Flectobacillus lacus sp. nov., isolated from a highly eutrophic pond in Korea

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A Gram-negative, almost straight, rod-shaped, non-motile bacterium, designated CL-GP79^T, was isolated from a highly eutrophic pond located within the campus of Seoul National University, Korea. Phylogenetic analyses based on 16S rRNA gene sequencing showed that strain CL-GP79^T belongs to the family *Flexibacteraceae*, with *Flectobacillus major* as its closest relative (similarity of 95.7%). The cellular fatty acids consist mainly of C_{16:1}ω5c (mean ± SD, 26.9 ± 10.8%), iso-C_{15:0} 2-OH and/or C_{16:1}ω7c (19.2 ± 2.3%) and iso-C_{15:0} (12.1 ± 1.3%). The DNA G + C content was found to be 38.3 mol%. Phenotypic, chemotaxonomic and phylogenetic analyses indicated that strain CL-GP79^T could be assigned to the genus *Flectobacillus*, but could be distinguished from *F. major*. The strain CL-GP79^T therefore represents a novel species, for which the name *Flectobacillus lacus* sp. nov. is proposed, with CL-GP79^T (=KCCM 42271^T = JCM 13398^T) as the type strain.

The genus *Flectobacillus* was proposed by Larkin *et al.* (1977). Later, two species ([*Flectobacillus*] *marinus* and [*Flectobacillus*] *glomeratus*) assigned to the genus *Flectobacillus* (Borrall & Larkin, 1978; McGuire *et al.*, 1987) were reclassified as *Cyclobacterium marinum* (Raj & Maloy, 1990) and *Polaribacter glomeratus* (Gosink *et al.*, 1998), respectively. At present, *Flectobacillus major* is the only accepted *Flectobacillus* species with a validly published name. The species was first described as '*Microcycylus major*' by Gromov (1963) and is the type species of the genus. In this study, we isolated a strain from a highly eutrophic pond and found that the 16S rRNA gene sequence of the isolate was related to those of species of *Flectobacillus*. Here we describe its phenotypic, physiological and chemotaxonomic features.

In November 2003, a surface freshwater sample was collected, using a sterile tube, from a highly eutrophic pond, Gongdae Pond, located in the campus of Seoul National University. The sample, at ambient temperature, was brought back to the laboratory within 15 min; 10–100 µl sample was spread on plates containing R2A agar [0.05% (w/v) yeast extract, 0.05% (w/v) peptone, 0.05% (w/v) Casamino acids, 0.05% (w/v) glucose, 0.05% (w/v) starch, 0.03% (w/v) sodium pyruvate, 0.03% (w/v) K₂HPO₄, 0.005% (w/v) MgSO₄ and 1.5% (w/v) agar, pH 7; Difco] and incubated at 20 °C for 1 week. Thirteen colonies were randomly selected and subsequently purified four times on R2A agar at 20 °C. The

isolates were initially characterized on the basis of their 16S rRNA gene sequences. Two of the isolates showed relatively low levels of sequence similarity (94–96%) to previously described species from different genera: one of these is strain CL-GP79^T, colonies of which are pink on R2A agar. The strain was stored at –80 °C in R2A broth (Difco) supplemented with 30% (v/v) glycerol until required for further analyses.

The temperature range for growth was tested on the basis of colony formation on R2A plates incubated at temperatures ranging from 5 to 45 °C, using increments of 5 °C. The pH range (pH 5–10, using increments of 1 pH unit) for growth was determined by assessing changes in OD₆₀₀ over the incubation period (up to 7 days) in R2A broth. The final pH was adjusted using NaOH and HCl solutions. To test the salt tolerance, R2A agar (Difco) and nutrient agar [0.3% (w/v) beef extract, 0.5% (w/v) peptone, 1.5% (w/v) agar, pH 7; Difco] containing various concentrations of NaCl (0, 0.1, 0.5, 1, 1.5, 2, 3, 5, 7%, w/v) were used. Growth of the isolate occurred at temperatures between 10 and 35 °C, with optimal growth around 25–30 °C. The isolate did not grow on either R2A agar or nutrient agar supplemented with 0.5–7% (w/v) NaCl. The isolate grew at pH values from 6 to 9; optimal growth occurred at pH 7.

Morphological and physiological tests were also performed. Gram-staining was performed as described by Smibert & Krieg (1994). The cellular morphology and the motility of the isolate were observed by phase-contrast microscopy. Transmission electron microscopy (EX2; JEOL) was used to establish whether flagella were present. Anaerobic growth was checked on R2A agar using the GasPak anaerobic system

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-GP79^T is DQ112352.

Selected phenotypic characteristics of strain CL-GP79^T and *Flectobacillus major* DSM 103^T are available in a supplementary table in IJSEM Online.

(BBL). The pigment absorption spectrum between 200 and 800 nm was determined by using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech) after ethanol extraction (Gosink *et al.*, 1998). The sample was further alkalized with 0.1 vol. 0.1 M NaOH, and the absorption spectrum was obtained again to check for the presence of flexirubin pigments (Gosink *et al.*, 1998). The cells were Gram-negative, aerobic, straight to slightly curved, non-motile rods with no flagella (Fig. 1). Other morphological characteristics are shown in Table 1. Pigment extracts of strain CL-GP79^T showed an absorption maximum at 477–481 nm and a spectrum similar to that of *F. major* DSM 103^T (i.e. the maximum absorption peak at 480–482 nm determined in this study; data not shown). Flexirubin pigments were not detected in either strain.

Catalase and oxidase activities were determined according to the protocols described by Smibert & Krieg (1994); gelatinase, amylase and nitrate reductase activities and the Voges–Proskauer test were investigated as described by Hansen & Sørheim (1991). The utilization of organic compounds as sole carbon sources was tested on the basis of colony formation on a minimal medium (Nikitin *et al.*, 2004) to which NaCl and CaCl₂ had not been added. Growth was scored as negative when it was equal to, or less than, that in the negative control (i.e. lacking a carbon source) after incubation at 25 °C for 1 month. Additional biochemical tests were performed by using API 20NE, API ZYM and API 50 CH strips (bioMérieux) according to the manufacturer's instructions. Sensitivities to antibiotics were assessed using disc-diffusion methodology (Bauer *et al.*, 1966). All phenotypic tests for strain CL-GP79^T were performed simultaneously with *F. major* DSM 103^T as a reference strain. Strain CL-GP79^T was positive for catalase, oxidase, gelatinase and

amylase, but negative in the Voges–Proskauer test. In the API tests, strain CL-GP79^T showed a physiological profile that was very similar to that of *F. major* DSM 103^T, i.e. they gave the same results for 25 out of 27 tests of enzyme activities, 39 out of 49 tests for acid production, and 8 out of 12 tests for carbon assimilation (Table 1; see also Supplementary Table S1 available in IJSEM Online). Features that differentiated strain CL-GP79^T from *F. major* DSM 103^T were as follows: acid was produced from D-arabitol and 5-ketogluconate, but not from DL-arabinose, D-xylose, L-rhamnose, amygdalin, arbutin, D-lyxose or L-fucose. Strain CL-GP79^T assimilated adipate, but did not assimilate L-arabinose, D-mannose, N-acetylglucosamine, D-maltose or gluconate. Strain CL-GP79^T was not capable of utilizing DL-cysteine or succinate as a sole carbon source. The other results of the biochemical and physiological tests are given in Table 1, Supplementary Table S1 and the species description.

The DNA G+C content of strain CL-GP79^T, determined by HPLC analysis (Mesbah *et al.*, 1989), was 38.3 mol%. This is similar to the values obtained for *F. major* (39.5–40.3 mol%; Larkin *et al.*, 1977). The whole-cell fatty acid methyl esters in strains CL-GP79^T and *F. major* DSM 103^T, grown under the same conditions (on R2A plates at 25 °C for 2 days), were analysed by gas chromatography according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms (Seoul, Korea). The fatty acid composition of strain CL-GP79^T was similar to that of *F. major* DSM 103^T (Table 2). The major fatty acids of strain CL-GP79^T were found to be C_{16:1}ω5c (mean ± SD, 26.9 ± 10.8 %), iso-C_{15:0} 2-OH and/or C_{16:1}ω7c (19.2 ± 2.3 %) and iso-C_{15:0} (12.1 ± 1.3 %). However, a significant amount (14.7 ± 0.1 %) of an unknown fatty acid with a carbon chain length equivalent to 15:731 was found in *F. major* DSM 103^T but not in strain CL-GP79^T (Table 2). These results support the affiliation of strain CL-GP79^T to the genus *Flectobacillus*. It is noteworthy that the fatty acid profile of *F. major* DSM 103^T obtained in this study is quite distinct from that of *F. major* reported by Urakami & Komagata (1986), possibly because of differences in the cultivation conditions and techniques used.

To establish the phylogenetic relationships within the family *Flexibacteraceae*, the sequence of the 16S rRNA gene of strain CL-GP79^T was determined: the 16S rRNA gene was PCR-amplified from a single colony by using *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using the AccuPrep PCR purification kit (Bioneer) and direct sequence determination of the purified 16S rRNA gene was performed with an Applied Biosystems automatic sequencer (ABI3730XL) at Macrogen Corp. (Seoul, Korea). The 16S rRNA gene sequence of strain CL-GP79^T was compared with 16S rRNA gene sequences available in GenBank, using a BLASTN (Altschul *et al.*, 1990) search. The sequence of strain CL-GP79^T was aligned manually with representative sequences of the family *Flexibacteraceae* obtained from the GenBank

Fig. 1. Transmission electron micrograph of negatively stained cells of strain CL-GP79^T. Bar, 5 μm.

Table 1. Differential characteristics of *Flectobacillus* species

Strains: 1, *F. lacus* CL-GP79^T; 2, *F. major* DSM 103^T. Data are from Larkin *et al.* (1977), Larkin & Borrall (1984), Raj & Maloy (1990), Nikitin *et al.* (1994), Gosink *et al.* (1998) and this study. Both strains comprise Gram-negative, strictly aerobic, almost straight rods, are non-motile and are able to grow on R2A and nutrient agar containing 0% or 0.1% (w/v) NaCl, but are unable to grow in the presence of 2–7% (w/v) NaCl. Colonies of both strains are pale pink to rose in colour, round, convex, smooth and entire. +, Positive; –, negative; w, weakly positive; v, variable.

Characteristic	1	2
Habitat	Highly eutrophic pond	Freshwater
Colony diameter (mm)	2–6	4–6
Cell size (length × width, µm)	4.7–10.0 × 0.3–0.6	2.0–5.0 × 0.6–1.0
Temperature range (optimum) (°C)	10–35 (25–30)	4–30 (20–30)
pH range (optimum)	6–9 (7)	6–8 (7)
Growth in the presence of NaCl at:		
0.5% (w/v)	–	+*
1.0% (w/v)	–	+*
1.5% (w/v)	–	+*
DNA G+C content (mol%)	38.3	39.5–40.3
Biochemical tests		
Catalase	+	w
Nitrate reductase	v†	–
β-Galactosidase	+	v
Urease	–	v
Aesculin hydrolysis	+	v
Acid production from:		
D-Arabinose	–	+
L-Arabinose	–	+
D-Ribose	–	v
D-Xylose	–	+
L-Rhamnose	–	+
D-Cellobiose	+	v
Utilization as a sole carbon source		
N-Acetylglucosamine	+	–
DL-Aspartate	–	v
DL-Cysteine	–	+
Ethanol	–	v
Pyruvate	+	v
Succinate	–	+
Antibiotics (µg per disc)‡		
Kanamycin (30)	R	S
Erythromycin (15)	R	S
Mitomycin (1)	S	R

*Test performed in this study.

†Tested according to Hansen & Sørheim (1991) in this study and showed a positive response; negative in API 20NE test.

‡R, Resistant; S, susceptible.

and Ribosomal Database Project (Cole *et al.*, 2003) databases, using known 16S rRNA secondary structure information. Phylogenetic trees were obtained by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes &

Cantor (1969). The robustness of tree topologies was assessed by using bootstrap analyses based on 1000 replications (for the neighbour-joining and maximum-parsimony methods) or 100 replications (for the maximum-likelihood method). Alignment analysis was carried out using the jPHYDIT program (Jeon *et al.*, 2005) and phylogenetic analyses were carried out using MEGA 3 (Kumar *et al.*, 2004).

Table 2. Cellular fatty acid compositions (%) of *Flectobacillus* species

Strains: 1, *F. lacus* CL-GP79^T; 2, *F. major* DSM 103^T. Both strains were cultured on R2A plates at 25 °C for 2 days. Values are percentages of total fatty acids, expressed as means $\pm$ SD. ECL, Equivalent chain-length; tr, trace amount (<1 %); –, not detected. Trace amounts of C_{17:0}, C_{18:0}, C_{14:1} ω 5c, iso-C_{17:0}, iso-C_{17:1} ω 9c, anteiso-C_{17:0}, C_{14:0} 2-OH, C_{15:0} 2-OH, iso-C_{16:0} 3-OH, C_{17:0} 2-OH, C_{13:0} 3-OH and/or iso-C_{15:1} I, ECL 11:543, ECL 13:565 and ECL 16:582 were observed in both strains.

Fatty acid	1	2
C _{13:0}	tr	–
C _{14:0}	1.7 $\pm$ 0.2	1.0 $\pm$ 0.01
C _{15:0}	4.3 $\pm$ 0.3	tr
C _{16:0}	1.3 $\pm$ 0.2	1.0 $\pm$ 0.3
C _{20:0}	–	tr
C _{15:1} ω 6c	4.2 $\pm$ 1.7	tr
C _{15:1} ω 8c	–	tr
C _{16:1} ω 5c	26.9 $\pm$ 10.8	36.4 $\pm$ 1.1
C _{17:1} ω 6c	1.5 $\pm$ 0.9	tr
C _{17:1} ω 8c	tr	–
C _{18:1} ω 5c	–	tr
C _{18:1} ω 9c	tr	–
iso-C _{13:0}	tr	–
anteiso-C _{13:0}	tr	–
iso-C _{15:0}	12.1 $\pm$ 1.3	9.5 $\pm$ 0.8
anteiso-C _{15:0}	2.4 $\pm$ 0.2	2.5 $\pm$ 0.4
iso-C _{16:0}	–	tr
iso-C _{14:0} 3-OH	–	tr
C _{15:0} 3-OH	3.5 $\pm$ 1.3	–
iso-C _{15:0} 3-OH	3.8 $\pm$ 5.3	3.7 $\pm$ 0.2
C _{16:0} 2-OH	–	tr
C _{16:0} 3-OH	2.9 $\pm$ 0.5	2.9 $\pm$ 0.4
C _{17:0} 3-OH	tr	–
iso-C _{17:0} 3-OH	3.6 $\pm$ 0.01	4.6 $\pm$ 0.8
C _{14:0} 3-OH and/or iso-C _{16:1} I	1.4 $\pm$ 0.4	tr
iso-C _{15:0} 2-OH and/or C _{16:1} ω 7c	19.2 $\pm$ 2.3	10.8 $\pm$ 1.2
anteiso-C _{17:1} B and/or iso-C _{17:1} I	tr	2.5 $\pm$ 0.1
ECL 14:959	5.0 $\pm$ 0.03	1.6 $\pm$ 0.4
ECL 15:731	–	14.7 $\pm$ 0.1

and PAUP* 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio tests in MODELTEST, version 3.04 (Posada & Crandall, 1998). An almost complete 16S rRNA gene sequence of strain CL-GP79^T (1369 bp) was obtained. Strain CL-GP79^T was closely related to *F. major* (95.7 % similarity). The tree topologies inferred from the three tree-making algorithms showed that strain CL-GP79^T formed a robust cluster with species of the genus *Flectobacillus* (Fig. 2). This grouping was supported by high bootstrap values (neighbour-joining and maximum-likelihood methods, 100 %; maximum-parsimony method, 98 %). Thus, it is clear that our isolate belongs to the genus *Flectobacillus*. However, the relatively low level of similarity (95.7 %) between the 16S rRNA gene sequences of strain

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between *F. lacus* CL-GP79^T and other related genera belonging to the *Flexibacteraceae*. Only those bootstrap values (expressed as percentages of 1000 replications) above 70 % are shown at nodes. Filled circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 0.02 nucleotide substitutions per site.

CL-GP79^T and *F. major* ATCC 29496^T indicated that strain CL-GP79^T represents a novel species in the genus (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001).

Phylogenetic analyses based on 16S rRNA gene sequences, chemotaxonomic data and physiological features suggest that strain CL-GP79^T belongs to the genus *Flectobacillus* and represents a novel species of the genus. Therefore, strain CL-GP79^T should be assigned to a novel species of the genus *Flectobacillus*, for which the name *Flectobacillus lacus* sp. nov. is proposed.

Description of *Flectobacillus lacus* sp. nov.

Flectobacillus lacus (la'cus. L. gen. n. *lacus* of a lake or pond, referring to the isolation of the type strain).

Gram-negative, aerobic, almost straight rods, approximately 0.3–0.6 μ m wide and 4.7–10.0 μ m long. Cells are non-motile and do not possess flagella. Colonies on R2A agar are pale pink to rose in colour and are round (2–6 mm in diameter), convex and smooth with entire margins. Growth occurs at 10–35 °C and pH 6–9; optimal temperature and pH for growth are 25–30 °C and pH 7, respectively. Grows well on R2A and nutrient agar without NaCl or with 0.1 % (w/v) NaCl; no growth occurs in the presence of $\geq$ 0.5 % (w/v) NaCl. Grows on R2A medium and nutrient medium, but not on MacConkey medium. Flexirubin pigments are absent. Positive for catalase, oxidase, gelatinase and amylase activities; negative in the Voges–Proskauer test. Positive for α -galactosidase, β -galactosidase, aesculin hydrolysis, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine, valine and cystine arylamidases, trypsin, α -chymotrypsin, naphthol-phosphohydrolase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Negative for indole

production, arginine dihydrolase, lipase (C14) and β -glucuronidase. Other phenotypic properties (e.g. acid production, utilization of carbon, sensitivity to antibiotics) are shown in Table 1 and Supplementary Table S1. The DNA G + C content is 38.3 mol%. The major fatty acids are C_{16:1} ω 5c (mean $\pm$ SD, 26.9 $\pm$ 10.8 %), iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c (19.2 $\pm$ 2.3 %) and iso-C_{15:0} (12.1 $\pm$ 1.3 %).

The type strain, CL-GP79^T (=KCCM 42271^T=JCM 13398^T), was isolated from a highly eutrophic pond, Gongdae Pond, located in the campus of Seoul National University, Korea.

Acknowledgements

We acknowledge the expert technical support of Ms M. J. Kang in electron microscopy carried out at the National Center for Inter-university Research Facilities at Seoul National University. This work was supported (in part) by the BK21 project of the Korean Government.

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