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Gilliamella intestini sp. nov., *Gilliamella bombicola* sp. nov., *Gilliamella bombi* sp. nov. and *Gilliamella mensalis* sp. nov.: Four novel *Gilliamella* species isolated from the bumblebee gut

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ABSTRACT

Spectra of five isolates (LMG 28358^T, LMG 29879^T, LMG 29880^T, LMG 28359^T and R-53705) obtained from gut samples of wild bumblebees of *Bombus pascuorum*, *Bombus lapidarius* and *Bombus terrestris* were grouped into four MALDI-TOF MS clusters. RAPD analysis revealed an identical DNA fingerprint for LMG 28359^T and R-53705 which also grouped in the same MALDI-TOF MS cluster, while different DNA fingerprints were obtained for the other isolates.

Comparative 16S rRNA gene sequence analysis of the four different strains identified *Gilliamella apicola* NCIMB 14804^T as nearest neighbour species. Average nucleotide identity values of draft genome sequences of the four isolates and of *G. apicola* NCIMB 14804^T were below the 96% threshold value for species delineation and all four strains and *G. apicola* NCIMB 14804^T were phenotypically distinct. Together, the draft genome sequences and phylogenetic and phenotypic data indicate that the four strains represent four novel *Gilliamella* species for which we propose the names *Gilliamella intestini* sp. nov., with LMG 28358^T as the type strain, *Gilliamella bombicola* sp. nov., with LMG 28359^T as the type strain, *Gilliamella bombi* sp. nov., with LMG 29879^T as the type strain and *Gilliamella mensalis* sp. nov., with LMG 29880^T as the type strain.

Introduction

Like many other pollinating insects, bumblebees are in a worldwide decline. Several studies highlighted the importance of the gut microbiota of honeybees and bumblebees in maintaining bee health [5,11]. The gut microbiota composition of honeybees and bumblebees is similar and consists of a specific and consistent set of bacteria of which a gammaproteobacterium (referred to as Gamma-1), a betaproteobacterium (referred to as Beta) and a firmicutes (referred to as Firm-5) phylotype occur in most bee individuals [12,15,17]. Some isolates belonging to each of these three phylotypes have already been characterised and have been formally named *Gilliamella apicola*, *Snodgrassella alvi* and *Lactobacillus bombicola*, respectively [13,20]. Some Gamma-1 phylotype bacteria are able to degrade pectin which is a compound of the pollen cell wall and ferment mannose, arabinose, xylose and rhamnose which are sugars present in nectar and which are toxic to bees [5,27]. This phylotype is also negatively associated with the presence of the bumblebee trypanosome parasite *Crithidia bombi* [3]. Since the Gamma-1 phylotype is both phylogenetically as well as functionally diverse, it has been suggested that it might comprise multiple taxa [5,13]. The latter was confirmed in the present study, which reports the isolation of four novel *Gilliamella* species from gut samples of three wild bumblebee species.

Materials and methods

Sampling of wild bumblebees and preparation of cell suspensions

Bumblebees were caught in the field near the city of Ghent, Belgium, and identified as *Bombus pascuorum*, *Bombus lapidarius* and *Bombus terrestris* by their colour pattern. Identifications as *B. lapidarius* and *B. terrestris* were confirmed through cytochrome oxidase I (COI) gene sequence analysis as the colour pattern of these species can be confused with that of some other *Bombus* species [4]. The COI gene was amplified as described by Carolan et al.

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[4] after Chelex® 100 resin (Bio-Rad) DNA-extraction from two bumblebee legs which were ground with a micropestle [24]. The bumblebees were immobilised at −20 °C for 10 min and surface sterilised with 2.5% Umonium38® Master (Laboratoire Huckert's International, Brussels, Belgium) before dissecting out their crop and gut. The crops and guts were homogenised in 125 μl saline solution (0.1% peptone, 0.1% Tween 80, 0.85% NaCl) with a sterile micro-pestle. Afterwards, 125 μl of a 10% DMSO solution was added to the cell suspensions which were stored at −80 °C until further use.

Isolation of bumblebee gut bacteria and dereplication

Cell suspensions were serially diluted to 10^{-4} in physiological saline (0.85% NaCl), plated on M144 (23 g/l special peptone, 1 g/l soluble starch, 5 g/l NaCl, 0.3 g/l cysteine hydrochloride, 5 g/l glucose and 15 g/l agar) and all culture (AC) agar (Sigma-Aldrich) and incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) for M144 agar or microaerobically (80% N_2, 15% CO_2 and 5% O_2) for AC agar at 37 °C. After five days of incubation, colonies were picked up from the agar plates and third generation axenic subcultures were dereplicated by MALDI-TOF MS followed by curve-based spectrum analysis [7] using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

Phylogenetic analysis

Representative isolates of each MALDI-TOF MS cluster were selected for further identification. The 16S rRNA gene sequences were determined as previously described [2] and EzTaxon-e [10] was used as a first step in the identification process. The MEGA6 software package was used to align the sequences obtained with the corresponding sequence of their phylogenetic neighbour species by MUSCLE and to obtain phylogenetic trees by using the maximum-likelihood method and the general time-reversible model with invariant sites [23]. The robustness of the tree topologies was estimated by bootstrap analysis with 1000 replicates [6]. MEGA6 was also used to calculate the sequence similarity levels.

RAPD fingerprinting

Random amplified polymorphic DNA (RAPD) analysis using primer RAPD-272 as described by Williams et al. [25] was performed on five isolates tentatively identified by 16S rRNA gene sequence analysis as *Gilliamella* sp.

Draft genome sequence analysis

The genomes of strains LMG 28358^T and LMG 28359^T were 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 was passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artifacts. The following steps were then performed for assembly: filtered Illumina reads were assembled using Velvet (version 1.2.07 [26]), 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 [8]). The genomes of strains LMG 29879^T and LMG 29880^T were sequenced using the Illumina HiSeq 4000 platform. Sequence assembly of raw reads into contigs was performed with CLCgenomics workbench 7.

The quality of the assemblies (draft genomes) and their DNA G+C content was assessed with Quality Assessment Tool for Genome Assemblies (QUAST (http://quast.bioinf.spbau.ru/)). The average nucleotide identity based on mummer (ANIm) values was calculated by JSpecies [22]. Genome annotation was performed by RAST [1].

Phenotypic analysis

Biochemical characteristics were determined for one *Gilliamella* isolate of each MALDI-TOF MS cluster, i.e. LMG 28358^T, LMG 28359^T, LMG 29879^T and LMG 29880^T, and for *G. apicola* NCIMB 14804^T. To test substrate utilisation and enzyme activity, API 20NE galleries (bioMérieux) were inoculated according to the manufacturer's instructions. API 20NE strips were read after 2 days of incubation at 37 °C. Gram-stain-reaction, verification of oxidase, catalase and DNase activity (DNase agar Difco™) and hydrolysis of Tween 20, Tween 40, Tween 60, Tween 80 (Tween 10 ml, peptone 10 g, NaCl 5 g, $CaCl_2.2H_2O$ 0.1 g and agar 9 g), starch (TSA agar supplemented with 10 g starch) and casein (PCA agar 23 g + dry skim milk 13 g) were performed using standard microbiological procedures [14]. Lactose fermentation was tested on MacConkey agar. Growth was checked on AC agar and M144 agar at different temperatures (20 °C, 28 °C, 37 °C and 42 °C). Growth was also tested on AC agar, M144 agar and Columbia blood agar at 28 °C and 37 °C in a microaerobic and anaerobic atmosphere. Cell morphology and motility were checked with phase contrast microscopy.

To test the ability to degrade pectin or polygalacturonic acid, the strains were streaked onto MP-7 agar plates (HiMedia Laboratories) at pH 5 or 7 containing either pectin from citrus peel (Sigma) or polygalacturonic acid (Sigma). After two days of incubation, 1% hexadecyltrimethylammonium bromide (CTAB) was poured onto the plates. After 20 min, pectin or polygalacturonic acid degradation zones were measured with a mauser digital 2 micrometer. *Dickeya chrysanthemi* LMG 2804^T was used as a positive control and as a negative control this strain was streaked on MP-7 agar plates without pectin. The ability to degrade polygalacturonic acid was also tested by growing the strains onto AC agar for two days after which an overlay agar layer containing polygalacturonic acid was poured onto the grown colonies as described by Engel et al. [5].

Fatty acid methyl ester (FAME) analysis was performed for strains LMG 28358^T, LMG 28359^T, LMG 29879^T, LMG 29880^T and NCIMB 14804^T using an Agilent Technologies 6890N gas chromatograph. Cultivation of the strains 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 cultures grown on M144 and AC agar at 37 °C for 48 h. The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI).

The respiratory quinone composition was analysed for strains LMG 28358^T, LMG 28359^T, LMG 29879^T and LMG 29880^T using the protocol described by da Costa et al.[18]. To improve the efficacy of the quinone extraction, the hexane and methanol phases were allowed to separate for two hours 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 high performance liquid chromatography (HPLC). The samples were eluted with 100% methanol at a rate of 1 min/ml and the quinones were detected at 269 nm. *G. apicola* NCIMB 14804^T which was reported to comprise ubiquinone 8 was used as a control strain [13].

Results and discussion

Spectra of five isolates obtained from gut samples of wild bumblebees of *B. pascuorum*, *B. lapidarius* and *B. terrestris* caught near the city of Ghent, Belgium (Table S1) grouped into four MALDI-TOF