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ORIGINAL PAPER

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A non-dechlorinating strain of *Dehalospirillum multivorans*: evidence for a key role of the corrinoid cofactor in the synthesis of an active tetrachloroethene dehalogenase

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Abstract A strain of *Dehalosprillum multivorans*, designated strain N, was isolated from the same source as the formerly described tetrachloroethene (PCE)-dechlorinating *D. multivorans*, herein after referred to as strain K. Neither growing cells nor cell extracts of strain N were able to dechlorinate PCE. The *pceA* and *pceB* genes encoding for the PCE-reductive dehalogenase were detected in cells of strain N; and they were 100% homologous to the corresponding genes of strain K. Since the PCE dehalogenase of *D. multivorans* strain K contains a corrinoid cofactor, the corrinoids of strain N cells were extracted. Analysis of the corrinoids revealed the absence of the specific corrinoid, which is the cofactor of the PCE dehalogenase of strain K cells. RT-PCR of mRNA indicated that the *pceA* gene was transcribed in strain N cells to a far lower extent than the *pceA* gene of strain K under the same experimental conditions. Western blot analysis of crude extracts of strain N showed that, if at all, an insignificant amount of the apoprotein of the PCE dehalogenase was present. The results indicate that the inability of strain N to dechlorinate is due to the absence of the corrinoid cofactor of the enzyme mediating PCE dechlorination.

Keywords Corrinoid · Dehalorespiration · *Dehalospirillum multivorans* · PCE · *pceA* · *pceB* · Strain K · Strain N · Tetrachloroethene · Tetrachloroethene reductive dehalogenase

Introduction

Dehalospirillum multivorans is a strictly anaerobic, gram-negative bacterium, which is able to grow at the expense of hydrogen plus tetrachloroethene (PCE) as sole energy sources (Scholz-Muramatsu et al. 1995). The enzyme mediating the dechlorination of PCE via trichloroethene (TCE) to *cis*-1,2-dichloroethene (DCE) is the PCE-reductive dehalogenase, a protein containing iron–sulfur clusters and a corrinoid cofactor (Neumann et al. 1995, 1996). In the past few years, several reductive dehalogenases have been isolated from anaerobic bacteria that show similar features (Holliger et al. 1999; Wohlfarth and Diekert 1999). Among these organisms, different species of the gram-positive *Desulfitobacterium* spp and *Dehalospirillum multivorans* are well characterized with respect to the dehalogenation of chlorinated ethenes and phenols. The ability to reduce chlorinated compounds was thought to be a major physiological characteristic of these species. However, the isolation of a non-dechlorinating *Desulfitobacterium* strain from human feces has been reported recently (van de Pas et al. 2001). Here, we report on the isolation and properties of a non-dechlorinating strain of *Dehalospirillum multivorans*, herein after designated strain N, which was isolated from the same source as the PCE-dechlorinating type strain of *D. multivorans*, herein referred to as strain K. The reason for the inability of strain N to dechlorinate PCE was investigated.

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Materials and methods

Cultivation procedures

D. multivorans strain N (non-dechlorinating; DSM 15119; this work) and strain K (PCE-dechlorinating; DSM 12446; Scholz-Muramatsu et al. 1995) were routinely grown under identical conditions at 25 °C in anoxic mineral medium with pyruvate (40 mM) in the presence of PCE (0.2 mM), as described by Neumann et al. (1994). *Sulfurospirillum deleyianum* (DSM 6946) was grown in the same medium. For analysis of cell components and enzyme activity, the pre-cultures were grown with pyruvate plus PCE and the medium was depleted of yeast extract. The main cultures (1 l) were inoculated with 10% and grown with pyruvate plus fumarate (40 mM) instead of PCE. Cells were harvested in the late exponential growth phase by centrifugation at 10,000×*g* and 4 °C. They were frozen in liquid nitrogen and stored at –70 °C. Cell extracts were prepared as described by Neumann et al. (1996).

Partial purification of the PCE-reductive dehalogenase

The PCE-reductive dehalogenase was enriched by fast performance liquid chromatography (FPLC) as described by Neumann et al. (1996). Crude extracts of 20 g of wet cells were loaded onto a HiLoad 16/10 Q-Sepharose HP column equilibrated with 50 mM Tris HCl (pH 7.5) containing 0.5 mM dithiothreitol (buffer A). After washing the column with 50 ml buffer A and subsequently with 200 ml buffer A containing 30 mM KCl, the protein was eluted with a linear gradient from 30 mM to 1 M KCl in buffer A (400 ml). The flow rate was set to 4 ml min^{-1}. The enzyme activity in the fractions (8 ml) was tested using the spectrophotometric assay described below. Active fractions were pooled and used for Western blot analysis and cyanide extraction of the corrinoids.

Partial purification of polyclonal antibodies against PCE-reductive dehalogenase

Polyclonal antibodies against PCE-reductive dehalogenase were obtained using purified PCE-reductive dehalogenase for immunization of a rabbit (3 months; SeqLab, Göttingen, Germany). The antibodies were partially purified from the serum using affinity chromatography with immobilized PCE dehalogenase. For preparation of the affinity chromatography column, CNBr-activated sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) was used as column material. Recombinant PCE dehalogenase (with leader peptide) (Neumann et al. 1998) was bound to the column, according to the manufacturer's protocol. The enzyme was obtained from inclusion bodies by denaturation with 8 M urea. Urea was removed by dialyzing against 20 mM Hepes (pH 7.5) containing 0.1% Triton X-100. The protein was diluted with 4 ml of 0.1 M $NaHCO_3$ (pH 8.5), added to the activated column material (3.5 ml), and the suspension was stirred overnight at 4 °C. The suspension was subsequently applied to an empty column (10 mm i.d., 43 mm long), and non-immobilized PCE dehalogenase was removed by washing the column with 0.1 M $NaHCO_3$ (pH 8.5). Remaining reactive groups were blocked, according to the manufacturer's protocol. The serum containing approximately 170 mg protein was diluted two-fold with PBS (Sambrook et al. 1989), applied to the column, and unbound protein was removed by washing the column with 15 ml PBS. The bound PCE dehalogenase antibodies were eluted with 10 ml 0.1 M glycine-HCl (pH 2.5). The protein-containing fractions were adjusted to (pH 7.0) by adding 1 M K_2HPO_4. The buffer was exchanged to PBS by centrifugation with PallFiltron 30K (Pall Gelman Laboratory, Dreieich, Germany) and diluted to a final volume of 1 ml. The antibodies were tested for cross reactivity with crude extracts of strain K cells. The optimal antibody concentration for the Western hybridization was determined using a dot-blot procedure with purified PCE dehalogenase as antigen.

Purification and analysis of corrinoids

For the quantification and analysis of the corrinoids, crude extracts or enriched PCE dehalogenase were extracted with cyanide, using the method described by Stupperich et al. (1986). The corrinoids were purified by reversed-phase HPLC equipped with a diode array detector (Merck, Darmstadt, Germany). Lichrosphere 100 RP-18, 5 µm (5 mm i.d., 250 mm long) served as stationary phase. Corrinoids were eluted with 23% (v/v) methanol in 0.08% (v/v) acetic acid (15 min) followed by a linear gradient to 100% (v/v) methanol within 10 min; and subsequently, the column was washed with 100% (v/v) methanol (7 min). The flow rate was 1 ml min^{-1}. The elution profiles were obtained by measuring the absorption at 360 nm. Peaks corresponding to corrinoids, as identified by their absorption spectra recorded with the diode array detector, were sampled and dried in a speed vacuum concentrator. The corrinoids were dissolved in water and further characterized.

The UV/Vis spectra of the corrinoids were recorded with a Varian Cary 100 spectrophotometer (Varian, Darmstadt, Germany). The corrinoid concentration was determined photometrically using a corrinoid solution in 10 mM KCN. In the presence of KCN, corrinoids are in the dicyanocobalamin form with an absorption maximum at 580 nm (Friedrich 1975). The corrinoid content was calculated assuming an absorption coefficient (ε_{580}) of 10.1 $mM^{-1}\,cm^{-1}$, as reported for dicyanocobalamins (Stupperich et al. 1988). The corrinoids were further characterized by their abiotic reductive dehalogenation activity, using essentially the spectrophotometric assay for the PCE dehalogenase activity (Neumann et al. 1996; see next section). However, the assays were conducted in 800 µl of assay buffer with 0.85 mM trichloroacetic acid as electron acceptor instead of chlorinated ethenes (Neumann et al. 2002). The reaction was started by addition of the corrinoid solution (corrinoid content in the assay ranged over 0.5–50 nM). Rate constants (K) were calculated from the decrease of absorbance at 578 nm and are given as moles of chloride released per mole of corrinoid per second.

Analytical methods

Dechlorination in growing cultures was followed by analysis of the chlorinated ethenes, which were quantified by gas chromatography with flame ionization detection, using a 2 m 10% Ucon LB column (i.d. 2 mm; Werner Günther Analysentechnik, Moers, Germany) and N_2 as carrier gas (gas flow 25–30 ml min^{-1}). The gas sample volume was 0.5 ml, taken from the headspace of heated samples (5 min at 98 °C). The following temperatures were applied: oven 80 °C, injector 150 °C, and detector 250 °C. Under these conditions, the retention times were as follows: DCE = 1.4 min, TCE = 2.2 min, and PCE = 3.6 min.

Enzyme activities were determined using the spectrophotometric assay described by Neumann et al. (1996), using methyl viologen as the artificial electron donor for PCE reduction. The protein concentration was determined according to Bradford (1976), using the Bio-Rad reagent (Bio-Rad Laboratories, Munich, Germany).

Western blot analysis of PCE-reductive dehalogenase

Both the crude extracts and the enriched PCE-reductive dehalogenase of strain K and both the crude extract and the corresponding FPLC fractions of strain N were analyzed using SDS-polyacrylamide gel electrophoresis (12% polyacrylamide). The proteins were blotted onto a polyvinylidene difluoride membrane (Ausubel et al. 1999). The membrane was then equilibrated for 1 h in PBST (PBS plus 0.05% (v/v) Tween 20) containing 10% blocking reagent. After washing the membrane three times for 10 min with PBST, PCE dehalogenase antibodies dissolved in PBST were added and the membrane was incubated at 24 °C for 16 h. Subsequently, the membrane was washed twice with PBST (10 min) and with 100 mM Tris HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$. The secondary antibody was a sheep anti-rabbit antibody-alkaline phosphatase conjugate (Boehringer, Mannheim, Germany) dissolved in the alkaline buffer described above and applied at a concentration of 0.2 units ml^{-1} for 1 h. After washing the membrane three times for 10 min with the same Tris buffer, the reaction mixture for the alkaline phosphatase reaction (alkaline Tris buffer containing, per milliliter: 0.34 mg nitrobluetetrazolium, 0.175 mg·5-bromo-4-chloro-3-indolyl phosphate) was applied. The reaction was stopped with TE buffer (Sambrook et al. 1989) after 3 min (strain K) and 6 min (strain N).

DNA extraction, PCR, and cloning of PCR products for sequence analysis

DNA was extracted from *D. multivorans* according to the miniprep protocol for extraction of bacterial genomic DNA (Ausubel et al. 1999). The PCR was performed with Ready-to-go PCR beads (Amersham Pharmacia Biotech, Freiburg, Germany). For the analysis of the PCE dehalogenase genes, each PCR contained 100 pg of template DNA and 5 pmol of each of two primers (see next section). The following conditions were applied: initial denaturation 3 min at 96 °C and then 30 cycles of 0.5 min at 96 °C, 1 min at 50 °C,

and 2 min at 72 °C. PCR products of strain N were purified using the Strataprep PCR purification kit (Stratagene, La Jolla, USA) and cloned into the pPCR-Script AmpSK+ cloning vector of the Stratagene PCR-Script-Amp-cloning kit, according to the manufacturers protocol. The resulting vector was transformed into XL10-Gold Kan^r-ultracompetent cells supplied with the cloning kit, isolated from an culture grown overnight, using the SeqLab Plasmid Prep kit, and sequenced at SeqLab (Göttingen, Germany), using the respective T7 and T3 primers.

The following primers were used for gene-specific PCR (see Fig. 3B; all primer data in these sections are presented as: primer name, direction, nucleotide (nt) position, sequence 5′→3′).

A, forward, 2,902, AAGCGAATAAAACATAAAATATTAGGGACG.

C, forward, 3,437, GGTTCTCCCATCATAGTTAACGACAAATTGG.

E, reverse, 3,688, ATAGTTAATATCTAAGGTCCATCCAGCA.

F, forward, 4,190, AGCTGTAATGGTGTTGGACAATCAGTTGCC.

G, reverse, 4,287, GGTCCAAATTCAGGAGTAATACAAGCACCC.

H, reverse, 5,145, TTCATCAGCGCTTTGGAGTCACTCC.

RNA extraction and RT-PCR

RNA was isolated from 10 mg of cells of both *D. multivorans* strains, using the Qiagen RNeasy mini-kit protocol (Qiagen, Hilden, Germany). DNA was removed from the preparation by DNaseI digestion in the buffer of the RT-PCR kit for 1 h at 37 °C. DNaseI was subsequently heat-inactivated by incubation for 15 min at 70 °C. The samples were used for RT-PCR with the Reverse iT-1st strand synthesis kit from ABgene (Hamburg, Germany). Gene-specific primers (10 pmol of each) for the *pceA* gene (see below) were used for reverse transcription. RT-PCR was carried out as a two-step procedure, according to the manufacturer's protocol. The PCR conditions described in the previous section were applied for the amplification of cDNA.

The following primers were used (see Fig. 3B): B, forward, 3,162, AACACATTAAAAAATAAATAACTGTACTTGGGG and D, reverse, 3,501, TGAGTAAACGCTGTTCGTACTTCAGC.

Typing of microbial strains by 16S rDNA analysis, repetitive extragenic palindromic sequence-PCR, and repetitive intergenic consensus sequence-PCR

For 16S rDNA analysis of *D. multivorans* strain N, PCR was performed using 200 ng of genomic DNA and 50 pmol of each primer specific for the 16S rDNA for strain K: 16S Dm1, forward, GGCGTGCTTAACACATGCAAGTC and 16S Dm2, reverse, GCAGGTTCTCCTACGGTTACC.

The conditions used for the PCR were identical to those used for detection of the *pceAB* operon (see above). The PCR products were purified using the Strataprep PCR purification kit (Stratagene, La Jolla, USA). Purified PCR product (160 ng) was cloned into the pCR4-TOPO vector of the pCR4-TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany), according to the manufacturer's protocol. The cells used for transformation were *Escherichia coli* TOP10 (Invitrogen, Karslruhe, Germany). Plasmid DNA was purified from an LB-Amp-culture grown overnight, as described by Birnboim and Doly (1979). Two clones were chosen for sequencing with the universal T3 and T7 primers. Sequences of both clones were compared and the consensus sequence is published in GenBank under accession number AF 524868.

DNA fingerprints of repetitive genomic sequences were produced with two sets of primers, one targeting repetitive extragenic palindromic sequences (REP-PCR) and the second targeting repetitive intergenic consensus sequences (ERIC-PCR). The primer sequences were designed according to Versalovic et al. (1991) and were purchased from Genset (Paris, France): REP1RI (IIIICGICGICATCIGGC), REP2I (ICGICTTATCIGGCCTAC), ERIC1R (ATGTAAGCTCCTGGGGATTCAC), and ERIC2 (AAGTAAGTGACTGGGGTGAGCG).

PCR was performed with Ready-to-go PCR beads (Amersham Pharmacia Biotech, Freiburg, Germany). The REP- and ERIC-PCR contained 100 ng of bacterial genomic DNA and 100 pmol of each of the corresponding primers. After initial denaturation of the reaction mixture for 2 min at 94 °C, a touchdown-PCR was performed (according to Johnson and O'Bryan 2000) under the following conditions: 10 cycles of 5 °C touchdown with denaturation for 30 s at 94 °C, ramping at 1.5 °C s^{-1} to the initial annealing temperature of 50 °C (0.5 °C decrease of the annealing temperature per cycle), annealing for 1 min, ramping at 0.1 °C s^{-1} to 72 °C, and holding at 72 °C for 4.5 min. The touchdown cycles were followed by 25 cycles of 30 s at 94 °C, 1 min at 45 °C, and 4.5 min at 72 °C. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

Source of materials

Gases (CO_2 grade 4.5, N_2 grade 5.0, N_2/H_2 at 95%/5%) were supplied by Linde (Leuna, Germany). All chemicals were of the highest available purity and purchased from Aldrich (Steinheim, Germany), Boehringer (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisendorf, Germany). Blocking reagent was from Boehringer (Mannheim, Germany). Primers were purchased from GibcoBRL (Karlsruhe, Germany). Pseudovitamin B_{12} and factor A were kindly provided by Prof. Dr. Bernhard Kräutler (University of Innsbruck, Austria).

Results

Isolation of the non-dechlorinating strain N of *D. multivorans*

The strictly anaerobic, PCE-dechlorinating *D. multivorans* was isolated from activated sludge, which was not exposed

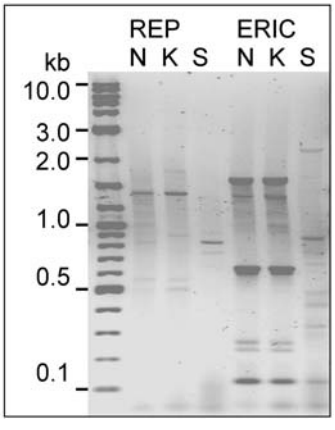

Fig. 1 Strain typing with repetitive extragenic palindromic sequence (REP)-PCR and repetitive intergenic consensus sequence (ERIC)-PCR of genomic DNA of *Dehalospirillum multivorans* strain N (*N*), strain K (*K*), and *Sulfurospirillum deleyianum* (*S*). *Lane 1* DNA marker (Log 2; New England Biolabs, Frankfurt, Germany) with band sizes *indicated at the left*, *lanes 2–4* REP-PCR of *D. multivorans* strain N (*lane 2*), strain K (*lane 3*), and *S. deleyianum* (*lane 4*), *lanes 5–7* ERIC-PCR of *D. multivorans* strain N (*lane 5*), strain K (*lane 6*), and *S. deleyianum* (*lane 7*). PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. The obtained fingerprint image was reversed for better visibility of the weak bands

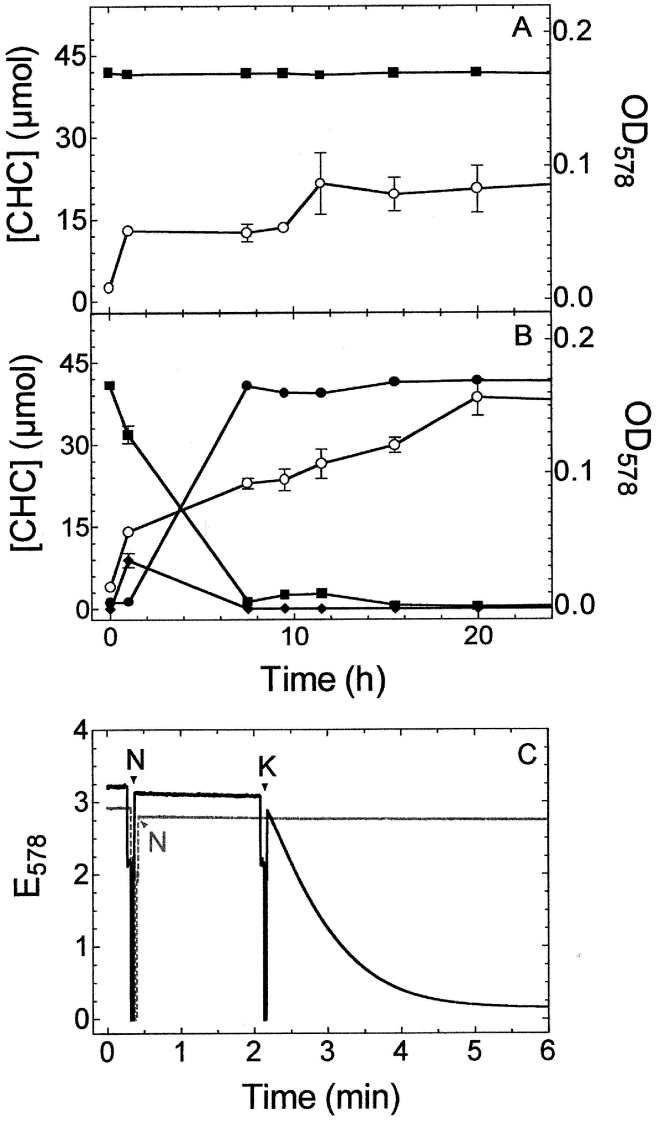

Fig. 2A–C Reductive dechlorination of tetrachloroethene (PCE) in growing cultures and cell extracts of *D. multivorans* strains N and K. **A** Growth (*circles*) and PCE (*squares*) dechlorination in strain N. **B** Growth (*white circles*), PCE (*squares*) dechlorination and formation of trichloroethene (*diamonds*) and cis-1,2-dichloroethene (*black circles*) in strain K. In **A** and **B**, the data shown are means of three replicates. Pyruvate served as electron donor in the cultures. **C** Kinetics of methyl viologen oxidation with PCE as electron acceptor, mediated by cell extracts of strain N (1 mg protein in the assay) or strain K (0.4 mg protein in the assay). The addition of cell extract of strains N and K is indicated by the correspondingly *labeled arrows*

to chlorinated ethenes (Scholz-Muramatsu et al. 1995). Along with the PCE-dechlorinating type strain (strain K), another strain of *D. multivorans* (designated strain N) was isolated from the same source and has been deposited in the DSMZ under accession number 15119. This strain exhibited more than 97% homology of the 16S-rDNA with the PCE-dechlorinating *D. multivorans* strain K. From an ERIC-PCR-based typing technique, the same fragment pattern was obtained for strain N and strain K (Fig. 1, lanes 5, 6). These patterns were different from the pattern obtained for the closely related *S. deleyianum* (lane 7), suggesting that both *D. multivorans* strains are closely related. The DNA-fragment patterns of the REP-PCR obtained for *Dehalospirillum* strain N and strain K were similar but not identical (lanes 2, 3) and differed significantly from the pattern of *S. deleyianum* (lane 4).

Cultures of strain N growing on pyruvate (40 mM) did not dechlorinate PCE (~40 µmol in 210-ml serum bottles with 100 ml liquid medium; Fig. 2A), whereas cultures of strain K rapidly dechlorinated PCE via TCE to DCE under the same experimental conditions (Fig. 2B). Cell extracts of strain N, in contrast to those of strain K, did not mediate the reductive dechlorination of PCE (0.7 mM) and TCE with methyl viologen (0.5 mM) as artificial electron donor (Fig. 2C). These findings indicated that strain N was not able to dechlorinate PCE. Dechlorination was also not observed in cell extracts of strain N with other chlorinated substrates such as 3-chlorobenzoate, 3-chloro-4-hydroxyphenylacetate, and pentachlorophenol (data not shown).

Presence of the PCE-reductive dehalogenase genes in strain N

Southern hybridization of digested genomic DNA of both *D. multivorans* strains with a gene probe for the PCE dehalogenase genes indicated that at least a major part of the

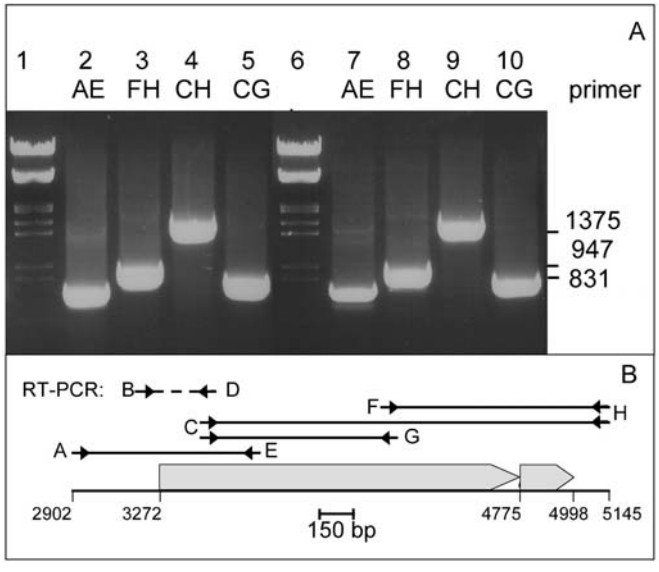

Fig. 3 PCR products (**A**) and structure of the PCE dehalogenase operon (**B**) in *D. multivorans* strain K. **A** *Lanes 1, 6* λ/*Eco*RI/*Hin*dIII DNA marker with sizes *indicated at the right*. *Lanes 2–5* PCR products of strain K obtained by PCR with bacterial genomic DNA, *lanes 7–10* PCR products of strain N DNA with identical primer combinations, as used for strain K. The expected sizes of the PCR products were 786, 955, 1,708, and 850 bp, respectively. **B** The sequence numbering of the genes *pceA* and *pceB* according to the GenBank entry and the position of the respective products *are indicated* [using primers A, C, E–H for PCR and B, D (*dotted line*) for RT-PCR]

genes was present in strain N (data not shown). Therefore, it was tested whether the complete PCE dehalogenase genes, comprising the *pceA* and *pceB* genes, are present in strain N. PCR with different primers was performed for the operon encoding the PCE dehalogenase. The sizes of the PCR products obtained were the same for strain K and strain N (Fig. 3A) and were in accordance with the known gene sequence (Neumann et al. 1998). From the gene sequence, sizes of 786, 955, 1,708, and 850 bp were expected. The PCR products of strain N obtained with the primers A and E (Fig. 3A, lane 7), F and H (lane 8), and C and G (lane 10) were cloned and sequenced. These PCR products comprised both *pceA* and *pceB* and DNA sequences upstream and downstream of the PCE dehalogenase genes. The sequences obtained were 100% identical to the published PCE dehalogenase operon sequence. From this finding, it is concluded that the lack of the ability of strain N to dechlorinate PCE is not due to the absence of the PCE dehalogenase genes.

Absence of the PCE dehalogenase corrinoid cofactor in strain N

Earlier investigations on the PCE-reductive dehalogenase of *D. multivorans* (Neumann et al. 2002) indicated that the enzyme might contain a novel type of corrinoid cofactor. It is feasible that the dechlorination inability of strain N is due to a lack of this specific corrinoid cofactor. Therefore, corrinoids were extracted from cell extracts of both strains and analyzed by HPLC (Fig. 4A, B). The resulting elution profiles were compared with those of corrinoid standards (Fig. 4C) and the corrinoid cofactor extracted from the purified PCE dehalogenase of strain K (data not shown). The PCE dehalogenase corrinoid is indicated by the PCE label in Fig. 4A; and the retention time under the chosen experimental conditions was 6 min. According to the UV-Vis absorption spectra, only peaks indicated by asterisks in Fig. 4A, Brepresent corrinoids.

From extracts obtained from strain N, the only corrinoid detected eluted after about 7.8 min (Fig. 4B); and this retention time was similar to that of cyanocobalamin (8.2 min; peak 2 in Fig. 4C). The assumption that this corrinoid was cyanocobalamin was supported by the mass of the compound determined by matrix-assisted laser-desorption ionization–time of flight (MALDI-TOF; 1,330.5 M2H$^+$-CN; data not shown) and by the K_{TCA}–value. The latter value is the rate of the corrinoid-mediated methyl viologen oxidation with trichloroacetate as electron acceptor (Neumann et al. 2002). It is given as x s^{-1}, corresponding to x mol chloride released mol^{-1} catalyst s^{-1}; and the value for cyanocobalamin is 14 s^{-1}. It cannot be excluded that the cyanocobalamin isolated from strain N cell extracts originated from the culture medium, which contained 80 nM cyanocobalamin.

In cell extracts of strain K, four corrinoid peaks were detected (Fig. 4A). The major peak eluted at about 6 min, which corresponded to the retention time of the PCE dehalogenase cofactor. The K value was about 720 s^{-1}

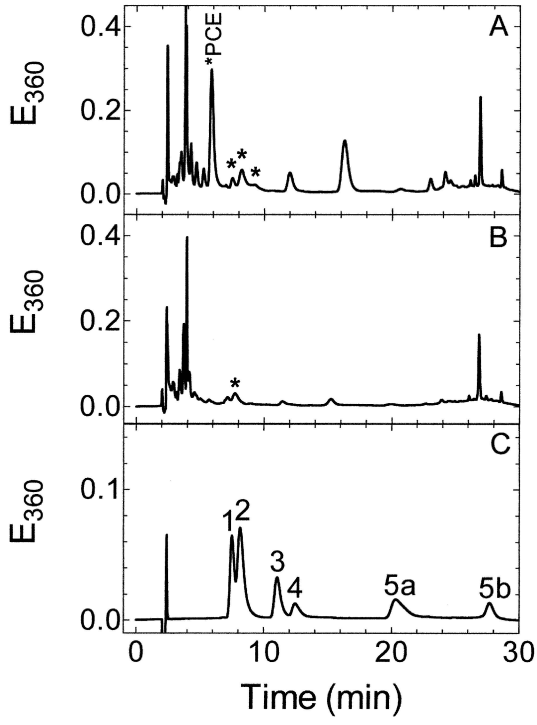

Fig. 4 HPLC elution profiles of corrinoid extracts of *D. multivorans* strains K (**A**) and N (**B**) and standard cobalamins (**C**). Peaks marked by *asterisks* in **A** and **B** were identified as corrinoids by their UV/Vis spectra. The peak labeled *PCE* was identified as the PCE dehalogenase cofactor. In **C**, the peaks *labeled with numbers* correspond to the following corrinoids: *1* pseudovitamin B_{12}, *2* cyanocobalamin, *3* factor A, *4* hydroxocobalamin, *5a* and *5b* derivatives originating from dicyanocobinamide

(Neumann et al. 2002) and the mass determined by MALDI-TOF was 1,304.5 MH$^+$-CN (data not shown), which is identical with the mass of the purified PCE dehalogenase cofactor determined by fast atom bombardment–mass spectrometry (B. Kräutler, University of Innsbruck, personal communication).

Cultures of strain N fed with the PCE dehalogenase corrinoid instead of cyanocobalamin were not able to dechlorinate PCE, when they were grown on pyruvate plus PCE (data not shown).

The data were taken to indicate that the reason for the inability of strain N to dechlorinate PCE is the lack of the specific PCE dehalogenase corrinoid cofactor.

Expression of the *pceA* gene in strain N

It cannot be excluded that, even though no active PCE-reductive dehalogenase could be synthesized by cells of strain N, these cells are able to synthesize the corrinoid-free apoprotein of the dehalogenase. Therefore, the expression of the *pceA* gene in strain N was investigated.

RT-PCR of the total bacterial RNA of strain N and strain K was performed with primers specific for *pceA* (see Materials and methods). The expected size of the PCR product was 339 bp. In strain N, a RT-PCR product of this

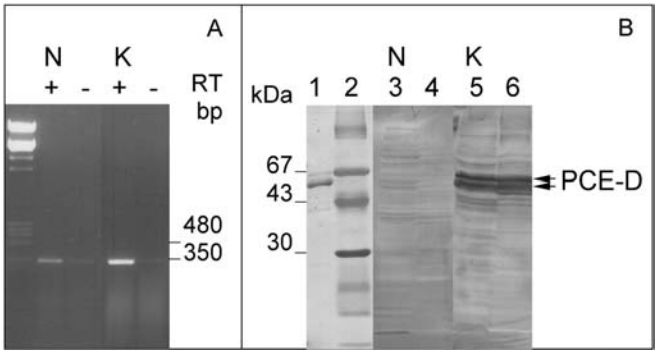

Fig. 5 RT-PCR of total bacterial RNA of *D. multivorans* strains N and K (**A**) and Western hybridization of cell extracts and enriched protein of both strains with PCE dehalogenase-specific antibodies (**B**). **A** RT-PCR with total bacterial RNA of strain N and strain K was performed under identical experimental conditions (lanes marked +). Control reactions for PCR without cDNA synthesis by reverse transcriptase are indicated by –. Bands of the expected size of 339 bp could be detected in both strains. The *left lane* shows a λ/*Pvu*II-DNA marker with band sizes *indicated at the right*. **B** Western blot analysis of cell extracts and enriched protein prepared according to the Materials and methods section. *Lanes 1, 2* Coomassie stain of purified PCE dehalogenase and protein marker on a polyvinylidene difluoride membrane, with sizes *indicated at the left*. *Lanes 3–6* Western hybridization of cell extract of strain N (*lane 3*), enriched protein of strain N (*lane 4*), cell extract of strain K (*lane 5*), and enriched protein of strain K (*lane 6*). Cell extracts (12 µl, ~250 µg protein) and enriched protein fractions (~60 µg) of both strains were used for analysis

size could be detected, although the band was by far weaker than for the RT-PCR product of strain K (Fig. 5A). This finding indicated that the *pceA* gene was transcribed at a lower level in strain N than in strain K.

Western blot hybridization was performed with PCE dehalogenase-specific polyclonal antibodies to find out whether the apoprotein of the dehalogenase was formed (see Materials and methods; Fig. 5B). The experiment was performed with cell extracts (Fig. 5B, lanes 3, 5) and with enzyme enriched by chromatography on Q-Sepharose (Fig. 5B, lanes 4, 6). Two bands were expected for strain K cell extracts or enriched protein, respectively, to correspond with the processed (53 kDa) and unprocessed enzyme (56 kDa), dependent on the absence or presence of the leader peptide (Neumann et al. 1998). These bands were found in cell extracts and in the enriched protein (Fig. 5B). In strain N, significant bands could not be detected, neither in cell extracts nor in enriched protein (Fig. 5B), indicating that the apoprotein was not formed in strain N cells in significant amounts.

Discussion

In this communication, the isolation of a non-dechlorinating strain N of *D. multivorans* is described. Based on the similar and identical fragment patterns of the REP-PCR and ERIC-PCR typing techniques, respectively, strain N is very closely related to the PCE-dechlorinating *D. multivorans* strain and its 16S rDNA is more than 97% homologous. *S. deleyianum*, which exhibits 98% homology of its 16S rDNA to that of *D. multivorans* strain K, gave significantly different fragment patterns with both techniques. Surprisingly, the genes encoding for the PCE dehalogenase, namely *pceA* and *pceB*, were present in strain N cells and were 100% identical to the corresponding genes of strain K. Therefore, attempts to trace enzymatic dehalogenating activity by dehalogenase-specific gene probes (Smidt et al. 2000) might lead to ambiguous results. 16S rDNA gene probes have also been used to monitor dechlorination potential at polluted sites (Fennell et al. 2001; Löffler et al. 2000). These gene probes indicate the presence of organisms that may have dechlorinating activity. Neither the detection of the dehalogenase genes nor the presence of organisms belonging to dechlorinating genera stringently implicates a dehalogenation potential in the investigated environments. Recently, the isolation of a non-dechlorinating strain of *Desulfitobacterium* was described (van de Pas et al. 2001). Formerly, it was assumed that the ability to dechlorinate chlorinated hydrocarbons was a common property of all species of this genus. The reason why this specific *Desulfitobacterium* strain DP7 is not able to dechlorinate chlorinated compounds is not known. Our finding of the *Dehalospirillum multivorans* strain N described here indicates that non-dechlorinating species or strains may also exist in other genera which were assumed to dehalogenate chlorinated compounds.

The inability of strain N to dechlorinate PCE was probably due to the absence of the *D. multivorans* PCE dehalogenase corrinoid cofactor, which – according to its properties – appears to be a novel type of corrinoid. This unique cofactor could not be detected in the PCE-dehalogenating *Desulfitobacterium frappieri* TCE1 and *Desulfitobacterium* strain PCE-S, nor in the chlorophenol-dehalogenating *D. hafniense* and *D. dehalogenans* (Siebert et al., unpublished data).

The absence of the PCE dehalogenase corrinoid is probably caused by the inability of strain N to synthesize this cofactor. Cell extracts of strain N exhibited enzymatic activities of δ-aminolevulinic acid dehydratase and porphobilinogen deaminase (data not shown), indicating that strain N was able to mediate the early steps in uroporphyrinogen biosynthesis. Therefore, it is assumed that one or more of the enzymes involved in the PCE dehalogenase corrinoid synthesis from hydroxymethylbilane is/are missing in strain N. Strain K is able to grow and dechlorinate PCE in the absence of yeast extract and cyanocobalamin (data not shown). Evidently, this strain is capable of a de novo synthesis of this corrinoid cofactor.

Attempts to restore the dechlorinating activity of strain N in vivo by supplementing the growth medium with corrinoids extracted from strain K cells or with the isolated dehalogenase cofactor failed until now. This may be explained by the inability of strain N cells to take up the corrinoid or by the lack of other factors involved in the assembly of the functional enzyme.

Since the dechlorinating activity could not be restored in vivo, it appeared feasible to restore the activity in vitro. A prerequisite for this experiment is that the PCE dehalo-

genase genes would be expressed in strain N cells and that the expression would lead to the formation of the cofactor-free apoprotein. Western blot analysis with PCE dehalogenase-specific antibodies showed that, if at all, extremely low amounts of apoprotein were detectable in cells of strain N. Therefore, the in vitro restoration experiment was not performed, since no measurable dehalogenase activity could be expected. The RT-PCR experiment indicated that the PCE dehalogenase gene of strain N was transcribed at least to a certain extent. No quantitative analysis was performed. However, it could be estimated that only low amounts of mRNA have been formed in strain N as compared with strain K, since the RT-PCR of both strains was performed under exactly the same experimental conditions. The findings indicate that the corrinoid cofactor might influence the synthesis of the apoprotein by regulation of the transcription and/or translation. Another possible explanation would be that the apoprotein formed cannot be folded correctly in the absence of the cofactor and might therefore be subject to rapid degradation by proteases. We favor the former hypothesis, since the latter assumption could not explain the lower amounts of mRNA detected in strain N cells.

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