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Novel Dehalogenase Mechanism for 2,3-Dichloro-1-Propanol Utilization in *Pseudomonas putida* Strain MC4

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A *Pseudomonas putida* strain (MC4) that can utilize 2,3-dichloro-1-propanol (DCP) and several aliphatic halocarbons and haloalcohols as sole carbon and energy source for growth was isolated from contaminated soil. Degradation of DCP was found to start with oxidation and concomitant dehalogenation catalyzed by a 72-kDa monomeric protein (DppA) that was isolated from cell lysate. The *dppA* gene was cloned from a cosmid library and appeared to encode a protein equipped with a signal peptide and that possessed high similarity to quinohemoprotein alcohol dehydrogenases (ADHs), particularly ADH IIB and ADH IIG from *Pseudomonas putida* HK. This novel dehalogenating dehydrogenase has a broad substrate range, encompassing a number of nonhalogenated alcohols and haloalcohols. With DCP, DppA exhibited a *k*<sub>catal</sub> of 17 s<sup>−1</sup>. *H*<sup>1</sup> nuclear magnetic resonance experiments indicated that DCP oxidation by DppA in the presence of 2,6-dichlorophenolindophenol (DCPIP) and potassium ferrocyanide [K<sub>4</sub>Fe(CN)<sub>6</sub>] yielded 2-chloroacrolein, which was oxidized to 2-chloroacrylic acid.

 Dichloropropanols are widely used in the chemical industry, particularly as intermediates for epichlorohydrin production. The classical epichlorohydrin manufacturing process proceeds via hydrochlorination of allylchloride, which yields both 2,3-dichloro-1-propanol and 1,3-dichloro-2-propanol (30). Because of the increasing availability of glycerol as a side product from biodiesel synthesis, this classical process is being replaced with the use of glycerol as a renewable feedstock for epichlorohydrin manufacture, again via chlorination to the same dichloropropanols or via 1-chloro-2,3-propanediol (6). Epichlorohydrin itself as well as the production intermediates 2,3-dichloropropanol (DCP) and 1,3-dichloropropanol are mutagenic, genotoxic, and carcinogenic, and therefore their release and the possibility of human exposure are of significant concern (38). DCP also occurs as a contaminant in cellulose and starch hydrolysates, soy sauce, and baked foods (27).

Microorganisms that metabolize dichloropropanols are of interest in view of their role in the removal of these compounds from waste streams and contaminated environments (14), from food and pulp products, and also carbohydrate hydrolysates (55) and because dichloropropanols occur as intermediates in a catabolic pathway for degradation of the emerging priority contaminant 1,2,3-trichloropropane (8). Of the dichloropropanols, 2,3-dichloro-1-propanol is chemically more stable and more difficult to degrade than 1,3-dichloropropanol (14). Furthermore, microorganisms that convert dichloropropanols can be used in the preparation of enantiopure building blocks for the pharmaceutical industry (27, 30). Several bacterial strains are known to grow on dichloropropanols, such as *Pseudomonas* sp. strain OS-K-29 (30), *Alcaligenes* sp. strain DS-K-S38 (29), *Mycobacterium* sp. strain GP1 (40), *Agrobacterium* sp. strain NHG3 (14, 20), and *Arthrobacter* sp. strain AD2 (51).

During the microbial conversion of vicinal haloalcohols, dehalogenation is usually the first step, and this reaction can be catalyzed by haloalcohol dehalogenases (24). These enzymes, also called halohydrin dehalogenases, are composed of 2 to 4 subunits of molecular masses between 28 and 35 kDa and are phylogenetically related to the short-chain dehydrogenase reductase superfamily (SDR proteins) (54), even though they do not possess a nicotinamide cofactor binding site. They catalyze the intramolecular displacement of a halogen by the vicinal hydroxyl group, yielding an epoxide, a halide ion, and a proton (52). At least six halohydrin dehalogenases have been found so far: two enzymes from *Corynebacterium* sp. strain N-1074 (HheA and HheB) (57) and homologs in *Arthrobacter* sp. strain AD2 (HheB<sub>AD2</sub>) (52), *Agrobacterium* sp. strain NHG3 (DehB) (20), *Arthrobacter erythri* H10a (DehA) (5), and *Agrobacterium radiobacter* strain AD1 (HheC) (51). The structures are known, and the catalytic mechanism is well understood (11, 12, 21). The halohydrin dehalogenases have a preference for substrates with the halogen group on a terminal (primary) carbon atom, which can be explained by analysis of X-ray structures (11, 21). However, important compounds, such as 2-chloro-1-propanol and DCP, are not easily converted. The potential importance of DCP as an intermediate in the degradation of 1,2,3-trichloropropane prompted us to search for new pathways of DCP metabolism (9).

A well-established mechanism for the conversion of alcohols is oxidative conversion by alcohol dehydrogenases (ADHs). Many ADHs are NAD- or NADP-dependent enzymes (34). Oxidation of alcohols by oxidases, which generates hydrogen peroxide, is also possible. A special class of alcohol dehydrogenases is formed by the periplasmic quinoprotein ADHs, which contain a quinoid cofactor, such as pyrroloquinoline quinone (PQQ), and Ca<sup>2+</sup>. A quinoprotein methanol dehydrogenase has been found to be responsible for 2-chloroethanol oxidation in the 1,2-dichloroethene catabolic pathway (26). PQQ-dependent ADHs have been discovered in a wide variety of bacteria, including *Acetobacter, Glucono-
bacter, Pseudomonas, and Comamonas strains (1, 2, 4, 19, 47). Some of these enzymes contain heme as a secondary prosthetic group and are known as quinohemoproteins (4). The periplasmic quinohemoproteins transfer electrons to the membrane-bound bacterial respiratory chain (4, 34).

In this paper we show that such a quinohemoprotein alcohol dehydrogenase may act as DCP dehalogenase. We started with the isolation of a DCP-degrading organism from a site polluted with chlorinated compounds. We report the properties of this new DCP-utilizing bacterium, analyze the gene encoding the quinohemoprotein alcohol dehydrogenase, and propose a pathway for DCP metabolism.

MATERIALS AND METHODS

Chemicals, reagents and enzymes. All chemicals were obtained from Alfa Aesar, Sigma-Aldrich, and Acros Organics. Oxidase test discs were obtained from Fluka. Plasmid DNA was isolated with the Qiagen plasmid isolation kit. Enzymes used for cloning were either from Roche or New England BioLabs. The PCR master mix for screening was purchased from Promega.

Isolation and characterization of strain MC4. The organism used in this work, Pseudomonas putida strain MC4, was isolated from mixed samples of soil collected from a site polluted with chlorinated hydrocarbons (close to Chemiehaven, Botlek, Rotterdam, Netherlands), using enrichment cultivation with 2 mM DCP as a sole carbon and energy source. Its growth spectra with different halogenated and nonhalogenated compounds were determined by replica plating on minimal medium (MMY) agar plates supplemented with the carbon source of choice (25). The organism is deposited at DSMZ under accession number 25823.

A segment of 16S rRNA was amplified from the genomic DNA of strain MC4 by PCR with the universal primers 27F and 1492R (33), cloned in pZero-2, and sequenced.

Growth and enzyme purification. Strain MC4 was grown in a 2.5-liter fermentor in MMY medium containing 5 mM DCP as the sole carbon source. The inoculum was prepared by growing strain MC4 overnight in LB at 30°C. After batch cultivation, cells were collected by centrifugation and washed in MMY medium. This mixture was added to the batch culture to an initial optical density at 600 nm (OD$_{600}$) of 0.05. The OD$_{600}$ and chloride release were monitored at regular intervals. The pH of the growing culture was maintained at 7.0 with 2 M NaOH, and the temperature was maintained at 30°C. An OD$_{600}$ of 0.45, more substrate was added, to a total input of 10 mM. At an OD$_{600}$ of 0.7, the cells were collected, centrifuged, and washed with 10 mM Tris-SO$_4$, pH 8.0. The cell pellet was resuspended in 5 volumes of 10 mM Tris-SO$_4$, pH 8.0, and stored at −80°C until further use. Five batches obtained in this manner were mixed and sonicated. The lysate was centrifuged at 23,000 × g for 20 min to remove cell debris. The supernatant was again centrifuged at 160,000 × g for 2 h to separate the membrane fraction and the cell extract.

The cell extract was subjected to ammonium sulfate fractionation. Fractions of 55%, 60%, and 65% precipitation were pooled together and desalted with a desalting column (EconoPac 10DG; Bio-Rad Laboratories). The pooled ammonium sulfate fractions were applied to a 0.6-m DEAE-Sepharose column (GE Healthcare) equilibrated with 10 mM Tris-SO$_4$, pH 8.0. A salt gradient of 1 M NaCl in 10 mM Tris-SO$_4$, pH 8.0, was used for elution. Active fractions were pooled and then concentrated with an Amicon filter (Millipore YM30), and the buffer was replaced with 10 mM phosphate buffer, pH 8.0. The concentrated fraction was further purified on a ceramic hydroxyapatite (HAP) column equilibrated with 10 mM potassium phosphate buffer, pH 8.0. The enzyme was eluted with a gradient of 0.01 to 0.5 M potassium phosphate buffer, pH 8.0, and concentrated, and the buffer was exchanged with 10 mM Tris-SO$_4$, pH 8.0. Fractions of high purity were pooled and stored at −20°C for further work.

Enzyme characterization. For molecular weight determination of native enzyme, purified dehydrogenase (DppA) was analyzed by gel filtration (see the supplemental material). The purity and molecular weight of the protein were determined by SDS-PAGE (12% gel) analysis. Heme staining of the SDS-PAGE gels was done by using the method of Francis and Becker (15).

To determine heme concentrations, we measured difference spectra of reduced and oxidized pyridine hemochrome according to a published protocol (53a).

Enzyme assays. All enzyme assays were performed at 25°C. The dehydrogenase (DppA) activity toward DCP in cell extracts was measured by following the reduction of the electron acceptor DCPIP with PMS as an intermediate electron carrier. The reaction mixture (1 ml) contained 50 mM potassium phosphate, pH 7.4, 35 μM 2,6-DCPIP, 5 mM DCP, and cell extract. After addition of 1.6 mM PMS, the absorbance was monitored at 600 nm. The absorption coefficient of DCPIP at 600 nm is 21.0 mM$^{-1}$ cm$^{-1}$ (16).

For routine measurements, the enzyme activity was measured by following the reduction of potassium ferricyanide, K$_3$Fe(CN)$_6$, to ferrocyanide, and a suitable amount of enzyme in 50 mM potassium phosphate buffer, pH 8.0. At several time points, a 3-ml sample was withdrawn and quenched with 10 μl of 5 M phosphoric acid. Samples were extracted with diethyl ether (1 ml) containing mesitylene as internal standard, and extracts were analyzed on a gas chromatograph containing an HP1 column (30 by 0.25 mm; 0.25 μm) according to the following method: 50°C for 5 min, with temperature increase from 50°C to 200°C over 20 min. The carrier gas was helium.

For chloride measurements, the remaining aqueous layers from diethyl ether extractions were analyzed on an ion chromatograph (DX 120; Dionex, Sunnyvale, CA) equipped with an Alltech A-2 anion column (100 by 4.6 mm; 7 μm) and an Alltech guard column (50 by 4 mm). A mixture of NaHCO$_3$ and Na$_2$CO$_3$ (3 mM each), pH 10, in deionized water was used as eluent at a flow rate of 1.0 ml/min.

To identify the expected aldehyde product of the dehydrogenase reaction, 1 ml of the reaction mixture underwent derivatization by adding dinitrophenyl hydrazine to 5 mM at pH 3.5 (3). Acateldehyde and propanal were used to standardize the derivatization procedure. The derived products were separated on a liquid chromatograph-mass spectrometer (LC-MS) system (LCQ Fleet ion trap MS; Thermo Scientific) equipped with a C$_{18}$ Lichrosorb (Agilent Technologies, Santa Clara, CA) reverse-phase column (150 by 3 mm [inner diameter]; 5 μm), an electrospray ionization (ESI) ion source, and a photodiode array detector set at 365 nm.

To determine the position of chlorine in the reaction product, we studied the conversion of DCP using nuclear magnetic resonance (NMR) spectroscopy by recording one-dimensional (1D) proton NMR spectra at 25°C on a Varian Unity Plus 500-MHz spectrometer. The NMR tube (1 ml) contained 5 mM DCP, 20 mM potassium ferricyanide, 100 mM potassium phosphate buffer, pH 8.0, and 20 μl of enzyme solution, all in D$_2$O. The reaction was started by adding the enzyme, followed by gentle mixing and recording of 1D $^1$H NMR spectra for 24 h. Each experiment was performed with 176 scans per transient reaction, an evolution time of 2 s, and an interscan delay of 3 s, giving rise to a net acquisition time of ~15 min per spectrum.
N-terminal sequencing and primer design. The purified DppA protein was subjected to N-terminal sequencing by automated Edman degradation (Eurosequence B.V., Groningen, Netherlands). The resulting sequence was used in a BLAST search with the NCBI database (https://blast.ncbi.nlm.nih.gov/) to identify homologous sequences. The primers prF1 and prR1, which are based on the N terminus of the protein and a conserved region of the homologous quinohemoprotein alcohol dehydrogenases, were used for PCR amplification. The amplified DNA was cloned in pZero-2 and sequenced. Next, the specific primers prR2, F22, based on the amplified sequence, were used for screening the gene libraries in pLAFR3 and pZero-2 vectors. Another primer, prR3, was used in conjunction with the M13FP primer (pZero-2) for screening a second sublibrary in pZero-2, obtained by HindIII digestion. Primer sequences are provided in the supplemental material.

Cloning and sequencing of the dichloropropanol dehalogenase gene region. General procedures for cloning and DNA isolation and manipulation were performed according to published protocols (42). The genomic DNA of strain MC4 strain was partially digested with Sau3A, and fragments of appropriate size (15 to 30 kb) were cloned into pLAFR3 and packaged (Promega) (44). Escherichia coli VC8257 cells were transduced with the packaged mixture, and colonies were selected on LB plates containing 12.5 μg/ml tetracycline. Next, colonies were arrayed in microtitre plates containing 100 μl of LB medium and incubated for 24 h. A PCR-based method (37) was used for screening the library, using DNA extracted from pooled clones. Positive microtitre plate pools were further screened by columns and rows. The identified positive clones were stored as glycerol stocks at −80°C.

EcoRI and HindIII were used to generate sublibraries in pZero-2. Ligation mixtures were transformed into E. coli TOP10 cells (Invitrogen) and selected with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and kanamycin (25 μg/ml) on LB plates. White colonies were screened by PCR as mentioned above. This yielded a positive EcoRI subclone with a 2.5-kb insert that was sequenced and found to contain an incomplete sequence of the target gene. From a HindIII sublibrary, another part of the dppA gene was sequenced. The complete gene sequence, including the putative ribosome binding site and promoter sequence, was assembled and analyzed by using BLAST tools of the NCBI database (http://blast.ncbi.nlm.nih.gov/).

Heterologous expression of DppA in E. coli. The deduced amino acid sequence of DppA was analyzed for possible subcellular localization and N-terminal cleavage sites by using PSORTb v.2.0 (17) and SignalP v.3.0 (7). Based on the predicted peptide cleavage site, a 114-bp forward primer (prF4) with an Ndel site was designed to replace the 24-amino-acid N-terminal sequence of DppA with the 21-amino-acid N terminus of E. coli alkaline phosphatase (accession no. AAA24358). This was used for PCR with a reverse primer containing a KpnI site (prR4). The PCR product was cut with Ndel/KpnI and cloned in a pBAD vector to give plasmid pNDL1. Primer sequences are provided in the supplemental material.

For protein expression, plasmid pNDL1 was cotransformed with plasmid pEC86, which constitutively produces cytochrome c maturation proteins (46), into E. coli TOP10 (Invitrogen) and JCB712 (23). Cells were grown in 1 liter of LB medium containing 50 μg/ml ampicillin and 175 μg/ml chloramphenicol under aerobic conditions at 30°C until the OD600 reached 0.5. Cultures were induced with 0.02% IPTG and incubated in a rotary shaker at 17°C and 200 rpm for 24 h. Cells were harvested by centrifugation, and the periplasmic fractions were obtained by using an osmotic shock procedure (17). The periplasmic fraction was incubated with 100 μM PQ and 1 mM CaCl2 at 30°C for 30 min to form the holoprotein. Enzyme activity of the recombinant enzyme was measured in the potassium ferricyanide reductase assay mentioned earlier. Heme staining of the periplasmic fraction was also performed to verify incorporation of heme into the active protein.

Nucleotide sequence accession numbers. The complete gene sequence of strain MC4, including the putative ribosome binding site and promoter sequence, was determined and the dppA gene was sequenced. The complete gene sequence of the DNA fragment has been deposited with GenBank under accession number JN162364. The 16S rRNA segment sequence was deposited with GenBank under accession number JF825523.

RESULTS

Isolation and characterization of strain MC4. Strain MC4 was isolated from contaminated soil by using enrichment cultivation with DCP as sole carbon and energy source. A growth curve and halide assays revealed that all chloride was liberated during DCP degradation (see Fig. S1 in the supplemental material). Cells of strain MC4 were Gram negative, motile, and rod shaped. The organism was oxidase positive and catalase positive and it hydrolyzed starch. Strain MC4 was able to grow on sugars (fructose, glucose, galactose, and ribose), citrate, acetate, succinate, benzoate, primary alcohols, and haloalcohols [3-bromo-2-methyl-1-propanol, (R)-3-chloro-1,2-propanediol, 3-chloro-1-propanol, rac-2-chloro-1-propanol, 2-chloroallyl alcohol, and rac-2,3-dichloro-1-propanol], while it did not utilize n-alkanes or 1-chloro-n-alkanes as growth substrates. Some haloalcohols, like 2-bromoacrylic acid, 2-chloroacrylic acid, rac-2-chloropropionic acid, rac-2,3-dichloropropionic acid, and 4-chlorobutyric acid, were also good growth substrates for strain MC4. Growth on DCP was not fast (ca. 0.02 h−1); with a 10% inoculum it took 3 days to consume 5 mM DCP in a fermentor (30°C).

The 16S rRNA gene sequence fragment of strain MC4 had 99% identity to the rRNA gene of Pseudomonas putida strains ATCC 17522, ATCC 17527, and ATCC 17536 (accession numbers AF094742.1, AF094743.1, and AF094747.1, respectively). This classifies the organism as a strain of P. putida.

Identification and purification of 2,3-dichloropropanol dehalogenase. For the conversion of vic-haloalcohols, several haloalcohol dehalogenases of the SDR superfamily of proteins have been described in the literature (54, 57). Therefore, we analyzed genomic DNA of strain MC4 for the presence of open reading frames similar to the respective genes for these enzymes. A series of PCR analyses with the primers derived from the sequences of the hheA, hheB, and hheC genes (54, 57) indicated that no similar gene was present in strain MC4. Assays with addition of DCP to cell extracts also failed to yield dehalogenase activity, whereas halohydrin dehalogenase activity was readily detected this way in control organisms.

Next, enzyme activity in cell extracts of strain MC4 grown on DCP was tested in a DCPIP reduction assay. The observed reduction of DCPIP was dependent on DCP. The specific activity of the enzyme in cell extract was 94 mU/mg of protein in the presence of the artificial electron acceptors DCPIP and PMS, which could not be replaced by NAD+ or NADP+. This suggests that the initial step in DCP conversion is catalyzed by a dehydrogenase that is suspected to simultaneously dechlorinate and transfer electrons to an acceptor that is not a nicotinamide coenzyme. Activity could also be monitored with ferricyanide as an artificial electron acceptor. We called the enzyme DppA.

The DppA protein was purified in three steps: ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose, and separation on a ceramic HAP column (Table 1; see also Fig. S2 in the supplemental material). DCIP-dependent ferricyanide reduction was measured at each purification step, in order to identify the protein responsible for the oxidation of the substrate. The molecular mass of DppA was estimated as 72 kDa by
TABLE 1 Purification of DppA

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein concn (mg/ml)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFE (NH₄)₂SO₄</td>
<td>15.3</td>
<td>6.6</td>
<td>265</td>
<td>0.4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>13.8</td>
<td>18.4</td>
<td>148</td>
<td>1.3</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>Ceramic HAP</td>
<td>0.5</td>
<td>5.8</td>
<td>69</td>
<td>10.9</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>13.2</td>
<td>53</td>
<td>14.1</td>
<td>20</td>
<td>32</td>
</tr>
</tbody>
</table>

*Enzyme activity was measured at 25°C in 50 mM Tris-SO₄ (pH 8.0) containing 1 mM KFe(CN)₆ and 5 mM 2,3-dichloropropanol.

SDS-PAGE analysis and as 73.5 kDa by gel filtration. This indicated that the DppA exists as a monomer in its native state. The enzyme was stable when stored at −20°C.

**Cloning and analysis of the dehalogenase gene.** To identify the gene responsible for DCP dehalogenation, the purified protein was subjected to N-terminal sequencing, which yielded NH₂-QV DQAAIIA, and the NCBI nonredundant protein database was scanned for homologs. This led to several hits annotated as quinohemoprotein dehydrogenases. Multiple sequence alignments showed that these enzymes possess a highly conserved region at about 1 kb downstream of the obtained N-terminal coding sequence. Two degenerate primers, prF1 based on the N terminus of the protein and prR1 based on the conserved region, were used to amplify a segment of the dehalogenase gene. The sequencing of this fragment confirmed that dppA encoded a quinohemoprotein.

To isolate the complete MC4 dehydrogenase/dehalogenase gene, a gene library of chromosomal DNA of MC4 was constructed in pLAFR3. The average size of pLAFR3 cosmids was between 45 and 50 kb, based on the restriction analysis of 10 random clones. PCR screening gave six positive clones. One of these was used to generate two separate sublibraries for sequencing, and assembly yielded a 3,555-bp contig containing the complete coding sequence for the DCP dehalogenase gene (dppA) along with its putative ribosome binding site and promoter sequence (Fig. 1).

The complete dppA-encoded protein sequence, including the signal peptide (698 amino acids [aa]), was very similar to type II quinohemoprotein alcohol dehydrogenases, particularly with the 2-chloroethanol dehydrogenase from *Pseudomonas stutzeri* (78% identity), the homologous alcohol dehydrogenases IIB (76%) and IIIG (53%) from *P. putida* HK5 (48, 49), a type I quinohemoprotein alcohol dehydrogenase from *Comamonas testesteroni* (51%) (39, 45), and a tetrahydrofurfuryl alcohol dehydrogenase from *Ralstonia eutropha* Bo (51% identity) (58). Homology with less than 50% identity was found with a quinohemoprotein alcohol dehydrogenase from *Rhodococcus erythropolis* (38% identity) (13, 31) and a methanol dehydrogenase from *Methylphilus methylotrophus* W3A1 (35% identity) (56). The first 25 amino acid residues of the encoded DppA protein constitute a typical signal sequence for the translocation of the protein to the periplasmic space. The presence of a signal peptide is a common characteristic of quinohemoproteins (50), which are located in the periplasm of Gram-negative bacteria. The predicted size of the mature protein without signal peptide is 72,978 kDa, in agreement with the SDS-PAGE analysis.

Sequence alignments with proteins of known structure indicated that the amino acids involved in PQQ and calcium binding in quinohemoproteins and quinoproteins are mostly conserved in DppA (Fig. 2). The PQQ and calcium binding domain, which corresponds to the N-terminal part of the sequence, contains several residues that are commonly conserved among quinoproteins and quinohemoproteins (e.g., Glu83, Cys129, Cys130, Arg135, Thr179, Gly195, Glu197, Trp256, Asn274, Trp318, and Asp319), whereas other residues (Gly194, Ala196, Thr254, Lys346, and Trp407) are only conserved in quinohemoproteins. An exception is Gly406 in the PQQ binding domain of DppA, which aligns with a conserved Asn present in most quinohemo/quinoproteins, with the exception of ADH IIIG, which has Asp at this position. The acidic residues in the PQQ and calcium domain that are involved in catalysis in quinohemo/quinoproteins are conserved as Asp319 and Glu197 in DppA (10). The heme binding residues Cys616, Cys619, and His620, which are conserved in the C-terminal heme domain of all quinohemoproteins, are also present in DppA, in agreement with the biochemically observed heme binding. Finally, the partially conserved tryptophan docking motifs (W1 to W8), a typical feature of quinohemo- and quinoproteins (4), are present in DppA as well.

A small open reading frame (ORF) encoding a peptide of 23 amino acids was present downstream of the dppA gene. BLAST analysis indicated that this peptide contains glutamate and tyrosine residues for PQQ biosynthesis (41). Upstream of the dppA gene, there is an ORF encoding 310 amino acids that constitute a hypothetical protein similar to a putative protein from *Azorarcus* sp. BH72 (accession number YP_934348) and QbdB from *Pseudomonas* spp. (accession number BAC15558). QbdB is a hypothetical protein believed to be involved in the meta-pathway of phenol degradation.

**Substrate range and kinetic parameters.** Using purified enzyme, the substrate profile of DppA was explored. Table 2 shows that the enzyme has a broad substrate range. The n-alcohols tested were well converted. The diols 1,2-propanediol and 1,3-propanediol gave considerably higher values than the other substrates, as also reported for ADH IIIG and ADH IIIB (47). The *K₉₅* value for DCP was 17.8 s⁻¹, and the *K₉₅*/*K₉₅* value was 2.3 s⁻¹ μM⁻¹, which indicates that DCP is well converted by DppA.
TABLE 2  Steady-state kinetic parameters and substrate specificities of DppA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
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<tr>
<td>Ethanol</td>
<td>14.9</td>
<td>788</td>
<td>0.02</td>
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<tr>
<td>Propanol</td>
<td>24.6</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>Butanol</td>
<td>20.2</td>
<td>1.5</td>
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<tr>
<td>Pentanol</td>
<td>25.3</td>
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<td>7.6</td>
</tr>
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<td>Hexanol</td>
<td>23.9</td>
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<td>1.1</td>
</tr>
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<tr>
<td>1,3-Propanediol</td>
<td>8.5</td>
<td>1,033</td>
<td>0.008</td>
</tr>
<tr>
<td>DCP</td>
<td>17.8</td>
<td>7.6</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The purification factor suggests that about 3% of the total protein in cell lysate is DCP dehalogenase which, in combination with the kinetic parameters of the dehalogenase and the assumption that about one-fourth of the total cell mass can be recovered as protein in the cell extract, suggests a possible DCP degradation rate of $(0.03 \times 0.25 \times 14.1)/(0.0076 + 0.0076)$ μmol/mg of cells/min. This would allow a growth rate of 0.35 h$^{-1}$ at 1 mM substrate, assuming that the yield on DCP is the same as on glycerol (ca. 0.06 mg cell [dry mass]/μmol [59]) and no energy generation from the dehalogenation reactions. Thus, the observed growth rate of less than 0.02 h$^{-1}$ appears not to be rate limited by the catalytic activity of the initial dehalogenase.

**Product identification.** To investigate the mechanism of dehalogenation, we examined the enzymatic conversion of DCP by purified DppA. Incubation of purified enzyme with substrate and potassium ferricyanide showed that DCP was converted with release of chloride, indicating that dechlorination and dehydrogenation take place simultaneously. During conversion of DCP (2.2 mM), 8 mM ferricyanide was reduced (see Fig. S3 in the supplemental material). As homologous quinohemoproteins are alcohol dehydrogenases that act on the terminal hydroxyl group of alcohols and diols to form the corresponding aldehydes (+), a plausible mechanism of DCP conversion would be the oxidation to 2,3-dichloropropanal. This aldehyde could undergo elimination of HCl to form 2- or 3-chloroacrolein.

We did not observe an aldehyde product by gas chromatography, which could be due to its reactivity or instability, and therefore we used dinitrophenylhydrazine (DNPH) for derivatization of the reaction samples and analyzed possible adducts by using LC-MS. In negative ionization mode, different adducts of DNPH with aldehydes should give different m/z values, viz m/z 305 (DNPH derivative of 2,3-dichloropropanal), m/z 271 (DNPH derivative of 2- and 3-chloropropanal), or m/z 269 (DNPH derivative of 2-chloroacrolein and 3-chloroacrolein). The negative-mode ESI mass spectra indicated the appearance of a peak at m/z 269 (Fig. 3) that disappeared later during the conversion. This indicated that either 2-chloroacrolein or 3-chloroacrolein was formed. A DNPH adduct of propanal was also seen in minute amounts during the initial phase of the enzyme reaction, which may have formed via an unidentified side reaction or could have been due to a substrate impurity or fragmentation in the LC-MS. No DNPH adducts indicating the formation of 2-chloropropanal, 3-chloropropanal, or 2,3-dichloropropanal were observed. This indicated that one chlorine was rapidly removed from the substrate during the oxidative reaction.

To establish the position of the chlorine released from DCP, proton NMR was performed, and chemical shifts were recorded for reaction mixtures containing enzyme, DCP, and ferricyanide. Three signals in the NMR spectra of an intermediate product were assigned to 2-chloroacrolein ([43]). The time course of its aldehyde proton (9.31 ppm) displayed the same trend as the two alkenic protons (6.72 and 6.57 ppm). This again suggested that the oxidation of the hydroxyl group was accompanied by swift elimination of HCl, either in the active site of the enzyme or very rapidly after product release from DppA. Two other $^1$H NMR signals (6.07 and 5.73 ppm) were assigned to the alkene protons of 2-chloroacrylic acid, which indicated further oxidation of 2-chloroacrolein to the acid, either by the same purified DppA enzyme or abiotically. The acid apparently was not converted further under these conditions.

The best-separated $^1$H NMR signals of the starting compound, as well as those of the intermediate product and the final product, were integrated in all samples and used to visualize substrate conversion over time (Fig. 4). The results showed that after 6 h, the reaction halted at approximately 72% conversion. This was probably due to complete consumption of the electron acceptor.
balances were not exactly stoichiometric, since only 25% of the final product was detected based on integration of proton signals. This may be due to the high substrate concentration and the fact that the intermediate 2-chloroacrolein is a very reactive compound that can form dimers or polymers in aqueous solution (43), especially when produced by a pure enzyme with few possibilities for further conversion. Some minor signals in the 1D 1H NMR spectra were indeed observed, indicating formation of side products, but these signals could not be related to a specific product.

**Heterologous expression.** Since the level of production of the native DppA in *Pseudomonas* strain MC4 was low, further work aimed at elucidating structure-function relationships in this novel dehalogenase would benefit from better enzyme production. To facilitate heterologous periplasmic expression in *E. coli* (18), we fused the *dppA* gene to the 21-mer signal sequence of *E. coli* alkaline phosphatase, yielding the construct pNDL1. As quinohemoproteins require heme c maturation (46), the fusion protein was expressed in *E. coli* JCB712 and *E. coli* TOP10 in the presence of cytochrome c maturation factors, encoded on plasmid pEC86. We found that the recombinantly produced DppA had a higher level of expression and heme incorporation in *E. coli* TOP10(pNDL1)(pEC86) than in *E. coli* JCB712(pNDL1)(pEC86) (Fig. 5), even though strain JCB712 is known to incorporate heme effectively in the periplasmic space (23). The specific activities of DppA in cell extracts of these recombinant *E. coli* strains were 1.3 U/mg and 0.1 U/mg, respectively, as measured in ferricyanide reduction assays. Heme staining confirmed that the DppA protein contained covalently bound heme and that the enzyme showed catalytic activity with DCP (Fig. 6). Qualitative analysis indicated that ferricyanide reduction was accompanied by chloride release.

**DISCUSSION**

We report the isolation of *P. putida* strain MC4 from a polluted site, and this organism is capable of growth on DCP as sole carbon source. The strain grew aerobically on many other compounds as well, including sugars, several halogenated aliphatics, and nonhalogenated alcohols. Bacterial cultures that utilize DCP and 1,3-dichloropropanol as growth substrates have been described previously, but often the substrate degradation has been incomplete due to enantioselectivity of the catalytic enzymes, which restricts the possibilities to use such organisms for bioremediation applications (8, 36), whereas they may be attractive for production of optically active compounds (27, 28, 29, 30).

It is obvious that dehalogenation is a key step for microbial utilization of halogenated organic compounds (24). The dehalogenation of haloalcohols is often catalyzed by haloalcohol dehalogenases (5, 36, 54), and we initially expected that strain MC4 would also contain such an enzyme, but no such activity could be detected and no homologous dehalogenase gene was present in strain MC4. Instead, activity measurements indicated that chloride release was electron acceptor dependent and led to the identification of a novel type of oxidative dehalogenase, which we called DppA. The enzyme had a broad substrate range encompassing a number of aliphatic alcohols and aldehydes. We also found that DppA accepts both the (+) and (−) enantiomers of DCP, since conversion goes to completion with no sign of biphasic kinetics.

A BLAST search of the sequence of the MC4 dehalogenase gene *dppA* in the NCBI database indicated that DppA was homologous to type II quinohemoprotein ADHs, which are mostly involved in the conversion of nonhalogenated alcohols and contain both PQQ and heme as cofactors (4). The native DppA sequence contains a 25-aa signal peptide at the N terminus that is cleaved off during maturation, which was apparent from the N-terminal sequence of the mature isolated protein. Furthermore, dehalogenase activity was detected in the periplasmic fraction prepared from strain MC4 by an osmotic shock method (data not shown). Other quinohemoproteins also reside in the periplasm (50). The presence of an enzyme in the periplasmic space may have functional implications, such as improved protein stability and reduced proteolytic degradation (18). Besides, the presence of a dehydrogenase that forms a reactive and toxic metabolite in the periplasm could suppress potential toxic effects that may occur when formation of a reactive product occurs in the cytoplasm. The conversion of 2-chloroethanol by a periplasmic quinoprotein was described earlier (53) and may have the same function: prevention of formation of highly reactive chlorinated aldehyde in the cytoplasm.

Since known quinohemoproteins convert alcohols into corresponding aldehydes (50), we also expected the formation of an aldehyde during DCP conversion. The results indeed indicated that the first step in the DCP catabolic pathway involves the conversion of DCP into 2-chloroacrolein, which is further converted into 2-chloroacrylic acid. Whether the same DppA is solely responsible for both steps was not certain, but NMR measurements indicated that purified DppA was active with 2-chloroacrolein as well. By analogy to the well-studied mechanism of quinohemo-

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**FIG 4** Conversion of DCP by DppA, followed by 1H NMR. Symbols: ○, 2,3-DCP; □, 2-chloroacrolein; Δ, 2-chloroacrylic acid.

**FIG 5** Coomassie stain (A) and heme stain (B) of an SDS-PAGE gel containing recombinant DppA expressed in *E. coli* strains JCB712 (lanes 1 and 2) and TOP10 (lane 3). Different concentrations of arabinose were used for induction: lane 1, 0.002%; lanes 2 and 3, 0.02%.

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proteins (50), we propose a catalytic mechanism of DppA (Fig. 6) that involves dehalogenation in the enzyme’s active site or immediately after product release. The $^3$H NMR experiments indeed suggested that release of chloride and a proton occur immediately upon formation of 2,3-dichloropropionaldehyde by hydride transfer to PQQ. This forms 2-chloroacrolein, with a structure that is more stable due to resonance delocalization of the $\pi$-electrons. Whether the DppA enzyme mechanistically participates in halide release, e.g., through specific stabilizing interactions that facilitate cleavage of the carbon-halogen bond, such as what occurs with haloalkane and halohydrin dehalogenases (11, 12), is uncertain at this moment.

A somewhat similar oxidative dehalogenation mechanism has been reported for a flavoenzyme from Alcaligenes sp. DS-S-7G, and this enzyme has been termed HDDase (28). The enzyme oxidatively dechlorinates (R)-3-chloro-1,2-propanediol and produces acetic acid and formaldehyde. It was suggested that this conversion starts with formation of 3-chloro-1,2-propanediol, which could be cleaved by the reductive action of the FADH$_2$-containing enzyme (27). The DppA-catalyzed dehalogenation is mechanistically completely different from the halohydrin dehalogenase-catalyzed dehalogenation of chloroalcohols, where the vicinal halogen is released and an epoxide is formed by an intramolecular nucleophilic substitution (11).

Further degradation of 2-chloroacrylic acid was not studied in MC4, but known pathways are hydrolytic dechlorination of 2-chloroacrylic acid, which yields pyruvate (32), or reduction of 2-chloroacrylic acid to 2-chloropropionic acid, which can be dehalogenated to lactate (35).

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