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*, *Gluconoacetobacter*, *Acidomonas* (Yamada *et al.*, 1997), *Asaia* (Yamada *et al.*, 2000), *Kozakia* (Lisdiyanti *et al.*, 2002), *Saccharibacter* (Jojima *et al.*, 2004), *Swaminathania* (Loganathan & Nair, 2004), *Neosaia* (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\(_2\)PO\(_4\), 0.02 % K\(_2\)HPO\(_4\), 0.02 % MgSO\(_4\), 0.002 % CaCl\(_2\), 0.001 % FeCl\(_3\), 0.0002 % Na\(_2\)MoO\(_4\), 10 % sucrose, pH 4.5; Cavalcante & Dobereiner, 1988) containing 150 mg cycloheximide l\(^{-1}\) (Jimenez-Salgado...
et al., 1997) and 150 mg nystatin 1 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 RG3T, a nitrogen-fixing bacterial strain that exhibited cellulose-producing ability even in nitrogen-free LGI broth, was isolated.

Strain RG3T 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 (Forgn 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 11196T, a gift of Y. Nakagawa and Y. Yamada, Gluconacetobacter intermedius LMG 18909T, Gluconacetobacter europeaus LMG 1518T, Gluconacetobacter oboediens LMG 18849T and Gluconacetobacter swingsii LMG 22125T, from the BCCM/ LMG, and Gluconacetobacter xylinus JCM 7644T 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 F254 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 5 μm 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 quinones 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 RG3T, 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 RG3T 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 1527T, 99.0% with G. entanii LTH 4560T, 98.6% with G. rheticus DST GL02T, 98.5% with G. swingsi DST GL01T, 98.3% with G. xylinus JCM 7644T and G. europeus JK2, 98.2% with G. saccharivorans LMG 1582T, 98.1% with G. oboediens LTH 2460T, G. intermedius TF2T and G. nataicola LMG 1536T, 96.7% with G. diazotrophicus LMG 7603T, 96.6% with G. azotocaptans CFN-Ca54T, 96.5% with G. johnnæ CFN-Cf55T and G. liquefaciens LMG 1382T 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. swimsii, G. rheticus and G. nataicola. The novel strain RG3T, which exhibits both these characteristics, clustered with the ‘cellulose-producing’ group, along the subbranch formed by G. hansenii and G. entanii.

Strain RG3T 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, RG3T could be differentiated from G. hansenii by the non-utilization of mannitol, 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 RG3T 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 dinitrogen reductase, nifH, was amplified from RG3T using degenerate primers 19F and 407R (Franke et al., 1998) and sequenced, confirming the presence of the nifH gene in RG3T.

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 Analyser software (Bio-Rad) by determining mean pixel densities in circles of equal size. The genomic DNA probe was prepared from strain RG3T; digested with
EcoRI 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 RG3ᵀ and closely related species of the genus Gluconacetobacter

| Strains/species: | 1, strain RG3ᵀ (data from this study); 2, G. hansenii (data from Gosselé et al., 1983; Navarro et al., 1999); 3, G. intermedius DSM 18909ᵀ (Boesch et al., 1998); 4, G. europaeus; 5, G. oboedens DSM 18849ᵀ; 6, G. xylinus (data in columns 4–6 from Sokollik et al., 1998); 7, G. swingsii DSM 22125ᵀ (Dellaglio et al., 2005); 8, G. entanii LTH 4560ᵀ (Schüller et al., 2000); 9, G. rhaeticus DSM 1382ᵀ (Dellaglio et al., 2005); 10, G. nataicola DSM 1536ᵀ; 11, G. saccharivorans DSM 1582ᵀ (data in columns 10 and 11 from Lisdiyanti et al., 2006). +, Positive; −, negative; W, weak; V, variable; ND, not determined; NR, not reported. |

<table>
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<th>Characteristic</th>
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<th>8</th>
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<td>2-Keto-D-gluconate</td>
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<td>+</td>
<td>−</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>−</td>
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<td>+</td>
<td>−</td>
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<tr>
<td>Growth in the presence of 30% D-glucose</td>
<td>+</td>
<td>−</td>
<td>NR</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>NR</td>
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<tr>
<td>Growth and pellicle formation in LGI medium</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND</td>
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<td>−</td>
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<td>DNA G+C content (mol%)</td>
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<td>58–63</td>
<td>NR</td>
<td>56–58</td>
<td>59.9</td>
<td>55–63</td>
<td>61.7</td>
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<td>62</td>
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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. europaeus LMG 1518$^T$), 5.2% (G. oboedens LMG 18849$^T$) and 23.4% (G. internus 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 kombucha$^e$ sp. nov. for strain RG3$^T$ isolated from Kombucha tea.

**Description of Gluconacetobacter kombucha$^e$ sp. nov.**

Gluconacetobacter kombucha$^e$ (kom.bu’c.ah. N.L. gen. fem. n. kombucha$^e$ 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 micro-aerophilic 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. 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 kombucha$^e$ sp. nov. for strain RG3$^T$ isolated from Kombucha tea.

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