Identification of *Acetobacter*, *Gluconobacter*, and *Asaia* Strains Isolated in Thailand Based on 16S-23S rRNA Gene Internal Transcribed Spacer Restriction and 16S rRNA Gene Sequence Analyses

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Twenty-six strains of acetic acid bacteria were isolated from fruits, flowers and related materials collected in Thailand. They were divided into three genera, *Acetobacter*, *Gluconobacter* and *Asaia*, by phenotypic characterization and 16S rRNA gene sequence analyses. On the basis of 16S-23S rRNA gene internal transcribed spacer (16S-23S rDNA ITS) restriction and 16S rRNA gene sequence analyses, fourteen isolates assigned to the genus *Acetobacter* were divided into five groups: 1) Group 1A or *A. tropicalis* (one isolate); 2) Group 2A or *A. orientalis* (four isolates); 3) Group 3A or *A. pasteurianus* (five isolates); 4) Group 4A or *A. syzygii* (one isolate); and 5) Group 5A or *A. ghanensis* (three isolates). The eleven isolates assigned to the genus *Gluconobacter* were divided into three groups: 6) Group 1B or *G. frateurii* (four isolates); 7) Group 2B or *G. japonicus* (six isolates); and 8) Group 3B or unidentified (one isolate). The remaining isolate was placed into: 9) Group 1C or unidentified, which was assigned to the genus *Asaia* and considered to constitute a new species on the basis of the 16S rRNA gene sequence analysis and DNA-DNA hybridization.

Key words: Acetic acid bacteria, *Acetobacter*, *Asaia*, *Gluconobacter*, 16S-23S rDNA ITS restriction analyses

Acetic acid bacteria (AAB) are Gram-negative, aerobic, rod-shaped bacteria assigned to the *Alphaproteobacteria*. These bacteria have undergone considerable changes, consequently increasing the number of novel genera and species. Presently, there are a total of 11 genera (Euzéby, 2008, URL: <http://www.bacterio.net>): *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, and *Tanticharoenia* (7, 10, 12, 13, 18, 19, 25, 28, 33, 38, 39, 42–45). These bacterial strains have been isolated from fruits, flowers, vinegar, grapes, wine, sake, cider, beer, soft drink, nata, sugarcane, bee hives, tea fungus, plant materials and so on (13).

Many new acetic acid bacteria have been reported in Thailand, including the genera *Neoasaia* and *Tanticharoenia*, and the species *Asaia krungthepensis*, *Gluconobacter albidus*, *Gluconobacter thailandicus*, *Neoasaia Chiangmaiensis*, *Gluconobacter kondonii*, *Gluconobacter roseus*, *Asaia lannaensis*, *Tanticharoenia sakaeratensis*, and *Gluconobacter japonicus* (11, 21–24, 31, 39, 40, 42–45). In addition, *Acetobacter pasteurianus*, *Gluconacetobacter liquefaciens*, *Asaia bogorensis*, *Asaia siamensis*, *Acetobacter orientalis*, *Acetobacter lovaniensis*, *Gluconobacter frateurii* (15, 20, 27, 41, 44), and all seven groups of *Gluconobacter* except *Gluconobacter*

cerinus (9) isolated from fruits, flowers and other materials collected in Thailand have been found. In the course of isolating, identifying and classifying acetic acid bacteria in Thailand, we have continued the taxonomic study of Thai isolates based on 16S-23S rRNA gene internal transcribed spacer (16S-23S rDNA ITS) restriction and 16S rRNA gene sequence analyses as well as phenotypic characterization and DNA-DNA hybridization. This paper is concerned with the identification of novel isolates from fruits, flowers, and other materials.

Materials and Methods

Isolation of acetic acid bacteria and reference strains

Thirteen fruits, two other materials collected at markets and two kinds of flowers that fell from trees were cut into small pieces and enriched using a glucose/ethanol/yeast extract (GEY) medium. An isolation source was incubated at pH 4.5 and 30°C for 3–5 days in a liquid medium (15 mL tube⁻¹) composed of 2.0% D-glucose, 5.0% ethanol and 1.0% yeast extract (all by w/v). When microbial growth was seen, the culture was streaked onto a GEY-agar plate containing 0.3% CaCO₃ (w/v) (36). Acetic acid bacteria were selected as acid-producing bacterial strains, which formed clear zones around colonies on the agar plate. *A. indonesiensis* NBRC 16471^T, *A. cibinongensis* NBRC 16605^T, *A. lovaniensis* NBRC 13753^T, *A. tropicalis* NBRC 16470^T, *A. orientalis* NBRC 16606^T, *A. pasteurianus* TISTR 1056^T, *A. aceti* NBRC 14818^T, *A. syzygii* NBRC 16604^T, *A. ghanensis* LMG 23848, *G. frateurii* NBRC 3264^T, *G. thailandicus* BCC 14116^T, *G. japonicus* NBRC 3271^T, *G.*

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cerinus NBRC 3267^T, *G. oxydans* NBRC 14819^T, *G. albidus* NBRC 3250^T, *G. kondonii* NBRC 3266^T, *Asaia lannaensis* BCC 15733^T, *Asaia bogorensis* BCC 12264^T, *Asaia siamensis* BCC 12268^T, and *Asaia krungthepensis* BCC 12978^T were used as reference strains.

Phenotypic characterization

Phenotypic characterization was carried out by incubating isolates at 30°C and pH 6.8 for two days on glucose/yeast extract/peptone/glycerol (GYPG) agar, which was composed of 1.0% D-glucose, 1.0% glycerol, 0.5% yeast extract, 1.0% peptone, and 1.5% agar (all by w/v), unless otherwise mentioned. For the Gram staining of bacterial cells, the Hucker-Conn modified method was used (8). Physiological and biochemical characterizations were made by the methods of Asai *et al.* (1), Gosselé *et al.* (6), and Yamada *et al.* (36). Isolates were grown in GEY medium on a rotary shaker (150–200 rpm) at 30°C for 24 h.

Chemotaxonomic characterization

AAB were grown in GEY broth on a rotary shaker (150–200 rpm) at 30°C for 24 h. Ubiquinone was extracted from freeze-dried cells by shaking with a mixture of chloroform-methanol (2:1, v/v). Cells were removed by filtration, and the combined filtrates were evaporated dry under reduced pressure on a rotary evaporator. The resulting residue was dissolved in a small volume of acetone,

and subjected to thin-layer chromatography on a silica gel plate (20×20 cm, silica gel 60F₂₅₄, Art 5715, E. Merck, Darmstadt, Germany) with a solvent system of pure benzene (35). The purified ubiquinone preparation was subjected to reversed-phase paper chromatography (35, 37) and to high performance liquid chromatography (29).

16S rRNA gene sequence analyses

The 16S rRNA genes of strains were amplified by PCR with *Taq* DNA polymerase and primers; 20F (5'-GAGTTTGATCCTGGCT-CAG'-3, the *Escherichia coli* numbering system (2) and 1500R (5'-GTTACCTTGTTACGACTT'-3) and sequenced, as described previously (31, 40, 43). The sequencing was carried out with an ABI PRISM BigDye Terminator v3.1 cycle sequencing kit on an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The following six primers were used for 16S rRNA gene sequencing; 20F, 1500R, 520F (5'-CAGCAG-CCGCG-GTAATAC-3', positions 519–536), 520R (5'-GTATTACCG-CGGCTGCTG-3', positions 536–519), 920F (5'-AAACTCAAAT-GAATTGACGG-3', positions 907–926), and 920R (5'-CCGT-CAATTCAT-TTGAAGTTT-3', positions 926–907). Multiple alignments of the 16S rRNA gene sequences determined were performed with the program CLUSTAL X (version 1.83) (32). Gaps and ambiguous bases were eliminated from the calculation. Distance

Fig. 1. A phylogenetic tree based on 16S rRNA gene sequences for Thai isolates assigned to the genus *Acetobacter*. The phylogenetic tree was constructed by the neighbor-joining method. Numerals (%) at nodes indicate bootstrap values derived from 1,000 replications.

Fig. 2. A phylogenetic tree based on 16S rRNA gene sequences for Thai isolates assigned to the genera *Gluconobacter* and *Asaia*. The phylogenetic tree was constructed by the neighbor-joining method. Numerals (%) at nodes indicate bootstrap values derived from 1,000 replications.

matrices for the aligned sequences were calculated by the two-parameter method of Kimura (14). A phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbor-joining method of Saitou and Nei (26) with the program MEGA (version 4.0) (30). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (5) based on 1,000 replications. Pair-wise 16S rRNA gene sequence similarities were calculated between phylogenetically related strains (1,381–1,476 bases of isolates GB23-1, KL13-3, LG5-1, SL22-1, PH108-1, GB222-1, GB132-1, RB88-1, and GB23-3, and 692–804 bases of the remaining isolates).

Restriction analysis of 16S-23S rDNA ITS

The purified PCR products were separately digested with restriction endonucleases; *Hpa*II and *Hae*III (New England BioLabs, Beverly, MA, USA) (15) in case of the *Acetobacter* strains, and *Mbo*II (Fermentas, Hanover, MD, USA), *Bsp*1286I (New England BioLabs), *Taq*I (Fermentas) and *Bst*NI (Fermentas) (20, 41) in the case of *Gluconobacter* strains. The resulting reaction products were analyzed by 2.5% (w/v) agarose gel electrophoresis developed at 100 V for 40 min in 1× Tris-acetate running buffer.

DNA-DNA hybridization

Bacterial DNAs were prepared, as described previously (31). DNA-DNA hybridization was carried out by the photobiotin-labeling method using microplate wells (4). Levels of DNA-DNA hybridization were determined colorimetrically (34). The color

intensity was measured at A_{450} on a model VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences for the twenty-six strains in this study have been deposited in DDBJ under the accession numbers AB470916 to AB470920, and AB489237 to AB489245 for *Acetobacter* strains; AB470467, AB470921, AB470922, and AB489246 to AB489253 for *Gluconobacter* strains; and AB469044 for the *Asaia* strain as indicated in the dendrogram (Figs. 1 and 2).

Results and Discussion

The twenty-six Thai isolates were Gram-negative and aerobic rods, produced catalase but not oxidase, and showed clear zones on GEY/CaCO₃ agar plates and growth at pH 3.5. Fourteen of the isolates were assigned to the genus *Acetobacter*, since acetate and lactate were intensely oxidized to CO₂ and H₂O, and Q-9 was recognized as a major quinone (39) (Table 1). Eleven isolates were assigned to the genus *Gluconobacter*, since acetate and lactate were not oxidized and Q-10 was recognized as a major quinone (39). One isolate was assigned to the genus *Asaia*, since the isolate grew on 30% D-glucose, showed weak oxidation of acetate and lactate, and had Q-10 as a major quinone (39). In addition, the

Table 1. Characteristics differentiating Thai isolates assigned to the genera *Acetobacter*, *Gluconobacter*, and *Asaia*

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Oxidation of																				
Acetate	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	w	w	w	w	w
Lactate	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	w	w	w	w	w
Acetic acid from ethanol on ethanol/CaCO ₃ agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	w
Growth on																				
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Mannitol agar	-	w	-	w	-	w	w	w	w	w	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 40°C	-	w	-	w	+	+	+	+	-	w	-	-	-	-	w	-	-	-	-	w
Production of																				
2-Keto-D-gluconate	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
5-Keto-D-gluconate	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Acid production from																				
meso-Erythritol	-	-	-	-	+	+	-	-	-	-	w	w	+	w	-	+	+	+	+	w
Maltose	w	-	-	-	-	-	+	+	-	-	w	w	-	-	-	-	-	-	w	w
Raffinose	w	-	-	w	w	w	-	-	-	-	-	-	w	w	-	w	+	+	w	w
Growth on																				
meso-Erythritol	-	-	-	-	+	+	w	w	-	-	-	-	+	+	w	+	+	+	+	w
D-Arabitol	-	-	-	-	-	-	-	-	-	-	+	+	+	+	w	w	+	+	+	w
L-Arabitol	-	-	-	-	-	-	-	-	-	-	+	+	w	w	w	w	+	+	+	w
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+

Strain: 1, *A. tropicalis* NBRC 16470^T; 2, Group 1A (1 isolate); 3, *A. orientalis* NBRC 16606^T; 4, Group 2A (4 isolates); 5, *A. pasteurianus* TISTR 1056^T; 6, Group 3A (5 isolates); 7, *A. syzygii* NBRC 16604^T; 8, Group 4A (1 isolate); 9, *A. ghanensis* LMG 23848^T; 10, Group 5A (3 isolates); 11, *G. frateurii* NBRC 3264^T; 12, Group 1B (4 isolates); 13, *G. japonicus* NBRC 3271^T; 14, Group 2B (6 isolates); 15, Group 3B (1 isolate); 16, *Asaia lannaensis* BCC 15733^T; 17, *Asaia bogorensis* BCC 12264^T; 18, *Asaia siamensis* BCC 12268^T; 19, *Asaia krungthepensis* BCC 12978^T; 20, Group 1C (1 isolate); +, positive; w, weakly positive; -, negative. Numbers in parentheses indicate the number of isolates showing a weakly positive or negative reaction.

16S rRNA gene sequence analysis supported that they belong to the genera *Acetobacter*, *Gluconobacter*, and *Asaia* (Figs. 1 and 2).

Isolates assigned to the genus *Acetobacter*

The fourteen isolates that were assigned to the genus *Acetobacter* were divided into five groups, 1A, 2A, 3A, 4A, and 5A, by the 16S rRNA gene sequence analysis (Fig. 1) and 16S-23S rDNA ITS restriction analysis with restriction endonucleases *Hpa*II and *Hae*III (Table 2 and Fig. 3).

In a phylogenetic tree based on 16S rRNA gene sequences (1,431 bp), the isolate GB23-1 (Group 1A) was located within the cluster of *A. tropicalis* (Fig. 1) and showed 99.8% pair-wise 16S rRNA gene sequence similarity with the type strain of *A. tropicalis*. It also gave restriction patterns that coincided with those of the type strain (Table 2 and Fig. 3). It tested positive for catalase activity, the oxidation of ethanol to acetic acid, and the oxidation of acetate and lactate to carbon dioxide and water. D-Gluconate and 2-keto-D-gluconate were produced from D-glucose. The isolate tested negative for oxidase activity and ketogenesis from glycerol. It produced acids from L-arabinose, D-galactose, D-glucose, and D-mannose, and to less of an extent, from maltose, raffinose, and D-xylose, but not at all from D-arabinose, dulcitol, meso-erythritol, D-fructose, glycerol, lactose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sorbitol, and sucrose. It did not grow on meso-erythritol, D-arabitol, L-arabitol or meso-ribitol. It showed almost the same phenotypic characteristics as *A. tropicalis* NBRC 16470^T (Table 1). Therefore, GB23-1 isolated from a flower of *Spathodea campanulata* was identified as *A. tropicalis* (16).

Isolates of Group 2A included LP120-2, KL13-2, KL13-3, and MM129-2. In a phylogenetic tree based on 16S rRNA gene sequences (1,381 bp), all the isolates were located within the cluster of *A. orientalis* (Fig. 1). Additionally, LP120-2, KL13-2, KL13-3, and MM129-2 had 99.8, 99.6, 99.9, and 99.5% pair-wise 16S rRNA gene sequence similarity with the type strain of *A. orientalis*, respectively. They also showed restriction patterns that coincided with those of the type strain of *A. orientalis* (Table 2 and Fig. 3). All the isolates of Group 2A produced 2-keto-D-gluconate from D-glucose. They produced acid from D-glucose, and in some cases, from mannose. Some isolates produced acids weakly from L-arabinose, raffinose, and xylose, but none produced acid from D-arabinose, dulcitol, meso-erythritol, D-galactose, D-fructose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sorbitol, or sucrose. The isolates did not grow on meso-erythritol, D-arabitol, L-arabitol and meso-ribitol. They showed almost the same phenotypic characteristics as *A. orientalis* NBRC 16606^T (Table 1). Therefore, the four isolates were identified as *A. orientalis* (17). They were found in fruits of kaffir lime, in musk-melon and in loog-pang khaomak (Table 2).

Isolates of Group 3A included LG5-1, LG6-1, MG70-1, SL21-2, and BB90-1. In a phylogenetic tree based on 16S rRNA gene sequences (1,460 bp), all of the isolates of Group 1A were located within the cluster of *A. pasteurianus* (Fig. 1). Additionally, LG5-1, LG6-1, MG70-1, SL21-2, and BB90-1 had 99.9% pair-wise 16S rRNA gene sequence similarity with the type strain of *A. pasteurianus*. They

Table 2. Identification of Thai isolates based on 16S-23S rDNA ITS restriction analysis

Isolate	Isolation source		Restriction pattern by digestion with					
	Source	Province	<i>Hpa</i> II	<i>Hae</i> III	<i>Mbo</i> II	<i>Bsp</i> 1286I	<i>Taq</i> I	<i>Bst</i> NI
GB23-1	<i>Spathodea campanulata</i> ¹		<i>At</i>	<i>At</i>				
LP120-2	Loog-pang khaomak ³	Pathumthani	<i>Ao</i>	<i>Ao, Ac, Aa</i>				
KL13-2, KL13-3	Kaffir lime ²	Bangkok	<i>Ao</i>	<i>Ao, Ac, Aa</i>				
MM129-2	Musk-melon ²	Bangkok	<i>Ao</i>	<i>Ao, Ac, Aa</i>				
LG5-1, LG6-1	Longan ²	Bangkok	<i>Ap</i>	<i>Ap, Ai, At</i>				
MG70-1	Mango ²	Bangkok	<i>Ap</i>	<i>Ap, Ai, At</i>				
SL21-2	Sapodilla ²	Bangkok	<i>Ap</i>	<i>Ap, Ai, At</i>				
BB90-1	Banana ²	Bangkok	<i>Ap</i>	<i>Ap, Ai, At</i>				
SL22-1	Sapodilla ²	Bangkok	<i>As</i>	<i>As, Ag, Al</i>				
TM7-1, TM7-3	Tomato ³	Bangkok	<i>Ag</i>	<i>As, Ag, Al</i>				
PH108-1	Peach ²	Pathumthani	<i>Ag</i>	<i>As, Ag, Al</i>				
AP59-1, AP59-2	Apple ²	Bangkok			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gc, Ga, Gk</i>	<i>Gf, Gt</i>
GB222-1	Guava ²	Bangkok			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gc, Ga, Gk</i>	<i>Gf, Gt</i>
GB223-3	Sugar apple ²	Bangkok			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gc, Ga, Gk</i>	<i>Gf, Gt</i>
F143-1	<i>Canna indica</i> ¹	Bangkok			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gj, Go</i>	<i>Gj</i>
GB132-1	Manila tamarind ²	Bangkok			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gj, Go</i>	<i>Gj</i>
GB125, GB126, GB127	Plum mango ²	Nakhonpathom			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gj, Go</i>	<i>Gj</i>
GB223-2	Sugar apple ²	Bangkok			<i>Gf, Gt, Gj</i>	<i>Gf, Gt, Gj</i>	<i>Gj, Go</i>	<i>Gf, Gt, Gj</i>
RB88-1	Rambutan ²	Bangkok			<i>Gu</i>	<i>Gu</i>	<i>Gj, Go</i>	<i>Gu</i>
GB23-3	<i>Spathodea campanulata</i> ¹	Bangkok			–	–	–	–

¹Flower, ²Fruit, ³Other materials; *Aa*, *A. aceti*; *Ac*, *A. cibinongensis*; *At*, *A. tropicalis*; *Ao*, *A. orientalis*; *As*, *A. syzygii*; *Ap*, *A. pasteurianus*; *Ag*, *A. ghanensis*; *Al*, *A. lovaniensis*; *Ai*, *A. indonesiensis*; *Go*, *G. oxydans*; *Gf*, *G. frateurii*; *Gt*, *G. thailandicus*; *Gj*, *G. japonicus*; *Gu*, unidentified; –, not tested.

(a) *Hpa*II **(b) *Hae*III**

Fig. 3. Restriction of 16S-23S rDNA ITS PCR products of Thai isolates assigned to the genus *Acetobacter*. The restriction patterns were obtained by digestion of 16S-23S rDNA ITS PCR products with *Hpa*II (a) and *Hae*III (b). For the estimation of digestion fragments produced from 16S-23S rDNA ITS PCR products, 50-bp DNA markers were used in the agarose gel electrophoresis. Strain: 1, *A. indonesiensis* NBRC 16471^T; 2, *A. cibinongensis* NBRC 16605^T; 3, *A. lovaniensis* NBRC 13753^T; 4, *A. tropicalis* NBRC 16470^T; 5, *A. orientalis* NBRC 16606^T; 6, *A. pasteurianus* TISTR 1056^T; 7, *A. aceti* NBRC 14818^T; 8, *A. syzygii* NBRC 16604^T; 9, *A. ghanensis* LMG 23848^T; 10, GB23-1 (Group 1A); 11, KL13-3 (Group 2A); 12, LG5-1 (Group 3A); 13, SL22-1 (Group 4A); 14, PH108-1 (Group 5A).

showed restriction patterns that coincided with those of *A. pasteurianus* TISTR 1056^T, when digested with *Hpa*II and *Hae*III (Table 2 and Fig. 3). They did not produce 2-keto-D-gluconate from D-glucose. All grew at 40°C. Acid was produced from L-arabinose, *meso*-erythritol, D-fructose, D-galactose, D-glucose, D-mannose, D-melibiose, and D-xylose, and weakly from D-arabinose, glycerol, D-sorbitol, and sucrose. Some produced acid weakly from dulcitol and raffinose but none produced acid from lactose, maltose, D-mannitol, L-rhamnose, or L-sorbose. They grew on *meso*-erythritol, but

not on D-arabitol, L-arabitol and *meso*-ribitol. They showed almost the same phenotypic characteristics as *A. pasteurianus* TISTR 1056^T (Table 1). The five isolates were therefore identified as *A. pasteurianus* (15). The isolates were found in fruits of longan fruit, mango, sapodilla and banana (Table 2).

Group 4A contained only one isolate SL22-1. In a phylogenetic tree based on 16S rRNA gene sequences (1,412 bp), the isolate was located within the cluster of *A. syzygii* (Fig. 1) and showed 99.9% pair-wise 16S rRNA gene sequence simi-

larity to the type strain of *A. syzygii*. It had restriction patterns that coincided with those of *A. syzygii* NBRC 16604^T (Table 2 and Fig. 3). All grew at 40°C. The isolate produced acids from D-glucose and maltose but not from L-arabinose, D-arabinose, dulcitol, *meso*-erythritol, D-fructose, D-galactose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, raffinose, L-sorbose, D-sorbitol, sucrose, and D-xylose. It grew weakly on *meso*-erythritol, but did not grow on D-arabitol, L-arabitol or *meso*-ribitol. It showed the same phenotypic characteristics as *A. syzygii* NBRC 16604^T (Table 1). Therefore, the isolate from sapodilla was identified as *A. syzygii* (17).

Isolates of Group 5A included TM7-1, TM7-3, and PH108-1. In a phylogenetic tree based on 16S rRNA gene sequences (1,442 bp), all the isolates were located within the cluster of *A. ghanensis* (Fig. 1). Additionally, TM7-1, TM7-3, and PH108-1 had 99.9% pair-wise 16S rRNA gene sequence similarity with the type strain of *A. ghanensis*. They showed restriction patterns that coincided with those of *A. ghanensis* LMG 23848^T (Table 2 and Fig. 3). The isolates produced acid from D-glucose and to a lesser extent, from D-arabinose and D-sorbitol. Some produced acids from L-arabinose, D-fructose, D-mannose, D-melibiose and D-xylose but none did so from *meso*-erythritol, dulcitol, D-galactose, glycerol, lactose, maltose, D-mannitol, L-rhamnose, raffinose (one weakly), L-sorbose, or sucrose. They did not grow on *meso*-erythritol, D-arabitol, L-arabitol and *meso*-ribitol (one weakly). They showed almost the same phenotypic characteristics as *A. ghanensis* LMG 23848^T (Table 1). The three isolates were therefore identified as *A. ghanensis* (3). They were found in fruits of peach and tomato (Table 2).

Isolates assigned to the genus *Gluconobacter*

The eleven isolates that were assigned to the genus *Gluconobacter* were divided into three groups, 1B, 2B, and 3B, by the 16S rRNA gene sequence analysis (Fig. 2) and 16S-23S rDNA ITS restriction analysis with the endonucleases *Mbo*II, *Bsp*1286I, *Taq*I, and *Bst*NI (Table 2 and Fig. 4).

Group 1B was composed of four isolates, GB222-1, GB223-3, AP59-1, and AP59-2. In a phylogenetic tree based on 16S rRNA gene sequences (1,476 bp), all the isolates were located within the cluster of *G. frateurii* (Fig. 2). GB222-1, GB223-3, AP59-1, and AP59-2 had 99.7, 99.9, 99.9, and 99.9% pair-wise 16S rRNA gene sequence similarity with the type strain of *G. frateurii*, respectively. All the isolates gave the same restriction patterns as the type strain of *G. frateurii* (Table 2 and Fig. 4) (20–22, 24, 31, 41). The isolates produced dihydroxyacetone from glycerol and grew at 30°C. Some did not grow at 37°C. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced from D-glucose, but not 2,5-diketo-D-gluconate. They produced acids from L-arabinose, D-galactose, D-glucose, *meso*-erythritol (weakly), and D-xylose (weakly), but not from D-arabinose, dulcitol, D-fructose, lactose, D-mannose, D-mannitol, D-melibiose, L-rhamnose, raffinose, L-sorbose, and D-sorbitol. Some produced acids from glycerol, maltose (weakly), and sucrose. The isolates showed growth on D-arabitol, L-arabitol and *meso*-ribitol (weakly) but not on *meso*-erythritol, being different from strains of *G. oxydans* and *G. cerinus* (31). They showed almost the same phenotypic characteristics as *G. frateurii* NBRC 3264^T (Table 1). The five isolates of Group 1B were therefore identified as *G. frateurii* (20–22, 24, 31, 41). They were found in fruits of guava, apple and sugar apple.

Fig. 4. Restriction of 16S-23S rDNA ITS PCR products of Thai isolates assigned to the genus *Gluconobacter*. The restriction patterns were obtained by digestion of 16S-23S rDNA ITS PCR products with *Mbo*II (a), *Bsp*1286I (b), *Taq*I (c) and *Bst*NI (d). For the estimation of the resulting restriction fragments, 50-bp DNA markers were used in agarose gel electrophoresis. Strain: 1, *G. frateurii* NBRC 3264^T; 2, *G. thailandicus* BCC 14116^T; 3, *G. japonicus* NBRC 3271^T; 4, *G. cerinus* NBRC 3267^T; 5, *G. oxydans* NBRC 14819^T; 6, *G. albidus* NBRC 3250^T; 7, *G. kondonii* NBRC 3266^T; 8, GB222-1 (Group 1B); 9, GB132-1 (Group 2B); 10, RB88-1 (Group 3B).

Group 2B was composed of six isolates, GB132-1, F143-1, GB125, GB127, and GB223-2. In a phylogenetic tree based on 16S rRNA gene sequences (1,409 bp), the isolates were located within the cluster of *G. japonicus* (Fig. 2). GB132-1, F143-1, GB125, GB126, GB223-2, and GB127 had 99.9, 99.8, 99.8, 99.9, 99.7, and 99.8% pair-wise 16S rRNA gene sequence similarity with the type strain of *G. japonicus* respectively. All the isolates gave the same restriction patterns as the type strain of *G. japonicus* (Table 2 and Fig. 4) (20, 24, 31, 41). The isolates produced dihydroxyacetone weakly from glycerol. They produced acids from D-glucose and *meso*-erythritol (weakly), and in some cases, from L-arabinose, D-galactose, glycerol, D-mannose, D-melibiose, sucrose, and D-xylose. Some isolates produced acids weakly from D-arabinose, dulcitol, D-fructose, lactose, and D-mannitol, but none produced acids from maltose, raffinose, L-rhamnose, L-sorbose, or D-sorbitol. They grew on *meso*-erythritol, D-arabitol, L-arabitol (weakly), and *meso*-ribitol. The isolates of Group 2B had a different type of restriction pattern, designated the *d* type, from the type strains of *G. frateurii* and *G. thailandicus*, all of which gave *c* type patterns in a 16S-23S rDNA ITS restriction analysis with the endonuclease *Taq*I. All the isolates were distinguished from *G. frateurii* by the ability to grow on *meso*-erythritol and produce acid (weakly) from raffinose. They showed almost the same phenotypic characteristics as *G. japonicus* NBRC 3271^T (Table 1) and were identified as *G. japonicus* (20, 24, 31, 41). The isolates were found in fruits of manila tamarind, plum mango, and sugar apple and in the flower of *Canna indica*.

Group 3B was composed of one isolate, RB88-1. The calculated pair-wise 16S rRNA gene sequence similarity of RB88-1 for 1,410 bases was 98.3, 98.2, 98.6, 98.6, 97, 97, 97.2, 97.2, and 97.2%, respectively, compared to the type strains of *G. thailandicus*, *G. cerinus*, *G. japonicus*, *G. frateurii*, *G. oxydans*, *G. roseus*, *G. albidus*, *G. kondonii*, and *G. sphaericus* (Fig. 2). The isolate was distinguished from the type strains of *G. oxydans*, *G. cerinus*, *G. albidus*, *G. frateurii*, *G. japonicus*, *G. thailandicus*, and *G. kondonii* by digestion with *Mbo*II, *Bsp*1286I, *Taq*I and *Bst*NI (Table 2 and Fig. 4). The isolate produced dihydroxyacetone weakly

from glycerol. It grew on mannitol agar and at 37°C but not on glutamate agar. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced from D-glucose, but not 2,5-diketo-D-gluconate. The isolate produced acids from D-arabinose, L-arabinose, dulcitol, D-fructose, D-glucose, D-melibiose, D-sorbitol, L-sorbose, sucrose and D-xylose, and to a lesser extent, from D-galactose, lactose, D-mannose, D-mannitol and L-rhamnose, but not at all on *meso*-erythritol, maltose, or raffinose. The isolate grew on *meso*-ribitol but only weakly on L-arabitol, D-arabitol, and *meso*-erythritol. It differed from *G. frateurii* NBRC 3264^T and *G. japonicus* NBRC 3271^T in its ability to grow and produce acid on *meso*-erythritol (Table 1). In addition, RB88-1 showed low DNA-DNA relatedness (6.2–17.7%) with the type strains of *Gluconobacter* (Table 3). From the data obtained, the isolate was considered to be a novel species of the genus *Gluconobacter* (20–22, 24). It was found in the fruit of rambutan.

Isolate assigned to the genus *Asaia*

Group 1C was composed of only one isolate, GB23-3, which was distinguished phylogenetically from the type strains of the four *Asaia* species on the basis of the 16S rRNA gene sequence analysis (1,414 bp) (Fig. 2). The isolate was located in the cluster of the genus *Asaia* and had 99.2, 99.2, 99.1, 99.0, and 98.7% pair-wise sequence similarity, respectively, to the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, *Asaia lannaensis*, and *Swaminathanian salitolerans*. The isolate produced acetic acid from ethanol on ethanol/calcium carbonate agar differing from the type strains of *Asaia bogorensis*, *Asaia siamensis* and *Asaia krungthepensis*, but similar to *Asaia lannaensis*. The isolate grew on glutamate agar and mannitol agar. It produced 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate. Acid was produced from D-arabinose, L-arabinose, dulcitol, D-fructose, D-glucose, glycerol, D-melibiose, D-sorbitol, L-sorbose, sucrose, and D-xylose, but only weakly from *meso*-erythritol, D-galactose, lactose, maltose, D-mannose, D-mannitol, raffinose, and L-rhamnose. It grew on *meso*-ribitol but grew weakly on *meso*-erythritol, D-arabitol, and L-arabitol. This strain could be differentiated from related *Asaia* species by the acid it produced from ethanol and maltose (Table 1). In addition, GB23-3 showed low DNA-DNA relatedness (35.0–58.7%)

Table 3. DNA-DNA relatedness of isolate RB88-1 and the type strains of *Gluconobacter* species

Isolate and strain	%DNA-DNA relatedness with labeled strains*			
	1	2	3	4
RB88-1	100	6.2	8.2	7.3
<i>G. thailandicus</i> BCC 14116 ^T	11.8	100	52.7	64.7
<i>G. cerinus</i> NBRC 3267 ^T	8.4	51.6	100	43.7
<i>G. frateurii</i> NBRC 3264 ^T	8.5	61.3	48.5	100
<i>G. japonicus</i> NBRC 3271 ^T	17.7	56	52	62.4
<i>G. roseus</i> NBRC 3990 ^T	13.8	46.3	26.2	39.9
<i>G. albidus</i> NBRC 3250 ^T	15.5	38.4	36.2	30.1
<i>G. kondonii</i> NBRC 3266 ^T	16.1	42.8	44.8	33.3
<i>G. sphaericus</i> NBRC 12467 ^T	14.4	47.4	25.5	37.2
<i>G. oxydans</i> NBRC 14819 ^T	13.1	45.9	28.2	35.8

*Strain: 1, RB88-1; 2, *G. thailandicus* BCC 14116^T; 3, *G. cerinus* NBRC 3267^T; 4, *G. frateurii* NBRC 3264^T.

Table 4. DNA-DNA relatedness of isolate GB23-3 and the type strains of *Asaia*, *Swaminathanian*, and *Neoasaia* species

Isolate and strain	%DNA-DNA relatedness with labeled strains*				
	1	2	3	4	5
GB23-3	100	28.3	45.5	55.7	40.6
<i>Asaia bogorensis</i> BCC 12264 ^T	35	100	51.6	47.5	67.9
<i>Asaia krungthepensis</i> BCC 12978 ^T	47.9	56.2	100	58.4	58.3
<i>Asaia siamensis</i> BCC 12268 ^T	52.6	59.9	51.9	100	61.6
<i>Asaia lannaensis</i> BCC 15733 ^T	58.7	46.3	39.7	40.2	100
<i>S. salitolerans</i> PA51 ^T	50.3	38	35.6	17.9	53.1
<i>N. chiangmaiensis</i> BCC 15763 ^T	26.9	14.5	10.6	10.3	17

*Strain: 1, GB23-3; 2, *Asaia bogorensis* BCC 12264^T; 3, *Asaia krungthepensis* BCC 12978^T; 4, *Asaia siamensis* BCC 12268^T; 5, *Asaia lannaensis* BCC 15733^T.

with the type strains of *Asaia* (Table 4). From the data obtained, the isolate was considered to constitute a novel species in the genus *Asaia* (11, 23, 38–40).

Seearunruangchai *et al.* (27) isolated forty acetic acid bacteria from fruits in Thailand. Thirty-one of the isolates, some 77% of the total were identified as *A. pasteurianus*. Three isolates (only 8%) were identified as *A. orientalis*, and the remaining six isolates (15%) from sugar cane, palm and coconut juice were identified as *Gluconacetobacter liquefaciens*.

Kommanee *et al.* (15) isolated ninety *Acetobacter* strains from fruits, flowers and other materials collected in Thailand. The isolates were divided into three groups, Group A containing fifty-three isolates identified as *A. pasteurianus*, Group B containing forty-two isolates identified as *A. orientalis*, and Group C containing two isolates identified as *A. lovaniensis*. The present study demonstrated similar results, with the Thai isolates mostly identified as two species, *A. pasteurianus* and *A. orientalis*, along with a very small number identified as *A. lovaniensis*.

Huong *et al.* (9) examined forty-four Thai isolates assigned to the genus *Gluconobacter* and classified them into seven groups. However, no isolate was identified as *G. cerinus*. The present study gave the same results.

Four species, viz., *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, and *Asaia lannaensis* are presently described in the genus *Asaia* (11, 23, 38–40). GB23-3 assigned to the genus *Asaia* was characterized by the production of acetic acid from ethanol, as found in the type strain of *Asaia lannaensis* but not in the type strains of *Asaia bogorensis*, *Asaia siamensis* and *Asaia krungthepensis*. The present study demonstrated that the Thai isolate constitutes a new species.

From the results obtained above, the Thai isolates of acetic acid bacteria assigned to the genera *Acetobacter*, *Gluconobacter* and *Asaia* were not successfully identified at the species level by the phenotypic characterization but the 16S rRNA gene sequence and 16S-23S rDNA ITS restriction analyses using *Hpa*II, *Hae*III, *Mbo*II, *Bsp*1286I, *Taq*I and *Bst*NI were useful. In addition, the DNA-DNA relatedness data made clear the taxonomic position of novel species. However, use of the 16S-23S rDNA ITS restriction analysis in the differentiation of *Asaia* species should be studied further.

In comparison with Indonesian isolates (16, 17) and African isolates (3), the Thai isolates of *Acetobacter* species were similar in phenotypic features but somewhat different in isolation sources. For example, the Thai isolates of *A. tropicalis* and *A. orientalis* were obtained respectively from a flower of *Spathodea campanulata* and fruits of kaffir lime, muskmelon and loog-pang khaomak (sweetened rice starter), however, the type strains of the species were from coconut and a canna flower in Indonesian sources (16, 17). In *A. ghanensis*, the Thai isolates were from tomato and peach in contrast to traditional heap fermentations of Ghanaian cocoa beans (3). It is of interest that *G. japonicus*, which was recently described for Japanese strains (24) was found in Thai isolates but not in Indonesian isolates (37). In the present study, possible new species of *Gluconobacter* from the fruit of rambutan and *Asaia* from the flower of *Spathodea campanulata*

were recognized.

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References

- Asai, T., H. Iizuka, and K. Komagata. 1964. The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. *J. Gen. Appl. Microbiol.* 10:95–126.
- Brosius, J., T.J. Dull, D.D. Sleeter, and H.F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* 148:107–127.
- Cleenwerck, I., N. Camu, K. Engelbeen, T. De Winter, K. Vandemeulebroecke, P. De Vos, and L. De Vuyst. 2007. *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. *Int. J. Syst. Evol. Microbiol.* 57:1647–1652.
- Ezaki, T., Y. Hashimoto, and E. Yabuuchi. 1989. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int. J. Syst. Bacteriol.* 39:224–229.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783–791.
- Gosselé, J., J. Swings, and J. De Ley. 1980. 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. *Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. C* 1:178–181.
- Greenberg, D.E., S.F. Porcella, F. Stock, A. Wong, P.S. Conville, P.R. Murray, S.M. Holland, and A.M. Zelazny. 2006. *Granulibacter thesedensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. *Int. J. Syst. Evol. Microbiol.* 56:2609–2616.
- Hucker, G.J., and H.J. Conn. 1923. Method of Gram staining. *N. Y. State. Agric. Exp. Stn. Tech. Bull.* 93:3–37.
- Huong, V.T.L., T. Malimas, P. Yukphan, W. Potacharoen, S. Tanasupawat, L.T.T. Loan, M. Tanticharoen, and Y. Yamada. 2007. Identification of Thai isolates assigned to the genus *Gluconobacter* based on 16S-23S rDNA ITS restriction analysis. *J. Gen. Appl. Microbiol.* 53:133–142.
- Jojima, Y., Y. Mihara, S. Suzuki, K. Yokozeki, S. Yamanaka, and R. Fudou. 2004. *Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen. *Int. J. Syst. Evol. Microbiol.* 54:2263–2267.
- Katsura, K., H. Kawasaki, W. Potacharoen, S. Saono, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata. 2001. *Asaia siamensis* sp. nov., an acetic acid bacterium in the α -*Proteobacteria*. *Int. J. Syst. Evol. Microbiol.* 51:559–563.
- Katsura, K., Y. Yamada, T. Uchimura, and K. Komagata. 2002. *Gluconobacter asaii* Mason and Claus 1989 is a junior subjective synonym of *Gluconobacter cerinus* Yamada and Akita 1994. *Int. J. Syst. Evol. Microbiol.* 52:1635–1640.
- Kerstens, K., P. Lisdiyanti, K. Komagata, and J. Swings. 2006. The family *Acetobacteraceae*: The genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. p. 163–200. In M. Dworkin, S. Falcow, E. Rosenberg, K.H. Schleifer, and E. Stackebrandts (ed.), *The Prokaryotes*, 3rd ed., vol. 5. Springer, New York, USA.
- Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111–120.
- Kommanee, J., A. Akaracharanya, S. Tanasupawat, T. Malimas, P. Yukphan, Y. Nakagawa, and Y. Yamada. 2008. Identification of *Acetobacter* strains isolated in Thailand based on 16S-23S rDNA gene ITS restriction and 16S rRNA gene sequence analyses. *Ann. Microbiol.* 58:319–324.
- Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura, and

- K. Komagata. 2000. Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg 1906) comb. nov., *Acetobacter lovaniensis* (Frateur 1950) comb. nov., and *Acetobacter estunensis* (Carr 1958) comb. nov. J. Gen. Appl. Microbiol. 46:147–165.
17. Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata. 2001. Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov., and *Acetobacter orientalis* sp. nov. J. Gen. Appl. Microbiol. 47:119–131.
 18. Lisdiyanti, P., H. Kawasaki, Y. Widyastuti, S. Saono, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata. 2002. *Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the *alpha-Proteobacteria*. Int. J. Syst. Evol. Microbiol. 52:813–818.
 19. Loganathan, P., and S. Nair. 2004. *Swaminathanian salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing, and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka). Int. J. Syst. Evol. Microbiol. 54:1185–1190.
 20. Malimas, T., P. Yukphan, M. Takahashi, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada. 2006. Heterogeneity of strains assigned to *Gluconobacter frateurii* Mason and Claus 1989 based on restriction analysis of 16S-23S rDNA internal transcribed spacer regions. Biosci. Biotech. Biochem. 70:684–690.
 21. Malimas, T., P. Yukphan, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada. 2007. *Gluconobacter kondonii* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*, an acetic acid bacterium in the *alpha-Proteobacteria*. J. Gen. Appl. Microbiol. 53:301–307.
 22. Malimas, T., P. Yukphan, M. Takahashi, and *et al.* 2008. *Gluconobacter roseus* (ex Asai 1935) sp. nov., nom. rev., a pink-colored acetic acid bacterium in the *Alphaproteobacteria*. J. Gen. Appl. Microbiol. 54:119–125.
 23. Malimas, T., P. Yukphan, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada. 2008. *Asaia lannaensis* sp. nov., a new acetic acid bacterium in the *Alphaproteobacteria*. Biosci. Biotechnol. Biochem. 72:666–671.
 24. Malimas, T., P. Yukphan, M. Takahashi, and *et al.* 2009. *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the *Alphaproteobacteria*. Int. J. Syst. Evol. Microbiol. 59:466–471.
 25. Mason, L.M., and G.W. Claus. 1989. Phenotypic characteristic correlated with deoxyribonucleic acid sequence similarity for three species of *Gluconobacter*: *G. oxydans* (Henneberg 1897) De ley 1961, *G. frateurii* sp. nov., and *G. asaii* sp. nov. Int. J. Syst. Bacteriol. 39:174–184.
 26. Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
 27. Seearunruangchai, A., S. Tanasupawat, S. Keeratipibut, C. Thawai, T. Itoh, and Y. Yamada. 2004. Identification of acetic acid bacteria isolated from fruits and related materials collected in Thailand. J. Gen. Appl. Microbiol. 50:47–53.
 28. Skerman, V.B.D., V. McGowan, and P.H.A. Sneath. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225–420.
 29. Tamaoka, J., Y. Katayama-Fujimura, and H. Kuraishi. 1983. Analysis of bacterial menaquinone mixtures by high-performance liquid chromatography. J. Appl. Bacteriol. 54:31–36.
 30. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599.
 31. Tanasupawat, S., C. Thawai, P. Yukphan, D. Moonmangmee, T. Itoh, O. Adachi, and Y. Yamada. 2004. *Gluconobacter thailandicus* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*. J. Gen. Appl. Microbiol. 50:159–167.
 32. Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876–4882.
 33. Urakami, T., J. Tamaoka, J., K. Suzuki, and K. Komagata. 1989. *Acidomonas* gen. nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. Int. J. Syst. Bacteriol. 39:50–55.
 34. Verlander, C.P. 1992. Detection of horseradish peroxidase by colorimetry. p. 185–201. In L.J. Kricka (ed.), Nonisotopic DNA Probe Techniques, Academic Press, New York, USA.
 35. Yamada, Y., K. Aida, and T. Uemura. 1969. Enzymatic studies on the oxidation of sugar and sugar alcohol. V. Ubiquinone of acetic acid bacteria and its relation to classification of *Gluconobacter* and *Acetobacter*, especially of the so-called intermediate strains. J. Gen. Appl. Microbiol. 15:186–196.
 36. Yamada, Y., Y. Okada, and K. Kondo. 1976. Isolation and characterization of “polarly flagellated intermediate strains” in acetic acid bacteria. J. Gen. Appl. Microbiol. 22:237–245.
 37. Yamada, Y., R. Hosono, P. Lisdiyanti, Y. Widyastuti, S. Saono, T. Uchimura, and K. Komagata. 1999. Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus *Gluconobacter*. J. Gen. Appl. Microbiol. 45:23–28.
 38. Yamada, Y., K. Katsura, H. Kawasaki, Y. Widyastuti, S. Saono, T. Seki, T. Uchimura, and K. Komagata. 2000. *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the *alpha-Proteobacteria*. Int. J. Syst. Evol. Microbiol. 50:823–829.
 39. Yamada, Y., and P. Yukphan. 2008. Genera and species in acetic acid bacteria. Int. J. Food Microbiol. 125:15–24.
 40. Yukphan, P., W. Potacharoen, S. Tanasupawat, M. Tanticharoen, and Y. Yamada. 2004. *Asaia krungthepensis* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*. Int. J. Syst. Evol. Microbiol. 54:313–316.
 41. Yukphan, P., W. Potacharoen, Y. Nakagawa, M. Tanticharoen, and Y. Yamada. 2004. Identification of strains assigned to the genus *Gluconobacter* Asai 1935 based on the sequence and the restriction analyses of the 16S-23S rDNA internal transcribed spacer regions. J. Gen. Appl. Microbiol. 50:9–15.
 42. Yukphan, P., M. Takahashi, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada. 2004. *Gluconobacter albidus* (ex Kondo and Ameyama 1958) sp. nov., nom. rev., an acetic acid bacterium in the *alpha-Proteobacteria*. J. Gen. Appl. Microbiol. 50:235–242.
 43. Yukphan, P., T. Malimas, W. Potacharoen, S. Tanasupawat, M. Tanticharoen, and Y. Yamada. 2005. *Neoasaia Chiangmaiensis* gen. nov., sp. nov., a novel osmotolerant acetic acid bacterium in the *alpha-Proteobacteria*. J. Gen. Appl. Microbiol. 51:301–311.
 44. Yukphan, P., T. Malimas, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada. 2006. Identification of strains assigned to the genus *Asaia* Yamada *et al.* 2000 based on 16S rDNA restriction analysis. J. Gen. Appl. Microbiol. 52:241–247.
 45. Yukphan, P., T. Malimas, Y. Muramatsu, and *et al.* 2008. *Tanticharoenia sakaeratensis* gen. nov., sp. nov., a new osmotolerant acetic acid bacterium in the *alpha-Proteobacteria*. Biosci. Biotechnol. Biochem. 72:672–676.