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, ferredoxin, 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 monoxygenases 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 monoxygenase, 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 tolune 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 (ipbA) 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 the Escherichia coli strains MM294 (Bachmann, 1987), MM294 (+) (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

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<th>Plasmid</th>
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<td>pKK223-3 recombinant plasmid encoding todC1C2BA</td>
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<td>pDA71 with 64 kb BstI fragment of pBD2 carrying ipbAC</td>
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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-hydroxyxynonic semialdehyde or 2-hydroxy-6-oxy-7-methylxynona-2,4-diene. 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 heterogeneous tolB-like 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/KS (+/−) 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, pMK36 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 0.06, the 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 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 μU). 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 0.066 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 BclI or BsrYI into the BglII-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 BsrYI (pMKD205), a 6.4 kb BclI (pMKD107) or a 7.5 kb BclI (pMKD119) insert in pDA71 (Fig. 1). Expression of ipbC in SQ1 carrying pMKD205, pMKD107 or pMKD119 was not stimulated by potential inducer substrates such as IPB or 3-IPC. Subcloning experiments proved that the BclI-HindIII fragment within the 1.6 kb overlapping region of the different clones was responsible for meta-cleavage activity. Retracer of the recombinant plasmids into E. coli MM294 and restriction analysis revealed that the two BclI fragments were overlapping within a 1.6 kb DNA region, while the BsrYI fragment was completely within the 6.4 kb BclI 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 tolune dioxygenase genes (todC1C2BA) from P. putida F1, which we have published recently (Dabrock et al., 1994), already indicated a significant homology between the tolune dioxygenase genes and the presumed IPB 2,3-
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**Fig. 7.** Cloning and subcloning of the *ipb* genes. *pBD2* fragments from *BclI* and *BstYI* 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*. *pBIIKS*, *pBluescript* II KS; *pBIIKS*, *pBluescript* II KS; *BamHl*; *BclI*; *Bglll*; *BstYl*; *Cclal*; *EcoRI*; *EcoRV*; *Hindlll*; *NotI*; *PstI*; *Sall*.

**Sequence analysis**

The 3.5 kb *EcoRI–NotI* and the 2.6 kb *NotI–EcoRV* 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 *EcoRI–Cclal* 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* (*Cclal–Bcll* 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* *pseudocaldigenes* KF707 (*Taira et al., 1992*). Amino acid identities with *BphACl* from *R. globertllus* 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. 2. Nucleotide sequence of the R. erythropolis BD2 ipbA1A2A3A4C genes and the corresponding amino acid sequences. Putative ribosome-binding sites are underlined.

Table 2. Comparison of the deduced amino acid sequences of the individual components of IPB dioxygenase and 3-IPC 2,3-dioxygenase from R. erythropolis BD2 encoded by the ipbA1A2A3A4 and ipbc genes with the corresponding dioxygenases of analogous pathways in Rhodococcus and Pseudomonas species, respectively.

Values give percentages of identical amino acids. Dioxygenases: biphenyl dioxygenase (BphA1A2A3A4) and 2,3-dihydroxybiphenyl dioxygenase (BphC) from R. erythropolis sp. RHA1 (Masai et al., 1995); biphenyl dioxygenase (BphA1A2A3A4) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC1) from R. globulans sp. P6 (Asturias et al., 1994, 1995); toluene dioxygenase (TodC1C2B2) and 3-methylcyclohexene 2,3-dioxygenase (TodE) from P. putida F1 (Zylstra & Gibson, 1989); 1,2,4-trichlorobenzene 5,6-dioxygenase (TcbAaAbAcAa) from Pseudomonas sp. PS1 (Van der Meer et al., 1991); benzene dioxygenase (BedC1C2BA) from P. putida ML2 (Tan et al., 1993); and biphenyl dioxygenase (BphA1A2A3A4) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) from P. pseudoalcaligenes KF707 (Taira et al., 1992).
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) and the toluene degradative pathway in P. putida F1 (Zylstra & Gibson, 1989). The arrow indicates the direction of transcription. ND, Not determined.

**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-HindII 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 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 (IpB), which precedes the dihydrodiol dehydrogenase gene ipbB. This gene order is only consistent with the ipb 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.

![Fig. 3. Organization of the ipb genes in R. erythropolis BD2 and comparison with the operon structures of analogous biphenyl degradative pathways in R. globulus 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.](image)

![Fig. 4. In vitro expression of ipbAC gene products. Polypeptides were synthesized and labelled with [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, 25 and 15 kDa); 6, pMK34 (52, 44, 39, 25 and 15 kDa); 7, molecular mass markers (66.0, 46.0, 30.0, 21.5 and 14.3 kDa).](image)
control of the hybrid promoter of the lactose and the tryptophan operon (Plac) is much more efficient than from Plac. 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 *NsiI–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 Plac. 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 *ipbA1B2C*). 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 *BgIII–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-dihydriodols 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

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<td><em>ipbA3</em></td>
<td>2891–3211</td>
<td>Ferredoxin&lt;sub&gt;IPB&lt;/sub&gt;</td>
<td>11-8</td>
<td>15-0</td>
</tr>
<tr>
<td><em>ipbA4</em></td>
<td>3211–4446</td>
<td>Ferredoxin&lt;sub&gt;IPB&lt;/sub&gt; reductase</td>
<td>45-3</td>
<td>44-0</td>
</tr>
<tr>
<td><em>ipbC</em></td>
<td>4774–5724</td>
<td>3-IPC 2,3-dioxygenase</td>
<td>34-9</td>
<td>39-0</td>
</tr>
</tbody>
</table>

Table 3. *ipbA1A2A3A4C* gene products from *R. erythropolis* BD2 and their molecular masses
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 extra-diol 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 *Pseudomonas* F1 (Zylstra & Gibson, 1989). The following lines of evidence led to the conclusion that the four ORFs designated *ipbA1A2A3A4* encode the components of a multicomponent 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. RH1; (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. RH1 (Masai et al., 1995) and *Rhodococcus* sp. 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. globulatus* P6. Based on the high-level sequence identity between equivalent *ipbA* genes from *R. erythropolis* BD2 and the *tcb, bed* and *bph* dioxygenase genes from *Pseudomonas* strains and the nontypical codon usage, a common non-*Rhodococcus* phylogenetic origin of these ring-activating dioxygenases in *Pseudomonas* and *Rhodococcus* is indicated.

The 6.4 kb *EcoRI* DNA fragment carrying the *ipb* genes in the *Rhodococcus–E. coli* shuttle vector pDA71, which only contained the A-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 nmol min⁻¹ mg⁻¹) than of the 3-IPC 2,3-dioxygenase (278 nmol min⁻¹ 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

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>E. coli</em> strain</th>
<th>Plasmid</th>
<th>Dioxygenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-IPC</td>
<td>XL1-Blue</td>
<td>pMK4</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>XL1-Blue</td>
<td>pMK2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>JM109</td>
<td>pMK34</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>XL1-Blue</td>
<td>pKKM2</td>
<td>246</td>
</tr>
<tr>
<td>Catechol</td>
<td>XL1-Blue</td>
<td>pKKM2</td>
<td>118</td>
</tr>
<tr>
<td>3-Methylecetechol</td>
<td>XL1-Blue</td>
<td>pKKM2</td>
<td>284</td>
</tr>
</tbody>
</table>

*Table 4. Specific activities and substrate specificities of the 3-IPC 2,3-dioxygenase in *E. coli* |
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) espacta* (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-Xaa-Gly-Xaa-Ala (or a homologous amino acid)-Xaa-Gly (where Xaa is any amino acid) predicted to bind NADH and FADH and a third conserved region located at the C-terminus, consisting of a Thr-Xaa-Ala-Xaa-Gly sequence predicted to bind FAD (Wierenga et al., 1986; Eggink et al., 1990). The consensus sequence CXHX,,-CXXH 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-fused ferredoxin oxidoreductase (protein IpbA4). Based on these characteristics in the electron transport chain, we conclude that the IPB 2,3-dioxygenase in *Rhodococcus* BD2 belongs to the Class IIIB 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. RH1A (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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REFERENCES


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