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***Alkaliphilus serpentinus* sp. nov. and *Alkaliphilus pronyensis* sp. nov., two novel anaerobic alkaliphilic species isolated from the serpentinite-hosted Prony Bay Hydrothermal Field (New Caledonia).**

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The GenBank accession numbers for the whole genome sequence and the 16S rRNA gene sequence of strain LacT<sup>T</sup> are respectively WBZB000000000 and KJ159208.

The GenBank accession numbers for the whole genome sequence and the 16S rRNA gene sequence of strain LacV<sup>T</sup> are respectively WBZC000000000 and KJ159207.

Keywords: *Firmicutes*, *Alkaliphilus*, anaerobic, hydrothermal vents, serpentization, New Caledonia

## Abstract

Two novel anaerobic alkaliphilic strains, designated as LacT<sup>T</sup> and LacV<sup>T</sup>, were isolated from the Prony Bay Hydrothermal Field (PBHF, New Caledonia). Cells were motile, Gram-positive, terminal endospore-forming rods, displaying a straight to curved morphology during the exponential phase. Strains LacT<sup>T</sup> and LacV<sup>T</sup> were mesophilic (optimum 30°C), moderately alkaliphilic (optimum pH 8.2 and 8.7, respectively) and halotolerant (optimum 2 % and 2.5 % NaCl, respectively). Both strains were able to ferment yeast extract, peptone and casamino acids, but only strain LacT<sup>T</sup> could use sugars (glucose, maltose and sucrose). Both strains disproportionated crotonate into acetate and butyrate. Phylogenetic analysis revealed that strains LacT<sup>T</sup> and LacV<sup>T</sup> shared 96.4 % 16S rRNA gene sequence identity and were most closely related to *A. peptidifermentans* Z-7036, *A. namsaraevii* X-07-2 and *A. hydrothermalis* FatMR1 (95.7%–96.3%). Their genome size was of 3.29 Mb for strain LacT<sup>T</sup> and 3.06 Mb for strain LacV<sup>T</sup> with a G+C content of 36.0 and 33.9 mol%, respectively. The ANI value between both strains was 73.2%. Finally, strains LacT<sup>T</sup> (= DSM 100337 = JCM 30643) and LacV<sup>T</sup> (= DSM 100017 = JCM 30644) are proposed as two novel species of the genus *Alkaliphilus*, order *Clostridiales*, phylum *Firmicutes*, *Alkaliphilus serpentinus* sp. nov. and *Alkaliphilus pronyensis* sp. nov., respectively. The genomes of the three *Alkaliphilus* species isolated from PBHF were consistently detected in the PBHF chimney metagenomes, although at very low abundance, but not significantly in the metagenomes of other serpentinizing systems (marine or terrestrial) worldwide, suggesting they represent indigenous members of the PBHF microbial ecosystem.

## Introduction

Serpentinization is a widespread geochemical process involving aqueous alteration of the mantle rock peridotite to serpentinite and reduction of inorganic CO<sub>2</sub>, resulting in an abundance of potential energy sources, in the form of H<sub>2</sub>, CH<sub>4</sub> and small organic molecules, that can be used by microbial life [1]. However, these reactions also generate challenging conditions: high pH, low availability of potential electron acceptors, and a low concentration of dissolved inorganic carbon source.

Over the last two decades, serpentinization-driven microbial ecosystems have been the object of increasing scientific interest, not only for the extremophilic nature of the microorganisms thriving in such harsh conditions, but also because these environments represent the best modern analogs of primitive hydrothermal systems existing at the Hadean where early proto-cellular life may have arisen [2]. Recent next-generation sequencing studies, targeting 16S rRNA gene, or true metagenomic analyses, have shown that all serpentinite-hosted ecosystems share a low microbial diversity, largely dominated by few abundant bacterial phyla/classes among which the *Betaproteobacteria*, the *Chloroflexi* and the *Clostridia*. The later one is thought to be associated with the anaerobic micro-habitats of serpentinizing sites, where highly reduced alkaline fluids are less mixed with oxygenated groundwater or seawater. *Clostridia* dominated in various terrestrial alkaline seeps, such as The Cedars (California) [3], Manleluag (Philippines) [4], Tablelands (Canada) [5], the deep alkaline groundwater of Cabeço de Vide Aquifer (CVA, Portugal) [6], and the anoxic deep borehole water of Outokumpu (Finland) [7]. They were also dominant at the oceanic site of Lost City Hydrothermal Field (LCHF) near the Mid Atlantic Ridge [8, 9] and in the marine shallow Prony Bay Hydrothermal Field (PBHF) in New Caledonia [10, 11].

Metagenomic studies of serpentinite-hosted systems have revealed a high diversity and abundance of [FeFe]-hydrogenases assigned to the *Firmicutes* orders *Thermoanaerobacterales* and *Clostridiales* [5, 12]. The [FeFe]-hydrogenases are typically linked to H<sub>2</sub> production by fermentations, or intra-cellular regulation of the reducing equivalents [13]. These enzymes thus play essential functions in the anaerobic challenging environments of the serpentinitizing systems.

Besides these ‘omics’ studies, a few cultivation-based approaches have been published. The earliest one, conducted on alkaline springs of the Oman ophiolite, reported the cultivation of aerobic heterotrophs belonging to *Firmicutes* [14], as did later similar studies on CVA (Portugal) and Yanartaş (Turkey) terrestrials alkaline springs [15, 16]. Only one new species was described, *Bacillus foraminis*, an aerobic, mesophilic, neutrophilic heterotroph, isolated from the alkaline ground water of CVA [17]. Its phenotypic characteristics, however, were not representative of typical serpentinite-hosted environment, and its belonging to the widespread genus *Bacillus* questions its indigeneity. That prompts us to look for better model organisms for physiological studies of adaptation to serpentinite-hosted environmental conditions by tempting to isolate novel anaerobic, alkaliphilic bacteria from our predilected site of study, the Prony Bay Hydrothermal Field in New Caledonia (PBHF). One target was the *Clostridiales* since they represented almost a third of the bacterial community in the submarine chimneys of PBHF [18]. The dominant phylotypes clustered with the *Clostridiales* from CVA (Portugal) [6] and The Cedars (California) [3]. A phylotype related to *Dethiobacter alkaliphilus*, dominant in juvenile chimneys of PBHF, was also proposed to be involved in the early organomineralization of carbonates during the edification of the chimneys [19]. Our cultivation efforts resulted in the isolation of several anaerobic strains belonging to *Clostridia*. They were described as novel species or genus: *Vallitalea pronyensis* [20], *Acetoanaerobium pronyense* [21], *Clostridium* sp.

PROH2 [22], *Serpentinicella alkaliphila* [23] and *Alkaliphilus hydrothermalis* [24]. The later was the first species described from a serpentinite-hosted system. Here we report the description of strains LacT<sup>T</sup> and LacV<sup>T</sup> isolated from PBHF representing two novel species of the genus *Alkaliphilus* (*Clostridiales*). We also investigated the distribution of the three PBHF *Alkaliphilus* species genomes in metagenomes of various serpentinite-hosted environments worldwide and discuss the potential role of the *Alkaliphilus* spp. in the PBHF ecosystem.

## Material and methods

### Sample collection

The Prony Bay Hydrothermal Field (PBHF) located in the south of New Caledonia is a serpentinizing marine hydrothermal system composed of brucite-carbonate chimneys discharging highly reduced alkaline fluids (up to pH 11.5) of low salinity enriched in hydrogen and methane in a shallow lagoon. In the east of the central bay, active chimneys were sampled during the 2011 HYDROPRONY campaign from site ST08 (22°21.598'S, 166°52.838'E) at 47 meters below sea level (mbsl) and site ST09 (22°21.653'S, 166°52.777'E) at 45 mbsl (Fig. S1) [10, 11, 25]. Aboard the RV *Alis*, internal chimney parts were aseptically subsampled to avoid contamination by the outer parts exposed to the surrounding seawater. Subsamples were further ground aseptically with a pestle in a mortar under a continuous flux of N<sub>2</sub> gas to preserve anaerobiosis and then distributed into sterile glass vials which were tightly closed with butyl rubber stoppers and crimped with aluminum seals. The head space in the vials was slightly pressurized (50 kPa) with 100% N<sub>2</sub> to prevent oxygen entrance. These samples were stored at 4°C until use.

### Enrichment, isolation and cultivation

For the enrichment cultures, about 0.5 g of chimney slurry (ST08 or ST09) was used to inoculate 5 mL of basal medium (noted BM) which contained (per litre of distilled water): 2 g NaCl, 0.1 g  $\text{KH}_2\text{PO}_4$ , 0.1 g  $\text{K}_2\text{HPO}_4$ , 1 g  $\text{NH}_4\text{Cl}$ , 0.1 g KCl, 0.1 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 g  $\text{CaCl}_2$ , 0.1 g Cystein-HCl, 3 g Tris Base, 3 g  $\text{Na}_2\text{S}_2\text{O}_3$ , 1 g yeast extract and 1 mL Widdel's elements. The pH was adjusted to 9.5 with 1M NaOH solution. The medium was boiled under a stream of  $\text{O}_2$ -free  $\text{N}_2$  gas and cooled to room temperature. Aliquots of 5 mL were dispensed into Hungate tubes, degassed under  $\text{N}_2:\text{CO}_2$  (80:20, v:v) and subsequently sterilized by autoclaving. Before inoculation, 0.1 mL of 1M Lactate, 0.3 mL of 8 % (w/v)  $\text{Na}_2\text{CO}_3$  and 0.1 mL of 2 % (w/v)  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  were injected from sterile stock solutions into the tubes. The Hungate technique was used throughout this study [26]. Isolation of colonies was performed three times consecutively using the roll-tube method and 1.6 % (w/v) agar containing BM [26].

#### Microscopic analyses

Cells were routinely observed under a Nikon ECLIPSE E600 phase-contrast microscope for culture monitoring. Cell structures and morphological characterization of strains LacT<sup>T</sup> and LacV<sup>T</sup> deposited on Cu-grids were performed using a FEI Tecnai G2 transmission electron microscope (TEM) operating at 200 kV. Exponentially grown cells were negatively stained during 15 min with 2 % (w/v) sodium phosphotungstate (pH 7.0) for observation of flagella. For observation of the cell wall, cells were first fixed 2 min with 10% (v/v) glutaraldehyde, stained overnight with 2% (w/v) osmium tetroxide, and then embedded in EMbed-812 resin. Embedded specimens were sliced into ultrathin sections (90 nm in thickness), placed on Cu TEM grid and stained during 3 min with 2% (w/v) uranyl acetate and 1 min with 1% (w/v) lead before observation.

## Physiological tests

The pH, temperature, and NaCl concentration ranges for growth were determined in Hungate tubes using basal medium (BM). Growth was determined by directly measuring the optical density at 600 nm in Hungate tubes using a spectrophotometer equipped with a tube holder (Cary 50, Varian). The BM pH was adjusted from 7.0 to 11.5 with anaerobic sterile stock solutions of 1M HCl (acidic pH values), 10 % (w/v) NaHCO<sub>3</sub> or 8 % (w/v) Na<sub>2</sub>CO<sub>3</sub> (basic pH values) and was verified after inoculation. Tris Base in the medium was replaced by HEPES (10 mM) for the low pH (7.0–8.0) values and by AMPSO (10 mM) for the high pH (10.0–11.5) values. Culture experiments to determine pH range and optimum growth conditions were performed at 30°C and 1 % NaCl. Temperature tests were performed at pH 9.0 and 1 % NaCl using water baths from 15 °C to 55 °C. For NaCl requirement, NaCl was weighted directly in the tubes prior to adding the medium (0 to 12 %) and cultures were performed at pH 9.0 and 30 °C. All cultures were performed in duplicate and reproduced twice. Growth rates were determined for subcultures under the same experimental conditions.

Substrate utilization experiments were performed for each strain under its optimal growth conditions (pH, temperature and NaCl) in a modified BM (yeast extract reduced to 0.5 g/L). Sugars, organic acids and alcohol were tested each individually at 20 mM and proteinaceous compounds at 5 g/L. Autotrophic growth was tested by replacing N<sub>2</sub> headspace by a H<sub>2</sub>:CO<sub>2</sub> (80:20) at 200 kPa. Each compound was tested in duplicate Hungate tubes and those for which positive growth was observed (OD significantly above that obtained on the inoculated duplicate controls on modified BM only) were checked by two successive subcultures under the same conditions. Bacterial growth was monitored by measuring the increase in turbidity at 600 nm as mentioned above. H<sub>2</sub>S production was determined measuring the formation of colloidal CuS photometrically at 480 nm following the method described by Cord-Ruwisch [27]. Metabolic



end-products were measured by HPLC using an Aminex HPX-87H (Bio-Rad) column with 2.5 mM H<sub>2</sub>SO<sub>4</sub> as mobile phase and gas-phase chromatography after 2-weeks of incubation at 30 °C. Fe(II) was measured as previously described [28].

#### Fatty acid and G+C content analyses

The determination of cellular fatty acids composition was performed at the DSMZ identification service (Braunschweig, Germany). Cells were harvested in the late exponential growth phase (c.a.  $\sim 10^8$  cells. ml<sup>-1</sup>). After extraction using a modified version of the method of Miller [29, 30], fatty acids were separated by gas chromatography and detected using the Microbial Identification system (MIDI, Sherlock Version 6.1) [31].

For experimental genomic G+C content determination of LacT<sup>T</sup> and LacV<sup>T</sup> (done at the DSMZ identification service), DNA was isolated and purified by chromatography on hydroxyapatite [32] and the G+C content was determined by using HPLC [33].

#### Phylogenetic and genomic analyses

For 16S rRNA gene analyses, genomic DNA isolation from LacT<sup>T</sup> and LacV<sup>T</sup> cultures (5 mL) and amplification by PCR (using 27F/1492r primer set; [34]) were performed as described previously [35]. Sanger sequencing of the PCR product was performed at GATC Biotech AG (Germany).

The 16S rRNA gene sequence was then compared with available sequences in the Genbank database using the BLASTN search [36] and RDP tools [37] as RDP classifier [38]. All evolutionary analyses were performed using MEGAX [39]. A multiple alignment was built using the MUSCLE program [40]. Sequence positions with alignment uncertainty and gaps were omitted from the analysis. Phylogenetic trees were reconstructed using three methods:

Neighbor-Joining, Maximum-Likelihood (Fig. 1) and Minimum Evolution methods [41-43]. The evolutionary distances were computed using the Kimura 2-parameter method [44]. The analysis involved 15 nucleotide sequences having a total of 1291 positions in the final dataset. Branch robustness was estimated by the non-parametric bootstrap procedure [45] (2000 replicates). The 16S rRNA sequences of strains LacT<sup>T</sup> and LacV<sup>T</sup> were deposited in Genbank under accession number KJ159208 and KJ159207, respectively.

For whole-genome analyses, genomic DNA extraction of strains LacT<sup>T</sup> and LacV<sup>T</sup> was performed as described previously [46]. A shotgun library was paired end sequenced on an Illumina HiSeq 2000 instrument (2x100 bp) at the Marine Biological Laboratory, Woods Hole, MA, USA. Reads of each data set were filtered, and high quality paired-end reads were *de novo* assembled using SPAdes v. 3.5.0 (<http://cab.spbu.ru/software/spades/>) [47]. The obtained whole genome draft sequences were annotated by using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and deposited at DDBJ/ENA/GenBank. For further comparative analyses, the draft genomes were also annotated by the IMG/M expert review system and incorporated in the IMG database (<https://img.jgi.doe.gov/>). The IMG Genome ID and GenBank accession numbers of both investigated strains are listed in Table 1. A whole genome-based taxonomic analysis of strains LacT<sup>T</sup> and LacV<sup>T</sup> genomes was performed using the bioinformatics platform Type Strain Genome Server (TYGS) (Fig. S3).

#### Biogeography of the *Alkaliphilus* species from PBHF

The presence and abundance of the *Alkaliphilus* species in various serpentinite-hosted systems worldwide were indirectly asserted by evaluating their genomes representation in the metagenomes of these environments. A total of 27 “Whole Genome Sequencing” (WGS) metagenomes from alkaline serpentinite-hosted ecosystems (Table S1) and 21 WGS

metagenomes from marine or terrestrial ecosystems (Table S2) were collected from NCBI Sequence Read Archive (SRA) database with the *fastq-dump* program from NCBI SRA toolkit (available at <https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software>) or with the CURL program for datasets in the MG-RAST system. Metagenomic paired-end reads were mapped to contigs of each *Alkaliphilus* genomes using the Anvi'o version 5.5 [48] snakemake metagenomic workflow with the reference mode (<http://merenlab.org/2018/07/09/anvio-snakemake-workflows/>). Raw paired-end reads were quality filtered using *illumina-utils* [49] with the Minoche's algorithm [50]. Then, quality filtered reads were mapped to each *Alkaliphilus* genome with Bowtie2 [51]. Results were normalized to the total quality filtered reads of each sample to highlight the percent read mapped to each reference genome across the serpentinite-hosted ecosystems. Additionally, results were visualized with Anvi'o version 5.5 to analyze the distribution of reads mapped along the genomes sequences [48].

## Results and discussion

### Strain isolation

Microbial growth was detected in enrichment cultures of chimney subsamples (ST08 and ST09) after 2-week incubation in the dark at 30°C. Several colonies obtained after 7-day incubation at 30°C were creamy yellow, round with smooth edges and a diameter of 0.5–1 mm. Several axenic cultures were obtained using the roll-tube method. Among them, strains LacT<sup>T</sup> (from ST08) and LacV<sup>T</sup> (from ST09) were selected for further characterization.

### Morphology

Strain LacT<sup>T</sup> is a straight to slightly curved bacilli measuring  $4.9 \pm 1.4 \mu\text{m}$  length and  $0.3 \pm 0.1 \mu\text{m}$  width during the exponential growth (Fig. 2A) and forms filamentous cells (up to 30  $\mu\text{m}$ )

during stationary growth phase (Fig. 2B). Strain LacV<sup>T</sup> is a straight, slightly shorter cell than LacT<sup>T</sup> ( $3.7 \pm 0.8 \mu\text{m}$  length and  $0.5 \pm 0.1 \mu\text{m}$  width; Fig. 3A). Both strains occur singly or in pairs, were motile, and several lateral flagella were observed (Fig. 2C and Fig. 3B). The presence of terminal endospores was observed by phase-contrast microscopy under stress conditions or in prolonged cultures (not shown). The cell wall structure observed by transmission microscopy revealed for both strains a Gram-positive structure with the presence of a jagged peptidoglycan and a multilayered cell wall in the absence of an outer membrane (Fig. 2D and Fig. 3C). Cell membrane staining also confirmed the Gram-positive structure of both strains.

#### Phylogenetic affiliation

The 16S rRNA sequences of strains LacT<sup>T</sup> and LacV<sup>T</sup> share 96.4% identity between them and 93.3–96.3 % identity with those of the closest species belonging to the *Alkaliphilus* genus (order *Clostridiales*), such as *A. peptidifermentans* Z-7036, *A. namsaraevii* X-07-2 and *A. hydrothermalis* FatMR1 (95.7–96.3%), *A. transvaalensis* SAGM1 and *A. crotonatoxidans* B11-2 (95.2–95.7%), and '*A. metalliredigens*' QYMF (not validated species), *A. oremlandii* OhILAs and *A. halophilus* E2R (93.3–94.5%).

#### Genome features

The genome size and G+C content are 3.29 Mb and 36.0% for strain LacT<sup>T</sup> and 3.06 Mb and 33.9 mol% for strain LacV<sup>T</sup> which is consistent with the G+C content of the genus *Alkaliphilus*. Strains LacT<sup>T</sup> and LacV<sup>T</sup> have a lower genome size than their closest phylogenetic neighbors, *A. peptidifermentans* (4.45 Mb, 34.09% G+C) and *A. transvaalensis* (4.02 Mb, 33.95% G+C), but close to *A. oremlandii* OhILAs (3.12 Mb, 36.3% G+C) [52]. The LacT<sup>T</sup> strain genome

consisted of 83 contigs, with a N50 length of 157444 pb, and a total of 3257 genes were predicted. Among them, 3183 were potential protein-coding genes (CDS). The LacV<sup>T</sup> strain genome consisted of 94 contigs, with a N50 length of 85817 pb, and a total of 3088 genes were predicted of which 3035 were CDS. Both strains possess genes encoding flagella-related proteins. The general genomic features of strains LacT<sup>T</sup> and LacV<sup>T</sup> are given in Table 1.

Value of average nucleotide identity (ANI) was calculated by using OrthoANI with usearch (<http://www.ezbiocloud.net/tools/ani>). Digital DNA-DNA hybridization (dDDH) was calculated using the Genome-to-Genome Distance Calculator (GGDC) 2.1 (<http://ggdc.dsmz.de/>) [53]. The ANI and dDDH values between LacT<sup>T</sup> and LacV<sup>T</sup> were found to be 73.2% and 14.1%, i.e. far below the species boundaries of 95~96% and 70%, respectively, recommended by Chun et al. [54] (Table 2). Additionally, ANI and dDDH values between the genomes of both strains and other available *Alkaliphilus* genomes are also below the species boundaries as presented in Table 2.

## Chemotaxonomy

The major cellular fatty acids of strain LacT<sup>T</sup> were C<sub>16:0</sub> (50.5 % of the total), C<sub>18:0</sub> (37.5 %), C<sub>14:0</sub> (6.9%) and C<sub>16:1 ω7c</sub> (5.1 %); those of strain LacV<sup>T</sup> were C<sub>16:0</sub> (24.2%), C<sub>14:0</sub> (20.3%), C<sub>18:0</sub> (14.4%), C<sub>16:1 ω7c</sub> (11.1%) and C<sub>17:0 isoI / anteisoB</sub> (6.6%) (Table S3). Genomic DNA of strains LacT<sup>T</sup> and LacV<sup>T</sup> contained respectively 37.0 mol % and 36.3 mol % of G+C as determined by HPLC at DSMZ. These values are 1.0 to 2.4% higher than those directly computed from the genome sequences (see above).

## Physiological characteristics and substrate utilization

287 Growth of strain LacT<sup>T</sup> occurred in 0–7 % NaCl, at 25–37 °C and pH 7.5–10.6, with optimal  
 288 growth observed at 2 % NaCl, at 30 °C and at pH 8.2. Growth was observed for a maximum  
 289 level of 0.5% O<sub>2</sub> in the initial gas phase. Growth of strain LacV<sup>T</sup> occurred in 0–8 % NaCl, at  
 290 20–40 °C and pH 8.2–10.0, with optimal growth at 2.5 % NaCl, at 30 °C and at pH 8.7. Growth  
 291 was observed for a maximum level of 0.1% O<sub>2</sub> concentration in the gas phase (Tables 3 and 4).  
 292 These data indicate that both strains are mesophilic, halotolerant, alkaliphilic and slightly  
 293 aerotolerant bacteria. The maximal growth rate obtained under optimal conditions was 0.035  
 294 h<sup>-1</sup> for strain LacT<sup>T</sup> and 0.07 h<sup>-1</sup> for strain LacV<sup>T</sup>.  
 295 Strain LacT<sup>T</sup> was able to use yeast extract, peptone, casamino acids, glucose, maltose, sucrose  
 296 and crotonate. Casein, tryptone, cellobiose, fructose, galactose, lactose, mannose, ribose,  
 297 threhalose, xylose, starch, acetate, butyrate, citrate, lactate, fumarate, malate, pyruvate,  
 298 succinate, tartrate, acetone, ethanol, methanol, glycerol and butanol did not improve growth.  
 299 Strain LacV<sup>T</sup> was able to use yeast extract, tryptone, peptone, casamino acids and crotonate but  
 300 none of the tested sugars. Arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose,  
 301 mannose, melibiose, threhalose, raffinose, ribose, sucrose, xylose, acetate, butyrate, citrate,  
 302 lactate, formate, fumarate, malate, pyruvate, tartrate, succinate, and glycerol did not improve  
 303 growth. HPLC analysis confirmed the use of substrates.  
 304 When grown on sugars, the main metabolic product of strain LacT<sup>T</sup> was acetate, accompanied  
 305 by H<sub>2</sub> and traces of CO<sub>2</sub>. When grown on yeast extract, the main metabolic product of both  
 306 strains was acetate, and minor fermentation products were propionate, butyrate and hydrogen,  
 307 while strain LacT<sup>T</sup> produced additionally isovalerate and CO<sub>2</sub>. Crotonate was disproportionated  
 308 to acetate and butyrate by both strains.  
 309 None of the strain could grow by autotrophy on H<sub>2</sub>:CO<sub>2</sub> (80:20, v:v) alone or on H<sub>2</sub>:CO<sub>2</sub> plus  
 310 acetate (20 mM) as sole carbon sources. Additionally, no growth was observed on H<sub>2</sub>:CO<sub>2</sub> or

311 on acetate (20 mM) combined each separately with Fe(OH)<sub>3</sub> (40 mM) or goethite α-  
312 Fe(III)O(OH) (40 mM) as insoluble iron (III) oxyhydroxide, Fe (III) (EDTA), sulfate or nitrate  
313 (20 mM). Both strains are thus strict organoheterotrophs.

314 The strains did not use any of the following electron acceptors tested in the BM medium with  
315 yeast extract at 2.5 g/L as electron donor: fumarate, thiosulfate, sulfate, sulfite, nitrite, nitrate,  
316 Fe(III)-citrate, Fe(III)-EDTA (20 mM each), Fe(OH)<sub>3</sub> (40 mM) or goethite α-Fe(III)O(OH) (40  
317 mM) and elemental sulfur (10 g/L) suggesting they both have a strictly fermentative type of  
318 energy metabolism. Thiosulfate slightly improved the growth of strain LacT<sup>T</sup> but without H<sub>2</sub>S  
319 production, suggesting that thiosulfate is not used as an electron acceptor for respiration but  
320 rather as a nutrient for anabolism.

321  
322 Both strains LacT<sup>T</sup> and LacV<sup>T</sup> were able to use various proteinaceous compounds and casamino  
323 acids, in accordance with their genomic data and similarly to *A. peptidifermentans* but unlike  
324 *A. namsaraevii*, *A. hydrothermalis* and *A. transvaalensis* [55-58] (Table 3). Strain LacT<sup>T</sup>  
325 displayed a quite large range of substrate utilization compared to other *Alkaliphilus* and it is the  
326 only one able to utilize glucose and sucrose among its closest relatives. It was also able to use  
327 maltose similarly to *A. crotonatoxidans* [59]. On the contrary, strain LacV<sup>T</sup> was not able to use  
328 any tested sugars similarly to *A. namsaraevii*, *A. peptidifermentans* and *A. transvaalensis*.  
329 Crotonate was disproportionated to acetate and butyrate by both strains LacT<sup>T</sup> and LacV<sup>T</sup>. The  
330 disproportion of crotonate, as well as fermentation, could constitute an ecological advantage in  
331 the anaerobic, highly reduced, high pH zones of serpentinizing environments known to be  
332 depleted in oxidants needed for respiratory metabolisms [1]. With *A. crotonatoxidans*, both  
333 strains LacT<sup>T</sup> and LacV<sup>T</sup> displayed slightly lower optimum growth pH compared to the closest  
334 *Alkaliphilus*. Compared to *A. hydrothermalis* which is the previously isolated *Alkaliphilus*

specie from the Prony Bay Hydrothermal Field, strains LacT<sup>T</sup> and LacV<sup>T</sup> display higher NaCl tolerance and prefer lower pH and temperature [55], suggesting a better adaptation to the mixing-zone of seawater with the warm, alkaline and poorly salted hydrothermal fluid.

Based on the phenotypic characteristics and phylogenetic analyses described above, and given the recommendations to recognize a new specie [54], strains LacT<sup>T</sup> and LacV<sup>T</sup> are considered to represent novel species of the genus *Alkaliphilus*, for which the name *Alkaliphilus serpentinus* sp. nov. and *Alkaliphilus pronyensis* sp. nov. are respectively proposed (Table 4).

#### Detection and abundance of *Alkaliphilus* species in serpentinizing hydrothermal systems

*Alkaliphilus* 16S rRNA gene sequences were consistently identified in several active chimneys from submarine sites of PBHF over a thirteen year period (2005-2018), first in clone libraries [11] and later by pyrosequencing of amplicons [18] or metagenomics [60]. Six *Alkaliphilus* OTUs, mostly originating from ST09 sites, form a cluster together with strain LacV<sup>T</sup>, and two of them, from ST09 and ST07 sites, displayed more than 99% sequence identity (Fig. S2). Moreover, two OTUs affiliated to the *Alkaliphilus* genus (96% identity over the complete 16S rRNA sequence with either *A. metalliredigens* strain QMFY or with strain LacV<sup>T</sup>) were found in LCHF metagenomes, in which they represent each 3.94% and 0.56%, and 8.55% and 8.82% of the 16S rRNA gene sequences of Marker 5 and Marker 3 metagenomes (LC.75 and LC.81), respectively [61, 62]. The biogeography of the three *Alkaliphilus* species isolated from PBHF was further evaluated by mapping the metagenomic paired-end reads from alkaline serpentinite-hosted ecosystems (Table S1) and marine and continental ecosystems worldwide (Table S2), to contigs of three *Alkaliphilus* species genomes (LacT<sup>T</sup>, LacV<sup>T</sup> and *A. hydrothermalis* FatMR1<sup>T</sup>). As expected, these novel *Alkaliphilus* genomes are well detected and distributed in PBHF metagenomes, especially LacV<sup>T</sup> and FatMR1<sup>T</sup> which recruited, uniformly on the whole genome

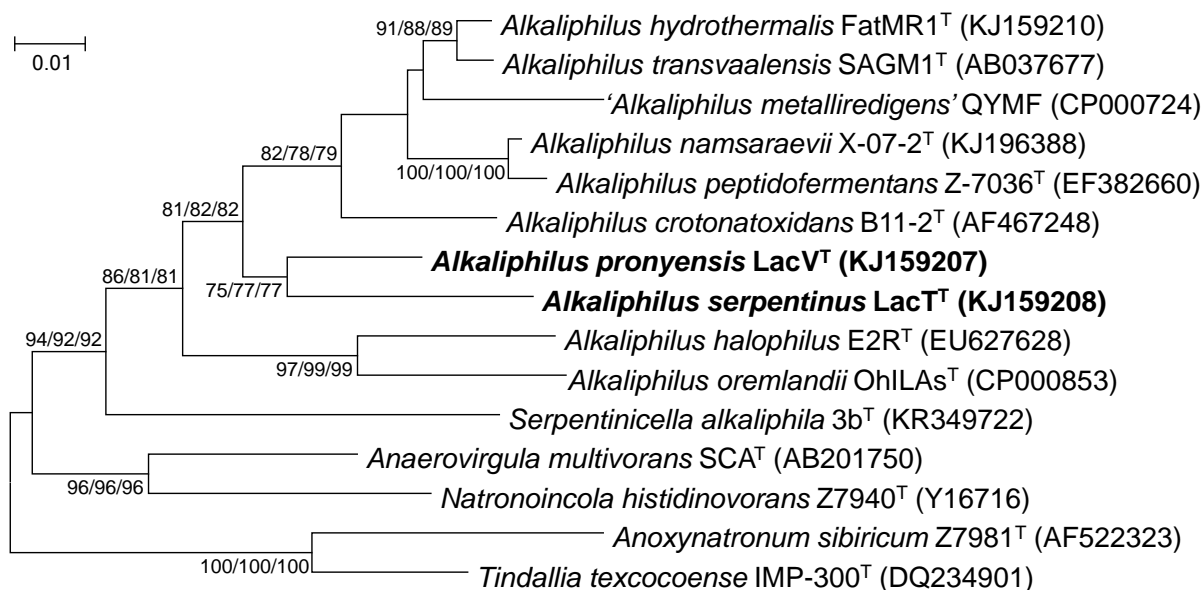


359 sequences, between 0.02% up to near 1% of the reads depending on the sampled chimney. The  
360 genome frequency was also dependent on the metagenomic reads coverage, even though the  
361 mapping was normalized for all metagenomic samples used in this study. For example, LacV<sup>T</sup>  
362 and FatMR1<sup>T</sup> genomes frequency in PBHF metagenomes are one order higher in the more  
363 recent metagenomes [63] which also have nearly 20 times more reads (77 million reads vs 4  
364 million reads) than the first metagenomes from ST09 chimneys (PBHF27 and PBHF28). This  
365 is probably because rare or low abundant genotypes are just ‘missed’ in low coverage  
366 metagenomic sequencing. In contrast, *Alkaliphilus* genomes are far less represented in other  
367 metagenomes, including those from serpentinite-hosted ecosystems, where the recruited-reads  
368 mapped only on a few, and limited regions, of the *Alkaliphilus* genomes. This could be  
369 explained by the presence, in these metagenomes, of other *Clostridia* sharing core genes with  
370 the novel *Alkaliphilus* genomes. Although *Alkaliphilus* species described so far have been  
371 isolated from various ecosystems (anaerobic digester, soda lake, deep gold mine water, river  
372 sediment, leachate pond) [52, 56-59, 64], the two novel *Alkaliphilus* species with *A.*  
373 *hydrothermalis* previously described from PBHF [55] might represent indigenous member of  
374 the PBHF ecosystem. Their prevalence and repeated detection in samples or cultures from all  
375 but one of the active submarine chimneys from PBHF (they were not detected in the deepest  
376 site ST012 of the Prony bay, sampled only once in 2011) are indicative of their ecological  
377 significance. The fermentative metabolism of PBHF *Alkaliphilus* lead to the production of H<sub>2</sub>  
378 and organic acids (i.e., acetate, butyrate, propionate) *in situ* at concentrations that might sustain  
379 the growth of microbial partners in syntrophy. This hypothetical partners could be  
380 hydrogenotrophic autotrophs such as the elusive *Methanosarcinales*, that co-occur within the  
381 same chimney samples, or hypothetical homoacetogens such as the candidate phyla  
382 *Bipolaricaulota* (formely named *Acetothermia*) which are dominant in PBHF and LCHF [65]

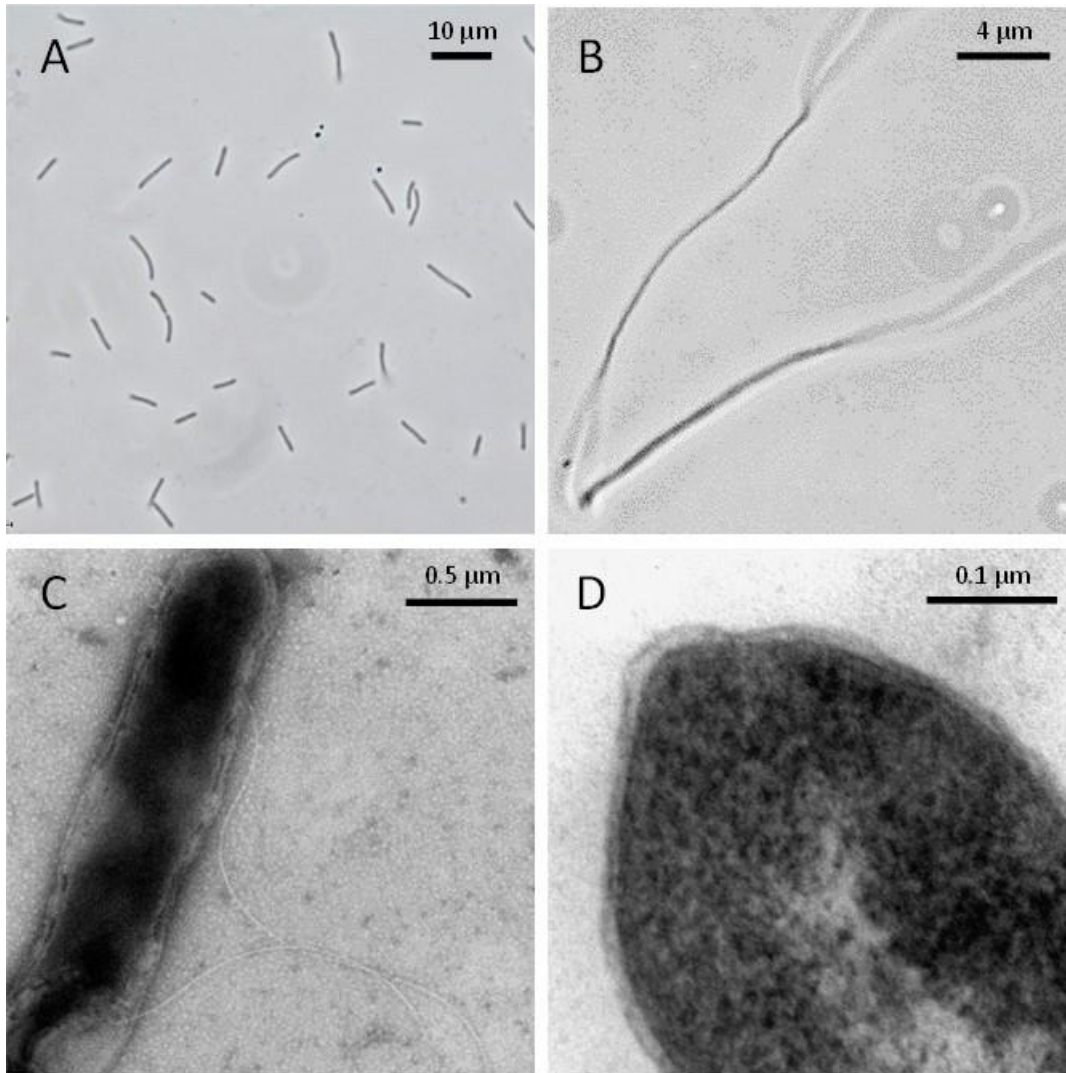
but also prevalent in terrestrial (ophiolites) serpentinized ecosystems [10, 66]. Thus, the *Methanosarcinales* could benefit, not only of the H<sub>2</sub> (also abiotically produced) but of the CO<sub>2</sub>, since the fluids are mostly depleted of DIC. Such an hypothesis has been recently formulated to tentatively explain the metabolism of the Lost City *Methanosarcinales* (LCMS), unable to use abiotically produced formate as dissolved carbon source, and thus would be dependent of anaerobic formate-oxidizing bacteria, present in the same micro-niche, feeding them with CO<sub>2</sub> [67, 68].

## Acknowledgments

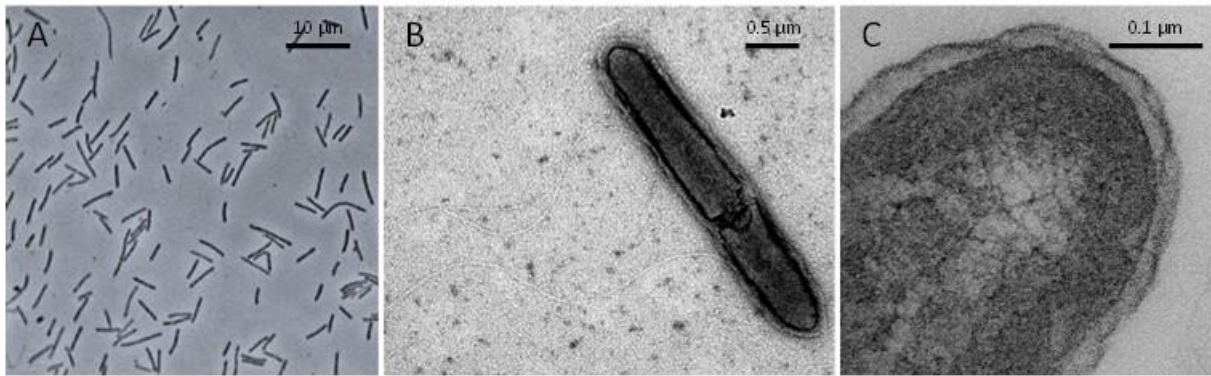
This project was financially supported by IRD (French National Research Institute for Sustainable Development) and ANR MICROPRONY (N°19-CE02-0020-02). We greatly acknowledge Kim Finnegan at the Marine Biological Laboratory, Woods Hole Oceanographic Institution, MA, USA) for performing the genomes sequencing and Noémie Robichon for genome assembly. Sequencing of *Alkaliphilus* genomes was supported by the Census of Deep Life of the Deep Carbon Observatory funded by Alfred Sloan Foundation. We thank Ms. Alison Strutt for proofreading and correcting English throughout the manuscript.



**Fig. 1.** Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequences showing the position of strains LacT<sup>T</sup> and LacV<sup>T</sup> within *Alkaliphilus*. Sequences of type species in the *Clostridiaceae* family are shown. The analysis involved a total of 1291 aligned nucleotide positions in the final dataset. Bootstrap values (> 70 % shown) were obtained with the maximum-likelihood / neighbor-joining / minimum evolution methods based on 2000 replicates. Bar: 0.01 substitutions per nucleotide.



**Fig. 2.** Microphotographs of strain LacT<sup>T</sup> showing (A) cells in exponential growth phase and (B) elongated (filamentous) cells in stationary growth phase, both observed in phase contrast; (C) peritrich lateral flagella and (D) thin section electron micrograph showing a jagged peptidoglycan and the absence of outer membrane, typical of a Gram-positive cell wall, both observed in MET microscopy.



**Fig. 3.** Microphotographs of strain LacV<sup>T</sup> showing (A) cells in exponential growth phase observed in phase contrast; (B) peritrich lateral flagella and (C) thin section electron microscopy showing a jagged peptidoglycan and the absence of outer membrane, typical of a Gram-positive cell wall, both observed in MET microscopy.

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433 **Table 1.** General features of the genomes of strains LacT<sup>T</sup> and LacV<sup>T</sup>.

<b>Genome Name / Sample Name</b>	<b><i>Alkaliphilus</i> sp. LacT<sup>T</sup></b>	<b><i>Alkaliphilus</i> sp. LacV<sup>T</sup></b>
<b>GenBank accession number</b>	WBZB000000000	WBZC000000000
<b>IMG Genome ID</b>	2639762554	2642422524
<b>Genome Size (bp)</b>	3291524	3061315
<b>Gene Count</b>	3257	3088
<b>Scaffold count</b>	83	94
<b>GC (%)</b>	36.00	33.87
<b>Coding Base Count %</b>	86.18	87.02
<b>CDS Count</b>	3183	3035
<b>RNA Count</b>	74	53
<b>rRNA Count</b>	1	2
<b>tRNA Count</b>	51	34

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**Table 2.** Digital DNA-DNA hybridization (dDDH: low left, green) and Average Nucleotide Identity (ANI: up right, red) values between strains LacT<sup>T</sup> and LacV<sup>T</sup> genomes and other *Alkaliphilus* species for which genome data were available: *A. peptidifermentans* Z-7036<sup>T</sup> (=DSM 18978) (FMUS000000000.1), *A. transvaalensis* SAGM1<sup>T</sup> (JHYF000000000.1), *A. metalliredigens* QYMF (CP000724) and *A. oremlandii* OhILAs<sup>T</sup> (CP000853). dDDH were calculated using the Genome-to-Genome Distance Calculator (GGDC) 2.1 (<http://ggdc.dsmz.de/>) [53]. ANI values were calculated using OrthoANI with usearch (<http://www.ezbiocloud.net/tools/ani>).

dDDH \ ANI (%)	A. sp. LacT	A. sp. LacV	<i>A. peptidifermentans</i>	<i>A. transvaalensis</i>	<i>A. metalliredigens</i>	<i>A. oremlandii</i>
A. sp. LacT		73.22	71.31	70.04	68.38	68.44
A. sp. LacV	14.10		68.74	70.54	68.97	68.74
<i>A. peptidifermentans</i>	13.40	15.50		70.83	70.20	69.16
<i>A. transvaalensis</i>	12.90	12.90	13.00		70.30	69.45
<i>A. metalliredigens</i>	12.60	12.70	12.90	13.00		69.80
<i>A. oremlandii</i>	12.60	12.70	12.70	12.80	13.00	

**Table 3.** Differential phenotypic and genotypic characteristics between strains LacT<sup>T</sup>, LacV<sup>T</sup> and type strains of recognized *Alkaliphilus* species: *A. peptidifermentans* Z-7036<sup>T</sup> [58], *A. namsaraevii* X-07-2<sup>T</sup> [57], *A. hydrothermalis* FatMR1<sup>T</sup> [24], *A. transvaalensis* SAGM1<sup>T</sup> [56] and *A. crotonatoxidans* B11-2<sup>T</sup> [59]. AFH: Amorphous ferric hydroxide, S<sub>0</sub>: elemental sulfur, nd: not determined.

Characteristics	Strain LacT <sup>T</sup>	Strain LacV <sup>T</sup>	<i>A. peptidifermentans</i> Z-7036 <sup>T</sup>	<i>A. namsaraevii</i> X- 07-2 <sup>T</sup>	<i>A. hydrothermalis</i> FatMR1 <sup>T</sup>	<i>A. transvaalensis</i> SAGM1 <sup>T</sup>	<i>A. crotonatoxidans</i> B11-2 <sup>T</sup>
<b>Origin</b>	Prony Bay Hydrothermal Field	Prony Bay Hydrothermal Field	Soda lake Verkhnee Beloe	Transbaikal steppe lake	Prony Bay Hydrothermal Field	Gold Mine, South Africa	Wastewater treating anaerobic digester
<b>Temperature growth range (opt.) (°C)</b>	25–37 (30)	20–40 (30)	6–40 (35)	25–47 (30)	20–55 (37)	20–50 (40)	15–45 (37)
<b>pH growth range (opt.)</b>	7.5–10.6 (8.2)	8.2–10.0 (8.7)	7.5–9.7 (9.1)	7.0–10.7 (9.6–10.3)	7.5–10.5 (8.8–9.0)	8.5–12.5 (10.0)	5.5–9 (8.0)
<b>NaCl growth range (opt.) (% w/v)</b>	0–7 (2)	0–8 (2.5)	0–5 (2)	0.5–15 (4)	0–3 (0.2–0.5)	0.1–3.3 (0.5) <sup>a</sup>	0–7 (1.0) <sup>b</sup>
<b>Electrons acceptors</b>	–	–	crotonate, AFH, Fe(III)-EDTA, quinone, fumarate, thiosulfate	AFH, S <sub>0</sub> , Fe(III) citrate	crotonate	crotonate <sup>c</sup> , S <sub>0</sub> , fumarate, thiosulfate	crotonate
<b>Crotonate disimultation</b>	+	+	+	–	+	nd	+
<b>Substrate use</b>							
<b>Casamino acids</b>	+	+	+	–	–	–	nd
<b>Glucose</b>	+	–	–	–	–	–	–
<b>Maltose</b>	+	–	–	–	–	–	+
<b>Sucrose</b>	+	–	–	–	–	–	–
<b>Main fermentation products</b>	Acetate	Acetate	Acetate, Formate	Acetate	Acetate, H <sub>2</sub> , CO <sub>2</sub>	nd	Acetate, Propionate
<b>Major fatty acids</b>	C16:0, C18:0	C16:0, C14:0, C18:0, C16:1ω7c	C16:0, C16:1ω7c, iso-C17, iso-C15, iso-C17:1	C16:1ω8, iso-C15:0, C14:0 3-OH, C16:0	C14:0, C16:0	iso-C15:0, iso-C17:0, iso-C15:1ω7c	C14:0, C16:0
<b>G + C content (mol%)</b>	36.0	33.9	33.8	32.6	37.1	36.4	30.6

<sup>a</sup> based on sea salts rather than NaCl addition; <sup>b</sup> from [64]; <sup>c</sup> from [59].



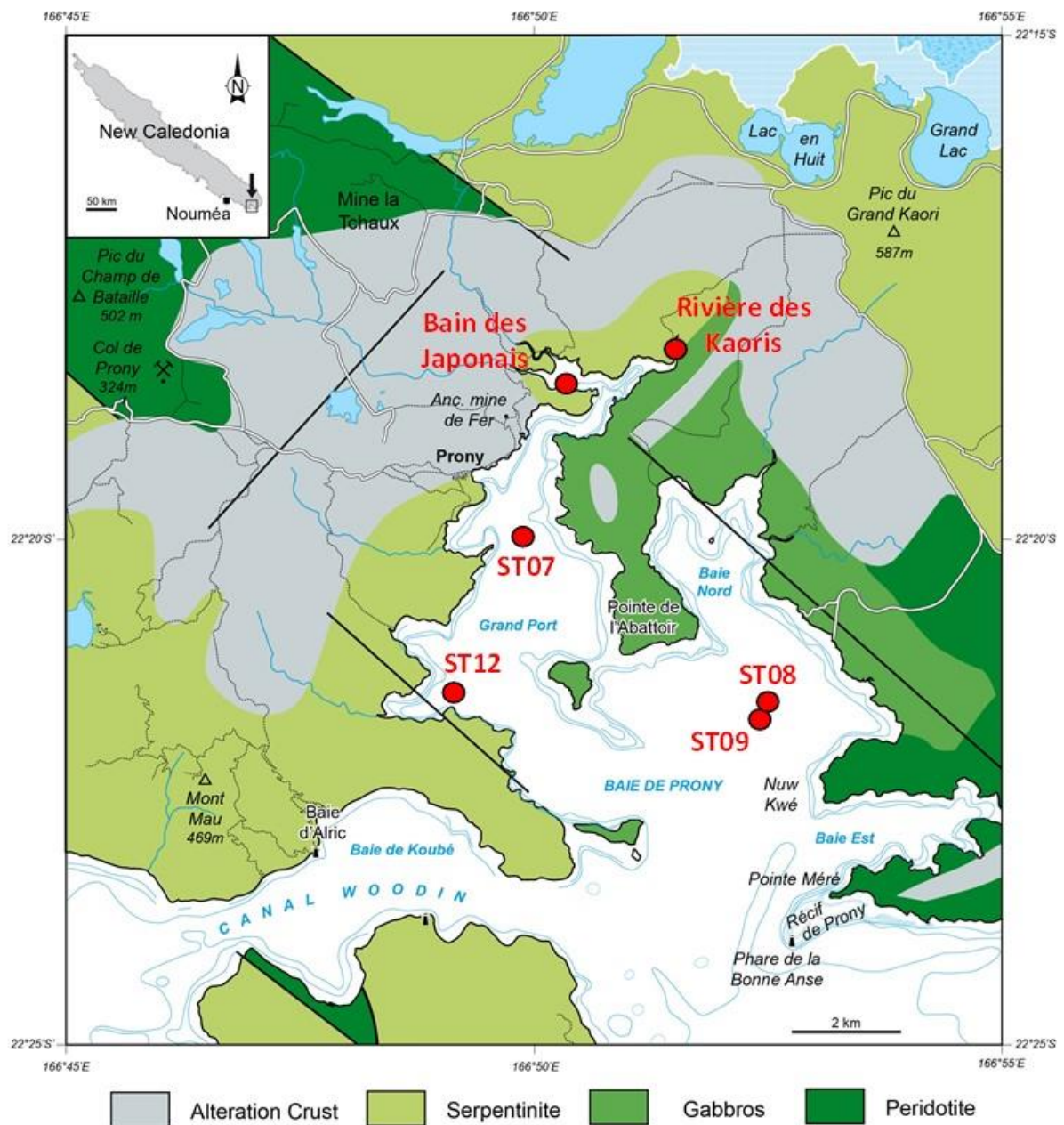
456 **Table 4.** Descriptions of *Alkaliphilus serpentinus* sp. nov. and *Alkaliphilus pronyensis* sp.  
457 nov..

<b>Genus name</b>	<i>Alkaliphilus</i>	<i>Alkaliphilus</i>
<b>Species name</b>	<i>Alkaliphilus serpentinus</i>	<i>Alkaliphilus pronyensis</i>
<b>Specific epithet</b>	<i>serpentinus</i>	<i>pronyensis</i>
<b>Species status</b>	<b>sp. nov.</b>	<b>sp. nov.</b>
<b>Species etymology</b>	(ser.pen.ti'nus N.L. masc. adj. <i>serpentinus</i> from a serpentinizing environment	pro.ny.en'sis. N.L. masc. adj. <i>pronyensis</i> of or belonging to Prony Bay, where the type strain was isolated
<b>Description of the new taxon and diagnostic traits</b>	Mesophilic anaerobic bacterium. Rod cells ( $4.9 \pm 1.4 \mu\text{m}$ length and $0.3 \pm 0.1 \mu\text{m}$ width during the exponential growth), forms filamentous cells (up to $30 \mu\text{m}$ ) during stationary growth phase. Gram-positive staining, forming terminal endospores, and motile by the means of lateral flagella. The temperature range for growth is 25–37°C, with an optimum at 30°C. The pH range for growth is 7.5–10.6, with an optimum at pH 8.2. Growth in the absence of NaCl (range 0–7 % NaCl, opt 2.0 %). Growth up to 0.5% O <sub>2</sub> in the gas phase. Able to ferment yeast extract, peptone, casamino acids, glucose, maltose, and sucrose. Fermentation products from growth on yeast extract were acetate, propionate, butyrate, isovalerate, CO <sub>2</sub> and H <sub>2</sub> . Fermentation products from growth on glucose were acetate, H <sub>2</sub> and CO <sub>2</sub> (trace). Crotonate is disproportionated into acetate and butyrate. None of the following electron acceptors were used (fumarate, thiosulfate, sulfate, sulfite, nitrite, nitrate, Fe(III)-citrate, Fe(III)-EDTA, Fe(OH) <sub>3</sub> , goethite $\alpha$ -Fe(III)O(OH) and elemental sulfur). The predominant fatty acids were C <sub>16:0</sub> and C <sub>18:0</sub> .	Mesophilic anaerobic bacterium. Rod cells (of $3.7 \pm 0.8 \mu\text{m}$ length and $0.5 \pm 0.1 \mu\text{m}$ width during the exponential growth), Gram-positive staining, forming terminal endospores, and motile by the means of lateral flagella. The temperature range for growth is 20–40°C, with an optimum at 30°C. The pH range for growth is 8.2–10.0, with an optimum at pH 8.7. Growth in the absence of NaCl (range 0–8 % NaCl, opt 2.5%). Growth up to 0.1% O <sub>2</sub> in the gas phase. Able to ferment yeast extract, tryptone, peptone, casamino acids. None of the sugars tested were used. Fermentation products from growth on yeast extract were acetate, propionate, butyrate and H <sub>2</sub> . Crotonate is disproportionated into acetate and butyrate. None of the following electron acceptors were used (fumarate, thiosulfate, sulfate, sulfite, nitrite, nitrate, Fe(III)-citrate, Fe(III)-EDTA, Fe(OH) <sub>3</sub> , goethite $\alpha$ -Fe(III)O(OH) and elemental sulfur). The predominant fatty acids were C <sub>14:0</sub> and C <sub>16:0</sub> .
<b>Country of origin</b>	New Caledonia	New Caledonia
<b>Region of origin</b>	Prony Bay Hydrothermal Field	Prony Bay Hydrothermal Field
<b>Source of isolation</b>	Shallow submarine hydrothermal chimney of the site ST08 of the Prony Bay, New Caledonia	Shallow submarine hydrothermal chimney of the site ST09 of the Prony Bay, New Caledonia
<b>Sampling date (dd/mm/yyyy)</b>	11/2011	11/2011
<b>Latitude (xx°xx'xx"N/S)</b>	22°21'35"S	22°21'39"S
<b>Longitude (xx°xx'xx"E/W)</b>	166°52'50"E	166°52'46"E
<b>16S rRNA gene accession nr.</b>	KJ159208	KJ159207
<b>Genome accession number [RefSeq; EMBL; ...]</b>	WBZB000000000	WBZC000000000

<b>Genome status</b>	Incomplete	Incomplete
<b>Genome size</b>	3,290 kpb	3,060 kpb
<b>GC mol% from genome analysis (from HPLC)</b>	36.0% (37.0 %)	33.9% (36.3%)
<b>Number of strains in study</b>	1	1
<b>Information related to the Nagoya Protocol</b>	Permits obtained from Province Sud, Nouvelle-Calédonie, République Française	Permits obtained from Province Sud, Nouvelle-Calédonie, République Française
<b>Designation of the Type Strain</b>	LacT <sup>T</sup>	LacV <sup>T</sup>
<b>Strain Collection Numbers</b>	DSM100337 <sup>T</sup> , = JCM30643 <sup>T</sup>	DSM 100017 <sup>T</sup> , =JCM 30644 <sup>T</sup>

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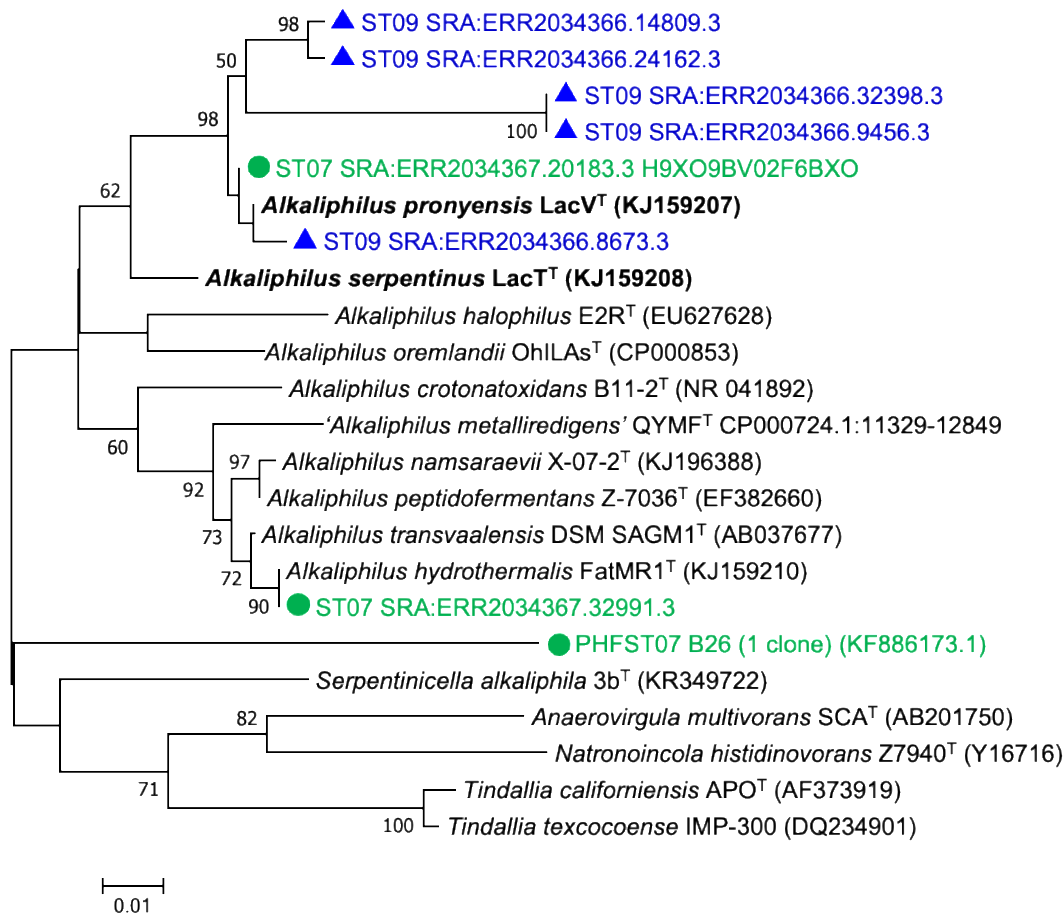
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463 **Fig. S1.** Simplified geological map of the Prony Bay, New Caledonia, South Pacific, modified from

464 [25, 69]. Strains LacT<sup>T</sup> and LacV<sup>T</sup> were isolated from samples of sites ST08 and ST09, respectively.

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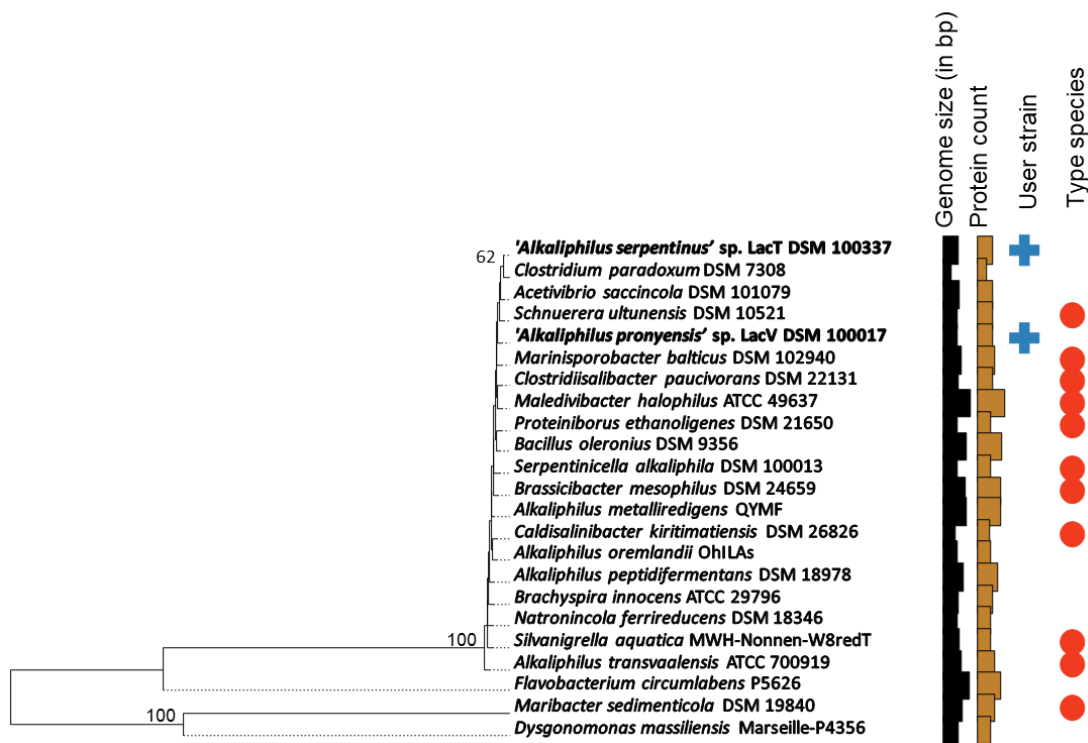
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**Fig. S2.** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains LacT<sup>T</sup> and LacV<sup>T</sup> within *Alkaliphilus* and related environmental sequences. In blue (triangle), OTUs from PBHF ST09 site, in green (circle) OTUs from PBHF ST07 site. The analysis involved a total of 397 aligned nucleotide positions in the final dataset. Bootstrap values ( $\geq 50\%$  shown) were based on 2000 replicates. Bar: 0.01 substitutions per nucleotide.



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**Fig. S3.** Whole genome-based taxonomic analysis of strains LacT<sup>T</sup> and LacV<sup>T</sup> genomes performed using the bioinformatics platform Type (Strain) Genome Server (TYGS) (<https://tygs.dsmz.de>) [70]. In brief, the TYGS analysis included a determination of the closest type strain genomes by (i) comparison against all type strain genomes available in the TYGS database *via* the MASH algorithm [71], and (ii) the closely related type strains determined *via* the 16S rDNA gene sequences. For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using the Genome BLAST Distance Phylogeny (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d5 [53]. The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1 [72]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [73].

491 **Table S1.** Mapping of metagenomic paired-end reads from alkaline serpentinite-hosted ecosystems to contigs of *Alkaliphilus* genomes of strains  
492 LacT<sup>T</sup>, LacV<sup>T</sup> and *A. hydrothermalis* FatMR1<sup>T</sup>.

Location, country	Type	Sample	Accession (SRA or MG-Rast)	Ref.	Total pairs passed (QC)	Alk_LacT	Alk_LacT (% mapped)	Alk_LacV	Alk_LacV (% mapped)	Alk_FatMR1	Alk_FatMR1 (% mapped)
Prony Bay, New Caledonia	Hydrothermal chimney	PBHF27 (ST09)	SRR1636516	[60]	4747529	47	0.0010	1704	0.0359	1067	0.0225
		PBHF28 (ST09)	SRR1636517		6012777	8	0.0001	1151	0.0191	1574	0.0262
		BdJ-11	NP	[63]	68376909	117966	0.1725	120271	0.1759	107973	0.1579
		BdJ-14			77737610	256760	0.3303	204727	0.2634	691304	0.8893
		RdK-14			68115190	123697	0.1816	127390	0.1870	114595	0.1682
		ST07-11			68444258	130824	0.1911	135275	0.1976	119136	0.1741
		ST07-14			63035475	123890	0.1965	225332	0.3575	102358	0.1624
Lost City hydrothermal field, MAR	Hydrothermal chimney	LCHF75	SRR1636508	[67]	12911379	69	0.0005	2517	0.0195	3605	0.0279
		LCHF81	SRR1636509		12484886	46	0.0004	2206	0.0177	3045	0.0244
Old City hydrothermal field, SWIR	Hydrothermal chimney	BaC	SRR9822619	[65]	19648677	3	0.0000	1447	0.0074	1905	0.0097
		PiMo	SRR9822616		19440539	0	0.0000	1066	0.0055	1425	0.0073
		Chan	SRR9822617		20478069	17	0.0001	1929	0.0094	2601	0.0127
		OCT	SRR9822614		21034520	6	0.0000	861	0.0041	1246	0.0059
		ToMo	SRR9822615		109686580	4	0.0000	3752	0.0034	5277	0.0048
The Cedars ophiolite, USA	Spring water	BS5B_11	DRR093101	[74]	24714554	14	0.0001	2309	0.0093	4586	0.0186
		BS5B_12	DRR093102		22551823	19	0.0001	2491	0.0110	5094	0.0226
		GPS1_11	DRR093103		26117910	18	0.0001	1902	0.0073	3337	0.0128
		GPS1_12	DRR093104		22036872	7	0.0000	1451	0.0066	2607	0.0118
Voltri Massif, Italy	Spring water	BR2_12	SRR2058405	[75]	14713490	1	0.0000	2030	0.0138	2905	0.0197
		BR2_13	SRR1636510		7616762	3	0.0000	736	0.0097	1192	0.0156
		GOR_12	SRR2058407		18290914	42	0.0002	1663	0.0091	3027	0.0165
		GOR_13	SRR1636513		25243838	105	0.0004	4276	0.0169	7465	0.0296

Cabeço de Vide aquifer, Portugal	Groundwater from a borehole	CVA	SRR1636515	NP	5871482	12	0.0002	1800	0.0307	2436	0.0259
Coast range ophiolite microbial observatory, USA	Groundwater from well	CSW1.1	4569549,3	[76]	9108623	1	0.0000	756	0.0083	1975	0.0217
		CSW1.3	4569551,3		16893824	345	0.0020	7839	0.0464	10688	0.0633
		QV1.1	4569550,3		15290683	2	0.0000	9805	0.0641	16685	0.1091
		QV1.2	4569552,3		12997149	730	0.0056	3064	0.0236	6947	0.0535
Santa Elena ophiolite, Costa Rica	Spring water	CM_SE_9	SRR4101185	[77]	7416565	0	0.0000	998	0.0135	1522	0.0205
		SE_9	SRR1636511	NP	51813498	216	0.0004	4387	0.0085	6495	0.0125
Peninsula Iberica	Deep aquifer	AC3	SRR6347719	NP	15897509	25	0.0002	3237	0.0204	3776	0.0238
		EBD	SRR6347720		12663159	116	0.0009	2600	0.0205	3774	0.0298
		EBPU	SRR6347718		12555955	22	0.0002	1227	0.0098	1683	0.0134

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496 **Table S2.** Mapping of metagenomic paired-end reads from marine or terrestrial ecosystems to contigs of *Alkaliphilus* genomes of strains LacT<sup>T</sup>,497 LacV<sup>T</sup> and *A. hydrothermalis* FatMR1<sup>T</sup>.

Location, country	Type	Sample	Accession (SRA or MG-Rast)	Ref.	Total pairs passed (QC)	Alk_LacT	Alk_LacT (% mapped)	Alk_LacV	Alk_LacV (% mapped)	Alk_FatMR1	Alk_FatMR1 (% mapped)
Axial Seamount, Juan de Fuca ridge	Basaltic hydrothermal fluids	Mkr33	ERR694199- ERR694206	[78]	25725851	6	0.0000	6210	0.0241	88417	0.3437
		Mkr113	ERR694207- ERR694213		22624331	12	0.0001	3336	0.0147	4464	0.0197
Piccard	Basaltic hydrothermal fluids	HC	ERR1662208- ERR1662232	[79]	78203317	24	0.0000	68060	0.0870	90624	0.1159
		MkrX19	ERR1662466- ERR1662503		141073647	2	0.0000	33365	0.0237	45580	0.0323
		SC	ERR1662233- ERR1662244		38058339	0	0.0000	208229	0.5471	295789	0.7772
		SG2	ERR868087- ERR868115		104273127	0	0.0000	21601	0.0207	30254	0.0290
Von Damm	Serpentinite-hosted hydrothermal fluids (non-alkaline)	GC	ERR868031- ERR868053	[79]	77403857	14	0.0000	18153	0.0235		
		MH	ERR1662199- ERR1662207		26539513	2	0.0000	6105	0.0230	8469	0.0319
		OMT	ERR1662181- ERR1662198		53972022	0	0.0000	17338	0.0321	25239	0.0468
		Rav2	ERR1662393- ERR1662413		64064672	0	0.0000	2926	0.0046	4566	0.0071
		SH	ERR1662414- ERR1662442		104733336	4	0.0000	14418	0.0138	20388	0.0195
Guaymas Basin	Hydrothermal sediments	4484 shallow	SRR6301444	[80]	233093488	306344	0.1314	312308	0.1340	284008	0.1218
		4484 deep	SRR6301445		179762089	462149	0.2571	470732	0.2619	426607	0.2373
		4572 shallow	SRR6301446		203371756	287320	0.1413	313182	0.1540	292335	0.1437
		4572 deep	SRR6301447		246429337	386166	0.1567	402130	0.1632	368436	0.1495
Gulf of Mexico	Methane seep sediments	S1	SRR7716469	[81]	40523149	13	0.0000	2556	0.0063	3494	0.0086
		S2	SRR7716470		69582704	0	0.0000	3356	0.0048	3999	0.0057



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		S3	SRR7716471		16553149	0	0,0000	1548	0,0094	3833	0,0232
South Africa Gold mine	Fracture fluids	MM51	SRR1633224	Not published	20525179	69	0,0003	5997	0,0292	7810	0,0381
		NO14	SRR1633225		29027742	156	0,0005	14094	0,0486	22127	0,0762
		TT10	SRR1633226		16553149	0	0,0000	1548	0,0094	3833	0,0232
NP; Not published.											

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502 **Table S3.** Fatty acids compositions of strains LacT<sup>T</sup> and LacV<sup>T</sup> and type strains of related  
 503 *Alkaliphilus* species: *A. peptidifermentans* Z-7036<sup>T</sup> [58], *A. namsaraevii* X-07-2<sup>T</sup> [57], *A.*  
 504 *hydrothermalis* FatMR1<sup>T</sup> [24], *A. transvaalensis* SAGM1<sup>T</sup> [56] and *A. crotonatoxidans* B11-2<sup>T</sup>  
 505 [59]. The two dominant fatty acids are indicated in bold for each strain. - ; not detected.

Fatty acids	LacT <sup>T</sup>	LacV <sup>T</sup>	Z-7036 <sup>T</sup>	X-07-2 <sup>T</sup>	FatMR1 <sup>T</sup>	SAGM1 <sup>T</sup>	B11-2 <sup>T</sup>
C <sub>14:0</sub>	6.9	<b>20.3</b>	-	10.9	<b>44.2</b>	1.7	<b>45.6</b>
C <sub>14:0</sub> <b>3-OH</b>	-	-	-	18.6	-	-	-
iso-C <sub>15:0</sub>	-	4.9	10.5	<b>36.2</b>	4.9	<b>51.6</b>	7.1
C <sub>16:0</sub>	<b>50.5</b>	<b>24.2</b>	<b>20.1</b>	18.6	<b>14.3</b>	3.9	<b>17.4</b>
C <sub>16:1</sub> <b>7c</b>	5.1	11.1	<b>14.5</b>	-	9	1.9	2.4
C <sub>16:1</sub> <b>8</b>	-	-	-	<b>37.0</b>	-	-	-
iso-C <sub>17:0</sub>	-	0.6	11.3	-	-	<b>12.2</b>	1.1
C <sub>17:0</sub> isoI / anteisoB	-	6.8	-	-	-	-	-
C <sub>18:0</sub>	<b>37.5</b>	14.4	1.9	4.2	-	2.0	0.8

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