Flavobacterium phragmitis sp. nov., an endophyte of reed (*Phragmites australis*)

Min Liu,^{1†} Yan Hong Li,^{1†} Yang Liu,¹ Jing Nan Zhu,¹ Qun Fang Liu,¹ Yin Liu,¹ Jin Gang Gu,² Xiao Xia Zhang² and Chun Li Li³

Correspondence

Yan Hong Li

liyh@mail.cnu.edu.cn

¹College of Life Science, Capital Normal University, Beijing 100048, PR China

²Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, PR China

³New Technique Centre, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China

A Gram-staining-negative bacterium, designated strain BLN2^T, was isolated from within the roots of reeds (*Phragmites australis*) in Beijing Cuihu Wetland (China) and characterized using a polyphasic taxonomic approach. The cells were yellow-pigmented, rod-shaped, strictly aerobic and devoid of flagella, but showed gliding motility. Strain BLN2^T produced yellow, translucent, circular and convex colonies, with optimal growth at 30 °C and pH 7.0. The major respiratory quinone was menaquinone 6 (MK-6) and the predominant fatty acids were iso-C_{15:0}, summed feature 3 (comprising C_{16:1ω7c} and/or C_{16:1ω6c}), C_{16:0} 3-OH, C_{16:0}, iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH. The G+C content of the genomic DNA was 34.8 mol%. The 16S rRNA gene sequence analysis showed that strain BLN2^T belonged to the genus *Flavobacterium* and was most closely related to *Flavobacterium anhuiense* CGMCC 1.6859^T (97.0% sequence similarity). The DNA–DNA relatedness between strain BLN2^T and *F. anhuiense* CGMCC 1.6859^T was 25.7%. Based on the phenotypic data and phylogenetic inference presented, it is concluded that strain BLN2^T represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium phragmitis* sp. nov. is proposed. The type strain is BLN2^T (=DSM 23314^T=CGMCC 1.10370^T).

Since the description of the genus *Flavobacterium* was emended by Bernardet *et al.* (1996) to group Gram-staining-negative, aerobic, predominantly gliding, yellow-pigmented bacteria with DNA G+C contents in the range of 32–37 mol%, many additional species have been described from diverse environments, such as a glacier (Xin *et al.*, 2009), a eutrophic lake (Qu *et al.*, 2009), a hard water rivulet (Ali *et al.*, 2009), a freshwater lake (Kim *et al.*, 2009), marine algae (Miyashita *et al.*, 2010), stream sediment (Lee *et al.*, 2010) and sludge (Liu *et al.*, 2010), but no endophytic members of the genus have been described so far. In this paper, the taxonomic characterization of a novel bacterial strain BLN2^T is reported. The strain was isolated from within the roots of reeds (*Phragmites australis*). Biochemical and chemotaxonomic data, 16S rRNA gene sequencing and DNA–DNA relatedness analysis showed that this bacterial isolate constitutes a novel species of the genus *Flavobacterium*.

†These authors contributed equally to this work.

Abbreviations: MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BLN2^T is GU564236.

Reed roots were collected 15 cm below the water surface from the constructed Beijing Cuihu Wetland, China. Surface disinfection of the roots was conducted as previously described (Li *et al.*, 2010). Roots were crushed in a ceramic mortar, diluted with sterile double distilled water and plated on Luria–Bertani (LB) agar using the standard dilution plating technique. Strain BLN2^T was isolated after incubation for 16 h at 30 °C. The new isolate was maintained on LB agar slants at 4 °C and as a glycerol suspension (25% v/v) in LB broth at –70 °C.

Preparation of genomic DNA was performed according to the method of Marmur (1961). The 16S rRNA gene sequence of strain BLN2^T was determined by PCR amplification using the primers 27f and 1492r (Lane, 1991). The amplification product (1475 bp) was purified and cloned into a pMD18-T vector (Takara). Sequencing was performed by using an ABI Big Dye Primer Cycle Sequencing Ready Reaction kit and an Applied Biosystems 3730 DNA sequencer. The 16S rRNA gene sequence of strain BLN2^T was first analysed using the EzTaxon server 2.1 (Chun *et al.*, 2007) to determine the closest type strains. The sequence was then aligned with those of related species and phylogenetic trees were constructed using the neighbour-joining (NJ) (Saitou &

Nei, 1987) and maximum-parsimony (MP) methods in the MEGA 4.0 software (Tamura *et al.*, 2007). Bootstrap analysis (1000 replications) was used to evaluate the topology of the trees. Comparative 16S rRNA gene sequence analysis showed that strain BLN2^T was most closely related to *Flavobacterium anhuiense* CGMCC 1.6859^T (97.0 % sequence similarity) (Liu *et al.*, 2008), and shared 96.8, 96.0 and 95.7 % 16S rRNA gene sequence similarity with *Flavobacterium denitrificans* DSM 15936^T (Horn *et al.*, 2005), *Flavobacterium defluvii* DSM 17963^T (Park *et al.*, 2007) and *Flavobacterium johnsoniae* JCM 8514^T (Bernardet *et al.*, 1996), respectively. The NJ phylogenetic tree is presented in Fig. 1. The MP tree showed essentially the same topology (data not shown). Both trees showed that strain BLN2^T is a member of the genus *Flavobacterium*, joining the cluster comprising the type strains with higher sequence similarity with a bootstrap value of 94 %. Since *F. anhuiense* CGMCC 1.6859^T, *F. denitrificans* DSM 15936^T, *F. defluvii* DSM 17963^T and *F. johnsoniae* JCM 8514^T were the closest relatives and clustered together with strain BLN2^T, they were used as reference strains for further taxonomic studies.

Cell morphology was examined by light and scanning electron microscopy (scanning fibre-optic microscope; FEI QUANTA) and colony morphology was observed on LB agar. Growth was assessed at 4, 15, 25, 28, 30, 35, 37 and 42 °C on LB agar. Tolerance to NaCl and pH was tested for 7 days at 30 °C in LB broth containing 0, 1, 2, 3, 5 and 7 % NaCl (w/v) or adjusted to pH 4.0–11.0 (at 0.5 pH unit intervals), respectively, according to Zhou *et al.* (2007). Growth was also evaluated on trypticase-soy agar (TSA) and nutrient agar (NA).

Gram staining was performed using a standard procedure (Hucker & Conn, 1923). Endospore and flagellum staining were conducted according to the methods described by Smibert & Krieg (1994). Gliding motility, the production of flexirubin-type pigments and the adsorption of Congo red by colonies were tested as previously described by Bernardet *et al.* (2002). Production of catalase, oxidase,

indole and H₂S, degradation of agar, CM-cellulose, gelatin, starch and L-tyrosine and anaerobic growth were assessed according to the methods of Lewin & Lounsbury (1969). Urease activity was determined as described by Cowan & Steel (1965). Reduction of nitrate and hydrolysis of Tween 80, Tween 20 and aesculin were determined according to the methods of Lányi (1987). Methyl red and Voges–Proskauer reactions, utilization of sodium citrate and arginine dihydrolase activity were assessed as described by Dong & Cai (2001). Carbon-source utilization was assessed by using the GN2 MicroPlate system (Biolog) and the test was read after incubation at 30 °C for 24 h. Antibiotic sensitivity tests were performed after incubation at 30 °C for 72 h on LB agar containing the following antibiotics (µg ml⁻¹): ampicillin (5, 50, 100), chloramphenicol (5, 50, 100), gentamicin (5, 50, 100), kanamycin (5, 50, 100), carbenicillin (5, 50, 100), rifampicin (5, 50, 100), streptomycin (5, 50, 100) and hygromycin (5, 50, 100).

All conventional phenotypic tests cited were performed in parallel on the new isolate and on the four reference strains. The phenotypic characteristics of strain BLN2^T are given in the species description and Table 1 shows the features that differentiate the new strain from other closely related species of the genus *Flavobacterium*.

To determine the whole-cell fatty acid composition, strain BLN2^T and the four reference strains were grown on TSA at 30 °C for 24 h. Extraction and analysis of the cellular fatty acids were performed according to the standard protocol of the Sherlock Microbial Identification system (MIDI; Sasser, 1990) version 6.0 and peaks were identified using the peak naming table TSBA6 6.00. The complete fatty acid profiles of strain BLN2^T and its phylogenetic relatives are given in Table 2. The major fatty acids of strain BLN2^T were: iso-C_{15:0} (20.7 %), summed feature 3 (comprising C_{16:1}ω7c and/or C_{16:1}ω6c, 19.6 %), C_{16:0} 3-OH (9.1 %), C_{16:0} (8.9 %), iso-C_{17:0} 3-OH (8.7 %) and iso-C_{15:0} 3-OH (7.0 %). This fatty acid profile was very similar to those of the four reference strains, although there

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain BLN2^T and representative members of the genus *Flavobacterium*. GenBank accession numbers are given in parentheses. Numbers at branching points are bootstrap values >70 %. *Myroides odoratus* ATCC 4651^T was used as an outgroup. Bar, 0.01 substitution per nucleotide position.

Table 1. Differential characteristics of strain BLN2^T and related species of the genus *Flavobacterium*

Strains: 1, BLN2^T; 2, *F. anhuiense* CGMCC 1.6859^T; 3, *F. defluvii* DSM 17963^T; 4, *F. denitrificans* DSM 15936^T; 5, *F. johnsoniae* JCM 8514^T. +, Positive result; –, negative result; w, weak or delayed reaction; i, intermediate resistance. All data from this study, except the DNA G+C content of the reference strains. Data from Liu *et al.* (2008), Park *et al.* (2007), Horn *et al.* (2005), and Bernardet *et al.* (1996) that differed from our own data are shown in parentheses.

Characteristic	1	2	3	4	5
Growth at 37 °C	–	+	– (+)	+	–
Tolerance to 2 % NaCl	+	+	–	+	+
pH range for growth	5.5–9.0	5–9 (4–10)	5.5–8.5 (5–10)	5.5–8.5 (5.5–8.2)	5.5–9.0
Degradation of:					
Tween 20	+	–	+	–	+
Tween 80	+	–	–	–	+
Urea	–	–	– (+)	–	+
CM-cellulose	w	–	+	+	+
L-Tyrosine	–	+	–	–	+
Resistant to:					
Streptomycin (100 µg ml ^{–1})	+	+	i (–)	–	+
Ampicillin (100 µg ml ^{–1})	+	+	i	–	+
DNA G+C content (mol%)	34.8	31.4	33.5	34.6	35.2

were significant differences in the proportions of some fatty acids (e.g. C_{16:0} 3-OH, iso-C_{17:0} 3-OH and summed feature 3).

Table 2. Fatty acid composition (%) of strain BLN2^T and related species of the genus *Flavobacterium*

Strains: 1, BLN2^T; 2, *F. anhuiense* CGMCC 1.6859^T; 3, *F. defluvii* DSM 17963^T; 4, *F. denitrificans* DSM 15936^T; 5, *F. johnsoniae* JCM 8514^T. tr, Traces (<1 % of the total). All data from this study. Fatty acids amounting to <1 % of the total fatty acids in all strains are not shown.

Fatty acid	1	2	3	4	5
C _{14:0}	1.3	1.5	tr	tr	1.1
C _{16:0}	8.9	11.6	4.7	4.6	11.3
iso-C _{15:0}	20.7	20.2	19.9	20.4	21.0
iso-C _{16:0}	tr	tr	1.9	3.3	1.3
anteiso-C _{15:0}	2.3	2.0	3.3	4.1	2.2
C _{15:1} ω6c	2.4	2.6	1.9	4.2	2.1
C _{17:1} ω6c	2.5	2.0	4.2	4.2	2.5
C _{17:1} ω8c	1.4	1.4	3.2	3.6	1.7
iso-C _{15:1} G	2.0	2.7	1.6	3.1	1.5
iso-C _{17:1} ω9c	1.5	1.5	9.0	5.9	2.6
C _{15:0} 3-OH	1.8	1.9	2.2	1.9	1.4
C _{16:0} 3-OH	9.1	9.3	tr	1.3	6.2
C _{17:0} 3-OH	1.0	1.0	1.1	1.3	tr
iso-C _{15:0} 3-OH	7.0	5.5	10.2	7.2	5.5
iso-C _{16:0} 3-OH	1.6	1.3	1.4	2.0	1.5
iso-C _{17:0} 3-OH	8.7	5.8	15.6	12.5	8.9
Summed feature 3	19.6	21.5	7.6	7.4	20.1

*Summed features represent groups of two or three fatty acids that could not be separated by GLC using the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or C_{16:1}ω6c.

Isoprenoid quinone analysis was performed according to Komagata & Suzuki (1987), using cells harvested from a LB broth culture incubated at 30 °C for 2 days. The predominant isoprenoid quinone was menaquinone 6 (MK-6), in line with all other recognized members of the family *Flavobacteriaceae*.

The G+C content of the genomic DNA was determined using the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as a control. The G+C content of the DNA of strain BLN2^T was 34.8 mol%, a value within the range (32–37 mol%) originally reported for members of the genus *Flavobacterium* by Bernardet *et al.* (1996). Higher values have been reported subsequently for newly described species (e.g. 41 mol% for *Flavobacterium croceum* and *Flavobacterium saliperosum*; Park *et al.*, 2006; Wang *et al.*, 2006).

DNA–DNA hybridization experiments were carried out at the China General Microbiological Culture Collection Center (CGMCC) to evaluate the DNA–DNA relatedness between strain BLN2^T and its most closely related neighbour (*F. anhuiense* CGMCC 1.6859^T) using the optical renaturation rate method (Gillis *et al.*, 1970) and a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. The DNA–DNA relatedness between strain BLN2^T and *F. anhuiense* CGMCC 1.6859^T was 25.7 %, a value clearly below the 70 % threshold value generally accepted for species delineation (Stackebrandt *et al.*, 2002).

In view of the combined morphological, physiological, and chemotaxonomic data and of the phylogenetic inference presented here, it is evident that strain BLN2^T belongs to the genus *Flavobacterium*. However, differentiating phenotypic characteristics as well as phylogenetic and genomic distinctiveness distinguish it from recognized species of the genus *Flavobacterium*. Hence, strain BLN2^T is considered

to represent a novel species in the genus *Flavobacterium*, for which the name *Flavobacterium phragmitis* is proposed.

Description of *Flavobacterium phragmitis* sp. nov.

Flavobacterium phragmitis [phrag.mi'tis. L. n. *phragmites* -is a kind of reed growing in hedges and also a scientific generic name (*Phragmites*); L. gen. n. *phragmitis* of a reed, of *Phragmites australis*, the wetland plant from which the type strain was isolated].

Cells are Gram-staining-negative rods approximately 1.0–3.5 µm in length and 0.3–0.6 µm in diameter, strictly aerobic, non-spore-forming and devoid of flagella, but motile by gliding. Cells are oxidase-negative and catalase-positive. Colonies are yellow, translucent, circular with regular edges and convex. Growth occurs on LB, TSA and NA. Growth occurs at 15–35 °C (optimum, 30 °C), in the presence of 0–3% (w/v) NaCl (optimum, 1%) and at pH 5.5–9 (optimum, pH 7.0). Flexirubin-type pigments are produced, but Congo red is not adsorbed by colonies. Nitrate is not reduced to nitrite. Urease activity is not detected, but arginine dihydrolase activity is present. Starch, Tween 20, Tween 80, aesculin and gelatin are hydrolysed, CM-cellulose is weakly hydrolysed, but agar and L-tyrosine are not hydrolysed. Sodium citrate is not utilized. Indole and H₂S are not produced and the methyl red and Voges–Proskauer reactions are negative. The following carbon sources are utilized in the GN2 MicroPlate: α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, lactulose, melibiose, methyl β-D-glucoside, raffinose, L-rhamnose, sucrose, acetic acid, D-galacturonic acid, D-glucuronide, α-butanoic acid, α-oxopentanoic acid, DL-lactic acid, propionic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, hydroxy-L-proline, L-proline, L-ornithine, L-serine, L-threonine and thymidine. The other substrates in the GN2 MicroPlate are not utilized. Susceptible to (µg ml⁻¹) rifampicin (5) and chloramphenicol (50), but resistant to (µg ml⁻¹) kanamycin (100), carbenicillin (100), streptomycin (100), hygromycin (50), gentamicin (100) and ampicillin (100). The major respiratory quinone is menaquinone-6. The predominant fatty acids (>6% of the total) are iso-C_{15:0}, summed feature 3 (comprising C_{16:1}ω7c and/or C_{16:1}ω6c), C_{16:0} 3-OH, C_{16:0} iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH.

The type strain, BLN2^T (=DSM 23314^T=CGMCC 1.10370^T), was isolated from within the roots of reeds (*Phragmites australis*) collected from the constructed Beijing Cuihu Wetland Park, China. The DNA G+C content of the type strain is 34.8 mol%.

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