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Marinobacter alkaliphilus sp. nov., a novel alkaliphilic bacterium isolated from subseafloor alkaline serpentine mud from Ocean Drilling Program Site 1200 at South Chamorro Seamount, Mariana Forearc

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Abstract Novel alkaliphilic, mesophilic bacteria were isolated from subseafloor alkaline serpentine mud from the Ocean Drilling Program (ODP) Hole 1200D at a serpentine mud volcano, South Chamorro Seamount in the Mariana Forearc. The cells of type strain ODP1200D-1.5^T were motile rods with a single polar flagellum. Growth was observed between 10 and 45–50°C (optimum temperature: 30–35°C, 45-min doubling time), between pH 6.5 and 10.8–11.4 (optimum: pH 8.5–9.0), and between NaCl concentrations of 0 and 21% (w/v) (optimum NaCl concentration: 2.5–3.5%). The isolate was a facultatively anaerobic heterotroph utilizing various complex substrates, hydrocarbons, carbohydrates, organic acids, and amino acids. Nitrate or fumarate could serve as an electron acceptor to support growth under anaerobic conditions. The G+C content of the genomic DNA was 57.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belonged to the genus *Marinobacter* and was the most closely related to *M. aquaeolei* strain VT8^T and *M. hydrocarbonoclasticus* strain SP.17^T, while DNA–DNA hybridization demonstrated that the new isolate could be genetically differentiated from the previously described species of *Marinobacter*. Based on the physiological and molecular properties of the new isolate, we propose the name *Marinobacter alkaliphilus* sp. nov., type strain: ODP1200D-1.5^T (JCM12291^T and ATCC BAA-889^T).

Keywords Alkaliphilic · Facultatively anaerobic · Ocean Drilling Program · Serpentine mud · Subseafloor biosphere

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Introduction

The genus *Marinobacter* accommodates Gram-negative, aerobic, motile, and rod-shaped bacteria within the γ-subclass of the *Proteobacteria* (Al-Mallah et al. 1990; Gauthier et al. 1992; Yakimov et al. 1998; Huu et al. 1999; Anzai et al. 2000; Gorshkova et al. 2003). It currently contains seven species. The type species *M. hydrocarbonoclasticus* strain SP.17^T was isolated from seawater near a petroleum refinery outlet in the Gulf of Fos along the French Mediterranean coast (Al-Mallah et al. 1990; Gauthier et al. 1992). From the same geographical site, a squalene-metabolizing *M. squalenivorans* strain 2A sq64^T has been recently obtained (Rontani et al. 2003). *M. aquaeolei* strain VT8^T, *M. litoralis* strain SW-45^T, and *M. excellens* strain KMM 3809^T were also isolated from marine habitats at an oil-producing well in Southern Vietnam (Huu et al. 1999) coastal seawater (Yoon et al. 2003) and sediments (Gorshkova et al. 2003) in the Japan Sea. In addition to marine habitats, *M. lipolyticus* strain SM19^T and *M. lutaoensis* strain T5054^T have been reported from saline soils in Spain (Martin et al. 2003) and a hot spring in Taiwan (Shieh et al. 2003), respectively. All of the

previously described members of *Marinobacter* are known to be halophilic or halotolerant, heterotrophic neutrophiles.

Serpentine-mud volcanism in the Mariana Forearc provides an opportunity to investigate novel geological and geochemical processes of the subduction factory in the Pacific–Philippine plates margin (Salisbury et al. 2002; Mottl et al. 2003). Throughout Ocean Drilling Program (ODP) activities, two drilling expeditions have been so far conducted to obtain subseafloor serpentine-mud materials in the Serpentine Seamounts (Conical Seamount in ODP Leg 125 and South Chamorro Seamount in ODP Leg 195, Fryer 1992a, b; Salisbury et al. 2002; Mottl et al. 2003). The geochemical characterization in both of the Serpentine Seamounts has revealed an outstanding feature of pore water in the subseafloor serpentine mud (Mottl 1992; Salisbury et al. 2002; Mottl et al. 2003). The pore-water pH of upwelling serpentine mud is as high as 12.5, and the extremely high pH is constant throughout the subseafloor environment below the mixing zone with seawater (Mottl 1992; Salisbury et al. 2002; Mottl et al. 2003). The subseafloor serpentine-mud pore waters represent the highest pH values ever measured in oceans (Salisbury et al. 2002; Mottl et al. 2003), equivalent to the highest pH limit for life reported (pH 12.4 of *Alakaliphilus transvaalensis* strain SAGM1^T, Takai et al. 2001c). Thus, the Serpentine Seamounts in the Mariana Forearc also provide an excellent opportunity to explore novel extremophiles and to investigate their phylogenetic diversity and ecological significance in the highly alkaline, subseafloor habitats.

In this study, we examined the culturability of the microorganisms in the subseafloor alkaline serpentine mud of the ODP Hole 1200D at South Chamorro Seamount, Mariana Forearc, which was retrieved during ODP Leg 195 in March 2001. A variety of alkaline media with organic and/or inorganic substrates was tested for cultivation of alkaliphilic microbial components in the core samples at different depths. Heterotrophic alkaliphiles were successfully cultivated from the relatively shallow zone of the core and then purified. The 16S rRNA gene sequence analysis of the alkaliphilic isolates revealed that most were very closely related. Two representative strains were further characterized. Based on the physiological and molecular properties of the new isolates, we propose a new species of *Marinobacter*, named *M. alkaliphilus*. This is the second new species taxonomically described from the core samples derived from the ODP in its long history, after *Desulfovibrio profundus* strain 500-1^T from the Japan Sea (Bale et al. 1997).

Materials and methods

Sample collection

Microbiological samples from ODP Hole 1200D were obtained from a cold-seep site on the northwest slope of South Chamorro Seamount, Mariana Forearc (13°47.0443′N, 146°00.1715′E), at a water depth of 2,942 m by means of the Advanced Piston Coring system equipped with the deep-sea drilling vessel (DSDV) *Joides Resolution* during ODP Leg 195 in March 2001. A total of 18 microbiological whole-round core samples were collected for microbiological analysis (Table 1), with a higher sampling frequency near the surface and a maximum sampled depth of 29.15 m below seafloor (mbsf). All microbiological samples were subject to a series of tracer tests to determine the presence and extent of possible contamination to the exterior and interior of whole-round cores. These included (1) analysis of adjacent whole-round core in situ pore-water chemistry (Mottl et al. 2003); (2) microscopic inspection of microbiological samples for the internal presence of fluorescent microspheres; and (3) high-performance liquid chromatography [(HPLC) Tamaoka and Komagata 1984] detection of a perfluorocarbon tracer (PFT), which was delivered in the drilling fluid (Smith et al. 2000). All three methods of detection positively confirmed the contamination of a single whole-round core sample from Hole 1200D (5H1, 20–30), showing relatively low alkalinity, interior fluorescent beads, and interior elevated levels of PFT, respectively. All other microbiological samples collected from Hole 1200D showed no contamination in terms of pore water chemistry, no internal occurrence of fluorescent microspheres, and no detectable levels of PFT above background. These results help to further demonstrate the potential for the collection of uncontaminated core samples to be used in microbiological research by ODP drilling in deep-ocean sediments. Subsamples from all microbiological cores were collected and processed shipboard using a laminar flow hood. Immediately after recovery, interior parts of the whole-round cores were subsampled using a sterile 3-ml syringe under an aerobic chamber with 95% N_2 and 5% H_2. Subsamples were placed in serum bottles under a gas phase of 100% N_2, tightly sealed with a butyl rubber stoppers, and preserved at 4°C prior to enrichment experiments.

Culturability test and purification

Immediately before the cultivation in the laboratory, the subsamples were suspended in 10 ml sterilized MJ synthetic seawater (Sako et al. 1996; Takai et al. 1999) containing 0.05% (w/v) sodium sulfide under a gas phase of 100% N_2 (100 kPa). These suspended slurries of the subsamples were used for the dilution–cultivation tests, using a series of media including MJYTGL medium as described below. Dilution–cultivation tests and subsequent cultivation and isolation of the microorganisms were conducted using the media and under conditions as follows:

1. For mesophilic to thermophilic methanogens, standard or alkaline MMJ medium with 5 mM calcium

Table 1 Physical and microbiological characteristics of subsamples in the ODP1200D core obtained from the South Chamorro Seamount, Mariana Forearc. *mbsf* Meters below seafloor

Subsample for microbiology[a] 195-1200D-	Subsample for geochemistry[a] 195-1200D-	Depth[a] (mbsf)	In situ temperature[a] (°C)	Pore-water pH[a]	Total cell counts (cells/cc) (± SD)	Viable population with standard MJYTGL medium at 37°C under an aerobic condition (cells/cc)	Viable population with standard MJYTGL medium at 37°C under an anaerobic condition (cells/cc)	Viable population with alkaline MJYTGL medium at 37°C under an aerobic condition (cells/cc)	Viable population with alkaline MJYTGL medium at 37°C under an anaerobic condition (cells/cc)
1H1, 0–10		0.05			$1.4 \pm 0.3 \times 10^7$	$1.0–5.0 \times 10^5$	$4.0–20 \times 10^2$ (strain A[b])	$2.3–11.5 \times 10^4$ (strain ODP1200D-0.1)	$1.5–7.5 \times 10^3$ (strain E[b])
1H1, 30–40		0.35			$6.0 \pm 1.5 \times 10^6$	$4.1–20.5 \times 10^4$	< 6	$4.1–20.5 \times 10^4$ (strain B[b])	$1.0–5.0 \times 10^4$ (strain F[b])
	1H1, 40–50	0.45	1.7	8.4					
1H1, 80–90									
	1H1, 90–100	0.95	1.7	8.9					
	1H1, 130–140	1.35	1.7	9.5					
1H1, 140–150		1.45			$2.5 \pm 0.7 \times 10^7$	$2.6–13.0 \times 10^3$	< 6	$2.6–13.0 \times 10^3$ (strain C[b])	$2.6–13.0 \times 10^3$ (strain ODP1200D-1.5[T])
1H2, 50–60		2.05							
	1H2, 60–70	2.15	1.7	9.9					
	1H2, 130–140	2.85	1.7	10.2					
1H2, 140–150		2.95			$5.1 \pm 2.0 \times 10^7$	< 6	< 6	$5.3–26.5 \times 10^1$ (strain D[b])	< 6
	1H3, 130–140	4.35	1.7	11.7					
1H3, 140–150		4.45							
	1H4, 130–140	5.85	1.7	11.9					
1H4, 140–150		5.95			$3.2 \pm 0.9 \times 10^7$	< 6	< 6	< 6	< 6
	1H5, 20–30	6.25	1.7	11.9					
1H5, 30–40		6.35			$7.2 \pm 2.5 \times 10^6$	< 6	< 6	< 6	< 6
	2H1, 130–140	8.25	1.7	12					
2H1, 130–140		8.35			$3.1 \pm 1.0 \times 10^6$	< 6	< 6	< 6	< 6
	2H2, 98–108	9.43	1.8	12.1					
2H2, 108–118		9.53			$1.8 \pm 0.4 \times 10^6$	< 6	< 6	< 6	< 6
	3H1, 130–140	11.25	1.8	12.3					
3H1, 140–150		11.35			$3.4 \pm 1.3 \times 10^6$	< 6	< 6	< 6	< 6
	3H2, 52–62	11.97	1.8	10.7					
3H2, 62–72		12.07			$6.6 \pm 1.4 \times 10^6$	< 6	< 6	< 6	< 6
5H1, 20–30		13.95			$3.0 \pm 2.2 \times 10^5$	< 6	< 6	< 6	< 6
	5H1, 30–40	14.05	1.8	12.4					
	6H1, 115–126	22.71	1.8	12.4					
6H1, 126–137		23.21			$2.0 \pm 0.9 \times 10^5$	< 6	< 6	< 6	< 6
7H1, 110–120		24.65			$3.0 \pm 1.4 \times 10^6$	< 6	< 6	< 6	< 6
	7H1, 120–130	24.75	1.9	12.4					
8H2, 97–107		27.42			$1.4 \pm 0.5 \times 10^6$	< 6	< 6	< 6	< 6
	8H2, 107–117	27.52	1.9	12.4					
	9H1, 90–100	29.05	1.9	12.4					
9H1, 100–110		29.15			$2.4 \pm 1.0 \times 10^6$	< 6	< 6	< 6	< 6

[a]These data were obtained from Salisbury et al. (2002)

[b]The partial rRNA gene sequences from these strains represented > 99% similarity to those of strain ODP1200D-1.5[T] and strain ODP1200D-0.1

chloride ($CaCl_2$) (Takai et al. 2002) was used and cultivation was performed at 37, 55, and 70°C.
2. For mesophilic to thermophilic hydrogen or sulfur oxidizers, standard or alkaline MMJHS medium (Takai et al. 2003) was used and cultivation was performed at 37, 55, and 70°C.
3. For psychrophilic to thermophilic heterotrophs, standard or alkaline MJYTGL medium (described blow) was used and cultivation was performed under either aerobic (a gas phase of air) or anaerobic (a gas phase of 100% N_2) conditions at 10, 37, 55, and 70°C.

To prepare the alkaline MMJ and MMJHS media, 1% (w/v) sodium carbonate, 0.1% (w/v) sodium silicate, and 0.02% (w/v) potassium hydroxide were added to, and sodium bicarbonate was removed from, the standard media. This treatment prepared the alkaline pH condition (approx. 10.5) of the media. A dilution series of cultivation was performed in duplicate to assess the viable population size of the targeted members of the microorganisms. The microorganism present in the most diluted series of the medium at each of temperatures was isolated by the subsequent extinction–dilution method (Takai and Horikoshi 2000). The partial sequences of the 16S rRNA genes (approx. 900 bp) from the isolates were determined (Takai et al. 2001a) and applied to sequence-similarity analysis. The strain, which was isolated from the culture grown at 37°C with the alkaline MJYTGL medium under anaerobic conditions including the subsample at a depth of 1.45 mbsf, was designated as ODP1200D-1.5^T, and the strain, from the culture grown at 37°C with the alkaline MJYTGL medium under aerobic conditions including the subsample at a depth of 0.05 mbsf, was designated as ODP1200D-0.1. These strains were further characterized. The purity of the isolates was ensured by the repeated plating technique using the alkaline MJYTGL medium solidified with 1.5% (w/v) agar and was routinely checked by microscopic examination and by repeated partial sequencing of the 16S rRNA gene, using several polymerase chain reaction (PCR) primers (Lane 1985).

Microbial cell counts in subsamples

Using the suspended subsamples, total microbial cell density was determined by staining with 4′,6-diamidino-2-phenylindole (DAPI) as described previously (Takai and Horikoshi 1999).

Sources of organisms

Marinobacter hydrocarbonoclasticus strain SP.17^T was obtained from the American Type Culture Collection [(ATCC), Manassas, Va., USA]. *M. aquaeolei* strain VT8^T and *M. lipolyticus* strain SM19^T were obtained from the Deutsche Sammlung von Mikrooganismen und Zellkulturen (Braunschweig, Germany). *M. litoralis* strain SW-45^T and *M. lutaoensis* strain T5054^T were purchased from the Japan Collection of Microorganisms [(JCM) Wako, Japan]. *M. excellens* strain KMM 3809^T was purchased from the Collection of Bacterial Strains of Institut Pasteur (Paris, France). All strains were cultivated with the Marine Broth 2216 (Difco) under optimal conditions.

Culture media and conditions

The strains ODP1200D-1.5^T and ODP1200D-0.1 were routinely cultivated in the alkaline MJYTGL medium under aerobic conditions. The alkaline MJYTGL medium consisted of 0.8 g of $CaCl_2$, 1 g of $NaNO_3$, 0.5 g of $Na_2SiO_3{\cdot}9H_2O$, 10 g of Na_2CO_3, 0.2 g of KOH, 2 g of yeast extract, 2 g of tryptone, 0.5 g of glucose, and 0.05% (w/v) of sodium lactate per liter of MJ(-N) synthetic seawater (Takai et al. 2000). To prepare the alkaline MJYTGL medium, all ingredients were dissolved in 1 l of MJ(-N) synthetic seawater, and the pH of the medium was adjusted with KOH to around 10.5 at room temperature before autoclaving. For the standard MJYTGL medium, $Na_2SiO_3{\cdot}9H_2O$, Na_2CO_3 and KOH were removed, and the pH was adjusted to 7.0 at room temperature before autoclaving. The medium was dispensed at 20% of the total bottle or tube volume. In case of anaerobic culture, the medium was purged with 100% N_2 for 10 min, and the bottle or tube was tightly sealed with a butyl rubber stopper under a gas phase of 100% N_2 (200 kPa).

All experiments described below were conducted in duplicate. In an attempt to examine the potential organic substrates, various complex organic substrates, carbohydrates, hydrocarbons, organic acids, and amino acids were tested as the sole energy and carbon sources instead of yeast extract, tryptone, glucose, and lactate in the alkaline MJYTGL medium under aerobic conditions. The gaseous hydrocarbons such as methane, propane, and butane were also tested with the alkaline MJYTGL medium in the absence of yeast extract, tryptone, glucose and lactate under a gas phase of 50% CH_4, 40% N_2 and 10% O_2 (200 kPa), 50% C_3H_8, 40% N_2 and 10% O_2 (200 kPa), and 10% C_4H_{10}, 80% N_2 and 10% O_2 (200 kPa), respectively. The potential electron acceptors such as nitrate, nitrite, elemental sulfur, sulfite, thiosulfate, soluble iron (III) (ferric citrate), and fumarate other than molecular oxygen were tested as sole electron acceptors instead of nitrate in the alkaline MJYTGL medium. These tests were performed under anaerobic conditions. The potential nitrogen sources such as 0.2% (w/v) yeast extract, 10 mM of $NaNO_3$ and NH_4Cl, and 1 mM of $NaNO_2$ were examined with the alkaline MJYTGL medium in the absence of yeast extract, tryptone, glucose, and nitrate under aerobic conditions. Molecular nitrogen was also tested

as a potential nitrogen source with the alkaline MJYTGL medium in the absence of yeast extract, tryptone, glucose, and nitrate under a gas phase of 90% N_2 and 10% O_2 (200 kPa).

For testing the effect of pH on growth, the pH of MJYTGL medium was adjusted at room temperature to various pH levels with H_2SO_4 or KOH (pH 6.5–8.0) in the standard MJYTGL medium, with decreasing amounts of Na_2CO_3 (pH 8.0–10.0) in the alkaline MJYTGL medium or by addition of KOH (> pH 10.0). After autoclaving, the pH of the medium was checked and readjusted at room temperature with KOH to the designed pH. The pH was found to be stable during the cultivation under aerobic conditions. For testing the effects of NaCl concentration on growth, the alkaline MJYTGL medium was prepared with a varying concentration of NaCl instead of 30 g l^{-1} NaCl.

Growth of the new isolates was measured by direct cell counting after staining with DAPI, using the Olympus BX51 microscope. Cultures were prepared in duplicate. The cultures were grown in 50-ml glass tubes (Iwaki glass), each containing 10 ml medium, with shaking (100 rpm) in a temperature-controlled dry oven. The pH growth curve was determined at 30°C, and the growth conditions for all other cultivation tests were 30°C and pH 9.0, unless otherwise noted.

Light and electron microscopy

Cells were routinely observed under a phase contrast Olympus BX51 microscope with the SPOT RT Slider CCD camera system (Diagnostic Instruments). Transmission electron microscopy of negatively stained cells and thin sections of the cells was carried out as described by Zillig et al. (1990) and Takai et al. (2001b), respectively. Cells grown in the alkaline MJYTGL medium (pH 9.0) at 30°C in the mid-exponential phase of growth were negatively stained with 2% (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope at an accelerating voltage of 120 kV. For ultrathin sectioning, cells grown in the alkaline MJYTGL medium (pH 9.0) at 30°C in the mid-exponential phase of growth were fixed with 4% (w/v) paraformaldehyde overnight at room temperature. Thin sections were prepared and stained with uranyl acetate and lead citrate and observed with a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV.

Antibiotic susceptibility

Sensitivity of strains ODP1200D-1.5^T and ODP1200D-0.1 to antibiotics such as chloramphenicol (50, 100, and 200 μg ml^{-1}), streptomycin (50, 100, and 200 μg ml^{-1}), kanamycin (50, 100, and 200 μg ml^{-1}), ampicillin (50, 100, and 200 μg ml^{-1}), and rifampicin (50 and 100 μg ml^{-1}) was tested at 30°C with the alkaline MJYTGL medium under aerobic conditions.

Isolation and base composition of DNA

Genomic DNA was prepared as described by Murmur and Doty (1962). The G + C content of DNA was determined by direct analysis of deoxyribonucleotides by HPLC. Nonmethylated DNA from bacteriophage λ (49.8 mol% G + C, TaKaRa, Kyoto, Japan) was used as a reference.

Amplification of 16S rRNA gene and sequence determination

The 16S rRNA gene was amplified by PCR using Bac27F and 1492R primers (DeLong 1992; Lane 1985). Approximately 1.5 kb of PCR product was directly sequenced by the dideoxynucleotide chain termination method using a DNA sequencer Model 3100 (Perkin Elmer/Applied Biosystems, Foster City, Calif., USA). The rRNA gene sequence was analyzed using the gapped-BLAST search algorithm (Altschul et al. 1997; Benson et al. 1998) to estimate the degree of similarity to other bacterial 16S rDNA sequences.

Data analysis

The almost-complete sequences (1,430 bp for strain ODP1200D-1.5^T and 1,435 bp for ODP1200D-0.1) of the new isolates were manually re-aligned to 16S rRNA gene data from the Ribosomal Data Project II (RDP-II) (Cole et al. 2003), based on the alignments determined by Sequence Aligner program of RDP-II. Phylogenetic analyses were restricted to nucleotide positions that could be unambiguously aligned. Evolutionary distance matrix analysis (using the Kimura two-parameter method, the least square distance method, and transition/transversion rate of 2.0) and a neighbor-joining analysis were performed using the PHYLIP package (version 3.5, obtained from J. Felsenstein, University of Washington, Seattle, Wash., USA). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

DNA–DNA hybridization analysis

DNA–DNA hybridization was carried out at 42°C for 3 h and was measured fluorometrically using photobiotin, according to the method of Ezaki et al. (1989). *M. hydrocarbonoclasticus* strain SP.17^T, *M. aquaeolei* strain VT8^T, *M. lipolyticus* strain SM19^T, *M. litoralis* strain SW-45^T, *M. excellens* strain KMM 3809^T, and *M. lutaoensis* strain T5054^T were used as reference strains.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the new isolates were deposited in the DDBJ/EMBL/GenBank nucleotide

sequence databases with the accession number AB125941 for strain ODP1200D-1.5^T and AB125942 for strain ODP1200D-0.1.

Results and discussion

Sample descriptions, culturability tests, and purification

In total, 18 subsamples for microbiological experiments were collected from the subcores at different depths up to 29.2 mbsf (Table 1). The in situ temperature of the subseafloor serpentine mud was 1.7–1.9°C, and the pH of the pore water increased to 12.5 with increasing depth (Table 1) (Salisbury et al. 2002; Mottl et al. 2003). Since the site for Hole 1200D was located at several meters' distance from the cold-seep epicenter, the degree of increasing pH and the potential mixing zone between upwelling alkaline seepage and infiltrating seawater in Hole 1200D was broader (up to approx. 4 mbsf) than in Hole 1200E (Salisbury et al. 2002; Mottl et al. 2003).

Based on the DAPI-staining direct cell counting of the subsamples, the surface serpentine mud contained approximately 1.0×10^7 cells cc^{-1}, while the mixing zone subsample at a depth of 1.45 mbsf represented the highest microbial community density of 5.0×10^7 cells cc^{-1} (Table 1). With increasing depth and increasing pH of the pore water, the microbial community density was decreased, while the deepest subsample still contained the microbial community density of more than 2.0×10^6 cells cc^{-1} (Table 1).

Using the anaerobically prepared slurries of the subsamples, the dilution-cultivation, using a series of media including MJYTGL medium, was conducted to assess the viable population size of the targeted members of the microorganisms. Throughout the dilution–cultivation tests under various conditions, the positive cultures were obtained from the standard or alkaline MJYTGL medium under both aerobic and anaerobic conditions at 10 and 37°C (Table 1). At 10°C, the positive cultures was recovered from the subsamples [(1H1, 0–10), (1H1, 30–40), and (1H1, 80–90) with the standard MJYTGL medium under an aerobic condition and (1H1, 0–10) with the alkaline MJYTGL medium under aerobic conditions], but the estimated population sizes were one or two order(s) of magnitude lower than those from the same media at 37°C (data not shown). The estimated population size of mesophilic (grown at 37°C), aerobic heterotrophs in the surface serpentine mud (1H1, 0–10) was greater at a neutral pH than at an alkaline pH, but the sizes in the deeper subsamples (1H1, 30–40 and 1H1, 80–90) were similar between under neutral and alkaline pH conditions (Table 1). The higher abundance of mesophilic, anaerobic heterotrophs was detected in the alkaline MJYTGL medium from the subsamples up to 0.85 mbsf rather than in the standard MJYTGL medium (Table 1). In addition, the estimated population size of mesophilic heterotrophs was increased in the subsample at a depth of 0.35 mbsf rather than in the surface serpentine mud. From the subseafloor serpentine-mud subsamples below 2.95 mbsf, no positive cultures were obtained in this study. The distribution profile of the cultivated microbial population may be associated with the formation of the mixing zone delineated by pore-water pH profiles (Table 1).

The aerobic or anaerobic heterotrophs present in the most diluted series of the standard or alkaline medium at each of temperatures was isolated by the subsequent extinction–dilution method (Takai and Horikoshi 2000). The partial sequences of the 16S rRNA genes (approx. 900 bp) from the isolates were determined as previously described (Takai et al. 2001a) and applied to sequence-similarity analysis. All the strains isolated from the cultures grown at 37°C with the alkaline MJYTGL medium, and the strain from the anaerobic culture with the standard MJYTGL medium including the surface serpentine mud were closely related to each other (>99% similarity of partial 16S rRNA gene sequences between any two of the strains, Table 1). In contrast, the strains obtained from aerobic cultures grown 10 and 37°C with the standard MJYTGL medium were phylogenetically associated with the members of the genus *Halomonas* (data not shown). Two representative alkaliphilic strains, designated as ODP1200D-1.5^T from the anaerobic culture of the relatively deep subsample (1H1, 140–150) and as ODP1200D-0.1 from the aerobic culture of the surface serpentine mud (1H1, 0–10), were applied for further taxonomic characterization.

Morphology

Cells of strains ODP1200D-1.5^T and ODP1200D-0.1 had similar morphology and were Gram-negative, straight-to-slightly bent rods, about 2.0–5.0 μm long and 0.3–0.8 μm wide in the exponential growth phase (Fig. 1a). Each of the cells in the exponential growth phase appeared to be motile and have a single polar flagellum. Both strains formed cream-colored, smooth, and circular colonies 1.0–2.0 mm in diameter on solidified alkaline MJYTGL medium after 3 days' cultivation at 30°C. These morphological features were quite similar to those of previously described *Marinobacter* species (Gauthier et al. 1992; Huu et al. 1999; Gorshkova et al. 2003; Martin et al. 2003; Rontani et al. 2003; Yoon et al. 2003).

Growth parameters

The new isolates (ODP1200D-1.5^T and ODP1200D-0.1) were strict heterotrophs capable of growing under either aerobic or anaerobic conditions. Both strains were able to utilize yeast extract (0.2%), tryptone (0.2%), casein (0.2%), casamino acid (0.2%), starch (0.2%), crude oil (0.5%), corn oil (0.5%), olive oil (0.5%), Tween 20 (0.1%), Tween 80 (0.1%), *n*-pentadecane [(C_{15}) 0.2%],

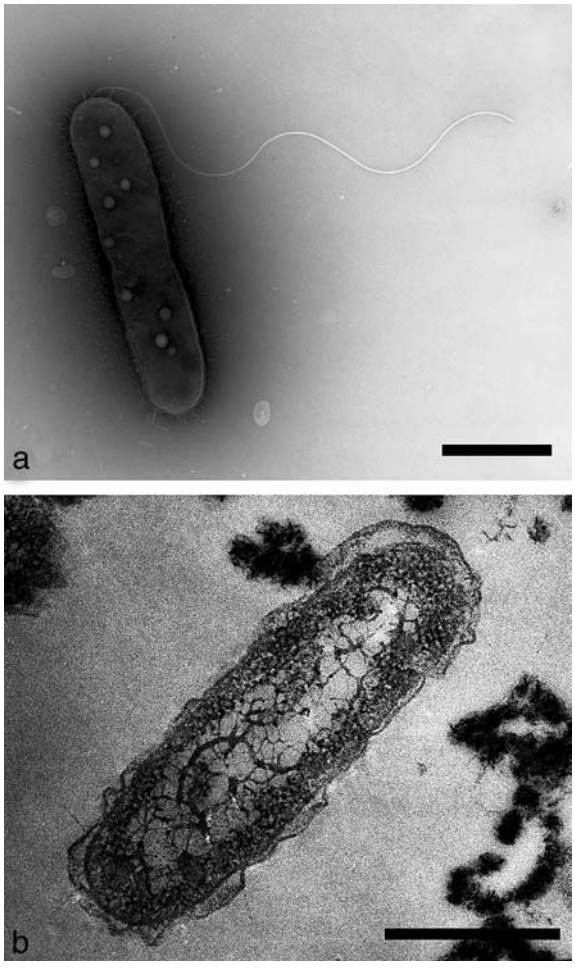

Fig. 1 Electron micrographs of a negatively stained cell (**a**) and a thin-section (**b**) of *Marinobacter alkaliphilus* strain ODP1200D-1.5^T. **a** Single cell in the mid-exponential phase of growth. *Bar* indicates 1.0 μm. **b** Thin-section of a cell in the mid-exponential phase of growth. *Bar* indicates 0.5 μm

n-tridecane [(C_{13}) 0.2%], *n*-undecane [(C_{11}) 0.2%], *n*-decane [(C_{10}) 0.2%], *n*-nonane [(C_9) 0.2%], kerosene (0.2%), ethanol (0.2%), glycerol (0.2%), maltose (0.2%), glucose (0.2%), lactose (0.2%), xylose (0.2%), fructose (0.2%), acetate (0.2%), lactate (0.2%), pyruvate (0.2%), alanine (0.2%), glutamate (0.2%), or proline (0.2%) as sole energy and carbon sources. None of the potential organic substrates tested such as agar (0.2%), crystalline cellulose (0.2%), carboxy methylcellulose (0.2%), lignin (0.2%), *n*-octane [(C_8) 0.2%], *n*-heptane [(C_7) 0.1%], *n*-hexane [(C_6) 0.1%], propane (50% of gas phase), methane (50% of gas phase), *p*-xylene (0.1%), cyclohexane (0.1%), phenol (0.1%), chloroform (0.1%), *n*-propanol (0.2%), methanol (0.1%), sucrose (0.2%), trehalose (0.2%), formate (0.1%), citrate (0.2%), succinate (0.2%), tartrate (0.2%), malate (0.2%), and 0.2% of any of amino acids other than alanine, glutamate, glutamine, and proline, supported the growth of both isolates. Although strain ODP1200D-0.1 was able to utilize glutamine (0.2%) and butane [(C_4) 10% of gas phase] as the sole energy and carbon sources, while strain ODP1200D-1.5^T was not. Higher growth yields of both strains were obtained from any of yeast extract, tryptone, starch, Tween 20, Tween 80, oils, casamino acid, lactate, pyruvate, glutamate, proline, kerosene, and *n*-pentadecane than from other energy and carbon sources, and the maximum cell yield was approx. 4.0×10^9. Under aerobic conditions, both strains used either nitrate or fumarate as an electron acceptor, and anaerobic fermentation did not occur without any of the potential electron acceptors. Yeast extract (0.2%), nitrate (10 mM), nitrite (1 mM), and ammonium ion (10 mM) served as nitrogen sources.

The optimum temperature and temperature range for growth were somewhat different between the two strains (Fig. 2a). Strain ODP1200D-1.5^T grew over the temperature range of about 10–45°C, showing an optimal growth at 30°C, and the generation time at 30°C was about 45 min at pH 9.0 (Fig. 2a). Strain ODP1200D-0.1 grew over between 10 and 50°C, showing an optimal growth at 35°C (Fig. 2a). Growth of the new isolates at 30°C occurred between pH < 6.5 and 11.4 (for strain ODP1200D-1.5^T) or 10.8 (for strain ODP1200D-0.1) with optimum growth at about pH 9.0 (for strain ODP1200D-1.5^T) or 8.5 (for strain ODP1200D-0.1) (Fig. 2b). The new isolates were halophilic but did not absolutely require NaCl for the growth. Both strains grew over the concentration range between 0 and 21%, with optimum growth at between 2.5 and 3.5% at 30°C and pH 9.0 (Fig. 2c). These physiological properties were generally similar to the previously described *Marinobacter* species (Gauthier et al. 1992; Huu et al. 1999; Gorshkova et al. 2003; Rontani et al. 2003; Yoon et al. 2003) other than the alkaliphily and alkalitolerance of the new isolates. In an attempt to ensure the difference in alkalitolerance between the new isolates and *Marinobacter* members reported, the simultaneous cultivation was performed with MJYTGL medium at a varying pH condition with *M. hydrocarbonoclasticus* strain SP.17^T, *M. aquaeolei* strain VT8^T, *M. lipolyticus* strain SM19^T, *M. litoralis* strain SW-45^T, and *M. excellens* strain KMM 3809^T at their optimum temperatures. In the alkaline MJYTGL medium, the highest pH limit for growth was pH 9.0 for *M. litoralis* strain SW-45^T and pH 9.4 for the other strains (Table 2). Hence, the alkaliphilic growth represented a distinctive physiological property of the new isolates from the previously described *Marinobacter* species.

Antibiotic susceptibility

Both strains revealed the identical antibiotics susceptibility that they were sensitive to chloramphenicol (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}), kanamycin (100 μg ml^{-1}), ampicillin (50 μg ml^{-1}), and rifampicin (50 μg ml^{-1}). The new isolates were resistant to kanamycin (50 μg ml^{-1}).

DNA base compositions

The G + C content of the genomic DNA of strains ODP1200D-1.5^T and ODP1200D-0.1 were almost identical at 57.5 ± 0.2 mol%.

Phylogenetic analyses and DNA–DNA hybridization

The almost-complete sequences (1,430 bp for strain ODP1200D-1.5^T and 1,435 bp for ODP1200D-0.1) of the 16S rRNA genes were determined. The 16S rRNA gene sequences of both strains were identical at the homologous positions compared and were most closely related to that of *M. aquaeolei* strain VT8^T (Huu et al. 1999, 98.0%), *M. hydrocarbonoclasticus* strain SP.17^T (Gauthier et al. 1992, 97.8%) or *M. excellens* strain KMM 3809^T (Gorshkova et al. 2003, 97.6%). This result indicated that the new isolates belonged to the genus *Marinobacter*.

The phylogenetic tree of the 16S rRNA gene sequences constructed by the neighbor-joining method also indicated that the new isolates were closely related to *M. aquaeolei* strain VT8^T (Huu et al. 1999) and *M. hydrocarbonoclasticus* strain SP.17^T (Gauthier et al. 1992) (Fig. 3). However, the bootstrap analysis revealed a relatively low confidence in the phylogenetic affiliation of the new isolates (Fig. 3).

The DNA–DNA hybridization analysis was conducted among the phylogenetically related strains within the genus *Marinobacter*, including the new isolates. The average hybridization was 96.7% between strains ODP1200D-1.5^T and ODP1200D-0.1, 18.9% between strain ODP1200D-1.5^T and *M. aquaeolei* strain VT8^T, 27.6% between ODP1200D-1.5^T and *M. hydrocarbonoclasticus* strain SP.17^T, 15.7% between ODP1200D-1.5^T and *M. litoralis* strain SW-45^T, 26.2% between ODP1200D-1.5^T and *M. lipolyticus* strain SM19^T, 45.1% between ODP1200D-1.5^T and *M. excellens* strain DMM 3809^T, and 21.9% between ODP1200D-1.5^T and *M. lutaoensis* strain T5054^T. These results indicated that the both strains could be genotypically identified as the same species but could also be differentiated from the previously described species of the genus *Marinobacter*.

Comparison with related species

The phylogenetic analysis indicated that both of the alkaliphilic isolates belonged to the genus *Marinobacter* and were the most closely related to *M. aquaeolei* strain VT8^T and *M. hydrocarbonoclasticus* strain SP.17^T (Fig. 3), which were isolated from an oil-producing well in the Southern Vietnam (Huu et al. 1999) and from seawater near a petroleum refinery outlet in the Gulf of Fos along the French Mediterranean coast (Al-Mallah et al. 1990; Gauthier et al. 1992), respectively. Most of the morphological, physiological, and molecular characteristics of the new isolate are similar with those of *M. aquaeolei* strain VT8^T and *M. hydrocarbonoclasticus* strain SP.17^T, and the other

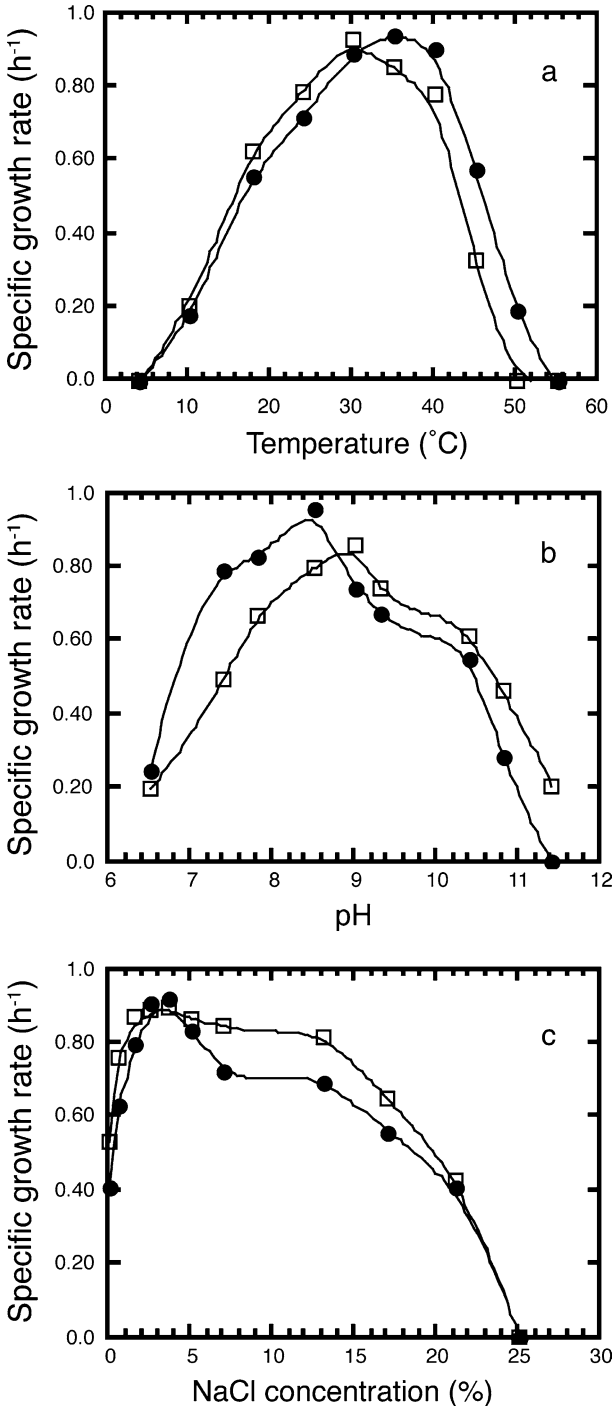

Fig. 2 Effects of temperature (**a**), pH (**b**), and NaCl concentration (**c**) on growth of *M. alkaliphilus* strains ODP1200D-1.5^T (*open squares*) and ODP1200D-0.1 (*close circles*). The growth curve at different temperatures was determined in the alkaline MJYTGL medium at pH 9.0. The pH growth curve was determined at 30°C over a range of pH values. The effect of NaCl concentration on growth was determined in the alkaline MJYTGL medium containing a varying concentration of NaCl instead of 30 g l^{-1} NaCl

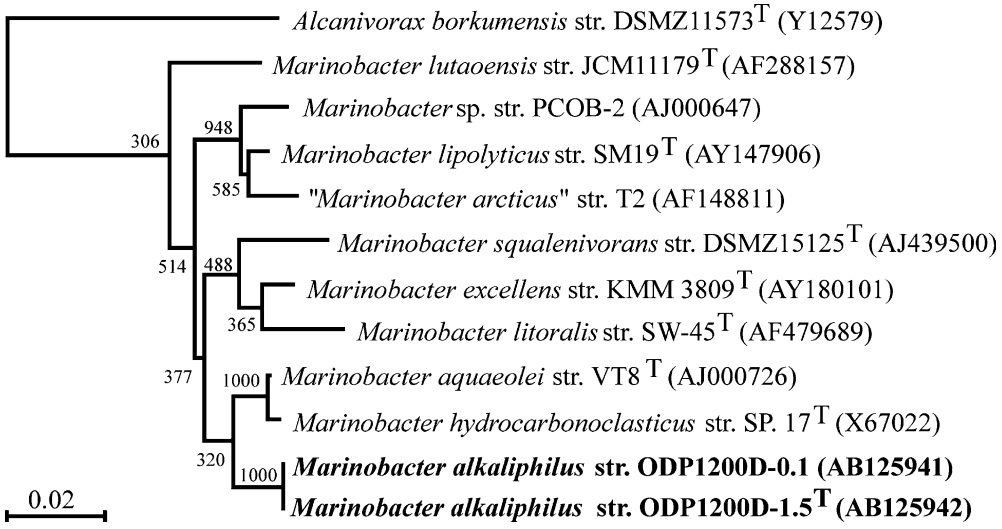

Fig. 3 Phylogenetic tree of representative *Marinobacter* species inferred from 16S rRNA gene sequences, using the neighbor-joining method on the 1,374 homologous sequence positions for each organism. The *number* at each node represents the bootstrap value (1,000 replicates). The *scale bar* indicates two substitutions per 100 nucleotides

previously described *Marinobacter* species (Table 2). However, the new isolates differ markedly from any other *Marinobacter* species with respect to several physiological features (Table 2). The most distinctive feature is the alkaliphily and alkalitolerance of the growth; the new isolates grew optimally at pH 8.5–9.0 and up to over pH 10.8. The optimum pH and the upper pH limit for growth of the new isolates were significantly higher than those of the other *Marinobacter* species (Table 2). In addition, the new isolates represent a different profile in capability of substrate utilization of starch, glucose, fructose, glycerol, and alanine from most of the other *Marinobacter* species (Table 2). The DNA–DNA hybridization analysis clearly reveals that the new isolates should be classified into the identical species and can be genetically differentiated from the previously described *Marinobacter* species on the species level. Based on these physiological and genetic properties of the new isolates, we propose that the isolates be classified as *M. alkaliphilus*, and the type strain is ODP1200D-1.5^T (JCM 12291^T and ATCC BAA-889^T).

Table 2 Characteristics of representative species of the genus *Marinobacter*

Characteristic	*M. alkaliphilus* strain ODP1200D-1.5^T	*M. alkaliphilus* strain ODP1200D-0.1	*M. hydrocarbonoclasticus* strain SP.17^T	*M. aquaeolei* strain VT8^T	*M. litoralis* strain SW-45^T
Temperature range for growth (°C)	10–45	10–50	10–45	13–50	4–46
Temperature optimum for growth (°C)	30	35	32	30	30–37
pH range for growth	< 6.5–11.4	< 6.5–10.8	6.0–9.5 (9.4)[a]	5–10 (9.4)[a]	> 4.5-(9.4)[a]
pH optimum for growth	9	8.5	7.0–7.5	7.3	7.0–8.5
NaCl concentration range for growth (%)	0–21	0–21	0.5–20.5	0–20	0.5–18
NaCl concentration optimum for growth (%)	2.5–3.5	2.5–3.5	3.5	5	2–7
Substrates					
Starch	+	+	–	ND[b]	–
Glucose and fructose	+	+	–	–	–
Glycerol	+	+	–	–	–
Alanine	+	+	–	+	–
Anaerobic growth	+	+	+	+	–
Electron acceptor					
Nitrate	+	+	+	+	+
Fumarate	+	+	ND	ND	ND
G + C content of genomic DNA (mol%)	57.5	57.5	52.7	55.7	55
Reference	This study	This study	Gauthier et al. (1992)	Huu et al. (1999)	Yoon et al. (2003)

[a]The highest pH limit for growth tested in this study
[b]*ND* Not determined

Ecological implication

Based on the dilution–cultivation tests, *M. alkaliphilus* and its relatives were likely one of the most predominant populations in the potentially viable microbial communities found in the subseafloor serpentine muds (Table 1). The distribution profile of *M. alkaliphilus* members represented a good accordance with the pH profile of the pore water in the subseafloor serpentine mud. The pH value in the deepest subsample providing a positive culture of *M. alkaliphilus* strain D at a depth of 2.95 mbsf (1H2, 140–150) was approximately 10.2, which is within the pH range of growth in *M. alkaliphilus*, but the pH value of the subsamples below 2.95 mbsf exceed 11.7, above the upper limit of pH for the growth (Table 1). The distribution of heterotrophic *M. alkaliphilus* members will be also strongly associated with the distribution of organic carbon sources. The organic carbon content exceeding 0.1 wt% was found only in the shallow zone of the serpentine mud above 3 mbsf (Salisbury et al. 2002; Mottl et al. 2003), which corresponds to the zone in which positive cultures of *M. alkaliphilus* members were recovered. In addition, the bacterial biomass estimation by using PLFA analysis (Mottl et al. 2003) also demonstrated the highest bacterial population at 0.05 mbsf. Thus, the occurrence of a cultivable population of *M. alkaliphilus* members might be strongly associated with the mixing of upwelling alkaline seepage and infiltrating seawater. The concomitant infiltration with the deep seawater probably provides the source of *M. alkaliphilus* members in the subseafloor serpentine mud. This may be one of the possible explanations for the unsuccessful cultivation of microorganisms from the deeper subseafloor serpentine mud below 2.95 mbsf, the pristine alkaline serpentine mud. Finally, the very low in situ temperature (1.7–1.9°C) may be a key environmental constraint to control the activity of *M. alkaliphilus* members as well. The temperature far below the lowest temperature limit for growth (10°C) determined in laboratory may reflect that most of the population of *M. alkaliphilus* members remains viable but dormant in the cold serpentine muds, and that the alkaline pH would allow for the enhanced survival of the potentially alkalitolerant *M. alkaliphilus* members than the other microbial species originally derived from seawater. Otherwise, *M. alkaliphilus* and its relatives may sustain themselves with extremely slow growth at low temperature and alkaline pH on low nutrients.

Description of *M. alkaliphilus* sp. nov.

- *M. alkaliphilus* (al.ka.li'phil.us N.L. n. *alkali*, from Arabic *al-qaliy* the ashes of saltwort; Gr. adj. *philos*, friendly; N.L. adj. *alkaliphilus*, liking alkaline environments).
- Each cell is a motile rod with a polar flagellum, with a mean length of 2.0–5.0 μm and a width of approximately 0.3–0.8 μm.
- Cells occur singly and in pairs.
- Gram negative.
- Facultative anaerobe.
- The temperature range for growth is 10°C to 45–50°C (optimum 30–35°C).
- The pH range for growth is <6.5 to 10.8–11.4 (optimum growth at pH 8.5–9.0).
- The concentration range of NaCl for growth is 0–21% (optimum growth occurs at 2.5–3.5%).
- Heterotrophic growth occurs with molecular oxygen, nitrate, or fumarate as an electron acceptor.
- The following compounds are utilized as the sole energy and carbon source: yeast extract (0.2%), tryptone (0.2%), casein (0.2%), casamino acid (0.2%), starch (0.2%), crude oil (0.5%), corn oil (0.5%), olive oil (0.5%), Tween 20 (0.1%), Tween 80 (0.1%), *n*-pentadecane [(C_{15}) 0.2%], *n*-tridecane [(C_{13}) 0.2%], *n*-undecane [(C_{11}) 0.2%), *n*-decane [(C_{10}) 0.2%], *n*-nonane [(C_9) 0.2%], butane [(C_4) 10% of gas phase], kerosene (0.2%) ethanol (0.2%), glycerol (0.2%), maltose (0.2%), glucose (0.2%), lactose (0.2%), xylose (0.2%), fructose (0.2%), acetate (0.2%), lactate (0.2%), pyruvate (0.2%), alanine (0.2%), glutamate (0.2%), glutamine (0.2%), and proline (0.2%).
- The G+C content of DNA is 57.5 ± 0.2 mol% (by HPLC).
- The organisms were isolated from alkaline serpentine mud at the South Chamorro Seamount in the Mariana Forearc.
- The type strain is *M. alkaliphilus* strain ODP1200D-1.5^T, JCM 12291^T, Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Japan, and ATCC BAA-889^T, American Type Culture Collection, Manassas, Va., USA.

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