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ORIGINAL PAPER

Mariprofundus micogutta sp. nov., a novel iron-oxidizing zetaproteobacterium isolated from a deep-sea hydrothermal field at the Bayonnaise knoll of the Izu-Ogasawara arc, and a description of *Mariprofundales* ord. nov. and *Zetaproteobacteria* classis nov.

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Abstract A novel iron-oxidizing chemolithoautotrophic bacterium, strain ET2^T, was isolated from a deep-sea sediment in a hydrothermal field of the Bayonnaise knoll of the Izu-Ogasawara arc. Cells were bean-shaped, curved short rods. Growth was observed at a temperature range of 15–30 °C (optimum 25 °C, doubling time 24 h) and a pH range of 5.8–7.0 (optimum pH 6.4) in the presence of NaCl at a range of 1.0–4.0 % (optimum 2.75 %). The isolate was a microaerophilic, strict chemolithoautotroph capable of growing using ferrous iron and molecular oxygen (O_2) as the sole electron donor and acceptor, respectively; carbon dioxide as the sole carbon source; and either ammonium or nitrate as the sole nitrogen source. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the new isolate was related to the only previously isolated *Mariprofundus* species, *M. ferrooxydans*. Although relatively high 16S rRNA gene similarity (95 %) was found between the new isolate and *M. ferrooxydans*, the isolate was distinct in terms of cellular fatty acid composition, genomic DNA G+C content and cell morphology. Furthermore, genomic comparison between ET2^T and *M. ferrooxydans* PV-1 indicated that the genomic dissimilarity of these strains met the standard for species-level differentiation. On the basis of its physiological and molecular characteristics, strain ET2^T (= KCTC 15556^T = JCM 30585 ^{T}) represents a novel species of *Mariprofundus*, for which the name *Mariprofundus micogutta* is proposed. We also propose the subordinate taxa *Mariprofundales* ord. nov. and *Zetaproteobacteria* classis nov. in the phylum *Proteobacteria*.

Keywords *Zetaproteobacteria* · *Mariprofundus* sp. · Iron-oxidizing bacteria · Hydrothermal field · Bayonnaise knoll

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Introduction

"Candidatus (Ca.) Zetaproteobacteria*"* have recently been recognized as a cosmopolitan group in global marine microbial ecosystems and likely play key roles in biogeochemical processes in many iron-rich redox-cline environments such as sediments and rocks in deep-sea hydrothermal fields and in shallow waters (Emerson et al. 2010). In addition, some *"Ca.* Zetaproteobacteria*"* members have been found in association with metallic corrosion moieties (McBeth et al. 2011). However, knowledge of the physiological and molecular characteristics of *"Ca.* Zetaproteobacteria*"* is limited because such information has been obtained from only a few isolates from the Loihi Seamount hydrothermal field (Emerson et al. 2007).

The marine iron-oxidizing chemolithoautotrophic bacterial strains PV-1^T and JV-1 were isolated by Emerson and Moyer (2002), Emerson et al. (2007) at the Loihi Seamount. Originally, strains PV-1^T and JV-1 were classified as *Gammaproteobacteria.* Later, detailed phylogenetic analysis showed that strains PV-1^T and JV-1 possibly represent a novel class of *Proteobacteria, "Ca.* Zetaproteobacteria", and belonged to a new species of the new genus *Mariprofundus*, named *Mariprofundus ferrooxydans* (Emerson et al. 2007; Moreira et al. 2014). Notably, strain PV-1^T forms ribbon-like iron oxyhydroxide stalks, an extracellular material secreted from the cell surface in a helical shape, whereas strain JV-1 does not form stalks. Stalks were first found in *Gallionella* sp. (Ehrenberg 1836) and became a defining feature. The genus *Gallionella* belongs to *Betaproteobacteria*. The genus *Leptothrix* also excretes extracellular material and is well known as a betaproteobacterial iron-oxidizing bacteria. While *Gallionella* forms helical stalks, *Leptothrix* forms tubular sheaths (van Veen et al. 1978; Emerson and Ghiorse 1992, 1993). Although these genera are found in the same environment, they are easily discerned based on their morphological differences under a microscope. *Mariprofundus ferrooxydans* PV-1 produces helical "stalks" (Emerson and Moyer 2002; Chan et al. 2011; Emerson et al. 2007) that morphologically resemble the stalks of *Gallionella* species (Ehrenberg 1836; Ghiorse 1984; Hallbeck and Pedersen 1990, 1991). Indeed, similar ribbon-like iron oxyhydroxide structures have been found globally in various iron-dominated deposits in deep-sea hydrothermal fields (Kennedy et al. 2003). Helical stalks in deep-sea hydrothermal environments were long regarded as potential products of deep-sea *Gallionella* populations (Halbach et al. 2001) until the isolation of *M. ferrooxydans* (Emerson and Moyer 2002; Emerson et al. 2007).

Recent culture-dependent and culture-independent microbiological characterizations have revealed the ecological concurrence between the distribution of *"Ca.* Zetaproteobacteria" members and multi-stalked iron-dominating deposits in low-temperature hydrothermal fields such as the Loihi Seamount (Emerson et al. 2007), the Northern Mariana arc (Davis and Moyer 2008), the Southern Mariana trough (Kato et al. 2009), the Vailulu'u seamount (Staudigel et al. 2006), the Kermadec arc (Hodges and Olson 2009) and the South Tonga arc submarine volcanoes (Forget et al. 2010). Mineralogical characterization has suggested that such natural biogenic iron oxyhydroxides, including the stalk structures, could be produced by in situ *"Ca.* Zetaproteobacteria" populations represented by *M. ferrooxydans* (Makita et al. 2016). Furthermore, using spatial autocorrelation analysis and molecular variance (AMOVA) analysis, McAllister et al. (2011) revealed the significant biogeographic diversity of *"Ca.* Zetaproteobacteria". Although the genome sequence analysis of *M. ferrooxidans* PV-1^T has been completed (Singer et al. 2011), the diversity, physiology and ecology of *"Ca.* Zetaproteobacteria" are still uncertain because very few strains are available for studies.

Here, we successfully isolated a novel iron-oxidizing strain belonging to *"Ca.* Zetaproteobacteria" from deep-sea sediment at a hydrothermal field in the Bayonnaise knoll at the Izu-Ogasawara arc. The new isolate was characterized as a mesophilic, neutrophilic, microaerophilic strict chemolithoautotroph. The phylogenetic analysis of its 16S rRNA gene sequence, molecular characteristics and morphological properties indicated that the isolate has features distinct from those of the previously described *M. ferrooxidans*. Through phenotypic and genomic comparison with related species, we propose a novel species of the genus *Mariprofundus*, named *Mariprofundus micogutta* sp. nov. Moreover, the taxonomic name *Zetaproteobacteria* had not been formally published to date. Therefore, taxonomic ranks between class and order are also proposed in this study.

Materials and methods

Sample collection, enrichment and purification

A deep-sea hydrothermal sediment sample was collected at the Bayonnaise knoll at the Izu-Ogasawara arc (31°57.432′N, 139°44.736′E; 772 m deep) using the JAMSTEC remotely operated vehicle (ROV) *Hyper-Dolphin* (dive #1649), in April 2014. Many dead chimney structures were present, but active chimney sites hosted diffusing hydrothermal fluids (>130 °C). The active chimneys were grey and orange and less than 20 cm tall. A sample of orange seafloor sediment was collected using an M-type sampler (Masuda et al. 2005). Immediately after recovery of the ROV, the subsample sediment was suspended in filter-sterilized (0.22 μm pore size) natural seawater containing 0.05 % (w/v) sodium sulphide in a 100-ml glass bottle and the bottle was tightly sealed with a butyl rubber cap under N_2 atmosphere.

In the laboratory after the cruise, the suspended slurry was inoculated onto an O_2 and Fe(II) gradient medium developed by Emerson and Moyer (1997, 2002) and modified in this study for use as an enrichment. In this method, opposite concentration gradients of molecular oxygen (O_2) and Fe(II) were formed by two solid medium layers (top and bottom) in the test tube (Fig. S1). The basal medium, a modified medium for ASW (Emerson and Moyer 2002; Emerson and Floyd 2005), was composed of 27.5 g NaCl, 5.38 g $MgCl_2{\cdot}6H_2O$, 6.78 g $MgSO_4{\cdot}7H_2O$, 0.72 g KCl, 0.2 g $NaHCO_3$, 1.4 g $CaCl_2{\cdot}2H_2O$, 1 g NH_4Cl and 10 ml trace element solution (MD-TMS; ATCC catalogue no. MD-TMS) per litre of distilled water. After autoclaving, 1 g

$NaHCO_3$ and 1 ml vitamin solution (MD-VS; ATCC catalogue no. MD-VS) were added aseptically. The top layer, consisting of agarose (0.15 % (v/v) Agarose S; NIPPON GENE, Tokyo, Japan)-stabilized medium, and the bottom layer, consisting of agarose (1.3 % v/v)-stabilized FeS, were prepared separately. The FeS was prepared according to Hanert (2006) and added as the Fe(II) source. The medium was prepared by purging the ASW medium containing $NaHCO_3$ and vitamin solution with CO_2 for an appropriate time to reduce the pH to 6.2–6.5, as determined with a pH meter. Microbial growth was visually confirmed by the formation of a brown colony band in the medium after culturing in the dark for approximately 10 days at 25 and 37 °C.

From a colony on solid gradient medium, which only grew at 25 °C, a pure culture was obtained with the dilution-to-extinction technique (Makita et al. 2012) using liquid medium at 25 °C. For the dilution series, 10 ml of liquid in 20 ml (16.5 φ × 16 cm) test tubes (Iwaki Glass, Tokyo, Japan) and screw caps with butyl rubber septa were used. Liquid medium was obtained by removing agarose from the top layer of the gradient medium described above. The medium was prepared under a gas phase of 80 % N_2 and 20 % CO_2. The final pH of medium was brought to approximately pH 6.4 by gas purging (measured with a pH meter). Immediately prior to inoculation, a sterile syringe with a 27-gauge needle was used to deliver approximately 200 µm $FeCl_2$ from the stock solution and O_2 (100 %, 100–300 µl, final O_2 concentrations; 1–3 %) to the headspace. The $FeCl_2$ stock solution was prepared according to Emerson and Moyer (2002). Unless otherwise indicated, growth tests were carried out with the above liquid medium at pH 6.4 and 25 °C. Non-motile bean shapes and curved rods dominated in the cultures. The purified strain was designated as strain ET2^T. Purity was confirmed by microscopic observation, repeated partial sequencing of the 16S rRNA gene using several PCR primer sets (Lane 1991) and genome analysis.

Morphology

Cells were routinely examined under a phase-contrast Olympus BX53 microscope with an Olympus DP72 colour CCD camera (Olympus Co. Ltd., Tokyo, Japan). Transmission electron micrographs (TEMs) of negatively stained cells were obtained as previously described (Zillig et al. 1990). The cells grown in liquid medium were harvested by centrifugation at the mid-exponential growth phase and stained with 1 % (w/v) neutral phosphotungstic acid for observation with a Tecnai-20 (FEI Company, Japan) electron microscope at an acceleration voltage of 120 kV. For scanning electron microscopic (SEM) observation, cells were prefixed 2 h in 2.5 % (w/v) glutaraldehyde in liquid medium at 25 °C. After being washed in fresh liquid media, cell suspensions were adhered to the poly-L-lysine (Sigma)-coated tip for 30 min at room temperature. Following this treatment, the cells were fixed in 2 % (w/v) osmium tetroxide dissolved in phosphate-buffered saline (PBS, pH 7.2; Nakarai Tesque, Japan). After rinsing with distilled water, conductive staining was performed by incubation with 0.2 % aqueous tannic acid (pH 6.8) for 30 min. The samples were washed with distilled water, treated with 1 % aqueous osmium tetroxide for 1 h, then dehydrated in a graded ethanol series and dried in a desiccator. The samples were finally coated with osmium using an osmium plasma coater (POC-3; Meiwafosis) and observed with a JSM-6700F field emission scanning electron microscope (JEOL, Japan) operated at 5 kV. Acid treatment (0.1 M HCl, 10 min) was performed to observe the association of cells with iron oxyhydroxides. Energy-dispersive spectrometry (EDS) was used to estimate the elemental composition of extracellular materials. The SEM–EDS and TEM–EDS analyses were conducted at an acceleration voltage of 15 keV for elemental analysis. EDS analyses were conducted using a JSM-6700F field emission SEM (JEOL, Japan) equipped with energy-dispersive X-ray spectroscopy (EDS, JED-2300; JEOL, Japan) and a Tecnai-20 (FEI Company, Japan) electron microscope equipped with EDAX EDS (EDAX Inc., USA).

Growth characteristics

The growth of strain ET2^T was examined by direct cell counting after staining with 4,6-diamidino-2-phenylindole (DAPI) using a phase-contrast Olympus BX53 microscope equipped for epifluorescence (Porter and Feig 1980). Before observation, acid treatment (1 M HCl of final concentration, 5 min) was performed to remove iron oxyhydroxides from cells in culture. To determine the temperature, pH and NaCl ranges for growth, strain ET2^T was grown in temperature-controlled dry ovens using 20-ml (16.5 φ × 16 cm) test tubes (Iwaki Glass, Tokyo, Japan) containing 10 ml of liquid medium. When the pH range for growth was determined, the pH of the liquid medium was adjusted to various values with 10 mM acetate/acetic acid buffer (pH 4–5), MES (pH 5–6), PIPES (pH 6–7), HEPES (pH 7–7.5), Tris (pH 7.5–8), TAPS (pH 8–8.5), CHES (pH 8.5–9.7) and CAPS (pH 9.7–10) at 25 °C. The pH was readjusted with HCl or NaOH immediately before inoculation. The NaCl requirement for growth was determined using various concentrations of NaCl (0–7.0 %, w/v) in the medium.

The new isolate was tested for its ability to grow with various combinations of electron donors and acceptors. ASW supplemented with 0.1 % (w/v) $NaHCO_3$ and vitamin solution (MD-VS) was used as the basal medium. To

examine growth with electron acceptors other than O_2, Fe^{2+} was used as an electron donor under N_2/CO_2 (80:20) gas phase (250 kPa). The potential electron acceptors S^0 (3 %, w/v), $Na_2S_2O_3{\cdot}5H_2O$ (0.1 %, w/v), Na2SO3·5H2O (0.01–0.1 %, w/v), $NaNO_3$ (0.1 %, w/v) and $NaNO_2$ (0.1 %, w/v) were tested. For testing growth on S^0 (3 %, w/v), $Na_2S_2O_3{\cdot}5H_2O$ (0.1 %, w/v) as an electron donor, $N_2/CO_2/O_2$ (77:20:3) was used as the gas phase (250 kPa). We also tested whether strain ET2^T could use zero-valent iron (Fe^0) for growth. Autoclaved Fe^0 particles were utilized as an iron source instead of the $FeCl_2$ solution. Approximately 50 mg of Fe^0 particles was added to 10 ml of liquid medium.

To examine heterotrophic growth, experiments were conducted using liquid medium without $NaHCO_3$ under a N_2/O_2 (97:3) gas phase (250 kPa). Each of the following potential carbon sources was tested at concentrations of 0.01 and 0.1 % (w/v): casamino acids, D(+)-glucose, maltose, formate, acetate, fumarate, lactate, citrate, pyruvate, tryptone peptone (Difco) and yeast extract (Difco).

To determine potential nitrogen sources for growth, utilization of N_2 as the nitrogen source was examined with liquid medium lacking all nitrogen compounds under a $N_2/CO_2/O_2$ (77:20:3) gas phase (250 kPa). After ET2^T was confirmed not to use N_2 as a nitrogen source, 0.1 % (w/v) NH_4Cl, $NaNO_2$, $NaNO_3$ or casamino acids were added to liquid media lacking all other nitrogen compounds under a $N_2/CO_2/O_2$ (77:20:3) gas phase (250 kPa). The O_2 preference for growth was determined by measuring the dissolved O_2 concentration at the location of colony band formation in the solid gradient culture using an oxygen sensor (Oxy-N; Unisense, Aarhus, Denmark).

The sensitivity of growth of strain ET2^T to antibiotics such as chloramphenicol (50 and 100 μg/ml), streptomycin (50 and 100 μg/ml), kanamycin (50 and 100 μg/ml), ampicillin (50 and 100 μg/ml) and rifampicin (50 and 100 μg/ml) was tested in liquid medium at 25 °C.

Fatty acid analysis

The cellular fatty acid composition was analysed via gas chromatography–mass spectrometer (GC/MS). Cells grown in liquid medium at 25 °C for 5 days (late exponential growth phase) were collected by centrifugation, and the iron hydroxide particles with cells were dissolved with a solution of dithionate (50 g/l) in 0.2 M citrate and 0.35 M acetic acid (Kostka and Luther 1994). The cells were collected by centrifugation and washed with a 2.75 % NaCl solution before lyophilization. The lyophilized cells (1.5 mg) were placed in test tubes containing 3 ml anhydrous methanolic HCl and heated at 100 °C for 3 h. Extraction and analysis of fatty acid methyl esters were as described elsewhere (Komagata and Suzuki 1987; Suzuki et al. 2005). The fatty acid methyl esters (FAMEs) were extracted thrice with n-hexane. Concentrated FAMEs were analysed using a GC/MS (JMS-Q1500GC; JEOL, Japan).

Nucleic acid analyses

Cells in the late exponential growth phase were harvested by centrifugation, and the cell and iron oxide pellet was washed three times with PBS. DNA was extracted using the MO BIO PowerMaxSoil DNA isolation kit (Carlsbad, CA, USA).

The 16S rRNA gene was amplified by PCR using primers of Bac 27F and 1492R (Lane 1991). Amplified 16S rRNA gene fragments directly served as a template for sequencing analysis. The sequence of the 16S rRNA gene was determined using the deoxynucleotide chain-termination method with a 3730XL DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). Sequence similarity analysis of 16S rRNA genes was conducted using BLAST (Altschul et al. 1997; Benson et al. 1998). The phylogenetic trees were constructed by the maximum likelihood (ML) method in the MEGA 5.0 package (Tamura et al. 2011), using the parameters for Jukes–Cantor model distance. Bootstrap confidence of branching was calculated using 1000 replications for the ML tree.

The genome of ET2^T was sequenced at FASMAC Co. Ltd., (Atsugi, Japan) using the Illumina MiSeq platform (San Diego, CA, USA). Genome assembly was performed using SPAdes version 3.5.0 (Bankevich et al. 2012), and the average coverage of pair-end reads was 100-fold. Gene annotations of the ET2^T assemblies were performed using Prokka version 1.11 (Seemann 2014). Protein annotation was further supported by KASS (KEGG Automatic Annotation Server) (Moriya et al. 2007). Average nucleotide identity (ANI) values were calculated with the OrthoANI algorithm (Lee et al. 2015). Digital DNA–DNA hybridization (DDH) values were determined using the in silico genome-to-genome distance calculator (GGDC2.1; http://ggdc.dsmz.de/distcalc2.php) using the alignment method blast+ (Meier-Kolthoff et al. 2013). Orthologous genes between strain ET2^T and *M. ferrooxydans* PV-1^T were identified with a bidirectional BLASTP search, and average amino acid identity (AAI) as well as percentage of conserved proteins (POCP) (Qin et al. 2014) were estimated.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *M. micogutta* strain ET2^T is LC107871. The genome sequence data of this strain are publicly available under the accession numbers BDFD01000001-BDFD01000059.

Results and discussion

Enrichment, purification and morphology

Cell growth was observed in a solid gradient culture after 5–10-day incubation only at 25 °C. Rust-coloured bands, at an intermediate distance between the FeS plug in the bottom of the tube and the air interface at the surface of the gel (Fig. S1), appeared as a brown sparkling granule drops. Once a stable band formed, usually after 10 days, it was subsampled and diluted into a new gradient medium. After the culture was cultivated several times in gradient media, it was transferred to a liquid medium. The non-motile bean-shaped cells and curved rods were purified from the first enrichment using the dilution-to-extinction technique at 25 °C.

Cells of strain ET2^T were gram-negative bean shapes or curved rods, approximately 0.5 μm wide and 1.0–1.6 μm long (Fig. 1a, b). Some cells had polar appendages, similar to prosthecate structures (Fig. 1b), that were 0.13 μm wide and 1.0–23 μm long. Cells occurred singly, and no sporulation was apparent under any culture condition tested.

Strain ET2^T produced many extracellular filamentous structures (Fig. 1c–f), which formed large structures by clumping together (Fig. 1f). These filamentous extracellular materials were morphologically different from the stalks formed by *M. ferrooxydans* (Emerson et al. 2007). SEM–EDS mapping analysis revealed major peaks for Fe and O and minor peaks for C in the extracellular materials (Fig. 2), suggesting that these materials mainly consist of iron oxides cemented together with trace carbon.

Acid treatment of the filamentous materials with 0.1 M HCl resulted in near-complete dissolution, suggesting that they are composed of the same poorly crystalline iron oxyhydroxides as *M. ferrooxydans* PV-1^T (Emerson et al. 2007). After acid treatment, residual filamentous extracellular materials were observed at several locations in the cell (Fig. 1g, h; black arrows). It has been reported that the stalk of PV-1 develops from a specific location in the cell (Emerson et al. 2007; Chan et al. 2011). TEM-EDS analysis revealed that the extracellular materials consisted mainly of carbon (Fig. S4). Interestingly, a polar appendage was no longer observed (possibly degraded by the acid treatment). The filaments produced by strain ET2^T may be an excretion mechanism of iron minerals, as in the stalks of *M. ferrooxydans* PV-1 and *Gallionella* sp.

Growth characteristics

In the liquid media, orange-brown deposits were produced in the bottom of test tubes during growth. The growth curves of strain ET2^T, shown in Fig. S3, indicate growth from 15 to 30 °C, with optimal growth at 25 °C. No growth was observed below 10 °C or above 35 °C. Growth occurred between pH 5.8 and pH 7.0, with an optimum at approximately pH 6.4. No growth was detected below pH 5.0 or above pH 8.3. The isolate showed an absolute requirement for NaCl at concentrations from 1.5 to 4.0 % with optimal growth at 2.75 %. No growth was observed below 1.0 % NaCl or above 4.5 % NaCl. The generation time and maximum cell yield at 25 °C were approximately 24 h and 3.0×10^7 cells/ml, respectively.

No combination other than Fe^{2+} and O_2 supported the growth of strain ET2^T, but growth was observed on zero-valent iron (Fe^0). The isolate was unable to use sulphur compounds of thiosulfate, bisulphite and elemental sulphur as either electron donors or acceptors and did not use nitrite or nitrate as electron acceptors, indicating that the new isolate grows via simple energy metabolism of iron-oxidizing O_2 reduction.

Strain ET2^T could not utilize any organic substrates as carbon sources using Fe^{2+} and O_2 as the electron donor and acceptor. In addition, no organic compounds sustained the growth of strain ET2^T as the electron donors instead of Fe^{2+}. The isolate ET2^T utilizes ammonium and nitrate as the sole nitrogen sources and could not utilize nitrite, N_2 or casamino acid. The optimal O_2 concentration for growth of ET2^T in the solid gradient medium was 2.6–2.8 mg/L, as determined by the oxygen sensor Oxy-N. Thus, strain ET2^T represents microaerophilic growth.

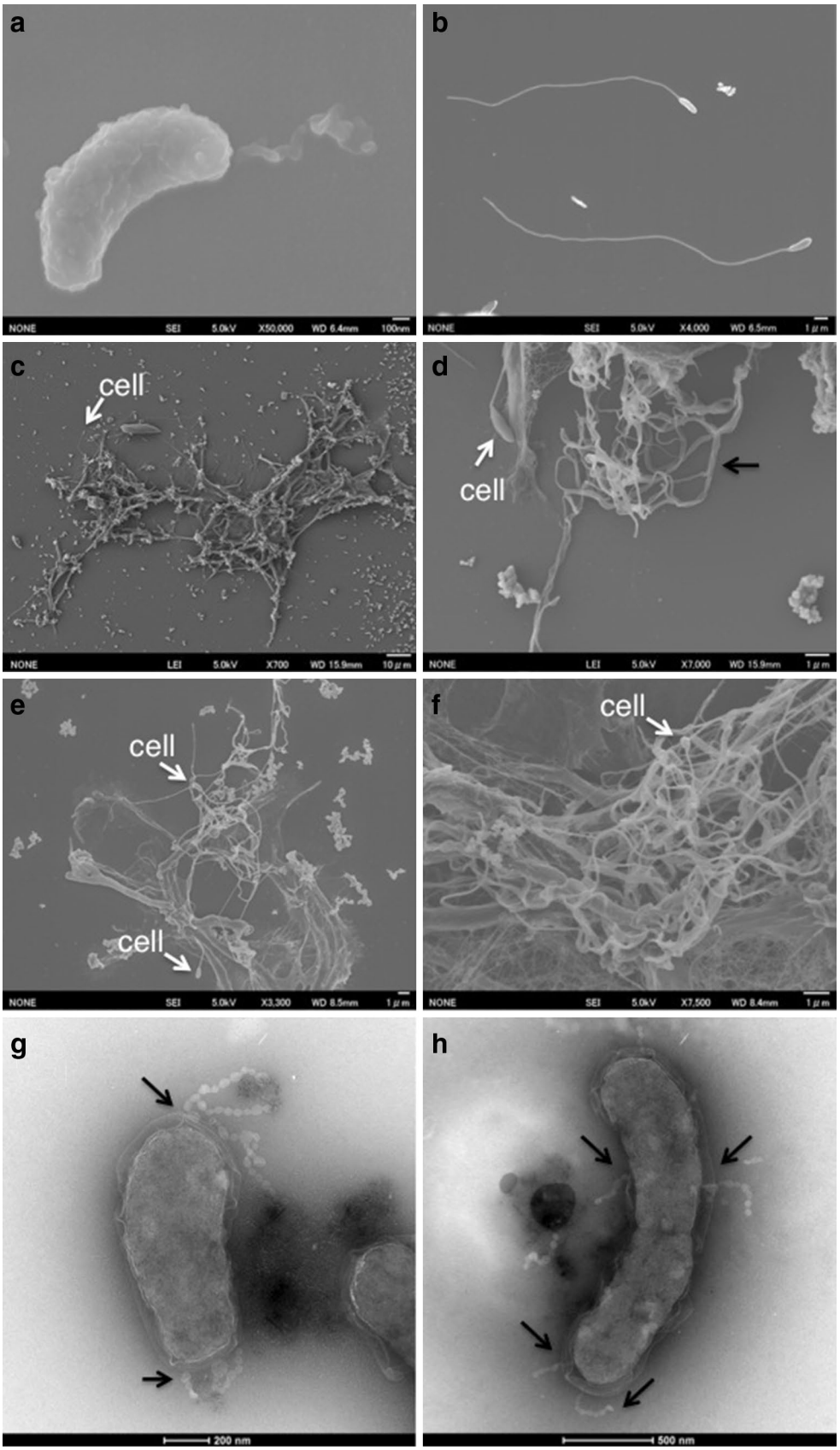
Antibiotics such as chloramphenicol (50 and 100 μg/ml), streptomycin (50 and 100 μg/ml), kanamycin (50 and 100 μg/ml), ampicillin (50 and 100 μg/ml) and rifampicin (50 and 100 μg/ml) completely inhibited the growth of strain ET2^T.

Genome sequencing

A draft genome of strain ET2^T was obtained via genome sequencing and assembly. The assemblies consisted of 59 contigs (total length of 2.497 Mbp), and the G+C content was 48.8 %. The ET2^T genome coded 2417 protein-coding sequences (CDSs). Two copies of the rRNA operons are encoded in the genome of strain ET2^T. A complete CDS set for carrying out carbon fixation using the Calvin–Benson–Bassham cycle was identified.

16S rRNA gene phylogenetic analysis

The 16S rRNA gene sequence of strain ET2^T was similar to the sequences of *M. ferrooxydans* strain PV-1^T (95 %), *Mariprofundus* sp. strain JV-1 (95 %) (Emerson


a
NONE SEI 5.0kV X50,000 WD 6.4mm 100nm
b
NONE SEI 5.0kV X4,000 WD 6.5mm 1 μm
c
cell
NONE LEI 5.0kV X700 WD 15.9mm 10 μm
d
cell
NONE LEI 5.0kV X7,000 WD 15.9mm 1 μm
e
cell
cell
NONE SEI 5.0kV X3,300 WD 8.5mm 1 μm
f
cell
NONE SEI 5.0kV X7,500 WD 8.4mm 1 μm
g
200 nm
h
500 nm

◂**Fig. 1** Electron micrographs of cell strain ET2^T. Scanning electron micrographs of a cell (**a**), cells producing polar prosthecates-like materials (**b**), cell with extracellular materials and iron oxide (**c**–**f**). Transmission electron micrographs of negatively stained single cell (**g**) and dividing cell (**h**) with extracellular iron oxyhydroxide filaments. *Black arrows* indicate extracellular materials. *Scale bars* indicate 0.1 μm (**a**), 1.0 μm (**b**, **d**–**f**), 10 μm (**c**), 0.2 μm (**g**) and 0.5 μm (**h**)

et al. 2007) and *Mariprofundus* sp. strain GSB-2 (94 %) (McBeth et al. 2011). The most similar sequences were environmental clones obtained from a colonization system deployed in an observatory at the Juan De Fuca ridge flank (FLOCS_1301A-GWPyrrhotite_C01; 97 %) (Baquiran et al. 2016) and from an inactive hydrothermal sulphide chimney at the mid-ocean ridge (MOR) spreading centre (3M34_081; 97 %) (Sylvan et al. 2012). The phylogenetic tree was constructed with 1329 homologous sequence positions and indicated that strain ET2^T is distinct from previously isolated strains of *M. ferrooxydans* within "*Ca.* Zetaproteobacteria" (Fig. 3).

Genomic comparison with other "*Ca.* Zetaproteobacteria"

Compared with *M. ferrooxydans* PV-1^T, strain ET2^T harbours a smaller genome with lower G+C content (2.99 Mb and 54 %, respectively) (Singer et al. 2011) (Table 2). The 16S rRNA gene similarity between strain ET2^T and *M. ferrooxydans* PV-1^T fell within the common index for genus-level differentiation (90–96 %) (Gillis et al. 2001). Genomic comparison with ANI and DDH calculations, however, indicated that genomic similarities between strain ET2^T and *M. ferrooxydans* strain PV-1^T were sufficient only for the threshold of species-level differentiation (95–96 % ANI, 70 % eDDH (Richter and Rosselló-Móra. 2009); 71.6 % of ANI and 13.4–19.8 % of DDH). The POCP value between ET2^T and *M. ferrooxydans* PV-1^T was 67.7 %, and the AAI of the shared CDSs was 68.5 %. These genomic similarity values are higher than the threshold of genus-level differentiation (50 %) (Qin et al. 2014). Combined, these results suggest that strain ET2^T should be characterized as a new species in the genus *Mariprofundus* within "*Ca.* Zetaproteobacteria".

Fatty acid composition

The major cellular fatty acid components of strain ET2^T were $C_{16:1\omega7c}$ (51.5 %), $C_{16:0}$ (27.1 %), $C_{18:0}$ (8.1 %), $C_{12:0}$ (7.7 %) and $C_{16:1\omega7t}$ (5.6 %). *Mariprofundus ferrooxydans* strain PV-1^T contains iso-C11:0-3OH as the most abundant component of cellular fatty acids (Emerson et al. 2007). Thus, the two strains differ greatly in fatty acid content (Table 1).

Phenotypic comparison with *Mariprofundus ferrooxydans*

The 16S rRNA gene phylogeny and genomic comparison indicate that strain ET2^T is a member of the genus *Mariprofundus*. Many physiological and metabolic properties are very similar between *M. ferrooxydans* and the new isolate. *Mariprofundus ferrooxydans* is a strict chemolithoautotroph, and it cannot grow on other inorganic energy sources, such as reduced sulphur compounds and H_2, or on organoheterotrophic substrates. Strain ET2^T is also a strict chemolithoautotroph that grows only with iron-oxidizing O_2 reduction and inorganic carbon. However, strain ET2^T shows different cellular fatty acid composition and genomic DNA G+C content from *M. ferrooxydans* strain PV-1^T (Table 1). More importantly, strain ET2^T has distinctive morphological features (Fig. 1; Table 1). Strain ET2^T forms appendages similar to those of *Caulobacter* spp. (Abraham et al. 1999) and *Hyphomicrobium* spp. (Abraham and Rhode 2014) in the class *Alphaproteobacteria* and is the first example of a prosthecate bacterium in "*Ca.* Zetaproteobacteria". The multiple iron oxyhydroxide filaments associated with ET2^T are morphologically different from the stalks composed of organic compounds and iron oxyhydroxides in the *M. ferrooxydans* strain PV-1^T. The cell division style is also quite different between strain ET2^T and *M. ferrooxydans* strain PV-1^T.

On the basis of these phylogenetic, genomic and phenotypic characteristics, we conclude that strain ET2^T represents a novel species within the *Mariprofundus* and propose a novel species named *Mariprofundus micogutta* sp. nov. Furthermore, the class "*Ca.* Zetaproteobacteria" containing the family *Mariprofundaceae* (Moreira et al. 2014) is a novel and distinctive taxon in the phylum *Proteobacteria*. However, the taxonomic name *Zetaproteobacteria* had not been formally published to date. The presence of "*Ca.* Zetaproteobacteria" has been confirmed in recent years a various environments (Emerson et al. 2007; Davis and Moyer. 2008; Kato et al. 2009; Staudigel et al. 2006; Hodges and Olson 2009; Forget et al. 2010). Moreover, the analysis of their genome is also progressing (Emerson et al. 2010; Singer et al. 2011). For the development of further research for these iron utilization microorganisms, there is a need to formally define their class and order. Therefore, taxonomic ranks between class and order are also proposed in this study.

Description of *Mariprofundus micogutta* sp. nov

Mariprofundus micogutta (*mic.o.gutta.* L. intr. *mico* sparkle. twinkle; L. f. *gutt.a* drop, spots; the type strain produces sparkling spot-like colonies under culture).

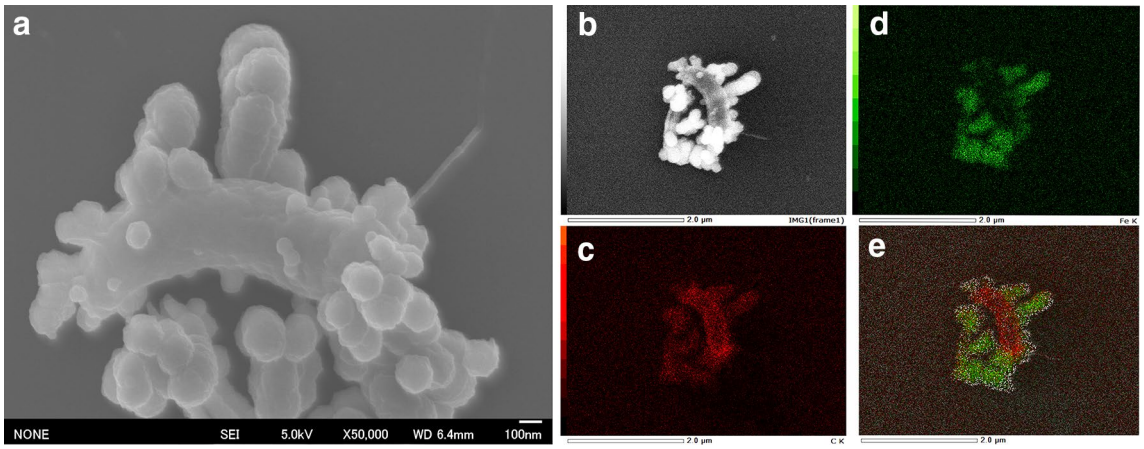


Fig. 2 SEM images and elemental maps showing the ET2^T cell and extracellular materials with iron oxide. **a** SEM image showing the cell and extracellular materials. **b** SEM–EDS image of the cell with extracellular material from **a**. Also shown are elemental maps: **c** Carbon shown in *red*, **d** Fe shown in *green* and **e** both elements overlaid. *Scale bars*, 0.1 μm (**a**) and 2 μm (**b**–**e**)

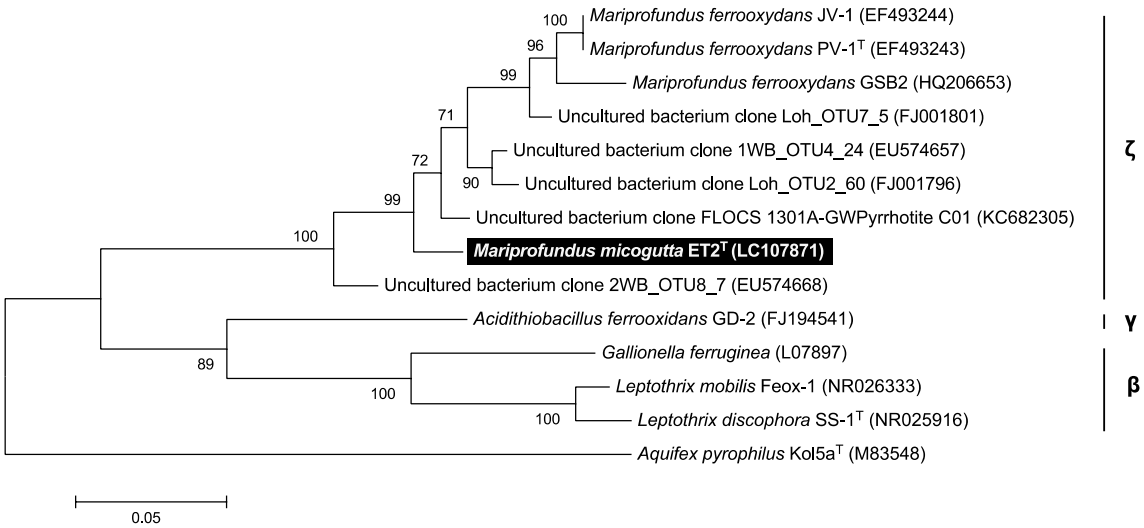


Fig. 3 Phylogenetic tree based on the 16S rRNA gene sequences of representative strains and environmental clones within the *"Ca.* Zetaproteobacteria*"*, including strain ET2^T. *Aquifex pyrophilus* strain Kol 5a^T was used as an outgroup. *Branch values* indicate the bootstrap confidence of branching in per cent. *Numbers in parentheses* are GenBank/EMBL/DDBJ database accession numbers. *Bar* indicates 1 substitution per 100 nucleotide positions

Cells are bean-shaped or curved rods of approximately 1.0–2.0 μm in diameter. Cells produce polar prosthecates-like materials, and appendages are 0.13 μm wide and 1.0–23 μm long. The temperature range for growth is 15–30 °C (optimum 25 °C). The pH range for growth is pH 5.8–7.0 (optimum pH 6.4). The NaCl concentration range for growth is 1.0–4.0 % (optimum 2.75 %). Fe^{2+} and Fe^0 are oxidized to Fe^{3+} during growth. Nitrate or ammonium is required as a nitrogen source. Organic compound is not available for their growth. The major fatty acids are $C_{16:1\omega7c}$ (51.5 %), $C_{16:0}$ (27.1 %), $C_{18:0}$ (8.1 %), $C_{12:0}$ (7.7 %) and $C_{16:1\omega7t}$ (5.6 %). The G+C content of DNA is 48.8 mol %.

The type strain is ET2^T (= KCTC 15556^T = JCM 30585^T), isolated from deep-sea sediment associated with hydrothermal activity at the Bayonnaise knoll of the Izu-Ogasawara Arc.

Table 1 Comparison of properties among strain ET2^T, PV-1^T and other iron-oxidizing genera and species

Strains	1 *M. micogutta* strain ET2^T (this study)	2 *M. ferrooxydans* strain PV-1^T	3 *G. ferruginea*	4 *F. amnicola* strain OYT1^T	5 *S. lithotrophicus* strain ES-1^T
Source	Deep-sea hydrothermal sediment	Deep-sea hydrothermal sediment, salt marsh	Groundwater seep, well water	Groundwater seep	Soil
Morphology	Bean shapes, curved rods	Curved rods	Bean shapes or curved rods	Curved rods	Curved rods
Extracellular materials	+	+	+	+	−
Length (μm)	1.0–1.6	1.0–4.0	1.5–2.5	0.8–1.9	1
Width (μm)	0.5	0.3–0.6	0.3–1.0	0.7–0.9	0.5–0.7
Motility	−	+	+	+	+
Temperature range (optimal temperature) for growth (°C)	15–30 (25)	10–30 (25–30)	5–25 (20)	8–30 (25–30)	21–30 (ND)
pH range (optimal) for growth	5.8–7.0 (6.4)	5.5–7.2 (6.2–6.5)	5.0–6.5 (ND)	5.6–7.3 (6.1–6.5)	5.5–7.0 (6.0–6.5)
Salinity range (optimal) for growth (% NaCl)	1.0–4.0 (2.75)	1.5–4.5 (2.5)	ND	0–0.8 (0)	ND
Energy sources:					
Fe(II)	+	+	+	+	+
Thiosulfate, sulphide	−	−	+	−	+
Other lithotrophy	−	−	−	−	−
Organic matter	−	−	−	−	−
Fatty acids*	16:1ω7*c*, 16:0, 18:0, 12:0 16:1ω7t	iso-11:0 -3OH, 16:1ω7*c*/iso-15:0 -2OH, iso-17:0 -3OH, 16:0, 19:0ω10*c*/16:1ω6*c*, iso-18:1-OH	ND	16:1ω7*c*/16:1ω6*c*, 16:0	16:1ω7*c*/iso-15:0 -2OH, 16:0, 18:0, 18:1ω9c
G+C content (mol%)	48.8	54	35.2–37.6	57.6	37.2

1. *Mariprofundus micogutta* strain ET2^T (this study); 2. *Mariprofundus ferrooxydans* strain PV-1^T (Emerson and Moyer 2002; Emerson et al. 2007); 3. *Betaproteobacteria, Gallionella ferruginea* (Hallbeck and Pederson 1990, 1991; Hallbeck et al. 1993); 4. *Betaproteobacteria, Ferriphaselus amnicola* strain OYT1^T (Kato et al. 2013, 2014); 5. *Betaproteobacteria, Sideroxydans lithotrophicus* strain ES-1^T (Emerson and Moyer 1997; Emerson et al. 2013)

+ Positive; − negative; *ND* not determined; * Components present at greater than 10 % of total fatty acids are given

Table 2 Features of the *Mariprofundus micogutta* ET2^T and *M. ferrooxydans* PV-1 ^{T} genomes and results of ANI, DDH (GGDC), AAI and POCP calculations[*1]

	M. micogutta ET2^T	*M. ferrooxydans* PV-1^{T*2}
Contigs	59	32
Total size (bp)	2,497,805	2,992,730
G+C content (%)	49	54
CDS	2417	2668
rRNA operon	2	2
tRNA	49	37
Accession no.	BDFD01000001–BDFD01000059	AATS00000001–AATS00000032
		vs *M. micogutta* ET2^T
ANI (OrthoANI)	ND	71.56
DDH (GGDC)		
F 1	ND	13.4
F 2	ND	19.8
F 3	ND	13.7
AAI	ND	68.5
POCP	ND	67.7

[*1] *ANI* average nucleotide identity, *DDH* DNA–DNA hybridization, *GGDC* genome-to-genome distance calculator, *AAI* average amino acid identity, *POCP* percentage of conserved proteins

[*2] Singer et al. (2011)

ND not determined

Description of *Mariprofundales* ord. nov

Mariprofundales (Mar.is.pro.fund'a.les. NL. masc. n. *Mariprofundus* type genus of the order; suff. -ales ending to denote an order; N. L. fem. pl. n. *Mariprofundales* the order of the genus *Mariprofundus*). The type genus is *Mariprofundus* (Emerson et al. 2007). The order is defined on the basis of a phylogenetic analysis of the 16S rRNA gene sequence analysis.

Description of *Zetaproteobacteria* classis nov

Zetaproteobacteria (Gr. n. zeta name of sixth letter of Greek alphabet; N.L. masc. n. *Mariprofundus* (Emerson et al. 2007) type genus of the family; N. L. fem. Pl. n. *Mariprofundaceae* (Moreira et al. 2014) the type family of the type order of the class; N. L. fem. pl. n. *Mariprofundales* the order of the class *Zetaproteobacteria*).

The class is defined on the basis of a phylogenetic analysis of 16S rRNA gene sequences of four isolated strains and uncultured representatives from various environments. The type genus: *Mariprofundus*. The type family: *Mariprofundaceae*. The type order: *Mariprofundales*.

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