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# Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium

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**Tetrachloroethene (PCE) and trichloroethene (TCE) are ideal solvents for numerous applications, and their widespread use makes them prominent groundwater pollutants. Even more troubling, natural biotic and abiotic processes acting on these solvents lead to the accumulation of toxic intermediates (such as dichloroethenes) and carcinogenic intermediates (such as vinyl chloride)[1-4]. Vinyl chloride was found in at least 496 of the 1,430 National Priorities List sites identified by the US Environmental Protection Agency, and its precursors PCE and TCE are present in at least 771 and 852 of these sites, respectively[5]. Here we describe an unusual, strictly anaerobic bacterium that destroys dichloroethenes and vinyl chloride as part of its energy metabolism, generating environmentally benign products (biomass, ethene and inorganic chloride). This organism might be useful for cleaning contaminated subsurface environments and restoring drinking-water reservoirs.**

Crucial to the detoxification of chloroethene-contaminated sites is the complete reductive dechlorination of these contaminants to non-chlorinated end products such as ethene, or their oxidation to carbon dioxide (mineralization) Microbial growth linked to the mineralization of *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) occurs under aerobic conditions[6, 7], but VC is generated frequently from polychlorinated ethenes in anoxic and reduced environments Thus, an anaerobic process that leads to complete detoxification would be most effective in achieving bioremediation *in situ* Substantial information describing bacteria that use polychlorinated ethenes as metabolic electron acceptors has accumulated[8, 9] but the populations capable of complete reductive dechlorination have remained elusive *Dehalococcoides ethenogenes* strain 195 (GenBank accession number AF004928 2) has been shown to dechlorinate PCE to ethene, but this organism failed to gain energy from VC, slowly producing ethene in a co-metabolic process[10] To exploit the reductive dechlorination process for environmental cleanup, finding organisms that efficiently reduce VC to ethene is a priority

Starting with a microcosm capable of dechlorinating PCE to ethene[11], we isolated a novel

bacterium that used VC as a growth-supporting electron acceptor, thereby transforming this carcinogenic compound to the benign products ethene and inorganic chloride The isolation procedure took advantage of the organism's ability to derive all its energy required for growth from the reduction of VC to ethene Continued transfers over 4 years in mineral salts medium amended with VC, hydrogen and acetate yielded a non-methanogenic, ethene-producing culture Dechlorination occurred with acetate as the sole electron donor, although at lower rates, apparently mediated in association with a syntrophic, acetate-oxidizing partner population[11, 12] Consecutive transfers without hydrogen achieved further enrichment of the dechlorinating population VC dechlorination activity was recovered repeatedly from $10^{-5}$ dilutions of consecutive dilution-to-extinction series in hydrogen-amended medium Microscopic examination after this enrichment procedure revealed the presence of three morphotypes a small, disc-shaped organism and two rod-shaped organisms, one short and one long Early attempts to tease out the VC-dechlorinating population in pure culture by using the dilution-to-extinction principle as well as cultivation in semisolid medium containing 0 5% low-melting agarose were unsuccessful Similarly to *Dehalococcoides* strains 195 (ref 10) and CBDB1 (ref 13), the addition of high concentrations of the peptidoglycan inhibitor ampicillin did not prohibit dechlorination, and after five consecutive transfers in liquid medium with 1 mg $ml^{-1}$ ampicillin, the rod-shaped organisms were no longer detectable by microscopic examination After treatment with ampicillin, dechlorinating activity was recovered repeatedly from $10^{-7}$ dilutions in defined basal salts medium amended with VC, hydrogen and acetate In addition, VC dechlorination occurred after transferring tiny opaque colonies that developed after 4–5 weeks in semisolid medium to liquid medium No growth occurred in complex medium, and the culture seemed microscopically homogeneous (Fig 1a) The isolate, designated BAV1, is a small non-motile organism of no more than 0 8 μm in diameter (Fig 1) Careful light-microscopic analysis suggested that BAV1 cells are disc-shaped rather than coccoid, supporting an earlier observation indicating that *Dhc ethenogenes* strain 195 cells might be flattened[10] Cells suspended in liquid seemed to tumble end over end, a phenomenon explained by a disc-shaped morphology High-resolution scanning electron microscopy also suggested that BAV1 cells have a disc-shaped rather than a coccoid morphology (Fig 1b) Figure 1b,c shows individual BAV1 cells and features peculiar filamentous appendages on the cell's surface Different 16S ribosomal RNA gene-based approaches corroborated the purity of the culture Terminal restriction-fragment-length polymorphism revealed single peaks of the expected sizes of 197, 442 and 512 base pairs (bp) after digestion of the amplicons with the restriction enzymes *Hha*I, *Msp*I and *Rsa*I, respectively Denaturing gradient gel electrophoresis analysis yielded a single 148-bp band, whose sequence exactly matched that of isolate BAV1 Amplified ribosomal DNA restriction analysis (ARDRA) of 80 clones from two 16S rRNA gene clone libraries established with genomic DNA obtained from a VC-grown culture generated patterns predicted by digestion *in silico*, indicating that all 16S rRNA gene inserts contained in the clone libraries belonged to isolate BAV1 The fact that the *Dhc ethenogenes* strain 195 genome (http //www tigr org) possesses a single ribosomal RNA operon suggested that a particular 16S rRNA gene-based analytical technique would not be complicated by the possible variations in multiple (slightly different) 16S rRNA gene sequences

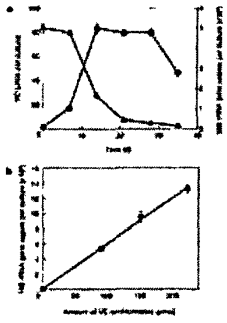
**Figure 1** Micrographs of isolate BAV1 Full legend

High resolution image and legend (102k)

Isolate BAV1 respired VC in defined, completely synthetic basal salts medium amended with acetate and hydrogen (Fig 2a) At room temperature (22–25 °C), BAV1 dechlorinated VC at up to 134 2 ± 10 nmol $min^{-1}$ per mg of protein, and grew with a doubling time of 2 2 days to yield 239 ± 27 mg (mean ± s d , $n = 6$) of protein per mole of chloride released Growth depended strictly on reductive dechlorination and the presence of hydrogen as an electron donor, which could not be replaced by organic substrates including formate, acetate, lactate, pyruvate, propionate, glucose, ethanol or yeast extract Besides VC, other growth-supporting electron acceptors included *cis*-DCE, *trans*-DCE, 1,1-DCE or 1,2-dichloroethane and vinyl bromide, stoichiometric amounts of ethene accumulated as the reduced end product It is notable that BAV1 is the first isolate capable of the metabolic dechlorination of all DCE isomers Chlorinated compounds not supporting growth included PCE, TCE, chlorinated propanes, 1,1,1-trichloroethane, 1,1-dichloroethane and chloroethane However, PCE and TCE were co-metabolized in the presence of a growth-supporting chloroethene and ethene was produced Other organic and inorganic electron acceptors such as nitrate, fumarate, ferric iron, sulphite, sulphate, thiosulphate, sulphur or oxygen were not utilized, and no fermentative growth was observed Respiratory growth was demonstrated conclusively by the chloroethene-dependent increase in cellular macromolecules (such as protein and DNA) Neither cell proliferation nor protein increase was detected in cultures lacking VC, acetate or hydrogen Real-time polymerase chain reaction (PCR) showed that the increase in cell numbers was concomitant with the consumption of VC (Fig 2a) A linear increase in biomass (that is, cells, as measured by the increase in 16S rRNA gene copies) occurred with increasing amounts of VC provided as electron acceptor, indicating a tight coupling between reductive dechlorination and growth (Fig 2b) The number of 16S rRNA gene copies measured in cultures without VC corresponded to the number of cells transferred with the inoculum, confirming that no growth occurred in the absence of VC Cultures that had consumed 80 μmol of *cis*-DCE contained about twice as many 16S rRNA gene copies than cultures grown with 80 μmol of VC (for example, (9 28 ± 0 41) × $10^9$ copies versus (4 99 ± 0 26) × $10^9$ copies) These findings show that BAV1 captured energy from both dechlorination steps when grown with *cis*-DCE

**Figure 2** VC-dependent growth of isolate BAV1 Full legend

High resolution image and legend (47k)



Phylogenetic analysis, performed by using double-stranded 16S rRNA gene sequencing, affiliated isolate BAV1 (AY165308) with the Pinellas group of the *Dehalococcoides* cluster[14], a deep branch within the phylum Chloroflexi (green non-sulphur bacteria)[13] The Pin-ellas group also includes *Dehalococcoides* sp strains CBDB1 (AF230641)[13] and FL2 (AF357918 2) [9] Metabolic ethene formation is not restricted to members of the Pinellas group and was described in a mixed culture containing a *Dehalococcoides* population of the Victoria group[15] Table 1 shows known metabolic electron acceptors along with the phylogenetic grouping of identified *Dehalococcoides*-like populations BAV1 shares a highly similar 16S rRNA gene sequence with *Dehalococcoides* populations that failed to dechlorinate chloroethenes or grow with VC as a metabolic electron acceptor (for example, strains CBDB1 and FL2) This high degree of 16S rRNA gene sequence similarity among members of the *Dehalococcoides* cluster implies that an analysis of 16S rRNA gene sequences cannot distinguish between populations of this group exhibiting different physiological activities Hence, focusing only on 16S rRNA gene sequence analysis is not sufficient for characterizing the dechlorinating community or for the reliable prediction of the dechlorination potential associated with a particular environment

Complete reductive dechlorination of chlorinated ethenes to non-toxic end products has been documented extensively in laboratory studies[12, 15, 16], and ethene formation was observed at some contaminated sites[14] However, until now no organisms that efficiently dechlorinate VC have been obtained in pure culture The isolation of the VC-respiring population BAV1 is a relevant milestone in chloroethene detoxification and provides deeper insight into poorly understood halogen cycles that involve the natural formation and utilization of chloroorganic compounds, including VC[17, 18] The comprehension of such cycles might explain why complete reductive dechlorination does not occur at all contaminated sites, even after alteration and optimization of geochemical conditions or supplying suitable electron donors to accelerate microbial activity A recent pilot demonstration in a hydraulically controlled recirculation test plot at the chloroethene-contaminated Bachman Road site in Oscoda, Michigan, supports bioaugmentation with BAV1 as a promising approach to achieve detoxification at sites where the indigenous microbiota to drive the reductive dechlorination process to completion is absent or the rates of contaminant removal are insufficient[19] Such innovative technologies are needed to clean up numerous chloroethene-contaminated aquifers at reasonable costs within acceptable time frames, and to protect threatened drinking-water reservoirs

## Methods

**Medium preparation** Completely defined, anaerobic mineral salts medium was prepared as described[12, 20, 21] The enrichment and isolation process was performed in 20-ml glass vials containing 10 ml (final volume) of growth medium, whereas the cultures used for kinetic studies or molecular analyses were grown in 160-ml serum bottles containing 100 ml of medium Unless indicated otherwise, cultures were incubated at 30 °C in the dark without shaking Soluble substrates were added at 5 mM Hydrogen was added by syringe to at least double the concentration required for complete reductive dechlorination (namely 8 5–17 kPa) Chlorinated compounds were added to final aqueous concentrations in the range of 0 47–1 33 mM Semisolid medium was prepared by adding 0 5% w/w low-melting agarose before autoclaving

**Analytical techniques** The protein content of liquid cultures was estimated as follows Cells were harvested from 8 ml of culture fluid by centrifugation (10 min, 10,000 *g*) After alkaline cell lysis[22], the Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, Illinois) was used in accordance with the manufacturer's recommendations Spectrophotometric determination at 595 nm was used to quantify protein by comparing the sample absorbance with protein standards of known concentration prepared in the same way as the samples Chloroethene and ethene concentrations were determined by gas chromatography as described[12]

**Electron microscopy** A Zeiss LSM 510 confocal microscope with a Plan-Neofluar objective (100 ×, numerical aperture, 1 3) was used to obtain micrographs of cell suspensions after staining with acridine orange (15–60 min in 0 01% aqueous solution) Scanning electron microscopy micrographs were obtained with a TOPCON DS-130 field-emission scanning electron microscope with samples staged 'in-lens' and photographed at 20 kV Samples were prepared as described[23], and coated with chromium (about 1 5 nm thickness) in a Denton DV-602 turbo magnetron sputter system

**Molecular analyses** DNA was extracted from actively growing cultures as described[12] Real-time PCR to quantify BAV1 cells used a probe targeted to the *Dehalococcoides* 16S rRNA gene, tagged with a 6-carboxyfluorescein reporter fluorochrome on the 5' end, and *N*,*N*,*N*',*N*'-tetramethyl-6-carboxyrhodamine quencher on the 3' end as described previously[12] Linear calibration curves ($r^2 > 0$ 99) were generated, spanning a template concentration range from 6 9 × $10^2$ to 6 9 × $10^6$ 16S rRNA gene copies per 30-µl reaction volume by using BAV1 genomic DNA or plasmid DNA containing the 16S rRNA gene from BAV1 Analysis of individual clones with ARDRA was performed as described previously[12, 24], except that the PCR-amplified 16S rRNA gene products from each clone were digested for 3 h with enzymes *Hha*I, *Msp*I and *Rsa*I at 37 °C The reactions were terminated by incubation at 65 °C for 10 min, in accordance with the manufacturer's recommendations (Gibco), and the resulting fragments were resolved by electrophoresis for 2 h on 2 5% low-melting agarose gel (Seaplaque, Cambrex, Rockland, Maine) The fluorescently labelled primer 8F-hex (5'-AGA GTT TGA TCC TGG CTC AG-3') and unlabelled 1492R (5'-GC(C/T) TAC CTT GTT ACG ACT T-3') were used to

amplify the 16S rRNA gene from pure culture DNA Fluorescently labelled terminal fragments obtained by digesting the PCR product with *Hha*I, *Msp*I and *Rsa*I were analysed at Michigan State University's Genomics Technology Support Facility Denaturing gradient gel electrophoresis was performed by Microbial Insights (Rockford, Tennessee) with the use of universal bacterial primers corresponding to *Escherichia coli* positions 341–534 as described[16]

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## References


1 Kielhorn, J , Melber, C , Wahnschaffe, U , Aitio, A & Mangelsdof, I Vinyl chloride still a cause for concern *Environ Health Perspect* **108**, 579-588 (2000) | PubMed | ISI | ChemPort |

2 Mohn, W W & Tiedje, J M Microbial reductive dehalogenation *Microbiol Rev* **56**, 482-507 (1992) | PubMed | ISI | ChemPort |

3 Roberts, A L *et al* Reductive elimination of chlorinated ethylenes by zero-valent metals *Environ Sci Technol* **30**, 2654-2659 (1996) | Article | ISI | ChemPort |

4 Vogel, T M , Criddle, C S & McCarty, P L Transformation of halogenated aliphatic compounds *Environ Sci Technol* **21**, 722-736 (1987) | ISI | ChemPort |

5 US Environmental Protection Agency Agency for Toxic Substances and Disease Registry, ToxFAQs for chlorinated ethenes, http //www atsdr cdc gov/tfacts70 html (1996)

6 Coleman, N V , Mattes, T E , Gossett, J M & Spain, J C Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites *Appl Environ Microbiol* **68**, 6162-6171 (2002) | Article | PubMed | ISI | ChemPort |

7 Coleman, N V , Mattes, T E , Gossett, J M & Spain, J C Biodegradation of *cis*-dichloroethene as the sole carbon source by a β-Proteobacterium *Appl Environ Microbiol* **68**, 2726-2730 (2002) | Article | PubMed | ISI | ChemPort |

8 Holliger, C , Wohlfarth, G & Diekert, G Reductive dechlorination in the energy metabolism of anaerobic bacteria *FEMS Microbiol Rev* **22**, 383-398 (1998) | Article | ISI | ChemPort |

9 Loffler, F E , Cole, J R , Ritalahti, K M & Tiedje, J M in *Dehalogenation Microbial Processes and Environmental Applications* (eds Haggblom, M M & Bossert, I D ) 53-87 (Kluwer Academic, New York, 2003)

10 Maymó-Gatell, X , Chien, Y -T , Gossett, J M & Zinder, S H Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene *Science* **276**, 1568-1571 (1997) | Article | PubMed | ISI | ChemPort |

11 He, J *et al* Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites *Environ Sci Technol* **36**, 3945-3952 (2002) | Article | PubMed | ISI | ChemPort |

12 He, J , Ritalahti, K M , Aiello, M R & Loffler, F E Complete detoxification of vinyl chloride (VC) by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* population *Appl Environ Microbiol* **69**, 996-1003 (2003) | Article | PubMed | ISI | ChemPort |

13 Adrian, L , Szewzyk, U , Wecke, J & Gorisch, H Bacterial dehalorespiration with chlorinated benzenes *Nature* **408**, 580-583 (2000) | Article | PubMed | ISI | ChemPort |

14 Hendrickson, E R *et al* Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe *Appl Environ Microbiol* **68**, 485-495 (2002) | Article | PubMed | ISI | ChemPort |

15 Cupples, A M , Spormann, A M & McCarty, P L Growth of a *Dehalococcoides*-like

microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR *Appl Environ Microbiol* **69**, 953-959 (2003) | Article | PubMed | ISI | ChemPort |

16 Duhamel, M *et al* Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, *cis*-dichloroethene and vinyl chloride *Water Res* **36**, 4193-4202 (2002) | Article | PubMed | ISI | ChemPort |

17 Gribble, G W in *Chlorinated Compounds in the Biosphere, Natural Production* (ed Meyers, R A ) 972-1035 (Wiley, New York, 1998)

18 Keppler, F , Borchers, R , Pracht, J , Rheinberger, S & Scholer, H F Natural formation of vinyl chloride in the terrestrial environment *Environ Sci Technol* **36**, 2479-2483 (2002) | Article | PubMed | ISI | ChemPort |

19 Lendvay, J M *et al* Bioreactive barriers bioaugmentation and biostimulation for chlorinated solvent remediation *Environ Sci Technol* **37**, 1422-1431 (2003) | Article | ISI | ChemPort |

20 Loffler, F E , Ritalahti, K M & Tiedje, J M Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens *Appl Environ Microbiol* **63**, 4982-4985 (1997) | ISI |

21 Loffler, F E , Tiedje, J M & Sanford, R A Fraction of electrons consumed in electron acceptor reduction and hydrogen threshold as indicators of halorespiratory physiology *Appl Environ Microbiol* **65**, 4049-4056 (1999) | PubMed | ISI |

22 Gerhardt, P (ed ) *Manual of Methods for General Bacteriology* (American Society for Microbiology, Washington DC, 1981)

23 Sung, Y *et al* Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp nov *Appl Environ Microbiol* **69**, 2964-2974 (2003) | Article | PubMed | ISI | ChemPort |

24 Loffler, F E , Sun, Q , Li, J & Tiedje, J M 16S rRNA gene-based detection of tetrachloroethene (PCE)-dechlorinating *Desulfuromonas* and *Dehalococcoides* species *Appl Environ Microbiol* **66**, 1369-1374 (2000) | Article | PubMed | ISI |

25 Bunge, M *et al* Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium *Nature* **421**, 357-360 (2003) | Article | PubMed | ISI | ChemPort |

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