

Deferribacter thermophilus gen. nov., sp. nov., a Novel Thermophilic Manganese- and Iron-Reducing Bacterium Isolated from a Petroleum Reservoir

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A thermophilic anaerobic bacterium, designated strain BMA^T (T = type strain), was isolated from the production water of Beatrice oil field in the North Sea (United Kingdom). The cells were straight to bent rods (1 to 5 by 0.3 to 0.5 μm) which stained gram negative. Strain BMA^T obtained energy from the reduction of manganese(IV), iron(III), and nitrate in the presence of yeast extract, peptone, Casamino Acids, tryptone, hydrogen, malate, acetate, citrate, pyruvate, lactate, succinate, and valerate. The isolate grew optimally at 60°C (temperature range for growth, 50 to 65°C) and in the presence of 2% (wt/vol) NaCl (NaCl range for growth, 0 to 5% [wt/vol]). The DNA base composition was 34 mol% G+C. Phylogenetic analyses of the 16S rRNA gene indicated that strain BMA^T is a member of the domain *Bacteria*. The closest known bacterium is the moderate thermophile *Flexistipes sinusarabici* (similarity value, 88%). Strain BMA^T possesses phenotypic and phylogenetic traits that do not allow its classification as a member of any previously described genus; therefore, we propose that this isolate should be described as a member of a novel species of a new genus, *Deferribacter thermophilus* gen. nov., sp. nov.

Metal-reducing microorganisms play important roles in the cycling of metals and organic matter, and iron- and manganese-reducing microorganisms are believed to be responsible for a substantial amount of carbon oxidation in marine sediments and other anaerobic environments (16, 17, 25).

Dissimilatory manganese- and iron-reducing microorganisms which can couple Mn(IV) and/or Fe(III) reduction to the oxidation of carbon compounds have been isolated from sediments and extreme environments, such as the deep subsurface (3, 17, 25). The isolates include strictly anaerobic bacteria belonging to the family *Geobacteraceae* (15), *Bacillus infernus* (3) and *Geovibrio ferrireducens* (6) strains, and facultatively anaerobic bacteria belonging to the genus *Shewanella* (23, 28). Nearly all of the known manganese- and iron-reducing isolates are mesophiles. While thermophilic iron reduction has been found in sediment samples from thermal springs (30), the only pure culture of a thermophilic iron reducer that has been described is a culture of *B. infernus*, an anaerobe from a deep terrestrial subsurface environment.

Petroleum reservoirs are frequently deep subsurface extreme environments having high temperatures, pressures, and levels of salinity, and several physiological groups of anaerobic bacteria, including thermophilic fermentative, sulfate-reducing, sulfur-reducing, and methanogenic microorganisms, have been isolated from these environments (7, 8, 12, 13, 26, 27, 32). Mesophilic iron-reducing bacteria have been detected in oil field fluids (24, 29) and have been identified as *Shewanella putrefaciens* (29). The presence of thermophilic metal-reducing microorganisms in petroleum reservoirs has not been reported previously.

Recent studies in our laboratory have shown that microbial Fe- and Mn-reducing activity is found frequently in production water samples from reservoirs. We speculate that iron and manganese oxides and hydroxides present in the petroleum

subsurface are conducive to the existence of metal-reducing microorganisms. This suggests that petroleum reservoirs represent a new source of metal-reducing microorganisms.

We describe in this paper the characteristics of a thermophilic manganese- and iron-reducing bacterium, designated strain BMA^T (T = type strain), which was isolated from the Beatrice oil reservoir (North Sea, United Kingdom).

MATERIALS AND METHODS

Source of bacterial strain. Bacterial strain BMA^T was isolated from produced formation water collected from well A01(07) in the Beatrice oil field. The Beatrice oil field is located in the British sector of the North Sea near the coast of Scotland at a depth of 2,058 m. The temperature of the reservoir ranges from 40 to 110°C across the field. The production water was anaerobic and had a level of salinity (NaCl) of 32 g/liter and a pH of 7.1. The details of sample collection have been described elsewhere (12).

Media and culture conditions. A medium designated MR and based on Beatrice oil field reservoir chemistry was formulated to enrich for manganese-reducing bacteria. MR medium was prepared anaerobically and contained (per liter of distilled water) 1 g of NH_4Cl , 0.08 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 4.5 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.375 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 32 g of NaCl, 3.6 g of NaHCO_3 , 2 g of yeast extract, and 1.3 g of MnO_2 . The MnO_2 was prepared by the method of Lovley and Phillips (19). The pH of the medium was adjusted to 7.1, and then the medium was boiled, cooled under a stream of N_2 , and dispensed into serum bottles under N_2 - CO_2 (80:20) to exclude oxygen; the bottles were capped with butyl rubber septa and autoclaved.

Isolation. Enrichment cultures were initiated by adding a 2-ml inoculum of production water to 20 ml of MR medium and were incubated at 60°C. Enrichment cultures that reduced MnO_2 were subcultured a number of times. These cultures were then tested for growth on nitrate (20 mM) as an alternative electron acceptor to MnO_2 . Pure cultures were obtained by the agar shake dilution method (14). The process involved serially diluting enrichment cultures in MR medium amended with 20 mM NaNO_3 in place of MnO_2 and fortified with agar (2%) at 50°C. The tubes then were cooled to solidify the agar and incubated at 60°C. Several colonies were selected, grown in MR medium, and tested for the ability to reduce MnO_2 . The purity of cultures was checked microscopically.

Physiological studies. Strain BMA^T was tested for its ability to use different terminal electron acceptors. Most electron acceptors were added from sterile stock solutions to MR medium lacking MnO_2 at a final concentration of 20 mM; the only exceptions were Mn(IV) (final concentration, 15 mM), Fe(III) (15 mM), oxygen (atmospheric concentration), and elemental sulfur (~1 g/liter).

The types of electron donors used by strain BMA^T were determined in the presence of MnO_2 , amorphous ferric oxyhydroxide (18), or nitrate as an electron acceptor in triplicate. Most of the electron donors tested were added at a final

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concentration of 20 mM; the only exceptions were hydrogen (80% H₂-20% CO₂ headspace), yeast extract (0.2%), peptone (0.2%), Casamino Acids (0.2%), tryptone (0.2%), and elemental sulfur (~1 g/liter). All electron donors were added from sterile stock solutions to MR medium that contained 0.02% yeast extract instead of 0.2% yeast extract.

Experiments to determine temperature, salinity, and pH optima for growth were performed in MR medium and MR medium amended with 20 mM nitrate instead of MnO₂.

The sensitivity of strain BMA^T to antibiotics was assessed in MR medium. The antibiotics tested were penicillin (150 µg/ml), vancomycin (150 µg/ml), streptomycin (150 µg/ml), tetracycline (100 µg/ml), and cycloserine (150 µg/ml).

All physiological studies were conducted either in water baths or in incubators without shaking at 60°C unless indicated otherwise.

Morphological characteristics. Gram reactions were determined by the method of Hucker (9) and the KOH lysis method of Buck (5). Cellular morphology was determined by phase-contrast microscopy. Electron microscopy was performed as described previously (2).

Analytical techniques. Manganese (Mn²⁺) contents were determined by a modification of the method of Brewer and Spencer (4). Samples (1 ml) were removed and treated with 0.25 M H₂SO₄ to remove Mn²⁺ adsorbed to MnO₂ particles and to dissolve carbonate particulates. The resulting solution was filtered immediately, diluted to a volume of 100 ml, and then adjusted to pH 8 to 9. A 4.6-ml aliquot was mixed with 0.4 ml of formaloxime-ammonia reagent, and the A₄₅₀ was determined within 2 to 30 min. Control tests were performed to ensure that acid treatment did not dissolve the MnO₂. The presence of Mn(IV) was assessed with a benzidine acetate reagent (10).

Reduced iron (Fe²⁺) contents were determined by the ferrozine method (31). A 0.1-ml sample was mixed with 3 ml of ferrozine reagent, and after 1 min the A₅₆₂ was determined. Reduction of nitrate was assessed by using Merckoquant nitrate test strips.

Growth was assessed by visual turbidity and microscopic observations, and numbers of cells were determined by the most-probable-number technique (1).

DNA base composition. DNA was purified by the method of Marmur (21). The DNA G+C content was determined by the thermal denaturation method (22).

16S rRNA analysis. PCR product of the 16S rRNA gene was sequenced directly with an automated model ABI sequencer by using a Prism dideoxy terminator cycle sequencing kit as recommended by the manufacturer (Applied Biosystems, Foster City, Calif.). The procedures used for phylogenetic analysis have been described previously (26, 27). The sequences used for phylogenetic comparison were obtained from the Ribosomal Database Project, version 5.0 (20), GenBank, and EMBL databases.

Nucleotide sequence accession number. The 16S rRNA sequence of strain BMA^T has been deposited in the GenBank data library under accession number U75602.

RESULTS

Enrichment and isolation. After 3 to 5 days of incubation at 60°C, a positive enrichment culture was obtained, as shown by the reduction of black solid manganese to a white precipitate. This precipitate was identified as an Mn²⁺ complex, most likely manganese carbonate, by a benzidine acetate test. A pure culture designated strain BMA^T was obtained from the enrichment culture by using the agar shake dilution technique.

Morphological characteristics. Strain BMA^T was a non-spore-forming, gram-negative, rod-shaped bacterium. The cells were straight to bent rods that were about 0.3 to 0.5 µm in diameter and 1 to 5 µm long (Fig. 1). Electron micrographs of thin sections of strain BMA^T revealed that the surface layer was separated from the cytoplasmic membrane by a periplasmic space. Colonies were 1 to 2 mm in diameter, orange-red pigmented, fairly flat, and round when they were grown in agar tubes with nitrate as an electron acceptor. Each strain BMA^T cell appeared to have a polar flagellum; however, motility was not evident in laboratory cultures.

Electron acceptors and donors. Strain BMA^T was grown routinely by using MnO₂ as the electron acceptor and yeast extract as the electron donor. Strain BMA^T grew on three terminal electron acceptors, Mn(IV), Fe(III), and nitrate, but not on nitrite, sulfate, sulfite, thiosulfate, trimethylamine *N*-oxide, fumarate, glycine, molybdate, and elemental sulfur. Both soluble Fe(III) citrate and insoluble Fe(III) oxide were reduced, although amorphous Fe(III) oxide was reduced more rapidly. The crystalline structure of Fe(III) influenced the reductive capacity of strain BMA^T. Poorly crystalline Fe(III)



FIG. 1. Transmission electron micrograph of a thin section of strain BMA^T. Bar = 0.2 µm.

oxide was reduced by strain BMA^T, whereas the more crystalline Fe(III) oxide hematite was not. Strain BMA^T was unable to grow with oxygen as a terminal electron acceptor at atmospheric or lower concentrations. Oxygen at atmospheric concentrations was toxic to strain BMA^T. However, strain BMA^T was able to grow in the presence of small amounts of air (up to 3% [vol/vol]), provided that either Mn(IV), Fe(III), or nitrate and a suitable electron donor were added.

Strain BMA^T grew best and reduced the electron acceptors most rapidly when yeast extract was provided as an electron donor. Other complex organic extracts, such as peptone, Casamino Acids, and tryptone, also supported growth and reduction of the electron acceptor. Strain BMA^T also used acetate, malate, citrate, pyruvate, lactate, succinate, valerate, and hydrogen as electron donors. While strain BMA^T was able to use extracts and some organic acids, it did not use carbohydrates, alcohols, and many other organic acids. Strain BMA^T was not able to use the following compounds as electron donors: elemental sulfur, glycine, formate, propionate, butyrate, isobutyrate, isovalerate, octanoate, oxalate, glutamate, fumarate, benzoate, salicylate, phenol, ethanol, propanol, butanol, pentanol, hexanol, octanol, nonanol, decanol, glycerol, 2,3-butandiol, sorbitol, mannitol, glucose, lactose, sucrose, raffinose, rhamnose, fructose, maltose, xylose, mannose, stearate, palmitate, decanoate, and dodecanoate.

Manganese and iron reduction. A time course of Mn(IV) reduction and cell growth is shown in Fig. 2a. After an initial lag period, the onset of Mn²⁺ release corresponded to the most rapid increase in cell numbers. Mn²⁺ production continued through the stationary phase of growth and up to about 40 h of incubation; after that both Mn²⁺ levels and cell numbers declined.

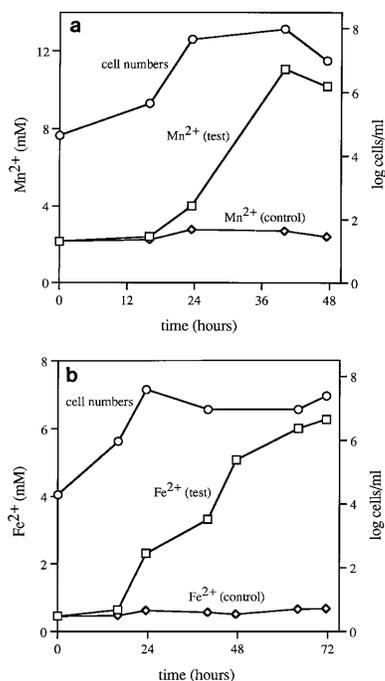


FIG. 2. (a) Time course of cell growth and Mn(IV) reduction by strain BMA^T. (b) Time course of cell growth and Fe(III) reduction by strain BMA^T.

The reduction of Fe(III) and the growth of cells over time are shown in Fig. 2b. The results were similar to the results obtained with the Mn-grown culture, although the initial Fe²⁺ release and the increases in cell numbers were more rapid. The Fe²⁺ levels continued to increase up to 72 h, while the cell numbers remained fairly constant after 24 h.

Reduction of MnO₂ by strain BMA^T yielded a whitish precipitate composed of the Mn(II) state. This precipitate was most likely an Mn carbonate or rhodocrosite compound formed due to the high carbonate and CO₂ levels present in the medium. Reduction of amorphous Fe oxide resulted in the formation of a black solid high in the Fe(II) state. It is possible that this solid was magnetite or a carbonate compound, although this could not be confirmed without using structural analysis techniques, such as X-ray diffraction.

Growth conditions. Figure 3 shows the relationship between temperature and growth. Strain BMA^T grew at temperatures between 50 and 65°C, and the optimum temperature was 60°C. Likewise, reduction of manganese occurred over the same

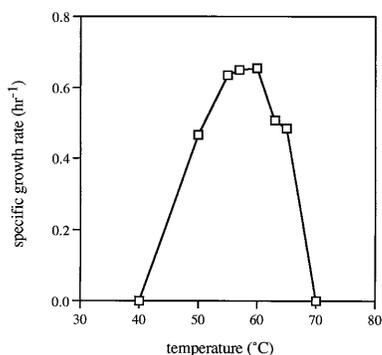


FIG. 3. Effect of temperature on the growth of strain BMA^T.

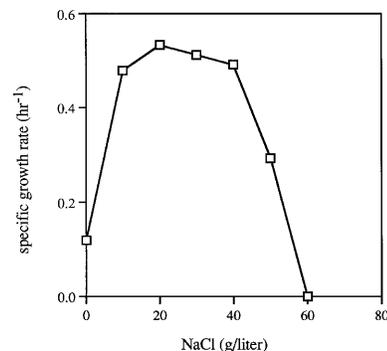


FIG. 4. Effect of salinity on the growth of strain BMA^T.

temperature range after 6 days of incubation and was most rapid at 60°C.

Figure 4 shows the relationship between salinity (NaCl) levels and growth. Growth occurred in the presence of NaCl concentrations ranging from 0 to 50 g/liter, and the optimum NaCl concentration was 20 g/liter. Manganese reduction occurred over the same salinity range as growth.

Figure 5 shows the relationship between pH and growth. Growth and reduction of manganese occurred at pH values ranging from 5 to 8. Strain BMA^T was slightly acidophilic, and the optimum pH for growth was 6.5.

Antibiotic susceptibility. Growth of strain BMA^T was inhibited by penicillin, vancomycin, streptomycin, and cycloserine. Growth and manganese reduction occurred in the presence of tetracycline.

DNA base composition. Strain BMA^T had a G+C content of 34 mol%.

Phylogenetic analysis. An almost complete sequence (1,526 bases) of strain BMA^T 16S rRNA was obtained and aligned with various sequences from representatives of the domain *Bacteria*. Figure 6 shows a dendrogram generated from a Jukes-Cantor evolutionary distance matrix, with the phylogenetic position of strain BMA^T indicated. The organism most closely related to strain BMA^T is *Flexistipes sinusarabici* (level of similarity, 88%). The level of similarity of the recently described organism *G. ferrireducens* (6) was 87%.

DISCUSSION

The prevailing physicochemical conditions, particularly the high temperatures and high levels of salinity, in many petroleum reservoirs mean that novel microorganisms such as strain

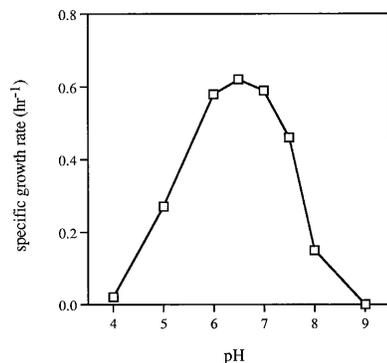


FIG. 5. Effect of pH on the growth of strain BMA^T.

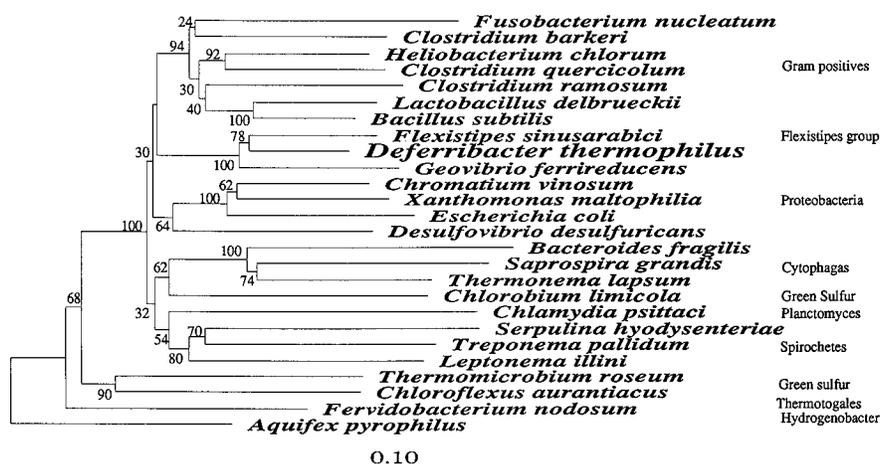


FIG. 6. Dendrogram showing the phylogenetic position of strain BMA^T. Most of the sequences used in the analysis were obtained from the Ribosomal Database Project, version 5.0 (20); the only exception was the sequence of *G. ferrireducens* ATCC 51996 (6), which was obtained from the EMBL (accession no. X95744).

BMA^T are likely to be present. Strain BMA^T is the first thermophilic Mn- and Fe-reducing isolate obtained from a petroleum reservoir.

Iron and manganese oxides and hydroxides are common in the subsurface environment and can be present in oil-bearing rock (24). The presence of these compounds provides an ecological niche for iron- and manganese-reducing microorganisms. We postulate that metal reduction has evolved as a mechanism by which many respiring microorganisms persist in the subsurface environment.

Strain BMA^T was able to use acetate and hydrogen as electron donors. Most other metal reducers use acetate or hydrogen. This is significant because acetate and hydrogen are common products of fermentation and fermentative microorganisms are common inhabitants of petroleum reservoirs, including the Beatrice field (12). The temperature and salinity optima of strain BMA^T, 60°C and 2% NaCl, respectively, are consistent with the in situ conditions in the Beatrice petroleum reservoir. It is probable that strain BMA^T actively uses acetate and/or hydrogen in situ.

The analysis of 16S rRNA sequences showed that *G. ferrireducens* is the only previously described metal-reducing microorganism related to strain BMA^T. Many of the previously described Fe and Mn reducers are mesophilic organisms belonging to the delta subdivision of the *Proteobacteria* (15). Other Fe and Mn reducers are found in the gamma subdivision of the *Proteobacteria*. The thermophile *B. infernus* is not related to other metal-reducing microorganisms and is placed in the *Bacillus-Lactobacillus-Streptococcus* subdivision of the gram-positive phylum. This shows the genetic diversity of metal reducers and supports the claim of Lonergan et al. (15) that metal reduction may be a characteristic that is widespread in the domain *Bacteria*. Reduction of Fe(III) or Mn(IV) is not tested routinely in physiological characterization studies, so it is possible that many microorganisms, particularly those isolated from the subsurface, have the capacity to reduce metals.

Phylogenetically, the closest known relatives of strain BMA^T are *F. sinuarabici*, an organism isolated from hot brine waters of the Red Sea (11), and *G. ferrireducens*, an organism isolated from surface sediment in a hydrocarbon-contaminated ditch (6). Table 1 shows a comparison of some phenotypic characteristics of these organisms. The three bacteria share few characteristics. Only strain BMA^T and *F. sinuarabici* appear to be

similar morphologically. Strain BMA^T and *G. ferrireducens* respire anaerobically, while *F. sinuarabici* has fermentative metabolism. Like *F. sinuarabici*, strain BMA^T is sensitive to penicillin, vancomycin, and streptomycin and is resistant to tetracycline. Strain BMA^T is thermophilic, while *F. sinuarabici* is only moderately thermophilic and *G. ferrireducens* is mesophilic. On the basis of phylogenetic and phenotypic traits, we propose that strain BMA^T should be described as a member of a novel species of a new genus.

Description of *Deferribacter* gen. nov. *Deferribacter* (De.fer.ri. bac'ter. L. pref. *de*, from; L. n. *ferrum*, iron; Gr. hyp. masc. n. *bacter*, rod; M. L. masc. n. *Deferribacter*, rod that reduces iron). Cells are gram negative and rod shaped and can vary in length. No spores are produced, and motility is not evident. Growth occurs under anaerobic conditions with iron(III), manganese(IV), and nitrate as electron acceptors. Complex organic extracts, such as yeast extract, and numerous organic acids can be used as electron donors. No fermentation occurs. Phylogenetically, the genus groups with the general cluster of eubacteria.

TABLE 1. Comparison of some characteristics of strain BMA^T, *F. sinuarabici*, and *G. ferrireducens*

Characteristic	Strain BMA ^T	<i>F. sinuarabici</i> ^a	<i>G. ferrireducens</i> ^b
Gram reaction	Negative	Negative	Negative
Morphology	Straight to bent rods	Straight to bent rods	Vibrio shaped
Metabolism	Respiratory	Fermentative	Respiratory
Optimum temp (°C)	60	45–50	35
Salinity range (% wt/vol)	0–6	3–18	0–2
G+C content (mol%)	34	39	43
Reduction of Mn(IV)	+	–	–
Reduction of Fe(III)	+	–	+
Antibiotic sensitivity			
Vancomycin	+	+	NT ^c
Penicillin	+	+	NT
Streptomycin	+	+	NT
Cycloserine	+	+	NT
Tetracycline	–	–	NT

^a Data from reference 11.

^b Data from reference 6.

^c NT, not tested.

Description of *Deferribacter thermophilus* sp. nov. *Deferribacter thermophilus* (ther.mo'phil.us. Gr. adj. *thermus*, warm, hot; Gr. adj. *philos*, loving; M. L. masc. adj. *thermophilus*, heat loving). Cells range from about 0.3 to 0.5 μm wide and from 1 to 5 μm long. Growth occurs at temperatures between 50 and 65°C (optimum temperature, 60°C), in the presence of NaCl concentrations ranging from 0 to 50 g/liter (optimum NaCl concentration, 20 g/liter), and at pH 5 to 8 (optimum pH, 6.5). Hydrogen, acetate, malate, citrate, pyruvate, lactate, succinate, and valerate are used as electron donors. The DNA base composition is 34 mol% G+C. Sensitive to penicillin, vancomycin, streptomycin, and cycloserine and resistant to tetracycline.

The type strain is *Deferribacter thermophilus* BMA, which was obtained from produced water from the Beatrice petroleum reservoir. This reservoir is a high-temperature, seawater-flooded reservoir and is located in the British sector of the North Sea. Strain BMA^T has been deposited in the Australian Collection of Microorganisms, University of Queensland, Brisbane, Australia as strain ACM 5093^T.

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