Hydrogenispora ethanolica gen. nov., sp. nov., an anaerobic carbohydrate-fermenting bacterium from anaerobic sludge

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An anaerobic, spore-forming, ethanol-hydrogen-coproducing bacterium, designated LX-B1T, was isolated from an anaerobic sludge treating herbicide wastewater. Cells of strain LX-B1T were non-motile rods (0.3–0.5×3.0–18.0 μm). Spores were terminal with a bulged sporangium. Growth occurred at 20–50 °C (optimum 37–45 °C), pH 5.0–8.0 (optimum pH 6.0–7.7) and 0–2.5 % (w/v) NaCl. The strain could grow fermentatively on glucose, maltose, arabinose, fructose, xylose, ribose, galactose, mannose, raffinose, sucrose, pectin, starch, glycerol, fumarate, tryptone and yeast extract. The major end-products of glucose fermentation were acetate, ethanol and hydrogen. Yeast extract was not required but stimulated growth. Nitrate, sulfate, thiosulfate, elemental sulfur, sulfite, anthraquinone-2,6-disulfonate, fumarate and Fe (III) nitrilotriacetate were not used as terminal electron acceptors. The G+C content of the genomic DNA was 56.1 mol%. The major cellular fatty acids were anteiso-C15 : 0, iso-C14 : 0 and C16 : 0. The most abundant polar lipids of strain LX-B1T were diphosphatidylglycerol and phosphatidylglycerol. 16S rRNA gene sequence analysis revealed that it belongs to an as-yet-unidentified taxon at the order- or class-level (OPB54) within the phylum Firmicutes, showing 86.5 % sequence similarity to previously described species of the Desulfotomaculum cluster. The name Hydrogenispora ethanolica gen. nov., sp. nov. is proposed to accommodate strain LX-B1T (=DSM 25471T=JCM 18117T=CGMCC 1.5175T) as the type strain.

The phylum Firmicutes is a phylogenetically and phenotypically divergent bacterial phylum, which plays an important role in biodegradation and the carbon cycle in nature (Ludwig et al., 2009). At the time of writing, there are four recognized classes of the phylum Firmicutes: the anaerobic class Clostridia, the obligately or facultatively aerobic class Bacilli, and the classes Erysipelotrichia and Thermotogae (http://www.bacterio.net/; Yutin & Galperin, 2013). Furthermore, many uncultured taxonomic groups exist in the phylum Firmicutes, such as candidate taxa OPB54, which was first detected from Obsidian Pool, a Yellowstone Park hot spring (Hugenholtz et al., 1998). Based on ARB-SILVA taxonomy, OPB54 is assigned to the class Clostridia at the order level (Dunfield et al., 2012) or represents a new class of the phylum Firmicutes (http://arbsilva.de). Environmental clones belonging to OPB54 have been found in a wide range of habitats, including soils (Brodie et al., 2006; Kanokratana et al., 2011), anaerobic wastewater and waste treatment systems (Tang et al., 2004), the skin microbiome (Kong et al., 2012) and aquatic moss pillars (Nakai et al., 2012). However, no cultivated representatives of this clade have been reported so far. In this study, an ethanol-type hydrogen-producing bacterium (strain LX-B1T), the first cultivated strain belonging to the OPB54 cluster of the phylum Firmicutes is described.

Strain LX-B1T was originally obtained from a mesophilic (35 °C) anaerobic sludge treating herbicide wastewater. The medium used for isolation and cultivation was prepared as described by Sekiguchi et al. (2000). The sludge was washed and transferred into liquid medium supplemented with 50 mg l−1 pyrazosulfuron, which was one of the major components of the original herbicide wastewater. After five transfers, the pyrazosulfuron enrichment culture was serially diluted in liquid medium supplemented with glucose (2 mM). Growth and hydrogen production were observed in higher dilution cultures within 2 weeks of incubation.
Cells from the highest dilution were further purified by repeated serial dilutions in glucose medium, then in glucose agar (2 %) roll tubes (Hungate, 1969). Small, dark brown, round colonies were formed after 2 weeks of incubation at 37 °C. The colonies were picked and retransferred to liquid medium supplemented with 2 mM glucose. This roll-tube isolation step involving transfer of single colonies from solid medium to liquid medium was repeated several times, resulting in the isolation of strain LX-B^T.

Cell morphology was examined under a fluorescent microscope (Olympus BX50F). Transmission electron microscopy (TEM) was performed with a Hitachi H-7000 transmission electron microscope as described by Sekiguchi et al. (2003). Gram staining was performed by the method of Hucker (Doetsch, 1981). Cells of strain LX-B^T were non-motile, straight or slightly curved rods, 0.3–0.5 μm in diameter and 3.0–18.0 μm in length (Fig. 1a). Spores were located terminally and had a bulged sporangium (Fig. 1a). Gram staining was negative, however, electron microscopy demonstrated that strain LX-B^T possessed a Gram-positive-type cell wall (Fig. 1b).

Physiological characteristics of strain LX-B^T were examined as described by Sekiguchi et al. (2000). The temperature range for growth was determined between 15 and 60 °C (5 °C intervals), the pH range was assessed at 37 °C over the range pH 3.5–8.5 (0.5 pH unit intervals) and NaCl tolerance was checked at 0–3.0 % (w/v, 0.5 % intervals). Cell growth was evaluated on the basis of the increase in OD at 400 nm and the production of hydrogen. Unless otherwise indicated, all cultivations were performed at 37 °C in serum vials (20 ml per 50 ml vial) under a gas phase of N2/CO2 (80 : 20, v/v) without shaking. Aerobic growth was tested in a glucose (2 mM) medium under aerobic conditions without reducing agents. Strain LX-B^T grew anaerobically on glucose medium at 20–50 °C (optimum, 37–45 °C), at pH 5.0–8.0 (optimum, 6.0–7.7), and with 0–2.5 % (w/v) NaCl, but did not grow at temperatures below 15 °C or above 55 °C after 8 weeks of incubation. The isolate was a strictly anaerobic organism: it could not grow in the presence of oxygen (20 %, v/v, in the gas phase) or after N2/CO2 purging only (without reducing agents). Vigorous growth and hydrogen production were observed with the following substrates (5 mM each unless otherwise specified): glucose, maltose, arabinose, fructose, xylose, ribose, galactose, mannose, raffinose, sucrose, pectin (0.5 g l\(^{-1}\)), starch (0.5 g l\(^{-1}\)), glycerol, fumarate, tryptone (0.5 %) and yeast extract (0.5 %). Yeast extract was not required but enhanced growth. In syntrophic substrate utilization tests, strain LX-B^T was co-cultured with the hydrogenotrophic methanogen *Methanospirillum hungatei* DSM 864^T_. None of the following substrates tested supported the growth of strain LX-B^T in pure culture or co-culture with *M. hungatei* DSM 864^T (5 mM each unless specified): crotonate, pyruvate, xylan (0.5 %), cellulose, Casamino acids, l-glutamate, serine, glycine, ethanol, methanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, ethylene glycol, citrate, formate, H\(_2\)/CO\(_2\) (80 : 20, v/v, head space) plus acetate, propionate, isobutyrate, butyrate, succinate, malate, benzoate, hydroquinone (2 mM), phenol (2 mM), 4-hydroxybenzoate, 2,5-dihydroxybenzoate or pyrazosulfuron (50 mg l\(^{-1}\)). In the medium supplemented with glucose (2 mM), the major end-products from glucose degradation were hydrogen, acetate and ethanol (1 mol glucose was converted to approximately 1 mol acetate, 0.86 mol ethanol and 2.52 mol hydrogen, electron recovery 98 %) (Fig. 2). Although the strain produced hydrogen, its growth was not stimulated in co-cultivation with *M. hungatei*. The ratio of ethanol/acetate from glucose degradation in co-culture was same with that in pure culture.

Strain LX-B^T did not use any of the following electron acceptors within 4 weeks of incubation with glucose (mM): nitrate (5), sulfate (5), thiosulfate (2), elemental sulfur (5), Fe (III) nitritoltriacetate (2), sulfite (1), fumarate (5).
or anthraquinone-2,6-disulfonate (AQDS) (2). Sensitivity to antibiotics was tested in glucose medium, containing filter-sterilized antibiotics at 50 mg l\(^{-1}\). Growth of strain LX-B\(^{T}\) was inhibited by gentamicin, kanamycin, lincomycin and tetracycline, but not ampicillin, chloramphenicol, streptomycin or penicillin.

For G+C content determination, DNA was extracted and purified according to the methods of Kamagata & Mikami (1991). The DNA G+C content of strain LX-B\(^{T}\) was determined from the melting point (\(T_m\)) by thermal denaturation (Mandel et al., 1970) using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer). DNA from *Escherichia coli* (Sigma D-2001) was used as a reference for the measurements. The DNA G+C content was 56.1 mol\% (SD: ±0.06 mol%). Fatty acids of cells were converted to methyl esters using HCl/methanol and identified by GC-MS (Agilent 7890A gas 140 chromatograph coupled to an Agilent 5975C MSD single quadrupole mass spectrometer) (Hanada et al., 2002). For fatty acid methyl ester and polar lipid analyses, cells were harvested at exponential growth phase from cultures grown aerobically on 10 mM glucose at 37 °C. The major cellular fatty acids were anteiso-C\(_{15:0}\) (58.6 %), iso-C\(_{14:0}\) (11.1 %), C\(_{16:0}\) (9.6 %), iso-C\(_{16:0}\) (6.9 %), iso-C\(_{15:0}\) (4.3 %) and C\(_{14:0}\) (4.2 %). Polar lipids were extracted and analysed by the methods of Tindall (1990) by using two-dimensional TLC (Merck silica gel 60 F254 plates, layer thickness 0.2 mm, no. 5554). The polar lipid patterns were characterized by the presence of diphosphatidylglycerol, phosphatidylglycerol and other unidentified phospholipids and amino phospholipids (Fig. S1, available in the online Supplementary Material).

For 16S rRNA gene sequencing, the genomic DNA of strain LX-B\(^{T}\) was extracted according to the method of Hiraishi (1992). 16S rRNA genes were amplified by PCR with *Taq* polymerase (Perkin Elmer). The PCR primers used in the amplification were the bacterial domain universal primer 8F and the prokaryote universal primer 1490R (Weisburg et al., 1991). The PCR product was sequenced directly on a Beckman CEQ-8000 DNA sequencer using a CEQ DTC quick start kit (Beckman Coulter). The phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbour-joining method (Saitou & Nei, 1987) implemented in the MEGA5 computer program (Tamura et al., 2007) and the distance matrix was calculated by the Jukes–Cantor model (Jukes & Cantor, 1969). Confidence values of branches in the phylogenetic tree were determined using bootstrap analysis based on 1000 resamplings (Felsenstein, 1985). A total of 1443 base pairs of the 16S rRNA gene from strain LX-B\(^{T}\) were sequenced and compared with other related sequences in the databases. Phylogenetic analysis revealed that strain LX-B\(^{T}\) is closely affiliated to members of the uncultured OPB54 clade at the order- or class-level based on the *ARB-SILVA* reference database (http://arb-silva.de). The closest relatives of strain LX-B\(^{T}\) based on 16S rRNA gene sequences were environmental clones, such as those from uranium contaminated soil (90–94 % similarity) (Brodie et al., 2006), and clone AC007 (93 % similarity) from a landfill leachate bioreactor (Burrell et al., 2004). The most closely related cultured species was *Clostridium* sp. 6-31 (94 % similarity), which was an as-yet-undescribed non-cellulolytic bacterium from biocompost (Sizova et al., 2011). The most closely related described species were *Desulfotomaculum hydrothermale* (86.5 % similarity), an anaerobic sulfate-reducing bacterium isolated from a hot spring (Haouari et al., 2008), *Pelotomaculum propionicicum* (86.1 %), a syntrophic propionate-degrading bacterium isolated from a methanogenic sludge (Imachi et al., 2007), and *Clostridium straminisolvens* (84.9 %), a thermophilic cellulolytic bacterium (Kato et al., 2004) (Fig. 3). Sequence similarity values for the 16S rRNA genes of strain LX-B\(^{T}\) and related genera such as *Desulfotomaculum* (<86.5 %), *Pelotomaculum* (<86.1 %) and *Clostridium* (<85 %) in the order Clostridiales of the phylum Firmicutes were in a range 84–86 %, justifying the creation of a new genus and novel species to accommodate the strain from a phylogenetic point of view.

Comparative phenotypic traits of strain LX-B\(^{T}\) and related genera in the order Clostridiales are shown in Table 1. Strain LX-B\(^{T}\) shared some common phenotypic traits with related organisms, such as spore formation and chemoheterotrophic growth. Major differences that distinguished strain LX-B\(^{T}\) from other related members of the genera *Desulfotomaculum*, *Pelotomaculum* and *Clostridium* were: (i) morphology, cells of strain LX-B\(^{T}\) are long thin rods, forming terminal endospores in a bulged sporangium, while the related organisms were short sausage-shaped rods with central or terminal endospores; (ii) metabolism, strain LX-B\(^{T}\) is an ethanol-type fermentation bacterium which could not perform syntrophic substrate oxidation in cooperation with hydrogenotrophic methanogens, or sulfate respiration. However, members of the genus *Pelotomaculum*...
could perform syntrophic substrate oxidation with hydro-
genotrophic methanogens (Imachi et al., 2002, 2007; Qiu
et al., 2006), and members of the genus Desulfotomaculum
could perform sulfate respiration (Haouari et al., 2008; Liu
et al., 1997; Parshina et al., 2005). Strain LX-B<sup>T</sup> showed similar
fermentative metabolism to <i>C. straminisolvens</i> in the family
Clostridiaceae, however, their substrate spectrums for growth
were different. For example, strain LX-B<sup>T</sup> was able to grow on
various carbohydrates and proteinaceous compounds, whereas
<i>C. straminisolvens</i> is a thermophilic cellulolytic bacterium,
which can only use cellulose and celllobiose (Kato et al., 2004).

Strain LX-B<sup>T</sup> is the first cultivable isolate accommodated in
the OPB54 lineage. On the basis of phylogenetic, genetic and
physiological properties, the strain represents a novel
species of a novel genus in the phylum <i>Firmicutes</i>. The name <i>Hydrogenispora ethanolica</i> is proposed.

**Description of Hydrogenispora gen. nov.**

<i>Hydrogenispora</i>[Hy.dro.ge.ni.spo'ra.N.L. n. hydrogenum (from
Gr. n. hudōr water; and Gr. v. gennaio to produce) hydrogen;
Gr. n. spora a seed, and, in biology, a spore; Hydrogenispora a
spore-forming, hydrogen-producing bacterium].

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**Fig. 3.** Phylogenetic relationships among strain LX-B<sup>T</sup> and representatives within the bacterial phylum <i>Firmicutes</i>. The 16S rRNA gene sequence of <i>Anaerolinea thermolimosa</i> DSM 16554<sup>T</sup> was used as an out-group. The tree was calculated on the basis of a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). Bar, 0.02 nucleotide changes per sequence position. Numbers at nodes show bootstrap values (%) obtained with 1000 resampling analyses.
Table 1. Characteristics of strain LX-B<sup>T</sup> and members of the related genera *Desulfotomaculum*, *Pelotomaculum* and *Clostridium* within the phylum *Firmicutes*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>OPB54</th>
<th>Order Clostridiales</th>
<th>Family Peptococcaceae</th>
<th>Family Clostridiaceae</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Similarity (%) with strain LX-B&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>86.5</td>
<td>85.6</td>
<td>85.3</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.3–0.5 × 3.0–18.0</td>
<td>1.0 × 3.0–6.0</td>
<td>1.0–1.1 × 2.0–5.0</td>
<td>0.5–1.5 × 5.0–15.0</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Spore location</td>
<td>Terminal</td>
<td>Subterminal to terminal</td>
<td>Paracentral</td>
<td>Subterminal or terminal</td>
</tr>
<tr>
<td>Optimum temperature (°C) for growth (range)</td>
<td>37–45 (20–50)</td>
<td>55 (40–60)</td>
<td>64 (40–65)</td>
<td>55 (30–68)</td>
</tr>
<tr>
<td>Optimum pH for growth (range)</td>
<td>6.0–7.7 (5.0–8.0)</td>
<td>7.1 (5.8–8.2)</td>
<td>7.5 (6.0–7.9)</td>
<td>6.8–7.2 (6.0–8.0)</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>0–2.5</td>
<td>0–1.5</td>
<td>0–2.0</td>
<td>0–1.7</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>56.1</td>
<td>46.8</td>
<td>47.1</td>
<td>46.9</td>
</tr>
<tr>
<td>Major cellular fatty acids</td>
<td>anteiso-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>ND</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, iso-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, iso-C&lt;sub&gt;16:0&lt;/sub&gt;</td>
</tr>
<tr>
<td>Substrate utilization</td>
<td>Carbohydrates</td>
<td>+</td>
<td>–</td>
<td>Only fructose</td>
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<tr>
<td></td>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Lactate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Metabolism</td>
<td>Fermentative</td>
<td>Fermentative, respiratory</td>
<td>Fermentative, respiratory</td>
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<tr>
<td></td>
<td>Electron acceptors</td>
<td>–</td>
<td>Sulfate</td>
<td>Sulfate</td>
</tr>
<tr>
<td></td>
<td>Habitat</td>
<td>Anaerobic sludge</td>
<td>Hot spring</td>
<td>Terrestrial subsurface</td>
</tr>
</tbody>
</table>

Strains: 1, LX-B<sup>T</sup>; 2, *Desulfotomaculum hydrothermale* DSM 18033<sup>T</sup>; 3, *Desulfotomaculum putei* DSM 12395<sup>T</sup>; 4, *Desulfotomaculum carboxydivorans* DSM 14480<sup>T</sup>; 5, *Pelotomaculum thermopropionicum* DSM 13744<sup>T</sup>; 6, *Pelotomaculum terephthalicum* DSM 16121<sup>T</sup>; 7, *Clostridium straminisolvens* DSM 16021<sup>T</sup>. +, Positive; –, negative; ND, no data available.
Strictly anaerobic, non-motile, spore-forming rods. Ferment various sugars but not amino acids or aromatic compounds. Oxygen, nitrate, sulfate, thiosulfate, elemental sulfur, sulfite, antheraquione-2,6-disulfonate, fumarate and Fe (III) nitritolactate do not serve as electron acceptors for growth. The type species is Hydrogenispora ethanolica.

Description of Hydrogenispora ethanolica sp. nov.

Hydrogenispora ethanolica (etha.no’li.ca. N.L. n. ethanol ethanol; L. fem. suff. -ica suffix used with the sense of pertaining to; N.L. fem. adj. ethanolica belonging to ethanol, referring to ethanol, which is produced by the species).

Shows the following characteristics in addition to those given for the genus. Cells are rods, 0.3–0.5 μm in diameter and 3.0–18.0 μm in length. Produces terminal endospores in bulged sporangium. Surface colonies on agar are dark brown and round after cultivation at 37 °C for 1–2 weeks. Grows at 20–50 °C (optimum 37–45 °C), pH 5.0–8.0 (optimum pH 6.0–7.7) and with 0–2.5 % (w/v) NaCl. Yeast extract is not required but stimulates growth. Ferments glucose, maltose, arabinose, fructose, xylose, ribose, sucrose, galactose, mannose, raffinose, pectin, starch, glycerol, fumarate, tryptophan and yeast extract in pure culture. The major end-products from glucose are acetate, ethanol and hydrogen. Sensitive to chloramphenicol, streptomycin and penicillin. The main fatty acids are anteiso-C_{15:0}, iso-C_{14:0} and C_{16:0}. The most abundant polar lipids are diphosphatidylglycerol and phosphatidylglycerol.

The type strain is LX-B^T (DSM 25471^T = JCM 18117^T = CGMCC 1.5175^T), isolated from a mesophilic anaerobic landfill leachate bioreactor.

\[ \text{The DNA G+C content of the type strain is 56.1 mol%.} \]

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References


nov, from a deep terrestrial subsurface, and *Desulfotomaculum luciae* sp. nov, from a hot spring. *Int J Syst Bacteriol* 47, 615–621.


