Thioprofundum hispidum sp. nov., an obligately chemolithoautotrophic sulfur-oxidizing gammaproteobacterium isolated from the hydrothermal field on Suiyo Seamount, and proposal of *Thioalkalispiraceae* fam. nov. in the order *Chromatiales*

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A novel mesophilic, facultatively anaerobic, sulfur-oxidizing bacterial strain, designated gps61^T, was isolated from a surface rock sample collected from the hydrothermal field of Suiyo Seamount on the Izu-Bonin Arc in the Western Pacific Ocean. Cells of the isolate were rod-shaped with a single sheathed polar flagellum. Neither extensive internal membranes nor storage materials were present in the cells. In a 20 % CO_2 atmosphere, strain gps61^T grew using thiosulfate, sulfur or tetrathionate as electron donors and oxygen or nitrate as electron acceptors. Other substrates, including organic acids and sugars, did not support growth, indicating that strain gps61^T was an obligate chemolithoautotroph. 16S rRNA gene sequence analysis revealed that strain gps61^T was closely related to *Thioprofundum lithotrophicum* 106^T (98.5 % sequence similarity) in the order *Chromatiales*. Phylogenetic trees grouped strain gps61^T and *Thioprofundum lithotrophicum* in the same cluster along with *Thioalkalispira microaerophila* and *Thiohalophilus thiocyanoxidans*, but it was apparent from the analysis that the novel strain had definitely departed from the family lineage. On the basis of its phylogenetic position along with its morphological and physiological characteristics, strain gps61^T (=NBRC 101261^T =DSM 18546^T) represents a novel species of the genus *Thioprofundum*, for which the name *Thioprofundum hispidum* sp. nov. is proposed. In addition, we propose a novel family name, *Thioalkalispiraceae*, in the order *Chromatiales*, to accommodate the genera *Thioalkalispira*, *Thiohalophilus* and *Thioprofundum*.

Hydrothermal vents have been discovered worldwide and host peculiar ecosystems that include chemolithoautotrophs as primary producers. Culture-independent analyses, based on 16S rRNA gene sequences, have revealed that various micro-organisms inhabit such environments (Corre *et al.*, 2001; Marteinsson *et al.*, 1995; Takai & Horikoshi, 1999; Takai *et al.*, 2001, 2003). To date, novel chemolithoautotrophic sulfur-oxidizing bacteria belonging to the phyla *Aquificae* and *Proteobacteria* have been found in hydrothermal vent systems. The class *Gammaproteobacteria* contains many environmental strains derived from symbiotic bacteria associated with invertebrates living in hydrothermal vents and cold-seep systems. Such symbionts are considered to be sulfur- or methane-oxidizing bacteria that supply energy to their host invertebrates (Cavanaugh *et al.*, 1981; Di Meo *et al.*, 2000; Distel *et al.*, 1994; Felbeck, 1981; Feldman *et al.*, 1997). In addition, several environmental strains belonging to the class *Gammaproteobacteria* have been retrieved from hydrothermal fields, cold-seep sediments and marine crusts (Arakawa *et al.*, 2006; Inagaki *et al.*, 2004; Li *et al.*, 1999; Santelli *et al.*, 2008), some of which have been

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain gps61^T is AB266389.

Two supplementary figures are available with the online version of this paper.

suggested as having sulfur-oxidizing metabolism (Hirayama *et al.*, 2007; Sunamura *et al.*, 2004). As for cultivated *Gammaproteobacteria*, some species of the genera *Halothiobacillus* and *Thiomicrospira* have been isolated from these environments (Brinkhoff *et al.*, 1999; Sievert *et al.*, 2000; Takai *et al.*, 2004).

Some previously uncharacterized bacterial strains from culture-independent analyses have been identified as possible sulfur-oxidizers belonging to the order *Chromatiales* of the class *Gammaproteobacteria*. At the time of writing, the order *Chromatiales* included three families, namely, *Chromatiaceae*, *Ectothiorhodospiraceae* and *Halothiobacillaceae*. Almost all members of these families are known to oxidize sulfur compounds, although there are some exceptions. Species belonging to the families *Chromatiaceae* and *Ectothiorhodospiraceae* are mainly anoxygenic photolithoautotrophic bacteria, which are able to oxidize sulfur compounds under anaerobic conditions by using light (Imhoff, 2005a, b). On the other hand, the family *Halothiobacillaceae* contains non-photosynthetic, chemolithoautotrophic, sulfur-oxidizing bacteria isolated from hypersaline, marine and terrestrial environments containing hydrothermal vent systems; its members can oxidize sulfur compounds under aerobic conditions (Durand *et al.*, 1993; Ito *et al.*, 2005; Mori & Suzuki, 2008; Sievert *et al.*, 2000). In this study, a group of previously uncharacterized bacterial strains found in hydrothermal fields and cold-seep sediments were found to be phylogenetically distant from the above families but formed a distinct clade within the order *Chromatiales*. This suggested that the creation of a new family was necessary to encompass these strains. To our knowledge, no such taxon has previously been proposed despite the fact that these isolates are clearly separate from recognized families (Sorokin *et al.*, 2007; Takai *et al.*, 2009). This is likely to be because of the highly complex nature of higher taxa in the class *Gammaproteobacteria*.

In this study, an obligately chemolithoautotrophic, sulfur-oxidizing bacterium, designated strain gps61^T, was isolated from a rock sample collected from the hydrothermal field on Suiyo Seamount. Based on 16S rRNA gene sequence analysis, the isolate belonged to the genus *Thioprofundum* of the order *Chromatiales*. Data from phylogenetic and phenotypic analyses suggested that strain gps61^T warrants classification as a novel species. On the basis of our phylogenetic analysis, it is also proposed that a new family be created to encompass the novel strain and its closest relatives.

Strain gps61^T was isolated from Suiyo Seamount, located in the Izu-Bonin Arc in the Western Pacific Ocean (28° 34′ N 140° 39′ E). The region has a submarine caldera with numerous hydrothermal vents at a depth of 1390 m (Glasby *et al.*, 2000). Rock samples were collected from the site, from July 19–23, 2002, using the benthic multicoring system (BMS; Metal Mining Agency of Japan), a tethered marine rock drill, to obtain cores from under the seafloor. The rock core samples were collected near the black smoker hydrothermal vents (~300 °C). For microbial cultivation, surface layers of the rock core were selected, crushed immediately with a vice in an anaerobic chamber (COY Laboratory Products) and resuspended in basal medium under an N_2/CO_2 (4 : 1, v/v) atmosphere. The basal medium was composed of (l^{-1}) 0.60 g KH_2PO_4, 0.11 g K_2HPO_4, 3.05 g $MgCl_2 . 6H_2O$, 0.15 g $CaCl_2 . 2H_2O$, 0.66 g $(NH_4)_2SO_4$, 30 g NaCl, 2.52 g $NaHCO_3$, and 2 ml each of trace element and vitamin solutions of NBRC medium 377 (NBRC, 2010). For the enrichment of sulfur-oxidizing micro-organisms, AP8SO1 medium, comprising basal medium supplemented with 5 mM $Na_2S_2O_3$, was used under an atmosphere of $N_2/CO_2/O_2$ (75 : 20 : 5, v/v/v; 150 kPa) in a vial sealed with a butyl rubber stopper and an aluminium cap; the enrichment was performed at 50 °C. After incubation for 1 week, thiosulfate was partly converted into elemental sulfur in the enrichment culture. The presence of elemental sulfur was determined during each transfer to fresh AP8SO1 medium and rod-shaped micro-organisms were observed in the culture using a light microscope (Olympus model AX70). These phenomena suggested that the small, but significant, production of elemental sulfur was due to biotic oxidation of thiosulfate by the rod-shaped micro-organisms present in the vials. Sulfur precipitation was observed in an enrichment culture incubated at 37 °C using AP8SO1 medium supplemented with 20 mM $Na_2S_2O_3$ (AP8SO2) under an atmosphere of $N_2/CO_2/O_2$ (60 : 20 : 20, 150 kPa). These culture conditions were used for the isolation of the thiosulfate-oxidizer by serial dilution. After multiple dilutions, incubation and transfer of the culture, a micro-organism exhibiting oxidation of sulfur compounds was successfully isolated as a pure culture and was designated strain gps61^T. The purity of the isolate was verified by microscopic observation, inoculation into media containing various heterotrophic substrates and determination of the 16S rRNA gene sequence, which was amplified using various primer sets (Mori & Suzuki, 2008).

Cells of strain gps61^T were rod-shaped, ~0.3 × 1.5–2.0 μm, and had single sheathed polar flagella (Fig. 1); however, motility was not observed under any growth conditions tested. Observation using electron microscopy revealed that the cells had neither storage compounds nor extensive internal membranes. Gram-staining was negative and oxidase and catalase activities (Tamaki *et al.*, 2003) were positive and negative, respectively.

Fatty acid methyl ester analysis was performed using the GC/MS method (Hanada *et al.*, 2002) and the MIDI microbial identification system. The major cellular fatty acids were $C_{16:0}$ (50 % of the total fatty acids) and branched $C_{17:0}$ (29 %). The branched $C_{17:0}$ could be distinguished from both the iso- and anteiso-branched fatty acids, although the binding position of the methyl group could not be precisely determined. The strain also contained $C_{16:1}\omega7c$ (16 %), $C_{18:1}\omega7c$ (6 %), $C_{15:0}$ (5 %) and $C_{14:0}$ (2 %) as minor fatty acid components. The

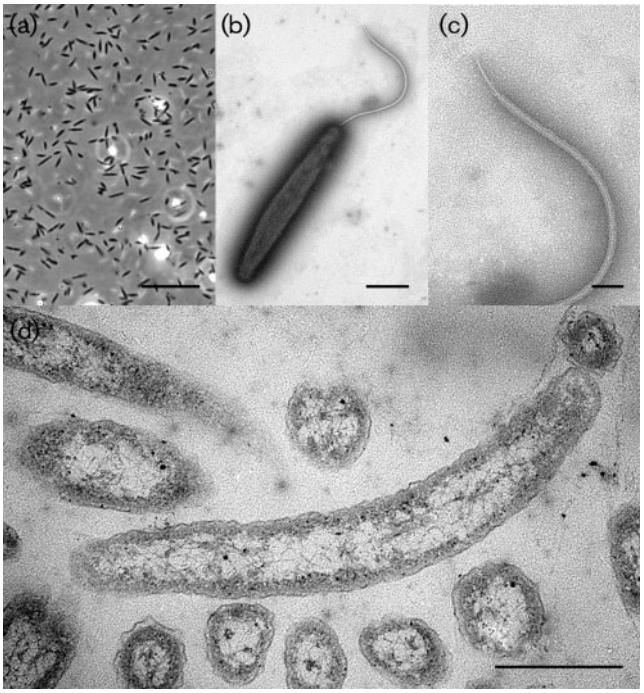

Fig. 1. Cell morphology of strain gps61^T. Phase-contrast micrograph (a) and negatively stained cells (b, c) of strain gps61^T and ultrathin section of strain gps61^T observed with a transmission electron microscope (model H-7600; Hitachi) (d). The cells have a single thick polar flagellum (~20 nm in diameter) and a concealing core was observed at the tip. Bars, 5 μm (a), 0.5 μm (b, d) and 100 nm (c).

genomic DNA G+C content (Mori *et al.*, 2000) of strain gps61^T was 62.9 mol%. An isoprenoid quinone was extracted from the cells according to the protocol outlined by Nakagawa & Yamasato (1993) and analysed using an LCMS-QP 8000 alpha spectrometer (Shimadzu). The isolate contained menaquinone but we were unable to determine the isoprenoid side chain length due to the low extraction amount.

Utilization of electron acceptors and donors was determined by measuring OD_{660} (spectrophotometer model U-2800; Hitachi), thiosulfate and sulfate concentration (HPLC model 2695 with conductivity detector model 432 and IC-Pak Anion column; Waters) (Mori *et al.*, 2008) and cell density via microscopic observation. Strain gps61^T grew using thiosulfate, elemental sulfur and tetrathionate as sole electron donors. However, the following substrates did not support growth (mM): sulfide (2 and 5), CH_4, H_2, H_2 + acetate (10), methanol (2 and 5), formate (10 and 30), acetate (10 and 30), butyrate (10), citrate (10), fumarate (10), glutamate (10), lactate (10), pyruvate (10), malate (10), succinate (10), L-arginine (10), L-asparagine (10), L-cysteine (10), L-histidine (10), L-leucine (10), L-methionine (10), arabinose (5), fructose (5), galactose (5), glucose (5), inositol (5), mannose (5), raffinose (5), sucrose (5) and xylose (10). The utilization of electron acceptors as a substitute for oxygen was tested by microscopic observation after 1 week of cultivation at 37 °C. Under anaerobic conditions (N_2/CO_2, 80 : 20, v/v; 150 kPa), strain gps61^T was able to use nitrate (10 mM) as an electron acceptor in the presence of thiosulfate or elemental sulfur. Nitrite was not detected in the culture on nitrate using a colorimetric assay (Hewitt & Nicholas, 1964). The following electron acceptors (mM) were not used, even in the presence of thiosulfate or elemental sulfur: nitrite (2.5 and 5), fumarate (10), iron(III) citrate (5) (Heising *et al.*, 1999), manganese (5), selenate (2.5 and 5), selenite (2.5 and 5) and arsenate (2.5 and 5). In the presence of thiosulfate under an atmosphere of N_2/CO_2 (80 : 20, v/v; 150 kPa), exposure to light from a halogen lamp did not induce growth of strain gps61^T; therefore, the isolate was not capable of anoxygenic photosynthesis. Because strain gps61^T was unable to use electron donors other than thiosulfate, elemental sulfur and tetrathionate, strain gps61^T was considered to be an obligate chemolithoautotroph that uses sulfur oxidation and carbon dioxide fixation.

Temperature and pH ranges for growth were determined using a temperature gradient incubator (model TN-2612; ADVANTEC). The pH of the AP8SO2 medium was adjusted by the addition of 10 % (w/v) Na_2CO_3 or 0.2 M HCl. The temperature range for growth of strain gps61^T was 29–43 °C (optimum 39 °C). Although slight growth was observed at 50 °C during the first enrichment process, microbial activity was not detected at that temperature. The initial pH range for growth at 37 °C was pH 6–8 (optimum pH 7). The NaCl concentration for growth ranged from 1–4 % (w/v) NaCl (optimum 2 %).

Cell density and concentration of thiosulfate and sulfate over time on either thiosulfate or elemental sulfur were determined (Supplementary Fig. S1, available in IJSEM Online). The maximum cell numbers of strain gps61^T grown on thiosulfate and elemental sulfur were almost identical (8.70×10^6 and 7.77×10^6 cells ml^{-1}, respectively). The doubling times on thiosulfate and elemental sulfur were 14.9 and 26.2 h, respectively. Cells grown on thiosulfate converted 13.3 mM thiosulfate into 19.2 mM sulfate and an undetermined amount of elemental sulfur.

The nearly complete sequence of the 16S rRNA gene of strain gps61^T was determined following methods described previously (Hattori *et al.*, 2000). After alignment of sequences using the ARB program (Ludwig *et al.*, 2004), phylogenetic trees were reconstructed using three methods: the neighbour-joining method using the CLUSTAL_X program (Saitou & Nei, 1987; Thompson *et al.*, 1997), the maximum-likelihood method with the NucML program in the MOLPHY software package (Adachi & Hasegawa, 1995; Hasegawa *et al.*, 1985; Mori *et al.*, 2003) and the maximum-parsimony method in PAUP version 4 using parameters as described previously (Mori *et al.*, 2003). Environmental clone sequences were used for analysis after screens for chimeras were performed using the Mallard program (Ashelford *et al.*, 2006). The phylogenetic analyses

indicated that strain gps61^T was a member of the genus *Thioprofundum* (Takai *et al.*, 2009) in the order *Chromatiales* in the class *Gammaproteobacteria* (Fig. 2 and Supplementary Fig. S2). The sequence similarity between strain gps61^T and its closest neighbour, *Thioprofundum lithotrophicum*, was 98.5 %. Furthermore, the isolate was closely related to environmental clone sequences (92.1–95.6 %) and uncharacterized marine denitrifying sulfur-oxidizing isolates found in hydrothermal systems such as strains OAII2 and NDII.1 (96.3 % and 93.6 %, respectively) (Meyer *et al.*, 2007). *Thioalkalispira microaerophila* and *Thiohalophilus thiocyanoxidans* were members of the same cluster albeit with somewhat lower similarity to the *Thioprofundum* strains (~93–94 %). This cluster was recovered in all phylogenetic trees reconstructed using different analytical methods, despite the fact that the bootstrap score at the node obtained from the neighbour-joining method was not particularly high. The phylogenetic analyses also suggested that the cluster, which included gps61^T, should be separated from the environmental clone cluster that included invertebrate symbionts. The genera *Nitrosococcus* and *Rheinheimera* were obviously separated from the families of the order *Chromatiales* in our analyses (Supplementary Fig. S2), which suggests the need for reclassification of this group.

Characteristics of strain gps61^T and *Thioprofundum lithotrophicum* are summarized in Table 1. *Thioprofundum lithotrophicum* is a moderately thermophilic, piezophilic, sulfur-oxidizing bacterium isolated from a black smoker chimney in the Mid Atlantic Ridge (Takai *et al.*, 2009) whose 16S rRNA gene sequence was very similar to that of strain gps61^T, as were some of its phenotypic features. However, strain gps61^T clearly differed from *Thioprofundum lithotrophicum* in the following characteristics. Optimum growth of *Thioprofundum lithotrophicum* was observed at 50 °C, whereas strain gps61^T grew optimally at 39 °C and could not grow at 50 °C. Strain gps61^T was able to grow in the presence of 20 % oxygen, whereas *Thioprofundum lithotrophicum* was unable to grow at oxygen concentrations >5 %. *Thioprofundum lithotrophicum* used sulfite as an electron donor, unlike strain gps61^T. The genomic DNA G+C content differed between strain gps61^T (63 mol%) and *Thioprofundum lithotrophicum* (66 mol%). Furthermore, DNA–DNA hybridization studies (Ezaki *et al.*, 1988, 1989)

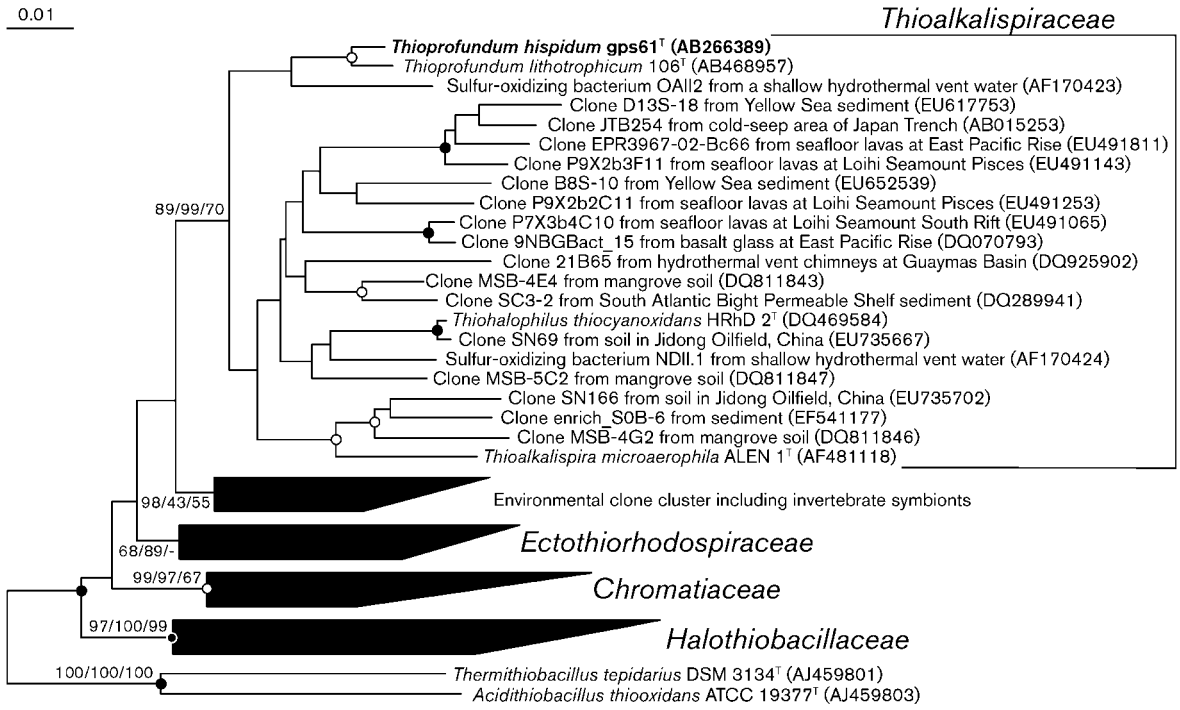

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of strain gps61^T, type species in the orders *Chromatiales* (excluding members of the genera *Nitrosococcus* and *Rheinheimera*) and *Acidithiobacillales* and uncharacterized environmental clones, inferred using the neighbour-joining method after alignment with the ARB program. The alignment dataset was extracted using the ARB default filter (gamma_2_rr5_dec4), and 1438 positions were used for reconstructing phylogenetic trees. Reference sequences used for the analysis but not shown in collapsed branches are shown in Supplementary Fig. S2 along with their accession numbers. Bootstrap values of neighbour-joining/maximum-likelihood/maximum-parsimony trees are indicated at the corresponding nodes. Probability scores at branching points obtained with three analysis methods are indicated by solid circles (>95 % by all methods) and open circles (>90 % by two methods). Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics of strain gps61^T, *Thioprofundum lithotrophicum*, *Thioalkalispira microaerophila* and *Thiohalophilus thiocyanoxidans*

Taxa: 1, gps61^T; 2, *Thioprofundum lithotrophicum* 106^T; 3, *Thioalkalispira microaerophila* ALEN 1^T; 4, *Thiohalophilus thiocyanoxidans* HRhD 2^T. All strains were chemolithoautotrophic. Reference data are from Sorokin *et al.* (2002, 2007) and Takai *et al.* (2009). +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4
Morphology	Rod	Short or spiral rod	Spiral rod	Rod
Intracellular deposit	−	ND	+	ND
Requirement for oxygen	Facultatively anaerobic and microaerobic	Facultatively anaerobic	Microaerobic	Facultatively anaerobic
Photosynthesis	−	ND	−	ND
Electron acceptor(s)	O_2, NO_3^-	O_2, NO_3^-	O_2	O_2, NO_2^-
Electron donors	S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$	S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}*	S^{2-}, S_8^{2-}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$	S^{2-}, $S_2O_3^{2-}$, thiocyanate
Optimum temperature for growth (°C)	39	50	30†	30†
Optimum pH for growth	7	7	10	7.5
Optimum NaCl concentration for growth	2 %	3 %	3 %	9 %
DNA G+C content (mol%)	62.9	66	58.9	58.2

**Thioprofundum lithotrophicum* was not able to use sulfite as an electron donor in our analysis.
†Growth temperature only, not optimum, was reported.

between strain gps61^T and *Thioprofundum lithotrophicum* showed relatedness values of only 11–21 %, strongly suggesting that they should be classified as different species. Based on its phylogenetic position as well as its phenotypic and chemotaxonomic properties, strain gps61^T represents a novel species, for which the name *Thioprofundum hispidum* sp. nov. is proposed.

Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain gps61^T and *Thioprofundum lithotrophicum* could be clearly distinguished from the families *Chromatiaceae*, *Ectothiorhodospiraceae* and *Halothiobacillaceae* of the order *Chromatiales* in the class *Gammaproteobacteria* (Fig. 2). Furthermore, this lineage contained *Thioalkalispira microaerophila* and *Thiohalophilus thiocyanoxidans*. Sorokin *et al.* (2002) reported that *Thioalkalispira microaerophila* is an alkaliphilic sulfur-oxidizing bacterium and the phylogenetic position was deeply branched and obscured in the class *Gammaproteobacteria*; subsequently, it was tentatively classed as a member of the family *Ectothiorhodospiraceae* based on the taxonomic outlines of Bergey's Manual of Systematic Bacteriology (http://www.bergeys.org/outlines.html). The phylogenetic position of *Thiohalophilus thiocyanoxidans*, a halophilic sulfur-oxidizing bacterium, was only indicated as a member of the class *Gammaproteobacteria* (Sorokin *et al.*, 2007). In our phylogenetic analyses, however, *Thioalkalispira microaerophila* and *Thiohalophilus thiocyanoxidans* were part of the same lineage, along with strain gps61^T and *Thioprofundum lithotrophicum*. Some phenotypic features of *Thioalkalispira microaerophila* and *Thiohalophilus thiocyanoxidans*, such as chemolithoautotrophy, sulfur-oxidation and moderately halophily, were similar to those of strain gps61^T and *Thioprofundum lithotrophicum* (Table 1). Accordingly, we propose the new family name *Thioalkalispiraceae* fam. nov. in the order *Chromatiales* to accommodate the genera *Thioalkalispira*, *Thiohalophilus* and *Thioprofundum*. In the order *Chromatiales*, the phylogenetic distances of members of the novel family and those of the families *Chromatiaceae*, *Ectothiorhodospiraceae* and *Halothiobacillaceae*, based on 16S rRNA gene sequences, were 93.1 %, 92.9 % and 90.2 %, respectively.

Description of *Thioalkalispiraceae* fam. nov.

Thioalkalispiraceae (Thi.o.al.ka.li.spi.ra′ce.a.e. N.L. fem. n. *Thioalkalispira* the type genus of family; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Thioalkalispiraceae* the family of the genus *Thioalkalispira*).

The cell wall is of the Gram-negative type. Mesophilic or moderately thermophilic. Strictly chemolithoautotrophic. Growth occurs by sulfur oxidation and carbon dioxide fixation. Members of the family are moderately halophilic and are isolated from marine and saline environments. The genomic DNA G+C content is 59–66 mol%. The phylogenetic position is in order *Chromatiales* in class *Gammaproteobacteria* of phylum *Proteobacteria*. The type genus of the family is *Thioalkalispira*.

Description of *Thioprofundum hispidum* sp. nov.

Thioprofundum hispidum (his′pi.dum. L. neut. adj. *hispidum* bristly).

Cells are Gram-reaction-negative, straight, non-motile rods, ~0.3 × 1.5–2.0 μm and have a single thick polar flagellum. Oxidase-positive and catalase-negative. Facultatively anaerobic and obligately chemolithoautotrophic. Grows at 29 and 43 °C (optimum 39 °C). pH range for

growth is 6–8 (optimum pH 7). The NaCl concentration for growth ranges from 1 to 4 % (optimum 2 %). Either oxygen or nitrate is used as an electron acceptor but nitrite, fumarate, iron(III) citrate, manganese, selenate, selenite and arsenate are not. Thiosulfate, elemental sulfur and tetrathionate are used as electron donors. Carbon dioxide is used as a sole carbon source; organic compounds are not used for growth. Major cellular fatty acids are $C_{16:0}$ and branched $C_{17:0}$. Minor components are $C_{16:1}\omega7c$, $C_{18:1}\omega7c$, $C_{15:0}$ and $C_{14:0}$.

The type strain, gps61^T (=NBRC 101261^T =DSM 18546^T), was isolated from a rock sample collected from the hydrothermal field on Suiyo Seamount, Izu-Bornin Arc, Western Pacific Ocean. The genomic DNA G+C content of the type strain is 62.9 mol% (determined by HPLC).

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