Bacillus galliciensis sp. nov., isolated from faeces of wild seahorses (*Hippocampus guttulatus*)

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A Gram-positive-staining, motile, rod-shaped, endospore-forming bacterium (BFLP-1^T) was isolated from faeces of wild long-snouted seahorses (*Hippocampus guttulatus*) captured in north-west Spain (Toralla, Galicia). Strain BFLP-1^T grew at 10–30 °C and pH 5.5–9 (optimally at 20 °C and pH 7.2) and with 0–7 % (w/v) NaCl (optimally with 2 % NaCl). The G + C content of the DNA was 48.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BFLP-1^T was a member of the genus *Bacillus* and was most closely related to *Bacillus herbersteinensis* D-1,5a^T (96.6 %), *B. shackletonii* LMG 18435^T (96.0 %) and *B. isabelliae* CVS-8^T (95.9 %). Chemotaxonomic data (peptidoglycan type, meso-diaminopimelic acid; major menaquinone, MK-7; predominant fatty acids, anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:1ω11}c; major polar lipids, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown aminoglycophospholipid) supported the affiliation of strain BFLP-1^T to the genus *Bacillus*. Comparative analysis of 16S rRNA gene sequences and chemotaxonomic and phenotypic features indicated that strain BFLP-1^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus galliciensis* sp. nov. is proposed. The type strain is BFLP-1^T (=DSM 21539^T =LMG 24668^T).

The genus *Bacillus* is an extensive, heterogeneous group, with members exhibiting a wide range of physiological and genetic characteristics (Ash *et al.*, 1991; Rössler *et al.*, 1991; Xu & Côté, 2003). Bacilli can occupy a wide variety of ecological niches including soil, water and some clinical samples (Logan *et al.*, 2004; Wieser *et al.*, 2005; Albuquerque *et al.*, 2008). Members of the genus also produce antibiotics, enzymes and other metabolites that are used extensively in the medical and pharmaceutical industries (Banat *et al.*, 2000; Balcázar & Rojas-Luna, 2007; Sorokulova *et al.*, 2008).

During the characterization of organisms isolated from faeces of wild long-snouted seahorses (*Hippocampus guttulatus*), strain BFLP-1^T was grown on tryptone soy agar (TSA) supplemented with 1.5 % (w/v) NaCl at 20 °C for 72 h. Subcultivation was done on the same medium at 20 °C for 48 h. On this agar, BFLP-1^T was able to grow at 10–30 °C, but not at 5 or 35 °C.

The Gram reaction was determined using the non-staining (KOH) method as described by Buck (1982). Cell morphology and motility were studied using phase-contrast

microscopy. NaCl tolerance and requirement for growth were investigated by using nutrient broth (0.5 % peptone from casein, 0.3 % meat extract, 0.3 % yeast extract; pH 7.2) supplemented with various concentrations of NaCl (0–15 % at intervals of 1 %). The pH range for growth was determined in nutrient broth adjusted to pH 2.0–12.5 at intervals of 0.5 pH units. Anaerobic growth was assessed at 20 °C in an anaerobic chamber with a H₂/CO₂ atmosphere (bioMérieux).

Catalase activity was determined by assessing bubble production in 3 % (v/v) H₂O₂; oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine as described by Lim *et al.* (2008). Some physiological characteristics were performed using API 20NE and API 20A strips (bioMérieux). Cells for inoculation of the strips were grown for 24 h at 20 °C on TSA and results were interpreted visually, according to the manufacturer's instructions.

Strain BFLP-1^T was found to consist of Gram-positive-staining, aerobic, motile, rod-shaped cells. Colonies on TSA were circular to slightly irregular and cream in colour after incubation at 20 °C for 48 h. The strain grew with 0–7 % (w/v) NaCl (optimum 2 %), but not with 8.0 % NaCl. Growth was observed at pH 5.5–9.0 (optimum pH 7.2). Oxidase and catalase reactions were positive. Other physiological characteristics of strain BFLP-1^T are shown in Table 1 and in the species description.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BFLP-1^T is FM162181.

A maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences is available as supplementary material with the online version of this paper.

Table 1. Characteristics of strain BFLP-1^T and the type strains of some related *Bacillus* species

Strains: 1, *Bacillus galliciensis* sp. nov. BFLP-1^T; 2, *B. herbersteinensis* D-1,5a^T; 3, *B. shackletonii* LMG 18435^T; 4, *B. isabeliae* CVS-8^T. All data were obtained in this study. +, Positive; (+), weakly positive; –, negative.

Characteristic	1	2	3	4
Sporangium shape	Swollen	Unswollen	Swollen	Swollen
Conditions for growth				
pH	5.5–9	7–12	4.5–9	6.5–8.5
NaCl (% w/v)	0–7	0–5	0–3	1–14
Temperature (°C)	10–30	4–28	15–55	20–40
Assimilation of <i>N</i> -acetylglucosamine	+	+	+	–
Acid production from:				
Cellobiose	–	–	+	+
D-Glucose	–	–	+	–
Lactose	–	–	(+)	–
Maltose	–	–	(+)	–
D-Mannitol	–	–	(+)	–
D-Mannose	–	–	(+)	–
Melezitose	–	–	–	+
Salicin	–	–	+	–
Sucrose	–	–	–	(+)
Trehalose	–	–	(+)	+
D-Xylose	–	–	–	(+)
Major fatty acids*	ai-C _{15:0} , ai-C _{17:0} , C _{16:1} ω11c	ai-C _{15:0} , i-C _{15:0}	ai-C _{15:0} , i-C _{15:0} , i-C _{16:0} , ai-C _{17:0}	i-C _{15:0} , ai-C _{15:0} , i-C _{16:0} , C _{16:0}

*ai, Anteiso-branched; i, iso-branched.

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995) and the G+C content was determined by the thermal denaturation method (Mandel & Marmur, 1968). DNA from *Bacillus subtilis* subsp. *subtilis* DSM 10^T was used as the reference for determination of the thermal-melting profile (T_m). The DNA G+C content was calculated to be 48.1 mol%. This value is within the range for the genus *Bacillus* (Xue *et al.*, 2008).

The 16S rRNA gene sequence was amplified by PCR with the universal primers 27F (5'-AGAGTTTGATCCTGGCT-CAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Amplification was carried out with a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) according to the following program: 95 °C for 10 min, 30 cycles of 94 °C for 0.5 min, 50 °C for 1 min and 72 °C for 2 min and final extension at 72 °C for 10 min. PCR products were purified by using ExoSAP-IT (USB). Sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit with an ABI PRISM 377 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions, and five primers (337F, 785F, 1225F, 518R, 1100R). The consensus sequences obtained were compared with reference 16S rRNA gene sequences available in the GenBank databases using BLASTN (Altschul *et al.*, 1990). After multiple alignments of data by CLUSTAL_X (Thompson *et al.*, 1997), phylogenetic analysis was performed using MEGA version 4.0 (Tamura *et al.*, 2007) with

distances calculated according to the Kimura-2 model and the neighbour-joining and maximum-parsimony methods. Bootstrap calculations were based on 1000 replications. The 16S rRNA gene sequence of strain BFLP-1^T was a continuous stretch of 1490 bp. The neighbour-joining analysis (Fig. 1) indicated that the closest relatives of strain BFLP-1^T were *Bacillus herbersteinensis* D-1,5a^T (96.6%), *B. shackletonii* LMG 18435^T (96.0%) and *B. isabeliae* CVS-8^T (95.9%). These similarity values are below the cut-off value of 97.0%, the level normally judged sufficient to justify the proposal of a novel bacterial species (Stackebrandt & Goebel, 1994; Janda & Abbott, 2002). Similar results were obtained with the maximum-parsimony algorithm (Supplementary Fig. S1, available in IJSEM Online).

Analysis of respiratory quinones was carried out by the DSMZ Identification Service and Dr Brian Tindall (DSMZ, Braunschweig, Germany). Peptidoglycan analysis was performed by using the method of Schleifer & Kandler (1972) and Schleifer (1985). Lipids were extracted and analysed according to Suresh *et al.* (2004). The predominant isoprenoid quinone in strain BFLP-1^T was unsaturated menaquinone with seven isoprene units (MK-7) and the diagnostic diamino acid in the cell wall was meso-diaminopimelic acid. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown aminoglycophospholipid were identified by TLC. These characteristics confirm that strain BFLP-1^T belongs to the genus *Bacillus* (Albert *et al.*, 2007).

Fig. 1. Phylogenetic dendrogram of strain BFLP-1^T with the most closely related *Bacillus* strains, based on 16S rRNA gene sequences and constructed by the neighbour-joining method. Bootstrap percentages (>70%) based on 1000 replications are shown at branch nodes. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 1% estimated sequence divergence.

Whole-cell fatty acids from the isolate were extracted from biomass grown on nutrient agar and analysed according to the standard protocol of the Sherlock Microbial Identification System version 4.5 (MIDI). The fatty acid composition was iso-C_{14:0} (3.1%), C_{14:0} (1.0%), iso-C_{15:0} (4.8%), anteiso-C_{15:0} (62.4%), C_{15:0} (1.1%), C_{16:1}ω7c (1.9%), iso-C_{16:0} (1.4%), C_{16:1}ω11c (7.3%), C_{16:0} (3.8%), iso-C_{17:1}ω10c (0.7%), iso-C_{17:0} (0.7%) and anteiso-C_{17:0} (9.0%). Thus, the major fatty acids in strain BFLP-1^T were anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:1}ω11c, which comprised approximately 79% of the cellular fatty acids extracted. Branched fatty acids, 14- to 17-carbon iso and anteiso series, are typically the major fatty acids found in *Bacillus* cell membranes (Kämpfer, 1994). However, strain BFLP-1^T and the most closely related strains, *B. herbersteinensis* D-1,5a^T, *B. shackletonii* LMG 18435^T and *B. isabelliae* CVS-8^T, could be clearly distinguished from each other based on relative fatty acid concentrations (Table 1).

Therefore, the phenotypic and genotypic properties of strain BFLP-1^T support its classification in a novel species within the genus *Bacillus*, for which the name *Bacillus galliciensis* sp. nov. is proposed.

Description of *Bacillus galliciensis* sp. nov.

Bacillus galliciensis (gal.li.ci.en'sis. L. masc. adj. *galliciensis* of Galicia, north-west Spain).

Cells are aerobic, Gram-positive-staining and spore-forming motile rods (0.8–1.2 × 2.5–3.5 μm). Primarily occur as single cells, although short chains are also seen. Oval spores develop subterminally in the cells and usually cause the sporangia to swell. Colonies on TSA after 48 h at 20 °C are cream-coloured, slightly irregular in shape and 1.5–3.0 mm in diameter. Optimum temperature for growth is 20 °C; no growth at 5 or 35 °C. Optimum pH for growth is pH 7.2;

grows at pH 5.5–9.0 but not at pH 5.0 or 9.5. Optimum NaCl concentration for growth is 2% (w/v) NaCl; grows with 0–7% (w/v) NaCl but not with 8% (w/v) NaCl. Positive for catalase, oxidase, *N*-acetylglucosamine, aesculin hydrolysis, β-galactosidase and assimilation of D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, maltose and potassium gluconate. Negative for indole production, nitrate production, urease, arginine dihydrolyase, gelatin hydrolysis and assimilation of D-mannose, caprate, adipate, malate, citrate and phenylacetate. Acid is not produced from L-arabinose, cellobiose, D-glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, melezitose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, trehalose or D-xylose. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:1}ω11c; smaller amounts of iso-C_{14:0}, C_{14:0}, iso-C_{15:0}, C_{15:0}, iso-C_{16:0}, C_{16:1}ω7c, C_{16:0}, iso-C_{17:1}ω10c and iso-C_{17:0} are present. The diamino acid in the cell wall is *meso*-diaminopimelic acid. The major respiratory menaquinone is MK-7. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown aminoglycophospholipid. The G + C content of the type strain is 48.1 mol% (T_m).

The type strain, BFLP-1^T (=DSM 21539^T =LMG 24668^T), was isolated from faeces of wild seahorses captured in north-west Spain (Toralla, Galicia).

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References

- Albert, R. A., Archambault, J., Lempa, M., Hurst, B., Richardson, C., Gruenloh, S., Duran, M., Worliczek, H. L., Huber, B. E. & other authors (2007). Proposal of *Viridibacillus* gen. nov. and reclassification of *Bacillus arvi*, *Bacillus arenosi* and *Bacillus neidei* as *Viridibacillus arvi* gen. nov., comb. nov., *Viridibacillus arenosi* comb. nov. and *Viridibacillus neidei* comb. nov. *Int J Syst Evol Microbiol* **57**, 2729–2737.
- Albuquerque, L., Tiago, I., Taborda, M., Nobre, M. F., Verissimo, A. & da Costa, M. S. (2008). *Bacillus isabeliae* sp. nov., a halophilic bacterium isolated from a sea salt evaporation pond. *Int J Syst Evol Microbiol* **58**, 226–230.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ash, C., Farrow, J. A. E., Wallbanks, S. & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett Appl Microbiol* **13**, 202–206.
- Balcázar, J. L. & Rojas-Luna, T. (2007). Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). *Curr Microbiol* **55**, 409–412.
- Banat, I. M., Makkar, R. S. & Cameotra, S. S. (2000). Potential commercial applications of microbial surfactants. *Appl Microbiol Biotechnol* **53**, 495–508.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.
- Janda, J. M. & Abbott, S. L. (2002). Bacterial identification for publication: when is enough enough? *J Clin Microbiol* **40**, 1887–1891.
- Kämpfer, P. (1994). Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *Syst Appl Microbiol* **17**, 86–98.
- Lim, J. M., Jeon, C. O., Jang, H. H., Park, D. J., Shin, Y. K., Yeo, S. H. & Kim, C. J. (2008). *Albimonas donghaensis* gen. nov., sp. nov., a non-photosynthetic member of the class *Alphaproteobacteria* isolated from seawater. *Int J Syst Evol Microbiol* **58**, 282–285.
- Logan, N. A., Lebbe, L., Verhelst, A., Goris, J., Forsyth, G., Rodríguez-Dias, M., Heyndrickx, M. & De Vos, P. (2004). *Bacillus shackletonii* sp. nov., from volcanic soil on Candlemas Island, South Sandwich archipelago. *Int J Syst Evol Microbiol* **54**, 373–376.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.
- Rössler, D., Ludwig, W., Schleifer, K. H., Lin, C., McGill, T. J., Wisotzkey, J. D., Jurtshuk, P., Jr & Fox, G. E. (1991). Phylogenetic diversity in the genus *Bacillus* as seen by 16S rRNA sequencing studies. *Syst Appl Microbiol* **14**, 266–269.
- Schleifer, K. H. (1985). Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Sorokulova, I. B., Pinchuk, I. V., Denayrolles, M., Osipova, I. G., Huang, J. M., Cutting, S. M. & Urdaci, M. C. (2008). The safety of two *Bacillus* probiotic strains for human use. *Dig Dis Sci* **53**, 954–963.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Suresh, K., Reddy, G. S. N., Sengupta, S. & Shivaji, S. (2004). *Deinococcus indicus* sp. nov., an arsenic-resistant bacterium from an aquifer in West Bengal, India. *Int J Syst Evol Microbiol* **54**, 457–461.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Wieser, M., Worliczek, H., Kämpfer, P. & Busse, H.-J. (2005). *Bacillus herbersteinensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 2119–2123.
- Xu, D. & Côté, J.-C. (2003). Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S–23S ITS nucleotide sequences. *Int J Syst Evol Microbiol* **53**, 695–704.
- Xue, Y., Ventosa, A., Wang, X., Ren, P., Zhou, P. & Ma, Y. (2008). *Bacillus aidingensis* sp. nov., a moderately halophilic bacterium isolated from Ai-Ding salt lake in China. *Int J Syst Evol Microbiol* **58**, 2828–2832.