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Desulfitobacterium sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols

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Abstract A strictly anaerobic bacterium, strain PCE1, was isolated from a tetrachloroethene-dechlorinating enrichment culture. Cells of the bacterium were motile curved rods, with approximately four lateral flagella. They possessed a gram-positive type of cell wall and contained cytochrome *c*. Optimum growth occurred at pH 7.2–7.8 and 34–38° C. The organism grew with L-lactate, pyruvate, butyrate, formate, succinate, or ethanol as electron donors, using either tetrachloroethene, 2-chlorophenol, 2,4,6-trichlorophenol, 3-chloro-4-hydroxy-phenylacetate, sulfite, thiosulfate, or fumarate as electron acceptors. Strain PCE1 also grew fermentatively with pyruvate as the sole substrate. L-Lactate and pyruvate were oxidized to acetate. Tetrachloroethene was reductively dechlorinated to trichloroethene and small amounts (< 5%) of *cis*-1,2-dichloroethene and *trans*-1,2-dichloroethene. Chlorinated phenolic compounds were dechlorinated specifically at the *ortho*-position. On the basis of 16S rRNA sequence analysis, the organism was identified as a species within the genus *Desulfitobacterium*, which until now only contained the chlorophenol-dechlorinating bacterium, *Desulfitobacterium dehalogenans*.

Key words *Desulfitobacterium* · Chlorinated ethenes · Chlorinated phenols · Reductive dechlorination

Abbreviations PCE Tetrachloroethene · TCE Trichloroethene · cDCE cis-1,2-Dichloroethene · VC Chloroethene

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Introduction

Halogenated aromatic and aliphatic compounds are notorious environmental pollutants. For an understanding of the fate of such chemicals in the environment and for the development of bioremediation techniques for the clean-up of polluted locations and water streams, it is important to gather detailed information about the physiology and ecology of the bacteria capable of degrading these compounds.

Reductive dechlorination is the most important mechanism for the microbial transformation of chlorinated aliphatic and aromatic environmental pollutants under anoxic conditions (Mohn and Tiedje 1992). Indeed, for some poly-chlorinated pollutants, e.g., tetrachloroethene, tetrachloromethane, hexachlorobenzene, and biphenyls with more than seven chlorine substituents, it is the only known mechanism of biodegradation (Mohn and Tiedje 1992). Reductive dechlorination can be a co-metabolic process catalyzed by reduced coenzymes of many anaerobic and aerobic bacteria. In addition, some anaerobic bacteria are able to use chlorinated compounds as electron acceptors for the oxidation of organic compounds or molecular hydrogen (Holliger and Schumacher 1994). In theory, such anaerobes have great potential for biological remediation procedures because they can use the energy of reductive dechlorination for growth and hence significantly increase the rate of in situ dechlorination.

Although reductive dechlorination of numerous compounds has been shown to occur in various anoxic soils and sediments, little is known about the biology of the bacteria that catalyze these reactions because it has been difficult to obtain and grow these anaerobic bacteria in pure culture. The best-studied organism is *Desulfomonile tiedjei*, a sulfate-reducing bacterium that can grow with *meta*-chlorinated benzoic acids as an electron acceptor (DeWeerd et al. 1990; Dolfing 1990; Mohn and Tiedje 1990). Recently, novel types of anaerobic bacteria that obtain energy from reductive dechlorination of chlorinated phenols (*Desulfitobacterium dehalogenans*) and tetra-

chloroethene (*Dehalospirillum multivorans*) have been isolated and characterized (Neumann et al. 1994; Utkin et al. 1994; Utkin et al. 1995).

In this paper, we describe a newly isolated anaerobic bacterium, strain PCE1, that grows on lactate or formate with tetrachloroethene or *ortho*-chlorinated phenolic compounds as electron acceptors.

Materials and methods

Organisms and cultivation

Desulfitobacterium dehalogenans JW/IU-DC1 (DSM 9161) was obtained from the German Collection of Microorganisms (DSM, Braunschweig, Germany). Strain PCE1 was isolated from a tetrachloroethene (PCE)-dechlorinating anaerobic enrichment obtained with a soil sample from a PCE-polluted location as an inoculum (Gerritse et al. 1995). This strain has been deposited in the DSM as strain DSM 10344. For further cultivation, a basal medium with the following components (per l) was used: $(NH_4)H_2PO_4$ (2.88 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.05 g), EDTA (1 mg), $FeSO_4 \cdot 7H_2O$ (2 mg), $ZnSO_4 \cdot 7H_2O$ (0.1 mg), $MnCl_2 \cdot 4H_2O$ (0.03 mg), H_3BO_3 (0.3 mg), $CoCl_2 \cdot 6H_2O$ (0.2 mg), $CuCl_2 \cdot 2H_2O$ (0.01 mg), $NiCl_2 \cdot 6H_2O$ (0.02 mg), $NaMoO_4 \cdot 2H_2O$ (0.03 mg), $Na_2SeO_3 \cdot 5H_2O$ (0.026 mg), $Na_2WO_4 \cdot 2H_2O$ (0.033 mg), resazurin (0.1 mg), and yeast extract (0.1 g). Basal medium was prepared under a N_2/CO_2 (80:20, v/v) gas phase in serum bottles or Hungate tubes that were sealed with butyl rubber septa. For cultures with chlorinated compounds, a Viton rubber septum was placed below the butyl rubber septum. After autoclaving, 1 ml l^{-1} of a filter-sterilized vitamins solution (Heijthuijsen and Hansen 1986) was added. KOH (1 g l^{-1}), $NaHCO_3$ (2.4 g l^{-1}), electron donors (20–50 mM), and electron acceptors (1–20 mM) were added to the medium from separately autoclaved or filter-sterilized anoxic stock solutions. PCE dissolved in an organic phase (0.5 M PCE in hexadecane) was added to a total amount of 10 mmol l^{-1} medium, which corresponded to an initial dissolved PCE concentration in the culture liquid of about 100 μM (Holliger et al. 1993). The hexadecane solution was sterilized by autoclaving for 30 min at 140° C, after which PCE was added through a PVFE membrane filter (pore size 0.2 μm, Alltech, Breda, The Netherlands). The pH of the complete medium was 7.2, and the initial chloride concentration was 0.3–0.5 mM. Finally the medium was reduced with Na_2S (70 mg l^{-1}).

Isolation techniques

Strain PCE1 was isolated by dilution of the enrichment culture in liquid medium and subsequent streaking of the highest PCE-dechlorinating dilution cultures on agar plates. For agar plates, solidified with 2% (w/v) agar, the yeast extract concentration of the medium was increased from 0.1 to 1.0 g l^{-1}. Agar media were plated in an anoxic glove box and incubated in a jar under N_2/CO_2 (80:20, v/v) at 30°C. PCE was added via a filter paper wetted with a drop of pure PCE placed in the lid of the petri dish. Agar plates were reduced by addition to the anoxic jar of 5 ml of a solution containing 2% (w/v) $Na_2S \cdot 9H_2O$ and 50 mM KH_2PO_4.

Electron microscopy

For electron micrographs, exponential-phase cells were negatively stained with 1% (w/v) uranyl acetate. For ultrathin sections, cells were fixed with 3% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, at 0° C, for 2 h) and post-fixed with 0.5% (w/v) OsO_4, 2.5% (w/v) $K_2Cr_2O_7$ in sodium cacodylate buffer (pH 7.2, at room temperature, for 2 h). After fixation, cells were post-stained overnight (en bloc) with 1% (w/v) uranyl acetate, dehydrated, and embedded in Epon 812 (Serva, Heidelberg, Germany).

Gram staining and cell wall murein analysis

The Gram character was determined according to Gregersen (1978) with *Pseudomonas aeruginosa* as a gram-negative control. For the analysis of the cell wall composition, cells were grown on pyruvate in a 20-l batch culture. Cells were harvested and washed in 20 mM potassium phosphate (pH 7.2) by centrifugation (15 min, 10,000 × *g*) and freeze-dried before analysis. Cell walls were prepared from cells by mechanical disruption with glass beads. The amino acid composition of complete wall hydrolysates was determined as described by Schleifer and Kandler (1972).

Cytochrome spectra

For the analysis of cytochromes, strain PCE1 was grown on pyruvate (40 mM) either in the absence or presence of PCE (10 mM). Cells were washed and suspended in 20 mM potassium phosphate (pH 7.2). Cytochrome spectra were determined in cell suspensions or in cell-free extracts prepared by sonication (8 × 30 s) and removal of cell debris by centrifugation (15 min, 4° C, 10,000 × *g*). Dithionite-reduced *minus* air-oxidized spectra were obtained at room temperature in a Shimadzu UV-1601 spectrophotometer (Shimadzu, Den Bosch, The Netherlands). Cytochrome *c* content was quantified from the absorbance difference between 553 and 538.5 nm using an extinction coefficient of 17.3 mM^{-1} cm^{-1} for cytochrome *c* (Smith 1978).

Temperature and pH optimum

Temperature optimum and pH ranges for growth were determined by following the increase in optical density in 10-ml cultures in Hungate tubes with 50 mM pyruvate as the growth substrate. Optical density values (OD_{660}) were obtained using a Biotron 101 colorimeter (Meyvis, Bergen op Zoom, The Netherlands). A temperature gradient from 18 to 42°C was obtained by heating one side and cooling the other side of a stainless steel block containing holes (diameter 1.7 cm, depth 6.0 cm) for the incubation of Hungate tubes at different temperatures. Media in the pH range from 6.0 to 9.0 were prepared by the addition of a buffer mixture (Mes, Pipes, phosphate, and Tris, 25 mM each). Before autoclaving, the pH was adjusted to the different values with KOH. To prevent precipitation, calcium and magnesium salts were added to the medium from separately autoclaved stock solutions. The pH optimum was determined at 30° C.

16S rRNA gene sequence analysis

Chromosomal DNA was isolated as described by Lawson et al. (1989), and 16S rRNA gene fragments were generated by polymerase chain reaction (PCR) amplification (Hutson et al. 1993). The amplified products were sequenced using a PRISM Dyedeoxy Terminator Sequencing Kit (Applied Biosystems, Warrington, UK), and the sequence reactions were electrophoresed using an Applied Biosystems model 373A automatic DNA Sequencer according to the manufacturer's protocols. The sequences were aligned with the sequences of a representative collection of (eu)bacterial 16S rRNAs obtained from the EMBL Data Library using the Wisconsin Molecular Biology Package (Devereux et al. 1984) on a Digital VAX-VMS (version V5.5). The distance matrix obtained was corrected for substitution rates by using Kimura's parameters (Kimura 1980). A phylogenetic tree was constructed by the neighbour-joining method of Saitou and Nei (1987).

Dechlorination in cultures of strain PCE1

A culture of strain PCE1 exponentially growing on L-lactate with PCE as the electron acceptor was used as the inoculum for batch cultures containing 1 mmol l^{-1} of a chlorinated aromatic or aliphat-

ic compound and a mixture of pyruvate and L-lactate as carbon and electron source (20 mM each). The culture bottles also contained 2% (v/v) hexadecane in order to dissolve and release hydrophobic substrates slowly into the culture medium. Dechlorination was scored positive when more than 0.5 mM chloride was produced within 50 days of incubation at 37° C. Uninoculated anoxic media containing the chlorinated compounds were used as controls.

Analytical techniques

Sulfide was analyzed colorimetrically as described by Trüper and Schlegel (1964). Chloride was measured with an ion-selective electrode as described previously (Gerritse et al. 1995). Protein was measured with the Bradford reagent with bovine serum albumin as a standard. Organic acids and H_2 were determined by gas chromatography (Nanninga and Gottschal 1985). Cell carbon was measured with a carbon analyzer (Gerritse et al. 1990). Chlorinated ethenes were determined by analysis of the head-space by capillary gas chromatography (Gerritse et al. 1995). Chlorinated phenolic compounds were quantified according to Pieper et al. (1988) on a Jasco HPLC (Tokyo, Japan) equipped with a UV975 UV/VIS detector and an Alltech Lichrospher 100RP8 column (Deerfield, Ill., USA).

Results

Enrichment and isolation of strain PCE1

An anaerobic tetrachloroethene (PCE)-dechlorinating enrichment culture was obtained from an inoculum of soil polluted by chlorinated ethenes (Gerritse et al. 1995). The primary carbon and energy substrate used in this enrichment was L-lactate. The enrichment culture dechlorinated PCE completely via trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), and chloroethene (VC) to ethene. Reductive dechlorination of PCE by subcultures of this batch enrichment was supported by various carbon and electron donors (Table 1). The rate and extent of dechlorination depended on the nature of the carbon substrate supplied. Relatively rapid dechlorination of PCE to TCE was obtained with formate, L-glutamate, and L-alanine as the primary substrates, whereas with propionate and succinate, dechlorination was slow, but more complete (i.e., dechlorination of PCE to VC and ethene). The addition of H_2 as a potential electron source inhibited reductive dechlorination in this enrichment culture.

A PCE-dechlorinating bacterium was isolated as follows: after two transfers in liquid L-lactate/PCE media, the enrichment was streaked on agar plates containing L-lactate as carbon source. A drop of pure PCE was added on a filter paper placed in the lid of the petri dish. Colonies formed were transferred into liquid media with either L-lactate, formate, or L-alanine as carbon substrate. Since rapid dechlorination of PCE to TCE was obtained in cultures with formate, a dilution series (up to 10^{10}-fold) was made in formate/PCE media. Dechlorination was obtained up to a dilution of 10^7. In these cultures, three types of bacteria could be distinguished microscopically: (1) thick rods, (2) vibrio-like bacteria, and (3) thin helical rods. In transferred cultures grown with formate plus PCE, the thin helical rods were numerically dominant. In subcultures grown on formate in the absence of PCE, in media in which magnesium sulfate and calcium nitrate were replaced by magnesium chloride and calcium chloride, the thick rods dominated. Significant amounts of acetate were formed in such cultures; therefore, these bacteria were probably acetogens. In cultures in which 20 mM sulfate was added as potential electron acceptor, the vibrio-like bacterium became dominant, suggesting that this was a sulfate-reducing organism. A pure culture of the thin helical rod was subsequently obtained on formate/PCE agar media containing pyruvate as an additional substrate. The purity of this PCE-dechlorinating bacterium, strain PCE1, was checked on agar media with pyruvate as the sole growth substrate, on which this organism formed small (diameter 0.5–2 mm) brown colonies. Purity was further confirmed by means of light- and electron-microscopic observations and through inoculation into liquid media with 1 g l^{-1} yeast extract and 10 mM glucose, on which strain PCE1 did not grow.

Table 1 Dechlorination of tetrachloroethene (*PCE*) by an anaerobic enrichment culture incubated with various carbon and energy sources. Temperature 30° C, initial tetrachloroethene concentration: 4 mmol l^{-1}. (*TCE* trichloroethene, *cDCE cis*-1,2-dichloroethene, *VC* chloroethene)

Substrate (mmol l^{-1})		Chloride (mmol l^{-1})		Chloroethenes formed[a] Day 166		
		Day 29	Day 217			
None[b]		0.8	3.3	TCE		
H_2	(29)	1.0	1.2	TCE	cDCE	
H_2	(29) + lactate (0.5)	1.3	1.5	TCE	cDCE	
Formate	(25)	3.9	4.9	TCE	cDCE	
Formate	(25) + lactate (0.5)	2.2	3.1	TCE	(cDCE)	
Pyruvate	(10)	2.6	6.6	(TCE)	cDCE	VC
Lactate	(10)	2.3	11.4		(cDCE)	VC
Propionate	(10)	0.6	11.1			VC
Butyrate	(10)	0.9	2.5	TCE	(cDCE)	
Succinate	(10)	0.8	7.6		cDCE	VC
Benzoate	(5)	1.5	3.9	TCE	(cDCE)	
Glutamate	(5)	3.3	4.7	(TCE)	cDCE	VC
Glycine	(10)	1.0	3.6	TCE	(cDCE)	
Alanine	(10)	4.3	10.4		cDCE	VC
Ethanol	(10)	1.5	3.9	TCE	(cDCE)	
2-Propanol	(10)	1.3	4.1	(TCE)	cDCE	
Glucose	(5)	2.1	9.8			VC
Fructose	(5)	1.7	12.6		(cDCE)	VC

[a] Compounds in parentheses were formed at concentrations < 10% of PCE added
[b] Medium contained 1 g l^{-1} of yeast extract

Cell morphology and cytological properties

Cells of strain PCE1 were helical (about 0.6–0.8 μm wide and 2–3 μm long) and motile by approximately four lateral flagella (Fig. 1 A). Sometimes up to 7-μm-long curved

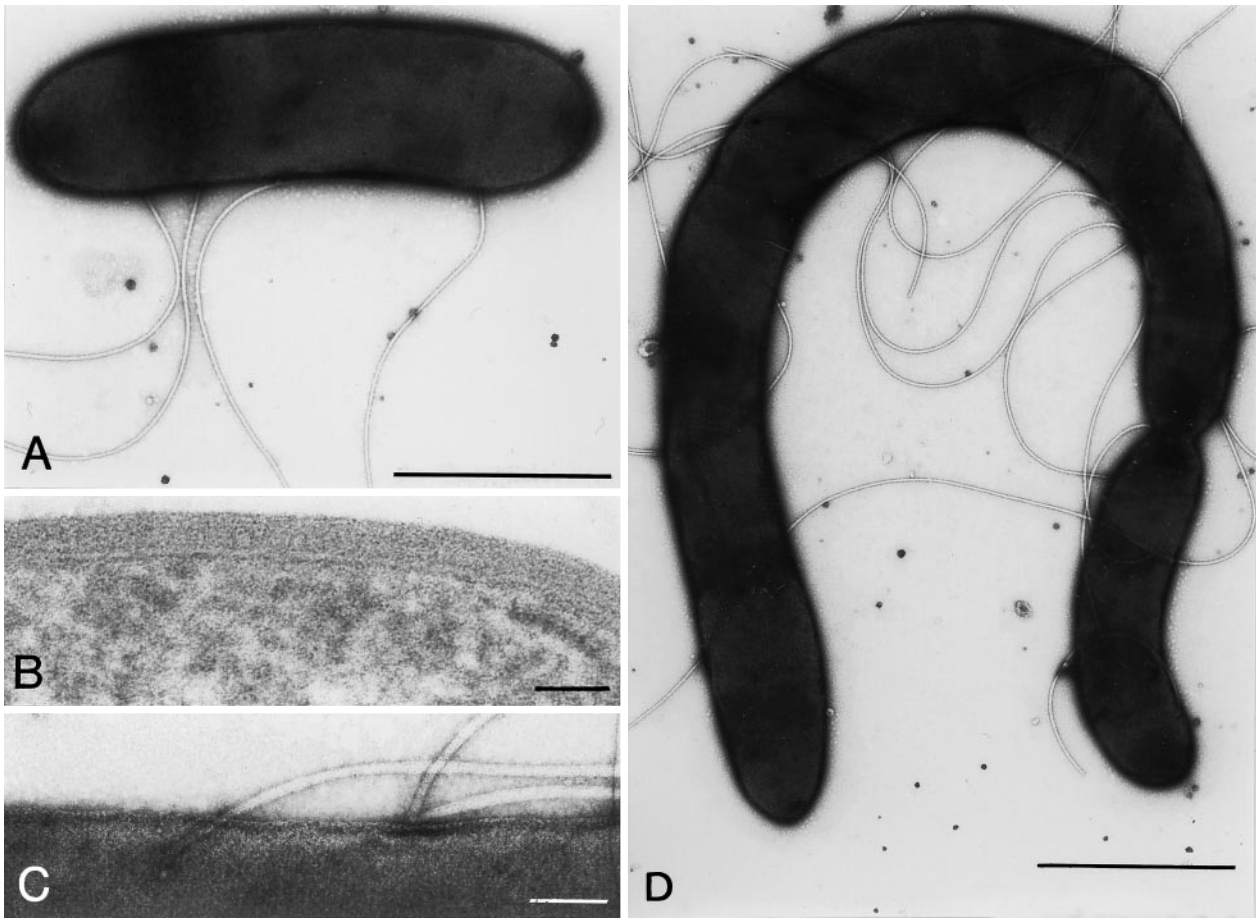

Fig. 1 A–D Electron micrographs of negatively stained exponential-phase cells of strain PCE1. **A** Cell with four laterally attached flagella, **B** ultrathin section revealing the thick gram-positive cell wall, **C** S-layer surrounding the cell wall, **D** dividing long curved cell. *Bar* 1 μm in **A** and **D**, 0.1 μm in **B** and **C**

cells were formed (Fig. 1 D). Electron micrographs revealed the presence of a 0.1-μm-thick, layered gram-positive cell wall (Fig. 1 B), surrounded by an 'S-layer' (Fig. 1 C). Both gram staining and the KOH method confirmed that strain PCE1 is a gram-positive bacterium. The formation of endospores was never observed in liquid or on solidified media. Pasteurization (incubation for 20 min at 85° C) irreversibly stopped the growth of cultures of strain PCE1. Dithionite-reduced *minus* air-oxidized spectra of washed suspensions and cell-free extracts revealed the presence of *c*-type cytochromes in strain PCE1 grown on pyruvate both with and without PCE (absorbance maxima at 421, 523, and 553 nm). The cytochrome *c* concentration determined in cell-free extracts of strain PCE1 grown on pyruvate plus PCE was 1.0 μmol (g protein)$^{-1}$.

Utilization of electron donors and acceptors

Strain PCE1 grew fermentatively with pyruvate as the carbon and energy source. For growth on either L-lactate, formate, butyrate, succinate, or ethanol, the presence of an external electron acceptor was required (see below). Strain PCE1 grew readily on L-lactate, whereas growth on formate, butyrate, succinate, and ethanol started after an initial lag phase of several weeks after transfer from a pyruvate-grown culture. The lag phase decreased to less than one week when the cultures were pregrown on these substrates. Acetate, propionate, fumarate, DL-malate, L-glutamate, L-alanine, L-glycine, methanol plus acetate, hydrogen plus acetate, glycerol, D-glucose, and D-fructose did not permit growth with or without sulfite as the electron acceptor. With L-lactate as an electron donor, strain PCE1 was capable of growth in the presence of various electron acceptors (Fig. 2 A, B). Sulfite and thiosulfate were reduced to sulfide, and fumarate was reduced to succinate. Strain PCE1 did not grow on L-lactate with either sulfate (20 mM), nitrate (20 mM), or nitrite (concentrations tested: 20 mM and 5 mM) within two months of incubation at 30° C. Oxygen (2% v/v added in the gas phase) completely inhibited growth on pyruvate. During growth with L-lactate as a carbon source and electron donor, PCE was used as an electron acceptor and was reductively transformed into TCE, chloride, and small amounts of *cis*- and *trans*-1,2-dichloroethene (< 5% of PCE consumed) (Fig. 3 A). With L-lactate or pyruvate as electron donors, several *ortho*-chlorinated phenolic compounds were utilized as electron acceptor by strain PCE1. 2,4,6-Trichlorophenol was reductively dechlorinated via 2,4-dichlorophenol to 4-chlorophenol (Fig. 3 B), and 2-

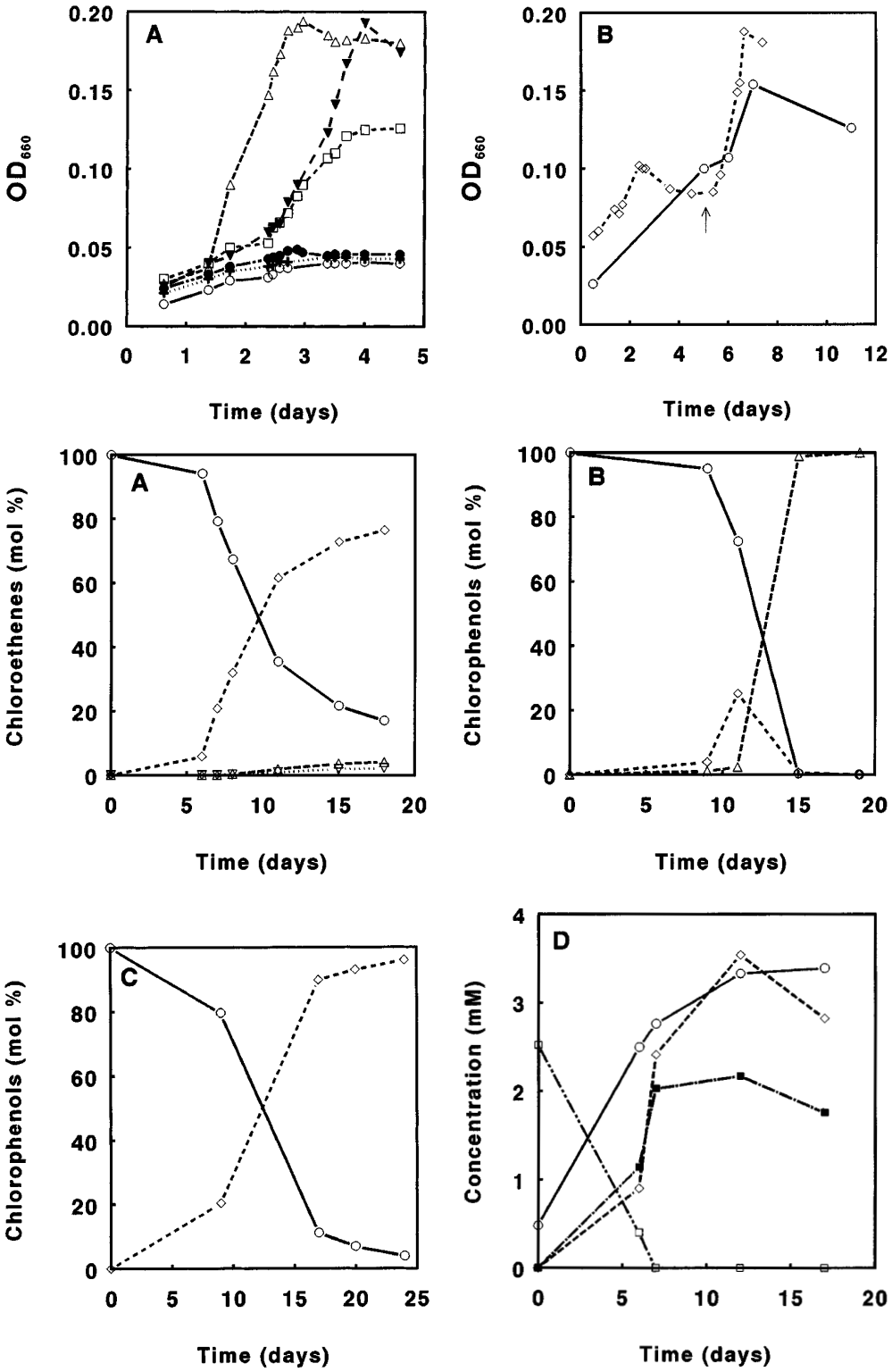

Fig. 2 A, B Growth of strain PCE1 on L-lactate (40 mM) in the presence of various electron acceptors. **A** Growth with 20 mM sulfite (*open triangles*), thiosulfate (*open squares*), sulfate (*crosses*), fumarate (*inverted filled triangles*), and nitrate (*filled circles*), or in the absence (*open circles*) of an electron acceptor. **B** Growth with either 4 mM tetrachloroethene (*open circles*), or with 1 mM 3-chloro-4-hydroxyphenylacetate (*open diamonds*) as electron acceptors. The *arrow* indicates the addition of 20 mM extra 3-chloro-4-hydroxy-phenylacetate

Fig. 3 **A** Reductive dechlorination of 10 mM of tetrachloroethene (*open circles*) to trichloroethene (*open diamonds*) and small amounts of *trans*-1,2-dichloroethene (*open inverted triangles*) and *cis*-1,2-dichloroethene (*open triangles*) in a batch culture of strain PCE1 grown with a mixture of formate (50 mM) and pyruvate (4 mM) as the electron donors. **B** Reductive dechlorination of 1 mmol l^{-1} 2,4,6-trichlorophenol (*open circles*) via 2,4-dichlorophenol (*open diamonds*) to 4-chlorophenol (*open triangles*) with a mixture of pyruvate (20 mM) and L-lactate (20 mM) as electron donors. Hexadecane (2%, v/v) was added to avoid toxicity of chlorophenols. **C** Reductive dechlorination of 1 mM of 2-chlorophenol (*open circles*) to phenol (*open diamonds*) in a culture with L-lactate (40 mM) as the electron donor. **D** Product formation during growth of strain PCE1 on L-lactate (40 mM) with 3-chloro-4-hydroxy-phenylacetate (*open squares*) (2.5 mM) as the electron acceptor, revealing formation of acetate (*open diamonds*), 4-hydroxy-phenylacetate (*filled squares*), and chloride (*open circles*)

chlorophenol to phenol (Fig. 3C). Also, reductive dechlorination of 3-chloro-4-hydroxy-phenylacetate to 4-hydroxyphenylacetate supported growth of strain PCE1 on L-lactate (μ_{max} = 0.030 h^{-1}) (Figs. 2B, 3D). Chlorinated compounds (1 mmol l^{-1}) that did not result in significant release of chloride in batch cultures with a mixture of pyruvate and L-lactate (20 mM each) as carbon sources and electron donors were: hexachlorobenzene, 2,5-dichlorobenzoate, 3,4-dichlorobenzoate, 2,3,6-trichlorobenzoate, trichloroacetate, trichloroethene, *trans*-1,2-dichloroethene, *cis*-1,2-dichloroethene, trichloromethane, dichloromethane, hexachloroethane, pentachlorophenol, and 4-chlorophenylacetate. No fermentative growth on pyruvate was observed in cultures incubated with pentachlorophenol and

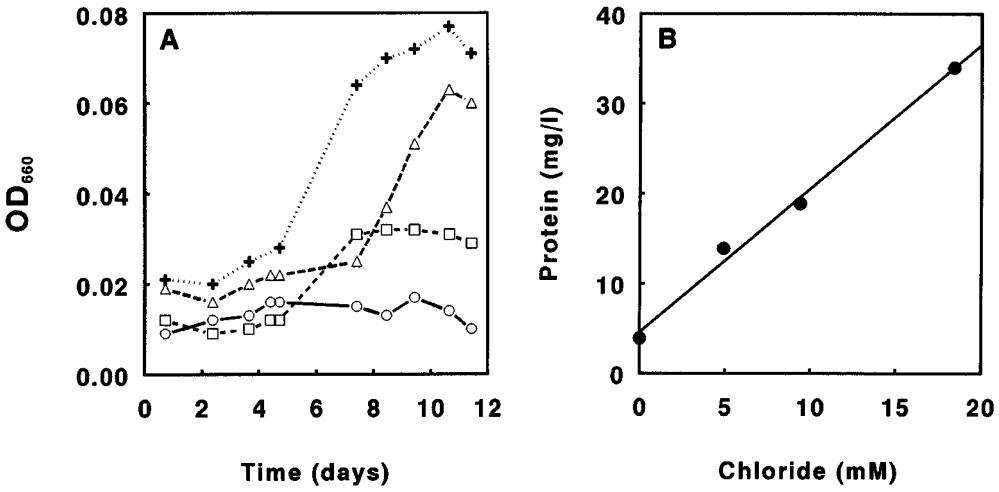

Fig.4 **A** Growth (OD_{660}) of strain PCE1 on formate (100 mM) with 0 (*open circles*), 5 (*open squares*), 10 (*open triangles*), and 20 (*crosses*) mM 3-chloro-4-hydroxyphenylacetate as electron acceptor, resulting in (**B**) increasing cell protein formed per mol chloride released (*filled circles*)

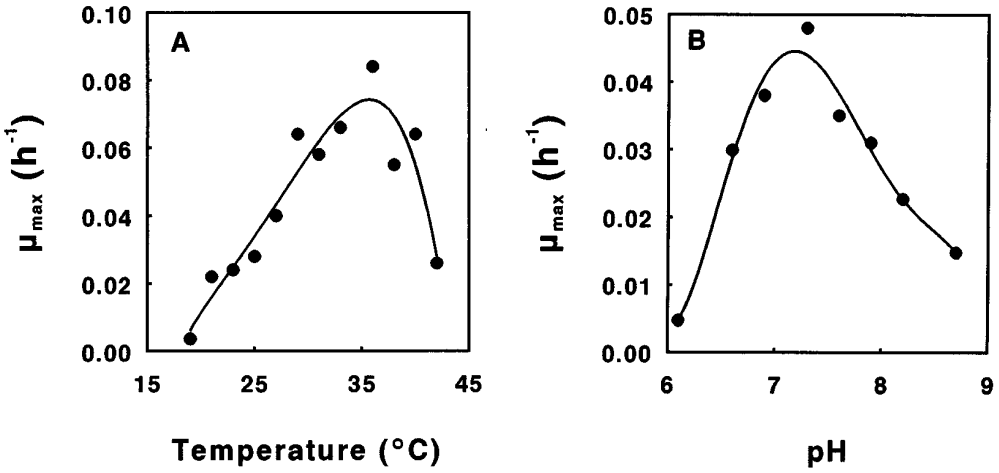

Fig.5 **A** Temperature optimum, and **B** pH optimum for growth of strain PCE1 on pyruvate

hexachloroethane. Apparently, the presence of these chlorinated compounds inhibited growth of strain PCE1.

The major products detected during growth of strain PCE1 on 10 mM pyruvate were acetate (10.6 mM), CO_2, and biomass. The amount of CO_2 formed could not be quantified because of the bicarbonate/CO_2 buffer used in the medium. Small amounts of formate, propionate, and butyrate (< 1 mM) were also formed. Approximately 0.7 mmol of PCE was reduced stoichiometrically to TCE and chloride per 10 mmol of pyruvate consumed by strain PCE1. The molar growth yield was 2.6 ± 0.4 or 4.0 ± 0.1 g cell carbon per mol pyruvate consumed in the absence or presence of PCE, respectively. In the presence of PCE, strain PCE1 oxidized L-lactate to acetate. With L-lactate as electron donor, a tenfold higher amount of PCE was dechlorinated than with pyruvate as electron donor (7 mmol of PCE per 10 mmol of L-lactate consumed). With 23.7 mmol of 3-chloro-4-hydroxy-phenylacetate as electron acceptor, 10.0 mmol of L-lactate was used resulting in the formation of 10.0 mmol of acetate, 18.9 mmol of chloride, 21.6 mmol of 4-hydroxy-phenylacetate and 1.8 mmol of cell carbon. Formation of H_2 (detection limit about 1 μmol l^{-1}) was never observed in cultures of this bacterium grown on pyruvate or on L-lactate. In cultures with formate (100 mM), biomass formation increased linearly with increasing amounts of 3-chloro-4-hydroxyphenylacetate supplied (0–20 mM), corresponding to a growth yield of 1.6 mol protein per mol chloride produced (Fig.4 A, B). In such cultures, no significant (< 0.5 mM) amounts of acetate were formed.

Influence of some growth conditions

On pyruvate and L-lactate, strain PCE1 grew well in a medium supplied with vitamins and yeast extract (0.1 g l^{-1}). Yeast extract was not essential for growth on pyruvate. However, the vitamin solution was required for growth of strain PCE1. With pyruvate as the growth substrate, strain PCE1 grew over a temperature range from 19 to 42° C, with an optimum between 34 and 37° C (μ_{max} = 0.085 h^{-1}) (Fig.5 A). The pH optimum obtained on pyruvate at 30° C was about 7.2 (Fig.5 B). No pigment formation was observed when strain PCE1 was grown in anoxic cultures in the light. Optimal growth on L-lactate with PCE as an electron acceptor in a shaking-incubator (150 rpm, 37° C, and pH 7.2) proceeded with a μ_{max} of 0.012 h^{-1}.

rRNA sequence analysis

The 16S rRNA gene of strain PCE1 was amplified by using PCR and sequenced directly. The determined sequence consisted of 1,484 bases (representing 96% of the total rRNA molecule) and has been deposited in the EMBL Data Library (accession no. X81032). The newly determined sequence was compared with other 16S RNA sequences available in GeneBank/EMBL using the Fasta program (Pearson and Lipman 1988). From this initial screening, it was evident that strain PCE1 is a member of the *Clostridium* subphylum of the gram-positive bacteria and specifically related to *Desulfitobacterium*. Pairwise comparison revealed that isolate PCE1 and *Desulfitobacterium dehalogenans* (strain JW/IU-DC1^T) were almost identical, exhibiting 99.3% (corresponding to 10 differences in a stretch of 1,484 nucleotides) 16S rRNA sequence similarity. This exceedingly high 16S sequence similarity indicates that the isolate is either a new strain of *Desulfitobacterium dehalogenans* or a new, closely related genomic species. The presence of a cell wall based on LL-diaminopimelic acid (LL-Dpm) in strain PCE1 is in accordance with its close phylogenetic affinity with *Desulfitobacterium dehalogenans* (Utkin et al. 1994). This cell wall murein type is relatively unusual amongst organisms of the *Clostridium* subphylum, which generally possess walls based on *meso*-Dpm.

Discussion

Strain PCE1 transformed PCE mainly to trichloroethene (TCE) and chloride and formed only trace amounts of *trans*-1,2-dichloroethene and *cis*-1,2-dichloroethene (cDCE). The complete dechlorination of PCE to ethene by the enrichment culture indicates that other bacteria were responsible for dechlorination of TCE and less-chlorinated ethenes. *Dehalospirillum multivorans* and strain PER-K23 (tentatively identified as *Dehalobacter restrictus*) are known to dechlorinate PCE via TCE to cDCE (Holliger et al. 1993; Neumann et al. 1994). Formation of TCE as an end product has been found for bacteria that dechlorinate PCE slowly in a co-metabolic process (e.g., homoacetogenic bacteria, *Methanosarcina* spp., and *Desulfomonile tiedjei*) (Fathepure et al. 1987; Terzenbach and Blaut 1994). Anaerobic bacteria that utilize di- or monochloroethenes as electron acceptors have not yet been isolated in pure culture.

Strain PCE1 grew fermentatively on pyruvate, whereas growth with other substrates depended on the availability of an external electron acceptor. Reductive dechlorination of PCE and 3-chloro-4-hydroxy-phenylacetate enabled growth of this strain on formate, demonstrating that the use of these chlorinated compounds as electron acceptors is coupled to ATP synthesis. To our knowledge, this is the first organism identified that is able to grow by means of reductive dechlorination of aliphatic or aromatic compounds. The range of different electron acceptors that support the growth of strain PCE1 on L-lactate is remarkable. Besides PCE and *ortho*-chlorinated phenolic compounds, also sulfite, thiosulfate, and fumarate were utilized. To reveal the mechanisms and regulation of the metabolism of these electron acceptors in strain PCE1, further physiological and biochemical studies are required.

The observation that during growth on L-lactate significantly more PCE was dechlorinated than during growth on pyruvate is not surprising since during the oxidation to acetate and CO_2, two more reducing equivalents are released from L-lactate than from pyruvate. Fermentative growth on L-lactate usually involves the oxidation of L-lactate via pyruvate to acetate and CO_2, along with the formation of reduced compounds such as propionate and/or H_2 (Gottschalk 1986). Fermentative growth of strain PCE1 on pyruvate, but not on L-lactate, indicates that the organism required an external electron acceptor for the oxidation of L-lactate to pyruvate. Assuming that per mol of acetate formed, one mol CO_2 was produced during the oxidation of 1 mol L-lactate, growth of this strain with 3-chloro-4-hydroxy-phenylacetate as electron acceptor proceeded according to the following equation:

$$0.5\ \text{L-lactate} + \text{3-chloro-4-hydroxy-phenylacetate} + 0.5\ H_2O \rightarrow 0.5\ \text{acetate} + 0.5\ CO_2 + \text{4-hydroxy-phenylacetate} + HCl$$

During growth on L-lactate or pyruvate with PCE as the electron acceptor, insufficient reduced products were recovered to make a full electron balance. Possibly, absorbance of substrates and/or products into hexadecane interfered with the construction of complete electron balances in cultures supplied with PCE.

Only few anaerobic bacteria, with diverse physiological and phylogenetic background, capable of using halogenated compounds have been obtained in pure culture, two of which can grow on chlorinated compounds as the sole source of carbon and electrons: a methyl chloride-utilizing homoacetogen, strain MC (Meßmer et al. 1993), and the photoheterotrophic *Rhodopseudomonas palustris* strain DCP3 that grows on 3-chlorobenzoate under anoxic conditions in the light (Van der Woude et al. 1994). *Desulfomonile tiedjei* is a gram-negative sulfate-reducing bacterium that is well known for its ability to use *meta*-chlorinated benzoic acids as electron acceptor (De Weerd et al. 1990; Dolfing 1990; Mohn and Tiedje 1990). Interestingly, this organism is also capable of catalyzing the reductive dechlorination of *meta*-chlorinated phenols and PCE (Fathepure et al. 1987; Mohn and Kennedy 1992). However, in contrast to strain PCE1, *Desulfomonile tiedjei* cannot use chlorophenols or PCE as a terminal electron acceptor for growth, whereas strain PCE1 appears not to be able to dechlorinate chlorobenzoates. At present, two bacteria besides strain PCE1 are known to grow with PCE as an electron acceptor. *Dehalospirillum multivorans* is a gram-negative spirillum belonging to the epsilon subdivision of Proteobacteria. Like strain PCE1, this organism utilizes pyruvate, L-lactate, ethanol, and formate as electron donors (Scholz-Muramatsu et a. 1995). An enrichment culture of an anaerobic bacterium, strain PER-K23, couples dechlorination of PCE to growth with H_2 or for-

mate as electron donors (Holliger et al. 1993). Strain PER-K23, tentatively described as *Dehalobacter restrictus*, appears to be related to the fourth subdivision of the gram-positive bacteria (Holliger and Schumacher 1994). Unlike strain PCE1, which produces TCE from PCE, both *Dehalospirillum multivorans* and strain PER-K23 dechlorinate PCE via TCE to cDCE. Reductive dechlorination of chlorophenols is carried out by *Desulfitobacterium dehalogenans* (Utkin et al. 1994, Utkin et al 1995) by strain 2CP-1, a facultative anaerobe related to the myxobacteria (Cole et al. 1994), and by the clostridium-like bacterium DCB-2 (Madsen and Licht 1992). Strain DCB-2 is capable of rapidly catalyzing *ortho*-dechlorination of pentachlorophenol, 2,4,5- and 2,4,6-trichlorophenol, and 2,4-dichlorophenol, and *meta*-dechlorination of 3,5-dichlorophenol, but it is not known whether this organism gains energy from this reaction. Strain 2CP-1, however, has been shown to be able to grow on acetate using 2-chlorophenol or 2,6-dichlorophenol as electron acceptor. *Desulfitobacterium dehalogenans* is a gram-positive sulfite-reducing bacterium that appears to be physiologically, chemically, and phylogenetically very closely related to strain PCE1. Comparative 16S rRNA sequence revealed less than 1% sequence divergence and clearly places these two bacteria within the same genus. However, since high rRNA sequence similarity does not necessarily guarantee species identity, chromosomal DNA-DNA hybridization studies are necessary to establish definitively whether isolate PCE1 is a member of *Desulfitobacterium dehalogenans* or whether it corresponds to a genetically separate species. Both organisms are capable of using pyruvate, L-lactate, and formate as the electron donor for *ortho*-dechlorination of phenolic compounds. Strain PCE1 and *Desulfitobacterium dehalogenans* are able to use fumarate, sulfite, and thiosulfate as electron acceptors; however, the following physiological properties distinguish strain PCE1 from *Desulfitobacterium dehalogenans*: (1) strain PCE1 is not capable of growth with nitrate as an electron acceptor, (2) strain PCE1 does not form L-lactate during fermentation of pyruvate, (3) strain PCE1 is able to dechlorinate 2-chlorophenol to phenol much more rapidly, and (4) strain PCE1 is able to grow on L-lactate with PCE as electron acceptor. *Desulfitobacterium dehalogenans* grew readily on L-lactate with sulfite as the acceptor in the medium used for strain PCE1. *Desulfitobacterium dehalogenans* neither dechlorinated nor grew in this medium with PCE as the potential electron acceptor; however, after the addition of yeast extract (1 g l^{-1}) and pyruvate (40 mM), dechlorination of PCE to TCE was observed (J. Gerritse, unpublished results). Similar results were obtained recently by M. Odom and J. Wiegel (personal communication). *Desulfitobacterium dehalogenans* and strain PCE1 are phylogenetically most closely related to members of the genus *Desulfotomaculum* (sulfate-reducing bacteria) and *Heliobacterium* (obligately anaerobic phototrophs) (Madigan 1992; Widdel 1992). We propose that strain PCE1 be placed within the genus *Desulfitobacterium*. The type species of this genus is *Desulfitobacterium dehalogenans* (Utkin et al. 1994).

Some ecological considerations

Strain PCE1 is capable of using chlorinated aliphatic (PCE) and chlorinated phenolic compounds, which are frequently encountered environmental pollutants, as terminal electron acceptors. Chlorinated ethenes, including PCE, are released into the environment through volcanic activity. However, in unpolluted soils, concentrations of PCE are generally very low. In contrast, chlorinated phenolic compounds occur at relatively high concentrations, mainly due to production by fungi (Gribble 1995). It is tempting to hypothesize that this natural production of chlorinated aromatics provides a niche enabling the evolution of bacteria, like strain PCE1, that can use chlorinated aliphatics. The isolation from distant locations of members of the genus *Desulfitobacterium* that are capable of dechlorination of various chlorinated aromatic and aliphatic compounds suggests that '*Desulfitobacterium*-like' bacteria may play an important role in the degradation of chlorinated pollutants in the environment.

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References

Cole JR, Cascarelli AL, Mohn WW, Tiedje JM (1994) Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl Environ Microbiol 60:3536–3542

Devereux J, Haeberli P, Smithies D (1984) A comprehensive set of sequence analysis programs for the Vax. Nucleic Acids Res 12: 387–395

DeWeerd KA, Mandelco L, Tanner RS, Woese CR, Suflita JM (1990) *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic dehalogenating, sulfate-reducing bacterium. Arch Microbiol 154:23–30

Dolfing J (1990) Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in an anaerobic bacterium, strain DCB-1. Arch Microbiol 153:264–266

Fathepure BZ, Nengu JP, Boyd SA (1987) Anaerobic bacteria that dechlorinate perchloroethene. Appl Environ Microbiol 53:2671–2674

Gerritse J, Gottschal JC (1990) Mixed chemostat cultures of obligately aerobic and fermentative or methanogenic bacteria under oxygen-limiting conditions. FEMS Microbiol Lett 66:87–94

Gerritse J, Renard V, Visser J, Gottschal JC (1995) Complete degradation of tetrachloroethene by combining anaerobic dechlorinating and aerobic methanotrophic enrichment cultures. Appl Microbiol Biotechnol 43:920–928

Gottschalk G (1986) Bacterial metabolism, 2nd edn. Springer, Berlin Heidelberg New York

Gregersen T (1978) Rapid method for distinction of gram-negative from gram-positive bacteria. Eur J Appl Microbiol Biotechnol 5:123–127

Gribble GW (1995) The natural production of chlorinated compounds. Environ Sci Technol 28:310–319

Heijthuijsen JHFG, Hansen TA (1986) Interspecies hydrogen transfer in co-cultures of methanol-utilising acidogens and sulfate-reducing or methanogenic bacteria. FEMS Microbiol Ecol 38:57–64

Holliger C, Schumacher W (1994) Reductive dehalogenation as a respiratory process. Antonie Van Leeuwenhoek 66:239–246

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

Hutson RA, Thompson DE, Collins MD (1993) Genetic interrelationships of saccharolytic *Clostridium botilinum* types B, E and F and related clostridia as revealed by small-subunit rRNA gene sequences. FEMS Microbiol Lett 108:103–120

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Lawson PA, Gharbia SE, Shah HN, Clark DR (1989) Recognition of *Fusobacterium nucleatum* subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restriction patterns. FEMS Microbiol Lett 65:41–46

Madigan MT (1992) The family Heliobacteriaceae In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, vol 2. Springer, Berlin Heidelberg New York, pp 1981–1992

Madsen T, Licht D (1992) Isolation and characterization of an anaerobic chlorophenol-transforming bacterium. Appl Environ Microbiol 58:2874–2878

Meßmer M, Wohlfarth G, Diekert G (1993) Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen strain MC. Arch Microbiol 160:383–387

Mohn WW, Kennedy KJ (1992) Reductive dehalogenation of chlorophenols by *Desulfomonile tiedjei* DCB1. Appl Environ Microbiol 58:1367–1370

Mohn WW, Tiedje JM (1990) Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. Arch Microbiol 153:267–271

Mohn WW, Tiedje JM (1992) Microbial reductive dehalogenation. Microbiol Rev 56:482–507

Nanninga HJ, Gottschal JC (1985) Amino acid fermentation and hydrogen transfer in mixed cultures. FEMS Microbiol Ecol 31:261–269

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

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448

Pieper DH, Reineke W, Engesser KH, Knackmuss H-J (1988) Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. Arch Microbiol 154:600–604

Saitu N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36: 407–477

Scholz-Muramatsu H, Neumann A, Meßner M, Moore E, Diekert G (1995) Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch Microbiol 163:48–56

Smith L (1978) Cytochrome spectra. Methods Enzymol 53:202–212

Terzenbach DP, Blaut M (1994) Transformation of tetrachloroethene to trichloroethene by homoacetogenic bacteria. FEMS Microbiol Lett 123:213–218

Trüper HG, Schlegel HG (1964) Sulfur metabolism in Thiorhodaceae. 1. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek 30:225–238

Utkin I, Woese C, Wiegel J (1994) Isolation and characterization of *Desulfitobacterium dehalogenans* gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int J Syst Bacteriol 44:612–619

Utkin I, Dalton DD, Wiegel J (1995) Specificity of reductive dehalogenation of substituted *ortho*-chlorophenols by *Desulfitobacterium dehalogenans* JW/IU-DC1. Appl Environ Microbiol 61:346–351

Van der Woude BJ, De Boer M, Van der Put NMJ, Van de Geld FM, Prins RA, Gottschal JC (1994) Anaerobic degradation of halogenated benzoic acids by photoheterotrophic bacteria. FEMS Microbiol Lett 119:199–208

Widdel F (1992) The genus *Desulfotomaculum*. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, vol 2. Springer, Berlin Heidelberg New York, pp 1792–1799