Cloning of new *Rhodococcus* extradiol dioxygenase genes and study of their distribution in different *Rhodococcus* strains

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Four extradiol dioxygenase genes which encode enzymes active against catechol and substituted catechols were cloned from two different *Rhodococcus* strains, and their nucleotide sequences were determined. A catechol 2,3-dioxygenase gene (*edoC*) was shown to be identical to the previously described *ipbC* gene from the isopropylbenzene operon of *Rhodococcus erythropolis*. Amino acid sequences deduced from the three other genes (*edoA*, *edoB* and *edoD*) were shown to have various degrees of homology to different extradiol dioxygenases. The *EdoA* and *EdoB* dioxygenases were classified as belonging to the third family of type I oxygenases and represented two new subfamilies, whereas the *EdoD* dioxygenase was a type II enzyme. Analysis of six *Rhodococcus* strains revealed a wide distribution of the above dioxygenase genes. *Rhodococcus* sp. I1 was shown to harbour all four of the analysed dioxygenase genes. Nucleotide sequences homologous to the *edoB* gene were present in all of the strains, including *R. erythropolis* NCIMB 13065, which did not utilize any of the aromatic compounds analysed. The latter finding points to the existence of a silent pathway(s) for degradation of aromatic compounds in this *Rhodococcus* strain.

Keywords: *Rhodococcus*, catechol, dioxygenase, PCR, biodegradation

INTRODUCTION

Gram-positive bacteria of the genus *Rhodococcus* demonstrate a remarkable ability to utilize a wide variety of xenobiotic compounds (Finnerty, 1992). However, despite a growing interest in the role of *Rhodococcus* spp. in the environment, relatively little is known of the genetic determinants which control the biodegradation routes in these micro-organisms and their distribution amongst different strains. Ring cleavage is an important step in the aerobic degradation of aromatic compounds and catechols are typical substrates for such reactions. This cleavage is catalysed by the enzymes belonging to one of two different classes: intradiol or extradiol dioxygenases (Harayama *et al.*, 1992). It is well-established that extradiol enzymes with activities against

catechol and substituted catechols are key enzymes in the bacterial degradation of aromatic compounds (Hayaishi, 1974; Harayama *et al.*, 1992). According to the most recent classification, based on a structure-validated alignment of 38 extradiol dioxygenase sequences and their subsequent phylogenetic analysis, these enzymes form two different types: type I includes 35 dioxygenases belonging to five families and type II includes only two enzymes which show low mutual sequence identity and probably belong to different families (Eltis & Bolin, 1996). Several extradiol dioxygenase genes from *Rhodococcus* spp. utilizing biphenyl have been cloned and characterized (Masai *et al.*, 1995; Maeda *et al.*, 1995). It has been shown that multiple *bphC* extradiol dioxygenase genes with different structural characteristics may be found in some biphenyl-degrading *Rhodococcus* strains (Asturias *et al.*, 1994; Maeda *et al.*, 1995).

A number of *Rhodococcus* biodegrading strains isolated in the Questor microbiology laboratory have been

The GSDB accession numbers for the sequences reported in this paper are L77225 (*edoA* gene), GSDB:S:1050684 (*edoB* gene), GSDB:S:1076510 (*edoC* gene) and GSDB:S:1076511 (*edoD* gene).

shown to degrade aromatic and aliphatic compounds (Larkin & Day, 1986; Curragh *et al.*, 1994). The aim of this study was to analyse the diversity of dioxygenase genes in *Rhodococcus*. We present cloning and sequence analysis of *Rhodococcus* dioxygenase genes from six strains which were isolated as naphthalene, cresol and 1-chloroalkane degraders. This analysis showed that highly homologous dioxygenase genes are probably widely distributed amongst *Rhodococcus* species.

METHODS

Bacterial strains and plasmids. *Escherichia coli* strain DH5 α (Sambrook *et al.*, 1989) and plasmid vectors pUC129 (Keen *et al.*, 1988) and pBluescript KS(+) were used for the cloning experiments. The Nah⁺ derivative P200 of *Rhodococcus rhodochrous* NCIMB 13064 (Kulakova *et al.*, 1996) and *Rhodococcus* sp. NCIB 12038 (Larkin & Day, 1986) have been described previously. The characteristics of other *Rhodococcus* strains isolated from soil are presented in Table 1.

Media and growth conditions. *Rhodococcus* and *E. coli* strains were propagated in a rich (2YT) or minimal (M9) medium (Miller, 1972). When required, Difco agar (1.8%, w/v) was added to the medium. When the cultures were grown on minimal agar plates, naphthalene, cresols, toluene, biphenyl, isopropylbenzene and 1-chloroalkanes were added as vapours. Ampicillin (100 μ g ml⁻¹), tetracycline (15 μ g ml⁻¹), IPTG (50 μ g ml⁻¹) and X-Gal (50 μ g ml⁻¹) were used for the detection of recombinant plasmids.

DNA techniques. Total DNA from *Rhodococcus* strains was isolated as described by Kulakova *et al.* (1995). Recombinant DNA work was done by using standard protocols as described by Sambrook *et al.* (1989). Restriction of both total DNA and recombinant plasmids was performed using enzymes obtained from Pharmacia, according to the manufacturer's instructions. The Sephaglas BandPrep kit (Pharmacia) was used for the recovery of restriction fragments and PCR products from agarose gels.

DNA fragments were transferred to Z-probe membranes (Bio-Rad) by Southern blotting, as described by Sambrook *et al.* (1989). The membranes were then treated according to the manufacturer's instructions. Hybridization probes were labelled with [³²P]dCTP using an oligolabelling kit (Pharmacia) and hybridization was carried out in 0.25 M Na₂HPO₄, pH 7.2, 7% (w/v) SDS at 60 °C for 10–12 h.

Enzyme assays. Cell-free extracts were prepared as described previously (Kulakova *et al.*, 1996). Catechol 2,3-dioxygenase and 3-methylcatechol and 4-methylcatechol 2,3-dioxygenase activities were assayed spectrophotometrically (Gibson, 1971) in 50 mM Tris/HCl buffer (pH 7.8), 0.1 mM DTT, 10% glycerol, 10% ethanol, and 2,3-dihydroxybiphenyl dioxygenase activity was assayed as described by Hauschild *et al.* (1996). The relative ring cleavage activities were determined from the absorption coefficients of the ring fission products formed from the following substrates: catechol (λ_{max} , 375 nm; ϵ = 33000 cm⁻¹ M⁻¹); 3-methylcatechol (λ_{max} , 388 nm; ϵ = 19400 cm⁻¹ M⁻¹); 4-methylcatechol (λ_{max} , 382 nm; ϵ = 28100 cm⁻¹ M⁻¹); 2,3-dihydroxybiphenyl (λ_{max} , 434 nm; ϵ = 13200 cm⁻¹ M⁻¹). 1,2-Dihydroxynaphthalene dioxygenase activity was measured as described by Patel & Barnsley (1980) and modified by Kuhm *et al.* (1991). Relative activities were expressed as percentages of the activity observed in cell-free extracts of DH5 α (pUP25) when catechol was the substrate, which was defined as 100%. Protein concentrations were determined using the BCA assay (Pierce).

Cloning. Total DNA isolated from *Rhodococcus* strains was digested with *Eco*RI, *Bam*HI or *Xho*I restriction endonucleases. The preparations were ligated with plasmid pUC129, digested with the corresponding enzyme and treated with alkaline phosphatase (Pharmacia) according to the manufacturer's instructions. The resulting recombinant plasmids were used to transform *E. coli* DH5 α . Clones that expressed catechol 2,3-dioxygenase and/or 3-methylcatechol 2,3-dioxygenase activities were identified by their yellow colour after spraying with a 0.1 M solution of corresponding ring cleavage substrate (Zukowski *et al.*, 1983). A Pharmacia kit utilizing exonuclease III and S1 nuclease was used to generate unidirectional deletion derivatives.

Table 1. Substrate utilization by *Rhodococcus* strains

Rhodococcus cultures were tested for growth on minimal agar (M9) plates at 30 °C with the corresponding compound as sole source of carbon and energy. All compounds listed were added as vapour. Plates were analysed for bacterial growth after 3–5 d. BP, biphenyl; Nah, naphthalene; IPB, isopropylbenzene; *m*-Cr, *m*-cresol; *o*-Cr, *o*-cresol; Tol, toluene; C₄Cl, 1-chlorobutane. +, Good growth; +/–, poor growth; –, no growth.

Strain	Selection substrate	Utilization of:						
		BP	Nah	IPB	<i>m</i> -Cr*	<i>o</i> -Cr	Tol	C ₄ Cl
<i>R. rhodochrous</i> P200	Nah	+ / –	+	–	+ / –	–	+	–
<i>Rhodococcus</i> sp. Acr33	<i>m</i> -Cr	+ / –	+	–	+	–	+ / –	–
<i>Rhodococcus</i> sp. A1	<i>m</i> -Cr	+ / –	–	–	+	+	–	–
<i>Rhodococcus</i> sp. I1	<i>m</i> -Cr	+ / –	+	+	+	+	+	–
<i>R. erythropolis</i> NCIMB 13065†	C ₄ Cl	–	–	–	–	–	–	+
<i>Rhodococcus</i> sp. NCIB 12038	Nah	–	+	–	+	+	–	–

* Identical results were obtained when *p*-cresol was used as growth substrate.

† Benzene, phenol, coumarin and phenanthrene were also tested as growth substrates for NCIMB 13065; no growth with any of these compounds was detected.

DNA sequencing. Plasmids pUC129 and pBluescript KS(+) with insertions containing extradiol dioxygenase genes were used as templates for the *Taq* Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems). DNA sequences were obtained using an automatic DNA sequencer (Applied Biosystems, model 373A). The nucleotide sequences of both strands were determined. Sequencing of PCR products was performed after template purification from agarose gels using corresponding PCR and/or internal primers. Primer synthesis was performed by Dr C. Stevenson (School of Biology and Biochemistry, The Queen's University of Belfast). Computer analysis of the sequences was performed using the DNASIS (Hitachi) software package. Alignment of the protein sequences was performed using the Higgins–Sharp algorithm (Higgins & Sharp, 1988), and the TREECON program (Van de Peer & De Wachter, 1994) was used for sequence analysis. Searches for nucleotide and amino acid sequence similarities were done by using the FASTA and BLAST programs (Pearson & Lipman, 1988) in the EMBL and GenBank databases.

PCR. PCR primers were synthesized to amplify the internal parts of the cloned and sequenced *Rhodococcus* dioxygenase genes. The primers were designed manually as well as by using the DNASIS package. Five sets of primers were so obtained: LK16, GAG GAC CCG AGC GGA ATC A (439–457 bp), and LK14, GAC CGC AGC GGC TAC GGG T (1071–1053 bp), producing an *edoA* PCR product of 633 bp; VD11, GGT TAC ATG GGC TTC GAG (54–71 bp), and VD14, CTC CGC CGA CTT CTC CAG (1149–1132 bp), producing an *edoC* PCR product of 1096 bp; LK33, CGC ATC GAG GCC ACC GAC (198–215 bp), and LK38, CGA CGG GTC CTC GAA CGT GA (503–484 bp), producing an *edoB* PCR product of 306 bp; LK33 and LK13, TCA GGT CGT TGA CGT GGC G (924–906 bp), producing an *edoB* PCR product of 727 bp; VD6, CTT CTA CAA GCT CAT GCC (1014–1032 bp), and VD3, CCG GTC GCT GCC TCG AGC (1416–1399 bp), producing an *edoD* PCR product of 403 bp. All primers were tested in PCR experiments using total DNA of the host strains from which corresponding dioxygenase genes were cloned. The authenticity of the products was confirmed by sequencing analysis.

Amplification of catechol dioxygenase gene sequences was done with *Taq* DNA polymerase (Stratagene) in a buffer supplied by the manufacturer. Reactions were carried out in volumes of 25 µl or 50 µl with concentrations of dNTPs at 200 µM and primers at 0.15 µM each. The following temperature cycle was used for PCR: denaturation at 95 °C for 3 min and then 95 °C for 40 s, 60 °C for 30 s, 72 °C for 1 min, for 30 cycles.

16S rRNA analysis. Amplification primers (27f, CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G; and 519r, GGG ATC CAA GCT TGT/A ATT ACC GCG GCG/T GCT G), located in the conserved region of the 16S rRNA gene, were adapted from Schuppler *et al.* (1995). The following temperature profile was used for the PCR amplification: denaturation at 95 °C for 2 min and then 92 °C for 20 s, 50 °C for 20 s, 72 °C for 1 min, for 30 cycles. PCR fragments were purified by agarose gel electrophoresis and the sequence of both strands was obtained as described previously. The partial 16S rDNA sequences (400 bp) were compared with all available 16S rRNA sequences in the EMBL and GenBank databases using the program BLAST. Sequences were then aligned with sequences from representative *Rhodococcus* spp. (EMBL and GenBank databases) using the DNASIS program. Distance matrices were produced by using the program TREECON (Van de Peer & De Wachter, 1994) and

trees were constructed by the neighbour-joining method with the same program. The reliability of tree nodes was analysed by bootstrapping. One hundred bootstrap trees were generated.

RESULTS

Classification and initial characterization of *Rhodococcus* strains

Partial 16S rDNA sequences (400 bp) were obtained for six *Rhodococcus* strains and these sequences were used for alignment and phylogenetic analysis. This analysis confirmed previous classification of *R. rhodochrous* NCIMB 13064 and *Rhodococcus erythropolis* NCIMB 13065. Three strains isolated from a cresol-contaminated site (A1, I1 and Acr33) were all classified as *Rhodococcus* spp. They showed the closest relationship to *Rhodococcus opacus* and *Rhodococcus marinonanscens*; however, complete sequence analysis of 16S rRNA genes and further taxonomic tests would probably be required for the final classification. The previously described naphthalene-degrading *Rhodococcus* sp. NCIB 12038 (Larkin & Day, 1986) was also shown to be closely related to *R. opacus*, *R. marinonanscens* and *Rhodococcus* strains studied in this work.

The *Rhodococcus* strains were tested for use of a range of substrates as carbon and energy sources. The results of the utilization of some of the aromatic compounds as well as 1-chlorobutane are presented in Table 1. Each strain differed in its ability to utilize aromatic compounds and *Rhodococcus* sp. I1 possessed the widest substrate range of all the strains. *R. erythropolis* NCIMB 13065, which was isolated on 1-chloroalkane, did not grow on any of the aromatic compounds tested.

Cloning of dioxygenase genes

R. rhodochrous P200 and *Rhodococcus* sp. I1 were chosen for the cloning of catechol oxygenase genes. From the pUC129 library of *Rhodococcus* sp. I1 *Bam*HI fragments, two positive clones were isolated from approximately 5×10^3 colonies sprayed with catechol and 3-methylcatechol. Fourteen positive clones were isolated from 2×10^4 clones of an *R. rhodochrous* P200 pUC129 library of *Bam*HI fragments. Yellow colouration of differing intensities developed in clones after incubation of between 3 and 30 min at room temperature. Both I1 clones were investigated, i.e. pUI11 (6.0 kbp insert) and pUI12 (3.2 kbp insert). Positive clones obtained from the P200 library were analysed using Southern hybridization (at 65 °C) and three non-homologous clones were chosen for further study. These clones were pUP25 (4.7 kbp insert), pUP22 (8 kbp insert) and pUP210 (3.5 kbp insert). Further analysis showed that the nucleotide sequence of pUP210 was 98% homologous to that of pUI12 (*Rhodococcus* sp. I1 clone).

Activities of the products of cloned dioxygenase genes against catechol and other compounds were determined

Table 2. Activities expressed by cloned *Rhodococcus* dioxygenase genes in *E. coli* DH5 α cells

DH5 α cells with recombinant plasmids were grown in 2YT medium. Activities of the enzymes were measured in cell-free extracts. The results are expressed as a mean of triplicate experiments. CAT, catechol; 3MC, 3-methylcatechol; 4MC, 4-methylcatechol; 23DHB, 2,3-dihydroxybiphenyl; DHN, 1,2-dihydroxynaphthalene. Activities are expressed as a percentage of the activity in DH5 α (pUP25) cell-free extracts (*edoA*) when catechol was the substrate, which was defined as 100% [corresponds to 0.95 nmol min⁻¹ (mg protein)⁻¹].

Gene (host)	Plasmid	Relative activity				
		CAT	3MC	4MC	23DHB	DHN
<i>edoA</i> (P200)	pUP25	100	444	2	2884	<1
<i>edoC</i> (I1)	pUI11	13958	66316	1661	599684	<1
<i>edoB</i> (P200)	pUP22	<1	323	<1	3620	<1
<i>edoD</i> (I1)	pUI12	41	337	138	640	<1
<i>edoD</i> (P200)	pUP210	12	72	27	150	<1

Table 3. Sequencing analysis of the *Rhodococcus* extradiol dioxygenase genes

DNA sequences were obtained using an automatic DNA sequencer (Applied Biosystems, model 373A). The nucleotide sequences of both strands were determined. Computer analysis of the sequences was performed using the DNASIS (Hitachi) software package. All four genes (*edoA*, *edoB*, *edoC* and *edoD*) are preceded by putative ribosome-binding sites (Shine & Dalgarno, 1974).

Gene (plasmid)	Host strain	Sequenced fragment (bp)	Coding region (bp)	Putative protein (aa/Da)	Accession no.
<i>edoA</i> (pUP25)	P200	1215	118–1072	318/35435	L77225
<i>edoB</i> (pUP22)	P200	1142	171–1071	300/33506	GSDB:S:1050684
<i>edoC</i> (pUI11)	I1	1338	36–987	317/35014	GSDB:S:1076510
<i>edoD</i> (pUI12)	I1	1995	844–1804	320/34426	GSDB:S:1076511

in cell-free extracts (Table 2). Surprisingly, 1,2-dihydroxynaphthalene dioxygenase activity was not detected in cell-free extracts of any clone obtained from strains P200 and I1, even though both strains utilized naphthalene as a growth substrate. However, all dioxygenases expressed activity against 2,3-dihydroxybiphenyl. Two genes encoding enzymes which expressed activities against catechol and 3-methylcatechol were designated *edoA* (pUP25) and *edoC* (pUI11). Two other genes which encoded enzymes with significantly lower (or no) activities against catechol than 3-methylcatechol were designated *edoB* (pUP22) and *edoD* (pUI12 and pUP210). In the case of *edoB* (plasmid pUP22), no activity was detected in *E. coli* cell-free extracts against catechol and 4-methylcatechol (Table 2).

Nucleotide sequence analysis of dioxygenase genes

The *edoA*, *edoB* and *edoD* genes from *R. rhodochrous* NCIMB 13064, as well as *edoC* and *edoD* genes from *Rhodococcus* sp. I1, were sequenced. The results of the analysis of the *Rhodococcus* dioxygenase genes are summarized in Table 3. The *edoC* gene showed high

homology (98% identity) with *Rhodococcus* sp. RHA1 2,3-dihydroxybiphenyl dioxygenase I (Masai *et al.*, 1995). Also, the nucleotide sequence of the *edoC* gene was identical to the *ipbC* gene from the isopropylbenzene operon of *R. erythropolis* BD2 (Kesseler *et al.*, 1996).

Several putative initiation codons in the same open reading frame (171–1071 bp) were found in the 1142 bp fragment of pUP22 encoding the *edoB* dioxygenase (Table 3). The analysis of unidirectional deletions of plasmid pUP22 produced with exonuclease III showed that the ORF of the *edoB* gene probably started at position 171 bp and encoded a protein of 300 aa with a calculated molecular mass of 33506 Da (results not shown).

Highly homologous sequences of the *edoD* gene were found in clones from *R. rhodochrous* P200 and *Rhodococcus* sp. I1 (plasmids pUI12 and pUP210; 98% homology). Only the nucleotide sequence of the *edoD* gene from strain I1 obtained from the plasmid pUI12 has been deposited in the database (Table 2). Two ORFs were found in the *edoD* region with the positions bp

BphC	MS-IRSLGYMGFAVSDVAWRSFLTQKLGLMEAGTTDNGDLF-RIDSRW	50
EdoA	MALVTGIGYIGIGVSDLPWEEF-AETIGFQIRERGEDGTYLRLMDKAHH	50
EdoC	MS-VQRLGYMGFEVADVPWAFMTEKLGAMEASSSENSARF-RIDSRW	50
EdoB	MS-IRSLAYMRIEATDMAAWREYGLKVLGMVEGKSGDPDALYLRMDFFPA	50
	* * * * *	
BphC	RIAVQOGEVDDLAFAGYEVADAAGLAQMAADKLKQAGIAVTTGDASLARRR	100
EdoA	RVAVHPTGEDDLTYVGWQVADENGFDLELRTLRAAGVPVEMAGEDDAELR	100
EdoC	RLMVEKGPSDDISLGSYEVESEDSLLAIKRLAEAGIEVTESGELADDR	100
EdoB	RLVIFPGEQDRLSVSGWETANAELQEVDRNLSAAGVAFKEGTAEQLQDR	100
	* * * * *	
BphC	GVTGLITFADPFGLPLEIYYGASEVFEKPFPLGAAVSGFLTGEQGLGHFV	150
EdoA	GVARLMRFEDPSGIKSEAYYGLVSEPEVPYVSPYAV-DFVTEDQGFHIV	150
EdoC	GVLGLISCTDTANTRVEIYYGATELFEKPFISPTGVSGFTTGDQGFHIV	150
EdoB	RVDELITFEDPSGNTLEAFHGAA-LEHRRVSPYGHK-FVTGEQGLGHV	150
	* * * * *	
BphC	RCVPDSKALAFYTDVLFQGLSDVIDMKMG-----PD-VTVPAYFLHCNE	200
EdoA	VMVDDYDETMRFYREVLGLQTSDLV--KVG-----AGGVQTRMAFMRCNP	200
EdoC	LAVPDIDAALDFYVKGGLGFHLSDIIDWKLN-----DE-LTVKLHFLHCNE	200
EdoB	LSTTDEASLRFYRDVLFGLRLRDSMLPQMVGRPADGKPAWLRFVFGCNP	200
	* * * * *	
BphC	RHHTLAIAAFPLPKRIHHEMLEVASLDDVGFADFRVDADGL-ITSTLGRH	250
EdoA	RQHSLAFWAGDSTTRLNHFMLQTQTLDQGTMDLRDRCFHGGI-PATNLGRH	250
EdoC	RHHTLALAGLPAGAKTHHEMLETKHMDVGLAYDKFDADGT-VVMTLGRH	250
EdoB	RHHSALFLPMPTPSGIVHLMIEVENSDDVGLCLDRALRKKVKMSATLGRH	250
	* * * * *	
BphC	TNDHMVSFYASTPSGVEVEYGWGSAVTVDRS-WVVVRHDSPSMWGHKSVRD	300
EdoA	VNDYAVSFYITTPSGFMIEYGWGVREVVSD-YPVDKYRSVSIWGHRLDG	300
EdoC	TNDHMLSFYGTATPSGFAVEYGWGARQVEPG-WSVVRDYKISIWGHKFVAE	300
EdoB	VNDLMLSFYMKTPGGFDIEFGCEGRQVEDESWARESTAVSLWGHDFS VG	300
	* * * * *	
BphC	K--A-----AARNKA	329
EdoA	IHYTQALFPPEAAESPAEQHLVEEPVAAA	329
EdoC	R--DRQVSSNAIEDELIDIDATLSAPAQA	329
EdoB	MQ-----P	329

Fig. 1. Alignment of the dioxygenases belonging to the type I, family 3 enzymes. EdoA, EdoC and EdoB dioxygenases were analysed in this work; BphC is *P. cepacia* LB400 2,3-dihydroxybiphenyl dioxygenase (Hofer *et al.*, 1993; accession no. X66122) of the same family. Residues conserved in all four proteins are indicated by asterisks. The conserved catalytic residues are given in bold and underlined.

190–787 (ORF1) and bp 844–1804 (ORF2). On the basis of the analysis of exoIII/S1 deletion derivatives of plasmid pUI12, ORF2 was shown to encode EdoD.

Amino acid sequences derived from the four dioxygenase genes were analysed. Alignment of EdoB, EdoA and EdoC with BphC LB400 2,3-dihydroxybiphenyl dioxygenase of *Pseudomonas (Burkholderia) cepacia* LB400 (Hofer *et al.*, 1993) of type I, family 3, for which X-ray structures have been analysed (Han *et al.*, 1995), is shown in Fig. 1. This alignment shows that three metal-binding ligands, His-146, His-210 and Glu-260, and three additional active site residues, His-195, His-241 and Tyr-250, of BphC LB400 are conserved in EdoB, EdoA and EdoC. The same degree of conservation was shown for all other type I extradiol dioxygenases (Eltis & Bolin, 1996). It is important to note that the detected level of amino acid identity did not allow assignment of EdoB or EdoA into any of the five existing subfamilies of family 3. According to Eltis & Bolin (1996), enzymes belonging to the same subfamilies in type I exhibit $\geq 54\%$ amino acid identity, significantly higher than similarities observed in comparisons between EdoA, EdoB and other type I.3 extradiolic dioxygenases. Fig. 2 is a dendrogram showing relationships between the *Rhodococcus* Edo sequences and other type I.3 extradiolic dioxygenases; EdoA and EdoB represent two new proposed subfamilies (F and G).

EdoD (*Rhodococcus* sp. I1) showed 47% amino acid

identity with the Mpc1 type II catechol 2,3-dioxygenase I from *Alcaligenes eutrophus* JMP222 (Kabisch & Fortnagel, 1990), and since it does not show similarity with any of the type I enzymes, EdoD evidently belongs to the type II dioxygenases. The alignment of EdoD with two type II dioxygenases (Mpc1 and MhpB) and one recent entry found in the database is presented in Fig. 3, and shows conservation of amino acid residues in the regions 54–67 and 195–208 aa. These three dioxygenases may be considered as belonging to the same type II family (II.2). A much lower level of similarity was observed between the above enzymes and LigB, subunit B of protocatechuate 4,5-dioxygenase from *Pseudomonas (Sphingomonas) paucimobilis* (Noda *et al.*, 1990). However, three histidines and four aspartates, which could possibly comprise the iron coordination sphere (Eltis & Bolin, 1996), are conserved in all four sequences (Fig. 3).

Distribution of extradiol dioxygenase genes in *Rhodococcus* strains

DNA fragments containing *Rhodococcus* dioxygenase genes *edoA* (1.2 kb fragment), *edoC* (1.3 kb), *edoB* (1.5 kb) and *edoD* (1.4 kb) were used as probes for hybridization with *EcoRI* and *BamHI* digests of total DNA obtained from all strains employed in this study. Internal primers designed on the basis of known

Fig. 2. Phylogram of the alignment of the family 3 (type I) extradiol dioxygenases. One enzyme from each of the five subfamilies (Eltis & Bolin, 1996) is present. BphC (RHA1), *Rhodococcus* sp. RHA1 2,3-dihydroxybiphenyl dioxygenase (Masai *et al.*, 1995; accession no. D32142); BphC (LB400), *P. cepacia* LB400 2,3-dihydroxybiphenyl dioxygenase (Hofer *et al.*, 1993); DbfB, *Sphingomonas paucimobilis* RW1 2,2',3-trihydroxybiphenyl dioxygenase (Happe *et al.*, 1993; accession no. X72850); NahC, *Pseudomonas putida* PpG7 1,2-dihydroxynaphthalene dioxygenase (Harayama & Rekik, 1989; accession no. J04994); PcbC, *Pseudomonas* sp. DJ12 2,3-dihydroxybiphenyl dioxygenase (Kim *et al.*, 1996; accession no. D44550). EdoA, EdoC and EdoB dioxygenases were analysed in this work; NahH, catechol 2,3-dioxygenase from *P. putida* PpG7 (Harayama *et al.*, 1987; accession no. X06412), was used as an outgroup. Distance matrices were produced with the program TREECON (Van de Peer & De Wachter, 1994) using Poisson correction (Zuckerkandl & Pauling, 1965), and trees were constructed by the neighbour-joining method with the same program. The reliability of tree nodes was analysed by bootstrapping. One hundred bootstrap trees were generated. Abbreviations, e.g. 3.A, designate family (3) and subfamily (A) in accordance with Eltis & Bolin (1996). Subfamilies I.3.F and I.3.G are proposed by this study to contain EdoA and EdoB, respectively. Bootstrap values (%) are indicated at the nodes.

sequences of *edoA*, *edoC*, *edoB* and *edoD* genes were used in PCR experiments to amplify the homologous sequences from the different *Rhodococcus* strains. The results of these experiments are presented in Table 4. The sizes of all PCR products corresponded to those obtained with DNA of strains P200 and I1 (hosts for cloned dioxygenase genes) and calculated from the sequence data. Complete nucleotide sequences of PCR fragments were obtained (633 nt for *edoA*; 727 nt for *edoB*; 1096 nt for *edoC*; and 403 nt for *edoD*) and compared with the sequences of the genes cloned from strains P200 and I1. The results (Table 4) revealed that highly homologous sequences of extradiol dioxygenase genes were present in the *Rhodococcus* strains. Four of the six strains contained more than one *edo* sequence and sequences homologous to *edoB* were found in all six strains. It is interesting to note that *R. erythropolis* NCIMB 13065 contained an *edoB*-type sequence even though it could not utilize any of the aromatic compounds tested (Table 1). Sequences corresponding to *edoA* were found in four *Rhodococcus* strains, and only

one gene (*edoC*) appeared to be uniquely represented; that is in strain I1.

DISCUSSION

Four *Rhodococcus* dioxygenase genes with activities against catechol and substituted catechols were cloned and sequenced. Only one gene (*edoC*) was shown to be homologous to previously analysed dioxygenases (Masai *et al.*, 1995; Kessler *et al.*, 1996), being assigned to the I.3.B subfamily according to the classification scheme of Eltis & Bolin (1996). The other three dioxygenase genes may be considered as essentially new in that they do not show high homology with any of the database entries. Amino acid sequences deduced from these genes were shown to have various degrees of homology to different extradiol dioxygenases. EdoA and EdoB were assigned to new subfamilies of the type I oxygenases (Eltis & Bolin, 1996), and EdoD dioxygenase was a type II enzyme.

The *Rhodococcus* strains analysed in this work showed a remarkable diversity. Three different dioxygenase genes, belonging to two different types of these enzymes, were cloned from *R. rhodochrous* P200 and all four oxygenase sequences analysed were found in *Rhodococcus* sp. I1. The analysis of six *Rhodococcus* strains revealed that sequences homologous to *edoA*, *edoB* and *edoD* were present in different *Rhodococcus* species. Only the *edoC* gene from *Rhodococcus* sp. I1 was not found in any of the other analysed strains (Table 4). Furthermore, this strain was the only one capable of utilizing isopropylbenzene, which, when considering the identity of *ipbC* and *edoC*, suggests that *edoC* belongs to the *ipb* operon described previously (Kessler *et al.*, 1996). The *edoD* gene was cloned from two strains belonging to different species of *Rhodococcus* and sequences highly homologous to *edoA* were found in four different strains.

The most interesting of the genes analysed in this work was probably *edoB*, which was found in all of the strains studied. A high degree of homology was found between the nucleotide sequence of this gene (cloned from strain P200) and corresponding PCR fragments obtained from the other *Rhodococcus* strains. The lowest homology observed was with NCIMB 13065 (Table 3). However, the translated amino acid sequence (obtained from a 900 bp PCR fragment, strain NCIMB 13065) exhibited 85% identity to that of the *edoB* gene. The finding of a gene homologous to *edoB* in *R. erythropolis* NCIMB 13065 is particularly noteworthy, since this strain was isolated as a 1-chloroalkane degrader and showed no ability to utilize any of the analysed aromatic compounds. The haloalkane dehalogenase (DhaA) of strain NCIMB 13064 was shown to be encoded by a 100 kbp plasmid pRTL1 (Kulakova *et al.*, 1995, 1997), and, remarkably, amongst a number of Dha⁻ derivatives from this strain, two (P200 and P400) unexpectedly acquired the ability to utilize naphthalene as a sole carbon and energy source (Kulakova *et al.*, 1996). The

EdoD	MTLALVCTSHSPLEFNNPPPEVRTEVD-----RAFAQARQFIEEYDPD	50
MhpB	MHAYLHCLSHSPLVGYVDPQAEVLDEVN-----GVIASARERIAAFSPE	50
Mpc1	MPIQLCELSHTPLHGYVDPPEVVAEVE-----RVQAAARDRVRAFDE	50
LigB	MARVTGTITSSHIPALGAAIQTGTSNDNDYWGPFVKGYQPIRDWIKQPGNM	50
	* + + + + + + + + + + + + + + + + + + +	
EdoD	L--VVSFAPDHYNGFFYKLMPSFCIGFEASGVGDFGSSA---GRLDVP--	100
MhpB	L--VVLFAPDHYNGFFYDVMPPFCLGVGATAIGDFGSAA---GELFVP--	100
Mpc1	LFTVVVFAPDHFNGFFYDVMPPFCIGAAATAIGDFKSLA---GKLPVP--	100
LigB	PDVVILVYNDHASAFDMNIIPTEAIGCAET---FKPADEGWGPRPVPDV	100
	+ *+ +++++ +++++ +* +* + + + + + + + + + + +	
EdoD	---SALAEQMAQSVLDQGVDAVSL---EMEVDHGAQVPLEILLGGIATKP	150
MhpB	---VELAEACAHAVMKSGIDLAVSYC---MQVDHGAQVPLEILLGGLDKVP	150
Mpc1	---ADLALSLESVMAADIDFTVALSHRMQVDHGACADALALTGSLHRYP	150
LigB	KGHDPDLAWHIAQSLILDEFDMTIM---NQMDVDHGCTVPLSMIFGEPEEWP	150
	** * + * + * **** * + * + *	
EdoD	--VIPIFVNSVAAPFAPMKRIRLLGEAVGTFLK--NFDGNVLLSGGGLS	200
MhpB	--VLPVFINGVATPLPGFQTRMLGEAIGRFTS--TLNKRVLFLSGGGLS	200
Mpc1	--VIPVFINSVAPPMTLRRARLLGDAVGRFLSFTRAGKRVLVVSGGGLS	200
LigB	CKVIPFPVNVVTYPPPSGKRCFALGDSIRAAVESFPEDLVHVWGTGGMS	200
	* * + * * + * + * + + + + + + + + + +	
EdoD	HDPPVPRLEAATGEQRKMLLGCNHP--TAAARAVRQQRVIDTAKAF--TRG	250
MhpB	HQPPVPELAKADAHMRDRLLLSGGKDLPASERELRQQRVISAAEKV--ED	250
Mpc1	HEPPVPELAGASEEVAERLIA--GRN--PSPESAARQARTVAAAKSFVFTAG	250
LigB	HQ-----LQGPRLAGLINK-----	250
	* + + + + * + + + + + + + + + + + + +	
EdoD	EAGIMDLAPEWDRQLLDILASGELERLDATAKEMATAGNSAHEVRTWV	300
MhpB	QRTLHPLNPIWQFMFTLEQGRIQELDAVSNEELSAIAGKSTHEIKTWV	300
Mpc1	DSHLPLNPEWDRAFLSLASGELTAVDGMTNDAITRDGGKSAHEIRTWV	300
LigB	-----EFDLNFIDKLISDP--EELSKMPHIQYLRSESGVELVMWL	300
	+ + + + * + + + + + + + + + + + + +	
EdoD	AAHNALK-AAAGQYTVTSEYYRPIPEYIAGFAV-----TTAVPVHKTA	350
MhpB	AAFAAIS--AFGNWRSEGRYYRPIPEWIAGFG-----SLSART	350
Mpc1	AAFGALAFATAYGPYRASLDYFRAIPEWIAGFA-----TMHAEP	350
LigB	IMRGALPEKVRDLYT----FYH--IPASNTALGAMILQPEETAGTPLEPRK	350
	++ * + + * + + + + + + + + + + + + +	
EdoD	GLR-----	360
MhpB	EN-----	360
Mpc1	AAV-----	360
LigB	VMSGHSLAQA	360

Fig. 3. Alignment of the type II dioxygenases. EdoD, extradiol dioxygenase from *Rhodococcus* sp. I1 (this work); MhpB, 3-(2,3-dihydroxyphenylpropionate) 1,2-dioxygenase of *E. coli* strain CS520 (accession no. X97450); Mpc1, catechol 2,3-dioxygenase I from *Alcaligenes eutrophus* JMP222 (Kabisch & Fortnagel, 1990); LigB, subunit B of protocatechuate 4,5-dioxygenase from *P. paucimobilis* (Noda *et al.*, 1990). The amino acid residues identical in all sequences are indicated by asterisks and those identical only in the dioxygenases of the family II.2 are indicated by +.

Table 4. Distribution of dioxygenase gene sequences in *Rhodococcus* strains

Dioxygenase gene sequences were amplified by PCR as described in Methods. Southern hybridization was carried out at 65 °C. +, Dioxygenase sequence was detected in hybridization experiments and PCR product obtained; —, no homologous sequence was found. PCR fragments produced were sequenced and the nucleotide sequences were analysed. Percentage similarities of the sequences by comparison with the corresponding genes (*edoA* and *edoB* from *R. rhodochrous* P200; *edoC* and *edoD* from *Rhodococcus* sp. I1) are given in parentheses.

Strain	Sequence (percentage homology)			
	<i>edoA</i>	<i>edoC</i>	<i>edoB</i>	<i>edoD</i>
<i>R. rhodochrous</i> P200	+(100)	—	+(100)	+(98)
<i>Rhodococcus</i> sp. I1	+(95)	+(100)	+(100)	+(100)
<i>Rhodococcus</i> sp. A1	+(90)	—	+(97)	—
<i>Rhodococcus</i> sp. Acr33	+(95)	—	+(99)	—
<i>R. erythropolis</i> NCIMB 13065	—	—	+(85)	—
<i>Rhodococcus</i> sp. NCIB 12038	—	—	+(91)	—

Nah⁺ phenotype in these derivatives appeared to be fairly stable; however, Dha⁺ revertants, which arose at a frequency of about 10⁻⁸, had lost the ability to utilize naphthalene (Kulakova *et al.*, 1996). These results may

suggest the presence of cryptic biodegradation pathways in *Rhodococcus* species. So far we have not been able to relate the dioxygenase genes *edoA*, *edoB* and *edoD* to specific aromatic degradation pathways.

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