academicJournals

Vol. 7(46), pp. 5219-5226, 21 November, 2013
DOI: 10.5897/AJMR2013.6172
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Thermoanaerobacter spp. recovered from hot produced water from the Thar Jath oil-field in South Sudan

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Accepted 8 October, 2013

Two bacterial isolates, designated S1.1 and S3.1, were recovered from oil-well produced water extracted from a deep and hot oil-well in the Thar Jath oil-field in South Sudan, and characterized. Based on their 16S rRNA gene sequences and phenotypic properties, the isolates were identified as members of the genus *Thermoanaerobacter*, possibly representing novel species. Both strains are strict anaerobes, grow optimally at 65 – 70°C at neutral pH and with ~0.3% NaCl, and can use various carbohydrates, proteinaceous compounds and organic acids as growth substrates. Strain S1.1 differs from strain S3.1 in its ability to grow on xylan and D-ribose, and S3.1 from S1.1 in its capacity to grow on acetate, arabinose, cellulose and lactate. Both produced acetate, ethanol, carbon dioxide and hydrogen as fermentation by-products from glucose, and growth was stimulated by thiosulphate. Strain S1.1 actively reduced Fe(III) as revealed by the formation of a dark paramagnetic precipitate and increased growth in cultures supplemented with Fe_2O_3. Cultures of both strains survived autoclaving at 121°C for 40 min, suggesting the formation of extremely heat-resistant endospores.

Key words: *Thermoanaerobacter*, oil-well, produced water, deep biosphere, petroleum microbiology, thermophiles, Thar Jath.

INTRODUCTION

Deep Oil-field reservoirs represent extreme anaerobic environments with high temperature and pressure, toxic compounds, and limited nutrient availability. Nevertheless, a number of anaerobic bacteria and archaeal isolates believed to be indigenous to these environments have been recovered and described, including fermentative organisms, methanogens, metal reducers, acetogens, and sulphate and nitrate reducers (Birkeland, 2004; Magot et al., 2000). Samples from oil-field reservoirs are usually taken from the well-head or at subsequent points in the production pipeline and the possible sources of sample contamination are therefore numerous (Magot et al., 2000; McInerney and Sublette, 1997). Contaminants can also be introduced into the production systems and oil-wells through the drilling process, well operations and from water re-injection of the wells. The frequent recovery of certain thermophiles, including members of the order Thermotogales, the fermentative genera *Thermoanaerobacter* and *Thermoanaerobacterium* and gram-positive sulphate-reducing *Desulfotomaculum* species from geographically widely separated high-temperature reservoirs, is taken as evidence for an indigenous microbial community in these habitats. This is also supported by the fact that members of the genera *Geotoga*, *Petrotoga* and *Thermovirga* have only been obtained from oil-well produced waters (Dahle and Birkeland, 2006; Davey et al., 1993; Lien et al., 1998; Miranda-Tello et al., 2007; Miranda-Tello et al., 2004). Subsurface microbial communities could represent a major part of the biosphere and

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possibly serve as analogous models for extraterrestrial life (Fredrickson and Balkwill, 2006). The main primary energy source for this microbial community is still uncertain, and information on the biogeochemical cycling of carbon and other nutrients is very limited. In reservoirs with temperatures below 80°C, the hydrocarbons tend to have been partially biodegraded over geological time scales (Head et al., 2003), but up to now, microorganisms capable of degrading hydrocarbons anaerobically under *in situ* conditions have not been identified. A number of microbial diversity analyses of high-temperature oil reservoirs using cultivation-independent methods have been carried out (Dahle et al., 2008; de Oliveira et al., 2008; Kaster et al., 2009; Kotlar et al., 2011; Li et al., 2007a; Li et al., 2006; Li et al., 2007b; Orphan et al., 2000; Sette et al., 2007), that basically confirm the presence of a diverse community including extremophilic populations unique to oil-bearing strata. Here we describe two thermophilic Gram-positive anaerobes belonging to the genus *Thermoanaerobacter* isolated from hot produced water from the Thar Jath oil-field in South Sudan. Members of this genus are common in oilfields worldwide and thus represent interesting organisms for analysis of bio-geographical structuring and special adaptations to this extreme deep biosphere habitat.

MATERIALS AND METHODS

Enrichment and isolation

Produced water from the Thar Jath oil-field (Block 5A) in the Unity State, South Sudan, was sampled from a sedimentation tank in December 2006. The water was collected in 100 ml sterile and anaerobic serum bottles and transported to Bergen, where they were kept at ~5°C. The reservoir, with an *in situ* temperature of ~70°C, had not been injected with any chemicals or reinjection water and had been operating for 6 months prior to sampling. Enrichment and growth was performed using an anaerobically prepared basal mineral salts medium (MMF) containing the following components (l^{-1} distilled water): 3 g NaCl, 0.7 g $MgSO_4$, 0.37 g KCl, 0.16 g NH_4Cl, 0.16 g $CaCl_2$, KH_2PO_4, 1 ml trace element solution SL-10 (Widdel et al., 1983), and 0.5 ml resazurin (0.02%). Following autoclaving in a dispenser (Lien and Beeder, 1997) and cooling to ~60°C under continuous flushing with dinitrogen, the medium was reduced by addition of 4 ml 0.5 M Na_2S (Lien et al., 1998). 10 ml of a vitamin solution (Balch et al., 1979) was added and the pH was adjusted to 6.8 with 1 M HCl. The medium was dispensed into 50 ml serum bottles. Substrates that are peptone, yeast extract and dextrin were added from stock solutions to give final concentrations of 0.3% (w/v) each prior to inoculation with 10% produced water. Pure cultures were obtained by dilution series using the shake tube culture method (Widdel and Pfennig, 1984) with anoxic Gelrite gellan gum (0.3%; Merck) as gelling agent.

Microscopy

Cells were observed with an Eclipse E400 (Nikon) phase microscope to determine purity, morphology and Gram staining.

Growth and metabolism

Growth was determined by monitoring increase in OD_{600} and total cell counts. For testing of substrate utilization, a fresh overnight culture that had been passed for at least five times in MMF containing 0.05% yeast extract (Sigma), was used as inoculum (10%). Growth experiments were performed in triplicates in MMF using 0.5% (w/v, final concentration) of the substrates as listed in Table 1, except for the following: yeast extract was used at 0.25% final concentration, lactate, sodium acetate and pyruvate at 40 mM. The polysaccharides used as substrates were maltodextrin from maize (Dextrin 20, Fluka), microcrystalline α-cellulose (Sigma) and xylan from birchwood (Sigma). Organic acids and alcohols, and hydrogen and carbon dioxide were determined using an HP 5890A and HP 6890 GC, respectively (Dahle and Birkeland, 2006). Growth rates at 45, 55, 65, 70, 75 and 80°C, and optimal pH and salinity, were determined in triplicates in MMF medium containing 0.5% glucose. Iron reduction was tested in medium supplemented with amorphic Fe(III) oxide (Slobodkin and Wiegel, 1997).

16S rRNA gene sequence determination and analysis

Genomic DNA was isolated using the cetyltrimethylammonium bromide method as modified by Lien et al. (1998). The 16S rRNA sequence was amplified using PCR with the universal primers, 5'-AGA GTT TGA TCC TGG CTC AG-3' (*Escherichia coli* 16S rRNA positions 8 to 27) and 5'-AAG GAG GTG ATC CAG CCG CA-3' (*E. coli* 16S rRNA positions 1541 to 1522) (Loffler et al., 2000). The PCR was performed with an initial denaturation at 96°C for 3 min, followed by 30 cycles of denaturation at 96°C for 45 s, annealing at 55°C for 30 s, extension at 72°C for 2.5 min and, finally, an extension at 72°C for 10 min. Purification and sequencing of the PCR product were performed as described by Dahle and Birkeland (2006).

The 16S rRNA gene sequences were compared with other sequences in the GenBank database using BLAST (Altschul et al., 1997). Alignments were made using CLUSTAL X (Thompson et al., 1997). The phylogenetic tree was constructed using the neighbor-joining algorithm as implemented in MEGA5 (Tamura et al., 2011).

RESULTS

Enrichment and isolation

Following overnight incubation at 70°C with a mixture of peptone, yeast extract and dextrin as substrates, the primary enrichment cultures became strongly turbid, with a dominance of rod- shaped bacteria. Enrichments incubated at 45°C in the same medium did not yield any visible growth. Dilution series prepared in Gelrite yielded single colonies in tubes diluted up to 10^8-fold after 3 – 5 days of incubation. Ten colonies were picked and successfully regrown in liquid medium. They were all pure cultures as revealed with phase contrast microscopy and sequencing of the 16S rRNA genes.

Phylogenetic analyses

The isolates were grouped into 2 OTUs sharing 95.5% sequence identity. Sequence identities within the groups were 100%, demonstrating that each group represented a distinct species. One isolate from each group, termed S1.1 and S3.1 (NCBI accession numbers KC994642 and KC994643, respectively), were chosen for further

Table 1. Substrate utilization range for strains S1.1 and S3.1 in comparison to their closest phylogenetic relatives shown in Figure 1.

Substrate	S1.1	*T. b. b*	*T. b. f*	*T. b. l*	*T. e*	*T. p*	S3.1	*T. i*	*T. m. m*	*T. m. a.*	*T. t.*
Acetate	-	N	N	N	N	N	+	N	N	N	N
Arabinose	-	-	-	-	N	N	+	+	+	+	+
Casamino acids	+	N	N	N	N	N	+	N	N	N	N
Cellobiose	+	N	N	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	+	-	-	-	+
D-Fructose	+	N	N	+	+	N	+	+	+	+	+
Galactose	+	+	+	+	+	N	+	+	-	+	+
D-Glucose	+	+	N	+	+	+	+	+	+	+	+
M-Inositol	+	N	N	N	-	N	+	N	-	N	N
Lactate	-	N	N	N	N	N	+	N	N	N	N
Lactose	+	N	N	+	+	N	+	+	+	N	+
Maltodextrin	+	N	N	N	N	N	+	N	N	N	N
Maltose	+	N	N	+	N	+	+	+	N	N	+
Mannitol	+	N	+	+	-	N	+	N	+	-	-
Mannose	+	-	+	+	+	N	+	N	+	N	+
Melezitose	+	N	N	N	-	N	+	+	+	-	-
Melibiose	+	N	N	-	-	N	+	+	+	-	-
Peptone	+	N	N	N	+	N	+	N	N	N	N
Pyruvate	+	N	N	+	+	N	+	N	N	N	N
L-Rhamnose	+	N	N	-	-	N	+	N	-	N	+
D-Ribose	+	N	N	+	+	N	-	N	+	+	-
Salicin	+	N	N	N	N	N	+	N	-	N	+
Sorbitol	+	N	N	N	-	N	+	N	-	N	+
Sucrose	+	N	N	+	+	+	+	+	+	+	-
Xylan	+	+	N	N	N	N	-	N	+	N	+
D-Xylose	+	-	N	+	+	+	+	N	+	N	N
Yeast extract	+	-	N	+	-	N	+	N	N	-	N

*N, not determined; +, growth; – , no growth; *T.m.m., Thermoanaerobacter mathranii* subsp. *mathranii* (Larsen et al., 1997); *T.m.a., Thermoanaerobacter mathranii* subsp. *Alimentarius* (Carlier et al., 2006); *T.t.*, *Thermoanaerobacter thermocopriae* (Collins et al., 1994); *T.i., Thermoanaerobacter italicus* (Kozianowski et al., 1997); *T.e, Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl, 1981); *T.p., Thermoanaerobacter pseudethanolicus* (Onyenwoke et al., 2007); *T.b.b., Thermoanaerobacter brockii* subsp. *brockii* (Cayol et al., 1995); *T.b.f., Thermoanaerobacter brockii* subsp. *finii* (Cayol et al., 1995); *T.b.l, Thermoanaerobacter brockii* subsp. *lactiethylicus* (Cayol et al., 1995). References are given in brackets. The dotted vertical line separates strain S1.1 and S3.1 and their respective closest relatives. The substrate utilization data for previously described species and subspecies are provided in the above citations.

analysis. They shared 99.3% 16S rRNA gene sequence identity with *Thermoanaerobacter ethanolicus* and *Thermoanaerobacter pseudoethanolicus*, and 99.1% with *Thermoanaerobacter mathranii*, respectively, as closest hits in Blast searches. Construction of a phylogenetic tree encompassing all the validly described species of the *Thermoanaereobacter* genus, placed strain S3.1 in a distinct lineage branching between *T. mathranii* and *Thermoanaerobacter italicus*, with a significant bootstrap value (Figure 1). Strain S1.1 branched within the *Thermoanaerobacter brockiil T. ethanolicus* clade, but with a low bootstrap value.

Morphology

Phase contrast microscopy of both cultures in exponential growth revealed rod-shaped cells, sometimes in chains (Figure 2). The cell size varied from 0.3 - 0.5 µm in width to 1 – 20 µm in length.

Motility was observed for both strains. Strain S3.1 formed highly refractive intra- and extracellular particles that could represent sulphur granules and/or endospores (Figure 2). Strain S1.1 formed terminal spore-like structures. Cultures of both strains survived autoclaving at 121°C for 40 min, indicating endospore-forming capa-

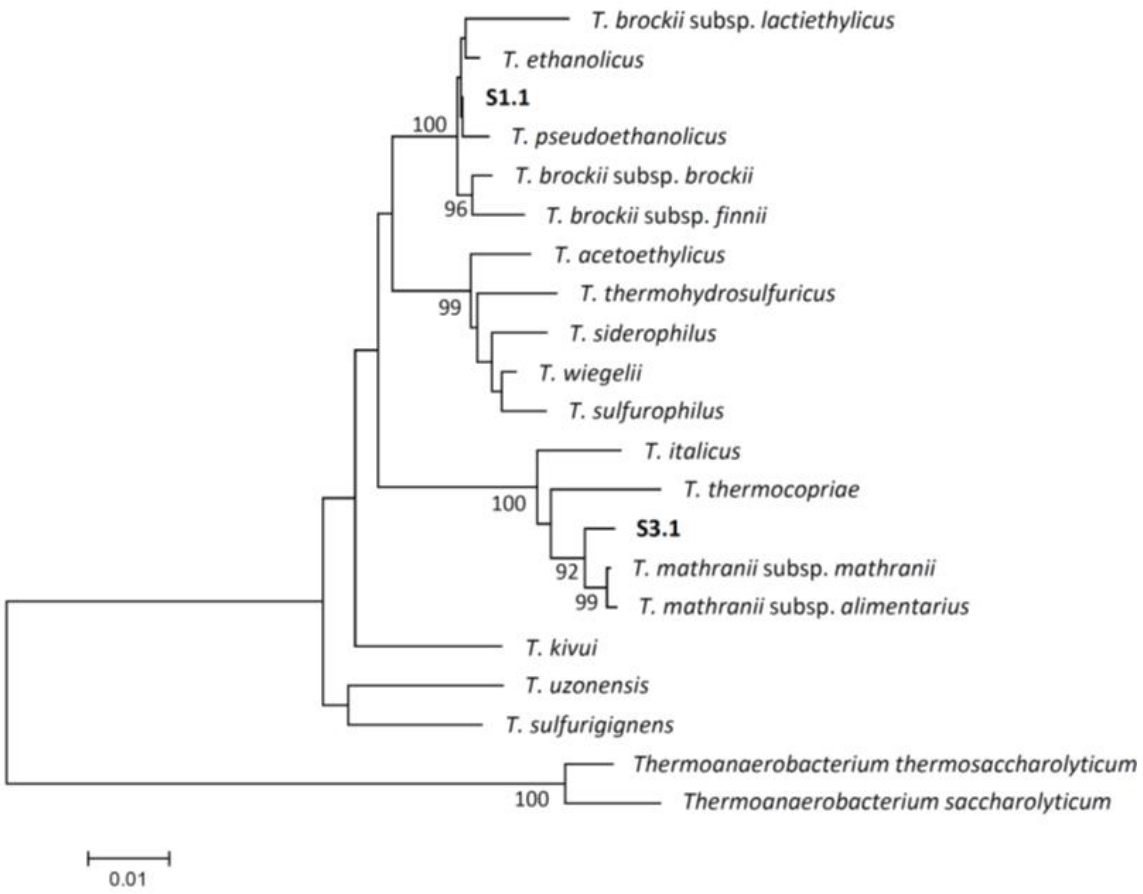

Figure 1. Phylogenetic dendrogram based on 16S rRNA gene sequences indicating the position of strains S1.1 and S3.1 within the *Thermoanaerobacter* genus. Only type strains of validly described species are included. Accession numbers for the sequences used in the analysis are as follows: Strain S1.1, Strain S3.1, *T. acetoethylicus,* L09163*; T. brockii subsp. brockii,* L09165*; T. brockii subsp. finnii,* CP002466; *T. brockii subsp. lactiethylicus,* U14330; *T. ethanolicus,* L09162; *T. italicus,* AJ250846; *T. kivui,* L09160; *T. mathranii subsp. alimentarius,* AY701758; *T. mathranii subsp. mathranii,* Y11279; *T. pseudoethanolicus,* CP000924; *T. siderophilus,* F120479; *T. sulfurigignens,* AF234164; *T. sulfurophilus,* Y16940; *T. thermocopriae,* L09167; *T. thermohydrosulfuricus,* L09161; *T. uzonensis,* EF530067; *T. wiegelii,* X92513. The 16S rRNA gene sequences of *Thermoanaerobacterium saccharolyticum* and *Thermoanaerobacterium thermosaccharolyticum* were used as outgroup. Bootstrap values ≥92% are indicated at nodes. The bar indicates the number of base substitutions per site.

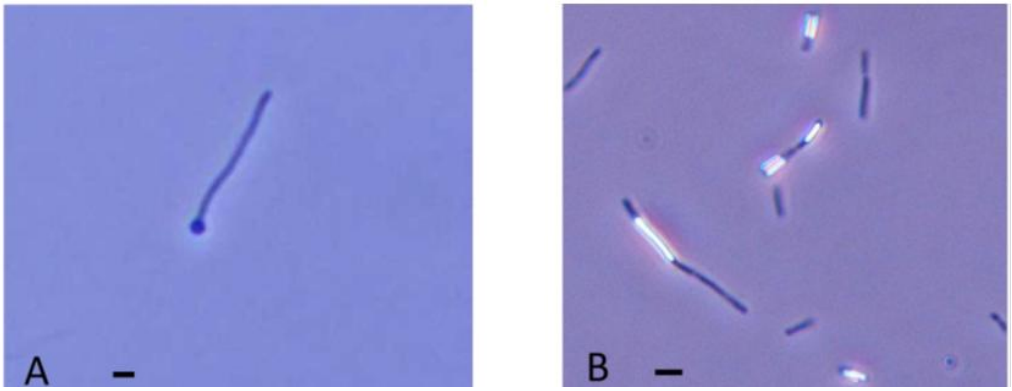

Figure 2. Phase contrast microscopy of (A) strain S1.1 indicating the presence of a terminal spore-like structure, and (B) strain S3.1 indicating the presence of sulphur granules. The size bar indicates 1 µm.

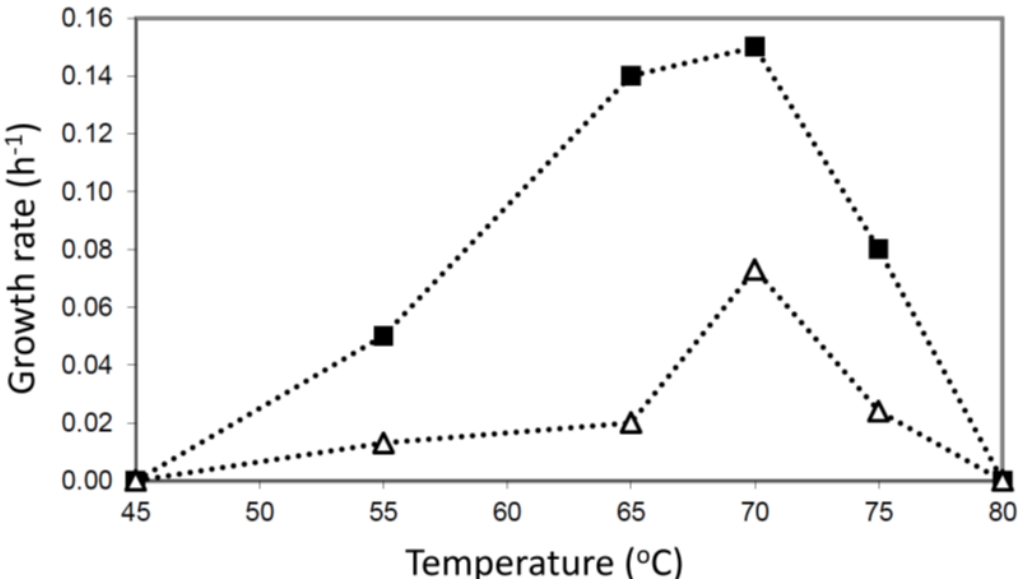

Figure 3. Growth rate of strain S1.1 (Δ) and S3.1 (▪) as a function of temperature in the 45 – 80°C temperature range.

bility. Both strains stained Gram-positive in early exponential growth phase while Gram-variable in late exponential phase and yielded a negative result for the KOH-test (Gregersen, 1978), indicating a Gram-positive cell wall structure.

Physiology

The isolates only grew under strict anaerobic conditions, with optimal growth between 65 - 70°C (Figure 3). Generation times were determined as 2 and 6 h for strain S3.1 and S1.1, respectively, at optimal temperature with glucose as carbon source. The strains were tested for growth on a large variety of carbon sources and were found to be extremely versatile. They could utilize a large number of carbohydrates, proteinaceous compounds and organic acids as substrates (Table 1). Strain S1.1 differed from strain S3.1 in its ability to grow on xylan and D-ribose, and S3.1 from S1.1 in capability to grow on acetate, arabinose, cellulose and lactate. Both strains displayed a fermentative metabolism, producing acetate, ethanol, carbon dioxide and hydrogen as fermentation products when grown on glucose. Growth of strain S3.1 was weakly stimulated by the addition of thiosulphate, which led to formation of suphur granules (Figure 2B). When strain S1.1 was grown on pyruvate, addition of Fe_2O_3 stimulated growth significantly, and the cultures yielded a strongly paramagnetic precipitate (Figure 4), indicating the capability of an iron-reducing anaerobic respiration. Growth of strain S3.1 was only slightly stimulated by Fe_2O_3 addition, and the development of paramagnetism was marginal.

DISCUSSION

The two *Thermoanaerobacter* spp. strains, designated S1.1 and S3.1, isolated from produced water from the deep and hot That Jath oil-field in South Sudan showed distinct but highly diverse substrate utilization patterns (Table 1). Out of 27 substrates tested, growth was observed on 21 substrates for both strains. Six substrates: acetate, arabinose, cellulose, lactate, ribose and xylan, were differentially utilized. A phylogenetic analysis placed strain S3.1 in a branch most closely related to *T. mathranii*, while the phylogenetic affiliation of strain S1.1 was less clear except for a close relationship to *T. ethanolicus* and *T. pseudoethanolicus* (Figure 1). S3.1 differs from *T. mathranii* subsp. *mathranii* and *T. mathranii* subsp. *alimentarius* by its ability to utilize cellulose, while many of the other substrates have not been tested for the *T. mathranii* subspecies, and a firm differentiation between *T. mathranii* and S3.1 based on substrate utilization thus cannot be made. Although strain S1.1 is phylogenetically closely related to *T. ethanolicus*, it differs significantly from the latter in substrate utilization pattern. S1.1, in contrast to *T. ethanolicus*, can utilize M-inositol, mannitol, melezitose, melibiose, L-rhamnose, sorbitol and yeast extract for growth. However with regards to substrate utilization, *T. pseudoethanolicus* has not been adequately characterized to be properly compared with strain S1.1. The results of phylogenetic and physiological analyses of S1.1 and S3.1 indicate that these isolates represent novel species, although a more thorough comparison with their closest relatives, using techniques such as genomic DNA: DNA hybridization

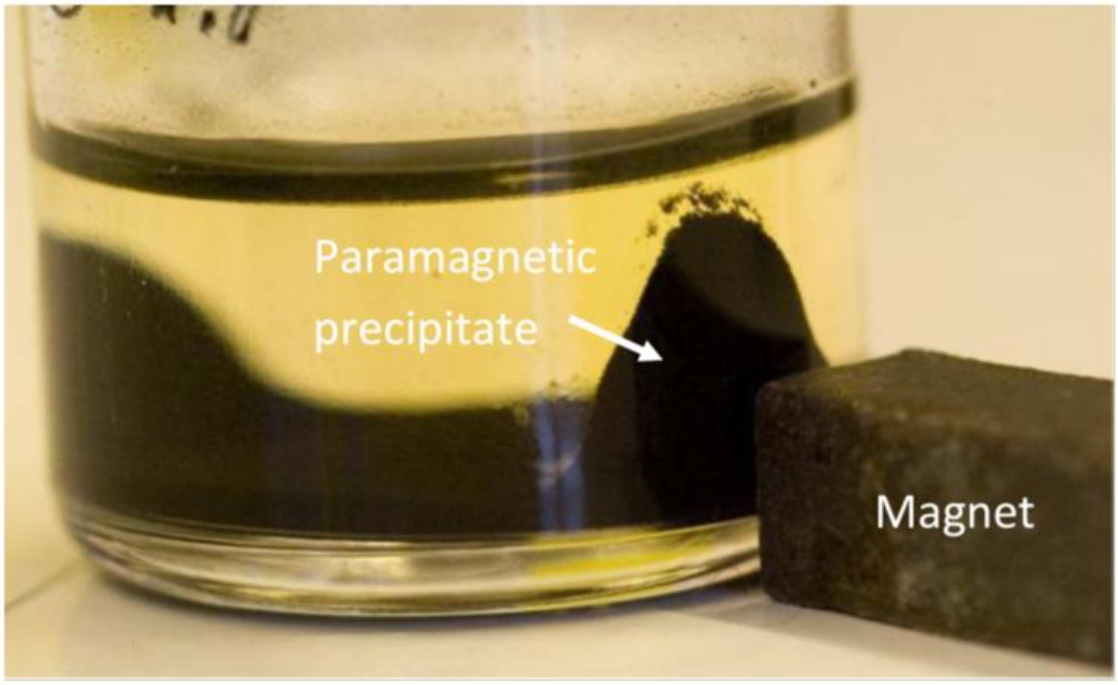

Figure 4. Demonstration of paramagnetic precipitate in a culture of strain S1.1 supplemented by Fe_2O_3.

and further substrate analyses are needed to provide further evidence. *Thermoanaerobacter* spp. growing at *in situ* temperatures have been recovered from oil wells around the world (Cayol et al., 1995; Fardeau et al., 2000; Grassia et al., 1996; Lan et al., 2012; Slobodkin et al., 1999) and is thus believed to represent one of the indigenous members of this subsurface community. Due to their physiological properties and potential capability for biological activities *in situ*, a mixed anaerobic culture dominated by *Thermoanaerobacter* spp. has been successfully used for microbially enhanced oil recovery (MEOR) of heavy oil fractions in an experimental simulated reservoir condition setting (Castorena-Cortes et al., 2012a; Castorena-Cortes et al., 2012b). Production of surface-active agents, solvents and gases, and degradation of heavy hydrocarbons by this mixed culture represent highly useful feature (Castorena-Cortes et al., 2012a). Thermoanaerobacters have also been noted for their anti- corrosion behaviour through their ability to reduce thiosulphate to elemental sulphur without sulphide formation (Lan et al., 2012). The above features underpin the importance of further analysis of the diversity of this microbial group due to its potential roles and applications in petroleum-related biotechnology. Moreover, magnetite biogenically formed by *Thermoanaerobacter* spp., a property confirmed for strain S1.1, also has potential as a biosignature for thermophilic iron-reducing bacteria in the deep hot biosphere as well as for the emergence of iron respiration on the early Precambrian earth (Li, 2012).

Conclusion

As part of an investigation of the bacterial diversity of the Thar Jaht oil-field in South Sudan, heterotrophic anoxic enrichments on a rich organic medium using produced water as inoculum, were made at reservoir *in situ* temperature of ~70°C. Two fermentative thermophilic anaerobes phylogenetically related to the genus *Thermoanaerobacter* were isolated and characterized. They utilize a variety of carbohydrates, proteinaceous compounds and organic acids, and may represent novel species. Reduction of thiosulphate to elemental sulphur by one of the isolates is a feature of interest for biological control of corrosion. One of the isolates efficiently reduced Fe(III) oxide. Further analysis is required to establish the exact taxonomic status and potential applications of the isolates.

ACKNOWLEDGEMENTS

We gratefully acknowledge the funding of this work by the Committee of the Faculty of Mathematics and Natural Sciences for Collaboration with Sudanese Universities (University of Bergen), and the Research Council of Norway (grant. 145854/110). We are thankful to the White Nile Petroleum Operating Company (Sudan) for their kind sponsorship of the round trip flight for FAS and NKB from Khartoum to Thar Jath, provision of accommodation, and assistance in sampling.

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