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Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium

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Abstract A strictly anaerobic bacterium dechlorinating tetrachloroethene (perchloroethylene, PCE) via trichloroethene (TCE) to *cis*-1,2-dichloroethene (DCE) was isolated from activated sludge with pyruvate plus PCE as energy substrates. The organism, called *Dehalospirillum multivorans*, is a gram-negative spirillum that does not form spores. The G+C content of the DNA was 41.5 mol%. According to 16S rRNA gene sequence analysis, *D. multivorans* represents a new genus and a new species belonging to the epsilon subdivision of *Proteobacteria*. Quinones, cytochromes *b* and *c*, and corrinoids were extracted from the cells. *D. multivorans* grew in defined medium with PCE and H_2 as sole energy sources and acetate as carbon source; the growth yield under these conditions was 1.4 g of cell protein per mol chloride released. Alternatively to PCE, fumarate and nitrate could serve as electron acceptors; sulfate could not replace fumarate, nitrate, or PCE in this respect. In addition to H_2, the organism utilized a variety of electron donors for dechlorination (pyruvate, lactate, ethanol, formate, glycerol). Upon growth on pyruvate plus PCE, the main fermentation products formed were acetate, lactate, DCE, and H_2. At optimal pH (7.3–7.6) and temperature (30°C), and in the presence of pyruvate (20 mM) and PCE (160 µM), a dechlorination rate of about 50 nmol min^{-1} (mg cell protein)$^{-1}$ and a doubling time of about 2.5 h were obtained with growing cultures. The ability to reduce PCE to DCE appears to be constitutive under the experimental conditions applied since cultures growing in the absence of PCE for several generations immediately started dechlorination when transferred to a medium containing PCE. The organism may be useful for bioremediation of environments polluted with tetrachloroethene.

Key words Anaerobic dehalogenation · Reductive dechlorination · Perchloroethylene · Tetrachloroethene Tetrachloroethene respiration · *cis*-1,2-Dichloroethene Trichloroethene · *Dehalospirillum multivorans*

Abbreviations *PCE* Perchloroethylene, tetrachloroethene · *TCE* Trichloroethene · *DCE* *cis*-1,2-Dichloroethene · *CHC* Chlorinated hydrocarbon

Introduction

Tetrachloroethene (perchloroethylene, PCE) is a volatile chlorinated hydrocarbon and is one of the pollutants most frequently found in ground water. Due to its toxic effects on biological systems, the application of PCE in dry cleaning in the textile industry, in the scouring of machines, and in fat extraction is no longer desirable. Therefore, efforts have been made to find substitutes for PCE and decontaminate polluted environments. For several years, the application of bioremediation for the decontamination of polluted environments has been discussed.

PCE is persistent under oxic conditions. During the last two decades, however, several reports on the complete dechlorination of tetrachloroethene in anaerobic enrichment cultures have been published (Vogel and McCarty 1985; Freedman and Gossett 1989; DiStefano et al. 1992; DeBruin et al. 1992). The main dechlorination products in these cultures are usually ethene or ethane. Recently, a highly enriched mixed culture of anaerobic bacteria has been obtained that is able to grow on an undefined medium with PCE plus H_2 or formate as sole energy sources (Holliger et al. 1993). The end product of PCE dechlorination in this system is *cis*-1,2-dichloroethene, which is formed via trichloroethene as intermediate. The

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organism responsible for the dechlorination is restricted to H_2 or formate as electron donor and to tetrachloroethene as electron acceptor in catabolism. In addition, the organism requires yeast extract for growth.

In this communication, we present the first report on the isolation of a pure culture of a tetrachloroethene-dechlorinating, strictly anaerobic bacterium growing on defined medium. In contrast to the organism formerly called PER-K23 enriched by Holliger et al. (1993), which is now available in pure culture (*Dehalobacter restrictus*; Holliger and Schumacher, in press), the bacterium described here was able to utilize a variety of electron donors and fumarate as an alternative electron acceptor in its energy metabolism. Due to its outstanding ability to dehalogenate tetrachloroethene, its morphology, and its relatively wide substrate spectrum, the bacterium was called *Dehalospirillum multivorans*. The characterization of the organism is described.

Materials and methods

Source of materials

All chemicals used were of the highest available purity and purchased from Fluka (Neu-Ulm, Germany) and Merck (Darmstadt, Germany). Tetrachloroethene was from Ferak (Berlin, Germany), *cis*-1,2-dichloroethene from Aldrich (Steinheim, Germany). Gases (CO_2 grade 4.8, H_2 grade 4.3, and N_2 grade 4.6) were supplied by Messer Griesheim (Düsseldorf, Germany).

Isolation and cultivation of *Dehalospirillum multivorans*

Dehalospirillum multivorans was enriched from activated sludge using the medium described below containing pyruvate (20 mM) plus tetrachloroethene (200 µM) as energy sources. The bacteria were routinely grown on anaerobic media composed of 1 l basal medium (see below), 0.5 ml vitamin solution, 2 ml trace element solution SL10, 1 ml B_{12} solution (cyanocobalamin, 50 mg/l), 0.2 ml selenite solution ($Na_2SeO_3 \cdot 5H_2O$, 26 mg/l), 0.1 ml tungstate solution ($Na_2WO_4 \cdot 2\ H_2O$, 33 mg/l), and 30 ml $NaHCO_3$ solution (84 g/l; autoclaved separately under CO_2). Where indicated, 20 ml yeast extract solution (yeast extract from Oxoid, Wesel, Germany, 100 g/l) and/or 4 ml sulfide solution ($Na_2S \cdot 9H_2O$, 120 g/l; autoclaved separately under N_2) were added. Pyruvate and fumarate, when used as substrates, were sterilized by filtration in 1 M solutions and added later to the medium to give a final concentration of 40 mM each. When tetrachloroethene was applied as electron acceptor instead of fumarate, it was added to the medium either directly (e.g., for the enrichment) or as an autoclaved solution in hexadecane. To obtain single colonies, solid media with 3% agar in roll-tubes were used.

The basal medium contained per liter: 70 mg Na_2SO_4, 200 mg KH_2PO_4, 250 mg NH_4Cl, 1 g NaCl, 400 mg $MgCl_2 \cdot 6H_2O$, 500 mg KCl, and 150 mg $CaCl_2 \cdot 2H_2O$. For chloride-poor media, the chlorides were replaced throughout by the corresponding bromides. The trace element solution was composed of 1 l H_2O, 10 ml HCl [25% (w/v)], 1 g $FeSO_4 \cdot 7H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_2O$, 6 mg H_3BO_3, 130 mg $CaCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, and 36 mg $Na_2MoO_4 \cdot 2H_2O$. The vitamin solution contained per liter: 80 mg 4-aminobenzoic acid, 20 mg D(+)biotin, 200 mg nicotinic acid, 100 mg Ca-D(+)pantothenate, 300 mg/l pyridoxamine-2HCl, and 200 mg thiamine-HCl. The final pH of the medium was between 7.2 and 7.4. The bacteria were grown in glass bottles stoppered with Teflon-lined rubber septa. The gas phase was N_2/CO_2 [80:20 (v/v); 150 kPa]. For the growth experiment on H_2 plus PCE, a gas phase of H_2/CO_2 [90:10 (v/v); 200 kPa] was used; PCE was added as a 0.5 M solution in hexadecane at a final concentration of 15 mM. Acetate was added as a carbon source at a final concentration of 5 mM. The medium was inoculated with 10% of a grown culture and incubated at 30°C and 200 rpm in a gyratory water bath shaker. Growth was determined either by the absorption at 578 nm, by the cell number per milliliter, or by the protein content of the cells.

Protein was determined essentially according to Bradford (1976) using the Bio-Rad reagent (Bio-Rad, Munich, Germany). The reagent was diluted 2:5 with quartz-distilled H_2O, and 45 ml HNO_3 was added prior to the addition of the sample (see below). NaOH (100 µl; 0.2 M) was added to 400-µl samples and the suspensions were incubated at 100°C for 5 min. After cooling at 0°C, the suspensions were centrifuged for 1 min at 13,000 × *g* in an Eppendorf centrifuge. Supernatant (455 µl) was added to 500 µl of the modified reagent (see above). The absorption of the solution was immediately measured at 620 nm and 465 nm; the ratio E_{620}/E_{465} was plotted versus the protein concentration. An albumin solution (concentrate for standard curve preparation with protein assays; Pierce, Rockford, Ill., USA) was used as a standard.

DNA analysis

The G+C content of the DNA was determined by HPLC by the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) essentially according to Mesbah et al. (1989).

Genomic DNA was isolated from about 0.1 g (wet weight) cells and by the method of Wilson (1987). The 16S rDNA was targeted for amplification by polymerase chain reaction (PCR; Saiki 1988), and DNA sequencing was performed as described by Karlson et al. (1993).

Extraction and determination of quinones, cytochrome *b*, cytochrome *c*, and corrinoids

Quinones were extracted from cells of *D. multivorans* and *Escherichia coli* according to Kröger (1978) and to Kröger and Innerhofer (1976a). Wet cells (1 g) were suspended in 2 ml H_2O and subjected to ultrasonic disintegration for 90 s at 0°C in intervals of 1 s. A sample was taken from the lysate for the protein determination (see above). The lysate was diluted with H_2O to a protein concentration of about 17–20 mg protein per ml. Then, 20 µl of 1% H_2O_2 (w/v) was added per milliliter. The extraction was performed after subsequent addition of 8 ml per ml lysate of 1:1 (v/v) methanol/petroleum benzene (bp between 40 and 60°C) and 4 ml acetone. The samples were incubated for 15 min at 22°C and 300 rpm in a gyratory shaker. The lysate was centrifuged for 5 min at 2,000 × *g*. The organic phase was carefully removed. The water phase was re-extracted with 4 ml methanol/petroleum benzene per ml. The organic phases were combined and subjected to rotary evaporation under reduced pressure. The residue was dissolved in 1.8 ml ethanol. The solution was diluted 1:2 with ethanol. Extract (1 ml) was transferred to a quartz cuvette and the absorption spectrum was recorded (200–400 nm). Then, 10 µl portions of a KBH_4 solution (5 mg/ml) were added. After each addition, the spectrum was recorded versus the spectrum of the oxidized extract. By the addition of KBH_4 (≥ 30 µl) the sample was alkalized; only reduced ubiquinone should be detected since ubiquinone is stable in the reduced state under alkaline conditions. Subsequently, after the addition of 40 µl KBH_4, 10 µl portions of sodium acetate buffer 0.5 M (pH 5.5) were added to acidify the solution. The spectrum was recorded again, now revealing the presence of menaquinone, the reduced state of which is stable under acidic conditions (Kröger 1978).

Cytochrome *b* was measured according to Kröger and Innerhofer (1976b). Pyruvate plus fumarate plus yeast-extract-grown cells [2.2 g (wet weight)] of *D. multivorans* or of aerobically grown *E. coli* were suspended in 3 ml 0.1 M Tris-HCl (pH 7.5) containing 1 mM $MgCl_2 \cdot 6H_2O$, 30 mg lysozyme, and 3 mg DNase I.

The cell suspension was incubated for 45 min at 37°C and subsequently centrifuged at 4°C and 10,000 × *g* for 10 min. The supernatant (3.5 ml) was centrifuged for 105 min at 100,000 × *g* and 4°C. The pellet (membrane fraction) was suspended in 2 ml 0.1 M Tris-HCl (pH 7.5), and the absorption spectrum was measured. A sodium dithionite solution was then added until a constant absorption spectrum was obtained. The spectrum of reduced versus oxidized cytochrome *b* was recorded. The concentration of cytochrome *b* was calculated from the absorbance difference ΔE_{561}–ΔE_{575}, assuming a $\Delta\varepsilon_{561-575}$ of 26.2 $mM^{-1}cm^{-1}$ (see Kröger and Innerhofer 1976b).

Cytochrome *c* was extracted essentially using the method of Kröger and Innerhofer (1976b) from 10 g cells (wet weight) of *D. multivorans* or *Wolinella succinogenes*. The cells were suspended in 20 ml of the same buffer (with lysozyme plus DNase I) used for the cytochrome *b* determination (see above). After incubation for 45 min at 37°C, the suspension was centrifuged for 10 min at 10,000 × *g* and 4°C. The supernatant was centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant was applied to a DEAE-Sepharose fast flow column (10 × 100 mm; Pharmacia, Uppsala, Sweden). The cytochromes were eluted with 0.1 M Tris-HCl (pH 7.5) plus 0.5 mM dithiothreitol. The fractions containing the soluble cytochromes were combined and subjected to ultrafiltration on Centricon (exclusion limit 10,000; Amicon, Beverly, Mass., USA) to a final volume of 6.5 ml. The concentrate was then dialyzed (tubing exclusion limit 10,000) overnight against H_2O and subsequently applied to a Serdolit column CG 50II (200–400 mesh; counter ion H^+; 10 × 100 mm; Serva, Heidelberg, Germany) . Elution was carried out with a linear gradient of H_2O : 0.5 M Tris-HCl (pH 7.6) (20 ml each); 4-ml fractions were collected. Fraction 7, which contained the soluble cytochrome, was analyzed photometrically by recording the reduced versus the oxidized spectrum as described above for the determination of cytochrome *b*. Cytochrome *c* was quantified by calculating the absorbance difference at 553 and 540 nm using a $\Delta\varepsilon_{553-540}$ of 10.8 $mM^{-1}cm^{-1}$ (see Kröger and Innerhofer 1976b).

Corrinoids were extracted as the dicyano-complex from 2 g of cells (wet weight) according to Scherer and Sahm (1981). As a control, cells of *Clostridium thermoaceticum* were used. Methanol (8 ml) containing 0.01% KCN (w/v) were added to the cells. The pH was adjusted to 6.0 with HCl. The suspension was incubated in a tightly closed bottle for 10 min at 100°C and then centrifuged at 25°C and 2,000 × *g* for 10 min. The pellet was re-extracted using the procedure described above. The supernatants were combined and methanol was removed by rotary evaporation under reduced pressure at 37°C. The residue was dissolved in 4 ml quartz-distilled H_2O. To this solution, 2.5 ml (bed volume) of a Q-Sepharose suspension (Q-Sepharose fast-flow from Pharmacia, Uppsala, Sweden) pre-equilibrated in 50 mM Tris-HCl (pH 8) was added. After stirring for 2 h at 0°C, the suspension was centrifuged for 10 min at 2,000 × *g*. The pellet was washed with 5 ml of the same buffer. The supernatants were combined and applied to a Q-Sepharose column (10 × 30 mm) pre-equilibrated with 50 mM Tris-HCl (pH 8). The corrinoids were eluted with the same buffer. The fractions containing the corrinoids were applied to an XAD column (10 × 40 mm) (Amberlite XAD-2, Serva, Heidelberg, Germany) pre-equilibrated with 40% (v/v) methanol and washed with 20 ml H_2O (degassed) prior to the application of the supernatant. The elution was performed sequentially with H_2O, 40% methanol, and 80% methanol, 10 ml each. The corrinoids were eluted with 80% methanol. The absorption spectrum was recorded and the corrinoid concentration was calculated from the extinction at 361 nm (ε_{361}=28.06 $mM^{-1}cm^{-1}$).

Determination of chlorinated ethenes

Tetrachloroethene, trichloroethene, and *cis*–1,2-dichloroethene were determined gas chromatographically by flame ionization detection using a 2 m column of 10% Ucon LB on WAW (WGA Analysentechnik, Düsseldorf, Germany) and N_2 as carrier gas. The following temperatures were applied: column, 80°C; injector, 150°C; detector, 250°C. The carrier gas flow was 25–30 ml/min (300 kPa), the gas sample volume was 500 µl. The retention times were: PCE, 2.76 min; TCE, 1.76 min; *cis*–1,2-DCE, 1.2 min; *trans*-1,2-DCE, 0.8 min; 1,1-DCE, 1.0 min. Standard solutions of PCE, TCE, or DCE were prepared by adding the chlorinated hydrocarbon (CHC) to H_2O (10 µl CHC/l H_2O) and stirring overnight. These standard solutions were then treated as described below for the samples.

For the analysis of the chlorinated hydrocarbons in the cultures, 1-ml samples were taken from the liquid phase and added with a syringe to 10 ml serum bottles stoppered with Teflon-lined butyl rubber septa and containing 1 g Na_2SO_4. The serum bottles were incubated for 1 h at 95°C. Samples (0.5 ml) were then taken from the gas phase, which contained the chlorinated hydrocarbons, and analyzed immediately for tetrachloroethene, trichloroethene, and dichloroethene.

The dechlorination activities given in this paper are calculated as amount of Cl^- released per min. Chloride release was determined either directly (see below) or calculated from the amount of tetrachloroethene (PCE) consumed and trichloroethene (TCE) formed or consumed, respectively, according to the following equation:

$$v = (2 \times \Delta[PCE]_{consumed} + \Delta[TCE])/t$$
$$(\Delta[PCE]_{consumed} = [PCE]_{t=t1} - [PCE]_{t=t2};$$
$$\Delta[TCE] = [TCE]_{t=t1} - [TCE]_{t=t2})$$

Determination of substrates and metabolites

The chloride concentration was quantified by means of ion chromatography (Ion chromatograph 690, Metrohm, Filderstadt, Germany) on Polyspher IC AN (10 m) (Merck, Darmstadt, Germany). Samples were passed through a guard column 10–4 and subsequently through a pre-packed RT 50–6 column (6 × 50 mm). Anions were determined by conductivity detection. The eluent flow was 1 ml/min; the eluent was a solution of 0.867 g potassium hydrogen phthalate plus 200 ml 2-propanol and 22.7 ml ethylene glycol in 5 l H_2O, adjusted to pH 4.65 with KOH. The column temperature was 35°C; the retention time of chloride, 4.3 min.

H_2 was determined gas chromatographically using thermal conductivity detection. Carbonic acids were determined by HPLC using a BioRad Aminex HPX-87H column (7.8 × 300 mm; BioRad, Munich, Germany), and a cation H guard column. Samples were taken from the liquid phase of the culture and acidified with concentrated H_2SO_4 (5 µl/ml sample volume). The sample was centrifuged at 16,000 × *g* for 10 min, and 50 µl of the supernatant was applied onto the column. A column temperature of 50°C was used; the eluent was 5 mM H_2SO_4 at a flow rate of 0.7 ml/min. The acids (e.g., formate, acetate, pyruvate, succinate, and lactate) were detected by their absorption at 220 nm. The retention times and areas of the peaks were related to standard solutions. The retention times under the conditions chosen were: pyruvate, 8.2 min; succinate, 10.5 min; lactate, 11.2 min; formate, 12.2 min; acetate, 13.4 min.

Results

Isolation of *Dehalospirillum multivorans*

Dehalospirillum multivorans was enriched from activated sludge that had not been exposed to chlorinated ethenes on a defined medium with pyruvate (20 mM) plus tetrachloroethene (PCE; 0.2 mM) as sole energy sources. A pure culture was obtained by repeated transfers in roll-tubes on agar medium containing 40 mM pyruvate as sole energy source. Approximately 5 days after inoculation, round, rough, and entire colonies with a diameter of almost 1 mm were detected. The colonies were resuspended and again transferred to roll-tubes. This procedure was repeated twice. Single colonies were then transferred to li-

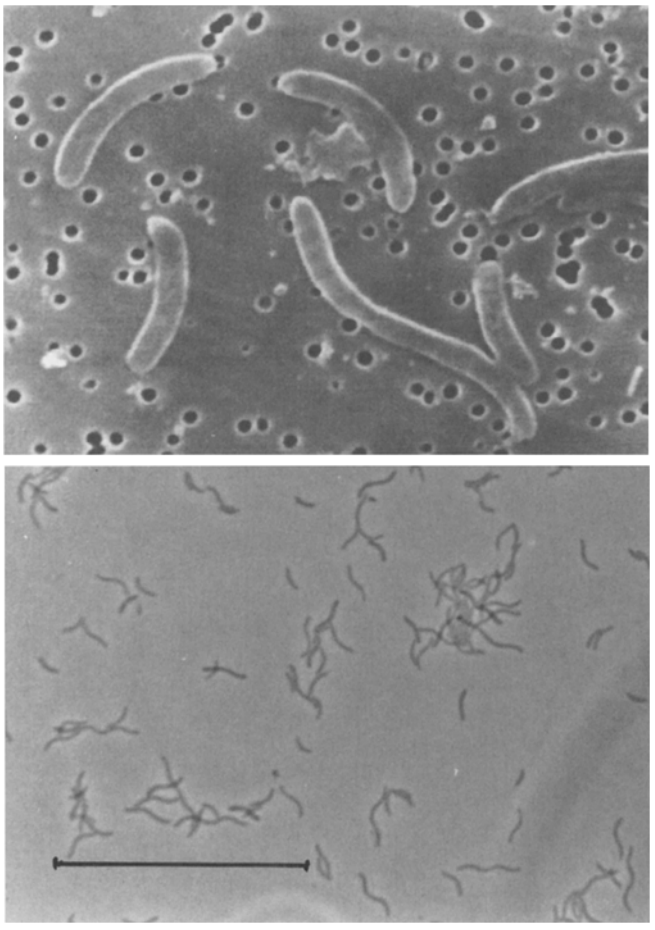

Fig. 1 **A** Scanning electron micrograph of *Dehalospirillum multivorans* fixed on polycarbonate filters with a pore size of 0.2 µm and sputtered with gold. **B** Phase-contrast micrograph of the bacterium (*bar* 20 µm)

quid media with 20 mM pyruvate plus 180 µM tetrachloroethene. Using this procedure, pure cultures of tetrachloroethene-dechlorinating bacteria were finally obtained that grew with a doubling time of about 2.5 h on this medium at 30°C. The bacteria grew in liquid media at temperatures between 15 and about 33°C, with an optimum near 30°C; no growth was observed at 37°C (data not shown). The optimal pH for growth was between 7.0 and 7.5.

Morphological characterization, phylogenetic classification, and other characteristics

Phase contrast microscopy, fluorescence microscopy (data not shown), and scanning electron microscopy (Fig. 1) of the isolate revealed curved or helical rods with a rapid darting motility in some of the strains isolated; the cells of the strain used in the studies described here appeared to be predominantly non-motile. In general, the cells occurred singly. Endospore formation was not observed. The bacteria were gram-negative. Under phase contrast microscopy, the cells exhibited a low contrast, even for a gram-negative bacterium. They were much paler than cells of *Wolinella succinogenes*, which resembled those of *D. multivorans* with respect to cell shape and size. The cell size of *D. multivorans* was 0.45 µm (diameter) by 2–5 µm (length).

The G+C content, determined after isolation of the DNA by HPLC, was determined to be 41.5 mol%. PCR amplification of the 16S rRNA gene, using primers annealing at positions 8–27 (*Escherichia coli* 16S rDNA numbering) and the complement of positions 1524–1541, allowed the determination of an estimated 97% of the complete gene (1464 nucleotide positions). The 16S rDNA sequence has been deposited with the EMBL under accession number X82931. Derived phylogenetic/taxonomic relationships of *Dehalospirillum multivorans* within the epsilon subgroup of *Proteobacteria* are depicted in Fig. 2.

Quinones and cytochromes were extracted from cells grown on a medium supplemented with 0.2% yeast extract with pyruvate plus fumarate as energy sources. For the extraction of quinones, 1 g of cells (wet weight) was used; cells of aerobically grown *E. coli* served as a control. The absorption spectrum of reduced versus oxidized quinones extracted from *D. multivorans* cells strongly resembled that of menaquinone obtained from *Wolinella succinogenes* as published by Kröger and Innerhofer (1976a) and was different from that of the *E. coli* extracts, indicating that the quinone extracted from cells of *D. multivorans* was menaquinone. This assumption was substantiated by the finding that the quinone was easily re-oxidized by oxygen under alkaline conditions (Kröger 1978). The amount of quinone in *D. multivorans* (Table 1) was comparable to that of *E. coli* cells (which contain ubiquinone rather than menaquinone) and about half the amount

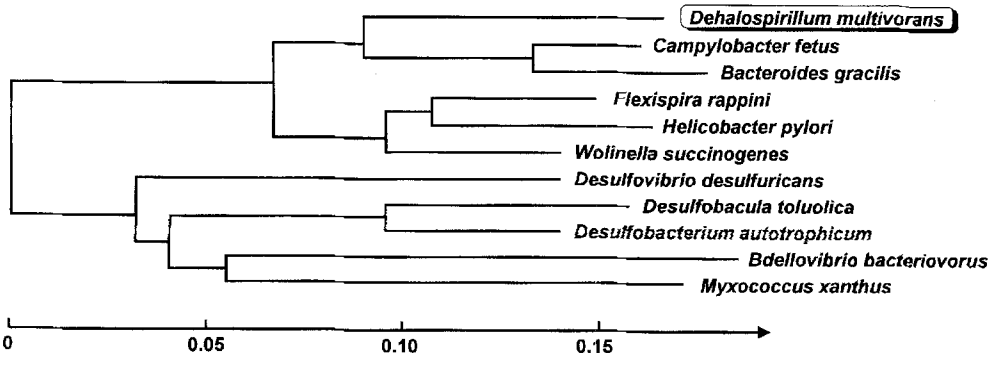

Fig. 2 Estimated phylogenetic position of *Dehalospirillum multivorans* among members of the epsilon and delta subgroups of *Proteobacteria* according to 16S rDNA analysis

Table 1 Amount of menaquinone, cytochrome b (membrane fraction), cytochrome c (soluble fraction), and corrinoids detected in cells of *D. multivorans*. The analysis of the compounds is described in detail in 'materials and methods'

Compound	Amount in the cells (nmol per g cell protein)
Menaquinone	1,800
Cytochrome b	40
Cytochrome c	550
Corrinoids	20

of menaquinone reported to be present in *W. succinogenes* (Kröger and Innerhofer 1976a).

Cytochromes were extracted from *D. multivorans* cells; *E. coli* (for cytochrome *b*) or *W. succinogenes* (for cytochrome *c*) served as a control. Cytochrome *b* was detected in the membrane fraction of *D. multivorans* after ultracentrifugation of the extracts (Table 1). The supernatant of the ultracentrifugation was subjected to column chromatography as described in Materials and methods for the detection of soluble cytochromes (cytochrome *c*). The absorption spectrum revealed the presence of a cytochrome of the *c*-type in *D. multivorans* (Table 1). It was almost identical to that of the column eluate obtained from extracts of *W. succinogenes*.

The corrinoid content of the cells was determined by extraction of the corrinoids as the dicyano-complex. *Clostridium thermoaceticum* served as a positive control. The absorption spectrum of the purified corrinoid revealed the presence of about 20 nmol per g of cell protein for *D. multivorans* (Table 1). This corresponded to about one-tenth of the corrinoid content of *C. thermoaceticum*.

Growth with pyruvate plus tetrachloroethene

D. multivorans was grown on a defined medium with 20 mM pyruvate as electron donor in the absence or presence of tetrachloroethene (18 µmol added directly to 50 ml culture; gas phase was 65 ml) or 20 mM fumarate as electron acceptors. The growth curves are depicted in Fig. 3A. The organisms were also able to utilize nitrate (10 mM) as electron acceptor (data not shown); growth with nitrate appeared to be dependent on yeast extract under the conditions applied. Although the organisms contained a nitrate as well as a nitrite reductase, nitrite rather than ammonia or N_2 was formed from nitrate. Growth with pyruvate as sole energy source under the conditions applied was poor. Tetrachloroethene significantly stimulated growth. The highest cell densities were obtained with fumarate or nitrate as electron acceptors (shown for fumarate in Fig. 3 A). The ability to dechlorinate PCE was not lost even after numerous transfers of the bacteria on medium containing pyruvate plus fumarate as energy sources and supplemented with yeast extract.

The kinetics of tetrachloroethene dechlorination during growth of *D. multivorans* with pyruvate plus tetrachloroethene are shown in Fig. 3B. During growth, tetrachloroethene is reductively dehalogenated via trichloroethene to *cis*-1,2-dichloroethene. For the evaluation of the stoichiometry of DCE formed per PCE reduced, it has to be kept in mind that the CHC concentration given in Fig. 3B represents the concentration in the liquid phase. Henry's constant H_c ($[CHC]_{gas}/[CHC]_{liq}$) at 30°C for PCE, TCE, and DCE was 0.92, 0.50, and 0.19, respectively. From the culture (50 ml liquid phase, 65 ml gas phase initially), 2-ml samples were taken from the liquid phase and analyzed for the CHC concentration. Due to the sampling (12 ml of the culture) and to CHC diffusion through the rubber septa (20% within 40 h of incubation), the CHC recovery in DCE was about 70%. It can be calculated from the experiment shown in Fig. 3B that the total amount of PCE added to the flask was about 18 µmol; at the end of the experiment about 12.5 µmol DCE was recovered in the culture (liquid phase plus gas phase).

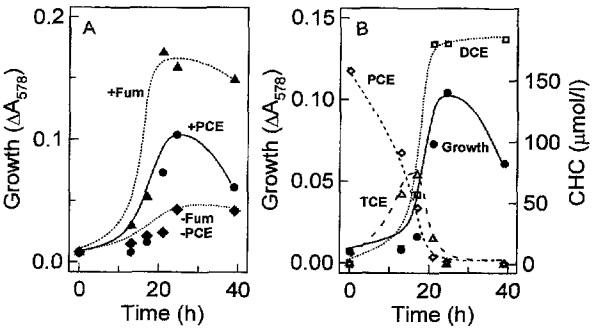

Fig. 3 **A** Growth of *Dehalospirillum multivorans* on defined media with pyruvate (20 mM) in the absence (*filled diamonds*) and presence of fumarate (20 mM; *filled triangles*) or tetrachloroethene (added directly to the culture; *filled circles*). **B** Dechlorination kinetics of *D. multivorans* growing with pyruvate plus tetrachloroethene. Growth temperature was 30°C, the pH was 7.3. At the time points indicated, samples were taken from the liquid phase and analyzed for the chlorinated ethenes. Since *cis*-1,2-dichloroethene is more soluble in water than tetrachloroethene (see text), the concentration of DCE in the liquid phase at the end of growth is higher than that of PCE. A ΔA_{578} value of 1 corresponds to a cell protein concentration of approximately 250 mg per l culture. *CHC* Chlorinated hydrocarbon [*PCE* (*open diamonds*), *TCE* (*open triangles*), *DCE* (*open squares*)], *growth* (*filled circles*)

D. multivorans was grown on chloride-poor mineral medium with pyruvate plus tetrachloroethene, and the concentrations of pyruvate and of the fermentation products as well as the biomass were determined at the start of the growth experiment and in the exponential growth phase. Acetate, lactate, H_2, and Cl^- (as the product of reductive dechlorination of PCE and TCE) were formed from pyruvate plus tetrachloroethene. Neither ethanol nor formate was detected; the detection limit was lower than 0.1 mM. CO_2 could not be measured since a carbonate buffer was used to ensure optimal growth conditions. The concentration of substrates and products measured at the start and in the log phase is given in Table 2. It should be noted that the ratio of products formed varied within a limited range in different experiments performed under identical experimental conditions. The variation was restricted mainly to the H_2, lactate, and Cl^- concentrations.

Table 2 Exemplary fermentation balance of *Dehalospirillum multivorans* upon growth on pyruvate plus tetrachloroethene. For details see text and 'Materials and methods'. [S] = substrate concentration, [P] = product concentration

S(ubstrate) or P(roduct)	[S] or [P] at t = 0 h	[S] or [P] at t = 47 h	Δ [S] or Δ [P]
Pyruvate	15.8 m*M*	7.8 m*M*	8.0 m*M*
Acetate	0 m*M*	6.1 m*M*	6.1 m*M*
Lactate	0 m*M*	1.4 m*M*	1.4 m*M*
H_2	0 m*M*	0.5 m*M*	0.5 m*M*
Cl^-	0.2 m*M*	1.6 m*M*	1.4 m*M*
Biomass	< 0.1 mg/l	22.8 mg/l	22.8 mg/l

The more Cl^- was released, the less H_2 and/or lactate was formed. Depending on the cultures, up to 50% of the reducing equivalents derived from pyruvate oxidation was recovered in the reduced chloroethenes or in the chloride released. In the experiment presented in Table 2, the recovery of carbon was 94% and the recovery of reducing equivalents about 91%, assuming the amount of CO_2 to be equivalent to that of acetate formed. When balancing the H and O recovery with respect to the H_2O required for substrate conversion, the following fermentation equation was deduced from the experiments shown in Table 2:

$$100\ \text{pyruvate}^- + 18\ \text{XCl} + 53\ H_2O \rightarrow$$
$$76\ \text{acetate}^- + 76\ CO_2 + 18\ \text{lactate}^- + 6H_2 + 18\ \text{XH}$$
$$+ 18\ H^+ + 18\ Cl^-$$

(XCl = chlorinated ethene = tetrachloroethene or trichloroethene).

For the reasons described above, this reaction equation must be considered as an example rather than as a constant fermentation balance.

In the absence of tetrachloroethene, essentially the same fermentation products were formed, with the exception of chloride. Under these conditions, H_2 and/or lactate formation, rather than tetrachloroethene reduction, served as an electron sink for metabolic oxidations (data not shown).

Utilization of other substrates

It was already stated above that *D. multivorans* was able to utilize fumarate or nitrate instead of tetrachloroethene as electron acceptors. The organism was also able to grow with electron donors other than pyruvate (see also Table 3). In this respect, it was of great interest to determine whether *D. multivorans* is able to utilize electron donors that do not supply ATP via substrate level phosphorylation upon metabolic oxidation, for example, H_2 or formate. *D. multivorans* was not able to grow with H_2 or formate plus tetrachloroethene without an additional carbon source, indicating that the organism is not capable of autotrophic growth with CO_2. Therefore, the organism was grown on a defined medium with H_2/CO_2 (90:10; v/v) plus tetrachloroethene (dissolved in hexadecane; 15 mmol

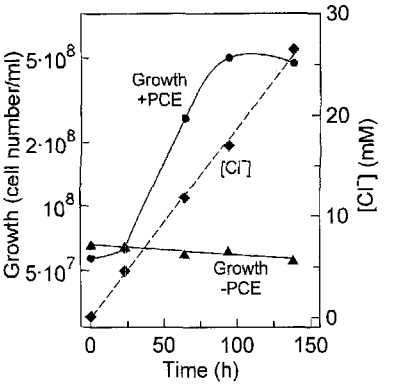

Fig. 4 Growth and chloride production of *Dehalospirillum multivorans* upon growth on chloride-poor defined media with H_2/CO_2 (90:10, v/v; 200 kPa) plus PCE (total concentration 15 mM, applied as a solution in hexadecane) as sole energy sources and acetate (5 mM) as carbon source. Inoculated medium without PCE served as a control. Growth temperature was 30°C. In the experiment, less than 1 mM acetate was consumed

total amount of PCE added to 1 l culture volume) as sole energy sources and with acetate (5 mM) as sole carbon source. (Hexadecane was not metabolized by the bacterium). The kinetics of growth and of chloride released are shown in Fig. 4. As a control, medium without tetrachloroethene containing H_2 plus acetate was inoculated and growth was determined (Fig. 4). In the presence of PCE, the bacteria grew to a cell density of approximately 5×10^8 cells per ml, corresponding to a cell protein concentration of about 15 mg/l; less than 1 mM of acetate was consumed in the experiment. From the chloride released and biomass (given as cell protein) formed in the late exponential growth phase (t = 64 h to t = 94 h), a specific growth yield of 1.4 g cell protein per mol Cl^- released was calculated.

In the absence of PCE, no growth was observed (Fig. 4). The same was valid for inoculated media without H_2 and with acetate plus PCE (data not shown). H_2 could be replaced by formate. The findings show that *D. multivorans* is able to couple the electron transfer from H_2 or formate to tetrachloroethene with energy conservation.

In addition to pyruvate, formate, or H_2, *D. multivorans* was able to utilize other electron donors for PCE, nitrate, or fumarate reduction. The organism grew with lactate, ethanol, and glycerol as electron donors; none of these substrates could be utilized in the absence of tetrachloroethene or fumarate as electron acceptors. It should be noted that the growth yield was much lower with lactate plus PCE than with pyruvate plus PCE. The sugars and sugar alcohols tested (with the exception of glycerol) were not fermentable (Table 3). Acetate and succinate were not utilized and they did not serve as electron donors for tetrachloroethene reduction (Table 3; see also Neumann et al. 1994). Pyruvate was the only substrate fermentable in the absence of an electron acceptor. On a complex medium with pyruvate plus fumarate supplemented with yeast extract (0.2%), *D. multivorans* grew within 12 to 15 h to an OD_{578} of about 0.7, corresponding

Table 3 Substrate spectrum of *Dehalospirillum multivorans* on defined medium. For details see 'materials and methods'. The substrates were applied in a concentration of 20 m*M* with the exception of H_2 (33%) and PCE (200 µ*M*). When H_2 or formate were used as energy sources, acetate (5 m*M*) was added as carbon source

Electron donor	Electron acceptor	Growth	Dechlorination
Pyruvate	None	+	
	Fumarate	+++	
	PCE	++	Yes
Lactate	None	–	
	Fumarate	++	
	PCE	+	Yes
Ethanol	None	–	
	Fumarate	+++	
	PCE	+	Yes
Formate	None	–	
	Fumarate	++	
	PCE	+	Yes
Glycerol	None	–	
	Fumarate	++	
	PCE	+	Yes
H_2	None	–	
	Fumarate	++	
	PCE	+	Yes
Acetate	None	–	
	Fumarate	ND	
	PCE	–	No
Na_2S	None	ND	
	Fumarate	++	
	PCE	ND	ND
Glucose	None	–	
	Fumarate	–	
	PCE	–	No
Fructose	None	–	
	Fumarate	–	
	PCE	–	No
Mannitol	None	–	
	Fumarate	–	
	PCE	–	No
Sorbitol	None	–	
	Fumarate	–	
	PCE	–	No

+++ = Good growth, ++ = reasonable growth, + = growth, – = No growth; ND = Not determined

to a cell protein concentration of about 175 mg/l. Since the ability to dechlorinate appeared to be constitutive (see above), large numbers of cells with dechlorination activity can be obtained. Yeast extract stimulated growth in the sense that higher growth yields, rather than higher growth rates, were obtained.

Discussion

In this communication, the first isolation of a pure culture of tetrachloroethene-dechlorinating bacteria, *Dehalospirillum multivorans*, capable of growing on mineral medium with H_2 plus tetrachloroethene as sole energy sources is described. Compared to *Dehalobacter restrictus* (strain PER-K23) described by Holliger et al. (1993), *D. multivorans* is versatile with respect to its substrate spectrum. *D. restrictus* is restricted to the use of H_2 or formate as electron donors and tetrachloroethene as the only utilizable electron acceptor and requires supplementation of the medium with yeast extract. *D. multivorans* grew on defined media with a variety of electron donors and at least fumarate and nitrate as alternative electron acceptors; preliminary experiments indicate that sulfur might also serve as electron acceptor for *D. multivorans* (Neumann et al. 1994). With respect to its morphological and physiological characteristics, the isolate closely resembles *W. succinogenes*, although the latter organism is not able to dechlorinate tetrachloroethene. Cytochrome *b* and menaquinone, both of which are components of the fumarate respiratory chain, and cytochrome *c* were found to be present in *D. multivorans* cells. In addition, the presence of corrinoids was confirmed. This is important, as the involvement of corrinoids in the abiotic dechlorination of chlorinated hydrocarbons has been reported (see for example Krone et al. 1989; Gantzer and Wackett 1991; Jablonski and Ferry 1992). Moreover, it has been shown that tetrachloroethene dechlorination in cell suspensions of *D. multivorans* is inhibited by propyl iodide (Neumann et al. 1994), which supports the idea of the involvement of a corrinoid in tetrachloroethene dechlorination, although the effect of propyl iodide is not absolute proof. An abiotic dechlorination of tetrachloroethene by corrinoids present in the cells was excluded by experiments with heat-treated cell extracts of the organism (Neumann et al. 1994). The high rates of dechlorination [near 50 nmol min^{-1} (mg cell protein)$^{-1}$ in growing cultures and about 500 nmol min^{-1} (mg cell protein)$^{-1}$ in cell extracts (Neumann et al. 1994)] also argue against abiotic dechlorination. The finding that *D. multivorans* was able to grow with H_2 plus tetrachloroethene as sole energy sources demonstrates that the organism couples the electron transfer from H_2 to tetrachloroethene with ATP synthesis. Since neither the oxidation of H_2 nor the reductive dechlorination of chlorinated ethenes can be mechanistically coupled to substrate level phosphorylation, ATP synthesis must proceed via a chemiosmotic mechanism.

The redox potential of the PCE/TCE pair is estimated to be +0.58 V, that of the TCE/DCE pair, +0.53 V (Vogel et al. 1987). From these data, the free energy under standard conditions ($\Delta G^{\circ\prime}$) for H_2 plus PCE conversion to DCE according to the equation

$$PCE + 2\ H_2 \rightarrow \textit{cis}\text{-}1,2\text{-}DCE + 2\ H^+ + 2\ Cl^-$$

can be estimated to be about –376 kJ/mol. The low growth yield (1.4 g cell protein per mol Cl^- released) with H_2 plus

tetrachloroethene as energy substrates indicates that the organism conserves only a minor part of the energy theoretically available for the synthesis of ATP. Holliger et al. (1993) reported a growth yield for *D. restrictus* of 2.1 g protein per mol Cl^- released. The higher growth yield of this organism as compared to that of *D. multivorans* may be due to the fermented yeast extract supplement to the medium used for cultivation of *D. restrictus*, whereas *D. multivorans* was grown on a defined medium with acetate plus CO_2 as sole carbon sources.

The question remains unanswered as to which reaction(s) of the electron transport chain, from H_2 to tetrachloroethene or trichloroethene, is (are) coupled to energy conservation. It is doubtful that the reduction of tetrachloroethene or trichloroethene or the reduction of trichloroethene to dichloroethene is directly coupled to proton extrusion, as evidence is available that the tetrachloroethene dehalogenase, measured by methyl viologen oxidation with PCE, is probably not membrane-associated, which would be a prerequisite for its activity as a proton pump (Neumann et al. 1994). Therefore, it is feasible that an extremely primitive electron transport chain is involved, wherein H_2 is converted to protons outside the cells, thus building up a proton gradient. The electrons are somehow translocated across the cytoplasmic membrane to an electron carrier that serves as electron donor for reductive dechlorination inside the cell, thus consuming one proton per chloride released (see also Holliger and Schumacher, in press). This mechanism would not require a membrane-bound dehalogenase. The only prerequisite for such a mechanism would be a localization of the hydrogenase either outside the cells (which would imply the involvement of an additional electron carrier in the membrane) or within the cytoplasmic membrane (which would imply the involvement of an electron carrier in the cytoplasm). Extensive studies on the localization and electron carrier specificity of enzymes possibly involved in energy conservation of *D. multivorans* are presently being carried out in our laboratory.

Description of *Dehalospirillum* gen. nov.

Dehalospirillum (De.ha.lo.spi.rillum. L. pref. *de* from; Gr. n. *halos* the sea, salt; gr. n. *spira* a spiral; M.L. dim. neut. n. *spirillum* a small spiral; *Dehalospirillum*, a dehalogenating small spiral). Cells forming small spirals that may be motile. Strictly anaerobic bacteria capable of using tetrachloroethene as terminal electron acceptor that is dehalogenated to *cis*-1,2-dichloroethene; alternative electron acceptors, such as fumarate or nitrate, are also reduced. A variety of organic and inorganic electron donors may be utilized. *Dehalospirillum* belongs to the epsilon subgroup of *Proteobacteria*.

Description of *Dehalospirillum multivorans* sp. nov.

Dehalospirillum multivorans (mul.ti.vorans. L. adj. *multus* many, numerous; L. v. *voro* to devour, swallow; M.L. part. adj. *multivorans* devouring numerous kinds of substrates). Cells are 0.4–0.5 µm in diameter and 2–5 µm in length. Motility may occur. H_2, Na_2S, formate, pyruvate, lactate, ethanol, and glycerol serve as electron donors; acetate is required as carbon source for growth with H_2 or formate plus tetrachloroethene. Sugars and sugar alcohols (with the exception of glycerol) are not utilized. The optimum pH for growth is between pH 7.0 and 7.5, optimum temperature is 30°C. No growth is observed at 37°C. The G+C content of the DNA is 41.5 mol%. Cells contain corrinoids, menaquinone, and cytochromes of the *b*- and *c*-type. The bacterium was isolated from activated sludge.

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References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

DeBruin WP, Kottermann MJJ, Posthumus MA, Schraa G, Zehnder AJB (1992) Complete biological reductive transformation of tetrachloroethene to ethane. Appl Environ Microbiol 58: 1996–2000

DiStefano TD, Gossett JM, Zinder SH (1992) Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. Appl Environ Microbiol 58:3622–3629

Freedman DL, Gossett JM (1989) Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl Environ Microbiol 55: 2144–2151

Gantzer CJ, Wackett LP (1991) Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ Sci Technol 25:715–722

Holliger C, Schumacher W (1994) Reductive dehalogenation as a respiratory process. In: Stouthamer AH, Schink B (eds) Antonie Van Leeuwenhoek, in press

Holliger C, Schraa G, Stams AJM, Zehnder AJB (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl Environ Microbiol 59:2991–2997

Jablonski PE, Ferry JG (1992) Reductive dechlorination of trichloroethylene by the CO-reduced CO dehydrogenase enzyme complex from *Methanosarcina thermophila*. FEMS Microbiol Lett 96:55–60

Karlson U, Dwyer DF, Hooper SW, Moore ERB, Timmis KN, Eltis LD (1993) Two independently regulated cytochromes *P-450* in a *Rhodococcus rhodochrous* strain that degrades 2-ethoxyphenol and 4-methoxybenzoate. J Bacteriol 175:1467–1474

Kröger A (1978) Determination of contents and redox states of ubiquinone and menaquinone. In: Colowick SP, Kaplan NO (eds) Methods of enzymology, vol 53. Academic Press, London New York, pp 579–591

Kröger A, Innerhofer A (1976a) The function of menaquinone, covalently bound FAD and iron-sulfur protein in the electron transport from formate to fumarate of *Vibrio succinogenes*. Eur J Biochem 69:487–495

Kröger A, Innerhofer A (1976b) The function of the *b* cytochromes in the electron transport from formate to fumarate of *Vibrio succinogenes*. Eur J Biochem 69:497–506

Krone UE, Thauer RK, Hogenkamp HPC (1989) Reductive dehalogenation of chlorinated C_1-hydrocarbons mediated by corrinoids. Biochemistry 28:4908–4914

Mesbah M, Premachandran U, Whitman W (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. Int J Syst Bacteriol 39: 159–167

Neumann A, Scholz-Muramatsu H, Diekert G (1994) Tetrachloroethene metabolism in *Dehalospirillum multivorans*. Arch Microbiol 162:295-301

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Scherer P, Sahm H (1981) Effects of trace elements and vitamins on the growth of *Methanosarcina barkeri*. Acta Biotechnol 1:57–65

Vogel TM, McCarty PL (1985) Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl Environ Microbiol 49:1080–1083

Vogel TM, Criddle CS, McCarty PL (1987) Transformation of halogenated aliphatic compounds. Environ Sci Technol 21: 722–736

Wilson K (1987) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Wiley, New York, pp 2.4.1–2.4.2