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^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^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 γ -pyrone from glucose and dihydroxyacetone from glycerol. Strain RG1^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^T represents a novel nitrogen-fixing species. The ubiquinone present was Q-9 and DNA G + C content was 64.1 mol%. Strain RG1^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 the basis of this data, the name *Acetobacter nitrogenifigens* sp. nov. is proposed, with the type strain RG1^T (=MTCC 6912^T = LMG 23498^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, cameroon grass, sweet potato, mango and banana (Muthukumarasamy *et al.*, 2002), while *Gluconacetobacter azotocaptans* and *Gluconacetobacter johannae* inhabit coffee plants (Fuentes-Ramirez *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 *Acetobacter peroxydans* along with *G. diazotrophicus* inhabiting cultivated wetland rice varieties demonstrated the presence of nitrogen-fixing property in the genus *Acetobacter* (Muthukumarasamy *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^T belonging to the genus *Acetobacter* of the family *Acetobacteraceae*. This family has been divided into eight genera; *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Acidomonas*, (Yamada

et al., 1997), *Asaia* (Yamada *et al.*, 2000), *Kozakia* (Lisdiyanti *et al.*, 2002), *Saccharibacter* (Jojima *et al.*, 2004) and *Swaminathanian* (Loganathan & Nair, 2004). The genus *Acetobacter* comprises, at present, 15 validly described species that were delineated mainly on the basis of DNA–DNA relatedness and phylogenetic relationships (Sokollek *et al.*, 1998; Lisdiyanti *et al.*, 2000, 2001; Cleenwerck *et al.*, 2002; Silva *et al.*, 2006). This report of the isolation of nitrogen-fixing strain RG1^T from the novel source Kombucha tea is only second in the genus *Acetobacter* after *A. peroxydans* (Muthukumarasamy *et al.*, 2005).

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

Aliquots of Kombucha mat suspension, after teasing the mat apart in the soup, were spread on to LGI (0.06 % KH₂PO₄, 0.02 % K₂HPO₄, 0.02 % MgSO₄, 0.002 % CaCl₂, 0.001 % FeCl₃, 0.0002 % Na₂MoO₄, 10 % sucrose, pH 4.5; Cavalcante & Döbereiner, 1988) agar plates containing 150 mg cycloheximide l⁻¹ (Jimenez-Salgado *et al.*, 1997) and 150 mg nystatin l⁻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

Abbreviation: MLEE, multilocus enzyme electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *nifH* gene sequences of strain RG1^T are AY669513 and AY952470, respectively.

A MLEE dendrogram showing the relationships between *Acetobacter nitrogenifigens* sp. nov. and type strains of closely related *Acetobacter* species is available as a supplementary figure in IJSEM Online.

shaking) were assayed for acetylene reduction activity (Stal, 1988). Nitrogenase-positive isolates were selected for further characterization. Nitrogen-fixing bacterial strain RG1^T 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 (Cavalcante & 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 5U C18 (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 RG1^T 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 RG1^T 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 RG1^T as template. The nucleotide sequence was similar to the extent of 98.2 % with *Acetobacter aceti*, 97.7 % with *Acetobacter estunensis*, 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 orleansis*, 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 RG1^T. The clustering of strain RG1^T with *A. aceti* was further confirmed by the formation of dihydroxyacetone from glycerol by both strains RG1^T and *A. aceti* ATCC 15973^T.

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

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

Strains/species: 1, *A. nitrogenifigens* RG1^T; 2, *A. cibinongensis* LMG 21418^T; 3, *A. orleanensis*; 4, *A. malorum* LMG 1746^T; 5, *A. aceti*; 6, *A. tropicalis*; 7, *A. oeni* B13^T; 8, *A. indonesiensis*; 9, *A. orientalis* LMG 21417^T; 10, *A. estunensis*; 11, *A. cerevisiae*. w, Weak; v, variable; ND, not determined; +, positive; -, negative. Data for species 1 and 5 are from this study. Motility data for species 4 and 11 are taken from Cleenwerck *et al.* (2002). All other data are from Silva *et al.* (2006) unless otherwise indicated.

Characteristic	1*	2	3	4	5*	6	7	8	9	10	11
Growth in ammonium with ethanol	+	w	-	-	+	-	-	-	-	+	-
Growth in presence of 10% ethanol	+	-	-	-	-	-	+	-	-	-	ND
Growth on carbon sources											
Maltose	-	-	v	-	v	+	-	+	w	v	-
Methanol	-	-	-	-	-	-	-	-	w	-	-
Growth on YE + 30% D-glucose	+	+	-	-	-	-	-	-	-	-	-
Growth on N-free LGI plate*	+	-	-	-	-	-	-	-	-	-	-
Motility	Polar-flagellation	ND	ND	Non-motile	ND	ND	Peritrichous flagellation	ND	ND	ND	Non-motile
DNA G + C content (mol%)	64.1	53.8–54.5	55.7–58.1	57.2	56.9–58.3	55.6–56.2	58.1	54.0–54.2	52.0–52.8	59.2–60.2	56.0–57.6

*This work.

Fig. 1. Phylogenetic relationships of acetic acid bacteria based on 16S rRNA gene sequences involving *Acetobacter nitrogenifigens* sp. nov. and other related species of the family *Acetobacteraceae*. Numbers at nodes indicate bootstrap values derived from 100 replications. GenBank accession numbers of 16S rRNA gene sequences are indicated in parentheses. Bar, 2 substitutions per 100 nucleotides.

previously (Selander *et al.*, 1986; Caballero-Mellado & Martínez-Romero, 1994). *A. cibinongensis* JCM 11196^T, *A. orleanensis* LMG 1583^T, *A. malorum* LMG 1746^T, *A. aceti* ATCC 15973^T, *A. tropicalis* LMG 19825^T, *A. oeni* B13^T, *A. orientalis* JCM 11195^T, *A. indonesiensis* LMG 19824^T, *A. estunensis* LMG 1626^T and *A. cerevisiae* LMG 1625^T were included as reference strains. The relationships among non-nitrogen-fixing species of *Acetobacter* and RG1^T 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 RG1^T exhibited highest similarity to *A. aceti* ATCC 15973^T, which is in agreement with its phylogenetic position (Fig. 1). Analysis of the dendrogram revealed that strain RG1^T formed a unique branch with *A. aceti* ATCC 15973^T 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 RG1^T 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 RG1^T using degenerate primers

19F and 407R (Franke *et al.*, 1998) and sequenced. Presence of *nifH* gene further confirmed the nitrogen-fixing ability of strain RG1^T.

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 RG1^T, 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 RG1^T 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. orleanensis* (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

(Fox *et al.*, 1992), it is suggested that strains sharing greater than 97 % similarity might belong to the same species but a lower similarity value could form the basis of delineation of bacterial species (Stackebrandt & Goebel, 1994). Also a DNA–DNA relatedness level below 70 % indicates a distinct species (Stackebrandt & Goebel, 1994). In the present study, these considerations were consistent with both the 16S rRNA gene sequence similarity levels and the low levels of DNA–DNA relatedness exhibited within the *Acetobacter* species. The genetic distance of different closely related acetobacters involving strain RG1^T was found to be greater than 0.5 in all comparisons and such a distance indicates a novel species; hence strain RG1^T 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 RG1^T 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'fi.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-pyrone 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 RG1^T can be differentiated from other *Acetobacter* species.

The type strain is strain RG1^T (=MTCC 6912^T=LMG 23498^T), isolated from Kombucha tea.

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