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-spor-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 α-subclass of the Proteobacteria, and, in particular, to the genus *Gluconacetobacter*, in the *Gluconacetobacter xylinus* branch (98–100 %). Chemotaxonomic data (major ubiquinone, Q10; predominant fatty acid, C<sub>18:1ω7C</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.

The species belonging to this genus, most of which have been proposed since 1998 (Boesch et al., 1998; Sokolke et al., 1998; Franke et al., 1999; Schüller et al., 2000; Yamada, 2000; 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 CO₂ and H₂O, 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<T> and DST GL02<T> 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 polysacchar-
ide 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⁻¹, 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 resampl-
ings 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 simi-
larity calculations after a neighbour-joining analysis showed
that the nearly complete 16S rRNA gene sequences of DST
GL01<T> and DST GL02<T> (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 simi-
larity 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<T> and *Gluconacetobacter raeticus* DST GL02<T> within the acetic acid bacteria. *Rhodopila globiformis* DSM 161<T> 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.](image)
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-butylmethyl ether (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 GL01T and DST GL02T 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 GL01T and DST GL02T contained large amounts of the fatty acid C18:1ω7c (approx. 50 % of the fatty acid content). The other fatty acids found in smaller but still significant amounts were C16:0 (13-1 %), C17:0 (9-8 %) and 2-OH C16:0 (6-6 %) for DST GL01T, and 2-OH C14:0 (19-5 %), 2-OH C16:0 (16-5 %) and C16:0 (6-9 %) for DST GL02T. These results are comparable to the results obtained for other Gluconacetobacter species (Urakami et al., 1989; Franke et al., 1999).

DST GL01T and DST GL02T were hybridized with the type strains of the ‘cellulose-producing’ Gluconacetobacter species: G. oboediens LMG 18849T, G. intermedius LMG 18909T, G. europaeus LMG 18890T, G. xylinus subsp. xylinus LMG 1515T, G. xylinus subsp. succrofermentans LMG 18788T, G. hansenii LMG 1527T and G. entanii LTH 4560T. 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 18909T, LMG 1515T and DST GL02T 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 $A_{260}/A_{280}$ and $A_{234}/A_{260}$ 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–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 GL01T and DST GL02T (45 %) and between these strains and the type strains of the G. xylinus branch ($\leq 60$ %) 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 Sokolke et al. (1998) (34 and 25 %, respectively). The discrepancy between these data are probably due to the fact that Sokolke 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. succrofermentans (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. succrofermentans should be elevated to the species level.

The G+C content of DST GL01T (61.7 mol%) and DST GL02T (63.4 mol%) was determined by HPLC according to the method of Mesbah et al. (1989). Non-methylated phase $\lambda$ DNA (Sigma) was used as the calibration reference.

Phenotypic characterization of DST GL01T and DST GL02T was performed. The production of 2- and 5-keto-D-gluconic acid was determined by the method described by Gosselé et al. (1980). Tolerance to 3 % (v/v) ethanol was tested in acid medium (5 g yeast extract l$^{-1}$, pH 3-0). The ability to grow on different carbon sources was tested using a
standardized and miniaturized assimilation test, ID 32C (Biomerieux), following the manufacturer’s guidelines, using bacterial suspensions with an OD_600 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 5g yeast extract l^{-1}. The phenotypic characteristics of DST GL01^T and DST GL02^T 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^T from DST GL02^T are available in Supplementary Table B in IJSEM Online). DST GL01^T 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-glucuronate as carbon sources and the inability to grow on D-ribose as carbon source. DST GL02^T 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^T and DST GL02^T 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^T and DST GL02^T 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’i.i. N.L. gen. n. swingsii of Swings, in honour of Jean Swings who studied and reviewed acetate 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. swingsi; 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 Gosselin 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.

<table>
<thead>
<tr>
<th>Character</th>
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<tr>
<td>Production of keto-D-gluconates from D-glucose:</td>
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<td>+</td>
<td>NR</td>
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<td>2-Keto-D-gluconate</td>
<td>+</td>
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<td>−</td>
<td>+</td>
<td>NR</td>
<td>+</td>
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<tr>
<td>5-Keto-D-gluconate</td>
<td>+</td>
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<td>−</td>
<td>+</td>
<td>NR</td>
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<tr>
<td>Growth without acetic acid</td>
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<td>+</td>
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<tr>
<td>Growth on 3% ethanol in the presence of 4–8% acetic acid</td>
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<td>NR</td>
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<td>Growth on carbon sources:</td>
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<td>Ethanol</td>
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<td>NR</td>
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<tr>
<td>D-Fructose</td>
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<td>Maltose</td>
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<td>NR</td>
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<td>D-Ribose</td>
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<tr>
<td>Sucrose</td>
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<td>Sorbitol</td>
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<td>D-Mannitol</td>
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<td>NR</td>
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<td>D-Glucuronate</td>
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<td>NR</td>
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<tr>
<td>Growth in the presence of 30% D-glucose</td>
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Catalase-positive. D-Glucose is oxidized to 2- and 5-keto-D-glucuronic acid 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. C18:1ω7c is the major fatty acid (50%); other fatty acids in smaller but still significant amounts are C16:0 (13-1%), C17:0 (9-8%) and 2-OH C16:0 (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 GL01T (=LMG 22125T = DSM 16373T).

Description of Gluconacetobacter rhaeticus sp. nov.

Gluconacetobacter rhaeticus (rhaeto’icus. 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-glucuronic 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 cellulose, 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-glucuronate, glucuronic and glucosamine. Cellulose is produced from glucose, ethanol, glycerol, fructose and sucrose. The ubiquinone system consists of Q-10 components. C18:1ω7c is the major fatty acid (50%); other fatty acids in smaller but still significant amounts are 2-OH C16:0 (19-5%), 2-OH C16:0 (16-5%) and C16:0 (6-9%). The DNA G+C content of the type strain is 62-4 mol%. Isolated from apple juice in South Tyrol region, Italy. Type strain is DST GL02T (=LMG 22125T = DSM 16373T).

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References


