# ORIGINAL ARTICLE

# Acetobacter thailandicus sp. nov., for a strain isolated in Thailand

Nittaya Pitiwittayakul • Pattaraporn Yukphan • Winai Chaipitakchonlatarn • Yuzo Yamada • Gunjana Theeragool

Received: 14 July 2014 / Accepted: 11 December 2014 / Published online: 10 January 2015 © Springer-Verlag Berlin Heidelberg and the University of Milan 2015

Abstract A Gram-negative, rod-shaped, and non-motile bacterium, designated as isolate  $AD25^{T}$ , was isolated from a flower of the blue trumpet vine (*Thunbergia laurifolia*) at Tong Pha Phum, Kanchanaburi, Thailand. Phylogenetic analyses of 16S rRNA gene, 16S-23S rRNA gene internal transcribed spacer (ITS) region, and *groEL* gene sequences showed that the isolate was quite remote and constituted a cluster independent from the type strains of other *Acetobacter cibinongensis*, one of the closest relatives, with 98.3 % 16S rRNA gene sequence similarity. The DNA G -+- C content of the isolate was 51.4 mol%. The isolate grew

**Electronic supplementary material** The online version of this article (doi:10.1007/s13213-014-1024-7) contains supplementary material, which is available to authorized users.

N. Pitiwittayakul · G. Theeragool Interdisciplinary Graduate Program in Genetic Engineering, The Graduate School, Kasetsart University, Ladyaow, Chatuchak Bangkok 10900, Thailand

P. Yukphan · W. Chaipitakchonlatam · Y. Yamada BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Klong Luang, Pathum Thani 12120, Thailand

#### Y. Yamada

Laboratory of Applied Microbiology (Professor Emeritus), Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Suruga-ku, Shizuoka 422-8529, Japan

#### Y. Yamada

Japan International Cooperation Agency (JICA Senior Overseas Volunteer), Shibuya-ku, Tokyo 151-8558, Japan

G. Theeragool (🖂)

Department of Microbiology, Faculty of Science, Kasetsart University, Ladyaow, Chatuchak Bangkok 10900, Thailand e-mail: fscignt@ku.ac.th intensely on 10 % ethanol with 1.5 % D-glucose in the presence of 0.3 % peptone and 0.3 % yeast extract, and grew weakly on 3.0 % D-glucose in the presence of 0.1 % ammonium sulfate as the sole source of nitrogen. The isolate produced only D-gluconic acid from D-glucose. Based on physiological, biochemical, and genotypic differences between the isolate and the type strains of the validly named species, it is proposed that the isolate be classified as a novel species of *Acetobacter*, for which the name *Acetobacter thailandicus* sp. nov. is introduced. The type strain is isolate AD25<sup>T</sup> (= BCC  $15839^{T} = NBRC \ 103583^{T}$ ).

**Keywords** Acetic acid bacterium  $\cdot$  *Acetobacter thailandicus* sp. nov  $\cdot$  *groEL* gene sequences  $\cdot$  16S rRNA gene sequences  $\cdot$  16S-23S rRNA gene ITS sequences

## Introduction

Acetic acid bacteria (AAB) that belong to the family *Acetobacteraceae*, known as *Alphaproteobacteria*, are commonly found and associated with different kinds of sugary and alcoholic materials. Strains of the genus *Acetobacter* are used for vinegar fermentation because of their intense ability to oxidize ethanol to acetic acid and their extremely high resistance to the resulting acetic acid (De Ley et al. 1984; Swings 1992). The genus *Acetobacter* is characterized by gram-negative aerobic rods and by the production of catalase, except for strains of *Acetobacter peroxydans*, and is differentiated from other genera by the intense oxidation of acetate and lactate to carbon dioxide and water and by the presence of the Q-9 system (Asai et al. 1964; Yamada et al. 1969; De

Ley et al. 1984; Cleenwerck and De Vos 2008). At the time of writing, the following 25 species have been reported: Acetobacter 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, A. fabarum, A. farinalis, A. okinawensis, A. papayae, A. persici, A. lambici, and A. sicerae (Skerman et al. 1980; Sokollek et al. 1998; Lisdiyanti et al. 2000, 2001a, b; Cleenwerck et al. 2002; Lisdiyanti et al. 2002; Dutta and Gachhui 2006; Silva et al. 2006; Cleenwerck et al. 2008; Tanasupawat et al. 2011a, b; Iino et al. 2012, 2013; Li et al. 2014; Spitaels et al. 2014).

In a previous study, 23 strains isolated in Thailand and assigned to the genus *Acetobacter* were identified at the species level by analysing the 16S rRNA and *groEL* gene sequences. The isolates were grouped into ten groups and identified as the species (Pitiwittayakul et al. 2014).

This paper proposes *Acetobacter thailandicus* sp. nov. as an additional Thai strain isolated at Tong Pha Phum, Kanchanaburi, Thailand on July 2, 2002 as the twenty-sixth species of the genus *Acetobacter*.

## Materials and methods

Bacterial isolation, reference strains, culture medium, and culture conditions

Isolate  $AD25^{T}$  (= BCC  $15839^{T}$  = NBRC  $103583^{T}$ ) was isolated from the flower of a blue trumpet vine by an enrichment culture approach using glucose/ethanol/yeast extract (GEY) medium, as briefly described below (Yamada et al. 1976, 1999; Kommanee et al. 2008; Muramatsu et al. 2009; Tanasupawat et al. 2011a). A sample source was incubated at pH 4.5 and 30 °C for 3-5 days in a liquid GEY medium (15 ml/tube) composed of 0.2 % D-glucose, 5.0 % ethanol and 1.0 % yeast extract. When microbial growth was observed, the culture was streaked onto a GEY-agar plate containing 0.3 % CaCO<sub>3</sub>. The acetic acid bacteria were selected as acidproducing bacterial strains that formed a clear zone around the colony on GEY-agar plate containing 0.3 % CaCO<sub>3</sub>. The reference strains of the genus Acetobacter were A. orientalis BCC 23127<sup>T</sup>, A. cibinongensis BCC 23126<sup>T-</sup>, and *A. tropicalis* BCC 23123<sup>T</sup>. Isolate AD25<sup>T</sup> and the reference strains used in this study were grown in a GEY broth on a rotary shaker (150-200 rpm) at 30 °C for 24 h.

PCR amplification of 16S rRNA, 16S-23S rRNA gene ITS and *groEL* gene sequences

Genomic DNA was extracted by the method described by Okumura et al. (1985). Primer sequences for the amplification and sequencing of the 16S rRNA genes were forward primer 27f; 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1525r; 5'-AAAGGAGGTGATCCAGCC-3' (Devereux and Wills 1995). Polymerase chain reaction (PCR) amplification was done, as described previously (Seearunruangchai et al. 2004).

For 16S-23S rRNA gene ITS amplification, primer 1522f (5'-TGCGGYTGGATCACCTCCT-3') and primer 38r (5' GTGCCWAGGCATCCACCG-3') were used (Ruiz et al. 2000). PCR analysis was conducted as described by Ruiz et al. (2000).

The groEL-specific primers for PCR amplification totalled nine (Table 1). In the present study, the primers for PCR amplification and sequencing of groEL genes were designed on the basis of the genome sequences of A. pasteurianus IFO 3283 (Azuma et al. 2009) and A. pomorum DM001 (Shin et al. 2011), except for primers groEL-10-F and groEL-11-R (Cleenwerck et al. 2010). The primers FgroEL and RgroEL amplified the nearly full length groEL genes of almost all strains, except for the type strains of A. peroxydans, A. cerevisiae, A. pomorum, A. aceti, A. oeni, A. estunensis, and A. nitrogenifigens, and isolate AD25<sup>T</sup>, in which an alternative primer combination, FgroELnew/RgroELnew, FgroEL89/RgroEL89, and groEL-10-F/groEL-11-R was used (Cleenwerck et al. 2010). Amplification and sequencing of all strains were done under the conditions described by Naser et al. (2005) and Cleenwerck et al. (2010). However, the optimized annealing temperature was changed to 54 °C for all primers.

Sequence data analysis and phylogenetic tree construction

Purified PCR products were sent to the First Base Laboratory (Selangor, Malaysia) for DNA sequencing. DNA sequences of the isolate obtained were edited by using the Chromas 2.33 program (http://www.technelysium.com. au/chromas.html). The DNA sequences of the isolate and the type strains of all the known species of the genus *Acetobacter* were aligned using CLUSTAL W (version 1. 83; Thompson et al. 1994). Gaps in the sequences were deleted using the BioEdit program (Hall 1999). The phylogenetic relationships among species using 16S rRNA gene, 16S-23S rRNA gene ITS, and *groEL* gene sequences were analyzed by the neighbor-joining approach (Saitou and Nei 1987) listed in MEGA (the Molecular Evolutionary Genetic Analysis, version-5.1 software; Tamura et al. 2011). For the neighbor-joining analysis, the distance FgroEL89

RgroEL89

FgroELcenter

groEL-10-F

groEL-11-R

Table 1	Primers used in this study	
Primer	Sequence (5' to 3')	Bacterial species to which primer applies
FgroEL RgroEL	CAATGGCTGCCAAAGACG GAAGGACTTAGAAGTCCAT	A. pasteurianus, A. orleanensis, A. lovaniensis, A. tropicalis, A. indonesiensis, A. syzygii, A. cibinongensis, A. orientalis, A. ghanensis, A. malorum, A. senegalensis, A. fabarum, A. farinalis, A. okinawensis, A. papayae and A. persici
FgroELne	w CTGGACAAGAGCTTCGGC	A. cerevisiae and A. peroxydans
RgroELne	W GGATAACGGCAACACCGC	A. thailandicus isolate AD25 <sup>T</sup>

A. nomorum

A. estunensis and A. oeni

A. estunensis and A. oeni

A. aceti, A. nitrogenifigens,

All species except for A. aceti, A. nitrogenifigens,

between the sequences was calculated by Kimura's twoparameter model (Kimura 1980). Bootstrap values were obtained for 1,000 randomly generated trees (Felsenstein

CCGTGCGGACAACCTTGG

CGGCACCACAACGGCTAC

GGTTGAAGAAGCCAAGCA

ACAAGTTCGAGAACATGGGC

TCCTTGCGCTCCTTCACCTC

1985). The pair-wise sequence similarity values (%) of 16S rRNA genes, 16S-23S rRNA gene ITS, and *groEL* genes were calculated without considering gaps in the

Size (bp)

1.600

1,400

1,000

1,200

900



**Fig. 1** Phylogenetic relationships of *Acetobacter thailandicus* isolate AD25<sup>T</sup>. The phylogenetic tree based on 16S rRNA gene sequences of 1,343 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

*Gluconacetobacter liquefaciens* NBRC 12388<sup>T</sup> and *Granulibacter bethesdensis* NIH1<sup>T</sup> were used as outgroups. Sequence accession numbers for 16S rRNA gene sequences are provided in *parentheses* 

Source or reference

The present study The present study

The present study

The present study

The present study

The present study

The present study

Cleenwerck et al. (2010)

Cleenwerck et al. (2010)

sequences with 1,343, 413, and 866 bases, respectively, among the species type strains.

# Phenotypic characterization

# DNA base composition and DNA-DNA hybridization

Chromosomal DNA was prepared by the method of Saito and Miura (1963). DNA base composition was determined by the method of Tamaoka and Komagata (1984).

DNA-DNA hybridization was performed by the photobiotin-labeling method with microplate wells, as described by Ezaki et al. (1989). Isolated, single stranded, and labeled DNA was hybridized with DNA from test strains in 2 × SSC and 50 % formamide at 45.0 °C for 15 h. The biotinylated DNA was quantitatively detected with streptavidin-POD and 3, 3', 5, 5'-Tetramethylbenzidine (TMB). Levels of DNA-DNA similarity (%) were determined colorimetrically (Verlander 1992). The color intensity was measured at  $A_{450}$  on a model Versa Max microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Isolate AD25<sup>T</sup> was examined for phenotypic features (Hucker and Conn 1923: Asai et al. 1964: Gosselé et al. 1980; Tanasupawat et al. 2011a). The isoprenoid guinone of the isolate was determined by the method of Yamada et al. (1969). The phenotypic features were mainly determined by incubating the isolate and test strains on glucose/yeast extract/peptone/glycerol (GYPG) agar or broth, which was composed of 10 g of D-glucose, 5 g of yeast extract, 10 g of peptone, and 10 g of glycerol, with or without 15 g of agar in one liter of water. The growth of the isolate and test strains was additionally tested on a glucose/ethanol/calcium carbonate/agar (GECA) medium which consisted of 1.5 % D-glucose, 10 % ethanol, 0.3 % peptone, 0.3 % yeast extract, 0.7 % calcium carbonate, and 1.2 % agar, or on Frateur's modified Hoyer medium, consisting of 3.0 % Dglucose, 0.1 % ammonium sulfate, 0.09 % potassium dihydrogen phosphate, 0.01 % dipotassium hydrogen phosphate, 0.025 % magnesium sulfate hydrated, and 0.0005 % hydrated ferric chloride.



**Fig. 2** Phylogenetic relationships of *Acetobacter thailandicus* isolate  $AD25^{T}$ . The phylogenetic tree based on 16S-23S rRNA gene ITS sequences of 413 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from

1,000 replications. *Gluconacetobacter liquefaciens* NBRC 12388<sup>T</sup> and *Granulibacter bethesdensis* NIH1<sup>T</sup> were used as outgroups. The sequence accession numbers for 16S-23S rRNA gene ITS sequences are provided in *parentheses* 

# **Results and discussion**

Phylogenetic analysis based on 16S rRNA, 16S-23S rRNA gene ITS, and *groEL* gene sequences

In a phylogenetic tree based on 16S rRNA gene sequences of 1,343 bases derived from the neighbor-joining method, the genus *Acetobacter* was divided into two major phylogenetic groups, i.e., Group I that corresponds to the *Acetobacter aceti* group and Group II that corresponds to the *Acetobacter aceti* group and Group With a bootstrap value of 100 % (Fig. 1) (Yamada and Yukphan 2008). Isolate AD25<sup>T</sup> was included in Group I and formed an independent cluster without any indications of bootstrap values and was quite remote from the type strains of any other species of the genus *Acetobacter*. The phylogenetic data obtained suggested that the isolate constitutes a new species within the genus *Acetobacter*.

In a phylogenetic tree based on 16S-23S rRNA gene ITS sequences of 413 bases derived from the neighbor-joining method, the two major phylogenetic groups mentioned above were also found in the genus *Acetobacter* with a bootstrap

value of 64 % (Fig. 2). However, the type strains of the four species, *A. aceti*, *A. nitrogenifigens*, *A. oeni*, and *A. estunensis*, which were once included in Group I, as well as the type strain of *Gluconacetobacter liquefaciens*, which was used as one of outgroups, were not located in the two major groups but in Sub-group I, differing from the two groups in the phylogenetic tree reported by González and Mas (2011) as well as the two groups in the phylogenetic trees of the genus *Gluconobacter* reported by Tanasupawat et al. (2004), Yukphan et al. (2004), and Malimas et al. (2009). Isolate AD25<sup>T</sup> was included in Group I and formed an independent cluster with a bootstrap value of 52 %.

In a phylogenetic tree based on *groEL* gene sequences of 866 bases derived from the neighbor-joining method, the resulting two major phylogenetic groups were similar to those based on 16S-23S rRNA gene ITS sequences (Fig. 3). The type strains of the four species *A. aceti*, *A. nitrogenifigens*, *A. oeni*, and *A. estunensis* were not located in the two major groups but in Sub-group I. In addition, the two species *A. peroxydans* and *A. papayae*, which were once included in Group II, were not located in



**Fig. 3** Phylogenetic relationships of *Acetobacter thailandicus* isolate AD25<sup>T</sup>. The phylogenetic tree based on *groEL* gene sequences of 866 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

*Gluconacetobacter liquefaciens* NBRC 12388<sup>T</sup> and *Granulibacter bethesdensis* NIH1<sup>T</sup> were used as outgroups. Sequence accession numbers for *groEL* gene sequences are provided in *parentheses* 

the two major groups but in Sub-group II. Isolate  $AD25^{T}$  was located in Group I and formed an independent cluster with a bootstrap value of 70 %.

16S rRNA gene, 16S-23S rRNA gene ITS, and groEL gene sequences similarities

The calculated pair-wise 16S rRNA gene sequence similarity values of isolate AD25<sup>T</sup> were 98.3, 98.0, 98.1, 97.8, 97.9, 97.9, 97.6, 98.2, 97.9, 97.6, 98.0, 97.5, 97.5, 97.5, 97.5, 97.7, 97.4, 97.0, 96.7, 96.9, 96.9, 98.0, and 97.6 %, respectively, to the type strains of *Acetobacter cibinongensis*, *A. indonesiensis*, *A. orientalis*, *A. aceti*, *A. malorum*, *A. cerevisiae*, *A. ghanensis*, *A. senegalensis*, *A. tropicalis*, *A. persici*, *A. nitrogenifigens*, *A. farinalis*, *A. fabarum*, *A. estunensis*, *A. oeni*, *A. pomorum*, *A. papayae*, *A. peroxydans*, *A. pasteurianus*, *A. sicerae*, and *A. lambici* (Supplementary table S1). Interestingly, there were no similarity values greater than 99 %. The phylogenetic data obtained indicated that the isolate obviously constitutes a separate species within the genus *Acetobacter*.

In 16S-23S rRNA gene ITS sequences, the calculated pair-wise sequence similarity values of isolate  $AD25^{T}$  were 83.7, 81.8, 84.0, 77.7, 82.0, 82.0, 83.0, 82.3, 82.3, 81.5, 79.9, 82.8, 83.2, 77.4, 80.8, 83.5, 82.0, 82.3, 76.9,

82.5, 77.7, 78.2, and 82.3 % as well, except for *A. sicerae* and *A. lambici* (Supplementary table S2). Similarly, the calculated pair-wise sequence similarity values of the isolate  $AD25^{T}$  were somewhat high in *groEL* gene sequences; they were 87.6, 85.6, 87.8, 80.6, 84.5, 84.0, 83.9, 85.5, 85.4, 84.6, 79.4, 83.7, 83.8, 78.9, 82.4, 83.7, 82.4, 84.0, 80.8, 83.7, 81.9, 84.5, and 85.7 %, respectively (Supplementary table S3).

DNA base composition and DNA-DNA hybridization

The DNA base composition of isolate  $AD25^{T}$  was 51.4 mol% G + C, which was lower in Group I or the *A. aceti* group. When single-stranded and labeled DNA from isolate  $AD25^{T}$  was hybridized with DNA from test strains, the calculated DNA-DNA similarities were  $100\pm0.04$ ,  $18.1\pm0.15$ ,  $17.6\pm0.1$  and  $6.7\pm0.1$  %, respectively, to isolate  $AD25^{T}$ , *A. orientalis* BCC 23127<sup>T</sup>, *A. cibinongensis* BCC 23126<sup>T</sup>, and *A. tropicalis* BCC 23123<sup>T</sup>, which were phylogenetically related. The labeled DNAs from *A. orientalis* BCC 23126<sup>T</sup>, *A. cibinongensis* BCC 23123<sup>T</sup> showed that the DNA-DNA similarities were  $13.2\pm0.1$ ,  $100\pm0.003$ ,  $22.9\pm0.1$ ,  $5.5\pm0.1$ ,  $6.8\pm0.15$ ,  $18.7\pm0.1$ ,  $100\pm0.01$ ,  $5.3\pm0.1$ ,  $6.8\pm0.06$ ,  $14\pm0.06$ , and  $100\pm0.01$  %, respectively. The genetic data obtained indicated that the isolate constitutes a separate species.

 Table2
 Differential characteristics of Acetobacter thailandicus isolate AD25<sup>T</sup>

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12 <sup>b</sup>
Production from D-glucose												
D-Gluconic acid	+	+	+	+ <sup>b</sup>	+ <sup>c</sup>	+ <sup>b</sup>	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>b</sup>	+ <sup>e</sup>	+ <sup>f</sup>	+ <sup>b</sup>
2-Keto-D-gluconic acid	-	+	+	+ <sup>b</sup>	+ <sup>c</sup>	+ <sup>b</sup>	+ <sup>d</sup>	$+^{d}$	+ <sup>b</sup>	+ <sup>e</sup>	+ <sup>f</sup>	+ <sup>b</sup>
5-Keto-D-gluconic acid	-	-	-	_b	_c	_b	_d	d	_b	_e	+ <sup>f</sup>	$+^{b}$
2,5-Diketo-D-gluconic acid		-	-	_b	_c	_b	d	_d	_b	_e	_f	_b
Growth in the presence of 10 % ethanol $v/v$ (GECA)		-	-	_b	+ <sup>c</sup>	_b	d	$+^{d}$	_b	_e	+ <sup>f</sup>	_b
Growth on ammoniac nitrogen (Frateur's modified Hoyer medium) with D-glucose Acid production from		_	_	_	W	+	_	_	W	+	+	_
D-Mannose	+	-	W	W	+	+	+	-	W	+	+	+
D-Galactose	+	-	VW	VW	+	VW	+	+	VW	+	+	+
D-Xylose	+	W	vw	v (+) <sup>b</sup>		v (+) <sup>b</sup>	+ <sup>d</sup>	+ <sup>d</sup>	v (+) <sup>b</sup>	_e	+ <sup>f</sup>	+ <sup>b</sup>
D-Ribose	vw	-	-	-	-	-	-	-	-	W	W	W
L-Arabinose		-	VW	v (+) <sup>b</sup>	+ <sup>c</sup>	v (+)	d	_d	v (-) <sup>b</sup>	w <sup>e</sup>	+ <sup>f</sup>	+ <sup>b</sup>
Mellibiose	vw	-	VW	-	-	VW	-	-	-	-	-	-
Ubiquinone		Q9	Q9	Q9 <sup>b</sup>	Q9 °	Q9 <sup>b</sup>	Q9 <sup>d</sup>	Q9 <sup>d</sup>	Q9 <sup>b</sup>	Q9 <sup>e</sup>	Q9 $^{\rm f}$	Q9 <sup>b</sup>
DNA base composition (mol%)		54.5 <sup>a</sup>	52.3 <sup>a</sup>	55.9 <sup>b</sup>	56.0 <sup>c</sup>	53.7 <sup>b</sup>	57.6 <sup>d</sup>	57.2 <sup>d</sup>	56.5 <sup>b</sup>	56.3 <sup>e</sup>	$58.7\ ^{\rm f}$	56.7 <sup>b</sup>

Abbreviation: 1, *A.thailandicus* isolate AD25<sup>T</sup>; 2, *A.cibinongensis* NBRC 16605<sup>T</sup>; 3, *A.orientalis* NBRC 16606<sup>T</sup>; 4, *A.tropicalis* NBRC 16470<sup>T</sup>; 5, *A.senegalensis* LMG 23690<sup>T</sup>; 6, *A.indonesiensis* NBRC 16471<sup>T</sup>; 7, *A.cerevisiae* LMG 1625<sup>T</sup>; 8, *A.malorum* LMG 1746<sup>T</sup>; 9, *A.orleanensis* NBRC 13752<sup>T</sup>; 10, *A.farinalis* G390-1<sup>T</sup>; 11, *A.persici* JCM 25330<sup>T</sup>; 12, *A.aceti* NBRC 14818<sup>T</sup>; +, positive; –, negative; *v* variable; *vw* very weak; *w* weak Cited from <sup>a</sup> Lisdiyanti et al. (2001); <sup>b</sup> Lisdiyanti et al. (2000); <sup>c</sup>Ndoye et al. (2007); <sup>d</sup> Cleenwerck et al. (2002); <sup>c</sup> Tanasupawat et al. (2011a); <sup>f</sup> lino et al. (2012)

# Phenotypic characteristics

Phenotypic and chemotaxonomic characteristics were described in the species description of the isolate.

Isolate AD25<sup>T</sup> was quite unique phenotypically (Table 2). In spite of being within Group I or the A. aceti group phylogenetically, the isolate was especially distinguisable from the type strains of A. cibinongensis and A. orientalis, which are phylogenetically related, by producing only D-gluconic acid, but not 2-keto-D-gluconic acid, from D-glucose (Table 2). The isolate was also discriminated from them by intense growth on GECA medium with 10 % ethanol and by weak growth on Frateur's modified Hoyer medium with 3 % glucose and by intense acid production from D-mannose, Dgalactose, and D-xylose. The weak acid production from melibiose also differentiated the isolate from the type strains of other Acetobacter, species except for A. orientalis and A. indonesiensis, which were distinguished from the isolate by the production of 2-keto-D-gluconic acid from D-glucose as well (Table 2).

From the experimental results obtained above, the new species can therefore be introduced in the genus *Acetobacter* with the name, *Acetobacter thailandicus* sp. nov.

# Description of Acetobacter thailandicus sp. nov

Acetobacter thailandicus (tha.i.lan'di.cus. N. L. masc. adj. thailandicus of Thailand, where the type strain was isolated).

Cells are Gram-negative and rod-shaped, measuring  $1.0 \times$ 1.6-2.6 µm and are non motile. Colonies are cream, smooth, glistening, non-pigmented, and raised with an entire margin on glucose/ethanol/calcium carbonate agar. Grows at pH 3.0 and 3.5 at 30 °C. No growth in the presence of 30 % Dglucose. Grows in the presence of 0.35 % acetic acid. Acetobacter thailandicus grows on GECA medium with 10 % ethanol and weakly on Frateur's modified Hoyer medium with 3 % glucose, but not on the medium when 3.0 % Dglucose (the carbon source) was replaced by 3.0 % Dmannitol or by 3.0 % ethanol. Ethanol is oxidized to acetic acid by Acetobacter thailandicus and it also oxidizes acetate and lactate to carbon dioxide and water. Acetic acid is produced on ethanol/calcium carbonate agar. Catalase is positive, and oxidase is negative. D-Gluconic acid is produced from Dglucose. Acetobacter thailandicus is unable to produce 2keto-D-gluconic acid, 5-keto-D-gluconic acid, and 2,5diketo-D-gluconic acid. Acetobacter thailandicus facilitates no production of dihydroxyacetone from glycerol. Acid is produced from D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose (weakly positive), D-ribose (very weakly positive), melibiose (very weakly positive), ethanol, and 1-butanol, but not from D-fructose, L-sorbose, D-arabinose, L-rhamnose, dulcitol, glycerol, methanol, trehalose,

sucrose, raffinose, and starch. The major ubiquinone is Q-9. DNA G + C content is 51.4 mol% G+ C.

The type strain is isolate  $AD25^{T}$  (= BCC  $15839^{T}$  = NBRC  $103583^{T}$ ), which was isolated from a flower of the blue trumpet vine (*Thunbergia laurifolia*) at Tong Pha Phum, Kanchanaburi, Thailand.

Acknowledgments This work was supported financially by the Strategic Scholarship/Fellowships Research Network from the Office of the Higher Education Commission, Ministry of Education (Grant no. 82/ 2549). Sincere thanks are also due to Mr. Richard James Goldrick, Department of Foreign Languages, Kasetsart University for English editing of this manuscript. A part of this work was carried out through collaboration of the Core to Core Program supported by the Japan Society for the Promotion of Science (JSPS) and the National Research Council of Thailand (NRCT).

#### References

- 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
- Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37:5768–5783
- Cleenwerck I, De Vos P (2008) Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int J Food Microbiol 125:2–14
- Cleenwerck I, Vandemeulebroecke K, Janssens D, Swing J (2002) Reexamination of the genus Acetobacter, with descriptions of Acetobacter cerevisiae sp. nov. and Acetobacter malorum sp. nov. Int J Syst Evol Microbiol 52:1551–1558
- 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 beans. Int J Syst Evol Microbiol 57:1647– 1652
- 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 58:2180–2185
- Cleenwerck I, De Vos P, De Vuyst L (2010) Phylogeny and differentiation of species of the genus *Gluconacetobacter* and related taxa based on multilocus sequence analyses of housekeeping genes and reclassification of *Acetobacter xylinus* subsp. *sucrofermentans* (Toyosaki et al. 1996) sp. nov., comb. nov. Int J Syst Evol Microbiol 60: 2277–2283
- De Ley J, Gillis M, Swings J (1984) In: Krieg NR, Holt JG (eds) Family VI. *Acetobacteraceae*, 1th Ed Bergey's Manual of Systematic Bacteriology, Vol. 1. Williams and Wilkins Co, Baltimore, pp 267–278
- Devereux R, Wills SG (1995) Amplification of ribosomal RNA sequences. In: Akkermans ADL, Van Elsas JD, De Bruijn FJ (eds) Molecular microbial ecology manual. Academic Publishers, Dordrecht, pp 3.3.1–3.3.2
- Dutta D, Gachhui R (2006) Novel nitrogen-fixing Acetobacter nitrogenifigens sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 56:1899–1903

- Ezaki T, Hashimoto Y, Yabuuchi E (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. Evol 39:783–791
- González A, Mas A (2011) Differentiation of acetic acid bacteria based on sequence analysis of 16S-23S rRNA gene internal transcribed spacer sequences. Int J Food Microbiol 147:217–222
- 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. Zbl Bakt Hyg I Abt Orig C 1(2):178–181
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98
- Hucker GJ, Conn HJ (1923) Method of gram staining. Tech Bull NY St Agric Exp Stn 93:3–37
- Iino T, Suzuki R, Kosako Y, Ohkuma M, Komagata K, Uchimura T (2012) Acetobacter okinawensis sp. nov., Acetobacter papayae sp. nov., and Acetobacter persicus sp. nov.; novel acetic acid bacteria isolated from stems of sugarcane, fruits, and a flower in Japan. J Gen Appl Microbiol 58:235–243
- Iino T, Suzuki R, Kosako Y, Ohkuma M, Komagata K, Uchimura T (2013) List of new names and new combinations previously effectively, but not validly, published. Validation List no. 149. Int J Syst Evol Microbiol 63:1–5
- 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, 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 58:319–324
- Li L, Wieme A, Spitaels F, Balzarini T, Nunes OC, Manaia CM, Van Landschoot A, De Vuyst L, Cleenwerck I, Vandamme P (2014) *Acetobacter sicerae* sp. nov., isolated from cider and kefir and identification of *Acetobacter species* by *dnaK*, *groEL* and *rpoB* sequence analysis. Int J Syst Evol Microbiol 64:2407–2415
- 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, 1996) comb. nov., Acetobacter lovaniensis (Frateur, 1950) comb. nov., and Acetobacter estunensis (Carr, 1958) comb. nov. J Gen Appl Microbiol 46:147–165
- Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (2001a) Validation of publication of new names and new combinations previously effectively published outside the IJSEM. Validation List no. 79. Int J Syst Evol Microbiol 51:263–265
- Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (2001b) 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
- Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (2002) Validation of publication of new names and new combinations previously effectively published outside the IJSEM. Validation List no. 84. Int J Syst Evol Microbiol 52:3–4
- Malimas T, Yukphan P, Takahashi M, Muramatsu M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2009) *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the α-*Proteobacteria*. Int J Syst Evol Microbiol 59: 466–471

- Muramatsu Y, Yukphan P, Takahashi M, Kaneyasu M, Malimas T, Potacharoen W, Yamada Y, Nakagawa Y, Tanticharoen M, Suzuki K (2009) 16S rRNA gene sequences analysis of acetic acid bacteria isolated from Thailand. Microbiol Cult Coll 25:13–20
- Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P, Vancanneyt M, Swings J (2005) Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. Microbiol 151:2141–2150
- Ndoye B, Cleenwerck I, Engelbeen K, Dubois-Dauphin R, Guiro AT, Van Trappen S, Willems A, Thonart P (2007) Acetobacter senegalensis sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (Mangifera indica L.). Int J Syst Evol Microbiol 57:1576–1581
- Okumura H, Uozumi T, Beppu T (1985) Construction of plasmid vectors and genetic transformation system for *Acetobacter aceti*. Agric Biol Chem 49:1011–1017
- Pitiwittayakul N, Yukphan P, Sintuprapa W, Yamada Y, Theeragool G (2014) Identification of acetic acid bacteria isolated in Thailand and assigned to the genus *Acetobacter* by *groEL* gene sequence analysis. Ann Microbiol. doi:10.1007/s13213-014-0994-9
- Ruiz A, Poblet M, Mas A, Guillamon JM (2000) Identification of acetic acid bacteria by RFLP of PCR amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int J Syst Evol Microbiol 150:1981–1987
- Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim Biophys Acta 72:619–629
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
- 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
- Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ (2011) Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Sci 334:670–674
- Silva LR, Cleenwerck I, Rivas R, Swings J, Trujillo ME, Willems A, Velázquez E (2006) *Acetobacter oeni* sp. nov., isolated from spoiled red wine. Int J Syst Evol Microbiol 56:21–24
- Skerman VBD, McGowan V, Sneath PHA (1980) Approved lists of bacterial names. Int J Syst Bacteriol 30:225–420
- Sokollek SJ, Hertel C, Hammes WP (1998) Description of *Acetobacter* oboediens sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations. Int J Syst Bacteriol 48:935–940
- Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014) *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer. Int J Syst Evol Microbiol 64:1083–1089
- Swings J (1992) The genera Acetobacter and Gluconobacter. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer H-K (eds) The prokaryotes, A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, vol. III. Springer, New York, pp 2268–2286
- Tamaoka J, Komagata K (1984) Determination of DNA base composition by reversed-phase high performance liquid chromatography. FEMS Microbiol Lett 25:125–128
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739
- Tanasupawat S, Thawai C, Yukphan P, Moonmangmee D, Itoh T, Adachi O, Yamada Y (2004) *Gluconobacter thailandicus* sp. nov., an acetic acid bacterium in the  $\alpha$ -*Proteobacteria*. J Gen Appl Microbiol 50: 159–167
- Tanasupawat S, Kommanee J, Yukphan P, Muramatsu Y, Nakagawa Y, Yamada Y (2011a) *Acetobacter farinalis* sp. nov., an acetic acid bacterium in the  $\alpha$ -*Proteobacteria*. J Gen Appl Microbiol 57:159–167

- Tanasupawat S, Kommanee J, Yukphan P, Muramatsu Y, Nakagawa Y, Yamada Y (2011b) List of new names and new combinations previously effectively, but not validly, published. Validation List no. 142. Int J Syst Evol Microbiol 61:2563–2565
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
- Verlander CP (1992) Detection of horseradish peroxidase by colorimetry. In: Kricka LJ (ed) Nonisotopic DNA probe techniques. Academic, New York, pp 185–201
- Yamada Y, Yukphan P (2008) Genera and species in acetic acid bacteria. Int J Food Microbiol 125:15–24
- Yamada Y, Aida K, Uemura T (1969) Enzymatic studies on the oxidation of sugar and sugar alcohol. V. Ubiquinone of acetic acid bacteria and

- Yamada Y, Okada Y, Kondo K (1976) Isolation and characterization of "polarly flagellated intermediate strains" in acetic acid bacteria. J Gen Appl Microbiol 22:237–245
- Yamada Y, Hosono R, Lisdiyanti P, Widyastuti Y, Saono S, Uchimura T, Komagata K (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
- Yukphan P, Potacharoen W, Nakagawa Y, Tanticharoen M, Yamada Y (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