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Nitrate reductase gene involvement in hexachlorobiphenyl dechlorination by *Phanerochaete chrysosporium*

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Abstract

Polychlorobiphenyl (PCB) degradation usually occurs through reductive dechlorination under anaerobic conditions and phenolic ring cleavage under aerobic conditions. In this paper, we provide evidence of nitrate reductase (NaR) mediated dechlorination of hexachlorobiphenyl (PCB-153) in *Phanerochaete chrysosporium* under non-ligninolytic condition and the gene involved. The NaR enzyme and its cofactor, molybdenum (Mo), were found to mediate reductive dechlorination of PCBs even in aerobic condition. Tungsten (W), a competitive inhibitor of this enzyme, was found to suppress this dechlorination. Chlorine release assay provided further evidence of this nitrate reductase mediated dechlorination. Commercially available pure NaR enzyme from *Aspergillus* was used to confirm these results. Through homology search using TBLASTN program, NaR gene was identified, primers were designed and the RT-PCR product was sequenced. The NaR gene was then annotated in the *P. chrysosporium* genome (GenBank accession no. AY700576). This is the first report regarding the presence of nitrate reductase gene in this fungus with the explanation why this fungus can dechlorinate PCBs even in aerobic condition. These fungal inoculums are used commercially as pellets in sawdust for enhanced bioremediation of PCBs at the risk of depleting soil nitrates. Hence, the addition of nitrates to the pellets will reduce this risk as well as enhance its activity.
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Keywords: Bioremediation; *P. chrysosporium*; PCB dechlorination; Nitrate reductase; NaR gene sequence

1. Introduction

Polychlorobiphenyls (PCBs) are a class of hazardous compounds which were widely used in industry. Though PCBs were banned from the USA in 1977, our biosphere contains approximately 750,000 tonnes of released PCBs (http://bio.nagaokaut.ac.jp) known to cause reproductive [1], neurological [2] and endocrinal defects [3]. PCBs also adversely affect fetal and infant developments [4] and are also carcinogenic [5,6]. Currently there are several physical and bioremediation technologies such as high temperature incineration [7], hazardous waste landfill, dredging [8], phytoremediation [9], bioaugmentation, biostimulation, etc. However, the physical techniques are extremely costly and generate toxic by-products (www.epa.gov). Bioremediation appears to be the most environmentally friendly solution. PCB degradation in microbes occurs through both reductive dechlorination under anaerobic conditions [10,11] and by breakdown of phenolic rings under aerobic conditions [12]. The importance of the white-rot fungus (*Phanerochaete chrysosporium* strain BKM-F-1767) lies in the presence of an extra-cellular ligninolytic system which is involved in the degradation of lignin and a wide variety of halogenated and non-halogenated organic compounds to CO_2 [13] under low nitrogen (ligninolytic) conditions.

New findings in our laboratory indicate that this fungus can also degrade toxic chemicals under nitrogen-rich (non-ligninolytic) conditions [14–16]. It was apparent that there must be another mechanism of PCB degradation by this fungus. Reductive dechlorination by *P. chrysosporium* has been shown to degrade 2,4,6-trichlorophenol [17] by unknown enzymes. It was also known that some microbes can utilize chlorophenols as electron acceptor with possible involvement of nitrate reductase (NaR) [18]. This lead to our hypothesis that reductive dechlorination could be mediated by the involvement of a nitrate reductase enzyme, which is usually responsible for converting nitrate to ammonia during the process of nitrogen

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assimilation. Certain bivalent ions such as molybdenum (Mo) are known cofactors of nitrate reductase activity, whereas tungsten (W) acts as an inhibitor for this enzyme [19]. This study presents first evidence that this fungus can degrade PCB aerobically under non-ligninolytic conditions with a substrate-level inducible nitrate reductase enzyme through involvement of specific genes.

2. Materials and methods

2.1. Source of the fungus

White-rot fungus (*P. chrysosporium* strain BKM-F-1767), a member of the class Basidiomycetes, was obtained from Professor C.A. Reddy's laboratory at Michigan State University, East Lansing, Michigan.

2.2. Medium and culture techniques

P. chrysosporium was grown on malt extract (ME) medium under non-ligninolytic (high nitrogen) conditions. Culturing and maintenance of these fungal mycelia were done as described before by us [14]. The fungus was grown in 250 ml flasks containing 50 ml of ME medium on shaker at 37 °C. Under these conditions in 3–4 days very small spherical mycelial balls were formed. For transferring the mycelia, improvisation was made by cutting the ends of the 5 ml pipette tips so that the mycelial balls can be pipetted. Sterile 12-well plates were taken and 2 ml of ME and 19 μl of 4000 ppm of 2,2′,4,4′,5,5′ hexachlorobiphenyl (PCB-153) (obtained from Ultra Scientific) dissolved in methanol was added to it to have a final concentration of 25 ppm (approximately 70 μM) of PCB-153. One millilitre of the very small mycelial ball suspensions was added (containing approximately 30 mycelial balls weighing 0.026 ± 0.006 g). 0.25 mM solutions of sodium molybdate or sodium tungstate were added separately in each of these wells. The liquid medium was pipetted out after 24 h and stored in new 1.5 ml eppendorf tubes at −20 °C.

2.3. High pressure liquid chromatography (HPLC) studies

For HPLC studies, PCB was extracted from the medium with equal volumes of hexane as described previously [20]. HPLC was done using a constant mobile phase composition of 90:10 (v/v) acetonitrile:water at a flow rate of 1.5 ml/min and UV light of wavelength 254 nm.

2.4. Chlorine release assay

For in vivo study of chlorine release by the nitrate reductase enzyme, 1 ml of the culture medium was taken and assay was done using the Bergmann and Sanik method [21]. For in vitro study, the mycelia (about 0.2 g in 1.5 ml eppendorf tube) were ground with 1 ml of extraction buffer [19] and mixed with 0.1 mM flavin adenine dinucleotide solution (FAD), 12 mM β-nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma–Aldrich protocol), 25 ppm PCB-153 and incubated at 30 °C for 2 h. For confirmation of the hypothesis of nitrate reductase mediated dechlorination, study with pure nitrate reductase enzyme (Sigma–Aldrich) was done similarly according to Sigma–Aldrich protocol.

2.5. Homology search and primer design

As the recently published genome of the *P. chrysosporium* (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) does not have any annotated genes and no published *P. chrysosporium* nitrate reductase gene sequence was obtained from GenBank, the nitrate reductase amino acid sequence of related Basidiomycota, *Hebeloma cylindrosporum* [22] was queried against the translated *P. chrysosporium* genome using TBLASTN program (http://www.ncbi.nlm.nih.gov/BLAST). Several primers were designed against the best match region using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) having a product size in between 350 and 600 bp; T_m values and GC percentage for forward and reverse primers almost equal and optimal primer length of 20. These primers were used for the RT-PCR reactions. One such primer sequence is given in the legend of Fig. 5.

2.6. RT-PCR

For RT-PCR, total RNA was obtained from the fungus using the Qiagen's Plant RNAeasy Mini Kit, and RT was done with the Invitrogen's AMV-RT enzyme and oligo(dT)$_{20}$ primers. PCR was done with the Qiagen PCR Core kit and nitrate reductase gene specific primers. The program setting used for PCR was one cycle of denaturation at 94 °C for 2 min followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 57.5 °C for 1 min and elongation at 72 °C for 2 min. The extracted RNA was measured spectrophotometrically and 5 μg of RNA was used for RT-PCR. Gel quantification was done using KODAK-1D software.

2.7. Sequencing and annotation

The RT-PCR product (413 bp) (Fig. 5) was cut from the agarose gel and eluted with the help of the Qiagen gel extraction kit. DNA sequencing was done at the University of Maryland genomic facility at College Park, Maryland, USA. The sequenced product was matched with the published raw sequence of the *P. chrysosporium* genome using two sequence BLAST program.

3. Results and discussion

3.1. HPLC studies

HPLC studies (Fig. 1) show that in the presence of *P. chrysosporium* PCB-153 is dechlorinated to about one-ninth of the original amount. In the presence of molybdenum, enhanced PCB dechlorination occurs to almost 1/27th, whereas with the addition of tungsten, PCB dechlorination is reduced to less than

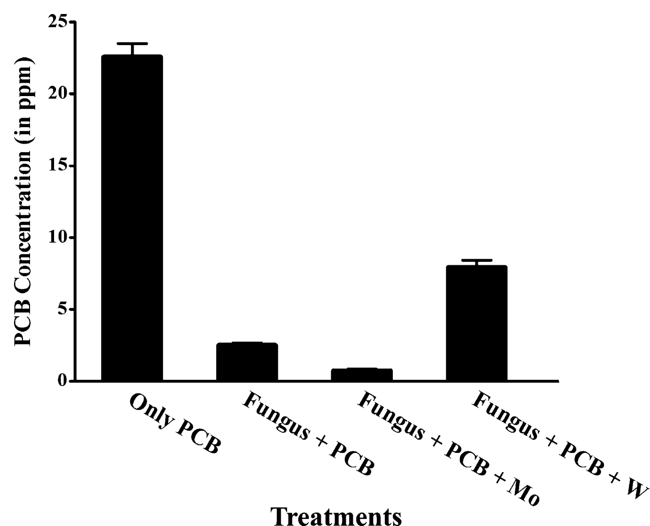

Fig. 1. Composite graphs of HPLC studies on the effect of the cofactor and the inhibitor of nitrate reductase mediated PCB dechlorination under non-ligninolytic condition (high nitrogen containing ME medium). The data indicate different degrees of PCB breakdown by *P. chrysosporium* in the presence of these two bivalent metal ions ($n = 3$; mean ± S.E.).

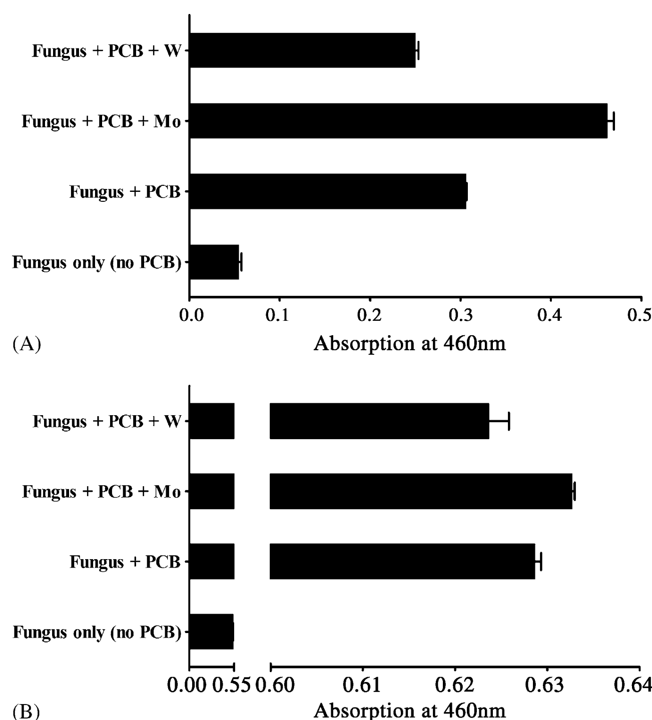

Fig. 2. Composite graphs of chlorine release assays induced by the fungus *P. chrysosporium.* These show that in the presence of molybdenum, chlorine-release increases dramatically, whereas, in the presence of tungsten chlorine release reduces dramatically. These findings suggest that Mo was an inducer of the specific PCB degrading enzymes while tungsten was an inhibitor ($n = 3$; mean ± S.E.) (A) relates to in vivo and (B) relates to in vitro studies.

one-third. For 'Only PCB', PCB-153 was added to the ME medium only and extracted similar to the samples. These observations were then statistically analyzed using ANOVA followed by Tukey's multiple comparison tests. These again showed that in malt extract medium (non-ligninolytic), there was a significant ($p < 0.05$) difference of the PCB dechlorination by the fungus in the presence and absence of tungsten as well molybdenum. All studies with the HPLCs were done at 1.5 ml/min flow rate using 25 cm columns for better resolution which made retention time higher (7 min). During the HPLC analysis Arochlor was not used as it produced multiple peaks which made it unsuitable. As tungsten is a known inhibitor of the nitrate reductase enzyme [23], these data show that in non-lignolytic conditions (ME medium) specific reductive enzymes did play a role, whereas in lignolytic conditions (MSM medium) generalized oxidative mechanisms were prevalent.

3.2. Chlorine release assay

Separate chlorine release assays were done using the fungal cultures (in vivo), the fungal cell extracts (in vitro), and with pure nitrate reductase enzyme. Fig. 2A summarizes the in vivo chlorine release assays with the addition of its substrate (KNO_3) and the effects of cofactor, molybdenum and inhibitor, tungsten. Fig. 2B presents composite data on in vitro studies with extracted proteins from PCB-153 treated fungal mycelia. Both the panels show significant ($p < 0.05$) increase in chlorine release with the addition of Mo and inhibition with W.

In order to establish our hypothesis that the nitrate reductase enzyme mediates dechlorination of hexachlorobiphenyl, we used purified nitrate reductase enzyme (obtained commercially from Sigma–Aldrich). Fig. 3 summarizes such data using Arochlor 1242 which is one of the well known environmental PCB contaminant, containing mixture of several congeners. This study shows that the nitrate reductase enzyme can significantly dechlorinate PCB in the presence of molybdenum and nitrates.

3.3. Nitrate reductase gene

The sequence of the nitrate reductase gene (GenBank accession no. AY700576) was matched with the *P. chrysosporium* genome database at Joint Genome Institute (JGI) and 97% homology was found with the scaffold 9 (Fig. 4). This is the

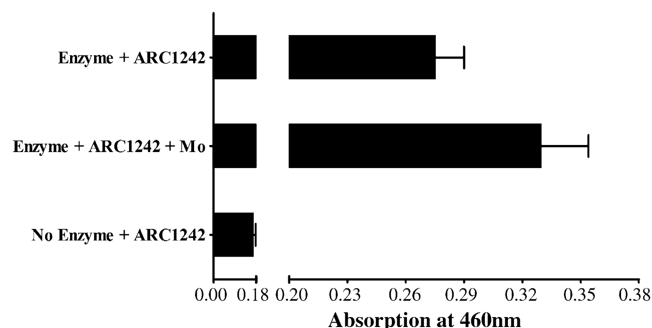

Fig. 3. Composite graphs showing chlorine release by pure nitrate reductase enzyme from 1000 ppm Arochlor 1242. Arochlor 1242 was used in this experiment since mixtures of different PCBs are present in the environment. Reaction volume of 500 μl was used containing 6 μM FAD (30 μl of stock 0.1 mM) and 120 μM KNO_3 (60 μl of stock 10 mM) ($n = 3$; mean ± S.E.).

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Sequenced product:        25 GCAGCGCCCTGAAAAACCGTACCAGTATCACATCGGTACCCTCTCGGGTGCGCTGTCCGC 84
                             ||||||||||||||| ||||||||||||||||||||| ||||||||||||||||||||||
Chromosome Scaffold:  91391 GCAGCGCCCTGAAAA-CCGTACCAGTATCACATCGGTGCCCTCTCGGGTGCGCTGTCCGC 91449

Sequenced product:        85 ACCCGTCGTGACAGCTCTAACCAACGATGATCCGGAGGCGCCTTTCTTGGACCCGAAAGT 144
                             |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Chromosome Scaffold:  91450 ACCCGTCGCGACAGCTCTAACCAACGATGATCCGGAGGCGCCTTTCTTGGACCCGAAAGT 91509

Sequenced product:       145 CTGGAAGTCGGTGACACTGACNAACATCGAGCGTGTGAACCACGACTCACTCGTATACCG 204
                             ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Chromosome Scaffold:  91510 CTGGAAGTCGGTGACACTGACGAACATCGAGCGTGTGAACCACGACTCACTCGTATACCG 91569

Sequenced product:       205 CTTTGCACTACCGCACGCGAGCACGCCGCTCGGTCTTCCCGTCGGGCAGCATGTCTTCGT 264
                             |||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||
Chromosome Scaffold:  91570 CTTTGCACTACCGCACGCGAGCACGCCGCTCGGTCTCCCCGTCGGGCAGCATGTCTTCGT 91629

Sequenced product:       265 GCGTCTCCGGCGAAAGGACACAGGTGAGGTCGTACAGCGCGCATACACGCCGGTGTCTCT 324
                             |||||||||||||||||||||||||||| |||||| ||||||||||||||||||||||||
Chromosome Scaffold:  91630 GCGTCTCCGGCGAAAGGACACAGGTGAGATCGTACTGCGCGCATACACGCCGGTGTCTCT 91689

Sequenced product:       325 TCAGGACGCGCGGGCGCGATCGACTTCCTGATAAAGTGCGTCTCTC-GTTGATGTCCAA 383
                             |||||| ||||||||||||||||||||||||||||||||||||||| |||||||||||||
Chromosome Scaffold:  91690 TCAGGATGCGCGGGCGCGATCGACTTCCTGATAAAGTGCGTCTCTCGGTTGATGTCCAA 91749

Sequenced product:       384 GTCATGCCA 392
                             |||||||||
Chromosome Scaffold:  91750 GTCATGCCA 91758
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Fig. 4. The nucleotide sequences of nitrate reductase gene aligned completely with the chromosomal DNA sequence of *P. chrysosporium* using the BLAST search. The GenBank accession number of this sequence is given as AY700576. The alignment produced a score = 646 bits (326), expect = 0.0, identities = 360/369, gaps = 2/369 (0%) and strand = Plus/Plus in the Scaffold 9 of *P. chrysosporium* genome.

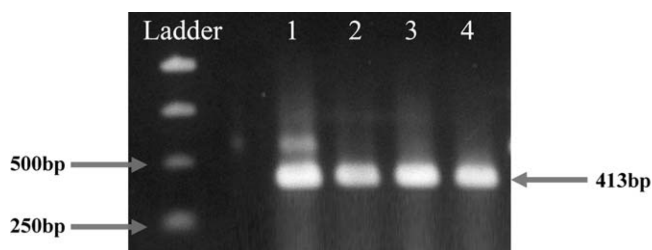

Fig. 5. The nitrate reductase gene expression in two replicates. Lanes marked as 1 and 2 were controls. Lanes 3 and 4 were PCB-153 treated. The Primers used were: ggcatgacttggacatcaac (forward) and aagcagaaactggcagaggt (reverse). The ladder used, was from Promega (cat #G7541.) The data show that the band intensities and integrated areas were found to be same.

first time that the nitrate reductase gene has been annotated in this fungus. The result of gene expression studies (Fig. 5) using KODAK-1D gel analysis software did not show significant hexachlorobiphenyl induced nitrate reductase gene expression. Controls versus treated integrated densities of the RT-PCR product (cDNA bands) were found to be 11.50 ± 0.63 versus 11.62 ± 0.85 which were insignificant ($p > 0.05$).

4. Conclusion

These studies have provided evidence for the first time that the white-rot fungus *P. chrysosporium* can dechlorinate PCB under non-lignolytic condition mediated through the nitrate reductase gene. Since nutrients (high nitrogen) were available in the ME medium, ligninolytic enzymes were not activated. Extensive molecular evidence has been provided for the presence of the nitrate reductase gene in this fungus by homology search, primer designing, RT-PCR and annotation of the *P. chrysosporium* genome. However, semi-quantitative RT-PCR analysis [24] showed no enhancement of the nitrate reductase gene expression when treated with hexachlorobiphenyl (PCB-153). This suggests that this gene is probably not induced by PCB-153 but in the presence of nitrates (such as KNO_3 used in this experiment) it can mediate PCB dechlorination.

HPLC and chlorine release studies after in vivo treatment with bivalent ions like molybdenum, which is a cofactor for nitrate reductase enzyme and tungsten, which is an inhibitor for this enzyme [23], confirmed the fact that the nitrate reductase enzyme was indeed involved in dechlorination. As expected, molybdenum enhanced and tungsten inhibited PCB degradation considerably. In vitro studies using pure nitrate reductase enzyme also confirmed the in vivo results.

Throughout the experiments we have used several controls such as "No Fungus, i.e. Only PCB" for HPLC studies, "No PCB, i.e. Fungus Only" for chlorine release assays, "no enzymes" for in vitro study with commercial nitrate reductase enzyme and water for RT-PCR reactions. As expected, all the control data were negative. Purity of the *P. chrysoporium* was monitored on a regular basis by microscopic examination of the unique *P. chrysosporium* basidiocarps [25,26].

This fungus can grow in varied temperatures, nutrient status and environmental conditions. The *P. chrysosporium* inoculums are used commercially as pellets in sawdust for enhanced bioremediation of PCBs (www.earthfax.com). However, sawdust has a risk of depleting the soil nitrates (www.mdc.mo.gov). Hence, the addition of nitrates to the pellets will reduce this risk as well as further enhance its activity. Thus, in practical applications, these fungal inoculums may be packed with sawdust pellets along with nitrate which will enhance the bioremediation of PCBs in the contaminated soils.

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