Herbivorax saccincola gen. nov., sp. nov., a cellulolytic, anaerobic, thermophilic bacterium isolated via in sacco enrichments from a lab-scale biogas reactor

Daniela E. Koeck,† Matthias Mechelke,¹ Vladimir V. Zverlov,¹,² Wolfgang Liebl¹ and Wolfgang H. Schwarz¹

¹Department of Microbiology, Technische Universität München, Emil-Ramann-Str. 4, D-85354 Freising-Weißenstephan, Germany
²Institute of Molecular Genetics, Russian Academy of Science, Kurchatov Sq. 2, 123182 Moscow, Russia

A novel Gram-stain-positive, rod-shaped, anaerobic, thermophilic bacterium, strain GGR1T, was isolated from a thermophilic lab-scale biogas fermenter. The novel organism was effectively degrading crystalline cellulose. It seems to play a role in remineralization of plant biomass by hydrolysing its polysaccharides. 16S rRNA gene comparative sequence analysis demonstrated that the isolate formed a hitherto unknown subline within the family Ruminococcaceae. The closest phylogenetic relative of GGR1T among the taxa with validly published names was Clostridiumthermocellum, sharing 94.3 % 16S rRNA gene sequence similarity. Strain GGR1T was catalase-negative, indole-negative and produced acetate and ethanol as major end-products during fermentative cellulose utilization. The major cellular fatty acids (>1 %) were 16:0 iso fatty acid and 16:0 fatty acid. Cells were rod shaped and grew optimally at 60°C and pH 7.0. The DNA G+C content was 34.9 mol%. A novel genus and species, Herbivoraxsaccincola gen. nov., sp. nov., is proposed on the basis of phylogenetic analysis and physiological properties of the novel isolate. Strain GGR1T (=DSM 101079T=CECT 9155T) represents the type strain for the novel genus and novel species Herbivoraxsaccincola gen. nov., sp. nov.

The recent taxonomic update of the phylum Firmicutes in Bergey’s Manual of Systematic Bacteriology divided the order Clostridiales into ten named families. The latest update from the LPSN (List of Prokaryotic Names with Standing in Nomenclature) includes 12 named families (Parte, 2014). Thereunder is the family Ruminococcaceae, which was described on the basis of phylogenetic analysis of 16S rRNA gene sequences (Euzéby, 2010; Ludwig et al., 2009). The family is morphologically diverse and includes long thin rods, rods, cocci and pleomorphic forms. All species are strictly anaerobic (Ludwig et al., 2009). Sixteen saccharolytic species of the genus Clostridium have been reassigned by Ludwig et al. (2009) to the family Ruminococcaceae. Therefore, the new genus Ruminiclostridium for the respective species of the genus Clostridium was proposed by Yutin & Galperin (2013), but this new genus name has not yet been validly published. Members of the families Lachnospiraceae and Ruminococcaceae are the most abundant bacteria in the microbial community of the gastrointestinal tract of mammals. The common characteristic of this otherwise diverse group is the effective degradation of plant biomass in various environments (Tap et al., 2009).

In this study, we succeeded in isolating a novel cellulose-degrading strain from a thermophilic biogas plant. The results of phylogenetic analyses based on 16S rRNA gene sequences revealed strain GGR1T as a member of a novel genus within the family Ruminococcaceae. In this communication, the taxonomic characterization of this new isolate is described and a novel genus and species is proposed.

Strain GGR1T has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the Spanish Type Culture Collection (CECT, Valencia, Spain). GS2 medium (Johnson et al., 1981) was used in all experimental cultures with the following composition per litre: 6 g yeast extract, 2.9 g K2HPO4,
2.1 g urea, 1.5 g KH₂PO₄, 2.9 g trisodium citrate dihydrate, 1.0 g cysteine hydrochloride monohydrate, 10 g MOPS, 0.1 g MgCl₂·6H₂O, 0.015 g CaCl₂·2H₂O, 0.125 mg FeSO₄·7H₂O and 0.01 % resazurin. Media were prepared in butyl-rubber-stoppered serum bottles under anaerobic conditions (N₂ atmosphere with 10 % hydrogen) and autoclaved.

A 20 l fermenter was operated with cow manure and fed with maize silage at 55 °C. Nylon bags with 50 µm pore size filled with 2 g shredded filter paper (Whatman No. 1) fixed on a special sample pipe were directly inserted into the thermophile lab-scale digester, to enrich cellulolytic bacteria in their natural environment. This in sacco method was originally developed to study the degradation of ruminant feeds in fistulated cows (Mohamed & Chaudhry, 2008). The bags were removed after 7 days and rinsed with 50 ml isotonic solution (0.9 g NaCl l⁻¹) to remove residues of fermenter content and non-adsorbed bacteria. The partly degraded filter paper was inoculated in GS2 medium with fresh filter paper (0.5 %, w/v) and serially diluted to 10⁻⁸. The cultures were incubated at 55 °C until the filter paper substrate showed obvious degradation. The highest dilution with filter paper degradation was diluted to 10⁻⁸ again and transferred into the same medium. After four repetitions of dilution and growth in fresh medium, the resultant enriched culture was serially diluted up to 10⁻⁸. Cultivation on solid medium and isolation of single colonies was performed as previously described (Koeck et al., 2015a).

Cells of strain GGR1T were inspected microscopically with an Axioskop optical microscope (Zeiss). Gram staining was performed in accordance with published procedures (Johnson et al., 1995). The following physiological and biochemical characteristics were studied as described by Koeck et al. (2015a): motility, colony morphology and catalase activity. Further biochemical tests were performed using the Enterobius-Pluri-Test (Liofilchem) in accordance with the manufacturer’s guidelines. Optimal growth temperature (37, 40, 45, 50, 55, 60, 65 and 70 °C) and pH (pH 5.0–9.0 at intervals of 0.5 pH units) were determined as described previously by measuring the cell protein yield of growing cultures (Koeck et al., 2015a). Total protein was measured using the Bradford Protein Assay kit (Thermo Scientific) with bovine serum albumin as standard. All data points represent the analysis of at least three replicates. Tolerance of NaCl concentration was tested in GS2 medium supplemented with 0.2–5 % NaCl (w/v) at intervals of 0.2 %. Growth was measured using the increase in optical density at 600 nm. For the analysis of cellular fatty acids, cell biomass of strain GGR1T was obtained from exponentially growing cells and freshly harvested after 2 days in GS2 medium with 0.5 % cellobiose. Analysis was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Fatty acid methyl esters were obtained by saponification, methylation and subsequent extraction. Identification of the fatty acids relied on the use of the Sherlock Microbial Identification System (MIDI) with fatty acids being identified against the TSBA 4.0 database.

Substrate utilization was tested in GS2 medium supplemented with various carbohydrates at 0.5 % (w/v) (obtained from Sigma Aldrich and listed in Table 1). Positive growth was assessed as described previously (Koeck et al., 2015a). Cell cultures with 0.6 % yeast extract were used as negative control. For each substrate, two technical replicates and a control without inoculation were included. To assess the production of volatile acids and alcohols, strain GGR1T was cultured for 10 days in GS2 medium with 0.5 % cellobiose (w/v). The volatile acid and alcohol content were analysed using a Knauer AZURA HPLC system equipped with an RI detector (ERC Refractomax 520) and a Knauer Eurokat H analytical column (300×8 mm i.d.) and pre-column (30×8 mm i.d.), both running at 60 °C with 5 mM H₂SO₄ as eluent at an isotropic flow of 0.8 ml min⁻¹. The sample volume injected was 50 µl. Hydrogen gas quantification was not possible due to the residual hydrogen in the gas atmosphere used for maintenance of anaerobiosis during media preparation.

For the measurement of extracellular enzyme activities, the strains were grown anaerobically in GS2 medium with 0.5 % (w/v) filter paper as carbon source. Proteins of the supernatant were precipitated with a 60 % saturated (NH₄)₂SO₄ solution as described previously (Koeck et al., 2014b). Aliquots of resolubilized protein (0.3–0.5 µg µl⁻¹) were added to substrate solutions (250 µl) (all substrates tested are listed in Table 2). Dinitrosalicylic acid assay according to Miller (1959) was performed as previously described (Koeck et al., 2014b; Miller, 1959). Assays with the respective substrate without addition of protein solution were used as negative control. Each assay was set up in triplicates and incubated at 55 °C for 24 h.

Genomic DNA was extracted from the culture broth using the innuPREP DNA Mini Kit (Analytik Jena). Amplification of the almost complete 16S rRNA gene of strain GGR1T was carried out as described by Koeck et al. (2015a). The PCR product was purified using the QiAquick gel extraction kit (Qiagen) and sequenced by Eurofins Genomics (Ebersberg, Germany). The resulting nucleotide sequences were compared with the GenBank/EMBL nucleotide sequence databases using the BLASTn algorithm (blast.ncbi.nlm.nih.gov) and the Ribosomal Database Project (RDP) database (http://rdp.cme.msu.edu/). Sequence similarity calculation was carried out using the EZTaxon program (Kim et al., 2012). The sequences were aligned by the CLUSTALW algorithm (Sievers et al., 2011). Analysis of the sequences was performed with the software package MEGA6 version 6.0 (Tamura et al., 2013). Maximum-likelihood-based treeing tools (Stamatakis, 2006) were used for tree generation, and bootstrapping (Felsenstein, 1985) was used to estimate the reliability of the phylogenetic reconstruction.

16S rRNA gene sequences of the most closely related type strains of species with validly published names were selected for tree reconstruction according to the Living Tree Project (LTP) (Munoz et al., 2011) and List of Prokaryotic Names (LPNS) with Standing in Nomenclature (Parte, 2014).
Table 1. Phenotypic characteristics that differentiate strain GGR1T from members of phylogenetically related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Biogas plant</td>
<td>Widespread</td>
<td>Soda lake</td>
<td>Rice straw</td>
<td>Sludge</td>
<td>Wood-processing fermenter</td>
<td>Freshwater</td>
<td>Compost</td>
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<tr>
<td>Temp. for growth (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Range</td>
<td>45–65</td>
<td>50–68</td>
<td>18–42</td>
<td>45–65</td>
<td>40–65</td>
<td>20–45</td>
<td>20–40</td>
<td>ND</td>
</tr>
<tr>
<td>Optimum</td>
<td>55–60</td>
<td>60–64</td>
<td>35–40</td>
<td>50–55</td>
<td>55–60</td>
<td>35</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>Variable</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>+/−</td>
<td>+/−</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Utilization of:</td>
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<tr>
<td>Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Xylan</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>35</td>
<td>38–39</td>
<td>30</td>
<td>41</td>
<td>37</td>
<td>40</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>16S rRNA gene sequence similarity (%) compared with strain 1</td>
<td>100</td>
<td>94.3</td>
<td>93.9</td>
<td>93.7</td>
<td>93.2</td>
<td>92.6</td>
<td>92.3</td>
<td>90.3</td>
</tr>
</tbody>
</table>

*Lower case letters indicate low concentration. F, formate; A, acetate; P, propionate; L, lactate; B, butyrate; E, ethanol.

The DNA G+C content of the microbial genome was determined by DNA extraction and subsequent whole genome sequencing, which was performed using an Illumina MiSeq system as described previously (Koeck et al., 2014a).

The cells of strain GGR1T were Gram-stain-positive, spore-forming rods. Motility could not be observed during light microscopy of different growth phases, however, without strict oxygen exclusion. Colonies on solid GS2 medium were circular, about 1–2 mm in diameter and dark yellow with an entire margin and a smooth surface. None of the several isolates of this species could grow under aerobic conditions (O2 concentrations >4 %).

The detailed results of substrate utilization, growth condition analysis and other phenotypic characteristics are summarized in Table 1 and in the species description. Strain GGR1T tested negative for the following biochemical properties: sulfate reduction, indole production, catalase expression, lactose fermentation, decarboxylation of ornithine and lysine, dulcitol fermentation, deamination of phenylalnine and degradation of citrate and urea. The major fatty acids (>1 %) of strain GGR1T were 16 : 0 iso fatty acid, 16 : 0 fatty acid and an unknown fatty acid, which together accounted for 62 % of the total fatty acids (Table 3).

16S rRNA gene sequence analysis showed that the novel strain is a member of the family Ruminococcaceae (phylum Firmicutes). Sequence similarity calculations indicated that strain GGR1T shared 94.3 % 16S rRNA gene sequence similarity with Clostridium thermocellum ATCC 27405T as the most closely related strain representing a species with a validly published name. The position in the phylogenetic tree and the low identity scores justify the description of strain GGR1T as a representative of a novel genus within the family Ruminococcaceae (Fig. 1). The 90.3–94.3 % range of overall 16S rRNA gene sequence similarity between strain GGR1T and the other related taxa of the family Ruminococcaceae supports this further. Their affiliation is clearly below the genus level (Table 1) (Tindall et al., 2010). Based on the phylogenetic analysis, strain GGR1T is proposed to be the
The cellulolytic and hemicellulolytic activities of culture supernatants from strain GGR1 were tested and compared with activities in *C. thermocellum* using several substrates, each supplied at 1 % (w/v) (Table 2). The cellulolytic activity of the GGR1 supernatant enzymes on crystalline cellulose was 0.05 U mg⁻¹, which is not too far from that for *C. thermocellum* ATCC 27405 (0.09 U mg⁻¹). Strain GGR1 differs from other recently isolated cellulolytic bacteria from biogas plants. It shows a significantly higher activity of the GGR1 supernatant enzymes on crystalline cellulose compared with other recently isolated cellulolytic bacteria from biogas plants. It shows a significantly higher activity of the GGR1 supernatant enzymes on crystalline cellulose compared with other recently isolated cellulolytic bacteria from biogas plants.

Analysis of the genome sequence and of the proteins of the GGR1 supernatant enzymes on crystalline cellulose revealed a cellulosome-like extracellular, macromolecular complex, which is also found with other related members of the family Ruminococaceae (for example *C. thermocellum, C. clariflavum*) (data not shown). The cellulosome is the most efficient cellulase system discovered to date (Schwarz, 2001). The genome sequence of *Herbinix hemicellulosilytica* T3/55 showed no cellulosomal genes (Koeck et al., 2015b). Due to its higher degradation ability, isolate GGR1 appears to play an important role in cellulose degradation in biogas plants and to be involved in the natural carbon cycle by actively degrading plant biomass.

**Description of Herbivorax gen. nov.**

*Herbivorax* (Her.bi.vo’rax. L. fem. n. herba plant; L. adj. vorax voracious; N.L. fem. n. *Herbivorax* feeding on plant biomass, pertaining to the degradation of plant cellulose fibers in anaerobic digesters).

Cells are Gram-stain-positive, regular-shaped rods with rounded ends. Spores are formed. Strictly anaerobic without growth under aerobic conditions. Catalase reaction is negative. Indole-negative and saccharolytic. The major metabolic end-products are ethanol and acetic acid. Phylogenetically the genus belongs to the family Ruminococaceae. The type species is *Herbivorax saccincola*.

**Table 2.** Specific activity (U mg⁻¹) of supernatant proteins from isolate GGR1 on various polymeric substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Avicel</th>
<th>Xylan</th>
<th>β-Glucan</th>
<th>CM-cellulose</th>
<th>PASC</th>
<th>Arabinxyolan</th>
<th>Xyloglucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGR1 T</td>
<td>0.05±0.003</td>
<td>4.57±0.12</td>
<td>5.88±0.16</td>
<td>0.17±0.009</td>
<td>0.18±0.034</td>
<td>5.83±0.16</td>
<td>4.33±0.16</td>
</tr>
</tbody>
</table>

Xylan was obtained from hardwood, xyloglucan from tamarind, β-glucan from barley (β-1,3-1,4-glucan); PASC, phosphoric acid swollen cellulose (prepared from Avicel).
The type strain is GGR1\textsuperscript{T}.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:0 ISO FA</td>
<td>3.3</td>
</tr>
<tr>
<td>15:0 ANTEISO FA</td>
<td>1.1</td>
</tr>
<tr>
<td>16:0 ALDE</td>
<td>1.2</td>
</tr>
<tr>
<td>15:0 ISO DMA</td>
<td>3.4</td>
</tr>
<tr>
<td>16.0 ISO FA</td>
<td>33.1</td>
</tr>
<tr>
<td>16:1 CIS 9 FA</td>
<td>2.8</td>
</tr>
<tr>
<td>16:0 FA</td>
<td>10.5</td>
</tr>
<tr>
<td>16:0 DMA</td>
<td>5.6</td>
</tr>
<tr>
<td>17:0 ISO FA</td>
<td>8.5</td>
</tr>
<tr>
<td>17:0 ANTEISO FA</td>
<td>2.1</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>1.3</td>
</tr>
<tr>
<td>Summed feature 10*</td>
<td>1.6</td>
</tr>
<tr>
<td>Unknown</td>
<td>18.4</td>
</tr>
</tbody>
</table>

\*Summed feature 3 contained 15:0 ISO ALDE and an unknown fatty acid; summed feature 10 contained 18:1 c11/0/9/6 FA and an unknown fatty acid.

**Description of Herbivorax saccincola sp. nov.**

*Herbivorax saccincola* (sacc.in’co.la. L. n. saccus a bag; L. n. incola an inhabitant; N.L. masc. n. saccincola inhabiting a bag, pertaining to isolation of the type strain by accumulation in nylon bags).

Gram-stain-positive, strictly anaerobic and thermophilic bacterium. Cells are long, thin rods, 5.0–10.0 μm long and 0.2 μm wide, form endospores and are not motile. Colonies on GS2 agar plates are circular, about 1–2 mm in diameter and dark yellow with an entire margin and a smooth surface. Cells grow at 45–65°C, at pH 6.5–9.0 and with 0–2% (w/v) NaCl; optimum growth occurs at 60°C, pH 7.0 and 0.2% NaCl. Catalase is absent and indole is not produced. Urea and casein are not hydrolysed. Glucose, galactose, sorbitol, xylan, xylose, cellobiose and cellulose are utilized as sole carbon source. Major fermentation products are ethanol, acetic acid and hydrogen. The following compounds are not utilized: mannose, fructose, arabinose, sucrose, starch and lactose. Negative for the following biochemical properties: sulfate reduction, decarboxylation of ornithine and lysine, dulcitol fermentation, deamination of phenylalanine and degradation of citrate. The major fatty acids (>1%) are 16:0 iso fatty acid, 16:0 fatty acid and an unknown fatty acid.

The type strain is GGR1\textsuperscript{T} (=DSM 101079\textsuperscript{T}=CECT 9155\textsuperscript{T}), isolated from a thermophilic lab-scale biogas fermenter near Munich, Germany. The G+C content of the genomic DNA of the type strain is 34.9 mol%.

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**References**


