Annals of Microbiology, **58 (2)** 319-324 (2008)

Identification of *Acetobacter* strains isolated in Thailand based on 16S-23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses

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Received 30 November 2007 / Accepted 3 March 2008

Abstract - Ninety-seven acetic acid bacteria, which were isolated from fruits, flowers and other materials collected in Thailand by an enrichment culture approach, were assigned to the genus *Acetobacter* by phenotypic and chemotaxonomic characterisations. On the basis of 16S-23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses, the ninety-seven isolates were grouped into three groups and identified at the specific level: 1) group A including fifty-three isolates, which were identified as *Acetobacter pasteurianus*, 2) group B including forty-two isolates, which were identified as *Acetobacter orientalis* and 3) group C including two isolates, which were identified as *Acetobacter lovaniensis*. There was no isolate to be assigned to other 15 species of the genus *Acetobacter*.

Key words: acetic acid bacteria; *Acetobacter*; Thai isolates; 16S-23S rRNA gene ITS restriction analysis.

INTRODUCTION

Acetic acid bacteria (AAB) are commonly found in nature, in fermented foods such as vinegar and in various plants such as fruits, flowers, etc. AAB have the ability to oxidise different kinds of alcohols and sugars in commercially important foods and chemical products such as vinegar, kombucha tea, L-sorbose, D-gluconic acid, etc. In addition, AAB do not only have the capability of producing considerable amounts of extracellular polysaccharides, but also spoiling beer, juice, wine and fruits (Kersters *et al*., 2006).

AAB are presently classified into ten genera: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia* and *Granulibacter* in the family *Acetobacteraceae* (Skerman *et al*., 1980; Loganathan and Nair, 2004; Jojima *et al*., 2004; Yukphan *et al*., 2005, 2006; Greenberg *et al*., 2006; Kersters *et al*., 2006).

The genus *Acetobacter* is characterised morphologically by Gram-negative, strictly aerobic rods with peritrichous flagella when motile, physiologically by oxidising acetate and lactate to CO_2 and H_2O and chemotaxonomically by ubiquinone-9 (Q-9) as the major quinone (Kersters *et al*., 2006).

According to the Approved Lists of Bacterial Names (Skerman *et al*., 1980), only three species were recognised in the genus *Acetobacter*: *Acetobacter aceti*, *Acetobacter pasteurianus* and *Acetobacter peroxydans*. At present, however, 18 species are reported in total: *A. aceti*, *A. pasteurianus*, *A. peroxydans*, *A. pomorum*, *A. lovaniensis*, *A. orleanensis*, *A. estunensis*, *A. indonesiensis*, *A. tropicalis*, *A. cibinongensis*, *A. cerevisiae*, *A. malorum*, *A. orientalis*, *A. syzygii*, *A. oeni*, *A. nitrogenifigens*, *A. senegalensis* and *A. ghanensis*.

AAB are conventionally identified at the species level by phenotypic characterisation (Asai *et al*., 1964; De Ley *et al*., 1984). However, the phenotypic identification is time-consuming, and the results obtained are often inaccurate and unreliable. Molecular methods are therefore required to resolve such inaccuracy and unreliability.

This paper deals with molecular identification of a large number of AAB isolated in Thailand on the basis of 16S-23S rRNA gene internal transcribed spacer (ITS) restriction and 16S rRNA gene

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sequence analyses, together with the phenotypic characterisation.

MATERIALS AND METHODS

Isolation of acetic acid bacteria and reference strains. Ninety-seven strains of AAB (Thai isolates) were isolated from 28 fruits, 19 flowers and four other materials collected in Bangkok and 18 provinces, Thailand by an enrichment culture approach using 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) composed of 2.0% D-glucose, 5.0% ethanol and 1.0% yeast extract (all by w/v). When microbial growth was found, the culture was streaked onto a GEY-agar plate containing 0.3% $CaCO_3$ (w/v) (Yamada *et al*., 1976). AAB were selected as an acid-producing bacterial strain that formed a clear zone around the colony on the GEY-agar plate containing 0.3% $CaCO_3$. *Acetobacter pasteurianus* TISTR 1056^T, *A. orientalis* NBRC 16606^T and *A. lovaniensis* NBRC 13753^T were used for reference strains.

Phenotypic characterisation. Phenotypic characterisation was carried out by incubating test strains 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 Gram stain of bacterial cells, the Hucker-Conn modified method was used (Hucker and Conn, 1923). Physiological and biochemical characterisations were made by the methods of Asai *et al*. (1964) and Gosselé *et al*. (1980).

Chemotaxonomic characterisation. AAB were grown in GYPG broths containing 10% potato extract on a rotary shaker (150-200 rpm) at 30 °C for 24 h (Moonmangmee *et al*., 2000). Ubiquinone was extracted from freeze-dry cells by shaking with a mixture of chloroform-methanol (2:1, v/v). Cells were removed by filtration, and the combined filtrates were evaporated to dryness under a reduced pressure on a rotary evaporator. The resulting residue was dissolved in a small volume of acetone, followed by thin-layer chromatography on a silica gel plate (20 x 20 cm, silica gel $60F_{254}$, Art 5715, E. Merck, Darmstdt, Germany) with a solvent system of pure benzene (Yamada *et al*., 1968). The purified ubiquinone preparation was applied to analysis for its homologues by reversed-phase paper chromatography (Yamada *et al*., 1968) and by high performance liquid chromatography (Tamaoka *et al*., 1983).

16S-23S rRNA gene ITS PCR amplification. 16S-23S rRNA gene ITS PCR amplification was made by using the two primers of Trãek and Teuber (2002), which were 5'TGCGG(C/T)TGGATCACCTC-CT-3' (position 1522-1540 on 16S rRNA by the *Escherichia coli* numbering system; Brosius *et al.*, 1981) and 5' GTGCC(A/T)AGGCATCCACCG-3' (position 38-22 on 23S rRNA).

Digestion of PCR products with restriction endonucleases. The purified PCR products were separately digested with restriction endonucleases, *Hpa*II and *Hae*III (Trček and Teuber, 2002) according to the manufacturer's instructions (New England BioLabs, Beverly, Massachusetts, USA). The resulting reaction products were analysed by 2.5% (w/v) agarose gel electrophoresis developed at 100 V for 40 min in 1X Tris-acetate running buffer.

16S rRNA gene sequencing. Bacterial 16S rRNA genes were amplified by PCR with *Taq* DNA polymerase and primers 20F (5'-GAGTTTGATCCTG-GCTCAG'-3, the *E. coli* numbering system, Brosius *et al*., 1981) and 1500R (5'GTTACCTTGTTACGACTT'-3) and sequenced as described previously (Tanasupawat *et al*., 2004). The 16S rRNA gene 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, California, USA). The following six primers were used for the 16S rRNA gene sequencing; 20F, 1500R, 520F (5'-CAGCAGCCGCG-GTAATAC-3'; positions 519-536), 520R (5'-GTAT-TACCGCGGCTGCTG-3'; positions 536-519), and 920F (5'AAACTCAAATGAATTGACGG-3'; positions 907-926), and 920R (5'-CCGTCAATTCATTTGAGTTT-3'; positions 926-907).

Sequence analyses. Multiple alignments of the sequences (ca. 1390 bases) determined were performed with a program CLUSTAL X (version 1.81) (Thompson *et al.*, 1997). Gaps and ambiguous bases were eliminated from calculation. Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree based on 16S rRNA gene sequences were constructed by the neighbour-joining method of Saitou and Nei (1987) with the program MEGA (version 2.1) (Kumar *et al*., 2001). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 replications. Pair-wise 16S rRNA gene sequence similarities were calculated for 1455 bases between phylogenetically related strains.

Base sequence deposition numbers. All the 16S rRNA gene sequences determined were deposited in the DDBJ database under the accession numbers,

AB308055, AB308058, and AB308060 respectively for isolates BBM91-1, PA3-3 and LBM3-1.

RESULTS AND DISCUSSION

The ninety-seven Thai isolates that were Gram-negative, aerobic rods, produced catalase but not oxidase and showed clear zones on GEY/$CaCO_3$ agar plates and growth at pH 3.5 were assigned to the genus *Acetobacter*, since the isolates oxidised acetate and lactate to CO_2 and H_2O and had Q-9 as the major quinone. The ninety-seven isolates were divided into three groups, *i.e*., groups A, B and C by 16S-23S rRNA gene ITS restriction analysis with *Hpa*II and *Hae*III (Table 1).

Isolates grouped into group A showed restriction patterns *f* and *h*, which showed restriction fragments comprised respectively of 450 and 330 bp and 470 and 300 bp (Trček and Teuber, 2002) and coincided with those of the type strain of *A. pasteurianus*, as found in isolate PA3-3 (Fig. 1). The isolates of group A were 53 in total (data not shown except for isolate PA3-3) and amounted to 55% of all the isolates assigned to the genus *Acetobacter*. In a phylogenetic tree based on 16S rRNA gene sequences, isolate PA3-3 was located within the cluster of *A. pasteurianus* (Fig. 2). Additionally, the isolate had 99.8% pair-wise 16S rRNA sequence similarity to the type strain of *A. pasteurianus*. Isolates PA3-3, SF61-2, SL89-1 and OR56-2 had Q-9 as the major quinone. These chemotaxonomic data were identical with those reported by Yamada *et al*. (1968). All the isolates of group A produced acetylmethyl carbinol from lactate and acid from cellobiose but not 2-keto-D-gluconate from D-glucose and grew at 40 °C and in the presence of 10% ethanol (v/v) (Table 2). From the data obtained above, the 53 isolates of group A were identified as *A. pasteurianus*. The group A isolates were found in fruits of rumbutan, jackfruit, starfruit, hog plum, watermelon, salas, cantaloup, custard apple, longan, strawbeery, pummelo, orange, tomato, apple, musk-melon, grape, guava, long gong, langsat, sapodilla, avocado, banana and rose apple, in flowers of zinnia, cananga, periwinkle, cordia, heliconia and siam tulip, in honey and in fermented starch.

Isolates grouped into group B showed restriction patterns *e* and *i*, which showed restriction fragments comprised respectively of 400 and 500 bp and 350 and 580 bp and coincided with those of the type strain of *A. orientalis*, when digested with *Hpa*II and *Hae*III, as found in isolate BBM91-1 (Fig. 1). The isolates of group B were 42 in total (data not shown except for isolate BBM91-1) and amounted to 43% of all the isolates assigned to the genus *Acetobacter*. In a phylogenetic tree based on 16S rRNA gene sequences, isolate BBM91-1 was located within the cluster of *A. orientalis* (Fig. 2). Additionally, the isolate had 99.6% pair-wise 16S rRNA sequence similarity to the type strain of *A. orientalis*. All the isolates of group B produced acetylmethyl carbinol from lactate weakly, acid from D-xylose and melibiose, and 2-keto-D-gluconate from D-glucose, but did not grow at 40 °C and in the presence of 10% ethanol (v/v) (Table 2). From the data obtained above, the 42 isolates of group B were identified as *A. orientalis*. The group B isolates were found in fruits of sugar apple, apple, starfruit, dragon fruit, musk-melon, tamarind, mangosteen, kaffir lime, jackfruit, jujube, banana and guava, in flowers of jasmine, ixora, rain tree, cordia, allamanda, quassia, spider, petunia, night jasmine, golden shower, cosmetic bark tree and chrismas, in khaomak, in thaivermicelli and in fermented starch.

Isolates grouped into group C showed restriction patterns *c* and *j*, which showed restriction fragments comprised respectively of 495 and 400 bp and 325 and 570 bp, and which coincided with those of the type strain of *A. lovaniensis*, when digested with *Hpa*II and *Hae*III, as found in isolate LMB3-1 (Fig. 1). The isolates of group C were two (data not shown except for isolate LMB3-1 and amounted to only 2%. In a phylogenetic tree based on 16S rRNA gene sequences, isolate LMB3-1 was located within the cluster of *A. lovaniensis* (Fig. 2). Additionally, the isolate had 99.8% pair-wise 16S rRNA sequence similarity to the type strain of *A. lovaniensis*. The isolates of group C produced acetylmethyl carbinol from lactate weakly, acid from D-xylose and cellobiose weakly, but did not produce 2-keto-D-gluconate from glucose, and did not grow at 40 °C and in the presence of 10% ethanol (v/v) (Table 2). From the data obtained above, the two isolates of group C were identified as *A. lovaniensis*. The group C isolates were found in flower of golden fig.

Seearunruangchai *et al*. (2004) isolated 40 AAB from fruits collected in Thailand. Among the 40 isolates, thirty-one strains that amounted to 77% of all the isolates were identified as *A. pasteurianus*. Three strains that amounted to only 8% were identified as *A. orientalis*, and the remaining six isolates that amounted to 15% were identified as *Gluconacetobacter liquefaciens*. The present study demonstrated that similar results were obtained that the isolates identified as the two species, *A. pasteurianus* and *A. orientalis* were mainly isolated from Thai sources along with a very small amount of isolates identified as *A. lovaniensis* except for no isolate to be identified as *Gluconacetobacter liquefaciens*.

From the results described above, a large number of Thai isolates can be assigned to the genus *Acetobacter* physiologically by the acetate and lactate oxidation and chemotaxonomically by the ubiquinone analysis and identified successfully at the species level by the molecular-biological techniques including 16S-23S rRNA gene ITS restriction

TABLE 1 - Identification of Thai isolates assigned to the genus Acetobacter based on 16S-23S rRNA ITS restriction analysis

Isolate	Source		Restriction pattern by digestion with	
	Sample	Province	*HpaII*	*HaeIII*
Group A *(Acetobacter pasteurianus)*				
OR55-1, OR55-2, OR56-2, OR95-1	Orange fruit	Bangkok	*f*	*h*
BB91-1	Banana fruit	Bangkok	*f*	*h*
GV74-1	Guava fruit	Bangkok	*f*	*h*
WM86-1	Watermelon fruit	Bangkok	*f*	*h*
AP94-1, AP60-1, AP94-2	Apple fruit	Bangkok	*f*	*h*
RB1-1, RB3-1	Rumbutan fruit	Bangkok	*f*	*h*
AK33-1	Musk-melon fruit	Bangkok	*f*	*h*
AM13-2	Heliconia flower	Bangkok	*f*	*h*
BA 28-2	Salas fruit	Rayong	*f*	*h*
LM26-1	Long gong fruit	Rayong	*f*	*h*
PW19-2	Periwinkle flower	Rayong	*f*	*h*
ZN22-1	Zinnia flower	Rayong	*f*	*h*
CS15-2, CS15-4	Cordia flower	Rayong	*f*	*h*
LK88-1	Long-gong fruit	Trad	*f*	*h*
SL89-1, SL89-2	Sapodilla fruit	Trad	*f*	*h*
WM77-1	Watermelon fruit	Trad	*f*	*h*
CT85-1, CT85-2	Cantaloup fruit	Saraburi	*f*	*h*
FCL4-5	Fermented starch	Saraburi	*f*	*h*
FBY4-3, FC4-3	Fermented starch	Saraburi	*f*	*h*
GR64-1, GR64-2	Grape fruit	Roie	*f*	*h*
KD66-1	Cananga flower	Roie	*f*	*h*
BB91-1	Banana fruit	Roie	*f*	*h*
PK48-1	Siam tulip flower	Khon Kaen	*f*	*h*
CR16-2, CR84-2	Custard apple fruit	Khon Kaen	*f*	*h*
RA3-1, RA30-1	Rose apple fruit	Pathumthani	*f*	*h*
ST107-1	Strawberry fruit	Pathumthani	*f*	*h*
PA3-3, TM58-1	Tomato fruit	Changmai	*f*	*h*
LG57-2	Longan fruit	Changmai	*f*	*h*
HP27-1	Hog plum fruit	Nongkhai	*f*	*h*
SM63-1, SM63-2	Jackfruit	Nongkhai	*f*	*h*
LS60-1, LS60-2	Langsat fruit	Chantaburi	*f*	*h*
SF61-2	Star fruit	Chantaburi	*f*	*h*
FP47-2	Siam tulip flower	Nontaburi	*f*	*h*
PS49-1	Siam tulip flower	Nontaburi	*f*	*h*
HG45-2	Honey	Phuket	*f*	*h*
AV28-1	Avocado fruit	Phetchabun	*f*	*h*
PM169-2	Pummelo fruit	Ubon	*f*	*h*
Group B (*Acetobacter orientalis*)				
CA76-2	Sugar apple fruit	Bangkok	*e*	*i*
SF18-1	Star fruit	Bangkok	*e*	*i*
MHM10-2	Jasmine flower	Bangkok	*e*	*i*
TE37-2	Allamanda flower	Bangkok	*e*	*i*
LM12-1	Spider flower	Bangkok	*e*	*i*
CM50-1, CM50-2	Chrismas flower	Chonburi	*e*	*i*
DM52-1	Cordia flower	Chonburi	*e*	*i*
JA54-1	Flower of rain tree	Chonburi	*e*	*i*
PN53-1	Petunia flower	Chonburi	*e*	*i*
RP55-1	Golden shower flower	Khon Kaen	*e*	*i*
TV83-2	Thaivermicelli	Khon Kaen	*e*	*i*
DA3-1	Dragon fruit	Khon Kaen	*e*	*i*
EN6-3, BL13-12	Khao-mak	Khon Kaen	*e*	*i*
CA127-1, CA127-2	Sugar apple fruit	Saraburi	*e*	*i*
MM86-1	Musk-melon fruit	Saraburi	*e*	*i*
FBM 4-3, FBM4-4	Fermented starch	Saraburi	*e*	*i*
KLM13-1	Kaffir lime fruit	Saraburi	*e*	*i*
LR41-1	Apple fruit	Rayong	*e*	*i*
NJ17-3	Night Jasmine flower	Rayong	*e*	*i*
CM3-1	Quassia flower	Rayong	*e*	*i*
HM12-1, HM12-2	Ixora flower	Rayong	*e*	*i*
JJ87-1	Jujube fruit	Trad	*e*	*i*
MT78-1, MT78-2	Mangosteen fruit	Trad	*e*	*i*
JF81-1	Jackfruit	Nongkhai	*e*	*i*
PN19-1	Jackfruit	Nongkhai	*e*	*i*
AM35, AM41, CD21-1	Tamarind fruit	Chantaburi	*e*	*i*
GA8-1, GA8-2, HN9-1, HN9-2	Guava fruit	Kanchanaburi	*e*	*i*
BN1-1, BN1-2	Kaffir lime fruit	Changmai	*e*	*i*
DT4-2	Flower of cosmetic bark tree	Khampangphet	*e*	*i*
BBM91-1	Banana fruit	Roie	*e*	*i*
Group C (*Acetobacter lovaniensis*)				
LBM3-1, LBM3-2	Golden Fig flower	Nongkhai	*c*	*j*

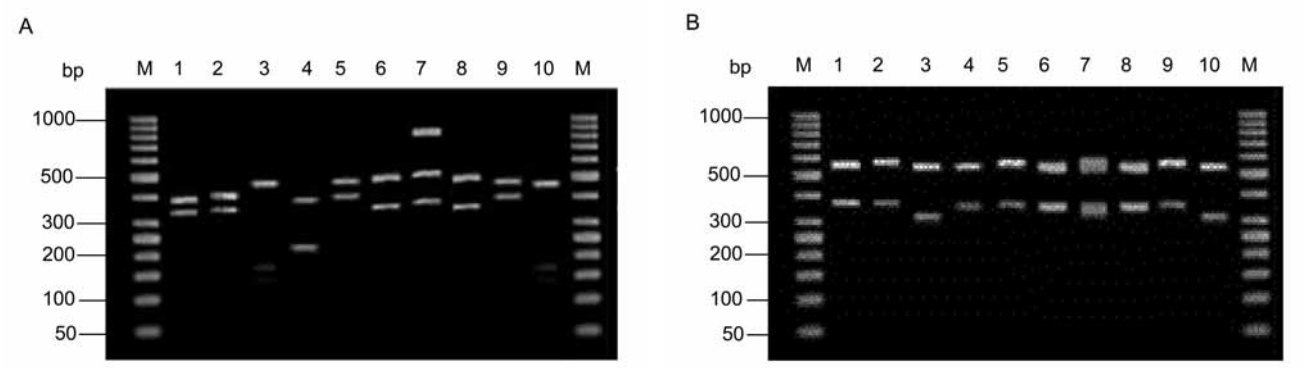

FIG. 1 - Restriction of 16S-23S rRNA ITS PCR products of Thai isolates assigned to the genera *Acetobacter*. The restriction analysis was made for Thai isolates assigned to the genus *Acetobacter* with *Hpa*II (A) and *Hae*III (B). For estimation of digestion fragments produced from 16S-23S rRNA ITS PCR products, 50 bp DNA markers were used in the agarose gel electrophoresis. 1: *A. indonesiensis* NBRC 16471^T, 2: *A. cibinongensis* NBRC 16605^T, 3: A. lovaniensis NBRC 13753T, 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: isolate PA3-3 of group A, 9: isolate BBM91-1 of group B, 10: isolate LBM3-1 of group C.

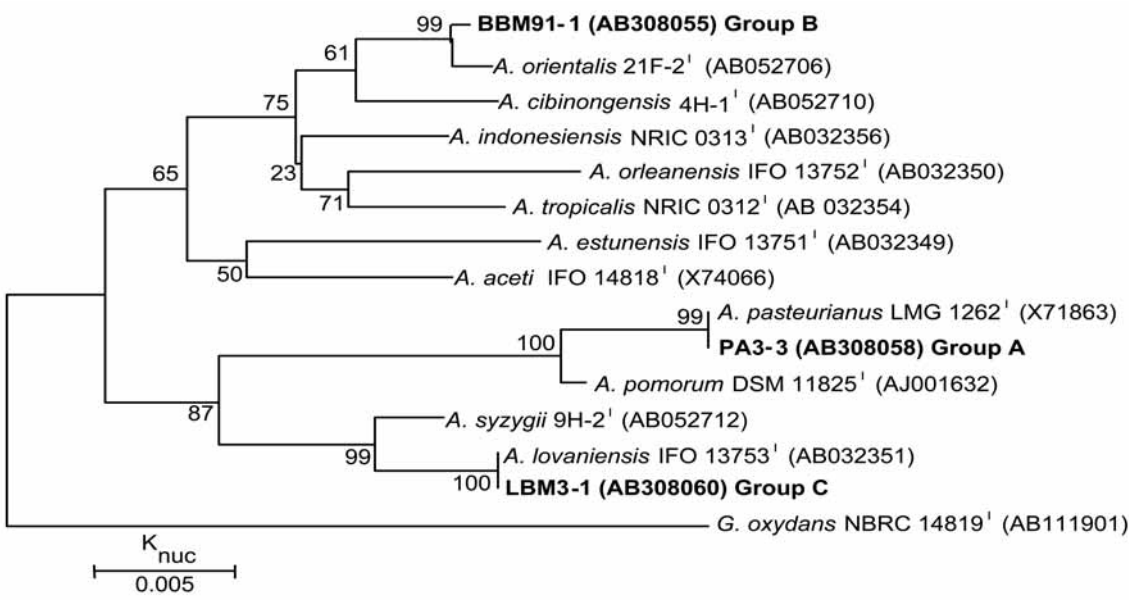

FIG. 2 - A phylogenetic tree based on 16S rRNA sequences for Thai isolates assigned to the genus *Acetobacter*. The phylogenetic tree was constructed by the neighbour-joining method. The type strain of *Gluconobacter oxydans* was used for an outgroup. Numbers (%) at nodes indicate bootstrap values derived from 1000 replications. Bar, 0.005 substitutions per 100 nucleotide positions.

TABLE 2 - Differential characteristics of Thai isolates assigned to the genus *Acetobacter*

Characteristic	Group A (53 isolates)	*Ap*	Group B (42 isolates)	*Ao*	Group C (2 isolates)	*Al*
Growth at 40 °C	+	+	-	-	-	-
Nitrate reduction	+	+	-	-	+	+
Growth in 10% ethanol (v/v)	+	+	-	-	-	-
Acetylmethyl carbinol from lactate	+	+	w	w	w	w
2-Keto-D-gluconate from D-glucose	-	-	+	+	-	-
Growth on L-Arabitol	-	-	-	-	w	w
Acid production from						
D-Xylose	-[a]	-	+	+	+	+
Cellobiose	+	+	-[b]	-	w	w
Melibiose	-	-	+	+	-	-

Ap: A. *pasteurianus* TISTR 1056^T, *Ao*: *A. orientalis* NBRC 16606^T, *Al*: *A. lovaniensis* NBRC 13753^T. +: positive, -: negative, w: weakly positive; [a] weakly positive in eight isolates, [b] weakly positive in six isolates.

analysis using *Hpa*II and *Hae*III and 16S rRNA gene sequence analysis.

Acknowledgements

This study was supported in part by the 90th Anniversary of Chulalongkorn University Research Grant (2006).

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