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Desulfovibrio longus sp. nov., a Sulfate-Reducing Bacterium Isolated from an Oil-Producing Well

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A novel type of sulfate-reducing bacteria with unusual morphology was isolated from an oil-producing well in the Paris Basin. The cells of this bacterium, strain SEBR 2582^T (T = type strain), are long, thin, flexible rods, contain desulfoviridin, and are physiologically similar to members of the genus *Desulfovibrio*. On the basis of 16S rRNA sequence data, this strain should be included in the genus *Desulfovibrio*. However, strain SEBR 2582^T differs from other members of this genus morphologically, physiologically, and phylogenetically. Thus, a new species, *Desulfovibrio longus* sp. nov., is proposed for this organism.

As an end product of anaerobic respiration, sulfate-reducing bacteria (SRB) produce hydrogen sulfide, a toxic compound which may accumulate in various anoxic sediments of ponds, lakes, coastal lagoons, or marine environments (14). SRB are also present in oil production facilities, where these bacteria cause severe economic losses due to iron corrosion in the absence of air, as well as to the in situ production of H_2S which results in the souring of oil reservoirs during secondary oil recovery (2, 6). Although a knowledge of the diversity of SRB in these industrial environments is necessary to improve treatments to prevent the detrimental effects of these organisms, little attention has been paid to identification of SRB, and few species of SRB isolated from oil field waters have been described (6). Recently, Rozanova et al. (21) isolated a new species of a new genus (*Desulfomicrobium apsheronum*) from an oil-bearing deposit. During the course of a microbiological study of several oil field waters, we isolated various unknown strictly anaerobic bacterial strains, including some SRB. The cells of one of these bacteria, strain SEBR 2582^T (T = type strain), are long, gracile rods that have an unusual morphology. Jones (13) first described this morphological type of SRB, and more recently, Widdel (23) isolated a similar bacterial type; however, these strains were never described as new species. Despite the morphology of strain SEBR 2582^T, the results of physiological and genetical analyses of this organism led us to assign it to the genus *Desulfovibrio*. However, strain SEBR 2582^T is not a member of a previously described species of this genus. In this paper we describe this strain and identify it as a new species. The name *Desulfovibrio longus* sp. nov. is proposed.

MATERIALS AND METHODS

Source of the isolate. Strain SEBR 2582^T was isolated from an oil-producing well in the Paris Basin (France). The production fluid (oil-water emulsion) was collected at the top of the well after extensive draining of the tubing. Sterile plasma bottles were used for sampling. Aliquots of the aqueous phase were anaerobically injected into SRB detection kits (type SEBR-A1), which have been described previously (15). The tubes were then incubated in the dark at 30°C. After 3 days of incubation, a black precipitate of FeS in the tubes revealed the presence of sulfide; then the isolation of SRB was begun.

Isolation and maintenance in pure culture. SRB were isolated by using the streaking method on agar medium in petri dishes that were incubated in an anaerobic glove box with a gas mixture containing N_2, H_2, and CO_2 (85:10:5). The culture medium used for isolation contained (per liter of distilled water) 2.0 g of Na_2SO_4, 0.3 g of $KHCO_3$, 1.0 g of $CaCl_2 \cdot 2H_2O$, 1.0 g of $MgSO_4 \cdot 7H_2O$, 1 ml of a 0.4% $FeSO_4 \cdot 7H_2O$ solution, 3.0 g of MOPS (morpholinepropanesulfonic acid) buffer, 1.0 g of NH_4Cl, 6.0 ml of a 60% sodium lactate solution, 1.0 g of sodium acetate, 1.0 g of yeast extract, 0.5 g of cysteine hydrochloride, and 16 g of agar. The pH was 7.4, and the medium was anaerobically distributed into bottles and autoclaved for 30 min at 105°C.

Colonies were purified by consecutive streaking onto the same medium.

The final liquid medium used for growth and maintenance of the strain was prepared as described by Pfennig et al. (19). This medium contained (per liter of distilled water) 3 g of Na_2SO_4, 0.3 g of KCl, 0.3 g of NH_4Cl, 1.2 g of NaCl, 0.4 g of $MgCl_2 \cdot 6H_2O$, 0.2 g of KH_2PO_4, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1 ml of trace element solution SL12 (9), 10 mmol of sodium lactate, 2.5 g of $NaHCO_3$, 4 ml of a vitamin solution (19), 1.0 g of yeast extract, and 0.2 g of $Na_2S \cdot 9H_2O$; the pH was 7.4.

The purity of strains was checked by using both phase-contrast microscopy and growth tests in sulfate-free TYG medium (3) (tested both aerobically and anaerobically). Pure cultures were maintained as stock cultures in 120-ml screw-cap bottles containing liquid medium supplemented with 1 ml of a growth factor solution (19) per liter.

Morphology. Microscopic observations were performed and photomicrographs were obtained by using a Zeiss photomicroscope as described by Pfennig and Wagener (18). Flagella were observed with a Hitachi model 600 transmission electron microscope after negative staining with 1% phosphotungstic acid.

Physiological tests. We tested the utilization of energy sources, carbon sources, and electron acceptors in basal liquid medium by using the substrate concentrations shown

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TABLE 1. Electron donors and acceptors used by *Desulfovibrio longus* SEBR 2582^{T}[a]

Electron donor or acceptor	Growth in the presence of:		
	Sulfate (30 mM)	No sulfate	Lactate
Electron donors			
Lactate (10 mM)	+	−	
Pyruvate (10 mM)	+	−	
Acetate (10 mM)	−		
Formate + CO_2 (10 mM)	+		
Formate + acetate (10 mM)	+		
Ethanol (10 mM)	−	−	
Propanol (10 mM)	−		
Fumarate (10 mM)	−		
Malate (10 mM)	−	−	
H_2 + CO_2 (10^5 Pa)	−	−	
H_2 (10^5 Pa) + acetate (10 mM)	+		
Electron acceptors			
Sulfate (30 mM)			+
Sulfite (5 mM)			+
Thiosulfate (10 mM)			+
Sulfur			+
Nitrate (10 mM)			−
Fumarate (10 mM)			+

[a] The following substrates were tested and were not utilized by strain SEBR 2582^{T}: succinate (10 mM), α-ketoglutarate (5 mM), citrate (5 mM), propionate (10 mM), butyrate (10 mM), isobutyrate (5 mM), glucose (5 mM), fructose (5 mM), gluconate (10 mM), methanol (10 mM), isopropanol (10 mM), butanol (10 mM), glycerol (10 mM), benzoate (5 mM), gallate (5 mM), catechol (2 mM), nicotinate (2 mM), lysine (10 mM), methionine (10 mM), cysteine (5 mM), glutamate (5 mM), aspartate (5 mM), acetone (5 mM), glycolate (2 mM), thioglycolate (2 mM), thioacetamide (2 mM), peptone (0.5 g/liter), Casamino Acids (0.5 g/liter), and yeast extract (0.5 g/liter). Glucose, fructose, succinate, and glycerol were not fermented. *n*-Alkanes from C_{15} to C_{40} were tested and were not utilized by strain SEBR 2582^{T} after 1 month of incubation under anaerobic conditions.

in Table 1. Fermentative growth was tested in the same medium except that sulfate was omitted. These tests, as well as the tests used to determine the optimum concentrations of NaCl and $MgCl_2$, the optimum pH, and the optimum temperature, were performed in completely filled 25-ml screw-cap tubes with rubber seals. Growth was measured over a period of 15 days by determining optical density at 450 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. At the end of the experiments, sulfide production was checked in each screw-cap tube by using the method of Cord-Ruwisch (5). The requirements for vitamins or growth factors were tested in 120-ml anaerobic bottles after five consecutive transfers by using the experimental system of Goupy (11). Eight duplicated experiments were done for seven vitamins (biotin, *p*-aminobenzoate, thiamine, panthothenic acid, pyridoxine, cyanocobalamin, and nicotinic acid).

Pigments. The presence of bisulfite reductase (desulfoviridin) in strain SEBR 2582^{T} was tested by using cell extracts and a Kontron Uvikon 860 spectrophotometer (24).

Disproportionation tests. The disproportionation of thiosulfate and sulfite was tested by using a basal synthetic medium that lacked sulfate and organic energy sources. The basal medium was supplemented with acetate (used as a carbon source by strain SEBR 2582^{T}) and either thiosulfate or sulfite as an energy source and electron acceptor (1). After incubation, disproportionation was tested by measuring the increases in optical density and sulfate and sulfide production (4a).

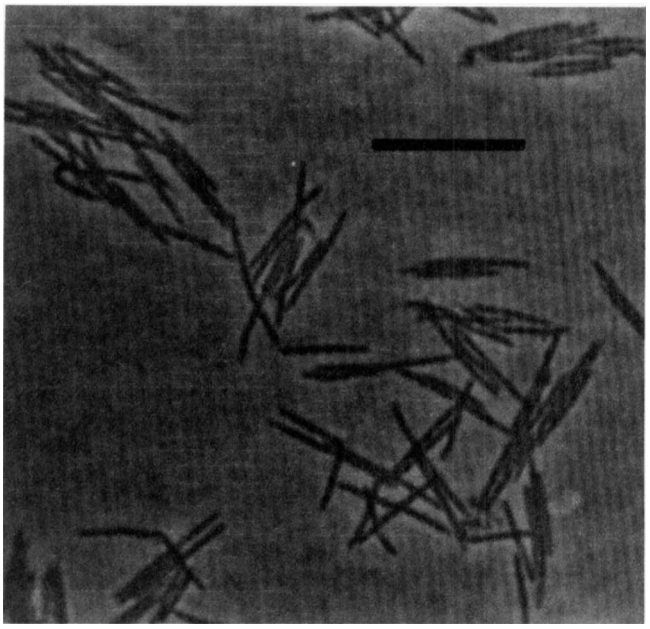

FIG. 1. Phase-contrast photomicrograph of *Desulfovibrio longus* SEBR 2582^{T} grown with lactate as a carbon and energy source. Bar = 10 μm.

G+C content of DNA and rRNA sequencing homology. The G+C content of strain SEBR 2582^{T} DNA was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. The DNA isolation and high-performance liquid chromatography procedures used have been described previously (4, 16).

rRNA extraction, primer-rRNA hybridization, the elongation reaction with reverse transcriptase, and electrophoresis of elongation products were performed as described previously (7). Of the 11 primers used, 8 were the same as those described by Embley et al. (10) (primers, 1, 2, and 4 through 9). Primer 11 (5′-ATTACTCACCCGTCCGCC-3′) has been described by Dauga et al. (7). The following two primers were selected to ease sequencing of the 5′ end of the rRNA molecule: primer 10a (5′-CCCACCAACAAGCTAATGA-3′) and primer 3a (5′-TCTACGGATTTCACGCCTAC-3′). The sequence of 16S rRNA from strain SEBR 2582^{T} was compared with the 16S rRNA sequences of several species belonging to the genera *Desulfovibrio*, *Desulfobulbus*, *Desulfotomaculum*, *Desulfococcus*, *Desulfobacter*, and *Desulfobacterium*; the data were kindly provided by D. Stahl (8). The sequences that were compared were aligned manually. Positions where nucleotides could not be determined unambiguously were not included in the calculation. The divergence (or distance) between two sequences was estimated as the number of nucleotide substitutions (λ). The K_{nuc} values were determined as described by Hori and Osawa (12), using the following formula: $K_{nuc} = -3/4 \ln (1 - 4/\lambda)$. A phylogenetic tree was constructed by using the neighbor-joining method (22), as recommended by a committee of experts (17).

Nucleotide sequence accession number. The nucleotide sequence (1,521 nucleotides) of *Desulfovibrio longus* 16S rRNA has been deposited with Genbank (EMBL) under accession number X63623.

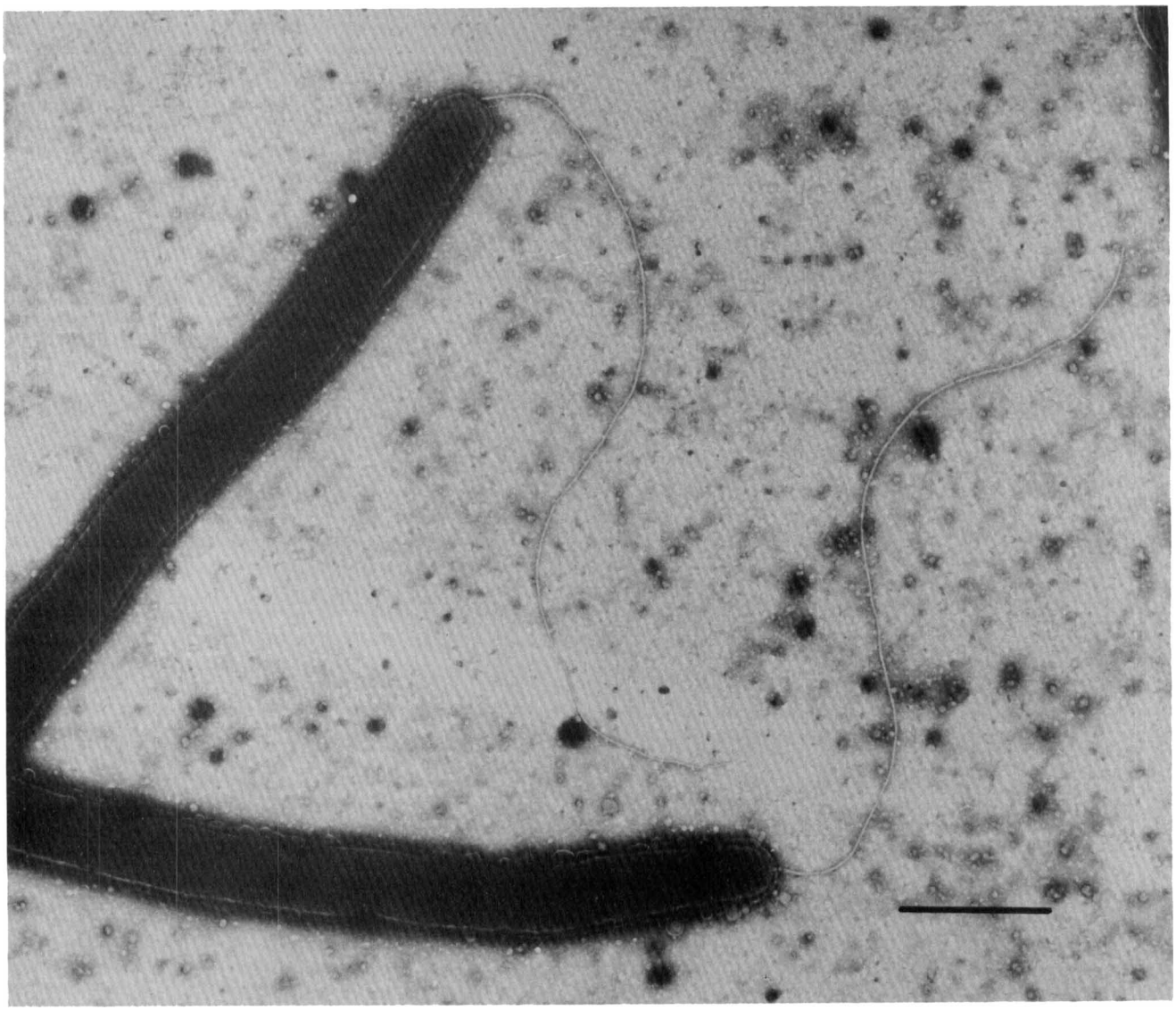

FIG. 2. Transmission electron micrograph after negative staining of *Desulfovibrio longus* SEBR 2582^T, showing the single polar flagellum. Bar = 1 μm.

RESULTS

Enrichment and isolation. From grey colonies that were 1 to 2 mm in diameter we obtained a pure culture after three consecutive transfers on solid media. The purity of this strain (strain SEBR 2582^T) was confirmed by the morphological homogeneity of cells observed under a phase-contrast microscope and by the absence of growth in liquid sulfate-free TYG medium under aerobic and anaerobic conditions.

Cell morphology. Individual cells of strain SEBR 2582^T were long, thin, nonsporulating rods which were sometimes slightly curved (Fig. 1). Whole cells were 0.4 to 0.5 μm wide and 5 to 10 μm long under optimal conditions. The cells moved slowly by means of a single polar flagellum (Fig. 2). Gram staining was negative.

Pigment composition. The spectrum of cell extracts exhibited the characteristic absorption band of desulfoviridin at 631.5 nm.

Physiological properties. The substrates that were tested as energy and carbon sources are shown in Table 1. Strain SEBR 2582^T used a rather limited number of substrates. Lactate, pyruvate, and formate were used as energy and carbon sources. H_2 was also used as an energy source. Acetate was used only as a carbon source. CO_2 was not used as a sole source of carbon by strain SEBR 2582^T.

None of the substrates which we tested was fermented.

Strain SEBR 2582^T used sulfate, sulfite, thiosulfate, sulfur, and fumarate as electron acceptors in the presence of lactate as an electron donor and carbon source.

After five transfers strain SEBR 2582^T did not grow without vitamins or yeast extract. Biotin, *p*-aminobenzoate, and thiamine significantly stimulated the growth of strain SEBR 2582^T. Associated biotin and *p*-aminobenzoate had a synergic effect.

In synthetic medium supplemented with lactate and sulfate, strain SEBR 2582^T grew at NaCl concentrations between 0 and 8%; optimum growth occurred NaCl concentrations of 1 to 2%. The optimum pH was 7.4. Strain SEBR 2582^T grew well between pH 6.5 and 8.5. The optimum

UUCCCUGAGGGUUUGAUCCUGGCUCAGGGUGAACGCUAGCGGCGCGCCUAACCCAUGCAAGUCNAACGAGAAAGGGUCCUUCG
GGACCUGAGUAGAGUGGCGCACGGGUGAGUAACGCGUAGGUGAUCUACCCUUAGGAUUGGGAUAACAGUUGGAAACGGCUGCU
AAUACCGGAUACGCUCCAUAUUUAGCUUUUUGGAUGAAAGGUGGCCUCUGCAUGCAAGCUACCACCUAAGGAUGAACCUGCGU
CUCAUUAGCUUGUUGGUGGGGUNAUGGCCUACNAAGGCGACGAUGAGUAGCUGAUCUGGGAGGAUGAUCAGCCACACUGGGAC
UGGAACACGGCCCAGACUCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNACGCAGCGACGCCGCG
UGAGGGAAGAAGGCUUCGGGUUCGUAAACCUCUGUCAGAAGGGAAGAAACCCUUGAGGGNGAAUAGGCCUCUUGGCUGACGGU
ACCUUCAGAGGAAGCACCGGCUAAUUCCGUGCCAGCAGCCGCGGUAAUACGGAGGGUGCAAGCGUUAAUCGGAAUCACUGGGC
GUAAAGCGCACGUAGGCGGUGCGGUCAGUCAGAUGUGAAACCUCGGCUCAACCGAGGAAUUGCAUUUGAUACUGCCGUGCUCG
AGUACUGGAGAGGCAGGCGGAAUUCCANNGUUAGGAGUGAAAUCCGUAGAUAUCUGGAGGAACACCAGUGGCGAAGGCGGCCU
GCUGGACAGAUACUGACGCUGAGGUNCGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCUGUAAACGAU
GGAUGCUAGGUGUCGGGGGGUUCUUCGGUGCCGCAGUUAACGCGUUAAGCAUCCCGCCUGGGGAGUACGGUCGCAAGGCUGNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCGCGAAGAANCCUUACCUAGGC
UUGACAUCCUNAGAACCCUCCCGAAAANGGAGGNNUGCCUUCGGGGNAUUCAGUGACAGGUGCUGCAUGGCUGUCGUCAGCUC
GUGCCGUGAGGUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUUGUUUCUUGUUGCCAGCGCGUAAUGGUGGGCACUCUAGU
GAGACUGCCGGGGUUAACCCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGGCCCUUACGCCUAGGGCUACACACGUACUA
CAAUGGCACGUACAAAGGGUUGCAAAGCCGCGAGGCAACGCCNAUCCCAAAAAACGUGUCCCAGUCCGGAUUGCAGUCUGCAA
CUCGACUGCAUGAAGUUGGAAUCGCUAGUAAUCCCAGAUCAGAAUGCUGGGGUGAAUACGUUCCCGGGCCUUGUACACACCGC
CCGUCACAUACCACGUAAAGCUGGUUCUACCCGACAUCGGCAAGCUAACUUCGGGAGGCAGCCGCCUACGGUAGGACCGGUGA
UUGGGGUGAAGUCGUAACAAGGUAGCC

FIG. 3. 16S rRNA sequence of *Desulfovibrio longus* SEBR 2582^T.

temperature was 35°C. The strain was a normal mesophilic organism that grew at temperatures between 10 and 40°C; it grew slowly at temperatures between 10 and 15°C.

Under optimal conditions of salinity, temperature, and pH with lactate as an energy and carbon source, the mean doubling time of strain SEBR 2582^T was about 3 h during exponential growth.

Sulfite was disproportionated, but thiosulfate was not.

The DNA base composition of strain SEBR 2582^T was 62.3 ± 0.6 mol% G+C (as determined by high-performance liquid chromatography).

16S rRNA sequence. A total of 1,522 nucleotides of the 16S rRNA sequence of strain SEBR 2582^T were determined, corresponding to about 98% of the whole molecule (Fig. 3).

The phylogenetic tree obtained by using the neighbor-joining method (22) is shown in Fig. 4.

Strain SEBR 2582^T clustered with *Desulfovibrio vulgaris*, *Desulfovibrio salexigens*, and *Desulfovibrio gigas*. This cluster was distinct from the cluster that contained the genera *Desulfobulbus*, *Desulfotomaculum*, *Desulfococcus*, *Desulfobacter*, and *Desulfobacterium*, as previously shown by Devereux et al. (8).

DISCUSSION

Strain SEBR 2582^T, which was isolated from an oil-producing well in the Paris Basin, is strictly anaerobic and grows with lactate as an energy and carbon source and with sulfate as an electron acceptor, thus reducing sulfate. Strain SEBR 2582^T is phylogenetically very closely related to members of the genus *Desulfovibrio*. On the basis of 16S rRNA sequence data, strain SEBR 2582^T clusters with *Desulfovibrio vulgaris* and *Desulfovibrio salexigens*. The G+C content of the DNA of strain SEBR 2582^T (62.3 ± 0.6 mol%) is also similar to the DNA G+C contents of members of the genus *Desulfovibrio* (47.5 to 65 mol%).

Thus, despite its morphology (motile, long, gracile, rod-shaped, nonsporulating cells), strain SEBR 2582^T should be included in the genus *Desulfovibrio* (20).

Strain SEBR 2582^T is the only *Desulfovibrio* strain with the morphology described above that is able to use formate but not fatty acids as both energy and carbon sources. Physiologically, strain SEBR 2582^T resembles *Desulfomicrobium apsheronum* (21). *Desulfomicrobium apsheronum* cells are rod shaped and differ from strain SEBR 2582^T cells by their size (small, short rods), their capacity for autotrophic growth, their fermentative metabolism, and their lack of desulfoviridin. Moreover, the G+C content of *Desulfomicrobium apsheronum* DNA (52 mol%) is lower than that of strain SEBR 2582^T DNA.

Among the species of the genus *Desulfovibrio*, *Desulfovibrio baculatus* is similar to strain SEBR 2582^T. Recently, *Desulfovibrio baculatus* has been transferred to the genus *Desulfomicrobium* as *Desulfomicrobium baculatum* (15). This short rod-shaped bacterium differs from strain SEBR 2582^T physiologically and genetically; *Desulfovibrio longus* SEBR 2582^T does not use malate but uses formate plus CO_2, in contrast to *Desulfomicrobium baculatum*, which uses malate but does not use formate plus CO_2. These organisms also differ in their DNA G+C contents (57 mol% for *Desulfomicrobium baculatum* and 62.3 ± 0.6 mol% for *Desulfovibrio longus* SEBR 2582^T).

A similar strain whose cells are long, gracile, and rod shaped was isolated and described by Jones (13). The energy and carbon sources used by this strain and strain SEBR 2582^T are similar, as are the G+C contents of their DNAs. However, the strain of Jones contains a special pigment in addition to desulfoviridin. Jones did not describe his organism as a new species of sulfate-reducing bacteria. Widdel (23) isolated and described a morphologically similar sulfate reducer and considered it to be a member of the genus

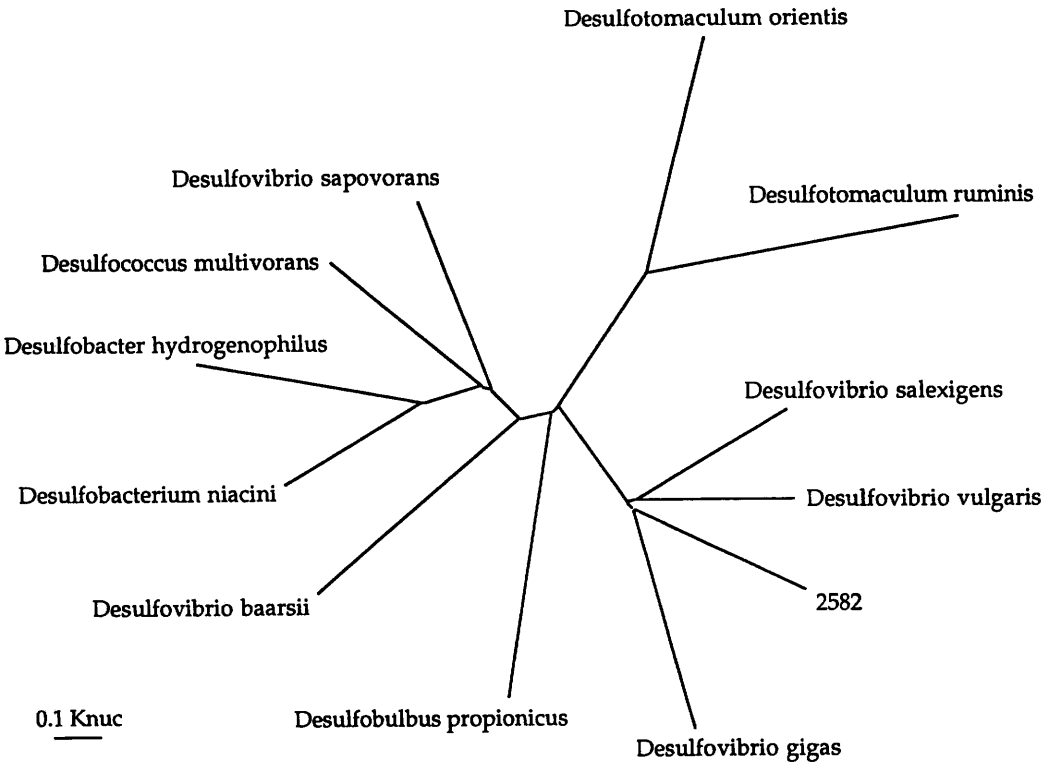

FIG. 4. Unrooted phylogenetic tree showing the relationships of *Desulfovibrio longus* SEBR 2582^T and some other species of sulfate-reducing bacteria, based on a comparison of 1,522 nucleotides (16S rRNA).

Desulfovibrio. Unfortunately this strain was lost recently (23a).

Strain SEBR 2582^T is a halotolerant organism which grows best in the presence of 1 to 2% NaCl in synthetic medium, as *Desulfovibrio salexigens* and *Desulfovibrio giganteus* do (10a). However, it differs from these species morphologically as well as physiologically, particularly in its inability to utilize alcohols and malate. Thus, on the basis of morphological, physiological, and phylogenetic differences compared with other members of the genus, below we describe this strain as a new species of the genus *Desulfovibrio*, *Desulfovibrio longus*.

Description of *Desulfovibrio longus* sp. nov. *Desulfovibrio longus* (long'us. L. adj. *longus*, long). Long, thin, straight, rod-shaped cells that are 0.4 to 0.5 μm wide and 5 to 10 μm long. Gram negative. Motile by means of a single polar flagellum. The pH range is 6.5 to 8.5; optimum growth occurs at 7.4. The temperature range is 10 to 40°C; optimum growth occurs at 35°C. The salinity range is 0 to 8% NaCl; optimum growth occurs in the presence of 1 to 2% NaCl.

Strictly anaerobic. Reduces sulfate, sulfite, thiosulfate, and elemental sulfur with production of sulfide. Fumarate is reduced to succinate. Nitrate is not used as an electron acceptor. The substrates that are oxidized by anaerobic respiration of sulfur compounds or fumarate are H_2, lactate, pyruvate, and formate. H_2 is used only in the presence of acetate or yeast extract. Organic substrates are not fermented. Biotin and *p*-aminobenzoate are required as growth factors.

Desulfoviridin is present. The G+C content of the DNA is 62.3 mol% (as determined by high-performance liquid chromatography).

Habitat: production fluid from an oil-producing well in the Paris Basin.

The type strain is strain SEBR 2582, which has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 6739.

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