Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov.

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An anaerobic, halorespiring bacterium (strain PCE-M2^T^ = DSM 13726^T^ = ATCC BAA-583^T^) able to reduce tetrachloroethene to cis-dichloroethene was isolated from an anaerobic soil polluted with chlorinated aliphatic compounds. The isolate is assigned to the genus *Sulfurospirillum* as a novel species, *Sulfurospirillum halorespirans* sp. nov. Furthermore, on the basis of all available data, a related organism, *Dehalospirillum multivorans* DSM 12446^T^, is reclassified to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov.

INTRODUCTION

Chlorinated ethenes are widespread soil and groundwater pollutants. Because of industrial activities, large amounts of chlorinated ethenes were discharged into the environment over the last few decades. Tetrachloroethene (perchloroethylene; PCE) is used mainly in dry-cleaning processes and as an organic solvent (DiStefano et al., 1991). It is a suspected carcinogen and is thought to be persistent under aerobic conditions (Bouwer & McCarty, 1983; Fathepure et al., 1987). However, Ryoo et al. (2000) recently reported the aerobic conversion of PCE by toluene-<i>o</i>-xylene monooxygenase of *Pseudomonas stutzeri* OX1. Under anaerobic conditions, PCE can be reductively dechlorinated via trichloroethene, dichloroethene and vinyl chloride to the non-toxic end-products ethene (DiStefano et al., 1991; Freedman & Gossett, 1989) and ethane (de Bruin et al., 1992).

Over the last decade, several bacteria that are able to couple the anaerobic reductive dechlorination of PCE to growth have been isolated. This respiratory process is also known as halorespiration. PCE is reduced to either trichloroethene or dichloroethene by, for example, *Dehalospirillum multivorans*, two *Dehalobacter* species and several *Desulfitobacterium* species (Scholz-Muramatsu et al., 1995; Holliger et al., 1993; Wild et al., 1996; Gerritse et al., 1996, 1999; Miller et al., 1997). One organism, *’Dehalococcoides ethenogenes’*, is able to reduce PCE to vinyl chloride, and couples these steps to energy conservation. Vinyl chloride is dechlorinated further to ethene by this organism, but this final reduction step is not coupled to growth (Maymo-Gatell et al., 1997, 1999). The ecological, physiological and technological aspects of halorespiring organisms have been reviewed in detail (El Fantroussi et al., 1998; Holliger et al., 1998; Middeldorp et al., 1999).

Here, we describe the isolation of a novel organism from soil from a polluted site in The Netherlands that is able to reduce PCE to cis-dichloroethene. Initial analysis showed our isolate to have high similarity to members of the genus *Sulfurospirillum* and to *Dehalospirillum multivorans*. Therefore, we included data for the type strains of *Sulfurospirillum barnesi*ii, *Sulfurospirillum deleyianum*, *Sulfurospirillum arsenophilum*, *Sulfurospirillum arcachonense* and *Dehalospirillum multivorans*. Evaluation of all physiological and phylogenetic properties makes it clear that the new isolate, strain PCE-M2^T^, is a member of the genus *Sulfurospirillum*. We propose strain PCE-M2^T^ as the type strain of a novel species within the genus *Sulfurospirillum*, *Sulfurospirillum halorespirans* sp. nov. Furthermore, on the basis of all available data, we propose to reclassify *Dehalospirillum multivorans* as *Sulfurospirillum multivorans* comb. nov.
METHODS

Inoculum source. A soil sample from a polluted site in Maassluis near Rotterdam Harbour in The Netherlands was used as the inoculum for laboratory-scale flow-through soil columns as described by Middeldorp et al. (1998). A liquid sample from one of these columns was used to start the enrichment culture.

Anaerobic medium and experimental set-up. A phosphate-/bicarbonate-buffered medium with a low chloride concentration, as described by Holliger et al. (1993), was used for the experiments. Electron acceptors and donors were added from aqueous, concentrated, sterile stock solutions to respective final concentrations of 10 and 25 mM, unless otherwise stated. PCE was added from a concentrated (1 M) stock solution in hexadecane. Hexadecane was not converted during experiments by the different bacteria. Yeast extract was omitted from the medium unless otherwise stated.

Incubations were carried out in 117 ml serum bottles containing 20 ml anaerobic medium. The headspace consisted of N2/CO2 (80:20) or H2/CO2 (80:20); the latter was used when molecular hydrogen was used as electron donor. Acetate was added as a carbon source when molecular hydrogen or formate was used as electron donor. For isolation purposes, the roll-tube method was used. The medium was solidified with Noble agar (22 g l−1; Difco).

Organisms. *Dehalospirillum multivorans* DSM 12446T, *S. deleyianum* DSM 6946T, *S. arcachonense* DSM 9755T and *S. arsenophilum* DSM 10659T were purchased from the DSMZ. *S. barnesi* ATCC 700032T was obtained from the ATCC.

*Escherichia coli* XL-1 Blue (Stratagene) was used as the host for cloning vectors. The strain was grown in Luria–Bertani medium at 37°C (Sambrook et al., 1989) and ampicillin was added at 100 µg ml−1 when appropriate.

DNA analyses. Both G+C-content analysis and DNA–DNA hybridization were performed at the DSMZ. DNA was isolated by chromatography with hydroxyapatite (Cashion et al., 1977). G+C contents were determined using HPLC, as described by Mesbah et al. (1989). DNA–DNA hybridizations were carried out as described by De Ley et al. (1970), with the modifications described by Huß et al. (1983) and Escara & Hutton (1980). Renaturation rates were computed according to Jahnke (1992).

Analytical techniques. Chloride anion concentrations were determined with a Micro-chlor-o-counter (Marius). Prior to analysis, 0–6 ml samples were acidified with 10 µl pure sulfuric acid and purged for 5 min with nitrogen gas to eliminate sulfide anions, which interfered with the chloride measurement. Volatile fatty acids were determined by HPLC, as described by Scholten & Stams (1995). Inorganic anions were separated on a dionex column as described by the same authors.

All chlorinated ethenes and ethene were determined qualitatively in headspace samples using a 438A Chrompack Packard gas chromatograph. The gas chromatograph was equipped with a flame-ionization detector connected to a capillary column [25 m × 0.32 mm inner diameter; df 10 µm; 100 kPa N2 (Poraplot Q; Chrompack)] and a splitter injector (ratio 1:10). The injector and detector temperatures were respectively 100 and 250°C. The column temperature was initially 50°C for 1 min and then increased by 39°C min−1 to 210°C; finally, the temperature was kept at 210°C for 7 min.

Fatty acid composition. Bacterial cultures were harvested by centrifugation (20 000 g, 20 min, 4°C) and pellets were extracted directly with a modified Bligh–Dyer extraction. The total lipid extract was fractionated on silicic acid and mild alkaline transmethylation was used to yield fatty acid methyl esters from the phospholipid fraction. Concentrations of individual polar-lipid fatty acids as fatty acid methyl esters were determined by using a capillary GC/flame-ionization detector. Identification of polar-lipid fatty acids was based on comparison of retention-time data with known standards (for further details, see Boschker et al., 1999).

Transmission electron microscopy (TEM). For TEM, cells were fixed for 2 h in 2.5%- (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 0°C. After the cells had been rinsed in the same buffer, they were subjected to post-fixation using 1% (w/v) OsO4 and 2.5%- (w/v) K2Cr2O7 for 1 h at room temperature. Finally, the cells were post-stained in 1% (w/v) uranyl acetate. After sectioning, micrographs were taken with a Philips EM400 transmission electron microscope.

Amplification of 16S rDNA, cloning and sequencing. Chromosomal DNA of strain PCE-M2T was isolated as described previously (Van de Pas et al., 1999). The 16S rDNA was amplified with a GeneAmp PCR System 2400 thermocycler (Perkin–Elmer–Cetus). After preheating to 94°C for 2 min, 35 amplification cycles of denaturation at 94°C for 20 s, primer annealing at 50°C for 30 s and elongation at 72°C for 90 s were performed. A final extension of 7 min at 68°C was performed. The PCR products (50 µl) contained 10 pmol primers 8f [5′-CACGGATCCAGATGTTTGAT(C/T)(A/C)] and TGGCTCAG-3′] and 1510r [5′-GTGAAGCTTACGG(C/T)TACCTGTGTACCTTG-3′] (Lane, 1991), 2 mM MgCl2, 200 µM each of dATP, dCTP, dGTP and dTTP and 1 U Expand Long Template enzyme mixture (Roche Diagnostics). PCR products were purified by the QIAquick PCR purification kit (Qiagen) and cloned into *E. coli* XL-1 Blue by using the pGEM-T plasmid vector (Promega). Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method, and standard DNA manipulations were performed according to published procedures (Sambrook et al., 1989) and manufacturers’ instructions. Restriction enzymes were purchased from Life Technologies.

DNA sequencing was performed using a LiCor DNA sequencer 4000L. Plasmid DNA used for sequencing reactions was purified with the QIAquick PCR Miniprep kit (Qiagen). Reactions were performed using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech). Infrared dye (IRD800)-labelled oligonucleotides were purchased from MWG Biotech. The sequence was determined using labelled primers 515f (5′-ACCGCGGCTCGTGGCAC-3′) and 338f (5′-CTCCTACCGGA-GGCACAGTGA-3′) and 968f (5′-AACGCGGAAACCTTTA-3′) (Nübel et al., 1996).

Sequences were analysed using the DNASTar software package and ARB software (Strunk & Ludwig, 1995). Initial sequence alignments were performed using the LALIGN utility at the GENESTREAM network server (http://www2.igh.cnrs.fr/bin/lalign-guess.cgi). The phylogenetic tree was constructed using the neighbour-joining method (*E. coli* positions 72–1419) (Saitou & Nei, 1987).

RESULTS

Isolation of strain PCE-M2T

A PCE-degrading culture was enriched from soil polluted with chlorinated ethenes by using, alternately, hydrogen and lactate as electron donor and PCE as electron acceptor. The enrichment degraded PCE via trichloroethene to (mainly) cis-dichloroethene. Minor amounts of vinyl chloride and ethene were also produced. A microscopically pure culture was obtained via serial dilution in liquid medium. This culture reduced PCE, via trichloroethene, to...
**Morphology**

Cells of strain PCE-M2T were slightly curved rods, 2–5–4 μm long by 0.6 μm wide (Fig. 1). The bacteria stained Gram-negative and formation of endospores was never observed. Cells in actively growing cultures were motile.

**Growth conditions**

Strain PCE-M2T was routinely cultivated with PCE as electron acceptor and lactate as electron donor. It was able to couple the oxidation of lactate, molecular hydrogen, formate and pyruvate to growth in the presence of PCE as terminal electron acceptor. Organic electron donors, except for formate, were oxidized incompletely to acetate. Formate and molecular hydrogen sustained growth only when acetate was present as carbon source. Strain PCE-M2T was able to couple the reduction of a number of electron acceptors to growth (Table 1). Oxygenated sulfur compounds (sulfate, sulfite and thiosulfate) could not replace PCE as electron acceptor, nor could 3-chloro-4-hydroxyphenylacetate or 1,2-dichloroethane.

Strain PCE-M2T grew fermentatively on both fumarate and pyruvate, whereas lactate could not be fermented. All known Sulfurospirillum species are also able to ferment fumarate (Stolz et al., 1999; Finster et al., 1997). Scholz-Muramatsu et al. (1995) reported that fumarate could not be fermented by Dehalospirillum multivorans DSM 12446T. However, we were able to grow Dehalospirillum multivorans fermentatively on fumarate. Pyruvate fermentation is also reported for S. deleyianum and Dehalospirillum multivorans, whereas S. arcachonense is not able to ferment pyruvate (Schumacher et al., 1992; Scholz-Muramatsu et al., 1995; Finster et al., 1997). No data on pyruvate fermentation have been reported for the other two Sulfurospirillum species.

**Table 1. Terminal electron acceptors used by strain PCE-M2T and related strains**

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>S&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>ND</td>
<td>+&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>AsO&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>ND</td>
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<tr>
<td>SeO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>ND</td>
</tr>
<tr>
<td>PCE</td>
<td>+</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+ (&lt;→NH&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>+&lt;sup&gt;a&lt;/sup&gt; (→NO&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>+&lt;sup&gt;f&lt;/sup&gt; (→NH&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>+&lt;sup&gt;f&lt;/sup&gt; (→NH&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>+&lt;sup&gt;d&lt;/sup&gt; (→NH&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>+</td>
<td>-&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Sulfite</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Thiosulfate</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Microaerophilic</td>
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<td>ND</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.

*Data for reference strains were taken from: a, Scholz-Muramatsu et al. (1995); b, Holliger et al. (1999); c, Finster et al. (1997); d, Oremland et al. (1994); e, Laverman et al. (1995); f, Schumacher et al. (1992); g, Stolz et al. (1999).
Strain PCE-M2T was able to grow at moderate temperatures. Optimal growth occurred between 25 and 30 °C.

Molecular analysis

The nucleotide sequence of a 16S rRNA gene of strain PCE-M2T was determined and analysis revealed that strain PCE-M2T is clustered in the ε-subclass of the Proteobacteria. A phylogenetic tree was constructed and showed that strain PCE-M2T groups within the genus Sulfurospirillum and is closely related to Dehalospirillum multivorans (Fig. 2). DNA–DNA hybridization values and levels of 16S rDNA sequence similarity between strain PCE-M2T, the different Sulfurospirillum species and Dehalospirillum multivorans are given in Table 2. These data show that both strain PCE-M2T and Dehalospirillum multivorans should be included within the genus Sulfurospirillum and that they are related most closely to S. arsenophilum.

The G+C content of strain PCE-M2T is 41.8 ± 0.2 mol%. With the exception of S. arcachonense (32-0 mol%; Finster et al., 1997), this agrees well with the G+C contents of other Sulfurospirillum species and Dehalospirillum multivorans (Table 2).

Fatty acid composition

Strain PCE-M2T and the other four strains analysed had similar polar-lipid fatty acid profiles, mainly comprising 16:1ω7c, 16:0 and 18:1ω7c (Table 3). The dominant fatty acids were similar in S. arcachonense, as reported by Finster et al. (1997): there were smaller amounts of an 18:1 fatty acid and larger amounts of 18:0 fatty acid. As discussed by Finster et al. (1997), the fatty acid composition detected is typical of bacteria belonging to the ε-subclass of the Proteobacteria.

DISCUSSION

An anaerobic bacterium able to use PCE, selenate, arsenate and some other compounds (Table 1) as terminal electron acceptors for growth was isolated. The organism was isolated from a soil polluted with chlorinated aliphatic compounds; this soil produced a rapid dechlorination of PCE in laboratory-scale flow-through columns (Middeldorp et al., 1998). Comparison of the physiological and phylogenetic features of strain PCE-M2T revealed a close relationship to members of the genus Sulfurospirillum and to Dehalospirillum multivorans (Finster et al., 1997; Holliger et al., 1998; Laverman et al., 1995; Oremland et al., 1994; Scholz-Muramatsu et al., 1995; Schumacher et al., 1992; Stolz et al., 1999). We used 16S rDNA sequences to construct a phylogenetic tree showing the position of strain PCE-M2T in relation to closely related organisms and other (dechlorinating) organisms (Fig. 2). DNA–DNA hybridizations between all species tested are below the critical value of 70 % (the approximate threshold for delineation of separate species; Stackebrandt & Goebel, 1994).

Table 2. DNA G+C content, DNA–DNA relatedness and 16S rDNA sequence similarity between strain PCE-M2T and related species

<table>
<thead>
<tr>
<th>Strain</th>
<th>G+C content (mol%)</th>
<th>16S rDNA sequence similarity (%) to/DNA–DNA hybridization (%) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Strain PCE-M2T</td>
<td>41.8 ± 0.2</td>
<td>98-8</td>
</tr>
<tr>
<td>2. D. multivorans DSM 12446T</td>
<td>41.5±1</td>
<td>65-7</td>
</tr>
<tr>
<td>3. S. arsenophilum DSM 10659T</td>
<td>40.9±5</td>
<td>33-2</td>
</tr>
<tr>
<td>4. S. deleyianum DSM 6946T</td>
<td>40.6±2</td>
<td>28-1</td>
</tr>
<tr>
<td>5. S. barnesii ATCC 700032T</td>
<td>40.8±1</td>
<td>29-2</td>
</tr>
<tr>
<td>6. S. arcachonense DSM 9755T</td>
<td>32-0±1</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Data obtained from: a, Schumacher et al. (1992); b, Scholz-Muramatsu et al. (1995); c, Finster et al. (1997); d, Stolz et al. (1999). ND, Not determined.
The original description of this genus was provided by Schumacher et al. (1992). Additionally, some species are able to use arsenate, selenate, PCE or trichloroethene as terminal electron acceptors. The type species is Sulfurospirillum deleyianum.

**Description of Sulfurospirillum halorespirans sp. nov.**

*Sulfurospirillum halorespirans* (ha.lo.res’pi.rans. N.L. part. adj. halorespirans halorespiring, respiring halogenated compounds).

Gram-negative. Slightly curved, rod-shaped cells, 2.5–4 μm long by 0.6 μm wide. Motile. Optimum growth between 25 and 30 °C. PCE, selenate, arsenate, nitrate, nitrite, sulfur and fumarate serve as terminal electron acceptors. Capable of microaerophilic growth. Nitrate and nitrite are reduced to ammonium. PCE is reduced to cis-dichloroethene. Selenate is reduced, via selenite, to elemental selenium. Hydrogen, formate, pyruvate and lactate serve as electron donors. Hydrogen and formate serve as electron donors only when acetate is present as carbon source. Can grow fermentatively on fumarate and pyruvate. The G+C content of the type strain is 41.8 ± 0.2 mol%.

The type and only strain, strain PCE-M2T (= DSM 13726T = ATCC BAA-583T), was isolated from a soil that was polluted with chlorinated aliphatic compounds in Maassluis, near Rotterdam Harbour, The Netherlands.

**Description of Sulfurospirillum multivorans comb. nov.**


The description was provided by Scholz-Muramatsu et al. (1995). Additionally, this species is able to use arsenate and selenate as electron acceptors. The type strain is DSM 12446T.

**ACKNOWLEDGEMENTS**

This work was financially supported by TNO Environment, Energy and Process Innovation, Apeldoorn, The Netherlands. The authors would like to thank A. Keizer-Gunnik (Laboratory for Electron Microscopy,
University of Groningen, The Netherlands) for the transmission electron micrographs. The authors would also like to thank Wim Roelofsen and Caroline Plugge for their assistance during this work.

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