Silvimonas iriomotensis sp. nov. and *Silvimonas amylolytica* sp. nov., new members of the class *Betaproteobacteria* isolated from the subtropical zone in Japan

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The taxonomic positions of two bacterial strains, ir6-1^T and ir6-4^T, isolated from soil collected in Iriomote Island in Japan, were determined by using a polyphasic taxonomic approach. The strains were facultatively anaerobic, motile and Gram-stain-negative rods and their optimum pH for growth was pH 4.0. Their major respiratory quinone was ubiquinone-8 and the predominant cellular fatty acids were C_{14:0}, C_{16:0}, C_{16:1ω7c} and C_{18:1ω7c}. The G+C content of the genomic DNA of ir6-1^T and ir6-4^T was 59.9 and 57.5 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strains clustered with the genus *Silvimonas* in the class *Betaproteobacteria*. DNA–DNA similarities were lower than 53% among ir6-1^T, ir6-4^T and *Silvimonas terrae* NBRC 100961^T, and these strains could be differentiated from each other by several phenotypic characters. Based on these results, we propose the emendation of the genus *Silvimonas* and inclusion of two novel species, *Silvimonas iriomotensis* sp. nov. (type strain ir6-1^T=NBRC 103188^T=CGMCC 1.8859^T=KCTC 22513^T) and *Silvimonas amylolytica* sp. nov. (ir6-4^T=NBRC 103189^T=CGMCC 1.8860^T=KCTC 22514^T).

In the course of isolation of free-living bacteria from subtropical areas in Japan by using a nitrogen-free medium, we obtained several bacterial strains included in the phylum *Proteobacteria* on the basis of partial 16S rRNA gene sequence analyses. Two isolates were phylogenetically related to the genus *Silvimonas* in the class *Betaproteobacteria*. The genus *Silvimonas* belongs to the family *Neisseriaceae* and accommodates only one species, *Silvimonas terrae*, until now (Yang *et al.*, 2005). *S. terrae* was isolated from forest soil in Korea in 2005 and is a Gram-negative, rod-shaped and facultatively anaerobic bacterium. It is distinguished from related genera by anaerobic growth, hydrolysis of chitin, nitrate reduction and growth at 40 °C (Stackebrandt *et al.*, 2007). In this study, we characterized the two novel isolates from genomic, chemotaxonomic and phenotypic points of view, and propose two novel species within the genus *Silvimonas* to accommodate these organisms.

Two strains, ir6-1^T and ir6-4^T were isolated from soil of the rhizosphere of bamboos collected in Iriomote Island, Japan. Approximately 10 mg soil sample was suspended in 0.5 ml sterilized distilled water. Soil suspension was diluted with sterilized distilled water and then inoculated

on a nitrogen-free agar plate (0.25 g KH₂PO₄, 0.125 g MgSO₄·7H₂O, 0.125 g NaCl, 0.005 g FeSO₄·7H₂O, 0.005 g Na₂MoO₄·2H₂O, 0.005 g MnSO₄·4H₂O, 0.1 g CaCO₃ and 10 g glucose per litre distilled water, pH 7.2). Clear colonies that appeared after 2 weeks at 25 °C were picked and purified by repeated restreaking on LB medium [10 g Bacto tryptone (Difco), 5 g yeast extract and 10 g sodium chloride per litre distilled water, pH 7.0). Attempts to isolate closely related strains from other sampling sites were unsuccessful.

Both strains ir6-1^T and ir6-4^T formed convex colonies, as described for the genus *Silvimonas* by Yang *et al.* (2005). Cellular morphology was observed under light and transmission electron microscopes. For the latter purpose, fresh bacterial cultures were stagnantly cultivated for 14 h in one-fifth-strength LB medium. Cells were then negatively stained with 1% phosphotungstic acid (pH 7.0) and observed under an Hitachi H-7600 transmission electron microscope at an accelerating voltage of 80 kV. Both strains ir6-1^T and ir6-4^T were motile and possessed a single polar flagellum similar to *S. terrae* (Fig. 1; Yang *et al.*, 2005).

Genomic DNA extraction and 16S rRNA gene sequencing were performed as previously described (Nakagawa *et al.*, 2002). Sequence data obtained were aligned with those of representative members of the class *Betaproteobacteria* by using CLUSTAL_X (Thompson *et al.*, 1997) and then

The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA gene sequences of strains ir6-1^T and ir6-4^T are AB326110 and AB326111, respectively.

Fig. 1. Transmission electron micrographs of negatively stained cells showing the single polar flagellum of strains ir6-1^T (a) and ir6-4^T (b). Bars, 0.5 μ m.

modified manually by referring to the 16S rRNA secondary structure of *Escherichia coli* (Gutell *et al.*, 1994) and using the BioEdit sequence alignment editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Phylogenetic trees were reconstructed by using the neighbour-joining method (Saitou & Nei, 1987) with calculated K_{nuc} values (Kimura, 1980). Topology of the trees was evaluated by using the bootstrap analysis method of Felsenstein (1985) with 1000 replicates. Almost complete sequences of the 16S rRNA gene from positions 28 to 1494 according to the *Escherichia coli* numbering system (Brosius *et al.*, 1978) were determined for strains ir6-1^T and ir6-4^T.

Analysis of the 16S rRNA gene sequences of the isolates resulted in their clustering with *Silvimonas terrae* within

the class *Betaproteobacteria* (Fig. 2). Similarity levels of the 16S rRNA gene sequences between strains ir6-1^T and *S. terrae* NBRC 100961^T, strains ir6-4^T and *S. terrae* NBRC 100961^T, and strains ir6-1^T and ir6-4^T were 98.8, 99.2 and 99.0 %, respectively. If two organisms show 98.7–99 % 16S rRNA gene sequence similarity, DNA–DNA reassociation experiments should be mandatory to reveal whether they belong to different species (Stackebrandt & Ebers, 2006). Thus, DNA–DNA hybridization experiments were carried out in microplate wells by using photobiotin-labelled probes, as described by Ezaki *et al.* (1989). The hybridization temperature was 47 °C in 2 $\times$ SSC containing 50 % formamide. Strains ir6-1^T and ir6-4^T showed 48–53 % and 23–35 % DNA–DNA relatedness with *S. terrae* NBRC 100961^T, respectively. DNA–DNA relatedness values between strains ir6-1^T and ir6-4^T were 40–45 %. G + C content of the DNA samples was subsequently determined according to Mesbah *et al.* (1989). The values for strains ir6-1^T and ir6-4^T were 59.9 and 57.5 mol%, respectively, similar to the value of 59.2 mol% obtained in this study for *S. terrae* NBRC 100961^T. These DNA–DNA similarity values indicated that the two isolates represented independent species.

To analyse whole-cell fatty acid composition, all strains were grown on both LB medium at 30 °C for 24 h and TSA (Difco) medium at 30 °C for 48 h. Fatty acid methyl esters were prepared and analysed according to the standard protocol described in the Microbial Identification System (Microbial ID). $C_{16:1\omega7c}$, which could not be separated by GLC with the MIDI system and forms a part of summed

Fig. 2. Neighbour-joining tree showing the phylogenetic positions of strains ir6-1^T and ir6-4^T based on 16S rRNA gene sequences. *Escherichia coli* K-12 (E05133) was used as an outgroup. Bar, 0.02 K_{nuc} . Bootstrap values greater than 700 in 1000 replicates are shown.

feature 3, was detected by GC-MS (GC system model 6890 and MSD model 5973; Agilent Technologies). Strains ir6-1^T and ir6-4^T showed similar fatty acid profiles, in which C_{14:0}, C_{16:0}, C_{16:1ω7c} and C_{18:1ω7c} were the dominant components (greater than 10 % of the total; Table 1) when strains were cultivated on LB. By contrast, the major fatty acids in *S. terrae* NBRC 100961^T were C_{16:0}, C_{17:0} cyclo, C_{16:1ω7c} and C_{18:1ω7c}. Strains ir6-1^T and ir6-4^T were clearly differentiated from *S. terrae* by the absence of C_{17:0} cyclo and the presence of C_{17:1ω6c} when grown on LB. When strains were cultivated on TSA, C_{17:0} cyclo was detected for strains ir6-1^T and ir6-4^T. C_{17:0} cyclo was one of the major fatty acids (greater than 10 %) in *S. terrae*, as described by Yang *et al.* (2005), but it was not a major component in the two isolates ir6-1^T and ir6-4^T (less than 5.8 %). Ubiquinone-8 was found to be a major respiratory quinone in strains ir6-1^T and ir6-4^T, as well as in *S. terrae*, when extracted according to Nakagawa & Yamasato (1993) and analysed by using an LC-MS (LC-MS 8000α, Shimadzu).

Anaerobic growth was examined after incubation at 25 °C for 3 weeks by using the Anaeropack (Mitsubishi Gas Chemical). Production of H₂S and oxidase was examined as described by Smibert & Krieg (1981). The ability of strains to degrade agar, alginate acid, casein, cellulose, CM-

cellulose, chitin, DNA, inulin, starch, Tween 80, tyrosine and yeast cells was investigated according to the methods of Lewin & Lounsbery (1969), Smibert & Krieg (1981) and Barrow & Feltham (1993) with one-fifth-strength LB medium. Utilization of ammonium sulfate, Bacto caseamino acids (Difco), sodium glutamate, sodium nitrate, Bacto peptone (Difco) and urea as nitrogen sources was examined by using basal medium (0.1 % glucose, 0.02 % sodium acetate, pH 7.2) supplemented with each compound. To analyse the growth response at different temperatures (5–45 °C, increments of 5 °C) and pH (3–11, increments of 1 pH unit), one-fifth-strength LB medium was used for cultivation with shaking. NaCl range for growth was determined by using one-fifth-strength LB medium supplemented with 0, 1, 3 or 5 % NaCl. The potential of the strains to metabolize 95 different carbon sources was examined by using GN2 Microplates (Biolog). Other phenotypic characteristics of the isolates were analysed with the API 20NE according to the manufacturer's protocol (bioMérieux). Phenotypic characteristics of strains ir6-1^T and ir6-4^T are summarized in the species descriptions and in Table 2.

Strains ir6-1^T and ir6-4^T were shown to belong to the genus *Silvimonas* and could be clearly differentiated from each other and from *S. terrae* by their cellular fatty acid compositions and phenotypic properties, summarized in Table 2. The two isolated strains evidently represent novel species within the genus *Silvimonas*. With the addition of two new species, the original genus description by Yang *et al.* (2005) cannot cover the characteristics of all *Silvimonas* species. Accordingly, we propose the emendation of the genus *Silvimonas* and the names *Silvimonas iriomotensis* sp. nov. to accommodate strain ir6-1^T and *Silvimonas amylolytica* sp. nov. to accommodate strain ir6-4^T.

Table 1. Cellular fatty acid compositions (%) of the genus *Silvimonas*

Strains: 1, *Silvimonas iriomotensis* sp. nov. ir6-1^T; 2, *Silvimonas amylolytica* sp. nov. ir6-4^T; 3, *Silvimonas terrae* NBRC 100961^T. All strains were grown both on LB medium at 30 °C for 24 h and on TSA (Difco) medium at 30 °C for 48 h. Data derived from cells cultivated on TSA are shown in parentheses. –, Not detected.

Fatty acid	1	2	3
Saturated			
C _{12:0}	0.6 (0.6)	0.6 (0.7)	3.3 (3.1)
C _{14:0}	10.3 (9.6)	10.0 (9.6)	4.9 (5.0)
C _{16:0}	28.2 (28.5)	30.5 (32.3)	32.1 (33.0)
C _{17:0}	1.3 (0.6)	1.1 (0.7)	1.1 (1.0)
C _{17:0} cyclo	– (5.8)	– (3.2)	10.3 (26.0)
C _{19:0} cyclo ω8c	– (0.1)	– (–)	– (1.6)
Unsaturated			
C _{15:1ω6c}	1.6 (1.0)	1.4 (0.9)	1.8 (0.4)
C _{16:1ω5c}	– (0.3)	– (0.2)	– (1.0)
C _{16:1ω7c}	38.2 (28.7)	36.6 (24.5)	31.5 (10.5)
C _{17:1ω6c}	1.9 (–)	1.6 (–)	– (–)
C _{18:1ω7c}	12.7 (18.1)	14.0 (22.2)	10.3 (13.1)
Summed feature*			
2	2.5 (3.5)	2.4 (2.8)	2.2 (2.5)
Unknown			
ECL 10.928	2.5 (4.0)	2.4 (3.7)	2.2 (3.3)

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 comprises C_{14:0} 3-OH and/or iso-C_{16:1} I.

Emended description of the genus *Silvimonas* Yang *et al.* 2005

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

The emended description is based on data from Yang *et al.* (2005) and this study. Cells are Gram-negative, rod-shaped (0.8–2.0 µm by 0.3–0.8 µm) and facultatively anaerobic. They occur singly or in pairs and are motile by means of a single polar flagellum. Colonies on R2A are circular, convex and milky white. Catalase is positive or weakly positive. Oxidase is positive. The predominant quinone is Q-8. Major cellular fatty acids are C_{16:0}, C_{16:1ω7c} and C_{18:1ω7c} when strains are cultivated on LB medium at 30 °C for 24 h. Some strains additionally contain C_{17:0} cyclo as a major fatty acid. The G + C content of the genomic DNA is 57–60 mol%. The type species is *Silvimonas terrae*.

Description of *Silvimonas iriomotensis* sp. nov.

Silvimonas iriomotensis (i.ri.o.mo.ten'sis. N.L. fem. adj. *iriomotensis* pertaining to Iriomote Island, Okinawa, Japan,

Table 2. Phenotypic differentiation of type strains of species of the genus *Silvimonas*

Strains: 1, *Silvimonas iriomotensis* sp. nov. ir6-1^T; 2, *Silvimonas amylolytica* sp. nov. ir6-4^T; 3, *Silvimonas terrae* NBRC 100961^T. +, Positive; –, negative; w, weakly positive. All strains gave positive reactions with dextrin, Tweens 40 and 80, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, maltose, D-mannitol, D-mannose, methyl β -D-glucoside, sucrose, D-trehalose, turanose, methyl pyruvate, D-galacturonic acid, D-gluconic acid, L-glutamic acid, uridine, and glucose 6-phosphate (Biolog). All strains gave negative reactions with α -cyclodextrin, glycogen, *N*-acetyl-D-galactosamine, adonitol, i-erythritol, L-fucose, *myo*-inositol, lactose, melibiose, D-raffinose, xylitol, succinic acid monomethyl ester, acetic acid, *cis*-aconic acid, citric acid, formic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyrroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ -aminobutyric acid, urocanic acid, inosine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol and D,L- α -glycerol phosphate (Biolog).

Characteristic	1	2	3
Cell size (μm , width $\times$ length)	0.3–0.5 $\times$ 1.0–1.5	0.4–0.5 $\times$ 0.8–1.3	0.4–0.5 $\times$ 0.9–1.3
Growth conditions			
pH range [optimum]	4–9 [4]	4–9 [4]	4–9 [7]
NaCl range [optimum]	0–3 [0]	0–1 [0]	0–2 [0]
Catalase production	+	w	+
Degradation of:			
Chitin	–	–	–*
Aesculin (API 20NE)	+	+	–
Metabolic activities with carbon source (Biolog)			
Lactulose	+	+	–
L-Rhamnose	–	+	+
D-Glucosaminic acid	–	w	w
D,L-Lactic acid	–	+	–
L-Asparagine	+	–	–
L-Aspartic acid	+	–	w
Thymidine	+	–	w
Glucose 1-phosphate	+	–	+
Utilization as sole nitrogen source			
NaNO ₃	–	+	+
(NH ₄) ₂ SO ₄	+	–	+
Urea	–	+	+
DNA G + C content (mol%)	59.9	57.5	59.2 (58†)

*Positive in the original description (Yang *et al.*, 2005).

†Data from Yang *et al.* (2005).

the origin of the soil from which the type strain was isolated).

Cells are Gram-stain-negative, facultatively anaerobic, motile rods that are 1.0–1.5 μm long and 0.3–0.5 μm wide. Growth is detected at 5–40 °C with an optimum at 30 °C. pH range for growth is 4–9 with an optimum at pH 4.0. Tolerates 0–3 % NaCl with optimum growth at 0 %. Oxidase and catalase activities are positive. Alginic acid, casein, aesculin, starch, Tween 80 and tyrosine are degraded, but agar, cellulose, chitin, gelatin, inulin, CM-cellulose, DNA and yeast cells are not. H₂S is produced from cysteine. Nitrate is reduced to nitrite but not reduced to gas. Ammonium sulfate is used as a sole nitrogen source for growth, but sodium nitrate and urea are not. Can metabolize lactulose, L-asparagine, L-aspartic acid, thymidine, and glucose 1-phosphate as sole carbon source

(Biolog GN2 microplate), but not L-rhamnose, D-glucosaminic acid or D,L-lactic acid. Major fatty acids are C_{14:0}, C_{16:0}, C_{16:1 ω 7c} and C_{18:1 ω 7c}. Major ubiquinone is Q-8. DNA G + C content of the type strain is 59.9 %.

The type strain, ir6-1^T (=NBRC 103188^T =CGMCC 1.8859^T =KCTC 22513^T), was isolated from soil of the rhizosphere of bamboos collected in Iriomote Island, Japan, in 2006.

Description of *Silvimonas amylolytica* sp. nov.

Silvimonas amylolytica (am.y.lo.ly'ti.ca. Gr. n. *amulon* starch; Gr. fem. adj. *lutikê* able to loosen, able to dissolve; N.L. fem. adj. *amylolytica* starch dissolving).

Cells are Gram-stain-negative, facultatively anaerobic, motile rods that are 0.8–1.3 μm long and 0.4–0.5 μm

wide. Growth is detected at 5–40 °C with an optimum at 30 °C. pH range for growth is 4–9 with an optimum at pH 4.0. Tolerates 0–1 % NaCl with optimum growth at 0 %. Oxidase activity is positive. Catalase activity is weakly positive. Alginic acid, casein, aesculin, starch, Tween 80 and tyrosine are degraded, but agar, cellulose, chitin, gelatin, inulin, CM-cellulose, DNA and yeast cells are not. H₂S is produced from cysteine. Nitrate is reduced to nitrite but not reduced to gas. Sodium nitrate and urea are used as sole nitrogen sources for growth, but ammonium sulfate is not. Positive metabolic activities with carbon sources of the Biolog GN2 microplate for lactulose, L-rhamnose and D,L-lactic acid and weakly positive activity for D-glucosaminic acid, but not L-asparagine, L-aspartic acid, thymidine or glucose 1-phosphate. Major fatty acids are C_{14:0}, C_{16:0}, C_{16:1ω7c} and C_{18:1ω7c}. Major ubiquinone is Q-8. DNA G+C content of the type strain is 57.5 %.

The type strain, ir6-4^T (=NBRC 103189^T =CGMCC 1.8860^T =KCTC 22514^T), was isolated from soil of the rhizosphere of bamboos collected in Iriomote Island, Japan, in 2006.

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