauvry E, Mathot A-G, Leguérinel I, Couvert O, Postollec F, Broussolle V, Coroller L (2017) Knowledge of the physiology of spore-forming bacteria can explain the origin of spores in the food environment. Res Microbiol 168:369–378. https://doi.org/10.1016/j.resmic.2016.10.006

Gomes E, de Souza AR, Orjuela GL, Da Silva R, de Oliveira TB, Rodrigues A (2016) Applications and benefits of thermophilic microorganisms and their enzymes for industrial biotechnology. In: Gene expression systems in fungi: advancements and applications. Springer, pp 459–492. https://doi.org/10.1007/978-3-319-27951-0_21

Gómez F, Parro V (2012) Applications of extremophiles in astrobiology: habitability and life detection strategies. In: Adaption of microbial life to environmental extremes. Springer, pp 199–229. https://doi.org/10.1007/978-3-211-99691-1_9

Gomri MA, Rico-Díaz A, Escuder-Rodríguez J-J, El Moulouk Khaldi T, González-Siso M-I, Kharroub K (2018) Production and characterization of an extracellular acid protease from thermophilic *Brevibacillus* sp. OA30 isolated from an Algerian hot spring. Microorganisms 6:31. https://doi.org/10.3390/microorganisms6020031

Gordon RE, Haynes W, Pang CH-N, Smith N (1973) The genus *Bacillus*. US Department of Agriculture handbook, vol 427

Gower JC (1971) A general coefficient of similarity and some of its properties. Biometrics 857–871. https://doi.org/10.2307/2528823

Hayashi M, Rosenberry DO (2002) Effects of ground water exchange on the hydrology and ecology of surface water. Groundwater 40:309–316. https://doi.org/10.1111/j.1745-6584.2002.tb02659.x

Hussein EI, Jacob JH, Shakhatreh MAK, Abd Al-razaq MA, Juhmani AF, Cornelison CT (2017) Exploring the microbial diversity in Jordanian hot springs by comparative metagenomic analysis. MicrobiologyOpen 6:e00521. https://doi.org/10.1002/mbo3.521

Jain D, Sunda SD, Sanadhya S, Nath DJ, Khandelwal SK (2017) Molecular characterization and PCR-based screening of cry genes from *Bacillus thuringiensis* strains. 3 Biotech 7:4. https://doi.org/10.1007/s13205-016-0583-7

Kambourova M, Radchenkova N, Tomova I, Bojadjieva I (2016) Thermophiles as a promising source of exopolysaccharides with interesting properties. In: Biotechnology of extremophiles. Springer, pp 117–139. https://doi.org/10.1007/978-3-319-13521-2_4

Khyami-Horani H (1996) Thermotolerant strain of *Bacillus licheniformis* producing lipase. World J Microbiol Biotechnol 12:399–401. https://doi.org/10.1007/bf00340219

Kikani B, Sharma A, Singh S (2015) Culture dependent diversity and phylogeny of thermophilic bacilli from a natural hot spring reservoir in the Gir Forest, Gujarat (India). Microbiology 84:687–700. https://doi.org/10.1134/S0026261715050124

Krishnamurthi S, Ruckmani A, Pukall R, Chakrabarti T (2010) *Psychrobacillus* gen. nov. and proposal for reclassification of *Bacillus insolitus* Larkin & Stokes, 1967, *B. psychrotolerans* Abd-El Rahman et al., 2002 and *B. psychrodurans* Abd-El Rahman et al., 2002 as *Psychrobacillus insolitus* comb. nov., *Psychrobacillus psychrotolerans* comb. nov. and *Psychrobacillus psychrodurans* comb. nov. Syst Appl Microbiol 33:367–373. https://doi.org/10.1016/j.syapm.2010.06.003

Kuisiene N, Raugalas J, Stuknyte M, Chitavichius D (2007) Identification of the genus *Geobacillus* using genus-specific primers, based on the 16S–23S rRNA gene internal transcribed spacer. FEMS Microbiol Lett 277:165–172. https://doi.org/10.1111/j.1574-6968.2007.00954.x

Kumar M, Yadav AN, Tiwari R, Prasanna R, Saxena AK (2014) Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs. Ann Microbiol 64:741–751. https://doi.org/10.1007/s13213-013-0709-7

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. https://doi.org/10.1093/molbev/msw054

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22: 1658–1659. https://doi.org/10.1093/bioinformatics/btl158

Logan NALA, Allan RNAN (2008) Aerobic, endospore-forming bacteria from antarctic geothermal soils. In: Microbiology of Extreme Soils, pp 155–175. https://doi.org/10.1007/978-3-540-74231-9_8

Logan NA, Halket G (2011) Developments in the taxonomy of aerobic, endospore-forming bacteria. In: Endospore-forming soil bacteria. Springer, pp 1–29. https://doi.org/10.1007/978-3-642-19577-8_1

Logan NA, Vos PD (2015a) Bacillaceae. In: Whitman WB et al (eds) Bergey's manual of systematics of archaea and bacteria. Wiley. https://doi.org/10.1002/9781118960608.fbm00112

Logan NA, Vos PD (2015b) Bacillus. In: Whitman WB et al (eds) Bergey's manual of systematics of archaea and Bacteria. Wiley. https://doi.org/10.1002/9781118960608.gbm00530

Logan NA et al (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. Int J Syst Evol Microbiol 59:2114–2121. https://doi.org/10.1099/ijs.0.013649-0

Manachini P, Fortina M, Parini C, Craveri R (1985) *Bacillus thermoruber* sp. nov., nom. rev., a red-pigmented thermophilic bacterium. Int J Syst Evol Microbiol 35:493–496. https://doi.org/10.1099/00207713-35-4-493

Mandic-Mulec I, Prosser JI (2011) Diversity of endospore-forming bacteria in soil: characterization and driving mechanisms. In: Endospore-forming Soil Bacteria. Springer, pp 31–59. https://doi.org/10.1007/978-3-642-19577-8_2

Mandic-Mulec I, Stefanic P, van Elsas JD (2016) Ecology of Bacillaceae. In: The bacterial spore: from molecules to systems. American Society of Microbiology, pp 59–85. https://doi.org/10.1128/microbiolspec.TBS-0017-2013

Maurer K-H (2004) Detergent proteases. Curr Opin Biotechnol 15:330–334. https://doi.org/10.1016/j.copbio.2004.06.005

McMullan G, Christie J, Rahman T, Banat I, Ternan N, Marchant R (2004) Habitat, applications and genomics of the aerobic, thermophilic genus *Geobacillus*. Portland Press Limited. https://doi.org/10.1042/bst0320214

Menzel P et al (2015) Comparative metagenomics of eight geographically remote terrestrial hot springs. Microb Ecol 70:411–424. https://doi.org/10.1007/s00248-015-0576-9

Miller D, Bryant J, Madsen E, Ghiorse W (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 65:4715–4724

Müller AL et al (2014) Endospores of thermophilic bacteria as tracers of microbial dispersal by ocean currents. ISME J 8:1153–1165. https://doi.org/10.1038/ismej.2013.225

Nazina T et al (2001) Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. th*. Int J Syst Evol Microbiol 51:433–446. https://doi.org/10.1099/00207713-51-2-433

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci 76:5269–5273. https://doi.org/10.1073/pnas.76.10.5269

Panda AK, Bisht SS, Kumar NS, De Mandal S (2015) Investigations on microbial diversity of Jakrem hot spring, Meghalaya, India using cultivation-independent approach. Genom Data 4:156–157. https://doi.org/10.1016/j.gdata.2015.04.016

Panosyan H (2017) Phylogenetic diversity of thermophilic bacilli isolated from geothermal springs of Nagorno Karabakh. Chem Biol 51:179–185

Pikuta E et al (2000) *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavitherms* comb. nov. Int J Syst Evol Microbiol 50:2109–2117. https://doi.org/10.1099/00207713-50-6-2109

Poli A, Romano I, Cordella P, Orlando P, Nicolaus B, Berrini CC (2009) *Anoxybacillus thermarum* sp. nov., a novel thermophilic bacterium isolated from thermal mud in Euganean hot springs, Abano Terme, Italy. Extremophiles 13:867–874. https://doi.org/10.1007/s00792-009-0274-y

Priest FG, Goodfellow M, Tood C (1988) A numerical classification of the genus *Bacillus*. Microbiology 134:1847–1882. https://doi.org/10.1099/00221287-134-7-1847

Raddadi N, Cherif A, Daffonchio D, Neifar M, Fava F (2015) Biotechnological applications of extremophiles, extremozymes and extremolytes. Appl Microbiol Biotechnol 99:7907–7913. https://doi.org/10.1007/s00253-015-6874-9

Reysenbach A, Pace N (1995) Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 101–107

Reysenbach A-L et al. (2001) Phylum BI. *Aquificae* phy. nov. In: Bergey's manual® of systematic bacteriology. Springer, pp 359–367. https://doi.org/10.1007/978-0-387-21609-6_18

Saibi H (2009) Geothermal resources in Algeria. Renew Sust Energ Rev 13:2544–2552. https://doi.org/10.1016/j.geothermics.2007.02.002

Saitou N, Nei M (1986) The neighbor-joining method—a new method for reconstructing phylogenetic trees. In: Japanese Journal of Genetics, vol 6. Genetics Soc Japan Natl Inst Genetics, Yata, Mishima, Shizuoka 411, Japan, pp 611–611. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Satyanarayana T, Littlechild J, Kawarabayasi Y (2013) Thermophilic microbes in environmental and industrial biotechnology. In: Biotechnology of Thermophiles. Springer. https://doi.org/10.1007/978-94-007-5899-5

Sayeh R, Birrien JL, Alain K, Barbier G, Hamdi M, Prieur D (2010) Microbial diversity in Tunisian geothermal springs as detected by molecular and culture-based approaches. Extremophiles 14:501–514. https://doi.org/10.1007/s00792-010-0327-2

Schallmey M, Singh A, Ward OP (2004) Developments in the use of *Bacillus* species for industrial production. Can J Microbiol 50:1–17. https://doi.org/10.1139/w03-076

Schleifer K-H (2009) Phylum XIII. Firmicutes Gibbons and Murray 1978, 5 (Firmacutes [sic] Gibbons and Murray 1978, 5). In: Bergey's manual® of systematic bacteriology. Springer, pp 19–1317. https://doi.org/10.1007/978-0-387-68489-5_3

Shida O, Takagi H, Kadowaki K, Komagata K (1996) Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int J Syst Evol Microbiol 46:939–946. https://doi.org/10.1099/00207713-47-1-248

Shivaji S, Srinivas T, Reddy G (2014) The family *Planococcaceae*. In: The Prokaryotes. Springer, pp 303–351. https://doi.org/10.1007/978-3-642-30120-9_351

Soares MM, Silva R, Gomes E (1999) Screening of bacterial strains for pectinolytic activity: characterization of the polygalacturonase produced by *Bacillus* sp. Rev Microbiol 30:299–303. https://doi.org/10.1590/S0001-37141999000400002

Song ZQ et al (2013) Bacterial and archaeal diversities in Yunnan and Tibetan hot springs, China. Environ Microbiol 15:1160–1175. https://doi.org/10.1111/1462-2920.12025

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 101:11030–11035. https://doi.org/10.1073/pnas.0404206101

Thompson JD, Higgins DG, Gibson TJ (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. https://doi.org/10.1093/nar/22.22.4673

Tindall BJ, Sikorski J, Smibert RA, Krieg NR (2007) Phenotypic characterization and the principles of comparative systematics. In: Methods for general and molecular microbiology, 3rd edn. American Society of Microbiology, pp 330–393. https://doi.org/10.1128/9781555817497.ch15

Tiwari K, Thakur HK (2014) Diversity and molecular characterization of dominant *Bacillus amyloliquefaciens* (JNU-001) endophytic bacterial strains isolated from native neem varieties of Sanganer region of Rajasthan. J Biodivers Biopros Dev 1:2. https://doi.org/10.4172/2376-0214.1000115

Trujillo ME (2001) Actinobacteria. In: Encyclopedia of life sciences. Wiley, pp 1–20. https://doi.org/10.1002/9780470015902.a0020366.pub2

Urbieta MS, Donati ER, Chan K-G, Shahar S, Sin LL, Goh KM (2015) Thermophiles in the genomic era: biodiversity, science, and applications. Biotechnol Adv 33:633–647. https://doi.org/10.1016/j.biotechadv.2015.04.007

Weigmann H (1898) Ueber zwei an der Käsefereifung beteiligte Bakterien. Bakteriol Parasitenkd Infektionskr Hyg Abt II Zentbl. Hyg. Abt. 820–834

Yoon J-H, Lee K-C, Weiss N, Kho YH, Kang KH, Park Y-H (2001) *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of th. Int J Syst Evol Microbiol 51:1079–1086. https://doi.org/10.1099/00207713-51-3-1079

Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. Int J Syst Evol Microbiol. https://doi.org/10.1099/ijsem.0.001755

Zhang Y, He S, Simpson BK (2018) Enzymes in food bioprocessing—novel food enzymes, applications, and related techniques. Curr Opin Food Sci. https://doi.org/10.1016/j.cofs.2017.12.007