Purification, cloning and sequencing of an enzyme mediating the reductive dechlorination of 2,4,6-trichlorophenol from *Desulfitobacterium frappieri* PCP-1

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A new membrane-associated 2,4,6-trichlorophenol reductive dehalogenase from *Desulfitobacterium frappieri* PCP-1 was isolated. Initial characterization of the crude preparation showed that the dechlorinating activity was sensitive to oxygen, and its optimum pH was 7.0. Its dechlorinating activity was not inhibited by sulphate, was completely inhibited by 1 mM sulphite, and partially inhibited by 5 mM sodium azide and by more than 5 mM nitrate. Several polychlorophenols were dechlorinated in the *ortho* position with respect to the hydroxy group. A dehalogenase was purified to apparent homogeneity. SDS gel electrophoresis revealed a single protein band with a molecular mass of 37 kDa. However, after two-dimensional gel electrophoresis, this band was composed of three isoforms. MS analyses showed that the three isoforms were from the same protein and the molecular mass of the most abundant isoform is 33 800 Da. A mixture of iodopropane and titanium citrate caused a light-reversible inhibition of the dechlorinating activity, suggesting the involvement of a corrinoid cofactor. The apparent K_m value for 2,4,6-trichlorophenol and pentachlorophenol were 18.3 ± 2.8 μM and 26.8 ± 2.9 μM respectively, at a methyl viologen concentration of 2 mM. The N-terminal amino acid sequence and an internal tryptic peptide sequence were determined. One open reading frame (ORF) was found in the *Desulfitobacterium hafniense* genome containing these peptides sequences. The corresponding ORF in *D. frappieri* PCP-1 was cloned and sequenced. This ORF, that we designated *crdA*, showed no homology with any known dehalogenase, suggesting a distinct reductive dehalogenase.

Key words: chlorophenols, dehalogenase, reductive dehalogenation.

INTRODUCTION

Halogenated compounds are generally known as toxic environmental pollutants. In anaerobic environments, reductive dehalogenation is one of the most important mechanisms involved in their transformation. Complete anaerobic degradation of highly halogenated compounds is usually achieved by microbial consortia. The key step of anaerobic degradation is the removal of halogens from the carbon skeleton of the molecule by reductive dehalogenation. This generates halogenated molecules that are less toxic and more easily biodegradable by the microbiota [1–3].

In microbial metabolism, halogenated compounds can be transformed under anaerobic conditions by dehalorespiration. Halogenated compounds are used as electron acceptors, and an electrochemical proton potential for ATP synthesis is generated to give energy for the micro-organisms. Few micro-organisms capable of dehalorespiration have been isolated in pure culture. These include *Desulfomonile tiedjei* DCB-1, *Dehalobacter restrictus* PER-K23, *Dehalospirillum multivorans* and all the members of the genus *Desulfitobacterium*, with the exception of one strain which apparently did not use chloroethenes or chlorophenols as the electron acceptor [4]. The range of substrates used differs between these bacteria but involves mostly chlorinated aromatic compounds, such as pentachlorophenol (PCP) and other chlorophenols, chlorobenzoate-derivative compounds, and/or tetrachloroethene (PCE) [1–3,5–8].

Hydrolytic and reductive dehalogenases involved in PCP degradation via tetrachlorohydroquinone, such as those of *Sphingobium chlorophenolicum*, have been well studied [9,10]. However, only few reductive dehalogenases from dehalorespiring bacteria have been fully characterized. The first purified anaerobic aryl dehalogenase was the 3-chlorobenzoate reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* [11]. This enzyme is a haem protein consisting of two subunits. The other reductive dehalogenases described in the literature consist of a single polypeptide with one corrinoid cofactor and two iron-sulphur clusters. PCE reductive dehalogenase was purified from *Dehalospirillum multivorans* [12] and the *pceA* gene encoding for this dehalogenase was reported [13]. From an enrichment culture containing *Dehalococcoides ethenogenes*, Magnuson et al. [14] have purified two membrane-bound reductive dehalogenases. This culture can completely dechlorinate PCE to ethene. PCE-reductive dehalogenase dechlorinated PCE to trichloroethene (TCE) and TCE-reductive dehalogenase dechlorinated TCE to ethene. Furthermore, Magnuson et al. [15] have cloned the *tceA* gene encoding for the TCE dehalogenase.

Löffler et al. [16] have initially characterized the dechlorinating enzyme system of *Desulfitobacterium chlororespirans* Co23. This

Abbreviations used: DTT, dithiothreitol; HIC, hydrophobic interaction chromatography; JGI, Joint Genome Institute; ORF, open reading frame; PCE, tetrachloroethene; PCP, pentachlorophenol; TCE, trichloroethene.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY043467.

system is inducible, membrane-associated and can dechlorinate 3-chloro-4-hydroxybenzoate and several chlorophenols exclusively at the *ortho* position with respect to the hydroxy group. Recently, the dehalogenase has been purified and mechanistic models for the corrinoid iron-sulphur reductive dehalogenase were proposed [17]. The PCE reductive dehalogenase of *Desulfitobacterium* sp. PCE-S [6], the 3-chloro-4-hydroxyphenylacetate reductive dehalogenase of *Desulfitobacterium hafniense* [18] and the *ortho*-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans* [19] were also purified and characterized. From this latter micro-organism, the presence of two closely linked genes was identified: *cprA* encoding the *o*-chlorophenol reductive dehalogenase and *cprB* encoding for an integral membrane protein that could act as a membrane anchor of the dehalogenase. Furthermore, a 11.5-kb genomic fragment containing *cprBA* revealed the presence of eight genes, designated *cprTKZEBACD*, involved in the expression and regulation of reductive dehalogenation [20].

Desulfitobacterium frappieri PCP-1 is a strictly anaerobic bacterium which can dechlorinate PCP to 3-chlorophenol and different chlorophenols at the *ortho*, *meta* and *para* positions [21,22]. Two inducible dehalogenase systems are involved in *D. frappieri* PCP-1; one for *ortho* dechlorination and a second for *meta* and *para* dechlorination. In this paper, we report the purification and the characterization of a reductive dehalogenase of *D. frappieri* PCP-1 that catalyses the *ortho* dechlorination of 2,4,6-trichlorophenol (2,4,6-TCP), and the isolation of the corresponding gene.

EXPERIMENTAL

Culture conditions and preparation of membrane fraction

D. frappieri PCP-1 (ATCC 700357) was cultivated anaerobically at 30 °C in 14-litre bottles containing 9.2 litres of mineral salt medium supplemented with 55 mM pyruvate, and 0.1 % yeast extract [21,22]. 2,4,6-TCP (50 μM) was added to induce the *ortho*-dechlorinating activity. The pH of the medium was adjusted to 8.0. The bottle was inoculated with 5 % (v/v) of an exponentially growing culture. After 5 h incubation, another 50 μM of 2,4,6-TCP was added. Cells were harvested 15 h later by centrifugation at 9000 ***g*** for 20 min at 4 °C, washed in 100 ml of 50 mM phosphate buffer, pH 7.5, with 1 mM dithiothreitol (DTT), and finally resuspended in 100 ml of 50 mM phosphate buffer, pH 7.5, with 1 mM DTT and 1 mg PMSF. Cells were broken by passing the suspension through a French press cell. The lysate was centrifuged at 17 000 ***g*** for 30 min. The supernatant was ultracentrifuged at 160 000 ***g*** for 90 min at 4 °C. The pellet, consisting of membranes, was resuspended in 10 ml of 50 mM phosphate buffer, pH 7.5, containing 1 mM DTT and 20 % (v/v) glycerol, and stored at – 80 °C. The crude membrane fraction (1.0 ml) was thawed and resuspended in 10 ml of 50 mM phosphate buffer, pH 7.0, containing 1 mM DTT and used in this state for some experiments, or further purified by adjusting the solution to 20 % (v/v) glycerol and 0.1 % Triton X-100. The preparation was then agitated for 45 min at 4 °C and centrifuged at 145 000 ***g*** for 90 min at 4 °C and the supernatant (solubilized dehalogenases) was used for characterization.

The work was performed in an anaerobic chamber (Bactron II, Sheldon Manufacturing, Cornelius, OR, U.S.A.) under a gas mixture containing 80 % N_2, 10 % H_2, and 10 % CO_2, or in serum bottles capped under this gas mixture. All the solutions used were made anoxic by repeated cycles of vacuum and flushing with oxygen-free gas mixture.

Purification of reductive dehalogenase

A crude membrane fraction was thawed and resuspended in 50 mM phosphate buffer, pH 7.5, containing 1 mM DTT and 10 % (v/v) glycerol. The suspension was agitated for 45 min at 4 °C and ultracentrifuged at 145 000 ***g*** for 60 min at 4 °C. The supernatant was mixed with a saturated ammonium sulphate solution to reach 0.75 M. The suspension was loaded on to a column (1.2 cm × 8.5 cm) of macro prep methyl hydrophobic interaction chromatography (HIC) support (Bio-Rad Laboratories, Mississauga, ON, Canada) previously equilibrated with 50 mM phosphate buffer, pH 7.5, with 1 mM DTT, 0.75 M ammonium sulphate and 5 % (v/v) glycerol. The bound proteins were eluted with a 0.75–0 M ammonium sulphate gradient and finally with the equilibrating buffer without ammonium sulphate. To this latter fraction that had the dehalogenating activity, 0.05 M Triton X-100 and 0.05 % trifluoroacetic acid were added. The suspension was incubated at 37 °C for 25 min and centrifuged at 17 000 ***g*** for 1 min to remove the precipitate. Supernatant (1 ml) was applied to a Protein Pak 300 SW column (8.0 mm × 30 cm) using a Waters 650 Advanced Protein Purification System. The column was previously equilibrated in 50 mM phosphate buffer, pH 7.5, containing 1 mM DTT, 0.02 % Triton X-100 and 5 % (v/v) glycerol. The peak with dehalogenating activity was collected in a serum bottle with oxygen-free gas mixture.

Enzyme assays

Dehalogenation assays were performed under anaerobic conditions in 12-ml serum bottles. The assay mixtures contained, in a total volume of 2.0 ml, 100 mM potassium phosphate buffer, pH 7.0, 2 mM titanium citrate, 2 mM methyl viologen, 20 % (v/v) glycerol, 1 mM 2,4,6-TCP and 50 μl of enzyme preparation. The assays were done in triplicate. After a 1-h incubation at 37 °C, the reaction was stopped by addition of 1.0 ml of acetonitrile containing 0.33 % (v/v) acetic acid. The mixture was centrifuged for 5 min at 5500 ***g***. A volume of 100 μl was injected on to an HPLC column for determination of 2,4-dichlorophenol, the product of the *ortho* dehalogenation.

The temperature stability of the enzyme in a standard assay mixture was also determined by first incubating the enzyme preparations without 2,4,6-TCP at the different temperatures for 2 h. The mixtures were then incubated at 37 °C, and the enzymic reaction was initiated by the addition of the substrate.

The optimum pH was determined by carrying out standard enzymic assays at different pH values between 5.5 and 8.0 in 100 mM potassium phosphate buffer at 37 °C. The pH values were verified immediately after incubation before HPLC analysis.

The K_m and V_{max} values for the 2,4,6-TCP and PCP were determined with a semi-purified preparation with a range of substrate concentrations varying from 5 to 250 μM. The values were calculated with Sigma Plot 2002 ver. 8.0, Enzyme kinetic module 1.1.

Analytical methods

The different substrates and products were analysed by HPLC with a reverse-phase NovaPak C_{18} column (3.9 mm × 150 mm). A water-acetonitrile gradient containing 0.1 % (v/v) acetic acid was used to elute the samples. Chlorophenols were analysed as described by Juteau et al. [23].

SDS/PAGE (10 % polyacrylamide) were carried out by the method of Laemmli [24] to analyse the protein purity and estimate the protein molecular mass. Protein concentration was estimated

by a modification of the Lowry assay [25] or by Bio-Rad Assay Protein kit (Bio-Rad Laboratories, Mississauga, On, Canada). Bovine serum albumin was used as the standard. Iron was assayed as described by Fish [26] using an iron volumetric standard. Two-dimensional gel electrophoresis was performed according to the method of Berkelman and Stentstedt [27]. The first dimension was done with IPGphor isoelectric focusing system (Amersham Pharmacia Biotech) using immobiline DryStrip, 18 cm, pH 4–7. Trypsin treatments of the protein bands were carried out with trypsin (Sequencing Grade Modified Trypsin from Promega). The protein and the tryptic peptides were analysed by a triple quadrupole mass spectrometer (Quattro II, Micromass, Pointe-Claire, Quebec, Canada) equipped with a Nanospray Z-Spray interface. Samples were introduced into the mass spectrometer using type D nanospray probe tips (Micromass), in positive mode. Capillary voltage was 1.0 kV and the cone was at 35 V. The protein mass was determined using the MaxEnt 1 software.

N-terminal sequencing by automated Edman degradation was performed with the 03RBLOT program on a 470A Gas-Phase sequencer from Applied Biosystems Inc (ABI). The protein was electroblotted to a polyvinylidene difluoride membrane and the 37 kDa protein was excised and loaded in a vertical cross-flow reaction cartridge (ABI). Phenylthiohydantoin amino acids derivatives were determined by comparison with standards (PTH Standards, ABI) and analysed on-line with a 120 PTH Analyser HPLC system from ABI.

Inactivation of dehalogenase with iodopropane

Light-reversible alkylation of corrinoids by iodopropane was based on the procedure of Brot and Weissbach [28]. Dehalogenase preparations were incubated for 30 min at 37 °C in the dark with 2 mM titanium(III)citrate with 0 (control) and 0.5 mM 1-iodopropane. The vials were placed on ice and exposed to a 300W slide projector for 10 min. Dehalogenase activities were determined on the different preparations before and after light exposure.

Gene isolation and sequence

Extraction of total DNA of strain PCP-1 was performed according to Li et al. [29]. Two oligonucleotides (DH1-G3: 5′-TTCATGAACTCGGGTTTGTG-3′ and DH1-D: 5′-GTA-TCACTGCGGAAAATGTGC-3′) were designed based on the *D. hafniense* genome sequence at the Joint Genome Institute (JGI) web site (http://www.jgi.doe.gov/) (see Results section) and synthesized with the Gene Assembler Plus (Pharmacia, Baie d'Urfé, Canada). PCRs were carried out with 50 μl reaction mixtures containing 10–30 ng of total strain PCP-1 DNA, deoxynucleoside triphosphates (200 μM each), *Taq* DNA polymerase buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 10 pmol of each oligonucleotide, and 2.5 units of *Taq* DNA polymerase (Pharmacia). This mixture was heated at 80 °C for 2 min before the addition of the DNA sample, after which it was incubated at 94 °C for 5 min then 55 °C for 5 min, followed by 30 cycles of 72 °C for 2 min, 94 °C for 40 s, and 55 °C for 1 min, and completed with a final elongation step of 10 min at 72 °C. PCR product was cloned into a T-vector according to the manufacturer specifications (pGEM T-easy vector, Promega, Madison, WI, U.S.A.) and both strands were sequenced (GenBank® accession no. AY043467). Sequencing at JGI was accomplished with support from the Department of Energy of the United States of America. Two independent clones of the PCR-amplified gene from separate experiment were sequenced.

RESULTS

Initial characterization of dehalogenase activity

Ortho-dechlorinating activity of 2,4,6-TCP to 2,4-dichlorophenol was observed mainly in the membrane preparation. The fractions were obtained from cell extract of cultures growing in the presence of 2,4,6-TCP. No dechlorination of 2,4,6-TCP was observed in cell extracts obtained from cultures growing in presence of 3,5-dichlorophenol, suggesting that the *ortho*-dehalogenating activity was inducible by 2,4,6-TCP. The *in vitro* dehalogenating activity was determined at 37 °C in presence of titanium (III) citrate and methyl viologen as the electron donor. A linear relationship between product formation, incubation time (up to 6 h for the non-purified enzyme), and protein concentration (up to 0.2 mg protein) was obtained in the standard assay system used in this study.

The enzyme was oxygen-sensitive and lost approx. 70 % of its activity when the enzyme preparations were incubated for 1 h at 4 °C in a vial where 10 % of the oxygen-free gas mixture was removed and replaced by 10 % air. The maximal initial rate of 2,4,6-TCP dechlorination was observed between pH 6.9 and 7.3 with an optimum at 7.0 (Figure 1). The enzymic system was stable under the assay conditions for at least 2 h at temperatures below 45 °C for the soluble enzyme and 50 °C for the enzyme in the membrane fraction. Rapid loss of enzyme activity was observed at higher temperatures. The crude enzyme preparations were stable for more than one month when stored at – 20 and – 70 °C.

The dechlorinating activity was completely inhibited by 1 mM sulphite, whereas in the presence of more than 5 mM nitrate, a rapid 50 % decrease of the initial activity was observed (Figure 2). Sulphate had no significant effect on dechlorination.

Adding 5 mM of $MgCl_2$, KCl, $ZnCl_2$, $MnCl_2$, NaCl, $CoCl_2$, or $FeNH_4(SO4)_2$, to the standard assay enzyme mixture had no significant effect on the dechlorination activity. However, 5 mM NaN_3 showed a 53 % inhibition of the dechlorinating activity. Incubation of the dehalogenase preparation with the metal chelator EDTA (10 mM) for 1 h had no effect on activity.

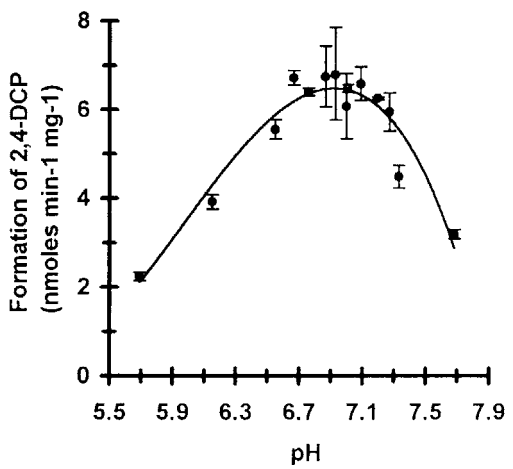

Figure 1 pH dependence of *ortho*-chlorophenol reductive dehalogenase activity of *D. frappieri*

Triton X-100 extract preparations were incubated with 2,4,6-TCP as substrate under standard conditions.

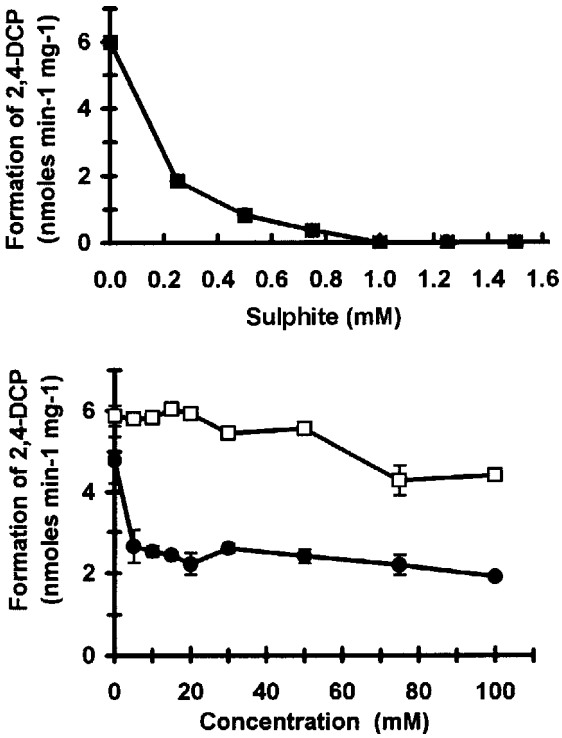

Figure 2 Effect of sulphate, sulphite and nitrate on *ortho*-chlorophenol reductive dehalogenase activity of *D. frappieri*

Triton X-100 extract preparations were incubated with 2,4,6-TCP as substrate under standard conditions. Curves for sulphate (□), sulphite (■) and nitrate (●) are shown.

Table 1 Substrate specificity profile of the crude solubilized dehalogenase preparation obtained from *D. frappieri* PCP-1

Activities were determined using the standard assay system. TCE was detected in the standard assay by GC analysis but was not quantified. TeCP, tetrachlorophenol; TCP, trichlorophenol; DCP, dichlorophenol; CP, chlorophenol; N.D., not determined.

Substrate	Product(s)	Position of dechlorination	Mean specific activity (nmol min^{-1} mg^{-1})
PCP	2,3,4,5-TeCP	*ortho*	204.1 ± 1.50
2,3,4,5-TeCP	3,4,5-TCP	*ortho*	97.77 ± 1.76
2,4,6-TCP	2,4-DCP	*ortho*	8.80 ± 0.28
3,4,5-TCP	3,5-DCP	*para*	0.93 ± 0.05
2,3,5-TCP	3,5-DCP	*ortho*	16.52 ± 0.80
2,3,4-TCP	3,4-DCP	*ortho*	23.58 ± 0.22
2,3,6-TCP	2,5-DCP	*ortho*	8.11 ± 0.28
2,4,5-TCP	3,4-DCP	*ortho*	Traces
2,3-DCP	3-CP	*ortho*	3.73 ± 0.11
2,4-DCP	4-CP	*ortho*	0.045 ± 0.006
PCE	TCE	–	N. D.

Several polychlorophenols were dechlorinated in the *ortho* position with respect to the hydroxy group (Table 1). The highest activity was observed with PCP and 2,3,4,5-tetrachlorophenol. Only 3,4,5-trichlorophenol was dechlorinated at the *para* position but with a relatively low rate. No dechlorination was detected with 2,5-dichlorophenol, 2,6-dichlorophenol, 3,4-dichlorophenol, 3,5-dichlorophenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol and 3-chloro-4-hydroxyphenylacetate.

Table 2 Purification scheme for the *ortho*-chlorophenol reductive dehalogenase

Purification step	Total activity* (nmol/min)	Yield (%)	Total protein† (mg)	Specific activity (nmol/min/mg)	Purification factor
Solubilized fraction	1062	100	9.5	111.8	1
Methyl-HIC	437.5	41.1	1.85	236.1	2.1
Treatment in buffer B	448.5	42.2	1.67	268.5	2.4
Gel filtration	88.4	8.3	0.23	377.8	3.4

* Amount (nmol) of 2,4-dichlorophenol produced per min at 37 °C with 2,4,6-trichlorophenol as substrate (see enzyme assays in the Experimental section for conditions).

† Total protein was determined with the Bio-Rad Assay Protein using serum albumin as a standard.

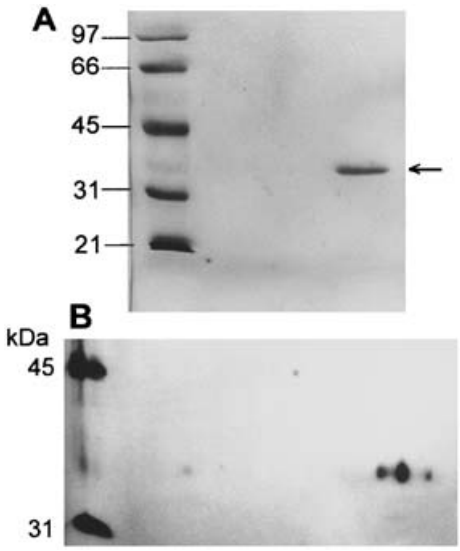

Figure 3 SDS and two-dimensional PAGE with the purified chlorophenol reductive dehalogenase

(**A**) Molecular size (kDa) markers are shown on the left-hand side of the gel. The arrow indicates the position of the purified chlorophenol reductive dehalogenase. The gel was stained for proteins with Coomassie Brilliant Blue R-250. (**B**) Two-dimensional electrophoresis of the purified dehalogenase. The pH gradient is in the horizontal direction and the SDS/PAGE separation is in the vertical direction. The gel (1.5 mm × 160 mm × 200 mm) was stained for proteins with silver.

Purification and characterization of the reductive dehalogenase

The dehalogenase was solubilized in buffer with DTT and glycerol but without Triton X-100. About 61 % of the dechlorinating activity was solubilized, with 39 % remaining in the membrane fraction. The glycerol was essential for solubilizing the dehalogenase from the membrane. The solubilized dehalogenase was purified by HIC and size exclusion chromatography. Specific activities increased 3.4-fold for a recovery of 8.3 % (Table 2). No loss of activity was observed when the preparation was incubated for 25 min at 37 °C with 0.05 % TFA and 0.05 % Triton X-100. However, important losses of activity were observed after HIC and gel filtration chromatography. These losses explain the low purification factor obtained. The purified protein solution had a yellowish colour.

SDS/PAGE analysis revealed only one band with a molecular mass estimated at 37 kDa (Figure 3). After two-dimensional gel electrophoresis, the purified protein was observed as three

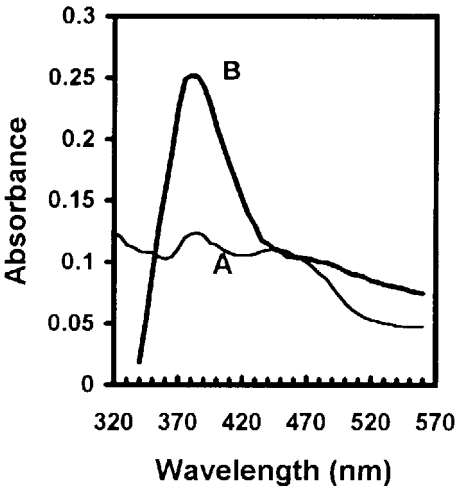

Figure 4 Visible absorption spectrum of the purified *ortho*-chlorophenol dehalogenase

The purified *ortho*-chlorophenol dehalogenase was in curve A: 50 mM phosphate buffer (pH 7.5) with 5% (v/v) glycerol, 0.01% Triton X-100 and 1 mM DTT; curve B shows the same preparation after addition of 2 mM titanium (III) citrate.

different spots with pI of 5.05, 5.20 and 5.25 respectively (Figure 3B). These spots were also detected after two-dimensional gel electrophoresis of a crude membrane preparation. Analysis by MS of each spot eluted from the gel and treated with trypsin showed similar tryptic peptide profiles, suggesting that they represent different isoforms of the same protein (results not shown). By MS, the molecular mass of the most abundant isoform was 33800 Da; a less intense peak corresponding to 33833 Da was also observed. No iron was detected in the purified enzyme.

As for the crude preparation, the purified dehalogenase was not inhibited by the addition of 20 mM sodium sulphate in the standard enzyme assays but was completely inhibited by sodium sulphite. The activity was 48.8% inhibited by 20 mM sodium nitrate. The dehalogenase lost 76% of its initial activity upon incubation in the dark with a mixture of 0.5 mM iodopropane and 2 mM titanium citrate. Subsequent exposure to light restored 92% of the activity of the control group, suggesting the presence of a cobalamin cofactor. Furthermore, the visible spectrum of the purified dehalogenase exhibited an absorption maximum near 385 nm and 455 nm (Figure 4). According to Gantzer and Wackett [30], the 385-nm peak corresponds to the typical absorption maximum for cob(I)alamin. Upon addition of titanium citrate, an increase of the 385-nm peak was observed due to the reduction of cob(III)alamin to its Co(I) form (Figure 4B). The 455-nm peak would correspond to the alkylated cobalamin.

In the conditions used, the purified dehalogenase dechlorinated PCP at *ortho* positions. The 3,5-dichlorophenol was not dechlorinated. The apparent K_m value for 2,4,6-TCP and PCP were $18.3 \pm 2.8\ \mu$M and $26.8 \pm 2.9\ \mu$M respectively at a methyl viologen concentration of 2 mM. With a semi-purified preparation, the V_{max} were 28.8 ± 1.5 nmol/min/mg for 2,4,6-TCP and 882.8 ± 33.6 nmol/min/mg protein for PCP.

The N-terminal amino acid of the purified dehalogenase was determined: AVDTTTSATVEAATPAXXXS (one-letter symbols used to denote amino acids). The amino acid sequence of one tryptic peptide was determined to be AAASTSTST by MS.

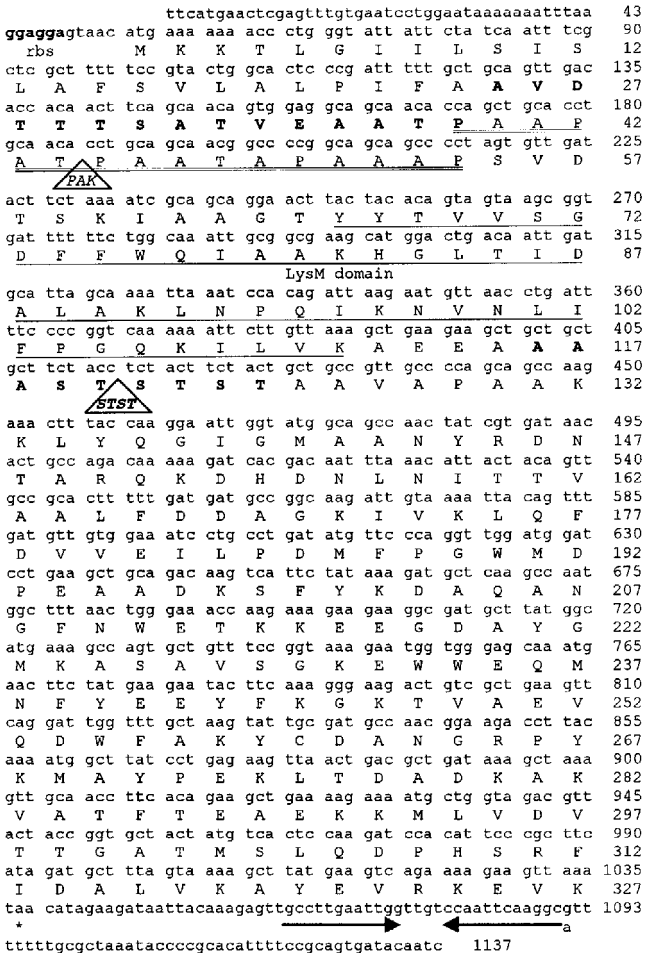

Figure 5 The *crdA* locus of *D. frappieri* PCP-1

Sequences in bold are the putative ribosomal binding site (rbs), and the N-terminal sequence (AVD...) and the internal peptide (AAASTSTST) determined by amino acid sequencing. Amino acid sequences indicated by triangles are the two insertions found in *D. hafniense* corresponding sequence. There is also one nucleotide substitution between the two sequences at nt 1091. The underlined sequence represents the LysM domain. The double underlined sequence is the $(PAX)_nTA(PAA)AP$ motif. The inverted repeat at the end of the nucleic sequence (indicated by arrows) may represent a transcriptional terminator.

Cloning and sequencing of the gene encoding the reductive dehalogenase

The JGI released the contig sequences of *D. hafniense* that cover more than 6 million bp. Niggemyer et al. [31] showed that the *D. frappieri* PCP-1 and *D. hafniense* DCB-2 genomes exhibit 88.7% DNA/DNA chromosomic re-association, indicating that these two strains belong to the same species. We found one open reading frame (ORF) in one of the *D. hafniense* contigs that contains the N-terminal sequence and the internal amino acid sequence (Figure 5) of the dehalogenase. From this, we designed and synthesized two oligonucleotides that were used to amplify the corresponding ORF from strain PCP-1 genomic DNA. A 1.1 kb fragment was amplified, cloned and sequenced, and compared with the corresponding sequence of *D. hafniense*. We designated this ORF the *crdA* gene for chloroaromatic reductive dehalogenase.

DNA analysis of the 1.1 kb fragment revealed an ORF of 327 codons. The N-terminal sequence is located 24 codons from the predicted start codon, suggesting that the *crdA* gene product has a signal peptide. Sequence analysis showed that this region is indeed

highly hydrophobic, as seen in most signal peptides, and sequence analysis with the SIGNALP program (http://ca.expasy.org) predicted the cleavage of this putative signal peptide at the observed N-terminal amino acid. The predicted mature protein has 303 amino acids and a molecular mass of 32 927 Da. The pI of the predicted mature protein is 5.1, which is around the value determined by two-dimensional gel electrophoresis. The *D. frappieri* PCP-1 *crdA* sequence is identical with the *D. hafniense crdA* sequence except for two small in-phase deletions (Figure 5). One of the deletions is located in a repetitive motif at the N-terminal region: $(PAX)_nTA(PAA)AP$; *D. frappieri* CrdA contains 3 PAX and *D. hafniense* 4. This motif was observed in other proteins, but no function was attributed to it. The other deletion is located in a di-amino acid repetitive motif ST: *D. frappieri* has 3 ST and *D. hafniense*, 5. The CrdA sequence has a LysM domain. Finally, hydrophobic profiles showed no transmembrane domain, no known cobalamin binding site motif, no iron-sulphur binding motif and no homology with other protein sequences in gene databases, except for the LysM domain.

DISCUSSION

The *ortho*-chlorophenol reductive dehalogenase was produced by *D. frappieri* PCP-1 in the presence of 2,4,6-TCP as inducer of *ortho*-dehalogenation. Most of the activity was found in the membrane fraction. This result corroborates with a peptide signal and a LysM domain found in the predicted amino acid sequence of the *crdA* gene encoding for this enzyme. The LysM domain is a widespread protein module involved in binding peptidoglycan [32]. The signal peptide will allow the dehalogenase to be translocated in the cell wall and to bind to peptidoglycan. However, the enzyme is probably not tightly bound to the membrane, since its extraction was possible in buffer containing glycerol but without Triton X-100 although the yield was better in the presence of 0.1% Triton X-100. This dehalogenase was completely inhibited by sulphite, as observed also for *Desulfomonile tiedjei* dehalogenase and *D. chlororespirans* Co23 dehalogenase. The enzyme was not inhibited by sulphate and EDTA and partially inhibited by nitrate. Miller et al. [33] have observed that PCE reductive dechlorination by crude extracts of *Desulfitobacterium* PCE-S was inhibited by EDTA, sodium azide and sulphite, but not by sulphate. The dehalogenase from *D. frappieri* PCP-1 was sensitive to oxygen as for the other reductive dehalogenases. The optimum pH was determined to be 7.0 and was 7.2 for extracts from *Desulfomonile tiedjei* [11] and 6.5 for membrane preparation from *D. chlororespirans* Co 23 [16].

The crude solubilized *D. frappieri* PCP-1 dehalogenase preparation was specific for *ortho*-chlorophenols, where the highest activity of dechlorination was for PCP, tetrachlorophenol and trichlorophenols. *Para*-dechlorination of 3,4,5-trichlorophenol and the PCE dechlorination to TCE were also observed but at a lower rate. As the *D. hafniens*e genome is highly similar to *D. frappieri* PCP-1 and contains several putative reductive dehalogenase genes [34], we cannot exclude the possibility that these two low dechlorination activities are produced by a different dehalogenase in our crude preparation. A PCE reductive dehalogenase, which cannot dechlorinate PCP and other chlorophenols, was already purified from *Desulfitobacterium* sp. strain PCE-S [35]. Also, van de Pas et al. [36] have shown recently that two distinct enzyme systems are responsible for PCE and chlorophenol reductive dehalogenation in *Desulfitobacterium* strain PCE1. Unlike the other *ortho*-chlorophenol reductive dehalogenases described already, the 3-chloro-4-hydroxyphenylacetate was not dechlorinated by the crude preparation of strain PCP-1. The highest activity for PCP and highly chlorinated phenols is consistent with the fact that the PCP-1 strain was isolated from a PCP-enriched consortium.

Light reversible inhibition by iodopropane, sulphite and sodium azide inhibition of dehalogenase activity and the visible absorption spectrum of the purified dehalogenase suggest that this enzyme contains a corrinoid cofactor. The presence of a corrinoid cofactor and of two iron/sulphur clusters was observed for the dehalogenases of *D. hafniense, D. dehalogenans, D. chlororespirans, Desulfitobacterium* PCE-S, *Dehalospirillum multivorans, Dehalococcoides ethenogenes* 195 and *Dehalobacter restrictus*. The presence of a haem cofactor was observed only with the 3-chlorobenzoate dehalogenase of *Desulfomonile tiedjei* [11]. However, the predicted amino acid sequence from the *crdA* gene does not contain the iron/sulphur Fe_4S_4 binding motif CXXCXXCXXXCP [13,15]. This result is confirmed by the absence of iron in the purified enzyme. The absence of iron/sulphur clusters suggests that an electron carrier located in the membrane and/or closely associated with the dehalogenase should be necessary.

Only one protein band was observed by SDS/PAGE of the purified protein; however, three spots were detected after two-dimensional electrophoresis. These spots represent different isoforms of the same protein, as suggested by the MS analysis, which showed a similar tryptic peptide profile for each spot. This was confirmed by the N-terminal sequencing of the band in SDS/PAGE, where only one N-terminal sequence was observed. As the protein contains a chromophore, the different isoforms probably represent different post-translationally modified enzymes, or different maturation steps of the enzyme or association with cofactors. The purified dehalogenase has an apparent molecular mass of 37 kDa by SDS/PAGE. This value is different from the molecular mass determined for the Cl-OHPA dehalogenase (47 kDa) of *D. hafniense* [18], the *ortho*-chlorophenol dehalogenase of *D. dehalogenans* (48 kDa) [19] and for the PCE or TCE reductive dehalogenases of *Desulfitobacterium* PCE-S (65 kDa) [35], of *Dehalococcoides ethenogenes* 195 (51 kDa and 61 kDa) [14] and of *Dehalospirillum multivorans* (57 kDa) [12]. The 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei* is also very different and consists of two subunits with an apparent molecular mass of 64 and 37 kDa [11].

The molecular masses of the enzyme determined by MS (33 800 Da) and predicted from the gene (32 927 Da) differ by 873 Da. This difference can be attributed to the corrinoid factor attached to the protein. However, most corrinoid molecules are bigger than 1000 Da. As biochemical assays detected the presence of a corrinoid cofactor, there is a possibility that a new type of corrinoid is attached to the protein or some modifications to the protein and/or to the cofactor occurred during the purification steps.

Recently, DNA–DNA hybridization and comparative physiological studies suggest that *D. hafniense* and *D. frappieri* should be united into one species [31]. *D. hafniense* DCB-2 and *D. frappieri* PCP-1 can reductively dechlorinate various chlorophenols but they are also capable of toxic metal and metalloid respiration. Both strains can use As(V), Fe(III), Fe pyrophosphate, Se (VI), nitrate, sulphite, elemental S and thiosulphate as the terminal electron acceptor [31]. We cannot exclude the possibility that some enzymes with transition-metal cofactors involved in the reductive dechlorination and in the metal or metalloid reduction could share some activities or fortuitous reactions, especially in the conditions used in the standard enzyme assays. Gantzer and Wackett [30] have already shown that bacterial transition-metal co-enzymes vitamin B_{12} (Co), co-enzyme F_{430} (Ni) and hematin

(Fe) catalysed the reductive dechlorination of polychlorinated ethenes, phenols and benzenes. Recently, Neumann et al. [37] have shown that several halogenated compounds were dehalogenated abiotically by the heat-inactivated PCE reductive dehalogenase and commercially available cyanocobalamin. The substrate specificity suggested that the corrinoid cofactor of this PCE dehalogenase is not a cyanocobalamin.

The characterization of the chlorophenol dehalogenase of *D. frappieri* PCP-1 indicates that this enzyme is different from the other corrinoid-containing dehalogenases although some properties are shared. Okeke et al. [38] have characterized an enzyme mediating the reductive dechlorination of PCE from *Clostridium bifermentans*. This enzyme has a molecular mass of approx. 35 kDa, may contain a corrinoid cofactor, as suggested by the light reversible inhibition of activity by propyl iodide, and has no iron/sulphur cluster, as suggested by the predicted amino acid sequence of the corresponding gene. This enzyme shows no homology with the *D. frappieri* dehalogenase and with any known dehalogenase.

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