

Thiogranum longum gen. nov., sp. nov., an obligately chemolithoautotrophic, sulfur-oxidizing bacterium of the family *Ectothiorhodospiraceae* isolated from a deep-sea hydrothermal field, and an emended description of the genus *Thiohalomonas*

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A novel, obligately chemolithoautotrophic, sulfur-oxidizing bacterial strain, designated strain *gps52*^T, was isolated from a rock sample collected near the hydrothermal vents of the Suiyo Seamount in the Pacific Ocean. The cells possessed a Gram-stain-negative-type cell wall and contained menaquinone-8(H₄) and menaquinone-9(H₄) as respiratory quinones, and C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c as major cellular fatty acids. Neither storage compounds nor extensive internal membranes were observed in the cells. Strain *gps52*^T grew using carbon dioxide fixation and oxidation of inorganic sulfur compounds with oxygen as electron acceptor. Optimal growth was observed at 32 °C, pH 6.5 and with 3 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain *gps52*^T belongs to the family *Ectothiorhodospiraceae* and is different from any other known bacteria, with sequence similarities of less than 93 %. Based on phenotypic and phylogenetic findings, the isolate is considered to represent a novel genus and species in the family *Ectothiorhodospiraceae*, and the name *Thiogranum longum* gen. nov., sp. nov. is proposed. The type strain is *gps52*^T (=NBRC 101260^T=DSM 19610^T). An emended description of the genus *Thiohalomonas* is also proposed.

Hydrothermal vents have been discovered in the ocean floor all over the world. These environments are home to unique ecosystems that include chemolithoautotrophs as primary producers, and sulfur-oxidizing prokaryotes often play a major role in the oxidation of sulfide from hydrothermal vents by using oxygen from the upper layer of the sea. Various kinds of chemolithoautotrophic, sulfur-oxidizing prokaryotes have been detected using culture-dependent or -independent analyses, and they have been shown to encompass phylogenetically diverse prokaryotes. Some mesophilic sulfur-oxidizing bacteria have been isolated from such environments, mainly belonging to the classes *Epsilonproteobacteria* and *Gammaproteobacteria*.

Takai *et al.* (2003) first reported that various kinds of sulfur-oxidizing epsilonproteobacteria inhabit hydrothermal vents, and many novel sulfur-oxidizing epsilonproteobacteria have subsequently been isolated from these environments (Inagaki *et al.*, 2003, 2004; Takai *et al.*, 2004). Several sulfur-oxidizing bacteria belonging to the class *Gammaproteobacteria* have also been detected in such environments (Dubilier *et al.*, 2008; Hirayama *et al.*, 2007; Nunoura *et al.*, 2012; Sunamura *et al.*, 2004) and they have actually been isolated (Brinkhoff *et al.*, 1999; Mori *et al.*, 2011; Sievert *et al.*, 2000; Takai *et al.*, 2006).

The order *Chromatiales* of the class *Gammaproteobacteria* contains five families – *Chromatiaceae*, *Ectothiorhodospiraceae*, *Halothiobacillaceae*, *Granulosicoccaceae* and *Thioalkalispiraceae*. The family *Chromatiaceae* consists of anaerobic phototrophic bacteria known as phototrophic purple sulfur bacteria (Imhoff, 2005a). The families *Halothiobacillaceae* and *Thioalkalispiraceae* are mainly composed

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain *gps52*^T is AB983478.

Three supplementary figures are available with the online Supplementary Material.

of strictly chemolithoautotrophic, sulfur-oxidizing bacteria (Ito *et al.*, 2005; Kelly & Wood, 2005; Mori & Suzuki, 2008; Mori *et al.*, 2011), and members of the genus *Granulosiccoccus*, the only genus in the family *Granulosoccaceae*, grow chemoheterotrophically (Lee *et al.*, 2007). By contrast, members of the family *Ectothiorhodospiraceae* show different types of metabolisms, e.g. photolithotrophic, photoheterotrophic, chemoheterotrophic, methylotrophic and chemolithotrophic using nitrite, sulfur compounds, arsenite and iron as inorganic electron donors (Hallberg *et al.*, 2011; Hoeft *et al.*, 2007; Imhoff, 2005b; Sorokin *et al.*, 2007b). Although strains of the order *Chromatiales* have been isolated from various environments such as lakes, ponds, rivers, hot springs, estuaries, marine habitats, salt lakes and soda lakes, all isolates from hydrothermal fields belonging to this order are sulfur-oxidizing bacteria (Durand *et al.*, 1993; Mori *et al.*, 2011; Sievert *et al.*, 2000; Takai *et al.*, 2009).

A submarine caldera of Suiyo Seamount in the Pacific Ocean has numerous black smoker hydrothermal vents (Glasby *et al.*, 2000), and sulfur-compound-utilizing prokaryotes have been observed and isolated there (Mori *et al.*, 2004, 2008, 2011; Nakagawa *et al.*, 2004; Sunamura *et al.*, 2004). Recently, an obligately chemolithoautotrophic, sulfur-oxidizing bacterium was isolated from the seamount, and phylogenetic analysis of 16S rRNA gene sequences suggested that the isolate represents a novel genus and species in the family *Ectothiorhodospiraceae*. In this paper, based on phenotypic characteristics as well as phylogenetic analysis, a novel taxon is proposed to accommodate the isolate – *Thiogranum longum* gen. nov., sp. nov.

Strain gps52^T was isolated from a rock sample collected from a deep-sea hydrothermal field on the Suiyo Seamount. Details of the sample collection, enrichment procedures, and composition of the media used for enrichment and isolation have been described previously (Mori *et al.*, 2011). AP8SO1 medium includes 5 mM thiosulfate in a basal medium under an atmosphere of N₂/CO₂/O₂ (75:20:5, by vol.; 150 kPa), and AP8SO2 medium is modified AP8SO1 medium supplemented with 20 mM thiosulfate under an atmosphere of N₂/CO₂/O₂ (60:20:20, by vol.; 150 kPa). After 1 week of incubation of the rock core sample in AO8SO1 medium at 30 °C, microbial growth was observed. Because better growth was observed in AP8SO2 than in AP8SO1 medium, AP8SO2 medium was subsequently used for isolation. Because growth of the enriched bacteria could not be achieved successfully on solid AP8SO2 medium, an attempt was made to isolate bacteria by serial dilution. After repeating the maximum dilution series several times, a sulfur-oxidizing bacterium, designated strain gps52^T, was obtained. 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 gps52^T were short rods but sometimes elongated without septa at the late exponential phase of

growth (0.7–3.0 µm in length and 0.3–0.4 µm in width) (Fig. 1a, b). Motility was not observed under a microscope. No flagellum was observed on cells negatively stained with 1% (w/v) phosphotungstic acid (data not shown). Observation of ultrathin sections of cells using an electron microscope (Mori & Suzuki, 2008) indicated that they possessed a Gram-negative type of cell wall with an outer membrane and contained neither storage compounds nor extensive internal membranes (Fig. 1c). The cells were also shown to be Gram-stain-negative using both conventional Gram staining and the 3% (w/v) KOH string test (Powers, 1995). Catalase (Holding & Collee, 1971) and oxidase (cytochrome oxidase paper; Nissui Pharmaceutical) activities were negative and positive, 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 of strain gps52^T were C_{16:1ω7c} (63% of total fatty acids), C_{16:0} (26%) and C_{18:1ω7c} (10%). The strain also contained C_{14:0} (3%), C_{15:0} (1%) and C_{17:1ω8c} (1%) as minor fatty acid components. Respiratory quinones were extracted from cells according to the protocol of Nakagawa & Yamasato (1993) and analysed with an LCMS-QP 800alpha spectrometer (Shimadzu). Strain gps52^T contained equivalent amounts of menaquinone-8(H₄) and menaquinone-9(H₄). The G+C content of the genomic DNA was determined by HPLC using a Shodex ODS pack F-411 (Showa Denko) after nuclease P1 treatment using a DNA-GC kit (Yamasa Shoyu) followed by alkaline phosphatase treatment (Kamagata & Mikami, 1991). The equimolar nucleotide mixture in the DNA-GC kit was used as a reference for quantitative analysis. The G+C content in the genomic DNA of strain gps52^T was 53.8 mol%.

Utilization of electron donors and acceptors was comprehensively determined by measuring the OD₆₆₀ (spectrophotometer U-2800; Hitachi), thiosulfate and sulfate concentrations by using HPLC (Mori *et al.*, 2008) and cell increase via microscopic observation. In medium under an N₂/CO₂/O₂ atmosphere, strain gps52^T oxidized thiosulfate (20 mM), sulfite (5 and 10 mM), elemental sulfur (5%), sulfide (2 and 5 mM) and tetrathionate (5 and 10 mM) as electron donors. The following substrates (mM) could not support growth of strain gps52^T: H₂ [N₂/CO₂/O₂, 60:20:20 (v/v), 150 kPa], H₂ + acetate (10), CH₄ [N₂/CO₂/O₂/CH₄, 50:20:20:10 (v/v), 150 kPa], methanol (2 and 5), formate (10 and 30), acetate (10 and 30), butyrate (10), citrate (10), fumarate (10), glutamate (10), lactate (10), malate (10), pyruvate (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) or xylose (5). In the presence of thiosulfate or sulfur as an electron donor, oxygen was the only usable electron acceptor for growth of strain gps52^T, and the following electron acceptors (mM) could not be used: nitrate (10), nitrite (2.5 and 5), fumarate (10), iron (III) hydroxide (5), iron (III) citrate (5) (Heising *et al.*,

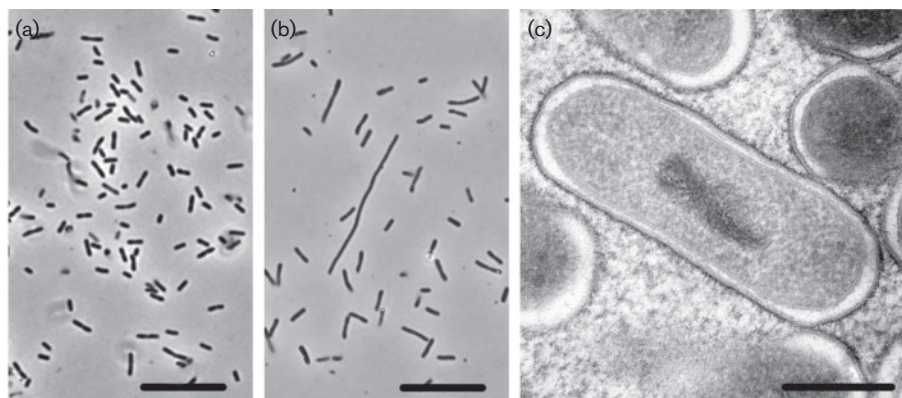


Fig. 1. Cell morphology of strain *gps52^T*. Phase-contrast micrograph (a, b) and ultrathin section of cells observed with a transmission electron microscope (c). The cells sometimes elongated at the late exponential phase of growth (b). Bars, 5 μ m (a, b) and 0.3 μ m (c).

1999), manganese (5), selenate (2.5 and 5), selenite (2.5 and 5) or 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 induced neither growth, nor decrease of thiosulfate, nor increase of sulfate, indicating that strain *gps52^T* was not capable of anoxygenic photosynthesis. Therefore, strain *gps52^T* is an obligate chemolithoautotroph that grows by sulfur oxidation and carbon dioxide fixation.

Temperature, initial pH, and NaCl concentration ranges for growth were determined by examining the time-course of OD_{660} with a temperature gradient incubator (model TN-2612; ADOVANTEC). The pH of the AP8SO2 medium was adjusted by the addition of 10% (w/v) Na_2CO_3 or 0.2 M HCl. The results are shown in Fig. S1 (available in the online Supplementary Material). Strain *gps52^T* grew at 19–35 °C, and the optimum temperature for growth was 32 °C. The initial pH range for growth was pH 6.0–7.5, with an optimum of pH 6.5. The strain grew with 2–4% (w/v) NaCl, with an optimum concentration of 3% NaCl. At NaCl concentrations of 1 and 5%, faint growth with increases in sulfate and decreases in thiosulfate were also observed. The specific growth rate under the optimum growth conditions was 0.064 h^{-1} .

An almost-complete 16S rRNA gene sequence for strain *gps52^T* was determined using a previously reported procedure (Hattori *et al.*, 2000). After sequence alignment using the ARB program (Ludwig *et al.*, 2004), phylogenetic trees were reconstructed using three methods: neighbour-joining using CLUSTAL X version 2.1 (Larkin *et al.*, 2007), maximum-likelihood using the NucML program in MOLPHY (Adachi & Hasegawa, 1995; Hasegawa *et al.*, 1985; Mori *et al.*, 2003) and maximum-parsimony using MEGA version 6.06 (Tamura *et al.*, 2013), using the tree-bisection-reconnection (TBR) search method. The phylogenetic analyses indicated that strain *gps52^T* is a member of the order *Chromatiales* in the class *Gammaproteobacteria*.

However, the sequence of strain *gps52^T* was distant from those of all known genera in the order, and the closest relatives were *Natronocella acetinitrilica* and *Methylo-natum kenyense* in the family *Ectothiorhodospiraceae*, with sequence similarities of 92.7 and 92.6%, respectively. The sequence similarities between strain *gps52^T* and species in the order with validly published names were 87.9–92.7% for the family *Ectothiorhodospiraceae*, 90.5–92.3% for the family *Thioalkalispiraceae*, 90.4–90.9% for the family *Granulosicoccaceae*, 86.6–90.2% for the family *Chromatiaceae* and 85.6–89.4% for the family *Halothiobacillaceae*. Phylogenetic trees were reconstructed, based on 16S rRNA gene sequences for strain *gps52^T*, type strains in the order *Chromatiales*, excluding members of the genera *Nitrosococcus* and *Rheinheimera* (Mori *et al.*, 2011), and the type strains of species of the genus *Thiohalomonas*, an unclassified gammaproteobacterial genus (Sorokin *et al.*, 2007a). The tree reconstructed using the neighbour-joining method (Fig. 2) indicated that strain *gps52^T* is a member of the family *Ectothiorhodospiraceae*, but the nodes for the clusters including members of the families *Ectothiorhodospiraceae* and *Thioalkalispiraceae* were ambiguous with low bootstrap scores. The tree reconstructed using the maximum-likelihood method (Fig. S2) was similar to that reconstructed using the neighbour-joining method. However, the tree reconstructed using the maximum-parsimony method (Fig. S3) indicated that the family *Ectothiorhodospiraceae* contains two distinct lineages, that the families *Ectothiorhodospiraceae* and *Thioalkalispiraceae* lack unity, and that the phylogenetic position of strain *gps52^T* is close to that of the genus *Thiopfundum*. Phylogenetic trees reconstructed using all methods showed that species in the genus *Thiohalomonas* belong to the family *Ectothiorhodospiraceae* (Figs 2, S2 and S3) and that the closest relative of the type strains of species of this genus is *Thioalbus denitrificans*, with a sequence similarity of 94.1–94.4%.

The genus *Thiohalomonas* accommodates moderately halophilic, obligately chemolithoautotrophic, sulfur-oxidizing

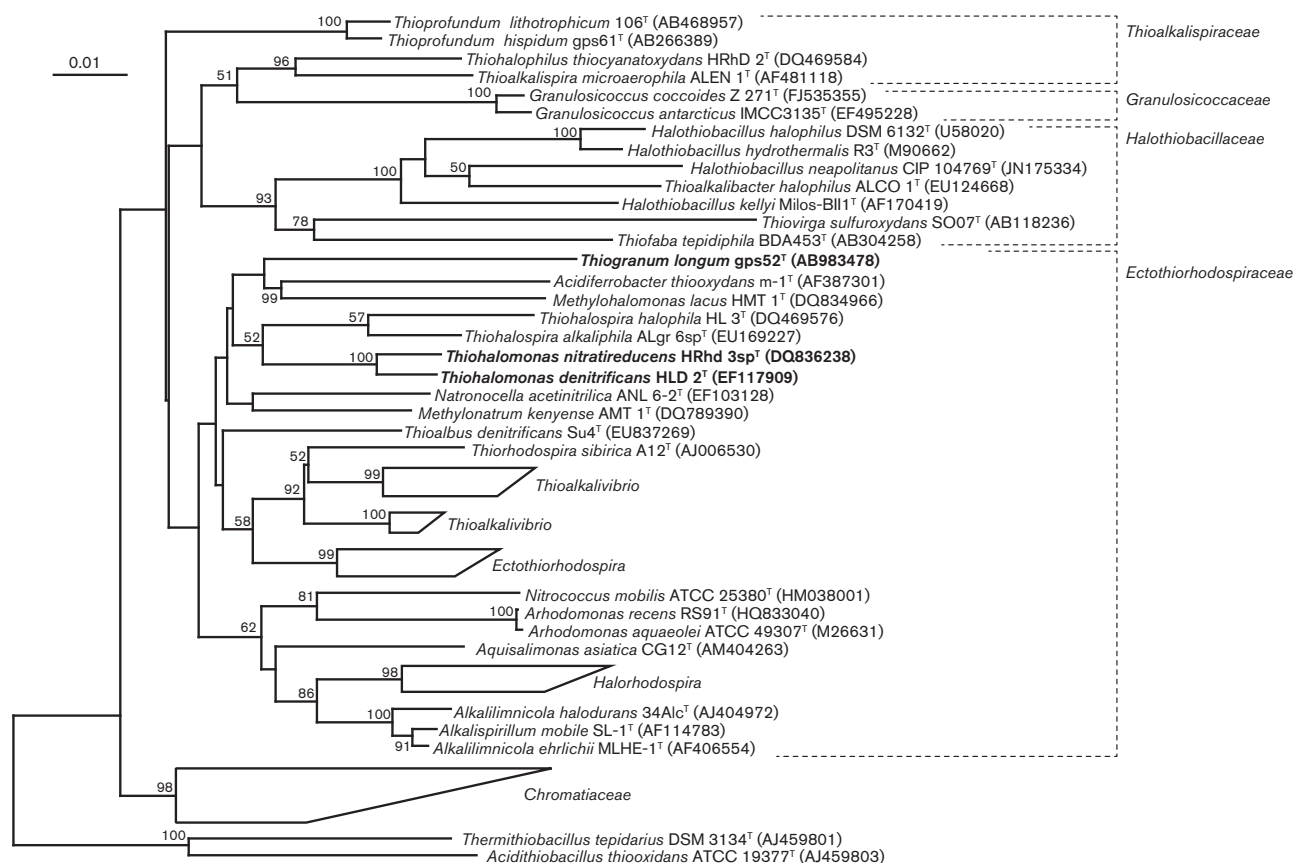


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of strain *gps52^T* and type strains in the order *Chromatiales* (excluding members of the genera *Nitrosococcus* and *Rheinheimera*) inferred using the neighbour-joining method after alignment with the ARB program. For tree reconstruction, 1047 positions were used. *Thermithiobacillus tepidarius* and *Acidithiobacillus thiooxidans* in the order *Acidithiobacillales* were used as outgroups. Probability scores greater than 50% are indicated at branching points. The trees reconstructed by maximum-likelihood and maximum-parsimony are shown in Figs S2 and S3. Bar, 0.01 substitutions per nucleotide position.

bacteria that belongs to the class *Gammaproteobacteria* (Sorokin *et al.*, 2007a). Sorokin *et al.* proposed the genus but did not propose a family to which it belongs. The results of our phylogenetic analyses using 16S rRNA gene sequences make it clear that the genus *Thiohalomonas* belongs to the family *Ectothiorhodospiraceae*.

An obligately chemolithoautotrophic, sulfur-oxidizing bacterium, named strain *gps52^T*, was isolated from a hydrothermal field in the Suiyo Seamount. Phylogenetic analyses based on 16S rRNA gene sequences using the neighbour-joining and maximum-likelihood methods indicated that the isolate belongs to the family *Ectothiorhodospiraceae*, although the bootstrap values at the nodes of the cluster *Ectothiorhodospiraceae* were low and the topology inferred using the maximum-parsimony method differed from that inferred using the other methods (Figs 2, S2 and S3). Although the possibility that strain *gps52^T* is affiliated with another family cannot be completely eliminated, at this point it seems reasonable to conclude that strain *gps52^T* belongs to the family *Ectothiorhodospiraceae*. The genera

Thioalbus, *Thioalkalivibrio*, *Thiohalomonas* and *Thiohalospira*, also belonging to the family *Ectothiorhodospiraceae*, are chemolithoautotrophic, sulfur-oxidizing bacteria (Banciu *et al.*, 2004; Imhoff, 2005b; Park *et al.*, 2011; Sorokin *et al.*, 2002a, b, 2003, 2004, 2007a, 2008, 2012), but strain *gps52^T* clearly differs from them in the following characteristics (Table 1). The G+C content of the genomic DNA differentiates strain *gps52^T* from members of these four genera. Species in the genus *Thioalkalivibrio* are alkaliphilic, whereas strain *gps52^T* was found to be neutrophilic. The members of the genera *Thiohalomonas* and *Thiohalospira* prefer extremely halophilic conditions for growth, but strain *gps52^T* grew optimally at 3% (w/v) NaCl. Given the unique phenotypic features of strain *gps52^T*, and considering its solitary lineage in the phylogenetic trees and its low sequence similarities with species of the order *Chromatiales*, it is clear that this novel strain cannot be assigned to any previously recognized genera. On the basis of physiological and phylogenetic findings, a novel taxon, *Thiogranum longum* gen. nov., sp. nov., belonging to the family *Ectothiorhodospiraceae*, is proposed.

Table 1. Characteristics of strain gps52^T and the various chemolithoautotrophic, sulfur-oxidizing genera of the family Ectothiorhodospiraceae

Genera: 1, *Thiogramum* gen. nov.; 2, *Thioalbus* (Park *et al.*, 2011); 3, *Thioalkalivibrio* (Banciu *et al.*, 2004; Imhoff, 2005b; Sorokin *et al.*, 2002a, b, 2003, 2004, 2012); 4, *Thiohalomonas* (Sorokin *et al.*, 2007a); 5, *Thiohalospira* (Sorokin *et al.*, 2008).

Characteristic	1	2	3	4	5
Requirement for O ₂	Aerobic	Facultatively anaerobic	Aerobic*	Facultatively anaerobic	Micro-aerobic
Electron acceptor(s)	O ₂	O ₂ , NO ₃ ⁻	O ₂ *	O ₂ , NO ₃ ⁻	O ₂
Optima for growth					
Temperature (°C)	32	28–32	28–30	6–15	32–35
pH	6.5	7.0–7.5	8.0–10.0	7.3–8.2	7.3–8.5
NaCl (% w/v)	3	3	2–3.5†	6–12	12–18
DNA G + C content (mol%)	53.8	64.5	61.3–66.9	58.0–62.9	65.6–65.8

*Only two species, *Thioalkalivibrio nitratireducens* and *Thioalkalivibrio thiocyanodenitrificans*, can grow under anaerobic conditions with nitrate.

†Optimum NaCl concentration of *Thioalkalivibrio halophilus* is, exceptionally, 12 %.

The order *Chromatiales* contains five families and includes various types of bacteria such as phototrophs, chemoautotrophs and chemoheterotrophs. The family *Granulosicoccaceae* in the order *Chromatiales* contains one genus, *Granulosicoccus*, and it is an obligately chemoheterotrophic bacterium (Kurilenko *et al.*, 2010; Lee *et al.*, 2007). Mori *et al.* (2011) proposed a fifth family, *Thioalkalispiraceae*, in the order *Chromatiales* while the members of the family *Granulosicoccaceae* were not included in their phylogenetic analysis. Subsequently, two other genera, *Acidiferrobacter* and *Thioalbus*, were added to the family *Ectothiorhodospiraceae*, but the phylogenetic analyses did not consider the family *Granulosicoccaceae* (Hallberg *et al.*, 2011; Park *et al.*, 2011). This study is the first taxonomic analysis of all the species of the order *Chromatiales* with validly published names. In our phylogenetic analyses based on 16S rRNA gene sequences, the boundaries of the families *Ectothiorhodospiraceae* and *Thioalkalispiraceae* were obscure, and the families could not be regarded as independent lineages. This is likely to arise from the deep branching of some genera of the families *Ectothiorhodospiraceae*, *Thioalkalispiraceae* and *Granulosicoccaceae* in the order *Chromatiales*. At this point, it is difficult to clearly divide these deep-branching genera, such as *Thiopfundum*, *Granulosicoccus*, *Acidiferrobacter*, *Methylohalomonas*, *Natronocella*, *Methylostratum*, *Thioalbus* and our proposed genus. More isolates will be required to fully clarify the relationships among the families in the order *Chromatiales*.

Description of *Thiogramum* gen. nov.

Thiogramum (Thi.o.gra'num. Gr. n. *theion* sulfur; L. neut. n. *granum* grain; N.L. neut. n. *Thiogramum* sulfur grain).

Obligately aerobic and chemolithoautotrophic. Gram-stain-negative cells. Grow by the oxidation of reduced sulfur compounds and the fixation of carbon dioxide. Non-phototrophic. Mesophilic, neutrophilic and slightly halophilic. Major cellular fatty acid is C_{16:1}ω7c. Respiratory

isoprenoid quinones are menaquinone-8(H₄) and menaquinone-9(H₄). Phylogenetic position based on 16S rRNA gene sequence is in the family *Ectothiorhodospiraceae* of the order *Chromatiales* of the class *Gammaproteobacteria*. The type species is *Thiogramum longum*.

Description of *Thiogramum longum* sp. nov.

Thiogramum longum (lon'gum. L. neut. adj. *longum* long).

Cells are short rods and sometimes elongate at the late-exponential phase of growth (0.7–3.0 μm in length and 0.3–0.4 μm in width). Cells have a Gram-stain-negative-type cell wall and possess neither storage compounds nor extensive internal membranes. Motility and a flagellum on cells are not observed. Catalase-negative. Oxidase-positive. Obligately chemolithoautotrophic. Grows aerobically by the oxidation of reduced sulfur compounds (thiosulfate, sulfite, elemental sulfur, sulfide and tetrathionate) and the fixation of carbon dioxide. Anaerobic or photosynthetic growth is not observed. Grows at 19–35 °C; optimal growth at 32 °C. The initial pH for growth is pH 6.0–7.5, with an optimum at pH 6.5. The NaCl concentration for growth ranges from 1 to 5 % (w/v), with an optimum at 3 %. Predominant cellular fatty acids are C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c. Menaquinone-8(H₄) and menaquinone-9(H₄) are almost equivalently contained.

The type strain, gps52^T (=NBRC 101260^T=DSM 19610^T), was isolated from a rock sample collected from the hydrothermal field on Suiyo Seamount, Izu-Bornin Arc, western Pacific Ocean. The DNA G + C content of the type strain is 53.8 mol% (determined by HPLC).

Emended description of the genus *Thiohalomonas* Sorokin *et al.* 2007

The description is as given by Sorokin *et al.* (2007a) with the following addition. Phylogenetic position based on 16S

rRNA gene sequence is in the family *Ectothiorhodospiraceae* of the order *Chromatiales* of the class *Gammaproteobacteria*.

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