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Sulfurimonas autotrophica gen. nov., sp. nov., a novel sulfur-oxidizing ε-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough

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A novel mesophilic, sulfur- and thiosulfate-oxidizing bacterium, strain OK10^T, was isolated from deep-sea sediments at the Hatoma Knoll in the Mid-Okinawa Trough hydrothermal field. Cells of strain OK10^T were short rods, each being motile by means of a single polar flagellum. The isolate grew at 10–40 °C (optimum 25 °C) and pH 4·5–9·0 (optimum pH 6·5). It grew chemolithoautotrophically with elemental sulfur, sulfide and thiosulfate as sole electron donors and oxygen as electron acceptor. Molecular hydrogen did not support growth. The G+C content of the genomic DNA of strain OK10^T was 35·2 mol%. Phylogenetic analysis, based on 16S rRNA gene sequences, indicated that the isolate belonged to the *ε-Proteobacteria*. On the basis of its physiological and molecular characteristics, strain OK10^T (=ATCC BAA-671^T=JCM 11897^T) represents the sole species of a new genus, *Sulfurimonas*, for which the name *Sulfurimonas autotrophica* is proposed.

Hydrothermal activity in the deep-sea floor provides a great variety of habitats for micro-organisms. Both culture-dependent and -independent microbiological investigation of deep-sea hydrothermal environments has revealed the distribution of phylogenetically and physiologically diverse micro-organisms within temperature and geochemical gradients (Takai & Fujiwara, 2002). Among the microbial components, an abundance of ε-proteobacteria has been demonstrated by frequent recovery of their rDNA sequences from global deep-sea hydrothermal vent systems (Moyer *et al.*, 1995; Polz & Cavanaugh, 1995; Reysenbach *et al.*, 2000; Longnecker & Reysenbach, 2001; Campbell *et al.*, 2001). However, the physiological properties and ecological impacts of the deep-sea hydrothermal vent *ε-Proteobacteria* have long been undefined because of their resistance to stable cultivation.

To date, two genera of deep-sea hydrothermal vent *ε-Proteobacteria*, *Caminibacter* and *Nautilia*, have been isolated from the tube-dwelling polychaete *Alvinella pompejana* on the East Pacific Rise and described (Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002). Both genera contained strictly anaerobic, moderately thermophilic hydrogen-oxidizers that used elemental sulfur as a primary electron acceptor. Based on 16S rRNA gene sequences, isolates of these genera were affiliated to the phylogenetic cluster of the previously uncultivated *ε-Proteobacteria* Group D (Corre *et al.*, 2001). Very recently, some ε-proteobacteria have been isolated from hydrothermal environments in the Mid-Okinawa Trough and the Central Indian Ridge (Takai *et al.*, 2003a). In this study, characterization of a mesophilic strain representing the most abundantly cultivated phylogenetic group of *ε-Proteobacteria* (Group B) from deep-sea hydrothermal environments is reported.

Deep-sea hydrothermal sediments were obtained from the inside of calderas in the Hatoma Knoll in the Mid-Okinawa Trough hydrothermal field, Japan (123° 50·573′ E, 24° 51·448′ N), at a depth of 1528 m using a grab sampler deployed from the mother vessel R/V *Kairei* during the KR01-09 geomicrobiology cruise carried out in June 2001. The subcore (10 cm depth from the sea-floor surface) was

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The DDBJ accession number for the 16S rDNA sequence of *Sulfurimonas autotrophica* OK10^T is AB088431.

Electron micrographs of a negatively stained cell and a thin-section of strain OK10^T are available as supplementary material in IJSEM Online.

collected immediately after recovery of the grab sampler. The surface of the sediment was composed of brown-grey coarse sand (0–1 cm) and the deeper zone (1–10 cm) was composed of grey and black angular coarse sand. Approximately 25 cm^3 of the surface (0–4 cm), middle range (4–7 cm) and deep range (7–10 cm) sediments were put separately into 100 ml sterilized glass bottles (Schott Glaswerke) with 50 ml sterilized MJ synthetic sea water containing 0·05 % (w/v) sodium sulfide and then tightly sealed with a butyl-rubber cap under a gas phase of 100 % N_2 (150 kPa). MJ synthetic sea water contained trace mineral solution (10 ml l^{-1}; Balch *et al.*, 1979) and the following compounds (l^{-1}): NaCl, 30·0 g; $CaCl_2.2H_2O$, 0·14 g; $MgSO_4$, 3·40 g; $MgCl_2$, 4·18 g; K_2HPO_4, 0·14 g; KCl, 0·33 g; NH_4Cl, 0·25 g; $NiCl_2.6H_2O$, 0·5 mg; $Na_2SeO_3.5H_2O$, 0·5 mg; and $FeCl_2$, 0·01 g. These slurry samples were inoculated directly into the medium (as described below) and stored at 4 °C on board the vessel.

Fundamental physico-chemical characteristics of the subcore were measured using a multi-parameter water quality monitoring system (Multi-Probe U-20; HORIBA) according to the manufacturer's recommendations. The pH and redox potential value in the middle range section (4–7 cm) were 6·5 and −187 mV, respectively. The sediments smelled strongly of H_2S and contained a few small tubeworms. These results indicated that the anoxic marine sediment was successfully collected from the deep-sea hydrothermal environment by the grab-sampling system.

The slurry samples (see above) were inoculated (10 % volume of the medium) into MMJHS medium (Takai *et al.*, 2003a) and incubated at room temperature (approx. 20–25 °C) on board the vessel. Enrichment growth was only observed in medium supplemented with 10 % oxygen in the gas phase that was inoculated with the slurry of surface sediment (0–4 cm) after 5 days incubation on board. This enrichment culture, which contained highly motile rods, was then purified by single colony isolation on MMJHS medium solidified with 1·5 % agar (Difco) incubated for 5 days. Cells were also further purified by the dilution-to-extinction technique (Baross, 1995) and the culture in the tube showing growth at the highest dilution was designated strain $OK10^T$. Purity was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers. Strain $OK10^T$ was routinely cultivated with the following MJ basal medium, made up in MJ synthetic sea water: 0·15 % (w/v) $NaHCO_3$, 0·15 % (w/v) $Na_2S_2O_3.5H_2O$ and 0·01 % (v/v) vitamin mixture (Balch *et al.*, 1979). Gas mixtures of $N_2/CO_2/O_2$ (77 : 17 : 6, 200 kPa) were used in the headspace. The ratio of liquid to gas phase in the test tube was 1 : 2 (v/v).

Cells were routinely observed under a phase-contrast Olympus BX51 microscope with the Olympus Camedia C3030 digital camera system. Cells grown in MJ basal medium at 24 °C in the mid-exponential phase of growth were negatively stained with 2 % (w/v) uranyl acetate and observed under a JEOL JEM-1210 transmission electron microscope at an accelerating voltage of 120 kV (Zillig *et al.*, 1990). Cells of strain $OK10^T$ were Gram-negative, slightly curved rods of $1·5–2·5 \times 0·5–1·0$ μm, with a single polar flagellum (an electron micrograph, A, is available as supplementary material in IJSEM Online; http://ijs.sgmjournals.org). Spore formation was not observed.

Thin-sections were prepared after fixation in 4·0 % (w/v) paraformaldehyde overnight at room temperature and then post-fixed with 1 % (v/v) OsO_4. The specimen was embedded overnight in Spurr resin and cut with an ultramicrotome. The thin-sections were stained in 2 % (w/v) uranyl acetate and observed with a JEOL JEM-1210 electron microscope. Micrographs revealed that strain $OK10^T$ had a cell-wall structure typical of Gram-negative bacteria (an electron micrograph, B, is available as supplementary material in IJSEM Online).

Effects of temperature, pH, and total salt and oxygen concentrations on the growth of strain $OK10^T$ were investigated. Growth of the isolate was monitored by a direct count of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained cells under an epifluorescence microscope (Porter & Feig, 1980). All experiments described below were conducted in duplicate. To determine the optimum growth temperature, cells were grown in MJ basal medium with continuous shaking as described above. Strain $OK10^T$ grew between 10 and 40 °C, showing optimal growth at 23–26 °C. No growth was observed below 5 °C or above 45 °C. To determine the effect of pH on growth, the pH of MJ basal medium was adjusted to various levels with 10 mM acetate/acetic acid buffer (pH 3·0–5·5), MES (pH 5·0–6·5), PIPES (pH 6·5–7·0), HEPES (pH 7·0–8·0), Tris and CAPSO (pH 8·0 and above). The pH was checked after autoclaving and was axenically readjusted with H_2SO_4 or NaOH at room temperature if necessary. Growth occurred between pH 4·5 and 9·0 and the optimum pH was around 6·0–7·5. Weak growth was observed at pH 4·5 and 9·0. The isolate required sea salts for growth. Strain $OK10^T$ grew over the total salt concentration range of 16 to 60 g l^{-1}, with optimum growth at 40 g total salt l^{-1} at 24 °C, pH 6·7. Oxygen sensitivity was examined using MJ basal medium and adjusting the oxygen concentration in the headspace gas during incubation at 24 °C, pH 6·7 and 4·0 % (w/v) sea salt concentration. To test for anaerobic, denitrifying growth, 10 mM nitrate was added to the MJ basal medium as an alternative electron acceptor. Growth of the isolate was observed in 1–15 % (v/v) oxygen, with optimal growth at 5–8 % (v/v). No growth was observed without oxygen or with over 20 % (v/v) in the headspace gas. Growth was also not observed with air saturation in the headspace. Under optimum growth conditions in MJ basal medium at 24 °C, pH 6·7, 4·0 % (w/v) sea salt and 10 % (v/v) oxygen, the final yield was approximately $4·0 \times 10^8$ cells ml^{-1} and the doubling time was approximately 1·4 h.

A variety of potential electron donors was tested in MJ basal medium supplemented with the following compounds:

3 % (v/v) elemental sulfur (S^0); 0·02 and 0·05 % (w/v) $Na_2S.9H_2O$ and cysteine.HCl; 5 mM each of $Na_2S_2O_3$, $Na_2S_2O_4$, $Na_2S_2O_5$, $Na_2S_2O_7$, $Na_2S_2O_8$ (the last three compounds were obtained from Wako Purechemical at purity of 64–67·4 %, >98 % and >97 %, respectively) and Na_2SO_3; 0·1 and 0·01 % (w/v) each of yeast extract, peptone, glucose maltose and sucrose; 0·1 and 0·01 % (v/v) each of methanol, ethanol, 2-propanol, formate, acetate, lactate, tartaric acid, fumarate, malate, pyruvate, ascorbic acid, succinate, nitrilo triacetic acid and thioglycolic acid; and 0·01 % methionine. The ability to use H_2 was examined using a gas mixture of $H_2/CO_2/O_2$ (67 : 27 : 6, 200 kPa) in the headspace. Strain OK10^T was able to use elemental sulfur, $Na_2S_2O_3$ and $Na_2S.9H_2O$ as sole energy sources for chemoautotrophic growth, but was unable to grow heterotrophically on any organic compounds. Final cell numbers of cultures grown on elemental sulfur or thiosulfate (5 mM) were over 10^8 cells ml^{-1}, whereas the medium containing sodium sulfide (<3 mM) produced accordingly lower cell numbers, below 2×10^7 cells ml^{-1}. No other substrate added to the medium as a potential electron donor supported growth. Potential electron acceptors such as 5 mM Na_2SO_4, Na_2SO_3, $NaNO_3$, $NaNO_2$ and fumarate, and 1 % (v/v) ferrihydrite were examined in place of oxygen in MJ basal medium with a gas mixture of N_2/CO_2 (70 : 30, 200 kPa). Ferrihydrite [$Fe(OH)_3$] was prepared as described by Kostka & Nealson (1998). However, none of these electron acceptors supported growth.

On the basis of the results described above, strain OK10^T was found to be an aerobic chemolithoautotrophic, sulfur-oxidizing bacterium capable of growth on sulfide, elemental sulfur and thiosulfate. To monitor thiosulfate oxidation, $S_2O_3^{2-}$ and SO_4^{2-} concentrations during growth were measured by HPLC (Shimadzu) (Takai *et al.*, 2002). Medium supplemented with 0·15 % (w/v) $NaHCO_3$, 5 mM $Na_2S_2O_3$, 0·01 % (v/v) vitamin mixture (Balch *et al.*, 1979) dissolved in sulfate-free MJ synthetic sea water containing $MgCl_2$ instead of $MgSO_4$ (i.e. containing 7·58 g $MgCl_2$ l^{-1}) was used in this experiment. The concentration of thiosulfate decreased with increasing cell density, whereas that of sulfate increased (Fig. 1), indicating that most thiosulfate was oxidized to sulfate.

The cellular fatty acid composition and the DNA base content of strain OK10^T were analysed as described previously (Takai *et al.*, 2003b). Cells grown in MJ basal medium at 24 °C in the late-exponential phase of growth were used for these analyses. The major cellular fatty acids of the isolate were $C_{14:0}$ (8·4 %), $C_{16:1cis}$ (45·2 %), $C_{16:0}$ (37·1 %) and $C_{18:1trans}$ (9·4 %). The G+C content of the genomic DNA of strain OK10^T was 35·2 ± 0·5 mol%, which is slightly higher than those of *Caminibacter hydrogeniphilus* AM1116^T (29·0 mol%) and *Nautilia lithotrophica* 525^T (34·7 mol%), which were isolated from a hydrothermal vent in the East Pacific Rise (Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002).

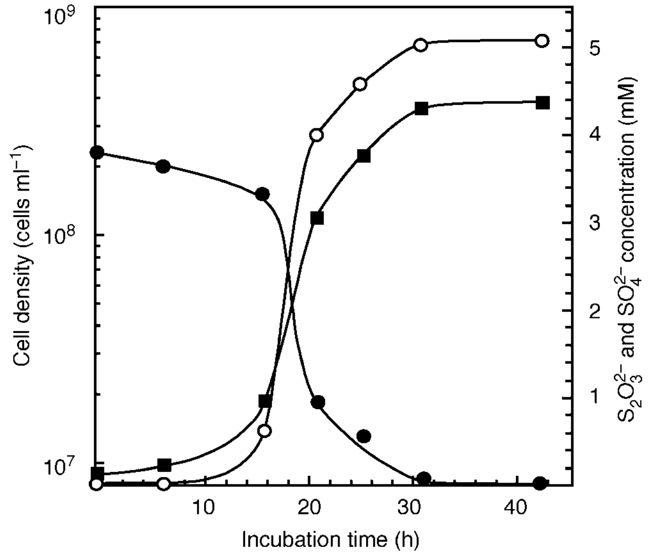

Fig. 1. Profile of changes in thiosulfate (●) and sulfate (○) concentrations during growth (■) of strain OK10^T.

The 16S rRNA gene of strain OK10^T was amplified by PCR using primers Bac27F and 1492R (DeLong, 1992; Lane, 1985). Both strands of the nearly complete rDNA sequence (1401 bp) from strain OK10^T were determined with a model 3100 automatic capillary sequencer (Applied Biosystems). The rDNA sequence of strain OK10^T was subjected to sequence similarity analysis against the prokaryotic SSU rRNA database and the non-redundant nucleotide sequence databases of GenBank, EMBL and DDBJ using the gapped-BLAST and FASTA search algorithms. Analysis indicated that the 16S rRNA gene sequence of strain OK10^T had high similarity with an uncultured environmental rDNA sequence of PVB-12 (99·1 %), obtained from a microbial mat near the deep-sea hydrothermal vent in the Loihi Seamount, Hawaii (Moyer *et al.*, 1995). Data suggest that the isolate is a member of the *ε-Proteobacteria*. Phylogenetic analysis was performed using 1191 homologous nucleotide positions that could be unambiguously aligned in all examined sequences. A least-squares distance matrix based on evolutionary distances was constructed using the correction of Kimura (1980). Neighbour-joining analysis was accomplished using the DDBJ CLUSTAL_X system (Thompson *et al.*, 1997) and bootstrap analysis was done to provide confidence estimates for phylogenetic tree topologies. The phylogenetic tree revealed that the rDNA sequence of strain OK10^T was affiliated with the uncultivated *ε-Proteobacteria* Group B, the cluster of which contains large numbers of environmental rDNA sequences obtained from deep-sea hydrothermal vent fields in the Loihi Seamount and the Mid-Atlantic Ridge (Corre *et al.*, 2001) (Fig. 2). The rDNA sequences of the recently isolated thermophilic *ε-Proteobacteria N. lithotrophica* and *C. hydrogeniphilus* were located in Group D and are distantly related to strain OK10^T (Fig. 2). The rDNA sequence of strain OK10^T was highly similar to that of the cultured ε-proteobacterium

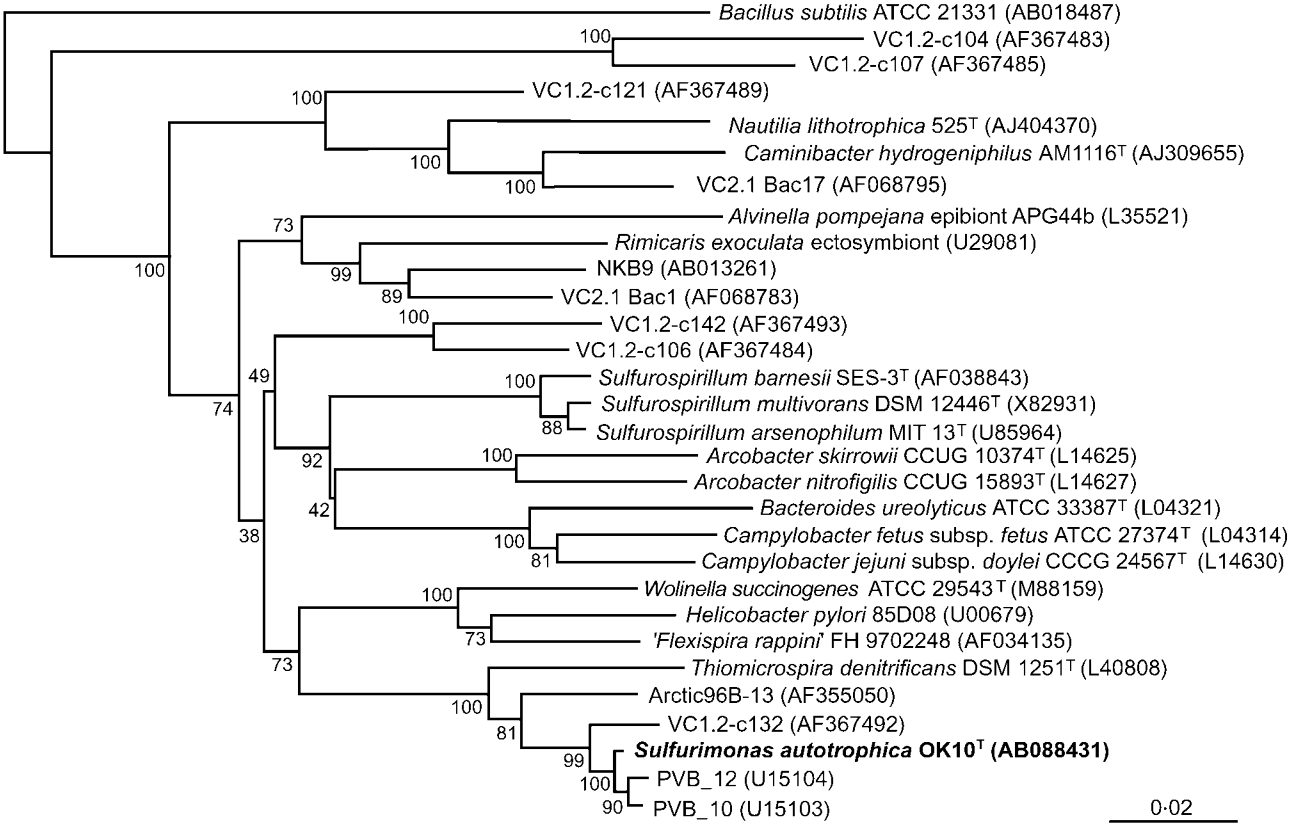

Fig. 2. Phylogenetic tree of representative members and environmental 16S rDNA sequences within the *ε-Proteobacteria*. The tree was constructed by the neighbour-joining method using 1191 homologous positions. EMBL/GenBank/DDBJ accession numbers are shown in parentheses. Numbers at the nodes represent bootstrap values (100 replicates). Bar, 2 substitutions per 100 nt.

Thiomicrospira denitrificans DSM 1251^T (93·5 %), which was isolated from coastal sea sediment (Timmer-ten Hoor, 1981).

On the basis of culture-independent molecular analyses in two hydrothermal vent fields in the Loihi Seamount and the 19° N Mid-Atlantic Ridge, Group B was the most predominant phylotype among the bacterial rDNA clone libraries (Loihi Seamount microbial mat, 61 %; Mid-Atlantic Ridge vent cap, 35 %) (Moyer *et al.*, 1995; Corre *et al.*, 2001). These data suggest that the isolate obtained from the Okinawa Trough might be one of the most abundant microbial components in the low-temperature microhabitats occurring in the ambient environment in global hydrothermal systems (Takai *et al.*, 2003a). In addition, the majority of ε-proteobacterial inhabitants represented by our isolate may have a significant biogeochemical role in recycling sulfur compounds by their metabolic activities (Inagaki *et al.*, 2002; Takai *et al.*, 2003a). Indeed, strain OK10^T represents the most frequently isolated ε-proteobacteria from the Okinawa hydrothermal fields (Takai *et al.*, 2003a). Further isolation and identification of previously uncultivated ε-proteobacterial inhabitants will clarify their ecological impacts on the hydrothermal geochemistry and ecosystem; these are our ongoing efforts.

Description of *Sulfurimonas* gen. nov.

Sulfurimonas (Sul.fu.ri.mo′nas. L. neut. n. *sulfur* sulfur; Gr. n. *monas* a unit, monad; N.L. fem. n. *Sulfurimonas* sulfur-oxidizing rod).

Cells are Gram-negative motile rods with a single polar flagellum. Mesophilic and aerobic. Require sea salts for growth. Growth occurs chemolithoautotrophically with sulfide, elemental sulfur and thiosulfate as electron donors, using CO_2 as a carbon source. 16S rDNA sequencing revealed that the genus falls within the *ε-Proteobacteria.*

The type species is *Sulfurimonas autotrophica.*

Description of *Sulfurimonas autotrophica* sp. nov.

Sulfurimonas autotrophica (au.to.tro′phi.ca. Gr. n. *autos* self; Gr. adj. *trophikos* nursing, tending or feeding; N.L. fem. adj. *autotrophica* autotroph).

Cells are Gram-negative short rods ($1{\cdot}5$–$2{\cdot}5 \times 0{\cdot}5$–$1{\cdot}0$ μm) that are motile by means of a polar flagellum. Strictly aerobic, requiring less than 15 % O_2 (i.e. 75 % air saturation) in the headspace gas (optimum 5–8 %). Forms white colonies on solid medium. Grows at 10–40 °C (optimum 23–26 °C) and pH 4·5–9·0 (optimum pH 6·0–7·5). Sea salts are required for growth; grows at 16–60 g sea salts l^{-1} (optimum 40 g l^{-1}). Growth occurs chemolithoautotrophically using sulfide, elemental sulfur and thiosulfate as electron donors and CO_2 as a carbon source. Organic substrates and H_2 are not utilized as electron donors and only oxygen is utilized as an electron acceptor. Major cellular fatty acids are $C_{14:0}$ (8·4 %), $C_{16:1cis}$ (45·2 %), $C_{16:0}$ (37·1 %) and $C_{18:1trans}$ (9·4 %).

The type strain is $OK10^T$ ($=$ ATCC BAA-671^T $=$ JCM 11897^T). The G + C content of its genomic DNA is $35{\cdot}2 \pm 0{\cdot}5$ mol% (HPLC method). Isolated from the surface of deep-sea hydrothermal sediment on the Hatoma Knoll in the Mid-Okinawa Trough hydrothermal field.

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