INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Baek *et al.*, *Int. J. Syst. Evol. Microbiol.* 2020;70:2026–2033

DOI 10.1099/ijsem.0.004008

MICROBIOLOGY SOCIETY

Acetobacter oryzoeni sp. nov., isolated from Korean rice wine vinegar

Ju Hye Baek[1]†, Kyung Hyun Kim[1]†, Ji Young Moon[2], Soo-Hwan Yeo[2,*] and Che Ok Jeon[1,*]

Abstract

A Gram-stain-negative, obligately aerobic bacterium, designated strain B6^T, was isolated from rice wine vinegar in the Republic of Korea. Cells were non-motile and oval short rods showing catalase-positive and oxidase-negative activities. Growth was observed at 15–45 °C (optimum, 30 °C) and pH 3.5–8.0 (optimum, pH 5.5–6.5). Strain B6^T contained summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), and $C_{16:0}$ as major fatty acids and ubiquinone-9 was identified as the sole isoprenoid quinone. The G+C content of the genomic DNA calculated from the whole genome was 53.1 mol%. Strain B6^T was most closely related to *Acetobacter pasteurianus* LMG 1262^T with very high 16S rRNA gene sequence similarity (100 %) and the strains formed a very close phylogenetic lineage together in phylogenetic trees based on 16S rRNA gene sequences. However, relatedness analyses based on concatenated amino acid sequences of 354 core genes and whole-cell MALDI-TOF profiles showed that strain B6^T may form a distinct phyletic lineage from *Acetobacter* species. In addition, average nucleotide identity and *in silico* DNA–DNA hybridization values between strain B6^T and the type strains of *Acetobacter* species were less than 93.3 and 51.4 %, respectively. The genomic features of strain B6^T were also differentiated from those of closely related *Acetobacter* type strains. Based on the phenotypic, chemotaxonomic and genomic features, strain B6^T clearly represents a novel species of the genus *Acetobacter*, for which the name *Acetobacter oryzoeni* sp. nov. is proposed. The type strain is B6^T (=KACC 21201^T=JCM 33371^T).

Acetic acid bacteria are Gram-stain-negative, obligately aerobic bacteria that produce acetic acid by ethanol oxidation and the genus *Acetobacter* is a representative acetic acid bacteria [1]. At the time of writing, the genus *Acetobacter* includes 28 validated published species that have been isolated from various alcoholic fermentation products such as in grapes, wine, water kefir, kombucha, beer, rice wine and cocoa bean and their vinegar products [2–9]. Cells of *Acetobacter* are motile or non-motile ellipsoidal to short rods usually showing catalase-positive and oxidase-negative activities and containing ubiquinone-9 (Q-9) as the predominant respiratory quinone. Their genomic DNA has a G+C content of 50.5–60.3 mol% [10]. Korean rice wine, called makgeolli in Korea, is made through alcohol fermentation by yeast from rice, along with nuruk, a Korean fermentation starter, with the growth of molds producing hydrolysable enzymes that hydrolyse rice starches into fermentable sugars. Korean rice wine vinegar is made by acetic acid fermentation from Korean rice wine and an indigenous seed vinegar called 'mother of vinegar' is generally added for the fermentation. In this study, we isolated an *Acetobacter* strain, designated strain B6^T, from a rice wine vinegar, which was very closely related to *Acetobacter* species type strains based on 16S rRNA gene sequence similarity. However, relatedness analyses based on core genomes, average nucleotide identity (ANI), *in silico* DNA–DNA hybridization (DDH) and whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) showed that strain B6^T may represent a novel species of the genus *Acetobacter*. Here we describe its taxonomic characteristics using a polyphasic approach.

Strain B6^T was isolated from a traditional rice wine (called makgeolli in Korea) vinegar. Briefly, a rice wine vinegar sample that was collected in Jeonju (35° 49′ 19″ N 127° 8′ 56″ E), Republic of Korea, was serially diluted in phosphate buffered saline (150 mM NaCl, 20 mM sodium phosphate, pH 7.0) and aliquots of each serial dilution were spread on

Author affiliations: [1]Department of Life Science, Chung-Ang University, Seoul 06974, Republic of Korea; [2]Department of Agrofood Resources, National Institute of Agricultural Sciences, RDA, Wanju-gun 55365, Republic of Korea.
***Correspondence:** Soo-Hwan Yeo, yeobio@korea.kr; Che Ok Jeon, cojeon@cau.ac.kr
Keywords: *Acetobacter oryzoeni*; new taxa; Korean rice wine; vinegar.
Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; Q-9, ubiquinone-9.
The GenBank accession numbers for the 16S rRNA gene and genome sequences of strain B6^T are MK367404 and CP042808–11, respectively.
†These authors contributed equally to this work
Two supplementary figures are available with the online version of this article.

YPGDE agar (0.5 g yeast extract, 0.5 g peptone, 0.5 g glycerol, 0.5 g D-glucose, 4 ml ethanol and 1.5 g agar per 100 ml) containing 2 % (w/v) $CaCO_3$. After 2 days of incubation at 30 °C, 16S rRNA genes of colonies with clear zones formed by dissolving of calcium carbonate on the agar plates were amplified using F1 (5′- AGAGTTTGATCMTGGCTCAG-3′) and R13 (5′- TACGGYTACCTTGTTACGACTT-3′) primers and the amplicons were sequenced using 340F (5′-CCTACGGGAGGCAGCAG-3′), 518R (5′-ATTACCGCGGCTGCTGG-3′) and 805F (5′-GATTAGATACCCTGGTAGTC-3′), as described previously [11]. The 16S rRNA gene sequences obtained by the 518R, 805F and 340F primers were assembled and compared with those of all reported type strains using the Nucleotide Similarity Search program in the EzBioCloud server (www.ezbiocloud.net/identify) [12]. From the results, strain B6^T was selected for further taxonomic characterization. Strain B6^T was routinely cultured under an aerobic condition in YPGDE broth at 30 °C for 2 days and was stored at –80 °C in YPGDE broth containing 15 % (v/v) glycerol for a long-term preservation. *Acetobacter pasteurianus* LMG 1262^T, '*Acetobacter ascendens*' LMG 1590^T, *Acetobacter oryzifermentans* SLV-7^T, *Acetobacter pomorum* LTH 2458^T and *Acetobacter aceti* KCTC 12290^T were used for the comparative genomic analysis, the whole-cell MALDI-TOF MS analysis and the comparisons (*Acetobacter aceti* KCTC 12290^T was not used for these comparisons) of phenotypic properties and fatty acid compositions.

The 16S rRNA gene sequence similarities between strain B6^T and closely related type strains were calculated using the EzBioCloud server [12]. The 16S rRNA gene sequences of strain B6^T and closely related strains were aligned using a fast secondary-structure aware infernal aligner which is available in the Ribosomal Database Project [13] and phylogenetic trees with bootstrap values (1000 replications) were reconstructed. Kimura's two-parameter model, the nearest-neighbour-interchange heuristic search method and pairwise deletion options were used for the reconstruction of neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) trees in the MEGA7 software version 7.026 [14].

Comparative analysis based on 16S rRNA gene sequence similarity showed that strain B6^T was most closely related to *A. pasteurianus* LMG 1262^T, *A. oryzifermentans* SLV-7^T, '*A. ascendens*' LMG 1590^T and *A. pomorum* LTH 2458^T with very high sequence similarities (99.78–100 %) while sequence similarities to other species were below 98.0 %. Phylogenetic analysis using 16S rRNA gene sequences based on the NJ algorithm showed that they formed a very close phylogenetic lineage that was not differentiated each other because of their high 16S rRNA gene sequence similarities (Fig. 1), which corresponded with the tree topologies based on the MP and ML algorithms (Fig. S1, available in the online version of this article). Strain Ab3 (registered as *A. pasteurianus* Ab3 in GenBank, not available because the strain was deposited for patent purposes at the China Center for Type Culture Collection, M2013116), which was reported to be able to be classified as a new species of the genus *Acetobacter* in a previous study [9], also clustered with them (Figs. 1 and S1).

The genomic DNA of strains B6^T was extracted according to a standard procedure using phenol–chloroform extraction and ethanol precipitation [15]. The genome of strain B6^T was completely sequenced and analysed under the proposed minimal standards for the taxonomy of prokaryotes [16]. In brief, the genome of strain B6^T was completely sequenced using a combination of PacBio RS single-molecule real-time (SMRT) sequencing with a 10 kb library and Illumina Hiseq 2500 sequencing at Macrogen (Republic of Korea). *De novo* assembly of sequencing reads derived from the PacBio SMRT sequencing was performed through the hierarchical genome assembly process using the HGAP3 software (ver. 3.11.1) [17], which generated four contigs with an average genome coverage of ×190.7. Paired-end raw reads with 991 Mb of 151 bp reads (314.3× average genome coverage) derived from the Illumina sequencing were mapped on complete genome sequences assembled from the PacBio sequencing reads for error corrections. The quality of the resulting genome was assessed based on its completeness and contamination rates using CheckM software (version 1.0.4) [18]. The completeness and contamination rates of the genome were 97.91 and 1.47 %, respectively, which clearly satisfied the criteria (≥90 and ≤10 %, respectively) to be considered as a high quality genome [18]. The complete genome of strain B6^T was composed of one circular chromosome [2 744 393 bp, 2425 protein coding sequences (CDS) and 53.1 mol% G+C content] and three circular plasmids (246 434 bp, 224 CDS and 53.7 mol%; 132 758 bp, 101 CDS and 50.8 mol%; 29 595 bp, 27 CDS and 56.1 mol%). The entire genome (3 153 180 bp) of strain B6^T contained 2989 total genes, five complete rRNA operons (16, 23 and 5S rRNA genes) and 57 tRNA genes encoding 20 amino acids. The DNA G+C content of the entire genome was 53.1 mol%, which is within the DNA G+C content range of *Acetobacter* species [10].

For the genome-based phylogenomic analysis, core genes of strain B6^T and closely related strains were obtained from their whole genomes using the USEARCH program (version 9.0) available in the Bacterial Pan-Genome Analysis (BPGA) pipeline (version 1.3), with a 50 % sequence identity cut-off [19]. The concatenated amino acid sequences of the obtained core genes were aligned using the MUSCLE program (version 3.8.31) [20], and a phylogenomic tree with 1000 replicate bootstrap values was reconstructed using the maximum-likelihood algorithm in the MEGA program. The relatedness for whole genomes including chromosomes and plasmids between strain B6^{T}and closely related strains was evaluated through average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (DDH) analyses, using a standalone program (www.ezbiocloud.net/sw/oat) [21] and the web-based Genome-to-Genome Distance Calculator version 2.1 (http://ggdc.dsmz.de/distcalc2.php) [22].

The results of genome-based phylogenomic analysis based on the concatenated amino acid sequences of 354 core-genes showed that strain B6^T formed a phylogenetic lineage with strain Ab3 and both strains were closely related to *A. pasteurianus* LMG 1262^T (Fig. 2), showing that they may be differentiated from other *Acetobacter* species. Therefore, pair-wise

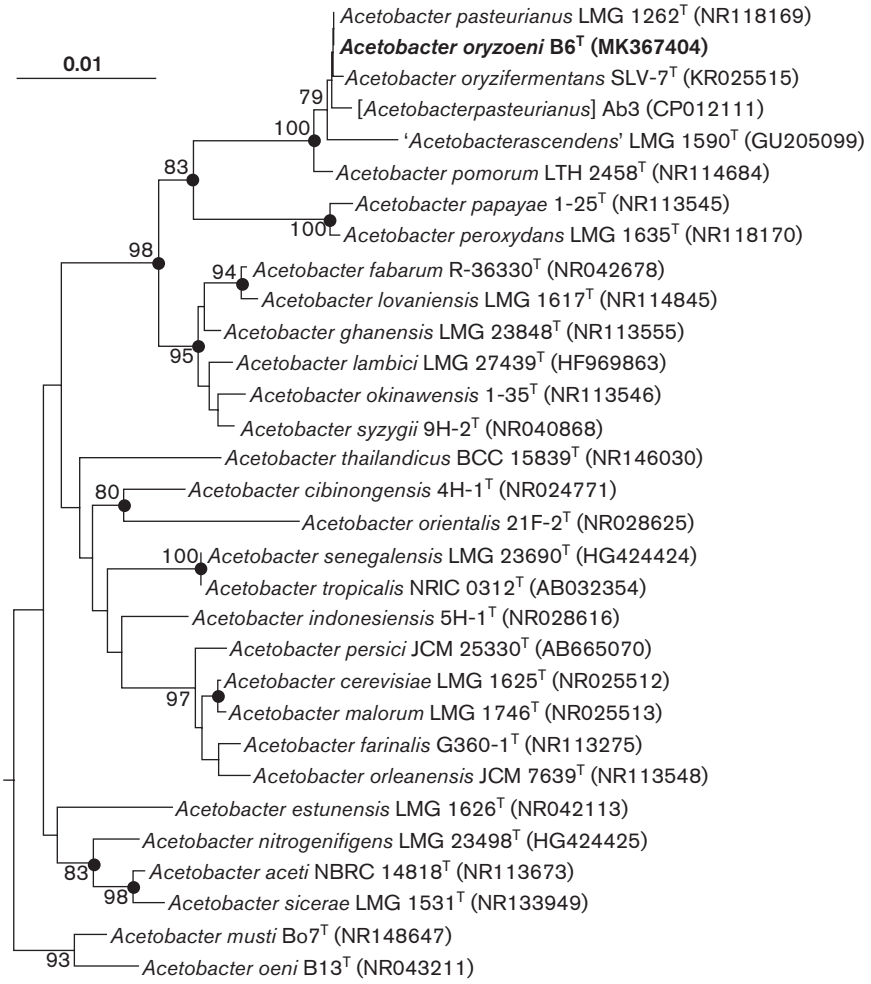

Fig. 1. A neighbour-joining tree based on 16S rRNA genes, showing phylogenetic relationships between strain B6^T and *Acetobacter* species. Bootstrap values above 70 % are shown on the nodes as percentages of 1000 replicates. Filled circles (●) indicate the corresponding nodes that were also recovered by the maximum-likelihood and maximum-parsimony algorithms. *Rhodopila globiformis* DSM 161^T (D86513) was used as outgroup (not shown). Bar, 0.01 changes per nucleotide position.

ANI and *in silico* DDH values among the genomes used for genome-based phylogenomic analysis were calculated (Table S1). The ANI and *in silico* DDH values between strains B6^T and Ab3 were 98.1 and 84.1 %, respectively, which were clearly higher than the thresholds, 95–96 and 70 %, respectively, for prokaryotic species delineation [21, 23–25], suggesting that strains B6^T and Ab3 should be considered as one species. The analyses showed that the ANI and *in silico* DDH values between strains B6^T and other closely related *Acetobacter* type strains were less than 93.3 and 51.4 %, which were clearly lower than the thresholds for prokaryotic species delineation, which suggests that strains B6^T represents a new species of the genus *Acetobacter*.

For the comparative genomic analysis of strain B6^T and closely related *Acetobacter* strains, their genomes were retrieved from GenBank and automatically annotated by using the NCBI prokaryotic genome annotation pipeline (www.ncbi.nlm.nih.gov/genome/annotation_prok/). Clustered regularly interspaced short palindromic repeat (CRISPR) gene sequences were searched using an online web service (http://crispr.i2bc.paris-saclay.fr) [26] and genomic islands were identified using the web-based IslandViewer 4 software (www.pathogenomics.sfu.ca/islandviewer/) [27]. All proteins derived from *Acetobacter* genomes were functionally classified based on their sequence similarity and domain features predicted by the InterProScan program [28] and their numbers were manually counted. Among alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH), membrane-bound proteins containing transmembrane helices were regarded as pyrroloquinoline quinone (PQQ)-ADH and PQQ-ALDH, respectively.

The general genomic features of strain B6^T and the reference *Acetobacter* strains are described in Table 1. The genome of strain B6^T was composed of one circular chromosome and three circular plasmids, like strains LMG 1590^T and SLV-7^T, while strain Ab3, the most closely related strain to strain B6^T,

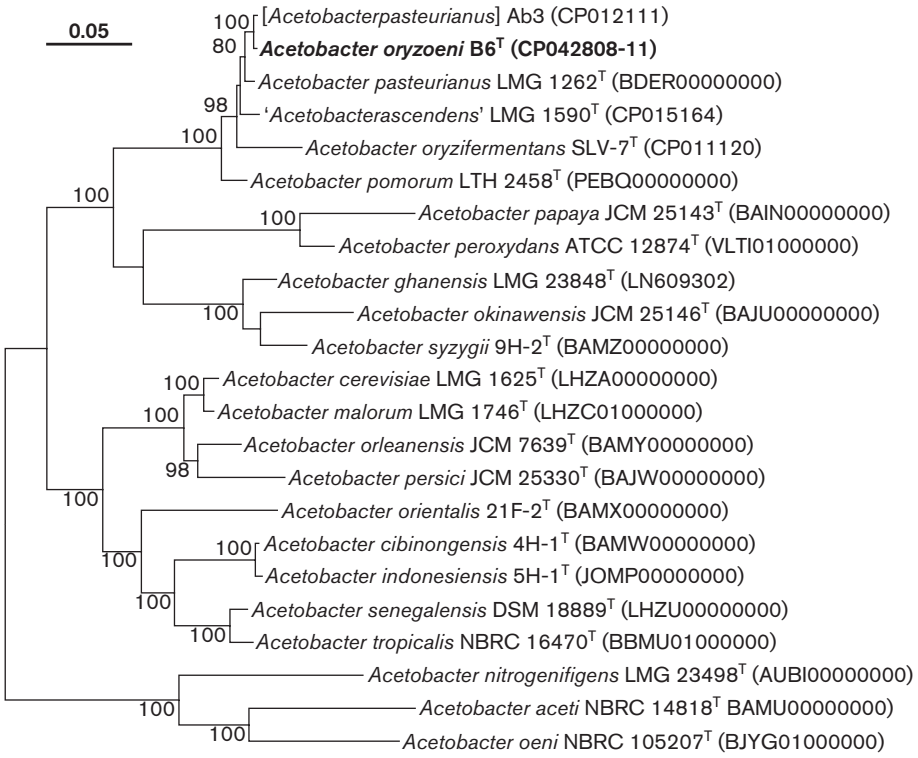

Fig. 2. A phylogenomic tree based on concatenated amino acid sequences of 354 core genes, showing phylogenetic relationships between strain B6^T and *Acetobacter* strains. Bootstrap values above 70 % are shown on the nodes as percentages of 1000 replicates. Bar, 0.05 changes per nucleotide position.

was composed of a single chromosome without plasmid. The genome size of strain B6^T was approximately 3.15 Mb with 2985 total genes and 2777 protein coding sequences, which was a little bigger than strain Ab3, *A. pasteurianus* LMG 1262^T and '*A. ascendens*' LMG 1590^T that were closely clustered with strain B6^T in the phylogenetic analyses (Figs 1 and 2). However, the genome size of strain B6^T was a little smaller than those of distantly related strains, *A. pomorum* LTH 2458^T and *A. aceti* KCTC 12290^T. Strain B6^T had five rRNA operons as did other all reference *Acetobacter* strains (Table 1, [29]). The G+C content and tRNA gene number of strain B6^T were 53.1 mol% and 57, respectively, which were in the ranges of the other reference *Acetobacter* strains. The genome of strain B6^T contained one confirmed CRISPR and 26 genomic islands, similar to the reference *Acetobacter* strains, indicating some occurrences of genetic alterations and exchanges during its evolutionary process.

The results of functional gene analyses of *Acetobacter* genomes showed that strain B6^T harboured 14 ADH- and 12 ALDH-coding genes that may be associated with acetate production from ethanol, which were a little higher than those of closely related *Acetobacter* strains including strain Ab3. However, it has been reported that the activities of membrane-bound PQQ-dependent ADH and ALDH in the periplasm of acetic acid bacteria play a major role in the conversion of ethanol to acetate during vinegar fermentation [29, 30]. Strain B6^T possessed five PQQ-ADH- and one PQQ-ALDH-coding gene, which was similar to those of other *Acetobacter* strains. It is well known that chaperone proteins, including GroES, GroEL, DnaK, DnaJ and GrpE, and ornithine/diaminopimelate decarboxylase, generating alkaline environment in the cytoplasm, confer tolerance to stressors such as acetic acid and ethanol in acetic acid bacteria [31]. Strain B6^T and the reference *Acetobacter* strains had the same numbers of genes encoding DnaK (1), DnaJ (4), GroEL (1), GroES (1) and GrpE (1), YbbN (1), DjlA (1), SecB (1) Hfq (1), HtpG (1), Hsp33 (1), ClpB (1) and ornithine/diaminopimelate decarboxylase (2). It has also been reported that urea metabolic systems contribute to acid tolerance in bacteria such as *Helicobacter pylori* and *Lactobacillus reuteri* by producing ammonia [32]. However, no urease gene cluster (*ureDABCEFG*) encoding the urease enzyme that produces ammonia by hydrolysing urea was identified in strain B6^T, but the other reference strains (except for *A. pasteurianus* LMG 1262^T and *A. pomorum* LTH 2458^T) harboured a urease gene cluster. In addition, strain B6^T did not harbour urea metabolic systems such as urea transporter (*urtABCDE*), urea carboxylase and allophanate hydrolase, unlike other reference *Acetobacter* strains, which suggests that strain B6^T may be less tolerant to acetic acid compared to the reference *Acetobacter* strains. In conclusion, the functional gene analyses of *Acetobacter* genomes showed that the general genomic and genotypic features of strain B6^T were a

Table 1. General genomic and genotypic features of strain B6^T and closely related *Acetobacter* strains*

Strains: 1, B6^T; 2, Ab3; 3, *Acetobacter pasteurianus* LMG 1262^T ; 4, '*Acetobacter ascendens*' LMG 1590^T; 5, *Acetobacter oryzifermentans* SLV-7^T; 6, *Acetobacter pomorum* LTH 2458^T; 7, *Acetobacter aceti* KCTC 12290^T. All strains have the same numbers of genes encoding the following chaperone proteins: DnaK (1), DnaJ (4), GroEL (1), GroES (1) and GrpE (1), YbbN (1), DjlA (1), SecB (1) Hfq (1), HtpG (1), Hsp33 (1) and ClpB (1), and ornithine/diaminopimelate decarboxylase (2). The information was obtained from GenBank annotated by the NCBI NCBI Prokaryotic Genome Annotation Pipeline.

Characteristic	1	2	3	4	5	6	7
Genome status (no. of contigs)*	C (4)	C (1)	D (141)	C (4)	C (4)	D (212)	D (209)
Total genome size (Mb)	3.15	2.80	2.98	3.00	3.10	3.33	3.55
DNA G+C content (mol%)	53.1	53.3	53.1	53.2	52.4	51.7	57.1
Total no. of genes	2985	2680	2864	2938	3009	3168	3274
No. of protein coding sequences	2777	2498	2688	2560	2787	2973	3122
No. of rRNA operons†	5	5	–	5	5	–	–
No. of tRNA genes	57	58	49	56	57	54	40
No. of confirmed CRISPRs (no. of questionable CRISPRs)‡	1	0	0 (1)	0	1	2 (2)	1
No. of genomic islands	26	23	23	23	26	40	25
Acetic acid production:							
Alcohol dehydrogenase (PQQ-ADH)§	14 (5)	11 (4)	12 (4)	9 (4)	11 (4)	11 (4)	16 (4)
Aldehyde dehydrogenase (PQQ-ALDH)§	12 (1)	9 (1)	12 (1)	10 (1)	8 (1)	10 (1)	11 (1)
Urea metabolism-associated:							
Urease gene cluster (*ureDABCEFG*)	0	1	0	1	1	0	1
Urea transporter (*urtABCDE*)	0	1	1	1	2	0	1
Urea carboxylase	0	0	1	1	1	0	1
Allophanate hydrolase	0	0	1	1	1	0	1

*C, complete; D, draft.
†Each rRNA operon consists of 16S, 23S and 5S rRNA genes and their numbers were counted for only complete genomes. –, not counted for draft genomes.
‡CRISPRs of more than two spacers with three or more perfect repeats are 'confirmed CRISPRs', whereas CRISPRs with fewer than three perfect repeats or nonidentical repeats are considered 'questionable' [26].
§Membrane-bound PQQ-alcohol dehydrogenase (ADH) and -aldehyde dehydrogenase (ALDH) were analysed based on the presence of transmembrane helices.

little different from those of the reference *Acetobacter* strains. In particular, the analyses clearly showed that strain B6^T can be differentiated by its genomic and genotypic features from strain Ab3 that was considered as the same species.

Various phenotypic analyses recommended for the description of new taxa in the genus *Acetobacter* were performed for the taxonomic characterization of strain B6^T. Growth of strain B6^{T}at different temperatures (5, 10, 15, 20, 25, 30, 37, 40 and 45 °C) and pH values (3.0–10.0 at 0.5 pH unit intervals) was assessed in YPGDE broth at 30 °C for 2 days. YPGDE broth with pH values below pH 5.5, pH 6.0–7.5 and pH 8.0–10.0 were prepared using citrate buffer, Na_2HPO_4–NaH_2PO_4, and Tris–HCl buffers, respectively, as described previously [33]. Cell morphology and motility of strain B6^T were investigated using transmission electron microscopy (JEM-1010, JEOL) and phase-contrast microscopy of cells grown on YPGDE agar at 30 °C for 2 days. The following properties of strain B6^T and three reference strains were investigated in parallel under the same conditions. Ethanol tolerance was assessed, as described previously [34]. Catalase and oxidase activities were checked by the production of oxygen bubbles in 3 % (v/v) aqueous H_2O_2 solution and the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), respectively [35]. Formation of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose was tested using the method described by Gosselé *et al.* [36]. Utilization of ammonium as the sole nitrogen source was checked using Frateur's modified Hoyer ethanol medium [37]. Growth in 0.5 % yeast extract solution containing 30 % D-glucose and in basal medium (0.05 % yeast extract and 0.3 % vitamin-free casamino acids) containing 0.3 % of each of carbon sources, D-fructose, D-sorbitol, glycerol, maltose and methanol was tested as described previously [37]. Acid production from L-arabinose, D-galactose, D-mannose, D-glucose and D-xylose was checked as described by Asai *et al.* [38].

Table 2. Comparison of phenotypic characteristics of strain B6^{T}and the type strains of closely related *Acetobacter* species

Strain: 1, B6^T; 2, *Acetobacter pasteurianus* LMG 1262^T; 3, '*Acetobacter ascendens*' LMG 1590^T; 4, *Acetobacter oryzifermentans* SLV-7^T; 5, *Acetobacter pomorum* KCTC 22319^T; 6, *Acetobacter aceti* KCTC 12290^T. All analyses were performed in this study. All strains are positive for catalase activity and growth in 10 % (v/v) ethanol. All strains are negative for the following characteristics: anaerobic growth, flagellar motility, oxidase activity, growth on maltose and methanol as a sole carbon source, ammonium utilization as the sole nitrogen source, growth on yeast extract with 30 % (w/v) D-glucose, and acid production from D-arabinose. +, Positive; –, negative.

Characteristic	1	2	3	4	5	6
Isolation source	Rice vinegar	Beer	Unknown	Rice vinegar	Vinegar	Vinegar
Growth on:						
D-Fructose	–	–	–	+	–	+
D-Sorbitol	–	–	+	–	–	–
Glycerol	+	–	–	+	–	+
Formation of ketogluconic acids from D-glucose:						
2-Keto-D-gluconic acid	–	–	–	–	–	+
5-Keto-D-gluconic acid	–	–	–	–	–	+
Acid production from:						
L-Arabinose	+	–	–	–	–	+
D-Galactose	+	–	–	–	–	+
D-Mannose	+	–	–	+	–	+
D-Glucose	+	+	–	+	+	+
D-Xylose	+	+	–	+	–	+

When cultured on YPGDE agar at 30 °C for 2 days, cells of strain B6^T were Gram-stain-negative, strictly aerobic and non-motile oval rods that occurred singly or in groups. Cells were 0.9–1.1 µm wide and 1.3–1.4 µm long (Fig. S2). Many phenotypic properties, including oxidase, catalase, flagellar motility, cell morphology, growth in 10 % (v/v) ethanol, formation of 5-keto-D-gluconic acid and 2-keto-D-gluconic acid from D-glucose and growth on maltose and methanol, were in common with those of the reference strains, while some phenotypic properties, including growth on D-fructose, D-sorbitol and glycerol and acid production from L-arabinose, D-galactose, D-mannose, D-glucose and D-xylose, differentiated strain B6^T from the reference *Acetobacter* strains (Table 2).

For the whole-cell MALDI-TOF MS analysis, cells of strain B6^T and four reference strains that were cultured on YPGDE agar at 30 °C for 3 days were harvested as described previously [39], and their MALDI-TOF MS spectra were obtained using an MALDI Biotyper (Bruker Daltonics), according to the manufacturer's suggested settings, with the detection of positive ions in a linear mode of a mass range of 2–20 kDa. Heatmap and hierarchical clustering analyses of the MALDI-TOF MS profiles were performed using one minus the Pearson correlation distances for the clustering in the GENE-E program (www.broadinstitute.org/cancer/software/GENE-E/). Isoprenoid quinones of strain B6^T were analysed using an HPLC (model LC-20A, Shimadzu) system equipped with a reversed-phase column (250Í4.6 mm, Kromasil, Akzo Nobel) and a diode array detector (SPD-M20A, Shimadzu) with methanol–isopropanol (2 : 1, v/v) as an eluent (1 ml min^{-1}), as described previously [40]. For the analysis of cellular fatty acids, strain B6^T and three reference strains were cultivated in YPGDE broth at 30 °C and their microbial cells were harvested at the same growth stage (exponential phase, OD600=0.6–0.8). The standard MIDI protocol was used for the analysis of cellular fatty acids of the microbial cells that were also saponified, methylated and extracted by following that protocol. For the detection of fatty acid methyl esters, gas chromatography (Hewlett Packard 6890) and the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B) were used as described previously [41].

The hierarchical clustering analysis based on MALDI-TOF MS profiles of *Acetobacter* strains showed that strain B6^T was clustered with *A. pasteurianus* LMG 1262^T, but their profiles were clearly different (Fig. 3). The only respiratory quinone detected from strain B6^T was Q-9, which was in accordance with members of the genus *Acetobacter* [10]. The major cellular fatty acids (>5 % of the total fatty acids) of strain B6^T were summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 64.1 %) and $C_{16:0}$ (10.0 %). The overall fatty acid profile of strain B6^T was similar to those of other *Acetobacter* species, but there were some differences in the respective proportions of fatty

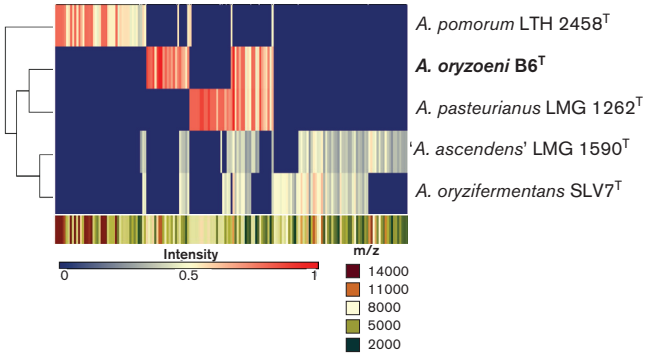

Fig. 3. Heat map profiling and hierarchical clustering of MALDI-TOF MS spectra showing relationships between strain B6^T and closely related *Acetobacter* strains. The MALDI-TOF MS spectra were obtained in triplicate from m/z peaks intensities of mass range 2000–20 000 m/z.

Table 3. Cellular fatty acid compositions (%) of strain B6^{T}and the type strains of closely related *Acetobacter* species

Strains: 1, B6^T; 2, *Acetobacter pasteurianus* LMG 1262^T; 3, '*Acetobacter ascendens*' LMG 1590^T; 4, *Acetobacter oryzifermentans* SLV-7^T. All data were obtained from this study. Data are expressed as percentages for the total fatty acids and fatty acids less than 0.5 % in all strains are not indicated. Major components (>5.0 %) are highlighted in bold. TR, Trace amount (<0.5 %).

Fatty acid	1	2	3	4
Saturated:				
$C_{12:0}$	1.2	–	–	–
$C_{14:0}$	TR	0.9	–	1.3
$C_{16:0}$	**10.0**	**15.5**	**11.3**	**10.2**
$C_{18:0}$	4.7	1.3	2.5	2.2
$C_{20:0}$	1.3	TR	–	TR
$C_{12:0}$ aldehyde	2.8	3.6	**5.5**	**5.7**
$C_{19:0}$ cyclo $\omega 8c$	1.5	0.8	1.7	1.4
Unsaturated:				
$C_{20:2}\omega 6,9c$	–	–	4.2	–
Hydroxy:				
$C_{14:0}$ 2-OH	2.9	3.6	4.3	4.2
$C_{16:0}$ 2-OH	3.0	4.9	4.3	**6.6**
$C_{16:0}$ 3-OH	1.3	0.9	3.0	1.6
$C_{18:0}$ 3-OH	3.1	**13.7**	**26.1**	1.0
Summed features:*				
2	1.9	–	1.3	–
8	**64.1**	**52.7**	**30.5**	**64.4**

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2, iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH; summed feature 8, $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$.

acid components such as $C_{12:0}$, $C_{20:0}$ and $C_{20:2}\omega 6,9c$ (Table 3). In conclusion, although the 16S rRNA gene sequence of strain B6^T was very similar with those of other *Acetobacter* type strains, the core genome-based phylogenomic tree, the ANI and *in silico* DDH values, and phenotypic features suggest that strain B6^T represents a novel species of the genus *Acetobacter*, for which the name *Acetobacter oryzoeni* sp. nov. is proposed.

DESCRIPTION OF *ACETOBACTER ORYZOENI* SP. NOV.

Acetobacter oryzoeni [o.ryz.oe'ni. Gr. fem. n. *oryza* rice; Gr. masc. n. *oenos* wine; N.L. gen. n. *oryzoeni* of rice wine (vinegar)].

Cells are Gram-stain-negative, obligately aerobic, non-motile and oval short rods (0.9–1.1 µm wide and 1.3–1.4 µm long). Catalase-positive and oxidase-negative. After incubation at 30 °C for 2 days, colonies on YPGDE agar are beige-coloured, round, convex, non-glossy and smooth. Growth occurs at 15–45 °C (optimum, 30 °C) and pH 3.5–8.0 (optimum, pH 5.5–6.5). Able to grow in the presence of 10 % ethanol. Unable to grow on yeast extract with 30 % D-glucose and on medium with ammonium as the sole nitrogen source. Does not produce 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose. Able to grow on glycerol as a sole carbon source, but not on D-fructose, D-sorbitol, maltose and methanol. Produces acids from D-mannose, D-glucose, D-xylose and D-galactose, but not from D-arabinose. Major isoprenoid quinone is Q-9. Major fatty acids are summed feature 8 (comprising $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$) and $C_{16:0}$.

The type strain is B6^T (=KACC 21201^T=JCM 33371^T), isolated from a Korean rice wine vinegar in South Korea. DNA G+C content of the type strain is 53.1 mol% (genome). GenBank accession numbers for the 16S rRNA gene and genome sequences of strain B6^T are MK367404 and CP042808–11, respectively.

Funding information
This work was supported by the National Research Foundation (2017M3C1B5019250) of the Ministry of Science and ICT and the Program for Collection of Domestic Biological Resources of the National Institute of Biological Resources (NIBR No. 2019-02-001) of the Ministry of Environment (MOE), Republic of Korea.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Yamada Y, Yukphan P. Genera and species in acetic acid bacteria. *Int J Food Microbiol* 2008;125:15–24.
2. Cleenwerck I, Gonzalez A, Camu N, Engelbeen K, De Vos P *et al*. *Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. *Int J Syst Evol Microbiol* 2008;58:2180–2185.
3. Iino T, Suzuki R, Kosako Y, Ohkuma M, Komagata K *et al*. *Acetobacter okinawensis* sp. nov., *Acetobacter papayae* sp. nov., and *Acetobacter persicus* sp. nov.; novel acetic acid bacteria isolated from stems of sugarcane, fruits, and a flower in Japan. *J Gen Appl Microbiol* 2012;58:235–243.

4. Chakravorty S, Bhattacharya S, Chatzinotas A, Chakraborty W, Bhattacharya D *et al*. Kombucha tea fermentation: microbial and biochemical dynamics. *Int J Food Microbiol* 2016;220:63–72.
5. Pitiwittayakul N, Theeragool G, Yukphan P, Chaipitakchonlatarn W, Malimas T *et al*. *Acetobacter suratthanensis* sp. nov., an acetic acid bacterium isolated in Thailand. *Ann Microbiol* 2016;66:1157–1166.
6. Ferrer S, Mañes-Lázaro R, Benavent-Gil Y, Yépez A, Pardo I. *Acetobacter musti* sp. nov., isolated from Bobal grape must. *Int J Syst Evol Microbiol* 2016;66:957–961.
7. Li L, Wieme A, Spitaels F, Balzarini T, Nunes OC *et al*. *Acetobacter sicerae* sp. nov., isolated from cider and kefir, and identification of species of the genus *Acetobacter* by *dnaK*, *groEL* and *rpoB* sequence analysis. *Int J Syst Evol Microbiol* 2014;64:2407–2415.
8. Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I *et al*. *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer. *Int J Syst Evol Microbiol* 2014;64:1083–1089.
9. Kim KH, Cho GY, Chun BH, Weckx S, Moon JY *et al*. *Acetobacter* oryzifermentans sp. nov., isolated from Korean traditional vinegar and reclassification of the type strains of *Acetobacter pasteurianus* subsp. *ascendens* (Henneberg 1898) and *Acetobacter pasteurianus* subsp. *paradoxus* (Frateur 1950) as *Acetobacter ascendens* sp. nov., comb. nov. *Syst Appl Microbiol* 2018;41:324–332.
10. Sievers M, Swings J. *Acetobacter*. *In Bergey's Manual of Of Systematics of Archaea and Bacteria*; 2015.
11. Khan SA, Jeong SE, Jung HS, Quan Z-X, Jeon CO. *Roseicella frigidaeris* gen. nov., sp. nov., isolated from an air-conditioning system. *Int J Syst Evol Microbiol* 2019;69:1384–1389.
12. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y *et al*. Introducing EzBioCloud: a taxonomically United database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
13. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73:5261–5267.
14. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1874.
15. Sambrook J, Russell DW. *Molecular Cloning: a Laboratory Manual*, 3rd edition. UK: Coldspring-Harbour Laboratory Press; 2001.
16. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR *et al*. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466.
17. Chin C-S, Alexander DH, Marks P, Klammer AA, Drake J *et al*. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nat Methods* 2013;10:563–569.
18. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015;25:1043–1055.
19. Chaudhari NM, Gupta VK, Dutta C. BPGA- an ultra-fast pan-genome analysis pipeline. *Sci Rep* 2016;6:24373.
20. Edgar RC. Muscle: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797.
21. Lee I, Ouk Kim Y, Park S-C, Chun J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* 2016;66:1100–1103.
22. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
23. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* 2009;106:19126–19131.
24. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P *et al*. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
25. Konstantinidis KT, Tiedje JM. Genomic insights that advance the species definition for prokaryotes. *Proc Natl Acad Sci U S A* 2005;102:2567–2572.
26. Grissa I, Vergnaud G, Pourcel C. CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res* 2007;35:W52–W57.
27. Bertelli C, Laird MR, Williams KP, Lau BY, Hoad G *et al*. IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res* 2017;45:W30–W35.
28. Jones P, Binns D, Chang H-Y, Fraser M, Li W *et al*. InterProScan 5: genome-scale protein function classification. *Bioinformatics* 2014;30:1236–1240.
29. Jia B, Chun BH, Cho GY, Kim KH, Moon JY *et al*. Complete genome sequences of two acetic acid-producing *Acetobacter pasteurianus* Strains (Subsp. *ascendens* LMG 1590^T and Subsp. *paradoxus* LMG 1591^T). *Front Bioeng Biotechnol* 2017;5:33.
30. Chinnawirotpisan P, Theeragool G, Limtong S, Toyama H, Adachi OO *et al*. Quinoprotein alcohol dehydrogenase is involved in catabolic acetate production, while NAD-dependent alcohol dehydrogenase in ethanol assimilation in *Acetobacter pasteurianus* SKU1108. *J Biosci Bioeng* 2003;96:564–571.
31. Wang B, Shao Y, Chen T, Chen W, Chen F. Global insights into acetic acid resistance mechanisms and genetic stability of *Acetobacter pasteurianus* strains by comparative genomics. *Sci Rep* 2015;5:18330.
32. Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. *Gastroenterology* 1990;99:697–702.
33. Gomori G. Preparation of buffers for use in enzyme studies. In: Colowick SP, Kaplan NO (editors). *Methods in enzymology*. New York: Academic Press; 1955. pp. 138–146.
34. Krisch J, Szajani B. Ethanol and acetic acid tolerance in free and immobilized cells of *Saccharomyces cerevisiae* and *Acetobacter aceti*. *Biotechnol Lett* 1997;19:525–528.
35. Smibert RM, Krieg NR. Phenotypic characterization. In: Gerhardt P (editor). *Methods for general and molecular bacteriology*. Washington, DC: American Society for Microbiology; 1994. pp. 607–654.
36. Gosselé F, Swings J, De Ley J, A rapid DLJ. A rapid, simple and simultaneous detection of 2-keto-, 5-keto-and 2,5-diketogluconic acids by thin-layer chromatography in culture media of acetic acid bacteria. *Zentralblatt für Bakteriologie: I. Abt. Originale C: Allgemeine, angewandte und ökologische Mikrobiologie* 1980;1:178–181.
37. Cleenwerck I, Vandemeulebroecke K, Janssens D, Swings J. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *Int J Syst Evol Microbiol* 2002;52:1551–1558.
38. Asai T, Iizuka H, Komagata K. The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. *J Gen Appl Microbiol* 1964;10:95–126.
39. Jeon JH, Oh H-W, Yun CS, Byun B-K, Park J-J *et al*. Rapid and reliable species identification of *Carposina sasakii* from its morphological homologues, by MALDI-TOF mass spectrometry. *J Asia Pac Entomol* 2017;20:411–415.
40. Komagata K, Suzuki K. Lipid and cell-wall analysis in bacterial Systematics. *Methods Microbiol* 1987;19:161–208.
41. Sasser M. Identification of bacteria by gas chromatography of cellular fatty acids; 1990.