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Identification and Characterization of a Novel CprA Reductive Dehalogenase Specific to Highly Chlorinated Phenols from *Desulfitobacterium hafniense* Strain PCP-1[▽]

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Desulfitobacterium hafniense **strain PCP-1 reductively dechlorinates pentachlorophenol (PCP) to 3-chlorophenol and a variety of halogenated aromatic compounds at the *ortho*, *meta*, and *para* positions. Several reductive dehalogenases (RDases) are thought to be involved in this cascade of dehalogenation. We partially purified a novel RDase involved in the dechlorination of highly chlorinated phenols from strain PCP-1 cultivated in the presence of 2,4,6-trichlorophenol. The RDase was membrane associated, and the activity was sensitive to oxygen, with a half-life of 128 min upon exposure to air. The pH and temperature optima were 7.0 and 55°C, respectively. Several highly chlorinated phenols were dechlorinated at the *ortho* positions. The highest dechlorinating activity levels were observed with PCP, 2,3,4,5-tetrachlorophenol, and 2,3,4-trichlorophenol. 3-Chloro-4-hydroxyphenylacetate, 3-chloro-4-hydroxybenzoate, dichlorophenols, and monochlorophenols were not dechlorinated. The apparent K_m value for PCP was 46.7 μM at a methyl viologen concentration of 2 mM. A mixture of iodopropane and titanium citrate caused a light-reversible inhibition of the dechlorinating activity, suggesting the involvement of a corrinoid cofactor. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the partially purified preparation revealed 2 bands with apparent molecular masses of 42 and 47 kDa. Mass spectrometry analysis using Mascot to search the genome sequence of *D. hafniense* strain DCB-2 identified the 42-kDa band as NADH-quinone oxidoreductase, subunit D, and the 47-kDa band as the putative chlorophenol RDase CprA3. This is the first report of an RDase with high affinity and high dechlorinating activity toward PCP.**

Halogenated compounds are generally known as toxic environmental pollutants. Hydrogenolytic reductive dehalogenation, a reaction involving the replacement of one halogen atom with one hydrogen atom, is the predominant mechanism for their transformation in anaerobic environments. This process can sustain microbial growth via electron transport-coupled phosphorylation (10, 26, 31). The majority of the known reductive dehalogenases (RDases) belong to the CprA/PceA family. These are single-polypeptide membrane-associated anaerobic enzymes that are synthesized as preproteins with a cleavable twin arginine translocation (TAT) peptide signal. They contain one corrinoid and two iron-sulfur clusters as cofactors.

CprA enzymes catalyzing the reductive dechlorination of chloroaromatics have been purified from *Desulfitobacterium hafniense* strain DCB-2 (6), *Desulfitobacterium dehalogenans* (30), *Desulfitobacterium chlororespirans* strain Co23 (12, 14), *Desulfitobacterium* sp. strain PCE1 (29), and *D. hafniense* strain PCP-1 (28) and characterized, and PceA enzymes have been purified from *Sulfurospirillum multivorans* (22, 23), *Desulfitobacterium* sp. strain PCE-S (18, 19), *D. hafniense* strain TCE1 (29), *Dehalococcoides ethenogenes* 195 (15, 16), *Desulfitobacterium* sp. strain PCE1 (29), *Dehalobacter restrictus* (17, 25), *Desulfitobacterium* sp. strain Y51 (27), and *Dehalococcoides* sp. strain VS (20) and characterized. However, none of these enzymes showed high dechlorinating activity toward highly chlorinated phenols such as pentachlorophenol (PCP).

D. hafniense strain PCP-1 is the only known strict anaerobic bacterium which reductively dechlorinates PCP to 3-chlorophenol (3-CP) and a variety of halogenated aromatic compounds at the *ortho*, *meta*, and *para* positions (2, 7). It dechlorinates PCP at the *ortho*, *ortho*, *para*, and *meta* positions in the following order: PCP → 2,3,5,6-tetrachlorophenol (2,3,5,6-TeCP) → 3,4,5-trichlorophenol (3,4,5-TCP) → 3,5-dichlorophenol (3,5-DCP) → 3-CP (7). Several RDases are thought to operate during this sequence of dechlorinations. Two RDases have already been purified from strain PCP-1. The first one, CrdA, is a membrane-associated enzyme, not related to CprA/PceA-type RDases, that mediates *ortho* dechlorination of 2,4,6-TCP and several chlorophenols (3). The second enzyme, CprA5, catalyzes the *meta* and *para* dechlorination of 3,5-DCP and several chlorophenols (28). Three other putative *cprA* genes were identified in strain PCP-1 (*cprA2*, *cprA3*, and *cprA4*), which suggests that other RDases with different specificities toward halogenated compounds exist in this strain (8, 31, 32). In this study, we have partially purified and characterized a new CprA-type RDase (CprA3) from strain PCP-1. CprA3 is the first reported RDase with high affinity toward PCP and with high *ortho*-dechlorinating activity toward PCP and other highly chlorinated phenols.

MATERIALS AND METHODS

Culture conditions and preparation of the membrane fraction. *D. hafniense* strain PCP-1 (ATCC 700357) was cultivated anaerobically in a 14-liter bottle containing 9 liters of mineral salt medium supplemented with 55 mM pyruvate

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TABLE 1. Purification scheme for the PCP RDase

Purification step	Total activity (nkat)[a]	Yield (%)	Total protein (mg)[b]	Sp act (nkat mg^{-1})	Purification factor
Cell extract	184	100	159.6	1.15	1
Membrane fraction	103	56	42.56	2.42	2.1
Solubilized fraction	62	34	27.04	2.31	2.0
DEAE-5PW column	27	15	4.09	6.59	5.7
Addition of $(NH_4)_2SO_4$	28	15	4.09	6.93	6.0
HiTrap butyl HP column	4.3	2.3	0.33	13.0	11.3

[a] Amount (nmol) of 2,3,4,5-TeCP produced per second at 37°C, with PCP as the substrate.
[b] The total protein concentration was determined with a Bio-Rad assay protein kit, using serum albumin as the standard.

and 0.1% yeast extract. 2,4,6-Trichlorophenol (2,4,6-TCP; 50 μM, final concentration) was added to induce the *ortho*-dechlorinating activity (2, 7). The medium was inoculated with 5% (vol/vol) of an exponentially growing culture and incubated at 37°C for 6 h and then at 28°C for the next 42 h. The pH of above 7.0 was maintained with a saturated solution of $NaHCO_3$. After approximately 8, 24, and 31 h of incubation, 2,4,6-TCP was added (25 μM, final concentration). Cells were harvested by centrifugation at 9,000 × *g* for 20 min at 4°C and washed in 300 ml of 50 mM phosphate buffer, pH 7.5, with 1 mM dithiothreitol (DTT). Cell pellets were stored at −80°C until further use.

Approximately 1.8 g of wet cell pellet was thawed, dispersed in 30 ml of 50 mM phosphate buffer, pH 7.5, with 1 mM DTT and 57 μM phenylmethylsulfonyl fluoride (PMSF), and fractionated by the method previously described (3). The membrane preparation was dispersed in 5 ml of 50 mM phosphate buffer, pH 6.5, containing 1 mM DTT, 20% (vol/vol) glycerol, and 3% Triton X-100. The preparation was agitated for 45 min at 4°C and centrifuged at 161,000 × *g* for 90 min at 4°C. The supernatant consisting of solubilized proteins was filtered through a membrane with a pore size of 0.2 μm and used immediately for purification.

The work was performed in an anaerobic chamber (Bactron II; Sheldon Manufacturing, Cornelius, OR) 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 were made anoxic in an anaerobic jar by repeated cycles of vacuuming and flushing with the oxygen-free gas mixture.

Partial purification of the reductive dehalogenase. A crude solubilized dehalogenase preparation was loaded onto a Protein-Pak DEAE-5PW column (8.0 by 75 mm; Waters Corporation, Milford, MA) equilibrated in 50 mM phosphate buffer, pH 6.5, containing 1 mM DTT, 0.1% Triton X-100, and 10% (vol/vol) glycerol. The dehalogenase was eluted with a 0 to 1 M NaCl gradient. Fractions with PCP-dehalogenating activity were pooled, and 0.5 M $(NH_4)_2SO_4$ (final concentration) was added. The suspension was filtered through a 0.2-μm membrane and loaded onto a HiTrap butyl HP column (1 ml; Bio-Rad Laboratories, Mississauga, Ontario, Canada) equilibrated in buffer A (50 mM phosphate buffer at pH 6.5, 1 mM DTT, 10% [vol/vol] glycerol) containing 0.5 M ammonium sulfate. The bound proteins were eluted with a 0.5 to 0 M ammonium sulfate gradient, followed by a 0 to 6 mM CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} gradient in buffer A. The fractions with PCP-dechlorinating activity were collected in serum bottles with the oxygen-free gas mixture.

Dehalogenase assays. Standard enzyme assays were performed under anaerobic conditions in 12-ml serum bottles containing 2.0 ml of the assay mixtures (3). The assay mixtures contained 100 mM potassium phosphate buffer, pH 7.0, 2 mM titanium citrate, 2 mM methyl viologen, 20% (vol/vol) glycerol, and 0.25 mM PCP or another chlorinated compound (1 mM). The reaction was initiated by addition of 100 μl of enzyme preparation. After 20 to 45 min of incubation at 37°C, the reaction was stopped by addition of 1 ml of acetonitrile containing 0.33% (vol/vol) acetic acid. The mixture was centrifuged for 5 min at 5,000 × *g* and analyzed by high-pressure liquid chromatography (HPLC).

Initial characterization was carried out with an enzyme preparation obtained after chromatography using a DEAE-5PW column, with PCP as the substrate. The optimum pH was determined by carrying out standard enzyme assays at pH values between 5.5 and 8.0 in 100 mM potassium phosphate buffer. The optimum enzymatic temperature assay was carried out with a standard enzyme assay mixture at between 5 and 70°C. The oxygen sensitivity assay was performed in 12-ml serum bottles exposed to air or in anoxic capped serum bottles (control). The dechlorinating activities of each bottle were determined from samples taken after 0, 45, 90, 135, 180, 225, and 250 min of incubation at 4°C. Light-reversible alkylation of corrinoids by iodopropane based on the procedure of Brot and Weissbach (4) was carried out with an enzyme preparation incubated for 30 min at 37°C in the dark with 2 mM titanium(III) citrate and 0 (control) or 0.5 mM 1-iodopropane. The vials were placed on ice and exposed to the light (300-W lamp) for 30 min. Standard enzyme assays were performed before and after light exposition. These experiments were performed in duplicate. The apparent K_m and apparent V_{max} values for PCP were determined with substrate concentrations ranging from 7.5 to 250 μM in triplicate. The values were calculated with SigmaPlot 2002, version 8.0, enzyme kinetic module 1.1. The substrate range was determined with enzyme preparations obtained after chromatography using a DEAE-5PW column and a butyl HP column. Standard enzyme assays were performed in triplicate.

Analytical methods. The products of the enzyme assays were determined by HPLC analysis, as described by Juteau et al. (11). Samples from assays with 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA), 3-chloro-4-hydroxybenzoate (3-Cl-4-OHBA), 4-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid, 3,5-dichloro-4-hydroxybenzoate, 2,4,6-trichlorobenzoate, and 3,4,5-trichlorobenzoate were eluted with a water-methanol gradient ranging from 100% water to 100% methanol for 10 min, followed by 10 min in 100% methanol. The flow rate was 1 ml/min, and the detection wavelength was 270 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) was carried out by the method of Laemmli (13). The protein concentration was determined with a Bio-Rad assay protein kit (bulletins 1069 and 1123; Bio-Rad Laboratories), using bovine serum albumin (2.5 to 10 μg) as the standard. Protein bands on SDS-PAGE were cut from the gel and treated with trypsin (Promega, Madison, WI) (9) for mass spectrometry analysis as described by Charbonneau et al. (5). The tryptic peptides were separated on a Agilent nano pump using a C_{18} Zorbax trap and a SB-C_{18} Zorbax 300 reversed-phase column (150 mm by 75 μm, 3.5-μm particle size) (Agilent Technologies, Inc., Germany). Mass spectra were recorded on a hybrid linear ion trap-triple quadrupole mass spectrometer (Q Trap; AB Applied Biosystems, MDS Sciex Instruments, CA) equipped with a nanoelectrospray ionization source. The accumulation of tandem mass spectrometry (MS-MS) data was performed with Analyst Software, version 1.4 (AB Applied Biosystems/MDS SCIEX Instruments, CA). MASCOT (Matrix Science, London, United Kingdom) was used to create peak lists from MS and MS-MS raw data (5).

Gene sequence. Extraction of *D. hafniense* strain PCP-1 total DNA was performed according to the method described by Gauthier et al. (8). Two oligonucleotides (CprA3_1047F, 5′ TGC CCG TCT GAA GCC ATA ACT CAT 3′, and CprA3_1399R, 5′ GCA AAG TTT GAG ACC GGC TA 3′) were designed based on the *D. hafniense* strain DCB-2 genome sequence (GenBank accession number CP001336) to amplify the region between nucleotides 1047 and 1399 from the start site of the *cprA3* gene. PCRs were carried out with 50-μl reaction mixtures containing 10 ng of *D. hafniense* strain PCP-1 total DNA, deoxynucleoside triphosphates (200 μM each), *Taq* DNA polymerase buffer, 10 pmol of each oligonucleotide, and 2.5 U of *Taq* DNA polymerase (GE Healthcare). The PCR parameters used were as follows: 94°C for 5 min and 57°C for 5 min, followed by 35 cycles of 72°C for 1 min, 94°C for 1 min, and 57°C for 1 min and a final elongation step of 10 min at 72°C. Both strands of the PCR product were sequenced.

RESULTS

Partial purification and characterization of the PCP RDase. The PCP RDase was partially purified by ion-exchange chromatography on a Protein-Pak DEAE-5PW column, followed

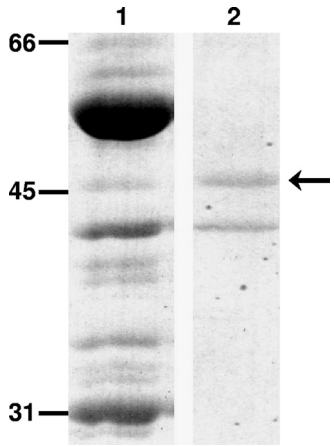

FIG. 1. SDS-PAGE with the partially purified PCP RDase. The gel was stained for proteins with Coomassie brilliant blue R-250. Molecular mass sizes (in kDa) are shown on the left-hand side of the gel. The arrow indicates the position of the chlorophenol RDase. Lane 1, fraction after using the DEAE-5PW column; lane 2, fraction after using the HiTrap butyl HP column.

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  1 MFRSSDRQNK PQEQKFQMNR RKFLKAGVAS ALTAGMVGAM RTLPVSA/AEA
 51 VASTGSSGSV NGARSKLHPK VDYGGASVRF VENNDQWLGT SQIVGTVNRT
101 HEAEQGFNLA LRGKLSSEAQ VAMYHYNFVM KHPFDGALGI FSNYVSAENI
151 VGGTPNQEKL PIPDPEQMSQ NIKDTAYFLR ADEVGIGKMP EYAYYSHKAP
201 FSHEELIRDD ISHSTPVTEK LPYVIVVMVD QHLETMLAST GYDGISSAQS
251 MRGYHATAVI SVILAQYIRN LGYNARAHHF ANYAAAMPPV TIAAGLGELS
301 RTGDCTVHPR LGYRHKVAAV TTDLPLLPDK PIDFGLQDFC RVCKKCADNC
351 PSEAITHDTD MVEYNGYLRW NSDMKKCAEF RLTNSEGSSC GRCMKVCPWN
401 SKEESWFHSA GIWIGSKGET SSRLLKQIDD MFGYGDEIIE KYKWWLEWPE
451 RYTLPKHL
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FIG. 2. Deduced amino acid sequence of *cprA3* from strain PCP-1. Underlined sequences are peptides that were detected by mass spectrometry with Mascot. Boldfaced sequences are the TAT motif (RRK FLK) and the two iron-sulfur binding motifs. A slash indicates the location of the cleavage of the peptide signal.

by hydrophobic interaction chromatography on a HiTrap butyl HP column. Specific activities increased 11.3-fold from the first step for a final recovery of 2.3% (Table 1). A dramatic loss (90%) of the activity for this preparation was observed after 5 h of incubation at 4°C. After 24 h of incubation, 5% of the activity remained. Addition of glycerol (20%) and bovine serum albumin (BSA; 10%) had a slight stabilizing effect. Triton X-100 (1%) and DTT (100 mM) had no effect. SDS-PAGE after the final chromatography revealed two bands with apparent molecular masses of 42 and 47 kDa, respectively (Fig. 1). These bands were analyzed by mass spectroscopy using Mascot to search the *D. hafniense* strain DCB-2 genome sequence. The 42-kDa band was identified as NADH-quinone oxidoreductase, subunit D (GenBank accession number YP_002460200), with a Mascot protein score of 810, and the 47-kDa band was identified as an RDase homologue, described as CprA3 by Villemur et al. (32) (GenBank accession number YP_002457213), with a Mascot score of 495. Further attempts to purify the RDase to homogeneity were unsuccessful due to strong losses of activity after use of the HiTrap butyl HP column and due to poor stability of the enzyme. Purification of CprA/PceA RDases has proved to be difficult, and a number of enzymes have not been purified to homogeneity (16, 20, 29). The enzyme preparation obtained after use of the DEAE column was used for the characterization of the PCP RDase because of the poor stability of the purified enzyme.

TABLE 2. Dechlorinating activity of partially purified preparations of the PCP RDase[b]

Substrate	Product	Sp act (nmol min^{-1} mg^{-1})[a]	
		Assay 1	Assay 2
PCP	2,3,4,5-TeCP	458.1 ± 30.2 (100)	318.1 ± 11.3 (100)
2,3,5,6-TeCP	2,3,5-TCP	9.4 ± 0.66 (2)	3.9 ± 0.2 (1)
2,3,4,5-TeCP	3,4,5-TCP	271.7 ± 2.6 (59)	273.3 ± 7.7 (86)
2,4,6-TCP	2,4-DCP	7 ± 0.67 (2)	3.4 ± 0.2 (1)
2,3,6-TCP	2,3-DCP	3.4 ± 0.92 (<1)	5.6 ± 00.9 (2)
2,3,4-TCP	3,4-DCP	153 ± 8.4 (33)	81.02 ± 5.8 (25)

[a] Each assay was performed with a different enzyme preparation after chromatography using a DEAE-5PW column and carried out in triplicate. In all cases, the dechlorination activity was at the *ortho* position relative to the hydroxyl group. For PCP dechlorination, the reaction was stopped before the formation of 3,4,5-TCP. Values in parentheses represent the percentages of specific activities relative to PCP as the substrate.

[b] Chlorophenols not dechlorinated: 2,4,5-TCP; 3,4,5-TCP; 2,3-DCP; 2,4-DCP; 2,5-DCP; 3,4-DCP; 3,5-DCP; 2-CP; 3-CP; and 4-CP. CP, monochlorophenol; DCP, dichlorophenol, TCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol.

The PCP RDase preparation had a pH optimum of 7.0. The optimum temperature for the dechlorinating activity was between 50 and 55°C. No activity was observed at 60°C, suggesting protein denaturation at this temperature. Because of poor yield of protein purification and the rapid lost of activity at 4°C, no attempts were made to determine temperature stability. The dehalogenase was oxygen sensitive, and it had a half-life of 128 min upon exposure to air. The dechlorinating activity was completely inhibited by 2.5 mM sulfite. Sulfate and nitrate at 100 mM had no effect on the dechlorinating activity. Addition of 10 mM KCN to the standard assay enzyme mixture caused a 90% inhibition, and addition of 5 mM NaN_3 caused a 32% inhibition. Incubation of the dehalogenase preparation with 10 mM EDTA for 1 h did not inhibit the dechlorinating activity. Addition of 5 mM $MnCl_2$, $MgCl_2$, KCl_2, or NaCl to the assay enzyme mixture had no effect. However, addition of 5 mM $CoCl_2$ or $Fe(NH_4)_2(SO_4)_2$ resulted in 15% and 31% increases of the dechlorinating activity, respectively. In the presence of 0.5 mM iodopropane and 2 mM titanium citrate, the dehalogenase lost 80% of the activity of the control group when incubated in the dark. Subsequent exposure to light restored 45% of the activity, suggesting the presence of a cobalamin cofactor.

The substrate specificity of the dehalogenase toward different chlorophenols is shown in Table 2. Several highly chlorinated phenols were dechlorinated at the *ortho* positions with respect to the hydroxyl group. The highest activity levels were observed with PCP, 2,3,4,5-TeCP, and 2,3,4-TCP. No dechlorinating activity was detected with 3,4,5-TCP, 2,4,5-TCP, dichlorophenols, monochlorophenols, 3-Cl-4-OHPA, 3-Cl-4-OHBA, 4-chlorophenoxy acetic acid, 2,4-dichlorophenoxy acetic acid, 3,5-dichloro-4-hydroxybenzoate, 2,4,6-trichlorobenzoate, and 3,4,5-trichlorobenzoate. The apparent K_m value for PCP was 46.7 ± 4.2 μM at a methyl viologen concentration of 2 mM, and the apparent V_{max} value was 1,145 ± 33.1 nmol of 2,3,4,5-TeCP min^{-1} mg of $protein^{-1}$ for PCP.

TABLE 3. Characteristics of chlorophenol RDases[a]

Enzyme	Organism	Position of dechlorination[b]	Apparent K_m (μM)	Other highly chlorinated substrates[c]	Reference(s)
PCP-CprA3	*D. hafniense* PCP-1	*ortho*	46.7 (PCP)	2,3,5,6-TeCP (1), 2,3,4,5-TeCP (73)	This study
3,5-DCP–CprA5	*D. hafniense* PCP-1	*meta*/*para*	49.3 (3,5-DCP)	PCP (30), 2,3,4,5-TeCP (70)	28
2,4,6-TCP–CrdA	*D. hafniense* PCP-1	*ortho*	18.3 (2,4,6-TCP), 26.8 (PCP)	2,3,4,5-TeCP (48)	3
3-Cl-4-OHPA–CprA	*D. hafniense* DCB-2	*ortho*	ND[d]	ND	6
3-Cl-4-OHPA–CprA	*D. dehalogenans*	*ortho*	20 (3-Cl-4-OHPA)	PCP (2)	30
3-Cl-4-OHPA–CprA	*Desulfitobacterium* sp. PCE1	*ortho*	ND	ND	29
3-Cl-4-OHBA–CprA	*D. chlororespirans*	*ortho*	12.4 (3-Cl-4-OHBA), 390 (PCP)	PCP (13), 2,3,5,6-TeCP (4), 2,3,4,5-TeCP (3)	12, 14
3-ClBA–RD	*Desulfomonile tiedjei*	*meta*	ND	ND	24

[a] Determined by SDS-PAGE. DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol; 3-Cl-4-OHBA, 3-chloro-4-hydroxybenzoate; 3-Cl-4-OHPA, 3-chloro-4-hydroxyphenylacetate; 3-ClBA, 3-chlorobenzoate.
[b] Position of the chlorine residue relative to the hydroxyl group.
[c] Values in parentheses represent the percentages of the ratios of activity compared to those for PCP for PCP-CprA3 (average values obtained from Table 2); 3,5-DCP for 3,5-DCP–CprA5; PCP for 2,4,6-TCP–CrdA; 3-Cl-4-OHPA for 3-Cl-4-OHPA–CprA; and 3-Cl-4-OHBA for 3-Cl-4-OHBA–CprA.
[d] ND, not determined.

Gene sequencing of the reductive dehalogenase. A 1.9-kb fragment containing the first 1,140 of 1,377 nucleotides of the *cprA3* gene (GenBank accession number AF321226) was previously isolated from a cosmid library of strain PCP-1 (32). In order to complete the *cprA3* gene sequence, two oligonucleotides were designed from the strain DCB-2 genome sequence to amplify the corresponding region between nucleotides 1047 and 1399. The complete sequence of strain PCP-1 *cprA3* is 100% identical to that of *cprA3* present in strain DCB-2.

cprA3 encodes a 458-amino-acid protein. The deduced amino acid sequence contains two iron-sulfur binding motifs characteristic of all CprA/PceA RDases (31). The N-terminal region is highly hydrophobic and contains the characteristic sequence motif RRXFXK of TAT signal peptides (1). The signal peptide cleavage site between residues 47 and 48 predicted with the SignalP program (http://ca.expasy.org) was confirmed by Mascot analysis (Fig. 2). The predicted molecular mass of the 411-amino-acid mature CprA3 protein is 45,796 Da, which is consistent with the value determined by SDS-PAGE. The theoretical pI is 6.1.

DISCUSSION

In this study, we have purified to near homogeneity a novel CprA-type RDase from *D. hafniense* strain PCP-1 specific toward *ortho*-chlorophenols with high affinity and high dechlorinating activity toward PCP. Mass spectrometry analysis related this enzyme to the putative chlorophenol RDase CprA3 of *D. hafniense* strain DCB-2 (32). The involvement of a CprA-type enzyme and its corrinoid cofactor in the dechlorination activity is suggested by the light-reversible inhibition by iodopropane and titanium citrate, as well as the inhibition by KCN and sulfite, which are known to react with cob(III)alamin (18, 21). The pH optimum, the oxygen sensitivity, and the optimum temperature of the dechlorinating activity were similar to those of the other CprA/PceA RDases already described (31).

A second protein with an apparent molecular mass of 42 kDa was copurified with CprA3. The protein is related to a subunit of NADH-quinone oxidoreductase, an enzyme complex catalyzing the electron transfer from NADH to menaquinone, or other quinones in the respiratory chain reaction (33). This dehydrogenase could be involved in the transfer of electrons to CprA3 (31).

Other CprA RDases such as the 3-Cl-4-OHPA RDase of *D. dehalogenans* (30), the 3-Cl-4-OHBA RDase of *D. chlororespirans* (12), and the 3,5-DCP RDase of *D. hafniense* strain PCP-1 (28) also dechlorinated PCP but at much lower rates than dichlorophenols and other lesser-chlorinated aromatic compounds (Table 3). For instance, the apparent K_m value determined for PCP with CprA3 is 8-fold greater (46.7 μM) than the K_m value for that with the 3-Cl-4-OHBA RDase of *D. chlororespirans* (390 μM) (12).

This is the third RDase that has been identified and characterized from strain PCP-1. CprA5 has *meta*- and *para*-dehalogenation activity toward lesser-chlorinated phenols. CrdA RDase, which is not a CprA-type RDase, has also a high rate of PCP *ortho*-dechlorination activity (3). However, the dechlorinating activity was measured with a crude solubilized dehalogenase preparation of *D. hafniense* strain PCP-1 cultivated with 2,4,6-TCP, and the presence of CprA3 or another unidentified dehalogenase in the crude extract could have accounted for the high level of PCP activity measured. CrdA or other CprA-type RDases were not found in the enzyme preparations after using the DEAE and butyl HP columns, ruling out the involvement of other copurified RDases.

In previous work, at least two other genes encoding CprA-type RDases were found in strain PCP-1, *cprA2* and *cprA4* (8, 32). Whether or not CprA2 and CprA4 are true RDases is still unknown, but based on the large spectrum of the reductive dehalogenation activity of this strain, it would not be surprising if they are.

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