Gluconacetobacter medellinensis sp. nov., cellulose- and non-cellulose-producing acetic acid bacteria isolated from vinegar

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The phylogenetic position of a cellulose-producing acetic acid bacterium, strain ID13488, isolated from commercially available Colombian homemade fruit vinegar, was investigated. Analyses using nearly complete 16S rRNA gene sequences, nearly complete 16S–23S rRNA gene internal transcribed spacer (ITS) sequences, as well as concatenated partial sequences of the housekeeping genes dnaK, groEL and rpoB, allocated the micro-organism to the genus Gluconacetobacter, and more precisely to the Gluconacetobacter xylinus group. Moreover, the data suggested that the micro-organism belongs to a novel species in this genus, together with LMG 1693T, a non-cellulose-producing strain isolated from vinegar by Kondo and previously classified as a strain of Gluconacetobacter xylinus. DNA–DNA hybridizations confirmed this finding, revealing a DNA–DNA relatedness value of 81 % between strains ID13488 and LMG 1693T, and values <70 % between strain LMG 1693T and the type strains of the closest phylogenetic neighbours. Additionally, the classification of strains ID13488 and LMG 1693T into a single novel species was supported by amplified fragment length polymorphism (AFLP) and (GTG)5-PCR DNA fingerprinting data, as well as by phenotypic data. Strains ID13488 and LMG 1693T could be differentiated from closely related species of the genus Gluconacetobacter by their ability to produce 2- and 5-keto-δ-gluconic acid from δ-glucose, their ability to produce acid

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Abbreviations: AFLP, amplified fragment length polymorphism; ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, 16S–23S ITS, dnaK, groEL and rpoB sequences of strain LMG 1693T are JX013852, HE802686, JX013854, JX013856 and JX013858, respectively; for the 16S rRNA gene, dnaK, groEL and rpoB sequences of strain ID13488 are JX013851, JX013853, JX013855 and JX013857, respectively; and for the dnaK, groEL and rpoB sequences of Gluconacetobacter kakiaceti LMG 26206T are JX022617–JX022619, respectively.

Four supplementary figures and a supplementary table are available with the online version of this paper.

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The genus *Gluconacetobacter*, classified in the family *Acetobacteraceae*, contains several strains that are regarded as efficient producers of bacterial cellulose. Bacterial cellulose is, in its primary chemical composition, the same as cellulose produced by higher plants and algae, but overall, it exhibits a higher chemical purity, a 3D network of nanoribbons and some unique physical properties, such as high hydrophilicity, great adsorbivity and high mechanical strength, which make it an interesting raw material for a variety of applications. At the time of writing, bacterial cellulose is mainly applied in food products (e.g. nata, kombucha), paper products, acoustics (e.g. acoustic diaphragms for headphones and loudspeakers), electronics and medical devices (e.g. membranes, temporary wound coverage). However, the prevalent potential application of bacterial cellulose is in the biomedical field, e.g. in the construction of artificial skin and vessels and controlled-drug-release carriers (Hoenich, 2006; Czaja et al., 2007). Nevertheless, extensive commercial use of bacterial cellulose is limited, partly due to the high cost of carbon sources for large-scale production.

In order to decrease the costs of bacterial cellulose production, research has focused on strain improvement (selection of more suitable strains and genetic engineering) and improvement of the production process (Bae et al., 2004; Bae & Shoda, 2005; Chien et al., 2006; De Muynck et al., 2007; Hutchens et al., 2007). Development of culture media based on fruits and vegetables as sources of sugars is one way to reduce the production costs (Castro et al., 2011).

Several species of the genus *Gluconacetobacter* harbour cellulose-producing strains, such as *Gluconacetobacter xylinus*, *Gluconacetobacter hansenii*, *Gluconacetobacter swin-gsi* and *Gluconacetobacter rhaeticus*. Taxonomically, these species belong to the *Gluconacetobacter xylinus* group, a set of phylogenetically closely related species, showing at least 98.2% 16S rRNA gene sequence similarity (Cleenwerck et al., 2010). Species identification of acetic acid bacteria belonging to this group is regarded as being difficult (Kersters et al., 2006; Cleenwerck & De Vos, 2008; Cleenwerck et al., 2010).

In this study, the taxonomic position of an acetic acid bacterium, isolated from homemade fruit vinegar acquired at the central market in Medellin, Colombia, and characterized as a cellulose-producing strain on media containing agro-industrial residues such as pineapple peel juice and sugar cane juice (Castro et al., 2011), was investigated. A micro-organism, designated strain ID13488, was isolated using Hestrin–Schramm (HS) medium, containing (w/v) 2% D-glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na₂HPO₄ and 0.11% citric acid (Hestrin & Schramm, 1954). After 8 days of incubation at 28°C without shaking, a film containing a bacterial culture (visible by light microscopy) was formed on the surface. This film was transferred into fresh medium with 0.01% fluconazole to inhibit proliferation of yeast. The process was repeated until no yeast could be detected by microscopy. After two days of film synthesis, serial dilutions of the culture medium, rich in cells, were plated onto HS agar. The bacterial growth looked pure and therefore a few colonies were picked for further cultivation and analyses. The culture was maintained on HS agar slants.

Initial phenotypic characterization was carried out using methods previously described (see Cleenwerck & De Vos, 2008 for review). Colonies of strain ID13488 grown on GYAE agar medium (Cleenwerck et al., 2010) at 28°C for 5 days were used to observe cell shape, size, Gram-staining reaction and motility (by the hanging drop method; Franke et al., 1999). The presence of catalase was verified by mixing a loopful of cells grown on HS medium on a slide with a drop of 3% hydrogen peroxide and checking for the immediate appearance of O₂ bubbles. The presence of oxidase was verified by flooding an agar plate containing colonies with Kovacs reagent and checking whether the colonies immediately turned dark purple or purple. Cells from beige, regular colonies were Gram-stain-negative, non-motile rods, approximately 1–3 µm long and 0.6–0.7 µm wide, occurring singly, in pairs or in chains. They were catalase-positive and oxidase-negative. Production of acetic acid was investigated through plating on CaCO₃-agar medium (0.05% D-glucose, 0.3% peptone, 0.5% yeast extract, 1.5% CaCO₃, 1.2% agar and 1.5% ethanol) and checking for formation of clear zones around the developed colonies. Over-oxidation of acetic acid to carbon dioxide and water was analysed by checking a colour change from yellow to blue on a medium containing 2% ethanol, 0.5% peptone, 0.5% yeast extract 0.27% Na₂HPO₄, 0.008% bromothymol blue and 1.3% agar. Acetic acid was formed from ethanol and oxidized further to carbon dioxide and water. A surface pellicle visible after incubation in HS medium for 3 days at 28°C without shaking was examined. It was harvested, washed with 5% KOH (w/v) for 14 h at 25°C and rinsed with distilled water to neutral pH. The morphology of the pellicle was investigated by scanning
electron microscopy (SEM) using a JSM 5910 LV microscope (JEOL) operated at 20 kV. Fig. 1(a) shows coiled cellulose fibris arising from the surface of rod-shaped Gram-negative cells and forming a three-dimensional network. Individual cellulose ribbons were also observed by transmission electron microscopy (TEM), after negative staining with 2 % uranyl acetate, using a CM200 microscope (Philips). Typical twisting ribbons with an average width of 50–70 nm are shown in Fig. 1(b).

A nearly complete 16S rRNA gene sequence of strain ID13488 (1443 bp) was determined using the protocol described by Vancanneyt et al. (2004) with the following modifications. The PCR-amplified 16S rRNA gene was purified using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). The primers *Gamma, *PD, *O, *3, *R, Gamma, 3 and BKL1 (Coenye et al., 1999; Cleenwerck et al., 2007) were used to obtain the sequence. Sequencing reactions were purified using a BigDye XTerminatorT Purification kit (Applied Biosystems), and electrophoresis of the sequence reaction products was performed using an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). The primers were concatenated partial sequences of the housekeeping genes dnaK, groEL and rpoB was performed. Both methods are useful for species identification and classification of acetic acid bacteria belonging to the genus Gluconacetobacter (Trček, 2005; Cleenwerck et al., 2010). A 16S–23S ITS sequence of strain LMG 1693T, and dnaK, groEL and rpoB sequences of strains ID13488, LMG 1693T and the type strain of G. kakiaceti, LMG 26206T (Iino et al., 2012), were determined by using the primers and conditions reported previously (Trček & Teuber, 2002; Trček, 2005; Cleenwerck et al., 2010). The sequences obtained were compared with partial sequences of reference strains of members of the genus Gluconacetobacter collected from the EMBL database. Phylogenetic trees were constructed with the software packagePHYLIP for the 16S–23S ITS sequences, and the BioNumerics 5.1 software package for the partial dnaK, groEL and rpoB gene sequences using the neighbour-joining method. The robustness of the branches was evaluated by bootstrap analysis (Felsenstein, 1985). 16S rRNA gene sequencing and phylogenetic analyses allocated strain ID13488 to the genus Gluconacetobacter, and more precisely to the G. xylinus group. Furthermore, the 16S rRNA gene sequence of strain ID13488 showed 100 % similarity with a nearly complete 16S rRNA gene sequence of LMG 1693T (1443 bp), a misclassified non-cellulose-producing G. [xylinus] strain (I. Cleenwerck, unpublished data), isolated from vinegar by Kondo about 60 years ago and intensively studied (Yamada et al., 1976; Tanaka et al., 1998; Navarro & Komagata, 1999). Since the end of 2011, the whole genome sequence of NBRC 3288T, a subculture of the same strain, has been available (Ogino et al., 2011), and the 16S rRNA gene sequence of LMG 1693T shows 100 % similarity with the five 16S rRNA gene copies present in that genome (Fig. 2). Based on the sequences of strains ID13488 and LMG 1693T, the following species were related to these strains with more than 98.7 % pairwise 16S rRNA gene sequence similarity, Gluconacetobacter intermedius TF2T, Gluconacetobacter oboediens LTH 2460T, G. swingsii DST GL01T, Gluconacetobacter europaeus DSM 6160T, Gluconacetobacter naticola LMG 1536T, G. rhaceticus DST GL02T, Gluconacetobacter saccharivorans BPR2001T, Gluconacetobacter medellinensis sp. nov.

To refine the taxonomic position of strains ID13488 and LMG 1693T, phylogenetic analyses using nearly complete 16S–23S internal transcribed sequence (ITS) and concatenated partial sequences of the housekeeping genes dnaK, groEL and rpoB was performed. Both methods are useful for species identification and classification of acetic acid bacteria belonging to the genus Gluconacetobacter (Trček, 2005; Cleenwerck et al., 2010). A 16S–23S ITS sequence of strain LMG 1693T, and dnaK, groEL and rpoB sequences of strains ID13488, LMG 1693T and the type strain of G. kakiaceti, LMG 26206T (Iino et al., 2012), were determined by using the primers and conditions reported previously (Trček & Teuber, 2002; Trček, 2005; Cleenwerck et al., 2010). The sequences obtained were compared with partial sequences of reference strains of members of the genus Gluconacetobacter collected from the EMBL database. Phylogenetic trees were constructed with the software packagePHYLIP for the 16S–23S ITS sequences, and the BioNumerics 5.1 software package for the partial dnaK, groEL and rpoB gene sequences using the neighbour-joining method. The robustness of the branches was evaluated by bootstrap analysis. Phylogenetic analyses based on nearly complete 16S–23S ITS sequences and concatenated partial dnaK, groEL and rpoB gene sequences confirmed that strains ID13488 and LMG1693T belonged to the G. xylinus group. In addition, the data indicated that the strains probably constituted a single novel species in that genus, as they showed sequence similarities below 95.1 % for dnaK, 95.6 % for groEL and 92.0 % for rpoB with these species (which is below the species cut-off values
previously reported for species of the *G. xylinus* group; Cleenwerck *et al.*, 2010). Also, they formed a branch separated from the known species of this group (Figs S1 and S2, available in IJSEM Online).

Strains ID13488 and LMG 1693\(^T\) were subjected to (GTG)\(_5\)-PCR and amplified fragment length polymorphism (AFLP) DNA fingerprinting using methods described previously (De Vuyst *et al.*, 2008; Cleenwerck *et al.*, 2009), with minor modifications for AFLP. Firstly, the selective amplification primer T03 was replaced by a tailed primer designated T13 (5’-GGTCTGAGTTGCCCCGAG-3’), secondly the selective PCR products were separated with an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems), thirdly tables with information on fragments of 40–580 bp were created using the program GeneMapper, and finally a slightly different data range for numerical analysis was used (50–500 bp). The techniques were performed to obtain a more detailed view of the genomic relatedness of strains ID13488 and LMG 1693\(^T\) with strains of the *G. xylinus* group. Cluster analysis of the obtained DNA fingerprints with DNA fingerprints of strains of the *G. xylinus* group, available in in-house BCCM/LMG databases, revealed that they formed a single cluster separate from the other profiles (Figs S3 and S4), again indicating that strains ID13488 and LMG 1693\(^T\) represent a distinct species of the genus *Gluconacetobacter*. In addition, the data revealed that strains ID13488 and LMG 1693\(^T\) have different patterns, showing that they are different strains.

DNA–DNA hybridizations were performed between strains LMG 1693\(^T\) and ID13488, and between LMG 1693\(^T\) and *G. intermedius* LMG 18909\(^T\), *G. oboediens* LMG 18849\(^T\), *G. swingsii* LMG 22125\(^T\), *G. europaeus* LMG 18890\(^T\), *G. nataicola* LMG 1536\(^T\), *G. rhaeticus* LMG 22126\(^T\), *G. saccharivorans* LMG 18788\(^T\), *G. xylinus* LMG 1515\(^T\), *G. sacchariflavourans* LMG 1582\(^T\) and *G. kakiaceti* LMG 26206\(^T\). DNA for DNA–DNA hybridizations was extracted at a large scale using the method described by Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). DNA–DNA hybridizations (four replications) were performed in the presence of 50 % formamide at 48 °C using a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the microplate method described by Ezaki *et al.* (1989). Reciprocal reactions (A × B and B × A) were performed for each DNA pair and their variation was generally within the limits of this method (Goris *et al.*, 1998). Strains LMG 1693\(^T\) and ID13488 exhibited 81 (± 5.5) % DNA–DNA relatedness, while values below the species level (< 70 %; Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002) were obtained between LMG 1693\(^T\) and the tested type strains: 36 (± 1) % with *G. intermedius* LMG 18909\(^T\), 27 (± 1.5) % with *G. oboediens* LMG 18849\(^T\), 29 (± 19) % with *G. swingsii* LMG 22125\(^T\), 31 (± 7) % with *G. europaeus* LMG 18890\(^T\), 32 (± 0.5) % with *G. nataicola* LMG 1536\(^T\), 33 (± 13) % with *G. rhaeticus* LMG 22126\(^T\), 43 (± 1.5) % with *G. saccharivorans* LMG 18788\(^T\), 25 (± 9) % with *G. xylinus* LMG 1515\(^T\), 20 (± 12.5) % with *G. sacchariflavourans* LMG 1582\(^T\) and 33 (± 10.5) % with *G. kakiaceti* LMG 26206\(^T\).
The DNA–DNA hybridization data revealed that strains LMG 1693\textsuperscript{T} and ID13488 indeed constitute a single novel species of the genus *Gluconacetobacter*.

The DNA base composition of strains ID13488 and LMG 1693\textsuperscript{T} was determined by HPLC according to the method described by Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as a calibration reference. The DNA G\textsubscript{+}C content of strains ID13488 and LMG 1693\textsuperscript{T} was 58.0 and 60.7 mol\%, respectively. These values fall within the DNA G\textsubscript{+}C content range of the species of the *G. xylinus* group (57–64 mol\%; Schüller *et al.*, 2000; Lisdiyanti *et al.*, 2006; Iino *et al.*, 2012), and that for strain LMG 1693\textsuperscript{T} is very close to the value reported previously (59.6 mol\%) by Navarro & Komagata (1999).

The whole-cell fatty acid composition of strains ID13488 and LMG 1693\textsuperscript{T} was determined using an Agilent Technologies 6890N gas chromatograph. *G. kakiaceti* LMG 26206\textsuperscript{T} was included as a control. Cultivation of the strains, extraction and analysis of the fatty acid methyl esters were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI), except that extraction was from cells harvested from the overlap between the second and third quadrant of cultures grown on GYP agar (Iino *et al.*, 2012) for two days at 28 °C. The peaks of the profiles were identified using the TSBA50 identification library version 5.0. Profiles obtained were compared with profiles of strains of the *G. xylinus* group generated previously (Iino *et al.*, 2012) under the same conditions, and were found to be similar. The major fatty acid of strains ID13488 and LMG 1693\textsuperscript{T} was C\textsubscript{18}:1\texttext{\textsubscript{O}}7c (52–62 \%), while fatty acids detected in minor amounts were C\textsubscript{16}:0 (9 \%), C\textsubscript{14}:0 \texttext{\textsubscript{2}}-OH (7 \%) and C\textsubscript{16}:0 \texttext{\textsubscript{2}}-OH (9–16 \%) (Table S1).

Additional phenotypic characterization was performed. Strains ID13488, LMG 1693\textsuperscript{T} and the type strains of the closely related species of the *G. xylinus* group were tested for growth on 3 % ethanol in the presence of 0, 4, 6 and 8 % acetic acid as described previously (Sokollek *et al.*, 1998). Growth on carbon sources such as ethanol,
D-fructose, maltose, D-ribose, D-xylene, sucrose, sorbitol, D-mannitol and D-glucanate was verified using a medium containing 0.5% yeast extract, 1.5% agar and 2% of the carbon source. The cultures were incubated at 28 °C for 8 days. Production of 2-keto- and 5-keto-D-gluconic acid from D-glucose for strains ID13488 and LMG 1693T was determined using the TLC method described by Gosselé et al. (1980). Growth in the presence of 30% D-glucose were verified for strains ID13488 and LMG 1693T using the method of Cleenwerck et al. (2002). Acid production from raffinose, sucrose and 1-propanol by strains ID13488 and LMG 1693T was determined using the method described by Asai et al. (1964), briefly, bacteria were incubated for 7 days at 28–30 °C in a medium consisting of 0.5% (w/v) yeast extract and 1% (w/v) carbon source. The formation of acid was checked by adding 0.002% (w/v) bromocresol purple to the cultured broths. The characteristics differentiating strains ID13488 and LMG 1693T from the closely related species of the G. xylinus group are shown in Table 1. Both strains can be differentiated from the other species of the genus Gluconacetobacter on the basis of their ability to produce 2- and 5-keto-D-gluconic acid from D-glucose, their ability to produce acid from sucrose, but not from 1-propanol, and their ability to grow on 3% ethanol in the absence of acetic acid and on ethanol, D-ribose, D-xylene, sucrose, sorbitol, D-mannitol and D-glucanate as carbon sources.

For strain LMG 1693T, additional features typical of members of the genus Gluconacetobacter (reviewed by Cleenwerck & De Vos, 2008) have been reported previously (Yamada et al., 1976; Tanaka et al., 1998), such as its ability to produce acetic acid from ethanol and dihydroxyacetone from glycerol, its ability to oxidize acetic acid and lactic acid to carbon dioxide and water and its possession of Q-10 as the major ubiquinone (93%) and Q-9 as the minor one (7%).

To conclude, based on the data presented, it is clear that strains ID13488 and LMG 1693T constitute a novel species of the genus Gluconacetobacter, for which the name Gluconacetobacter medellinensis is proposed.

**Description of Gluconacetobacter medellinensis sp. nov.**

Gluconacetobacter medellinensis (me.del.lin.en’sis. N.L. masc. adj. medellinensis of or belonging to Medellin, referring to the city, the origin of fruit vinegar from which an isolate was obtained).

Cells are Gram-stain-negative rods, approximately 1–3 μm long and 0.6–0.7 μm wide. They occur singly, in pairs or in chains, are oxidase-negative and catalase-positive. On GYAE-agar colonies are beige, round, raised, rough and opaque. Ethanol is oxidized to acetic acid, and acetic acid is oxidized to carbon dioxide and water. Capable of producing 2- and 5-keto-D-gluconic acid from D-glucose. Growth is observed on 3% ethanol. Acetic acid is not required for growth. Ethanol, D-fructose, maltose, sucrose, sorbitol, D-mannitol, D-glucanate, D-ribose and D-xylene can be used as carbon sources, although growth on the latter two can be weak

Acid is produced from sucrose but not from 1-propanol. Acid production from raffinose and cellulose production are variable (negative for the type strain).

The type strain is LMG 1693T (=NBRC 3288T = Kondo 51T), isolated from vinegar by K. Kondo. The DNA G+C content of the type strain is 60.7 mol%. An additional strain of the species is ID13488 with a DNA G+C content of 58 mol %.

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