Study of diversity of the gene encoding benzenetriol dioxygenase involved in $p$-nitrophenol degradation
Study of diversity of the gene encoding ‘benzenetriol dioxygenase’ involved in p-nitrophenol degradation

Introduction

Assessment of community functions need reliable tools to analyze the functions rather than taxonomical composition. PCR-based techniques have been used to detect functional/ catabolic genes in environmental isolates or environmental DNA, and diversity is usually assessed by sequencing of genes from isolates or PCR clone libraries (Yeates et al., 2000; Buchan et al., 2001; Duarte et al., 2001; Hamelin et al., 2002). Recent reports showed that PCR-amplified fragments of catabolic genes from environmental DNA can be separated by Density Gradient Gel Electrophoresis (Henckel et al., 1999; Nicolaisen and Ramsing, 2002) and Restriction Fragment Length Polymorphism (RFLP) analyses to select distinctive restriction patterns of single amplicons for further sequence determinations (Braker et al., 2000; Bakermans and Madsen, 2002; Yan et al., 2003). A more detailed knowledge on catabolic genes, retrieved by culture-independent methods and from isolates, can significantly improve our understanding of microbial functioning and degradation processes in the environment, which would in turn help to design new bioremediation strategies (Widada et al., 2002; Lovley, 2003).

Degradation of p-nitrophenol (PNP) usually follows two different metabolic routes: PNP is either converted to 4-nitrocatechol followed by the formation of benzenetriol and maleylacetate (Kitagawa et al., 2004) or is converted to p-benzoquinone, hydroquinone, 4-hydroxymuconic semialdehyde and finally maleylacetate (Chauhan et al. 2000), which is then metabolized to β-ketoadipate and enters the TCA cycle (Spain and Gibson, 1991; Spain et al., 2000). 1,2,4-Benzenetriol 1,2-dioxygenase (BtD) plays an important role in PNP degradation, wherein it converts benzenetriol to maleylacetate that is subsequently metabolized to form TCA cycle intermediates. This enzyme is also involved in several bacterial metabolic pathways including nitrophenols, chloroaromatic compounds and catechols (Stolz and Knackmuss, 1993; Meulenberg et al., 1996; Armengaud et al., 1999; Chauhan et al., 2000 a, b). Although BtD is important for understanding metabolic pathways of various aromatic compounds and has been characterized, very little information is available about the
gene(s) encoding this enzyme (Ferraroni et al., 2005). This study mainly focuses on the establishment of a phylogenetic relationship of BtD with other ARHDOs and to determination of the genetic diversity among BtDs amplified from various p-nitrophenol (PNP) degrading strains.

**Significance of aromatic-ring-hydroxylating hydrocarbon dioxygenases in environmental biotechnology**

Aromatic-ring-hydroxylating dioxygenases (ARHDOs) are key enzymes of the aerobic bacterial metabolism of aromatic compounds. The prototype reaction they catalyze is the addition of two hydroxy groups to vicinal carbons (Fig. 1), thereby, destroying the aromatic system and yielding dihydrodiol compounds of cis, cis stereochemistry (Butler and Mason, 1997; Boyd and Sheldrake, 1998; Kahl and Hofer, 2003).

![Figure 1](image)

**Figure 1.** Bacterial aromatic ring hydroxylating dioxygenases incorporate two atoms of dioxygen (O₂) into their substrates in the hydroxylation reaction. The product is (substituted) cis1,2-dihydroxycyclohexadiene, which is subsequently converted to (substituted) benzene glycol by a cis-diol dehydrogenase.

Bacterial ARHDOs are quite versatile both in terms of the substrates they accept and the oxidations they catalyze. Thus, even small aliphatic compounds such as trichloroethylene may serve as substrates (Furukawa et al., 1994; Wackett and Gibson, 1988) and different types of oxidations including dehydrogenation of vicinal carbons and mono-oxygenation of carbons and hetero atoms have been observed (Resnick et al., 1996; Boyd and
They also catalyze extradiol and intradiol ring cleavage of aromatic rings by dihydroxylation (Fig. 2).

**Figure 2.** Intradiol enzymes cleave the aromatic ring between two hydroxyl groups, whereas extradiol enzymes cleave the aromatic ring between a hydroxylated carbon and another adjacent non-hydroxylated carbon.

There are two principal reasons for the current interest in the structure and function of aromatic hydrocarbon dioxygenases. First, ARHDOs catalyze the breakdown of aromatic hydrocarbons that are common contaminants of soils and ground water and their removal from polluted environments by microbes represents a potential solution to the environmental problems posed by such pollutants (Spain et al., 2000; Rieger et al., 2002; Furukawa, 2003). Dihydroxylation of the aromatic ring is a prerequisite for oxidation of the aromatic nucleus by bacterial ring-fission dioxygenases. Studies on the multicomponent dioxygenases that oxidize aromatic hydrocarbons to vicinal arene cis-diols are of paramount importance in providing the scientific foundation necessary for the development of bioremediation technology (Buckel, 2005; Taylor and Janssen, 2005). The second reason for interest in aromatic hydrocarbon dioxygenases is related to the industry’s search for environmentally benign procedures for the synthesis of useful chemicals (Gibson and Parales, 2000). The dioxygenases fulfill this ‘green chemistry’ requirement as they are a source of new enantiopure arene cis-diols that are not attainable by conventional chemical synthesis. The diols are used as synthons in the development of new compounds of industrial and medical importance (Gibson and Parales, 2000; Taylor and Janssen, 2005).

The subunit characteristics and compositions of such dioxygenase systems vary considerably. However, all of these systems consist of a hydroxylase, which binds and oxidizes the organic substrate, and of a short transport chain for the electron supply of the hydroxylase (Butler and Mason, 1997). Based on the number and some characteristic
features of the subunits, ARHDOs have been grouped into five classes, namely IA, IB, IIA, IIB and III (Butler and Mason, 1997). Previous studies with class II ARHDO systems have demonstrated that the major determinants of the fundamental catalytic properties such as substrate and product spectra reside within the C-terminal part of the large or α-subunit (Kimura et al., 1997; Mondello et al., 1997; Beil et al., 1998; Zielinski et al., 2002), although the small or β-subunit has occasionally been reported to exert some influence on these properties (Furukawa et al., 1994). Recently, it has been shown that the C-terminal 60 amino acids of the α-subunit are of minor importance for these properties (Zielinski et al., 2002). Thus, if consensus oligonucleotide primers could be derived from conserved sequences flanking the part of the gene that encodes the catalytic centre, such segments could be rapidly amplified from, for example, bacterial isolates, microbial consortia or nucleic acid samples.

1,2,4-Benzenetriol 1,2-dioxygenase(s): A key enzyme involved in degradation of various aromatic compounds

Benzenetriol or hydroxyquinol (HQ) is one of the central intermediates in the degradation of a large variety of aromatic compounds. It has been detected as an intermediate in the metabolism of 4-hydroxybenzoate, resorcinol, salicylate, vanillate, benzoate, protocatechuate and gentisate by fungi such as Trichosporon cutaneum and Phanerochaete chrysosporium (Anderson and Dagley, 1980; Sze and Dagley, 1984; Rieble et al., 1994). In bacteria HQ has been identified as an intermediate in the degradation of mononuclear hydroxyaromatic compounds such as resorcinol and 2,4-dihydroxybenzoate (Chapman and Ribbons, 1976; Stolz and Knackmuss, 1993) or amino-hydroxyaromatic compounds such as 4-aminophenol as well as in the degradation of hydroxylated biaryl ethers such as 2-hydroxydibenzo-p-dioxin and 3-hydroxypbenzo[alpha]furan (Armengaud et al., 1999). HQ also occurs in the catabolic pathways of aromatic compounds carrying nitro groups such as in 3- and 4-nitrophenol or 4-nitrocatechol (Meulenberg et al., 1996; Jain et al., 1994; Chauhan et al., 2000; Kitagawa et al., 2004).

Hydroxyquinol (HQ) and its chloro-substituted derivatives 6-hydroxyquinol and 5-chlorohydroxyquinol (6CHQ) play an especially important role in the bacterial degradation of phenols or phenoxyacetates carrying a chloro-substituent in para position to the OH- or OCH_2COO^- group, respectively. Thus, pentachlorophenol by rhodococci and
mycobacteria has been reported to be degraded via HQ (Apajalahti and Salkinoja-Salonen, 1987; Haggblom et al., 1989; Uotila et al., 1995). In *Sphingobium chlorophenolicum* already the 2,6-dichloroquinol appears to be subject to ring cleavage however, in contrast to earlier reports, no 6CHQ is formed (Xun et al., 1999). On the contrary, for 2,4,6-trichlorophenol breakdown 6CHQ has been suggested as an intermediate for *Streptomyces rochei* 303 as well as several Gram-negative bacteria (Latus et al., 1995; Padilla et al., 2000; Louie et al., 2002; Xun and Webster, 2004). 2,6-Dichlorophenol can be degraded via 6CHQ, whereas 2,4-dichlorophenol, 4- and 2-chlorophenol by 2,4,6-trichlorophenol-induced cells may be transformed via the same pathway, although here HQ is the substrate for ring cleavage (Zaborina et al., 1995). In 2,4,5-trichlorophenoxyacetate degradation by *Burkholderia cepacia* AC1100 5CHQ and HQ are formed sequentially as intermediates (Daubaras et al., 1996). Although 3,5-dichlorohydroxyquinol was found to be an intermediate of 2,4-dichlorophenoxyacetate degradation by *Nocardioides simplex* 3E, HQ may also be involved as a ring cleavage substrate.

HQs are degraded aerobically by specialized intradiol ring-cleaving dioxygenases; the most studied enzymes from this family are the protocatechuate 3,4-dioxygenases (3,4-PCDOs), the catechol 1,2-dioxygenases (1,2-CDOs), and the chlorocatechol 1,2-dioxygenases (1,2-CCDOs) which generally possess distinctive substrate specificities (Lange and Que, 1998). The 1,2,4-benzenetriol 1,2-dioxygenases (BtD) catalyze the intradiol cleavage of hydroxyquinols to form 3-hydroxy-cis,cis-muconate, which occur in solution in the keto form, i.e. as maleylacetate (Fig. 3) (Pieper et al., 2002).

![Figure 3](image.png)

**Benzenetriol** → **3-Hydroxy-cis,cis muconate** → **Maleylacetate**

*Figure 3.* Benzenetriol is converted to maleylacetate via the formation of 3-hydroxy-cis, cis muconate by the action of 1,2,4-benzenetriol-1,2 dioxygenase. The enzyme cleaves benzenetriol between the two hydroxyl groups at position 1 and 2.
Some BtDs have been purified and characterized from a variety of microorganisms yet little is known about the factors controlling substrate specificity for this novel group of intradiol dioxygenases. Structural information of this enzyme was recently elucidated by Ferraroni et al. (2005) wherein they purified this enzyme from *Nocadiooides simplex* 3E that degraded polychlorinated aromatic compounds. DNA sequencing showed that the enzyme was closely related to catechol and chlorocatechol dioxygenases. Since BtDs and CDOs/CCDOs belong to different catabolic pathways, and correspondingly, the respective genes belong to different operons, the development of BtD substrate-specificity is a very important step in the evolution of pathways for efficient biodegradation of natural aromatic compounds as well as xenobiotics.

It is known that differences in the nucleotide sequence(s) of important enzymes involved in metabolism may be correlated to the functional adaptation of degradative bacteria and to the evolution of various metabolic routes. The objective of the present study was to establish the phylogenetic or evolutionary relationship of BtD with similar dioxygenases and to study the relationship of BtD sequences isolated from various organisms. For this, the partial gene(s) of BtD amplified from various PNP-degrading organisms were cloned and sequenced. The evolutionary relationships of the various dioxygenases were deduced by constructing a phylogenetic tree. RFLP pattern of the cloned sequences provided a quick assessment of the diversity of BtD in a community of PNP-degrading bacteria.

**Materials and Methods**

**Sampling sites, isolation and characterization of microorganisms, and culture conditions**

Soil samples were collected from various agricultural fields from various states of India (Haryana, Punjab, Assam, etc.) that were sprayed with organophosphate pesticides e.g. parathion and methyl parathion that hydrolyze in soil forming PNP. PNP degrading organisms were isolated using the ‘enrichment technique’ wherein soil samples were first inoculated in flasks containing 2% sodium succinate and PNP (0.2 mM) and incubated overnight at 30°C under shaking conditions. Inocula taken from these flasks were then used to inoculate in fresh flasks containing 0.4 mM PNP and this was repeated thrice in order to enrich the PNP-degrading strains. Thereafter, the cultures were diluted and...
plated on to nutrient agar plates containing PNP. The cultures that decolorized the yellow color of PNP were picked and patched onto plates containing 0.4 mM PNP as sole source of carbon and energy. The PNP-degrading isolates were identified and preserved. Strain SJ98 (Samanta et al., 2000) and RKJ4 (Samanta et al., 1999) used in the study are laboratory strains. Biochemical characterization was performed according to the Bergey’s manual of Systematic Bacteriology (1984).

**Fatty Acid Methyl Ester Analysis (FAME)**

This analysis was performed for identification of the PNP-degrading isolates. Cellular fatty acids were methylated (hydrochloric acid in methanol), extracted (hexane in methyl tert-butyl ether), and cleaned (sodium hydroxide) as specified by the users protocol (MIDI Technical note). Chromatographic analysis was performed using the Microbial Identification system (MIS) that includes a gas chromatograph (6890 series; Hewlett-Packard, Avondale, Pa.) with a flame ionization detector along with an autosampler and an integrator, coupled to a computer system. The Sherlock computer software (version 2.95; MIDI, Inc.) automatically sets the operating parameters of the gas chromatograph each time a sample is processed. Coupled to Sherlock is the ChemStation software (version 4.02; Hewlett-Packard) used for operating sampling, analysis, and integration of the chromatographic samples.

**DNA isolation and Polymerase Chain Reaction**

Genomic DNA was isolated from the PNP-degrading organisms using QIAGEN Genomic-tip 20/G (QIAGEN, Germany) and its concentration was determined spectrophotometrically. In order to amplify the 16S ribosomal RNA (rRNA) gene PCR was performed using 20-80 ng template DNA and universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T -3′) which target universally conserved regions and permit the amplification of an app. 1,500 bp fragment. The oligonucleotide primers were obtained from Biobasics, Canada. PCR amplification was carried out in a personal thermocycler (Eppendorf, Hamburg, Germany). Reaction tubes contained 50 ng of DNA, 1 U of *Taq* DNA Polymerase (New England Biolabs, Beverly, Mass.), 1X buffer (10mM Tris-HCl [pH 9.0], 1.5mM MgCl$_2$, 500mM KCl, 10mM deoxynucleoside triphosphate, and 20 pmol of each primer/µl. Initial DNA denaturation and enzyme activation steps were performed at 95°C for 30s, annealing at 50°C for 1 min and extension at 72°C for 2 min, and a final
extension for 10 min at 72°C. The presence and yield of PCR product was monitored by 1% agarose gel electrophoresis at 200V for 1h in 1X Tris-acetate-EDTA buffer.

In order to amplify the BtD gene from these organisms, the amino acid sequences of four hydroxyquinol (HQ) 1,2-dioxygenases available from GenBank e.g. TftH from B. cepacia AC1100 involved in 2,4,5-trichlorophenoxyacetate degradation (Daubaras et al. 1996), HadC from Ralstonia (formerly Burkholderia) pickettii DTP0602 involved in 2,4,6-TCP degradation (Hatta et al., 1999), DxnF from Sphingomonas sp. strain RWI involved in dibenzo-p-dioxin degradation (Armengaud et al., 1999), and an HQ 1,2-dioxygenase from Arthrobacter sp. strain BA-5-17 (Murakami et al., 1999) were aligned in CLUSTAL-X version 1.8 (Thompson et al., 1997).

Using the conserved regions from the N termini and the C termini, degenerate primers Hq_F and Hq_R (Table 1) were designed (Biobasics, Canada) such that the expected size of the amplicon was ~540 bp. The thermal profile for the amplification of this fragment of BtD gene was: 30 s at 95°C, 30 s at 55°C, 1 min at 75°C for 30 cycles, followed by extension at 75°C for 1 min. Gradient PCR was performed at a combination of two different MgSO₄ concentrations i.e. 2.0 mM and 2.5 mM, and three different annealing temperatures, i.e. 50°C, 53°C and 56°C. The reaction mixture consisted of 50 ng of genomic DNA, 1 U of DeepVent Polymerase (New England Biolabs, Mass.), 1X reaction buffer, 10 mM deoxynucleoside triphosphate, and 100 ng (16 pmol) of each primer (BtF and BtR).

### Table 1. Sequences of conserved amino acid residues and corresponding degenerate oligonucleotide primers used to amplify partial BtD gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Conserved protein sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hq_F</td>
<td>QEFILLS</td>
<td>5′-Agg AgT TCA TCC TgC T(g/C)(A/T) g-3′</td>
</tr>
<tr>
<td>Hq_R</td>
<td>DAVFGVR</td>
<td>5′-CcG AC(gC) CCg AAC AC(A/T) gCg TC-3′</td>
</tr>
</tbody>
</table>

**Cloning and Sequencing**

In order to clone the PCR product of BtD gene (~540 bp), pBluescriptII KS (+) (Novagen) was used as vector and blunt-end cloning was performed at the SmaI site; E. coli DH5α was used as a host for transformation. Recombinant plasmid DNA carrying the partial BtD gene(s) was isolated using the Miniprep kit (QIAGEN, Germany) for plasmid DNA isolation. Sequencing was performed with vector specific primers (in case of partial BtD gene) and 27F universal primer (in case of 16S rRNA gene) using an
automated DNA sequencer (ABI PRISM, model 377, version 3; Applied Biosystems). The sequences have been submitted to GenBank (NCBI). Data base searches were performed using the BLASTX service of the National Center for Biotechnology Information (NCBI). Using the ‘ORF finder’ tool of NCBI followed by translated BLAST search (BLASTX) the ORF(s) encoding the dioxygenase gene sequence were determined for each of the submitted nucleotide sequences.

**In silico Amplified Functional DNA Restriction Analysis (AFDRA)**

Predictions and simulations of the restriction fragment length of BtD (~540 bp) DNA sequences from the amplified sequences was performed by a restriction mapping software, Sci Ed Central Clone Manager Professional Suite (Bjerkan et al., 2004). Around 495 different endonuclease recognition sites were screened out of which two restriction enzymes that potentially produced phylogenetically informative fragments were selected for AFDRA analysis after comparison of the predicted restriction positions and calculation of the fragment lengths thus produced.

**Phylogenetic analysis**

Phylogenetic studies were performed using CLUSTAL-X (Heringa, 1999), PHYLIP (Retief, 2000) and TreeView (Win 16; University of Glasgow [http://taxonomy.zoology.gla.ac.uk/rod/treeview.html]). For alignment of amino acid sequences by CLUSTAL-X the pairwise parameters used were a gap opening penalty of 35 and a gap extension penalty of 0.75, whereas the multiple alignment parameters used were a gap opening penalty of 15 and gap extension penalty of 0.3 (Siew et al., 2004). Each phylogenetic subgroup was generated by using the SEQBOOT program of PHYLIP 3.63 (Felsenstein, 1987, 2001). Statistical reliability of the trees was assessed using 100 bootstrap replications. Maximum likelihood method was chosen because this program tend to be robust to many violations of the assumptions in the evolutionary model and even with very short sequences they tend to outperform alternative methods such as parsimony or distance methods. The consensus trees were obtained using the CONSENSE program (Felsenstein, 2001). The rooted tree diagrams were generated with the TREEVIEW program.

Distance estimations were performed using Treecon software (Van de Peer and De Wachter, 1993) by the method of Jukes and Cantor, 1969. Tree topology for the 16S rRNA nucleotide sequences and amino acid sequences of BtD were inferred by the
distance matrix using Neighbour Joining method (Saitou and Nei, 1987). The tree topologies for dioxygenase(s) involved in degradation, obtained with maximum parsimony, maximum likelihood and distance matrices were compared and phylogenetic relationships between the different dioxygenases were predicted.

**Nucleotide sequence accession numbers**

The GenBank Accession numbers of the partial 16S rRNA gene sequences and partial BtD nucleotide sequences have been tabulated (Table 2).

**Chemicals**

*p*-Nitrophenol and ampicillin were purchased from Sigma, USA. All other chemicals were of highest purity grade available locally.

**Results and Discussion**

**Phenotypic and genotypic characterization of the isolates**

Using enrichment culture technique fifteen morphologically different isolates were obtained that utilized PNP as sole sources of carbon and energy. Biochemical characterization of the cultures and microscopy indicated that they differed from each other (data not shown). For the molecular characterization of isolates, genomic DNA was isolated and PCR was carried out to amplify the partial BtD gene from each of them. Among the fifteen isolates, an approximately 540 bp gene could be amplified from nine isolates, however, after sequencing and database search seven amplicons out of nine showed homology with benzenetriol dioxygenase(s); the remaining two were spurious amplicons. The strains were thereafter designated as strain PNP1-7. In addition, two of the laboratory isolates that degraded PNP i.e. *Ralstonia* sp. SJ98 and *Arthrobacter* sp. RKJ4 were also used for PCR amplification of the partial BtD gene.

Isolates PNP1-7 were identified using FAME analysis and 16S rRNA gene sequencing. Results of identification of the strains have been indicated in Table 2. It may be inferred from these results that although the fifteen isolates utilized PNP as sole carbon and energy sources, yet BtD gene was not amplified in all of them. There could be two possibilities to explain the same: (i) The PNP degradation pathway was different in these organisms and benzenetriol might not be a pathway intermediate in cases where amplification was not obtained; (ii) The specificity of the primers used for amplification
could be another possible explanation. In this case, the gene sequence might have been so divergent that they did not permit the annealing of the degenerate primers. Amplification of BtD gene failed even upon altering the PCR conditions.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>BtD Gene Acc. No.</th>
<th>16S rRNA Gene Acc. No.</th>
<th>Organism/16S rDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNP 1</td>
<td>AY866518</td>
<td>DQ282187</td>
<td>Arthrobacter sp.</td>
</tr>
<tr>
<td>PNP 2</td>
<td>AY866524</td>
<td>DQ282188</td>
<td>Arthrobacter sp.</td>
</tr>
<tr>
<td>PNP 3</td>
<td>AY866520</td>
<td>DQ282189</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>PNP 4</td>
<td>AY866521</td>
<td>DQ282190</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>PNP 5</td>
<td>AY866522</td>
<td>DQ282191</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>PNP 6</td>
<td>DQ282192</td>
<td></td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>PNP 7</td>
<td>DQ282193</td>
<td></td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>SJ98</td>
<td>AY866519</td>
<td></td>
<td>Ralstonia sp.</td>
</tr>
<tr>
<td>RKJ4</td>
<td>AY866523</td>
<td>AY729888</td>
<td>Arthrobacter sp.</td>
</tr>
</tbody>
</table>

Table 2. List of the GenBank Accession numbers obtained for partial 16S rRNA gene(s) and BtD gene(s). The identification of the strains according to FAME analysis and 16S rRNA sequencing has also been listed.

Sequence analysis and Amplified Functional DNA Restriction Analysis

Sequence analysis of the PCR products amplified using the primer set Hq_F and Hq_R and subsequent homology search analysis using BLASTX programme (NCBI) showed that the deduced amino acid sequences of BtD were closely related to each other in strains PNP6 and PNP7 (98% identity); PNP3, PNP4, PNP5 (94-96% identity); PNP2, RKJ4 (76% identity). Identity refers to the extent to which two (nucleotide or amino acid) sequences are invariant during their alignment using the BLAST tool of NCBI. These results, therefore, indicate that BtD sequences in strain PNP6 and PNP7 are more similar than that in strain PNP2 and RKJ4. This may also suggest that the BtD from strains PNP6 and PNP7 are evolutionarily not much distant as in the case of PNP2 and RKJ4. Strains PNP3-5 also share a fair degree of homology and seemed to be evolutionarily related. The nucleotide sequence of BtD from strain SJ98 has been used for comparison of the above sequences. The amino acids encoded by the nucleotide sequence were determined using the ORF Finder tool in which the exact frame in which the protein is encoded could be identified. Amino acid sequence of the BtD amplified from each of the isolates as determined via ORF Finder tool (NCBI) are as follows:

>`>PNP1 ORF:1..527 Frame -2
MLSDTLGSLISVLDDINQHDEAATESSLLGPFYRDGVKESAFGANIAQSDGEPVFFHGTVGDVNG
KPVADALIEVWTAPNGMYEQGDQPDPEQEGNLRGFRRTNADGAYAFHSHIKPSTMPIPTDGPVQGM
LVATGRHPMRPAHIHFRAIDAPGYQPLTALTLYSDDPYVNFRCVSG

48
16S rRNA gene sequencing was carried out in order to identify the strains and also to determine their phylogenetic relationship with each other since 16S rRNA gene has been reported to be conserved through evolution (Torsvik and Overseas, 2002; Junca and Pieper, 2003). For this, partial 16S rRNA gene(s) (~500 bp) were analyzed using the BLASTN programme (NCBI) and the strains were identified up to genus level (Table 2). A phylogenetic tree was constructed (Fig. 4) based on the sequences obtained where two major groups belonging to Gram positive and Gram negative bacteria could be detected. The Gram negative bacteria again showed two distinct clades; one consisting of PNP3-5 and the other consisting of PNP6-7.
In order to study the phylogenetic relationship of BtD gene(s) with other ARHDOs, the amino acid sequence of partial BtD gene were aligned with those of the other dioxygenase(s) involved in degradation of pollutants. A phylogenetic tree was constructed using the distance matrix of TREECON and also parsimony and maximum likelihood trees were constructed using PHYLIP and TREEVIEW. The dendrogram created in TREECON (Fig. 5) suggested closer phylogenetic relationship between BtD and catechol and chlorocatechol dioxygenase(s) as compared to other ring cleaving dioxygenases. This relationship has also been reported by Ferraroni et al. (2005). Figures 5, 6 and 7 show the three different tree topologies as predicted by the various matrices used. The parsimony tree (Fig. 7) did not group the BtD sequences in a single phylogenetic cluster; therefore, the tree topology obtained by maximum likelihood and distance estimations have been discussed further. It is reported that maximum parsimony uses only some relevant sites in the sequence, so when the number of informative sites is not large this method is often less efficient than distance methods (Saitou and Nei, 1986). Maximum parsimony is not dependable for its sensitivity to codon bias and unequal rates of evolution.
Figure 5. Phylogenetic tree representing dioxygenases catalyzing intradiol ring-cleavage reactions. CLUSTAL-X was used for multiple alignment of amino acid sequences and TREECON was used for tree construction based on distance based matrix. Maleylacetate reductase (mal), an enzyme involved in the lower pathway of PNP degradation has been selected as the outgroup. The numbers indicate GenBank accession numbers. [Abbr: BtD, benzenetriol dioxygenase; CDO/CCDO, catechol dioxygenase/ chlorocatechol dioxygenase; PCDO, protocatechuate dioxygenase].

The distance methods are very rapid and easily permit statistical tests e.g. bootstrapping. They derive some measure of similarity or difference between the input sequences by making assumptions that (i) rate of change is equal among all sequences; (ii) branch lengths correlate with the expected phenotypic distance between sequences which corresponds to a proportional measure of time. The maximum likelihood method is on the other hand, very slow but preferred as it maximizes the likelihood of observing the data and almost always produces a single best possible tree (Hall, 2001). This method
estimates the probability of having a given nucleotide at a particular site and this decision is based on the transition/transversion ratio; the process is repeated for all nucleotide positions in a sequence. These probabilities are summed over the whole sequence for all branches of a tree. When there are many sequences, the estimation is done recursively at every branch point. Since each site evolves independently, the likelihood of the phylogeny can be estimated at every site. This process can only be executed in a reasonable amount of time with four sequences. If there are more than four sequences, basic trees can be made for sets of four sequences, and then extra sequences added to the tree and the process of finding the maximum likelihood would be re-estimated. The order in which the sequences are added and the initial sequences chosen to start the process critically influences the resulting tree. To prevent any bias, the whole process is repeated multiple times with random choices for the order of the sequences. A majority rule consensus tree is then chosen as the final tree.

![Phylogenetic tree](image)

**Figure 6.** 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 BtD sequences reported in the database. The tree was constructed based on 'maximum likelihood' using the PROML programme of Phylip. Maleylacetate reductase (MaR), an enzyme involved in PNP degradation has been selected as the outgroup. The numbers at the nodes represent bootstrapping values.

In this study the tree based on maximum likelihood and distance estimations showed similar topology, however, the former tree was more accurate as also confirmed by the AFDRA pattern (Fig. 8). Also, in the distance based dendrogram the position of
BtD obtained from strain PNP4 is unexpected as it has been clustered with protocatechuate dioxygenases. The other predictions based on these trees are similar and they also match the results of AFDRA (discussed later). The maximum likelihood based tree (Fig. 6) further re-confirms that there are two distinct evolutionary lines for BtD which is also evident from the distance based tree (Fig. 5).

Figure 7. Phylogenetic tree constructed on the basis of alignment of the amino acid sequences of benzenetriol dioxygenase. The tree was constructed using the PROTPARS programme of Phylip. The numbers at each node represents bootstrap values.

In silico restriction digestions of the BtD sequences using all tetra-base cutters amongst 495 restriction enzymes listed in the Clone Manager software suggest that Sau3AI and RsaI were the most suitable enzymes for generation of an AFDRA pattern with characteristic restriction fragments of suitable sizes. Additionally, it gives idea about the diversity of the amplified sequences based on similar restriction patterns with respect to particular restriction enzyme(s). The restriction patterns of the BtD sequences from each of the strains have been shown in figure 8. Based on the restriction patterns the isolates have been assigned four different groups for easy comparison of the sequences.
Group 1

\[ \text{Sau3AI} \quad \boxed{100} \quad \text{Rsal} \quad \boxed{200} \quad \text{Sau3AI} \]

**RKJ4** (529 bps)

\[ \text{Sau3AI} \quad \boxed{100} \quad \text{Rsal} \quad \boxed{300} \quad \text{Sau3AI} \]

**PNP2** (541 bps)

Group 2

\[ \text{Sau3AI} \quad \boxed{100} \quad \text{Rsal} \quad \boxed{200} \quad \text{Sau3AI} \]

**PNP1** (537 bps)

\[ \text{Sau3AI} \quad \boxed{100} \quad \text{Rsal} \quad \boxed{300} \quad \text{Sau3AI} \]

**SJ98** (541 bps)

Group 3

\[ \text{Sau3AI} \quad \boxed{100} \quad \text{Rsal} \quad \boxed{200} \quad \text{Sau3AI} \]

**PNP6** (533 bps)

\[ \text{Sau3AI} \quad \boxed{100} \quad \text{Rsal} \quad \boxed{300} \quad \text{Sau3AI} \]

**PNP7** (535 bps)
Chapter 3

Group 4

Figure 8. Amplified functional DNA restriction digestion pattern of the partial gene of BtD as generated by using Clone Manager. The size of the amplified gene has been indicated in brackets. The sequences having similar restriction patterns have been grouped.

Analyses of the restriction fragment profiles obtained from isolates showed four different patterns, hence four groups were formed (i) PNP2 and RKJ4; (ii) PNP1 and SJ98; (iii) PNP3, PNP4 and PNP7; (iv) PNP5 and PNP6. The differences in restriction pattern may also be correlated to the phylogenetic tree (constructed using amino acid sequences in maximum likelihood based program) which also showed four different clades in agreement with the restriction analysis data. Three of the clades were monophyletic and their members showed very similar restriction patterns (corresponding to group 1, 2 and 3) and one clade was polyphyletic (corresponds to group 4) wherein the strains show varying restriction patterns of the BtD nucleotide sequence(s). Indeed, in order to determine the phylogenetic diversity of any functional gene from strain collections or environmental DNA amplifications, new and quicker methods are required. This technique (AFDRA) has provided a quick assessment of BtD diversity.
among the nine different strains studied and when the results are compared with
dendrograms constructed on the basis of sequencing results, an insight of the
phylogenetic affiliation of BtD could be obtained in comparison with other known
sequence(s)/organisms.

The technique AFDRA has been successfully used by Junca and Pieper (2003) to
study the diversity of catechol 2,3-dioxygenase gene in soil bacteria. The authors
reported that cluster analyses of the restriction fragment profiles obtained from isolates
showed patterns with distinct similarities to the reference strain profiles, allowing to
distinguish four different groups. Also, sequences of PCR fragments from isolates were
in close agreement with the phylogenetic correlation predicted by the AFDRA approach.
The results therefore indicate that in combination with amplified ribosomal DNA
restriction analysis (ARDRA) and sequencing, AFDRA may be suitably used for
subsequent analysis of functional diversity in environmental samples wherein the soil
metagenome is used as template during amplification of the functional gene or rRNA
gene.

From the results obtained in this study it is also apparent that although strain
PNP1 (Arthrobacter sp.) differed substantially from Ralstonia sp. SJ98 in the 16S rRNA
gene, the BtD nucleotide sequences in these organisms showed a high degree of
relatedness (98% identity). Therefore, the identity of the microorganism (as determined
by the 16S rRNA gene sequence) had some correlation with the sequence of BtD gene.
However, there are exceptions because theoretically according to 16S rRNA gene
sequence, strain PNP1 that should have been grouped with PNP2 and RKJ4 but the
results of AFDRA and phylogenetic analysis suggest a different position for strain PNP1.
Recently, Nordin et al. (2005) showed that the MaR gene cloned from A.
chlorophenolicus A6 showed 45.8% identity with that of Ralstonia sp. SJ98. This
evidence also supports the above observation that functional gene(s) may be similar in
sequence although they may be encoded by organisms that are evolutionarily distant or
unrelated. This result may also suggest the possibility that these gene(s) have been
horizontally transferred from one organism to the other, irrespective of their genetic
makeup.

It is well documented that horizontal gene transfer is responsible for the tolerance
of soil microflora involved in the degradation of xenobiotics such as
hexachlorocyclohexane, atrazine, toluene, naphthalene, etc (Werlen et al., 1995; Wilson
et al., 2003; Trefault et al., 2004; Devers et al., 2005; Boltner et al., 2005). Gene(s)
involved in mineralization or transformation of such compounds are transferred from the
donor to recipient strains and may be rearranged in the recipient cell; the selection
pressure maintained in soil due to the continuous influx of xenobiotics facilitates this
process. Once the gene(s) are rearranged in the recipient organism, they may form a part
of the same pathway or a different one, as suggested by the fact that BtD gene(s) cloned
from nitroaromatics degrading organism(s) is evolutionary close to gene(s) involved in
the degradation of chloroaromatic compounds.

The partial BtD gene sequences submitted to GenBank (NCBI) are as follows:

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**VERSION**  AY866520.1 GI:61220989

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**TITLE**  Diversity of gene(s) encoding 1,2,4-benzenetriol 1,2-dioxygenase enzyme isolated from various p-nitrophenol degrading bacteria

**JOURNAL**  Unpublished

**REFERENCE**  2 (bases 1 to 528)


**TITLE**  Direct Submission

**JOURNAL**  Submitted (27-DEC-2004) Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India

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**ACCESSION**  AY866521

**VERSION**  AY866521.1 GI:61220991

**KEYWORDS**  .

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**ORGANISM**  Pseudomonas sp. PNP4  
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.

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58
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JOURNAL Unpublished
REFERENCE 2 (bases 1 to 528)
TITLE Direct Submission
JOURNAL Submitted (27-DEC-2004) Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India
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ORGANISM Pseudomonas sp. PNP5; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 519)
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JOURNAL Unpublished
REFERENCE 2 (bases 1 to 519)
TITLE Direct Submission
JOURNAL Submitted (27-DEC-2004) Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India
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Chapter 3

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References


