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Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene

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Abstract

Strain TEA, a strictly anaerobic, motile rod with one to four lateral flagella and a crystalline surface layer was isolated from a mixed culture that completely reduces chlorinated ethenes to ethene. The organism coupled reductive dehalogenation of tetrachloroethene or trichloroethene to *cis*-1,2-dichloroethene to growth, using molecular hydrogen as the electron donor. It was unable to grow fermentatively or in the presence of tri- or tetrachloroethene with glucose, pyruvate, lactate, acetate or formate. The 16S rDNA sequence of strain TEA was 99.7% identical to that of *Dehalobacter restrictus*. The two organisms thus are representatives of the same species or the same genus within the *Bacillus/Clostridium* subphylum of the gram-positive bacteria.

Introduction

Chlorinated ethenes are among the most frequently found contaminants in ground water. Anaerobic dehalogenation of these compounds by bacterial mixed cultures via sequential reductive dehalogenation to ethene (DiStefano et al. 1991; Freedman & Gossett 1989) or ethane (De Bruin et al. 1992; Utkin et al. 1994) as end products is well documented. Pure cultures of two strictly anaerobic bacteria coupling the reduction of chlorinated ethenes to growth have been described (Holliger et al. 1993; Scholz-Muramatsu et al. 1994). Both isolates oxidize an electron donor with concomitant reduction of trichloroethene (TCE) or tetrachloroethene (PCE) to *cis*-dichloroethene (*cis*-1,2-DCE). The chlorinated ethenes thus serve as terminal electron acceptors in a novel respiratory process (Holliger & Schumacher 1994). In a recently isolated facultatively anaerobic bacterium that transforms PCE to *cis*-1,2-DCE, coupling of PCE dehalogenation to growth is very inefficient or does not occur at all (Sharma & McCarty 1996). One of the strictly anaerobic PCE dehalogenating organisms, *Dehalospirillum multivorans*, utilizes a variety of electron donors such as pyruvate, lactate, hydrogen and formate (Scholz-Muramatsu et al. 1995). *Dehalobacter restrictus*, the other anaerobe, uses only hydrogen as an electron donor and depends on the presence of TCE or PCE for growth (Holliger et al. 1993). No other electron acceptor is known and the organism does not grow fermentatively. Here we describe the isolation and characterization of strain TEA, a respiratory-dehalogenating isolate closely related to *Dehalobacter restrictus*.

Materials and methods

Growth conditions and assay of dehalogenation activity

The growth medium used in the present work (medium A) was similar to the medium described previously (Wild et al. 1995), except that 55 mM carbonate was used as buffer, 1.0 mM of titanium citrate (Zehnder & Wuhrmann 1976) as reductant and H_2/CO_2 (80%/20%, v/v) at 2.8 bar as the gas phase. 5% (v/v) of filter-sterilized spent medium from the fixed-bed reactor was added as a source of growth factors. The fixed-bed

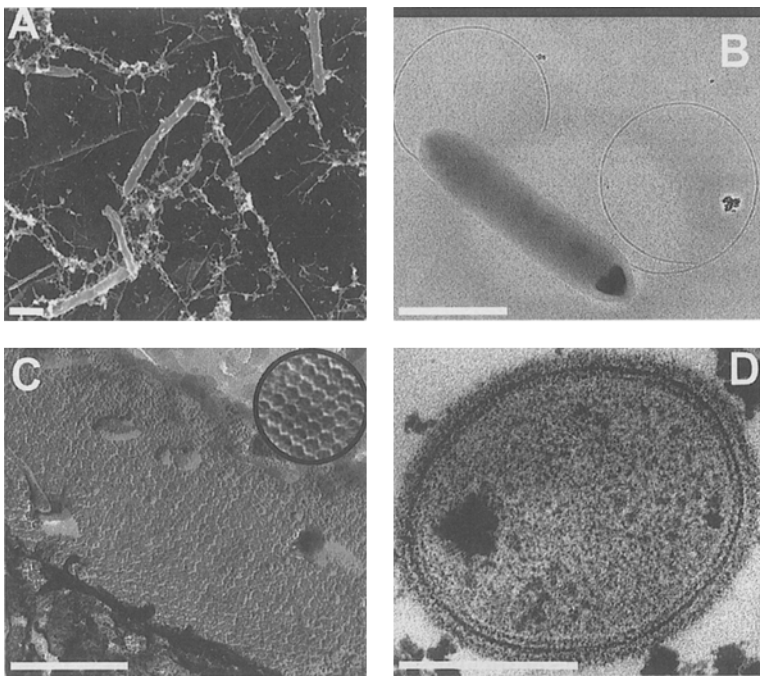

Figure 1. Electron micrographs of strain TEA. (A) Scanning electron micrograph. (B) Transmission electron micropgraph, flagella preparation. (C) Transmission electron micrograph, surface structure. The round inset shows an enlarged, Fourier-filtered image of the displayed area. The lattice constant is 14 nm. (D) Transmission electron micrograph, thin-section. The white bars equal 1 µm in A and B or 0.2 µm in C and D.

reactor (1.2 liter) providing the spent medium has been described (Wild et al. 1995). It was continuously fed at 20 °C with 2 mM glucose and 55 µM tichloroethene at a flow rate of 50 ml/h. All cultures were incubated on a rotary shaker at 30 °C in the dark. Dechlorination activity was monitored by analyzing headspace samples for TCE dehalogenation products as described previously (Wild et al. 1995).

Electron microscopy

For the preparation of thin-sections, bacteria were high-pressure frozen in cellulose capillary tubes (Hohenberg et al. 1994; Müller & Moor 1984). The tubes containing the suspended bacteria were then freeze-substituted in anhydrous acetone containing 2% osmium tetroxide and subsequently embedded in Epon/Araldite (Hohenberg et al. 1994). Whole cells and flagella were visualized by rotary shadowing with platinum/carbon at an angle of 30° after adsorption onto carbon-coated copper grids and subsequent air-drying. Replica of samples that had been frozen in a propane jet and subsequently freeze-etched were used to analyze surface structures (Müller et al. 1980). For scanning electron microscopy bacteria were adsorbed onto glow-discharged carbon-platelets, frozen by rapid plunging into liquid propane and freeze-substituted as described above. After washing in acetone, samples were critical point dried and planar-magnetron sputtered with 6 nm platinum in a Baltec MED 010 (Baltec, Balzers, Fürstentum Liechtenstein).

16S rDNA sequence analysis

Extraction of genomic DNA, PCR-mediated amplification of 16S rDNA, purification of PCR products and sequencing were carried out as described previously (Rainey et al. 1992; Rainey & Stackebrandt 1992). The 16S rDNA sequences were aligned manually against representatives of the *Clostridia* and related taxa. Pairwise evolutionary distances were computed using the correction of Jukes and Cantor (Jukes & Cantor 1969).

Results and discussion

Enrichment and isolation

Dechlorinating organisms were enriched from a laboratory fixed-bed reactor (Wild et al. 1995) that had

Table 1. Electron balance for the transformation of TCE to *cis*-1,2 DCE by a culture of strain TEA[a]

Parameter[b]	Concn. (μM) present on day		Difference	Factor[c]	Educts used	Products formed
	0	8	(μM)	(mol/e$^-$/mol)	(μM e$^-$)[b]	(μM e$^-$)[c]
Hydrogen	2419	64	- 2355	2	- 4710	
Acetate	335	294	- 41	8	- 328	
Formate	10	7	- 3	2	< - 6	
Biomass	< 0.2	2.2	2.2	20		44
PCE	0	0		4		
TCE	3809	1197	- 2612	6	- 15672	
c-DCE	408	2931	2523	8		20184
Vinyl chloride	0	0		10		
Ethene	0	0		12		

[a] The value represent the average from two independent cultures.
[b] Hydrogen, chlorinated ethenes and ethene were determined by gas chromatography (Wild et al. 1995), acetate and formate by ion chromatography (Wild et al. 1995). The amount of protein formed was measured as described (Mägli et al. 1995) and used to calculate the molarity of biomass according to Holliger et al. (1993).
[c] Electron equivalents released upon complete oxidation of the molecule to CO_2, H_2O and inorganic chloride.

been inoculated with material from a full size anaerobic charcoal reactor. This charcoal reactor was located in Sindelfingen (Germany) and used to eliminate dichloromethane and traces of TCE and PCE from contaminated groundwater (Stromeyer et al. 1991).

When medium A was inoculated (5% v/v) with a 10^{-6} fold diluted culture of the fixed-bed reactor, 1 mM TCE was transformed to *cis*-1,2-DCE within 9–14 days. After seven consecutive cultures, each inoculated with a 10^{-6} dilution of the previous culture, *cis*-1,2-DCE was the only dehalogenation product observed. The highly enriched culture consisted of two morphologically distinct organisms: a small rod named strain TEA and a motile, vibroid bacterium named TEB. Strain TEB grew well in Wilkins-Chalgren broth (Oxoid, Basingstoke, UK) and in medium A containing 10 mM lactate plus 10 mM sulfate (medium B). To separate strain TEB from strain TEA, sodium molybdate, a selective inhibitor of sulfate-reducing bacteria (Oremland & Capone 1988), was added to medium A. It was also shown that the spent reactor medium could be replaced by 1 mM acetate. After three more cycles of growth in medium A containing 0.5 mM of molybdate, a pure culture of strain TEA was obtained. The purity of the culture was demonstrated by the absence of growth upon inoculation (5% v/v) of Wilkins-Chalgren broth and of medium B.

Characterization of strain TEA

As shown in Figure 1, strain TEA exhibits a gram-negative cell wall. It is a motile rod with a diameter of 0.2–0.3 μm and a variable length of 2–5 μm. The organism caries 1 to 4 lateral flagella, and it features a distinct crystalline surface-layer (Sleytr & Messner 1988), a property we have also observed for *Dehalobacter restrictus*.

16S rDNA analysis indicated that strain TEA belongs to the subphylum of the gram-positive bacteria with low GC content. Comparison of its 16S rRNA sequence to the 16S rRNA sequence of *Dehalobacter restrictus* (Holliger 1992, C. Holliger personal communication) indicated that these two organisms are closely related (99.7% sequence identity) to each other. Strain TEA is also related to other strictly anaerobic bacteria such as *Desulfotomaculum orientis* (94% sequence identity), an acetogenic sulfate reducer (Devereux et al. 1989), and it also shares 92.5% sequence identity with *Desulfitobacterium dehalogenans* (Utkin et al. 1994), a respiratory-dehalogenating bacterium reducing chlorophenols. The 16S rDNA sequence of strain TEA has been deposited in the EMBL data library under accession number Y10164.

Electron balance and stoichiometry of dehalogenation

Table 1 shows the electron balance in a culture of strain TEA. The organism stoichiometrically reduced TCE to *cis*-1,2-DCE, using hydrogen as the electron donor and acetate and/or carbon dioxide as carbon source(s). The electron recovery of 97.6 ± 4.6% indicates that oxidation of hydrogen coupled to reductive dehalogenation of TCE to *cis*-1,2-DCE was the only relevant energy generating redox process in the system. The

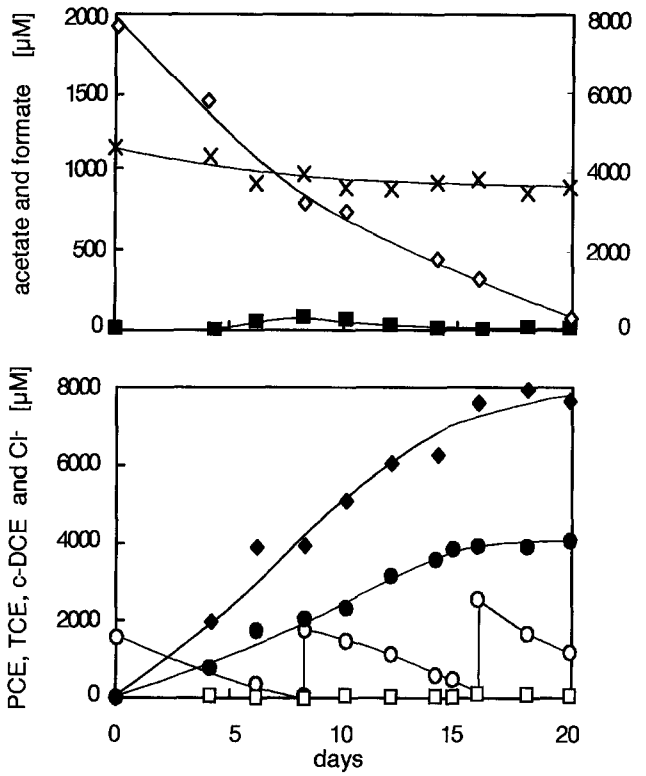

Figure 2. Consumption of H_2 (◊), PCE (○) and acetate (×) and formation of formate (■), TCE (□), *cis*-1,2-DCE (•) and chloride (♦) in a batch culture of strain TEA.

growth yield of strain TEA in this and other experiments ranged between 0.1 and 0.25 g of biomass per mol of chloride released. Although media of closely similar composition were employed for growing strain TEA and *Dehalobacter restrictus*, the growth yield of Strain TEA was ten- to twentyfold below that observed for *Dehalobacter restrictus* (Holliger et al. 1993) and for *Dehalospirillum multivorans* (Scholz-Muramatsu et al. 1994). In strain TEA the energy of reductive dehalogenation thus is inefficiently conserved. However, since propagation of the organism was strictly dependent on the presence of TCE or PCE in the medium, this inefficient process appears essential for providing energy for growth. Lactate, pyruvate, acetate, formate, and glucose did not serve as electron donors for strain TEA. Although the original habitat of the organism contained only 0.03 µM PCE and the enrichment was performed with TCE as an electron acceptor, the organism readily transformed PCE to *cis*-1,2-DCE without intermittent accumulation of TCE (Figure 2).

In conclusion, we have isolated from a geographically distant site an organism representing either a new strain of the species *Dehalobacter restrictus* or a new species of the genus *Dehalobacter*. Strain TEA originated from an enrichment originally inoculated with contaminated groundwater from a site near Stuttgart, Germany (Wild et al. 1995) while *Dehalobacter restrictus*, formerly designated strain PER-K23, was enriched from a column that had been wet packed with anaerobic sediment from the Rhine river near Wageningen, The Netherlands (Holliger et al. 1993). This suggests that bacteria of the genus *Dehalobacter* (Holliger and Schumacher 1994) are well adapted to growth in anaerobic systems contaminated with PCE and TCE. It will be interesting to explore the parameters that determine competitiveness of the highly specialized *Dehalobacter* with the metabolically more flexible *Dehalospirillum multivorans* and the recently discovered facultatively aerobic bacterium transforming PCE to *cis*-1,2-DCE (Sharma & McCarty 1996).

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