Thermosipho melanesiensis sp. nov., a New Thermophilic Anaerobic Bacterium Belonging to the Order *Thermotogales*, Isolated from Deep-Sea Hydrothermal Vents in the Southwestern Pacific Ocean

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A new thermophilic, anaerobic rod-shaped bacterium, strain BI429^T was isolated from the gills of a deep-sea vent hydrothermal mussel, *Bathymodiolus brevior*, from the Lau Basin (Southwestern Pacific Ocean). Phenotypically, this isolate exhibited characteristics similar to those described for members of the order *Thermotogales*. This organism was identified as a member of the genus *Thermosipho* on the basis of the presence of the typical outer sheath-like structure (toga), its 16S rRNA sequence, and its ability to grow on carbohydrates (sucrose, starch, glucose, maltose, lactose, cellobiose, and galactose). The cells of this organism were gram negative and rod shaped and generally occurred singly or in pairs, rarely occurring as chains with a maximum of five rods. At the optimum temperature for growth (70°C), optimum pH (6.5), and optimum salinity (30 g of NaCl per liter), the doubling time was 100 min. In spite of the high percentage of similarity of its 16S rRNA sequence with that of *Thermosipho africanus* (98.6%), the weak level of DNA-DNA reassociation with this strain (2%) and particular physiological characteristics allowed us to differentiate this new organism from the sole species of the genus *Thermosipho* previously described (*T. africanus*). On the basis of these observations, we propose that the new organism should be described as a new species, *Thermosipho melanesiensis*. The type strain of *T. melanesiensis* is BI429.

Members of the order *Thermotogales* thrive within extreme geological environments. They occur in shallow marine hydrothermal systems as well as in continental springs and in petroleum reservoirs. Up to now the *Thermotogales* have been represented by five genera. The genus *Thermotoga* is represented by the species *Thermotoga maritima* (11), found so far only within marine coastal hydrothermal systems; *Thermotoga neapolitana* (15, 37), isolated from both marine and low-salt continental hot springs; *Thermotoga thermarum* (37), obtained exclusively from continental hot springs; and *Thermotoga subterranea* (16) and *Thermotoga elfii* (26), isolated from continental oil reservoirs. The genus *Thermosipho* is represented only by the species *Thermosipho africanus* (12, 28), isolated from a marine coastal hydrothermal system in Djibouti. The genus *Fervidobacterium* is represented by the species *F. nodosum* (22), isolated from a hot spring in New Zealand; *F. islandicum* (13), isolated from an Icelandic hot spring; and *F. gondwanense*, isolated from an artesian basin in Australia (1). The two last genera, *Geotoga* and *Petrogoga*, with the species *G. petraea*, *G. subterranea*, and *P. miortherma* (4), were isolated from brines collected from oil fields in Oklahoma and Texas.

In this work, we describe the isolation and characterization of a new strain of thermophilic bacterium isolated from the gills of a deep-sea vent hydrothermal mussel and present evidence for its phylogenetic position within the *Thermotogales*. Based on phenotypical and phylogenetical analysis, this new strain can be described as a new species of *Thermosipho* that we have named *Thermosipho melanesiensis*.

MATERIALS AND METHODS

Bacterial cultures. Strain BI429 was isolated from the gills of a deep-sea hydrothermal mussel, *Bathymodiolus brevior* (34), collected at the bottom of a black smoker in the Lau Basin (latitude, 22°32'S; longitude, 176°43'W; depth, 1,832 to 1,887 m). *Thermotoga maritima* DSM 3109^T, *Thermotoga neapolitana* DSM 4359^T, *Thermotoga thermarum* DSM 5069^T, *Thermosipho africanus* DSM 5309^T, and *F. islandicum* DSM 5733^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). All the reference strains were routinely cultured by using media and culture conditions recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Isolation and further cultures of strain BI429 were performed on Zobell medium (38), pre-reduced with Na₂S, as described below.

Sample collection and sample source. Various samples of rocks, chimney fragments, hydrothermal fluids, invertebrates, and titanium preimmersed colonization substrates were collected during the 12 dives of the Biolau cruise (12 to 27 May 1989; A.-M. Alayse, chief scientist) on the hydrothermal site of Hine Hina, Southwestern Pacific Ocean (Lau Basin). Dives were operated with the French deep-sea manned submersible *Nautille*, and in situ discrete temperature measurements were made simultaneously with a temperature probe. The site of Hine Hina forms a massive deposit of highly vesiculated and brecciated andesite through which the hydrothermal fluid is released. The fluid temperature is generally low and under 20°C (5, 7).

During the dives, the samples were placed directly into an insulated box with thick walls to minimize the temperature variations during the ascent, but there were no means for maintaining them at the hydrostatic pressure occurring at the bottom of the ocean. On board, immediately upon retrieval, samples were treated according to their nature. Mineral samples were ground in sterile seawater and stored at ambient temperature in glass sealed bottles reduced with 20 ml of an Na₂S (Sigma Chemical Co., St. Louis, Mo.) 2.5% (wt/vol) solution per liter, with resazurine (1 mg/liter) being added as a reduction indicator. Hydrothermal fluids were filtered through sterile 0.22-μm-pore-size filters. Invertebrates were dissected. Filters and parts of dissected animals were stored in liquid nitrogen in microtubes filled with sterile seawater containing a cryopreservative, dimethyl sulfoxide (5%, vol/vol; Sigma Chemical Co.). All the steps mentioned above were performed under sterile conditions at atmospheric pressure.

Enrichment, isolation, and growth conditions. Enrichment cultures for anaerobic thermophilic bacteria were initiated by inoculation of 0.1 ml of ground samples in 10-ml portions of Zobell medium (38). The pH of the medium was adjusted to 7.5 with 1 N NaOH at ambient temperature before sterilization. It was then reduced with Na₂S at a final concentration of 500 mg/liter under N₂:H₂:CO₂ (5:5:95, vol/vol/vol). After inoculation, the tubes were incubated at

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60°C without agitation for one night. Isolation of the strain was conducted by streaking the positive enrichments onto solid medium plates (Zobell medium) hardened with Gelrite (Sigma Chemical Co.) as the gelling agent. After 3 or 4 days of incubation in anaerobic jars, the plates were examined under an N₂:H₂:CO₂ (5:5:95, vol/vol/vol) gas phase in an anaerobic chamber (La Calhène, Velizy, France). Each well-isolated colony was picked up and incubated in fresh liquid medium. In order to be sure to obtain a pure isolate, streaking and isolation steps were repeated at least two times with samples from the first purification tube.

Growth parameters, carbon sources, and oxygen susceptibility. All experiments were performed in triplicate. Bacterial growth was directly monitored in Hungate tubes (2) by absorbance at 660 nm (Uvikon 940; Kontron Instruments, Montigny le Bretonneux, France). Previous correlation between the absorbance at 660 nm and cell numeration was simultaneously established by direct counting of the cells by epifluorescence microscopy after staining with acridine orange (10).

The determination of optimum growth parameters was made in Zobell medium. Temperature ranges were studied in Zobell medium (pH 7.5, 30 g of NaCl per liter). pH ranges from 2.5 to 9.5 were studied (Zobell medium; 70°C; 30 g of NaCl per liter). Except for pH 2.5, 3.5, and 4.5, the medium was buffered with MES (morpholineethanesulfonic acid) for pH 5.5, PIPES for pH 6.5, HEPES for pH 7.5, and AMPSO for pH 8.5 and 9.5 (Sigma Chemical Co.). For the determination of salinity ranges, the salinity of the medium (Zobell medium; 70°C; pH 7.5) was adjusted by increasing the amount of sea salt (Sigma Chemical Co.).

The determination of carbon sources usable for the strain was performed in a basic medium supplemented with yeast extract (1 g/liter) or in basic medium alone. In both cases, carbon substrates were added at the concentration 5 g/liter. Basic medium was composed of sea salt (30 g/liter) supplemented with vitamin and trace mineral solutions (10 ml/liter each) of Balch et al. (2) and with NH₄Cl (1 g/liter) as the mineral nitrogen source. The carbon substrates tested were brain heart infusion, meat extract, yeast extract, malt extract, tryptone, Casamino Acids, glycine, glucose, sucrose, lactose, fructose, xylose, galactose, cellobiose, maltose, starch, glycogen, sorbitol, mannitol, ethanol, acetate, and formate (Difco Laboratories, Detroit, Mich., or Sigma Chemical Co.).

Susceptibility to oxygen was tested by comparative assays. The strain was grown in reduced and deaerated Zobell medium and concurrently in the same medium reoxygenated prior to inoculation or reoxygenated after 8.5 h of normal anaerobic growth.

Effects of electron acceptors on growth and on glucose metabolism and hydrogen susceptibility. Thiosulfate (20 mM), sulfate (20 mM), and sulfur (2%) were tested as electron acceptors (27). Their influence on growth and on the amounts of acetate and L-alanine produced during glucose fermentation was investigated according to the method of Ravot and colleagues (28, 29). Amounts of acetate and L-alanine were quantified by high-performance liquid chromatography (HPLC) (18, 23).

The influence of hydrogen on the growth of the new isolate was investigated by growing it in Zobell medium supplemented or not with elemental sulfur under N₂:CO₂ (80:20, vol/vol) and under H₂:CO₂ (80:20, vol/vol). H₂S formation was detected by the addition of 500 µl of 5 mM CuSO₄–50 mM HCl to 0.2 ml of the culture. A brown precipitate demonstrated the presence of H₂S.

Antibiotic susceptibility. Chloramphenicol, vancomycin, streptomycin, and rifampin (Sigma Chemical Co.) from filter-sterilized stock solutions were each added at a final concentration of 100 µg/ml to sterile prereduced Zobell medium.

Light and electron microscopy. For light microscopy, cells were routinely observed in phase contrast or by UV epifluorescence illumination after staining with acridine orange (10), with a BH2 Olympus microscope (oil immersion; objective, ×40).

For negative staining-transmission microscopy, 3 ml of a grown culture was centrifuged at 3,500 rpm (Microcentrifugeur A12; Jouan, Saint Herblain, France) for 15 min. The pellet was washed with NaCl (15 g/liter) and then concentrated 15-fold in NaCl (15 g/liter). A drop of the suspension was laid down on a grid for 1 min and then washed two times in ammonium acetate and stained with uranyl acetate for 1 min. Observations and microphotographs were made with a JEOL JEM-100 CX II electron microscope.

Determination of G+C content. DNA of harvested cells was extracted according to the procedure of Marmur and Doty (19). The moles percent of guanine plus cytosine was determined by melting point analysis (20). *Micrococcus lysodeikticus* DNA (G+C, 72 mol%), *Escherichia coli* DNA (G+C, 50 mol%) and *Clostridium welchii* DNA (G+C, 26.5 mol%) (Sigma chemical Co.) were used as references.

16S rRNA sequence studies. Partially purified DNA was used for 16S rRNA gene (rDNA) amplification in a ThermoCycler II (Coy, Grass Lake, Mich.) with *Taq* DNA polymerase (Appligene Oncor, Illkirch, France). The forward 16S rDNA gene primer was 5'-AGAGTTTGATCATGGCTCAG<3', and the reverse primer was 5'-AAGTCGTAACAAGGTAACCG<3', corresponding, respectively, to the following positions in the *E. coli* rRNA sequence: 8 to 28 and 1493 to 1509. The contents of 15 50-µl PCR tubes were pooled, and a 1,500-bp band corresponding to the amplification product of the 16S rRNA-encoding gene was recovered from agarose gel and purified by the GeneClean II Kit purification method (BIO 101 Inc., La Jolla, Calif.). The sequencing (32) was performed directly on the PCR products by Euro Séquences Gènes Services (Paris, France), and 1,497 bp were sequenced.

Phylogenetic analysis. The phylogenetic data described below were obtained by manual alignment of the different small-subunit rRNA sequences used. The sequences were obtained from the EMBL and GenBank databases and from the Ribosomal Database Project. The organisms used in this analysis and their small-subunit rRNA sequence accession numbers or source were as follows: *Thermotoga maritima* DSM 3109^T, M21774; *Thermotoga elfii* DSM 9442^T, X80790; *Thermotoga thermarum* DSM 5069^T, Ribosomal Database Project; *Thermosipho africanus* DSM 5309^T, M83140; *F. islandicum* DSM 5733^T, M59176; *F. nodosum* ATCC 35602^T, M59177; *Geotoga petraea* ATCC 51226^T, L10658; *G. subterranea* ATCC 51225^T, L10659; *P. miotherma* ATCC 51224^T, L10657; *Hydrogenobacter thermophilus* T3, Z30189; *Caldobacterium hydrogenophilum* Z-829, Z30242; and *Aquifex pyrophilus* Ko15a, M83548. All operations were done by using computer programs developed in R. Christen's laboratory (Centre National de la Recherche Scientifique, Villefranche sur mer, France). Domains used for deriving phylogenies were restricted to parts of the sequences for which homologies exist without doubt and did not include too many undetermined nucleotides.

Phylogeny analyses and programs. (i) **Neighbor joining.** An algorithm like that developed by Saitou and Nei (30) was used. The program was rewritten to include inputs and outputs compatible with the ribosomal database and other programs developed by R. Christen.

(ii) **Maximum parsimony.** The PAUP program (33) for Macintosh computers was used. All topologies were obtained by using the heuristic options. The robustness of the topology was evaluated under maximum parsimony conditions (heuristic search) through 100 bootstrap replications.

(iii) **Maximum likelihood.** The fDNAmI program, which was derived from DNAmI program (6) and was rewritten by G. J. Olsen (University of Illinois, Urbana), was used with a Sun station. All analyses were performed with the global option (in fact, with the F, Y, and G options).

All trees obtained with the three above-mentioned phylogenetic methods were plotted by using a Macintosh computer and a program (njplot) developed by M. Gouy (Unité de Recherche Associée 243, Centre National de la Recherche Scientifique, Université Claude Bernard, Villeurbanne, France) that allows transformation of a normal tree representation (Newick's format) into Claris Draw drawings. Only topologies that were found to be similar by all three methods were retained for this analysis. Theoretical works have indeed demonstrated that the convergence of the results of all three methods is a very strong indication that the correct phylogeny has been determined and that the tree topology found was robust.

DNA reassociation. Genetic relatedness was investigated by dot blot qualitative genomic DNA-DNA hybridizations (31) with reference strains of *Thermotogales*, by using the ECL random-prime system (Amersham Life Sciences, Les Ulis, France). Quantitative genomic DNA-DNA hybridization (8, 25) was then determined with the closest strain, *T. africanus*, and with *T. maritima*.

Lipid analysis. Lipids from the lyophilized cells (50 to 100 mg) were extracted by a modified Bligh-Dyer method (3, 35). The extracted lipids were fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography using appropriate volumes of chloroform, acetone, and methanol, respectively. The methanol fraction was subjected to a mild alkaline methanolysis, purified by thin-layer chromatography, and analyzed as fatty acid methyl esters by gas chromatography (GC) and GC-mass spectrometry (9). The position and the geometry of the double bond of monounsaturates were identified by using the dimethylsulfoxide derivatives and the procedure previously described (21).

GC analyses were performed on a Carlo Erba (Rodano, Italy) HRGG 5300 gas chromatograph equipped with a fused-silica column coated with a nonpolar phase (60 m × 0.2 mm [inner diameter]; film thickness, 0.25 µm) and a flame ionization detector. Hydrogen was used as the carrier gas (33 cm/s). The temperature program was as follows: 50°C for 1 min, 20°C min⁻¹ till 140°C, and then 2°C min⁻¹ till 300°C.

Fatty acid nomenclature. A shorthand nomenclature is used which is in the form of numbers separated by a colon. The number before the colon indicates the carbon chain length, and the number after the colon corresponds to the number of double bonds. The position of the double bond is defined by the symbol ω followed by the number of carbons from the methyl end. The prefixes i and a refer to iso and anteiso, respectively. The geometry of the double bonds is indicated by c (*cis*) and t (*trans*).

Nucleotide sequence accession number. The nucleotide sequence of the 16S rDNA of strain BI429 has been deposited in the EMNEW database under accession no. Z70248.

RESULTS

Enrichment and isolation. Of the 41 samples examined, 5 gave rise to positive enrichment cultures after 24 h of incubation at 60°C. The samples that resulted in positive enrichment cultures were parts of dissected gastropod or bivalve mollusks. The temperatures of the sites from which these samples were obtained ranged from 18 to 30°C, meaning that the cultured microorganisms might be trapped and remain dormant in these filtering and grazing invertebrates. Observations of the

FIG. 1. Negatively stained *Thermosipho melanesiensis* cell, showing the toga ballooning over the ends. Bar, 0.5 μ m.

positive enrichment cultures on Nuclepore filters after acridine orange staining showed the presence of rod-shaped microorganisms. From the five positive enrichment cultures, five pure isolates were obtained, BI429, BI430, BI433, BI434, and BI487. Unless stated otherwise, all experiments were performed with isolate BI429.

Morphology and cell structure. Cells of strain BI429 appeared as rod-shaped organisms occurring singly or in pairs and rarely in chains with a maximum of five rods. They stained gram negative. By scanning electron microscopy the rods have been measured and found to be 1 to 3.5 μ m in length and 0.4 to 0.6 μ m in width. Transmission electron microscopy showed an internal organism structure very similar to that of *Thermosipho africanus* (12) and revealed the presence of a "toga," a sheath-like structure ballooning over the ends, characteristic of members of the order of *Thermotogales* (14) (Fig. 1).

Optimal growth conditions. Strain BI429 grew at temperatures ranging from 50 to 75°C, with an optimum around 70°C. No growth was observed at temperatures below 45°C and above 80°C. Growth was obtained between pH 4.5 and 8.5, with optimum growth at pH 6.5 to 7.5. The organism grew in the presence of sodium chloride concentrations ranging from 10 to 60 g/liter with an optimum at 30 g/liter (Fig. 2). At the optimum temperature for growth (70°C), optimum pH (6.5), and optimum salinity (30 g of NaCl per liter), the doubling time was 100 min.

Strain BI429 was not able to grow in the presence of oxygen. Growth did not occur when the strain was inoculated in oxygenated medium and was interrupted if reoxygenation of the medium happened.

Substrate utilization. Only brain heart infusion and yeast extract were able to support growth when provided alone in the basic medium, but under these conditions the growth of strain BI429 was weak. Utilization of other substrates required the addition of yeast extract. When yeast extract was added, growth was obtained with brain heart infusion, malt extract, and tryptone. For the carbohydrates, decreasing yields of growth were obtained with sucrose or starch, glucose or maltose, and lactose, cellobiose, or galactose. Meat extract, xylose, fructose, ethanol, mannitol, sorbitol, glycine, glycogen, formate, acetate, and Casamino Acids did not support growth, even when combined with yeast extract.

Influence of hydrogen and of electron acceptors. Increasing the hydrogen partial pressure of the medium by growing strain

FIG. 2. Temperature, pH, and salinity dependence of growth of *Thermosipho melanesiensis*. The doubling times were calculated from the slopes of the growth curves (not shown).

BI429 under an $H_2:CO_2$ gas phase (80:20, vol/vol) inhibited growth. This inhibition could be overcome by lowering the hydrogen partial pressure of the medium by growing the strain under an appropriate gas phase, like N_2 or $N_2:CO_2$ (80:20, vol/vol), or by the addition of S^0 . When S^0 was added to the culture medium under an $N_2:CO_2$ or an $H_2:CO_2$ gas phase, strain BI429 reduced elemental sulfur and formed H_2S . Neither sulfate nor thiosulfate could be used as electron acceptors during growth of strain BI429. The addition of elemental sulfur increased the growth density and especially the growth rate from that achieved in control cultures to which no electron acceptors had been added or in cultures to which thiosulfate or sulfate had been added (Fig. 3). These results showed the sensitivity of strain BI429 to hydrogen and how the reduction of S^0 might act as an electron trap to prevent growth inhibition by high levels of H_2 that would be produced otherwise.

The presence of S^0 as an electron acceptor, lowering the hydrogen partial pressure of the medium, also had an effect on the alanine/acetate ratio produced during glucose fermentation. When S^0 is absent, an alanine/acetate ratio of about 0.3 (0.28 in the control cultures without electron acceptors and 0.35 with the inutilized thiosulfate) was found. In presence of S^0 , this ratio was found to be 0.028. When the hydrogen concentration increased, strain BI429 shifted its metabolism to

FIG. 3. Influence of electron acceptors on growth of *Thermosipho melanesiensis* as determined by optical density (OD). □, growth without electron acceptor; ○, growth with S^0 as electron acceptor; ▲, growth with sulfate; ☆, growth with thiosulfate.

alanine production and consequently less acetate was produced. This shifting metabolism depending on the hydrogen partial pressure was first described for the archaeon *Pyrococcus furiosus* (17) and then for members of the order *Thermotogales* (29).

Antibiotic susceptibility. Growth of isolate BI429 was completely inhibited by rifampin, chloramphenicol, and streptomycin, each at a final concentration of 100 μ g/ml. However, slight growth was observed with the same concentration of vancomycin.

DNA base composition. The DNA base composition (G+C) of strain BI429 was determined to be 30.5 mol%.

DNA reassociation. Genomic DNA-DNA dot blot qualitative hybridizations were low with the three strains of *Thermotoga*, *Thermotoga maritima*, *Thermotoga neapolitana*, and *Thermotoga thermarum*, and with *F. islandicum*. The strongest response was with the only species of the genus *Thermosipho*, *Thermosipho africanus*. Quantitative genomic DNA-DNA hybridizations resulted in 0% reassociation with *Thermotoga maritima* and 2% reassociation with *Thermosipho africanus*.

16S rRNA sequence analyses. The phylogenetic position of strain BI429 was investigated in detail by including some representatives of thermophilic bacterial species. The analyses were performed by three methods: the neighbor-joining method, the maximum-parsimony method, and the maximum-likelihood method using Jukes and Cantor corrections. The results of these analyses were always consistent, regardless of which method was used. The results of these analyses are summarized in Fig. 4.

This result showed two monophyletic groups. (i) The first group clustered together the *Thermosipho*, *Thermotoga*, *Fervidobacterium*, *Geotoga*, and *Petrotoga* species and strain BI429; (ii) the second group was formed by *Aquifex* and *Hydrogenobacter* and was used as the external group. The first monophyletic group, including members of the order *Thermotogales* and strain BI429, was well supported by the maximum-likelihood method and by the maximum-parsimony method, with which a bootstrap replication was investigated and the value was 100%. In all analyses, strain BI429 clustered with *Thermosipho africanus*. This branch was well supported by the maximum-likelihood method (the branch was significantly positive at $P < 0.01$) and by the maximum-parsimony method (heuristic search option), by which one most-parsimonious tree was obtained (length, 329; consistency index [excluding noninformative characters], 0.723; retention index, 0.818) and the

FIG. 4. Phylogenetic position of strain BI429^T (*Thermosipho melanesiensis*). The analysis was investigated with some thermophilic bacteria of the orders *Thermotogales* and *Aquificales*. The topology shown is an unrooted tree obtained by a neighbor-joining algorithm (Jukes and Cantor corrections). Branches labelled with double asterisks are significantly positive at $P < 0.01$ by the maximum-likelihood method. Supports from a bootstrap analysis using parsimony are shown as percentages of replications above each branch (only percentages above 50% are indicated); these numbers indicate also that these branches are retrieved within the most-parsimonious tree obtained.

bootstrap replication value was 69%. The percentages of similarity between species were calculated for domains including all nucleotides aligned without any ambiguity. We found 95.4, 95.2, and 98.6% similarity, respectively, between BI429 and *Thermotoga maritima*, BI429 and *F. islandicum*, and BI429 and *Thermosipho africanus*.

Lipid analysis. The phospholipid fatty acid profile of strain BI429 is listed in Table 1. This profile is characterized by the predominance of saturates, accounting for 90% of the total fatty acids, with C16:0 and C18:0 as major acids. Monounsaturated fatty acids were present in low concentrations, with C18:1 ω 7 and C16:1 ω 7 accounting for 2.38 and 5.48% of the total fatty acids, respectively.

TABLE 1. Fatty acid profile of *Thermosipho melanesiensis*

Fatty acid(s)	% Total acid
C14:0	1.15
iC15:0	0.07
aC15:0	0.15
C15:0	2.35
iC16:0	0.73
C16:1 ω 7c	2.38
C16:1 ω 7t	0.72
C16:0	71.69
iC17:0	0.45
aC17:0	0.85
C17:0	4.7
C18:1 ω 7c	5.48
C18:1 ω 7t	1.48
C18:0	7.8
Saturates	89.84
Monounsaturates	10.06

DISCUSSION

Deep-sea hydrothermal vent ecosystems represent an original environment with respect to their physicochemical conditions (high pressures, high temperatures, low pH, high concentrations of metals, such as zinc, iron, manganese, and silica, high concentrations of dissolved gases, such as hydrogen sulfide, methane, carbon monoxide, carbon dioxide, and hydrogen) and where primary production is totally based on chemosynthesis. They have provided an exciting source for the isolation of unusual microbes. Previous microbiological studies of such environments have led to the identification of several thermophilic microorganisms, but since this time they were members of the kingdom *Archaea* (24). Up to now, representatives of the order *Thermotogales* have not been isolated from deep-sea hydrothermal environments. *Thermotogales* have been found previously in numerous terrestrial or coastal extremophilic environments, and the strain BI429 described in this work extends their known habitats.

The new isolate BI429 is a gram-negative, anaerobic, heterotrophic thermophilic rod-shaped bacterium with an outer sheath-like structure characteristic of genera belonging to the order *Thermotogales*. As its minimal temperature for growth is 45°C, it was dormant in the gills of the hydrothermal mussel, *B. breviar* (34), from which it was isolated (in situ temperature ranging from 18 to 30°C). Phenotypically, this isolate exhibits characteristics similar to those described for representatives of the order *Thermotogales* (14, 36). The phospholipid fatty acid profile of strain BI429 is characterized by the presence of large amounts of saturated fatty acids, with C16 accounting for 70% of the total fatty acids. This is a trait commonly found among thermophilic bacteria as a response to high temperature. This profile is consistent with other profiles of *Thermotoga* species, with the exception of the presence of C16:1 ω 7 and C18:1 ω 7 as monounsaturates.

Physiologically, strain BI429 differs from the species *Thermotoga maritima* and *Thermotoga neapolitana* by its lower optimal temperature, from the species *Thermotoga thermarum*, *Thermotoga elfii*, and *Thermotoga subterranea* by its higher salinity range and optimum, from the genus *Fervidobacterium* by its higher salinity requirement, and from the genera *Geotoga* and *Petrotoga* by its higher growth temperature. Its physiological properties (temperature and pH optima and salinity requirement) are closest to those of *Thermosipho africanus*.

Strain BI429 shares with *Thermotogales* isolated from volca-

nic hot springs the capacity to reduce elemental sulfur to sulfide, but unlike them it is unable to reduce thiosulfate. As described for the archaeon *Pyrococcus furiosus* (17) and for members of the order *Thermotogales* (29), BI429 is able to shift its metabolism when grown with or without an electron acceptor (i.e., elemental sulfur). Ravot et al. (29) have distinguished two groups of *Thermotogales* according to the amounts of L-alanine and acetate produced by glucose fermentation. Data presented by the authors allow the calculation of an alanine/acetate ratio. For group I (*F. islandicum*, *Thermosipho africanus*, *Thermotoga elfii*, *Thermotoga* strain SEBR 7054), this ratio ranges from 0.3 to 0.6 when an electron acceptor is absent and from 0 to 0.06 when an electron acceptor is present. For group II (*Thermotoga maritima*, *Thermotoga neapolitana*, *Thermotoga* strain SEBR 2665), the ratio is 0.04 when an electron acceptor is absent and ranges from 0.02 to 0.05 when an electron acceptor is present. Results obtained for strain BI429 allow the classification of this new isolate in group I, with an alanine/acetate ratio of 0.3 in the absence of elemental sulfur and an alanine/acetate ratio of 0.028 when S⁰ is present. These results are in agreement with the previous findings of Ravot et al. for various members of *Thermotogales* and strengthen the argument that strain BI429 belongs to the *Thermosipho* genus. Further features affiliate isolate BI429 with the genus *Thermosipho*: (i) a DNA G+C content of 30.5 mol% and (ii) the phylogenetic analyses (BI429 belongs to the monophyletic group represented by the *Thermotogales* order, and its 16S rRNA sequence presents 98.6% similarity with the unique representative of the genus *Thermosipho*, *Thermosipho africanus*.)

However, based on genomic DNA-DNA hybridization (2% reassociation) and particular physiological differences (i.e., inability to use thiosulfate) and considering that the high level of 16S rRNA similarity with *Thermosipho africanus* is attributable to the phylogenetic analysis performed only between domains of the sequences which aligned without ambiguity (insofar as with this analysis the two species of *Fervidobacterium* present a percentage of similarity of 98.7%), we consider that isolate BI429 is not closely enough related to *Thermosipho africanus* to be considered a member of that species. Thus, the new isolate represents a new species of *Thermosipho* which we name *Thermosipho melanesiensis* with reference to its site of isolation.

Description of *Thermosipho melanesiensis* sp. nov. *Thermosipho melanesiensis* (me. la. ne. si. en'sis. L. gen. n. *melanesiensis*, originating from Melanesia, describing its site of isolation). Cells are gram-negative rods with a length of 1 to 3.5 μ m and a width of 0.4 to 0.6 μ m. The rods are surrounded by a sheath-like structure, ballooning over the ends. They occur singly, in pairs, or, rarely, in chains with a maximum of five rods. They are anaerobic. Growth occurs between 45 and 80°C, with an optimum at 70°C; between pH 3.5 and 9.5, with an optimum at pH 6.5 to 7.5; and at NaCl concentrations between 5 and 60 g/liter, with an optimum at 30 g/liter (doubling time at optimum temperature and salinity and at pH 6.5, 100 min). For heterotrophic growth under anaerobic conditions, complex organic material, such as yeast extract or brain heart infusion, is necessary. Growth is supported by yeast extract or brain heart infusion alone or by malt extract, tryptone, and carbohydrates supplemented by yeast extract. For carbohydrates, decreasing yields of growth are obtained with sucrose and starch, glucose and maltose, lactose, cellobiose, and galactose. Meat extract, xylose, fructose, ethanol, mannitol, sorbitol, glycine, glycogen, formate, acetate and Casamino Acids do not support growth, even combined with yeast extract. Hydrogen inhibits growth, but this inhibition is overcome by the reduction of elemental sulfur. Under this condition, hydrogen sulfide is produced and the growth yield and more precisely the growth rate are in-

creased. Neither thiosulfate nor sulfate can be used as electron acceptors. Shifting metabolism to alanine production in the absence of elemental sulfur. Completely inhibited by rifampin, chloramphenicol, and streptomycin, each at a final concentration of 100 µg/ml. Slight growth with the same concentration of vancomycin.

The DNA base composition (G+C content) is 30.5 mol%. Phylogenetic analysis based on the 16S rRNA sequence affiliates the strain with the *Thermotogales* and, more specifically, with the genus *Thermosipho*, but genomic DNA-DNA similarity analysis and physiological properties offer clear evidence for significant divergence from the species *Thermosipho africanus*. Isolated from the gills of a deep-sea hydrothermal mussel (*B. breviar*) collected at the bottom of a black smoker in the Lau Basin (latitude, 22°32'S; longitude, 176°43'W; depth, 1,832 to 1,887 m), Southwest Pacific Ocean. Type strain is *Thermosipho melanesiensis* BI429^T (= CIP 104789^T [Collection de Bactéries de l'Institut Pasteur, Paris, France]).

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