Desulfovibrio carbinoliphilus sp. nov., a benzyl alcohol-oxidizing, sulfate-reducing bacterium isolated from a gas condensate-contaminated aquifer

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Phenotypic and phylogenetic studies were performed on a novel sulfate-reducing bacterium, strain D41^T, isolated as part of a methanogenic syntrophic culture from a gas condensate-contaminated aquifer undergoing intrinsic bioremediation. The bacterium was a Gram-negative, non-spore-forming, curved rod, motile by a single polar flagellum, which oxidized several alcohols incompletely, including methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 3-methyl-1-butanol (isoamyl alcohol), ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,4-butanediol, phenylethanol and benzyl alcohol. Additionally, the strain oxidized H₂/CO₂, formate, lactate, pyruvate, maleate, malate and fumarate. Sulfate, thiosulfate and sulfite were used as electron acceptors. The DNA G+C content was 63 mol%. Based on phylogenetic and phenotypic evidence, the novel species *Desulfovibrio carbinoliphilus* sp. nov. is proposed. The type strain is D41^T (=ATCC BAA-1241^T =DSM 17524^T).

In hydrocarbon-contaminated groundwater systems, the monocyclic aromatic hydrocarbons benzene, toluene, ethylbenzene and isomers of xylene (BTEX) are of particular concern because of their relatively low molecular mass, high aqueous solubility and their toxicity (carcinogenicity in the case of benzene) (Sittig, 1991). However, BTEX compounds can be naturally attenuated in contaminated aquifers by indigenous soil micro-organisms (Gieg *et al.*, 1999). The indigenous anaerobic microbiota of BTEX-contaminated groundwater systems is important for *in situ* bioremediation because contaminated sites quickly become oxygen-depleted, and stimulating anaerobic degradation of contaminants (via the addition of sulfate or nitrate) is easier than stimulating aerobic degradation (Lovley, 2001). Studies have shown that aerobic and anaerobic oxidation of toluene (a model compound for study of BTEX degradation) can produce partially oxidized intermediates including benzyl alcohol, cresol isomers and

3-methylcatechol (Altenschmidt & Fuchs, 1992; Grbić-Galić & Vogel, 1987; Vanderberg *et al.*, 2000).

During a study using partially oxidized intermediates of toluene as enrichment substrates, a sulfate-reducing bacterium (SRB) strain designated D41^T was isolated using benzyl alcohol. The strain was isolated from a syntrophic, methane-producing culture from a gas condensate-contaminated aquifer undergoing intrinsic bioremediation (Gieg *et al.*, 1999). Phenotypic and phylogenetic analysis indicated that isolate represents a novel species of *Desulfovibrio*.

Anaerobic sediments were collected from a gas condensate-contaminated aquifer near Fort Lupton, CO, USA, and diluted using an anaerobic pyrophosphate dilution buffer (pH 7.2) reduced with a cysteine-sulfide agent (Tanner, 2002). The enrichment and cultivation medium (pH 7.3–7.5) (used for all syntrophic experiments with a hydrogen-using methanogen, strain M41) contained the following (l⁻¹): 10 ml mineral solution (Tanner, 2002), 10 ml vitamin solution (Tanner, 2002), 5 ml trace metal solution (Tanner, 2002); 1.0 g *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 0.05 g yeast extract (Becton Dickinson) and 2.5 g NaHCO₃. Sodium acetate (1.0 g) was added to stimulate growth (Tanner, 2002) and the medium was reduced with a cysteine-sulfide agent. Benzyl alcohol (1.0–2.0 mM) was added as a substrate.

Abbreviations: BTEX, benzene, toluene, ethylbenzene and isomers of xylene; SRB, sulfate-reducing bacteria(um).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain D41^T is DQ186200.

A transmission electron micrograph of a cell of strain D41^T and graphs showing conversion of benzyl alcohol to benzoate in pure culture and co-culture are available as supplementary material with the online version of this paper.

After dilution on agar roll tubes (Hungate, 1969), the resulting indigenous, syntrophic culture contained a hydrogen-using methanogen, *Methanobacterium formicum* strain M41 (identified by 16S rRNA gene sequence analysis), and the sulfate-reducing strain D41^T. The pure co-culture was used for all syntrophic experiments. The SRB medium used for isolation and routine culture was derived from one published previously (Tanner, 1996) and contained (l⁻¹) 5 g NaCl, 0.5 g (NH₄)₂SO₄, 0.3 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.04 g CaCl₂·2H₂O, 10 ml vitamin solution, 5 ml trace metal solution, 0.1 g yeast extract and 0.5 g TES (final pH 7.5). Cysteine (50 mg l⁻¹) was used as the reducing agent and benzyl alcohol (5 mM) was used as the substrate. Strain D41^T was isolated from the methanogenic co-culture by transfer to the SRB medium with benzyl alcohol (5 mM) as substrate and by dilution in agar roll tubes (Hungate, 1969). All incubations were at 22 °C unless specified otherwise. Cells growing on lactate/sulfate were used for pH and temperature experiments. Cellular morphology was examined using a JEOL JEM 2000 FX transmission electron microscope as described previously (Liou *et al.*, 2005). Utilization of electron donors and acceptors was determined. Electron donors were assayed with and without sulfate and with a hydrogen-using methanogen, strain M41. For the sulfate-free medium, the basal medium was used except that sulfate salts were replaced with chloride salts and sulfate was omitted. Positives were scored by an increase in optical density and serial passage, or by assaying for hydrogen sulfide production (Cline, 1969) compared with negative controls without substrate. For nutrition experiments, triplicate cultures were incubated at 30 °C. The presence of desulfovibrin was determined by the fluorescence test described by Postgate (1959).

Strain D41^T was a Gram-negative, non-spore-forming, curved rod, motile by a single, polar flagellum (see Supplementary Fig. S1 available in IJSEM Online). Cells were 2.4–3.0 µm long and 0.6–0.7 µm wide. Cells grew between 22 and 36 °C. Growth was not observed at 4 or 37 °C. The optimum temperature for growth was 30 °C. Cells grew at pH 5.8–8.4. Growth was not observed at pH 5.0 or 9.2. The optimum pH for growth was between pH 7.0 and 8.0. Strain D41^T grew in the absence of vitamins and yeast extract. Growth occurred on the following compounds with sulfate (or a hydrogen-using methanogen) as the electron acceptor: H₂/CO₂ (80:20), formate, lactate, pyruvate, malate, maleate, fumarate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 3-methyl-1-butanol (isoamyl alcohol), ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,4-butanediol, benzyl alcohol and phenylethanol. Methanol and 2-propanol were oxidized without an increase in optical density in the presence of sulfate. Strain D41^T fermented pyruvate in the absence of sulfate. Doubling times at 30 °C were 5.8 h on ethanol, 12.9 h on benzyl alcohol and 15.2 h on H₂/CO₂. The following compounds were not used: acetate, propionate, butyrate, crotonate, malonate, succinate,

oxalate, L-tartrate, glutamate, L-alanine, L-serine, benzylamine, choline, fructose, glycerol, 2-methyl-2-butanol (*tert*-amyl alcohol), 2,3-butanediol, phenol, *o*-hydroxy cresol, *m*-hydroxy cresol, *p*-hydroxy cresol, 3-methylcatechol, benzoate, benzylsuccinate, 4-hydroxy methylbenzoate, toluene, *o*-toluate, *m*-toluate and *p*-toluate. Sulfate, thiosulfate and sulfite were used as electron acceptors. Elemental sulfur, nitrate, fumarate and maleate were not reduced. After treatment with 2 M NaOH, the fluorescence test of cell suspensions for desulfovibrin was positive.

Aromatic alcohols and acids were measured using HPLC with an Econosphere C18 reversed-phase column (Alltech), with a mobile phase of 50 mM sodium acetate (pH 4.5)/acetonitrile (75:25). Organic acids were quantified by HPLC using a Prevail organic acid column (Alltech) with a mobile phase of 25 mM KH₂PO₄ (pH 2.5)/methanol (75:25). Aliphatic alcohols were quantified using gas chromatography (GC) with a flame-ionization detector (FID) using a 80/120 Carbowax B-DA/4 % Carbowax 20M packed column (Supelco) heated to 155 °C. Methane was quantified using GC-FID with a Porapak Q column at 60 °C (Supelco). The role of strain D41^T as an intermediary in the microbial mineralization of toluene in hydrocarbon-contaminated sediments was investigated. Strain D41^T oxidized benzyl alcohol stoichiometrically to benzoate in the presence of sulfate (0.95 mmol benzyl alcohol to 1.03 mmol benzoate; 108 % carbon recovery) or in co-culture with a hydrogen-using methanogen (1.08 mmol benzyl alcohol to 0.97 mmol benzoate and 0.41 mmol methane; 90.3 % carbon recovery) (Supplementary Figs S2 and S3), corresponding to the theoretical yield (below). Lactate, pyruvate and ethanol were oxidized incompletely to acetate. 1-Butanol and 1-propanol were oxidized to butyrate and propionate, respectively.

Benzyl alcohol:

(equation 1) $2\text{C}_7\text{H}_8\text{O} + 2\text{H}_2\text{O} \rightarrow 2\text{C}_7\text{H}_5\text{O}_2^- + 4\text{H}_2 + 2\text{H}^+$; $\Delta G^{0'} = +7.12$ kJ per 2 mol benzyl alcohol

(equation 2) $\text{HCO}_3^- + \text{H}^+ + 4\text{H}_2 \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$; $\Delta G^{0'} = -135.57$ kJ per reaction

(equation 3) $2\text{C}_7\text{H}_8\text{O} + \text{HCO}_3^- \rightarrow 2\text{C}_7\text{H}_5\text{O}_2^- + \text{CH}_4 + \text{H}^+ + \text{H}_2\text{O}$; $\Delta G^{0'} = -128.45$ kJ per 2 mol benzyl alcohol

The transformation of benzyl alcohol to benzoate under standard conditions is a thermodynamically unfavourable hydrogen-producing reaction (equation 1). Hydrogen is consumed rapidly by autotrophic growth of a methanogen (equation 2), maintaining a very low H₂ partial pressure and allowing a now energetically favourable reaction to proceed (equation 3). Gibbs' free energies of formation were calculated from values in Thauer *et al.* (1977) or from Kaiser & Hanselmann (1982).

Benzyl alcohol (and its derivatives) have been observed as reduction products of benzoate (and its derivatives) by *Desulfomicrobium escambiense* (Genthner *et al.*, 1997) and by *Desulfovibrio vulgaris* (Bock *et al.*, 2000). *Desulfovibrio* strains have also been shown to oxidize benzaldehyde to

benzoate (Zellner *et al.*, 1990). *Desulfovibrio inopinatus* (Reichenbecher & Schink, 1997), like strain D41^T, oxidized aromatic alcohols; however, strain D41^T exhibited aromatic alcohol oxidation without ring cleavage. In addition, strain D41^T oxidized secondary alcohols and diols. Relatively few reports exist regarding strains of *Desulfovibrio* with a similar range of alcohol metabolism (Oppenberg & Schink, 1990; Qatibi *et al.*, 1991, 1998; Ouattara *et al.*, 1992, 1999; Tanaka, 1992).

The DNA G+C content was determined according to the method of Mesbah *et al.* (1989) to be 63 mol%. The 16S rRNA genes of the isolate were amplified by PCR using universal primers pA (positions 8–28; *Escherichia coli* numbering) and pH (positions 1542–1522) (Hutson *et al.*, 1993). The amplified products were purified by using a QIAquick PCR purification kit (Qiagen) and sequenced directly with primers directed towards conserved positions of the 16S rRNA gene and a dRhodamine terminator cycle sequencing kit using an automatic DNA sequencer. The closest known relatives of the new isolate were determined by performing database searches using the program FASTA (Pearson & Lipman, 1985). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequence using the program SEQtools (Rasmussen, 2002). The resulting multiple sequence alignment was corrected manually and approximately 100 bases at the 5' end of the rDNA were omitted from further analysis because of alignment uncertainties due to the highly variable region V1, determined using the program GeneDoc (Nicholas *et al.*, 1997). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the programs SEQtools and TreeView (Page, 1996). The stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same programs. Sequence searches of the GenBank and Ribosomal Database Project libraries revealed that the unknown organism was a member of the

family *Desulfovibrionaceae* of the *Deltaproteobacteria*. A phylogenetic tree, constructed by the neighbour-joining method, depicting the phylogenetic affinity of the novel bacterium is shown in Fig. 1. It is evident from the phylogenetic analysis that the novel curved rod-shaped organism represents a previously unknown sublineage within the genus *Desulfovibrio* and in particular forms a subline of decent with a subcluster of species consisting of *Desulfovibrio alcoholivorans* (96.3% sequence similarity with the type strain; Qatibi *et al.*, 1991), *Desulfovibrio burkinensis* (96.2%; Ouattara *et al.*, 1999), *Desulfovibrio carbinolicus* (96.6%; Nanninga & Gottschal, 1987) and *Desulfovibrio fructosivorans* (96.2%; Ollivier *et al.*, 1988) and is supported by a bootstrap value of 100%. Strain D41^T shares a branching node with *Desulfovibrio fructosivorans*, although this relationship is not supported by a statistically significant bootstrap value (65%), demonstrating that this is not a particularly specific relationship. The maximum-parsimony algorithm confirmed the major branching order (not shown). Tree topology and sequence divergence values (>3%) are consistent with closely related, albeit phylogenetically distinct, species (Stackebrandt & Goebel, 1994). Further support for the separateness of the unknown bacterium from its nearest phylogenetic relatives also comes from phenotypic characterization. Traits useful for distinguishing strain D41^T from its closest phylogenetic relatives are shown in Table 1. Therefore, based on the presented phenotypic and phylogenetic findings, it is proposed that the unknown curved rod-shaped bacterium represents a novel species, *Desulfovibrio carbinoliphilus* sp. nov.

Description of *Desulfovibrio carbinoliphilus* sp. nov.

Desulfovibrio carbinoliphilus (car.bi.no.li.phi'lus. N.L. neut. n. *carbinolum* alcohol; N.L. part. adj. *philus* from Gr. adj. *philos* friendly to, loving; N.L. part. adj. *carbinoliphilus* alcohol-loving).

Fig. 1. Unrooted phylogenetic tree showing the phylogenetic relationship of strain D41^T (*Desulfovibrio carbinoliphilus* sp. nov.) and some members of the genus *Desulfovibrio*. The tree was constructed using the neighbour-joining method based on a comparison of approx. 1350 nucleotides. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar, 1% sequence divergence.

Table 1. Differentiating characteristics between strain D41^T and type strains of other alcohol-utilizing *Desulfovibrio* species

Strains: 1, *Desulfovibrio carbinoliphilus* sp. nov. D41^T; 2, *Desulfovibrio alcoholivorans* SPSN^T (data from Qatibi *et al.*, 1991); 3, *Desulfovibrio burkinensis* HDv^T (Ouattara *et al.*, 1999); 4, *Desulfovibrio carbinolicus* EDK82^T (Nanninga & Gottschal, 1987); 5, *Desulfovibrio fructosivorans* JJ^T (Ollivier *et al.*, 1988; Qatibi *et al.*, 1991). (+), Utilized without an increase in optical density.

Characteristic	1	2	3	4	5
Growth at 37 °C	–	+	+	+	ND
Vitamins required:	–	–	+	ND	–
Electron donors					
Succinate	–	+	+	+	–
Fructose	–	–	–	–	+
Methanol	(+)	+	–	+	+
Glycerol	–	+	+	+	+
1,2-Propanediol	+	+	+	+	+
Ethylene glycol	+	(+)	(+)	+	+
DNA G + C content (mol%)	63	64.5	67	65	64.1

*1,2-Propanediol was degraded only in the presence of glycerol (Qatibi *et al.*, 1998).

Cells are Gram-negative, non-spore-forming, curved rods, 2.4–3.0 µm long by 0.6–0.7 µm wide, motile by a single polar flagellum. Grows at 22–36 °C and pH 5.8–8.4. Sulfate, thiosulfate and sulfite serve as electron acceptors. Sulfur, nitrate, fumarate and maleate are not reduced. H₂/CO₂ (80:20), formate, lactate, pyruvate, maleate, DL-malate, fumarate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isoamyl alcohol, ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,4-butanediol, benzyl alcohol and phenylethanol are used as electron donors. Methanol and 2-propanol are used without an increase in optical density. Lactate is oxidized incompletely to acetate. Ethanol, 1-propanol, 1-butanol and benzyl alcohol are oxidized incompletely to the corresponding acids. Pyruvate is fermented in the absence of sulfate. Vitamins not required for growth. Acetate, propionate, butyrate, crotonate, malonate, succinate, oxalate, L-tartrate, glutamate, L-alanine, L-serine, benzylamine, choline, fructose, glycerol, *tert*-amyl alcohol, 2,3-butanediol, phenol, isomers of cresol, 3-methylcatechol, benzoate, benzylsuccinate, 4-hydroxymethylbenzoate, toluene and isomers of toluate are not utilized. Desulfovibrin is present. The G + C content of the genomic DNA is 63 mol%.

The type strain, D41^T (=ATCC BAA-1241^T =DSM 17524^T), was isolated from sediment from a gas condensate-contaminated aquifer.

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