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Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants

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Diazotrophic bacteria were isolated, in two different years, from the rhizosphere and rhizoplane of coffee (*Coffea arabica* L.) plants cultivated in Mexico; they were designated as type DOR and type SAd isolates. They showed characteristics of the family *Acetobacteraceae*, having some features in common with *Gluconacetobacter* (formerly *Acetobacter*) *diazotrophicus*, the only known N_2-fixing species of the acetic acid bacteria, but they differed from this species with regard to several characteristics. Type DOR isolates can be differentiated phenotypically from type SAd isolates. Type DOR isolates and type SAd isolates can both be differentiated from *Gluconacetobacter diazotrophicus* by their growth features on culture media, their use of amino acids as nitrogen sources and their carbon-source usage. These results, together with the electrophoretic mobility patterns of metabolic enzymes and amplified rDNA restriction analysis, suggested that the type DOR and type SAd isolates represent two novel N_2-fixing species. Comparative analysis of the 16S rRNA sequences revealed that strains CFN-Cf55^T (type DOR isolate) and CFN-Ca54^T (type SAd isolate) were closer to *Gluconacetobacter diazotrophicus* (both strains had sequence similarities of 98·3%) than to *Gluconacetobacter liquefaciens, Gluconacetobacter sacchari* (similarities < 98%) or any other acetobacteria. Strain CFN-Cf55^T exhibited low levels of DNA–DNA reassociation with type SAd isolates (mean 42%) and strain CFN-Ca54^T exhibited mean DNA–DNA reassociation of 39·5% with type DOR isolates. Strains CFN-Cf55^T and CFN-Ca54^T exhibited very low DNA reassociation levels, 7–21%, with other closely related acetobacterial species. On the basis of these results, two novel N_2-fixing species are proposed for the family *Acetobacteraceae*, *Gluconacetobacter johannae* sp. nov. (for the type DOR isolates), with strain CFN-Cf55^T (= ATCC 700987^T = DSM 13595^T) as the type strain, and *Gluconacetobacter azotocaptans* sp. nov. (for the type SAd isolates), with strain CFN-Ca54^T (= ATCC 700988^T = DSM 13594^T) as the type strain.

Keywords: *Gluconacetobacter johannae* sp. nov., *Gluconacetobacter azotocaptans* sp. nov., acetic acid bacteria, nitrogen fixation, coffee plants

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Abbreviations: ARDRA, amplified rDNA restriction analysis; MLEE, multilocus enzyme electrophoresis.

The GenBank accession numbers for the 16S rDNA sequences of strains CFN-Cf55^T and CFN-Ca54^T are AF111841 and AF192761, respectively.

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INTRODUCTION

Bacteria of the family *Acetobacteraceae* are characterized phenotypically by their ability to grow at low pH and by their ability to oxidize ethanol to acetic acid (De Ley *et al.*, 1984a; Swings, 1992). This family has been divided historically into the genera *Acetobacter* and *Gluconobacter* (De Ley *et al.*, 1984a; Swings, 1992). However, the classification of the acetic acid bacteria group has been subject to controversy. For instance, the transfer of the methylotrophic species *Acetobacter methanolicus* to a new genus, *Acidomonas*, has been proposed (Urakami *et al.*, 1989) and is supported by 5S rRNA sequence data (Bulygina *et al.*, 1992), but the creation of this new genus has been criticized and was therefore not recognized (Sievers *et al.*, 1994; Swings, 1992). Similarly, the establishment of the subgenus *Gluconoacetobacter* (Yamada & Kondo, 1984) has been questioned (Swings, 1992). More recently, Yamada *et al.* (1997) proposed the division of the acetic acid bacteria into four genera, *Acetobacter*, *Gluconobacter*, *Gluconoacetobacter* and *Acidomonas*, on the basis of an analysis of partial 16S rRNA sequences. In that proposal, only the species *Acetobacter aceti* and *Acetobacter pasteurianus* were retained in the genus *Acetobacter*, whilst the species *Acetobacter diazotrophicus*, *Acetobacter europaeus*, *Acetobacter hansenii*, *Acetobacter liquefaciens* and *Acetobacter xylinus* were transferred to the genus *Gluconoacetobacter*, which has subsequently been corrected to *Gluconacetobacter* (Yamada *et al.*, 1998). In addition to the species referred to in the study by Yamada *et al.* (1997), two further species, *Acetobacter oboediens* and *Acetobacter pomorum*, have been described (Sokollek *et al.*, 1998). However, *Acetobacter oboediens*, together with *Acetobacter intermedius*, has been reassigned to the genus *Gluconacetobacter* (Franke *et al.*, 1999; Yamada, 2000). More recently, *Gluconacetobacter sacchari*, a novel species of acetic acid bacterium, has been described (Franke *et al.*, 1999).

Gluconacetobacter (formerly *Acetobacter*) *diazotrophicus*, an endophytic bacterium (Cavalcante & Döbereiner, 1988; Fuentes-Ramírez *et al.*, 1993), is the only known N_2-fixing species belonging to the acetic acid bacteria (Gillis *et al.*, 1989) and is suggested to be a nitrogen contributor for sugar cane crops (Boddey *et al.*, 1991). For this reason, therefore, the search for this species has been extended to other plants. Our search for N_2-fixing bacteria associated with coffee plants led to the isolation of *Gluconacetobacter diazotrophicus* (Jiménez-Salgado *et al.*, 1997). In addition, other acetic-acid-producing, diazotrophic bacteria were recovered from the rhizospheres of coffee plants. These diazotrophs, referred to in our previous study as type DOR, SAd, NAP and APL isolates, shared features with the genus *Gluconacetobacter* but differed from *Gluconacetobacter diazotrophicus* with respect to morphological and biochemical traits, as well as genetic and molecular features.

In this study, we present an extended taxonomic analysis of type DOR and type SAd isolates, including new isolates recovered from the rhizospheres and rhizoplanes of coffee plants. We present evidence that indicates that these isolates represent two novel N_2-fixing species within the genus *Gluconacetobacter*. We propose the names *Gluconacetobacter johannae* sp. nov. for the type DOR isolates and *Gluconacetobacter azotocaptans* sp. nov. for the type SAd isolates.

METHODS

Isolation and reference strains. Rhizosphere soil around the root and root samples from coffee plants (*Coffea arabica* L. var. Caturra, collected in Motozintla, Chiapas State, Mexico) were treated as described previously (Jiménez-Salgado *et al.*, 1997) and were inoculated into vials containing 5 ml N-free semi-solid LGI medium (Cavalcante & Döbereiner, 1988). Vials were incubated at 30 °C for 5 d; thereafter, vials were replicated under the same conditions and assayed for acetylene-reduction activity as described previously (Mascarúa-Esparza *et al.*, 1988). N_2-fixing type DOR and SAd isolates were recovered as described previously (Jiménez-Salgado *et al.*, 1997); N_2-fixing type DOR and SAd isolates and strains representative of the different species of the family *Acetobacteraceae* used in this study are shown in Table 1.

Phenotypic characterization. Strains were grown at 29 °C unless otherwise indicated. An inoculum was prepared by growing type DOR or SAd isolates and *Gluconacetobacter diazotrophicus* strains for 12 h in SYP medium (Caballero-Mellado & Martínez-Romero, 1994) modified by increasing the amount of yeast extract to 0·3% (w/v). Other acetobacteria species were grown in MESMA liquid medium (Fuentes-Ramírez *et al.*, 1999). The cultures were centrifuged twice and resuspended in 10 mM $MgSO_4$. Each culture was streaked on solid media to determine phenotypic characteristics. Four replicates were used for each characteristic examined; growth was recorded after 5 d of incubation. Colony morphology was examined on LGI (Cavalcante & Döbereiner, 1988) agar plates supplemented with 0·005% yeast extract and on potato agar (Cavalcante & Döbereiner, 1988) containing 5, 10 or 15% (w/v) cane sugar. Compounds used as sole carbon sources were tested in the presence and absence of growth factors from yeast extract (0·005%), using LGI medium supplemented with 0·1% NH_4Cl, and with cane sugar replaced by individual filter-sterilized (0·22 µm pore) carbon substrates (0·5%). Acetic acid and citric acid were also tested at 0·1%. Butanol, ethanol and methanol were tested at 0·1 and 0·5% (v/v). Sucrose was used as a positive control; the negative control did not contain a carbon substrate. When amino acids were tested as sole nitrogen sources, the LGI medium was modified by omitting cane sugar and adding sorbitol at a final concentration of 0·5% (w/v). Modified LGI medium containing NH_4Cl (0·1%, w/v) was used as a positive control; the negative control lacked a nitrogen source. The same LGI basal medium (without cane sugar) was used to test L-amino acids as carbon and nitrogen sources. LGI basal media containing sucrose (0·5%) and NH_4Cl (0·1%) or lacking both carbon and nitrogen sources were used as positive and negative controls. Filter-sterilized (0·22 µm pore) L-amino acids were added (final concentration 0·1%) in both assays described above. Unless stated otherwise, LGI culture medium was adjusted with HCl to a final pH of 5·5 for the

Table 1. Representative strains of novel N_2-fixing acetobacteria species associated with coffee plants and strains of related species used in this study

Strain	Source of isolate	Reference/source
Type DOR isolates		
Gluconacetobacter johannae CFN-Cf55^T	Rhizosphere	Jiménez-Salgado *et al.* (1997)
Gluconacetobacter johannae UAP-Cf57	Rhizosphere	Jiménez-Salgado *et al.* (1997)
Gluconacetobacter johannae CFN-Cf75	Rhizosphere	This study
Gluconacetobacter johannae UAP-Cf76	Rhizoplane	This study
Type SAd isolates		
Gluconacetobacter azotocaptans CFN-Ca54^T*	Rhizosphere	Jiménez-Salgado *et al.* (1997)
Gluconacetobacter azotocaptans UAP-Ca97	Rhizosphere	This study
Gluconacetobacter azotocaptans UAP-Ca99	Rhizoplane	This study
Gluconacetobacter diazotrophicus PAl 5^T	Sugar cane	Cavalcante & Döbereiner (1988)
Gluconacetobacter diazotrophicus UAP-5560	Sugar cane	Fuentes-Ramírez *et al.* (1993)
Gluconacetobacter diazotrophicus UAP-Cf05	Coffee	Jiménez-Salgado *et al.* (1997)
Acetobacter aceti ATCC 15973^T	–	ATCC
Acetobacter pasteurianus ATCC 33445^T	–	ATCC
Gluconacetobacter hansenii ATCC 35959^T	–	ATCC
Gluconacetobacter liquefaciens ATCC 14835^T	–	ATCC
Gluconobacter oxydans ATCC 19357^T	–	ATCC

* This strain was formerly designated CFN-Cf54 (Jiménez-Salgado *et al.*, 1997).

tests described above, and production of acid was recorded. In LGI medium, bromcresol green (0·0025%) was used as the pH indicator instead of bromothymol blue. Additional tests included the production of water-soluble brown pigments, the oxidation of ethanol to acetic acid and the oxidation of acetate or lactate to CO_2 and water on GYC medium (De Ley *et al.*, 1984b). Growth at 29 and 37 °C at various pH values and tolerance of NaCl at up to 1·5% (w/v) were determined in LGI liquid medium supplemented with 0·005% yeast extract.

Multilocus enzyme electrophoresis (MLEE). Cell extracts for MLEE assays were prepared as described previously (Caballero-Mellado & Martínez-Romero, 1994). Starch gel electrophoresis and the selective staining of indophenol oxidase, lysine dehydrogenase, leucine dehydrogenase, xanthine dehydrogenase, alcohol dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, esterases and an unidentified dehydrogenase were done by using methods described previously (Selander *et al.*, 1986). These enzymes were analysed under the conditions described previously (Caballero-Mellado *et al.*, 1995). Distinctive combinations of alleles for the 10 enzyme loci were designated as different electrophoretic types (Selander *et al.*, 1986). The dendrogram illustrating the relatedness among strains was obtained from the programs ETDIV and ETCLUS from T. S. Whittam, kindly provided by B. D. Eardly (Pennsylvania State University). *Gluconacetobacter diazotrophicus* strains PAl 5^T, UAP 5560 and UAP-Cf 05 and the type strains of *Gluconacetobacter hansenii*, *Gluconacetobacter liquefaciens*, *Acetobacter aceti* and *Acetobacter pasteurianus* were included as references in MLEE assays.

DNA isolation, RFLP analysis of 16S rRNA genes and DNA–DNA reassociation analysis. Cultures were grown in SYP or MESMA liquid media for 16 h and centrifuged at 12000 ***g*** and total DNA was prepared by using a DNA–RNA isolation kit (USB Amersham). To distinguish the family *Acetobacteraceae* from other *α-Proteobacteria*, hybridization patterns of 16S rRNA genes were analysed as described previously (Jiménez-Salgado *et al.*, 1997). Total DNA from the N_2-fixing acetobacteria was restricted with *Sph*I and *Nco*I and Southern blots were hybridized with a 16S rDNA probe. In addition, DNA was digested with *Eco*RI and electrophoresed in vertical 1·0% agarose gels; total DNA digests were transferred from gels to nylon filters by Southern blotting (Caballero-Mellado & Martínez-Romero, 1994). DNA relatedness was based on relative levels of reassociation to ^{32}P-labelled DNA, using the *redi*prime DNA-labelling system (Amersham). Labelled DNAs in independent experiments were from strains CFN-Cf55^T (type DOR isolate) and CFN-Ca54^T (type SAd isolate). DNA–DNA reassociation was for 12 h at 65 °C and the nylon filters were washed once in 2× SSC at room temperature for 10 min and once in 1× SSC for 5 min at 65 °C. Autoradiography was performed for 4 h; filter lanes were cut and the radioactivity estimated with a Beckman scintillation counter. Percentage reassociation was calculated for each strain tested in relation to the homologous control.

Amplified rDNA restriction analysis (ARDRA) and nucleotide sequence of 16S rRNA genes. The 16S rRNA genes from strains CFN-Cf55^T and CFN-Ca54^T were amplified by PCR with the primers fD1 and rD1 (Weisburg *et al.*, 1991) using the proof-reading *Pwo* DNA polymerase (Boehringer-Roche). The PCR conditions were an initial denaturing cycle (95 °C, 3 min), 35 amplification cycles (95 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min) and a final elongation cycle (72 °C, 3 min). Approximately 400 ng PCR-amplified 16S rRNA gene fragment (approx. 1·5 kb) was restricted with 10 U of each endonuclease (*Alu*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I, *Nci*I, *Rsa*I, *Sau*3AI and *Taq*I). The lengths of the restriction fragments were determined from their electrophoretic separation in 3% agarose gels and the restriction patterns from each isolate were compared. To obtain nucleotide sequences, PCR products from strains CFN-Cf55^T and CFN-Ca54^T

Table 2. Comparison of the novel N_2-fixing acetobacteria with *Gluconacetobacter diazotrophicus*

The number of strains of each species analysed is shown. Characters are scored as: +, good growth; ±, slight growth; −, no growth. Ranges are indicated by e.g. +/±. All taxa shown were catalase-positive and oxidase-negative and oxidized ethanol to acetic acid and acetate and lactate to CO_2 and water; were positive for acetylene reduction, even with 10 mM nitrate, and negative for nitrate reduction. All taxa shown grew on sucrose, D-glucose, L-fructose and gluconate, used L-alanine and L-aspartic acid as nitrogen sources with sorbitol as the carbon source and showed slight growth on L-leucine or L-lysine and no growth on L-glycine, L-methionine or L-threonine. None of the taxa used D-lactose, L-rhamnose, dulcitol, *myo*-inositol, citric acid, fumaric acid, D-glucuronic acid, DL-malic acid, starch or methanol (0·1 or 0·5%) as sole carbon sources or used single amino acids (L-alanine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glycine, L-leucine, L-lysine, L-proline, L-methionine, L-phenylalanine, L-threonine or L-tryptophan) as sole sources of carbon and nitrogen. *G.*, *Gluconacetobacter*.

Characteristic	Type DOR (*n* = 4) (*G. johannae*)	Type SAd (*n* = 3) (*G. azotocaptans*)	*G. diazotrophicus* (*n* = 3)
Dark brown colonies on potato agar with 5, 10 or 15% sugar	No	No	Yes
Growth on:*			
D-Galactose	+	±	+
D-Xylose	+	−	±/−
D-Raffinose	+/±	−	+
D-Arabinose	±/−	−	+
Melibiose	±	±/−	±
Maltose	+/±	±/−	±/−
Mannose	−	−	±
D-Sorbitol	+/±	+/±	+
Glycerol	±/−	−	+
D-Mannitol	±/−	−	+
Ethanol	+	+/±	±
Butanol	±	−	−
Growth on L-amino acids in the presence of sorbitol as carbon source:			
L-Cysteine	−	+	+
L-Glutamic acid	−	+	+
L-Proline	−	−	+
L-Tryptophan	+	−	+

* Regardless of the presence or absence of growth factors from yeast extract.

were cloned initially with a PCR cloning kit (Boehringer-Roche) in the pCAPs vector and subcloned in pUC19. Nucleotide sequences of the 16S rDNA genes were determined with an ALF automated sequencer (Pharmacia Biotech) using fluorescent primers for M13. The 16S rDNA sequences corresponded to positions 17–1524 of the sequence of *Escherichia coli* K-12 (accession no. AE000460).

ARDRA and nucleotide sequence analyses. The nucleotide sequences obtained in this study were compared with different 16S rDNA sequences of acetic acid bacteria obtained from GenBank. Multiple alignment of the sequences was performed by progressive pairwise alignments with the Wisconsin package (GCG version 8), based on the method of Feng & Doolittle (1987). Corrected evolutionary distances were calculated with the GCG version 8 using the method of Jukes & Cantor (1969). Taxonomic tree files from ARDRA and DNA sequences were constructed with the neighbour-joining method, using CLUSTAL W (Thompson *et al.*, 1994). The trees were bootstrapped with CLUSTAL W and displayed with the TREEVIEW program (Page, 1996). Over 50 16S rDNA sequences (accession numbers not shown) from acetic acid bacteria were aligned in order to search for discriminatory *Sph*I and *Nco*I restriction sites, as described previously (Jiménez-Salgado *et al.*, 1997).

Design of specific primers and PCR conditions. To obtain a rapid identification test for the type DOR and SAd isolates, specific primers were designed. The aligned 16S rDNA sequences from acetic acid bacteria showed conserved and variable regions. A region that did not show variability among the *Acetobacteraceae* species was selected for use in the design of a universal oligonucleotide. The variable regions were selected for use in the design of DOR- and SAd-specific oligonucleotides. Primers were designed with the help of the software OLIGO 4.0. PCR amplifications of 16S rDNA were performed both with purified DNA and with supernatants of cells heated at 95 °C for 8 min and centrifuged for 2 min at 12000 ***g***. PCRs contained 2·5 mM $MgCl_2$, 20 nM primers, 1 μM dNTPs and 0·06 U *Taq* polymerase μl^{-1}. PCR amplifications were performed with the primer U475 and one of the specific primers, using the following protocol: 95 °C for 3 min, 32 cycles of 95 °C for 1 min, specific primer annealing temperature for 1 min and 72 °C for 1 min and finally 72 °C for 3 min. Amplifications with the selected primers were tested both with purified DNA and with cell extracts of the strains described in Table 1, as well as *Acidomonas methanolica* ATCC 43581, *Gluconacetobacter xylinus* ATCC 700178, *Gluconobacter cerinus* ATCC 19441[T] and *Gluconobacter asaii* ATCC 43781.

RESULTS

Phenotypic analysis of novel N_2-fixing acetobacterial species

Typical phenotypic characteristics of the N_2-fixing type DOR and type SAd isolates were compared with phenotypic features of *Gluconacetobacter diazotrophicus* (Table 2). The three N_2-fixing acetobacteria were Gram-negative and motile by means of peritrichous flagella (data not shown). They were capable of producing water-soluble brown pigments; however, the production of these pigments was variable among isolates of *Gluconacetobacter diazotrophicus*. Growth of type DOR and type SAd isolates in N-free semi-solid LGI medium resulted in the formation of a yellow surface pellicle similar to that described for *Gluconacetobacter diazotrophicus* (Cavalcante & Döbereiner, 1988), and isolates showed acetylene-reduction activity. However, these isolates did not exhibit growth typical of *Gluconacetobacter diazotrophicus* on LGI agar plates. The colony morphology of type DOR isolates has been described previously (Jiménez-Salgado *et al.*, 1997). DOR isolates in pure culture formed yellow–orange colonies, but, in contrast to those of *Gluconacetobacter diazotrophicus*, they were very irregular, smooth, flat colonies, 3–5 mm in diameter after 5 d growth. Type SAd colonies were similar to those of *Gluconacetobacter diazotrophicus* with regard to their orange colour, but formed round, mucous, smooth, convex colonies, 3–5 mm in diameter and with translucent margins. On potato agar with 5, 10 or 15% cane sugar, *Gluconacetobacter diazotrophicus* formed very characteristic dark-brown colonies after 5 d, as described elsewhere (Cavalcante & Döbereiner, 1988), whereas type SAd colonies were light brown (one isolate was reddish) but turned brownish after 10 d, and produced a brownish liquid pigment. In contrast, type DOR isolates formed only beige, or very light-brownish, colonies, even after 10 d incubation on potato agar plates.

Type DOR and SAd isolates differed from *Gluconacetobacter diazotrophicus* in their ability to utilize some substrates as sole carbon sources; regardless of the presence of growth factors from yeast extract, only a very few of the carbon substrates supported growth of the type DOR and type SAd isolates (Table 2). Growth of the type DOR and SAd isolates was slight on succinic acid. However, type DOR isolates can be differentiated from SAd isolates by their ability to grow on D-xylose, D-raffinose and 0·1% butanol. In addition, in the presence of sorbitol as the carbon source, type SAd isolates can be differentiated from the DOR isolates by their ability to utilize L-cysteine and L-glutamic acid (but not L-tryptophan) as nitrogen sources (Table 2). Type DOR isolates produced abundant acid on D-galactose, D-xylose and ethanol, but gave only slight acid production on D-raffinose, D-arabinose, maltose, D-sorbitol, glycerol and butanol. Type SAd isolates, like DOR isolates, produced abundant acid on 0·1 and 0·5% ethanol, but, in contrast, they showed only slight acid production when grown on D-galactose and D-sorbitol.

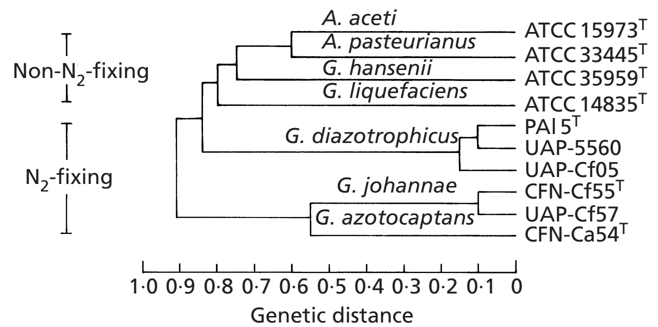

Fig. 1. Relationships among the N_2-fixing type DOR (*Gluconacetobacter johannae*) and type SAd (*Gluconacetobacter azotocaptans*) isolates and reference species of *Acetobacter* (*A.*) and *Gluconacetobacter* (*G.*) determined by MLEE. The electrophoretic types of strains UAP-Cf76 and CFN-Cf75 corresponded to those of strains CFN-Cf55^T and UAP-Cf57, respectively. The electrophoretic types of strains UAP-Ca97 and UAP-Ca99 corresponded to that of strain CFN-Ca54^T.

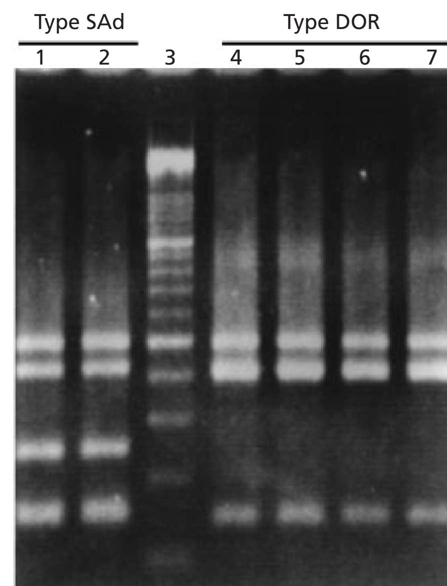

Fig. 2. ARDRA profiles of the N_2-fixing type DOR (*Gluconacetobacter johannae*) and type SAd (*Gluconacetobacter azotocaptans*) isolates digested with *Rsa*I. Lanes: 1, CFN-Ca54^T; 2, UAP-Ca97; 3, 100 bp molecular size marker; 4, CFN-Cf55^T; 5, UAP-Cf57; 6, CFN-Cf75; 7, UAP-Cf76.

MLEE assays

The relationships among the N_2-fixing type DOR and type SAd isolates and reference species of *Acetobacter* and *Gluconacetobacter* are illustrated by a dendrogram based on the electrophoretic mobility of metabolic enzymes (Fig. 1). The analysis revealed that the N_2-

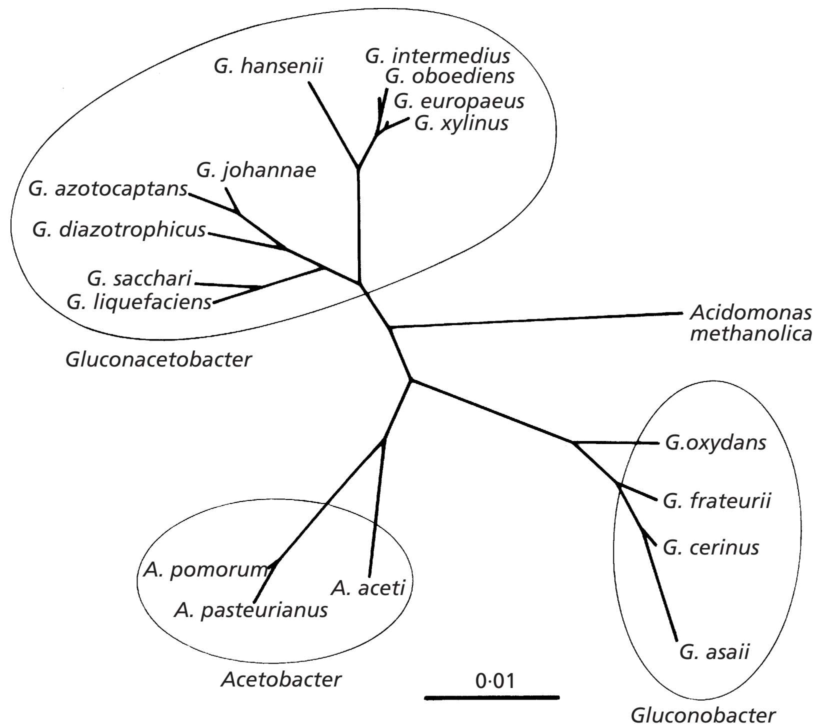

Fig. 3. Phylogenetic tree showing the relationships of the N_2-fixing type DOR (*Gluconacetobacter johannae*) and type SAd (*Gluconacetobacter azotocaptans*) isolates among the *Acetobacteraceae*. The tree was based on 16S rRNA gene sequences from acetic acid bacteria, and represents the maximum-likelihood tree that was obtained by progressive alignment. GenBank accession numbers were X75618 (*Gluconacetobacter diazotrophicus* PAl 5^T), Z21936 (*Gluconacetobacter europaeus* DSM 6160^T), X75620 (*Gluconacetobacter hansenii* NCIB 8746^T), Y14694 (*Gluconacetobacter intermedius* $TF2^T$), X75617 (*Gluconacetobacter liquefaciens* IFO 12388^T), AF127407 (*Gluconacetobacter sacchari* SRI 1794^T), AJ007698 (*Gluconacetobacter xylinus* BPR 2001), X75619 (*Gluconacetobacter xylinus* NCIB 11664^T), AJ001631 (*Gluconacetobacter oboediens* LTH 2460^T), X77468 (*Acidomonas methanolica* LMG 1668^T), X74066 (*Acetobacter aceti* NCIB 8621^T), X71863 (*Acetobacter pasteurianus* LMD 22.1^T), AJ001632 (*Acetobacter pomorum* LTH 2478^T), X80165 (*Gluconobacter asaii* IFO 3276^T), X80775 (*Gluconobacter cerinus* IFO 3267^T), X82290 (*Gluconobacter frateurii* IFO 3264^T) and X73820 (*Gluconobacter oxydans* DSM 3503^T). Bar, 1% estimated sequence divergence.

fixing type DOR and type SAd isolates formed two unique clusters, at a genetic distance of 0·560, which diverged significantly at distances of 0·920 from the *Gluconacetobacter diazotrophicus* cluster as well as from the non-N_2-fixing *Acetobacter–Gluconacetobacter* cluster.

RFLPs of 16S rDNA genes

Genomic DNA from the type DOR and type SAd isolates digested with *Sph*I and *Nco*I showed 1·3 and 1·24 kb bands, respectively, when hybridized with the 16S rDNA probe (data not shown). Such hybridization bands were observed previously in the *Acetobacteraceae* (Jiménez-Salgado *et al.*, 1997).

ARDRA analysis and nucleotide sequence of 16S rRNA genes

Type DOR, type SAd and *Gluconacetobacter diazotrophicus* isolates showed different restriction patterns for PCR products of 16S rDNA. Patterns from *Alu*I, *Dde*I, *Msp*I, *Nci*I and *Taq*I digestions of 16S rDNA differentiated type DOR and type SAd isolates from those of *Gluconacetobacter diazotrophicus* (data not shown). Similarly, the patterns from *Alu*I, *Dde*I, *Sau*3AI, *Taq*I and *Rsa*I digestions of 16S rDNA differentiated DOR and SAd isolates from those of *Gluconacetobacter liquefaciens* (data not shown). Type DOR isolates can be differentiated from type SAd strains by riboprinting only with *Rsa*I digestion (Fig. 2). The 16S rDNA sequence of *Gluconacetobacter johannae* (DOR isolates) shows three *Rsa*I sites, giving fragments of 403, 405, 504 and 135 bp. *Gluconacetobacter azotocaptans* (SAd isolates) possesses four *Rsa*I sites, generating fragments of 404, 159, 246, 504 and 134 bp. The 403 and 405 bp fragments in *Gluconacetobacter johannae* and those of 159 and 134 bp in *Gluconacetobacter azotocaptans* migrated together in agarose gel electrophoresis. In addition, the 16S rDNA sequences of the strains CFN-$Cf55^T$ and CFN-$Ca54^T$ were aligned and compared with those of closely related bacteria present in GenBank. The phylogenetic tree obtained with the 16S rDNA sequence data of the acetic-acid-producing bacteria is illustrated in Fig. 3. The genus *Gluconacetobacter* clearly constituted a cluster separate from the clusters formed by the genera *Acetobacter*, *Acidomonas* and *Gluconobacter*. Two subclusters were clearly evident within the genus *Gluconacetobacter*: one contained only non-diazotrophic species (*Gluconacetobacter europaeus*, *Gluconacetobacter hansenii*, *Gluconacetobacter xylinus*, *Gluconacetobacter oboediens* and *Gluconacetobacter intermedius*) and the other included both the non-diazotrophic species *Gluconacetobacter liquefaciens* and *Gluconacetobacter sacchari* as well as *Gluconacetobacter diazotrophicus* and the novel N_2-fixing type DOR (strain CFN-$Cf55^T$) and type SAd (strain CFN-$Ca54^T$) isolates. On the basis of the 16S rDNA sequence analysis, strains CFN-$Cf55^T$ and CFN-$Ca54^T$ (99·31% similarity) were closer to *Gluconacetobacter diazotrophicus* PAl 5^T (both strains

Table 3. DNA–DNA hybridization levels between representative strains of the novel N_2-fixing acetobacteria species and type strains of related species

Strain	DNA relatedness (%) with:	
	CFN-Cf55^T	CFN-Ca54^T
Gluconacetobacter johannae CFN-Cf55^T	100	42
Gluconacetobacter johannae UAP-Cf57	97	49
Gluconacetobacter johannae UAP-Cf76	95	33
Gluconacetobacter johannae CFN-Cf75	79	34
Gluconacetobacter azotocaptans CFN-Ca54^T	45	100
Gluconacetobacter azotocaptans UAP-Ca97	50	79
Gluconacetobacter azotocaptans UAP-Ca99	31	80
Gluconacetobacter diazotrophicus PAl 5^T	19	12
Gluconacetobacter diazotrophicus UAP-5560	21	20
Gluconacetobacter liquefaciens ATCC 14835^T	21	18
Acetobacter aceti ATCC 15973^T	7	8
Gluconobacter oxydans ATCC 19357^T	7	7

having similarities of 98·3 %) than to either *Gluconacetobacter liquefaciens* (similarities of 97·83 and 97·76 %, respectively) or *Gluconacetobacter sacchari* (similarities of 97·83 and 97·68 %, respectively).

Primer sequences

The following primers showed specific amplification of a fragment of the 16S rRNA genes of type DOR and type SAd isolates: primer U475 (5′-AATGACTGGGCGTAAAG-3′, universal primer); primer L927Gj (5′-GAAATGAACATCTCTGCT-3′, *Gluconacetobacter johannae* specific); primer L923Ga (5′-AATGCTCATCTCTGAACA-3′, *Gluconacetobacter azotocaptans* specific). The annealing temperatures were 62 °C (oligonucleotide L927Gj) and 67 °C (primer L923Ga). Under the conditions described, these primers allowed the amplification of a fragment of approximately 400 bp from the targeted species only (results not shown).

DNA relatedness

The results of DNA–DNA reassociation experiments are shown in Table 3. Type DOR isolates constituted a homogeneous group with high levels of DNA–DNA reassociation (mean 92·75 %) with reference strain CFN-Cf55^T. Strain CFN-Cf55^T exhibited a relatively low level of DNA–DNA reassociation with the type SAd isolates (mean 42 %). DNAs from other related acetobacterial species, including *Gluconacetobacter diazotrophicus*, *Gluconacetobacter liquefaciens*, *Acetobacter aceti* and *Gluconobacter oxydans*, exhibited very low DNA–DNA reassociation levels, ranging from 7 to 21 %, with total DNA from strain CFN-Cf55^T. In addition, a homogeneous group with relatively high levels of DNA–DNA reassociation (mean 86·3 %) was found among type SAd isolates with reference strain CFN-Ca54^T. The DNA relatedness of strain CFN-Ca54^T with type DOR isolates exhibited a mean of 39·5 % and the same strain (CFN-Ca54^T) exhibited less than 20 % reassociation (range 7–20 %) with the other related acetobacterial species mentioned above.

DISCUSSION

It is well known that Gram-negative, rod-shaped, aerobic bacteria that oxidize ethanol to acetic acid in neutral or acid media are candidates for membership of the family *Acetobacteraceae* (Swings, 1992). Additionally, the family *Acetobacteraceae* can be distinguished from other α-*Proteobacteria* by two internal *Sph*I and *Nco*I restriction sites in their 16S rDNA genes, except in the case of *Gluconobacter oxydans*, which lacks one of the *Nco*I restriction sites (because of a change in the base corresponding to nucleotide 110 of *Gluconacetobacter diazotrophicus*) (Caballero-Mellado *et al*., 1999; Jiménez-Salgado *et al*., 1997). Analysis of the 16S rDNA nucleotide sequence of the majority of acetobacterial strains reported in GenBank revealed that only a few strains lack the *Nco*I restriction site (nucleotide 110). This *Nco*I restriction site is present in all of the species included in the subcluster formed by the N_2-fixing type DOR and type SAd isolates, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter sacchari*. On the basis of the characteristics described above and the phenotypic features (Gram-negative aerobic bacteria, oxidation of ethanol to acetic acid, oxidation of acetate and lactate to CO_2 and water), it can be concluded that the N_2-fixing type DOR and type SAd isolates belong to the family *Acetobacteraceae*.

Over-oxidation of ethanol, first to acetic acid and then further to CO_2 and water, has been considered historically to be a fundamental phenotypic characteristic

of the genus *Acetobacter* (De Ley *et al*., 1984b; Swings, 1992) and it differentiates the genus from *Gluconobacter* (members of which are incapable of over-oxidizing ethanol). Members of the genus *Acidomonas*, as proposed by Yamada *et al*. (1997), can be distinguished phenotypically from closely related genera by their ability to grow on methanol. As type DOR and type SAd isolates were able to over-oxidize ethanol but did not show methylotrophic growth, these N_2-fixing bacteria can be classified as belonging to the genus *Acetobacter* according to Swings (1992). Otherwise, they might be assigned to the genus *Gluconacetobacter*, according to the proposal of Yamada *et al*. (1997).

A genetic distance greater than 0·5 in MLEE analysis has been used as a criterion to suggest species limits (Musser *et al*., 1987; Selander *et al*., 1985). On this basis, the MLEE results strongly support the notion that the type DOR and type SAd isolates could represent two novel N_2-fixing species within the family of acetic acid bacteria. The results of ARDRA support the notion that the type DOR and type SAd isolates represent two novel N_2-fixing species within the family *Acetobacteraceae*, since they can be clearly differentiated from the closely related species *Gluconacetobacter diazotrophicus* and *Gluconacetobacter liquefaciens*.

Although the limitations of 16S rRNA sequencing for the differentiation of closely related species have been documented (Fox *et al*., 1992), it is suggested that 97 % is the threshold 16S rRNA similarity level for the delineation of bacterial species (Stackebrandt & Goebel, 1994). In addition, DNA–DNA reassociation levels below 70 % are indicative of distinct species (Stackebrandt & Goebel, 1994). Nevertheless, some bacteria that have shown 16S rRNA sequence similarities above 98 % have been considered different species, since they had DNA–DNA reassociation levels lower than 50 %. For instance, the recent descriptions of *Acetobacter pomorum* and [*Acetobacter*] *oboediens* (Sokollek *et al*., 1998) and *Gluconacetobacter sacchari* (Franke *et al*., 1999) were based partially on 16S rDNA sequence similarity higher than 97·9 or 99·0 % and levels of DNA–DNA reassociation below 50 %. In the present study, these considerations were consistent both with the 16S rRNA similarity levels and with the low levels of DNA relatedness exhibited within the *Gluconacetobacter* N_2-fixing cluster. In this case, the 16S rDNA sequence similarity among the type DOR and type SAd isolates and *Gluconacetobacter diazotrophicus* ranged from 98·3 to 99·31 % and DNA–DNA reassociation levels were never higher than 50 %, in spite of the fact that the hybridization conditions were not highly stringent. Comparison of the type DOR and type SAd isolates with other species of acetic acid bacteria produced reassociation values that did not exceed 21 %.

On the basis of the data obtained from biochemical and genomic analyses, we recommend that the N_2-fixing type DOR and type SAd isolates described herein should be assigned to two novel species of the family *Acetobacteraceae*. We propose the names *Gluconacetobacter johannae* for the type DOR isolates, strain CFN-Cf55^T being the type strain, and *Gluconacetobacter azotocaptans* for the type SAd isolates, CFN-Ca54^T being the type strain.

Description of *Gluconacetobacter johannae* sp. nov.

Gluconacetobacter johannae (jo.han′nae. N.L. gen. n. *johannae* of Johanna, in honour of the Brazilian microbiologist Johanna Döbereiner, who isolated the first nitrogen-fixing species of the genus *Gluconacetobacter* and discovered several other nitrogen-fixing bacteria).

Cells are straight rods with rounded ends, approximately 1·5–1·9 μm long and 0·5–0·6 μm wide, and occur singly, in pairs or in short chains. Motile by means of peritrichous flagella. Isolates are Gram-negative, oxidase-negative and catalase-positive. Colonies on potato agar with 5, 10 or 15 % cane sugar are light brown, but, after 10 d, turn brownish and produce a brownish liquid pigment. Strains are aerobic and fix atmospheric nitrogen microaerophilically, even in the presence of 10 mM nitrate. Nitrates are not reduced to nitrite, but isolates grow well with ammonium. Regardless of the presence of growth factors from yeast extract, very few carbon sources support growth. Strains grow and produce abundant acid on sucrose, D-glucose and L-fructose and grow in 30 % D-glucose or sucrose. Ethanol is oxidized to acetic acid and acetate and lactate are oxidized to CO_2 and water. Isolates grow on 0·1 and 0·5 % ethanol or 0·1 % butanol, but not with 0·1 or 0·5 % methanol. Growth occurs at 29 °C, but not at 37 °C, in LGI liquid medium at pH 4–7. Growth occurs with 0·25 and 0·5 % NaCl, but not with 1·0 % NaCl, in LGI liquid medium. Single amino acids cannot be used as sole sources of carbon and nitrogen. Characteristics that differentiate this species from other N_2-fixing acetobacteria are shown in Table 2. This species can be differentiated from other N_2-fixing acetobacteria by ARDRA patterns, in addition to DNA–DNA reassociation data, and by means of PCR analysis using specific primers. Strain CFN-Cf55^T (= ATCC 700987^T = DSM 13595^T) is the type strain and has a G+C content of 57·96 mol %. This strain was recovered from the rhizospheres of coffee plants.

Description of *Gluconacetobacter azotocaptans* sp. nov.

Gluconacetobacter azotocaptans (a.zo.to.cap′tans. N.L. n. *azotum* nitrogen; L. part. adj. *captans* catching; N.L. adj. *azotocaptans* nitrogen-catching, referring to the ability to assimilate atmospheric nitrogen).

Cells are straight rods with rounded ends, approximately 1·6–2 μm long and 0·5–0·6 μm wide, and occur

singly or in pairs. Motile by means of peritrichous flagella. Isolates are Gram-negative, catalase-positive and oxidase-negative. Growth occurs at 29 °C, but not at 37 °C, in LGI liquid medium at pH 4–7. Colonies on potato agar with 5, 10 or 15 % cane sugar are beige or very light-brownish, even after 10 d. Strains are aerobic and fix atmospheric nitrogen microaerophilically, even in the presence of 10 mM nitrate. Nitrates are not reduced to nitrite, but isolates grow well with ammonium. Regardless of the presence of growth factors from yeast extract, very few carbon sources support growth. Strains grow and produce abundant acid on sucrose, D-glucose and L-fructose and grow in 30 % D-glucose or sucrose. Ethanol is oxidized to acetic acid and acetate and lactate are oxidized to CO_2 and water. Isolates grow on 0·1 and 0·5 % ethanol, but not with 0·1 or 0·5 % methanol. Growth occurs with 0·25 and 0·5 % NaCl, but not with 1·0 % NaCl, in LGI liquid medium. Single amino acids cannot be used as sole sources of carbon and nitrogen. Characteristics that differentiate this species from other N_2-fixing acetobacteria are shown in Table 2. This species can be differentiated from other N_2-fixing acetobacteria by ARDRA patterns, in addition to DNA–DNA reassociation data, and by means of PCR analysis using specific primers. Strain CFN-Ca54^T (= ATCC 700988^T = DSM 13594^T) is the type strain and has a G+C content of 64·01 mol %. This strain was recovered from the rhizospheres of coffee plants.

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