Oceanithermus desulfurans sp. nov., a novel thermophilic, sulfur-reducing bacterium isolated from a sulfide chimney in Suiyo Seamount

Koji Mori,^{1,2} Takeshi Kakegawa,³ Yowsuke Higashi,¹ Ko-ichi Nakamura,⁴ Akihiko Maruyama¹ and Satoshi Hanada¹

Correspondence

Satoshi Hanada
s-hanada@aist.go.jp

¹Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

²Biological Resource Centre (NBRC), National Institute of Technology and Evaluation (NITE), 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan

³Graduate School of Science, Tohoku University, Sendai, Miyagi 980-8578, Japan

⁴Institute for Marine Resource and Environment, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 7, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8567, Japan

A novel thermophilic, microaerophilic, sulfur-reducing bacterium designated strain St55B^T was isolated from a sulfide chimney in the hydrothermal field of Suiyo Seamount (Izu-Bonin Arc, Western Pacific). Cells of the isolate were rod-shaped and tended to form a chain-link circular structure (a rotund body) at exponential phase under good growth conditions. The isolate was a chemoheterotroph requiring yeast extract for growth. Although strain St55B^T used oxygen as an electron acceptor, it could not form colonies in an oxygen concentration of more than 5% (v/v). The isolate also used nitrate, nitrite or elemental sulfur in the absence of oxygen. A phylogenetic analysis based on the 16S rRNA gene sequence revealed that the isolate was closely related to *Oceanithermus profundus*, belonging to the phylum 'Deinococcus-Thermus' (sequence similarity 99.5%). However, strain St55B^T differed from *O. profundus* in terms of usage of electron donors, cellular fatty acid profile and DNA G+C content. In addition, a DNA–DNA hybridization test indicated low relatedness between the isolate and *O. profundus*. For the reasons given above, the name *Oceanithermus desulfurans* sp. nov. is proposed for strain St55B^T (=NBRC 100063^T=DSM 15757^T).

Deep-sea hydrothermal vents have been discovered globally. In such environments, there are the peculiar ecosystems that are completely independent of sunlight and which include thermophilic chemoautotrophs as primary producers. Chimney structures and neighbouring sulfide mounds in hydrothermal areas are able to support metabolically diverse micro-organisms because of the fact that mixing of reduced hydrothermal fluid with oxic deep-sea water provides sharp physical and chemical gradients. Culture-independent analyses based on a 16S rRNA gene clone sequence revealed that various micro-organisms inhabit such environments (Corre *et al.*, 2001; Marteinsson *et al.*, 1995; Takai & Horikoshi, 1999; Takai *et al.*, 2001, 2003). In recent years,

many novel thermophilic bacteria have been found in and around these hydrothermal vents (Alain *et al.*, 2002b; Götz *et al.*, 2002; Huber *et al.*, 2002; Jeanthon *et al.*, 2002; Miroshnichenko *et al.*, 2003a, b; Nakagawa *et al.*, 2003; Sako *et al.*, 2003; Vetriani *et al.*, 2004; Wery *et al.*, 2001).

Investigations of thermophilic isolates in the domain *Bacteria* have focused on chemoautotrophs (e.g. oxidizers of hydrogen and sulfur compounds and anaerobes that reduce sulfur and nitrogen compounds). The ecosystem in hydrothermal vents does not consist solely of chemoautotrophs, but probably contains many heterotrophic thermophiles as decomposers or scavengers. In fact, many novel chemoheterotrophic bacteria have been isolated recently from hydrothermal environments. Among the heterotrophs isolated are three species belonging to the family *Thermaceae*: *Marinithermus hydrothermalis* (Sako *et al.*, 2003), *Oceanithermus profundus* (Miroshnichenko *et al.*, 2003a) and *Vulcanithermus mediatlanticus* (Miroshnichenko

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et al., 2003b). These novel species of the *Thermaceae*, recently proposed in rapid succession, are all 'true' marine organisms that require NaCl for growth. Before these marine species were found, all species in this family (belonging to the genera *Thermus* and *Meiothermus*) were inhabitants of freshwater environments, or were isolated from marine environments but were merely halotolerant and not halophilic (Marteinsson *et al.*, 1995). These marine species were rod-shaped micro-organisms able to grow by respiration with oxygen as a terminal electron acceptor. However, the two species *O. profundus* and *V. mediatlanticus* are relatively sensitive to oxygen, and grow well at oxygen concentrations of less than 6% (v/v) and 4–8% (v/v), respectively (Miroshnichenko *et al.*, 2003a, b). The micro-aerophiles are also able to use nitrate as an electron acceptor under anaerobic conditions.

Recently, a novel thermophilic, sulfur-reducing bacterium, designated strain St55B^T, was isolated from a chimney in a vent deposit sample in a submarine hydrothermal field. The chimney sample containing strain St55B^T was collected from Suiyo Seamount in Izu-Bonin Arc, Western Pacific (28° 34' N 140° 39' E; depth, 1380 m) by the vessel ROV *Hakuyo 2000* (Shin Nippon Kaiji) on 9 August 2001. The chimney sample was approximately 50 cm in diameter and 30 cm in length. A black smoker (300 °C) was discharging from this chimney. Mineralogical zoning was recognized in this chimney sample: the central part is essentially composed of chalcopyrite (CuFeS₂), the middle part is composed of sphalerite (ZnS), pyrite (FeS₂) and anhydrite (CaSO₄), while sphalerite and pyrite are essential constituents of the outer part, together with arsenic sulfides. Elemental sulfur was also found in the middle and outer parts by X-ray diffraction analyses. The middle part of this chimney was used for this study.

For enrichment, vials (each with a butyl-rubber stopper and an aluminium cap) with basal medium were used. The basal medium under a N₂/CO₂ (4:1, v/v) atmosphere was composed of the following (l⁻¹): KH₂PO₄, 0.75 g; K₂HPO₄, 0.78 g; MgCl₂·6H₂O, 0.36 g; CaCl₂·2H₂O, 0.1 g; NH₄Cl, 0.54 g; NaHCO₃, 5 g; NaCl 30 g; trace-element solution DSM 334 (DSMZ, 1993), 10 ml; vitamin solution DSM 141 (DSMZ, 1993), 10 ml. A fragment of the chimney was incubated at 55–85 °C in the enrichment medium, which was basal medium supplemented with sulfur (10 g l⁻¹) and yeast extract (2 g l⁻¹), and supplied with O₂ (N₂/CO₂/O₂, 75:20:5, by vol.). After a 1-week incubation, growth of micro-organisms was observed at 55 °C; the culture was transferred to fresh enrichment medium several times. A similar enrichment medium solidified with 2% (w/v) agar was used for isolation. An inoculated agar plate was incubated at 55 °C in a sealed nylon bag with an oxygen-absorbing agent (Anaero Pack Campylo; Mitsubishi Gas Chemical Co.) for microaerophiles. Colourless colonies formed on the agar plate after 4 days incubation; strain St55B^T was then isolated. The isolate was generally maintained in basal medium supplied

with 2 g yeast extract l⁻¹ and 20 mM sodium nitrate under anaerobic conditions (see below).

Strain St55B^T was characterized morphologically as comprising non-motile rods (about 0.5 µm wide and 1.5–2.0 µm long; Fig. 1a, b). Occasionally (at exponential phase under good growth conditions), cells tended to connect to each other forming chain-linked circular structures, called 'rotund bodies', that are often observed in some species of the genus *Thermus* (Brock & Edwards, 1970) and in *O. profundus* (Miroshnichenko *et al.*, 2003a) (Fig. 1c, d). The isolate had negatively Gram-stained cells with a thick cell-wall structure (Fig. 1b, d). Spore formation and pigmentation were not observed. Oxidase and catalase activities (Tamaki *et al.*, 2003) were positive and weakly positive, respectively. However, when strain St55B^T grew anaerobically with nitrate as an electron acceptor (see below), oxidase was not produced.

An almost complete 16S rRNA gene sequence (Hattori *et al.*, 2000) of strain St55B^T was determined (AB107956). After alignment with the ARB program (<http://www.arb-home.de/>), the phylogenetic tree (Fig. 2) was constructed by the neighbour-joining method with the CLUSTAL W program (Saitou & Nei, 1987; Thompson *et al.*, 1994). Strain St55B^T was found to be a member of the family *Thermaceae* in the phylum 'Deinococcus-Thermus' and was very close to *O. profundus* (Miroshnichenko *et al.*, 2003a), with a sequence similarity of 99.5%.

Strain St55B^T could grow by oxygen respiration but did not support itself in an atmosphere of air (20%, v/v, oxygen). The isolate was not able to form colonies at oxygen concentrations above 5% (v/v); colony formation was observed at concentrations between 1 and 3% (v/v). Although no fermentative growth was observed, the isolate showed good growth under anaerobic conditions in the presence of a suitable electron sink. Tests for utilization of electron acceptors in the presence of yeast extract (2 g l⁻¹) as a substrate (Hattori *et al.*, 2000) revealed that the isolate could use nitrate (5 mM), nitrite (2.5 mM) and elemental sulfur (10 g l⁻¹) as electron acceptors in place of oxygen. However, it could not grow with the following acceptors: sulfate (5 mM), thiosulfate (5 mM), sulfite (2.5 mM), DMSO (5 mM), fumarate (5 mM), Fe(III) citrate (5 mM) (Heising *et al.*, 1999) and selenate (5 mM). The culture with elemental sulfur showed slow growth, but sulfur certainly supported growth under anaerobic conditions. Miroshnichenko *et al.* (2002) reported that *O. profundus* could not use elemental sulfur as an electron acceptor. However, we also observed faint growth of *O. profundus* in our basal medium supplemented with elemental sulfur (10 g l⁻¹), sucrose (10 mM) and yeast extract (0.1 g l⁻¹). The turbidity of the culture was apparently lower than that of a culture with nitrate or oxygen as an electron acceptor; growth of *O. profundus* on elemental sulfur was similar to that in strain St55B^T.

The isolate required a small amount of yeast extract

Fig. 1. Cell morphology of strain St55B^T grown on yeast extract and nitrate (at 60 °C and pH 7.0). (a, c) Phase-contrast micrographs of strain St55B^T. (b, d) Ultrathin sections of strain St55B^T, observed with a transmission electron microscope (model H-7000; Hitachi) (Hattori *et al.*, 2000). 'Rotund bodies' were observed in a well-grown culture at exponential phase (c, d). Bars, 10 μm (a, c) and 1.0 μm (b, d).

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of strain St55B^T and relatives. Bootstrap percentages are indicated at branch points. Accession numbers of reference sequences are shown in parentheses. Bar, 0.03 substitutions per compared nucleotide.

(0.1 g l⁻¹) for growth under any growth conditions. In the presence of 5 mM nitrate and 0.1 g yeast extract l⁻¹, the isolate grew on each of the following as a sole source of energy and carbon: yeast extract (1 g l⁻¹), tryptone (1 g l⁻¹), Casamino acids (1 g l⁻¹), butyrate (20 mM), formate (40 mM), glutamate (20 mM), lactate (20 mM), propionate (20 mM), pyruvate (20 mM), succinate (20 mM), L-alanine (20 mM) and L-cysteine (20 mM). The following substrates could not support growth: H₂/CO₂ (4:1, v/v), acetate (40 mM) + H₂/CO₂ (4:1, v/v), arabinose (10 mM), fructose (10 mM), galactose (10 mM), glucose (10 mM), inositol (10 mM), mannose (10 mM),

raffinose (10 mM), sucrose (10 mM), xylose (10 mM), acetate (20 and 40 mM), citrate (20 mM), fumarate (20 mM), malate (20 mM), L-arginine (20 mM), L-asparagine (20 mM), L-histidine (20 mM), L-leucine (20 mM), L-methionine (20 mM), L-serine (20 mM), ethanol (20 mM), 2-propanol (20 mM) and methanol (20 mM).

Temperature, pH and NaCl-concentration ranges for growth were determined using 25 ml Hungate tubes containing 10 ml medium (Hattori *et al.*, 2000). The medium contained 2 g yeast extract l⁻¹ as an energy source and 20 mM nitrate as an electron acceptor under

N₂/CO₂ (4:1, v/v). The temperature for growth of strain St55B^T ranged from 30 to 65 °C, with an optimum at 60 °C. The pH range for growth at 60 °C was 6.0–8.0, with an optimum at pH 6.5. The isolate required NaCl for growth and grew at NaCl concentrations of 1–5 % (w/v), with optimum growth at 3 % (w/v).

Fatty acid methyl ester analysis with a GC/MS system (Hanada *et al.*, 2002) revealed that the main cellular fatty acids of strain St55B^T grown at 55 °C were iso-C_{15:0} (48 % of total fatty acids) and anteiso-C_{15:0} (13 %). The strain also contained iso-C_{15:1} (5 %), C_{16:1} (6 %), iso-C_{16:0} (4 %), iso-C_{17:1} (6 %) and two kinds of branched C_{17:0} (18 %) as minor components. All members of the genera *Thermus*, *Meiothermus* or *Marinithermus* contain predominantly iso- and anteiso-branched saturated fatty acids (Da Costa & Rainey, 2001; Sako *et al.*, 2003). The cellular fatty acids of strain St55B^T consisted mainly of iso- and anteiso-branched, odd-carbon-numbered fatty acids, like other related species. The closest relative, *O. profundus*, is known to include significant amounts (33 % of total fatty acids) of unsaturated fatty acids (Miroshnichenko *et al.*, 2003a). While such a high content of unsaturated fatty acids is rarely observed in thermophilic bacteria, our fatty acid methyl ester analysis of *O. profundus* grown at 55 °C also indicated that the related species clearly contains a large amount of them (approx. 37 %). In our isolate, strain St55B^T, unsaturated fatty acids such as iso-C_{15:1}, C_{16:1} and iso-C_{17:1} were also detected, but they represented only 18 % of the total cellular fatty acids, equivalent to half that present in *O. profundus*. The fatty acid methyl ester profile of strain St55B^T grown at 45 °C was almost identical to that of cells grown at 55 °C. In general, the unsaturated fatty acid content tends to increase when cells are grown at lower temperatures, but the content in strain St55B^T was stable even if it was grown at low temperatures.

MK-8 was detected as the major quinone of strain St55B^T by using HPLC (Shintani *et al.*, 2000). Trace amounts of MK-6(H₂), MK-7, MK-7(H₈) and MK-9 were also detected. The genomic DNA G+C content (Mori *et al.*, 2000) of strain St55B^T was 71.1 mol%. The G+C content of the reference species, *O. profundus*, was 68.6 mol% when measured by the same method, whereas the value reported by Miroshnichenko *et al.* (2003a) was 62.9 mol%.

Differential characteristics of St55B^T are summarized in Table 1 in comparison with those of the most related species, *O. profundus* (Miroshnichenko *et al.*, 2003a). *O. profundus* is a thermophilic, microaerophilic micro-organism isolated from a deep-sea hydrothermal vent site in East Pacific Rise. The 16S rRNA gene sequence and also some phenotypic features were very similar to those of strain St55B^T. However, strain St55B^T clearly differed from *O. profundus* in the following respects: (i) strain St55B^T grew with nitrite as an electron acceptor under anaerobic conditions, unlike *O. profundus*; (ii) *O. profundus* utilized various sugars and alcohols, but strain St55B^T was unable to use these substrates and preferred complex compounds such

as yeast extract, tryptone and Casamino acids; (iii) the genomic DNA G+C content differentiated the isolate (71.1 mol%) from *O. profundus* (68.9 mol%); (iv) the unsaturated fatty acid content of the isolate (18 %) was clearly lower than that in *O. profundus* (33–37 %); and (v) the pH for optimum growth of the isolate was slightly acid (pH 6.5).

The results of the DNA–DNA hybridization study (Tamaki *et al.*, 2003) are also shown in Table 1. The DNA relatedness between strain St55B^T and *O. profundus* was less than 20 %, suggesting strongly that these two organisms should be classified as different species (Wayne *et al.*, 1987). For the reasons described above, we propose strain St55B^T as a novel species with the name *Oceanithermus desulfurans* sp. nov.

A lot of bacteria able to use elemental sulfur in anaerobic respiration that have been isolated, including *Caminibacter hydrogeniphilus* (Alain *et al.*, 2002a), *Nautilia lithotrophica* (Miroshnichenko *et al.*, 2002), *Desulfurobacterium thermolithotrophum* (L'Haridon *et al.*, 1998) and members of the genus *Marinitoga* (Alain *et al.*, 2002b), are strictly anaerobic sulfur-reducing bacteria that live in deep-sea hydrothermal vents. *Persephonella marina* (Götz *et al.*, 2002) and some strains in the ϵ -Proteobacteria (Takai *et al.*, 2003) are facultatively anaerobic bacteria that are capable of reducing elemental sulfur. Members of the genus *Oceanithermus* can use elemental sulfur as an electron acceptor under anaerobic conditions, and this is the first report of a sulfur-reducer in the family Thermaceae. A shortage of oxygen easily occurs in deep-sea environments where reduced sulfur compounds are supplied constantly. The ability to reduce elemental sulfur is advantageous to *Oceanithermus* species under such conditions. *Oceanithermus* species may be significant contributors to the sulfur cycle in deep-sea hydrothermal vents.

Emended description of the genus *Oceanithermus* Miroshnichenko *et al.* 2003

Cells are non-motile, Gram-negative rods, and 'rotund bodies' are observed. Moderately thermophilic. Non-sporulating. Microaerophilic. Growth also occurs by anaerobic respiration with nitrate and elemental sulfur. Some species use nitrite as an electron acceptor. MK-8 is the major respiratory quinone. The major cellular fatty acids are iso- and anteiso-branched types. Unsaturated fatty acids were also detected (18–37 %). The genomic DNA G+C content is 62.9–71.1 mol%. The phylogenetic position, based on 16S rRNA gene sequences, is in the family Thermaceae. The type species is *Oceanithermus profundus*.

Description of *Oceanithermus desulfurans* sp. nov.

Oceanithermus desulfurans (de.sul'fu.rans. N.L. part. adj. *desulfurans* making free of/reducing sulfur).

Cells are rods, about 0.5 µm wide and 1.5–2.0 µm long, and 'rotund bodies' are observed under good growth conditions. Gram reaction is negative. Non-motile, non-pigmented and

Table 1. Characteristics of strain St55B^T and *O. profundus*

Data for *O. profundus* 506^T were taken from Miroshnichenko *et al.* (2003a). +, Positive; –, negative. Cells of both taxa are rods, with the formation of 'rotund bodies', and the same results were obtained for both strains for the following characteristics: spore formation (–); pigmentation (–); Gram-staining (–); sensitivity to O₂ (microaerophilic and anaerophilic); major respiratory quinone (MK-8); NaCl concentration for optimum growth (3 %, w/v).

Characteristic	Strain St55B ^T	<i>O. profundus</i> 506 ^T
Habitat	Hydrothermal vent site (Western Pacific)	Hydrothermal vent site (Eastern Pacific)
DNA G + C content (mol%)	71.1	68.6*
Cellular fatty acids:		
Main fatty acids	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , iso-C _{16:1}
Unsaturated fatty acid content (%)	18	37*
Optimum growth conditions:		
Temperature (°C)	60	60
pH	6.5	7.5
Electron acceptors:		
O ₂	+	+
NO ₃ [–]	+	+
NO ₂ [–]	+	–
S ⁰	+	+
Carbon and energy sources		
Yeast extract	+	–
Tryptone	+	+
Casamino acids	+	–
Fructose	–	+
Galactose	–	+
Glucose	–	+
Sucrose	–	+
Xylose	–	+
Acetate	–	+
Propionate	+	+
Pyruvate	+	+
Ethanol	–	+
Methanol	–	+
DNA–DNA hybridization (%) with:		
St55B ^T	100	19
<i>O. profundus</i> 506 ^T	17	100

*Results for G + C content, cellular fatty acids and utilization of elemental sulfur in *O. profundus* 506^T were obtained in this study.

non-sporulating. Chemoheterotrophic. Microaerophilic and facultatively anaerophilic. Anaerobic growth occurs in the presence of elemental sulfur, nitrate or nitrite as an electron acceptor, but sulfate, thiosulfate, sulfite, DMSO, fumarate, Fe(III) citrate and selenate do not support growth. Yeast extract is necessary for growth. Yeast extract, tryptone, Casamino acids, butyrate, formate, glutamate, lactate, propionate, pyruvate, succinate, L-alanine and L-cysteine are used as carbon and energy sources. No growth occurs with H₂/CO₂, acetate + H₂/CO₂, arabinose, fructose, galactose, glucose, inositol, mannose, raffinose, sucrose, xylose, acetate, citrate, fumarate, malate, L-arginine, L-asparagine, L-histidine, L-leucine, L-methionine, L-serine, ethanol, 2-propanol or methanol. Grows at 30–65 °C; optimum growth is at 60 °C. The pH range for growth is 6.0–8.0, with optimum growth at pH 6.5. Growth occurs at

1–5 % (w/v) NaCl and the optimum growth concentration is 3 % (w/v). MK-8 is the major quinone. The major cellular fatty acid is iso-C_{15:0}. Unsaturated fatty acids (18 %) are also present. The genomic DNA G + C content is 71.1 mol%.

The type strain is St55B^T (=NBRC 100063^T=DSM 15757^T), isolated from a chimney in the Suiyo Seamount (at a depth of 1390 m) in the Izu-Bonin Arc, Western Pacific.

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