Novel nitrogen-fixing Acetobacter nitrogenifigens sp. nov., isolated from Kombucha tea

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The four nitrogen-fixing bacteria so far described in the family Acetobacteraceae belong to the genera Gluconacetobacter and Acetobacter. Nitrogen-fixing bacterial strain RG1\textsuperscript{T} was isolated from Kombucha tea and, based on the phylogenetic analysis of 16S rRNA gene sequence which is supported by a high bootstrap value, was found to belong to the genus Acetobacter. Strain RG1\textsuperscript{T} differed from Acetobacter aceti, the nearest member with a 16S rRNA gene sequence similarity of 98.2\%, and type strains of other Acetobacter species with regard to several characteristics of growth features in culture media, growth in nitrogen-free medium, production of \ensuremath{\gamma}-pyrone from glucose and dihydroxyacetone from glycerol. Strain RG1\textsuperscript{T} utilized maltose, glycerol, sorbitol, fructose, galactose, arabinose and ethanol, but not methanol as a carbon source. These results, along with electrophoretic mobility patterns of nine metabolic enzymes, suggest that strain RG1\textsuperscript{T} represents a novel nitrogen-fixing species. The ubiquinone present was Q-9 and DNA G+C content was 64.1 mol\%. Strain RG1\textsuperscript{T} exhibited a low value of 2–24 % DNA–DNA relatedness to the type strains of related acetobacters, which placed it as a separate taxon. On this basis, the name Acetobacter nitrogenifigens sp. nov. is proposed, with the type strain RG1\textsuperscript{T} (\textsuperscript{=}MTCC 6912\textsuperscript{T}=LMG 23498\textsuperscript{T}).

Endophytic bacteria colonize the internal tissues of the host plant for mutual benefits. The nitrogen-fixing endophyte Gluconacetobacter diazotrophicus has been found to be associated with sugarcane, pineapple, grass, sweet potato, mango and banana (Muthukumarasamy \textit{et al.}, 2002), while Gluconacetobacter azotocaptans and Gluconacetobacter johannae inhabit coffee plants (Fuentes-Ramirez \textit{et al.}, 2001) and provide host plants with useful fixed nitrogen and growth stimulants. The first report of the occurrence and association of nitrogen-fixing \textit{Acetobacter peroxydans} along with \textit{G. diazotrophicus} inhabiting cultivated wetland rice varieties demonstrated the presence of nitrogen-fixing property in the genus \textit{Acetobacter} (Muthukumarasamy \textit{et al.}, 2005). Based on the striking resemblance of the high sugar and low pH habitats of sugarcane and Kombucha tea (Blanc, 1996), we explored and isolated a nitrogen-fixing strain RG1\textsuperscript{T} belonging to the genus \textit{Acetobacter} of the family \textit{Acetobacteraceae}. This family has been divided into eight genera; \textit{Acetobacter}, \textit{Gluconacetobacter}, \textit{Gluconobacter}, \textit{Acidomonas}, (Yamada \textit{et al.}, 1997), \textit{Asaia} (Yamada \textit{et al.}, 2000), \textit{Kozakia} (Lisdiyanti \textit{et al.}, 2002), \textit{Saccharibacter} (Jojima \textit{et al.}, 2004) and \textit{Swaminathan} (Loganathan \& Nair, 2004). The genus \textit{Acetobacter} comprises, at present, 15 validly described species that were delineated mainly on the basis of DNA–DNA relatedness and phylogenetic relationships (Sokolkel \textit{et al.}, 1998; Lisdiyanti \textit{et al.}, 2000, 2001; Cleenwerck \textit{et al.}, 2002; Silva \textit{et al.}, 2006). This report of the isolation of nitrogen-fixing strain RG1\textsuperscript{T} from the novel source Kombucha tea is only second in the genus \textit{Acetobacter} after \textit{A. peroxydans} (Muthukumarasamy \textit{et al.}, 2005).

We present morphological, biochemical and genetic evidence which indicates that this isolate represents a novel nitrogen-fixing species within the genus \textit{Acetobacter} isolated from a novel source. We propose the name \textit{Acetobacter nitrogenifigens} for the RG1\textsuperscript{T} isolate.

Aliquots of Kombucha mat suspension, after teasing the mat apart in the soup, were spread on to LGI (0–06 % KH\textsubscript{2}PO\textsubscript{4}, 0–02 % K\textsubscript{2}HPO\textsubscript{4}, 0–02 % MgSO\textsubscript{4}, 0–002 % CaCl\textsubscript{2}, 0–001 % FeCl\textsubscript{3}, 0–0002 % Na\textsubscript{2}MoO\textsubscript{4}, 10 % sucrose, pH 4–5; Cavalcante \& Dobereiner, 1988) agar plates containing 150 mg cycloheximide l\textsuperscript{–1} (Jimenez-Salgado \textit{et al.}, 1997) and 150 mg nystatin l\textsuperscript{–1}. Plates were incubated at 30 °C for 5 days. The bacterial isolate was purified by repeated streaking on to LGI plates, which have no combined nitrogen source. Gas tight vials of bacteria-inoculated LGI medium (under microaerophilic environment, without...
shaking) were assayed for acetylene reduction activity (Stal, 1988). Nitrogenase-positive isolates were selected for further characterization. Nitrogen-fixing bacterial strain RG1T was isolated. Other reference strains and the novel isolates were grown in mannitol broth for further study. Colony morphology was examined on LGI agar plates and on potato agar plates containing 10% sucrose. Various phenotypic and morphological studies were performed using standard techniques described elsewhere (Cavalante & Döbereiner, 1988; Caballero-Mellado et al., 1995; Jimenez-Salgado et al., 1997; Cleenwerck et al., 2002). Isoprenoid quinone of the isolate was extracted with chloroform/methanol [2:1, (v/v)], and then purified by TLC on silica gel 60 F254 (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 reverse-phase column [Luna 5 μC18 (2) 100A, 250 × 4.6 mm; Phenomenex] and a mixture of methanol/2-propanol [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 coenzymes Q-9 and Q-10 standards from Sigma-Aldrich. Ubiquinone Q-9 was present in strain RG1T and fitted with the previous observations that showed the presence of this ubiquinone type in the genus Acetobacter (Cleenwerck et al., 2002). The type strain RG1T deviated biochemically and morphologically from other species of the genus Acetobacter as shown in Table 1.

A 1451 bp fragment of 16S rRNA gene was PCR amplified with bacteria-specific primers fD1 and rD1 (Weisburg et al., 1991) using Taq polymerase and genomic DNA from strain RG1T as template. The nucleotide sequence was similar to the extent of 98.2% with Acetobacter aceti, 97.7% with Acetobacter estuensis, 97.4% with Acetobacter indonesiensis, 97.2% with Acetobacter tropicalis, 97.2% with Acetobacter oeni, 97.1% with Acetobacter cibinongensis, 97.0% with Acetobacter cerevisiae, 97.0% with Acetobacter malorum, 96.9% with Acetobacter orleanensis, 96.6% with Acetobacter orientalis and 97.0% with Acetobacter syzygii after performing a similarity search with FASTA (ungapped). The phylogenetic tree (Fig. 1) was deduced using MEGA version 3.1 (Kumar et al., 2004) software after multiple alignment of 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 was determined by using bootstrap values (Felsenstein, 1985) based on 100 replications. Two subclusters were evident in the genus Acetobacter: one composed of Acetobacter pomorum, Acetobacter pasteurianus, A. syzygii, Acetobacter lovaliensis and A. peroxydans and the other included Acetobacter aceti and other acetobacters along with the novel nitrogen-fixing strain RG1T. The clustering of strain RG1T with A. aceti was further confirmed by the formation of dihydroxyacetone from glycerol by both strains RG1T and A. aceti ATCC 15973T.

Cell extracts were prepared for multilocus enzyme electrophoresis (MLEE) and run on a starch gel as described

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Table 1: Differential characteristics between Acetobacter nitrogenifigens sp. nov. and closely related members of the genus Acetobacter

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1*</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5*</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tr>
<td>Growth on ammonium with ethanol</td>
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<td>+</td>
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<tr>
<td>Growth in presence of 10% ethanol</td>
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<td>+</td>
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<td>Maltose</td>
<td>+</td>
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<tr>
<td>Methanol</td>
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<td>Growth on YE + 3% D-glucose</td>
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<td>Growth on N-free LGI plate*</td>
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<tr>
<td>Motility</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>55.7–58.1</td>
<td>57.2</td>
<td>56.9–58.3</td>
<td>55.5–65.2</td>
<td>58.1</td>
<td>54.0–54.2</td>
<td>52.9–52.8</td>
<td>59.2–60.2</td>
<td>56.9–57.6</td>
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*This work.
previously (Selander et al., 1986; Caballero-Mellado & Martínez-Romero, 1994). *A. cibinongensis* JCM 11196T, *A. orleansis* LMG 1583T, *A. malorum* LMG 1746T, *A. aceti* ATCC 15973T, *A. tropicalis* LMG 19825T, *A. oeni* B13T, *A. orientalis* JCM 11195T, *A. indonesiensis* LMG 19824T, *A. estunensis* LMG 1626T and *A. cerevisiae* LMG 1625T were included as reference strains. The relationships among non-nitrogen-fixing species of *Acetobacter* and RG1T are illustrated by the dendrogram (Supplementary Fig. S1 available in IJSEM Online) derived by program ETMEGA (T. S. Whittam; http://foodsafe.msu.edu/whittam/programs/index.html) and MEGA version 3.1 software based on the electrophoretic mobility of nine metabolic enzymes, indophenol oxidase, alcohol dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, xanthine dehydrogenase, leucine dehydrogenase, lysine dehydrogenase, hexokinase and esterase. In MLEE, strain RG1T exhibited highest similarity to *A. aceti* ATCC 15973T, which is in agreement with its phylogenetic position (Fig. 1). Analysis of the dendrogram revealed that strain RG1T formed a unique branch with *A. aceti* ATCC 15973T that deviated at a genetic distance of more than 0.5. This value has been used as a criterion to suggest species limits (Selander et al., 1985; Musser et al., 1987). Thus, MLEE results strongly support the existence of strain RG1T as a novel *Acetobacter* species.

The nitrogenase enzyme complex, responsible for nitrogen-fixation, is composed of *nifHDK* and other gene fragments. A 336 bp region encoding dinitrogenase reductase, *nifH*, was amplified from strain RG1T using degenerate primers 19F and 407R (Franke et al., 1998) and sequenced. Presence of *nifH* gene further confirmed the nitrogen-fixing ability of strain RG1T.

To determine genomic relatedness of the novel 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 and 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 equal sized circles. A genomic DNA probe was prepared from the novel strain RG1T, digested with EcoRI and run on 0.7% agarose gel. Total DNA digests were transferred from gels to nylon membrane by Southern blotting. Hybridization was performed at a temperature of 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). A low level of genomic DNA relatedness (DNA–DNA hybridization values less than 50%) was observed between the phylogenetically closest species and genera. Strain RG1T showed low DNA–DNA relatedness with the type strains of *A. oeni* (24%), *A. aceti* (22%), *A. cerevisiae* (19%), *A. cibinongensis* (18-91%), *A. orientalis* (18-9%), *A. estunensis* (11%), *A. orleansis* (10-37%), *A. malorum* (7-31%), *A. indonesiensis* (3-71%) and *A. tropicalis* (2%).

Although the limitations of 16S rRNA gene sequencing to differentiate closely related species have been documented...
Cells are straight rods with rounded ends, approximately nitrogenifigens adj. nitrogenium nov. species, hence strain RG1T represent a novel species of the genus Acetobacter. In view of low physiological, biochemical, phylogenetic and genetic similarities among different closely related members of the genus Acetobacter, we recommend that nitrogen-fixing strain RG1T described here should be assigned to a novel species of the genus Acetobacter, for which the name Acetobacter nitrogenifigens sp. nov. is proposed.

**Description of Acetobacter nitrogenifigens sp. nov.**

Acetobacter nitrogenifigens (ni.tro.gen.‘i’ff.gens. N.L. n. nitrogenium nitrogen; L. part. adj. figens fixing; N.L. part. adj. nitrogenifigens nitrogen-fixing).

Cells are straight rods with rounded ends, approximately 1.5–2.0 μm in length and 0.1–0.2 μm in width and occur singly or in chains. These Gram-negative bacteria are motile with polar flagellation, catalase-positive and oxidase-negative. Growth occurs on nitrogen-free LGI plates at 30 °C, but not at 37 °C and in LGI broth under microaerophilic conditions. After incubation for 5 days, colonies grown on LGI plates are smooth, round, glistening, transparent and 2–3 mm in diameter, while 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. Strains are aerobic and fix atmospheric nitrogen microaerobically. Growth occurs well in the presence of ammonium, but nitrate is not reduced. In absence of yeast extract, strains can utilize different carbon sources such as D-galactose, D-xylose, D-fructose, D-arabinose, D-mannitol, D-sorbitol, and grow on 30% sucrose and 30% glucose. Dihydroxyacetone is produced from glycerol and γ-pyrene from D-glucose. Ethanol is oxidized to acetic acid, and acetate and lactate over oxidize to CO₂ and water. Methanol is not utilized. Single amino acids like L-alanine, L-cysteine and L-phenylalanine can be used as a sole source of carbon and nitrogen. L-Threonine is not utilized as the sole source of carbon and nitrogen. The ubiquinone present is of the type Q-9 and DNA G+C content is 64-1 mol%. Based on data from the MLEE assay along with DNA–DNA relatedness and nifH gene sequence, the type strain RG1T can be differentiated from other Acetobacter species.

The type strain is strain RG1T (=MTCC 6912 = LMG 23498T), isolated from Kombucha tea.

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**References**


