Desulfocurvus vexinensis gen. nov., sp. nov., a sulfate-reducing bacterium isolated from a deep subsurface aquifer

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A novel anaerobic, chemo-organotrophic bacterium, designated VNs36^T, was isolated from a well that collected water from a deep saline aquifer used for underground gas storage at a depth of 830 m in the Paris Basin, France. Cells were curved motile rods or vibrios (3.0–5.0×0.5 μm). Strain VNs36^T grew at temperatures between 20 and 50 °C (optimum 37 °C) and at pH values between 5.0 and 9.0 (optimum 6.9). It did not require salt for growth, but tolerated up to 20 g NaCl l^{-1} (optimum 2 g l^{-1}). In the presence of sulfate, strain VNs36^T used lactate, formate and pyruvate as carbon and energy sources. The main fermentation products from lactate were acetate, H_2 and CO_2. Sulfate, thiosulfate and sulfite were used as electron acceptors, but not sulfur. The genomic DNA G+C content of strain VNs36^T was 67.2 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain VNs36^T was affiliated with the family *Desulfovibrionaceae* within the class *Deltaproteobacteria*. On the basis of 16S rRNA gene sequence comparisons, DNA G+C content and the absence of desulfoviridin in cell extracts, it is proposed that strain VNs36^T be assigned to a new genus, *Desulfocurvus* gen. nov., as a representative of a novel species, *Desulfocurvus vexinensis* sp. nov. The type species of this genus is *Desulfocurvus vexinensis* with the type strain VNs36^T (=DSM 17965^T=JCM 14038^T).

Sulfate-reducing bacteria (SRB) are widely distributed in deep subsurface environments. Considering only some of the recently published papers, SRB have been found in many deep geological formations including a 4–5 km deep fault (Moser *et al.*, 2005), deep aquifers in Australia (Kimura *et al.*, 2005) and France (Basso *et al.*, 2005b, 2009), deeply buried marine sediments (Schippers & Neretin, 2006) and oil reservoirs (Grabowski *et al.*, 2005; Magot, 2005; Birkeland, 2005). From the numerous observations already published, it appears that SRB may constitute an important, or even major, component of the subterrestrial biosphere, which is considered to be possibly the largest prokaryotic habitat on our planet (Whitman *et al.*, 1998; Parkes *et al.*, 2005). Up to now, this hidden biodiversity has not been described in great detail and, although molecular studies have shown that the deep subsurface harbours many undescribed species, very few strains have been fully described.

The microbial community collected from a deep saline aquifer in the close vicinity of an underground gas storage aquifer was described recently by Basso *et al.* (2009). The dominant microbial populations were hydrogen-utilizing autotrophic bacteria including SRB and homoacetogens, suggesting that CO_2 and H_2 are the main carbon and energy sources sustaining a nutrient-limited subsurface lithoautotrophic microbial ecosystem (Stevens & McKinley, 1995). As well as these autotrophic bacteria, several other previously uncultured anaerobic heterotrophs representing a minor component of the microbial community were isolated. These included several sulfate-reducing isolates that were sufficiently phylogenetically

Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of VNs36^T is DQ841177.

distant from recognized species to suggest that they could be representatives of a novel species in a new genus; one of these isolates is described here.

Water samples were retrieved from an artesian well approximately 830 m deep located in the north-west of the Paris Basin, France. This aquifer belongs to the Rauracian geological formation (approximately 150 million years old). The aquifer water is moderately saline, with a total mineral content of 10 g l^{-1}. The *in situ* conditions are 38 °C and pH 7.7 (Basso *et al.*, 2009). Many precautions were taken during sampling to ensure that the micro-organisms present in the water sample were indigenous to the geological formation. The detailed sampling procedure and its validation for the recovery of deep subsurface indigenous bacteria have been described previously (Basso *et al.*, 2005a).

Water samples were used to inoculate various culture media which were then incubated at 37 °C, close to the temperature of the geological formation. Several previously undescribed bacterial species were isolated from these experiments, including the recently described *Geosporobacter subterraneus* (Klouche *et al.*, 2007). Several novel sulfate-reducing isolates that shared the same 16S rRNA gene sequence were purified from different enrichment cultures in media designed for oligotrophic anaerobes or heterotrophic SRB (Basso *et al.*, 2009). Experiments were performed in an anaerobic glove box (La Calhène) under a gas phase composed of $N_2/H_2/CO_2$ (85 : 10 : 5). One of these isolates, designated VNs36^T, was studied and characterized.

The Hungate technique was then used throughout the studies. The basal medium contained (l^{-1}): 0.3 g KH_2PO_4; 0.3 g K_2HPO_4; 1.0 g NH_4Cl; 10 g NaCl; 0.1 g KCl; 0.1 g $CaCl_2 . 2H_2O$; 1 ml trace mineral element solution (Widdel, 1988) and 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_2-free N_2 gas, cooled to room temperature and 5 ml aliquots were distributed in Hungate tubes under a stream of O_2-free N_2 gas. The N_2 gas phase was replaced with N_2/CO_2 (80 : 20) and the tubes were autoclaved. Prior to inoculation, 0.05 ml 2 % $Na_2S . 9H_2O$, 0.1 ml 10 % $NaHCO_3$ and 0.1 ml $MgCl_2 . 6H_2O$ (150 g l^{-1}) were added.

Cells of strain VNs36^T were curved motile rods (3.0–5.0 × 0.5 μm) that stained Gram-negative when grown on basal medium supplemented with lactate, sulfate and yeast extract. Cell morphology in the oligotrophic synthetic medium from which the strain was first isolated was that of a typical vibrio. Ultrathin sections showed a typical Gram-negative cell wall with a thin peptidoglycan layer and an outer membrane.

Strain VNs36^T was strictly anaerobic. The optimal physiological growth conditions were determined in duplicate experiments conducted in basal medium containing lactate (20 mM) and sodium sulfate (20 mM) as described previously (Fardeau *et al.*, 2000). The optimal temperature for growth was 37 °C (range 20–50 °C), which is consistent with the *in situ* temperature of the geological formation. For pH studies, the medium was adjusted to the desired pH using anaerobically prepared stock solutions of $NaHCO_3$ (10 %) or Na_2CO_3 (10 %). The optimum pH was 6.9, but it is worth noting that growth occurred over an unusually wide pH range (from pH 5.0 to 9.0). Because of H_2S toxicity, growth of SRB at moderately acidic pH is uncommon, but has been reported recently (Rampinelli *et al.*, 2008). For studies determining NaCl requirements, NaCl was weighed directly in the tubes at concentrations ranging from 0 to 5 % NaCl before dispensing a basal medium free from NaCl. The isolate could grow without added NaCl, but tolerated up to 20 g NaCl l^{-1}. These physiological traits illustrate the adaptation of strain VNs36^T to the physico-chemical conditions of its environment (Basso *et al.*, 2009).

Substrate utilization was tested with 1 g yeast extract added to 1 l basal medium. Very few of the tested compounds were metabolized; only lactate, formate and pyruvate (20 mM) were used as carbon and energy sources with sulfate as electron acceptor. Methanol, ethanol, propanol, butanol, glycerol, glucose, fructose, acetate, propionate, butyrate, succinate, fumarate, malate, citrate and Casamino acids were also tested, but did not support growth. Lactate was also fermented; the end products from fermentation were acetate, H_2 and CO_2. Although growth occurred in mineral medium with lactate as the only organic substrate, yeast extract did improve growth. Hydrogen, a potential energy source in deep environments, was not used. This restricted heterotrophic metabolism suggests that this bacterial species does not contribute to the primary production of organic matter in the aquifer, as other species, e.g. *Desulfovibrio aespoeensis* and *Acetobacterium carbinolicum*, are able to do (Basso *et al.*, 2009). Despite representing 1–10 % of the cultivable bacterial population, i.e. less than 0.1 % of the total microbial count (Basso *et al.*, 2009), the novel strain could contribute efficiently to the recycling of carbon and energy in this nutrient-limited extreme environment.

Using lactate as electron donor, sulfate (20 mM), thiosulfate (20 mM) and sulfite (2 mM) were used as electron acceptors, but not elemental sulfur, nitrate (20 mM), nitrite (2 mM) or $FeCl_3$. Under optimal conditions with lactate as electron donor and sulfate as electron acceptor, the maximum growth rate of VNs36^T was 0.21 h^{-1} and the doubling time was 3.3 h.

Visible absorption spectra of a cell-free extract of strain VNs36^T showed the presence of low redox potential *c*-type cytochromes (very likely the tetrahaemic cytochrome c_3) with absorption peaks at 522, 551 and 418 nm in the dithionite reduced form. The characteristic absorption band of desulfoviridin (the dissimilatory high-spin bisulfite reductase characteristic of the genus *Desulfovibrio*) at 628 nm was not detected in the cell-free extract. The

reddish colour of the crude extract may possibly indicate the presence of desulforubidin, the other cytoplasmic dissimilatory bisulfite reductase isolated in mesophilic non-sporulating species of SRB (Fauque *et al.*, 1991).

The DNA G+C content of VNs36^T, determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) using the method of Mesbah *et al.* (1989), was 67.2 mol%.

The genomic DNA of VNs36^T was extracted using the Wizard Genomic DNA Purification kit, according to the manufacturer's protocol (Promega). The 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 1532 bp sequence was aligned manually with representatives of the class *Deltaproteobacteria* from the family *Desulfovibrionaceae* using the BIOEDIT sequence alignment editor (Hall, 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 1999). Sequence and alignment uncertainties were omitted from the analysis. The pairwise evolutionary distances based on 1384 unambiguous nucleotides were computed by the Jukes & Cantor (1969) method. The phylogenetic tree obtained by the neighbour-joining method (Fig. 1) showed that strain VNs36^T was included in the wide cluster of the genus *Desulfovibrio*. Its closest relatives were *Desulfovibrio senezii* DSM 8436^T with 91.5 % similarity and '*Desulfovibrio ferrophilus*' strain IS5 with 93.9 % similarity. These high phylogenetic distances indicate that VNs36^T represents a novel species of SRB and suggest that it could be included in a new genus. This possibility is supported by the DNA G+C content of VNs36^T, which is higher (67.2 mol%) than those of all *Desulfovibrio* species (46–61 mol%) (Kuever *et al.*, 2005). Moreover, although the presence of desulfoviridin is considered a characteristic trait of members of the genus *Desulfovibrio* (Kuever *et al.*, 2005), this pigment was not detected in cell-free extracts of the novel isolate. It is thus proposed that strain VNs36^T represents a novel species in a new genus, for which the name *Desulfocurvus vexinensis* sp. nov. is proposed. A comparison of the main characteristics of strain VNs36^T and the distantly related species *Desulfovibrio senezii* is given in Table 1.

Description of *Desulfocurvus* gen. nov.

Desulfocurvus (De.sul.fo.cur′vus. L. pref. *de* from; L. n. *sulfur* sulfur; N.L. pref. *desulfo-* desulfuricating, used to characterize a dissimilatory sulfate-reducing prokaryote; L. adj. *curvus* curved; N.L. masc. n. *Desulfocurvus* a curved sulfate-reducing bacterium).

Motile curved rods or vibrios. Gram-negative and non-sporulating. Strictly anaerobic heterotroph. Genomic DNA G+C content is about 67 mol%. Phylogenetically, included in the domain '*Bacteria*', phylum '*Proteobacteria*', class *Deltaproteobacteria*, order *Desulfovibrionales*, family *Desulfovibrionaceae*. Most closely related to the genus *Desulfovibrio*, sharing most of characteristics with this genus. Desulfoviridin is absent. The type species is *Desulfocurvus vexinensis*.

Description of *Desulfocurvus vexinensis* sp. nov.

Desulfocurvus vexinensis (ve.xi.nen′sis. N.L. masc. adj. *vexinensis* pertaining to the geographical origin of the isolate, the Vexin, an area of the Paris Basin, France).

Cells are anaerobic, motile rods or vibrios (3.0–5.0 × 0.5 μm). Neutrophilic and slightly halotolerant. The temperature range for growth is 20–50 °C (optimum 37 °C). The optimum pH is 6.9 (range 5.0–9.0). Lactate, formate and pyruvate are used as carbon and energy sources. The main end product of lactate catabolism is acetate. Methanol, ethanol, propanol, butanol, glycerol, glucose, fructose, acetate, propionate, butyrate, succinate,

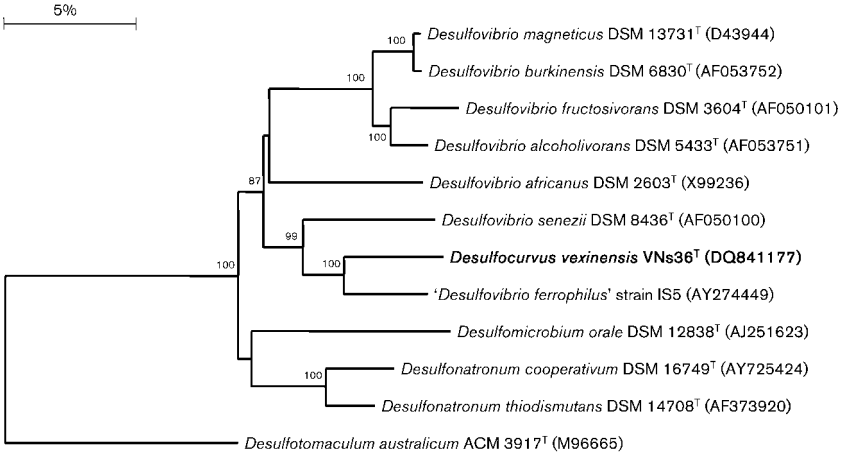

Fig. 1. Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of *Desulfocurvus vexinensis* VNs36^T and strains of related species. The tree was constructed by the neighbour-joining method and rooted using *Desulfotomaculum australicum* ACM 3917^T as an outgroup. Bootstrap values for 1000 replicates are shown. Bar, 5 nt changes per 100 nt.

Table 1. Comparison of the main characteristics of strain VNs36^T and *Desulfovibrio senezii* DSM 8436^T

Both strains use sulfur, sulfate, sulfite and thiosulfate as electron acceptors, use lactate and pyruvate as electron donors and contain cytochrome c_3.

Characteristic	VNs36^T	*D. senezii* DSM 8436^T
Morphology	Vibrios or curved rods	Vibrios
Size (μm)	0.5 × 3.0–5.0	0.3 × 1.0–1.3
DNA G+C content (mol%)	67.2	62
Temperature range for growth (optimum) (°C)	20–50 (37)	25–45 (37)
pH range for growth (optimum)	5.0–9.0 (6.9)	6.4–8.3 (7.6)
Salinity range for growth (%)	0–2	0–12.5
Electron donors:		
Formate	+	−
Hydrogen	−	+
Presence of desulfoviridin	−	+

fumarate, malate, citrate and Casamino acids are not used. Sulfate, sulfite and thiosulfate are utilized as electron acceptors. Desulfoviridin is absent and *c*-type cytochromes are present.

The type strain, VNs36^T (=DSM 17965^T=JCM 14038^T), was isolated from a deep artesian well in France. The DNA G+C content of the type strain is 67.2 mol%.

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