Marinitoga camini gen. nov., sp. nov., a rod-shaped bacterium belonging to the order *Thermotogales*, isolated from a deep-sea hydrothermal vent

Nathalie Wery,† Françoise Lesongeur, Patricia Pignet, Véronique Derennes,‡ Marie-Anne Cambon-Bonavita, Anne Godfroy and Georges Barbier

Author for correspondence: Nathalie Wery. Tel: +44 1225 826407. Fax: +44 1225 826779. e-mail: bssnaw@bath.ac.uk

Laboratoire de Caractérisation des Micro-organismes Marins, Département de Valorisation des Produits, IFREMER, Centre de Brest, BP 70, 29280 Plouzané, France

A thermophilic, anaerobic, chemo-organotrophic sulfur-reducing bacterium, designated MV1075^T, was isolated from a deep-sea hydrothermal chimney sample collected on the Mid-Atlantic Ridge. Cells were rod-shaped with a sheath-like outer structure, motile with polar flagella and stained Gram-negative. They appeared singly, in pairs or in short chains. The temperature range for growth was 25–65 °C, with an optimum at 55 °C. Growth was observed from pH 5 to pH 9, and the optimum pH was around 7. The salinity range for growth was 15–70 g sea salt l^{-1} (corresponding to 10–45 g NaCl l^{-1}), with an optimum at 30 g l^{-1} (20 g NaCl l^{-1}). The isolate was able to grow on a broad spectrum of carbohydrates or complex proteinaceous substrates. Sulfur was not necessary for growth. Growth was inhibited by H$_2$, but, in presence of sulfur, this inhibition was removed and H$_2$S was produced. The G+C content of the genomic DNA was 29 mol%. Phylogenetic analyses of the 16S rRNA gene located the strain within the order *Thermotogales*, in the domain *Bacteria*. On the basis of 16S rDNA sequence comparisons, in combination with morphological and physiological characteristics, it is proposed that the isolate should be described as a novel species of a new genus, *Marinitoga* gen. nov., of which *Marinitoga camini* sp. nov. is the type species. The type strain is MV1075^T (= CNCM I-2413^T = DSM 13578^T).

INTRODUCTION

The order *Thermotogales* was first described as an order containing hyperthermophilic and extremely thermophilic bacteria (Huber & Stetter, 1992). This order was then expanded by the discovery of new species, extending both the temperature and salinity ranges for growth. Currently, it contains thermophilic

bacteria growing at up to 90 °C, such as *Thermotoga maritima*, as well as moderate thermophiles such as *Geotoga subterranea*, which grows at an optimum temperature of 45 °C. The order *Thermotogales* is represented by five genera: *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Geotoga* and *Petrotoga*. The strains have various origins: oil-producing wells or oil reservoirs (Davey *et al.*, 1993; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a; Lien *et al.*, 1998), geothermally heated continental waters (Patel *et al.*, 1985; Windberger *et al.*, 1989; Huber *et al.*, 1990; Andrews & Patel, 1996; Friedrich & Antranikian, 1996) and geothermally heated marine waters (Huber *et al.*, 1986, 1989; Jannasch *et al.*, 1988; Antoine *et al.*, 1997). Duckworth *et al.* (1996) have also isolated an an-

aerobic thermophile from a soda lake, and have proposed that it could be assigned to a new genus within the order *Thermotogales* because of its 16S rDNA sequence and its alkaliphilic phenotype. Among the described species of the *Thermotogales*, only *Thermosipho melanesiensis* was isolated from deep-sea hydrothermal vents. It was isolated from the gills of a deep-sea hydrothermal vent mussel from the Lau Basin (south-western Pacific Ocean) (Antoine *et al.*, 1997). Other anaerobic, heterotrophic sulfur-reducing strains were isolated from deep-sea vents and assigned to the genus *Thermotoga* (Marteinsson *et al.*, 1997).

Members of the order *Thermotogales* are studied for their industrial applications in the production and use of thermophilic enzymes. Xylanolytic enzymes or amylases are located inside the 'toga' of some *Thermotoga* species and *Petrotoga mobilis* (Sunna & Antranikian, 1996; Lien *et al.*, 1998). Thermostable cellulases, β-glucosidases, α-galactosidase, β-mannanase and β-mannosidase were also found in some *Thermotoga* species (Liebl *et al.*, 1996; Duffaud *et al.*, 1997; Zverlov *et al.*, 1997). *Fervidobacterium pennivorans* was isolated for its ability to degrade native feather meal, and a thermostable keratinase was purified (Friedrich & Antranikian, 1996). Amylolytic and pullulanolytic activities were also detected in this strain (Canganella *et al.*, 1994; Bertoldo *et al.*, 1999).

In September 1997, samples were collected from deep-sea vent fields of the Azores Triple Junction, on the Mid-Atlantic Ridge. In this paper, we report the isolation, from one of these samples, and characterization of a novel rod-shaped bacterium belonging to the order *Thermotogales*.

METHODS

Collection of samples. Samples were collected by the man-operated submersible *Nautile* during the MARVEL cruise, on the following deep-sea vent fields on the Mid-Atlantic Ridge: Lucky Strike (32° 16′ W, 37° 17′ N; −1600 to −1700 m) and Menez–Gwen (31° 31′ W, 37° 51′ N; −800 to −1000 m). Samples of chimney wall, rock debris and mussels were collected using an insulated box filled with sterilized water. Samples were sheltered in an anaerobic chamber and stored at 4 °C in serum vials filled with sterile sea water under anaerobic conditions (N_2 headspace gas and 0·5 g sodium sulfide l^{-1}).

Growth conditions. Most of the experiments were performed on the enrichment medium YPCS, but the media YGS and YTS were also used. YPCS medium contained the following (l^{-1}): 0·5 g yeast extract (Difco), 1 g peptone (Difco), 5 g cellobiose (Sigma), 10 g sulfur (Prolabo), 30 g sea salt (Sigma), 6·05 g PIPES buffer (Sigma) and 1 mg resazurin (Sigma). YGS medium contained the following (l^{-1}): 1 g yeast extract, 2·5 g glucose (Sigma), 10 g sulfur, 30 g sea salt, 6·05 g PIPES buffer and 1 mg resazurin. In YTS medium, the glucose was replaced by 2 g tryptone l^{-1}. YPC and YG media were YPCS and YGS media, respectively, but without the sulfur. YGC medium was YGS medium in which the sulfur was replaced by 10 g cystine l^{-1}. The pH was adjusted to 7·5 in YPCS medium and to 7 in the other media. The

media containing sulfur were sterilized at 100 °C for 30 min on two successive days. When sulfur was omitted, sterilization was obtained after 20 min at 121 °C. Sterile media were transferred into an anaerobic chamber containing $N_2/H_2/CO_2$ (90 : 5 : 5), reduced by the addition of sodium sulfide to a final concentration of 0·5 g l^{-1}, and then distributed into Hungate tubes (6 ml in a total volume of 20 ml) or in serum vials with butyl rubber stoppers (20 ml in a total volume of 60 ml). Unless indicated otherwise, cultures were incubated at atmospheric pressure under the anaerobic-chamber gas mixture, at 50 °C. All growth experiments were carried out in triplicate, except for analyses of organic acids and amino acids, which were performed in duplicate.

Enrichment and purification procedures. The enrichment procedure was performed using 1 ml of the sheltered sample from the hydrothermal chimney inoculated into YPCS medium incubated at 65 °C at atmospheric pressure under the anaerobic-chamber gas mixture. The presence of cells was revealed by microscopic observation. Cultures were purified by streaking enrichment samples onto the same medium solidified with Gelrite (Scott Laboratories) and incubating them at 65 °C in anaerobic jars (Godfroy *et al.*, 1997).

Storage. Cultures from the exponential growth phase were stored at 4 °C. For long-term storage, cultures from the exponential growth phase were stored anaerobically in cryotubes at −20 and −70 °C, after the addition of DMSO (5%, v/v; Sigma).

Observation of the culture and determination of cell numbers. Enumeration of cells was performed by means of direct cell counting using a Thoma chamber (depth, 0·02 mm). Alternatively, cells were stained with 4′,6′-diamidino-2-phenylindole added directly to the samples at a final concentration of 1 mg l^{-1}. Cells were observed with an Olympus model BH-2 microscope.

Morphology. Gram staining was realized using the Bacto 3-step Gram stain Set-S (Difco). Endospores were examined by phase-contrast microscopy. For scanning electron microscopy, cells were fixed with 10% (v/v) formalin for 1 h and then harvested by centrifugation for 20 min at 2000 *g*. Pellets were resuspended in 23 g NaCl l^{-1}, displayed on 0·22 µm filters (Nucleopore) and dried overnight at room temperature. Samples were then coated with gold (SCD040; Balzers) and examined with an XL 30 LaB6 scanning electron microscope (Philips). For transmission electron microscopy, cells were harvested at the end of exponential phase. The pellet was resuspended and fixed for 1 h at room temperature in cacodylate buffer [0·4 M cacodylate, pH 7·4; 5·5% (w/v) NaCl; 8% (v/v) glutaraldehyde; 2 : 1 : 1] and then post-fixed for 1 h at 4 °C in another cacodylate buffer [0·4 M cacodylate, pH 7·4; 10% (w/v) NaCl; 2% (w/v) OsO_4; 1 : 1 : 2]. After dehydration by immersion in different solutions with increasing ethanol concentrations, cells were embedded in Spurr's resin, thin-sectioned, contrasted with 1% (w/v) uranyl acetate and 1% (w/v) lead citrate (Bio-Rad) and then examined with an EM201 transmission electron microscope (Philips).

Analyses of amino acids, organic acids and aromatic acids. Each sample was centrifuged for 30 min at 8000 *g* to eliminate cell debris. Half of the supernatant was transferred to an ultrafree-CL-PLGC 10000 NMWL unit (Millipore) and centrifuged at 4500 *g* for several hours; 20 µl of the filtrate was mixed with 30 µl of ethanol/water/triethylamine (2 : 2 : 1) and vacuum-dried. A derivatizing solution (20 µl)

containing ethanol/water/triethylamine/phenylisothiocya-nate (7:1:1:1) was added and, after 10 min at room temperature, the sample was vacuum-dried. Analysis of amino acids by HPLC (Alliance 2690; Waters) was then performed using the conditions and products of the Waters PicoTag method (WAT007360; Waters). In the other part of the supernatant, proteins were precipitated with a 2 % (w/v) solution of 5-sulfo-salicylic acid overnight at 4 °C and eliminated by centrifugation at 8000 g for 10 min. The supernatant was then used for HPLC analyses of glucose, aromatic acids and linear organic acids. For glucose and linear organic acid analysis, 20 µl supernatant was eluted on an H$^+$ exclusion column (polyspher OAKC 1.51270; Merck) at 60 °C with 9 mM H$_2$SO$_4$ at a flow rate of 0·35 ml min^{-1} and components were detected using a differential refrac-tometer (refractometer 410; Waters). For analysis of arom-atic acids, 20 µl supernatant was eluted on an H$^+$ exclusion column (column 28352; Chrompack) at 65 °C using 4·5 mM H$_2$SO$_4$ at a flow rate of 0·5 ml min^{-1} and components were detected by absorbance at 210 nm (UV detector 486; Waters).

Determination of growth parameters. To determine the optimum temperature for growth, cells were grown in Hungate tubes containing YPCS medium. Temperatures were maintained with aluminium heating blocks (Bioblock) and monitored with temperature probes placed in control tubes. To determine the effect of pH on growth, YPCS medium was modified by using the following buffers (Sigma), each at a concentration of 20 mM: for pH 2, 3 and 4, no buffer; for pH 5, 5·5 and 6, MES buffer; for pH 6·5 and 7, PIPES buffer; for pH 7·5 and 8, HEPES buffer; for pH 8·5 and 9, AMPSO buffer; for pH 10, no buffer. In the anaerobic chamber, sodium sulfide was added and the pH was controlled at room temperature and adjusted, if necessary, with 0·1 M HCl and 0·1 M NaOH. To determine the re-quirement for salt, YPCS medium was prepared with different concentrations of sea salt. The effects of tem-perature, pH and salinity were determined by measuring the cellular concentration when all of the different cultures were in the exponential phase of growth.

Determination of growth requirements. The ability of isolate MV1075^T to use various carbon sources was tested in medium containing the following (l^{-1}): 30 g sea salt, 10 ml mineral solution (Balch *et al.*, 1979), 10 ml vitamin solution (Balch *et al.*, 1979), 5 ml KH$_2$PO$_4$ (7%, w/v), 6·05 g PIPES buffer and the carbon source. Carbohydrates were tested at 5 g l^{-1}, alcohols at 5 ml l^{-1} and acids at 2 g l^{-1}. Sugar and vitamin solutions were sterilized by filtration and added just before inoculation. For monosaccharides, polysaccharides, acids and alcohols, the medium was supplemented with 0·1 g yeast extract l^{-1} as the nitrogen source. The monosaccharides tested were: D(+)-sucrose, D(+)-glucose, D(−)-fructose, maltose, D(+)-cellobiose, D(+)-mannose, L(−)-sorbose, D(−)-arabinose, D(+)-galactose, D(+)-xylose, lactose, D(−)-ribose. The following polysaccharides were tested: starch, cellulose, carboxymethylcellulose, chitin, pectin, dextran and xylan. The following acids and alcohols were tested: glycerol, methanol, ethanol, formate, acetate, pro-pionate, pyruvate, succinate and lactate. The other carbon sources, tested at 5 g l^{-1}, were as follows: yeast extract, brain–heart infusion (BHI), gluten, peptone, tryptone, beef extract, casein, collagen, keratin, elastin, albumin and Casamino acids. A solution of 20 amino acids, each at 0·1 g l^{-1}, and combinations of 1 g peptone/0·5 g yeast extract l^{-1} and 1 g tryptone/0·5 g yeast extract l^{-1} were also tested as carbon sources. Nitrogen sources were tested with 5 g

glucose l^{-1} as the carbon source. Peptone, yeast extract, tryptone and BHI were tested at 0·1 g l^{-1}; sodium nitrate, urea and NH$_4$Cl were tested at 10 mM. Tests were performed in serum vials. To avoid growth on the substrates carried over with the inoculum, positive cultures were transferred once (10% inoculum). The final concentration of cells was determined by direct counting and compared with the concentration in the control without the carbon or nitrogen source tested. Autotrophic growth with H$_2$/CO$_2$ (80:20) gas as the sole carbon and energy source was also tested. To compare growth on sugar with growth on proteinaceous substrates, amino, organic and aromatic acids were analysed during cultivation on YGS and YTS media. The concen-trations of amino acids and organic acids were compared with uninoculated medium. H$_2$, CO$_2$ and H$_2$S were detected using an MTI micro-gas chromatograph equipped with a thermal conductivity detector. A molecular sieve with argon as the carrier gas and a temperature of 30 °C were used to detect H$_2$. H$_2$S and CO$_2$ were determined using a paraplotU column, at 100 °C, with helium as the gas carrier.

Effects of possible electron acceptors. The ability of the isolate to grow in the presence of different electron acceptors was tested on YPC medium. Sulfur and cystine were tested at 10 g l^{-1}, polysulfides at 10 mM (Blumentals *et al.*, 1990) and thiosulfate at 20 mM. Tests were performed in Hungate tubes. Complementary analyses were performed to deter-mine the effects of sulfur and cystine: the consumption and production of organic acids and amino acids were de-termined on YG, YGC and YGS media. The variations in concentration of amino acids and organic acids were compared with the concentrations in uninoculated media.

Influence of the gas headspace. To test the influence of the gas composition of the headspace, cells were cultured on YG and YGS media with various headspace gases, as follows: N$_2$, N$_2$/CO$_2$ (80:20), H$_2$/CO$_2$ (80:20) and N$_2$/H$_2$/CO$_2$ (90:5:5). The production of H$_2$S was examined using lead acetate paper (Whatman).

Susceptibility to antibiotics. Susceptibility to antibiotics at 50 µg ml^{-1} was investigated. Concentrated solutions of antibiotics were added to YPCS medium just before in-oculation. When the antibiotic was diluted in ethanol (chloramphenicol) or DMSO (rifampicin), the same volume of solvent was added to control cultures. When the strain was insensitive, the concentration of antibiotic was increased to 100 µg ml^{-1}; when it was sensitive, susceptibility at 25 µg m l^{-1} was determined.

DNA extraction and purification. Genomic DNA was extracted by the following procedure. Aliquots (200 ml each) of late-exponential-phase culture were harvested at 10 000 g for 30 min, washed in 23 g NaCl l^{-1} and resuspended in 5 ml lysis buffer (100 mM Tris/HCl, 100 mM NaCl, 50 mM EDTA; pH 8). SDS (1%, w/v) was added; cell lysis was immediate. One phenol/chloroform/isoamyl alcohol (24:24:1) extraction was performed and the aqueous phase was treated with RNase A (30 µg l^{-1} final) for 1 h at 60 °C. A second phenol/chloroform/isoamyl alcohol (24:24:1) ex-traction and a chloroform extraction were then performed. The DNA was precipitated by adding 2 vols absolute ethanol at −20 °C and resuspended in TE buffer (10 mM Tris/HCl, 2 mM EDTA; pH 7·4) or in milliQ-sterilized water. The concentration and purity of the genomic DNA obtained were estimated using a GenQuant II spectrophotometer (Pharmacia) at 260 nm. The quality of the extraction was checked on a 0·8% (w/v) agarose gel containing 0·8 µg ethidium bromide ml^{-1}.

DNA base composition. The DNA was purified by CsCl gradient centrifugation. After precipitation, the DNA was resuspended in TE buffer containing 1·075 g CsCl ml⁻¹ and 10 mg ethidium bromide ml⁻¹ and then centrifuged in a preparative ultracentrifuge for 15 h at 65000 r.p.m. (rotor 70.1 Ti, model CO-L70K; Beckman). The DNA band was removed with a syringe. The ethidium bromide was then extracted using 2-propanol saturated with CsCl. Finally, the solution was dialysed overnight against 1 litre TE buffer. The G + C content of the DNA was determined by thermal denaturation (Marmur & Doty, 1962) under the conditions reported by Raguénès *et al.* (1997). A calibration curve was obtained by using ultrapure DNAs from *Escherichia coli* strain B (50 mol% G + C), *Clostridium perfringens* (26·5 mol% G + C) and *Micrococcus luteus* (72 mol% G + C) as standards (Sigma).

Amplification of the 16S rDNA. The 16S rDNA was selectively amplified from purified genomic DNA by using a PCR with oligonucleotide primers designed to anneal to conserved positions in the 3′- and 5′-regions of the 16S rRNA genes. The bacterial forward primer SAdir (5′-AGAGTT-TGATCATGGCTCAGA-3′) corresponded to positions 8–28 of *E. coli* 16S rRNA and the bacterial reverse primer S17rev (5′-GTTACCTTGTTACGACTT-3′) corresponded to the complement of positions 1493–1509 of *E. coli* 16S rRNA. The following components were combined in a total volume of 50 μl: 100 ng template DNA, 5 μl 10 × PCR buffer [100 mM Tris/HCl, pH 9·0, 50 mM KCl, 1·5 mM MgCl₂, 0·1% (w/v) Triton X-100, 0·2 ml BSA or gelatin ml⁻¹; Appligene-Oncor], 2·5 U *Taq* DNA polymerase (Appligene-Oncor), 200 μM each dNTP, 2 mM forward primer and 2 mM reverse primer. The complete reaction mixture was overlaid with mineral oil (Sigma) and incubated in a 96-gradient thermocycler (Stratagene). The following temperature profile was used for PCR: 94 °C for 3 min and then 30 cycles of 94 °C for 1 min, 50 °C for 90 s and 72 °C for 2 min and, finally, an extension step at 72 °C for 6 min. PCR results were analysed by electrophoresis on 0·8% (w/v) agarose/TAE gels (0·04 M Tris/acetate, 0·001 M EDTA) containing 0·8 μg ethidium bromide ml⁻¹. All gel images were realized using a Fluor-S multiImager (Bio-Rad).

16S rDNA sequence analysis. The PCR product was sequenced using the primers described by Raguénès *et al.* (1996). This work was done at Euro Sequence Gene Service (France) with an automatic DNA-analysis system (Applied Biosystems); 1469 positions of the 16S rDNA were determined. The sequence was then compared with others available in GenBank, using the BLAST program (Altschul *et al.*, 1990). A multiple-sequence file, corresponding to positions 33–1356 of *E. coli* 16S rRNA, was obtained by using the MEGALIGN program of the DNASTAR package (Promega). Alignments and similarity levels were obtained by using the CLUSTAL W method with weighted residues (Thompson *et al.*, 1994). Alignment was then performed manually using the multiple sequence alignment editor SEAVIEW. The phylogenetic reconstruction was produced using PHYLO_WIN (Galtier *et al.*, 1996) with the following set-up: the Jukes–Cantor distance matrix and, in succession, the neighbour-joining (Saitou & Nei, 1987), maximum parsimony (Lake, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap values were determined according to Felsenstein (1985). The following 16S rDNA sequences (accession numbers in parentheses) were used for the phylogenetic analysis: *Thermus thermophilus* ATCC 27634ᵀ (TTHB27), *Thermosipho africanus* DSM 5309ᵀ (M83140), *Thermosipho melanesiensis* CIP 104789ᵀ (Z70248), *Thermo-*

toga hypogea DSM 11164ᵀ (U89768), *Thermotoga elfii* DSM 9442ᵀ (X80790), *Thermotoga subterranea* DSM 5069ᵀ (U22664), *Thermotoga maritima* DSM 3109ᵀ (Z11839), '*Thermopallium natronophilum*' (X91822), *Fervidobacterium nodosum* ATCC 35602ᵀ (M59177), *Fervidobacterium islandicum* DSM 5733ᵀ (M59176), *Fervidobacterium gondwanense* ACM 5017ᵀ (Z49117), *Geotoga petraea* ATCC 51226ᵀ (L10658), *Geotoga subterranea* ATCC 51225ᵀ (L10659), *Petrotoga miotherma* ATCC 51224ᵀ (L10657) and *P. mobilis* DSM 10674ᵀ (Y15479).

RESULTS

Isolation

During the MARVEL cruise, YPCS medium was inoculated with pulverulent mineral material from a chimney at the Menez–Gwen hydrothermal site (−980 m) on the Mid-Atlantic Ridge. The enrichment culture, incubated at 65 °C, contained non-motile, coccoid cells, single or in pairs, and also short rod-shaped cells that were either single or in chains. After repeated streaking on solidified YPCS medium, isolated colonies were used to inoculate liquid YPCS medium. The two different morphologies were still observed in these cultures. PCR–RFLP analysis of the 16S rDNA (Antoine *et al.*, 1995) revealed that the rod-shaped cells were in a mixture with a *Thermococcus* species. Streaking on plates and serial dilutions (1:10) at 65 °C in YPCS medium failed to separate the rod-shaped cells from the coccoid cells. The rod-shaped bacteria were purified by subculturing at 40 °C, without sulfur, diluted 1:10 every day for a week. The last subculture was purified by streaking on solidified YPCS medium. The isolate obtained was named MV1075ᵀ.

Morphology

Microscopic observation indicated that cells of isolate MV1075ᵀ were motile rods that stained Gram-negative. Polar flagella were visible by scanning (Fig. 1) and
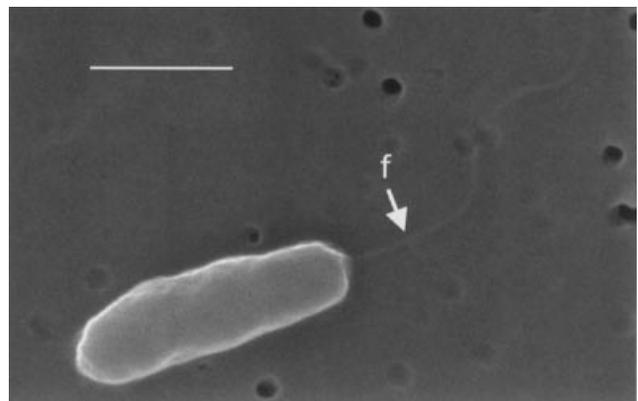

Fig. 1. Scanning electron micrograph of strain MV1075ᵀ, showing the flagellum (f). Bar, 1 μm.
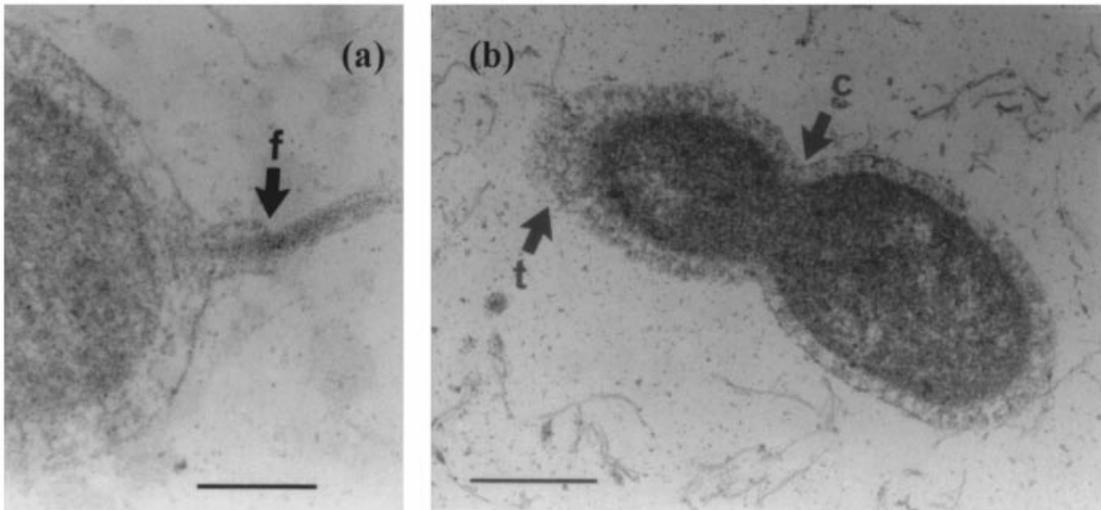
Fig. 2. Transmission electron micrographs of strain MV1075[T]. (a) The flagellum (f) and insertion point are shown. Bar, 0·2 μm. (b) Cells dividing by means of a constriction (c) and surrounded by a sheath-like membrane or 'toga' (t). Bar, 0·5 μm.
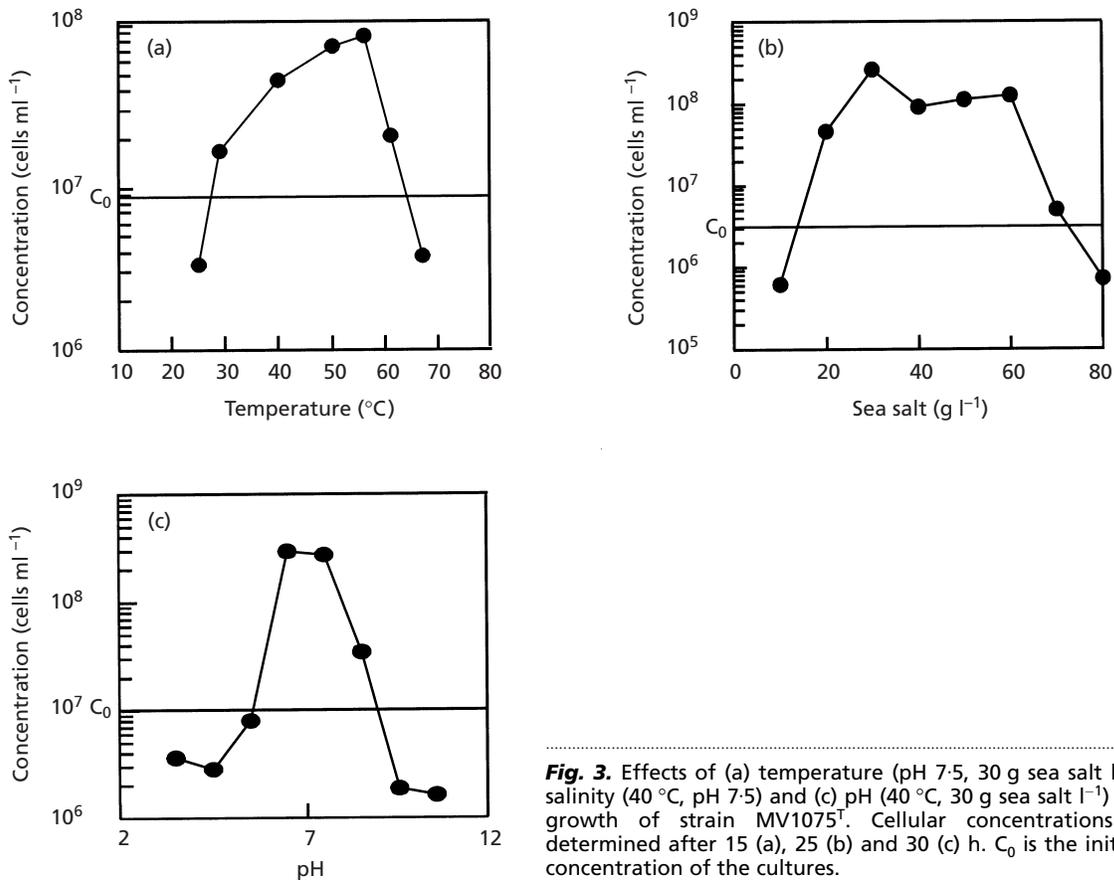

Fig. 3. Effects of (a) temperature (pH 7·5, 30 g sea salt l[−1]), (b) salinity (40 °C, pH 7·5) and (c) pH (40 °C, 30 g sea salt l[−1]) on the growth of strain MV1075[T]. Cellular concentrations were determined after 15 (a), 25 (b) and 30 (c) h. C_0 is the initial cell concentration of the cultures.

transmission electron microscopy (Fig. 2a). Cells were surrounded by a 'toga', an outer sheath-like structure specific to members of the order *Thermotogales* (Fig. 2b). This envelope was visible in all phases of growth by phase-contrast microscopy. The cells appeared singly, in pairs or in chains within the sheath. The

smaller single rods moved more rapidly than the longer cells. During the exponential growth phase under optimal conditions in YGS medium, the rods were 2–3 μm long by 0·5–1 μm wide. The cells divided by constriction (Fig. 2b). During the stationary phase of growth, rods became shorter to spherical. Chains of up to 10 cells were occasionally observed. The cells grew longer and the proportion of chains increased under non-optimal conditions. Endospores were not observed in old cultures.

Determination of growth parameters

Isolate MV1075^T grew at 25–65 °C and the optimum temperature for growth was around 55 °C (Fig. 3a). Growth was observed at sea-salt concentrations ranging from 15 to 70 g l^{-1} (corresponding to 10–45 g NaCl l^{-1}), the optimum concentration being around 30 g l^{-1} (20 g NaCl l^{-1}) (Fig. 3b). No growth was detected at salt concentrations of 10 or 80 g l^{-1}. At high salinity, i.e. 60–70 g sea salt l^{-1}, changes in morphology were visible: motile, short, rod-shaped cells, single or in short chains, became longer and thinner and formed into non-motile, long chains. Growth was observed at pH 5–9, the optimum pH being around 7 (Fig. 3c).

Growth requirements and metabolic products

Strain MV1075^T is an obligate chemo-organotroph. No growth was detected on mineral media complemented with vitamins, sulfur with an H$_2$/CO$_2$ (80:20) headspace. Growth was observed both on proteinaceous substrates and on carbohydrates. BHI or gluten successfully supported growth, final concentrations being between 5×10^7 and 5×10^8 cells ml^{-1}. Final concentrations from 2×10^7 to 5×10^7 cells ml^{-1} were reached with yeast extract. Peptone and tryptone were also used at 1 g l^{-1}, but only in the presence of 0·5 g yeast extract l^{-1}. The corresponding final concentrations were greater than 10^8 cells ml^{-1}. The other proteinaceous carbon sources tested did not support growth. When 0·1 g yeast extract l^{-1} was added, growth was observed on D(+)-glucose, D(−)-fructose, maltose, D(+)-cellobiose and D(+)-sucrose, final cell concentrations being from 5×10^7 to 10^8 cells ml^{-1} for D(+)-sucrose and 2×10^7–5×10^7 cells ml^{-1} for the other sugars. Isolate MV1075^T also used polysaccharides such as starch, cellulose, carboxymethylcellulose, pectin and chitin, but not xylan or dextran. Among the acids and alcohols tested, significant growth was detected only on pyruvate. Yeast extract was the only nitrogen source tested that supported growth in combination with glucose. The final cell concentrations reached in the controls without added carbon or nitrogen sources ranged from 5×10^6 to 2×10^7 cells ml^{-1}. Maximum growth rates and cell yields were achieved in YGS medium. Under optimal conditions (pH 7, 55 °C, 20 g NaCl l^{-1}), the final cell density was over 2×10^8 cells ml^{-1} after 12 h and 5×10^8 cells ml^{-1} after 48 h. The doubling time was around
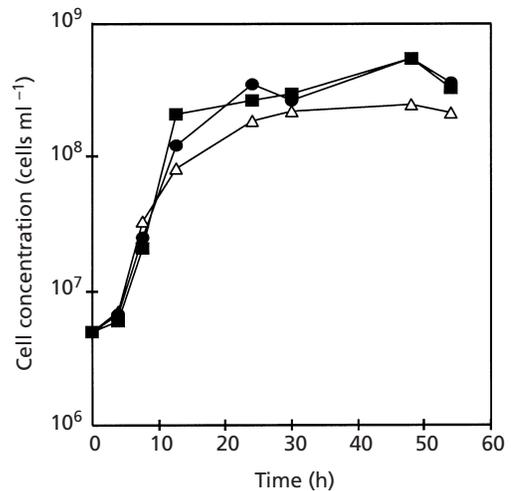

Fig. 4. Effects of sulfur (■) and cystine (●) on the cell concentration during growth of MV1075^T on YGS medium. The control curve was obtained in medium without sulfur or cystine (△).

1·7 h. During growth, the concentrations of arginine, isoleucine, leucine and phenylalanine decreased in YGS medium, whereas the concentrations of alanine, proline and lysine increased. Glucose was consumed and acetate, lactate, isovalerate and isobutyrate were produced. H$_2$, CO$_2$ and H$_2$S (absent in the uninoculated control) were detected. Hydroxyphenylacetate and phenylacetate were produced at concentrations of less than 0·01 mM. When glucose (medium YGS) was replaced by tryptone (medium YTS), indole-3-acetate was produced and the concentrations of hydroxyphenylacetate and phenylacetate increased 3-fold. Less acetate and lactate but more isovalerate and isobutyrate were produced. The proline concentration after 48 h in YTS medium was 7-fold higher than that in YGS medium. In cultures on YGS and YTS media, the concentrations of arginine decreased dramatically.

Effect of electron acceptors and influence of the gas headspace

The addition of thiosulfate or polysulfides to the culture medium did not stimulate growth. A small increase in the final cell concentration was obtained with L-cystine and sulfur (Fig. 4). After 24 h in the presence of elemental sulfur, 2·5-fold more acetate and 5·5-fold more alanine were produced and 4-fold more glucose and 8-fold more leucine were consumed. In the presence of L-cystine, 3-fold more acetate and 10-fold more alanine were produced and 6·5-fold more glucose and 6·5-fold more leucine were consumed. Of the amino acids and organic acids, the concentrations of alanine, leucine and acetate were those most affected by growth of the strain. Growth was not affected when the gas headspace was replaced by N$_2$ or N$_2$/CO$_2$
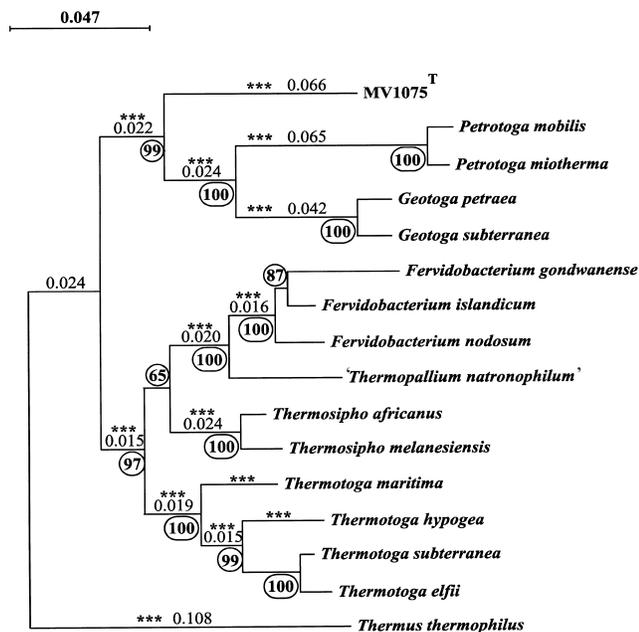
Fig. 5. Phylogenetic position of strain MV1075[T] within the order *Thermotogales*; 1017 sites were used for the phylogenetic analysis. Alignment was performed with representatives of the genera *Fervidobacterium*, *Thermotoga*, *Thermosipho*, *Geotoga* and *Petrotoga*. *Thermus thermophilus* was chosen as the outgroup. The topology shown is an unrooted tree obtained with a neighbour-joining algorithm (Jukes–Cantor corrections) established using PHYLO_WIN and performed manually with SEAVIEW. Bootstrap percentages are encircled and evolutionary distances are presented on the branches. Branches labelled with triple asterisks have been confirmed by maximum-likelihood and parsimony methods. Bar, 4·7 nucleotide substitutions per 100 nucleotides.

(80:20), but it was strongly inhibited by H_2/CO_2 (80:20). Inhibition by H_2 was partially eliminated by the addition of sulfur, but the growth rate remained lower than those obtained with the other headspace gases: after 8·5 h, the concentration was $2·7 \times 10^7$ cells ml^{-1} with H_2/CO_2 (80:20) and 7×10^7–$1·5 \times 10^8$ with the other headspace gases. In all cases, when elemental sulfur was present in the culture medium, growth of strain MV1075[T] was accompanied by the production of H_2S, which was not detected in the uninoculated control incubated in the same conditions.

Susceptibility to antibiotics

Growth of isolate MV1075[T] was inhibited by the addition of streptomycin, vancomycin, chloramphenicol, rifampicin and penicillin G (at 25 µg ml^{-1} in each case). The strain was not sensitive to kanamycin at 100 µg ml^{-1}.

DNA base composition

The G+C content of the DNA of isolate MV1075[T] was 29 ± 1 mol%.

16S rDNA sequence analysis

Comparisons (using BLAST) with 16S rDNA sequences available in GenBank revealed that isolate MV1075[T] belonged to the order *Thermotogales*. Consequently, the 16S rDNA sequences of strain MV1075[T] and 14 representatives of the order *Thermotogales* were compared. The sequence of MV1075[T] grouped with the *Geotoga/Petrotoga* lineage with all three algorithms used (neighbour-joining, maximum-parsimony and maximum-likelihood), having corresponding bootstrap values of 99 or 100% (Fig. 5). The level of 16S rDNA sequence similarity between strain MV1075[T] and the other strains was less than 83% in all cases. The highest 16S rDNA sequence similarity, 82%, was to *G. petraea*, *G. subterranea* and *Thermotoga maritima*.

DISCUSSION

The novel, marine, thermophilic strain MV1075[T] belongs to the bacterial domain according to its sensitivity to antibiotics and its 16S rDNA sequence. The major physiological traits of isolate MV1075[T] are consistent with assignment to the order *Thermotogales*, which includes other anaerobic, thermophilic bacteria. Transmission electron microscopy has shown the presence of an external sheath surrounding the cells, which is a morphological characteristic of the members of this order. The strain is Gram-negative, chemo-organotrophic and able to ferment several sugars and growth is inhibited by H_2 in the absence of sulfur. Isolate MV1075[T] produces L-alanine from glucose fermentation, which has been proposed by Ravot *et al.* (1996) as an ancestral metabolism shared by the orders *Thermotogales* and *Thermococcales*. Thiosulfate and sulfur were both reported to improve the growth rates and cell yields of all members of the *Thermotogales* tested: *F. islandicum*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Thermotoga elfii* (Ravot *et al.*, 1995b), *Thermotoga subterranea* (Jeanthon *et al.*, 1995), *P. mobilis*, *P. miotherma* (Lien *et al.*, 1998) and *Thermosipho melanesiensis* (Antoine *et al.*, 1997). In the case of strain MV1075[T], neither sulfur nor thiosulfate had a major stimulatory effect on growth rates and final cell concentrations. Indeed, the most prominent effect of sulfur was on amino acid and organic acid production. The presence of thiosulfate, for some members of the *Thermotogales*, results in increased production of acetate and decreased production of alanine (Ravot *et al.*, 1996). This metabolic shift, which is linked to the effect of the electron acceptor on H_2 concentration, also occurred in the presence of sulfur, but not with thiosulfate, for *Thermosipho melanesiensis* (isolated from deep-sea hydrothermal vents). This was not observed with isolate MV1075[T], since, in the presence of sulfur, more alanine – but also more acetate – was produced. Of the genera within the order *Thermotogales*, MV1075[T] is closest to the genera *Petrotoga* and *Geotoga*. MV1075[T] and the species of *Petrotoga* and *Geotoga* are moderate thermophiles with optimum temperatures between 45

and 60 °C. The other species of the *Thermotogales* grow optimally at temperatures above 65 °C. Furthermore, phylogenetic analysis based on 16S rDNA showed a stronger affiliation to the species of *Geotoga* and *Petrotoga* than to other species. Unlike the growth of species isolated from oil wells, such as *P. mobilis* or *P. miotherma*, growth of MV1075[T] is not stimulated by thiosulfate. The effect of thiosulfate on the growth of *Geotoga* spp. has never been reported. Unlike the species of *Petrotoga* and *Geotoga*, which are described as fermentative bacteria able to grow on various polysaccharides, isolate MV1075[T] is capable of growth on both sugars and proteinaceous substrates. The salinity range for growth of species belonging to the genera *Petrotoga* and *Geotoga* is much wider (0·5–9 %, w/v, NaCl) than the salinity range of MV1075[T] (1–4·5 %, w/v, NaCl). This can be correlated with their respective origins: brines from oilfields for *Geotoga* and *Petrotoga* species, deep-sea hydrothermal vents for MV1075[T]. MV1075[T] is well adapted to a salinity of 2 % (w/v) NaCl because of its marine origin. Unlike that of isolate MV1075[T], the generation times of the *Geotoga* and *Petrotoga* species are long: 7 h for *P. miotherma* and more than 10 h for the other species. Because of the evolutionary distance between strain MV1075[T] and the species of the genera *Geotoga* and *Petrotoga*, its shorter generation time, its deep-marine origin and the physiological differences, we propose that MV1075[T] should be assigned to a new genus within the order *Thermotogales*.

Description of *Marinitoga* Wery, Cambon-Bonavita, Godfroy and Barbier gen. nov.

Marinitoga (Ma.ri.ni.to′ga. L. n. *marinus* of the sea; L. fem. n. *toga* Roman outer garment; N.L. fem. n. *Marinitoga* a marine toga, referring to the marine isolation of the organism and the presence of a 'toga'-like sheath).

Cells are rod-shaped with a sheath-like outer structure, motile with polar flagella and stain Gram-negative. Cell division occurs by constriction. Moderate thermophile, adapted to the pH and salinity of ocean. Anaerobic, chemo-organotrophic and able to ferment a broad spectrum of carbohydrates and proteinaceous substrates. Sulfur is not essential for growth. The presence of sulfur prevents inhibition by H_2, and H_2S is produced. The G+C content is 29 mol%. 16S rDNA sequence comparisons locate *Marinitoga* in the bacterial domain, within the order *Thermotogales*, close to the *Geotoga*/*Petrotoga* lineage. The type species is *Marinitoga camini*.

Description of *Marinitoga camini* Wery, Cambon-Bonavita, Godfroy and Barbier sp. nov.

Marinitoga camini (ca′mi.ni. L. gen. n. *camini* of a chimney, relating to its isolation from a hydrothermal chimney).

Cells are rod-shaped with a sheath-like outer structure, motile with polar flagella and stain Gram-negative.

Under optimal conditions, cells appear as short rods (2–3 μm long by 0·5–1 μm wide), singly, in pairs or in short chains (of fewer than five cells). Spherical bodies are formed in the stationary phase. Grows at 25–65 °C (optimum at 55 °C), at pH 5–9 (optimum at pH 7) and in 15–70 g sea salt l[-1], corresponding to 10–45 g NaCl l[-1] (optimum at 30 g sea salt l[-1], corresponding to 20 g NaCl l[-1]). The doubling time under optimal conditions is 1·7 h and the maximum cell yield is 2×10^8 cells ml^{-1}. Anaerobic. Chemo-organotrophic. Able to ferment gluten, BHI, peptone, tryptone, sucrose, glucose, fructose, maltose and cellobiose in the presence of yeast extract. CO_2, H_2, acetate, isovalerate and iso-butyrate are formed during glucose fermentation. The G+C content is 29 ± 1 mol%. The 16S rDNA similarity to *Geotoga petraea* and *Geotoga subterranea* is 82%. Isolated from chimney rocks collected from the Menez–Gwen hydrothermal site on the Mid-Atlantic Ridge (31° 31′ W, 37° 51′ N; depth 980 m). The type strain is MV1075[T] (= CNCM I-2413[T] = DSM 13578[T]).

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