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

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Three bacterial strains were isolated from flowers collected in Bangkok, Thailand, by an enrichment-culture approach for acetic acid bacteria. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolates were located in the lineage of the genus *Asaia* but constituted a cluster separate from the type strains of *Asaia bogorensis* and *Asaia siamensis*. The DNA base composition of the isolates was 60·2–60·5 mol% G+C, with a range of 0·3 mol%. The isolates constituted a taxon separate from *Asaia bogorensis* and *Asaia siamensis* on the basis of DNA–DNA relatedness. The isolates had morphological, physiological, biochemical and chemotaxonomic characteristics similar to those of the type strains of *Asaia bogorensis* and *Asaia siamensis*, but the isolates grew on maltose. The major ubiquinone was Q_{10}. On the basis of the results obtained, the name *Asaia krungthepensis* sp. nov. is proposed for the isolates. The type strain is isolate AA08^T (=BCC 12978^T=TISTR 1524^T=NBRC 100057^T=NRIC 0535^T), which had a DNA G+C content of 60·3 mol% and was isolated from a heliconia flower ('paksaasawan' in Thai; *Heliconia* sp.) collected in Bangkok, Thailand.

The genus *Asaia* was introduced with a single species, *Asaia bogorensis*, as the fifth genus of the family *Acetobacteraceae* (Yamada *et al.*, 2000). *Asaia siamensis*, the second species, was described by Katsura *et al.* (2001). Strains assigned to the genus *Asaia* are characterized by poor or non-existent production of acetic acid from ethanol and by the absence of growth in the presence of 0·35 % acetic acid (v/v). On the other hand, strains of the genera *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter* and *Kozakia* produce large amounts of acetic acid from ethanol and grow in the presence of 0·35 % acetic acid. According to Yamada *et al.* (2000) and Katsura *et al.* (2001), strains of the genus *Asaia* have been isolated mostly from tropical flowers. During the course of our studies on acetic acid bacteria isolated from flowers collected in Thailand, we found that three isolates constitute a novel species; this paper describes *Asaia krungthepensis* sp. nov.

Three bacterial strains were isolated from heliconia flowers ('paksaasawan' in Thai; *Heliconia* sp.) collected in Bangkok, Thailand, by an enrichment-culture approach using a sucrose medium, which was composed of 2·0 % sucrose, 0·3 % peptone, 0·3 % yeast extract and 0·3 % acetic acid (v/v) and adjusted to pH 3·5. When bacterial growth was found in the sucrose medium, micro-organisms were streaked on agar plates containing 2·0 % D-glucose, 0·5 % ethanol (v/v), 0·3 % peptone, 0·3 % yeast extract, 0·7 % $CaCO_3$ and 1·2 % agar (w/v). These acetic acid bacteria were selected as acid-producing bacterial strains that formed clear zones around colonies on agar plates. The three isolates, AA08^T (=BCC 12978^T), AA09 (=BCC 12979) and AA06 (=BCC 12977), were cultured at 30 °C on agar slants comprising glucose/ethanol/$CaCO_3$/agar medium (GECA medium) that contained 2·5 % D-glucose, 0·5 % ethanol,

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0·5 % peptone, 0·3 % yeast extract, 0·7 % $CaCO_3$ and 1·2 % agar and were maintained at 4 °C. *Asaia bogorensis* IFO 16594^T and *Asaia siamensis* IFO 16457^T were used as reference strains, together with *Acetobacter aceti* IFO 14818^T, *Gluconobacter oxydans* IFO 14819^T and *Gluconacetobacter liquefaciens* IFO 12388^T.

Morphological, physiological and biochemical characteristics were examined by using the methods reported by Asai *et al.* (1964), Yamada *et al.* (1999, 2000) and Katsura *et al.* (2001). The phenotypic characteristics of the three isolates are described in the species description. The isolates were different from the type strains of *Asaia bogorensis* and *Asaia siamensis* in acid production from maltose and growth on maltose: the isolates produced acid weakly from maltose, with one exception, and grew on maltose, but their growth was not intense (Table 1).

Isoprenoid quinones were extracted from bacterial cells and purified by the method of Yamada *et al.* (1969). Ubiquinone isoprenologues were quantified by reversed-phase HPLC (Tamaoka *et al.*, 1983). The three isolates (AA08^T, AA09 and AA06) had Q_{10} as the major quinone. The ubiquinone system of isolate AA08^T was composed of 1 % Q_7, 2 % Q_8, 16 % Q_9 and 81 % Q_{10}.

Gene fragments specific for 16S rRNA-encoding regions were amplified by means of a PCR as described previously (Yamada *et al.*, 2000; Katsura *et al.*, 2001; Kawasaki *et al.*, 1993). Two primers, 20F (5′-GAGTTTGATCCTGGCTCAG-3′, positions 9–27) and 1500R (5′-GTTACCTTGTTACGACTT-3′, positions 1509–1492), were used. 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 sequenced directly with an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit on an ABI PRISM model 310 Genetic Analyzer (both from Applied Biosystems). The following primers were used for sequencing: 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 X (version 1.81) (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). Comparison of the sequence data obtained was made on 1376 bases (Yamada *et al.*, 2000) and the robustness for individual branches was estimated by bootstrapping with 1000 replications (Felsenstein, 1985). *Asaia krungthepensis* sp. nov. isolates AA08^T, AA09 and AA06 were located in the lineage of the genus *Asaia*, but constituted a cluster separate from the type strains of the known species of the genus *Asaia*, *Asaia bogorensis* and *Asaia siamensis* (Fig. 1).

Pairwise sequence similarities (%) of 16S rRNA genes among acetic acid bacteria, including the novel isolates, were determined for 1382 bases. Among the three isolates, the sequence similarities were 100 %. *Asaia krungthepensis* AA08^T (=BCC 12978^T) showed respectively 99·6, 99·5, 97·3, 96·2, 96·2, 95·9 and 95·4 % sequence similarity to the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Kozakia baliensis*, *Acetobacter aceti*, *Gluconacetobacter liquefaciens*, *Acidomonas methanolica* and *Gluconobacter oxydans*.

Extraction and isolation of bacterial DNA were performed using the modified method of Marmur (1961) (Saito & Miura, 1963; Ezaki *et al.*, 1983). DNA base composition was determined by the method of Tamaoka & Komagata (1984). The DNA G+C contents of the three isolates (AA08^T, AA09 and AA06) were respectively 60·3, 60·2 and 60·5 mol%, with a range of 0·3 mol%.

Table 1. Characteristics that differentiate *Asaia krungthepensis*, *Asaia bogorensis* and *Asaia siamensis*

Strains: 1, *Asaia krungthepensis* AA08^T; 2, *Asaia krungthepensis* AA09; 3, *Asaia krungthepensis* AA06; 4, *Asaia bogorensis* IFO 16594^T; 5, *Asaia siamensis* IFO 16457^T. +, Positive; −, negative; W, weak. All strains showed weak oxidation of acetate and lactate and no growth in the presence of acetic acid (0·35 %, v/v).

Characteristic	1	2	3	4	5
Dihydroxyacetone formation from glycerol	+	+	+	+	W
Acid production from:					
Dulcitol	+	+	+	+	−
Maltose	W	W	−	−	−
Ethanol	−	−	−	−	W
Growth on maltose	W	W	W	−	−
G+C content of DNA (mol%)	60·3	60·2	60·5	60·2*	59·3*

*Data from Yamada *et al.* (2000) and Katsura *et al.* (2001).

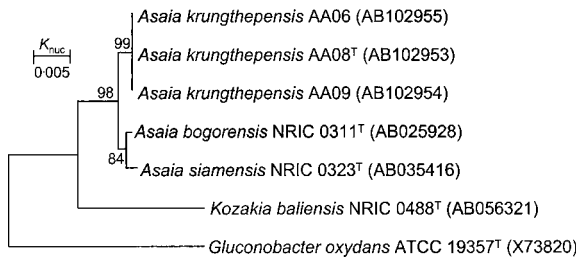

Fig. 1. Phylogenetic relationships of *Asaia krungthepensis* sp. nov. based on 16S rRNA gene sequences. *Gluconobacter oxydans* ATCC 19357^T was used as an outgroup. Numbers at nodes indicate bootstrap percentages derived from 1000 replications.

DNA–DNA hybridization was carried out using the photobiotin-labelling method with microdilution wells as described by Ezaki *et al.* (1989). DNA–DNA relatedness (%) was determined using the colorimetric method (Verlander, 1992). Isolated single-stranded and labelled DNAs were hybridized with DNAs from test strains in 2× SSC and 50 % formamide at 48·0 °C for 12 h. The colour intensity was measured as A_{450} on a Bio-Rad model 3550 microplate reader. Labelled DNA from *Asaia krungthepensis* AA08^T (=BCC 12978^T) showed respectively 100, 100, 100, 38, 40 and 9 % DNA–DNA relatedness to isolates AA08^T, AA09 and AA06, *Asaia bogorensis* IFO 16594^T, *Asaia siamensis* IFO 16457^T and *Gluconobacter oxydans* IFO 14819^T. When isolate AA06 DNA was labelled, the DNA–DNA similarities were respectively 100, 100, 100, 31, 36 and 5 %. *Asaia bogorensis* IFO 16594^T and *Asaia siamensis* IFO 16457^T had respectively 27 and 17 %, 36 and 22 %, 29 and 18 %, 100 and 22 %, 32 and 100 % and 6 and 4 % DNA–DNA relatedness to isolates AA08^T, AA09 and AA06, *Asaia bogorensis* 16594^T, *Asaia siamensis* IFO 16457^T and *Gluconobacter oxydans* IFO 14819^T.

The acetic acid bacteria are currently classified into six genera: *Acetobacter* (the type genus), *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia* and *Kozakia* (Yamada *et al.*, 2000; Lisdiyanti *et al.*, 2002). In the phylogenetic tree based on 16S rRNA-gene sequences, the three isolates (AA08^T, AA09 and AA06) were located in the lineage of the genus *Asaia*, but constituted a cluster separate from the cluster comprising *Asaia bogorensis* IFO 16594^T and *Asaia siamensis* IFO 16457^T. The DNA base compositions of the isolates (60·2–60·5 mol% G+C) were almost identical to that of *Asaia bogorensis* IFO 16594^T (60·2 mol% G+C). The DNA–DNA relatedness (40–17 %) of the isolates revealed a taxon separate from *Asaia bogorensis* and *Asaia siamensis*. Phenotypically, the three isolates can be distinguished from the type strains of the known species (Table 1). The isolates were different from *Asaia siamensis* IFO 16457^T in terms of acid production from dulcitol. The three isolates showed varying growth responses on maltose, which also differed from those of the type strains of the known species, and the growth was not intense for any strain. Consequently, the three isolates can be distinguished genetically and phenotypically from the known species of the genus *Asaia*, and should be classified as a novel species. The name *Asaia krungthepensis* sp. nov. is proposed for the three isolates.

Description of *Asaia krungthepensis* sp. nov.

Asaia krungthepensis (krung.thep.en′sis. N.L. fem. adj. *krungthepensis* of or pertaining to Krungthep, the Thai name of Bangkok, Thailand, where the type strain was isolated).

Cells are Gram-negative rods, measuring 0·6–1·0×1·0–2·5 μm on GECA medium. Motile with peritrichous flagella. Colonies are pink, shiny, smooth and raised with an entire margin on GECA medium. Strictly aerobic. Grows at pH 3·0 and 3·5 at 30 °C on $CaCO_3$- and agar-free GECA medium. Oxidizes acetate and lactate to carbon dioxide and water, but the activity is not intense. No production of acetic acid from ethanol. No growth on methanol. Produces 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate. Grows on glutamate agar and mannitol agar. Vigorous growth on a vitamin-free glucose/$(NH_4)_2SO_4$ medium, but not on a vitamin-free ethanol/$(NH_4)_2SO_4$ medium. Produces dihydroxyacetone. Acid is produced from D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, D-xylose, L-arabinose, L-rhamnose, D-mannitol, D-sorbitol, dulcitol, *meso*-erythritol, glycerol, melibiose and sucrose. Acid production is weak from D-arabinose, maltose and raffinose and acid is not produced from lactose or ethanol. Grows on D-glucose, D-galactose, D-fructose, L-sorbose, D-xylose, L-arabinose, D-mannitol, D-sorbitol, dulcitol, *meso*-erythritol, glycerol and sucrose, but grows hardly at all on D-mannose, D-arabinose, L-rhamnose, melibiose and raffinose. No growth is shown on lactose and ethanol. Grows on maltose, but the growth is not intense. The major ubiquinone is Q_{10}. The G+C content of the DNA is 60·2–60·5 mol%, with a range of 0·3 mol%.

The type strain, isolate AA08^T, has a DNA G+C content of 60·3 mol% and was isolated from a heliconia flower collected in Bangkok, Thailand. The type strain has been deposited in the BIOTEC Culture Collection, BIOTEC Central Research Unit, National Centre for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, Thailand, as BCC 12978^T, in the culture collection of the National Biological Resource Centre, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba-ken, Japan, as NBRC 100057^T, in the NODAI Culture Collection, Tokyo University of Agriculture, Setagaya-ku, Tokyo, Japan, as NRIC 0535^T and in the culture collection of the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand, as TISTR 1524^T.

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