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**TAXONOMIC DESCRIPTION**

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# *Frischella japonica* sp. nov., an anaerobic member of the *Orbales* in the *Gammaproteobacteria*, isolated from the gut of the eastern honey bee, *Apis cerana japonica* Fabricius

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**Abstract**

The gut of honey bees is characterized by a stable and relatively simple community of bacteria, consisting of seven to ten phylotypes. Two closely related honey bees, *Apis mellifera* (western honey bee) and *Apis cerana* (eastern honey bee), show a largely comparable occurrence of those phylotypes, but a distinct set of bacterial species and strains within each bee species. Here, we describe the isolation and characterization of Ac13$^T$, a new species within the rare proteobacterial genus *Frischella* from *A. cerana japonica* Fabricius. Description of Ac13$^T$ as a new species is supported by low identity of the 16S rRNA gene sequence (97.2%), of the average nucleotide identity based on orthologous genes (77.5%) and digital DNA–DNA hybridization relatedness (24.7%) to the next but far related type strain *Frischella perrara* PEB0191$^T$, isolated from *A. mellifera*. Cells of Ac13$^T$ are mesophilic and have a mean length of 2–4 µm and a width of 0.5 µm. Optimal growth was achieved in anoxic conditions, whereas growth was not observed in oxic conditions and strongly reduced in microaerophilic environment. Strain Ac13$^T$ shares several features with other members of the *Orbaceae*, such as the major fatty acid profile, the respiratory quinone type and relatively low DNA G+C content, in accordance with its evolutionary relationship. Unlike *F. perrara*, strain Ac13$^T$ is susceptible to a broad range of antibiotics, which could be indicative for an antibiotic-free *A. cerana* bee keeping. In conclusion, we propose strain Ac13$^T$ as a novel species for which we propose the name *Frischella japonica* sp. nov. with the type strain Ac13$^T$ (=NCIMB 15259=JCM 34075).

Social corbiculate bees, including honey bees and bumble bees, have previously been shown to harbour specialized gut microbiota, which can be detected in bee guts or the hive environment but not elsewhere [1, 2]. This bacterial community is transmitted between bees by social interactions [3] and consists of seven to ten phylotypes with five taxa consistently found in most host species and thus referred to as core microbiota. Core genera are *Gilliamella* and *Snodgrassella* of the Gram-stain-negative '*Proteobacteria*', two Gram-stain-positive *Lactobacillus* species of the '*Firmicutes*' phylum (Firm-4 and Firm-5) and, less abundant, *Bifidobacterium* of the '*Actinobacteria*'. Less numerous and prevalent rare phylotypes (i.e. only in some bee species and therein not in every individual) within honey bees are *Commensalibacter*, *Bartonella* and *Frischella* of the '*Proteobacteria*' and *Apibacter*, belonging to the '*Bacteroidetes*' [4].

The gut microbiome has major effects on host health and nutrition, including the degradation of (toxic) carbohydrates, immune system stimulation and protection against pathogens [5]. Especially interesting with respect to host interaction is the rare species *Frischella perrara*, presence of which in the distal pyloric part of the midgut in the western honey bee *Apis mellifera* co-occurs with a local melanization response of the host tissue [6], caused by strong activation of the immune system with potential implications for *A. mellifera* health [7]. Yet, research has focused on the globally important and widely distributed honey bee species *A. mellifera*, with many

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**Keywords:** *Apis cerana*; *Frischella*; gut microbiota; honey bee.
**Abbreviations:** ANI, average nucleotide identity; BHIA, brain–heart infusion agar; CBA, Columbia blood agar; dDDH, digital DNA–DNA hybridization; GAM, Gifu anaerobic medium; OrthoANIb, BLAST-based Orthologous Average Nucleotide Identity; PNPG, p-nitrophenyl-beta-d-galactoside (β-galactosidase substrate); PYG, peptone–yeast extract–glucose; TSA, trypticase soy agar.
The GenBank/ENA/DDBJ accession number for the partial 16S rRNA gene sequence of Ac13$^T$ is MT560688. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JABURY000000000. The version described in this paper is version JABURY01.
Three supplementary tables are available with the online version of this article.

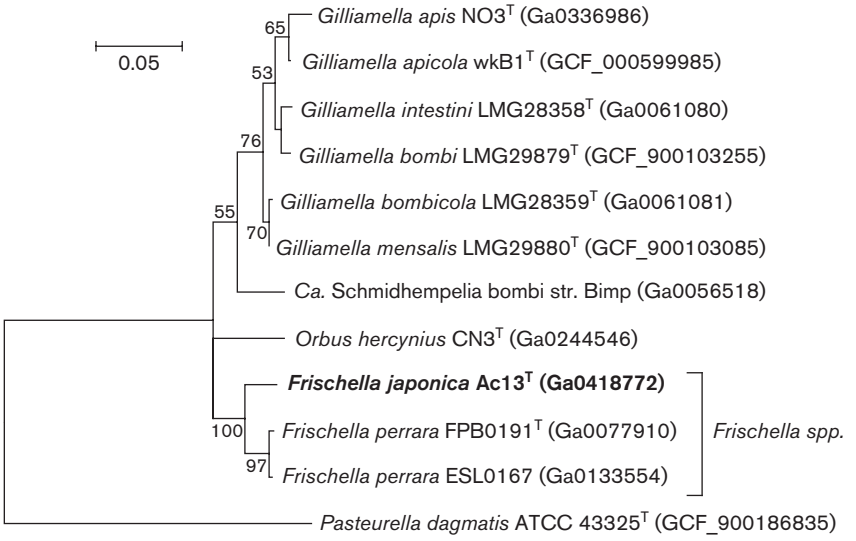


**Fig. 1.** Phylogenetic placement based on 16S rRNA sequence alignment (1512 sites) of Ac13$^T$ and other type strains of the *Orbaceae*, including the yet-described type species of the genus *Gilliamella*, *F. perrara*, *Orbus hercynius* and '*Ca*. Schmidhempelia bombi' aligned using MAFFT. For rooting the tree, *Pasteurella dagmatis* ATCC 43325 of the closest related family to the *Orbaceae* was included. The maximum-likelihood tree was inferred using IQ-TREE using the TIM3 substitution model with four discrete Gamma categories and invariable sites (TIM3+I+G4) with 1000 bootstrap replicates; values >50% are shown. Bar, 0.05 substitutions per nucleotide position. Accession numbers (NCBI or IMG) of genomic data from which 16S rRNA sequences were retrieved are shown in parentheses.

bacterial isolates available, while analysis of the microbiome of the eastern honey bee *Apis cerana* is still scarce. A recent study comparing the microbiome of *A. mellifera* and *A. cerana* in Japan using shotgun metagenomics demonstrated that, despite an overall consistent composition on phylotype level, both honey bee species show a distinct bacterial composition with different species and variable rare taxa [8]. This suggests that unique gut microbes have co-evolved with *A. cerana* and may have a profound impact on the host physiology.

Here, we present the isolation, cultivation and physiological characterization of a new species of *Frischella* isolated from the gut of eastern honey bee *A. cerana japonica* Fabricius specimens from Japan, and propose the name *Frischella japonica* sp. nov.

Strain Ac13$^T$ was isolated from the gut homogenate of inhive bees of an *A. cerana japonica* colony in Isumi, Chiba, Japan (35° 13′ 00.9″ N, 140°23′12.9″ E) in September 2017. Bees were immobilized by chilling at 4 °C before their guts were dissected with sterile forceps. Three guts were pooled in a 2 ml reaction tube and homogenized in PBS (137 mM NaCl, 1.47 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, 2.7 mM KCl) by bead-beating with 1 mm glass-beads in a MicroSmash MS-100 homogenizer (TOMY) at 5500 r.p.m. for 30 s. The gut homogenate was stored at −80 °C supplemented with 15% w/v glycerol. Thawed gut homogenate was plated on Columbia blood agar (CBA; Difco BD) with 5% v/v defibrinated sheep blood (Nippon Bio-test Lab), incubated at 37 °C with 5% v/v $CO_2$ in air until bacterial colonies became visible after 4 days.

Comparative sequence analyses based on the 16S rRNA gene and single-copy core genes were performed between strain Ac13$^T$ and other described type species of the *Orbaceae*. DNA of strain Ac13$^T$ for whole genome sequencing was extracted using NucleoSpin Tissue Kit (Macherey-Nagel). The genomic sequence of strain Ac13$^T$ (accession number: JABURY01) was obtained by Illumina NovaSeq6000 2×150 bp pair-end platform with 670× coverage. The trimmed reads could be assembled into 39 contigs with a total length of 2656327 bp and an N50 value of 219086. Of the total 2416 genes, 2342 were identified as protein-coding and 56 identified as RNAs. DNA G+C content of 34.5 mol% and total genome assembly length of 2.66 Mb resemble the characteristics of *F. perrara* PEB0191$^T$. The genome-derived complete 16S rRNA gene sequence of Ac13$^T$ was phylogenetically compared to the almost-complete 16S rRNA genes of relevant type species within the *Orbaceae* including *Gilliamella* species, *Orbus hercynius* DES39, '*Candidatus* Schmidhempelia bombi' O970 and *Pasteurella dagmatis* ATCC 43325 of the closest-related family to the *Orbaceae* for rooting the tree (1512 sites). The 16S rRNA gene sequences were aligned using MAFFT version 7.427 [9], the best substitution model was computed using jModelTest version 2 [10] and the selection confirmed with Modeltest-NG [11]. The suggested model was the TIM3 substitution model with four discrete Gamma categories and invariable sites based on AIC, AICc and BIC. The maximum-likelihood phylogeny with 1000 bootstrap replicates was calculated using the IQ-TREE software version 2.1.2 [12] and visualized using MEGA-X version 10.0.5 [13]. Whole-genome phylogeny was performed with same described type species on amino acid sequences of 890 single-copy orthologous genes, retrieved using Orthofinder version 2.3.3 [14], aligned using MAFFT and concatenated. The concatenated alignment

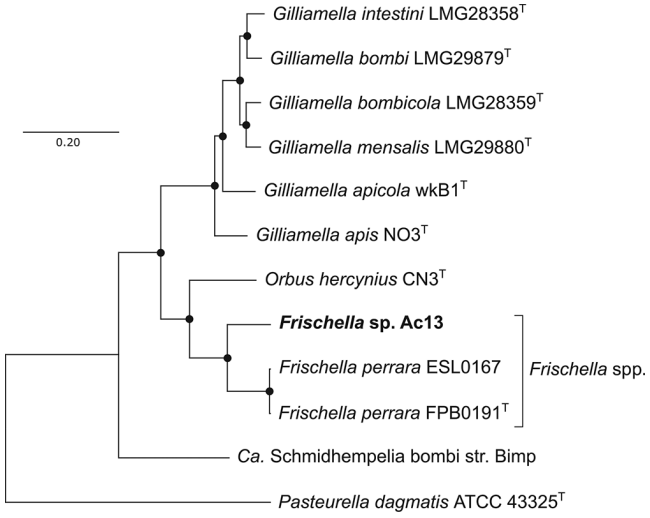


**Fig. 2.** Maximum-likelihood phylogeny based on 890 single-copy orthologous genes, retrieved from amino acid sequences of the whole genomes of the *Orbaceae*, including the yet-described type species of the genus *Gilliamella*, *F. perrara*, *Orbus hercynius* and '*Ca*. Schmidhempelia bombi' aligned using MAFFT. For rooting the tree, *Pasteurella dagmatis* ATCC 43325 of the closest related family to the *Orbaceae* was included. The tree was inferred using IQ-TREE with the LG substitution model with four discrete Gamma categories and invariable sites on the amino acid sequences (LG+I+G4+F) with 100 bootstrap replicates. Dots represent nodes supported by 100% of bootstrap replications. Bar, 0.2 substitutions per site.

of the core genes was automatically curated for blocks represented by >50% sequence information using Gblocks with the providers' parameters for protein sequences [15]. The best substitution model was computed using Modeltest-NG. The improved general amino acid replacement matrix (LG) [16] with four discrete Gamma categories and invariable sites was suggested based on AIC, AICc and BIC. The maximum-likelihood phylogeny of the concatenated single-copy gene alignments with 100 bootstrap replicates was calculated using IQ-TREE and visualized using MEGA-X. Phylogenetic analysis based on 16S rRNA gene sequences and 890 single-copy orthologous genes identified the new isolate Ac13$^T$ as a member of the *Orbaceae* of the *Orbales* in the *Gammaproteobacteria*. Ac13$^T$ deeply branched into a distinct species within the genus *Frischella*, supported by 100% of bootstrap replications for 16S rRNA gene sequences (Fig. 1) and whole-genome phylogeny (Fig. 2).

Average nucleotide identity (ANI) values were calculated based on the complete genome assemblies used for phylogenetic analysis, chopped into 1024 bp fragments using the BLAST-based Orthologous Average Nucleotide Identity (OrthoANIb) algorithm [17]. Digital DNA–DNA hybridization (dDDH) values were calculated by the Genome-to-Genome Distance Calculator GGDC 2.1 [18]. OrthoANIb values of 77.46 and 77.56% to the type strain *F. perrara* PEB0191$^T$ and isolate ESL0167 (Tables 1 and S1, available in the online version of this article) and dDDH values of 24.7 and 25.5% (Tables 1 and S2), respectively, support the branching of Ac13$^T$ into a new species in the genus *Frischella* with values well below the accepted thresholds of 95% for ANI and 70% for dDDH [19, 20].

To determine optimal growth conditions, we tested different culture methods for Ac13$^T$. The cells were resuspended in PBS, and equal volumes of bacterial suspension were used to inoculate cultures in trypticase soy agar (TSA; Difco BD), Columbia blood agar (CBA; Difco BD), brain–heart infusion agar (BHIA) and Gifu anaerobic medium (GAM; Nissui). Strain Ac13$^T$ did not grow under oxic conditions and growth was reduced in 5% v/v $CO_2$ conditions. In anoxia, growth was observed after 2–3 days on TSA and CBA with 5% v/v defibrinated sheep blood. Very weak growth was observed on BHIA with defibrinated sheep blood but not without it. Ac13$^T$ also grew well on GAM agar. No haemolytic activity was observed when incubated on media containing defibrinated sheep blood. The initial pH range for anaerobic growth in peptone–yeast extract–glucose (PYG) broth (Hardy Diagnostics) was determined from pH 4 to 10 in steps of 0.5 pH units. The pH was adjusted by HCl or NaOH. We observed growth at pH 7.0–8.5 and weak growth at pH 6.5 and 9.0, which would allow growth of the strain in the proximal pyloric part of the midgut, comparable to *F. perrara* in *A. mellifera* [21]. For optimal temperature, a range from 15–40 °C in steps of 5 °C was tested and strain Ac13$^T$ grew best at 30 and 35 °C, corroborating relatively high preferred *in vitro* growth temperatures of other bee gut symbionts [22]. For long-term preservation, strain Ac13$^T$ was harvested from CBA supplemented with 5% v/v defibrinated sheep blood plates and stored in PBS with final 15% w/v glycerol at −80 °C.

Using transmission electron microscopy (H-7600, Hitachi) and light microscopy (AxioImager, Zeiss), we characterized the morphology of strain Ac13$^T$ grown in PYG broth at 35 °C for 48 h under anoxic conditions (Fig. 3). Rod-shaped cells, 2–4 µm long and 0.5 µm wide, were the predominant morphological type of strain Ac13$^T$.

To describe the isolate further, a number of biochemical and metabolic analyses were performed. Results are summarized in Table 1, in comparison with published results for *F. perrara* PEB0191$^T$, *G. apicola* wkB1$^T$ and *Orbus hercynius* CN3$^T$ [23]. Using API20A, API ZYM and API20 NE kit (bioMérieux), we found that strain Ac13$^T$ was negative for catalase (EC 1.11.1.6), nitrate reduction, indole production and cytochrome *c* oxidation and hydrolysis of urea, gelatin and PNPG (*β*-galactosidase substrate). Strain Ac13$^T$ was also negative for hydrolysis of aesculin (*β*-glucosidase substrate), while the three compared type strains were positive. Acid production from fermentation was observed with glucose, lactose and mannitol. Although strain Ac13$^T$ was negative for *β*-galactosidase (PNPG) activity, it could produce acid from fermentation of lactose, suggesting that the enzyme may not be secreted into the assay medium or may recognize lactose but not the artificial substrate PNPG.

Cellular fatty acid composition was determined by fatty acid methyl ester analysis (Sherlock MIS version 6.0,

**Table 1.** Differential biochemical characteristics of strain Ac13$^T$ compared to type strains of the *Orbaceae*

Strain: 1, Ac13$^T$; 2, *Frischella perrara* PEB0191$^T$; 3, *Gilliamella apicola* wkB1$^T$; 4, *Orbus hercynius* CN3$^T$. +, positive; –, not detected; w, weak production; AN, anaerobe; MA, microaerobe; A, aerobe; R, rod-shaped cells; C, coccoid cells. Major fatty acid components are marked in bold. 16S rRNA gene sequence identities are based on the MAFFT alignment. For dDDH, results using Formula 1 (length of all high-scoring segment pairs divided by total genome length) are shown. Results using other formulas are summarized in Table S2.

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Aerobic growth | – | – | – | + |
| Microaerophile | + | – | + | – |
| Optimum growth temperature (°C) | 37 | 37 | 37 | 20–30 |
| Morphology | R | R | R | R, C |
| DNA G+C content (mol%) | 34.5 | 34.1 | 33.6 | 38.8 |
| Genome size (Mb) | 2.66 | 2.69 | 3.14 | 2.36 |
| 16S rRNA gene sequence identity (%) | 100 | 97.2 | 96.0 | 94.6 |
| OrthoANIb (%) | 100 | 77.5 | 73.8 | 71.1 |
| dDDH (Formula 1) (%) | 100 | 24.7 | 14.1 | 13.4 |
| Nitrate reduction | – | – | – | + |
| Catalase | – | + | – | + |
| Cytochrome *c* oxidation | – | – | – | + |
| Urea hydrolysis | – | – | – | + |
| *β*-Glucosidase (aesculin) | – | + | + | + |
| *β*-Galactosidase (PNPG) | – | – | + | – |
| Acid production from fermentation of: | | | | |
| D-Glucose | + | + | + | + |
| L-Arabinose | – | w | + | – |
| D-Galactose | – | – | + | – |
| Lactose | + | – | + | – |
| D-Mannitol | + | – | + | + |
| Raffinose | – | – | + | + |
| D-Sorbitol | – | – | + | – |
| Fatty acids (%): | | | | |
| $C_{14:0}$ | 7.5 | 5.2 | 7.5 | 6.9 |
| $C_{16:0}$ | **42.0** | **35.1** | **31.7** | **33.7** |
| $C_{18:0}$ | 2.2 | 3.3 | 1.3 | 0.4 |
| $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c* | 4.7 | 2.0 | 9.4 | **10.7** |
| $C_{18:1}$ ω9*c* | – | 0.8 | – | – |
| $C_{18:1}$ ω7*c*/$C_{18:1}$ ω6*c* | **33.2** | **44.4** | **41.3** | **38.5** |
| Others | **10.6** | 9.4 | 8.8 | 9.4 |

Microbial ID) from a culture of Ac13$^T$ grown in PYG broth at 35 °C for 48 h under anoxia, corresponding to the late-exponential phase. From the same exponential phase culture, predominant respiratory quinones were extracted according to Minnikin *et al.* [24], and identified using an Acquity UPLC H-Class system (Waters). Major fatty acids detected in Ac13$^T$ were palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}$ ω7*c*/$C_{18:1}$ ω6*c*; Table 1). The major quinone was ubiquinone-8, a common feature of the *Orbaceae* [23, 25, 26].

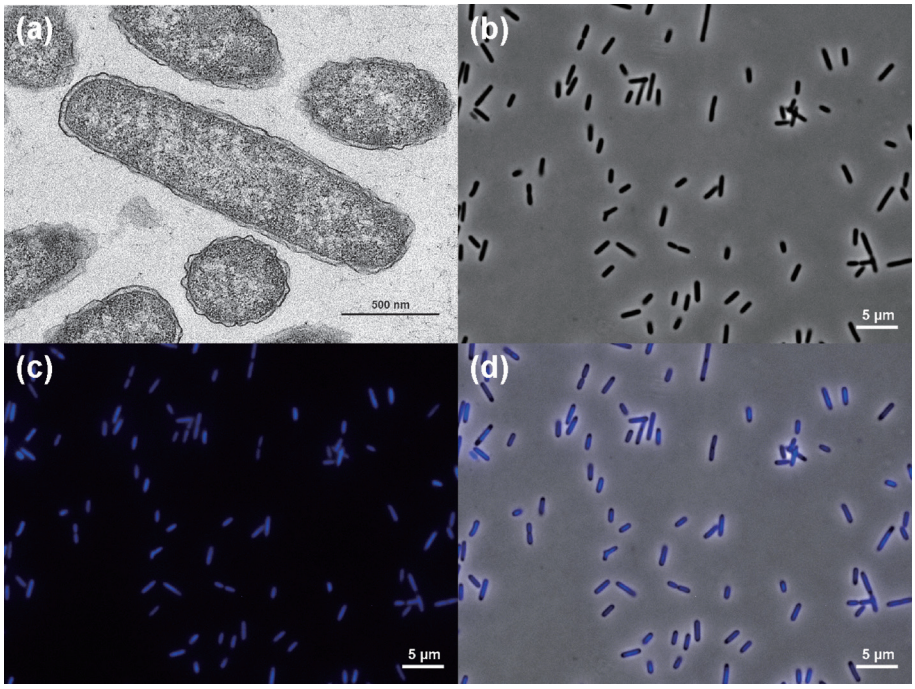


**Fig. 3.** Transmission electron microscopy (TEM) and light microscopy of cells of Frischella *japonica* Ac13$^T$. Images of TEM (a), phase contrast (b), and fluorescence stained with Hoechst33342 (c) are shown. An overlay of the two light microscopy channels is shown in (d).

Because a shared feature of *F. perrara* PEB0191$^T$ and *G. apicola* wkB1$^T$ is the resistance to oxytetracycline and other antibiotics, antibiotic susceptibility of isolate Ac13$^T$ was investigated. To this end, the strain was grown for 2 days on TSA supplemented with 5% v/v defibrinated sheep blood, collected in PBS, and diluted until 0.5 McFarland. The cell suspension was spread on a fresh plate of the same medium. Using the disc diffusion assay, the following antibiotics were tested: ampicillin (20 µg), carbenicillin (30 µg), ceftazidime (25 µg), chloramphenicol (30 µg), gentamicin (25 µg), kanamycin (30 µg), nalidixic acid (30 µg), oxytetracycline (30 µg), rifampicin (30 µg), spectinomycin (50 µg), streptomycin (10 µg) and tylosin (30 µg) and the inhibition zone measured after 2 days. Strain Ac13$^T$ showed no resistance to the tested antibiotics (Table S3). Antibiotic resistance in the honey bee microbiota was shown to reflect the history of antibiotic use in the habitat of the host bee [27] and susceptibility of Ac13$^T$ to all tested antibiotics thus might reflect a low usage of antibiotics to treat *A. cerana* colonies in Japan due to their rather low significance for economic production of honey [28].

The phenotypic, phylogenetic and biochemical analysis of strain Ac13$^T$ indicates that it represents a new species in the genus *Frischella* within the *Orbaceae*. We propose the name *Frischella japonica* based on its isolation from eastern honey bee *A. cerana* from Japan. Ac13$^T$ shares many characteristics to other members of the *Orbaceae*, including the respiratory quinone, a similar fatty acid profile and a low DNA G+C content (~34 mol%). However, ANI and dDDH values based on orthologs and the 16S rRNA gene sequence identity of 77.5, 24.7 and 97.2%, respectively, to the closest relative *F. perrara* PEB0191$^T$, clearly separates Ac13$^T$ from *F. perrara* PEB0191$^T$ through phylogenetic comparison. Phenotypically, Ac13$^T$ can be differentiated from *F. perrara* PEB0191$^T$ by utilizing lactose and D-mannitol for fermentation, missing catalase and *β*-glucosidase activities, and the broad susceptibility to a range of antibiotics.

## DESCRIPTION OF *FRISCHELLA JAPONICA* SP. NOV.

*Frischella japonica*, (ja.po′ni.ca. N.L. fem. adj. *japonica* pertaining to Japan).

Cells have a mean length of 2–4 µm and a width of 0.5 µm. Optimal growth is observed on TSA, CBA supplemented with 5% v/v defibrinated sheep blood and GAM after 2–3 days in anoxia. On TSA with 5% v/v sheep blood for 3 days, colonies of Ac13$^T$ are smooth, round, semi-translucent colonies with a shiny surface and a diameter of about 1 mm. No haemolysis is observed on media supplemented with sheep blood. Negative for catalase activity, nitrate reduction, indole production and cytochrome *c* oxidation and hydrolysis of urea, gelatin, *β*-galactosidase and *β*-glucosidase substrates.

Acid is produced from fermentation of D-glucose, lactose and D-mannitol. Susceptible to a broad range of antibiotics.

The type strain, Ac13$^T$ (=NCIMB 15259=JCM 34075), was isolated from the gut of an eastern honey bee, *A. cerana japonica* from Isumi, Chiba, Japan. The genomic DNA

G+C content of the type strain is 34.5 mol% (by genome sequencing).

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Author contributions
Conceptualization: R. M. Data curation: L. A. W. and R. M. Formal analysis: L. A. W. Funding acquisition: R. M. Investigation: L. A. W., S. S. and R. M. Methodology: L. A. W., S. S. and R. M. Project administration: R. M. Resources: R. M. Software: L. A. W. Supervision: R. M. Validation: L. A. W., S. S. and R. M. Visualization: L. A. W. and R. M. Writing – original draft: L. A. W. Writing – review and editing: L. A. W., S. S. and R. M.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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