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Halomonas sp. strain DT-W, a halophile from the 11,000 m-depth of the Mariana Trench

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Abstract Moderately halophilic strain DT-W was isolated from the mud of the Mariana Trench. Cells of the organism were rod-shaped (1.5-2 μm x 0.5-0.8 μm) with some flagella extruding from the cells. Growth occurred in an NaCl concentration of about 0.1-15% (optimal: 3-5%), at pH of 6-9, and at temperatures ranging from 4-51 °C (optimal: 30-37 °C). The results of 16S rDNA analysis and DNA-DNA hybridization analyses showed that DT-W was closely related to *Halomonas aquamarina*, *Halomonas axialensis* and *Halomonas meridiana*. Furthermore, additional physiological properties and the cytochrome contents of DT-W were also analyzed.

Key words: *Halomonas* sp. strain DT-W, halophile, Mariana Trench

Introduction

Microorganisms have been studied extensively because of their close relationship with humans. To date, many microorganisms have been isolated from the environment and most of them were obtained from land. However, land surfaces occupy only about 30% of the earth and another 70% of the earth is occupied by oceans. Thus, the mud and water from oceans should be an attractive source for the isolation of novel microorganisms. The deep-sea is especially of interest because it is still a relatively unknown area because of the difficulty in exploring these regions. In Japan, many submersibles have been operated by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) and many microorganisms have been isolated from recovered deep-sea samples. These explorations have made significant contributions to the development of the studies of deep-sea microorganisms. The mud of the Mariana Trench, one of the deepest trenches in the world, was collected by a sterilized mud sampler using the unmanned submersible KAIKO operated by JAMSTEC in 1996. Genetic analysis concerning biodiversity was carried out [6], and many novel bacteria were isolated from the mud [4, 10, 16, 17, 19, 20].

Among the microorganisms isolated from the mud of the Mariana Trench, we observed some halophilic strains. Halophilic and halotolerant bacteria are typically isolated from salt lakes, saline soils, salted foods, and other saline environments [22]. Moderate halophilles are also quite abundant in the water and mud of seas. Sometimes they compose up to 10% of the total marine microbial community [7]. Many halophiles were isolated from the sea samples collected at the sea of 0-3000 m depths [7] and some of them were identified as novel species of the genus *Halomonas*.

In the present study, we isolated one of the halophilic strains from the mud of the Mariana Trench and investigated the genetic and physiological properties of the strain. This is the first report concerning halophiles from the Mariana Trench. An examination of such strains should provide novel insights regarding life in the depths of deep-seas.

Materials and Methods

Isolation of the bacterium

Mud from the Mariana Trench (11°22.10' N, 142°25.85' E, 10898 m depth) [19] was used as the source for isolation of organisms. The mud was collected by a sterilized mud sampler using the unmanned submersible KAIKO. Approximately 5 g of mud were suspended in MT-1 medium [20] and the solids were removed by decantation. A 0.5 ml aliquot of the supernatant was seeded on the plate of MT-1 agar medium (MT-1 medium containing 1.5% agar). The plate was incubated at 30°C overnight and the colonies that formed were isolated for further study.

Organisms and cultivation conditions

Strain DT-W was grown in MT-1 medium [20], normally at 30 °C. The reference strains used in this study, *Halomonas aquamarina* DSM 30161^T, *Halomonas axialensis* DSM 15723^T, and *Halomonas venusta* DSM 4743^T were obtained from DSMZ, while *Halomonas meridiana* NBRC 15608^T was obtained from National Institute of Technology and Evaluation Biological Resource Center (NBRC). These bacterial strains were maintained on MT-1 agar medium at 30 °C. Growth under various pressures for 20 h was monitored as previously described [5].

Physiological analyses

Physiological tests were performed with slight modifications of the general procedures as described previously [1]. Acid production from sugar was assessed using MT-1 medium containing 1% of each substrate and 0.03% of bromothymol blue.

Physical and Chemical analyses

Morphology of the cells of DT-W was determined by transmission electron microscopy as described previously [12]. Cellular fatty acids and isoprenoid quinones were analyzed according to the methods also described previously [12].

Molecular biological studies

Chromosomal DNA was extracted from each strain by the method of Saito and Miura [14]. The G+C content was determined by the method of Tamaoka and Komagata [18]. DNA-DNA hybridization was carried out by the method of Ezaki *et al*. [3] at 40 °C for 3h and the results were assessed fluorometrically. 16S rDNA sequences were determined as described previously [19]. The determined sequence was deposited in DDBJ, EMBL and GenBank with the accession number AB249883.

Phylogenetic analysis

A phylogenetic tree was constructed based on the 16S rDNA sequences. Database searches were carried out by FASTA [13] on the Internet. Nucleotide substitution rates [9] were determined and a distance-matrix tree was constructed by the neighbour-joining method [15] using the CLUSTAL_W program [21] on the Internet.

Analysis of cytochrome content

The cells of DT-W (2g, wet weight) were suspended in 25 mM Na-phosphate buffer (pH 7.0) containing 0.1 M EDTA and disrupted by a sonic oscillator (22.5 kHz, 100 W). The suspension was centrifuged (10,000 x *g*, 10 min) to remove unbroken cells and the cell-free extract obtained was again centrifuged (100,000 x *g*, 1 h). The supernatant was then used as the soluble fraction. The pellet was resuspended in 25 mM Na-phosphate buffer (pH 7.0), and the resulting suspension was used as the membrane fraction. Reduced minus oxidized difference spectra of each fraction were measured spectrophotometrically. Protein concentrations of each fraction were determined by the method of Lowry et al. [11] with slight modifications [2].

Results and Discussion

Cells of the strain DT-W were rod-shaped with some flagella apparent on the cell surface (Fig. 1). The cells were 1.5-2 μm long and 0.5-0.8 μm wide. Growth occurred in an NaCl concentrations ranging from 0.1-15% (optimal: 3-5%), at pH of 6-9, and at temperatures from 4-51 °C (optimal: 30-37 °C). These results indicated that DT-W was a moderate halophile, and can grow under low temperature conditions as found in the deep sea. Table 1 shows the effects of pressure on the growth of DT-W. It appears that strain DT-W is not a piezophilic bacterium, but is tolerant to high pressures to some extent.

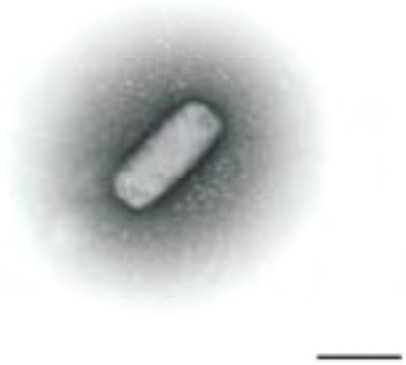

Fig. 1 Transmission electron micrograph of negatively stained cells of strain DT-W. Bar, 1.0 μm

Table 1 Effect of pressure on the growth of DT-W.

Pressure (MPa)	0.1	30	50	70
Optical density at 660 nm after 20h	0.180	0.094	0.056	0.018

A phylogenetic tree was constructed on the basis of 16S rDNA sequences (Fig. 2). The tree indicated that DT-W could be classified in the genus *Halomonas*. Specifically, it is closely related to *H. axialensis*, *H. aquamarina* and *H. meridiana*. In order to analyze the relationship between each strain, DNA-DNA hybridization was carried out (Table 2). DT-W showed comparatively high level of DNA-DNA relatedness with *H. axialensis*, *H. aquamarina* and *H. meridiana* (>70%). These values were higher than those accepted for the phylogenetic definition of a species [23]. Therefore, these strains might be classified in the same species. Strain DT-W appeared to belong to a subspecies of these Halomonads. This result is not consistent with a recent study which showed that *H. axialensis* is distinguishable from *H. aquamarina* and *H. meridiana* [8]. However DNA-DNA relatedness between *H. venusta* and the other strains was low enough to show that there was no technical errors in our procedure. *H. aquamarina* was isolated from shallow sea samples, *H. axialensis* was from a deep sea depth of 1,530 m depth and *H. meridiana* was from a lake. It is interesting that a bacterium living in the Mariana Trench (11,000 m depth) is closely related with such bacteria isolated from water depth quite distinct from its normal habitat.

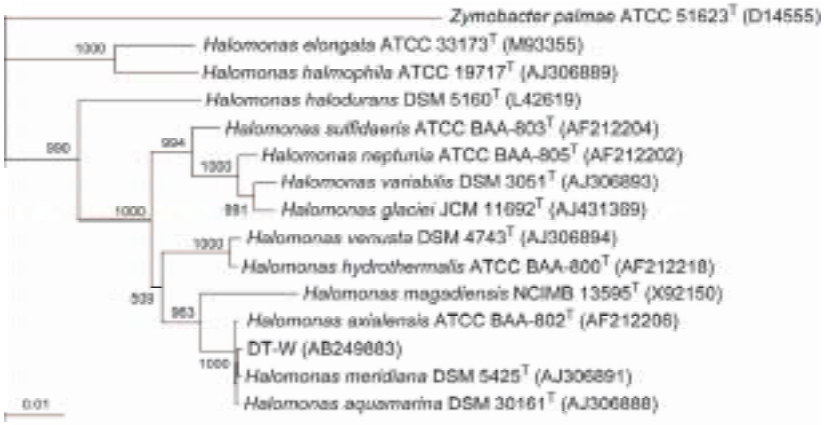

Fig. 2. Phylogenetic tree showing the relationships of strain DT-W with related strains.
The tree was constructed by the neighbor-joining method and based on 16S rDNA sequences. The data for *Zymobacter palmae* was used as the outgroup for the phylogenetic tree. Numbers indicated bootstrap values greater than 500. Bar, 0.1 nucleotide substitutions per site.

Table 2 DNA-DNA hybridization using chromosomal DNA of DT-W and related strains.

Strain	% Homology with DNA from:				
	1	2	3	4	5
1	100	87	83	88	25
2	85	100	83	94	31
3	84	87	100	90	31
4	77	86	81	100	26
5	20	22	24	23	100

Strains: 1; Strain DT-W, 2; *H. aquamarina* DSM 30161^T, 3; *H. axialensis* DSM 15723^T, 4; *H. meridiana* NRBC 15608^T, 5; *H. venusta* DSM 4743^T.

Table 3 Phenotypic characteristics of strain DT-W and reference strains

Characteristic	1	2	3	4
Growth properties				
Temperature (°C)				
range	4 - 51	5 – 40	-1 - 35	-5 - 45
optimum	30 - 37	20 – 25	30	28 - 40
pH				
range	6 - 9	5 – 10	5 - 12	5 - 10
Salinity				
range	0.1 - 15	0 – 20	0.5 - 24	0.01 - 25
optimum	3 - 5	7.5 – 10	4	1 - 3
Other properties				
gelatin hydrolysis	-	-	-	-
casein hydrolysis	-	-	-	-
starch hydrolysis	-	-	-	+
Tween 80 hydrolysis	-	+	-	+
Catalase test	+	+	+	+
Oxidase test	+	+	+	+
GC content (%)	58.8	56.7	54.7	56.9
Acid production from:				
cellobiose	-	ND	ND	ND
fructose	+	+	-	+
galactose	+	+	-	+
glucose	+	+	+	+
lactose	+	+	-	+
mannitol	-	+	ND	+
mannose	-	+	-	+
raffinose	-	ND	ND	ND
rhamnose	-	+	ND	+
sucrose	+	+	-	+
xylose	-	+	-	+

Strains: 1; Strain DT-W, 2; *H. aquamarina* DSM 30161^T, 3; *H. axialensis* DSM 15723^T, 4; *H. meridiana* NRBC 15608^T. Data of substrate hydrolysis, catalase test, oxidase test and GC content for all strain were determined in this study. Other data for 1 were determined in this study, for 2 and 4 were from reference and for 3 were from reference . ND; no data available.

Characteristics of strain DT-W and the reference strains are shown in Table 3. The results suggest that strain DT-W appears to be an aerobic chemoorganotroph. Furthermore, neither nitrate nor nitrite supported growth of the organism with concomitant denitrification (data not shown). The G+C content of the DNA was calculated to be 58.8 mol%. In addition, the major quinone isolated from strain DT-W was ubiquinone-9 (Q-9). The fatty acid compositions of DT-W and the reference strains are summarized in Table 4. The major fatty acids in strain DT-W are C16:0 (hexadecanoic acid) and C18:1 (octadecenoic acid).

Table 4 Fatty acid composition of strain DT-W and reference strains

Fatty acid	1	2	3	4
3-OH-12:0	5	5	6	8
14:0	3	3	4	5
16:0	22	21	21	24
16:1	7	10	8	8
18:1	62	61	62	55
19:1				2

Values are percentages of total fatty acids. Strains: 1; Strain DT-W, 2; *H. aquamarina* DSM 30161^T, 3; *H. axialensis* DSM 15723^T, 4; *H. meridiana* NRBC 15608^T. Empty cells; not detected.

The cytochrome content of strain DT-W was analyzed spectrophotometrically as shown in Fig. 3. These difference spectra showed that *b*-type (or/and *o*-type) and *c*-type cytochromes were expressed in the membrane fraction of the organism, and *c*-type cytochrome(s) in the soluble fraction. There were no *a*-type or *d*-type cytochromes detected. This suggests that the terminal oxidase of DT-W is a *b*-type or *o*-type cytochrome.

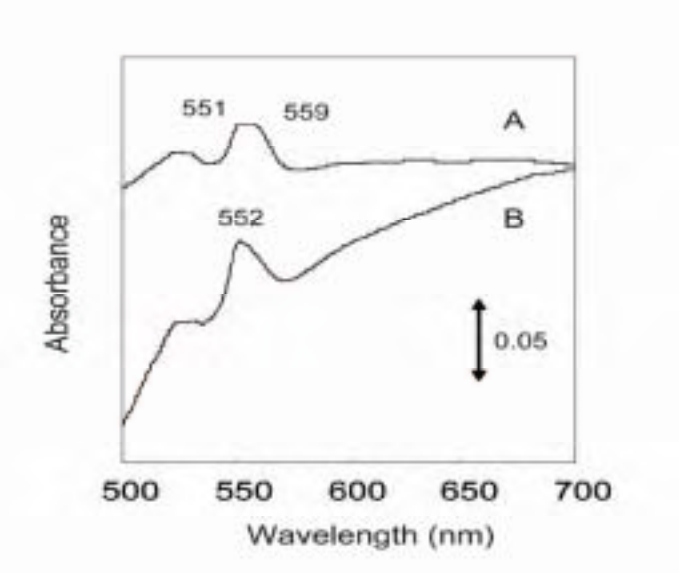

Fig. 3 Reduced minus oxidized difference spectra of membrane fraction (6.1 mg/ml of protein, spectrum A) and soluble fraction (2.2 mg/ml of protein, spectrum B) of strain DT-W.
Reduced forms were prepared by adding a small amount of $Na_2S_2O_4$ and oxidized forms were prepared by adding a small amount of $(NH_4)_2S_2O_8$.

In the present study, we isolated *Halomonas* sp. strain DT-W from the mud of the Mariana Trench. Furthermore, we characterized some of the physiological properties on DT-W in detail. These results represent the first report concerning Halomonads from the Mariana Trench.

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References

1) Barrow G.I. and Feltham R.K.A. 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd edn. Cambridge University Press, New York.

2)Dulley J.R. and Grieve P.A. 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. 64:136-141.

3)Ezaki T., Hashimoto Y. and Yabuuchi E. 1989. Fluorometric deoxyribonucleic acid- deoxyribonucreic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39:224-229.

4) Kaneko H., Takami H., Inoue A. and Horikoshi K. 2000. Effects of hydrostatic pressure and temperature on growth and lipid composition of the inner membrane of barotolerant *Pseudomonas* sp. BT1 isolated from the deep-sea. Biosci. Biotechnol. Biochem. 64:72-79.

5) Kato C., Sato T., Smorawinska M. and Horikoshi K. 1994. High pressure conditions stimulate expression of chloramphenicol acetyltransferase regulated by the *lac* promoter in *Escherichia coli*. FEMS Microbiol. Lett. 122:91-96.

6) Kato C., Li L., Tamaoka J. and Horikoshi K. 1997. Molecular analyses of the sediment of the 11000-m deep Mariana Trench. Extremophiles 1:117-123.

7) Kaye J.Z. and Baross J.A. 2000. High incidence of halotolerant bacteria in Pacific hydrothermal-vent and pelagic environments. FEMS Microbiol. Ecol. 32:249-260.

8) Kaye J.Z., Márquez M.C., Ventosa A. and Baross J.A. 2004. *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov. and *Halomonas hydrothermalis* sp. nov.: halophilic bacteria isolated from deep-sea hydrothermal-vent environments. Int. J. Syst. Evol. Microbiol. 54:499-511.

9) Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparable studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

10) Kobayashi H., Takaki Y., Kobata K., Takami H. and Inoue A. 1998. Characterization of α-maltotetraohydrolase produced by *Pseudomonas* sp. MS300 isolated from the deepest site of the Mariana Trench. Extremophiles 2:401-407.

11) Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

12) Nogi Y., Kato C. and Horikoshi K. 1998. Taxonomic studies of deep-sea barophilic *Shewanella* strains and description of *Shewanella violacea* sp. nov. Arch. Microbiol. 170:331-338.

13) Pearson W.R. and Lipman D.J. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444-2448.

14) Saito H. and Miura K. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta 72:619-629.

15) Saitou N. and Nei M. 1987. The neighbor-joining method: a new method for reconstituting phylogenetic trees. Mol. Biol. Evol. 4:406-425.

16) Takai K., Inoue A. and Horikoshi K. 1999. *Thermaerobacter marianensis* gen. nov., sp. nov., an aerobic extremely thermophilic marine bacterium from the 11000 m deep Mariana Trench. Int. J. Syst. Evol. Microbiol. 49:619-628.

17) Takami H., Nishi S., Lu J., Shimamura S. and Takaki Y. 2004. Genomic characterization of thermophilic *Geobacillus* species isolated from the deepest sea mud of the Mariana Trench. Extremophiles 8:351-356.

18) Tamaoka J. and Komagata K. 1984. Determination of DNA base composition by reversed phase high-performance liquid chromatography. FEMS Microbiol. Lett. 25:125-128.

19) Tamegai H., Li L., Masui N. and Kato C. 1997. A denitrifying bacterium from the deep sea at 11000-m depth. Extremophiles 1:207-211.

20) Tamegai H., Nakamura S., Miyazaki M., Nogi Y., Kasahara R., Kato C. and Horikoshi K. 2005. Physiological properties of *Pseudomonas* sp. strain

MT-1, denitrifier from the 11,000 m-depth of Mariana Trench. J. Jpn. Soc. Extremophiles 4:25-31.

21) Thompson J.D., Higgins D.G. and Gibson T.J. 1994. CLUSTAL_W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

22) Ventosa A., Nieto J.J. and Oren A. 1998. Biology of moderately halophilic aerobic bacteria. Microbiol. Mol. Biol. Rev. 62:504-544.

23) Wayne L.G., Brenner D.J., Colwell R.R., Grimont P.A.D., Kandler O., Krichevsky M.I., Moore L.H., Moore W.E.C., Murray R.G.E., Stackebrandt E., Starr M.P. and Trüper H.G. 1987. Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463-464.