International Journal of Systematic and Evolutionary Microbiology (2003), **53**, 669–673 DOI 10.1099/ijs.0.02298-0

Rhodoferax ferrireducens sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III)

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To further investigate the diversity of micro-organisms capable of conserving energy to support growth from dissimilatory Fe(III) reduction, Fe(III)-reducing micro-organisms were enriched and isolated from subsurface sediments collected in Oyster Bay, VA, USA. A novel isolate, designated T118^T, was recovered in a medium with lactate as the sole electron donor and Fe(III) as the sole electron acceptor. Cells of T118^T were Gram-negative, motile, short rods with a single polar flagellum. Strain T118^T grew between pH 6·7 and 7·1, with a temperature range of 4–30 °C. The optimal growth temperature was 25 °C. Electron donors utilized by strain T118^T with Fe(III) as the sole electron acceptor included acetate, lactate, malate, propionate, pyruvate, succinate and benzoate. None of the compounds tested was fermented. Electron acceptors utilized with either acetate or lactate as the electron donor included Fe(III)–NTA (nitrilotriacetic acid), Mn(IV) oxide, nitrate, fumarate and oxygen. Phylogenetic analysis demonstrated that strain T118^T is most closely related to the genus *Rhodoferax*. Unlike other species in this genus, strain T118^T is not a phototroph and does not ferment fructose. However, phototrophic genes may be present but not expressed under the experimental conditions tested. No *Rhodoferax* species have been reported to grow via dissimilatory Fe(III) reduction. Based on these physiological and phylogenetic differences, strain T118^T (=ATCC BAA-621^T=DSM 15236^T) is proposed as a novel species, *Rhodoferax ferrireducens* sp. nov.

INTRODUCTION

Fe(III) is often an abundant electron acceptor for microbial respiration in subsurface environments and aquatic sediments (Lovley, 2000a). Until recently, there has been much less investigation into the diversity of Fe(III)-reducing micro-organisms than that of micro-organisms carrying out other forms of respiration. However, it is becoming increasingly apparent that there is a wide phylogenetic diversity of *Bacteria* and *Archaea* capable of conserving energy to support growth from electron transport to Fe(III) (Lovley, 2000a).

Fe(III)-reducing micro-organisms that can use acetate as an electron donor are of interest because acetate is an important intermediate in the anaerobic degradation of organic matter in sedimentary environments (Lovley & Chapelle, 1995). Micro-organisms capable of oxidizing acetate with the reduction of Fe(III) include *Geobacter* and *Desulfuromonas* species within the family *Geobacteraceae* in the *δ-Proteobacteria* (Lovley, 2000a), as well as *Geothrix fermentans* (Coates *et al.*, 1999) and *Geovibrio ferrireducens* (Caccavo *et al.*, 1996). The Fe(III)-reducing hyperthermophilic *Archaea* species *Geoglobus ahangari* (Kashefi *et al.*, 2001) and *Ferroglobus placidus* (Tor *et al.*, 2001) are also capable of acetate oxidation. These organisms are all strict anaerobes. However, a facultatively anaerobic *γ-Proteobacterium* capable of acetate oxidation, *Pantoea agglomerans* SP1, was recently described (Francis *et al.*, 2000).

Most previously studied Fe(III)-reducing micro-organisms have an optimal growth temperature of 20–30 °C, but thermophilic and hyperthermophilic Fe(III)-reducing micro-organisms have also been described (Greene *et al.*, 1997; Kashefi & Lovley, 2000; Lovley, 2000b; Tor *et al.*, 2001; Vargas *et al.*, 1998). There is less information on Fe(III)-reducing micro-organisms growing at lower temperatures, but several psychrophilic enrichment cultures were

Published online ahead of print on 4 October 2002 as DOI 10.1099/ijs.0.02298-0.

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Abbreviations: AQDS, anthraquinone-2,6-disulfonate; NTA, nitrilotriacetic acid; PHA, poly-hydroxyalkanoate; RDP, Ribosomal Database Project.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain T118^T is AF435948.

recently reported to reduce Fe(III) at temperatures as low as 0 °C (Zhang *et al.*, 1999). However, pure cultures of psychrotolerant, Fe(III)-reducing micro-organisms have not previously been reported.

As part of a study to characterize the diversity of metal-reducing micro-organisms in subsurface environments, aquifer material from a Department of Energy subsurface study site in Oyster Bay, VA, USA, was used as an inoculum for the enrichment and isolation of Fe(III)-reducing micro-organisms. Several isolates were obtained from various enrichments. Here we report on one such isolate, which is a novel facultatively anaerobic, acetate-oxidizing, Fe(III)-reducing micro-organism capable of growing at temperatures as low as 4 °C. It is most closely related to micro-organisms in the genus *Rhodoferax*, but unlike *Rhodoferax* species, it did not grow as a photosynthetic micro-organism under the experimental conditions provided.

METHODS

Origin of enrichment cultures and isolate. Sediment from a variety of locations and depths at a site in Oyster Bay, VA, was collected as part of an ongoing collaboration to characterize metal-reducing micro-organisms at Department of Energy facilities. Sediment was sealed in anaerobic canisters for transport, and enrichments were begun upon its arrival in the laboratory. The sediment that served as an inoculum for this culture was designated site T1, depth 18 feet (5·5 m). Hence the first strain isolated from this sediment was designated T118^T.

Media and growth conditions. Techniques for strict anaerobic culture were used throughout. The enrichment medium was a defined freshwater medium (Lovley *et al.*, 1993) that contained 10 mM lactate as the electron donor and 100 mmol l^{-1} poorly crystalline Fe(III) oxide as the sole electron acceptor. The medium (10 ml) was dispensed in anaerobic pressure tubes and bubbled with N_2/CO_2 (80 : 20, v/v) to remove dissolved oxygen. The final pH was approximately 6·7. The enrichment culture was initiated with a 1 g sediment inoculum. The cultures were incubated at 20 °C in the dark. Positive Fe(III)-reducing enrichments were transferred (10 % inoculum) at least five times.

To obtain pure-culture isolates, the enrichment was streaked on a similar medium solidified with purified agar (1·5 %) in wide-mouthed glass tubes (Bellco Glass) which were then sealed with a butyl stopper. The slant medium differed in that Fe(III) chelated with nitrilotriacetic acid (NTA) was used in lieu of poorly crystalline Fe(III) oxide. Distinct colonies were picked and restreaked at least three times on solid agar slants, before being suspended in liquid media. Tests for phototrophic growth utilized two different types of medium. The first was a standard phototrophic growth medium adapted from Brock *et al.* (1994). The second was adapted from the original characterization of the genus *Rhodoferax* (Hiraishi *et al.*, 1991). Electron donors utilized to test for phototrophic growth included fructose, acetate, hydrogen and succinate.

Characterization of anaerobic growth and electron donor and acceptor utilization. Cells were incubated at 4 or 25 °C for all growth and donor/acceptor utilization experiments. Cells were enumerated with epifluorescent microscopy (Hobbie *et al.*, 1977). Acetate was quantified by using HPLC. Electron donor utilization was evaluated with 10 mM Fe(III)–NTA as the sole terminal electron acceptor. Lactate and acetate were tested separately as electron donors for studies on the range of electron acceptors reduced. Fe(II) was assayed with ferrozine as described previously (Lovley & Phillips, 1987). Reduction of all other electron acceptors was determined visually by observing precipitation, colour change or turbidity.

Identification of poly-hydroxyalkanoate (PHA) inclusions. PHA inclusion bodies were identified by staining with Nile blue A, as described by Rees *et al.* (1992).

16S rDNA and phylogenetic analysis. Cells grown on lactate and Fe(III)–NTA were collected by centrifugation, and genomic DNA was extracted using the GNOME DNA Isolation Kit (Bio 101). Almost the entire 16S rDNA of strain T118^T was amplified using primers 8 Forward (5′-AGAGTTTGATCCTGGCTCAG-3′) (Eden *et al.*, 1991) and 1492 Reverse (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification mixtures (total 100 µl) contained 10 µl 10 × buffer, 8 µl dNTPs (200 µm), 2 µl BSA (400 ng μl^{-1}), 5 µl DMSO, 3 µl primer, 0·5 µl genomic DNA template and 2·5 U AmpliTaq (PerkinElmer Cetus). Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) with an initial denaturation step at 96 °C for 2 min, followed by 20 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, concluded by a final extension at 72 °C for 10 min. PCR amplification products were prepared for sequencing using a QIAquick PCR Purification kit (Qiagen). DNA sequencing was performed by the Orono DNA Sequencing Facility at the University of Maine. Complete bidirectional sequences were obtained from the PCR amplification product.

The sequences were compared to the GenBank and Ribosomal Database Project (RDP) databases using the BLAST (National Center for Biotechnology Information) and SIMILARITY_RANK (RDP) algorithms. The secondary structure was verified manually. The sequences were aligned with related 16S rDNA sequences from GenBank and the RDP using the Wisconsin Package version 10 sequence editor (Genetics Computer Group). Phylogenetic trees were inferred using the distance, maximum-likelihood and parsimony tools of PAUP* (Swofford, 1998).

RESULTS AND DISCUSSION

Enrichment and isolation

Sediments from the Oyster Bay site were inoculated into anaerobic medium that contained lactate as the electron donor and poorly crystalline Fe(III) oxide as the potential electron acceptor. A positive enrichment, designated T118^T, reduced the Fe(III) oxide; this was visually apparent by the colour change from reddish-brown to black and the formation of Fe(II), as determined by the ferrozine assay. This enrichment culture was transferred five consecutive times with continued Fe(III) reduction. An aliquot of the enrichment culture was then streaked onto the solidified medium in which Fe(III) was provided as Fe(III)–NTA. Only one type of colony grew on these slants; the colonies were glossy white, smooth, round and convex. A single colony was resuspended in liquid medium with lactate as the electron donor and Fe(III)–NTA as the electron acceptor. This culture continued to reduce Fe(III)–NTA in consecutive transfers.

Cells that grew in this medium were short, straight, motile rods, approximately 3–5 µm long and 1 µm wide (Fig. 1). All cells had a single, polar flagellum (Fig. 1). Cells stained Gram-negative during all growth phases, and did not form visible spores under any of the growth conditions tested.

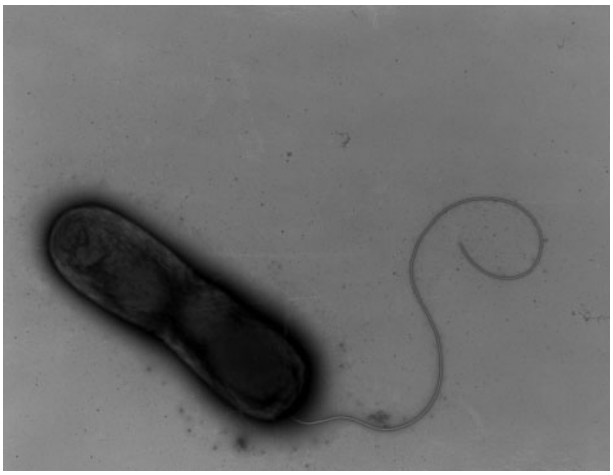

Fig. 1. Negatively stained electron micrograph of strain T118^T showing the rod shape and the single polar flagellum.

However, cells in carbon-rich medium did produce inclusion bodies that were apparent under phase-contrast and electron microscopy. These inclusions were later identified as PHA by staining with Nile blue A and imaging with UV light, as described by Rees *et al.* (1992).

Electron donors and acceptors utilized

Strain T118^T also grew with acetate as the electron donor (Fig. 2). Fe(III)–NTA reduction was accompanied by an increase in cell number and a loss of acetate (Fig. 2). The stoichiometry of acetate consumption and Fe(III) reduction was consistent with the metabolism of acetate according to the reaction:

$$CH_3COO^- + 2H_2O + 8Fe(III) \rightarrow 2CO_2 + 7H^+ + 8Fe(II)$$

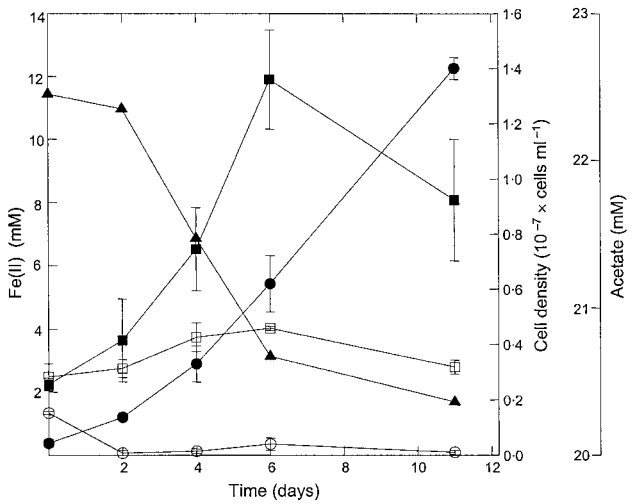

Fig. 2. Increase in cell number, production of Fe(II) and acetate consumption in cultures of T118^T at 25 °C. ●, Fe(II) with acetate; ○, Fe(II) without acetate; ■, cell density with acetate; □, cell density without acetate; ▲, acetate. Results are the means of triplicate analyses. Error bars represent one standard deviation.

The electron donors utilized in media with 20 mM Fe(III)–NTA serving as the sole electron acceptor included acetate, lactate, propionate, pyruvate and malate (all 20 mM), succinate (10 mM) and benzoate (1 mM). Under the same conditions, the following compounds were not utilized: formate, butyrate, ethanol, methanol or glycerol (all 20 mM), caproate, isobutyrate, valerate, butanol or propanol (all 10 mM), or hydrogen (160 kPa). Yeast extract (0·1 %) did not support growth. Strain T118^T did not grow by converting H_2 and CO_2 to acetate and it did not grow phototrophically in either of the phototroph media that were tested. Strain T118^T did not ferment any of the compounds tested.

The electron acceptors utilized in media with 20 mM lactate serving as the electron donor included Fe(III)–NTA and Mn(IV) oxide (both 10 mM), fumarate and nitrate (both 20 mM) and atmospheric oxygen. The following electron acceptors were not utilized: poorly crystalline Fe(III) oxide (100 mM), Fe(III) citrate (50 mM), anthraquinone-2, 6-disulfonate (AQDS) or chromium(VI) (both 5 mM), cobalt-EDTA (0·05 mM), uranium(VI) (2·5 mM), elemental sulfur (1 g l^{-1}), nitrite, selenate or selenite (all 10 mM), or sulfate, sulfite or thiosulfate (all 20 mM). A similar pattern was observed with acetate as the electron donor, with the exception that nitrate did not serve as an electron acceptor for growth on acetate.

T118^T is only the second facultatively anaerobic micro-organism known to oxidize acetate with the reduction of Fe(III), the first being '*Shewanella saccharophilia*' strain GC-29 (Coates *et al.*, 1998). It is the first facultatively anaerobic organism found to use benzoate as an electron donor for Fe(III) reduction. Strain T118^T is unusual amongst Fe(III)-reducing micro-organisms in its inability to reduce AQDS, as most Fe(III)-reducing micro-organisms, including hyperthermophilic *Archaea* species, can use this electron acceptor (Lovley, 2000a; Lovley *et al.*, 1996, 1998, 2000).

Temperature optimum and growth at 4 °C

The optimum growth temperature for strain T118^T was 25 °C (Fig. 3), but in long-term incubations, there was significant growth at temperatures as low as 4 °C (Fig. 4). Fe(III)-reducers capable of growth at such low temperatures have a competitive advantage in cold, Fe(III)-rich subsurface environments. Far northern aquifers and permafrost areas have sediment temperatures that remain at 0–8 °C (Zhang *et al.*, 1999). Fe(III)-reducing enrichment cultures from marine sediment and Alaskan tundra permafrost reduced Fe(III) faster at 10 °C than at 25 °C, indicating that some organisms may prefer cold temperatures for Fe(III) reduction (Zhang *et al.*, 1999). However, strain T118^T is only psychrotolerant, not psychrophilic.

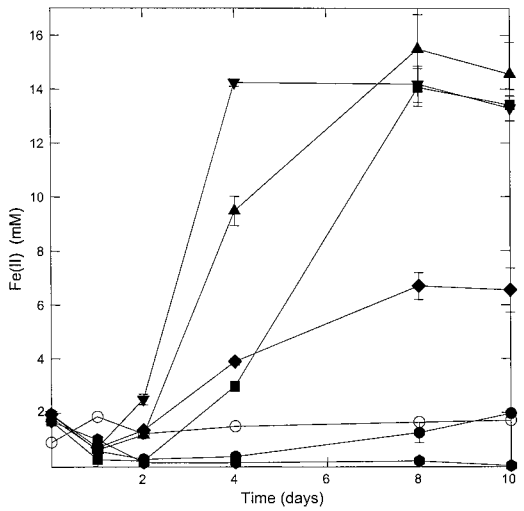

Fig. 3. Production of Fe(II) in cultures of T118^T with lactate (20 mM) as the electron donor and Fe(III)–NTA (10 mM) as the electron acceptor. ●, 4 °C; ■, 15 °C; ▲, 20 °C; ▼, 25 °C; ◆, 30 °C; ⬢, 37 °C; ○, no cells. Results are the means of triplicate analyses. Error bars represent one standard deviation.

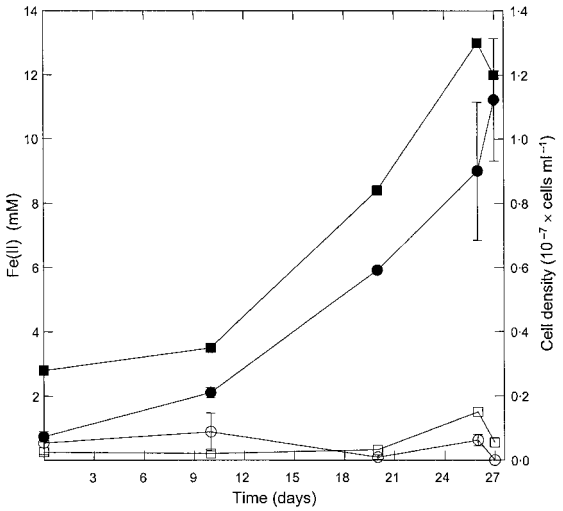

Fig. 4. Growth of T118^T at 4 °C. ●, Fe(II) with lactate; ○, Fe(II) without lactate; ■, cells with lactate; □, cells without lactate. Results are the means of triplicate analyses. Error bars represent one standard deviation.

Phylogeny

Analysis of the 16S rDNA sequence of strain T118^T indicated that its closest known relatives are *Rhodoferax fermentans*, *Aquaspirillum delicatum* and *Rhodoferax antarcticus*, with DNA similarity values of 97·3, 96·5 and 96·4 %, respectively; 1420 bases were considered in all cases (Fig. 5). Neither the morphology nor the physiology of strain T118^T is consistent with the genus *Aquaspirillum* (Eden *et al.*, 1991). Although strain T118^T is morphologically similar to previously described *Rhodoferax* species, these other species are not reported to grow via anaerobic respiration. Furthermore, strain T118^T did not grow phototrophically under conditions that support the growth of *Rhodoferax* species, nor could it ferment fructose, as has been reported in both *Rhodoferax* species that have been characterized to date (Hiraishi *et al.*, 1991; Madigan *et al.*, 2000). These physiological and phylogenetic differences are significant enough to warrant placing strain T118^T as a novel species within the genus *Rhodoferax*. The proposed name is *Rhodoferax ferrireducens* sp. nov.

Description of *Rhodoferax ferrireducens* sp. nov.

Rhodoferax ferrireducens (fer.ri.re.du′cens. L. n. *ferrum* iron; L. part. adj. *reducens* converting to a reduced oxidation state; N.L. part. adj. *ferrireducens* converting iron to a reduced oxidation state).

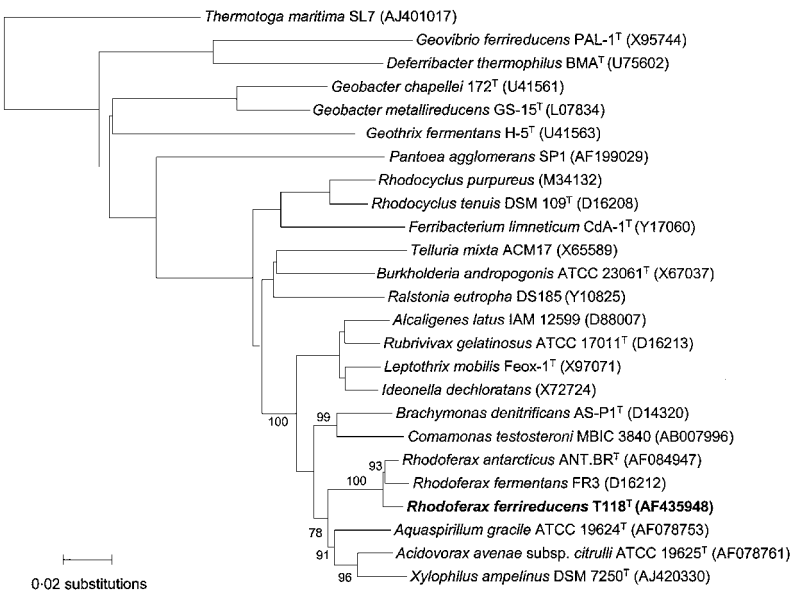

Fig. 5. Phylogenetic tree inferred from the neighbour-joining of distances calculated by the Kimura two-parameter model in TREECON for Windows with 1420 bases considered. Bootstrap values at nodes were calculated using 100 replicates. Bar, 0·02 nucleotide substitutions.

Cells are Gram-negative, short rods, 3–5 μm long by 1 μm wide, that are motile via a single polar flagellum. Colonies are glossy white, smooth, round and convex. Optimum temperature and pH are 25 °C and 7·0, respectively. Grows at and reduces Fe(III) at temperatures as low as 4 °C. There is no fermentative or phototrophic growth. Facultatively anaerobic: respires with Fe(III)–NTA, Mn(IV) oxide, fumarate, nitrate and atmospheric oxygen. AQDS, chromium(VI), cobalt-EDTA, elemental sulfur, poorly crystalline Fe(III) oxide, Fe(III) citrate, nitrite, 1 % oxygen, selenate, selenite, sulfate, sulfite, thiosulfate and uranium(VI) are not reduced. Electron donors that are utilized include acetate, lactate, malate, propionate, pyruvate, benzoate and succinate. Does not utilize butanol, butyrate, caproate, ethanol, formate, glycerol, hydrogen, isobutyrate, methanol, propanol or valerate. PHA inclusion bodies fluoresce under UV light when stained with Nile blue A.

The type strain is T118^T (= ATCC BAA-621^T = DSM 15236^T). Isolated from coastal aquifer sediment in Oyster Bay, VA, USA.

ACKNOWLEDGEMENTS

We would like to thank Elizabeth Blunt for her assistance with obtaining the Oyster Bay sediment and initiating the various enrichment cultures. We acknowledge Brian Pastens of Old Dominion University for samples from the Oyster Bay, VA, site. We also thank Lucy Yin of the University of Massachusetts microscopy facility for the electron microscopy, which is supported by NSF grant # BBS 8714235. This research was funded by the Natural and Accelerated Bioremediation Research (NABIR) program, Biological and Environmental Research (BER), US Department of Energy (grant # DE-FG02-00ER62985), and the Cooperative State Research Extension, Education Services, US Department of Agriculture, Massachusetts Agricultural Experiment Station, under project no. MASS00787.

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