The pentachlorophenol-dehalogenating
*Desulfitobacterium hafniense* strain PCP-1

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In this report, a complete description of *Desulfitobacterium hafniense* strain PCP-1 is presented. The *D. hafniense* strain PCP-1 was isolated from a methanogenic consortium for its capacity to dehalogenate pentachlorophenol (PCP) into 3-chlorophenol. This strain is also capable of dehalogenating several other chloroaromatic compounds and tetrachloroethene into trichloroethene. Four gene loci encoding putative chlorophenol-reductive dehalogenases (CprA2 to CprA5) were detected, and the products of two of these loci have been demonstrated to dechlorinate different chlorinated phenols. Strain PCP-1 was used in laboratory-scale bioprocesses to degrade PCP present in contaminated environments. *Desulfitobacterium hafniense* strain PCP-1 is an excellent candidate for the development of efficient bioprocesses to degrade organohalide compounds.

1. Pentachlorophenol

Pentachlorophenol (PCP) is a molecule that was invented in the 1930s and has been used as a very effective biocide [1]. Its widespread use, mostly as a wood-preserving agent, has led to contamination of the environment. For instance, in many wood-preserving facilities, the soil was heavily contaminated with PCP and other chlorophenols (CPs), impacting surrounding ground waters and lake waters [2]. PCP and other CPs are very toxic molecules, as they uncouple oxidative phosphorylation and make cell membranes permeable to protons, resulting in dissipation of the transmembrane pH gradients and electrical potentials [2,3]. These molecules are also suspected to be carcinogens, teratogens and embryotoxins [1]. PCP and other CPs have been listed as priority pollutants by the US Environmental Protection Agency [4], and many developed countries have banned the usage of PCP or severely restricted its use [5]. However, the disposal of PCP-treated wood and PCP-contaminated soils are still a threat to the environment and to human health.

PCP biodegradation occurs via hydroxylation, oxygenolysis and reductive dehalogenation [2]. Microbial anaerobic dehalogenation of organic halogenated molecules generally occurs by reductive dehalogenation, which results in the replacement of halogen by hydrogen. This process generates energy for the micro-organisms, and the halogenated compounds serve as electron donors [6,7]. Anaerobic bacterial species have been isolated for their capacity for reductive dehalogenation by halorespiration; several members of the genus *Desulfitobacterium* are among these species.

2. *Desulfitobacterium*

*Desulfitobacterium* spp. are strictly anaerobic bacteria that belong to the Firmicutes, Clostridia, Clostridiales and Peptococcaceae (see review by Villemur et al. [8]). The first members of the genus were isolated from soil or sediment waste-water sludge following enrichment on chlorinated phenolic compounds, chlorinated alkanes or alkenes (figure 1). Later, these strains were found in very diverse environments ([30], and references in figure 1). Most of the *Desulfitobacterium* strains can dehalogenate halogenated organic compounds, but very few of these strains were studied specifically for PCP degradation, which is most probably owing to the difficulty of growing these bacteria on such a toxic compound.
Although some Desulfitobacterium strains can dehalogenate PCP, this molecule can have a toxic effect on these strains, which reduces the biomass yield that is needed for the purification of enzymes involved in reductive dehalogenation.

(a) Desulfitobacterium hafniense strain PCP-1

Desulfitobacterium hafniense strain PCP-1 was isolated from an anaerobic consortium degrading PCP by methanogenic fermentation [31]. This consortium came from an initial inoculum that consisted of activated anaerobic sewage sludge and soil, which was historically contaminated with PCP. It was enriched after several culture passages with medium containing PCP and glucose and/or sodium formate as carbon sources. This consortium was established for the development of a continuous-flow fixed-film reactor for the degradation of PCP-contaminated soils or lixiviates. This laboratory-scale reactor was capable of complete dehalogenation of PCP and removal of phenol from a PCP liquor, which was extracted from wood chip powder contaminated with PCP [32].

(b) The relationship of strain PCP-1 to other desulfitobacteria

Upon heat treatment, it was obvious that the dehalogenation of PCP to 3-CP was carried out by a spore-forming bacterium in the consortium [33], which was later isolated and identified as D. frappieri strain PCP-1 [10]. It was later renamed D. hafniense strain PCP-1 as DNA–DNA reassociation assays between strain PCP-1 and D. hafniense strain DCB-2, the type strain, revealed 88 per cent identity [11]. As illustrated in figure 1, 10 strains belonging to the Desulfitobacterium spp. Sequences of 16S rRNA genes available from Desulfitobacterium species and strains were aligned by the ClustalW v. 2 program. The 5' end of the gene that includes the 100–200 nt insertion was excluded. GenBank accession numbers are indicated in parentheses. The origin of the strains is indicated with the type of enrichment. Evolutionary analyses were conducted in MEGA5 [9]. The neighbour-joining trees were inferred from a matrix of pairwise distances using 1266 aligned positions and a bootstrap analysis of 1000 replicates. The numbers at the forks indicate the percentage of species or a group of species originated at each fork. Percentages below 50% are not shown.

Figure 1. Phylogenetic tree of Desulfitobacterium spp. Sequences of 16S rRNA genes available from Desulfitobacterium species and strains were aligned by the ClustalW v. 2 program. The 5’ end of the gene that includes the 100–200 nt insertion was excluded. GenBank accession numbers are indicated in parentheses. The origin of the strains is indicated with the type of enrichment. Evolutionary analyses were conducted in MEGA5 [9]. The neighbour-joining trees were inferred from a matrix of pairwise distances using 1266 aligned positions and a bootstrap analysis of 1000 replicates. The numbers at the forks indicate the percentage of species or a group of species originated at each fork. Percentages below 50% are not shown. Desulfosporosinus orientis was used as the outspecies. Bar: 0.01 nucleotide substitutions per site. References: [10 – 29].

3. The dechlorinating ability of strain PCP-1

Desulfitobacterium hafniense strain PCP-1 can dehalogenate PCP and other CPs at the ortho, para and meta positions when grown with pyruvate and yeast extract [10]. However, this strain cannot dehalogenate monochlorophenols (MCPs). The kinetics of dechlorination of PCP by strain PCP-1 revealed that PCP...
is rapidly dechlorinated into 3,4,5-trichlorophenol (TCP), followed by a lag period before this compound is dechlorinated into 3,5-dichlorophenol (DCP) and 3-CP. These results suggested that two different enzymatic systems are involved in TCP dechlorination. The first system dechlorinates TCP rapidly at the ortho positions to generate 3,4,5-TCP, and the second system dechlorinates 3,4,5-TCP at the meta and para positions to generate 3-CP. PCP, 2,4,6-TCP, 2,3,4-TCP, 2,3,5 TCP, 2,6-DCP and 2,4-DCP were found to be inducers of the ortho-dechlorinating activity, and 3,4,5-TCP and 3,5-DCP are inducers of the meta- and para-dechlorinating activities.

Desulfitobacterium hafniense strain PCP-1, with D. dehalogenans, was the most studied for the substrate spectrum of dehalogenation [8,35]. Figure 2 summarizes the different forms of halogenated-aromatic compounds that can be dehalogenated. Other than chlorinated phenols, strain PCP-1 can dehalogenate 2,4,6-tribromophenol into 4-bromophenol without the presence of inducers. Complete dehalogenation of tetrachlorocatechol can occur in the presence of dehalogenating activity inducers. O-demethylation of tetrachloroguaiacol, tetrachloroveratrole and pentachloroanisole was observed after a first step of dechlorination, with further dehalogenation after the demethylation. Strain PCP-1 dechlorinates 3,5-dichloro-4-hydroxybiphenyl into 3-chloro-4-hydroxybiphenyl. The replacement of the hydroxyl function by an amino group does not interfere with the dechlorination, as observed with dechlorination of pentachloroaniline and 2,3,5,6-tetrachloroaniline into different tri- and dichloroanilines. With pentachloronitrobenzene, strain PCP-1 reduces the nitro group into an amino group, resulting in pentachloroaniline, which is then dechlorinated. Even when the phenolic structure is replaced by a pyridine structure, dechlorination can occur as demonstrated with the dehalogenation of pentachloropyridine into trichloropyridine. Tetrachloroethylene (PCE) can be dechlorinated into trichloroethene (TCE) but not further. In contrast to strain DCB-2, 3-chloro-4-hydroxyphenyl acetate (3Cl4OHPA) is not dehalogenated. However, this compound is deacetylated into 2-CP.

Strain PCP-1 can also dehalogenate 2,3,5,6-tetrachloro-4-methoxyphenol and tetrachlorohydroquinone [36].

4. Reductive dehalogenases in strain PCP-1

Reductive dehalogenation of chloroaromatic and chloroalkyl compounds is usually carried out by reductive dehalogenases (Rdases). Among these Rdases, chloroaromatic Rdases (CprA) or PCE/TCE Rdases (PceA/TceA) are the most studied [8]. Rdase genes are associated with gene clusters that encode several accessory proteins (e.g. CprA-anchor protein [37], chaperones, regulators) [8]. In the currently available complete genome sequence of D. hafniense strain DCB-2, seven Rdase gene loci have been identified [38,39]. Two of them appear to be non-functional: one disabled by an insertion of a transposable element and the other by a nonsense mutation. PCR primers were designed from the sequences of the five complete Rdase genes in strain DCB-2, which allowed the detection of four of these genes in D. hafniense strain PCP-1 [40]. Only the gene that encodes for the 3OH4ClPA Rdase was not detected. This result is consistent with the inability of strain PCP-1 to dehalogenate this compound. I named this Rdase CprA1 because it was closely related to the first CprA characterized from D. dehalogenans [41] (figure 3) and the four other Rdases, CprA2 to CprA5.

Among the putative Rdases encoded by the four gene loci present in D. hafniense strain PCP-1, we characterized the Rdase activities of CprA3 and CprA5 [43,44]. In D. hafniense strain DCB-2, these two CprAs correspond to the rdhA5 and rdhA3 gene products, respectively [38]. The nucleic acid sequences of cprA3 and cprA5 are essentially identical to the corresponding genes in strain DCB-2, with one nucleotide substitution between cprA5 and rdhA3. We can therefore assume similar dechlorinating properties of these two gene products in the D. hafniense PCP-1 and DCB-2 strains.

CprA3 is involved in the dechlorination of highly chlorinated phenols (table 1) [43,44]. This enzyme was produced upon the inclusion of strain PCP-1 in cultures of inducers of

Figure 2. Halogenated-aromatic compounds dehalogenated by strain PCP-1. Chlorinated aromatic compounds where deacetylation and O-demethylation activities were observed by strain PCP-1 are illustrated.

dehalogenation at ortho, para and meta positions

halogenated phenols, chlorocatecols, chloropyridines, 3,5-dichloro-4-hydroxybiphenyl

deacetylation O-demethylation

3-chloro-4-hydroxyphenyl acetate 3-Cl-4-hydroxyphenylacetate chlorinated guaiacol, veratrole and anisole

tetrachlorohydroquinone, 2,3,5,6-tetrachloro-4-methoxyphenol

OH, NO₂, NH₂ OH OH Cl Cl Cl Cl Cl Cl Cl OH, O-CH₃ OH Cl Cl OH CH₂COO⁻
Ortho-dechlorinating activity. The highest activity levels were observed with PCP, 2,3,4,5-tetrachlorophenol (TeCP) and 2,3,4-TCP. No activity was observed towards any DCPs and MCPs. CprA5 catalyses the dechlorination of several CPs at the meta and para positions (table 1) [44]. It is only produced upon the inclusion of D. hafniense strain PCP-1 in cultures of inducers of the meta- and para-dechlorinating activities. The highest activity was observed at the meta position with 3,5-DCP and 2,3,5-TCP. Dechlorination at the para position was also detected with PCP, 2,3,4,5-TeCP and 3,4,5-TCP. Dechlorination at the ortho position was observed only with 2,4,6-TCP, 2,4,5-TCP and 2,4-DCP.

Interestingly, CprA5 shows 66 per cent identity (76–77% similarity) with PceA from D. hafniense strain Y51, Desulfotobacterium sp. strain PCE-S and Dehalobacter restrictus. CprA5 shares less than 40 per cent similarity with the other CprAs (figure 3). However, these other CprAs were all derived from Desulfotobacterium strains cultured in the presence of 3Cl4OHphenylacetate Rdases (ortho), PCP Rdases (ortho) or 2,4,6-TCP, which are all inducers of the ortho-dechlorinating activity [8,44]. The nature of the meta and para positions of the chlorine residues relative to the hydroxyl residues suggests that the catalytic site of CprA5 requires a structure more similar to the substrate of PceA than of CprA.

Evidence of dehalogenation activity by the putative CprA2 and CprA4 Rdases has not yet been demonstrated. Phylogenetic analysis has grouped CprA2 and CprA4 with the ortho-dechlorinating CprA (figure 3), suggesting similar activities for these two putative CprAs.

5. Transcriptional regulation of crpA genes in strain PCP-1

Transcripts of cprA2, cprA3 and cprA4 were detected in strain PCP-1 cultured in the absence of CPs, suggesting basal expression of these genes [40]. Quantitative assays by reverse transcriptional analysis and primer extension showed that transcript levels of cprA2, cprA3 and cprA4 were low in the absence of CPs but increased in the presence of PCP. This suggests that the transcriptional regulation of cprA genes is under the control of the inducer PCP.
transcription–quantitative PCR (RT-qPCR) with strain PCP-1 in cultures amended with 2,4,6-TCP, which induced the ortho-dechlorinating activity, showed that cprA2 and cprA3 were strongly upregulated [50]. This strong upregulation still occurred in cultures containing as little as 0.125 mM 2,4,6-TCP. Furthermore, 700-fold and 33 500-fold increases in the cprA2 and cprA3 transcript levels were observed, respectively, in PCP-amended cultures (figure 4).

Transcripts of cprA5 were not detected from strain PCP-1 cultured in the absence of CPs, but they were detected in the presence of 2,4,6-TCP or 3,5-DCP (an inducer of the meta- and para-dechlorinating activities) [40]. RT-qPCR revealed that cprA5 expression was strongly upregulated in the presence of 3,5-DCP in strain PCP-1 cultures [50]. For 2,4,6-TCP-amended cultures, the occurrence of 2,4-DCP was associated with the upregulation of cprA5 in strain PCP-1 cultures. Kim et al. [38] also observed this upregulation with the D. hafniense strain DCB-2 for rdhA3 (corresponding to cprA5) by microarrays. Upregulation of cprA5 occurred in cultures containing as little as 0.15 mM 3,5-DCP [50]. No upregulation was observed in PCP-amended cultures after 12 h (figure 4).

Significant upregulation of cprA4 was not observed in strain PCP-1 cultures amended with 2,4,6-TCP or 3,5-DCP. However, in PCP-amended cultures, we noticed a 13-fold increase in cprA4 transcript levels, which was just above the level of significance (arbitrarily, set at 10) (figure 4).

Kim et al. [38] observed similar results with the ortho-dechlorinating inducer 3Cl4OHPA for the corresponding cprA2 and cprA3 genes (rdhA4 and rdhA5) in D. hafniense strain DCB-2. The nature of this shared upregulation can be attributed to the fact that cprBA2 and cprBA3 are positioned head to tail in the PCP-1 and DCB-2 genomes [38,39]. These results suggest that cprA2, as cprA3, is involved in the dehalogenation of highly chlorinated phenolic compounds. Phylogenetic analysis (figure 3) appears to confirm this hypothesis, as CprA2 (but also CprA4) groups more closely with CprA3 than with the other ortho CprAs. The basal

Table 1. Properties of chlorophenol-reductive dehalogenases. nd, not determined; o, p, m, dehalogenation at the ortho, para or meta position, respectively; PCP, pentachlorophenol; TeCP, tetrachlorophenol; TCP, trichlorophenol; DCP, dichlorophenol; MCP, monochlorophenol; CP, chlorophenol; PCE, perchloroethene; TCE, trichloroethene; 3Cl4OHPA, 3-chloro-4-hydroxyphenylacetate; 3,5Cl4OHBA, 3,5-dichloro-4-hydroxybenzoate; MCBAs, monochloro-benzoates; Br, bromide; F, fluoride.

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<th></th>
<th>MW kDa</th>
<th>pH optimum</th>
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<td>CrdA</td>
<td>37</td>
<td>7.0</td>
<td>nd</td>
<td>α: PCP, TCps, 2,3-DCP, 2,4-DCP, p: 3,4,5-DCP, PCE to TCE</td>
<td>other DCPs, MCPs,</td>
<td>[45]</td>
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<td>CprA5</td>
<td>57</td>
<td>6.8–7.0</td>
<td>50</td>
<td>α: 3,5-DCP, p: PCP, 2,3,4,5-TeCP, 3,4,5-DCP, p: 2,4,6-DCP, 2,4,5-DCP.</td>
<td>other DCPs, MCPs,</td>
<td>[44]</td>
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<tr>
<td>CprA3</td>
<td>47</td>
<td>7.0</td>
<td>50–55</td>
<td>α: PCP, 2,3,4,5-TeCP, 2,3,4-TCP, 2,4,6-TCp, 2,3,6-TCp</td>
<td>3,4,5-TCp, 2,4,5-TCp, MCPs, DCPs, 3Cl4OHPA, 3,5Cl4OHBA</td>
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| **D. hafniense strain DCB-2** |        |            |               |                                   |                   |            |
| CprA1                | 47     | nd         | nd            | 3Cl4OHPA                          | PCE               | [46]       |
| **D. dehalogenans**  |        |            |               |                                   |                   |            |
| CprA                 | 48     | 8.1        | 52            | 3Cl4OHPA, 2CP, 2,3-DCP, PCP, 2,4-DCP, 2,6-DCP, 2Br4CP | PCE, TCE, 3CP, 4CP, 2,5DCP, 2F4CP | [41]       |
| **Desulfitoabacterium sp. PCE1** |        |            |               |                                   |                   |            |
| CprA                 | 48     | nd         | nd            | 3Cl4OHPA                          |                   | [47]       |
| **D. chlororespirans** |        |            |               |                                   |                   |            |
| CprA                 | 50     | 6.8        | 59            | PCP, TeCPs, TCps, DCPs, MCBAs, DCPs, MCPs, PCE | 3Cl4OHPA, 3,5Cl4OHBA | [48,49]    |

a Determined by SDS-PAGE.

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**Figure 4.** Effect of PCP on cprA2–5 expression. PCP (30 mM) was added to exponentially growing cultures that were incubated at 30°C for 12 h. Total RNA was extracted from the cultures, and RT-qPCR was performed. The results were obtained from duplicate separate experiments. Relative values that fall between 0.1 and 10 (grey zone) were not considered significantly different from the unexposed cultures, upon considering the s.d. See Bisaillon et al. [50] for details regarding the experimental procedure.
expression of 

expression of cprA2, cprA3 and cprA4 as well upregulation of their expression in the presence of CPs could allow strain PCP-1 to respond rapidly to the presence of highly toxic PCP in the medium by its dechlorination into TCP/DCP. When TCPs/DCPs appear, cprA5 expression is induced to transform these compounds into MCP.

It was shown that, in strain D. hafniense DCB-2, 3CH4OHPA can induce the expression of cprA1, cprA2 and cprA3 (rdhA6, rdhA4 and rdhA5 based on the Kim et al. [38] nomenclature). In addition, several halogenated phenolic compounds, such as 2,4-DCP, 2-bromo-4-CP and 3CH4OHPA, can bind to strain D. hafniense DCB-2 CprK1, a CPR/FNR-type transcriptional regulator involved in the transcriptional activation of cprA1 [51,52]. Broad CprK substrate-binding specificity could explain the finding that several CPs (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 the strain PCP-1 [35].

6. Occurrence of Rdase by horizontal gene transfer

As mentioned earlier, the genome of D. hafniense strain DCB-2 contained seven loci associated with Rdases [38]. Six of these are located in close proximity within the chromosome. Among these six Rdase loci, a CprA-like sequence is interrupted by a transposable element. None of the six loci associated with this region in strain DCB-2 was found in D. hafniense strain Y51. A transposable element also flanks the PCE gene locus (pceABCT) in strain Y51. This strain can lose its PCE-dechlorinating activity upon cultivation in the absence of cis-DCE or PCE but not in the presence of TCE. This activity correlated with the loss of the pceABCT gene locus [53,54]. Futagami et al. [54] observed circular forms containing transposable elements and the pceABCT locus in strain Y51, suggesting that this locus is part of a catabolic transposon. All these results suggest that the Rdase gene loci can be transmitted by horizontal gene transfer, and that selective pressure, which occurs after several passages of the original consortium in the presence of PCP, may have favoured the genetic acquisition of different Rdase gene loci by D. hafniense strain PCP-1. The nature of the hot spot or Rdase ‘island’ in strains DCB-2 and PCP-1 remains unknown.

7. The CrdA Rdase

Another Rdase, named CrdA, was purified from D. hafniense strain PCP-1 cultures induced for ortho-dechlorinating activities with 2,4,6- TCP (table 1) [45]. This Rdase, which is not a CprA-type Rdase, can dechlorinate PCP and 2,4,6-TCP at the ortho positions. There is evidence that CrdA contains a corrinoid cofactor. Furthermore, the deduced amino acid sequence of crdA revealed a LysM domain. This domain is a widespread protein module that is involved in binding peptidoglycan [55]. The crdA gene was detected in D. hafniense strains DCB-2, DP7, TCP-1 and TCE1; Desulfitobacterium sp. strain PCE-1; D. dehalogenans and D. chlororespirans; and in the genome of D. hafniense strain Y51. Transcripts of crdA were observed in strain PCP-1 in the presence of the absence of CPs. However, crdA transcripts were only detected in D. hafniense strains PCP-1, DCB-2 and TCE1 [40]. Such experiments have not been conducted with strain Y51. The mechanism of action of CrdA remains unknown.

8. Pilot-scale testing of PCP-1 for biodegradation

Strain PCP-1 was used in laboratory-scale bioprocesses to degrade PCP-contaminated environments. Inoculation of a PCP-contaminated soil slurry with strain PCP-1 accelerated the degradation of PCP, and this activity was observed at levels as high as 500 mg kg⁻¹ soil [56,57]. Strain PCP-1 was also inoculated in an anaerobic upflow sludge bed reactor for the degradation of PCP-contaminated effluents. The proliferation of strain PCP-1 allowed for an increase of the volumetric PCP load from 5 to 80 mg l⁻¹·day⁻¹ with a PCP removal efficiency of 99 per cent and a dechlorination efficiency of not less than 90 per cent [58]. Analysis by FISH with D. hafniense-specific probes showed colonization of the external layer of the granules by D. hafniense, suggesting that strain PCP-1 was present at the surface of the granules to perform dechlorination of PCP into less toxic CPs, such as 3-CP [59]. Finally, PCP-degrading, methanogenic fixed-film reactors derived from biomass inoculated with D. hafniense strain PCP-1 were monitored by FISH with D. hafniense-specific probes, and this analysis revealed that up to 19 per cent of the biofilm was composed of D. hafniense [60]. These results demonstrated that dehalogenating bacteria can be an important component of efficient bioprocesses for the degradation of organohalide compounds.

9. Outlook for pentachlorophenol biodegradation

Although the production of PCP has been banned and its use is restricted, PCP is persistent in contaminated environments, especially in soil. As soil is an anoxic environment, reductive dehalogenation mechanisms would favour in situ bioremediation either by biostimulation (inclusion of dehalogenating-activity inducers or specific nutrients) or by bioaugmentation with specialized bacterial strains, such as strain PCP-1. Furthermore, as the upregulation of Rdase genes still occurs at low concentrations of CPs (below μM), the presence of strain PCP-1 would promote the bioremediation process via the dehalogenation of most PCP in soil to 3-CP, a less toxic and more biodegradable substrate for other microorganisms.

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