Thermodesulfatator indicus gen. nov., sp. nov., a novel thermophilic chemolithoautotrophic sulfate-reducing bacterium isolated from the Central Indian Ridge

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A thermophilic, marine, anaerobic, chemolithoautotrophic, sulfate-reducing bacterium, strain CIR29812T, was isolated from a deep-sea hydrothermal vent site at the Kairei vent field on the Central Indian Ridge. Cells were Gram-negative motile rods that did not form spores. The temperature range for growth was 55–80°C, with an optimum at 70°C. The NaCl concentration range for growth was 10–35 g l⁻¹, with an optimum at 25 g l⁻¹. The pH range for growth was 6–6.7, with an optimum at approximately pH 6.25. H₂ and CO₂ were the only electron donor and carbon source found to support growth of the strain. However, several organic compounds were stimulatory for growth. Sulfate was used as electron acceptor, whereas elemental sulfur, thiosulfate, sulfite, cystine, nitrate and fumarate were not. No fermentative growth was observed with malate, pyruvate or lactate. The phenotypic characteristics of strain CIR29812T were similar to those of Thermodesulfobacterium hydrogeniphilum, a recently described thermophilic, chemolithoautotrophic sulfate-reducer. However, phylogenetic analyses of the 16S rRNA gene sequences showed that the new isolate was distantly related to members of the family Thermodesulfobacteriaceae (similarity values of less than 90%). The chemotaxonomic data (fatty acids and polar lipids composition) also indicated that strain CIR29812T could be distinguished from Thermodesulfobacterium commune, the type species of the type genus of the family Thermodesulfobacteriaceae. Finally, the G+C content of the genomic DNA of strain CIR29812T (46·0 mol%) was not in the range of values obtained for members of this family. On the basis of phenotypic, chemotaxonomic and genomic features, it is proposed that strain CIR29812T represents a novel species of a new genus, Thermodesulfatator, of which Thermodesulfatator indicus is the type species. The type strain is CIR29812T (≡ DSM 15286T = JCM 11887T).

In the latest edition of Bergey’s Manual of Systematic Bacteriology, the class Thermodesulfobacteria (Garrity & Holt, 2001) contained two species: Thermodesulfobacterium commune and Thermodesulfobacterium mobile (recently renamed Thermodesulfobacterium thermophilum) (Judicial Commission of the International Committee on Systematics of Prokaryotes, 2003). In the past few years, two novel species of the genus Thermodesulfobacterium, Thermodesulfobacterium hveingerdense (Sonne-Hansen & Ahring, 1999) and Thermodesulfobacterium hydrogeniphilum (Jeanthon et al., 2002), have been described and classified in this genus. All Thermodesulfobacterium species are anaerobic, thermophilic, non-spore-forming, deeply branching, sulfate-reducing bacteria. With the exception of the marine chemolithoautotrophic organism T. hydrogeniphilum, all Thermodesulfobacterium spp. are chemo-organotrophs that thrive in terrestrial and subterrestrial environments (Zeikus et al., 1983; Rozanova & Khudyakova, 1974;


We describe here the isolation and characterization of another novel thermophilic, strictly chemolithoautotrophic, sulfate-reducing bacterium. The new isolate was obtained from a sample of an active black smoker collected at a depth of 2420 m at the Kairei vent field (25°19′ S, 70°02′ E) on the Central Indian Ridge (Van Dover et al., 2001) in April 2001. The chimney fragment was collected by the ROV Jason and was placed in an isolated container for the trip to the surface. Subsamples of the chimney fragment were ground in a mortar and the slurry was stored under an atmosphere of nitrogen at 4 °C until used as an inoculum.

Initial enrichments were done using the following medium that contained (1−1 distilled water): 29 g NaCl; 7 g MgSO_{4}.7H_{2}O; 4 g NaOH; 0.5 g KCl; 2 g Na_{2}SO_{3}.5H_{2}O; 1.66 g MgCl_{2}.6H_{2}O; 0.4 g CaCl_{2}.2H_{2}O; 0.2 g NH_{4}Cl; 0.3 g K_{2}HPO_{4}.3H_{2}O; and 10 ml of a trace element stock solution according to Boone et al. (1989) (http://methanogens.pdx.edu/OCM_media.html). The medium was prepared with anoxic water and, prior to autoclaving, the pH was adjusted to pH 6 at room temperature with sulfuric acid. The medium was dispensed under a CO_{2} atmosphere into Bellco tubes and capped with butyl-rubber stoppers. After inoculation with the sulfide slurry [10 % (v/v) inoculum], the tubes were pressurized with H_{2} (100 %; 138 kPa) and incubated at 70 °C without shaking.

After 4 days, cultures of small motile rods producing sulfide were observed. Enrichments that produced sulfide were subsequently transferred to a sulfate-reducing bacteria (SRB) medium that consisted of (1−1 distilled water): 20 g NaCl; 4 g Na_{2}SO_{4}; 3 g MgCl_{2}.6H_{2}O; 0.2 g KH_{2}PO_{4}; 0.5 g KCl; 0.25 g NH_{4}Cl; 3.46 g PIPES; 0.15 g CaCl_{2}.2H_{2}O; 1 mg resazurin; 2 mg sodium tungstate; 0.5 mg sodium selenate; 1 ml vitamin mixture (Widdel & Bak, 1992); 1 ml thiamin solution (Widdel & Bak, 1992); and 0.05 mg vitamin B_{12}. The pH of the medium was adjusted to pH 6.7 at room temperature using 5 M HCl. After autoclaving under N_{2} (100 %), the pH had decreased to 6.5. Medium (10 ml) was dispensed anaerobically in 50 ml vials sealed with butyl-rubber stoppers and reduced with 0.1 ml of a 10 % (w/v) Na_{2}S.9H_{2}O sterile solution; H_{2}/CO_{2} (80:20; 200 kPa) was used as the gas phase. Cultures were incubated at 70 °C with shaking (150 r.p.m.). The pH of the medium in uninoculated vials checked at room temperature after incubation at 70 °C decreased from 6.5 to 6.3.

One pure culture, strain CIR29812^{T}, was obtained by using shake dilution tubes (Widdel & Bak, 1992) of SRB solidified medium, where agar was replaced by 0.7 % (w/v) Phytagar (Sigma). After 6 days incubation at 70 °C, smooth, brown, spindle-shaped colonies of approximately 1 mm in diameter were transferred into SRB medium and checked for purity microscopically. Furthermore, the purity of the isolate was checked at 55 and 70 °C. SRB medium supplemented with 2 g Difco yeast extract l−1, 2 g tryptone l−1 and 10 mM glucose with air in the headspace was used to check for aerobic contaminants. The latter medium prepared anaerobically with N_{2} (100 %; 200 kPa) or H_{2} (100 %; 100 kPa) as the gas phase was used to detect anaerobic contaminants. The presence of possible autotrophic contaminants was checked in SRB medium where sulfate was omitted but where 2 g Difco yeast extract l−1 and 2 mM acetate were added. Stock cultures of strain CIR29812^{T} were stored in SRB medium at 4 °C. However, frequent transfers (twice per month) with 10 % (v/v) of inoculum in freshly prepared culture medium were found optimal to ensure re-growth. Alternatively, the isolate was stored in liquid nitrogen in the same medium containing 5 % (w/v) DMSO.

Cells of strain CIR29812^{T} were small rods, approximately 0.8–1 µm in length and 0.4–0.5 µm in width, with a single polar flagellum (Fig. 1a, b). Cells occurred singly, in pairs or in chains of three cells, and elongated during the stationary phase of growth. Occasionally, visible creamy aggregates that corresponded to large clumps of cells could be observed in the liquid medium. No spores were produced.

Unless otherwise stated, growth experiments were performed in duplicate in SRB medium supplemented with

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**Fig. 1.** (a) Phase-contrast micrograph of strain CIR29812^{T}; bar, 5 µm. (b) Electron micrograph of negatively stained cell (method as described by Jeanthon et al., 2002); bar, 500 nm.
0.5 g tryptone 1\textsuperscript{–1} and 2 mM acetate. Growth was monitored by measuring the increase in optical density at 600 nm with a Spectronic 401 spectrophotometer (Biolab). The temperature range for growth was determined without agitation with 20 g NaCl 1\textsuperscript{–1} at pH 6.5. The NaCl range was obtained at 70°C and pH 6.5 under agitation (150 r.p.m.). To determine the pH range for growth, SRB medium was buffered with 20 mM MES (pH adjusted to 6) or 20 mM PIPES (pH adjusted to 6.7 and 7.2). After autoclaving, these pH values decreased to pH 5.9 (with MES as buffer), pH 6.5 and pH 7 (with PIPES as buffer). The pH ranges from 5.9 to 6.75 (with MES) and 6 to 7 (with PIPES) were obtained by the addition of varying concentrations of NaHCO\textsubscript{3}. The pH of the media was checked at room temperature after overnight incubation of uninoculated tubes under H\textsubscript{2}/CO\textsubscript{2} at 70°C.

Under these conditions, strain CIR29812\textsuperscript{T} grew between 55 and 80°C, with an optimum at 70°C. No growth was observed at 50 or 82°C. Growth occurred between 10 and 35 g NaCl 1\textsuperscript{–1}, with a growth optimum at 25 g NaCl 1\textsuperscript{–1}. No growth was detected after 96 h in media containing 5 and 40 g NaCl 1\textsuperscript{–1}. Growth occurred between pH 6 and 6.7 in PIPES buffered medium, with an optimum at approximately pH 6.25. Growth occurred in MES buffered medium from pH 6 to 6.25. Under optimal growth conditions with shaking (150 r.p.m.), the doubling time of strain CIR29812\textsuperscript{T} was around 2 h (maximal OD\textsubscript{600} 0.11).

The new isolate was a strict anaerobe and was transferred at least six times under strict chemolithoautotrophic conditions, using H\textsubscript{2} as the electron donor and sulfate as the electron acceptor. Hydrogen sulfide was produced during growth. Elemental sulfur (1%), thiosulfate (10 mM), cysteine (1%), nitrate (5 mM), fumarate (10 mM) and sulfite (2 mM) were not used as electron acceptors. CO\textsubscript{2} was the sole carbon source used by strain CIR29812\textsuperscript{T}. In the presence of H\textsubscript{2}/CO\textsubscript{2} and sulfate, growth was stimulated by acetate (2 mM), methanol (0.5%), monomethylamine (0.2%), glutamate (5 mM), peptone (0.1%), fumarate (15 mM), tryptone (0.1%), isobutyrate (5 mM), 3-CH\textsubscript{3} butyrate (5 mM), ethanol (10 mM) and propanol (5 mM). In the presence of H\textsubscript{2}/CO\textsubscript{2} and sulfate, growth was not affected by isovalerate (5 mM), glucose (5 mM), fructose (5 mM) or succinate (10 mM), whereas acetic acid (15 mM), propionate (10 mM), butyrate (10 mM), 2-CH\textsubscript{3} butyrate (5 mM) and yeast extract (0.2%) were slightly inhibitory. Growth was completely inhibited by lactate (15 mM), caprylate (2.5 mM), caproate (5 mM), caprate (2.5 mM), formate (15 mM), malate (10 mM), valerate (5 mM), pyruvate (10 mM) and heptanoate (5 mM). In sulfate-free medium, no fermentative growth was observed with malate, pyruvate or lactate. The strain preferentially used ammonium (5 mM) as the nitrogen source but peptone (0.5%), nitrate (5 mM) and tryptone (0.1%) also supported growth.

Unlike the control culture of Desulfovibrio fructosovorans DSM 3604\textsuperscript{T} (Ollivier et al., 1988), strain CIR29812\textsuperscript{T} did not contain desulfoviridin (Postgate, 1959).

Sensitivity to antibiotics (at 25, 50, 100 and 200 μg ml\textsuperscript{–1}) was tested at 70°C. Strain CIR29812\textsuperscript{T} was resistant to penicillin and kanamycin (200 μg ml\textsuperscript{–1}) and streptomycin (100 μg ml\textsuperscript{–1}), but was inhibited by tetracycline (50 μg ml\textsuperscript{–1}), ampicillin, chloramphenicol and rifampicin (all at 25 μg ml\textsuperscript{–1}).

Respiratory lipoquinones and polar lipids were extracted from 100 mg of freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane (Tindall, 1990a, b) and the polar lipids were extracted by adjusting the remaining methanol/0.3% aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3% aqueous NaCl mixture (1:2:0.8, by vol.). The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel art. no. 805 023), using hexane/tert-butylmethyl ether (9:1, v/v) as solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical (Thermo Separation Products) HPLC apparatus fitted with a reverse phase column (2 mm × 125 mm, 3 μm, RP18; Macherey-Nagel) using methanol/heptane as the eluant. Respiratory lipoquinones were detected at 269 nm.

Examination of the respiratory lipoquinone composition of strain CIR29812\textsuperscript{T} indicated that menaquinones were the sole respiratory quinones present. The major component was a menaquinone with seven isoprenologues, i.e. menaquinone 7 (MK-7). MK-7 had also been identified as the major menaquinone in T. commune and T. thermophilum (Collins & Widdel, 1986).

Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.). They were separated by two-dimensional silica-gel TLC (Macherey-Nagel art. no. 818 135). The first direction was developed in chloroform/methanol/water (65:25:4, by vol.) and the second was developed in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolylbenzophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (quaternary nitrogen) and anisaldehyde-sulfuric acid (glycolipids).

The polar lipids of strain CIR29812\textsuperscript{T} were predominantly phospholipids (Fig. 2). The two major lipids were identified initially on the basis of their R\textsubscript{f} values and staining behaviour as phosphatidylinositol and phosphatidylethanolamine. In addition, one of the minor phospholipids was identified as phosphatidylglycerol. Additional phospholipids
Fig. 2. Two-dimensional thin-layer chromatogram of the polar lipids of strain CIR29812T. All polar lipids were stained with 5% ethanolic molybdophosphoric acid. Solvents: chloroform/methanol/water (65:25:4, by vol.), first direction; chloroform/methanol/acetic acid/water (80:12:15:4, by vol.), second direction. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL1, PL2 and PL3, phospholipids of unknown structure.

(PL1, PL2, PL3) were present in small amounts and could not be identified unambiguously. Confirmation of the head-group structures was made by ESI-MS/MS studies, details of which will be reported elsewhere. The lipid composition of T. commune was similar. However, a third phospholipid identified in T. commune was not present in strain CIR29812T. This phospholipid had the \( R_f \) value and staining behaviour of the phosphatidyl aminopentetrol, which has also been reported in Hydrogenobacter thermophilus (Yoshino et al., 2001), the head-group structure having originally been described in the methanogenic members of the Archaea (Ferrante et al., 1987, 1988).

Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg of dry cell material. Cells were subjected to differential hydrolysis in order to detect ester-linked and non-ester-linked (amide-bound) fatty acids (B. J. Tindall, unpublished). Fatty acid methyl esters were analysed by GC using a 0.25% SP-2330 column and flame-ionization detection. The run conditions consisted of a temperature programme from 130 to 310°C at a rate of 4°C min\(^{-1}\).

The fatty acids of strain CIR29812T comprised both saturated and unsaturated straight-chain, as well as hydroxylated, fatty acids. The major fatty acids of strain CIR29812T consisted of \( \text{C}_{18:0} \) (42.7–50.9%) and \( \text{C}_{18:1} \) (19.2–23.6%) (Table I, available from IJSEM Online). By comparison, Langworthy et al. (1983) reported the presence of iso-, anteiso- and straight-chain fatty acids in T. commune, a pattern which could be confirmed in this study. Although Langworthy et al. (1983) examined the fatty acid composition of the lipid fraction, a re-examination of the fatty acid composition from whole cells confirmed these results, but also indicated the presence of hydroxyl fatty acids (data not shown). We assume that the hydroxyl fatty acids are bound to the cell, perhaps in the form of lipopolysaccharide-bound fatty acids.

For the determination of the G+C content, DNA was isolated after disruption of cells using a French pressure cell (Thermo Spectronic) and purified by hydroxyapatite chromatography (Cashion et al., 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The G+C content of the DNA of strain CIR29812T determined by the HPLC method described by Tamaoka & Komagata (1984) was 46 mol%.

A total of 1496 nt from the 16S rRNA gene were sequenced as described previously (Götze et al., 2002). The sequence was
re confirmed using the Thermo Sequenase TM Primer Cycle Sequencing Kit (Amersham) and the reactions were run on a LI-COR automatic sequencer (model 4200) using the LI-COR BASE IMAGEIR software (Science Tec) for analysis.

Distance and maximum-likelihood analyses (De Soete 1983; Olsen et al., 1994) (1301 nt were used) revealed that strain CIR29812\(^{1}\) clustered with all other members of the family Thermodesulfobacteriaceae and was most closely related to \textit{T. hydrogeniphilum} (10-3\% distant) (Fig. 3).

The metabolic and physiological properties of strain CIR29812\(^{1}\) are very similar to those of \textit{T. hydrogeniphilum} SL6\(^{2}\). Contrary to other members of the family Thermodesulfobacteriaceae, both organisms are thermophilic, chemolithoautotrophic, sulfate-reducing bacteria, which are non-fermenting, unable to reduce thiosulfate or sulfite, and require NaCl for growth (Table 1). However, their optimal temperature and NaCl range for growth and their resistance to streptomycin and penicillin represent phenotypic characteristics that distinguish these sulfate-reducing chemolithoautotrophs from one another. In addition, the 16S rRNA gene sequences of strain CIR29812\(^{1}\) and \textit{T. hydrogeniphilum} SL6\(^{2}\) are very different (10-3\% distance). Moreover, when analysed using the same method (HPLC), a 15\% difference discriminates the G + C content of their DNA. Lastly, the major fatty acids present in strain CIR29812\(^{1}\) differed from those of \textit{T. commune}, the type species of the type genus of the family Thermodesulfobacteriaceae. They were more similar to the fatty acid patterns reported for members of the \textit{Aquifex–Hydrogenobacter} group (Stöhr et al., 2001), although the longer-chain components found in the latter group were not found in strain CIR29812\(^{1}\).

Based on a combination of 16S rRNA, chemotaxonomic and physiological data, we propose that strain CIR29812\(^{1}\) be placed into a new genus within the family Thermodesulfobacteriaceae, for which we propose the name \textit{Thermodesulfatator}, as a new species, \textit{Thermodesulfatator indicus}, which is the sole and type species of this genus.

**Description of \textit{Thermodesulfatator} gen. nov.**

\textit{Thermodesulfatator} (Ther.mo.de.sul.fa.ta’tor. Gr. masc. n. thermos heat; N.L. n. desulfatator sulfate-reducer; N.L. masc. n. Thermodesulfatator thermophilic sulfate-reducer).

Thermophilic. Strictly anaerobic. Marine. Cells are Gram-negative, rod-shaped (0.8–1 \(\mu\)m long and 0.4–0.5 \(\mu\)m wide) and motile by means of a single polar flagellum. They occur singly, in pairs, in chains of three cells and may form cell aggregates in stationary-phase cultures. Do not form spores. Chemolithoautotrophs growing exclusively with hydrogen as the sole electron donor and sulfate as the sole electron acceptor. 16S rRNA gene sequence comparison differentiates \textit{Thermodesulfatator} from the other genera of the family Thermodesulfobacteriaceae.

**Table 1. Differentiating characteristics of cultivated members of the family Thermodesulfobacteriaceae**

Data were obtained from Zeikus et al. (1983), Rozanova & Pivovarova (1988), Henry et al. (1994), Sonne-Hansen & Ahring (1999), Jeanthon et al. (2002) and Kashefi et al. (2002). Electron donors were tested with CO\(_2\) as the carbon source. ND, Not determined. Species: 1, strain CIR29812\(^{1}\); 2, \textit{T. hydrogeniphilum}; 3, \textit{G. ferrireducens}; 4, \textit{T. commune}, \textit{T. thermophilum} and \textit{T. hveragerdense}.

<table>
<thead>
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<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>G + C content (mol%)</td>
<td>46</td>
<td>28 (31.5)*</td>
<td>ND</td>
<td>31–40</td>
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<td>NaCl range (g l(^{-1}))</td>
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<td>5–55</td>
<td>0–7–5</td>
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<tr>
<td>Optimal salinity (g l(^{-1}))</td>
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<td>30</td>
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<td>Temperature range ((^{\circ})C)</td>
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<td>50–80</td>
<td>65–100</td>
<td>45–85</td>
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<tr>
<td>Optimal temperature ((^{\circ})C)</td>
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<td>75</td>
<td>85–90</td>
<td>65–74</td>
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<td>pH range for growth</td>
<td>6–6–7</td>
<td>6–3–6–8</td>
<td>ND</td>
<td>6–8 for \textit{T. commune}, ND for the other species</td>
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<tr>
<td>(\text{H}_2)</td>
<td>+(\ddagger)</td>
<td>+(\ddagger)</td>
<td>+(\ddagger)</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<td>–</td>
<td>+</td>
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<td>Pyruvate fermentation</td>
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<td>Thiosulfate</td>
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<td>Streptomycin (200 (\mu)g ml(^{-1}))</td>
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<td>+</td>
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<td>Penicillin (200 (\mu)g ml(^{-1}))</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
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</table>

*The value in parentheses was obtained in this study by HPLC.
\(\ddagger\)Autotrophic growth.
The type species is *Thermodesulfator indicus*.

**Description of Thermodesulfator indicus sp. nov.**

*Thermodesulfator indicus* (in.di´cus. L. adj. indicus referring to the Indian Ocean, from where the strain was isolated).

Gram-negative rods (0.8–1 μm long by 0.4–0.5 μm wide), motile by means of a single polar flagellum. Cells occur singly, in pairs or in chains of three cells in early cultures. Growth occurs between 55 and 80 °C (optimum 70 °C), pH 6 and 6.7 (optimum at pH 6.25) and in the presence of 10 and 35 g NaCl l⁻¹ (optimum 25 g l⁻¹). Anaerobic. Strictly chemolithoautotrophic using sulfate as electron acceptor and H₂ as electron donor. No fermentative metabolism. With H₂/CO₂ and sulfate, growth is stimulated by methanol, monomethylamine, glutamate, peptone, fumarate, tryptophane, isobutyrate, 3-CH₃ butyrate, ethanol, propanol and low amounts of acetate. Unable to use sulfur, cystine, thiosulfate, sulfate, fumarate and nitrate as electron acceptor. Ammonium is the preferred nitrogen source. Sensitive to ampicillin, chloramphenicol and rifampicin (25 μg ml⁻¹). Resistant to tetracycline and streptomycin (25 μg ml⁻¹), penicillin and kanamycin (200 μg ml⁻¹). The major lipoquinone is MK-7. Predominant polar lipids are phosphatidylethanolamine and phosphatidylinositol. Small amounts of phosphatidylglycerol and three unknown phospholipids (PL1, PL2, PL3) are detected. Fatty acid profile is mainly composed of C₁₈:0 and C₁₈:1.

The type strain (CIR29812^T^ = DSM 15286^T^ = JCM 11887^T^) was isolated from an active hydrothermal sulfide chimney deposit at the Kairei vent field on the Central Indian Ridge. The G+C content of its DNA is 46.0 mol%.

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