***Anaerobranca gottschalkii* sp. nov., a novel thermoalkaliphilic bacterium that grows anaerobically at high pH and temperature**

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A novel thermoalkaliphilic, obligately anaerobic bacterium was isolated from a humid soil sample of a hot inlet of Lake Bogoria, Kenya. The newly isolated strain grows optimally at pH 9.5 and 50–55 °C and its growth range is pH 6.0–10.5 and 30–65 °C. Unlike the already known thermoalkaliphiles, the strain grows heterotrophically on a variety of mono- and polysaccharides (glucose, ribose, mannose, fructose, sucrose, maltose, starch, pullulan, xylan and cellulose) and on proteinaceous substrates such as yeast extract, peptone and tryptone. No dissimilatory sulfate reduction was observed, whereas thiosulfate was found to enhance growth when glucose or starch were used as substrates. Under optimal conditions, the doubling time is 48 min. Sodium ions are necessary for growth, with an optimal concentration of 230 mM (1% NaCl, w/v) at pH 9.5. The rod-shaped cells are motile in the exponential growth phase under optimal growth conditions. Despite the Gram-negative staining and negative KOH assay, the strain is a Gram-positive organism, having an atypically thin cell wall. A sheath-like structure occurs at the cell separation area and parts of a surface layer-like structure were also observed. Based on physiological properties and molecular biological analysis, the strain falls within the radiation of the clostridia and represents a new species of *Anaerobranca* within the *Clostridium/Bacillus* subphylum of the Gram-positive bacteria. Strain LBS3^T (= DSM 13577^T) is named *Anaerobranca gottschalkii* sp. nov. and is designated as the type strain.

Keywords: *Anaerobranca gottschalkii* sp. nov., alkaliphile, thermophile, extremophile

INTRODUCTION

Alkaliphilic prokaryotes have been isolated from a variety of habitats such as soils, sewage plants and seawater (Grant & Tindall, 1980; Horikoshi, 1991a; Maeda & Taga, 1980). A relatively restricted range of mesophilic prokaryotes, mainly representing species of *Bacillus*, have been investigated intensively and reclassified taxonomically (Fritze *et al.*, 1990; Nielsen *et al.*, 1995). True alkaline environments are only present at special locations distributed over the world (Grant & Horikoshi, 1992). Such sites represent habitats for a

highly diversified microbial community (Jones *et al.*, 1998). The Great Rift Valley soda lakes are geologically and geochemically well examined (Engle *et al.*, 1995; Grant *et al.*, 1990; Jones *et al.*, 1998), including the low-saline northern lakes (Bogoria, Nakuru, Elmenteita, Sonachi) and the arid, carbonate-saturated southern lakes (Magadi, Natron). Of special interest are the sites in and around Lake Bogoria, the origin of our samples, where a number of hot springs are found with temperatures of up to 96 °C.

Recent studies have shown that the phylogenetic diversity in these lakes is high (Duckworth *et al.*, 1996; Jones *et al.*, 1998). However, only one anaerobic, thermophilic strain growing at pH 9.5 and 70 °C, belonging to the order *Thermotogales*, '*Thermopallium natronophilum*', has been detected. The following isolates are the only identified anaerobic isolates to

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date: *Clostridium paradoxum* (Li *et al.*, 1993), *Clostridium thermoalkaliphilum* (Li *et al.*, 1994), *Thermococcus alkaliphilus* (Keller *et al.*, 1995), *Anaerobranca horikoshii* (Engle *et al.*, 1995) and one mesophile, *Spirochaeta asiatica* (Zhilina *et al.*, 1996). Some new, less-halotolerant mesophilic isolates and a few haloalkaliphilic strains have been described (Jones *et al.*, 1998). Based on genetic techniques, however, there is further evidence for the existence of a wide range of strictly anaerobic micro-organisms (Grant *et al.*, 1990; Zhilina & Zavarzin, 1994). The newly described genus *Anaerobranca* comprises only one species to date, *A. horikoshii* (Engle *et al.*, 1995). The common features of this genus were described as the formation of branched-type cells, obligately anaerobic, heterotrophic and proteolytic growth and a low DNA G + C content of 30–34 mol%. Here we describe a new species within this genus.

There is great interest in understanding the mechanisms that allow growth of micro-organisms at high pH and temperature. In this report, we describe the isolation, characterization and identification of a new anaerobic thermoalkaliphile, LBS3^T, isolated from a hot alkaline inlet from the alkaline Lake Bogoria, Kenya. Previously, we reported on the role of sodium ions in the process of energy transduction in this extremophile (Prowe *et al.*, 1996).

METHODS

Sample collection. Soil samples from Lake Bogoria in Kenya were collected in 1992 from the shore of the lake and from the inlet of a hot spring. The temperatures of the sample sites ranged from 50 to 80 °C and the pH was around 9–10. The samples were kept at ambient temperature until inoculation in the laboratory.

Media and culture conditions. Hungate anaerobic technique (Hungate, 1969; Macy *et al.*, 1972) was used to isolate and cultivate strain LBS3^T. Isolation was done on a medium containing (g l⁻¹): (NH₄)₂SO₄, 1.5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.05; NaCl, 3.0; tryptone, 1.0; yeast extract, 1.0; FeCl₃, 0.01; resazurin, 0.001; trace element solution 141 (DSMZ), 10 ml; vitamin solution 141 (DSMZ), 10 ml; NaHCO₃, 2.2; Na₂CO₃, 2.2; cysteine hydrochloride, 0.5; with 0.5% (w/v) starch (Merck) or xylan (Roth) as a carbon source. The medium without carbonates and cysteine was boiled, concentrated to 90% of the original volume and cooled to 4 °C under continuous gassing with O₂-free N₂. Cysteine was added prior to distribution in 15 ml Hungate tubes (9 ml medium per tube) or 100 ml serum bottles (20 or 50 ml per bottle). The sealed tubes were subsequently autoclaved. The carbonate stock solution was concentrated 10-fold and sterilized separately. One millilitre of the carbonate solution was added aseptically to 9 ml sterile medium prior to incubation; the final pH was pH 9.5. Higher or lower pH values were obtained by varying the concentration of stock carbonate solution.

Cultivation of strain LBS3^T was performed in optimized medium containing (g l⁻¹): (NH₄)₂SO₄, 1.0; NH₄Cl, 0.4; Na₂S₂O₃, 0.1; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.05; NaCl, 10.0; tryptone, 0.25; yeast extract, 0.25; FeCl₃, 0.01; resazurin, 0.001; trace element solution

141 (DSMZ), 10 ml; vitamin solution 141 (DSMZ), 10 ml; NaHCO₃, 2.2; Na₂CO₃, 2.2; cysteine hydrochloride, 0.5. Additional carbon sources (0.5% w/v) were added prior to boiling or added separately after sterilization.

Isolation and purification. Serum bottles containing anaerobic medium at pH 8–10 were inoculated with samples and incubated at temperatures from 50 to 70 °C. Starch, glucose, peptone or xylan (each 0.5% w/v) were added as carbon sources. After growth was observed, samples of the culture were plated on solidified medium (15 g agar l⁻¹) in an anaerobic chamber (Microflow Anaerobic System) under a N₂/H₂ (95:5) atmosphere and incubated at 50 °C in an anaerobic jar for 4 d. Single colonies were picked and again spread on agar-solidified medium until one colony type was observed. The cultures were checked routinely for purity every 2 months. Due to media turbidity caused by different carbon sources, the cell number was determined by counting the cells in a Neubauer counting chamber in order to use a comparable standard method for growth control.

Growth conditions. The influence of temperature on growth was studied by incubation of inoculated media (5%, v/v) containing starch or peptone as carbon source at temperatures between 30 and 70 °C for 24 h. The cell number was counted every 2 h in a Neubauer counting chamber. The influence of pH on growth was studied in media with pH values varying from 5 to 11. For pH 5.0–7.5, the pH was adjusted by the addition of 1 M KOH or 1 M HCl after adding 1 g NaHCO₃ l⁻¹. For pH 8.0–11.0, different amounts of carbonate were used. During growth at 50 °C, the pH was adjusted by the addition of sterile 1 M NaOH.

In order to test for aerobic growth, the medium, which contained starch or glucose as the carbon source at pH 9.5, was prepared aerobically without the addition of reducing agent and placed in Erlenmeyer shake flasks. In addition, anaerobically prepared media in Hungate tubes, containing starch or glucose as carbon source, were flushed aseptically with air until the resazurin red colour was stable, indicating aerobic conditions. Culture flasks or Hungate tubes were incubated at 50 °C without shaking.

In all studies concerning the growth conditions (pH, temperature, substrates, salt), the cell number of a third inoculum was counted after two transfers of culture material under the same conditions.

Substrate spectrum. Growth of strain LBS3^T was tested on different substrates as the sole carbon source by transferring the strain three times to medium containing 0.5% (w/v) arabinose, glucose, fructose, galactose, mannose, ribose, xylose, cellobiose, lactose, maltose, sucrose, sorbitol, glycerol, pyruvate, succinate, urea, Tween 80, casein, peptone, tryptone, yeast extract, starch, xylan, pullulan or cellulose (filter paper) and subsequent counting of the cell number after 6, 12 and 24 h.

Dissimilatory sulfate reduction. Hungate tubes were prepared with the above-described medium, pH 9.5, without Na₂S₂O₃ and containing NH₄Cl instead of (NH₄)₂SO₄. In addition, 1% (w/v) glucose, 0.02% (w/v) lactate or 0.02% (w/v) pyruvate, supplemented with 0.01% (w/v) FeSO₄, 0.1% (w/v) Na₂S₂O₃ or 0.1% Na₂SO₄, was added. Medium containing additional elemental sulfur was supplemented with either glucose at 1% (w/v), pyruvate at 0.02% (w/v) or lactate (0.02% w/v). In addition, the medium containing sulfur was flushed anaerobically with H₂ in the presence of glucose as carbon source. Production of sulfide was

visualized by the addition of FeSO_4 or CuSO_4 (from a stock solution of 0.6% w/v) resulting in a blackening of the medium (modified method of Smibert & Krieg, 1994).

Cellular characterization. Gram staining of strain LBS3^T was determined by staining cells using the Hucker method (in Gerhardt *et al.*, 1994) and KOH lysis (Buck, 1982), using cells from 4 and 20 h cultures. For the sporulation test, cells were grown for up to 7 d in a medium containing 0.5% (w/v) glucose as carbon source. The presence of spores was determined by phase-contrast microscopy (Zeiss Axioplan).

Scanning electron microscopy of whole cells was performed with a Leitz ISI-SR-50 scanning electron microscope. Cell material from a 50 ml culture grown for 8 h was prepared according to Lamed *et al.* (1987) without ferritin.

Transmission electron microscopy was performed in a Phillips EM 01 at 80 kV. For ultrathin sections, cells were treated according to Behn & Arnold (1974). Cell material was harvested from a 50 ml culture grown for 8 h and washed twice with a 100 mM PBS buffer pH 8.0 (K_2HPO_4 , 8.7 g l⁻¹; KH_2PO_4 , 6.8 g l⁻¹; NaCl, 9.0 g l⁻¹; glycine, 0.75 g l⁻¹). Embedding was done by the method of Spurr (1969) and polymerization was performed for 8 h at 70 °C. Ultrathin sectioning and contrasting with lead-citrate solution was performed as described previously (Venable & Coggeshall, 1965). Negative staining was performed according to Valentine *et al.* (1968).

Salt tolerance. Salt concentrations (NaCl or KCl) were varied between 0 and 5% (855 mM) in a medium containing 0.5% (w/v) starch. Growth was followed at optimal pH by counting the cell number every 2 h.

Antibiotic resistance. Antibiotics were prepared as concentrated stock solutions, filter-sterilized and added to a freshly 10% (v/v) inoculated liquid medium at pH 8.0; the lower pH was chosen in order to prevent inactivation of antibiotics at elevated pH. Monensin was added at 25 µg ml⁻¹, rifampicin at 50 µg ml⁻¹, nalidixic acid at 50 µg ml⁻¹, chloramphenicol at 25 µg ml⁻¹, penicillin at 25 and 100 µg ml⁻¹ and streptomycin at 25 and 250 µg ml⁻¹. Cultures were incubated at 50 °C for 24 h.

Fermentation products. Analysis of fermentation products was performed by HPLC (Knauer) using a Bio-Rad HPX-87 H column with 5 mM H_2SO_4 as the liquid phase.

16S rDNA sequence analysis. DNA analysis was performed at the DSMZ. Genomic DNA extraction from lyophilized cell mass, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out as described previously (Rainey *et al.*, 1992; Rainey & Stackebrandt, 1993). The 16S rDNA sequence data were compared with all currently available sequences of organisms belonging to the *Clostridium*/*Bacillus* subphylum of the Gram-positive bacteria. Pairwise evolutionary distances (Jukes & Cantor, 1969) and the least-squares distance method of De Soete (1983) were used in the construction of the phylogenetic dendrogram from distance matrices.

DNA isolation, DNA-DNA hybridization and determination of G + C content. DNA from the new strain LBS3^T and *A. horikoshii* was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA-DNA hybridization and computation were carried out at the DSMZ using the methods of De Ley *et al.* (1970) and Jahnke (1992), with the modification described (Escara & Hutton, 1980; Huss *et al.*, 1983).

DNA for the determination of G + C content was isolated as described above. The DNA was digested enzymically and the G + C content was determined by HPLC by separating the nucleotides as described by Mesbah *et al.* (1989).

Analysis of lipid components. Analysis of the respiratory lipoquinone, fatty acids and the polar lipid pattern of LBS3^T and *A. horikoshii* were carried out by B. J. Tindall (DSMZ, Braunschweig, Germany) using methods described previously (Tindall, 1990a, b).

RESULTS AND DISCUSSION

Isolation of thermoalkaliphilic bacteria

Enrichment cultures (pH 9.0) containing starch or glucose and inoculated with sample material from a hot lake inlet of Lake Bogoria showed bacterial growth after 24 h of anaerobic incubation at 50 °C. Microscopy revealed the presence of rod-shaped cells (Fig. 1) that all seemed to belong to the same phenotype. After incubation for 24 h under anaerobic conditions, culture material was plated on an agar-solidified medium containing 0.5% (w/v) starch. After a number of transfers, uniform small, pale-whitish, lens-shaped colonies with smooth edges and diameters of 3–5 mm were observed. These colonies and the resulting liquid cultures were shown to exhibit the same characteristics. One culture, strain LBS3^T, was selected as the culture for the type strain.

Cellular characteristics

Cells of strain LBS3^T (*Anaerobranca gottschalkii*) from the exponential phase were rod-shaped (0.3–0.5 µm wide and 2–3 µm long). They occurred singly or as short chains of up to four cells. Using glucose as substrate, the cells were motile, especially in the early exponential growth phase around the pH optimum of pH 9.5 (see Fig. 3a). Morphological changes were observed in the stationary growth phase. Protoplast-like structures and pleiomorphic, irregular cell chains were observed after 24 and 48 h. Cells from the late

Fig. 1. Scanning electron micrograph of cells of *A. gottschalkii* LBS3^T. Bar, 1.4 µm.

Fig. 2. (a)–(b) Electron micrographs of ultrathin-sectioned cells of *A. gottschalkii* LBS3^T from the exponential growth phase showing the atypically thin cell wall (a) (indicated by arrow) and branch formation (b); no separation was found at the branch junction. (c)–(d) Electron micrographs of negatively contrasted cells of *A. gottschalkii* LBS3^T showing the sheath-like structure (Sh) at the separation area (c) and part of a surface layer-like structure (S) (d). Bars, 0.1 μ m (a, d) and 0.3 μ m (b, c).

phase of growth were less motile. In addition, a few Y-type branched cells were also observed (Fig. 2b). The ultrathin sections shown in Fig. 2(b) indicate that these forms consisted of only one cell. Similar cell morphology of primary branched cells was reported for the alkali-tolerant thermophilic bacterium *A. horikoshii* (Engle *et al.*, 1995). The KOH assay and Gram staining of cells of *A. gottschalkii* LBS3^T were negative, regardless of the age of the culture. This property, which was also observed for the thermoalkaliphiles *Clostridium paradoxum* (Li *et al.*, 1993) and *Clostridium thermoalkaliphilum* (Li *et al.*, 1994), seems to be widespread in Gram-positive alkaliphiles (Grant & Horikoshi, 1992). *A. horikoshii*, on the other hand, showed a positive Gram stain and a typical Gram-

positive-type cell wall in ultrathin sections (Engle *et al.*, 1995), which allowed distinction of the strain from *A. gottschalkii* LBS3^T. *A. gottschalkii* LBS3^T is, nevertheless, regarded as Gram-positive, due to the data obtained from 16S rDNA analysis (see below). This was also supported by the fact that protoplasts were obtained easily by lysozyme treatment (Prowe *et al.*, 1996). Ultrathin sectioning of cells also showed an atypical thin cell wall (Fig. 2a), as reported already for *Clostridium paradoxum* (Li *et al.*, 1993). Negative-contrast electron microscopy of whole cells showed the presence of a sheath-like structure around the cells (Fig. 2c, d). The presence of an outer surface layer has been reported for alkaliphilic *Bacillus* species (Kruschwitz, 1982) and a sheath-like structure was

Fig. 3. (a) Effect of pH on growth (●) and motility (○) of *A. gottschalkii* LBS3^T. Incubation was at 50 $^{\circ}\text{C}$ in the medium described containing starch (0.5% w/v) or peptone (1% w/v). Results were similar regardless of the substrate used. (b) Effect of temperature on growth of *A. gottschalkii* LBS3^T. The curve was obtained by cultivation of the strain for up to 24 h in a medium containing starch (0.5% w/v) or peptone (1% w/v), pH 9.5. Results were similar independent of the substrate used. (c) Effect of various salt concentrations on growth of *A. gottschalkii* LBS3^T. The medium at pH 9.5, supplemented with 0.5% (w/v) starch, contained additional amounts of NaCl (●) or KCl (○). Incubation was performed at 50 $^{\circ}\text{C}$ for 12 h.

observed in *Clostridium thermoalcaliphilum* (Li *et al.*, 1994).

A. gottschalkii LBS3^T did not grow on solid or liquid medium supplemented with MnSO_4 . Endospores were not observed either in fresh cultures or in old cultures stored at 4 $^{\circ}\text{C}$ or those kept at room temperature for months. Such stored cultures lost their viability after 4 weeks, indicating the absence of spores. Cultures incubated at 50 $^{\circ}\text{C}$ for 8 h at pH 9.5 were not resistant to heat (80 or 90 $^{\circ}\text{C}$ for 5–10 min). No growth was observed after inoculation of these cells into fresh medium, thus indicating the absence of heat-resistant spores. Similar observations were made for the non-spore-forming *Clostridium thermoalcaliphilum* (Li *et al.*, 1994).

Growth conditions

No growth was observed under aerobic conditions, with starch or glucose as substrate. Growth occurred only anaerobically between pH 6.0 and 10.5, with an optimum pH for growth at 9.5 (Fig. 3a) when incubated at 50 $^{\circ}\text{C}$ with starch or glucose as carbon source. The largest number of motile cells was observed around the optimal pH (Fig. 3a). Since the strain was also able to grow at neutral pH, it should be considered

as a facultative alkaliphile, according to a proposed definition (Grant & Horikoshi, 1992; Horikoshi, 1991a; Krulwich & Guffanti, 1989).

In experiments performed in pH-controlled fermentations with starch or peptone as energy sources, *A. gottschalkii* LBS3^T exhibited a doubling time of 48 min at pH 9.5 and 50 $^{\circ}\text{C}$. At pH 10, the doubling time increased to 100 min.

The temperature range for growth was 30–65 $^{\circ}\text{C}$ with an optimum temperature for growth of 50–55 $^{\circ}\text{C}$ (Fig. 3b). Cells grown at temperatures below 40 $^{\circ}\text{C}$ or above 60 $^{\circ}\text{C}$ were pleiomorphic and did not correspond to the shape of cells grown at optimum temperatures. Thus, *A. gottschalkii* LBS3^T is regarded as a moderately thermophilic micro-organism.

Salt tolerance

The effect of NaCl under optimal growth conditions (pH 9.5, 50 $^{\circ}\text{C}$) showed a concentration range for growth of 0–4% (w/v), with an optimum concentration of 1% NaCl (w/v) (Fig. 3c). The tolerated Na^+ concentration is therefore 60–560 mM. The amount of Na^+ resulting from sodium carbonates (2.2 g l^{-1}) at pH 9.5 is 60 mM. Accordingly, the Na^+ concentration for optimal growth at 1% NaCl is 230 mM. These data

differ from the concentrations tolerated by *Clostridium paradoxum* (Li *et al.*, 1993) and *Clostridium thermoalcaliphilum* (Li *et al.*, 1994), where Na⁺ concentrations of 750 mM and 1.25 M, respectively, were tolerated. The optimal concentration of Na⁺ ions for *Clostridium paradoxum* and *Clostridium thermoalcaliphilum* is 50–100 mM and that for *Clostridium thermoalcaliphilum* is 200 mM in the presence of K⁺ ions. These values are lower than those obtained for *A. gottschalkii* LBS3^T. In the case of *A. gottschalkii* LBS3^T, Na⁺ could not be replaced by K⁺, resulting in a dramatic decrease of the growth yield at K⁺ concentrations above 27 mM (0.2% w/v KCl) (Fig. 3c). As reported previously, Na⁺ ions play a major role in the energy transduction of this micro-organism; K⁺ ions have no such effect (Prowe *et al.*, 1996). Based on these results, it can be concluded that *A. gottschalkii* LBS3^T depends strictly on Na⁺ ions for growth.

The fact that alkaliphiles require Na⁺ for their growth is well documented in earlier reports (Duckworth *et al.*, 1996; Horikoshi, 1991a, b; Li *et al.*, 1993, 1994; Zhilina & Zavarzin, 1994; Zhilina *et al.*, 1996). Most of the environments of alkaliphilic micro-organisms have high Na⁺ concentrations. The function of this ion in neutrophiles (Lolkema *et al.*, 1994), as well as alkaliphiles (Krulwich & Guffanti, 1989; Krulwich *et al.*, 1990) and some extremophiles (Speelmans *et al.*, 1995), is either as a coupling ion for energy transduction or in pH homeostasis. We have demonstrated previously that Na⁺ ions play a crucial role in the energy transduction of strain LBS3^T (Prowe *et al.*, 1996). This was also supported by the fact that Na⁺ could not be replaced by K⁺ in the growth medium.

Substrates and fermentation products

A. gottschalkii LBS3^T grew on a variety of substrates including carbohydrates, proteins and peptides. The addition of 0.1 g yeast extract and 0.1 g tryptone l⁻¹ was required for optimal growth. In their absence, changes in cellular morphology were observed. Good growth with cell densities of up to 5×10^8 cells ml⁻¹ was observed in batch cultures (serum bottles) without stirring in the presence of 0.5% (w/v) glucose, fructose, mannose, maltose, sucrose, peptone, tryptone or yeast extract. Starch and pullulan were also hydrolysed. Growth (1×10^8 cells ml⁻¹) was observed with ribose, casein, xylan and cellulose (filter paper). Moderate growth (5×10^7 cells ml⁻¹) was obtained with galactose, xylose, cellobiose, lactose and glycerol (0.5% w/v each). The main fermentation product during growth on starch or glucose at pH 9.5 was acetate; small amounts of ethanol (< 1 µmol ml⁻¹) were also detected. Gaseous products were not detected by HPLC.

Dissimilatory sulfate reduction

Compared with a control (without additional Na₂S₂O₃), growth of *A. gottschalkii* LBS3^T on glucose or starch was enhanced in the presence of Na₂S₂O₃.

Table 1 Susceptibility of *A. gottschalkii* LBS3^T to antibiotics

Growth conditions were 50 °C and pH 9.0. Cells were grown without antibiotics for one generation and then inoculated to a medium preincubated with the drug at the concentrations shown. Growth inhibition is scored as: +, inhibition of growth; –, no inhibition of growth.

Antibiotic	Concentration (µg ml ⁻¹)	Inhibition of growth
Chloramphenicol	25	+
Monensin	25	+
Rifampicin	50	–
Nalidixic acid	50	+
Penicillin	25	–
	100	–
Streptomycin	25	–
	250	+

(0.01% w/v). Good growth of *A. gottschalkii* LBS3^T (1×10^9 cells ml⁻¹) was also observed when elemental sulfur was used. Thus, sulfur does not inhibit growth. Under these conditions, elemental sulfur was partially solubilized, resulting in a yellow-coloured medium, which may be due to the formation of polysulfide at pH 9.5. During growth in the presence of sulfur-containing compounds (Na₂S₂O₃ and S⁰), H₂S was detected by the addition of FeSO₄ or CuSO₄, resulting in a deep blackening of the medium due to the precipitation of FeS or CuS; the controls did not show this effect. Growth on sulfur with glucose as carbon source was inhibited when the medium was gassed with H₂ prior to inoculation. Thus, it is suggested that thiosulfate can be used as electron acceptor. This was also reported for members of the order *Thermotogales* (Ravot *et al.*, 1995) when glucose or other fermentable carbohydrates were used for growth. The addition of Na₂SO₄ or FeSO₄ did not stimulate or inhibit growth. No dissimilatory sulfate reduction was detected, excluding the organism from the genus *Desulfotomaculum*.

Sensitivity to antibiotics

As shown in Table 1, inhibition of growth was observed with chloramphenicol, monensin and nalidixic acid. No inhibition was observed with rifampicin or penicillin. Streptomycin showed an inhibitory effect at higher concentrations only. This is in agreement with the bacterial sensitivity pattern towards antibiotics.

Phylogenetic position

A complete sequence (1433 bases) of the 16S rDNA of strain *A. gottschalkii* LBS3^T was analysed and compared with all currently available sequences of organisms belonging to the *Clostridium*/*Bacillus* sub-

Fig. 4. Phylogenetic tree for *A. gottschalkii* LBS3^T and related organisms based on results of 16S rDNA sequence analysis. Bar, 10 inferred changes per 100 nucleotides.

phylum of the Gram-positive bacteria (Fig. 4). The closest relationship was to *A. horikoshii*, at the level of 97.5% 16S rDNA similarity. The DNA G + C content

of *A. gottschalkii* LBS3^T is 30.9 mol%, which differs from the G + C content reported for *A. horikoshii* (34.3 mol%). DNA–DNA hybridization with *A. horikoshii* showed only 60.4% relatedness. In addition, the lipid composition of the strains also differed. The main fatty acids in the lipids of *A. horikoshii* are 14:0 (26%), i-15:0 (7.5%), 15:0 (11%) and 16:0 (28%). The lipids in *A. gottschalkii* LBS3^T consist mainly of the fatty acids i-13:0 (4.3%), i-15:0 (42%), ai-15:0 (12%) and 16:0 (11%), with smaller amounts of ai-13:0 (2.6%) and ai-17:0 (3.4%) fatty acids.

These data demonstrate clearly that the new isolate is a new species within the genus *Anaerobranca*. This fact is also supported by the physiological and morphological data presented in Table 2. In contrast to *A. horikoshii*, the new isolate is able to grow on a variety of complex proteinaceous substrates (yeast extract, peptone, tryptone), sugars and polysaccharides. *A. horikoshii* is only able to utilize a restricted number of substrates for growth. The morphological feature of branched cells was also observed for *A. horikoshii* (Engle *et al.*, 1995), but the negative Gram-type staining and the thin cell wall of *A. gottschalkii* LBS3^T reflect the differences between the two species. The ability of *A. gottschalkii* LBS3^T to reduce thiosulfate suggests that these are phylogenetically distinct strains.

Table 2 Characteristics that distinguish *A. gottschalkii* LBS3^T from *A. horikoshii*

Data for *A. horikoshii* were taken from Engle *et al.* (1995). Utilization of compounds is scored as: ++, growth to $2-5 \times 10^8$ cells ml⁻¹; +, growth to $0.5-1 \times 10^8$ cells ml⁻¹; –, no growth. Both taxa produce Y-shaped, branched cells and show strong growth ($2-5 \times 10^8$ cells ml⁻¹) on yeast extract, peptone and tryptone. Neither taxon forms spores. ND, Not determined.

Characteristic	<i>A. gottschalkii</i> LBS3 ^T	<i>A. horikoshii</i>
DNA G + C content (mol %)	30.9	33–34
pH range for growth	6.0–10.5	6.5–10.3
Optimum pH for growth	9.5	8.5–8.8
Optimum temperature (°C)	50–55	57
Optimum Na ⁺ concentration (mM)	230	8.5
Gram staining	–	+
Gram type	+ (Thin cell wall)	+ (Thick cell wall)
S ₂ O ₃ → S ²⁻	+	–
Fermentation products	Acetate, ethanol	Acetate, CO ₂ , H ₂
Utilization of:		
Starch	++	–
Pullulan	+	ND
Xylan	–	ND
Cellulose	+	ND
Glucose	++	–
Fructose	++	–
Mannose	++	ND
Maltose	++	–
Sucrose	++	–
Ribose	+	–
Galactose	+	–
Cellobiose	+	–
Lactose	+	–
Glycerol	+	–

No observations were reported on the effect of thio-sulfate on growth of *A. horikoshii*.

A. gottschalkii LBS3^T exhibits a large evolutionary distance from other thermoalkaliphilic members of the clostridium subphylum. The 16S rDNA similarity values to *Clostridium paradoxum* (Li *et al.*, 1993) and *Clostridium thermoalkaliphilum* (Li *et al.*, 1994) are only 83.3 and 83.1 %. The distinct differences in their physiological and structural features clearly separate these strains from *A. gottschalkii* LBS3^T. Closer similarities were found to two *Moorella* species, *Caloramator fervidus* and *Clostridium thermocellum* (Fig. 4). These bacteria clearly differ from *A. gottschalkii* LBS3^T in their physiological features (pH and temperature range of growth, substrate utilization) and cellular characteristics (*A. gottschalkii* LBS3^T possesses an atypically thin cell wall, has almost no spore formation and exhibits branched cells) as well as their phylogenetic position (Fig. 4). Thus, strain LBS3^T is not a member of any previously described species within the *Clostridium/Bacillus* subphylum. Based on phylogenetic analysis by 16S rDNA, DNA–DNA homology and G+C content, the strain was found to be closely related to *A. horikoshii*, but represents a new species within the recently described genus *Anaerobranca* (Engle *et al.*, 1995).

Description of *Anaerobranca gottschalkii* sp. nov.

Anaerobranca gottschalkii (gott.schalk'i.i. M.L. gen. n. *gottschalkii* after Gerhard Gottschalk, in recognition of his pioneering contributions to our knowledge of the physiology and metabolism of anaerobes).

Cells are rod-shaped, Gram-positive, 0.3–0.5 µm thick and 3–5 µm long. Colonies are 3–5 mm in diameter, pale-whitish and lens-shaped. Obligately anaerobic. Temperature range for growth is 30–65 °C, with an optimum around 50–55 °C. Range of pH for growth is pH 6–10.5, with an optimum at pH 9.5. Growth from 0 to 4 % NaCl (w/v) with an optimum around 1 % NaCl (w/v; final Na⁺ concentration 230 mM). Grows heterotrophically with glucose, fructose, mannose, maltose, sucrose, starch, pullulan, peptone, tryptone and yeast extract. Growth is observed in the presence of sulfate, thiosulfate or sulfur. Thiosulfate enhances growth in the presence of glucose and starch, resulting in the formation of H₂S. Fermentation products (on starch with thiosulfate) are acetate and ethanol. Cell wall is thin, but the strain belongs to the Gram-positive type. Branched cells are regularly present as 2–10 % of the total number of cells.

Strain LBS3^T (= DSM 13577^T) is the type strain of *A. gottschalkii* and was isolated from a hot lake inlet at Lake Bogoria, Kenya.

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