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A denitrifying bacterium from the deep sea at 11 000-m depth

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Abstract The denitrifying bacterium strain MT-1 was isolated from the mud of the Mariana Trench. The optimal temperature and pressure for growth of this bacterium were found to be 30°C and 0.1 MPa, respectively. However, it showed greater tolerance to low temperature (4°C) and high hydrostatic pressure (50 MPa) as compared with denitrifiers obtained from land. From the results, it can be said that this organism is adapted to the environment of the deep sea. Strain MT-1 was shown to belong to the genus *Pseudomonas* by analysis of its 16S rDNA. The cytochrome contents of the bacterium were similar to those of *Ps. stutzeri* in spectrophotometric studies.

Key words Deep-sea · Mariana Trench · Denitrification Extreme environment · *Pseudomonas*

Introduction

The deep sea is a world of high pressure and low temperature, and the detailed properties of this remote world are almost unknown because of the difficulty in reaching it. Manned and unmanned submersibles operated by the Japan Marine Science and Technology Center have been employed to investigate the deep-sea environment and the organisms living there. In our laboratory, many bacteria have been isolated from samples collected from the deep sea (Abe et al. 1995; González et al. 1995; Kato et al. 1995).

Recently, mud from the bottom of the Mariana Trench, one of the deepest trenches in the world, was collected in an operation involving the use of *KAIKO*, an unmanned submersible operated by the Japan Marine Science and Technology Center. Many kinds of organisms were isolated from this mud, and some genetic analyses were performed (Kato et al. 1997). Among these organisms, we found a bacterium that can grow by means of denitrification.

Denitrification is a form of anaerobic respiration, and it is thought to represent an ancient form of aerobic respiration (Saraste and Castresana 1994). Nitric oxide reductase, one of the enzymes in the denitrification process, shows similarity in primary structure to *cb*-type cytochrome *c* oxidase (Saraste and Castresana 1994; Zumft et al. 1994). *cb*-Type cytochrome *c* oxidase is usually functional under microaerobic conditions (García-Horsman et al. 1994; Gray et al. 1994; Preisig et al. 1993; Tamegai and Fukumori 1994), and is thought to be an ancient type of cytochrome *c* oxidase evolved from nitric oxide reductase (Saraste and Castresana 1994). Further, the copper center of nitrous oxide reductase, another enzyme involved in denitrification, shows properties similar to the Cu_A domain of cytochrome *c* oxidase (Zumft et al. 1992). Many researchers are interested in the evolution of respiration and the relationship between anaerobic and aerobic respiratory systems.

In the present study, we isolated a denitrifying bacterium, *Pseudomonas* sp. strain MT-1, from the mud of the Mariana Trench. Such a bacterium obtained from an isolated world might provide a novel view of the evolution of respiratory systems.

Materials and methods

Mud sample from the Mariana Trench

Mud from the Mariana Trench (11° 22.10′ N, 142° 25.85′ E, 10 898 m depth) was collected by means of a sterilized mud

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sampler using the unmanned submersible *KAIKO*, operated by the Japan Marine Science and Technology Center. The mud collected was placed at 4°C on the support vessel *M. S. Yokosuka*.

Isolation of the bacterium

About 5 g of mud from the Mariana Trench was suspended in 50 ml of 3% (w/v) NaCl. The solids were removed by decantation. A 0.5-ml aliquot of the supernatant was added to 50 ml of LBII medium (5 g yeast extract, 10 g tryptone, 30 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, and 1 g $CaCl_2$ per 1 liter) containing 10 mM $NaNO_3$. A cultivation bottle was filled with this inoculated medium, and incubated without agitation at 30°C for 48 h. After this period of cultivation, the formation of bubbles was observed. Plates of LBII medium containing 1.5% agar were seeded with inocula from this culture, and the plates were incubated at 30°C overnight. The colonies that formed were picked, and cultured in LBII medium containing 10 mM $NaNO_3$. A bacterial isolate which generated bubbles during growth was thereby selected.

Bacterial strains

Pseudomonas stutzeri IFO 14165 and *Paracoccus denitrificans* IFO 13301 were purchased from the Institute for Fermentation, Osaka (Osaka, Japan).

Conditions for growth of the bacterium

For the study of growth under different conditions of hydrostatic pressure, strain MT-1, *Ps. stutzeri*, and *P. denitrificans* were cultured in 2.5-ml soft plastic vials at 30°C, pressurized using pressure vessels. LBII medium containing 10 mM $NaNO_3$ was used for this study. The optimal temperature for growth of strain MT-1 was determined by using a temperature gradient bio-photorecorder TN-112D (Advantec Toyo, Tokyo, Japan). LBII medium was used, and the bacterium was cultivated under aerobic conditions.

Analysis of the cytochrome content

Cultivation of strain MT-1 and *Ps. stutzeri* under aerobic conditions for spectrophotometric analysis was performed using 3-l shake flasks containing 750 ml of LBII medium with incubation at 30°C for 18 h. To obtain nitrate-grown cells of each strain, the bacteria were cultivated in 11 of LBII medium containing 10 mM $NaNO_3$ in a 1-l glass bottle at 30°C for 18 h. The cells of each strain were suspended in 10 mM Tris-HCl buffer, pH 8, and disrupted in a sonic oscillator (20 kHz, 200 W). The suspension was centrifuged (10000 × *g*, 15 min) to remove unbroken cells. The cell-free extract obtained in this manner was centrifuged (143000 × *g*, 1 h), and the pellet was washed with 10 mM Tris-HCl buffer, pH 8 containing 1 M NaCl. The suspension was stirred for 1 h, and centrifuged at 143000 × *g* for 1 h. The pellet was suspended in 10 mM Tris-HCl buffer, pH 8, and dialyzed against the same buffer for 3 h. This suspension was used as the membrane fraction. The two supernatants resulting from the ultracentrifugation steps were combined and dialyzed against 10 mM Tris-HCl buffer, pH 8 for 3 h. This solution was used as the soluble fraction.

Physical and chemical measurements

Protein concentrations were determined by the method of Lowry et al. (1951) with slight modifications (Dulley and Grieve 1975). Spectrophotometric analyses were performed using a Shimadzu UV-2400PC spectrophotometer. All chemicals were of the highest grade commercially available.

Genetic analysis

Total DNA was prepared from strain MT-1 according to the method described previously (Saito and Miura 1963). The polymerase chain reaction (PCR) amplification of 16S rDNA with synthesized oligonucleotide primers corresponding to forward primer Eubac 27F (AGAGTTTGATCCTGGCTCAG) and reverse primer Eubac 1492R (GGTTACCTTGTTACGACTT) (Lane 1991) was performed using 50 µl PCR reaction mixtures under the conditions recommended by Takara (Otsu, Japan) by the method of DeLong (1992). The amplified 16S rDNA was purified from the reaction components by means of a DNA recovery filter SUPRECTM-02 (Takara) and sequenced directly by the dideoxynucleotide chain termina-

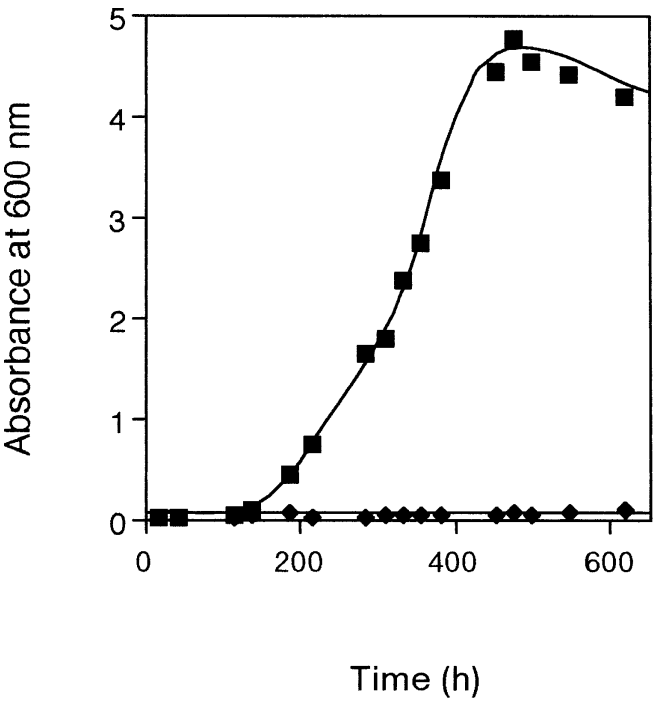

Fig. 1. Growth curves of *Pseudomonas* sp. strain MT-1 (*squares*) and *Ps. stutzeri* (*diamonds*) at 4°C. Cells were cultured in LBII medium under aerobic conditions

tion method using a model 373A DNA sequencer (Perkin Elmer/Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. Sequencing was performed using the primers of Kato et al. (1997) for bacterial 16S rDNA sequencing.

The determined 16S rDNA sequence was checked for similarity with sequences in the DNA databases, GenBank and EMBL, using the GENETYX-CD program (version 33.0, Software Co., Tokyo, Japan). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL W program.

The 16S rDNA sequence of *Pseudomonas* sp. strain MT-1 determined in the present study has been deposited in the DDBJ (Mishima, Japan), EMBL (Heidelberg, Germany), and GenBank (Mountain View, CA, USA) nucleotide sequence databases. The accession number of the DNA sequence amplified from strain MT-1 is AB004241.

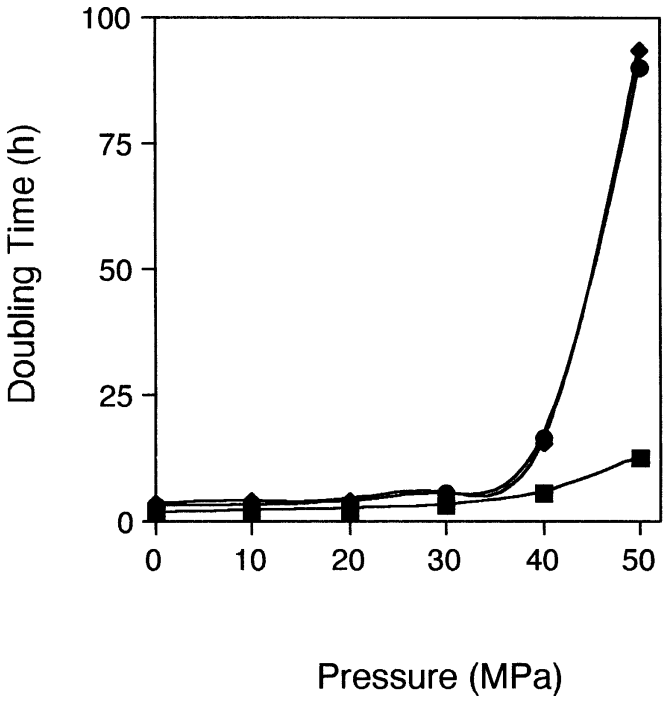

Fig. 2. Effect of hydrostatic pressure on growth of *Pseudomonas* sp. strain MT-1 (*squares*), *Ps. stutzeri* (*diamonds*), and *P. denitrificans* (*circles*). Culture conditions are described in Materials and Methods

Results and discussion

Properties of the isolated bacterium

The isolated bacterium, named strain MT-1, was found to grow with oxygen or nitrate as the terminal electron acceptor. The optimal temperature for growth was 30–35°C, and the optimal pressure for growth was 0.1 MPa. Compared with the properties of *Ps. stutzeri* (Palleroni 1984), a terrestrial denitrifying bacterium, no obvious difference was evident. However, the lower limit of temperature for growth was found to be different. Even at 4°C, strain MT-1 displayed growth, although it was very slow, while *Ps. stutzeri* did not grow at this temperature (Fig. 1). This observation suggests that strain MT-1 is adapted to the deep-sea environment. *Pseudomonas* sp. strain MT-1 formed yellow colonies on the agar plates, while the colonies of *Ps. stutzeri* were white.

Effect of hydrostatic pressure on growth of the bacterium

The effect of hydrostatic pressure on the growth of strain MT-1 was examined (Fig. 2). This bacterium did not display barophilic properties; however, it grew even under a pressure of 50 MPa. *Ps. stutzeri* and *P. denitrificans*, typical denitrifiers, showed more sensitivity to hydrostatic pressure than strain MT-1. This result further supports the view that strain MT-1 is adapted to the deep-sea environment.

Phylogenetic analysis

The 16S rDNA of strain MT-1 was sequenced and compared with other bacteria (Fig. 3). *Shewanella* sp. DB6705,

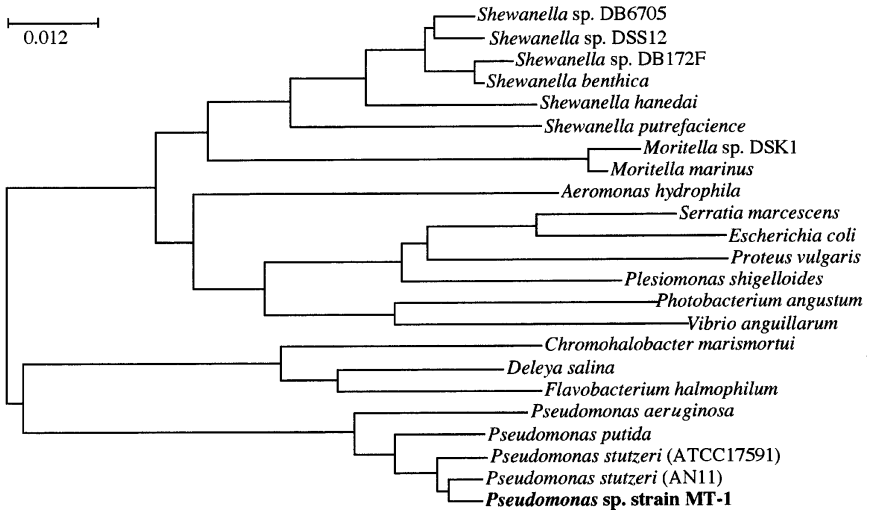

Fig. 3. Phylogenetic tree showing the relationships of strain MT-1 within the Proteobacteria γ subgroup, as determined by a 16S rDNA sequence comparison, using the neighbor-joining method. The *scale* indicates the average number of nucleotide substitutions per site

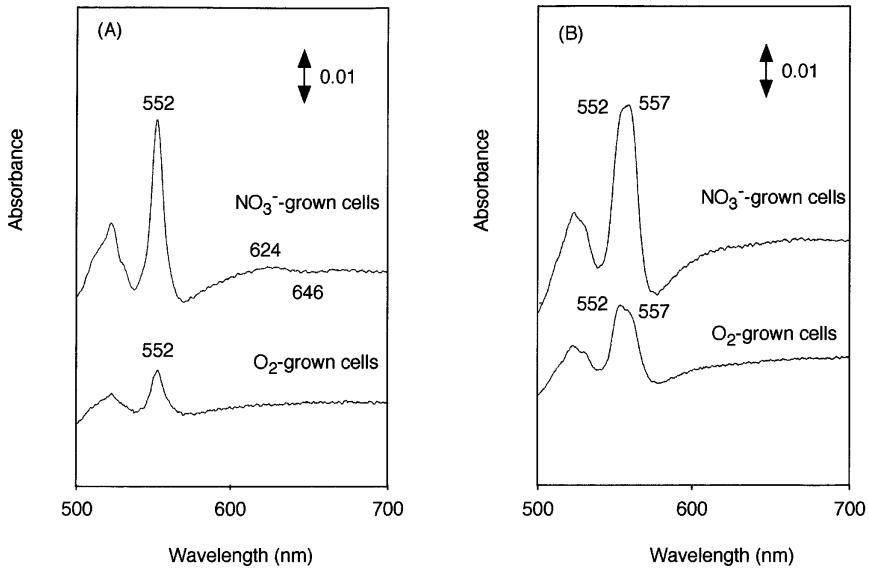

Fig. 4. Reduced minus oxidized difference spectrum of (A) soluble fractions and (B) membrane fractions of *Pseudomonas* sp. strain MT-1. Each fraction contained 1 mg/ml of protein. Reduced forms were prepared by adding a small amount of $Na_2S_2O_4$, and oxidized forms were prepared by adding a small amount of $(NH_4)_2S_2O_8$. *Utter traces*, NO_3^--grown cells; *lower traces*, O_2-grown cells; *double arrow*, scale in absorbance units

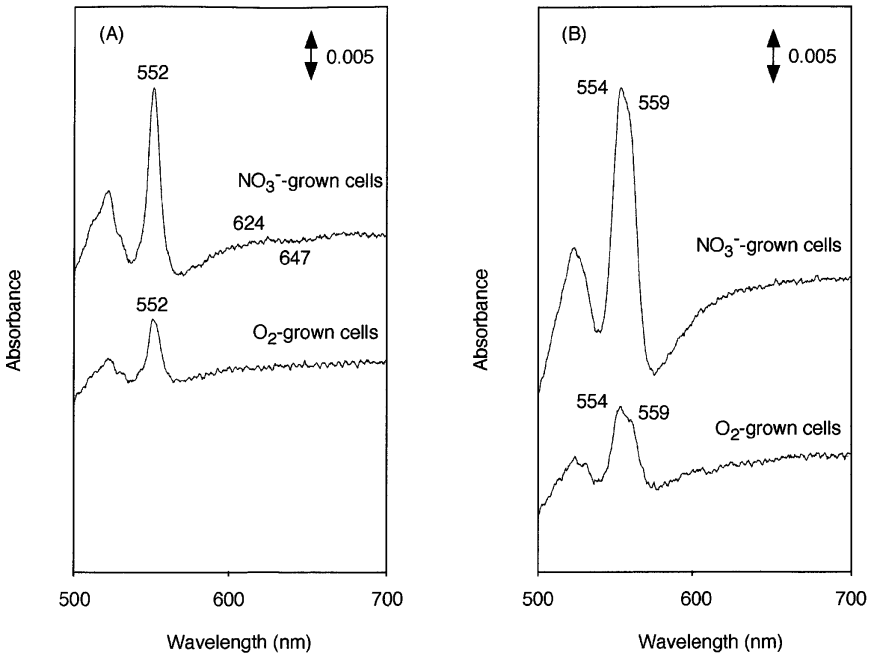

Fig. 5. Reduced minus oxidized difference spectrum of (A) soluble fractions and (B) membrane fractions of *Pseudomonas stutzeri*. Each fraction contained 1 mg/ml of protein. Reduced forms were prepared by adding a small amount of $Na_2S_2O_4$, and oxidized forms were prepared by adding a small amount of $(NH_4)_2S_2O_8$. *Traces* as in Fig. 4

DSS12, and DB172F and *Moritella* sp. DSK1 were isolated from deep-sea sediments obtained from depths in the range of 5000–6500 m (Kato et al. 1995), and these strains show barophilic properties. The 16S rDNA sequence comparisons showed that strain MT-1 does not belong to the subbranch of barophiles, but rather belongs to the genus *Pseudomonas*, and has an especially close relationship with *Ps. stutzeri*.

Spectral analyses of cytochrome composition

The cytochrome content of *Pseudomonas* sp. strain MT-1 was analyzed spectrophotometrically (Fig. 4). Cells grown under aerobic conditions contained *c*-type and *b*-type cytochromes in the soluble fraction, and *c*-type and *b*- (*o*-)type cytochromes in the membrane fraction. No *a*-type cytochrome was detected. Cells grown under conditions of denitrification contained *c*-type, *b*-type, and d_1-type cytochromes in the soluble fraction, and *c*-type and *b*- (*o*-)type cytochromes in the membrane fraction. These properties are quite similar to those of *Ps. stutzeri* (Fig. 5). Although the ratio of amounts of cytochrome *b* and *c* differs between the two strains, with respect to the cytochrome content of strain MT-1, no special features were evident upon spectrophotometric analysis. However, it cannot be denied that the respiratory system of strain MT-1 appears to display no novel features as suggested by this study. Further studies will be needed to fully elucidate the respiratory system of this bacterium.

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