Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov.

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Ten strains previously assigned to *Acetobacter hansenii* (= *Gluconacetobacter hansenii*), *Acetobacter pasteurianus* LMG 1584 and eight reference strains of the genus *Gluconacetobacter* were reclassified by 16S rRNA gene sequencing, DNA–DNA similarity, DNA base composition and phenotypic characteristics. The *A. hansenii* strains and *A. pasteurianus* LMG 1584 were included in the cluster of acetic acid bacteria (family *Acetobacteraceae*) by 16S rRNA gene sequences. Further, they were separated into seven distinct groups by DNA–DNA similarity. DNA–DNA similarity group I was identified as *G. hansenii*. DNA–DNA similarity group II was retained as *Gluconacetobacter* sp., because DNA–DNA similarity between the strain and *Gluconacetobacter entanii* LTH 4560^T could not be determined. This was due to a lack of availability of the type strain from any source. DNA–DNA similarity group III was regarded as a novel species, for which the name *Gluconacetobacter saccharivorans* sp. nov. (type strain, LMG 1582^T = NRIC 0614^T) is proposed. DNA–DNA similarity group IV included the type strains of *Gluconacetobacter oboediens* and *Gluconacetobacter intermedius*, and three *A. hansenii* strains. This group was identified as *G. oboediens* because high values of DNA–DNA similarity were obtained between the type strains and *G. oboediens* has priority over *G. intermedius*. DNA–DNA similarity group V was identified as *Gluconacetobacter europaeus*. DNA–DNA similarity group VI was regarded as a novel species, for which the name *Gluconacetobacter nataicola* sp. nov. (type strain, LMG 1536^T = NRIC 0616^T) is proposed. DNA–DNA similarity group VII was reclassified as *Gluconacetobacter xylinus*. The description of *G. hansenii* is emended.

The species *Acetobacter hansenii* was established by numerical analysis of phenotypic features and protein gel electropherograms (Gosselé *et al.*, 1983b). The species was then transferred to the genus *Gluconacetobacter* on the basis of ubiquinone systems and partial 16S rRNA gene sequences (Yamada *et al.*, 1997, 1998). However, protein gel electropherograms and some phenotypic features (Gosselé *et al.*, 1983b) had already suggested the heterogeneity of the strains comprising *A. hansenii*. Further, four distinct DNA–DNA homology groups were recognized in *Gluconacetobacter*

hansenii strains (Navarro *et al.*, 1999). Homology group I was identified as *G. hansenii* and the other three groups remained unnamed. Two *G. hansenii* strains (LMG 1517 = NBRC 14822 and LMG 1689) were identified as *Gluconacetobacter intermedius* by partial 16S rRNA gene sequences and *Hae*III and *Hpa*II restriction profiles of the PCR-amplified 16S–23S rDNA spacer region (Trček & Teuber, 2002). *G. hansenii* LMG 1582^T was suggested to be a novel species on the basis of the above-mentioned sequences and profiles (Trček, 2002).

The aim of the present study was to reclassify ten strains previously identified as *A. hansenii* (= *G. hansenii*) (Gosselé *et al.*, 1983b; Navarro *et al.*, 1999) and *Acetobacter pasteurianus* LMG 1584 (= *A. pasteurianus* subsp. *pasteurianus* LMD 39.5) (Table 1). *A. pasteurianus* LMG 1584 was used throughout the present study because the strain was transferred to the genus *Gluconacetobacter* (Lisdiyanti *et al.*, 2000). *Gluconacetobacter entanii* LTH 4560^T was not used in the present study because its transportation made it very

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this study of strains NBRC 14816, NBRC 14817, NBRC 14820^T, LMG 1688, NBRC 14822, LMG 1689, LMG 1582^T, LMG 1584, NBRC 3261, LMG 1536^T, NBRC 14815, JCM 9730^T, JCM 7644^T, JCM 10150, DSM 6160^T and DSM 11826^T are AB166734–AB166744 and AB205217–AB205221, respectively.

Table 1. List of strains studied

Strain no.	Received as	History	Source
NBRC* 14820 ^T	<i>G. hansanii</i>	< Shizuoka Univ. (Y. Yamada) < NCIB 8746 ^T < J. L. Shimwell < M. Schramm	Local vinegar, Jerusalem, Israel
NBRC 14817	<i>G. hansanii</i>	< Shizuoka Univ. (Y. Yamada) < NCIB 8246 < J. Senez < M. Aschner	Local vinegar, Jerusalem, Israel
NBRC 14816	<i>G. hansanii</i> homology group unknown	< Shizuoka Univ. (Y. Yamada) < NCIB 8747 < J. L. Shimwell < M. Schramm	Local vinegar, Jerusalem, Israel
NBRC 14815	<i>G. hansanii</i> homology group IV†	< Shizuoka Univ. (Y. Yamada) < NCIB 8752 < T. K. Walker	Malt vinegar brewery acetifiers
LMG 1582	<i>A. hansanii</i>	< LMD (' <i>A. mesoxydans</i> subsp. <i>saccharovorans</i> ') < J. Frateur (' <i>A. mesoxydans</i> var. <i>sucrasiferum</i> ') < Institut für die Essigindustrie Berlin (' <i>Bacterium dihydroxyaceticum</i> ') < K. Bernhauer (' <i>A. dioxyceticum</i> var. <i>pectinatum</i> ') < NCTC 4940 < A. C. Thaysen	Beet juice
LMG 1584	<i>A. pasteurianus</i>	< LMD (' <i>A. mesoxydans</i> subsp. <i>saccharovorans</i> ') < K. Bernhauer (' <i>A. dioxyceticum</i> var. <i>pectinatum</i> ') < NCTC 4940 < A. C. Thaysen	
NBRC 14822	<i>G. hansanii</i> homology group II†	< Shizuoka Univ. (Y. Yamada) < NCIB 4940 < NCTC 4940 < A. C. Thaysen	
LMG 1688	<i>A. hansanii</i>	< LMD	
LMG 1689	<i>A. hansanii</i>	< LMD (' <i>A. xylinum</i> var. <i>xylinoides</i> ') < W. Henneberg (' <i>Bacterium xylinoides</i> ') < NBRC (K. Kondo, 42)	
NBRC 3261	<i>G. hansanii</i> homology group II†	< NBRC (K. Kondo, 42)	<i>Myrica rubra</i> (fruit), Nishinomiya, Hyogo, Japan
LMG 1536	<i>A. hansanii</i>	< E. Magno (' <i>A. xylinum</i> ') < M. Sievers	Nata, Manila, Philippines
DSM 6160 ^T	<i>G. europaeus</i>	< J. Trček	Vinegar, Germany
JK2	<i>G. europaeus</i>	< C. Boesch, ETH; TF2	Vinegar
DSM 11804 ^T	<i>G. intermedius</i>	< S. J. Sokollek & W. P. Hammes	Tea fungus beverage (kombucha), Switzerland
DSM 11826 ^T	<i>G. oboediens</i>	< NCIB 11664 ^T < NCTC 4112 < G. Bertrand < T. Tsuchida BPR 2001 ^T	Industrial red wine vinegar fermentation, Germany
JCM 7644 ^T	<i>G. xylinus</i>	< NCIB 11664 ^T < NCTC 4112 < G. Bertrand < T. Tsuchida BPR 2002	Cherry, Japan
JCM 9730 ^T	<i>G. xylinus</i> subsp. <i>sacrofermentans</i>	< T. Tsuchida BPR 2001 ^T	
JCM 10150	' <i>G. xylinus</i> subsp. <i>nonacetoxidans</i> '	< T. Tsuchida BPR 2002	Grape, Japan
NBRC 12388 ^T	<i>G. liquefaciens</i>	< Shizuoka Univ. < IAM 1834 ^T < IAM (T. Asai, G-1)	<i>Diospyros</i> sp. (dried persimmon), Japan
NBRC 14818 ^T	<i>A. aceti</i>	< Shizuoka Univ. (Y. Yamada) < NCIB 8621 ^T < W. Verhoeven < J. Frateur	Alcohol turned to vinegar

*IFO cultures were transferred to NBRC (NITE Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu-shi, Chiba 292-0818, Japan) with the same accession numbers.

†Homology groups by Navarro *et al.* (1999).

difficult to obtain (C. Hertel, Institut für Lebensmitteltechnologie, Universität Hohenheim, Stuttgart, Germany, personal communication) and it was not available from any culture collections.

Sequencing of the 16S rRNA gene and the construction of a phylogenetic tree were carried out as reported previously (Lisdiyanti *et al.*, 2000; Yamada *et al.*, 2000). The 16S rRNA gene was amplified by PCR with two primers: 20F (5'-GATTTTATCTCTGGCTCAG-3', positions 9–27) and 1500R (5'-GTTACCTTGTTACGACTT-3', positions

1509–1429). The numbering of positions was based on the *Escherichia coli* numbering system (GenBank accession no. V00348; Brosius *et al.*, 1981). The purified PCR products were sequenced directly by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 310 genetic analyser. The following six primers were used: 20F, 1500R, 520F (5'-CAGCAGC-CGCGGTAATAC-3', positions 519–536), 520R (5'-GTATTACCGCGGCTGCTG-3', positions 536–519), 920F (5'-AAACTAAATGAATTGACGG-3', positions 907–926) and 920R (5'-CCGTCATTCATTTGAGTTT-3', positions

Fig. 1. Phylogenetic relationships of acetic acid bacteria deduced from 16S rRNA gene sequence clustering by neighbour joining. Numbers indicate the bootstrap value derived from 1000 replications. Sequences determined in this study are shown in bold. *Rhodospirillum rubrum* ATCC 11170^T (GenBank accession no. D30778) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

926–907). Multiple alignments were performed in the program CLUSTAL_W (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by the two-parameter method (K_{nuc}) (Kimura, 1980). Neighbour-joining (NJ) (Saitou & Nei, 1987), maximum likelihood (ML) (Felsenstein, 1981) and maximum parsimony (MP) (Felsenstein, 1983) were employed for constructing phylogenetic trees. The robustness of individual branches of the NJ tree was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Alignment gaps and unidentified base positions were not taken into account for the calculations. The 16S rRNA gene sequences of ten *G. hansenii* strains, *A. pasteurianus* LMG 1584, *Gluconacetobacter oboediens* DSM 11826^T, *Gluconacetobacter europaeus* DSM 6160^T, *Gluconacetobacter xylinus* JCM 7644^T, *G. xylinus* subsp. *sucrofermentans* JCM 9730^T (Toyosaki *et al.*, 1995) and '*G. xylinus* subsp. *nonacetoxidans*' JCM 10150 (Kojima *et al.*, 1998) were determined in the present study. InforBIO, an e-workbench for the databasing, classification and identification of microbes, was used for the analysis of the phylogenetic trees (Sugawara *et al.*, 2003; www.wdcm.org). Available 16S rRNA gene sequences were obtained from GenBank/EMBL/DBJ. Species, type strains, strains and GenBank accession numbers are presented in Figs 1–3.

Phylogenetic trees of the strains studied and reference strains of the genus *Gluconacetobacter* were rather similar to each other when produced by NJ and MP (Figs 1, 2). A

phylogenetic tree of the strains produced by ML was a little different from those produced by NJ and MP (Fig. 3). However, all of the strains were included in the cluster of the genus *Gluconacetobacter*, in a broad cluster of the acetic acid bacteria (family *Acetobacteraceae*). Further, the species of the genus *Gluconacetobacter* seemed likely to be separated into two subclusters. One consisted of *Gluconacetobacter swingsii*, *G. europaeus*, *Gluconacetobacter nataicola*, *G. xylinus*, *G. oboediens*, *G. intermedius*, *Gluconacetobacter rhaeticus*, *Gluconacetobacter saccharivorans*, *G. hansenii* and *G. entanii*, and 16S rRNA gene sequence similarity was 98.1–99.9% between the type strains of the above species. The other cluster consisted of *Gluconacetobacter liquefaciens*, *Gluconacetobacter sacchari*, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter azotocaptans* and *Gluconacetobacter johannae*, and the 16S rRNA gene sequence similarity was 98.4–99.16% between the type strains of these species. Further, the 16S rRNA gene sequence similarity between the two subclusters was 95.7–97.6%.

DNA was extracted and purified as reported by Saito & Miura (1963). The medium was the same as that described previously (Navarro & Komagata, 1999). A filter-sterilized cellulase (cellulase of *Trichoderma viride*; Wako) solution was added to the liquid medium at a final concentration of 0.025% for the extraction of DNA from cellulose-producing strains. The cellulase was added before cultivation. This enabled the extraction of DNA from the strains because

Fig. 2. Phylogenetic relationships of acetic acid bacteria deduced from 16S rRNA gene sequence clustering by maximum likelihood. Numbers indicate the bootstrap value derived from 1000 replications. Sequences determined in this study are shown in bold. *Rhodospirillum rubrum* ATCC 11170^T (GenBank accession no. D30778) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

cellulose production was prevented by cellulase. DNA–DNA hybridization was performed as described by Ezaki *et al.* (1989) at 50 °C, a stringent hybridization temperature. G + C contents were determined as described by Tamaoka & Komagata (1984). DNA base compositions of the strains studied fell into a range of 57–63 mol% G + C (Table 2).

According to the phylogenetic relationships of 16S rRNA gene sequences, DNA–DNA similarity was determined between the strains of *G. europaeus*, *G. xylinus*, *G. nataicola*, *G. oboediens*, *G. intermedius*, *G. saccharivorans*, *G. hanseni* and *Gluconobacter* sp. NBRC 14815, and seven DNA–DNA similarity groups were delineated in the present study (Table 2). DNA–DNA similarity group I contained *G. hanseni* NBRC 14820^T, NBRC 14816 and NBRC 14817, and was identified as *G. hanseni* because these strains showed high levels of DNA–DNA similarity (80–100 %) to the type strain and one another. These strains showed 99.9–100 % 16S rRNA gene sequence similarity to one another. DNA–DNA similarity group II contained a single strain, NBRC 14815. This strain showed 99.9 % 16S rRNA gene sequence similarity to *G. entanii* LTH 4560^T. However, NBRC 14815 should be retained as *Gluconacetobacter* sp. because the type strain of *G. entanii* was not available from any source and the DNA–DNA similarity could not be determined. This case raises a problem about the identification of such strains in the future. DNA–DNA similarity group III included LMG 1582^T and LMG 1584. These strains were identical to each

other and separate from other groups based on DNA–DNA similarity. Therefore, the strains were regarded as a novel species, for which the name *Gluconacetobacter saccharivorans* sp. nov. is proposed. LMG 1582^T was previously suggested to belong to a novel species based on a partial 16S rRNA gene sequence and *Hae*III and *Hpa*II restriction profiles of the PCR-amplified 16S–23S rRNA gene spacer region (Trček, 2002). This author's suggestion was confirmed in the present study. DNA–DNA similarity group IV contained *G. oboediens* DSM 11826^T, *G. hanseni* NBRC 14822, LMG 1688 and LMG 1689 and *G. intermedius* DSM 11804^T. Their 16S rRNA gene sequence similarity was 99.9–100 % to one another. High levels of DNA–DNA similarity and the similarity of 16S rRNA gene sequence revealed a synonymous relationship between *G. oboediens* and *G. intermedius*. *G. oboediens* has priority over *G. intermedius* (Boesch *et al.*, 1998a, b; Sokollek *et al.*, 1998) and *G. intermedius* is a later heterotypic synonym of *G. oboediens*. As a result, this group was identified as *G. oboediens*. LMG 1517 (= NBRC 14822) and LMG 1689 were identified previously as *G. intermedius* by partial 16S rRNA gene sequences and *Hae*III and *Hpa*II restriction profiles of the PCR-amplified 16S–23S rDNA spacer region (Trček & Teuber, 2002). The present study confirmed their identification. DNA–DNA similarity group V included *G. europaeus* DSM 6160^T, *G. europaeus* JK2 and NBRC 3261 and was identified as *G. europaeus* because these strains showed high levels of DNA–DNA similarity (70–100 %) to

Fig. 3. Phylogenetic relationships of acetic acid bacteria deduced from 16S rRNA gene sequence clustering by maximum parsimony. Numbers indicate the bootstrap value derived from 1000 replications. Sequences determined in this study are shown in bold. *Rhodospirillum rubrum* ATCC 11170^T (GenBank accession no. D30778) was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

the type strain and one another. A high value of DNA–DNA similarity (98 %) was reported between *G. europaeus* DSM 6160^T and strain JK2 (Trček *et al.*, 2000). It is interesting that NBRC 3261 was maintained without notation of requirement of acetic acid in the culture collection. DNA–DNA similarity group VI contained only LMG 1536^T. This strain was located together with *Acetobacter* sp. ITDI 2.1, which was isolated from nata de coco (Bernardo *et al.*, 1998), and close to *G. europaeus* and *G. xylinus* strains (Figs 1, 2) with 99.7–99.9 % 16S rRNA gene sequence similarity. LMG 1536^T was differentiated from *G. europaeus* and *G. xylinus* by DNA–DNA similarity. Therefore, this strain was regarded as a novel species, for which the name *Gluconacetobacter nataicola* sp. nov. is proposed, according to its isolation source. DNA–DNA similarity group VII included *G. xylinus* JCM 7644^T, *G. xylinus* subsp. *sucrofermentans* JCM 9730^T and 'G. *xylinus* subsp. *nonacetoxidans*' JCM 10150, and was identified as *G. xylinus* because these strains showed high levels of DNA–DNA similarity (67–100 %) to the type strain and one another. This study confirmed high levels of DNA–DNA similarity of *G. xylinus* strains and *G. xylinus* subsp. *sucrofermentans* as reported by Tanaka *et al.* (2000). Recently, *G. swingsii* and *G. rhaeticus* were described and these species were separated from the type strains of the *G. xylinus* branch (Dellaglio *et al.*, 2005).

The present study emphasized that when a bacterial species was established without DNA–DNA similarity data between

the strains used, such a bacterial species should be re-examined for the similarity between the strains comprising the species for the stability of nomenclature. DNA–DNA similarity is still a reliable criterion for distinguishing bacterial species when clear-cut, differential phenotypic characteristics have not yet been found among the species (Cleenwerck *et al.*, 2002; Katsura *et al.*, 2002; Navarro *et al.*, 1999; Lisdiyanti *et al.*, 2000).

Isoprenoid quinones were extracted as described by Yamada *et al.* (1969) and determined by HPLC (Komagata & Suzuki, 1987). Three strains (NBRC 14816, LMG 1536^T and LMG 1584) and two reference strains (DSM 11804^T and JK2) had ubiquinones, with Q-10 accounting for > 90 % and Q-9 for < 10 % of the total ubiquinones. Thus, all *Gluconacetobacter* strains used in the present study had Q-10 as the major quinone, in agreement with data reported previously (Navarro *et al.*, 1999).

Phenotypic characterization of the strains studied was mostly carried out as described previously (Lisdiyanti *et al.*, 2000). Production of acid from ethanol and oxidation of ethanol were examined by clearing around colonies (Frature, 1950; Swings *et al.*, 1992). Growth in the presence of 0.35 % acetic acid was examined by using AG medium. This medium was used for the separation of the species in the genera *Asaia* and *Saccharibacter* from those of the genera *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Kozakia* and *Swaminathanian* (Jojima *et al.*, 2004; Katsura

Table 2. DNA base compositions and levels of DNA–DNA similarity of strains studied

Strains: 1, *G. hansenii* NBRC 14820^T; 2, *Gluconacetobacter* sp. NBRC 14815; 3, *G. saccharivorans* LMG 1582^T; 4, *G. saccharivorans* LMG 1584; 5, *G. oboediens* DSM 11826^T; 6, *G. oboediens* NBRC 14822; 7, *G. oboediens* LMG 1688; 8, *G. intermedius* DSM 11804^T; 9, *G. europaeus* DSM 6160^T; 10, *G. europaeus* JK2; 11, *G. europaeus* NBRC 3261; 12, *G. nataicola* LMG 1536^T; 13, *G. xylinus* JCM 7644^T; 14, *G. xylinus* subsp. *sucrofermentans* JCM 9730^T; 15, '*G. xylinus* subsp. *nonacetoxidans*' JCM 10150. ND, Not determined.

DNA–DNA similarity group	Strain no.	G+C content (mol%)	Levels of DNA–DNA similarity with strain (probe)														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
I	NBRC 14820 ^T	59	100	9	6	5	8	4	6	9	10	18	5	11	12	15	20
	NBRC 14816	58	100	11	6	4		3	7	10			6				
	NBRC 14817	59	100	12	7	4		3	8				7				
II	NBRC 14815	62	23	100	29	5	15		18	22	17	37	22	26	15	21	30
III	LMG 1582 ^T	61		21	100	83	30		20	23	34	53	20	25	25	13	29
	LMG 1584	61	25		88	100			11								
IV	DSM 11826 ^T	60†		42	44		100			77	50		53	53	52	37	58
	NBRC 14822	61		8	26	14	9		100	83	71		34	52			
	LMG 1688	61		12	34	13	10	68	100	100	75	45	41	58	31	32	48
	LMG 1689	60		11	40	14	13		100	94	71		49	59			
	DSM 11804 ^T	61*			40		94	73		100	59	60	47	51	42	32	66
V	DSM 6160 ^T	57‡	15	30	23		39			48	100		70	56			24
	JK2	ND										100					
	NBRC 3261	58	9	45	22	10	28	29	38	48	68	75	100	43	15	12	38
VI	LMG 1536 ^T	62	8	46	13	3	55	3	7	23	40		37	100	29	31	59
VII	JCM 7644 ^T	62	12	45	38		47			34	50		47	42	100	67	98
	JCM 9730 ^T	62§		47	30		33			42	46		56	51	73	100	98
	JCM 10150	63		13	10		16			30	24		16	16	69	69	100
<i>G. liquefaciens</i>	NBRC 12388 ^T	65¶		9	7		6			5	8		5	6	8	5	14
<i>A. aceti</i>	NBRC 14818 ^T	58¶		10	5		8			8	9		12	3	9	8	5

*Boesch *et al.* (1998).

†Sokollek *et al.* (1998).

‡Sievers *et al.* (1992).

§Toyosaki *et al.* (1995).

||Kojima *et al.* (1998).

¶Lisdiyanti *et al.* (2000).

et al., 2001; Lisdiyanti *et al.*, 2002; Loganathan & Nair, 2004; Yamada *et al.*, 2000). Growth at 0, 1 and 5 % acetic acid was determined by using AE broth (Entani *et al.*, 1985) as a basal medium because *G. europaeus* and *G. entanii* strains require acetic acid for growth and these species exhibit a strong tolerance to acetic acid (Entani *et al.*, 1985; Schüller *et al.*, 2000; Sievers *et al.*, 1992). Growth on ethanol was determined by using the medium described by Gosselé *et al.* (1983a) (Gosselé's medium). Utilization of ammoniacal nitrogen was determined by using Hoyer–Fratureur medium (De Ley & Fratureur, 1974), Fratureur's modified Hoyer's medium (De Ley *et al.*, 1984) and the medium described by Asai *et al.* (1964) (Asai's medium). Ethanol, D-glucose or D-mannitol was used as sole carbon source for the media. Acid production from sugars and sugar alcohols was examined by using the medium described by Asai *et al.* (1964).

Cells of all strains studied were Gram-negative and rod-shaped, measured 0.5–1.0 by 1.0–3.0 µm and were

non-motile. They were aerobic, catalase-positive and oxidase-negative. The strains did not produce a water-soluble brown pigment on the culture media used, oxidized acetate and lactate, produced acid from ethanol and grew well on mannitol agar and glutamate agar. They grew in the presence of 0.35 % acetic acid in AG medium and grew without acetic acid, but not at 1 or 5 % acetic acid, in AE broth. However, NBRC 3261 grew at 1 % acetic acid and DSM 6160^T and JK2 grew even at 5 % acetic acid. Production of 5-keto-D-gluconate from D-glucose varied with the strain studied. *G. hansenii* strains produced acid from galactitol. *G. saccharivorans* produced acid from propan-1-ol. Data of other characteristics are shown in Table 3.

The terms 'production of acid from ethanol' and 'oxidation of ethanol' may bring about some confusion in the identification of acetic acid bacteria. Acid production from ethanol and oxidation of ethanol involve basically the same biochemical reaction by acetic acid bacteria, the production of acetic acid from ethanol. This has been tested

Table 3. Phenotypic characteristics of *Gluconacetobacter* strains studied

Abbreviations: KGA, keto-D-gluconate; +, positive; –, negative; w, weak; vw, very weak; ND, not determined. Note: all strains were Gram-negative, rod-shaped bacteria, positive for catalase, production of acetic acid from ethanol, growth on mannitol agar and glutamate agar, growth at 0.35% acetic acid in AG medium, production of dihydroxyacetone, production of D-gluconate from D-glucose and production of acid from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, ethanol, butan-1-ol and butan-2-ol. All strains are negative for production of a water-soluble brown pigment, oxidase, production of 2,5-diketo-D-gluconate from D-glucose and production of acid from L-sorbose, lactose and starch. Strains: 1, *G. hansenii* NBRC 14820^T; 2, *G. hansenii* NBRC 14817; 3, *G. hansenii* NBRC 14816; 4, *Gluconacetobacter* sp. NBRC 14815; 5, *G. saccharivorans* LMG 1582^T; 6, *G. saccharivorans* LMG 1584; 7, *G. oboediens* DSM 11826^T; 8, *G. oboediens* NBRC 14822; 9, *G. oboediens* LMG 1688; 10, *G. oboediens* LMG 1689; 11, *G. oboediens* DSM 11804; 12, *G. europaeus* DSM 6160^T; 13, *G. europaeus* JK2; 14, *G. europaeus* NBRC 3261; 15, *G. nataicola* LMG 1536^T; 16, *G. xylinus* JCM 7644^T; 17, *G. xylinus* JCM 9730; 18, *G. xylinus* JCM 10150.

Characteristic	DNA–DNA similarity group																		
	I			II	III		IV					V			VI		VII		
	1	2	3	4	5	6	7*	8	9	10	11†	12‡	13§	14	15	16	17	18¶	
Oxidation of:																			
Acetate	+	+	+	+	+	+	ND	+	+	+	ND	+	+	+	+	+	+	–	
Lactate	+	+	+	+	+	+	ND	+	+	+	ND	+/–	+	+	+	+	+	–	
Growth in the presence of acetic acid at:																			
0 %	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	ND	ND	ND	
1 %	–	–	–	–	–	–	+	–	–	–	+	+	+	+	–	ND	ND	ND	
5 %	–	–	–	–	–	–	+	–	–	–	–	+	+	–	–	ND	ND	ND	
Utilization of ammoniacal N₂ in:																			
Hoyer–Frature medium:																			
D-Glucose	+	+	+	–	+	+	ND	–	–	–	ND	ND	ND	–	–	ND	ND	ND	
D-Mannitol	+	+	+	+	+	+	ND	+	–	–	ND	ND	ND	–	+	ND	ND	ND	
Ethanol	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	–	–	ND	ND	ND	
Asai’s medium:																			
D-Glucose	+	+	+	–	+	+	ND	–	–	–	ND	ND	ND	–	–	+	+	ND	
D-Mannitol	+	+	+	+	+	+	ND	+	–	–	ND	ND	ND	–	+	ND	ND	ND	
Ethanol	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	–	–	ND	ND	ND	
Production of KGAs from glucose:																			
2-KGA	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+	
5-KGA	+	+	+	+	–	–	–	+	–	–	–	+	+	+	+	+	–	+	
Acid production from:																			
D-Fructose	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	+	–	vw	–	ND	
Maltose	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	+	–	–	–	–	
Raffinose	–	–	+	+	–	–	ND	+	–	–	ND	ND	ND	+	–	ND	ND	ND	
Glycerol	+	+	+	+	+	+	ND	–	–	–	ND	ND	ND	+	–	–	w	–	
Galactitol	+	+	+	–	–	–	ND	–	–	–	ND	ND	ND	–	–	ND	ND	–	
D-Mannitol	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	+	–	–	–	ND	
D-Sorbitol	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	+	–	–	–	ND	
Sucrose	–	–	–	–	–	–	ND	–	–	–	ND	ND	ND	–	–	–	+	–	
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	vw	+	+	
Propan-1-ol	–	–	–	–	+	+	ND	–	–	–	ND	ND	ND	–	–	ND	ND	ND	
Cellulose production	–	–	+	+	–	–	–	+	–	–	+	–	–	–	+	+	+	+	

*Sokollek *et al.* (1998).†Boesch *et al.* (1998).‡Sievers *et al.* (1992).§Trček *et al.* (2000).||Toyosaki *et al.* (1995).¶Kojima *et al.* (1998).

by the titration of acetic acid produced with 0.1 M KOH (Asai *et al.*, 1964), respirometry (Asai *et al.*, 1964; Kondo & Ameyama, 1958), the dissolution of CaCO₃ around colonies (Asai *et al.*, 1964; Shimwell *et al.*, 1960; Swings *et al.*, 1992) or the colour change of the indicator (Carr, 1968; Swings *et al.*, 1992). Media used for the test usually contain yeast extract, peptone or other organic substances as nitrogen sources with ethanol. However, when small amounts of acetic acid are produced in liquid media and on solid media, the acid may be masked by diffusion into the media or the buffer action of the above nitrogenous substances and may give a false-negative result. Therefore, acid production from ethanol should be evaluated cautiously and recorded correctly for the identification of acetic acid bacteria. The dissolution of CaCO₃ is appropriate for the production of acid from ethanol in the light of the history of acetic acid bacteria.

The strains studied did not grow on ethanol in Gosselé's broth. In contrast, a false growth was recognized on Gosselé's agar plates because the strains studied showed scant growth on agar plates with and without ethanol. The utilization (assimilation) of or growth on organic carbon compounds means the biochemical uptake of the compounds into cellular materials, and the increase in cellular mass has been regarded as the uptake of the compounds. The increase in turbidity in liquid cultures and in colonial mass on agar media has been used as a parameter for the utilization of organic compounds. However, the media used for the utilization of carbon compounds have not been specified and some media contain yeast extract, peptone or other organic substances as nitrogen sources and growth factors, such as Gosselé's agar medium. When the medium contains much larger amounts of yeast extract or peptone than of the carbon source, these organic, nitrogenous substances may serve as the carbon source as well and often give a false-positive result. Therefore, the results obtained should be examined carefully. Media containing agar cause much more difficulty in determination of growth (Cleenwerck *et al.*, 2002; Gosselé *et al.*, 1983a; Swings *et al.*, 1992). When the bacteria examined require some growth factors, defined media would be preferable to complex media for the determination of utilization of carbon compounds. Acid production from sugars and sugar alcohols is often confusing with the utilization of the organic carbon compounds. This may cause false-positive growth because basal media usually contain considerable amounts of yeast extract, such as the medium reported by Asai *et al.* (1964).

The strains of *G. hansenii* and *G. saccharivorans* grew well at the expense of D-glucose and D-mannitol on Hoyer-Frateur medium, Frateur's modified Hoyer's medium and Asai's medium, but did not grow at the expense of ethanol. The utilization of ammoniacal nitrogen of acetic acid bacteria has been reported to vary with carbon compound used (Gosselé *et al.*, 1983b; Navarro *et al.*, 1999; Shimwell, 1957). *Asaia* strains grew well on Hoyer-Frateur medium with

D-glucose or D-mannitol as sole carbon source, but not with ethanol (Katsura *et al.*, 2001; Lisdiyanti *et al.*, 2000; Yamada *et al.*, 2000; Yukphan *et al.*, 2004). *Frateruria aurantia* strains are biochemically similar to acetic acid bacteria and they grow on Hoyer-Frateur medium and Frateur's modified Hoyer's medium with D-mannitol as sole carbon source, but not with ethanol or D-glucose (Lisdiyanti *et al.*, 2003). In addition, the utilization of ammoniacal nitrogen on Hoyer-Frateur medium and Frateur's modified Hoyer's medium was recognized to be inappropriate for the identification of *Acetobacter* strains because some strains gave false-positive growth (Lisdiyanti *et al.*, 2000). Therefore, the taxonomic value of the utilization of ammoniacal nitrogen is rather limited to some species or genera of acetic acid bacteria and should be examined by using several kinds of carbon compounds on defined media.

The production of cellulose varied with strains studied. Cellulose production was reported to be useful for the separation of acetic acid bacteria, particularly *G. xylinus* in the old descriptions of acetic acid bacteria (De Ley & Frateur, 1974; Vaughn, 1957), but some strains of *G. hansenii*, *G. oboediens*, *G. xylinus* and *G. nataicola* and *Gluconacetobacter* sp. NBRC 14815 also produced cellulose in the present study. Therefore, the production of cellulose is not useful for the differentiation of the species in the genus *Gluconacetobacter*. Pellicles produced by acetic acid bacteria do not always mean real cellulose and the production of real cellulose should be confirmed by boiling the pellicles with a dilute NaOH solution (Forng *et al.*, 1989; Navarro *et al.*, 1999).

Description of *Gluconacetobacter saccharivorans* sp. nov.

Gluconacetobacter saccharivorans (sac.cha.ri.vo'rans. L. neut. n. *saccharum* sugar; L. part. adj. *vorans* devouring; N.L. masc. adj. *saccharivorans* sugar-devouring).

Cells are Gram-negative and rod-shaped, measure 0.5–0.8 by 1.0–1.5 µm, occur singly or in pairs and are non-motile. Strictly aerobic, catalase-positive and oxidase-negative. Cellulose and a water-soluble brown pigment are not produced. Acetate and lactate are oxidized to CO₂ and H₂O. Acetic acid is produced from ethanol. Growth occurs in the presence of 0.35 % acetic acid in AG medium, but not at 1 or 5 % acetic acid in AE broth. D-Glucose and D-mannitol are assimilated in Hoyer-Frateur medium, Frateur's modified Hoyer's medium and Asai's broth, but not ethanol. Dihydroxyacetone is produced from glycerol and growth occurs on mannitol agar and glutamate agar. D-Gluconate and 2-keto-D-gluconate are produced from D-glucose, but 5-keto-D-gluconate and 2,5-diketogluconate are not. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, glycerol, ethanol, propan-1-ol, butan-1-ol and butan-2-ol, but not from D-fructose, L-sorbose, lactose, maltose, sucrose, raffinose, galactitol, D-mannitol, D-sorbitol or starch. The major quinone is Q-10 and the minor quinone is Q-9.

The type strain is LMG 1582^T (=NRIC 0614^T) and its G + C content is 61 mol%. Isolated from beet juice in Germany in 1927.

Description of *Gluconacetobacter nataicola* sp. nov.

Gluconacetobacter nataicola [na.ta.i'co.la. N.L. neut. n. *nataum* nata (a food composed of cellulose produced by acetic acid bacteria in South-East Asia); L. masc. suff. *-cola* inhabitant; N.L. masc. n. *nataicola* nata inhabitant, referring to the isolation source, nata de coco, of the type strain).

Cells are Gram-negative and rod-shaped, measure 0.5–0.8 by 1.0–1.5 µm, occur singly or in pairs and are non-motile. Strictly aerobic, catalase-positive and oxidase-negative. Cellulose is produced, but water-soluble brown pigment is not. Acetate and lactate are oxidized to CO₂ and H₂O. Acetic acid is produced from ethanol. Growth occurs in the presence of 0.35 % acetic acid in AG medium, but not at 1 or 5 % acetic acid in AE broth. D-Mannitol is assimilated in Hoyer–Frater medium, Frater's modified Hoyer's medium and Asai's broth, but not D-glucose or ethanol. Dihydroxyacetone is produced from glycerol and growth occurs on mannitol agar and glutamate agar. D-Gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate are produced from D-glucose, but 2,5-diketogluconate is not. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, ethanol, butan-1-ol and butan-2-ol, but not from D-fructose, L-sorbose, lactose, maltose, sucrose, raffinose, glycerol, galactitol, D-mannitol, D-sorbitol, propan-1-ol or starch. The major quinone is Q-10 and the minor quinone is Q-9.

The type strain is LMG 1536^T (=NRIC 0616^T) and its G + C content is 62 mol%. Isolated from nata de coco in the Philippines.

Emended description of *Gluconacetobacter hansenii* (Gosselé et al. 1983) Yamada et al. 1998

Cells are Gram-negative and rod-shaped, measure 0.5–0.8 by 1.0–1.5 µm, occur singly or in pairs and are non-motile. Strictly aerobic, catalase-positive and oxidase-negative. Cellulose (with the exception of one strain) and a water-soluble brown pigment are not produced. Acetate and lactate are oxidized to CO₂ and H₂O. Acetic acid is produced from ethanol. Growth occurs in the presence of 0.35 % acetic acid in AG medium, but not at 1 or 5 % acetic acid in AE broth. D-Mannitol and D-glucose are assimilated in Hoyer–Frater medium, Frater's modified Hoyer's medium and Asai's broth, but not ethanol. Dihydroxyacetone is produced from glycerol and growth occurs on mannitol agar and glutamate agar. D-Gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate are produced from D-glucose, but not 2,5-diketogluconate. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, galactitol, ethanol, butan-1-ol and butan-2-ol, but not from D-fructose, L-sorbose,

lactose, maltose, sucrose, glycerol, D-mannitol, D-sorbitol, propan-1-ol or starch. The major quinone is Q-10 and the minor quinone is Q-9.

The type strain is NBRC 14820^T (=LMG 1527^T=NCIB 8745^T) and its G + C content is 62 mol%. Isolated from local vinegar in Jerusalem, Israel.

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