Frischella perrara gen. nov., sp. nov., a gammaproteobacterium isolated from the gut of the honeybee, *Apis mellifera*

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The gut of the Western honeybee, *Apis mellifera*, is colonized by a characteristic set of bacteria. Two distinct gammaproteobacteria are consistent members of this unique microbial community, and one has recently been described in a new genus and species with the name *Gilliamella apicola*. Here, we present the isolation and characterization of PEB0191^T, a strain belonging to the second gammaproteobacterial species present in the honeybee gut microbiota, formerly referred to as 'Gammaproteobacterium-2'. Cells of strain PEB0191^T were mesophilic and had a mean length of around 2 μm, and optimal growth was achieved under anaerobic conditions. Growth was not obtained under aerobic conditions and was reduced in a microaerophilic environment. Based on 16S rRNA gene sequence analysis, strain PEB0191^T belongs to the family *Orbaceae*, and its closest relatives, with around 95 % sequence similarity, are species of the genera *Orbus* and *Gilliamella*. Phylogenetic analyses suggest that PEB0191^T is more closely related to the genus *Orbus* than to the genus *Gilliamella*. In accordance with its evolutionary relationship, further similarities between strain PEB0191^T and other members of the family *Orbaceae* were revealed based on the respiratory quinone type (ubiquinone 8), the fatty acid profile and the DNA G+C content. Interestingly, like strains of the genus *Gilliamella*, PEB0191^T exhibited a high level of resistance to oxytetracycline. The similar levels of sequence divergence from the genera *Gilliamella* and *Orbus* and its uncertain phylogenetic position within the family *Orbaceae* indicate that strain PEB0191^T represents a novel species of a new genus, with the proposed name *Frischella perrara* gen. nov., sp. nov. The type strain of *Frischella perrara* is PEB0191^T (=NCIMB 14821^T=ATCC BAA-2450^T).

The Western honeybee, *Apis mellifera*, and species of the related genus *Bombus* (bumblebees) have recently been shown to harbour a stably associated gut microbiota consisting of bacterial taxa found neither in solitary bees nor in the environment (Babendreier *et al.*, 2007; Cox-Foster *et al.*, 2007; Koch & Schmid-Hempel, 2011a; Martinson *et al.*, 2011). As in other animals, these bacteria might possess important symbiotic functions in the gut and, hence, substantially influence the health of their host. In contrast to the complex composition of other gut-associated communities, the honeybee gut microbiota consists of only eight major bacterial species (based on 16S rRNA gene sequence similarity >97 %): two alphaproteobacterial and two gammaproteobacterial species, two members of the phylum *Firmicutes*, one betaproteobacterium and one species of the genus *Bifidobacterium* (Moran *et al.*, 2012; Sabree *et al.*, 2012). Although culturing of some of these bacteria has been reported (Engel & Moran, 2013; Engel *et al.*, 2012; Killer *et al.*, 2009, 2011; Koch & Schmid-Hempel, 2011b; Olofsson & Vásquez, 2008), their characterization is based mainly on 16S rRNA gene and metagenomic sequencing (Engel *et al.*, 2012). Recently, the betaproteobacterium and one of the two gammaproteobacteria were assigned to two novel genera and species with the names *Snodgrassella alvi* and *Gilliamella apicola*, respectively (Kwong & Moran, 2013). Different strains of both species were isolated from *A. mellifera* and from two bumblebee species. *G. apicola* was further shown to belong to a novel order and family of the class *Gammaproteobacteria* with the names *Orbales* and *Orbaceae*, respectively. The nomenclature for this deeply branching lineage originated from *Orbus hercynius* CN3^T, the first species characterized from the order *Orbales* (Volkmann *et al.*,

Abbreviation: DICM, differential interference contrast microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PEB0191^T is JX878306. The accession numbers for the protein-encoding genes of *G. apicola* strains wkB1^T, wkB11 and wkB30 and strain PEB0191^T sequenced in this study are JX878307–JX878338.

Two supplementary figures and a supplementary table are available with the online version of this paper.

2010). Recently, a second species of the genus *Orbus* has been described; *Orbus sasakiae* C7^T was isolated from the gut of the butterfly *Sasakia charonda* (Kim *et al.*, 2013). Sequencing-based methods revealed the existence of additional taxa within the family *Orbaceae*. These sequences originated from different insect samples, suggesting that bacteria of the family *Orbaceae* might be predominantly associated with arthropod hosts. Strikingly, besides *G. apicola*, the second gammaproteobacterium present in the gut of honeybees also belongs to the family *Orbaceae*. This gammaproteobacterium (formerly known as the 'Gammaproteobacterium-2' phylotype) is only distantly related to the genus *Gilliamella* and has not been found in surveys of bumblebee species. In honeybees, however, it seems to be a consistent member of the gut community, since corresponding 16S rRNA gene sequences have been found in most studies conducted so far (Babendreier *et al.*, 2007; Cox-Foster *et al.*, 2007; Moran *et al.*, 2012; Sabree *et al.*, 2012).

Here we report the cultivation and characterization of the 'Gammaproteobacterium-2' phylotype from *A. mellifera* and propose the name *Frischella perrara* gen. nov., sp. nov.

Strain PEB0191^T was isolated from homogenized guts of the Western honeybee, *A. mellifera*. Two adult worker bees were collected from lab-raised hives (West Haven, CT, USA) and immobilized by chilling at 4 °C. Subsequently, guts were dissected with sterile forceps and homogenized in PBS by bead-beating. The homogenate was plated on tryptic soy agar (TSA; Difco BD) supplemented with 5 % defibrinated sheep blood (HEMA Resource and Supply) and incubated at 37 °C in 5 % CO_2. Bacterial colonies became visible after 2–3 days of growth and were screened by PCR with primers amplifying a sequence specific to the 'Gammaproteobacterium-2' phylotype (prPE47, 5′-GATGCCGCATTTTTCAATATTCC-3′; prPE48, 5′-TTCCCCCTAAACCAAATGAAC-3′). DNA amplification was carried out in a thermocycler by denaturing the DNA from boiled colonies for 5 min at 95 °C, followed by 32 cycles of amplification (95 °C for 20 s, 54 °C for 30 s and 72 °C for 30 s) and 5 min of final elongation at 72 °C. Amplicons were sequenced as described by Kwong & Moran (2013). A single colony gave a positive PCR signal and was passaged to plates with fresh medium. However, repeated passaging in 5 % CO_2 resulted in the loss of the positive PCR signal, despite the presence of bacterial growth. Apparently, the original colony contained genotypes other than 'Gammaproteobacterium-2' that were favoured under the growth conditions used. Amplification and sequencing of the 16S rRNA gene (pr928F, 5′-TAAAACTYAAAKGAATTGACGGG-3′; pr1492R, 5′-GGTTACCTTGTTACGACTT-3′), using the protocol described above, confirmed that most colonies were strains of *G. apicola* and not 'Gammaproteobacterium-2'. Only after passage into anaerobic conditions in AnaeroGen sachets (Oxoid) or in an anaerobic chamber at 37 °C were we able to isolate and transfer a colony representing an isogenic population of 'Gammaproteobacterium-2'. This isolate was designated strain PEB0191^T. PCR amplification and sequencing of the 16S rRNA gene with primers pr928F and pr1492R revealed that PEB0191^T shared 99 % sequence similarity in its 16S rRNA gene with 'Gammaproteobacterium-2' sequences from non-culture-based studies previously deposited in databases (e.g. GenBank accession numbers EF187250, DQ837610 and DQ837611). For long-term preservation, strain PEB0191^T was harvested from TSA plates, resuspended in tryptic soy broth (TSB; Difco BD) supplemented with 20 % (v/v) glycerol and stored at −80 °C.

To determine optimal growth conditions, we tested different culturing methods for strain PEB0191^T. For all experiments, bacteria were resuspended in PBS. Equal volumes of bacterial suspension were then used to inoculate cultures. Strain PEB0191^T did not grow in air, and growth was greatly reduced in 5 % CO_2. Under anaerobic conditions, growth could be observed after 1 day on TSA, heart infusion agar (HIA; Difco BD) and brain heart infusion agar (BHIA; Difco BD), with and without defibrinated sheep blood. PEB0191^T also grew on lysogeny broth agar (Difco BD), but growth was much weaker than on the other media tested. No haemolytic activity was observed when incubated on media containing defibrinated sheep blood. The pH range for growth in TSB was determined by measuring OD_{600} after 48 h of incubation at 37 °C. TSB was buffered with 0.1 M sodium acetate/acetic acid or 0.1 M Na_2HPO_4 to analyse a pH range from 6.50 to 8.00 in steps of 0.25 pH units. Although we generally observed only weak growth, the highest OD_{600} was obtained for pH 7.00. In the temperature range 24–42 °C, strain PEB0191^T grew best at 37 °C.

Phylogenetic trees and comparative sequence analysis of strain PEB0191^T were based on the 16S rRNA gene and concatenated deduced amino acid sequences of eight single-copy genes (RadA, COG1066; TypA, COG1217; AlaS, COG0013; RecN, COG0497; Srp, COG541; PyrG, COG0504; UvrB, COG0556; UvrC, COG0322). All sequences were obtained from an ongoing genome-sequencing project of strain PEB0191^T (Illumina HISeq, paired-end library; data not shown). Pairwise CLUSTAL W alignments of full-length 16S rRNA gene sequences showed that strain PEB0191^T (16S rRNA gene length 1534 bp) shares 95.3, 95.6, 95.4, 95.3 and 94.1 % similarity with *G. apicola* strains wkB1^T (JQ936674), wkB11 (JQ936675) and wkB30 (JQ936676), *O. hercynius* CN3^T (FJ612598) and *O. sasakiae* C7^T (JN561614), respectively. Sequence similarities to other closely related gammaproteobacterial species were less than 91 %.

Phylogenetic analysis of 16S rRNA gene sequences positioned strain PEB0191^T in a monophyletic group with the genus *Orbus* (Fig. 1). However, the corresponding clade (*Orbus*+PEB0191^T) was not supported by the bootstrap analysis (i.e. support values <80 %). Furthermore, varying the number and type of taxa included in the analysis also affected the position of strain PEB0191^T. Together, these results indicated that, based on our 16S rRNA gene

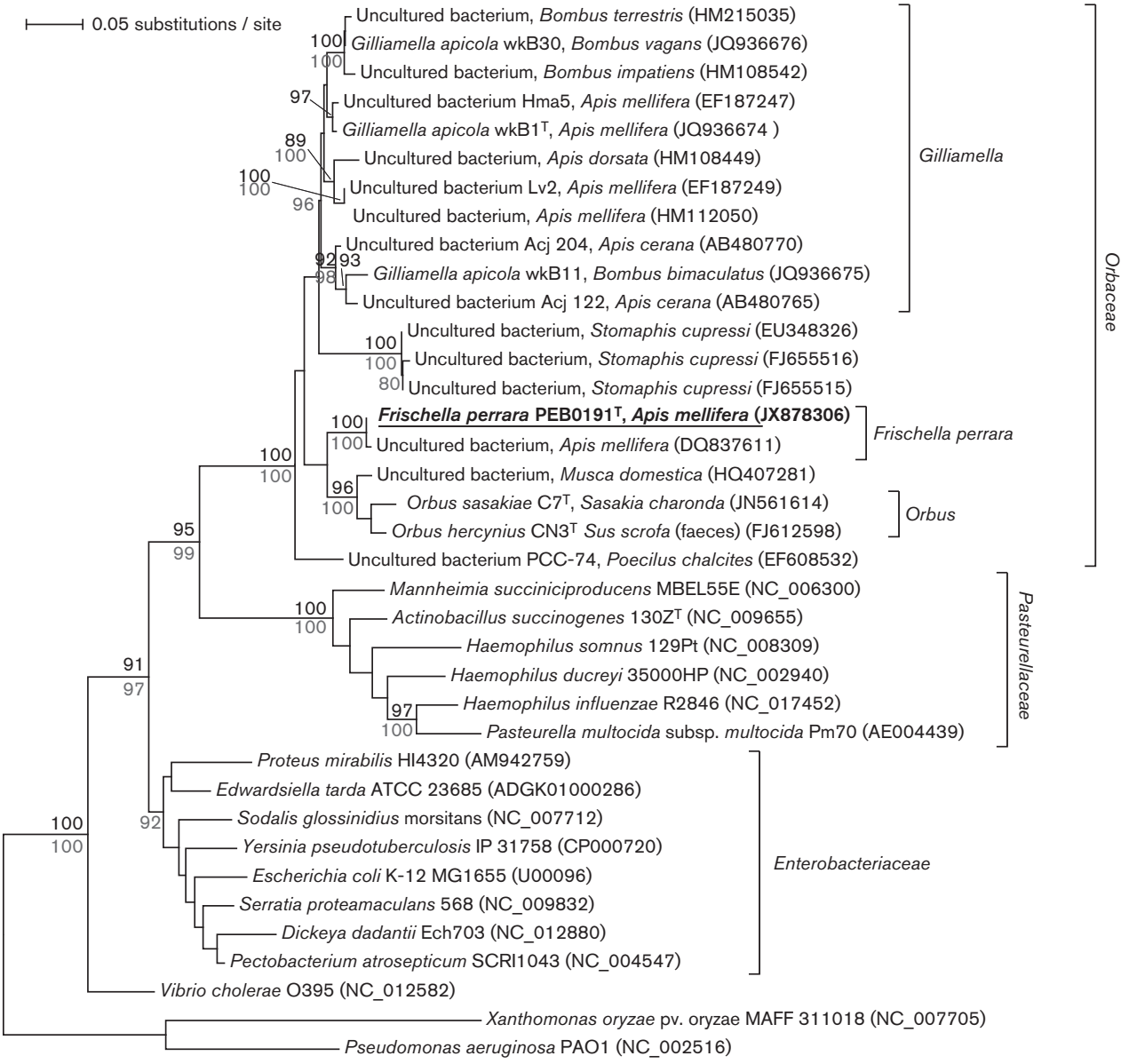

Fig. 1. Maximum-likelihood phylogeny based on a 16S rRNA gene sequence alignment (1258 sites) generated with CLUSTAL W. The tree was inferred with MEGA5 (Tamura *et al.*, 2011) using the general time reversible (GTR) model with proportion of invariable sites (+I) and gamma distribution (+G). The model was selected with ProtTest 3.0 (Darriba *et al.*, 2011). Bootstrap values are indicated in black for 100 replicates; numbers in grey indicate bootstrap values (1000 replicates) for a neighbour-joining analysis conducted on the same alignment with MEGA5 using the maximum composite likelihood method for computing evolutionary distances. Host species in which bacterial taxa were identified are indicated for members of the family *Orbaceae*. Bar, 0.05 substitutions per site.

sequence analysis, strain PEB0191^T cannot be assigned with confidence to the clade of the genus *Orbus*. We therefore analysed its phylogenetic position using concatenated deduced amino acid sequences of the eight previously mentioned single-copy genes. Consistent with the 16S rRNA gene tree, strain PEB0191^T formed a monophyletic group with *O. hercynius* CN3^T, which was supported by high bootstrap values (Fig. S1, available in IJSEM Online). However, fewer taxa were included in the amino acid sequence-based analysis because of the limited number of sequences available. This could have biased the tree topology and bootstrap analysis. In conclusion, the phylogenetic position of strain PEB0191^T within the family *Orbaceae* is uncertain, and further sampling of bacteria belonging to this family will be necessary in order to resolve the evolutionary relationships. The similar levels of sequence divergence from the genera *Gilliamella* and *Orbus* suggest that strain PEB0191^T might belong to a novel genus

within the family *Orbaceae*. This is also supported by the fact that bacteria with 16S rRNA gene sequences closely related to that of strain PEB0191^T are present in honeybees all over the world (Babendreier *et al.*, 2007; Cox-Foster *et al.*, 2007; Moran *et al.*, 2012; Sabree *et al.*, 2012). This makes it likely that additional species and strains of this genus will be characterized in future.

Based on transmission electron microscopy (EM-900, Zeiss) and differential interference contrast microscopy (DICM; Eclipse 80i, Nikon), we characterized the morphology of bacteria of strain PEB0191^T grown on TSA supplemented with 5 % sheep blood for 2 days (Figs 2 and S2). Rod-shaped cells, ~2 μm long and 0.5 μm wide, were the predominant morphological type of strain PEB0191^T. However, we also observed filamentous cells (>10 μm long) and coccoid-like morphotypes (Fig. 2).

To characterize the isolate further, we conducted a number of biochemical and metabolic analyses (see also Table 1). Using test reactions provided in the API20 NE kit (bioMérieux), we found that strain PEB0191^T was negative for activity of nitrate and nitrite reductases, arginine dihydrolase, urease, gelatinase and *β*-galactosidase (PNPG) and for glucose fermentation and indole production, but positive for *β*-glucosidase (aesculinase) activity. We also tested the presence of catalase activity by adding cells to 3 % H_2O_2 and cytochrome *c* oxidase activity by smearing cells onto filter paper wetted with Gordon–McLeod reagent (Sigma). Formation of bubbles after adding H_2O_2 indicated that PEB0191^T harboured catalase activity. No cytochrome *c* oxidase activity was observed. Acid production from carbohydrates was determined using the oxidation/fermentation test as described by MacFaddin (2000), and tests were performed in triplicate and monitored for at least 4 days. Results for strain PEB0191^T and *G. apicola* wkB1^T are summarized in Table 1 in comparison with published results for *O. hercynius* CN3^T and *O. sasakiae* C7^T (Kim *et al.*, 2013). Strain PE0191^T clearly produced acid from fermentation of D-fructose, D-glucose and D-mannose. Weak acid production was also observed from the fermentation of D-arabinose,

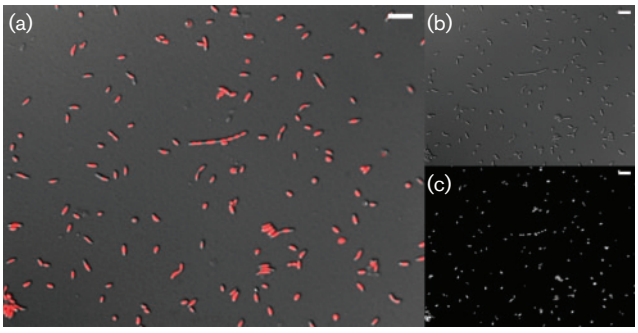

Fig. 2. DICM images of strain PEB0191^T grown for 2 days on HIA supplemented with 5 % sheep blood. Cells were resuspended in PBS and the cell density was adjusted to OD_{600}=1, Hoechst stain was added (1 : 1000) and a 1 μl aliquot was spotted onto a thin agarose pad poured onto a microscopy slide. Image analysis was carried out with a Nikon 80i microscope using DIC mode for visualization of bacterial cell shapes (b) and UV mode for DNA detection (c). An overlay of the two channels is shown in (a). The UV channel is shown in red. Bars, 5 μm.

Table 1. Differential characteristics of type strains of species of the family *Orbaceae*

Strains: 1, PEB0191^T; 2, *G. apicola* wkB1^T; 3, *O. hercynius* CN3^T; 4, *O. sasakiae* C7^T. Data for *G. apicola* wkB1^T, *O. hercynius* CN3^T and *O. sasakiae* C7^T were taken from Kwong & Moran (2013) and Kim *et al.* (2013) unless indicated. All four strains were negative for maltose fermentation and positive for fermentation of D-glucose, D-fructose, D-mannose, D-ribose, sucrose and DL-xylose. For the latter three sugars, PEB0191^T showed only weak acid production; W, weak fermentation activity. Fermentation tests of *O. hercynius* CN3^T and *O. sasakiae* C7^T were conducted with the API50 CH method (bioMérieux) (Kim *et al.*, 2013); fermentation data for PEB0191^T and *G. apicola* wkB1^T were obtained in this study under the same laboratory conditions using the method described by MacFaddin (2000). −, Not detected.

Characteristic	1	2	3	4
Oxygen requirement*	AN	MA	A	A
Optimum growth temperature (°C)	37	37	20–30	20–25
Morphology†	R	R	R, C	C
DNA G+C content (mol%)	33.9	33.5	36.4	32.1
Nitrate reductase	−	−	+	+
Catalase	+	−	+	+
Cytochrome *c* oxidase	−	−	+	−
Urease	−	−	+	−
β-Galactosidase (PNPG)	−	+	−	+
Acid production from fermentation of:				
D-Arabinose	W	+	−	−
D-Galactose	−	+	−	+
Lactose	−	+	−	+
D-Mannitol	−	+	+	−
Melibiose	−	−	−	+
Raffinose	−	+	+	+
D-Sorbitol	−	+	−	−
Fatty acids (%)‡				
$C_{14:0}$	5.15	7.52	6.88	11.2
$C_{16:0}$	**35.05**	**31.69**	**33.73**	**28.0**
$C_{18:0}$	3.29	1.31	0.35	<0.5
$C_{16:1}\omega7c/C_{16:1}\omega6c$	1.96	9.41	10.70	9.3
$C_{18:1}\omega9c$	0.78	−	−	−
$C_{18:1}\omega7c/C_{18:1}\omega6c$	**44.41**	**41.32**	**38.45**	**29.4**
Others	9.36	8.75	9.37	19.9

*A, Aerobe; AN, anaerobe; MA, microaerobe. The environment for optimal growth is given. PEB0191^T can grow under microaerophilic conditions, but growth was weaker than under anaerobic conditions. Both species of the genus *Orbus* can also grow in an anaerobic environment.

†C, Coccoid; R, rod.

‡Major components are indicated in bold.

D-ribose, sucrose and DL-xylose. No acid production was found for D-galactose, lactose, maltose, D-mannitol, melibiose, raffinose or D-sorbitol. Interestingly, both strains were negative for D-glucose fermentation with the API20 NE kit, but produced acid by fermentation in our oxidation/fermentation tests.

To characterize the cellular fatty acid composition, a fatty acid methyl ester analysis (Sherlock MIS-MIDI; Microbial ID) was conducted. Strain PEB0191^T was grown in peptone-yeast-glucose broth at 35 °C for 20–24 h under anaerobic conditions, corresponding to the late-exponential phase of physiological growth. Similar to other members of the family *Orbaceae* (Kwong & Moran, 2013; Kim *et al.*, 2013; Volkmann *et al.*, 2010), palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) were found to be the major fatty acids of strain PEB0191^T (Table 1). Respiratory quinones were extracted and identified as described by Kwong & Moran (2013); the major quinone detected in PEB0191^T was ubiquinone 8, which is also consistent with other members of the family *Orbaceae*.

We tested the susceptibility of isolate PEB0191^T to a range of antibiotics using the disc diffusion assay. To this end, the strain was grown for 2 days on TSA supplemented with 5 % defibrinated sheep blood and half a plate was used to inoculate a new plate of the same medium. Discs containing defined amounts of antibiotics were put on top and inhibition zones around discs were measured after 3 days of growth. The MIC was determined for antibiotics to which strain PEB0191^T had shown resistance in the disc diffusion assay. Strain PEB0191^T was found to be resistant to ampicillin (MIC >20 μg ml^{-1}), carbenicillin (MIC >20 μg ml^{-1}), streptomycin (MIC >10 μg ml^{-1}) and oxytetracycline (MIC >50 μg ml^{-1}). These results, including the size of clearance zones and control experiments conducted with *Escherichia coli* K-12 MG1655, are summarized in Table S1.

The phenotypic and phylogenetic data presented here indicate that strain PEB0191^T represents a novel member of the family *Orbaceae*, for which we propose the name *Frischella perrara* gen. nov., sp. nov. Although PEB0191^T shares many characteristics with other members of the family *Orbaceae*, including a common respiratory quinone, similar fatty acid profiles and a low DNA G+C content (~34 mol%), the 16S rRNA gene sequence similarity to its closest relatives, the type strains of *O. hercynius* and *O. sasakiae*, was only 95.3 and 94.1 %, respectively. Phenotypically, PEB0191^T can be differentiated from the *Orbus* strains by a lack of nitrate reduction. Furthermore, PEB0191^T appeared unique in its preference for growth under anaerobic conditions, as this has not been observed in species of the genera *Gilliamella* or *Orbus*. The antibiotic resistance profile may vary among isolates of *F. perrara* gen. nov., sp. nov., since the presence of antibiotic resistance determinants in the honeybee microbiota has been shown to reflect the history of antibiotic use, which varies among countries (Tian *et al.*, 2012).

Description of *Frischella* gen. nov.

Frischella (Frisch.el′la. N.L. dim. fem. n. *Frischella* named after Karl Ritter von Frisch, 1889–1982, for his prominent role in the study of honeybee behaviour).

Cells are mesophilic, Gram-negative rods. An anaerobic environment provides optimal growth conditions, but growth can also be observed in 5 % CO_2. The major cellular fatty acids are palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). The main isoprenoid quinone is ubiquinone 8. Based on 16S rRNA gene sequence similarity, the genus is most closely related to the genera *Gilliamella* and *Orbus*, sharing about 95 % sequence similarity with both. Based on phylogenies inferred from 16S rRNA gene sequences and eight concatenated deduced amino acid sequences, the genus *Frischella* seems to be more closely related to the genus *Orbus* than to the genus *Gilliamella*. The type species of the genus is *Frischella perrara*.

Description of *Frischella perrara* sp. nov.

Frischella perrara (per.ra′ra. L. fem. adj. *perrara* very rare/exceptional, referring to its restricted occurrence in the honeybee gut).

Exhibits the following properties in addition to those given in the genus description. Cells have a mean length of around 2 μm and a width of 0.5 μm. Optimal growth is achieved on HIA, BHIA and TSA supplemented with 5 % defibrinated sheep blood at 37 °C. After 3 days of incubation on TSA supplemented with 5 % sheep blood at 37 °C, forms smooth, round, flat, semi-translucent colonies with a diameter of about 1 mm. No growth occurs under aerobic conditions. Catalase-positive. Negative for nitrate reductase, cytochrome *c* oxidase and urease. The type strain is resistant to ampicillin (MIC >20 μg ml^{-1}), carbenicillin (MIC >20 μg ml^{-1}), streptomycin (MIC >10 μg ml^{-1}) and oxytetracycline (MIC >50 μg ml^{-1}). Acid is produced from fermentation of D-glucose, D-fructose and D-mannose, but not D-galactose, lactose, maltose, D-mannitol, melibiose or raffinose.

The type strain, PEB0191^T (=NCIMB 14821^T=ATCC BAA-2450^T), was isolated from the gut of a honeybee, *A. mellifera*, from West Haven, CT, USA. The genomic DNA G+C content of the type strain is 33.9 mol%.

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