Electron donor and acceptor utilization by halorespiring bacteria

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Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
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Front cover: Modified EM picture of *Sulfurospirillum halorespirans* PCE-M2

Stellingen

1. Elk nadeel heb zijn voordeel.
   Dit proefschrift.

2. De ene volledige reductie van PCE is de andere nog niet.
   Dit proefschrift.

3. Sectorale communicatie reikt niet ver genoeg.
   HRH the Prince of Orange, Wat. Sci. Technol. 2002, 45, 19-21.

4. Carnaval en wetenschap hebben samen een hoge impact.
   Hoffmann, R., Nature, 2004, 424, 21.

5. Het gebruik van probiotica is niet zonder gevaar.
   Abbott, A., Nature, 2004, 427, 284-286.

6. Dit proefschrift is logisch ingedeeld.

7. "De hoogtepunten van het nieuws" kunnen een macaber karakter hebben.

Stellingen behorend bij het proefschrift
'Electron donor and acceptor utilization by halorespiring bacteria'.
Maurice Luijten
Wageningen, 11 juni 2004.

Vieze grond
is een schone grond
voor een grondig onderzoek

Contents

Preface

Tetrachloroethene (PCE) is, mainly due to human activity, a common pollutant in soil and groundwater. PCE has been used in dry cleaning and industrial cleaning resulting in (accidental) spillage into soil and groundwater. Because of the toxicity of PCE and its degradation products, the clean-up of soils polluted with these compounds has been studied extensively since the last two decades. Chlorinated ethenes can be converted via both chemical and (micro)biological processes. PCE is considered to be persistent to microbial conversion under aerobic conditions, though this dogma has been questioned (Ryoo *et al.*, *Nature Biotechnol.* 2000, 18, 775-778). Under aerobic conditions lower chlorinated ethenes can be degraded via metabolic, growth-coupled processes and gratuitously via co-metabolic processes. Under anaerobic conditions PCE and its degradation products can be reductively dechlorinated by anaerobic microorganisms. In this process, the chloride atoms are stepwise replaced by hydrogen atoms to form the dechlorinated end product ethene (Figure 1). In this anaerobic reduction the chlorinated compound acts as electron acceptor.

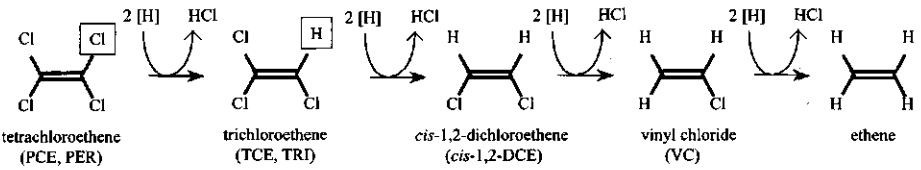

Figure 1: Anaerobic microbial reduction of tetrachloroethene

A number of bacteria that couple this anaerobic reductive dechlorination of PCE to growth has been isolated. Only one of these bacteria, "*Dehalococcoides ethenogenes*" dechlorinates PCE completely to ethene, the others dechlorinate PCE only partially to produce trichloroethene (TCE) or dichloroethene (DCE).

Complete reduction of chlorinated ethenes has been observed at many polluted sites. However, at other sites no or only partial dechlorination of chlorinated ethenes has been found. Especially partial dechlorination may cause significant problems since the gaseous intermediate vinyl chloride is more toxic than PCE itself. Partial dechlorination of PCE may be due to a number of reasons. The absence of microorganisms able to reduce the pollutant completely is often postulated. Another reason may be that the electron donor required for the reduction of PCE is either not present in sufficient amounts or consumed by other microorganisms present.

Halorespiring bacteria present may also preferentially use other possible electron acceptors.

The experimental work described in this dissertation focuses on microorganisms able to degrade chlorinated ethenes. Chapter 1 reports on the phylogenetic position and physiological features of a newly isolated halorespiring bacterium *Sulfurospirillum halorespirans* PCE-M2. This bacterium turned out to be phylogenetically related to *Sulfurospirillum* species that are known as sulphur- and metal-reducing bacteria. Based on this finding, *Sulfurospirillum halorespirans* and a number of different other halorespiring and related bacteria were screened for their ability to reduce oxidized metals as well (Chapter 2). A screening for the oxidation and reduction capacity of $A(H_2)QDS$, a humic acid analogue, is also described in this chapter. Humic substances play an important role as electron shuttles in soil. Our work and research by others shows that many halorespiring bacteria are able to reduce a broad spectrum of other electron acceptors. The ability of halorespiring bacteria to use other electron acceptors raises the question whether the presence of these alternative electron acceptors influences the degradation of PCE. In the third chapter we describe the effect of some alternative electron acceptors, such as nitrate, sulphate and oxidized metals on the reduction of PCE.

The efficiency of anaerobic reductive dechlorination depends on the type of electron acceptor, but is also influenced by the electron donors available. Molecular hydrogen is thought to be an important electron donor of possible electron-accepting processes in soil. Each electron-accepting process appears to have a distinct hydrogen threshold concentration. Chapter 4 describes hydrogen threshold values for several reducing processes by bacteria that are able to halorespire. These threshold values are compared with hydrogen concentrations measured during an *in-situ* bioremediation project.

No microorganisms had been isolated able to metabolically reduce vinyl chloride under anaerobic conditions at the start of the research described in this thesis. The isolation of such an organism will help to expand the knowledge on the complete anaerobic (*in-situ*) biodegradation of chlorinated ethenes in polluted environments. The last experimental chapter (Chapter 5) describes the enrichment of two bacterial cultures able to reduce vinyl chloride at relatively high rates. Unfortunately, this work has not resulted in the isolation of an anaerobic vinyl chloride respiring microorganism.

This final experimental chapter is followed by a review (Chapter 6) that combines our results with the available literature data. Finally, this dissertation closes with some concluding remarks and future perspectives.

1

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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Int. J. Syst. Evol. Microbiol. 2003, 53, 787-793

Abstract

An anaerobic, halorespiring bacterium (strain PCE-M2^T = DSM 13726^T = ATCC BAA-583^T) able to reduce tetrachloroethene to *cis*-1,2-dichloroethene was isolated from a 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 (perchloroethene; 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 and McCarty, 1983; Fathepure *et al.*, 1987). However, Ryoo *et al.* (2000) reported the aerobic conversion of PCE by toluene-*o*-xylene mono-oxygenase 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 and 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 (Maymó-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*-1,2-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 barnesii*, *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.

Materials and methods

Inoculum source

A soil sample from a polluted site in Maassluis near Rotterdam harbor in The Netherlands was used as inoculum for laboratory-scale flow-through 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 N_2/CO_2 (80:20) or H_2/CO_2 (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; Difco).

Organisms

Sulfurospirillum multivorans (former *Dehalospirillum multivorans*) DSM 12446^T, *S. deleyianum* DSM 6946^T, *S. arcachonense* DSM 9755^T and *S. arsenophilum* DSM 10659^T were purchased from the DSMZ. *S. barnesii* ATCC 700032^T 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 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 Huβ *et al.* (1983) and Escara and 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 minutes with nitrogen gas to eliminate sulfide anions, which interfered with the chloride measurement. Volatile fatty acids were determined by HPLC, as described by Scholten and Stams (1995). Inorganic anions were separated on a dionex column as described by these same authors.

All chlorinated ethenes and ethene were determined qualitatively in headspace samples using a 438A Chrompack Packard gas chromatograph (GC). The gas chromatograph was equipped with a flame-ionization detector connected to a capillary column (25 m x 0.32 mm inner diameter; df 10 µm; 100 kPa N_2 (Poraplot Q; Chrompack)) and a splitter injector (ration 1:10). The injector and detector temperatures were 100 and 250 °C, respectively. The column temperature was initially 50 °C for 1 minute and was then increased by 39 °C per minute to 210 °C; finally, the temperature was kept at 210 °C for 7 minutes.

Fatty acid composition

Bacterial cultures were harvested by centrifugation (20000 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 hours 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) OsO_4 and 2.5 % (w/v) $K_2Cr_2O_7$ for 1 hour 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-M2^T 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 minutes, 35 amplification cycles of denaturation at 94 °C for 20 seconds, primer annealing at 50 °C for 30 seconds and elongation at 72 °C for 90 seconds were performed. The PCRs (50 µl) contained 10 pmol primers 8f [5'-CACGGATCCAGAGTTTGAT(C/T)-(A/C)TGGCTCAG-3'] and 1510r [5'-GTGAAGCTTACGG(C/T)TACCTTGTTA-CGACTT-3'] (Lane, 1991), 2 mM $MgCl_2$, 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 lyses method, and standard DNA manipulations were performed according to established 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 QIAprep spin miniprep kit (Qiagen). Reactions were performed using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Infrared dye (IRD800)-labeled oligonucleotides using labeled primers 515r [5'-ACCGCGGCTGC-TGGCAC-3'] (Lane, 1991), 338f [5'-ACTCCTACGGGAGGCAGCAGGTA-3'] and 968f [5'-AACGCGAAGAACCTTA-3'] (Nübel *et al.*, 1996).

Sequences were analyzed using the DNAstar software package and ARB software (Strunk and 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 neighbor-joining method (*E. coli* positions 72-1419) (Saitou and Nei, 1987).

Results

Isolation of strain PCE-M2^T

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*-1,2-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 *cis*-1,2-dichloroethene. No other reduced products could be found in this enrichment. This culture was used to prepare a dilution series in roll tubes. In these roll tubes, only lens-shaped colonies were observed. Single colonies from these roll tubes were transferred back into liquid medium. Immediately, a new dilution series in liquid medium was prepared and incubated in parallel on solidified medium in roll tubes. After growth, single colonies were again transferred back to, and maintained in, liquid medium. One of these cultures was checked for purity by incubation in a rich Wilkins-Chalgren medium (Oxoid). This resulted in growth of, presumably, the PCE-reducing organism only. This culture, strain PCE-M2^T, was used for further characterization.

Morphology

Cells of strain PCE-M2^T were slightly curved rods, 2.5-4 µm long by 0.6 µm wide (Figure 1). The bacteria stained Gram-negative and formation of endospores was never observed. Cells in actively growing cultures were motile.

Figure 1: Transmission electron micrograph of cells of *Sulfurospirillum halorespirans* sp. nov. PCE-M2^T. Bar 0.5 µm.

Growth conditions

Strain PCE-M2^T 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-M2^T was able to couple the reduction of a number of electron acceptors to growth (Table 1). Oxygenated sulphur compounds (sulphate, sulphite and thiosulphate) could not replace PCE as electron acceptor, nor could 3-chloro-4-hydroxyphenylacetate or 1,2-dichloroethane.

Strain PCE-M2^T 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.*, 1995; Finster *et al.*, 1997). Scholz-Muramatsu *et al.* (1995) reported that fumarate could not be fermented by *Dehalospirillum multivorans* DSM

12446^T. However, we were able to grow *Dehalospirillum multivorans* fermentatively on fumarate. Pyruvate fermentation is also reported for *S. deleyianum* and *D. 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.

Strain PCE-M2^T was able to grow at moderate temperatures. Optimal growth occurred between 25 and 30 °C.

Table 1: Terminal electron acceptors used by strain PCE-M2^T and related strains. Strains are indicated as: 1 = PCE-M2^T; 2 = *Dehalospirillum multivorans* DSM 12446^T; 3 = *S. arsenophilum* DSM 10659^T; 4 = *S. deleyianum* DSM 6946^T; 5 = *S. barnesii* ATCC 700032^T; 6 = *S. arcachonense* DSM 9755^T.

Acceptor	1	2	3	4	5	6
S^0	+	nd	+[g]	+[f]	+[e]	+[c]
AsO_4	+	+[b]	+[g]	-[g]	+[e]	nd
SeO_4	+	+[b]	-[g]	-[g]	+[d]	nd
PCE	+	+[a]	nd	nd	-	nd
NO_3	+($\rightarrow NH_4$)	+[a]($\rightarrow NO_2$)	+[g]($\rightarrow NH_4$)	+[f]($\rightarrow NH_4$)	+[d]($\rightarrow NH_4$)	-[c]
NO_2	+	-[a]	+[g]	+[f]	+[e]	-[c]
Sulphite	-	nd	nd	+[f]	-[g]	-[c]
Thiosulphate	-	nd	+[g]	+[f]	+[e]	-[c]
Microaerophilic	+	nd	+[g]	+[f]	+[e]	+[c]
Fumarate	+	+[a]	nd	+[f]	+[e]	nd

nd = Not determined. Data obtained 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.

Molecular analysis

The nucleotide sequence of a 16S rRNA gene of strain PCE-M2^T was determined and analysis revealed that strain PCE-M2^T is clustered in the ε-subclass of the *Proteobacteria*. The GenBank accession number for the 16S rDNA sequence of strain PCE-M2^T is AF218076. A phylogenetic tree was constructed and showed that strain PCE-M2^T groups within the genus *Sulfurospirillum* and is closely related to *Dehalospirillum multivorans* (Figure 2). DNA-DNA hybridization values and levels

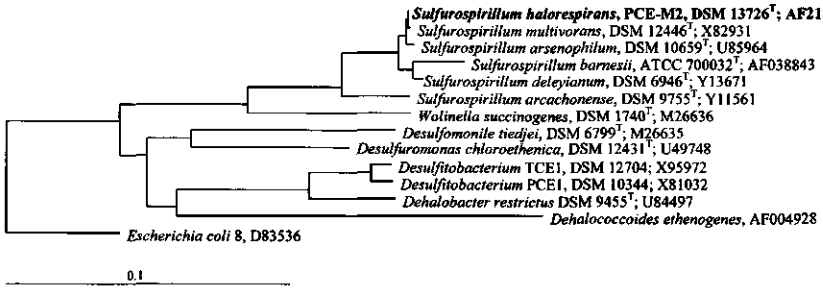

Figure 2: Phylogenetic tree constructed by the neighbour-joining method (Saitou and Nei, 1987), using 1347 nucleotides from 16S rDNA sequences, showing the position of strain PCE-M2^T in relation to members of the genus *Sulfurospirillum* and other (dechlorinating) organisms. Bar = 10 % divergence.

of 16S rDNA sequence similarity between strain PCE-M2^T, the different *Sulfurospirillum* species and *Dehalospirillum multivorans* are given in Table 2. These data show that both strain PCE-M2^T and *Dehalospirillum multivorans* should be included within the genus *Sulfurospirillum* and that they are related most to *S. arsenophilum*.

The G + C content of strain PCE-M2^T 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 content of other *Sulfurospirillum* species and *Dehalospirillum multivorans* (Table 2).

Table 2: DNA G + C content, DNA-DNA relatedness and 16S rDNA sequence similarity between strain PCE-M2^T and related species. Values above the diagonal are 16S rDNA sequence similarity (%); values below the diagonal are DNA-DNA hybridization (%).

Strain	G + C content (mol%)	16S rDNA sequence similarity (%) to / DNA-DNA hybridization (%) with:					
		1	2	3	4	5	6
PCE-M2^T	41.8 ± 0.2	-	98.8	99.0	97.0	95.0	93.0
D. multivorans	41.5[b]	65.7	-	99.0	98.0	98.0	92.0
S. arsenophilum	40.9[d]	33.2	35.4	-	97.0	97.0	93.0
S. deleyianum	40.6[a]	28.1	30.8	30.7[d]	-	98.0	92.0
S. barnesii	40.8[d]	29.2	31.5	49.7[d]	55.0[d]	-	91.7
S. arcachonense	32.0[c]	nd	nd	nd	nd	nd	-

Strains are indicated as: 1 = PCE-M2^T, 2 = *D. multivorans* DSM 12446^T, 3 = *S. arsenophilum* DSM 10659^T, 4 = *S. deleyianum* DSM 6946^T, 5 = *S. barnesii* ATCC 700032^T, 6 = *S. arcachonense* DSM 9755^T. 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.

Fatty acid composition

Strain PCE-M2^T and the other four strains analyzed had similar polar-lipid fatty acid profiles, mainly comprising 16:1ω7*c*, 16:0 and 18:1ω7*c* (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.*

Table 3: Polar-lipid fatty acid composition of strain PCE-M2^T and related strains. Values are mol% of polar lipid fatty acids.

Fatty acid	PCE-M2^T	*D. multivorans*	*S. arsenophilum*	*S. deleyianum*	*S. barnesii*
14:0	3.3	5.1	4.5	-	1.8
i15:0	-	-	2.1	-	-
15:0	2.1	1.5	1.3	1.1	0.6
i16:1	1.4	0.6	1.1	0.6	0.6
16:1ω7*c*	47.4	47.4	47.5	52.0	43.2
16:1ω5	0.3	0.3	0.6	-	0.5
16:0	38.3	40.7	24.5	29.2	30.2
i17:1	-	1.1	2.1	-	1.1
17:0	-	-	1.9	-	0.2
18:1ω7*c*	6.5	2.8	12.2	17.2	21.6
18:0	0.5	-	0.4	-	-
Minor components	0.2	0.6	1.7	-	0.1

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-M2^T revealed a close relationship to members of the genus *Sulfurospirillum* and to *Dehalospirillum multivorans* (Schumacher *et al.*, 1992; Oremland *et al.*, 1994; Scholz-Muramatsu *et al.*, 1995; Laverman *et al.*, 1995; Finster *et al.*, 1997; Holliger *et al.*, 1998; Stolz *et al.*, 1999). We used 16S rDNA sequences to construct a phylogenetic tree showing the position of strain PCE-M2^T in relation to

closely related organisms and other (dechlorinating) organisms (Figure 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)).

Schumacher *et al.* (1992) established the genus *Sulfurospirillum* to describe '*Spirillum*' 5175 as *S. deleyianum*, a Gram-negative, elemental sulphur-reducing spirillum. Since then, several bacteria have been identified as additional members of the genus *Sulfurospirillum* (Finster *et al.*, 1997; Stolz *et al.*, 1999). The data presented here justify the addition of our isolate, strain PCE-M2^T, to the *Sulfurospirillum* clade. Since our strain differs from the other described species, e.g. in using PCE as a terminal electron acceptor for growth, we propose that strain PCE-M2^T represents a novel species, *Sulfurospirillum halorespirans* sp. nov.

Strain PCE-M2^T is very similar to *Dehalospirillum multivorans*, especially with respect to the reduction of chlorinated ethenes. At the time of publication, the data that Scholz-Muramatsu *et al.* (1995) presented on *Dehalospirillum multivorans* justified the establishment of a new genus. However, over the last decade, more physiological data on *Dehalospirillum multivorans* have become available, such as the ability of this microorganism to reduce selenate and arsenate (Holliger *et al.*, 1998). Combining these new data with the phylogenetic data presented here, it is necessary to reclassify *Dehalospirillum multivorans* DSM 12446^T in the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov.

Dehalobacter restrictus was the first organism isolated that is able to reduce PCE metabolically (Holliger *et al.*, 1993). This organism is limited to the use of PCE and trichloroethene as electron acceptors and molecular hydrogen as electron donor. Several *Desulfitobacterium* strains that are more diverse in their substrate spectrum have also been isolated from distinct environment. This diversity could indicate that strains of the genus *Desulfitobacterium* play an important role in the attenuation of chlorinated compounds in nature. Members of the genus *Sulfurospirillum* also have a more diverse substrate spectrum. They are known specifically for the reduction of sulphur and oxidized metals such as arsenate and selenate. The isolation of strain PCE-M2^T, a novel halorespiring species, and the addition of *Dehalospirillum multivorans* indicate the importance of the genus *Sulfurospirillum* in biotransformations in soils polluted with chlorinated ethenes and metal ions.

Emended description of Sulfurospirillum (Schumacher et al., 1993)

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, sulphur and fumarate serve as terminal electron acceptors. Capable of microaerophilic growth. Nitrate and nitrite are reduced to ammonium. PCE is reduced to *cis*-1,2-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 mol%.

The type and only strain, strain PCE-M2^T (= DSM 13726^T = ATCC BAA-583^T), was isolated from a soil polluted with chlorinated aliphatic compounds in Maassluis, near Rotterdam Harbor, The Netherlands.

Description of Sulfurospirillum multivorans comb nov.

Basonym: *Dehalospirillum multivorans* (Scholz-Muramatsu *et al.*, 2002)

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 12446^T.

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2

Anaerobic reduction and oxidation of quinone moieties and the reduction of oxidized metals by halorespiring and related organisms

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Abstract

Halorespiring bacteria have been detected in soils that were not polluted with chlorinated compounds. In this study, we describe alternative electron acceptor utilization by some halorespiring bacteria and phylogenetically related bacteria. It appears that oxidized metals like selenate, arsenate and manganese are rather common electron acceptors for halorespiring species of *Desulfitobacterium* and *Sulfurospirillum* and related bacteria. All tested bacteria are able to reduce AQDS and 4 tested organisms (*Desulfitobacterium hafniense* DP7, *Sulfurospirillum barnesii*, *S. deleyianum* and *S. arsenophilum*) are able to oxidize AH_2QDS as well.

The characteristic to reduce oxidized metals, and to reduce and oxidize quinone moieties coupled to energy conservation is a likely explanation for the presence of halorespiring microorganisms in unpolluted soils.

Introduction

Over the last decade a number of bacteria able to use chlorinated aromatics and ethenes as terminal electron acceptors for growth has been isolated from a range of different environments contaminated with chlorinated compounds (Holliger *et al.*, 1993; Utkin *et al.*, 1994; Scholz-Muramatsu *et al.*, 1995; Sanford *et al.*, 1996; Wild *et al.*, 1996; Bouchard *et al.*, 1996; Gerritse *et al.*, 1996; Maymó-Gatell *et al.*, 1997; Gerritse *et al.*, 1999; Finneran *et al.*, 2002; Luijten *et al.*, 2003). Most of these organisms are known to respire oxidized sulfur or nitrogen compounds as well. Recently, two non-dechlorinating members of the otherwise halorespiring genus *Desulfitobacterium* have been isolated (Niggemeyer *et al.*, 2001; Van de Pas *et al.*, 2001). Furthermore, by using molecular techniques it was shown that halorespiring bacteria are also present at sites where chlorinated compounds are absent (Löffler *et al.*, 2000; Lanthier *et al.*, 2001). These two findings raised the question whether halorespiration is the only mechanism of energy conservation of these halorespiring bacteria in nature.

Recently, the possibility to reduce oxidized metals and humic acids was reported for some halorespiring bacteria (Holliger *et al.*, 1999; Niggemeyer *et al.*, 2001; Finneran *et al.*, 2002; Cervantes *et al.*, 2002; Luijten *et al.*, 2003). Here we report the results of an extended screening of halorespiring and related bacteria for their capacity to reduce oxidized metals and the humic acid analogue anthraquinone-2,6-disulfonate (AQDS). We have also tested the possibility of bacteria to oxidize reduced anthrahydroquinone-2,6-disulfonate (AH_2QDS). Our results show that these features are common among halorespiring species of the genera *Desulfitobacterium* and *Sulfurospirillum*. They may represent an alternative growth mode for these bacteria at locations not contaminated with chlorinated pollutants.

Material and Methods

Organisms

Various pure cultures of anaerobic bacteria were used in this study. *Sulfurospirillum halorespirans* PCE-M2 (DSM 13726; ATCC BAA-583) and *Desulfitobacterium hafniense* DP7 (DSM 13498) were isolated at the Laboratory of Microbiology, Wageningen University, The Netherlands (Van de Pas *et al.*, 2001; Luijten *et al.*, 2003). *Sulfurospirillum multivorans* (formerly known as *Dehalospirillum multivorans* (Luijten *et al.*, 2003)) (DSM 12446), *Sulfurospirillum deleyianum* (DSM 6964), *Sulfurospirillum arsenophilum* (DSM 10659), *Desulfitobacterium dehalogenans* (DSM 9161), *Desulfitobacterium* strain PCE1 (DSM 10344) and *Desulfitobacterium chlororespirans* Co23 (DSM 11544) were purchased form the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Sulfurospirillum barnesii* (ATCC 700032) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Geobacter metallireducens (DSM 7210) was a kind gift from F.J. Cervantes of the sub-department of Environmental Technology from Wageningen University, The Netherlands. *Desulfitobacterium hafniense* TCE1 (DSM 12704) was a kind gift from J. Gerritse (TNO Environment, Energy and Process Innovation, Apeldoorn, The Netherlands).

Anaerobic medium and experimental set-up

Incubations were carried out in 117-ml serum bottles containing 20 ml anaerobic medium. A phosphate-bicarbonate buffered medium with a low chloride concentration as described by Holliger *et al.* (1993) was used. The medium was enriched with 3 µg/l Na_2SeO_4 and 8 µg/l $Na_2WO_4.H_2O$. Electron acceptors and electron donors were added from aqueous concentrated stock solutions, unless otherwise stated. All stock solutions were prepared anaerobically. Yeast extract (1 g/l) was added to experiments with *Desulfitobacterium* species. The headspace in the bottles consisted of N_2/CO_2 (80/20) or H_2/CO_2 (80/20), the latter when molecular hydrogen was used as electron donor. Acetate (5 mM) was added as carbon source when molecular hydrogen was used as electron acceptor. All experiments were

incubated at 30 °C and started with a 10 % inoculum. In all experiments performed to study the possible reductions, lactate was used as electron donor, except for experiments with *S. deleyianum*. This organism can not use lactate as electron donor (Schumacher *et al.*, 1992) and pyruvate was used instead. To prove unambiguously that the tested metal ions were used as terminal electron acceptor the bacteria were sub-cultured several sequential times with hydrogen as sole electron donor present.

Amorphous manganese(IV)oxide (δMnO_2) was prepared as described by Langenhoff *et al.* (1996). The final concentration of Mn(IV) in our experiments was approximately 10 mM.

Anthrahydroquinone-2,6-disulfonate (AH_2QDS) was produced biologically from AQDS with *Geobacter metallireducens*. An AQDS saturated solution (25 mM) was inoculated with *G. metallireducens* at 30 °C, molecular hydrogen was added as electron donor and acetate as carbon source. After reduction, the AH_2QDS was added to a final concentration of approximately 5 mM via a 0.2 µm filter into the serum bottles for the experiments with AH_2QDS as electron donor. To test the oxidation of AH_2QDS the organisms were incubated with the following electron acceptors: nitrate for *Sulfurospirillum arsenophilum*, *S. deleyianum*, *S. barnesii* and *Desulfitobacterium hafniense* DP7; tetrachloroethene (PCE) for *S. halorespirans* PCE-M2 and *S. multivorans*; and 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) for *Desulfitobacterium* strain PCE1, *D. dehalogenans* and *D. chlororespirans* Co23.

Analyses

Volatile fatty acids were determined by high-pressure liquid chromatography (HPLC) as described by Scholten and Stams (1995). Inorganic anions (nitrate, nitrite, arsenate, selenate) were separated on a dionex column and detected with suppressed conductivity as described by Scholten and Stams (1995).

Fe(II) was determined quantitatively using the ferrozine method (Lovley and Phillips, 1987) and visually assessed via the production of black FeS precipitates. The reduction of selenate and arsenate was also (non-quantitatively) visually confirmed via the production of red Se(0) precipitates and the production of yellow precipitates, presumably orpiment (As_2S_3), respectively (Niggemeyer *et al.*, 2001). The reduction of Mn(IV) was assessed visually via a color change from the black MnO_2 to the white $MnCO_3$ precipitates (Niggemeyer *et al.*, 2001). The oxidation and reduction of

$A(H_2)QDS$ was also qualitatively determined. The reduced AH_2QDS has a bright red color, whereas the oxidized AQDS has a brownish color.

Cell numbers were determined by phase-contrast microscopy using a Bürker-Türk counting chamber at a magnification of 1250 times.

Transmission electron microscopy

For transmission electron microscopy (TEM) cells were fixed for two hours in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2) at 0 °C. After rinsing in the same buffer a post fixation was done in 1 % (w/v) OsO_4 and 2.5 % (w/v) $K_2Cr_2O_7$ for 1 hour 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.

Results and discussion

Reduction of oxidized metals

Recently, we have isolated a tetrachloroethene respiring organism that phylogenetically groups in the *Sulfurospirillum* clade. This organism, *Sulfurospirillum halorespirans* PCE-M2, is also able to reduce oxidized metals like arsenate and selenate (Luijten *et al.*, 2003). To study the relationship between respiration with chlorinated ethenes and oxidized metals we screened a number of halorespiring and related organisms for their capacity to reduce metals.

The results show that the ability to metabolically reduce metals is a common feature among different halorespiring microorganisms of the genera *Desulfitobacterium* and *Sulfurospirillum* (Table 1). The growth of *Desulfitobacterium* strain PCE1 in medium with selenate as electron acceptor and lactate as electron donor is shown in Figure 1. In all positive experiments for selenate reduction a red precipitate was formed indicating at least partial reduction to elemental selenium.

In contrast to the results obtained for the reduction of selenate, only one of the *Desulfitobacterium* species tested, *Desulfitobacterium hafniense* TCE1, was able to reduce arsenate (Table 1). Three other *Desulfitobacterium* isolates (strain GBFH, *D.*

hafniense and *D. frappieri*) have been reported positive for arsenate reduction as well (Niggemeyer *et al.*, 2001). During arsenate reduction yellow precipitates were formed, presumably orpiment (As_2S_3). Reduction of arsenate was also observed for all tested *Sulfurospirillum* species (Table 1).

Table 1: Utilization of oxidized metals as terminal electron acceptor by halorespiring and related organisms. ++ = Significant reduction within 2 weeks; + = significant reduction within one month; - = no significant reduction within one month.

	Se(VI)	As(V)	Fe(III)	Mn(IV)
Desulfitobacterium dehalogenans	++	-	-	-
Desulfitobacterium strain PCE1	++	-	-	-
Desulfitobacterium chlororespirans Co23	++	-	+	++
Desulfitobacterium hafniense DP7	++	-	+/-[1]	++
Desulfitobacterium hafniense TCE1	++	++	+	++
Sulfurospirillum deleyianum	-	++	-	++
Sulfurospirillum barnesii SES3	+	++	-	+
Sulfurospirillum arsenophilum	+	++	-	+
Sulfurospirillum halorespirans PCE-M2	+	++	+/-[1]	+
Sulfurospirillum multivorans	+	++	+/-[1]	-

[1]: Fe(III) reduction did occur in initial incubations. The iron reducing activity was lost in sequential transfers.

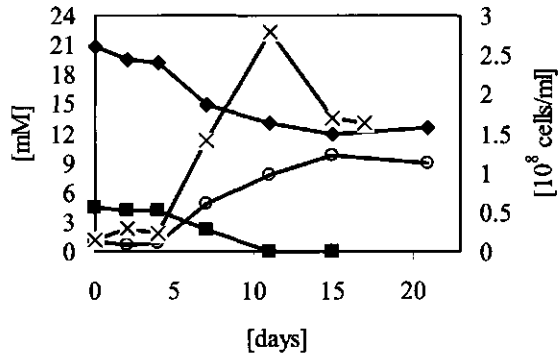

Figure 1: The reduction of selenate by *Desulfitobacterium* strain PCE1.
x = Cell number, ■ = selenate; ♦ = lactate; o = acetate.

Figure 2 shows a TEM picture of *Sulfurospirillum halorespirans* PCE-M2 grown on arsenate and lactate, where the accumulation of precipitates, presumably orpiment, around the cells can be seen.

Desulfitobacterium hafniense TCE1, *D. hafniense* DP7 (Figure 3) as well as *D. chlororespirans* Co23 reduced δMnO_2 (Table 1). Growth on and reduction of δMnO_2

was accompanied by the production of white precipitates that are likely to be $MnCO_3$. *Desulfitobacterium* strain PCE1, *D. dehalogenans* and *Sulfurospirillum multivorans* did not reduce manganese. All other *Sulfurospirillum* species did reduce manganese, albeit at a lower rate than the tested *Desulfitobacterium* species except for *S. deleyianum*. This bacterium reduced manganese at a rate comparable to that of *Desulfitobacterium* species.

Desulfitobacterium hafniense TCE1 and *D. chlororespirans* Co23 reduced iron that was added as Fe(III)citrate (Table 1). *D. hafniense* DP7, *Sulfurospirillum halorespirans* PCE-M2 and *S. multivorans* were in initial incubations also able to reduce iron, but they lost this activity after transfer to following incubations.

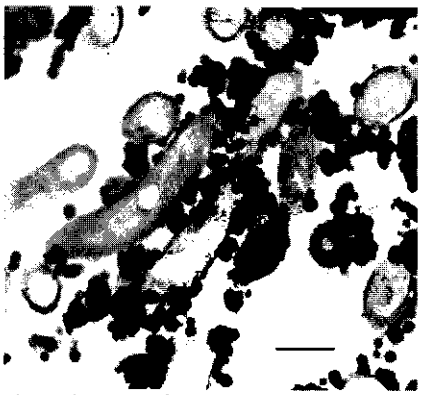

Figure 2: TEM picture from *Sulfurospirillum halorespirans* PCE-M2 grown on arsenate and lactate. Accumulation of precipitates, presumably orpiment, around the cells can be seen. Magnification is 17000. Bar represents 1 µm.

All screening experiments were performed with either lactate or pyruvate as electron donor, substrates that may generate ATP via substrate level phosphorylation. To demonstrate energy conservation and growth via electron transport phosphorylation, several organisms were incubated and sub-cultured with molecular hydrogen as electron donor. Both *Desulfitobacterium hafniense* TCE1 and *D. hafniense* DP7 were able to grow with δMnO_2 and H_2. The growth of *Sulfurospirillum deleyianum* and *Sulfurospirillum halorespirans* PCE-M2 was tested with H_2 and As(V) and Se(VI),

respectively. For all tested combinations, growth and thus energy conservation via electron transport phosphorylation took place.

Recently, Niggemeyer *et al.* (2001) have reported the isolation of a new non-halorespiring *Desulfitobacterium* species, strain GBFH, able to reduce arsenate metabolically. These authors found that *Desulfitobacterium hafniense* DCB-2 (DSM 10664), *D. hafniense* PCP-1 (DSM 12420), *D. dehalogenans* JW/IU-DC1 (DSM 9161) and *D. chlororespirans* Co23 (DSM 11544) are able to reduce different oxidized metals as well. Their results are in agreement with ours, showing that the reduction of metals is a common property of many (halorespiring) bacteria of the genera *Desulfitobacterium* and *Sulfurospirillum*.

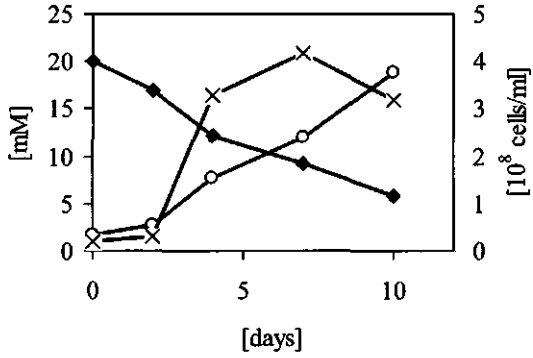

Figure 3: The reduction of manganese by *Desulfitobacterium hafniense* DP7. x = Cell number; ♦ = lactate; o = acetate.

Nevertheless, data reported in literature show some contradicting results compared to our findings. *S. arsenophilum*, which was tested positive in our experiments on selenate, was tested negative in experiments by Stolz *et al.* (1999). Arsenate reduction by *S. deleyianum* was reported to be negative (Stolz *et al.*, 1999) while *D. dehalogenans* was found to reduce manganese (Niggemeyer *et al.*, 2001). These results are in contrast to ours. These differences may be caused by adaptation of the bacteria to certain growth conditions, which may influence the outcome of screening experiments. A possible loss of genes encoding specific processes may cause these differences as well (Stotzky and Babich, 1986; Van de Meer *et al.*, 1987). Contradicting results for iron reduction have also been reported for

Desulfitobacterium hafniense TCE1 (negative) (Gerritse *et al.*, 1999) and for *Sulfurospirillum barnesii* SES3 (positive) (Laverman *et al.*, 1995). In experiments studying iron reduction the chelating agent used, citrate in our experiments, may also influence the results obtained.

Reduction and oxidation of quinone moieties

Until recently, humic substances were thought to be inert to microbial conversion. However, Lovley *et al.* (1996) showed that anaerobic microorganisms are able to use humic substances as electron acceptor. Recently, three *Desulfitobacterium* species (*D. dehalogenans*, *D.* strain PCE1 and *D. metallireducens*) were reported to couple the reduction of humic acids and/or AQDS to energy conservation (Finneran *et al.*, 2002; Cervantes *et al.*, 2002). Anthraquinone-2,6-disulfonate (AQDS) is often used in lab scale experiments to mimic the quinone moieties of humic substances (Lovley *et al.*, 1999). Lovley *et al.* (1999) showed that AQDS is a suitable model compound for natural occurring, quinone containing humic substances. Our more extended screening of nine halorespiring and related bacteria for their ability to reduce AQDS showed that all tested microorganisms are able to use the quinone moiety in AQDS as terminal electron acceptor. This indicates that this may be a widespread feature amongst (halorespiring) bacteria of the genera *Desulfitobacterium* and *Sulfurospirillum* (Table 2). AQDS reduction could be sustained for at least two transfers by the tested microorganisms, whereas in different control experiments (without cells or electron donor) no reduction occurred.

Table 2: The reduction and oxidation of $A(H_2)QDS$ by halorespiring and related organisms.
+ = Significant reduction/oxidation within one month; - = no significant reduction/oxidation within one month.

	AQDS reduction	AH_2QDS oxidation
Desulfitobacterium dehalogenans	+	-
Desulfitobacterium strain PCE1	+	-
Desulfitobacterium chlororespirans Co23	+	-
Desulfitobacterium hafniense DP7	+	+
Sulfurospirillum deleyianum	+	+
Sulfurospirillum barnesii SES3	+	+
Sulfurospirillum arsenophilum	+	+
Sulfurospirillum halorespirans PCE-M2	+	-
Sulfurospirillum multivorans	+	-

The chemical oxidation of reduced anthrahydroquinone-2,6-disulfonate (AH_2QDS) coupled to the reduction of oxidized metals such as iron and manganese has been described by several researchers (Stone and Morgan, 1984; Lovley *et al.*, 1996; Field *et al.*, 2000). The biological oxidation of reduced AH_2QDS has been reported with fumarate or nitrate as electron acceptors (Lovley *et al.*, 1997). Lovley *et al.* (1997) have shown that *Sulfurospirillum barnesii* could oxidize AH_2QDS but that it was not able to couple this oxidation to growth. Our experiments show that both the oxidation of AH_2QDS and the reduction of AQDS can be performed by *Desulfitobacterium hafniense* DP7 and by several *Sulfurospirillum* species. We were able to sustain growth of all organisms marked positive in table 2, including *S. barnesii*, for at least two transfers in liquid medium.

Implication for in-situ bioremediation

Several of the studied bacteria are expected to be involved in the degradation of chlorinated pollutants at contaminated sites since they have been isolated from such sites. The potential of these bacteria to use oxidized metals and quinone moieties as electron acceptors instead of the (chlorinated) contaminants may therefore have negative effects on the degradation of chlorinated solvents. Sites that are polluted with chlorinated compounds may have an excess of these electron acceptors under anaerobic conditions. When the amount of electron donor present at such a polluted site is not sufficient, the addition of an electron donor is required to stimulate the bioremediation of these pollutants. Since the oxidized metals and AQDS will be reduced as well, they may consume part of the added electron donor. To calculate the theoretical/minimal amount of electron donor required, one has to take into account the amount of oxidized metals and AQDS as well as other possible electron acceptors present at the site. Only then one can obtain efficient and complete reductive dechlorination of the chlorinated compounds next to other possible (electron donor consuming) reducing processes.

The apparent widespread possibility of bacteria to reduce quinone moieties is important for the reduction of metals as discussed before. Oxidized metal species, that are abundant in soils, are poorly available for microorganisms because of their low solubility. The presence of reduced quinone moieties may lead to a chemical reduction of these iron species. Our results indicate that all tested halorespiring

bacteria are able to recycle quinone moieties and thus able to reduce oxidized iron species indirectly. This includes those bacteria that cannot reduce iron metabolically.

Occurrence at non-polluted sites

The ability of halorespiring bacteria of the genera *Desulfitobacterium* and *Sulfurospirillum* to use chlorinated compounds and oxidized metals as electron acceptor sheds new light on the presence of these bacteria in the environment. Until recently, halorespiring bacteria were mainly found at sites polluted with chlorinated ethenes. The use of molecular techniques has also demonstrated the presence of halorespiring microorganisms at a variety of habitats including those that apparently do not contain chlorinated compounds (Löffler *et al.*, 2000; Lanthier *et al.*, 2001). Löffler *et al.* (2000) have speculated that the presence of halorespiring bacteria at sites not polluted with chlorinated compounds by human activity may be the result of biotic and/or a-biotic production of chlorinated compounds. However, the characteristic of several halorespiring bacteria to reduce oxidized metals, such as iron, manganese, selenate and arsenate as well as to reduce and oxidize quinone moieties is an additional and maybe a more likely explanation for the occurrence of halorespiring bacteria in non-polluted soils.

In this view, it is very interesting to test halorespiring species of other genera such as *Dehalobacter* and *Dehalococcoides* for their ability to reduce metals and quinones. To date only a limited number of electron acceptors for these microorganisms is known. A possible ability of these bacteria to use other electron acceptors can help us to understand their niche in natural habitats and can facilitate studying these bacteria.

Acknowledgements

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3

Electron acceptor utilization by halorespiring *Sulfurospirillum* species

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Abstract

Most halorespiring bacteria are able to use a wide variety of compounds as electron acceptors. Not only chlorinated compounds, but also nitrate, sulphate, oxidized metals and humic acids are reduced. Here, we report on the usage of mixtures of electron acceptors by *Sulfurospirillum* species.

Nitrate, which is reduced to ammonium by *S. halorespirans*, is reduced preferentially before PCE by this organism. After reduction of nitrate and the intermediate nitrite, PCE reduction by this bacterium recovers. In contrast, the closely related *S. multivorans* only reduces nitrate to nitrite. When nitrate is added to PCE reducing batches, PCE reduction ceases and does not recover. Sulphate does not influence the dechlorination by *Sulfurospirillum* species, but sulphite inhibits the dechlorination via a reversible chemical inhibition.

Also, the physiological properties of *S. halorespirans* adapted to certain electron acceptors was tested. It appears that PCE is only reduced by cells pre-grown on PCE. The same was found for the reduction of selenate. Arsenate and nitrate, however, are reduced by cells pre-grown on all substrates tested (PCE, selenate, arsenate and nitrate). This indicates that the arsenate and nitrate reducing enzymes are constitutively present.

Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are major soil and groundwater pollutants, which can be degraded under anaerobic conditions via the process of reductive dechlorination (Vogel and McCarty, 1985; Middeldorp *et al.*, 1999; Holliger *et al.*, 1999). Nowadays, this reductive process is often used in *in-situ* bioremediation projects. By the addition of an appropriate electron donor the indigenous dechlorinating bacteria present at the polluted site are stimulated (Middeldorp *et al.*, 2002).

A number of halorespiring bacteria belonging to different genera that can couple the (partial) reduction of PCE to growth, has been isolated (Middeldorp *et al.*, 1999; Holliger *et al.*, 1999; Luijten *et al.*, 2003; Sung *et al.*, 2003). All halorespiring bacteria, except *Dehalobacter restrictus* (Holliger *et al.*, 1993; Wild *et al.*, 1996; Holliger *et al.*, 1998) can reduce chlorinated alkenes, as well as other oxidized compounds like nitrate, sulphite, chlorinated aromatics and oxidized metals (Holliger *et al.*, 1999; Middeldorp *et al.*, 1999; Niggemeyer *et al.*, 2001; Luijten *et al.*, 2003; Luijten *et al.*, 2004).

Sites polluted with chlorinated ethenes may contain other oxidized compounds as well. The presence of nitrate and/or sulphate, but also the presence of metals like iron, manganese, arsenate or selenate may affect the anaerobic reductive dechlorination at polluted sites. Competition for the available electron donor between halorespiring and other, e.g. denitrifying or sulphate-reducing bacteria may occur. Since most halorespiring bacteria are able to use different terminal electron acceptors, competition for the available electron donor may also occur within these bacteria. Therefore, high concentrations of nitrate or sulphate at a particular site will require the addition of an excess of electron donor to efficiently stimulate the dechlorination.

To effectively enhance biodegradation at polluted sites using halorespiring bacteria, one has to understand the influence of different alternative electron acceptors on the dechlorinating capacity of these bacteria. Here, we report on the effect of nitrate and oxidized sulphur oxyanions on the reductive dechlorination of PCE by halorespiring species of the genera *Sulfurospirillum* and *Desulfitobacterium*. Furthermore, we

present data about the adaptation of *Sulfurospirillum halorespirans* PCE-M2 to different electron acceptors.

Material and Methods

Organisms

Sulfurospirillum halorespirans PCE-M2 (DSM 13726 = ATCC BAA-583) was isolated at the Laboratory of Microbiology from a soil polluted with chlorinated ethenes (Luijten *et al.*, 2003). *Sulfurospirillum multivorans* (DSM 12446), formerly known as *Dehalospirillum multivorans*, was obtained from the DSMZ (Braunschweig, Germany). *Desulfitobacterium* sp. PCE1 (DSM 10344) was a kind gift from J. Gerritse (University of Groningen, Groningen, The Netherlands).

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 electron donors were added from anaerobic, aqueous concentrated stock solutions. Tetrachloroethene was added from a concentrated (1 M) stock solution in hexadecane. Yeast extract (1 g/l) was routinely added in experiments with *Desulfitobacterium* species. Incubations were carried out in 117-ml serum bottles containing 20 ml anaerobic medium. The headspace consisted of N_2/CO_2 (80/20 v/v). For the adaptation experiments, cells were pre-grown for at least two generations on the studied electron acceptor (10 mM) (PCE, arsenate, selenate or nitrate). Lactate (29 mM) was used as electron donor.

Analytical techniques

Chloride anion concentrations were determined with a Micro-chlor-o-counter (Marius, Utrecht, The Netherlands) as described before (Luijten *et al.*, 2003). Volatile fatty acids and inorganic anions were determined by high-pressure liquid chromatography and ion chromatography, respectively, as described by Scholten and Stams (1995).

Results and Discussion

Effect of nitrate

Sulfurospirillum halorespirans strain PCE-M2 reduces nitrate stoichiometrically to ammonium in the presence of a proper electron donor (Luijten *et al.*, 2003). In the presence of lactate as electron donor, nitrate is preferentially used instead of PCE. When strain PCE-M2, pre-grown on PCE, is incubated with a mixture of PCE (10 mM) and nitrate (5 mM), a delay of the PCE reduction occurs until all nitrate (and the intermediate nitrite) is reduced (Figure 1). The same preferential reduction of nitrate before PCE occurs when nitrate is added to actively dechlorinating cultures of *Sulfurospirillum halorespirans* strain PCE-M2. Dechlorination stops within a few hours after the addition of nitrate and only resumes after nitrate and nitrite have disappeared.

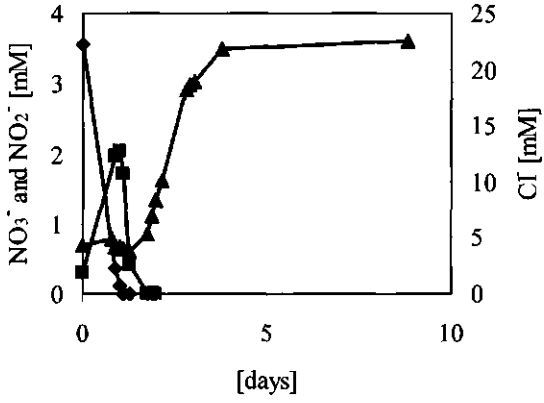

Figure 1: The reduction of nitrate and PCE, both present before incubation, by *Sulfurospirillum halorespirans* strain PCE-M2. (♦) = Nitrate; (■) = nitrite; (▲) = chloride.

Sulfurospirillum multivorans reduces nitrate mainly to nitrite (Scholz-Muramatsu *et al.*, 1995). This halorespiring bacterium does not reduce PCE when prior to incubation both nitrate and PCE are present. After addition of nitrate to an actively dechlorinating culture, PCE reduction ceases and *S. multivorans* only reduces the available nitrate stoichiometrically to nitrite (Figure 2). The PCE reduction by *S. multivorans* does not recover in these experiments.

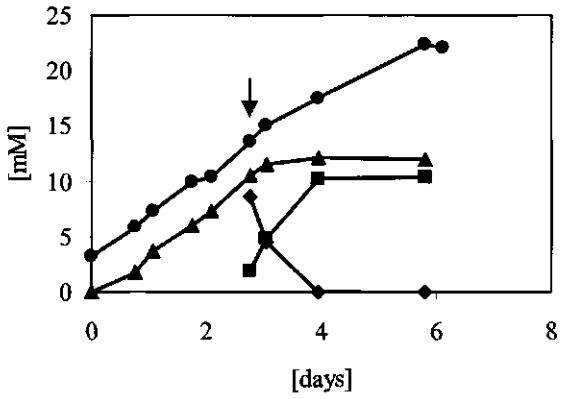

Figure 2: The reduction of PCE by *Sulfurospirillum multivorans*. After 2.8 days, nitrate (10 mM) was added. (♦) = Nitrate; (■) = nitrite; (▲) = chloride; (●) = chloride in control experiment without nitrate addition.

Dechlorination of PCE by *Desulfitobacterium* sp. PCE1 is not inhibited by the presence of up to 10 mM nitrate (data not shown). This bacterium is not able to reduce nitrate (Gerritse *et al.*, 1996). *Desulfitobacterium frappieri* strain TCE1, a bacterium that does reduce nitrate, was not inhibited by the presence of 2 mM nitrate (Gerritse *et al.*, 1999). Dechlorination and nitrate reduction occurred simultaneously in their chemostat studies when an electron donor was present in excess. Under electron donor limitation, the dechlorination was completely blocked. Towsend and Suflita (1997) found that dechlorination of 3-chlorobenzoate by *Desulfomonile tiedjei* was not inhibited by nitrate, and that nitrate is not used for respiration by *D. tiedjei*. Van de Pas (2000) reported that nitrate did inhibit the 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) reduction in cell extracts but not in cell suspensions of *Desulfitobacterium*

dehalogenans, an bacterium that is able to reduce nitrate. Actually, *D. dehalogenans* reduced nitrate and Cl-OHPA simultaneously (Mackiewicz and Wiegel, 1998; Van de Pas, 2000).

Effect of sulphur oxyanions

Sulphate is often found at sites polluted with chlorinated compounds (Middeldorp *et al.*, 2002). Reductive dechlorination is possible under sulphate-reducing conditions (Bagley and Gossett, 1990), but most known halorespiring bacteria are not able to use sulphate as terminal electron acceptor (Van de Pas, 2000), except for some species belonging to the δ-subdivision of the *Proteobacteria*. Therefore, different groups of microorganisms will be active under these conditions. We tested the influence of sulphate on the reductive dechlorination of PCE by *Sulfurospirillum halorespirans* strain PCE-M2. Sulphate concentrations up to 20 mM had no effect on the PCE (or nitrate) reducing activity of strain PCE-M2. The reductive dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. PCE1 was also not influenced by the presence of sulphate. Sulphate itself was not used as an electron acceptor by these bacteria, which may explain why sulphate has no effect on the PCE reduction. There was no inhibition of the dechlorination when sulphate was already present before inoculation nor when sulphate was added to actively dechlorinating cultures. Towsend and Suflita (1997) found that sulphate does inhibit the reduction of 3-chlorobenzoate by the sulphate-reducing *Desulfomonile tiedjei* under growth conditions but not under non-growth conditions.

Sulphite is a possible terminal electron acceptor for many halorespiring bacteria but not for *Sulfurospirillum* species. Sulphite (1 mM and higher) completely inhibited the dechlorination of PCE by both *Sulfurospirillum halorespirans* strain PCE-M2 and *S. multivorans*. Also, the reductive dehalogenase of *Desulfitobacterium chlororespirans* Co23 was strongly inhibited by sulphite (Löffler *et al.*, 1996). Studies on purified tetrachloroethene reductive dehalogenases showed that these are cobalamine containing enzymes (Miller *et al.*, 1998; Magnuson *et al.*, 1998; Van de Pas, 2000; Smidt, 2001). Sulphite chemically reacts with cobalamine, thus affecting the reductive dehalogenase, which may explain the inhibition of the reductive dechlorination (Miller *et al.*, 1997). Gerritse *et al.* (1999) showed in studies with *Desulfitobacterium frappieri* strain TCE1, a sulphite-reducing bacterium, that sulphite indeed inhibited its

dechlorination. Dechlorination was restored after reduction of sulphite, indicating to a reversible chemical inhibition.

Adaptation of Sulfurospirillum halorespirans

Most halorespiring bacteria are able to use a broad range of electron acceptors. We investigated the physiological properties of *Sulfurospirillum halorespirans* PCE-M2 adapted to various electron acceptors. Cells of strain PCE-M2 were pre-grown on several electron acceptors (PCE, nitrate, selenate and arsenate) and then transferred to incubations with alternative electron acceptors. Table 1 shows that only cells pre-grown on PCE are able to reduce PCE. The same was observed for the reduction of selenate. By contrast, arsenate and nitrate are reduced independently of the electron acceptor used to pre-grown the cells (Table 1). This indicates that the enzymes for the reduction of nitrate and arsenate are constitutively present in *Sulfurospirillum halorespirans*.

Table 1: Adaptation of *Sulfurospirillum halorespirans* PCE-M2 to alternative electron acceptors. ++ = Complete reduction within 1 week; + = complete reduction within 2 weeks; - = no reduction within 1 month.

	PCE	nitrate	arsenate	selenate
PCE pre-grown	++	++	+	-
Nitrate pre-grown	-	++	++	-
Arsenate pre-grown	-	+	+	-
Selenate pre-grown	-	++	++	+

Conclusion

Halorespiring bacteria have been found in soils that are not polluted with chlorinated compounds (Löffler *et al.*, 2000; Lanthier *et al.*, 2001). Recently it was found that most halorespiring bacteria can use a broad spectrum of electron acceptors (Niggemeyer *et al.*, 2001; Finneran *et al.*, 2002; Luijten *et al.*, 2004). This may explain the occurrence of these halorespiring bacteria in unpolluted environments. We showed that *Sulfurospirillum halorespirans* reduces PCE only when adapted to this substrate. Cells pre-grown on other electron acceptors (nitrate, arsenate, selenate)

seem to have lost their dechlorinating capacity. This indicates that the presence of halorespiring bacteria in a polluted soil does not necessarily mean that they grow via halorespiration. Still, at many sites, indigenous bacteria that are actively respiring chlorinated compounds, make it interesting to learn more about the evolutionary aspects of halorespiring bacteria and their dehalogenases.

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4

Hydrogen threshold concentrations in pure cultures of halorespiring bacteria and at a site polluted with chlorinated ethenes

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Abstract

Halorespiring bacteria are able to oxidize organic electron donors such as formate, acetate, pyruvate and lactate, but also H_2. Since these bacteria have a high affinity for H_2, this may be the most important electron donor for halorespiration in the environment. We have studied the role of H_2-threshold concentrations in pure halorespiring cultures and compared them with mixed cultures and field data. We have found H_2-threshold values between 0.05 and 0.08 nM for *Sulfurospirillum halorespirans*, *S. multivorans* and *Dehalobacter restrictus* under PCE and nitrate reducing conditions.

The reduction of PCE and TCE can proceed at H_2 concentrations below 1 nM at a polluted site. However, for the reduction of lower chlorinated ethenes a higher H_2 concentration is required. This indicates that the measured H_2 concentration *in-situ* can be an indicator of the extend of anaerobic reductive dechlorination.

Introduction

Over the last decade halorespiring bacteria were studied extensively. This has resulted in a better understanding of the degradation of toxic chlorinated ethenes. For bioremediation purposes this knowledge opened the possibility to effectively stimulate the *in-situ* clean-up of polluted soils by using halorespiring bacteria. To optimize this process research was done to understand the enhancement of reductive dechlorination by halorespiring bacteria in soils and their competition with other microorganisms for available electron donors. Organic matter is degraded under anaerobic conditions to small organic molecules and H_2 by various fermenting bacteria. The H_2 and lower fatty acids produced can be oxidized and coupled to the reduction of different terminal electron acceptors like nitrate, ferric iron, sulphate or CO_2 by respiring bacteria. Halorespiring bacteria have been found to oxidize organic electron donors such as formate, acetate, pyruvate and lactate, but also H_2. Several authors speculate that H_2 is the most important electron donor for halorespiration in the environment (Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Ballapragada *et al.*, 1997; Löffler *et al.*, 1999; Mazur and Jones, 2001). A high affinity for H_2 results in a low H_2-threshold, a value that represents the minimal H_2 concentration that can be consumed under defined reducing conditions by a bacterium (Lovley and Goodwin, 1988; Löffler *et al.*, 1999). Table 1 lists H_2-threshold concentrations for different metabolic processes reported in literature. The data for the dechlorination processes have been measured in sediment samples. Löffler and co-workers (1999) reported H_2-thresholds of below 0.3 nM for different *Desulfitobacterium* species reducing either tetrachloroethene (PCE), 3-Cl-4-hydroxybenzoate or 2-chlorophenol. To our knowledge, these data are the only data known for pure cultures of halorespiring bacteria.

In this paper, we present H_2-threshold values for different reducing processes from laboratory batch studies with different halorespiring bacterial strains. We compare these results with H_2 concentrations and dechlorination efficiency from a soil polluted with chlorinated ethenes during an active *in-situ* bioremediation.

Table 1: H_2-threshold concentrations for different processes.

Process	H_2-threshold [nM]	References
Acetogenesis	>300	1, 2
Methanogenesis	5-100	1, 2, 3, 4, 5, 6
Sulphate reduction	1-10	1, 2, 3, 4, 6
Iron reduction	0.1-0.8	1, 3, 4, 6
Manganese reduction	<0.05	6
Ammonification	0.015-0.025	1, 2
Nitrate reduction	<0.05	1, 6
Halorespiration*	<0.3	1, this study
PCE & TCE reduction**	0.6-0.9	4
Cis-1,2-DCE reduction**	0.1-2.5	4, 5
VC reduction**	2-24	4, 5

* = Pure culture studies; ** = mixed culture/field studies.
References: 1 = Löffler *et al.*, 1999; 2 = Cord-Ruwisch *et al.*, 1988; 3 = Chapelle *et al.*, 1996; 4 = Lu *et al.*, 2001; 5 = Yang and McCarty, 1998; 6 = Lovley and Goodwin, 1988.

Materials and methods

Organisms

Sulfurospirillum halorespirans PCE-M2 (DSM 13726; ATCC BAA-583) was isolated in the Laboratory of Microbiology (Luijten *et al.*, 2003). *Sulfurospirillum multivorans* (DSM 12446) (formerly known as *Dehalospirillum multivorans*), *Dehalobacter restrictus* (DSM 9455) and *Desulfovibrio* strain G11 (DSM 7057) were obtained from the DSMZ, Braunschweig, Germany.

Set-up H_2-threshold experiments

The threshold concentrations for H_2 were determined in batch experiments at 30 °C using an anaerobic phosphate-bicarbonate buffered medium with a low chloride concentration (Holliger *et al.*, 1993). Initially, the bacteria were pre-grown with an excess of H_2 present in the headspace. After growth of the organisms, the headspace was changed under sterile conditions from H_2/CO_2 to N_2/CO_2 to remove the remaining H_2. To avoid limitation during the experiment, the electron acceptor (10 mM) was re-added to the medium. Finally, 2 ml of pure H_2 was added to restart and the hydrogen concentration was followed in time. This experimental set-up was chosen to allow accurate measurements at low H_2 concentrations.

Field data

The Rademarkt site, located in the center of the city of Groningen in the north of The Netherlands, is polluted with PCE and trichloroethene (TCE). The site is characterized by mixed (iron- and sulphate reducing) redox conditions. Intrinsic reductive degradation takes place as evidenced by the detection of degradation products (*cis*-1,2-dichloroethene (*cis*-1,2-DCE) and vinyl chloride (VC)). However, the intrinsic degradation rates were too low to prevent spreading of PCE and TCE. The *in-situ* microbiological degradation of PCE and TCE is stimulated at this site at a pilot of 20 by 50 meter via the addition of electron donors in an infiltration, extraction and recirculation system. The pilot system, located in the source zone, consists of 10 injection wells (5 m below surface level) in a row on one site of the pilot and a row of 5 extraction wells situated at the other site. Part of the extracted groundwater is reinfiltrated via the infiltration wells, and a mixture of electron donors (methanol and compost extract) and NH_4Cl is added simultaneously. By circulating part of the extracted groundwater without purification, the soil is used as a bioreactor. This circulation system is located perpendicular to the groundwater flow (20 m/yr). Furthermore, a large number of monitoring wells has been installed. At the Rademarkt site various parameters have been monitored including the H_2 and chlorinated ethenes concentrations.

Analytical techniques

Inorganic anions were measured after separation on a dionex column as described by Scholten and Stams (1995). H_2 was analyzed on a RGA3 reduction gas analyzer equipped with a 60/80 unibeads pre-column and a 60/80 molecular sieve 5A column. The detector and column temperature were 265 °C and 105 °C, respectively. The detection limit was approximately 0.015 nM H_2.

Determination of the H_2 concentration is done at the Rademarkt site according to the "bubble strip" method (Chapelle *et al.*, 1996).

Results

H_2-threshold concentrations in pure cultures

We determined the H_2-threshold concentration of *Sulfurospirillum halorespirans* PCE-M2 with PCE as electron acceptor (Figure 1). After two months the H_2 concentration had slowly leveled off to approximately 0.05 nM H_2. The addition of yeast extract, a possible source of H_2, did not alter the H_2-threshold concentration of 0.05 nM (Table 2).

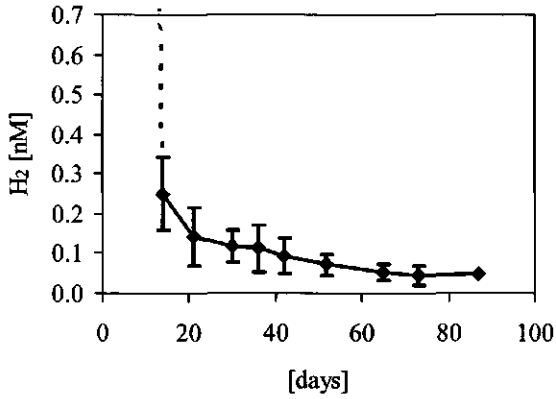

Figure 1: The H_2 concentration versus time for *Sulfurospirillum halorespirans* PCE-M2 incubated with PCE as electron acceptor in the presence of yeast extract.

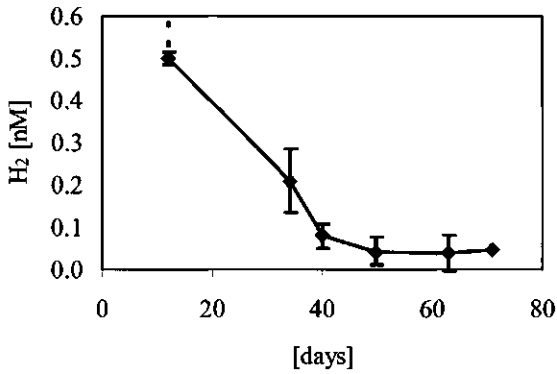

Figure 2: The H_2 concentration versus time for *Sulfurospirillum halorespirans* PCE-M2 incubated with nitrate as electron acceptor in the presence of yeast extract.

Table 2: H_2-threshold concentrations determined in this study. n = The number of batches of which the reported threshold is the average value. *S.* = *Sulfurospirillum*, y.e. = yeast extract.

Organism	Electron acceptor	H_2-threshold [nM]	n
S. halorespirans PCE-M2	PCE	0.05 ± 0.01	4
	PCE + y.e.	0.05 ± 0.02	4
	nitrate	0.04 ± 0.02	4
	nitrate + y.e.	0.04 ± 0.03	5
	nitrate + hexadecane	0.05 ± 0.01	4
S. multivorans	PCE	0.08 ± 0.04	5
	nitrate	0.06 ± 0.03	5
Dehalobacter restrictus	PCE	0.06	1
Desulfovibrio G11	sulphate	1.76 ± 0.15	5

The H_2-threshold concentration under nitrate reducing conditions is comparable to the H_2-threshold concentration under halorespiring conditions and also independent of the addition of yeast extract (Figure 2; Table 2). Tetrachloroethene was added from a concentrated solution dissolved in hexadecane. Since hexadecane was not added in incubations with nitrate, we confirmed that hexadecane did not affect the H_2-threshold values for incubations with nitrate (Table 2).

The H_2-threshold concentrations for *Sulfurospirillum multivorans* and *Dehalobacter restrictus* were comparable to those of *Sulfurospirillum halorespirans* PCE-M2 (Table 2). All halorespiring bacteria studied are able to reduce PCE to *cis*-1,2-DCE.

To validate our H_2-threshold concentration method against literature data, we included a sulphate reducing microorganisms, *Desulfovibrio* G11. We measured a H_2-threshold of 1.8 nM, which is in agreement with literature data for sulphate reducing bacteria (Cord-Ruwisch *et al.*, 1988; Chapelle *et al.*, 1996).

Relation between dechlorination and hydrogen concentrations at a field site

The Rademarkt site has been studied intensively during active stimulation of in-situ bioremediation. Figure 3 shows both the H_2 concentration and the molar ratio of (chlorinated) ethenes in two monitoring wells at the Rademarkt site. In well 11, the H_2 concentration was below 1 nM during the pilot test, and accumulation of the partial dechlorinated intermediate *cis*-1,2-DCE occurred. This monitoring well is located in between the infiltration and extraction wells. We concluded that the infiltration well

near monitoring well 11 was not functioning properly, because only a small part of the added tracer (bromide) in the infiltration solution reached monitoring well 11 compared to the amount of tracer in other monitoring wells. This also means that only a part of the added electron donor had reached monitoring well 11. Well 15 is located less than one meter downstream from an infiltration well, and had a 10 times higher H_2 concentration than well 11. In well 15, a complete sequential dechlorination to the non-chlorinated ethene had occurred.

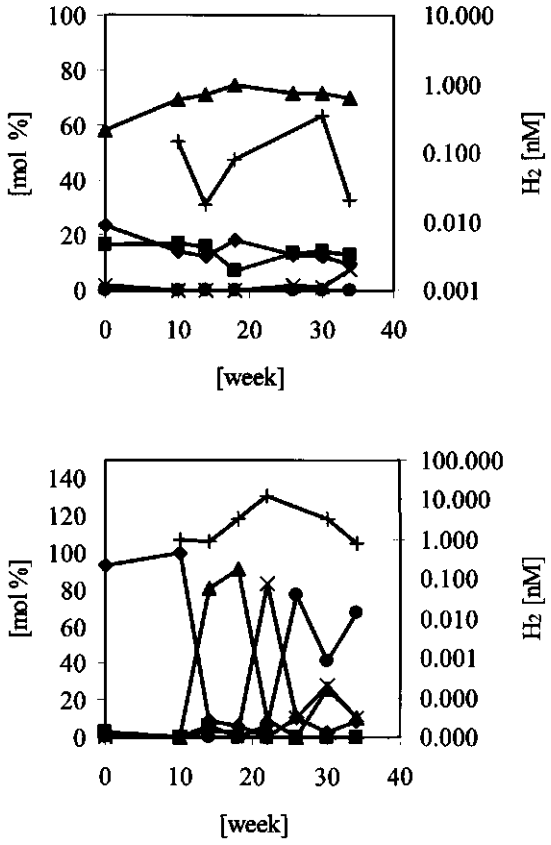

Figure 3: The H_2 concentration and molar ration of (chlorinated) ethenes measured over time in well 11 (upper graph) and well 15 (lower graph) at the Rademarkt site. Please note the scales on the different axes. (♦) = PCE; (■) = TCE; (▲) = *cis*-1,2-DCE; (x) = VC; (●) = ethene, and (+) = H_2.

Discussion

Halorespiring bacteria are able to use H_2 and a number of organic compounds such as formate, acetate, pyruvate and lactate as electron donor (Holliger *et al.*, 1999; Middeldorp *et al.*, 1999; Sung *et al.*, 2003; He *et al.*, 2003). Several authors have concluded that H_2 may be the most important electron donor for the process of anaerobic reductive dechlorination in soil (DiStefano *et al.*, 1992; Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Mazur and Jones, 2001). For the halorespiration process, Gibbs Free energies are still exergonic at very low H_2 concentrations (Löffler *et al.*, 1999; Mazur and Jones, 2001). Therefore, halorespiring bacteria can outcompete methanogenic archaea, acetogenic bacteria, and sulphate-reducing bacteria at low H_2 concentrations. However, they have to compete for the available H_2 with for example nitrate- and iron-reducing bacteria. These bacteria are also able to use H_2 at low concentrations (Table 1).

Several studies have been done to determine H_2 concentrations in mixed batch cultures under dechlorinating conditions with polluted sediments as inocula. These studies show that the reductive dechlorination can proceed at H_2 concentrations lower than 20 nM (Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Yang and McCarty, 1998; Lu *et al.*, 2001). The H_2 concentrations were the result of ongoing H_2-producing (organic matter fermentation) and H_2-consuming (halorespiration) processes. However, the H_2-threshold concentration for individual halorespiring strains is much lower than the values found in the previously mentioned batch studies with mixed cultures. The H_2-threshold values for *Sulfurospirillum halorespirans*, *S. multivorans* and *Dehalobacter restrictus* under PCE reducing conditions were between 0.05 and 0.08 nM (Table 2). These values are in agreement with the H_2-threshold data of below 0.3 nM reported by Löffler *et al.* (1999) for *Desulfitobacterium* species. We also found that the H_2-threshold concentrations for different reducing processes within halorespiring microorganisms are comparable (Table 2). This, together with the exergonic Gibbs Free energies, confirms that these halorespiring bacteria may have to compete with other anaerobic, respiring bacteria for the available H_2.

At sites polluted with chlorinated ethenes, accumulation of lower chlorinated ethenes has often been encountered (DiStefano, 1999; Bradley, 2000; Mazur and Jones, 2001;

Villarante *et al.*, 2001). The actual H_2 concentration may play an important role in this accumulation. The Rademarkt site data show that lower H_2 concentrations are often accompanied by a partial dechlorination whereas at higher H_2 concentrations often a complete dechlorination is observed. In batch studies, comparable results were obtained by Lu *et al.* (2001). They reported the requirement of a higher (up to 24 nM) H_2 concentration for the reduction of lower chlorinated ethenes compared to the reduction of PCE to TCE (Table 1). In conclusion, the reduction of PCE to *cis*-1,2-DCE can occur at low H_2 concentrations as demonstrated by our pure culture and field studies. However, a high(er) H_2 concentration is most likely required for the reduction of *cis*-1,2-DCE and VC as demonstrated by our field study. Thus, the measured (*in-situ*) H_2 concentration can be an indicator of the extent of anaerobic reductive dechlorination. This is in contrast with the conclusion of Löffler and co-workers (1999), who concluded that for complete and incomplete dechlorination similar H_2 concentrations occur in mixed culture experiments.

Unfortunately, no bacterial strains are available in commercial culture collections to study the reduction of lower chlorinated ethenes such as *cis*-1,2-DCE and VC. Such studies would result in a better understanding of the complete reductive dechlorination reactions during bioremediation processes at polluted sites.

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5

High rate anaerobic reductive dechlorination of *cis*-1,2-dichloroethene and vinyl chloride to ethene

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Abstract

Two bacterial enrichment cultures (DCE-1 and DCE-2) able to reduce *cis*-1,2-dichloroethene and vinyl chloride under anaerobic conditions have been obtained. Both hydrogen and formate support the stoichiometric production of ethene from *cis*-1,2-dichloroethene and vinyl chloride. The addition of (fermented) yeast extract is required to sustain dechlorination in both enrichments. Vinyl chloride is reduced at high rates (0.8 – 1.4 mmol/l/d) by both enrichments. High concentrations of vinyl chloride (up to 4.9 mM) are not toxic for the enrichments. *Cis*-1,2-dichloroethene is reduced at approximately 20-30 fold lower rates and lower concentrations appear to be inhibitory for both enrichments. Our results indicate that the dehalogenating organisms present in the enrichments gain energy from the reduction of vinyl chloride. The reduction of *cis*-1,2-dichloroethene seems to be the bottleneck in the complete reduction of higher chlorinated ethenes. The slow reduction of *cis*-1,2-dichloroethene cannot be explained theoretically by the hydrogen threshold concentration required for the reduction of *cis*-1,2-dichloroethene. The poor reduction of *cis*-1,2-dichloroethene by reduced cobalamine may explain why *cis*-1,2-dichloroethene dechlorination is the limiting step in reductive conversion of chlorinated ethenes.

Introduction

Chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) are produced in large quantities during a number of industrial activities. Due to (accidental) spills PCE and TCE are among the most abundant pollutants of soils and groundwater worldwide. PCE is microbiologically resistant under aerobic conditions but TCE and lower chlorinated ethenes can be oxidized co-metabolically. Anaerobically, PCE can undergo a microbial, stepwise reduction via TCE, *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and vinyl chloride (VC) to ethene (Lee *et al.*, 1998; El Fantroussi *et al.*, 1998; Middeldorp *et al.*, 1999) and sometimes ethane (De Bruin *et al.*, 1992). This reduction pathway has been observed at many anaerobic polluted sites and is attributed to the indigenous microorganisms. However, sequential dechlorination is often incomplete and results in concomitant accumulation of mainly *cis*-1,2-DCE and sometimes VC (see Wickramanayake *et al.*, (2000) for numerous examples). The same partial dechlorination pattern of PCE to either TCE or *cis*-1,2-DCE has been observed with pure cultures of bacteria able to use PCE as terminal electron acceptor (Holliger *et al.*, 1993; Scholz-Muramatsu *et al.*, 1995; Gerritse *et al.*, 1996; Lee *et al.*, 1998; El Fantroussi *et al.*, 1998; Middeldorp *et al.*, 1999; Holliger *et al.*, 1999). Only one, recently isolated, microorganism, "*Dehalococcoides ethenogenes*" strain 195, is able to dechlorinate PCE completely to ethene. In contrast to the reduction of PCE to VC is the final reduction step from VC to ethene not coupled to growth by this organism (Maymó-Gatell *et al.*, 1997; Maymó-Gatell *et al.*, 1999).

When we performed our studies, no organisms had been isolated that are able to couple the anaerobic reduction of *cis*-1,2-DCE and/or VC to microbial growth. However, recently He *et al.* (2003) isolated *Dehalococcoides* strain BAV1, which is able to couple the reduction of *cis*-1,2-DCE and VC to growth. Furthermore, a number of reports have been published, in which enrichment cultures that are able to convert lower chlorinated ethenes under anaerobic conditions are described. Anaerobic oxidation of *cis*-1,2-DCE and VC to CO_2 was reported under Fe(III)-reducing and sulphate-reducing conditions (Bradley and Chapelle, 1997; Bradley and Chapelle, 1998). Anaerobic reductive dechlorination of *cis*-1,2-DCE and/or VC to

ethene was reported to occur in mixed cultures (Komatsu *et al.*, 1994; Rosner *et al.*, 1997; Bradley and Chapelle, 1997; Bradley and Chapelle, 1998; Middeldorp *et al.*, 1999) and in cell extracts (Rossner *et al.*, 1997). In this paper we report the anaerobic reduction of VC at relatively high concentrations in enrichment cultures. Our results show conversion rates of VC at apparent metabolic rates, indicating that the responsible organisms are able to use lower chlorinated ethenes as terminal electron acceptors and derive energy for growth from these conversions.

Materials and Methods

Chemicals

Cis-1,2-DCE was obtained from Aldrich, Zwijndrecht, The Netherlands. Vinyl chloride (gaseous) and ethene were obtained from Hoekloos, Schiedam, The Netherlands.

Enrichment cultures

The dechlorination of lower chlorinated ethenes was carried out by two different anaerobic enrichment cultures.

The first culture, tentatively called DCE-1, originated from a fixed bed column reducing PCE to ethane. The original inoculum consisted of a mixture of anaerobic sludge and sediment from the river Rhine near Wageningen in The Netherlands (De Bruin *et al.*, 1992). Enrichment DCE-1 has been routinely transferred using VC as electron acceptor and molecular hydrogen as electron donor. Acetate was added as carbon source in these experiments.

The second enrichment, tentatively called DCE-2, originated from a laboratory anaerobic packed bed reactor, which was fed with PCE as electron acceptor and acetate as electron donor and carbon source. Complete anaerobic reduction of PCE to ethene took place in this reactor (Henssen *et al.*, 1997). A sample from this reactor was kindly provided by Bioclear Environmental Biotechnology BV, Groningen, The Netherlands.

Batch dechlorination assays

A phosphate-bicarbonate buffered medium with a low chloride concentration as described before was used (Holliger *et al.*, 1993). Incubations were performed in 117-ml serum bottles containing 20 ml anaerobic medium. The headspace consisted of N_2/CO_2 (80/20) or H_2/CO_2 (80/20); the latter when molecular hydrogen was used as electron donor. Acetate was added as carbon source when either molecular hydrogen or formate was used as electron donor. Electron donors and electron acceptors were added from aqueous concentrated sterile stock solutions, unless stated otherwise. VC was added as a gas through a sterile 0.2 µm filter. Yeast extract (1.25 g/l final concentration) or fermented yeast extract were added to the medium, unless otherwise stated. Yeast extract was fermented by adding anaerobic sludge and incubating at 30 °C for at least 2 weeks. All experiments were performed at 30 °C or 20 °C for enrichment DCE-1 and DCE-2, respectively.

For isolation purposes the roll tube method was used. The medium was solidified by adding 22 g/l agar noble (Difco, Detroit, MI, USA).

Analysis

All chlorinated ethenes, ethene, ethane and methane were determined in headspace samples by a 438A Chrompack Packard gas chromatograph. The gas chromatograph was equipped with a flame ionization detector (FID) connected to a capillary column (Poraplot Q, Chrompack, The Netherlands; 25 m by 0.32 mm inner diameter; df 10 µm; 100 kPa N_2) and a splitter injector (ratio 1:10). The injector and detector temperatures were 100 and 250 °C, respectively. For calculating the distribution between gas and liquid phase, dimensionless Henry's law constants for VC at 20 °C and 30 °C were extracted from literature values (Gossett, 1987). Unless otherwise stated, all reported concentrations are expressed as nominal concentrations, i.e., the gas-liquid distribution is ignored and the component is considered to be solely in the liquid phase.

Volatile fatty acids were determined by high-pressure liquid chromatography (HPLC) as described by Scholten and Stams (1995).

Results

DCE-1

Enrichment DCE-1, obtained from a lab-scale fixed bed column (De Bruin *et al.*, 1992), was enriched with H_2 as electron donor. This enrichment is able to reduce both *cis*-1,2-DCE and VC to ethene. Incubations were regularly started with a 1 % (v/v) inoculum. Dechlorination was achieved until 10^{-5} dilutions in liquid medium. In initial incubations, bromoethanesulphonate (Bres) had been added to inhibit methanogenic activity. After several transfers methane production ceases, even in the absence of Bres indicating the disappearance of methanogenic microorganisms. The addition of yeast extract or fermented yeast extract was necessary to maintain the dechlorinating activity of enrichment DCE-1. Neither peptone, acetate, nor a mixture of lower volatile fatty acids did support dechlorination and could therefore not replace the (fermented) yeast extract. The addition of filter sterile medium from full-grown incubations did not enhance dechlorination rates. Both molecular hydrogen and formate were suitable electron donors for the reduction of lower chlorinated ethenes. No acetate production was found during the experiments, indicating that (homo)acetogenic bacteria were absent in this culture. Figure 1 shows the stoichiometric reduction of VC to ethene by enrichment DCE-1. The maximum VC reduction rate observed in this experiment was 0.97 mmol/l/day.

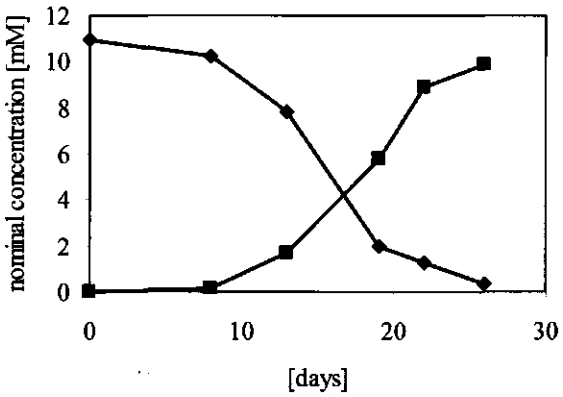

Figure 1: Stoichiometric anaerobic reduction of vinyl chloride (♦) to ethene (■) by enrichment DCE-1 using H_2 as electron donor.

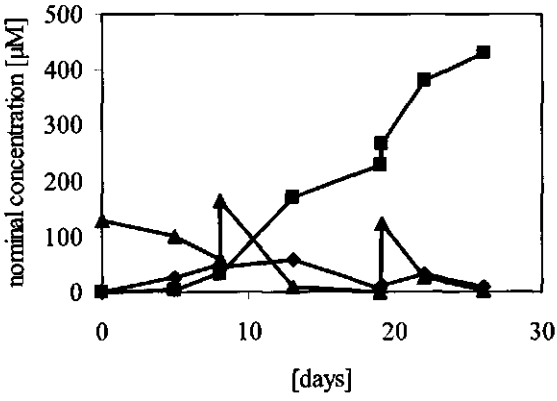

Figure 2: Reduction of *cis*-1,2-DCE (▲) by enrichment DCE-1. Traces of VC (♦) were produced and a stoichiometric amount of ethene (■) was produced.

Figure 2 shows the reduction of *cis*-1,2-DCE to ethene. In this experiment the *cis*-1,2-DCE reduction rate was 32 µmol/l/day. VC reduction by enrichment DCE-1 was not inhibited by initial nominal VC concentrations up to 10 mM. Nominal concentrations above 200 µM *cis*-1,2-DCE caused a significant decrease of the *cis*-1,2-DCE reduction rate. After several culture transfers using VC as electron acceptor the culture could still reduce *cis*-1,2-DCE indicating that the same bacteria were possibly responsible for the dechlorination of both VC and *cis*-1,2-DCE.

DCE-2

Initially a sludge sample from the laboratory reactor (Henssen *et al.*, 1997) was incubated with PCE and acetate. This incubation showed complete reduction of PCE to ethene as well as formation of methane. A liquid sample from this incubation was transferred into fresh medium. New incubations were always started with a 10 % (v/v) inoculum. Initially, the culture was maintained using acetate (and yeast extract) as electron donor and *cis*-1,2-DCE as electron acceptor. Figure 3 shows the stoichiometric reduction of *cis*-1,2-DCE via VC to ethene by enrichment DCE-2 using acetate as electron donor. After several generations, the culture was transferred into medium containing VC in stead of *cis*-1,2-DCE and we started using molecular hydrogen as electron donor.

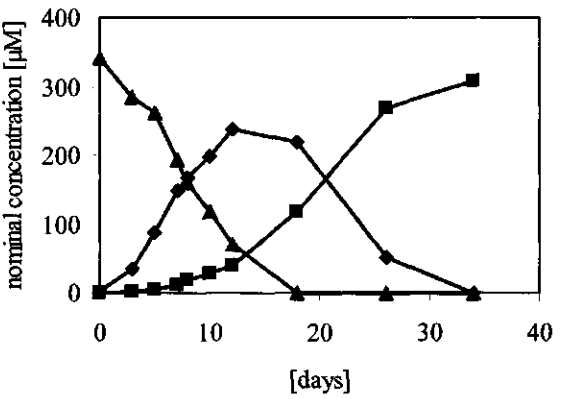

Figure 3: Reduction of *cis*-1,2-DCE (▲) to VC (♦) and ethene (■) by enrichment DCE-2 using acetate as electron donor

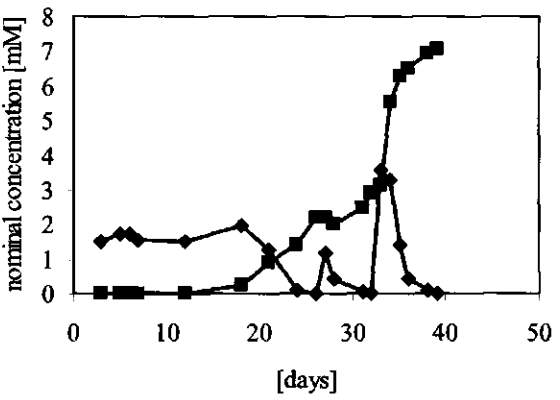

Figure 4: Reduction of VC (♦) by enrichment DCE-2 using H_2 as electron donor. VC was twice added after its depletion. Stoichiometric amounts of ethene (■) were produced.

By that time the enrichment had lost its ability to produce methane. Whereas no Bres had been added, toxic effects of the chlorinated ethenes possibly killed the methanogens. As in enrichment DCE-1, the addition of yeast extract was necessary to sustain dechlorinating activity. Yeast extract could be replaced by fermented yeast extract without losing the dechlorinating capacity. Analysis of the lower fatty acids

revealed the production of acetate in the enrichment indicating the presence of (homo)acetogenic bacteria, in contrast to our findings for enrichment DCE-1.

Figure 4 shows the reduction of VC by enrichment DCE-2 with H_2 as electron donor. Over time VC was added to this culture after its depletion. Stoichiometric conversion of VC to ethene occurred in these incubations. Enrichment DCE-2 could reduce nominal VC concentrations of up to 25 mM (Figure 5). Maximal VC reduction rates measured in enrichment DCE-2 were between 0.8 and 1.4 mmol/l/day. Nominal *cis*-1,2-DCE concentrations up to 400 µM could be reduced by enrichment DCE-2; measured reduction rates for *cis*-1,2-DCE were around 30 µmol/l/day.

Isolation

To date we have not been able to isolate the bacteria responsible for the dechlorination of either *cis*-1,2-DCE or VC in these two enrichment cultures by using serial dilution in liquid medium or the roll tube method. The addition of (fermented) yeast extract seems to be required to sustain dechlorinating activity in these two enrichment cultures. The added (fermented) yeast extract may also cause the survival of other bacteria like (homo)acetogens.

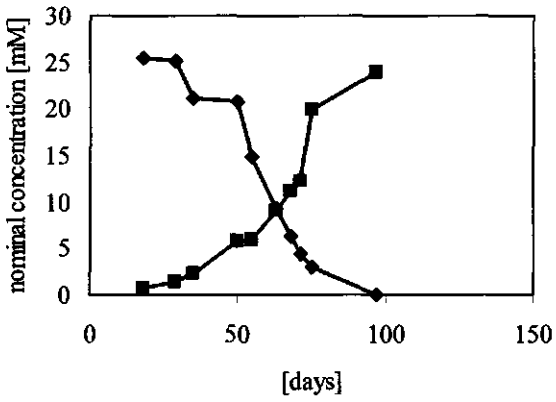

Figure 5: Reduction of VC (♦) to ethene (■) by enrichment DCE-2 using H_2 as electron donor. The initial nominal VC concentration of 25 mM corresponds to an aqueous VC concentration of 4.9 mM.

Discussion

Analyses of soils polluted with chlorinated ethenes (PCE and TCE) show the occurrence of reductive dechlorination via trichloroethene, dichloroethene and vinyl chloride to ethene under anaerobic conditions. However, partial dechlorination with the concomitant accumulation of mainly *cis*-1,2-DCE and sometimes VC is often found at these polluted sites. The accumulation of *cis*-1,2-DCE and/or VC may be caused by the lack of bacteria able to reduce lower chlorinated ethenes or by unfavorable environmental conditions for the bacteria involved in these biotransformations.

Here, we report of two anaerobic bacterial cultures, DCE-1 and DCE-2, enriched form two different sources that are able to reduce *cis*-1,2-DCE and VC to ethene. To date we have not been able to isolate the bacteria responsible for the dechlorination of the lower chlorinated ethenes. Neither dilution series in liquid medium nor incubation on solidified medium resulted in a pure dechlorinating culture. Use of relatively high levels of chlorinated ethenes did also not result in isolation of the dechlorinating organism(s). The requirement for (fermented) yeast extract may have prevented the isolation of the responsible bacteria. Another possibility that cannot be excluded is that a syntrophic relation between microorganisms is required to reduce these lower chlorinated ethenes.

In contrast to other studies which have reported conversion rates between 0.2 and 83 µmol/l/day achieved by enrichment cultures (Table 1) (Bradley and Chapelle, 1997; Haston and McCarty, 1999; DiStefano, 1999; Wickramanayake *et al.*, 2000; Flynn *et al.*, 2000), our experiments show conversion rates for VC that are 10- to 1000-fold higher. The nominal conversion rates of VC in our enrichment cultures were around 1 mmol/l/day. Conversion rates comparable to our VC reduction rates were also reported for the metabolic reduction of PCE by pure cultures of *Dehalobacter restrictus*, *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE1 (Table 1) (Holliger *et al.*, 1993; Scholz-Muramatsu *et al.*, 1995; Gerritse *et al.*, 1996).The high VC conversion rates achieved by our enrichment cultures suggest that the reduction of VC may be used to obtain metabolic energy.

Table 1: Reductive dechlorination rates for lower chlorinated ethenes by enrichment cultures and for PCE by pure cultures (rates calculated from reported literature data).

		Reduction rate µmol/l/day	Reference
VC → ethene	Sediment	0.2	1
VC → ethene	Enrichment culture	13	2
VC → ethene	Enrichment culture	42.5 - 82.5	3
VC → ethene	Enrichment culture	0.2	4
VC → ethene	Enrichment DCE-1	970	This study
VC → ethene	Enrichment DCE-2	800 - 1400	This study
PCE → TCE	*Desulfitobacterium* PCE1	1200	5
PCE → *cis*-1,2-DCE	*Dehalobacter restrictus*	1800	6
PCE → *cis*-1,2-DCE	*Sulfurospirillum multivorans*	4400	7

References: 1 = Bradley and Chapelle, 1997; 2 = Haston and McCarty, 1999 ; 3 = Flynn *et al.*, 2000; 4 = DiStefano, 1999; 5 = Gerritse *et al.*, 1996; 6 = Holliger *et al.*, 1993; 7 = Scholz-Muramatsu *et al.*, 1995.

It is noteworthy that we determined for both enrichment cultures 20- 30-fold lower conversion rates for *cis*-1,2-DCE than for VC. Comparable differences in rates between *cis*-1,2-DCE and VC (20-fold) were also reported by Rosner *et al.* (1997). Besides the lower conversion rates for *cis*-1,2-DCE as compared to VC we also found that the latter can be transformed at about 10- to 100-fold higher concentrations. The initial nominal VC concentration of 25 mM (Figure 5) used in our study corresponds to an actual aqueous concentration of 4.9 mM.

The low rates for the reduction of *cis*-1,2-DCE and VC reported to date are often used to explain the accumulation of these compounds at polluted sites. However, the VC and *cis*-1,2-DCE reduction rates measured in our enrichment cultures indicate that the reduction of *cis*-1,2-DCE and not that of VC may be the rate-limiting step for the complete anaerobic dehalogenation of chlorinated ethenes. This may also explain the frequently encountered accumulation of mainly *cis*-1,2-DCE in polluted sites.

A theoretical explanation for the accumulation of *cis*-1,2-DCE may be found in the thermodynamical aspects of these conversions in relation to the availability of hydrogen (H_2). Calculations of the thermodynamical critical H_2 concentrations, which are the calculated H_2 concentrations for $\Delta G_r = 0$ kJ/mol, show that there is a difference between the different dechlorination steps (Table 2). The calculations show that the highest H_2 concentration ($2.9 * 10^{-31}$ M) is required for the reduction of *cis*-1,2-DCE to VC. A comparable pattern is found for a $\Delta G_r = -70$ kJ/mol (Stams, 1994), the free energy required for biological systems to synthesize 1 mole of ATP (Table 2).

However, the H_2 concentrations calculated for both the biological and thermodynamical critical point are too low to explain the accumulation of lower chlorinated ethenes in terms of H_2 threshold values. Several authors have reported H_2 threshold levels for dechlorination of 1 – 10 nM (10^{-9} M) H_2 in mixed cultures (Ballapragada *et al.*, 1997; Yang and McCarty, 1998; Löffler *et al.*, 1999). The difference between this measured threshold (10^{-9} M H_2) and the theoretical threshold (10^{-31} M H_2) (Table 2) has to be the result of some other limitations. To date it is not known what these limitations are, but one could speculate about the efficiency of the dehalogenating enzyme system and/or mass transfer limitations for both H_2 and chlorinated ethenes.

Several authors have reported the involvement of cobalamine in dehalogenating enzymes (Neumann *et al.*, 1996; Miller *et al.*, 1998; Van de Pas, 2000). In homogeneous aqueous solution, Glod *et al.* (1997) found that *cis*-1,2-DCE is the less reactive chlorinated ethene in reduction studies with cob(I)alamin. When cobalamine dependent enzymes are involved in reductive dechlorination of *cis*-1,2-DCE, this could explain the lower reduction rates for *cis*-1,2-DCE found in our and other studies.

Table 2: Calculated critical H_2 concentrations for the separate reduction steps of PCE. H_2 thresholds are calculated for the thermodynamical critical point and for the biological critical point at which 1 ATP can be produced (-70 kJ/mol).

	$[H_2]$ ΔG_r = -70 kJ/mol	$[H_2]$ ΔG_r = 0 kJ/mol
PCE → TCE	$5.9 * 10^{-25}$	$3.3 * 10^{-37}$
TCE → *cis*-1,2-DCE	$3.8 * 10^{-24}$	$2.1 * 10^{-36}$
Cis-1,2-DCE → VC	$5.3 * 10^{-19}$	$2.9 * 10^{-31}$
VC → ethene	$9.4 * 10^{-21}$	$5.2 * 10^{-33}$

ΔG_f^0 values for PCE, TCE, *cis*-1,2-DCE, VC, ethene and Cl^- are 27.6, 25.4, 27.8, 59.6, 81.4 and –131.2 kJ/mol, respectively (Hanselmann, 1991; Dolfing and Janssen, 1994). The following assumptions were made: concentrations of chlorinated ethenes and ethene $1 * 10^{-4}$ mol/l, Cl^- $1 * 10^{-3}$ mol/l, pH 7 and T 298 K.

Acknowledgements

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6

Anaerobic respiration of chlorinated ethenes: the role of alternative electron acceptors and hydrogen as electron donor

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Abstract

Chlorinated ethenes are common soil and groundwater pollutants. They are of major concern because of their toxicity. Under aerobic conditions, higher chlorinated ethenes can only be degraded via co-metabolic reactions. In contrast, about fifteen anaerobic bacteria have been isolated which couple the degradation of higher chlorinated ethenes to growth. This degradation is called halorespiration, and consists of a (partial) reduction of chlorinated ethenes. For the lower, more reduced chlorinated ethenes this anaerobic reduction is relatively slow. Aerobic bacteria are able to mineralize these lower chlorinated ethenes and these lower chlorinated ethenes can also be oxidized under Mn(IV)- and Fe(III)-reducing conditions. All chlorinated ethenes can be co-metabolically reduced by methanogenic, acetogenic and sulphate reducing bacteria under anaerobic conditions.

Halorespiring bacteria have to compete with other respiring bacteria in soils, where alternative electron acceptors may be present. Nitrate and sulphite appear to influence dechlorination by halorespiring bacteria, but sulphate does not influence the reductive dechlorination by halorespiring bacteria.

Molecular hydrogen (H_2) is thought to be an important electron donor for anaerobic reductive dechlorination by halorespiring bacteria. Halorespiring bacteria are very efficient in using H_2. Threshold values below 1 nM H_2 have been measured. This value is in the same range as for iron and nitrate reducing bacteria.

Introduction

Six chlorinated ethenes exist, tetrachloroethene or perchloroethene (PCE), trichloroethene (TCE), 3 isomers of dichloroethene, 1,1-dichloroethene (1,1-DCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and *trans*-1,2-dichloroethene (*trans*-1,2-DCE), and vinyl chloride (VC). Physical and chemical characteristics of these chlorinated ethenes are given in table 1.

Worldwide PCE is used as dry cleaning solvent, in the production of chlorofluorcarbons, in vapor degreasing and metal cleaning. The chemical production of the colorless, nonflammable liquid PCE in the US is 400 million pounds (http://www.epa.gov/opptintr/chemfact/s_perchl.txt). This annual production decreases due to recycling and the use of alternative solvents. Although this chemical production is the main source of PCE in the environment, natural formation of chlorinated ethenes occurs as well. Abrahamson *et al.*, (1995) reported the formation of PCE and TCE by various algae. Keppler *et al.* (2002) described the natural formation of VC during the oxidative degradation of organic matter in a reaction between humic acids, iron and chloride.

Due to the use of PCE and TCE as solvents, large amounts have been spilled into the environment, and they have become major soil and groundwater pollutants.

Aerobic microbial conversion of chlorinated ethenes

Growth related conversions

To date no aerobic microorganisms have been isolated able to grow by the oxidation of PCE and TCE. Aerobic mineralization of 1,2-dichloroethene (a mixture of the *cis* and *trans* isomers) in an enrichment culture has been found by Bradley and Chapelle (2000). *Cis*-1,2-DCE can function as sole carbon and energy source for aerobic growth of strain JS666, a ß-*Proteobacterium* (Coleman *et al.*, 2002). This organism is not able to grow on VC or ethene.

Table 1: Chemical and physical properties of the chlorinated ethenes.

	PCE	TCE	1,1-DCE	*cis*-1,2-DCE	*trans*-1,2-DCE	VC
Cas	127-18-4	79-01-6	75-35-4	156-59-2	156-60-5	75-01-4
Formula	C_2Cl_4	C_2HCl_3	$C_2H_2Cl_2$	$C_2H_2Cl_2$	$C_2H_2Cl_2$	C_2H_3Cl
Water solubility [g/l]	0.2	1.2	2.5	4-6	4-6	3
Boiling point [° C]	121	87	32	48-60	48-60	-14
Melting point [° C]	-19	-86	-122	< -57	< -57	-154
Mass	165.8	131.4	96.9	96.9	96.9	62.5
Density [g/ml]	1.6	1.5	1.2	1.3	1.3	-
MAC [ppm]	35	35	5	200	200	3

Data from "Chemiekaarten" 19th edition, numbers C-0037 (PCE), C0042 (TCE), C-1204 (*cis*-1,2-DCE and *trans*-1,2-DCE), C-0299 (1,1-DCE) and C-0044 (VC). MAC = maximal acceptable concentration.

Vinyl chloride can be mineralized by *Mycobacterium aurum* L1 under aerobic conditions, thereby serving as carbon and energy source (Hartmans and De Bont, 1992). Vinyl chloride can also be used as sole carbon and energy source under aerobic conditions by *Pseudomonas aeruginosa* MF1 (Verce *et al.*, 2002).

Co-metabolic conversion

Co-metabolic conversion is a non-specific fortuitous reaction, where conversion of a non-growth substrate occurs via a non-energy gaining reaction by an enzyme or co-factor active in the conversion of the primary substrate (Alexander, 1994; Van Eekert, 1999).

PCE has long been considered to be persistent under aerobic conditions. However, aerobic co-metabolic conversions have now been reported for all chlorinated ethenes. Ryoo *et al.* (2000) reported the aerobic co-metabolic conversion of PCE by *Pseudomonas stutzeri* OX1. PCE is degraded by this organism through a toluene-*o*-xylene monooxygenase. Mixtures of chlorinated ethenes, including PCE, are also degraded by a recombinant strain of *Escherichia coli* expressing constitutively the toluene-*o*-xylene monooxygenase of *P. stutzeri* OX1 (Shim *et al.*, 2001).

Co-metabolic aerobic conversion of all other chlorinated ethenes has been reported to occur by mono- and dioxygenases during growth on methane, propane, propene, isopropene, ammonia and aromatic compounds like phenol and toluene (Sipkema, 1999; Van Hylckama Vlieg, 1999). Chlorinated ethenes themselves can also act as primary substrate for co-metabolic conversions. For example, *Pseudomonas aeruginosa* MF1 is an organism that co-metabolizes *cis*-1,2-DCE when grown aerobically on VC as sole carbon and energy source (Verce *et al.*, 2002).

Anaerobic microbiological conversion of chlorinated ethenes

Chlorinated ethenes can microbiologically be converted via different mechanisms under anaerobic conditions. They can be reductively dechlorinated via an energy gaining respiratory process or via a fortuitous co-metabolic reaction. They can also be degraded via an oxidative process. These three processes will be discussed in more

detail with emphasis on the energy gaining respiratory process (halorespiration), which is the main topic of this thesis.

Co-metabolic conversion

Anaerobic reductive conversion of higher chlorinated ethenes, such as PCE and TCE is already known for two decades (Bouwer and McCarty, 1983; Barrio-Lage *et al.*, 1986). Initially, reduction of chlorinated ethenes was attributed to non-energy gaining co-metabolic processes occurring under methanogenic, sulphate reducing and homoacetogenic conditions (Vogel and McCarty, 1985; Fathepure *et al.*, 1987; Fathepure and Boyd, 1988; Fathepure and Boyd, 1988a; Freedman and Gossett, 1989; Van Eekert *et al.*, 2001). Pure cultures of methanogenic and homoacetogenic bacteria were shown to couple the conversion of a primary substrate like methanol and acetate to the reduction of PCE (Fathepure and Boyd, 1988a; Terzenbach and Blaut, 1994). This co-metabolic conversion is catalyzed by co-factors like co-enzyme F_{430} (Ni), vitamin B_{12} (Co) and hematin (Fe) (Gantzer and Wackett, 1991; Terzenbach and Blaut, 1994). The electrons needed for dechlorination are provided by the transition metal in the co-factor.

Anaerobic oxidative conversions

Vogel and McCarty (1985) speculated already in the mid eighties on the possible anaerobic oxidation of chlorinated ethenes. They found that labeled ^{14}C-PCE was reductively converted, but that part of the labeled carbon also appeared in CO_2. This indicated that an anaerobic oxidation of PCE or one of the more reduced intermediates (TCE, DCE or VC) took place. By now, vinyl chloride and *cis*-1,2-dichloroethene mineralization has been observed under various redox conditions (Bradley and Chapelle, 1998; Bradley *et al.*, 1998a; Bradley *et al.*, 1998b; Bradley, 2000)

The anaerobic oxidation of vinyl chloride is possible in the presence of a sufficient strong acceptor such as Fe(III) of Mn(IV) (Bradley and Chapelle, 1996; Bradley *et al.*, 1998a; Bradley, 2000). Anaerobic oxidation of vinyl chloride can also be coupled to the reduction of humic acids (Bradley *et al.*, 1998b). The mineralization rate of VC seems to decrease with a decrease in redox potential (aerobic > Fe(III) > sulphate > CO_2). Alternatively, vinyl chloride may be oxidized to acetate (oxidative acetogenesis) and then be converted to CO_2 and CH_4 (acetotrophic methanogenesis) (Bradley and Chapelle, 2000a).

The anaerobic oxidation of 1,2-dichloroethene has been observed under Fe(III)-, sulphate-, carbon dioxide and humic acid reducing conditions. The mineralization rate of DCE was lower than that of vinyl chloride under all electron accepting conditions tested (Bradley and Chapelle, 1998; Bradley *et al.*, 1998b). Since the observed dichloroethene oxidation rates were comparable under all reducing conditions, these authors speculated that dichloroethene is first reduced to vinyl chloride in a rate limiting step, followed by the oxidation of vinyl chloride to CO_2 (Bradley and Chapelle, 1998). Another explanation may be that Fe(III) and sulphate are not such strong oxidants as Mn(IV) (Bradley and Chapelle, 1998; Bradley, 2000). This last explanation was supported by the observation that under Mn(IV)-reducing conditions dichloroethene was mineralized without the intermediate production of vinyl chloride (Bradley *et al.*, 1998a).

Halorespiration

Halorespiration is the process in which bacteria are able to use halogenated organic compounds as terminal electron acceptor for growth. Via this anaerobic reductive process these bacteria are able to gain energy for growth by electron transport phosphorylation. 'Dehalorespiration', 'chlororespiration' and 'chloridogenesis' are other terms used for the same process (Holliger *et al.*, 1999; Bradley, 2003; Löffler *et al.*, 2003).

In the early nineties, Holliger *et al.* (1993) described strain PER-K23, an organism that completely depends for its growth on the presence of PCE or TCE as terminal electron acceptor. Strain PER-K23, later described as *Dehalobacter restrictus* (Holliger *et al.*, 1998), was the first bacterium isolated able to use chlorinated ethenes as terminal electron acceptor. Strain PER-K23 is restricted in its substrate spectrum: only PCE and TCE are reduced by this organism, while molecular hydrogen is the only electron donor used. Other tested electron acceptors and electron donors were not used by this bacterium, nor by the closely related *D. restrictus* TEA (Table 3) (Holliger *et al.*, 1993; Wild *et al.*, 1996; Holliger *et al.*, 1998). Hereafter, Scholz-Muramatsu *et al.* (1994) isolated *Dehalospirillum multivorans*, another tetrachloroethene dechlorinating bacterium with a more versatile substrate spectrum than that of *Dehalobacter restrictus*. *Dehalospirillum multivorans* was recently renamed to *Sulfurospirillum multivorans* (Chapter 1; Luijten *et al.*, 2003). Table 2 gives an overview of the isolated bacteria that are able to couple the reduction of

chlorinated ethenes to growth via halorespiration. This table shows that a partial reduction/dechlorination of PCE can be performed by halorespiring bacteria that belong to six different genera. Figure 1 shows the phylogenetic position of known halorespiring bacteria.

Table 3 summarizes features of halorespiring bacteria able to reduce chlorinated ethenes. The genera *Desulfitobacterium*, *Dehalococcoides* and *Sulfurospirillum* are not included in table 3, but are discussed separately in the following paragraphs.

Table 2: Overview on bacteria able to reduce chlorinated ethenes via the process of halorespiration.

	Bacterium	Reference
PCE → TCE	*Desulfitobacterium frappieri* PCE1	Gerritse *et al.*, 1996
	Desulfitobacterium sp. Viet1	Löffler *et al.*, 1997
PCE → *cis*-1,2-DCE	*Sulfurospirillum multivorans*	Scholz-Muramatsu *et al.*, 1995; Luijten *et al.*, 2003; Chapter 1
	Sulfurospirillum halorespirans PCE-M2	Luijten *et al.*, 2003; Chapter 1
	Desulfitobacterium frappieri TCE1	Gerritse *et al.*, 1999
	Desulfitobacterium sp. PCE-S	Miller *et al.*, 1997
	Desulfitobacterium metallireducens 853-15A	Finneran *et al.*, 2002
	Dehalobacter restrictus PER-K23	Holliger *et al.*, 1993; Holliger *et al.*, 1998
	Dehalobacter restrictus TEA	Wild *et al.*, 1996
	Desulfuromonas chloroethenica TT4B	Krumholz *et al.*, 1996; Krumholz, 1997
	Desulfuromonas michiganensis BB1	Löffler *et al.*, 1997; Löffler *et al.*, 1999; Sung *et al.*, 2003
	Desulfuromonas michiganensis BRS1	Sung *et al.*, 2003
	Enterobacter strain MS-1	Sharma and McCarty, 1997; Sharma and McCarty, 1999
PCE → VC	*Dehalococcoides ethenogenes* 195	Maymó-Gatell *et al.*, 1997; Maymó-Gatell *et al.*, 1999
DCE → ethene	*Dehalococcoides* strain BAV1	He *et al.*, 2003

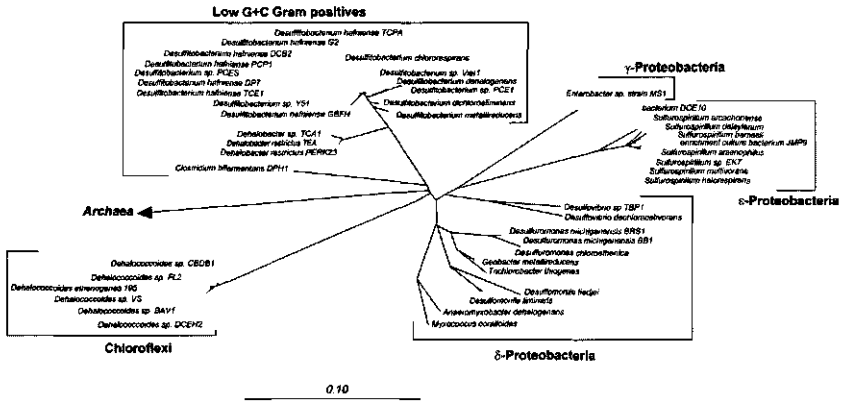

Figure 1: Phylogenetic tree constructed by the neighbour-joining method using 16S rDNA sequences, showing the position of halorespiring and related bacteria. Bar = 10 % divergence.

The genus Desulfitobacterium

This genus groups phylogenetically within the low G+C gram positive bacteria and was established by Utkin *et al.* (1994). They described *Desulfitobacterium dehalogenans*, an anaerobic bacterium that is able to use chlorinated phenols as well as other oxidized compounds as terminal electron acceptor (Table 4). Since then, several *Desulfitobacterium* strains have been isolated that are not only able to reduce chlorinated phenols, but also chlorinated ethenes and chlorinated benzoates (Table 4). The occurrence and diversity of *Desulfitobacterium* species indicates that this genus plays an important role in the dechlorination of PCE and other chlorinated compounds at polluted sites.

The genus *Desulfitobacterium* also contains species that are not able to reduce chlorinated compounds (Table 4). The isolation of *Desulfitobacterium metallireducens* and *Desulfitobacterium* strain GBFH shows that the diversity in terminal electron acceptors is even broader than initially thought. Nowadays, oxidized metals and humic acids are common electron acceptors for *Desulfitobacterium* species (Niggemeyer *et al.*, 2001; Finneran *et al.*, 2002; Luijten *et al.*, 2004; Chapter 2).

Table 3: Features of various tetrachloroethene respiring bacteria not belonging to the genera *Sulfurospirillum*, *Desulfitobacterium* or *Dehalococcoides*.

	origin	phylogenetic position	electron donors	electron acceptors		fermentable substrates	references
				chlorinated	non-chlorinated		
Dehalobacter restrictus PER-K23	river Rhine sediment mixed with granular sludge	low G+C Gram positives	H_2	PCE, TCE	-	-	Holliger *et al.* 1993; Holliger *et al.*, 1998
Dehalobacter restrictus TEA	contaminated groundwater	low G+C Gram positives	H_2	PCE, TCE	-	-	Wild *et al.*, 1996
Desulfuromonas chloroethenica TTB4	sediment polluted with chlorinated ethenes	δ-subdivision *Proteobacteria*	acetate, pyruvate	PCE, TCE	fumarate, Fe(III), polysulfide	nr*	Krumholz *et al.*, 1996; Krumholz, 1997
Desulfuromonas michiganensis BB1	pristine Père Marquette river sediment	δ-subdivision *Proteobacteria*	acetate, lactate, pyruvate, succinate, malate, fumarate	PCE, TCE	fumarate, malate, Fe(III), sulphur	malate, fumarate	Löffler *et al.*, 1997; Löffler *et al.*, 1999; Sung *et al.*, 2003
Desulfuromonas michiganensis BRS1	polluted Père Marquette river sediment	δ-subdivision *Proteobacteria*	acetate, lactate, pyruvate, succinate, malate, fumarate	PCE, TCE	fumarate, malate, Fe(III), sulphur	malate, fumarate	Sung *et al.*, 2003
Enterobacter MS-1	contaminated aquifer material	γ-subdivision *Proteobacteria*	glucose, pyruvate, formate, lactate, acetate, yeast extract	PCE, TCE	nitrate, oxygen	carbohydrates, fatty acids, amino acids, purines, pyrimidines	Sharma and McCarty, 1996

*: nr = not reported.

Table 4: Features of the genus *Desulfitobacterium*. The *Desulfitobacteria* cluster phylogenetically into the low G+C Gram positive bacteria.

	origen	electron donors	electron acceptors		fermentable substrates	References
			chlorinated	non-chlorinated		
D. dehalogenans JW/IU-DC1	methanogenic lake sediment	lactate, pyruvate, formate, H_2	ClOHPA	sulphite, thiosulphate, sulphur, nitrate, selenate, fumarate	pyruvate	Utkin *et al.*, 1994; Luijten *et al.*, 2004, Chapter 2
D. chlororespirans Co23	compost soil	lactate, pyruvate, formate, H_2, butyrate, crotonate	3-Cl-4OHbenzoate, DCP, TCP, ClOHPA	sulphite, thiosulphate, sulphur, selenate, Fe(III), Mn(IV)	pyruvate	Sanford *et al.*, 1996; Luijten *et al.*, 2004; Chapter 2
D. hafniense DCB-2	municipal sludge	pyruvate	TCP, PCP, DCP, ClOHPA	sulphite, thiosulphate, nitrate, Fe(III)	pyruvate, tryptophan	Christiansen and Ahring, 1996
D. hafniense GBFH	arsenic enriched sediment	lactate, pyruvate, formate, fumarate	-	sulphite, thiosulphate, sulphur, selenate, Mn(IV), fumarate, arsenate, Fe(III)	pyruvate	Niggemeyer *et al.*, 2001
D. frappieri PCP-1*	methanogenic consortium	pyruvate	PCP, TeCP, TCP, DCP	sulphite, thiosulphate, nitrate	pyruvate	Bouchard *et al.*, 1996
D. frappieri TCE1	Cl-ethene polluted soil	H_2, formate, lactate, butyrate, crotonate, ethanol	PCE, TCE	sulphite, thiosulphate, nitrate, fumarate, selenate, arsenate, Fe(III), Mn(IV)	pyruvate, serine	Gerritse *et al.*, 1999; Luijten *et al.*, 2004; Chapter 2
D. frappieri DP7	human feces	lactate, pyruvate, formate, H_2, butyrate, ethanol	-	sulphite, thiosulphate, nitrate, fumarate, selenate, Fe(III), Mn(IV)	pyruvate	Van de Pas *et al.*, 2001; Luijten *et al.*, 2004; Chapter 2
Desulfitobacterium sp. PCE1	polluted soil	lactate, pyruvate, butyrate, formate, succinate, ethanol	PCE, 2-Clphenol, ClOHPA, TCP	sulphite, thiosulphate, fumarate, selenate	pyruvate	Gerritse *et al.*, 1996; Luijten *et al.*, 2004; Chapter 2
Desulfitobacterium sp. PCE-S	polluted soil	H_2, pyruvate	PCE, TCE	fumarate	nr**	Miller *et al.*, 1997
D. metallireducens	contaminated soil	lactate, formate, ethanol, butanol, butyrate, malate, pyruvate	PCE, TCE, ClOHPA	AQDS, Fe(III), humics, sulphur, Mn(IV), thiosulphate	-	Finneran *et al.*, 2002
D. dichloroeliminans DCA1	1,2-dichloroethane polluted soil	H_2, formate, lactate	1,2-dichloroethane, dichloroepropanes, dichlorobutanes	sulphite, $S_2O_3^{2-}$, nitrate		De Wildeman *et al.*, 2003
Desulfitobacterium sp. Viet1	river sediment	lactate, pyruvate		nr	nr	Löffler *et al.*, 1997; Löffler *et al.*, 1999

*: Niggemeyer *et al.* (2001) suggest the reclassification of this species to *D. hafniense* PCP-1; **: nr = not reported; DCP = dichlorophenol; TCP = trichlorophenol; ClOHPA = 3-Cl-4-hydroxyphenylacetate; PCP = pentachlorophenol; TeCP = tetrachlorophenol.

The genus Dehalococcoides

Maymó-Gatell *et al.* (1997) described strain 195, the first isolated organism able to dechlorinate PCE completely to ethene. Phylogenetically, strain 195 groups within the eubacteria without close affiliation to any known groups. The new genus "*Dehalococcoides*" was established and strain 195 was tentatively called "*Dehalococcoides ethenogenes*" (Maymó-Gatell *et al.*, 1997). The final reduction of VC to ethene, as well as the reduction of *trans*-1,2-dichloroethene by strain 195, were found to be non-halorespiring reactions (Maymó-Gatell *et al.*, 1999).

Besides chlorinated ethenes, "*Dehalococcoides ethenogenes*" strain 195 is also able to reduce 1,2-dichloroethane to ethene (Maymó-Gatell *et al.*, 1999). Similar as *Dehalobacter restrictus*, this organism uses only molecular hydrogen as electron donor. Cultivation of strain 195 is still difficult since several undefined additions such as extracts from mixed microbial cultures are required (Maymó-Gatell *et al.*, 1997).

Recently, a second chloroethene reducing member of the genus "*Dehalococcoides*" was isolated (strain BAV1). This isolate is, in contrast to strain 195, able to couple the reduction of VC to growth (He *et al.*, 2003). It also reduces all DCE isomers, vinyl bromide and 1,2-dichloroethane. PCE and TCE do not support growth but can be co-metabolized in the presence of a growth supporting substrate (He *et al.*, 2003). Strain BAV1 is able to grow in a defined synthetic medium and does not require the addition of complex organic substances. It grows via the reduction of chlorinated ethenes with molecular hydrogen as electron donor.

The physiologically different strain CBDB1 of the genus "*Dehalococcoides*" couples the oxidation of molecular hydrogen to the reduction of 1,2,3-trichlorobenzene (1,2,3-TCB), 1,2,4-TCB, 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), 1,2,3,5-TeCB and 1,2,4,5-TeCB to dichlorobenzenes or 1,3,4-TCB (Adrian *et al.*, 2000). Strain CBDB1 is the first and to date only bacterium isolated able to derive energy from the reductive dechlorination of higher chlorinated benzenes via a respiratory process. This bacterium cannot use any other electron donors and electron acceptors. Strain CBDB1 is also, in contrast to "*Dehalococcoides ethenogenes*" 195, able to grow in a synthetic medium (Adrian *et al.*, 2000).

The genus "*Dehalococcoides*" exists of 3 isolated members: two are able to reduce chlorinated ethenes (strain 195 and strain BAV1) and one is able to reduce chlorinated benzenes (strain CBDB1). Several (to date) unculturable members of the genus "*Dehalococcoides*" have been found by molecular techniques (Pulliam Holoman *et*

al., 1998; Adrian *et al.*, 2000; Löffler *et al.*, 2000; Cutter *et al.*, 2001; Fennell *et al.*, 2001; Hendrickson *et al.*, 2002). These detected "*Dehalococcoides*" relatives are not only able to reduce chlorinated ethenes and chlorinated benzenes, but also chlorinated biphenyls.

The importance of this group of organisms is indicated by the detection of increasing numbers of "*Dehalococcoides*" species in diverse environments. "*Dehalococcoides*" species degrade a wide range of chlorinated pollutants and they have a widespread distribution in soils. Hendrickson and co-workers (2002) showed, via molecular techniques, a link between the presence of "*Dehalococcoides*" species and complete degradation of PCE at polluted sites all over the world. Members of the "*Dehalococcoides*" group were detected at 21 sites and complete dechlorination occurred at all sites. No "*Dehalococcoides*" sequences were found at 3 sites, where only partial dechlorination occurred (Hendrickson *et al.*, 2002). This functional relationship indicates that molecular techniques may be useful to assess the full dechlorination potential at sites polluted with chlorinated ethenes. however, one should realize that not all "*Dehalococcoides*" species are able to reduce chlorinated ethenes.

The genus Sulfurospirillum

Schumacher *et al.* (1992) established the genus *Sulfurospirillum* by describing "*spirillum*" 5175 as *Sulfurospirillum deleyianum*, an elemental sulphur-reducing bacterium. With the designation of strain SES-3 (Oremland *et al.*, 1994; Laverman *et al.*, 1995) to the genus *Sulfurospirillum* as *S. barnesii* strain SES-3 and the isolation of *S. arsenophilum* (Stolz *et al.*, 1999) the genus became known for its metal reducing capacities (Table 5). We described *S. halorespirans* PCE-M2, the first halorespiring member within this genus (Luijten *et al.*, 2003; Chapter 1), and we also included *Dehalospirillum multivorans* into the genus *Sulfurospirillum* as *S. multivorans*. The range of different electron acceptors that can be used by *Sulfurospirillum* species is broad (Schumacher *et al.*, 1992; Oremland *et al.*, 1994; Laverman *et al.*, 1995; Stolz *et al.*, 1999; Luijten *et al.*, 2003; Luijten *et al.*, 2004; Chapter 1 and 2). Table 5 shows that *Sulfurospirillum* species can convert chlorinated ethenes and other compounds such as nitrate, AQDS, oxidized metals, arsenate and selenate. This broad range of electron acceptors makes the bacteria from this genus interesting for bioremediation purposes and also gives them the chance to survive in many different habitats.

Table 5: Features of the genus *Sulfurospirillum*. The *Sulfurospirilla* cluster phylogenetically within the ε-subclass of the *Proteobacteria*.

	origin	electron donors	electron acceptors		fermentable substrates	references
			Chlorinated	non-chlorinated		
S. halorespirans PCE-M2	polluted soil	H_2, lactate, pyruvate, formate	PCE, TCE	nitrate, nitrite, fumarate, selenate, arsenate, microaerophiilic, Mn(IV), Fe(III)	pyruvate, fumarate	Luijten *et al.*, 2003; Luijten *et al.*, 2004; Chapter 1 and 2
S. multivorans	activated sludge	H_2, lactate, pyruvate, formate, glycerol, ethanol	PCE, TCE	nitrate, fumarate, selenate, arsenate, Fe(III)	pyruvate, fumarate	Scholz-Muramatsu *et al.*, 1995; Holliger *et al.*, 1999; Luijten *et al.*, 2004; Chapter 2
S. deleyianum	anoxic mud	H_2, formate, sulphide, succinate, fumarate, malate, aspartate, oxoglutarate, pyruvate, oxaloacetate	nr*	nitrate, nitrite, sulphur, sulphite, thiosulphate, microaerophilic, fumarate, DMSO**, arsenate, Mn(IV)	pyruvate, fumarate, malate	Schumacher *et al.*, 1992; Stolz *et al.*, 1999; Luijten *et al.*, 2004; Chapter 2
S. barnesii SES-3	freshwater marsh	pyruvate, fumarate, formate, lactate	-	nitrate, nitrite, sulphur, arsenate, thiosulphate, microaerophilic, fumarate, Fe(III), Mn(IV), selenate, trimethylamine-N-oxide	fumarate	Oremland *et al.*, 1994; Laverman *et al.*, 1995; Stolz *et al.*, 1999; Luijten *et al.*, 2004; Chapter 2
S. arsenophilum	watershed sediments	pyruvate, fumarate, formate, lactate	nr	nitrate, nitrite, sulphur, arsenate, selenate, Mn(IV), thiosulphate, microaerophilic	fumarate	Stolz *et al.*, 1999; Luijten *et al.*, 2004; Chapter 2
S. arcachonense	marine surface sediment	H_2, pyruvate, lactate, α-ketoglutarate, glutarate, yeast extract, fumarate	nr	sulphur, microaerophilic	fumarate	Finster *et al.*, 1997; Stolz *et al.*, 1999

*: nr = not reported. **: DMSO = dimethyl sulphoxide.

Influence of alternative electron acceptors on halorespiration

Dehalobacter restrictus PER-K23 depends completely on PCE or TCE as terminal electron acceptor. Other tetrachloroethene respiring bacteria are able to use a broader range of terminal electron acceptors (Table 3-5). This versatility results in a competition for available reducing equivalents between different terminal electron accepting processes. This may lead to an inhibition of the dechlorinating activity by halorespiring bacteria.

Nitrate is a common terminal electron acceptor for halorespiring bacteria, and it is preferred above PCE by *Sulfurospirillum halorespirans* PCE-M2 (Chapter 3). After reduction of nitrate and the intermediate nitrite, PCE reduction is resumed by strain PCE-M2. In contrast, *S. multivorans* has been found to reduce nitrate to nitrite in the presence of PCE, but upon the depletion of nitrate, PCE reduction did not resume (Chapter 3). Nitrate does not inhibit PCE reduction by *Desulfitobacterium* strain PCE1, a strain that cannot use nitrate (Gerritse *et al.*, 1996). PCE reduction by *D. frappieri* strain TCE1 was not inhibited by nitrate. Simultaneous reduction of PCE and nitrate, fumarate and sulphite was observed in chemostat studies when the electron donor (lactate) was present in excess, while under electron donor limitation the dechlorination was blocked completely (Gerritse *et al.*, 1999).

Sulphate, which is often present at sites polluted with chlorinated ethenes, has not been found to be used as terminal electron acceptor by most known halorespiring bacteria. Sulphate also does not influence PCE reduction by *Sulfurospirillum* and *Desulfitobacterium* species (Neumann *et al.*, 1994; Löffler *et al.*, 1996; Chapter 3).

Sulphite, which can be used as electron acceptor by *Desulfitobacterium* species, but not by e.g. *Sulfurospirillum* species, does inhibit tetrachloroethene dechlorination in many halorespiring bacteria, such as *Desulfuromonas*, *Desulfitobacterium* and *Sulfurospirillum* species (Löffler *et al.*, 1996; Gerritse *et al.*, 1999; Sung *et al.*, 2003; Chapter 3). The known tetrachloroethene reductive dehalogenases are cobalamine containing enzymes (Miller *et al.*, 1998; Magnuson *et al.*, 1998; Van de Pas, 2000; Smidt, 2001). Sulphite reacts chemically with cobalamine, and can thus affect the PCE reductive dehalogenases and inhibit reductive dechlorination (Miller *et al.*, 1997). Dechlorination by *Desulfitobacterium frappieri* TCE1 was completely

inhibited until all sulphite was reduced indicating that it concerns a reversible chemical inhibition (Gerritse *et al.*, 1999).

Importance of hydrogen as electron donor for reductive dechlorination

In the early nineties several researchers hypothesized that molecular hydrogen (H_2) may be the actual electron donor for the reductive dehalogenation of chlorinated ethenes in mixed cultures (Gibson and Sewell, 1992; DiStefano *et al.*, 1992). They suggested that organic electron donors such as short-chain organic acids and alcohols were fermented, and that the H_2 produced served as electron donor for reductive dehalogenation. This hypothesis was supported by the isolation of *Dehalobacter restrictus* PER-K23, the first bacterium isolated able to reduce tetrachloroethene metabolically (Holliger *et al.*, 1993; Holliger *et al.*, 1998). This bacterium uses only H_2 as electron donor, just like the isolated "*Dehalococcoides*" species. Despite the fact that other halorespiring bacteria can use a broad range of electron donors (Table 3-5), it seems that H_2 plays a key-role in the process of reductive dechlorination.

Assuming that H_2 is an important electron donor for dechlorination, it is expected that tetrachloroethene reducing bacteria have to compete for the available H_2 with other hydrogenotrophic bacteria such as methanogens and sulphate reducers. Roughly a 10-fold higher half-velocity constant ($K_s(H_2)$), which is the concentration at which the rate is half of the maximum rate, was found for methanogenesis (960 ± 180 nM) compared to the dechlorination of PCE (100 ± 50 nM) (Smatlak *et al.*, 1996). Similarly, an approximately 10 times lower $K_s(H_2)$ for the reduction of PCE to ethenes was reported by Ballapragada *et al.* (1997). Due to this lower K_s for hydrogen, dechlorinating bacteria may outcompete methanogens at low H_2 concentrations. A low H_2 partial pressure would therefore be favorable for dechlorination. Consequently, butyrate and propionate may be better electron donors than lactate and ethanol since the fermentation of the latter two results in 2-3 times higher H_2 partial pressure and thus in more methane production than with butyrate and propionate (Fennell *et al.*, 1997). In long-term experiments, however, reductive

dechlorination can be sustained equally good regardless of the electron donor fed (Fennell *et al.*, 1997; Carr and Hughes, 1998).

A high H_2 affinity has been found for dechlorinating bacteria, which coincides with relatively low threshold H_2 concentrations at which reductive dechlorination can proceed. Mixed culture studies show that reductive dechlorination can proceed at H_2 concentrations below 20 nM (Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Yang and McCarty, 1998; Lu *et al.*, 2001). These steady state H_2-concentrations are the result of both H_2-producing and H_2-consuming processes. It was shown that the reached steady state is controlled by the physiological characteristics of the H_2-consuming bacteria (Cord-Ruwisch *et al.*, 1988; Lovley and Goodwin, 1988; Löffler *et al.*, 1999). Different terminal electron accepting processes have different affinities for H_2 and different H_2-threshold concentrations. These threshold concentrations depend on the redox potential of the terminal electron acceptor (Cord-Ruwisch *et al.*, 1988). Löffler *et al.* (1999) found H_2-threshold values below 0.3 nM H_2 in pure cultures of *Desulfitobacterium* species. Comparable low H_2-threshold concentrations were measured in long term batch studies for *Sulfurospirillum halorespirans* PCE-M2, *S. multivorans* and *Dehalobacter restrictus* (Luijten *et al.*, 2004a, Chapter 4). The measured H_2-threshold values suggest that the dechlorinating bacteria can outcompete methanogens and (homo)acetogens at H_2 concentrations below approximately 5 nM (Table 6) (Smatlak *et al.*, 1996; Yang and McCarty, 1998; Löffler *et al.*, 1999; Luijten *et al.*, 2004a; Chapter 4).

Table 6: H_2-threshold concentrations for different reducing processes.

Process	H_2-threshold [nM]	References
Acetogenesis	>350	1,2
Methanogenesis	5-100	1,2,3,4,5,6
Sulphate reduction	1-10	1,2,3,4,6, Chapter 4
Ammonification	0.015-0.06	1,2, Chapter 4
Nitrate reduction	<0.05	1,6
Manganese reduction	<0.05	6
Iron reduction	0.1-0.8	1,3,4,6
Halorespiration*	<0.3	1, Chapter 4
PCE & TCE reduction**	0.6-0.9	4, Chapter 4
Cis-1,2-DCE reduction**	0.1-2.5	4,5, Chapter 4
VC reduction**	2.24	4,5, Chapter 4

References: 1 = Löffler *et al.*, 1999; 2 = Cord-Ruwisch *et al.*, 1988; 3 = Chapelle *et al.*, 1996; 4 = Lu *et al.*, 2001; 5 = Yang and McCarty, 1998; 6 = Lovley and Goodwin, 1988.

* = Pure culture studies; ** = mixed culture/field studies.

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7

Concluding remarks and future perspectives

The total number of halorespiring bacterial species able to (partially) reduce chlorinated ethenes is about 15, distributed over 6 genera. Some of these bacteria are restricted in their electron acceptor usage, such as *Dehalobacter restrictus*, which can dechlorinate only tetrachloroethene (PCE). However, our work and that of others shows that most halorespiring bacteria are able to use a broad range of electron acceptors, as is the case for *Sulfurospirillum* species as described in this thesis (chapter 1). This gives these bacteria the opportunity to survive in diverse environments, also in those where chlorinated ethenes are absent. Due to the introduction/appearance of chlorinated ethenes in the environment, dehalogenating enzymes can get induced resulting in the degradation of the chlorinated pollutant. This process is actually recognized and referred to as natural attenuation. However, the tetrachloroethene-reducing activity is not always induced when alternative electron acceptors are present, and the dechlorinating activity can even be inhibited by e.g. the presence of nitrate. This would mean that the ability of halorespiring bacteria to use a broad range of electron acceptors is not always advantageous. Competition between the chlorinated compounds and alternative electron acceptors, or even the loss of the dechlorinating capacity, could occur when exposed to other electron acceptors. To understand this better, more detailed studies are required on the regulation mechanisms for the use of alternative electron acceptors by halorespiring bacteria, and the evolutionary aspects of the dehalogenating enzymes.

The application of *in-situ* degradation of chlorinated ethenes is growing in importance. The Dutch Ministry of Spatial Planning, Housing and the Environment formulates in her latest policy-making document (beleidsbrief bodem BWL/2003 096 250) that more emphasis should be placed on the application of *in-situ* bioremediation techniques. Many Dutch aquifers are anoxic, which offers the possibility to apply reductive dechlorination of chlorinated ethenes. However, a number of drawbacks are encountered in the field making *in-situ* bioremediation not yet an established and accepted technology. At some polluted sites, halorespiring bacteria may be absent, whereas at other sites natural attenuation is observed, indicating to the presence of active, indigenous bacteria capable of reducing chlorinated ethenes. In the latter case sometimes only partial dechlorination is observed, resulting in the accumulation of *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and vinyl chloride (VC), which is even more toxic than the higher chlorinated ethenes. This indicates that bacteria able to convert

these lower chlorinated ethenes are absent, or that the proper conditions are not met to reduce these lower chlorinated intermediates. The specific conditions for bacteria to dechlorinate lower chlorinated ethenes are yet unknown, but seem to be different from the conditions needed to degrade tetrachloroethene (PCE) and trichloroethene (TCE). Recently, the first halorespiring bacteria able to reduce *cis*-1,2-DCE and VC have been isolated (e.g. *Dehalococcoides ethenogenes*) and they appear to have stringent growth requirements. Furthermore, the type of electron donor for reductive dechlorination is another important aspect. Halorespiring bacteria are able to use relatively low H_2 concentrations very efficiently. They are more efficient than methanogenic archaea and acetogenic bacteria at low H_2 concentrations. However, they are found to be outcompeted at higher H_2 concentrations by methanogens and acetogens. That makes the type of organic electron donor added during an *in-situ* bioremediation project an important tool to enhance the efficiency of reductive dechlorination. Substrates that are fermented slowly will result in a relatively low H_2 concentration over a longer time. This allows an efficient utilization of the available electron donor by halorespiring bacteria.

To make *in-situ* bioremediation of chlorinated ethenes a reliable technique, insight is required in the competition between electron acceptors present (including the chlorinated ethenes) and the (organic) substrates required. Additionally, emphasis on the reduction of lower chlorinated ethenes is desired. More knowledge on these final reduction steps will increase the efficiency of *in-situ* anaerobic reductive dechlorination projects and provide the knowledge to completely transform chlorinated ethenes to harmless products. When this information is available *in-situ* bioremediation projects may become accepted for a broad application.

New developments, that have not been addressed in this thesis, such as anaerobic, oxidative degradation of lower chlorinated ethenes may also become important for completely degrading chlorinated pollutants under anoxic conditions. In the field, higher chlorinated ethenes can be degraded *in-situ* via an anaerobic, reductive process to lower chlorinated ethenes. Further downstream, the lower chlorinated ethenes produced may be oxidized under e.g. iron-reducing conditions.

8

Summary

Chlorinated ethenes are widespread soil and groundwater pollutants. Over the last 2 decades a lot of effort has been made to understand the degradation mechanisms for these pollutants. In the early eighties reduction of tetrachloroethene (PCE) was observed in anaerobic soil samples, which was shown to be mediated by microorganisms. The first microorganism able to couple the anaerobic reduction of PCE to growth in a process called halorespiration (alternative terms are chlororespiration, chloridogenesis or dehalorespiration) was isolated in 1993. Since then, about 15 bacteria able to reduce PCE metabolically have been isolated. This thesis describes research on different aspects influencing the reductive dechlorination of chlorinated ethenes by anaerobic halorespiring bacteria.

A new halorespiring bacterium is described in chapter 1. This bacterium, *Sulfurospirillum halorespirans* PCE-M2, was isolated from a polluted soil near Rotterdam harbor. Strain PCE-M2 is a metabolically versatile bacterium able to use a variety of electron acceptors and electron donors. This new strain is closely related to *Dehalospirillum multivorans*, but more detailed studies indicated that strain PCE-M2 belongs to the genus *Sulfurospirillum*. It also appeared that *Dehalospirillum multivorans* had to be included in this genus. Consequently, it was reclassified to *Sulfurospirillum multivorans*.

Members of the genus *Sulfurospirillum* were originally known for their sulphur, selenate and arsenate respiring properties. Therefore, we screened a number of halorespiring and related bacteria for their metal reducing properties (Chapter 2). It was shown that the reduction of metals such as ferric iron, manganese, selenate and arsenate is a common property amongst halorespiring bacteria. We also investigated the quinone reducing and oxidizing abilities. All tested bacteria are able to reduce AQDS, a quinone-bearing humic acid analogue. Some of the tested bacteria (*Desulfitobacterium hafniense* DP7, *Sulfurospirillum barnesii*, *S. deleyianum* and *S. arsenophilum*) are also able to oxidize AH_2QDS coupled to nitrate reduction.

The influence of some alternative electron acceptors on the reductive dechlorination is discussed in chapter 3. *Sulfurospirillum halorespirans* preferably reduces nitrate (to ammonium) and then PCE. In contrast, *Sulfurospirillum multivorans* reduces nitrate only to nitrite, and PCE reduction is blocked irreversibly in the presence of nitrate. In

Desulfitobacterium frappieri TCE1, PCE and nitrate are reduced simultaneously in excess of electron donor. Under electron donor limitation PCE reduction was inhibited (Gerritse *et al., Appl. Environ. Microbiol.* 1999, 65, 5212-5221). The influence of nitrate on the reduction of chlorinated ethenes by halorespiring bacteria differs between species and may also depend on the availability of electron donor. Sulphate, which is not used as electron acceptor by chlorinated ethenes respiring bacteria is often found at polluted sites. We have tested the influence of sulphate on halorespiring bacteria (Chapter 3). It appeared that sulphate does not influence these microorganisms. Sulphite however, a possible electron acceptor for *Desulfitobacterium* species, inhibits the reduction of PCE. This inhibition may be the result of a chemical interaction between sulphite and cobalamine containing dehalogenases. We also studied the adaptation of *Sulfurospirillum halorespirans* PCE-M2 to different alternative electron acceptors (Chapter 3). Both nitrate and arsenate are reduced by cells pre-grown on PCE, nitrate, arsenate and selenate. This indicates that the enzymes responsible for the reduction of nitrate and arsenate are constitutively present in *S. halorespirans*. In contrast, PCE and selenate are only reduced by cells pre-grown on PCE or selenate respectively.

Halorespiring bacteria have a high affinity for hydrogen (H_2). H_2 may even be the most important electron donor for these organisms in natural environments. We have studied H_2-threshold concentrations in pure cultures of halorespiring bacteria (Chapter 4). H_2-threshold values between 0.05 and 0.08 nM under PCE-reducing and nitrate-reducing conditions were measured. Furthermore, we measured H_2 concentrations at a field site polluted with chlorinated ethenes. PCE and trichloroethene (TCE) reduction can occur at H_2 concentrations below 1 nM. However, for the reduction of lower chlorinated ethenes a higher H_2 concentration seems to be required.

Accumulation of *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and vinyl chloride (VC) under anaerobic conditions is often observed. The enrichment of two cultures (DCE-1 and DCE-2) able to reduce VC at relative high rates is described in chapter 5. *Cis*-1,2-DCE is reduced at approximately 20-30 fold lower rates than VC. Our results suggest that these two enrichment cultures are able to gain energy from the reduction of lower chlorinated ethenes. When we performed these studies, no microorganisms had been isolated able to grow by the reduction of VC. However, recently He *et al.* (*Nature.* 2003,

424, 62-65) isolated *Dehalococcoides* strain BAV1, which is able to couple the reduction of DCE and VC to ethene to growth.

Finally, the results obtained are combined with available literature data to obtain a state-of-the-art on chlorinated ethenes respiring microorganisms, the influence of alternative electron acceptors on these microorganisms and the role of H_2 and H_2-threshold values in halorespiration.

9

Samenvatting

In dit onderzoek is gekeken naar de afbraak van gechloreerde verbindingen (ethenen) door anaërobe (zuurstofloze) bacteriën. Gechloreerde ethenen worden onder andere gebruikt in chemische wasserijen en in fabrieken. Daar vroeger minder zorgvuldig met afval werd omgegaan komen gechloreerde ethenen nu veel als verontreinigingen in grond en grondwater voor. De afgelopen 20 jaar is veel energie gestopt in het inzichtelijk maken van de afbraakmogelijkheden van deze verontreinigingen. Al in het begin van de tachtiger jaren werd de afbraak van tetrachlooretheen (PCE ofwel PER) waargenomen in zuurstofloze grondmonsters. Deze afbraak werd veroorzaakt door bacteriën. Het afbraakproces in deze bacteriën wordt ook wel halorespiratie genoemd. In dit proces gebruiken bacteriën gechloreerde verbindingen om adem te halen net zoals wij mensen zuurstof gebruiken. Pas in 1993 werd de eerste bacterie geïsoleerd die deze verontreinigingen kan afbreken en er via halorespiratie energie uit kan halen om te groeien. Inmiddels zijn zo'n 15 bacteriën bekend die dit kunnen. In dit proefschrift hebben we gekeken naar factoren die de groei van deze bacteriën beïnvloeden, met als achterliggend doel om deze bacteriën beter te kunnen gebruiken om verontreinigde bodems efficiënt schoon te maken.

In het eerste hoofdstuk van dit proefschrift wordt een nieuwe bacterie beschreven die tetrachlooretheen kan afbreken. Deze bacterie, *Sulfurospirillum halorespirans* PCE-M2 genaamd, komt uit een verontreinigde bodem bij de Rotterdamse haven. PCE-M2 is in staat om op meerdere substraten (voedingsstoffen) te groeien. Uit ons onderzoek bleek dat PCE-M2 verwant is aan bacteriën van het geslacht *Sulfurospirillum*, en ook veel leek op een andere bacterie, namelijk *Dehalospirillum multivorans*. Uit ons onderzoek bleek dat deze laatste bacterie een verkeerde (familie) naam heeft en ook een *Sulfurospirillum* is. Daarom hebben we hem een nieuwe naam gegeven, namelijk *Sulfurospirillum multivorans*.

Het oorspronkelijke kenmerk van *Sulfurospirillum* is dat ze op verschillende verbindingen kunnen groeien, zoals zwavel en verschillende metalen (selenaat en arsenaat). PCE-M2 bleek naast gechloreerde ethenen ook op dergelijke verbindingen te kunnen groeien (hoofdstuk 2). Ook hebben we andere bacteriën die gechloreerde ethenen kunnen afbreken getest of ze op deze verbindingen kunnen groeien. Meerdere bacteriën blijken naast gechloreerde verontreinigingen ook zwavel en verschillende metalen te kunnen gebruiken als substraat. Daarnaast is getest of deze bacteriën

quinonen kunnen gebruiken. Quinonen zijn onderdeel van humus zuren die veel in de grond voorkomen. Het bleek dat alle door ons geteste bacteriën inderdaad quinonen kunnen gebruiken.

In hoofdstuk 3 hebben wordt beschreven hoe sommige van de verbindingen uit hoofdstuk 2 de afbraak van gechloreerde ethenen beïnvloeden. We hebben gevonden dat PCE-M2 nitraat (zit o.a. in kunstmest) liever gebruikt dan de gechloreerde ethenen en dat de verontreiniging pas wordt afgebroken nadat alle nitraat weg is. *Sulfurospirillum multivorans* heeft eveneens een voorkeur voor nitraat, maar breekt gechloreerde ethenen niet meer af nadat nitraat weg is. Het blijkt (en dat wordt ook door andere onderzoekers gevonden) dat verschillende tetrachlooretheen afbrekende bacteriën verschillend reageren op de aanwezigheid van nitraat.
Sulfaat komt eveneens vaak in de bodem voor. Het blijkt dat nagenoeg geen enkele bacterie die gechloreerde ethenen kan afbreken ook in staat is om sulfaat als substraat te gebruiken. Verder is gebleken dat de aanwezigheid van sulfaat de halorespirerende bacteriën niet stoort in het afbreken van de verontreiniging.
Sulfiet, een verbinding die veel op sulfaat lijkt, kan wel door sommige dechlorerende bacteriën (o.a. *Desulfitobacterium* soorten) gebruikt worden als substraat. Er is gevonden dat de aanwezigheid van sulfiet de afbraak van gechloreerde ethenen onmiddellijk stillegt. Sulfiet reageert chemisch met de enzymen die verantwoordelijk zijn voor de afbraak van gechloreerde ethenen. Hierdoor kunnen de gechloreerde ethenen niet meer afgebroken worden.
Tenslotte hebben we gekeken of bacteriën nog altijd gechloreerde ethenen kunnen afbreken wanneer ze op een andere substraat zijn doorgekweekt. Het blijkt dat PCE-M2 gechloreerde ethenen niet meer kan afbreken na groei op nitraat, selenaat of arsenaat.

Halorespirerende bacteriën hebben een hoge affiniteit voor waterstof. Dat betekent dat ze al bij zeer lage waterstof concentraties kunnen groeien. In hoofdstuk 4 hebben we bepaald welke waterstof concentratie (0.05-0.08 nM) halorespirerende bacteriën kunnen bereiken in het laboratorium onder gecontroleerde omstandigheden. We hebben ook onderzocht bij welke hoeveelheden waterstof de bacteriën de verontreinigingen nog afbreken in een verontreinigde bodem. Daar blijkt de concentratie zo'n 20 keer hoger te zijn. Het verschil met die lage waarde uit de

laboratorium proeven wordt onder andere veroorzaakt doordat in een bodem ook andere bacteriën aanwezig zijn die continu waterstof produceren. Halorespirerende bacteriën hebben waterstof nodig om verontreinigingen af te breken. Het blijkt dat de lage concentraties waterstof waaraan halorespirerende bacteriën genoeg hebben niet genoeg is voor andere bacteriën in de bodem. Hierdoor zijn halorespirerende bacteriën in het voordeel wanneer er maar weinig waterstof is. Hiervan wordt gebruik gemaakt wanneer men een verontreinigde bodem wil schoonmaken met bacteriën.

Wanneer bacteriën gechloreerde ethenen afbreken onder zuurstofloze omstandigheden, dan doen ze dat stapsgewijs. Het blijkt dat het niet altijd lukt om PCE volledig af te breken, vaak worden tussenproducten zoals dichlooretheen (DCE) en vinyl chloride (VC; hiervan wordt PVC gemaakt) niet verder afgebroken. Als gevolg hiervan hopen deze stoffen op in de bodem. Dat is een probleem omdat VC giftiger is dan PCE. Er is gekeken naar de afbraak van deze tussenproducten (hoofdstuk 5). We hebben 2 verschillende ophopingscultures verkregen die deze tussenproducten relatief snel kunnen afbreken onder zuurstofloze omstandigheden. Het is ons echter niet gelukt om te achterhalen welke bacterie verantwoordelijk was voor de relatief snelle afbraak. Op het moment dat we deze proeven deden was er nog geen bacterie geïsoleerd die dit kon. Inmiddels zijn enkele bacteriën geïsoleerd die op deze tussenproducten kunnen groeien.

Tenslotte hebben we in hoofdstuk 6 een overzicht gemaakt van wat er tot nu toe bekend is over de afbraak van gechloreerde ethenen door bacteriën onder zuurstofloze omstandigheden. We hebben hier onze resultaten gecombineerd met de resultaten van andere onderzoekers.

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Curriculum vitae

Op een zeer hete maandag, de 28e juli in de welbekende 'Summer of 69' zag Maurice Louis Gerard Clara Luijten het levenslicht.

Na de Petrus Canisius (lagere) school in Puth en het Albert Schweitzer Scholengemeenschap (VWO) in Geleen succesvol te hebben afgerond vertrok Maurice uit het mooie Limburg naar Wageningen. Daar werd van 1988 tot 1994 de studie Milieuhygiëne aan de Landbouw Universiteit doorlopen. De studie werd afgesloten met twee afstudeervakken met als specialisaties milieu-technologie/waterzuivering (optimalisatie van de verzurende stap in de thermofiele anaërobe afbraak van complex materiaal in UASB reactors) en microbiologie (isolatie en karakterisatie van een sulfaatreducerende bacterie). Daarnaast heeft Maurice twee stages gelopen, de eerste op het Ecotoxicologisch Laboratorium van AKZO-NOBEL in de groep van Dr. Van Ginkel (bestudering van slibleeftijd en adaptatie op de afbraak van o.a. detergenten in door de OECD voorgeschreven standaardtesten (Closed-bottle- en SCAS-test)) en de tweede op het lab van Prof. Bouwer (Department of Geography and Environmental Engineering) van de Johns Hopkins University (Baltimore, MD, USA) (afbraak van aromatische koolwaterstoffen onder micro-aërofiele en denitrificerende condities).

Na zijn studie werkte Maurice gedurende een jaar bij de vakgroep Milieutechnologie (Wageningen Universiteit) aan de anaërobe afbraak van azo-dyes. Hierna verbleef Maurice vanaf 1996 tot 2002 bij het Laboratorium voor Microbiologie (Wageningen Universiteit). In verschillende aanstellingen werkte hij aan de anaërobe afbraak van gechloreerde ethenen. Een van deze projecten was zijn promotie onderzoek zoals beschreven in dit proefschrift.

Inmiddels is Maurice sinds eind 2002 werkzaam bij de afdeling Milieubiotechnologie van TNO-MEP (Apeldoorn) waar hij als PostDoc werkt binnen het EU-CORONA project aan de chemische en microbiologische karakterisatie van een met gechloreerde ethenen verontreinigde grondwater pluim.

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