Anaerobium acetethylicum gen. nov., sp. nov., a strictly anaerobic, gluconate-fermenting bacterium isolated from a methanogenic bioreactor

Yogita Patil, 1 Madan Junghare, 1, 2 Michael Pester, 1 Nicolai Müller 1 and Bernhard Schink 1

A novel strictly anaerobic, mesophilic bacterium was enriched and isolated with gluconate as sole substrate from a methanogenic sludge collected from a biogas reactor. Cells of strain GluBS11T stained Gram-positive and were non-motile, straight rods, measuring 3.0–4.5 × 0.8–1.2 mm. The temperature range for growth was 15–37°C, with optimal growth at 30°C, the pH range was 6.5–8.5, with optimal growth at pH 7, and the generation time under optimal conditions was 60 min. API Rapid 32A reactions were positive for α-galactosidase, α-glucosidase and β-glucosidase and negative for catalase and oxidase. A broad variety of substrates was utilized, including gluconate, glucose, fructose, maltose, sucrose, lactose, galactose, melezitose, melibiose, mannitol, erythritol, glycerol and aesculin. Products of gluconate fermentation were ethanol, acetate, formate, H2 and CO2. Neither sulfate nor nitrate served as an electron acceptor. Predominant cellular fatty acids (>10%) were C14:0, C16:0, C16:1ω7c/iso-C15:0 2-OH and C18:1ω7c. The DNA G+C content of strain GluBS11T was 44.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequence data revealed that strain GluBS11T is a member of subcluster XIVa within the order Clostridiales. The closest cultured relatives are Clostridium herbivorans (93.1% similarity to the type strain), Clostridium populeti (93.3%), Eubacterium uniforme (92.4%) and Clostridium polysaccharolyticum (91.5%). Based on this 16S rRNA gene sequence divergence (>6.5%) as well as on chemotaxonomic and phenotypic differences from these taxa, strain GluBS11T is considered to represent a novel genus and species, for which the name Anaerobium acetethylicum gen. nov., sp. nov. is proposed. The type strain of Anaerobium acetethylicum is GluBS11T (=LMG 28619T =KCTC 15450T =DSM 29698T).

The genus Clostridium is one of the largest genera known among prokaryotes and comprises anaerobic, Gram-positive-staining, endospore-forming bacteria. In past decades, numerous species of the genus Clostridium capable of fermenting gluconate have been studied, e.g. Clostridium aceticum, C. pasteurianum, C. roseum, C. butyricum, ‘C. rubrum’ (C. beijerinckii ATCC 14949) and ‘C. butylicum’ (C. beijerinckii ATCC 14823) (Andreesen & Gottschalk, 1969; Bender et al., 1971). Members of the genus Clostridium have been isolated from soil, sediment, decomposing biological material and the lower gut of mammals (Suresh et al., 2007). Recently, Servinsky et al. (2014) studied a complete metabolic network in Clostridium acetobutylicum for utilization of glucose, gluconate and galacturonate using online databases, previous studies, genomic information and experimental data. Besides members of the genus Clostridium, Escherichia coli (Eisenberg & Dobrógosz, 1967), lactic acid bacteria such as Lactobacillus reuteri and L. mucosae and acid-utilizing bacteria such as Megasphaera elsdenii and Mitsuokella multacida (Tsukahara...
et al., 2002) have been found to grow with gluconate. In the present study, we report the isolation of a gluconate-fermenting, strictly anaerobic bacterium from a methanogenic sludge sample. To determine the taxonomic position of strain GluBS11T, it was subjected to a detailed physiological, chemotaxonomic and phylogenetic characterization.

Strain GluBS11T was isolated from a sludge sample obtained from a biogas reactor in Odendorf, Germany. The reactor converts corn waste to methane gas at 40 °C. An anaerobic slurry/sludge sample obtained from the reactor was diluted directly with anoxic medium and the enrichment culture was started by inoculating a few milliliters of approximately 10-fold-diluted sludge sample into a freshwater medium containing gluconate (10 mM) as a carbon source. Isolation, cultivation and growth experiments were performed in anoxic, bicarbonate-buffered, sulfide-reduced freshwater mineral medium containing (unless indicated otherwise, in g l−1): NaCl, 1.0; MgCl2.6H2O, 0.4; KH2PO4, 0.2; NH4Cl, 0.25; KCl, 0.5; CaCl2.2H2O, 0.15; NaHCO3, 2.5; Na2S9H2O, 1 mM (Widdel & Bak, 1992). The medium (excluding Na2S9H2O and NaHCO3) was autoclaved at 121 °C for 25 min and cooled under an oxygen-free mixture of N2/CO2 (80 : 20). Resazurin (0.4 mg l−1) was added as a redox indicator. Further, 1 ml trace element solution SL-10 (Widdel et al., 1983), 1 ml sele-nate-tungstate (Tschech & Pfennig, 1984) and 1 ml seven-vitamin solution (Pfennig, 1978) were added from concentrated stock solutions. The medium was adjusted to an initial pH of 7.3±0.1 with sterile 1 M NaOH or 1 M HCl. Cultivations and transfer of the strain were performed under a N2/CO2 (80 : 20) atmosphere. The strain was cultivated in the dark at 30 °C.

Pure cultures were obtained by repeated agar (1 %) shake dilutions (Widdel & Bak, 1992). Agar shake tubes were incubated in an inverted position for 1–2 weeks until isolated, visible colonies appeared (Junghare & Schink, 2015). The strain was routinely examined for purity by the KOH test (pasteurization at 80 °C for 2 min followed by 32 cycles of 94 °C for 40 s and 72 °C for 60 s, and final extension at 72 °C for 5 min. The amplified 16S rRNA gene fragment of approximately 1.4 kb was purified using a DNA purification kit (DNA clean and concentrator; Zymo Research) and sequenced at GATC Biotech AG (Konstanz, Germany). The 16S rRNA gene sequences of closely related cultured micro-organisms were obtained using the EzTaxon-e service (Kim et al., 2012), BLAST searches against the non-redundant GenBank database (Altschul et al., 1990) and
the Sequence_Match tool of the Ribosomal Database Project (RDP) (Cole et al., 2014). The taxonomic assignment of strain GluBS11T was performed within the ARB program package (Ludwig et al., 2004). Phylogenetic trees were reconstructed based on 1242 unambiguously aligned sequence positions using the RAxML maximum-likelihood method (Stamatakis et al., 2008) as implemented in ARB. Furthermore, the results of maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods also supported the phylogenetic placement of strain GluBS11T within subcluster XIVa of the order Clostridiales (data not shown). Confidence in the resulting tree topology was evaluated by resampling 1000 bootstrap trees using the RAxML algorithm (Stamatakis et al., 2008).

Growth was monitored by measuring the OD_{600} using a Jenway 6300 spectrophotometer. Utilization of each substrate and fermentation products were analysed and quantified with an HPLC system fitted with an RID detector (LC-prominence; Shimadzu) equipped with an Aminex HPX-87H ion-exchange column (Bio-Rad) and analysed at 60 °C, using 5 mM H_{2}SO_{4} as the mobile phase at a flow rate of 0.6 ml min⁻¹. Analysis of traces of hydrogen gas was performed with a high-sensitivity Peak Performer 1 gas chromatograph (Peak Laboratories) equipped with a reducing compound photometer (RCP). Nitrogen was used as the carrier gas. Qualitative determination of nitrate and nitrite was performed by using colour-developing commercial strips (Qantofix; Macherey-Nagel). Sulfide and nitrite was performed by using colour-developing commercial strips (Qantofix; Macherey-Nagel). Sulfide was determined according to the method described by Cord-Ruwisch (1985). All tests were conducted independently in duplicate.

After 1 week of incubation in agar shake tubes at 30 °C, colonies were opaque, convex and circular with an entire margin and appeared yellow–white in the centre as the colony matured (Fig. S1, available in the online Supplementary Material). The strain did not grow aerobically on LB agar plates, and anoxic, reduced conditions were required for growth. Cells grown with 10 mM gluconate were 3.0–4.5 μm long (sometimes even 10 μm long) and 0.8–1.2 μm wide, non-motile, and occurred in chains, pairs or as single cells (Fig. 1). Spore formation was never observed, not even if cultures were grown with 1 mM manganese sulfate and 30 mM glucose for 2 weeks (further incubation led to cell lysis and death) or at < pH 6. Cells stained Gram-positive, as determined both by the Gram-staining reaction and by the KOH test. The strain reacted negative in tests for catalase and oxidase.

Strain GluBS11T grew at 15–37 °C, with an optimum at 30 °C, and at pH 6.5–8.5, with an optimum at pH 7.2 ± 0.2. When grown on gluconate (10 mM), the doubling time was approximately 1 h at 30 °C (Fig. 2). Glucose, lactose, sucrose, fructose, maltose, xylose, galactose, melibiose, melezitose, gluconate, mannitol, erythritol, glycerol and ascorbic acid were used as carbon sources. Starch, cellulose, cellobiose, pectin and glycogen were not utilized. No growth was observed with ribose, arabinose, rhamnose, raffinose, trehalose, inositol, gelatin, casein, Casamino acids, gum arabic, pyruvate or lactate. Sulfate and nitrate did not serve as electron acceptors. Strain GluBS11T produced acetate (8.4 mM), formate (4.6 mM), ethanol (4.9 mM) and H_{2} (2.1 mM) from the fermentation of gluconate (8.4 mM), as shown in Table 1 (lactate was occasionally detected in the range 0.5–1 mM). No butyrate, propionate, butanol, propanol or methane was formed. Cellular fatty acids included C_{12}:0 (0.68 %), C_{13}:0 3-OH/iso-C_{15}:1 (4.88 %), C_{13}:1 at 12–13 (1.11 %), C_{14}:0 (27.74 %), C_{15}:0 (0.36 %), C_{16}:0 (21.74 %), C_{16}:1o9c (4.85 %), C_{16}:1o5c (0.89 %), C_{16}:1o7c alcohol (0.50 %), C_{16}:1o7c/iso-C_{15}:0 2-OH (16.71 %), C_{17}:1o8c (1.12 %), iso-C_{17}:1 anteiso-C_{17}:1 B (2.81 %), C_{18}:0 (0.55 %), C_{18}:1o9c (11.24 %) and C_{18}:1o9c (2.86 %). The DNA G+C content of the strain was 44.1 mol%.

![Fig. 1. Phase-contrast micrograph of cells of strain GluBS11T grown with gluconate. Bar, 10 μm.](image1)

![Fig. 2. Growth curve, gluconate consumption and fermentation product formation with time for strain GluBS11T.](image2)
Phylogenetic analysis of the almost-complete 16S rRNA gene sequence of strain GluBS11T (1402 bp) using the maximum-likelihood method RAxML (Stamatakis et al., 2008) revealed that it is a member of cluster XIVa within the order Clostridiales (Fig. 3). This was corroborated independently by using the neighbour-joining and maximum-parsimony algorithms as provided in ARB as well as by the EzTaxon server (Kim et al., 2012) and RDP (Wang et al., 2007) classifiers. On the basis of 16S rRNA gene sequence similarity, the type strains of Clostridium populeti (93.3 %), Clostridium herbivorans (93.1 %), Eubacterium uniforme (92.4 %) and Clostridium polysaccharolyticum (91.5 %) are most closely related to strain GluBS11T. All of them belong to cluster XIVa within the order Clostridiales as defined by Collins et al. (1994), and our isolate represents a distinct phyletic line within this cluster. Strain GluBS11T was not associated with the type species of the genus Clostridium, Clostridium butyricum (cluster I). The terminal branching between strain GluBS11T and its closest relatives (as stated above) was not well resolved, as revealed by low bootstrap support (Fig. 3) and alternative phylogenetic tree reconstruction using the neighbour-joining and maximum-parsimony algorithms as implemented in ARB (Ludwig et al., 2004) (data not shown). This indicates that the resolution of the 16S rRNA gene as a phylogenetic marker is not high enough to distinguish speciation events reliably within clostridial cluster XIVa (Warnick et al., 2002). Clostridial cluster XIVa (Collins et al., 1994) contains a diverse assortment of organisms, including the genera Acetitomaculum, Anaerostipes, Butyrivibrio, Catonella, Coprococcus, Dorea, Hespella, Johnsonella, Lachnospira, Lachnobacterium, Moryella, Oribacterium, Parasporobacterium, Pseudobutyribrio, Roseburia, Shuttleworthia, Sporohacterium and Syntrophococcus in addition to a few misclassified clostridial species (Cotta et al., 2009). Strain GluBS11T forms a distinct lineage within this cluster, specifically within the family Lachnospiraceae, and does not display a particularly close affiliation to any of the aforementioned taxa (Fig. 3).

It is currently accepted that 5.5 % 16S rRNA gene sequence divergence is necessary for separation of novel genera from existing ones (Yarza et al., 2014). Our pairwise comparison shows >6.5 % sequence divergence between strain GluBS11T and the most closely related type strains. On the basis of this high 16S rRNA gene sequence divergence and the topologies of the phylogenetic trees, we propose strain GluBS11T to represent a novel genus within the family Lachnospiraceae.

Differential characteristics of strain GluBS11T from the most closely related type strains in clostridial subcluster XIVa (Collins et al., 1994) are summarized in Table 2. Strain GluBS11T may be distinguished from three of its closest cellulolytic relatives, C. herbivorans (Varel et al., 1995), C. polysaccharolyticum (van Gylswyk, 1980; van Gylswyk et al., 1980) and C. populeti (Sleat & Mah, 1985), by its inability to utilize cellulose and cellobiose and its fermentation end product pattern (no butyrate produced). C. herbivorans, C. polysaccharolyticum and C. populeti produce butyrate as a fermentation major product, whereas strain GluBS11T produces formate, acetate and ethanol. Strain GluBS11T can be differentiated phenotypically from the type strains of these three species by its lack of motility (absence of flagella) and its inability to form spores. Strain GluBS11T is also distantly related to E. uniforme (van Gylswyk & van der Toorn, 1985), but can be readily distinguished from it by the fact that E. uniforme has a much lower DNA G+C content (35 mol%), hydrolyses starch and cellobiose and produces lactate as a major fermentation product in addition to formate, acetate and ethanol (Table 2). According to our 16S rRNA gene phylogenetic analyses, strain GluBS11T is more closely related to C. herbivorans and C. populeti (as many members of the Lachnospiraceae are polysaccharolytic and typically produce butyrate as one of the fermentation end products) but, in terms of fermentation pattern, strain GluBS11T appears to be more similar to E. uniforme (van Gylswyk & van der Toorn, 1985), as neither taxon produces butyrate at all. The fatty acid patterns of C. herbivorans and strain GluBS11T show remarkable differences, in particular the presence of a higher percentage of C14:0 (21.7 %) fatty acid in strain GluBS11T, which was present at only 5.3 % in cells of C. herbivorans (Varel et al., 1995). Later, C16:0

<table>
<thead>
<tr>
<th>Substrate utilized (mM)</th>
<th>Max. OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Cell dry mass (mg)</th>
<th>Substrate assimilated (mM)</th>
<th>Substrate dissimilated (mM)</th>
<th>Fermentation products (mM)</th>
<th>Electron recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconate 8.42 (agitated)</td>
<td>1.091</td>
<td>272.7</td>
<td>2.04</td>
<td>5.24</td>
<td>8.40</td>
<td>4.64</td>
</tr>
<tr>
<td>7.03 (static)</td>
<td>0.958</td>
<td>239.5</td>
<td>1.79</td>
<td>3.33</td>
<td>6.99</td>
<td>4.64</td>
</tr>
<tr>
<td>Glucose 3.71 (static)</td>
<td>0.958</td>
<td>239.5</td>
<td>1.79</td>
<td>3.14</td>
<td>2.87</td>
<td>2.80</td>
</tr>
</tbody>
</table>

Table 1. Stoichiometry of substrate fermentation and product formation by strain GluBS11T at 30 °C after ~8 h of incubation.
aldehyde (10.2 %) and C_{14} : 0 dimethyl acetal (22.1 %) (Varel et al., 1995) fatty acids were present in cells of *C. herbivorans* in considerable amounts, but these were completely absent from strain GluBS11\textsuperscript{T}. Besides this, *C. pasteurianum* and *C. butyricum* ferment glycerol to butanol and 1,3-propanediol as main fermentation products, whereas strain GluBS11\textsuperscript{T} ferments glycerol to acetate, formate, ethanol and hydrogen (no butanol formation was detected). Rarely, a very small amount of 1,3-propanediol was detected if the fermentation time was extended for several days.

In conclusion, the novel strain GluBS11\textsuperscript{T} described in our study shows significant differences in phenotype, phylogeny and chemotaxonomic characteristics from previously described closely related type strains within clostridial subcluster XIVa and in particular within the family Lachnospiraceae (Collins et al., 1994). Therefore, we propose that strain GluBS11\textsuperscript{T} represents a novel genus within the family Lachnospiraceae in the order Clostridiales, for which the name *Anaerobium acetethylicum* gen. nov., sp. nov. is proposed.

### Description of Anaerobium gen. nov.

**Anaerobium** (An.a.e.ro‘bi.um. Gr. pref. an indicating a negation; Gr. n. aer air; Gr. n. bios life; N.L. neut. n. Anaerobium an anaerobic life form).

Cells are strictly anaerobic, long rods, found singly or in pairs. Non-spore-forming. Growth is observed with glucose and fructose in addition to a wide range of carbohydrates. The major end products of fermentation include formate, acetate and ethanol. The whole-cell fatty acids consist mainly of a mixture of straight-chain saturated and monounsaturated acids. The DNA G + C content of the type strain of the type species is 44.1mol\%. Comparative analysis of the 16S rRNA gene sequence shows that the genus *Anaerobium* represents a separate lineage...
within the family \textit{Lachnospiraceae}. The type species of the genus is \textit{Anaerobium acetethylicum}.

\textbf{Description of \textit{Anaerobium acetethylicum} sp. nov.}

\textit{Anaerobium acetethylicum} (a.ce.te.thy’l.cum. L. n. \textit{acetum} vinegar; N.L. root \textit{ethyl}- referring to the ethyl moiety, e.g. ethyl alcohol, ethanol; N.L. neut. adj. \textit{acetethylicum} referring to its main fermentation products, acetate and ethanol).

Displays the following properties in addition to those given for the genus. Cells form opaque, circular, convex colonies (Fig. S1) when grown in agar-shake tubes. Cells stain Gram-positive. They measure $3.0 \pm 0.8-1.2$ mm (sometimes cells even 10 mm long are observed; Fig. 1). Cells are non-motile. With 10 mM gluconate at 30 $\degree C$, the doubling time is about 1 h (Fig. 2).

\textbf{Table 2.} Physiological and chemotaxonomic characteristics that differentiate strain GluBS11 $^T$ from its nearest phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source*</td>
<td>Biogas slurry</td>
<td>Intestinal tract of pig</td>
<td>Woody biomass digester</td>
<td>Sheep rumen</td>
<td>Sheep rumen</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Straight rods</td>
<td>Straight rods</td>
<td>Slightly curved rods</td>
<td>Rod-shaped</td>
<td>Short rods</td>
</tr>
<tr>
<td>Motility</td>
<td>Non-motile</td>
<td>Positive</td>
<td>Motile</td>
<td>Motile</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Gram-stain type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum growth temperature ($\degree C$)</td>
<td>30</td>
<td>39-42</td>
<td>36</td>
<td>30-38</td>
<td>38-45</td>
</tr>
<tr>
<td>Sporulation</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>44.1</td>
<td>38*</td>
<td>28*</td>
<td>41.6*</td>
<td>35.2 ± 2</td>
</tr>
<tr>
<td>16S rRNA gene sequence similarity to strain GluBS11 $^T$ (%)*</td>
<td>(100)</td>
<td>93.1</td>
<td>93.3</td>
<td>91.5</td>
<td>92.4</td>
</tr>
</tbody>
</table>

\*Data for reference taxa are from the type strains.

†A/a, Acetate; B/b, butyrate; E/e, ethanol; F/f, formate; L/l, lactate. Upper-case letters indicate major end products and lower-case letters indicate minor end products.
API Rapid 32A reactions are obtained for α-galactosidase, α-glucosidase and β-glucosidase, and weakly positive reactions are observed for β-galactosidase, α-arabinofuranosidase and β-glucuronidase. Negative API Rapid 32A reactions for urease, arginine dihydrolase, β-galactosidase-6-phosphate, N-acetyl-β-glucosaminidase, mannose fermentation, raffinose fermentation, glutamic acid decarboxylase, α-fucosidase, nitrate reduction, indole production, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyrogalactomannan arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Grows with glucose, lactose, sucrose, fructose, maltose, xylose, galactose, melibiose, melezitose, gluconate, mannnitol, erythritol, glycerol and aesculin. No growth with starch, cellulose, cellobiose, pectin, ribose, arabinose, rhamnose, raffinose, trehalose, inositol, gelatin, casein, Casamino acids, gum arabic, glycogen, pyruvate or lactate. Sulfate and nitrate are not reduced. Products of gluconate fermentation are acetate, formate, ethanol and H₂. Major cellular fatty acids (>10 %) are C₁₄:₀, C₁₆:₀, C₁₆:₁ω7c/iso-C₁₅:₀ 2-OH and C₁₈:₁ω7c.

The type strain is GluBS11ᵀ (=LMG 28619ᵀ=KCTC 15450ᵀ=DSM 29698ᵀ), which was isolated from a methanogenic biogas reactor fed with corn silage.

Acknowledgements

The authors thank Dr Dominik Montag for providing the biogas sludge sample and for helpful discussions in analytics. We also thank Antje Wiese for technical support. The authors also thank the DAAD, Bonn, Germany, for a fellowship for M.J.

References


