

Reclassification of the sulfate- and nitrate-reducing bacterium *Desulfovibrio vulgaris* subsp. *oxamicus* as *Desulfovibrio oxamicus* sp. nov., comb. nov.

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Desulfovibrio vulgaris subsp. *oxamicus* (type strain, DSM 1925^T) was found to use nitrate as a terminal electron acceptor, the latter being reduced to ammonium. Phylogenetic studies indicated that strain DSM 1925^T was distantly related to the type strain of *Desulfovibrio vulgaris* (95.4% similarity of the small-subunit rRNA gene) and had as its closest phylogenetic relatives two other nitrate- and sulfate-reducing bacteria, namely *Desulfovibrio termitidis* (99.4% similarity) and *Desulfovibrio longreachensis* (98.4% similarity). Additional experiments were conducted to characterize better strain DSM 1925^T. This strain incompletely oxidized lactate and ethanol to acetate. It also oxidized butanol, pyruvate and citrate, but not glucose, fructose, acetate, propionate, butyrate, methanol, glycerol or peptone. The optimum temperature for growth was 37 °C (range 16–50 °C) and the optimum NaCl concentration for growth was 0.1% (range 0–5%). Because of significant genotypic and phenotypic differences from *Desulfovibrio termitidis* and *Desulfovibrio longreachensis*, reclassification of *Desulfovibrio vulgaris* subsp. *oxamicus* as *Desulfovibrio oxamicus* sp. nov., comb. nov., is proposed. The type strain is strain Monticello 2^T (= DSM 1925^T = NCIMB 9442^T = ATCC 33405^T).

In addition to sulfate, sulfate-reducing bacteria (SRB) have been found to use many other mineral electron acceptors to oxidize H₂ and organic compounds (Fauque & Ollivier, 2004). Among them, the dissimilatory reduction of nitrate to ammonium has been poorly studied as few SRB have been reported to reduce nitrate as an alternative to sulfate (Moura *et al.*, 1997). Within the SRB, members of the genera *Desulfohalobus* (e.g. *Desulfohalobus propionicus*; Widdel & Pfennig, 1982), *Desulforhopalus* (e.g. *Desulforhopalus singaporensis*; Lie *et al.*, 1999) and *Desulfobacterium* (e.g. *Desulfobacterium catecholicum*; Szewzyk & Pfennig, 1987) and *Desulfovibrio* species have been shown to be able to utilize nitrate as a terminal electron acceptor. The latter species include *Desulfovibrio desulfuricans*, which has been isolated from various

ecosystems and particularly within the gut of a soil-feeding termite (Liu & Peck, 1981; Steenkamp & Peck, 1981; Keith & Herbert, 1983; Seitz & Cypionka, 1986; Brauman *et al.*, 1990; Krekeler & Cypionka, 1995; Costa *et al.*, 1996), *Desulfovibrio simplex* isolated from an anaerobic sour whey digester (Zellner *et al.*, 1989), *Desulfovibrio furfuralis* isolated from a continuous fermenter culture treating the organic constituents of a sulfite evaporator condensate (Brune *et al.*, 1983; Folkerts *et al.*, 1989) and *Desulfovibrio termitidis* isolated from the hindgut of a termite (Brauman *et al.*, 1990; Trinkerl *et al.*, 1990). In all cases reported so far, dissimilatory reduction of nitrate by SRB leads to the formation of ammonium, with nitrite being an intermediary product of metabolism (Moura *et al.*, 1997). Despite the fact that this reductive process prevails energetically over sulfate reduction (Thauer *et al.*, 1977; Pietzsch & Babel, 2003), little attention has been paid to it ecologically. One explanation for the lack of attention given to dissimilatory nitrate

Abbreviations: SRB, sulfate-reducing bacteria; SSU, small subunit.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Desulfovibrio oxamicus* DSM 1925^T is DQ122124.

reduction by SRB may be that, in some cases, sulfate is preferred for use as a terminal electron acceptor when both sulfate and nitrate are present in the culture medium (Widdel & Pfennig, 1982; Pietzsch & Babel, 2003), thereby making nitrate reduction by SRB a marginal respiratory system within the ecosystems that they inhabit. Nevertheless, it has also been established that some SRB may (i) have a preference to use nitrate over sulfate as a terminal electron acceptor (Seitz & Cypionka, 1986) or (ii) use the two electron acceptors simultaneously (Keith & Herbert, 1983), thus making the real significance of nitrate reduction by SRB ambiguous in marine or terrestrial sediments.

We screened our collection of micro-organisms for SRB with the ability to use nitrate as a terminal electron acceptor. Interestingly, *Desulfovibrio vulgaris* subsp. *oxamicus* DSM 1925^T was found to reduce nitrate to ammonium. This metabolic feature has only been attributed recently to this subspecies, as well as to another metabolically related SRB that can detoxify U(VI) (Pietzsch & Babel, 2003). Therefore, we undertook additional experiments to characterize strain DSM 1925^T phylogenetically and metabolically. Our results indicate that this subspecies is not phylogenetically related to the type strain of *Desulfovibrio vulgaris* and should be considered as a novel species of the genus *Desulfovibrio*.

Desulfovibrio vulgaris subsp. *oxamicus* DSM 1925^T and *Desulfovibrio termitidis* DSM 5308^T were used as reference cultures and were obtained from our laboratory collection of micro-organisms.

The Hungate technique (Hungate, 1969) was used throughout this study. Experiments were done using a basal medium containing per litre of distilled water: 1 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 2.0 g MgCl₂.6H₂O, 0.1 g CaCl₂, 0.1 g KCl, 1 g NaCl, 0.5 g cysteine hydrochloride, 0.5 g yeast extract (Difco), 1 ml trace mineral solution (Widdel & Pfennig, 1982) and 1 mg resazurin. The pH was adjusted to 7 with 10 M KOH and the medium was boiled under a stream of O₂-free N₂ gas and then cooled to room temperature. The medium was dispensed into serum bottles and Hungate tubes (20 and 5 ml, respectively), under a stream of O₂-free N₂ gas that was subsequently replaced by a mixture of N₂/CO₂ (80/20%, v/v). The vessels were autoclaved for 45 min at 121 °C and sterile stock solutions of Na₂S.9H₂O, NaHCO₃ and lactate (Difco) were injected prior to the inoculation of bacteria, to give final concentrations of 0.02%, 0.4% and 20 mM, respectively. Sodium sulfate or sodium nitrate (20 mM) was used as the terminal electron acceptor.

Water baths were used to obtain incubation temperatures of up to 55 °C. For studies on NaCl requirement, NaCl was weighed directly in tubes prior to the medium being dispensed. The strains were subcultured at least once under the same experimental conditions prior to determination of growth rates. Substrates were tested at a final concentration of 20 mM.

Unless indicated otherwise, duplicate culture tubes were used throughout. Growth was measured by inserting the tubes directly into a model Cary 50 Scan spectrophotometer (Varian Corp.) and measuring the optical density at 580 nm. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Fermentation products were determined as described by Miranda-Tello *et al.* (2003).

The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977) and the G+C content was determined by using HPLC, as described by Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as a standard.

DNA–DNA hybridization was performed at the DSMZ as described by De Ley *et al.* (1970), with the modification described by Escara & Hutton (1980) and Huß *et al.* (1983), using a Gilford System model 2600 equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed using the TRANSFER.BAS program (Jahnke, 1992).

The genomic DNA of strain DSM 1925^T was extracted using a Wizard Genomic DNA Purification kit (Promega), and the almost complete small-subunit (SSU) 16S rRNA gene (positions 8–1494; *Escherichia coli* numbering) was amplified by PCR using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GTCGTAACAAGGTAACCGTA-3'). The purified product (Nucleo Spin Extract kit; Macherey Nagel) was cloned using a pGEM-T Easy cloning kit (Promega). A plasmid containing the correct length insert was isolated using a Wizard Plus SV Minipreps DNA Purification System kit (Promega) and was sequenced by Genome Express.

The SSU rRNA gene sequence of strain DSM 1925^T was aligned with sequences of related *Desulfovibrio* species obtained from GenBank (Benson *et al.*, 1999), using the sequence aligner software from the Ribosomal Database Project II (Maidak *et al.*, 2001) and the sequence alignment editor BioEdit (Hall, 1999). Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1513 unambiguous nucleotides were computed using the method of Jukes & Cantor (1969). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), with the SSU rRNA gene sequence of *Desulfohalobium retbaense* DSM 5692^T as the outgroup. Confidence in the tree topology was determined by bootstrap analysis of 1000 resamplings of the sequences (Felsenstein, 1985). The phylogenetic programs were all implemented in the TREECON package (Van de Peer & De Wachter, 1994).

A strain of *Desulfovibrio* that could use oxamate was isolated by J. R. Postgate in 1963 (Postgate, 1963) and was later

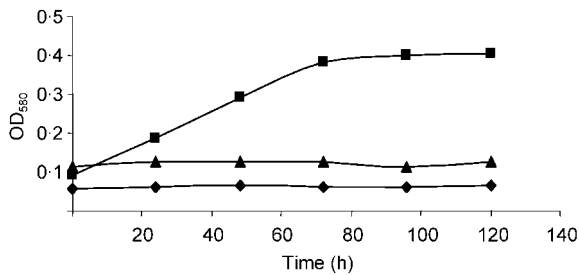


Fig. 1. Effect of nitrate reduction on growth of *Desulfovibrio oxamicus* sp. nov. DSM 1925^T. ▲, Lactate + nitrate without bacteria; ◆, nitrate without lactate + bacteria; ■, lactate + nitrate + bacteria.

recognized as a novel variety of *Desulfovibrio vulgaris*, *Desulfovibrio vulgaris* var. *oxamicus* (Postgate & Campbell, 1966); the name *D. vulgaris* subsp. *oxamicus* was included in the Approved List (Skerman *et al.* 1980). In 1966, this strain (Monticello 2^T) presented a taxonomic problem to Postgate. Indeed, despite its DNA base composition, resistance to hibitane and its morphology, which clearly placed it in the *Desulfovibrio vulgaris* group, strain Monticello 2^T had metabolic features (e.g. fermentation of choline and pyruvate) that resembled those of *Desulfovibrio desulfuricans* (Postgate & Campbell, 1966). In addition, strain Monticello 2^T differed from the type strain of *Desulfovibrio vulgaris* by its ability to metabolize oxamate and oxalate (Postgate & Campbell, 1966).

Desulfovibrio vulgaris subsp. *oxamicus* DSM 1925^T was recently reported to be a dissimilatory nitrate-reducing bacterium (Pietzsch & Babel, 2003). We believe that this reductive process that we have confirmed in this study (Fig. 1) is of taxonomic relevance, as it is not shared by *Desulfovibrio vulgaris* Hildenborough^T (Pietzsch & Babel, 2003; this study). We have therefore undertaken additional experiments to characterize phenotypically, phylogenetically and genetically *Desulfovibrio vulgaris* subsp. *oxamicus* DSM 1925^T. In addition to hydrogen and formate, which are only oxidized in the presence of acetate as a carbon source as

reported previously (Postgate & Campbell, 1966), lactate, pyruvate, butanol, citrate and ethanol, but not glucose, fructose, acetate, propionate, butyrate, methanol, glycerol or peptone, were used as energy sources in the presence of sulfate as terminal electron acceptor. Lactate and ethanol were incompletely oxidized to acetate. In addition, strain DSM 1925^T grew optimally at 37 °C (temperature range for growth 16–50 °C) and in the presence of 0.1 % NaCl (NaCl range for growth 0–5 %). Strain DSM 1925^T was only distantly related to the type strain of *Desulfovibrio vulgaris* subsp. *vulgaris* (95.4 % similarity of the SSU rRNA gene) and had *Desulfovibrio termitidis* (99.4 % similarity; Trinkerl *et al.*, 1990) and *Desulfovibrio longreachensis* (98.4 % similarity; Redburn & Patel, 1994) as its closest relatives (Fig. 2). Interestingly, both *Desulfovibrio termitidis* and *Desulfovibrio longreachensis* (Trinkerl *et al.*, 1990; Redburn & Patel, 1994), together with *Desulfovibrio vulgaris* subsp. *oxamicus*, reduce nitrate to ammonium, suggesting that comparative sequence analysis of SSU rRNA genes is highly discriminatory for distinguishing a clade of nitrate-SRB comprising the three isolates cited above within the genus *Desulfovibrio*.

As yet, we do not know the ecological significance of the ability of some SRB to reduce nitrate to ammonium, but we can hypothesize that, most probably, such SRB display this reductive process in sediments at lower redox potentials than those for sulfate reduction. In this respect, the role of nitrate-SRB in the global nitrogen cycle within the ecosystems they inhabit might have been underestimated. Of interest was the isolation from the hindgut of termites of two SRB that reduced nitrate (*Desulfovibrio termitidis* and '*Desulfovibrio desulfuricans* subsp. *termitidis*') (Brauman *et al.*, 1990; Trinkerl *et al.*, 1990). In such an ecosystem, it has been hypothesized that these two organisms could act as hydrogen scavengers through interspecies hydrogen transfer with methanogens, during the degradation of highly reduced compounds such as benzoate and its relatives (Brauman *et al.*, 1990). However, taking into account that nitrogen fixation has been demonstrated in termites (Breznak *et al.*, 1973), our results suggest that nitrate-reducing *Desulfovibrio* species may interfere with nitrogen cycling in termites by incompletely oxidizing organic

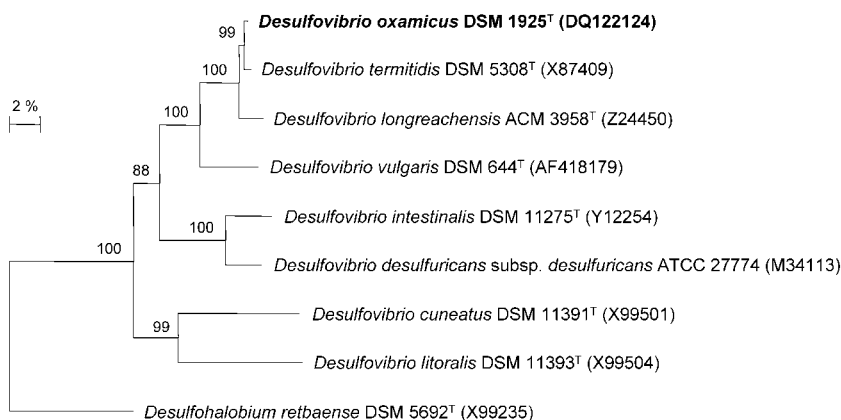


Fig. 2. Phylogenetic position of strain DSM 1925^T within the genus *Desulfovibrio*, based on SSU rRNA gene sequences. Accession numbers of reference organisms are included. Percentage bootstrap values, based on 1000 replications, are shown at branch points. Bar, 2 nucleotide substitutions per 100 nucleotides.

Table 1. Characteristics that differentiate *Desulfovibrio oxamicus* sp. nov. from *Desulfovibrio termitidis* and *Desulfovibrio longreachensis*

Taxa: 1, *Desulfovibrio oxamicus* sp. nov. DSM 1925^T (data from this study); 2, *Desulfovibrio termitidis* (Trinkerl *et al.*, 1990); 3, *Desulfovibrio longreachensis* (Redburn & Patel, 1994). ND, Not determined.

Characteristic	1	2	3
Growth on sulfate with:			
Hydrogen	+	-	+
Sugars	-	+	-
Fumarate	-	ND	+
Temperature range for growth (°C)	16–50	18–45	20–48
Optimum temperature (°C)	37	35	37
Highest NaCl concentration for growth (%)	5	2	ND
DNA G+C content (mol%)	65.2	67–68	69

compounds to acetate, which is an essential compound for the respiration of these insects (Brauman *et al.*, 1990). Despite the existence of close phylogenetic similarities between *Desulfovibrio termitidis*, *Desulfovibrio longreachensis* and *Desulfovibrio vulgaris* subsp. *oxamicus*, the latter differs genomically from the other two *Desulfovibrio* species by having a lower G+C content of the DNA [65.2 mol% for *Desulfovibrio vulgaris* subsp. *oxamicus* DSM 1925^T (this study) versus 69 mol% for *Desulfovibrio longreachensis* (Redburn & Patel, 1994) and 67–68 mol% for *Desulfovibrio termitidis* (Trinkerl *et al.*, 1990)]. In addition, a DNA–DNA relatedness value of only 58% was obtained between *Desulfovibrio vulgaris* subsp. *oxamicus* DSM 1925^T and its closest phylogenetic relative, *Desulfovibrio termitidis* DSM 5308^T. There are also major phenotypic differences that distinguish the two strains (Table 1). In contrast to *Desulfovibrio termitidis*, *Desulfovibrio vulgaris* subsp. *oxamicus* does not use sugars as an energy source and oxidizes hydrogen in the presence of acetate as a carbon source. *Desulfovibrio longreachensis* differs from *Desulfovibrio vulgaris* subsp. *oxamicus* by using fumarate in the presence and absence of sulfate as terminal electron acceptor and by the temperature range for growth.

Our results clearly indicate that *Desulfovibrio vulgaris* subsp. *oxamicus* is not a subspecies of *Desulfovibrio vulgaris*. With regard to genomic and phenotypic differences from its closest relatives, we propose that *Desulfovibrio vulgaris* subsp. *oxamicus* should be reclassified as *Desulfovibrio oxamicus* sp. nov., comb. nov.

Description of *Desulfovibrio oxamicus* sp. nov., comb. nov.

Desulfovibrio oxamicus (ox.am'i.cus. N.L. masc. adj. *oxamicus* pertaining to oxamic acid).

Basonym: *Desulfovibrio vulgaris* subsp. *oxamicus* Postgate and Campbell 1966.

The description of the species is the same as that given by Postgate (1984). Incompletely oxidizes lactate and ethanol to acetate. Oxidizes butanol, pyruvate and citrate, but not glucose, fructose, acetate, propionate, butyrate, methanol, glycerol or peptone. Optimum temperature for growth is 37°C (range 16–50°C). Optimum NaCl concentration for growth is 0.1% (range 0–5%). Reduces nitrate to ammonium.

The type strain is strain Monticello 2^T (=DSM 1925^T = NCIMB 9442^T = ATCC 33405^T).

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