

## ***Caloranaerobacter azorensis* gen. nov., sp. nov., an anaerobic thermophilic bacterium isolated from a deep-sea hydrothermal vent**

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**A thermophilic, anaerobic, chemo-organotrophic bacterium, designated MV1087<sup>T</sup>, was isolated from a deep-sea hydrothermal chimney sample collected from the Mid-Atlantic Ridge. The cells were straight, motile and stained Gram-negative. Growth was observed from 45 to 65 °C, with an optimum around 65 °C. No growth was observed at 40 or 70 °C. Growth was observed from pH 5.5 to 9.0 and the optimum pH was around 7. The salinity range for growth was 10–100 g sea salt l<sup>-1</sup> (corresponding to 6.5–65 g NaCl l<sup>-1</sup>) with an optimum at 30 g sea salt l<sup>-1</sup> (20 g NaCl l<sup>-1</sup>). Strain MV1087<sup>T</sup> was heterotrophic, able to ferment proteinaceous substrates, such as brain/heart infusion and gluten, and carbohydrates, such as glucose, xylan and starch. The DNA G+C content was 27 mol%. Phylogenetic analyses using 16S rDNA sequences indicated that strain MV1087<sup>T</sup> belonged to cluster XII of the *Clostridium* subphylum. Due to its phenotypic and genotypic characteristics, isolate MV1087<sup>T</sup> is proposed as a novel species of a new genus, *Caloranaerobacter azorensis* gen. nov., sp. nov. The type strain is MV1087<sup>T</sup> (= CNCM I-2543<sup>T</sup> = DSM 13643<sup>T</sup>).**

**Keywords:** bacteria, *Caloranaerobacter*, *Caloranaerobacter azorensis*, deep-sea vent, thermophile

### **INTRODUCTION**

Thermophilic micro-organisms have been isolated from various environments, especially for biotechnological interests, as a source of more thermostable biocatalysts (Kristjansson, 1992). These environments include oil reservoirs, deep aquifers, continental hot springs, shallow marine hydrothermal systems and deep-sea vents. Within the domain *Bacteria*, various thermophilic and hyperthermophilic marine species have been isolated and described. These bacteria are aerobic heterotrophs (*Thermus thermophilus*, *Rhodothermus marinus*, *Thermaerobacter marianensis*), microaerophilic autotrophs (*Aquifex pyrophilus*), anaerobic autotrophs (*Desulfurobacterium thermolitho-*

*trophum*) and anaerobic heterotrophs (*Thermotogales*) (Alfredsson *et al.*, 1988; Manaia & Da Costa, 1991; Huber *et al.*, 1992; Antoine *et al.*, 1997; Takai *et al.*, 1999). Both aerobic and anaerobic thermophilic heterotrophic bacterial strains have been isolated from deep-sea hydrothermal vents. The aerobic strains have been assigned to the genera *Thermus* and *Bacillus* (Marteinsson *et al.*, 1995, 1996) and the anaerobic strains to the genera *Thermosipho* and *Thermotoga* within the order *Thermotogales*, a deep branch of the small-subunit rRNA universal phylogenetic tree (Antoine *et al.*, 1997; Marteinsson *et al.*, 1997; Takai & Horikoshi, 2000). Finally, an anaerobic strain isolated from deep-sea hydrothermal vents of the Mid-Atlantic Ridge was recently assigned to a new species of a new genus of the order *Thermotogales*, *Marinitoga camini* (Wery *et al.*, 2001).

Collins *et al.* (1994) used 16S rDNA phylogenetic analysis to define 19 clusters within the genus *Clostridium* and they subsequently proposed five new

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genera: *Caloramator*, *Filifactor*, *Moorella*, *Oxobacter* and *Oxalophagus* (Collins *et al.*, 1994). Evidence for the placement of further genera within the *Clostridium* subphylum of the Gram-positive bacteria was then presented, leading to the proposal of *Johnsonella*, *Catonella* (Willems & Collins, 1995), *Tissierella* (Farrow *et al.*, 1995), *Fusibacter* (Ravot *et al.*, 1999) and *Thermohalobacter* (Cayol *et al.*, 2000). Members of some of these genera, such as *Fusibacter*, *Thermohalobacter*, *Caloramator* and *Moorella*, are anaerobic thermophiles. Anaerobic heterotrophic thermophiles of the order *Clostridiales* have already been isolated from continental hot springs and shallow hydrothermal vents; for example, *Moorella glycerini*, *Caldicellulosiruptor kristjanssonii*, *Thermoanaerobacter siderophilus* and *Thermoterrabacterium ferrireducens* (Slobodkin *et al.*, 1997a, b, 1999; Bredholt *et al.*, 1999). However, to date, no thermophilic anaerobic heterotrophic species of the order *Clostridiales* has been isolated and described from deep-sea hydrothermal vents.

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 first characterization of a thermophilic anaerobic bacterium isolated from deep-sea hydrothermal vents and affiliated to the subphylum *Clostridium*.

## METHODS

**Collection of samples.** Samples were collected by the man-operated submersible *Nautille* during the MARVEL cruise, on deep-sea vent fields of 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 walls, rock debris and mussels were collected using an insulated box filled with sterilized sea water. They were prepared in an anaerobic chamber and stored at 4 °C in serum vials filled with sterile seawater under anaerobic conditions (N<sub>2</sub> headspace gas and 0.5 g sodium sulfide l<sup>-1</sup>).

**Growth conditions.** The medium YPXS used during the enrichment and the isolation contained (l<sup>-1</sup>): 0.5 g yeast extract (Difco), 1 g peptone (Difco), 5 g oat spelt xylan (Sigma), 10 g sulfur (Prolabo), 30 g sea salt (Sigma), 6.05 g PIPES buffer (Sigma) and 1 mg resazurin (Sigma). YTG medium contained (l<sup>-1</sup>): 1 g yeast extract, 1 g tryptone, 2.5 g glucose (Sigma), 30 g sea salt, 6.05 g PIPES buffer and 1 mg resazurin. Glucose was omitted in YT medium. The pH was adjusted to 7.5 in YPXS 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 achieved after 20 min at 121 °C. Sterile media were transferred into an anaerobic chamber containing N<sub>2</sub>/H<sub>2</sub>/CO<sub>2</sub> (90:5:5), reduced by adding sodium sulfide to a final concentration of 0.5 g l<sup>-1</sup> and then distributed into Hungate tubes (6 ml in a total volume of 16 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 65 °C.

**Enrichment and isolation procedures.** Enrichment culture was performed in YPXS medium, inoculated with 1 ml of

the sheltered sample of hydrothermal chimney and incubated at 65 °C, at atmospheric pressure under the anaerobic chamber gas mixture. Cultures were purified by streaking enrichment samples onto YPXS medium solidified with Gelrite (Scott Laboratories) as gelling agent and incubated in jars as reported by Godfroy *et al.* (1997).

**Storage.** Cultures in exponential growth phase were stored at 4 °C or room temperature. For long-term storage, cultures in exponential growth phase were stored anaerobically in cryotubes at -20 and -80 °C after the addition of 5% (v/v) DMSO (Sigma).

**Observation of the culture and determination of cell number.** Enumeration of cells was performed by direct cell counting using a Thoma chamber (depth 0.02 mm). Alternatively, cells were stained with 4',6'-diamidino-2-phenylindole (DAPI), which was added to the samples at a final concentration of 1 mg l<sup>-1</sup>. Cells were observed with an Olympus model BH-2 microscope. The turbidity of the cells in YTG medium was also quantified with a spectrophotometer (Spectronic 301; Milton Roy), measuring the optical density at 600 nm. A linear correlation was found between the number of cells per ml (C) and OD<sub>600</sub> from 0.1 to 0.25:  $C = 1.25 \times 10^9 \times OD_{600}$  ( $r^2 = 0.87$ ).

**Morphology.** Gram staining was done using the Bacto 3-step Gram stain Set-S (Difco). 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 sodium 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 sodium cacodylate, pH 7.4/10% (w/v) NaCl/2% (w/v) OsO<sub>4</sub> (1:1:2)]. Following 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 examined in a 100CXII transmission electron microscope (JEOL).

**Tests for sporulation.** The presence of spores was determined by phase-contrast microscope observation of cultures stained with malachite solution at different phases of growth. The presence of heat-resistant spores was tested by heating cultures obtained previously on YTG and YT media. On each medium, different cultures were heated at 80, 90 and 100 °C for 10, 20 and 60 min. Samples of the cultures were then observed. The heat resistance of cells was also tested as reported by Cayol *et al.* (2000).

**Analyses of amino acids, organic acids and aromatic acids.** Each sample was centrifuged for 30 min at 8000 g to eliminate cells. Half of the supernatant was transferred to an ultrafree-CL-PLGC 10000 NMWL unit (Millipore) and centrifuged at 4500 g for several hours. The filtrate (20 µl) was mixed with 30 µl of a solution of ethanol/water/triethylamine (2:2:1) and vacuum-dried. A derivatizing solution (20 µl) containing ethanol/water/triethylamine/phenylisothiocyanate (7:1:1:1 by vol.) was then added. After 10 min at room temperature, the sample was vacuum-dried. Analysis of amino acids by HPLC (Alliance 2690; Waters) was then conducted under the conditions and with the products of the Waters PicoTag method (WAT007360; Waters). In the other part of the supernatant, proteins were precipitated with a solution of 5-sulfosalicylic acid at 2% (w/v) overnight at 4 °C and removed 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 analysis of glucose and linear organic acids, 20 µl

supernatant was eluted on an H<sup>+</sup> exclusion column (polyspher OAKC 1.51270; Merck) at 60 °C by a 9 mM H<sub>2</sub>SO<sub>4</sub> solution with a flow rate of 0.35 ml min<sup>-1</sup> and detected using a differential refractometer (refractometer 410; Waters). For analysis of aromatic acids, 20 µl supernatant was eluted on an H<sup>+</sup> exclusion column (column 28352; Chrompack) at 65 °C by a 4.5 mM H<sub>2</sub>SO<sub>4</sub> solution with a flow rate of 0.5 ml min<sup>-1</sup> and detected by absorbance at 210 nm (UV detector 486; Waters).

**Determination of growth parameters.** All experiments were conducted in triplicate. Cells were grown in Hungate tubes on YTG 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, YTG 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; for pH 6.5 and 7, PIPES; for pH 7.5 and 8, HEPES; for pH 8.5 and 9, AMPSO [3-(1,1-dimethyl-2-hydroxyethyl)-amino-2-hydroxypropanesulfonic acid]; for pH 10, no buffer. In the anaerobic chamber, sodium sulfide was added and the pH was measured at room temperature and adjusted, if necessary, with 0.1 M HCl and 0.1 M NaOH. To determine the salt requirement, YTG medium was prepared with different dilutions of sea salt. The effect of salinity was determined at 65 °C and pH 7. The effect of pH was determined at 65 °C and 30 g sea salt l<sup>-1</sup>. The effect of temperature was determined at pH 7 and 30 g sea salt l<sup>-1</sup>. Growth rates ( $\mu$ ) were obtained after linear regression of the curve ln(OD) versus time and the doubling time ( $t_d$ ) was calculated as  $t_d = \ln(2)/\mu$ . Confidence intervals and related standard error were calculated as reported by Barbier *et al.* (1999).

**Determination of growth requirements.** All experiments were carried out in duplicate. The ability of isolate MV1087<sup>T</sup> to use gluten, brain/heart infusion, yeast extract, peptone or tryptone was tested at 2 g l<sup>-1</sup> in a medium containing (l<sup>-1</sup>): 30 g sea salt, 10 ml minimal solution (Balch *et al.*, 1979), 10 ml vitamin solution (Balch *et al.*, 1979), 5 ml 7% (w/v) KH<sub>2</sub>PO<sub>4</sub> and 6.05 g PIPES. A solution of 20 amino acids each at 0.1 g l<sup>-1</sup> was also tested. A stock solution containing each amino acid at a concentration of 1 g l<sup>-1</sup> was sterilized separately by filtration. The ability of isolate MV1087<sup>T</sup> to use other substrates was tested in a medium containing (l<sup>-1</sup>): 30 g sea salt, 10 ml mineral solution, 10 ml vitamin solution, 5 ml 7% (w/v) KH<sub>2</sub>PO<sub>4</sub>, 6.05 g PIPES, 1 g yeast extract, 1 g tryptone and the substrate tested. The substrates tested were: D(+)-sucrose, D(+)-glucose, D(+)-cellobiose, D(+)-xylose, starch, cellulose, dextran, xylan, glycerol, methanol, ethanol, acetate, propionate, pyruvate and lactate. Polysaccharides were tested at 5 g l<sup>-1</sup>, sugars at 2.5 g l<sup>-1</sup>, alcohols at 5 ml l<sup>-1</sup> and acids at 2 g l<sup>-1</sup>. Sugar and vitamin solutions were sterilized by filtration and added just before inoculation. Tests were performed in Hungate tubes. To avoid growth on substrates brought with the inoculum, positive cultures were transferred once (10% inoculum) and the final concentration of the cells was determined by direct counting. Amino, organic and aromatic acids were analysed during culture on YTG medium. The variations in concentration of amino acids and organic acids were controlled in uninoculated media. API50CH strips (bioMérieux) with medium 50CHE (bioMérieux) were used to test the consumption of other carbohydrates and APIZYM strips (bioMérieux) were used to test enzyme activities, following the instructions of the supplier. The strips were incubated at 65 °C, instead of 37 °C.

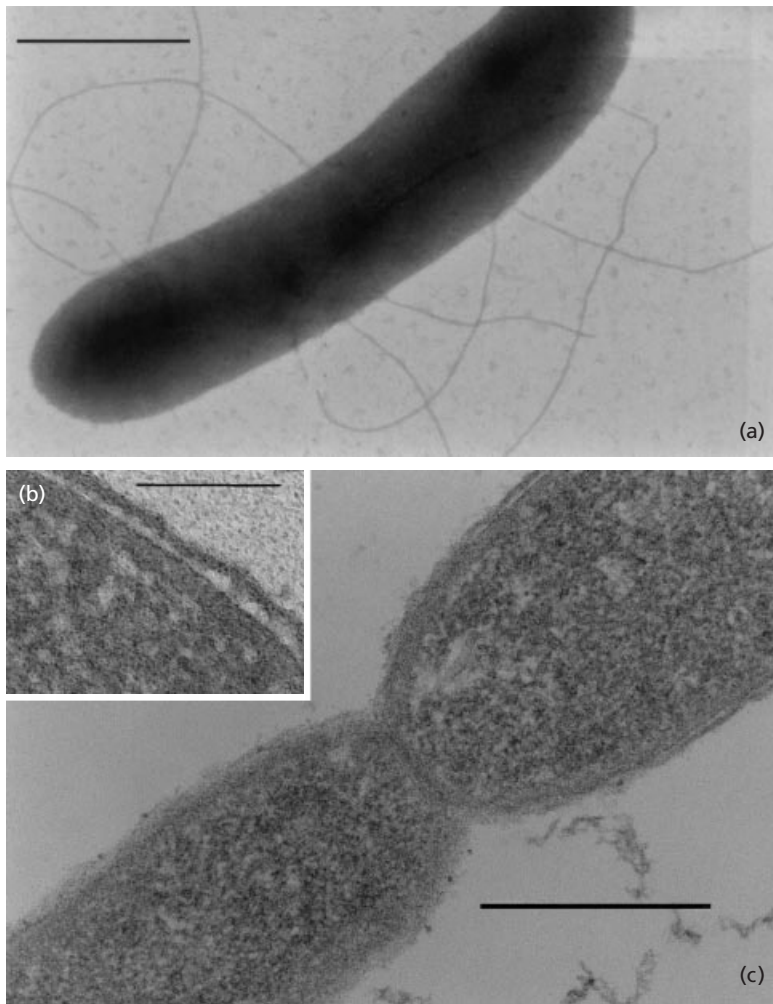
**Effect of possible electron acceptors.** The effects on growth of sulfur (10 g l<sup>-1</sup>), sodium thiosulfate (20 mM), sodium sulfite (20 mM), FeCl<sub>3</sub> (5 mM), sodium nitrite (20 mM) and sodium nitrate (20 mM) were tested on YTG medium. The experiments were performed in triplicate. The cell concentration was determined by direct cell counting. Production of H<sub>2</sub>S was examined using lead acetate paper (Whatman).

**DNA extraction and purification.** Genomic DNA was extracted as reported by Barbier *et al.* (1999), with incubation at 45 °C for 0.5 h instead of for 3 h at 40 °C. The quality of the extraction was checked on a 0.8% (w/v) agarose gel containing 0.8 µg ethidium bromide ml<sup>-1</sup>.

**DNA base composition.** The DNA was purified by CsCl gradient centrifugation. After precipitation, the DNA was suspended in TE buffer containing 1.075 g CsCl ml<sup>-1</sup> and 10 mg ethidium bromide ml<sup>-1</sup> and centrifuged for 15 h at 65000 r.p.m. (Beckman preparative ultracentrifuge, model CO-L70K; rotor 70.1 Ti). The DNA band was removed with a syringe. The ethidium bromide was then extracted by using 2-propanol saturated with CsCl. Finally, the solution was dialysed overnight against 1 l TE buffer. The G + C content of the DNA was determined by thermal denaturation under the conditions reported by Raguénès *et al.* (1997). A calibration curve was obtained by using ultrapur 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 the bacterial forward primer SAdir (5'-AGAGTTTGATC-ATGGCTCAGA-3'), corresponding to positions 8–28 of the *Escherichia coli* 16S rRNA, and the bacterial reverse primer S17rev (5'-GTTACCTTGTACGACTT-3'), corresponding to the complement of positions 1493–1509. The following reagents 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 MgCl<sub>2</sub>, 0.1% (w/v) Triton X-100, 0.2 ml BSA or gelatin ml<sup>-1</sup>; Appligene-Oncor], 2.5 U *Taq* DNA polymerase (Appligene-Oncor), 200 µM of 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 PCR temperature profile used was: 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 1.5 min 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<sup>-1</sup>. All images were obtained with 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 by Genome Express (France) and 1443 positions of the 16S rDNA sequence were determined. The sequence was then compared with others available in GenBank using BLAST (Altschul *et al.*, 1990). A multiple sequence file was obtained by using the MEGALIGN program of the DNASTAR package (Promega). Alignments and similarity levels were obtained by the CLUSTAL W method with weighted residues (Thompson *et al.*, 1994). The alignment was then corrected manually using the multiple sequence alignment editor SEAVIEW and the phylogenetic reconstruction was produced using PHYLO-WIN (Galtier *et al.*, 1996) with the following algorithms: Jukes–Cantor



**Fig. 1.** Transmission electron micrograph of strain MV1087<sup>T</sup> showing (a) cell shape and flagella (negatively stained), (b) Gram-negative type cell wall and (c) pinching mechanism of cell division. Bars, 0.5 (a), 0.1 (b) and 0.4 (c)  $\mu\text{m}$ .

distance matrix and successively the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Lake, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap values were determined according to Felsenstein (1985).

## RESULTS

### Isolation

During the MARVEL oceanographic cruise, YPXS medium was inoculated with a sample from the Lucky Strike deep-sea hydrothermal site of the Mid-Atlantic Ridge. The sample was composed of rocks from the bottom of a diffuser supporting mussels, collected at a depth of 1650 m. After a few days at 65 °C under anaerobic conditions, motile straight rods were observed. The strain was purified by streaking on plates and the isolate was called MV1087<sup>T</sup>.

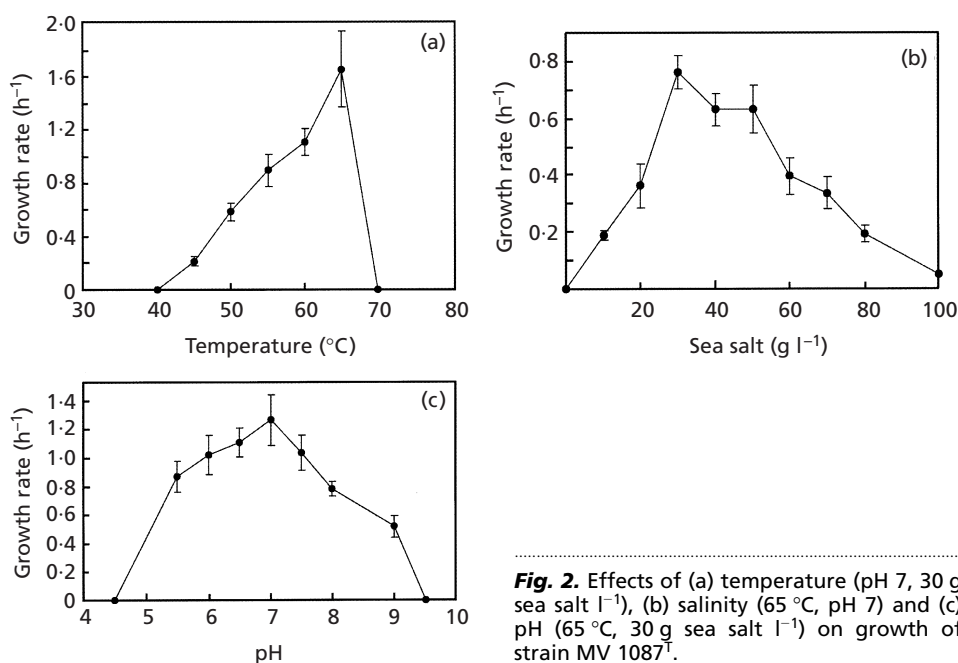
### Morphology

Microscope observations indicated that cells of isolate MV1087<sup>T</sup> were motile, round-ended rods; flagella were observed (Fig. 1a). The cells stained Gram-negative in

all phases of growth. Electron microscopy of thin sections of strain MV1087<sup>T</sup> revealed a cell wall ultrastructure typical of Gram-negative bacteria (Fig. 1b) and cell division with a pinching mechanism (Fig. 1c). The cells appeared singly, in pairs or in short chains (fewer than five cells). Under optimal growth conditions on YTG medium, cells were 0.3–0.5  $\mu\text{m}$  wide and 0.5–2  $\mu\text{m}$  long. Cells more than 20  $\mu\text{m}$  long were formed occasionally. Spores were not observed, but cells survived pasteurization at 100 °C for 20 min. In cultures stored for a few days at 4 °C, in poor media or at high pH, some terminal, round, external bodies were observed. Their significance remains unknown.

### Determination of growth parameters

Growth was observed from 45 to 65 °C and the optimum temperature for growth was around 65 °C (Fig. 2a). No growth was observed at 40 or 70 °C. Growth was observed at sea salt concentrations ranging from 10 to 100  $\text{g l}^{-1}$  (corresponding to 6.5–65  $\text{g NaCl l}^{-1}$ ) and the optimum sea salt concentration was around 30  $\text{g l}^{-1}$  (20  $\text{g NaCl l}^{-1}$ ) (Fig. 2b). No growth was detected without sea salt. Isolate MV1087<sup>T</sup> grew



**Fig. 2.** Effects of (a) temperature (pH 7, 30 g sea salt l<sup>-1</sup>), (b) salinity (65 °C, pH 7) and (c) pH (65 °C, 30 g sea salt l<sup>-1</sup>) on growth of strain MV 1087<sup>T</sup>.

from pH 5.5 to 9 and the optimum pH was around 7 (Fig. 2c). Under optimal growth conditions on YTG medium, the shortest generation time observed was 15 min and the maximum cell yield was  $2 \times 10^9$  cells ml<sup>-1</sup>.

### Growth requirements

Strain MV1087<sup>T</sup> is heterotrophic. Growth was observed both on proteinaceous substrates and on carbohydrates. MV1087<sup>T</sup> grew on brain/heart infusion or gluten at 2 g l<sup>-1</sup>. Isolate MV1087<sup>T</sup> was able to use glucose and starch with cell yields greater than 10<sup>9</sup> ml<sup>-1</sup>, xylan with a cell yield between 10<sup>8</sup> and 10<sup>9</sup> cells ml<sup>-1</sup> and xylose with a cell yield between 5 × 10<sup>7</sup> and 10<sup>8</sup> cells ml<sup>-1</sup>. It was not able to use sucrose, cellobiose, cellulose or dextran. Cell concentrations of 4 × 10<sup>8</sup> cells ml<sup>-1</sup> were reached on pyruvate but none of the other organic acids or alcohols tested (methanol, ethanol, propionate and lactate) supported growth. API50CH strips showed that the strain was also able to use arabinose, ribose, galactose, fructose and sorbose, but not maltose, lactose or mannitol. Using APIZYM tests, alkaline and acid phosphatase activities were detected but not α-mannosidase, α-fucosidase, α-galactosidase, α-glucosidase, β-galactosidase or β-glucosidase activities. These last results were congruent with the inability to use lactose, maltose and cellobiose. During fermentation on YTG medium, 8 mM glucose was consumed and 8 mM acetate, 0.2 mM isovalerate and 1 mM alanine were produced in the medium. Concentrations of proline, butyrate, propionate, phenylacetate and hydroxyphenylacetate also increased. The concentration of lactate remained unchanged during growth. The 0.2 mM arginine pres-

ent in the medium after inoculation was consumed rapidly and no arginine was then detected.

### Effect of electron acceptors

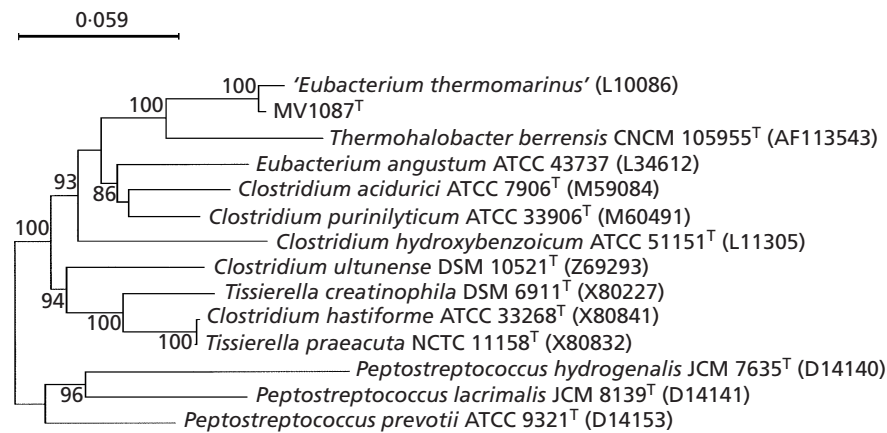
Sulfur, thiosulfate, nitrate and ferric ions did not enhance growth. No growth was observed in the presence of nitrite or sulfite. Production of H<sub>2</sub>S was detected when isolate MV1087<sup>T</sup> was cultivated with sulfur, but not with thiosulfate or in the control without thiosulfate or sulfur. This production of H<sub>2</sub>S was significant compared with production in uninoculated medium containing sulfur and incubated under the same conditions.

### DNA base composition

The G + C content of the DNA of isolate MV1087<sup>T</sup> was 27 ± 1 mol %.

### 16S rDNA sequence analysis

A total of 1443 bp of the 16S rDNA sequence was determined. Comparison using BLAST with 16S rDNA sequences available in GenBank and EMBL revealed that strain MV1087<sup>T</sup> was phylogenetically affiliated to the low-G + C-content Gram-positive bacteria. The nearest published relatives were *Thermohalobacter berrensis*, *Clostridium acidurici*, *Clostridium purinilyticum* and *Eubacterium angustum*, with respective sequence similarity values of 92, 91, 91 and 89%. These species belong to cluster XII of the genus *Clostridium* (nomenclature of Collins *et al.*, 1994). The highest percentage similarity, 98.5%, was obtained to the 16S rDNA sequence of an undescribed strain,



**Fig. 3.** Phylogenetic position of strain MV1087<sup>T</sup> within cluster XII of the *Clostridium* subphylum of Gram-positive bacteria. A total of 1302 sites were used for the phylogenetic analysis. Alignment was performed with representatives of cluster XII, with representatives of cluster XIII as an outgroup. The topology shown is an unrooted tree obtained by a neighbour-joining algorithm (Jukes & Cantor corrections) established using PHYLONWIN and performed manually using SEAVIEW. Significant bootstrap values (calculated from 500 trees) are indicated as percentages at the branching points. Bar, 5.9 nt substitutions per 100 nt.

'*Eubacterium thermomarinus*' (accession no. L10086). This strain was isolated from the gut contents of a polychaete worm, *Paralvinella* sp., collected on a hydrothermal vent from the Juan de Fuca Ridge in the north-east Pacific (J. A. Baross, personal communication). An alignment was generated using 16S rDNA sequences of anaerobic species of clusters XII and XIII of the genus *Clostridium* (*sensu* Collins *et al.*, 1994) (Fig. 3). The position of MV1087<sup>T</sup> within cluster XII of the genus *Clostridium*, close to *Thermohalobacter berrensis*, *Clostridium acidurici*, *Clostridium purinilyticum* and *Eubacterium angustum*, was supported by the three algorithms used: neighbour-joining, maximum-parsimony and maximum-likelihood.

## DISCUSSION

Isolate MV1087<sup>T</sup> is an anaerobic, heterotrophic thermophile of the domain *Bacteria*. The growth rate and DNA base composition showed the distinctiveness of isolate MV1087<sup>T</sup> among the thermophilic, anaerobic bacteria. The usual doubling times of anaerobic, thermophilic bacteria are around 50–120 min (Wiegel, 1992); strain MV1087<sup>T</sup> has a short doubling time of 15 min. The G+C content of the DNA, 27 mol%, is one of the lowest for a thermophilic, anaerobic bacterium. The previously observed values range from 25.6 (*Caloramator indicus*) to 56 mol% (*Clostridium thermoautotrophicum*) (Wiegel, 1992; Chrisostomos *et al.*, 1996).

The 16S rDNA sequence similarity analysis and 16S rDNA phylogenetic analyses placed MV1087<sup>T</sup> close to *Thermohalobacter berrensis*, *Clostridium acidurici*, *Clostridium purinilyticum* and *Eubacterium angustum*, within cluster XII of the *Clostridium* subphylum in the Gram-positive bacteria lineage (nomenclature of Collins *et al.*, 1994). The DNA G+C contents were

also similar between MV1087<sup>T</sup> (27 mol%), *Clostridium acidurici* (28 mol%) and *Clostridium purinilyticum* (29 mol%) (Dürre *et al.*, 1981; Cato *et al.*, 1986). As with MV1087<sup>T</sup>, *Thermohalobacter berrensis* is an anaerobic, thermophilic, non-sporulating, rod-shaped bacterium and ferments sugars (Cayol *et al.*, 2000). However, the 16S rDNA similarity values with the 16S rDNA sequences of these four species were less than 93% and some phenotypic characteristics of strain MV1087<sup>T</sup> are completely distinct. Isolate MV1087<sup>T</sup> is adapted to the salinity of the ocean, with optimum growth at 2% NaCl. *Thermohalobacter berrensis*, isolated from a solar saltern, is a moderate halophile, growing in the presence of 2–15% NaCl with an optimum of 5% NaCl (Cayol *et al.*, 2000). Moreover, the DNA G+C content of *Thermohalobacter berrensis*, 33 mol%, is 6 mol% higher than that of isolate MV1087<sup>T</sup>. Strain MV1087<sup>T</sup> and *Thermohalobacter berrensis* do not use the same carbohydrates. In contrast to *Thermohalobacter berrensis*, strain MV1087<sup>T</sup> is able to use arabinose, ribose, xylose, galactose and sorbose, but not cellobiose, maltose, sucrose, glycerol or mannitol. Isolate MV1087<sup>T</sup> is also distinct from *Clostridium acidurici*, *Clostridium purinilyticum* and *Eubacterium angustum*. It is thermophilic, with an optimum growth temperature of 65 °C, while the other three bacterial species grow optimally at 36–37 °C (Beuscher & Andreesen, 1984). Furthermore, in contrast to isolate MV1087<sup>T</sup>, which is unable to grow without NaCl, these species are not known to require sea salt or NaCl for growth. Some morphological characteristics also distinguish the marine isolate MV1087<sup>T</sup> from the three terrestrial species. It stained Gram-negative in all phases of growth, while *Clostridium purinilyticum* stained Gram-positive and *Clostridium acidurici* stained Gram-variable. *Eubacterium angustum* is non-motile. MV1087<sup>T</sup> is

also metabolically more diverse than *Eubacterium angustum*, *Clostridium acidurici* and *Clostridium purinilyticum*. These three species, although they belong to two different genera, are all specialists. They are neither proteolytic nor saccharolytic and grow on purines such as uric acid and adenine but not on sugars or amino acids (except for glycine). In complete contrast, MV1087<sup>T</sup> is saccharolytic, is able to grow on carbohydrates such as glucose and starch and is proteolytic.

The present work is the first description of an anaerobic strain of the *Clostridium* subphylum isolated from deep-sea hydrothermal vents. Isolate MV1087<sup>T</sup> is distinct from its phylogenetically affiliated species and also has very novel characteristics among the anaerobic thermophilic bacteria. We propose that it should be assigned to a new species of a new genus of the low-G+C-content Gram-positive lineage, *Caloranaerobacter azorensis* gen. nov., sp. nov.

#### Description of *Caloranaerobacter Wery*, *Cambon-Bonavita* and *Barbier* gen. nov.

*Caloranaerobacter* (Ca.lor.an.ae.ro.bac'ter. L. n. calor heat; Gr. pref. an not; Gr. n. aer air; N.L. bacter masc. equivalent of Gr. neut. n. bakterion rod or staff; N.L. masc. n. Caloranaerobacter a thermophilic, anaerobic rod).

Rod-shaped bacterium that stains Gram-negative. Thermophilic, adapted to the pH and salinity of ocean waters. Anaerobic, chemo-organotrophic, able to ferment carbohydrates and proteinaceous substrates. Sulfur is not necessary for growth. The G+C content is 27 mol%. 16S rDNA sequence comparisons locate *Caloranaerobacter* in the lineage of the low-G+C-content Gram-positive bacteria, within cluster XII of the *Clostridium* subphylum. The type species is *Caloranaerobacter azorensis*.

#### Description of *Caloranaerobacter azorensis* Wery, *Cambon-Bonavita* and *Barbier* sp. nov.

*Caloranaerobacter azorensis* (a.zo.ren'sis. N.L. masc. adj. azorensis of the Azores).

Cells are rod-shaped, motile and stain Gram-negative. Under optimal conditions, cells appear as short rods (0.5–2 µm long by 0.3–0.5 µm wide), singly, in pairs or in short chains (fewer than five cells). Growth occurs between 45 and 65 °C (optimum 65 °C), pH 5.5 and 9 (optimum 7) and 10 and 100 g sea salts l<sup>-1</sup> (optimum 30 g sea salts l<sup>-1</sup>). The optimal doubling time and maximum cell yield are respectively 15 min and 2 × 10<sup>9</sup> cells ml<sup>-1</sup>. Anaerobic. Chemo-organotrophic. Able to ferment gluten, brain/heart infusion, glucose, starch, xylan and pyruvate. The G+C content is 27 ± 1 mol%. 16S rDNA sequence similarity of 92% to *Thermohalobacter berrensensis* and 91% to *Clostridium acidurici* and *Clostridium purinilyticum*. The GenBank/EMBL accession number for the 16S rDNA sequence is AJ272422.

The type strain, MV1087<sup>T</sup> (= CNCM I-2543<sup>T</sup> = DSM 13643<sup>T</sup>), was isolated from chimney rocks collected on the Lucky Strike hydrothermal site on the Mid-Atlantic Ridge (32° 16' W, 37° 17' N; depth 1650 m).

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