Geoalkalibacter subterraneus sp. nov., an anaerobic Fe(III)- and Mn(IV)-reducing bacterium from a petroleum reservoir, and emended descriptions of the family *Desulfuromonadaceae* and the genus *Geoalkalibacter*

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A strictly anaerobic Fe(III)-reducing bacterium, designated strain Red1^T, was isolated from the production water of the Redwash oilfield, USA. The cells were motile rods (1–5×0.5–0.6 µm) that stained Gram-negative and possessed polar flagella. Strain Red1^T obtained energy from the reduction of Fe(III), Mn(IV), nitrate, elemental sulfur and trimethylamine *N*-oxide in the presence of a wide range of electron donors, including a variety of organic acids, alcohols, biological extracts and hydrogen. Strain Red1^T was incapable of fermentative growth. The novel isolate grew optimally at 40 °C (temperature range for growth, 30–50 °C) and at pH 7 (pH range, 6–9) with 2 % (w/v) NaCl (NaCl range, 0.1–10 %, w/v). The DNA G + C content was 52.5 mol%.

Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain Red1^T was a member of the order *Desulfuromonadales* within the class *Deltaproteobacteria* and most closely related to *Geoalkalibacter ferrihydriticus* Z-0531^T (95.8 %), *Desulfuromonas palmitatis* SDBY1^T (92.5 %) and '*Desulfuromonas michiganensis*' BB1 (92.4 %). On the basis of phenotypic and phylogenetic differences, the novel strain is proposed to represent a novel species, *Geoalkalibacter subterraneus* sp. nov. (type strain Red1^T=JCM 15104^T=KCTC 5626^T).

In the last two decades, dissimilatory Fe(III)-reduction has been recognized as an important and common mechanism of anaerobic respiration carried out by a wide diversity of bacteria from a variety of environments (Lovley, 2000; Slobodkin, 2005). As new strains have been recognized, increasing phylogenetic, morphological, metabolic and ecological diversity has been seen amongst metal reducers. Phylogenetic analyses have revealed that many of the metal reducers still belong to the class *Deltaproteobacteria* within the order *Desulfuromonadales* (Lovley *et al.*, 2004). While the majority of the members of this order are from freshwater and marine sediments, a number have been isolated more recently from extreme environments such as soda lake and Arctic sediments (Zavarzina *et al.*, 2006; Vandieken *et al.*, 2006), hot springs (Kashefi *et al.*, 2002) and hydrothermal vents (Kashefi & Lovley, 2003). Besides the class *Deltaproteobacteria*, metal-reducing isolates have been found in numerous other lineages throughout both

the domain *Bacteria* and *Archaea* (Lovley *et al.*, 2004). These include members of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria*, *Firmicutes* (Boone *et al.*, 1995; Slobodkin *et al.*, 1997), *Acidobacteria* (Coates *et al.*, 1999), *Thermodesulfobacteria* (Kashefi *et al.*, 2002), *Deinococcus-Thermus* (Kieft *et al.*, 1999), *Deferribacteres* (Caccavo *et al.*, 1996; Greene *et al.*, 1997), *Thermotogae* (Vargas *et al.*, 1998; Slobodkin *et al.*, 1999) and numerous genera within both the *Crenarchaeota* and *Euryarchaeota* (Vargas *et al.*, 1998; Slobodkin 2005). The increasing genetic and ecological diversity amongst metal reducers supports the claims by Lonergan *et al.* (1996) that metal reduction may be a characteristic widespread across the domain *Bacteria* as well as *Archaea*.

In this paper, we describe the characteristics of an Fe(III)- and Mn(IV)-reducing bacterium designated strain Red1^T isolated from an oilfield in Utah, USA. Petroleum reservoirs are known to contain an array of anaerobic micro-organisms (Grassia *et al.*, 1996; Magot *et al.*, 2000) but relatively few iron-reducers have been isolated. To date only *Deferribacter thermophilus* (Greene *et al.*, 1997) and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Geoalkalibacter subterraneus* sp. nov. Red1^T is EU182247.

A supplementary figure showing the growth of strain Red1^T on acetate and Fe(III) oxyhydroxide is available with the online version of this paper.

several species of the genera *Thermanaerobacter*, *Thermococcus*, *Thermotoga* (Slobodkin *et al.*, 1999) and *Shewanella* (Semple & Westlake, 1987) have been isolated from reservoir environments.

Bacterial strain Red1^T was isolated from produced formation water collected from well 41-21B in the Redwash oilfield. Redwash is an on-shore oilfield located in Utah, USA, at a depth of 1540 m. The *in situ* temperature of the reservoir was 52 °C. The production water collected was anaerobic with a salinity level of 25 g l⁻¹ and a pH of 7.9. Samples were collected from oil wells through sampling valves. Sampling lines were flushed for at least 10 min and then sterile 1 l glass bottles were completely filled and tightly sealed to prevent oxygen diffusion.

A medium designated FRR and based on Redwash oilfield reservoir chemistry was formulated to enrich for Fe(III)-reducing bacteria. FRR medium was prepared anaerobically and contained (l⁻¹ distilled water): 1 g NH₄Cl, 0.08 g K₂HPO₄ · 3H₂O, 4.5 g MgCl₂ · 6H₂O, 0.375 g CaCl₂ · 2H₂O, 17 g NaCl, 3.6 g NaHCO₃, 3 g yeast extract, 10 mM sodium acetate and 20 mM Fe(III) citrate or Fe(III) oxyhydroxide. The pH of the medium was adjusted to 7.0 and was prepared as described previously (Greene *et al.*, 1997).

Enrichment cultures were initiated by adding 0.5 ml production water inoculum to 10 ml FRR medium and then incubated at 40, 50 or 60 °C. Enrichment cultures that reduced Fe(III) were streaked aseptically onto FRR agar (2%) plates in an anaerobic chamber and incubated in anaerobic jars under N₂/CO₂ (80:20) at their enrichment temperature. Colonies of strain Red1^T were selected from agar plates and grown in FRR liquid medium. Electron donors utilized by strain Red1^T were determined using FRR medium with Fe(III) oxyhydroxide and amended with 0.1 g l⁻¹ yeast extract and no sodium acetate. Most of the electron donors were added at a final concentration of 20 mM; the only exceptions were hydrogen (80% H₂/20% CO₂), yeast extract and peptone (each at 2 g l⁻¹). Alternative electron acceptors (20 mM) were tested in FRR medium lacking Fe(III) and with acetate (10 mM) as the electron donor. Experiments to determine the pH and salinity optima for growth were performed using FRR medium amended to the appropriate pH and salt concentrations. The optimum temperature was determined using FRR medium. For electron microscopy examinations, cells were negatively stained with 0.5% (w/v) uranyl acetate and viewed with a transmission electron microscope (1200SX, JEOL). The DNA G+C content measurement and other analytical techniques were performed as described previously (Greene *et al.*, 1997). Phylogenetic analysis of 16S rRNA gene sequence was performed as described previously (Kanso *et al.*, 2002).

Enrichment cultures were positive for Fe(III)-reduction after incubation at 40 °C and 50 °C for 4 to 5 days. No positive Fe(III)-reducing enrichment cultures occurred at 60 °C. A pure culture designated strain Red1^T was obtained

from the 40 °C enrichment culture by picking a colony from an agar plate. Colonies were 0.5–1 mm in diameter, uniformly round and red pigmented. Strain Red1^T was a non-spore-forming, Gram-negative, rod-shaped bacterium. The cells were generally short to medium in length, 1.0–5.0 µm, and 0.5–0.6 µm in diameter (Fig. 1). The cells occurred singly and were motile by polar flagella.

Strain Red1^T grew routinely by using Fe(III) citrate or Fe(III) oxyhydroxide as the electron acceptor and acetate as the electron donor. Apart from Fe(III), strain Red1^T was able to use MnO₂, nitrate, elemental sulfur and trimethylamine *N*-oxide as electron acceptors. Strain Red1^T did not reduce sulfate, sulfite, thiosulfate, glycine, fumarate, molybdate, chromium or selenium. Oxygen was not used as a terminal electron acceptor at atmospheric or lower oxygen concentrations. Strain Red1^T was able to use a wide variety of electron donors, for details see the species description. Strain Red1^T was not able to use the following compounds as electron donors: malate, tartrate, glutarate, benzoate, glucose, sucrose, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol or decanol. The growth of strain Red1^T was not inhibited by penicillin, rifampicin, vancomycin, streptomycin, tetracycline or cycloserine.

Strain Red1^T was able to grow by the complete oxidation of acetate coupled to the reduction of Fe(III) (see Supplementary Fig. S1 in IJSEM Online). Various types of iron(III) were tested for reduction by strain Red1^T; ferrihydrite, Fe(III) citrate and Fe(III) oxyhydroxide were all used readily by strain Red1^T. However, two of the most common iron oxide minerals found in nature, goethite and haematite, were not used. Likewise, strain Red1^T was

Fig. 1. Electron micrograph of cells of strain Red1^T showing the short rod shape and flagella (arrow). Bar, 0.2 µm.

selective in the forms of MnO_2 that it reduced. The crystalline mineral, pyrolusite, was not reduced whereas amorphous forms of vernadite and synthetic MnO_2 were used readily.

An almost complete 16S rRNA gene sequence (1555 bases) of strain Red1^T was obtained and aligned with various sequences from representatives of the domain Bacteria (Fig. 2). Phylogenetic analysis showed that strain Red1^T was aligned within the class *Deltaproteobacteria* and in the order *Desulfuromonadales*. All members of this order are anaerobic bacteria that reduce Fe(III) and/or sulfur. The organisms most closely related to strain Red1^T are *Geoalkalibacter ferrihydriticus* (95.8% sequence similarity), *Desulfuromonas palmitatis* (92.5%) and '*Desulfuromonas michiganensis*' (92.4%). All have a similar rod-shaped morphology and are able to use acetate linked to the reduction of Fe(III). While the genus *Geoalkalibacter* is tentatively included along with the genera *Geobacter*, *Geopsychrobacter* and *Geothermobacter* in the family *Geobacteraceae* (mean similarity of 89.5%), the current study reveals that this genus is more closely related to members of the family *Desulfuromonadaceae*, particularly those within the *Desulfuromonas*–*Pelobacter* cluster (91.3% mean sequence similarity). Therefore, we propose that the genus *Geoalkalibacter* be classified in the family *Desulfuromonadaceae*.

Numerous characteristics distinguished strain Red1^T from its closest taxonomic relatives (Table 1). Unlike *Geoalkalibacter ferrihydriticus*, strain Red1^T has an optimum pH around neutrality, though it can grow under alkaline conditions up to pH 9. Part of the genus description for *Geoalkalibacter* is that the species are obligately alkaliphilic, which is not the case for strain Red1^T. Therefore we suggest that the genus description should be emended to indicate that species could be either alkaliphilic or alkalitolerant. A number of other characteristics also distinguish *Geoalkalibacter ferrihydriticus* from

the novel strain. *Geoalkalibacter ferrihydriticus* is unable to use Fe(III) citrate, has lower temperature requirements and has a slightly higher DNA G + C content. A clear difference between strain Red1^T and all of the closest relatives is the ability to use nitrate but not fumarate as electron acceptors. Strain Red1^T is able to use a wide range of substrates, considerably more than the other three closely related bacteria. In addition, yeast extract, formate, methanol and butyrate are used only by strain Red1^T. Strain Red1^T is similar to *Desulfuromonas palmitatis* in that it can use long-chain fatty acids and hydrogen while the others cannot. Based on the phenotypic and phylogenetic traits, we suggest that strain Red1^T represents a novel species of the genus *Geoalkalibacter*, *Geoalkalibacter subterraneus* sp. nov.

Studies in our laboratory suggest that metal reducers are widespread in petroleum reservoirs. Strain Red1^T is the first member of the order *Desulfuromonadales* to be isolated from a petroleum reservoir and one of only a few from deep subsurface environments. The most closely related bacteria to strain Red1^T are from marine or freshwater sediments and not the deep subsurface. The use of the waterflooding processes in oil recovery can result in substantial variability in the physico-chemical conditions within a single reservoir and can introduce new micro-organisms into reservoirs. Redwash production waters in particular have revealed a wide diversity of anaerobic microorganisms (Grassia *et al.*, 1996; Rees *et al.*, 1997). Whether many of these organisms and strain Red1^T are indigenous to the reservoir environment remains unknown. It is possible that many of the micro-organisms have been introduced to the reservoir during waterflooding, particularly as river water was used at Redwash. However, the characteristics of the growth of strain Red1^T such as the optimum temperature and salinity and the use of hydrogen and acetate reflect the conditions within the Redwash reservoir. The use of both hydrogen and acetate is unusual for Fe(III)-reducers, though there are several other

Fig. 2. Phylogenetic tree of strain Red1^T and its closest relatives in the order *Desulfuromonadales* within the class *Deltaproteobacteria*. The tree was constructed by using the neighbour-joining method and based on 16S rRNA gene sequences. GenBank accession numbers are given in parentheses. Bar, 2 nucleotide exchanges per 100 nucleotides.

Table 1. Characteristics that differentiate strain Red1^T from the most closely related members of the order *Desulfuromonadales*

Taxa: 1, strain Red1^T (data from this study); 2, *Geoalkalibacter ferrihydriticus* (Zavarzina *et al.*, 2006); 3, *Desulfuromonas palmitatis* (Coates *et al.*, 1995); 4, '*Desulfuromonas michiganensis*' (Sung *et al.*, 2003). All tests were performed under comparable conditions. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4
Temperature optimum (°C)	40	35	40	25
Temperature range (°C)	30–50	18–39	ND	5–45
pH optimum	7	8.5	ND	7.0–7.5
pH range	6–9	7.8–10	ND	6.8–8
Motility	+	+	−	+
DNA G + C content (mol%)	52.5	55.3	54.7	ND
Electron acceptors				
Fe(III) citrate	+	−	+	+
Fumarate	−	+	+	+
Mn(IV)	+	+	+	ND
Nitrate	+	−	−	−
Electron donors				
Butyrate	+	−	−	−
Ethanol	+	+	−	−
Formate	+	−	−	−
Glycerol	+	−	ND	−
Hydrogen	+	−	+	−
Malate	−	ND	ND	+
Malonate	+	−	ND	ND
Methanol	+	−	−	−
Palmitate	+	ND	+	ND
Peptone	+	−	ND	ND
Propionate	+	+	−	−
Pyruvate	+	+	−	+
Stearate	+	ND	+	ND
Succinate	+	−	+	+
Tartrate	−	+	ND	ND
Yeast extract	+	−	−	ND

strains in the order *Desulfuromonadales* that are capable of using both. The use of hydrogen as an electron donor is a trait common with all Fe(III)-reducing strains isolated so far from petroleum reservoirs. Hydrogen is commonly found in reservoirs, often produced in part by the activities of fermentative micro-organisms.

Emended description of the family *Desulfuromonadaceae*

The description is based on that given by Kuever *et al.* (2005) with the following amendment. The family includes the genera *Desulfuromonas*, *Desulfuromusa*, *Malonomonas*, *Pelobacter* and *Geoalkalibacter*. The type genus is *Desulfuromonas*.

Emended description of the genus *Geoalkalibacter*

The description is based on that given by Zavarzina *et al.* (2006) with the following amendment. The genus contains

alkaliphilic and alkalitolerant species. The type species is *Geoalkalibacter ferrihydriticus*.

Description of *Geoalkalibacter subterraneus* sp. nov.

Geoalkalibacter subterraneus (sub.ter.ra'ne.us. L. adj. *subterraneus* underground, subterranean).

Cells are non-spore-forming, strictly anaerobic and slightly rod-shaped. Cells are Gram-negative and 0.5–0.6 × 1–5 µm. Motile by polar flagella. Able to use iron(III), manganese(IV), nitrate, elemental sulfur and trimethylamine *N*-oxide as electron acceptors in the presence of a wide variety of electron donors, including formate, acetate, malonate, propionate, pyruvate, lactate, butyrate, isobutyrate, succinate, fumarate, valerate, isovalerate, citrate, salicylate, octanoate, *p*-anisate, decanoate, dodecanoate, palmitate, stearate, methanol, ethanol, glycerol, peptone, yeast extract and hydrogen. No fermentation occurs. Growth occurs at temperatures between 30 and 50 °C (optimum 40 °C), in the presence of NaCl concentrations ranging from 1 to 100 g l^{−1} (optimum 20 g l^{−1}) and at pH 6–9 (optimum pH 7).

The type strain, Red1^T (=JCM 15104^T=KCTC 5626^T), was obtained from produced water from the Redwash petroleum reservoir in Utah, USA. The DNA G + C content of the type strain is 52.5 mol%.

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