Reclassification of [*Flavobacterium*] *ferrugineum* as *Terrimonas ferruginea* gen. nov., comb. nov., and description of *Terrimonas lutea* sp. nov., isolated from soil

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Strain DY^T, which was isolated from garden soil in Japan, was subjected to a polyphasic taxonomic study. Sequence analysis of the 16S rRNA gene and the GyrB protein revealed that the closest relative of strain DY^T was [*Flavobacterium*] *ferrugineum* Sickles and Shaw 1934, with 94.8 and 90.1 % similarity, respectively. The two strains had similar chemotaxonomic characteristics, with menaquinone 7 as the major quinone system, 47.2–48.9 mol% DNA G + C content and 15:0 iso, 15:1 iso, 17:0 iso 3-OH and summed feature 3 as the major fatty acids. Based on genotypic and phenotypic characteristics, [*Flavobacterium*] *ferrugineum* IAM 15098^T could be clearly differentiated from other members of the genus *Flavobacterium*. Strain DY^T and [*Flavobacterium*] *ferrugineum* IAM 15098^T could be easily distinguished from neighbouring taxa by morphological features (non-motile, non-gliding and non-filamentous single cells). Therefore, it is proposed that [*Flavobacterium*] *ferrugineum* IAM 15098^T and strain DY^T represent two separate species of a new genus, *Terrimonas* gen. nov., with the names *Terrimonas ferruginea* comb. nov. (type species; type strain IAM 15098^T = ATCC 13524^T) and *Terrimonas lutea* sp. nov. (type strain DY^T = IAM 15284^T = CCTCC AB205006^T), respectively.

During screening of nitrogen-fixing bacteria in a soil sample from the garden of the University of Tokyo (Japan), a novel yellow-pigmented bacterium, designated DY^T, was isolated. A polyphasic study demonstrated that strain DY^T was closely related to [*Flavobacterium*] *ferrugineum* IAM 15098^T, a member of the phylum *Bacteroidetes*, which had been isolated from soil from an unknown locality. Previous data from DNA–rRNA hybridization (Steyn *et al.*, 1992), 16S rRNA gene sequence analysis (Nakagawa *et al.*, 2002) and chemotaxonomic studies (Takeuchi & Yokota, 1992) had shown that [*Flavobacterium*] *ferrugineum* IAM 15098^T was not related to other members of the genus *Flavobacterium* and represented an independent taxon within the phylum *Bacteroidetes*. Therefore, [*Flavobacterium*] *ferrugineum* IAM 15098^T should be reclassified (Sly *et al.*, 1999; Nakagawa & Yamasato, 1996; Bernardet *et al.*, 1996). New data [sequences of the 16S rRNA and *gyrB* (B subunit of DNA gyrase) genes, respiratory quinone system, cellular fatty acids and phenotypic characteristics] indicated that the soil isolate DY^T and [*Flavobacterium*] *ferrugineum* IAM 15098^T represent two separate species of a novel genus.

[*Flavobacterium*] *ferrugineum* IAM 15098^T and strain DY^T grew well in nutrient broth (NB; Difco), IAM medium 802 [polypeptone (Nihon Pharmaceutical Co.), 10 g; yeast extract, 2.0 g; MgSO₄·7H₂O, 1 g; distilled water, 1.0 l] and nitrogen-free medium (Xie & Yokota, 2005) at 29 °C. API 20E, API 50 CH and API ZYM kits (bioMérieux) were used to determine physiological and biochemical characteristics. The API strips were incubated for 2 days at 30 °C, except for API ZYM, the results of which were recorded after 4 h incubation at 37 °C, according to the manufacturer's instructions. Acid production from carbohydrates was determined using O/F basal medium (peptone, 2.0 g; NaCl, 5.0 g; K₂HPO₄, 0.3 g; bromothymol blue, 0.03 g; agar, 3.0 g; distilled water, 1 l; pH 7.1). A carbohydrate solution (sterilized by filtration) was added at a concentration of 1 % (w/v) and the results were recorded after 7 days of incubation. Tolerance of salinity was determined by inoculating the strains into NB supplemented with 0–4.0 % NaCl (w/v). Hydrolysis of chitin was assessed using the following medium: colloidal chitin, 4.0 g; (NH₄)₂SO₄, 1.0 g; KH₂PO₄, 1.36 g; MgSO₄·7H₂O, 0.3 g; NaCl, 1.0 g; and distilled water, 1 l; pH 7.3. The colloidal chitin was prepared as follows: 4.0 g chitin was dissolved in 40 ml of 36 % HCl by stirring for 30 min, precipitated in cold water and washed with water until the pH reached 3.5. Chitosan

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain DY^T are AB192292 and AB192293, respectively.

degradation was assessed using the method described by Park *et al.* (1999) and *Mitsuaria chitosanitabida* IAM 14711^T was used as a chitosanase-positive control. Cells grown on trypticase soy agar (TSA; Becton Dickinson) for 3 days at 29 °C were used for cellular fatty acid analysis. Fatty acid methyl esters were prepared and identified using the Microbial Identification System (MIDI; Sasser, 1990). Cells grown in NB for 5 days at 29 °C were used for the determination of the respiratory quinone system and of the DNA G+C content using HPLC (Shimadzu), as described by Xie & Yokota (2003). Genomic DNA was extracted using the method of Marmur (1961) from cells cultured for 2 days in NB.

Since strain DY^T was able to grow well in nitrogen-free medium, the presence of nitrogenase activity was assessed. The sensitive, rapid and inexpensive acetylene reduction assay (ARA) (Hardy *et al.*, 1968) was first used. [*Flavobacterium*] *ferrugineum* IAM 15098^T and strain DY^T were grown in 7 ml LB broth for 24 h at 30 °C. The cells were centrifuged and washed three times with nitrogen-free medium and 0.1 ml suspensions (OD₆₀₀ = 1.0) were transferred to gas vials (20 ml) containing 2 ml semi-solid nitrogen-free medium (0.3 % agar), in an atmosphere of 2 % oxygen, 10 % acetylene and 88 % nitrogen. After incubation at 30 °C for 18–24 h, 0.1 ml gas was withdrawn from the vials and analysed by gas chromatography (GC-17A; Shimadzu; equipped with a Porapak N column of 30 m in length) at 50 °C. Helium was used as a carrier gas at a flux of 3.4 ml min⁻¹ and H₂ flame ionization was used for detection. As ethylene was not produced from acetylene in the ARA test, nitrogenase activity was further assessed by attempting to amplify the *nifH* gene (encoding the iron protein of nitrogenase) using the primers IGK-AQE, PolF-PolR or IGK-R750 (Xie & Yokota, 2005). However, the *nifH* gene could not be amplified from strain DY^T or [*Flavobacterium*] *ferrugineum* IAM 15098^T. Hence, the fact that nitrogenase activity could not be detected using two different tests demonstrated that the two strains did not have nitrogen-fixing ability, although they could grow well on nitrogen-free medium. They may have used atmospheric nitrogen for growth or may possess some unusual

nitrogen-fixing system, as reported for *Streptomyces thermoautotrophicus* (Gadkari *et al.*, 1992).

An approximately 1500-bp fragment of the 16S rRNA gene sequence was amplified by PCR and sequenced as described by Xie & Yokota (2003). The *gyrB* gene sequences were amplified and sequenced using a set of primers described by Yamamoto & Harayama (1995) and a newly designed primer, *gyrB*840 (CTTCACCAACAACATCCC). The sequences obtained in this study and from GenBank were aligned using the CLUSTAL W software package (Thompson *et al.*, 1994) and evolutionary distances and the *K*_{nuc} value (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were excluded from the calculations. A phylogenetic tree based on a comparison of 1189 bases was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates, whereas similarity values were calculated using PAUP 4.0b1 (Swofford, 1998). Using the same method, 399 aa sequences of GyrB were also aligned and a phylogenetic tree was constructed.

Comparison of the 16S rRNA gene sequences showed that strain DY^T and [*Flavobacterium*] *ferrugineum* IAM 15098^T had a sequence similarity of 94.8 %, forming a branch that shared less than 92 % sequence similarity with the cluster comprising [*Cytophaga*] *arvensicola*, [*Flexibacter*] *japonensis*, [*Flexibacter*] *sancti*, [*Flexibacter*] *filiformis* and *Chitinophaga pinensis* (Oyaizu *et al.*, 1982; Fujita *et al.*, 1996; Sly *et al.*, 1999) (Fig. 1).

The GyrB amino acid sequence similarity between strain DY^T and [*Flavobacterium*] *ferrugineum* IAM 15098^T was 90.1 %, whereas the sequence similarity between the two strains and other members of the related cluster was less than 78 %. A phylogenetic tree (Fig. 2) based on 399 aa of GyrB was consistent with the tree based on 16S rRNA gene sequences. The level of 16S rRNA (94.8 %) and *gyrB* (77.5 %) gene sequence similarity shared by strain DY^T and [*Flavobacterium*] *ferrugineum* IAM 15098^T suggests that they represent two separate species in the same genus, which

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1189 bp) showing the phylogenetic relationships of the soil isolate DY^T and [*Flavobacterium*] *ferrugineum* IAM 15098^T. The type species of the genus *Flavobacterium*, *Flavobacterium aquatile*, was used as an outgroup. Numbers at nodes indicate percentage occurrence in 100 bootstrapped trees; only values >50 % are shown. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. Neighbour-joining tree based on GyrB sequences (399 aa) showing the phylogenetic relationships of strain DY^T and [Flavobacterium] ferrugineum IAM 15098^T. Numbers at nodes indicate percentage occurrence in 100 bootstrapped trees; only values >60% are shown. Bar, 0.05 substitutions per amino acid.

can be easily differentiated from each other by several phenotypic characteristics (Table 1).

The major cellular fatty acids of strain DY^T and [Flavobacterium] ferrugineum IAM 15098^T were 15:0 iso, 15:1 iso, 17:0 iso 3-OH and summed feature 3 (comprising 15:0 iso 2-OH and/or 16:1ω7c, which could not be separated by the MIDI system) (Table 2). This fatty acid composition differed significantly from that of [Cytophaga] arvensicola (major fatty acids 15:0 iso, 15:0 iso 2-OH, 15:0 iso 3-OH, 16:1ω11c and 17:0 iso 3-OH; Oyaizu *et al.*, 1982). Several

characteristics (non-filamentous cell morphology; absence of gliding motility, chitin degradation and urease activity and presence of catalase activity) distinguished strain DY^T and [Flavobacterium] ferrugineum IAM 15098^T from [Flexibacter] japonensis, [Flexibacter] sancti, [Flexibacter] filiformis and Chitinophaga pinensis (Table 3). On the other hand, strain DY^T and [Flavobacterium] ferrugineum IAM 15098^T possessed MK-7 as the major menaquinone and MK-6 as a minor component, similar to [Cytophaga] arvensicola (Oyaizu *et al.*, 1982) and [Flexibacter] japonensis (Fujita *et al.*, 1996). The G+C content of the DNA of strain DY^T (48.9 mol%) and [Flavobacterium] ferrugineum IAM 15098^T (47.2 mol%) was similar to that of their close relatives. Our data confirmed that [Flavobacterium] ferrugineum differs

Table 1. Differential characteristics of *Terrimonas ferruginea* comb. nov. IAM 15098^T and *Terrimonas lutea* sp. nov. strain DY^T

Both strains are positive for Voges–Proskauer test, gelatin liquefaction and nitrate reduction, but are negative for indole and H₂S production, citrate utilization and urease and arginine dihydrolase activities. w, Weakly positive.

Characteristic	IAM 15098 ^T	DY ^T
Colony pigmentation	Salmon red	Yellow
Acid production from:		
Rhamnose	+	–
Raffinose	–	+
Sucrose	–	+
Melibiose	+	–
Galactose	–	+
Fructose	–	+
Assimilation of:		
Arabinose	–	+
Trehalose	–	+
D-Xylose	+	–
Methyl α-D-mannoside	–	+
N-Acetylglucosamine	–	+
Melibiose	–	+
Sucrose	–	+
Starch	+	–
Glycogen	+	–
Gentiobiose	–	+
Enzymic activities in API ZYM test:		
β-Galactosidase	+	–
α-Chymotrypsin	+	–
α-Fucosidase	w	–

Table 2. Cellular fatty acid composition (%) of *Terrimonas ferruginea* comb. nov. IAM 15098^T and *Terrimonas lutea* sp. nov. strain DY^T

Summed feature 3 comprises 15:0 iso 2-OH and/or 16:1ω7c, which could not be separated using the MIDI system. ECL, Unknown fatty acids (numbers indicate equivalent chain length). ND, Not detected.

Fatty acid	IAM 15098 ^T	DY ^T
13:0 iso	0.5	0.5
13:1	0.2	0.7
ECL 13:565	1.27	3.2
14:0 iso	1.1	4.3
14:0	0.5	0.4
15:1 iso	26.2	21.8
15:0 iso	28.4	34.8
15:0 anteiso	0.6	ND
15:0	2.7	0.4
16:0 iso	1.4	ND
Summed feature 3	11.2	14.9
16:0	1.7	1.1
15:0 iso 3-OH	2.2	2.3
15:0 2-OH	0.8	0.3
15:0 3-OH	0.3	ND
ECL 16:582	1.3	1.8
16:0 iso 3-OH	0.7	ND
16:0 3-OH	2.5	3.3
17:0 iso 3-OH	15.3	14.2

Table 3. Differential characteristics of the genus *Terrimonas* and related taxa

Taxa: 1, *Terrimonas*; 2, [*Cytophaga*] *arvensicola* IAM 12650^T; 3, [*Flexibacter*] *japonensis* NBRC 16041^T; 4, [*Flexibacter*] *sancti* NBRC 15057^T; 5, [*Flexibacter*] *filiformis* NBRC 15056^T; 6, *Chitinophaga pinensis* ACM 2034^T. Data from Takeuchi & Yokota (1992), Oyaizu *et al.* (1982), Bernardet *et al.* (2002) and Fujita *et al.* (1996). All strains were isolated from soil and are negative for the production of acid from glucose and of H₂S. ND, Not determined; w, weakly positive.

Characteristic	1	2	3	4	5	6
Pigment	Red or yellow	Yellow–orange	Yellow–orange	Golden yellow	Golden yellow	Yellow
Cell length (µm)	1–2	0.6–4	2–18	2–15	30–80	< 40
Gliding motility	–	–	+	+	+	+
Catalase	w	+	+	–	–	+
Urease	–	–	ND	ND	ND	+
Gelatin liquefaction	+	–	+	+	+	+
Chitin degradation	–	–	–	–	+	+
Growth at 37 °C	+	–	+	–	+	+
Highest NaCl concentration tolerated (%)	1.0	2.0	2.0	1.0	0.3	ND
G + C content (mol%)	47.2–48.9	46.0	49.8	43.3	45.0	45.2

considerably from other members of the genus *Flavobacterium*, where the major menaquinone is MK-6 and the G + C content is 32–37 mol% (Bernardet *et al.*, 1996).

Based on phylogenetic and phenotypic analyses, it is proposed that [*Flavobacterium*] *ferrugineum* IAM 15098^T and the soil isolate DY^T represent two independent species in a new genus, *Terrimonas* gen. nov., with the names *Terrimonas ferruginea* comb. nov. and *Terrimonas lutea* sp. nov., respectively.

Description of *Terrimonas* gen. nov.

Terrimonas (Ter.ri.mo' nas. L. n. terra soil; L. fem. n. monas a unit, monad; N.L. fem. n. *Terrimonas* soil monad).

Cells are strictly aerobic, Gram-negative, non-motile, non-gliding, non-filamentous single rods. Growth is inhibited by >1.0% NaCl. Oxidase-positive and weakly positive for catalase. Positive for gelatin liquefaction, Voges–Proskauer test and nitrate reduction, but negative for urease production and chitin degradation. Major cellular fatty acids are 15:0 iso, 15:1 iso, 17:0 iso 3-OH and summed feature 3. MK-7 is the predominant respiratory quinone and MK-6 is a minor component. The G + C content of the DNA is 47.2–48.9 mol%. The type species is *Terrimonas ferruginea*.

Description of *Terrimonas ferruginea* comb. nov.

Terrimonas ferruginea (fer.ru.gi' ne.a. L. fem. adj. *ferruginea* rust-coloured).

Basonym: *Flavobacterium ferrugineum* Sickles and Shaw 1934.

The characteristics are the same as those given in the genus description. Cells are single rods, 0.3–0.5 µm in width and 1–3 µm in length. Colonies are circular, about 1 mm in diameter and non-spreading. Growth occurs at 10–37 °C, with optimum growth at 25–32 °C. Colonies on nutrient

agar and IAM medium 802 are salmon red. Grows well on TSA and nitrogen-free medium. Chitin and chitosan are not degraded on conventional media. Acid is produced from glucose, cellobiose, maltose, mannose, melibiose, rhamnose and xylose, but not from sucrose, dulcitol, glycerol, inositol, mannitol, sorbitol, raffinose, fructose or galactose. Glucose, mannose, aesculin, xylose, maltose, cellobiose, rhamnose, lactose, 5-ketogluconate, starch and glycogen are assimilated, but sucrose, trehalose, dulcitol, arabinose, galactose, fructose, glycerol, inositol, mannitol, methyl α-D-mannoside, raffinose, sorbitol and gentiobiose are not. Activities of β-galactosidase, alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, α-chymotrypsin, naphthol phosphohydrolase, C4 esterase, C8 esterase lipase and N-acetyl-β-glucosaminidase are strongly positive; activities of cystine arylamidase, trypsin, α-glucosidase and α-fucosidase are weakly positive. α-Galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and C14 lipase activities are absent. The G + C content of the DNA of the type strain is 48.9 mol%.

The type strain is IAM 15098^T (=NBRC 14992^T = LMG 4021^T = ATCC 13524^T), which was isolated from soil from an unknown locality.

Description of *Terrimonas lutea* sp. nov.

Terrimonas lutea (lu.te'a. L. fem. adj. *lutea* golden-yellow).

The characteristics are the same as those given in the genus description. Cells are single rods, 0.3–0.5 µm in width and 1–3 µm in length. Colonies are circular, about 1 mm in diameter and non-spreading. Growth occurs at 10–37 °C, with optimum growth at 25–32 °C. Colonies are yellow on nutrient agar and IAM medium 802. Grows well on TSA and nitrogen-free medium. Chitin and chitosan are not degraded on conventional media. Acid is produced from glucose, cellobiose, maltose, mannose, fructose, galactose, raffinose and sucrose, but not from dulcitol, glycerol,

inositol, mannitol, melibiose, rhamnose, xylose or sorbitol. Glucose, mannose, aesculin, maltose, lactose, 5-ketoglucuronate, arabinose, galactose, fructose, methyl α -D-mannoside, cellobiose, *N*-acetylglucosamine, sucrose, raffinose, gentiobiose and melibiose are assimilated, but starch, D-xylose, glycogen, glycerol, inositol, mannitol, sorbitol, rhamnose and dulcitol are not. Alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol phosphohydrolase, C4 esterase, C8 esterase lipase and *N*-acetyl- β -glucosaminidase activities are strongly positive; C14 lipase, cystine arylamidase, α - and β -glucosidase and trypsin activities are weakly positive; β -glucuronidase, α - and β -galactosidases, α -mannosidase, α -chymotrypsin and α -fucosidase activities are absent. The fatty acid composition is only slightly different from that of the type species. The DNA G + C content of the type strain is 47.2 mol%.

The type strain is DY^T (= IAM 15284^T = CCTCC AB205006^T), which was isolated from garden soil in Japan.

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