**Varunaivibrio sulfuroxidans** gen. nov., sp. nov., a facultatively chemolithoautotrophic, mesophilic alphaproteobacterium from a shallow-water gas vent at Tor Caldara, Tyrrhenian Sea

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A mesophilic, facultatively anaerobic, facultatively chemolithoautotrophic bacterium, designated strain TC8\(^T\), was isolated from a sulfidic shallow-water marine gas vent located at Tor Caldara, in the Tyrrhenian Sea, Italy. Cells were Gram-stain-negative curved rods with one or more polar flagella. Cells were approximately 1–1.5 µm in length and 0.6 µm in width. Strain TC8\(^T\) grew between 20 and 35 °C (optimum 30 °C), with between 5 and 45 g NaCl l\(^{-1}\) (optimum 15–20 g l\(^{-1}\)) and between pH 4.5 and 8.5 (optimum pH 6.0–7.0). The generation time under optimal conditions was 8 h. Strain TC8\(^T\) was a facultative chemolithoautroph also capable of using organic substrates as electron donors and carbon sources. Chemolithoautotrophic growth occurred with sulfur and thiosulfate as the electron donors, CO\(_2\) as the carbon source, and nitrate, oxygen (5 %, v/v) and ferric iron as the electron acceptors. Chemoorganoheterotrophic growth occurred with tryptone, peptone, Casamino acids, pyruvate and glycerol as substrates, while chemolithoherotrophic growth occurred with D\(^{(+)}\)-glucose, sucrose, yeast extract, acetate, lactate, citrate and L-glutamine. The G+C content of the genomic DNA was 59.9 mol%.

Phylogenetic analysis of the 16S rRNA gene sequence of strain TC8\(^T\) showed that this organism formed a lineage within the family **Rhodospirillaceae**, which branched separately from the two closest relatives, *Magnetovibrio blakemorei* MV1\(^T\) (91.25 % similarity) and *Magnetospira thiophila* MMS-1\(^T\) (90.13 %). Based on phylogenetic, physiological and chemotaxonomic characteristics, it is proposed that the organism represents a novel species of a new genus within the family **Rhodospirillaceae**.

Sediment samples were collected from a submarine coastal gas vent at Tor Caldara (41°29’ 9” N 12°35’ 23” E) in August 2013. The sediment samples were stored in the surrounding ambient seawater at 4 °C until further processing.

The family **Rhodospirillaceae**, which comprises over 40 genera, belongs to the order **Rhodospirillales** within the class **Alphaproteobacteria** (Garrity et al., 2005). This family is morphologically and metabolically diverse and comprises chemooorganoheterotrophs (e.g. *Dongia* and *Elstera*; Liu et al., 2010; Rahalkar et al., 2012) as well as chemoautotrophs (e.g. *Magnetovibrio* and *Magnetospira*; Bazylinski et al., 2013; Williams et al., 2012), and phototrophs (e.g. *Rhodospirillum* and *Rhodovibrio*; Imhoff et al., 1988; Pfennig & Trüper, 1971) adapted to a range of different habitats.

In this study, we describe a novel mesophilic, facultatively chemolithoautotrophic, sulfur-oxidizing alphaproteobacterium that was isolated from the marine sediment of a sulfidic coastal submarine gas vent at Tor Caldara, in the Tyrrhenian Sea. Based on 16S rRNA gene sequence phylogeny, physiological traits and a distinct chemotaxonomic profile, this strain is considered to represent a novel species of a new genus within the family **Rhodospirillaceae**.
Primary enrichment cultures were performed in the laboratory by inoculating a slurry containing 1 g of the sediment sample re-suspended in 1 ml of anoxic artificial seawater into 10 ml of liquid modified 1011 medium (Inagaki et al., 2004) supplemented with 10 % (w/v) potassium nitrate under an N₂/CO₂ gas phase (80 : 20; 200 kPa). Medium 1011 contained (per litre): 30 g NaCl, 0.14 g K₂HPO₄, 0.14 g CaCl₂·2H₂O, 3.4 g MgSO₄·7H₂O, 4.18 g MgCl₂·6H₂O, 0.33 g KCl, 0.5 mg NiCl₂·6H₂O, 0.5 mg Na₃SeO₃·5H₂O, 0.01 g Fe(NH₄)₂(SO₄)·6H₂O, 0.25 g NH₄Cl, 1.5 g NaHCO₃, 1.5 g Na₂S₂O₃·5H₂O, 10 ml trace mineral solution 151 and 1 ml of trace vitamins 197 (http://www.dsmz.de/). The primary enrichment was diluted to extinction and incubated at 30 °C.

An aliquot from a 10⁻⁶ dilution of the primary enrichment was inoculated into fresh medium and pure cultures were obtained by performing three consecutive series of dilutions to extinction, confirmed with microscopic observations and 16S rRNA gene sequencing. During the isolation procedures, the cultures were incubated at 30 °C. The pure culture obtained using this procedure was designated strain TC8ᵀ. Long-term stocks of isolates were prepared by adding 50 µl DSMO (Fisher Scientific) to 1 ml of culture and were immediately stored at –80 °C.

Transmission electron micrographs of cells of strain TC8ᵀ (Fig. 1a) were obtained as thin sections showing cell morphology and negatively stained planktonic cells showing the presence of single or multiple polar flagella (Fig. 1b). Cells were Gram-stain-negative. The presence of endospores was not observed.

Growth rates (µ; h⁻¹) were estimated as µ=(ln N₂ – ln N₁)/ (T₂ – T₁), where N₂ and N₁ represent the cell densities (cells ml⁻¹) using direct microscopic counts at times (hours of incubation) T₂ and T₁, respectively. Generation times (tₚ; measured in h) were calculated as tₚ=ln(2)/µ. All growth experiments were performed in duplicate modified 1011 medium supplemented with 20 mM potassium nitrate under N₂/CO₂ (80 : 20; 200 kPa) unless stated otherwise.

Strain TC8ᵀ was incubated at temperatures between 20 and 40 °C, at 5 °C intervals. Growth was observed between 20 and 35 °C, with optimal growth at 30 °C. No growth was detected at 40 °C. Subsequent experiments were performed at 30 °C. Optimum salinity was established by varying the concentration of NaCl between 0 and 50 g l⁻¹, at 5 g l⁻¹ intervals. Growth was observed at NaCl concentrations between 5 and 45 g l⁻¹ with optimal growth at 15–20 g l⁻¹. No growth was detected at 50 g l⁻¹. The pH optimum for strain TC8ᵀ was determined as previously described (Vetriani et al., 2004). Growth occurred between pH 4.5 and pH 8.5, with an optimum at pH between 6 and 7. The generation time of strain TC8ᵀ under the determined optimal conditions (pH 6.5, 15 g NaCl l⁻¹, 30 °C) was 8 h. Further experiments were carried out at pH 6.5, 15 g NaCl l⁻¹ and 30 °C.

Antibiotic sensitivity was tested in liquid cultures containing streptomycin, ampicillin, chloramphenicol or kanamycin (all 100 µg ml⁻¹). All antibiotics were added aseptically before incubation at 30 °C. An ethanol control was performed in parallel to the chloramphenicol resistance tests. All four antibiotics inhibited growth of strain TC8ᵀ.

The presence of catalase activity was determined by resuspending concentrated cells collected during exponential growth in 70 µl of a 3 % solution of H₂O₂ at room temperature. Strain TC8ᵀ did not form gas bubbles on addition of hydrogen peroxide, indicating absence of catalase activity.

The effect of organic substrates (all supplemented at 2 g l⁻¹ unless otherwise specified) on the growth of strain TC8ᵀ showed that this bacterium could use tryptone, peptone, Casamino acids, pyruvate and glycerol as carbon and energy sources (chemo-organoheterotrophic growth), while it used
d(+)-glucose, sucrose, yeast extract (final concentrations: 0.1 and 1 g l−1), acetate, lactate, citrate and l-glutamine as carbon sources when thiosulfate was used as an electron donor (chemolithoautotrophic growth; Table S1, available in the online Supplementary Material). Formate and l-cysteine inhibited growth of strain TC8T (Table S1).

Strain TC8T conserved energy by coupling the oxidation of thiosulfate with the reduction of nitrate. Formation of gas as the end product of nitrate respiration was detected by the formation of bubbles in an inverted Durham tube placed in the culture medium, implying that strain TC8T is a denitrifier. In addition to thiosulfate, strain TC8T used elemental sulfur (3 %, w/v) as an alternative electron donor. The ability of strain TC8T to use alternative electron acceptors in addition to nitrate was investigated by adding sulfate (7 mM), thiosulfate (4 mM), sulfite (4.1 mM), arsenate (5 mM), selenate (5 mM), ammonium ferric citrate (500 mM) and oxygen (5 %, v/v) to nitrate-depleted media. Growth occurred in the presence of oxygen and ammonium ferric citrate. Slow growth occurred when arsenate and selenium were the sole electron acceptors.

Strain TC8T was able to grow in the absence of fixed nitrogen (nitrate and ammonia) under a headspace of N2/C02/O2 gas phase (53 : 45 : 2; 200 kPa), which implies that it is able to fix gaseous nitrogen.

Genomic DNA was extracted from cells of strain TC8T, and the 16S rRNA gene was amplified from the genomic DNA and sequenced as described previously (Vetriani et al., 2004). The jmodeltest software was used to statistically determine the best-fit model for nucleotide substitution to determine phylogeny (Darriba et al., 2012; Guindon & Gascuel, 2003). 16S rRNA gene-based maximum-likelihood phylogenetic trees, reconstructed using PhyML (Guindon et al., 2010) with the general time reversible (GTR) model and 1000 bootstrap re-samplings, indicated that strain TC8T belonged to the family Rhodospirillaceae within the Alphaproteobacteria (Fig. 2). However, strain TC8T formed a lineage distinct from the two genera Magnetovibrio and Magnetospira. When compared to its closest relatives, the pairwise nucleotide similarity of the 16S rRNA gene sequence of strain TC8T, calculated using the EzTaxon server (http://www.ezbiocloud.net/eztaxon), was 91.25 % to Magnetovibrio blakemorei MV1T (Bazylinski et al., 2013) and 90.13 % to Magnetospira thiophila MMS-1T (Williams et al., 2012). These values are within the range (90–96 %) indicative of genus-level differentiation (Gillis et al., 2001).

The closest 16S rRNA gene sequence to strain TC8T in GenBank was that of clone 7M24.051 (99 % similarity), which was recovered from the exterior of an inactive sulfide chimney collected near a vent field at 9 N on the East Pacific Rise (Fig. 2; Sylvan et al., 2012).

The G+C content of the genomic DNA of strain TC8T was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) by HPLC analysis of deoxyribonucleosides as described by Mesbah et al. (1989). The genomic DNA of strain TC8T had a G+C content of 59.9 mol%, which is considerably higher than that of its closest cultured relatives, Magnetovibrio blakemorei MV1T (52.9 mol%) and Magnetospira thiophila MMS-1T (47.2 mol%; Table 1).

**Fig. 2.** Maximum-likelihood phylogenetic tree derived from 16S rRNA gene sequences showing the position of strain TC8T within the Alphaproteobacteria. Bootstrap values higher than 50 % were based on 1000 replicates and are shown at each node. Bar, 0.05 % substitutions per position. Sequences belonging to the family Rickettsiaceae were used as the outgroup.
Chemotaxonomic analyses of strain TC8\textsuperscript{T}, including cellular fatty acid composition, polar lipids and respiratory quinones, were carried out by the Identification Service of the DSMZ using 200 mg of freeze-dried cells grown to early stationary phase under optimal culture conditions. The cellular fatty acid composition of strain TC8\textsuperscript{T} was analysed as the methyl ester derivatives using the Sherlock Microbial Identification System (MIDI, Microbial ID) and an Agilent model 6890N gas chromatograph (Labrenz et al., 1998). The fatty acid composition of strain TC8\textsuperscript{T}, analysed using version 6.1 of the MIDI Sherlock MIS software, consisted primarily of C\textsubscript{18}:1\textit{ω}7c/C\textsubscript{18}:1\textit{ω}6c (57.47 %), C\textsubscript{16}:0 (21.9 %) and C\textsubscript{16}:1\textit{ω}7c/C\textsubscript{16}:1\textit{ω}6c (14.39 %) (Table S2). Small amounts of C\textsubscript{19}:0 cyclo ω8c (1.4 %), C\textsubscript{18}:0 3-OH (1.13 %) and C\textsubscript{18}:0 (1.08 %) were also present. Compared to Magnetovibrio blakemorei and Magnetospira thiophila (Bazylinski et al., 2013; Williams et al., 2012), strain TC8\textsuperscript{T} had a unique fatty acid profile with significantly divergent ratios of shared fatty acids.

Polar lipids were identified by staining with molybdophosphoric acid to visualize lipids (Tindall, 1990a, b). The polar lipids were classified as phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmonomethylethanolamine, aminophospholipid and an unidentified phospholipid (Fig. S1). Lipoquinones of strain TC8\textsuperscript{T} were identified using TLC followed by HPLC of the eluted products (Tindall, 1990a, b). The lipoquinones of strain TC8\textsuperscript{T} consisted of ubiquinone-9 (Q-9) and ubiquinone-8 (Q-8) at 71 and 29 %, respectively.

Formation of magnetosomes in strain TC8\textsuperscript{T} under optimal anaerobic conditions was not observed in thin sections of cells. This could be for the following reasons: (a) ferric quinate, a major iron source for cultured magnetotactic bacteria, was not present in the medium; (b) iron provided to strain TC8\textsuperscript{T} through the trace element solution in the medium was at an order of magnitude lower concentration than that provided to both its closest relatives Magnetovibrio blakemorei and Magnetospira thiophila; and (c) formation was not tested under microaerobic conditions.

Strain TC8\textsuperscript{T} exhibited several divergent characteristics from its closest relatives, Magnetovibrio blakemorei and Magnetospira thiophila. The 16S rRNA gene sequence phylogeny

<table>
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<th>Characteristic</th>
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<th>2</th>
<th>3</th>
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<td>Morphology</td>
<td>Curved rod</td>
<td>Curved rod–helicoid</td>
<td>Curved rod</td>
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<td>Temperature range for growth (°C)</td>
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<td>4–31</td>
<td>5–37</td>
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<tr>
<td>Optimum temperature for growth (°C)</td>
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<td>26–28</td>
<td>24–26</td>
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<td>Optimum pH for growth</td>
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<td>NaCl concentration range for growth (g l\textsuperscript{-1})</td>
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<td>8–40</td>
<td>8–40</td>
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<tr>
<td>Optimum NaCl concentration for growth (g l\textsuperscript{-1})</td>
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<td>16.5</td>
<td>16.4</td>
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<tr>
<td>Doubling time under optimal conditions (h)</td>
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<td>11</td>
<td>96</td>
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<td>G+C content of genomic DNA (mol%)</td>
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<td>52.9</td>
<td>47.2</td>
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<td>Catalase activity</td>
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<td>−</td>
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<td>Sulfide-rich sediments, salt marsh of Neponset river, USA</td>
<td>Mud from marsh, Woods Hole, MA, USA</td>
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<td>Terminal electron acceptors</td>
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<td>Monotrichous</td>
<td>Amphitrichous</td>
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Table 1. Differential characteristics between strain TC8\textsuperscript{T} and related members of the family Rhodospirillaceae

Strains: 1, TC8\textsuperscript{T} (data from this study); 2, Magnetovibrio blakemorei MV-1\textsuperscript{T} (Bazylinski et al., 2013); 3, Magnetospira thiophila MMS-1\textsuperscript{T} (Williams et al., 2012).
placed strain TC8<sup>T</sup> in a separate lineage from both *Magneto-
vibrio blakemorei* and *Magnetospira thiophila* clades. In addi-
tion to genetic distinctions, strain TC8<sup>T</sup> also had the most
diverse metabolism, highest DNA G+C content, broadest pH
range for growth and displayed a unique fatty acid profile.

The phylogenetic position of strain TC8<sup>T</sup>, as well as its
physiological characteristics and unique chemotaxonomic
profile, are different from those of other members of the
family *Rhodospirillaceae* and are indicative of genus-level
differentiation. Hence, we propose the name *Varunaivibrio*
gen. nov. The type species is *Varunaivibrio sulfuroxidans*
sp. nov., with TC8<sup>T</sup> (=DSM 101688<sup>T</sup>=JCM 31027<sup>T</sup>) as the
type strain.

The shallow-water gas vents of Tor Caldara are characterized
by reducing conditions in the proximity of the gas emissions
as well as oxidizing conditions in the surrounding water
column. Since these gas vents are exposed to wave motion
and mixing, micro-organisms inhabiting them experience both
reducing and oxidizing conditions on a temporal as well as
a spatial scale. Furthermore, besides the availability of CO<sub>2</sub>
and C<sub>1</sub>-C<sub>3</sub> donors, electron acceptors and carbon sources, might be an
adaptation to the characteristic dynamic environment found
at these shallow-water gas vents.

**Description of Varunaivibrio gen. nov.**

*Varunaivibrio* [Var.u.nai.vibrio. Varuna the God of water and
the celestial ocean surrounding the world in Hindu
mythology; L. v. *vibrate* to vibrate; N.L. masc. n. *vibrio* that
vibrates and also a genus name of bacteria possessing a
curved rod shape (*Vibrio*); N.L. masc. n. *Varunaivibrio* a
curved rod that inhabits the ocean].

Cells are motile, vibrio-shaped (1–1.5 μm long, 0.6 μm
wide) with polar flagella. Spores are absent. Gram-stain-
negative cell-wall structure. Mesophilic, facultatively anaero-
bic, microaerophilic chemautotrophic and chemohetero-
trophic metabolism. Growth by oxidation of sulfur, coupled
with the reduction of nitrate. Catalase-negative. The principal
cellular fatty acid components are C<sub>16:0</sub>, C<sub>18:1ω7c</sub>, C<sub>18:1ω6c</sub>,
C<sub>16:1</sub>ω7c, C<sub>16:1</sub>ω6c, and C<sub>18:0</sub>. The polar lipids are composed
of phosphatidylethanolamine, phosphatidylglycerol and
aminolipid. Habitat is shallow-water gas vents. The type spe-
cies is *Varunaivibrio sulfuroxidans*.

**Description of Varunaivibrio sulfuroxidans**

*Varunaivibrio sulfuroxidans* (sulfur.oxi.dans. L. n. sulfur
sulfur; N.L. part. adj. oxidans oxidizing; N.L. part. adj. sul-
furoxidans pertaining to the ability to oxidize sulfur).

General morphological and chemotaxonomic characteris-
tics are as given above for the genus. Growth occurs
between 20 and 35 °C, with between 5 and 45 g NaCl l<sup>−1</sup>
and at between pH 4.5 and 8.5. Under optimal growth condi-
tions (30 °C, 15–20 g NaCl l<sup>−1</sup> and pH 6.0–7.0) the gener-
tation time is 8 h. Chemolithoautotrophic growth occurs
with sulfur and thiosulfate as the electron donors, CO<sub>2</sub> as
the carbon source, and nitrate, oxygen (5 %, v/v) and ferric
iron as the electron acceptors. Also capable of chemo
organoheterotrophic and chemolithoheterotrophic growth.
Sensitive to streptomycin, ampicillin, chloramphenicol and
kanamycin (100 mg ml<sup>−1</sup>).

The type strain, TC8<sup>T</sup> (=DSM 101688<sup>T</sup>=JCM 31027<sup>T</sup>), was
isolated from surface sediment of an active shallow-water
gas vent at Tor Caldara in the Tyrrenian Sea, Italy. The
genomic DNA G+C content of the type strain is 59.9 mol%.

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research.

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