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Komagataeibacter cocois sp. nov., a novel cellulose-producing strain isolated from coconut milk

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Abstract

Phylogenetic analysis was performed on a cellulose-producing strain, designated WE7^T, isolated from contaminated coconut milk. The analysis utilized nearly complete 16S rRNA gene sequences, as well as concatenated partial sequences of the housekeeping genes *dna*K, *gro*EL and *rpo*B, and allowed identification of the strain as belonging to the genus *Komagataeibacter*. DNA–DNA correlation or average nucleotide identity analysis was performed between WE7^T and its closest phylogenetic neighbours, and the resulting values were below the species level (<70 % and <95 %), suggesting that the strain represents a novel species in genus *Komagataeibacter*. Strain WE7^T was coupled with *Komagataeibacter* species more tightly than with *Gluconacetobacter* species in a 16S rRNA gene sequence phylogenetic tree. Strain WE7^T can be differentiated from closely related *Komagataeibacter* and *Gluconacetobacter entanii* species by the ability to grow on the carbon sources D-mannitol, sodium D-gluconate and glycerol, the ability to form acid by D-fructose, sucrose, D-mannitol, D-galactose and ethanol, and the ability to grow without acetic acid. The major fatty acid of WE7^T is $C_{18:1}\omega 9c$ (52.3 %). The DNA G+C content of WE7^T is 63.2 mol%. The name *Komagataeibacter cocois* sp. nov. is proposed, with the type strain WE7^T (=CGMCC 1.15338^T=JCM 31140^T).

Many bacterial cellulose-producing strains have been characterized and mostly are classified as part of the genera *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter* of the family *Acetobacteraceae* [1]. The members of this family are obligate aerobes with the ability to convert ethanol to acetic acid, allowing them to grow at low pH levels [2]. The genus *Gluconacetobacter* is subdivided into two groups, the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group [3]. Species belonging to the *Gluconacetobacter xylinus* group were classified into the novel genus *Komagataeibacter* in 2013 [4]. However, *Gluconacetobacter entanii* was not included in the genus *Komagataeibacter*, since the type strain is not available in any culture collections including the German Collection of Microorganisms and Cell Cultures (DSMZ) [5, 6].

There are several differences between the genera *Komagataeibacter* and *Gluconacetobacter*. The genus *Komagataeibacter* is characterized morphologically by a lack of motility and a lack of flagellation. *Komagataeibacter* strains are unable to produce 2,5-diketo-D-gluconate, γ-pyrone compounds, and a water-soluble brown pigment from glucose, but *Gluconacetobacter* strains have peritrichous flagella and good motility, produce a water-soluble brown pigment when grown on glucose/yeast extract/calcium carbonate medium, and produce 2,5-diketo-D-gluconate and γ-pyrone compounds from D-glucose [6]. Both *Komagataeibacter* and *Gluconacetobacter* strains produce acetic acid from ethanol, oxidize acetate and lactate to carbon dioxide and water, and can grow in the presence of 0.35 % acetic acid (v/v). The genus *Gluconacetobacter* is plant-associated, and strains of this genus have mostly been isolated from flowers, fruits, sugarcane and coffee plants, but *Komagataeibacter* strains are not plant-associated, and have been isolated mostly from fermented foods such as vinegar, nata de coco and tea fungus beverages [7–12]. Currently, 14 named species are classified in the genus *Komagataeibacter* [4, 6, 13, 14], some of which are not cellulose-producing strains, such as *Komagataeibacter hansenii* and *Komagataeibacter europaeus*, and some are cellulose-producing strains, such as

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Keywords: *Komagataeibacter cocois* sp. nov.; acetic acid bacteria; cellulose-producing strain; coconut milk .

Abbreviations: ANI, Average Nucleotide Identity; CICC, China Centre of Industrial Culture Collection; HF, Hoyer–Frateur; HPLC, High Performance Liquid Chromatography; LC/ MS, Liquid Chromatograph Mass Spectrometer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *dnaK*, *groEL* and *rpoB* sequences of *Komagataeibacter cocois* WE7^T are KR998072, KR998073, KR998074 and KR998075, respectively.

Two supplementary figures are available with the online version of this article.

Komagataeibacter xylinus, *Komagataeibacter nataicola* and *Komagataeibacter rhaeticus* [15].

Strain WE7^T was isolated from a gel pellicle sample of naturally fermented coconut milk, and primary isolation and purification were on Hestrin–Schramm (HS) media at 28 °C [16]. The type strains *Komagataeibacter maltaceti* LMG 1529^T, *K. nataicola* JCM 25120^T and *Komagataeibacter swingsii* JCM 17123^T were selected as reference strains.

Gram-staining was performed as described by Cristina *et al.* (2013) [17]. Cell morphology was observed under an optical microscope (×100) and transmission electron microscopy (×4000), with cells grown for 3d at 28 °C on HS medium. The ability to use different carbon sources and the ability to form acid were analysed as previously reported [17, 18]. The over-oxidation of acetic acid to carbon dioxide and water was analysed by detection of a colour change from yellow to blue on medium containing 2 % ethanol, 0.5 % peptone, 0.5 % yeast extract, 0.27 % Na_2HPO_4, 0.008 % bromothymol blue and 1.3 % agar [17].

The ability to use D-glucose, D-mannitol and ethanol as sole carbon sources was analysed on Hoyer–Frateur (HF) medium with ammonium as the sole nitrogen source (0.1 % $(NH_4)_2SO_4$, 0.09 % KH_2PO_4, 0.01 % K_2HPO_4, 0.025 % $MgSO_4{\cdot}7H_2O$, 0.002 % $FeCl_3{\cdot}6H_2O$, 3 % carbon source, 1.5 % agar, pH 6.8) and on Asai medium (0.3 % $(NH_4)_2SO_4$, 0.3 % KH_2PO_4, 0.2 % $MgSO_4{\cdot}7H_2O$, 0.5 % carbon source, pH 6.8) [18, 19]. Growth without acetic acid and ethanol and the ability to grow at different concentrations of acetic acid and ethanol were analysed in RAE medium [20]. The production of 2- and 5-keto-D-gluconic acid was determined by triple quadrupole liquid chromatography–mass spectrometry. The production of cellulose was checked by boiling the pellicles in 5 % NaOH after harvesting the pellet from RAE media (5 % glucose, 0.5 % yeast extract) [5, 21].

Respiratory quinone measurements [determined by high-performance liquid chromatography (HPLC) at the China Centre of Industrial Culture Collection (CICC)] and fatty acid measurements [18, 22] were performed as described previously. Strains were cultivated as described by Slapšak *et al.* and fatty acid methyl esters were measured as reported by O'Fallon *et al.* [22].

Genomic DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen) following the manufacturer's instructions. The 16S rRNA gene sequence of strain WE7^T was amplified with primers 7F and 1540R using genomic DNA as template. The *dna*K, *gro*EL and *rpo*B sequences were determined by using the primers and conditions reported previously [23]. The purified PCR-amplified genes were sequenced by using Applied Biosystems 3730XL apparatus (BigDye Terminator version 3.1 Cycle Sequencing Kit, Life Technologies).

The sequences were compared with the 16S rRNA, *dna*K, *gro*EL and *rpo*B gene sequences of the reference strains of acetic acid bacteria taxa collected from the NCBI database and the EzTaxon server database (www.ezbiocloud.net/) [24]. Phylogenetic trees were reconstructed using the MEGA 5.0 software package using the neighbour-joining method. The robustness of the branches was evaluated by bootstrap analysis. Numbers at branching points indicate bootstrap percentage values based on 1000 replications. The bootstrap value of WE7^T was a high value (shown in the phylogenetic trees).

The genomic correlation between *K. maltaceti* NBRC 14815^T and strain WE7^T was determined by DNA–DNA hybridization carried out with denaturalization in liquid phase for molecular hybridization (as tested by the CICC) [25, 26]. Average nucleotide identity (ANI) analysis was done by using the draft genome of WE7^T (unpublished) and the genomes of some strains belonging to genus *Komagataeibacter* (Table 1) using Jspecies [27]. The DNA base composition of WE7^T was determined by HPLC in the CICC [28].

The nearly complete 16S rRNA gene sequence (1454 bp) of strain WE7^T was amplified and sequenced. In the 16S rRNA-based phylogenetic tree (Fig. 1a), the novel proposed species WE7^{T}coupled tightly with *G. entanii* LTH 4560^T (99.7 %) [5]. Similar clustering was observed in a 16S rRNA gene sequence phylogenetic tree derived from the maximum-likelihood and neighbour-joining methods, and the same results were obtained from the replicates. Strains belonging to the *Komagataeibacter* group share high 16S rRNA identity, from 98.2 to 100 %, making it difficult to identify strains on a species level based only on the results of the 16S rRNA sequence. As an additional complication, the type strain of *G. entanii* is not available in any culture collection, including the DSMZ [6], and was originally isolated from submerged high-acid industrial vinegar fermentation by Schuller [5].

Recently, multilocus sequence analysis based on concatenated partial *dna*K (encoding a heat-shock protein), *gro*EL (encoding a chaperonin protein) and *rpo*B (encoding the β-subunit of bacterial RNA polymerase) gene sequences was reported as a useful method for phylogeny and species differentiation of acetic acid bacteria [23, 29–32]. Among genus *Komagataeibacter*, the values of the *dna*K, *gro*EL and *rpo*B genes between different species were usually below 99.0, 99.2, and 98.8 %, respectively [18, 23].

To refine the taxonomic position of strain WE7^T, phylogenetic analyses were performed using partial sequences of the housekeeping genes *dna*K (Fig. S1a, available in the online version of this article), *rpo*B (Fig. S1b) and *gro*EL (Fig. S1c), and concatenated partial sequences of 16S rRNA, *dna*K, *rpo*B and *gro*EL (Fig. 1b). Phylogenetic analyses based on the partial 16S rRNA, *dna*K, *gro*EL and *rpo*B gene sequences indicated that strain WE7^T belongs to the a single novel species in the genus *Komagataeibacter*, with sequence similarities of 96.2 % for *dna*K, 91.2 % for *gro*EL and 91.7 % for *rpo*B with these species in the genus *Komagataeibacter* [23].

Thus, the sequences suggested that strain WE7^T formed a separate branch from the known species of this group (Figs 1b and S1), and the same results were obtained from

Table 1. ANI values between *Komagataeibacter cocois* sp. nov. and closely related members of the genus *Komagataeibacter*

Genome A, means the genome of WE7^T; Genome B, the genome of reference strain.

Genomes	Accession number	DNA G+C content (mol%)	OrthoANIu value (%)	Average aligned length (bp)	Genome A coverage (%)	Genome B coverage (%)
Komagataeibacter cocois WE7^T	QEXL00000000	62.7	100	3406 946	100	100
Komagataeibacter maltaceti LMG 1529^T	GCA_002906255.1	63.2	84.29	2 411 062	70.77	67.41
Komagataeibacter hansenii JCM 7643^T	NZ_BAIO00000000	62.1	82.66	1 591 534	47.24	45.48
Komagataeibacter hansenii ATCC 23769	NZ_CP021467	61.7	82.63	1 549 301	45.99	43.2
Komagataeibacter kombuchae LMG 23726	GCA_001938755.1	59.40	81.63	2 203 427	61.34	63.11
Komagataeibacter kakiaceti JCM 25156	NC_016027	60.6	79.60	1 018 937	30.24	38.78
Komagataeibacter europaeus LMG 18890^T	NZ_CM000920	59.4	79.02	1 374 392	40.79	33.69
Komagataeibacter xylinus E25	NZ_BANE00000000	59.3	79.02	1 374 392	40.79	33.69
Komagataeibacter oboediens 174Bp2	NZ_BANF00000000	61.6	78.62	1 353 503	40.17	33.15
Komagataeibacter europaeus SRCM101446	NZ_BCTP00000000	62.3	78.61	1 346 319	39.96	35.47
Komagataeibacter rhaeticus AF1	NZ_JDTI00000000	62.5	78.53	1 286 646	38.19	33.6
Komagataeibacter medellinensis NBRC 3288	NZ_CP004360	62.1	77.97	1 271 935	37.75	36.24
Komagataeibacter intermedius TF2	NZ_CADT00000000	61.3	77.76	1 272 207	37.76	35.44
Komagataeibacter xylinus NBRC 15237^T	NZ_CP019875	62.4	77.59	1 259 208	37.38	35.53
Komagataeibacter nataicola RZS01	NZ_CADP00000000	61.3	77.38	1 308 041	38.83	34.82

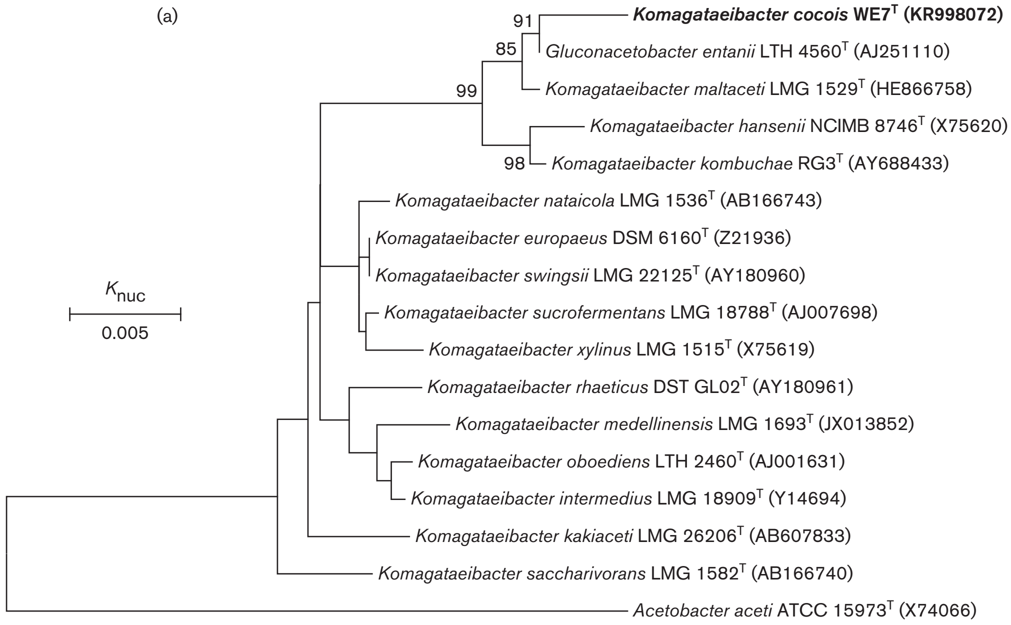
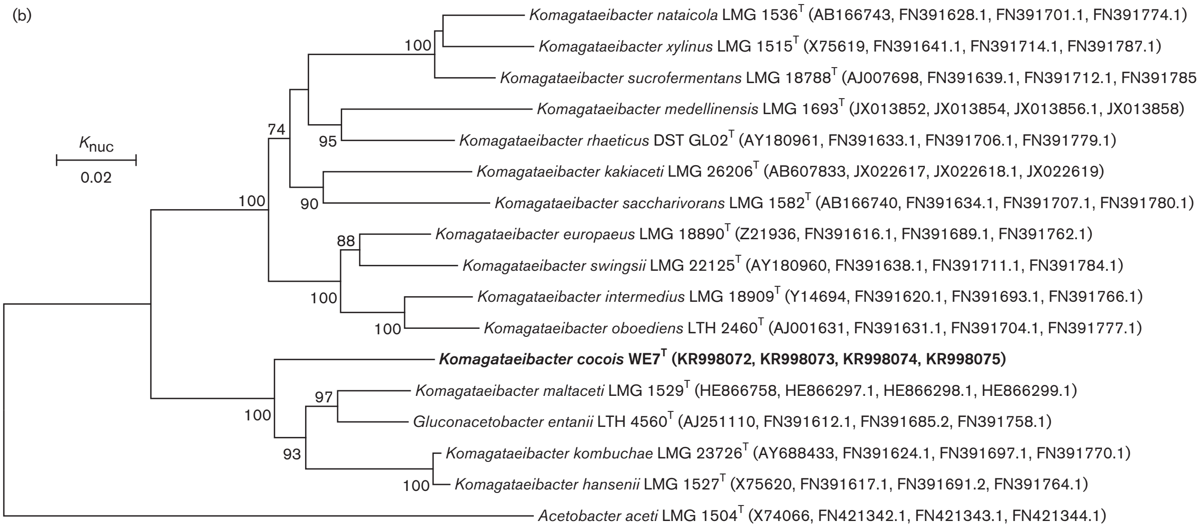


Fig. 1. Phylogenetic trees reflecting the relationships between *Komagataeibacter cocois* sp. nov. and type strains of the known species of the genus *Komagataeibacter*. The trees based on the 16S rRNA sequences (a) and concatenated partial sequences of 16S rRNA, *dna*K, *rpo*B and *gro*EL (b) are reconstructed by the neighbour-joining method. The type strain of *Acetobacter aceti* is included as an out-group. Bootstrap values are indicated at branching points.

Fig. 1. (cont.)



the replicates. According to previous phylogenetic analysis of 16S rRNA, *G. entanii* LTH 4560^{T} was in a branch with *Komagataeibacter* species. The physiological and biochemical characteristics of *G. entanii* LTH 4560^{T} were also consistent with *Komagataeibacter* [14]. However, because no samples of *G. entanii* LTH 4560^{T} are available, it remains classified as part of the genus of *Gluconacetobacter*.

K. maltaceti NBRC 14815^{T} and WE7^{T} exhibited 45.2 % DNA–DNA correlation, with values below the species level (<70 %) [33, 34]. The genome ANI values between WE7^{T} and *K. maltaceti* LMG 1529^{T}, *K. europaeus* SRCM101446, *K. europaeus* LMG 18890^{T}, *K. xylinus* E25, *K. xylinus* NBRC 15237^{T}, *K. intermedius* TF2, *K. hansenii* ATCC 23769, *K. hansenii* JCM 7643^{T}, *K. kombuchae* LMG 23726, *K. medellinensis* NBRC 3288, *K. rhaeticus* AF1, *K. kakiaceti* JCM 25156, *K. oboediens* 174Bp2 and *K. nataicola* RZS01 were determined and are shown in Table 1. All the ANI values were below the species level (<95 %) [27]. The hybridization and ANI values indicate that the isolate was a different species from these strains.

The DNA G+C content of WE7^{T} is 63.2 mol%, within the 55.8–63.4 % range reported for species belonging to the genus *Komagataeibacter* [5, 18, 35, 36].

Cells of WE7^{T} were Gram-negative rods, 0.4–0.5 µm wide and 1.6–3.5 µm long (Fig. S2), and aerobic, non-motile, catalase-positive and oxidase-negative. The colonies were smooth, shiny, beige and with a diameter of approximately 0.5 mm after 3 days of growth. Differential phenotypic characteristics of the proposed novel species and the other phylogenetically closely related species in the genus *Komagataeibacter* are listed in Table 2.

The major ubiquinone of WE7^{T} was Q-10, the same as other strains in the genus *Komagataeibacter,* as reported previously [18, 36, 37]. The whole-cell fatty acid composition of WE7^{T} grown in RAE broth without ethanol or acetic acid was analysed (Table 2) [20]. The major fatty acid of WE7^{T} was $C_{18:1}\omega 9c$ (52.3 %), and the minor fatty acids (above 4 %) were $C_{17:0}$ (9.9 %), $C_{16:0}$ (9.1 %) and $C_{18:0}$ (5.0 %). The major fatty acids of *K. swingsii* JCM 17123^{T} identified were $C_{18:1}\omega 7c$ (71.9 %), $C_{16:0}$ (13.1 %) and $C_{18:0}$ (4.4 %), as determined previously [7].

In contrast to *G. entanii* LTH 4560^{T}, WE7^{T} grew well in RAE medium without ethanol and acetic acid. Additionally, the ability to grow on the carbon sources D-mannitol, D-gluconate and glycerol, and the ability to form 2-keto-D-gluconic acid from D-glucose differentiated WE7^{T} from the closely related species *G. entanii* LTH 4560^{T}. Strain WE7^{T} exhibited resistance to 3 % acetic acid in the presence of 3 % ethanol, and *K. maltaceti* LMG 1529^{T} is resistant to 2 % acetic acid. In contrast to *K. hansenii* LMG 1527^{T}, strain WE7^{T} could form acid from D-fructose, sucrose, D-mannitol and ethanol. Using the phenotypic tests listed in Table 3, strain WE7^{T} can be differentiated from its phylogenetic neighbours.

In conclusion, the results of this polyphasic study demonstrate that strain WE7^{T} represents a novel *Komagataeibacter* species that can be distinguished from its nearest phylogenetic neighbours. Therefore, we propose classification of the strain as *Komagataeibacter cocois* sp. nov. This is the first novel species of genus *Komagataeibacter* since the combination of this genus.

DESCRIPTION OF *KOMAGATAEIBACTER COCOIS* SP. NOV.

Komagataeibacter cocois (co.co′is. N.L. fem. gen. n. *cocois* of Cocos, referring to the origin of contaminated coconut milk from which the type strain was isolated).

Table 2. The physiology and biochemistry characteristics of CGMCC 1.15338 and reference strains

Strains: 1, WE7^T; 2, *K. maltaceti* LMG 1529^T; 3, *K. nataicola* JCM 25120^T; 4, *G. entanii* LTH 4560^T; 5, *K. hansenii* LMG 1527^T; 6, *K. kombuchae* RG3^T; 7, *K. xylinus* JCM 7644^T. +, Positive; –, negative; W, reduced growth; V, variable; ND, no data available. Strains 4–7, data from Dutta *et al* [38] Lisdiyanti *et al.* [36]; Schuller *et al.* [5] and Slapsak *et al.* [18].

Characteristic	1	2	3	4	5	6	7
Motility	–	–	–	–	–	+	–
Formation from D-glucose:							
2-Keto-D-gluconic acid	+	+	+	–	+	–	+
5-Keto-D-gluconic acid	+	+	+	–	+	+	+
Growth on carbon sources:							
Glycerol	+	+	+	–	+	+	+
D-Xylose	W	+	+	ND	+	–	W
D-Mannitol	–	+	+	–	+	+	+
D-Galactitol	–	+	–	ND	+	–	–
D-Gluconate	+	+	+	–	+	W	+
1-Propanol	–	+	–	+	+	ND	–
Acid production on carbon sources:							
D-Fructose	+	+	+	ND	–	ND	+
D-Mannitol	+	–	+	ND	–	ND	+
1-Propanol	+	+	+	ND	–	ND	–
Maltose	–	–	+	ND	–	ND	+
Sorbitol	–	–	–	ND	–	ND	+
Sucrose	+	–	+	ND	–	ND	+
Ethanol	+	+	+	ND	–	ND	–
Utilization of ammoniacal N2 in:							
HF medium with:							
D-Mannitol	+	–	+	ND	+	ND	+
Ethanol	–	–	+	ND	–	ND	–
Asai medium with:							
D-Mannitol	+	–	+	ND	+	ND	+
Ethanol	–	–	+	ND	–	ND	–
Growth in AE (4a/3e) broth	–	–	–	+	–	–	–
Growth on RAE medium in the absence of ethanol and acetic acid	+	+	–	–	+	+	+
Growth on RAE medium in the presence of 1 % ethanol and acetic acid at:							
1.0 %	+	+	+	ND	+	ND	+
2.0 %	+	+	–	ND	+	ND	+
3.0 %	–	–	–	ND	–	ND	+
4.0 %	–	–	–	ND	–	ND	–
Growth on RAE medium in the presence of 3 % ethanol and acetic acid at:							
0 %	+	+	+	–	+	ND	+
1.0 %	+	+	–	ND	+	ND	+
2.0 %	+	+	–	ND	+	ND	+
3.0 %	+	–	–	ND	–	ND	+
4.0 %	–	–	–	+	–	ND	+
Cellulose production	+	–	+	–	V	+	+

Cells are Gram-negative rods, 0.4–0.5 µm wide, 1.6–3.5 µm long, aerobic, non-motile, catalase-positive and oxidase-negative. Colonies are smooth, shiny, beige and have a diameter of approximately 0.5 mm after 3 days of growth. The strain grows on HF and Asai media containing D-glucose or mannitol, but not on either medium containing ethanol. The strain oxidizes acetate, weakly oxidizes ethanol, and produces cellulose, gluconic acid, and 2-and 5-keto-D-gluconic from D-glucose. The strain grows at 15–37 °C and at pH 3.0–6.5, but not at pH 2.5 or 7.0. Acetic acid and ethanol are not required for growth. Growth is observed with 3 % acetic acid in the presence of 3 % ethanol. Acid is produced from D-fructose, D-mannose, sucrose, galactose, D-glucose, raffinose, D-xylose, n-propyl alcohol, n-butyl

Table 3. Cellular fatty acid compositions of *Komagataeibacter cocois* sp. nov. and closely related members of the genus *Komagataeibacter*

Strains: 1, *K. cocois* sp. nov. WE7^T; 2, *K. maltaceti* LMG 1529^T; 3, *K. nataicola* JCM 25120^T; 4, *K. swingsii* JCM 17123^T; 5, *K. europaeus* JCM 16935^T; 6, *K. xylinus* JCM 7644^T; 7, *K. kakiaceti* LMG 26206^T; 8, *K. medellensis* LMG 1693^T; Strains 1 and 4, data taken from this study; 2, 3 and 5–8, data from Iino *et al*. [35]. Fatty acids comprising <1 % of the total are not shown. ND, no data available.

Fatty acid (%)	1	2	3	4	5	6	7	8
Saturated straight-chain:								
$C_{12:0}$	–	–	2.0	–	–	–	–	–
$C_{14:0}$	4.2	4.4–5.5	5.8	4.3	3.2	3.8	3.7	4.1
$C_{16:0}$	9.1	6.9–8.5	14.5	13.1	14.3	23.1	9.9	8.6
$C_{17:0}$	9.9	ND	–	3.2	–	–	7.2	ND
$C_{18:0}$	5.0	ND	3.7	4.4	3.8	4.1	2.8	1.0
Unsaturated straight-chain:								
$C_{13:1}$ AT 12–13	–	–	1.8	–	1.4	–	–	–
$C_{18:1}\omega 7c$	–	60.2–64.8	65.8	71.9	75.2	67.4	53.9	62.1
$C_{18:1}\omega 9c$	52.3	–	–	–	–	–	–	–
$C_{20:1}$ 7c	–	–	1.3	–	1.0	–	–	–
Hydroxy fatty acids:								
$C_{14:0}$ 2-OH	–	–	2.6	–	–	–	3.9	7.4
$C_{16:0}$ 2-OH	–	–	2.5	–	–	–	6.4	9.2
$C_{16:0}$ 3-OH	–	–	–	–	–	–	1.2	3.7

alcohol and maltose, but not from lactose, starch, glycerol, methanol or sodium D-gluconate. The major cellular fatty acid is $C_{18:1}\omega 9c$. The major isoprenoid quinone is Q-10. The genomic DNA G+C content is 63.2 mol% (HPLC).

Type strain is WE7^T (CGMCC 1.15338^T=JCM 31140^T), isolated from a gel pellicle sample of naturally fermented coconut milk, collected from the College of Food Science and Technology, Hainan University, Haikou, China.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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