

## Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian apple fruit

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Two Gram-negative, rod-shaped, non-spore-forming bacteria (DST GL01<sup>T</sup> and DST GL02<sup>T</sup>) were isolated from apple fruit juice in the region of the Italian Alps. On the basis of 16S rRNA gene sequence similarities, strains DST GL01<sup>T</sup> and DST GL02<sup>T</sup> were shown to belong to the  $\alpha$ -subclass of the *Proteobacteria*, and, in particular, to the genus *Gluconacetobacter*, in the *Gluconacetobacter xylinus* branch (98.5–100%). Chemotaxonomic data (major ubiquinone, Q10; predominant fatty acid, C<sub>18:1 $\omega$ 7c</sub>, accounting for approximately 50% of the fatty acid content) support the affiliation of both strains to the genus *Gluconacetobacter*. The results of DNA–DNA hybridizations, together with physiological and biochemical data, allowed genotypic and phenotypic differentiation between strains DST GL01<sup>T</sup> and DST GL02<sup>T</sup> and from the 11 validly published *Gluconacetobacter* species. They therefore represent two new species, for which the names *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov. are proposed, with the type strains DST GL01<sup>T</sup> (=LMG 22125<sup>T</sup> = DSM 16373<sup>T</sup>) and DST GL02<sup>T</sup> (=LMG 22126<sup>T</sup> = DSM 16663<sup>T</sup>), respectively.

Species of the genus *Gluconacetobacter* can be phylogenetically subgrouped into two clusters: ‘N<sub>2</sub>-fixing’ (*Gluconacetobacter diazotrophicus*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter sacchari*, *Gluconacetobacter azotocaptans* and *Gluconacetobacter johanna*) and ‘cellulose-producing’ (*Gluconacetobacter xylinus*, *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens*, *Gluconacetobacter hansenii* and *Gluconacetobacter entanii*). Characterization of a new isolate on the basis of these phenotypic traits might, however, lead to an erroneous identification, as each cluster contains strains lacking these properties (Bernardo *et al.*, 1998; Franke *et al.*, 1999; Fuentes-Ramirez *et al.*, 2001). For instance, the ability to synthesize cellulose is easily lost in many strains and cannot be used as a determinative feature (Swings, 1992).

The species belonging to this genus, most of which have been proposed since 1998 (Boesch *et al.*, 1998; Sokollek *et al.*, 1998; Franke *et al.*, 1999; Schüller *et al.*, 2000; Yamada, 2000;

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Gluconacetobacter swingsii* DST GL01<sup>T</sup> and *Gluconacetobacter rhaeticus* DST GL02<sup>T</sup> are AY180960 and AY180961, respectively.

Tables of DNA–DNA hybridization data and additional distinguishing characteristics are available as supplementary material in IJSEM Online.

Fuentes-Ramirez *et al.*, 2001), are strongly correlated at the phylogenetic level.

In this study we present a comprehensive taxonomic analysis of two cellulose-producing strains of acetic acid bacteria (DST GL01<sup>T</sup> and DST GL02<sup>T</sup>), isolated from apple fruit juice in the South Tyrol region of Italy (Val Venosta), by means of the study of phenotypic and chemotaxonomic properties, the analysis of the phylogenetic marker 16S rRNA gene, the DNA base composition and DNA relatedness. We provide evidence that the two new isolates represent two different new cellulose-producing species within the genus *Gluconacetobacter*, for which we propose the names *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov.

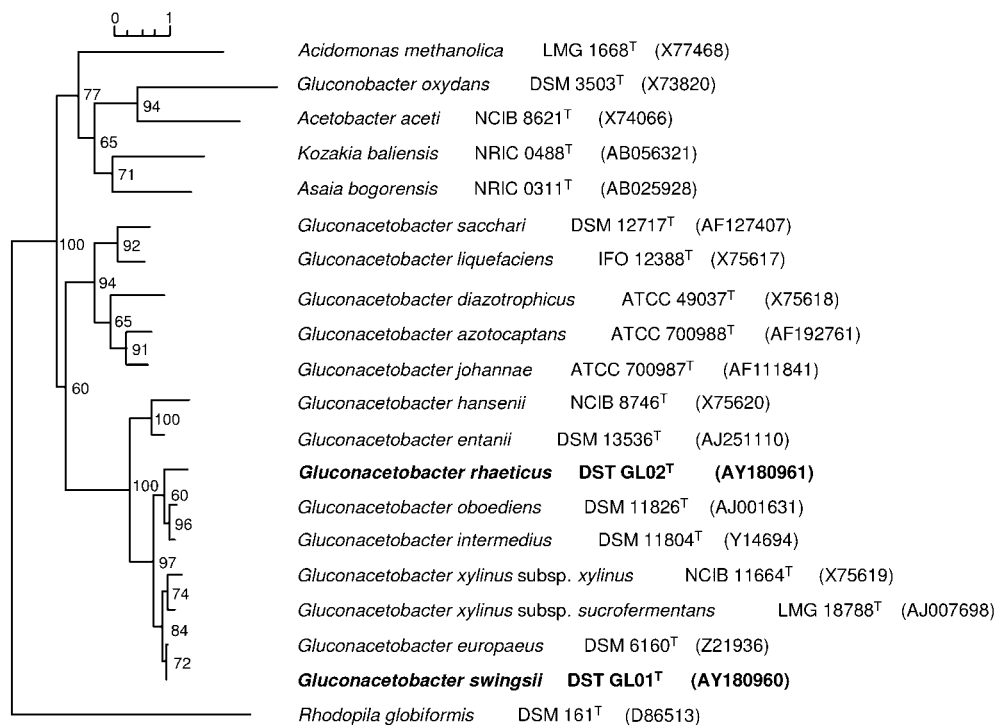
Two cellulose-producing strains, DST GL01<sup>T</sup> and DST GL02<sup>T</sup>, were isolated from organic apple juice prepared with fruits from the Val Venosta region in Italy. Both strains were propagated in liquid culture without shaking, using the synthetic medium ACE (50 g glucose l<sup>-1</sup>, 5 g yeast extract l<sup>-1</sup>, pH 6.5) and incubated aerobically at 28 °C for 6 days. The isolation and identification of superoxidizing acetic acid bacteria were performed in Medium 2 (30 g yeast extract l<sup>-1</sup>, 0.22 g bromocresol green l<sup>-1</sup>, 2%, v/v, ethanol, 15 g agar l<sup>-1</sup>, pH 6.5). The colonies showed a yellow

acidification halo that turns to blue after acetic acid super-oxidation to CO<sub>2</sub> and H<sub>2</sub>O, indicating that they belonged to *Acetobacter/Gluconacetobacter* and not to *Gluconobacter* (Swings, 1992). Cell shape and cell size were determined from cells grown aerobically at 28 °C for 3 days on ACE agar medium. Gram staining, oxidase and catalase activity were determined as described previously (Cleenwerck *et al.*, 2002). Cells of DST GL01<sup>T</sup> and DST GL02<sup>T</sup> were Gram-negative, non-motile coccoid rods. Both isolates were catalase-positive and oxidase-negative.

DNA for PCR amplification was isolated from cellulose-producing cultures (10 ml), preliminarily homogenized by an UltraTurrax T25 (IKA) at 8500 r.p.m. for 15 s and filtered through sterile gauzes to eliminate the polysaccharide matrix. Cells were collected by centrifugation, washed three times, resuspended in 0.5 ml TE buffer (pH 8.0), supplemented with 10 g lysozyme l<sup>-1</sup>, and incubated at 37 °C for 2 h. The DNA was isolated by the CTAB method (Cleenwerck *et al.*, 2002). PCR amplification of the 16S rRNA genes was conducted as described by Boesch *et al.* (1998). Amplification products were purified from a 1% (w/v) agarose gel by the QIAEX II Gel Extraction System (Qiagen). Sequencing was carried out on purified PCR amplicons at the Bio Molecular Research Center (BMR), University of Padua, Italy. The 16S rRNA gene sequences determined and sequences of strains belonging to the same

phylogenetic group, retrieved from the EMBL library, were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics 3.50 software package (Applied Maths). Unknown bases were discarded from the calculations. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 1000 bootstrap resamplings of the data. A maximum-parsimony analysis was also performed with the program DNAPARS of the PHYLIP package (version 3.5c), using the default options. Sequence similarity calculations after a neighbour-joining analysis showed that the nearly complete 16S rRNA gene sequences of DST GL01<sup>T</sup> and DST GL02<sup>T</sup> (1446 and 1336 bp, respectively) had the highest similarities to sequences of reference strains of the *G. xylinus* branch (98.5–100%). The levels of similarity to the 16S rRNA genes of other validly described species of the *Acetobacteraceae* family were below 97.2%. A neighbour-joining tree, reflecting the positions of these strains within the acetic acid bacteria lineage, is shown in Fig. 1. Parsimony analysis confirmed the phylogenetic placement of the strains in the 'cellulose-producing' *Gluconacetobacter* species cluster.

Analyses of respiratory quinones were carried out by the Identification Service of the DSMZ and Dr Brian Tindall, Braunschweig, Germany. Respiratory lipoquinones were determined from cells grown on Sabouraud-glucose (2%)



**Fig. 1.** Phylogenetic tree obtained by neighbour-joining analysis of the almost complete 16S rRNA gene sequences, showing the positions of *Gluconacetobacter swingsii* DST GL01<sup>T</sup> and *Gluconacetobacter rhaeticus* DST GL02<sup>T</sup> within the acetic acid bacteria. *Rhodopila globiformis* DSM 161<sup>T</sup> was used as outgroup in the analysis. Bar, 1% sequence dissimilarity. Numbers at branching points indicate bootstrap percentages derived from 1000 samples. Accession numbers are given in parentheses.

liquid medium, pH 5.7, at 29 °C. The respiratory lipoquinones were extracted from 100 mg freeze-dried cell material based on the two-stage method described by Tindall (1990a, b) using methanol:hexane. They were separated into their different classes (menaquinones and ubiquinones) by thin layer chromatography on silica gel (Art. NO. 805 023; Macherey–Nagel), using hexane:tert-butylmethylether (9:1, v/v) as solvent. UV absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed on an LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (2 × 125 mm, 3 µm particle size, RP18; Macherey–Nagel) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm. DST GL01<sup>T</sup> and DST GL02<sup>T</sup> contained Q-10 as the major component, a characteristic that is common to strains belonging to the genus *Gluconacetobacter* (Yamada, 2000).

Fatty acid profiles were determined from cells grown for 48 h at 28 °C under aerobic conditions on M129 medium from the Catalogue of Cultures of the BCCM/LMG Bacteria Collection (Janssens *et al.*, 1998), containing 5.0% D-glucose, 0.5% yeast extract and 1.5% agar (final pH 6.5). Fatty acids were prepared and identified following the instructions of the MIDI (Microbial Identification) system. DST GL01<sup>T</sup> and DST GL02<sup>T</sup> contained large amounts of the fatty acid C<sub>18:1ω7c</sub> (approx. 50% of the fatty acid content). The other fatty acids found in smaller but still significant amounts were C<sub>16:0</sub> (13.1%), C<sub>17:0</sub> (9.8%) and 2-OH C<sub>16:0</sub> (6.6%) for DST GL01<sup>T</sup>, and 2-OH C<sub>14:0</sub> (19.5%), 2-OH C<sub>16:0</sub> (16.5%) and C<sub>16:0</sub> (6.9%) for DST GL02<sup>T</sup>. These results are comparable to the results obtained for other *Gluconacetobacter* species (Urakami *et al.*, 1989; Franke *et al.*, 1999).

DST GL01<sup>T</sup> and DST GL02<sup>T</sup> were hybridized with the type strains of the 'cellulose-producing' *Gluconacetobacter* species: *G. oboediens* LMG 18849<sup>T</sup>, *G. intermedius* LMG 18909<sup>T</sup>, *G. europaeus* LMG 18890<sup>T</sup>, *G. xylinus* subsp. *xylinus* LMG 1515<sup>T</sup>, *G. xylinus* subsp. *sucrofermentans* LMG 18788<sup>T</sup>, *G. hansenii* LMG 1527<sup>T</sup> and *G. entanii* LTH 4560<sup>T</sup>. High-molecular-mass DNA for the DNA–DNA hybridization study was prepared by the method of Wilson (1987), with minor modifications (Cleenwerck *et al.*, 2002). The type strains were cultured as recommended by the BCCM/LMG Bacteria Collection, except for *G. entanii* for which a pellet of cells was kindly supplied to us by Dr Hertel (University of Hohenheim, Germany). Strains LMG 18909<sup>T</sup>, LMG 1515<sup>T</sup> and DST GL02<sup>T</sup> were subjected to a mild alkaline hydrolysis step before cell lysis, as described by Willems *et al.* (2001). DNA quantity and quality were determined by measuring absorption at 260, 280 and 234 nm. Only high quality DNA with A<sub>260</sub>/A<sub>280</sub> and A<sub>234</sub>/A<sub>260</sub> ratios of 1.8–2.0 and 0.40–0.60, respectively, was selected for further use. The size of the DNA was estimated by agarose gel electrophoresis. Only high-molecular-mass DNA was used. DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) (Goris *et al.*,

1998; Cleenwerck *et al.*, 2002). The hybridization temperature was 48 °C. Reciprocal reactions (e.g. A × B and B × A) were performed. The DNA-binding values reported are the mean values of a minimum of four hybridization experiments, the reciprocal reactions included. The level of DNA–DNA binding between DST GL01<sup>T</sup> and DST GL02<sup>T</sup> (45%) and between these strains and the type strains of the *G. xylinus* branch (≤60%) demonstrates a relatedness below the species level.

In this study the DNA–DNA binding values between the type strains of the *G. xylinus* branch were also determined (except for *G. entanii* for which it is very difficult to obtain enough DNA for hybridizations) to obtain a clear view of the DNA relatedness between these strains (see Supplementary Table A in IJSEM Online). From the current literature data, this was not really possible due to multiple factors. The reported DNA binding values are difficult to compare with each other as in many cases different DNA hybridization methods were used. DNA-binding values between some of these species are only vaguely reported, such as the DNA-binding values between *G. intermedius* and *G. xylinus* and between *G. intermedius* and *G. hansenii* (DNA-binding values are below 60%). But most of all, DNA-binding values between some species have not been reported, such as those between *G. oboediens* and *G. intermedius*, *G. oboediens* and *G. hansenii*, *G. entanii* and *G. intermedius*. The DNA-binding value between *G. intermedius* and *G. europaeus* (57%) is comparable to the 60% reported by Boesch *et al.* (1998). The values between *G. oboediens* and *G. intermedius* (63%), and between *G. oboediens* and *G. hansenii* (24%) demonstrate that they represent different species. The DNA-binding values between *G. oboediens* and *G. xylinus* (68%), and between *G. oboediens* and *G. europaeus* (51%) are higher than the values reported by Sokollek *et al.* (1998) (34 and 25%, respectively). The discrepancy between these data are probably due to the fact that Sokollek *et al.* (1998) did not perform reciprocal reactions, which are very important to obtain unequivocal results. The DNA homology value between *G. xylinus* subsp. *xylinus* and *G. xylinus* subsp. *sucrofermentans* (56%) is comparable to the 58.2% reported by Toyosaki *et al.* (1995). This value is lower than DNA-binding values found between some other species in the *G. xylinus* branch and therefore the question could be raised whether *G. xylinus* subsp. *sucrofermentans* should be elevated to the species level.

The G+C content of DST GL01<sup>T</sup> (61.7 mol%) and DST GL02<sup>T</sup> (63.4 mol%) was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage λ DNA (Sigma) was used as the calibration reference.

Phenotypic characterization of DST GL01<sup>T</sup> and DST GL02<sup>T</sup> was performed. The production of 2- and 5-keto-D-gluconic acid was determined by the method described by Gossele *et al.* (1980). Tolerance to 3% (v/v) ethanol was tested in acid medium (5 g yeast extract l<sup>-1</sup>, pH 3.0). The ability to grow on different carbon sources was tested using a

standardized and miniaturized assimilation test, ID 32C (Biomérieux), following the manufacturer's guidelines, using bacterial suspensions with an OD<sub>600</sub> of 0.7 to inoculate the galleries. The composition of the minimal medium and the carbon substrates tested are reported in the protocol from the kit. The ability to grow on different carbon sources was determined after 15 days incubation at 28 °C. Cellulose production was tested in the presence of 1% (w/v) D-glucose, 1% (w/v) D-fructose, 1% (w/v) sucrose, 2% (w/v) ethanol and 2% (v/v) glycerol in a liquid medium containing 5 g yeast extract l<sup>-1</sup>. The phenotypic characteristics of DST GL01<sup>T</sup> and DST GL02<sup>T</sup> are given in the species descriptions below. The characteristics that differentiate the two strains from each other and from their phylogenetically closest neighbours are given in Table 1 (additional characteristics differentiating DST GL01<sup>T</sup> from DST GL02<sup>T</sup> are available in Supplementary Table B in IJSEM Online). DST GL01<sup>T</sup> is distinguished from *G. xylinus* by the ability to grow on 3% ethanol in the presence of 4–8% acetic acid, the ability to grow on ethanol as carbon source and the ability to grow in the presence of 30% D-glucose. It is distinguished from *G. europaeus* by the ability to grow in the absence of acetic acid, the ability to grow on ethanol, sucrose, sorbitol, D-mannitol and D-gluconate as carbon sources and the inability to grow on D-ribose as carbon source. DST GL02<sup>T</sup> is distinguished from *G. oboediens* by the ability to produce 5-keto-D-gluconic acid from D-glucose, the ability to grow on ethanol,

D-xylose, sorbitol and D-mannitol as carbon sources and the inability to grow on D-gluconate as carbon source. It is distinguished from *G. intermedius* by the ability to produce 2- and 5-keto-D-gluconic acid from D-glucose. DST GL01<sup>T</sup> and DST GL02<sup>T</sup> did not grow in liquid medium shaken on a rotary shaker. Cell proliferation seems to be strictly correlated to cellulose production (Kamide *et al.*, 1990).

Overall, the results obtained indicate that DST GL01<sup>T</sup> and DST GL02<sup>T</sup> represent two novel species of the genus *Gluconacetobacter*, for which we propose the names *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., respectively.

### Description of *Gluconacetobacter swingsii* sp. nov.

*Gluconacetobacter swingsii* (swing'si.i. N.L. gen. n. *swingsii* of Swings, in honour of Jean Swings who studied and reviewed acetic acid bacteria).

Cells are Gram-negative, coccoid, approximately 0.9 × 1.5–2.5 µm in size, occurring singly or in pairs. Cells are non-motile. Endospores are not detected. Colonies are beige, regular, convex and smooth with a diameter of 0.8 mm on standard medium. Oxidase-negative. Catalase-positive. D-Glucose is oxidized to 2- and 5- keto-D-gluconic acid. Acetic acid is not required for growth. Growth is observed in 3% (v/v) ethanol in acid medium, pH 3.0. Growth occurs on

**Table 1.** Characteristics differentiating *G. swingsii* sp. nov. and *G. rhaeticus* sp. nov. from each other and from the phylogenetically closely related *Gluconacetobacter* species

Species: 1, *G. swingsii*; 2, *G. rhaeticus*; 3, *G. oboediens* (data from Sokollek *et al.*, 1998); 4, *G. intermedius* (data from Boesch *et al.*, 1998); 5, *G. xylinus* (data from Sokollek *et al.*, 1998); 6, *G. europaeus* (data from Sokollek *et al.*, 1998); 7, *G. hansenii* [data from Gosselé *et al.* (1983) and Navarro *et al.* (1999)]; 8, *G. entanii* (data from Schüller *et al.*, 2000). Symbols: +, positive; –, negative; v, variable; NR, not reported.

Character	1	2	3	4	5	6	7	8
Production of keto-D-gluconates from D-glucose:								
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-Fructose	+	+	+	NR	+	+	v	+
Maltose	+	+	+	NR	+	+	v	+
D-Ribose	–	+	+	NR	–	+	–	NR
D-Xylose	+	+	–	NR	+	+	–	NR
Sucrose	+	+	+	NR	+	–	v	+
Sorbitol	+	+	–	NR	+	–	–	–
D-Mannitol	+	+	–	NR	+	–	v	–
D-Gluconate	+	–	+	NR	+	–	–	–
Growth in the presence of 30% D-glucose	+	+	+	NR	–	–	–	–

cellobiose, D-xylose, maltose, D-mannitol, melezitose, raffinose, sorbitol, sorbose, trehalose, D-gluconate, glucuronate and glucosamine as carbon sources, but not on erythritol, galactose, L-arabinose, melibiose, palatinose, rhamnose and D-ribose. Cellulose is produced from glucose, ethanol and glycerol, but not from fructose and sucrose. The ubiquinone system consists of Q-10 as the major component and Q-9 as a minor component. C<sub>18:1 $\omega$ 7c</sub> is the major fatty acid (50%); other fatty acids in smaller but still significant amounts are C<sub>16:0</sub> (13.1%), C<sub>17:0</sub> (9.8%) and 2-OH C<sub>16:0</sub> (6.6%). The DNA G+C content of the type strain is 61.7 mol%. Isolated from apple juice in South Tyrol region, Italy. Type strain is DST GL01<sup>T</sup> (=LMG 22125<sup>T</sup>=DSM 16373<sup>T</sup>).

### Description of *Gluconacetobacter rhaeticus* sp. nov.

*Gluconacetobacter rhaeticus* (rhae'ti.cus. L. masc. adj. *rhaeticus* pertaining to Rhaetia, Latin denomination of South Tyrol region in Italy, where the type strain was isolated).

Cells are Gram-negative, coccoid, approximately 0.9 × 1.5–2.5 µm in size, occurring singly, in pairs or in short chains. Cells are non-motile. Endospores are not detected. Colonies are beige, regular, convex and weakly rough with a diameter of 1.0 mm on standard medium. Oxidase-negative. Catalase-positive. D-Glucose is oxidized to 2- and 5-keto-D-gluconic acid. Acetic acid is not required for growth. Growth is observed in 3% (v/v) ethanol in acid medium, pH 3.0. Growth occurs on cellobiose, D-xylose, maltose, D-mannitol, melezitose, raffinose, sorbitol, sorbose, trehalose, erythritol, galactose, L-arabinose, melibiose, palatinose, rhamnose and D-ribose as carbon sources, but not on D-gluconate, glucuronate and glucosamine. Cellulose is produced from glucose, ethanol, glycerol, fructose and sucrose. The ubiquinone system consists of Q-10 components. C<sub>18:1 $\omega$ 7c</sub> is the major fatty acid (50%); other fatty acids in smaller but still significant amounts are 2-OH C<sub>14:0</sub> (19.5%), 2-OH C<sub>16:0</sub> (16.5%) and C<sub>16:0</sub> (6.9%). The DNA G+C content of the type strain is 63.4 mol%. Isolated from apple juice in South Tyrol region, Italy. Type strain is DST GL02<sup>T</sup> (=LMG 22126<sup>T</sup>=DSM 16663<sup>T</sup>).

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