Sulfurovum aggregans sp. nov., a hydrogen-oxidizing, thiosulfate-reducing chemolithoautotroph within the Epsilonproteobacteria isolated from a deep-sea hydrothermal vent chimney, and an emended description of the genus Sulfurovum

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A novel mesophilic, strictly hydrogen-oxidizing, sulfur-, nitrate- and thiosulfate-reducing bacterium, designated strain Monchim33T, was isolated from a deep-sea hydrothermal vent chimney at the Central Indian Ridge. The non-motile, rod-shaped cells were Gram-stain-negative and non-sporulating. Growth was observed between 15 and 37 °C (optimum 33 °C; 3.2 h doubling time) and between pH 5.4 and 8.6 (optimum pH 6.0). The isolate was a strictly anaerobic chemolithoautotroph capable of using molecular hydrogen as the sole energy source and carbon dioxide as the sole carbon source. The G+C content of the genomic DNA was 42.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the novel isolate belonged to the genus Sulfurovum and was closely related to Sulfurovum sp. NBC37-1 and Sulfurovum lithotrophicum 42BK7 (95.6 and 95.4 % similarity, respectively). DNA–DNA hybridization demonstrated that the novel isolate could be differentiated genotypically from Sulfurovum sp. NBC37-1 and Sulfurovum lithotrophicum. On the basis of the molecular and physiological traits of the new isolate, the name Sulfurovum aggregans sp. nov. is proposed, with the type strain Monchim33T (=JCM 19824T =DSM 27205T).

Deep-sea hydrothermal vents represent one of the most productive marine ecosystems. The ecosystem is supported primarily by microbial chemosynthesis (Jannasch, 1985). Deep-sea vent chemolithoautotrophs are able to use a range of reductive substrates as energy sources (Campbell et al., 2006; Fisher et al., 2007; Sievert & Vetriani, 2012). Molecular hydrogen is one of the most abundant reducing gas components in vent fluids. A diversity of hydrogen-oxidizing, chemolithoautotrophic, thermophilic and hyperthermophilic genera have been isolated from deep-sea hydrothermal fields (Sievert & Vetriani, 2012). In addition, recent research indicated that H2 could be an important energy source for symbiotic psychrophilic to mesophilic bacteria (Petersen et al., 2011). Various mesophilic, hydrogen-oxidizing Epsilonproteobacteria have been isolated from deep-sea vents, including Sulfurimonas paralvinellae GO25T (Takai et al., 2006), Sulfurovum sp. NBC37-1 (Nakagawa et al., 2007), Thioferdoct micantiosi BKB25Ts-Y1T (Nakagawa et al., 2005a) and ‘Thiofactor thioaminus’ 496Chim (Makita et al., 2012). In particular, members of the genus Sulfurovum are frequently found as endo- and/or epi-symbionts of various vent animals in global deep-sea hydrothermal fields (Haddad et al., 1995; Polz & Cavanaugh, 1995; Cary et al., 1997; Goffredi et al., 2004; Suzuki et al., 2005; Urakawa et al., 2005; Watsuji et al., 2010). However, the description of the genus Sulfurovum is based on just two strains originated from the
Okinawa Trough hydrothermal vent fields (Inagaki et al., 2004; Nakagawa et al., 2007). Here we report the characterization of a novel mesophilic, hydrogen-oxidizing member of the genus *Sulfurovum* from the hydrothermal vent field of the Central Indian Ridge.

Strain Monchim33<sup>T</sup> was isolated from a deep-sea hydrothermal vent chimney collected from the Kairei field in the Central Indian Ridge (25°19’2186”S 02°42’19”’E), at a depth of 2422 m, by DSV *Shinkai 6500* during the YK09-13 leg 2 scientific cruise aboard the *R/V Yokosuka* in November 2009. The interior part of the chimney sample was mixed anaerobically with 25 ml sterilized seawater containing 0.05 % (w/v) neutralized sodium sulfide in 100 ml glass bottles (Schott Glaswerke) soon after the vehicle was recovered. Each bottle was then tightly sealed with a butyl-rubber stopper under a gas phase of 100 % N<sub>2</sub> (0.2 MPa) and stored at 4 °C until use. Then, 100 µl of the resultant slurry was inoculated into 3 ml MMJHS medium (Takai et al., 2003), and the serial dilution technique (1 : 10 dilution series) was employed at 33 °C to evaluate the abundance of culturable micro-organisms. MMJHS medium contained 1 g each of NaHCO<sub>3</sub>, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O and NaNO<sub>3</sub>, 3 g S<sub>0</sub> and 10 ml trace mineral solution per litre of MJ synthetic seawater (Sako et al., 1996). The medium was prepared under 80 % H<sub>2</sub> + 20 % CO<sub>2</sub> (0.3 MPa). The final pH of the medium with the headspace gas was adjusted to approximately pH 6.5. After successful enrichment with MMJHS medium, strain Monchim33<sup>T</sup> was obtained as a pure culture using the dilution-to-extinction technique (Baross, 1995). Purity was confirmed routinely by microscopic observation and by sequencing of the 16S rRNA gene using several PCR primers (Lane, 1991).

The cell morphology of strain Monchim33<sup>T</sup> was observed by phase-contrast microscopy with a Zeiss Axiophot microscope and by electron microscopy with a JSM-6010 LA scanning electron microscope (JEOL) and JEM-1011 transmission electron microscope (JEOL). Cells of strain Monchim33<sup>T</sup> were Gram-stain negative, rod-shaped, approximately 0.4–0.6 µm in width and 0.6–3.6 µm in length (Fig. 1). No motility was observed in MMJHS medium. When grown in MMJHS medium lacking elemental sulfur, black aggregates were observed after the early stationary growth phase (Fig. S1a, available in the online Supplementary Material). The aggregates consisted of bacterial cells and amorphous materials (Fig. 2). No sporulation was apparent under any culture conditions.

Growth of strain Monchim33<sup>T</sup> was measured by direct cell counting after staining with 4’,6-diamidino-2-phenylindole, using a Zeiss Axioskop microscope (Porter & Feig, 1980). To determine temperature, pH and NaCl ranges for growth, duplicate cultures were grown in 15 ml test tubes (Iwaki Glass), each containing 3 ml medium in an incubator. The strain grew at 15–37 °C, showing optimum growth at 33 °C. No growth was observed below 10 °C or above 40 °C. When the pH optimum was examined, pH of the medium was readjusted immediately before inoculation with H<sub>2</sub>SO<sub>4</sub> and NaOH by using a compact pH meter (Horiba AS-212) at 33 °C. pH was stable during the cultivation period. Growth occurred between pH 5.4 and 8.6, with optimum growth at pH 6.0. No growth was found below pH 5.0 or above pH 9.6. NaCl requirement for growth was determined by using various concentrations of NaCl (1.0–5.0 %, w/v) in the medium. The isolate grew in the concentration range of about 2.0–4.0 % (w/v) NaCl, showing optimum growth at 2.5 % (w/v) NaCl (Fig. S2). The maximum cell yield and generation time at 33 °C, 2.5 % (w/v) NaCl and pH 6.0 were about 1.5 × 10<sup>8</sup> cells ml<sup>−1</sup> and 3.2 h, respectively.

**Fig. 1.** Transmission electron micrograph of cells of strain Monchim33<sup>T</sup>. Bar, 1.0 µm.

**Fig. 2.** Scanning electron micrograph of cell aggregates of strain Monchim33<sup>T</sup>. Arrowheads, cells; arrows, amorphous components. Bar, 2.0 µm.
The new isolate was tested for its ability to grow on combinations of a single electron donor and acceptor. MJ synthetic seawater containing 0.1 % (w/v) NaHCO₃ was used as the basal medium. To examine growth on hydrogen as an electron donor, H₂/CO₂ (80:20) was used as the gas phase (0.3 MPa). To determine electron acceptors used for growth, each of the potential electron acceptors, such as SO₄⁰ (1 %, w/v), Na₂S₂O₃ • 5 H₂O (0.1 %, w/v), NaN₂O₃ (0.1 %, w/v), NaNO₂ (0.1 %, w/v), O₂ (0.1–20 %, v/v), 5 mM selenate or selenite, and 10 mM ferric citrate, was tested. For testing growth on SO₄⁰ (1 %, w/v), Na₂S₂O₃ • 5 H₂O (0.1 %, w/v) or selenite (5 mM) as an electron donor, N₂/CO₂ (80 : 20) was used as the gas phase (0.3 MPa). Cell growth was determined by microscopic observation. None of the electron donors other than H₂ supported growth of strain Monchim33T, while nitrate, thiosulfate and SO₄⁰ were able to serve as electron acceptors.

Heterotrophic growth of strain Monchim33T was tested in MMJHS medium without NaHCO₃ under a gas phase of 100 % H₂ (0.3 MPa). Each of the following potential organic carbon sources was tested at concentrations of 0.01 and 0.1 % (w/v): L-cysteine, L-phenylalanine, L-proline, Casamino acids, D(-)-glucose, lactose, maltose, chitin, starch, cellulose, formate, formaldehyde, acetate, citrate, pyruvate, propionate, 2-propanol, methanol, tryptone peptone and yeast extract (all from Difco). Strain Monchim33T was not able to grow heterotrophically using H₂ as a potential energy source and SO₄⁰, thiosulfate or nitrate as an electron acceptor. In addition, to assess the utilization of these organic compounds as an energy source, substrates were added to MMJHS medium under an N₂/CO₂ (80:20) gas phase (0.3 MPa). None of the organic compounds sustained growth of strain Monchim33T as the energy source.

To determine nitrogen source utilization, 0.025 and 0.25 % (w/v) NH₄Cl, NaNO₂ or NaNO₃, urea and Casamino acids was added to MMJHS medium lacking all nitrogen sources, under an H₂/CO₂ (80 : 20) gas phase (0.3 MPa). Utilization of N₂ was examined under an H₂/N₂/CO₂ (60:20:20) gas phase (0.3 MPa). The strain was able to use ammonium or nitrate as a sole nitrogen source but not nitrite, Casamino acids, urea or molecular nitrogen. To examine a sulfur source for growth of strain Monchim33T, potential sources such as sulfate (5 mM), thiosulfate (5 mM), SO₄⁰ (1 %, w/v), sodium sulfide (2 mM), cysteine hydrochloride (2 mM), Casamino acids (0.1 % w/v) and yeast extract (0.1 % w/v) were examined in MMJHS medium in which sulfur compounds were removed and replaced with the chloride salts under an H₂/CO₂ (80:20) gas phase (0.3 MPa). Strain Monchim33T grew using SO₄⁰, thiosulfate or yeast extract as the sole sulfur source.

Susceptibility to antibiotics (at 50, 100 and 200 µg ml⁻¹) such as ampicillin, kanamycin, rifampicin, streptomycin and chloramphenicol was tested in MMJHS medium at 33 °C. Strain Monchim33T was sensitive to chloramphenicol at 50 µg ml⁻¹, but was resistant to ampicillin and kanamycin at 100 µg ml⁻¹ and to rifampicin at 50 µg ml⁻¹ (but sensitive to ampicillin, kanamycin and rifampicin at 200 and 100 µg ml⁻¹, respectively). Resistance to kanamycin or rifampicin has also been reported in deep-sea vent chemolithotrophs including Nitratiruptor salsuginis E9137-1T and Nitratiruptor tergarcus M155-1T (Nakagawa et al., 2005b).

In addition, the oxygen sensitivity of growth was examined with MMJHS medium under various H₂/CO₂/O₂ gas phase ratios (79:20:1, 78.5:20:1.5, 78:20:2, 77:20:3, 75:20:5, 70:20:10 or 60:20:20). O₂ above 3 % (v/v) inhibited growth of strain Monchim33T.

The respiratory lipoquinones and polar lipids of strain Monchim33T were extracted from 100 mg freeze-dried cells and analysed according to Minnikin et al. (1984). Cells grown at the late exponential phase of growth in MMJHS
medium (without elemental sulfur) at 33 °C were used. Respiratory quinones were dissolved in petroleum ether and applied to TLC plates (silica gel). The purified respiratory quinones were analysed using a Shimadzu HPLC system with a reversed-phase Kinetex C18 column and methanol-2-propanol (3:1, v/v) as the mobile phase at 1 ml min⁻¹ at 37 °C and were detected at 269 nm (Tamaoka et al., 1983). Polar lipids were separated by two-dimensional silica gel TLC. The respiratory quinone of strain Monchim33T was menaquinone-6 (MK-6), as in other members of the class Epsilonproteobacteria (Garrity et al., 2005; Makita et al., 2012). As shown by TLC, strain Monchim33T possessed phosphatidylethanolamine, phosphatidylglycerol, unidentified aminophospholipids, unidentified aminophosphoglycolipids, an unidentified phospholipid and unidentified lipids (Fig. S3). Diphosphatidylglycerol detected in ‘Thiofactor thioacuminus’ 496Chim (Makita et al., 2012) was not found in strain Monchim33T. Respiratory quinones were analysed using a Shimadzu HPLC system with a reversed-phase Kinetex C18 column and methanol-2-propanol (3:1, v/v) as the mobile phase at 1 ml min⁻¹ at 37 °C and were detected at 269 nm (Tamaoka et al., 1983). Polar lipids were separated by two-dimensional silica gel TLC. The respiratory quinone of strain Monchim33T was menaquinone-6 (MK-6), as in other members of the class Epsilonproteobacteria (Garrity et al., 2005; Makita et al., 2012). As shown by TLC, strain Monchim33T possessed phosphatidylethanolamine, phosphatidylglycerol, unidentified aminophospholipids, unidentified aminophosphoglycolipids, an unidentified phospholipid and unidentified lipids (Fig. S3). Diphosphatidylglycerol detected in ‘Thiofactor thioacuminus’ 496Chim (Makita et al., 2012) was not found in strain Monchim33T. For analysis of total cellular fatty acids, cells were cultivated in MMJHS (lacking elemental sulfur) medium at 33 °C in the late exponential growth phase. Lyophilized cells were placed in a Teflon-lined, screw-capped tube containing 1 ml of anhydrous methanolic HCl and heated at 100 °C for 3 h as described previously (Izumi et al., 2012). Fatty acid methyl esters were analysed using a Shimadzu GCMS-QP2010 system. The cellular fatty acids of strain Monchim33T were C₁₆:₀ (40.0 %), C₁₈:₀ (18.9 %), C₁₆:₁ (18.1 %), C₁₈:₁ (9.4 %), C₁₄:₀ (7.7 %) and C₁₄:₁ (5.9 %). The dominance of even-chain fatty acids is shared with other deep-sea Epsilonproteobacteria, including Sulfitovum lithothrophicum 42BT (Inagaki et al., 2004), Sulfitovum autotrophicica 42OK (Inagaki et al., 2003), Sulfitovum paralvinellae GO25 (Takai et al., 2006), Nitratifractor salsuginis E9317 (Nakagawa et al., 2005b) and ‘Thiofactor thioacuminus’ 496Chim (Makita et al., 2012).

Genomic DNA of strain Monchim33T was prepared using standard phenol/chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989). The G+C content of the DNA was determined by direct analysis of deoxyribonucleotides using HPLC with a DNA-GC kit (Yamasa Shouyu) as described by Tamaoka & Komagata (1984). The G+C content of the genomic DNA of strain Monchim33T was 42.6 mol%, a value similar to those of Sulfitovum lithothrophicum 42BT (48.0 mol%) and Sulfitovum sp. NBC37-1 (43.8 mol%).

DNA–DNA hybridization between the genomic DNA of strain Monchim33T and Sulfitovum sp. NBC37-1 or Sulfitovum lithothrophicum 42BT was performed using the microplate method of Ezaki et al. (1989) at 44 °C. DNA–DNA hybridization values between strain Monchim33T and Sulfitovum sp. NBC37-1 (26.1 %) and Sulfitovum lithothrophicum 42BT (25.2 %) were low, indicating that the new isolate could be genotypically differentiated from previously described species of the genus Sulfitovum.

The 16S rRNA gene was amplified by PCR using primers Eubac 27F and 1492R (Lane, 1991). The sequence of the PCR products (1384 bp) was determined directly in both strands using the dideoxynucleotide chain-termination method. 16S rRNA gene sequence similarity analysis was conducted using the BLAST search algorithm with all nucleotides (Altschul et al., 1997). The sequence of strain Monchim33T shared highest similarity with those of Sulfitovum sp. NBC37-1 (95.6 %) and Sulfitovum lithothrophicum ATCC BAA-797 (95.4 %). These similarity values indicated that the new isolate potentially represented a novel species of the genus Sulfitovum based on the evolutionary distance (97 %) for differentiation at the species level (Stackebrandt & Goebel, 1994). More closely related sequences were derived from environmental clones from the Juan de Fuca Ridge (AV07-CP-53; 97.9 % 16S rRNA gene sequence similarity). To determine the phylogenetic position of strain Monchim33T, the sequence was aligned with a subset of 16S rRNA gene sequences using the ARB software (Ludwig et al., 2004). The resulting alignment was verified against known secondary regions, and only unambiguously aligned nucleotide positions (1178 bases) were used for phylogenetic analyses with the MEGA 5.05 package (Tamura et al., 2011). A phylogenetic tree was reconstructed by the maximum-likelihood method (Felsenstein, 1981) with Kimura’s two-parameter calculation model (Kimura, 1980). Bootstrap analysis was used for 1000 replications to provide confidence estimates for the phylogenetic tree topologies.

Phylogenetic analysis indicated that strain Monchim33T belonged to the genus Sulfitovum (Fig. 3). In addition, the fatty acid composition of the new isolate was similar to that of Sulfitovum lithothrophicum (Inagaki et al., 2004). However, the new isolate could be differentiated from previously described species of the genus Sulfitovum based on several physiological properties (Table 1). Strain Monchim33T is the only representative of a strictly anaerobic species within the family ‘Thiovulaceae’ (genera Sulfitovum, Nitratifractor, Sulfitovum and Sulfitovum) (Campbell et al., 2006). In addition, Monchim33T formed aggregates when grown in MMJHS medium without elemental sulfur (Figs 2 and S1). We have not determined the composition of the amorphous materials, although they might be formed by sulfide produced by the microbial reduction of thiosulfate. The formation of aggregates is reported here for the first time within deep-sea vent mesophilic chemolithoautotrophs. Furthermore, DNA–DNA hybridization analysis clearly showed that strain Monchim33T could be differentiated genetically from Sulfitovum sp. NBC37-1 and Sulfitovum lithothrophicum at the species level. On the basis of these physiological and genetic properties, strain Monchim33T is considered to represent a novel species of the genus Sulfitovum, for which the name Sulfitovum aggregans sp. nov. is proposed.

**Emended description of the genus Sulfitovum**

The description is based on that by Inagaki et al. (2003). Cells are Gram-stain-negative, non-motile, cocoid to rods. Strictly to facultatively anaerobic. Sea salts are required for growth. Growth occurs chemolithoautotrophically with hydrogen, elemental sulfur and thiosulfate as an electron donor.
Table 1. Comparison of the major characteristics of strain Monchim33\(^{T}\) with those of other strains within the *Epsilonproteobacteria*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>Origin*</td>
<td>Kairei field, CIR</td>
<td>Iheya North field, OT</td>
<td>Iheya North field, OT</td>
<td>Iheya North field, OT</td>
<td>Iheya North field, OT</td>
<td>Hatoma Knoll field, OT</td>
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<td>Nikko Seamount field, NMA</td>
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<td>Rod</td>
<td>Coccoid to oval</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
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<td>Rod or curved rod</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>33</td>
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<td>23–26</td>
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<td>Electron donor(s)</td>
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<td>NO(_3)(^–), O(_2)</td>
<td>NO(_3)(^–), O(_2)</td>
<td>NO(_3)(^–), O(_2)</td>
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<td>MK-6</td>
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<td>C(<em>{16:0}) (53.7), C(</em>{16:0}) (31.3), C(_{16:1}) (15.0),</td>
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<td>C(<em>{18:1}) (37.1), C(</em>{16:0}) (22.4), C(_{16:1}) (5.1),</td>
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<td>DNA G+C content (mol%)</td>
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<td>48.0</td>
<td>43.8</td>
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<td>35.2</td>
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* CIR, Central Indian Ridge; NMA, North Mariana Arc; OT, Okinawa Trough.
donor, and with oxygen, nitrate, thiosulfate and elemental sulfur as an electron acceptor using CO₂ as the carbon source. 16S rRNA gene sequence analysis places the genus within the Epsilonproteobacteria. The type species is *Sulfurovum lithotrophicum* (Inagaki et al., 2003).

**Description of Sulfurovum aggregans sp. nov.**


Cells are Gram-stain-negative, non-motile and rod-shaped. Growth occurs at 15–37 °C (optimum, 33 °C), at pH 5.4–8.6 (optimum, 6.0) and with 2.0–4.0 % (w/v) NaCl (optimum, 2.5 %). Optimal doubling time is about 3.2 h. Strictly anaerobic. Strictly chemolithoautotrophic growth occurs with H₂ as an electron donor and with S⁰, thiosulfate or nitrate as an electron acceptor. Elemental sulfur, thiosulfate or yeast extract serve as a sulfur source for growth. Nitrate or ammonium is required as a nitrogen source. DNA–DNA relatedness to *Sulfurovum* sp. NBC37-1 and *Sulfurovum lithotrophicum* 42BKT is low.

The type strain is Monchim33T (=JCM 19824T=DSM 27205T), isolated from a deep-sea hydrothermal vent in the Central Indian Ridge. The DNA G + C content of the type strain is 42.6 mol% (by HPLC).

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**References**


