Flectobacillus roseus sp. nov., isolated from freshwater in Taiwan

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Two Gram-negative, aerobic, pale pink/rose-coloured, rod-shaped, non-motile bacterial strains, designated GFA-11^T and RG-4, were isolated from a freshwater fish culture pond and a freshwater spring sample, respectively. Based on characterization by using a polyphasic approach, the two strains showed highly similar phenotypic, physiological and genetic characteristics. They shared 99.9% 16S rRNA gene sequence similarity and 89–94% DNA–DNA relatedness, suggesting that they represent a single genomic species. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains GFA-11^T and RG-4 formed a monophyletic branch at the periphery of the evolutionary radiation occupied by the genus *Flectobacillus* within the family *Flexibacteraceae*. Their closest neighbours were *Flectobacillus major* DSM 103^T (97.7% 16S rRNA gene sequence similarity) and *Flectobacillus lacus* CL-GP79^T (95.9%). Levels of DNA–DNA relatedness between the two novel strains and the type strains of *F. major* and *F. lacus* were less than 70%. The results of physiological and biochemical tests allowed clear phenotypic differentiation of strains GFA-11^T and RG-4 from recognized members of the genus *Flectobacillus*. The predominant fatty acid constituents of strain GFA-11^T were C_{16:1}ω5c (40.2% of the total) and iso-C_{15:0} (15.0%). The DNA G+C content of strain GFA-11^T was 39.7 mol%. On the basis of 16S rRNA gene sequence analysis and chemotaxonomic and physiological data, strains GFA-11^T and RG-4 are considered to represent a novel species of the genus *Flectobacillus*, for which the name *Flectobacillus roseus* sp. nov. is proposed. The type strain is GFA-11^T (=BCRC 17834^T=LMG 24501^T).

The genus *Flectobacillus*, a member of the family *Flexibacteraceae*, was proposed by Larkin *et al.* (1977). Two species ([*Flectobacillus*] *marinus* and [*Flectobacillus*] *glomeratus*) that were assigned initially to this genus (Borrall & Larkin, 1978; McGuire *et al.*, 1987) were reclassified as *Cyclobacterium marinum* (Raj & Maloy, 1990) and *Polaribacter glomeratus* (Gosink *et al.*, 1998), respectively. At the time of writing, the genus comprised two recognized species, *Flectobacillus major* and *Flectobacillus lacus*. *F. major*, the type species of the genus,

was first described as ‘*Microcycylus major*’ by Gromov (1963). *F. lacus* was isolated from a highly eutrophic pond in Korea (Hwang & Cho, 2006). The present study focuses on the polyphasic taxonomic study of two *Flectobacillus*-like strains isolated from two different freshwater ecosystems in Taiwan.

Strain RG-4 was isolated from a freshwater spring located in Ligang Township, Pingtung County, southern Taiwan, on R2A agar (BD Difco) at 25 °C. Strain GFA-11^T was isolated from a freshwater fish culture pond located in Taoyuan County, northern Taiwan, on R2A agar at 25 °C. The two isolates were routinely maintained on R2A agar at 25 °C. Strains GFA-11^T and RG-4 on R2A agar showed

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains GFA-11^T and RG-4 are EU420062 and EU921645, respectively.

similar colony morphology with pale pink to rose colouration. *F. major* DSM 103^T and *F. lacus* CL-GP79^T, obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and KCCM (Korean Culture Center of Microorganisms), respectively, were used as reference strains. Strains were preserved at -80°C in R2A broth with 20 % (v/v) glycerol or by lyophilization with 20 % (w/v) skimmed milk.

The morphology of bacterial cells was observed during the lag, exponential and stationary phases of growth under a phase-contrast microscope (DM 2000; Leica). Flagellar staining was performed by using the Spot Test Flagella Stain (BD Difco). The Gram reaction was determined by using the Gram Stain Set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Accumulation of poly- β -hydroxybutyrate granules was investigated by light microscopy after staining the cells with Sudan black (Gerhardt *et al.*, 1994). Colony morphology was examined under a stereo microscope (SMZ 800; Nikon) after incubating the cells on R2A agar at 25°C for 2 days. Cellular pigments were extracted with acetone/methanol (7:2, v/v) from cultures grown on R2A agar, and the absorption spectra were determined with a scanning UV/visible spectrophotometer (Helios Delta; Thermo Fisher Scientific). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992) and Bernardet *et al.* (2002).

The optimum pH range for growth was examined by measuring the OD₅₉₅ of the cultures grown in R2A broth, adjusted to various pH (4–10 at intervals of 1.0 pH units) by using appropriate biological buffers (Chung *et al.*, 1995). Tolerance to NaCl was tested in R2A broth, adjusted to various NaCl concentrations (0, 0.5 and 1.0–4.0 %, w/v, at 1.0 % intervals). The temperature range for growth (4, 10, 15, 20, 25, 30, 35, 40 and 45°C) was examined in R2A broth. Growth under anaerobic conditions was checked using the Oxoid AnaeroGen system. Catalase, oxidase, DNase and lipase activities, and hydrolysis of starch, casein, and Tweens 20, 40, 60 and 80 were determined by using standard techniques (Gerhardt *et al.*, 1994). Additional biochemical tests were performed with the API 20NE, API ZYM, API 50 CH (all bioMérieux) and Biolog GN2 (Biolog) microtest systems according to the methods outlined by the manufacturers. Sensitivity of the strains to different antibiotics was tested after spreading cells (0.5 McFarland) on R2A agar plates. The antibiotic discs (Oxoid) used contained ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 μg), streptomycin (10 μg), sulfamethoxazole (23.75 μg), plus trimethoprim (1.25 μg) or tetracycline (30 μg). The effect of antimicrobial agents on cellular growth was assessed after 3 days incubation and susceptibility was scored based on the distance between the edges of the clear zone and the disc.

Cells of strains GFA-11^T and RG-4 were Gram-negative, aerobic, non-spore-forming, straight to slightly curved,

non-motile rods. Pigment extracts of strains GFA-11^T and RG-4 showed a major absorption peak at 475–480 nm, similar to the absorption peak values reported for *F. major* DSM 103^T (480–482 nm) and *F. lacus* CL-GP79^T (477–481 nm) (Hwang & Cho, 2006). Flexirubin-type pigments were not detected. Detailed morphological, physiological and biochemical characteristics of strains GFA-11^T and RG-4 are provided in the species description below and in Table 1. Carbon substrate utilization tests with various sugars and organic acids as substrates revealed few positive results.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). The resultant 16S rRNA gene sequences were compared with sequences from the GenBank database. Multiple sequence alignments of the 16S rRNA genes of strains GFA-11^T, RG-4 and their closest relatives were performed by using BioEdit software (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred via the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. Evolutionary distance matrices were generated for the neighbour-joining tree by using the Kimura two-parameter distance model (Kimura, 1983) and bootstrap analysis was performed based on 1000 resamplings.

Nearly complete 16S rRNA gene sequences were obtained for strains GFA-11^T and RG-4 (1385 nt for the two strains). 16S rRNA gene sequence similarity between the two strains was 99.9 %. Strains GFA-11^T and RG-4 were related most closely to *F. major* DSM 103^T (97.7 % 16S rRNA gene sequence similarity) and *F. lacus* CL-GP79^T (95.9 % similarity). Phylogenetic analysis based on 16S rRNA gene sequences indicated that the novel strains belonged to the family *Flexibacteraceae*, phylum *Bacteroidetes*, and fell within the evolutionary radiation of the genus *Flectobacillus* (Fig. 1). Levels of 16S rRNA gene sequence similarity between strains GFA-11^T and RG-4 and the type strains of all other recognized species in the *Bacteroidetes* were not more than 95 %. Overall tree topologies obtained by using the neighbour-joining, maximum-parsimony and maximum-likelihood methods were similar (data not shown).

Whole-genome DNA–DNA hybridization experiments were carried out with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The level of DNA–DNA relatedness was calculated from triplicate experiments. Levels of DNA–DNA relatedness between strains GFA-11^T and RG-4 were 89–94 %, indicating that they are members of the same genomic species (Wayne *et al.*, 1987). However, strain GFA-11^T showed only 31 ± 17 % (mean $\pm$ SD) and 22 ± 14 % DNA–DNA relatedness to *F. major* DSM 103^T and *F. lacus* CL-GP79^T, respectively.

For chemotaxonomic analyses, strain GFA-11^T was grown on R2A agar for 48 h at 25°C . Fatty acid methyl esters

Table 1. Differential characteristics between strains GFA-11^T and RG-4 (*F. roseus* sp. nov.) and recognized *Flectobacillus* species

All data were obtained in the present study, except where indicated. +, Positive; –, negative; v, variable. All are positive for utilization of methyl β -D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, methyl α -D-glucopyranoside, aesculin, D-salicin, maltose, D-lactose, α -melibiose, sucrose, trehalose, inulin, α -melezitose, α -raffinose, starch, gentiobiose and turanose, but negative for utilization of glycerol, erythritol, D-adonitol, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, xylitol, D-tagatose, D-fucose, L-arabitol, gluconate and 2-ketogluconate (API 50 CH). All are susceptible to gentamicin (10 μ g).

Characteristic	Strains GFA-11 ^T and RG-4*	<i>F. major</i> DSM 103 ^T	<i>F. lacus</i> CL-GP79 ^T
Indole production	–	+	–
α -Mannosidase	v(–)	+	+
α -Fucosidase	v(–)	+	+
Assimilation of (API 20NE):			
Arabinose	–	+	–
N-Acetylglucosamine	–	+	–
Gluconate	–	+	–
Adipate	–	–	+
Assimilation of (API 50 CH):			
D-Xylose	+	–	–
Methyl α -D-mannopyranoside	v(–)	+	+
Amygdalin	v(–)	–	+
Arbutin	+	–	+
Glycogen	+	–	–
D-Lyxose	–	–	+
L-Fucose	v(+)	–	+
D-Arabitol	–	+	–
5-Ketogluconate	+	+	–
Susceptibility to (μ g per disc):†			
Nalidixic acid (30)	S	S	R
Kanamycin (10)	R	S	R
DNA G + C content (mol%)	39.7–40.1 (39.7)	39.5–40.3‡	38.3‡

*Data in parentheses are for the type strain.

†S, Susceptible; R, resistant.

‡Data from Hwang & Cho (2006).

were prepared, separated and identified by using the Microbial Identification System (Microbial ID; Sasser, 1990). The major cellular fatty acid constituents of strain GFA-11^T were C_{16:1} ω 5c (40.2 % of the total) and iso-C_{15:0} (15.0 %) (Table 2). The overall cellular fatty acid profile of strain GFA-11^T was consistent with those reported for *F. major* and *F. lacus*. However, strain GFA-11^T could be differentiated from the type strains of *F. major* and *F. lacus* by the absence of ECL 14.959 and ECL 15.731 (Table 2). The DNA G + C content was determined from triplicate experiments by using HPLC as described by Mesbah *et al.* (1989). The DNA G + C contents of strains GFA-11^T and RG-4 were 39.7 $\pm$ 1.5 mol% (mean $\pm$ SD) and 40.1 $\pm$ 1.2 mol%, respectively (Table 1).

Based on 16S rRNA gene sequence comparisons, strains GFA-11^T and RG-4 occupied a separate phylogenetic position in the genus *Flectobacillus*. This was confirmed by their unique combination of chemotaxonomic char-

acteristics (Table 2) and biochemical traits (Table 1). It is clear from the genotypic and phenotypic data presented that strains GFA-11^T and RG-4 represent a single novel species of the genus *Flectobacillus*, for which the name *Flectobacillus roseus* sp. nov. is proposed.

Description of *Flectobacillus roseus* sp. nov.

Flectobacillus roseus (ro.se'us. L. masc. adj. *roseus* rose-coloured, pink).

Cells are Gram-negative, aerobic, non-motile, straight rods (0.3–0.5 μ m in diameter and 1.5–2.5 μ m in length). Accumulation of poly- β -hydroxybutyrate granules is not observed. Colonies on R2A agar are pale pink to rose coloured, circular (1.5–2.5 mm in diameter), convex and smooth with entire margins. Growth occurs at 10–40 °C and pH 7–8; optimal temperature and pH for growth are 30 °C and pH 7. Good growth is observed on R2A and nutrient agar without NaCl or with 0.5 % (w/v) NaCl; no

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank/EMBL/DBJ (accession numbers are given in parentheses) constructed after multiple alignments of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distance calculations (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were performed by using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar *et al.*, 2004). Bootstrap values based on 1000 replications are given as percentages at branch points (only values >50 % are shown). Bar, 0.01 substitutions per nucleotide position.

growth is seen in the presence of 2.0 % (w/v) NaCl or on MacConkey agar. Flexirubin-type pigments are not detected. Positive for catalase and oxidase. Negative for DNase and lipase (corn oil), and hydrolysis of Tweens 20, 40, 60, 80 and skimmed milk. Hydrolysis of starch is strain specific. Positive reactions (API 20NE) are seen for β -glucosidase and β -galactosidase, but negative reactions for nitrate reduction, indole production, glucose acidification, arginine dihydrolase, urease, protease, and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl acetate. Positive enzyme reactions (API ZYM) are seen for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase and *N*-acetyl- β -glucosaminidase; negative enzyme reactions are seen for lipase (C14) and β -glucuronidase. Enzyme reactions for α -mannosidase and α -fucosidase are strain specific. The following compounds are utilized in the API 50 CH test system: D-arabinose, D-xylose, methyl β -D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, methyl α -D-glucopyranoside, arbutin, aesculin, D-salicin, cellobiose, maltose, D-lactose, α -melibiose, sucrose, trehalose, inulin, α -melezitose, α -raffinose, starch, glycogen, gentiobiose, turanose and 5-ketogluconate. Negative for assimilation of glycerol, erythritol, D-ribose, L-xylose, adonitol, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate and 2-ketogluconate. Utilization of the

remaining substrates (L-fucose, L-arabinose, methyl α -D-mannopyranoside, *N*-acetylglucosamine and amygdalin) is strain specific. The following compounds are oxidized in the Biolog GN II test system: α -cyclodextrin, dextrin, glycogen, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, α -D-lactose, lactulose, maltose, D-mannose, melibiose, D-psicose, raffinose, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, DL-lactic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, uridine and α -D-glucose 1-phosphate. The following compounds are not oxidized: Tweens 40 and 80, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, *myo*-inositol, D-mannitol, L-rhamnose, D-sorbitol, xylitol, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketoglutaric acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglyutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol and DL- α -glycerol phosphate. Sensitive to tetracycline, rifampicin, gentamicin, penicillin G, chloramphenicol, nalidixic acid, novobiocin, streptomycin, ampicillin, and sulfamethoxazole

Table 2. Fatty acid profiles of strain GFA-11^T (*F. roseus* sp. nov.) and recognized *Flectobacillus* species

Strain GFA-11^T was cultivated on R2A plates at 25 °C for 48 h, similar to the conditions described for *F. lacus* CL-GP79^T and *F. major* DSM 103^T by Hwang & Cho (2006). Values are percentages of total fatty acid content. ECL, equivalent chain-length; —, not detected; tr, trace amount (<1%). For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The *cis* isomer is indicated by the suffix *c*.

Fatty acid	Strain GFA-11 ^T	<i>F. major</i> DSM 103 ^T	<i>F. lacus</i> CL-GP79 ^T
C _{13:0}	—	—	tr
C _{14:0}	5.7	1.0	1.7
C _{15:0}	tr	tr	4.3
C _{16:0}	6.5	1.0	1.3
C _{20:0}	—	tr	—
C _{15:1ω6<i>c</i>}	tr	tr	4.2
C _{15:1ω8<i>c</i>}	—	tr	—
C _{16:1ω5<i>c</i>}	40.2	36.4	26.9
C _{17:1ω6<i>c</i>}	—	tr	1.5
C _{17:1ω8<i>c</i>}	—	—	tr
C _{18:1ω5<i>c</i>}	—	tr	—
C _{18:1ω9<i>c</i>}	—	—	tr
iso-C _{13:0}	tr	—	tr
anteiso-C _{13:0}	—	—	tr
iso-C _{15:0}	15.0	9.5	12.1
anteiso-C _{15:0}	tr	2.5	2.4
iso-C _{16:0}	—	tr	—
iso-C _{14:0} 3-OH	—	tr	—
C _{15:0} 3-OH	—	—	3.5
iso-C _{15:0} 3-OH	6.9	3.7	3.8
C _{16:0} 2-OH	1.7	tr	—
C _{16:0} 3-OH	7.0	2.9	2.9
C _{17:0} 3-OH	—	—	tr
iso-C _{17:0} 3-OH	3.9	4.6	3.6
C _{14:0} 3-OH and/or iso-C _{16:1} I	1.3	tr	1.4
iso-C _{15:0} 2-OH and/or C _{16:1ω7<i>c</i>}	8.9	10.8	19.2
anteiso-C _{17:1} B and/or iso-C _{17:1} I	—	2.5	tr
ECL 14.959	—	1.6	5.0
ECL 15.731	—	14.7	—

plus trimethoprim. Resistant to kanamycin. Major cellular fatty acids are C_{16:1 ω 5*c*} and iso-C_{15:0}. The DNA G+C content of the type strain is 39.7 mol%.

The type strain, GFA-11^T (=BCRC 17834^T=LMG 24501^T), was isolated from a fish culture pond, Taoyuan County, northern Taiwan. RG-4, isolated from a freshwater spring, Pingtung County, southern Taiwan, is a second strain of the species.

Acknowledgements

This study was funded by grants from the National Science Council (96-2313-B-022-001-MY3) and Academia Sinica (Thematic Grant 2008-2010), Taipei, Taiwan, ROC.

References

Bernardet, J.-F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049–1070.

Borral, R. & Larkin, J. M. (1978). *Flectobacillus marinus* (Raj) comb. nov., a marine bacterium previously assigned to *Microcycus*. *Int J Syst Bacteriol* **28**, 341–343.

Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., de Vos, P., Mergeay, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**, 1729–1735.

Chung, Y. C., Kobayashi, T., Kanai, H., Akiba, T. & Kudo, T. (1995). Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. *Appl Environ Microbiol* **61**, 1502–1506.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

- Gosink, J. J., Woese, C. R. & Staley, J. T. (1998).** *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group and reclassification of '*Flectobacillus glomeratus*' as *Polaribacter glomeratus* comb. nov. *Int J Syst Bacteriol* **48**, 223–235.
- Gromov, B. V. (1963).** A new bacterium of the genus *Microcycclus*. *Dokl Akad Nauk SSSR* **152**, 733–734 (in Russian).
- Hall, T. A. (1999).** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Hwang, C. Y. & Cho, B. C. (2006).** *Flectobacillus lacus* sp. nov., isolated from a highly eutrophic pond in Korea. *Int J Syst Evol Microbiol* **56**, 1197–1201.
- Kimura, M. (1983).** *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kluge, A. G. & Farris, F. S. (1969).** Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.
- Kumar, S., Tamura, K. & Nei, M. (2004).** MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Larkin, J. M., Williams, P. M. & Taylor, R. (1977).** Taxonomy of the genus *Microcycclus* Ørskov 1928: reintroduction and emendation of the genus *Spirosoma* Migula 1894 and proposal of a new genus, *Flectobacillus*. *Int J Syst Bacteriol* **27**, 147–156.
- McGuire, A. J., Franzmann, P. D. & McMeekin, T. A. (1987).** *Flectobacillus glomeratus* sp. nov., a curved, nonmotile, pigmented bacterium isolated from Antarctic marine environments. *Syst Appl Microbiol* **9**, 265–272.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Powers, E. M. (1995).** Efficacy of the Ryu nonstaining KOH technique for rapidly determining gram reactions of food-borne and waterborne bacteria and yeasts. *Appl Environ Microbiol* **61**, 3756–3758.
- Raj, H. D. & Maloy, S. R. (1990).** Proposal of *Cyclobacterium marinus* gen. nov., comb. nov., for a marine bacterium previously assigned to the genus *Flectobacillus*. *Int J Syst Bacteriol* **40**, 337–347.
- Reichenbach, H. (1992).** The order *Cytophagales*. In *The Prokaryotes*, 2nd edn, vol. 4, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990).** *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- 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.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.