Degradation of 2-methylaniline in *Rhodococcus rhodochrous*: cloning and expression of two clustered catechol 2,3-dioxygenase genes from strain CTM

ANGELIKA SCHREINER, KLAUS FUCHS,† FRIEDRICH LOTTSPEICH,‡ HARALD POTH§ and FRANZ LINGENS*

Institut für Mikrobiologie, Universität Hohenheim, Garbenstraße 30, D-7000 Stuttgart 70, FRG

(Received 31 December 1990; revised 2 April 1991; accepted 13 May 1991)

Rhodococcus rhodochrous strain CTM degrades 2-methylaniline mainly via the *meta*-cleavage pathway. Conversion of the metabolite 3-methylcatechol was catalysed by an M_r 156 000 catechol 2,3-dioxygenase (C23OI) comprising four identical subunits of M_r 39 000. The corresponding gene was detected by using an oligonucleotide as a gene probe. This oligonucleotide was synthesized on the basis of a partial amino acid sequence obtained from the purified enzyme from *R. rhodochrous*. The structural gene of C23OI was located on a 3.5 kb *Bgl*/II restriction fragment of plasmid pTC1. On the same restriction fragment the gene for a second catechol 2,3-dioxygenase, designated C23OII, was found. This gene coded for the synthesis of the M_r 40 000 polypeptide of the M_r 158 000 tetrameric C23OII. More precise mapping of the structural genes showed that the C23OI gene was located on a 1.2 kb *Bgl*/II–*Sma*I fragment and the C23OII gene on the adjacent 1.15 kb *Sma*I fragment. Comprehensive substrate range analysis showed that C23OII accepted all the substrates that C23OI did, but additionally cleaved 2,3-dihydroxybiphenyl and catechols derived from phenylcarboxylic acids. C23OI exhibited highest activity towards methylcatechols, whereas C23OII cleaved unsubstituted catechol preferentially.

Introduction

In the preceding paper (Fuchs *et al.*, 1991) we described the catabolism of 2-methylaniline by *Rhodococcus rhodochrous* wild-type strain CTM and a spontaneous mutant CTM2. Strain CTM metabolized 2-methylaniline chiefly via the *meta*-cleavage pathway. In strain CTM2, however, cleavage of 3-methylcatechol was exclusively intradiol (*ortho*-cleavage). Enzyme assays on crude extracts showed the presence of catechol 2,3-dioxygenase as an inducible enzyme in *R. rhodochrous* strain CTM, but not in strain CTM2.

The only genetic data on the metabolism of methylanilines that have been published previously (McClure & Venables, 1987) concerned a conjugative plasmid pTDN1 in *Pseudomonas* strain UCC2. This plasmid harboured the degradative genes for the catabolism of

aniline and 3- and 4-methylaniline. The C23O structural gene was located on a 1.9 kb *Hind*III restriction fragment (Saint *et al.*, 1990) and coded for an enzyme which preferentially cleaved methylcatechols (Wallis & Chapman, 1990).

In this paper we describe the cloning of two C23O genes and some properties of the corresponding enzymes from *R. rhodochrous* CTM.

Methods

Bacterial strains and culture conditions. Isolation and culture of *R. rhodochrous* strains CTM and CTM2 are described in the preceding paper (Fuchs *et al.*, 1991). For enzyme purification, cells of *R. rhodochrous* CTM were cultivated in a 500 l fermenter on mineral medium supplemented with sorbitol. After induction with 2-methylaniline (1 mM), bacteria were harvested when the culture fluid reached its absorption maximum at 387 nm due to the formation of 2-hydroxy-6-oxoheptadienoic acid. For plasmid DNA preparations the strain was cultivated in LB-medium (Maniatis *et al.*, 1989) with 10 mM-DL-threonine. Recombinant *E. coli* strains were grown in Oxoid antibiotic medium no. 3 (AM3) containing 100 µg ampicillin ml⁻¹. Details of strains used in this work are shown in Table 1.

† Present address: Kronwald Separationstechnik, D-6920 Sinsheim, FRG.

‡ Present address: Genzentrum, D-8033 Martinsried, Am Klopfer-spitz, FRG.

§ Present address: GBF, Microbiology Division, D-3300 Braunschweig, FRG.

Abbreviation: C23O, catechol 2,3-dioxygenase.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmids and relevant phenotypes/genotypes	Source
<i>R. rhodochrous</i>		
CTM	pTC1 (C23OI ⁺ C23OII ⁺), pTC2 (cryptic)	This study
CTM2	pTC1-2 (C23OI ⁻ C23OII ⁻), pTC2	
<i>E. coli</i>		
TG1	$\Delta(lac-pro) thi supE (r_K^- m_K^-)$	Maniatis <i>et al.</i> (1989)
DH1	<i>hsdR17 (r_K^- m_K^-)</i>	
DH1	pMVS301 (Ap ^r Thio ^r)	Vogt Singer & Finnerty (1988)
DH1	pUC18/pUC19 (Ap ^r <i>lacZ</i>)	
DH1	pJOE875 (Ap ^r Thio ^r Mel ⁺)	Vieira & Messing (1982)
DH1	pAS1360 (Ap ^r C23OI ⁺ C23OII ⁺)	
DH1	pAS1361 (Ap ^r C23OI ⁺ C23OII ⁺)	This study
TG1	pAS2600 (Ap ^r C23OI ⁻ C23OII ⁺)	
DH1	pAS1700 (Ap ^r C23OI ⁻ C23OII ⁺)	
DH1	pAS1200 (Ap ^r C23OI ⁻ C23OII ⁻)	
DH1	pAS1150 (Ap ^r C23OI ⁻ C23OII ⁺)	
TG1	pAS2400 (Ap ^r C23OI ⁺ C23OII ⁺)	

Detection of C23O activity.

(i) *In whole cells.* *E. coli* clones expressing C23OI were detected by the catechol spray test (Worsey *et al.*, 1978) using ferrous ammonium sulphate (10 mM) followed by 3-methylcatechol (5 mM) in buffer A (20 mM-potassium phosphate, pH 7.5). For detection of C23OII activity, a solution of 5 mM-2,3-dihydroxybiphenyl, dissolved in acetone, was sprayed on the colonies.

(ii) *In crude extracts.* Samples of crude extracts (0.5–2 mg protein) were pre-incubated with 0.2 mM (final concentration) 3-methylcatechol. The amount of 2-hydroxy-6-oxoheptadienoic acid formed was estimated as described by Rast *et al.* (1980). The same extinction coefficient at 390 nm was used to determine *meta*-cleavage products from chloromethylcatechols. On polyacrylamide gels, protein bands carrying C23O activity were detected by an activity-staining procedure according to Schmitt *et al.* (1984).

C23OII activity was assayed using biphenyl-2,3-diol as substrate. The reaction mixture (final vol. 1 ml) contained 3–5 mg protein. Pre-incubation with Fe²⁺ was omitted and the reaction was started by addition of 0.5 mM (final concentration) 2,3-dihydroxybiphenyl dissolved in acetone. The formation of 2-hydroxy-6-oxo-6-phenylhexadienoic acid was followed at the absorption maximum of 432 nm, and quantified using the extinction coefficient described by Catelani & Colombi (1974).

Purification of C23O from R. rhodochrous. All purification steps were done at 4 °C using buffer A. Cells were harvested at the end of the exponential growth phase. Disruption of the cells was achieved by three passages through a French press at 20000 p.s.i. (about 138 MPa). After centrifugation (48000 g) the supernatant was used as crude extract. The cell-free extract was then applied onto a DEAE cellulose column (15 × 5.5 cm), previously equilibrated with buffer A. Enzyme was eluted with a linear gradient of 0–600 mM-KCl in buffer A. Fractions containing active enzyme were combined and passed through a Superose-12 gel filtration column (type HR 10/30, Pharmacia/LKB) with a flow rate of 0.3 ml min⁻¹. Fractions with C23O activity were again combined and brought to 1.7 M-(NH₄)₂SO₄. Further purification was obtained by hydrophobic interaction chromatography on phenyl-Superose (MonoP, type HR 5/5). The enzyme was eluted with a linear (NH₄)₂SO₄ gradient (1.7–0 M).

Molecular mass determination. Molecular masses of subunits were determined using SDS-PAGE according to Laemmli (1970). Native molecular masses were determined by gel filtration using buffer A. The flow rate was 0.4 ml min⁻¹.

Isoelectric focusing. The isoelectric point of the purified enzyme was determined on Ampholine-PAGE plates (Pharmacia/LKB) with a pH range of 3.5–9.3 at 10 °C on a horizontal LKB 2117 Multiphor apparatus.

pH optimum. C23OI activity was assayed in Britton–Robinson buffer (Britton & Robinson, 1931) at different pH values with 3-methylcatechol as substrate.

Iron content. The iron content of purified C23OI was determined by energy dispersive X-ray fluorescence spectroscopy in relation to an internal iron standard.

Influence of metal ions and other agents. Samples (160 µg) of purified C23OI were pre-incubated in buffer A for 10 min at 25 °C with various amounts (up to 3 mM) of different metal ions, 2,2'-bipyridyl (1.3–6.4 mM), 1,10-phenanthroline (11 mM), ascorbate (up to 1 mM in 0.1 mM steps) and 0.03% (v/v) H₂O₂. 3-Methylcatechol (1 mM) was used as enzyme substrate without addition of Fe²⁺.

N-terminal amino acid sequence. A gas-phase amino acid sequence analysis according to the method of Edman was done on an Applied Biosystems 477 sequencer. The N-terminal sequence obtained by this method, however, was not suitable for the synthesis of an oligonucleotide. Therefore, partial amino acid sequencing was carried out, using cyanogen bromide.

Chromatographic separation of C23OI and C23OII from E. coli clones. Crude extracts from *E. coli* containing cloned C23O genes were prepared as described above. After an ammonium sulphate precipitation (30% saturation) and dialysis of the supernatant against buffer A (containing 1 mM-Fe²⁺) overnight, aliquots of the protein samples were loaded onto an anion-exchange column (MonoQ, type HR 5/5). A linear KCl gradient (0–1 M) was applied (flow rate 1 ml min⁻¹; 0–6 ml washing-off unbound protein; 6–21 ml, 0–1 M-KCl; 21–26 ml, 1 M-KCl). Active fractions were desalted, concentrated by ultrafiltration (PM10 membrane, Amicon), loaded onto a MonoQ column again, and

eluted with a stepwise KCl gradient (0–6 ml, 0 M-KCl; 6–12 ml, 0–0.4 M-KCl; 16–21 ml 0.4–1 M-KCl). Substrate specificity of the enzymes was determined with various catechols including 2,3-dihydroxyphenylpropionic acid and 2,3-dihydroxycinnamic acid. The reaction mixtures contained a suitable amount of protein and the corresponding substrate (1.5 mM) in a final volume of 1 ml. For determination of C23OI activity the mixture was supplemented with 0.2 mM-Fe²⁺ and preincubated at 25 °C for 1 min.

DNA isolation. For analytical purposes plasmid DNA from *R. rhodochrous* was isolated as described by Anderson & McKay (1983), with the following modifications. The incubation time of the cells with lysozyme (10 mg ml⁻¹) was extended to 120 min. Complete cell lysis was achieved after addition of SDS (20%, w/v) and incubation for 30 min at 55 °C. Rapid preparations of plasmid DNA of *E. coli* clones was done according to Holmes & Quigley (1981).

For preparative isolation of plasmid DNA from *R. rhodochrous* the cell pellet from a 50 ml bacterial culture was resuspended in 7.6 ml buffered sucrose (6.7%, w/v, sucrose, in 50 mM-Tris/HCl, 1 mM-EDTA, pH 8.0). After addition of 2.0 ml lysozyme [40 mg ml⁻¹ in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0)] the cell suspension was incubated at 37 °C for 15 min. Then 970 µl 250 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, was added and incubation was continued for another 105 min. Cells were lysed by addition of 600 µl SDS (20% w/v in 50 mM-Tris/HCl, 20 mM-EDTA, pH 8.0) and incubating at 55 °C for 30 min. The lysate was sheared by vigorous vortexing for 30 s. Then alkaline denaturation was achieved by addition of 560 µl freshly prepared 3 M-NaOH followed by gentle mixing for 10 min. The pH was decreased by adding 1 ml 2.0 M-Tris/HCl, pH 7.0, followed by gentle mixing for 5 min. After addition of 2.1 ml of SDS (20%, w/v in TE buffer), 4.2 ml of ice-cold 5.0 M-NaCl was added and the tubes were incubated at 4 °C for several hours or overnight. The precipitate was collected by centrifugation (4 °C, 48000 g, 90 min) and the clear supernatant containing the DNA was carefully removed. The DNA was precipitated by addition of 1 vol. cold propan-2-ol followed by incubation at -20 °C for 30 min. After centrifugation (4 °C, 10000 g, 20 min) the precipitate was dissolved in 1.5 ml TE buffer.

Vector DNA from *E. coli* was isolated by the method of Kieser (1984). Large-scale isolation of plasmid DNA from *E. coli* clones was performed by a brij lysis as described by Clewell & Helinski (1969). All preparative plasmid DNA isolates were further purified by ultracentrifugation on caesium chloride gradients. Total DNA was prepared according to the method of Hopwood *et al.* (1985).

Recovery of DNA from agarose gels. Restriction fragments were recovered by the freeze-squeeze method described by Trautz & Renz (1983).

Nucleic acid hybridization. A mixed oligonucleotide (5'->GCTGXTTXXAAXAANGTGTC<-3'; Y = T/C, X = A/G, N = A/G/C/T) was synthesized by the β -cyanoethylphosphoramidite method on an Applied Biosystems 380A instrument using the protocol provided by the manufacturer. This oligonucleotide was selected according to the partial amino acid sequence ¹³⁰Asp-Thr-Phe-Phe-Asn-Glu-Ala¹³⁶ of a cyanogen bromide peptide of C23OI obtained by gas-phase amino acid sequence analysis. The oligonucleotide was labelled with [γ -³²P]ATP by using T4 polynucleotide kinase and hybridized with electrophoretically fractionated DNA transferred to nylon membrane filters (Southern, 1975). Hybridizations were carried out at room temperature overnight. Membranes were washed twice in 6 × SSC buffer (20 × SSC is 3.0 M-NaCl, 0.3 M-sodium citrate, pH 7.0) at room temperature followed by washes at 45 °C and 50 °C. The membranes were exposed to X-ray film at -70 °C for 12–48 h.

Cloning experiments. The *Rhodococcus-E. coli* shuttle vector pMVS301 was used (Vogt Singer & Finnerty, 1988) and plasmid DNA (10 µg) from *R. rhodochrous* strains CTM and CTM2 was digested with

*Bgl*II and size-fractionated by agarose gel electrophoresis. The desired restriction fragments were ligated to the vector pMVS301 after extraction from the agarose gel. pMVS301 (2 µg) was digested with *Bgl*II and treated with calf intestinal alkaline phosphatase. Ligation was carried out with 2 µg plasmid DNA, 0.5 µg vector DNA and 1 Unit of T4-DNA ligase. Competent cells of *E. coli* DH1 and *E. coli* TG2 were prepared according to Mandel & Higa (1970). For subcloning in *E. coli* TG2 the *Streptomyces-E. coli* shuttle vector pJOE875 and the vector pUC18 were used.

Results

Properties of C23OI

C23OI purified (Table 2) from *R. rhodochrous* strain CTM showed a single band on SDS-PAGE, corresponding to a subunit M_r of 39000 ± 2000 . The M_r of the native enzyme, determined by gel filtration, was 156000 ± 3600 , suggesting that the native enzyme is a tetramer of four identical subunits. The isoelectric point of the enzyme was pH 4.4. The addition of acetone, as described for the stabilization of other metapyrocatechases (Nozaki, 1979), did not affect the stability of C23OI. The pH optimum of the enzyme was 8.0–8.5. Enzyme activity was highest between 50 and 65 °C. This high temperature is indicative of the heat stability of the enzyme which was still active after incubation at 80 °C for 5 min.

Microbial *meta*-cleavage enzymes usually contain ferrous iron (Nozaki, 1979). Therefore the effect of complexing agents, reducing and oxidizing agents and different metal ions on enzyme activity was tested. Complexing agents like 2,2'-bipyridyl and 1,10-phenanthroline inhibited C23OI activity. Hg²⁺ (0.01 mM) and 3 mM-Fe³⁺ totally inhibited enzyme activity; 0.2 mM-Fe³⁺ caused 35% inhibition, 1 mM-Ag⁺ 53%, 0.1 mM-Cu²⁺ 43% and 0.1 mM-Co²⁺ 32%. The enzyme was activated by ascorbate (0.1 mM for 50%) and by Fe²⁺ (1 mM for 94%).

Analysis of the iron content revealed that 0.13 nmol of the native enzyme contained 0.510 nmol iron. On the basis of an M_r of 156000 and four identical subunits these data imply 0.98 mol of iron per subunit. Thus we conclude that one atom of Fe is bound per subunit of C23OI.

The N-terminal amino acid sequence of C23OI from *R. rhodochrous* strain CTM was found to be ¹Glu-Ala-Ile-Thr-Ser-Asp-Ser-Asn-Ile-Val-Glu-Val-Ser-Val-Pro¹⁵. Comparison with the published sequence of 3-methylcatechol 2,3-dioxygenase from *Pseudomonas putida* strain UCC2 (Wallis & Chapman, 1990) showed that only the valine residues at position 12 was identical in these two *meta*-cleavage enzymes.

Fig. 1. (a) Agarose gel electrophoresis of *Bgl*II (lanes 2–5), *Pst*I (lanes 6–9) and *Bam*HI (lanes 10–13) restriction enzyme digests of total plasmid DNA and total DNA from *R. rhodochrous* strains CTM and CTM2. Lane 1, λ /*Hind*III size standards; lane 14, λ /*Pst*I size standards. Lanes 2, 6 and 10 show the plasmid DNA of strain CTM and lanes 3, 7 and 11 the plasmid DNA of strain CTM2. Lanes 4, 8 and 12 show total DNA from strain CTM, and lanes 5, 9 and 13 total DNA from CTM2. (b) Autoradiogram of a Southern blot hybridization of the DNA shown in (a) with ³²P-labelled oligonucleotide derived from C23OI.

Table 2. Purification of C23O from *R. rhodochrous* CTM

Fraction	Vol. (ml)	Protein (mg)	Total activity (mU)	Specific activity [mU (mg protein) ⁻¹]	Purification (–fold)
Crude extract*	89	712	1780	2.5	1.0
DEAE-52	100	305	1000	3.3	1.32
Superose-12	18	0.72	515	720	286
Phenyl-Superose	12	0.058	97	1690	676

* Prepared from 80 g cells (wet wt).

Cloning of C23OI genes

R. rhodochrous strain CTM harbours two plasmids of about 111 kb (pTC1) and 20.5 kb (pTC2). We probed plasmid DNA and total DNA of strain CTM with a mixed oligonucleotide synthesized on the basis of the amino acid sequence of a peptide with seven amino acids from the purified C23OI of *R. rhodochrous* strain CTM (see Methods). The probe hybridized strongly (Fig. 1)

with a 3.5 kb *Bgl*II fragment, a 5.7 kb *Pst*I fragment and a 1.45 kb *Bam*HI fragment of plasmid DNA from strain CTM. No hybridization was observed with plasmid and total DNA of strain CTM2.

Cloning the hybridizing 3.5 kb *Bgl*II fragment in the vector pMVS301 with subsequent selection of transformants for C23O activity by the catechol spray test yielded 27 positive clones. Analysis of the recombinant

Fig. 2. Crude cell extract from *E. coli* DH1(pAS1360) eluted from a MonoQ ion-exchange column with a linear gradient of 0–1.0 M KCl. The fractions in which cloned C23OI and C23OII eluted are marked by arrows. ▲, Absorption of the fractions at 280 nm; ■, molarity of salt gradient.

Table 3. Substrate specificity of C23OII compared with C23OI purified from *R. rhodochrous* CTM

The activity of C23OI was measured after pre-incubating of the sample with Fe^{2+} for 1 min; 100% activity $\equiv 4.7$ mU (mg protein) $^{-1}$. The activity of C23OII was determined without addition of Fe^{2+} ; 100% activity $\equiv 1.0$ mU (mg protein) $^{-1}$.

Substrate	Relative activity (%)	
	C23OI	C23OII
Catechol	100	100
3-Methylcatechol	360	63
4-Methylcatechol	165	45
5-Chloro-3-methylcatechol	144	10.5
3-Chlorocatechol	0	0
4-Chlorocatechol	51	31
2,3-Dihydroxybenzoate	0	9.5
3,4-Dihydroxybenzoate	0	3
3,4-Dihydroxybenzoateethyl ester	0	15
3,4-Dihydroxybenzoatemethyl ester	0	0
2,3-Dihydroxyphenylpropionic acid	0	8
2,3-Dihydroxycinnamic acid	0	33
2,3-Dihydroxybiphenyl	0	29
3,4-Dihydroxybiphenyl	0	0

plasmid DNA in these clones showed that the 3.5 kb *Bgl*II fragment inserted in the vector pMVS301 randomly with respect to orientations as determined by digestion of the recombinant plasmids (pAS1360/pAS1361) with *Hind*III. All clones showed constitutive expression of C23O. These findings suggested that no regulatory genes were present on the cloned *Bgl*II fragment. Insertion of the 1.45 kb *Bam*HI fragment into the vector pUC18 did not yield clones with C23O activity.

Characterization of the gene products from cloned DNA.

We partially purified the C23O from recombinant *E. coli* cells harbouring pAS1360. Chromatography of the crude

extract through an anion-exchange column (MonoQ) yielded two distinct protein peaks with C23O activity when tested with 3-methylcatechol as substrate (see Fig. 2). Crude extracts from cells of *E. coli* strain DH1 carrying no recombinant plasmid, when separated on a MonoQ column, showed no protein peaks with C23O activity. Partial purification and characterization of the two proteins indicated that one protein was identical with C23OI from *R. rhodochrous* CTM with respect to the M_r values of the native enzyme and its subunits, the substrate range and activation by Fe^{2+} . The second protein represented an additional *meta*-cleaving enzyme, designated C23OII. The M_r of native C23OII was found by gel-filtration to be $158\,000 \pm 3400$. Under denaturing conditions, a single protein band of M_r $40\,000 \pm 2000$ was demonstrated, indicating four identical subunits. The activity of purified C23OII decreased when pre-incubated with Fe^{2+} for 1 min. This was in contrast to C23OI which was activated by such treatment. Comparison of the substrate specificity of the two enzymes from recombinant *E. coli* yielded the following results. The enzyme which was identical with C23OI from *R. rhodochrous* strain CTM converted 3-methylcatechol much better than non-substituted catechol. C23OII, however, preferentially cleaved catechol. This enzyme accepted additional substrates like protocatechuic acid which was cleaved at the 2,3-position and 2,3-dihydroxybiphenyl which was cleaved at the 1,2-position. Neither compound was converted by C23OI (Table 3). By exploiting this different substrate specificity of the two catechol dioxygenases, we were able to detect both active protein peaks in extracts from wild-type *R. rhodochrous* strain CTM by using 3-methylcatechol and 2,3-dihydroxybiphenyl as discerning substrates (data not shown).

Subcloning

In order to locate the structural genes for C23OI and C23OII more precisely, the 3.5 kb *Bgl*II insert of plasmid pAS1360 was mapped and subcloning experiments were done as shown in Fig. 3. Insertion of the 1.2 kb *Bgl*II–*Sma*I fragment into pUC18 and pUC19 yielded no positive clones. Probing the clones with the oligonucleotide gave a strong signal with the inserted fragment. However, the 1.2 kb insert had suffered a deletion within the *Hind*III restriction site as determined by restriction enzyme analysis. The protein peak of C23OI could be detected in the elution profile of crude extract from the MonoQ column. Obviously, this deletion led to the expression of inactive enzyme.

Expression of C23OI and C23OII

Results (Table 4) of experiments on *R. rhodochrous* extracts showed that both enzymes were induced during

Fig. 3. Restriction map of the 3.5 kb *Bgl*II fragment from plasmid pTC1 cloned in pMVS301 and its derivative subclones. The phenotype of the clones harbouring the corresponding plasmid is marked as follows: C23OI⁺ (●); C23OII⁺ (▲). The abbreviations for the restriction enzymes are as follows: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Eo, *Eco*RV; H, *Hind*III; M, *Mlu*I; N, *Nae*I; Nr, *Nru*I; P, *Pvu*I; S, *Sst*I; Sm, *Sma*I.

Table 4. Specific activities of C23OI and C23OII in crude extracts of different *E. coli* clones and in *R. rhodochrous* CTM

Strain	Phenotype	Specific activity [mU (mg protein) ⁻¹]				Induction
		Substrate... 3-Methylcatechol		2,3-Dihydroxybiphenyl		
		– Fe ²⁺	+ Fe ²⁺	– Fe ²⁺	+ Fe ²⁺	
<i>R. rhodochrous</i> CTM	C23OI ⁺ C23OII ⁺	0	0	0	0	None
<i>R. rhodochrous</i> CTM	C23OI ⁺ C23OII ⁺	3.6	8.6	0.8	<0.1	<i>o</i> -Toluidine
<i>R. rhodochrous</i> CTM	C23OI ⁺ C23OII ⁺	0.75	2.4	0.2	<0.1	Biphenyl
<i>E. coli</i> (pAS1360)	C23OI ⁺ C23OII ⁺	8.8	11.4	8.2	7.2	None
<i>E. coli</i> (pAS1700)	C23OI [–] C23OII ⁺	72.0	51.6	43.6	15.3	None
<i>E. coli</i> (pAS1150)	C23OI [–] C23OII ⁺	29.7	25.6	12.9	5.1	None

cultivation in the presence of 2-methylaniline or biphenyl. As C23OII also converted 3-methylcatechol (see Table 3), the data for enzyme activities with 3-methylcatechol as substrate are the sum of C23OI and C23OII activities in *R. rhodochrous* strain CTM and also in *E. coli* clones harbouring the genes for both enzymes (pAS1360). The enzyme activities measured with dihydroxybiphenyl as substrate can be attributed exclusively to C23OII activity. In *E. coli* clones the activities of *meta*-cleaving enzymes were significantly higher than in *R. rhodochrous* due to the presence of a strong promoter in these clones.

Discussion

Our studies on the differences between wild-type *R. rhodochrous* strain CTM and the spontaneous mutant strain CTM2 at the enzymic and genetic level confirmed that the mutant strain CTM2 has lost a 3.5 kb fragment of plasmid pTC1 which carries the genes for C23OI and

C23OII, and therefore exhibits the C23O[–] phenotype. Our studies included cloning, expression and investigating some properties of two isofunctional catechol 2,3-dioxygenases in *R. rhodochrous* CTM. One of them (C23OI) exhibits a high affinity towards methylcatechols.

Two kinds of *meta*-cleavage enzymes have been described which are able to convert methylcatechols. One type prefers unsubstituted catechol as substrate (Murray *et al.*, 1972; Latorre *et al.*, 1984) and the second shows a preference for methylcatechols (Bayly *et al.*, 1977; Wallis & Chapman, 1990).

Up until now, only one *meta*-cleavage enzyme from a *Rhodococcus* species has been purified (Rast *et al.*, 1980). It belongs to the second enzyme-type, like the C23OI of *R. rhodochrous* strain CTM. Both enzymes, however, differ widely in most of their properties such as substrate specificity, the number of subunits, activation by Fe²⁺ and sensitivity towards metal ions (Rast *et al.*, 1980). The main difference between *R. rhodochrous* strain CTM, *R. rubrus* and other rhodococci that are known to have a

C23O is the presence of two *meta*-cleavage enzymes in *R. rhodochrous* strain CTM.

C23OI converts 3-methylcatechol at higher rates than unsubstituted catechol. Recently, a *meta*-cleavage enzyme of the same substrate specificity type from the methylaniline-degrading *Pseudomonas putida* strain UCC2 (McClure & Venables, 1986, 1987), was described by Wallis & Chapman (1990). Although the first 15 amino acid residues of the N-termini of the enzymes from *P. putida* strain UCC2 and *R. rhodochrous* strain CTM showed no homology, both enzymes are comparable regarding their native M_r values, number of subunits, optimal pH and the activating effect of Fe^{2+} . Treatment of both enzymes with oxidants and H_2O_2 resulted in irreversible loss of enzyme activity. The restriction endonuclease maps of the C23O gene from *P. putida* strain UCC2 (Saint *et al.*, 1990) and the C23OI gene from *R. rhodochrous* strain CTM, however, showed no similarity.

C23OII from *R. rhodochrous* strain CTM is very similar to C23OI with respect to the M_r of the native enzyme and the number and size of the subunits, but C23OII shows less pronounced substrate specificity. C23OII preferentially converts non-substituted catechol and additionally catechols derived from phenyl carboxylic acids and 2,3-dihydroxybiphenyl. C23OII is completely different in size and number of subunits from previously described 2,3-dihydroxybiphenyl dioxygenases, which either had more than four subunits (Furukawa & Arimura, 1987) or consisted of non-identical subunits (Selivonov *et al.*, 1988). C23OII, like all other dihydroxybiphenyl dioxygenases, accepts 2,3-dihydroxybiphenyl but not 3,4-dihydroxybiphenyl as a substrate. A further characteristic of C23OII of *R. rhodochrous* strain CTM is the cleavage of protocatechuate at the 2,3-position and not at the 4,5-position, which is the case for most other *meta*-cleavage enzymes. Up until now, cleavage at the 2,3-position has been observed only for the protocatechuate dioxygenase from *Bacillus* spp. (Crawford, 1975; Crawford *et al.*, 1979), and this was believed to be a specific taxonomic feature.

The existence of more than one *meta*-pyrocatechase in xenobiotic-degrading micro-organisms is not uncommon. In various biphenyl-degrading bacteria as well as in *Pseudomonas* strains harbouring TOL plasmids (Chatfield & Williams, 1986; Keil *et al.*, 1985) two *meta*-pyrocatechases have been observed (Selivonov *et al.*, 1988; Furukawa *et al.*, 1983; Furukawa & Arimura, 1987). *Pseudomonas* MT15 harbouring plasmid pWW15 has two non homologous C23O enzymes (Keil *et al.*, 1985) which convert catechol and 3-methylcatechol to a similar extent, like the dioxygenases of *R. rhodochrous* strain CTM. In contrast to *P. putida* MT15, where the two dioxygenase genes are located at different positions

(Keil *et al.*, 1985), in *R. rhodochrous* CTM the genes for both dioxygenases are clustered on a 2.4 kb *Bgl*II-*Mlu*I restriction fragment of plasmid pTC1.

This work was supported by the Bundesministerium für Forschung und Technologie and the Fonds der Chemischen Industrie. We thank Dr J. Eberspächer for critical reading of the manuscript.

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