Phylogeny and differentiation of species of the genus *Gluconacetobacter* and related taxa based on multilocus sequence analyses of housekeeping genes and reclassification of *Acetobacter xylinus* subsp. *sucrofermentans* as *Gluconacetobacter sucrofermentans* (Toyosaki *et al.* 1996) sp. nov., comb. nov.

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Three housekeeping genes (*dnaK*, *groEL* and *rpoB*) of strains belonging to the genus *Gluconacetobacter* (37 strains) or related taxa (38 strains) were sequenced. Reference strains of the 15 species of the genus *Gluconacetobacter* were included. Phylogenetic trees generated using these gene sequences confirmed the existence of two phylogenetic groups within the genus *Gluconacetobacter*. These groups clustered separately in trees constructed using concatenated sequences of the three genes, indicating that the genus *Gluconacetobacter* should not remain a single genus and should be split, as suggested previously. Multilocus sequence analysis (MLSA) of the three housekeeping genes also proved useful for species differentiation in the family *Acetobacteraceae*. It also suggested that *Gluconacetobacter xylinus* LMG 18788, better known as the type and only strain of *Acetobacter xylinus* subsp. *sucrofermentans*, represents a distinct species in the genus *Gluconacetobacter*, and is not a true *G. xylinus* strain. In previous studies, this strain showed less than 70 % DNA relatedness to the type strains of *G. xylinus* and *Gluconacetobacter nataicola*, the phylogenetically nearest relatives, and could be distinguished from them phenotypically. Additionally, AFLP and (GTG)₅-PCR DNA fingerprinting data supported its reclassification within a distinct species. The name *Gluconacetobacter sucrofermentans* (Toyosaki *et al.* 1996) sp. nov., comb. nov. is proposed.

The genus *Gluconacetobacter* belongs to the family *Acetobacteraceae* (Gillis & De Ley, 1980) of the *Alphaproteobacteria* and was proposed by Yamada *et al.* (1997, 1998) for strains assigned to the genus *Acetobacter* at that time that contained ubiquinone Q-10 as the major respiratory

Abbreviations: AAB, acetic acid bacteria; AFLP, amplified fragment length polymorphism; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the *dnaK*, *groEL* and *rpoB* sequences reported in this paper are FN391590–FN391808 and FN421342–FN421344, as detailed in Fig. 1.

Strain details, a summary of sequence similarity for various genes and additional phylogenetic trees are available as supplementary material with the online version of this paper.

quinone. As a result, *Acetobacter xylinus*, *A. liquefaciens*, *A. hansenii*, *A. diazotrophicus* and *A. europaeus* were reclassified in the genus *Gluconacetobacter* as new combinations (Yamada *et al.* 1997, 1998). However, the two subspecies of *A. xylinus*, namely *A. xylinus* subsp. *xylinus* and *A. xylinus* subsp. *sucrofermentans* (Toyosaki *et al.*, 1995, 1996), were not mentioned in the reclassification. At the time of writing, 15 species with validly published names exist in the genus *Gluconacetobacter*, which is divided phylogenetically into two groups based on 16S rRNA gene sequence analysis, namely the *Gluconacetobacter xylinus* species group and the *Gluconacetobacter liquefaciens* species group (Dellaglio *et al.*, 2005; Yamada & Yukphan, 2008). These two groups both consist of closely related species, sharing

more than 98.1 % 16S rRNA gene sequence similarity, but the two groups themselves are not so tightly linked. For this reason, the question has been raised whether *Gluconacetobacter* should remain a single genus (Yamada & Yukphan, 2008). At present, confusion also exists regarding the taxonomic status of *G. xylinus* LMG 18788 (=JCM 9730 =DSM 15973), better known as the type and only known strain of *A. xylinus* subsp. *sucrofermentans*. Based on its 16S rRNA gene sequence, this strain is a member of the *G. xylinus* species group (Dellaglio *et al.*, 2005; Lisdiyanti *et al.*, 2006), but its allocation at the species level is unclear. Firstly, there are discrepancies in the DNA relatedness values reported for this strain with the type strain of *G. xylinus*. According to Lisdiyanti *et al.* (2006), the two strains share a DNA relatedness of 70 %, whereas, according to Toyosaki *et al.* (1995) and Dellaglio *et al.* (2005), they share DNA relatedness of 56–58 %. Secondly, the (GTG)₅-PCR DNA fingerprint of LMG 18788 is different from those of other true *G. xylinus* strains (De Vuyst *et al.*, 2008). Thirdly, according to Toyosaki *et al.* (1995) and Kojima *et al.* (1998), the strain shows phenotypic characteristics that differentiate it from other *G. xylinus* strains.

In the present study, additional analyses were performed on reference strains of the species of the genus *Gluconacetobacter* (37 strains) and related taxa (38 strains) (Supplementary Table S1, available in IJSEM Online) in order to obtain a better view of their phylogeny and to clarify the taxonomic position of strain LMG 18788.

The strains were cultivated on GY agar (5 % glucose, 1 % yeast extract, 1.5 % agar; w/v) or GYAE agar (5 % w/v glucose, 1 % w/v yeast extract, 1.5 % w/v agar, 2 % v/v ethanol, 1 % v/v acetic acid; the latter two components were sterilized separately by filtration and added aseptically to the autoclaved medium) and, in a few cases, on the medium suggested by the provider, by incubation at 28 °C under aerobic conditions. DNA was extracted using the method described previously (Cleenwerck *et al.*, 2009).

A phylogenetic tree based on 16S rRNA gene sequences, retrieved from EMBL, of type strains of the species of the family *Acetobacteraceae* was constructed with the BioNumerics 4.61 software package (Applied Maths) using the neighbour-joining method (Saitou & Nei, 1987). The robustness of the branches was evaluated by bootstrap resamplings of the data (Felsenstein, 1985). The topology of the tree was found to be similar to that of trees constructed previously (Cleenwerck & De Vos, 2008; Yamada & Yukphan, 2008) and confirmed the existence of the two, not so tightly linked species groups within the genus *Gluconacetobacter* (Supplementary Fig. S1). The analysis also confirmed that strain LMG 18788 is a member of the *G. xylinus* species group, with strains of *Gluconacetobacter swingsii*, *G. europaeus*, *G. xylinus*, *G. nataicola*, *G. oboediens* and *G. intermedius* as its closest relatives, sharing 99.3–99.8 % 16S rRNA gene sequence similarity (Supplementary Table S2).

Several studies have shown that sequences of housekeeping genes are superior to 16S rRNA gene sequences for determining phylogenetic relationships of closely related species (Naser *et al.*, 2005; Martens *et al.*, 2007; De Bruyne *et al.*, 2007; Brady *et al.*, 2008). To avoid uncovered horizontal gene transfer or recombinations in a single gene resulting in a misleading phylogenetic signal, it has been recommended to examine a minimum of three housekeeping genes (Konstantinidis *et al.*, 2006). In this study, ten genes (*recA*, *dnaK*, *rpoB*, *atpD*, *groEL*, *glnA*, *thrC*, *gyrA*, *gltA* and *infB*) were evaluated for the construction of a multilocus sequence analysis (MLSA) framework for acetic acid bacteria (AAB). The genes *atpD*, *glnA*, *gyrA* and *infB* were found in multiple copies in the complete genome sequences of AAB available at the start of this study (*Gluconobacter oxydans* 621H and *Granulibacter bethesdensis* CGDNIH1^T; <http://www.ncbi.nlm.nih.gov/>) and were, for this reason, discarded from further analyses. For the remaining genes, primers for their amplification were designed on the basis of the two genome sequences of AAB (Table 1) and tested in PCRs on a subset of reference strains using the amplification conditions described by Naser *et al.* (2005) and an annealing temperature of 55 °C. These PCRs resulted in a single amplification product of the expected size for nearly all tested strains for *dnaK*, *rpoB* and *groEL*, whereas, for the other three genes (*recA*, *thrC* and *gltA*), a relatively large number of the tested strains yielded multiple amplification products or no product at all (not shown). In the present study, it was decided to investigate the 75 strains shown in Supplementary Table S1, by MLSA of the genes *dnaK* (encoding a heat-shock protein), *rpoB* (encoding the β -subunit of bacterial RNA polymerase) and *groEL* (encoding a chaperonin protein).

Primers for sequencing of the three selected genes (Table 1) were, as for the amplification primers, designed on the basis of the genome sequences of *Gluconobacter oxydans* 621H and *Granulibacter bethesdensis* CGDNIH1^T. Amplification and sequencing of the 75 strains were performed using the conditions described by Naser *et al.* (2005) but with optimized annealing temperatures for amplification, namely 55 °C for *dnaK* and *groEL* and 50 °C for *rpoB* for most strains. For strains of *G. azotocaptans*, *G. diazotrophicus*, *G. johannae* and *G. frateurii*, an annealing temperature of 46 or 58 °C was used. The primer combinations *dnaK*-01-F/*dnaK*-02-R, *groEL*-10-F/*groEL*-11-R and *rpoB*-01-F/*rpoB*-02-R amplified the target genes of most strains. Where necessary, alternative primer combinations were used, namely *dnaK*-01-F/*dnaK*-04-R for strains of *G. azotocaptans*, *G. diazotrophicus* and *G. johannae*, *rpoB*-01-F/*rpoB*-07-R for strains of *G. thailandicus* and *Kozakia baliensis* LMG 21812^T and *rpoB*-01-F/*rpoB*-07-R together with *rpoB*-03-F/*rpoB*-02-R for strains of *G. oxydans*. For *Saccharibacter floricola* LMG 23170^T, none of the genes could be sequenced with the primers listed in Table 1. Raw sequence data were transferred to the AutoAssembler 1.4.0 software (Applied Biosystems) for assembly of consensus sequences by manual alignment. Phylogenetic trees based

Table 1. Amplification and sequencing primers used in this study

Primer positions are given relative to the genome sequence of *G. oxydans* 621H.

Primer	Sequence (5'–3')	Position
dnaK-01-F	CTGCGCATCATCAACGAGCC	925719–925738
dnaK-02-R	CTCACGCTCGCCTGATAGA	926546–926527
dnaK-03-F	TTCGACGTSTCCATCCTCGA	925818–825837
dnaK-04-R	TTGGTCGGGATCGTCGTGTT	926470–925451
groEL-10-F	ACAAGTTCGAGAACATGGGC	2083114–2083133
groEL-11-R	TCCTTGCGCTCCTTCACCTC	2084104–2084085
groEL-12-F	TSAAGCGCGCATCGACAA	2083270–2083288
groEL-13-F	CTGATCCACGAAAAGAAGCT	2083584–2083603
groEL-14-R	TGCAACGGCCCTTGATGTC	2083962–2083944
groEL-15-R	AGCTTCTTTTCGTGGATCAG	2083603–2083584
rpoB-01-F	GATAACGGCACCTTCATCAT	405644–405625
rpoB-02-R	AGATTGTGATATCGTCGAT	404620–404639
rpoB-03-F	GATCAGACAAGGGCAAGAC	405566–405547
rpoB-04-F	CCAAGCTGACCGCSCGTA	405157–405140
rpoB-05-R	ATGTTTCATCTTCACACGACCG	404743–404763
rpoB-06-R	TACSGCGGTGAGCTTGG	405140–405157
rpoB-07-R	AGRCGCATGTTCATCTTSA	404737–404755
recA_A_F	AAGGCTCGATCATGCGCATG	1676846–1676827
recA_A_R	AYCTTGCTGATGCCTTCWCC	1676104–1676121
thrC_A_F	GAAGTGTTCACGGCCCGAC	2048229–2048210
thrC_A_R	TCCAGCARTTCGAACAGCA	2047559–2047577
gltA_A_F	CTTCCGCCGCGATGCSCATCC	2188601–2188581
gltA_A_R	ACAGCACSGTGAACATGCT	2187820–2187838

on concatenated and individual *dnaK*, *groEL* and *rpoB* gene sequences were constructed with the BioNumerics 4.61 software package using the neighbour-joining and maximum-parsimony (Felsenstein, 1985) methods. The robustness of the branches was evaluated by bootstrap resamplings of the data (Felsenstein, 1985).

The neighbour-joining and maximum-parsimony trees based on concatenated *dnaK*, *groEL* and *rpoB* gene sequences (Fig. 1 and Supplementary Fig. S2) showed very similar topologies, and were also largely in agreement with the constructed 16S rRNA gene sequence-based tree (Supplementary Fig. S1), illustrating an overall consistency of the data. The *G. xylinus* and *G. liquefaciens* species groups were visible in both concatenated housekeeping gene-based trees, but they did not cluster together, indicating that the genus *Gluconacetobacter* should not remain as a single genus, as suggested previously (Yamada & Yukphan, 2008). At present, several phenotypic features have been suggested to differentiate strains of the two *Gluconacetobacter* species groups, such as flagella, motility and production of 2,5-diketogluconic acid, γ -pyrone and a water-soluble brown pigment. However, some of these features have previously been reported to be of little use for the differentiation of AAB (Cleenwerck & De Vos, 2008). For this reason, other differentiating features should be looked for before splitting the genus, following the

recommendations of Tindall *et al.* (2010) regarding descriptions of new genera.

Phylogenetic trees based on the concatenated *dnaK*, *groEL* and *rpoB* gene sequences also delineated all species, and revealed high bootstrap values at the species level, namely $\geq 99\%$ in the neighbour-joining tree and $\geq 96\%$ in the maximum-parsimony tree, except for *G. xylinus* (Fig. 1 and Supplementary Fig. S2). These results show the usefulness of MLSA for species differentiation in the family *Acetobacteraceae*. Trees based on the individual *dnaK*, *groEL* or *rpoB* gene sequences (Supplementary Figs S3–S5) also appeared useful for this purpose, as they showed similar topologies to those based on the concatenated housekeeping gene sequences. However, the bootstrap values at the species level were generally lower in the single-gene-based trees, indicating that they are less reliable for allocation of AAB at the species level.

For *G. xylinus*, low bootstrap values (58 and 52 %, respectively) were obtained in the neighbour-joining and maximum-parsimony trees based on the concatenated housekeeping gene sequences. These lower bootstrap values were caused by LMG 18788, and were reflected in the sequence similarities for *dnaK*, *groEL* and *rpoB* obtained for this strain (Supplementary Table S2). LMG 18788 showed sequence similarities of 96.6 % (*dnaK*), 98.0–98.3 % (*groEL*) and 95.5–95.7 % (*rpoB*) with *G. xylinus* strains LMG 1515^T and LMG 1518, whereas strains within the other species of the *G. xylinus* species group showed at least 99.0 % *dnaK*, 99.2 % *groEL* and 98.8 % *rpoB* gene sequence similarity. In addition, the sequence similarities obtained for LMG 18788 were lower than or comparable to sequence similarities obtained between strains of *G. oboediens* and *G. intermedius* (97.0–97.7 % for *dnaK*, 98.0–98.3 % for *groEL* and 95.6–95.9 % for *rpoB*) and between strains of *G. azotocaptans* and *G. johannae* (99.0–99.2 % for *dnaK*, 98.1–98.6 % for *groEL* and 95.9–96.5 % for *rpoB*). Moreover, LMG 18788 showed greater *rpoB* gene sequence similarity to *G. nataicola* LMG 1536^T (96.0 %) than with *G. xylinus* strains LMG 1515^T and LMG 1518 (95.7 and 95.5 %, respectively). Taken together, these results suggest clearly that LMG 18788 represents a distinct species in the *G. xylinus* species group.

LMG 18788 and other strains of the *G. xylinus* species group were subjected to (GTG)₅-PCR and amplified fragment length polymorphism (AFLP) DNA fingerprinting, as described previously (De Vuyst *et al.*, 2008; Cleenwerck *et al.*, 2009), to obtain a more detailed view of their genomic relatedness. Cluster analysis of the DNA fingerprints obtained revealed a separate position for LMG 18788 (Fig. 2), again indicating that the strain represents a distinct *Gluconacetobacter* species.

The DNA base composition of LMG 18788 was determined following the method described by Mesbah *et al.* (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02 M NH₄H₂PO₄ (pH 4.0) and 1.5 % (v/v) acetonitrile. Non-methylated

Fig. 1. Neighbour-joining tree based on concatenated *dnaK*, *groEL* and *rpoB* gene sequences, showing the relationships of species of the genus *Gluconacetobacter* and related species. *Granulibacter bethesdensis* LMG 24392^T was used as an outgroup. Numbers at branching points are percentage bootstrap values based on 500 replications. Bar, 5 % sequence divergence.

lambda phage DNA (Sigma) and DNA from *Escherichia coli* LMG 2093 were used as calibration reference and control, respectively. The DNA G+C content of LMG 18788 was 62.7 mol%, which was very similar to the value

reported previously (62.3 mol%; Toyosaki *et al.*, 1995). The value was also within the expected G+C content range of the *G. xylinus* species group (57–64 mol%) (Dellaglio *et al.*, 2005; Lisdiyanti *et al.*, 2006).

Fig. 2. (GTG)₅-PCR (a) and AFLP (b) DNA fingerprints of strain LMG 18788^T and other strains of the *G. xylinus* species group. The dendrograms were constructed with the UPGMA method after calculation of the band pattern similarity (%) using Pearson's correlation coefficient (a) and the Dice coefficient (b).

Phenotypic and chemotaxonomic characterization of LMG 18788 has been performed previously (Toyosaki *et al.*, 1995; Kojima *et al.*, 1998). Results of this characterization are given in the species description. Phenotypic characteristics that differentiate LMG 18788 from phylogenetically related *Gluconacetobacter* species are presented in Table 2. Strain LMG 18788 could be differentiated from *G. xylinus* and *G. nataicola*, the phylogenetically nearest relatives, by the combination of its inability to produce 5-keto-D-gluconic acid from D-glucose and its ability to produce acid from sucrose and trehalose.

In conclusion, the results of the present study demonstrate that LMG 18788 should be classified in a distinct species in the genus *Gluconacetobacter*. The name *Gluconacetobacter sucrofermentans* sp. nov., comb. nov. is proposed. The results also indicate that the genus *Gluconacetobacter* should be split into two genera, if additional differentiating

phenotypic characteristics can be identified. Finally, MLSA of the housekeeping genes *dnaK*, *groEL* and *rpoB* was shown to be useful for species differentiation in the family *Acetobacteraceae*.

Description of *Gluconacetobacter sucrofermentans* (Toyosaki *et al.* 1996) sp. nov., comb. nov.

Gluconacetobacter sucrofermentans (su.cro.fer.men'tans. N.L. part. adj. *sucrofermentans* sucrose-fermenting).

Basonym: *Acetobacter xylinus* subsp. *sucrofermentans* corrig. Toyosaki *et al.* 1996.

This description is based on the description of *Acetobacter xylinus* subsp. *sucrofermentans* given by Toyosaki *et al.* (1995) and the results of Kojima *et al.* (1998). Cells are Gram-negative, non-motile, short rods, approximately 0.6–0.8 × 1.0–1.2 µm, occurring singly, in pairs or in chains. Colonies grown on GY agar for 3 days at 28 °C are beige, round, raised, rough, shiny and approximately 0.5 mm in diameter. Oxidase-negative, catalase-positive. Grows at 28 and 37 °C and at pH 3.0. Produces acetic acid from ethanol and dihydroxyacetone from glycerol. Oxidizes acetic acid and lactic acid to carbon dioxide and water and D-glucose to 2-keto-D-gluconic acid, but not to 5-keto-D-gluconic acid. The ubiquinone system consists mainly of ubiquinone-10. Grows on ethanol, D-xylose, D-galactose, D-glucose, glycerol, *meso*-erythritol, dulcitol, sucrose and trehalose as carbon sources. Produces acid from D-xylose, D-galactose, D-glucose, glycerol, *meso*-erythritol, sucrose and trehalose.

The type strain, LMG 18788^T (=JCM 9730^T =DSM 15973^T), was isolated from a cherry. This strain is able to produce a large amount of cellulose from sucrose in agitated cultures. The DNA G + C content of LMG 18788^T is 62.7 mol%.

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Table 2. Characteristics that differentiate strain LMG 18788^T from its phylogenetically nearest relatives

Strains: 1, *G. sucrofermentans* sp. nov., comb. nov. LMG 18788^T; 2, *G. xylinus* (4 strains); 3, *G. nataicola* LMG 1536^T; 4, *G. swingsii* LMG 25125^T; 5, *G. europaeus* (3 strains); 6, *G. intermedius* LMG 18909^T; 7, *G. oboediens* (4 strains); 8, *G. rhaeticus* LMG 22126^T; 9, *G. saccharivorans* (2 strains). +, Positive; –, negative; v, variable; w, weakly positive; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9
Production from D-glucose of:*									
2-Keto-D-gluconic acid	+	+	+	+	v	–	+	+	+
5-Keto-D-gluconic acid	–	+	+	+	+	–	v	+	–
Acid production from:†									
Glycerol	w	v	–	NR	+	NR	–	NR	+
Sucrose	+	–	–	NR	–	NR	–	NR	–
Trehalose	+	–	NR	NR	NR	NR	NR	NR	NR
Cellulose production‡	+	+	+	+	–	+	v	+	–

*Data in columns 4 and 8 were taken from Dellaglio *et al.* (2005); the other data were taken from Lisdiyanti *et al.* (2006). For *G. xylinus*, the result given is that of the type strain. Production of ketogluconic acids from D-glucose was determined for all strains using the TLC method described by Gosselé *et al.* (1980).

†Data in columns 1 and 2 were taken from Kojima *et al.* (1998); the other data were taken from Lisdiyanti *et al.* (2006). When reported, acid production from carbon sources was determined using the method described by Asai *et al.* (1964). Briefly, bacteria were incubated for 7 days at 28 °C in a medium consisting of 0.5% (w/v) yeast extract and 1% (w/v) carbon source. The formation of acid was checked by adding 0.002% (w/v) bromocresol purple to the cultured broths.

‡Data were taken from Lisdiyanti *et al.* (2006). Production of cellulose was verified for all strains by incubation in a growth medium containing D-glucose and by boiling formed pellicles in 5% (v/v) NaOH. Cellulose production was confirmed if the pellicles did not dissolve after 2 h of boiling.

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