Studies on the isopropylbenzene 2,3-dioxygenase and the 3-isopropylcatechol 2,3-dioxygenase genes encoded by the linear plasmid of *Rhodococcus erythropolis* BD2

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The enzymes responsible for the degradation of isopropylbenzene (IPB) and co-oxidation of trichloroethene (TCE) by *Rhodococcus erythropolis* BD2 are encoded by the linear plasmid pBD2. Fragments containing IPB catabolic genes were cloned from pBD2 and the nucleotide sequence was determined. By means of database searches and expression of the cloned genes in recombinant strains, we identified five clustered genes, *ipbA1A2A3A4C*, which encode the three components of the IPB 2,3-dioxygenase system, reductase_{IPB} (*ipbA4*), ferredoxin_{IPB} (*ipbA3*) and the two subunits of the terminal dioxygenase (*ipbA1A2*), as well as the 3-isopropylcatechol (IPC) 2,3-dioxygenase (*ipbC*). The protein sequences deduced from the *ipbA1A2A3A4C* gene cluster exhibited significant homology with the corresponding proteins of analogous degradative pathways in Gram-negative and Gram-positive bacteria, but the gene order differed from most of them. IPB 2,3-dioxygenase and 3-IPC 2,3-dioxygenase could both be expressed in *Escherichia coli*, but the IPB 2,3-dioxygenase activities were too low to be detected by polarographic and TCE degradative means. However, inhibitor studies with the *R. erythropolis* BD2 wild-type are in accordance with the involvement of the IPB 2,3-dioxygenase in TCE oxidation.

Keywords: *Rhodococcus erythropolis*, isopropylbenzene 2,3-dioxygenase, 3-isopropylcatechol 2,3-dioxygenase, trichloroethene co-oxidation

INTRODUCTION

Multicomponent oxygenases often play a dual crucial role in the bacterial degradation of aromatic and chlorinated aliphatic compounds (Ensley, 1991). They catalyse the oxygenation of their inducer substrates and in addition oxidize chloroalkenes such as trichloroethene (TCE), a toxic groundwater contaminant with suspected carcinogenic activity which is widely distributed throughout the world (Miller & Guengerich, 1983). Since the first documentation by Nelson *et al.* (1986) of a pure culture capable of metabolizing TCE under aerobic conditions, a number of TCE-degrading micro-organisms have been

isolated (Ensley, 1991). Most investigations on TCE degradation have addressed Gram-negative bacteria and revealed that TCE is only biodegradable through co-metabolic transformation processes mediated by oxygenases which exhibit broad substrate specificities, such as monooxygenases acting on methane (Oldenhuis *et al.*, 1989), propane (Wackett *et al.*, 1989), isoprene (Evers *et al.*, 1990), ammonia (Arciero *et al.*, 1989), toluene (Whited & Gibson, 1991) or phenol (Folsom *et al.*, 1990). By analogy with metabolism of chlorinated alkenes by mammalian cytochrome P-450 monooxygenase, bacterial oxygenases might also activate TCE and release intermediate reaction products, such as TCE epoxide and chloral, which produce cytotoxic effects. So far, only one dioxygenase of a Gram-negative micro-organism, the toluene dioxygenase of *Pseudomonas putida* F1, is known to oxidize TCE (Nelson *et al.*, 1988; Wackett & Householder, 1989). Two representatives of Gram-positive genera, isoprene-oxidizer *Rhodococcus erythropolis* strain JE77 and the propane-utilizer *Mycobacterium vaccae*, have

Abbreviations: IPB, isopropylbenzene; IPC, isopropylcatechol; *Plac*, lactose operon promoter; *Ptac*, hybrid promoter of the lactose and the tryptophan operon; TCE, trichloroethene.

The GenBank accession number for the sequences reported in this paper is U24277.

been reported to co-oxidize TCE (Wackett *et al.*, 1989; Evers *et al.*, 1990).

Recently, we described *R. erythropolis* strain BD2, which is able to grow with isopropylbenzene (IPB) and to co-oxidize TCE (Dabrock *et al.*, 1992). This observation suggested that an inducible enzyme of the IPB degradative pathway is involved in TCE degradation. *R. erythropolis* BD2 transforms IPB by oxidation of the aromatic ring to give 3-isopropylcatechol (IPC), which subsequently undergoes a *meta*-cleavage reaction leading to the formation of 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate. Interestingly, all three characteristics, the IPB-oxidizing activity, the *meta*-cleavage activity and the degradation of TCE, are encoded by a linear plasmid, named pBD2 (a 208 kb linear DNA plasmid recently detected in *R. erythropolis* strain BD2; Dabrock *et al.*, 1994).

To analyse the molecular and biochemical basis of IPB oxidation and TCE co-oxidation, the genes encoding the IPB 2,3-dioxygenase (*ipbA1A2A3A4*) and the 3-IPC 2,3-dioxygenase (*ipbC*) of BD2 have been cloned and sequenced. Comparisons of the deduced amino acid sequences were made with those encoded by analogous genes of other catabolic pathways. They revealed differences in arrangement but a high degree of homology, so that a common origin of these pathways is indicated.

METHODS

Bacterial strains, growth conditions and plasmids. *Rhodococcus erythropolis* strain BD2, which was recently isolated in our laboratory, was grown in M3 minimal salt medium with IPB as carbon and energy source as described previously (Dabrock *et*

al., 1994). *R. erythropolis* strain SQ1 (Quan & Dabbs, 1993) was grown on Luria-Bertani (LB) medium (Miller, 1972) at 30 °C as were the *Escherichia coli* strains MM294 (Bachmann, 1987), MM294 (λ) (Dabbs *et al.*, 1990), JM109 (Yanisch-Perron *et al.*, 1985) and XL1-Blue (Bullock *et al.*, 1987). *E. coli* strains carrying recombinant plasmids derived from pBluescript II KS/SK(+/-) (Stratagene), pKK223-3 (Pharmacia Biosystems) or pDA71 (E. R. Dabbs) were selected on LB agar containing ampicillin (100 µg ml⁻¹). The plasmids used in this study are listed in Table 1.

DNA manipulations and transformation of rhodococci.

Standard recombinant DNA techniques were performed according to Sambrook *et al.* (1989). The *R. erythropolis* BD2 linear plasmid was isolated as described previously (Dabrock *et al.*, 1994). For transformation of *R. erythropolis* SQ1, 100 ml cultures were grown on LB medium containing 1.8% (w/v) sucrose, 1.5% (w/v) glycine and 0.01% isonicotinic acid hydrazide to an OD₆₀₀ of 3.0, harvested, washed twice with ice-cold, sterile distilled water and resuspended in 1 ml 30% (w/v) PEG to reach an OD₆₀₀ of 300. Aliquots of 100 µl were stored at -70 °C. For transformation, *Rhodococcus* cells were thawed on ice, mixed with ≤ 1 µg DNA, transferred into a chilled electroporation cuvette (0.2 cm; Bio-Rad) and incubated on ice for 10 min. Electroporation was performed at 2.5 kV, 25 µF and 400 Ω, yielding time constants of about 6.8 ms (Gene pulser; Bio-Rad). LB medium (1 ml) was added and cells were spread on LB agar. After 6 h, 250 µl of a chloramphenicol stock solution was added underneath the agar (40 µg ml⁻¹ final concentration) to select for transformants carrying pDA71 or derivatives.

Screening for IPB- and IPC-transforming activities. To screen for IPB-2,3-dioxygenase-catalysed indole oxidation, *R. erythropolis* SQ1 transformants were grown on LB medium containing chloramphenicol (40 µg ml⁻¹) and indole (1 mM). IPB was

Table 1. Plasmids used in this work

Plasmid	Relevant characteristics	Source/reference
pBluescript II KS/SK(+/-)	Cloning vectors, Ap ^r	Stratagene
pKK223-3	Expression vector, Ap ^r , P _{tac}	Pharmacia Biosystems
pDA71	<i>E. coli</i> - <i>Rhodococcus</i> shuttle vector, Ap ^r Cm ^r	E. R. Dabbs
pBD2	Linear megaplasmid of IPB degrader <i>R. erythropolis</i> BD2	Dabrock <i>et al.</i> (1994)
pDTG601	pKK223-3 recombinant plasmid encoding <i>todC1C2B.A</i> from <i>P. putida</i> F1	Zylstra & Gibson (1989)
pMKD107	pDA71 with 6.4 kb <i>Bcl</i> I fragment of pBD2 carrying <i>ipbAC</i>	This work
pMKD119	pDA71 with 7.5 kb <i>Bcl</i> I fragment of pBD2 carrying <i>ipbCB</i>	
pMKD205	pDA71 with 4.2 kb <i>Bst</i> YI fragment of pBD2 carrying <i>ipbC</i>	
pMK1 (+/-)	pBluescript II KS with 3.5 kb <i>Eco</i> RI- <i>Not</i> I fragment of pMKD107	
pMK2 (+/-)	pBluescript II KS with 2.6 kb <i>Not</i> I- <i>Eco</i> RV fragment of pMKD107 carrying <i>ipbC</i>	
pMK3 (+/-)	pBluescript II SK with 4.2 kb <i>Bgl</i> II- <i>Eco</i> RI fragment of pMKD107 carrying <i>ipbA</i> under control of <i>Plac</i>	
pMK4 (+/-)	pBluescript II SK with 2.6 kb <i>Not</i> I- <i>Eco</i> RV fragment of pMKD107 carrying <i>ipbC</i> under control of <i>Plac</i>	
pMK34 (+/-)	pMK3 with 2.0 kb <i>Eco</i> RI fragment of pMKD119 carrying <i>ipbACB</i> under control of <i>Plac</i>	
pKKM2	pKK223-3 with 2.6 kb <i>Pst</i> I- <i>Hind</i> III fragment of pMKD107 carrying <i>ipbC</i> under control of <i>P_{tac}</i>	
pKKID5	pKK223-3 with 4.9 kb <i>Eco</i> RI fragment of pMKD107 carrying <i>ipbA</i> under control of <i>P_{tac}</i>	

supplied via the gas phase to guarantee induction of the *ipb* genes. In the presence of indole, colonies with IPB 2,3-dioxygenase (IpbA) activity turn blue due to the formation of indigo from indole. Transformants carrying DNA fragments encoding 3-IPC 2,3-dioxygenase (IpbC) activity were detected by a catechol or 3-IPC spray assay (Furukawa & Miyazaki, 1986) for the production of the yellow-coloured *meta*-cleavage products, such as 2-hydroxymuconic semialdehyde or 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate. For activity staining of *E. coli* transformants, IPTG was added to a final concentration of 0.1 mM to solid LB media and 0.5 mM to liquid LB media. Indole, the substrate for indigo production, could be omitted from media for growth of *E. coli* recombinant strains because it was provided by the *E. coli* tryptophanase.

Southern hybridization. Southern blots of the cloned pBD2 fragments were performed with nylon membranes (GeneScreen Plus; DuPont, NEN Research products). Hybridizations with ³²P-labelled DNA were carried out according to Anderson & Young (1985). Experiments were done under low-stringency conditions as described previously by Dabrock *et al.* (1994). The heterologous toluene dioxygenase genes from *P. putida* F1 (*todC1C2BA*) carried on plasmid pDTG601 (Zylstra & Gibson, 1989) were kindly provided by D. T. Gibson, University of Iowa, USA.

Subcloning and nucleotide sequencing. DNA fragments carrying *ipb* genes were subcloned into pBluescript II KS/SK(+/-) or pKK223-3 by making use of the corresponding restriction maps and an Erase-a-base kit (Promega) to obtain a series of deletion derivatives. Single-stranded DNA of these clones was isolated (Vieira & Messing, 1987) and nucleotide sequences were determined by the dideoxy termination method (Sanger *et al.*, 1977) with an automated sequencing kit (Pharmacia) and analysed with the GCG package referring to SWISS-PROT and EMBL data.

Identification of gene products. Expression of the cloned *ipb* genes was performed *in vitro* with the corresponding pKK223-3 derivatives using an *E. coli* S30 extract system (Promega) and L-[³⁵S]methionine (Hartmann). After SDS-PAGE analysis according to the method of Schägger & von Jagow (1987), the gel was stained with Coomassie Brilliant Blue (Serva), dried for 40 min at 60 °C in a vacuum gel dryer (Bio-Rad) and exposed to X-ray film.

Enzyme assays and analysis of substrate specificity. For IPB 2,3-dioxygenase and 3-IPC 2,3-dioxygenase activity assays, *R. erythropolis* BD2 cells were grown in 60 ml M3 minimal medium with a supply of IPB in the vapour phase as described previously by Dabrock *et al.* (1994), harvested at an OD₆₀₀ of 1.0, washed twice and resuspended in 300 µl 50 mM phosphate buffer (pH 7.0). The 3-IPC 2,3-dioxygenase activity in *E. coli* recombinant cells carrying pBluescript II KS (negative control), pMK2, pMK4, pMK34 or pKKM2 was induced with IPTG (0.5 mM) in the early-exponential phase during growth (OD₆₀₀ 0.15–0.2) in 30 ml LB medium containing ampicillin (50 µg ml⁻¹). At an OD₆₀₀ of 0.6, cells were harvested, washed and resuspended in 100 µl 50 mM phosphate buffer (pH 7.0). Recombinant *R. erythropolis* SQ1 cells carrying pMKD107 were similarly treated but grown in the presence of chloramphenicol (40 µg ml⁻¹) instead of ampicillin, induced by IPB via the vapour phase and harvested at varying OD₆₀₀ values of 0.5–1.9 with an optimal activity at the early-exponential phase (OD₆₀₀ 0.5–1.0).

Substrate-dependent oxygen-uptake rates of the cells were followed by a Clark oxygen electrode (Rank Brothers) at 30 °C in 2 ml 50 mM phosphate buffer (pH 7.0). Substrate-independent oxygen consumption was measured for 3 min before IPB

or *meta*-cleavage substrates like 3-IPC, catechol or 3-methylcatechol were added to a final concentration of 200 µM and the total oxygen uptake was followed for 6 min. The difference in the substrate-dependent oxygen-uptake rate and substrate-independent oxygen-uptake rate was defined as the IPB or 3-IPC 2,3-dioxygenase activity, respectively (1 nmol min⁻¹ mg⁻¹ = 1 mU). Protein content was measured by the method of Schmidt *et al.* (1963).

Inhibitor studies. Cultures (100 ml) of *R. erythropolis* BD2 were grown for 24 h in minimal medium in the presence of IPB as sole carbon source and re-fed as described previously by Dabrock *et al.* (1992). Cells were harvested by centrifugation, washed once and resuspended in 1.5 ml sodium/potassium phosphate buffer supplemented with 5 mM MgSO₄. Cell suspensions were incubated for 5 min in the presence of 0–0.66 µmol 3-chlorocatechol (mg protein)⁻¹ before substrate-dependent oxygen uptake was measured with IPB and 3-IPC, respectively. The degradation of TCE by IPB-induced BD2 cells in the presence of the *meta*-cleavage inhibitor 3-chlorocatechol [0.33 µmol (mg protein)⁻¹] was quantified by headspace gas chromatography as described previously by Dabrock *et al.* (1992).

RESULTS

Cloning and mapping of the *ipb* genes

Two libraries of the *R. erythropolis* BD2 linear plasmid pBD2 (208 kb) were constructed by ligation of pBD2 DNA partially digested with *Bcl*I or *Bst*YI into the *Bgl*II-linearized *E. coli*–*Rhodococcus* shuttle vector pDA71. After transformation of *E. coli* MM294, plasmid DNA of pooled colonies was isolated and transformed into *R. erythropolis* SQ1 as described in Methods. When approximately 2 × 10⁴ colonies were screened for *meta*-cleavage activity by spraying with both 3-IPC and catechol, 50 colonies turned yellow, indicating *meta*-cleavage activity of the 3-IPC 2,3-dioxygenase (IpbC). Recombinant plasmids from 30 colonies were isolated and found to carry a 4.2 kb *Bst*YI (pMKD205), a 6.4 kb *Bcl*I (pMKD107) or a 7.5 kb *Bcl*I (pMKD119) insert in pDA71 (Fig. 1). Expression of *ipbC* in SQ1 carrying pMK205, pMK107 or pMKD119 was not stimulated by potential inducer substrates such as IPB or 3-IPC. Subcloning experiments proved that the *Bcl*I–*Hind*III fragment within the 1.6 kb overlapping region of the different clones was responsible for *meta*-cleavage activity. Retransfer of the recombinant plasmids into *E. coli* MM294 and restriction analysis revealed that the two *Bcl*I fragments were overlapping within a 1.6 kb DNA region, while the *Bst*YI fragment was completely within the 6.4 kb *Bcl*I fragment (Fig. 1). Examination of the recombinant *E. coli* clones for 3-IPC 2,3-dioxygenase activities by using the 3-IPC and catechol spray assay revealed no 3-IPC 2,3-dioxygenase activities, indicating that the *R. erythropolis* BD2 *ipbC* gene is not expressed in *E. coli* in pDA71.

Southern hybridization experiments performed with linear pBD2 DNA and heterologous DNA probes of the toluene dioxygenase genes (*todC1C2BA*) from *P. putida* F1, which we have published recently (Dabrock *et al.*, 1994), already indicated a significant homology between the toluene dioxygenase genes and the presumed IPB 2,3-

Fig. 1. Cloning and subcloning of the *ipb* genes. pBD2 fragments from *Bcl*I and *Bst*YI partial digests were ligated into the suicide shuttle vector pDA71, generating pMKD107, pMKD119 and pMKD205. Corresponding recombinant *R. erythropolis* SQ1 colonies and *E. coli* XL1-Blue carrying derived recombinant pBluescript II plasmids were sprayed with catechol (100 mM) to detect *meta*-cleavage activity (yellow colour; +). The arrow indicates the direction of transcription deduced from nucleotide sequence analysis and expression of the extradiol dioxygenase IpbC. pBIISK, pBluescript II SK; pBIKS, pBluescript II KS; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; Bs, *Bst*YI; C, *Cla*I; E, *Eco*RI; Ec, *Eco*RV; H, *Hind*III; N, *Not*I; P, *Pst*I; S, *Sal*I.

dioxygenase genes of *R. erythropolis* BD2. In order to identify IPB 2,3-dioxygenase genes, insert DNA of pMKD107, pMKD119 and pMKD205 was hybridized with the 32 P-labelled toluene dioxygenase gene probe. Clear hybridization signals were observed with pMKD107 and pMKD205, but not with pMKD119. These results provided strong evidence for the localization of the IPB 2,3-dioxygenase genes on pMKD107 and pMKD205.

Sequence analysis

The 3.5 kb *Eco*RI–*Not*I and the 2.6 kb *Not*I–*Eco*RV fragments of pMKD107 were cloned into pBluescript II KS, yielding pMK1 and pMK2, respectively (Fig. 1). pMK1 and pMK2 were then subjected to partial digestion with exonuclease III to construct overlapping deletion series for sequence analysis of one strand. Making use of the predicted restriction sites, deletion plasmids for sequence analysis of the other strand were constructed. Complete sequence data of the 5.7 kb *Eco*RI–*Cla*I DNA region are presented in Fig. 2. Five ORFs were found with significant homology to genes encoding aromatic-ring-activating multicomponent dioxygenases and extradiol dioxygenases of aromatic hydrocarbon degraders. Based on the homology and the hybridization data as well

as on the *meta*-cleavage activity, ORFs 1–4 were identified as the cistrons for the α - and β -subunit of the iron-sulfur protein, the ferredoxin, and the ferredoxin reductase components of a multicomponent IPB 2,3-dioxygenase (IpbA1A2A3A4), while ORF5 was found to encode the 3-IPC 2,3-dioxygenase (IpbC). The potential Shine–Dalgarno sequences preceding the *ipb* genes are underlined in Fig. 2. Further sequence analysis of the DNA downstream of *ipbC* (*Cla*I–*Bcl*I fragment) strongly suggested the presence of *ipbB* encoding the IPB 2,3-dihydrodiol dehydrogenase (data not shown).

Sequence comparisons and organization of the *ipb* genes

IpbAC of *R. erythropolis* BD2 showed high homologies (54–78% amino acid identity) with other ring-hydroxylating and *meta*-cleaving dioxygenases of toluene, benzene and biphenyl degradative pathways in *Pseudomonas* strains (Table 2), such as *P. putida* F1 (Zylstra & Gibson, 1989), *Pseudomonas* sp. P51 (Van der Meer *et al.*, 1991), *P. putida* ML2 (Tan *et al.*, 1993) and *Pseudomonas pseudoalcaligenes* KF707 (Taira *et al.*, 1992). Amino acid identities with BphAC1 from *R. globerulus* P6 (Asturias *et al.*, 1994, 1995) are 59.4–79.5% and with the biphenyl dioxygenase and the 2,3-dihydroxybiphenyl dioxygenase from *Rhodococcus*

Fig. 3. Organization of the *ipb* genes in *R. erythropolis* BD2 and comparison with the operon structures of analogous biphenyl degradative pathways in *R. globerulus* P6 (Asturias *et al.*, 1994, 1995) and *Rhodococcus* sp. RHA1 (Masai *et al.*, 1995) and the toluene degradative pathway in *P. putida* F1 (Zylstra & Gibson, 1989). The arrow indicates the direction of transcription. ND, Not determined.

sp. RHA1 even 87.4–98.9 % (Masai *et al.*, 1995). The latter scores are surprisingly high and, therefore, growth of *R. erythropolis* BD2 with biphenyl in liquid culture was tested. No increase of the optical density was observed. BD2 cells grown on IPB and spread on plates with a supply of biphenyl in the vapour phase gave a slight yellow colouration of the agar but again no growth occurred. This indicates that the IpbACB enzymes of BD2 are able to oxidize biphenyl but that further degradation of the oxidized products of biphenyl does not take place. Analysis of deduced amino acid sequence data and alignments of the IPB 2,3-dioxygenase subunits with the corresponding subunits of BphA1A2A3A4 from *Rhodococcus* sp. RHA1 (Masai *et al.*, 1995), TodC1C2BA from *P. putida* F1 (Zylstra & Gibson, 1989), BedC1C2BA from *P. putida* ML2 (Tan *et al.*, 1993) and BphA1A2A3A4 from *P. pseudoalcaligenes* KF707 (Taira *et al.*, 1992) revealed three conserved regions in the reductase_{IPB} component (IpbA4) likely to bind FAD and NAD⁺: two of the conserved regions were located at the N-terminus and the middle region of the reductase component IpbA4, respectively, and are centred around a $\beta\alpha\beta$ -fold (Mason & Cammack, 1992). A third conserved sequence forming a β -sheet was found at the C-terminus of IpbA4 and supposed to bind the O-3 group of the ribityl chain of the flavin moiety of FAD (amino acids 265–275) (Wierenga *et al.*, 1986; Eggink *et al.*, 1990). Conserved sequences with two cysteines and two histidines as putative [2Fe–2S] Rieske-type cluster ligands were identified in the ferredoxin IpbA3 as well as in the α -subunit of the terminal dioxygenase IpbA1 (amino acids 43–65 and 98–121, respectively).

A comparison of the organization of the *ipb* genes and the corresponding genes of analogous degradative pathways showing the highest homologies is presented in Fig. 3. The operon structure of the biphenyl degradative pathway in *R. globerulus* P6 (Asturias *et al.*, 1994, 1995) and the toluene degradative pathway in *P. putida* F1 (Zylstra & Gibson, 1989) is consistent with the metabolic order of

Fig. 4. *In vitro* expression of *ipbAC* gene products. Polypeptides were synthesized and labelled with L-[³⁵S]methionine by an *E. coli* S30 extract system (Promega), separated by SDS-PAGE and detected on an autoradiogram. Lanes: 1, pBestLuc as a positive control (60.7 kDa); 2, no DNA template added; 3, pKK223-3 as a negative control; 4, pKKM2 (39 kDa); 5, pKKID5 (52, 44, 23.5 and 15 kDa); 6, pMK34 (52, 44, 39, 23.5 and 15 kDa); 7, molecular mass markers (66.0, 46.0, 30.0, 21.5 and 14.3 kDa).

the gene products. In contrast to this, the IPB 2,3-dioxygenase genes (*ipbA1A2A3A4*) are followed by a noncoding region of about 300 bp and the gene encoding the *meta*-cleavage 3-IPC 2,3-dioxygenase (IpbC), which precedes the dihydrodiol dehydrogenase gene *ipbB*. This gene order is only consistent with the *bph* genes of the biphenyl degradative pathway in *Rhodococcus* sp. RHA1 (Masai *et al.*, 1995). Therefore, interestingly, the order of the genes for the *meta*-cleaving dioxygenase and the dihydrodiol dehydrogenase in BD2 is reversed with regard to their metabolic order.

Identification of *ipbA* and *ipbC* gene products

The gene products of *ipbAC* were identified by an *in vitro* transcription/translation assay using derivatives of the expression vector pKK223-3 as DNA templates. The derivative pKKID5 carries the 4.9 kb *EcoRI* fragment of pMKD107 containing the IPB 2,3-dioxygenase genes (*ipbA1A2A3A4*), whereas pKKM2 contains the 2.5 kb *PstI*–*HindIII* fragment of pMKD107 encoding *ipbC* (Table 1). The results of SDS-PAGE analysis are shown in Fig. 4. The molecular masses of the expressed polypeptides are consistent with the theoretical values based on the deduced amino acid sequences as described in Table 3. Comparison of lanes 4 (pKKM2) and 5 (pKKID5) with lane 6, which represents transcription/translation analysis with the plasmid pMK34 carrying *ipbABC* under the control of the lactose operon promoter (*Plac*), shows that expression of *ipbA* and *ipbC* under the

Table 3. *ipbA1A2A3A4C* gene products from *R. erythropolis* BD2 and their molecular masses

Gene	Region of nucleotide sequence	Protein	Estimated molecular mass (kDa)	Determined molecular mass (kDa)
<i>ipbA1</i>	855–2234	Terminal dioxygenase (iron–sulfur protein), large subunit	50.6	52.0
<i>ipbA2</i>	2319–2879	Terminal dioxygenase (iron–sulfur protein), small subunit	20.6	23.5
<i>ipbA3</i>	2891–3211	Ferredoxin _{IPB}	11.8	15.0
<i>ipbA4</i>	3211–4446	Ferredoxin _{IPB} reductase	45.3	44.0
<i>ipbC</i>	4774–5724	3-IPC 2,3-dioxygenase	34.9	39.0

control of the hybrid promoter of the lactose and the tryptophan operon (*P_{tac}*) is much more efficient than from *P_{lac}*. Some slight bands, undetectable in the negative control lanes 2 and 3, probably represent truncated polypeptides resulting from internal translational starts. The ferredoxin containing only two methionines, including the one at the start, and the ferredoxin reductase nearly escaped detection.

IPB and 3-IPC 2,3-dioxygenase activities in *E. coli* carrying a recombinant plasmid

The 2.6 kb *NotI*–*EcoRV* fragment of pMKD107 carrying the 3-IPC 2,3-dioxygenase gene of strain BD2 was cloned in pBluescript II KS and pBluescript II SK, respectively. The resulting plasmids, designated pMK2 and pMK4 (Fig. 1), were transformed into *E. coli* XL1-Blue. After induction with IPTG and spraying with 3-IPC, *E. coli* XL1-Blue colonies carrying the recombinant plasmid pMK4 showed *meta*-cleavage activity while those harbouring pMK2 did not, suggesting that the 3-IPC 2,3-dioxygenase was expressed under control of *P_{lac}*. These recombinant cells exhibited a specific 3-IPC 2,3-dioxygenase activity of 25 nmol min⁻¹ mg⁻¹. Similar expression levels were obtained with *E. coli* JM109 carrying the recombinant plasmid pMK34 (encoding *ipbABC*). No further increase of the specific 3-IPC 2,3-dioxygenase activity was found in the presence of the wild-type inducer substrate IPB. Insertion of the 2.6 kb *PstI*–*HindIII* fragment carrying the *ipbC* gene into the expression vector pKK223-3 strongly improved the expression, as indicated by the oxygen-uptake rates of *E. coli* XL1-Blue cells carrying the recombinant plasmid pKKM2 (Table 4).

The recombinant plasmid pMK34, which carried the 4.2 kb *BglII*–*EcoRI* fragment of pMKD107 containing the IPB 2,3-dioxygenase genes plus the 2.0 kb *EcoRI* fragment of pMKD119 encoding the 3-IPC 2,3-dioxygenase (Fig. 1), was transferred into *E. coli* XL1-Blue. During growth on LB medium, the *E. coli* XL1-Blue recombinant

colonies carrying pMK34 exhibited the blue colouration characteristic of indigo production while those harbouring pMK4 carrying the 3-IPC 2,3-dioxygenase gene did not. Previous publications have shown that several bacteria able to oxidize aromatic hydrocarbons to *cis*-dihydrodiols also catalyse the oxidation of indole to give indigo (Ensley *et al.*, 1983; Clarke & Laverack, 1984). This indole oxidation was found to be brought about by the dioxygenases mediating the initial activation of the aromatic nucleus. In accordance with this, we recently published data on the IPB-induced indigo formation from indole by *R. erythropolis* BD2 (Dabrock *et al.*, 1994). The indigo production by recombinant *E. coli* strains carrying IPB 2,3-dioxygenase genes from strain BD2 was found to be independent of induction with IPTG or IPB. Unfortunately, the rates of the IPB 2,3-dioxygenase were too low to be detected by the polarographic method in the *E. coli* background.

Inhibitor studies

Because of the low IPB 2,3-dioxygenase activities in *E. coli* recombinant strains carrying the cloned IPB 2,3-dioxygenase, an IPB-2,3-dioxygenase-catalysed TCE degradation could not be demonstrated. To identify the TCE-oxidizing enzyme of the IPB degradative pathway in *R. erythropolis* BD2, the TCE oxidation by IPB-induced BD2 wild-type cells was determined in the presence of 3-chlorocatechol, a competitive inhibitor of *meta* ring-fission dioxygenases (Bartels *et al.*, 1984). After addition of 0.66 µmol 3-chlorocatechol (mg protein)⁻¹, the 3-IPC-2,3-dioxygenase-catalysed *meta* ring-fission of 3-IPC was completely inhibited, while 30–60% of the IPB 2,3-dioxygenase activity was still detected in fully induced wild-type cells. Examination of the TCE oxidation activity in the presence of 3-chlorocatechol revealed that cells completely inhibited in *meta* ring-fission activity still exhibited a TCE oxidation rate of 1.1 nmol min⁻¹ (mg protein)⁻¹, representing 70% of the maximum TCE oxidation rates [1.6 nmol min⁻¹ (mg protein)⁻¹] in cells of

Table 4. Specific activities and substrate specificities of the 3-IPC 2,3-dioxygenase in *E. coli*

The 3-IPC 2,3-dioxygenase activity [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] was determined polarographically. The strains were induced with IPTG as described in Methods.

Substrate	<i>E. coli</i> strain	Plasmid	Dioxygenase activity
3-IPC	XL1-Blue	pMK4	25
	XL1-Blue	pMK2	0
	JM109	pMK34	22
	XL1-Blue	pKKM2	246
Catechol	XL1-Blue	pKKM2	118
3-Methylcatechol	XL1-Blue	pKKM2	284

strain BD2 fully induced by IPB. This result, in accordance with the correlation of the linear-plasmid-encoded IPB 2,3-dioxygenase and TCE oxidation activity (Dabrock *et al.*, 1994), strongly suggests that the IPB-induced TCE degradation in strain BD2 is catalysed by the IPB 2,3-dioxygenase of *R. erythropolis* BD2.

DISCUSSION

We cloned and sequenced a 5.7 kb fragment from the linear plasmid of *R. erythropolis* BD2 encoding dioxygenases involved in IPB degradation. Sequence analysis of *ipbC* revealed characteristics of genes encoding extradiol dioxygenases; the strongest homologies were found to functionally equivalent enzymes of biphenyl degradation in *Rhodococcus* species (Asturias *et al.*, 1994, 1995; Masai *et al.*, 1995) and toluene degradation in *P. putida* F1 (Zylstra & Gibson, 1989). The following lines of evidence led to the conclusion that the four ORFs designated *ipbA1A2A3A4* encode the components of a multi-component IPB 2,3-dioxygenase: (i) significant homologies of the products of *ipbA1A2A3A4* with functionally related aromatic dioxygenases; (ii) characteristic unidirectional tandem organization of *ipbA1A2A3A4* analogous to biphenyl and toluene multicomponent aromatic dioxygenases; (iii) consistent organization of *ipbA1A2A3A4CB* and the equivalent *bph* genes in *Rhodococcus* sp. RHA1; (iv) production of indigo from indole by *E. coli* recombinant strains carrying *ipbA1A2A3A4C*; (v) sizes of the *ipbA1A2A3A4* and *ipbC* gene products, which were consistent with analogous dioxygenase proteins. The most significant degrees of homology were found between the *ipbA* genes in *R. erythropolis* BD2 and the analogous *bph* genes from *Rhodococcus* sp. RHA1 (Masai *et al.*, 1995) and *R. globerulus* P6 (Asturias *et al.*, 1995). These results suggest that the *ipb* and *bph* genes are derived from the same ancestral origin. Interestingly, no predominance of the *Rhodococcus*-typical GAA codon over GAG for Glu was found. This was also reported for the *bph* genes in *R. globerulus* P6. Based on the high-level sequence identity between equivalent *ipbA* genes from *R. erythropolis* BD2 and the *tod*, *tcb*, *bed* and *bph*

dioxygenase genes from *Pseudomonas* strains and the non-typical codon usage, a common non-*Rhodococcus* phylogenetic origin of these ring-activating dioxygenases in *Pseudomonas* and *Rhodococcus* is indicated.

The 6.4 kb *BclI* DNA fragment carrying the *ipb* genes in the *Rhodococcus-E. coli* shuttle vector pDA71, which only contained the λ -promoter of the *EcoRI* gene, did not express either IPB 2,3-dioxygenase or 3-IPC 2,3-dioxygenase activities in *E. coli* when grown in the presence of the inducer substrate IPB. This suggests that the natural promoter is not present or not active or that a positive regulator of the *ipb* genes is missing on the cloned DNA fragment. Since 0.85 kb of 5'-DNA localized upstream of *ipbA1* did not match with the consensus sequences for *E. coli* promoters, we favour the explanation that a *Rhodococcus* promoter is present but not active in *E. coli*. Evidence supporting this hypothesis is that 3-IPC 2,3-dioxygenase activity was detected in *R. erythropolis* SQ1(pMKD107) carrying the cloned *ipb* genes inserted in the shuttle vector pDA71. Independent of the presence of IPB, SQ1 recombinant strains carrying the 3-IPC 2,3-dioxygenase gene only revealed approximately 10% of the maximal 3-IPC 2,3-dioxygenase activity detected in fully induced BD2 cells. An incomplete promoter site or a missing regulator gene may account for this low basic level of 3-IPC 2,3-dioxygenase activity and the undetectable, low or missing IPB 2,3-dioxygenase activity in SQ1. Another explanation for the low level of 3-IPC 2,3-dioxygenase could be a transcription of the *ipb* genes from multiple promoters; a promoter site induced by IPB might be located far upstream of the *ipbA1* start codon and therefore might be missing on the cloned fragment. A transcription from multiple promoters has already been demonstrated for the analogous biphenyl dioxygenase in *P. putida* LB400, where a biphenyl-induced promoter was identified approximately 1.0 kb upstream of the *ipbA* start codon and two additional promoter sites were mapped very close to the *ipbA* start codon (Erickson & Mondello, 1992). The tight clustering and the head-to-tail organization of the *ipb* genes in BD2 suggests that *ipbA1A2A3A4* and *ipbC* are cotranscribed. However, no IPB 2,3-dioxygenase activity was detected in SQ1 cells expressing low 3-IPC 2,3-dioxygenase activity. This might be due to lower specific activities of the IPB dioxygenase as compared to 3-IPC 2,3-dioxygenase activities. This hypothesis is supported by the result that fully induced wild-type cells reveal lower specific activities of the IPB 2,3-dioxygenase ($145 \text{ nmol min}^{-1} \text{ mg}^{-1}$) than of the 3-IPC 2,3-dioxygenase ($278 \text{ nmol min}^{-1} \text{ mg}^{-1}$).

After subcloning of *ipbA1A2A3A4* and *ipbC* genes in different vectors downstream of *Plac* or *Ptac* and transfer into *E. coli* cells, polarographic measurements revealed high 3-IPC 2,3-dioxygenase activities in these recombinant *E. coli* strains, whereas IPB dioxygenase activities were only detectable by the IPB-2,3-dioxygenase-mediated oxidation of indole to indigo. As the indole oxidation is known to be more sensitive than polarographic methods (Jenkins & Dalton, 1985) and very specific for aromatic dioxygenases (Ensley *et al.*, 1983; Clarke & Laverack, 1984), we conclude that *ipbA1A2A3A4* gene products

are only poorly active in *E. coli*. Since the IpbA1, IpbA2, IpbA3 and IpbA4 proteins were expressed *in vitro*, we suggest that post-translational modifications influencing the binding of NAD(P)H and flavin to the apoenzyme or affecting the coordination of Rieske type iron-sulfur clusters might be responsible for the very low IPB 2,3-dioxygenase activities. A modified assembly of the IPB 2,3-dioxygenase components in *E. coli* could change domain packing, which is important for the orientation of the prosthetic groups of multicomponent enzymes, as has been shown for the phthalate dioxygenase reductase in *Burkholderia* (*Pseudomonas*) *cepacia* (Correll *et al.*, 1992). A change in the assembly could also affect the orientation of the components of the IPB dioxygenase. This might inhibit the rapid distribution of electrons (Correll *et al.*, 1992). As edge-to-edge distances and electronic coupling between donor and receptor components are crucial parameters determining the rates of biological electron transfer reactions (Correll *et al.*, 1985), a change in subunit interaction could drastically reduce the IPB 2,3-dioxygenase activity. The rather short putative Shine-Dalgarno sequence found upstream of *ipbA4* as well as the strong stem-loop structure of approximately 60 bonds per stem which has been mapped upstream of the *ipbA4* start codon could account for the poor expression of *ipbA4* during *in vitro* expression.

Analysis of the deduced protein sequence of the product of *ipbA4* (reductase_{IPB}) revealed two conserved regions with a consensus sequence of Xaa-Gly-Xaa₂-Gly-Xaa₃-Ala (or a homologous amino acid)-Xaa₆-Gly (where Xaa is any amino acid) predicted to bind NAD⁺ and FAD⁺ and a third conserved region located at the C-terminus, consisting of a Thr-Xaa₆-Ala-Xaa-Gly-Asp consensus sequence predicted to bind FAD (Wierenga *et al.*, 1986; Eggink *et al.*, 1990). The consensus sequence CXHX₁₆₋₁₇CXXH of Rieske iron-sulfur proteins for the binding of a [2Fe-2S] cluster (Rieske *et al.*, 1964) was present in the amino acids of the deduced *ipbA1* and *ipbA3* products. These observations suggest that the IPB 2,3-dioxygenase in BD2 consists of three protein components, the terminal oxygenase (protein IpbA1 and A2), the intermediate Rieske iron-sulfur ferredoxin (IpbA3) and an NADH- (Rieske iron-sulfur) ferredoxin oxidoreductase (protein IpbA4). Based on these characteristics in the electron transport chain, we conclude that the IPB 2,3-dioxygenase in *R. erythropolis* BD2 belongs to the Class IIB ring-activating dioxygenases (Batie *et al.*, 1987).

Very recently, a multicomponent biphenyl dioxygenase (BphA) was reported to be involved in biphenyl degradation by *Rhodococcus* sp. RHA1 (Masai *et al.*, 1995); it exhibits significant amino acid identity to the products of the *ipbA* genes of BD2. As *R. erythropolis* BD2 did not grow with biphenyl as sole carbon and energy source but was able to convert biphenyl to the yellow *meta*-cleavage product, different substrate specificities of the lower IPB and BPH pathway enzymes can be assumed. The genes encoding lower IPB pathway enzymes have not been mapped so far and it is not known whether they are located on the linear transmissible plasmid pBD2 like the IPB and the 3-IPC 2,3-dioxygenase genes.

The IPB-induced TCE degradation and the correlation of the plasmid-encoded IPB degradative pathway with the capability of TCE co-oxidation strongly suggested an IPB pathway dioxygenase being responsible for the oxidation of TCE. As the biochemical and the molecular analysis of the IPB pathway in strain BD2 revealed two dioxygenases, the ring-activating IPB 2,3-dioxygenase and the *meta*-cleavage 3-IPC 2,3-dioxygenase, to be implicated in IPB oxidation, one of these two key dioxygenases was suggested to catalyse TCE co-oxidation. Inhibitor studies, together with the previous results on the IPB-induced TCE degradation (Dabrock *et al.*, 1992), led to the conclusion that the IPB 2,3-dioxygenase catalyses TCE oxidation in BD2. To our knowledge, this interesting enzyme is the first aromatic dioxygenase in a Gram-positive bacterium capable of TCE oxidation. Further biochemical studies are underway in our laboratory to shed more light on the structure-function relationship of the linear-plasmid-encoded IPB 2,3-dioxygenase and to elucidate the mechanism of IPB-2,3-dioxygenase-catalysed TCE oxidation.

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