Analysis of a new dimeric extradiol dioxygenase from a naphthalenesulfonate-degrading sphingomonad

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A new extradiol dioxygenase was cloned by screening a gene bank from the naphthalenesulfonate-degrading bacterial strain BN6 for colonies with 2,3-dihydroxybiphenyl dioxygenase (DHBPDO) activity. A 1.6 kb DNA fragment was sequenced and an ORF of 954 bp identified. Comparison of the deduced amino acid sequence of DHBPDO II from strain BN6 with previously published sequences showed the closest relationship to a metapyrocatechase (MpcII) from *Alcaligenes eutrophus* JMP 222. Thus, the enzyme was only distantly related to the main groups of catechol 2,3-dioxygenases or DHBPDOs. The dioxygenase was expressed using a T7 expression vector and the enzymic characteristics of the protein were examined. The enzyme oxidized 2,3-dihydroxybiphenyl, 3-isopropylcatechol, 3-methylcatechol, 4-fluorocatechol and 1,2-dihydroxynaphthalene. Comparison of the UV/visible spectrum of the product formed from 3,5-dichlorocatechol with previous reports suggested that this substrate is oxidized by different extradiol dioxygenases either by proximal or distal ring cleavage. The enzyme required Fe²⁺ for maximal activity. In contrast to most other extradiol dioxygenases, the enzyme consisted of only two identical subunits.

Keywords: strain BN6, extradiol dioxygenases, 2,3-dihydroxybiphenyl dioxygenase, *Sphingomonas*

INTRODUCTION

The aerobic bacterial degradation of aromatic compounds usually proceeds via the formation of intermediates which carry two hydroxyl groups attached either in the *ortho* or *para* position to each other on the aromatic ring. If two vicinal hydroxyl groups are present, the ring fission occurs either between the two hydroxyl groups (intradiol cleavage) or at a bond proximal to one of the hydroxyl groups (extradiol cleavage). Extradiol dioxygenases play a key role in the degradation of various aromatic compounds such as toluene, naphthalene, biphenyl and polyaromatic hydrocarbons (Cerniglia, 1992; Dagley, 1975; Furukawa, 1994; Harayama *et al.*, 1992; Harayama & Timmis, 1992). Initially, it was assumed that the extradiol dioxygenases were a rather uniform evolutionarily

related group of enzymes which all contain Fe²⁺ ions and belong to one superfamily (Harayama & Rekik, 1989). Recently, evidence has accumulated from DNA sequencing experiments that certain extradiol dioxygenases, e.g. catechol 2,3-dioxygenase from *Alcaligenes eutrophus* JMP 222, protocatechuate 4,5-dioxygenase from *Sphingomonas* (*Pseudomonas*) *paucimobilis* or 3,4-dihydroxyphenylacetate 2,3-dioxygenase from *Escherichia coli* C, do not belong to the major superfamily of extradiol dioxygenases (Harayama *et al.*, 1992; Kabisch & Fortnagel, 1990a; Noda *et al.*, 1990; Roper & Cooper, 1990). Moreover, the genes for two 2,3-dihydroxybiphenyl 1,2-dioxygenases (DHBPDOs) have been cloned and sequenced (*bphC2* and *bphC3*) from the biphenyl-degrading strain *Rhodococcus globululus* P6, which according to their small subunit size, the composition of the holoenzymes and their amino acid sequences, constitute a separate group of extradiol dioxygenases (Asturias *et al.*, 1994; Asturias & Timmis, 1993). Furthermore, there have been reports on extradiol dioxygenases which are dependent on manganese or magnesium ions for their catalytic activity (Boldt *et al.*,

Abbreviations: 2,3-DHBP 2,3-dihydroxybiphenyl; DHBPDO, 2,3-dihydroxybiphenyl dioxygenase.

The GenBank accession number for the nucleotide sequence reported in this paper is U38978.

1995; Gibello *et al.*, 1994; Que *et al.*, 1981). Thus, it appears that the group of enzymes which catalyse the extradiol cleavage of aromatic compounds is more diverse than was previously believed.

We have recently reported the cloning and characterization of an extradiol dioxygenase from the bacterium strain BN6, which exhibited the highest degree of identity to DHBPDOs II and III from *R. globerulus* P6 and thus did not belong to the major group of extradiol dioxygenases (Heiss *et al.*, 1995). Strain BN6 degrades amino- and hydroxynaphthalenesulfonates via substituted 1,2-dihydroxynaphthalenes to substituted salicylates (Kuhm *et al.*, 1991, 1993; Nörtemann *et al.*, 1986, 1994). Although clones derived from strain BN6 were obtained which were able to oxidize various catechol derivatives, the corresponding protein was not involved in the degradation of naphthalenesulfonates. In this study, another extradiol dioxygenase was obtained, from a gene bank of strain BN6, which was also significantly different from the members of the major superfamily of extradiol dioxygenases.

METHODS

Bacterial strains, culture conditions, plasmids and DNA manipulation techniques. The isolation and characterization of strain BN6 and the growth conditions have been described previously (Nörtemann *et al.*, 1986). The strain has been deposited at DSM, Braunschweig, Germany, as *Sphingomonas* sp. BN6 DSM 6383. *Escherichia coli* strains were routinely cultured in Luria-Bertani medium. The *E. coli* strains used as host strains for recombinant DNA work, the techniques for DNA manipulations, the construction of the plasmid library and the detection of recombinants oxidizing 2,3-dihydroxybiphenyl (2,3-DHBP) have been described previously (Heiss *et al.*, 1995).

Nucleotide sequence analysis. The DNA sequence was determined by the dideoxy chain-termination method with dsDNA of overlapping subclones in an automated DNA sequencing system (ALF Sequencer, Pharmacia) with fluorescently labelled primers (Heiss *et al.*, 1995).

Sequence analysis and comparisons were done with the PC/GENE software package, version 6.7 (IntelliGenetics). The dendrogram based on the pairwise alignment of the extradiol dioxygenases was produced with the program CLUSTAL using the default parameters. Database searches were performed online using the programs BLASTN, BLASTP and TBLASTN (Altschul *et al.*, 1990) on the BLAST electronic mail server from the National Center for Biotechnology Information, Bethesda, MD, USA.

Hyperexpression of DHBPDO. The strategy used for expression of *bphC2* in *E. coli* BL21(DE3) under the control of the T7 promoter has been described previously (Heiss *et al.*, 1995). The upstream primer (5' CGTCATATGAACCAGAACAGCCCGTCG 3') incorporated a *NdeI* site (underlined) and the downstream primer (5' CGCGGATCCTCAGGCCTCCGCCGGTTC 3') incorporated a *BamHI* site (underlined). The amplified product was restricted with *NdeI* and *BamHI* and ligated into pET11a to create pGHS201.

Relative activities of the enzyme preparation with (substituted) catechols. The preparation of cell extracts and the

enzyme assay with 2,3-DHBP were performed as described previously (Heiss *et al.*, 1995). For determination of the enzyme activities with catechol, 3-methyl-, 4-methyl-, 3-isopropyl- and 4-chlorocatechol, and 2,3-DHBP the same reaction conditions and extinction coefficients as previously described were used (Heiss *et al.*, 1995). The extinction coefficient for the oxidation product of 1,2-dihydroxynaphthalene was $\epsilon_{331\text{nm}} = 2.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Kuhm *et al.*, 1991). The extinction coefficients of the oxidation products of 3,5-dichlorocatechol, 3-bromo-5-chlorocatechol, 4-fluorocatechol and 3,4-DHBP were experimentally determined as $\epsilon_{337\text{nm}} = 10.0 \text{ mM}^{-1} \text{ cm}^{-1}$, $\epsilon_{335\text{nm}} = 8.3 \text{ mM}^{-1} \text{ cm}^{-1}$, $\epsilon_{382\text{nm}} = 18.4 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{383\text{nm}} = 12.0 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively. The apparent enzyme activities were determined with freshly prepared solutions of these substrates.

Stability of the enzyme in cell-free extracts from *E. coli* BL21(DE3)(pGHS201). To determine the optimal conditions for storage and purification of the enzyme, crude extracts were incubated in different buffer systems alone, in the presence of protease inhibitors or compounds which are known to stabilize extradiol dioxygenases (Patel & Barnsley, 1980; Scopes, 1982). After 4 d storage at 4 °C in sodium/potassium phosphate buffer or Tris/HCl (each 100 mM, pH 7.5), 70 or 50% of the initial activity was recovered, respectively. The addition of PMSF (1 mM), pepstatin A (0.1 µM), EDTA (5 mM), glycerol (30%, v/v), β -mercaptoethanol (9 mM), dithiothreitol (1 mM), glutathione (4 mM), ethanol (10 or 20%, v/v) or acetone (10% or 20%, v/v) did not stabilize the enzyme activity.

Enzyme purification and determination of molecular mass. The protein was purified at room temperature by use of a FPLC system, as previously described (Heiss *et al.*, 1995).

Crude extract from *E. coli* BL21(DE3)(pGHS201) was applied to a Q-Sepharose fast-flow column (HR 16/10) and eluted at a flow rate of 4 ml min⁻¹ with Tris/HCl (50 mM, pH 7.5) and a linear NaCl gradient (0–2 M over 300 ml). Fractions (5 ml each) were collected and those containing DHBPDO activity (eluting at an NaCl concentration of about 0.28–0.32 M) were pooled and transferred to a Phenyl-Superose fast flow column (HR 10/10). Protein was eluted with 150 ml of a linear gradient of Tris/HCl (50 mM, pH 7.5) containing (NH₄)₂SO₄ (500 mM in 50 mM Tris/HCl, pH 7.5). Both buffers contained ethyleneglycol (5%, v/v). The flow rate was 1 ml min⁻¹ and fractions of 4 ml were sampled. The activity of DHBPDO was determined and those fractions with the highest activity [fractions 39–44, eluting at approximately 50 mM (NH₄)₂SO₄] were pooled and concentrated by ultrafiltration (Centricon-10, Amicon). The sample was applied to a Hiload Superdex 200 (HR 16/60) column and eluted with 130 ml Tris/HCl buffer (50 mM, pH 7.5) plus NaCl (100 mM) at a flow rate of 0.7 ml min⁻¹. Fractions (2 ml each) were collected and the active fractions combined.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (1970). Protein staining of the gels was performed with Coomassie brilliant blue G 250 (Merck).

Chemicals. The sources of 2,3-DHBP, 3,5- and 4,5-dichlorocatechol, 3-bromo-5-chlorocatechol, 3-isopropylcatechol and 3,4,6-trichlorocatechol were as described previously (Heiss *et al.*, 1995). 3,4-DHBP was purchased from Promochem and 3-chlorocatechol from Tokyo Kasei TCI. 3,6-Dichlorocatechol was obtained from Helix Biotech and 1,2,4-trihydroxybenzene from Aldrich. 4-Fluorocatechol was kindly provided by M. Schlömann (this laboratory). All other chemicals were obtained from Merck, Fluka, Sigma or Aldrich.

RESULTS

Cloning and subcloning of the DNA fragment encoding the 2,3-DHBP oxidizing enzyme activity

The construction of the plasmid library and the spray-test for the identification of clones with the ability to oxidize 2,3-DHBP to the *meta* cleavage compound 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate have been described previously (Heiss *et al.*, 1995). In this gene bank, two types of recombinant plasmids encoding 2,3-DHBP oxidizing activity were found. In a previous publication (Heiss *et al.*, 1995), a clone containing a 9.75 kb insert was analysed (on pGHS1) which har-

boured a gene encoding a DHBPDO (BphC). In this study, the second clone was examined. The gene for DHBPDO activity was originally located on a 4.75 kb *Bgl*II fragment (pGHS2). In further subcloning experiments the gene (designated *bphC2*) was located on a 1.6 kb *Eco*RV fragment.

Sequencing of the 1.6 kb fragment carrying the dioxygenase gene and sequence comparisons with other extradiol dioxygenases

Various overlapping subclones were sequenced. The DNA and deduced amino acid sequences of the 1.6 kb

1	<u>EcoRV</u> <u>GATATCAGCAAGGACGACGCGCTGTCGCATATCGTCGCCTATTCGGTGTTCACGACGGTTCGATCCGCGACTATCAGCTCAAGACGCCG</u> D I S K D D A L S H I V A Y S V F N D G S I R D Y Q L K T P
91	CAGTGGACGGTCGGCAAGAAATTCGACGATACCGCGCCTTCGGTCCCTGGCTGGTAACGGCCGACGAGCTGCCTGCGGGCGGCGCCGGG Q W T V G K N F D D T G A F G P W L V T A D E L P A G G A G
181	CTCAAGATCGAAACCCGCTCAACGGCCAGGTAGTGCAGAGCGCAACACATCCGACATGGTGTTCGATGTGTCGACACGGTCGCCCTC L K I E T R L N G Q V V Q S A N T S D M V F D V V D T V A L
271	CTGTCCACCTGCTTCACGCTGGAAGCGGGCGACGTTCTGGTCAATGGGAACCCCGTCGGGCATCGGCCTTGCCCGCAAGCCTCCGCTGTTT L S T C F T L E A G D V L V M G T P S G I G L A R K P P L F
361	ATGAAGGATGGCGATGTGTGCGAAGTGGAAATCGAAAAGATCGGCCTGTTGGTGAACCCCATCAAGGCAGCATGATCGAAAGGCCGGCGC M K D G D V C E V E I E K I G L L V N P I K A A *
451	<u>RBS</u> CGTCCTGTGCGCGCCCGCCAGCCAGGAGCGAAGGATGAACCAGAACAGCCCGTCGGGACGCCCGCCCAATAGTTTGGGCGTCCATTCC BphC2 → M N Q N S P S G R R A N S L G V H S
541	ATCGATCATTTTCGGGTGGAAGTCCCGATCTGGAAGCGCGCGGACATTCTACACGCTCTTCGGACTTGACGTCCGCGAGCGGGATGAC I D H F A V E V P D L E A A R T F Y T L F G L D V R E R D D
631	ACGCTGGAATTATATGCGTTCGGCAATCCGCATCGCTGGGCCGTGCTGAGCCAGGGCGGCGACGCCAAGCGGTGCGCTATCTGAGCTTC T L E L Y A F G N P H R W A V L S Q G G D A K R L R Y L S F
721	GGCATCTATCCGGACGAAGCGGACGCCCTTTGCCGCTCATCTCGACGGCTCGGGCGTCAACGGATCGATCCGCCCCAAGCGCGGAAGCC G I Y P D E A D A F A A H L D G C G V T R I D P P E G A E A
811	GACGGCATCTGGTTCGCAAGCTTCGATGGCCTGCCGATCAACGTCCGCGTCCGAGACAAGGCGACGCCATCGGAAAAGAGCCATTTCACT D G I W F A G F D G L P I N V R V A D K A T P S E K S H F T
901	TTGTCGACGCGCGCGCGGGCCAATCCGGCGGATCCCTAACAGCAAGCGCCCAAGGTTACCCCGCGCGCTTTTCGATTTCCGCATC F V S A A P G Q S G A I P N S K A P K V H P R R L S H F A I
991	TTGCGGACCGACGTGAATGCCGCGATCGACTTCTATGAGAAGACGCTCGGCCTGCGCCTGTCCGACAAGAGCGAGCCGCGCGTTCGCCTTC F A T D V N A A I D F Y E K T L G L R L S D K S E P A V A F
1081	CTGCACGGCCCCCATGGCAGCGATCATCACCTGCTGGCCCTCGTCTGTCCGATCGGCGGGCATGCATCACAATAGCTGGGACGTGCGG L H G P H G S D H H L L A L V L S D R R G M H H N S W D V G
1171	TCGGTGATGGAAGTGGGCTGGGCGGCGGACCATGGCCCGGCGGCTATGGCCCCAAGTGGGCGTGGGCCAGCAGTATTGGGCGCC S V M E V G L G G A T M A R A G Y G P N W G V G Q H V L G A
1261	AATTATTTCTATTATGTGCGCGATCCTTGGGGCAGTTTCAGCGAATATTCGGCCGATATTGACTTCATCCCGATGACGTGAGTGGCGC N Y F Y Y V R D P W G S F S E Y S A D I D F I P H D V D W P
1351	<u>RBS</u> GCCGCCAACCATGCGCCAGAGGACAGCATGTTCTCTGTGGGACCCGATCCATTCGCCAATTCATCCAGAACACTGAACCGGCGGAGGCC A A N H A P E D S M F L W G P D P F P E F I Q N T E P A E A
1441	TGATCGTCATGACCACATTCTGACTTCTTCACGGCGGCGGATGGGCGGCTGGACATGGAATATGTCCGTGAAATCTGGAGGCCGGG * M T T F V L L H G G G M G G W T W K Y V R E I L E A G G
1531	GCCACAAGGTCTACACGCGACCTTCACCGGCTTTGGCGAGCGGACATCTGATCGGCCGCGATGTGGGCAATGCGACTCATGTGCGCG H K V Y T P T F T G F G E R E H L I G R D V G N A T H V R D
1621	<u>EcoRV</u> <u>ATATC</u> I

Fig. 1. Nucleotide sequence of the 1.6 kb *Eco*RV fragment containing *bphC2*. Ribosomal binding sites (RBS) and the *Eco*RV restriction sites are underlined. Asterisks mark the ends of the ORFs.

Fig. 2. Dendrogram resulting from pairwise alignments of amino acid sequences. NahH, catechol 2,3-dioxygenase from *P. putida* NAH7 (Harayama et al., 1987); XylE, *P. putida* mt-2(TOL) (Nakai et al., 1983); DmpB, *Pseudomonas* sp. CF600(pVI150) (Shingler et al., 1992); TodE, *P. putida* F1 (Zylstra & Gibson, 1989); MpcI, *A. eutrophus* JMP222 (Kabisch & Fortnagel, 1990a); MpcII, *A. eutrophus* JMP222 (Kabisch & Fortnagel, 1990b); NahC, 1,2-dihydroxynaphthalene dioxygenase from *P. putida* NAH7 (Harayama & Rekik, 1989); CmtC, 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase from *P. putida* F1 (Eaton, 1996); BphC, BphC1, BphC2 and BphC3, DHBPDs from *S. paucimobilis* Q1 (Taira et al., 1988), *R. globerulus* P6 (Asturias et al., 1994), *Pseudomonas* sp. LB 400 (Hofer et al., 1993), *Pseudomonas* sp. KKS102 (Kimbara et al., 1989), *P. putida* KF715 (Hayase et al., 1990) and strain BN6 (Heiss et al., 1995).

EcoRV fragment are shown in Fig. 1. A putative start codon (ATG) was found at positions 487–489 of the nucleotide sequence. A putative ribosome binding site was found 6 bp upstream (AAGGAG). A termination codon (TGA) was identified at positions 1441–1443. This suggested an ORF of 954 bp, encoding a peptide with 318 aa and a molecular mass of 34802 Da. The protein sequence was pairwise aligned with the amino acid sequences of other extradiol dioxygenases. The resulting dendrogram is shown in Fig. 2. The enzyme from strain BN6 showed the highest degree of identity to a metapyrocatechase from *A. eutrophus* JMP 222 (MpcII). A recently described 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase from *Pseudomonas putida* F1 (CmtC) (Eaton, 1996) appears to belong to the same group. The dendrogram suggested that the *bphC2* gene did not belong to the major superfamily of extradiol dioxygenases as found previously for the *bphC* gene from the same strain. The pairwise alignment of the amino acid sequences of BphC2 and MpcII showed 46% identity (Fig. 3). Sequence analysis of the complete 1.6 kb *EcoRV* fragment revealed two further putative ORFs flanking *bphC2*, which were separated from *bphC2* by short intervening sequences. One deduced ORF begins with an ATG 5 bp downstream of the *bphC2* stop codon. The deduced amino acid sequence corresponded to 59 aa (Fig. 1) and showed 39–42% identity to sequences determined previously for 2-hydroxy-6-phenylhexa-2,4-dienoate (HOPDA) hydrolases (BphD) from *P. putida* KF715, *Pseudomonas* sp. LB 400 and *Comamonas testosteroni* B-356 (Ahmad et al., 1995; Hayase et al., 1990; Hofer et al., 1993).

A second putative ORF was identified by translating the nucleotide sequence upstream of the *bphC2* putative start site in all three reading frames, followed by a homology search with each deduced amino acid sequence. A sequence was identified which showed homology to the 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase/5-oxopent-3-ene-1,2,5-tricarboxylate de-

BPHC2_BN6	-	MNQNSPSGRRANSL-----GVHSDHFAVEVFDLEAARTFYTLFGLDVL	-43
MPCII_JMP	-	MDTHRADASQRSQAPARPHAVHSIDHYALEVFDLAVAFRLDAFGLTV	-50
BPHC2_BN6	-	RERDDTLELYAFGNPHRWAVLSQGGDAKRLRLVSPGIYPDEADAFAAHLD	-93
MPCII_JMP	-	ARTPECLEVYA-ADQRCWARFYEY-ERKRLAYLSFSCFEGDFAGIRQQLA	-98
BPHC2_BN6	-	GCQVTRIDPPEGAEEADGIWFAFGDGLPINRVADKATPSEKSHFTFVSAA	-143
MPCII_JMP	-	ASGATLVEDPRYGDSESGVWFDFPDGNLVQVKGPKTSPSSKSPARLEGAP	-148
BPHC2_BN6	-	PGQSGAIPNSKAPKVPRLRLSHFAIFATDVNAADIFYEKTGLRLSDKSE	-193
MPCII_JMP	-	GGQRCQAVVRSQVQVRLPRLSHVLLFTFSPVQALDFYDALGLRLSDRS	-198
BPHC2_BN6	-	PAVAFHLGPHGSDHLLALVLSDRRGMHNSWDVGSVMEVGLGGATMARA	-243
MPCII_JMP	-	DVIAFTHAPYGSDDHLLALVKSARGWHHAADVADVNEVGGASQMAKA	-248
BPHC2_BN6	-	GYGPNWVGQHVGLGANYFYVVRDPWGSFSEYSADIDFIPHDVDPWAANHA	-293
MPCII_JMP	-	GYTQGWGTGRHVLGNSNYFFVLDPWGSFCEYSADIDYIPAGQAWFAGDFA	-298
BPHC2_BN6	-	PDSDMFLWGPDPFPEFIQNTPEAEA	-318
MPCII_JMP	-	ABDSLYQWGPDPVEYFVRNTE--A	-320

Fig. 3. Comparison of the amino acid sequences of BphC2 from strain BN6 and MpcII from *A. eutrophus* JMP222 (Kabisch & Fortnagel, 1990b). Identical amino acids are indicated by asterisks.

carboxylase from *E. coli* C, which is part of the degradative pathway for homoprotocatechuate in this organism (Roper & Cooper, 1990, 1993). From an overlap of 115 aa, 36 residues were identical.

Analysis of the gene product

To improve the expression of *bphC2*, the gene was cloned into the T7 expression vector pET11a. *Nde*I and *Bam*HI sites were introduced by PCR to enable ligation of the resulting fragment into pET11a, producing pGHS201, which was subsequently transformed into *E. coli* BL21(DE3). Expression of the *Nde*I–*Bam*HI fragment in pGHS201 yielded an additional peptide in the crude extracts with an apparent molecular mass on an SDS-PAGE gel of 39 ($\pm$ 2) kDa (n = 3).

Table 1. Purification of BphC2 from strain BN6

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Recovery factor (%)	Purification (-fold)
Crude extract	10	413	438	1.06	100	1.0
Q-Sepharose	30	97	424	4.36	97	4.1
Phenyl-Superose	24	24	262	10.9	60	10.3
Gel filtration	6	24	158	6.57	36	6.2

Table 2. Relative activities for the oxidation of various aromatic diols by cell extracts of *E. coli* BL21(DE3)(pGHS201) encoding DHBPDO II (BphC2) from strain BN6 in comparison to those of BphC from the same strain

The specific activities with 2,3-DHBP (= 100 %) were 1.7 and 2.1 U (mg protein)⁻¹ for BphC and BphC2, respectively. ND, Not determined.

Substrate (0.2 mM each)	Enzyme			
	BphC2		BphC	
	$\lambda_{\max}$ of product (nm)	Relative activity	$\lambda_{\max}$ of product (nm)	Relative activity
2,3-DHBP	434	100.0	434	100.0
3,4-DHBP	383	0.05	383	0.6
1,2-Dihydroxynaphthalene	ND	1.8	ND	0.0
Catechol	375	0.7	375	0.7
3-Methylcatechol	385	6.2	384	10.0
4-Methylcatechol	382	0.03	382	0.2
3-Isopropylcatechol	388	86.0	384	12
3-Chlorocatechol	ND	0.0	378	16
4-Chlorocatechol	379	0.3	379	0.9
3,4-Dichlorocatechol	ND	0.0	380	ND
3,5-Dichlorocatechol	337	0.6	389	6.0
3-Bromo-5-chlorocatechol	335	0.5	384	13
4-Fluorocatechol	382	2.6	ND	ND
4-Hydroxycatechol (= 1,2,4-trihydroxybenzene)	346	ND	ND	0.0

Several attempts were made to purify the enzyme by FPLC under aerobic or anaerobic conditions in different buffer systems but did not yield homogeneous preparations of the enzyme. A typical purification scheme is shown in Table 1. The molecular mass of the enzyme was determined by gel filtration and estimated to be 65 (± 5) kDa ($n = 3$). From this, it can be assumed that the enzyme consists of two subunits. In the chromatograms from the gel filtration step, only a single protein peak was observed which co-eluted with the enzyme activity. SDS-PAGE gels of samples from various purification steps characteristically showed an enrichment of a peptide with the expected mass of the DHBPDO subunit (39 kDa). In addition, two further bands of different intensities corresponding to peptides with masses of approximately 14 and 30 kDa were observed. These peptides could not be removed by further chromatography using different columns and their formation was

observed in different buffer systems (Tris/HCl, sodium/potassium phosphate or imidazole) in the presence of different protease inhibitors, enzyme stabilizing agents and also during FPLC under anaerobic conditions. The sequence data demonstrated that the two peptides (30 and 14 kDa) were direct products of the 39 kDa peptide and were formed presumably prior to cell disruption (see Methods). Because of the unavailability of homogeneous preparations of DHBPDO, further experiments were performed with cell extracts from *E. coli* BL21(DE3)(pGHS201).

Iron as cofactor

The amino acid sequence of BphC2 suggested that the enzyme was not, or only marginally related to the major group of extradiol dioxygenases, which almost invariably contain Fe²⁺ ions as cofactor. Furthermore,

Fig. 4. Oxidation of (a) 1,2,4-trihydroxybenzene, (b) 3,4-DHBP, (c) 4-fluorocatechol and (d) 3,6-dichlorocatechol by BphC2 from strain BN6. The reaction mixtures contained 4–1400 µg cell extract and the respective substrate (0.2 mM each) in 1 ml sodium/potassium phosphate buffer (100 mM, pH 7.5). A spectrum was recorded every 1–5 min.

manganese- and magnesium-dependent extradiol dioxygenases have been described recently (Boldt *et al.*, 1995; Gibello *et al.*, 1994; Que *et al.*, 1981). The cell extract from *E. coli* BL21(DE3)(pGHS201) [60 mg protein ml⁻¹; specific activity of DHBPDO = 1.43 U (mg protein)⁻¹] was incubated for 15 min with various Fe²⁺-chelating agents such as 8-hydroxyquinoline, *o*-phenanthroline or 2,2'-dipyridyl (1 mM each), or with the oxidizing agent H₂O₂ (0.1 and 2%, v/v). The treatment with 8-hydroxyquinoline, *o*-phenanthroline, 2,2'-dipyridyl or H₂O₂ (0.1%) reduced the activity of DHBPDO to 67–86% of the control. With 2% H₂O₂ a complete inhibition was observed (< 2% of the original activity). This inactivated enzyme preparation was separated from residual H₂O₂ by ultrafiltration (Centricon-10, Amicon), rediluted to the original volume by the addition of 200 µl buffer, divided into aliquots (50 µl each) and incubated with (NH₄)₂Fe(SO₄)₂ (2 mM), (NH₄)₂Fe(SO₄)₂ (2 mM) plus dithiothreitol (5 mM), MnCl₂ (2 mM), MgCl₂ (2 mM) or without any supplements. After 10 min incubation at room temperature, 31 and 44% of the original enzyme activity was recovered in the samples which had been treated with (NH₄)₂Fe(SO₄)₂ or (NH₄)₂Fe(SO₄)₂ plus dithiothreitol, respectively. In contrast, only 9, 14 or 10% of the original

Table 3. Substrate specificity of BphC2 from strain BN6

The values were determined with cell-free extracts from *E. coli* BL21(DE3)(pGHS201) which expressed BphC2. The kinetic data were calculated using the computer program Enzfitter (Biosoft, Cambridge, UK, 1987), assuming Michaelis–Menten kinetics.

Substrate	$V_{\max}$ (U mg ⁻¹)	K_m (mM)
2,3-DHBP	1.5 ± 0.12	0.003 ± 0.00092
Catechol	0.08 ± 0.0012	0.8 ± 0.029
3-Methylcatechol	0.27 ± 0.0057	0.12 ± 0.0097
3-Isopropylcatechol	1.37 ± 0.028	0.0056 ± 0.00044
4-Fluorocatechol	0.10 ± 0.0020	0.19 ± 0.0092

activity was found in the samples which had been incubated with MnCl₂, MgCl₂ or with no additives, respectively.

Oxidation of 2,3-DHBP, alkylcatechols and 1,2,4-trihydroxybenzene by cell-free extracts of *E. coli* BL21(DE3) carrying the recombinant plasmid

Several different aromatic 1,2-diols were tested as substrates with crude extracts from *E. coli* BL21(DE3)(pGHS201) and the substrate specificity compared to that obtained previously for BphC from strain BN6. The products from 2,3-DHBP, catechol, and 3-methyl- and 3-isopropylcatechol showed the same absorption spectra as the products formed by BphC (Table 2). As for BphC, the enzyme encoded by pGHS201 showed the highest specific activity with 2,3-DHBP and was therefore called BphC2. In comparison to BphC, BphC2 showed a much higher relative activity with 3-isopropylcatechol as substrate. In addition to the substrates oxidized by BphC, BphC2 also converted 1,2,4-trihydroxybenzene (Table 2, Fig. 4).

The enzyme activity of BphC2 in the cell-free extracts was not inhibited by higher substrate concentrations. Therefore the K_m values and relative $V_{\max}$ values for different substrates were compared with the cell extracts using spectrophotometric assays (Table 3).

The crude extracts did not oxidize protocatechuate, protocatechualdehyde, homoprotocatechuate, 3,4-dihydroxycinnamate, 3,4-dihydroxymandelate, 3,4-dihydroxyphenylglycol, 4-sulfocatechol or 4-nitrocatechol.

Oxidation of halocatechols by BphC and BphC2

When BphC2 was incubated with 3-chlorocatechol, no reaction was observed spectrophotometrically. Furthermore, the enzyme was inactivated after incubation with 3-chlorocatechol as reported for extradiol dioxygenases from different bacteria (Adams *et al.*, 1992; Arensdorf & Focht, 1994; Bartels *et al.*, 1984; Klecka & Gibson, 1981; Wasserfallen *et al.*, 1991).

With 3,5-dichlorocatechol as substrate, the formation of

a new absorption maximum ($\lambda_{\max}$) was observed. A surprising result was obtained when the absorption spectra of the products formed by BphC and BphC2 from 3,5-dichlorocatechol and 3-bromo-5-chlorocatechol were compared. The product formed by BphC2 from 3,5-dichlorocatechol had a $\lambda_{\max}$ at 337 nm and the product from 3-bromo-5-chlorocatechol at 335 nm. These products were obviously different from the products obtained with BphC, which showed $\lambda_{\max}$ at 388 and 384 nm for 3,5-dichlorocatechol and 3-bromo-5-chlorocatechol, respectively.

The reaction mixtures obtained from the oxidation of 3,5-dichlorocatechol by BphC or BphC2 were acidified to pH 2 where both products lost their $\lambda_{\max}$ at 384 nm and 337 nm, respectively. The solutions were immediately reneutralized and another set of spectra was recorded. Thus the $\lambda_{\max}$ at 337 nm did not reappear, while that of the product formed from BphC ($\lambda_{\max}$ = 388 nm) was at least partly restored. Both BphC and BphC2 oxidized 3,6-dichlorocatechol to a product which had a $\lambda_{\max}$ at 364 nm (Fig. 4). BphC and BphC2 did not oxidize 4,5-dichlorocatechol or 3,4,6-trichlorocatechol.

BphC2 oxidized 4-fluorocatechol to a product with a $\lambda_{\max}$ at 382 nm (Fig. 4, Table 2). This absorption maximum disappeared reversibly after acidification and irreversibly after incubation at pH 13.

DISCUSSION

Analysis of gene banks from bacterial strains with the ability to degrade aromatic compounds for clones with DHBPDO activity often results in the isolation of different clones with DHBPDO activity of unknown physiological function. Using this method, three to four different DHBPDOs were found in *R. globerulus* P6 and *R. erythropolis* TA421 (Asturias & Timmis, 1993; Maeda *et al.*, 1995). Also, strain BN6 encodes at least three different extradiol dioxygenases, BphC, BphC2 and the 1,2-dihydroxynaphthalene dioxygenase which participates in the degradation of naphthalenesulfonates. According to the structure of the holoenzyme (dimer vs octamer), the amino-terminal amino acid sequence and the substrate specificity, BphC2 is clearly distinct from the 1,2-dihydroxynaphthalene dioxygenase from strain BN6 (Kuhm *et al.*, 1991).

The deduced amino acid sequence of BphC2 from strain BN6 was significantly different to the corresponding sequences of the members of the main superfamily of extradiol dioxygenases which is represented by enzymes like catechol 2,3-dioxygenase from TOL or NAH7 plasmids, DHBPDO from *Pseudomonas* sp. LB400 or *P. putida* KF715, and 1,2-dihydroxynaphthalene dioxygenase from NAH7 (Harayama *et al.*, 1992). The highest degree of amino acid identity was found between BphC2 and MpcII from *A. eutrophus* JMP 222. The dendrogram obtained by pairwise alignment of the amino acid sequences suggested that BphC2 clustered with MpcII and CmtC in a separate group (Fig. 2). Unfortunately, there is little published information about MpcII from *A. eutrophus* JMP 222 other than the nucleotide

sequence and limited data about its oxidation of catechol, 3-methylcatechol and 4-methylcatechol (Kabisch & Fortnagel, 1990b). Induction of a catechol 2,3-dioxygenase has been found in *A. eutrophus* JMP 134 (= JMP 222 harbouring plasmid pJP4) after growth of the strain with 2-methylphenoxyacetate (Pieper *et al.*, 1988). Consequently, MpcI or MpcII could be functional in the degradation of this substrate.

It has been suggested that the members of the major class of extradiol dioxygenases all contain an amino acid consensus sequence ([G,T,N,R]X[H,A]XXXXXXXX-[L,I,V,M,F]YXX[D,E,T,N,A]PX[G,P]X[2,3]E) (Boldt *et al.*, 1995). A part of this consensus sequence has also been identified in BphC from strain BN6 (IYFYD-PDGHMLE) (which is equivalent to the underlined part of the proposed consensus sequence shown above) (Heiss *et al.*, 1995). The alignment of the deduced amino acid sequences from BphC2 from strain BN6 and MpcII from *A. eutrophus* JMP 222 demonstrated an almost perfect conservation of the sequence G[Q,R]HVLG-[A,S]NYF[Y,F],YV[R,L]DPWGSF[S,C]EYSADID (aa 252–279 in BphC2 and 257–284 in MpcII, Fig. 3) in both proteins, which is (at least for the sequence of BphC2) included in the proposed consensus sequence for extradiol dioxygenases (the corresponding amino acids are shown in bold letters above). This suggests that the catalytic centre of BphC2 resembles those of the major class of extradiol dioxygenases. This was also suggested by the observation that the enzyme was inactivated by H_2O_2 and could at least partially be reactivated by the addition of Fe^{2+} ions.

Comparison of the UV/visible spectra of the products formed from 3,5-dichlorocatechol by BphC and BphC2 ($\lambda_{\max}$ at 389 and 337 nm, respectively) clearly demonstrated that two different products were formed. It seems reasonable to assume that one of the products results from a proximal mode of dioxygenolytic cleavage and the other product from a distal cleavage of the substrate. The product of the proximal cleavage would be the acyl chloride of a chlorohydroxy-substituted muconic acid. For the structurally related product (= 2-hydroxymuconate) of the 1,2-dioxygenolytic cleavage of 1,2,3-trihydroxybenzene (pyrogallol), a $\lambda_{\max}$ at 290 nm has been described (Saeki *et al.*, 1980). In contrast, the distal cleavage of 3,5-dichlorocatechol should give a dichlorinated muconic acid semialdehyde with the characteristic $\lambda_{\max}$ at 375–390 nm. Thus 3,5-dichlorocatechol was probably oxidized by BphC by a distal cleavage mechanism, whereas BphC2 oxidized this compound in a proximal manner. This is in good agreement with the fact that BphC oxidized 3-chlorocatechol to a product with $\lambda_{\max}$ at 378 nm which can only be explained by a distal cleavage of 3-chlorocatechol (Heiss *et al.*, 1995). Presumably, BphC2 oxidized 3-chlorocatechol and 3,5-dichlorocatechol with low initial enzyme activities by proximal cleavage to the corresponding acyl chlorides which hydrolyse spontaneously to the muconic acids. Because the product from 3-chlorocatechol (2-hydroxymuconic acid) has a $\lambda_{\max}$ at 290 nm it cannot be detected spectrophotometrically.

metrically in the presence of cell-free extract. In contrast, the reaction of 3,5-dichlorocatechol results in the formation of a $\lambda_{\max}$ at 337 nm, which can be monitored sensitively using a spectrophotometer. Wasserfallen *et al.* (1991) showed that the catechol 2,3-dioxygenase from *P. putida* converted approximately 1000 molecules of 3-chlorocatechol before being inactivated. We are currently attempting to isolate the reaction products to definitively prove their structures.

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