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Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov.

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Thirty-four *Acetobacter* strains, representing *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Acetobacter peroxydans*, *Acetobacter lovaniensis*, *Acetobacter estunensis*, *Acetobacter orleanensis*, *Acetobacter indonesiensis* and *Acetobacter tropicalis*, were subjected to a polyphasic study that included DNA–DNA hybridizations, DNA base ratio determinations, 16S rDNA sequence analysis and phenotypic characterization. Two novel species are proposed, *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. The type strains of these species are respectively LMG 1625^T (= DSM 14362^T = NCIB 8894^T = ATCC 23765^T) and LMG 1746^T (= DSM 14337^T).

Keywords: *Acetobacter cerevisiae* sp. nov., *Acetobacter malorum* sp. nov., acetic acid bacteria, taxonomy, classification

INTRODUCTION

Acetic acid bacteria are classified into the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas* and the recently described genus *Asaia* (Yamada *et al.*, 2000). These genera join phylogenetically into a broad rRNA cluster within the α-subclass of the *Proteobacteria*, the acetic acid bacteria lineage.

The genus *Acetobacter* is differentiated from the other genera by its Q9 ubiquinone system and by the oxidation of acetate and lactate to CO_2 and H_2O (De Ley & Frateur, 1974; Yamada *et al.*, 1997, 2000). At the time of writing, the genus consisted of nine species: *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Acetobacter peroxydans*, *Acetobacter lovaniensis*, *Acetobacter estunensis*, *Acetobacter orleanensis*, *Acetobacter indonesiensis* and *Acetobacter tropicalis*. These species were delineated mainly on the basis of DNA–DNA relatedness and phylogenetic relationships (Sokollek *et al.*, 1998; Lisdiyanti *et al.*, 2000).

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The EMBL accession numbers for the 16S rDNA sequences of strains LMG 1629, LMG 18848^T, LMG 1633, LMG 1617^T, LMG 1626^T, LMG 1572, LMG 1531, LMG 1588, LMG 1663, LMG 1625^T, LMG 1746^T and LMG 1583^T are respectively AJ419834–AJ419845.

In the course of a larger study set up to improve the identification of *Acetobacteraceae*, 16 strains from the heterogeneous species *A. pasteurianus*, as well as 17 reference strains representing all known *Acetobacter* species, were analysed genotypically and phenotypically. DNA homology data demonstrate that five strains currently allocated to *A. pasteurianus* could not be assigned to any known *Acetobacter* species. Four of these strains constituted a separate but homogeneous taxon, for which the name *Acetobacter cerevisiae* sp. nov. is proposed. The name *Acetobacter malorum* sp. nov. is proposed for strain LMG 1746^T.

METHODS

Bacterial strains. Strains used in this study are listed in Table 1. They were checked for purity by plating on medium 13 from the *Catalogue of Cultures* of the BCCM/LMG Bacteria Collection (Janssens *et al.*, 1998) containing 2·5% D-mannitol, 0·5% yeast extract, 0·3% peptone and 1·5% agar.

DNA isolation. Total DNA for the determination of the DNA base composition and for DNA–DNA hybridizations was prepared by the method of Wilson (1987), with minor modifications. Cells were washed with RS buffer (0·15 M NaCl, 10 mM EDTA, pH 8·0) and then suspended and lysed in a Tris/EDTA buffer (10 mM Tris/HCl with up to 200 mM EDTA, pH 8·0) containing RNase A (Sigma), SDS (Serva) and proteinase K (Merck) to final concentrations of 400 μg ml^{-1}, 2% (w/v) and 200 μg ml^{-1}, respectively. NaCl (5 M stock solution) and CTAB/NaCl solution [10% (w/v) hexadecyltrimethylammonium bromide in 0·7 M NaCl] were added to final concentrations of 1 M and 13·3% (v/v),

Table 1. List of strains studied

Strains are listed under their proposed species designations. LMG, BCCM/LMG Bacteria Collection, Laboratorium Microbiologie, Universiteit Gent, Belgium; NCIB, National Collections of Industrial and Marine Bacteria, Aberdeen, UK; LMD, Laboratorium voor Microbiologie, Technische Universiteit, Delft, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; NRIC, NODAI Research Institute Culture Collection, Tokyo University of Agriculture, Tokyo, Japan.

Strain	As received	Current species designation	Source (if known)
A. pasteurianus			
LMG 1555	NCIB 8163	*A. pasteurianus*	
LMG 1686	LMD 31.4	*A. pasteurianus*	
LMG 1262^{T}t1*	LMD 22.1^{T}t1*	*A. pasteurianus*	Beer
LMG 1630	EQ	*A. pasteurianus*	Sugar cane bagasse, Brazil
LMG 1629	A	*A. pasteurianus*	Fermented *Agave sisalina* juice, Brazil
LMG 1658	MM 80	*A. pasteurianus*	Toddy palm, Burma
LMG 1659	MM 73	*A. pasteurianus*	Toddy palm, Burma
A. pomorum			
LMG 18848^T	LTH 2458^T	*A. pomorum*	Submerged cider vinegar fermentation, Germany
A. peroxydans			
LMG 1633	LMD 53.9	*A. pasteurianus*	Ditch water
LMG 1635^T	NCIB 8618^T	*A. peroxydans*	Ditch water, the Netherlands
A. lovaniensis			
LMG 1617^T	NCIB 8620^T	*A. lovaniensis*	Sewage on soil, Belgium
A. estunensis			
LMG 1626^T	NCIB 8935^T	*A. estunensis*	Cider, UK
LMG 1572	LMG E	*A. pasteurianus*	Cider, UK
LMG 1580	LMD 50.6	*A. estunensis*	Beer, the Netherlands
A. aceti			
LMG 1531	NCIB 8941	*A. aceti*	Quick vinegar, the Netherlands
LMG 1535	LMG Ch31	*A. aceti*	Vinegar plant, Belgium
LMG 1504^T	NCIB 8621^T	*A. aceti*	Beech-wood shavings of vinegar plant
LMG 1496	LMG 24WR	*A. aceti*	
***A. cerevisiae* sp. nov.**			
LMG 1625^T	NCIB 8894	*A. pasteurianus*	Beer (ale) in storage, Toronto, Canada
LMG 1599	NCIB 6425	*A. pasteurianus*	
LMG 1699	MARTIN 2	*A. pasteurianus*	Brewery, UK
LMG 1682	C101	*A. pasteurianus*	Beer, Ireland
***A. malorum* sp. nov.**			
LMG 1746^T	LMG 76.10	*A. pasteurianus*	Rotting apple, Ghent, Belgium
A. orleanensis			
LMG 1583^T	NCIB 8622^T	*A. orleanensis*	Beer, Belgium
LMG 1592	NCIB 2224	*A. orleanensis*	
LMG 1608	NCIB 8088	*A. orleanensis*	Beer
LMG 1545	IFO 3296	*A. orleanensis*	Film in fermenter of rice vinegar, Japan
A. indonesiensis			
LMG 19824^T	NRIC 0313^T	*A. indonesiensis*	Fruit of *Annona muricata*, Indonesia
LMG 1588	LMD 39.6	*A. indonesiensis*	
LMG 1571	LMD 39.2	*A. indonesiensis*	
A. tropicalis			
LMG 19825^T	NRIC 0312^T	*A. tropicalis*	Coconut juice, Indonesia
LMG 19826	NRIC 0321	*A. tropicalis*	Lime, Indonesia
LMG 1754	LMG 79.18	*A. pasteurianus*	Fruit of *Ficus capensis*, Ivory Coast
LMG 1663	592	*A. pasteurianus*	Fermenting putrefied meat sample, UK

* The type strain of *A. pasteurianus* shows two stable colonial variations, t1 and t2, that give identical protein profiles by SDS-PAGE.

respectively. To obtain DNA solutions free of RNA, a second RNase treatment was performed. RNase A was added to the DNA solutions to a final concentration of 100 μg ml^{-1} and incubated at 37 °C for 1 h. Finally, the degraded RNA was removed by chloroform extraction. The DNA was dissolved in 0·1 × SSC (0·15 M NaCl, 0·015 M

citric acid, 0·4 M NaOH, pH 7·0) to obtain a concentration of 0·3–0·8 mg ml^{-1}. DNA quantity and quality were determined by measuring the 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 was selected for further use. The size of the DNA was checked by agarose gel electrophoresis. Only high-molecular-mass DNA was used.

DNA–DNA hybridizations. DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) and Goris *et al.* (1998). Briefly, biotinylated probe DNA was sheared by ultrasonication, denatured and then hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 50 °C in a hybridization solution containing 50% formamide (2× SSC, 5× Denhardt's solution, 50% formamide, 2·5% dextran sulfate, low-molecular-mass denatured salmon sperm DNA to a final concentration of 100 μg ml^{-1}, 1·25 μg biotinylated probe DNA ml^{-1}). After 3 h, the hybridization solution was removed and streptavidin/β-D-galactosidase (Sigma) was added to the wells. After 10 min of incubation at 37 °C, the wells were washed and 4-methylumbelliferyl β-D-galactoside (Sigma) was added. The fluorescence intensity was measured with an HTS7000 Bio Assay reader (Applied Biosystems). Salmon sperm DNA (Sigma) was used as a negative control in all experiments. The DNA relatedness percentages presented are means, based on at least two independent hybridization experiments. Reciprocal reactions (e.g. A × B and B × A) were performed and also considered as independent hybridization experiments.

Determination of the DNA G + C content. The G + C content of DNA was determined by HPLC according to the method of Mesbah *et al.* (1989) using a Waters Symmetry Shield RP_8 column thermostatted at 37 °C. The solvent was 0·02 M $NH_4H_2PO_4$ with 1·5% acetonitrile (pH 4·0). Non-methylated phage lambda DNA (Sigma) was used as the calibration reference.

Sequencing of 16S rDNA. 16S rDNA was amplified using oligonucleotide primers complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer was 5′-AGAGTTTGATCCTGGCTCAG-3′ (hybridizing at positions 8–27, according to the *Escherichia coli* numbering system) and the reverse primer was 5′-AAGGAGGTGATCCAGCCGCA-3′ (hybridizing at positions 1541–1522). PCR products were purified using a QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions. Purified PCR products were sequenced by using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an Applied Biosystems 377 DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). The eight sequencing primers used are listed in Coenye *et al.* (1999). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems).

Phylogenetic analysis. The 16S rRNA gene sequences determined and sequences of strains belonging to the same phylogenetic group, retrieved from EMBL, were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the Bionumerics 1.01 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 500 bootstrap resamplings of the data. The strain numbers, species names and accession numbers of 16S rDNA sequences retrieved from EMBL for use in the phylogenetic analysis are presented in Fig. 1.

Phenotypic characterization. Cell shape and size were determined for cells grown aerobically at 28 °C for 2–4 days on medium 13. For the type strain of *A. pomorum*, cell shape and size were also determined for cells grown aerobically at 28 °C for 2 days on RAE agar (Sokollek & Hammes, 1997). Gram staining was carried out by the method of Hucker & Conn (1923). Oxidase activity was tested using 1% N,N,N′,N′-tetramethyl *p*-phenylenediamine (Kovacs, 1956). Catalase activity was tested by adding young cells to a drop of a 10% H_2O_2 solution and observing production of O_2. The production of 2- and 5-keto-D-gluconic acid was determined by the method described by Gosselé *et al.* (1980). The utilization of ammonium as the sole nitrogen source in the presence of ethanol as carbon source was tested using Frateur's modified Hoyer ethanol/vitamins medium (De Ley *et al.*, 1984) containing 2·5% agar. Growth was checked after 7 days incubation at 28 °C. Growth on the carbon sources glycerol, maltose and methanol was tested in basal medium (0·05% yeast extract, 0·3% vitamin-free Casamino acids, 2·5% agar) with the carbon source to be tested added at a final concentration of 0·3%. Medium without the carbon source was used as a control. Growth was evaluated after 7 days incubation at 28 °C. Growth in 30% D-glucose was tested in a medium containing 0·5% yeast extract and 30% D-glucose. Growth was checked after 7 days incubation at 28 °C under stationary conditions. Growth with *n*-propanol as carbon source was examined as described by Sokollek *et al.* (1998).

RESULTS AND DISCUSSION

DNA relatedness

The results of DNA–DNA hybridizations of all strains examined are shown in Table 2. DNA–DNA hybridization data revealed that four strains, LMG 1625^T, LMG 1599, LMG 1699 and LMG 1682, displayed a high level of DNA relatedness (66–85%) and low levels of relatedness to the known *Acetobacter* species. The name *Acetobacter cerevisiae* sp. nov. is proposed for this taxon. Strain LMG 1746^T showed DNA relatedness at an intermediate level (50–53%) to the *A. cerevisiae* strains and at low levels to the known *Acetobacter* species. The name *Acetobacter malorum* sp. nov. is proposed for this strain. The DNA–DNA hybridization data also revealed that the type strain of *A. pomorum*, LMG 18848^T, is related to strains of *A. pasteurianus* at an intermediate level (51–58%). The latter result clearly differs from the results obtained by Sokollek *et al.* (1998), who found only 17% DNA relatedness between the type strain of *A. pomorum*, LTH 2458^T, and the type strain of *A. pasteurianus*, DSM 3509^T. The discrepancy between these data could be explained by the fact that Sokollek *et al.* (1998), who used the membrane method, did not perform reciprocal reactions, which are important to obtain unequivocal results. The hybridization data also showed that four strains, LMG 1633, LMG 1572, LMG 1754 and LMG 1663, currently classified as *A. pasteurianus*, have DNA–DNA binding values of less than 22% to the type strain of *A. pasteurianus* and should be allocated to *A. peroxydans* (LMG 1633), *A. estunensis* (LMG 1572) and *A. tropicalis* (LMG 1754, LMG 1663).

Table 2. DNA–DNA binding values and G+C content of *Acetobacter* strains studied

Mean standard deviation of DNA–DNA hybridization is ±7% (see Goris *et al*., 1998).

Strain	G+C content (mol%)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
A. pasteurianus																																			
1. LMG 1555	54.3	100																																	
2. LMG 1686	53.5		100																																
3. LMG 1262^T t1	53.4	78	82	100																															
4. LMG 1630	53.7		79	71	100																														
5. LMG 1629	53.6		90	70		100																													
6. LMG 1658	53.2		66	62	59		100																												
7. LMG 1659	53.7			69			66	100																											
A. pomorum																																			
8. LMG 18848^T	52.1	51	58	53	51	55		55	100																										
A. peroxydans																																			
9. LMG 1633	60.7			6					9	100																									
10. LMG 1635^T	59.7			13					23	73	100																								
A. lovaniensis																																			
11. LMG 1617^T	57.4		21	14		18			20	17	16	100																							
A. estunensis																																			
12. LMG 1626^T	59.2			20					13	9		10	100																						
13. LMG 1572	60.2	19	21	21	19	3			12	7		9	98	100																					
14. LMG 1580	59.5												92	92	100																				
A. aceti																																			
15. LMG 1531	58.3		16	18		6			12			16				100																			
16. LMG 1535	57.0		14	10		8			8			14				59	100																	
17. LMG 1504^T	56.9	7	8	7	9	6	5	6	6	10		13	17	17		53	100	100																	
18. LMG 1496	56.9		6	9		7			7			12				55	104	95	100															
***A. cerevisiae* sp. nov.**																																			
19. LMG 1625^T	57.6		37	32		19			27	10		11	15	17		18	16	12	15	100														
20. LMG 1599	56.8			30					23				25					11		77	100													
21. LMG 1699	56.0			35					24				27					8		74	76	100												
22. LMG 1682	57.4		22	32		13			19			18	15			18	10	9	9	85	80	66	100											
***A. malorum* sp. nov.**																																			
23. LMG 1746^T	57.2	10	11	7	8	6			9	7		9	5	5				3		53	50			100										
A. orleanensis																																			
24. LMG 1583^T	55.7			12					12	11		10	6	7				4		33	37	37	40	28	100									
25. LMG 1592	58.0*																								90*	100								
26. LMG 1608	58.1*																								98*		100							
27. LMG 1545	57.3*																								77*	89*	85*	100						
A. indonesiensis																																			
28. LMG 19824^T	54.0																			18									100					
29. LMG 1588	54.2			17					16	7		15	13	18				9		20	26	32	20	10	12				88*	100				
30. LMG 1571	54.1																												85*	83	100			
A. tropicalis																																			
31. LMG 19825^T	56.0																			34									18			100		
32. LMG 19826	55.6																												19			81	100	
33. LMG 1754	56.2			18			11	12										7															100	
34. LMG 1663	55.9		22	20		14	6	14	10	6		14	13	13		10	10	9	8	27			24	12	14				20	17		86	93	76	100

*Data taken from Lisdiyanti *et al.* (2000).

Table 3. Characteristics that differentiate the species of the genus *Acetobacter*

Taxa are listed as: 1, *A. cerevisiae* sp. nov. ($n = 4$); 2, *A. malorum* sp. nov. ($n = 1$); 3, *A. pasteurianus* ($n = 7$); 4, *A. pomorum* ($n = 1$); 5, *A. peroxydans* ($n = 2$); 6, *A. lovaniensis* ($n = 1$); 7, *A. orleanensis* ($n = 4$); 8, *A. indonesiensis* ($n = 2$; strains LMG 1588 and LMG 1571); 9, *A. tropicalis* ($n = 2$; strains LMG 1754 and LMG 1663); 10, *A. estunensis* ($n = 3$); 11, *A. aceti* ($n = 4$). Characters are scored as: +, positive; v, variable; −, negative. Abbreviation: YE, yeast extract.

Feature	1	2	3	4	5	6	7	8	9	10	11
Formation from D-glucose:											
5-Keto-D-gluconic acid	−	−	−	−*	−	−	−	−	−	−	+
2-Keto-D-gluconic acid	+	+	v (2+, −)†	−*	−	+	+	+	+	+	+
Growth on ammonium with ethanol	−	−	−	−	+	+	−	−	−	+	+
Growth on carbon sources:											
Glycerol	+	+	v (2+, −)	+	−	+	+	+	+	v (1+, +)	+
Maltose	−	−	v (2+, −)	−	+	−	v (1+, −)	+	+	−	v (1+, −)
Methanol	−	+	−	−	−	+	−	−	−	−	−
Growth on YE + 30% D-glucose	−	+	v (1+, −)	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	−	+	+	+	+	+	+
G+C content of DNA (mol%)	56·0–57·6	57·2	53·2–54·3	52·1	59·7–60·7	57·1–58·9‡	55·7–58·1	54·0–54·2	55·6–56·2	59·2–60·2	56·9–58·3

* Data taken from Sokollek *et al.* (1998).

† For variable characters, the number of strains testing positive and the result for the type strain are given in parentheses.

‡ Data taken from Lisdiyanti *et al.* (2000).

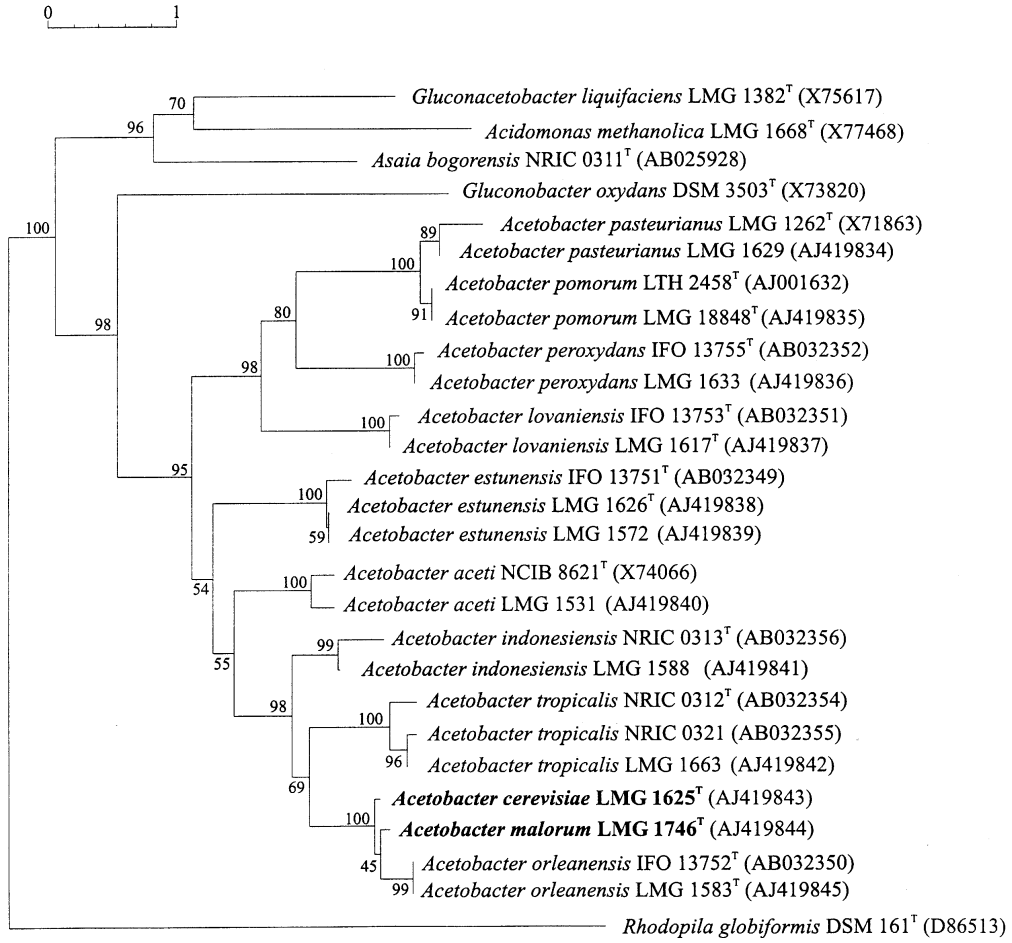

Fig. 1. Neighbour-joining tree reflecting the phylogenetic position of *A. cerevisiae* sp. nov. LMG 1625^T and *A. malorum* sp. nov. LMG 1746^T within the acetic acid bacteria based on almost complete 16S rRNA gene sequences. *Rhodopila globiformis* DSM 161^T was used as an outgroup in this analysis. Bar, 1% sequence dissimilarity. Numbers at branching points indicate bootstrap percentages derived from 500 samples.

DNA G+C content

The G+C contents of the *Acetobacter* strains studied are given in Table 2 and ranged from 52·1 to 60·7 mol%, whereas the G+C range of a species was limited to 2·4 mol%. The latter result shows that the G+C content of *Acetobacter* species is no longer too broad for a species, as was previously the case, especially for *A. pasteurianus*, which had a G+C range of 9·7 mol% (Gosselé *et al.*, 1983b; Swings *et al.*, 1992). Analogous results were obtained by Lisdiyanti *et al.* (2000). The G+C content of the strains belonging to *A. cerevisiae* varied from 56·0 to 57·6 mol%. The *A. malorum* strain LMG 1746^T had a G+C content of 57·2 mol%. The distribution of G+C content for each *Acetobacter* species is given in Table 3.

Phylogenetic analysis based on 16S rRNA gene sequences

The nearly complete 16S rRNA gene sequences (1436–1444 nucleotides) of *A. cerevisiae* LMG 1625^T, *A. malorum* LMG 1746^T and ten strains representing the known *Acetobacter* species were determined and compared with deposited 16S rRNA gene sequences of strains of the known *Acetobacter* species and of the type species of the other genera in the family *Acetobacteraceae*. A phylogenetic tree, reflecting the positions of these strains within the acetic acid bacteria lineage, was generated using the neighbour-joining method and is shown in Fig. 1. Bootstrap values supported the topology of the tree. The tree showed that the genus *Acetobacter* formed two major rRNA groups, one containing the species *A. pasteurianus*, *A. pomorum*, *A. peroxydans* and *A. lovaniensis* and the second containing *A. cerevisiae* LMG 1625^T, *A. malorum* LMG 1746^T and the species *A. estunensis*, *A. aceti*, *A. indonesiensis*, *A. tropicalis* and *A. orleanensis*. It is noteworthy that the *Acetobacter* species within each rRNA group showed more than 97·2% 16S rDNA sequence similarity and that strains belonging to the same *Acetobacter* species showed more than 99·6% 16S rDNA sequence similarity. *A. cerevisiae* LMG 1625^T and *A. malorum* LMG 1746^T showed 99·9% 16S rDNA sequence similarity, indicating that

they are phylogenetically very closely related. Both strains shared 99·6 % 16S rDNA sequence similarity to *A. orleanensis*. Somewhat lower values were observed to members of *A. estunensis*, *A. aceti*, *A. indonesiensis* and *A. tropicalis* (97·6–98·9 %). The 16S rDNA sequence of *A. pomorum* LMG 18848^T was compared to the sequence deposited for *A. pomorum* LTH 2458^T (EMBL accession no. AJ001632). The two sequences were 100 % identical over 1440 bases, reinforcing the assumption that we used the same strain as Sokollek *et al.* (1998).

Phenotypic characteristics

Table 3 gives characteristics useful in the differentiation of the species of the genus *Acetobacter*. *A. cerevisiae* and *A. malorum* could not be differentiated from the known *Acetobacter* species by an exclusive phenotypic characteristic. However, the combination of growth on methanol as a carbon source, the inability to grow on ammonium as a nitrogen source with ethanol as the carbon source and the ability to grow on 30 % D-glucose allowed the differentiation of *A. malorum* from the other *Acetobacter* species. *A. cerevisiae* could be differentiated from the species *A. pomorum*, *A. peroxydans*, *A. lovaniensis*, *A. indonesiensis*, *A. tropicalis*, *A. estunensis* and *A. aceti* by the combination of the following phenotypic characteristics: production of 2-keto-D-gluconic acid (but not 5-keto-D-gluconic acid) from D-glucose, the inability to grow on ammonium as a nitrogen source with ethanol as the carbon source and the inability to grow on maltose as a carbon source. It is important to mention that some strains of *A. pasteurianus*, *A. pomorum*, *A. orleanensis*, *A. indonesiensis* and *A. tropicalis*, even without the addition of *A. cerevisiae*, have phenotypic characteristics that are similar to one another. For identification of these strains to the species level, genotypic characterization (as determining DNA similarity) is required.

The type strains of *A. pomorum* and *A. pasteurianus* were evaluated for growth in the presence of 30 % D-glucose, growth on methanol as carbon source and growth on *n*-propanol with ammonium as the sole nitrogen source, three features useful in the differentiation of the two species according to Sokollek *et al.* (1998). In our hands, however, strain LMG 18848^T could not be distinguished from the type strain of *A. pasteurianus* by any of these features.

Some controversial reports exist on the growth of *Acetobacter* strains on mannitol agar (Gosselé *et al.*, 1983b; Franke *et al.*, 1999; Boesch *et al.*, 1998; Yamada *et al.*, 1997; Yamada, 2000; Lisdiyanti *et al.*, 2000). In our hands, all *Acetobacter* strains grew on medium 13 (Janssens *et al.*, 1998), also known as YPM agar (0·5 % yeast extract, 0·3 % peptone, 2·5 % D-mannitol and 1·5 % agar), although in most cases this growth was not abundant. Medium 13 is, however, very useful to maintain *Acetobacter* strains viable on plates for a more extended period.

In conclusion, on the basis of DNA–DNA reassociation values, DNA base compositions, phylogenetic relationships and phenotypic characteristics, this study revealed the existence of two novel *Acetobacter* species, *A. cerevisiae* (type strain LMG 1625^T) and *A. malorum* (type strain LMG 1746^T). It also showed that four *A. pasteurianus* strains should be renamed: LMG 1633 should be allocated to *A. peroxydans*, LMG 1572 should be allocated to *A. estunensis* and LMG 1754 and LMG 1663 should be assigned to *A. tropicalis*.

Furthermore, this study confirms that *Acetobacter* strains should be classified and identified mainly on the basis of genotypic characteristics, as identification on the basis of phenotypic tests does not always lead to clear-cut results. The results above show that DNA–DNA hybridization is a recommended technique for accurate identification of *Acetobacter* strains.

Description of *Acetobacter cerevisiae* sp. nov.

Acetobacter cerevisiae (ce.re.vi′si.a.e. L. fem. gen. n. *cerevisiae* of beer, referring to the source from which most strains have been isolated).

Cells are Gram-negative, ellipsoidal to rod-shaped, approximately 1·0 × 2·0–4·0 μm in size, occurring singly, in pairs or occasionally in short chains. Involution forms like swollen cells occur in some strains. Cells are non-motile. Endospores are not detected. Colonies are beige to brown, round, regular to wavy, raised and smooth with a diameter of 0·3–0·5 mm on YPM agar. Obligately aerobic. Oxidase-negative. Catalase-positive. Characterized by the combination of the following phenotypic features: 2-keto-D-gluconic acid is produced from D-glucose, 5-keto-D-gluconic acid is not produced from D-glucose, no growth with ammonium as the sole nitrogen source on ethanol as carbon source, no growth on maltose or methanol as carbon source (Table 3). The range of G+C content of the DNA is 56·0–57·6 mol %. The type strain is LMG 1625^T (= DSM 14362^T = NCIB 8894^T = ATCC 23765^T), which has a G+C content of 57·6 mol % and was isolated from beer (ale) in storage at Toronto, Canada (Kozulis & Parsons, 1958).

Description of *Acetobacter malorum* sp. nov.

Acetobacter malorum (ma.lo′rum. L. neut. gen. pl. n. *malorum* of apples, referring to the isolation of the type strain from a rotting apple).

Cells are Gram-negative, ellipsoidal, approximately 0·9 × 1·1–1·3 μm in size, occurring singly or in pairs. Cells are non-motile. Endospores are not detected. Colonies are beige, round, regular to wavy, convex and smooth with a diameter of 0·5 mm on YPM agar. Obligately aerobic. Oxidase-negative. Catalase-positive. Characterized by the combination of the following phenotypic features: 2-keto-D-gluconic acid is produced from D-glucose, 5-keto-D-gluconic acid is

not produced from D-glucose, no growth with ammonium as the sole nitrogen source on ethanol as carbon source, no growth on maltose as carbon source, growth on methanol as carbon source (Table 3). The type strain is LMG 1746^T (= DSM 14337^T), which has a G+C content of 57·2 mol% and was isolated from a rotting apple in Ghent, Belgium (Gosselé *et al.*, 1983a, b).

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