Silvimonas terrae gen. nov., sp. nov., a novel chitin-degrading facultative anaerobe belonging to the 'Betaproteobacteria'

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A taxonomic study was carried out on a bacterial strain, designated KM-45^T, isolated from forest soil collected near Daejeon, South Korea. Comparative 16S rRNA gene sequence analysis indicated a clear affiliation of this bacterium to the 'Betaproteobacteria' and that it was related most closely to *Chitinibacter tainanensis* BCRC 17254^T, *Formivibrio citricus* DSM 6150^T and *Iodobacter fluvialis* ATCC 33051^T (92.4, 91.2 and 88.9% 16S rRNA gene sequence similarity, respectively). Cells were Gram-negative, facultatively anaerobic, motile and rod-shaped. The strain grew well on R2A medium and utilized a broad spectrum of carbon sources. The G + C content of the genomic DNA was 58 mol% and the predominant ubiquinone was Q-8. Major fatty acids were C_{16:0}, C_{16:1ω7c}/iso-C_{15:0} 2-OH, C_{18:1ω7c}/ω9t/ω12t and C_{17:0} cyclo. On the basis of the evidence presented, it is proposed that strain KM-45^T should be placed in a novel genus and species, for which the name *Silvimonas terrae* gen. nov., sp. nov. is proposed. The type strain is KM-45^T (=KCTC 12358^T=NBRC 100961^T).

During the course of a study on the culturable aerobic and facultatively anaerobic bacterial community of forest soil and rice fields near Daejeon, South Korea, a large number of novel bacterial strains were isolated. One of these isolates, strain KM-45^T, was identified as belonging to the family *Neisseriaceae* in the 'Betaproteobacteria' lineage and was the subject of further taxonomic investigation. We report on its taxonomic position, including genotypic, chemotaxonomic and classical phenotypic characteristics, in order to establish its phylogenetic affiliation, and propose that strain KM-45^T should be placed in a novel genus and species, for which the name *Silvimonas terrae* gen. nov., sp. nov. is proposed.

Strain KM-45^T was isolated from forest soil collected near the Korea Advanced Institute of Science and Technology (KAIST) in Daejeon, South Korea. The soil was homogenized by using an Ace Homogenizer (Nihonseiki Kaisha Ltd). The suspension was spread on R2A agar plates (Difco) after being diluted serially with 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30 °C for 2 weeks. Single colonies on the plates were purified by transferring them onto new plates and incubating again under the same conditions. The isolate was cultured routinely on R2A agar

at 30 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C.

Extraction of genomic DNA was performed by using a commercial genomic DNA extraction kit (Core Biosystem), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to the methods given by Kim *et al.* (2005). The full-length 16S rRNA gene sequences were compiled by using SeqMan software (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed by using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BIOEDIT program (Hall, 1999). Evolutionary distances were calculated by using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods, using the MEGA3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

A nearly complete 16S rRNA gene sequence of strain KM-45^T was obtained (1491 bp). Preliminary comparison against the 16S rRNA gene sequences in GenBank indicated that strain KM-45^T belonged to the order 'Neisseriales' of the 'Betaproteobacteria'. On the basis of 16S rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KM-45^T is AB194302.

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of *Silvimonas terrae* gen. nov., sp. nov. and its nearest neighbours based on 16S rRNA gene sequence analysis. Dots indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1000 replications) >70% are shown at the branch points. Bar, 1 substitution per 100 nucleotide positions.

sequence similarity, the closest recognized relatives were *Chitinibacter tainanensis* BCRC 17254^T (92.4%), *Formivibrio citricus* DSM 6150^T (91.2%) and *Iodobacter fluvialis* ATCC 33051^T (88.9%). This relationship between strain KM-45^T and other members of the order 'Neisseriales' was also evident in the phylogenetic tree (Fig. 1). Strain KM-45^T and the three strains discussed above formed a monophyletic clade with a high bootstrap value (99%), which was supported by the neighbour-joining and maximum-parsimony methods employed.

Gram reaction was identified by the non-staining method as described by Buck (1982). Cell morphology was examined by light microscopy (Nikon) and transmission electron microscopy (Carl Zeiss) after negative staining with 1% (w/v) phosphotungstic acid. Catalase and oxidase tests were performed as outlined by Cappuccino & Sherman (2002). Substrate utilization as sole carbon source and other physiological characteristics were determined with the API 32GN and API 20NE galleries according to the manufacturer's instructions (bioMérieux). Tests for anaerobic growth were performed in a serum bottle containing R2A broth supplemented with thioglycolate (1 g l⁻¹) and the upper air layer was substituted with nitrogen gas. Nitrate- and nitrite-reduction tests were performed in serum bottles containing R2A broth supplemented with KNO₃ (10 mM) and NaNO₂ (10 mM), respectively, and reduction of nitrate and nitrite was monitored by using an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and anion-exchange column (Metrosep Anion Supp 4; Metrohm). Degradation of DNA [using DNA agar (Difco) supplemented with 0.01% toluidine blue (Merck)], chitin, CM-cellulose, starch (Atlas, 1993), lipids (Kouker & Jaeger, 1987) and xylan (Ten *et al.*, 2004) was also investigated; reactions were read after 5 days. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10% (w/v) NaCl after 5 days incubation. Duplicate antibiotic-sensitivity tests were performed by using filter-paper discs containing the following: streptomycin (5, 10 and 15 µg ml⁻¹), tetracycline (5, 10 and 15 µg ml⁻¹), kanamycin (1.0, 1.5 and 2.0 mg ml⁻¹) and ampicillin (20, 30 and 50 µg ml⁻¹) (Sigma). Discs were placed on R2A plates spread with KM-45^T culture and were then incubated at 30 °C for 5 days.

Cells of strain KM-45^T are Gram-negative, rod-shaped, facultatively anaerobic and motile by means of a single polar flagellum (Fig. 2b). The colony grown on R2A agar plates (Difco) for 3 days was circular (1.3–2.0 mm in diameter), convex and milky white. On R2A agar, strain KM-45^T was able to grow at 10–40 °C, but not at 4 or 45 °C. Physiological characteristics of strain KM-45^T are summarized in the genus and species descriptions and a comparison of selective

Fig. 2. (a) Phase-contrast photomicrograph of cells of *Silvimonas terrae* gen. nov., sp. nov. KM-45^T and (b) negatively stained transmission electron micrograph of a single cell, showing one polar flagellum. Bars, (a) 10 µm; (b) 2 µm.

characteristics with those of its nearest phylogenetic neighbours is given in Table 1.

Quinones were extracted from cells grown in nutrient broth (Difco) and analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC; cellular fatty acids were analysed in cells grown on trypticase soy agar (TSA, Difco) for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification system (MIDI, 1999). The fatty acids analysed by GC (Hewlett Packard 6890) were identified by the Microbial Identification software package. Mean values for each component were obtained from duplicate experiments.

Chromosomal DNA for determination of G + C content was extracted from cells grown on a nutrient agar plate (Difco) as described by Moore (1995). RNA in the DNA solution was removed by incubation with a mixture of RNases A and T1 (each at 20 U ml⁻¹) at 30 °C for 1 h. The G + C content of the chromosomal DNA was analysed as described by Mesbah *et al.* (1989) using reversed-phase HPLC.

Strain KM-45^T had Q-8 as its major quinone. The fatty acid profile of strain KM-45^T included C_{16:0} (33.5 ± 0.8%), summed feature 4 (C_{16:1}ω7*cl*/iso-C_{15:0} 2-OH, 21.8 ± 1.3%), summed feature 7 (C_{18:1}ω7*cl*/ω9*t*/ω12*t*, 14.7 ± 0.2%), C_{17:0} cyclo (15.3 ± 1.6%), C_{14:0} (6.4 ± 0.3%), C_{12:0} (3.6 ± 0.3%) and summed feature 3 (C_{14:0} 3-OH/iso-C_{16:1} I, 3.4 ± 0.2%). The G + C content of the genomic DNA of strain KM-45^T was 58 mol%.

Phylogenetically, strain KM-45^T formed a novel lineage of descent within the 'Betaproteobacteria', and clustered with *C. tainanensis* BCRC 17254^T (92.4% 16S rRNA gene sequence similarity), *F. citricus* DSM 6150^T (91.2%) and *I. fluviatilis* ATCC 33051^T (88.9%). These low 16S rRNA gene sequence similarities support the affiliation of strain KM-45^T to a novel genus, which is also supported by its phenotypic and chemotaxonomic characteristics (Table 1). Strain KM-45^T can be differentiated from *C. tainanensis* BCRC 17254^T by its anaerobic growth, nitrate reduction and fatty acid profile. Distinguishing phenotypic characteristics of *F. citricus* DSM 6150^T are inability to reduce nitrate into nitrite, inability to hydrolyse chitin and no β-glucosidase activity. *I. fluviatilis* ATCC 33051^T can be differentiated from strain KM-45^T on the basis of its relatively low G + C content and other phenotypic characteristics (Table 1). On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence comparisons, strain KM-45^T is considered to represent a novel genus and species, for which the name *Silvimonas terrae* gen. nov., sp. nov. is proposed.

Description of *Silvimonas* gen. nov.

Silvimonas (Sil.vi.mo'nas. L. fem. n. *silva* forest; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Silvimonas* forest monad).

Cells are Gram-negative, rod-shaped (1.3–2.0 μm by 0.5–0.8 μm) and facultatively anaerobic. They occur singly or in pairs (Fig. 2a) and are motile by means of a single polar flagellum (Fig. 2b). Colonies on R2A are circular, convex

Table 1. Selective differential phenotypic and chemotaxonomic characteristics of *Silvimonas terrae* gen. nov., sp. nov. and its nearest phylogenetic neighbours

Taxa: 1, *Silvimonas terrae* KM-45^T; 2, *Chitinibacter tainanensis* BCRC 17254^T; 3, *Formivibrio citricus* DSM 6150^T; 4, *Iodobacter fluviatilis* NCTC 11159^T. +, Positive; –, negative result; Tr, trace.

Characteristic	1	2*	3†	4‡
Anaerobic growth	+	–	+	+
Nitrate reduction to NO ₂ [–]	+	–	–	+
Nitrite reduction to N ₂	–	–	–	+
Chitin hydrolysis§	+	+	–	Weak (delayed)
β-Glucosidase	+	–	–	+
Cellular fatty acid (% of total):				
C _{12:0} 3-OH	–	4.1 ± 0.2	Tr	2.5 ± 0.5
C _{14:0}	6.4 ± 0.3	Tr	2.2	8.4
C _{14:0} 3-OH	–	–	9.5	–
10-Methyl C _{19:0}	–	1.7 ± 0.3	–	–
Growth at 40 °C	+	+	–	–
DNA G + C content (mol%)	58	56	61	50–52
Major quinone	Q-8	Q-8	Q-8	Q-8

*Data taken from Chern *et al.* (2004).

†Data taken from Tanaka *et al.* (1991), except for chitin hydrolysis and cellular fatty acids (this study).

‡Data taken from Logan (1989), except for chitin hydrolysis and cellular fatty acids (this study).

§Data for chitin hydrolysis and cellular fatty acids, except those for *Silvimonas terrae*, were taken from Chern *et al.* (2004).

and milky white. Catalase and oxidase are positive. The predominant quinone is Q-8. Major cellular fatty acids are C_{16:0}, summed feature 4 (C_{16:1}ω7c/iso-C_{15:0} 2-OH), summed feature 7 (C_{18:1}ω7c/ω9t/ω12t) and C_{17:0} cyclo. Based on 16S rRNA gene sequence analysis, *Silvimonas* belongs to the 'Betaproteobacteria'. The type species is *Silvimonas terrae*.

Description of *Silvimonas terrae* sp. nov.

Silvimonas terrae (ter'rae. L. gen. n. *terrae* of the earth).

In addition to the characteristics that define the genus, it has the following characteristics. Growth is observed at temperatures of 15–40 °C (optimum 25–30 °C) and at pH values of 6.0–8.0 (optimum pH 7.0). Growth occurs in the absence of NaCl and in the presence of 2.0 % (w/v) NaCl; no growth is observed above 4.0 % (w/v) NaCl. Positive for production of acid from glucose, reduction of nitrate to nitrite and production of β-glucosidase and β-galactosidase. It can degrade chitin, starch, CM-cellulose and DNA, but cannot degrade lipid or xylan. Negative for reduction of nitrite to nitrogen, production of indole, arginine dihydrolase, urease and protease activities (gelatin hydrolysis). The following compounds are assimilated as sole carbon sources: L-fucose, L-arabinose, maltose, D-mannose, L-rhamnose, D-sucrose, mannitol, N-acetylglucosamine and gluconate. The following compounds are not assimilated as sole carbon sources: D-ribose, 3-hydroxybutyrate, acetate, adipate, citrate, 3-hydroxybenzoate, malate, valerate, 4-hydroxybenzoate, 5-ketogluconate, caprate, itaconate, D-sorbitol, lactate, malonate, phenylacetate, suberate, L-alanine, L-proline, L-serine, inositol, D-melibiose, glyco-gen and salicin. Resistant to 30 µg ampicillin ml⁻¹, 15 µg tetracycline ml⁻¹ and 15 µg streptomycin ml⁻¹, but sensitive to 1 mg kanamycin ml⁻¹. Major cellular fatty acids are C_{16:0}, summed feature 4 (C_{16:1}ω7c/iso-C_{15:0} 2-OH), summed feature 7 (C_{18:1}ω7c/ω9t/ω12t) and C_{17:0} cyclo. The G + C content of the genomic DNA of the type strain is 58 mol%.

The type strain, KM-45^T (=KCTC 12358^T=NBRC 100961^T), was isolated from forest soil collected near KAIST, Daejeon, South Korea.

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