Arch Microbiol (1997) 168 : 520–527

ORIGINAL PAPER

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Purification, characterization and gene sequence analysis of a novel cytochrome *c* co-induced with reductive dechlorination activity in *Desulfomonile tiedjei* DCB-1

Received: 30 May 1997 / Accepted: 29 July 1997

Abstract The sulfate-reducing bacterium, *Desulfomonile tiedjei* DCB-1, conserves energy for growth from reductive dechlorination of 3-chlorobenzoate via halorespiration. To understand this respiratory process better, we examined electron carriers from different cellular compartments of *D. tiedjei*. A 50-kDa cytochrome from the membrane fraction was found to be co-induced with dechlorination activity. This inducible cytochrome was extracted from the membrane fractions by Tris-HCl buffer containing ammonium sulfate at 35% saturation and was purified to electrophoretic homogeneity by phenyl superose, Mono Q, and hydroxyapatite chromatography. The purified cytochrome had a high-spin absorption spectrum. In a pH titration experiment, the absorption spectrum of the inducible cytochrome shifted to low spin at pH 13.2. The midpoint potential of the inducible cytochrome at pH 7.0 was –342 mV. The NH_2-terminal amino acid sequence of the inducible cytochrome was determined and was used to obtain inverse PCR products containing the sequence of the gene encoding the inducible cytochrome. The ORF was 1398 bp and coded for a protein of 52.6 kDa. Two *c*-type heme-binding domains were identified in the COOH-terminal half of the protein. A putative signal peptide of 26 residues was found at the NH_2-terminal end. The protein sequence was not found to have substantial sequence similarity to any other sequence in GenBank. We conclude that this is a *c*-type cytochrome substantially different from previously characterized *c*-type cytochromes.

Key words *Desulfomonile tiedjei* DCB-1 · Halorespiration · Reductive dechlorination · Electron transport · Cytochrome *c*

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Introduction

Reductive dechlorination is important for pollutant biodegradation. A number of highly chlorinated aromatic environmental contaminants, such as polychlorinated biphenyls, pentachlorophenol, and hexachlorobenzene, are toxic and recalcitrant. These pollutants are resistant to oxidative transformation in natural environments. Reductive dechlorination is the only known mechanism for biological removal of halogen substituents from certain highly halogenated aromatic compounds. Dechlorination generally makes these compounds less toxic and more amenable for further degradation (Holliger and Schraa 1994; Mohn and Tiedje 1992).

Reductive dechlorination has been shown to support growth via halorespiration in several bacterial isolates. *Desulfitobacterium dehalogenans* JW/IU-DC1 cell yield increases concurrently as 3-chloro-4-hydroxy-phenylacetate is reductively dechlorinated, suggesting the bacterium may gain energy via halorespiration (Utkin et al. 1994). A related strain, *Desulfitobacterium* sp. PCE1, has been shown to grow via halorespiration by coupling tetrachloroethene reductive dechlorination to formate oxidation, and to grow only on lactate in the presence of external electron acceptors such as tetrachloroethene and 3-chloro-4-hydroxy-phenylacetate (Gerritse et al. 1996). Growth by halorespiration was also demonstrated in *Desulfitobacterium chlororespirans*, which reductively dechlorinates 3-chloro-4-hydroxybenzoate (Sanford et al. 1996), and *Dehalospirillum multivorans* (Scholz-Mura-

matsu et al. 1995) and "*Dehalobacter restrictus*" (Holliger and Schumacher 1994), which both use tetrachloroethene as terminal electron acceptors. *Desulfomonile tiedjei* DCB-1 (DeWeerd et al. 1990), a Gram-negative sulfate-reducing bacterium, is the most-studied pure culture capable of coupling reductive dechlorination to growth by halorespiration. This organism specifically removes chlorine substituents at the *meta* positions of chlorobenzoates (Shelton and Tiedje 1984) and chlorophenols (Mohn and Kennedy 1992). *D. tiedjei* obtains energy for growth by coupling reductive dechlorination of 3-chlorobenzoate, as electron acceptor, to formate oxidation (Dolfing 1990; Mohn and Tiedje 1990a), suggesting that energy is conserved by electron transport phosphorylation. Subsequently, Mohn and Tiedje (1991) showed that *D. tiedjei* was capable of coupling reductive dechlorination and ATP synthesis via an ATPase driven by a proton motive force, which is probably generated by an uncharacterized electron transport chain during reductive dechlorination. In addition, reductive dechlorination in *D. tiedjei* cell extract is inhibited by addition of sulfite or thiosulfate (DeWeerd and Suflita 1990). In a related study using washed *D. tiedjei* cell suspensions, the presence of sulfite or thiosulfate also inhibited dehalogenation of 3-chlorobenzoates (DeWeerd et al. 1991). Linkfield and Tiedje (1990) observed *D. tiedjei* dechlorination activity being inhibited by thiosulfate, sulfite, and sulfate. These observations suggest that reduction of sulfoxy anions may potentially compete with reductive dechlorination for reducing equivalents or electron carriers. Recently, a membrane-bound, inducible reductive dehalogenase was purified from *D. tiedjei* (Ni et al. 1995). This enzyme is proposed to be the terminal reductase of the electron transport chain.

All of the above evidence suggests that reductive dechlorination in *D. tiedjei* is a respiratory process. Unfortunately, little is known about the biochemical mechanism of halorespiration. Further understanding of the mechanism of reductive dechlorination and halorespiration will come from identification and localization of the electron carriers involved in the process. We report here the purification, characterization and the gene sequence of a cytochrome *c* purified from the *D. tiedjei* cytoplasmic membrane. This cytochrome *c* is co-induced with reductive dechlorination and might be part of the electron transport chain involved in reductive dechlorination.

Materials and methods

Organism and growth conditions

D. tiedjei DCB-1 (ATCC 49306) was grown on reduced anaerobic medium as previously described by Mohn and Tiedje (1991) with the following modifications and additions: 20 mM pyruvate was used as electron donor, 0.1 mM titanium citrate was the only reductant, 2 g yeast extract/l plus 2 g tryptone/l were added as supplements, the phosphate concentration was raised from 4 mM to 10 mM, and the vitamin solution was changed to the vitamin mixture reported by Wolin et al. (1963) plus 200 µg 1,4-naphthoquinone/l. The gas phase was N_2/CO_2 (95:5, v/v), and the pH was adjusted to 7.5. When the cells were grown under dechlorinating conditions, 500 µM of 3-chlorobenzoate was added from a 100-mM anoxic filter-sterilized stock solution. When depleted, 3-chlorobenzoate was replenished. When the cells were grown under fermenting conditions, no 3-chlorobenzoate was added. When the cells were grown under sulfate-respiring conditions, 20 mM sodium formate plus 5 mM sodium sulfate were added instead of pyruvate and 3-chlorobenzoate. Inocula were 10%, and cultures were incubated stationary at 37° C.

Preparation of the membrane fraction

Late-exponential-growth phase cultures were harvested either by centrifugation at 10,000 × *g* for 20 min or by a bench-scale cross-flow filtration unit equipped with a 0.3-µm pore-size microfiltration membrane (Filtron). Cells were resuspended in 10 mM Tris-HCl (pH 7.7) and were broken by passing the cell suspensions 4 times through a French pressure cell at a cell pressure of 103 MPa. Cell lysates were then centrifuged at 12,000 × *g* for 20 min. The pelleted, unbroken cells and debris were resuspended in 10 mM Tris-HCl (pH 7.7), and passed through the French pressure cell four more times and centrifuged as described above. The combined supernatants were centrifuged for 2 h at 180,000 × *g*. The pellets were washed and resuspended in the Tris buffer, and centrifuged at 180,000 × *g* for another 2 h. The pellets were considered to be membrane fractions and were stored at –20° C.

Purification of the 50-kDa inducible cytochrome *c*

Unless indicated otherwise, the entire purification procedure was carried out at room temperature without protection from oxygen. The membrane fractions were thawed and resuspended in 10 mM Tris-HCl (pH 7.7) to approximately 13 mg protein/ml. Ammonium sulfate was added to reach 35% saturation and the mixture was shaken for 30 min. The mixture was then centrifuged at 12,000 × *g* for 10 min, and the supernatant was collected.

The ammonium sulfate membrane extract was concentrated by Centriplus-30 (Amicon) to 2.5 mg protein/ml. The concentrate was divided into three 3.5-ml aliquots. Each aliquot was loaded independently onto an FPLC Phenyl Superose HR 5/5 (1 ml) column (Pharmacia Biotech) equilibrated with 10 mM Tris-HCl (pH 7.7) plus ammonium sulfate at 35% saturation. The column was first eluted with a decreasing linear gradient (10 ml) from 35% to 25% saturation of ammonium sulfate in the Tris buffer followed by successive 7-ml steps of 25%, 15% and 0% saturation of ammonium sulfate in the same buffer. The flow rate was kept at 0.25 ml/min. The inducible cytochrome was eluted at 25% saturation of ammonium sulfate. Fractions containing the inducible cytochrome were combined, dialyzed against 10 mM Tris-HCl (pH 7.7) overnight at 4° C in dialysis tubing with a molecular weight cutoff of 6,000–8,000 (Spectrum Medical Industries), and then concentrated by Centricon-10 (Amicon) to 1.3 mg of protein in 1 ml. The concentrate was loaded onto an FPLC Mono Q HR 5/5 (1 ml) column (Pharmacia Biotech) equilibrated with 10 mM Tris-HCl (pH 7.7). The inducible cytochrome did not bind to the Mono Q column and was eluted with same buffer. The unbound Mono Q fraction was exchanged into 10 mM sodium phosphate buffer (pH 6.5) by dialysis. About 507 µg of protein was recovered and loaded onto a 5-ml hydroxyapatite column (Biorad) equilibrated with 10 mM sodium phosphate buffer (pH 6.5). The inducible cytochrome was eluted at 300 mM sodium phosphate during an increasing linear gradient (50 ml) from 10 to 500 mM sodium phosphate (pH 6.5) at 1 ml/min. Fractions containing the inducible cytochrome were pooled, concentrated by Centricon-10, and stored at 4° C for subsequent analyses.

Redox and pH titration of the inducible cytochrome

The midpoint potential of the inducible cytochrome was determined by two methods. A redox titration was performed under

a nitrogen atmosphere, using a cuvette sealed with a butyl rubber stopper (Dutton 1978). *N*,*N*,*N*′,*N*′-Tetramethyl phenylenediamine, hexammineruthenium (III) chloride, pyocyanine, 2-hydroxyl-1,4-naphthoquinone, anthraquinone-2-sulfonate, benzyl viologen, and methyl viologen in 100 mM sodium phosphate buffer (pH 6.5) were used as redox mediators. Anoxic, freshly dissolved sodium dithionite was used as reductant. Concentrations of purified inducible cytochrome and individual redox mediators were 1.5 μM and 0.3 μM, respectively. The redox potential of the mixture was measured by a miniature Pt-Ag/AgCl combination redox electrode (Microelectrodes) calibrated against freshly prepared pH 4.0 and 7.0 buffers saturated with quinhydrone. The absorbances of the inducible cytochrome at 399 and 555 nm were monitored as a function of the measured redox potential. Midpoint potential was also determined using equilibrium reaction between 8-hydroxyriboflavin, which has a midpoint potential of –332 mV (Müller 1983), and the inducible cytochrome. A solution of 1.6 μM of inducible cytochrome, 50 μM 8-hydroxyriboflavin, and 10 mM EDTA in 100 mM sodium phosphate buffer (pH 7.0) was sealed in a cuvette with a butyl rubber stopper and kept in the dark. The mixture was made anoxic by flushing with oxygen-free nitrogen and was then gradually photoreduced by direct exposure to sunlight. Progress of reduction of the inducible cytochrome and 8-hydroxyriboflavin was monitored spectrophotometrically after each period of irradiation. 8-Hydroxyriboflavin reduction was measured at 475 nm, which was an isosbectic point of the oxidized and reduced inducible cytochrome. Reduction of the inducible cytochrome was measured at 384 nm and 555 nm.

A pH titration of the inducible cytochrome was done under an air atmosphere. Small aliquots of sodium hydroxide solution (1 M) were added to a 1.5 μM solution of purified inducible cytochrome. UV-visible absorption spectra were recorded and pH in the solution was monitored by a miniature Pt-Ag/AgCl combination pH electrode (Microelectrodes). An Orion 290A pH/mV meter was used to measure pH of the sample and redox potential in chemical redox titration.

NH_2-terminal protein sequence analysis

After resolving the purified inducible cytochrome on SDS-PAGE, the gel was soaked in transfer buffer [10 mM CAPS (pH 9.0) containing 0.5 mM dithiotheritol]. The protein was electroblotted to a polyvinylidene difluoride membrane (Millipore) and stained with Coomassie brilliant blue R250 in 50% methanol. After destaining with 50% methanol, the membrane was air-dried. The inducible cytochrome band was excised from the membrane and sequenced. The NH_2-terminal sequencing was done by the Nucleic Acid - Protein Service Unit at the University of British Columbia.

Genomic DNA isolation

About 5 g (wet weight) of cells were washed 3 times in 15 ml of 50 mM Tris-HCl, 50 mM EDTA, and 170 mM sodium carbonate buffer (pH 9.0) at 35°C for 30 min. The washed cell pellets were resuspended in 30 ml 10 mM Tris-HCl, 1 mM EDTA buffer pH 8.0 (TE buffer). Genomic DNA was extracted from the cell suspension as previously described by Ausubel et al. (1992), with the following modifications. The final concentration of SDS was 0.33%, 3 mg self-digested Pronase E/ml (Sigma) was used instead of proteinase K, and the suspension was incubated at 56°C for 4 h. The SDS-Pronase E lysis procedure followed by freezing at –20°C was repeated 3 times to achieve adequate cell lysis. Cell lysates were incubated in sodium chloride and cetyltrimethylammonium bromide (Sigma) at 65°C for 1 h, dissolved nucleic acids were treated with 2 mg RNase A/ml (Sigma) at 37°C for 1 h, and the RNase A was inactivated by 0.1 mg protease K/ml at 37°C for 1 h.

Inverse PCR and cloning of inverse PCR product

D. tiedjei genomic DNA was completely digested by an appropriate restriction enzyme, self-ligated and used for inverse PCR as previously described by You et al. (1996) with the following modifications. Five micrograms of DNA/ml was self-ligated by 200 U T4 DNA ligase (New England Biolab) at 16°C, and the inverse PCR reaction contained 1 μg of self-ligated DNA, 0.5 μM of each primer, and 1 mM $MgCl_2$. Two pairs of primers TA2F2 (5′-CAGTGATGACAACCGGAG) plus TA2R2 (5′-TTGATTCGATTATGACTC), and 18a (5′-GCCATGAAATGCACCTG) plus 18c (5′-TTCCAGGAGCTTCTTGA) were used in the two independent inverse PCRs to amplify the complete gene sequence. Inverse PCR was performed as follows: (1) denaturation at 95°C for 3 min; (2) 0.5 min at 95°C, 1 min at 45°C, and 3.5 min at 72°C for 5 cycles; (3) 0.5 min at 95°C, 1 min at 40°C, and 3.5 min at 72°C for 30 cycles; (4) a final incubation at 72°C for 10 min to complete any unfinished single-stranded products. Fresh inverse PCR products were ligated to pCR II vector in TA PCR cloning kit (Invitrogen), under conditions recommended by the manufacturer. Competent *Escherichia coli* DH5α was transformed with ligation mixtures by a standard procedure (Ausubel et al. 1992). The primers used were custom synthesized by the Nucleic Acid - Protein Service Unit at the University of British Columbia.

Analytical methods

SDS-PAGE was performed according to the method of Laemmli (1970) using 5% stacking-10% separating acrylamide gels. The gels were stained with Coomassie brilliant blue R250. Heme staining of SDS-PAGE gels was done using dimethoxybenzidine hydrochloride (Sigma) according to the method of Francis and Becker (1984), except protein samples were incubated at 50°C for 15 min. Reductive dechlorination of 3-chlorobenzoate to benzoate was analyzed by the HPLC method of Mohn and Tiedje (1990b). Sulfate consumption in sulfate-respiring cultures was analyzed by HPLC with an anion chromatography column (Hewlett Packard, 125×4 mm) under conditions recommended by the manufacturer. Protein was determined by the bicinchoninic acid method (Smith et al. 1985) with bovine serum albumin as the standard. UV-visible absorption spectra were obtained by a Cary 1E spectrophotometer (Varian).

Plasmid DNA of inverse PCR clones was used as templates for double-stranded DNA sequencing, using the dye-terminator cycle sequencing kit (Perkin-Elmer). DNA sequencing was done by the Nucleic Acid – Protein Service Unit at the University of British Columbia. The nucleotide sequence of the *D. tiedjei* inducible cytochrome has been deposited in GenBank under accession number AF015192.

Results

Cytochromes in *D. tiedjei* membrane fractions

Two *D. tiedjei* membrane cytochromes were detected by heme staining of SDS-PAGE gels loaded with membrane fractions solubilized by 1% SDS. A cytochrome with an apparent molecular mass of 50 kDa, was present only in the membrane fractions of *D. tiedjei* induced for reductive dechlorination (Fig. 1). A more abundant constitutive cytochrome of apparent molecular mass 17 kDa was present in membrane fractions of cells grown under reductive-dechlorinating, pyruvate-fermenting, and sulfate-respiring conditions.

Purification of the inducible cytochrome

The majority of membrane cytochromes were extracted from membrane fractions of dechlorinating cells by 10 mM Tris-HCl (pH 7.7) with 35% saturation of ammonium

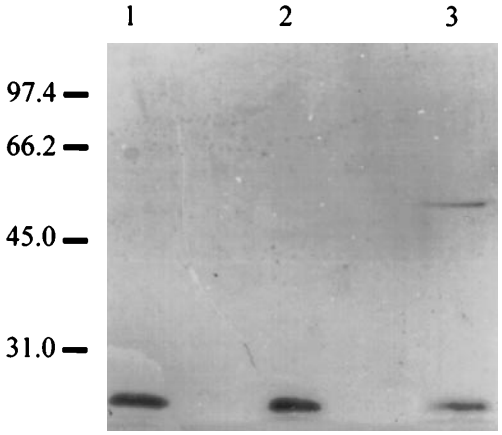

Fig. 1 Heme staining of membrane cytochromes of *Desulfomonile tiedjei* grown under the following conditions: (*1*) sulfate-reducing; (*2*) pyruvate-fermenting; and (*3*) reductive dechlorinating. Whole membrane fractions, solubilized by 1% SDS, were loaded onto the gel. Cytochrome content in each membrane fraction was estimated by absorption spectroscopy and calibrated against horseheart cytochrome *c*. The amounts of the three types of membrane fractions loaded onto the gel were adjusted such that each lane contained an equal amount of cytochrome (equivalent to about 11 μg horseheart cytochrome *c*). The numbers on the *left* are molecular size markers in Kilodaltons

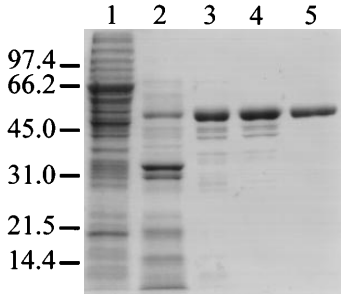

Fig. 2 SDS-PAGE of the successive purification steps of the *D. tiedjei* inducible cytochrome. *Lane 1* Crude membrane fraction solubilized by 1.0 % SDS (10 μg protein); *lane 2* 35 % $(NH_4)_2SO_4$ membrane extract (3.0 μg protein); *lane 3* phenyl superose 25% $(NH_4)_2SO_4$ eluent (3.0 μg protein); *lane 4* Mono Q unbound eluent (3.4 μg protein); *lane 5* hydroxyapatite 300 mM NaP_i eluent (1.0 μg protein). The numbers on the *left* are molecular size markers in Kilodaltons

sulfate. Left-over membrane materials were solubilized by 1% SDS, loaded onto SDS-PAGE gels and heme-stained to check the extraction efficiency. Only a small amount of the 17-kDa constitutive cytochrome, but none of the 50-kDa inducible cytochrome, remained in membranes after the extraction (data not shown). The inducible cytochrome was purified until electrophorectically homogenous by phenyl superose hydrophobic interaction chromatography, Mono Q anionic exchange, followed by hydroxyapatite chromatography, as shown in Fig. 2. About 105 μg purified protein was recovered from 20 g wet cells. In SDS-PAGE, the purified protein migrated as a single band corresponding to an apparent molecular mass of 50 kDa (Fig. 2). When the purified protein was chromatographed on an FPLC Superose-6 gel filtration column, the apparent molecular mass of the inducible cytochrome was determined to be 155 kDa (data not shown).

Characterization of the inducible cytochrome

The inducible cytochrome displayed high-spin absorption spectra. The purified inducible cytochrome was in the oxidized form with a broad Soret peak at 399 nm (Fig. 3a). Reduction of this cytochrome by sodium dithionite resulted in a shift of the Soret peak to 423 nm. The shape of the Soret peak also changed. A shoulder at 430 nm appeared. In addition, a broad peak at 555 nm appeared upon reduction. A pH titration was performed with the purified inducible cytochrome. No significant changes in absorption spectra occurred until the pH was raised above 12 (Fig. 3b, c). When the pH reached 13.2, the inducible cytochrome had an oxidized absorption spectrum similar to that of low-spin eukaryotic ferricytochrome *c*, with a narrow and symmetric Soret peak at 413 nm plus a broad α-peak at 540 nm. When reduced by sodium dithionite at pH 13.2, the spectrum changed to that of a reduced typical low spin cytochrome *c*, with the Soret peak shifted to 416 nm. Distinctive α and β peaks at 549 and 521 nm, respectively, also appeared.

The purified inducible cytochrome was titrated chemically with sodium dithionite over a wide range of redox potentials and the midpoint potential was estimated to be –360 mV (data not shown). By photoreducing an equilibrated mixture of inducible cytochrome and 8-hydroxyriboflavin, the inducible cytochrome midpoint potential was calculated to be –342 mV at pH 7.0 (Fig. 4).

NH_2-terminal sequence of the inducible cytochrome

The NH_2-terminal amino acid sequence of the inducible cytochrome was determined to be ESKKVPSSYSPVVITEPFDSIMTRMKAAKP. Based on this amino acid sequence, a pair of degenerate primers were designed from both ends of the NH_2-terminal protein sequence. The primer mixtures were used to amplify the corresponding DNA sequence from *D. tiedjei* genomic DNA by PCR. An expected 89-bp PCR product was cloned. Two clones with the right insert were isolated, and the inserts were sequenced. In both cases, the encoded protein sequences of the inserts matched the NH_2-terminal protein sequence.

Gene sequence of the inducible cytochrome

A Southern blot of *D. tiedjei* DNA cleaved with various restriction enzymes was probed with the 89-bp cloned fragment. The end-labeled 89-bp probe hybridized to a single 1.2-kb *Sst*I fragment (data not shown). However, attempts to clone this *Sst*I fragment from a genomic cos-

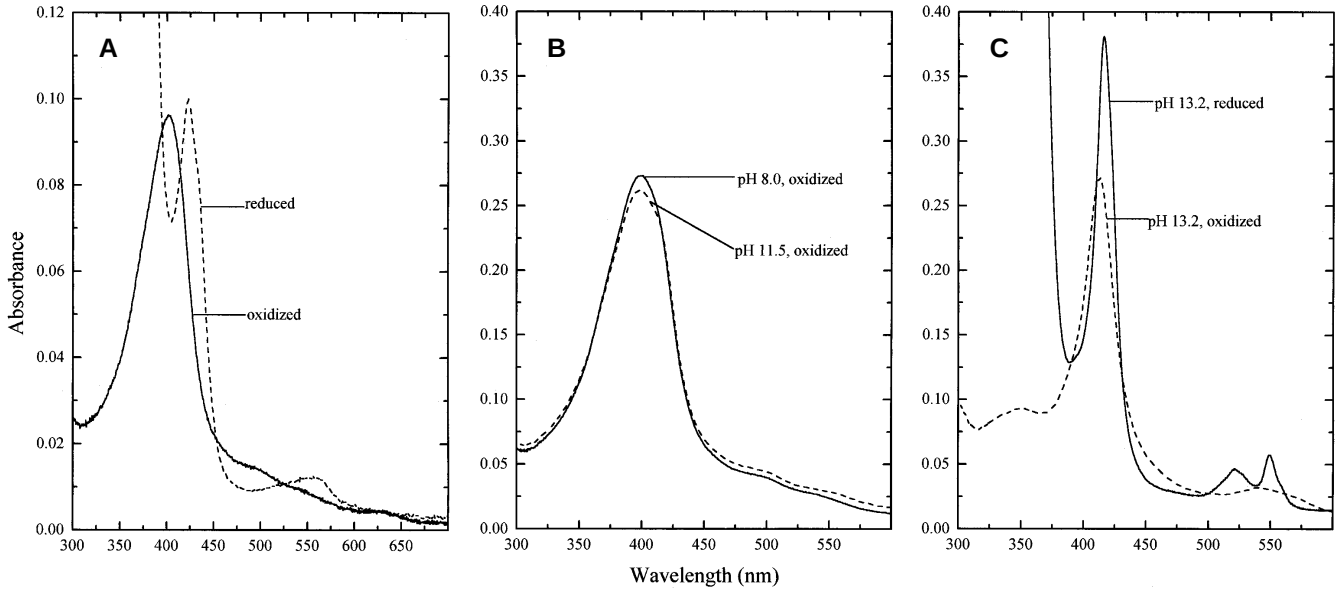

Fig. 3A–C Oxidized and reduced absorption spectra of 0.75 μM purified *D. tiedjei* inducible cytochrome in 100 mM sodium phosphate pH 6.5 buffer. The reduced form of the cytochrome was obtained by the addition of sodium dithionite (**A**). Oxidized absorption spectra of 1.5 μM purified *D. tiedjei* inducible cytochrome at pH 8.0 and 11.5 (**B**). Oxidized and reduced absorption spectra of 1.5 μM purified *D. tiedjei* inducible cytochrome at pH 13.2 (**C**). All of the absorption spectra are recorded in a 1-cm path length quartz cuvette

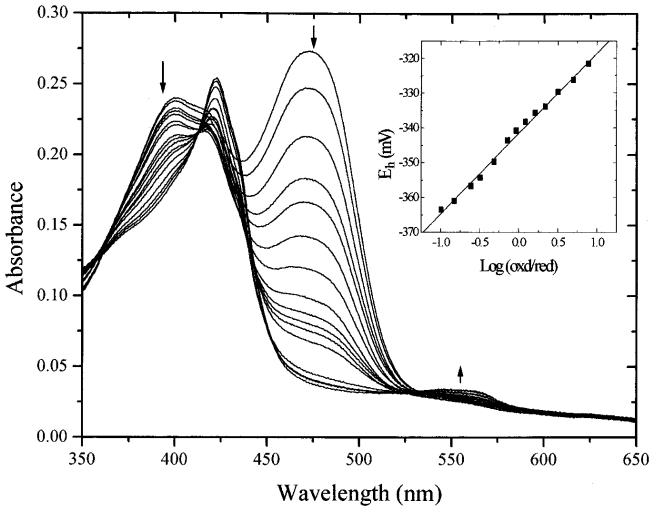

Fig. 4 Spectral changes observed from 350 to 650 nm during photoreduction, by 8-hydroxyriboflavin, of the *D. tiedjei* inducible cytochrome. *Arrows* show direction of change in regions of the absorption spectrum as the inducible cytochrome and 8-hydroxyriboflavin were reduced. *Inset* Nernstian plot of the redox titration. The experimental points are denoted by *solid squares*. The *straight line* was drawn according to a theoretical Nernst equation with $n = 2$. The midpoint redox potential was determined to be –342 mV at pH 7.0

mid library and a plasmid library by colony hybridization with the same 89-bp probe were unsuccessful. Therefore, the gene and flanking DNA sequences were cloned by inverse PCR. *D. tiedjei* genomic DNA was cleaved by *Sst*I and self-ligated. The circularized DNA was then used as the template for inverse PCR with primers TA2F2 and TA2R2. A linear inverse PCR product of expected size was cloned and named ISST18. DNA sequencing of clone ISST18 revealed a 1151-bp insert flanked by *Sst*I sites. The DNA sequence encoding the NH_2-terminal amino acid sequence of the inducible cytochrome was identified in the insert. Directly 5′ to this DNA sequence was a stretch of DNA sequence encoding a putative 26-amino-acid signal peptide, which began with a putative GUG start codon. A putative ribosome binding site was identified about 10 bp 5′ to the start codon. In addition, a *c*-type heme-binding site, CFDCH, was identified (Fig. 5). No stop codon was identified within this ORF. Also, the encoded product of this inverse PCR insert was smaller than the apparent molecular mass of the inducible cytochrome determined from SDS-PAGE. We concluded that the 3′ end of the gene was not present in clone ISST18.

A second DNA fragment containing the entire sequence of the inducible cytochrome gene was cloned by a second inverse PCR. A 719-bp PCR product amplified from pISST18 was labeled as a probe, and hybridized to a 2.2 kb *Mun*I-cleaved genomic DNA fragment (data not shown) which contained the complete gene sequence. A second inverse PCR with self-ligated *Mun*I-cleaved DNA as template and primers 18a and 18c yielded an expected 2.2-kb inverse PCR product. Two recombinant clones with this product, IMUN3 and IMUN22, were obtained. Plasmid DNA extracted from these two clones hybridized strongly to the 719-bp probe in a Southern blot analysis (data not shown). The insert of pIMUN3 was sequenced and contained the DNA sequence identified previously from the insert of clone ISST18, plus 1183 bp of 3′ sequence. A second *c*-type heme-binding domain, CAVCH (Fig. 5), and a UAA stop codon was identified. The complete ORF was therefore 1398 bp long, encoding a 466-amino-acid product. The molecular mass of the encoded

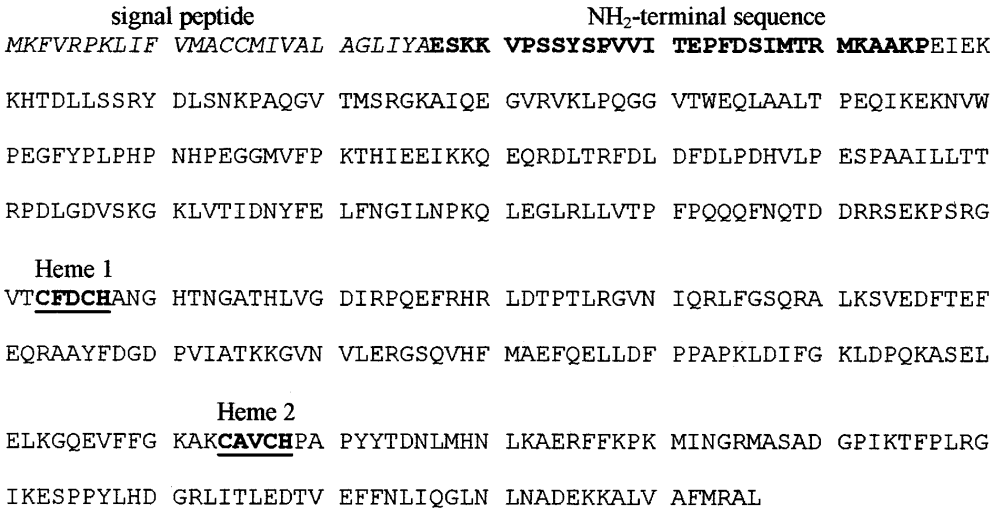

Fig. 5 The protein sequence encoded by the *D. tiedjei* inducible cytochrome gene. The encoded protein sequence is shown using the one-letter amino acid code. A putative 26-amino-acid signal peptide is italicized. The NH_2-terminal protein sequence is *bold* and the two heme-binding domains are *bold* and *underlined*

product, excluding the putative signal peptide, was calculated to be 49,722 Da and closely matched the apparent molecular mass of 50 kDa determined by SDS-PAGE. The protein sequence was not found to have substantial sequence similarity to published sequences in GenBank. The NH_2-terminal half of the protein sequence had been compared to GenBank sequences independently. It was also not similar in sequence to any previously published sequences. No other conserved redox-center binding domain could be identified within the complete protein sequence. In addition, hydropathy analysis did not identify any apparent transmembrane domain within the protein sequence. A 335-bp DNA sequence with significant sequence similarity to published ferric uptake regulator (FUR) protein sequences in GenBank was identified about 275 bp 3′ to the inducible cytochrome gene (data not shown). This suggests the presence of another ORF encoding a FUR-like protein directly downstream of the inducible cytochrome gene.

Discussion

We purified a membrane cytochrome from *D. tiedjei*, which was co-induced with reductive dechlorination activity. The protein should be a peripheral membrane protein, as it was washed off the membrane by high salt buffer, and no transmembrane domain was identified within the protein sequence. From the encoded protein sequence, two *c*-type heme-binding domains were identified in the COOH-terminal half of the protein, suggesting this is a diheme cytochrome *c*. The molecular mass of the protein was about 50 kDa, according to the encoded protein sequence and SDS-PAGE. The native molecular mass determined by gel-permeation chromatography was 155 kDa, suggesting the native inducible cytochrome was a homotrimer. However, we believe this is probably an artifact, depending on the chromatographic and electrophoretic conditions. Most of the purified proteins aggregated and stayed in the 5% stacking gel of SDS-PAGE, when the proteins were heated at 50° C for 15 min (heme-staining conditions), indicating the apparent molecular mass was greater than 200 kDa. However, all of the proteins converted to monomeric form after being heated at 95° C for 5 min.

This inducible cytochrome displayed high-spin UV-visible absorption spectra, similar to those of cytochromes *c*′ (Bartsch and Kamen 1960; Imai et al. 1969; Saraiva et al. 1995; Yamanaka and Imai 1972). However, the *D. tiedjei* inducible cytochrome differs from cytochromes *c*′ in molecular weight and heme content (Moore and Pettigrew 1990), and spectral changes at different pHs (Imai et al. 1969; Monkara et al. 1992). Amino acid residues conserved in cytochromes *c*′ are also absent in *D. tiedjei* inducible cytochrome (Moore and Pettigrew 1990). The pH titration experiment showed that both hemes were high spin at neutral pH and then switched to low spin at alkaline pH, suggesting the two hemes might have similar spectroscopic properties. However, when we aligned amino acid sequences around the two heme-binding domains of the *D. tiedjei* inducible cytochrome with each other, we did not observe any significant sequence similarity, indicating the two heme-binding domains did not likely arise from gene duplication or gene fusion, as in some diheme cytochromes (Ambler et al. 1984). All of the data above suggest that the inducible cytochrome of *D. tiedjei* is a novel high-spin cytochrome *c*.

The midpoint redox potential of the inducible cytochrome at pH 7.0 was calculated to be –342 mV. The results fitted very well to a theoretical Nernstian curve for a two-electron transfer reaction ($n = 2$). Despite the fact that the inducible cytochrome is a diheme protein, a theoretical Nernstian curve for a one-electron transfer reaction ($n = 1$) is expected, if the two heme centers are identical and independent. It has been demonstrated with a polymer of vinylhydroquinone that the redox titration of a macromolecule containing many independent redox centers of the same midpoint potential results in an n value identical to that obtained for a single redox center (Cassidy 1949). On the other hand, the Nernstian curve should resolve into

two one-electron transfer reactions if the two heme centers have different midpoint potentials. One possible explanation for the discrepancy between our result and the expected result is that the two heme centers are not independent and there are interactions between them. Leitch et al. (1985) pointed out that positive cooperativity between two heme centers of a diheme cytochrome could result in a Nernstian curve with an *n* value greater than 1. Moreover, both positive and negative cooperativity were demonstrated between the four heme centers of cytochrome c_3 isolated from several species of *Desulfovibrio* (Benosman et al. 1989; Niki et al. 1984; Santos et al. 1984). An alternative explanation is that the two heme centers vary in contributions to spectral changes when the inducible cytochrome is reduced. Under simulated conditions, the slope of a Nernstian plot of two independent redox centers with different midpoint potential would vary, depending on the contribution of each redox center to the total change in absorbance (Kristensen et al. 1991).

Whether or not there are interactions between the two heme centers, our results indicate that the *D. tiedjei* inducible cytochrome has a very low midpoint potential, compared to an E_0' of +297 mV for the 3-chlorobenzoate/benzoate half reaction (Dolfing and Harrison 1992). The low redox potential suggests that this inducible cytochrome is not likely to be the direct electron donor for the reductive dehalogenase in vivo. However, its co-induction with reductive dechlorination suggests it does function in the chlororespiration process. If the reduced inducible cytochrome can replace reduced methyl viologen as the reductant of the in vitro dehalogenase assay (DeWeerd and Suflita 1990; Ni et al. 1995), it would indicate possible involvement of this inducible cytochrome in the electron transport chain coupled to the membrane-bound reductive dehalogenase.

In conclusion, we purified a high-spin cytochrome *c*, which is co-induced with reductive dechlorination, from *D. tiedjei* membrane fraction. This is a diheme cytochrome with a very negative midpoint potential of –342 mV. Spectral and sequence data suggest that this cytochrome *c* is substantially different from previously characterized ones. The gene sequence of this cytochrome is the first known gene sequence from *D. tiedjei*, except for that of the 16S rRNA gene.

Acknowledgements We thank Dr. Vincent Massey (Department of Biological Chemistry, University of Michigan, Ann Arbor) for providing us with 8-hydroxyriboflavin for the redox titration experiment, Meng Markillie for technical assistance in genomic DNA preparation, and Vincent Martin for critically reviewing this manuscript. We are also grateful to Grant Mauk, Dean Hildebrand, and Federico Rosell (Deptartment of Biochemistry, University of British Columbia) for helpful discussions and invaluable suggestions. This work was supported by the National Science and Engineering Research Council of Canada (NSERC), the Council of Forestry Industries of British Columbia and the Ministry of the Environment of British Columbia through an Industrial Research Chair in Forest Products Waste Management, and the Microbial Biotechnology Initiative at the Pacific Northwest National Laboratory. T. M. Louie was supported by an NSERC postgraduate scholarship.

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