Sulfuricurvum kujiense gen. nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity

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A facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium, strain YK-1^T, was isolated from an underground crude-oil storage cavity at Kuji in Iwate, Japan. The cells were motile, curved rods and had a single polar flagellum. Optimum growth occurred in a low-strength salt medium at pH 7.0 and 25 °C. It utilized sulfide, elemental sulfur, thiosulfate and hydrogen as the electron donors and nitrate as the electron acceptor under anaerobic conditions, but it did not use nitrite. Oxygen also served as the electron acceptor under the microaerobic condition (O₂ in the head space 1 %). It did not grow on sugars, organic acids or hydrocarbons as carbon and energy sources. The DNA G + C content of strain YK-1^T was 45 mol%. Phylogenetic analysis, based on the 16S rRNA gene sequence, showed that its closest relative was *Thiomicrospira denitrificans* in the 'Epsilonproteobacteria', albeit with low homology (90 %). On the basis of physiological and phylogenetic data, strain YK-1^T should be classified into a novel genus and species, for which the name *Sulfuricurvum kujiense* gen. nov., sp. nov. is proposed. The type strain is YK-1^T (=JCM 11577^T=MBIC 06352^T=ATCC BAA-921^T).

In one of our previous studies, molecular ecological approaches showed that a group of bacteria (designated cluster-1 bacteria) affiliated with the *Thiovulum* subgroup (Maidak *et al.*, 1999) in the 'Epsilonproteobacteria' constituted the major bacterial population in groundwater accumulated at the bottom of an underground crude-oil storage cavity at Kuji in Iwate Prefecture, Japan (Watanabe *et al.*, 2000). The *Thiovulum* subgroup is a phylogenetic group that includes several sulfur-oxidizing bacteria, such as *Thiomicrospira denitrificans* (isolated from marine mud; Timmer-ten Hoor, 1975) and *Thiomicrospira* sp. strain CVO (isolated from the production water of an oil well; Gevertz *et al.*, 2000), and many environmental clones of 16S rRNA genes (obtained from hydrothermal vents; Corre *et al.*, 2001; Moyer *et al.*, 1995), marine sediments (Madrid *et al.*, 2001) and cave springs (Engel *et al.*, 2003). Later, a bacterial strain (strain YK-1^T) that represented the cluster-1 bacteria was isolated and physiologically characterized (Kodama & Watanabe, 2003). Strain YK-1^T is the first and sole bacterium in the

Thiovulum subgroup and was isolated from a freshwater environment. It is a sulfur-oxidizing chemolithotroph capable of growth by oxidizing sulfur compounds in crude oil as the energy sources (Kodama & Watanabe, 2003). Based on its physiological and phylogenetic characteristics, we describe strain YK-1^T as *Sulfuricurvum kujiense* gen. nov., sp. nov.

Strain YK-1^T was isolated by directly streaking the groundwater on agar plates containing DSM medium 113 (used for cultivating nitrate-reducing thiosulfate-oxidizing bacteria) (DSMZ catalogue). Colonies formed on the plates were analysed by sequencing their 16S rRNA genes, and a strain, named YK-1^T, was found to have a 16S rRNA gene sequence identical to that of a cluster-1 bacterium (Kodama & Watanabe, 2003). The growth of strain YK-1^T in DSM medium 113 was, however, not efficient, and we have developed a low-ion-strength medium (MBM) suitable for freshwater sulfur-oxidizing chemolithotrophs. MBM comprised (l⁻¹) 0.2 g KH₂PO₄, 0.2 g NH₄Cl, 0.4 g MgCl₂·6H₂O, 0.2 g KCl, 0.1 g CaCl₂·2H₂O, 0.2 g NaNO₃, 2 mg resazurin and 2 ml trace element solution SL-4 (DSMZ catalogue). Solid media contained 1.5 % Bacto-agar (Difco). For anaerobic cultivation, freshly prepared Na₂S·9H₂O (2 mM) was used as a reducing agent unless otherwise specified, and it could also serve as the energy source. Routine cultivation was conducted without shaking

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YK-1^T is AB053951.

A sequence similarity matrix for strain YK-1^T and related genera is available as supplementary material in IJSEM Online.

at 25 °C in a bottle capped with a Teflon-coated butyl-rubber septum and sealed with an aluminium crimp seal. The vapour phase in the bottle was filled with N₂/CO₂ (80:20 %, v/v), N₂/CO₂/H₂ (80:10:10, by vol.) or pure N₂. For monitoring growth, cells in liquid culture were counted by epifluorescence microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI) as described previously (Kodama & Watanabe, 2003). Cells of YK-1^T were stored at -80 °C in liquid MBM supplemented with 15 % (v/v) glycerol.

Cell morphology was examined by transmission and scanning electron microscopy (Beveridge *et al.*, 1994). Motility was checked by phase-contrast microscopy. Gram staining, oxidase and catalase tests were conducted according to standard procedures (Smibert & Krieg, 1994). Effects of temperature, pH and salinity (NaCl concentration) on growth were examined in MBM. Buffer systems used for changing pH of the medium have been described elsewhere (Gevertz *et al.*, 2000). G+C content was determined by HPLC according to the method of Mesbah *et al.* (1989).

Aerobic growth was examined in MBM without nitrate (the head space was sterilized air) supplemented with each of the following electron donors (2 mM except for elemental sulfur): sulfide, thiosulfate, acetate, pyruvate, succinate, fumarate, lactate, glucose, formate, malate, glutamate, benzoate, phenol, octane, toluene, benzene and elemental sulfur (1 %, w/v). Microaerobic growth [oxygen in the head space was 1 % (v/v)] was tested in MBM without nitrate (the head space was filled with N₂/CO₂ = 80:20). Electron donors tested included sulfide (2 mM), thiosulfate (2 mM), H₂ (10 % in the head space) and elemental sulfur (1 %). Titanium(III) citrate (1.3 mM) was used as a reducing agent (Lomans *et al.*, 1999), when the medium did not contain sulfide. Anaerobic growth (the head space was N₂:CO₂ = 80:20) was examined in modified MBM, containing sulfide (2 mM), thiosulfate (2 mM), H₂ (the head space was N₂:CO₂:H₂ = 80:10:10) or elemental sulfur (1 %) as the electron donor and nitrate (0.6 mM) or nitrite (0.7 mM) as the electron acceptor. Titanium(III) citrate (1.3 mM) was used as a reducing agent. Fermentative growth was examined in MBM without nitrate and sulfide (the head space was N₂:CO₂ = 80:20) supplemented with one of the following substrates (2 mM); acetate, pyruvate, succinate, fumarate, malate, aspartate, lactate or glucose. Ascorbate (2 mM) was added as a reducing agent for this test; strain YK-1^T was unable to utilize ascorbate. In growth test with organic compounds (2 mM methanol, formate, acetate, pyruvate, succinate, fumarate, lactate, glucose, malate, glutamate, phenol, benzoate or octane) as electron donor, MBM (head space was N₂:CO₂ = 80:20) was used in which nitrate served as an electron acceptor. Carbon-source utilization was examined in MBM containing sulfide as the electron donor and nitrate as the electron acceptor under anaerobic conditions (the head space of the bottle was filled with deoxygenated N₂). Test compounds

(2 mM) included bicarbonate, acetate, glucose, octane, toluene and benzene.

Phylogenetic analysis based on the 16S rRNA gene sequence was conducted as described previously (Watanabe *et al.*, 2000). The profile alignment technique of CLUSTAL W version 1.7 (Thompson *et al.*, 1994) was used to align the sequences, and the alignments were refined by visual inspection; secondary structures were considered for the refinement (Gutell, 1994). The pairwise evolutionary distances based on 1232 unambiguous bases of the YK-1^T sequence were determined by the method of Jukes & Cantor (1969) using PHYLIP version 3.572. For reference strains described below, the distances were estimated based on sequences of shorter fragments; *Thiovulum* sp. (639 bp), *Hydrogenimonas thermophila* (862 bases), strain CVO (1102 bases) and FWKO B (1102 bases). A phylogenetic dendrogram was constructed by using the njplot program in CLUSTAL W version 1.7.

The optimum growth conditions of strain YK-1^T were 25 °C and pH 7.0. Addition of NaCl suppressed growth, and the best growth was observed in MBM in the absence of NaCl. This feature was quite different from the salt requirement of *Thiomicrospira denitrificans* and *Thiomicrospira* sp. CVO. This difference is, however, understandable because YK-1^T was isolated from the freshwater environment, while *Thiomicrospira denitrificans* was isolated from marine sediment and strain CVO from a petroleum reservoir undergoing produced-water re-injection.

Cells of strain YK-1^T were Gram-negative, curved rods (spiral cells were also observed in the exponential phase of growth), 0.4 µm wide and 1–2 µm long (Fig. 1). They are motile and have one polar flagellum (Fig. 1). The oxidase reaction was positive, while the catalase reaction was negative. Strain YK-1^T was an obligate chemolithotroph, utilizing nitrate and oxygen as electron acceptors, and sulfide, thiosulfate, elemental sulfur and hydrogen as electron donors under microaerobic and anaerobic conditions. It could not grow on nitrite, and nitrite was the final product of nitrate reduction. Sugars, organic acids (including

Fig. 1. Scanning (a) and transmission (b) electron micrographs of strain YK-1^T. Bar is 1.2 µm, which also applies to part (b).

Fig. 2. Phylogenetic dendrogram based on 16S rRNA gene sequence comparison, showing the position of strain YK-1^T and related genera within the 'Epsilonproteobacteria'. Accession numbers for the sequences retrieved from the databases are given in parentheses. Numbers at the branch nodes are bootstrap values (per 100 trials); only values greater than or equal to 50 are shown. Bar, 0.021 substitution per site.

acetate) and hydrocarbons could not serve as carbon and energy sources. The DNA G + C content of strain YK-1^T was 45 mol% as determined by HPLC.

On the basis of 16S rRNA gene sequence similarity values (data available in IJSEM Online), strain YK-1^T falls within the 'Epsilonproteobacteria' and is most closely related to *Thiomicrospira* species [*Thiomicrospira denitrificans* (90.1%) and *Thiomicrospira* sp. strain CVO (90.0%)]. A phylogenetic dendrogram based on 16S rRNA gene sequences (Fig. 2) shows the relationship of strain YK-1^T to other strains in the 'Epsilonproteobacteria'. Previously, we have classified the 16S rRNA gene sequences of the *Thiovulum* subgroup into four assemblages, namely *Thiovulum*, marine symbiont, groundwater bacteria and *Thiomicrospira denitrificans* assemblages (Watanabe *et al.*, 2000). Strain YK-1^T is the sole isolate belonging to the groundwater-bacteria assemblage.

The reason for creating a new genus for strain YK-1^T was the high sequence divergence from related genera. Moreover, we found several distinct features of strain YK-1^T that could clearly separate it from strains of *Thiomicrospira* (*Thiomicrospira denitrificans* and *Thiomicrospira* sp. CVO); these features include habitat, motility, NaCl sensitivity, inability to utilize acetate as carbon source, ability to utilize hydrogen gas as electron donor, end product from sulfide oxidation and inability to utilize nitrite as electron acceptor. Another isolated member of the *Thiovulum* subgroup, *Thiovulum* sp., is also a sulfur-oxidizing bacterium; however, its features described in the literature (Riviere & Schmidt, 1992) are quite different from those of strain YK-1^T. *Thiovulum* sp. grows only under microaerobic conditions by reducing molecular oxygen, and cells are round or ovoid (5–25 µm in diameter). From these results, strain YK-1^T is considered to represent a new genus in the 'Epsilonproteobacteria'.

Description of *Sulfuricurvum* gen. nov.

Sulfuricurvum (Sul.fu.ri.cur'vum. L. neut. n. *sulfur* -uris sulfur; L. adj. *curvus* -a -um curved; N.L. neut. n. *Sulfuricurvum* curved bacterium that utilizes sulfur).

Chemolithoautotrophic and sulfur-oxidizing, capable of thriving under microaerobic and anaerobic conditions. Based on 16S rRNA gene sequence analysis, it represents a new lineage in the 'Epsilonproteobacteria'.

The sole and type species is *Sulfuricurvum kujiense*.

Description of *Sulfuricurvum kujiense* sp. nov.

Sulfuricurvum kujiense (ku.ji.en'se. N.L. neut. adj. *kujiense* referring to Kuji, Iwate Prefecture, Japan, where the bacterium was isolated).

Cells are curved rods (spiral cells are also observed in the exponential phase of growth) of 1–2 × 0.4 µm in size; motile by one polar flagellum. Gram-negative and non-spore-forming. Oxidase-positive and catalase-negative. Growth is observed only under low NaCl concentrations (below 1%). Optimum growth temperature is 25 °C (range 10–35 °C); optimum pH is 7.0 (range 6.0–8.0). Grows anaerobically and microaerobically by oxidizing reduced sulfur species, such as sulfide, elemental sulfur and thio-sulfate, and hydrogen. Molecular oxygen and nitrate serve as sole electron acceptors. Grows autotrophically on carbon dioxide and bicarbonate; no growth occurs with organic acids, such as acetate, lactate, pyruvate, succinate and formate, or sugars. Does not utilize nitrite as an electron acceptor, and nitrite is the terminal product of nitrate reduction. DNA G + C content is 45 mol%. Isolated from drain water from an underground crude-oil storage cavity in Kuji, Iwate, Japan.

The type strain is YK-1^T (=JCM 11577^T=MBIC 06352^T=ATCC BAA-921^T).

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