Terrimonas aquatica sp. nov., isolated from a freshwater spring

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A yellow-pigmented bacterial strain, designated RIB1-6^T, was isolated from a freshwater spring in Taiwan. Strain RIB1-6^T was aerobic, Gram-negative, rod-shaped, non-motile and non-spore-forming. Growth occurred at 10–37 °C, at pH 7–8 and with 0–1 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain RIB1-6^T belonged to the genus *Terrimonas* and its two closest neighbours were *Terrimonas ferruginea* ATCC 13524^T and *Terrimonas lutea* DY^T (16S rRNA gene sequence similarity 97.4 % and 93.5 %, respectively). Strain RIB1-6^T contained iso-C_{15:0} (33.4 %), iso-C_{17:0} 3-OH (18.2 %), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 14.7 %) and iso-C_{15:1} (11.5 %) as the predominant fatty acids. The major isoprenoid quinone was MK-7. The DNA G + C content of strain RIB1-6^T was 47.3 mol%. On the basis of the genotypic and phenotypic data, strain RIB1-6^T represents a novel species in the genus *Terrimonas*, for which the name *Terrimonas aquatica* sp. nov. is proposed. The type strain is RIB1-6^T (=BCRC 17941^T=LMG 24825^T).

The genus *Terrimonas* was proposed by Xie & Yokota (2006a). At the time of writing, this genus comprises only two recognized species: *Terrimonas ferruginea*, the type species, previously [*Flavobacterium*] *ferrugineum*, and *Terrimonas lutea*. Based on detailed phylogenetic analyses of the 16S rRNA gene sequence, Ludwig *et al.* (2008) proposed a new family, *Chitinophagaceae*, to consist of the genera *Chitinophaga* (Sly *et al.*, 1999), *Terrimonas* (Xie & Yokota, 2006a), *Niastella* (Weon *et al.*, 2006), *Niabella* (Kim *et al.*, 2007), *Flavisolibacter* (Yoon & Im, 2007), *Segetibacter* (An *et al.*, 2007) and *Sediminibacterium* (Qu & Yuan, 2008). Three other recently described genera, *Filimonas* (Shiratori *et al.*, 2009), *Ferruginibacter* (Lim *et al.*, 2009) and *Lacibacter* (Qu *et al.*, 2009), also belong to the family. The present study was carried out to clarify the taxonomic position of a *Terrimonas*-like bacterial strain, RIB1-6^T.

During the characterization of micro-organisms from a freshwater spring in Kaohsiung County, Taiwan (22° 36' 9.2" N 120° 22' 52.5" E), a yellow strain, RIB1-6^T, was

selected for detailed analysis. Strain RIB1-6^T was isolated on R2A agar (BD Difco) after incubation at 30 °C for 3 days. The isolate was subcultivated under the same conditions for 2–3 days and stored at –80 °C in R2A broth with 20 % (v/v) glycerol or by lyophilization. On R2A agar, strain RIB1-6^T was able to grow at 20–32 °C. *T. ferruginea* JCM 21559^T and *T. lutea* JCM 21735^T were obtained from the Japan Collection of Microorganisms and used as reference strains for phenotypic and fatty acid analyses.

The morphology of cells from the lag, exponential and stationary phases of growth was observed by phase-contrast microscopy (DM 2000; Leica). Flagellar and gliding motility was tested by the hanging drop method. Gram reaction was determined using the Gram stain Set S kit (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Cells were stained with Sudan black and observed using light microscopy to determine the presence of poly-β-hydroxybutyrate granules. Colony morphology was observed on R2A agar using a stereoscopic microscope (SMZ 800; Nikon).

Growth at pH 4–10 (at intervals of 1 pH unit) was determined by measuring the optical density (wavelength 600 nm) of cultures in nutrient broth (NB; BD Difco) with the pH adjusted prior to sterilization using appropriate biological buffers (Chung *et al.*, 1995). Verification of the pH values after autoclaving revealed only minor changes.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RIB1-6^T is FJ347757.

A table showing sole carbon source utilization patterns of strain RIB1-6^T and its closest phylogenetic neighbours is available with the online version of this paper.

Growth at 4, 10, 15, 20, 25, 30, 32, 37, 40, 45 and 50 °C was determined on nutrient agar (NA; BD Difco). Growth with 0, 0.5 and 1.0–10.0 %, w/v, NaCl (at intervals of 1.0 %) was determined in NB prepared according to the formula of the medium but with the NaCl concentration adjusted. Growth under anaerobic conditions was investigated by incubating strain RIB1-6^T on R2A agar in the Oxoid AnaeroGen system. Strain RIB1-6^T was examined for a broad range of phenotypic properties. Catalase, oxidase, DNase, urease and lipase (corn oil) activities and hydrolysis of starch, casein, chitin, gelatin, agar and Tweens 20, 40, 60 and 80 were determined using standard methods (Gerhardt *et al.*, 1994). Hydrolysis of CM-cellulose was tested as described by Bowman (2000) using R2A agar as the basal medium. The presence of flexirubin-type pigments was determined using 20 % KOH (Fautz & Reichenbach, 1980). Additional biochemical tests were performed using API ZYM, API 20E and API 20NE kits (bioMérieux) and carbon source utilization was evaluated using GN2 MicroPlates (Biolog), according to the manufacturers' recommendations.

Antibiotic sensitivity of strain RIB1-6^T and the reference strains was determined by the disc diffusion method after spreading cells (0.5 McFarland standard) on R2A agar, using discs (Oxoid) containing the following antibiotics (µg per

disc): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), nalidixic acid (30), novobiocin (30), rifampicin (5), penicillin G (10), streptomycin (10), sulfamethoxazole (23.75) plus trimethoprim (1.25) and tetracycline (30). The effect of antibiotics on cell growth was assessed after 2 days at 30 °C and a strain was considered susceptible if the diameter of the inhibition zone was >13 mm, intermediate if 10–12 mm and resistant if <10 mm. The phenotypic characteristics of strain RIB1-6^T are given in Table 1, Supplementary Table S1 (available in IJSEM Online) and the species description.

The 16S rRNA gene sequence of strain RIB1-6^T was analysed as described previously (Chen *et al.*, 2001). After multiple alignments of the data using CLUSTAL_X (Thompson *et al.*, 1997), analysis of the sequence data was performed using the BioEdit software package (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Distances were calculated and corrected according to Kimura's two-parameter model (Kimura, 1983) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) phylogenetic trees were generated using the algorithms contained in the PHYLIP software package (Felsenstein, 1993). Bootstrap values were calculated on 1000 replications. An almost-complete

Table 1. Differential characteristics of species of the genus *Terrimonas*

Taxa: 1, RIB1-6^T; 2, *T. ferruginea* JCM 21559^T; 3, *T. lutea* JCM 21735^T. All data were taken from this study except for DNA G + C content in columns 2 (*T. ferruginea* IAM 15098^T) and 3 (*T. lutea* DY^T; Xie & Yokota, 2006a, b). +, Positive; w, weakly positive; –, negative; s, sensitive; R, resistant.

Characteristic	1	2	3
Colony pigmentation	Yellowish orange	Salmon red	Yellow
Flexirubin-type pigments	+	+	–
Hydrolysis of:			
Casein	+	–	–
Starch	+	+	w
Lipid	–	–	w
Enzyme activities (API ZYM)			
Trypsin	–	–	w
α-Chymotrypsin	–	–	+
β-Glucuronidase	+	–	–
α-Mannosidase	–	w	w
α-Fucosidase	+	–	w
Assimilation of (API 20NE):			
N-Acetylglucosamine	+	+	–
Glucose	+	+	–
Arabinose	+	+	–
Mannose	–	w	w
Maltose	+	+	–
Malate	w	–	–
Susceptibility to:			
Gentamicin	s	R	s
Ampicillin	R	R	s
Chloramphenicol	R	s	s
Nalidixic acid	s	s	R
DNA G + C content (mol%)	47.3	48.9	47.2

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain RIB1-6^T and related taxa in the family *Chitinophagaceae*. Bootstrap values (>70%) based on 1000 replications are shown at branch nodes for neighbour-joining (above) and maximum-parsimony (below) algorithms. Filled circles indicate that the corresponding nodes were also recovered in trees generated using the maximum-likelihood and maximum-parsimony algorithms. *Flavobacterium aquatile* DSM 1132^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

16S rRNA gene sequence (1435 bp) of strain RIB1-6^T was compared against 16S rRNA gene sequences available from the EzTaxon server (Chun *et al.*, 2007), the Ribosomal Database Project (Maidak *et al.*, 2001) and GenBank (<http://www.ncbi.nlm.nih.gov/>). The 16S rRNA gene sequence analysis indicated that strain RIB1-6^T belonged to the family *Chitinophagaceae* of the class *Sphingobacteria*, phylum *Bacteroidetes* (Fig. 1). Strain RIB1-6^T formed a distinct subline within the genus *Terrimonas* in the neighbour-joining tree. The topologies of the maximum-likelihood and maximum-parsimony phylogenetic trees were similar. Sequence comparisons (over 1400 bp) indicated that strain RIB1-6^T was closely related to *T. ferruginea* ATCC 13524^T (97.4% 16S rRNA gene sequence similarity) and *T. lutea* DY^T (93.5%). Lower sequence similarities (<93.0%) were found with other representatives of the family *Chitinophagaceae*.

DNA–DNA hybridization experiments were performed using the method of Ezaki *et al.* (1989). The level of DNA–DNA relatedness of strain RIB1-6^T with *T. ferruginea* JCM 21559^T and *T. lutea* JCM 21735^T was 53.6 ± 3.2 and 4.7 ± 3.4 %, respectively. Since the recommended DNA–DNA relatedness threshold for the definition of a species is 70% (Wayne *et al.*, 1987), these results indicate that strain RIB1-6^T does not belong to any known species of the genus *Terrimonas*.

The fatty acid profiles of strain RIB1-6^T, *T. ferruginea* JCM 21559^T and *T. lutea* JCM 21735^T were determined using cells grown on NA at 30 °C for 2 days. The fatty acid methyl esters were prepared, separated and identified

according to the instructions of the Microbial Identification System (Sasser, 1990). The fatty acid profile of strain RIB1-6^T was similar to those of the other species of the genus *Terrimonas*, although there were differences in the proportions of some components (Table 2). The major fatty acids of strain RIB1-6^T were iso-C_{15:0}, iso-C_{17:0} 3-OH, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1} ω7c) and iso-C_{15:1}. Isoprenoid quinones were extracted and purified according to the method of Collins (1985) and were analysed by HPLC. The major respiratory quinone of strain RIB1-6^T was MK-7. The DNA G+C content of strain RIB1-6^T, determined by HPLC according to Mesbah *et al.* (1989), was 47.3 ± 1.0 mol%.

The 16S rRNA gene sequence similarity, fatty acid profile, major respiratory quinone, DNA G+C content and some phenotypic properties of strain RIB1-6^T are consistent with properties of members of the genus *Terrimonas* (Xie & Yokota, 2006a). However, strain RIB1-6^T could be differentiated from *T. ferruginea* JCM 21559^T and *T. lutea* JCM 21735^T by a combination of physiological and biochemical characteristics. Hence, strain RIB1-6^T represents a novel species of the genus *Terrimonas*, for which the name *Terrimonas aquatica* sp. nov. is proposed.

Description of *Terrimonas aquatica* sp. nov.

Terrimonas aquatica (a.qua'ti.ca. L. fem. adj. *aquatica* living, growing or found in the water, aquatic).

Cells are strictly aerobic, Gram-negative, non-spore-forming, non-motile and rod-shaped. Poly-β-hydroxybutyrate

Table 2. Cellular fatty acid compositions of species of the genus *Terrimonas*

Taxa: 1, RIB1-6^T; 2, *T. ferruginea* JCM 21559^T; 3, *T. lutea* JCM 21735^T. All data were taken from this study. Fatty acids amounting to <1% in all strains are not shown. tr, Trace (<1%); –, not detected.

Fatty acid (%)	1	2	3
Saturated straight-chain			
C _{12:0}	tr	1.0	tr
C _{14:0}	2.1	2.6	tr
C _{16:0}	4.2	4.9	2.9
C _{18:0}	2.1	3.0	1.9
Saturated branched			
iso-C _{13:0}	tr	1.1	tr
iso-C _{14:0}	1.2	tr	tr
iso-C _{15:0}	33.4	34.7	25.8
iso-C _{15:1}	11.5	14.9	21.7
iso-C _{16:0}	1.7	1.8	tr
iso-C _{19:0}	–	–	1.2
Unsaturated			
anteiso-C _{17:1ω9c}	–	–	1.0
Hydroxy			
iso-C _{15:0} 3-OH	3.2	2.3	3.0
iso-C _{16:0} 3-OH	1.0	tr	1.6
C _{16:0} 3-OH	3.1	3.1	3.1
iso-C _{17:0} 3-OH	18.2	14.9	13.9
Summed feature 3*	14.7	11.9	20.0

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1ω7c}.

granules are not accumulated. Flexirubin-type pigments are present. After 24 h on R2A agar at 30 °C, the mean cell size is approximately 0.3–0.5 µm in width and 1.8–3.0 µm in length. Colonies are yellow, convex, round and smooth with entire edges, approximately 1.0 mm in diameter on R2A agar after 72 h at 30 °C. Growth occurs at 10–37 °C (optimum 30 °C), at pH 7–8 (optimum pH 7) and with 0–1% NaCl (optimum 0%). Positive for oxidase, catalase and DNase activities and hydrolysis of casein, gelatin and starch. Negative for lipase and urease activities and hydrolysis of chitin, agar, Tweens 20, 40, 60 and 80 and CM-cellulose. With API 20NE, positive for β-glucosidase and β-galactosidase activities, gelatin hydrolysis and assimilation of glucose, arabinose, N-acetylglucosamine, maltose and malate, but negative for nitrate reduction, indole production, glucose acidification, arginine dihydrolyase and urease activities and for assimilation of mannose, mannitol, gluconate, caprate, adipate, citrate and phenylacetate. With API 20E, positive for β-galactosidase activity and gelatin hydrolysis. With API ZYM, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, N-acetyl-β-glucosaminidase and α-fucosidase, but negative

for lipase (C14), trypsin, α-chymotrypsin and α-mannosidase. With GN2 MicroPlates, the following are utilized as sole carbon sources: glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, cellobiose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, melibiose, β-methyl D-glucoside, trehalose, turanose, D-galacturonic acid, DL-lactic acid, L-glutamic acid, L-ornithine and L-proline; all other substrates are not utilized. Resistant to penicillin G, ampicillin and chloramphenicol, but sensitive to gentamicin, rifampicin, kanamycin, tetracycline, novobiocin, streptomycin, nalidixic acid and sulfamethoxazole plus trimethoprim. The major fatty acids (>10%) are iso-C_{15:0}, iso-C_{17:0} 3-OH, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1ω7c}) and iso-C_{15:1}. The major respiratory quinone is MK-7.

The type strain is RIB1-6^T (=BCRC 17941^T=LMG 24825^T), which was isolated from a freshwater spring, Kaoshiung County, Taiwan. The DNA G + C content of the type strain is 47.3 mol%.

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