International Journal of Systematic and Evolutionary Microbiology (2001), **51**, 559–563 Printed in Great Britain

NOTE

Asaia siamensis sp. nov., an acetic acid bacterium in the α-*Proteobacteria*

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Five bacterial strains were isolated from tropical flowers collected in Thailand and Indonesia by the enrichment culture approach for acetic acid bacteria. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolates were located within the cluster of the genus *Asaia*. The isolates constituted a group separate from *Asaia bogorensis* on the basis of DNA relatedness values. Their DNA G+C contents were 58·6–59·7 mol%, with a range of 1·1 mol%, which were slightly lower than that of *A. bogorensis* (59·3–61·0 mol%), the type species of the genus *Asaia*. The isolates had morphological, physiological and biochemical characteristics similar to *A. bogorensis* strains, but the isolates did not produce acid from dulcitol. On the basis of the results obtained, the name *Asaia siamensis* sp. nov. is proposed for these isolates. Strain S60-1^T, isolated from a flower of crown flower (dok rak, *Calotropis gigantea*) collected in Bangkok, Thailand, was designated the type strain (= NRIC 0323^T = JCM 10715^T = IFO 16457^T).

Keywords: *Asaia siamensis* sp. nov., acetic acid bacteria, *Acetobacteraceae*, *Proteobacteria*

The genus *Asaia* was introduced with a single species, *Asaia bogorensis*, in the family *Acetobacteraceae* (Yamada *et al*., 2000). In contrast with strains of the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*, the strains assigned to this genus are characterized by no or very weak capability for oxidizing ethanol to acetic acid and no growth in the presence of acetic acid (0·35%, v/v). This paper describes the proposal of *Asaia siamensis* sp. nov., the second species of the genus *Asaia*, for strains isolated from tropical flowers collected in Thailand and Indonesia.

Five bacterial strains (S60-1^T, D4-1, Y85, i36 and B28S-3) were isolated from tropical flowers collected in Thailand and Indonesia by the enrichment culture approach using a sorbitol medium and a dulcitol medium at pH 3·5 (Table 1) (Yamada *et al*., 2000). These strains were maintained on agar slants of AG medium composed of 0·1% D-glucose, 1·5% glycerol, 0·5% peptone, 0·5% yeast extract, 0·2% malt extract, 0·7% $CaCO_3$ and 1·5% agar (w/v). *Acetobacter aceti* IFO 14818^T, *Gluconobacter oxydans* IFO 14819^T, *Gluconacetobacter liquefaciens* IFO 12388^T and *Asaia bogorensis* JCM 10569^T, NRIC 0317 and NRIC 0318 were used as reference strains.

Morphological, physiological and biochemical characteristics were examined according to the methods reported by Asai *et al*. (1964) and Yamada *et al*. (1976, 1999, 2000). Cells of all the isolates were Gram-negative, strictly aerobic and rod-shaped, measuring 0·6–1·0 × 1·0–4·5 μm. The cells were motile by means of peritrichous flagella. Colonies were pink, shiny, smooth and raised with an entire margin on AG agar plates. All the isolates grew well at pH 3·0 and 3·5 and at 30 °C on a $CaCO_3$-free AG medium. The isolates oxidized acetate and lactate to carbon dioxide and water but, as with strains of *Asaia bogorensis*, their

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The DDBJ accession numbers for the 16S rRNA gene sequences of isolates S60-1^T and Y85 are AB035416 and AB025932, respectively.

Table 1 Strain designations and isolation sources

NRIC, NODAI Culture Collection Center, Tokyo University of Agriculture (NODAI), 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan; JCM, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; IFO, Institute for Fermentation, Osaka, 2-17-85 Juso Honmachi, Yodogawa-ku, Osaka 532-8686, Japan.

Isolate	Source	NRIC	JCM	IFO
S60-1^T	Flower of crown flower (dok rak, *Calotropis gigantea*), Bangkok, Thailand	0323^T	10715^T	16457^T
D4-1	Flower of spider lily (plub-plueng, *Crinum asiaticum*), Bangkok, Thailand	0324		
Y85	Flower of spider lily (krinum bakung, *Crinum asiaticum*), Bogor, Indonesia	0325	10716	
i36	Flower of spider lily (krinum bakung, *Crinum asiaticum*), Bogor, Indonesia	0326		
B28S-3	Flower of ixora (soka, *Ixora chinensis*), Bogor, Indonesia	0327		

Table 2 Physiological and biochemical characteristics, and ubiquinone composition of isolates

+, Positive; w, weakly positive; −, negative. All strains were positive for growth at pH 3·5 and production of acid from D-glucose; all strains were negative for growth on methanol. Strains: 1, *Asaia siamensis* S60-1^T; 2, *Asaia siamensis* Y85; 3, *Asaia siamensis* D4-1; 4, *Asaia siamensis* i36; 5, *Asaia siamensis* B28S-3; 6, *Asaia bogorensis* NRIC 0317; 7, *Asaia bogorensis* NRIC 0318; 8, *Asaia bogorensis* JCM 10569^T; 9, *Acetobacter aceti* IFO 14818^T; 10, *Gluconobacter oxydans* IFO 14819^T; 11, *Gluconacetobacter liquefaciens* IFO 12388^T.

Character	1	2	3	4	5	6	7	8	9	10	11
Oxidation of acetate and lactate	w	w	w	w	w	w	w	w	+	−	+
Production of water-soluble brown pigment on glucose/$CaCO_3$ medium	−	−	−	−	−	−	−	−	−	−	+
Dihydroxyacetone formation from glycerol	+	+	w	w	w	w	w	w	+	+	+
Growth on dulcitol	+	+	+	+	+	+	+	+	−	−	−
Assimilation of ammonium sulfate on vitamin-free glucose medium	+	+	+	+	+	+	+	+	w	−	w
Growth at pH 3·5 in the presence of acetic acid (0·35 %, v/v)	−	−	−	−	−	−	−	−	+	+	+
Acid production from:											
Ethanol	w	−	−	−	−	w	−	−	+	+	+
Dulcitol	−	−	−	−	−	+	+	+	−	−	−
D-Mannitol and D-sorbitol	+	+	+	+	+	+	+	+	−	+	−
Glycerol	+	+	+	+	+	+	+	+	−	+	w
Ubiquinone composition (%):*											
Q-10	98	98	99	100	96	97	97	93	2	98	93
Q-9	2	1	1	0	3	3	3	7	79	2	7
Q-8	0	1	0	0	1	0	0	0	19	0	0

* The ubiquinone compositions of the type strains of *Acetobacter aceti*, *Gluconobacter oxydans* and *Gluconacetobacter liquefaciens* were cited from Urakami *et al*. (1989). The ubiquinone isoprenologues below 1 % were omitted.

oxidation capability was not intense (Table 2). The production of dihydroxyacetone from glycerol was variable depending upon the isolates; positive or weakly positive strains were present. All the isolates grew on glutamate agar and mannitol agar, but did not grow on methanol. They showed vigorous growth on a vitamin-free glucose/$(NH_4)_2SO_4$ medium, but no growth was found on a vitamin-free ethanol/$(NH_4)_2SO_4$ medium. The isolates produced 2-keto-D-gluconate and 5-keto-D-gluconate, but not 2,5-diketo-D-gluconate, and a water-soluble brown pigment was observed on a glucose/yeast extract/$CaCO_3$ medium. Acetic acid was not produced on an ethanol/yeast extract/$CaCO_3$ agar. The presence of 0·35 % (v/v) acetic acid completely inhibited growth of the isolates at pH 3·5 on $CaCO_3$-free AG medium. Acid was produced from D-glucose, D-mannose, D-fructose, L-sorbose, D-xylose, L-arabinose, D-ribose, *myo*-inositol, ribitol, D-arabitol, xylitol, *meso*-erythritol, glycerol, melibiose and sucrose, but not from lactose. The isolates did not produce acid from dulcitol, but assimilated dulcitol for growth.

Isoprenoid quinones were extracted from bacterial cells and purified by the method of Yamada *et al*. (1969). Ubiquinone isoprenologues were quantitatively determined by reversed-phase HPLC (Tamaoka *et al*., 1983). Standard preparations of Q-10, Q-9 and Q-8 were obtained from the cells of *Gluconobacter cerinus* NRIC 0229^T, *Acetobacter aceti* IFO 14818^T

Table 3 DNA base compositions and values of DNA relatedness

NT, Not tested.

Species	Strain	G+C content (mol%)	DNA relatedness (%) with:		
			S60-1^T	Y85	JCM 10569^T
Asaia siamensis	S60-1^T	59·3	100	84	20
	D4-1	58·7	94	92	24
	Y85	59·7	86	100	20
	i36	58·6	79	97	19
	B28S-3	59·6	100	93	23
Asaia bogorensis	JCM 10569^T	60·2	33	27	100
	NRIC 0317	59·7	NT	27	68
	NRIC 0318	59·3	NT	36	87
Acetobacter aceti	IFO 14818^T	58·3	12	7	4
Gluconobacter oxydans	IFO 14819^T	61·7	15	11	8
Gluconacetobacter liquefaciens	IFO 12388^T	65·0	11	6	4

and *Frateuria aurantia* IFO 3245^T, respectively. The major quinone isoprenologue for the isolates was Q-10, as shown in Table 2. The quinone system was composed of 96–100 % Q-10, 0–3 % Q-9 and 0–1 % Q-8.

Extraction and isolation of bacterial DNA were performed by the modified method of Marmur (Marmur, 1961; Ezaki *et al*., 1983). DNA base composition (G+C content) was determined by reversed-phase HPLC (Tamaoka & Komagata, 1984). The DNA G+C composition of the isolates was 58·6–59·7 mol%, with a range of 1·1 mol%. These values were slightly lower than those of *Asaia bogorensis* strains (59·3–61·0 mol%) (Table 3).

DNA–DNA hybridization was carried out at 48·9 °C for 2–3 h by a microdilution well technique using photobiotin for labelling (Ezaki *et al*., 1989). Isolated, single-stranded and labelled DNAs were hybridized in 2× SSC and 50 % formamide. DNA relatedness values for four of the isolates to S60-1^T were 79–100 % and those to Y85 were 84–97 % (Table 3). *Asaia bogorensis* JCM 10569^T showed low DNA relatedness values of 33 and 27 %, respectively, to isolates S60-1^T and Y85. When the DNA of *Asaia bogorensis* JCM 10569^T was used as a probe, low DNA relatedness values (19–24 %) were found. These data indicate that the isolates constitute a group separate from *Asaia bogorensis* strains.

Gene fragments specific for the 16S rRNA-coding regions were amplified by PCR as described previously (Kawasaki *et al*., 1993; Yamada *et al*., 2000). Two primers, 20F (5′-GAGTTTGATCCTGGCTCAG-3′; positions 9–27) and 1500R (5′-GTTACCTTGTTACGACTT-3′; positions 1509–1492), were used. The numbers of positions in the rRNA gene fragments were based on the *Escherichia coli* numbering system (accession number V00348; Brosius *et al*., 1981). Amplified 16S rRNA genes were directly sequenced using an ABI PRISM Bigdye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM model 310 Genetic Analyzer. 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 alignments of the sequences were carried out with the program CLUSTAL W (version 1.7) (Thompson *et al*., 1994). The distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The neighbour-joining method was used for constructing a phylogenetic tree (Saitou & Nei, 1987). Since two kinds of bases (T/C) were found at positions 91 and 139 in the strains of *Asaia bogorensis*, the bases at these positions were excluded in the construction of the phylogenetic tree. The comparison of the sequence data obtained was made on 1402 bases (Yamada *et al*., 2000). The robustness for individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). The species, type strains and the accession numbers of the base sequences taken from databases are presented in Fig. 1. Percentage similarities among acetic acid bacteria, including isolate S60-1^T, were calculated in pairs of sequences of the 1402 bases (Yamada *et al*., 2000). Isolates S60-1^T and Y85 were subjected to phylogenetic analysis based on 16S rRNA gene sequences. When compared with *Asaia bogorensis* JCM 10569^T (C, G and G at positions 593, 989 and 1327, respectively), base differences were found at positions 989 and 1327 (A and A, respectively) in isolate S60-1^T and at positions 593, 989 and 1327 (T, A and A, respectively) in isolate Y85. The rRNA gene sequences of the two isolates had 99·9 % similarity to

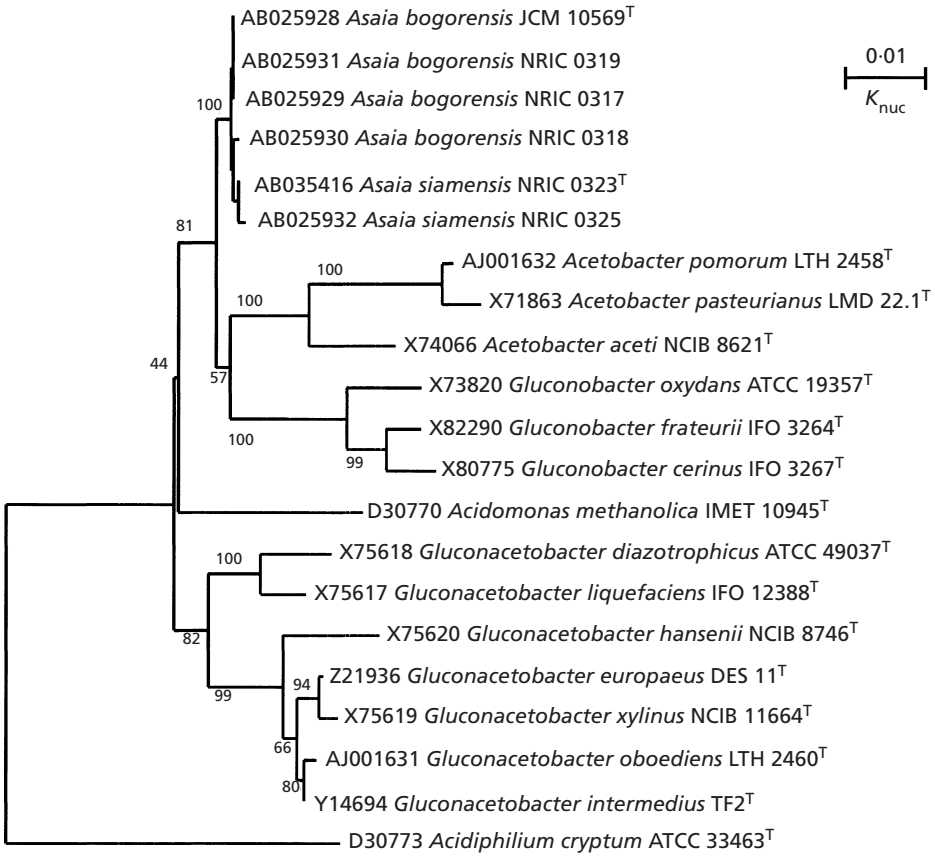

Fig. 1. Phylogenetic relationships of isolates based on 16S rRNA gene sequences. *Acidiphilium cryptum* ATCC 33463^T was used as an outgroup. Numbers at nodes indicate the bootstrap percentages derived from 1000 samples.

each other and the two isolates constituted a cluster along with the strains of *Asaia bogorensis* in the 16S rRNA phylogenetic tree (Fig. 1). Isolate S60-1^T had 99·9 % similarity to the type strain of *Asaia bogorensis* and isolate Y85 had 99·8 % similarity. The calculated similarities of isolate S60-1^T were 96·4, 96·0, 95·9 and 96·5 % to the type strains of *Acetobacter aceti*, *Gluconobacter oxydans*, *Acidomonas methanolica* and *Gluconacetobacter liquefaciens*, respectively. The phylogenetic data obtained indicated that the isolates should be accommodated in the genus *Asaia*.

Acetic acid bacteria are currently classified in the following five genera: *Acetobacter* (the type genus), *Gluconobacter*, *Acidomonas*, *Gluconacetobacter* and *Asaia* (Yamada *et al*., 1997a, 1997b, 2000). The two isolates S60-1^T and Y85 could be contained in the same cluster as the strains of *Asaia bogorensis* on the basis of 16S rRNA gene sequences, and accommodated in the large cluster of acetic acid bacteria. DNA relatedness values showed a taxon separate from *Asaia bogorensis*. The isolates had peritrichous flagella and conformed with members of the genera *Acetobacter*, *Gluconacetobacter* and *Asaia*. The isolates oxidized acetate and lactate to carbon dioxide and water, but their oxidizing capability, and that of strains of *Asaia bogorensis*, was not as intense as that of the genera *Acetobacter* and *Gluconacetobacter*. The isolates did not utilize methanol as a sole source of carbon. They grew at pH 3·0. Their growth was completely inhibited at pH 3·5 on a $CaCO_3$-free AG medium containing 0·35 % (v/v) acetic acid. Since the isolates showed vigorous growth on a vitamin-free glucose/$(NH_4)_2SO_4$ medium and a vitamin-free mannitol/$(NH_4)_2SO_4$ medium but not on an ethanol/$(NH_4)_2SO_4$ medium, they are quite different from strains of the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*. These characteristics are consistent with those of strains of *Asaia bogorensis*. The isolates produced acid from a number of sugars and sugar alcohols. In contrast with *Asaia bogorensis* strains, the isolates did not produce acid from dulcitol. Consequently, all the five isolates can be distinguished genetically and phenotypically from *Asaia bogorensis* and should be classified into a separate species (Tables 2 and 3). The name *Asaia siamensis* sp. nov. is proposed for the five isolates.

Description of *Asaia siamensis* sp. nov.

Asaia siamensis [si.a.m′en.sis. *Siam* old name of Thailand; M.L. fem. suffix -*ensis* indicating geographical origin; M.L. fem. gen. n. *siamensis* of or pertaining to Siam (Thailand), where the type strain was isolated].

Cells are Gram-negative and rod-shaped, measuring 0·6–1·0 × 1·0–4·5 μm. Peritrichous flagella. Colonies are pink, shiny, smooth and raised with an entire margin on AG agar plates. Strictly aerobic. Grows at pH 3·0 and 30 °C. Oxidizes acetate and lactate to carbon dioxide and water, but the activity is not intense. Grows on glutamate agar and mannitol agar. No or very weak production of acetic acid from ethanol. Growth is inhibited by 0·35% (v/v) acetic acid. No growth on methanol. Production of dihydroxyacetone from glycerol is positive or weakly positive. Assimilates ammonium sulfate for growth on vitamin-free glucose medium and vitamin-free mannitol medium. Produces 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate, and a water-soluble brown pigment. Acid is produced from D-glucose, D-mannose, D-fructose, L-sorbose, D-xylose, L-arabinose, D-ribose, D-mannitol, D-sorbitol, *myo*-inositol, ribitol, D-arabitol, xylitol, *meso*-erythritol, glycerol, melibiose and sucrose, but not from lactose. Does not produce acid from dulcitol. Major ubiquinone is Q-10. DNA G+C composition is 58·6–59·7 mol%, with a range of 1·1 mol%; G+C composition of the type strain is 59·3 mol%. The type strain is isolate S60-1^T (= JCM 10715^T = IFO 16457^T = NRIC 0323^T), isolated from a flower of crown flower (dok rak, *Calotropis gigantea*) collected in Bangkok, Thailand.

Acknowledgements

The authors wish to thank the Japan Bioindustry Association, Tokyo, Japan for the support of this study and Institute for Fermentation, Osaka (IFO), Osaka, Japan for supplying reference cultures.

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.

Brosius, J., Dull, T. J., Sleeter, D. D. & Noller, H. F. (1981). Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J Mol Biol* **148**, 107–127.

Ezaki, T., Yamamoto, N., Ninomiya, K., Suzuki, S. & Yabuuchi, E. (1983). Transfer of *Peptococcus indolicus*, *Peptococcus asaccharolyticus*, *Peptococcus prevotii*, and *Peptococcus magnus* to the genus *Peptostreptococcus* and proposal of *Peptostreptococcus tetradius* sp. nov. *Int J Syst Bacteriol* **33**, 683–698.

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. *Evolution* **39**, 783–791.

Kawasaki, H., Hoshino, Y., Hirata, A. & Yamasato, K. (1993). Is intracytoplasmic membrane structure a generic criterion? It is not parallel to phylogenetic interrelationships among photosynthetic purple non-sulfur bacteria. *Arch Microbiol* **160**, 358–362.

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.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Tamaoka, J., Katayama-Fujimura, Y. & Kuraishi, H. (1983). Analysis of bacterial menaquinone mixtures by high-performance liquid chromatography. *J Appl Bacteriol* **54**, 31–36.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (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.

Urakami, T., Tamaoka, J., Suzuki, K. & Komagata, K. (1989). *Acidomonas* gen, nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. *Int J Syst Bacteriol* **39**, 50–55.

Yamada, Y., Aida, K. & Uemura, T. (1969). Enzymatic studies on the oxidation of sugar and sugar alcohol. V. Ubiquinone of acetic acid bacteria and its relation to classification of *Gluconobacter* and *Acetobacter*, especially of the so-called intermediate strains. *J Gen Appl Microbiol* **15**, 186–196.

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., Hoshino, K. & Ishikawa, T. (1997a). The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. *Biosci Biotechnol Biochem* **61**, 1244–1251.

Yamada, Y., Hoshino, K. & Ishikawa, T. (1997b). The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA. *Bull Fac Agric Shizuoka Univ* **47**, 37–44.

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.

Yamada, Y., Katsura, K., Kawasaki, K., Widyastuti, Y., Saono, S., Seki, T., Uchimura, T. & Komagata, K. (2000). *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-*Proteobacteria*. *Int J Syst Evol Microbiol* **50**, 823–829.