Bifidobacterium bombi sp. nov., from the bumblebee digestive tract

J. Killer,^{1,2} J. Kopečný,² J. Mrázek,² V. Rada,¹ O. Benada,³ I. Koppová,² J. Havlík¹ and J. Straka⁴

Correspondence

J. Kopečný

kopecnyj@iapg.cas.cz

¹Czech University of Life Sciences, Department of Microbiology, Nutrition and Dietetics, Kamýcká 129, Prague 6 – Suchbát, 165 21, Czech Republic

²Institute of Animal Physiology and Genetics v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4 – Krč, 142 20, Czech Republic

³Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4 – Krč, 142 20, Czech Republic

⁴Charles University in Prague, Department of Zoology, Albertov 6, Prague 2, 128 43, Czech Republic

Gram-positive-staining, anaerobic, non-spore-forming, lactate- and acetate-producing bacterial strains were isolated from the digestive tracts of different bumblebee species (*Bombus lucorum*, *Bombus pascuorum* and *Bombus lapidarius*). All of the isolates produced fructose-6-phosphate phosphoketolase activity. A representative strain, BluCI/TP^T, was characterized further. Cells of strain BluCI/TP^T showed occasional bifurcation and irregular constrictions. The bacterium utilized a wide range of carbohydrates. Glucose was fermented to acetate and lactate. The DNA base composition was 47.2 mol% G + C. Complete 16S rRNA and partial *hsp60* gene sequences were obtained and phylogenetic relationships were determined. Strain BluCI/TP^T and related isolates were located in the actinobacterial cluster and were closely related to the genera *Bifidobacterium*, *Scardovia*, *Aeriscardovia* and *Parascardovia*. The results presented support the proposal of a novel species to accommodate strain BluCI/TP^T, with the name *Bifidobacterium bombi* sp. nov.; the type strain is BluCI/TP^T (=DSM 19703^T =ATCC BAA-1567^T).

Bumblebees are social insects that live in nests or colonies. Adults are represented by workers, which are sterile females, queens and males (drones), which come from unfertilized eggs and usually appear in late summer. The nest temperature is regulated to about 30 °C. Each worker forages independently, and bumblebees never exchange food. These facts can influence the structure and metabolism of the bumblebee gut microbial population (Mohr & Tebbe, 2006). These authors compared gut bacteria from *Apis mellifera* subsp. *carnica* (Carniolan honeybee) and *Bombus terrestris* (buff-tailed bumblebee), as well as from larvae of *Osmia bicornis* (red mason bee). The microflora was dominated by members of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* in all bee species. Bacterial cell densities were in the range 10⁸–10⁹ cells per g gut contents, and the gut bacteria of adult bees were more diverse than in larvae.

The counts of anaerobic bacteria in honeybees were higher than the counts of aerobic species (Kačániová *et al.*, 2004). The dominant bacteria in honeybee intestines were species of the genus *Bacillus* (Gilliam, 1997). *Propionibacterium acnes* was found in larvae of *Apis mellifera* and *Osmia bicornis* (Mohr & Tebbe, 2006). This species was also very common in this study in the bumblebee digestive tract (not shown). Three bifidobacterial species have already been described from the honeybee digestive tract: *Bifidobacterium asteroides*, *Bifidobacterium coryneforme* and *Bifidobacterium indicum* (Felis & Dellaglio, 2007). Because there were no reports on the presence of bifidobacteria in the bumblebee, the aim of this study was to describe the bifidobacterial population in the bumblebee digestive tract.

Bacteria were isolated from the digestive tracts of the bumblebee species *Bombus lucorum*, *Bombus pascuorum* and *Bombus lapidarius*. Worker bumblebees were collected in central Bohemia during summer 2006, and their intestinal tracts were weighed and transferred aseptically into tubes containing sterile MRS broth (Oxoid) supplemented with soybean peptone (5 g l⁻¹) and cysteine hydrochloride (0.5 g l⁻¹). The tubes were flushed with O₂-

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and partial *hsp60* gene sequences of strain BluCI/TP^T are EU127549 and EU869281.

free CO₂ and closed with rubber stoppers. The same broth was used for serial dilutions of all samples. Aliquots (0.1 ml) were plated on TPY agar with mupirocine as described by Rada & Petr (2000). *Propionibacterium acnes* and representatives of several novel species with relatively low levels of 16S rRNA gene sequence similarity (92–95 %) to the genus *Bifidobacterium* were present in the cultures.

Estimation of the G + C content in bacterial chromosomal DNA was done after extraction and enzymic hydrolysis by HPLC chromatography on a C18 reversed-phase column (Supelcosil LC18) with the same guard column (Mesbah *et al.*, 1989). DNA from *Escherichia coli* strain B (50 mol%) and calf thymus (42 mol%) was used as reference samples for G + C content. The G + C content of the representative strain BluCI/TP^T was 47.2 mol% (Table 1).

DNA of five strains was isolated (Gregg *et al.*, 1994) and regions carrying the 16S rRNA gene (1490 bp) were amplified by PCR with the aid of primers fD1 and rP2 (Weisburg *et al.*, 1991; Barcenilla *et al.*, 2000) and then analysed by electrophoresis in 2 % (w/v) agarose gels (Sambrook *et al.*, 1989). In three strains, 16S rRNA gene sequences were obtained after amplifying 500 bp regions of bacterial DNA with primers FP27-515R, FP3-RP16 and FP19-rP2 (Forster *et al.*, 1996). These fragments were sequenced on a 3100-Avant Genetic Analyzer (Applied Biosystems) with a BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems). After editing, the full 16S rRNA gene sequence was compared with published sequences of related bacteria from the EMBL and GenBank nucleotide databases using BLAST and with data from the Ribosomal Database Project (Maidak *et al.*, 1994). The

sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) in the BioEdit program (Hall, 1999). Nucleotide divergences among 16S rRNA gene sequences were calculated with the PAUP software, version 4.0b10 (Swofford, 2003), using a corrected distance based on the GTR + I + G model of sequence evolution (Tavaré, 1986). The GTR + I + G model was determined to be the appropriate model for our dataset by the hierarchical likelihood ratio test of goodness-of-fit of 56 different nested models to the data, as implemented in the MODELTEST program, version 3.7 (Posada & Crandall, 1998). Phylogenetic relationships among the sequences were reconstructed using PAUP by analysis of the distance matrix by the neighbour-joining algorithm and by using the maximum-parsimony (heuristic search) optimality criterion. Phylogenetic analyses using the maximum-likelihood criterion were performed with PAUP and using the GTR + I + G evolutionary model with the following base frequencies: A = 0.2105, C = 0.2350, G = 0.3431 and T = 0.2114 (Fig. 1). To quantify the confidence in the partitioning within the maximum-likelihood tree, we performed the non-parametric bootstrap test as applied to phylogeny by Felsenstein (1985) using 1000 replications.

The highest 16S rRNA gene sequence similarity of isolate BluCI/TP^T was to *B. asteroides* CCUG 24607^T (93 %). Because of the low G + C content, the phylogenetic location of the novel strains was verified by another phylogenetic analysis. In this case, the *hsp60* gene was selected and analysed with the same method, with the exception that the TrN + I + G evolutionary model was selected (Fig. 2). The

Table 1. Characteristics of members of the genus *Bifidobacterium* isolated from the insect digestive tract

Strains: 1, strain BluCI/TP^T; 2, *B. asteroides* DSM 20089^T; 3, *B. coryneforme* DSM 20216^T; 4, *B. indicum* DSM 20214^T. Some data for reference type strains were obtained from Simpson *et al.* (2004) and Huys *et al.* (2007). +, Positive; –, negative; v, variable; ND, no data available. All taxa are positive for acid production from raffinose.

Characteristic	1	2	3	4
Temperature range for growth (°C)	10–37	21–42	ND	ND
Acid from:				
Arabinose	–	+	+	–
Cellobiose	–	+	+	+
Gluconate	–	v	+	+
Maltose	–	v	+	v
Mannose	+	v	–	–
Melezitose	+	–	–	v
Salicin	+	+	+	–
Sucrose	–	+	+	+
Trehalose	–	–	–	v
Xylose	–	+	+	–
DNA G + C content (mol%)	47	59	55	60

Fig. 1. Phylogenetic tree showing relationships of 16S rRNA gene sequences of isolate BluCI/TP^T and species of *Bifidobacterium* and related genera. Bar, 1 substitution per 100 nucleotides. The tree was constructed using neighbour-joining analysis of a distance matrix obtained from a multiple-sequence alignment in a model of sequence evolution. *Gardnerella vaginalis* ATCC 14018^T was used as the outgroup sequence.

Fig. 2. Phylogenetic tree showing relationships of *hsp60* gene sequences of isolate BluCI/TP^T and species of *Bifidobacterium* and related genera. Bar, 1 substitution per 100 nucleotides. See legend to Fig. 1 for further details.

highest *hsp60* gene sequence similarity of isolate BluCI/TP^T was to *B. asteroides* JCM 8230^T (82 %).

Bifidobacteria degrade hexoses via the fructose-6-phosphate phosphoketolase pathway. The key enzyme in this pathway is fructose-6-phosphate phosphoketolase. The enzyme is considered a taxonomic marker for identification of the genus *Bifidobacterium* (Biavati & Mattarelli, 2006). The novel strains produced fructose-6-phosphate phosphoketolase (data not shown).

Substrate utilization (Table 1) and enzyme production by the isolates were tested with API 50 CH and Rapid ID 32 test kits (bioMérieux). None of the isolates produced catalase, urease or indole. All strains utilized glucose, ribose, aesculin, salicin, arbutin and gentiobiose. The isolates produced β -galactosidase, α - and β -glucosidases, proline arylamidase, arginine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, histidine arylamidase and serine arylamidase. Glycerol, erythritol, arabinose, D- and L-xylose, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, cellobiose, maltose, lactose, inulin, glycogen and turanose were not utilized. The novel strains utilized mannose and melibiose and produced α -galactosidase. All growth parameters are in good agreement with the descriptions of *Bifidobacterium* species and related genera (Jian & Dong, 2002; Simpson *et al.*, 2004).

Fermentation products (short-chain fatty acids) were analysed by GLC. After dilution and centrifugation, the short-chain fatty acid sample was ultrafiltered, transferred to 2 ml vials and analysed on a gas chromatography capillary column with FID detector. Hydrogen was used as the carrier gas.

All strains tested fermented sugars to acetate and lactate (Table 2). Production of acetate and lactate was compared between the novel strains and bifidobacteria from the honeybee digestive tract. These bacteria, *B. asteroides*, *B. coryneforme* and *B. indicum*, are taxonomically distinct from other bifidobacteria (Sakata *et al.*, 2006; Jian *et al.*, 2001). Production of both lactate and acetate was similar to

B. asteroides, with the acetate/lactate ratio close to 3.3. No production of formate was observed in cultures of strain BluCI/TP^T.

Cellular fatty acid profiles have been used previously to establish taxonomic relationships among anaerobic bacteria (Moore, 1994) and have also proved useful in the classification of Gram-positive bacteria (Van Den Velde *et al.*, 2006). Cellular fatty acids were determined by GLC. Preparation of FAMES by saponification, methylation and extraction into hexane and sample cleanup followed the procedure of Moore (1994). Methyl esters were analysed on a 100 m SP-2560 column using an Agilent GC 6890N chromatograph equipped with FID detector and Chromatopac C-R6A for data processing. A calibration standard (Supelco 37 component FAME mix) was used for identification of fatty acids and equivalent chain lengths were calculated for unknown peaks (Moore, 1994). Relative concentrations (% w/v) of individual fatty acids were calculated.

The fatty acid profile of cultures of isolate BluCI/TP^T contained small proportions of branched-chain fatty acids. The dominant fatty acids were oleic (C_{18:1}; 7.49 %) and arachidic (C_{20:0}; 7.18 %) acids. Isolate BluCI/TP^T contained a relatively high level of linoleic acid (C_{18:2}; 7.34 %). Heneicosanoic acid (C_{21:0}) was not present. Four other fatty acids were present: palmitic (C_{16:0}; 7.14 %), stearic (C_{18:0}; 5.91 %), behenic (C_{22:0}; 5.87 %) and tricosanoic (C_{23:0}; 5.38 %) acids. The content of odd-numbered fatty

Table 2. Fermentation products of insect bifidobacteria grown on glucose in TPY medium

Strain	Acetate (mM)	Lactate (mM)	Ratio
<i>B. asteroides</i> DSM 20089 ^T	45.0	13.6	3.3
<i>B. coryneforme</i> DSM 20216 ^T	45.0	4.9	9.3
<i>B. indicum</i> DSM 20214 ^T	46.0	6.9	6.7
BluCI/TP ^T	59.0	17.0	3.5

acids was high (41.2%, w/v). Increased content of unsaturated fatty acids corresponded with the lower optimal temperature for growth of isolate BluCI/TP^T (Suutari & Laakso, 1994).

Morphological properties were examined by scanning electron microscopy. Samples were fixed with 3% (w/v) glutaraldehyde in cacodylate buffer, pH 7.2, washed with a cacodylate buffer and allowed to settle for 48 h at 4 °C onto an SPI-pore filter (0.2 µm) treated with poly-L-lysine (Sanders *et al.*, 1975). The samples were dehydrated in an alcohol series followed by absolute acetone and dried in a critical-point device (Balzers 010). Alternatively, fixed and dehydrated clusters of aggregated bacterial cells were mounted onto aluminium stubs. Finally, all samples were sputter-coated with gold in a Polaron sputter-coater (Series 11HD) and examined in a Philips CM12/STEM electron microscope at 80 kV or in an Aquasem scanning electron microscope (TESCAN) at 15 kV. All recorded images were digitally processed in Analysis 3.2 software (Olympus Soft Imaging Solutions).

We propose the novel species *Bifidobacterium bombi* sp. nov. for the strains from the bumblebee digestive tract because of their unique metabolic and morphological properties, supported by distinctive lipid composition and gene sequences.

Description of *Bifidobacterium bombi* sp. nov.

Bifidobacterium bombi (bom'bi. L. n. *bombus* a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumblebee; N.L. gen. n. *bombi* of *Bombus*, of a bumblebee).

Cells are Gram-positive-staining, catalase- and oxidase-negative, non-motile, non-spore-forming, short and irregularly shaped rods (0.4–0.5 µm wide and 0.9–1.1 µm long). In culture, the appearance resembles dividing bacilliform cells, frequently forming filaments, with irregular contractions along the cells and bifurcations (Fig. 3). Strictly anaerobic. Colonies on TPY agar under anaerobic conditions are white, circular and low convex with sharp, undulate edges. Colonies reach 1.0–1.7 mm in diameter after 3 days of incubation. Mannose and melibiose are utilized. Good growth occurs at 10–37 °C (optimum 30 °C; maximum 37 °C); very limited growth at 47 °C. Minimal initial pH for growth is 4.5. The DNA G + C content of the type strain is 47.3 mol%.

The type strain is BluCI/TP^T (=ATCC BAA-1567^T =DSM 19703^T), isolated from the digestive tract contents of *Bombus lucorum* from central Bohemia, Czech Republic.

Acknowledgements

This work was supported by the Grant Agency of the Czech Academy of Sciences (project no. GA AV ČR 1QS500200572), IRP IAPG (no. AV0Z50450515), IRP IMB (no. AV0Z50200510) and grant no. MSM 6046070901 of the Grant Agency of the Ministry of Education, Youth

Fig. 3. Scanning electron photomicrograph of cells of strain BluCI/TP^T showing occasional bifurcations and irregular constrictions. Bar, 1 µm.

and Sports of Czech Republic. We appreciate the help of Dr J. P. Euzéby (École Nationale Vétérinaire, Toulouse) with nomenclature.

References

- Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C. & Flint, H. J. (2000). Phylogenetic relationship of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* **66**, 1654–1661.
- Biavati, B. & Mattarelli, P. (2006). The family Bifidobacteriaceae. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 322–382. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.
- Felis, G. E. & Dellaglio, F. (2007). Taxonomy of lactobacilli and bifidobacteria. *Curr Issues Intest Microbiol* **8**, 44–61.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Forster, R. J., Teather, R. M., Gong, J. & Deng, S. J. (1996). 16S rRNA analysis of *Butyrivibrio fibrisolvens*: phylogenetic position and relation to butyrate producing anaerobic bacteria from the rumen of a short-tailed deer. *Lett Appl Microbiol* **23**, 218–222.
- Gilliam, M. (1997). Identification and roles of non-pathogenic microflora associated with honeybees. *FEMS Microbiol Lett* **155**, 1–10.
- Gregg, K., Cooper, C. L., Schafer, D. J., Sharpe, H., Beard, C. E., Allen, G. & Xu, J. (1994). Detoxification of the plant toxin fluoroacetate by a genetically modified rumen bacterium. *Biotechnology* **12**, 1361–1365.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Huys, G., Vancanneyt, M., D'Haene, K., Falsen, E., Wauters, G. & Vandamme, P. (2007). *Alloscardovia omnicoles* gen. nov., sp. nov., from human clinical samples. *Int J Syst Evol Microbiol* **57**, 1442–1446.
- Jian, W. & Dong, X. (2002). Transfer of *Bifidobacterium inopinatum* and *Bifidobacterium denticolens* to *Scardovia inopinata* gen. nov.,

comb. nov., and *Parascardovia denticolens* gen. nov., comb. nov., respectively. *Int J Syst Evol Microbiol* **52**, 809–812.

Jian, W., Zhu, L. & Dong, X. (2001). New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. *Int J Syst Evol Microbiol* **51**, 1633–1638.

Kačániová, M., Chlebo, R., Kopernický, M. & Trakovická, A. (2004). Microflora of the honeybee gastrointestinal tract. *Folia Microbiol (Praha)* **49**, 169–171.

Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, G. J., Fogel, K., Blandy, J. & Woese, C. R. (1994). The Ribosomal Database Project. *Nucleic Acids Res* **22**, 3485–3487.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Mohr, K. I. & Tebbe, C. C. (2006). Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. *Environ Microbiol* **8**, 258–272.

Moore, W. E. C. (1994). Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic gram-negative bacilli. *Int J Syst Bacteriol* **44**, 338–347.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Rada, V. & Petr, J. (2000). A new selective medium for the isolation of glucose nonfermenting bifidobacteria from hen caeca. *J Microbiol Methods* **43**, 127–132.

Sakata, S., Ryu, C. S., Kitahara, M., Sakamoto, M., Hayashi, H., Fukuyama, M. & Benno, Y. (2006). Characterization of the genus *Bifidobacterium* by automated ribotyping and 16S rRNA gene sequences. *Microbiol Immunol* **50**, 1–10.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanders, S. K., Alexander, E. L. & Braylan, R. C. (1975). A high-yield technique for preparing cells fixed in suspension for scanning electron microscopy. *J Cell Biol* **67**, 476–480.

Simpson, P. J., Ross, R. P., Fitzgerald, G. F. & Stanton, C. (2004). *Bifidobacterium psychraerophilum* sp. nov. and *Aeriscardovia aeriphila* gen. nov., sp. nov., isolated from a porcine caecum. *Int J Syst Evol Microbiol* **54**, 401–406.

Suutari, M. & Laakso, S. (1994). Microbial fatty-acids and thermal adaptation. *Crit Rev Microbiol* **20**, 285–328.

Swofford, D. L. (2003). PAUP: phylogenetic analysis using parsimony (and other methods), version 4. Sunderland, MA: Sinauer Associates.

Tavaré, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. In *Some Mathematical Questions in Biology: DNA Sequence Analysis* (Lectures on Mathematics in the Life Sciences, vol. 17), pp. 57–86. Edited by R. M. Miura. Providence, RI: American Mathematical Society.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Van Den Velde, S., Lagrou, K., Desmet, K., Wauters, G. & Verhaegen, J. (2006). Species identification of corynebacteria by cellular fatty acid analysis. *Diagn Microbiol Infect Dis* **54**, 99–104.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.