Geosporobacter subterraneus gen. nov., sp. nov., a spore-forming bacterium isolated from a deep subsurface aquifer

Nihel Klouche,^{1,2} Marie-Laure Fardeau,¹ Jean-François Lascourrèges,^{3,4} Jean-Luc Cayol,¹ Hocine Hacene,⁵ Pierre Thomas¹ and Michel Magot³

Correspondence

Michel Magot
michel.magot@univ-pau.fr

¹IRD, UR 101 Extrêmophiles, IFR-BAIM, Universités de Provence et de la Méditerranée, ESIL, 163 Avenue de Luminy, F-13288 Marseille cedex 09, France

²Laboratoire d'écologie et de gestion des écosystèmes, Faculté des Sciences, Université Aboubekr-Belkaid, Tlemcen, Algeria

³Université de Pau et des Pays de l'Adour, IBEAS, Laboratoire d'Ecologie Moléculaire, EA3525, F-64013 Pau, France

⁴APESA, Hélioparc, 2 avenue P. Angot, F-64053 Pau cedex 09, France

⁵Unité de recherche sur les zones arides, URZA, Université Bab ElZouar, Alger, Algeria

A novel, strictly anaerobic, chemo-organotrophic bacterium, designated strain VNs68^T, was isolated from a well that collected water from a deep aquifer at a depth of 800 m in the Paris Basin, France. Cells were thin, non-motile, Gram-positive rods forming terminal endospores (3.0–5.0×0.5 µm). Strain VNs68^T grew at temperatures between 30 and 55 °C (optimum 42 °C) and at pH 5.6–8.4 (optimum pH 7.3). It did not require salt for growth but tolerated up to 40 g NaCl l⁻¹. Strain VNs68^T was an obligate heterotroph fermenting carbohydrates such as glucose, xylose, fructose, ribose and cellobiose. Casamino acids and amino acids (arginine, serine, lysine, alanine, aspartate, asparagine, isoleucine, histidine) were also fermented. The main fermentation products from glucose were acetate with H₂ and CO₂. Sulfate, sulfite, thiosulfate, elemental sulfur, nitrate and nitrite were not used as electron acceptors. The G+C content of the genomic DNA was 42.2 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain VNs68^T was affiliated to cluster XI, order *Clostridiales*, domain *Bacteria*. On the basis of 16S rRNA gene sequence comparisons and physiological characteristics, strain VNs68^T is considered to represent a novel species of a new genus, for which the name *Geosporobacter subterraneus* gen. nov., sp. nov. is proposed. The type strain of *Geosporobacter subterraneus* is VNs68^T (=DSM 17957^T =JCM 14037^T).

It is now widely accepted that the biosphere extends deep beneath the surface of the Earth, to depths that could exceed 3000 m. The subterranean biosphere is in fact considered to be the largest prokaryotic habitat, possibly representing a biomass equivalent to that found on the Earth's surface (Whitman *et al.*, 1998; Parkes *et al.*, 2005). In spite of the scientific and potential economic interest in this huge unrevealed biodiversity, our knowledge of the deep subsurface microbiology is still extremely scarce, because these habitats are generally beyond the reach of most sampling methods.

We have recently developed an improved sampling procedure to collect clean, microbiologically representative

water samples from deep wells. The efficiency of this procedure was tested on an approximately 800-m-deep artesian well located in the Paris Basin, France (Basso *et al.*, 2005). Briefly, the well-cleaning protocol consisted of: (i) mechanical brushing of the steel tubing to detach the biofilm colonizing the pipe wall, which was then discarded by an extensive purge, and (ii) sterilization of the tubing by three successive chlorine injections, followed by another extensive purge of the line. A water sample, representative of the deep subsurface aquifer, was then collected (sample S4; Basso *et al.*, 2005) and shown to contain 3×10⁴ bacterial cells ml⁻¹ via epifluorescence microscopy direct counting.

S4 subsamples were inoculated in various culture media, which were then incubated at 37 °C, a temperature close to that of the rock formation at 800 m depth (38 °C). Several

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of VNs68^T is DQ643978.

previously undescribed bacterial species were isolated directly from these enrichment cultures (data not shown). Isolation of one strain, designated VNs68^T, was more indirect: the presence of bacterial spores was first observed in an enrichment culture of sulfate-reducers. An aliquot was collected, heated for 10 min at 80 °C, and inoculated at a series of dilutions in different culture media. A highly turbid culture was obtained in NIH thioglycollate broth (Difco) incubated anaerobically at 37 °C. Strain VNs68^T was obtained from the last positive dilution by three successive streakings on solid medium in Petri dishes under anaerobic conditions.

Strain VNs68^T produced thin, long (3.0–5.0 × 0.5 µm), non-motile, straight or curved rods, staining Gram-positive, and forming terminal endospores when grown on a medium containing glucose and yeast extract (Fig. 1). Ultrathin sections obtained as described by Fardeau *et al.* (1997) revealed a thin (about 12 nm), atypically stratified, Gram-positive cell wall (without any outer membrane) (Fig. 2).

The Hungate technique was then used throughout the study for physiological and metabolic characterization. The basal medium contained (per litre): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1.0 g NH₄Cl, 10 g NaCl, 0.1 g KCl, 0.1 g CaCl₂·2H₂O, 10 ml trace mineral element solution (Balch *et al.*, 1979) plus 1 ml 0.1% resazurin. The pH was adjusted to 7.2 with 10 M KOH. The basal medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature and 5-ml aliquots were distributed in Hungate tubes under a stream of O₂-free N₂ gas. The N₂ gas phase was replaced with N₂/CO₂ (80:20) and the tubes were autoclaved. Prior to inoculation, 0.05 ml 2% Na₂S·9H₂O, 0.1 ml 10% NaHCO₃ and 0.1 ml MgCl₂·6H₂O (150 g l⁻¹) were added.

The physiological optimal growth conditions for strain VNs68^T were determined in duplicate experiments conducted in basal medium containing glucose (20 mM) and yeast extract (0.1%) as described by Fardeau *et al.* (2000).

Fig. 1. Phase-contrast micrograph of cells of strain VNs68^T. Bar, 10 µm.

Fig. 2. Transmission electron micrograph of a transverse thin section of a cell of strain VNs68^T, showing the atypically stratified Gram-positive wall. Bar, 0.2 µm.

Strain VNs68^T was strictly anaerobic. Its optimal temperature for growth was 42 °C (range 30–55 °C); growth was not observed at 25 °C. For pH studies, the medium was adjusted to the desired pH by using anaerobically prepared stock solutions of NaHCO₃ (10%) or Na₂CO₃ (10%). Optimum pH was 7.3 (range pH 5.6–8.4). For studies of NaCl requirements, NaCl was weighed directly in the tubes for concentrations ranging from 0 to 50 g NaCl l⁻¹ before dispensing a basal medium lacking NaCl. Strain VNs68^T did not require salt for growth, but tolerated up to 40 g NaCl l⁻¹.

Substrate utilization was studied in the presence of 1 g yeast extract l⁻¹ added to the basal medium. The following carbohydrates (20 mM) were used as carbon and energy sources: D-xylose, cellobiose, ribose, fructose and glucose. Casamino acids (0.1%) and some amino acids [arginine, serine, lysine, glutamine, glutamate, aspartate, asparagine, histidine (from which acetate was formed) and isoleucine (from which 2-methyl butyrate was formed)] (20 mM) were also used. The Stickland reaction (alanine/arginine) was positive.

Methanol, mannitol, ethanol, propanol, butanol, glycerol, sorbose, mannose, formate, acetate, propionate, succinate, fumarate, lactate, pyruvate, starch (0.1%), threonine, glycine, proline, cysteine, tyrosine, phenylalanine, valine and leucine were also tested but did not support growth.

The end products from glucose fermentation were mainly acetate, formate, H₂ and CO₂. Traces of ethanol were also detected. Acetate was not produced from H₂ and CO₂.

Sulfate (20 mM), thiosulfate (20 mM), elemental sulfur (0.1%), sulfite (2 mM), nitrate (20 mM) and nitrite (2 mM) were not used as electron acceptors.

Under optimal conditions with glucose as electron donor, the maximum growth rate of strain VNs68^T was 0.14 h⁻¹ and the doubling time was 4.9 h.

Growth of strain VNs68^T was inhibited by the addition of chloramphenicol (200 µg ml⁻¹), penicillin G (300 µg ml⁻¹) and ampicillin (300 µg ml⁻¹).

The DNA G+C content determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) according to the method of Mesbah *et al.* (1989) was 42.2 mol%.

Genomic DNA of strain VNs68^T was extracted by using the Wizard Genomic DNA Purification kit, according to the manufacturer's protocol (Promega). Universal primers Fd1 (5'-CAGAGTTTGATCCTGGCTCAG-3', positions 7–27 according to the *Escherichia coli* numbering system) and R6 (5'-TACGTTACCTTGTACGAC-3', positions 1513–1494) were used to amplify the 16S rRNA gene. The nucleotide sequence grouped with members of cluster XI within the order *Clostridiales* (Collins *et al.*, 1994), the most closely related sequences being those of either uncultured or as yet undescribed species. Interestingly, several of these related micro-organisms, as with strain VNs68^T, originated from deep subsurface water samples. This was the case for uncultured bacterial clones 47mm70, retrieved from a South African gold mine deep borehole (GenBank accession no. AY796047.1; 96.6 % 16S rRNA gene sequence similarity), and R82, retrieved from Australia's Great Artesian Basin (accession no. AF407695.1; 95.2 % similarity). The most closely related type strains of recognized species were *Caminicella sporogenes* AM 1114^T (91.5 % similarity), isolated from a deep-sea hydrothermal vent

(Alain *et al.*, 2002), and the two species of the genus *Alkaliphilus*, *Alkaliphilus transvaalensis* SAGM1^T, isolated from an ultradeep gold mine (91.2 % similarity) (Takai *et al.*, 2001), and *Alkaliphilus crotonatoxidans* B11-2^T (90.8 % similarity) (Cao *et al.*, 2003).

The nucleotide sequence of strain VNs68^T was aligned manually with reference sequences of various bacteria belonging to cluster XI of the *Clostridiales*, with *Bacillus subtilis* as an outgroup, by using the sequence alignment editor BioEdit (Hall, 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak *et al.*, 2001) and GenBank databases (Benson *et al.*, 1999). Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1291 unambiguous nucleotides were computed according to the method of Jukes & Cantor (1969). The phylogenetic tree obtained by the neighbour-joining method (Saitou & Nei, 1987) is shown in Fig. 3. The topology of this tree was also supported by using the maximum-parsimony and maximum-likelihood algorithms.

According to its phylogenetic position, strain VNs68^T is likely to represent a novel species in cluster XI of *Clostridiales*; moreover, its phylogenetic distance from members of the genera *Caminicella* and *Alkaliphilus* (91.5 % 16S rRNA gene sequence similarity or less) suggests that this isolate represents a new genus within this cluster. This is strongly supported by significant genetic and physiological traits, as detailed in Table 1. The G+C content of the DNA of strain VNs68^T was much higher (42.2 mol%) than values for related species, which do not exceed 36.4 mol% (*A. transvaalensis*). VNs68^T differs from *C. sporogenes* by the structure of its cell envelope; it is a true Gram-positive bacterium with a cytoplasmic membrane and a thick

Fig. 3. Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strain VNs68^T and most species of cluster XI of the *Clostridiales*. The tree was constructed by the neighbour-joining method and rooted by using the type strain of *Clostridium butyricum* (the type species of the genus *Clostridium*) as an outgroup. Bootstrap values based on 1000 replicates are shown. Bar, 5 changes per 100 nucleotide positions.

Table 1. Differential characteristics between strain VNs68^T and related members of cluster XI of the order *Clostridiales*

Characteristic	Strain VNs68 ^T	<i>C. sporogenes</i> AM1114 ^T	<i>A. crotonatoxidans</i> B11-2 ^T	<i>A. transvaalensis</i> SAGM1 ^T
DNA G + C content (mol%)	42.2	24.2	30.6	36.4
Temperature growth range (optimum) (°C)	25–55 (42)	45–65 (60)	15–45 (37)	20–50 (40)
pH growth range (optimum)	5.6–8.4 (7.3)	4.5–8.0 (7.5)	5.5–9.0 (7.5)	8.5–12.5 (10.0)
Motility	–	+	+	+
Use of electron acceptors				
S ⁰	–	–	–	+
Thiosulfate	–	–	–	+
Fumarate	–	–	–	+
Substrate utilization				
Fructose	+	–	+	–
Cellobiose	+	–	+	–
Xylose	+	–	+	–
Ribose	+	–	+	–
Glucose	+	+	–	–
End products from glucose fermentation*	Acetate, formate, H ₂ , CO ₂ , (ethanol)	Butyrate, acetate, ethanol, H ₂ , CO ₂	–	–

*Trace products in parentheses.

peptidoglycan layer, whereas, although clustering with low-G+C Gram-positive bacteria, *C. sporogenes* has a characteristic Gram-negative cell wall. Strain VNs68^T can be differentiated from *A. transvaalensis* based on major phenotypic traits, such as the use of carbon sources, electron acceptors and pH range for growth (Table 1). By contrast, it is phenotypically quite similar to *A. crotonatoxidans*, differing mainly by its ability to use glucose and to grow at temperatures above 45 °C, its more restricted pH range and the absence of motility.

On the basis of the data reported here, we suggest that strain VNs68^T represents a novel species of a new genus, for which the name *Geosporobacter subterraneus* gen. nov., sp. nov. is proposed.

Description of *Geosporobacter* gen. nov.

Geosporobacter (Ge.o.spo.ro.bac'ter. Gr. n. *ge* the earth; Gr. n. *spora* a seed and, in biology, a spore; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Geosporobacter* a sporulated rod from the earth).

Straight or slightly curved, long and thin rods. Non-motile. Gram-positive and spore-forming. Strictly anaerobic heterotrophs. The G + C content of the genomic DNA is about 42 mol%. The genus is phylogenetically included in cluster XI of the order *Clostridiales*, most closely related to the genera *Caminicella* and *Alkaliphilus*. The type species is *Geosporobacter subterraneus*.

Description of *Geosporobacter subterraneus* sp. nov.

Geosporobacter subterraneus (sub.ter.ra'ne.us. L. masc. adj. *subterraneus* underground, subterranean).

Cells are 3.0–5.0 × 0.5 µm. Neutrophilic and slightly halo-tolerant. Temperature range for growth is 30–55 °C, with optimum growth at 42 °C. The optimum pH is 7.3. Carbohydrates are fermented. Xylose, cellobiose, ribose, fructose, glucose, Casamino acids and some amino acids (arginine, serine, lysine, glutamine, alanine, glutamate, isoleucine, aspartate, asparagine, histidine) serve as growth substrates. The main end product of glucose catabolism is acetate. Methanol, mannitol, ethanol, propanol, butanol, glycerol, sorbose, mannose, formate, acetate, propionate, succinate, fumarate, lactate, pyruvate, starch and some amino acids (cysteine, proline, threonine, glycine, phenyl-alanine, valine) are not used. Sulfate, sulfite, thiosulfate and elemental sulfur are not utilized as electron acceptors. The DNA G + C content of the type strain is 42.2 mol%.

The type strain, VNs68^T (=DSM 17957^T =JCM 14037^T), was isolated from a water-producing deep artesian well in France.

References

- Alain, K., Pignet, P., Zbinden, M., Quillevere, M., Duchiron, F., Donval, J.-P., Lesongeur, F., Raguene, G., Crassous, P. & other authors (2002). *Caminicella sporogenes* gen. nov., sp. nov., a novel thermophilic spore-forming bacterium isolated from an East-Pacific Rise hydrothermal vent. *Int J Syst Evol Microbiol* 52, 1621–1628.
- Balch, W. E., Fox, G. E., Magrum, L. J. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* 43, 260–296.
- Basso, O., Lascourrèges, J.-F., Jarry, M. & Magot, M. (2005). The effect of cleaning and disinfecting the sampling well on the microbial communities of deep subsurface water samples. *Environ Microbiol* 7, 13–21.
- Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J., Ouellette, B. F., Rapp, B. A. & Wheeler, D. L. (1999). GenBank. *Nucleic Acids Res* 27, 12–17.

- Cao, X., Liu, X. & Dong, X. (2003).** *Alkaliphilus crotonatoxidans* sp. nov., a strictly anaerobic, crotonate-dismutating bacterium isolated from a methanogenic environment. *Int J Syst Evol Microbiol* **53**, 971–975.
- 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 the five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Fardeau, M.-L., Patel, B. K. C., Magot, M. & Ollivier, B. (1997).** Utilization of serine, leucine, isoleucine, and valine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methanobacterium* sp. as electron acceptors. *Anaerobe* **3**, 405–410.
- Fardeau, M.-L., Magot, M., Patel, B. K. C., Thomas, P., Garcia, J.-L. & Ollivier, B. (2000).** *Thermoanaerobacter subterraneus* sp. nov., a novel thermophile isolated from oilfield water. *Int J Syst Evol Microbiol* **50**, 2141–2149.
- Hall, T. A. (1999).** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Jukes, T. H. & Cantor, C. R. (1969).** Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Jr, Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M. & Tiedje, J. M. (2001).** The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29**, 173–174.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Parkes, R. J., Webster, G., Cragg, B. A., Weightman, A. J., Newberry, C. J., Ferdelman, T. G., Kallmeyer, J., Jorgensen, B. B., Aiello, I. W. & Fry, J. C. (2005).** Deep sub-seafloor prokaryotes stimulated at interfaces over geological time. *Nature* **436**, 390–394.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- 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.
- Whitman, W. B., Coleman, D. C. & Wiebe, W. J. (1998).** Prokaryotes: the unseen majority. *Proc Natl Acad Sci U S A* **95**, 6578–6583.