Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family *Neisseriaceae* of the *Betaproteobacteria*, and *Gilliamella apicola* gen. nov., sp. nov., a member of *Orbaceae* fam. nov., *Orbales* ord. nov., a sister taxon to the order '*Enterobacteriales*' of the *Gammaproteobacteria*

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Gut-associated bacteria were isolated in axenic culture from the honey bee *Apis mellifera* and the bumble bees *Bombus bimaculatus* and *B. vagans* and are here placed in the novel genera and species *Snodgrassella alvi* gen. nov., sp. nov. and *Gilliamella apicola* gen. nov., sp. nov. Two strains from *A. mellifera* were characterized and are proposed as the type strains of *Snodgrassella alvi* (type strain wkB2^T =NCIMB 14803^T =ATCC BAA-2449^T =NRRL B-59751^T) and *Gilliamella apicola* (type strain wkB1^T =NCIMB 14804^T =ATCC BAA-2448^T), representing, respectively, phylotypes referred to as 'Betaproteobacteria' and 'Gammaproteobacteria-1'/ 'Gamma-1' in earlier publications. These strains grew optimally under microaerophilic conditions, and did not grow readily under a normal atmosphere. The predominant fatty acids in both strains were palmitic acid (C$_{16:0}$) and *cis*-vaccenic acid (C$_{18:1}\omega 7c$ and/or C$_{18:1}\omega 6c$), and both strains had ubiquinone-8 as their major respiratory quinone. The DNA G+C contents were 41.3 and 33.6 mol% for wkB2^T and wkB1^T, respectively. The *Snodgrassella alvi* strains from honey bees and bumble bees formed a novel clade within the family *Neisseriaceae* of the *Betaproteobacteria*, showing about 94 % 16S rRNA gene sequence identity to their closest relatives, species of *Stenoxybacter*, *Alysiella* and *Kingella*. The *Gilliamella apicola* strains showed the highest 16S rRNA gene sequence identity to *Orbus hercynius* CN3^T (93.9 %) and several sequences from uncultured insect-associated bacteria. Phylogenetic reconstruction using conserved, single-copy amino acid sequences showed *Gilliamella apicola* as sister to the order '*Enterobacteriales*' of the *Gammaproteobacteria*. Given its large sequence divergence from and basal position to the well-established order '*Enterobacteriales*', we propose to place the clade encompassing *Gilliamella apicola* and *O. hercynius* in a new family and order, *Orbaceae* fam. nov. and *Orbales* ord. nov.

Honey bees and bumble bees are generalist insect pollinators that play a key role in maintaining healthy ecosystem function. In addition, the Western honey bee, *Apis mellifera*, is of great importance to human agriculture, producing products such as honey and pollinating many of the world's crops. Despite substantial interest in bee health and disease, little is known about the normal microbial flora in bees. Bacteria that live in close, symbiotic association with insects can perform beneficial functions

including nutrient synthesis, digestion and provision of disease resistance (Dillon & Dillon, 2004; Koch & Schmid-Hempel, 2011b). Honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.) have recently been found to possess a highly specific gut microbial composition, suggesting they too may participate in an intimate host–microbe symbiosis (Martinson *et al.*, 2011; Koch & Schmid-Hempel, 2011a).

The unique bee gut microbiota consists mainly of eight bacterial phylotypes: two from the *Alphaproteobacteria*, two from the *Gammaproteobacteria*, two from *Lactobacillus*, one from *Bifidobacterium* and one from the *Betaproteobacteria* (Martinson *et al.*, 2011; Moran *et al.*, 2012). Although there has been a long history of culture-based studies of bee microbes (Gilliam, 1997), the characteristic bacteria that make up most of the bee gut microbiota are mostly known from 16S rRNA gene sequencing (Jeyaprakash *et al.*, 2003; Babendreier *et al.*, 2007; Martinson *et al.*, 2011; Koch & Schmid-Hempel, 2011a; Disayathanoowat *et al.*, 2012; Moran *et al.*, 2012) and from a recent metagenomic survey (Engel *et al.*, 2012). Some culturing of these bacteria has been reported (Olofsson & Vásquez, 2008; Yoshiyama & Kimura, 2009; Killer *et al.*, 2009, 2010; Koch & Schmid-Hempel, 2011b; Engel *et al.*, 2012), but most of these phylotypes remain undescribed.

Here, we report the isolation and characterization of members of the 'Betaproteobacteria' and 'Gammaproteobacteria-1'/ 'Gamma-1' groups (Babendreier *et al.*, 2007; Martinson *et al.*, 2011) from *A. mellifera* and *Bombus* species, and propose novel genera and species, *Snodgrassella alvi* gen. nov., sp. nov. and *Gilliamella apicola* gen. nov., sp. nov., respectively, in which to place members of these groups. These names are consistent with those proposed by Martinson *et al.* (2012), '*Candidatus* Snodgrassella alvi' and '*Candidatus* Gilliamella apicola'. Strains wkB2[T] and wkB1[T] from *A. mellifera* are proposed as the respective type strains of *Snodgrassella alvi* and *Gilliamella apicola*. We also provide evidence to support the position of the *Gilliamella apicola* clade as sister to the family *Enterobacteriaceae*/order '*Enterobacteriales*'. Given the genetic and phenotypic similarity of *Gilliamella apicola* to *Orbus hercynius* CN3[T] (Volkmann *et al.*, 2010), we propose a novel family and order in which to place this group, *Orbaceae* fam. nov. and *Orbales* ord. nov.

Methods

Bacterial strains were isolated from the gut of the Western honey bee, *A. mellifera*, and the bumble bees *Bombus bimaculatus* and *Bombus vagans*. Adult worker bees, *A. mellifera* from lab-raised hives and *Bombus* species wild foragers, were captured in West Haven, CT, USA, in May and June 2011. Bees were immobilized by chilling at 4 °C before the guts were dissected out and homogenized in 10 mM $MgSO_4$ by bead beating. The homogenates were plated on HIA (heart infusion agar; Difco BD) and blood agar (tryptic soy agar supplemented with 5 % sheep's blood; Hardy Diagnostics) and incubated at 37 °C in a CO_2-enriched atmosphere. The atmosphere was provided by either a CO_2 incubator set at 5 % CO_2 or CO_2Gen Compact sachets (Oxoid) in airtight pouches, which produced a 6 % CO_2, 15 % O_2 atmosphere.

Colonies were visible after 2 days and were identified by sequencing of the 16S rRNA gene. DNA was extracted using the Qiagen DNeasy Blood & Tissue kit according to its protocol for Gram-negative bacteria. PCR amplicons were generated using the universal 16S primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTT-ACCTTGTTACGACTT-3′) with 35 cycles of amplification (95 °C for 20 s, 52 °C for 30 s and 72 °C for 40 s) after an initial incubation for 10 min at 95 °C. Amplicons were sequenced using the dideoxy chain-termination method with the Big Dye Terminator version 3.1 kit and subsequent capillary gel electrophoresis on an Applied Biosystems 3730xl DNA Genetic Analyzer (DNA Analysis Facility, Yale University). In order to determine the species identity of wild-captured bumble bees, the COI gene was sequenced from bee tissue with primers LEP-F1 and LEP-R1, as described previously (Hebert *et al.*, 2004), and compared using BLAST against annotated sequences in GenBank.

As part of a multigenome sequencing project, the genomes of strains wkB2[T], wkB12, wkB29, wkB1[T], wkB11 and wkB30 were sequenced using the Illumina platform from mate-pair libraries (data not shown). Sequence assembly of raw reads into contigs was performed with Velvet 1.1.06 (Zerbino & Birney, 2008). Accession numbers of sequences from this study used to produce Fig. 5 are listed in Table S1 (available in IJSEM Online). All phylogenetic analysis was done with the MEGA 5 software package (Tamura *et al.*, 2011).

For transmission electron microscopy imaging, 2-day-old cultures from blood-agar plates were harvested in trypticase soy broth (TSB), pelleted and fixed with 4 % glutaraldehyde in 0.1 M Sorensen's phosphate buffer (PB) (162 mM Na_2HPO_4, 32 mM NaH_2PO_4), pH 7.4, for 1 h. Cells were then washed with Sorensen's PB and fixed with 1 % osmium tetroxide in distilled water for 1 h. Following rinsing with distilled water, the samples were dehydrated with successive 70, 80, 90 and 100 % ethanol washes. Samples were then washed with propylene oxide before incubating in Embed-812/propylene oxide mixture (1 : 1) for 1 h and then overnight in 2 : 1 Embed-812/propylene oxide mixture. Finally, samples were immersed in pure Embed-812 and cured under vacuum overnight at 50 °C. Sections of 60–100 nm thickness were cut and stained with uranyl acetate and lead citrate, and these were viewed with a Zeiss EM-900 transmission electron microscope.

Biochemical and metabolic tests were conducted to characterize the isolates further. Catalase activity was tested by adding cells directly to 3 % H_2O_2 and observed for the generation of gas. Presence of cytochrome *c* oxidase was determined by smearing cells onto filter paper wetted with Gordon–McLeod reagent (Sigma). Sulfide-indole-motility (SIM) medium (Hardy Diagnostics) was used to test for H_2S production and motility. The API 20NE kit (bioMérieux) was used to determine activities of nitrate and nitrite reductases, arginine dihydrolase, urease, *β*-glucosidase (aesculinase), gelatinase

and β-galactosidase (substrate PNPG) and for glucose fermentation and indole production according to the manufacturer's instructions. The pH range for growth in TSB was determined by measuring the OD_{600} after 48 h of incubation at 37 °C. TSB was buffered with 0.1 M sodium acetate/acetic acid for pH 5.0–5.5 or 0.1 M Na_2HPO_4/NaH_2PO_4 for pH 6.0–7.0. Long-term stocks of strains were prepared by resuspending plated cultures in TSB plus 15 % glycerol and stored at −80 °C.

The antibiotic susceptibility of strains was tested via disc diffusion assays on trypticase soy agar (TSA) plates using the following antibiotics spotted on Whatman filter paper no. 1: ampicillin (20 μg), carbenicillin (50 μg), ceftazidime (30 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). Plates were incubated at 5 % CO_2 at 37 °C for 2 days and inspected for inhibition zones. Strains were considered resistant if zones were less than half the size of those of controls (*Escherichia coli* DH5α and *Bacillus cereus* UW85).

Cellular fatty acid composition was determined by fatty acid methyl ester (FAME) analysis (Sherlock MIS-MIDI; Microbial ID Inc.). Cultures were grown on 5 % sheep's blood agar at 35 °C for 24 h under 3–5 % CO_2, and FAME analysis was performed on the late-exponential-phase portion of the cultures. For identification of isoprenoid quinones and polar lipids, cells were grown on blood agar at 37 °C in 5 % CO_2 for 48 h, pelleted in water and freeze-dried. Extraction of predominant quinones was carried out according to Minnikin *et al.* (1984), and quinones were identified using a Bruker Apex Qe 9.4T FT-ICR mass spectrometer (Keck Biotechnology Resource Laboratory, Yale University). Polar lipid analysis of strain wkB1^T was carried out by the Identification Service of the DSMZ, according to Tindall *et al.* (2007).
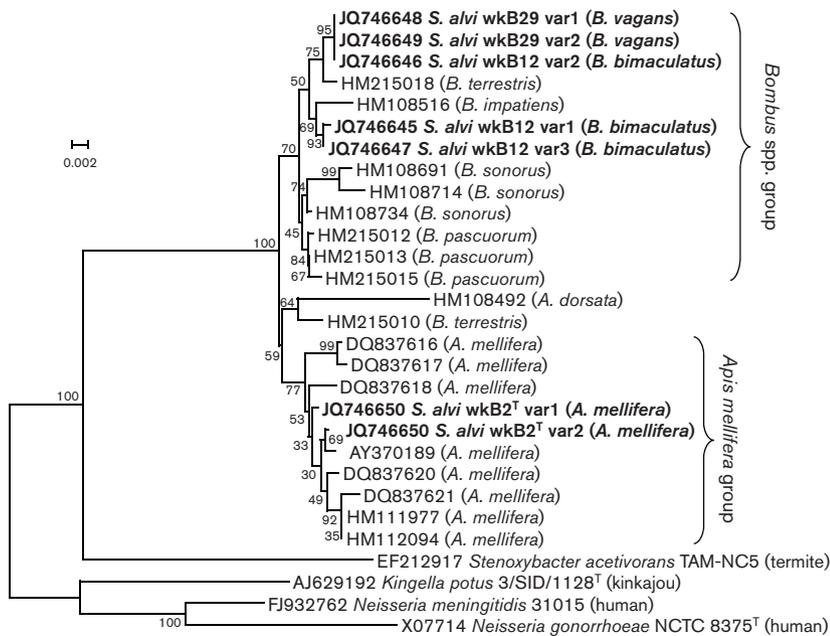
Characterization of *Snodgrassella alvi* gen. nov., sp. nov.

Colonies matching the 'Betaproteobacteria' phylotype (Martinson *et al.*, 2011) were readily recovered from bee guts and could be repeatedly passaged in culture using the above conditions. Three strains were examined in detail: wkB2^T, wkB12 and wkB29. Strain wkB2^T, from *A. mellifera*, was isolated on blood agar and formed smooth, white, round colonies, approximately 1 mm in diameter after 2 days. Strain wkB12, from *B. bimaculatus*, was isolated on HIA and passaged on blood agar, where it formed small colonies, <0.5 mm in diameter, which were white, but less opaque than those of wkB2^T. Strain wkB29, from *B. vagans*, exhibited similar morphology to wkB12.

Sequencing of the 16S rRNA gene showed wkB2^T, wkB12 and wkB29 to branch within the family *Neisseriaceae* of the class *Betaproteobacteria* (Fig. 1). All three strains contained multiple variants (alleles) of the 16S rRNA gene. Strain wkB2^T contained two variants, labelled var1 and var2 in Fig. 2, which shared 99.7 % identity. Strain wkB12 (var1) shared 99.1 and 99.4 % 16S rRNA gene sequence identity with wkB2^T var1 and wkB29 var1, respectively. It was previously reported that bee 'Betaproteobacteria' sequences from the same host species tend to cluster together, suggesting the possibility of codivergence or coevolution with their *Bombus* or *Apis* host lineages (Martinson *et al.*, 2011; Koch & Schmid-Hempel, 2011a). This holds for our isolates: wkB2^T and wkB12 grouped with other sequences from *Apis* and *Bombus*, respectively (Fig. 2). Their closest relative was the type strain of *Stenoxybacter acetivorans* (93.8 % identity to wkB2^T var1, GenBank accession no. EF212897), a species found in guts of the termite *Reticulitermes flavipes* (Wertz & Breznak, 2007a). A maximum-likelihood tree generated from the amino acid sequence of the acetate kinase gene, *ack*, supported this grouping of *Stenoxybacter* with wkB2^T and wkB12 to the exclusion of other members of the *Neisseriaceae* (Fig. S1).
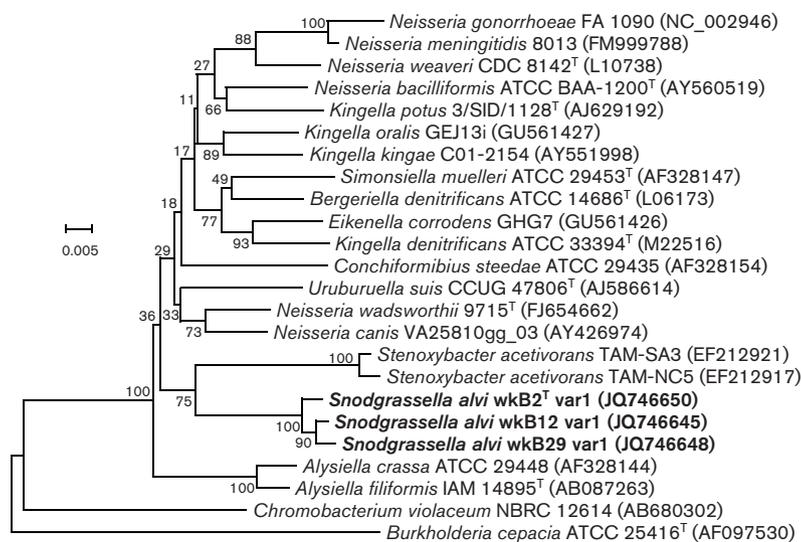
Fig. 1. 16S rRNA gene phylogeny of *Snodgrassella alvi* strains wkB2^T, wkB12 and wkB29 and other members of the *Neisseriaceae*. *Burkholderia cepacia* ATCC 25416^T is the outgroup. Alignment (length 1355 bp) was done with CLUSTAL W. The neighbour-joining algorithm was used with the maximum composite likelihood model and bootstrapped 1000 times; bootstrap values are shown as percentages. Bar, 0.5 % divergence.

Other close relatives included *Alysiella filiformis* ATCC 15532[T] (95.0 %; GenBank accession no. AF487710), *Kingella kingae* ATCC 23330[T] (93.7 %; AY551999) and *Neisseria gonorrhoeae* NCTC 8375[T] (93.0 %; X07714).

Genome characteristics were discerned from whole-genome sequencing. The genome of wkB2[T] was approximately 2.53 Mb in size and had a G+C content of 41.3 mol%, while wkB12 had a 2.35 Mb genome with a G+C content of 42.7 mol%.

Strain wkB2[T] showed good growth on blood agar, TSA, HIA, lysogeny broth agar (LBA) and brain heart infusion agar, while wkB12 grew on blood agar and HIA and grew weakly on LBA and TSA. At 42 °C, growth of strain wkB2[T] was retarded, while wkB12 did not grow. Strain wkB2[T] grew in TSB with incubation at 37 °C. Cells in liquid medium resisted suspension, even when grown in a shaker, and instead adhered to the bottom of the vessel. Growth was observed for strain wkB2[T] in TSB buffered to initial pH 6.0–6.5. Cells of strain wkB2[T] were visualized by transmission electron microscopy (Fig. 3a) and were found to be rod-shaped, approx. 1.0 μm long and 0.4 μm wide. No flagella were observed.

Strains required a microaerophilic atmosphere for optimal growth. No growth was observed in a normal atmosphere (0.04 % CO_2, 20.95 % O_2) on either blood agar or TSA after 4 days of incubation at 37 °C. Growth under anaerobic conditions was tested by incubating plates in AnaeroGen sachet pouches (Oxoid), which provided an 8–14 % CO_2, <1 % O_2 atmosphere. Compared with cultures in CO_2Gen pouches (6 % CO_2, 15 % O_2) or a 5 % CO_2 incubator, growth of strains under anaerobic conditions was greatly reduced. After 4 days of incubation at 37 °C, wkB2[T] showed no growth, while wkB12 developed only

sporadic colonies. Microaerophily is also characteristic of members of *Stenoxybacter*, which grow optimally between 1 and 2 % O_2 and growth of which is stimulated by the presence of 5 % CO_2 (Wertz & Breznak, 2007a). There is an oxygen gradient in the termite gut, from hypoxic levels near the gut wall to anaerobic conditions in the lumen,
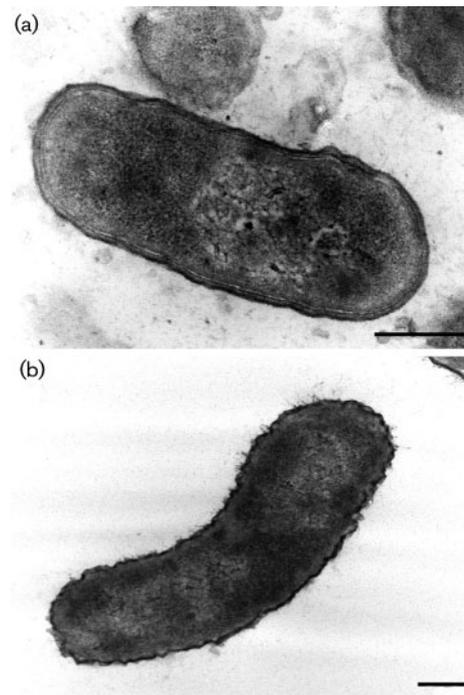
Fig. 3. Transmission electron micrographs of cells of *Snodgrassella alvi* wkB2[T] (a) and *Gilliamella apicola* wkB1[T] (b). Bars, 200 nm.

which may play a role in confining *Stenoxybacter* to the gut wall (Wertz & Breznak, 2007b). Although the O$_2$ and CO$_2$ conditions in the bee gut have not been studied in detail, there is evidence from microscopy that the honey bee 'Betaproteobacteria' phylotype is also found close to the gut wall (Martinson *et al.*, 2012), suggesting they too may occupy a specific hypoxic niche within the gut. Microaerophilic members of the gut flora, such as *Snodgrassella alvi*, may play a role in modulating the gut environment by consuming O$_2$, maintaining anaerobic conditions deeper in the gut lumen (Brune *et al.*, 1995; Wertz & Breznak, 2007b).

Results of biochemical characterization tests are summarized in Table 1, in comparison with other members of the *Neisseriaceae*. Strains wkB2^T and wkB12 were positive for nitrate reductase and catalase activity and negative for cytochrome *c* oxidase, *β*-glucosidase, gelatinase, *β*-galactosidase, glucose fermentation, indole production, H$_2$S production and motility. Strain wkB2^T was urease-positive and strain wkB12 had arginine dihydrolase activity.

Strains did not grow in the provided API AUX medium (bioMérieux) for the API 20NE carbon substrate assimilation tests. Instead, supplemented M9 agar containing 1× M9 salts, 10 mM MgSO$_4$, 1 mM CaCl$_2$, 0.2 % (w/v) casein digest and 10 mM single carbon source (L-arabinose, citric acid, D-fructose, D-galactose, D-glucose, L-glutamine, glycerol, glycine, DL-malic acid, maltose, D-mannitol, D-mannose or succinic acid) was used to test for carbon source utilization. Strain wkB2^T showed moderate growth on citric and malic acids, with weak or no growth on the others. Strain wkB12 did not grow on any of these media.

Strain wkB2^T was susceptible to all antibiotics tested except oxytetracycline and tylosin. Strain wkB12 was resistant to tylosin and completely susceptible to all other antibiotics. Additional antibiotic dilution assays showed that the MICs of oxytetracycline and tylosin for wkB2^T were >50 mg l^{-1} on TSA. Many *A. mellifera* apicultural practices include prophylactic treatment with oxytetracycline and tylosin to protect against the pathogen *Paenibacillus larvae* (Alippi *et al.*, 2005); hence, it may not be surprising that members of the normal gut flora either have developed resistance or are intrinsically resistant to these antibiotics.

The main fatty acids of our strains were palmitic acid (C$_{16:0}$) and *cis*-vaccenic acid (C$_{18:1}\omega7c$/C$_{18:1}\omega6c$), followed by lauric acid (C$_{12:0}$). However, the major fatty acid of wkB2^T was *cis*-vaccenic acid (45.7 %), whereas the main constituent in wkB12 was palmitic acid (47.5 %). Related genera for which fatty acid data were available (Table 2) showed different FAME profiles from our strains. The major isoprenoid quinone of wkB2^T was ubiquinone-8, which is consistent with its classification among the *Neisseriaceae*.

The genetic and phenotypic data presented here demonstrate that strains wkB2^T, wkB12 and wkB29 are divergent enough from other members of the *Betaproteobacteria* to justify their classification as members of a novel genus and species. *Snodgrassella alvi* forms a distinct clade within the *Neisseriaceae*, a group containing many animal commensals

Table 1. Differential characteristics of *Snodgrassella alvi* strains wkB2^T and wkB12 and related strains

Strains: 1, *Snodgrassella alvi* wkB2^T; 2, *Snodgrassella alvi* wkB12; 3, *Stenoxybacter acetivorans* TAM-DN1^T (data from Wertz & Breznak, 2007a); 4, *Alysiella filiformis* IAM 14895^T (Steed, 1962; Kuhn *et al.*, 1977); 5, *Kingella potus* 3/SID/1128^T (Lawson *et al.*, 2005). ND, No data available.

Characteristic	1	2	3	4	5
DNA G+C content (mol%)	41.3	42.7	53.7	45.4	58.4
Growth at 42 °C	+	−	−	ND	ND
Nitrate reductase	+	+	ND	−	−
Catalase	+	+	+	+	−
Cytochrome *c* oxidase	−	−	+	ND	+
Urease	+	−	ND	−	−
β-Glucosidase (aesculinase)	−	−	ND	−	−
Indole production	−	−	ND	−	−
Gelatinase	−	−	ND	+	ND
β-Galactosidase (PNPG)	−	−	ND	ND	−
Arginine dihydrolase	−	+	ND	ND	−
Glucose fermentation	−	−	ND	+	−
H$_2$S production	−	−	ND	+	ND
Haemolysis	−	−	ND	+	−

Table 2. Fatty acid compositions of *Snodgrassella alvi* strains and related strains of the *Neisseriaceae*

Strains: 1, *Snodgrassella alvi* wkB2^T; 2, *Snodgrassella alvi* wkB12; 3, *Alysiella filiformis* IAM 4895^T (data from Xie & Yokota, 2005); 4, *Kingella potus* 3/SID/1128^T (Lawson *et al.*, 2005). Fatty acids comprising <1 % of the total for all listed strains have been omitted.

Fatty acid	1	2	3	4
C$_{10:0}$ 3-OH	0	0.11	0	3.0
C$_{11:0}$	0	0	0	2.0
C$_{12:0}$	6.97	10.34	6.8	5.2
C$_{12:0}$ 3-OH	4.67	5.38	4.0	3.6
C$_{14:0}$	5.74	9.63	9.5	2.7
C$_{15:0}$	0	0	0	2.1
C$_{16:0}$	27.59	47.47	19.4	34.8
C$_{16:0}$ 3-OH	0	0.25	0	1.5
C$_{18:0}$	0.72	1.81	3.9	1.0
C$_{16:1}\omega5c$	0.48	0.12	1.2	0
C$_{16:1}\omega7c$/C$_{16:1}\omega6c$	3.85	6.45	24.5	14.4*
C$_{18:1}\omega9c$	0	0.61	4.1	2.4
C$_{18:2}\omega6,9c$/anteiso-C$_{18:0}$	0.22	0.35	3.8	3.3
C$_{18:1}\omega7c$/C$_{18:1}\omega6c$	45.66	12.87	18.3	18.5
Other†	3.26	4.36	2.5	3.0

*This value could also be due to iso-C$_{15:0}$ 2-OH.
†Includes C$_{12:0}$ aldehyde?/unknown 10.9525, unknown ECL 15.487, iso-C$_{16:1}$ I and C$_{14:0}$ 3-OH.

and pathogens that live in the oral cavity (*Simonsiella*, *Alysiella*, *Kingella*) and other mucosal surfaces (*Neisseria*) (Hedlund & Staley, 2002). Cells of the novel strains are short rods, unlike the elongated rods of *Stenoxybacter* (Wertz & Breznak, 2007a), the diplococcoid cells of *Neisseria* (Brenner *et al.*, 2005) or the ribbon-like filaments of *Alysiella* and *Simonsiella* (Xie & Yokota, 2005). In contrast to the closely related genera *Stenoxybacter*, *Neisseria*, *Alysiella*, *Simonsiella*, *Kingella*, *Eikenella* and *Uruburuella*, members of *Snodgrassella* are oxidase-negative, and, unlike *Kingella* and *Eikenella*, *Snodgrassella* strains are also catalase-positive (Wertz & Breznak, 2007a; Brenner *et al.*, 2005; Vela *et al.*, 2005). Another defining characteristic of *Snodgrassella* is the lack of growth in fully aerobic or anaerobic culture; members of most genera of the *Neisseriaceae*, including *Neisseria*, *Alysiella*, *Simonsiella*, *Kingella*, *Eikenella* and *Uruburuella*, are aerobes or facultative anaerobes (Brenner *et al.*, 2005; Vela *et al.*, 2005). Based on phylogenetic evidence, *Snodgrassella alvi* is most closely related to *Stenoxybacter acetivorans*, and these species may comprise an as-yet unrecognized group of gut-specific betaproteobacteria.

Characterization of *Gilliamella apicola* gen. nov., sp. nov.

Two of the characteristic members of the bee microbiota, previously called 'Gamma-1' and 'Gamma-2', have been found to cluster phylogenetically together with other insect-associated bacteria identified from 16S rRNA gene sequencing (Martinson *et al.*, 2011; Koch & Schmid-Hempel, 2011a; Chandler *et al.*, 2011). 'Gamma-1' has recently been proposed as '*Candidatus* Gilliamella apicola' (Martinson *et al.*, 2012). Members of this candidate species have been cultured previously: by Olofsson & Vásquez (2008), where it was called '*Pasteurellaceae*', by Yoshiyama & Kimura (2009), where it was called '*Enterobacteriaceae*', and by Koch & Schmid-Hempel (2011b), where it was cultured from bumble bees and suggested to confer a protective function against an intestinal parasite, *Crithidia bombi*.

The phylogenetic position of the 'Gamma-1'/'Gamma-2' clade within the *Gammaproteobacteria* is uncertain. This is partly due to the paucity of fully characterized members of this group. The only described strains are *Orbus hercynius* CN3^T, a transient faecal isolate from wild boar (Volkmann *et al.*, 2010), and *Orbus sasakiae* C7^T, found recently in the gut of a butterfly (Kim *et al.*, 2013). Volkmann *et al.* (2010) determined that its closest relatives were in the families *Enterobacteriaceae* and *Pasteurellaceae*. However, *O. hercynius* CN3^T exhibited a phenotype that was inconsistent with membership of either family, and it was suggested that this strain may be sufficiently divergent to warrant its placement in a new family and order.

Strain characterization. 'Gamma-1' (*Gilliamella apicola*) isolates were readily recovered from bee guts and could be repeatedly passaged in culture. Three representative strains

were studied in detail: wkB1^T, wkB11 and wkB30. Strain wkB1^T, from *A. mellifera*, was isolated on blood agar and formed smooth, white, round colonies, approximately 2.5 mm in diameter after 2 days of growth at 37 °C and 5 % CO$_2$. Strain wkB11, from *B. bimaculatus*, was isolated on HIA and passaged on blood agar, where it formed smooth, semi-translucent white colonies, <1 mm in diameter. Strain wkB30, from *B. vagans*, was isolated on blood agar, and formed smooth, white colonies, <1 mm in diameter.

The genome of wkB1^T was approximately 3.14 Mb in size and had a G + C content of 33.6 mol%, while wkB11 had a 2.19 Mb genome with a G + C content of 34.1 mol% and wkB30 had a 2.29 Mb genome and a G + C content of 34.5 mol%. The G + C content of these genomes is close to the value of 36.4 mol% reported for *O. hercynius* CN3^T and is, as noted by Volkmann *et al.* (2010), more A + T-rich than is typical for species of the *Enterobacteriaceae* or *Pasteurellaceae*.

Strains wkB1^T, wkB11 and wkB30 showed good growth on blood agar, TSA, HIA and LBA, but grew poorly on brain heart infusion agar. Strains were not haemolytic on blood agar and did not grow at 42 °C. Strain wkB1^T grew well in TSB with a pH range of about 6.0–6.5, but exhibited clumping behaviour and formation of dense cell aggregates. These aggregates resisted suspension and tended to settle quickly if left without agitation. Transmission electron microscopy showed that cells of wkB1^T were rod-shaped and approx. 1.5 μm long and 0.5 μm wide. Dividing cells frequently displayed a pliable, curving morphology (Fig. 3b), and filamentous forms growing to lengths >10 μm were also present. Short, hair-like surface projections seen in micrographs of cells grown in liquid culture may be fimbriae used for cell-to-cell attachment; this could explain the observed clumping behaviour.

Strains wkB1^T, wkB11 and wkB30 are facultative anaerobes, but did not grow optimally under fully aerobic conditions. No growth was observed in a normal atmosphere after 4 days of incubation at 37 °C. Strains grew well in CO$_2$Gen pouches and AnaeroGen pouches and in a 5 % CO$_2$ incubator.

Results of biochemical characterization tests are summarized in Table 3, in comparison with *O. hercynius* CN3^T. Strain wkB1^T was positive for β-glucosidase and β-galactosidase activities. Neither strain wkB1^T nor wkB11 grew in SIM medium. In contrast to *O. hercynius* CN3^T, glucose fermentation, cytochrome *c* oxidase, nitrate reductase and urease activity were not detected. As was observed for *O. hercynius* CN3^T (Volkmann *et al.*, 2010), our strains showed no growth in the API 20NE carbon substrate assimilation tests. However, this may be due to an inability to grow in the provided API AUX medium (bioMérieux), rather than true negative phenotypes. Attempts to grow strain wkB1^T in M9-based minimal media also failed.

Table 3. Differential characteristics of *Gilliamella apicola* strains and *O. hercynius* CN3[T]

Strains: 1, *Gilliamella apicola* wkB1[T]; 2, *Gilliamella apicola* wkB11; 3, *O. hercynius* CN3[T] (data from Volkmann *et al.*, 2010). All three strains were negative for indole production, gelatinase and arginine dihydrolase.

Characteristic	1	2	3
DNA G+C content (mol%)	33.6	34.1	36.4
Nitrate reductase	−	−	+
Catalase	−	+	+
Cytochrome *c* oxidase	−	−	+
Urease	−	−	+
β-Glucosidase (aesculinase)	+	−	+
β-Galactosidase (PNPG)	+	−	−
Glucose fermentation	−	−	+

Strain wkB1[T] was susceptible to all antibiotics tested except oxytetracycline and tylosin. Antibiotic dilution assays showed the MICs of oxytetracycline and tylosin in wkB1[T] to be 12 and 30 mg l^{-1}, respectively.

The main fatty acids of strains wkB1[T] and wkB11 were palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}\omega7c$/$C_{18:1}\omega6c$), with each comprising >30% of the total composition (Table 4). This FAME profile was similar to that reported for *O. hercynius* CN3[T] (Volkmann *et al.*, 2010), further supporting the grouping of *Orbus* and *Gilliamella* into a novel clade. Like *O. hercynius* CN3[T], the major isoprenoid quinone of wkB1[T] was ubiquinone-8 and the major polar lipids were phosphatidylethanolamine and phosphatidylglycerol (Kim *et al.*, 2013). A phosphoaminoglycolipid and an unidentified phospholipid (PL1) were also present (Fig. S2).

Table 4. Fatty acid compositions of *Gilliamella apicola* strains and *O. hercynius* CN3[T]

Strains: 1, *Gilliamella apicola* wkB1[T]; 2, *Gilliamella apicola* wkB11; 3, *O. hercynius* CN3[T] (data from Volkmann *et al.*, 2010). Fatty acids comprising <1% of the total for all listed strains have been omitted.

Fatty acid	1	2	3
$C_{14:0}$	7.52	9.07	6.88
$C_{16:0}$	31.69	37.97	33.73
$C_{18:0}$	1.31	6.10	0.35
$C_{16:1}\omega7c$/$C_{16:1}\omega6c$	9.41	3.19	10.70*
$C_{18:1}\omega9c$	0	1.67	0
$C_{18:1}\omega7c$/$C_{18:1}\omega6c$	41.32	35.80	38.45
Other†	8.75	4.37	9.37

*This value could also be from iso-$C_{15:0}$ 2-OH.
†Includes $C_{12:0}$ aldehyde?/unknown 10.9525, iso-$C_{16:1}$ I and $C_{14:0}$ 3-OH.

Phylogenetic position. Phylogenetic analysis of 16S rRNA gene sequences showed strains wkB1[T], wkB11 and wkB30 to cluster with previously reported members of the bee gut symbiont 'Gamma-1' (Fig. 4). We propose the name *Gilliamella apicola* gen. nov., sp. nov. for members of this clade. These strains were also closely related to 'Gamma-2', *O. hercynius* CN3[T] and other uncultured bacteria from insect guts. Strain wkB1[T] had 93.9% (1441/1534 bp) 16S rRNA gene sequence identity to *O. hercynius* CN3[T]. The 16S rRNA gene sequences of the clade containing *Gilliamella*, *Orbus* and the uncultured insect-associated bacteria clustered separately from both families *Enterobacteriaceae* and *Pasteurellaceae*, with strong bootstrap support. Although Fig. 4 suggests a closer relation of this group with the *Pasteurellaceae*, the determination of phylogenies based solely on 16S rRNA gene sequence data can be unreliable (Ludwig *et al.*, 1998).

To better ascertain the phylogenetic position of strains wkB1[T], wkB11 and wkB30 in relation to the *Enterobacteriaceae* and *Pasteurellaceae*, preliminary data from our multigenome sequencing project were used. To reconstruct the concatenated-protein tree shown in Fig. 5, 10 proteins were chosen based on the criteria that they: (i) were present in all three *Gilliamella apicola* genomes, (ii) represented a diversity of cell functions, (iii) were present in the genome in a single copy and (iv) have been used previously in building a phylogeny of the *Gammaproteobacteria* (Williams *et al.*, 2010). Sequences of these 10 proteins were retrieved from 17 other genomes representing diverse members of the families *Enterobacteriaceae* and *Pasteurellaceae* and from *O. hercynius* CN3[T] (ftp://ftp.sanger.ac.uk/pub4/pathogens/Orbus/hercynius/CN3/). The protein sequences were concatenated and aligned and a tree was reconstructed using the maximum-likelihood algorithm. Trees were also reconstructed using each of the ten proteins individually (not shown).

Fig. 5 shows support for the placement of the *Gilliamella apicola*/*O. hercynius* clade at the base of the *Enterobacteriaceae*, and not with the *Pasteurellaceae*. Although incongruent with the 16S rRNA gene tree (Fig. 4), this topology was also observed for seven of the ten single-protein trees, and only one, *uvrA*, showed strong support (74% bootstrap) for placement of *Gilliamella apicola* at the base of the *Pasteurellaceae* (not shown). An identical branching pattern was observed whether using members of *Pseudomonas* (GenBank accession nos NC_002516 and NC_004129) or *Vibrio* (NC_002505 and CP000020) as outgroups. Given the large divergence of *Gilliamella apicola* and its basal position to the *Enterobacteriaceae*, and considering its close phylogenetic relationship and phenotypic similarities to *O. hercynius* CN3[T], we propose that the group encompassing *Gilliamella* and *Orbus* be placed in a new family and order, *Orbaceae* fam. nov. and *Orbales* ord. nov.

Current evidence supports *Gilliamella* and *Snodgrassella* as monophyletic groups wherein all members inhabit the
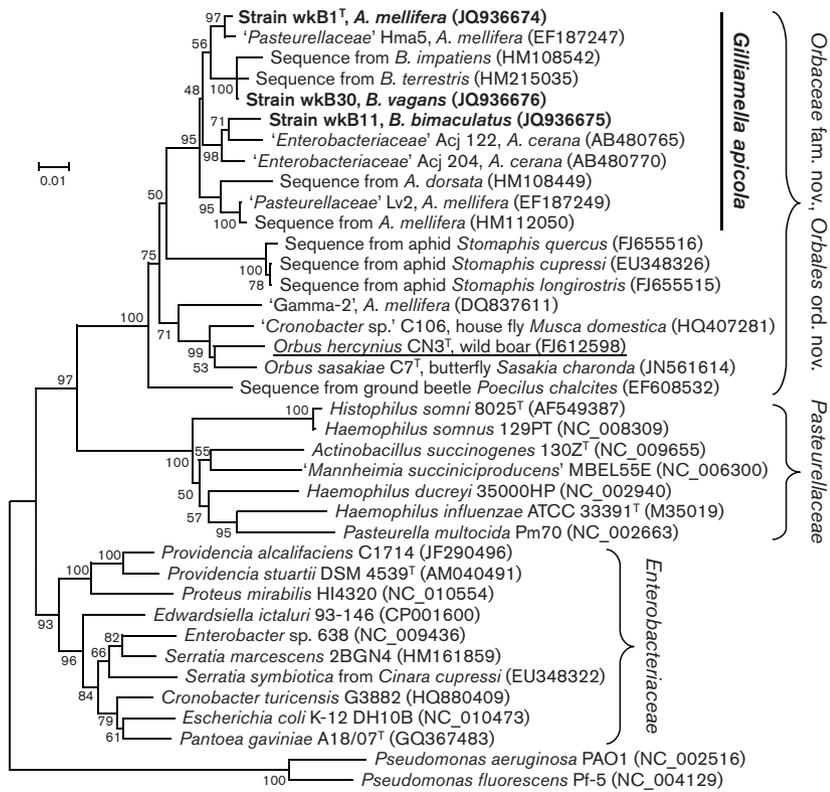
Fig. 4. 16S rRNA gene phylogeny of *Gilliamella apicola* strains wkB1[T], wkB11 and wkB30. Two members of the genus *Pseudomonas* were used as the outgroup. Alignment (length 1206 bp) was made using MUSCLE. The neighbour-joining algorithm was used with the maximum composite likelihood model and bootstrapped 1000 times; bootstrap values are shown as percentages. *O. hercynius* CN3[T] (underlined) was the first described strain in the proposed order *Orbales* ord. nov. Bar, 1 % divergence.

same ecological niche – the bee gut. Although we have proposed two novel species designations for our isolates, it should be recognized that substantial intraspecific variation exists, as shown by the strains' differing biochemical traits, genomic properties and phylogenetic divergence. Previous studies based on 16S rRNA gene sequence data have also

found considerable diversity within these groups (Martinson *et al.*, 2011; Koch & Schmid-Hempel, 2011a; Moran *et al.*, 2012). It is possible that distinct lineages exist within what we have defined as a 'species'; this is strongly suggested by the apparent host-specificity seen in *Snodgrassella* (Fig. 2) and the functional diversification in
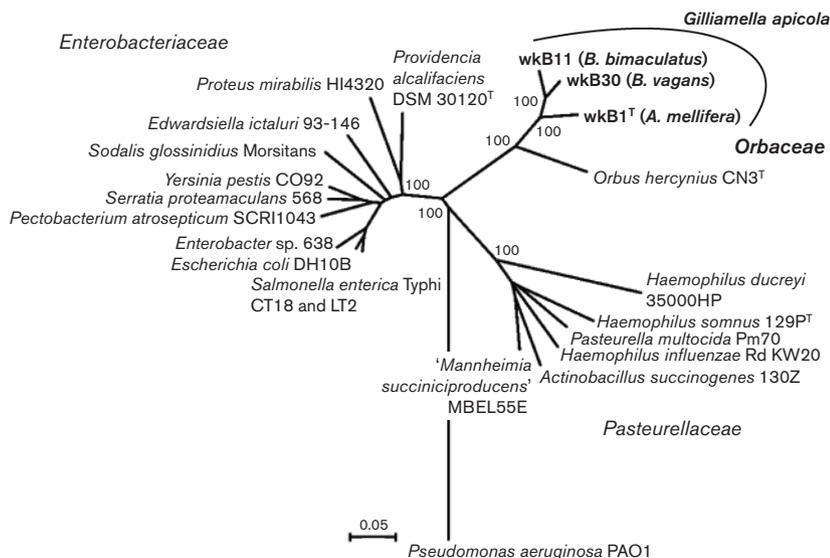
Fig. 5. Phylogeny of the *Enterobacteriaceae*, *Pasteurellaceae* and *Orbaceae* fam. nov. reconstructed using 10 single-copy proteins. The gene sequences (*dnaE*, *ftsH*, *glyS*, *mutS*, *parC*, *recA*, *rpoB*, *secA*, *serS* and *uvrA*) were retrieved from sequenced genomes using TBLASTN against the annotated copy of the gene in the genome sequence of *E. coli* K-12. Deduced amino acid sequences were con-catenated and then aligned using MUSCLE, resulting in a 7891 amino acid alignment. The tree was reconstructed using maximum-likelihood with the Jones–Taylor–Thornton amino acid substitution model. Bootstrap values (*n*=500, shown as percentages) are displayed for selected nodes. *Pseudomonas aeruginosa* PAO1 is the outgroup. The bee species from which *Gilliamella apicola* strains were isolated are given in parentheses. Bar, 0.05 substitutions per site.

Gilliamella reported by Engel *et al.* (2012). Future studies should take a genomic or, at least, a multigene approach in order to best characterize and assign taxonomy within these clades.

Description of *Snodgrassella* gen. nov.

Snodgrassella (Snod.gras′sel.la. N.L. dim. fem. n. *Snodgrassella* named after Robert Evans Snodgrass, a pioneer in the study of insect physiology in the early 20th century).

Comprises a clade of symbiotic bee-gut betaproteobacteria within the family *Neisseriaceae*. Microaerophilic, positive for catalase and nitrate reductase and negative for cytochrome *c* oxidase. Cells are short rods. The DNA G + C content is 41–43 mol%. The main isoprenoid quinone is ubiquinone-8. The type species of the genus is *Snodgrassella alvi*.

Description of *Snodgrassella alvi* sp. nov.

Snodgrassella alvi (al′vi. L. gen. n. *alvi* of the bowels, referring to the location of the species in the gut of bees).

In addition to the characteristics of the genus, the following properties apply. A 5 % CO_2 atmosphere at 37 °C provides optimal conditions for growth. Strains can grow on blood agar, TSA, HIA and LBA, and form smooth, white, round colonies, approximately 1 mm in diameter or smaller after 2 days. Can utilize citric and malic acids as main carbon sources. Very weak or no growth in air or under anaerobic conditions. In TSB, the growth range is approximately pH 6.0–6.5. Cells are non-motile, approximately 1.0×0.4 μm. Strains are negative for β-glucosidase, indole production, gelatinase, β-galactosidase, glucose fermentation and haemolysis and show variable reactions for the presence of urease and arginine dihydrolase. The main constituent fatty acids are palmitic acid ($C_{16:0}$), *cis*-vaccenic acid ($C_{18:1}\omega7c$/$C_{18:1}\omega6c$) and lauric acid ($C_{12:0}$).

The type strain is wkB2[T] (=NCIMB 14803[T] =ATCC BAA-2449[T] =NRRL B-59751[T]), isolated from the gut of the Western honey bee, *A. mellifera*, in Connecticut, USA. The DNA G + C content of the type strain is 41.3 mol%.

Description of *Orbales* ord. nov.

Orbales (Or.ba′les. N.L. masc. n. *Orbus* type genus of the order; L. pl. suff. -*ales* ending to denote an order; N.L. fem. pl. n. *Orbales* the order of the genus *Orbus*).

The order *Orbales* is mainly defined by 16S rRNA gene sequence similarity to *O. hercynius* CN3[T] and the *Gilliamella apicola* strains described here. Members of this order are characterized by low genomic DNA G + C content (about 32–37 mol%), a fatty acid profile dominated by palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and an association with animal (mainly insect) intestinal tracts. The predominant isoprenoid quinone is ubiquinone-8 and the major polar lipids are phosphatidylethanolamine and phosphatidylglycerol. The type genus is the genus *Orbus* Volkmann *et al.* 2010.

Description of *Orbaceae* fam. nov.

Orbaceae (Or.ba.ce′a.e. N.L. masc. n. *Orbus* type genus of the order; L. pl. suff. -*aceae* ending to denote a family; N.L. fem. pl. n. *Orbaceae* the family of the genus *Orbus*).

Description is the same as for the order *Orbales*. The type genus is the genus *Orbus* Volkmann *et al.* 2010.

Description of *Gilliamella* gen. nov.

Gilliamella (Gil.li.am′el.la. N.L. dim. fem. n. *Gilliamella* named after Martha A. Gilliam, for her contributions to the early study of honey bee microbes).

Comprises a clade of symbiotic bee-gut gammaproteobacteria in the family *Orbaceae*. Metabolism is microaerophilic and facultatively anaerobic. Cells are rod-shaped and may be filamentous. Show negative reactions for nitrate reductase, cytochrome *c* oxidase, urease and glucose fermentation and variable reactions for catalase, β-glucosidase and β-galactosidase. The type species of the genus is *Gilliamella apicola*.

Description of *Gilliamella apicola* sp. nov.

Gilliamella apicola [a.pi′co.la. L. fem. n. *apis* bee; L. suff. -*cola* from L. n. *incola* inhabitant, dweller; N.L. n. (nominative in apposition) *apicola* bee-dweller].

In addition to the characteristics of the genus *Gilliamella*, family *Orbaceae* and order *Orbales*, the following properties also apply. Strains can grow on blood agar, TSA, HIA and LBA. They form smooth, white, round colonies, approximately 2.5 mm in diameter or smaller after 2 days of incubation at 37 °C and 5 % CO_2. Strains do not grow at 42 °C. In TSB, the growth range is pH 6.0–6.5. Cells are approximately 1.5×0.5 μm, but can also form filaments over 10 μm long. Negative for indole production, gelatinase, arginine dihydrolase and haemolysis. Phosphatidylethanolamine, phosphatidylglycerol and phosphoaminoglycolipid are the major polar lipids. The main constituent fatty acids are palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$).

The type strain is wkB1[T] (=NCIMB 14804[T] =ATCC BAA-2448[T]), isolated from the gut of the Western honey bee, *A. mellifera*, in Connecticut, USA. The DNA G + C content of wkB1[T] is 33.6 mol%.

Acknowledgements

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