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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.

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ABSTRACT

Twelve *Acetobacter pasteurianus*-related strains with publicly available genomes in GenBank shared high 16S rRNA gene sequence similarity (>99.59%), but average nucleotide identity (ANI) and *in silico* DNA-DNA hybridization (DDH) values and multilocus sequence- and genome-based relatedness analyses suggested that they were divided into four different phylogenetic lineages. Relatedness analyses based on multilocus sequences, 1,194 core genes and whole-cell MALDI-TOF profiles supported that strains LMG 1590^T and LMG 1591 (previously classified as the type strains of *A. pasteurianus* subsp. *ascendens* and *paradoxus*, respectively) and strain SLV-7^T do not belong to *A. pasteurianus*. Strain SLV-7^T, isolated from Korean traditional vinegar, shared low ANI (<91.0%) and *in silico* DDH (44.2%) values with all other *Acetobacter* type strains analyzed in this study, indicating that strain SLV-7^T represents a new *Acetobacter* species. The phenotypic and chemotaxonomic analyses confirmed these results and therefore a new species named *Acetobacter oryzifermentans* sp. nov. is proposed with SLV-7^T (= KACC 19301^T = JCM 31096^T) as the type strain. Strains LMG 1590^T and LMG 1591 shared high ANI (99.4%) and *in silico* DDH (96.0%) values between them, but shared low ANI (<92.3%) and *in silico* DDH (<49.0%) values with other type strains analyzed in this study, indicating that strains LMG 1590^T and LMG 1591 should be reclassified into a new single species that should be named *Acetobacter ascendens* sp. nov., comb. nov., with LMD 51.1^T (= LMG 1590^T = NCCB 51001^T) as its type strain.

Acetic acid bacteria (AAB) are Gram-negative, obligate aerobic bacteria that mainly oxidize ethanol to acetic acid [59]. Besides acetate production, AAB produce various organic acids, cellulose, surfactants, and pigments, and display interesting physiological and phenotypic properties including biomaterial production, nitrogen fixation, and even animal pathogenicity [45,7,57]. The genus *Acetobacter*, a representative of AAB, belongs to the family *Acetobacteraceae* within the class *Alphaproteobacteria*, and at the time of writing, it comprises 48 validated published species and subspecies, which are usually found in sugary, alcoholic, and acidic habitats including several traditional fermented foods and alcoholic beverages, such as vinegar, water kefir, kombucha, sour beers, and in the cocoa bean fermentation process [49,8,60]. Recently, they have been used as starters in the commercial production of vinegars, kombucha, or in cocoa bean fermentation [17,34].

Acetobacter pasteurianus is one of the most well-studied *Acetobacter* species that has been used to brew diverse vinegars as starter cultures worldwide [17,34]. In the past, *A. pasteurianus* contained several subspecies named *estunensis*, *lovaniensis*, *pasteurianus*, *ascendens*, and *paradoxus* [11], but it was later pro-

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posed that *A. pasteurianus* subsp. *ascendens* and *A. pasteurianus* subsp. *paradoxus* were heterotypic synonyms of *A. pasteurianus* [50,18]. In addition, *A. pasteurianus* subsp. *estunensis* and *A. pasteurianus* subsp. *lovaniensis* were reclassified as new species of the genus *Acetobacter*, namely as *Acetobacter estunensis* and *Acetobacter lovaniensis*, respectively [35]. However, the *A. pasteurianus* subspecies *pasteurianus*, *ascendens* and *paradoxus* have been used to classify *A. pasteurianus* strains included in the NCBI taxonomic database, which suggests that a clearer taxonomic description of *A. pasteurianus* strains is necessary.

Sequencing of 16S rRNA genes has been widely used for bacterial identification and classification, but in some cases it is not possible to distinguish between closely related species with high 16S rRNA gene sequence similarities, as is the case for AAB [26,9]. As 16S rRNA gene sequence-based approaches alone are not appropriate to determine the phylogenetic relationships between AAB strains, various other approaches such as 16S-23S rDNA internally transcribed spacer region sequences, $(GTG)_5$-PCR, multilocus sequence analysis, and whole-cell matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), have been used for this purpose [57,36,2,12,37]. In particular, the introduction of MALDI-TOF MS for the identification and classification of AAB has significantly reduced the time required for analysis. Although these approaches have considerably contributed to progress in the identification and classification of AAB [2,37], they have limited use in the investigation of comprehensive phylogeny to resolve genotypic and phenotypic diversities and differences between AAB, because these methods do not reflect genotypic and phenotypic properties [26,10]. Recently, taxonomic approaches based on the whole bacterial genome facilitated by the development of high-throughput and low-cost sequencing technologies are able to identify and classify bacteria into taxa, more accurately and with higher resolution than the previous traditional taxonomic approaches [23,61]. In this study, we isolated strain SLV-7^T, which is very closely related to *A. pasteurianus* based on 16S rRNA gene sequence similarity, from a Korean traditional vinegar and sequenced its complete genome along with those of *A. pasteurianus* LMG 1590^T and LMG 1591 that were previously classified as the type strains of *A. pasteurianus* subsp. *ascendens* and *A. pasteurianus* subsp. *paradoxus*, respectively [24]. Here, relatedness analyses based on average nucleotide identity (ANI), and wet-lab and *in silico* DNA-DNA hybridization (DDH) values, multilocus sequence analysis (MLSA) of *dnaK*, *groEL*, and *rpoB* genes, core genome-based relatedness, and whole-cell MALDI-TOF MS profiles were performed, and a new species of the genus *Acetobacter*, *Acetobacter oryzifermentans*, was proposed. In addition, we proposed to reclassify *A. pasteurianus* strains LMG 1590 and LMG 1591 as a single novel species, *Acetobacter ascendens* with LMG 1590^T as the type strain.

Strain SLV-7^T was isolated from a Korean traditional rice vinegar sample. Briefly, 38 Korean traditional rice vinegar samples were collected from all over the Korean peninsula and were serially diluted in phosphate buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.0). Aliquots of each serial dilution were spread on YPGD agar (0.5 g yeast extract, 0.5 g peptone, 0.5 g glycerol, 0.5 g D-glucose, and 1.5 g agar per 100 ml) containing 2% (w/v) $CaCO_3$ and 4% (v/v) ethanol. After two days of incubation at 30 °C, a strain, designated SLV-7^T, with great acetate-producing ability, was selected. *Acetobacter aceti* KCTC 12290^T (= NCIMB 8621^T), *Acetobacter* strains KACC 13994^T (= LMG 1262^T), LMG 1590, and LMG 1591 (previously classified as the type strains of *A. pasteurianus* subsp. *pasteurianus*, *A. pasteurianus* subsp. *ascendens*, and *A. pasteurianus* subsp. *paradoxus*, respectively), *Acetobacter pomorum* KCTC 22319^T (= LHT 2458^T), and *A. pomorum* DM001 (a gift from prof. W.J. Lee at Seoul National University) were used as reference strains for wet-lab DNA–DNA hybridization (DDH) and/or comparison of phenotypic properties, fatty acid compositions and whole-cell MALDI-TOF MS profiles.

The 16S rRNA gene of strain SLV-7^T was amplified using the F1 (5′- AGAGTTTGATCMTGGCTCAG-3′) and R13 (5′-TACGGYTACCTTGTTACGACTT-3′) primers and the amplicon was sequenced, as described previously [27]. The obtained sequence was compared with the 16S rRNA gene sequences available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). The 16S rRNA gene sequences of strain SLV-7^T, of the closely related type strains and of closely related strains with the whole genome sequences in GenBank (Table S1) were aligned using the Infernal secondary-structure aware aligner, available within the Ribosomal Database Project [43]. Phylogenetic trees were constructed using the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) algorithms of the PHYLIP software (version 3.695) [15]. The resulting tree topology was evaluated using bootstrap analysis based on a 1000 times resampled dataset in the PHYLIP package. A MLSA was performed using concatenated nucleotide sequences (7728 bp) of three housekeeping genes [*dnaK* (1911 bp), *groEL* (1644 bp), and *rpoB* (4173 bp)] of *Acetobacter* strains derived from their whole genome sequences. The concatenated gene sequences were aligned using a web-based tool (CLUSTAL Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/) [51] and a phylogenetic tree with bootstrap values was constructed using the NJ algorithm in the MEGA7 software [28].

A 16S rRNA gene sequence-based phylogenetic tree including the type strains of all *Acetobacter* species showed that strain SLV-7^T was very closely related with strains descirbed in GenBank as *A. pasteurianus* or *A. pomorum*, and formed with them a distinct phylogenic lineage from other *Acetobacter* species (Fig. S1), suggesting that they may have evolved independently from a common ancestor. A phylogenetic tree of *A. pasteurianus*-related strains using the NJ algorithm showed that strain SLV-7^T was clustered with strains DmCS_004 and DM001 (described as *A. pomorum* in GenBank), with 100% 16S rRNA gene sequence similarities (Fig. 1A) and the tree topologies generated with the ML and MP algorithms were same (data not shown). However, the latter three strains were not well separated from *A. pasteurianus* strains LMG 1262T, LMG 1590 and LMG 1591, to which they showed >99.66% 16S rRNA gene sequence similarities. Yet, strains LMG 1590 and LMG 1591, previously known as the type strains of *A. pasteurianus* subsp. *ascendens* and subsp. *paradoxus*, respectively, formed a cluster with strain 3P3, separated from the other *A. pasteurianus*-related strains. Phylogenetic analysis based on the concatenated sequences of *dnaK*, *groEL*, and *rpoB* genes supported this findings, revealing the same phylogenetic lineages.

Recently, it has been suggested that less than 98.65–98.7% 16S rRNA gene sequence similarity can be used as a threshold to avoid laborious DDH for qualifying different species in bacterial classification [52,29,46]. Therefore, wet-lab DDH were performed among *A. pomorum* KCTC 22319^T, strain SLV-7^T, and *A. pasteurianus* strains KACC 13994^T, LMG 1590, and LMG 1591, showing more than 98.7% 16S rRNA gene sequence similarities in the phylogenetic tree. DDH experiments were carried out in triplicate using the DIG High Prime DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the procedure described previously [33]. Hybridization signals produced by the hybridization of probes to the homologous target DNA were considered 100%, and signal intensities derived from the hybridizations of serial dilutions were used as a standard for calculating DNA-DNA relatedness. The wet-lab DDH was performed reciprocally (*e.g.*, A × B and B × A). DDH values between strain SLV-7^T and the *A. pomorum* KCTC 22319^T and *A. pasteurianus* strains KACC 13994^T, LMG 1590, and LMG 1591 were 35.2 ± 4.5%, 21.3 ± 4.5%, 25.1 ± 10.9%,

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