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1 ***Alkalicella caledoniensis* gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium**
2 **isolated from ‘La Crouen’ alkaline thermal spring, New Caledonia**

3

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16

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18

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21

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26 **Abstract**

27

28 A novel anaerobic, alkaliphilic, mesophilic, Gram-stain-positive, endospore-forming bacterium
29 was isolated from an alkaline thermal spring (42°C, pH 9.0) in New Caledonia. This bacterium
30 designated strain LB2^T grew at 25–50°C (optimum 37°C) and pH 8.2–10.8 (optimum 9.5).
31 Added NaCl was not required for growth (optimum 0–1%) but was tolerated up to 7%. Strain
32 LB2^T utilized a limited range of substrates, such as peptone, pyruvate, yeast extract and xylose.
33 End products detected from pyruvate fermentation were acetate and formate. Both ferric citrate
34 and thiosulfate were used as electron acceptors. Elemental sulfur, nitrate, nitrite, fumarate,
35 sulfate, sulfite, and DMSO were not used as terminal electron acceptors. The two major cellular
36 fatty acids were iso-C15:0 and C16:0. The genome consists of a circular chromosome (3.7 Mb)
37 containing 3626 predicted protein-encoding genes with a G+C content of 36.2 mol%.
38 Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolate is a
39 member of the family *Proteinivoraceae*, order *Clostridiales* within the phylum *Firmicutes*.
40 Strain LB2^T was most closely related to the thermophilic *Anaerobranca gottschalkii* LBS3^T
41 (93.2% 16S rRNA gene sequence identity). Genome-based analysis of average nucleotide
42 identity (ANI) and digital DNA–DNA hybridization (dDDH) of strain LB2^T with *A.*
43 *gottschalkii* LBS3^T showed respective values of 70.8% and 13.4%. Based on phylogenetic,
44 genomic, chemotaxonomic, and physiological properties, strain LB2^T is proposed to represent
45 the first species of a novel genus, for which the name *Alkalicella caledoniensis* gen. nov., sp.
46 nov. is proposed (type strain LB2^T = DSM 100588^T = JCM 30958^T).

47

48

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

59 In New Caledonia (Southwest Pacific), several thermal springs are located in the southeastern
60 half of the main island [6] (see the map in [7]). Such springs are found down south in the Prony
61 Bay area and the area close to the city of Canala [7]. A sampling campaign was conducted in
62 November 2014 during which water and sediment samples were collected at one of the springs
63 of the Canala region, namely the spring feeding the abandoned spa facility of ‘La Crouen’ (165°
64 53' 20.6" E 21° 32' 06.6" S). On-site measurements showed that its waters are characterized by
65 high pH (9.0), low Eh value (-174 mV, ref H₂), low dissolved O₂ (0.71 mg/L), low conductivity
66 (314 μS/cm), and moderate temperature (42°C) (data from our study). Deville and Prinzhofer
67 [8] reported that the gas emitted at the springs is dinitrogen (N₂) with traces of methane (CH₄
68 <3%), but we did not observe degassing in the spring during our visit. Our dissolved gases
69 analyses showed that N₂ is dominant, followed by O₂, but no CH₄ or hydrogen (H₂) was
70 detected. Both the geological setting and geochemistry differ from those of the well-known
71 hyperalkaline springs located in the peridotite nappe in the Prony Bay Hydrothermal Field
72 (PBHF). These on-shore or submarine springs discharge a warm (<40°C), highly reduced, high-
73 pH (up to 11.2) fluid enriched in N₂, H₂, and CH₄ [9], produced by the serpentinization process

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

81 For culturing experiments, a sterile glass bottle was filled with sediment slurry sample,
82 then hermetically sealed to preserve anoxic conditions, and stored at 4°C. In the laboratory,
83 anaerobic enrichment cultures were carried out at 37°C using 0.5 mL of the slurry sample
84 (corresponding to 10% inoculum) in Hungate tubes containing 5 mL of nutrient-rich LB (Luria-
85 Bertani) medium (i.e., 10g/L Tryptone, 5 g/L yeast extract, and 5 g/L NaCl), supplemented with
86 25 mM Tris-Base. Before inoculation, the culture medium was adjusted to pH 9.5, boiled for 5
87 min, and then cooled to room temperature under a flow of O₂-free N₂ gas. The medium was
88 dispensed into Hungate tubes, degassed under a flow of N₂, and subsequently autoclaved (45
89 min, 120°C). The following sterile solutions were injected in each tube: 0.1 mL of 2%
90 Na₂S·9H₂O (reducing agent) and 0.5 mL of 8% Na₂CO₃ (to adjust and buffer the final pH to
91 9.5). The Hungate technique for anaerobic cultivation was used throughout this study [22].

92 Temperature, pH, and NaCl concentration ranges (respectively tested: 20–70°C, 6.0–12.0, 0–
93 10%) for growth of the isolate were determined in duplicate using Hungate tubes containing
94 LB medium, supplemented with 25 mM Tris-Base (except for testing the optimal pH for
95 growth, which was determined using different buffer solutions: 0 mM Tris-Base for pH 6.0–
96 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
97 media was adjusted with anaerobic sterile stock solutions of 8% Na₂CO₃ or 1M NaOH. The pH
98 was always checked both after inoculation and at the end of incubation. Water baths were used

99 for incubation at temperatures up to 70°C. For studies of NaCl requirements, NaCl was weighed
100 and added directly into the tubes before culture medium addition. Each test culture was sub-
101 cultured at least once under the same experimental conditions before determining growth rates.
102 A range of inorganic and organic substrates was tested as substrate for the isolated growth in
103 the basal medium containing (per liter of distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1 g
104 NH₄Cl, 1 g NaCl, 0.1 g KCl, 0.5 g cysteine.HCl, and 10 mL of trace elements solution [23].
105 The pH was adjusted to 9.5 with a 1M NaOH solution. The following compounds were tested
106 as potential substrates (at a final concentration of 20 mM for simple substrates and 2g/L for
107 complex substrates): adenine, adenosine 5'-diphosphate, albumin, arabinose, casamino-acids,
108 casein, cellobiose, fructose, galactose, gelatin, glucose, glycerol, guanine, lactate, lactose,
109 mannose, peptone, pyruvate, ribose, starch, sucrose, tryptone, uracil, xylose, yeast extract,
110 H₂/CO₂ (80/20, v/v) and H₂/CO₂ (80/20, v/v) in the presence of acetate (2 mM) as carbon
111 source. Elemental sulfur (1% w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM),
112 nitrate (10 mM), nitrite (2 mM), fumarate (20 mM), FeIII-citrate (10 mM), and DMSO (10 mM)
113 were tested as terminal electron acceptors using pyruvate as electron donor. Sensitivity to
114 antibiotics (ampicillin, bacitracin, chloramphenicol, cycloserine, kanamycin, metronidazole,
115 novobiocin, penicillin, rifampicin, streptomycin, tetracycline, vancomycin) was tested by
116 adding them separately to the LB medium (supplemented with 25 mM Tris-Base) at a final
117 concentration of 25 µg/mL or 50 µg/mL. Each test and control, carried out in parallel, was run
118 in duplicate Hungate tubes. Controls were (i) inoculated tubes without tested compounds and
119 (ii) uninoculated tubes with tested compounds. Bacterial growth was monitored by measuring
120 the turbidity increase at 600 nm by inserting Hungate tubes into the tube holder of a
121 spectrophotometer (Cary 50, Varian). H₂S production was measured photometrically as
122 colloidal CuS, as described by Cord-Ruwisch [24]. End-products of metabolism were measured

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

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

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

135 Near-complete 16S rRNA genes were amplified from genomic DNA extracted as described
136 by Quéméneur *et al.* [28], using the primers 27F and 1492R [29], then sequenced by GATC-
137 Biotech (Konstanz, Germany). The 16S rRNA gene sequence was deposited and aligned with
138 available sequences from NCBI database using the MUSCLE program [30] implemented in
139 MEGA7 software [31]. Evolutionary analyses were conducted from 16 nucleotide sequences
140 using three different methods: maximum-likelihood (ML) [32], maximum-parsimony (MP)
141 [33], and neighbor-joining (NJ) [34].

142 Whole-genome analysis was performed from high molecular weight DNA of the isolate,
143 extracted using a phenol-chloroform method, then sequenced at the GeT-PlaGe platform
144 (INRAe Toulouse, France) by combining long reads (Oxford Nanopore) and short-reads
145 (Illumina MiSeq) technologies, as described by Quéméneur *et al.* [25] with some modifications
146 because it was sequenced by multiplex sequencing according to the manufacturer's instructions
147 "1D Native barcoding genomic DNA" (EXP-NBD104 and SQK-LSK109)". Base-calling was

148 carried out by MinKNOW-Live-Base-calling software version 3.6.0 (Oxford Nanopore
149 Technologies, Oxford, UK). Read demultiplexing and adapter trimming were performed using
150 qcat v1.1.0 (<https://github.com/nanoporetech/qcat>) and genome assembly with Unicycler v0.4.6
151 [35]. The genome sequence was deposited in the NCBI database and annotated using the MaGe
152 (Microscope Genome Annotation) [36] and the Integrated Microbial Genomes (IMG) systems
153 [37]. The value of average nucleotide identity (ANI) was calculated using OrthoANI with
154 usearch (<http://www.ezbiocloud.net/tools/ani>). Digital (or *in silico*) DNA-DNA hybridization
155 (dDDH) was calculated using the Genome-to-Genome Distance Calculator (GGDC) 2.1
156 (<http://ggdc.dsmz.de/>) [38]. A whole genome-based taxonomic analysis was performed using
157 the bioinformatics platform Type (Strain) Genome Server (TYGS) (<https://tygs.dsmz.de>) [39].
158 In brief, the TYGS analysis included a determination of the closest type strain genomes by (i)
159 comparison against all type strain genomes available in the TYGS database *via* the MASH
160 algorithm [40] and (ii) the closely related type strains determined *via* the 16S rDNA gene
161 sequences. For the phylogenomic inference, all pairwise comparisons among the genomes were
162 conducted using Genome BLAST Distance Phylogeny (GBDP) and accurate intergenomic
163 distances inferred under the algorithm 'trimming' and distance formula d5. The resulting
164 intergenomic distances were used to infer a balanced minimum evolution tree with branch
165 support *via* FASTME 2.1.6.1 [41]. Branch support was inferred from 100 pseudo-bootstrap
166 replicates each. The trees were rooted at the midpoint [42].

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

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

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

180 Cells of strain LB2^T stain Gram-positive are motile and endospore-forming bacterium (Fig.
181 1A). Endospores are terminal and oval (0.8–1 x 1.0–1.5 μm). Rods are approximately 6.0–10.0
182 μm long and 0.5–0.6 μm wide. They occur singly or in pairs and possessed peritrichous flagella
183 (Fig. 1B). They are pleiomorphic, i.e., rod-shaped during the exponential growth phase (Fig.
184 S1A), they turn coccoid in the stationary phase (after 24h, Fig. S1B).

185 Strain LB2^T is mesophilic and grows only under anaerobic conditions, ranging from 25°C
186 to 50°C (Table 1). The optimum temperature for growth is 37°C. The pH range for growth is
187 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
188 growth, and the NaCl range for growth is 0–7% (optimum at 0–1%) but is tolerated up to 7%,
189 indicating that strain LB2^T is halotolerant and adapted to the low-salinity and high-pH fluid
190 emitted by the alkaline spring of ‘La Crouen’ (New Caledonia).

191 Peptone, pyruvate, xylose, and yeast extract were the only substrates used for growth
192 (Table 1). Yeast extract was degraded to acetate, formate, H₂, and propionate. Growth on yeast
193 extract was improved by the addition of thiosulfate used as a terminal electron acceptor, reduced
194 to sulfide. End products detected from pyruvate fermentation were acetate and formate. Both
195 ferric citrate and thiosulfate were used as electron acceptors using pyruvate at pH 9.5. Elemental
196 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-13S^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

222 originated from low-salt alkaline ecosystems: anaerobic fermentation reactor with waste
223 activated sludge under mesophilic condition (pH 9.0; GU455162) [51] and surface
224 rock/sediment of Lonar Crater Lake (pH 8.9–10.0; JQ738949) [52]. 16S rRNA genes affiliated
225 to the family *Proteinivoraceae* (formerly known as the family *Anaerobranaceae*) were also
226 detected in the subsurface hyperalkaline fluids of the Semail Ophiolite (Oman) [53] and other
227 oligotrophic, rock-hosted ecosystems, such as deep crystalline aquifer of Outokumpu (Finland)
228 [54]. Still, their potential functional roles in the subsurface remain unclear.

229 The complete genome sequence of strain LB2^T is a unique circular chromosome of 3.7 Mb
230 long with a 36.2 mol% G+C content (Table 2), close to the G+C content measured by HPLC
231 (36.4 mol%, Table 2). The G+C content of strain LB2^T is higher than that of its closest
232 *Anaerobranca* relatives (*A. californiensis*, 30.0–33.4 mol%; *A. gottschalkii*, 30.0–32.9 mol%;
233 *A. horikoshii*, 32.0–34.0 mol%; *A. zavarzini*, 32.5 mol%), and *P. tanatarense* (31.9–32.2
234 mol%) within the family *Proteinivoraceae*. The genome size of strain LB2^T is much larger than
235 that of its closest phylogenetic neighbor for which genomes are available, *A. californiensis*
236 (2.0 Mb) and *A. gottschalkii* (2.3 Mb), but is similar to those of the mesophilic and alkaliphilic
237 genus *Alkaliphilus* (>3 Mb). The ANI and dDDH values between LB2^T and *A. californiensis*
238 PAOHA-1^T were 70.61% and 13.50%. The ANI and dDDH values between LB2^T and *A.*
239 *gottschalkii* LBS3^T were 70.78% and 13.40% (Supplementary Table S1), i.e., far below the
240 species boundaries of 95~96% and 70%, respectively, recommended by Chun *et al.* [55]. The
241 LB2^T strain genome comprises a single chromosome and 3,777 genes, including 3,626 protein-
242 encoding genes and 94 RNA encoding genes. Amino acids, carbohydrates, and inorganic ion
243 transport and metabolisms represented 7.4%, 5.7%, and 4.9% of the function-predicted genes.
244 The distribution of genes into COGs functional categories is shown in Supplementary Table
245 S2. In a phylogenomic analysis (Fig S3), strain LB2 clustered with other alkaliphilic *Firmicutes*.

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

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

252 Table 1 summarizes the physiological and biochemical differences between strain LB2^T
253 and the type species of all genera of the family *Proteinivoraceae*, including *A. horikoshii* and
254 *P. tanatarense*. Strain LB2^T shares similar physiological and biochemical properties with this
255 latter (e.g., proteinaceous substrate utilization and high-pH optimal growth ranging between 8.5
256 and 9.5) but differs by growing optimally higher pH (up to 10.8) compared to other
257 *Proteinivoraceae* members. Unlike *Anaerobranca* and *Proteinivorax* genera, strain LB2^T
258 displays optimal growth in the absence of NaCl addition, similarly to another alkaliphilic
259 anaerobe of the family *Clostridiaceae* *Serpentinicella alkaliphila* 3b^T, isolated from the
260 serpentinizing Prony Bay Hydrothermal Field of New Caledonia [20]. In contrast to the
261 alkalithermophilic *Anaerobranca* spp., displaying optimal growth temperatures between 50–
262 60°C, strain LB2^T was mesophilic (the optimum temperature at 37°C) similarly to *P.*
263 *tanatarense* and *P. hydrogeniformans* that grew optimally at 32–38°C and 30–32°C,
264 respectively [44, 49]. Like other *Proteinivoraceae* members, strain LB2^T utilizes proteinaceous
265 substrates for growth (i.e., yeast extract). It can also use pyruvate as the sole carbon source,
266 similarly to *A. californiensis* (or *A. zavarzinii* that co-metabolizes pyruvate with yeast extract).
267 As reported for *A. zavarzinii*, both formate and acetate were the major end products detected. It
268 suggests that pyruvate is mainly metabolized *via* pyruvate:formate lyase and not *via* the
269 pyruvate:ferredoxin oxidoreductase as commonly reported in *Firmicutes*. In the latter case,
270 acetate plus H₂ would be the major metabolic end-products. Genomic data supported this

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

278 In conclusion, the strain LB2^T differs from other species of the closest genera
279 *Anaerobranca* and *Proteinivorax* (belonging to the family *Proteinivoraceae*) by several
280 features: (i) it forms a distinct branch among the family *Proteinivoraceae* in the 16S rRNA gene
281 phylogenetic trees, (ii) it is only distantly related to *Anaerobranca* spp. in phylogenomic
282 analyses, (iii) it displays a larger genome size and G+C content than other *Proteinivoraceae*
283 members, (iv) it grows optimally at higher pH (up to 10.8) than other *Proteinivoraceae*
284 members, (v) it grows optimally in the absence of NaCl addition, (vi) it has a much lower
285 optimal growth temperature (37°C) than the closest *Anaerobranca* members, (vii) it is the only
286 one in the family *Proteinivoraceae* (including pleomorphic bacteria) occurring as rods or cocci
287 depending on the growth phase, as observed for the actinobacterial genus *Arthrobacter* [56]. In
288 contrast, cells of the genus *Anaerobranca* are rod-shaped, sometimes showing branching [46,
289 48]. Based on morphological, phenotypic, phylogenetic, and genomic, strain LB2^T is proposed
290 to represent a novel species of the new genus *Alkalicella*, *A. caledoniensis* gen. nov., sp. nov.

291

292 Description of *Alkalicella* gen. nov.

293

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

296 environment).

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

306

307 Description of *Alkalicella caledoniensis* sp. nov.

308

309 *Alkalicella caledoniensis* (ca.le.do.ni.en'sis. N.L. fem. adj. *caledoniensis* pertaining to New
310 Caledonia, where strain LB2^T was first isolated).

311 Displays the following characteristics in addition to those listed in the genus description. Cells
312 are approximately 6.0–10.0 µm long and 0.5–0.6 µm wide, occurring singly or in pairs. Cells
313 are motile and possess multiple flagella. They are pleiomorphic depending on the growth phases
314 (from rod to cocci). Colonies are white and circular and about 1–2 mm in diameter after seven
315 days of incubation at 30°C. Growth occurs at 25–50°C (optimum 37°C), at pH 8.2–10.8
316 (optimum pH 9.5) with 0–7% NaCl (optimum 0–1%). Added NaCl was not required for growth.
317 Pyruvate, yeast extract, xylose, and peptone were used as electron donors. Still, adenine,
318 adenosine 5'-diphosphate, albumin, arabinose, casamino-acids, casein, cellobiose, fructose,
319 galactose, gelatin, glucose, glycerol, guanine, lactate, lactose, mannose, ribose, starch, sucrose,
320 tryptone, uracil, and H₂/CO₂ were not. End products detected from pyruvate fermentation were

321 acetate and formate. Both ferric citrate and thiosulfate were used as electron acceptors using
322 pyruvate and reduced to ferrous iron and sulfide. Yeast extract was degraded into acetate,
323 formate, H₂, and propionate. Elemental sulfur, nitrate, nitrite, fumarate, sulfate, sulfite, and
324 DMSO were not used as terminal electron acceptors. The predominant fatty acids were iso-
325 C15:0 (15.3%), C16:0 (10.2%), C14:0 (8.0%), cyclo-C17:0 (6.9%), iso-C17:1 (8.8%), and
326 C18:0 (6.1%). The GenBank accession number for the 16S rRNA gene sequence of strain LB2^T
327 is KR349724, and that for the whole genome sequence is CP058559.

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

330

331 **Conflicts of interest**

333 The authors declare that there are no conflicts of interest.

334

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342

343

344 **Figures legends**

345

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

349

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

356

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518 **Table 1.** Differential characteristics between strain LB2^T (obtained in this study) and data obtained from previous studies for all type species of all
 519 genera of the family *Proteinivoraceae*. Strains: 1, Strain LB2^T; 2, *Anaerobranca horikoshii* JWIYL-13S^T [46]; 3, *Anaerobranca gottschalkii* LBS3^T
 520 [45]; 4, *Anaerobranca californiensis* PAOHA-1^T [47]; 5, *Anaerobranca zavarzinii* JW/VK-KS5Y^T [48]; 6, *Proteinivorax tanatarense* Z-910^T [44];
 521 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, H ₂ (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

522 +, Positive; -, negative; ND, not data available.

523 ¹ G+C content determined from genomic data.

524 ² G+C content determined by using HPLC.

525 * 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	<i>Anaerobranca californiensis</i> PAOHA-1 ^T	<i>Anaerobranca gottschalkii</i> 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

NA, data not applicable.

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529