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Thermophilic bacteria from Peruvian hot springs with high potential application in environmental biotechnology

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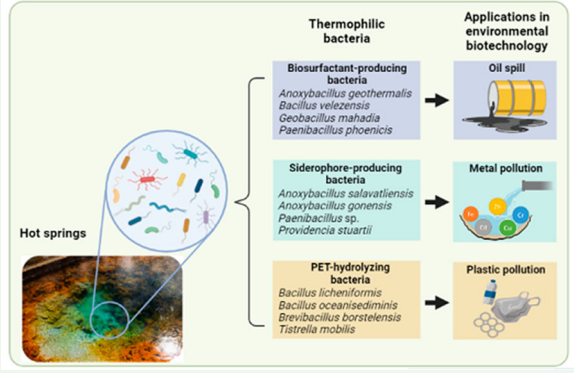
ABSTRACT

Hot springs are extreme environments in which well-adapted microorganisms with biotechnological applications can thrive naturally. These thermal environments across Peruvian territory have, until now, remained poorly investigated. In this study, two hot springs, El Tragadero and Quilcate, located in Cajamarca (Peru) were selected in order to investigate the biotechnological potential of indigenous thermophilic bacteria. Enrichment and isolation processes were carried out using microbial mats, sediments, biofilms, and plastic polymers as samples. Screening for biosurfactants and siderophores production, as well as for polyethylene terephthalate (PET) hydrolysis was done using culture-dependent techniques. After molecular identification, *Bacillus* was found as the most abundant genus in both hot springs. *Bacillus velezensis* was found producing biosurfactants under high-level temperature. *Anoxybacillus* species (*A. salavatliensis* and *A. gonensis*) are here reported as siderophore-producing bacteria for the first time. Additionally, *Brevibacillus* and the less-known bacterium *Tistrella mobilis* were found demonstrating PET hydrolysis activity. Our study provides the first report of thermophilic bacteria isolated from Peruvian hot springs with biotechnological potential for the bioremediation of oil-, metal- and plastic-polluted environments.



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Thermophiles; biosurfactants; siderophores; hydrolysis; polyethylene terephthalate

Introduction

Extreme environments such as hot springs are the natural culture media for the development of well-adapted microorganisms suitable for being used in biotechnology. As an example of this, we can find thermophiles, microorganisms with optimal growth temperature of 45°C or higher, whose thermostable enzymes (thermozymes) are valuable resources not only in biocatalysis but also in bioremediation. Biosurfactants [1], siderophores [2], and hydrolases such as PETases, cutinases, lipases, and proteases [3], are some examples of thermozymes with extensive applications in environmental biotechnology. Additionally, phosphatases and oxidoreductases, with potential application in the degradation of phosphate- and aromatic-like compounds, have also been reported in microorganisms isolated from hot spring environments [4]. Currently, different thermophilic bacteria have been retrieved and identified from hot springs worldwide. *Bacillus, Paenibacillus, Anoxybacillus, Geobacillus, Lysinibacillus* and *Brevibacillus* are some examples of thermophilic bacterial genera with different applications in biotechnology [1,4–8].

Thermophilic bacteria can be used as workhorses for bioremediation purposes. In recent decades, a plethora of studies have applied a bioremediation approach

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using mesophilic microorganisms under standard pH and temperature conditions to decrease the negative effects of long-term environmental problems such as metal [9], hydrocarbon [10,11] or even petro-polymer [12] pollution. Nevertheless, we know that the biodegradation of some recalcitrant pollutants can be improved dramatically by manipulating environmental factors (e.g. temperature). At higher temperatures, structural and chemical features in some pollutants like plastics are changed that might enhance their enzymatic degradation [13]. Further, when temperature increase, the rate of metal biosorption by microorganisms increase as well, and the viscosity of some liquids (such as hydrocarbons) decreases [9]. Under these temperature conditions, using thermophiles as biotechnological tools offer an invaluable opportunity to get better results in bioremediation processes.

Cajamarca, a region of the northern mountain range of Peru, contains various hot springs such as El Tragadero and Quilcate throughout its territory. According to the Geological, Mining and Metallurgical Institute of Peru (INGEMMET, by its acronym in Spanish), the waters of these places emerge from the Goyllarisquizga and Calipuy geological groups and have an average temperature of 51–71°C. Both hot springs belong to the chlorinated waters due to the presence of chloride ions from 101 to 370 mg L^{-1}, and owing to their geochemical composition both are metal-rich waters, especially by their higher concentration of Mn and As [14]. Nowadays, and due to the belief of these waters are medicinal, these hot springs are only exploited as touristic sites, with little or no scientific research. Nevertheless, the occurrence of microbial complexes such as biofilms and microbial mats, are indicatives of the well-adapted microbial activity in these extreme environments [15,16], making hot springs suitable places for the study of adapted microorganisms with potential applications in biotechnology.

This study was conducted in order to investigate the biotechnological potential of thermophilic bacteria that naturally occur in two hot springs (El Tragadero and Quilcate) located in Cajamarca, Peru. Biosurfactants and siderophores production, but also the PET hydrolysis capability was evaluated on these bacteria, which were then identified using the 16S rRNA gene sequencing analysis. The metabolites produced and the hydrolysis capability by these thermophilic bacteria have potential applications in environmental biotechnology and could contribute to the development of innovative bioremediation strategies to help solve environmental pollution problems in Cajamarca or even worldwide.

Materials and methods

Zone description and sample collection

Two hot springs located in the Cajamarca region were selected for this study, El Tragadero (Baños del Inca) and Quilcate (San Miguel) (Figure 1A). Access to both sites was by foot. El Tragadero hot spring has an area of 46.3 m^2 and is located near a tourist site. Despite the site being modified by concrete and brickworks, microbial complexes (biofilms and microbial mats) occurred naturally (Figure 1B). The geological activity at the site gave rise to reddish and grey sediments across the whole area. Quilcate hot spring has an area of 1818 m^2 and its natural features, contrary to El Tragadero, remain. However, plastic polymers were more visible here (Figure 1B). Physicochemical parameters (temperature and pH) were measured *in situ* in both sites using a multimeter (Hanna instruments). Microbial mats, biofilms, and sediments were collected from each site (Figure 1B). Microbial mats were recovered from the surface of the water using a sterile spatula, then stored in sterile plastic bags and sealed. Biofilms were collected with plastic sterile syringes of 10 mL and stored in 15 mL falcon tubes. Sediment samples were collected with 60 mL sterile syringes which were cut off and introduced into the sediment until 10 cm of depth, then sealed with parafilm and stored in plastic bags. For plastic biodegradation assays, plastic polymers (bottles and bags) were collected from each hot spring and stored in sterile plastic bags with water to maintain the biofilm's integrity. All samples were collected in triplicates and stored in a cooler box to maintain the hot spring's temperature until shipping. Additional water and sediment samples were collected for chemical analysis. Samples were shipped to the Laboratorio de Microbiología at the Universidad Nacional de Cajamarca; there, microbial mats and sediment samples collected from each place were pooled and blended in sterile flasks and maintained at 55–60°C for microbiological assays. Biofilms were mixed and pelleted, to be resuspended later in sterile saline solution (SS) (0.85% (w/v) NaCl) prior to use.

Chemical analysis

Total metal content was analyzed in surface and pore water samples. The pore water was recovered after sediment centrifugation according to U.S. EPA [17]. Analysis was carried out using Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) in a Thermo Scientific iCAP 7000 series after acid digestion.

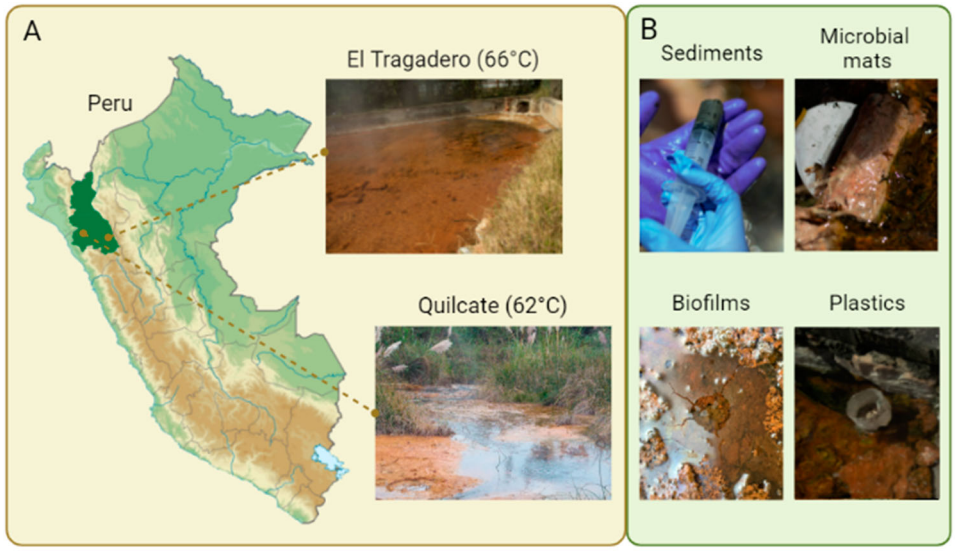

Figure 1. Location of El Tragadero and Quilcate hot springs in Cajamarca (Peru) (A), and sample types collected (B) (created with Biorender).

Enrichment and isolation

For biosurfactant-producing bacteria

Samples undergo three successive cycles of enrichment in 250 mL glass bottles containing 100 mL of minimal salt medium (MSM) supplemented with 1% (v/v) diesel or gasoline as carbon sources [10]. The composition of MSM was the following (g L^{-1}): $NaNO_3$, 7.5; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; K_2HPO_4, 4.4; KH_2PO_4, 3.4; $MgSO_4.7H_2O$, 0.5; yeast extract, 0.5; and glucose, 10.0. To 1 L of this mixture, 5 mL of a modified trace element solution was added according to the following composition (g L^{-1}): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17 [18]. The pH of the medium was adjusted to 6.5 with NaOH or HCl 1N and then autoclaved at 121°C at 1 atm for 20 min. Hydrocarbons and trace element solution were added by filtration using a 0.2 µm pore size filter. Inoculation was done with 10% (w/v) of each mixed sample under sterile conditions. Flasks were incubated at 55–60°C for 7 days. A new enrichment was set up after the incubation time by transferring 10 mL of the old enrichment supernatant into a bottle with a fresh medium.

Isolation was performed in Bushnell Haas (BH) medium supplemented with 1% (v/v) hydrocarbons as the sole carbon source [10]. The composition of BH was the following (g L^{-1}): $MgSO_4.7H_2O$, 0.2; $CaCl_2.4H_2O$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $FeCl_3$, 0.05 and agar, 17.0. Medium pH was adjusted to 6.5 and then autoclaved. Hydrocarbons were sterilized and supplemented as described above but this time in Petri dishes. Inoculation was done with 10 µL of the final enrichment and spread on the surface with a Drigalsky spatula. Plates were incubated at 55–60°C for 24–48 h. To avoid excessive agar desiccation, Petri dishes were placed in moistened plastic bags and sealed. Phenotypically different colonies were collected and sub-cultured (three times) into nutrient agar (NA) to get purity.

For siderophore-producing bacteria

Samples were passed through two enrichment cycles. 100 mL Luria Bertani (LB) broth (Micromedia Trading House Ltd, Hungary) into 250 mL glass bottles supplemented with two concentrations of EDTA (50 and 100 µM) was used to produce iron deficient conditions for the enrichments in this study. The pH of the medium was adjusted to 6.5 and the medium was then autoclaved. Inoculation was carried out with 10% (w/v) of each sample, flasks were incubated at 55–60°C for 24–48 h in an orbital shaker (120 rpm). Serial dilutions were done from the last enrichment and 10 µL of the last dilution (10^{-3}) was spread on LB agar (1.7% (w/v) agar). Phenotypically different colonies were collected and cultured again into NA to get purity.

For PET-hydrolyzing bacteria

Biofilms adhered to plastic polymers were recovered [19]. Briefly, plastics were aseptically cut into 5 × 5 cm pieces with sterile scissors and placed in 50 mL centrifuge tubes containing 20 mL of sterile SS. Tubes were vigorously agitated by hand for 20 min. The supernatant (containing biofilms) was centrifuged at 5000 rpm for 5 min at 4°C. Pellets were resuspended into 10 mL SS, vortexed, and serially diluted. Isolation was performed on LB agar using 10 µL of each suspension spread on

the surface of the plate. Plates were incubated at 55–60° C for 24–48 h. Phenotypically different colonies were picked out and sub-cultured into NA to get purity.

Screening for metabolic activity

Biosurfactants production

Cultures were reactivated in 4 mL LB broth for 48 h, then centrifuged at 16200 g for 20 min in a microcentrifuge. The supernatant was eliminated and pellets were resuspended in 0.5 mL of SS. Inoculation was done into 50 mL serum bottles containing 15 mL of MSM with 1% hydrocarbons (sterilized and supplemented as mentioned above) (Figure 2A). Each culture was incubated at 55–60°C for 7 days without agitation. After incubation, cultures were filtered and the free-cell solution was used for the screening as follows.

The drop collapse method (DCM) and the oil spread method (OSM) were performed to check biosurfactant activity, while the emulsification activity (EA) and emulsifying index 24 (EI-24) assays were used to screen the production of bioemulsifiers. DCM was done with 20 µL of the free-cell solution, which was placed on the hydrophobic surface of parafilm M [20]. Drops collapse indicated a positive result for biosurfactant production and was classified according to the following pattern: +, low; ++, regular; and +++, total collapse (Figure 2B). OSM protocol [21] consisted in adding 10 µL of filtered culture on the surface of a thick hydrocarbon film, disruption in surface tension of hydrocarbon was measured and considered a positive result. EA was performed with a 3 mL cell-free solution that was mixed with 0.5 mL of each hydrocarbon and vortexed at maximum speed for 2 min [22]. The final solution was allowed to settle for 1 h at room temperature. The aqueous phase was recovered and analyzed at 400 nm using a UV/VIS 2100 spectrophotometer (UNICO™). Emulsifying units (EU) were calculated according to the following expression: EU = absorbance at 400 nm/0.01. EI-24 analysis was done with 7 mL of the cell-free solution and vortexed at maximum speed with 3 mL of each hydrocarbon for 1 min. After 24 h, the EI-24 was calculated as EI-24 = Height of emulsifying layer/ Total height of the liquid x 100 [23] (Figure 2C-D).

Siderophores production

The chrome azurol S (CAS) assay [24] was used to detect whether these iron-chelating metabolites were produced.

Qualitative assay

We used CAS agar plates, in which two stock solutions were prepared. Minimal medium 9 (MM9) stock solution (solution I) was prepared by mixing the following components (g L^{-1}): KH_2PO_4, 0.3; NaCl, 0.5; NH_4Cl, 1; $MgSO_4$, 0.25; $CaCl_2$, 0.011. The pH of the medium was adjusted to 7.4 with NaOH or HCL 1N and deferrated using 10% 8-hydroxyquinoline in chloroform [25]. CAS solution (solution II) was composed of three solutions: CAS, iron and hexadecyltrimethylammonium bromide (HDTMA) solutions. 1 L of CAS agar was prepared by mixing 750 mL of distilled water with 32.34 g of piperazine-N,N′-bis(2-ethanesulfonic acid) PIPES -maintaining pH 6.8 of the solution with 16 g of NaOH 50% (w/w)-, 100 mL of solution I and 17 g of bacto agar; the mixture was autoclaved at the conditions described above and cooled to 50°C prior to the addition of 30 mL of casamino acid solution (previously deferrated) and 10 mL of 20% glucose solution by filtration. Finally, 100 mL of solution II (CAS solution) was added slowly to

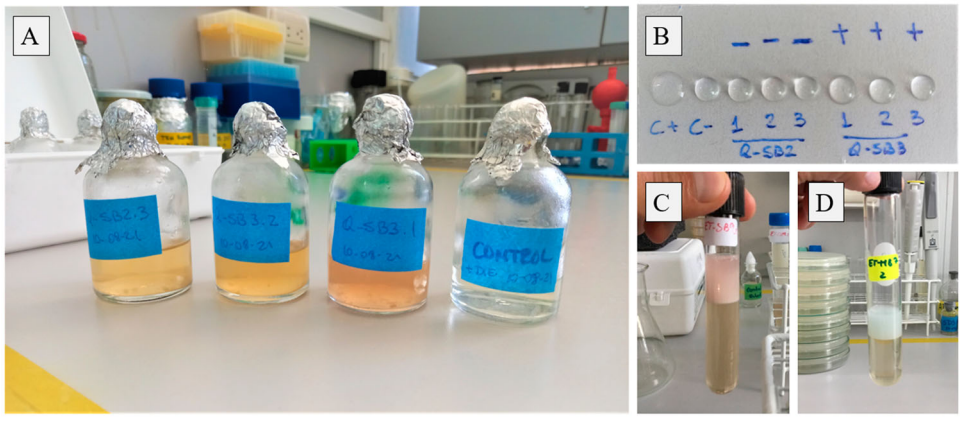

Figure 2. Screening assays for biosurfactant production of thermophilic bacteria isolated from El Tragadero and Quilcate hot springs. (A) MSM supplemented with hydrocarbons (diesel/gasoline) for biosurfactant production. Assays were done by triplicates including a control. (B) DCM showing negative and positive results. Positive results (emulsification) of the EI-24 assay with diesel (C) and gasoline (D) as hydrocarbons.

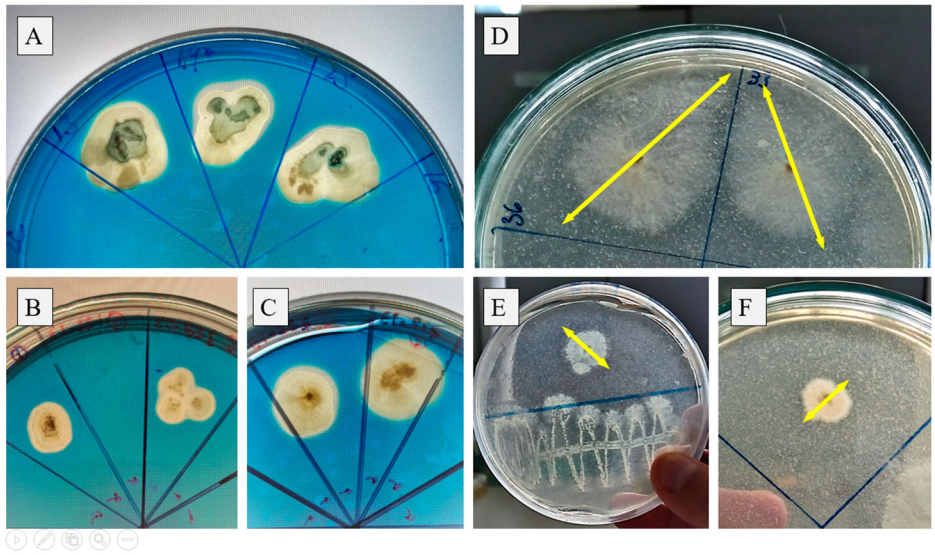

Figure 3. Screening assays of thermophilic bacteria isolated from El Tragadero and Quilcate hot springs for siderophore production using CAS-agar (A, B, and C) and for PET hydrolysis using PET-agar (D, E, and F). Yellowish-orange halos indicate positive siderophores production. Yellow arrows indicate the diameter of the clear zone in PET-agar.

the mixture along the glass wall to mix thoroughly. Plates were poured with 20 mL of CAS medium. Inoculation was done with 10 µL of previously reactivated cultures in deferrated LB broth (d-LB). In cultures where biofilms were formed, the entire biofilm was placed on the plate to enhance microbial growth. The siderophore-producing strain *Burkholderia vietnamiensis* la1a4, which was kindly donated by the Laboratorio de Microbiología Agrícola 'Raúl Ríos Reátegui' of the Universidad Nacional de San Martín-Tarapoto, was used as a positive control. Plates were incubated at 55–60°C for 24–48 h. A change of colour from blue to orange around the colony was considered a positive result (Figure 3A-C).

Quantitative assay

We followed the CAS liquid assay [26] with some modifications for isolates with positive siderophore production after the qualitative assay. We used 1 mL d-LB (pH 6.8) to stimulate the siderophore production by thermophilic bacteria isolates. After 24–48 h of incubation, cultures were centrifuged at 10000 rpm for 5 min, and the supernatant was discarded. Pellets were resuspended in d-LB and washed out 2–3 times. Resuspended cells were inoculated at the rate of 1% (v/v) in glass vials containing 5 mL of d-LB and incubated at 55–60°C for 24–48 h. The fermented broth was centrifuged at 10000 rpm for 15 min and 1 mL was mixed with 1 mL of CAS solution [27]. We measured the absorbance in samples at 630 nm after 24 h of incubation (absorbance was stable at this time) at room temperature. Siderophore units (SU) in samples were measured as follows: A – Ab (A: absorbance of the sample; Ab: absorbance of the blank), considering the uninoculated culture as the blank [28].

PET hydrolysis

The clear zone assay [29] was used to visualize the utilization of PET as the sole carbon source by the isolated cultures through enzymatic degradation. The medium was composed of two solutions. The polymer solution was double concentrated in the following way: 1.2 g of PET powder was mixed into a 250 mL glass bottle containing 200 mL of distilled water, and the solution was shaken at 170 rpm for 1 h. In a 500 mL glass flask, 7.2 g of agar was added to 200 mL of distilled water, agar was melted and cooled to 50°C. PET solution was added to the agar solution and autoclaved. Mineral salts solution consisted of MSM prepared as specified above (biosurfactant section), but this time without the carbon source. The medium was autoclaved under the same conditions. Plates were composed of two solid medium layers; the lower layer consisted of 15 mL of mineral salts solution and the upper layer of 10 mL of the polymer solution. Cultures were inoculated by the puncture technique using a maximum of 4 cultures per plate. A clear zone (halo) around the colony was considered a positive result (Figure 3D-F).

Microbiological characterization of the isolates

Isolates, which were positive for metabolic activity, were characterized based on their stains, their cell and colony morphology, but also on their physiological features.

The temperature range of growth in fresh NA medium was established by performing culture experiments from 45 to 70°C. Pure cultures were subjected to Gram and malachite green stains (0.5% (w/v) malachite green). Catalase and oxidase activities were tested as well. The utilization of citrate as the sole carbon source was evaluated in Simmons Citrate agar. The Voges-Proskauer test was used to determine whether thermophilic bacteria produce acetoin as a product of glucose fermentation. Chapman agar (7.5% (w/v) NaCl) was used to test the tolerance of isolates to salinity. Physiological analyses were performed at 50°C and were evaluated at 24, 48 and 72 h.

16S rRNA sequencing and phylogenetic analysis

DNA extraction and PCR

Bacterial genomic DNA was extracted from each isolate using a standard protocol [30]. The quantity and quality of the genomic DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). 16S rRNA genes were amplified using universal primer pairs: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR mixture used for amplification contained: 1 µL DNA, 5 µL 10X buffer, 5 µL 2mM dNTPs, 1.5 µL of each primer (10 µM), 25 mM MgSO4, 1 µL KOD Hot Start DNA Polymerase (1 U/µL) and PCR grade water to reach a final volume of 50 µL. PCR was conducted using a S1000™ Thermal Cycler (BIO-RAD). PCR setup consisted of 35 cycles of denaturation at 95°C for 20 s, annealing at 54°C for 30 s, elongation at 70°C for 20 s and a final extension at 70°C for 5 min. PCR products were loaded in 1% (w/v) agarose gel to check the size of the amplicons through electrophoresis. Amplicons were visualized in Visi-Blue™ Transilluminator (UVP, Analytik Jena) and stored until sequencing.

Sequencing and phylogenetic analysis

Sequencing of bacterial 16S rRNA amplicons was performed on a ABI 3730XL Capillary Electrophoresis Sequencing System by Macrogen (South Korea). Raw data of the DNA sequences were edited and analyzed using the Chromas (v. 2.6.6) and the AliView software (v. 1.28). BLASTn was used to obtain information on the phylogenetically closest bacteria strains in the NCBI database (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/Blast) [31]. The phylogenetic trees were constructed using the Neighbor-Joining method [32] with the MEGA X software [33]. Confidence in branching points was determined by 1000 bootstrap analyses. The 16S rRNA gene sequences of each isolate were deposited into the GenBank in which accession numbers were assigned (see Table 4).

Results

Geochemical parameters of El Tragadero and Quilcate hot springs

Physicochemical parameters of El Tragadero and Quilcate hot springs are displayed in Table 1. Temperature and pH were similar in both hot springs. Chemical analysis showed high variability of metals, metalloids, and non-metals in surface and pore water samples (Table 1). These compounds were found in the following order of concentration in El Tragadero: Ca > Si > Fe > Al > *P* > Mn > Zn > As, and Quilcate: Ca > Si > Fe > Mn > Al > As > *P* > Zn. It is worth noticing that Fe, Al, Mn, and As, in El Tragadero, and Fe, Mn, and As, in Quilcate, surpass the limiting values of the environmental quality standards (ECA, by its acronym in Spanish) for Peruvian norms (subcategory B-surface water intended for recreation).

Isolation and metabolic capability of thermophilic bacteria

After enrichment, we isolated seventy-eight thermophilic bacteria, 34 from El Tragadero and 44 from Quilcate hot spring. We coded each strain considering the place of isolation (ET, El Tragerdero; Q, Quilcate), the sample (S, sediment; M, microbial mat; B, biofilm), and their metabolic capability (B, biosurfactants; S, siderophores; P, plastic biodegradation).

Table 1. Physico-chemical parameters -pH, temperature (T)- and chemical composition (metals, metalloids, and non-metals concentration) measured in surface water and pore water (sediments) samples of El Tragadero and Quilcate hot springs. WT: water type; S: surface water; P: pore water.

				Chemical composition (mM)							
Site	WT	pH	T (^{0}C)	Al	Fe	Zn	Mn	Ca	Si	As	*P*
El Tragadero	S	6.4	66.3	9.9×10^{-1}	13.3×10^{-1}	3.9×10^{-3}	6.2×10^{-2}	26.1×10^{-1}	17.2×10^{-1}	3.5×10^{-4}	7.5×10^{-2}
	P	6.5	–	28×10^{-1}	9.0×10^{-1}	2.0×10^{-2}	1.0×10^{-1}	18.0×10^{-1}	39×10^{-1}	4.3×10^{-3}	1.1×10^{-2}
Quilcate	S	6.6	62	2.4×10^{-3}	1.8×10^{-2}	4.4×10^{-4}	2.5×10^{-3}	26.8×10^{-1}	9.5×10^{-1}	2.4×10^{-3}	1.9×10^{-3}
	P	6.6	–	6.6×10^{-3}	1.9×10^{-1}	5.8×10^{-5}	7.0×10^{-3}	66.0×10^{-1}	2.4×10^{-1}	3.4×10^{-3}	3.5×10^{-3}

Table 2. Screening for biosurfactants and siderophores production, as well as for PET hydrolysis capability of thermophilic bacteria obtained from El Tragadero (ET) and Quilcate (Q) hot springs. D: diesel; G: gasoline; DCM: drops-collapse method (+, low; ++, regular; and +++, total collapse); OSM: oil-spread method; EA: emulsifying assay (EU: emulsifying units); EI-24: Emulsifying index-24 (*: only one replicate was positive); SU: siderophore units (NG: no growth).

Biosurfactants						Siderophores			PET hydrolysis	
Isolate	Hydrocarbon	DCM	OSM (mm)	EA (EU)	EI-24 (%)	Isolate	CAS agar	% SU	Isolate	PET agar
ETSB2	D	+	0.6 ± 0.5	65.5 ± 47.1	–	ETSS1	+	50.6 ± 0.04	ETP1	+
ETSB6	G	+	0.5 ± 0.2	19.5 ± 13.3	–	ETSS2	–	–	ETP2	+
ETSB7	G	+	0.3 ± 0.1	45.7 ± 43.4	–	ETSS7	+	62.4 ± 0.1	ETP3-6	–
ETSB9	D	++	0.7 ± 0.4	77.3 ± 15.5	32.4*	ETSS8	+	56.5 ± 0.1	QP1-2	–
ETMB3	D	++	0.3 ± 0.1	41.2 ± 9	27.9 ± 1.0	ETSS9-11	–	–	QP3	+
ETMB6	G	++	0.5 ± 0.3	43.4 ± 32.8	28.6*	ETSS12	+	54.3 ± 0.1	QP4	–
ETMB7	G	++	0.5 ± 0.2	158.2 ± 76.9	30.8 ± 4.7	ETSS14	–	–	QP6	+
ETMB8	G	+	0.2 ± 0.2	113.1 ± 25.2	–	ETBS1	+	NG	QP7	–
ETMB9	G	+	0.3 ± 0.1	45.4 ± 28.3	27.7 ± 2.7	ETBS4	+	NG	QP8	+
ETBB1	D	++	0.6 ± 0.4	37.2 ± 5.3	–	ETBS5	+	60.3 ± 0.1	QP9	–
ETBB2	D	+	0.2 ± 0.1	56.6 ± 20.7	16.7 ± 3.4	ETBS8-9	–	–	QP12	+
QMB1	D	+	0.4 ± 0.2	13.2 ± 6.4	–	ETMS1-3	–	–	QP13-14	–
QSB1	D	+++	0.6 ± 0.2	55.4 ± 9.8	–	QSS1-2	–	–	QP15	+
QSB2	D	-	-	97.6 ± 15.6	–	QSS4	–	–	QP16	–
QSB3	D	++	0.7 ± 0.4	87.7 ± 50.7	–	QSS5	+	39.1 ± 0.04	QP18	–
						QSS6-7	–	–	QP19	+
						QSS9-10	–	–	QP20	–
						QMS2	+	15.5 ± 0.3	QP22	+
						QMS5-6	–	–	QP23-26	–
						QMS7	+	46.2 ± 0.2	QP27	+
									QP28	+
									QP29-33	–

Biosurfactant production

Fifteen isolates were retrieved, 11 from El Tragadero and 4 from Quilcate. Biosurfactant production was confirmed in all these isolates after the screening with diesel or gasoline as hydrocarbons (Table 2). All isolates were positive for DCM and OSM, except QSB2. EA was positive in all isolates, with values from 13.2 ± 6.4 to a maximum of 158.2 ± 76.9. Only six isolates were positive for the EI-24 assay, in which one of the highest values corresponded to the same isolate with the highest EA (ETMB7) (Table 2).

Siderophore production

Twenty-nine isolates were obtained after LB enrichment, 17 of El Tragadero and 12 from Quilcate. From that 29, only 10 isolates were positive for siderophore production (Table 2), indicating that, unlike biosurfactant enrichment, siderophore enrichment was not very selective. After the quantitative assay, SU ranged from 15.5% up to a maximum of 62.4% (Table 2). Although we only consider as positive results the change of colour on CAS agar medium, we also noticed variability in strain colony size, halo size, and even in the colour intensity of the halo. These features could be the focus of new research projects to clarify how thermophilic bacteria grow in this medium, and how siderophores are produced/released under extreme conditions. Biofilm-producing strains were those that produced better growth in this medium (Figure 3A-C).

PET hydrolysis

Thirty-four bacteria were recovered from plastic-adhered biofilms, 6 from El Tragadero and 28 from Quilcate. From that number, only 11 isolates produced visible halos in PET-agar medium (Table 2) indicating that they are able to hydrolyze this polymer (Figure 3D-F). Although isolation was not conducted throughout the enrichment approach, unlike for the other two metabolites, the number of positive isolates indicates that the isolation approach used in this study, which consisted in recovering these bacteria from the environment on LB agar, has a good performance as demonstrated in other studies [34].

Characteristics of the isolates

Microbiological characterization of thermophilic bacteria is displayed in Table 3. In general, most isolates were Gram-stain-positive (some were Gram variable), rod-shaped, spore-forming bacteria, except for isolates QMS2 and QP27, which were Gram-stain-negative, non-spore-forming bacteria. It is worth noticing that some strains showed filamentous shapes, creating cell mass complexes (ETMB3, ETMB9, ETSS7, ETSS12, and QMS7). Colony characteristics were highly variable in form, elevation, margin, texture; and colour. All bacteria showed a general temperature range of 50–60°C with faster-growing rates (from 24 to 48 h) as well. All the isolates were positive for the catalase test (not displayed in Table 3). After the oxidase test, we found that the

Table 3. Microbiological characterization of thermophilic bacteria from El Tragadero and Quilcate hot springs with biosurfactant- and siderophore- production capability, as well as with PET-hydrolyzing activity. var, variable; Arrang: arrangement; nd, not determined.

	Stains		Cell morphology		Colony morphology					Physiological features				
Isolate	Gram	Spore	Shape	Arrang.	Form	Elevation	Margin	Texture	Colour	Temp. range (°C)	Citrate utilization	Oxidase Test	Voges Test	7.5% NaCl Grow
ETSB2	+	+	short rods	pairs	circular	crateriform	entire	moist	cream	50–55	–	–	–	–
ETSB6	+	+	short rods	chains/group	circular	raised	entire	dry	cream	50–55	–	+	–	–
ETSB7	+	+	short rods	chains/group	circular	crateriform	entire	moist	cream	50–55	–	+	–	–
ETSB9	+	+	tiny rods	diplobacilli	circular	convex	undulate	mucoid	No	50–55	+	+	+	+
ETMB3	var	+	filamentous	cell masses	circular	raised	entire	moist	cream	50–55	–	+	–	–
ETMB6	+	+	rods	palisades	circular	raised	entire	moist	cream	50–55	–	–	–	–
ETMB7	+	+	rods	palisades	circular	raised	entire	moist	cream	50–55	–	+	–	–
ETMB8	var	+	tiny rods	palisades	circular	raised	entire	moist	cream	50–60	–	+	–	–
ETMB9	var	+	filamentous	cell masses	circular	raised	entire	moist	cream	50–60	–	+	–	–
ETBB1	+	+	short rods	single/chains	irregular	umbonate	entire	moist	white	50–55	+	+	+	+
ETBB2	+	+	short rods	single/chains	irregular	umbonate	entire	moist	white	50–55	+	+	–	+
QMB1	+	+	short rods	single/chains	irregular	umbonate	entire	moist	white	50–55	–	+	+	+
QSB1	+	+	rods	single/group	rhizoid	flat	filiform	moist	white	50–55	+	+	+	+
QSB2	+	+	short rods	single/chains	irregular	crateriform	entire	moist	white	50–55	–	+	–	+
QSB3	+	+	short rods	single/chains	irregular	umbonate	entire	moist	white	50–55	–	+	+	+
ETSS1	+	+	short rods	pairs	circular	crateriform	entire	moist	cream	50–55	–	+	–	–
ETSS7	var	+	filamentous	cell masses	circular	umbonate	entire	dry	cream	50–60	–	+	–	–
ETSS8	+	+	short rods	single/chains	irregular	umbonate	entire	moist	white	50–55	+	+	+	+
ETSS12	var	+	filamentous	cell masses	circular	raised	entire	moist	orange	50–60	–	+	–	–
ETBS1	var	+	short rods	single/group	filamentous	flat	filiform	moist	cream	50–55	nd	nd	nd	nd
ETBS4	var	+	short rods	single/chains	irregular	raised	filiform	dry	cream	50–55	nd	nd	nd	nd
ETBS5	var	+	short rods	single/pairs	circular	convex	entire	moist	cream	50–55	–	+	+	+
QSS5	+	+	short rods	single/chains	irregular	umbonate	entire	moist	white	50–55	+	+	+	+
QMS2	–	–	rods	group	irregular	raised	entire	moist	cream	50–60	nd	nd	nd	nd
QMS7	var	+	filamentous	cell masses	circular	raised	entire	moist	orange	50–60	–	–	–	–
ETP1	+	+	rods	group	circular	flat	undulate	moist	cream	50–55	–	+	–	+
ETP2	+	+	short rods	single/chains	irregular	convex	lobate	moist	cream	50–55	–	–	+	+
QP3	+	+	short rods	single/chains	irregular	umbonate	undulate	dry	white	50–55	–	–	+	+
QP6	+	+	short rods	single/chains	circular	umbonate	undulate	dry	white	50–55	–	–	–	+
QP8	+	+	rods	group	irregular	flat	undulate	moist	cream	50–55	–	+	–	–
QP12	+	+	short rods	single/chains	irregular	flat	entire	dry	cream	50–55	–	–	+	+
QP15	+	+	rods	group	irregular	raised	filiform	dry	white to pink	50–55	–	–	+	+
QP19	+	+	short rods	single/chains	irregular	raised	filiform	dry	white to pink	50–55	–	+	+	+
QP22	+	+	rods	group	irregular	raised	filiform	dry	white to pink	50–55	+	+	+	+
QP27	–	–	rods	single/pairs	circular	flat	entire	moist	cream	50–55	–	+	–	–
QP28	+	+	short rods	single/chains	irregular	flat	filiform	moist	white to pink	50–55	–	+	+	+

cytochrome c oxidase is produced by most of the isolates. The ability to use citrate as the sole carbon source was only detected in 7 bacteria. Halotolerant bacteria were also detected due to their ability to grow up to 7.5% NaCl (1.3 M). Most of the isolates produced acetoin during the Voges-Proskauer test.

Taxonomy and phylogeny of bacteria

After analysis, sequences showed high similarity (> 99%) with their homologous in the Genbank (Table 4). The closest evolutionary relationship of each isolate is displayed in phylogenetic trees (Figures 4–6). *Bacillus* was found as the most abundant genus in both hot springs. Biosurfactant-producing bacteria were affiliated to the following genera (Figure 4): *Bacillus*, *Parageobacillus*, *Geobacillus*, *Paenibacillus* and *Anoxybacillus*. An unexpected isolate of *Bacillus velezensis* was recovered from El Tragadero under high-temperature conditions. Regarding siderophores, *Bacillus*, *Paenibacillus* and *Anoxybacillus* were found as siderophore-producing bacteria as well. It is worth noticing that the latest genus has not been reported producing siderophores so far. Furthermore, an isolate affiliated to *Providencia stuartii* was the only strain with siderophore production capability that clustered with the *Proteobacteria* phylum (Figure 5). Concerning plastic hydrolysis, *Bacillus* was the predominant genus again, but also two isolates affiliated to *Brevibacillus* and to, the less-known bacterial genus, *Tistrella* (*Proteobacteria*) (Figure 6) were found.

Discussion

Geochemical characteristics of the hot springs

Due to their geochemical features, El Tragadero and Quilcate hot springs can be considered neutral thermal waters. The occurrence of Fe, Al and Si (Table 1) in water samples could be due to the dissolution processes promoted by the constant interaction of water with minerals such as halloysites ($Al_2Si_2O_5(OH)_4{\cdot}2H_2O$), kaolinites

Table 4. Taxonomic identification of thermophilic bacteria from El Tragadero and Quilcate hot springs with biosurfactant- and siderophore-production capability, as well as with PET-hydrolyzing activity. AN, accession number; S: sediment; B: biofilm; MM: microbial mats; PP: plastic polymers.

Capability	Sample type	Isolate	NCBI AN	Bacterial phylum	Bacterial genus	NCBI homologous/AN	Similarity (%)
Biosurfactants production	S	ETSB2	ON004976	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus salavatliensis*/LT835101.1	99.9
	S	ETSB6	ON004977	*Firmicutes*	*Paenibacillus*	*Paenibacillus phoenicis*/MW686866.1	99.7
	S	ETSB7	ON004978	*Firmicutes*	*Paenibacillus*	*Paenibacillus ginsengihumi*/LC588569.1	99.6
	S	ETSB9	ON004979	*Firmicutes*	*Bacillus*	*Bacillus velezensis*/MN365041.1	100
	MM	ETMB3	ON004969	*Firmicutes*	*Parageobacillus*	*Parageobacillus thermoglucosidasius*/ NR043022.2	99.8
	MM	ETMB6	ON004970	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus rupiensis*/KJ842629.1	99.8
	MM	ETMB7	ON004971	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus rupiensis*/MK418866.1	99.9
	MM	ETMB8	ON004972	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus geothermalis*/MK615936.1	99.9
	MM	ETMB9	ON004973	*Firmicutes*	*Geobacillus*	*Geobacillus mahadia*/KY744705.1	100
	B	ETBB1	ON004964	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/KT720299.1	99.9
	B	ETBB2	ON004965	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/KX768314.1	100
	MM	QMB1	ON004984	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/MW148421.1	99.7
	S	QSB1	ON004996	*Firmicutes*	*Bacillus*	*Bacillus sp.*/MG515303.1	99.6
	S	QSB2	ON004997	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/ MK418415.1	97.1
	S	QSB3	ON004998	*Firmicutes*	*Bacillus*	*Bacillus licheniformis* MN410531.1	99.7
Siderophores production	S	ETSS1	ON004980	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus salavatliensis*/LT835101.1	99.9
	S	ETSS7	ON004981	*Firmicutes*	*Paenibacillus*	*Paenibacillus* sp./JX848635.1	99.4
	S	ETSS8	ON004982	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/MK859953.1	100
	S	ETSS12	ON004983	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus gonensis*/CP012152.1	99.9
	B	ETBS1	ON004966	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/FJ266313.1	99.4
	B	ETBS4	ON004967	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/KJ842633.1	99.7
	B	ETBS5	ON004968	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/KX768314.1	100
	S	QSS5	ON004999	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/KJ842639.1	99.5
	MM	QMS2	ON004985	*Proteobacteria*	*Providencia*	*Providencia stuartii*/AP022374.1	99.8
	MM	QMS7	ON004986	*Firmicutes*	*Anoxybacillus*	*Anoxybacillus gonensis*/MT772202.1	97.3
PET hydrolysis	PP	ETP1	ON004974	*Firmicutes*	*Bacillus*	*Bacillus oceanisediminis*/KM374766.1	99.7
	PP	ETP2	ON004975	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/MN410531.1	99.9
	PP	QP3	ON004987	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/FJ266313.1	96.2
	PP	QP6	ON004988	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/MK859957.1	99.3
	PP	QP8	ON004989	*Firmicutes*	*Brevibacillus*	*Brevibacillus borstelensis*/KX783601.1	99.9
	PP	QP12	ON004990	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/MW148420.1	95.4
	PP	QP15	ON004991	*Firmicutes*	*Bacillus*	*Bacillus paralicheniformis*/OL824873.1	99.8
	PP	QP19	ON004992	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/GU323372.1	99.7
	PP	QP22	ON004993	*Firmicutes*	*Bacillus*	*Bacillus sp.*/MT505502.1	96.9
	PP	QP27	ON004994	*Proteobacteria*	*Tistrella*	*Tistrella mobilis*/KF783213.1	99.8
	PP	QP28	ON004995	*Firmicutes*	*Bacillus*	*Bacillus licheniformis*/KU983867.1	98.7

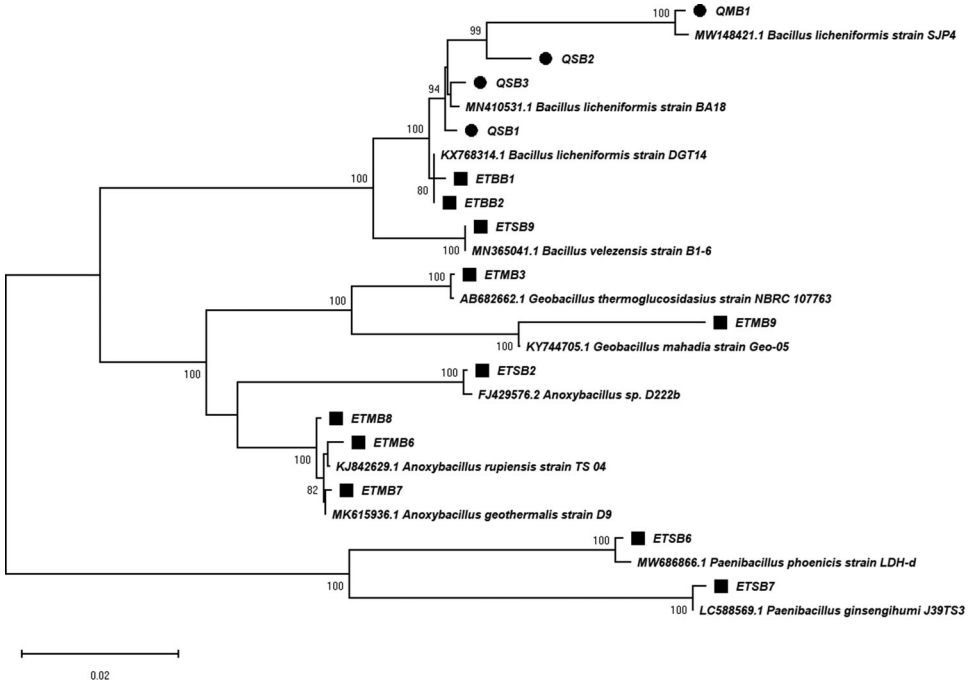

Figure 4. Phylogenetic tree of biosurfactant-producing thermophilic bacteria isolated from El Tragadero (■) and Quilcate (●) hot springs. This tree was assembled by comparison of the full-length 16S rRNA gene sequences of bacterial isolates with closely related bacteria using the Neighbor-Joining approach. Bootstrap values expressed as percentages from 1000 replications are depicted at each node. The scale bar represents a 2% divergence.

($Al_2Si_2O_5(OH)_4$), biotites ($K(Mg,Fe)_3\ AlSi_3O_{10}(OH,\ F)_2$) or albites ($NaAlSi_3O$), which are normally reported in hot springs [35]. Total Fe indicates a great reservoir of this element, although maybe it is present as complex-like compounds, thus its bioavailability being limited [5]. Furthermore, iron was in high enough concentrations to cause reddish-brown colorations ($> 5.4 \times 10^{-3}$ mM) [36], which are typical in the sediments of both hot springs. Ca ions in water samples from El Tragadero were found as high as in Quilcate, showing a moderate hardness of the waters in both sites. Detection of trace metals such as Zn, Mn, Fe, and Ca, and even other non-metals such as P, indicate an important source of micronutrients in both hot springs supporting the growth of different microbial metabolisms in both sites [37].

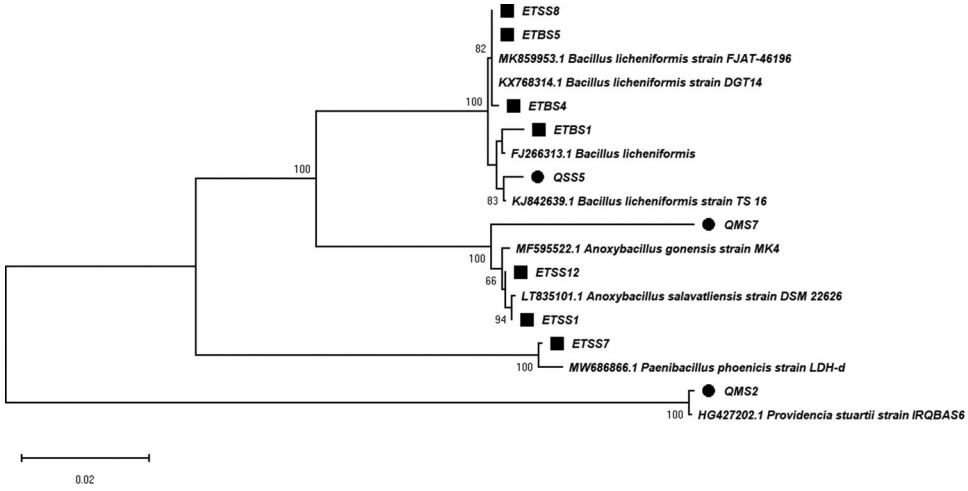

Figure 5. Phylogenetic tree of siderophore-producing thermophilic bacteria isolated from El Tragadero (■) and Quilcate (●) hot springs. This tree was assembled by comparison of the full-length 16S rRNA gene sequences of bacterial isolates with closely related bacteria using the Neighbor-Joining approach. Bootstrap values expressed as percentages from 1000 replications are depicted at each node. The scale bar represents a 2% divergence.

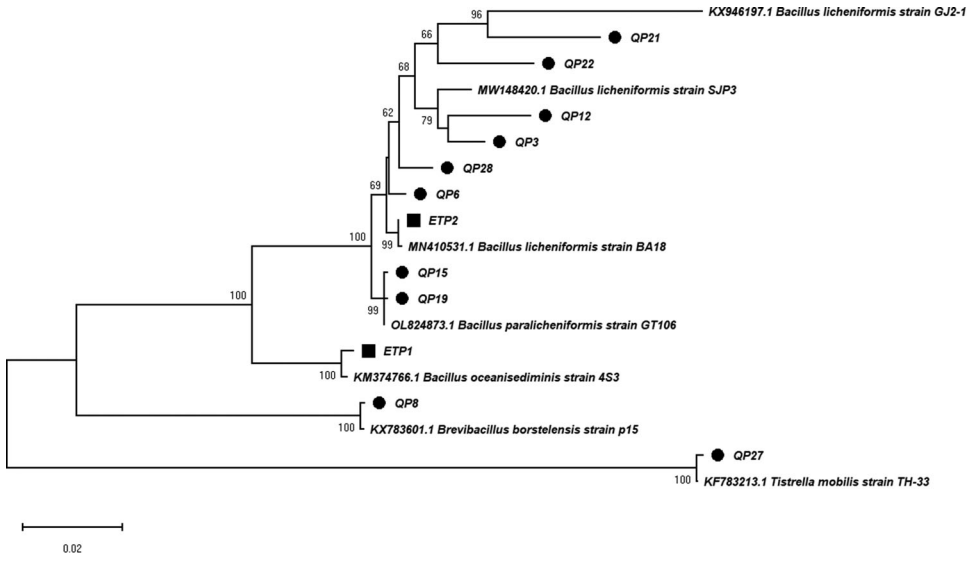

Figure 6. Phylogenetic tree of PET-hydrolyzing thermophilic bacteria isolated from El Tragadero (■) and Quilcate (●) hot springs. This tree was assembled by comparison of the full-length 16S rRNA gene sequences of bacterial isolates with closely related bacteria using the Neighbor-Joining approach. Bootstrap values expressed as percentages from 1000 replications are depicted at each node. The scale bar represents a 2% divergence.

Metabolic capability of thermophilic bacteria

Biosurfactants production

Biosurfactants (generally including biosurfactants proper and bioemulsifiers) are organic molecules that can be produced as by-products for microorganisms in extreme conditions [1]. Among their main features, these metabolites are amphipathic, so that they can reduce surface tension between different phases (biosurfactants) or stabilize/emulsify two immiscible liquids enhancing their mixing (bioemulsifiers) [38]. Microorganisms use these molecules to obtain otherwise insoluble carbon sources in their environments. However, due to their properties, biosurfactants can also help in bioremediation by promoting the bioavailability of hydrophobic organic pollutants, such as hydrocarbons [1,39]. In this study, we tested different screening methods to determine biosurfactant/bioemulsifier production (Table 2). In particular, DCM and OSM values showed that 14 isolates can reduce interfacial tension (from regular to total collapse) (Figure 2B) and demonstrate displacement activity (from 0.2 ± 0.1–0.7 ± 0.4 mm), respectively, when growing with gasoline or diesel as carbon sources. Similar results have been previously reported for thermophilic bacteria [10] indicating the biosurfactant production capability of the thermophilic strains isolated from El Tragadero and Quilcate hot springs.

Bioemulsification activity was also demonstrated in this study by EA and EI-24 screening methods (Table 2). The highest values in the EA (EU: 158.2 ± 76.9) and EI-24 test (32.4%), which were obtained with gasoline and diesel as hydrocarbons (Figure 3C-D), respectively, are similar to values of some isolates retrieved from oil-polluted environments [10,22] something that might be explained by the higher temperature at which this assay was carried out. Under elevated temperature, viscosity decreases, and thus the solubility and bioavailability of hydrocarbons are enhanced [39], making possible the development of relatively high emulsification indexes. The occurrence of hydrocarbon-degrading bacteria in non-polluted environments such as El Tragadero and Quilcate could be also explained by the human intervention at both sites, which could be passively supplying chemical pollutants, such as petro-polymers into the water promoting the establishment of these bacteria. Furthermore, in this study we found that bioemulsifying activity occurred with both gasoline and diesel as hydrocarbons, however, more uniform results were viewed with gasoline than with diesel. This correlates with the biodegradability of each carbon source, in which gasoline is relatively more biodegradable due to its chemical composition (isoalkanes, n-alkanes, cycloalkanes and aromatic compounds) that are similar to fatty acids and the kinds of paraffin ubiquitous in nature [37] in contrast to diesel (branched alkanes) [40].

The genera *Bacillus*, *Anoxybacillus*, *Geobacillus* and *Parageobacillus* were identified after 16S rRNA amplicon analysis (Table 4, Figure 4) showing biosurfactant activity. *Bacillus* sp. is maybe the most abundant genus found through biosurfactant-based studies [41],

reported as powerful micro-factories of surfactin, and normally isolated from non-extreme [42,43] and extreme environments [41,44]. Here, *Bacillus* spp. were isolated from both hot springs, including one isolate with the highest activity in the reduction of interfacial tension (Table 2), a desirable trait for bioremediation of oil-polluted environments. On the other hand, *B. velezensis*, a biosurfactant-producing bacterium firstly reported from saline environments (Vélez river, Spain [45]), was found in this study growing at high-temperature conditions and showing biosurfactant and bioemulsifying capabilities. To the best of our knowledge, this bacterium has not been isolated from hot springs to date, but its characteristics make this genus a suitable option in bioremediation as well. In contrast, the genus *Anoxybacillus* has already been isolated from hot springs [39] including *A. rupiensis* [4,46]. The high emulsifying activity at extreme temperatures of this genus has been reported before and is a matter of interest for environmental applications [47]. Alternatively, additional roles of *Anoxybacillus* in biotechnology such as biosorption of toxic metals, azo-dyes biodegradation and biohydrogen production are attributed [4,39,48]. Similarly, *Geobacillus* and *Parageobacillus* are well-known thermophilic bacteria with biotechnological applications, the former being reported as a suitable option for hydrocarbon biodegradation [49].

Siderophores production

Siderophores are high-affinity ferric ion-specific chelators with low molecular weight excreted under iron starvation by microorganisms [50]. These secondary metabolites are released into the environment for iron scavenging, thus supplying the iron requirements of the cell. Under extreme conditions, such as high temperature, siderophore-producing bacteria can occur [51]. Here, we identify four genera of thermophilic bacterial able to produce siderophores from both El Tragadero and Quilcate hot springs (Table 2, Table 4). *Bacillus*, which was aforementioned in the biosurfactant section, was found as the most abundant genus with siderophore production capability following CAS agar assay (Figure 3A-C). Although we have not identified the siderophore produced, we know *Bacillus* species can produce bacillibactin, an extensively studied siderophore belonging to the catecholate group [52].

Although additional functions of siderophores beyond the perspective of iron chelation such as the acquisition of zinc (zincophores) or manganese have been established [53,54], little is known about the application of siderophores in bioremediation processes of metal-polluted environments, especially at high temperatures [55]. Notably, under this extreme condition, metal biosorption can be enhanced due to the decrease of liquid viscosity and the simultaneous increase of kinetic energy and surface activity for metal sequestration [9]. However, some other factors could play crucial roles in metal chelation mediated by siderophores, such as the number of SU produced, something that can be influenced by the identity and concentration of the metals to which the bacteria are exposed. For instance, the thermophilic bacterium *Aeribacillus pallidus* is able to increase its SU production when Pb concentration increases as well (near to 40% of SU under 0.48 mM of Pb exposition), suggesting an immobilization of this element by metal-siderophore complex formation, decreasing its exposure and enhancing its removal [28]. Important SU values were found in our study after screening (from 39.1% to 60.3% for *Bacillus* species) (Table 2) and considering the natural exposure of thermophilic bacteria to higher levels of metals species (Table 1), we can infer that the siderophores production by thermophilic bacteria could be improved after controlled metal exposition, something that would be convenient for bioremediation purpose.

Anoxybacillus (*A. salavatliensis* and *A. gonensis*) and *Paenibacillus* were found as additional siderophore-producing bacteria in this study (Table 4, Figure 5). To the best of our knowledge, there are no reports on siderophores production by thermophilic *Anoxybacillus* species so far. However, *Anoxybacillus* has been reported to have interactions with metals [39,56]. Similar to *Bacillus*, *Anoxybacillus* has been also found in this study as a biosurfactant-producing bacteria. There is evidence that both metabolites, siderophores and biosurfactants, can be produced by the same microorganisms, and can even be related to oil biodegradation [55]. In contrast, *Paenibacillus* (with the highest % SU produced (Table 2)) seems to be a widely recognized siderophore-producing bacterium (*P. polymyxa*) [57,58]. Currently, there are no reports of *Paenibacillus* siderophores being used in metal sequestration. However, metal removal assays from wastewater by the additional production of organic acids (e.g. oxalic acid) [59] and lipopeptides (e.g. colistin) [60] are reported, pointing to this bacterium as a potential tool for the bioremediation of metal-polluted environments.

PET hydrolysis

PET is a petro-polymer with heteroatoms in the main chain and forms part of our daily life. Its extensive use, lack of control and structural features of this polymer promotes its persistence in the environment (including in hot springs, Figure 1B) [3]. Enzymatic degradation of PET is a matter of current research, using different microorganisms that naturally occur in the environment. Here

we found *Bacillus*, as the most representative thermophilic bacteria with PET-hydrolyzing capability following the screening method (PET-agar assay) (Table 4, Figure 3A, B, C). *Bacillus* species are associated with PET materials (such as *B. oceanisediminis*) [34], also used in PET-degrading assays [61], and even as part of a microbial consortium at mesophilic conditions [62]. At high temperatures, *Bacillus* spp. are reported with major enzymatic degradation capability of other polymers such as polyethylene (PE), low-density polyethylene (LDPE), and high-density polyethylene (HDPE) [63]. However, there is a lack of information on PET hydrolysis (with just one study using an engineered *Bacillus* strain as far as we are aware [64]). Enzymatic degradation of PET at elevated temperatures offers many advantages. Under this condition, changes in the physical and optical polymer properties, higher rates of enzyme activities and the enhanced diffusion rates of organic compounds into the cell can enhance the degradation of this polymer [13]. It seems likely that the dearth of studies related to PET hydrolysis with thermophilic *Bacillus*-like bacteria may reflect the low level of plastic pollutants in many thermal environments such as hot springs.

The thermophilic bacterium *Brevibacillus borstelensis* was found in this study with the unusual hydrolytic capability of PET. *B. borstelensis* has been found and isolated from different thermal sites [46,65,66], and its isolation in this study confirms that the PET-agar assay can be used as an efficient screening method for the detection of thermophilic PET-hydrolyzing bacteria (Figure 3D, E, F). Interestingly, *B. borstelensis*, which is normally reported with PE enzymatic degradation capability [7], grew well in a culture media supplemented with PET as a sole carbon source. This finding suggests that *B. borstelensis* could have the suite of metabolic machinery to metabolize more complex polymers than PE under *in vitro* conditions. However, in its natural environment, PET hydrolysis by *B. borstelensis* could be carried out by co-metabolism, working with some other microbial partners, in which they could be making the first steps of PET degradation [61], producing intermediates that could be used later by *B. borstelensis*. There is evidence of thermophilic *Brevibacillus* species forming part of a microbial consortium for plastic degradation [67], indicating that this rationale is viable.

An unexpected finding in this study was *Tistrella mobilis* as a thermophilic and PET-hydrolyzing bacterium (Table 4, Figure 6). *T. mobilis* has been reported as a mesophile (optimal growth temperature of 30°C) able to produce polyhydroxyalkanoates (PHA) [68]. To the best of our knowledge, this bacterium has not been reported to grow under high-temperature conditions. However, *T. mobilis* was isolated from the Quilcate hot spring at 55–60°C in this study. Additional roles of *Tistrella* species are associated with biodegradable plastic [69] and hydrocarbon biodegradation [70]. This is not surprising given the nature of plastic (petro-polymers) and the necessity of producing biosurfactants by plastic-hydrolyzing bacteria to decrease the hydrophobicity of polymers [7], showing that there is a narrow demarcation between hydrocarbon- and plastic-degrading metabolisms, something that can be exploited from a biotechnological point of view.

Conclusion

El Tragadero and Quilcate hot springs located in Cajamarca (Peru) are natural resources of thermophilic microorganisms with potential applications in biotechnology. The special geochemical characteristics of both hot springs allow the occurrence of well-adapted bacteria despite the extreme temperature conditions. Throughout our study, we isolated 36 diverse thermophilic bacteria able to produce biosurfactants and siderophores, and to hydrolase PET. These extremophilic bacteria offer a valuable resource for biotechnological application, especially for bioremediation purposes of oil-, metal- and plastic-polluted environments.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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