International Journal of Systematic and Evolutionary Microbiology
June 2009; Volume 59 : Pages 1508-1512
http://dx.doi.org/10.1099/ijs.0.006767-0

Deferribacter autotrophicus sp. nov., an iron(III)-reducing bacterium from a deep-sea hydrothermal vent

G. B. Slobodkina[1, *], T. V. Kolganova[2], N. A. Chernyh[1], J. Querellou[3], E. A. Bonch-Osmolovskaya[1] and A. I. Slobodkin[1]

[1] Winogradsky Institute of Microbiology, Russian Academy of Sciences, Prospect 60-letiya Oktyabrya 7/2, 117312 Moscow, Russia
[2] Bioengineering Center, Russian Academy of Sciences, Prospect 60-letiya Oktyabrya 7/1, 117312 Moscow, Russia
[3] UMR 6197, Microbiology of Extreme Environments, Ifremer, Centre de Brest, 29280 Plouzane, France

*: Corresponding author : G. B. Slobodkina, email address : gslobodkina@mail.ru

Abstract:

A thermophilic, anaerobic, chemolithoautotrophic bacterium (designated strain SL50^T) was isolated from a hydrothermal sample collected at the Mid-Atlantic Ridge from the deepest of the known World ocean hydrothermal fields, Ashadze field (1 ° 58' 21" N 4 ° 51' 47" W) at a depth of 4100 m. Cells of strain SL50^T were motile, straight to bent rods with one polar flagellum, 0.5–0.6 µm in width and 3.0–3.5 µm in length. The temperature range for growth was 25–75 °C, with an optimum at 60 °C. The pH range for growth was 5.0–7.5, with an optimum at pH 6.5. Growth of strain SL50^T was observed at NaCl concentrations ranging from 1.0 to 6.0 % (w/v) with an optimum at 2.5 % (w/v). The generation time under optimal growth conditions for strain SL50^T was 60 min. Strain SL50^T used molecular hydrogen, acetate, lactate, succinate, pyruvate and complex proteinaceous compounds as electron donors, and Fe(III), Mn(IV), nitrate or elemental sulfur as electron acceptors. The G+C content of the DNA of strain SL50^T was 28.7 mol%. 16S rRNA gene sequence analysis revealed that the closest relative of strain SL50^T was *Deferribacter abyssi* JR^T (95.5 % similarity). On the basis of its physiological properties and phylogenetic analyses, the isolate is considered to represent a novel species, for which the name *Deferribacter autotrophicus* sp. nov. is proposed. The type strain is SL50^T (=DSM 21529^T=VKPM B-10097^T). *Deferribacter autotrophicus* sp. nov. is the first described deep-sea bacterium capable of chemolithoautotrophic growth using molecular hydrogen as an electron donor and ferric iron as electron acceptor and CO_2 as the carbon source.

Fe(III)-reducing micro-organisms play an important role in the cycling of carbon and metals in various anaerobic ecosystems including the thermal environments. Thermophilic iron reducers have been found in continental hot springs, deep terrestrial subsurface, and submarine petroleum reservoirs (Slobodkin, 2005). However, the diversity of this group of prokaryotes in deep-sea hydrothermal ecosystems is much less studied (Miroshnichenko and Bonch-Osmolovskaya, 2006). At present thermophilic and hyperthermophilic iron-reducing micro-organisms recovered from deep-sea habitats include three representatives of *Archaea* (Slobodkin et al., 2001, Kashefi et al., 2002, Reysenbach et al., 2006) and two species of *Bacteria*, *Geothermobacter ehrlichii* (Kashefi et al., 2003), and *Deferribacter abyssii* (Miroshnichenko et al., 2003).

The order *Deferribacterales* represents a deep lineage in the domain *Bacteria* (Cole et al., 2007). The family *Deferribacteracea* (Huber & Stetter, 2002) is composed of three genera, *Deferribacter*, *Flexistipes* and *Geovibrio*. Currently, the genus *Deferribacter* consists of three species, *D. thermophilus* (Greene et al., 1997), *D. desulfuricans* (Takai et al., 2003) and *D. abyssii* (Miroshnichenko et al., 2003). The type species of the genus, *D. thermophilus*, was isolated from a high-temperature sea-water-flooded oil reservoir located in the North Sea. The other two micro-organisms were isolated from the deep-sea hydrothermal vents. *D. desulfuricans* was obtained from Suiyo Seamount hydrothermal chimney and *D. abyssii* was isolated from the Rainbow hydrothermal vent field of the Mid-Atlantic Ridge. All described species of the genus *Deferribacter* are strictly anaerobic, thermophilic organisms capable of the oxidation of a variety of complex organic compounds and organic acids in the presence of diverse electron acceptors. In this article, we report the isolation and characterization of a novel species of this genus originating from the deepest of the known World Ocean hydrothermal field.

Strain SL 50^T was isolated from a sample of the fragment of the hydrothermal structure. The sample was collected in March 2007 during the SERPENTINE cruise at the Ashadze hydrothermal field (12 58' 21" N, 44 51' 47" W) on the Mid-Atlantic Ridge at a depth of 4100 m. For the samples collection sterilized microbiological boxes filled with sterile freshwater were prepared onboard. Active chimney samples were collected by the ROV Victor. On site, after opening the box lid, the freshwater was replaced by in situ seawater, the chimney fragment introduced and the lid closed. All following operations were done onboard in sterile conditions. Boxes with samples were stored at 4^{o}C. An enrichment culture was initiated by inoculation of 10% (w/v) of the sample into anaerobically prepared, bicarbonate-buffered, sterile (135^{o}C, 1 h) liquid medium with lactate (1.5 g l^{-1}) as an electron donor and poorly crystalline Fe(III) oxide (90 mM) as an electron acceptor. Medium composition and preparation techniques were described earlier (Slobodkin et al., 1999). Isolate L50 was purified from the enrichment by serial dilution in the same medium. The pure culture was obtained with sodium acetate (18mM) as electron donor and potassium nitrate (10 mM) as electron acceptor by agar-shake dilution technique with an agar block in the tube (1.5% agar in growth medium) in the medium of the following composition (per litre of distilled water): 0.34 g KCl, 4.00 g $MgCl_2.6\ H_2O$, 0.25 g NH_4Cl, 0.14g $CaCl_2.2\ H_2O$, 0.14 g K_2HPO_4, 18.00 g NaCl, 5.00 g $NaHCO_3$, 0.20 g yeast extract (Difco), 0.002 g $Fe(NH_4)_2(SO_4)_2.7\ H_2O$, 1 ml trace-element solution (Slobodkin et al., 1997), 10 ml vitamin solution (Wolin et al., 1963), 0.001 g resazurin, 0.50 g $Na_2S.9\ H_2O$, gas phase CO_2 (100%). Physiological studies on substrate and electron acceptor utilization, temperature, pH and salinity ranges for growth, light and electron microscopy, analytical techniques, DNA extraction and determination of G+C content were performed as described previously (Slobodkin et al., 1999). Growth of the strain with poorly crystalline Fe(III) was determined by direct cells count using light microscopy after dissolving the iron precipitate in solution of ammonium oxalate (28 g l^{-1}) /

93 oxalic acid (15 g l^{-1}). pH measurements and pH-meter calibration were carried out at 60°C.
94 NO, N_2O and N_2 were detected by GC (Molsiv 5A column at 40°C, Ar). Ammonium and
95 nitrite were determined by HPLC with conductivity detector (Aquilon C1P column, 4 mM
96 HNO_3 for ammonium and Aquilon A1.2 column, 3.5 mM carbonate buffer for nitrite). 16S
97 rRNA gene amplification, sequencing and sequence analysis were done as described
98 previously (Zavarzina et al., 2002).
99
100 In agar-shake cultures, brown lens-shaped colonies (0.2 to 0.5 mm in diameter) of strain
101 $SL50^T$ appeared after 7-10 days of incubation at 50°C. Vegetative cells of strain $SL50^T$ were
102 straight to bent rods, 0.5-0.6 μm in diameter and 3.0-3.5 μm in length. The cells occurred
103 singly, in pairs or in short chains, and had one polar flagellum. Spores were not observed.
104 Ultrathin sectioning of strain SL 50^T revealed that the cell wall had a typical Gram-negative
105 structure.
106 The temperature range for growth of strain SL 50^T was 25-75°C, with an optimum at 60°C.
107 No growth was detected at 80°C or at temperatures up to 22°C after incubation for 3 weeks.
108 The pH range for growth was pH 5.0-7.5, with an optimum at pH 6.5. No growth was noticed
109 at pH 4.5 or 8.0. Growth of strain SL 50^T was observed at NaCl concentrations ranging from
110 1.0 to 6.0% (w/v) with an optimum at 2.5% (w/v), but no growth was evident at 0 and 7.0%
111 NaCl (w/v). Yeast extract was not necessary for growth of the strain $SL50^T$ but strongly
112 stimulated it. Potential electron acceptors were tested with sodium acetate (18 mM) or sodium
113 lactate (1.5 g l^{-1}) as an energy source in the presence of 0.20 g l^{-1} yeast extract. Nitrate
114 (potassium salt, 10mM), elemental sulfur (10 g l^{-1}), ferric citrate (5mM), poorly crystalline
115 Fe(III) oxide (90 mM), Mn(IV) supplied as 25 mM of MnO_2 and 9,10-anthraquinone 2,6-
116 disulfonate (AQDS (20 mM)) was used as an electron acceptor for growth of strain SL 50^T.
117 Sulfate (14 mM), thiosulfate and fumarate (20 mM each) were not reduced and did not

support growth. Poorly crystalline Fe(III) oxide was reduced to black magnetic precipitate with high Fe(II) content. During Mn(IV) reduction black insoluble MnO_2 turned to light-brown precipitate that almost disappeared after prolonged incubation. No changes in color and precipitate amount were observed in uninoculated controls with 0.20 gl-1 yeast extract in the growth media containing poorly crystalline Fe(III) oxide, Mn(IV) or AQDS during the incubation period at 60^{o}C. Elemental sulfur was reduced to hydrogen sulfide (Cord-Ruwisch, 1985). Nitrate was reduced to ammonium; NO, N_2O or nitrite were not produced in measurable amounts.

Strain SL 50^{T} was able to grow on peptone, yeast extract, (10 g l^{-1} each), formate, acetate, lactate, pyruvate, fumarate, malate, propionate, succinate, maleinate, maltose (25 mM each) as electron donors and potassium nitrate (10 mM) as electron acceptor in the presence of 0.20 g l^{-1} yeast extract. Maltose was completely oxidized to CO_2 without formation of soluble fermentations products. Strain SL50^{T} could grow chemolithoautotrophically in the absence of yeast extract, using molecular hydrogen as an electron donor and poorly crystalline Fe(III) oxide (90 mM) as electron acceptor and CO_2 as the carbon source. When Mn(IV) was used as an electron acceptor, H_2/CO_2 (80/20 v/v) was utilized and supported growth in the presence of yeast extract (0.20 g l^{-1}). With nitrate or sulfur as an electron acceptor molecular hydrogen did not support the growth neither chemolithoautotrophically nor in a presence of 0.20 g l^{-1} yeast extract. Strain SL 50^{T} was not able to utilize casein, tryptone, starch (10 g l^{-1} each), methanol, ethanol, n-propanol, iso-propanol, n-buthanol, (20 mM each), fructose, xylose, cellobiose, sucrose, L-arabinose (25 mM each), glycerol, butyrate, benzoate (20 mM each) with potassium nitrate (10 mM) as electron acceptor.

The G+C content of the genomic DNA of strain SL 50^{T} was 28.7 mol % (T_m). A comparison of 1543 nucleotides of 16S rDNA sequence of strain SL 50^{T} with those available in GenBank

database showed that strain SL 50^T belonged to the genus *Deferribacter* and related genera (Fig.1). Only 16S rRNA sequences of the type strains of validly published species were included in the analyses. The 16S rRNA sequence of the new isolate had the highest identity with that of *D. abyssi* JR^T (95.5%). The levels of 16S rRNA gene sequence similarity with other members of the genus *Deferribacter* were 94.3-94.6%. The trees constructed by maximum likelihood and by maximum parsimony algorithms displayed the same topology (data not shown). Transversion analysis (Woese *et al*., 1991) did not affect the phylogenetic position of the new strain.

The new isolate described in this report, represents a micro-organism capable of reduction of Fe(III) and Mn(IV) as well as nitrate and sulfur which are also common substances in deep-sea hydrothermal environments. Strain SL 50^T shares many phenotypic features with the described representatives of the genus *Deferribacter* (Table 1). Species of the genus *Deferribacter* with the exception of *D. thermophilu*s, were isolated from the deep-sea hydrothermal vents. According to the electron microscopic analysis all of them have a Gram-negative type of cell wall structure. All *Deferribacter* species are rod shaped cells with polar flagellum growing under anaerobic conditions by the oxidation of a variety of complex organic compounds and organic acids in the presence of diverse electron acceptors and unable to fermentation. However, strain SL 50^T differs from all representatives of the genus *Deferribacter* by its ability to grow chemolithoautotrophically, utilizing hydrogen as an electron donor, CO_2 as a carbon source and poorly crystalline Fe(III) oxide as an electron acceptor. The new isolate has the widest range of utilized electron acceptors in this genus. Unlike the type species of the genus, *D. thermophilu*s, strain SL 50^T is able to reduce sulfur. The ability of the strain SL 50^T to reduce both Fe(III) and Mn(IV) differentiate it from *D. abyssi* which cannot utilize Mn(IV) and *D. desulfuricans* which is unable to use either of

these metals as electron acceptors. In contrast to the type species of the genus and the phylogenetically closest species, *D. abyssi*, isolate SL 50^T can grow on formate and propionate as substrates. Another significant characteristics that differentiate strain SL 50^T from all described representatives of the genus are nitrate reduction to ammonium, not to nitrite and the ability to use disaccharide - maltose as electron donor with nitrate as electron acceptor. On the basis of the phylogenetic, phenotypic, physiological properties that clearly differentiate strain SL 50^T from known species of the genus *Deferribacter*, we propose strain SL 50^T as the type strain of the new species, *Deferribacter autotrophicus*.

Description of *Deferribacter autotrophicus* sp.nov.

Deferribacter autotrophicus (au.to.tro'.phi.cus. N.L. masc. adj. autotrophicus, autotrophic). Cells are motile by means of one polar flagellum, straight to bent rods, 0.4-0.6 μm in diameter and 2.0-3.0 μm in length, spores were not observed. Cells form brown lens-shaped colonies (0.2 to 0.5 mm in diameter) in agar-shake cultures. The temperature range for growth is 25-75^{o}C, with an optimum at 60^{o}C. The pH range for growth is 5.0-7.5, with an optimum at pH 6.5. Growth occurs at NaCl concentrations ranging from 1.0 to 6.0% (w/v) with an optimum at 2.5% (w/v). Anaerobic. Capable of chemolithoautotrophic growth using molecular hydrogen as an electron donor and ferric iron as electron acceptor and CO_2 as the carbon source. Anaerobically oxidizes peptone, yeast extract, (10 g l^{-1} each), formate, acetate, lactate, pyruvate, fumarate, malate, propionate, succinate, maleinate, maltose with sulfur, nitrate, Mn(IV) or Fe(III) as electron acceptor in the presence of 0.20 g l^{-1} yeast extract. Casein, tryptone, starch, methanol, ethanol, n-propanol, iso-propanol, n-buthanol, fructose, xylose, cellobiose, sucrose, L-arabinose, glycerol, butyrate, benzoate are not utilized with nitrate as electron acceptor. Does not reduce fumarate, sulfate, thiosulfate, and oxygen (20%, v/v in the

gas phase). The G+C content of DNA is 28.7 mol % (T_m). Isolated from hydrothermal vent field of the Mid-Atlantic Ridge. The type strain is SL 50^T, that has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen under the accession number DSMZ 21529^T and in the Russian National Collection of Industrial Microorganisms (VKPM) under the accession number VKPM-10097 ^{T}.

ACKNOWLEDGEMENTS

This work was supported by grant 06-04-48684-a from the Russian Foundation for Basic Research and by federal programs “Molecular and cell biology” and “The origin and evolution of the biosphere” of the Russian Academy of Science. We acknowledge the officers and the crew of the R/V Pourquoi Pas? and the ROV Victor operation team. We are also grateful to Dr. Yves Fouquet, Chief Scientist of the SERPENTINE cruise, for his very effective contribution to the success of Ashadze hydrothermal field exploration.

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279 Table 1. Differentiating characteristics for strain SL50 from other *Deferribacter* species.

280

Characteristic	Strain SL50	*D. abyssi*	*D. thermophilus*	*D. desulfuricans*
Temperature range (^{o}C)	25-75	45-65	50-65	40-70
Optimal temperature (^{o}C)	60	60	60	60-65
Electron acceptor:				
Elemental sulfur	+	+	−	+
Fe(III)	+	+	+	−
Mn(IV)	+	−	+	−
Electron donor:				
Ethanol	−	−	−	+
Formate	+	−	−	+
Propionate	+	−	−	+
Malate	+	−	−	+
Maltose	+	−	−	−
Chemolithoautotrophic growth with different electron acceptors:				
Fe(III)	+	−	−	−
Elemental sulfur	−	+	−	−
Potassium nitrate	−	+	−	−
G+C content (mol%)				
	28.7	30.8	34.0	38.6

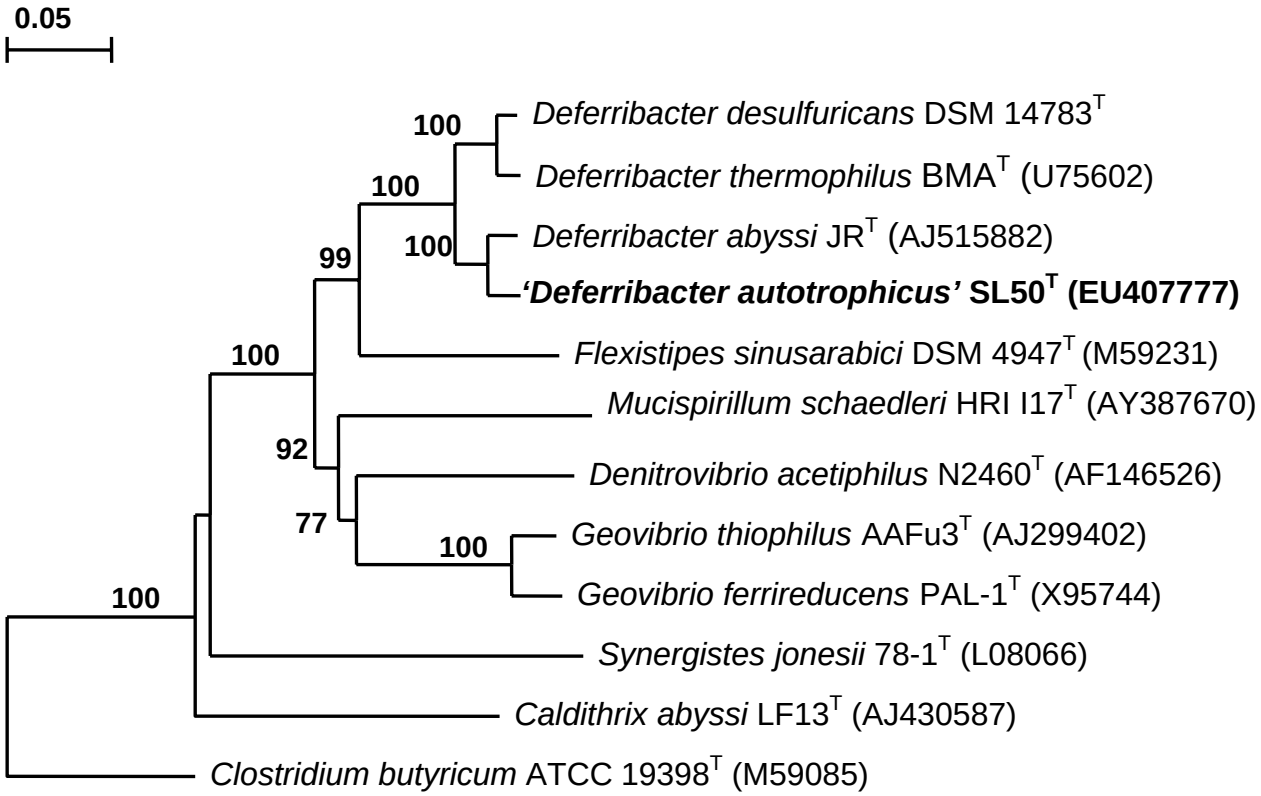
281

282 +, positive; -, negative.

283

Figure Legends

Fig.1. Phylogenetic tree based on 16S rRNA gene sequences indicating the position of isolate SL 50^T within the representative members of the order *Deferribacteres*. The 16S rRNA gene sequence of *Clostridium butyricum* was included as outgroup. GenBank accession numbers are given in parentheses. Bar, 5 substitutions per 100 nt. Only the bootstrap values higher 70% are indicated.


0.05
100
100
100
99
100
92
77
100
100
Deferribacter desulfuricans DSM 14783T
Deferribacter thermophilus BMAT (U75602)
Deferribacter abyssi JRT (AJ515882)
'Deferribacter autotrophicus' SL50T (EU407777)
Flexistipes sinusarabici DSM 4947T (M59231)
Mucispirillum schaedleri HRI I17T (AY387670)
Denitrovibrio acetiphilus N2460T (AF146526)
Geovibrio thiophilus AAFu3T (AJ299402)
Geovibrio ferrireducens PAL-1T (X95744)
Synergistes jonesii 78-1T (L08066)
Caldithrix abyssi LF13T (AJ430587)
Clostridium butyricum ATCC 19398T (M59085)