

Geobacter uraniireducens sp. nov., isolated from subsurface sediment undergoing uranium bioremediation

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A Gram-negative, rod-shaped, motile bacterium, strain Rf4^T, which conserves energy from dissimilatory Fe(III) reduction concomitant with acetate oxidation, was isolated from subsurface sediment undergoing uranium bioremediation. The 16S rRNA gene sequence of strain Rf4^T matched sequences recovered in 16S rRNA gene clone libraries constructed from DNA extracted from groundwater sampled at the same time as the source sediment. Cells of strain Rf4^T were regular, motile rods, 1.2–2.0 µm long and 0.5–0.6 µm in diameter, with rounded ends. Cells had one lateral flagellum. Growth was optimal at pH 6.5–7.0 and 32 °C. With acetate as the electron donor, strain Rf4^T used Fe(III), Mn(IV), anthraquinone-2,6-disulfonate, malate and fumarate as electron acceptors and reduced U(VI) in cell suspensions. With poorly crystalline Fe(III) oxide as the electron acceptor, strain Rf4^T oxidized the following electron donors: acetate, lactate, pyruvate and ethanol. Phylogenetic analysis of the 16S rRNA gene sequence of strain Rf4^T placed it in the genus *Geobacter*. Strain Rf4^T was most closely related to '*Geobacter humireducens*' JW3 (95.9% sequence similarity), *Geobacter bremensis* Dfr1^T (95.4%) and *Geobacter bemidjensis* Bem^T (95.1%). Based on phylogenetic analysis and phenotypic differences between strain Rf4^T and closely related *Geobacter* species, this strain is described as a representative of a novel species, *Geobacter uraniireducens* sp. nov. The type strain is Rf4^T (=ATCC BAA-1134^T =JCM 13001^T).

Geobacter species are Gram-negative bacteria of the family *Geobacteraceae* within the class *Deltaproteobacteria*. Based on phylogenetic analysis of conserved genes (*nifD*, *recA*, *gyrB*, *rpoB* and *fusA*), members of the family *Geobacteraceae* have been grouped into three phylogenetic clusters: *Geobacter*, *Desulfuromonas* and *Desulfuromusa* (Holmes *et al.*, 2004). The *Geobacter* cluster hosts freshwater mesophilic dissimilatory iron-reducing bacteria (DIRB) of the genus *Geobacter* that are capable of complete oxidation of acetate and other multi-carbon organic substrates to carbon dioxide. Microorganisms of the *Desulfuromonas* cluster are capable of growth in marine media and include psychrophilic, mesophilic and thermophilic DIRB of the genera *Desulfuromonas*, *Geoalkalibacter* and *Geothermobacter*

(Holmes *et al.*, 2004; Zavarzina *et al.*, 2006; Vandieken *et al.*, 2006). The *Desulfuromusa* cluster contains S⁰- and Fe(III)-reducing psychrophilic and mesophilic *Desulfuromusa*, *Malomonas* and *Geopsychrobacter* species (Holmes *et al.*, 2004; Vandieken *et al.*, 2006). Species of the genus *Pelobacter* are scattered through all three phylogenetic clusters of the *Geobacteraceae*. With one exception, the members of this genus have a primarily fermentative metabolism and can grow in syntrophic association with methanogens (Schink & Pfennig, 1982; Schink & Stieb, 1983; Schink, 1984, 1985). The recently isolated *Pelobacter seleniigenes* can grow via complete oxidation of acetate to carbon dioxide with selenate, S⁰, Fe(III), fumarate, anthraquinone-2,6-disulfonate (AQDS) and nitrate serving as electron acceptors and also via fermentation of pyruvate, citrate and lactate (Narasingarao & Häggblom, 2007).

Geobacter strains are often the dominant members of subsurface sediments under metal-reducing conditions (Lovley *et al.*, 2004). The goal of this isolation effort was to recover an environmentally relevant *Geobacter* strain from subsurface sediments undergoing *in situ* uranium bioremediation. The

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Abbreviations: AQDS, anthraquinone-2,6-disulfonate; DIRB, dissimilatory iron-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Geobacter uraniireducens* strain Rf4^T is EF527427.

criterion for relevancy was that the 16S rRNA gene sequence of the isolate should match the sequence(s) in a 16S rRNA gene clone library derived from the same source at the same time. Source sediment for isolation was collected in September 2002 during the third month of acetate injection into the subsurface at the Old Rifle uranium bioremediation field site (Anderson *et al.*, 2003). The PO5 core section from depth 4.5 m was used for isolation.

To recover *Geobacter* strains, a direct isolation approach without an enrichment step was chosen. Direct isolation avoids overgrowth of micro-organisms that grow rapidly in laboratory media, but are minor constituents in the environment. Isolate Rf4^T was recovered using a roll-tube method (Hungate, 1969). BBL agar (1.5%; Becton Dickinson) was the solidifying agent. An inoculum (1 ml) from 10-fold serial dilutions of sediment in a liquid FW (freshwater) medium (Lovley & Phillips, 1988) was added to 27 ml pressure tubes containing 7 ml melted medium and the pressure tubes were rolled with a tube spinner (Bellco Glass). Acetate (20 mM) served as the electron donor and clay size fraction minerals from the site (5% by wt) served as the source of Fe(III). The clay size fraction minerals were isolated from background Rifle sediment (core PO1) (Chandler *et al.*, 2006) as described previously (Shelobolina *et al.*, 2004). Roll-tubes were incubated vertically at 16 °C, the groundwater temperature. Strain Rf4^T was recovered from an individual colony in the last positive dilution (10⁵) roll-tube. The colony was transferred to a pressure tube with 2 ml liquid FW medium containing 20 mM acetate as the electron donor and poorly crystalline Fe(III) oxide (100 mmol l⁻¹) (Lovley & Phillips, 1986) as the electron acceptor. Purity of the culture was checked microscopically and by DNA analysis after growing the culture in medium with yeast extract.

DNA was extracted from strain Rf4^T using the FastDNA SPIN kit (Bio 101). The 16S rRNA gene was amplified as described previously (Shelobolina *et al.*, 2007b). The 16S rRNA gene sequence of strain Rf4^T had 97.3–99.2%

similarity to *Geobacter* sequences 14o40, 27o254 and 34o239 found in a 16S rRNA gene clone library from groundwater taken at the same time as the source sediment (Fig. 1). Phylogenetic analysis of the 16S rRNA gene sequence of strain Rf4^T confirmed that it belongs to the *Geobacteraceae* (Fig. 1). Strain Rf4^T was most similar to '*Geobacter humireducens*' JW3 (95.9% sequence similarity), *Geobacter bremensis* Dfr1^T (95.4% sequence similarity) and *Geobacter bemidjiensis* Bem^T (95.1% sequence similarity). The DNA G+C content of strain Rf4^T was 54 mol%, determined by total genome analysis courtesy of the DOE Joint Genome Institute (http://genome.jgi-psf.org/finished_microbes/geour/geour.home.html).

Physiological characteristics were determined by previously described methods (Shelobolina *et al.*, 2007b). Results are given in the species description. The phenotypic differences between strain Rf4^T and closely related *Geobacter* species are listed in Table 1. Unlike other described *Geobacter* species, cells of strain Rf4^T had two to four very long flagella (up to 16 µm long) (Fig. 2). Cell suspension experiments were carried out as described previously (Shelobolina *et al.*, 2007a). U(VI)-reducing activity of strain Rf4^T was 0.18 µmol U(VI) (mg protein)⁻¹ min⁻¹, which is comparable to the U(VI)-reducing activity of *Geobacter sulfurreducens* (Shelobolina *et al.*, 2007a).

The fatty acid contents of strain Rf4^T and strains of two closely related *Geobacter* species, *G. bemidjiensis* Bem^T and '*G. humireducens*' JW3, were determined after growth with acetate as the electron donor and fumarate as the electron acceptor. Independent triplicate cultures of each strain were grown under identical conditions, washed and lyophilized and whole-cell fatty acid profiles were determined following standard protocols (Sasser, 1990). The whole-cell fatty acid profile of strain Rf4^T comprised mainly straight-chain, even-numbered fatty acids, several branched, odd-chain fatty acids, and several hydroxy fatty acids (Table 2). The four most abundant fatty acids were 16:1ω7c, 16:0, i15:0 and 14:0.

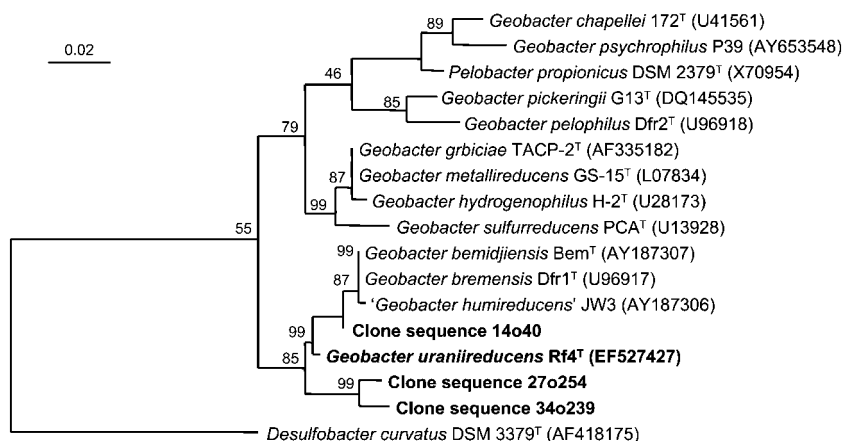


Fig. 1. Neighbour-joining phylogenetic tree with Jukes–Cantor correction based on 16S rRNA gene sequences. The tree, which was generated using the ARB software environment (<http://www.arb-home.de>), shows the relationships between strain Rf4^T and related members of the family *Geobacteraceae*. *Desulfobacter curvatus* DSM 3379^T was used as the outgroup. The shorter environmental clone sequences (448 bases; not deposited in GenBank) were recovered from groundwater sampled at the same time as the source sediment for strain Rf4^T isolation. Bootstrap values (%) from 1000 iterations are shown at nodes. Bar, 2 substitutions per 100 nucleotide positions.

Table 1. Properties of strain Rf4^T and type strains of closely related *Geobacter* species and the type species, *Geobacter metallireducens*

All tests were performed under comparable conditions. Strains: 1, *Geobacter uraniireducens* sp. nov. Rf4^T (data from this study); 2, '*G. humireducens*' JW3 (Coates *et al.*, 1998); 3, *G. bremensis* Dfr1^T (Straub *et al.*, 1998; Straub & Buchholz-Cleven, 2001); 4, *G. bemidjiensis* Bem^T (Nevin *et al.*, 2005); 5, *G. metallireducens* GS-15^T (Lovley *et al.*, 1993). For strain Rf4^T, electron acceptors were tested with acetate as the electron donor and electron donors were tested with poorly crystalline Fe(III) oxide as the electron acceptor. +, Positive; -, negative, ND, no data available.

Characteristic	1	2	3	4	5
DNA G + C content (mol%)	54	ND	60	60.9	56.6
Optimal temperature (°C)	32	30	30–32	30	30–35
Electron acceptors					
Ferric citrate	-	+	ND	+	+
Elemental sulfur	-	+	+	-	-
Nitrate	-	+	-	-	+
Malate	+	-	+	+	ND
Fumarate	+	+	+	+	-
Electron donors					
Lactate	+	+	+	+	-
H ₂	-	+	+	+	-
Formate	-	+	+	-	-
Propionate	-	-	+	+	+
Butyrate	-	-	+	+	+
Valerate	-	ND	-	+	+
Succinate	-	-	+	+	ND
Butanol	-	ND	+	+	+

Based on phenotypic and genotypic differences between strain Rf4^T and other *Geobacter* species (Tables 1 and 2, Fig. 1), it is suggested that this isolate represents a novel species, *Geobacter uraniireducens* sp. nov.

Description of *Geobacter uraniireducens* sp. nov.

Geobacter uraniireducens (u.ra.ni.i.re.du'cens. N.L. n. *uranium* uranium; L. part. adj. *reducens* leading back, bringing back and, in chemistry, converting to a reduced oxidation state; N.L. part. adj. *uraniireducens* reducing uranium).

Table 2. Whole-cell fatty acid compositions of *G. uraniireducens* sp. nov. Rf4^T, *G. bemidjiensis* Bem^T and '*G. humireducens*' JW3

Data are presented as means ± SD (*n*=3) of total fatty acids (percentages by weight); -, fatty acid not found; tr, present at less than 1% of total fatty acids. Fatty acids were identified by co-elution with known standards and MS analysis. Fatty acids are listed in order of elution on a DB-1 column and named using the format *x:y_{ωz}*, where *x* is the number of carbons in the molecule, *y* is the number of double bonds present and *z* is the position of the double bond in reference to the aliphatic or 'ω' end of the molecule. i, iso branching pattern; a, anteiso branching pattern; br, unknown branching pattern; 3-OH, β-hydroxy fatty acid. MS analysis suggests that UNK 1 is probably a summed feature of branched, unsaturated 15-carbon fatty acids; its equivalent chain length (ECL) is 14.32. The mass spectrum of UNK 2 (ECL 16.15) has a base peak typical of a 3-OH fatty acid (*m/z* 103), but its ECL is inconsistent with any known straight-chain, saturated β-hydroxy fatty acid.

Fatty acid	Rf4 ^T	<i>G. bemidjiensis</i> Bem ^T	' <i>G. humireducens</i> ' JW3
12:0	-	tr	tr
13:0	tr	tr	tr
14:1	2.16 ± 0.03	tr	tr
14:0	10.20 ± 0.25	4.64 ± 0.67	6.58 ± 0.93
UNK 1	3.63 ± 0.017	1.11 ± 0.15	2.67 ± 0.34
i15:0	11.46 ± 0.49	15.01 ± 0.82	12.25 ± 0.77
a15:0	tr	tr	tr
15:1	-	tr	tr
15:0	3.43 ± 0.02	3.99 ± 1.06	3.99 ± 0.15
3-OH	tr	tr	1.68 ± 0.08
14:0			
16:1 _{ω7}	26.06 ± 0.71	31.06 ± 0.83	29.67 ± 1.41
16:1 _{ω5}	1.16 ± 0.02	1.82 ± 0.07	2.57 ± 0.11
16:0	21.50 ± 0.12	20.53 ± 0.41	19.46 ± 0.98
UNK 2	6.70 ± 0.75	6.57 ± 1.10	7.11 ± 0.55
br17:1	5.82 ± 0.10	10.99 ± 1.09	7.23 ± 1.39
a17:1 _{ω6}	-	tr	-
a17:0	-	tr	-
3-OH	5.17 ± 0.028	tr	3.64 ± 0.93
16:0			
18:1 _{ω7}	1.23 ± 0.45	tr	1.15 ± 0.07
18:0	1.04 ± 0.38	1.08 ± 0.84	tr

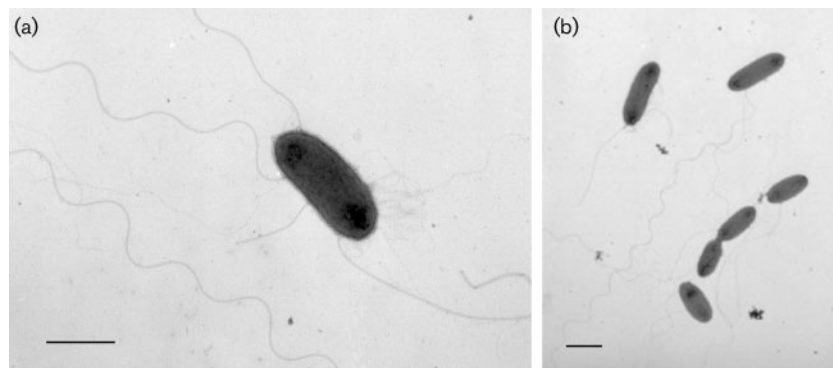


Fig. 2. Morphology of negatively stained cells of Rf4^T. Strain Rf4^T was grown with acetate as the electron donor and fumarate as the electron acceptor. Cells were harvested in the middle of the exponential growth phase. Cells exhibit long pili and tubular projections (blebs) on the cell surface (a) as well as long flagella (b). Bars, 1 μm.

Cells are Gram-negative, motile, regular or slightly curved rods, 1.2–2.0 µm long and 0.5–0.6 µm in diameter. When grown on 20 mM fumarate/10 mM acetate or 20 mM malate/10 mM acetate medium, cells have two to four flagella (up to 16 µm long), pili and vesicles. On 20 mM fumarate/10 mM acetate medium, growth is most rapid at pH 6.5–7.0; no growth is observed below pH 6.0 or above pH 7.7. Optimal growth is at 32 °C; no growth is observed at initial temperatures lower than 10 °C or higher than 34 °C. With 100 mmol poorly crystalline Fe(III) oxide I⁻¹ as electron acceptor, the following electron donors are utilized: acetate, lactate, pyruvate and ethanol. Hydrogen (H₂/CO₂; 80:20, v/v), formate, methanol, butanol, butyrate, valerate, propionate, succinate and citrate are not utilized as electron donors. With 20 mM acetate, the following electron acceptors are utilized: poorly crystalline Fe(III) oxide, ferruginous smectite SWa-1, Fe(III) nitrilotriacetate, Fe(III) pyrophosphate, birnessite (MnOOH), AQDS, malate and fumarate. Oxygen, Fe(III) citrate, sulfate, sulfite, thiosulfate, elemental sulfur and nitrate are not utilized as electron acceptors. Cell suspensions reduce U(VI). The major fatty acids are 16:1ω7c, 16:0, i15:0 and 14:0.

The type strain is Rf4^T (=ATCC BAA-1134^T =JCM 13001^T), isolated from subsurface sediment undergoing uranium bioremediation. The DNA G+C content of the type strain is 54.0 mol%.

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