Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea

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A few members of the family *Acetobacteraceae* are cellulose-producers, while only six members fix nitrogen. Bacterial strain RG3^T, isolated from Kombucha tea, displays both of these characteristics. A high bootstrap value in the 16S rRNA gene sequence-based phylogenetic analysis supported the position of this strain within the genus *Gluconacetobacter*, with *Gluconacetobacter hansenii* LMG 1527^T as its nearest neighbour (99.1 % sequence similarity). It could utilize ethanol, fructose, arabinose, glycerol, sorbitol and mannitol, but not galactose or xylose, as sole sources of carbon. Single amino acids such as L-alanine, L-cysteine and L-threonine served as carbon and nitrogen sources for growth of strain RG3^T. Strain RG3^T produced cellulose in both nitrogen-free broth and enriched medium. The ubiquinone present was Q-10 and the DNA base composition was 55.8 mol% G+C. It exhibited low values of 5.2–27.77 % DNA–DNA relatedness to the type strains of related gluconacetobacters, which placed it within a separate taxon, for which the name *Gluconacetobacter kombuchae* sp. nov. is proposed, with the type strain RG3^T (=LMG 23726^T=MTCC 6913^T).

Endophytic bacteria colonize the internal tissues of the host plant for mutual benefit. The nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* was found to be associated with sugar cane, pineapple, Cameroon grass, sweet potato, mango and banana (Muthukumarasamy *et al.*, 2002), while *Gluconacetobacter azotocaptans* and *Gluconacetobacter johannae* inhabit coffee plants (Fuentes-Ramírez *et al.*, 2001); these organisms might provide host plants with useful fixed nitrogen and growth stimulants (Lee *et al.*, 2000; Muthukumarasamy *et al.*, 2002). Based on the strikingly similar high-sugar and low-pH environments presented by the habitats of sugar cane and Kombucha tea (Blanc, 1996), we explored the bacterial flora of Kombucha tea and isolated a nitrogen-fixing strain, *Acetobacter nitrogenifigens* RG1^T (Dutta & Gachhui, 2006), and another nitrogen-fixing and cellulose-producing strain, RG3^T, belonging to the family *Acetobacteraceae*.

The family *Acetobacteraceae* has been divided into 10 genera: *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Acidomonas* (Yamada *et al.*, 1997), *Asaia* (Yamada *et al.*, 2000), *Kozakia* (Lisdiyanti *et al.*, 2002), *Saccharibacter* (Jojima *et al.*, 2004), *Swaminathania* (Loganathan & Nair, 2004), *Neoasaia* (Yukphan *et al.*, 2005) and *Granulibacter* (Greenberg *et al.*, 2006). Only six members of the family, *G. diazotrophicus* (Gillis *et al.*, 1989), *G. johannae* (Fuentes-Ramírez *et al.*, 2001), *G. azotocaptans* (Fuentes-Ramírez *et al.*, 2001), *Acetobacter peroxydans* (Muthukumarasamy *et al.*, 2005), *Swaminathania salitolerans* (Loganathan & Nair, 2004) and *Acetobacter nitrogenifigens* (Dutta & Gachhui, 2006), are known to fix nitrogen. The genus *Gluconacetobacter* comprises 15 species with validly published names at present, differentiated on the basis of DNA–DNA relatedness, phylogenetic relationships and morphological characteristics. Isolation of strain RG3^T from Kombucha tea is the first instance of a strain within the family having both nitrogen-fixing and cellulose-producing activity. We present morphological, biochemical and genetic evidence that indicates that RG3^T represents a novel nitrogen-fixing species within the genus *Gluconacetobacter*.

Kombucha tea is a fermented tea that contains an association of yeast and bacteria. A jelly-like membrane floats in the nutrient solution of tea and sugar exposed to oxygen. At the right temperature, it multiplies continuously. It first spreads over the entire surface of the tea, and then thickens. Kombucha tea was subcultured every 7–10 days by mixing 10 % of old soup with 10 % sucrose dissolved in brewed black tea. After teasing the mat apart in the soup, aliquots of Kombucha mat suspension were spread on LGI agar plates (0.06 % KH_2PO_4, 0.02 % K_2HPO_4, 0.02 % $MgSO_4$, 0.002 % $CaCl_2$, 0.001 % $FeCl_3$, 0.0002 % Na_2MoO_4, 10 % sucrose, pH 4.5; Cavalcante & Döbereiner, 1988) containing 150 mg cycloheximide l^{-1} (Jimenez-Salgado

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strain RG3^T are respectively AY688433 and DQ141200.

et al., 1997) and 150 mg nystatin l^{-1}. Plates were incubated at 30 °C for 5 days. Repeated streaking on LGI plates, which contain no combined nitrogen, purified the bacterial isolate. Gas-tight vials of LGI medium inoculated with bacteria (under a microaerophilic environment, without shaking) were assayed for acetylene reduction activity (Stal, 1988). Nitrogenase-positive isolates were selected for further characterization. Strain RG3^T, a nitrogen-fixing bacterial strain that exhibited cellulose-producing ability even in nitrogen-free LGI broth, was isolated.

Strain RG3^T produced cellulose when grown in HS medium (Hestrin & Schramm, 1954) under stationary as well as shaking culture conditions at 30 °C after incubation for 3 days. The cellulosic character of the pellicle was confirmed by boiling the pellicles with a dilute NaOH solution (Forng *et al.*, 1989; Navarro & Komagata, 1999). The polymer was a simple carbohydrate in nature, as indicated by the greenish-blue colour of the supernatant with *o*-toluidine. In acid-hydrolysed bacterial pellicle samples, the amount of reducing sugars released was found to be comparable to the amount of glucose residues estimated by using glucose oxidase. Thus, the pellicle had repetitive glucose units. Liberation of glucose units upon enzymic digestion of the pellicle with cellulase confirmed it to be composed of cellulose.

Reference strains (*Gluconacetobacter hansenii* JCM 11196^T, a gift of Y. Nakagawa and Y. Yamada, *Gluconacetobacter intermedius* LMG 18909^T, *Gluconacetobacter europeaus* LMG 1518^T, *Gluconacetobacter oboediens* LMG 18849^T and *Gluconacetobacter swingsii* LMG 22125^T, from the BCCM/LMG, and *Gluconacetobacter xylinus* JCM 7644^T from the JCM) were grown in different media according to the instructions of the culture collections. Colony morphology was examined on LGI agar plates and on potato agar plates containing 10 % sucrose. Various phenotypic and morphological characters were tested using standard techniques described previously (Franke *et al.*, 1999; Schüller *et al.*, 2000; Dellaglio *et al.*, 2005; Fuentes-Ramírez *et al.*, 2001). Isoprenoid quinones of the isolate were extracted with chloroform/methanol (2 : 1, v/v) and purified by TLC on silica gel 60 F_{254} plates (20 × 20 cm; Merck) by using benzene as the developing solvent. Quinones recovered from the TLC plates were dissolved in acetone and analysed by HPLC (Lu *et al.*, 1999). The HPLC system was equipped with a reversed-phase column [Luna 5U C18 (2) 100A, 250 × 4.6 mm; Phenomenex] and a mixture of methanol and isopropanol (2 : 1, v/v) was used as the mobile phase at a flow rate of 1 ml min^{-1}. Types of quinone were identified by absorption at 275 nm and compared with coenzyme Q-9 and coenzyme Q-10 standards from Sigma-Aldrich. Ubiquinone Q-10 was present in strain RG3^T, in agreement with previous observations of the presence of this ubiquinone type in the genus *Gluconacetobacter* (Cleenwerck *et al.*, 2002).

A 1442 bp fragment of the 16S rRNA gene was amplified by PCR with bacteria-specific primers fD1 and rD1 (Weisburg *et al.*, 1991) using *Taq* polymerase and genomic DNA from RG3^T as the template. The nucleotide sequence showed the following levels of similarity to sequences from strains of the genus *Gluconacetobacter* after performing similarity searches with FASTA (ungapped): 99.1 % with *G. hansenii* LMG 1527^T, 99.0 % with *G. entanii* LTH 4560^T, 98.6 % with *G. rhaeticus* DST GL02^T, 98.5 % with *G. swingsii* DST GL01^T, 98.3 % with *G. xylinus* JCM 7644^T and *G. europeaus* JK2, 98.2 % with *G. saccharivorans* LMG 1582^T, 98.1 % with *G. oboediens* LTH 2460^T, *G. intermedius* TF2^T and *G. nataicola* LMG 1536^T, 96.7 % with *G. diazotrophicus* LMG 7603^T, 96.6 % with *G. azotocaptans* CFN-Ca54^T, 96.5 % with *G. johannae* CFN-Cf55^T and *G. liquefaciens* LMG 1382^T and 96.4 % with *G. sacchari* IF 2-6. The phylogenetic tree was deduced using MEGA version 3.1 (Kumar *et al.*, 2004) software after multiple alignment with 16S rRNA gene sequences of other acetic acid bacteria with CLUSTAL W (Thompson *et al.*, 1994). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were determined by using bootstrap values (Felsenstein, 1985) based on 100 replications. As evident from the tree (Fig. 1), the species of this genus are subgrouped phylogenetically into two clusters, comprising nitrogen-fixers such as *G. diazotrophicus* and cellulose-producers such as *G. xylinus*, *G. swingsii*, *G. rhaeticus* and *G. nataicola*. The novel strain RG3^T, which exhibits both these characteristics, clustered with the 'cellulose-producing' group, along the subbranch formed by *G. hansenii* and *G. entanii*.

Strain RG3^T differed biochemically from the phylogenetically closely related species of the genus *Gluconacetobacter* (Table 1). It could be differentiated from *G. hansenii* and *G. entanii*, its phylogenetically closest neighbours, in utilizing sorbitol as a sole source of carbon. Additionally, RG3^T could be differentiated from *G. hansenii* by the non-utilization of ethanol, mannitol and sucrose as carbon sources and from *G. entanii* by growth in the absence of acetic acid and utilization of D-mannitol as a carbon source. The major difference between RG3^T and the related 'cellulose-producing' gluconacetobacters is the absence of nitrogen-fixing behaviour in the remaining species. Of the genes responsible for nitrogen fixation, the structural genes *nifHDK* of the nitrogenase enzyme complex are the most important. A 536 bp region encoding dinitrogenase reductase, *nifH*, was amplified from RG3^T using degenerate primers 19F and 407R (Franke *et al.*, 1998) and sequenced, confirming the presence of the *nifH* gene in RG3^T.

To determine the genomic relatedness of the new isolate, dot-blot hybridization experiments were carried out with DIG-labelled DNA as described previously (Labrenz *et al.*, 2000) using the detection kit from Roche Applied Sciences following the manufacturer's instructions. Colorimetric quantification of dot intensities was done using the Molecular Analyst software (Bio-Rad) by determining mean pixel densities in circles of equal size. The genomic DNA probe was prepared from strain RG3^T; digested with

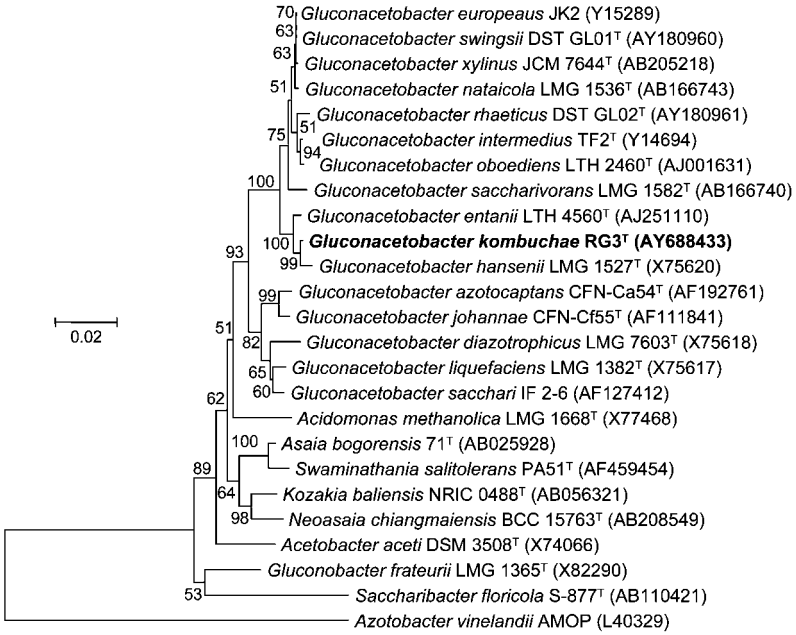

Fig. 1. Phylogenetic relationships based on 16S rRNA gene sequences of acetic acid bacteria including strain RG3^T and related members of the family *Acetobacteraceae*. Numerals at nodes indicate bootstrap values derived from 100 replications. GenBank accession numbers are indicated in parentheses. Bar, 2 substitutions per 100 nucleotides.

*Eco*RI and separated on a 0.7 % agarose gel. Total DNA digests were transferred from gels to nylon membrane by Southern blotting. Hybridization was performed at 75 °C for 16 h and the membrane was washed under high-stringency conditions (twice with 2× SSC/0.1 % SDS at room temperature for 10 min, once with 0.1× SSC/0.1 % SDS at 75 °C for 15 min). Low levels of genomic DNA relatedness (DNA–DNA hybridization values of less than 30 %) were

Table 1. Differential characteristics between strain RG3^T and closely related species of the genus *Gluconacetobacter*

Strains/species: 1, strain RG3^T (data from this study); 2, *G. hansenii* (data from Gosselé *et al.*, 1983; Navarro *et al.*, 1999); 3, *G. intermedius* LMG 18909^T (Boesch *et al.*, 1998); 4, *G. europeaus*; 5, *G. oboediens* LMG 18849^T; 6, *G. xylinus* (data in columns 4–6 from Sokollek *et al.*, 1998); 7, *G. swingsii* LMG 22125^T (Dellaglio *et al.*, 2005); 8, *G. entanii* LTH 4560^T (Schüller *et al.*, 2000); 9, *G. rhaeticus* LMG 22126^T (Dellaglio *et al.*, 2005); 10, *G. nataicola* LMG 1536^T; 11, *G. saccharivorans* LMG 1582^T (data in columns 10 and 11 from Lisdiyanti *et al.*, 2006). +, Positive; −, negative; W, weak; V, variable; ND, not determined; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Production from D-glucose of:											
2-Keto-D-gluconate	−	+	−	NR	+	+	+	−	+	+	+
5-Keto-D-gluconate	+	+	−	NR	−	+	+	−	+	+	−
Growth without acetic acid	+	+	+	−	+	+	+	−	+	+	+
Growth on 3 % ethanol in the presence of 4–8 % acetic acid	−	−	NR	+	+	−	+	+	+	−	−
Growth on carbon sources											
Ethanol	+	−	NR	−	−	−	+	NR	+	−	−
D-Xylose	−	V	NR	+	−	+	+	NR	+	NR	NR
D-Fructose	+	V	NR	+	+	+	+	+	+	NR	NR
Maltose	+	V	NR	+	+	+	+	+	+	NR	NR
Sucrose	+	−	NR	−	+	+	+	+	+	NR	NR
Sorbitol	+	V	NR	−	−	+	+	W	−	NR	NR
D-Gluconate	W	−	NR	−	+	+	+	−	+	NR	NR
D-Mannitol	+	−	NR	−	−	+	+	−	+	+	+
Growth in the presence of 30 % D-glucose	+	−	NR	−	+	−	+	−	+	NR	NR
Growth and pellicle formation in LGI medium	+	−	−	−	−	−	−	ND	ND	ND	ND
Cellulose production	+	V	+	V	−	+	+	−	+	+	−
DNA G+C content (mol%)	55.8	58–63	NR	56–58	59.9	55–63	61.7	58	63.4	62	61

observed between strain RG3^T and its phylogenetically closest relatives; DNA–DNA relatedness values of 24.8 % (with *G. hansenii* JCM 7643^T), 21.8 % (*G. swingsii* LMG 22125^T), 27.7 % (*G. xylinus* JCM 7644^T), 24.6 % (*G. europeaus* LMG 1518^T), 5.2 % (*G. oboediens* LMG 18849^T) and 23.4 % (*G. intermedius* LMG 18909^T) were obtained.

The limitations of 16S rRNA gene sequencing for the differentiation of closely related species have been documented (Fox *et al.*, 1992), but a DNA–DNA reassociation level below 70 % indicates a distinct species (Stackebrandt & Goebel, 1994). Although the 16S rRNA gene sequence similarity levels were greater than 97 %, low levels (below 30 %) of DNA relatedness were found among the closely related *Gluconacetobacter* species studied. In view of the low physiological, biochemical, phylogenetic and genetic similarities among members of the genus *Gluconacetobacter*, we recommend that strain RG3^T should be assigned to a novel species of the genus *Gluconacetobacter*. We propose the name *Gluconacetobacter kombuchae* sp. nov. for strain RG3^T isolated from Kombucha tea.

Description of *Gluconacetobacter kombuchae* sp. nov.

Gluconacetobacter kombuchae (kom.bu′chae. N.L. gen. fem. n. *kombuchae* from Kombucha, a kind of fermented tea).

Cells are straight rods, approximately 2.0–3.0 μm long and 0.1–0.2 μm wide, and occur singly or in bunches. Gram-negative, motile with polar flagellation, catalase-positive and oxidase-negative. Growth occurs on nitrogen-free LGI plates at 30 and 37 °C and in LGI broth under microaerophilic conditions with formation of a cellulosic pellicle on the surface. Colonies grown on LGI plates are smooth, round, dull, dry, white and opaque, 0.5–1.0 mm in diameter after incubation for 5 days. Dark-yellow colonies are formed on LGI agar supplemented with 0.001 % bromothymol blue. Colonies on potato agar are light brown after 5 days of incubation, but the intensity increases after 10 days. The type strain is aerobic and fixes atmospheric nitrogen microaerobically. It oxidizes ethanol to acetic acid, turning EYC or GYC opaque plates transparent, and overoxidizes acetate and lactate to CO_2 and water. L-Alanine supports growth, as well as the formation of a pellicle, as the sole source of carbon and nitrogen in LGI broth. Can utilize the single amino acids L-cysteine and L-threonine as sole sources of carbon and nitrogen but not L-phenylalanine. Butanol (0.1 %) can not support growth in nitrogen-free medium. In the absence of yeast extract, the type strain can utilize different carbon sources such as D-arabinose, D-mannitol, D-sorbitol and glycerol, but not D-galactose or D-xylose, and can grow on 30 % sucrose and 30 % glucose. Formation of a cellulosic pellicle is prevalent in the presence of almost all the general carbon sources used except sorbitol and gluconate. Can not utilize D-cellobiose, maltose or lactose in LGI medium for growth. The ubiquinone present is of the type Q-10 and the DNA G+C content of the type strain is 55.8 mol%.

The type strain is strain RG3^T (=LMG 23726^T=MTCC 6913^T), isolated from Kombucha tea.

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