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Report of eight unrecorded *Acetobacter* species in Korea, discovered during the survey in 2018–2019

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Acetic acid bacteria (AAB) convert ethanol to acetic acid through oxidation, and the fermentation pathway of AAB is important in the vinegar industry. The genus *Acetobacter* is the representative one of AAB, and several Korean traditional vinegars are produced using *Acetobacter* strains. Until now, four species in the genus *Acetobacter* were reported as native species in Korea. During the past two years, we isolated several AAB strains from fruits, flowers and fermented foods, and several AAB species unrecorded in Korea were found on the basis of 16S rRNA gene sequence analyses. In this study, we report eight *Acetobacter* species as native ones which are *A. fabarum* C10-3 (=KACC 21483) isolated from plumcot fruit (Naju-si), *A. lovaniensis* KDG-EC1 (=KACC 22697) isolated from diced radish kimchi (Naju-si), *A. okinawensis* GAM12-M2 (=KACC 22696) isolated from persimmon fruit (Sangju-si), *A. orientalis* FR32C4 (=KACC 22370) isolated from fruit of *Cudrania tricuspidata* (Jeonju-si), *A. papaya* FR35B3 (=KACC 22046) isolated from grape fruit (Yeongdong-gun), *A. suratthaniensis* GAM15-R2 (=KACC 22694) isolated from persimmon fruit (Gimje-si), *A. syzygii* C25-1 (=KACC 22048) isolated from peach fruit (Namwon-si) and *A. thailandicus* JDF1-M1 (=KACC 22693) isolated from plum fruit (Seoul).

Keywords: Acetic acid bacteria, *Acetobacter*, unrecorded species

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Introduction

Acetic acid bacteria (AAB) are commonly found in diverse sources including carbohydrate-, acid-, and alcohol-rich environments (Crotti *et al*., 2010). The most prominent feature of AAB is the capability to convert ethanol to acetic acid through oxidation (Wang *et al*., 2015). Acetic acid-producing capability of AAB makes them important to vinegar industry (Sengun and Karabivikli, 2011). The genus *Acetobacter* is the representative taxa of AAB and the type genus of the family *Acetobacteraceae* (Gillis and De Ley, 1980). *Acetobacter* comprises 33 species with the validly published names and *A. aceti* is the type species of the genus (https://lpsn.dsmz.de/). In Korea, two *Acetobacter* species *A. aceti* and *A. pasteurianus* are permitted as GRAS (generally recognized as safe) by the Ministry of Food and Drug Safety and can be used limitedly in the vinegar industry. On the other hand, some *Acetobacter* species are known to be phytopathogenic (Gosselé and Swings, 1986; Pal and Jalali, 2003) and import of them is under strict control by the Korean Animal and Plant Quarantine Agency. Four species, *A. ghanensis*, *A. oryzoeni*, *A. pasteurianus* and *A. tropicalis*, have been recorded as native species to date in Korea. We isolated *Acetobacter* strains from fruits, flowers and fermented foods in Korea over two years, and identified eight unrecorded species using the 16S rRNA gene sequence analysis. In this study, the taxonomic properties of eight *Acetobacter* species are presented.

Materials and Methods

Several environmental samples including fruits, flowers and fermented foods were collected and diluted in 0.85% NaCl (w/v) solution. The diluted samples were spread to isolate AAB strains on AAB medium [2.5% (w/v) D-mannitol, 0.5% (w/v) yeast extract, 0.3% (w/v) peptone, 0.2% (w/v) bromocresol purple and 1.5% (w/v) agar] and incubated at 28°C for five days. The isolates were then subcultured on the YPM medium [0.5% (w/v)

yeast extract, 0.3% (w/v) peptone, 2.5% (w/v) D-mannitol and 1.5% (w/v) agar] to get pure culture. All strains were maintained in glycerol suspension (15%, v/v) at −80°C.

The 16S rRNA gene sequencing was conducted by Genotech (Daejeon, Republic of Korea). The sequence similarity was calculated through the EzBioCloud (http://www.ezbiocloud.net/identify) (Yoon *et al*., 2017). Type strains of the *Acetobacter* species were obtained from EzBioCloud database (https://www.ezbiocloud.net/) (Yoon *et al*., 2017) and NCBI database (https://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic trees were reconstructed with three different algorithms, neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-likelihood (ML) (Felsenstein, 1981) and maximum-parsimony (MP) (Fitch, 1971) algorithm, in MEGA X (Kumar *et al*., 2018). Evolutionary distance matrices for the neighbor-joining and maximum likelihood analyses were evaluated using Tamura-Nei model. Bootstrap analyses with 1000 replicates were implemented for stability evaluation of tree topology (Felsenstein, 1985). Cellular morphology and cell size were examined using a phase-contrast microscope (AX10; Carl Zeiss) and a transmission electron microscope (LEO 912AB; LEO Electron) after being grown on YPM agar at 28°C for 2−3 days. Gram staining was carried out using a Gram staining kit (Sigma), according to the manufacturer's instructions. The biochemical characteristics were performed using catalase and oxidase test kits, API 20NE and API 50CH (bioMérieux) according to the manufacturer's instructions.

Results and Discussion

Eight bacterial strains isolated from fruits and kimchi were identified as unrecorded *Acetobacter* species in Korea on the basis of the 16S rRNA gene sequence analysis (Table 1). Seven *Acetobacter* species were isolated from fruit samples harvested between July and September. One unrecorded *Acetobacter* species was isolated from diced radish kimchi. The neighbor-joining phylogenetic tree for the isolated strains and type strains of the *Acetobacter* species are presented in Fig. 1. Cellular morphology and flagella presence, which were examined by phase-contrast microscope and TEM, were presented in Fig. 2. Eight *Acetobacter* species are coccoid-rod, rod-shaped or long rods. Flagellum or flagella were observed on six *Acetobacter* species including *Acetobacter fabarum* C10-3, but were not on *Acetobacter suratthaniensis* GAM15-R2 and *Acetobacter thailandicus* JDF1-M1 (Fig. 2). The absence of flagellum of two species was consistent with results from type strains of two *Acetobacter* species (Pitiwittayakul *et al*., 2015; 2016). Morphological and biochemical differences among eight *Acetobacter* species were sum-

Table 1. The taxonomic affiliations of isolated strains belonging to the genus *Acetobacter*.

Order	Family	Genus	Strain code	KACC ID	Identification	Isolation source	Location	Culture conditions
Rhodospirillales	*Acetobacteraceae*	*Acetobacter*	C10-3	21483	*Acetobacter fabarum*	Plumcot fruit	Naju-si, Jeollanam-do	YPM medium, 28°C, 2−3 days
			KDG-EC1	22697	*Acetobacter lovaniensis*	Diced radish kimchi	Naju-si, Jeollanam-do	
			GAM12-M2	22696	*Acetobacter okinawensis*	Persimmon fruit	Sangju-si, Gyeongsangbuk-do	
			FR32C4	22370	*Acetobacter orientalis*	*Cudrania tricuspidata* fruit	Jeonju-si, Jeollabuk-do	
			FR35B3	22046	*Acetobacter papaya*	Muscat grape fruit	Yeongdong-gun, Chungcheongbuk-do	
			GAM15-R2	22694	*Acetobacter suratthaniensis*	Persimmon fruit	Gimje-si, Jeollabuk-do	
			C25-1	22048	*Acetobacter syzygii*	Peach fruit	Namwon-si, Jeollabuk-do	
			JDF1-M1	22693	*Acetobacter thailandicus*	Plum fruit	Seoul-si	

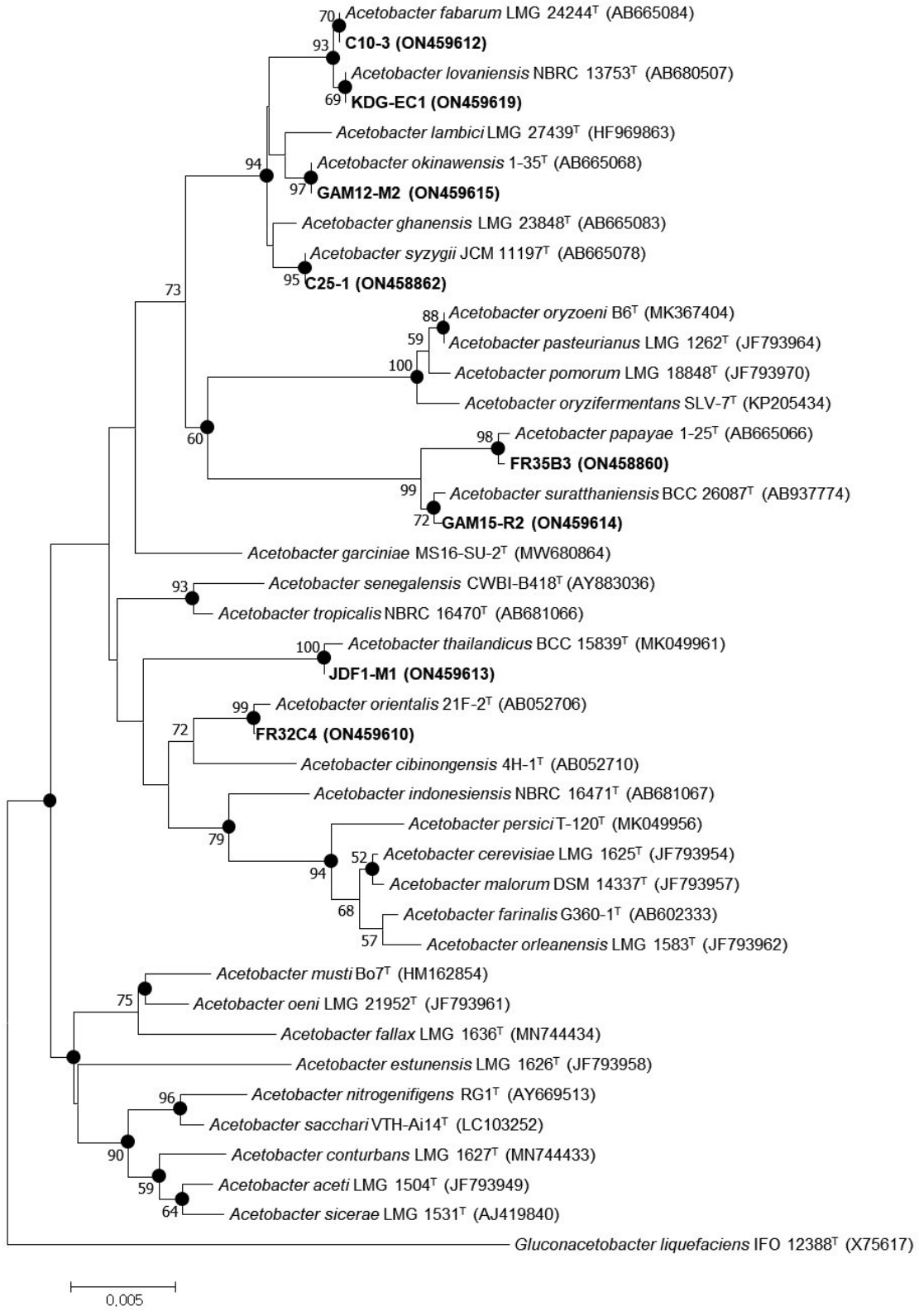

Fig. 1. Neighbor-joining phylogenetic tree showing the phylogenetic relationships of strains reported in this study and related species of *Acetobacter*, based on 16S rRNA gene sequences. Numbers on nodes correspond to bootstrap values for branches (1,000 replicates); only values over 50% are shown. Filled circles indicate the corresponding nodes that were also recovered in trees constructed using the maximum-likelihood and maximum-parsimony algorithms. *Gluconacetobacter liquefaciens* IFO 12388^T (X75617) was used as an outgroup. Scale bar, 0.005 substitutions per nucleotide.

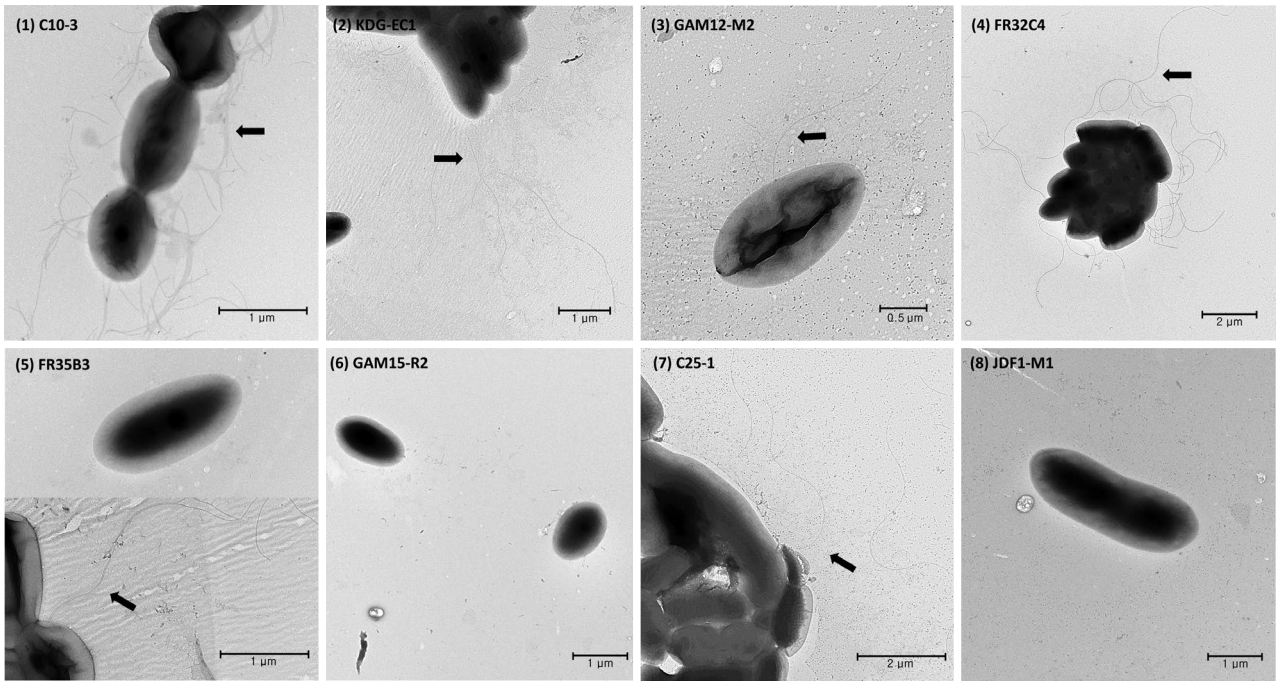

Fig. 2. Transmission electron micrographs of the isolated strains. Strains: 1, *Acetobacter fabarum* C10-3; 2, *Acetobacter lovaniensis* KDG-EC1; 3, *Acetobacter okinawensis* GAM12-M2; 4, *Acetobacter orientalis* FR32C4; 5, *Acetobacter papaya* FR35B3; 6, *Acetobacter suratthaniensis* GAM15-R2; 7, *Acetobacter syzygii* C25-1; 8, *Acetobacter thailandicus* JDF1-M1. Arrows indicate flagellum.

marized in Table 2. The detailed description of each of *Acetobacter* strains were described below.

Description of *Acetobacter fabarum* C10-3 (=KACC 21483)

Cells are Gram-stain-negative, motile, non-pigmented and coccoid rod (0.7−0.8 μm × 1.2−1.9 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5 mm. Catalase-positive and oxidase-negative. Positive for aesculin hydrolysis, but negative for indole production, arginine dihydrolase, urease and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose, D-melibiose, gentiobiose and D-fucose, and weakly produced from potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain C10-3 (=KACC 21483) was isolated from plumcot fruit sampled at Naju-si. The GenBank accession number for the 16S rRNA gene sequence of strain C10-3 is ON459612.

Description of *Acetobacter lovaniensis* KDG-EC1 (=KACC 22697)

Cells are Gram-stain-negative, motile, non-pigmented and rod (0.6−0.7 μm × 1.2−2.2 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5 mm. Catalase-positive and oxidase-negative. Positive for aesculin hydrolysis, but negative for indole production, arginine dihydrolase, urease and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose and D-fucose, and weakly produced from D-melibiose, gentiobiose and potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, D-turanose,

Table 2. Differential phenotypic characteristics among the isolated strains.

Characteristics	1	2	3	4	5	6	7	8
Cellular morphology	Coccoid rod	Rod	Coccoid rod	Rod	Coccid rod	Coccoid rod	Coccid rod	Rod or long rod
Cell size (μm)	0.7−0.8 × 1.2−1.9	0.6−0.7 × 1.2−2.2	0.85−0.95 × 1.5−2.5	0.8−1.0 × 1.5−2.4	0.7−0.8 × 1.2−2.0	0.8−0.9 × 1.3−2.3	0.8−0.9 × 1.2−1.8	0.9−1.2 × 2.0−8.0
Flagellum	+	+	+	+	+	−	+	−
Urease	−	−	−	+	+	+	−	−
Acid production								
D-Ribose	+	+	+	+	+	w	+	w
D-Mannose	+	+	+	+	+	w	+	+
Salicin	−	−	−	w	−	−	−	−
D-Melibiose	+	w	w	−	w	−	w	+
Gentiobiose	+	w	w	−	w	−	−	−
Potassium 5-ketogluconate	w	w	w	w	w	w	w	+

Strains: 1, *Acetobacter fabarum* C10-3; 2, *Acetobacter lovaniensis* KDG-EC1; 3, *Acetobacter okinawensis* GAM12-M2; 4, *Acetobacter orientalis* FR32C4; 5, *Acetobacter papaya* FR35B3; 6, *Acetobacter suratthaniensis* GAM15-R2; 7, *Acetobacter syzygii* C25-1; 8, *Acetobacter thailandicus* JDF1-M1. All strains are Gran-stain-negative, catalase-positive and oxidase-negative. +, Positive; w, weakly positive; −, negative.

D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain KDG-EC1 (=KACC 22697) was isolated from diced radish kimchi sampled at Naju-si. The GenBank accession number for the 16S rRNA gene sequence of strain KDG-EC1 is ON459619.

Description of *Acetobacter okinawensis* GAM12-M2 (=KACC 22696)

Cells are Gram-stain-negative, motile, non-pigmented and coccoid rod (0.85−0.95 μm × 1.5−2.5 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5 mm. Catalase-positive and oxidase-negative. Positive for aesculin hydrolysis, but negative for indole production, arginine dihydrolase, urease and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose and D-fucose, and weakly produced from D-melibiose, gentiobiose and potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain GAM12-M2 (=KACC 22696) was isolated from persimmon fruit sampled at Sangju-si. The GenBank accession number for the 16S rRNA gene sequence of strain GAM12-M2 is ON459615.

Description of *Acetobacter orientalis* FR32C4 (=KACC 22370)

Cells are Gram-stain-negative, motile, non-pigmented and rod (0.8−1.0 μm × 1.5−2.4 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5−1.0 mm. Catalase-positive and oxidase-negative. Positive for urease and aesculin hydrolysis, but negative for indole production, arginine dihydrolase and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose and D-fucose, and weakly produced from salicin and potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain FR32C4 (=KACC 22370) was isolated from fruit of *Cudrania tricuspidata* sampled at Jeonju-si. The GenBank accession number for the 16S rRNA gene sequence of strain FR32C4 is ON459610.

Description of *Acetobacter papaya* FR35B3 (=KACC 22046)

Cells are Gram-stain-negative, motile, non-pigmented and coccoid rod (0.7−0.8 μm × 1.2−2.0 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5 mm. Catalase-positive and oxidase-negative. Positive for urease and aesculin hydrolysis, but negative for indole production, arginine dihydrolase and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose and D-fucose, and weakly produced from D-melibiose, gentiobiose and potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain FR35B3 (=KACC 22046) was isolated from grape fruit sampled at Yeongdong-gun. The GenBank accession number for the 16S rRNA gene sequence of strain FR35B3 is ON458860.

Description of *Acetobacter suratthaniensis* GAM15-R2 (=KACC 22694)

Cells are Gram-stain-negative, non-motile, non-pigmented and coccoid rod (0.8−0.9 μm × 1.3−2.3 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5 mm. Catalase-positive and oxidase-negative. Positive for urease and aesculin hydrolysis, but negative for indole production, arginine dihydrolase and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-xylose, D-galactose, D-glucose and D-fucose, and weakly produced from D-ribose, D-mannose and potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain GAM15-R2 (=KACC 22694) was isolated from persimmon fruit sampled at Gimje-si. The GenBank accession number for the 16S rRNA gene sequence of strain GAM15-R2 is ON459614.

Description of *Acetobacter syzygii* C25-1 (=KACC 22048)

Cells are Gram-stain-negative, motile, non-pigmented and coccoid rod (0.8−0.9 μm × 1.2−1.8 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 0.5 mm. Catalase-positive and oxidase-negative. Positive for aesculin hydrolysis, but negative for indole production, arginine dihydrolase, urease and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose and D-fucose, and weakly produced from D-melibiose and potassium 5-ketogluconate, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain C25-1 (=KACC 22048) was isolated from peach fruit sampled at Namwon-si. The GenBank accession number for the 16S rRNA gene sequence of strain C25-1 is ON458862.

Description of *Acetobacter thailandicus* JDF1-M1 (=KACC 22693)

Cells are Gram-stain-negative, non-motile, non-pigmented and rod or long rod (0.9−1.2 μm × 2.0−8.0 μm) after 2 days incubation on YPM medium at 28°C. Colonies are round, raised and beige colored with a diameter of 1.0 mm. Catalase-positive and oxidase-negative. Positive for aesculin hydrolysis, but negative for indole production, arginine dihydrolase, urease and gelatin hydrolysis. Does not assimilate any of the substrates embedded on API 20 NE test strip. Acid is produced from L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, D-melibiose, D-fucose and potassium 5-ketogluconate, and weakly produced from D-ribose, but not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygd-

alin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. Strain JDF1-M1 (=KACC 22693) was isolated from plum fruit sampled at Seoul. The GenBank accession number for the 16S rRNA gene sequence of strain JDF1-M1 is ON459613.

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