Moorella humiferrea sp. nov., a thermophilic, anaerobic bacterium capable of growth via electron shuttling between humic acid and Fe(III)

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An anaerobic, thermophilic, spore-forming bacterium (strain 64-FGQ^T) was isolated from a terrestrial hydrothermal spring from the Kamchatka peninsula, Russia. This strain utilized lactate as an electron donor, insoluble poorly crystalline Fe(III) oxide incorporated into alginate beads as a potential electron acceptor and 9,10-anthraquinone-2,6-disulfonate (AQDS) as an electron-shuttling compound. Vegetative cells of strain 64-FGQ^T were Gram-stain-positive, peritrichously flagellated, motile, straight rods, 0.3–0.5 µm in diameter and 2.0–5.0 µm long, growing singly or forming short chains. Cells formed round refractive endospores in terminal swollen sporangia. The temperature range for growth was 46–70 °C, with an optimum at 65 °C. The pH range for growth was 5.5–8.5, with an optimum at pH 7.0. The substrates utilized by strain 64-FGQ^T in the presence of AQDS as an electron acceptor included lactate, malate, succinate, glycerol and yeast extract. The strain fermented galactose, fructose, maltose, sucrose, pyruvate and peptone. Strain 64-FGQ^T used AQDS, humic acid, thiosulfate, nitrate and perchlorate as electron acceptors for growth. Fe(III) was not directly reduced, but strain 64-FGQ^T was able to grow and reduce Fe(III) oxide in the presence of small amounts of AQDS or humic acid as electron-shuttling compounds. The G+C content of the DNA of strain 64-FGQ^T was 51 mol%. 16S rRNA gene sequence analysis placed the isolate in the genus *Moorella*, with the type strain of *Moorella glycerini* as its closest relative (97.2 % similarity). Based on phylogenetic analysis and physiological characteristics, strain 64-FGQ^T is considered to represent a novel species of the genus *Moorella*, for which the name *Moorella humiferrea* sp. nov. is proposed; the type strain is 64-FGQ^T (=DSM 23265^T=VKM B-2603^T).

Microbial Fe(III) reduction is a global biogeochemical process that contributes significantly to the cycling of iron and carbon in different biotopes, including thermal ecosystems (Lovley *et al.*, 2004; Slobodkin, 2005). At neutral pH, Fe(III) exists as insoluble, solid-phase minerals, mostly iron oxides. Electron transfer from dissimilatory iron-reducing micro-organisms to iron(III) oxides can be achieved either by direct contact between the cell and the mineral or by soluble electron shuttles (Lovley *et al.*, 2004). One of the most studied exogenous electron-shuttling compounds is humic acid or its synthetic analogue 9,

10-anthraquinone-2,6-disulfonate (AQDS); even small quantities of these substances significantly stimulate Fe(III) reduction (Lovley *et al.*, 1996). It is assumed that humic substances derived from the surrounding soil are present in terrestrial hydrothermal systems. Several thermophilic and hyperthermophilic micro-organisms are known to use humic acid or AQDS as an electron acceptor (Slobodkin *et al.*, 1997a; Lovley *et al.*, 2000). However, direct enrichment and isolation of thermophiles in the presence of these compounds has not been performed, and the ecological role of different taxonomic groups of thermophilic prokaryotes in the reduction of Fe(III) and humic compounds in natural ecosystems is virtually unknown. In this paper, we describe a novel anaerobic, thermophilic bacterium (designated strain 64-FGQ^T) isolated from a

Abbreviation: AQDS, 9,10-anthraquinone-2,6-disulfonate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 64-FGQ^T is GQ872425.

terrestrial hydrothermal spring. Although strain 64-FGQ^T cannot directly reduce Fe(III), it is capable of reducing humic acid and thus could be involved in the electron flow from organic matter to ferric iron compounds in thermal environments.

Strain 64-FGQ^T was isolated from a sample of sediment collected in a freshwater hot spring originating from the Grot geyser located near the Geysernaya river, Geyser Valley, Kamchatka, Russia. The banks of the spring 20–30 cm from the sampling site (54° 26.288' N 160° 08.433' E) were covered with grass. The water at the sampling site was at 78 °C, pH 7.7 and E_h varied from –10 to –60 mV. The sample of sediment used for inoculation consisted of three layers that were ochre, grey and black in colour; each layer had a thickness of about 0.5 cm. To isolate micro-organisms with the ability to reduce insoluble Fe(III) compounds without direct contact of the cells with the mineral, poorly crystalline iron(III) oxide (ferrihydrite) was incorporated into alginate beads and AQDS was provided as an electron shuttle. An enrichment culture was initiated by inoculating 10% (w/v) of the sample into anaerobically prepared, bicarbonate-buffered, sterile (135 °C, 1 h) liquid medium with lactate (12 mM) as an electron donor, ferrihydrite [90 mmol Fe(III) l⁻¹] incorporated into microporous alginate beads as a potential electron acceptor and AQDS (0.1 mM) as an electron shuttle. Inoculation was performed anaerobically under 100% CO₂. Composition of the medium and preparation techniques were as described previously (Slobodkin *et al.*, 1999). Alginate beads were prepared as described by Nevin & Lovley (2000). After incubation of enrichment cultures at 65 °C for 7–30 days the colour of the beads changed from brown to black, indicating Fe(III) reduction. A pure culture was obtained from an enrichment positive for Fe(III) reduction by means of serial dilutions in the same medium followed by the selection of well-separated colonies that had developed in anaerobic agar blocks [1.5% agar in growth medium with 20 mM AQDS from which Fe(III)-containing alginate beads were omitted]. The process of obtaining separate colonies was subsequently repeated four times. Finally, the single colony that had developed in the highest positive agar shake dilution (10⁻⁸) was designated strain 64-FGQ^T and was used for further studies. Denaturing gradient gel electrophoresis of the outgrown cultures of strain 64-FGQ^T performed as described by Slobodkina *et al.* (2009) revealed a single band and thus additionally confirmed the purity of the culture. Physiological studies on substrate utilization, and on temperature, pH and salinity ranges for growth, were carried out in medium containing 20 mM AQDS unless noted otherwise. All electron donors and acceptors were tested in the presence of 0.2 g yeast extract l⁻¹. AQDS was omitted in experiments examining utilization of electron acceptors.

Light and electron microscopy, Gram staining, analytical techniques for Fe(II) determination and analysis of other metabolic products, genomic DNA extraction, determination

of the G + C content and DNA–DNA hybridization studies were performed as described by Slobodkin *et al.* (1999). The 16S rRNA gene was selectively amplified from genomic DNA by PCR using forward and reverse primers 11F and 1492R (Lane, 1991). PCR was carried out in a 50 µl reaction mixture containing 50 ng DNA template, 5 pmol (each) primer, 12.5 nmol (each) dNTP and 3 U *Taq* DNA polymerase (Fermentas) in *Taq* DNA polymerase reaction buffer (Fermentas). The temperature cycling programme consisted of a first cycle of 9 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by 30 amplification cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and a final extension at 72 °C for 7 min. PCR products were purified by using the Wizard PCRPreps kit (Promega) as recommended by the manufacturer. The 16S rRNA gene was sequenced in both directions with Big Dye Terminator v.3.1 (Applied Biosystems) as described in the manufacturer's instructions by using an ABI PRISM 3730 sequencer (Applied Biosystems). The sequences were assembled and checked for accuracy manually by using the alignment editor BioEdit v5.0.9 (Hall, 1999). The full-length 16S rRNA gene sequence of strain 64-FGQ^T was compared with other sequences in GenBank (Benson *et al.*, 1999) via BLAST (Altschul *et al.*, 1997) to identify its closest relatives. Alignment with a representative set of other 16S rRNA gene sequences in GenBank was carried out by using the CLUSTAL W program provided by the phylogenetic analysis package MEGA version 4 (Tamura *et al.*, 2007) and was then adjusted manually. Pairwise similarity values were calculated by means of EzTaxon (Chun *et al.*, 2007). A phylogenetic dendrogram was constructed by the neighbour-joining method (Saitou & Nei, 1987) with the Maximum Composite Likelihood model (Tamura *et al.*, 2004) by using MEGA version 4. Reference strain *Moorella glycerini* JW/AS-Y6^T (=DSM 11254^T) used in physiological and DNA–DNA hybridization studies was obtained from the culture collection of the Laboratory of Hyperthermophilic Microbial Communities, Winogradsky Institute of Microbiology; this strain was routinely cultured in medium with glycerol as described by Slobodkin *et al.* (1997b).

Colonies of strain 64-FGQ^T appearing in agar shake cultures after 48–120 h incubation at 65 °C were white, circular and 1.5–2.0 mm in diameter. Vegetative cells of strain 64-FGQ^T were straight rods, 0.3–0.5 µm in diameter and 2.0–5.0 µm long. Cells occurred singly or in short chains and exhibited tumbling motility due to peritrichous flagella. Strain 64-FGQ^T formed round, refractive endospores in terminally swollen sporangia. Cells stained Gram-positive in both exponential and stationary growth phases. Ultrathin sectioning of cells of strain 64-FGQ^T revealed a distinct peptidoglycan layer in the cell wall. The temperature range for growth of strain 64-FGQ^T was 46–70 °C, with optimum at 65 °C. No growth was detected at 37 or 75 °C after incubation for 2 weeks. The pH range for growth was 5.5–8.5, with optimum at pH 7.0. No growth was detected at pH 5.0 or 9.0. Growth of strain 64-FGQ^T

was observed at NaCl concentrations ranging from 0 to 1.5 % (w/v), with optimum at 0 % (w/v), but no growth was evident at 2.0 % (w/v). The generation time under optimal conditions in medium with lactate and AQDS was 126 min. Strain 64-FGQ^T utilized lactate (12 mM), malate, succinate, glycerol (each substrate at 20 mM) and yeast extract (0.2 g l⁻¹) with AQDS as electron acceptor. Lactate was incompletely oxidized to acetate as the only metabolic product; no molecular hydrogen was produced. In the absence of AQDS, no growth on these substrates was observed. Yeast extract at a concentration of at least 50 mg l⁻¹ was required for growth. In the absence of AQDS, strain 64-FGQ^T fermented galactose, fructose, maltose, sucrose, pyruvate (each at 20 mM) and peptone (10 g l⁻¹). The main product of fructose fermentation was acetate. Carbohydrates chemically reduced AQDS in sterile controls, and thus tests for growth with AQDS and carbohydrates were not performed. Strain 64-FGQ^T grew on pyruvate and peptone in the presence as well as in the absence of AQDS; growth rate and cell yield were the same under the two conditions. The following substrates were tested but were not utilized for growth in the absence or presence of AQDS: H₂/CO₂ (80:20, v/v), formate, acetate, propionate, butyrate, oxalate, fumarate, methanol, ethanol, n-propanol, butanol, benzoate, acetone, glycine, valine, alanine, glucose, mannose, cellobiose, xylose, arabinose (each substrate at 20 mM), filter paper, alginate, starch and olive oil (each at 10 g l⁻¹). Strain 64-FGQ^T reduced AQDS (20 mM) to 9,10-anthrahydroquinone-2,6-disulfonate, thiosulfate (10 mM) to sulfide, nitrate (10 mM) to nitrite and ammonia, and perchlorate (10 mM) to chloride with lactate as electron donor. The isolate was able to grow (maximal cell concentration 1.0 × 10⁸ cells l⁻¹) with humic acid (humic acid, sodium salt; Aldrich, 1.0 g l⁻¹) as an electron acceptor and lactate as electron donor. Strain 64-FGQ^T was not able to reduce ferrihydrite [90 mmol Fe(III) l⁻¹], Fe(III)-EDTA (10 mM), iron(III) citrate (10 mM), MnO₂ (25 mmol l⁻¹), fumarate (10 mM), sulfate (14 mM), sulfite (5 mM), elemental sulfur (150 mmol l⁻¹) or oxygen [0.5, 1.0, 3.0 or 20 % (v/v) in the gas phase] with lactate or glycerol as electron donors. The isolate was able to grow and reduce ferrihydrite [90 mmol Fe(III) l⁻¹] suspended in the cultivation medium or incorporated into

alginate beads in the presence of AQDS or humic acid as electron-shuttling compounds. The minimal concentrations of AQDS and humic acid that supported growth (3–7 × 10⁷ cells ml⁻¹) and Fe(III) reduction [4.0–7.0 mmol Fe(II) l⁻¹ produced] were 0.05 mM and 0.25 g l⁻¹, respectively. Ampicillin, chloramphenicol, erythromycin, rifampicin and tetracycline completely inhibited growth at a concentration of 100 µg ml⁻¹. Streptomycin (100 µg ml⁻¹) did not inhibit growth.

Strain 64-FGQ^T provides a first example of an organism that was enriched and isolated by using a soluble compound providing the electron transfer between organic substances and insoluble iron(III) oxide. Incorporation of ferrihydrite into alginate beads and thus preventing direct contact of the cell with the mineral allowed us to select for micro-organisms involved in Fe(III) reduction via shuttling of electron-transferring molecules. The important role of humic acid-reducing bacteria in indirect Fe(III) reduction was suggested for freshwater lake sediments (Kappler *et al.*, 2004). Our results indicate that in thermal environments, relatively low concentrations of humic acid and possibly other quinone-containing compounds can provide sustainable microbial growth and oxidation of organic matter coupled to Fe(III) reduction.

The G + C content of the genomic DNA of strain 64-FGQ^T was 51 mol% (T_m). Phylogenetic analysis showed that highest levels of 16S rRNA gene sequence similarity were found with species of the genus *Moorella* within the *Firmicutes* (Fig. 1). A comparison of 1488 nt of the 16S rRNA gene sequence of strain 64-FGQ^T showed that it was related most closely to the type strain of *M. glycerini* (Slobodkin *et al.*, 1997b) with which it shared 97.2 % similarity. Strain 64-FGQ^T was similar to all recognized *Moorella* species in cell morphology, endospore formation and thermophilic nature, but it shared only 94.2–94.9 % 16S rRNA gene sequence similarity with the type strains of other members of the genus *Moorella* except *M. glycerini*. Therefore, strain 64-FGQ^T cannot be assigned to *Moorella thermoacetica* (Fontaine *et al.*, 1942), *Moorella thermoautotrophica* (Wiegel *et al.*, 1981) or *Moorella mulderi* (Balk *et al.*, 2003), irrespective of its physiological traits. Given that the level of 16S rRNA gene sequence similarity

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences indicating the position of strain 64-FGQ^T among members of the genus *Moorella*. The tree was constructed by using the neighbour-joining algorithm; application of the maximum-parsimony and minimal-evolution methods did not change the topology of the tree. The sequence from *Thermoanaerobacter ethanolicus* DSM 2246^T was used as the outgroup. GenBank accession numbers are given in parentheses. Numbers at nodes are bootstrap values (500 replications); only values >70 % are shown. Bar, 2 substitutions per 100 nt.

between strain 64-FGQ^T and the type strain of *M. glycerini* was more than 97 %, quantitative DNA–DNA hybridization experiments were performed. The level of DNA–DNA relatedness between strain 64-FGQ^T and *M. glycerini* JW/AS-Y6^T was 17 %. These data indicate that strain 64-FGQ^T represents a novel species of the genus *Moorella*. Furthermore, strain 64-FGQ^T could be differentiated from *M. glycerini* based on a number of physiological characteristics such as temperature range and optimum for growth and utilization of electron donors and acceptors (Table 1). *M. glycerini* is not able to use humic acid or AQDS as electron acceptors for growth, which is a hallmark feature of strain 64-FGQ^T. Therefore, on the basis of physiological properties and the results of phylogenetic analysis we suggest that strain 64-FGQ^T represents a novel species of the genus *Moorella*, for which the name *Moorella humiferrea* sp. nov. is proposed.

Description of *Moorella humiferrea* sp. nov.

Moorella humiferrea (hu.mi.fer're.a. L. n. *humus* humus; L. adj. *ferreus* made of or referring to iron; N.L. fem. adj. *humiferrea* pertaining to humic substances and iron).

Cells are straight rods, 0.3–0.5 µm in diameter and 2.0–5.0 µm long. Cells occur singly or in short chains and exhibit tumbling motility due to peritrichous flagella. Gram-stain-positive. Produces round, refractive endospores in terminally swollen sporangia. The temperature range for growth is 46–70 °C, with optimum at 65 °C. The pH range for growth is 5.5–8.5, with optimum at pH 6.8–7.0. Growth occurs at NaCl concentrations of 0–1.5 % (w/v). Anaerobic. Substrates utilized in the presence of AQDS include lactate, malate, succinate, glycerol and yeast extract. Lactate is incompletely oxidized to acetate as the only metabolic product. In the absence of AQDS, no growth

on these substrates is observed. Yeast extract is required for growth. Ferments galactose, fructose, maltose, sucrose, pyruvate and peptone. Grows on pyruvate and peptone in the presence as well in the absence of AQDS. H₂/CO₂, formate, acetate, propionate, butyrate, oxalate, methanol, ethanol, propanol, butanol, benzoate, acetone, glucose, mannose, cellobiose, xylose, arabinose, filter paper, alginate, starch and olive oil are not utilized with or without an exogenous electron acceptor. The main product of fructose fermentation is acetate. Reduces AQDS, thiosulfate, nitrate and perchlorate, but not ferrihydrite, iron(III) citrate, MnO₂, fumarate, sulfate, sulfite, elemental sulfur or oxygen [0.5, 1.0, 3 or 20 % (v/v) in the gas phase]. Able to grow and reduce Fe(III) in the presence of small amounts of AQDS or humic acid as electron-shuttling compounds.

The type strain, 64-FGQ^T (=DSM 23265^T=VKM B-2603^T), was isolated from a terrestrial hot spring in Kamchatka, Russia. The DNA G+C content of the type strain is 51 mol%.

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Table 1. Differential characteristics of strain 64-FGQ^T and *Moorella glycerini*

All characteristics were determined in this study.

Characteristic	Strain 64-FGQ ^T	<i>M. glycerini</i>
Temperature range for growth (°C)	46–70	43–65
Optimum temperature (°C)	65	58
Utilization of:		
Glucose	–	+
Sucrose	+	–
Mannose	–	+
Reduction of:		
Nitrate	+	–
AQDS	+	–
Fumarate	–	+
End product of thiosulfate reduction	Sulfide	Elemental sulfur

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