Herbinix hemicellulosilytica gen. nov., sp. nov., a thermophilic cellulose-degrading bacterium isolated from a thermophilic biogas reactor

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Phenotypic and phylogenetic studies were performed on new isolates of a novel Gram-stain-positive, anaerobic, non-sporulating, rod-shaped bacterium isolated from a thermophilic biogas plant. The novel organisms were able to degrade crystalline cellulose. 16S rRNA gene comparative sequence analysis demonstrated that the isolates formed a hitherto unknown subline within the family Lachnospiraceae. As a representative of the whole group of isolates, strain T3/55T was further characterized. The closest relative of T3/55T among the taxa with validly published names is Mobilitalea sibirica, sharing 93.9 % 16S rRNA gene sequence similarity. Strain T3/55T was catalase-negative, indole-negative, and produced acetate, ethanol and propionic acid as major end products from cellulose metabolism. The major cellular fatty acids (≥1 %) were 16 : 0 dimethyl acetal, 16 : 0 fatty acid methyl ester and 16 : 0 aldehyde. The DNA G + C content was 36.6 mol%. A novel genus and species, Herbinix hemicellulosilytica gen. nov., sp. nov., is proposed based on phylogenetic analysis and physiological properties of the novel isolate. Strain T3/55T (DSM 29228T = CECT 8801T), represents the type strain of Herbinix hemicellulosilytica gen. nov., sp. nov.

The order Clostridiales has traditionally been described by its morphological characteristics, such as spore formation and anaerobic growth. The classification and naming of novel species on the basis of 16S rRNA gene sequences has led to severe discrepancies concerning the relationship between taxa within the order Clostridiales (Collins et al., 1994). The recent taxonomic update of the phylum Firmicutes in Bergey’s Manual of Systematic Bacteriology divided the order Clostridiales into 10 named families. Nine additional families were identified as incertae sedis in an effort to regroup species positioned outside the named families (Collins et al., 1994; Ludwig et al., 2009). In the past decades, studies of 16S rRNA gene sequence diversity in metagenomic approaches have led to the identification of thousands of novel taxa, while knowledge about the roles of novel species in their environments is still limited (DeLong, 2009). For various groups with confusing taxonomic structure, such as the class Clostridia, linking the phylogeny of novel, uncultured taxa to possible ecological roles and physiological characteristics requires extensive isolation of new strains and their characterization – these are time-consuming experiments (Biddle et al., 2013).

Members of the family Lachnospiraceae, within the order Clostridiales, are often identified in mammalian gastrointestinal tract microbiomes (Meehan & Beiko, 2014), particularly in ruminants (Kittelmann et al., 2013) and humans (Gosalbes et al., 2011). The family Lachnospiraceae was first proposed by Rainey (2009) and is described on the basis of phylogenetic analysis of 16S rRNA gene sequences (Euzéby, 2010; Ludwig et al., 2009). It currently comprises 25 genera (Parte, 2014). The family is morphologically diverse and includes rods, vibrios and cocci. All species are strictly anaerobic and primarily non-spor-forming. As shown in the ‘Road map’ of Bergey’s Manual of Systematic Bacteriology, a number of the type strains of species with validly published names of the genera Acetivibrio, Anaerospirobacter, Bacteroides, Blautia, Clostridium, Desulfovomaculum,
Eubacterium, Fusobacterium, Howardella, Lactobacillus, Lactonifactor and Ruminococcus — currently classified in families other than Lachnospiraceae — are phylogenetically intermingled with members of the latter family. Yutin & Galperin (2013) proposed a new genus ‘Lachnoclostridium’ for the respective species of the genus Clostridium.

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 that strain T3/55T and other closely related isolates were members of a novel genus within the family Lachnospiraceae. In this communication, the taxonomic characterization of this new isolate is described and a novel genus and species are proposed.

The strain T3/55T has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the Belgium Coordinated Collections of Microorganisms (BCCM/LMG, Gent, Belgium). The strains Clostridium thermocellum DSM 1237T (=ATCC 27405T) and Clostridium stercorarium DSM 8532T were used for reference. They were obtained from the DSMZ.

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 and autoclaved.

New strains were isolated from a thermophilic biogas reactor in Viersen, Germany (GPS coordinates: 51° 13’ 36.5” N 6° 19’ 35.8” E). A total of 1 ml of the sludge was suspended in 50 ml NaCl solution (0.2 M) and serially diluted to 10−8. Each dilution was freshly inoculated into autoclaved, anaerobic GS2 medium with 0.5 % (w/v) filter paper (Whatman No. 1) as the main carbon source and 0.5 % (v/v) rumen extract to enrich cellulolytic microbes. The culture was incubated at 55 °C until the filter paper 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 10−4. An aliquot (0.1 ml) of the dilution was plated on a GS2 agar plate (2 % agar) containing 0.05 % (w/v) cellobiose as the carbon source and overlaid with GS2 agar (2 %, w/v) containing 0.5 % (w/v) PASC (phosphoric acid swollen cellulose) (Wood, 1988) in an anaerobic chamber (95 % N2 + 5 % H2; Coy Laboratory Products). The agar plates were incubated anaerobically at 55 °C. Colonies producing clear haloes due to cellulolytic activity were streaked to single colonies three times. Single colonies were picked and inoculated into GS2 medium with filter paper to confirm cellulose degradation and purity.

Genomic DNA was extracted from the culture broth using a bacterial genomic DNA kit (Molzym). PCR amplification and sequencing of the 16S rRNA genes were carried out as described previously (Koeck et al., 2014). The resulting nucleotide sequences were inserted into a 16S rRNA ARB (Ludwig et al., 2004) seed database containing an alignment of a non-redundant dataset comprising about 40 000 complete high-quality primary structures extracted from the SILVA 119 SSU Ref NR release (Quast et al., 2013). All available 16S rRNA gene sequences representing type strains of species with validly published names were selected according to the Living Tree Project (LTP) (Munoz et al., 2011) and List of Prokaryotic Names with Standing in Nomenclature (LPSN; Parte, 2014). Quality-checking and filtering were performed by applying the quality, chimera and operational taxonomic unit (OTU) tools of the ARB software package (Westram et al., 2011); these were also performed manually. A subset of about 3000 sequences comprising all sequences representing members of the family Lachnospiraceae in the seed database as well as primary structures from selected representatives of other major groups of the phylum Firmicutes and the other bacterial and archaeal phyla was used for tree reconstruction. A selection of different conservation profile-based (alignment) column filters, alternative models (of evolution) in combination with distance matrix-, maximum-parsimony-, and maximum-likelihood-based treeing tools (Ludwig et al., 2004; Stamatakis, 2006) available within the ARB package were used for tree generation. Evaluation of significance and support of tree topologies, as well as tree presentation, was performed by applying the consensus tree tool of the ARB package. Positioning of branches representing lower quality type strain sequences of relevance for the family Lachnospiraceae in evaluated and optimized trees was performed by applying the ARB parsimony tool with the option of maintaining the initial tree topology.

Cells of strain T3/55T were inspected microscopically with an Axioskop optical microscope (Zeiss). Gram staining was performed according to published procedures (Johnson et al., 1995). The motility of strain T3/55T was observed by light microscopy. Endospore formation was assessed using light microscopy and recultivability after pasteurization (15 min, 80 °C). Colony morphology was observed using cells grown on GS2 agar plates for 4 days at 55 °C. For high-resolution field emission scanning electron microscopy, preparations of freshly harvested cells were fixed with 2.5 % glutaraldehyde in 75 mM cacodylate buffer containing 25 mM NaCl and 2 mM MgCl2 (pH 7.0). Drops of the fixed samples were placed onto carbon-coated glass slides, covered with a coverslip, and rapidly frozen with liquid nitrogen. The coverslip was removed with a razor blade, and the glass slide was immediately post-fixed with 2.5 % (w/v) glutardialdehyde in fixative buffer, washed, post-fixed with 1 % osmium tetroxide, washed with buffer and then with distilled water, dehydrated in a graded series of acetone solutions, and then critical-point dried after transfer to liquid CO2. Specimens were mounted on stubs, coated with 3 nm...
platinum using a magnetron sputter coater, and then
examined with a Zeiss Auriga scanning electron micro-
scope operated at 1 kV (Wanner et al., 1989).

The presence of catalase was determined by dropping 10 %
(v/v) H2O2 on top of bacterial cells on a microscope slide.
Biochemical tests were performed using the EnteroPluri-
Test (Liofilchem) in accordance with the manufacturer’s
instructions. Casein hydrolysis was tested with GS2 agar
plates containing 0.25 % casein. Optimal growth tempera-
ture and pH were determined by measuring the cell protein
yield of growing cultures in GS2 medium containing 0.2 %
(w/v) Avicel after incubation for 6 days (Reveneau et al.,
2003). The cell protein yield was determined after recover-
ing bacterial cells by centrifugation, washing the pellets
twice with isotonic NaCl, and boiling in 1 % (w/v)
CHAPS detergent containing 0.3 M NaCl for 20 min.
Total protein was measured using a Bradford Protein
Assay kit (Thermo Scientific) with BSA as standard. All
data points represent the analysis of at least three replicates.
Tolerance of salinity was tested in GS2 medium sup-
plemented with 0.2–2.2 % (w/v) NaCl at intervals of
0.2 %. Growth was measured using the increase in optical
density at 600 nm. Cellular fatty acid analysis was per-
formed by the Identification Service of DSMZ. Cells freshly
harvested after 2 days of growth in GS2 medium contain-
ing 0.5 % cellobiose were used. The methyl esters were
extracted and analysed in accordance with the standard
protocol of the Sherlock Microbial Identification System
(version 6.1; MIDI).

To assess the production of volatile acids and alcohols,
strain T3/55T was cultured for 10 days in GS2 medium
supplemented with various carbohydrates at 0.5 % (w/v)
obtained from Sigma Aldrich. Positive growth was
assessed visually by comparing the turbidity of the
medium with a non-inoculated control of the same sub-
strate. To confirm positive results, the isolates were trans-
ferred into medium with the same substrate. The cell
protein yield of every growth reaction was measured as
described above. For each substrate, two technical replicates
and a control without inoculation were included. The
volatile acid and alcohol content was measured with a gas
chromatography system (GC-2010; Shimadzu) equipped
with a flame-ionization detector. The samples were separ-
ated using a temperature gradient from 70 to 260 °C with
a Phase-Stabilwax-DA capillary column (Shimadzu) in
accordance with the manufacturer’s protocol. Hydrogen
gas quantification was not possible due to the residual
hydrogen in the gas atmosphere used for maintenance of
anaerobiosis during media preparation.

To determine hydrolytic activity of secreted proteins, all
newly isolated strains were grown in 50 ml GS2 medium
with filter paper as the sole carbon source until substrate
depletion. The bacterial culture was centrifuged (5000
g, 30 min, 4 °C), and the clarified culture supernatant was
incubated overnight at 4 °C with a 60 % saturated
(NH4)2SO4 solution. The precipitated protein was har-
vested by centrifugation (5000 g, 30 min, 4 °C), redissolved
in 0.1 M MOPS buffer (pH 6.5), and concentrated to
0.2–0.7 mg ml−1. As described above, total protein was
measured with a Bradford Protein Assay kit (Thermo
Scientific). Aliquots (15 μl) of the protein solutions (con-
taining 0.3–0.5 μg protein μl−1) were added to substrate
solution (250 μl) containing 0.5 % (w/v) Avicel and 10 ×
reaction buffer (50 μl) and diluted with water to a total
volume of 500 μl. All hydrolytic reactions, which were per-
formed in triplicate, were incubated at 60 °C for 24 h. The
samples were analysed for the presence of reducing sugar
using the DNSA assay with glucose for assay standardiz-
ation (Miller, 1959).

The DNA G+C content of the microbial genome was
determined by DNA extraction and subsequent whole
gene sequencing, which was established on the Illumina
MiSeq system (Koeck and others, unpublished results).
Analysis and interpretation of the genome sequence was
performed with GenDB (Meyer et al., 2003).

As a representative of the whole group of isolates, strain T3/55T
was further characterized. The cells were Gram-stain-positive,
Table 1. Phenotypic characteristics that differentiate strain T3/55\textsuperscript{T} from phylogenetically related species

Strains: 1, T3/55\textsuperscript{T}; 2, \textit{Mobilitalea sibirica} DSM 26468\textsuperscript{T} (data from Podosokorskaya et al., 2014); 3, \textit{Clostridium jejuense} DSM 15929\textsuperscript{T} (Jeong et al., 2004); 4, \textit{Clostridium xylanovorans} DSM 12503\textsuperscript{T} (Mechichi et al., 1999); 5, \textit{Clostridium aminovalericum} DSM 1283\textsuperscript{T} (Hardman & Stadtman, 1960); 6, \textit{Clostridium phytofermentans} ATCC 700394\textsuperscript{T} (Warnick et al., 2002); 7, \textit{Clostridium herbivorans} ATCC 49925\textsuperscript{T} (Varel et al., 1995); 8, \textit{Clostridium polysaccharolyticum} ATCC 33142\textsuperscript{T} (Van Gylswyk, 1980); 9, \textit{Clostridium populeti} ATCC 35295\textsuperscript{T} (Sleat & Mah, 1985); 10, \textit{Eubacterium xylanophilum} ATCC 35991\textsuperscript{T} (Van Gylswyk & Vandertoorn, 1985); 11, \textit{Anaerosporobacter mobilis} DSM 15930\textsuperscript{T} (Jeong et al., 2007). All taxa are anaerobic rods that utilize glucose and cellobiose but do not utilize sorbitol. W, Weakly positive; ND, no data.

<table>
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<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>Hot water well</td>
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<td>Methanogenic digester</td>
<td>Sewage sludge</td>
<td>Forest soil</td>
<td>Intestinal tract of pigs</td>
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<td>37</td>
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<td>7.0</td>
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<td>7.0</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
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<td>Gram reaction</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>–</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>Galactose</td>
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<td>+</td>
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<td>–</td>
<td>W</td>
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<td>–</td>
<td>+</td>
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<td>–</td>
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<td>Sucrose</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>37</td>
<td>33</td>
<td>41</td>
<td>40</td>
<td>28</td>
<td>36</td>
<td>38</td>
<td>42</td>
<td>28</td>
<td>39</td>
<td>41</td>
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<tr>
<td>16S rRNA gene sequence similarity (%) compared with T3/55\textsuperscript{T}</td>
<td>100</td>
<td>93.9</td>
<td>92.7</td>
<td>92.3</td>
<td>91.5</td>
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<td>91.5</td>
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*Filter paper (Whatman No. 1).
non-spore-forming rods. Rods had an irregular shape and lost their cell integrity during prolonged incubation, which resulted in a complete collapse of the cells after only 5 days of cultivation in GS2 medium (Fig. 1). Peritrichous flagella were observed in high-resolution scanning electron micrographs. This result was supported by preliminary genomic analysis, which revealed all genes essential for flagella synthesis and cell motility. However, motility could not be observed during light microscopy of different growth phases. Colonies on solid GS2 medium were convex, about 2–5 mm in diameter, circular, and had a glossy white colour with a translucent area towards the slightly undulating margin. None of the isolates 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. Examination of strain T3/55T for the following properties yielded a negative result: sulfate reduction, indole production, catalase expression, lactose fermentation, decarboxylation of ornithine and lysine, dulcitol fermentation, deamination of phenylalanine and degradation of citrate. The major cellular fatty acids (>1 %) detected in the dried cell material of strain T3/55T were 16:0 dimethyl acetal (DMA), 16:0 fatty acid methyl ester (FAME) and 16:0 aldehyde (ALDE) (Table S1).

The results of the 16S rRNA-based analyses clearly identify biogas plant isolate T3/55T as a member of the family Lachnospiraceae. The position of the respective branch within the edges of the radiation representing the taxa assigned to the family in the consensus tree in Fig. S1 indicates no closer relationship to any of them. Fig. 2 shows an extract of the comprehensive consensus tree (Fig. S1). The 86.2–93.9 % range of overall 16S rRNA gene sequence similarity between strain T3/55T and the other taxa of the family Lachnospiraceae support the situation. The highest overall 16S rRNA gene sequence similarity (93.9 %) is shared by the strains T3/55 and Mobilitalea sibirica DSM 26468T (Podosokorskaya et al., 2014). According to the present and previous (Ludwig et al., 2009) 16S rRNA gene based phylogenetic analyses, a number of the type strains of species with validly published names of the genera Acetivibrio, Anaerospirobacter, Bacteroides, Blautia, Clostridium, Desulfovomaculum, Euibacterium, Fusobacterium, Howardella, Lactobacillus, Lactonifactor and Ruminococcus – currently classified in families other than Lachnospiraceae – are found in the radiation of branches representing members of the latter family. For some of them, rooting in the periphery of the Herbinix gen. nov. branch is supported by the majority of treeing analyses (Fig. S1). Their affiliations, however, are clearly below the genus level (Table 1). Thus, the phylogenetic analysis and the moderate identity scores justify the description of a novel genus and species, Herbinix hemicellulosilytica gen. nov., sp. nov., with isolate T3/55T as the representative type strain.

There are also several phenotypic characteristics that separate strain T3/55T from phylogenetically related species. In particular, the optimal growth temperature of closely related strains such as M. sibirica DSM 26468T is mesophilic, whereas the new isolate showed optimal growth at 55 °C. T3/55T also had a significantly lower DNA G+C content (36.6 mol%) than Clostridium xylanovorans DSM 12503T (40 mol%) and Clostridium jejuense DSM 15929T (41 mol%). Fatty acid content profiling also clearly differentiated the novel isolate from its relatives (Table 1). The
major cellular fatty acid of *M. sibirica* DSM 26468<sup>T</sup> for example is 16:0 (Podosokorskaya et al., 2014).

Strain T3/55<sup>T</sup> hydrolysed various cellulosic substrates (Whatman filter paper No. 1, PASC, CM-cellulose). Additionally, the hemicellulolytic activity of culture supernatants was tested using several hemicellulosic substrates, each supplied at 1 % (w/v) (Table S1). High hemicellulolytic activity of T3/55<sup>T</sup> could be detected on xylans. Cellulolytic activity of T3/55<sup>T</sup> culture supernatant proteins on crystalline cellulose (Avicel) was present but 11-fold lower than that of *C. thermocellum* and 3.75-fold lower than that of *C. stercorarium* (data not shown).

### Description of *Herbinix* gen. nov.

*Herbinix* (Heribi.nix. L. fem. n. herba plant; L. fem. n. nix snow; N.L. fem. n. *herbinix* snow-white of plants, pertaining to incomplete degradation of plant cellulose fibres to white powder resembling a snow-like appearance).

Cells are Gram-stain-positive, irregularly shaped rods with rounded, sometimes cigar-shaped ends. No spores are formed. Strictly anaerobic and no growth is observed under aerobic conditions. The catalase reaction is negative. Indole-negative and saccharolytic. Capable of hydrolysing urea but not casein. The major metabolic end-products are ethanol, acetic acid, and small amounts of propionic acid. *Herbinix hemicellulosilytica* nov.

Description of *Herbinix hemicellulosilytica* sp. nov.

*Herbinix hemicellulosilytica* | he.mi.cell.lu.lo.si.ly’t.i.ca. N.L. neut. n. *hemicellulosilytica* hemicellulose; N. L. adj. *lyticus* -a -um (from Gr. adj. *lytikos* -e -on) able to loosen, able to dissolve; N.L. adj. *hemicellulosilytica* hemicellulose dissolving].

Gram-stain-positive, strictly anaerobic and thermophilic bacterium. Cells are straight to irregularly shaped rods, 2.0–6.0 μm long and 0.5 μm wide, which do not form endospores. Non-motile, although peritrichous flagella are present (however, microscopic inspection was not conducted under strictly anaerobic conditions). Colonies on GS2 agar plates are convex, about 2–5 mm in diameter, circular, and have a glossy white colour with a translucent area towards the slightly undulated margin. Cells grow at 45–62 °C, at pH 6.5–9.5 and with 0–0.9 % (w/v) NaCl; optimum growth occurs at 55 °C and pH 7.0. Catalase is absent, and indole is not produced. Urea is hydrolysed, but not casein. Glucose, fructose, mannose, arabinose, sucrose, soluble starch, xylan, xylose, cellobiose and cellulose can be utilized as a sole carbon source. Major fermentation products of these substrates are ethanol, acetic acid and propionic acid (0.001–0.002 %). The following compounds are not utilized: inulin, galactose, sorbitol, pectin and lactose.

The type strain is T3/55<sup>T</sup> (=DSM 29228<sup>T</sup>=CECT 8801<sup>T</sup>), isolated from a thermophilic biogas plant in Viersen, Germany. The G+C content of genomic DNA of the type strain is 36.6 mol%.

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### References


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