The catechol 2,3-dioxygenase gene of *Rhodococcus rhodochrous* CTM: nucleotide sequence, comparison with isofunctional dioxygenases and evidence for an active-site histidine

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In cell-free extracts of *Escherichia coli* clones harbouring the 3.5 kb *Bgl*II fragment of plasmid pTC1 from *Rhodococcus rhodochrous* CTM a catechol 2,3-dioxygenase (C23O) accepting both 3-methylcatechol and 2,3-dihydroxybiphenyl as substrates could be detected. The plasmid-encoded gene for C23O of *R. rhodochrous* CTM and its flanking regions were sequenced. In front of the gene a sequence resembling an *E. coli* promoter was identified, which led to constitutive expression of the cloned gene in *E. coli* TG1. The derived amino acid sequence of the C23O was compared to that of nine other enzymes, which all catalyse the extradiol cleavage of an aromatic ring. These nine sequences were from different *Pseudomonas* strains, in contrast to the sequence described here, from a Gram-positive bacterium. The role of four strongly conserved histidines was examined by chemical modification of the histidyl residues of the native enzyme by diethylpyrocarbonate. For that purpose the C23O was purified to homogeneity from *E. coli* harbouring pSC1701. However, the enzyme lost its activity during the purification. Activity could partially be restored by treatment with Fe^{2+} and reducing agents.

Keywords: *Rhodococcus rhodochrous*, catechol 2,3-dioxygenase, aromatic catabolism, extradiol dioxygenases

INTRODUCTION

The Gram-positive bacterium *Rhodococcus rhodochrous* CTM is able to degrade 2-methylaniline via the *meta*-cleavage pathway (Fuchs *et al.*, 1991). The enzyme which catalyses the cleavage of the aromatic ring is catechol 2,3-dioxygenase (C23O) (EC 1.13.11.2). This enzyme consists of four identical subunits, each of M_r 39 000, and contains 1 g atom of Fe^{2+} per subunit (Schreiner *et al.*, 1991). The structural gene for this enzyme is located on a 3.5 kb *Bgl*II restriction fragment of plasmid pTC1. We report here the nucleotide sequence of the structural gene for C23O. As described by Harayama & Rekik (1989), the extradiol-cleaving enzymes belong to one gene family. All members of this gene family described up to now are produced by different *Pseudomonas* strains (see Table 1).

Abbreviations: C23O, catechol 2,3-dioxygenase; DEPC, diethylpyrocarbonate; *cdo*, gene encoding catechol 2,3-dioxygenase.

The GenBank accession number for the nucleotide sequence reported in this paper is X69504.

We therefore compared the derived amino acid sequence of C23O from the Gram-positive *R. rhodochrous* CTM with the rest of the gene family, with the aim of obtaining information concerning the active site.

METHODS

Bacterial strains and vectors. *Rhodococcus rhodochrous* CTM was isolated from garden soil with 2-methylaniline as sole carbon source (Appel *et al.*, 1984). *Escherichia coli* TG1 (Sambrook *et al.*, 1989) was the recipient strain in transformation and transduction experiments. The vectors were pUC18, M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985). The source of DNA for subcloning and nucleotide sequencing was the plasmid pTC1 of *R. rhodochrous* CTM.

Media and growth conditions. *R. rhodochrous* CTM was grown at 27 °C in the minimal medium described by Fuchs *et al.* (1991), with 0.5% (w/v) sorbitol as carbon source. For plasmid DNA preparations *R. rhodochrous* CTM was cultivated at 27 °C in complex LB medium (Sambrook *et al.*, 1989) with 10 mM DL-threonine. *E. coli* TG1 was grown at 37 °C in M9 minimal medium (Miller, 1972), containing 0.2% (w/v) glucose as

carbon source. Recombinant *E. coli* strains were grown at 37 °C in 2 × TY medium or H broth solidified if necessary with 1.5 % (w/v) agar (Miller, 1972). Ampicillin at a concentration of 100 µg ml⁻¹ was used for selection of *E. coli* TG1 containing hybrid plasmids derived from pUC18. For protein purification recombinant *E. coli* clones were grown overnight at 37 °C in LB medium with ampicillin.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and the T7 Sequencing kit and Deaza T7 Sequencing mixes were from Pharmacia LKB; shrimp alkaline phosphatase was from United States Biochemicals; the GeneClean II kit was from Dianova; [³⁵S]dATP-αS was from Amersham; 2,3-dihydroxybiphenyl was from Wako Chemicals; 3-methylcatechol was from EGA-Chemie; diethylpyrocarbonate was from Sigma.

DNA manipulations and transformations. Preparative amounts of plasmid DNA from *R. rhodochrous* were obtained by the method described previously by Schreiner *et al.* (1991). For analytical purposes recombinant plasmid DNA of *E. coli* was isolated by the alkaline lysis method (Sambrook *et al.*, 1989). Agarose gel electrophoresis, DNA digestion with restriction enzymes, treatment with alkaline phosphatase and ligation were done by standard procedures (Sambrook *et al.*, 1989). DNA fragments were isolated from agarose gels with the GeneClean II kit according to the recommendations of the supplier. Transformation of *E. coli* with plasmid DNA was performed by the CaCl₂ procedure (Mandel & Higa, 1970).

Recombinant plasmid construction. Plasmid DNA from *R. rhodochrous* CTM was digested with the appropriate restriction endonucleases and size-fractionated by agarose gel electrophoresis. After extraction from the agarose gel the desired restriction fragments were ligated into the linearized vector pUC18 (Fig. 1). pUC18 was digested with either the equivalent enzyme or an enzyme producing compatible ends (e.g., *Bam*HI/*Bgl*II) and then treated with shrimp alkaline phosphatase. Ligation was carried out with 2.5 µg insert DNA, 0.5 µg vector DNA and 1 unit T4 DNA ligase. After transformation of competent *E. coli* TG1, ampicillin-resistant white colonies on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside plates were screened for the presence of C23O activity by spraying the plates with a solution of 5 mM 2,3-dihydroxybiphenyl in acetone (Worsey *et al.*, 1978). Colonies of cells that express the gene turn yellow due to the formation of the corresponding *meta*-cleavage product, 2-hydroxy-6-oxo-6-phenylhexadienoic acid.

DNA sequencing. The 3.5 kb *Bgl*II fragment of plasmid pTC1 from *R. rhodochrous* CTM was isolated for subcloning into M13mp18 and M13mp19. Fragments of the 3.5 kb *Bgl*II fragment generated by digestion with different restriction enzymes were introduced into vector M13 (Fig. 1). Transfection of *E. coli* with M13 phage DNA, preparation of single-stranded DNA from selected phage plaques and dideoxy sequencing reactions were performed according to the instruction manual provided by Pharmacia with the sequencing kit for the use of [³⁵S]dATP-αS. All stretches of DNA were sequenced in both directions by the dideoxynucleotide sequencing method of Sanger *et al.* (1977). Sequence ladders were resolved on gels containing 6 % (w/v) polyacrylamide. The nucleotide sequences were analysed with the GENMON program (Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG).

Purification of C23O from *E. coli* clones. Frozen cells (1 g wet weight per ml buffer) of *E. coli* clones were suspended in 50 mM potassium phosphate buffer, pH 7.5, and disrupted for 20 min by sonification with a Branson Sonifier 450. After centrifugation (48000 g) the cell-free extract was heated to 45 °C for 5 min and centrifuged again. All following purification steps were done at

20 °C. The supernatant was applied onto a Q Sepharose Fast Flow column (14 × 2.3 cm), previously equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Enzyme was eluted with a 0–0.8 M linear KCl gradient in 50 mM potassium phosphate buffer, pH 7.5. Fractions containing active C23O were combined and brought to 20 % saturation with ammonium sulphate. Further purification was obtained by hydrophobic interaction chromatography on Butyl-Sepharose CL-4B (7.5 × 3 cm). The enzyme was eluted with a linear ammonium sulphate gradient (20–0 % saturation). Fractions with C23O activity were combined and desalted by dilution with 50 mM potassium phosphate buffer, pH 7.5, and ultrafiltration with a PM 10 membrane (Amicon). The pooled fractions were then again applied onto the Q Sepharose Fast Flow column and eluted with a 0–0.8 M linear KCl gradient as described above. The active fractions were collected, concentrated to 3 ml by ultrafiltration and stored at 4 °C. This enzyme solution was used for modification with diethylpyrocarbonate.

Reactivation of C23O. The incubation mixture contained 50 mM Tris/acetate buffer, pH 7.5, and 1.4 mg of enzyme in a final volume of 1.0 ml. The following additions to the incubation mixture were made as indicated: 2 mM ferrous ammonium sulphate (Fe²⁺), 5 mM ascorbic acid or 2 mM cysteine.HCl. Incubation was carried out at 24 °C. An aliquot of the mixture (0.1 ml) was used for the spectrophotometric assay of enzyme activity. Reactivation of C23O after every step of purification was done in the same way, using Fe²⁺ and ascorbic acid.

Partial purification of C23O. To check for the presence of two C23Os in *E. coli* clones, fractions from the first Q Sepharose Fast Flow column described above were examined for enzyme activity with 2,3-dihydroxybiphenyl and 3-methylcatechol as substrates. Active fractions were combined and desalted by dilution with 50 mM potassium phosphate buffer, pH 7.5, and ultrafiltration with an Amicon PM 10 membrane. The sample was subjected to a second anion-exchange chromatography using a MonoQ HR5/5 column connected with an FPLC system and eluted with a 0–0.8 M linear KCl gradient in 50 mM potassium phosphate buffer, pH 7.5. Fractions were analysed for enzyme activity with both substrates.

Frozen cells (0.5 g wet weight per ml buffer) of *R. rhodochrous* CTM were suspended in 50 mM potassium phosphate buffer, pH 7.5, and disrupted by three passages through a French press at 20000 p.s.i. (about 138 MPa). After centrifugation (48000 g) the cell-free extract was analysed as described above for extracts of *E. coli* clones.

Enzyme assay. C23O activity was determined at 25 °C. The standard assay volume of 1 ml contained 50 mM potassium phosphate buffer, pH 7.5, and 100 µl 5 mM 2,3-dihydroxybiphenyl dissolved in acetone or 300 µl 5 mM 3-methylcatechol in water. The reaction was started by adding an appropriate amount of enzyme. The formation of 2-hydroxy-6-oxo-6-phenylhexadienoic acid, the *meta*-cleavage product of 2,3-dihydroxybiphenyl, was followed spectrophotometrically at the absorption maximum of 432 nm, and quantified using the absorption coefficient reported by Catelani & Colombi (1974). The amount of 2-hydroxy-6-oxohepta-2,4-dienoic acid formed from 3-methylcatechol was estimated as described by Rast *et al.* (1980). One unit (U) of activity was defined as the amount of enzyme which cleaves 1 µmol 2,3-dihydroxybiphenyl or 3-methylcatechol min⁻¹. Specific activity was based on protein determined by the method of Bradford (1976) with bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was carried out according to the method of Laemmli (1970) using a

Fig. 1. Restriction map of the *R. rhodochrous* plasmid DNA region containing *cdo*. The bold line below *cdo* indicates the sequenced region shown in Fig. 3, which was established by sequencing the M13 subclones shown above the map. In the plasmid constructs below the map, the open arrowheads denote the position and direction of the *lac* promoter of pUC18. Expression of C23O in clones harbouring the corresponding plasmid is shown in parenthesis. Abbreviations: A, *Apal*; B, *Bam*HI; Bg, *Bgl*II; (Bg), *Bgl*II site lost after cloning; E, *Eco*RI; Eo, *Eco*RV; H, *Hind*III; M, *Mlu*I; Nr, *Nru*I; P, *Pvu*I; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I.

10% (w/v) polyacrylamide gel and Tris/glycine buffer, pH 8.3. Protein staining of the gels was performed with Coomassie Brilliant Blue R-250.

Chemical modification of histidyl residues by diethylpyrocarbonate (DEPC). Modification of histidyl residues (Miles, 1977) was carried out at 25 °C by incubating the purified, non-reactivated C23O (1.4 mg) with 5, 10 or 25 µl of 0.3 M DEPC in 50 mM potassium phosphate buffer, pH 7.5. DEPC was freshly diluted in distilled ice-cold ethanol prior to each experiment. Samples (50 µl) were removed from the incubation mixture at various intervals and added to cuvettes containing the assay mixture for determination of enzyme activity. The formation of *N*-carbethoxyhistidyl residues, which have an absorption maximum between 230 and 250 nm, was followed spectrophotometrically by the increase in absorbance. The number of modified histidyl residues was calculated using the difference absorption coefficient, $\Delta\epsilon_{240} = 3200 \text{ cm}^{-1} \text{ M}^{-1}$, for carbethoxy-histidine (Miles, 1977). Recovery of enzyme activity from DEPC inactivation was performed with 0.02 M neutral hydroxylamine.

RESULTS AND DISCUSSION

Detection of C23O activity after partial purification

Schreiner *et al.* (1991) described the expression of two C23Os from the 3.5 kb *Bgl*II fragment (pSC3501, pSC3502; Fig. 1). However, using ion-exchange chromat-

Fig. 2. Anion-exchange chromatography on Mono Q. —, A_{280} ; ---, KCl. Arrow, activity with 3-methylcatechol and 2,3-dihydroxybiphenyl.

ography (FPLC) as described by them, only one C23O could be found (Fig. 2). In cell-free extracts of *R. rhodochrous* CTM partially purified by ion-exchange chromatography, again only one C23O could be detected. This enzyme accepted 3-methylcatechol as well as 2,3-

<i>EcoRI</i>	
1 GAATTCTGTTGGAGGAAGTGTTCACAGTCTGGCCCTCACTAATGCCACGAATGAAGATCATCGTCGCATTGAGTCGGCGCTACGGAAGA	90
91 TGGAAATCAGACTCGGAGGATCCGACTGGGTTCTTCAATGCGAATGTTGAGTTTACCCGGATGATCGCCCGGTCTACCGGGGTACCCGTCC	180
181 TAGTCGGAGTTTACGAAAGTTTGGTCACCGTTCTCACCGGCGCATTGGTAAGGGCGGCGTGGACTTCGGGTACACGAGAACTGTGCAAC	270
271 ACAATGTCATGGTTACCCGGGCAATTTTGAAGCGATTCAATCCGGTGATCGAAAGAGACTGGAAGAAGCCACTTTGTTGACCCGCGTCG	360
361 ATATGGTCAATATAAGTGATCCTGATCTGTACCAATCAACCACCTGAAAACAGCACTCAGAACTGAAAGGCCGATTACGGTCTAGTC	450
-35 -10 SD MetSerAsnIleThrSerAspSerAsnIleValGluValSerVal	
451 GACCGTCTGTGCGAATTTCAATAACTACGCCAGGAGATGACTCTCATGTCCAACATCAGTCCGATTCTAATATCGTCGAGGTATCTGTC	540
ProArgValHisAsnLeuHisHisValGluLeuLeuThrProLysProAsnGluSerLeuAspPhePheThrArgValLeuGlyLeuHis	
541 CCTCGCGTCCACAATTTGCACCATGTGGAGTTGCTGACTCCGAAGCCAAACGAATCTCTCGATTCTTACCCGAGTCTTAGGACTGCAC	630
GluThrHisArgGluGlyGlnSerValTyrLeuArgGlySerGlyGluTrpGlyGlnTyrSerThrIleLeuThrGluSerProThrAla	
631 GAGACCCATCGTGAAGGCCAGTCCGTCTACCTGCGTGGCTCCGAGAGTGGGGCCAGTACTCGACCATCTGACTGAGTCCCCACCGCC	720
GlyLeuGlyHisMetGlyTrpGlnValAlaGluProGluHisValGlnGlyTrpGlyArgArgLeuLysAspAlaGlyValGluHisArg	
721 GGTCTGGGACATATGGGCTGGCAGGTGGCAGAGCCGAGCAGTCCAGGGATGGGGCGACGGCTCAAGGATGCTGGCGTGGAGCACCGC	810
PheGluAlaGlyGlySerSerPheAlaGlnGlyAspThrValSerPheGluGlyProTyrGlyHisLysMetGluLeuPheTyrAspPhe	
811 TTTGAGGCAGGGGATCCTCATTTGCGCAGGGCGATACGGTCAAGTTCGAAGGCCCTACGGGCACAAGATGGAGCTGTTCTACGACTTC	900
GluArgPheValProGluAspArgSerLysLeuLeuSerGlnProValLysPheProThrProGlyIleGlyAlaArgArgLeuAspHis	
901 GAGCGCTTTGTCCCCGAGGATCGGTCAAAGCTCCTGAGCCAGCCAGTCAAGTTCCCCACCCCGGGATTGGCGCCCTCGACTAGATCAC	990
LeuAsnIleThrAlaLysAspValAspAspAlaArgAsnTrpTyrSerAspValLeuGlyPheLysLeuArgGluAlaLeuArgThrPro	
991 CTCAACATCACAGCGAAGGACGTTGACGACGCCCGTAACCTGGTATTCCGATGTTCTCGGGTTCAAACCTGCGCGAAGCACTTCGGACCCCG	1080
AspGlyAspLeuGlyAlaTrpMetSerValSerSerGlnValHisAspMetAlaIleMetArgAspGlyMetGlyGluGluGlyArgLeu	
1081 GACGGCGATCTCGGAGCTTGATGTGCGTCAGCTCGCAGGTGCACGACATGGCGATCATGCGCGACGGGATGGGAGAGGAAGGACGCTC	1170
HisHisIleAlaTyrTrpLeuGluThrProGluThrLeuLeuArgAlaValAspThrPheValGluGluGlyThrProIleAspValGly	
1171 CATCACATCGCTACTGGCTCGAGACCCCGAGACCCCTTCTCCGTGCGGTGACACGTTTGTGCGAGGAAGGCACTCCAATCGATGTGCGT	1260
ProGlyLysHisGlyLeuThrGlnAlaPheTyrValTyrValPheGluProGlyGlyAsnArgValGluLeuPheThrGlyGlyTyrPro	
1261 CCGGGAAGACACGGACTGACCAAGCTTCTACGTGTACGTGTTGAGCCCGCGGTAAACCGTGTGAGCTCTTACCGGCGGCTACCCC	1350
IleTyrGlyProAspTrpAspProValIleTrpGluGlyGluAsnMetAspArgAlaIleValTrpTyrGlyGlyGlnLeuProAspThr	
1351 ATCTACGGCCCCGACTGGGATCCGGTGATCTGGGAAGCGAGAATGGAACCGCGCGATCGTCTGGTACGGCGGTACGCTTCCCGACACC	1440
PhePheAsnGlnAlaThr***	
1441 TTCTTCAACAGGCGACCTAAATGATAGACAGTGGCGGTTGCGCTCAGGAAGAGCGCTATTCCGCGGCGCTACTAGGAGCGTTGAGCCTC	1530
1531 AAACCTCAACGCGCTCGAGAACTCGGATGGTCTGTTTCTAGGAGGCATAAATGACTACCCAAAAGCATCTAGATGGTGGGCGCAGCTCCG	1620
1621 TTGACGATCTCAACGAATTCGTGCGCGAAGATAGAGTGCACCGAAAGCTCTACACCGACCCAGCATCTTCTCGAAGAGATGACTAAAA	1710
1711 TCTTCGGTGGACAATCATGGGTCTACCTCGCTCACGAATCACAGCTTCTGAGCCCACTCATCTCTCACTGTCCACATGGGCTTGCGGC	1800
1801 CCGTCATCGTCACCCGCGATCGACGTGGGGGACTCAACGCGGTTTCAACCGTTGCACTCACCGGGCTGCGACCGTCTGTCGCGAAGAGA	1890
1891 GCGGCGTTCGCGAAGAGCTTCCAATGCCCTATCACGGATGGACTTTCAGAAACACCGGCGAGCTAGTCGGAACGCCCTGGCCACGCGCT	1980
1981 ACTCGGACAGCTTCGACCAAGTCAAAACATGGGCTACTTCGAGTCACTCGAGTTGAGTCTTACCGAGGCTTCATCTTCGGGACCATGAACG	2070
2071 ATGCCGCCCATCAGTTCGGAGTGGTTAGGGCCAGCACTAAATGGCTCGACTACTGGATTGACCGCGCGCCAAACGGCGAGATCT	2157
<i>BglII</i>	

Fig. 3. Nucleotide sequence of the 2157 bp *EcoRI*–*BglII* fragment from the *R. rhodochrous* plasmid pTC1 containing the *cdo* gene. The ORF commences at base 496 and terminates at 1458. The predicted amino acid sequence is indicated above the DNA sequence. Underlined amino acid sequences are identical with those determined by Edman degradation. A potential ribosome binding site (marked as SD) and an *E. coli* promoter-like sequence (marked as –10 and –35) upstream of the ORF are indicated. The arrows designate inverted repeat sequences downstream of the termination codon (indicated by asterisks).

dihydroxybiphenyl and was thus identified as C230 II described by Schreiner *et al.* (1991). From these findings we conclude that C230 I observed by them was probably an artefact.

Subcloning

In order to elucidate the contradiction between our results and those of Schreiner *et al.* (1991), we repeated a subcloning experiment already described by them. The 1.2 kb *BglII*–*SmaI*, the 1.15 kb *SmaI* and the 2.35 kb *BglII*–*SmaI* fragments (pSC1200, pSC1150, pSC2350; Fig. 1) were cloned in pUC18. In contrast to the results of

Schreiner *et al.* (1991), only insertion of the 2.35 kb *BglII*–*SmaI* fragment into pUC18 led to expression of C230. The recombinant plasmids pSC1200 and pSC1150 yielded no positive clones. This suggests that only one gene for C230 was present in the region between the first *BglII* and the second *SmaI* restriction site (Fig. 1), and that the first *SmaI* restriction site was situated inside the structural gene.

Clones with the 3.5 kb *BglII* or the 1.7 kb *EcoRI* fragment constitutively expressed a single C230. The enzyme was expressed in both orientations with regard to the *lac* promoter of pUC18 (Fig. 1). This means that the structural

Table 1. Extradiol dioxygenases belonging to the same gene family

Gene	Gene product	Bacterium	Plasmid	References
<i>cdo</i>	Catechol 2,3-dioxygenase	<i>Rhodococcus rhodochrous</i> CTM	pTC1	Schreiner <i>et al.</i> (1991) This study
<i>dmpB</i>	Catechol 2,3-dioxygenase	<i>Pseudomonas</i> CF600	pVI150	Bartilson & Shingler (1989)
<i>nabH</i>	Catechol 2,3-dioxygenase	<i>Pseudomonas putida</i>	NAH7	Ghosal <i>et al.</i> (1987) Harayama <i>et al.</i> (1987)
<i>xylE1</i>	Catechol 2,3-dioxygenase	<i>Pseudomonas putida</i> mt-2	TOL plasmid pWW0	Nakai <i>et al.</i> (1983) Zukowski <i>et al.</i> (1983)
<i>xylE2</i>	Catechol 2,3-dioxygenase	<i>Pseudomonas putida</i> HS1	TOL plasmid pDK1	Benjamin <i>et al.</i> (1991)
<i>todE</i>	3-Methylcatechol 2,3-dioxygenase	<i>Pseudomonas putida</i> F1		Zylstra & Gibson (1989)
<i>bphC1</i>	2,3-Dihydroxybiphenyldioxygenase	<i>Pseudomonas pseudoalcaligenes</i> KF707		Furukawa <i>et al.</i> (1987)
<i>bphC2</i>	2,3-Dihydroxybiphenyldioxygenase	<i>Pseudomonas paucimobilis</i> Q1		Taira <i>et al.</i> (1988)
<i>bphC3</i>	2,3-Dihydroxybiphenyldioxygenase	<i>Pseudomonas</i> sp. strain KKS102		Kimbara <i>et al.</i> (1989)
<i>nabC</i>	1,2-Dihydroxynaphthalene dioxygenase	<i>Pseudomonas putida</i>	NAH7	Harayama & Rekik (1989)

gene of C23O was expressed by its own promoter in *E. coli* and that no regulatory genes were present on the cloned fragments.

Nucleotide sequence of the C23O gene *cdo*

A restriction map of the cloned region with the location of the *cdo* gene and the sequencing strategy are shown in Fig. 1. The determined nucleotide sequence and the deduced amino acid sequence of C23O are shown in Fig. 3. A total of 2157 bp, corresponding to the region between the first *Bgl*II and the second *Eco*RI restriction site, was sequenced. The nucleotide sequence was determined in both directions, and all the restriction sites used for cloning were verified by determination as part of an overlapping sequence (Fig. 1). A 966 bp open reading frame (ORF) initiating at ATG (bp 496) and terminating at TAA (bp 1461) was identified within this sequence. This ORF was confirmed as that encoding C23O by its predicted N-terminal amino acid sequence, which corresponds exactly to the N-terminal amino acid sequence obtained from the purified protein. The methionine at position 1 was absent from the purified protein. The partial amino acid sequence reported by Schreiner *et al.* (1991) was in good agreement with the derived C-terminal amino acid sequence. The ORF predicts a 321 amino acid residue protein with an M_r of 36078. This value is consistent with that of 39000 ± 2000 per subunit determined by SDS-PAGE (Schreiner *et al.*, 1991).

Nine base pairs upstream from the initiation codon is a putative Shine–Dalgarno site, the ribosome-binding sequence 5'-AGGAG-3' (Shine & Dalgarno, 1974). Further upstream a sequence homologous to the consensus sequence of *E. coli* promoters (Rosenberg & Court, 1979) was identified. We presume that this sequence functions as a promoter in *E. coli*, but it is not known whether it has the same function in the Gram-positive *R. rhodochrous*.

Inverted repeat sequences, which may act as transcription terminators (Rosenberg & Court, 1979), were found in the 3' flanking region downstream from the termination codon.

The 60.7 mol % G + C content of the *cdo* gene is similar to the 63–72 mol % G + C content of the genus *Rhodococcus* (Goodfellow, 1986). Thus the chromosomal G + C content of the host appears to be maintained even at the level of a structural gene carried on a plasmid. As would be predicted from the high G + C content of the *cdo* gene, its codon usage is highly biased (75 %) in favour of G or C in the wobble base.

Similarity of C23O with other meta-cleavage enzymes

The comparison of amino acid sequences of related enzymes is of particular interest, because conserved amino acid residues may be important for enzyme function. Therefore the derived amino acid sequence of C23O was compared to that of the various extradiol dioxygenases reported already. As described by Harayama & Rekik (1989) some of these enzymes are members of the same gene family. Since 1989 the sequences of another four extradiol-cleaving enzymes have been published, which are similar to those of the above-mentioned family. Together with that of the C23O of *R. rhodochrous* CTM, it is now possible to compare the amino acid sequences of 10 extradiol dioxygenases (Table 1).

In Fig. 4 these amino acid sequences are shown optimally aligned by introducing gaps to maximize identities using the GENMON program. C23O of *R. rhodochrous* CTM is about 30 % homologous to the derived amino acid sequences of NAHH, XYLE1, XYLE2 and DMPB. The remaining sequences (TODE, BPHC1, BPHC2, BPHC3, NAHC; for references see Table 1) could be aligned with that of C23O with about 20 % identical residues. The

Fig. 4. Amino acid alignment of the extradiol-cleaving dioxygenases. The top sequence shows the consensus, in which the upper-case letters mean 100% conservation in all sequences examined and the lower-case letters conservation in more than 50% of the sequences. The numbering system refers only to the amino acid arrangement in this figure and not to any of the sequences. The sequences were obtained from the sources indicated in Table 1.

Table 2. Degree of homology between C230 and the gene family members

Comparison between C230 and:	Identical amino acids	Degree of homology (%)	Identical nucleotides	Degree of homology (%)
NAHH	94/307	31	425/921	46
XYLE1	95/307	31	443/921	48
XYLE2	93/307	30	430/921	47
DMPB	94/307	31	424/921	46
TODE	56/290	19	304/870	35
BPHC1	50/302	17	329/906	36
BPHC2	53/299	18	336/897	38
BPHC3	50/292	17	319/876	36
NAHC	62/302	21	341/906	38

degrees of homology based on comparison of the amino acid sequences and of the nucleotide sequences are summarized in Table 2. In all cases the homology between the nucleotide sequences is higher than that of the amino acid sequences. This may indicate a common genetic origin of all these enzymes. However, it is remarkable that the extradiol dioxygenase of a Gram-positive organism

has such high similarities to isofunctional enzymes from Gram-negative *Pseudomonas* species.

The gene family of extradiol dioxygenases can be divided into two subfamilies comprising dioxygenases for monocyclic and bicyclic compounds, respectively. The members of each subfamily exhibit greater homology among one another than to members of the other subfamily (Harayama & Rekik, 1989). C230 of *R. rhodochrous* CTM accepts both catechol (monocyclic) and 2,3-dihydroxybiphenyl (bicyclic) as substrates. The amino acid sequence of C230 has more similarities to dioxygenases cleaving monocyclic compounds, but there are some similarities with the bicyclic dioxygenases (Fig. 4). Catalytically indispensable amino acid residues must be highly conserved in all 10 sequences, and the difference in substrate specificity between the dioxygenases for monocyclic and bicyclic catechols must be hidden in the nonconserved regions.

Besides these extradiol-cleaving enzymes belonging to one gene family, four unrelated extradiol dioxygenases have been described. Kabisch & Fortnagel (1990a, b) reported the nucleotide and the derived amino acid sequences of two C230s (MPCI and MPCII) from *Alcaligenes eutrophus* JMP222. On the basis of the nucleotide sequence, MPCI has less than 10% homology and MPCII has between 16 and 20% homology with the above-

mentioned gene family members. However, their amino acid sequences did not match to the alignment of the gene family of dioxygenases. The same applies to the derived amino acid sequence of the homoprotocatechuate 2,3-dioxygenase from *E. coli* C (Roper & Cooper, 1990). The extent of similarity of this enzyme with any of the aromatic ring-fission dioxygenases (extradiol and intradiol) varied between 8% and 14%, indicating that there was little or no relationship.

These three extradiol dioxygenases do not belong to the gene family, but they consist of identical subunits of M_r about 30000–40000, like the members of the extradiol dioxygenase gene family. In contrast, the protocatechuate 4,5-dioxygenase from *Pseudomonas paucimobilis* consists of an equal number of two different subunits, α and β , of M_r 18000 and 34000, respectively (Noda *et al.*, 1990). The amino acid sequences of these subunits are not related to those of the main C23O family, but the β -subunit of protocatechuate 4,5-dioxygenase exhibits sequence similarity to that of the C23O from *Alcaligenes eutrophus* (Harayama *et al.*, 1992).

Thus it can be suggested that some of the *meta*-cleavage dioxygenases originated from a common ancestor. However, besides this gene family, other isofunctional dioxygenases must have evolved separately. This suggestion, based on comparison of amino acid sequences, is supported by the finding that each naturally occurring TOL plasmid examined by Keil *et al.* (1985) and Chatfield & Williams (1986) appeared to carry either two homologous or two nonhomologous genes for C23O. Furthermore, Olson *et al.* (1992) described a 3,4-dihydroxyphenylacetate 2,3-dioxygenase in soil *Arthrobacter* strains not having any immunological similarity to several C23Os of *Bacillus* and *Pseudomonas* strains. This unique type of extradiol-cleaving dioxygenase seemed to be confined to *Arthrobacter* soil bacteria.

Conserved amino acid residues

Residues that are important for enzyme activity may be found among the conserved amino acids. Altogether 18 amino acid residues have been conserved among the compared extradiol dioxygenase sequences (Fig. 4). It is noteworthy that five glycines and two prolines are among these. The contribution of glycyl and prolyl residues is structural, and the high frequency of their conservation in the primary sequences suggests that the tertiary structures will prove to be highly conserved among the extradiol dioxygenases.

The active site of C23O contains a mononuclear high-spin Fe^{2+} centre that has been implicated in the binding and catalytic activation of molecular oxygen (Mabrouk *et al.*, 1991). It has been suggested that histidine and/or tyrosine may be the iron-binding residues in C23O (Tatsuno *et al.*, 1980), as is the case for protocatechuate 3,4-dioxygenase, an intradiol-cleaving enzyme (Ohlendorf *et al.*, 1988). Therefore the high conservation of four histidines and one tyrosine in all 10 enzymes compared is very interesting. Williams *et al.* (1990) constructed hybrid *xylE* genes to define the region important for enzyme activity.

Fig. 5. SDS-PAGE (10% polyacrylamide gel) of C23O during purification from *E. coli* clones. Lane 1 (from top to bottom), phosphorylase *b* (M_r 94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100). Lane 2, crude extract; lane 3, after heat precipitation; lane 4, after Q Sepharose Fast Flow; lane 5, after Butyl-Sepharose CL-4B; lane 6, after second Q Sepharose Fast Flow. The protein band of C23O corresponds to an M_r of 39000.

Their results suggested that the main active-site amino acids were located in the C-terminal region. Three of the four conserved histidines and the conserved tyrosine are situated in the C-terminal region, indicating their putative importance for enzyme activity.

Taira *et al.* (1988) suggested that cysteine might be functionally important due to the formation of disulphide linkages between the subunits of extradiol dioxygenases. However, this cannot be the case with C23O, as this enzyme does not contain any cysteine.

Purification of C23O from *E. coli* clones

E. coli clones harbouring the recombinant plasmid pSC1701 (Fig. 1) were used for enzyme purification, because these clones produced the highest amount of C23O per g cells. The purification procedure allowing the isolation of 2.6 mg homogeneous C23O (Fig. 5) per g cells (wet weight) is summarized in Table 3.

C23O from *R. rhodochrous*, like that from other bacteria, is easily inactivated during the purification procedure (see Table 3). This inactivation appears to be due to oxidation of Fe^{2+} to Fe^{3+} . The enzyme, thus inactivated, can be partially reactivated by incubation with ferrous ion and a reducing agent (Nozaki *et al.*, 1968). In this way an

Table 3. Purification of C23O from *R. rhodochrous* CTM, cloned in *E. coli* TG1

Values in parentheses: purification with reactivation of C23O after every step.

Fraction	Vol. (ml)	Protein (mg)	Total activity (U)	Specific activity [mU (mg protein) ⁻¹]	Purification (-fold)
Crude extract*	26 (32)	1482 (1776)	258 (288)	174 (162)	1.0 (1.0)
Heat precipitation	23 (27)	1166 (1161)	253 (268)	217 (231)	1.3 (1.4)
Q Sepharose Fast Flow	49 (48)	403 (122)	56 (123)	138 (1003)	0.8 (6.2)
Butyl-Sepharose CL-4B	165 (139)	147 (53)	14 (54)	97 (1024)	0.6 (6.3)
2nd Q Sepharose Fast Flow	46 (45)	49 (33)	1.1 (32)	22 (972)	0.1 (6.0)

* Prepared from 16 g cells (wet wt).

Table 4. Activation and reactivation of C23O

Enzyme preparation and addition	Specific activity (mU mg ⁻¹)	Activation (-fold)
Untreated*	29	1.0
Fe ²⁺	162	5.6
Ascorbate + Fe ²⁺	179	6.2
Cysteine + Fe ²⁺	205	7.1
Hydroxylamine	170	5.9
Inactivated with DEPC	0	0
Hydroxylamine	178	6.1

* Untreated enzyme preparation means enzyme partially inactivated by the purification procedure.

enzyme activity six to seven times higher than that found after the purification procedure could be obtained (Table 4). Even if reactivation was carried out after every step of purification, the C23O could not be isolated in a fully reactivated form (see Table 3, values in parentheses).

Modification of histidine by diethylpyrocarbonate

To elucidate the role of the conserved histidyl residues, we studied the effect of chemical modification by DEPC on the activity of C23O. For this purpose, we used C23O purified without reactivation after every step.

Inactivation of an enzyme by DEPC may be correlated with the modification of histidyl residues if hydroxylamine reactivates the enzyme. C23O was inactivated by DEPC in a time-dependent and concentration-dependent manner. The time-dependent formation of *N*-carbethoxyhistidine can be monitored by the increase in absorption between 230 and 250 nm (Miles, 1977). The absorption spectra at various times after addition of 25 µl DEPC are shown in Fig. 6(a). In Fig. 6(b) the calculated number of *N*-carbethoxyhistidines formed after addition of different amounts of DEPC is plotted against time. Parallel to the modification, concentration-dependent inactivation of C23O could be measured (Fig. 6c). The enzyme was

totally inactivated within 10 min after addition of 25 µl DEPC (7.5 µmol) and within 45 min after addition of 10 µl DEPC (3 µmol). Addition of 5 µl DEPC (1.5 µmol) led to a decrease of enzyme activity to 10% residual activity within 60 min.

C23O could be reactivated by hydroxylamine. Two hours after addition of hydroxylamine the enzyme was fully active again. (The term 'fully active' means here the maximal activity obtainable if the purified untreated enzyme was reactivated with Fe²⁺ and reducing agents.) Addition of hydroxylamine to either DEPC-inactivated or untreated C23O led to an activation up to the same extent within 2 h. Longer incubation with hydroxylamine resulted in a total and irreversible loss of enzyme activity.

Although DEPC reacts preferentially with histidyl groups, under certain conditions it may also react with cysteine, lysine and tyrosine (Miles, 1977). In contrast to the reaction with histidine and tyrosine, the reaction with cysteine and lysine is not reversible by addition of hydroxylamine. Furthermore, the formation of modified tyrosyl residues would be indicated by a decrease of absorption at 278 nm, but only an increase of absorption between 230 and 250 nm due to *N*-carbethoxyhistidine was observed (Fig. 6a). Thus the inactivation of C23O by incubation with DEPC is exclusively due to the modification of histidyl residues.

Our results imply that at least one of the four strictly conserved histidines is indeed important for enzyme activity. This finding is supported by a site-specific mutagenesis experiment reported by Taira *et al.* (1992). They exchanged the strictly conserved His²³⁴ of TODE, BPHC1, BPHC2 and BPHC3 (for references see Table 1) and the nonconserved His²³³ and His³¹⁶ of BPHC1 with alanine (for the numbering of histidines see Fig. 4). The exchange of the conserved His²³⁴ completely abolished enzyme activity. On the other hand, mutagenesis of the nonconserved histidines had no effect on enzyme activity. They concluded His²³⁴ to be part of the binding site for Fe²⁺.

Further investigations, in particular site-directed mutagenesis experiments, will show whether all four conserved

Fig. 6. (a) Effects of DEPC on the absorption spectrum of C23O. Spectra were recorded 1, 3, 5, 10, 15, 20, 30, 45 and 60 min after addition of 25 µl DEPC (7.5 µmol). (b) Time-dependent formation of *N*-carboxyhistidine. (c) Time-dependent inactivation of C23O; 100% activity is equivalent to 29 mU mg⁻¹. Effects due to addition of 5 µl (1.5 µmol) (●), 10 µl (3 µmol) (■) and 25 µl (7.5 µmol) (▲) DEPC are shown. The figures shown are representative plots chosen from two independent experiments.

histidines are essential for enzyme activity. The possible participation of the strictly conserved tyrosine (Tyr²⁷⁵, Fig. 4) in the binding of Fe³⁺ also merits further study.

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REFERENCES

- Appel, M., Raabe, T. & Lingens, F. (1984). Degradation of *o*-toluidine by *Rhodococcus rhodochrous*. *FEMS Microbiol Lett* **24**, 123–126.
- Bartilson, M. & Shingler, V. (1989). Nucleotide sequence and expression of the catechol 2,3-dioxygenase-encoding gene of phenol-catabolizing *Pseudomonas* CF600. *Gene* **85**, 233–238.
- Benjamin, R. C., Voss, J. A. & Kunz, D. A. (1991). Nucleotide sequence of *xylE* from the TOL pDK1 plasmid and structural comparison with isofunctional catechol 2,3-dioxygenase genes from TOL pWW0 and NAH7. *J Bacteriol* **173**, 2724–2728.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Catelani, D. & Colombi, A. (1974). Metabolism of biphenyl. *Biochem J* **143**, 431–434.
- Chatfield, L. K. & Williams, P. A. (1986). Naturally occurring TOL plasmids in *Pseudomonas* strains carry either two homologous or two nonhomologous catechol 2,3-oxygenase genes. *J Bacteriol* **168**, 878–885.
- Fuchs, K., Schreiner, A. & Lingens, F. (1991). Degradation of 2-methylaniline and chlorinated isomers of 2-methylaniline by *Rhodococcus rhodochrous* strain CTM. *J Gen Microbiol* **137**, 2033–2039.
- Furukawa, K., Arimura, N. & Miyazaki, T. (1987). Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *Pseudomonas pseudoalcaligenes*. *J Bacteriol* **169**, 427–429.
- Ghosal, D., You, I.-S. & Gunsalus, I. C. (1987). Nucleotide sequence and expression of gene *nabH* of plasmid NAH7 and homology with gene *xylE* of TOL pWW0. *Gene* **55**, 19–28.
- Goodfellow, M. (1986). Genus *Rhodococcus*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1472–1481. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Harayama, S. & Rekik, M. (1989). Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *J Biol Chem* **264**, 15328–15333.
- Harayama, S., Rekik, M., Wasserfallen, A. & Bairoch, A. (1987). Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequences of catechol oxidation genes of pWW0 and NAH7 plasmids. *Mol & Genet* **210**, 241–247.
- Harayama, S., Kok, M. & Neidle, E. L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46**, 565–601.
- Kabisch, M. & Fortnagel, P. (1990a). Nucleotide sequence of metapyrocatechase I (catechol 2,3-dioxygenase I) gene *mpcI* from *Alcaligenes eutrophus* JMP222. *Nucleic Acids Res* **18**, 3405–3406.
- Kabisch, M. & Fortnagel, P. (1990b). Nucleotide sequence of metapyrocatechase II (catechol 2,3-dioxygenase II) gene *mpcII* from *Alcaligenes eutrophus* JMP222. *Nucleic Acids Res* **18**, 5543.
- Keil, H., Lebens, M. R. & Williams, P. A. (1985). TOL plasmid pWW15 contains two nonhomologous, independently regulated catechol 2,3-oxygenase genes. *J Bacteriol* **163**, 248–255.
- Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M. & Yano, K. (1989). Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to

- benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. *J Bacteriol* **171**, 2740–2747.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Mabrouk, P. A., Orville, A. M., Lipscomb, J. D. & Solomon, E. I. (1991).** Variable-temperature variable-field magnetic circular dichroism studies of the Fe(II) active site in metapyrocatechase: implications for the molecular mechanism of extradiol dioxygenases. *J Am Chem Soc* **113**, 4053–4061.
- Mandel, M. & Higa, A. (1970).** Calcium dependent bacteriophage DNA infection. *J Mol Biol* **53**, 159–162.
- Miles, E. W. (1977).** Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol* **47**, 431–442.
- Miller, J. H. (1972).** *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Nakai, C., Kagamiyama, H., Nozaki, M., Nakazawa, T., Inouye, S., Ebina, Y. & Nakazawa, A. (1983).** Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2. *J Biol Chem* **258**, 2923–2928.
- Noda, Y., Nishikawa, S., Shiozuka, K.-I., Kadokura, H., Nakajima, H., Yoda, K., Katayama, Y., Morohoshi, N., Haraguchi, T. & Yamasaki, M. (1990).** Molecular cloning of the protococatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. *J Bacteriol* **172**, 2704–2709.
- Nozaki, M., Ono, K., Nakazawa, T., Kotani, S. & Hayaishi, O. (1968).** Metapyrocatechase. II. The role of iron and sulfhydryl groups. *J Biol Chem* **243**, 2682–2690.
- Ohlendorf, D. H., Lipscomb, J. D. & Weber, P. C. (1988).** Structure and assembly of protococatechuate 3,4-dioxygenase. *Nature* **336**, 403–405.
- Olson, P. E., Qi, B., Que, L. Jr. & Wackett, L. P. (1992).** Immunological demonstration of a unique 3,4-dihydroxyphenylacetate 2,3-dioxygenase in soil *Arthrobacter* strains. *Appl Environ Microbiol* **58**, 2820–2826.
- Rast, H. G., Engelhardt, G. & Wallnöfer, P. R. (1980).** 2,3-Cleavage of substituted catechols in *Nocardia* sp. DSM 43251 (*Rhodococcus rubrus*). *Zentralbl Bakteriol Hyg (Abt I, Orig C)* **1**, 224–236.
- Roper, D. I. & Cooper, R. A. (1990).** Subcloning and nucleotide sequence of the 3,4-dihydroxyphenylacetate (homoprotocatechuate) 2,3-dioxygenase gene from *Escherichia coli* C. *FEBS Lett* **275**, 53–57.
- Rosenberg, M. & Court, D. (1979).** Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu Rev Genet* **13**, 319–353.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schreiner, A., Fuchs, K., Lottspeich, F., Poth, H. & Lingens, F. (1991).** Degradation of 2-methylaniline in *Rhodococcus rhodochrous*: cloning and expression of two clustered catechol 2,3-dioxygenase genes from strain CTM. *J Gen Microbiol* **137**, 2041–2048.
- Shine, J. & Dalgarno, L. (1974).** The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc Natl Acad Sci USA* **71**, 1342–1346.
- Taira, K., Hayase, N., Arimura, N., Yamashita, S., Miyazaki, T. & Furukawa, K. (1988).** Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. *Biochemistry* **27**, 3990–3996.
- Taira, K., Hirose, J., Hayashida, S. & Furukawa, K. (1992).** Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. *J Biol Chem* **267**, 4844–4853.
- Tatsuno, Y., Saeki, Y., Nozaki, M., Otsuka, S. & Maeda, Y. (1980).** Mössbauer spectra of metapyrocatechase. *FEBS Lett* **112**, 83–85.
- Williams, P. A., Assinder, S. J. & Shaw, L. E. (1990).** Construction of hybrid *xylE* genes between the two duplicate homologous genes from TOL plasmid pWW53: comparison of the kinetic properties of the gene products. *J Gen Microbiol* **136**, 1583–1589.
- Worsey, M. J., Franklin, F. C. H. & Williams, P. A. (1978).** Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWW0) from *Pseudomonas putida* mt-2. *J Bacteriol* **134**, 757–764.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zukowski, M. M., Gaffney, D. F., Speck, D., Kauffmann, M., Findeli, A., Wisecup, A. & Lecocq, J.-P. (1983).** Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. *Proc Natl Acad Sci USA* **80**, 1101–1105.
- Zylstra, G. J. & Gibson, D. T. (1989).** Toluene degradation by *Pseudomonas putida* F1. *J Biol Chem* **264**, 14940–14946.

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