Desulfacinum infernum gen. nov., sp. nov., a Thermophilic Sulfate-Reducing Bacterium from a Petroleum Reservoir

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A thermophilic sulfate-reducing bacterium, *Desulfacinum infernum*, is described. This bacterium was isolated from produced formation water from a North Sea petroleum reservoir. In liquid culture, the cells are oval, 1.5 by 2.5 to 3 µm, nonmotile and gram negative. Spore formation has not been observed. Growth occurs at temperatures ranging from 40 to 65°C, with optimum growth occurring at 60°C, and at levels of salinity ranging from 0 to 50 g of NaCl per liter, with optimum growth occurring in the presence of 10 g of NaCl per liter. *D. infernum* grows on a range of organic acids, including formate, acetate, butyrate, and palmitate, and alcohols. *D. infernum* can grow autotrophically with H₂. A vitamin supplement is required for growth. Sulfite and thiosulfate are used as electron acceptors. Sulfur and nitrate are not reduced. The DNA base composition is 64 mol% G+C. Phylogenetically, *D. infernum* clusters with members of the delta subdivision of the *Proteobacteria*. Its closest relatives are *Syntrophobacter wolinii* (level of similarity, 90.6%) and *Desulfomonile tiedjei* (level of similarity, 87.1%).

Sulfate-reducing bacterial activity in petroleum reservoirs often is initiated through the introduction of water that is injected to improve oil recovery. This bacterial activity results in the production and accumulation of hydrogen sulfide. Crude oil containing significant levels of hydrogen sulfide is regarded as soured and has greatly reduced commercial value because of increased refining and processing costs. Hydrogen sulfide also has been recognized as a cause of corrosion damage in structures used for oil recovery and storage and as a cause of blockage of formation strata by iron sulfide, resulting in reduced oil production. In addition, hydrogen sulfide is an occupational health and safety hazard which has resulted in death of oil industry workers (2). The problems resulting from hydrogen sulfide production are believed to be primarily caused by introduced sulfate-reducing bacteria (17). However, recent evidence suggests that indigenous sulfate-reducing bacteria may be involved in souring (36).

An understanding of the ecology of sulfate-reducing bacteria is essential if the adverse effects associated with hydrogen sulfide formation are going to be controlled. In this pursuit, a number of sulfate-reducing bacteria have been isolated from petroleum reservoirs. These include thermophilic sulfate-reducing bacteria, such as *Thermodesulfobacterium mobile* (formerly *Desulfovibrio thermophilus* [34]), *Desulfotomaculum* spp. (25, 32), and *Archaeoglobus* spp. (39), and mesophilic sulfate-reducing bacteria, such as *Desulfovibrio* spp. (22, 33), *Desulfobacterium cetonicum* (14), and “*Desulfobacterium oleovorans*” (1), as well as *Desulfomicrobium baculatus* and *Desulfomicrobium apsheronum* (35).

The target reservoir used in this study (Beatrice, United Kingdom, North Sea) has been injected with seawater as a routine oil recovery practice. This has created a temperature gradient (40 to 115°C) between the wells used to inject the seawater and the wells used to produce formation fluids. Continuous injection of seawater has created a cool zone around the injection wells which is expanding toward the

producing wells. The temperature of the reservoir prior to the introduction of seawater was about 115°C.

As part of an on-going study to investigate the microbial ecology of high-temperature petroleum reservoirs, a novel thermophilic, sulfate-reducing bacterium, strain BαG1^T (T = type strain), was isolated from produced formation waters obtained from the Beatrice petroleum reservoir. In this paper we describe strain BαG1^T.

MATERIALS AND METHODS

Bacterial strain. Strain BαG1^T was isolated from produced formation water collected directly from well A08(10) on the Beatrice field platform. The Beatrice oil field is located in the British sector of the North Sea near the coast of Scotland.

Media and culture conditions. Strain BαG1^T was enriched and maintained on Postgate medium B (30) supplemented with 20 g of NaCl per liter and the vitamin solution of Wolin et al. (45). Purification and physiological studies were carried out in bicarbonate-buffered, sulfide-reduced medium containing 20 g of NaCl per liter and 3 g of MgCl₂ · 6H₂O g per liter (42), as well as trace element solution SL-10 (18) and either the vitamin solution of Wolin et al. or 0.1 g of yeast extract per liter. Media were prepared as described by Patel et al. (26) and then reduced after dispensing. Cultures were grown in screw-cap tubes or serum bottles fitted with butyl rubber septa. Substrate tests were carried out in medium supplemented with vitamins, and the preparations were incubated for 2 to 4 weeks. Carbon and energy sources were added to media from sterile stock solutions. Hydrocarbons were filter sterilized directly into culture media, and the bottles were inverted to reduce hydrocarbon contact with the septa. Selenite and tungstate trace element solutions (4) were added when aromatic compounds were used as carbon sources. Conditions for autotrophic growth were established by dispensing 20-ml portions of medium supplemented with vitamins into 125-ml serum bottles containing an 80% N₂-20% CO₂ headspace and overpressuring the medium with 1 atm (101.29 kPa) of 80% H₂-20% CO₂. To confirm autotrophy, strain BαG1^T was subjected to three consecutive transfers (inoculum, 1%) in medium by using the H₂-CO₂ and N₂-CO₂ conditions described above. Unless indicated otherwise, all cultures were incubated at 60°C.

Isolation and purity checks. Enrichment cultures for sulfate-reducing bacteria were prepared by adding produced formation water to culture media. Enrichment cultures that produced sulfide were subcultured into agar shake dilution tubes (2 g of purified agar per liter), and colonies were removed and subcultured until a pure culture was obtained (42).

Purity was checked at 40, 60, and 75°C aerobically on nutrient agar plates (Oxoid) and anaerobically in thioglycolate broth (Difco). In addition, purity was confirmed microscopically by examining cultures growing in anaerobic mineral salts medium containing yeast extract (1 g/liter), pyruvate (10 mM), and glucose (10 mM).

Cellular characterization. Gram-staining characteristics were determined by using the Hucker method as described by Doetsch (11) and KOH lysis (5).

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FIG. 1. Phase-contrast micrograph of strain BαG1^T. Bar = 10 μm.

Escherichia coli and *Staphylococcus aureus* were used as reference microorganisms. Negative stains and thin sections were prepared as described by Patel et al. (27). To facilitate detection of spores, cultures were subjected to heating at 95°C for 5 mins and then transferred to Postgate medium containing lactate and yeast extract. The resulting cultures were incubated for 4 weeks and examined periodically for sulfide production and growth. The Schaeffer-Fulton spore stain and Sudan black lipid stain (11) were used to characterize cell inclusions.

DNA was purified as described by Marmur (23), and the denaturation temperature was used to calculate the DNA base composition (24).

To detect desulfoviridin, cell extracts were examined for an absorption maximum at 630 nm (28). Cells also were examined for the presence of desulfoviridin following alkaline lysis (29).

16S rRNA sequence studies. Purification of genomic DNA and amplification and purification of the 16S rRNA gene were carried out as described previously (21, 31). The purified PCR product was sequenced directly or cloned and sequenced as described previously (21). Sequences were determined with an ABI automated DNA sequencer by using a Prism dideoxy terminator cycle sequencing kit and the recommendations of the manufacturer (Applied Biosystems, Ltd.). The primers used for sequencing have been described previously (31).

Sequence editor ae2 (20) was used to align data obtained from the Ribosomal Database Project and GenBank and the 16S rRNA sequence of strain BαG1^T. Positions at which the sequence and alignment were uncertain were omitted from the analysis. Pairwise evolutionary distances based on 851 unambiguous nucleotides were computed by using the method of Jukes and Cantor (19). Dendrograms were constructed from the evolutionary distances by using the neighbor-joining method. A transversion analysis was performed by using the DNAPARS program. Tree topology was reexamined by using 100 bootstrapped data sets. The computer programs used to do this were SEQBOOT, DNADIST, FITCH, and CONSENSE. These programs are part of the PHYLIP version 3.51c software package (13). Programs available on TREECON (40) also were used to reexamine tree topology.

RESULTS

Isolation of thermophilic sulfate-reducing bacteria. Enrichment cultures containing lactate produced sulfide after 5 to 7 days of incubation at 60°C. Microscopy revealed the presence of vibrios, as well as rod-shaped, spiral, and oval bacteria. The oval organism which dominated some enrichment cultures was purified by serial transfer and was designated strain BαG1^T.

Cellular characteristics and nutrition. Strain BαG1^T cells were oval (1.5 by 2.5 to 3 μm) (Fig. 1). Motility was not observed, and flagella were not seen in negatively stained electron microscopy preparations. Gram staining and the KOH lysis test produced a gram-negative reaction. An examination of thin sections confirmed that this organism has a gram-negative type of cell wall.

Poorly refractile bodies were observed occasionally in sta-

tionary-phase cultures. Cultures containing these cellular inclusions did not survive heating at 95°C for 5 min and did not react with the spore stain. The cellular inclusions did not react with the Sudan black stain for lipids and were not disrupted by centrifugation; thus, these inclusions were not gas vesicles.

Strain BαG1^T cells did not contain desulfoviridin. The DNA base composition was 64 mol% G+C.

Strain BαG1^T did not grow after three successive transfers in medium lacking vitamins. The vitamin requirement of strain BαG1^T could be replaced by 0.1 g of yeast extract per liter.

Physiological characteristics. Strain BαG1^T was able to use the following compounds as electron donors and carbon sources: formate (10 mM), acetate (10 mM), propionate (10 mM), *n*-butyrate (10 mM), isobutyrate (10 mM), *n*-valerate (5 mM), isovalerate (5 mM), octanoate (2.5 mM), decanoate (0.5 mM), dodecanoate (0.5 mM), hexadecanoate (1 mM), octadecanoate (1 mM), lactate (10 mM), pyruvate (10 mM), malate (10 mM), fumarate (5 mM), succinate (5 mM), glycerol (10 mM), ethanol (10 mM), propanol (10 mM), butanol (10 mM), hexanol (5 mM), and alanine (10 mM). Good growth was maintained following subculture into carbon-free medium with a H₂-CO₂ headspace. No growth was detected after a second subculture into carbon-free medium with a N₂-CO₂ headspace.

Growth on octanoate, decanoate, and dodecanoate was dependent on substrate concentration. When octanoate was the substrate, growth was more rapid with 2.5 mM octanoate than with 5 mM octanoate. No growth occurred when 10 mM octanoate was added to media. Growth on decanoate or dodecanoate did not occur when the concentration was greater than 0.5 mM.

Benzoate (5 mM), 3-chlorobenzoate (2 mM), 4-methoxybenzoate (5 mM), cresol (1 mM), citrate (10 mM), glutamate (10 mM), glucose (5 mM), sucrose (5 mM), fructose (5 mM), dodecane (1 mM), hexadecane (1 mM), and crude oil (1 ml/liter) were not utilized.

Strain BαG1^T grew when pyruvate was present and sulfate was absent. No growth occurred on lactate, 4-methoxybenzoate, fumarate, ethanol, or glycerol in the absence of sulfate.

Sulfite and thiosulfate were used as alternative electron acceptors when either lactate, formate, or ethanol was used as a carbon and energy source. Sulfur and nitrate could not be used as electron acceptors.

Growth occurred at temperatures ranging from 40 to 65°C, and optimum growth occurred at 60°C. Growth occurred in media containing 0 to 50 g of NaCl per liter, and optimum growth occurred in medium containing 10 g of NaCl per liter. Growth occurred at pHs ranging from 6.6 to 8.4, and the optimum pH was between 7.1 to 7.5.

Growth on medium containing lactate and 20 mM sulfate was markedly affected by the presence of molybdate. The generation times for strain BαG1^T during normal growth and growth in the presence of 0.01 mM molybdate were 23 and 25 h, respectively. Strain BαG1^T was inhibited completely by 0.03 mM molybdate. The inhibition of growth by molybdate is consistent with the finding that this organism is a sulfate-reducing bacterium.

Phylogeny. A nearly complete sequence (1,544 bases) of the 16S rRNA gene of strain BαG1^T was obtained by using 10 primers. This sequence (positions 8 to 1542; *E. coli* numbering as described by Winker and Woese [44]) had all 70 of the required signature nucleotides and nucleotide pairs which indicate that an organism is a member of the bacterial domain (44). An analysis of the 16S rRNA gene revealed that strain BαG1^T belongs in the delta subdivision of the *Proteobacteria* and that this organism is related to groups 2 to 7 of the sulfate-reducing bacteria (7, 15). Strain BαG1^T also possessed

TABLE 1. Evolutionary distance matrix for strain BαG1^T and related bacteria

Organism	Evolutionary distance from:													
	<i>Escherichia coli</i>	Strain BαG1 ^T	<i>Syntrophobacter wolinii</i>	<i>Desulfomonile tiedjei</i>	<i>Desulfoarculus baarsii</i>	<i>Desulfuromonas acetoxidans</i>	<i>Desulfobacter latus</i>	<i>Desulfobacter postgatei</i>	<i>Desulfobacterium autotrophicum</i>	<i>Desulfobacterium vacuolatum</i>	<i>Desulfococcus multivorans</i>	<i>Desulfobotulus sapovorans</i>	<i>Desulfobulbus propionicus</i>	<i>Desulfovibrio vulgaris</i>
Strain BαG1 ^T	25.5													
<i>Syntrophobacter wolinii</i>	24.2	9.4												
<i>Desulfomonile tiedjei</i>	22.4	12.9	13.5											
<i>Desulfoarculus baarsii</i>	21.8	15.3	14.5	13.3										
<i>Desulfuromonas acetoxidans</i>	21.8	13.6	15.2	13.3	16.3									
<i>Desulfobacter latus</i>	25.2	19.5	19.6	20.1	19.5	17.8								
<i>Desulfobacter postgatei</i>	24.0	16.9	17.8	17.8	18.4	15.2	4.0							
<i>Desulfobacterium autotrophicum</i>	23.6	17.2	16.5	16.3	18.3	14.0	11.5	111.1						
<i>Desulfobacterium vacuolatum</i>	26.5	18.6	20.1	18.7	18.9	17.2	13.1	12.1	6.8					
<i>Desulfococcus multivorans</i>	21.6	14.5	15.0	14.0	13.8	16.1	15.3	14.2	12.9	13.8				
<i>Desulfobotulus sapovorans</i>	23.2	17.2	17.4	17.1	16.1	16.2	16.6	14.8	12.4	14.2	11.0			
<i>Desulfobulbus propionicus</i>	22.6	14.3	15.9	15.5	17.2	13.6	18.1	15.8	16.8	19.5	17.2	18.3		
<i>Desulfovibrio vulgaris</i>	21.6	19.9	20.4	21.6	16.8	18.1	21.8	20.7	19.9	21.0	19.2	18.6	19.3	
<i>Desulfomicrobium baculatus</i>	20.9	18.3	19.0	19.9	18.9	17.4	18.4	16.2	16.6	18.6	16.2	17.4	17.8	15.0

the signature sequences which define the phylum *Proteobacteria* (37). The most closely related organisms were *Syntrophobacter wolinii* and *Desulfomonile tiedjei* (levels of similarity, 90.6 and 87.1%, respectively). Strain BαG1^T was distantly related to members of other groups (Table 1). Relationships derived from sequence data are shown in Fig. 2.

The base composition of the 16S rRNA gene of strain BαG1^T was 58.7 mol% G+C; this value is similar to the values obtained for *Syntrophobacter wolinii* (57.5 mol% G+C), *Desulfoarculus baarsii* (56 mol% G+C), and *Desulfomonile tiedjei* (55.3 mol% G+C). The average level of similarity between the 16S rRNA G+C content of strain BαG1^T and the 16S rRNA G+C contents of the other sulfate-reducing bacteria in Table 1 was only 52.5%; consequently, a bias in tree construction can be introduced. No change in tree topology was observed when

we used transversion analysis. In addition, a parsimony analysis produced the same tree topology. The bootstrap analysis revealed a robust relationship (100%).

Two important differences between the 16S rRNA sequence of strain BαG1^T and the 16S rRNA sequences of all other members of the delta subdivision of the *Proteobacteria* were (i) an absence of the common signature sequences in strain BαG1^T (8, 9), and (ii) a difference in the higher-order structure at positions 179 to 190 (*E. coli* numbering of Winker and Woese [44]).

DISCUSSION

On the basis of 16S rRNA sequence data, strain BαG1^T falls into the delta subdivision of the *Proteobacteria* and clusters with members of the family *Desulfobacteriaceae* (as proposed by Widdel and Bak [42]). The closest relatives of strain BαG1^T are *Desulfomonile tiedjei* (10) and *Syntrophobacter wolinii* (15). Previously described thermophilic sulfate reducers either branch near the root of the eubacterial tree (16, 43) or are members of the *Archaea* (38). There also are thermophilic members of the genus *Desulfotomaculum* which branch with the gram-positive line of eubacteria (41). In contrast, strain BαG1^T is related to mesophilic gram-negative sulfate-reducing bacteria (7) which belong to the delta subdivision of the *Proteobacteria*.

Strain BαG1^T was isolated by enrichment with lactate and could grow on a range of fatty acids, including acetate and propionate. Compounds such as acetate and propionate are known to occur in oil field waters (6) and therefore are more likely to be the substrates used by BαG1^T in situ.

Strain BαG1^T differs physiologically and morphologically from its closest phylogenetic relatives in several respects. Strain BαG1^T is thermophilic, whereas its relatives are mesophilic. It differs from *Desulfomonile tiedjei* by having a higher DNA G+C content, by not producing desulfovibridin, by not having a "collared" morphology, and by not being able to use benzoate, demethylate methoxybenzoate, or dechlorinate 3-chlorobenzoate. *Syntrophobacter wolinii* originally was classified on the basis of its morphology and ability to grow only on

FIG. 2. Dendrogram indicating the position of strain BαG1^T among the sulfate-reducing bacteria belonging to the delta subdivision of the *Proteobacteria*. The dendrogram was derived from the distance matrix shown in Table 1. Scale bar = 10 substitutions per 100 nucleotides.

propionate in syntrophic association with an H_2 -using organism (3). Since the original classification, Harmsen et al. have described a purified culture of *Syntrophobacter wolinii* that grows on pyruvate (15); the ability to oxidize propionate to acetate by using sulfate as an electron acceptor also has been reported (12). The metabolic capabilities of *Syntrophobacter wolinii* are not understood fully; however, physiologically this organism appears to differ significantly from strain BαG1^T. Strain BαG1^T was similar to *Desulfoarculus baarsii* the following characteristics: DNA G+C content, ability to use monocarboxylic acids with 3 to 18 carbon atoms, and absence of desulfovibrin. Strain BαG1^T differed from *Desulfoarculus baarsii* in its ability to grow autotrophically with H_2 and its ability to use a range of dicarboxylic acids and alcohols as electron donors. On the basis of the results of phylogenetic analyses and physiological studies, we propose that strain BαG1^T represents a new species of a new genus. We propose the name *Desulfacinum infernum* gen. nov., sp. nov. for this organism.

Description of *Desulfacinum* gen. nov. *Desulfacinum* (De. sulf.a.ci'num. L. pref. *de*, from; L. n. *sulfur*, sulfur; L. n. *acinum*, a berry [especially a grape]; L. n. *Desulfacinum*, a berry-shaped sulfate-reducing bacterium). Oval cells which are nonmotile. Strict anaerobes which use sulfate as an electron acceptor; the sulfate is reduced to sulfide. Nutritionally versatile, oxidizing a number of organic acids and alcohols. The genus *Desulfacinum* belongs to the delta subunit of the *Proteobacteria*. The closest relatives are *Syntrophobacter wolinii* and the genus *Desulfomonile*.

Description of *Desulfacinum infernum* sp. nov. *Desulfacinum infernum* (in. fer' num. L. adj. *infernum*, that which is, or comes from, below, especially the lower world). Oval cells that are 1.5 by 2.5 to 3 μ m. Nonmotile. Gram-negative type of cell wall structure. Vitamins are required for growth. Temperature range, 40 to 65°C. Optimum growth occurs at 60°C. pH range, 6.6 to 8.4. The optimum pH is between 7.1 and 7.5. Salinity range, 0 to 50 g of NaCl per liter. Optimum growth occurs in the presence of 10 g of NaCl per liter. Formate, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, hexanoate, octanoate, decanoate, dodecanoate, hexadecanoate, octadecanoate, lactate, pyruvate, malate, fumarate, succinate, glycerol, ethanol, propanol, butanol, hexanol, and alanine are used as electron donors and carbon sources. Autotrophic growth occurs with H_2 -CO₂. Benzoate, cresol, citrate, glutamate, glucose, sucrose, fructose, dodecane, and hexadecane are not used. Sulfate, sulfite, and thiosulfate are used as electron acceptors and are reduced to sulfide. DNA base composition, 64 mol% G+C (as determined by thermal denaturation). The habitat is a high-temperature, seawater-flooded petroleum reservoir in the British sector of the North Sea.

The type strain is BαG1, which has been deposited in the Australian Collection of Microorganisms, University of Queensland, Brisbane, Australia, under accession number ACM 3991.

ACKNOWLEDGMENTS

This work was funded by Live Oil Services N.V., Melbourne, Australia.

We thank J. Bruce for technical assistance, The Australian National University and Griffith University for use of electron microscopy facilities, and the assistance of workers at British Petroleum Exploration (UK) for sample collection.

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