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

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\textit{Rhodococcus rhodochrous} strain CTM degrades 2-methylaniline mainly via the meta-cleavage pathway. Conversion of the metabolite 3-methylcatechol was catalysed by an \(M, 156000\) catechol 2,3-dioxygenase (C230I) comprising four identical subunits of \(M, 39000\). 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 \textit{R. rhodochrous}. The structural gene of C230I was located on a 3.5 kb \(BgfII\) restriction fragment of plasmid pTC1. On the same restriction fragment the gene for a second catechol 2,3-dioxygenase, designated C230II, was found. This gene coded for the synthesis of the \(M, 40000\) polypeptide of the \(M, 158000\) tetrameric C230II. More precise mapping of the structural genes showed that the C230I gene was located on a 1.2 kb \(BgfII\)-\(SmaI\) fragment and the C230II gene on the adjacent 1.15 kb \(SmaI\) fragment. Comprehensive substrate range analysis showed that C230II accepted all the substrates that C230I did, but additionally cleaved 2,3-dihydroxybiphenyl and catechols derived from phenylcarboxylic acids. C230I exhibited highest activity towards methylcatechols, whereas C230II cleaved unsubstituted catechol preferentially.

Introduction

In the preceding paper (Fuchs \textit{et al.}, 1991) we described the catabolism of 2-methylaniline by \textit{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 \textit{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 \textit{Pseudomonas} strain UCC2. This plasmid harboured the degradative genes for the catabolism of aniline and 3- and 4-methylaniline. The C230 structural gene was located on a 1.9 kb \(HindIII\) restriction fragment (Saint \textit{et al.}, 1990) and coded for an enzyme which preferentially cleaved methylcatechois (Wallis & Chapman, 1990).

In this paper we describe the cloning of two C230 genes and some properties of the corresponding enzymes from \textit{R. rhodochrous} CTM.

Methods

\textit{Bacterial strains and culture conditions.} Isolation and culture of \textit{R. rhodochrous} strains CTM and CTM2 are described in the preceding paper (Fuchs \textit{et al.}, 1991). For enzyme purification, cells of \textit{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 cultured in LB-medium (Maniatis \textit{et al.}, 1989) with 10 mM DL-threonine. Recombinant \textit{E. coli} strains were grown in Oxoid antibiotic medium no. 3 (AM3) containing 100 \(\mu\)g ampicillin ml\(^{-1}\). Details of strains used in this work are shown in Table 1.
Detection of C230 activity.

(i) In whole cells. E. coli clones expressing C230I 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 C230II activity, the same extinction coefficient at 390 nm was used to determine meta-cleavage products from chloromethylcatechols. On polyacrylamide gels, protein bands carrying C230 activity were detected by an activity-staining procedure according to Schmitt et al. (1984).

(ii) In crude extracts. Samples of crude extracts (0.5-2 mg protein) were pre-incubated with 0.2 mM (final concentration) 3-methylcatechol and 0.5 mM Fe2+ was sprayed on the colonies. 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-oxoheptadienoic acid was followed at the absorption maximum of 432 nm, and quantified using the extinction coefficient described by Catelani & Colombi (1974).

Purification of C230 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 x 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 C230 activity were again combined and brought to 1 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. C230I 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 C230I 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 C230I 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 mM), 1,10-phenanthroline (11 mM), ascorbate (up to 1 mM in 0.1 M steps) and 0.01% (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 C230I and C230II from E. coli clones. Crude extracts from E. coli containing cloned C230 genes were prepared as described above. After an ammonium sulphate precipitation (30% saturation) and dialysis of the supernatant against buffer A (containing 1 mM-FeCl₃) 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

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmids and relevant phenotypes/genotypes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. rhodochrous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTM</td>
<td>pTC1 (C230I+C230II⁺), pTC2 (cryptic)</td>
<td>This study</td>
</tr>
<tr>
<td>CTM2</td>
<td>pTC1-2 (C230I⁻C230II⁻), pTC2</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>Δlac-pro thi supE (rΔm₅₈)</td>
<td>Maniatis et al. (1989)</td>
</tr>
<tr>
<td>DH1</td>
<td>hsdR17 (rΔm₅₈)</td>
<td>Vogt Singer &amp; Finnerty (1988)</td>
</tr>
<tr>
<td>DH1</td>
<td>pMVS301 (Ap'Thio)</td>
<td>Vieira &amp; Messing (1982)</td>
</tr>
<tr>
<td>DH1</td>
<td>pUC18/pUC19 (Ap'lacZ)</td>
<td>J. Altenbuchner Stuttgart, FRG</td>
</tr>
<tr>
<td>DH1</td>
<td>pIOE875 (Ap'Thio' Met⁺)</td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>pAS1360 (Ap'C230I+C230II⁺)</td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>pAS1361 (Ap'C230I+C230II⁺)</td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>pAS2600 (Ap'C230I+C230II⁺)</td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>pAS1700 (Ap'C230I⁻C230II⁺)</td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>pAS1200 (Ap'C230I⁻C230II⁺)</td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>pAS1150 (Ap'C230I⁻C230II⁺)</td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>pAS2400 (Ap'C230I⁻C230II⁺)</td>
<td></td>
</tr>
</tbody>
</table>
eluted with a stepwise KCl gradient (0–6 ml, 0.5 M-KCl; 6–12 ml, 0–
0.4 M-KCl; 16–21 ml 0.4–1 M-KCl). Substrate specificity of the en-
zeymes was determined with various catechols including 2,3-dihydrorx-
phenylpropionic acid and 2,3-dihydrorxycinnamic 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
C230I activity the mixture was supplemented with 0.2 mM-Fe$^{3+}$ 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 (67%, w/v, sucrose, in 50 mM-Tris/HCl, 1 mM-
EDTA, pH 8.0) and incubation was continued for 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 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 precipitat-
ed by addition of 1 vol. cold propan-2-01 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 ultracentrif-
ugation on cesium 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–squeez method described by Trautz & Renz
(1983).

**Nucleic acid hybridization.** A mixed oligonucleotide (5'->GCTTGXXAAAXAAGTGTGTC<3'; Y = T/C, X = A/G, 
N = A/G/C/T) was synthesized as 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 $^{130}$Asp-Thr-Phe-Phe-
Asn-Glu-Ala$^{136}$ of a cyanogen bromide peptide of C230I 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

*BglII* 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
*BglII* 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 C230I**

C230I purified (Table 2) from *R. rhodochrous* strain CTM showed a single band on SDS-PAGE, correspond-
ning 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 metapyrocate-
chases (Nozaki, 1979), did not affect the stability of
C230I. 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-phen-
anthroline inhibited C230I activity. Hg$^{2+}$ (0.01 mm) and
3 mM-Fe$^{3+}$ totally inhibited enzyme activity; 0.2 mM-
Fe$^{3+}$ caused 35% inhibition, 1 mM-Ag$^{+}$ 53%, 0.1 mM-
Cu$^{2+}$ 43% and 0.1 mM-Co$^{2+}$ 32%. The enzyme was
activated by ascorbate (0.1 mM for 50%) and by Fe$^{2+}$
(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 the $M_{r}$ 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
C230I.

The N-terminal amino acid sequence of C230I from
*R. rhodochrous* strain CTM was found to be 1-Glu-Ala-Ile-
Thr-Ser-Asp-Ser-Asn-Glu-Ala-Pro1. Comparison with the published sequence of 3-methyl-
catechol 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 BglII (lanes 2–5), PstI (lanes 6–9) and BamHI (lanes 10–13) restriction enzyme digests of total plasmid DNA and total DNA from R. rhodochrous strains CTM and CTM2. Lane 1, λ/HindIII size standards; lane 14, λ/PstI 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 C230I.

Table 2. Purification of C230I from R. rhodochrous CTM

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (mU)</th>
<th>Specific activity [mU (mg protein)]^-1</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract*</td>
<td>89</td>
<td>712</td>
<td>1780</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-52</td>
<td>100</td>
<td>305</td>
<td>1000</td>
<td>3.3</td>
<td>1.32</td>
</tr>
<tr>
<td>Superose-12</td>
<td>18</td>
<td>0.72</td>
<td>515</td>
<td>720</td>
<td>286</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>12</td>
<td>0.058</td>
<td>97</td>
<td>1690</td>
<td>676</td>
</tr>
</tbody>
</table>

* Prepared from 80 g cells (wet wt).

Cloning of C230I 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 C230I of R. rhodochrous strain CTM (see Methods). The probe hybridized strongly (Fig. 1) with a 3.5 kb BglII fragment, a 5.7 kb PstI fragment and a 1.45 kb BamHI 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 BglII fragment in the vector pMVS301 with subsequent selection of transformants for C230 activity by the catechol spray test yielded 27 positive clones. Analysis of the recombinant
plasmid DNA in these clones showed that the 3.5 kb BglII fragment inserted in the vector pMVS301 randomly with respect to orientations as determined by digestion of the recombinant plasmids (pAS1360/pAS1361) with HindIII. All clones showed constitutive expression of C23O. These findings suggested that no regulatory genes were present on the cloned BglII fragment. Insertion of the 1.45 kb BamHI 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 158000 ± 3400. Under denaturating conditions, a single protein band of $M_r$ 40000 ± 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).

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

The activity of C23O was measured after pre-incubating the sample with Fe$^{2+}$ for 1 min; 100% activity = 4.7 mU (mg protein)$^{-1}$. The activity of C23OII was determined without addition of Fe$^{2+}$; 100% activity = 1.0 mU (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C23O</th>
<th>C23OII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3-Methylcatehol</td>
<td>360</td>
<td>63</td>
</tr>
<tr>
<td>4-Methylcatehol</td>
<td>165</td>
<td>45</td>
</tr>
<tr>
<td>5-Chloro-3-methylcatehol</td>
<td>144</td>
<td>10.5</td>
</tr>
<tr>
<td>3-Chlorocatehol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4-Chlorocatehol</td>
<td>51</td>
<td>31</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoate</td>
<td>0</td>
<td>9.5</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoate</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoateethylster</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoateethylster</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2,3-Dihydroxyphenylpropaninic acid</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>2,3-Dihydroxycinnamic acid</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>3,4-Dihydroxybiphenyl</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Subcloning

In order to locate the structural genes for C23OI and C23OII more precisely, the 3.5 kb BglII insert of plasmid pAS1360 was mapped and subcloning experiments were done as shown in Fig. 3. Insertion of the 1.2 kb BglII–SmaI 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 HindIII 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...
cultivation in the presence of 2-methylaniline or biphenyl. As C230II also converted 3-methylcatechol (see Table 3), the data for enzyme activities with 3-methylcatechol as substrate are the sum of C230I and C230II 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 C230II 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 promotor 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 C230I and C230II, and therefore exhibits the C230- phenotype. Our studies included cloning, expression and investigating some properties of two isofunctional catechol 2,3-dioxygenases in R. rhodochrous CTM. One of them (C230I) 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 C230I 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

![Fig. 3. Restriction map of the 3.5 kb BglII fragment from plasmid pTCl cloned in pMVS301 and its derivative subclones. The phenotype of the clones harbouring the corresponding plasmid is marked as follows: C230I+ (●); C230II+ (▲). The abbreviations for the restriction enzymes are as follows: A, ApaI; B, BamHI; Bg, BglII; E, EcoRI; Eo, EcoRV; H, HindIII; M, MluI; N, NaeI; Nr, NruI; P, PstI; S, SsrI; Sm, SmaI.]

Table 4. Specific activities of C230I and C230II in crude extracts of different E. coli clones and in R. rhodochrous CTM

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Phenotype</th>
<th>-Fe2+</th>
<th>+Fe2+</th>
<th>-Fe2+</th>
<th>+Fe2+</th>
<th>Induction</th>
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<tr>
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<td>3-Methylcatechol</td>
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<td>R. rhodochrous CTM</td>
<td>C230I+  C230II+</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>None</td>
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<td>8.6</td>
<td>0.8</td>
<td>&lt;0.1</td>
<td>0.1</td>
<td>α-Toluidine</td>
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<tr>
<td>E. coli (pAS1360)</td>
<td>C230I+  C230II+</td>
<td>0.75</td>
<td>2.4</td>
<td>0.2</td>
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<td>0.1</td>
<td>Biphenyl</td>
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<td>11.4</td>
<td>8.2</td>
<td>7.2</td>
<td>7.2</td>
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<td>0</td>
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<tr>
<td>R. rhodochrous CTM</td>
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<td>12.9</td>
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![Plasmid pAS1360 ● ▲

pAS2600 ●

pAS2400 ● ▲

pAS1700 ▲

pAS1150 ▲]
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 $M$, 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 $M$, 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, strain UCC2 (Saint et al., 1990). C23OII 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-pyrocatenate 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-pyrocatenate 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 BgII–MluI restriction fragment of plasmid pTC1.

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**References**


