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SHORT COMMUNICATION

I-Hsien Tsu · Chi-Yu Huang · Jean-Louis Garcia
Bharat K. C. Patel · Jean-Luc Cayol · Larry Baresi
Robert A. Mah

# Isolation and characterization of *Desulfovibrio senezii* sp. nov., a halotolerant sulfate reducer from a solar saltern and phylogenetic confirmation of *Desulfovibrio fructosovorans* as a new species

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**Abstract** A new halotolerant *Desulfovibrio*, strain CVL$^T$ (T = type strain), was isolated from a solar saltern in California. The curved, gram-negative, nonsporeforming cells (0.3 × 1.0–1.3 μm) occurred singly, in pairs, or in chains, were motile by a single polar flagellum and tolerated up to 12.5% NaCl. Strain CVL$^T$ had a generation time of 60 min when grown in lactate-yeast extract medium under optimal conditions (37°C, pH 7.6, 2.5% NaCl). It used lactate, pyruvate, cysteine, or $H_2/CO_2$ + acetate as electron donors, and sulfate, sulfite, thiosulfate, or fumarate as electron acceptors. Elemental sulfur, nitrate, or oxygen were not used. Sulfite and thiosulfate were disproportionated to sulfate and sulfide. The G+C content of the DNA was 62 mol%. Phylogenetic analysis revealed that *Desulfovibrio fructosovorans* was the nearest relative. Strain CVL$^T$ is clearly different from other *Desulfovibrio* species, and is designated *Desulfovibrio senezii* sp. nov. (DSM 8436).

**Key words** *Desulfovibrio senezii* · *Desulfovibrio fructosovorans* · Desulfovibrionaceae · Halotolerant · Sulfate reduction

J.-L. Cayol · J.-L. Garcia (✉)
Laboratoire ORSTOM de Microbiologie des Anaérobies,
Université de Provence, CESB-ESIL case 925,
163 avenue de Luminy, F-13288 Marseille cedex 9, France
e-mail: garcia@esil.univ-mrs.fr
Tel. +33-49182-8572; Fax +33-49182-8570

I.-H. Tsu · C.-Y. Huang · L. Baresi · R. A. Mah
University of California at Los Angeles, School of Public Health,
Department of Environmental Health Sciences,
Los Angeles, CA 90024–1772, USA

B. K. C. Patel
School of Biomolecular and Biomedical Sciences,
Faculty of Science, Griffith University, Brisbane,
Queensland 4111, Australia



## Introduction

Sulfate-reducing bacteria form a physiological group with the common property of using sulfate as the main electron acceptor during anaerobic metabolism (Widdel and Bak 1992; Widdel and Hansen 1992). They can be differentiated on the basis of numerous criteria including complete or incomplete oxidation of electron donors. Species that exhibit incomplete oxidation produce low-molecular-weight fatty acids, mainly acetate, as the end product of their metabolism. Bacterial sulfate reduction is an important process of mineralization of organic matter in anoxic environments, especially in marine and hypersaline systems (Jørgensen 1982; Oren 1988; Caumette 1993; Ollivier et al. 1994). However, only a limited range of substrates including $H_2$, formate, and lactate are found to be involved in this process in hypersaline environments (Oren 1988). To date only one example of the oxidation of volatile fatty acids such as acetate by sulfate reducers has been documented at high salinities (Brandt and Ingvorsen 1997). All other strains isolated to date at high salinities are mainly incomplete oxidizers. At salinities above 15%, rates of sulfate reduction and methanogenesis decrease dramatically (Oren 1988).

Fluctuating salt concentrations result in an abnormal accumulation of $H_2$ (Zeikus 1983) and diverse volatile fatty acids in sediments (Oren 1988), suggesting that oxidation of organic matter is incomplete at high levels of NaCl as compared to other ecosystems where acetate and $H_2 + CO_2$ are used to produce $CH_4$. Accumulation of acetate or other volatile fatty acids indicates that anaerobic mineralization of natural organic compounds via interspecies hydrogen transfer hardly occurs in hypersaline environments. Even if the possibility of the oxidation of volatile fatty acids exists, the process is slow as compared to the fermentation of carbohydrates by most anaerobes (Oren 1988).

In the course of a study of the degradation of organic matter in hypersaline environments, we isolated and characterized a new strain of sulfate reducer from a solar

saltern, *Desulfovibrio senezii* sp. nov., and describe its properties in this communication.

## Materials and methods

### Sample source and strains

Samples of sediment subsequently analyzed and found to contain 12% NaCl were collected from the Western Salt Company solar saltern in the San Diego Bay area (Chula Vista, Calif., USA) and were kept under anoxic conditions in serum bottles. *Desulfovibrio fructosovorans* was obtained from the DSMZ (DSM 3604) and was grown as previously described (Ollivier et al. 1988).

### Media, isolation, and culture techniques

The anaerobic Hungate techniques (Hungate 1969; Macy et al. 1972) were used. The culture medium was a modification of medium HS-1 (Liaw and Mah 1992). The basal mineral medium contained (g/l): $NH_4Cl$ (1), $MgCl_2 \cdot 6\ H_2O$ (7), $MgSO_4 \cdot 7\ H_2O$ (9.6), $CaCl_2 \cdot 2\ H_2O$ (0.5), KCl (3.8), $K_2HPO_4 \cdot 3\ H_2O$ (0.4), $Na_2CO_3$ (3) NaCl (120), trace mineral solution (Balch et al. 1979; 10 ml/l), L-cysteine (0.50, and resazurin (0.001). The medium was autoclaved at 120°C for 20 min. For enrichments and routine cultivation of axenic cultures, the following autoclaved stock solutions were added to the basal medium (final concentration in g/l): sodium lactate (10), yeast extract (1), $NaHCO_3$ (3) $Na_2S \cdot 9\ H_2O$ (0.5), and $Na_2SO_4$ (3). Medium (10 or 25 ml) was dispensed under $N_2/CO_2$ (70:30, v/v) into Hungate tubes and serum bottles (Wheaton Scientific, Milleville, N.J., USA), respectively. Enrichments were initiated by adding a few grams of the sediment sample to 25 ml of culture medium followed by incubation at 37°C without shaking. Enrichment cultures showing growth were transferred subsequently five more times every week (10% of inoculum). Colonies were isolated by the agar (1.8%) roll tube method (Hungate 1969).

### Morphology and physiological studies

Gram reaction was determined by using Difco kit reagents. Cell characteristics were examined using a Zeiss phase-contrast microscope. Flagella were stained using Gray's method (Gerhardt 1994). Growth at various temperatures (15–55°C), pH values (pH 6–8.5), and NaCl concentrations (0–30%) was determined by calculating the growth rates from this exponential early phase in triplicate. Growth on carbon substrates was tested in triplicate by adding test substrates (10 mM final concentration) to lactate-free growth medium. Substrate utilization was recorded as positive if a minimum growth rate of at least 0.2 $h^{-1}$ was obtained.

Oxygen was tested as the electron acceptor in liquid and solid media. Fumarate, nitrate, sulfate, sulfite, thiosulfate, tetrathionate (each 10 mM) and elemental sulfur (added as a slurry) with lactate as the carbon source were tested as alternate electron acceptors by replacing sulfate in the growth medium. Growth on $H_2/CO_2$ (80:20%) was examined in anaerobic liquid medium with 0.1% sodium acetate as the carbon source. Fermentative growth was tested in the same medium, but with the omission of sulfate.

### Analytical techniques

Growth rates were determined by measuring the optical densities of cultures at a wavelength of 560 nm. Volatile fatty acids and alcohols were analyzed as described by Ollivier et al. (1988), and $H_2S$ was determined photometrically as described by Cord-Ruwisch (1985). The disproportionation of thiosulfate and sulfite was tested in basal medium free of sulfate and organic energy source. The medium was supplemented with acetate as a carbon source and with either thiosulfate or sulfite as an energy source. After incubation, disproportionation was tested by measuring the increase in optical density, and the sulfate and sulfide production. DNA was extracted (Pitcher et al. 1989), and the G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) using the methods of Meshbah et al. (1989) and Tamaoka and Komagata (1984). Cell extract from a culture grown in lactate-sulfate medium was examined for the presence of cytochromes and sulfite reductase (desulfoviridin) according to the method of Postgate (1959).

### Phylogeny

DNA extraction from strain $CVL^T$ and from *D. fructosovorans*, and the methods used for 16S rRNA gene amplification, purification, sequencing, and sequence analysis have been described previously (Redburn and Patel 1993; Andrews and Patel 1996). All sequences were obtained from the Ribosomal Database Project (Maidak et al. 1996) with the exception of sequences for *Desulfovibrio oxyclinae* and *Desulfovibrio profundus*, which were extracted from the GenBank (accession nos. U33316 and U90726, respectively). Pairwise evolutionary distances based on 890 unambiguous nucleotides were computed using DNADIST and neighbor-joining programs that form part of the PHYLIP package (Felsenstein 1993). The nucleotide sequences of the 16S rRNA genes of strain $CVL^T$ and *D. fructosovorans* have been deposited in the Genbank database under accession nos. AF050100 and AF050101, respectively.

## Results and discussion

Positive enrichments developed within 1 week of incubation at 37°C. Microscopic examination revealed the presence of rods and curved, spiral-shaped cells. Four strains of highly motile, short vibrios (0.3 × 1.0–1.3 μm) were isolated from colonies that developed in roll tubes. One strain, designated strain $CVL^T$, was chosen for further characterization. Cells of strain $CVL^T$ stained gram-negative, occurred singly, in pairs, or – rarely – in chains, and were motile by means of a single polar flagellum, but old cultures lacked motility. The strain grew with NaCl concentrations ranging from 0 to 12.5% (optimum, 2.5%), at temperatures ranging from 25 to 45°C (optimum, 37°C), and at pH values ranging from 6.4 to 8.3 (pH optimum, 7.6). Growth occurred in mineral medium with lactate as an organic substrate, but yeast extract improved growth. The doubling time under optimal conditions in lactate-yeast extract medium was 60 min. Strain $CVL^T$ used $H_2/CO_2$ with acetate, DL-lactate, pyruvate, and cysteine but not fumarate, succinate, L-malate, citrate, oxalate, formate, acetate, propionate, butyrate, D-glucose, fructose, methanol, ethanol, isopropanol, glycerol, acetone, lysine, methionine, glutamate, aspartate, casamino acids, benzoate, nicotinate, yeast extract, or trypticase. Sulfate, sulfite, thiosulfate, or fumarate served as electron acceptors, but sulfur, oxygen, and nitrate did not. Strain $CVL^T$ could use an organic (tripticase) or inorganic ($NH_4Cl$) nitrogen source. Cytochrome $c_3$ and desulfoviridin were present (Postgate 1959). The G+C content of DNA of strain $CVL^T$ was 62 mol%.

The presence of desulfoviridin and cytochrome *c*3 suggests that strain $CVL^T$ is a *Desulfovibrio* species. With re-

# FICHE DESCRIPTIVE

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**Titre original** : Isolation and characterization of *Desulfovibrio senezii* sp. nov., a halotolerant sulfate reducer from a solar saltern and phylogenetic confirmation of *Desulfovibrio fructosovorans* as a new species

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**Titre en Français** : Isolement et caractérisation de *Desulfovibrio senezii* sp. nov., une bactérie sulfato-réductrice halotolérante d'un marais salant et confirmation phylogénétique de *Desulfovibrio fructosovorans* comme nouvelle espèce

**Mots-clés matières** : *Desulfovibrio senezii*, *Desulfovibrio fructosovorans*, Desulfovibrionaceae, halotolérance, sulfato-réduction, anaérobiose, taxonomie
**(10 au plus)**

**Résumé en Français :**
**(150 mots maximum)**

**Plan de classement** : Monde végétal et Animal - Fermentations

**Table 1** Characteristics of strain CVL$^T$ and its close phylogenetic neighbors from the genus *Desulfovibrio* (*nd* not determined)

| Species | Morphology | Flagella | Optimal growth conditions | | Source | Electron donors | | | | | | G+C% DNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Temperature (°C) | NaCl (g/l) | | $H_2$ | Formate | Ethanol | Fumarate | Malate | Others | |
| *D. africanus*[a] | Vibrio | Lophotrichous | 34–37 | 0 | Salt and fresh waters | + | + | + | – | + | | 61 |
| *D. halophilus*[b] | Vibrio | Polar | 35 | 30–180 | Solar saltern | + | + | + | – | – | Propanol | 61 |
| *D. longus*[c] | Rod | Polar | 35 | 10–20 | Oil well | + | + | – | + | – | | 62 |
| *D. oxyclinae*[d] | Rod/vibrio | Polar | nd | 5–10 | Cyanobacterial mat | + | + | + | – | + | Alcohols | nd |
| *D. profundus*[e] | Vibrio | Polar | 25 | 6–80 | Deep marine sediment | + | – | – | – | – | | 53 |
| *D. salexigens*[f] | Vibrio | Polar | 34–37 | 20 | Sea water, marine muds | + | + | + | – | + | | 46 |
| *D. fructosovorans*[g] | Vibrio | Polar | 35 | 0 | Estuarine sediment | + | + | – | + | + | Fructose | 64 |
| Strain CVL$^T$ | Vibrio | Polar | 37 | 25 | Solar saltern | + | – | – | – | – | Cysteine | 62 |

[a] Campbell et al. (1966)
[b] Caumette et al. (1991)
[c] Magot et al. (1992)
[d] Krekeler et al. (1997)
[e] Bale et al. (1997)
[f] Postgate and Campbell (1996)
[g] Ollivier et al. (1988)

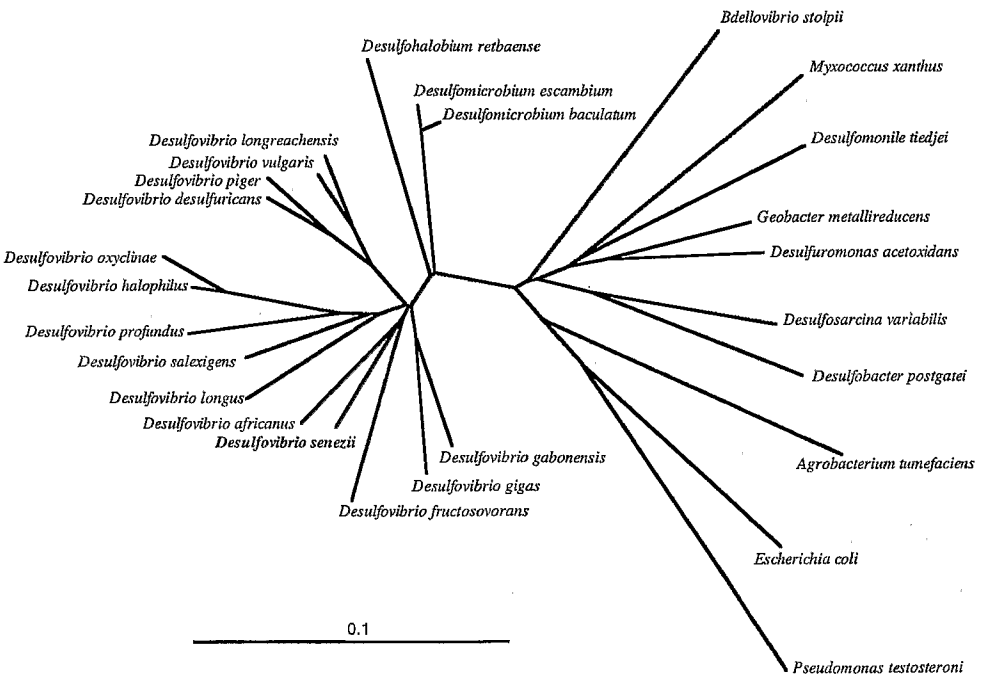


**Fig. 1** Unrooted phylogenetic tree showing the position of strain CVL$^T$ in relationship to the genus *Desulfovibrio* and other genera. The tree was generated using methods described in Materials and methods (*bar* 10%)

spect to its requirement for 2.5% NaCl for optimal growth, it resembles isolates such as *D. salexigens* (Postgate and Campbell 1966), *D. africanus* (Campbell et al. 1966), *D. profundus* (Bale et al. 1997), *D. longus* (Magot et al. 1992), and *D. oxyclinae* (Krekeler et al. 1997). However, phenotypic characteristics that include flagellation, the utilization of formate, ethanol, malate, and cysteine, and the G+C content of the DNA can be used to differentiate it from *D. salexigens*, *D. africanus*, and other marine halophiles described above (Table 1).

Sequence alignment and subsequent comparison of 1,540 nucleotides for strain CVL$^T$ and 1,522 nucleotides for *D. fructosovorans* (whose phylogenetic position is unknown) corresponding to positions 9–1542 and 28–1542, respectively [*Escherichia coli* numbering; according to Winker and Woese (1991)] confirmed that both strains were members of the genus *Desulfovibrio* (Fig. 1). The closest relative of strain CVL$^T$ and *D. fructosovorans* was *D. africanus* (similarity of 91 and 88%, respectively). Strain CVL$^T$ was less closely related to *D. profundus*, *D. halophilus*, *D. oxyclinae*, and *D. longus* (similarity lower than 89%). The clustering of strain CVL$^T$ with the sole lineage represented by *D. africanus* is interesting but cannot be ascertained with confidence since the result of bootstrap analysis (100 datasets) was poor (31%). The introduction of *D. fructosovorans*, which also clustered in the vicinity of *D. africanus* lineage, did not improve the poor confidence value.

The phylogenetic and phenotypic differences warrant the inclusion of strain CVL$^T$ as a new species of the genus *Desulfovibrio*. The new isolate is therefore designated *Desulfovibrio senezii* sp. nov. Strain CVL$^T$ is designated the type species. The phylogenetic data on *D. fructosovorans* indicate that it is sufficiently distinct from other *Desulfovibrio* species that have been described to date and concur with the phenotypic characteristics published by Ollivier et al. (1988), indicating its validity as a taxon.

Description of *Desulfovibrio senezii* sp. nov.

*Desulfovibrio senezii*, se.ne′ zi.i M.L.gen.n., in honor of the French microbiologist Jacques C. Senez, who greatly stimulated research on sulfate reduction. Vibrios 0.3 × 1.0–1.3 μm, highly motile by single polar flagella; occur singly, in pairs, or rarely in chains. Gram-negative, nonsporeforming. Colonies (up to 5 mm) are round with entire edges, smooth, convex, opaque, and brownish. Halotolerant. Optimum NaCl concentration for growth, 2.5%; range for growth, 0–12.5% NaCl. Mesophilic. Optimal growth temperature, 37°C; range for growth, 25–45°C. pH range from 6.4 to 8.3; optimum, pH 7.6. No growth factors are required for growth, but yeast extract stimulates growth. Uses organic or inorganic nitrogen source. Strictly anaerobic. Sulfate, sulfite, thiosulfate, and fumarate serve as electron acceptors; elemental sulfur and nitrate are not utilized. No growth with $H_2$+$CO_2$ without acetate. Lactate, pyruvate, cysteine, and $H_2$/$CO_2$ with acetate are used as growth substrates. Does not ferment lactate, pyruvate, fumarate, malate, succinate, glucose, fructose, or glycerol. Desulfoviridin present. The G+C content of the DNA is 62 mol% (as determined by HPLC). The type strain is CVL$^T$ (DSM 8436); it was isolated from the solar saltern of Chula Vista (San Diego Bay area, California, USA).

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