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EXPERIMENTAL ARTICLES

Desulfacinum subterraneum sp. nov., a New Thermophilic Sulfate-Reducing Bacterium Isolated from a High-Temperature Oil Field

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Abstract—A new thermophilic sulfate-reducing bacterium isolated from the high-temperature White Tiger oil field (Vietnam) is described. Cells of the bacterium are oval (0.4–0.6 by 0.6–1.8 μm), nonmotile, non-spore-forming, and gram-negative. Growth occurs at 45 to 65°C (with an optimum at 60°C) at NaCl concentrations of 0 to 50 g/l. In the course of sulfate reduction, the organism can utilize lactate, pyruvate, malate, fumarate, ethanol, salts of fatty acids (formate, acetate, propionate, butyrate, caproate, palmitate), yeast extract, alanine, serine, cysteine, and $H_2 + CO_2$ (autotrophically). In addition to sulfate, the bacterium can use sulfite, thiosulfate, and elemental sulfur as electron acceptors. In the absence of electron acceptors, the bacterium can ferment pyruvate and yeast extract (a yet unrecognized capacity of sulfate reducers) with the formation of acetate and H_2. The G+C content of DNA is 60.8 mol %. The level of DNA–DNA hybridization of the isolate (strain 101^T) and *Desulfacinum infernum* (strain BαG1^T) is as low as 34%. Analysis of the nucleotide sequence of 16S rDNA places strain 101^T in the phylogenetic cluster of the *Desulfacinum* species within the sulfate reducer subdivision of the delta subclass of Proteobacteria. All these results allowed the bacterium studied to be described as a new species, *Desulfacinum subterraneum* sp. nov., with strain 101 as the type strain.

Key words: thermophile, sulfate reduction, *Desulfacinum subterraneum* sp. nov., autotrophy, oxidation of yeast extract, reduction of elemental sulfur.

Over the last several years, the interest in the microbiology of subsurface ecosystems (*subsurface microbiology*) has considerably increased [1]. Anaerobic microorganisms, including sulfate-reducing bacteria (SRB), are widespread components of subsurface microflora. At great depths, where the temperature is high, thermophilic bacteria reside, whose properties and activities have been insufficiently studied. Their aboriginal nature is currently subject to debate [2]. A list of thermophilic SRB recognized to date can be found in the review by Slobodkin *et al.* [3]. The search for new thermophilic microorganisms remains urgent, since it can result in the isolation of forms with previously unknown properties.

We have studied the high-temperature (100°C and higher) White Tiger oil field (South China Sea, Vietnam), whose exploitation involves its flooding with seawater, which results in somewhat decreased temperatures (50–100°C) in the near-bottom zones of injection wells. Thus, the strata of this oil field exhibit conditions appropriate for the development of thermophilic microorganisms. The formation gas contains low amounts of hydrogen sulfide. SRB are the main representatives of the thermophilic microflora [4].

The aim of the present work was to investigate the properties and provide a description of a new thermophilic SRB that was isolated from the White Tiger oil field.

MATERIALS AND METHODS

The isolation source was a water sample taken during a backflow of an injection well reaching the base rock of the White Tiger oil field. The base rock is fractured granitoid rock containing formation water with a low mineralization [4]. The composition of the sampled water was close to seawater, which must have replaced the formation water in the near-bottom zone of the injection well. The mineralization of the sampled water was 35.5 g/l and the sulfate content was 2.62 g/l.

Cultivation methods and methods of analysis of metabolic products. The utilization of various electron donors and acceptors was determined in batch cultures grown on the mineral base of Widdel marine II medium [5] supplemented with various compounds. The organic substrates tested were sugars, yeast extract, tryptone, ethanol, butanol, methanol, amino acids, ace-

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tate, propionate, butyrate, crotonate (1 g/l), caproate, palmitate (0.4 g/l), lactate, malate, and fumarate (2 g/l). The medium was supplemented with trace elements [6] and vitamins [5]. Cultivation was performed in Hungate tubes at 60°C. The capacity for autotrophic growth was tested in Hungate tubes filled by one-third with Widdel marine II medium and by two-thirds with a mixture of either H_2/CO_2 or N_2/CO_2 (volume ratio, 80 : 20 in both cases). The growth on various media was judged from hydrogen sulfide formation (> 150 mg/l) determined with *N*,*N*-dimethyl-*para*-phenylenediamine [7] in the third culture passage on the given medium.

To establish the salinity range in which growth is possible, Na_2SO_4 in the Widdel marine II medium (2.8 g/l) was replaced with $CaSO_4$ (2.67 g/l). The organic substrate was sodium lactate (2 g/l) plus yeast extract (0.5 g/l). The same substrate was used in the experiments conducted to determine the growth temperature range.

During the fermentation of organic substrates, acetate in the liquid phase and H_2 and CO_2 in the gas phase were analyzed by gas–liquid chromatography (GLC) [8]. To test the capacity for acetogenesis, cultivation was performed in Hungate tubes filled by one-third with the sulfate-free mineral base of Widdel marine II medium under an atmosphere of H_2/CO_2. Acetate was determined by GLC [8]. The same method was used to determine the production of acetate in medium with sulfate and butyrate (1 g/l).

When utilization of electron acceptors was determined, $Na_2S_2O_3$ (1 g/l), Na_2SO_3 (0.5 g/l), S^0 powder (1.5 g/l), or $NaNO_3$ (1 g/l), were substituted for sulfate in butyrate-containing medium. The reduction of sulfur and nitrate was judged from hydrogen sulfide production and ammonium production, respectively [9].

To detect desulfoviridin, the Postgate method [10] was used.

The fine structure of cells was studied as described earlier [11]. Micrographs of ultrathin sections were taken under a JEM 100 electron microscope.

DNA analysis. DNA was isolated from cells by the Marmur procedure [12]. The G+C content was calculated from thermal denaturation curves (recorded on a Pye Unicum SP 1800 spectrophotometer) using the formula G+C (mol %) = T_m – 106.4 [13]. DNA–DNA homology levels were determined using the optical reassociation method [14].

Sequencing of the 16S rRNA gene. Amplification and sequencing of 16S rDNA was performed using primers that are universal for most prokaryotes [15]. The buffer used for amplification was composed of 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.001% gelatin. The reaction mixture (100 μl) contained standard amounts of dNTP and equimolar amounts of the pA and pH' primers. The 30 amplification cycles had the following temperature profile: DNA denaturation at 94°C, 30 s; primer annealing at 40°C, 1 min; elongation at 72°C, 2 min 30 s. The amplified 16S rRNA gene was purified using low-gelling-temperature agarose and Promega columns and sequenced in both directions using forward and reverse universal primers and Sequenase (Biochemicals, Cleveland, Ohio).

The complete sequence of the 16S rRNA gene of strain 101 was deposited in GenBank under the accession number AF 385080.

Analysis of the sequence of the 16S rRNA gene. The obtained nucleotide sequence of the 16S rRNA gene of strain 101 was manually aligned with the 16S rDNA sequences of sulfate-reducer species belonging to the delta subclass of Proteobacteria (the latest GenBank database version was used). The positions in the alignment for which not all nucleotides had been determined were omitted, and the remaining 1405 nucleotides were compared. A phylogenetic tree was constructed using the methods implemented in the TREECON software package [16].

RESULTS

Pure culture isolation. A pure culture of the thermophilic strain 101 was isolated from an enrichment culture obtained on Widdel marine II medium containing 1 g/l of butyrate. The enrichment culture was first grown at 30°C and then transferred to the same medium and incubated at 60°C, which resulted in the domination of the thermophilic component. To complete the isolation, separate colonies grown on solid medium with butyrate at 60°C were transferred to liquid medium. The culture purity was judged from the negative results of peptone and sugar fermentation tests and from the cell morphology examined under a Jeneval (Germany) phase contrast microscope at a magnification of 1000×.

Morphology of strain 101. Cells of strain 101 are oval (0.4–0.6 by 0.6–1.8 μm), nonmotile, and nonspore-forming; they occur singly or in pairs (Fig. 1a). Gram staining is negative. On the whole, the cells are morphologically similar to cells of *Desulfacinum infernum* [17]. The fine structure of the cells that was revealed in thin sections is typical of gram-negative bacteria. Three-layered outer and inner membrane, periplasm, nucleoid, polyribosomes, and inclusions surrounded by a membrane can be seen (Fig. 1b). In the stationary phase, the inclusions were present in all cells. The mechanism of cell division is constriction.

Physiological characteristics. The bacterium formed hydrogen sulfide during cultivation at 45–65°C; the optimal temperature was 60°C. Hydrogen sulfide production was detected at NaCl concentrations from 0 to 5% with an optimum at 0.5%. Sulfate reduction did not require the addition of vitamins to the medium and could occur at the expense of lactate, pyruvate, malate, fumarate, ethanol, alanine, serine, cysteine, acetate, propionate, butyrate, valerate, caproate, and palmitate.

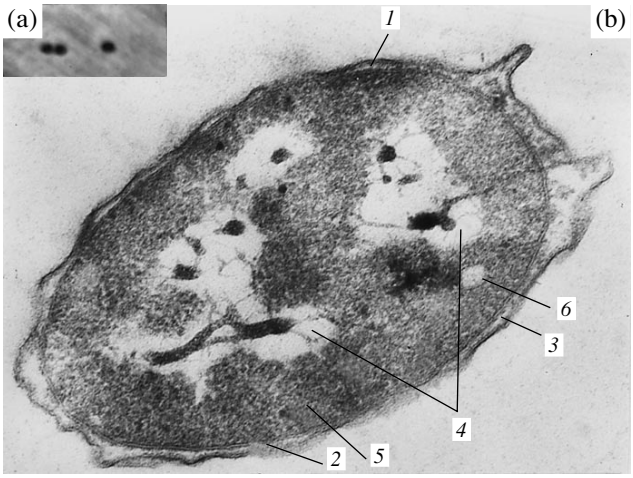

Fig. 1. Morphology of the thermophilic sulfate-reducing bacterial strain 101. (a) Cells under a light microscope, 3000×. (b) Cell ultrastructure under an electron microscope (61950×): *1*, outer membrane; *2*, inner membrane; *3*, periplasm; *4*, the nucleoid zone; *5*, polyribosomes; *6*, an inclusion surrounded by a membrane.

In addition, hydrogen sulfide formation (166 mg/l in 72 h at 60°C) was observed in medium with 1 g/l of yeast extract. The bacterium was able to grow autotrophically, reducing sulfate at the expense of molecular hydrogen. During butyrate utilization (initial concentration, 1 g/l), up to 100 mg/l of H_2S was formed; no acetate accumulation occurred. Growth was not supported by citrate, formate, methanol, benzoate, glycine, phenylalanine, proline, asparagine, valine, arginine, tyrosine, crotonate, glucose, fructose, or tryptone.

In the sulfate-free medium, the bacterium failed to use H_2/CO_2; i.e., it exhibited no acetogenic capacity.

Pyruvate and yeast extract were fermented with the formation of acetate, CO_2, and H_2. The fermentation of yeast extract is a distinguishing characteristic of the bacterium studied. Alanine, tryptone, and glucose were not fermented; however, the addition of tryptone and glucose (1 g/l) to sulfate-free medium containing yeast extract the production of acetate twofold (from 150 mg/l after 7 days to 330 mg/l).

Apart from sulfate, sulfite, thiosulfate, and elemental sulfur could be utilized as electron acceptors. They were reduced to H_2S in butyrate- or lactate-containing media. In medium with lactate and elemental sulfur, 170 mg/l H_2S was formed after 76 h of cultivation. Nitrate did not promote growth, and no production of NH_4^+ was detected. H_2S production occurred at pH 6.5–8.4, with an optimum at 7.2–7.4. The minimum generation time in medium with 2 g/l of lactate and 0.5 g/l of yeast extract was 32 h as determined by the counting of cells under a light microscope. Desulfoviridin was not revealed in the cells.

Genotypic characteristics. The DNA G+C content of cells (grown in medium with 2 g/l lactate and 0.5 g/l yeast extract) was 60.8%. The DNA–DNA hybridization level between strain 101 and the type strain *Desulfacinum infernum* BαG1 did not exceed 34%.

Phylogenetic analysis. A considerable part of the 16S rRNA gene (1446 nucleotides corresponding to *E. coli* positions 22 to 1459) was sequenced. Comparative analysis of 16S rRNA sequences showed (Fig. 2) that strain 101 belongs to the sulfate reducers of the delta subclass of Proteobacteria, exhibiting a 81.3–97.8% similarity with them. It was most close to *Desulfacinum* species (97.8% sequence similarity with the type species of the genus, *D. infernum*, and 96.1% similarity with the recently described *D. hydrothermale* [2]). In the phylogenetic tree, the above two species and strain 101 form a coherent cluster with a high significance of the branching point position (a bootstrap value of 100). Among other genera of the delta subclass sulfate-reducers, *Desulforhabdus amnigenus* and *Thermodesulforhabdus norvegicus* were most close to the cluster under consideration (90.6–91.1 and 89.7–89.9% sequence similarity, respectively). The affiliation of strain 101 to the genus *Desulfacinum* is also substantiated by *Desulfacinum*-specific peculiarities of the 16S rRNA secondary structure (between positions 180 and 200 and positions 820 and 880 in *E. coli* numbering).

DISCUSSION

The thermophilic strain 101 studied in this work is similar to thermophilic eubacteria of the genus *Desulfacinum* in its oval cell shape and cell size. The cells of strain 101 are nonmotile, which is characteristic of *D. infernum* representatives (table). Strain 101, like the thermophilic eubacteria of the genera *Desulfacinum* [2, 17] and *Thermodesulforhabdus* [18] and the mesophilic bacteria of the genus *Desulforhabdus* [19], can reduce sulfates at the expense of a wide range of substrates, such as lactate, ethanol, and fatty acids with both even and odd number of carbon atoms, including acetate. In addition, strain 101 can reduce sulfate at the expense of molecular hydrogen under autotrophic conditions (table). Thus, the bacterium exhibits mixotrophic properties; it produces hydrogen sulfate under both heterotrophic and autotrophic conditions. As distinct from *D. infernum* [18] and *D. hydrothermale* [2], strain 101 could not reduce sulfate in formate-containing medium (table).

Of interest is the ability of strain 101 to reduce sulfate at the expense of the utilization of yeast extract or the amino acids alanine, serine, and cysteine. Alanine is also utilized by other representatives of the genus *Desulfacinum* (table); however, their ability to utilize other amino acids and yeast extract has not been previously studied.

The ability of our novel sulfate-reducing isolate to reduce sulfate at the expense of yeast extract and to fer-

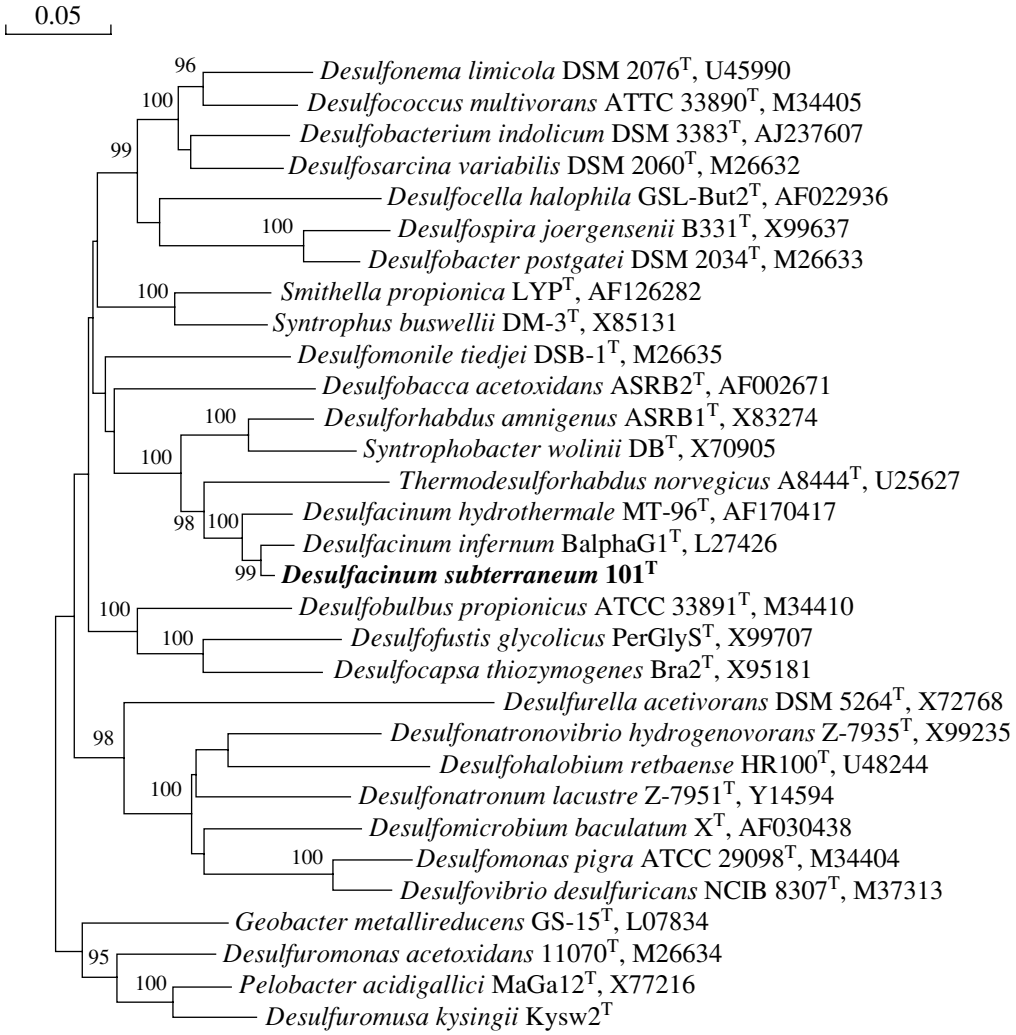

Fig. 2. Phylogenetic tree showing the position of strain 101 among sulfate reducers belonging to the delta subclass of Proteobacteria. The tree was constructed based on comparison of the nucleotide sequences of 16S rRNA genes. *Myxococcus xanthus* DR 1622 was used as the outgroup. Scale bar corresponds to 5 nucleotide substitutions per 100 nucleotides. Numerals show the branching order significance determined by bootstrap analysis (bootstrap values higher than 95% are considered significant).

ment yeast extract (a yet unrecognized capacity of sulfate reducers) can be important for understanding of the processes of bacterial biomass degradation both in sulfate-rich and sulfate-depleted bodies of water.

A distinguishing feature of strain 101 is its capacity for the reduction of elemental sulfur: none of the thermophilic sulfate-reducing bacteria studied so far possesses such a capacity [3].

The DNA–DNA hybridization level of strain 101 and the type species *Desulfacinum infernum* BαG1 was 34%, indicating the affiliation of these organisms to separate species of the same genus. The analysis of nucleotide sequences of the 16S rRNA genes also demonstrated the affiliation of strain 101 with the genus *Desulfacinum* and its nonidentity with the earlier described species of this genus, *D. infernum* and *D. hydrothermale* (96.1–97.8% sequence similarity). All these data allows strain 101 to be described as a new species, *Desulfacinum subterraneum* sp. nov. (Fig. 2).

Currently, the question of the indigenous origin of this oil field microorganism is subject to debate. Magot *et al.* [20], who isolated several sulfate reducers from oil reservoirs, consider the fact that the subsurface anaerobic microflora is well adapted to the salinity and temperature of the habitat to be indicative of its aboriginal nature (i.e., viable bacteria reside in intact unexploited oil reservoirs). It should be, however, recalled that *D. infernum* [17] and *Thermodesulforhabdus norvegicus* [18] were isolated from oil fields flooded with seawater. On the other hand, Sievert and Kuever [2], who described *Desulfacinum hydrothermale*, emphasize that thermophilic eubacteria are peculiar to the subsurface biosphere: they occur in oceanic hydrothermal vents and can be introduced into oil fields flooded

Comparison of strain 101 with two clusters of physiologically similar sulfate-reducing bacteria

Characteristics	Bacteria				
	Desulfacinum infernum [17]	*D. hydrothermale* [2]	*Desulforhabdus amnigenus* [19]	*Thermodesulforhabdus norvegicus* [18]	Strain 101
Morphology:					
cell shape	Oval	Oval	Rod	Rod	Oval
motility	–	+ (one polar)	–	+ (one polar)	–
cell size μm	1.5 × 2.5–3.0	0.8–1.0 × 1.5–2.5	1.4–1.9 × 2.5–3.4	1.0 × 2.5	0.4–0.6 × 0.6–1.8
G+C, mol %	64.0	59.5	52.5	51.0	60.8
Optimal salinity, g/l	10 (0–50)	32–36 (15–78)	0	16	5 (0–50)
Optimal temperature, °C	60 (40–65)	60 (37–64)	37 (25–45)	60 (44–74)	60 (45–70)
Electron donors:					
formate	+	+	+	–	–
acetate	+	+	+	+	+
fatty acids (C number)	3–18	3–5, 7–, 12, 16, 18	3–4	4–18	3–6, 16
ethanol	+	+	+	+	+
lactate	+	+	+	+	+
pyruvate	+	+	+	+	+
fumarate	+	–	–	+	+
malate	+	–	–	+	+
yeast extract	ND	ND	ND	ND	+
alanine	+	+	–	ND	+
serine	ND	ND	ND	ND	+
cysteine	ND	ND	ND	ND	+
$H_2 + CO_2$ (autotr.)	+	+	+	–	+
Fermentation of					
pyruvate	+	+ (weak)	–	–	+ (weak)
yeast extract;	ND	ND	ND	ND	+
Electron acceptors additional to SO_4^{2-}					
SO_3^{2-}	+	+	+	+	+
$S_2O_3^{2-}$	+	+	+	–	+
S^0	–	–	–	–	+
Growth factors required	Vitamins	No	ND	No	No

Note: "–" signifies that the character is negative; "+" signifies that the character is positive; ND means that no data are available.

with seawater. Thermophilic bacteria can also be found in cold marine sediments [21].

Our isolate, strain 101, was not found in the seawater that was injected into the oil field we studied [4]. However, we feel that a discussion of its possible aboriginal nature would be premature until additional data have been accumulated.

Description of *Desulfacinum subterraneum* sp. nov. *Desulfacinum subterraneum* (sub.ter.ra'ne.um. Lat. neutr. adj. *subterraneum*, subsurface). Cells are oval (0.4–0.6 by 0.6–1.8 μm), nonmotile, non-spore-forming. Gram staining is negative. The cell ultrastructure is typical of gram-negative bacteria. Strict anaerobe. Grows by reducing sulfates at the expense of $H_2 + CO_2$, acetate, propionate, butyrate, valerate, caproate, palmitate, lactate, pyruvate, malate, fumarate, ethanol, alanine, serine, cysteine, and yeast extract. During butyrate utilization, up to 100 mg/l of H_2S is formed; no

acetate accumulation occurs. Growth is not supported by formate, methanol, crotonate, benzoate, citrate, glycine, proline, phenylalanine, asparagine, arginine, tyrosine, glucose, fructose, or tryptone. Fermentation of yeast extract and weak fermentation of pyruvate are possible. Sulfite, thiosulfate, and elemental sulfur can be used as electron acceptors in addition to sulfate. Vitamins are not required for growth. Growth occurs in NaCl-free media and at 5% NaCl (with an optimum at 0.5% NaCl), in a temperature range of 45–65°C (with an optimum at 60°C), and a pH range of 6.5–8.4 (with an optimum at 7.2–7.4). The G+C content of DNA is 60.8 mol %.

Isolated from a high-temperature oil field flooded with seawater.

The type strain is 101 (=DSMZ 14185).

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