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Quantitative Analysis of the Relative Transcript Levels of Four Chlorophenol Reductive Dehalogenase Genes in *Desulfitobacterium hafniense* PCP-1 Exposed to Chlorophenols[▽]

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Relative to those of unexposed cultures, the transcript levels of the four CprA-type reductive dehalogenase genes (*cprA2*, *cprA3*, *cprA4*, and *cprA5*) in *Desulfitobacterium hafniense* PCP-1 were measured in cultures exposed to chlorophenols. In 2,4,6-trichlorophenol-amended cultures, *cprA2* and *cprA3* were upregulated, as was *cprA5*, but concomitantly with the appearance of 2,4-dichlorophenol (DCP). In 3,5-DCP-amended cultures, only *cprA5* was upregulated. In pentachlorophenol-amended cultures grown for 12 h, *cprA2* and *cprA3* were upregulated but not *cprA5*. *cprA4* was not upregulated significantly in cultures containing any tested chlorophenols.

Desulfitobacterium hafniense PCP-1 is able to dehalogenate pentachlorophenol (PCP) and several chlorophenols (3) by hydrogenolytic reductive dehalogenation (5, 9, 15, 16, 19). This dehalogenation involves two different enzymatic systems: the *ortho*-dechlorinating system and the *meta*-/*para*-dechlorinating system (2). At least four open reading frames (*cprA2*, *cprA3*, *cprA4*, and *cprA5*) encoding CprA-type reductive dehalogenases (RDases) were identified in this strain (8). We previously showed that *cprA3* in strain PCP-1 encodes an RDase with *ortho*-dechlorinating activity toward highly chlorinated phenols such as PCP, tetrachlorophenols, and several trichlorophenols (TCPs) (1). We also determined that *cprA5* encodes an RDase with *meta*- and *para*-dechlorinating activities toward several TCPs and dichlorophenols (DCPs) (17). The functions of the putative RDases encoded by the *cprA2* and *cprA4* genes have yet to be determined. We hypothesized that 3,5-DCP (a *meta*-/*para*-dechlorinating activity inducer) and 2,4,6-TCP (an *ortho*-dechlorinating activity inducer) modulate differently the level of transcription of these genes in strain PCP-1. In this study, we examined by real-time reverse transcription quantitative PCR (RT-qPCR) changes in the transcript levels of *cprA2* to *cprA5* following the addition of inducer 2,4,6-TCP or 3,5-DCP to *D. hafniense* PCP-1 cultures and their response to PCP exposure.

Culture conditions. *D. hafniense* PCP-1 (ATCC 700357) was cultivated anaerobically in either 70-ml serum vials or 1-liter bottles containing, respectively, 35 or 500 ml of mineral salt medium with pyruvate and yeast extract as described previously (8). In all experiments, the medium was inoculated with 5% (vol/vol) of an exponentially growing culture. For 2,4,6-TCP and 3,5-DCP exposure experiments, strain PCP-1 was grown without chlorophenol for two successive transfers. The last culture was used to inoculate three cultures (500 ml) without chlorophenol. After an incubation at 30°C for 30 h (optical density at 600 nm [OD_{600}], 0.25 to 0.27), the first culture received 2,4,6-TCP at 50 μM (final concentration) and the second 3,5-DCP at 60 μM (final concentration). No chlorophenol was added to the third culture (unexposed culture). Aliquots of the cultures (20 ml) were transferred into 70-ml sterile serum bottles. After various incubation periods (0, 4, 8, 18, 28, and 44 h) at 30°C, a 2-ml sample was collected for measuring the OD_{600} and the chlorophenol concentrations. For the exposure experiments with different concentrations of 2,4,6-TCP or 3,5-DCP and with PCP, strain PCP-1 was grown without chlorophenol for three successive transfers. The last culture (500 ml) was incubated at 30°C for 30 h (OD_{600}, 0.25 to 0.27). A volume of 20 ml of the cultures was transferred into 70-ml sterile anaerobic serum bottles. PCP was then added at 30 μM, whereas 2,4,6-TCP was added at 0.125, 5, 25, and 50 μM and 3,5-DCP at 0.15, 3, 6, 30, and 60 μM (final concentration). The cultures were incubated at 30°C for 12 h. For the temperature experiment, the bottles were incubated for 3 h at 22, 30, or 37°C prior to addition of 3,5-DCP at 60 μM. The bottles were further incubated for 12 h at these temperatures. For each type of experiment, an additional bottle was treated similarly but without chlorophenol and served as an unexposed culture.

For analysis of chlorophenols, 0.5 ml of acetonitrile containing 0.33% (vol/vol) acetic acid was added to a 1-ml culture sample. The mixture was centrifuged for 5 min at 5,000 × *g*, and the supernatant was collected for high-pressure liquid chromatography (HPLC) analysis as described previously (10).

RNA extraction and RT-qPCR. Cells were collected from a 12-ml culture sample by centrifugation at 6,000 × *g* for 4 min at 4°C and dispersed in 450 μl of ice-cold RNAwiz reagent (Ambion, Austin, TX). Total RNA was extracted using a RiboPure Bacteria kit (Ambion) according to the manufacturer's recommendations. Contaminant DNA was removed by two successive treatments with a DNA-free kit (Ambion). Primers (Table 1) were designed using the *D. hafniense* DCB-2 genomic sequence currently deposited in the NCBI GenBank database (www.ncbi.nlm.nih.gov; accession number NC_011830)

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TABLE 1. Primer sequences used in this study

Gene target	Primer name[a]	Sequence (5′–3′)
cprA2	cprA2-f	AAGGCCAGTGGTCCAAGTTAAAGC
	cprA2-r	AAATCCGCCGTCGTCTTCATTGGT
cprA3	cprA3-f	AAGAAATGCGCCGAATTCCGTCTG
	cprA3-r	ATCCAAATGCCGGCTGAATGGAAC
cprA4	cprA4-f	TGACCAATGGCTTGGTACCTCGAA
	cprA4-r	TAGCCATTCCCATGGCATCACTCA
cprA5	cprA5-f	TTAAAGCATCTCTGGTGAGCGTCG
	cprA5-r	ACTTGGTAAGGAAATTCCGCGGTC
rpoB	rpob-f	TCCTGAAGGTCCCAACATCGGTTT
	rpob-r	ATTTCTCTTCTTCGTCGGCGGTCA

[a] f, forward primer; r, reverse primer.

to target the genes *cprA2*, *cprA3*, *cprA4*, and *cprA5* and the gene carrying the RNA polymerase beta subunit *rpoB* (locus tags, Dhaf_0711, Dhaf_0713, Dhaf_0689, Dhaf_0696, and Dhaf_0414, respectively). Each of the primer sets was tested for specificity and efficiency of amplification by qPCR with serial dilutions of strain PCP-1 total DNA as the standard. The PCR efficiency (E) for each primer pair was calculated from the slope of the standard curve according to the following formula: $E = (10^{-1/\text{slope}}) - 1$. The RT-qPCR analyses were performed using an iScript One-Step RT-PCR kit with SYBR green (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) according to the manufacturer's instructions, with 50 ng total RNA or diethylpyrocarbonate-treated water (negative control) and 10 nM each primer (forward and reverse) in a 25-μl volume. Thermocycling conditions for reverse transcription and PCR amplification were as follows: 10 min at 50°C and 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 55°C. Transcript levels of the *cprA* genes were calculated by relative quantification using the ΔΔCT method described by Livak and Schmittgen (12), with *rpoB* as the normalizing gene (4, 13). Cultures exposed to chlorophenols were compared to unexposed cultures. Threshold cycle (C_T) expression data were reported as $2^{-(\Delta\Delta CT)}$. An induction factor higher than 10 with respect to the unexposed cultures was arbitrarily set as representing significant induction. Variations in *rpoB* C_T were minimal (1 C_T or less) whatever the PCP-1 culture conditions.

Exposure to 2,4,6-TCP, 3,5-DCP, and PCP. Dechlorination of 2,4,6-TCP and 3,5-DCP occurred after an 8-h lag period in both cases. 2,4,6-TCP was almost entirely converted into 2,4-DCP after 18 h and into 4-chlorophenol (4-CP) after 44 h (Fig. 1A). 3,5-DCP was almost entirely converted into 3-CP after 28 h (Fig. 2A). Relative to the results seen with unexposed cultures, *cprA3* transcription was upregulated 400-fold within 4 h in 2,4,6-TCP-amended cultures (Fig. 1B). Upregulation reached its highest (18,000-fold) level within 18 h and then decreased. Similarly, *cprA2* transcription was upregulated (40-fold) within 4 h; upregulation reached a maximum (160-fold) within 18 h and then decreased. *cprA5* transcription was upregulated 60-fold after 18 h, and upregulation reached 1,200-fold after 44 h (Fig. 1B). Exposure to 3,5-DCP did not significantly affect the transcript levels of *cprA2* and *cprA3* (Fig. 3B). *cprA5* transcription upregulation reached 100-fold within 4 h and reached its highest level (1,200-fold) within 18 h (Fig. 2B). No significant increase in *cprA4* transcript levels was observed after exposure to either 2,4,6-TCP or 3,5-DCP.

Concentrations of naturally occurring chlorophenols in environment are minute (at approximately picomolar levels) (14) but can reach millimolar levels in contaminated soil (11). To see the effect of the chlorophenol concentrations on the transcript levels of *cprA3* and *cprA5*, cultures were exposed for 12 h to 2,4,6-TCP at concentrations ranging from 0.125 to 50 μM and to 3,5-DCP at concentrations ranging from 0.15 to 60 μM. The *cprA3* transcript level increased by 1,000-fold at the lowest concentration of 2,4,6-TCP (0.125 μM) compared to unexposed culture results and reached a maximum (4,000-fold increase) at 25 μM (Fig. 3A). The *cprA5* transcript level was upregulated more than 200-fold in the presence of 3,5-DCP at 0.15 μM. The induction leveled off at 6 to 60 μM with a 900-fold increase (Fig. 3B). These results suggest that dehalogenating activities in strain PCP-1 would be induced in soil or

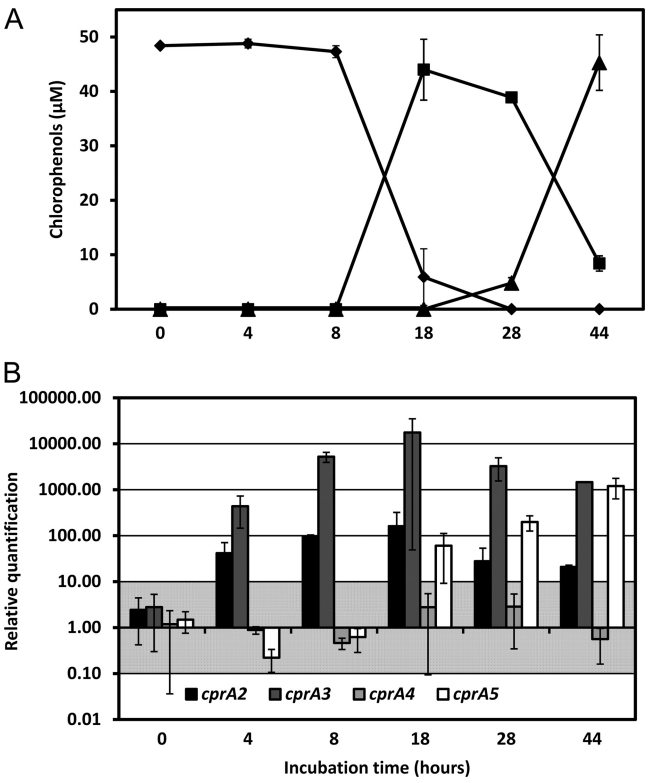

FIG. 1. Kinetics of dehalogenation of 2,4,6-TCP and relative quantification of transcripts of *cprA2*, *cprA3*, *cprA4*, and *cprA5* in 2,4,6-TCP-amended PCP-1 cultures. (A) 2,4,6-TCP (50 μM) was added to exponential cultures at 30°C. The cultures were then sampled at different times in hours (*x* axis) to measure the concentrations of chlorophenols and to extract total RNA. Diamonds, 2,4,6-TCP; squares, 2,4-DCP; triangles, 4-CP. (B) RT-qPCR were performed for each *cprA* gene. The relative quantification data represent ratios of $2^{-(\Delta\Delta CT)}$ values between the chlorophenol-exposed cultures and the unexposed cultures. Relative values that fall between 0.1 and 10 (the gray zone) were not considered to represent significant differences in the results obtained for the unexposed cultures, taking the standard deviation into account. Results represent data from duplicated separate experiments.

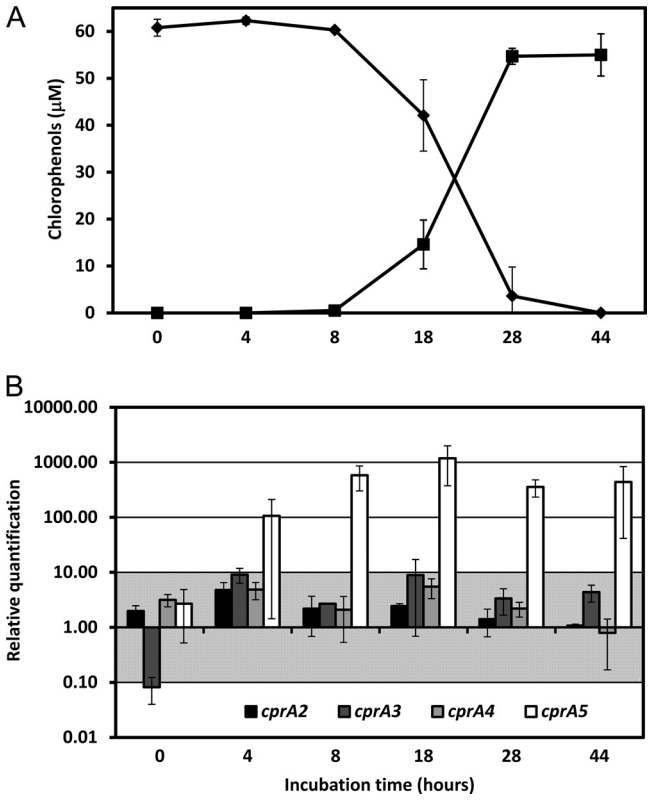

FIG. 2. Kinetics of dehalogenation of 3,5-DCP and relative quantification of transcripts of *cprA2*, *cprA3*, *cprA4*, and *cprA5* in 3,5-DCP-amended PCP-1 cultures. 3,5-DCP (60 μM) was added to exponential cultures at 30°C. See Fig. 1 legend for details. Diamonds, 3,5-DCP; squares, 3-CP.

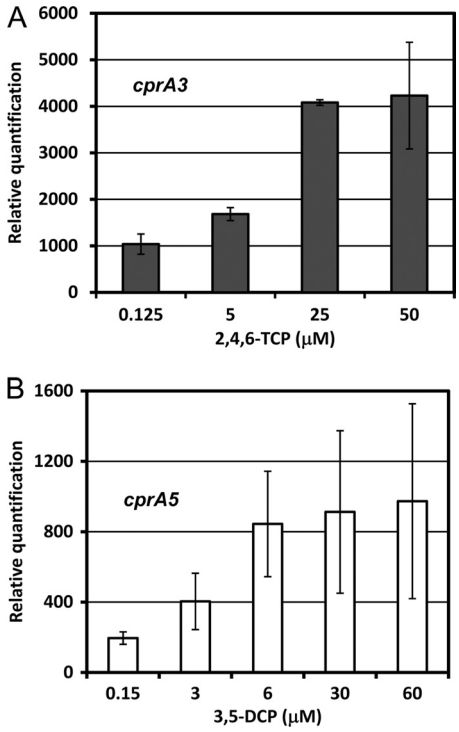

FIG. 3. Effect of the concentrations of 2,4,6-TCP and 3,5-DCP on *cprA3* and *cprA5* transcript levels. Different concentrations of 2,4,6-TCP (A) or 3,5-DCP (B) were added to exponentially growing cultures that were incubated at 30°C for 12 h. Total RNA was extracted from cultures, and RT-qPCR analyses were performed. Results represent data from duplicated separate experiments.

water environments upon an addition of a small amount of chlorophenols.

Exponentially growing cultures were incubated with PCP for 12 h. Bouchard et al. (2) showed that PCP was completely transformed in 3,4,5-TCP in less than 12 h, followed by a 36-hour lag period before 3,4,5-TCP dehalogenation began to occur. The transcript levels of *cprA2* and *cprA3* increased by 700-fold and 33,500-fold, respectively. *cprA4* transcription was upregulated by about 13-fold, while the *cprA5* transcript level was not significantly affected (data not shown).

Effect of temperature. Thibodeau et al. (17) showed that the growth temperature can affect the production of CprA5 in strain PCP-1. Although the optimal growth temperature for strain PCP-1 is 38°C, the CprA5-specific activity was 2.2- and 9.6-fold higher in strain PCP-1 cultured at 22°C than at 30°C and at 37°C, respectively (17). To determine the effect of the temperature on the *cprA5* transcript level, exponentially growing cultures were placed at 22, 30, and 37°C and exposed to 3,5-DCP for 12 h. The *cprA5* transcript levels were similar at 30°C and 37°C but were 50-fold higher than those seen at 22°C (data not shown). This suggests a posttranscriptional regulation of CprA5 expression (such as transcript stability) at 22°C. As observed at 30°C, no significant upregulation of *cprA2*, *cprA3*, and *cprA4* transcript levels was observed at 22°C or 37°C after exposure to 3,5-DCP.

***cprA2* and *cprA3*.** *cprA2* and *cprA3* transcript levels were upregulated in 2,4,6-TCP- and PCP-amended cultures but not in 3,5-DCP-amended cultures. In 2,4,6-TCP-amended cultures, the two genes showed similar patterns of expression over time. Upregulation was clearly apparent in both cases 8 h after addition of 2,4,6-TCP. However, 2,4,6-TCP dechlorination was observed only after this period. Similarly, addition of 3-chloro-4-hydroxyphenylacetate (3Cl4OHPA) to *D. dehalogenans* cultures increased the expression of *cprA*, a gene encoding the 3Cl4OHPA RDase, by 15-fold after 30 min, but significant dechlorination was observed only after 2 h (16). These results suggest that a lag period could be needed for *de novo* protein synthesis, which is consistent with a lack of dechlorinating activity in cultures supplemented with chloramphenicol (3, 18). Furthermore, other factors may have to be produced before dechlorination can occur. Such factors could include components of the organohalide respiration chain and proteins involved in the maturation and translocation of the enzyme. CprA proteins contain cobalamin and iron-sulfur cofactors, as well as a twin-arginine signal sequence that may be involved in targeting the enzyme to the TAT secretion pathway for translocation across the cytoplasmic membrane in a folded conformation (9, 15, 19).

Our results showed that more than one chlorophenol can upregulate *cprA2* and *cprA3*. It has previously been shown that, in *D. hafniense* strain DCB-2, 3Cl4OHPA can induce expression of *cprA1*, *cprA2*, and *cprA3* and that several haloge-

nated phenolic compounds such as 2,4-DCP, 2Br-4-CP, and 3Cl4OHPA can bind to *D. hafniense* DCB-2 CprK1, a CRP-FNR-type transcriptional regulator involved in transcriptional activation of *cprA1* in strain DCB-2 (6, 7). Broad CprK substrate-binding specificity could explain the finding that several chlorophenols (PCP, 2,4,6-TCP, 2,3,4-TCP, 2,3,5-TCP, 2,6-DCP, and 2,4-DCP) can act as inducers of *ortho*-dechlorinating activity in strain PCP-1 (3).

Based on the fact that *cprA2* and *cprA3* are upregulated similarly and that their deduced amino acid sequences are highly similar (69%), this suggests that the *cprA2* gene product is involved in *ortho*-dechlorination of highly chlorinated phenols.

***cprA5*.** 3,5-DCP upregulated only *cprA5* expression in PCP-1 cultures. The kinetics of induction of 3,5-DCP dechlorination was similar to that observed with 2,4,6-TCP. Upregulation (580-fold) of *cprA5* expression was clearly apparent at 8 h after addition of 3,5-DCP, but 3,5-DCP dechlorination was observed only after this period. In the 2,4,6-TCP-amended cultures, *cprA5* upregulation occurred concomitantly with the appearance of 2,4-DCP, suggesting that this molecule can induce *cprA5* expression. The levels of upregulation induced by 2,4-DCP (1,200-fold increase in transcription) were similar to the levels measured in the 3,5-DCP-amended cultures.

***cprA4*.** *cprA4* transcription was not upregulated in the presence of 2,4,6-TCP or 3,5-DCP and was barely (13-fold) upregulated above the arbitrary limit threshold in the presence of PCP. Phylogenic analyses showed that deduced amino acid sequences of *cprA2*, *cprA3*, and *cprA4* grouped together (69 to 76% similarity) (19), suggesting related activities, such as the dehalogenation of highly chlorinated compounds, among these 3 Rdases, although further experiments are needed to confirm this.

In conclusion, the findings with respect to transcriptional upregulation of *cprA3* by 2,4,6-TCP and PCP and of *cprA5* by 3,5-DCP concur with the CprA3- and CpA5-specific activities toward different chlorophenols, where CprA3 preferentially dechlorinates highly chlorinated phenols and CprA5 preferentially dechlorinates DCPs. *D. hafniense* strain PCP-1 was isolated from a PCP-degrading microbial consortium (2). The presence of several genes involved in dehalogenation of highly chlorinated phenols and their induction by several chlorophenols would allow strain PCP-1 (and the consortium) to response efficiently to the presence of these toxic compounds by producing versatile enzymes to carry the dehalogenation reactions. The dehalogenation products (DCPs) could in turn induce expression of *cprA5* for the dehalogenation of the lesser chlorinated phenol to monochlorophenols.

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