Diversity of ‘benzenetriol dioxygenase’ involved in *p*-nitrophenol degradation in soil bacteria

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Abstract Ring hydroxylating dioxygenases (RHDOs) are one of the most important classes of enzymes featuring in the microbial metabolism of several xenobiotic aromatic compounds. One such RHDO is benzenetriol dioxygenase (BtD) which constitutes the metabolic machinery of microbial degradation of several mono- phenolic and bi-phenolic compounds including nitrophenols. Assessment of the natural diversity of benzenetriol dioxygenase (*btd*) gene sequence is of great significance from basic as well as

applied study point of view. In the present study we have evaluated the gene sequence variations amongst the partial *btd* genes that were retrieved from microorganisms enriched for PNP degradation from pesticide contaminated agriculture soils. The gene sequence analysis was also supplemented with an *in silico* restriction digestion analysis. Furthermore, a phylogenetic analysis based on the deduced amino acid sequence(s) was performed wherein the evolutionary relatedness of BtD enzyme with similar aromatic dioxygenases was determined. The results obtained in this study indicated that this enzyme has probably undergone evolutionary divergence which largely corroborated with the taxonomic ranks of the host microorganisms.

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Introduction

Microbial decontamination of environments polluted with xenobiotic organic chemicals promises to be a benign, eco-friendly and cost-effective mechanism [1, 2]. Microorganisms have acquired enormous metabolic potential as a consequence of their ability to undergo rapid genetic evolution [3, 4]. Substituted aromatic compounds e.g. nitroaromatic compounds and chloroaromatic compounds have been reported to be the major contaminants of soil, sediments and groundwater [2, 5]. Several bacterial strains belonging to diverse taxonomic groups have been isolated

Fig. 1. Conversion of benzenetriol into maleylacetate via the formation of 3-hydroxy-*cis, cis* muconate by the action of BtD. The enzyme cleaves benzenetriol between the two hydroxyl groups at position 1 and 2.

Table 1. Enzymes (amino acid sequences) used for designing degenerate primers for partial *btd* gene amplification.

Enzyme	Origin	Degradation pathway	Reference
Hydroxyquinol 1,2 dioxygenase (TfH)	<i>Burkholderia cepacia</i> AC1100	2,4,5-Trichlorophenoxy acetate	29
Hydroxyquinol 1,2 dioxygenase (HadC)	<i>Ralstonia pickettii</i> DTP0602	2,4,6-Trichlorophenol	30
Hydroxyquinol 1,2 dioxygenase (DxnF)	<i>Sphingomonas</i> sp. RW1	Dibenzo- <i>p</i> -dioxin	15
Hydroxyquinol 1,2 dioxygenase	<i>Arthrobacter</i> sp. BA-5-17	Benzamide	31

and characterized for degradation of the above compounds [5, 6]. The molecular regulation of some of these degradation pathways have also been elucidated in detail [7–9]. Aromatic ring hydroxylating dioxygenases (RHDOs) are one of the most important classes of enzymes that catalyze aromatic ring cleavage for complete degradation of these compounds. Therefore, understanding the sequence variation and evolution of such dioxygenase(s) is crucial to develop successful bioremediation strategies [4, 10].

Benzenetriol dioxygenase (BtD), a multi-component enzyme, belongs to a large family of non-heme iron dioxygenases that play an important role in PNP degradation; this enzyme acts as the RHDO to convert benzenetriol (BT) to maleylacetate (MA) which is subsequently metabolized via TCA cycle (Fig. 1). Microbial degradation of PNP usually occurs by the formation of either 4-nitrocatechol (4NC) and BT [11], or benzoquinone (BQ) and hydroquinone (HQ) [12, 13] as the upper pathway intermediates. Although the biochemistry of PNP degradation pathways has been fairly well characterized, information pertaining to the molecular regulation is scarce. Amongst all the enzymes involved in PNP degradation in either of the above pathways, BtD is an important target for detailed characterization. Furthermore, this enzyme is also involved in microbial degradation of several other mono- and bi-phenolic compounds including chlorophenols and catechols [9, 14, 15]. One of the recent approaches for characterization of functional/ catabolic genes is to evaluate their sequence diversity amongst various sources [10]. The present work aims to elucidate the

variations of *btd* gene sequence from different PNP degrading microorganisms and decipher its putative evolutionary relation with the taxonomic diversity of the host microorganisms.

Materials and Methods

Sampling sites and characterization of bacterial isolates: Soil samples were collected from an agricultural field in Punjab, India that was sprayed with organophosphate pesticides such as parathion and methyl parathion. Both these pesticides are known to undergo chemical hydrolysis in soil to form PNP [13]. PNP degrading organisms from above samples were isolated using the ‘enrichment culture technique’ as described earlier [13], and their biochemical characterization was performed according to the Bergey’s Manual of Systematic Bacteriology (1984). Two of the laboratory strains viz. *Burkholderia* sp. SJ98 and *Arthrobacter* sp. RKJ4 [16] were used as the known PNP degraders in the study. Earlier strain SJ98 was tentatively identified as *Ralstonia* sp. on the basis of the morphological and biochemical characterization [17], however, 16S rRNA gene sequence (1449 bp) ascertained its identity as a *Burkholderia* sp. This was further confirmed by FAMES analysis and genomic mol% G+C content (data not shown).

DNA isolation and PCR: Genomic DNA was isolated from microorganisms using QIAGEN Genomic-tip 20/G (Qiagen GmbH, Germany) according to the manufacturer’s

instruction. The concentration and purity of DNA was measured spectrophotometrically. For amplification of partial *btd* gene from the above organisms, a set of degenerate primers Hq_F (5'-AGG AGT TCA TCC TGC T(G/C)(A/T) G-3') and Hq_R (5'-CGC AC(GC) CCG AAC AC(A/T) GCG TC-3') were designed on the basis of the conserved regions (Q84 EFILLS and D264 AVFGVR) of BtD amino acid sequences available from the GenBank and Pubmed (Table 1). The PCR parameters were standardized for concentrations of DNA template and magnesium salt as well as for annealing temperature on Mastercycler gradient (Eppendorf, Hamburg, Germany). PCR conditions used were: initial denaturation and enzyme activation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C $\pm$ 4 for 1 min, extension at 75 °C for 1 min and a final extension at 75 °C for 5 min. The reaction mixture contained 20–80 ng of genomic DNA, 1 U of Deep Vent polymerase (New England Biolabs, MA, USA), 1X Thermopol buffer, 200 μ M of each dNTPs and 20 pmol of each primer (BioBasic Inc. Ontario, Canada). In order to characterize the microorganisms at the molecular level 16S rRNA gene amplification was performed with universal eubacterial primers viz. 27F and 1492R using *Taq* DNA polymerase as described earlier [18].

Cloning and sequencing: PCR products for partial *btd* gene were cloned in pBlueScript II KS (+) cloning vector (Novagen, EMD Chemical Inc., CA, USA) following a blunt-end ligation at *Sma*I restriction digestion site. 16S rRNA gene amplicons were cloned in T/A cloning vector pGEM®-T Easy (Promega Corporations, MD, USA) according to the manufacturer's instructions. Recombinants were transformed into *E. coli* DH5 α and clones were screened with blue-white selection. Recombinant plasmids were isolated using the Miniprep kit (QIAGEN, GmbH, Germany). Sequencing was performed using vector specific primers with an automated DNA sequencer (ABI PRISM, model 377; Applied Biosystems, Foster City, CA, USA). The obtained sequences were submitted to GenBank (NCBI) and database searches were performed using the BLAST [19].

In silico Amplified Functional DNA Restriction Analysis (AFDRA): An *in silico* simulations of the restriction digestion pattern was performed on all the partial *btd* gene amplicons using restriction mapping software viz., Sci. Ed. Central Clone Manager Professional Suite (Scientific & Educational Software, NC, USA).

Phylogenetic analysis: Phylogenetic analyses including the calculation of bootstrap values were carried out using CLUSTAL_X [20], PHYLIP [21] and TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). For alignment of deduced amino acid sequences by CLUSTAL_X the alignment parameters for penalties were used as

Table 2. GenBank accession numbers for the partial *btd* gene(s) and 16S rRNA gene(s) from PNP degrading strains along with their identification up to genus level.

Strain name	Accession numbers		Organism
	<i>btd</i> gene	16S rRNA gene	
PNP1	AY866518	DQ282187	<i>Arthrobacter</i> sp.
PNP2	AY866524	DQ282188	<i>Arthrobacter</i> sp.
PNP3	AY866520	DQ282189	<i>Pseudomonas</i> sp.
PNP4	AY866521	DQ282190	<i>Pseudomonas</i> sp.
PNP5	AY866522	DQ282191	<i>Pseudomonas</i> sp.
PNP6	DQ286583	DQ282192	<i>Pseudomonas</i> sp.
PNP7	DQ286584	DQ282193	<i>Pseudomonas</i> sp.
SJ98	AY866519	DQ986324	<i>Burkholderia</i> sp.
RKJ4	AY866523	AY729888	<i>Arthrobacter</i> sp.

described earlier [22]. Phylogenetic subgroup was generated by using the SEQBOOT program of PHYLIP 3.63 [23, 24]. Statistical reliability of the trees was assessed using 100 bootstrap replications. A phylogenetic tree was constructed based on 'maximum likelihood estimation' algorithm using PHYLIP software. The consensus trees were obtained using the CONSENSE program [24] and rooted tree diagrams were generated with the TreeView program. In addition phylogenetic analysis was also performed using the 'distance matrix' algorithm wherein distance calculations were performed with Treecon software [25] by Jukes and Cantor model of evolution and the tree topology was determined using Neighbour-Joining method [26].

Nucleotide sequence accession numbers: The GenBank accession numbers for the partial 16S rRNA and *btd* gene sequences obtained in this study are shown in Table 2.

Results and Discussion

Phenotypic and genotypic characterization of the microorganisms: Using enrichment culture technique fifteen morphologically distinct microorganisms were isolated that were capable of utilizing PNP as sole source of carbon and energy. To investigate the presence of BtD-mediated PNP degradation pathway and to decipher the BtD amino acid sequence variation amongst the microorganisms, a *btd* gene specific PCR was performed. An ~ 540 bp amplicon (corresponding to 84th – 264th positions of BtD amino acid sequences that were targeted by degenerate primers) could be amplified from ten of the fifteen microorganisms. However, following DNA sequencing and database search, amplicons from only seven microorganisms showed sequence homology with *btd* gene; the remaining three were found to be spurious amplicons. The seven microorganisms that gave

btd gene specific amplification were designated as strains PNP1-7. Morphological and biochemical characterization of these microorganisms had indicated that they differed from each other (data not shown). Partial *btd* gene could also be amplified from strains SJ98 and RKJ4. All the partial *btd* gene sequences were submitted to GenBank and the accession numbers obtained are listed in Table 2. It is noteworthy that although fifteen enriched microorganisms were capable of utilizing PNP as sole source of

carbon and energy, *btd* gene could not be amplified from eight of the above microorganisms. This may be due to the fact that either PNP degradation may follow a different pathway wherein BT is not an intermediate, or the *btd* gene sequence(s) in these microorganisms might have been so divergent that did not permit the annealing of degenerate primers.

For further characterization of strains PNP1-7, SJ98 and RKJ4, partial 16S rRNA gene sequencing was

Fig. 2. Amplified functional DNA restriction digestion pattern of the clones containing partial *btd* gene as generated using Clone Manager. The clones have been grouped based on the similarities of their restriction patterns along with the corresponding strain names.

performed and sequences were submitted to GenBank. Partial sequences of 16S rRNA gene amplicons ascertained their identity up to the genus level (Table 2). Results of 16S rRNA gene sequence analysis showed that the enriched PNP degraders belonged to genera *Arthrobacter*, *Burkholderia* and *Pseudomonas*.

Sequence analysis and AFDR: Analysis of partial *btd* gene sequences and subsequent homology search using

BLASTX program (NCBI) showed that the deduced amino acid sequences of partial *btd* gene were closely related to each other in strains PNP1 and SJ98 (98 % identity); PNP6 and PNP7 (98 % identity); PNP3, PNP4 and PNP5 (94–96 % identity) and PNP2 and RKJ4 (76 % identity). An in silico AFDR analysis of the *btd* gene sequences was performed using Clone Manager software with an array of 34 restriction enzymes wherein *Sau3AI* and *RsaI* could

Fig. 3. A 'distance matrix' phylogenetic tree showing the position of BtD (obtained in this study) among some of the related dioxygenases. The tree was constructed using Treecon software. Maleylacetate reductase (mal), an enzyme involved in the lower pathway of PNP degradation, was used as the outgroup. The numbers within parentheses indicate GenBank accession numbers. Abbr: BtD, benzenetriol dioxygenase; CDO/ CCDO, catechol dioxygenase/ chlorocatechol dioxygenase; PCDO, protocatechuate dioxygenase. The numbers at the nodes represent bootstrapping values.

Fig. 4. Phylogenetic tree constructed on the basis of alignment of the amino acid sequences of BtD obtained from the organisms used in this study and other reported in the database. The tree was generated based on ‘maximum likelihood’ using the PROML programme of PHYLIP. Maleylacetate reductase (MaR) gene encoding another enzyme involved in PNP degradation was used as outgroup. The numbers at the nodes represent bootstrapping values.

generate phylogenetically informative digestion patterns. Based on the restriction fragment profile the microorganisms could be categorized into four distinct groups viz. (i) PNP1 and SJ98; (ii) PNP2 and RKJ4; (iii) PNP6 and PNP7; and (iv) PNP3, PNP4 and PNP5 (Fig. 2). The AFDRA based grouping of the above microorganisms corroborated with that based on deduced amino acid sequences. These results therefore indicate that AFDRA could provide a quick and reliable assessment of *btd* gene diversity among the PNP degrading strains (PNP1-7, SJ98 and RKJ4).

Phylogenetic analysis: The phylogenetic tree constructed according to the 16S rRNA gene sequences showed presence of two major groups belonging to Gram-positive bacteria (consisting of strains PNP1-2 and RKJ4) and Gram-negative bacteria. The Gram-negative group included three distinct clades: one consisting of strains PNP3-5, another consisting of strains PNP6-7 and the third consisting of strain SJ98 (data not shown).

The amino acid sequence deduced from the partial *btd* gene was also subjected to phylogenetic analysis using ‘maximum likelihood estimation’ and ‘distance matrix’ algorithms. The results obtained with phylogenetic analy-

sis were in close agreement with those obtained using AFDRA analysis since both the approaches categorized PNP degrading microorganisms in a similar manner. However, the phylogenetic study illustrated a few minor deviations that were not evident from the AFDRA analysis. Amongst the algorithms used ‘maximum likelihood estimation’ performed better as compared to ‘distance matrix’. This observation is quite apparent since it is reported that the ‘maximum likelihood method’ is statistically substantial even with very short sequences. As expected the grouping of the microorganisms based on 16S rRNA gene relatedness was comparable with that based on BtD amino acid sequences. The only exception being strain PNP1 (*Arthrobacter* sp.) which differed substantially from strain SJ98 (*Burkholderia* sp.) at the 16S rRNA gene sequence level, however, both of them exhibited a high degree of BtD amino acid sequence similarity (98 % identity). Recently, Nordin et al [9], also reported a similar observation with maleylacetate reductase (MaR); another enzyme involved in the degradation of aromatic compounds. The MaR gene cloned from *Arthrobacter chlorophenolicus* A6 showed 45.8 % identity with that of a taxonomically distantly

related microorganism *Ralstonia* sp. SJ98. Based on these evidences it may be concluded that functional genes may have similar sequences despite of being encoded by distantly related organisms. This phenomenon could be explained on the basis of the fact that these functional genes have possibly undergone horizontal transfer from one organism to the other irrespective of their taxonomic ranks. It is well documented that horizontal gene transfer is responsible for diversity and distribution of catabolic gene(s) involved in the degradation of naphthalene, atrazine etc [27, 28].

In order to evaluate the relatedness of BtD with some selected RHDOs phylogenetic trees were constructed as described above. The dendrogram created with 'distance matrix' method suggested a close relationship between BtD and catechol/chlorocatechol dioxygenases as compared to other RHDOs (Fig. 3). Figure 4 shows the phylogenetic relationship of BtD according to the 'maximum likelihood estimation'. The tree topologies as predicted by the above algorithms were in close agreement and both of them confirmed two distinct evolutionary lineages for BtD. The sequence information of BtD obtained from nitroaromatic-degrading microorganisms in this study is of significance since BtD plays a key role in degradation of aromatic compounds. The understanding of variations in sequence may enable rational improvement in its activity and specificity by site-directed mutagenesis and/or protein engineering in future.

In summary, the diversity of BtD, an enzyme involved in PNP degradation, has been studied at nucleotide sequence level and deduced amino acid sequence level by isolating different PNP degrading microorganisms from a pesticide contaminated agricultural soil. In a systematic approach encompassing AFDRA and phylogenetic analyses it was determined that BtD has two distinct sources of evolution and is also phylogenetically related to catechol and chlorocatechol dioxygenases. Moreover, the diversity of *btd* gene(s) showed explicit co-relation with the diversity of microorganisms.

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References

- Watanabe K (2001) Microorganisms relevant to bioremediation. *Curr Opin Biotechnol* 12:237–241
- Dua M, Singh A, Sethunathan N & Johri AK (2002) Biotechnology and bioremediation: successes and limitations. *Appl Microbiol Biotechnol* 59:143–152
- Timmis KN & Pieper DH (1999) Bacteria designed for bioremediation. *Trends Biotechnol* 17:200–204
- Watanabe K, Futamata H & Harayama S (2002) Understanding the diversity in catabolic potential of microorganisms for the development of bioremediation strategies. *Antonie Van Leeuwenhoek* 81:655–663
- Peres CM & Agathos SN (2000) Biodegradation of nitroaromatic pollutants: from pathways to remediation. *Biotechnol Annu Rev* 6:197–220
- Holliger C, Gaspard S, Glod G, Heijman C, Schumacher W, Schwarzenbach RP & Vazquez F (1997) Contaminated environments in the subsurface and bioremediation: organic contaminants. *FEMS Microbiol Rev* 20:517–523
- Takeo M, Yasukawa T, Abe Y, Niihara S, Maeda Y & Negoro S (2003) Cloning and characterization of a 4-nitrophenol hydroxylase gene cluster from *Rhodococcus* sp. PN1. *J Biosci Bioeng* 95:139–145
- Hofmann KW, Knackmuss HJ & Heiss G (2004) Nitrite elimination and hydrolytic ring cleavage in 2,4,6-trinitrophenol (picric acid) degradation. *Appl Environ Microbiol* 70:2854–2860
- Nordin K, Unell M & Jansson JK (2005) Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in *Arthrobacter chlorophenolicus* A6. *Appl Environ Microbiol* 71:6538–6544
- Junca H & Pieper DH (2003) Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria. *J Microbiol Methods* 55:697–708
- Kitagawa W, Kimura N & Kamagata Y (2004) A novel *p*-nitrophenol degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101. *J Bacteriol* 186:4894–4902
- Spain JC (1995) Biodegradation of nitroaromatic compounds. *Annu Rev Microbiol* 49:523–555
- Chauhan A, Chakraborti AK & Jain RK (2000) Plasmid-encoded degradation of *p*-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormiae*. *Biochem Biophys Res Commun* 270:733–740
- Meulenberg R, Pepi M & de Bont JA (1996) Degradation of 3-nitrophenol by *Pseudomonas putida* B2 occurs via 1,2,4-benzenetriol. *Biodegradation* 7:303–311
- Armengaud J, Timmis KN & Wittich RM (1999) A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. *J Bacteriol* 181:3452–3461
- Samanta SK, Chakraborti AK & Jain RK (1999) Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the formation of 1-naphthol. *Appl Microbiol Biotechnol* 53:98–107
- Samanta SK, Bhushan B, Chauhan A & Jain RK (2000) Chemotaxis of a *Ralstonia* sp. SJ98 toward different nitroaromatic compounds and their degradation. *Biochem Biophys Res Commun* 269:117–123
- Ghosh A, Paul D, Prakash D, Mayilraj S & Jain RK (2006) *Rhodococcus imtechensis* sp. nov., a nitrophenol-degrading actinomycete. *Int J Syst Evol Microbiol* 56:1965–1969

19. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
20. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–82
21. Retief JD (2000) Phylogenetic analysis using PHYLIP. *Methods Mol Biol* 132:243–258
22. Siew JP, Khan AM, Tan PT, Koh JL, Seah SH, Koo CY, Chai SC, Armugam A, Brusica V & Jeyaseelan K (2004) Systematic analysis of snake neurotoxins' functional classification using a data warehousing approach. *Bioinformatics* 20: 3466–3480
23. Felsenstein J (1987) Estimation of hominoid phylogeny from a DNA hybridization data set. *J Mol Evol* 26:123–131
24. Felsenstein J (2001) The troubled growth of statistical phylogenetics. *Syst Biol* 50:465–467
25. Van de Peer Y & De Wachter R (1993) TREECON: a software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* 9:177–182
26. Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
27. Wilson MS, Herrick JB, Jeon CO, Hinman DE & Madsen EL (2003) Horizontal transfer of *phnAc* dioxygenase genes within one of two phenotypically and genotypically distinctive naphthalene-degrading guilds from adjacent soil environments. *Appl Environ Microbiol* 69:2172–2181
28. Devers M, Henry S, Hartmann A & Martin-Laurent F (2005) Horizontal gene transfer of atrazine-degrading genes (*atz*) from *Agrobacterium tumefaciens* St96-4 pADP1::Tn5 to bacteria of maize-cultivated soil. *Pest Manag Sci* 61: 870–880
29. Daubaras DL, Saido K & Chakrabarty AM (1996). Purification of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase: the lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. *Appl Environ Microbiol* 62:4276–4279
30. Hatta T, Nakano O, Imai N, Takizawa N & Kiyohara H (1999) Cloning and sequence analysis of hydroxyquinol 1,2-dioxygenase gene in 2,4,6-trichlorophenol-degrading *Ralstonia pickettii* DTP0602 and characterization of its product. *J Biosci Bioeng* 87:267–272
31. Murakami S, Okuno T, Matsumura E, Takenaka S, Shinke R & Aoki K (1999) Cloning of a gene encoding hydroxyquinol 1,2-dioxygenase that catalyzes both intradiol and extradiol ring cleavage of catechol. *Biosci Biotechnol Biochem* 63: 859–865