Tepidicaulis marinus gen. nov., sp. nov., a marine bacterium that reduces nitrate to nitrous oxide under strictly microaerobic conditions

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A moderately thermophilic, aerobic, stalked bacterium (strain MA2T) was isolated from marine sediments in Kagoshima Bay, Japan. Phylogenetic analysis of 16S rRNA gene sequences indicated that strain MA2T was most closely related to the genera Rhodobium, Parvibaculum, and Rhodoligotrophos (92–93 % similarity) within the class Alphaproteobacteria. Strain MA2T was a Gram-stain-negative and stalked dimorphic bacteria. The temperature range for growth was 16–48 °C (optimum growth at 42 °C). This strain required yeast extract and NaCl (≥ 1%, w/v) for growth, tolerated up to 11% (w/v) NaCl, and was capable of utilizing various carbon sources. The major cellular fatty acid and major respiratory quinone were C18:1ω7c and ubiquinone-10, respectively. The DNA G+C content was 60.7 mol%. Strain MA2T performed denitrification and produced N2O from nitrate under strictly microaerobic conditions. Strain MA2T possessed periplasmic nitrate reductase (Nap) genes but not membrane-bound nitrate reductase (Nar) genes. On the basis of this morphological, physiological, biochemical and genetic information a novel genus and species, Tepidicaulis marinus gen. nov., sp. nov., are proposed, with MA2T (=NBRC 109643T =DSM 27167T) as the type strain of the species.

Nitrous oxide (N2O) is an important ozone-depleting gas that significantly contributes to global warming (Ravishankara et al., 2009). The ocean is one of the major sources of N2O, and the mean annual N2O emissions from both open oceans and coastal areas is estimated to be 5.4 Tg N yr−1; this represents 29% of the total natural and anthropogenic N2O sources (US EPA, 2010). The highest N2O concentrations are typically found in oxygen minimum zones (OMZs) (Rönnér & Sörensson, 1985; Yoshinari, 1976), and N2O in the OMZs
has mainly been attributed to nitrifiers (Nevison et al., 2003). However, microbes in some pure cultures have been shown to produce N$_2$O during denitrification performed under aerobic or microaerobic conditions (Bazylinski & Blakemore, 1983; Frette et al., 1997; Okada et al., 2005; Patureau et al., 1998; Robertson et al., 1995; Scholten et al., 1999; Takaya et al., 2003; Torres et al., 2011). Nevertheless, the majority of previous isolates capable of aerobic or microaerobic denitrification were obtained from terrestrial not marine environments (Blakemore et al., 1979; Frette et al., 1997; Okada et al., 2005; Patureau et al., 1998; Robertson & Kuenen, 1983; Scholten et al., 1999; Torres et al., 2011). At the time of writing the only known aerobic denitrifier that has been derived from a marine environment is strain F6 of a species of the genus Marinobacter (Zheng et al., 2012), which was isolated from a marine aquaculture system; however, the end denitrification product of strain F6 is unknown. To date, very little is known about how much N$_2$O is produced during aerobic or microaerobic denitrification in the ocean, and which marine microbes are involved in these processes. Recently, we succeeded in isolating an aerobic marine bacterium that produces N$_2$O from nitrate under microaerobic conditions, but not under obligatory anaerobic or aerobic conditions. In the present study, we describe the morphological, physiological, biochemical and genetic characteristics of strain MA2$^T$, and propose a new genus and species, Tepidicaulis marinus gen. nov., sp. nov., to accommodate the isolate.

In Kagoshima Bay, Japan, multiple hydrothermal vents that emit high-temperature fluid containing methane were identified at the Wakamiko crater (Ishibashi et al., 2008; Yamanaka et al., 2013). Marine surface sediments were collected near the Wakamiko crater at a water depth of 161 m (31°41.09’ N 130°47.124’ E) in 2001 and were used as an inoculum. A methane-utilizing mixed culture (Takeuchi et al., 2014a, b) was established from the marine sediments and strain MA2$^T$ was isolated by plating the mixed culture on marine agar 2216 (Difco).

Cells of strain MA2$^T$ were investigated after growth in marine broth 2216 (Difco). Cell morphology and motility were examined under a phase-contrast microscope (Olympus BX51). After negative staining with 1 % (w/v) aqueous uranyl-acetate, cells were observed using a Hitachi H7000 transmission electron microscope. Biochemical tests were carried out using API 20NE, API 50CH and APIZYM strips (bioMérieux). For API 20NE and APIZYM tests, cells were suspended in 2 % (w/v) NaCl solution. Two different culture media were used for the API 50CH and part of API 20NE assays: NMS medium (Bowman, 2006) that was supplemented with 0.01 % (w/v) yeast extract, vitamin solution (Widdel & Pfennig, 1981) and 2 % (w/v) NaCl, and CHB/E medium (bioMérieux). Strips were incubated in a desiccator at 38°C for 2 weeks. Other growth substrates tested in vials included methanol, methylamine, dimethylamine, trimethylamine, methanesulfonic acid, dimethylsulfide, methylsuccinic acid, formate, acetate, lactate, succinate, propionate, ethanol, lactate, acetone, toluene, vanillin, aspartate, choline, betaine, oxalic acid, glycolate, pyruvate, sarcosine, arginine, serine, histidine, valine, methionine, alanine, asparagine and yeast extract. These substrates were added to NMS medium [supplemented with 2 % (w/v) NaCl and 0.01 % (w/v) yeast extract] at a final concentration of 0.1 % (w/v) (except for acetone, toluene, and ethanol, which were added at a final concentration of 0.03 % (w/v) and incubated at 35°C. The ability of strain MA2$^T$ to utilize methane was also determined by adding 20 % methane to the headspace. Growth of strain MA2$^T$ was tested at various temperatures (10, 16, 20, 25, 35, 38, 42, 45, 48, and 50°C) and pH values (pH 5, 6, 7, 8, 9, and 10). The effect of NaCl on growth was examined using NMS medium (Bowman, 2006) supplemented with 0.3 % (w/v) peptone, 0.01 % (w/v) yeast extract, vitamin solution (Widdel & Pfennig, 1981) and various concentrations (0, 1, 2, 3, 6, 11, and 13 %, w/v) of NaCl. Cellular fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl. The methyl esters were analysed with a Hitachi M7200A GC/3DQMS system (Hanada et al., 2002). Quinones were analysed by HPLC equipped with a Hewlett Packard Zorbox ODS column (Zhang et al., 2000). The total DNA of strain MA2$^T$ was extracted using the ArchivePure DNA Tissues kit (5 Prime) and DNA base composition was determined by reverse-phase HPLC (Shimadzu model LC-6A) (Kamagata & Mikami, 1991). The ability of strain MA2$^T$ to reduce nitrate under aerobic, microaerobic and anaerobic conditions was examined using $^{15}$NO$_3^-$ according to previously described methods (Katsuyama et al., 2008; Yoshinaga et al., 2011). In brief, 20 ml NMS medium supplemented with 0.3 % (w/v) sodium acetate, 0.01 % (w/v) yeast extract and vitamin solution (Widdel & Pfennig, 1981) was prepared in 50 ml vials. $[^{15}N]$NaNO$_3$ was added to NMS media to a final concentration of 15 mM, and the headspace gas was replaced with He by repeatedly vacuuming and purging. For aerobic and microaerobic conditions to be established in vials, pure O$_2$ was added to the headspace to produce 15 % and 5 % O$_2$ in the headspace, respectively (each in triplicate). Vials were incubated at 37°C with stirring. Concentrations of O$_2$, N$_2$ and N$_2$O in the headspace gas were monitored periodically by GC/MS as described previously (Katsuyama et al., 2008; Yoshinaga et al., 2011). Nitrate and nitrite concentrations in the liquid phase were determined by ion chromatography. Chromosomal DNA was extracted using the ArchivePure DNA Tissues kit (5 Prime). The 16S rRNA gene from strain MA2$^T$ was amplified using specific primers for bacteria, fd1 (Weisburg et al., 1991) and Eub1389R (Osborn et al., 2000) as described by Takeuchi et al. (2011), and sequenced with an ABI 3130 × 1 Genetic Analyzer (Applied Biosystems). The draft genome sequence of strain MA2$^T$ was determined using 454 pyrosequencing by single-end (SE) and paired-end (PE) data. We generated 725 877 reads by SE and 177 136 reads by PE, representing a single-end (SE) and paired-end (PE) data. We generated. The sequence of the 16S rRNA gene of strain MA2$^T$ was aligned with relatives in the SILVA database (Pruesse et al., 2007) using MOTHUR v.1.29 (Schloss et al., 2009). The sequences of the napA and napB genes, which encode the periplasmic nitrate reductase, were deduced into the amino acid sequence. Other nucleotide sequences for periplasmic nitrate reductase were retrieved from the GenBank sequence
sequences deduced from concatenated sequences of napA and napB were aligned to reconstruct a phylogenetic tree. Amino acid genera for which nap operon structures are known were database in March 2014. Representative sequences from each genera for which nap operon structures are known were selected to reconstruct a phylogenetic tree. Amino acid sequences indicated that strain MA2T was affiliated with the order Rhizobiales in the class Alphaproteobacteria (Fig. 1). Closely related species with validly published names were Rhodobium orientis (Hiraishi et al., 1995) (93.0 % sequence similarity), Parvibaculum hydrocarbonolasticum (Rosario- Passapera et al., 2012) (92.4 % similarity) and Rhodoligos- trophos appendicifer (Fukuda et al., 2012) (92.1 % similarity). Strain MA2T was distantly related to other stalked bacteria such as Caulobacter crescentus, Maricaulis maris and Oceanicaulis alexandri (Fig. 1). A phylogenetic tree based on 16S rRNA gene sequences demonstrated that strain MA2T formed a separate branch distant from related species within the order Rhizobiales (Fig. 1).

Strain MA2T could grow under aerobic and microaerobic conditions, but could not grow under anaerobic conditions with nitrate or nitrite added as an electron acceptor.

Growth occurred at pH values of pH 6–10, but not at pH 5; the optimum pH was 8. Strain MA2T required NaCl for growth over a range of 1–11% (w/v) with an optimum of 3% (w/v). Strain MA2T additionally required yeast extract (0.01%). Nitrate and ammonium, but not nitrite, were used as nitrogen sources. Strain MA2T grew on acetate, lactate, malate, succinate, gluconate, maltose, pyruvate, yeast extract, alanoline, ethanol, glycerol and capric acid. Analysis of the cellular fatty acid composition showed the predominant fatty acid to be C₁₈:1ω7c (87.3 %). Other major fatty acids were C₁₆:0 (5.4 %), C₁₈:0 (4.9 %) and C₁₈:1 (2.4 %). The major respiratory quinone was ubiquinone-10 (90.8 % of the total). The genomic DNA of strain MA2T contained 60.7 mol% G + C. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain MA2T was affiliated with the order Rhizobiales (Fig. 1). Closely related species with validly published names were Rhodobium orientis (Hiraishi et al., 1995) (93.0 % sequence similarity), Parvibaculum hydrocarbonolasticum (Rosario- Passapera et al., 2012) (92.4 % similarity) and Rhodoligos- trophos appendicifer (Fukuda et al., 2012) (92.1 % similarity). Strain MA2T was distantly related to other stalked bacteria such as Caulobacter crescentus, Maricaulis maris and Oceanicaulis alexandri (Fig. 1). A phylogenetic tree based on 16S rRNA gene sequences demonstrated that strain MA2T formed a separate branch distant from related species within the order Rhizobiales (Fig. 1).

Strain MA2T could grow under aerobic and microaerobic conditions, but could not grow under anaerobic conditions with nitrate or nitrite added as an electron acceptor.

Cells of strain MA2T were dimorphic and appeared as curved rods of 0.3–0.4 × 0.6–3.0 μm in size. Stalked cells were non-motile (Fig. S1a, available in the online Supplementary Material), while non-stalked cells were motile by means of a single polar flagellum (Fig. S1b). Cells reproduced by binary fission (Fig. S1a). When grown on marine agar 2216 at 37 °C, colonies measuring 0.6–0.8 mm in diameter were observed after 6 days. Colonies were round, convex with entire edges and cream. Strain MA2T was Gram-stain-negative, catalase-positive and oxidase-positive. APIZYM test results were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α- and β-glucosidase, and N-acetyl-β-glucosaminidase. Strain MA2T was positive for β-galactosidase and for the hydrolysis of both aesculin and cream. Strain MA2T was Gram-stain-negative, catalase-
Denitrification of nitrate to N₂O (but not to N₂) was observed only under microaerobic conditions (Fig. S2), while nitrate reduction to nitrite was observed under both aerobic and microaerobic conditions (Fig. S2). Production of N₂ was not observed under any conditions tested. N₂O was detected at 20 p.p.m.v. after 2 days of incubation when 3% O₂ remained in the headspace (27 µM in the liquid phase; Fig. S2a). The N₂O concentration reached 4170 p.p.m.v. after 8 days of incubation, which represented 25% of the total amount of nitrate added. To the best of our knowledge, strain MA2T is the first strictly microaerobic denitrifying bacterium that produces N₂O but not N₂. We searched for denitrifying genes in the draft genome sequence. Two types of dissimilatory nitrate reductase are known: a membrane-bound (Nar) and a periplasmic-bound (Nap) nitrate reductase. Only genes encoding Nap (napEDABC) were found in the draft genome of strain MA2T. PCR amplification using specific primers for the Nar-encoding gene (narG 1960F, narG 2659R) (Smith et al., 2007) was also negative (data not shown). Use of Nap and not Nar is a common trait in microaerobic and aerobic denitrifying bacteria (Bell et al., 1960; Sears, 1990; Sears et al., 1997; Taoka et al., 2003; Torres et al., 2011). However, Nap is not exclusively involved in microaerobic and aerobic denitrification (Stewart et al., 2002). As all previously identified aerobic or microaerobic denitrifying bacteria belong to the phylum Proteobacteria, we reconstructed a phylogenetic tree using the concatenated napA gene and napB gene sequences, which are commonly found among Nap operons in members of the phylum Proteobacteria (Fig. S3). When compiled with operon structure information, three major groups were recognized in the phylogenetic tree of napAB genes, i.e. FDA-, E- and AGH-Groups (Fig. S3). napAB genes of the aerobic and microaerobic denitrifying bacteria Sinorhizobium meliloti, Paracoccus pantotrophus and Paracoccus denitrificans, and strain MA2T, were closely related and fell into a certain cluster in the E-Group (designated the MA2 group in Fig. S3); however, Magnetospirillum magnetotacticum was excluded from this cluster.

The differential characteristics of strain MA2T and related taxa are listed in Table 1. Strain MA2T is distinguished from the genera Parivibaculum, Rhodoligotrophos, Rhodobium, Tepidamorphus and Anderseniella by the formation of stalked cells, optimal growth temperature and fatty acid composition. Based on morphological, biochemical, physiological and genetic data, we propose a novel genus and species, Tepidicaulis marinus gen. nov., sp. nov., for strain MA2T.

**Description of Tepidicaulis gen. nov.**

*Tepidicaulis* (Te.pi.di.ca.u'lis. L. adj. tepidus tepid; L. n. masc. n. caulis stalk, referring to a prostheca; N.L. n. masc. *Tepidicaulis* a moderately thermophilic stalked bacterium).

Gram-stain-negative, aerobic and reproduce by binary fission. Cells are dimorphic. Stalked cells are non-motile, while non-stalked cells are motile by means of a single polar flagellum. Aerobic and chemo-organotrophic metabolism. Utilize nitrate or ammonia as a nitrogen source. The major respiratory quinone is ubiquinone-10. The major fatty acids are C₁₈:₁ω7c and C₁₆:₀. Habitat is a marine environment. Phylogenetically, a member of the order Rhizobiales in the class Alphaproteobacteria. The type species is *Tepidicaulis marinus*. The DNA G+C content of the type species is 60.7 mol%.

**Description of Tepidicaulis marinus sp. nov.**

*Tepidicaulis marinus* (ma.ri’nus. L. masc. adj. marinus of the sea, marine).

Cells are 0.3–0.4 x 0.6–3.0 µm. Colonies on marine agar 2216 are cream, round and convex with entire edges. Yeast extract and NaCl are required for growth. Able to grow at 16–48 °C and with 1–11% (w/v) NaCl. Optimal conditions

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Table 1. Main differentiating characteristics of strain MA2T and other genera

Taxa: 1, strain MA2T (this study); 2, Parivibaculum (data from Schleheck et al., 2004; Lai et al., 2011; Rosario-Passapera et al., 2012); 3, Rhodoligotrophos (Fukuda et al., 2012); 4, Rhodobium (Hiraishi et al., 1995; Srinivas et al., 2007); 5, Tepidamorphus (Albuquerque et al., 2010); 6, Anderseniella (Brettar et al., 2007). +, Positive; –; negative; ND, no data.
for growth are 42 °C, pH 8 and 3.0 % (w/v) NaCl. Denitriﬁes nitrate only under microaerobic conditions and produces N₂O. Utilizable carbon sources are acetate, lactate, malate, succinate, gluconate, maltose, pyruvate, yeast extract, alanine, ethanol, glycerol and capric acid. The type strain is MA2T (=NBRC 109643T=DSM 27167T), which was isolated from a methane-utilizing mixed culture obtained from marine sediments of Kagoshima Bay, Japan.

Acknowledgements
We are grateful to Yoshimichi Ogamino and Aya Akiba for their technical assistance. We are grateful to the captain, crew and other onboard scientists of the Kagoshima-maru 2001 cruise for their technical expertise and support onboard. This work was partly supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan by a Grant in Aid for Scientiﬁc Research on Innovative Areas ‘Genome Science’ (no. 22IS0002) and a Grant-in-Aid for Scientiﬁc Research (C) (no. 225450263), and by the Cooperative Program of Atmosphere and Ocean Research Institute, The University of Tokyo (no. 005, 2013).

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