Starkeya koreensis sp. nov., isolated from rice straw

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A facultatively chemolithoautotrophic, Gram-negative, aerobic, highly curved rod-shaped, non-motile, non-spore-forming bacterium, strain Jip08^T, was isolated from rice straw in Daejeon, South Korea. Strain Jip08^T produced colourless colonies (1.0–1.5 mm) on R2A medium after 3 days. On the basis of 16S rRNA gene sequence similarity, strain Jip08^T was shown to belong to the α -2 subclass of the *Proteobacteria*, and was most closely related to *Starkeya novella* IAM 12100^T (98.6%), *Angulomicrobium tetradale* DSM 5895^T (98.1%), *Angulomicrobium amanitiforme* NCIMB 1785^T (98.0%), *Ancylobacter aquaticus* ATCC 25396^T (96.6%) and *Methylothermobacter multivorans* DM13^T (95.8%), all of which belong to the family *Hyphomicrobiaceae*. The genomic DNA G + C content of strain Jip08^T was 69 mol%. Chemotaxonomic data [major ubiquinone, Q-10; major fatty acids, C_{18:1} isomer (summed feature of C_{18:1} ω 7cl ω 9tl ω 12t), C_{19:0} cyclo ω 8c and C_{16:0}], 16S rRNA gene sequence analyses and some morphological and physiological features supported the affiliation of strain Jip08^T to the genus *Starkeya*. However, DNA–DNA hybridization data and some phenotypic properties showed that strain Jip08^T could be distinguished from the single known *Starkeya* species and represented a novel species, for which the name *Starkeya koreensis* sp. nov. is proposed. The type strain is Jip08^T (= KCTC 12212^T = NBRC 100963^T = IAM 15215^T).

The genus *Starkeya* was first described by Kelly *et al.* (2000), who proposed that *Thiobacillus novellus* should be reclassified as *Starkeya novella*; the genus *Starkeya* currently comprises only one species, *S. novella* IAM 12100^T (Kelly *et al.*, 2000). The genus *Starkeya* belongs to the family *Hyphomicrobiaceae* of the α -2 subclass of the *Proteobacteria*, which also contains the genera *Ancylobacter* (Raj, 1983), *Angulomicrobium* (Vasil'eva *et al.*, 1979), *Azorhizobium* (Dreyfus *et al.*, 1988) and *Methylothermobacter* (Doronina *et al.*, 1995). All of these genera form monophyletic clades in the phylogenetic tree based on 16S rRNA sequences (Fig. 1).

The genus *Starkeya* was created for the reclassification of *T. novellus* (Kelly *et al.*, 2000). Ever since the genus *Thiobacillus*

was first described by Beijerinck (1904), the ability to grow with a reduced sulfur compound as a source of energy has been considered sufficiently important taxonomically to merit classifying all Gram-negative, sulfur-oxidizing, non-phototrophic rods in this genus. However, as studies using modern taxonomic methods have begun to show that some of the species are only superficially related, virtually every paper describing a novel *Thiobacillus* species in the last decade has mentioned the need to reorganize the genus. Most of this reorganization has now been done (Kelly & Wood, 2000).

During the course of a study on the culturable aerobic bacterial community in environmental samples (Im *et al.*, 2003) such as rice straw, anaerobic granular sludge (An *et al.*, 2006; Aslam *et al.*, 2005a; Bae *et al.*, 2005; La *et al.*, 2005), aerobic activated sludge (Aslam *et al.*, 2005b; Quan *et al.*, 2006), compost (H.-C. Yang *et al.*, 2006) and soil from a ginseng field (D.-C. Yang *et al.*, 2005, 2006) near Daejeon in Korea, a large number of novel bacterial strains were isolated

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Jip08^T is AB166877.

A comparison of selected characteristics of strain Jip08^T and *Starkeya novella* IAM 12100^T is available as supplementary material in IJSEM Online.

Fig. 1. Phylogenetic tree, constructed from a comparative analysis of 16S rRNA gene sequences, showing the relationships between *S. koreensis* Jip08^T and related taxa. This tree was constructed from unambiguously aligned nucleotides by using the neighbour-joining and maximum-parsimony methods. Filled circles indicate generic branches that were also recovered using maximum-parsimony algorithms. Bootstrap values are expressed as percentages of 1000 replications. Bar, 1 substitution per 100 nucleotide positions.

that belonged to the *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. The taxonomic status of these bacteria is gradually being explored.

In this study, polyphasic approaches, including phylogenetic analyses based on 16S rRNA gene sequences, chemotaxonomic data and phenotypic properties, were used to determine the precise taxonomic position of strain Jip08^T, which was isolated in South Korea from rice straw. The results obtained in this study indicated that strain Jip08^T can be designated a member of the genus *Starkeya*, but it can be clearly distinguished from *S. novella*. Therefore, we propose Jip08^T as the type strain of a novel species, namely *Starkeya koreensis* sp. nov.

Strain Jip08^T was isolated from rice straw at a farm near Daejeon, South Korea: the rice straw was cut into small pieces and thoroughly suspended in 50 mM phosphate buffer (pH 7.0). This suspension was spread on R2A agar (Difco) after serial dilution with 50 mM phosphate buffer (pH 7.0) and then the plates were incubated at 30 °C for 3 weeks. Strain Jip08^T was one of the dominant morphotypes appearing on the plates in aerobic conditions; it was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C. Strain Jip08^T was then deposited in the Korean Collection for Type Cultures as strain KCTC 12212^T, and at the National Institute of Technology and Evaluation, Japan, as strain NBRC 100963^T.

The Gram reaction was determined using the non-staining method, as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at $\times 1000$ and in a transmission electron microscope (CM20; Philips) at $\times 27\,500$. Catalase activity was determined by assessing bubble production in 3 % (v/v) H₂O₂, and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. The utilization of various substrates as sole carbon sources was determined, along with some physiological characteristics, using API 32GN and API 20NE galleries according to the instructions of the manufacturer (bioMérieux). For the degradation of casein, starch and

L-tyrosine (Atlas, 1993; Lewin & Lounsbury, 1969), reactions were read after 5 days. The hydrolysis of cellulose and xylan was tested as described by Ten *et al.* (2004). Growth with formate, methanol, methylamine, succinate and oxalate was tested in minimal medium B (Lingens *et al.*, 1985). Tests for growth on sulfur, tetrathionate and thiosulfate were done with sulfur medium, tetrathionate broth, thiocyanate utilization medium and thiosulfate-oxidizing medium (Atlas, 1993). Negative and positive controls were obtained by inoculating *Flavobacterium granuli* and *S. novella*, respectively, and observing the turbidity after 10 days. Growth at different temperatures and pH values was assessed after 5 days incubation. Salt tolerance was measured in R2A broth medium supplemented with 1–10 % (w/v) NaCl after 7 days incubation. To determine the heat resistance of strain Jip08^T, 0.1 ml aliquots of 8- and 16-h-old cultures that had been treated at three different temperatures (80 °C for 20 min, 90 °C for 10 min and 100 °C for 10 min) were inoculated into R2A medium; growth was observed for up to 72 h at 30 °C. Duplicate antibiotic-sensitivity tests were done using filter-paper discs containing the following: streptomycin (5, 10 and 15 µg ml^{–1}) (Mast Diagnostics), tetracycline (5, 10 and 15 µg ml^{–1}), kanamycin (1.0, 1.5 and 2.0 mg ml^{–1}) and ampicillin (30, 50 and 60 µg ml^{–1}) (Sigma). Discs were placed on R2A plates spread with Jip08^T culture and were then incubated at 30 °C for 7 days.

The extraction of genomic DNA was performed using a commercial genomic DNA extraction kit (Core Biosystem). The 16S rRNA gene sequence was amplified from the chromosomal DNA by using the universal bacterial primer set (primers 9F and 1512R) and the purified PCR products were sequenced by GenoTech in Daejeon, South Korea (Kim *et al.*, 2005). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were obtained using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura

two-parameter model (Kimura, 1983). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and the maximum-parsimony method (Fitch, 1971) with the MEGA3 program (Kumar *et al.*, 2004) and bootstrap values based on 1000 replications (Felsenstein, 1985).

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified via TLC and subsequently analysed by HPLC, as described previously (Collins & Jones, 1981; Shin *et al.*, 1996). The cellular fatty acids were analysed using cells grown on R2A agar (Difco) for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI; Sasser, 1990). The fatty acids were analysed by GC (Hewlett Packard 6890) and identified by using the software package of the Microbial Identification System.

Genomic DNA of strain Jip08^T was extracted and purified with the 100/G genomic-tip system (Qiagen) and then degraded enzymically into nucleosides, as described previously (Mesbah *et al.*, 1989). DNA–DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Five replicate hybridizations were conducted for each sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were used to calculate similarity values. The DNA relatedness values quoted are the means of these three values.

Strain Jip08^T was found to comprise Gram-negative, highly curved rods (0.4–0.8 × 1.2–2.0 µm) (Fig. 2) that occurred singly, in pairs and sometimes in clumps of three to four cells; no filaments were found, even in old cultures. Colonies grown on R2A agar plates for 3 days were smooth, circular, convex and colourless. Spores were never observed and the cells were found to be heat-sensitive. The physiological characteristics of strain Jip08^T are summarized in the species description. The distinguishing characteristics of strain Jip08^T and related type strains are shown in Table 1.

The 16S rRNA gene sequence of strain Jip08^T was a continuous stretch of 1406 bp. Sequence-similarity calculations obtained after a neighbour-joining analysis indicated that the closest relatives of strain Jip08^T were *S. novella* IAM 12100^T (98.6%), *Angulomicrobium tetradale* DSM 5895^T (98.1%), *Angulomicrobium amanitiforme* NCIMB 1785^T (98.0%), *Ancylobacter aquaticus* ATCC 25396^T (96.6%) and *Methylobacterium multivorans* DM13^T (95.8%) (Fig. 1). DNA–DNA hybridization experiments were performed between the type strain of *S. novella* (IAM 12100^T) and Jip08^T for confirmation of a novel species: the DNA–DNA relatedness value was 15%, which showed that strain Jip08^T belongs to a distinct genomic species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Moreover, the two strains could be easily differentiated using tests for the utilization of substrates as sole carbon sources (see Supplementary

Fig. 2. Transmission electron micrograph of strain Jip08^T from a 3-day-old culture cultivated on R2A agar. Bar, 0.2 µm.

Table S1 available in IJSEM Online). Although the genera *Angulomicrobium*, *Ancylobacter* and *Methylobacterium* show high levels of 16S rRNA gene sequence similarity with respect to strain Jip08^T, they are clearly distinguishable in the phylogenetic tree (Fig. 1) and on the basis of phenotypic characteristics (Table 1) (Vasil'eva *et al.*, 1979; Raj, 1983; Doronina *et al.*, 1995; Kelly *et al.*, 2000).

Strain Jip08^T had a genomic DNA G+C content of 69 mol% and a ubiquinone of the Q-10 type. The major fatty acids were C_{18:1} isomer (summed feature of C_{18:1}ω7c/ω9t/ω12t) (66.1%), C_{19:0} cyclo ω8c (23.7%), C_{16:0} (7.5%) and C_{18:0} (2.7%). The major fatty acids of strain Jip08^T were similar to those of the type strain of *S. novella* (Kelly *et al.*, 2000).

A phylogenetic analysis based on 16S rRNA gene sequence similarity, chemotaxonomic characteristics [G+C content of genomic DNA, ubiquinone (Q-10) type, and cellular fatty acid profile] and some ecophysiological and biochemical characteristics (such as the oxidation of thiosulfate and tetrathionate for growth, the optimum temperature and pH for growth, and the absence of gas vacuoles) clearly shows that strain Jip08^T belongs to the genus *Starkeya*. However, strain Jip08^T can be distinguished by its low level of DNA–DNA relatedness (15%) with respect to *S. novella* IAM 12100^T and its cell morphology (Fig. 2). On the basis of the data and observations described above, strain Jip08^T should be assigned to the genus *Starkeya* as the type strain of a novel species, for which the name *Starkeya koreensis* sp. nov. is proposed.

Table 1. Comparison of distinguishing characteristics of strain Jip08^T, *S. novella* IAM 12100^T and related taxa

Strains: 1, strain Jip08^T (data from this study); 2, *S. novella* IAM 12100^T (data from Kelly *et al.*, 2000); 3, *Ancylobacter aquaticus* ATCC 25396^T (data from Raj, 1983, and Xin *et al.*, 2004); 4, *Angulomicrobium amanitiforme* NCIMB 1785^T (data from Vasil'eva *et al.*, 1979); 5, *Azorhizobium caulinodans* ATCC 43989^T (data from Dreyfus *et al.*, 1988); 6, *M. multivorans* DM13^T (data from Doronina *et al.*, 1995). All species shown are Gram-negative, catalase-positive and positive for growth on glucose. +, Positive; (+), weakly positive or delayed response; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
DNA G+C content (mol%)	69	67.3–68.4	66.3–67.7	67.7	66–68	66.2
Cell morphology	Highly curved rods	Short rods, coccoidal or ellipsoidal	Curved rods	Mushroom-shaped	Rods	Rods
Cell size (µm)	0.4–0.8 × 0.8–2.0	0.4–0.8 × 0.8–2.0	0.3–1.0 × 1.0–3.0	1.0 × 1.5	0.5–0.6 × 1.5–2.6	0.4–0.6 × 1.2–2.0
Curved/vibrioid rods, rings	+	–	+	–	–	Slightly curved rods
Arrangement of cells	Single	Single or pairs	Single	Single	Single	Single or pairs
Motility	–	–	–	–	+	–
Oxidase	+	+	+	+	+	–
Temperature range (°C)	Range, 4–40; optimum, 28–30	Range, 10–37; optimum, 25–30	Range, 5–43; optimum, 22–37	Range, 15–40; optimum, 28–30	Range, 12–43; optimum, ND	Range, 10–45; optimum, 28–34
pH range for growth	6.5–8.0	7.0–8.0	6.8–7.0	5.2–8.0	5.5–7.8	6.8–7.4
Growth with 3% NaCl	(+)	–	–	ND	–	–
Cyclopropane acid of C ₁₉	+	+	–	+	ND	+
Cellular hydroxy fatty acid	None	None	C _{10:0} 3-OH	None	ND	None
Growth on:						
Formate	+	+	+	+	–	+
Methanol	+	+	+	–	–	+
Methylamine	–	–	+	–	–	+
Sucrose	–	–	–	–	–	+
Maltose	–	–	–	–	–	+
Citrate	+	–	+	+	+	ND
Malate	–	–	–	–	+	ND
Succinate	–	–	+	+	+	ND

Description of *Starkeya koreensis* sp. nov.

Starkeya koreensis (ko.re.en'sis. N.L. fem. adj. *koreensis* pertaining to Korea, from where the novel micro-organism was isolated).

Cells are strictly aerobic, Gram-negative, non-spore-forming, non-motile, highly curved rods 0.4–0.8 µm wide and 1.2–2.0 µm long. Cells occur singly, in pairs and sometimes in clumps comprising three or four cells, but do not form filaments. Colonies on R2A and complex medium are circular, convex and colourless and are 0.7–1.2 mm in diameter after 3 days. The temperature range for growth is 4–40 °C; no growth occurs at 42 °C. The optimum temperature for growth is 28–30 °C. The pH range for growth is between pH 6.5 and 8.5, with an optimum between pH 7 and 8. Growth occurs in the absence of NaCl and in the presence of 3.0% (w/v) NaCl, but not with 4% (w/v) NaCl. Positive for catalase, oxidase, urease, β-glucosidase and β-galactosidase. H₂S is not produced. Nitrate is not reduced. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and gelatinase. Does not produce any acid or gas from glucose. Casein, xylan, chitin, tyrosine, CM-cellulose, DNA and starch are not

degraded. The following compounds are utilized as sole carbon sources: glucose, formate, methanol, oxalate, citrate, L-alanine, L-arabinose, propionate, 3-hydroxybutyrate, acetate, DL-lactate and L-proline. The following compounds are not utilized as sole carbon sources: adipate, methylamine, 4-hydroxybenzoate, valerate and caprate, mannose, N-acetylglucosamine, maltose, propionate, mannitol, gluconate, malate, phenylacetate, salicin, D-melibiose, L-fucose, D-sorbitol, histidine, 2-ketogluconate, 4-hydroxybutyrate, rhamnose, D-ribose, inositol, D-sucrose, itaconate, malonate, lactate, 5-ketogluconate, glycogen and L-serine. As this species can also utilize thiosulfate or tetrathionate as an energy source, it is facultatively chemolithoautotrophic. Sensitive to 20 µg ampicillin ml^{–1}, 5 µg tetracycline ml^{–1} and 1.0 mg kanamycin ml^{–1}; resistant to 15 µg streptomycin ml^{–1}. The major cellular fatty acids are C_{18:1} isomer (66.1%), C_{19:0} cyclo ω8c (23.7%), C_{16:0} (7.5%) and C_{18:0} (2.7%). The G+C content of the genomic DNA of strain Jip08^T is 69 mol% (as determined by HPLC).

The type strain, Jip08^T (=KCTC 12212^T=NBRC 100963^T=IAM 15215^T), was isolated from rice straw in Daejeon, South Korea.

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