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Desulfurobacterium indicum sp nov., a thermophilic sulfur-reducing bacterium from the Indian Ocean

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Abstract :

A novel sulfur-reducing bacterium, strain K6013T, was isolated from a sulfide sample collected at a depth of 2771 m from a high-temperature hydrothermal vent in the Indian Ocean. Cells were Gram-stain-negative, anaerobic, motile rods (0.9–2.2×0.4–0.6 µm). The strain grew at NaCl concentrations ranging from 1 to 4.5% (w/v) (optimum 2.5%), at pH 5 to 8 (optimum pH 6), and at temperatures between 40 and 75°C (optimum 65°C). K6013T was an obligate chemolithoautotroph, using thiosulfate, sulfur and nitrate as terminal electron acceptors in the presence of H_2 but not sulfate, sulfite nor nitrite. The major cellular fatty acids were $C_{16:0}$ (17.4%), $C_{18:1}\omega7c/C_{18:1}\omega6c$ (ummed feature 8, 37.91%), $C_{18:0}$ (18.29%) and $C_{14:0}$ 3-OH/iso-$C_{16:1}$I (summed feature 2, 8.56%). The DNA G+C content was 38.2 mol%. The results of phylogenetic 16S rRNA gene sequence analyses indicated that K6013T represents a member of the genus *Desulfurobacterium* within the class *Aquificae* , with highest sequence similarity of 96.93% to *Desulfurobacterium atlanticum* SL22T. On the basis of genotypic and phenotypic data, K6013T is considered to represent a novel species of the genus *Desulfurobacterium* , for which the name *Desulfurobacterium indicum* sp. nov. is proposed, with the type strain K6013T (=DSM 101677T=MCCC 1A01868T).

Keywords : Desulfurobacterium, thermophilic, hydrothermal vent, chemolithoautotroph, sulfur-reducer

Sulfur-reducing prokaryotes are anaerobic or facultative anaerobic prokaryotes, using sulfur (or other lower oxidation states of this element, S^0, S_8) as a terminal electron acceptor but not sulfate (Rabus *et al.*, 2013). Members of the genus *Desulfurobacterium* are sulfur-reducing chemolithoautotrophs using hydrogen as sole electron donor (Alain *et al.*, 2003; L'Haridon *et al.*, 1998; L'Haridon *et al.*, 2006). They represent a deep-branching lineage of the bacterial phylogenetic tree which play an important role in deep-sea hydrothermal ecosystems as primary producers of organic matter in anaerobic zones (Miroshnichenko & Bonch-Osmolovskaya, 2006). At the time of writing, the genus *Desulfurobacterium*, within the family *Desulfurobacteriaceae*, comprises 4 species, *D. pacificum*, *D. atlanticum*, *D. thermolithotrophum* and '*D. crinifex*', which have been isolated exclusively from deep-sea hydrothermal systems (Alain *et al.*, 2003; L'Haridon *et al.*, 1998; L'Haridon *et al.*, 2006).

In this study, we describe a novel thermophilic sulfur-reducer, strain K6013^T, isolated from a hydrothermal sulfide sample in the Indian Ocean. Phenotypic and 16S ribosomal RNA (rRNA) gene sequences phylogenetic studies indicate that it represents a novel species of the genus *Desulfurobacterium*.

A deep-sea sulfide sample was collected at a depth of 2,771 m from a hydrothermal vent in the Indian Ocean (37° 78’S, 49° 65’E; site JL-Dive94-S01) in January 2015, during the DY35 cruise of the research vessel “*Xiang Yang Hong Jiu Hao*”. The sample was collected using a benthic seabed grab and anaerobically preserved in sealed sterile vials at 4 °C onboard. Once in

the lab, a subsample was used to inoculate a completely mineral medium (KA22 medium; Alain *et al*., 2003), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubated anoxically at 60 °C. After 5 days of incubation, populations were composed of short rod-shaped cells. They were subcultured under the same conditions, and purified by 7 repeated dilutions-to-extinction series. One isolate, strain K6013^T, was obtained. The purity of this isolate was confirmed routinely by microscopic examination, by repeated partial sequencing of the 16S rRNA gene using 4 different primers (see below) and by sequencing of its genome. Stock cultures were stored at -80 °C with 5% (v/v) DMSO.

Genomic DNA was extracted with the QIAGEN Genomic-tip 20/G (QIAGEN, Düsseldorf, Germany) kit following the manufacturer's standard protocol. The 16S rRNA gene was sequenced by Sanger method using the primers Bac8F (5'-AGA GTT TGA TCA TGG CTC AG-3'), S8dir (5'-GTA GCG GTG AAA TGC GTA GA-3'), U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') and W34 (5'-TTA CCG CGG CTG CTG GCA C-3') (Alain *et al.*, 2002). Pairwise 16S rRNA gene sequence similarity was determined using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim *et al.*, 2012)). Phylogenetic analysis was performed using the software MEGA version 5.0 (Tamura *et al.*, 2011). Distances were calculated using the Kimura two-parameters model and clustering was performed with the neighbor-joining algorithm (Saitou & Nei, 1987). The robustness of the inferred topology was assessed by bootstrap analyses based on 1,000 replications.

Almost a full-length 16S rRNA gene sequence (1517 bp) of strain K6013^T was determined. Based on the 16S rRNA gene phylogenetic analysis, the novel isolate was affiliated with the class *Aquificae,* in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain K6013^T formed a robust cluster with the genus *Desulfurobacterium,* within the family *Desulfurobacteriaceae* (Fig. 1). Strain K6013^T shared the highest sequence similarity of 96.93% with *Desulfurobacterium atlanticum* SL22^T, followed by *D. pacificum* SL17^T (95.46%), *Thermovibrio guaymasensis* SL19^T (93.99 %) and *D. thermolithotrophum* DSM 11699^T (93.74 %). The level of 16S rRNA gene sequence similarity with *D. atlanticum* showed that strain K6013^T displayed sufficient molecular differences for delineation at the species level, because it falls well below the threshold value (98.65-98.7%) currently recommended for two species demarcation (Kim *et al.,* 2014; Stackebrandt & Ebers, 2006).

The draft genome of the novel isolate was recently sequenced by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China), using Solexa paired-end (500 bp library) sequencing technology. The draft genome (1,607,407 bp with 62 contigs) accession number for strain K6013^T is MOEN00000000. The DNA G+C content of strain K6013^T is 38.2 mol%, as determined from the draft genome sequence.

Morphological characteristics of strain K6013^T were observed by using light microscopy (Olympus BX60 and CX40) and scanning electron microscopy (FEI Quanta 200). Cells were Gram-negative, motile rods (0.9-2.2 μm in length and 0.4-0.6 μm in diameter, n=10,

Supplementary Fig. 1) with a polar flagellum, that occurred generally singly. Some cells became spherical in the late stationary growth phase.

Unless noted otherwise, physiological tests were carried out anaerobically in the totally mineral SO4PNsalts medium (Alain *et al.*, 2010) depleted of sulfate, in duplicate, using elemental sulfur as a terminal electron acceptor, and a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) as energy and carbon sources. Growth tests were generally carried out as described previously (Alain *et al.*, 2003). Cells were routinely counted by direct cell counting by using a modified Thoma chamber (depth 10 μm). Determination of the temperature range for growth was carried out at 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. The isolate was thermophilic and grew between 40 and 75 °C with an optimum around 65 °C. Salt tolerance was tested at 65 °C with various concentrations of NaCl (0, 0.2, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 %, w/v). Strain K6013^T required NaCl for growth and grew at NaCl concentrations between 1.0 and 4.5% (optimum: 2.5%). The pH range for growth was tested from pH 4.0 to pH 9.0 (initial pH at 20 °C) with increments of 1 unit. Growth of the isolate was observed between pH 5.0 and 8.0 with an optimum around 6.0.

Strain K6013^T was a strictly anaerobic bacterium using hydrogen and sulfur as primary electron donor and acceptor, respectively. It could not grow heterotrophically on peptone or yeast extract. The ability of the isolate to use various electron acceptors was tested with sulfite (2 mM), thiosulfate (20 mM), elemental sulfur (12 g L^{-1}), nitrate (10 mM), nitrite (2 mM) or

oxygen (1% v/v). Hydrogen sulfide production was tested as described previously (Cord-Ruwisch, 1985). Ammonia/ammonium production was determined with the Nessler's reagent (Sigma-Aldrich) as described elsewhere. Sulfur, thiosulfate and nitrate were used as terminal electron acceptors, but not sulfate, sulfite, oxygen nor nitrite. Hydrogen sulfide was formed from sulfur or thiosulfate reduction, and ammonia was formed from nitrate reduction. Strain K6013^T grew exclusively with hydrogen and carbon dioxide, when using elemental sulfur as a terminal electron acceptor. Its growth was not stimulated by yeast extract.

Chemotaxonomic analyses were performed on mid- to late-exponential-phase of growth cultures grown for 90 hours on KA22 medium prepared with S° as a terminal electron acceptor, hydrogen as an electron donor and carbon dioxide as a carbon source. The cellular fatty acids in whole cells were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The cellular fatty acids were analyzed by GC (Agilent Technologies 6850) and identified by using the TSBA6.0 database of the Microbial Identification System (Sasser, 1990). The major fatty acids were $C_{16:0}$ (17.4%), $C_{18:1}$ *ω7c* (Summed feature 8, 37.91%), $C_{18:0}$ (18.29%) and $C_{14:0}$ 3-OH (Summed feature 2, 8.56%), which were in the same range than the values got from *D. pacificum*, *D. atlanticum* and *D. thermolithotrophum* (Table 1). Under our growth conditions, the novel isolate K6013^T did not contain $C_{18:1}$, but this fatty acid was detected in low amounts in *D. pacificum*, *D. atlanticum* and *D. thermolithotrophum*.

The phylogenetic, phenotypic, physiological and chemotaxonomic data shown in this article support the view that strain K6013^T should be assigned to the genus *Desulfurobacterium* in the family *Desulfurobacteriaceae*. However, there are several important phenotypic differences, detailed in Tables 1 and 2, between the novel isolate and its closely related species. Strain K6013^T and *D. pacificum* are both able to reduce sulfur, nitrate and thiosulfate, but *D. atlanticum* is unable to reduce sulfur, *D. thermolithotrophum* is unable to reduce nitrate and '*D. crinifex*' is unable to reduce thiosulfate. Strain K6013^T and *D. thermolithotrophum* can grow at 40 °C, while *D. pacificum, D. atlanticum* and '*D. crinifex*' cannot grow at temperature lower than 50 °C. Therefore, from the phylogenetic, phenotypic, physiological and chemotaxonomic evidence, we proposed to assign strain K6013^T to a novel species of the genus *Desulfurobacterium*, for which the name *Desulfurobacterium indicum* sp. nov. is proposed.

Description of *Desulfurobacterium indicum* sp. nov.

Desulfurobacterium indicum (in'di.cum. L. neut. adj. *indicum*. Indian, referring to the Indian Ocean, from where the type strain was isolated).

Cells were Gram-negative, anaerobic, motile rods (0.9-2.2 × 0.4-0.6 μm). Growth was observed at temperatures between 40 and 75 °C (optimum 65 °C), at NaCl concentration from 1.0 to 4.5% (optimum 2.5%) and at pH from 5 to 8 (optimum 6.0). Strain K6013^T used sulfur, thiosulfate and nitrate as terminal electron acceptors, but not sulfate, sulfite nor nitrite. The predominant fatty acids were $C_{16:0}$ (17.4%), $C_{18:1}$ $\omega 7c$ (Summed feature 8, 37.91%), $C_{18:0}$

(18.29%) and $C_{14:0}$ 3-OH (Summed feature 2, 8.56%). The genomic DNA G+C content was 38.2 mol%.

The type strain K6013^T (= DSM 101677^T= MCCC 1A01868^T) was isolated from a deep-sea sulfide sample collected at a depth of 2771 m from a hydrothermal area in the Indian Ocean (37° 78'S, 49° 65'E; site JL-Dive94-S01).

Author statements

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Figure Legends

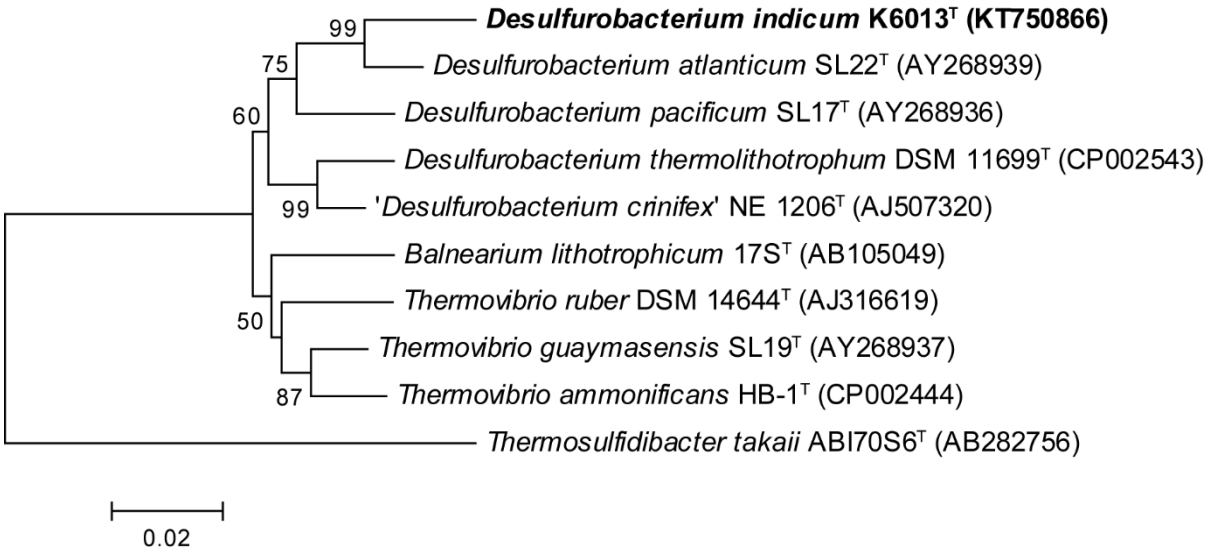


Fig. 1. Neighbor-joining tree showing the phylogenetic positions of strain K6013^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch nodes. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units.

Table 1. Whole-cell fatty acid profiles of strain K6013^T and related species of genus *Desulfurobacterium*.

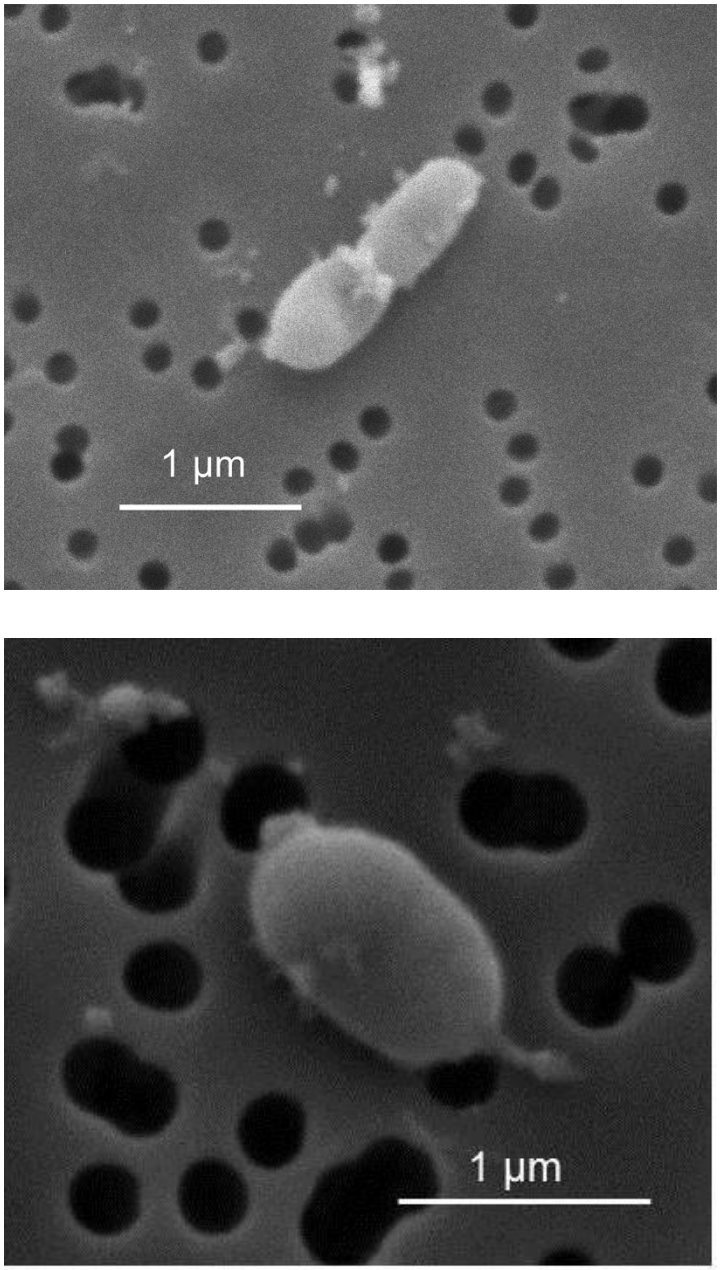
Strains: 1, K6013^T; 2, *D. pacificum* SL17^T (L'Haridon *et al.*, 2006); 3, *D. atlanticum* SL22^T (L'Haridon *et al.*, 2006); 4, *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998). Values are percentages of total fatty acids. Data for strain K6013^T were obtained in this study. Data for strains 2, 3 and 4 were obtained from L'Haridon *et al.* (2006).

Fatty acid	1	2	3	4
$C_{9:0}$	1.37			
Summed feature 2 ($C_{14:0}$ 3-OH/iso-$C_{16:1}$ I)	8.56	6.87	10.98	6.82
Summed feature 3 ($C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*)	2.13		1.42	1.29
$C_{16:0}$	17.4	14.38	16.16	5.7
Summed feature 5 ($C_{18:2}$ ω6,9*c*/ante-$C_{18:0}$)	4.14			
$C_{18:1}$	ND	2.68	2.16	3.28
$C_{18:1}$ ω9*c*	4.84	3.37	1.95	
Summed feature 8 ($C_{18:1}$ ω7*c*/$C_{18:1}$ ω6*c*)	37.91	31.99	42.08	44.98
$C_{18:0}$	18.29	31.01	26.85	35.31

Table 2. Differential characteristics of strain K6013^T and related species of the genus *Desulfurobacterium.*

Strains: 1, K6013^T; 2, *D. pacificum*; 3, *D. atlanticum*; 4, *D. thermolithotrophum*; 5, '*D. crinifex*'. Characteristics are scored as: +, positive; -, negative. Data for strain 1 were obtained in this study; data for strains 2 or 3, 4 and 5 were obtained from L'Haridon *et al.* (2006), L'Haridon *et al.* (1998) and Alain *et al.* (2003), and differences were confirmed in this study.

Characteristics	1	2	3	4	5
Cell shape	Straight to curved rods	Straight to curved rods	Straight rods	Straight rods	Straight to curved rods
Length (μm)	0.9-2.2	1-2	2.5-3.5	1-2	0.9-3.5
Width (μm)	0.4-0.6	0.4-0.5	0.4-0.5	0.4-0.5	0.4-0.7
T (°C) (optimal)	40-75 (65)	55-85 (75)	50-80 (70-75)	40-75 (70)	50-70 (60-65)
pH (optimal)	5-8 (6)	5.5-7.5 (6-6.2)	5.5-7 (5.8-6)	4.4-8 (6)	5-7.5 (6-6.2)
NaCl (%) (optimal)	1-4.5 (2.5)	1.5-5 (3)	1.5-5 (3)	1-4.6 (2.3)	2-4 (3)
Flagellation	Monopolar	Monopolar	Monopolar	Monopolar	Bipolar
G+C (mol%)	38.3	42	41	36	37
Electron acceptor					
S^0	+	+	–	+	+
NO_3^-	+	+	–	–	+
$S_2O_3^{2-}$	+	+	+	+	–
SO_3^{2-}	–	–	–	+	–



Supplementary Fig. 1. Scanning electron micrographs of cells of strain K6013^{T}.

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