

Caminicella sporogenes gen. nov., sp. nov., a novel thermophilic spore-forming bacterium isolated from an East-Pacific Rise hydrothermal vent

^{1,4,5} Laboratoire de Microbiologie et de Biotechnologie des Extrémophiles, Département de Valorization des Produits¹, Laboratoire de Géochimie et de Métallogénie, Département Géosciences Marines⁴, Laboratoire 'Biotechnologie et Molécules Marines', Département de Valorization des Produits⁵, IFREMER, Centre de Brest, BP 70, 29280 Plouzané, France

² UMR 7622, Laboratoire de Biologie Cellulaire et Moléculaire du Développement, 7 quai St Bernard, 75252 Paris Cédex 05, France

³ Laboratoire de Microbiologie Industrielle, URCA, UFR Sciences, BP 1039, Moulin de la Housse, 51687 Reims Cedex 2, France

⁶ Laboratoire Environnement Profond, Direction des Ressources Océaniques, IFREMER, Centre de Brest, BP 70, 29280 Plouzané, France

Karine Alain,¹ Patricia Pignet,¹ Magali Zbinden,² Morgane Quillevere,¹ Francis Duchiron,³ Jean-Pierre Donval,⁴ Françoise Lesongeur,¹ Gérard Ragueneas,⁵ Philippe Crassous,⁶ Joël Querellou¹ and Marie-Anne Cambon-Bonavita¹

Author for correspondence: Karine Alain. Tel: +33 2 98 22 45 53. Fax: +33 2 98 22 47 57. e-mail: karine.alain@ifremer.fr

A novel thermophilic, anaerobic, strictly chemoorganoheterotrophic bacterium, designated as AM1114^T, was isolated from a deep-sea hydrothermal vent sample from the East-Pacific Rise (EPR 13° N). The cells were long (3–10 µm) rods, motile with peritrichous flagella, and exhibited a Gram-negative cell wall ultrastructure. In the late stationary phase of growth, cells formed an ovoid, refractile, terminal endospore. They grew at 45–65 °C inclusive (optimum 55–60 °C; doubling time approx. 45 min), at pH 4.5–8.0 inclusive (optimum pH 7.5–8.0) and at sea salt concentrations of 20–60 g l⁻¹ inclusive (optimum 25–30 g l⁻¹). Strain AM1114^T was an obligately heterotrophic bacterium able to ferment a mixture of 20 amino acids, complex proteinaceous substrates (such as yeast extract, brain–heart infusion or peptone), and carbohydrates such as glucose, galactose or maltose. The main fermentation products on glucose/yeast extract/peptone/sulfur medium were hydrogen, carbon dioxide, butyrate, ethanol, acetate, formate and L-alanine. The G+C content of the genomic DNA (determined by thermal denaturation) was 24.2±1 mol%. Phylogenetic analyses of the 16S rRNA gene located the strain within cluster XI of the lineage encompassing the genus *Clostridium* and related genera (*sensu* Collins *et al.*, 1994), in the bacterial domain. On the basis of 16S rDNA sequence comparisons and physiological and biochemical characteristics, it is proposed that the isolate should be described as a novel genus, namely *Caminicella* gen. nov., of which *Caminicella sporogenes* sp. nov. is the type species. The type strain is AM1114^T (= DSM 14501^T = CIP 107141^T).

Keywords: deep-sea hydrothermal vent, thermophile, *Clostridiales*, *Caminicella sporogenes*

INTRODUCTION

Over the past decade, microbiological investigations of deep-sea hydrothermal vents from a number of geographically distant sites have revealed the presence of a

phylogenetically and physiologically diverse community of thermophilic and hyperthermophilic microorganisms thriving in the hottest part of the hydrothermal vent ecosystem. Representative species of both the *Bacteria* and the *Archaea* have been isolated from deep-sea vents. Physiological types isolated from this biotope include chemolithoautotrophs, chemoorganoheterotrophs and mixotrophs (Jeanthon, 2000). Recently, several molecular phylogenetic approaches conducted by means of the comparison of 16S rRNA gene sequences have revealed a plethora of novel

Published online ahead of print on 18 January 2002 as DOI 10.1099/ijms.0.02142-0.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain AM1114^T is AJ320233.

microbial lineages previously unknown for deep-sea hydrothermal vents (Polz *et al.*, 1995; Moyer *et al.*, 1995, 1998; Takai *et al.*, 1999; Reysenbach *et al.*, 2000; Longnecker *et al.*, 2001). In this work, we report the isolation and characterization of a strain belonging to cluster XI of the 'Clostridia and relatives' group (according to the scheme of Collins *et al.*, 1994).

The *Clostridiales* constitute one of the largest of the eubacterial taxa. It encompasses a complex range of Gram-negative or Gram-positive, psychrophilic, mesophilic or thermophilic, spore-forming or non-spore-forming, chemoorganoheterotrophic or chemolithotrophic bacteria that can be found in a variety of habitats, and includes a large number of eukaryotic pathogens (Cato *et al.*, 1986). Among this group of micro-organisms, 19 clusters have been defined by Collins *et al.* within the genus *Clostridium* and related genera, on the basis of phylogenetic 16S rDNA sequence analysis (Collins *et al.*, 1994). However, the inability to identify clear-cut phenotypic discriminatory properties essential for circumscribing clusters defined only by phylogenetic analysis hinders the reclassification process and leads to 'artificial' clustering. Consequently, the genus *Clostridium*, as well as other genera placed in the *Clostridium* subphylum of the low-G+C Gram-positive bacteria, is always in revision in order to make the taxonomy consistent with the phylogeny (Rainey *et al.*, 1993; Stackebrandt *et al.*, 1999).

Members of cluster XI of the 'Clostridia and relatives' group exhibit a wide range of phenotypes (Andreesen *et al.*, 1970; Adamse, 1980; Braun *et al.*, 1981; Lux *et al.*, 1992; Jalava *et al.*, 1999; Ravot *et al.*, 1999). Notably, this cluster comprises few extreme micro-organisms such as alkaliphiles (Li *et al.*, 1993, 1994; Collins *et al.*, 1994; Kevbrin *et al.*, 1998; Takai *et al.*, 2001), moderate thermophiles (Li *et al.*, 1993, 1994) and halophiles (Fendrich *et al.*, 1990). Although this cluster encompasses an extremely diverse range of metabolic and physiological properties, this group is phylogenetically well defined (Collins *et al.*, 1994).

In 1999, hydrothermal samples were collected from a deep-sea vent located on the East-Pacific Rise (EPR 13° N). We describe the isolation (from one of these samples) and characterization of a thermophilic, spore-forming bacterium having phenotypic and phylogenetic characteristics consistent with its assignment to a new genus within the *Clostridiales*.

METHODS

Collection of samples. In 1999, a deep-sea vent field located on the East-Pacific Rise [Elsa (HOT 3) 103° 56' 326 W, 12° 48' 200 N] was explored during the 'Advanced Microbiological Studies of Thermophiles: Adaptations and Diversity' oceanographic cruise. White entire tube samples of the hydrothermal vent polychaete *Alvinella pompejana*, which inhabits the surface of active sulfide structures ('chimneys', 'smokers'), attached to small fragments of chimney rocks, were collected by the man-operated *Nautilus*

submersible. After collection, the samples were immediately transferred into an anaerobic chamber and then, after removal of the tubes' inner contents, placed in 50 ml serum vials filled with a sterile solution of 3% sea salts (v/v) (Sigma). They were stored at 4 °C until enrichment in the laboratory.

Enrichment and purification procedures. Cultures were enriched and purified anaerobically in a medium containing the following (per l): 30 g sea salts (Sigma), 6.05 g PIPES buffer (Sigma), 0.2 g yeast extract (Difco), 0.2 g D(+)-glucose (Sigma), 0.2 g dextran (produced by *Leuconostoc mesenteroides* strain B-512, mol. wt 148000; Sigma), 2 g sterile *A. pompejana* pulverized tubes, 0.5 ml vitamin mixture (Balch *et al.*, 1979), 10 ml trace-element solution (Balch *et al.*, 1979) and 1 mg resazurin (Sigma). The medium was adjusted to pH 7.0, at room temperature, before being autoclaved. All manipulations preceding the enrichment and isolation experiments were performed in an anaerobic chamber, under an atmosphere of N₂/CO₂/H₂ (90:5:5). The sterile medium was reduced using 0.5 g l⁻¹ sodium sulfide, then distributed into serum vials before inoculation. Enrichment cultures were performed at 60 °C without agitation by inoculating a 1 ml sample directly into each vial filled with 25 ml medium. After the enrichment experiments, positive cultures were subcultured and then purified by using the dilution-to-extinction technique (Baross, 1995). The purity of the strain was confirmed by microscopic observations and by cloning and sequencing of 10 16S rDNA clone genes.

Culture medium and conditions. The new isolate was grown on glucose/yeast extract/peptone/sulfur (GYPS) medium containing the following (per litre): 30 g sea salts (Sigma), 6.05 g PIPES buffer (Sigma), 0.5 g yeast extract (Difco), 1 g bacto-peptone (Difco), 5 g D(+)-glucose (Sigma), 12 g elemental sulfur (Prolabo) and 0.1% (v/v) resazurin solution. Its pH was adjusted to 7.5 before autoclaving, and the medium was reduced by 0.5 g sodium sulfide l⁻¹ before inoculation. Unless indicated otherwise, cultures were incubated at 60 °C under the anaerobic chamber gas mixture, at atmospheric pressure.

Observation of the culture, and quantification. Cells were observed under a light microscope (model BH2; Olympus) equipped with a phase-contrast oil-immersion objective (× 100 magnification). Cells were quantified by direct cell counting using a Thoma chamber (depth, 0.02 mm). Alternatively, cells were fixed with 1% glutaraldehyde (v/v) for 45 min at room temperature and stored at -20 °C before being counted in a Thoma chamber.

Morphology. The cells were Gram-stained by using the Bacto 3-step Gram stain Set-S (Difco). The Gram type was determined by using the Ryu non-staining KOH test (Ryu, 1940; Buck, 1982; Powers, 1995). The SpotTest flagella stain (Difco) was used for flagella detection.

For scanning electron microscopy, cells were fixed with 10% formaldehyde (v/v) for 1 h, displayed on filters (0.22 µm pore size; Nucleopore) and dried overnight at room temperature. Samples were then coated with gold (SCD040; Balzers) and examined with a scanning electron microscope (XL 30 LaB6; Philips).

For transmission electron microscopy, cultures were fixed at room temperature in a fixative solution containing 3% glutaraldehyde (v/v), 0.2 M cacodylate buffer, pH 7.4, and 0.35 M sucrose. After centrifugation at 2000 g for 20 min, the fixative was carefully removed and replaced with the

washing solution. Post-fixation in osmium tetroxide (1 vol. in 1 vol. of 0.2 M cacodylate buffer) was performed. After post-fixation and washing, the pellets were wrapped in agar (2% solution), according to Pottu-Boumendil (1989). Agar blocks containing the cells were cut into small cubes (1 mm³). These cubes were then dehydrated in ethanol and propylene oxide series and further embedded in Araldite CY212 (TAAB) according to a routine procedure. Ultrathin sections (50 nm) were obtained on a Reichert–Jung ultramicrotome. Transmission electron microscopy observations were performed on an LEO 912 electron microscope, operated at 120 kV with a LaB₆ source.

Determination of growth parameters. To determine the optimum temperature, pH and sea-salts concentration, cells were grown in Hungate tubes (15 ml; Bellco) containing 6 ml GYPS medium. Experiments were performed in thermostatic aluminium heating blocks (Bioblock) monitored with temperature probes placed in control tubes. To determine the effect of pH on the growth, the GYPS medium was modified with the following 10 mM buffers (Sigma): for pH 3.0, 4.0 and 4.5, no buffer; for pH 5.0, 5.5 and 6.0, MES buffer; for pH 6.5 and 7.0, PIPES buffer; for pH 7.5 and 8.0, HEPES buffer; and for pH 8.5 and 9.0, AMPSO [3-(1,1-dimethyl-2-hydroxyethyl)-amino-2-hydroxypropanesulfonic acid] buffer. Sodium sulfide was added in the anaerobic chamber and, if necessary, the pH was adjusted with 0.1 M HCl or 0.1 M NaOH. To determine the concentration of salts required for growth, the GYPS medium was prepared with different dilutions of sea salt. Cells were then incubated at the optimal temperature and pH for growth. The effects of temperature, pH and salinity were determined by measuring growth rates calculated using linear regression analysis from four to seven points along the logarithmic portions of the growth curves. All growth experiments were carried out in triplicate.

Determination of growth requirements. The ability of the new isolate to grow in the presence of different electron acceptors was investigated on GYPS medium in which elemental sulfur had been replaced with one of the following electron acceptors: L-cystine was tested at 50 mM, polysulfides (Blumentals *et al.*, 1990) and ferric iron were tested at 10 mM, and thiosulfate, sulfate, sulfite, nitrate and nitrite were tested at 20 mM. Growth in the absence of elemental sulfur was also tested.

The ability of strain AM1114^T to use a single carbon source was investigated by adding one of the following organic compounds to the GYPS medium from which carbon sources had been omitted, and which was supplemented with 0.5 ml l⁻¹ vitamin solution (Balch *et al.*, 1979): 2 g l⁻¹ NaHCO₃, 10 ml l⁻¹ trace-element solution (Balch *et al.*, 1979) and 1 ml l⁻¹ selenite/tungstate solution (6 mg l⁻¹ Na₂SeO₃·5H₂O; 0.8 mg l⁻¹ Na₂WO₄·2H₂O; 0.4 g l⁻¹ NaOH). D(+)-Glucose and maltose were added at a final concentration of 0.5% (w/v), while yeast extract and brain–heart infusion were tested at 0.2% (w/v). The ability of the strain to grow on pairs of amino acids (a wide range of amino acid pairs capable of being used via the Stickland reaction was tested) or on a mixture of 20 amino acids was also tested (for these experiments, the concentration of each amino acid was 0.1 g l⁻¹). The following amino acids, which were rapidly consumed when the strain was cultivated on a mixture of 20 amino acids, were also tested singly at 0.2 g l⁻¹: L-serine, L-proline, L-asparagine, L-tryptophan, L-cysteine, L-isoleucine, L-leucine and L-phenylalanine. For the other carbon sources tested, a small amount of yeast extract

(0.01% w/v) was used in GYPS basal medium for culture induction. The following carbon sources were tested: gelatin, glycogen (from oyster), maltose, D(+)-glucose, D(-)-fructose, D(+)-galactose, starch (soluble), dextran (from *L. mesenteroides* strain B-512, *M_r* 148 000; Sigma), cellulose, xylan (from oat spelts), chitin, D(+)-cellobiose were tested to a final concentration of 0.5% (w/v), while peptone, tryptone, formate and acetate were added at a final concentration of 0.2% (w/v), and methanol at a final concentration of 0.5% (v/v).

The ability of the strain to grow by homoacetogenesis was tested on a medium that had the following composition: sea salts, 30 g l⁻¹; PIPES buffer, 6.05 g l⁻¹; NH₄Cl, 1 g l⁻¹; NaHCO₃, 10 g l⁻¹; yeast extract, 0.1 g l⁻¹; resazurin solution, 0.1% (v/v); KH₂PO₄, 0.35 g l⁻¹; K₂HPO₄, 0.35 g l⁻¹; Na₂S·9H₂O, 0.5 g l⁻¹; selenite/tungstate solution, 1 ml l⁻¹; trace-element solution (Balch *et al.*, 1979), 10 ml l⁻¹; vitamin solution (Balch *et al.*, 1979), 0.5 ml l⁻¹. This experiment was performed under an H₂/CO₂ (80:20; 200 kPa) gas phase.

All these tests were performed in duplicate, and positive cultures were transferred to confirm growth. Growth was determined by direct cell counting in a Thoma chamber (depth, 0.02 mm) with a phase-contrast microscope.

Analytical techniques. All of the analyses of the metabolic end-products were carried out in triplicate. The amino acid, organic acid and aromatic acid metabolic end-products were analysed by means of HPLC (Alliance 2690; Waters) as described by Wery *et al.* (2001b). Proteins from the supernatant of the culture were precipitated with a solution of 5-sulfosalicylic acid at 2% (w/v). Amino acids were studied after derivatization [with an ethanol/water/triethylamine/phenylisothiocyanate (7:1:1:1 by vol.) solution] under the conditions specified for the Waters Pico Tag method (WAT007360; Waters). Alcohols, glucose and linear organic acids were studied on an H⁺ exclusion column (polyspher OAKC 1.51270; Merck) at 60 °C with a 18 mM H₂SO₄ elution (0.35 ml min⁻¹) and with differential refractometer (refractometer 410; Waters) detection. For analysis of aromatic acids, the supernatant was eluted on an H⁺ exclusion column (column 28352; Chrompack) at 65 °C by a 4.5 mM H₂SO₄ solution (0.5 ml min⁻¹) and detected by absorbance at 210 nm (UV detector 486; Waters).

The gas composition was analysed using a AGILENT M200 gas microchromatograph equipped with two parallel analysis tracks [a molecular sieve-packed column (10 m) and a Poraplot U column (8 m)] and a thermal conductivity detector. Helium was used as the carrier gas.

H₂S determination. H₂S formation was detected by the addition of 500 µl 5 mM CuSO₄/50 mM HCl to 0.2 ml culture. A brown precipitate demonstrated the presence of H₂S.

Susceptibility to antibiotics. Sensitivity to antibiotics was estimated by using 25, 50, 75 and 100 µg ml⁻¹ solutions of the following antibiotics: chloramphenicol, kanamycin, penicillin G, rifampicin, streptomycin and vancomycin. Antibiotic solutions were added to GYPS medium just before inoculation. When the antibiotic was diluted in ethanol (chloramphenicol) or DMSO (rifampicin), the same volume of solvent was added to the control cultures.

DNA extraction and purification. Genomic DNA was extracted as described by Wery *et al.* (2001a). The concentration and purity of the genomic DNA obtained were estimated by using a GenQuant II spectrophotometer

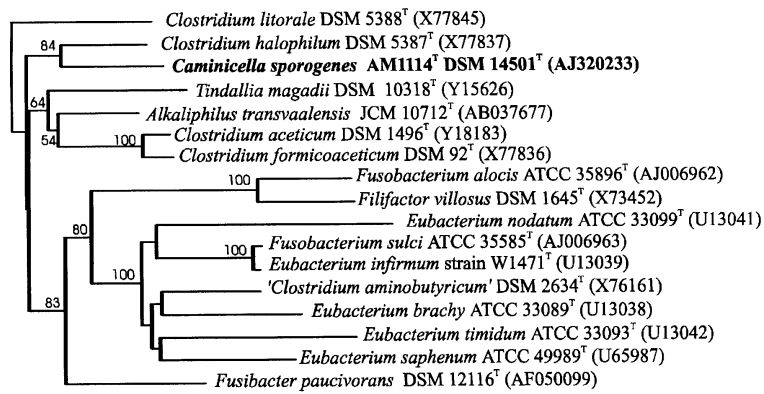


Fig. 1. Phylogenetic position of strain AM1114^T within the *Clostridiales*. The alignment was performed with 16S rDNA sequences of related species of *Clostridium* cluster XI. *Clostridium litoreale* was used as an outgroup. Accession numbers are indicated in parentheses. The topology shown corresponds to an unrooted tree obtained using a neighbour-joining algorithm (Kimura corrections) established using PHYLO_WIN and manually refined using SEAVIEW. Bootstrap values are displayed on their relative branches. The scale bar indicates 6.0 nt substitutions per 100 nt.

(Pharmacia) at 260, 280 and 320 nm. The quality of the extraction was checked on a 0.8% (w/v) agarose gel containing 0.5 µg ethidium bromide ml⁻¹.

DNA base content. The DNA was purified by caesium chloride gradient centrifugation (Sambrook *et al.*, 1989), and its purity was checked spectrophotometrically. The G+C content of the genomic DNA was determined from the melting point, according to the methods of Marmur *et al.* (1962), under the conditions reported by Raguénès *et al.* (1997). A calibration curve was constructed by using ultrapure DNA, as described by Wery *et al.* (2001a).

Amplification of the 16S rDNA. The 16S rDNA was selectively amplified from purified genomic DNA by a PCR with oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of the 16S rRNA genes. The forward primer was SAdir (5'-AGAGTTTGATCATGGC-TCAGA-3', corresponding to positions 8–28 in the *Escherichia coli* 16S rRNA) and the reverse primer was S17rev (5'-GTTACCTTGTTACGACTT-3', corresponding to positions 1493–1509). The initial denaturation step consisted of heating of the reaction mixture to 94 °C for 3 min. This was followed by 30 cycles as follows: denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min 30 s, and extension at 72 °C for 2 min. A final extension step was carried out at 72 °C for 6 min. The PCR products were analysed 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, and recorded with a Fluor-S multiImager (Bio-Rad).

16S rDNA sequence analysis. The 16S rRNA gene was double-strand sequenced with the primers described by Raguénès *et al.* (1996). This work was done by Genome Express (Grenoble, France) with an automatic DNA-analysis system (Applied Biosystems); 1456 positions of the 16S rDNA sequence were determined. The CLUSTAL W method with weighted residues was used to align the sequences of representative *Clostridiales* and to calculate similarity levels (Thompson *et al.*, 1994). The 16S rDNA sequence alignment was refined manually by using the multiple sequence alignment editor SEAVIEW, and a phylogenetic reconstruction was produced using PHYLO_WIN (Galtier *et al.*, 1996) with the following settings: neighbour-joining (Saitou *et al.*, 1987) with the correction of Kimura (1980), maximum-parsimony (Lake, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap values were determined according to Felsenstein (1985). The strains and 16S rDNA sequences used for phylogenetic analysis are given in Fig. 1.

RESULTS AND DISCUSSION

Enrichment and isolation

Growth of motile, rod-shaped micro-organisms was observed in enrichment cultures after 37 h incubation at 60 °C. The enriched cultures were successfully subcultured, and one isolate was purified and referenced as strain AM1114^T (= DSM 14501^T = CIP 107141^T).

Phylogenetic analysis

The almost complete sequence (1456 bp) of the 16S rDNA gene of strain AM1114^T was determined. The phylogenetic position of this organism was determined by comparing its 16S rDNA sequence with those of representative ones from the 19 *Clostridiales* clusters (according to the scheme of Collins *et al.*, 1994). For this phylogenetic reconstruction, *Bacillus subtilis* (GenBank/EMBL accession no. AB016721) was used as an outgroup. According to this first reconstruction, it was concluded that strain AM1114^T belonged to cluster XI of the lineage encompassing the genus *Clostridium* and related genera. A second phylogenetic tree was constructed, performed with species of cluster XI and one species of cluster XII (*Caloranaerobacter azorensis* DSM 13643^T, accession no. AJ272422; Wery *et al.*, 2001b) as an outgroup. It showed that the sequence of AM1114^T was related to the *Clostridium halophilum* subcluster. Cluster XI is very large, and, consequently, the final phylogenetic reconstruction (Fig. 1) was performed only with closely related species among this cluster (with *Clostridium litoreale* as an outgroup). The pairwise evolutionary distances based on 1441 unambiguous nucleotides were computed using the Kimura two-parameter model (Kimura, 1980), and a dendrogram was constructed from these distances using the neighbour-joining method. The species most closely related to AM1114^T was *Clostridium halophilum* (91% 16S rDNA sequence similarity). It was followed by *Clostridium formicoaceticum* (90%), *Clostridium aceticum* (90%), *Alkaliphilus transvaalensis* (90%) and *Tindallia magadii* (90%). The positioning of strain AM1114^T was supported by the results of the three algorithms used: neighbour

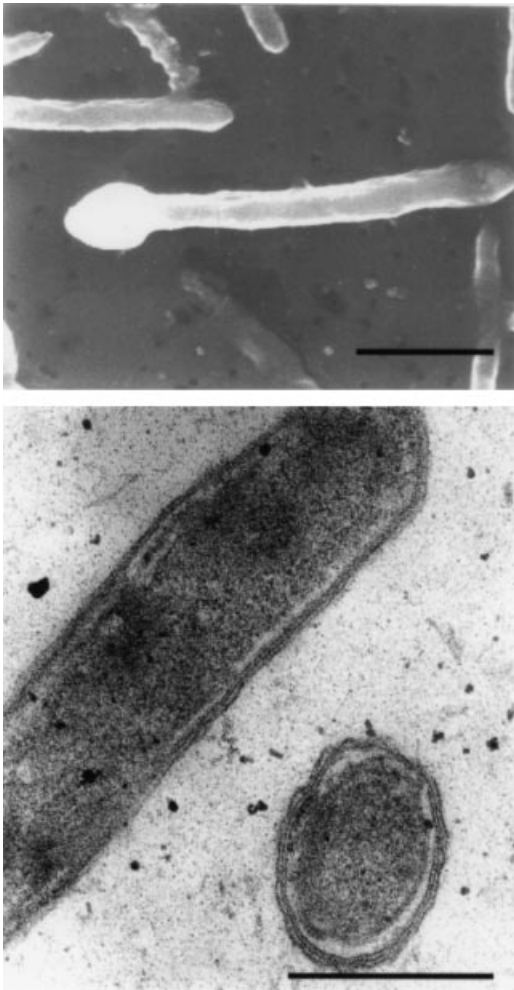


Fig. 2. (top) Scanning electron micrograph of strain AM1114^T in the mid-exponential phase of growth, showing a sporulated cell. Bar, 2 μm . (bottom) Transmission electron micrograph of strain AM1114^T in the mid-exponential phase of growth, showing a cell wall with (from the inside to the outside) a cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane – the characteristic cell wall ultrastructure of Gram-negative bacteria. Bar, 1 μm .

joining, maximum parsimony and maximum likelihood. The topology of the phylogenetic tree (Fig. 1) was also supported by the three algorithms used, except in the case of some branches (bootstraps < 50%). To our knowledge, this is the first report of the isolation of a member of cluster XI (*sensu* Collins *et al.*, 1994) within the lineage of the genus *Clostridium* and related genera from a deep-sea hydrothermal vent.

The genus *Clostridium* is intermixed with members of different genera, exhibiting – or not exhibiting – a combination of *Clostridium*-type properties. The taxonomic definition of the genus *Clostridium*, proposed by Prazmowski (Hippe *et al.*, 1992), comprised only four (combined) criteria, i.e (1) an ability to form endospores; (2) an anaerobic type of energy metabolism;

(3) an inability to carry out the dissimilatory reduction of sulfate; and (4) a Gram-positive cell wall ultrastructure. Strain AM1114^T does not come close to meeting this definition.

Morphological and physiological characteristics

Scanning electron microscopy and phase-contrast microscopy indicated that cells of isolate AM1114^T were thin, straight to slightly curved rods approximately 0.5–0.7 μm wide and 3–10 μm long (mean length, 6 μm) during the exponential phase of growth. They appeared as single cells (Fig. 2, top) or in pairs. Under unfavourable growth conditions and in the stationary phase of growth, cells elongated; occasionally, cells that were 17 μm long were observed. The cells stained Gram-negative, and electron microscopy of thin sections of strain AM1114^T revealed the presence, in the cell wall, of a thin peptidoglycan layer characteristic of Gram-negative bacteria (Fig. 2, bottom). Moreover, the KOH reaction was positive, confirming the Gram-negative type of AM1114^T cells. In the late stationary phase of growth, cells formed an ovoid, refractile, terminal endospore (Fig. 2, top). Both non-sporulated and sporulated cells were motile by means of peritrichous flagella.

This isolate is not an exception among Gram-negative bacteria of the *Firmicutes*. In fact, the genus *Clostridium sensu lato* is polyphyletic and is related phylogenetically to several Gram-negative-staining taxa (for example Broda *et al.*, 2000; Wery *et al.*, 2001b).

Strain AM1114^T grew at 45–65 °C, the optimum being around 55–60 °C. No growth was observed at 40 or 70 °C. The strain required NaCl for growth, and grew at sea-salt concentrations of between 20 and 60 g l⁻¹ (corresponding to 15–46 g NaCl l⁻¹). The optimum sea-salt concentration was approximately 25–30 g l⁻¹ (corresponding to 19–23 g NaCl l⁻¹). Growth occurred at pH 4.5–8.0, the optimum pH being around 7.5–8.0.

Metabolic properties

Strain AM1114^T is an anaerobic, obligately heterotrophic bacterium unable to grow by homoacetogenesis. The isolate was able to grow on complex proteinaceous substrates and carbohydrates. Very active growth was observed on yeast extract as the sole energy and carbon source. Although weak growth occurred on a mixture of 20 amino acids, the strain was unable to grow on amino acid pairs via the Stickland reaction.

On the other hand, the other substrates tested as sole carbon sources did not allow growth. In the presence of 0.01% (w/v) yeast extract, growth on brain–heart infusion, D(+)-glucose and maltose was supported. Poor growth was observed on peptone and on D(+)-galactose (in the presence of 0.01%, w/v, yeast extract). None of the other organic acids, alcohols or

carbohydrates tested in combination with 0.01% (w/v) yeast extract supported growth. No requirement for an external electron acceptor was observed. Despite the fact that elemental sulfur, L-cystine and thiosulfate did not clearly enhance growth, they were reduced to hydrogen sulfide. Conversely, no growth was observed in the presence of nitrites, sulfites and polysulfides. Ferric iron, sulfates and nitrates did not enhance growth. These data suggest that the isolated strain is an obligately fermentative bacterium.

The lineage that encompasses the genus *Clostridium* and related genera is metabolically heterogeneous, containing a large proportion of fermentative micro-organisms (Hippe *et al.*, 1992; Rainey *et al.*, 1993; Takai *et al.*, 2001). The isolate does not stand out from the majority. It is a strictly chemoorganoheterotrophic bacterium able to ferment complex proteinaceous substrates and carbohydrates.

Under optimal growth conditions (with yeast extract as a carbon and energy source, sulfur as an external electron acceptor, and at optimal temperature, pH and salinity), the final concentration in vials was around 8×10^8 cells ml⁻¹, and the generation time was approximately 45 min.

During fermentation on GYPS medium, strong growth was observed. Notably, a high consumption of glucose (i.e. 2.3 g l⁻¹) concomitant with high levels of production of hydrogen, carbon dioxide, acetate (7.50 mM), butyric acid (0.82 mM) and ethanol (10.7 mM) was observed (the values given above and below were performed at the end of the exponential phase of growth). Weak production of butanol was also observed (0.1 mM). Moreover, 0.05 mM succinate and the small amounts of free amino acids (aspartate, glutamate, asparagine, serine, histidine, methionine, cysteine, isoleucine, leucine, phenylalanine, tryptophan and lysine) available in the medium were consumed. At the same time, formate (3 mM) and L-alanine (0.08 mM) were produced. Weak increases in the hydroxyphenylacetate, phenylacetate, propionate, isobutyrate, isovalerate and pyruvate concentrations in the medium were also measured, together with small increases in the glycine and proline concentrations (probably obtained by degradation of the yeast-extract proteins). When the strain was cultivated in a medium containing a mixture of 20 amino acids (each at a concentration of 0.1 g l⁻¹), weak growth was observed, and serine, proline, asparagine, tryptophan, cysteine, isoleucine, leucine and phenylalanine were partially consumed.

Thus, the results of the analysis of the metabolic end-products clearly indicated that the strain is a heterofermentative bacterium that degrades glucose via the butyric acid fermentation pathway. This fermentation pathway is widely distributed among the genus *Clostridium* and related genera. Catabolic end-products of glucose fermentation included the butyrate produced via the butyrate pathway, and acetate, ethanol, hydrogen and carbon dioxide produced via secondary

parallel pathways. However, acetone and 2-propanol were not produced, indicating that the acetone/2-propanol parallel pathway was not activated. The inhibition of this pathway could be due to the high concentrations of carbon dioxide produced in the medium, as has been described previously for many clostridia.

On the other hand, a significant amount of L-alanine was excreted into the medium during glucose fermentation. This phenomenon has been previously reported for some thermophilic micro-organisms belonging to the *Thermotogales* (Ravot *et al.*, 1996), the *Thermococcales* (Kengen *et al.*, 1994; Kobayashi *et al.*, 1995) and the *Clostridiales* (Fardeau *et al.*, 2001). It has been correlated, in most cases, with an increase in the H₂ partial pressure. The production of L-alanine could be a way of avoiding the possible 'toxic' or inhibiting effect of H₂ excesses, i.e. by attaching them to a carbon structure that would be secreted. Under cultivation in the presence of S⁰, an additional mechanism for preventing this inhibition could be the production of H₂S, as has been observed in this work.

Susceptibility to antibiotics

Growth of isolate AM1114^T was inhibited by vancomycin, chloramphenicol, rifampicin and penicillin G at the lowest concentrations tested (25 µg ml⁻¹), and by streptomycin at 50 µg ml⁻¹. The strain was resistant to 100 µg kanamycin ml⁻¹.

DNA base composition

The G+C content of the genomic DNA of strain AM1114^T was 24.2 ± 1 mol%.

Taxonomic position of the novel isolate

The closest phylogenetic relatives of strain AM1114^T were *Clostridium halophilum* (Fendrich *et al.*, 1990) (91% similarity) followed by *Clostridium formicoaceticum* (Andresen *et al.*, 1970), *Clostridium aceticum* (Braun *et al.*, 1981), *Alkaliphilus transvaalensis* (Takai *et al.*, 2001) and *T. magadii* (Kevbrin *et al.*, 1998) (mean similarity of 90%). In addition to the phylogenetic distances (9–10%), strain AM1114^T exhibited significant genotypic and phenotypic differences from its nearest phylogenetic neighbours: (1) cells of strain AM1114^T stain Gram-negative, whereas cells of *Clostridium halophilum*, *T. magadii* and *Alkaliphilus transvaalensis* stain Gram-positive; (2) *Clostridium aceticum* and *Clostridium formicoaceticum* are acetogens capable of chemolithoautotrophic and aromatic-dependent growth; (3) *T. magadii* and *Alkaliphilus transvaalensis* are true alkaliphilic micro-organisms; (4) *Clostridium halophilum* is halophilic; (5) the temperature ranges for growth are very different: the new isolate is thermophilic, and its closest phylogenetic relatives are mesophiles; (6) the G+C content of the genomic DNA of the isolate is 24.2 mol%, while those of the closest relatives range from 26.9 to 37.6 mol% (for

Clostridium halophilum and *T. magadii*, respectively). In addition, *T. magadii* was an asporogenous bacterium.

On the basis of the overall 16S rDNA dissimilarity value between strain AM1114^T and the closest described relatives (9–10%) as well as the major phenotypic and genotypic differences, we propose that AM1114^T should be assigned to a new genus within cluster XI of the *Clostridium* subphylum. Because of its origin in a hydrothermal vent, we name this genus *Caminicella*, and, because of its ability to form spores, we name the new species *Caminicella sporogenes*.

Description of *Caminicella* gen. nov.

Caminicella (Ca.mi'ni.cel'la. L. gen. n. *camini* of a chimney, relating to the hydrothermal chimney origin; L. fem. n. *cella* cell; N.L. fem. n. *caminicella* cell from a hydrothermal chimney).

Cells are rod-shaped, motile, and stain Gram-negative. Spore-forming thermophile, adapted to the pH and salinity of the ocean. Anaerobic and heterotrophic; able to ferment proteinaceous substrates and carbohydrates. 16S rDNA sequence comparisons locate the *Caminicella* genus in the domain *Bacteria*, within cluster XI of the lineage encompassing the genus *Clostridium* and related genera (Collins *et al.*, 1994).

Description of *Caminicella sporogenes* sp. nov.

Caminicella sporogenes (spo.rog.en'es. M.L. n. *spora* a spore; Gr. v. *genmaio* produce; M.L. part. adj. *sporogenes* spore-producing).

Cells are rod-shaped (3–10 µm long × 0.5–0.7 µm wide), motile by means of peritrichous flagella, and exhibit a Gram-negative cell wall ultrastructure. Sporulation is observed in the late stationary phase of growth. Growth occurs at 45–65 °C (optimum, 55–60 °C), at pH 4.5–8.0 (optimum, pH 7.5–8.0), and at sea salt concentrations of 20–60 g l⁻¹ (optimum, 25–30 g sea salt l⁻¹). The optimal doubling time is around 45 min; the maximum cell yield is 8 × 10⁸ cells ml⁻¹ in vials. Anaerobic. Able to ferment yeast extract, brain-heart infusion, D(+)-glucose, maltose, and, to a much lesser extent, peptone, galactose and a mixture of 20 amino acids. The main fermentation products on GYPS medium are H₂, CO₂, butyrate, ethanol, acetate, formate and L-alanine. The G+C content of the genomic DNA is 24.2 ± 1 mol%. The 16S rDNA similarity to *Clostridium halophilum* is 91%. The GenBank/EMBL accession number for the 16S rDNA sequence is AJ320233. The type strain, AM1114^T (= CIP 107141^T = DSM 14501^T), was isolated from young entire *Alvinella pompejana* tubes attached to small fragments of chimney rocks that were collected from the hydrothermal site Elsa (HOT3) in the East-Pacific Rise (103° 56' 326 W, 12° 48' 200 N).

ACKNOWLEDGEMENTS

We thank the chief scientists of the French oceanographical cruise AMISTAD ('Advanced Microbiological Studies of Thermophiles: Adaptations and Diversity', 1999), C. Jeanthon, the Captain and the crew of *NO Atalante*, and the *DSV Nautilie* pilots and support crew. This work was supported by the Région Bretagne and the French Dorsales Programme. Electron microscopy was performed at the 'service de Microscopie Electronique', IFR de biologie integrative, CNRS/Paris VI.

REFERENCES

- Adamse, A. D. (1980). New isolation of *Clostridium aceticum* (Wieringa). *Antonie Leeuwenhoek* **46**, 523–531.
- Andreesen, J. R., Gottschalk, G. & Schlegel, H. G. (1970). *Clostridium formicoaceticum* nov. spec. isolation, description and distinction from *C. aceticum* and *C. thermoaceticum*. *Arch Microbiol* **72**, 154–174.
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Baross, J. A. (1995). Isolation, growth and maintenance of hyperthermophiles. In *Archaea: a Laboratory Manual. Thermophiles*, pp. 15–23. Edited by F. T. Robb, A. R. Place, K. R. Sowers, H. J. Schreier, S. DasSarma & E. M. Fleischmann. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Blumentals, I. I., Itoh, M., Olson, G. J. & Kelly, R. M. (1990). Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl Environ Microbiol* **56**, 1255–1262.
- Braun, M., Mayer, F. & Gottschalk, G. (1981). *Clostridium aceticum* (Wieringa), a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. *Arch Microbiol* **128**, 288–293.
- Broda, D. M., Saul, D. J., Bell, R. G. & Musgrave, D. R. (2000). *Clostridium algidixylanolyticum* sp. nov., a psychrotolerant, xylan-degrading, spore-forming bacterium. *Int J Syst Evol Microbiol* **50**, 623–631.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium* Prazmowski 1880, 23^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Fardeau, M. L., Ollivier, B., Garcia, J. L. & Patel, B. K. C. (2001). Transfer of *Thermobacteroides leptospartum* and *Clostridium thermolacticum* as *Clostridium stercorarium* subsp. *leptospartum* subsp. nov., comb. nov. and *C. stercorarium* subsp. *thermolacticum* subsp. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 1127–1131.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **30**, 783–791.
- Fendrich, C., Hippe, H. & Gottschalk, G. (1990). *Clostridium halophilum* sp. nov. and *C. litorale* sp. nov., an obligate halophilic and a marine species degrading betaine in the Stickland reaction. *Arch Microbiol* **154**, 127–132.
- Galtier, N., Gouy, M. & Gautier, C. (1996). SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *CABIOS* **12**, 543–548.

- Hippe, H., Andreesen, J. R. & Gottschalk, G. (1992). The genus *Clostridium* – nonmedical. In *The Prokaryotes*, vol. 2, pp. 1800–1866. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Jalava, J. & Eerola, E. (1999). Phylogenetic analysis of *Fusobacterium alocis* and *Fusobacterium sulci* based on 16S rRNA gene sequences: proposal of *Filifactor alocis* (Cato, Moore and Moore) comb. nov. and *Eubacterium sulci* (Cato, Moore and Moore) comb. nov. *Int J Syst Bacteriol* **46**, 1375–1379.
- Jeanthon, C. (2000). Molecular ecology of hydrothermal vent microbial communities. *Antonie Leeuwenhoek* **77**, 117–133.
- Kengen, S. W. M. & Stams, A. J. M. (1994). Formation of L-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. *Arch Microbiol* **161**, 168–175.
- Kevbrin, V. V., Zhilina, T. N., Rainey, F. A. & Zavarzin, G. A. (1998). *Tindallia magadii* gen. nov., sp. nov.: an alkaliphilic anaerobic ammonifier from soda lake deposits. *Curr Microbiol* **37**, 94–100.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. *J Mol Evol* **16**, 111–120.
- Kobayashi, T., Higuchi, S., Kimura, K., Kudo, T. & Horikoshi, K. (1995). Properties of glutamate dehydrogenase and its involvement in alanine production in a hyperthermophilic archaeon, *Thermococcus profundus*. *J Biochem* **118**, 587–592.
- Lake, J. A. (1987). A rate-independent technique for analysis of nucleic acid sequences: evolutionary parsimony. *Mol Biol Evol* **4**, 167–191.
- Li, Y., Mandelco, L. & Wiegel, J. (1993). Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum* sp. nov. *Int J Syst Bacteriol* **43**, 450–460.
- Li, Y., Engle, M., Weiss, N., Mandelco, L. & Wiegel, J. (1994). *Clostridium thermoalkaliphilum* sp. nov., an anaerobic and thermo-tolerant facultative alkaliphile. *Int J Syst Bacteriol* **44**, 111–118.
- Longnecker, K. & Reysenbach, A. L. (2001). Expansion of the geographic distribution of a novel lineage of ϵ -*Proteobacteria* to a hydrothermal vent site on the Southern East Pacific Rise. *FEMS Microbiol Ecol* **35**, 287–293.
- Lux, M. F. & Drake, H. L. (1992). Re-examination of the metabolic potentials of the acetogens *Clostridium acetium* and *Clostridium formicoaceticum*: chemolithoautotrophic and aromatic-dependent growth. *FEMS Microbiol Lett* **74**, 49–56.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Moyer, C. L., Dobbs, F. C. & Karl, D. M. (1995). Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl Environ Microbiol* **61**, 1555–1562.
- Moyer, C. L., Tiedje, J. M., Dobbs, F. C. & Karl, D. M. (1998). Diversity of deep-sea hydrothermal vent *Archaea* from Loihi Seamount, Hawaii. *Deep-Sea Res Part II* **45**, 303–317.
- Polz, M. F. & Cavanaugh, C. M. (1995). Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. *Proc Natl Acad Sci U S A* **92**, 7232–7236.
- Pottu-Boumendil, J. (1989). Principes et méthodes de préparation. In *Techniques en Microscopie Electronique*, pp. 51–57. Paris: INSERM Editions.
- Powers, E. M. (1995). Efficacy of the Ryu nonstaining KOH technique for rapidly determining Gram reactions of food-borne and waterborne bacteria and yeasts. *Appl Environ Microbiol* **61**, 3756–3758.
- Raguénès, G., Pignet, P., Gauthier, G., Peres, A., Christen, R., Rougeaux, H., Barbier, G. & Guezennec, J. (1996). Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. *fijiensis*, and preliminary characterization of the polymer. *Appl Environ Microbiol* **62**, 67–73.
- Raguénès, G., Christen, R., Guézennec, J., Pignet, P. & Barbier, G. (1997). *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *Int J Syst Bacteriol* **47**, 989–995.
- Rainey, F. A. & Stackebrandt, E. (1993). 16S rDNA analysis reveals the phylogenetic diversity among the polysaccharolytic clostridia. *FEMS Microbiol Lett* **113**, 125–128.
- Rainey, F. A., Ward, N. L., Morgan, H. W., Toalster, R. & Stackebrandt, E. (1993). Phylogenetic analysis of anaerobic thermophilic bacteria: aid for their reclassification. *J Bacteriol* **175**, 4772–4779.
- Ravot, G., Ollivier, B., Fardeau, M.-L., Patel, B. K. C., Andrews, K. T., Magot, M. & Garcia, J.-L. (1996). L-alanine production from glucose fermentation by hyperthermophilic members of the domains *Bacteria* and *Archaea*: a remnant of an ancestral metabolism? *Appl Environ Microbiol* **62**, 2657–2659.
- Ravot, G., Magot, M., Fardeau, M. L., Patel, B. K. C., Thomas, P., Garcia, J. L. & Ollivier, B. (1999). *Fusibacter paucivorans* gen. nov., an anaerobic, thiosulfate-reducing bacterium from an oil-producing well. *Int J Syst Bacteriol* **46**, 1141–1147.
- Reysenbach, A. L., Longnecker, K. & Kirshtein, J. (2000). Novel bacterial and archaeal lineages from an *in situ* growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Appl Environ Microbiol* **66**, 3798–3806.
- Ryu, E. (1940). A simple method of differentiation between Gram-positive and Gram-negative organisms without staining. *Kiasso Arch Exp Med* **17**, 58–63.
- Saitou, M. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Stackebrandt, E., Kramer, I., Swiderski, J. & Hippe, H. (1999). Phylogenetic basis for a taxonomic dissection of the genus *Clostridium*. *FEMS Immunol Med Microbiol* **24**, 253–258.
- Takai, K. & Horikoshi, K. (1999). Genetic diversity of *Archaea* in deep-sea hydrothermal vent environments. *Genetics* **152**, 1285–1297.
- Takai, K., Moser, D. P., Onstott, T. C., Spoelstra, N., Piffner, S. M., Dohnalkova, A. & Fredrickson, J. K. (2001). *Alkaliphilus transvaalensis* gen. nov., sp. nov., an extremely alkaliphilic bacterium isolated from a deep South African gold mine. *Int J Syst Evol Microbiol* **51**, 1245–1256.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wery, N., Lesongeur, F., Pignet, P., Derennes, V., Cambon-Bonavita, M. A., Godfroy, A. & Barbier, G. (2001a). *Marinitoga camini* gen. nov., sp. nov., a rod-shaped bacterium belonging to the order *Thermotogales*, isolated from a deep-sea hydrothermal vent. *Int J Syst Evol* **51**, 495–504.
- Wery, N., Moricet, J. M., Cuff, V., Jean, J., Pignet, P., Lesongeur, F., Cambon-Bonavita, M. A. & Barbier, G. (2001b). *Caloranaerobacter azorensis* gen. nov., sp. nov., an anaerobic thermophilic bacterium, isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **51**, 1789–1796.