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Taxonomic studies of extremely barophilic bacteria isolated from the Mariana Trench and description of *Moritella yayanosii* sp. nov., a new barophilic bacterial isolate

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Abstract We have isolated two strains of extremely barophilic bacteria from sediment collected from the world's deepest ocean floor in the Mariana Trench, Challenger Deep, at a depth of 10898 m [Kato C, Li L, Nogi Y, Nakamura Y, Tamaoka J, Horikoshi K (1998) Appl Environ Microbiol 64:1510-1513]. One strain, DB21MT-2, was identified as a strain of *Shewanella benthica*, and the other strain, DB21MT-5, is closely affiliated with members of the genus *Moritella* on the basis of 16S rDNA sequence analysis. The hybridization values for DNA-DNA relatedness between DB21MT-5 and the *Moritella* reference strains were significantly lower than that accepted as the phylogenetic definition of a species. Based on this and other taxonomic differences, strain DB21MT-5 appears to represent a novel obligately barophilic deep-sea *Moritella* species. The name *Moritella yayanosii* (JCM 10263) is proposed. This is the first proposed species of obligately barophilic bacteria of the genus *Moritella*.

Key words Extremely barophilic bacteria · Deep Sea · *Shewanella benthica* · *Moritella yayanosii* · Mariana Trench

Introduction

The deepest ocean bottom in the world is the Mariana Trench, Challenger Deep, and only a few microorganisms have been recovered from this site. *Pseudomonas bathycetes* was the first bacterial isolate obtained from a sediment sample collected from the Mariana Trench (Morita 1976), although apparently *P. bathycetes* is not a barophilic bacterium (Pope et al. 1975; Schwarz and Colwell 1975). Barophilic bacteria are characterized by enhanced growth at pressures greater than 1 atmosphere. Since the report by Yayanos et al. (1979), numerous deep-sea barophilic bacterial strains have been isolated and characterized in an effort to understand the interaction between the deep-sea environment and its microbial inhabitants (Kato et al. 1995, 1996, 1998). With respect to barophilic bacteria isolated from the Mariana Trench, Yayanos et al. (1979) were successful in collecting an amphipod (*Hirondella gigas*) at a depth of 10476 m by using an insulated trap. An obligately barophilic bacterium, strain MT41, that could grow only at pressures of approximately 50 MPa or more, was isolated from this amphipod (Yayanos et al. 1981). Strain MT41 was found to be closely related to the genus *Colwellia* (DeLong et al. 1997).

Most barophilic bacteria isolated thus far fall into the Proteobacteria γ-subgroup according to phylogenetic classification based on 16S ribosomal RNA gene sequence information (Kato et al. 1995, 1996, 1998; DeLong et al. 1997; Nogi et al. 1998a; Li et al. 1998). DeLong et al. (1997) reported that 11 cultivated psychrophilic and barophilic deep-sea bacteria were affiliated with one of five genera within the γ-subgroup: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* , and an unidentified genus. The deep-sea barophilic species of five of these genera were named as *S. benthica* and *S. violacea* in the genus *Shewanella* (Deming et al. 1984; MacDonell and Colwell 1985; Nogi et al. 1998b), *P. profundum* in the genus *Photobacterium* (Nogi et al. 1998a), *C. hadaliensis* in the genus *Colwellia* (Deming et al. 1988), and *M. japonica* in the genus *Moritella* (Nogi et al. 1998c). Members of the genera *Shewanella*, *Photobacterium*, and *Moritella* are not unique to deep-sea marine environments. Most of these isolates are not obligately barophilic, and before the present report *C. hadaliensis* strain BNL-1 (Deming et al. 1988) was the only known obligately barophilic strain within this group.

In this paper, we describe taxonomic studies of extremely barophilic strains isolated from a sample of deep-sea sediment collected from the Mariana Trench at a depth of 10898 m. Several lines of evidence indicate that one of these isolates, strain DB21MT-5, represents a novel species within

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the genus *Moritella*. Our results suggest that this strain should be classified as a new species, proposed as *Moritella yayanosii*, and its description is presented here. The species name is in honor of A. Aristides Yayanos, an American biologist who, among other accomplishments, has made numerous and valuable contributions to our understanding of deep-sea microbiology. This is the first report of obligately barophilic bacteria belonging to the genus *Moritella*.

Materials and methods

Bacterial strains and culture conditions

The extremely barophilic *Moritella* sp. strain DB21MT-5 and *Shewanella benthica* strain DB21MT-2 were isolated from the Mariana Trench, Challenger Deep (11°22.10'N, 142°25.85'E), at a depth of 10898 m as described previously (Kato et al. 1998). The reference strains used in this study, *Moritella marina* ATCC 15381^T (^{T}= type strain) and *Shewanella benthica* ATCC 43992^T, were obtained from the American Type Culture Collection (Rockville, MD, USA). *Shewanella hanedai* IAM 12641^T was obtained from the Institute of Applied Microbiology (Tokyo, Japan). *Moritella japonica* JCM 10249 (Nogi et al. 1998c) and *Shewanella violacea* JCM 10179 (Nogi et al. 1998b) were isolated in our laboratory. These bacteria were maintained on Marine Agar 2216 (Difco, Detroit, MI, USA). *M. marina* and *S. hanedai* were grown at 15°C, *M. japonica* and *S. violacea* were grown at 8°C, *S. benthica* was grown at 4°C, and the extremely barophilic strains were grown at 10°C. All the bacteria were grown at atmospheric pressure, except for extremely barophilic isolates, which were grown at 70 MPa. High-pressure cultivation was performed according to the procedure reported previously (Kato et al. 1995).

Phenotypic characterization

Physiological tests were performed by slight modification of the general procedures described by Barrow and Feltham (1993) and DeLong et al. (1997). All high-pressure physiological tests were performed in tandem with uninoculated blank controls according to the procedure described as follows.

Acid production from sugars was assessed using a modified OF medium (Hugh and Leifson 1953) containing 0.5× artificial seawater (ASW; 1× ASW consists of 3% NaCl, 0.07% KCl, 1.08% $MgCl_2 \cdot 6H_2O$, 0.54% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$), 1% sugar, 0.2% peptone, 0.1% yeast extract, 0.03% K_2HPO_4, 0.5% low-melting agar, and 0.003% bromothymol blue (pH was adjusted to 7.1 at 20°C). Fermentation test cultures were inoculated as stabs and sealed with molten OF media. Oxidization test cultures were inoculated as stabs and covered with fluorinert (FC-72; Sumitomo-3M, Tokyo, Japan). After capping the tube, it was sealed with parafilm, and incubated at 70 MPa at 10°C. After decompression, the stabs were examined for growth and acid production.

Hydrogen sulfide production from thiosulfate and production of indole were assessed using SIM (sulfide indole motility) agar (Nissui Pharmaceutical, Tokyo, Japan) stabs prepared with 0.5× ASW instead of water. Gelatinase activity was tested by inoculation of stab cultures prepared using Marine Broth 2216 medium containing 1% agar and 0.5% gelatin, then sealed with parafilm, and incubated at 70 MPa at 10°C. After decompression, the agar stab culture was pulled out of the tube and cut open to assess cell growth. The agar stab culture was placed in 30% trichloroacetic acid solution, and then examined for the presence of a clear zone. Oxidase and catalase tests were performed using cells cultivated in Marine Broth 2216 medium. After centrifugation at 8000 × *g* for 10 min, the cell pellet was removed by means of a plastic inoculation loop, tested for oxidase by spreading oxidase test paper (Nissui) with the cell pellet, and tested for catalase by putting 3% H_2O_2 on the cell pellet.

Cellular fatty acids and isoprenoid quinones

Extremely barophilic isolates were grown at 70 MPa for 4 days, and reference strains were grown at atmospheric pressure for 2–3 days, in Marine Broth 2216. The cells were washed twice with ASW at 4°C by centrifugation at 8000 × *g*, and freeze-dried. Cellular fatty acids were analyzed by means of a gas-liquid chromatograph/mass spectrometer, and isoprenoid quinones were analyzed by reversed-phase high-performance liquid chromatography according to methods described previously (Nogi et al. 1998a).

Phylogenetic characterization

DNA was purified by a standard method (Saito and Miura 1963). The guanine-plus-cytosine (G+C) content was determined by reversed-phase high-performance liquid chromatography (Tamaoka and Komagata 1984).

16S rRNA sequences were obtained by direct sequencing of PCR-amplified DNA as described previously (Kato et al. 1998). Nucleotide substitution rates (K_{nuc}) (Kimura 1980) were determined, and a distance matrix tree was constructed by the neighbor-joining method (Saitou and Nei 1987), using the CLUSTAL W program (Thompson et al. 1994). Alignment gaps and unidentified base positions were not taken into consideration for the calculations. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 trials. The accession numbers of the 16S rDNA sequences of *Shewanella benthica* strain DB21MT-2 (JCM 10264) and *Moritella yayanosii* strain DB21MT-5 (JCM 10263) are AB008796 and AB008797, respectively.

Results

Phylogenetic relationships based on 16S rDNA sequences

We isolated six extremely barophilic strains from a sample of sediment collected from the Mariana Trench at a depth

of 10898 m. These comprised two groups of strains, identified as *Moritella* sp. and *Shewanella* sp., as reported previously (Kato et al. 1998). For further study, we chose strain DB21MT-2 from among the *Shewanella* sp. and strain DB21MT-5 from among the *Moritella* sp. The results of phylogenetic analyses are shown in Fig. 1. Strain DB21MT-2 belongs in a cluster with all previously described members of the genus *Shewanella*, and is particularly related to *S. benthica* and *S. violacea*, of the *Shewanella* barophile branch (Li et al. 1998). The other strain, DB21MT-5, falls into the genus *Moritella*, and is closely related to the psychrophilic strain *M. marina* (Urakawa et al. 1998) and the moderately barophilic strain *M. japonica* (Nogi et al. 1998c).

Biochemical and physiological characteristics

The characteristics of the extremely barophilic strains and the reference strains are shown in Tables 1 and 2. Strain DB21MT-2 is a facultatively anaerobic chemoorganotroph displaying both respiratory and fermentative types of metabolism. Cells of the deep-sea strain, DB21MT-2, were found to be gram-negative rods, 2–4 μm long and 0.8–1.0 μm wide, motile by means of a single unsheathed polar flagellum (an electron micrograph of this strain was presented in Kato et al. 1998). This strain is unable to grow at atmospheric pressure at any temperature, but grows under hydrostatic pressures in the range of 60–100 MPa at 10°C; the optimal pressure and temperature condition for growth were found to be 70 MPa and 10°C (Kato et al. 1998). Acid, but not gas, is produced from D-glucose, D-lactose, D-mannose and L-rhamnose. Catalase and cytochrome oxidase are positive, gelatin is hydrolyzed, and nitrate is reduced to nitrite but not to nitrogen. This strain is negative for amylase, H_2S production, and indole production.

The following compounds are not utilized: L-arabinose, cellobiose, D-fructose, D-galactose, glycerol, myo-inositol, maltose, D-mannitol, D-raffinose, D-sorbitol, sucrose, D-trehalose, and xylose. The G+C content of the DNA was found to be 48.7 mol%. The major isoprenoid quinone in strain DB21MT-2 was found to be Q-8 (ubiquinone-8), and a minor quinone was Q-7 (ubiquinone-7), which accounted for 60% and 40% of the total isoprenoid quinones, respectively, while MK-7 (menaquinone-7) was not detected in this strain. Among the reference strains, *Shewanella benthica* was found to share the most physiological characteristics with strain DB21MT-2. However, unlike the *S. benthica* type strain, this Mariana strain does not produce H_2S, utilizes quite different carbohydrates, and is unable to grow at atmospheric pressure at any temperature. Nevertheless, the results of DNA-DNA hybridization analysis (Kato et al. 1998) indicate that this strain is a member of the species *S. benthica*.

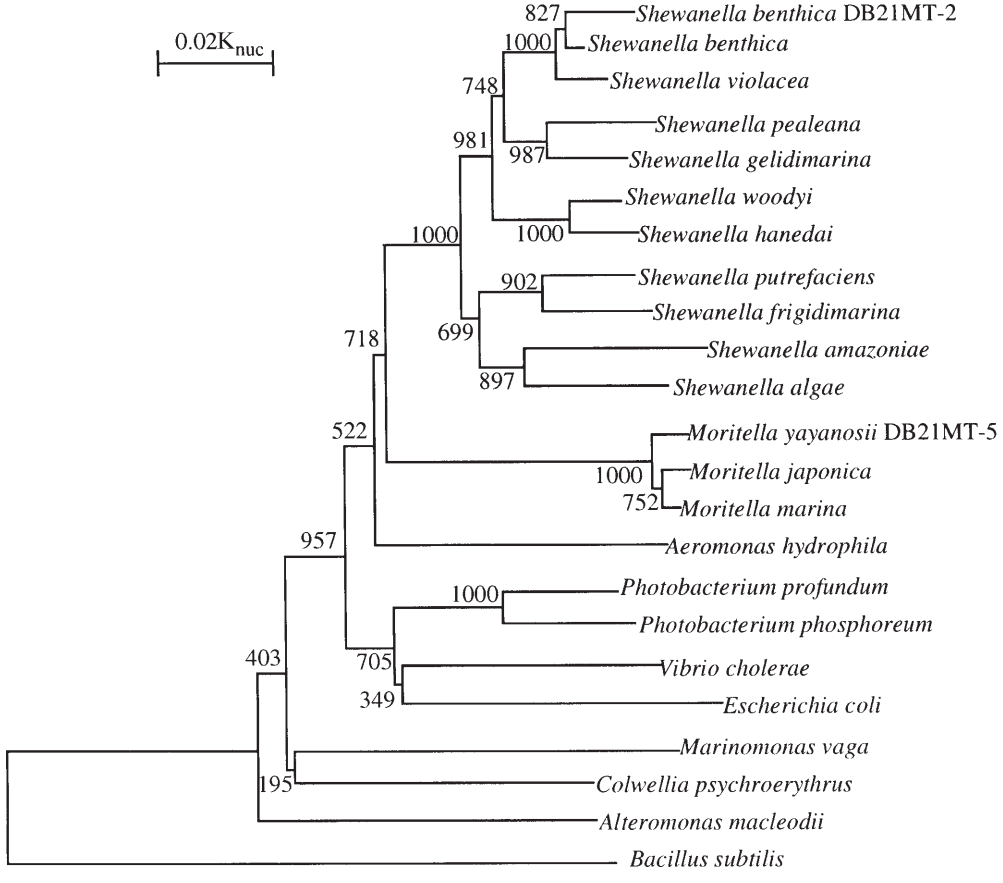

Fig. 1. Phylogenetic tree shows the relationships of amplified DB21MT-2 and DB21MT-5 16S rDNA within the Proteobacteria γ-subgroup as determind using the neighbor-joining method. The *scale* represents the average number of nucleotide substitutions per site. Bootstrap values were calculated from 1000 trees

Table 1. Characteristics of the isolated extremely barophilic strain and related type strains

Characteristics	*M. yayanosii* DB21MT-5^T	*M. marina* ATCC15381^T	*M. japonica* JCM10249^T	*S. benthica* DB21MT-2	*S. benthica* ATCC43992^T	*S. hanedai* IAM12641^T	*S. violacea* JCM10179^T
Gram stain	−	−	−	−	−	−	−
Optimum growth temp at 0.1 MPa (°C)	NG	18	10	NG	4	14	8
Optimum pressure (MPa)	80	0.1	50	70	40	0.1	30
Motility	+	+	+	+	+	+	+
Fermentation of glucose or mannose[a]	+	+	+	+	+	+	+
Gas produced with growth on glucose or mannose[a]	−	−	−	−	−	−	+
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+
Amylase	−	−	−	−	−	−	−
Production of H_2S	−	−	−	−	+	+	−
Production of indole	−	−	−	−	−	−	−
Nitrate reduced	−	+	+	+	+	+	+
Nitrite reduced	−	−	−	−	−	−	−
GC content	44.6%	42.5%	45.0%	48.7%	47.9%	45.4%	47.0%
Quinone	Q-8	Q-8	Q-8	Q-7 (40%) Q-8 (60%)	Q-7 (70%) Q-8 (30%) MK-7	Q-7 (50%) Q-8 (50%) MK-7	Q-7 (30%) Q-8 (70%)

M., *Moritella*; *S*., *Shewanella*; +, positive; −, negative; NG, no growth; Q, ubiquinone; MK, menaquinone
[a] Only extremely barophilic bacteria were tested in the case of mannose

Table 2. Carbohydrates from which acid was produced by the isolated extremely barophilic strain and related type strains

	M. yayanosii DB21MT-5^T	*M. japonica* JCM10249^T	*M. marina* ATCC15381^T	*S. benthica* DB21MT-2	*S. benthica* ATCC43992^T	*S. hanedai* IAM12641^T	*S. violacea* JCM10179^T
L-Arabinose	−	−	−	−	−	−	−
Cellobiose	−	−	+	−	+	+	+
D-Fructose	−	+	+	−	−	−	−
D-Galactose	−	−	+	−	−	+	+
D-Glucose	−	+	+	+	+	+	+
Glycerol	−	+	+	−	−	−	−
myo-Inositol	−	−	−	−	−	−	−
D-Lactose	−	−	−	+	−	−	−
Maltose	+	−	+	−	−	−	−
D-Mannitol	−	−	−	−	−	−	−
D-Mannose	+	−	−	+	−	−	−
D-Raffinose	−	−	−	−	−	−	−
L-Rhamnose	−	−	−	+	−	−	−
D-Sorbitol	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−
D-Trehalose	−	−	−	−	−	−	−
Xylose	+	−	−	−	−	−	−

+, positive; −, negative

Strain DB21MT-5 is a facultatively anaerobic chemoorganotroph displaying both respiratory and fermentative types of metabolism. Cells of the deep-sea strain DB21MT-5 were found to be gram-negative rods, 2–4 μm long and 0.8–1.0 μm wide, motile by means of a single unsheathed polar flagellum (Kato et al. 1998). This strain is unable to grow at atmospheric pressure at any temperature; however, it is able to grow in pressure vessels under hydrostatic pressures in the range of 60–100 MPa at 10°C and the optimal pressure and temperature condition for growth were found to be 80 MPa and 10°C (Kato et al. 1998). Acid, but not gas, is produced from maltose, D-mannose, and xylose. Catalase and cytochrome oxidase test are positive, gelatin is hydrolyzed, but nitrate and nitrite are not reduced. Test results for amylase, H_2S, production and indole production are negative for this strain. The following compounds are not utilized: L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, myo-inositol, D-lactose, D-mannitol, D-raffinose, L-rhamnose, D-sorbitol, sucrose, and D-trehalose. The G+C content of the DNA was found to be 44.6 mol%. The major isoprenoid quinone was found to be Q-8. Among the reference strains, *Moritella* species share the most physiological characteristics with DB21MT-5. This strain did not reduce nitrate, utilized quite different carbohydrates than those used by the *Moritella* reference strains, and was unable to grow at atmospheric pressure at any temperature.

Table 3. Fatty acid compositions of the extremely barophilic strain and of the *Moritella* and *Shewanella* reference strains

	% of total fatty acids[a]						
	M. yayanosii DB21MT-5^T	*M. japonica* JCM10249^T	*M. marina* ATCC15381^T	*S. benthica* DB21MT-2	*S. benthica* ATCC43992^T	*S. hanedai* IAM12641^T	*S. violacea* JCM10179^T
11:0				1	2		2
12:0			1	2	5	5	4
iso-13:0				1	11	12	8
13:0						1	
14:0	15	18	16	3	17	10	6
14:1	6	2	1				1
iso-15:0				3	5	7	14
15:0	1	1	1	1		4	7
iso-16:0			1				
16:0	13	21	21	15	15	24	15
16:1	5	5	6			1	
16:1	48	45	39	35	37	19	19
17:0						1	1
17:1						2	1
18:1	1	2	2	13	1	1	5
20:5				25	8	15	15
22:6	9	6	12				

[a] Main components

Considered together with the results of DNA-DNA hybridization analysis (Kato et al. 1998), these results suggest that strain DB21MT-5 represents a novel *Moritella* species.

Fatty acid composition

The whole-cell fatty acid compositions of strains DB21MT-2 and DB21MT-5 and of selected reference strains are shown in Table 3. The major fatty acids in strain DB21MT-2 were 16:0 (hexadecanoic acid), 16:1 (hexadecenoic acid), 18:1 (octadecenoic acid), and 20:5 ω3 (eicosapentaenoic acid, EPA). In general, this fatty acid profile showed a low level of similarity to that of the *S. benthica* type strain. For example, the dominant components in the fatty acid profile of strain DB21MT-2 were different from those in the profile of the *S. benthica* type strain, which contained substantial amounts of *iso*-13:0 (11-methyldodecanoic acid), 14:0 (tetradecanoic acid), and only small amounts of 18:1 and 20:5. The major fatty acids in strain DB21MT-5 were 14:0, 14:1 (tetradecenoic acid), 16:0, 16:1, and 22:6 (docosahexaenoic acid, DHA). The fatty acid profile of strain DB21MT-5 was basically similar to that of the *Moritella* type strains employed as reference strains. Approximately 70% of the membrane lipids in this strain were unsaturated fatty acids, and compared with the reference strains, higher amounts of 14:1 in strain DB21MT-5 were evident.

On the basis of phenotypic, genotypic, and phylogenetic data, it is logical to conclude that the two extremely barophilic isolates from the Mariana Trench examined in the present study are members of the genera *Shewanella* and *Moritella*. We propose the name *Moritella yayanosii* sp. nov. for strain DB21MT-5 (JCM 10263) as the type strain.

Discussion

The first obligately barophilic bacterium isolated from the Mariana Trench was strain MT41 (Yayanos et al. 1981). This strain was unable to grow at pressures less than 50 MPa, and its optimal pressure for growth was approximately 100 MPa (Yayanos 1986, 1995). This strain was an extremely barophilic bacterium closely related to the genus *Colwellia* (DeLong et al. 1997). Deming et al. reported that the optimal pressure for growth of the obligately barophilic bacterium strain BNL-1 was about 90 MPa at 10°C, and this strain was designated *Colwellia hadaliensis* (Deming et al. 1988).

We have isolated two different strains of obligately barophilic bacteria from the world's deepest ocean bottom, the Mariana Trench, Challenger Deep, at a depth of 10898 m (Kato et al. 1998). From the results of DNA-DNA relatedness study, these strains were identified as *S. benthica* (strain DB21MT-2) and a novel *Moritella* species (strain DB21MT-5) (Kato et al. 1998); adding other taxonomic results, we then named strain DB21MT-5 as *M. yayanosii*. The optimal pressure conditions for growth of *S. benthica* strain DB21MT-2 and *M. yayanosii* strain DB21MT-5 were 70 and 80 MPa, respectively. Neither of these strains was able to grow at a pressure of less than 50 MPa, but both were able to grow well at higher pressures, even at 100 MPa (Kato et al. 1998). The dominant fatty acid profile of strain DB21MT-2 is different from that of the *S. benthica* type strain. Approximately 70% of the membrane lipids in strain DB21MT-2 were found to be polyunsaturated fatty acids (PUFAs). DeLong and Yayanos (1985, 1986) reported that the fatty acid composition of the barophilic strain changed as a function of pressure, and in general, greater amounts of PUFAs were synthesized at

higher growth pressures. The different fatty acid profiles of the *S. benthica* type strain and strain DB21MT-2 appear to reflect the greater pressure adaptation of the extremely barophilic strain, as its fatty acid composition showed fewer short-chain fatty acids and more long-chain PUFA.

The other extremely barophilic isolate, DB21MT-5, which we propose to be named *Moritella yayanosii* sp. nov., falls into the genus *Moritella* as indicated by comparison of DNA-DNA hybridization values, and it is closely related to the psychrophilic strain *M. marina* and the barophilic strain *M. japonica*. The fatty acid profile of strain DB21MT-5 was found to be basically similar to that of the *Moritella* type strains employed as reference strains. Approximately 70% of the membrane lipids in strain DB21MT-5 were PUFAs, and this finding suggests that this strain is also well adapted to life in a high-pressure environment.

Description of *Moritella yayanosii* sp. nov.

Moritella yayanosii (yayanos.ii. M. L. gen. n. yayanosii, of Yayanos, in honor of American deep-sea biologist, A. Aristides Yayanos). Cells are rod shaped; cell width ranges from 0.8 to 1.0 μm, and cell length ranges from 2 to 4 μm. Cells are gram-negative and motile by means of a single unsheathed polar flagellum. The bacterium is halophilic and psychrophilic. Best growth occurs at an NaCl concentration of about 3%. No growth occurs in the absence of NaCl, and the organism is not able to grow at atmospheric pressure at any temperature. However, this strain is able to grow under hydrostatic pressures in the range of 60–100 MPa at 10°C, and the optimal pressure for growth is 80 MPa at 10°C. It is a facultatively anaerobic chemoorganotroph displaying both respiratory and fermentative types of metabolism. Catalase and cytochrome oxidase test results are positive. Acid is produced from D-mannose but no gas is produced. The organism does not produce H_2S. Nitrate and nitrite are not reduced. Acid is formed oxidatively from maltose, D-mannose, and xylose. No acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, myo-inositol, D-lactose, D-mannitol, D-raffinose, L-rhamnose, D-sorbitol, sucrose, or D-trehalose. The G+C content of the DNA is about 44.6 mol%. The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are 14:0, 14:1, 16:0, 16:1, and 22:6 (DHA).

The type strain is *Moritella yayanosii* strain DB21MT-5, which has been deposited in the Japan Collection of Microorganisms (Wako-shi, Saitama, Japan) as strain JCM 10263.

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