Flavobacterium caeni sp. nov., isolated from a sequencing batch reactor for the treatment of malachite green effluents

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A Gram-stain-negative, heterotrophic, aerobic, non-spore-forming and non-motile bacterial strain, designated LM5^T, was isolated from activated sludge from a sequencing batch reactor for the treatment of effluents contaminated by malachite green. The taxonomy of strain LM5^T was studied by phenotypic and phylogenetic methods. Strain LM5^T formed orange colonies on R2A and YP plates. Cells were rods, 0.4–0.6 µm in diameter and 0.8–1.2 µm in length. Growth occurred at 10–35 °C (optimum, 20–25 °C), at pH 5.5–9.5 (optimum, pH 6.5–7.5) and in the presence of 0–2 % (w/v) NaCl (optimum, 0.5 %). Oxidase and catalase activities were present. Flexirubin-type pigments were present, but extracellular glycans were absent. MK-6 was the major respiratory quinone. The major fatty acids were iso-C_{15:0} (28.3 %) and iso-C_{17:1ω9c} (13.8 %). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain LM5^T was a member of the genus *Flavobacterium* with highest sequence similarity to *Flavobacterium soli* DS-6^T (93.2 %) and *Flavobacterium lindanitolerans* IP-10^T (92.9 %). Together with *F. lindanitolerans* IP-10^T, strain LM5^T formed a distinct lineage in the phylogenetic tree. The DNA G + C content was 52 ± 0.6 mol% (HPLC), which is significantly higher than that of other species of the genus *Flavobacterium* (30–41 mol%). Based on phylogenetic and phenotypic evidence, strain LM5^T is considered to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium caeni* sp. nov. is proposed; the type strain is LM5^T (=CGMCC 1.7031^T=NBRC 104239^T).

The genus *Flavobacterium* belongs to the family *Flavobacteriaceae* in the phylum ‘*Bacteroidetes*’ (Ludwig & Klenk, 2001). It was proposed by Bergey *et al.* (1923) and emended by Bernardet *et al.* (1996). Recently, Bernardet & Bowman (2006) provided an overview of the taxonomy and ecology of members of the genus *Flavobacterium*. Species of the genus are widespread in nature and have been isolated from many freshwater and soil habitats. In recent years, many novel species belonging to the genus *Flavobacterium* have been identified. At the time of writing, the genus *Flavobacterium* comprises about 50 species with validly published names. A *Flavobacterium* strain, designated LM5^T, was isolated from activated sludge from a sequencing batch reactor for the treatment of malachite green effluents and subjected to a polyphasic taxonomic study; the results are presented in this report.

Malachite green is an *N*-methylated diamino-triphenyl-methane dye used for dyeing silk, leather, cotton, wool and jute. Traditionally, malachite green has also been used to treat fungal infections in fishes (Alderman, 1985). Malachite green can be toxic to human cells and promotes liver tumour formation in rodents (Srivastava *et al.*, 2004). Due to its potentially harmful effects, the US Food and Drug Administration nominated malachite green as a priority chemical for toxicity and carcinogenicity testing in 1993. Strain LM5^T was isolated from activated sludge in an anaerobic/aerobic sequential batch reactor used for the treatment of malachite green-contaminated effluents. The sludge sample was suspended in normal saline by vigorous vortexing and part of the suspension was spread onto low-organic Luria–Bertani [containing (l⁻¹): 1.0 g tryptone (Difco), 0.5 g yeast extract and 10.0 g NaCl, pH 7.0] agar plates and incubated at 30 °C for 1 week. Single colonies on the plates were picked up and strain LM5^T was purified by repeated streaking on new plates. Subcultures were performed on R2A agar (Difco) and the strain was stored in R2A broth (Difco) supplemented with 15 % glycerol (v/v) at –80 °C.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LM5^T is EU313814.

The following tests were performed in parallel on strain LM5^T and on its closest phylogenetic neighbours, *Flavobacterium soli* DS-6^T, *Flavobacterium lindanitolerans* IP-10^T and *Flavobacterium terrae* R2A1-13^T. Growth was tested on R2A agar, marine 2216 agar (MA; laboratory prepared), nutrient agar (NA; laboratory prepared), trypticase soy agar (TSA; Difco), 0.1 × TSA and YP agar [containing (l⁻¹): 3.0 g tryptone (Difco), 3.0 g yeast extract (Difco), 0.5 g MgSO₄·7H₂O, 0.3 g NaCl, 15 g agar, pH 7.0]. Cell morphology was examined using phase-contrast microscopy and transmission electron microscopy (H600; Hitachi). Gram staining was performed as described by Gerhardt *et al.* (1994). Catalase and oxidase activities and H₂S production were determined as described by Dong & Cai (2001). Gliding motility was assessed as described by Bernardet *et al.* (2002). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with anaerobically prepared R2A broth. Salt tolerance was tested in R2A broth supplemented with 0–2.5 % (w/v) NaCl (at intervals of

0.5 %). The pH range for growth was tested in R2A broth adjusted to pH 5.0–10.0 (at intervals of 0.5 pH). The temperature range for growth was tested in R2A broth at 5–40 °C (at intervals of 5 °C). Hydrolysis of casein [50 % (v/v) skimmed milk], L-tyrosine (0.5 %, w/v), starch (0.2 %, w/v), Tweens 20, 40, 60 and 80 (1 %, v/v), carboxymethyl-cellulose (0.5 %, w/v), egg yolk (5 %, w/v), pectin (0.5 %, w/v), agar (1.5 %, w/v) and DNA (0.1 %, w/v) was tested according to Gerhardt *et al.* (1994) using R2A agar as a basal medium.

The presence of flexirubin-type pigments and extracellular glycans was assessed by using the KOH and Congo red tests, respectively (Fautz & Reichenbach, 1980; Bernardet *et al.*, 2002).

Carbon source assimilation was studied by using the GN2 MicroPlate (Biolog) according to the manufacturer's instructions. Acid production from various substrates was assessed by using API 50CH test strips (bioMérieux) and 0.5 × CHB/E medium (bioMérieux) according to Yoon

Table 1. Phenotypic characteristics of strain LM5^T and closely related species of the genus *Flavobacterium*

Strains: 1, LM5^T; 2, *F. soli* DS-6^T; 3, *F. lindanitolerans* IP-10^T; 4, *F. terrae* R2A1-13^T. All data are from this study (except for the DNA G + C content of the three reference strains). All strains are negative for absorption of Congo red, DNA degradation, H₂S production and nitrate reduction. In GN2 MicroPlate assays, all strains (except *F. terrae* R2A1-13^T, which does not utilize any GN2 MicroPlate substrates) utilize dextrin, α-D-glucose, maltose, α-ketovaleric acid, L-alanyl glycine, L-proline, L-threonine and uridine. In the API ZYM system, all strains are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Oxidase activity	+	–	+	+
Catalase activity	w	+	+	–
Gliding motility	–	+	–	–
Growth on NA, TSA	w	+	+	+
Degradation of:				
Aesculin	+	+	+	–
Casein	–	–	–	+
Gelatin	+	–	+	+
Starch	+	–	–	+
Tyrosine	+	–	+	+
Utilization of:				
α-Cyclodextrin, glycogen, succinic acid monomethylester	+	+	–	–
Tween 40, erythritol, pyruvic acid methyl ester, acetic acid, L-leucine, inosine, thymidine, α-ketobutyric acid	–	+	–	–
Cellobiose, glycyl-L-aspartic acid	–	–	+	–
D-Galactose, α-D-lactose, formic acid, propionic acid	+	–	–	–
Gentiobiose, L-glutamic acid	–	+	+	–
D-Mannose, turanose, glycyl-L-glutamic acid	+	–	+	–
API ZYM results:				
Valine arylamidase	+	+	–	+
Trypsin	+	–	+	–
α-Glucosidase	+	–	+	+
Cystine arylamidase	+	–	–	+
α-Chymotrypsin	+	–	–	–
DNA G + C content (mol%)	52 ± 0.6	31	37	34

et al. (2006). Additional biochemical properties and enzyme activities were detected by using API 20NE and API ZYM strips (bioMérieux). Susceptibility to antibiotics was determined using filter-paper discs (Beijing Pharmaceutical Company) containing various antibiotics as specified in the species description. The phenotypic properties of strain LM5^T are given in Table 1 and in the species description. *F. terrae* R2A1-13^T was unable to utilize any GN2 MicroPlate substrates.

Genomic DNA extraction, PCR and sequencing of the 16S rRNA gene were performed according to Kim *et al.* (1998). The sequence of the amplified fragment (1387 nt) was determined by direct sequencing and compared with available 16S rRNA gene sequences in GenBank using the BLAST program (Altschul *et al.*, 1990) available at NCBI. Strain LM5^T showed highest 16S rRNA gene sequence similarity to *F. soli* DS-6^T (93.2%), *F. lindanitolerans* IP-10^T (92.9%), *F. terrae* R2A1-13^T (92.7%) and *Flavobacterium flevense* ATCC 27944^T. Strain LM5^T shared 91.2% sequence similarity with the type strain of the type species of the genus *Flavobacterium*, *Flavobacterium aqua-*

tile ATCC 11947^T. A multiple alignment with closely related members of the genus *Flavobacterium* was performed by using the CLUSTAL W program (Thompson *et al.*, 1994). Ambiguous and unalignable bases were removed manually and a neighbour-joining phylogenetic tree was constructed from the evolutionary distance matrix calculated using the MEGA program version 3.1 (Kumar *et al.*, 2004). The stability of the relationships was assessed by means of bootstrapping (based on 1000 replicates). Strain LM5^T, together with *F. lindanitolerans* IP-10^T, formed a distinct phylogenetic lineage within the genus *Flavobacterium* (Fig. 1), indicating that it probably represents a novel member of the genus.

Isoprenoid quinones were extracted and analysed as described by Komagata & Suzuki (1987). For fatty acid analysis, cell mass of strain LM5^T, *F. soli* DS-6^T, *F. lindanitolerans* IP-10^T and *F. terrae* R2A1-13^T was obtained from R2A plates after cultivation for 2 days at 30 °C. Cellular fatty acids were extracted, methylated and analysed following the instructions of the Sherlock Microbial Identification System (MIDI). The major respiratory

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Flavobacterium caeni* LM5^T and representative members of the genus *Flavobacterium*. Numbers at nodes are bootstrap values >50% (1000 replications). *Cellulophaga lytica* ATCC 23178^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

quinone was MK-6, as in all members of the family *Flavobacteriaceae*. The dominant fatty acid of strain LM5^T was iso-C_{15:0} (28.3%), in line with the three most closely related *Flavobacterium* type strains grown under the same conditions (Table 2). It differed from them, however, by a significantly higher content of iso-C_{17:1}ω9c.

The DNA G + C content of strain LM5^T was determined by the thermal denaturation (Sly *et al.*, 1986) and HPLC (Mesbah *et al.*, 1989) methods using DNA from *Escherichia coli* K-12 as a control. Both methods gave almost the same results, i.e. 52 mol% (*T_m*) and 52 ± 0.6 mol% (HPLC; mean and SD of five experiments), values that are significantly higher than those of other species of the genus *Flavobacterium* (30–41 mol%; Bernardet & Bowman, 2006; Park *et al.*, 2006; Wang *et al.*, 2006).

Table 2. Cellular fatty acid profiles of strain LM5^T and closely related species of the genus *Flavobacterium*

Strains: 1, LM5^T; 2, *F. soli* DS-6^T; 3, *F. lindanitolerans* IP-10^T; 4, *F. terrae* R2A1-13^T. All data are from this study. Values are percentages of the total fatty acid content. Only fatty acids representing at least 1% of the total fatty acids in all strains are shown. —, Not detected; tr, trace (<1.0%).

Fatty acid	1	2	3	4
C _{13:0}	tr	1.4	tr	tr
C _{14:0}	1.0	1.4	tr	tr
C _{16:0}	6.5	1.6	1.5	tr
iso-C _{13:0}	tr	2.2	tr	1.8
iso-C _{14:0}	tr	2.2	2.7	8.8
iso-C _{15:0}	28.3	25.8	18.2	15.3
iso-C _{16:0}	9.0	3.1	5.6	8.8
C _{15:0} 2-OH	tr	tr	4.7	1.6
C _{15:0} 3-OH	—	1.6	3.1	2.3
C _{16:0} 3-OH	tr	3.2	1.2	1.3
iso-C _{14:0} 3-OH	tr	1.1	tr	3.2
iso-C _{15:0} 3-OH	6.7	10.6	4.7	11.7
iso-C _{16:0} 3-OH	1.1	2.6	3.7	4.9
iso-C _{17:0} 3-OH	6.0	4.5	6.3	9.4
iso-C _{15:1} G	7.9	14.9	13.5	12.7
iso-C _{16:1} G	—	—	—	2.7
iso-C _{16:1} H	1.5	1.7	1.7	—
iso-C _{17:1} ω9c	13.8	2.3	3.0	4.2
C _{15:1} ω6c	1.8	5.2	2.9	tr
C _{17:1} ω6c	tr	tr	3.9	tr
C _{17:1} ω8c	1.5	tr	1.1	tr
C _{17:1} ω9c	—	—	—	4.2
Summed feature 3*	4.6	5.7	15.6	1.1
anteiso-C _{15:0}	2.5	3.2	1.8	2.2
anteiso-C _{15:1} A	tr	tr	tr	1.2
anteiso-C _{17:1} ω9c	1.5	—	—	—

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

The phylogenetic tree clearly showed that strain LM5^T belongs to the cluster comprising *Flavobacterium columnare* and several recently described species of the genus *Flavobacterium* that share rather low levels of 16S rRNA gene sequence similarity with the type species of the genus, *F. aquatile*, and with the *Flavobacterium* species that form the main cluster (Fig. 1). Moreover, the DNA G + C content of strain LM5^T clearly fell outside of the range reported for the genus (Bernardet & Bowman, 2006). Yet, as the overall phenotypic characteristics of strain LM5^T were in accordance with those of the genus *Flavobacterium*, it was decided to describe it as a novel species in this genus, *Flavobacterium caeni* sp. nov., pending further taxonomic research. Strain LM5^T could be differentiated from other species of the genus by means of the characteristics listed in Tables 1 and 2.

Description of *Flavobacterium caeni* sp. nov.

Flavobacterium caeni (cae'ni. L. gen. n. *caeni* of sludge).

Strictly aerobic and heterotrophic. Cells are Gram-stain-negative, non-motile, non-flagellated and non-spore-forming rods, 0.4–0.6 µm in diameter and 0.8–1.2 µm in length. Abundant growth occurs on YP agar, R2A and 0.1 × TSA; weak growth occurs on TSA, MA and NA. Colonies are orange, circular, smooth with entire margins and about 2–3 mm in diameter after incubation on R2A at 30 °C for 2 days. Growth occurs at 10–35 °C (optimum, 20–25 °C), at pH 5.5–9.5 (optimum, pH 6.5–7.5) and in the presence of 0–2% (w/v) NaCl (optimum, 0.5%). Oxidase and catalase activities are present. Arginine dihydrolase activity is absent. Acid is not produced from glucose. L-Tyrosine, starch and Tweens 20 and 80 are hydrolysed. Tweens 40 and 60 are weakly hydrolysed. Casein, pectin, carboxymethylcellulose, agar, DNA and urea are not hydrolysed. A brown pigment is produced on tyrosine agar. No precipitate is formed on egg yolk agar. Flexirubin-type pigments are present, but extracellular glycans are absent. In the API ZYM test, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acidic phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase activities are present, but lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. In GN2 MicroPlate assays, α-cyclodextrin, dextrin, glycogen, D-galactose, α-D-glucose, α-D-lactose, maltose, D-mannose, turanose, succinic acid monomethylester, formic acid, α-ketovaleic acid, propionic acid, glycyl L-glutamic acid, L-threonine, uridine, L-alanyl glycine and L-proline are utilized; all other substrates in the MicroPlate are not utilized. Acid is produced from aesculin and produced weakly from maltose, D-lactose, starch and glycogen in the API 50CH strip; acid is not produced from the other substrates in the strip. Sensitive (µg per disc unless otherwise stated) to vancomycin (30), gentamicin (10), carbenicillin (100), streptomycin (10), tetracycline (30), ampicillin (10), erythromycin (15),

novobiocin (30), novobiocin (5), chloramphenicol (30), ciprofloxacin (5), corfloxacin (10), rifampicin (5) and penicillin (10 U); intermediate resistance is observed for polymyxin B (300 U) and kanamycin (30). The major respiratory quinone is MK-6. The major fatty acids (>10 % of the total fatty acids) are iso-C_{15:0} and iso-C_{17:1}ω_{9c}.

The type strain, LM5^T (=CGMCC 1.7031^T=NBRC 104239^T), was isolated from activated sludge from a sequencing batch reactor for the treatment of malachite green effluents. The DNA G+C content of the type strain is 52 ± 0.6 mol% (HPLC).

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