Annals of Microbiology, **58 (4)** 741-747 (2008)

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

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Received 23 May 2008 / Accepted 23 September 2008

ABSTRACT - Forty-five 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 *Gluconobacter* by phenotypic and chemotaxonomic characterisations. On the basis of 16S-23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses, the forty-five isolates were grouped into five groups and identified at the specific level as follows: 1) seventeen isolates were grouped into Group A and identified as *G. oxydans*; 2) twelve isolates were grouped into Group B and identified as *G. cerinus*; 3) nine isolates were grouped into Group C and identified as *G. frateurii*; 4) six isolates were included into Group D and identified as *G. thailandicus*; 5) one isolate was grouped into Group E, characterised by a restriction pattern comprised of 667 and 48-bp fragments in *Ava*II digestion, differing from those of strains of Group C or *G. frateurii* and Group D or *G. thailandicus*, and unidentified.

Key words: acetic acid bacteria, *Gluconobacter*, Thai isolates, 16S-23S rRNA gene ITS restriction analyses, 16S rRNA gene sequence analyses.

INTRODUCTION

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

AAB are presently classified into eleven genera: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter* and *Tanticharoenia* in the family *Acetobacteraceae* (Skerman *et al*., 1980; Jojima *et al*., 2004; Loganathan and Nair, 2004; Greenberg *et al*., 2006; Kersters *et al*., 2006; Yukphan *et al*., 2005a, 2006, 2008a, 2008b).

In the genus *Gluconobacter* Asai 1935, eight species were once described: *Gluconobacter oxydans* De Ley 1961 (the type species), *Gluconobacter cerinus* Yamada and Akita 1984, *Gluconobacter frateurii* Mason and Claus 1989, *Gluconobacter asaii* Mason and Claus 1989, *Gluconobacter albidus* Yukphan *et al*. 2005a, *Gluconobacter thailandicus* Tanasupawat *et al*. 2005, *Gluconobacter kondonii* Malimas *et al*. 2008 and *Gluconobacter roseus* Malimas *et al*. 2008 (De Ley, 1961; Skerman *et al*., 1980; Yamada and Akita, 1984a, 1984b; Mason and Claus, 1989; Yukphan *et al*., 2004a, 2005b; Tanasupawat *et al*., 2004, 2005; Malimas *et al*., 2007, 2008a, 2008b, 2008c). Of the eight species, *G. asaii* was a junior subjective synonym of *G. cerinus* (Tanaka *et al*., 1999; Yamada *et al*., 1999; Katsura *et al*., 2002).

AAB have traditionally been classified and identified on the basis of morphological, physiological, biochemical and chemotaxonomic characteristics. For species classification and identification of *Gluconobacter* strains, phenotypic features characterised morphologically by Gram-negative, strictly aerobic, polar-flagellated rods, physiologically by no oxidisation of acetate and lactate, requirement of nicotinic acid and growth on D-arabitol, L-arabitol and *meso*-ribitol and chemotaxonomically by ubiquinone-10 (Q-10) are generally utilised (Asai *et al*., 1964, Gosselé *et al*., 1983; Yamada and Akita, 1984a; Yamada *et al*., 1969, 1999; Tanaka *et al*., 1999; Katsura *et al*., 2002). However, the phenotypic classification and identification are not so easy but rather difficult, and the results obtained are sometimes not only inaccurate but also time-consuming. The application of molecular methods based, for example, on 16S-23S rRNA gene internal transcribed spacer (ITS) restriction and sequence analyses achieves quick and accurate classification and identification

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of these microorganisms, along with 16S rRNA gene sequence analysis (Tanasupawat *et al*., 2004; Yukphan *et al*., 2004a; Malimas *et al*., 2007, 2008b; Kommanee *et al.,* 2008).

This paper deals with a large number of *Gluconobacter* strains isolated in Thailand on the basis of 16S-23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses as well as the phenotypic characterisation.

MATERIALS AND METHODS

Isolation of acetic acid bacteria and reference strains. Forty-five strains of AAB were isolated from 14 fruits, 20 flowers and three other materials collected in Bangkok and nine 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 agar plate. *Gluconobacter oxydans* NBRC 14819^T, *G. cerinus* NBRC 3267^T, *G. frateurii* NBRC 3264^T, *G. albidus* NBRC 3250^T, *G. thailandicus* NBRC 100600^T, *G. kondonii* NBRC 3266^T and *G. roseus* NBRC 3990^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), Gosselé *et al*. (1983), Yamada and Akita (1984a), Mason and Claus (1989), Yamada *et al.* (1999) and Katsura *et al*. (2002).

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*., 1969). The purified ubiquinone preparation was applied to analysis for its homologues by reversed-phase paper chromatography (Yamada *et al*., 1969) and by high performance liquid chromatography (Tamaoka *et al*., 1983).

16S-23S rRNA gene ITS PCR amplification. The 16S-23S rRNA gene ITS PCR amplification was made by using the two primers of Trček and Teuber (2002), which were 5'TGCGG(C/T) TGGATCACCTCCT-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). Isolate AN1-1 of Group E was sequenced for 16S-23S rRNA gene ITS (Yukphan *et al*., 2004b), and the sequence determined was analysed with the program NEBcutter (ver. 2.0, New England BioLabs, Beverly, Massachusetts, USA) for *Mbo*II, *Bsp*1286I and *Ava*II digestions.

Digestion of PCR products with restriction endonucleases. The purified PCR products were separately digested with restriction endonucleases *Bsp*1286I, *Mbo*II and *Ava*II (New England BioLabs). The resulting reaction products were analysed by 2.5% (w/v) agarose gel electrophoresis developed at 100V for 40 min in 1x Tris-acetate running buffer.

16S rRNA gene sequencing. The bacterial 16S rRNA genes were amplified by PCR with *Taq* DNA polymerase and primers 20F (5'-GAGTTTGATCCTGGCTCAG'-3, the *Escherichia 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'-CAGCAGCCGCGGTAATAC-3'; positions 519-536), 520R (5'-GTATTACCGCGGCTGCTG-3'; positions 536-519), and 920F (5'AAACTCAAATGAATTGACGG-3'; positions 907-926), and 920R (5'-CCGTCAATTCATTTGAGTTT-3'; positions 926-907).

Sequence analyses. Multiple sequence alignments (ca. 1382 bases) were performed with a program CLUSTAL_X (version 1.83) (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 was constructed by the neighbor-joining method of Saitou and Nei (1987) with the program MEGA 4 (Tamura *et al*., 2007). 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 1435 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, AB436557, AB436556, AB436558, AB436559 and AB436555 respectively for isolates JR70-1, AK33-2, LD51-1, MG71-2 and AN1-1.

RESULTS AND DISCUSSION

The 45 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 *Gluconobacter*, since the isolates did not oxidise acetate and lactate and had Q-10 as the major quinone. The isolates showed growth on mannitol agar, but not on glutamate agar. Production of dihydroxyacetone from glycerol was positive. The 45 isolates were divided into five groups, *i.e*., Groups A, B, C, D and E on the basis of the restriction patterns obtained in *Mbo*II, *Bsp*1286I and *Ava*II digestions.

Group A was composed of 17 isolates, which included AG21-1, BA28-1, etc. (Table 1). The 17 isolates gave the same restriction patterns as *G. oxydans* NBRC 14819^T, when digested with restriction endonucleases *Bsp*1286l and *Mbo*ll, which showed respectively 362, 177, 92 and 82-bp fragments and 585, 58, 35 and 35-bp fragments (Fig. 1) (Yukphan *et al*., 2004a), In a

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

Isolate	Isolation source		Restriction pattern by digestion with		
	Source	Province	*Bsp*1286I	*Mbo*II	*Ava*II
Group A or *G. oxydans*					
AM26, AM28, AM46	Pagoda flower	Rayong	*Go*	*Go*	-
AM48, AM68	Baccaurea	Rayong	*Go*	*Go*	-
AG21-1	Petunia	Rayong	*Go*	*Go*	-
BA28-1	Salas	Rayong	*Go*	*Go*	-
SB20-2	Chaba	Rayong	*Go*	*Go*	-
JJ87-2	Jujube	Trad	*Go*	*Go*	-
LK88-2	Long-gong	Trad	*Go*	*Go*	-
ST79-1	Strawberry	Trad	*Go*	*Go*	-
LP92-1	Look-pang	Khon Kaen	*Go*	*Go*	-
RP55-2	Flower of golden shower tree	Khon Kaen	*Go*	*Go*	-
JR70-1	Mango	Nontaburi	*Go*	*Go*	-
LD51-2	Plumeria flower	Chonburi	*Go*	*Go*	-
SF61-1	Star fruit	Chantaburi	*Go*	*Go*	-
HG45-1	Honey	Phuket	*Go*	*Go*	-
Group B or *G. cerinus*					
MR40-1	Spider flower	Bangkok	*Gc*	*Gc*	-
PH32-2	Peach	Bangkok	*Gc*	*Gc*	-
PP42-1	Bottle tree	Bangkok	*Gc*	*Gc*	-
AK33-2	Musk-melon	Bangkok	*Gc*	*Gc*	-
AG21-2	Petunia	Rayong	*Gc*	*Gc*	-
AM10-3	Cordia	Rayong	*Gc*	*Gc*	-
AM14-1	Baccaurea	Rayong	*Gc*	*Gc*	-
MM86-2	Musk-melon	Saraburi	*Gc*	*Gc*	-
AN34-2	Night jasmine	Saraburi	*Gc*	*Gc*	-
DM52-2	Cordia	Chonburi	*Gc*	*Gc*	-
MK44-1	Papaya	Phuket	*Gc*	*Gc*	-
PS49-2	Petunia	Nontaburi	*Gc*	*Gc*	-
Group C or *G. frateurii*					
AM10-1	Cordia	Rayong	*Gf*	*Gf*	*Gf*
CR16-1	Caricature plant	Rayong	*Gf*	*Gf*	*Gf*
CR84-1	Custard apple	Khon Kaen	*Gf*	*Gf*	*Gf*
LP92-2	Look-pang	Khon Kaen	*Gf*	*Gf*	*Gf*
KT43-2	Ixora	Bangkok	*Gf*	*Gf*	*Gf*
LR4-1	Heliconia	Bangkok	*Gf*	*Gf*	*Gf*
HP27-2	Hog plum	Nongkhai	*Gf*	*Gf*	*Gf*
JR70-2	Mango	Nontaburi	*Gf*	*Gf*	*Gf*
LD51-1	Plumeria	Chonburi	*Gf*	*Gf*	*Gf*
Group D or *G. thailandicus*					
AM29, AM 47, AM24	Little yellow star	Rayong	*Gf*	*Gf*	*Gt*
ZN22-2	Zinnia	Rayong	*Gf*	*Gf*	*Gt*
MG71-2	Mango	Bangkok	*Gf*	*Gf*	*Gt*
CK36-2	Flower of golden shower tree	Khon Kaen	*Gf*	*Gf*	*Gt*
Group E (unidentified)					
AN1-1	Flower of rain tree	Rayong	*Gf*	*Gf*	unident.

Go: *G. oxydans*, *Gc*: *G. cerinus*, *Gf*: *G. frateurii*, *Gt*: *G. thailandicus*, -: not tested, unident.: unidentified.

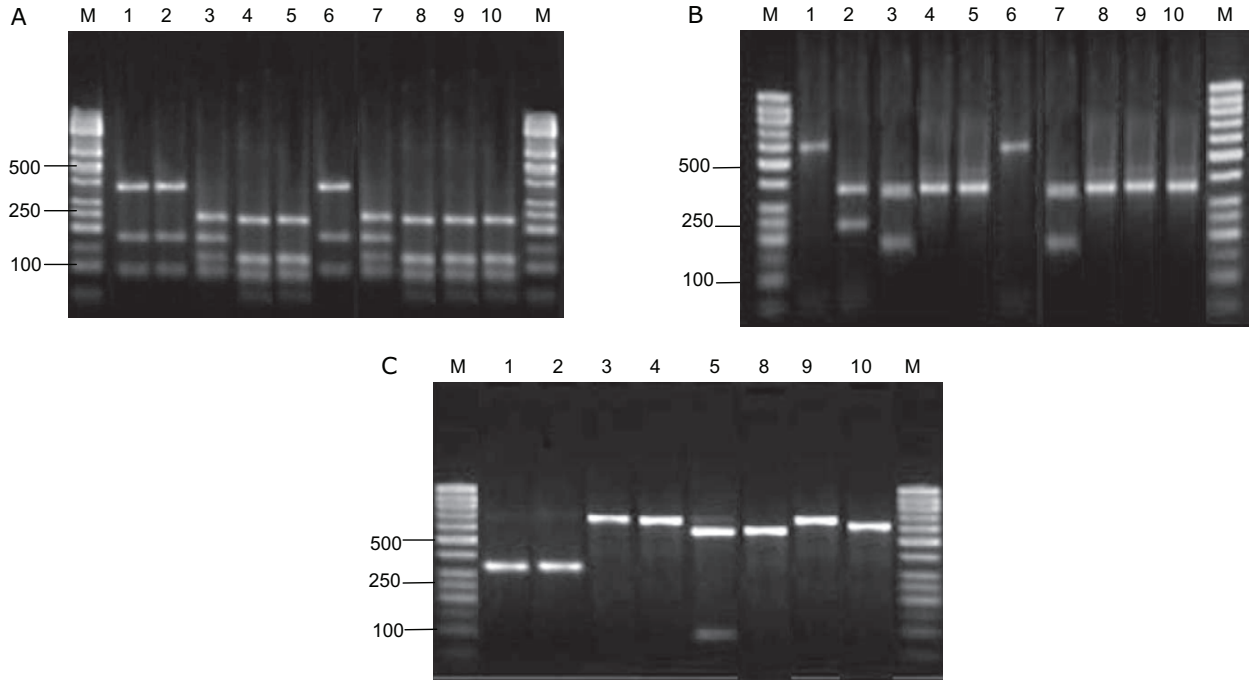

FIG. 1 - Restriction of 16S-23S rRNA gene ITS PCR products of Thai isolates assigned to the genus *Gluconobacter*. The restriction analysis was made for Thai isolates assigned to the genus *Gluconobacter* with restriction endonucleases *Bsp*1286I (A), *Mbo*II (B) and *Ava*II (C). For estimation of digestion fragments, 50 bp DNA markers were used in the agarose gel electrophoresis. Lane 1: *G. oxydans* NBRC 14819^T, lane 2: *G. albidus* NBRC 3250^T, lane 3: *G. cerinus* NBRC 3267^T, lane 4: *G. thailandicus* NBRC 100600^T, lane 5: *G. frateurii* NBRC 3264^T, lane 6: isolate JR70-1 of Group A, lane 7: isolate AK33-2 of Group B, lane 8: isolate LD51-1 of Group C, lane 9: isolate MG71-2 of Group D, lane 10: isolate AN1-1 of Group E, M: 50 bp DNA marker.

phylogenetic tree based on 16S rRNA gene sequences, isolate JR70-1 of Group A was located within the cluster of *G. oxydans* (Fig. 2) and had 99.8% pair-wise 16S rRNA gene sequence similarity to the type strain of *G. oxydans*. The isolates produced acetyl methyl carbinol from lactate and grew at 30 and 37 °C. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced, but 2,5-diketo-D-gluconate was not. The isolates did not show any growth on D-arabitol, L-arabitol and *meso*-ribitol, being different from strains of other groups (Table 2). They produced acid from D-mannose (weakly positive), *myo*-inositol (weakly positive), maltose and melibiose (weakly positive), but not from D-sorbitol and sucrose. The 17 isolates of Group A were therefore identified as *G. oxydans.* They were found in fruits of mango, jujube, long-gong, salas, strawberry, star fruit, baccaurea, in flowers of

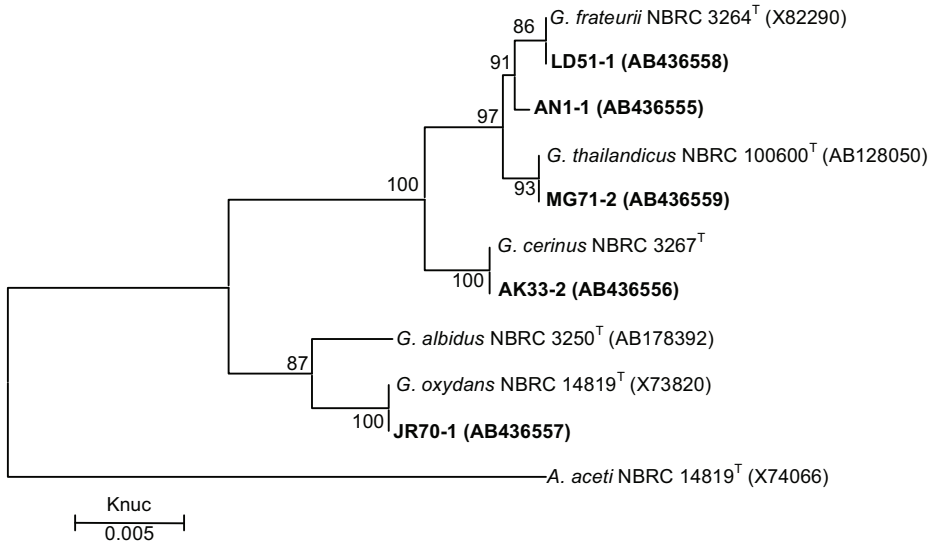

FIG. 2 - A phylogenetic tree based on 16S rRNA gene sequences for Thai isolates assigned to the genus *Gluconobacter*. The phylogenetic tree was constructed by the neighbor-joining method. The type strain of *Acetobacter aceti* NBRC14818^T was used for an outgroup. Numerals (%) at nodes indicate bootstrap values derived from 1000 replications. Bar: 0.005 substitutions per 100 nucleotide position.

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

Characteristics	Group A 17 isolates	*G. oxydans* NBRC 14819^T	Group B 12 isolates	*G. cerinus* NBRC 3267^T	Group C 9 isolates	*G. frateurii* NBRC 3264^T	Group D 6 isolates	*G. thailandicus* NBRC 100600^T	Group E 1 isolate
Acetate oxidation	-	-	-	-	-	-	-	-	-
Lactate oxidation	-	-	-	-	-	-	-	-	-
Ubiquinone	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
Growth at 37 °C	+	+	-	-	-	-	w	w	+
Growth at pH 3.0	+	+	-	-	+	+	+	+	+
Growth on:									
D-Arabitol	-	-	+	+	+	+	+	+	-
L-Arabitol	-	-	-	-	w	w	w	w	w
meso-Ribitol	-	-	-	-	w	w	w	w	w
Acid production from:									
D-Mannose	w	w	+	+	+	+	-	-	+
D-Sorbitol	-	-	+	+	-	-	-	-	-
myo-Inositol	w	w	+	+	+	+	-	-	-
Maltose	+	+	-	-	-	-	-	-	+
Melibiose	w	w	+	+	+	+	-	-	+
Sucrose	-	-	-	-	+	+	-	-	+

+: positive, w: weakly positive, -: negative.

petunia, plumeria, chaba, pagoda, golden shower tree, in honey and in look-pang.

Group B was composed of 12 isolates, which included DM52-2, MK44-1, etc. (Table 1). The 12 isolates gave the same restriction patterns as *G. cerinus* NBRC 3267^T, when digested with restriction endonucleases *Bsp*1286I and *Mbo*II, which showed respectively 241, 177, 121, 92 and 82-bp fragments and 356, 186 and 171-bp fragment (Fig. 1) (Yukphan *et al*., 2004a). In a phylogenetic tree based on 16S rRNA gene sequences, isolate AK33-2 of Group B was located within the cluster of *G. cerinus* (Fig. 2) and had 99.9% pair-wise 16S rRNA gene sequence similarity to the type strain of *G. cerinus*. The isolates produced acetyl methyl carbinol from lactate and grew at 30 °C but not at 37 °C. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced, but 2,5-diketo-D-gluconate was not. The isolates showed growth on D-arabitol, being different from strains of *G. oxydans*, but not any growth on L-arabitol and *meso*-ribitol, being different from strains of *G. frateurii* and *G. thailandicus* (Table 2). They produced acid from D-mannose, D-sorbitol, *myo*-inositol and melibiose, but not from maltose and sucrose. The 12 isolates of Group B were therefore identified as *G. cerinus.* They were found in fruits of baccaurea, peach and musk-melon and in flowers of petunia, papaya, spider, night jasmine, cordia and bottle tree.

Group C was composed of nine isolates, which included AM10-1, CR16-1, CR84-1, etc. (Table 1). The nine isolates gave the same restriction patterns as *G. frateurii* NBRC 3264^T, when digested with restriction endonucleases *Bsp*1286I, *Mbo*II and *Ava*II, which showed respectively 244, 125, 121, 92, 82 and 51-bp fragments, 360 and 355-bp fragments and 610 and 105-bp fragments (Fig. 1) (Tanasupawat *et al*., 2004; Yukphan *et al*., 2004a; Malimas *et al*., 2006). In a phylogenetic tree based on 16S rRNA gene sequences, isolate LD51-1 of Group C was located within the cluster of *G. frateurii* (Fig. 2) and had 99.8% pair-wise 16S rRNA gene sequence similarity to the type strain of *G. frateurii*. The isolates produced acetyl methyl carbinol from lactate and grew at 30 °C but not at 37 °C. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced, but 2,5-diketo-D-gluconate was not. The isolates showed growth on D-arabitol, L-arabitol (weakly positive) and *meso*-ribitol (weakly positive), being different from strains of *G. oxydans* and *G. cerinus*. They produced acid from D-mannose, *myo*-inositol, melibiose and sucrose, but not from D-sorbitol and maltose. The nine isolates of Group C were therefore identified as *G. frateurii.* They were found in fruits of custard apple, mango, hog plum, in flowers of cordia, caricature, ixora, plumeria and heliconia and in look-pang.

Group D was composed of six isolates, which included MG71-2, ZN22-2, AM24, etc. (Table 1). The six isolates gave the same restriction patterns as *G. thailandicus* NBRC 100600^T, when digested with restriction endonucleases *Bsp*1286I, *Mbo*II and *Ava*II, which showed respectively 244, 125, 121, 92, 82 and 51-bp fragments, 360 and 355-bp fragments and a 715-bp band without any fragmentation (Fig. 1) (Yukphan *et al*., 2004a; Tanasupawat *et al*., 2004; Malimas *et al*., 2006). In a phylogenetic tree based on 16S rRNA gene sequences, isolate MG71-2 of Group D was located within the cluster of *G. thailandicus* (Fig. 2) and had 99.8% pair-wise 16S rRNA gene sequence similarity to the type strain of *G. thailandicus*. The isolates produced acetyl methyl carbinol from lactate and grew at 30 °C but weakly at 37 °C. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced, but 2,5-diketo-D-gluconate was not. The isolates showed growth on D-arabitol, L-arabitol (weakly positive) and *meso*-ribitol (weakly positive), being different from strains of *G. oxydans* and *G. cerinus*, but not from *G. frateurii*. The isolates did not produce acid from D-mannose, D-sorbitol, *myo*-inositol, maltose, melibiose and sucrose, differing from strains of *G. frateurii*. The six isolates of Group D were therefore identified as *G. thailandicus.* They were found in flowers of golden shower tree, little yellow star, zinnia and in fruit of mango.

Group E was composed of one isolate AN1-1 (Table 1). The isolate gave the same restriction patterns as *G. frateurii* NBRC 3264^T and *G. thailandicus* NBRC 100600^T, when digested with restriction endonucleases *Bsp*1286I and *Mbo*II. However, a different restriction pattern was given in *Ava*II digestion (Fig. 1). The computerized calculation of restriction patterns showed 360 and 355-bp fragments in *Mbo*II digestion, 244, 125, 121, 92, 82, 51-bp fragments in *Bsp*1286I digestion and showed 667 and 48-bp fragments in *Ava*II digestion, when analysed with the program NEBcutter (ver. 2.0). In a phylogenetic tree based on 16S rRNA gene sequences, isolate AN1-1 was located in the sublineage of *G. frateurii* and *G. thailandicus* but formed an inde-

pendent cluster, which was phylogenetically related to the type strain of *G. thailandicus* (Fig. 2). The calculated sequence similarities of the isolate were 98.0% to *G. frateurii* NBRC 3264^T and 99.5% to *G. thailandicus* NBRC 100600^T. The isolates produced acetyl methyl carbinol from lactate and grew at 30 and 37 °C. 2-Keto-D-gluconate and 5-keto-D-gluconate were produced, but 2,5-diketo-D-gluconate was not. The isolate grew on L-arabitol (weakly positive) and *meso*-ribitol (weakly positive), but not on D-arabitol, being different from strains of other groups. Acid was produced from D-mannose, maltose, melibiose and sucrose, but not from D-sorbitol and *myo*-inositol. The isolate was found in a flower of rain tree. From the data obtained, the isolate was not identified, and further studies are required to elucidate whether the isolate constitutes a new taxon.

In a previous study, the presence of three groups or three species assigned to the genus *Acetobacter*, *i.e*., Group A or *A. pasteurianus*, Group B or *A. orientalis* and Group C or *A. lovaniensis* was reported in 97 Thai isolates (Kommanee *et al.,* 2008). In the present study, however, four groups or four species were recognised in the 45 Thai isolates assigned to the genus *Gluconobacter* except for one isolate, which was grouped into Group E and supposed to constitute a new taxon.

Yamada *et al*. (1999) isolated a large number of acetic acid bacteria from Indonesian sources. Among the 11 isolates of the genus *Gluconobacter*, seven isolates were identified as *G. oxydans*, the remaining four isolates were identified as *G. frateurii*, and there were no isolates to be identified as *G. cerinus*. Tanaka *et al*. (1999) obtained similar results in *Gluconobacter* strains isolated from Japanese isolation sources. Huong *et al*. (2007) examined 44 Thai isolates assigned to the genus *Gluconobacter* and grouped the isolates into seven groups. However, there were no isolates to be identified as *G. cerinus*. In this study, however, the 12 isolates were actually identified as *G. cerinus*, which was one of the most unusual acetic acid bacteria.

From the results obtained above, a large number of Thai isolates assigned to the genus *Gluconobacter* physiologically by no oxidation of acetate and lactate and chemotaxonomically by Q-10 were successfully identified at the species level by the molecular-biological techniques including 16S-23S rRNA gene ITS restriction analysis using three restriction endonucleases, *Bsp*1286I, *Mbo*II and *Ava*II as well as 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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