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Short Communication

Asaia astilbes sp. nov., *Asaia platycodi* sp. nov., and *Asaia prunellae* sp. nov., novel acetic acid bacteria isolated from flowers in Japan

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During a study of bacterial flora of acetic acid bacteria in Japan, eight bacterial strains were isolated from various kinds of flowers by the enrichment culture approach to acetic acid bacteria, and they were allocated to the genus *Asaia* in the family *Acetobacteraceae* based on the phylogenetic analysis of 16S rRNA gene sequences. Further, the strains were classified into three DNA groups on the basis of DNA–DNA relatedness, and they were assigned to novel species in the genus *Asaia*.

Currently, strains of the *Asaia* species, *A. bogorensis* (Yamada et al., 2000), *A. siamensis* (Katsura et al., 2001), *A. krungthepensis* (Yukphan et al., 2004), and *A. lannensis* (Malimas et al., 2008; The specific epithet *lannaensis* [*sic*] has been corrected according to Rule 61. Validation Lists No. 122, 2008) have been isolated from flowers and fruits in the tropical countries, Indonesia (*A. bogorensis* and *A. siamensis*) and Thailand (*A. siamensis*, *A. krungthepensis*, and *A. lannensis*). Therefore, the genus *Asaia* had been assumed an acetic acid bacterium with specific niches in the tropical region. However, *Asaia* strains were isolated from flowers collected in Akita, Niigata, Tokyo, and Yamanashi in this study, which are located in the temperate region in Japan. (Details will be reported elsewhere.) Therefore, the genus *Asaia* is not an acetic acid bacterium of which distribution is limited to tropical countries, and can be regarded as a cosmopolitan bacterium.

Further, the genus *Asaia* has been studied with interests focusing on the pathogenicity to humans. *Asaia* strains were isolated from a peritoneal dialysis fluid of a patient with a medical history of end-stage renal disease secondary to diabetes (Snyder et al., 2004), and from blood of a man with a history of intravenous drug abuse (Tuuminen et al., 2006).

In addition, *Asaia* strains were isolated from an Asian malarial mosquito vector, *Anopheles stephensi* (Favia et al., 2007). According to our study, the finding of *Asaia* cells in adult mosquitoes is logical because *Asaia* strains are widely distributed in nectar-bearing flowers. When adult mosquitoes visit flowers to collect, lay eggs, hatch larvae, and then new offspring come into

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DDBJ accession numbers for the 16S rRNA gene sequences of isolates T-153^T, T-671, T-683^T, T-692, T-694, T-6123, T-6124, and T-6133^T are AB485741, AB485742, AB485739, AB485745, AB485746, AB485744, AB485743, and AB485740, respectively.

being, young mosquitoes will suck up the nectar; in consequence, *Asaia* cells will move to the organs of the mosquitoes during their life spans. Therefore, members of the genus *Asaia* deserved attention for taxonomic interests and ecological behaviors.

This paper deals with the descriptions of three novel species, *Asaia astilbes* sp. nov., *Asaia platycodi* sp. nov., and *Asaia prunellae* sp. nov., isolated from flowers collected in several prefectures in Japan.

Acetic acid bacteria used in this study and their sources and reference strains are shown in Table 1.

Out of 345 strains isolated from 776 sources including flowers, fruits, fermented foods, and others collected from 2004 to 2007 in Japan, eight strains were selected and subjected to this study on the basis of phylogenetic analysis. They were isolated by using enrichment medium I (EM I, Lisdiyanti et al., 2003) and enrichment medium VI (EM VI) at pH 3.5. EM I was composed of 1.0% glucose, 0.5% ethanol (v/v), 1.5% peptone, 0.8% yeast extract, 0.3% acetic acid (v/v), and 0.01% cycloheximide, and was designed for isolating a variety of acetic acid bacteria; EM VI consisted of 1.0% sorbitol, 1.0% dulcitol, 0.5% peptone, 0.3% yeast extract, and 0.01% cycloheximide, and was appropriate for isolation of *Asaia* strains because of their assimilation of these sugar alcohols.

Of the eight strains, seven were isolated with EM VI, and one was isolated with EM I (Table 1). This indicates usefulness of EM VI for the isolation of *Asaia* strains.

When microbial growth occurred in the enrichment media, bacterial strains were isolated by a method described previously (Yamada et al., 2000). The isolation medium contained 1.0% glucose, 1.0% glycerol, 0.5% yeast extract, 1.0% peptone, 0.5% ethanol, 0.7% $CaCO_3$, and 1.5% agar. Isolates were maintained on agar slants of GYP medium consisting of 1.0% glucose, 1.0% glycerol, 1.0% peptone, 0.5% yeast extract, 0.7% $CaCO_3$, and 1.5% agar.

Sequencing of the 16S rRNA gene and the construction of phylogenetic trees were carried out as reported previously (Lisdiyanti et al., 2000; Yamada et al., 2000). The 16S rRNA gene was amplified by PCR with two primers: 20F (5′-GAGTTTGATCCTGGCTCAG-3′, positions 9–27) and 1500R (5′-GTTACCTTGTTACGACTT-3′, positions 1509–1492). The numbering of positions was based on the *Escherichia coli* numbering system (accession number V00348; Brosius et al., 1981). The purified PCR products were sequenced directly with an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM® 3130 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′-AAACTCAAATGAATTG ACGG-3′, positions 907–926) and 920R (5′-CCGTCA ATTCATTTGAGTTT-3′, positions 926–907).

Multiple alignments were performed by the program CLUSTAL_X (ver. 1.81) (Thompson et al., 1997). Distance matrices for the aligned sequences were calculated by the two-parameter method (K_{nuc}, Kimura, 1980). Neighbor joining (NJ, Saitou and Nei, 1987), maximum likelihood (ML, Felsenstein, 1981), and maximum parsimony (MP, Felsenstein, 1983) were employed for constructing phylogenetic trees. The robustness of individual branches of the NJ tree was estimated by bootstrapping with 1,000 replicates (Felsenstein, 1985). Alignment gaps and unidentified base positions were not taken into account for calculation. Available 16S rRNA gene sequences were obtained from GenBank/EMBL/DDBJ. Species, type strains, strain numbers, and accession numbers of the sequences are presented in Figs. 1–2.

Phylogenetic trees of the isolates are produced with NJ, MP, and ML, and the trees are similar to one another (Figs. 1–2. The tree produced by MP is not shown.). All of the isolates were in the cluster of the genus *Asaia* in the above three phylogenetic trees and in a broad cluster of the family *Acetobacteraceae*. Further, the isolates were allocated to three independent subclusters, and the isolates in each subcluster showed high similarities to one another. The three subclusters were separated from the four known *Asaia* species, *Asaia bogorensis*, *A. siamensis*, *A. krungthepensis*, and *A. lannensis*.

Each of the three subclusters consisted of strains with a similarity of 99.7 to 100%, and the subclusters were separated from the type strain of *A. bogorensis* with a similarity of 99.3 to 99.6%, of *A. siamensis* with 99.5 to 99.7, of *A. krungthepensis* with 99.2–99.5, and of *A. lannensis* with 99.3–99.6%.

DNAs were extracted by a previous method (Saito and Miura, 1963). DNA base composition was determined by using HPLC (Tamaoka and Komagata, 1984). DNA–DNA hybridization was carried out by the fluorometric DNA–DNA hybridization method as described previously (Ezaki et al., 1989).

The isolates were separated into three DNA groups,

Table 1. Isolates and reference strains used in this study.

Strain numbers	Other designations	Sources	Enrichment medium used	Groups on DNA–DNA relatedness	Identification of isolates
T-6133^T	JCM 15831^T, DSM 23030^T	astilbe (*Astilbe thunbergii* var. *congesta*), Yamanashi, Japan	VI	I	*A. astilbes*
T-6123	JCM 25445	manyspiny knotweed (*Persicaria senticosa*), Niigata, Japan	VI	I	*A. astilbes*
T-6124	JCM 25446	Asian dayflower (*Commelina communis*), Niigata, Japan	VI	I	*A. astilbes*
T-683^T	JCM 25414^T, DSM 23029^T	balloon flower (*Platycodon grandiflorum*), Akita, Japan	VI	II	*A. platycodi*
T-671	JCM 25411	unidentified flower, Tokyo, Japan	VI	II	*A. platycodi*
T-153^T	JCM 25354^T, DSM 23028^T	self-heal (*Prunella vulgaris*), Akita, Japan	I	III	*A. prunellae*
T-692	JCM 25423	self-heal (*Prunella vulgaris*), Akita, Japan	VI	III	*A. prunellae*
T-694	JCM 25425	unidentified flower, Akita, Japan	VI	III	*A. prunellae*
A. bogorensis NRIC 0311^T	71^T (Yamada), JCM 10569^T, LMG 21650^T, NBRC 16594^T				
A. siamensis NRIC 0323^T	S60-1^T (Katsura), JCM 10715^T, LMG 21657^T, NBRC 16457^T				
A. krungthepensis NRIC 0535^T	AA08^T (Yukphan), LMG 23083^T, NBRC 100057^T				
A. lannensis NBRC 102526^T	AB92^T (Malimas)				

^{T}, type strain; *A.*, *Asaia*. Abbreviations of the culture collection: DSM, German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany; JCM, Japan Collection of Microorganisms, Wako, Japan; LMG, BCCC/LMG Bacteria, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; NBRC, NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Japan; NRIC, NODAI Research Institute Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan.

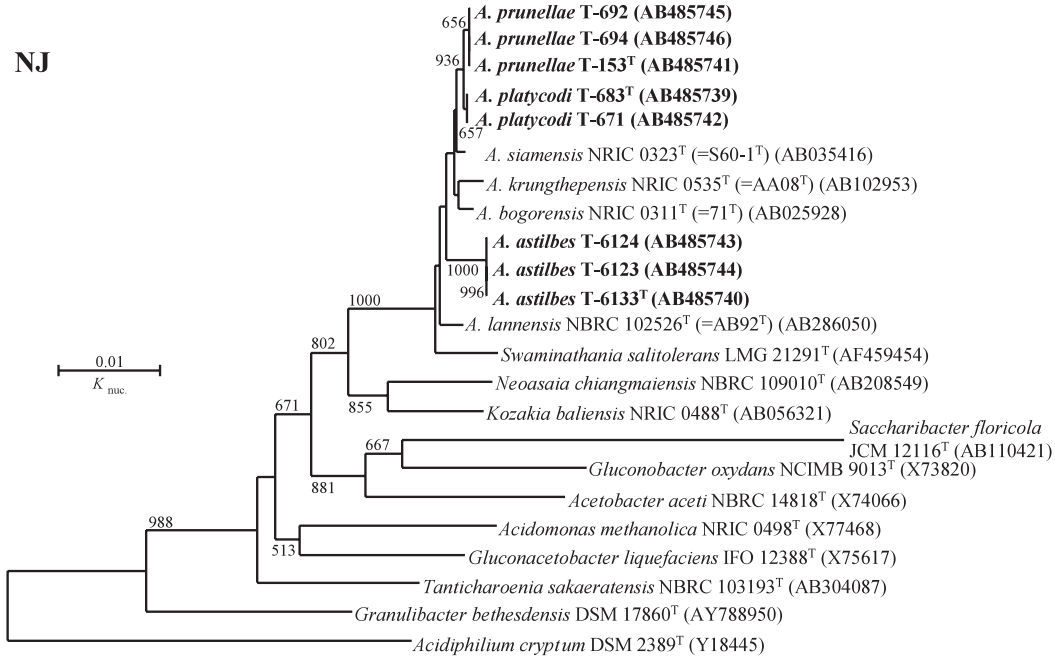

Fig. 1. Phylogenetic relationships of acetic acid bacteria deduced from 16S rRNA gene sequence clustering by neighbor-joining (NJ).

Numerals indicate the bootstrap value derived from 1,000 replications. *Acidiphilium cryptum* DSM 2389^T (Y18445) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. Abbreviations: ^{T}, type strain; *A.*, *Asaia*.

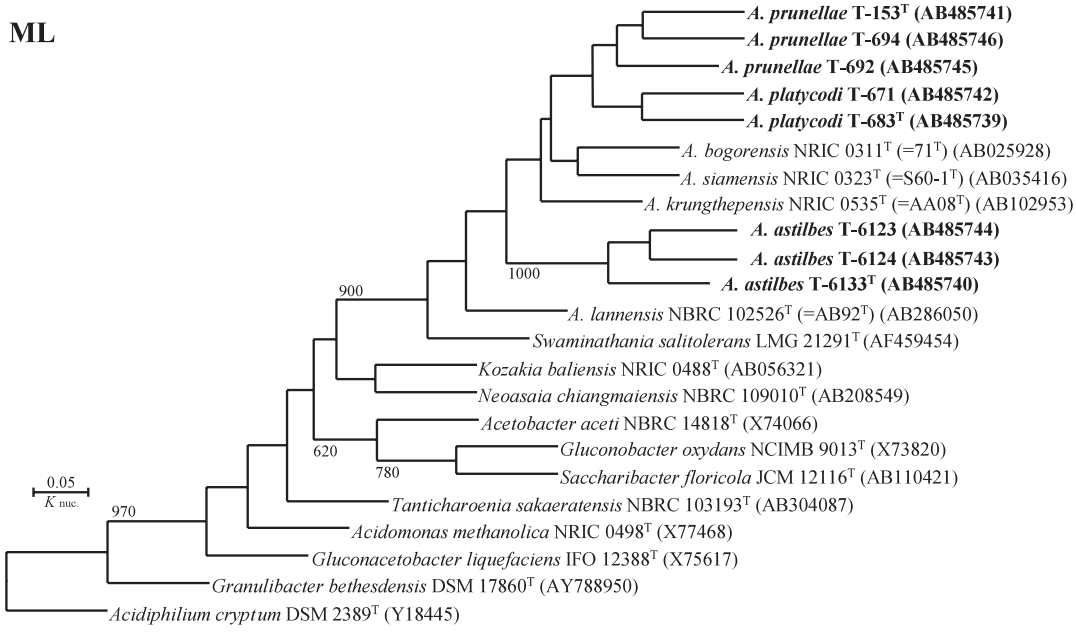

Fig. 2. Phylogenetic relationships of acetic acid bacteria deduced from 16S rRNA gene sequence clustering by maximum likelihood (ML).

Numerals indicate the bootstrap value derived from 1,000 replications. *Acidiphilium cryptum* DSM 2389^T (Y18445) was used as an outgroup. Bar, 0.05 substitutions per nucleotide position. Abbreviations: ^{T}, type strain; *A.*, *Asaia*.

I, II, and III, and delineated from the four known *Asaia* species on the basis of DNA–DNA relatedness. Each group showed a high level of relatedness over 70% (Table 2). DNA base compositions ranged from 58.8 to 59.4 mol% of G+C for group I, 60.0 to 60.1% for group II, and 58.9 to 59.3% for group III. A good correlation was found between the three clusters based on 16S rRNA gene sequences and the three DNA groups.

Isoprenoid quinones were extracted as reported previously (Yamada et al., 1969), and determined by using HPLC (Komagata and Suzuki, 1987).

The eight *Asaia* strains tested had ubiquinones with Q-10 accounting for more than 96% and Q-9 for less than 4.0% (except for T-671 with 16.9%) of the total ubiquinones.

Phenotypic characterization was carried out mostly by the methods described previously (Lisdiyanti et al., 2000, 2001).

The isolates were Gram-negative short rods measuring 0.6 by 1.0–1.5 μm, aerobic, and motile with lateral flagella. They were positive for catalase, and negative for oxidase. They grew well on GYP medium, and produced smooth, entire, shiny, raised, and light brown to pinkish orange colonies; strains in DNA group I produced a darker tint than other strains did. All the isolates grew on glutamate agar and mannitol agar. Further, they grew on Hoyer-Frateur medium (De Ley and Frateur, 1974) and Frateur modified Hoyer medium (De Ley et al., 1984) at the expense of glucose and mannitol. However, they did not grow on either medium at the expense of ethanol.

The isolates produced dihydroxyacetone from glycerol. They oxidized lactate, but oxidation of acetate varied with strains. They produced 2-ketogluconate and 5-ketogluconate from glucose, but not 2,5-diketogluconate. Good growth occurred at temperatures between 10°C and 30°C. However, strains in DNA group III grew at 37°C and only weakly at 10°C. None of the strains tested grew at 42°C. The isolates grew at pHs between 3.0 and 8.5, and developed at concentrations of glucose between 5% and 35%. They did not grow in the presence of 0.35% acetic acid.

The isolates produced acid from considerable numbers of sugars, sugar alcohols, and alcohols, but did not from lactose, maltose, dextrin, dulcitol, or 2-propanol. Production of acid from raffinose, sorbitol, ethanol, and 1-butanol varied with strains. Production of acid from trehalose was delayed in T-153^T, T-652, and T-694, and was read after a 7-day incubation. In addition, data on acid production and growth at 37°C by and of *A. bogorensis*, *A. siamensis*, *A. krungthepensis*, and *A. lannensis* obtained in this study are included in Table 3.

The genus *Asaia* has some curious characteristics compared with other acetic acid bacteria, of which one is the production of acid from ethanol. To date, the *Asaia* strains were reported to produce no or a little acid from ethanol (Katsura et al., 2001; Malimas et al.,

Table 2. DNA base compositions and levels of DNA–DNA relatedness of strains studied.

Groups on DNA–DNA relatedness	Strain numbers	G+C content (mol%)	Values of DNA–DNA relatedness (%) with the strain						
			1	2	3	4	5	6	7
I	T-6133^T	58.9	100	28	23	48	28	34	10
	T-6123	59.4	73	23	17	18	22	59	—
	T-6124	58.8	70	17	11	21	10	13	—
II	T-683^T	60.0	24	100	28	23	47	24	17
	T-671	60.1	21	83	26	43	32	32	—
III	T-153^T	58.9	14	22	100	15	45	12	10
	T-692	59.3	16	38	102	15	43	11	10
	T-694	59.2	11	25	98	11	40	10	16
A. bogorensis	NRIC 0311^T	60.2[a]	9	17	8	100	49	21	20
A. siamensis	NRIC 0323^T	59.3[b]	4	8	20	3	100	4	27
A. krungthepensis	NRIC 0535^T	60.3[c]	10	17	15	16	39	100	24
A. lannensis	NBRC 102526^T	60.8[d]	26	46	37	49	34	28	100

Abbreviations: ^{T}, Type strain; *A., Asaia.* Strains: 1, *A. astilbes* T-6133^T; 2, *A. platycodi* T-683^T; 3, *A. prunellae* T-153^T; 4, *A. bogorensis* NRIC 0311^T; 5, *A. siamensis* NRIC 0323^T; 6, *A. krungthepensis* NRIC 0535^T; and 7, *A. lannensis* NBRC 102526^T. Data were taken: [a]from Yamada et al. (2000); [b]from Katsura et al. (2001); [c]from Yukphan et al. (2004); and [d]from Malimas et al. (2008).

Table 3. Differential characteristics of *Asaia* species.

Characteristics	Species and strains						
	1	2	3	4	5	6	7
Growth at 37°C	−	−	+	+[e]	+[e]	w[e]	+[e]
Acid from							
L-rhamnose	−	+	−	+[e]	+[e]	+[c]	+[d]
trehalose	−	−	+d	−[e]	−[e]	−[e]	−[e]
raffinose	−	+	w	+[e]	+[e]	w[c]	w[d]
dulcitol	−	−	−	+[a]	−[b]	+[c]	w[d]
1-propanol	−	+	+	+[e]	+[e]	+[e]	+[e]
DNA G+C content (mol%)	58.9	60.0	58.9	60.2[a]	59.3[b]	60.3[c]	60.8[d]
Quinone	Q-10	Q-10	Q-10	Q-10[a]	Q-10[b]	Q-10[c]	Q-10[d]

Species and strains: 1, *A. astilbes* T-6133^T; 2, *A. platycodi* T-683^T; 3, *A. prunellae* T-153^T; 4, *A. bogorensis* 71Ta; 5, *A. siamensis* S60-1^{Tb}; 6, *A. krungthepensis* AA08^{Tc}; 7, *A. lannensis* AB92^{Td}. Abbreviations: +, positive; −, negative; w, weakly positive; and d, delayed. Data were taken: [a]from Yamada et al. (2000), [b]from Katsura et al. (2001), [c]from Yukphan et al. (2004), [d]from Malinas et al. (2008), and [e]in this study.

2008; Yamada et al., 2000; Yukphan et al., 2004). Further, *A. bogorensis* NRIC 0311^T was described as not producing alcohol dehydrogenase (Ano et al., 2008). Actually, only *A. prunellae* T-683^T produced acid from ethanol in this study. Nevertheless, the strains of six species of the seven known *Asaia* species produced acid from 1-propanol (Table 3). In addition, growth was found on 1-propanol in the test of acid production. Further, *Gluconacetobacter saccharivorans* was reported to produce acid from 1-propanol (Lisdiyanti et al., 2006). This is worthy of attention from the viewpoint of the metabolic activity of acetic acid bacteria.

All of the known *Asaia* species are barely differentiated with phenotypic characteristics. Growth at 37°C would be useful for separation of *A. astilbes* and *A. platycodi* strains from *A. prunellae* strains and other *Asaia* species, and the type strains of the known *Asaia* species did not grow at 42°C. Further, acid production from L-rhamnose, trehalose, raffinose, dulcitol, and 1-propanol would serve to differentiate the *Asaia* species as well (Table 3). However, the similarity of 16S rRNA gene sequences and DNA−DNA relatedness are a reliable way to identify the species.

On the basis of results obtained, the three DNA groups were regarded as novel species in the genus *Asaia*, and *Asaia astilbes* sp. nov. is proposed for DNA group I strains, *Asaia platycodi* sp. nov. for DNA group II strains, and *Asaia prunellae* sp. nov. for DNA group III strains.

Description of *Asaia astilbes* sp. nov.

Asaia astilbes (*a.stilb'es*. N. L. fem. n. *astilbes*, a generic name of saxifragaceous plant from which the type strain was isolated; N. L., fem. gen. n. *astilbes*, of *Astilbes*.)

Gram-negative rods, measuring 0.6 × 1.2−2.0 μm, aerobic, and motile with lateral flagella (type strain). Catalase positive, and oxidase negative. Grows on glutamate agar and mannitol agar. Grows well on GYP medium, and produces smooth, entire, shiny, raised, and dark pinkish colonies. Further, grows on Hoyer-Frateur medium and Frateur modified Hoyer medium at the expense of D-glucose and mannitol, but not on either medium at the expense of ethanol. Produces dihydroxyacetone from glycerol. Oxidizes lactate, but not acetate (one strain weakly oxidized). Produces 2-ketogluconate and 5-ketogluconate from D-glucose, but not 2,5-diketogluconate. Growth occurs at temperatures between 10°C and 30°C but not at 37°C, and does at pHs between 3.0 and 8.5. Grows at the concentration of 35% D-glucose. Does not grow in the presence of 0.35% acetic acid.

Produces acid from L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, D-fructose, L-sorbose, melibiose, sucrose, glycerol, mannitol, and sorbitol, but not from L-rhamnose, lactose, maltose, trehalose, raffinose (one strain grew), dextrin, dulcitol, ethanol, 1-propanol, 2-propanol, or 1-butanol.

The major quinone is Q-10, and the G+C content of DNA of the type strain is 58.9%.

The type strain is T-6133^T (= JCM 15831^T = DSM

23030^T), which was isolated from astilbe, *Astilbe thunbergii* var. *congesta*, Yamanashi, Japan.

Description of *Asaia platycodi* sp. nov.

Asaia platycodi (*pla.ty.co'di.* N. L. neut. n. *Platycodon*, a generic name of balloon flower from which the type strain was isolated; N. L. neut. gen. n. *platycodi*, of *Platycodon*.)

Gram-negative rods, measuring 0.6 × 0.8–2.0 μm, aerobic, and motile with lateral flagella (type strain). Catalase positive, and oxidase negative. Grows on glutamate agar and mannitol agar. Grows well on GYP medium, and produces smooth, entire, shiny, raised, and light brown to pinkish orange colonies. Further, grows on Hoyer-Frateur medium and Frateur modified Hoyer medium at the expense of D-glucose and mannitol, but not on either medium at the expense of ethanol. Produces dihydroxyacetone from glycerol. Oxidizes lactate and acetate (one strain weakly oxidized). Produces 2-ketogluconate and 5-ketogluconate from D-glucose, but not 2,5-diketogluconate. Growth occurs at temperatures between 10°C and 30°C but not at 37°C, and does at pHs between 3.0 and 8.5. Grows at the concentration of 40% D-glucose. Does not grow in the presence of 0.35% acetic acid.

Produces acid from L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, D-fructose, L-sorbose, L-rhamnose, melibiose, sucrose, trehalose, raffinose, glycerol, mannitol, sorbitol, ethanol (one strain produced), 1-propanol, and 1-butanol (one strain weakly produced), but not from lactose, maltose, dextrin, dulcitol, or 2-propanol.

The major quinone is Q-10, and the G+C content of DNA of the type strain is 60%.

The type strain is T-683^T (= JCM 25414^T = DSM 23029^T), which was isolated from balloon flower, *Platycodon grandiflorum*, Akita, Japan.

Description of *Asaia prunellae* sp. nov.

Asaia prunellae (*pru.nel'lae*. N. L. fem. n. *Prunella*, a generic name of self-heal from which the type strain was isolated; N. L. gen. n. *prunellae*, of *Prunella*.)

Gram-negative rods, measuring 0.6 × 1.0–1.5 μm, aerobic, and motile with lateral flagella (type strain). Catalase positive, and oxidase negative. Grows on glutamate agar and mannitol agar. Grows well on GYP medium, and produces smooth, entire, shiny, raised, and light brown to pinkish orange colonies. Further, grows on Hoyer-Frateur medium and Frateur modified Hoyer medium at the expense of D-glucose and mannitol, but not on either medium at the expense of ethanol. Produces dihydroxyacetone from glycerol. Oxidizes lactate and acetate. Produces 2-ketogluconate and 5-ketogluconate from D-glucose, but not 2,5-diketogluconate. Growth occurs at temperatures between 15°C and 37°C but not at 42°C, and does at pHs between 3.0 and 8.5. Grows at the concentration of 35% D-glucose. Does not grow in the presence of 0.35% acetic acid.

Produces acid from L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, D-fructose, L-sorbose, melibiose, sucrose, trehalose (delayed), glycerol, mannitol, 1-propanol, and 1-butanol (one strain grew weakly), but not from L-rhamnose, lactose, maltose, raffinose, dextrin, dulcitol, sorbitol (one strain grew), ethanol, or 2-propanol.

The major quinone is Q-10, and the G+C content of DNA of the type strain is 58.9%.

The type strain is T-153^T (= JCM 25354^T = DSM 23028^T), which was isolated from self-heal, *Prunella vulgaris*, Akita, Japan.

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