Desulfovibrio bastinii sp. nov. and *Desulfovibrio gracilis* sp. nov., moderately halophilic, sulfate-reducing bacteria isolated from deep subsurface oilfield water

Michel Magot,¹ Odile Basso,¹ Christèle Tardy-Jacquenod²
and Pierre Caumette¹

Correspondence

Michel Magot
michel.magot@univ-pau.fr

¹Université de Pau et des Pays de l'Adour, IBEAS, Laboratoire d'Ecologie Moléculaire, EA3525, F-64013 Pau, France

²11 Place Garibaldi, F-42000 Saint Etienne, France

Two moderately halophilic, mesophilic, sulfate-reducing bacteria were isolated from production-water samples from Emeraude Oilfield, Congo. Motile, vibrioid cells of SRL4225^T grew optimally at a concentration of 4 % NaCl, at pH 5.8–6.2, with a minimal pH for growth of 5.2, showing that it is a moderately acidophilic bacterium. Cells of SRL6146^T were motile, curved or vibrioid, long and thin rods. Optimal growth was obtained at a concentration of 5–6 % NaCl, at pH 6.8–7.2. The nutritional requirements showed that many of the characteristics of these strains overlap with those of known *Desulfovibrio* species. On the basis of 16S rRNA gene sequence analysis and DNA–DNA hybridization studies, both strains are members of the genus *Desulfovibrio*. However, they are not closely related to any species of the genus that have validly published names. It is therefore proposed that the two strains are members of two novel species of the genus *Desulfovibrio* with the names *Desulfovibrio bastinii* sp. nov. (type strain SRL4225^T = DSM 16055^T = ATCC BAA-903^T) and *Desulfovibrio gracilis* sp. nov. (type strain SRL6146^T = DSM 16080^T = ATCC BAA-904^T).

To understand the unusually fast corrosion failure of the Emeraude Oilfield (Congo) undersea oil pipeline in 1990, investigations into corrosion, water and oil chemistry, and microbiology were performed (Crolet & Magot, 1996). Among other approaches, it was decided to isolate, characterize and identify all cultivable bacteria from a set of representative samples of production water. During this work, a large number of previously undescribed moderately halophilic, mesophilic, strictly anaerobic bacterial species have been isolated and described (Magot *et al.*, 1997a, b; Ollivier *et al.*, 1997; Ravot *et al.*, 1997, 1999). Several strains of sulfate-reducing bacteria that could not be related to any known species on a phylogenetic basis have also been isolated. Among them, strains SRL4225^T and SRL6146^T were included in the genus *Desulfovibrio*; however, they are distant enough from known *Desulfovibrio* species, either phylogenetically or physiologically, to be described as novel species, for which we propose the names *Desulfovibrio bastinii* sp. nov. (SRL4225^T) and *Desulfovibrio gracilis* sp. nov. (SRL6146^T).

Strain SRL4225^T was isolated from a water sample collected in the oil pipeline linking offshore production platforms of the Emeraude offshore oilfield (Congo) to onshore treatment facilities. The total bacterial count in the water sample (acridine orange direct count) indicated the presence of 2.5×10^4 bacteria ml⁻¹. Most probable number counts of sulfate-reducing bacteria were done as described previously (Magot *et al.*, 1992) and showed the presence of 2.5×10^2 cultivable sulfate-reducing bacteria ml⁻¹. In this experiment, the total salinity of the culture medium and the incubation temperature were adjusted so that they corresponded to the *in situ* conditions: total salinity of 53 g l⁻¹ and a temperature of 37 °C. Isolation of pure strains from the highest dilution of the most probable number count was done on solid medium in Petri dishes. Round black colonies (2–3 mm in diameter) were obtained after 2 weeks incubation. Several colonies were picked and their isolation was repeated twice. Cell morphology and restriction patterns of the amplified 16S rRNA genes allowed detection of strain SRL4225^T, potentially representing a previously unknown species. Cells of strain SRL4225^T were actively motile vibrios occurring singly or in pairs and were 2.0–3.0 × 0.5 µm in size (Fig. 1a).

The same procedure was applied for the isolation of strain

Published online ahead of print on 26 March 2004 as DOI 10.1099/ijs.0.02977-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of SRL4225^T, SRL6146^T and *Desulfovibrio longus* DSM 6739^T are AY359868, U53464 and AY359867, respectively.

Fig. 1. Phase-contrast micrographs of strains SRL4225^T (a) and SRL6146^T (b). Bars, 5 µm.

SRL6146^T. It was isolated from an oil and water emulsion collected at production well E153 of Emeraude Oilfield. The water sample contained 2.5×10^5 bacteria ml⁻¹ (acridine orange direct count), from which 9.5×10^3 sulfate-reducing bacteria ml⁻¹ were cultivated. Cells of strain SRL6146^T were long, thin, motile curved or vibrioid cells, $4.5\text{--}9.0 \times 0.3$ µm in size (Fig. 1b).

Electron microscopic observations indicated that cells of both strains were motile by means of a single polar flagellum (not shown). Gram staining of both strains was negative.

The physiological and metabolic characterization of the strains included tests to determine utilization of different carbon substrates and energy sources, utilization of a variety of electron acceptors, fermentative growth, temperature, pH and salinity optima, vitamin requirements and pigment content; the tests used have been described previously (Magot *et al.*, 1992; Tardy-Jacquenod *et al.*, 1996).

The energy and carbon sources used by SRL4225^T and SRL6146^T are shown in Table 1 and indicate that both isolates used a rather limited number of substrates. After five transfers, neither strain grew in the absence of vitamins or yeast extract.

Spectra of cell extracts of SRL4225^T and SRL6146^T both exhibited the characteristic absorption band of desulfoviridin at 631.5 nm.

Both strains are moderately halophilic bacteria displaying a similar salt requirement that is in agreement with the salinity of their oilfield-water habitat (Table 1). Strain SRL4225^T can grow between pH 5.2 and 7.4, with an optimum at pH 5.8–6.2: such tolerance of low pH is uncommon among sulfate-reducing bacteria. According to these characteristics, this strain can be considered as a moderately acidophilic sulfate-reducing bacterium, a physiological description that corresponds with the pH of Emeraude Oilfield water *in situ* (pH 5.5–6.5), but which is quite uncommon in this bacterial group.

The optimum temperatures for growth of both strains (see descriptions of the species) are also in accordance with the *in situ* temperature, which varies from 35 to 42 °C in the different production zones of the oilfield.

Under optimal conditions with lactate as electron donor and sulfate as electron acceptor, the doubling times of strains SRL4225^T and SRL6146^T were 15 and 22 h, respectively.

Determination of the G + C content of the DNA of strains SRL4225^T and SRL6146^T was done by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. The results are reported in Table 1.

The 16S rRNA genes of SRL4225^T and SRL6146^T were amplified and their sequences determined and analysed as previously described (Tardy-Jacquenod *et al.*, 1996). A new sequence of the 16S rRNA gene of *Desulfovibrio longus* DSM 6739^T was determined, for the purpose of this work, by using the same procedure.

The results of FASTA analysis of the 1416-nt 16S rRNA gene sequence of strain SRL4225^T against sequences in GenBank, and the construction of a phylogenetic tree as previously described (Tardy-Jacquenod *et al.*, 1996), revealed that strain SRL4225^T forms a distinct cluster with the undescribed *Desulfovibrio* species strain ASPO3 (93.6% similarity, distance matrix value), *Desulfovibrio hydrothermalis* (92.6%), *Desulfovibrio salexigens* (91.8%) and *Desulfovibrio zosterae* (91.7%) (Fig. 2). These results suggested that SRL4225^T could represent a novel species within the genus *Desulfovibrio*, although many of its characteristics (except its pH tolerance) overlap with those of known *Desulfovibrio* strains.

The 1476 nt 16S rRNA gene sequence of strain SRL6146^T is more closely related (98.6% similarity) to that of the undescribed *Desulfovibrio* sp. strain LVform6 isolated from carbonate sediments (Van Lith *et al.*, 2003), and to the recently described species '*Desulfovibrio capillatus*' (98.2% similarity), isolated from an oilfield separator (Miranda-Tello *et al.*, 2003). Since it is also closely related to *Desulfovibrio longus* (97.5% similarity), the results of

Table 1. Discriminating characteristics between strains SRL4225^T and SRL6146^T, halophilic desulfovibrios and other related *Desulfovibrio* species

Taxa: 1, *Desulfovibrio halophilus*; 2, *Desulfovibrio desulfuricans* subsp. *aestuarii*; 3, *D. salexigens*; 4, *Desulfovibrio giganteus*; 5, *Desulfovibrio gabonensis*; 6, *D. longus*; 7, '*D. capillatus*'; 8, *Desulfovibrio profundus*; 9, *Desulfovibrio aespoeensis*; 10, *D. zosteriae*; 11, *Desulfovibrio oxyclinae*; 12, *D. hydrothermalis*. Data were taken from Postgate (1984), Esnault *et al.* (1988), Caumette *et al.* (1991), Magot *et al.* (1992), Tardy-Jacquenod *et al.* (1996a), Bale *et al.* (1997), Krekeler *et al.* (1997), Motamedi & Pedersen (1998), Nielsen *et al.* (1999), Alazard *et al.* (2003) and Miranda-Tello *et al.* (2003). +, Positive test reaction; (+), weak test reaction; −, negative test reaction; ND, not determined. The following other substrates were tested but are not used by strains SRL4225^T and SRL6146^T with sulfate as electron acceptor: butyrate, isobutyrate, valerate, crotonate, nonanoate, octanoate, palmitate, tartrate, benzoate, succinate, citrate, 2-oxoglutarate, gluconate, glutarate, gallate, glycollate, thioglycollate, thioacetamide, methanol, 2-propanol, acetone, glucose, fructose, catechol, alanine, lysine, methionine, glutamate, aspartate, betaine, glycine, indole, phenol, nicotinate, peptone, Casamino acids and yeast extract. Glucose, fructose, succinate, fumarate, lactate, cysteine and glycerol were not fermented by either strain in the absence of any electron acceptor. All strains/species used H₂ + acetate and lactate as electron donors, none used acetate as electron donor and all were able to ferment sulfate.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	SRL4225 ^T	SRL6146 ^T
Morphology	Vibrios	Vibrios	Vibrios	Curved rods	Curved rods	Long, thin vibrios	Vibrios	Vibrios	Vibrios	Curved rods	Rods, curved rods	Vibrios	Vibrios	Long, thin vibrios
Size (µm)	0.6 × 2.5–5	0.5–1 × 3–5	0.5–1 × 3.5	1 × 5–10	0.4 × 2–4	0.4–0.5 × 5–10	0.2 × 1–2	0.5–1 × 1–2	0.5 × 1.7–2.5	0.5 × 3.0	0.5 × 2.3	0.5–1.2 × 1–2	0.5 × 2–3	0.3 × 4.5–9
Salinity range (g l ^{−1})	30–180	5–60	5–120	2–50	10–170	8	5–100	6–100	0–30	0–35	25–225	Up to 40	10–120	20–120
Salinity optimum (g l ^{−1})	60–70	25	20–40	2–25	50–60	10–20	30	10–80	7	12	50–100	25	40	50–60
DNA G + C content (mol%)	60.7	ND	46–49	55.5	59.5	62.3	58.7	53	61	42.7	ND	47	44.6	59.0
Electron donors														
H ₂ + CO ₂	−	−	−	−	−	−	−	(+)	ND	−	−	−	−	−
Pyruvate	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
Malate	−	+	−	−	+	−	−	−	+	+	+	+	−	−
Fumarate	−	+	+	−	+	−	−	−	−	+	−	+	−	−
Formate + acetate	+	+	−	+	+	+	−	ND	ND	(+)	+	+	−	+
Propionate	−	−	−	−	−	−	−	−	ND	−	−	−	−	−
Ethanol	+	+	−	+	+	−	−	−	−	+	+	+	+	−
Butanol	−	+	−	+	(+)	−	−	ND	ND	ND	+	ND	+	−
Glycerol	−	−	−	+	−	−	−	ND	ND	ND	ND	+	+	−
Cysteine	−	−	−	+	−	−	ND	−	ND	ND	ND	ND	−	−
Fermentation of:														
Pyruvate	−	+	−	+	+	−	+	+	+	+	+	+	+	−
Malate	−	−	−	−	+	−	−	ND	ND	ND	ND	ND	−	−
Electron acceptors														
Sulfite	+	+	ND	+	+	+	+	+	ND	+	+	+	+	+
Thiosulfate	+	+	ND	+	+	+	+	−	+	+	+	+	+	+
Sulfur	(+)	(+)	ND	−	+	+	+	−	+	+	+	−	+	+
Nitrate	−	+	ND	−	−	−	−	+	−	−	−	−	−	−
Fumarate	−	−	ND	−	ND	+	−	(+)	ND	ND	+	ND	−	+

Fig. 2. Unrooted phylogenetic tree based on 16S rRNA gene sequence comparison (1217 conserved positions) indicating the position of strains SRL4225^T and SRL6146^T among related members of the genus *Desulfovibrio*. Bootstrap values, expressed as percentages of 1000 replications, are shown at the nodes. Bar, 2 nucleotide substitutions per 100 nucleotides.

DNA–DNA reassociation experiments, done by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, were analysed to assess the species status of SRL6146^T. The relatedness between SRL6146^T and *D. longus* (23.1%; this study) and that between SRL6146^T and '*D. capillatus*' (28.6%; Miranda-Tello *et al.*, 2003) clearly indicated that the three strains represent three distinct species.

Thus, SRL6146^T, *D. longus* and '*D. capillatus*' form a distinct cluster of closely related bacteria isolated from oilfield waters. Strain SRL6146^T closely resembles *D. longus* in terms of its morphological, physiological and biochemical characteristics. Apart from the 3.3 mol% difference in the G + C contents of their DNAs, the main difference between these species lies in their salt tolerance. SRL6146^T is a true moderately halophilic bacterium, since it requires at least 20 g NaCl l⁻¹, whereas *D. longus* can grow in the absence of salt (Table 1). This major physiological difference is also reflected by the upper limit of salt tolerance of 120 g l⁻¹, as opposed to 80 g l⁻¹ for *D. longus*, and its optimum salinity of 50–60 g NaCl l⁻¹, which compares with 10–20 g l⁻¹ for *D. longus*.

SRL6146^T is phenotypically different from the other related species, '*D. capillatus*' (Miranda-Tello *et al.*, 2003). These two bacteria differ in morphology, use different electron donors, differ in the fermentation of pyruvate and differ in the use of fumarate as electron acceptor (Table 1).

On the basis of these observations, we propose to assign strains SRL4225^T and SRL6146^T to the genus *Desulfovibrio* as novel species, *Desulfovibrio bastinii* sp. nov. and *Desulfovibrio gracilis* sp. nov., respectively.

The two novel bacterial strains isolated during this work display several characteristics that suggest that they might be indigenous to the oil reservoir. Their moderately halophilic character, with optimal salinities for growth between 40 and 60 g l⁻¹, correspond perfectly to the *in situ* water salinity of 53 g l⁻¹. In addition, the optimal growth temperatures and pH values are in good agreement with the

physico-chemical conditions prevailing in the reservoir, i.e. 37–42 °C and pH 5.5–6.5. For these reasons, we consider that SRL4225^T and SRL6146^T represent novel bacterial strains originating from a deep subsurface environment (Magot *et al.*, 2000).

Description of *Desulfovibrio bastinii* sp. nov.

Desulfovibrio bastinii (bas.ti'ni.i. N.L. gen. n. *bastinii* of Bastin, named after the American microbiologist Edson S. Bastin, who studied sulfate-reducing bacteria from oilfields at the beginning of the 20th century).

Cells are vibrioid, 0.5 × 2–3 µm, occur singly or in pairs and are motile by means of a single polar flagellum. Gram-negative and non-spore-forming. Colonies are round with entire edges, smooth, and grey to dark grey. Moderately halophilic. Optimal NaCl concentration for growth is 40 g l⁻¹; growth occurs between 10 and 120 g NaCl l⁻¹. Mesophilic. Optimal growth temperature is 35–40 °C; range for growth is 20–50 °C. Moderately acidophilic. pH range is 5.2–7.4; optimum is pH 5.8–6.2. Vitamins are required for growth. Strictly anaerobic. Sulfate, sulfite, thiosulfate and elemental sulfur serve as electron acceptors, whereas nitrate and fumarate do not. No autotrophic growth. Lactate, pyruvate, malate, fumarate, ethanol, butanol, glycerol and hydrogen in the presence of acetate are used as growth substrates. Lactate is oxidized to acetate and CO₂. Pyruvate is fermented. Desulfovibrin is present. The G + C content of the DNA is 44.6 mol%.

The type strain is SRL4225^T (=DSM 16055^T=ATCC BAA-903^T), isolated from oilfield production water collected from a pipeline of Emeraude Oilfield, Congo.

Description of *Desulfovibrio gracilis* sp. nov.

Desulfovibrio gracilis (gra'cil.is. L. masc. adj. *gracilis* slim, slender, thin).

Cells are rod-shaped, 0.3 × 4.5–9.0 µm, straight, curved or vibrioid and thin, occur singly and are motile by means of a single polar flagellum. Gram-negative and non

spore-forming. Colonies are round with entire edges, smooth and grey to dark grey. Moderately halophilic. Optimal NaCl concentration for growth is 50–60 g l⁻¹; growth occurs between 20 and 120 g NaCl l⁻¹. Mesophilic. Optimal growth temperature is 37–40 °C; range for growth is 20–40 °C. pH range is 5.4–8.4; optimum is pH 6.8–7.2. Vitamins are required for growth. Strictly anaerobic. Sulfate, sulfite, thiosulfate, elemental sulfur and fumarate serve as electron acceptors; nitrate is not used. No autotrophic growth. Lactate and pyruvate are used as growth substrates. Lactate is oxidized to acetate and CO₂. Fumarate and hydrogen are used in the presence of acetate. Glucose, fructose, succinate, malate, fumarate, pyruvate, lactate, cysteine and glycerol are not fermented. Desulfovibrin is present. The G + C content of the DNA is 59.0 mol%.

The type strain is SRL6146^T (=DSM 16080^T=ATCC BAA-904^T), isolated from oilfield production water collected at the E153 wellhead of Emeraude Oilfield, Congo.

Acknowledgements

Part of this work was supported by a grant from ELF-Aquitaine. We greatly thank F. Laigret for the determination of the 16S rDNA sequence of strain SRL6146^T.

References

- Alazard, D., Dukan, S., Urios, A., Verhé, F., Bouabida, N., Morel, F., Thomas, P., Garcia, J.-L. & Ollivier, B. (2003). *Desulfovibrio hydrothermalis* sp. nov., a novel sulfate-reducing bacterium isolated from hydrothermal vents. *Int J Syst Evol Microbiol* **53**, 173–178.
- Bale, S. J., Goodman, K., Rochelle, P. A., Marchesi, J. R., Fry, J. C., Weightman, A. J. & Parkes, R. J. (1997). *Desulfovibrio profundus* sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. *Int J Syst Bacteriol* **47**, 515–521.
- Caumette, P., Cohen, Y. & Matheron, R. (1991). Isolation and characterization of *Desulfovibrio halophilus* sp. nov., a halophilic sulfate-reducing bacterium isolated from Solar Lake (Sinai). *Syst Appl Microbiol* **14**, 33–38.
- Crolet, J.-L. & Magot, M. (1996). Non-SRB sulfidogenic bacteria in oilfield production facilities. *Mater Perform* **35**, 60–64.
- Esnault, G., Caumette, P. & Garcia, J.-L. (1988). Characterization of *Desulfovibrio giganteus* sp. nov., a sulfate-reducing bacterium isolated from a brackish coastal lagoon. *Syst Appl Microbiol* **10**, 147–151.
- Krekeler, D., Sigalevich, P., Teske, A., Cypionka, H. & Cohen, Y. (1997). A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxycliniae* sp. nov. *Arch Microbiol* **167**, 369–375.
- Magot, M., Caumette, P., Desperrier, J. M., Matheron, R., Dauga, C., Grimont, F. & Carreau, L. (1992). *Desulfovibrio longus* sp. nov., a sulfate-reducing bacterium isolated from an oil-producing well. *Int J Syst Bacteriol* **42**, 398–403.
- Magot, M., Fardeau, M.-L., Arnauld, O., Lanau, C., Ollivier, B., Thomas, P. & Patel, B. K. C. (1997a). *Spirochaeta smaragdinae* sp. nov., a new mesophilic strictly anaerobic spirochete from an oilfield. *FEMS Microbiol Lett* **155**, 185–191.
- Magot, M., Ravot, G., Campagnolle, X., Ollivier, B., Patel, B. K. C., Fardeau, M.-L., Thomas, P., Crolet, J.-L. & Garcia, J.-L. (1997b). *Dethiosulfovibrio peptidovorans* gen. nov., sp. nov., a new anaerobic, slightly halophilic, thiosulfate-reducing bacterium from corroding offshore oil wells. *Int J Syst Bacteriol* **47**, 818–824.
- Magot, M., Ollivier, B. & Patel, B. K. C. (2000). Microbiology of petroleum reservoirs. *Antonie van Leeuwenhoek* **77**, 103–116.
- Miranda-Tello, E., Fardeau, M.-L., Fernández, L., Ramirez, F., Cayol, J.-L., Thomas, P., Garcia, J.-L. & Ollivier, B. (2003). *Desulfovibrio capillatus* sp. nov., a novel sulfate-reducing bacterium isolated from an oil field separator located in the Gulf of Mexico. *Anaerobe* **9**, 97–103.
- Motamedi, M. & Pedersen, K. (1998). *Desulfovibrio aesopaeensis* sp. nov., a mesophilic sulfate-reducing bacterium from deep groundwater at Äspö hard rock laboratory, Sweden. *Int J Syst Bacteriol* **48**, 311–315.
- Nielsen, J. T., Liesack, W. & Finster, K. (1999). *Desulfovibrio zosterae* sp. nov., a new sulfate reducer isolated from surface-sterilized roots of the seagrass *Zostera marina*. *Int J Syst Bacteriol* **49**, 859–865.
- Ollivier, B., Cayol, J.-L., Patel, B. K. C., Magot, M., Fardeau, M.-L. & Garcia, J.-L. (1997). *Methanoplanus petrolearius* sp. nov., a novel methanogenic bacterium from an oil producing well. *FEMS Microbiol Lett* **147**, 51–56.
- Postgate, J. R. (1984). Genus *Desulfovibrio* Kluyver and van Niel 1936, 397^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 666–672. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Ravot, G., Magot, M., Ollivier, B., Patel, B. K. C., Ageron, E., Grimont, P. A. D., Thomas, P. & Garcia, J.-L. (1997). *Haloanaerobium congolense* sp. nov., an anaerobic, moderately halophilic, thiosulfate- and sulfur-reducing bacterium from an African oilfield. *FEMS Microbiol Lett* **147**, 81–88.
- Ravot, G., Magot, M., Fardeau, M.-L., Patel, B. K. C., Thomas, P., Garcia, J.-L. & Ollivier, B. (1999). *Fusibacter paucivorans* gen. nov., sp. nov., an anaerobic, thiosulfate-reducing bacterium from an oil-producing well. *Int J Syst Bacteriol* **49**, 1141–1147.
- Tardy-Jacquenod, C., Magot, M., Laigret, F., Kaghad, M., Patel, B. K. C., Guezennec, J., Matheron, R. & Caumette, P. (1996). *Desulfovibrio gabonensis* sp. nov., a new moderately halophilic sulfate-reducing bacterium isolated from an oil pipeline. *Int J Syst Bacteriol* **46**, 710–715.
- Van Lith, Y., Warthmann, R., Vasconcelos, C. & McKenzie, J. A. (2003). Microbial fossilization in carbonate sediments: a result of the bacterial surface involvement in dolomite precipitation. *Sedimentology* **50**, 237–245.