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

Kevin T. Finneran,† Claudia V. Johnsen and Derek R. Lovley

Correspondence
Derek R. Lovley
dlovley@microbio.umass.edu
Department of Microbiology, University of Massachusetts, Amherst, MA 01003, USA

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 T118T, was recovered in a medium with lactate as the sole electron donor and Fe(III) as the sole electron acceptor. Cells of T118T were Gram-negative, motile, short rods with a single polar flagellum. Strain T118T grew between pH 6.5–7 and 7.5–1, with a temperature range of 4–30°C. The optimal growth temperature was 25°C. Electron donors utilized by strain T118T 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 T118T is most closely related to the genus Rhodoferax. Unlike other species in this genus, strain T118T 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 T118T (=ATCC BAA-621T = DSM 15236T) 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 Desulfitomonas 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
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 scaled 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°.

**Media and growth conditions.** Techniques for strict anaerobic culture were used throughout. The enrichment medium was a defined freshwater medium (Lovley et al., 1997) 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 a mixture of 10 mM lactate as the electron donor and poorly crystalline Fe(III) oxide as the sole electron acceptor. The medium was then solidified with purified agar (1.5% w/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% w/v) in wide-mouthed glass tubes (Bellco Glass) which were then scaled 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 phototropic growth utilized two different types of medium. The first was a standard phototropic 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 phototropic 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 inclusions 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° 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, 0.5-5 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°, 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.
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 T118ᵀ 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:

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\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} + 8\text{Fe(III)} \rightarrow 2\text{CO}_2 + 7\text{H}^+ + 8\text{Fe(II)}
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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 propionate (all 10 mM), or hydrogen (160 kPa). Yeast extract (0-1 %) did not support growth. Strain T118ᵀ did not grow by converting H₂ and CO₂ to acetate and it did not grow phototrophically in either of the phototroph media that were tested. Strain T118ᵀ 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-disulphonate (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.

T118ᵀ is only the second facultatively anaerobic microorganism 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 T118ᵀ 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 T118ᵀ 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 T118ᵀ is only psychrotolerant, not psychrophilic.
Phylogeny

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

\textbf{Description of \textit{Rhodoferax ferrireducens} sp. nov.}

\textit{Rhodoferax ferrireducens} (fer.ri.re.du’ens. L. n. ferrum iron; L. part. adj. \textit{reducens} converting to a reduced oxidation state; N.L. part. adj. \textit{ferrireducens} converting iron to a reduced oxidation state).

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

\textbf{Fig. 4.} Growth of T118\textsuperscript{T} at 4 \textdegree 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.

\textbf{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 T118T (= ATCC BAA-62T = DSM 15236T). Isolated from coastal aquifer sediment in Oyster Bay, VA, USA.

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