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

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

Abstract

A Gram-positive, motile, rod-shaped, endospore-forming bacterium (BFLP-1^T) was isolated from faeces of wild seahorses (*Hippocampus guttulatus*) captured in northwest 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 at salt concentrations in the range 0–7% (w/v) NaCl. The G+C content of the DNA was 48.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequence shown that strain BFLP-1^T was a member of the genus *Bacillus*, being most closely related to *Bacillus herbersteinensis* (96.6 %), *B. shackletonii* (96.0 %) and *B. isabeliae* (95.9 %). Chemotaxonomic data (peptidoglycan type, *meso*-diaminopimelic; major menaquinone, MK-7; predominant fatty acids, $C_{15:0}$ anteiso, $C_{17:0}$ anteiso and $C_{16:1}\omega 11c$; 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 the 16S rRNA gene sequence data, chemotaxonomy and phenotypic features of the novel isolate and related species of *Bacillus* 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). It 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 this genus also produce antibiotics, enzymes, and other metabolites, which 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 seahorses (*Hippocampus guttulatus*), strain BFLP-1^T was grown on tryptone soy agar (TSA) supplemented with 1.5 % NaCl at 20 ºC for 72 h. Subcultivation was done on the same medium at 20 ºC for 48 h. On this agar, BFLP-1^T was able to grow at 10–30 °C, but not at 5 or 35 °C.

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 growth tolerance and requirements were investigated by using nutrient broth [0.5% peptone from casein, 0.3% meat extract, 0.3% yeast extract, and adjusted to 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 that was adjusted to various pH values (pH 2.0–12.5 at intervals of 0.5 pH units). Anaerobic growth was assessed at 20 ºC in anaerobic chambers with an H_2/CO_2 atmosphere (bioMérieux).

Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2; 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 (bioMérieux). Cells for inoculation of the strips were grown for 24 h at 20 ºC on TSA and results were visually interpreted according to the manufacturer's instructions.

Strain BFLP-1^T was found to consist of Gram-positive, aerobic, motile, and rod-shaped cells. Colonies on TSA are circular to slightly irregular and cream coloured after incubation at 20 ºC for 48 h. The strain grew at salt concentrations in the range 0–7% (w/v) NaCl, with optimum growth occurring at 2.0% (w/v) NaCl. The strain did not grow in the presence of ≥8.0% (w/v) NaCl. Growth was observed at pH 5.5–9.0 with optimum at pH 7.2. Oxidase and catalase reactions were positive. Other physiological characteristics of strain BFLP-1^T are shown in Table 1 and also in the species description.

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

The 16S rRNA gene was amplified by PCR with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-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, followed by 30 cycles consisting of 94 °C for 0.5 min, 50 °C for 1 min and 72 °C for 2 min, and a final extension period 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 on ABI PRISM 377 Genetic Analyzer (Applied

Biosystems), as recommended by the manufacturer, and five primers (337F, 785F, 1225F, 518R, 1100R). The consensus sequences obtained were compared to reference 16S rRNA gene sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center of Biotechnology Information database using the BLASTN (Alschul *et al.*, 1990). Phylogenetic analysis was performed using the software MEGA version 4.0 (Tamura *et al.*, 2007) after multiple alignments of data by CLUSTAL X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining (Fig. 1) and maximum-parsimony (Supplementary Fig. S1 in IJSEM online) methods were determined by using bootstrap values based on 1000 replications. The 16S rRNA sequence of strain BFLP-1^T was a continuous stretch of 1490 bp. Sequence similarity calculations after a neighbour-joining analysis indicated that the closest relatives of strain BFLP-1^T were *Bacillus herbersteinensis* (96.6 %), *B. shackletonii* (96.0 %) and *B. isabeliae* (95.9 %). Similar results were obtained for strain BFLP-1^T when the maximum-parsimony algorithm was used (Supplementary Fig. S1 in IJSEM online). These gene sequence 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).

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). Unsaturated menaquinone with seven isoprene units (MK-7) was the predominant isoprenoid quinone found in strain BFLP-1^T, and the diagnostic diamino acid in their cell walls was *meso*-diaminopimelic acid. Diphosphatidylglycerol, phosphatidylglycerol,

phosphatidylethanolamine and an unknown aminoglycophospholipid were identified by using TLC. These characteristics confirm that strain BFLP-1^T belongs to the genus *Bacillus* (Albert *et al*., 2007).

Whole-cell fatty acids from the isolate were extracted from biomass grown on nutrient agar [0.5% peptone from casein, 0.3% meat extract, 0.3% yeast extract, 1.5% agar, and adjusted to pH 7.2] and were analysed according to the standard protocol of the Sherlock Microbial Identification System (MIDI version 4.5). The major fatty acids in strain BFLP-1^T were $C_{15:0}$ anteiso, $C_{17:0}$ anteiso and $C_{16:1}\omega 11c$, which comprise 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 most closely related type strains, *B. herbersteinensis*, *B. shackletonii* and *B. isabeliae*, could be clearly distinguished from each other based on the relative fatty acid concentration.

Therefore, the phenotypic and genotypic properties of strain BFLP-1^T support its description as 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, northwest Spain].

Cells are aerobic, Gram-positive 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 growth temperature is 20 °C. No growth occurs below 5 °C or above 35 °C. Growth occurs at pH 5.5–9.0, but not below pH 5.0 or above pH 9.5.

Growth occurs at NaCl concentrations between 0 and 7 % (w/v), but not in the presence of 8 % (w/v) NaCl. Positive for catalase, oxidase; *N*-acetyl-glucosamine; aesculin hydrolysis; β-galactosidase, assimilation of D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, D-maltose and potassium gluconate. Negative for indole production; nitrate production; urease, arginine dihydrolase; gelatine hydrolysis; assimilation of D-mannose, caprate, adipate, malate, citrate and phenyl-acetate. Acid is not produced from L-arabinose, D-cellobiose, D-glucose, glycerol, D-lactose, D-maltose, D-mannitol, D-mannose, D-melezitose, D-raffinose, L-rhamnose, salicin, D-sorbitol, D-sucrose, D-trehalose or D-xylose. The fatty acid profile consists of $C_{14:0}$ iso (3.1 %), $C_{14:0}$ (1.0 %), $C_{15:0}$ iso (4.8 %), $C_{15:0}$ anteiso (62.4 %), $C_{15:0}$ (1.1 %), $C_{16:1}$ $\omega 7c$ (1.9 %), $C_{16:0}$ iso (1.4 %), $C_{16:1}$ $\omega 11c$ (7.3 %), $C_{16:0}$ (3.8 %), $C_{17:1}$ $\omega 10c$ iso (0.7 %), $C_{17:0}$ iso (0.7 %), and $C_{17:0}$ anteiso (9.0 %). 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 thermal denaturation temperature of DNA from BFLP-1^T is 89.0 °C, and the G+C content determined from this value is 48.1 mol%.

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

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herbersteinensis D-1,5a^T, *B. shackletonii* LMG 18435^T and *B. isabeliae* CVS-8^T, respectively.

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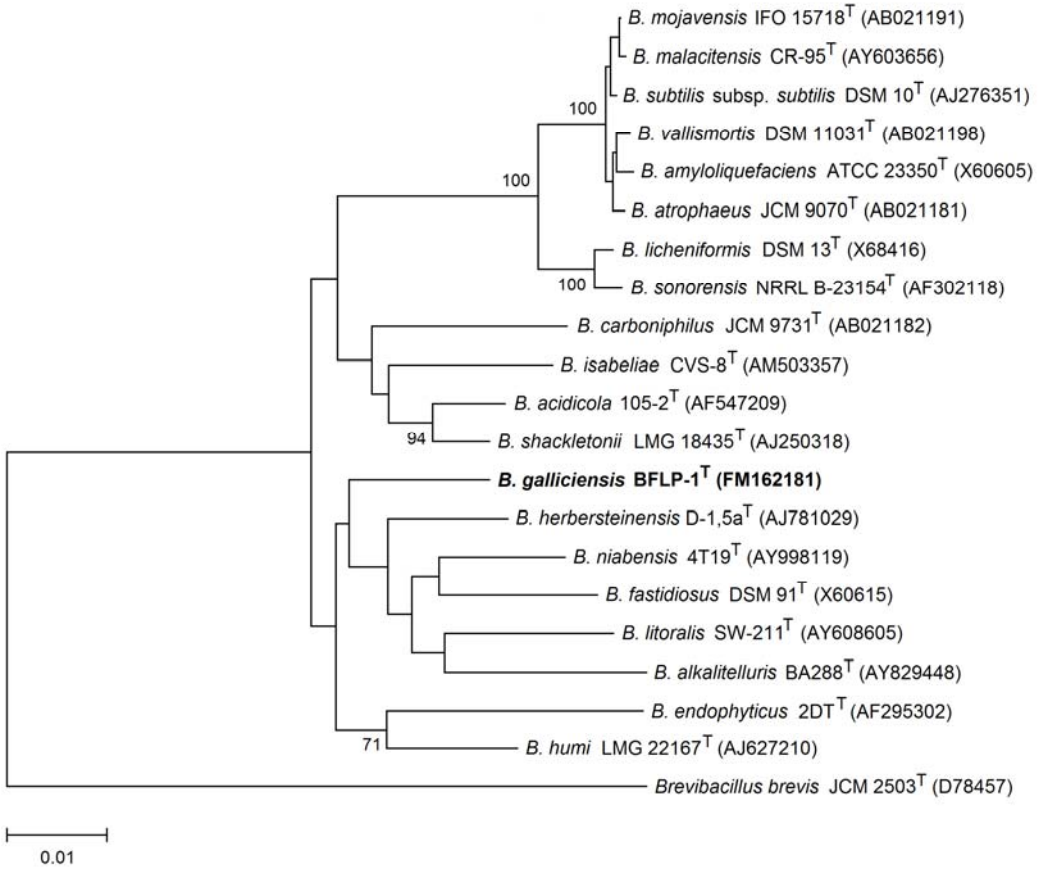

Fig. 1. Phylogenetic dendrogram of *Bacillus galliciensis* sp. nov. with the most closely related *Bacillus* species, based on 16S rRNA gene sequences and constructed by the neighbour-joining method. Bootstrap percentages (based on 1000 replications) greater than 70 % are shown at branch points. *Brevibacillus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

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

Characteristic	**1**	**2**	**3**	**4**
Sporangium shape	Swollen	Unswollen	Swollen	Swollen
pH tolerance	5.5–9	7–12	4.5–9	6.5–8.5
NaCl (%) range	0–7	0–5	0–3	1–14
Temperature (°C) range	10–30	4–28	15–55	20–40
N-acetyl-glucosamine	+	+	+	–
Acid production from:				
D-Cellobiose	–	–	+	+
D-Glucose	–	–	+	–
D-Lactose	–	–	(+)	–
D-Maltose	–	–	(+)	–
D-Mannitol	–	–	(+)	–
D-Mannose	–	–	(+)	–
D-Melezitose	–	–	–	+
Salicin	–	–	+	–
Sucrose	–	–	–	(+)
D-Trehalose	–	–	(+)	+
D-Xylose	–	–	–	(+)
Major fatty acids	ai-$C_{15:0}$, ai-$C_{17:0}$, $C_{16:1}\omega 11c$	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-$C_{15:0}$, $C_{16:0}$, ai-$C_{15:0}$, i-$C_{16:0}$

278

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