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 gps52T, 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 C16:1ω7c, C16:0 and C18:1ω7c as major cellular fatty acids. Neither storage compounds nor extensive internal membranes were observed in the cells. Strain gps52T 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 gps52T 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 gps52T (=NBRC 101260T=DSM 19610T). 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...
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 *Granulosicoccus*, the only genus in the family *Granulosicoccaceae*, 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 (Glasye 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 – *Thiogramum longum* gen. nov., sp. nov.

Strain gps52T 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 A08SO1 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 gps52T, 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 gps52T 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 gps52T were C16:1ω7c (63 % of total fatty acids), C16:0 (26 %) and C18:1ω7c (10 %). The strain also contained C14:0 (3 %), C15:0 (1 %) and C17:0iso8c (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 gps52T 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 (Yamasu 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 gps52T 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 gps52T oxidized thiosulfate (20 mM), sulfite (5 and 10 mM), elemental sulfur (5 %), sulfide (2 and 5 mM) and tetrahionate (5 and 10 mM) as electron donors. The following substrates (mM) could not support growth of strain gps52T: H2 [H2/CO2/O2, 60 : 20 : 20 (v/v/v), 150 kPa], H2 + acetate (10), CH4 [N2/CO2/O2/CH4, 50 : 20 : 20 : 10 (v/v/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 gps52T, 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.,...
An almost-complete 16S rRNA gene sequence for strain gps52T 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: neighbouring 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 gps52T is a member of the order Chromatiales in the class Gammaproteobacteria.

However, the sequence of strain gps52T was distant from those of all known genera in the order, and the closest relatives were Natronocella aceticitrifilica and Methylnatronum kenyaense in the family Ectothiorhodospiraceae, with sequence similarities of 92.7 and 92.6 %, respectively. The sequence similarities between strain gps52T 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 gps52T, type strains of species 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 gps52T 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 gps52T is close to that of the genus Thioprofundum. 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 Thiobus denitrificans, with a sequence similarity of 94.1–94.4 %.

The genus Thiohalomonas accommodates moderately halophilic, obligately chemolithoautotrophic, sulfur-oxidizing
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, 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, *Thiogramnum longum* gen. nov., sp. nov., belonging to the family *Ectothiorhodospiraceae*, is proposed.

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**Table 1**

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<th>Genus</th>
<th>Type Strain</th>
<th>Salt Tolerance</th>
<th>Growth Temperature</th>
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<td><em>Thiogramnum</em></td>
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**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 *Rheinegheria*) inferred using the neighbour-joining method after alignment with the ARB program. For tree reconstruction, 1047 positions were used. *Thermithiobacillus tepidarius* and *Acidithiobacillus thiooxidans* 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.
Table 1. Characteristics of strain gps52T and the various chemolithoautotrophic, sulfur-oxidizing genera of the family Ectothiorhodospiraceae

The order Chromatiales contains five families and includes various types of bacteria such as phototrophs, chemoheterotrophs 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, Thiokyoviraceae, 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 Thiokyoviraceae 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, Thiokyoviraceae and Granulosicoccaceae in the order Chromatiales. At this point, it is difficult to clearly divide these deep-branching genera, such as Thiopseudomonadum, Granulosicoccus, Acidiferrobacter, Methylallobacter, Natronocella, Methylobacterium, Thioalbus and our proposed genus. More isolates will be required to fully clarify the relationships among the families in the order Chromatiales.

Description of Thiogranum gen. nov.

Thiogranum (Thi.o.gra’num. Gr. n. theion sulfur; L. neut. n. granum grain; N.L. neut. n. Thiogranum 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 C16:1ω7c. 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 Thiogranum longum.

Description of Thiogranum longum sp. nov.

Thiogranum 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, sulfate, 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 C16:0, C16:1ω7c and C18:1ω7c. Menaquinone-8(H4) and menaquinone-9(H4) are almost equivalently contained.

The type strain, gps52T (=NBRC 101260T =DSM 19610T), 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 Ecothiorhodospiraceae of the order Chromatiales of the class Gammaproteobacteria.

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