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Abstract

A novel anaerobic, alkaliphilic, mesophilic, Gram-stain-positive, endospore-forming bacterium was isolated from an alkaline thermal spring (42°C, pH 9.0) in New Caledonia. This bacterium designated strain LB2^T grew at 25–50°C (optimum 37°C) and pH 8.2–10.8 (optimum 9.5). Added NaCl was not required for growth (optimum 0–1%) but was tolerated up to 7%. Strain LB2^T utilized a limited range of substrates, such as peptone, pyruvate, yeast extract and xylose. End products detected from pyruvate fermentation were acetate and formate. Both ferric citrate and thiosulfate were used as electron acceptors. Elemental sulfur, nitrate, nitrite, fumarate, sulfate, sulfite, and DMSO were not used as terminal electron acceptors. The two major cellular fatty acids were iso-C15:0 and C16:0. The genome consists of a circular chromosome (3.7 Mb) containing 3626 predicted protein-encoding genes with a G+C content of 36.2 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolate is a member of the family *Proteinivoraceae*, order *Clostridiales* within the phylum *Firmicutes*. Strain LB2^T was most closely related to the thermophilic *Anaerobranca gottschalkii* LBS3^T (93.2% 16S rRNA gene sequence identity). Genome-based analysis of average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) of strain LB2^T with *A. gottschalkii* LBS3^T showed respective values of 70.8% and 13.4%. Based on phylogenetic, genomic, chemotaxonomic, and physiological properties, strain LB2^T is proposed to represent the first species of a novel genus, for which the name *Alkalicella caledoniensis* gen. nov., sp. nov. is proposed (type strain LB2^T = DSM 100588^T = JCM 30958^T).

Over the last twenty years, alkaliphilic bacteria have been extensively studied because they are sources of biomolecules and enzymes stable at extreme conditions, precious in biotechnology and bioremediation [1, 2, 3]. Alkaliphiles are microorganisms that optimally grow at pH values above 9 but cannot grow or slowly grow at near-neutral pH [3]. They inhabit various alkaline ecosystems (e.g., soda lakes, thermal springs, oceans, industrial or mining-impacted areas). Some of them can grow under multiple extreme conditions like a combination of high salt concentrations and high temperature [4]. Anaerobic alkaliphiles, especially members of the order *Clostridiales* (e.g., the genera *Alkaliphilus*, *Anaerobranca*, *Natronoincola*, *Serpentinicella*, or *Tindallia*), have been isolated from different sites around the world and include halo-, meso-, and thermo-alkaliphiles [4, 5].

In New Caledonia (Southwest Pacific), several thermal springs are located in the southeastern half of the main island [6] (see the map in [7]). Such springs are found down south in the Prony Bay area and the area close to the city of Canala [7]. A sampling campaign was conducted in November 2014 during which water and sediment samples were collected at one of the springs of the Canala region, namely the spring feeding the abandoned spa facility of 'La Crouen' (165° 53' 20.6" E 21° 32' 06.6" S). On-site measurements showed that its waters are characterized by high pH (9.0), low Eh value (-174 mV, ref H_2), low dissolved O_2 (0.71 mg/L), low conductivity (314 µS/cm), and moderate temperature (42°C) (data from our study). Deville and Prinzhofer [8] reported that the gas emitted at the springs is dinitrogen (N_2) with traces of methane (CH_4 <3%), but we did not observe degassing in the spring during our visit. Our dissolved gases analyses showed that N_2 is dominant, followed by O_2, but no CH_4 or hydrogen (H_2) was detected. Both the geological setting and geochemistry differ from those of the well-known hyperalkaline springs located in the peridotite nappe in the Prony Bay Hydrothermal Field (PBHF). These on-shore or submarine springs discharge a warm (<40°C), highly reduced, high-pH (up to 11.2) fluid enriched in N_2, H_2, and CH_4 [9], produced by the serpentinization process

(i.e., a hydrothermal alteration that transforms ferromagnesian minerals contained in ultramafic rocks into serpentine minerals [10]). Prokaryotic communities of PBHF have been studied [11-15] and several anaerobic *Clostridiales* strains have been isolated and described as new taxa [15-21]. On the contrary, microbial communities of 'La Crouen' spring had not been studied previously. Here we report the isolation and characterization of a novel bacterium isolated from 'La Crouen' spring, which original features allow the proposition of a novel species within a novel genus belonging to the family *Proteinivoraceae*, phylum *Firmicutes*.

For culturing experiments, a sterile glass bottle was filled with sediment slurry sample, then hermetically sealed to preserve anoxic conditions, and stored at 4°C. In the laboratory, anaerobic enrichment cultures were carried out at 37°C using 0.5 mL of the slurry sample (corresponding to 10% inoculum) in Hungate tubes containing 5 mL of nutrient-rich LB (Luria-Bertani) medium (i.e., 10g/L Tryptone, 5 g/L yeast extract, and 5 g/L NaCl), supplemented with 25 mM Tris-Base. Before inoculation, the culture medium was adjusted to pH 9.5, boiled for 5 min, and then cooled to room temperature under a flow of O_2-free N_2 gas. The medium was dispensed into Hungate tubes, degassed under a flow of N_2, and subsequently autoclaved (45 min, 120°C). The following sterile solutions were injected in each tube: 0.1 mL of 2% $Na_2S.9H_2O$ (reducing agent) and 0.5 mL of 8% Na_2CO_3 (to adjust and buffer the final pH to 9.5). The Hungate technique for anaerobic cultivation was used throughout this study [22].

Temperature, pH, and NaCl concentration ranges (respectively tested: 20–70°C, 6.0–12.0, 0–10%) for growth of the isolate were determined in duplicate using Hungate tubes containing LB medium, supplemented with 25 mM Tris-Base (except for testing the optimal pH for growth, which was determined using different buffer solutions: 0 mM Tris-Base for pH 6.0–7.0, 25 mM Tris-Base for pH 7.0–9.0, or 20 mM CAPS for pH 9.0–12.0). The pH of the culture media was adjusted with anaerobic sterile stock solutions of 8% Na_2CO_3 or 1M NaOH. The pH was always checked both after inoculation and at the end of incubation. Water baths were used

for incubation at temperatures up to 70°C. For studies of NaCl requirements, NaCl was weighed and added directly into the tubes before culture medium addition. Each test culture was sub-cultured at least once under the same experimental conditions before determining growth rates. A range of inorganic and organic substrates was tested as substrate for the isolated growth in the basal medium containing (per liter of distilled water): 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 1 g NaCl, 0.1 g KCl, 0.5 g cysteine.HCl, and 10 mL of trace elements solution [23]. The pH was adjusted to 9.5 with a 1M NaOH solution. The following compounds were tested as potential substrates (at a final concentration of 20 mM for simple substrates and 2g/L for complex substrates): adenine, adenosine 5'-diphosphate, albumin, arabinose, casamino-acids, casein, cellobiose, fructose, galactose, gelatin, glucose, glycerol, guanine, lactate, lactose, mannose, peptone, pyruvate, ribose, starch, sucrose, tryptone, uracil, xylose, yeast extract, H_2/CO_2 (80/20, v/v) and H_2/CO_2 (80/20, v/v) in the presence of acetate (2 mM) as carbon source. Elemental sulfur (1% w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM), nitrite (2 mM), fumarate (20 mM), FeIII-citrate (10 mM), and DMSO (10 mM) were tested as terminal electron acceptors using pyruvate as electron donor. Sensitivity to antibiotics (ampicillin, bacitracin, chloramphenicol, cycloserine, kanamycin, metronidazole, novobiocin, penicillin, rifampicin, streptomycin, tetracycline, vancomycin) was tested by adding them separately to the LB medium (supplemented with 25 mM Tris-Base) at a final concentration of 25 µg/mL or 50 µg/mL. Each test and control, carried out in parallel, was run in duplicate Hungate tubes. Controls were (i) inoculated tubes without tested compounds and (ii) uninoculated tubes with tested compounds. Bacterial growth was monitored by measuring the turbidity increase at 600 nm by inserting Hungate tubes into the tube holder of a spectrophotometer (Cary 50, Varian). H_2S production was measured photometrically as colloidal CuS, as described by Cord-Ruwisch [24]. End-products of metabolism were measured

by high-performance liquid chromatography (HPLC) and gas chromatography (GC) after 1 day of incubation at 37°C [19].

Cellular morphology and purity of the strain and the presence of spores were assessed by observation using a phase-contrast microscope (Optiphot; Nikon). For transmission electron microscopy (using a MET FEI Tecnai G2), exponentially grown cells were negatively stained with sodium phosphotungstate (to observe flagella) or fixed with glutaraldehyde and osmium tetraoxide, epon-embedded and stained with uranyl acetate and lead citrate (to watch the cell wall), as described by Quéméneur *et al.* [25].

Cellular fatty acids were determined in the late exponential growth phase (after 24-hour incubation at 37°C using LB-Tris medium at pH 9.5) by the Identification Service of DSMZ with modified Miller extraction protocol [26] using the MIDI Microbial Identification system (version 4.0, MIS operating manual March 2001) [27].

Near-complete 16S rRNA genes were amplified from genomic DNA extracted as described by Quéméneur et al. [28], using the primers 27F and 1492R [29], then sequenced by GATC-Biotech (Konstanz, Germany). The 16S rRNA gene sequence was deposited and aligned with available sequences from NCBI database using the MUSCLE program [30] implemented in MEGA7 software [31]. Evolutionary analyses were conducted from 16 nucleotide sequences using three different methods: maximum-likelihood (ML) [32], maximum-parsimony (MP) [33], and neighbor-joining (NJ) [34].

Whole-genome analysis was performed from high molecular weight DNA of the isolate, extracted using a phenol-chloroform method, then sequenced at the GeT-PlaGe platform (INRAe Toulouse, France) by combining long reads (Oxford Nanopore) and short-reads (Illumina MiSeq) technologies, as described by Quéméneur *et al.* [25] with some modifications because it was sequenced by multiplex sequencing according to the manufacturer's instructions "1D Native barcoding genomic DNA" (EXP-NBD104 and SQK-LSK109)". Base-calling was

carried out by MinKNOW-Live-Base-calling software version 3.6.0 (Oxford Nanopore Technologies, Oxford, UK). Read demultiplexing and adapter trimming were performed using qcat v1.1.0 (https://github.com/nanoporetech/qcat) and genome assembly with Unicycler v0.4.6 [35]. The genome sequence was deposited in the NCBI database and annotated using the MaGe (Microscope Genome Annotation) [36] and the Integrated Microbial Genomes (IMG) systems [37]. The value of average nucleotide identity (ANI) was calculated using OrthoANI with usearch (http://www.ezbiocloud.net/tools/ani). Digital (or *in silico*) DNA-DNA hybridization (dDDH) was calculated using the Genome-to-Genome Distance Calculator (GGDC) 2.1 (http://ggdc.dsmz.de/) [38]. A whole genome-based taxonomic analysis was performed using the bioinformatics platform Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de) [39]. 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 [40] and (ii) the closely related type strains determined *via* the 16S rDNA gene sequences. For the phylogenomic inference, all pairwise comparisons among the genomes were conducted using Genome BLAST Distance Phylogeny (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d5. The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support *via* FASTME 2.1.6.1 [41]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [42].

The G+C content of the DNA was also determined at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) using HPLC, as described by Mesbah et al. [43].

The enrichment culture of the ‘La Crouen’ slurry sample showed significant growth on LB-Tris-Base medium after two days of incubation at 37°C and at pH 9.5. This enrichment was then subcultured several times under the same growth conditions before isolation. The Roll-

tube method described originally by Hungate [22] was used for strain isolation. The culture was serially diluted tenth in roll tubes containing molten 1.6% agar medium. Single colonies were transferred into the liquid medium and immediately serially diluted tenth in roll tubes a second time. The colonies grown in roll tubes were white and circular (1–2 mm in diameter after one-week incubation at 30°C). After the third round of roll tubes isolation, a single colony was finally transferred into the liquid medium, resulting in the strain designated LB2^T that was further physiologically and metabolically characterized.

Cells of strain LB2^T stain Gram-positive are motile and endospore-forming bacterium (Fig. 1A). Endospores are terminal and oval (0.8–1 x 1.0–1.5 µm). Rods are approximately 6.0–10.0 µm long and 0.5–0.6 µm wide. They occur singly or in pairs and possessed peritrichous flagella (Fig. 1B). They are pleiomorphic, i.e., rod-shaped during the exponential growth phase (Fig. S1A), they turn coccoid in the stationary phase (after 24h, Fig. S1B).

Strain LB2^T is mesophilic and grows only under anaerobic conditions, ranging from 25°C to 50°C (Table 1). The optimum temperature for growth is 37°C. The pH range for growth is 8.2–10.8, with an optimum at an initial pH of 9.5; the final pH is 9.2. NaCl is not required for growth, and the NaCl range for growth is 0–7% (optimum at 0–1%) but is tolerated up to 7%, indicating that strain LB2^T is halotolerant and adapted to the low-salinity and high-pH fluid emitted by the alkaline spring of 'La Crouen' (New Caledonia).

Peptone, pyruvate, xylose, and yeast extract were the only substrates used for growth (Table 1). Yeast extract was degraded to acetate, formate, H_2, and propionate. Growth on yeast extract was improved by the addition of thiosulfate used as a terminal electron acceptor, reduced to sulfide. End products detected from pyruvate fermentation were acetate and formate. Both ferric citrate and thiosulfate were used as electron acceptors using pyruvate at pH 9.5. Elemental sulfur, sulfate, nitrate, nitrite, fumarate, and DMSO were not used as terminal electron

197 acceptors, using pyruvate as an electron donor. The maximal growth rate found was 0.07 h^{-1}
198 using pyruvate and thiosulfate as electron donor and acceptor, respectively.

199 Strain LB2^T was not sensitive to the presence of antibiotics cycloserine and novobiocin
200 added at 25 µg/mL and 50 µg/mL in the culture medium. It also grew in the presence of 25
201 µg/mL of chloramphenicol and kanamycin, but it was sensitive to these antibiotics at 50 µg/mL.
202 Ampicillin, bacitracin, metronidazole, penicillin, rifampicin, streptomycin, tetracycline, and
203 vancomycin at 25 µg/mL completely inhibited cell growth.

204 Phylogenetic analysis based on the almost full-length 16S rRNA gene sequence (1245 nt)
205 indicated that strain LB2^T is a member of the family *Proteinivoraceae* [44], within the order
206 *Clostridiales*, phylum *Firmicutes* (Fig. 2). It forms a distinct branch among alkaliphilic
207 clostridia of the family *Proteinivoraceae*, including the genera *Anaerobranca* and
208 *Proteinivorax* (91.2–93.2% 16S rRNA gene sequence identity) (Fig. 2). The phylogenetic
209 position of strain LB2^T was confirmed by using three different methods: maximum-likelihood
210 (ML, Fig 2), maximum-parsimony (MP, Fig S2A), and neighbor-joining (NJ, Fig S2B). The
211 closest relatives of strain LB2^T are type species: *Anaerobranca gottschalkii* LBS3^T (93.2%
212 identity) isolated from a hot lake inlet (Lake Bogoria, Kenya) [45], *Anaerobranca horikoshii*
213 JWIYL-l3S^T (92.6% identity) isolated from Yellowstone National Park (USA) [46],
214 *Anaerobranca californiensis* PAOHA-1^T (91.7% identity) isolated from alkaline hot spring
215 sediment (Mono Lake, CA, USA) [47], *Anaerobranca zavarzinii* JW/VK-KS5Y^T (92.2%
216 identity) isolated from a small shallow geothermally heated pool (Kamchatka, Russia) [48],
217 *Proteinivorax hydrogeniformans* Z-710 ^{T} (91.8% identity) and *Proteinivorax tanatarense* Z-
218 910^T (91.5% identity) isolated from a decaying algal bloom in the alkaline lake Tanatar (Russia)
219 [44, 49].

220 The 16S rRNA gene sequence of strain LB2^T was closely related (>97% identity) to few
221 environmental sequences retrieved in August 2020 by a NCBI web BLAST search [50] and

originated from low-salt alkaline ecosystems: anaerobic fermentation reactor with waste activated sludge under mesophilic condition (pH 9.0; GU455162) [51] and surface rock/sediment of Lonar Crater Lake (pH 8.9–10.0; JQ738949) [52]. 16S rRNA genes affiliated to the family *Proteinivoraceae* (formerly known as the family *Anaerobrancaceae*) were also detected in the subsurface hyperalkaline fluids of the Semail Ophiolite (Oman) [53] and other oligotrophic, rock-hosted ecosystems, such as deep crystalline aquifer of Outokumpu (Finland) [54]. Still, their potential functional roles in the subsurface remain unclear.

The complete genome sequence of strain LB2^T is a unique circular chromosome of 3.7 Mb long with a 36.2 mol% G+C content (Table 2), close to the G+C content measured by HPLC (36.4 mol%, Table 2). The G+C content of strain LB2^T is higher than that of its closest *Anaerobranca* relatives (*A. californiensis*, 30.0–33.4 mol%; *A. gottschalkii*, 30.0–32.9 mol%; *A. horikoshii*, 32.0–34.0 mol%; *A. zavarzinii*, 32.5 mol%), and *P. tanatarense* (31.9–32.2 mol%) within the family *Proteinivoraceae*. The genome size of strain LB2^T is much larger than that of its closest phylogenetic neighbor for which genomes are available, *A. californiensis* (2.0 Mb) and *A. gottschalkii* (2.3 Mb), but is similar to those of the mesophilic and alkaliphilic genus *Alkaliphilus* (>3 Mb). The ANI and dDDH values between LB2^T and *A. californiensis* PAOHA-1^T were 70.61% and 13.50%. The ANI and dDDH values between LB2^T and *A. gottschalkii* LBS3^T were 70.78% and 13.40% (Supplementary Table S1), i.e., far below the species boundaries of 95~96% and 70%, respectively, recommended by Chun *et al.* [55]. The LB2^T strain genome comprises a single chromosome and 3,777 genes, including 3,626 protein-encoding genes and 94 RNA encoding genes. Amino acids, carbohydrates, and inorganic ion transport and metabolisms represented 7.4%, 5.7%, and 4.9% of the function-predicted genes. The distribution of genes into COGs functional categories is shown in Supplementary Table S2. In a phylogenomic analysis (Fig S3), strain LB2 clustered with other alkaliphilic *Firmicutes*.

It positions in a branch containing members of the genera *Alkalibacterium* (class *Bacilli*) and *Serpentinicella* (class *Clostridia*) distantly related to the *Anaerobranca* species.

The predominant cellular fatty acids (>5% of total fatty acids) were iso-C15:0 (15.3%), C16:0 (10.2%), C14:0 (8.0%), cyclo-C17:0 (6.9%), iso-C17:1 (8.8%), and C18:0 (6.1%). The proportion of iso-C15:0 and C16:0 was lower than that of *A. gottschalkii* and *P. tanatarense,* while the iso-C15:0 content was higher than that of *A. horikoshii* (Supplementary Table S3).

Table 1 summarizes the physiological and biochemical differences between strain LB2^T and the type species of all genera of the family *Proteinivoraceae,* including *A. horikoshii* and *P. tanatarense*. Strain LB2^T shares similar physiological and biochemical properties with this latter (e.g., proteinaceous substrate utilization and high-pH optimal growth ranging between 8.5 and 9.5) but differs by growing optimally higher pH (up to 10.8) compared to other *Proteinivoraceae* members. Unlike *Anaerobranca* and *Proteinivorax* genera, strain LB2^T displays optimal growth in the absence of NaCl addition, similarly to another alkaliphilic anaerobe of the family *Clostridiaceae Serpentinicella alkaliphila* 3b^T, isolated from the serpentinizing Prony Bay Hydrothermal Field of New Caledonia [20]. In contrast to the alkalithermophilic *Anaerobranca* spp., displaying optimal growth temperatures between 50–60°C, strain LB2^T was mesophilic (the optimum temperature at 37°C) similarly to *P. tanatarense* and *P. hydrogeniformans* that grew optimally at 32–38°C and 30–32°C, respectively [44, 49]. Like other *Proteinivoraceae* members, strain LB2^T utilizes proteinaceous substrates for growth (i.e., yeast extract). It can also use pyruvate as the sole carbon source, similarly to *A. californiensis* (or *A. zavarzinii* that co-metabolizes pyruvate with yeast extract). As reported for *A. zavarzinii,* both formate and acetate were the major end products detected. It suggests that pyruvate is mainly metabolized *via* pyruvate: formate lyase and not *via* the pyruvate: ferredoxin oxidoreductase as commonly reported in *Firmicutes.* In the latter case, acetate plus H_2 would be the major metabolic end-products. Genomic data supported this

hypothesis. The gene encoding the formate lyase, also known as formate C-acetyltransferase (EC 2.3.1.54), is found in the genome of strain LB2^T and not the gene of the pyruvate: ferredoxin oxidoreductase (PFOR, EC 1.2.7.1). The ability of strain LB2^T to use both ferric citrate and thiosulfate as electron acceptors were also shared with *A. californiensis*. Sugars (i.e., xylose) was used by strain LB2^T, similarly to *A. gottschalkii*, as well as *A. californiensis, A. zavarzinii, 'P. hydrogeniformans'* and *P. tanatarense* ferment some sugars, but in the presence of tryptone or yeast extract.

In conclusion, the strain LB2^T differs from other species of the closest genera *Anaerobranca* and *Proteinivorax* (belonging to the family *Proteinivoraceae*) by several features: (i) it forms a distinct branch among the family *Proteinivoraceae* in the 16S rRNA gene phylogenetic trees, (ii) it is only distantly related to *Anaerobranca* spp. in phylogenomic analyses, (iii) it displays a larger genome size and G+C content than other *Proteinivoraceae* members, (iv) it grows optimally at higher pH (up to 10.8) than other *Proteinivoraceae* members, (v) it grows optimally in the absence of NaCl addition, (vi) it has a much lower optimal growth temperature (37°C) than the closest *Anaerobranca* members, (vii) it is the only one in the family *Proteinivoraceae* (including pleomorphic bacteria) occurring as rods or cocci depending on the growth phase, as observed for the actinobacterial genus *Arthrobacter* [56]. In contrast, cells of the genus *Anaerobranca* are rod-shaped, sometimes showing branching [46, 48]. Based on morphological, phenotypic, phylogenetic, and genomic, strain LB2^T is proposed to represent a novel species of the new genus *Alkalicella*, *A. caledoniensis* gen. nov., sp. nov.

Description of *Alkalicella* gen. nov.

Alkalicella (Al.ka.li.cel'la. N.L. n. *alkali* from Arabic *al-qaliy* ashes of saltwort; L. fem. n. *cella* a storeroom, chamber, and in biology a cell; N.L. fem. n. *Alkalicella*, a cell living in an alkaline

environment).

Gram-stain-positive (terminal and oval) endospore-forming bacterium with anaerobic chemoorganotrophic metabolism. Occurs as rods or cocci under different conditions. Able to utilize sugars and proteinaceous substrates. Use ferric citrate and thiosulfate as electron acceptors. Alkaliphilic and mesophilic. Displays optimal growth in the absence of NaCl addition. Belongs to the family *Proteinivoraceae*, order *Clostridiales*, class *Clostridia* within the phylum *Firmicutes*. Most closely related to *Anaerobranca gottschalkii* LBS3^T (93.2% 16S rRNA gene sequence identity). The G+C content of the genomic DNA is 36.2%. The type species is *Alkalicella caledoniensis*, and the genus description is based on that of the type species, currently the only species ascribed to this genus.

Description of *Alkalicella caledoniensis* sp. nov.

Alkalicella caledoniensis (ca.le.do.ni.en′sis. N.L. fem. adj. *caledoniensis* pertaining to New Caledonia, where strain LB2^T was first isolated).

Displays the following characteristics in addition to those listed in the genus description. Cells are approximately 6.0–10.0 µm long and 0.5–0.6 µm wide, occurring singly or in pairs. Cells are motile and possess multiple flagella. They are pleiomorphic depending on the growth phases (from rod to cocci). Colonies are white and circular and about 1–2 mm in diameter after seven days of incubation at 30°C. Growth occurs at 25–50°C (optimum 37°C), at pH 8.2–10.8 (optimum pH 9.5) with 0–7% NaCl (optimum 0–1%). Added NaCl was not required for growth. Pyruvate, yeast extract, xylose, and peptone were used as electron donors. Still, adenine, adenosine 5'-diphosphate, albumin, arabinose, casamino-acids, casein, cellobiose, fructose, galactose, gelatin, glucose, glycerol, guanine, lactate, lactose, mannose, ribose, starch, sucrose, tryptone, uracil, and H_2/CO_2 were not. End products detected from pyruvate fermentation were

acetate and formate. Both ferric citrate and thiosulfate were used as electron acceptors using pyruvate and reduced to ferrous iron and sulfide. Yeast extract was degraded into acetate, formate, H_2, and propionate. Elemental sulfur, nitrate, nitrite, fumarate, sulfate, sulfite, and DMSO were not used as terminal electron acceptors. The predominant fatty acids were iso-$C_{15:0}$ (15.3%), $C_{16:0}$ (10.2%), $C_{14:0}$ (8.0%), cyclo-$C_{17:0}$ (6.9%), iso-$C_{17:1}$ (8.8%), and $C_{18:0}$ (6.1%). The GenBank accession number for the 16S rRNA gene sequence of strain LB2^T is KR349724, and that for the whole genome sequence is CP058559.

The type strain LB2^T (= DSM 100588^T = JCM 30958^T) was isolated from a sediment slurry of 'La Crouen' thermal spring near Canala, New Caledonia.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figures legends

Fig 1: Phase-contrast micrographs of cells of strain LB2^T with terminal and oval endospore formation (A; bar 10 µm), and transmission electron micrograph showing cells of strain LB2^T with flagella (B; bar 1 µm).

Fig. 2: Maximum-likelihood (ML) phylogenetic tree based on 1245 aligned bp of 16S rRNA gene sequences showing the position of strain LB2^T among the other family members *Proteinivoraceae* within the order *Clostridiales*. *Clostridium butyricum* (M59085) was used as an outgroup (not shown). Bootstrap values (based on 1000 replicates) are shown at branch nodes. Accession numbers are indicated in parentheses. Bar: 0.02 substitutions per 100 nucleotides.

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Table 1. Differential characteristics between strain LB2^T (obtained in this study) and data obtained from previous studies for all type species of all genera of the family *Proteinivoraceae.* Strains: 1, Strain LB2^T; 2, *Anaerobranca horikoshii* JWIYL-l3S^T [46]; 3, *Anaerobranca gottschalkii* LBS3^T [45]; 4, *Anaerobranca californiensis* PAOHA-1^T [47]; 5, *Anaerobranca zavarzinii* JW/VK-KS5Y^T [48]; 6, *Proteinivorax tanatarense* Z-910^T [44]; 7, *Proteinivorax hydrogeniformans* Z-710^T [49].

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Origin	La Crouen spring, New Caledonia	Yellowstone National Park, USA	Lake Bogoria, Kenya	Mono Lake, USA	Kamchatka, Russia	Lake Tanatar VI, Russia	Lake Tanatar III, Russia
Cell size (µm)	0.5–0.6 x 6–10	0.5–0.6 x 11–20	0.3–0.5 x 3–5	0.26–0.31 x 2.4–5.0	0.36–0.45 x 3.6–7.7	0.5 x 1.6–4	0.5–0.6 x 2.0–4.5
Spore formation	+	-	-	-	-	+	-
Motility	+	+	+	+	-	+/-	+
Temperature for growth (°C)							
Range	25–50	34–64	30–65	45–70	34–64	16–52	12–45
Optimum	37	57	50–55	58	54–60	32–38	30–32
pH for growth							
Range	8.2–10.8*	6.7–10.3	6.0–10.5	8.6–10.4	7.7–9.9	7.6–10.5	7.7–10.4
Optimum	9.5	8.5	9.5	9.0–9.5	8.5–9.0	8.8	8.3–8.9
NaCl for growth (%, w/v)							
Range	0–7	ND	0–4	0–6	ND	0.4–3.6	0.7–3.0
Optimum	0*–1	ND	1	1–2.5	ND	2–3	1.0–1.4
DNA G+C content (mol%)[1]	36.2[1*]	34[2]	30.9[2]	30[2]	32.5[2]	32.2[2]	33.9[2]
Substrates	Peptone, pyruvate, xylose, yeast extract	Peptone, tryptone, yeast extract	Peptone, tryptone, yeast extract, mono- and polysaccharides	Peptone, soytone, malt extract, Casamino acids	Soytone and yeast extract	Albumine, casein, gelatin, peptone, soytone, tryptone, yeast extract	Peptone, soytone, tryptone, yeast extract
Fermentation products	From yeast extract: acetate and propionate; from pyruvate: acetate and formate	From yeast extract: acetate, H_2, CO_2	From glucose or starch: Acetate, ethanol (traces)	ND	From YE: Formate, acetate, H2 (major), propionate (minor)	From tryptone: acetate, formate, NH_4, H_2, propionate, succinate, n-butyrate, iso-butyrate, 2-methylbutyrate, iso-valerate	From tryptone: acetate, hydrogen, ammonium (major), formate, propionate, n-butyrate, iso-butyrate, iso-valerate and succinate (minor).
Electron acceptors	Ferric citrate, thiosulfate	Fumarate	Thiosulfate	Sulfur, polysulfide, thiosulfate, ferric citrate	Fumarate, thiosulfate, sulfur	Fumarate, DMSO, thiosulfate, sulfur	Fumarate, thiosulfate, sulfur

+, Positive; -, negative; ND, not data available.
[1] G+C content determined from genomic data.
[2] G+C content determined by using HPLC.
* Characteristics distinguishing the strain LB2^T from others presented.

526 **Table 2**. Genome statistics of strain LB2^T, *Anaerobranca californiensis* PAOHA-1^T [47], and

527 *Anaerobranca gottschalkii* LBS3^T [45].

Genome Name / Sample Name	LB2 ^{T}	*Anaerobranca californiensis* PAOHA-1^T	*Anaerobranca gottschalkii* LBS3^T
GenBank accession number	CP058559	NZ_FRAI00000000	NZ_FOIF01000000
IMG Genome ID	2888992949	2585428191	2602042032
Genome Size (bp)	3,695,371	2,034,462	2,300,172
Contig count	NA	51	115
Scaffold count	1	NA	110
GC (%)	36.2	33.4	32.9
Gene Count	3,777	2,132	2,382
Protein count	3,626	2,006	2,246
rRNA Count	27	22	16
tRNA Count	62	52	55
Other RNA	5	5	4

528 NA, data not applicable.
529