RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2012.38.044

ScienceAsia 38 (2012): **44–53**

Identification of *Acetobacter* strains isolated in Thailand based on the phenotypic, chemotaxonomic, and molecular characterizations

Jintana Kommanee[a], Somboon Tanasupawat[a,*], Pattaraporn Yukphan[b], Nuttha Thongchul[c], Duangtip Moonmangmee[d], Yuzo Yamada[b,e]

[a] Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330 Thailand

[b] BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani 12120 Thailand

[c] The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330 Thailand

[d] Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140 Thailand

[e] JICA Senior Overseas Volunteer, Japan International Cooperation Agency (JICA), Shibuya-ku, Tokyo 151-8558, Japan, Professor Emeritus, Shizuoka University, Shizuoka 422-8529, Japan

[*] Corresponding author, e-mail: Somboon.T@chula.ac.th

Received 14 Sep 2011
Accepted 14 Dec 2011

ABSTRACT: The restriction analysis of 16S-23S rRNA gene internal transcribed spacer regions (ITS) using *Taq*I, *Alu*I, *Hpa*II, and *Ava*II revealed that forty-seven bacterial isolates found in fruits and flowers collected in Thailand belong to the genus *Acetobacter*. They were identified as follows: Group 1 containing 11 isolates was identified as *A. pasteurianus*, Group 2 containing nine isolates was identified as *A. orientalis*, Group 3 containing eight isolates was identified as *A. lovaniensis*, Group 4 including eight isolates was identified as *A. indonesiensis*, Group 5 containing three isolates was identified as *A. tropicalis*, Group 6 containing five isolates was identified as *A. ghanensis*, and Group 7 containing four isolates was identified as *A. orleanensis*. The differentiation of *Acetobacter* species by the 16S-23S rRNA gene ITS restriction analysis was discussed. The phenotypic, chemotaxonomic, and molecular characteristics including 16S rRNA gene sequences supported the above-mentioned identification. All Thai isolates were screened for their alcohol dehydrogenase activity and exhibited an activity of 2.05–7.52 units/mg protein at 30 °C. Isolate PHD-23 produced the highest quantity of 1.50% acetic acid (v/v) from 4.0% ethanol (v/v).

KEYWORDS: acetic acid bacteria, acetic acid production, alcohol dehydrogenase, 16S-23S rRNA gene ITS restriction analysis

INTRODUCTION

The genus *Acetobacter* contains 19 species comprising *A. aceti*, *A. indonesiensis*, *A. cerevisiae*, *A. cibinongensis*, *A. pasteurianus*, *A. lovaniensis*, *A. orleanensis*, *A. estunensis*, *A. malorum*, *A. orientalis*, *A. peroxydans*, *A. pomorum*, *A. syzygii*, *A. tropicalis*, *A. oeni*, *A. ghanensis*, *A. nitrogenifigens*, *A. senegalensis*, and *A. fabarum*[1–5]. *Acetobacter* strains exhibit the capability of producing acetic acid from ethanol, oxidize acetate and lactate to CO_2 and water, and contain the major ubiquinone with nine isoprene units (Q-9)[3,6]. They are well known as vinegar producers from ethanol by two sequential catalytic reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase[7].

Thailand is located in the tropical area and there are diverse fruits and flowers from which acetic acid bacteria were isolated[8,9]. In Europe, *Acetobacter* strains, the most popular strains for making acetic acid in vinegar factories, are mesophilic, with optimum temperature for growth at about 30 °C[10]. The industrial vinegar production is strictly controlled at 30 °C. In many countries, the fermentation rate and fermentation efficiency decrease when the temperature increases by 2–3 °C. Therefore, it would be desirable to find strains of acetic acid bacteria (AAB) that can work optimally at temperatures above 30 °C.

This study identifies *Acetobacter* isolates isolated in Thailand by 16S-23S rRNA gene internal transcribed spacer region (ITS) restriction analysis. It includes phenotypic and chemotaxonomic characterizations and 16S rRNA gene sequence analysis, along with screening for acetic acid production by isolates using ADH activity.

MATERIALS AND METHODS

Isolation of acetic acid producing bacteria

The forty-seven isolates were isolated from fruits and flowers collected in Thailand by an enrichment culture approach using glucose/ethanol/yeast extract (GEY) medium[8]. A sample source was incubated at pH 4.5 and 30 °C for 3–5 days in a liquid medium. When microbial growth was found, the culture was streaked onto a GEY-agar plate containing 0.3% $CaCO_3$ (w/v)[11]. AAB were selected as acid-producing bacterial strains that form a clear zone around colonies growing on the agar plate.

Identification methods

Phenotypic and chemotaxonomic characterizations: Phenotypic characterizations were carried out by incubating test strains at 30 °C and pH 6.8 for two days on glucose/yeast extract/peptone/glycerol (GYPG) agar[8]. For Gram staining of bacterial cells, the Hucker-Conn modified method[12] was used. Physiological and biochemical characterizations were made by the methods of Asai et al[13] and Gosselé et al[14]. The selected isolates were grown in GYPG media on a rotary shaker (150–200 rpm) at 30 °C for 24 h. Ubiquinone was extracted and purified, as reported by Yamada et al[6]. The purified ubiquinone preparation was distinguished from its homologues by reversed-phase paper chromatography[6] and by high performance liquid chromatography[15]. DNA was extracted by the method described by Saito and Miura[16]. DNA base composition was determined by the method of Tamaoka and Komagata[17].

16S rRNA gene sequencing and phylogenetic analyses: The extracted DNA of an isolate was amplified for 16S rRNA genes with two primers, 20F (5′-GAGTTTGATCCTGGCTCAG-3′; positions 9–27 by the *Escherichia coli* numbering system, accession number V00348)[18] and 1500R (5′-GTTACCTTGTTACGACTT-3′; positions 1509–1492). The amplified 16S rRNA genes were sequenced with an ABI PRISM BigDye Terminator V3.1[8]. For the sequencing of 16S rRNA genes, the following six primers were used: 20F, 1500R, 520F (5′-CAGCAGCCGCGGTAATAC-3′; positions 519–536), 520R (5′-GTATTACCGCGGCTGCTG-3′; positions 536–519), 920F (5′-AAACTCAAATGAATTGACGG-3′; positions 907–926), and 920R (5′-CCGTCAATTCATTTGAGTTT-3′; positions 926–907). Multiple sequence alignments were performed with the program CLUSTAL X (version 1.83)[19]. Gaps and ambiguous bases were eliminated from the calculations. Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura[20]. A phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbour-joining method[21] with the program MEGA 4[22]. The confidence values at individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein[23] based on 1000 replications.

Restriction analysis of 16S-23S rRNA gene ITS regions: The 16S-23S rRNA gene ITS PCR amplification was made with two primers[24], which were 5′-TGCGG(C/T)TGGATCACCTCCT-3′ (position 1522–1540 on 16S rRNA by the *Escherichia coli* numbering system)[18] and 5′-GTGCC(A/T)AGGCATCCACCG-3′ (position 38–22 on 23S rRNA). The purified PCR products for *Acetobacter* isolates were separately digested with restriction endonucleases *Taq*I, *Alu*I, *Hpa*II, and *Ava*II (New England BioLabs, Beverly, Massachusetts, USA) according to the manufacturer's instructions. The resulting reaction products were analysed by 2.5% (w/v) agarose gel electrophoresis[8].

Acetic acid production

Alcohol dehydrogenase activity assay: ADH activity was measured colourimetrically with potassium ferricyanide as an electron acceptor, as described by Adachi et al[25]. *Acetobacter* isolates were inoculated into 200 ml of potato medium, which contained 0.5% D-glucose, 1% yeast extract, 1% peptone, 2% glycerol, 10% potato extract at 30 °C for 48 h on a rotary shaker (200 rpm). Cells were harvested by centrifugation at 8000*g* for 10 min and washed twice with cooled 5 mM K_3PO_4 buffer, pH 6.0. The washed cells were re-suspended in the same buffer and placed in a sonicator at 16 000 lb/min for 10 min. Cell debris was removed by centrifugation at 8000*g* for 10 min, and then the supernatant was used for ADH assay. The reaction mixture (1 ml) contained enzyme solution, McIlvaine buffer (McB), pH 5.0, substrate (100 µl of 1 M ethanol) and 100 µl of 0.1 M ferricyanide solution. After 5 min, 500 µl of Dupanol reagent was added, and incubated for 20 min. Then, 3.5 ml of dH_2O was added and mixed well. The absorbance at 660 nm was measured on a UV spectrophotometer.

One unit of enzyme activity was defined as the quantity of enzymes catalysing the oxidation of 1 μmol of ethanol per min under the operating conditions. The specific activity was expressed as units per mg protein, and the protein content was determined by the Lowry method[26] with bovine serum albumin as standard.

Effect of ethanol and initial acetic acid concentration on acetic acid production: The selected isolate that showed the highest ADH activity was inoculated and cultivated in 100 ml potato medium at 30 °C on a rotary shaker (200 rpm) for 24 h.

Effects of initial ethanol concentration: 10 ml of the culture mentioned before was transferred to 90 ml YE medium, which contained 0.3% yeast extract and 0–12% ethanol in a 500 ml embossed flask and cultivated at 30 °C on a rotary shaker (200 rpm) for three days. In the YE medium, ethanol concentration was varied as 0, 2, 4, 6, 8, 10, and 12% (v/v).

Effects of initial acetic acid concentration: 10 ml of the culture mentioned before was transferred to 90 ml YEA medium, which contained 0.3% yeast extract, 4% ethanol, and 1–3% acetic acid in a 500 ml embossed flask and cultivated at 30 °C on a rotary shaker at 200 rpm for three days. In the YEA medium, acetic acid concentration was varied as 0, 1, 1.5, 2, 2.5, and 3% (v/v). Samples were taken for biomass evolution and acetic acid analysed as described below.

Analytical methods

The total biomass evolution was determined by the turbidimetric method (absorbance at 600 nm measured on a spectrophotometer). Acetic acid determination using 1–10% absolute ethanol as standard was carried out by a gas chromatograph equipped with a capillary column and an FID detector. All the assays were performed in triplicate to obtain valid statistical evaluation of results, expressed as mean ± s.e.m.

RESULTS AND DISCUSSION

Strain identification

All isolates had characteristics of Gram-negative, aerobic, and rod-shaped. They produced catalase but not oxidase and showed clear zones on GEY/$CaCO_3$ agar plates. All isolates oxidized acetate and lactate and showed a major ubiquinone of Q-9. Therefore, they were assigned to the genus *Acetobacter*[6,13] (Table 1 and Table 2).

The *Acetobacter* isolates were divided into seven groups based on the restriction analysis using the four restriction endonucleases; *Taq*I, *Alu*I, *Hpa*II, and *Ava*II.

Table 1 Identification and ADH activity of isolates assigned to the genus *Acetobacter*.

Isolate	Source	Location	ADH activity* (unit/mg)
Group 1 or *A. pasteurianus*			
PHD-23	Little Yellow Star[1]	Rayong	7.01 ± 0.01
PHD-24	Little Yellow Star[1]	Rayong	5.89 ± 0.03
PHD-32	Mango[2]	Khon Kaen	4.63 ± 0.04
PHD-33	Mango[2]	Khon Kaen	5.08 ± 0.02
PHD-56	Peach[2]	Bangkok	5.68 ± 0.01
PHD-57	Peach[2]	Bangkok	5.06 ± 0.01
PHD-70	Red Grape[2]	Rayong	4.96 ± 0.02
PHD-71	Red Grape[2]	Rayong	5.33 ± 0.01
PHD-76	Salas[2]	Rayong	4.89 ± 0.03
PHD-77	Salas[2]	Rayong	4.75 ± 0.04
PHD-78	Salas[2]	Rayong	3.89 ± 0.03
Group 2 or *A. orientalis*			
PHD-12	Jujube[2]	Trad	3.16 ± 0.02
PHD-34	Mango[2]	Bangkok	3.89 ± 0.01
PHD-35	Mango[2]	Bangkok	3.66 ± 0.01
PHD-37	Mango[2]	Nontaburi	3.12 ± 0.02
PHD-38	Mango[2]	Nontaburi	2.89 ± 0.01
PHD-51	Orange[2]	Khon Kaen	3.45 ± 0.04
PHD-73	Rumbutan[2]	Khon Kaen	3.33 ± 0.01
PHD-74	Rumbutan[2]	Khon Kaen	4.56 ± 0.03
PHD-75	Rumbutan[2]	Khon Kaen	4.12 ± 0.02
Group 3 or *A. lovaniensis*			
PHD-16	Makrut[2]	Changmai	4.05 ± 0.01
PHD-17	Makrut[2]	Saraburi	4.45 ± 0.02
PHD-18	Makrut[2]	Saraburi	3.66 ± 0.01
PHD-25	Longan[2]	Rayong	2.58 ± 0.03
PHD-26	Longan[2]	Rayong	2.65 ± 0.02
PHD-63	Pineapple[2]	Chantaburi	3.12 ± 0.01
PHD-91	Tamarind[2]	Chantaburi	3.48 ± 0.04
PHD-92	Tamarind[2]	Chantaburi	2.89 ± 0.02
Group 4 or *A. indonesiensis*			
PHD-3	Guava[2]	Kanchanaburi	2.05 ± 0.02
PHD-5	Guava[2]	Kanchanaburi	2.54 ± 0.01
PHD-7	Guava[2]	Ubon	2.28 ± 0.03
PHD-8	Hog Plum[2]	Nongkhai	2.72 ± 0.02
PHD-9	Ixoria/Ixora[1]	Rayong	2.58 ± 0.01
PHD-13	Jujube[2]	Trad	2.75 ± 0.04
PHD-44	Musk-melon[2]	Saraburi	2.99 ± 0.01
PHD-45	Musk-melon[2]	Saraburi	2.56 ± 0.01
Group 5 or *A. tropicalis*			
PHD-4	Guava[2]	Kanchanaburi	2.24 ± 0.02
PHD-6	Guava[2]	Ubon	3.14 ± 0.01
PHD-42	Musk-melon[2]	Bangkok	2.34 ± 0.02

(Continues on next page.)

Group 1 included 11 isolates: PHD-23, PHD-24, PHD-32, PHD-33, PHD-56, PHD-57, PHD-70, PHD-71, PHD-76, PHD-77, and PHD-78 (Table 1). They produced acid from L-arabinose, *meso*-erythritol, D-fructose, D-galactose, D-glucose, D-mannose,

Table 1 (Cont.)

Isolate	Source	Location	ADH activity* (unit/mg)
	Group 6 or *A. ghanensis*		
PHD-14	Jujube[2]	Trad	2.12 ± 0.01
PHD-15	Makrut[2]	Changmai	3.02 ± 0.03
PHD-61	Pineapple[2]	Chantaburi	2.88 ± 0.02
PHD-62	Pineapple[2]	Chantaburi	3.12 ± 0.01
PHD-72	Rose apple[2]	Ubon	2.11 ± 0.02
	Group 7 or *A. orleanensis*		
PHD-86	Strawberry[2]	Trad	3.11 ± 0.04
PHD-87	Strawberry[2]	Trad	2.14 ± 0.01
PHD-84	Star fruit[2]	Chantaburi	3.16 ± 0.03
PHD-85	Star fruit[2]	Chantaburi	2.88 ± 0.01

[1] Flower, [2] Fruit

* Values are the mean of three determinations.

D-melibiose, or D-xylose, and weakly from D-arabinose, glycerol, D-sorbitol, or sucrose. Some strains produced acid weakly from dulcitol or raffinose but not from lactose, maltose, D-mannitol, L-rhamnose, or L-sorbose. They grew on *meso*-erythritol but not on D-arabitol, L-arabitol, or *meso*-ribitol. They did not produce 2-keto-D-gluconate from D-glucose. All isolates grew at 37 °C. They showed almost the same phenotypic characteristics as the type strain of *A. pasteurianus*[2] (Table 2). All the isolates were located within the cluster of *A. pasteurianus* in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) and had 100% pairwise 16S rRNA gene sequence similarity to the type strain of *A. pasteurianus*. A representative isolate PHD-23 showed the restriction patterns that coincided with those of the type strain of *A. pasteurianus* when digested with *Taq*I and *Alu*I. In *Taq*I digestion, the isolate showed the same restriction pattern as the type strains of *A. orientalis* and *A. pasteurianus*, but differed from the type strain of *A. orientalis* when digested with *Alu*I (Fig. 2). DNA G+C content of isolate PHD-23 was 53.3 mol%. From the data obtained above, all isolates accommodated to Group 1 were identified as *A. pasteurianus*[2].

Group 2 included nine isolates: PHD-12, PHD-34, PHD-35, PHD-37, PHD-38, PHD-51, PHD-73, PHD-74, and PHD-75 (Table 1). They produced acid from L-arabinose, D-glucose, D-mannose, raffinose, or D-xylose but not from D-arabinose, dulcitol, *meso*-erythritol, D-galactose, D-fructose, glycerol, lactose, maltose, D-mannitol, melibiose, L-rhamnose, L-sorbose, D-sorbitol, or sucrose. The isolates did not grow on *meso*-erythritol, D-arabitol, L-arabitol, or *meso*-ribitol. They produced 2-keto-D-gluconate from D-glucose. They showed almost

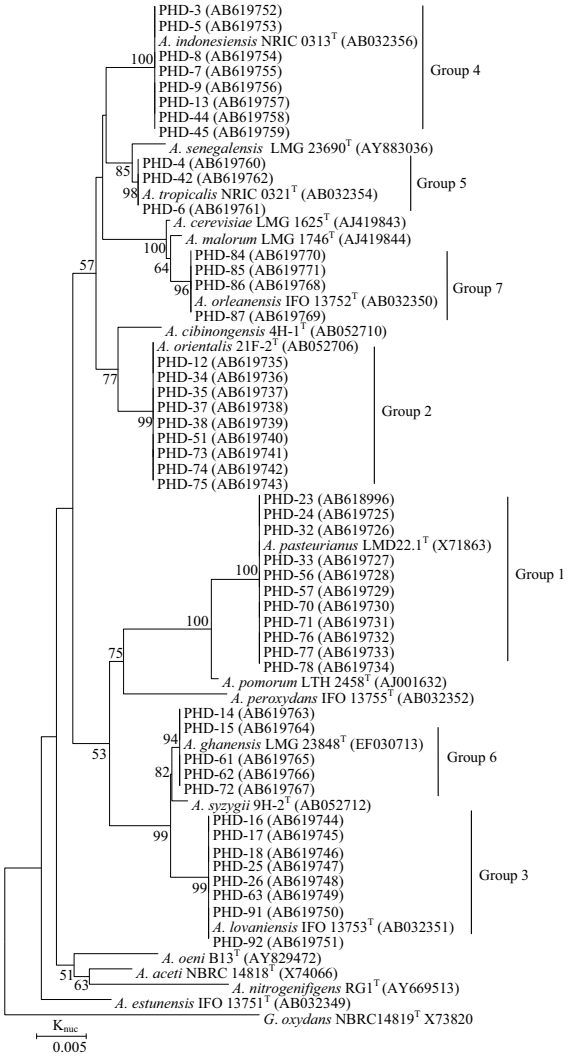

Fig. 1 Phylogenetic relationships of isolates of Groups 1–7 based on 16S rRNA gene sequences. The phylogenetic tree was constructed by the neighbour-joining method. Numbers at nodes indicate bootstrap percentages derived from 1000 replications.

the same phenotypic characteristics as the type strain of *A. orientalis*[4] (Table 2). They were located within the cluster of *A. orientalis* in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) and had 100% pairwise 16S rRNA gene sequence similarity to the type strain of *A. orientalis*. A representative isolate PHD-12 showed the same restriction pattern as the type strains of *A. orientalis* and *A. pasteurianus* when digested with *Taq*I, but differed from the type strain of *A. pasteurianus* when digested with *Alu*I (Fig. 2). Isolate PHD-12 had DNA G+C content of 52.2 mol%. From the data obtained above, all

Table 2 Differential characteristics of isolates assigned to the genus *Acetobacter*.

Characteristic	G1	*Ap*	G2	*Aori*	G3	*Al*	G4	*Ai*	G5	*At*	G6	*Ag*	G7	*Aorl*
Oxidation of														
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37 °C	+(w4)	w	−(w2)	−	−(w3)	−	−	−	−	−	−	−	−	−
Growth at 40 °C	−(w2)	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth on mannitol agar	−(w3)	−	−(w2)	−	−(w2)	−	−(w1)	−	−	−	−(w3)	w	w	−
Production of														
2-keto gluconic acid	−	−	+	+	+	+	+	+	+	+	−	−	+	+
5-keto gluconic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Major quinone	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9
Acid production from														
meso-Erythritol	+(w4)	+	−(w2)	−	−	−	−(w1)	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	w	−	−	w	−	−	−	−
Raffinose	w	w	−(w4)	−	−	−	−	−	w	w	−	−	−	−
DNA G+C (mol%)	53.3	52.7*	52.2	52.3*	58.3	58.6*	54.2	53.7*	56.0	55.9*	57.1	57.3†	55.8	56.5*

Ap, *A. pasteurianus* TISTR 1056^T; G1, Group 1 (11 isolates); *Aori*, *A. orientalis* NBRC 16606^T; G2, Group 2 (9 isolates); *Al*, *A. lovaniensis* NBRC 13753^T; G3, Group 3 (8 isolates); *Ai*, *A. indonesiensis* NBRC 16471^T; G4, Group 4 (8 isolates); *At*, *A. tropicalis* NBRC 16470^T; G5, Group 5 (3 isolates); *Ag*, *A. ghanensis* LMG 23848^T; G6, Group 6 (5 isolates); *Aorl*, *A. orleanensis* NBRC 13752^T; G7, Group 7 (4 isolates); +, positive; w, weakly positive; −, negative; numbers in parentheses indicate the isolates showing the reaction. *Data cited from Lisdiyanti et al[4]; †Data cited from Cleenwerck et al[1].

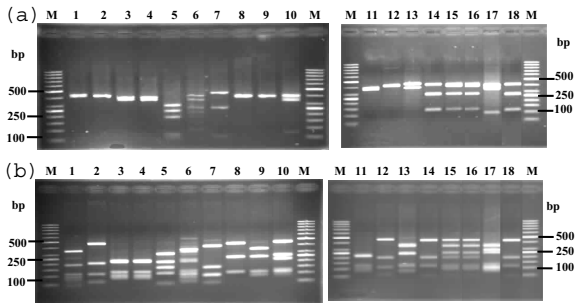

Fig. 2 Restriction analysis of 16S-23S rRNA gene ITS PCR products of isolates of Group 1 and Group 2 by digestion with (a) *Taq*I and (b) *Alu*I. 1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisiae* LMG 1625^T; 3, *A. syzygii* NBRC 16604^T; 4, *A. ghanensis* LMG 23848^T; 5, *A. cibinongensis* NBRC 16605^T; 6, *A. estunensis* NBRC 13751^T; 7, *A. peroxydans* NBRC 13755^T; 8, *A. senegalensis* LMG 23690^T; 9, *A. tropicalis* NBRC 16470^T; 10, *A. indonesiensis* NBRC 16471^T; 11, *A. lovaniensis* NBRC 13753^T; 12, *A. malorum* LMG 1746^T; 13, *A. nitrogenifigens* LMG 23498^T; 14, *A. orientalis* NBRC 16606^T; 15, *A. pasteurianus* TISTR 1056^T; 16, isolate PHD-23 (Group 1); 17, *A. aceti* IFO 14818^T; 18, isolate PHD-12 (Group 2); M, 50 bp DNA markers.

isolates accommodated to Group 2 were identified as *A. orientalis*[4].

Group 3 included eight isolates: PHD-16, PHD-17, PHD-18, PHD-25, PHD-26, PHD-63, PHD-91, and PHD-92 (Table 1). They produced acid from L-arabinose, D-glucose, D-mannose, raffinose, or D-xylose but not 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. They did not grow on *meso*-erythritol, D-arabitol, L-arabitol, or *meso*-ribitol. They showed almost the same phenotypic characteristics as *A. lovaniensis*[3] (Table 2). They were located within the cluster of *A. lovaniensis* in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) and had 100% 16S rRNA gene sequence similarities, respectively, to the type strain of *A. lovaniensis*. A representative isolate PHD-16 showed the same restriction patterns as the type strain of *A. lovaniensis* when digested with *Taq*I, *Hpa*II, and *Ava*II (Fig. 3). In *Taq*I digestion, the representative isolate also showed the same restriction pattern as the type strains of *A. lovaniensis*, *A. syzygii*, and *A. ghanensis*, but differed from the type strains of *A. ghanensis* and *A. syzygii* when digested with *Hpa*II and *Ava*II. Isolate PHD-16 had 58.3 mol% G+C. From the data obtained above, all isolates accommodated to Group 3 were identified as *A. lovaniensis*[3].

Group 4 included eight isolates: PHD-3, PHD-5, PHD-7, PHD-8, PHD-9, PHD-13, PHD-44, and PHD-45 (Table 1). They produced acid from L-arabinose, D-glucose, D-xylose, D-galactose, or D-mannose. Some isolates produced acids from D-fructose, D-mannose, D-melibiose, and D-xylose but not from *meso*-erythritol, dulcitol, lactose, maltose, D-mannitol, L-rhamnose, raffinose, L-sorbose, or sucrose. They produced D-gluconate and 2-keto-D-gluconate from D-glucose, and dihydroxyacetone from glycerol. They showed almost the same phe-

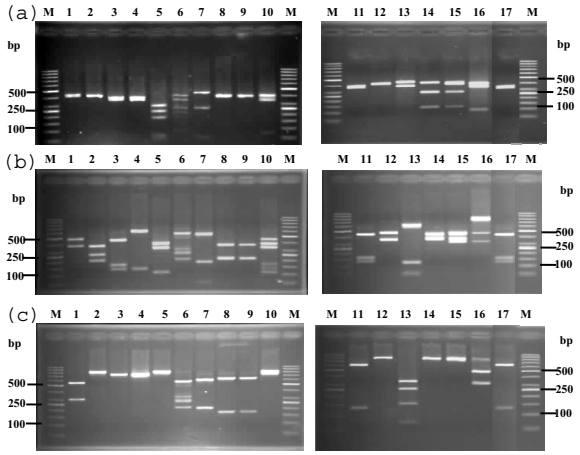

Fig. 3 Restriction analysis of 16S-23S rRNA gene ITS PCR products of isolates of Group 3 by digestion with (a) *Taq*I, (b) *Hpa*II, and (c) *Ava*II. 1, *A. orleanensis* NBRC 13752^{T}; 2, *A. cerevisiae* LMG 1625^{T}; 3, *A. syzygii* NBRC 16604^{T}; 4, *A. ghanensis* LMG 23848^{T}; 5, *A. cibinongensis* NBRC 16605^{T}; 6, *A. estunensis* NBRC 13751^{T}; 7, *A. peroxydans* NBRC 13755^{T}; 8, *A. senegalensis* LMG 23690^{T}; 9, *A. tropicalis* NBRC 16470^{T}; 10, *A. indonesiensis* NBRC 16471^{T}; 11, *A. lovaniensis* NBRC 13753^{T}; 12, *A. malorum* LMG 1746; 13, *A. nitrogenifigens* LMG 23498^{T}; 14, *A. orientalis* NBRC 16606^{T}; 15, *A. pasteurianus* TISTR 1056^{T}; 16, *A. aceti* IFO 14818^{T}; 17, isolate PHD-16 (Group 3); M, 50 bp DNA markers.

notypic characteristics as *A. indonesiensis*[3] (Table 2). Phylogenetically, they were located within the cluster of *A. indonesiensis* in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) and had 16S rRNA gene sequence similarities around 99.9% to the type strain of *A. indonesiensis*. A representative isolate PHD-3 showed the same restriction pattern as the type strain of *A. indonesiensis* and differed in this respect from the type strains of other *Acetobacter* species when digested with *Alu*I (Fig. 4). DNA G+C content of isolate PHD-3 was 54.2 mol%. From the data obtained above, all isolates accommodated to Group 4 were identified as *A. indonesiensis*[3].

Group 5 included three isolates: PHD-4, PHD-6, and PHD-42 (Table 1). The isolates produced acids from L-arabinose, D-galactose, D-glucose, and D-mannose, and to a less extent from maltose, raffinose, and D-xylose, but not from D-arabinose, dulcitol, *meso*-erythritol, D-fructose, glycerol, lactose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sorbitol, and sucrose. They did not grow on *meso*-erythritol, D-arabitol, L-arabitol, or *meso*-ribitol. They produced D-gluconate and 2-keto-D-gluconate

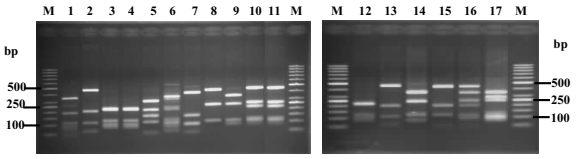

Fig. 4 Restriction analysis of 16S-23S rRNA gene ITS PCR products of isolates of Group 4 by digestion with *Alu*I. 1, *A. orleanensis* NBRC 13752^{T}; 2, *A. cerevisiae* LMG 1625^{T}; 3, *A. syzygii* NBRC 16604^{T}; 4, *A. ghanensis* LMG 23848^{T}; 5, *A. cibinongensis* NBRC 16605^{T}; 6, *A. estunensis* NBRC 13751^{T}; 7, *A. peroxydans* NBRC 13755^{T}; 8, *A. senegalensis* LMG 23690^{T}; 9, *A. tropicalis* NBRC 16470^{T}; 10, *A. indonesiensis* NBRC 16471^{T}; 11, isolate PHD-3 (Group 4); 12, *A. lovaniensis* NBRC 13753^{T}; 13, *A. malorum* LMG 1746; 14, *A. nitrogenifigens* LMG 23498; 15, *A. orientalis* NBRC 16606^{T}; 16, *A. pasteurianus* TISTR 1056^{T}; 17, *A. aceti* IFO 14818^{T}; M, 50 bp DNA markers.

from D-glucose, and dihydroxyacetone from glycerol. They showed almost the same phenotypic characteristics as the type strain of *A. tropicalis*[3] (Table 2). All isolates were located within the cluster of *A. tropicalis* in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) and showed 99.9% pairwise 16S rDNA sequence similarity with the type strain of *A. tropicalis*. A representative isolate PHD-4 showed the same restriction pattern as the type strain of *A. tropicalis* and differed in this respect from the type strains of other *Acetobacter* species when digested with *Alu*I and *Hpa*II (Fig. 5). DNA G+C content of isolate PHD-4 was 56.0 mol%. From the data obtained above, all isolates accommodated to Group 5 were identified as *A. tropicalis*[3].

Group 6 included five isolates: PHD-14, PHD-15, PHD-61, PHD-62, and PHD-7 (Table 1). The isolates produced acid from D-arabinose, D-glucose, and D-sorbitol. Some isolates produced acids from L-arabinose, D-fructose, D-mannose, D-melibiose, and D-xylose but none did 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 did not produce 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, or 2,5-diketo-D-gluconic acid from D-glucose and grew on glycerol weakly but not on maltose or methanol as a carbon source. They showed the same phenotypic characteristics as the type strain of *A. ghanensis*[1] (Table 2). They were located within the cluster of *A. ghanensis* in the phylogenetic tree based on 16S

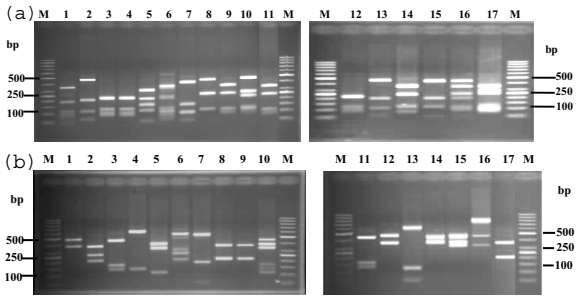

Fig. 5 Restriction analysis of 16S-23S rRNA gene ITS PCR products of isolates of Group 5 by (a) digestion with *Alu*I. 1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisiae* LMG 1625^T; 3, *A. syzygii* NBRC 16604^T; 4, *A. ghanensis* LMG 23848^T; 5, *A. cibinongensis* NBRC 16605^T; 6, *A. estunensis* NBRC 13751^T; 7, *A. peroxydans* NBRC 13755^T; 8, *A. senegalensis* LMG 23690^T; 9, *A. tropicalis* NBRC 16470^T; 10, *A. indonesiensis* NBRC 16471^T; 11, isolate PHD-4 (Group 5); 12, *A. lovaniensis* NBRC 13753^T; 13, *A. malorum* LMG 1746; 14, *A. nitrogenifigens* LMG 23498^T; 15, *A. orientalis* NBRC 16606^T; 16, *A. pasteurianus* TISTR 1056^T; 17, *A. aceti* IFO 14818^T. (b) Digestion with *Hpa*II. 1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisiae* LMG 1625^T; 3, *A. syzygii* NBRC 16604^T; 4, *A. ghanensis* LMG 23848^T; 5, *A. cibinongensis* NBRC 16605^T; 6, *A. estunensis* NBRC 13751^T; 7, *A. peroxydans* NBRC 13755^T; 8, *A. senegalensis* LMG 23690^T; 9, *A. tropicalis* NBRC 16470^T; 10, *A. indonesiensis* NBRC 16471^T; 11, *A. lovaniensis* NBRC 13753^T; 12, *A. malorum* LMG 1746; 13, *A. nitrogenifigens* LMG 23498^T; 14, *A. orientalis* NBRC 16606^T; 15, *A. pasteurianus* TISTR 1056^T; 16, *A. aceti* IFO 14818^T; 17, isolate PHD-4 (Group 5); M, 50 bp DNA markers.

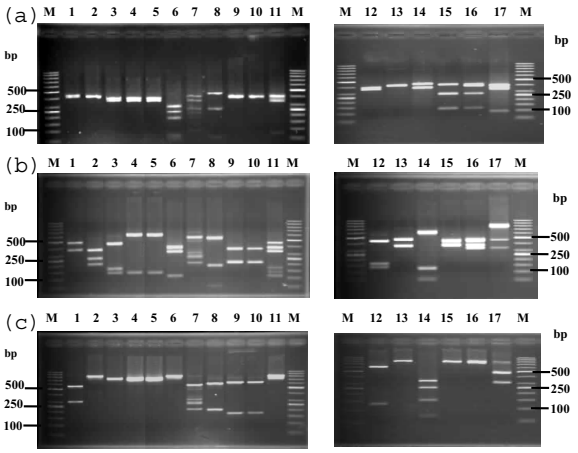

Fig. 6 Restriction analysis of 16S-23S rRNA gene ITS PCR product of isolates of Group 6 by digestion with (a) *Taq*I, (b) *Hpa*II, and (c) *Ava*II. 1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisiae* LMG 1625^T; 3, *A. syzygii* NBRC 16604^T; 4, *A. ghanensis* LMG 23848^T; 5, isolate PHD-14 (Group 6); 6, *A. cibinongensis* NBRC 16605^T; 7, *A. estunensis* NBRC 13751^T; 8, *A. peroxydans* NBRC 13755^T; 9, *A. senegalensis* LMG 23690^T; 10, *A. tropicalis* NBRC 16470^T; 11, *A. indonesiensis* NBRC 16471^T; 12, *A. lovaniensis* NBRC 13753^T; 13, *A. malorum* LMG 1746^T; 14, *A. nitrogenifigens* LMG 23498^T; 15, *A. orientalis* NBRC 16606^T; 16, *A. pasteurianus* TISTR 1056^T; 17, *A. aceti* IFO 14818^T; M, 50 bp DNA markers.

rRNA gene sequences (Fig. 1) and had 16S rRNA gene sequence similarities around 99.9% to the type strain of *A. ghanensis*. A representative isolate PHD-14 showed the same restriction patterns as the type strain of *A. ghanensis* when digested with *Taq*I, *Hpa*II, and *Ava*II. It was discriminated from the type strains of *A. syzygii* and *A. lovaniensis* when digestion with *Hpa*II and *Ava*II, respectively (Fig. 6). DNA G+C content of isolate PHD-14 was 57.1 mol%. From the data obtained above, all isolates accommodated to Group 6 were identified as *A. ghanensis*[1].

Group 7 included four isolates: PHD-84, PHD-85, PHD-86, and PHD-87 (Table 1). They produced acid from L-arabinose, D-glucose, maltose, but not from 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. They grew on *meso*-erythritol, but did not grow on D-arabitol, L-arabitol, or *meso*-ribitol. They produced D-gluconic acid from D-glucose but did not produce 5-keto-D-gluconic acid from D-glucose. They showed the same phenotypic characteristics as *A. orleanensis*[3] (Table 2). All isolates were located within the cluster of *A. orleanensis* in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) and showed 99.9% pairwise 16S rRNA gene sequence similarities to the type strain of *A. orleanensis*. A representative isolate PHD-85 gave the restriction patterns that coincided with those of the type strain of *A. orleanensis* when digested with *Hpa*II and *Ava*II (Fig. 7). DNA G+C content of isolate PHD-85 was 55.8 mol%. From the data obtained above, all isolates accommodated to Group 7 were identified as *A. orleanensis*[3].

In the restriction analysis of 16S-23S rRNA gene ITS PCR products, the four restriction endonucleases, *Taq*I, *Alu*I, *Hpa*II, and *Ava*II were useful for differentiating the *Acetobacter* strains at the species level. The type strains of *A. orleanensis*, *A. cibinongensis*, *A. estunensis*, *A. peroxydans*, *A. senegalensis*, *A. tropicalis*, *A. indonesiensis*, *A. nitrogenifigens*, *A. orientalis*, *A. pasteurianus*, and *A. aceti* were distinguished

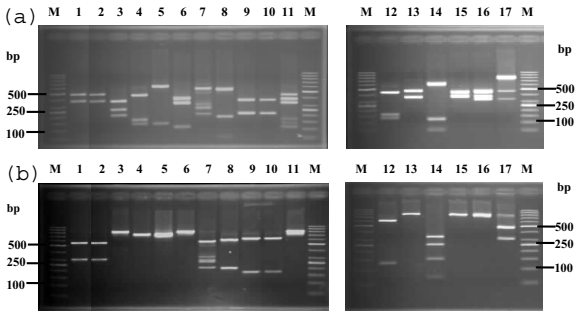

Fig. 7 Restriction analysis of 16S-23S rRNA gene ITS PCR products of isolates of Group 7 by digestion with (a) *Hpa*II and (b) *Ava*II. 1, *A. orleanensis* NBRC 13752^{T}; 2, isolate PHD-85 (Group 7); 3, *A. cerevisiae* LMG 1625^{T}; 4, *A. syzygii* NBRC 16604^{T}, 5, *A. ghanensis* LMG 23848^{T}; 6, *A. cibinongensis* NBRC 16605^{T}; 7, *A. estunensis* NBRC 13751^{T}; 8, *A. peroxydans* NBRC 13755^{T}; 9, *A. senegalensis* LMG 23690^{T}; 10, *A. tropicalis* NBRC 16470^{T}; 11, *A. indonesiensis* NBRC 16471^{T}; 12, *A. lovaniensis* NBRC 13753^{T}; 13, *A. malorum* LMG 1746^{T}; 14, *A. nitrogenifigens* LMG 23498^{T}; 15, *A. orientalis* NBRC 16606^{T}; 16, *A. pasteurianus* TISTR 1056^{T}; 17, *A. aceti* IFO 14818^{T}; M, 50 bp DNA markers.

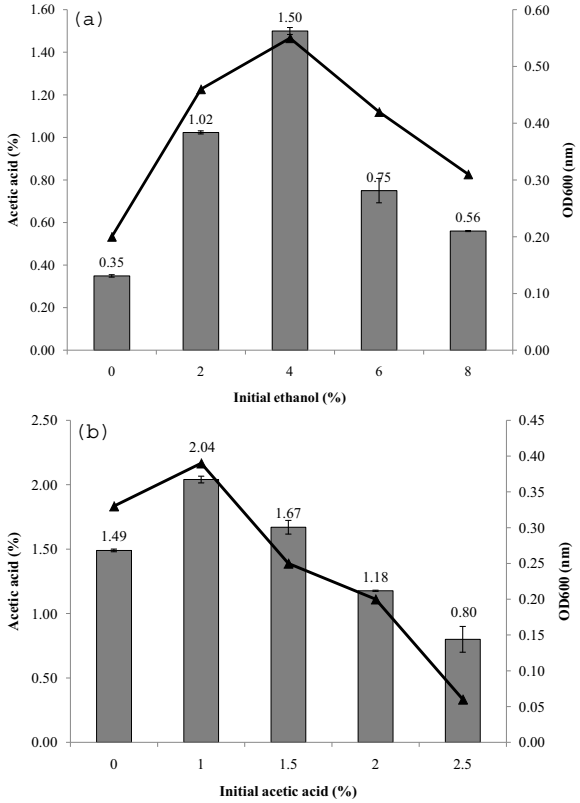

Fig. 8 (a) Effect of initial ethanol concentrations on acetic acid production of *A. pasteurianus* isolate PHD-23 at 0, 2, 4, 6, 8% ethanol and (b) effect of initial acetic acid concentration on acetic acid production of *A. pasteurianus* isolate PHD-23 at 0, 1, 1.5, 2, 2.5% acetic acid. Symbols: acetic acid (column), growth (line), the values are as percentages and growth is expressed as Optical Density (nm). Tests were in triplicate to obtain valid statistical evaluation of the results, expressed as mean ± s.e.m.

from one another by *Taq*I and *Alu*I digestions, while the type strains of *A. cerevisiae*, *A. ghanensis*, and *A. malorum* were differentiated from the type strains of other *Acetobacter* species by *Hpa*II digestion. In addition, the type strains of *A. syzygii* and *A. lovaniensis* were distinguished from the type strains of other *Acetobacter* species by *Ava*II and *Hpa*II digestion.

The 16S-23S rRNA gene ITS restriction analysis described above is therefore one of the most rapid methods for molecular identification, when used together with phenotypic and chemotaxonomic characterizations as well as 16S rRNA gene sequence analysis.

Acetic acid production by *Acetobacter* strains isolated in Thailand

The 47 isolates assigned to the genus *Acetobacter* showed that ADH activity ranged from 2.05–7.01 units/mg protein at 30 °C (Table 1). Isolate PHD-23 identified as *A. pasteurianus* showed the highest ADH activity, and was therefore selected for optimization of acetic acid production. *A. pasteurianus* isolate PHD-23 produced 0.35, 1.02, 1.50, 0.75, and 0.56% acetic acid (v/v) without lag time when ethanol concentrations were changed, respectively, to 0, 2.0, 4.0, 6.0, and 8.0% (v/v) (Fig. 8a). The growth increased maximally at ethanol concentration of 4.0% (v/v). The strain produced 1.5% acetic acid when 4.0% ethanol was used as a carbon source. The effect of initial acetic acid concentration (0–2% v/v) on acetic acid production in isolate PHD-23 showed that the isolate oxidized ethanol and accumulated acetic acid until the initial concentrations of acetic acid that was less than 2% (Fig. 8b). The data obtained suggested that isolate PHD-23 could produce the highest acetic acid concentration when there was no addition of acetic acid. The growth of the isolate decreased when the initial acetic acid concentration was more than 1.0% (v/v). At 2.5% acetic acid (v/v), the bacterial growth was not observed (Fig. 8b).

Saeki et al[27] reported that a thermotolerant acetic acid bacterium, isolate SKU1180 isolated from Thailand, could grow at 37–40 °C and produced 5.06–

4.42 g/l/h of acetic acid using higher concentrations of ethanol up to 9%, without any appreciable lag time.

Lu et al[28] reported that a thermotolerant acetic acid bacterium, *Acetobacter* isolate I14-2 isolated from a spoiled banana in Taiwan, produced 41 g/l acetic acid at 37 °C during six-day cultivation in a medium containing 2 g/l acetic acid and 5% (v/v) ethanol. The isolate retained 68% acetic acid-producing activity when compared with cultivation at 30 °C.

Ndoye et al[29] reported two isolates, *A. tropicalis* CWBI-B418 and *A. pasteurianus* CWBIB419, which were isolated, respectively, from mango and cereal. The two isolates showed their growth without any appreciable lag phase and a high level of acetic acid production at 35 and 38 °C, respectively. At 30 °C, isolates CWBI-B418 and CWBI-B419 produced 1.5% and 2% acetic acid w/v, respectively. Isolate CWBI-B419 produced 2% acetic acid (w/v) at 35 and 38 °C, and isolate CWBI-B418 produced 2% and 1.8% acetic acid (w/v) at 35 and 38 °C, respectively.

In comparison with the acetic acid production mentioned above, a new isolate, *A. pasteurianus* isolate PHD-23 reported in this study oxidized higher concentrations of ethanol up to 8% without any appreciable lag time, which was similar to the thermotolerant acetic acid bacteria reported by Saeki et al[27]. In the acetic acid production at 30 °C, isolate PHD-23 produced acetic acid similar to isolates CWBI-B418 and CWBI-B419[29].

Considering the data obtained above, *A. pasteurianus* isolate PHD-23 has advantages of (1) resistance to ethanol, (2) high acetic acid productivity, and (3) easy preservation by lyophilization, so that the isolate is suitable for vinegar making.

Acknowledgements: This study was supported by the Faculty of Pharmaceutical Sciences Research Fund, 2008, Chulalongkorn University, Thailand and Graduate Institute of Science and Technology, TGIST, 2007, National Science and Technology Development Agency, Thailand.

REFERENCES

1. Cleenwerck I, Camu N, Engelbeen K, de Winter T, Vandemeulebroecke K, de Vos P, de Vuyst L (2007) *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa bean. *Int J Syst Evol Microbiol* **57**, 1647–52.
2. Cleenwerck I, Gonzalez A, Camu N, Engelbeen K, de Vos P, de Vuyst L (2008) *Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. *Int J Syst Evol Microbiol* **58**, 2180–5.
3. Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (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**, 147–65.
4. Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (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**, 119–31.
5. Skerman VBD, McGowan V, Sneath PHA (1980) Approved lists of bacterial names. *Int J Syst Bacteriol* **30**, 225–420.
6. Yamada Y, Aida K, Uemura T (1968) Distribution of ubiquinone 10 and 9 in acetic acid bacteria and its relation to the classification of genera *Gluconobacter* and *Acetobacter*, especially of so-called intermediate strains. *Agr Biol Chem* **32**, 786–8.
7. Matsushita K, Tomaya H, Adachi O (1994) Respiratory chain and bioenergetics of acetic acid bacteria. In: Rose AH, Tempest DW (eds) *Advances in Microbial Physiology*, Academic Press, London, pp 247–301.
8. Kommanee J, Akaracharanya A, Tanasupawat S, Malimas T, Yukphan P, Nakagawa Y, Yamada Y (2008) Identification of *Acetobacter* strains isolated in Thailand based on 16S-23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses. *Ann Microbiol* **58**, 319–24.
9. Seearunruangchai A, Tanasupawat S, Keeratipibut S, Thawai C, Itoh T, Yamada Y (2004) Identification of acetic acid bacteria isolated from fruits and related materials collected in Thailand. *J Gen Appl Microbiol* **50**, 47–53.
10. Sievers M, Sellmer S, Teuber M (1992) *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in Central Europe. *Syst Appl Microbiol* **15**, 386–92.
11. Yamada Y, Okada Y, Kondo K (1976) Isolation and characterization of "polarly flagellated intermediate strains" in acetic acid bacteria. *J Gen Appl Microbiol* **22**, 237–45.
12. Hucker GJ, Conn HJ (1923) Method of Gram staining. *NY State Agric Exp Stn Tech Bull* **93**, 3–37.
13. Asai T, Iizuka H, Komagata K (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.
14. Gosselé J, Swings J, De Ley J (1980) A rapid, simple and simultaneous detection of 2-keto, 5-keto- and 2,5-diketogluconic acid by thin layer chromatography in culture media of acetic acid bacteria. *Zentralbl Bakteriol Parasitenk Infektionskrankh Hyg I Abt Orig C* **1**, 178–81.

15. Tamaoka J, Katayama-Fujimura Y, Kuraishi H (1983) Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. *J Appl Bacteriol* **54**, 31–6.
16. Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–29.
17. Tamaoka J, Komagata K (1984) Determination of DNA base composition by reversed-phase high performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–8.
18. Brosius J, Dull TJ, Sleeter DD, Noller HF (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J Mol Biol* **148**, 107–27.
19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–82.
20. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–20.
21. Saitou N, Nei M (1987) The neighboring-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–25.
22. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–9.
23. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–91.
24. Trček J, Teuber M (2002) Genetic and restriction analysis of the 16S–23S rDNA internal transcribed spacer regions of the acetic acid bacteria. *FEMS Microbiol Lett* **208**, 69–75.
25. Adachi O, Miyagawa E, Shinagawa E, Matsushita K, Ameyama M (1978) Purification and properties of particulate alcohol dehydrogenase from *Acetobacter aceti*. *Agr Biol Chem* **42**, 2331–40.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**, 265–75.
27. Saeki A, Theeragool G, Matsushita K, Toyama H, Lotong N, Adachi O (1997) Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. *Biosci Biotechnol Biochem* **61**, 138–45.
28. Lu SF, Lee FL, Chen HK (1999) A thermotolerant and high acetic acid-producing bacterium *Acetobacter* sp. I14-2. *J Appl Microbiol* **86**, 55–62.
29. Ndoye B, Lebecque S, Dubois-Dauphin R, Tounkara L, Guiro AT, Kere C, Diawara B, Thonart P (2006) Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. *Enzym Microb Tech* **39**, 916–23.