Inmirania thermothiophila gen. nov., sp. nov., a thermophilic, facultatively autotrophic, sulfur-oxidizing gammaproteobacterium isolated from a shallow-sea hydrothermal vent

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A novel thermophilic, facultatively autotrophic bacterium, strain S2479T, was isolated from a thermal spring located in a tidal zone of a geothermally heated beach (Kuril Islands, Russia). Cells of strain S2479T were rod-shaped and motile with a Gram-negative cell-wall type. The temperature range for growth was 35–68 °C (optimum 65 °C), and the pH range for growth was pH 5.5–8.8 (optimum pH 6.5). Growth of strain S2479T was observed in the presence of NaCl concentrations ranging from 0.5 to 3.5 % (w/v) (optimum 1.5–2.0 %). The strain oxidized sulfur and thiosulfate as sole energy sources for autotrophic growth under anaerobic conditions with nitrate as electron acceptor. Strain S2479T was also capable of heterotrophic growth by reduction of nitrate with oxidation of low-chain fatty acids and a limited number of other carboxylic acids or with complex proteinaceous compounds. Nitrate was reduced to N2. Sulfur compounds were oxidized to sulfate. Strain S2479T did not grow aerobically during incubation at atmospheric concentration of oxygen but was able to grow microaerobically (1 % of oxygen in gas phase). Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain was a member of the family Ectothiorhodospiraceae, order Chromatiales, class Gammaproteobacteria. On the basis of phylogenetic and phenotypic properties, strain S2479T represents a novel species of a new genus, for which the name Inmirania thermothiophila gen. nov., sp. nov. is proposed. The type strain of the type species is S2479T (=DSM 100275T=VKM B-2962T).

Microbial oxidation of reduced inorganic sulfur compounds to sulfate is one of the major reactions of the global sulfur cycle. Sulfur-oxidizing prokaryotes are phenotypically and taxonomically diverse. They include bacteria with different types of metabolisms, e.g. photolithotrophic, photoheterotrophic, chemolithotrophic, chemoheterotrophic and methylotrophic as well as chemolithotrophic archaea (Friedrich et al., 2001, Mori et al., 2015). Sulfur-oxidizing micro-organisms inhabit diverse environments including soils, meromictic lakes and brackish lagoons, bacterial mats, intertidal mud flats, activated sludges and wastewater biofilms. These micro-organisms have also been isolated from extreme ecosystems such as acidic mine drainage, hypersaline and/or hyperalkaline lakes, volcanic hot springs, solfataras, marine geothermal fields and deep-sea hydrothermal vents (for references see Ghosh & Dam, 2009). Most sulfur-oxidizing prokaryotes are mesophilic; thermophilic and hyperthermophilic species belong to the bacteria of the order Aquificales (Reysenbach, 2001) and the crenarchaeota of the order Sulfolobales (Stetter, 1989). Additionally two moderately thermophilic species are known among the bacteria of the order

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S2479T is KT159732.
Chromatiales (Imhoff, 2005). In this paper, we describe a facultatively anaerobic and microaerophilic, thermophilic bacterium capable of chemolithoautotrophic oxidation of sulfur compounds.

Strain S2479\textsuperscript{T} was isolated from a sample of sand and water from a thermal spring located in a tidal zone of a hydrothermally heated beach (43° 59.742' N 145° 48.166' E) on Kunashir Island (Kurils, Russia). Samples were taken anaerobically in tightly stoppered bottles and transported to the laboratory. An enrichment culture was initiated by inoculation of the sample (10%, v/v) into anaerobically prepared, bicarbonate-buffered liquid medium of the following composition (per litre distilled water): 0.33 g NH\textsubscript{4}Cl, 0.33 g KCl, 0.33 g CaCl\textsubscript{2}, 6H\textsubscript{2}O, 0.33 g KH\textsubscript{2}PO\textsubscript{4}, 18.0 g NaCl, 4.33 g MgCl\textsubscript{2}, 6H\textsubscript{2}O, 2.0 g NaHCO\textsubscript{3}, 1 ml trace element solution (Slobodkin \textit{et al.}, 2012) and 1 ml vitamin solution (Wolin \textit{et al.}, 1963). No reducing agents were added. The isolation medium had a pH of 6.5–6.8 (measured at 25°C). The medium was dispensed in 10 ml portions into 17 ml Hungate tubes and sterilized at 121°C for 60 min; the head space was filled with CO\textsubscript{2} (100%). Elemental sulfur (5 g l\textsuperscript{-1}) and potassium nitrate (10 mM) were added as electron donor and acceptor, respectively. After incubation in the dark for 10–14 days at 65°C, growth of rod-shaped cells was observed. After three subsequent transfers and following serial 10-fold dilutions in the same medium, only one morphological type was observed in the highest growth-positive dilution (10\textsuperscript{-9}). A pure culture of strain S2479\textsuperscript{T} was obtained by means of multiple serial dilutions in the same medium and purity was confirmed by routine microscopic examination.

Bacterial growth was determined by direct counting with a phase-contrast microscope (CX-41; Olympus). Transmission electron microscopy was performed with a model JEM-100 electron microscope (JEOL) as described previously (Bonch-Osmolovskaya \textit{et al.}, 1990). Gram-staining, and oxidase and catalase activity tests were performed using standard methods (Gerhardt \textit{et al.}, 1994). Growth experiments were performed in duplicate, using Hungate tubes. Determination of temperature and pH ranges for growth and potential electron donors and acceptors was carried out in the same medium as used for isolation. For the pH experiments, the medium was adjusted to different pH values (3.0–10.0) with sterile anaerobic solutions of HCl (2 M) or NaOH (5%, w/v). The NaCl requirement for growth was determined in the medium of the same mineral composition lacking NaCl and containing 0.33 g l\textsuperscript{-1} MgCl\textsubscript{2}, 6H\textsubscript{2}O. Varying amounts of NaCl (0–7.0%, w/v) were added directly into the Hungate tubes. Electron donors and acceptors were added from sterile anaerobic stock solutions before inoculation. Elemental sulfur was added directly into each test tube with liquid medium prior to sterilization. Medium with poorly crystaline Fe(III) oxide (ferrihydrite) was prepared as described previously (Slobodkin \textit{et al.}, 1999). The ability of the strain to grow aerobically was determined in 60 ml flasks sealed with a rubber stopper and aluminum screw cap containing 10 ml aerobically prepared medium (100% air in the gas phase). To check for microaerobic growth, various amounts of air were injected in the headspace of flasks containing anaerobically prepared medium (with no dissolved oxygen in the liquid medium) and lacking electron acceptor.

Cells for chemotaxonomic analyses were grown in the presence of 0.1 g l\textsuperscript{-1} yeast extract with thiosulfate and nitrate as electron donor and acceptor, respectively. Cellular fatty acids composition was analysed by GC-MS according to Zhilina \textit{et al.} (2012). The genomic DNA G+C content was determined from the melting point according to Marmur & Doty (1962) with DNA of Escherichia coli K-12 as a reference. Nitrate and sulfate concentrations were monitored with a Stayer ion chromatograph (Aquanil) equipped with an IonPack AS4-ASC column ( Dionex) and conductivity detector. Determination of gaseous products of metabolism was performed by GC equipped with a HayeSep N 80/100 mesh column at 40°C and flow rates of 20 ml min\textsuperscript{-1} (argon as carrier gas). Nitrite was determined using Nitrate-Test strips (MQquantTM, Merck KGaA); ammonium was determined by phenol-hypochlorite reaction (Chaney & Marbach, 1962). Sulfide was measured colorimetrically with dimethyl-p-phenylenediamine (Trüper & Schlegel, 1964).

Genomic DNA isolation and 16S rRNA gene amplification and sequencing were performed as described previously (Slobodkina \textit{et al.}, 2013). Pairwise similarity values were calculated by means of the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim \textit{et al.}, 2012). Alignment with a representative set of related 16S rRNA gene sequences from the GenBank database (Benson \textit{et al.} 1999) was carried out with the \textit{clustal w} program implemented in the phylogenetic analysis package \textit{MEGA} version 6 (Tamura \textit{et al.}, 2013). Evolutionary analysis and phylogenetic tree reconstruction employed the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993) provided by \textit{MEGA} version 6.

Cells of strain S2479\textsuperscript{T} were straight or slightly bent rods with rounded ends, 0.6–0.7 μm in diameter and 1.0–2.0 μm in length. Cells were motile due to a single polar flagellum (Fig. 1a). Endospore formation was not observed. The cells stained Gram-negative in both exponential and stationary growth phases. Ultrathin sections of strain S2479\textsuperscript{T} revealed a Gram-negative cell-wall type with an outer membrane (Fig. 1b). No intracellular membranes were observed. The temperature range for growth of strain S2479\textsuperscript{T} was 35–68°C (optimum 65°C); no growth was detected at and below 30°C, or at or above 70°C after incubation for 3 weeks. The pH range for growth was 5.5–8.8 (optimum pH 6.5); no growth was detected at pH 5.0 and below, or at pH 9.0 and above. Strain S2479\textsuperscript{T} was obligately Na\textsuperscript{+}-dependent with optimum growth in the presence of 1.5–2.0% (w/v) NaCl. The minimal sodium ion concentration for growth was 0.5% (w/v) and growth was possible in the presence of up to 3.5% (w/v) NaCl. The isolate grew chemolithoautotrophically.
under anaerobic conditions coupling oxidation of elemental sulfur (5 g l\(^{-1}\)) or thiosulfate (15 mM) with nitrate (10 mM) reduction. The end-product of sulfur compounds oxidation was sulfate; nitrate was reduced to \(\text{N}_2\), and nitrite or ammonia did not accumulate. Addition of at least 0.1 g l\(^{-1}\) yeast extract stimulated growth. The doubling time for strain S2479\(^T\) growing on thiosulfate and nitrate in the presence and in the absence of yeast extract was 2.0 h and 3.6 h, respectively. Growth with \(\text{H}_2\) [\(\text{H}_2/\text{CO}_2\) (80 : 20; \(\text{v}/\text{v}\))] as electron donor and nitrate as electron acceptor was observed only when 0.1 g l\(^{-1}\) yeast extract was added. The isolate also grew chemooorganoheterotrophically with nitrate as electron acceptor in the presence of 0.1 g l\(^{-1}\) yeast extract with the following substrates: formate, acetate, propionate, butyrate, pyruvate, lactate, malate, succinate (10 mM each), yeast extract and peptone (2 g l\(^{-1}\) each). No growth was observed with sulfite (5 mM), sulfide (0.2, 0.5 or 2.0 mM), methanol, ethanol, glycerol, fumarate (10 mM each), glucose, fructose, arabinose, maltose and sucrose (2 g l\(^{-1}\) each) in the presence of 0.1 g l\(^{-1}\) yeast extract and with nitrate as electron acceptor. Strain S2479\(^T\) was not able to grow without external electron acceptors by fermentation of pyruvate, lactate, malate, succinate (10 mM each), fructose or yeast extract (2 g l\(^{-1}\) each). Potential electron acceptors were tested with acetate (10 mM) as electron donor in the presence of 0.1 g l\(^{-1}\) yeast extract. Nitrate supported growth of the isolate whereas sulfate (14 mM), sulfite (5 mM), elemental sulfur (5 g l\(^{-1}\)), thiosulfate (15 mM), nitrite (2.5 mM) and ferricydrite [90 mmol Fe(III) l\(^{-1}\)] were not utilized as electron acceptors. Strain S2479\(^T\) was able to grow microaerobically using molecular oxygen as an electron acceptor (1% \(\text{v}/\text{v}\) \(\text{O}_2\) in the gas phase) with elemental sulfur, thiosulfate, acetate or succinate, but no growth was observed during incubation with atmospheric concentration of oxygen. The isolate did not grow phototrophically under anoxic conditions with elemental sulfur (5 g l\(^{-1}\)), thiosulfate (15 mM), hydrogen [\(\text{H}_2/\text{CO}_2\) (80 : 20; \(\text{v}/\text{v}\)], acetate or pyruvate (10 mM each) in the presence of 0.1 g l\(^{-1}\) yeast extract. Oxidase activity was positive and catalase activity was negative. Cellular fatty acid analysis demonstrated a dominance of saturated C\(_{16:0}\) (48.5%) and unsaturated C\(_{18:1\omega 7\gamma}\) (30.1%) with less amounts of C\(_{18:0}\) (8.7%) and C\(_{19:0}\) (5.5%). The G+C content of the genomic DNA of strain S2479\(^T\) was 71.5 mol% (\(T_m\)).

A comparison of 1521 nt of 16S rRNA gene sequence of strain S2479\(^T\) with those available in the GenBank and EzTaxon server databases showed that the strain belonged to the family *Ectothiorhodospiraceae* of the order *Chromatiales*, class *Gammaproteobacteria* (Fig. 2). This family is metabolically diverse and contains phototrophic purple sulfur bacteria that perform anoxygenic photosynthesis, strictly chemolithotrophic, chemoheterotrophic and facultatively methylotrophic bacteria (Oren, 2013). Strain S2479\(^T\) shared the highest pairwise 16S rRNA gene similarity with the type strain of phototrophic *Ectothiorhodospira salini* (92.34%) (Ramana et al., 2010) and strictly chemotrophic *Thioalkalivibrio denitrificans* (92.26%) (Sorokin et al., 2001). The representatives of the genera *Ectothiorhodospira* and *Thioalkalivibrio*, in common with strain S2479\(^T\), are rod-shaped, motile, non-spore-forming, Gram-negative bacteria, capable of lithoautotrophic anoxic oxidation of sulfur compounds. Other shared features include Na\(^+\) requirement for growth and respiratory type of metabolism with nitrate or oxygen as electron acceptors. Substrates consumed chemoheterotrophically by the novel isolate are much the same as the members of the genus *Ectothiorhodospira* utilize for photoheterotrophic growth. Composition of the fatty acids of strain S2479\(^T\) is similar to cellular fatty acid profiles of other representatives of the family *Ectothiorhodospiraceae* (Sorokin et al., 2012; Thiemann & Imhoff, 1996; Oren, 2013). The inability of phototrophic growth and the lack of colour of cell cultures caused by the absence of intracellular photosynthetic membranes clearly distinguish the novel isolate from members of the genus *Ectothiorhodospira* (Table 1). Species of the genus *Thioalkalivibrio* are obligately alkaliphilic not growing below pH 7.5, whereas strain S2479\(^T\) was found to be neutrophilic. Unlike members of the genus *Thioalkalivibrio*
Ectothiorhodospira magna B7-7T (HM149323)
Ectothiorhodospira shaposhnikovii DSM 243T (FR733667)
Ectothiorhodospira salini JA430T (FM244738)
Thioalkalivibrio sulfidiphilus HL-EbGR7T (CP001339)
Thioalkalivibrio denitrificans ALID1T (AF126545)
Thioalkalivibrio thiocyanodenitrificans ARhD 1T (KB900536)
Thiorhodospira sibirica ATCC 700588T (AGFD01000020)
Thioalkalivibrio nitratireducens DSM 14787T (CP003989)
Inmirania thermothiophila S2479T (KT159732)
Thioalkalivibrio denitrificans ALJDT (AF126545)
Thioalkalivibrio thiocyanodenitrificans ARhD 1T (KB900536)
Thiorhodospira sibirica ATCC 700588T (AGFD01000020)
Thioalkalivibrio nitratireducens DSM 14787T (CP003989)

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain S2479T among members of the order Chromatiales. The tree was reconstructed using the maximum-likelihood method. Trees reconstructed by neighbour-joining, minimum-evolution and maximum-parsimony algorithms displayed the same topology. Numbers at nodes are bootstrap values from 500 trials. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differentiating characteristics of strain S2479T and the closest related genera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Thermal spring</td>
<td>Estuaries, salt flats, salt lakes, soda lakes</td>
<td>Soda lakes</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>35–68</td>
<td>20–45</td>
<td>Up to 41</td>
</tr>
<tr>
<td>Optimum</td>
<td>65</td>
<td>25–40</td>
<td>30–35</td>
</tr>
<tr>
<td>Growth pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>5.5–8.8</td>
<td>7.0–11.0</td>
<td>7.5–10.65</td>
</tr>
<tr>
<td>Optimum</td>
<td>6.5</td>
<td>7.5 – (9.0–10.0)</td>
<td>(8.0–9.0) – (10.0–10.2)</td>
</tr>
<tr>
<td>NaCl for growth (%), w/v</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.5–3.5</td>
<td>0–20</td>
<td>1.2–10.5, some strains up to 29.2</td>
</tr>
<tr>
<td>Optimum</td>
<td>1.5–2.0</td>
<td>0.5–8.0</td>
<td>2.3–11.7</td>
</tr>
<tr>
<td>Phototrophic growth</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Heterotrophic growth</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sulfide oxidation</td>
<td>–</td>
<td>+*</td>
<td>+†</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>–</td>
<td>+/–†</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>71.5</td>
<td>59.2–68.4</td>
<td>61.3–66.9</td>
</tr>
</tbody>
</table>

*All strains except Ectothiorhodospira salini.
†Some species.
that are strict chemolithoautotrophs, strain S2479 is capable of organotrophic growth. All members of the family Ectothiorhodospiraceae are mesophiles, while strain S2479T grows optimally at 65 °C. Along with two species of the genus Methylothermus (Tsubota et al., 2005; Hirayama et al., 2011), strain 2479T has one of the highest growth temperatures reported for the representatives of the class Gammaproteobacteria.

On the basis of morphological, physiological and chemotaxonomic properties, and the low degree of 16S rRNA gene sequence similarity with related taxa, strain S2479T is proposed to represent a novel species of a new genus, Inmirania thermothiophila gen. nov., sp. nov.

**Description of Inmirania gen. nov.**

*Inmirania* [In.mi.rā’ni.a N.L. fem. n. *Inmirania* arbitrary name formed from the acronym of the Institute of Microbiology of Russian Academy of Sciences (Russian transliteration: Institut Mikrobiologii Rossijskoj Akademii Nauk; INMI RAN), which has made significant contributions to microbiology, particularly in research of sulfur and nitrogen cycles].


The type species is *Inmirania thermothiophila*.

**Description of Inmirania thermothiophila sp. nov.**


Has the following properties in addition to those given in the genus description. Cells (0.6–0.7 μm × 1.0–2.0 μm) are motile due to a single pole flagellum. Growth occurs at 35–68 °C (optimum 65 °C), pH 5.5–8.8 (optimum pH 6.5) and NaCl concentrations of 0.5–3.5% (w/v) (optimum 1.5–2.0%). Grows chemolithoautotrophically using sulfur or thiosulfate as an electron donor, nitrate as an electron acceptor and HCO₃⁻/CO₂ as a carbon source. Yeast extract stimulates growth. Sulfur and thiosulfate are oxidized to sulfate, nitrate is reduced to nitrogen. Utilizes hydrogen, formate, acetate, propionate, butyrate, pyruvate, lactate, malate, succinate, yeast extract and peptone with nitrate as electron acceptor. Capable of microaerobic growth. Does not use sulfate, sulfite, elemental sulfur, thiosulfate, nitrite and ferricydrite as electron acceptors. Phototrophic growth is not observed.

The type strain, S2479T (=DSM 100275T=VKM B-2962T), was isolated from a thermal spring (Kuril Islands, Russia). The DNA G+C content of the type strain is 71.5 mol% (Tm).

**Acknowledgements**

This work was supported by the Russian Science Foundation (auto-trophy) (grant 14-24-00165) and the Russian Foundation for Basic Research (sulfur metabolism) (grant 15-04-00405). Cellular fatty acid determination was supported by President of Russia (grant MK-4530.2015.4). We thank N.A. Kostrikina (INMI RAS) for the help with the electron microscopy of the novel organism.

**References**


