# *Bifidobacterium actinocoloniiforme* sp. nov. and *Bifidobacterium bohemicum* sp. nov., from the bumblebee digestive tract

J. Killer,[1] J. Kopečný,[1] J. Mrázek,[1] I. Koppová,[1] J. Havlík,[2] O. Benada[3] and T. Kott[4]

Correspondence
J. Killer
killer.jiri@seznam.cz

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

[2]Czech University of Life Sciences, Department of Microbiology, Nutrition and Dietetics, Kamýcká 129, Prague 6 – Suchdol 165 21, Czech Republic

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

[4]Institute of Animal Science, Přátelství 815, Prague – Uhříněves 104 00, Czech Republic

Our previous study, based primarily on PCR-denaturing gradient gel electrophoresis and 16S rRNA gene sequencing, focused on the isolation of four bifidobacterial groups from the digestive tract of three bumblebee species. In that study, we proposed that these isolated groups potentially represented novel species of the family *Bifidobacteriaceae*. One of the four, *Bifidobacterium bombi*, has been described recently. Strains representing two of the other groups have been classified as members of the genus *Bifidobacterium* on the basis of positive results for fructose-6-phosphate phosphoketolase activity and analysis of partial 16S rRNA and heat-shock protein 60 (*hsp60*) gene sequences. Analysis of 16S rRNA gene sequence similarities revealed that the isolates of the first group were affiliated to *Bifidobacterium asteroides* YIT 11866$^T$, *B. indicum* JCM 1302$^T$ and *B. coryneforme* ATCC 25911$^T$ (96.2, 96.0 and 95.9 % sequence similarity, respectively), together with other bifidobacteria showing lower sequence similarity. Additional representatives of the second group were found to be affiliated to *Bifidobacterium minimum* YIT 4097$^T$ and *B. coryneforme* ATCC 25911$^T$ (96.0 and 96.3 % sequence similarity) and also to other bifidobacteria with lower sequence similarity. These results indicate that the isolates of the two groups belong to novel species within the genus *Bifidobacterium*. This observation was further substantiated by the results of partial sequencing of *hsp60*. On the basis of phylogenetic and phenotypic analyses and analysis of 16S rRNA and partial *hsp60* gene sequences, we propose two novel species, *Bifidobacterium actinocoloniiforme* sp. nov. (type strain LISLUCIII-P2$^T$ =DSM 22766$^T$ =CCM 7728$^T$) and *Bifidobacterium bohemicum* sp. nov. (type strain JEMLUCVIII-4$^T$ =DSM 22767$^T$ =CCM 7729$^T$).

Bifidobacteria are Gram-positive, anaerobic, non-spore-forming, lactate- and acetate-producing bacteria belonging to the class *Actinobacteria* and, together with lactobacilli, they are the main representatives of animal and human probiotics (Reuter, 2001). At the time of writing, 28 species and nine subspecies of the genus *Bifidobacterium* have been isolated from the gastrointestinal tracts of humans (Scardovi & Crociani, 1974; Lauer, 1990; Hoyles *et al.*, 2002) and other animals (Scardovi *et al.*, 1979; Watabe *et al.*, 1983; Biavati *et al.*, 1991; Zhu *et al.*, 2003), milk products (Watanabe *et al.*, 2009) and sewage (Biavati *et al.*, 1982). Three species of bifidobacteria were also found in honeybees (Scardovi & Trovatelli, 1969). In addition, Mrázek *et al.* (2008) detected bifidobacteria through molecular techniques in other insects, including wasps (*Vespula vulgaris*), hornets (*Vespa crabro*) and cockroaches (*Nauphoeta cinerea*).

We have recently described the heterogeneous bifidobacterial population in the digestive tract of bumblebees

Abbreviation: F6PPK, fructose-6-phosphate phosphoketolase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LISLUCIII-P2$^T$, LUCL-P3, JEMLUCVIII-4$^T$ and JEMLUCVII-1 are FJ858731, FJ858735, FJ858736 and FJ858737; those for the partial *hsp60* gene sequences of the same strains are GU223108, GU238419, GU223107 and GU238418.

Three supplementary figures are available with the online version of this paper.

(Killer *et al.*, 2010), which was found to be distinct from the bifidobacterial populations in honeybees. The first named representative of the bumblebee bifidobacteria, *Bifidobacterium bombi*, has been described recently (Killer *et al.*, 2009). Here, we report the results of a taxonomic study of two further representatives of the bumblebee bifidobacteria. Bacterial strains were collected from the digestive tracts of three species of bumblebee, as described previously (Killer *et al.*, 2010). Isolates LISLUCIII-P2$^T$, LUCL-P3, JEMLUCVIII-4$^T$ and JEMLUCVII-1 were taken as representatives of the two newly described species.

Chromosomal DNA of the selected strains was extracted using the DNeasy Blood & Tissue kit (Qiagen). 16S rRNA gene sequences (1490 bp) were amplified by PCR with the aid of primers fP1 and rP2 and sequenced after dividing the sequences into shorter, 500 bp regions (Weisburg *et al.*, 1991; Forster *et al.*, 1996). The *hsp60* gene has been proposed as an alternative phylogenetic marker for the family *Bifidobacteriaceae* (Jian *et al.*, 2001; Huys *et al.*, 2007). Hence, partial sequences (~590 bp) of the *hsp60* gene were determined as described by Okamoto *et al.* (2007). Fragments were then sequenced on a 3100-Avant Genetic Analyzer with a BigDye Terminator version 3.1 cycle sequencing kit (both from Applied Biosystems). Sequence data were aligned with the CLUSTAL_X package (Thompson *et al.*, 1997) in the BioEdit program (Hall, 1999) and the full 16S rRNA gene and partial *hsp60* sequences were then compared with published sequences of related bacteria from the EMBL and GenBank nucleotide databases using the BLAST program (Maidak *et al.*, 1994). Sequence similarities were then calculated using the PHYDIT program (Chun, 2001). The 16S rRNA gene sequences of strains LISLUCIII-P2$^T$ and LUCL-P3 were similar to those of *Bifidobacterium asteroides* YIT 11866$^T$, *B. indicum* JCM 1302$^T$ and *B. coryneforme* ATCC 25911$^T$ (96.2, 96.0 and 95.9 % sequence similarity, respectively). The highest *hsp60* gene sequence similarities of these strains were found to *B. asteroides* JCM 8230$^T$ (88.4 %) and *B. indicum* JCM 1302$^T$ (88.5 %). Strains JEMLUCVIII-4$^T$ and JEMLUCVII-1 showed the highest 16S rRNA gene sequence similarity to *Bifidobacterium minimum* YIT 4097$^T$ (96 %) and *B. coryneforme* ATCC 25911$^T$ (96.3 %) and other bifidobacteria. These genotypic results are in agreement with the classification of strains of different species within the genus *Bifidobacterium* (Stackebrandt & Goebel, 1994; Jian *et al.*, 2001). Affinity of strains LISLUCIII-P2$^T$ and LUCL-P3 to honeybee bifidobacteria and strains JEMLUCVIII-4$^T$ and JEMLUCVII-1 to *B. minimum* was substantiated through the phylogenetic tree of the family *Bifidobacteriaceae* (Fig. 1) based on 16S rRNA gene sequences (Simpson *et al.*, 2003). A similar phylogenetic tree topology reconstructed using the neighbour-joining method was found based on partial *hsp60* gene sequences (Fig. 2). In addition, the tree topologies based on both gene sequences were confirmed using maximum-likelihood cluster analysis (Supplementary Figs S1 and S2, available in IJSEM Online).

The DNA G+C contents of the representative strains LISLUCIII-P2$^T$ and JEMLUCVIII-4$^T$ were determined using the enzymic degradation method described by Mesbah *et al.* (1989). The nucleotide mixture was then 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.). A linear gradient using eluents A (50 mM $KH_2PO_4$, adjusted to pH 4.5 with phosphoric acid) and B (100 % acetonitrile), changing from 100 % buffer A to 80 : 20 buffer A/buffer B over 15 min, led to separation of all nucleotides in 13 min. The flow rate was set to 1 ml min$^{-1}$, column temperature 32 °C, injection volume 5 μl and detection wavelength 250 nm. Internal calibration references used were calf thymus, salmon sperm (both Sigma) and *E. coli* DNA, with DNA G+C contents of 44.0, 43.4 and 50.5 mol%, respectively. The DNA G+C contents of strains LISLUCIII-P2$^T$ and JEMLUCVIII-4$^T$ were 52.7 mol% (mean of five experiments, SD=0.5) and 51.2 mol% (SD=0.3), respectively. These values are lower than those of described members of the genus *Bifidobacterium* (53–67 mol%) (Scardovi, 1986; Okamoto *et al.*, 2008), but higher than values for other genera within the family *Bifidobacteriaceae*, including *Scardovia* (45–55 mol%) and *Alloscardovia* (47.3–48.3 mol%) (Crociani *et al.*, 1996; Huys *et al.*, 2007; Downes *et al.*, 2011). We found that *B. bombi* BluCI/TP$^T$, the type strain of the first described *Bifidobacterium* species isolated from the digestive tract of bumblebees (Killer *et al.*, 2009), also has a low DNA G+C content of 50.5 mol%.

Biochemical profiles for strains of both proposed species were obtained with API 50 CHL and Rapid ID 32A kits (bioMérieux) according to the manufacturer's instructions. Growth of the strains was tested in anaerobic TPY broth at 5, 10, 25, 37 and 47 °C for 24–48 h. The sensitivity of the type strains to low pH was determined at 37 °C in anaerobic TPY broth at pH 3.5, 4, 4.5 and 5 for 24 h. Growth of representative strains was also tested on TPY agar at 37 °C under aerobic, microaerophilic (CampyGen generating system; Oxoid) and anaerobic conditions for 24–48 h. Table 1 shows the major phenotypic characteristics and DNA G+C contents of the tested strains and related bacteria. In contrast to bifidobacteria isolated from the digestive tract of honeybees, the novel bumblebee bifidobacteria have much lower DNA G+C contents (59–60 and 50.5–52.7 mol%). All bee bifidobacteria except *B. bombi* BluCI/TP$^T$ and strain JEMLUCVIII-4$^T$ were able to grow under microaerophilic conditions. None of the strains grew under aerobic conditions. The strains were found to differ in the utilization of 14 substrates and production of alanine arylamidase.

The key enzyme of hexose catabolism in members of the family *Bifidobacteriaceae* is fructose-6-phosphate phosphoketolase (F6PPK) (de Vries & Stouthamer, 1967; Jian & Dong, 2002), through which hexoses are degraded to the end products acetic and lactic acids in a theoretical final ratio of 1.5 : 1.0. Hence, we assessed the end products of hexose catabolism for the representative strains through

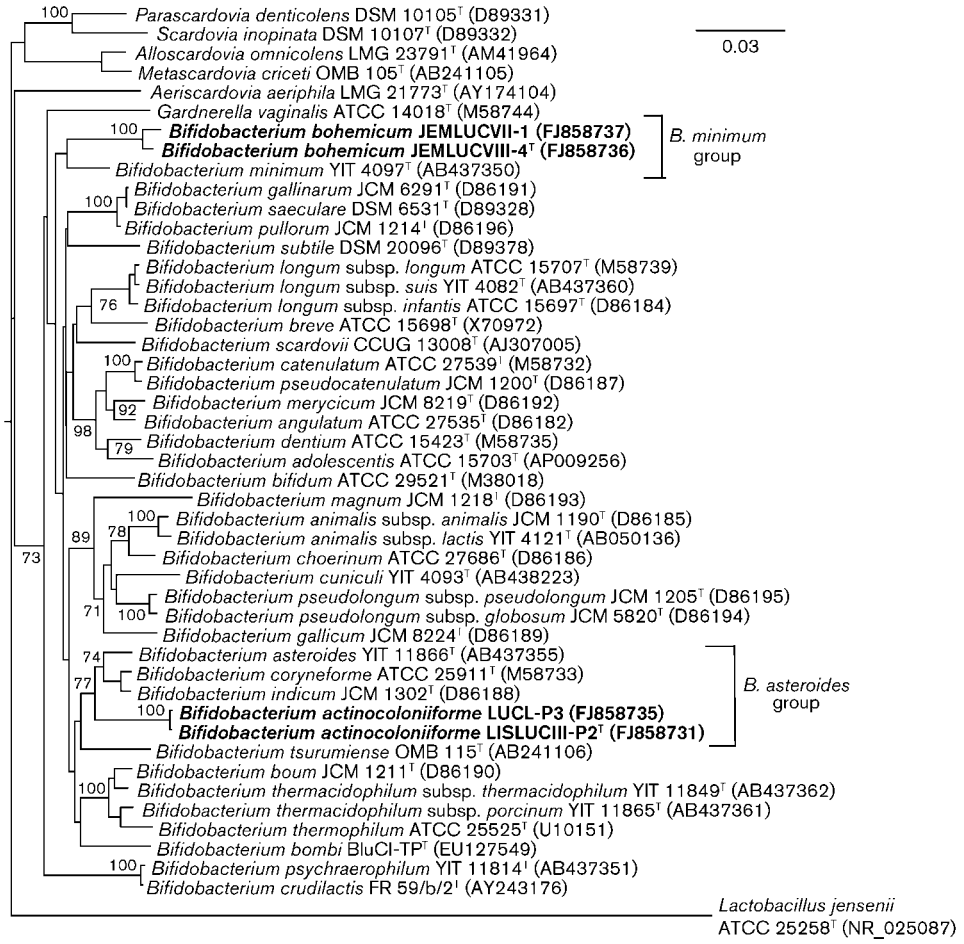


**Fig. 1.** Phylogenetic tree of the family *Bifidobacteriaceae* based on 16S rRNA gene sequences (mean length 1313 nt) showing the position of the four novel strains from the bumblebee digestive tract. The tree was rooted with *Lactobacillus jensenii* ATCC 25258$^T$ and constructed using the neighbour-joining algorithm. Bootstrap values >70 %, expressed as percentages of 1000 datasets, are given at nodes. GenBank accession numbers are given in parentheses. The phylogenetic tree was reconstructed using sequences from the type strains of bifidobacterial and scardovial species. Bar, 0.03 substitutions per nucleotide position.

capillary isotachophoresis. The strains were first cultivated in anaerobic MRS broth (Oxoid) supplemented with soybean peptone (5 g $l^{-1}$) at 37 °C for 18 h. The cultures were then centrifuged (13 000 r.p.m., 8 min) and the supernatants were used for the determination of fermentation products (acetic, lactic and propionic acids) with a column-coupling instrument EA 101 (Villa Labeco Co.). Strains LISLUCIII-P2$^T$ and JEMLUCVIII-4$^T$ produced lactic and acetic acids from glucose at molar ratios of 1 : 1.4 and 1 : 1.5, respectively. Small amounts of propionic acid were also observed in both strains (2.2–4.6 mmol $l^{-1}$). Some previous studies have also found propionic acid in growing cultures of bifidobacteria (Han *et al.*, 2005).

More detailed phenotypic characterization of *B. bombi* BluCI/TP$^T$ was carried out previously using cellular fatty acid profiling (Killer *et al.*, 2009). The same method was also used for characterization and differentiation of the representative strains LISLUCIII-P2$^T$ and JEMLUCVIII-4$^T$. In comparison to *B. bombi* BluCI/TP$^T$, both strains contained much larger amounts of palmitic acid ($C_{16:0}$) (Table 2) and did not contain linoleic ($C_{18:2}$), behenic ($C_{22:0}$), tricosanoic ($C_{23:0}$), lignoceric ($C_{24:0}$) or pentadecenoic ($C_{15:1}$) acids. Similar results for the cellular fatty acid compositions of bifidobacteria were reported by Veerkamp (1971). In comparison to the latter study, we did not detect palmitoleic ($C_{16:1}$) acid in strain LISLUCIII-P2$^T$ or JEMLUCVIII-4$^T$ or *B. bombi* BluCI/TP$^T$.

Detailed cell morphology of strains LISLUCIII-P2$^T$ and JEMLUCVIII-4$^T$ was examined by scanning electron microscopy, as described previously (Killer *et al.*, 2009). Morphological characteristics of colonies of strains related to LISLUCIII-P2$^T$ growing on TPY agar under anaerobic conditions were examined using a Leica DFC480 microscope.

On the basis of genotypic, phylogenetic and phenotypic studies supported by distinctive cellular fatty acid compositions and

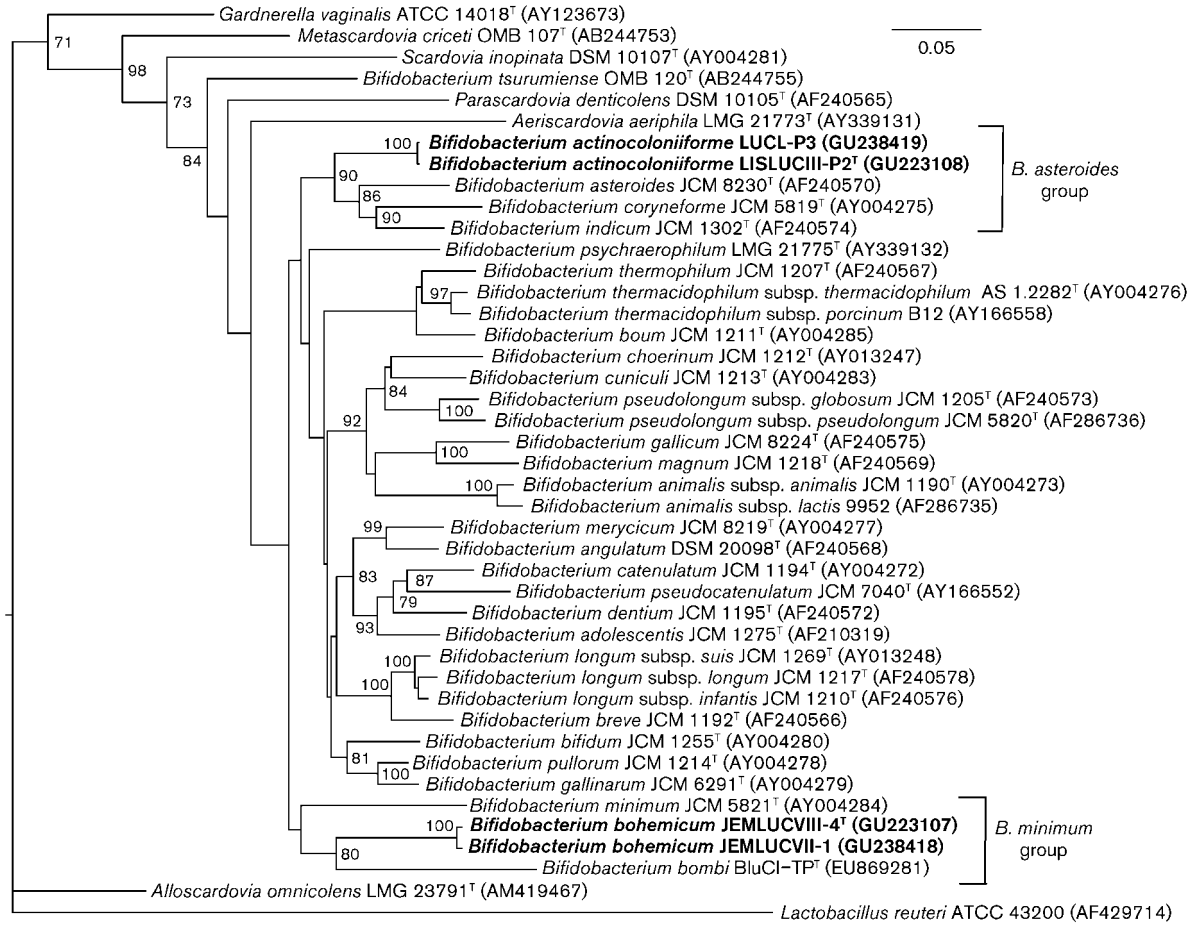


**Fig. 2.** Neighbour-joining phylogenetic tree, derived from analysis of partial *hsp60* gene sequences (552 bp), showing the position of the four novel strains from the bumblebee digestive tract. Bootstrap values >70 %, expressed as percentages of 1000 datasets, are given at nodes. GenBank accession numbers are given in parentheses. Bar, 0.05 substitutions per nucleotide position.

morphological characteristics, strains LISLUCIII-P2$^T$, LUCL-P3, JEMLUCVIII-4$^T$ and JEMLUCVII-1, together with related strains, represent two novel species of the genus *Bifidobacterium*, for which we propose the names *Bifidobacterium actinocoloniiforme* sp. nov. and *Bifidobacterium bohemicum* sp. nov.

## Description of *Bifidobacterium actinocoloniiforme* sp. nov.

*Bifidobacterium actinocoloniiforme* (ac.ti′no.co.lo′ni.i.for′me. Gr. fem. n. *actis*, *-inos* a ray; L. n. *colonia* a dwelling group, a colony; L. neut. adj. *forme* formed, shaped. N.L. neut. part. adj. *actinocoloniiforme* shaped like a ray-shaped colony).

Cells are Gram-type-positive, F6PPK-positive, non-motile, non-spore-forming, irregularly shaped rods (0.3–0.6 μm wide and 0.4–1.2 μm long) with enlarged or tapered ends (Fig. 3a, b). They grow singly or in short chains. Colonies on TPY agar under anaerobic conditions are cream in colour, sometimes irregularly circular with entire edges and rigid cores, and reach 2.05–3.97 mm in diameter after 2 days of incubation. Some colonies have filamentous parts growing around the solid core (Supplementary Fig. S3) when they are incubated for 72 h under anaerobic conditions. Colonies are also formed under microaerophilic conditions, when they reach 1.79–2.62 mm in diameter after 2 days of incubation. Growth in TPY broth occurs at 25 and 37 °C, but not at 47 °C (after 24–48 h). In TPY broth, the lowest pH attained is 4.5; minimum initial pH for growth is pH 5. Cells contain relatively large amounts of palmitic, oleic and stearic acids. DNA G+C content and biochemical parameters are shown in Table 1.

The type strain, LISLUCIII-P2$^T$ (=DSM 22766$^T$ =CCM 7728$^T$), was isolated from the digestive tract contents of a bumblebee (*Bombus lucorum*) sampled from Central Bohemia, Czech Republic, in 2006.

**Table 1.** Differential phenotypic characteristics and DNA G+C contents of insect bifidobacteria and *B. minimum* ATCC 27538$^T$

Strains: 1, *B. actinocoloniiforme* sp. nov. LISLUCIII-P2$^T$; 2, *B. bohemicum* sp. nov. JEMLUCVIII-4$^T$; 3, *B. bombi* BluCI/TP$^T$; 4, *B. asteroides* DSM 20089$^T$; 5, *B. coryneforme* DSM 20216$^T$; 6, *B. indicum* DSM 20214$^T$; 7, *B. minimum* ATCC 27538$^T$. Data are from this study and previous studies (Scardovi, 1986; Scardovi & Trovatelli, 1969; Killer *et al.*, 2009, 2010). All insect strains ferment ribose, salicin, glucose, gentiobiose and arbutin. None of the insect bifidobacteria ferment glycerol, erythritol, adonitol, methyl β-D-xylopyranoside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, *N*-acetylglucosamine, inulin, starch, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 5-ketogluconate. In contrast to the insect bifidobacteria, *B. minimum* ATCC 27538$^T$ ferments starch and does not ferment ribose or salicin. All insect strains are positive for α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-arabinosidase, glycine arylamidase, arginine arylamidase, proline arylamidase, leucine arylamidase (except *B. bombi* BluCI/TP$^T$), phenylalanine arylamidase (except *B. bombi* BluCI/TP$^T$), tyrosine arylamidase and histidine arylamidase. All are negative for urease, indole production, nitrate reduction, arginine dihydrolase, β-galactosidase-6-phosphate, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, glutamic acid decarboxylase, alkaline phosphatase, pyroglutamic acid arylamidase, glutamyl glutamic acid arylamidase, catalase and oxidase. +, Positive; (+), weakly positive; −, negative; ND, no data available.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| DNA G+C content (mol%) | 52.7 | 51.2 | 50.5* | 59 | ND | 60 | 61.5 |
| Growth on agar under microaerophilic conditions | + | − | − | + | + | + | ND |
| Temperature range for growth (°C) | 25–37 | 10–37 | 10–37 | 22–43 | 22–43 | 22–43 | 22–40 |
| Fermentation of (API 50 CHL): | | | | | | | |
| L-Arabinose | + | + | − | (+) | + | + | − |
| D-Xylose | + | + | − | + | − | − | − |
| D-Galactose | + | + | − | (+) | + | + | − |
| D-Fructose | − | (+) | + | + | + | + | + |
| D-Mannose | − | + | + | + | − | − | − |
| Methyl α-D-glucopyranoside | − | − | (+) | − | − | (+) | ND |
| Amygdalin | + | − | + | + | (+) | + | ND |
| Cellobiose | (+) | − | + | − | + | + | − |
| Maltose | − | − | − | − | + | + | + |
| Melibiose | (+) | + | + | − | + | (+) | − |
| Sucrose | + | − | − | + | (+) | (+) | + |
| Trehalose | + | − | − | − | − | − | − |
| Melezitose | − | − | − | + | − | − | − |
| Raffinose | − | + | + | − | + | − | − |
| Alanine arylamidase (Rapid ID 32A) | + | − | − | − | + | + | ND |

*Revised value from this study.

**Table 2.** Cellular fatty acid profiles of bumblebee bifidobacteria

Strains: 1, *B. actinocoloniiforme* sp. nov. LISLUCIII-P2$^T$; 2, *B. bohemicum* sp. nov. JEMLUCVIII-4$^T$; 3, *B. bombi* BluCI/TP$^T$ (data from Killer *et al.*, 2009). Relative concentrations (%, w/v) of total fatty acids were calculated.

| Fatty acid | Common name | 1 | 2 | 3 |
|---|---|---|---|---|
| $C_{16:0}$ | Palmitic acid | 20.17 | 15.97 | 7.14 |
| $C_{18:1}$ | Oleic acid | 9.99 | 4.69 | 7.49 |
| $C_{18:0}$ | Stearic acid | 7.05 | 6.56 | 5.91 |
| $C_{20:0}$ | Arachidic acid | 3.25 | 4.62 | 7.18 |
| $C_{17:0}$ | Margaric acid | 2.11 | 2.56 | 4.21 |
| $C_{14:0}$ | Myristic acid | 1.97 | 2.86 | <0.01 |
| $C_{18:2}$ | Linoleic acid | <0.01 | <0.01 | 7.34 |
| $C_{22:0}$ | Behenic acid | <0.01 | <0.01 | 5.87 |
| $C_{23:0}$ | Tricosanoic acid | <0.01 | <0.01 | 5.38 |
| $C_{24:0}$ | Lignoceric acid | <0.01 | <0.01 | 4.68 |
| $C_{15:1}$ | Pentadecenoic acid | <0.01 | <0.01 | 2.47 |

## Description of *Bifidobacterium bohemicum* sp. nov.

*Bifidobacterium bohemicum* (bo.he′mi.cum. M.L. neut. adj. *bohemicum* from Bohemia, referring to the Czech Republic, where the bacterium was first isolated).

Cells are Gram-type-positive, F6PPK-positive, non-motile, non-spore-forming, very irregularly shaped rods (0.2–0.4 μm wide and 0.6–1.0 μm long) with frequent constrictions and deformities. They are organized in chains in filament forms (Fig. 3c, d). Strictly anaerobic colonies on TPY agar under anaerobic conditions are cream in colour, circular in shape with sharp, entire edges, frequently with small irregular cores. They appear in variable shapes and sizes (0.13–2.28 mm in diameter after 2 days of incubation). Optimum temperature for growth is 37 °C, with a minimum of 10 °C and a maximum of 40 °C. No growth occurs at 5 or 45 °C. In TPY broth, the minimum initial pH for growth is pH 5 within a period of 24–48 h (weak growth observed at pH 4.5, and no growth observed at

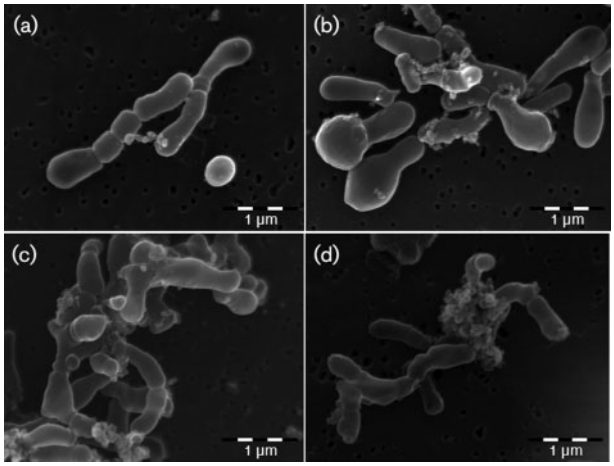


**Fig. 3.** Scanning electron photomicrographs of cells of *Bifidobacterium actinocoloniiforme* sp. nov. LISLUCIII-P2$^T$ (a, b) and *Bifidobacterium bohemicum* sp. nov. JEMLUCVIII-4$^T$ (c, d). (a, b) Chains of curved irregular cells and clubbed cells; (c, d) chains of very irregular cells and cells with occasional constrictions. Bars, 1 μm.

lower pH). Cells contain relatively large amounts of palmitic, oleic and stearic acids. DNA G+C content and biochemical parameters are shown in Table 1.

The type strain, JEMLUCVIII-4$^T$ (=DSM 22767$^T$ =CCM 7729$^T$), was isolated from the digestive tract contents of a bumblebee (*Bombus lucorum*) sampled from South Bohemia, Czech Republic, in 2007.

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