Apibacter mensalis sp. nov.: a rare member of the bumblebee gut microbiota

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Isolates LMG 28357T (＝R-53146T) and LMG 28623 were obtained from gut samples of Bombus lapidarius bumblebees caught in Ghent, Belgium. They had identical 16S rRNA gene sequences which were 95.7 % identical to that of Apibacter adventoris wkB301T, a member of the family Flavobacteriaceae. Both isolates had highly similar matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and randomly amplified polymorphic DNA (RAPD) profiles. A draft genome sequence was obtained for strain LMG 28357T (Gold ID Gp0108260); its DNA G + C content was 30.4 %, which is within the range reported for members of the family Flavobacteriaceae (27 to 56 mol%) and which is similar to that of the type strain of A. adventoris (29.0 mol%). Whole-cell fatty acid methyl ester analysis of strain LMG 28357T revealed many branched-chain fatty acids, a typical characteristic of bacteria of the family Flavobacteriaceae and a profile that was similar to that reported for A. adventoris wkB301T. MK6 was the major respiratory quinone, again conforming to bacteria of the family Flavobacteriaceae. The isolates LMG 28357T and LMG 28623 could be distinguished from A. adventoris strains through their oxidase activity. On the basis of phylogenetic, genotypic and phenotypic data, we propose to classify both isolates as representatives of a novel species of the genus Apibacter, Apibacter mensalis sp. nov., with LMG 28357T (=DSM 100903T＝R-53146T) as the type strain.

Insect pollination is of major importance for global food production with a total economic value of €153 billion in 2005 (Gallai et al., 2009). In Europe, 84 % of the cultivated crops depend directly on insect pollination (Gallai et al., 2009) to which especially bumblebees, honeybees and solitary bees contribute. Bumblebees have experienced distribution declines in various parts of the world, with many species living in restricted areas and some critically endangered on a larger scale (http://www.iucnredlist.org). These declines, presumably caused by a combination of factors such as pesticide use, pathogen emergence and changes in climate and agricultural practices (Goulson et al., 2008; Meeus et al., 2011) may have a detrimental impact on agriculture and ecosystem integrity (Klein et al., 2007).

To reverse or avoid further declines, governments have stimulated farmers to improve the agricultural landscape and environment for bees (http://www.ec.europa.eu). In addition, the importance of the bumblebee gut microbiota for bee health has been established (Cariveau et al., 2014; Koch & Schmid-Hempel, 2011b, 2012). The cultivable microbiota of these insects is gradually being characterized (Killer et al., 2009, 2010, 2011; Kwong & Moran, 2013; Praet et al., 2015) and is a first step towards understanding of their functionality with regard to their host, environment and/or associated microbiota.

The gut microbiota of bumblebees resembles that of honeybees and often comprises different species of the same genus (Koch & Schmid-Hempel, 2011b). It consists of a core set of genera belonging to the families Neisseriaceae (Snodgrassella), Orbaceae (Gilliamella), Lactobacillaceae (Lactobacillus) and Bifidobacteriaceae (Bifidobacterium) and several non-core bacteria. Koch & Schmid-Hempel (2011a) reported the presence of a bumblebee gut phylotype belonging to the phylum Bacteroidetes which has only sporadically been detected in bumblebees and therefore its prevalence is presumably low (Koch & Schmid-Hempel, 2011b).
In August 2013, Bombus lapidarius bumblebees were caught in the field in the region of Ghent, Belgium, and identified by their colour pattern. The bumblebees were immobilized at −20 °C for 10 min and surface sterilized with 2.5% Umonium (Laboratoire Huckert’s International) before dissecting out their crop and gut. The crops and guts were homogenized in 250 μl saline solution (0.1% peptone, 0.1% Tween 80, 0.85% NaCl, 5% DMSO) with a sterile micro-pistle. Subsequently, the cell suspensions were serially diluted to 10⁻⁴ in physiological saline (0.85% NaCl), plated on all culture (AC) agar (Sigma-Aldrich) and incubated microaerobically at 37°C. After 5 days, colonies were picked up from the agar plates and third-generation axenic isolates were dereplicated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS followed by curve-based data analysis (Ghyselinck et al., 2011) using BioNumerics 5.1 software (Applied Maths). Representative isolates of each MALDI-TOF MS cluster were selected for further identification. The MALDI-TOF MS profiles of two isolates (LMG 28357T and LMG 28623) from two different B. lapidarius bumblebees sampled in Bourgoyen in Ghent, Belgium, were highly similar (Fig. S1, available in the online Supplementary Material) and clustered separately from those of all other isolates in our database (data not shown).

Both isolates were routinely grown microaerobically on AC agar at 37°C. Random amplified polymorphic DNA (RAPD) analysis was performed on both isolates with primer RAPD-272 as described by Williams et al. (1990). The RAPD profiles obtained were highly similar and differed only in the intensity of some bands (Fig. S2).

To identify both isolates their 16S rRNA gene sequences were determined as previously described (De Bruyne et al., 2007) and were identical. MEGAS analysis designated A. adventoris as the most closely related neighbour taxon with a validly published name, yet only 9.57% 16S rRNA gene sequence identity was calculated towards its type strain, wkB301T (Kwong & Moran, 2016). The 16S rRNA gene sequence of isolates LMG 28357T and LMG 28623 was also 98.4% similar to that of a second A. adventoris strain, wkB309, highlighting a considerable genetic divergence within the latter species. Based on the threshold of 98.65% 16S rRNA gene sequence identity for species differentiation, the isolates LMG 28357T and LMG 28623 represent a novel species of the genus Apibacter (Kim et al., 2014). A 16S rRNA gene sequence based phylogenetic tree comprising the isolates LMG 28357T and LMG 28623 and their closest neighbours based on an EzTaxon-e analysis is shown in Fig. 1.

Whole genomic DNA of isolate LMG 28357T was obtained as described by Pitcher et al. (1989). A draft genome sequence (Gold ID Gp0108260) was generated at the DOE Joint Genome Institute (JGI) using Illumina technology. An Illumina std shotgun library was constructed and sequenced using the Illumina HiSeq 2000 platform. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. Raw Illumina sequence data were passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artefacts. The following steps were then performed for assembly: filtered Illumina reads were assembled using Velvet (version 1.2.07; Zerbino & Birney, 2008), 1–3 kb simulated paired-end reads were created from Velvet contigs using wgsim (version 0.3.0; https://github.com/lh3/wgsim) and Illumina reads were assembled with simulated read pairs using Allpaths–LG (version r46652; Gnerre et al., 2011). Genome annotation was performed by RAST (Aziz et al., 2008). The genome size of strain LMG 28357T was 2.3 Mb and consisted of 2095 coding sequences. Its DNA G+C content was determined by using GC calculator (http://www.genomicsplace.com/gc_calc.html) and was 30.4 mol%. This conforms to values reported for members of the family Flavobacteriaceae (27 to 56 mol%) and is similar to the value of the type strain of A. adventoris (29.0 mol%) (Bernardet, 2010).

The genome statistics of strain LMG 28357T are summarized in Table 1. Most annotated coding sequences belonged to the following RAST subsystems (Aziz et al., 2008): cofactors, vitamins, prosthetic groups, pigments (12%); protein metabolism (12%); amino acids and derivatives (17%); and carbohydrates (10%). Complete [riboflavin (=vitamin B2) and lipoic acid] or nearly complete [pyridoxal 5-phosphate (=vitamin B6), menaquinone 6 (=vitamin K2), vitamin B12, niacin (=vitamin B3) and folate] biosynthesis pathways of several vitamins are present. These vitamins may provide extra nutrients for bumblebees that feed on pollen, which is difficult to digest and often depleted in fat-soluble vitamins such as vitamins K, D and E (Schmidt, 1997). Protein and amino acid metabolism are also two abundant subsystems with only a minority of genes involved in protein degradation (11%) and amino acid degradation (13%). The biosynthesis pathways for phenylalanine and tryptophan, two essential amino acids for bumblebees which are sometimes lacking in pollen (Roulston & Cane, 2000), could be reconstructed completely. The assigned functions of the genes of the vitamin and amino acid production pathways were checked with InterProScan, and the position of the proteins in the cell was checked with PSORT and TMpred. Tblastn was used to search for missing genes by aligning the translated genome to homologous protein sequences of a close relative.
Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of isolates LMG 28357T and LMG 28623 and their closest neighbours. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; +G, parameter=0.7823). The rate variation model allowed for some sites to be evolutionarily invariable (+I, 55.1926 % sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 47 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 1334 positions in the final dataset.

Genomes of obligate or early symbionts are often very small and contain few coding sequences (McCutcheon & Moran, 2012). Genes involved in replication, transcription, translation and serving the host are relatively dominant in such genomes while other functions are in the process of being lost. Although the genome of strain LMG 28357T is relatively small, degeneracy was not observed.

To obtain a higher phylogenetic resolution, a phylogenetic tree based on 20 protein sequences of isolate LMG 28357T and its closest neighbours was reconstructed (Fig. 2). The 20 protein sequences (encoded by genes alaS, atpA, dnaA, dnaN, fisZ, fusA, groEL, gyrB, lepA, metK, nusG, pflA, pyrG, recA, rpLA, rpLB, rpsB, sdiA and secA) were obtained from the genome sequence of strain LMG 28357T and from its closest relatives to produce a tree based on 10 444 positions. The MEGAS software package was used to align the sequences by MUSCLE and to obtain phylogenetic trees by using the maximum-likelihood method and the general time-reversible model with invariant sites for the 16S rRNA gene based tree (Tamura et al., 2013) and the JTT matrix-based model for the protein-sequences-based tree. The robustness of the topology of the trees was estimated by bootstrap analysis with 100 replicates (Felsenstein, 1985).

Biochemical characteristics were determined for isolates LMG 28357T and LMG 28623 and compared with those reported for A. adventoris (Table 2). To test substrate utilization and enzyme activity, API 20NE and API ZYM galleries (bioMérieux) were inoculated with dense cell suspensions (McFarland 0.5). The API 20NE strips were read after 2 days of incubation at 37 °C. Gram-stain-reaction, and verification of oxidase, catalase and DNase activity and hydrolysis of Tween 60, Tween 80, starch, casein and gelatin were performed using standard microbiological procedures (MacFaddin, 1980). Lactose fermentation was tested on MacConkey agar. Growth was determined on AC agar at different temperatures (20, 28, 37 and 45 °C) and in AC broth at different pH values (pH 3, 5, 7 and 9) and NaCl concentrations (5, 6, 7 and 8%). Growth was also tested on AC agar and Columbia blood agar (Oxoid) at 37 °C in aerobic, microaerobic (80% N2, 15% CO2 and 5% O2) and anaerobic (80% N2, 10% H2, 10% CO2) atmospheres. Cell morphology and motility was checked with phase-contrast microscopy (BX40F 4; Olympus). Both isolates could be differentiated from the type strain of A. adventoris by means of their oxidase activity and ability to grow anaerobically and aerobically; they also differed from strain wkB309 by means of their oxidase and nitrate reduction activity, ability to grow anaerobically and aerobically, and lack of motility and β-galactosidase activity (Table 2).

Fatty acid methyl ester (FAME) analysis was performed for strain LMG 28357T using an Agilent Technologies 6890N gas chromatograph. Cultivation of the strain and extraction of the fatty acids were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI), except that fatty acids were extracted from a culture grown on AC agar at 37 °C under microaerobic conditions for 48h. The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI). FAME analysis revealed iso-C15:0 3-OH (24.25 %), iso-C16:0 3-OH (16.92 %) and C16:0 (15.15 %) were also the predominant fatty acids while several other fatty acids were present in lesser amounts. The fatty acids iso-C17:0 3-OH (18.0 %), iso-C15:0 (23.0 %) and C16:0 (15.1 %) were also the major fatty acids reported in the type strain of A. adventoris (Kwong & Moran, 2016).

The respiratory quinone composition was determined for strain LMG 28357T using the protocol described by da Costa et al. (2011). To improve the efficacy of the quinone extraction, the hexane and methanol phases were allowed to separate for 2 h at −80 °C. An XBridge BEH phenyl column (pore diameter 130 Å, particle size 5 μm, 4.6 mm width × 250 mm length) was used to separate the respiratory quinones via HPLC. The samples were

<table>
<thead>
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<th>Table 1. Genome statistics of A. mensalis sp. nov. LMG 28357T</th>
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<tr>
<td><strong>Genome statistic</strong></td>
</tr>
<tr>
<td>Number of scaffolds</td>
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<td>Size (Mb)</td>
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<tr>
<td>DNA G+C content (mol%)</td>
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<tr>
<td>Scaffold N/LS0</td>
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<tr>
<td>Read coverage</td>
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<tr>
<td>Number of CDS calls</td>
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<td>Number of tRNA calls</td>
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eluted with 100 % methanol at a rate of 1 min ml\(^{-1}\) and the quinones were detected at 269 nm. *Flavobacterium granuli* LMG 23365\(^T\), which contains MK6, was used as a control strain (Aslam et al., 2005). The major respiratory quinone of strain LMG 28357\(^T\) was MK6, which is a typical characteristic of bacteria of the family *Flavobacteriaceae* in which MK6 is either the only or major respiratory quinone (Bernardet & Nakagawa, 2006).

Based on the phylogenetic, genotypic and phenotypic data, we propose to classify isolates LMG 28357\(^T\) and LMG 28623 as representatives of a novel species of the genus *Apibacter*, *Apibacter mensalis* sp. nov., with LMG 28357\(^T\) (=DSM 100903\(^T\)) as the type strain. Members of this novel species and their close relatives have only recently been detected in bumblebees and honeybees and with a low prevalence. Also, in our study, only two isolates were obtained from the 67 bumblebees investigated (data not shown). The 16S rRNA gene sequences of both isolates revealed 99.4 and 99.3 % sequence identity to those of uncultivated clones (HM215036 and HM215037, respectively) obtained from *Bombus terrestris* and *Bombus hortorum* bumblebees (Koch & Schmid-Hempel, 2011b) suggesting that this species may not be restricted to *B. lapidarius*.

### Description of *Apibacter mensalis* sp. nov.

*Apibacter mensalis* (men.sa’lis. L. adj. mensalis of a table; because this gut bacterium and its host are using the same nutrients and thus share the same ‘dinner table’).

### Table 2. Differential phenotypic characteristics of strain LMG 28357\(^T\) and *A. adventoris* wkB301\(^T\) and wkB309

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LMG 28357(^T)</th>
<th>wkB301(^T)</th>
<th>wkB309</th>
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<tr>
<td>Enzymic activity</td>
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<tr>
<td>(\beta)-Galactosidase (PNPG)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth</td>
<td>Microaerobic, aerobic (weakly) and anaerobic</td>
<td>Microaerobic</td>
<td>Microaerobic</td>
</tr>
<tr>
<td>Genome size (Mb)</td>
<td>2.3</td>
<td>2.7</td>
<td>2.3</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>30.4</td>
<td>29.0</td>
<td>30.6</td>
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Cells are Gram-stain-negative, non-motile, rod-shaped, slightly curved and 1 μm wide and 3 to 5 μm long. Colonies are 1 mm, round, shiny and colourless after 2 days of growth on AC agar. Growth is observed after 2 days when incubated microaerobically and anaerobically at 37 °C on AC agar and Columbia blood agar. No growth is observed at 20, 28 or 45 °C. Weak growth is observed in the presence of 5 to 8% NaCl, when grown aerobically or on AC agar at pH 3 and 5. Grows at pH 7 but not at pH 9. No growth is observed on MacConkey agar or on the basal medium used to test Tween 60 and Tween 80 hydrolysis. Catalase and oxidase activities are present. Hydrolyses aesculin but not gelatin, starch or casein. Activity of alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and β-glucosidase is detected. Ferments glucose and reduces nitrate to nitrite. No production of lipase (C14), α- and β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, urease, arginine dihydrolase, gelatinase, tryptophanase, casease, amylase or DNase. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid. The type strain, LMG 28357T (DSM 100903T), is 30.4 mol%.

The type strain, LMG 28357T (DSM 100903T = R-53146T), was isolated from the gut of a B. lapidarius bumblebee sampled in 2013 in Gent, Belgium. Its DNA G + C content is 30.4 mol%.

Acknowledgements

The authors acknowledge support by Ghent University-Special Research Fund (BOF). The genome sequence data were produced by the Office of Science of the US Department of Energy under the Office of Science of the US Department of Energy, a DOE Office of Science User Facility, is supported by the Office of Science of the US Department of Energy under contract no. DE-AC02-05CH11231.

References


