Systematic and Applied Microbiology 33 (2010) 359–366

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Bombiscardovia coagulans gen. nov., sp. nov., a new member of the family *Bifidobacteriaceae* isolated from the digestive tract of bumblebees☆

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ARTICLE INFO

Article history:
Received 6 January 2010

Keywords:
Scardovia
Bifidobacteriaceae
Bumblebees
Digestive tract
Bombiscardovia

ABSTRACT

One hundred and eighty-seven fructose-6-phosphate phosphoketolase positive strains were isolated from the digestive tract of three different bumblebee species. Analyses of the partial 16S rRNA gene sequences of the representative strains showed only 92.8% and 92.5% similarity to *Bifidobacterium coryneforme* YIT 4092^T and *Bifidobacterium indicum* JCM 1302^T, 92.2% similarity to *Alloscardovia omnicolens* CCUG 18650 and slightly reduced similarity of 91% to other members of the family *Bifidobacteriaceae*. On the other hand, analyses of the partial heat-shock protein 60 (*hsp60*) gene sequence revealed that the proposed type strain BLAPIII-AGV^T was affiliated only to the 60 kDa chaperonin sequence of uncultured bacteria from human vagina (79–80%) and the *hsp60* gene sequence of *A. omnicolens* CCUG 31649^T (75.5%). The peptidoglycan type was A4α with an L-Lys-D-Asp interpeptide bridge. The polar lipids contained diphosphatidylglycerol, an unknown phospholipid, six glycolipids and two phosphoglycolipids. The major fatty acids were $C_{18:1}$, $C_{20:0}$ and $C_{18:0}$. These and other analyses indicated that the isolates represented a new genus within the family *Bifidobacteriaceae*. This observation was further substantiated by determination of the DNA G + C contents (46.1–47.1 mol%). Affinity of the strains to some scardovial genera (*Aeriscardovia*, *Alloscardovia* and *Metascardovia*) was also confirmed by their ability to grow under aerobic conditions. Besides the above mentioned differences, *Bombiscardovia coagulans* was found to differ from all scardovial genera in the ability to grow at temperatures as low as 5 °C, which was another major phenotypically different characteristic of this new member of the family *Bifidobacteriaceae*. Hence, on the basis of phylogenetic analyses using partial 16S rRNA and *hsp60* gene sequence data, and the temperature related phenotypic difference, we propose a novel taxa, *B. coagulans* gen. nov., sp. nov. (type strain = BLAPIII-AGV^T = DSM 22924^T = ATCC BAA-1568^T).

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Introduction

Currently, the family *Bifidobacteriaceae* is composed of 28 species and 9 subspecies of the genus *Bifidobacterium*. In addition, 5 scardovial genera (*Aeriscardovia*, *Alloscardovia*, *Metascardovia*, *Parascardovia* and *Scardovia*) and the genus *Gardnerella*, each containing only one species, are also included in this family [18,20,31,51]. Family *Bifidobacteriaceae* within the class Actinobacteria was established on the basis of 16S rDNA/rRNA sequence-based phylogenetic clustering and the presence of taxon-specific 16S rDNA/RNA signature nucleotides [41]. Family *Bifidobacteriaceae* consists of pleomorphic rods that occur singly or in many-celled chains or clumps. Cells have no capsule, and are non-spore-forming and non-motile. They are Gram-positive except for *Gardnerella vaginalis* (Gram-variable). They are anaerobic or facultatively anaerobic. They are negative for the following: indole, gelatine hydrolysis, catalase (except for *Bifidobacterium indicum* and *Bifidobacterium asteroides*) and oxidase. The optimum growth temperature is 35–39 °C. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt in which fructose-6-phosphoketolase (EC 4.1.2.2) cleaves fructose-6-phosphate into acetyl-phosphate and erythrose-4-phosphate. End products of glucose fermentation, acetic and lactic acids, are formed primarily in the molar ratio of 3:2. The G + C content varies from 42 to 67 mol%. They are chemoorganotrophs and have a fermentative type of metabolism. They produce acid but no gas from a variety

☆ The 16S rRNA gene sequences of strains BLAPIII-AGV^T and LISPASI-P3 are available from the GenBank database under the accession numbers EU127550 and FJ858733. The partial *hsp60* gene sequence of strain BLAPIII-AGV^T is available in the GenBank database under the accession number GU111354.

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doi:10.1016/j.syapm.2010.08.002

of carbohydrates [2,36]. Scardovial genera members are seen especially outside the intestines, mainly in human and animal dental caries, as well as in other human clinical samples including tonsils and lung abscesses.

Together with *G. vaginalis*, they could therefore be considered as opportunistic pathogens [5,13,18,20]. On the other hand, most members of the genus *Bifidobacterium* mainly inhabit the human [46] and animal intestines [14] and, as such, are considered to be non-pathogenic probiotic bacteria [35].

In comparison to bifidobacteria, most of the representatives of the scardovial genera are able to grow under aerobic conditions [18,31,40]. Another difference between the bifidobacterial and scardovial species is the DNA G + C content. Bifidobacteria have a DNA G + C content ranging from 53 to 67 mol% [3,35], whereas scardovial species have a relatively lower content ranging from 45 to 54 mol% [5,18,39]. All members of the family *Bifidobacteriaceae* are known to degrade hexoses via the specific pathway where the key enzymes involved are fructose-6-phosphate phosphoketolase (F6PPK) and xylulose phosphoketolase. Of the two, it is the activity of F6PPK that is considered to be a taxonomic marker for identification of the family *Bifidobacteriaceae* [36,40].

To date, four *Bifidobacterium* species have been isolated from the insect digestive tract. Of the four species, two, *B. asteroides* and *Bifidobacterium coryneforme*, are found in the *Apis mellifera* intestine, whereas *B. indicum* is found in the intestine of *Apis cerana* and *Apis dorsata* from the Philippines and Malaysia, respectively [37]. The fourth species, *Bifidobacterium bombi*, known to be isolated only from the digestive tract of bumblebees, has been characterized recently [22]. Our study here describes the isolation and identification of new bacterial strains related to the family *Bifidobacteriaceae*.

Materials and methods

Isolation of bifidobacterial strains

A total of 187 new phosphoketolase-positive bacterial strains were isolated from the digestive tract of three bumblebee species thriving in three localities of the Czech Republic [23]. The bacterial strains were isolated using two selective media; MTPY [34] and another selective medium with pollen extract [23]. Integration of the strains into the family *Bifidobacteriaceae* was confirmed via a positive F6PPK test [33] and other analyses. This study describes the representatives of the group of forty-three strains which were isolated from the specimens of the three different bumblebee species, of which two strains, BLAPIII-AGV^T and LISPASI-P3, were representatives of the new isolates.

Genotypic characteristics

DNA of the representative strains was isolated using DNeasy Blood & Tissue Kit (Qiagen, USA). Regions carrying the 16S rRNA (1490 bp) gene were amplified by PCR with the aid of primers fD1 and rP2 [52]. In addition, partial sequences (~590 bp) of the gene encoding heat shock protein, 60 kDa, were determined for the type strain using primers MO-157 and MO-158 [31]. Subsequently, the 16S rRNA gene sequences were obtained after amplifying 500 bp regions of bacterial DNA with the aid of several primers [12]. These fragments were sequenced on a 3100-Avant Genetic Analyser with a BigDye Terminator v3.1 cycle sequencing kit (all from Applied Biosystems, USA). Sequence data were aligned with the CLUSTAL X package [45] in BioEdit program [16] and then the full 16S rRNA gene (accession numbers of strains BLAPIII-AGV^T and LISPASI-P3 in GenBank are EU127550 and FJ858733, respectively) and partial *hsp60* sequence of BLAPIII-AGV^T (GU111354) were compared with published sequences of related bacteria from the EMBL (EBI) and GenBank (NCBI) nucleotide databases using the BLAST program [26]. The sequence similarities were then calculated using the PHYDIT program [19]. The 16S rRNA and *hsp60* gene sequences of the representative strains were deposited in GenBank nucleotide sequence databases by means of the Banklt program in the National Center for Biotechnology Information database (NCBI; www.ncbi.nlm.nih.gov) website.

The DNA G + C contents (mol%) of the two chosen strains were determined using the enzymatic degradation method described by Mesbah et al. [27]. The nucleotide mixture was later analysed on a Phenomenex Gemini C-18 column (250 × 4.6 mm, particle size 5 μm) using a Dionex Summit System with diode array detection (Dionex Corp., USA). A linear gradient using A: 50 mM KH_2PO_4 adjusted to pH 4.5 with *ortho*-phosphoric acid, and B: 100%, A/B: 100/0–80/20 in 15 min led to separation of all nucleotides in 13 min. The flow rate was set to 1 mL min^{-1}, column temperature was 32 °C, injection volume was 5 μL and the detection wavelength 250 nm. Internal calibration references used were calf thymus, salmon testes (both Sigma) and *E. coli* DNA containing 44.0, 43.4 and 50.5 mol% G + C, respectively.

Phylogenetic analysis

The 16S rRNA and partial *hsp60* gene sequences obtained in the present study and those for each strain of the family *Bifidobacteriaceae* (obtained from the NCBI) were aligned using CLUSTAL X. Using the PHYLIP package version 3.6 (obtained from http://evolution.genetics.washington.edu/phylip.html) [11], evolutionary distance matrices were generated with the Cantor and Juke coefficient by the DNADIST program. A phylogenetic tree was constructed according to the neighbour joining method using the program NEIGHBOR. The stability of the grouping was estimated by bootstrap analysis (1000 replicons) using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE (PHYLIP package). The tree topology was also confirmed using maximum parsimony and maximum likelihood cluster analysis using the PHYLIP package, PAUP 4.0b10 (Sinauer Associates, USA), and MrBayes version 3.1.2 software (http://mrbayes.csit.fsu.edu). The phylogenetic trees were viewed using TreeView software and revised using FigTree software (http://tree.bio.ed.ac.uk/software/figtree).

Phenotypic characteristics

The carbohydrate fermentation and enzyme production patterns of the strains were determined using API 50 CHL and Rapid ID 32A test strips (bioMérieux), according to the manufacturer's instructions. Additionally, catalase activity was tested using 3% H_2O_2 and oxidase activity according to the procedure described by Vanderzant and Nickelson [48]. Ability to hydrolyse gelatine was determined using the API 20E system (bioMérieux).

The temperature growth range of the strains was tested using an anaerobic TPY broth at a temperature gradient of 5, 10, 25, 37 and 47 °C for 24–48 h. The sensitivity of the strains to low values of pH was determined at 37 °C in anaerobic TPY broth (pH gradient 3.5, 4, 4.5 and 5) for 24 h. Growth of two chosen strains and the type strains of honeybee bifidobacteria was also tested on TPY agar at 37 °C under aerobic, microaerophilic conditions using the CampyGen generating system (Oxoid, UK) and anaerobic conditions using Anaerostat (Anaerobic Plus System HP11, Oxoid) for 24–48 h. The sensitivity of the strains to the different concentrations of oxygen was examined after a period of cultivation by measuring bacterial colony size.

The end products of hexose catabolism were determined using capillary isotachophoresis. Strains were cultivated in anaerobic MRS broth (Oxoid, UK) supplemented with soybean peptone (5 g L^{-1}) at 37 °C for 18 h. The cultures were centrifuged

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