Acetobacter lambici sp. nov., isolated from fermenting lambic beer

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An acetic acid bacterium, strain LMG 27439ᵀ, was isolated from fermenting lambic beer. The cells were Gram-stain-negative, motile rods, catalase-positive and oxidase-negative. Analysis of the 16S rRNA gene sequence revealed the strain was closely related to Acetobacter okinawensis (99.7 % 16S rRNA gene sequence similarity with the type strain of this species), A. ghanensis (99.6 %), A. syzygii (99.6 %), A. fabarum (99.4 %) and A. lovaniensis (99.2 %). DNA–DNA hybridization with the type strains of these species revealed moderate DNA–DNA hybridization values (31–45 %). Strain LMG 27439ᵀ was unable to grow on glycerol or methanol as the sole carbon source, on yeast extract with 10 % ethanol or on glucose-yeast extract medium at 37 °C. It did not produce acid from L-arabinose, D-galactose or D-mannose, nor did it produce 2-keto-D-gluconic acid, 5-keto-D-gluconic acid or 2,5-diketo-D-gluconic acid from D-glucose. It did not grow on ammonium as the sole nitrogen source and ethanol as the sole carbon source. These genotypic and phenotypic data distinguished strain LMG 27439ᵀ from established species of the genus Acetobacter, and therefore we propose this strain represents a novel species of the genus Acetobacter. The name Acetobacter lambici sp. nov. is proposed, with LMG 27439ᵀ (=DSM 27328ᵀ) as the type strain.

The genus Acetobacter belongs to the family Acetobacteraceae within the class Alphaproteobacteria and currently comprises 23 species with validly published names. The ability of species of the genus Acetobacter and all other acetic acid bacteria (AAB), except for members of the genus Asaia, to oxidize ethanol to acetic acid or to carbon dioxide and water under neutral or slightly acidic conditions enables their growth in fermented foods and beverages (Cleenwerck et al., 2002). This growth capacity can be detrimental, for instance when it leads to spoilage of lager or ale beers, wines or ciders, as well as beneficial, for instance in the production of vinegar, fermented cocoa, kombucha, red sour ales or lambic beers (Bartowsky & Henschke, 2008; Bokulich et al., 2012; Martens et al., 1991; Martens et al., 1997; Papalexandratou et al., 2011; Raspor & Goranovic, 2008; Vaughan et al., 2005).

Strain LMG 27439ᵀ was isolated during a study of the fermentation process of acidic lambic beers. The latter beers are the product of a spontaneous fermentation, which progresses for at least two years in wooden casks. Strain LMG 27439ᵀ was isolated on acetic acid medium (AAM), an AAB enrichment medium which consists of 1 %

Abbreviations: AAB, acetic acid bacteria; FAME, fatty acid methyl esters; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, dnaK, groEL and rpoB gene sequences of Acetobacter lambici sp. nov. LMG 27439ᵀ are HF969863, HG329531, HG329543 and HG329555, respectively; those for the 16S rRNA gene sequences of Acetobacter lambici sp. nov. LMG 27440, R-50193 and R-50194 are HG329567–HG329569, respectively. The GenBank/EMBL/DDBJ accession numbers for other sequence data generated in this study are HG329532–HG329542 for dnaK gene sequences; HG329544–HG329554 for groEL gene sequences; and HG329556–HG329566 for rpoB gene sequences (see Fig. 2 for details).

Three supplementary figures are available with the online version of this paper.
glucose, 0.5% ethanol, 1.5% peptone, 0.8% yeast extract and 0.3% acetic acid (Lisdiyanti et al., 2003). The medium had a pH of 3.5 and contained 5 p.p.m. amphotericin B and 200 p.p.m. cycloheximide to prevent fungal growth. Isolates grown on AAM were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Wieme et al., 2012). MALDI-TOF MS was shown to be useful for the identification of AAB involved in the production of vinegar (Andrés-Barrao et al., 2013) and was used as a dereplication tool in the present study. A total of 187 AAB lambic isolates obtained from two different breweries (an industrial and a traditional type) had identical mass spectra that differed from those of established AAB species, which suggested a unique taxonomic position. Random amplified polymorphic DNA (RAPD) analysis of a selection of 13 isolates representing the two breweries was performed as described by Williams et al. (1990) and revealed that all isolates were clonal derivatives of a single strain (data not shown). Subsequently, two isolates of the industrial type of brewery (LMG 27439T and R-50194) and two from the traditional type of brewery (LMG 27440 and R-50193) were chosen as representatives for further analyses.

The 16S rRNA gene sequence of strain LMG 27439T was determined as described previously (Snauwaert et al., 2013). EzBioCloud analysis (Kim et al., 2012) of the obtained sequence revealed that it was an AAB strain, closely related to Acetobacter okinawensis (99.7%), Acetobacter ghanensis (99.6%), Acetobacter syzygii (99.6%), Acetobacter fabarum (99.4%) and Acetobacter lovaniensis (99.2%); values in parentheses are pairwise similarity values towards the type strains of these species. All sequences were aligned using the SINA Incremental Aligner (SINA v1.2.11) (http://www.arb-silva.de/aligner/) (Pruesse et al., 2012), with the corresponding SILVA SSURef 111 database (Pruesse et al., 2007), and a dendrogram was reconstructed using the MEGA 5.2 software package (Tamura et al., 2011). The tree topologies were shown.

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Neighbour-joining tree based on the nearly full-length 16S rRNA gene sequences (1401 bp) showing the phylogenetic relationship of isolates LMG 27439T, LMG 27440, R-50193 and R-50194 and of the type strains of all species of the genus Acetobacter. Gluconacetobacter liquefaciens NBRC 12388T was used as an outgroup. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. GenBank accession numbers are given in parentheses. Bootstrap percentages (>50%) are shown next to the branch points. Bar, 1% sequence divergence.
statistically analysed using 1000 bootstrapping replications. The maximum-likelihood and maximum-parsimony trees (data not shown) showed the same topology as the neighbour-joining tree (Fig. 1).

Due to the limited taxonomic resolution of the 16S rRNA gene in this group of bacteria, the phylogenetic position of the taxon represented by strains LMG 27439<sup>T</sup> and LMG 27440 and of type and other taxonomic reference strains of its nearest phylogenetic neighbours (A. okinawensis, A. ghanensis, A. syzygii, A. fabarum and A. lovaniensis) were analysed using sequence analysis of the housekeeping genes dnaK (encoding chaperone protein DnaK), groEL (encoding the 60 kDa chaperonin) and rpoB (encoding DNA-directed RNA polymerase subunit beta) (Cleenwerck et al., 2010). Sequences of at least 627 nt, 715 nt and 582 nt were generated for dnaK, groEL and rpoB, respectively. All gene sequences were aligned at the amino acid level using the MEGA 5.2 software (Tamura et al., 2011). The trees were built using the maximum-likelihood model. A discrete gamma distribution was used to model evolutionary rate differences among sites and the rate variation model allowed for some sites to be evolutionarily invariable. Tree topologies were analysed statistically using 1000 bootstrapping replications. Numerical analysis of the individual (Figs S1–3, available in the online Supplementary Material) and concatenated (Fig. 2) gene sequences revealed that the novel taxon represented by strains LMG 27439<sup>T</sup> and LMG 27440 could be clearly differentiated from its nearest neighbours.

DNA–DNA hybridizations were performed between strain LMG 27439<sup>T</sup> and the type strains of its nearest phylogenetic neighbours as described previously (Cleenwerck et al., 2008). DNA–DNA relatedness values are presented as means of reciprocal reactions (A × B and B × A), where each reciprocal reaction was performed at least in three-fold. The level of DNA–DNA relatedness between strain LMG 27439<sup>T</sup> and the type strains of its nearest neighbours was intermediate: 42% towards A. okinawensis LMG 26457<sup>T</sup>, 35% towards A. ghanensis LMG 23848<sup>T</sup>, 31% towards A. syzygii LMG 21419<sup>T</sup>, 41% towards A. fabarum LMG 24244<sup>T</sup> and 45% towards A. lovaniensis LMG 1617<sup>T</sup>. The DNA G + C content of strain LMG 27439<sup>T</sup> was determined as described previously (Cleenwerck et al., 2008) and was 56.2 mol%.

The phenotypic characteristics of strain LMG 27439<sup>T</sup> and of three additional isolates (LMG 27440, R-50193 and R-50194) were determined as described previously (Cleenwerck et al., 2002). Type strains of closely related AAB were included as positive or negative controls. For microscopy and colony morphology, strains were grown aerobically at 28 °C for 48 h on AAM agar. The biochemical characteristics tested included Gram-stain reaction, analysis of catalase and oxidase activity, growth on glucose (30%), glycerol (0.3%) or methanol (0.3%) as the sole carbon sources, growth on ammonium as the sole nitrogen source and ethanol as the sole carbon source, and growth at 37 °C on GY agar medium (5% glucose, 1% yeast extract and 1.5% agar). In addition, acid production from 1% L-arabinose, D-galactose, D-mannose and D-glucose was determined as described previously (Asai et al., 1964). We observed that Acetobacter farinalis LMG 26772<sup>T</sup> did not exhibit catalase activity in contrast with previously reported data (Tanasupawat et al., 2011). Analysis of additional strains of A. farinalis, i.e. LMG 27045 and LMG 27046, confirmed that strains of this species lack catalase activity. We also observed that A. farinalis LMG 26772<sup>T</sup> exhibited strong growth at 37 °C instead of weak growth as reported previously (Tanasupawat et al., 2011).

For testing the production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, cells were grown as described by Gosselé...
Table 1. Differential characteristics for *Acetobacter lambici* sp. nov. and established species of the genus *Acetobacter*

| Taxa: | 1, *Acetobacter lambici* sp. nov. (n=4); 2, *A. okinawensis* (n=7); 3, *A. ghanensis* (n=3); 4, *A. syzygii* LMG 21419^T; 5, *A. fabarum* (n=4); 6, *A. lovaniensis* LMG 1617^T; 7, *A. aceti* (n=4); 8, *A. peroxidas* (n=2); 9, *A. cerevisiae* (n=4); 10, *A. cibinongensis* LMG 21418^T; 11, *A. estunensis* (n=3); 12, *A. orleanensis* (n=4); 13, *A. persici* (n=2); 14, *A. malorum* LMG 1746^T; 15, *A. orientalis* LMG 21417^T; 16, *A. farinalis* (n=3); 17, *A. tropicalis* (n=2); 18, *A. indonesiensis* (n=2); 19, *A. oeni* B13^T; 20, *A. papayae* (n=2); 21, *A. pomorum* LMG 18848^T; 22, *A. pasteurianus* (n=7); 23, *A. senegalensis* (n=3); 24, *A. nitrogenifigens* RG1^T. n is number of strains; the type strain is included for all taxa. +, Positive; −, negative; w, weakly positive; v, strain-dependent (the result of the type strain is given in parentheses). Data for taxon 1 were obtained in the present study; data for taxa 2, 13 and 20 and the data for acid production of different carbon sources were taken from Iino *et al.* (2011); and data for taxa 3–12, 14–15, 17–19 and 21–24 were taken from Cleenwerck *et al.* (2008).

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</table>
In conclusion, the present study provides polyphasic evidence that demonstrates the taxon represented by Acetobacter sp. nov. and type strains of all established species of the genus Acetobacter could be differentiated from the species described by Cleenwerck et al. (2007) using 16S rRNA gene analysis. The differences between the spectra could also be examined visually (Fig. 3). Some of the peaks previously not reported for 2-keto-D-gluconic acid were present in the mass spectra of other species of the genus Acetobacter, but not in the isolates LMG 27439T, LMG 27440, R-50193 and R-50194.

**Table 2.** Cellular fatty acid contents (%) of *Acetobacter lambici* sp. nov. and type strains of all established species of the genus *Acetobacter*

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| C14:0      | 4.05 | 4.6 | 4.25 | 4.05 | 4.62 | 4.46 | 6.06 | 5.96 | 5.98 | 5.55 | 2.03 | 0.94 | 1.36 | 1.18 | 1.07 | - | 2.22 | - | 1.46 | 1.91 | 1.2 | 5.48 | 6.32 | 3.89 | 2.81 | - |
| C18:0      | 1.92 | 2.06 | 1.68 | 1.57 | 2.94 | 2.58 | 2.39 | 3.1 | 2.16 | 4.44 | 2.23 | 2.22 | 3.82 | 2.61 | 2.58 | 2.3 | 2.63 | 4.71 | 2.37 | 4.48 | 4.57 | 4.37 | 3.74 | 7.6 | 6.85 | 7.35 | 5.46 |
| C18:1ω7c   | 59.33 | 57.74 | 60.49 | 59.83 | 59.28 | 61.43 | 60.92 | 61.47 | 65.43 | 35.12 | 60.03 | 62.98 | 62.08 | 61.7 | 64.57 | 64.8 | 61.49 | 61.93 | 58.12 | 52.77 | 53.83 | 48.08 | 46.27 | 41.44 | 42.75 | 33.82 | 33.38 |
| C19:0 cyclo | 1.2 | 1.42 | 1.05 | 1.26 | 1.5 | 1.47 | 1.24 | 1.02 | - | 1.57 | - | 3.58 | 4.05 | 3.29 | 1.82 | 1.87 | 3.13 | 1.64 | 3.02 | 3.69 | 4 | 8.04 | 1.55 | 4.75 | 2.97 | 6.11 | 2.28 |
| C19:0 cyclo | 2.14 | 3.6 | 1.93 | 2.68 | 2.22 | 1.95 | 2.23 | 3.06 | 1.75 | - | 2.63 | 1.03 | 1.87 | 1.93 | 0.59 | - | - | 1.58 | 3.09 | - | 1.02 | 2.63 | - | - | - | 1.76 |
by LMG 27439<sup>T</sup>, LMG 27440, R-50193 and R-50194 could be differentiated from its nearest phylogenetic neighbours, *A. okinawensis*, *A. ghanensis*, *A. syzygii*, *A. fabarum* and *A. lovaniensis*, by multiple genotypic and phenotypic characteristics and methodologies. We therefore propose to name this taxon *Acetobacter lambici* sp. nov., with LMG 27439<sup>T</sup> (=DSM 27328<sup>T</sup>) as the type strain.

**Description of *Acetobacter lambici* sp. nov.**

*Acetobacter lambici* (lam"bi.ci. N.L. gen. n. *lambici* of lambic, an acidic spontaneously fermented beer).

Cells are Gram-stain-negative, motile rods and are approximately 0.7 μm wide and 1.5–4.0 μm long. Cells occur separately or in pairs. Catalase-positive but oxidase-negative. After incubation for 48 h on AAM agar at 28 °C, colonies are round, rough, brownish-beige and slightly raised, with a diameter of approximately 1 mm. Ethanol is oxidized to acetic acid. D-Gluconic acid is produced from D-glucose but not 2-keto-D-gluconic acid or 5-keto-D-gluconic acid. Unable to grow on glycerol or methanol as the sole carbon source, on 30 % glucose or on GY medium at 37 °C. Unable to produce acid from L-arabinose, D-galactose and D-mannose. No growth with ammonium as the sole nitrogen source and on glycerol or on yeast extract with 10 % ethanol.

The type strain is strain LMG 27439<sup>T</sup> (=DSM 27328<sup>T</sup>), which was isolated from fermenting lambic beer. The G+C content of strain LMG 27439<sup>T</sup> is 56.2 mol%.

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**Fig. 3.** Visualization of MALDI-TOF MS profiles of *Acetobacter lambici* sp. nov. and its closest phylogenetic neighbours. Asterisks indicate the set of six peaks by which the novel strains could be differentiated from other species of the genus *Acetobacter*. The profiles are visualized using mMass 5.5.0 (Strohalm et al., 2010).

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*Acetobacter lambici* LMG 27439<sup>T</sup>

*Acetobacter okinawensis* LMG 26457<sup>T</sup>

*Acetobacter ghanensis* LMG 23848<sup>T</sup>

*Acetobacter syzygii* LMG 21419<sup>T</sup>

*Acetobacter fabarum* LMG 24244<sup>T</sup>

*Acetobacter lovaniensis* LMG 1617<sup>T</sup>
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References


