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

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A novel, strictly anaerobic, chemo-organotrophic bacterium, designated strain VNs68T, 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 VNs68T 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 VNs68T 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 VNs68T was affiliated to cluster XI, order Clostridiales, domain Bacteria. On the basis of 16S rRNA gene sequence comparisons and physiological characteristics, strain VNs68T 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 VNs68T (=DSM 17957T =JCM 14037T).

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
previously undescribed bacterial species were isolated directly from these enrichment cultures (data not shown). Isolation of one strain, designated VNs68<sup>T</sup>, 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 thiglycollate broth (Difco) incubated anaerobically at 37 °C. Strain VNs68<sup>T</sup> was obtained from the last positive dilution by three successive streakings on solid medium in Petri dishes under anaerobic conditions.

Strain VNs68<sup>T</sup> 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 KH<sub>2</sub>PO<sub>4</sub>, 0.3 g K<sub>2</sub>HPO<sub>4</sub>, 1.0 g NH<sub>4</sub>Cl, 10 g NaCl, 0.1 g KCl, 0.1 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 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 O<sub>2</sub>-free N<sub>2</sub> gas and cooled to room temperature and 5-ml aliquots were distributed in Hungate tubes under a stream of O<sub>2</sub>-free N<sub>2</sub> gas. The N<sub>2</sub> gas phase was replaced with N<sub>2</sub>/CO<sub>2</sub> (80 : 20) and the tubes were autoclaved. Prior to inoculation, 0.05 ml 2 % Na<sub>2</sub>S·9H<sub>2</sub>O, 0.1 ml 10 % NaHCO<sub>3</sub> and 0.1 ml MgCl<sub>2</sub>·6H<sub>2</sub>O (150 g l<sup>−1</sup>) were added.

The physiological optimal growth conditions for strain VNs68<sup>T</sup> 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).

Strain VNs68<sup>T</sup> 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 NaHCO<sub>3</sub> (10 %) or Na<sub>2</sub>CO<sub>3</sub> (10 %). Optimun 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<sup>−1</sup> before dispensing a basal medium lacking NaCl. Strain VNs68<sup>T</sup> did not require salt for growth, but tolerated up to 40 g NaCl l<sup>−1</sup>.

Substrate utilization was studied in the presence of 1 g yeast extract l<sup>−1</sup> 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, H<sub>2</sub> and CO<sub>2</sub>. Traces of ethanol were also detected. Acetate was not produced from H<sub>2</sub> and CO<sub>2</sub>. 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 VNs68T was 0.14 h\(^{-1}\) and the doubling time was 4.9 h.

Growth of strain VNs68T was inhibited by the addition of chloramphenicol (200 \(\mu\)g ml\(^{-1}\)), penicillin G (300 \(\mu\)g ml\(^{-1}\)) and ampicillin (300 \(\mu\)g 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 VNs68T 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’-TACGGTTACCTTGTTACGAC-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 VNs68T, 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 transvaalenensis SAGM1\(^T\), isolated from an ultradepth 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 VNs68T 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 VNs68T 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 VNs68T was much higher (42.2 mol%) than values for related species, which do not exceed 36.4 mol% (A. transvaalenensis). VNs68T 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

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Fig. 3. Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strain VNs68T 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.
peptidoglycan layer, whereas, although clustering with low-G+C Gram-positive bacteria, *C. sporogenes* has a characteristic Gram-negative cell wall. Strain VNs68<sup>T</sup> 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<sup>T</sup> 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 halotolerant. 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, phenylalanine, 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<sup>T</sup> (=DSM 17957<sup>T</sup> = ICMP 14037<sup>T</sup>), was isolated from a water-producing deep artesian well in France.

**References**


