Flavobacterium glycines sp. nov., a facultative methylotroph isolated from the rhizosphere of soybean

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An aerobic, yellow-pigmented, facultatively methylotrophic, Gram-staining-negative, non-spore-forming bacterium, designated strain Gm-149^T, was isolated from the rhizosphere of cultivated soybean in India. Cells were motile by gliding. The predominant cellular fatty acids were iso-C_{15:0}, summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1ω7c}), C_{16:0} 3-OH and anteiso-C_{15:0}, and the major isoprenoid quinone was MK-6. The G+C content of the genomic DNA of strain Gm-149^T was 35.6 mol%. Comparative 16S rRNA gene sequence analysis showed that strain Gm-149^T formed a distinct phyletic line within the genus *Flavobacterium*. Based on levels of pairwise 16S rRNA gene sequence similarity, strain Gm-149^T was related most closely to the type strain of *Flavobacterium daejeonense* (97.1 %), but the level of DNA–DNA relatedness between these two strains was about 11.2 %. On the basis of phenotypic and genotypic data, strain Gm-149^T is considered to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium glycines* sp. nov. is proposed. The type strain is Gm-149^T (=ICMP 17618^T=NBRC 105008^T).

The genus *Flavobacterium*, first proposed by Bergey *et al.* (1923) and the description of which was emended considerably by Bernardet *et al.* (1996), is the type genus of the family *Flavobacteriaceae* in the phylum *Bacteroidetes* (formerly the *Cytophaga–Flavobacterium–Bacteroides* group) (Ludwig *et al.*, 2008). *Flavobacterium* species have been isolated from polar environments, seawater, fresh water and river sediment, but relatively few have been isolated from soil environments (Kirchman, 2002; Kim *et al.*, 2006; Yoon *et al.*, 2006). Twenty-six recognized species of *Flavobacterium* were listed by Bernardet & Bowman (2006), but many novel species have since been described (<http://www.bacterio.cict.fr/f/flavobacterium.html>). Methylotrophic bacteria can turn over one-carbon (C₁) compounds in the

environment. They represent a diverse group of organisms that have many potential practical applications due to their unique metabolic capacities. Methylotrophy is widespread in the bacterial world and is found within members of the phyla *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* (Lidstrom, 2006). Facultative methylotrophy has been reported in *Flavobacterium* strains isolated from sediment of the River Thames (London, UK), but the strains were not characterized fully (Boden *et al.*, 2008). Recent studies in our laboratory focusing on the methylotrophic bacterial population associated with different agriculturally important crops, such as rice, red pepper and soybean collected from various locations, have yielded a large number of isolates. In the present study, one novel isolate, designated strain Gm-149^T, with an ability to produce 1-aminocyclopropane-1-carboxylate deaminase (ACCD) was investigated. Based on the data presented here, this strain is shown to represent a novel species of the genus *Flavobacterium*.

Strain Gm-149^T was isolated from rhizosphere soil of soybean (*Glycine max* L. cv. CO1) collected from an

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ACCD, ACC deaminase; IAA, indole-3-acetic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mxrF* gene sequences of strain Gm-149^T are EU672803 and EU912490, respectively.

A supplementary figure showing a scanning electron micrograph of cells of strain Gm-149^T is available with the online version of this paper.

experimental plot at the Tamilnadu Agricultural University, Coimbatore, India. Selective ammonium mineral salts (AMS) medium (Whittenbury *et al.*, 1970) supplemented with filter-sterilized cycloheximide ($10 \mu\text{g ml}^{-1}$) and methanol (0.5%, v/v) was used for isolation at 28°C by the serial-dilution technique. Strain Gm-149^T was able to grow slowly on AMS or R2A agars. It was cultured routinely on the same medium at 30°C and preserved as a suspension in R2A broth with 50% glycerol at -80°C . *Flavobacterium daejeonense* KACC 11422^T, *Flavobacterium johnsoniae* DSM 2064^T, *Flavobacterium defluvii* DSM 17963^T and *Flavobacterium soli* DSM 19725^T were grown under the same conditions and were studied in parallel with the new isolate by using the same phenotypic tests. Phenotypic properties were studied according to standard procedures (Gerhardt *et al.*, 1994). Biolog GN2 microplates were used to assess utilization of carbon compounds; utilization of carbon compounds not included with the GN2 plates was assessed by using the methods described by Madhaiyan *et al.* (2007a) and Green & Bousfield (1982). Other physiological and biochemical characteristics were tested by using the API ZYM and API 20NE galleries (bioMérieux) following the manufacturer's instructions. Scanning electron microscope observations were performed as described by Bozzola & Russell (1998) by using a Hitachi S-2500C microscope with GEMINI column equipped with a field-emission electron source. Gliding motility and absorption of Congo red by the colonies were determined by using the methods of Bernardet *et al.* (2002). Flexirubin-type pigments were detected by flooding the plates with 20% (w/v) potassium hydroxide (Fautz & Reichenbach, 1980). Pigments were extracted by methanol from a 5-day-old R2A broth culture. The resultant supernatant was scanned for absorbance at a wavelength of 190–1100 nm at room temperature on a UV-1601 spectrophotometer (Shimadzu). The minimal inhibitory concentrations of heavy metals and antibiotics were determined as described by Madhaiyan *et al.* (2007b) and Chanprame *et al.* (1996). A quantitative assay for indole-3-acetic acid (IAA) and plate assays for detecting the presence of siderophore production, ACCD activity, acetylene reduction and sulfur oxidation were carried out as described by Poonguzhali *et al.* (2006). Quantitative estimation of methanol dehydrogenase activity was carried out according to the procedure of Dunfield *et al.* (2003).

Phenotypic features of strain Gm-149^T are given in Table 1 and in the species description below. Some of these characteristics were in accordance with those of members of the genus *Flavobacterium*, whereas others allowed the differentiation of strain Gm-149^T from closely related *Flavobacterium* species (Table 1). Flexirubin-type pigments were produced, as shown by the colour shift of the colonies when flooded with 20% KOH. Carotenoid pigments were also produced, as revealed by absorption maxima at 213, 261, 449 and 476 nm of the methanol extract (Asker *et al.*, 2007). Strain Gm-149^T was able to utilize 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source when

tested by plate assay, and considerable activity of ACCD was detected in cell-free extracts. *F. daejeonense* KACC 11422^T, *F. johnsoniae* DSM 2064^T, *F. defluvii* DSM 17963^T and *F. soli* DSM 19725^T were tested under the same conditions by preliminary plate assay on AMS supplemented with $30 \mu\text{mol ACC}$ as a nitrogen source. These four strains were negative for ACCD activity. Strain Gm-149^T showed no activity for sulfur oxidation and was negative in the siderophore assay, but was positive for nitrogenase activity [$4.4 \text{ nmol C}_2\text{H}_4 (\text{mg protein})^{-1} \text{ h}^{-1}$], IAA production ($1.8 \mu\text{g ml}^{-1}$) and ACCD activity [$18.5 \text{ nmol } \alpha\text{-ketobutyrate } (\text{mg protein})^{-1} \text{ h}^{-1}$]. Strain Gm-149^T produced methanol dehydrogenase at a concentration of $8.4 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. Seed inoculation with strain Gm-149^T significantly increased the root length of canola (*Brassica campestris* L.; 22.4% increase versus control) and tomato (*Lycopersicon esculentum* Mill.; 26.3% increase versus control) (data not shown).

Chromosomal DNA was extracted by using a QIAamp DNA mini kit (Qiagen). The 16S rRNA gene was amplified by using the universal primers 27F and 1492R as described by Madhaiyan *et al.* (2009) and the full gene sequence was determined by the fluorescent dye-terminator method by using a sequencing kit (ABI PRISM Big Dye terminator cycle sequencing ready reaction kit version 3.1). The amplified

Table 1. Differential characteristics between strain Gm-149^T and the type strains of related *Flavobacterium* species

Strains: 1, Gm-149^T; 2, *F. daejeonense* KACC 11422^T; 3, *F. defluvii* DSM 17963^T; 4, *F. johnsoniae* DSM 2064^T; 5, *F. soli* DSM 19725^T. All are positive for catalase activity and growth on nutrient and trypticase soy agars at 25°C . All are negative for urease activity and precipitation on egg-yolk agar. +, Positive; –, negative; w, weakly positive. All data are from the present study.

Characteristic	1	2	3	4	5
Flexirubin-type pigment	+	–	+	+	–
Gliding motility	+	–	+	+	–
Highest NaCl concentration (%) tolerated	1	3	<2	1	2
Acid from carbohydrate	+	+	+	+	–
Glucose utilization	–	+	+	+	+
Oxidase	w	+	–	+	+
Degradation of:					
Gelatin	–	–	+	+	+
Casein	+	–	+	+	–
Starch	+	+	–	+	–
CM-cellulose	–	–	+	+	–
Pectin	–	–	–	+	w
Chitin	–	–	–	+	w
Aesculin	–	+	+	+	+
Tyrosine	–	–	+	+	–
β-Galactosidase activity	+	+	w	+	–
Nitrate reduction	+	+	–	+	–
DNA G + C content (mol%)	35.6	35.0	33.5	34.0	36.9

products were analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The resultant 16S rRNA gene sequence was compared with those of the type strains of representative species of the genus *Flavobacterium* and related genera retrieved from GenBank, and the sequences were aligned by using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic relationships were determined with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods by using the program MEGA4.1 (Tamura *et al.*, 2007), with bootstrap values based on 1000 replications (Felsenstein, 1985). Sequence similarity values were computed by using two tools: the FASTA nucleotide similarity search program (Pearson & Lipman, 1988) and the EzTaxon Server (Chun *et al.*, 2007). The methanol dehydrogenase (*mxoF*) and ACCD (*acdS*) genes were amplified from DNA extracted from strain Gm-149^T and the four reference strains by using the primer pairs *mxoF*

f1003/mxoF r1561 and F1936/F1939, respectively (McDonald & Murrell, 1997; Blaha *et al.*, 2006). The presence of the *nifH* gene was assessed by PCR amplification of a 390 bp fragment of this gene by using the specific primers 19F and 407R (Ueda *et al.*, 1995). The products were sequenced directly and analysed as described above.

The neighbour-joining phylogenetic tree with selected 16S rRNA gene sequences showed that strain Gm-149^T was related to members of the genus *Flavobacterium* (Fig. 1). 16S rRNA gene sequence similarity calculations and phylogenetic analysis revealed that strain Gm-149^T was related most closely to *F. daejeonense* KACC 11422^T (97.1 %), *F. johnsoniae* DSM 2064^T (96.9 %) and *F. defluvi* DSM 17963^T (96.9 %). Pairwise 16S rRNA gene sequence comparisons via FASTA nucleotide similarity searches and the EzTaxon Server showed that strain Gm-149^T was related most closely to *F. daejeonense* KACC 11422^T. The

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of strain Gm-149^T among *Flavobacterium* species. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values ≥ 70 % are shown. Bar, 0.005 substitutions per nucleotide position.

topologies of the trees generated with the neighbour-joining, maximum-parsimony and maximum-likelihood methods were almost identical (data not shown). An amplification product of the expected size for the *mxoF* gene (572 bp) was detected in strain Gm-149^T and was similar to the *mxoF* genes of *Methylobacterium* species. Sequencing of the *mxoF* gene of strain Gm-149^T revealed 97.2 % sequence similarity to that of the type strain of *Methylophilus methylotrophus*. The *mxoF* gene could not be amplified from the four reference *Flavobacterium* strains. The *acdS* gene was not detected in strain Gm-149^T or the four reference *Flavobacterium* strains. The *nifH* gene was detected in all five strains (data not shown).

For analysis of cellular fatty acids, strain Gm-149^T and the four reference strains were grown on TSA at 28 °C for 48 h. Fatty acids were extracted, esterified, analysed by GC (Hewlett Packard 6890) and identified by using the Microbial Identification System (MIDI; Microbial ID) software package, according to standard protocols (Sasser, 1990). The G + C content of the genomic DNA of strain Gm-149^T was determined by HPLC as described by Mesbah *et al.* (1989) by using a Supelcosil LC-18-S reversed-phase column (Supelco). DNA–DNA hybridization experiments between strain Gm-149^T and the four reference strains were carried out on a nitrocellulose membrane according to the method of Seldin & Dubnau (1985). The DIG-High prime system and the DIG luminescent detection kit (Roche Diagnostics GmbH) were used to label the DNA probe and for visualization. Hybridization temperatures were 60 and 65 °C and the level of DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad Laboratories). Respiratory quinones were extracted and analysed according to Kroppenstedt (1982).

The major fatty acids (>9 %) of strain Gm-149^T were iso-C_{15:0}, summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), C_{16:0} 3-OH and anteiso-C_{15:0}. This profile was similar to those of the type strains of its four closest phylogenetically related species, but there were clear differences in the respective proportions of some components between the five strains (Table 2). The DNA G + C content of strain Gm-149^T was 35.6 mol%, a value within the range reported for the genus *Flavobacterium* (Bernardet & Bowman, 2006). Strain Gm-149^T showed a low level of DNA–DNA hybridization of 7.2–11.2 % with its four closest relatives, well below the 70 % threshold generally accepted for species delineation (Stackebrandt *et al.*, 2002). Therefore, on the basis of the data presented here, strain Gm-149^T is considered to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium glycines* sp. nov. is proposed.

Description of *Flavobacterium glycines* sp. nov.

Flavobacterium glycines (gly.ci'nes. N.L. n. *Glycine* a botanical genus name; N.L. gen. n. *glycines* of *Glycine max*, from which the type strain was isolated).

Cells are Gram-staining-negative rods, 0.2–0.3 µm in diameter and 0.8–1.0 µm in length (see Supplementary

Table 2. Cellular fatty acid compositions of strain Gm-149^T and of the type strains of related *Flavobacterium* species

Strains: 1, Gm-149^T; 2, *F. daejeonense* KACC 11422^T; 3, *F. defluvii* DSM 17963^T; 4, *F. johnsoniae* DSM 2064^T; 5, *F. soli* DSM 19725^T. Values are percentages of total fatty acids. Fatty acids representing <1 % in all strains were omitted. All data are from the present study. tr, Trace (<1 %); ND, not detected.

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	3.0	1.0	ND	ND	ND
C _{15:0}	ND	ND	7.1	6.5	8.1
C _{16:0}	6.2	6.5	4.5	3.3	4.5
Hydroxy					
C _{15:0} 2-OH	1.3	tr	tr	tr	ND
C _{15:0} 3-OH	2.8	2.6	2.2	2.1	ND
C _{16:0} 3-OH	11.2	7.5	4.6	5.2	1.2
C _{17:0} 3-OH	1.4	1.2	1.4	1.2	ND
Branched					
iso-C _{14:0}	1.9	tr	1.2	1.2	ND
anteiso-C _{15:0}	9.2	5.2	4.3	4.1	4.6
iso-C _{15:0}	15.5	20.1	18.5	24.0	27.1
iso-C _{15:1} G	3.3	5.1	6.5	5.4	5.1
iso-C _{15:0} 3-OH	6.4	8.6	9.3	7.5	8.2
iso-C _{16:0}	tr	tr	2.9	3.2	3.3
iso-C _{16:0} 3-OH	3.8	1.2	6.6	5.3	5.1
iso-C _{17:0} 3-OH	4.5	9.4	12.9	10.5	7.1
iso-C _{17:1} ω9c	tr	ND	2.6	2.7	7.4
Unsaturated					
C _{15:1} ω6c	5.9	6.0	1.3	1.3	1.9
C _{17:1} ω6c	2.5	2.6	2.3	2.4	2.6
C _{17:1} ω8c	tr	tr	ND	ND	1.3
Summed features*					
2	1.1	1.0	1.1	1.0	ND
3	15.8	17.2	9.9	12.5	11.3

*Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 2 comprised C_{12:0} ALDE and/or unknown ECL 10.928; summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Fig. S1 in IJSEM Online), that are motile by gliding. Growth occurs at 10–35 °C (optimum, 28 °C), at pH 6–8 and in the presence of 0–1 % NaCl. Grows optimally on R2A medium without the addition of NaCl; growth is severely inhibited on R2A medium containing >1 % (w/v) NaCl. Growth occurs on AMS, R2A, trypticase soy and nutrient agars, but not on MacConkey agar. Colonies are yellow, convex and circular with regular margins. Catalase- and oxidase-positive. Starch and casein are degraded, but gelatin, CM-cellulose, pectin, chitin, aesculin, tyrosine and egg yolk are not. Congo red is not absorbed by colonies. Flexirubin and carotenoid pigments are produced. Tolerates up to 2 mM NiCl₂ and 0.5 mM CdCl₂. Methanol, ethanol, succinate and dichloromethane are utilized as sole carbon sources, but methylamine, trimethylamine, diethanolamine, formaldehyde, dimethylamine and methane are not. Utilizes

ammonium chloride, ACC, potassium nitrate, ammonium sulfate, sodium nitrate, L-glutamine, glycine and potassium thiocyanate as sole nitrogen sources, but not L-alanine, L-glutamate, urea, L-aspartate, L-tryptophan, methylamine, diethylamine, diphenylamine or potassium cyanate. Positive for nitrate reduction and β -galactosidase activity, but negative for glucose fermentation, arginine dihydrolase and urease activities, and aesculin and gelatin hydrolysis (API 20NE). In the same gallery, assimilates L-arabinose, D-mannose and maltose, but not D-glucose, D-mannitol, N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid. In the API ZYM gallery, positive for alkaline phosphatase, esterase C4 (weak), esterase lipase C8 (weak), leucine arylamidase, valine arylamidase, acid phosphatase (weak), naphthol-AS-BI-phosphohydrolase, α -galactosidase (weak) and β -galactosidase activities, but negative for lipase C14, cystine arylamidase, trypsin, α -chymotrypsin, α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase, α -fucosidase and N-acetyl- β -glucosaminidase activities. The type strain is highly resistant (up to 500 $\mu\text{g ml}^{-1}$) to spectinomycin, ampicillin, carbenicillin, nalidixic acid, bacitracin, cefotaxime, chloramphenicol, penicillin G, polymyxin B, novobiocin, vancomycin, doxycycline, rifampicin, cephalosporin and trimethoprim, but sensitive to tetracycline (500 $\mu\text{g ml}^{-1}$), gentamicin (300 $\mu\text{g ml}^{-1}$), kanamycin (300 $\mu\text{g ml}^{-1}$) and erythromycin (500 $\mu\text{g ml}^{-1}$). The following substrates are utilized in the GN2 microplate system: α -cyclodextrin, dextrin, glycogen, L-arabinose, cellobiose, D-fructose, L-fucose, D-galactose, α -D-lactose, lactulose, maltose, D-mannose, melibiose, D-psicose, raffinose, L-rhamnose, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, D-galacturonic acid, D-glucuronic acid, α -ketobutyric acid, DL-lactic acid and L-glutamic acid. All other substrates in the GN2 microplate system are not utilized. The major isoprenoid quinone is MK-6. The major cellular fatty acids are iso-C_{15:0}, summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c), C_{16:0} 3-OH and anteiso-C_{15:0}. The G+C content of the genomic DNA of the type strain is 35.6 mol%.

The type strain, Gm-149^T (=ICMP 17618^T=NBRC 105008^T), was isolated from the rhizosphere of field-grown soybean in Tamilnadu, India.

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References

Asker, D., Beppu, T. & Ueda, K. (2007). Unique diversity of carotenoid-producing bacteria isolated from Misasa, a radioactive site in Japan. *Appl Microbiol Biotechnol* **77**, 383–392.

Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W. & Huntoon, F. M. (editors) (1923). *Bergey's Manual of Determinative Bacteriology*. Baltimore, MD: Williams & Wilkins.

Bernardet, J.-F. & Bowman, J. P. (2006). The genus *Flavobacterium*. In *The Prokaryotes*, vol. 7, pp. 481–531. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

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.

Blaia, D., Prigent-Combaret, C., Sajjad Mirza, M. & Moënné-Loccoz, Y. (2006). Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene *acdS* in phyto-beneficial and pathogenic Proteobacteria and relation with strain biogeography. *FEMS Microbiol Ecol* **56**, 455–470.

Boden, R., Thomas, E., Savani, P., Kelly, D. P. & Wood, A. P. (2008). Novel methylotrophic bacteria isolated from the River Thames (London, UK). *Environ Microbiol* **10**, 3225–3236.

Bozzola, J. J. & Russell, L. D. (1998). *Electron Microscopy*, 2nd edn. Sudbury, MA: Jones & Bartlett.

Chanprame, S., Todd, J. J. & Widholm, J. M. (1996). Prevention of pink-pigmented methylotrophic bacteria (*Methylobacterium mesophilicum*) contamination of plant tissue cultures. *Plant Cell Rep* **16**, 222–225.

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.

Dunfield, P. F., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A. & Dedysh, S. N. (2003). *Methylocella silvestris* sp. nov., a novel methanotroph isolated from an acidic forest cambisol. *Int J Syst Evol Microbiol* **53**, 1231–1239.

Fautz, E. & Reichenbach, H. (1980). A simple test for flexirubin-type pigments. *FEMS Microbiol Lett* **8**, 87–91.

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

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

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

Green, P. N. & Bousfield, I. J. (1982). A taxonomic study of some Gram-negative facultatively methylotrophic bacteria. *J Gen Microbiol* **128**, 623–638.

Kim, B. Y., Weon, H. Y., Cousin, S., Yoo, S. H., Kwon, S. W., Go, S. J. & Stackebrandt, E. (2006). *Flavobacterium daejeonense* sp. nov. and *Flavobacterium suncheonense* sp. nov., isolated from greenhouse soils in Korea. *Int J Syst Evol Microbiol* **56**, 1645–1649.

Kirchman, D. L. (2002). The ecology of Cytophaga–Flavobacteria in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP18) and a silver loaded ion exchanger as stationary phases. *J Liq Chromatogr* **5**, 2359–2367.

- Lidstrom, M. E. (2006).** The aerobic methylotrophic prokaryotes. In *The Prokaryotes*, vol. 2, pp. 618–634. Edited by M. Dworkin, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Ludwig, W., Euzéby, J. & Whitman, W. B. (2008).** Draft Taxonomic Outline of the *Bacteroidetes*, *Planctomycetes*, *Chlamydiae*, *Spirochaetes*, *Fibrobacteres*, *Fusobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Dictyoglomi*, and *Gemmatimonadetes* (*Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 4). http://www.bergeys.org/outlines/Bergeys_Vol_4_Outline.pdf
- Madhaiyan, M., Kim, B.-Y., Poonguzhali, S., Kwon, S.-W., Song, M.-H., Ryu, J.-H., Go, S.-J., Koo, B.-S. & Sa, T.-M. (2007a).** *Methylobacterium oryzae* sp. nov., an aerobic, pink-pigmented, facultatively methylotrophic, 1-aminocyclopropane-1-carboxylate deaminase-producing bacterium isolated from rice. *Int J Syst Evol Microbiol* **57**, 326–331.
- Madhaiyan, M., Poonguzhali, S. & Sa, T. M. (2007b).** Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). *Chemosphere* **69**, 220–228.
- Madhaiyan, M., Poonguzhali, S., Kwon, S. W. & Sa, T. M. (2009).** *Methylobacterium phyllosphaerae* sp. nov., a pink-pigmented facultatively methylotrophic bacterium from rice. *Int J Syst Evol Microbiol* **59**, 22–27.
- McDonald, I. R. & Murrell, J. C. (1997).** The methanol dehydrogenase structural gene *mxoF* and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl Environ Microbiol* **63**, 3218–3224.
- 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.
- Pearson, W. R. & Lipman, D. J. (1988).** Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- Poonguzhali, S., Madhaiyan, M. & Sa, T. M. (2006).** Cultivation-dependent characterization of rhizobacterial communities from field grown Chinese cabbage *Brassica campestris* ssp *pekinensis* and screening of potential plant growth promoting bacteria. *Plant Soil* **286**, 167–180.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990).** Identification of bacteria through fatty acid analysis. In *Methods in Phytobacteriology*, pp. 199–204. Edited by Z. Klement, K. Rudolph & D. C. Sands. Budapest: Akademiai Kiado.
- Seldin, L. & Dubnau, D. (1985).** Deoxyribonucleic acid homology among *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans*, and other nitrogen-fixing *Bacillus* strains. *Int J Syst Bacteriol* **35**, 151–154.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002).** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- 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., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Ueda, T., Suga, Y., Yahiro, N. & Matsuguchi, T. (1995).** Phylogeny of Sym plasmids of rhizobia by PCR-based sequencing of a *nodC* segment. *J Bacteriol* **177**, 468–472.
- Whittenbury, R., Davies, S. L. & Wilkinson, J. F. (1970).** Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61**, 205–218.
- Yoon, J.-H., Kang, S.-J. & Oh, T.-K. (2006).** *Flavobacterium soli* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **56**, 997–1000.