Desulfovibrio putealis sp. nov., a novel sulfate-reducing bacterium isolated from a deep subsurface aquifer

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A novel sulfate-reducing bacterium was isolated from a well that collected water from a deep aquifer at a depth of 430 m in the Paris Basin, France. The strain, designated B7-43^T, was made up of vibrioid cells that were motile by means of a single polar flagellum. Cells contained desulfoviridin. In the presence of sulfate, the following substrates were used as energy and carbon sources: lactate, pyruvate, malate, fumarate, ethanol, butanol, acetate/H₂ and glycine. Sulfite and thiosulfate were also used as electron acceptors in the presence of lactate. In the absence of electron acceptors, pyruvate, malate and fumarate were fermented. Optimal growth was obtained in 1 g NaCl l⁻¹ and at pH 7. On the basis of 16S rRNA gene sequence analysis, the isolate was most closely related to members of the genus *Desulfovibrio* (90 % similarity). It is thus proposed that strain B7-43^T (=DSM 16056^T=ATCC BAA-905^T) represents a novel species within this genus, *Desulfovibrio putealis* sp. nov.

During the last two decades, an increasing number of novel sulfate-reducing bacteria (SRB) have been isolated from a wide variety of environments such as fresh to saline and hypersaline waters or sediments, either from surface or subsurface habitats (Voordouw, 1995; Widdel & Bak, 1992). Among them, strains of the genus *Desulfovibrio* are the most commonly isolated. Subsequently, a great number of novel *Desulfovibrio* species has been described during the last 10 years. Recently, several strains of SRB were isolated from a subsurface aquifer water sample. Among them, strain B7-43^T was phylogenetically related, although distantly, to a large cluster of *Desulfovibrio* species. Considering its very low similarity to the closest existing species of the genus *Desulfovibrio* (less than 90 % 16S rRNA gene sequence similarity), strain B7-43^T could possibly be described as a representative of a new genus of SRB. However, due to its phylogenetic position within the large *Desulfovibrio* cluster and its physiological traits, which are similar to those of most *Desulfovibrio* species, it is proposed that strain B7-43^T should be classified in the genus *Desulfovibrio*. Therefore, because of its phylogenetic distance to the most closely related validly published members of the genus and its differing physiological traits, strain B7-43^T is proposed as a

representative of a novel species, *Desulfovibrio putealis* sp. nov.

Strain B7-43^T was isolated from a sample of deep subsurface water from the Paris Basin, France. Samples of formation water were aseptically collected at the wellhead under anoxic conditions as described previously (Basso *et al.*, 2004). The total number of bacteria in a water sample (counted after staining with acridine orange) was 3×10^4 cells ml⁻¹. Handling and enrichment cultures of sulfate-reducers were accomplished as described previously (Magot *et al.*, 1992). The final salinity of the culture media was adjusted by addition of NaCl (1 g l⁻¹) to mimic *in situ* conditions. Incubations were carried out at 30 °C, corresponding to the *in situ* temperature in the aquifer. Enrichment cultures were obtained by serial dilutions in Hungate tubes. The last positive tube was used for isolation by streaking on solid medium in Petri dishes under anaerobic conditions. Colonies obtained after 2 weeks incubation were picked and their isolation was repeated twice. The purity of strains was checked by phase-contrast microscopy. Strain B7-43^T was selected for detailed investigation. It was composed of vibrioid cells that were actively motile with a single polar flagellum. The cells were generally 2.3–4.1 µm in length and 0.7 µm wide. Cells occurred either singly or in pairs and stained Gram-negative.

To obtain a picture of the physiological properties of strain B7-43^T, the following tests were carried out: utilization of carbon and energy sources, utilization of a variety of

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Abbreviation: SRB, sulfate-reducing bacteria(-um).

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electron acceptors, fermentative growth tests and determination of temperature, pH and salinity optima (Magot *et al.*, 1992; Pfennig *et al.*, 1981; Tardy-Jacquenod *et al.*, 1996; Widdel & Bak, 1992). All tests were performed in triplicate. Strain B7-43^T was a strictly anaerobic micro-organism; no growth was observed when it was incubated in the presence of air. The substrates tested as energy and carbon sources are listed in Table 1. Strain B7-43^T used the following substrates as energy and carbon sources in the presence of sulfate as electron acceptor: lactate, pyruvate, malate, fumarate, ethanol, butanol, glycine and acetate/H₂. Acetate was produced from lactate oxidation. No growth was observed on

acetate, propionate, butyrate, benzoate, succinate, pyruvate, citrate, methanol, glycerol, glucose, fructose, alanine, cysteine, glutamate, aspartate, formate, toluene, propanol or H₂/CO₂. The spectrum of cell extracts exhibited the characteristic absorption band of desulfovibridin at 631 nm (Postgate, 1956). Strain B7-43^T grew at 20–40 °C, with optimal growth at 30 °C. No growth was observed at 15 or 45 °C. Strain B7-43^T was unable to grow in NaCl concentrations higher than 6 g l⁻¹. Optimal growth was observed between 0 and 1 g NaCl l⁻¹. pH 7.0 was optimal for growth, but the isolate grew well between pH 6.25 and 7.5. Under optimal conditions, with lactate as electron donor and sulfate as

Table 1. Characteristics that can discriminate between strain B7-43^T and other related *Desulfovibrio* species

Taxa: 1, *Desulfovibrio putealis* B7-43^T; 2, *D. vulgaris*; 3, *D. alcoholivorans*; 4, *D. gabonensis*; 5, *D. longus*; 6, *D. sulfodismutans*; 7, *D. profundus*; 8, *D. fructosivorans*; 9, *D. magneticus*; 10, *D. burkinensis*; 11, sulfate-reducing bacterium F1-7b. Data were taken from Postgate & Campbell (1966), Qatibi *et al.* (1991), Tardy-Jacquenod *et al.* (1996), Magot *et al.* (1992), Bak & Pfennig (1987), Bale *et al.* (1997), Ollivier *et al.* (1988), Sakaguchi *et al.* (2002), Ouattara *et al.* (1999) and Wind *et al.* (1999). The following substrates were also tested but were not used by strain B7-43^T with sulfate as electron acceptor: butyrate, benzoate, succinate, citrate, methanol, glucose, fructose, alanine, glutamate, aspartate, formate, toluene and propanol. +, Positive test reaction; (+), weak test reaction; –, negative test reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Morphology	Vibrio	Vibrio	Curved rods	Curved rods	Long, thin vibrios	Curved cells	Vibrio	Curved rods	Vibrio-shaped	Curved rods	Curved rods
Cell diameter (µm)	0.7	0.5–1.0	0.7–0.9	0.4	0.4–0.5	0.75	0.5–1	0.5–0.7	1	0.8–1.2	0.8–1.2
Cell length (µm)	2.3–4.1	3.0–5.0	2.8–3.2	2–4	5.0–10.0	2.5–3.5	1–2	2–4	3–5	2.2–3.1	2.1–2.8
Salinity range (g l ⁻¹)	0–6		0–2	10–170	0–80	Maxima of 20	2–100	0–40	0–5	0–10	ND
Salinity optimum (g l ⁻¹)	0–1	0	0.75	50–60	10–20	14	6–80	0	0	0	ND
G + C content (mol%)	57.8	61	64.5 ± 0.3	59.5	62.3	64.1	53	64.13	66	67	ND
Electron donors:											
H ₂ + CO ₂	–		+	–	–		–	+	–	–	+
H ₂ + acetate	(+)		+	+	+	–	+	+	–	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	–	+	+	+	+	+
Malate	+	–	+	+	–	–	–	+	+	+	–
Fumarate	+		+	+	–	–	–	+	+	+	(+)
Acetate	–	–	–	–	–	–	+	–	–	–	–
Propionate	–		–	–	–	–	–	–	–	–	–
Ethanol	(+)		+	+	–	+	–	(+)	+	+	+
Butanol	(+)		+	(+)	–	+			ND	+	–
Glycerol	–		+	–	–			+	+	+	ND
Cysteine	–		ND	–	–		–	–	ND	ND	ND
Glycine	(+)		ND				–	–	–	ND	ND
Fermentation:											
Pyruvate	+	+	+	+	–			+	+	+	
Malate	+		–	+	–			+	–	+	
Lactate	–				–		+	–	–		
Fumarate	+		+					+	–	+	
Electron acceptors:											
Sulfate	+	+	+	+	+	–	+	+	+	+	+
Sulfite	+		+	+	+	+	+	+	–	+	+
Thiosulfate	+		+	+	+	+	+	+	+	+	+
Sulfur	–		+	+	+	–	–	+	+	+	(+)
Nitrate	–		–	–	–	–	+	–	ND	–	(+)
Fumarate	+		ND	ND	+	–	(+)		+	+	–

electron acceptor, the maximum growth rate of strain B7-43^T was 0.115 h⁻¹ and the doubling time was 6 h.

The G+C content of strain B7-43^T DNA was determined by HPLC at the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The G+C content of the DNA was 57.8 mol%.

The 16S rRNA gene of strain B7-43^T was amplified and its sequence was determined and analysed as described previously (Maidak *et al.*, 2001; Tardy-Jacquenod *et al.*, 1996; Weisburg *et al.*, 1991). A search for related sequences was performed using the GenBank and EMBL databases and the BLAST (Altschul *et al.*, 1990) and FASTA (Pearson & Lipman, 1988) software packages.

A total of 1498 nt of the 16S rRNA gene sequence of strain B7-43^T was determined. The sequence was aligned with representatives of the δ -Proteobacteria from the family 'Desulfovibrionaceae'. Phylogenetic analysis revealed that strain B7-43^T was included in the wide cluster of the genus *Desulfovibrio*. The phylogenetic tree obtained using the neighbour-joining method is shown in Fig. 1. Strain B7-43^T clustered with *Desulfovibrio sulfodismutans* (Bak & Pfennig,

1987) with a similarity of 90.7 % and *Desulfovibrio alcoholivorans* (Qatibi *et al.*, 1991) with a similarity of 90.6 %. Its closest relative was F1-7b (99.7 % similarity), a partially described SRB isolated from rice field soil (Wind *et al.*, 1999). This high level of similarity suggested that these two strains may belong to the same species. Unfortunately, strain F1-7b has been lost (R. Conrad, personal communication) and further comparison of their physiological characters or DNA-DNA hybridization experiments cannot be achieved.

Strain B7-43^T differed from *D. sulfodismutans* by its ability to grow with pyruvate, malate and fumarate as electron donors. Unlike *D. sulfodismutans*, it is not halotolerant (Table 1). It differed from *D. alcoholivorans* by its inability to use glycerol as an electron donor and its ability to ferment malate. Strain B7-43^T also differed from its closest relative, strain F1-7b, in utilization of different energy and carbon sources (Table 1), such as malate, butanol or H₂/CO₂.

Although strain B7-43^T was phylogenetically distant from known *Desulfovibrio* species, suggesting that it could even be a member of a new genus, its physiological characteristics are essentially the same as those displayed by *Desulfovibrio* species. Although B7-43^T was a typical freshwater isolate, with an optimal salinity for growth of 1 g NaCl l⁻¹ or less, its

Fig. 1. Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of *Desulfovibrio putealis* B7-43^T and selected species of the δ -Proteobacteria. The tree was constructed by the neighbour-joining method and rooted using *Escherichia coli* as an outgroup. Bootstrap values (expressed as percentages) for 1000 replicates are shown. Bar, 2 nt changes per 100 nt.

DNA G + C content of 57.8 mol% was closer to that of most halophilic *Desulfovibrio* species (46.1–62.0 mol%) such as *Desulfovibrio profundus* (Bale *et al.*, 1997) or *Desulfovibrio gabonensis* (Tardy-Jacquenod *et al.*, 1996). Generally, freshwater isolates such as *D. sulfodismutans* have a higher G + C content (62–67 mol%) (Table 1). Strain B7-43^T was also phylogenetically distinct from *Desulfovibrio* species isolated from the deep subsurface, e.g. *D. profundus*, *Desulfovibrio longus* (Magot *et al.*, 1992) and *D. gabonensis*, and by its inability to grow at NaCl concentrations above 6 g l⁻¹. On the basis of its physiological and phylogenetic properties compared with those of other members of the genus *Desulfovibrio*, it is proposed that strain B7-43^T represents a novel species, *Desulfovibrio putealis* sp. nov.

Description of *Desulfovibrio putealis* sp. nov.

Desulfovibrio putealis (pu.te.a'lis. L. adj. *putealis* from a well).

Vibrioid cells, 2.3–4.1 × 0.7 µm. Gram-negative. Motile by means of a single polar flagellum. Optimum growth occurs at 30 °C and pH 7.0. Grows in 0–6 g NaCl l⁻¹, with optimum growth at 1 g NaCl l⁻¹. Strictly anaerobic. Able to use sulfate, sulfite and thiosulfate, with production of sulfide. Nitrate is not used as an electron acceptor. Substrates that are oxidized by anaerobic respiration of a sulfate compound are H₂, lactate, pyruvate, malate, fumarate, ethanol, butanol and glycine. H₂ is only utilized in the presence of acetate. Pyruvate, fumarate and malate are fermented. Acetate, butyrate, benzoate, succinate, citrate, methanol, glucose, fructose, alanine, glutamate, aspartate, formate, toluene and propanol are not utilized. Desulfoviridin is present.

The type strain is B7-43^T (=DSM 16056^T=ATCC BAA-905^T), which was isolated from a water-producing well in France. The DNA G + C content of the type strain is 57.8 mol%.

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