Serpentinicella alkaliphila gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from the serpentinite-hosted Prony hydrothermal field, New Caledonia.

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A novel anaerobic, alkaliphilic, Gram-stain-positive, spore-forming bacterium was isolated from a carbonaceous hydrothermal chimney in Prony Bay, New Caledonia. This bacterium, designated strain 3b[T], grew at temperatures from 30 to 43 °C (optimum 37 °C) and at pH between 7.8 and 10.1 (optimum 9.5). Added NaCl was not required for growth (optimum 0–0.2 %, w/v), but was tolerated at up to 4 %. Yeast extract was required for growth. Strain 3b[T] utilized crotonate, lactate and pyruvate, but not sugars. Crotonate was dismutated to acetate and butyrate. Lactate was disproportionated to acetate and propionate. Pyruvate was degraded to acetate plus trace amounts of hydrogen. Growth on lactate was improved by the addition of fumarate, which was used as an electron acceptor and converted to succinate. Sulfate, thiosulfate, elemental sulfur, sulfite, nitrate, nitrite, $FeCl_3$, Fe(III)-citrate, Fe(III)-EDTA, chromate, arsenate, selenate and DMSO were not used as terminal electron acceptors. The G+C content of the genomic DNA was 33.2 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolate is a member of the family *Clostridiaceae*, order *Clostridiales* within the phylum *Firmicutes*. Strain 3b[T] was most closely related to '*Alkaliphilus hydrothermalis*' FatMR1[T] (92.2 % 16S rRNA gene sequence similarity), and was positioned approximately equidistantly between the genera *Alkaliphilus*, *Anaerovirgula* and *Natronincola*. On the basis of phylogenetic, genetic, chemotaxonomic and physiological properties, strain 3b[T] is proposed to represent a novel species of a new genus, for which the name *Serpentinicella alkaliphila* gen. nov., sp. nov. is proposed. The type strain of *Serpentinicella alkaliphila* is 3b[T] (=DSM 100013[T]=JCM 30645[T]).

The hydrothermal field of Prony Bay (PHF; New Caledonia, South Pacific) comprises several intertidal and shallow submarine hyperalkaline springs located at less than 50 m below sea level (mbsl). Similarly to the famous deep-sea Lost City hydrothermal field (LCHF, located at ~800 mbsl, off the Mid-Atlantic Ridge, 30°N), PHF discharges into the seawater high-pH (~11) fluids enriched in hydrogen (H_2) and methane (CH_4) whose production results from serpentinization reactions (Kelley *et al.*, 2005; Monnin *et al.*, 2014). In contact with seawater, minerals emitted by these hyperalkaline fluids precipitate to form chimney structures tens of metres high mainly composed of carbonates ($CaCO_3$, $MgCO_3$) and brucite [$Mg(OH)_2$] (Launay & Fontes, 1985). The PHF and LCHF display similar geochemical and mineralogical features, but PHF notably differs from LCHF by the low temperature range (<40 °C) and low salinity of the fluids discharged in a shallow submarine environment.

The PHF ecosystem has only recently been investigated for microbial diversity (Postec *et al.*, 2015; Quéméneur *et al.*, 2014). Archaeal diversity was much lower than bacterial diversity and was dominated by uncultured *Methanosarcinales*. Bacterial communities were mainly composed of

Chloroflexi, *Deinococcus thermus*, *Firmicutes* and *Proteobacteria*. Several anaerobic bacteria belonging to the phylum *Firmicutes*, order *Clostridiales*, have been successfully isolated from submarine chimneys of the PHF (Postec *et al.*, 2015; Mei *et al.*, 2016). They include members of the genera *Acetoanaerobium*, *Alkaliphilus*, *Clostridium* and *Vallitalea* (Ben Aissa *et al.*, 2014, 2015; Mei *et al.*, 2014; Bes *et al.*, 2015).

This study reports on the isolation and characterization of a novel anaerobic, alkaliphilic bacterium isolated from an intertidal spring of the PHF, and this bacterium has phenotypic and genotypic traits that allow the proposition of a novel species within a new genus belonging to the family *Clostridiaceae*, phylum *Firmicutes*.

A small carbonate chimney (~10 cm in height and ~3 cm in diameter) was collected in October 2012 at the hydrothermal site 'Rivière des Kaoris' (RK) of the PHF (New Caledonia). The intertidal RK site, located at the eastern end of Carenage Bay within Prony Bay (22° 17.969′ S 166° 51.709′ E), consisted of a multitude of small vents and centimetre-scale chimneys discharging high-pH (~10.8), H_2-rich fluids at moderate temperature (~30 °C). In the laboratory (Centre IRD de Nouméa), chimney subsamples were crushed, transferred to a hermetically sealed serum bottle with a nitrogen (N_2) gas headspace and stored overnight at 4 °C until culturing attempts.

Slurry materials (0.5 g) from the chimneys were transferred to Hungate tubes containing 5 ml of culture medium that were subsequently incubated at 37 °C. The basal culture medium contained (per litre of distilled water): 0.1 g KH_2PO_4, 0.1 g K_2HPO_4, 0.1 g NH_4Cl, 2 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 0.1 g cysteine hydrochloride and 10 ml trace mineral element solution (Balch *et al.*, 1979). The initial pH was adjusted to 9.5 with NaOH. The medium was boiled for 5 min, and then cooled under a flow of O_2-free N_2 gas to room temperature. The medium was dispensed into Hungate tubes, degassed under a flow of N_2/CO_2 (80 : 20, v/v) and subsequently autoclaved (45 min, 120 °C). Prior to inoculation, the following sterile solutions were injected into each tube: 0.1 ml of 2 % $Na_2S.9H_2O$ (reducing agent) and 0.1 ml of 8 % Na_2CO_3 (to adjust and buffer the final pH to 9.5); biotrypcase (2 g l^{-1}), yeast extract (2 g l^{-1}) and glucose (2 g l^{-1}) were used as substrates. This final medium was referred as BYG medium. The Hungate technique for anaerobic cultivation was used throughout this study (Hungate, 1969).

Growth experiments were performed in duplicate using Hungate tubes containing basal culture medium. Temperature, pH and NaCl concentration ranges (respectively: 10–60 °C, 6–12, 0–60 g l^{-1}) for growth were determined using basal culture medium supplemented with yeast extract (2 g l^{-1}) and lactate at 20 mM as the electron donor. Three buffers were used to test the optimal pH for growth: (i) 10 mM PIPES for pH 6–6.5, (ii) 10 mM HEPES for pH 6.6–8.5 and (iii) 20 mM CAPS for pH 9–12. The pH of the culture media was adjusted with anaerobic sterile stock solutions of

8 % Na_2CO_3 or 1 M NaOH and the pH was always checked both after inoculation and at the end of incubation. Water baths were used for incubation at temperatures up to 60 °C. For studies of NaCl requirements, NaCl was weighed and added directly into the tubes prior to addition of culture medium. Each test-culture was sub-cultured at least once under the same experimental conditions prior to determination of growth rates. The following substrates were tested at a concentration of 20 mM in the basal medium containing 1 g yeast extract l^{-1}: acetate, lactate, glucose, fructose, pyruvate, glycerol, ribose, galactose, maltose, xylose, peptone, lactose, mannose, raffinose, sucrose, arabinose, cellobiose, rhamnose, formate, fumarate, crotonate, ethanol, Casamino acids, biotrypticase, 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 (without addition of yeast extract). Elemental sulfur (1 %, w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM), nitrite (2 mM), fumarate (20 mM), $FeCl_3$ (10 mM), Fe(III)-citrate (10 mM), Fe(III)-EDTA (10 mM), arsenate (10 mM), selenate (10 mM), chromate (10 mM) and DMSO (10 mM) were tested as terminal electron acceptors.

Bacterial growth was monitored by measuring the increase in turbidity at 600 nm by insertion of Hungate tubes into the tube-holder of a spectrophotometer (Cary 50; Varian). H_2S production was measured photometrically as colloidal CuS following the method described by Cord-Ruwisch (1985). End-products of metabolism were measured by HPLC and GC after 1 week of incubation at 37 °C (Mei *et al.*, 2014).

The presence of spores was examined by phase-contrast microscopic observations of cultures. Gram coloration staining of the cell wall was performed with heat-fixed liquid cultures with the Difco kit reagents. Cellular morphology and purity of the strain 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 observe the cell wall), as previously described (Fardeau *et al.*, 1997).

The G+C content of the DNA was determined at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977), and the G+C content was determined by using HPLC as described by Mesbah *et al.* (1989). Cellular fatty acids were measured (after 3 days of incubation at 37 °C using lactate as an energy source) by the Identification Service of the DSMZ with a modified extraction protocol (Kuykendall *et al.*, 1988) of the method of Miller (1982). Fatty acids were identified using the MIDI Microbial Identification system (version 4.0, MIS operating manual March 2001) (Sasser, 1990).
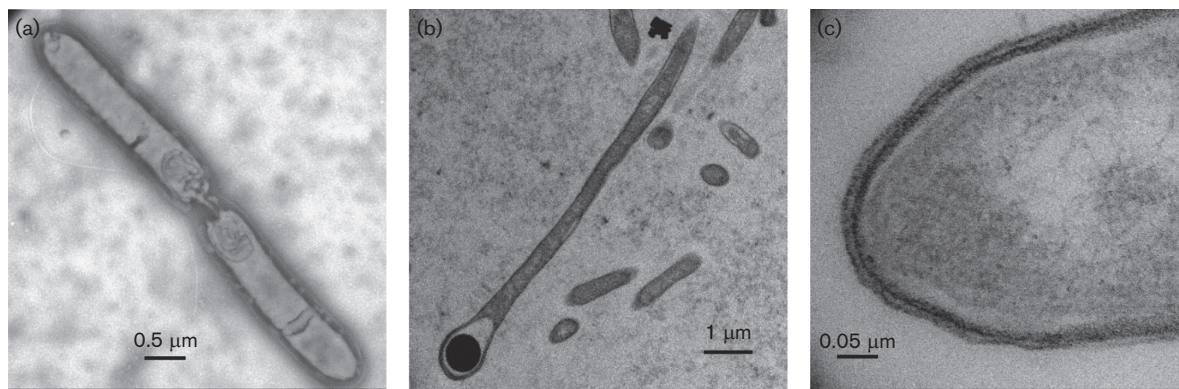
Fig. 1. Transmission electron micrographs showing cells of strain 3b^T with (a) flagella (bar, 0.5 μm), and (b) terminal spore formation (bar, 1 μm). (c) Thin-section electron micrograph of strain 3b^T showing the Gram-positive type of cell wall (bar, 0.05 μm).

PCR amplification of the 16S rRNA genes was carried out using as template genomic DNA prepared as described by Quéméneur *et al.* (2015) and primers 27F and 1492R (Lane, 1991). Sanger sequencing of the PCR product was performed by GATC-Biotech (Konstanz, Germany) using primers 27F and 1492R. The 16S rRNA gene sequence was then compared with available sequences in the GenBank database using the BLAST N search program (Altschul *et al.*, 1997). A multiple alignment was built using the MUSCLE program (Edgar, 2004) implemented in MEGA 6 software (Tamura *et al.*, 2013). Sequence positions with alignment uncertainty and gaps were omitted from the analysis. Evolutionary analyses were conducted in MEGA 6 using the neighbour-joining and maximum-likelihood methods, which gave similar tree topologies. For the neighbour-joining method, evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). The analysis involved 18 nucleotide sequences. There were a total of 1312 positions in the final dataset. Branch robustness of the resulting neighbour-joining tree was estimated by the non-parametric bootstrap procedure implemented in MEGA 6 (1000 replicates of the original dataset). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

An enrichment culture of an RK chimney sample showed significant growth on BYG medium after 1 week of incubation at 37 °C and at pH 9.5. This enrichment was then sub-cultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted 10-fold in roll-tubes. Several colonies that developed were picked separately. The colonies grown in roll-tubes were faint yellow and circular. They were 1–2 mm in diameter after 1 week of incubation at 37 °C. Several strains were isolated and were shown to use only yeast extract, but not glucose in the enrichment culture medium. Among them, strains 1ac and 3b^T were found to be phylogenetically closely related based on their 16S rRNA gene sequences (99.2 % similarity) and assacharolytic, growing optimally at pH 9.5 and 37 °C. Strain 3b^T was selected and used for further physiological and metabolic characterization.

Cells of strain 3b^T were Gram-stain-positive, motile, terminally spore-forming rods (1–10 μm long and 0.4–0.6 μm wide). They possessed peritrichous flagella and occurred singly or in pairs (Fig. 1).

Strain 3b^T was mesophilic and grew only under anaerobic conditions at temperatures ranging from 30 to 43 °C (Fig. 2). Optimum temperature for growth was 37 °C. The pH range for growth was 7.8–10.1, with an optimum at pH 9.5. NaCl was not required for growth (optimum at 0–
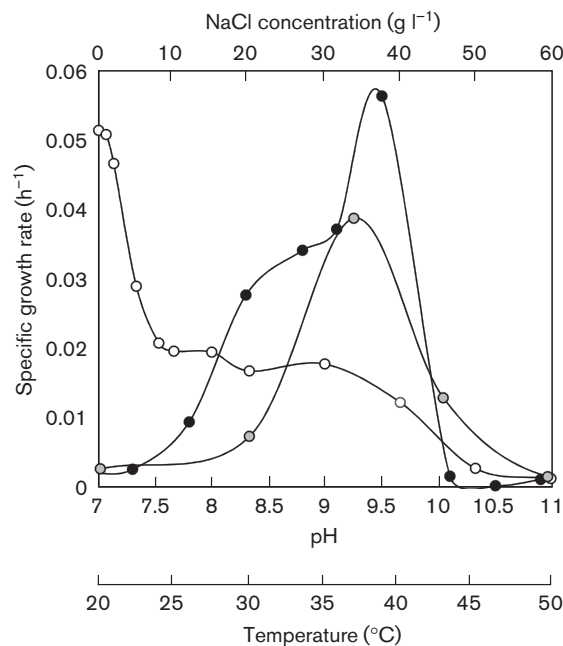

Fig. 2. Influence of temperature (◐), pH (●) and NaCl concentration (○) on growth of strain 3b^T. Growth was determined in culture basal medium supplemented with yeast extract and lactate at pH 9.5. No growth was observed below pH 7.8 or above pH 10, at temperatures below 30 °C or above 43 °C, or at NaCl concentrations above 40 g l^{-1}.

0.2 %, w/v), and was tolerated at up to 4 %, thus clearly indicating that strain 3b[T], isolated from an intertidal hydrothermal spring, was more adapted to the low-salinity and high-pH fluid emitted by hydrothermal chimneys than to the higher salinity of the surrounding seawater.

The only substrates utilized for growth were lactate, pyruvate and crotonate (Table 1). The isolate required yeast extract for growth. Lactate was disproportionated to acetate and propionate. Pyruvate was metabolized to acetate and hydrogen. Crotonate was dismutated to acetate and butyrate. Growth on lactate was significantly improved by the addition of fumarate used as a terminal electron acceptor, with succinate production resulting from its reduction. Sulfate, thiosulfate, sulfite, nitrate, nitrite, FeCl$_3$, Fe(III)-citrate, Fe(III)-EDTA, arsenate, selenate, DMSO and chromate were not used as terminal electron acceptors, using lactate as an electron donor. Elemental sulfur (1 %, w/v), nitrite (2 mM), FeCl$_3$ (10 mM), Fe(III)-EDTA (10 mM), selenite (10 mM) and chromate (10 mM) inhibited growth on lactate. Thus, the ability of strain 3b[T] to use only a small number of organic acids suggests that it represents a secondary anaerobe using dissipated products of primary anaerobes inhabiting this extreme environment.

Phylogenetic analysis of the almost full-length 16S rRNA gene sequence (1417 nt) indicated that strain 3b[T] was a member of the family *Clostridiaceae*, phylum *Firmicutes* (Fig. 3). Strain 3b[T] formed a distinct branch among alkaliphilic and mesophilic clostridia of this family and was positioned approximately at equidistance between the genera *Alkaliphilus*, *Natronincola*, *Anaerovirgula* and *Clostridium*

Table 1. Differential characteristics between strain 3b[T] and its closest relatives

Strains: 1, 3b[T]; 2, 'Alkaliphilus hydrothermalis' FatMR1[T] (Ben Aissa et al., 2015); 3, *Alkaliphilus transvaalensis* SAGM1[T] (Takai et al., 2001); 4, *Natronincola histidinovorans* Z-7940[T] (Zhilina et al., 1998); 5, *Anaerovirgula multivorans* SCA[T] (Pikuta et al., 2006). +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5
Origin	Prony hydrothermal field, New Caledonia	Prony hydrothermal field, New Caledonia	Gold mine, South Africa	Magadi soda lake, Kenya	Owens Lake, CA, USA
Spore formation	+	−	+	+/−	+
Motility	+	+	+	+	+
Temperature for growth (°C)					
Range	30–43	20–55	20–50	37–45	10–45
Optimum	37	37	40	37-40	35
pH for growth					
Range	7.8–10.1	7.5–10.5	8.5–12.5	8–10.5	6.7–10.0
Optimum	9.5	9.0	10.0	9.4	8.5
NaCl for growth (%, w/v)					
Range	0–4.0	0–3	0–3.3	4–16	0.5–9.0
Optimum	0	0.2–0.5	0.5	8–10	2
DNA G+C content (mol%)*	33.2	37.1	36.4	31.9	34.2
Electron acceptors:					
Elemental sulfur	−	−	+	ND	−†
Thiosulfate	−	−	+	ND	−†
Fumarate	+	−	+	+	−†
Nitrate	−	−	ND	−	−
DMSO	−	ND	−	ND	−†
Ferric iron	−	ND	−	ND	−†
Electron donors:					
Lactate	+	−	−	−	+
Yeast extract	+	+	+	+	+
Peptone	−	+	+	+	+
Biotrypticase	−	+	+	ND	ND
Casamino acids	−	−	−	+	+
Fructose	−	+	−	−	+
Pyruvate	+	+	−	+/−	+
Crotonate disproportionation	+	+	+	ND	ND

*The G+C content was determined by using HPLC.
†Personal communication from the authors.

(91.5–92.2 % 16S rRNA gene sequence similarity) (Fig. 3). The closest relatives to strain 3b^T were 'Alkaliphilus hydrothermalis' FatMR1^T (92.2 % 16S rRNA gene sequence identity) isolated from another PHF submarine hydrothermal chimney (Ben Aissa et al., 2015), Anaerovirgula multivorans SCA^T (91.5 %) isolated from black mud sediments of a soda lake in California, USA (Pikuta et al., 2006), and Natronoincola histidinovorans Z-7940^T (91.5 %), isolated from soda deposits in Lake Magadi, Kenya (Zhilina et al., 1998).

The G+C content of the genomic DNA of strain 3b^T was 33.2 mol%. This is lower than that of its closest Alkaliphilus relative, 'A. hydrothermalis' (37.1 mol%), but is between those of Anaerovirgula multivorans SCA^T and N. histidinovorans Z-7940^T (34.2 and 31.9 mol%, respectively). The predominant cellular fatty acids were C$_{16:1}\omega$7c/C$_{16:1}\omega$6c (31.7 %), C$_{16:0}$ (21.8 %), C$_{14:0}$ (16.0 %) and iso-C$_{17:1}$/anteiso-C$_{17:1}$ (12.9 %). The proportion of C$_{14:0}$ was lower than that of 'A. hydrothermalis' while the proportion of C$_{16:0}$ was higher than that of the latter bacterium (Table S1, available in the online Supplementary Material).

Table 1 summarizes the physiological and biochemical differences between strain 3b^T and other closely related alkaliphiles within the family Clostridiaceae, including A. lkaliphilus hydrothermalis, Anaerovirgula multivorans and N. histidinovorans. Despite strain 3b^T sharing similar physiological properties (e.g. low salt and high pH requirements)

with Alkaliphilus species, it is, to our knowledge, the first highly alkaliphilic anaerobe to display optimal growth in the absence of added NaCl. Consequently, this isolate can be considered as an indigenous micro-organism of serpentinite-hosted hydrothermal vents, and therefore endemic, as was previously recently proposed for Alkaliphilus hydrothermalis, the first alkaliphilic anaerobe isolated from such an ecosystem (Ben Aissa et al., 2015). In contrast to Alkaliphilus hydrothermalis and Anaerovirgula multivorans SCA^T, strain 3b^T was assacharolytic and grew optimally at higher pH (up to 10.1). This inability to ferment sugars is shared with other alkaliphilic bacteria, such as Alkaliphilus transvaalensis SAGM1^T (Takai et al., 2001) and N. histidinovorans Z-7940^T. Unlike Alkaliphilus transvaalensis, strain 3b^T can use organic acids as substrates, but cannot use sulfur compounds as terminal electron acceptors. Among these alkaliphilic bacteria, the production of propionate as the major end product of fermentation was specific to strain 3b^T. Therefore, on the basis of phenotypic, phylogenetic, genetic and chemotaxonomic features, strain 3b^T is proposed to represent a novel species of a new genus, for which the name Serpentinicella alkaliphila gen. nov., sp. nov. is proposed.

Description of Serpentinicella gen. nov.

Serpentinicella (Ser.pen.ti.ni.cel′la. N.L. neut. n. serpentinum serpentine; L. fem. n. cella a cell; N.L. fem. n. Serpentinicella a cell from serpentine).
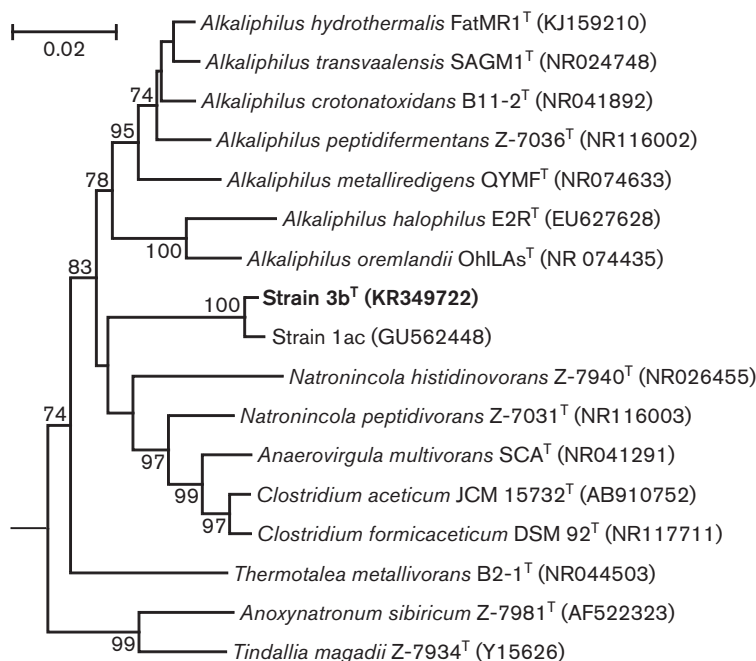

Fig. 3. Neighbour-joining phylogenetic tree based on 1154 aligned base pairs of 16S rRNA gene sequences showing the position of strain 3b^T among other members of the family Clostridiaceae. Bacillus subtilis (AJ276351) and Acetoanaerobium spp. (GU562448 and KJ174056) were used as the outgroup (not shown). Bootstrap values higher than 70 % (based on 1000 replicates) are shown at branch nodes. Accession numbers are indicated in parentheses. Bar, 0.02 substitutions per 100 nt.

Gram-stain-positive, spore-forming, alkaliphilic, mesophilic rods. Obligate anaerobes using organic acids, but not sugars. Belongs to the family *Clostridiaceae* (phylum *Firmicutes*). The G+C content of the genomic DNA is around 33.2 mol%. The type species is *Serpentinicella alkaliphila*.

Description of *Serpentinicella alkaliphila* sp. nov.

Serpentinicella alkaliphila (al.ka.li′phi.la. N.L. n. *alkali* alkali; Gr. adj. *philos* loving; N.L. fem. adj. *alkaliphila* loving alkaline conditions).

Displays the following characteristics in addition to those listed in the genus description. Cells are approximately 1–10 µm long and 0.4–0.6 µm wide, occurring singly or in pairs. Cells are motile and possess multiple flagella. Spores are terminal without swollen sporangia, 0.5–0.7 µm in diameter. Colonies are circular and yellow and about 1–2 mm in diameter after 7 days of incubation at 37 °C. Growth occurs at 30–43 °C (optimum 37 °C), at pH 7.8–10.1 (optimum pH 9.5) and with 0–4 % (w/v) NaCl (optimum 0–0.2 %). Added NaCl is not required for growth. Yeast extract is required for growth. Lactate, crotonate and pyruvate are used as electron donors, but acetate, glucose, fructose, glycerol, ribose, galactose, maltose, xylose, peptone, lactose, mannose, raffinose, sucrose, arabinose, cellobiose, rhamnose, formate, fumarate, ethanol, Casamino acids, biotrypticaseand H_2/CO_2 are not. Lactate is disproportionated to acetate and propionate. Crotonate is disproportionated to acetate and butyrate. Pyruvate is metabolized to acetate and trace amounts of hydrogen. Fumarate is used as a terminal electron acceptor. None of the following electron acceptors tested is used: elemental sulfur, sulfate, thiosulfate, sulfite, nitrate, nitrite, $FeCl_3$, Fe(III)-citrate, Fe(III)-EDTA, arsenate, selenite, DMSO and chromate. The predominant cellular fatty acids are $C_{16:1}\omega 7c/C_{16:1}\omega 6c$, $C_{16:0}$, $C_{14:0}$ and iso-$C_{17:1}$/anteiso-$C_{17:1}$.

The type strain, $3b^T$ (=DSM 100013^T=JCM 30645^T), was isolated from a hyperalkaline spring in the serpentinite-hosted PHF, New Caledonia.

Acknowledgements

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