In vivo reactivation of catechol 2,3-dioxygenase mediated by a chloroplast-type ferredoxin: a bacterial strategy to expand the substrate specificity of aromatic degradative pathways

Alessandra Polissi and Shigeaki Harayama

Department of Medical Biochemistry, Faculty of Medicine, 1, rue Michel-Servet, 1211 Geneva 4, Switzerland

Communicated by K.N.Timmis

The *meta*-cleavage operon of the TOL plasmid pWW0 of *Pseudomonas putida* contains 13 genes responsible for the oxidation of benzoate and toluates to Krebs cycle intermediates via extradiol (*meta*) cleavage of (methyl)catechol. The functions of all the genes are known with the exception of *xylT*. We constructed pWW0 mutants defective in the *xylT* gene, and found that these mutants were not able to grow on *p*-toluate while they were still capable of growing on benzoate and *m*-toluate. In the *xylT* mutants, all the *meta*-cleavage enzymes were induced by *p*-toluate with the exception of catechol 2,3-dioxygenase whose activity was 1% of the *p*-toluate-induced activity in wild-type cells. Addition of 4-methyl-catechol to *m*-toluate-grown wild-type and *xylT* cells resulted in the inactivation of catechol 2,3-dioxygenase in these cells. In the wild-type strain but not in the *xylT* mutant, the catechol 2,3-dioxygenase activity was regenerated in a short time. The regeneration of the catechol 2,3-dioxygenase activity was also observed in H_2O_2-treated wild-type cells, but not in H_2O_2-treated *xylT* cells. We concluded that the *xylT* product is required for the regeneration of catechol 2,3-dioxygenase.

Key words: catechol 2,3-dioxygenase/enzyme reactivation/*Pseudomonas putida*/TOL plasmid/*xylT*

Introduction

The TOL plasmid pWW0, originally found in *Pseudomonas putida* mt-2, carries a set of genes responsible for the mineralization of toluene, *m*-xylene and *p*-xylene (Worsey and Williams, 1975). The genes are organized into two operons 'upper' and '*meta*' (Harayama *et al.*, 1984; Harayama and Rekik, 1990). The upper operon encodes three enzymes which transform toluene, *m*-xylene and *p*-xylene to benzoate, *m*-toluate and *p*-toluate, respectively (Harayama *et al.*, 1986, 1989). The *meta* operon, on the other hand, comprises 13 genes, *xylXYZLTEGFJQKIH* (Nakazawa *et al.*, 1980; Harayama *et al.*, 1987; Harayama and Rekik, 1990). The first four genes of the *meta* operon, *xylXYZL*, are involved in the oxidation of benzoate, *m*-toluate and *p*-toluate to catechol, 3-methylcatechol and 4-methyl-catechol, respectively, while the *xylEGFJQKIH* genes are required for the transformation of these catechols into Krebs cycle intermediates. As shown in Figure 1, the functions of all the *meta* operon genes are known except *xylT* which is located between the *xylXYZL* and *xylEGFJQKIH* clusters (Harayama *et al.*, 1991a). Isofunctional genes to *xylTEGF-JQKIH* are found in the *sal* operon on the NAH7 plasmid

responsible for the mineralization of salicylate (Yen and Gunsalus, 1982; You *et al.*, 1991), and in the *dmp* operon on the pVI150 plasmid responsible for the mineralization of (dimethyl)phenol (Bartilson *et al.*, 1990; Nordlund *et al.*, 1990; Shingler *et al.*, 1992). The gene orders and DNA sequences are highly conserved between *xylTEGFJQKIH* and their homologues in the *sal* and *dmp* operons (Shingler *et al.*, 1992; Harayama and Rekik, 1993). The *nahT* gene on the NAH7 plasmid and the *dmpQ* gene on the pVI150 plasmid are equivalent to the *xylT* gene on the pWW0 plasmid, and the amino acid sequences of these gene products all possess a motif characteristic of chloroplast-type ferredoxins (Harayama *et al.*, 1991a; You *et al.*, 1991; Shingler *et al.*, 1992).

Since the *xylT*, *nahT* and *dmpQ* genes have been evolutionarily conserved in three different operons involved in the degradation of catechols, we expected that the *xylT*/*nahT*/*dmpQ* products may have some important role in the mineralization of catechols. Here we describe the isolation and characterization of pWW0 mutants defective in *xylT*. We found that the *xylT* product was involved in the regeneration of inactivated catechol 2,3-dioxygenase, and that the regeneration was indispensable for the growth of the host cells on *p*-xylene, *p*-methylbenzyl alcohol and *p*-toluate.

Results

Construction of xylT mutants of pWW0

The 6 kb *Eco*RI−*Pst*I fragment containing the *xylXYZLTEG* sequence of TOL plasmid pWW0 (Figure 2) was subcloned into pACYC184 to form pGA1. The pGA1 plasmid is resistant to tetracycline (Tc^R). The 1.2 kanamycin (Km) cassette from pRME1 was inserted into the *Nco*I site of pGA1 within the *xylT* gene to create the *xylT1*::Km^R mutation. The pGA2 plasmid thus constructed is Tc^R, Km^R. This plasmid was used to transform *Escherichia coli* strain LE392 harboring pWW0-161. The pWW0-161 plasmid carries transposon Tn*401*, and confers resistance to ampicillin (Ap^R). LE392(pWW0-161, pGA2) transformants were therefore selected for Ap^R, Km^R. LE392(pWW0-161, pGA2) was then mated with *E.coli* GSH3491 to isolate spectinomycin resistant (Sp^R, selection for GSH3491), Ap^R (selection for pWW0) and Km^R (selection for pGA2) transconjugants. Since pACYC184 is neither conjugative nor mobilizable, the transfer of pGA2 from LE392(pWW0-161, pGA2) to the GSH3491 recipient may have resulted from cointegrate formation between the conjugative pWW0-161 and plasmid pGA2. Theoretically, a cointegrate of these two plasmids can be formed either by homologous recombination or by the transposition of Tn*401* onto pGA2. If a cointegrate was formed by the homologous recombination, its resolution into two plasmids through the second homologous recombination may have eventually exchanged the

xylT1::Km^R allele of pGA2 for the *xylT*$^+$ allele of pWW0-161.

Ten independent Sp^R, Ap^R, Km^R transconjugants of GSH3491 were mated with *P.putida* KT2440, and Km^R transconjugants were selected on benzoate M9 minimal plates. KT2440 is a prototroph and encodes an *ortho* pathway that allows this strain to grow on benzoate (but not on *m*-toluate and *p*-toluate). The Km^R transconjugants of KT2440 were Tc^S indicating that the wild-type *xylT* allele was replaced by the *xylT1*::Km^R allele in these transconjugants.
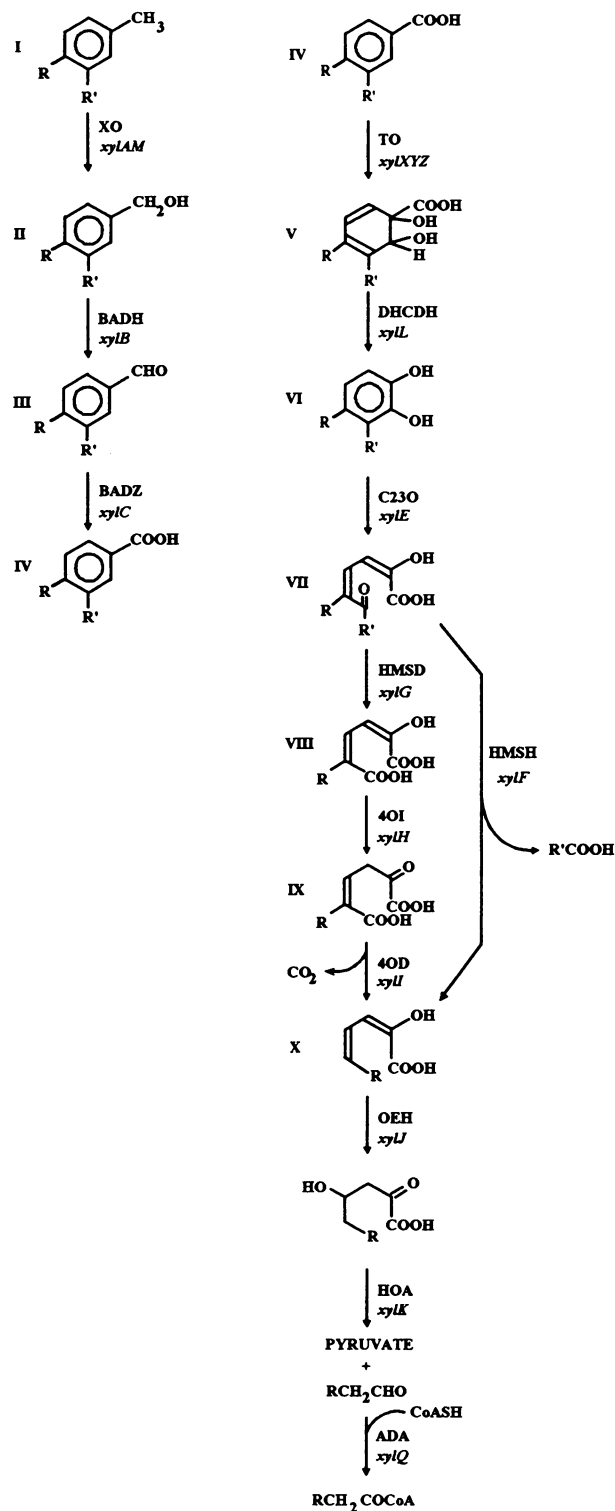
These *P.putida* Km^R transconjugants grew on toluene, benzyl alcohol, benzoate, *m*-xylene, *m*-methylbenzyl alcohol and *m*-toluate, but did not grow on *p*-xylene, *p*-methylbenzyl alcohol and *p*-toluate.

The enzymes of the *meta* pathway, namely catechol 2,3-dioxygenase, hydroxymuconic semialdehyde dehydrogenase and hydroxymuconic semialdehyde hydrolase coded for by *xylE*, *xylG* and *xylF*, respectively, were not induced by *m*-toluate, but expressed constitutively in these *P.putida* Km^R transconjugants (data not shown). This observation indicated that the *xylT1*::Km^R mutation on pWW0 exhibits a polar effect on the genes downstream of *xylT*. Therefore the observed phenotype of *P.putida* KT2440(pWW0-161 *xylT1*::Km^R), namely the absence of growth on *p*-xylene and its alcohol and carboxylate derivatives, may not necessarily be due to the defect in the *xylT* gene itself, but to the altered expression of the *meta* operon genes downstream of *xylT*. In order to construct non-polar *xylT* mutations, plasmid pGA1 was cleaved at the unique *Nco*I site located within *xylT*, the *Nco*I cohesive ends thus created were filled with Klenow polymerase, and the linearized DNA was either directly ligated to form pGA4 plasmid, or ligated with a 7 bp long linker (5'-AAGCTTG-3') to form pGA3 plasmid. The *xylT* mutations thus constructed in pGA3 and pGA4 were called *xylT2* and *xylT3* respectively.

Escherichia coli strain LE392(pWW0-161 *xylT1*::Km^R) was transformed either by pGA3 or pGA4, and the Tc^R marker of pGA3 and pGA4 in LE392(pWW0-161 *xylT1*::Km^R, pGA3) or LE392(pWW0-161 *xylT1*::Km^R, pGA4), was conjugally transferred into GSH3491. Tc^R transconjugants derived from GSH3491 were further conjugated with *P.putida* KT2440, and KT2440 derivatives that grow on *m*-xylene (*m*-Xyl$^+$) were selected [the growth test of KT2440(pWW0-161 *xylT1*::Km^R) described above indicated that the *xylT* mutation on pWW0 does not affect the growth of *P.putida* on *m*-xylene]. Among the *m*-Xyl$^+$ transconjugants, those containing pWW0-161 carrying the *xylT2* or *xylT3* allele instead of the *xylT1*::Km^R allele were expected to exist. The *m*-Xyl$^+$ transconjugants were

Fig. 1. The *xyl* operons for the catabolism of *m*-toluate, *p*-toluate and benzoate encoded by TOL plasmid pWW0 of *P.putida*. Enzyme abbreviations: XO, xylene monooxygenase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate 1,2-dioxygenase; DHCDH, 1,2-dihydroxycyclohexa-3,5-dienecarboxylate dehydrogenase; C23O, catechol 2,3-dioxygenase; HMDS, hydroxymuconic semialdehyde dehydrogenase; HMSH, hydroxymuconic semialdehyde hydrolase; 4OI, 4-oxalocrotonate isomerase; 4-OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase; ADA, acetaldehyde dehydrogenase (acetylating). *xylE−xylZ* are the designations of the structural genes for the catabolic enzymes. Compounds: for R = H, R' = H, (I) toluene; (II) benzyl alcohol; (III) benzaldehyde; (IV) benzoate; (V) 1,2-dihydroxycyclohexa-3,5-dienecarboxylate; (VI) catechol; (VII) 2-hydroxymuconic semialdehyde; (VIII) 2-hydroxy-hexa-2,4-diene-1,6-dioate; (IX) 2-oxohex-4-ene-1,6-dioate; (X) 2-oxopent-4-enoate; for R = H, R' = CH₃, (I) *m*-xylene; (II) *m*-methylbenzyl alcohol; (III) *m*-methylbenzaldehyde; (IV) *m*-toluate; (V) 1,2-hydroxy-3-methylcyclohexa-3,5-dienecarboxylate; (VI) 3-methylcatechol; (VII) 2-hydroxy-6-oxohepta-2,4-dienoate; (X) 2-oxopent-4-enoate; for R = CH₃, R' = H, (I) *p*-xylene; (II) *p*-methylbenzyl alcohol; (III) *p*-methylbenzaldehyde; (IV) *p*-toluate; (V) 1,2-dihydroxy-4-methylcyclohexa-3,5-dienecarboxylate; (VI) 4-methylcatechol; (VII) 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate; (VIII) 2-hydroxy-5-methylhexa-2,4-diene-1,6-dioate; (IX) 5-methyl-2-oxo-hex-4-ene-1,6-dioate; (X) 2-oxo-*cis*-hex-4-enoate.

therefore tested for their sensitivity to Km, and Km^S clones were found at a frequency of 2 × 10^{-2}.

All the Km^S transconjugants of KT2440 showed the same phenotype as KT2440(pWW0-161 xylT1::Km^R): they grew on toluene, m-xylene and their alcohol and carboxylate derivatives, but did not grow on p-xylene and its alcohol and carboxylate derivatives. Each one of the putative pWW0-161 xylT2 and pWW0-161 xylT3 plasmids was isolated and their xylT sequences were determined: they carried the expected xylT mutations.

The meta pathway enzyme activities in the xylT2 and xylT3 mutants

The activities of four *meta* operon enzymes, catechol 2,3-dioxygenase, hydroxymuconic semialdehyde hydrolase, hydroxymuconic semialdehyde dehydrogenase and 4-oxalocrotonate tautomerase were assayed in KT2440(pWW0-161), KT2440(pWW0-161 xylT2) and KT2440(pWW0-161 xylT3) grown in glucose M9 minimal medium containing either m-toluate or p-toluate as an inducer. In the xylT mutants induced by m-toluate, the activities of the tested enzymes were comparable with those of the wild-type strain with the exception of the catechol 2,3-dioxygenase activity which was half the wild-type activity (Table I). More strikingly, the xylT mutants grown in the presence of p-toluate showed a catechol 2,3-dioxygenase activity <1% of the wild-type activity although the levels of other *meta* pathway enzymes were similar to those in the wild-type strain (Table I). The lower catechol 2,3-dioxygenase activity in the xylT mutants upon the growth with p-toluate was not explained by the failure of the induction of catechol 2,3-dioxygenase by p-toluate because other *meta* pathway enzymes encoded in the
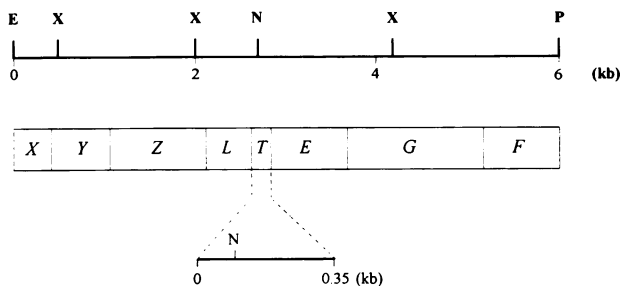

Fig. 2. Physical and genetic map of the xylT region on TOL plasmid pWW0. Restriction sites are: E, *Eco*RI; N, *Nco*I; P, *Pst*I; and X, *Xho*I. Open boxes represent structural genes (Harayama and Rekik, 1990).

same operon were induced. The possibility that the xylT product is required for the post-translational activation of catechol 2,3-dioxygenase was ruled out since a significant catechol 2,3-dioxygenase activity was observed in the xylT mutants grown in the presence of m-toluate. We therefore expected that catechol 2,3-dioxygenase in the xylT mutants was inactivated during the growth on p-toluate.

The inactivation and reactivation of catechol 2,3-dioxygenase in vivo

The results described above suggested that catechol 2,3-dioxygenase in the xylT mutants was inactivated by a metabolite of p-toluate. We tested the possibility that 4-methylcatechol could inactivate catechol 2,3-dioxygenase in the xylT2 mutant. When the wild-type and xylT2 cells were grown in m-toluate M9 minimal medium, catechol 2,3-dioxygenase activity expressed in the xylT2 cells was ~50% of the wild-type level (Figure 3B). When 2 mM 4-methylcatechol was added to the xylT2 strain grown at a mid-exponential growth phase, the catechol 2,3-dioxygenase activity in the xylT2 cells rapidly dropped to zero, and the activity lost was not recovered during the next 18 h cultivation (Figure 3D). The culture of the xylT2 mutant turned to black 6 h after the addition of 4-methylcatechol; this may be due to the spontaneous polymerization of 4-methylcatechol. The addition of 4-methylcatechol to the wild-type culture also resulted in the rapid decrease in the catechol 2,3-dioxygenase activity within 2 h, but the activity started to reincrease 4 h after the addition of 4-methylcatechol. The wild-type culture become yellow after the addition of 4-methylcatechol suggesting the accumulation of the ring-cleavage product of 4-methylcatechol, but unlike the xylT2 culture, the development of the black color was not observed. Hence most of the exogenous 4-methylcatechol was rapidly metabolized in the wild-type culture. When 3-methylcatechol was added, only slight and temporal decrease in the catechol 2,3-dioxygenase activity was observed both in the wild-type and xylT2 cultures (Figure 3F). These observations explained why the xylT mutants do not grow on p-xylene, p-methylbenzyl alcohol or p-toluate but do grow on m-xylene, m-methylbenzyl alcohol and m-toluate.

In vivo reactivation of catechol 2,3-dioxygenase

Above, we observed that the addition of 4-methylcatechol to the wild-type and xylT cultures resulted in the inactivation of catechol 2,3-dioxygenase, and that the catechol 2,3-dioxy-

Table I. Activities[a] of *meta* pathway enzymes in wild-type and xylT mutant strains

Strain	C230 (xylE)[b]				HMSH (xylF)				HMSD (xylG)				40I (xylH)	
	Substrate													
					Ring-cleavage product of									
	Catechol		4-methyl-catechol		Catechol		4-methyl-catechol		Catechol		4-methyl-catechol		4-oxalocrotonate	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
WT	5700	4100	5200	3500	59	22	21	10	71	47	78	42	1300	1200
xylT2	2800	10	2800	8	33	23	19	11	92	23	75	22	1500	1300
xylT3	2900	14	2900	34	40	32	23	16	87	27	73	32	1200	1400

[a]Activities in crude extracts are expressed as nmol of product formed (or substrate consumed) per min per mg of protein.
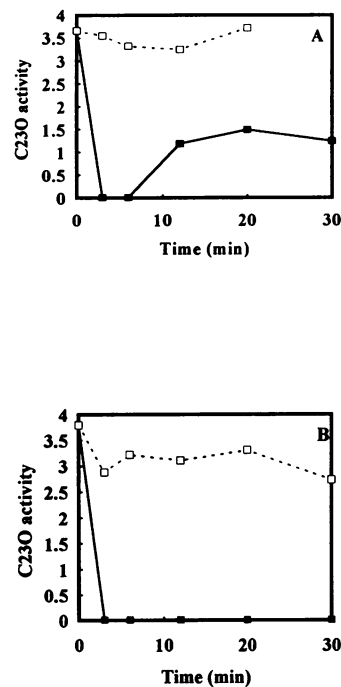[b]For abbreviation of *meta* pathway enzymes, see Figure 1.
A, m-toluate induced cells.
B, p-toluate induced cells.
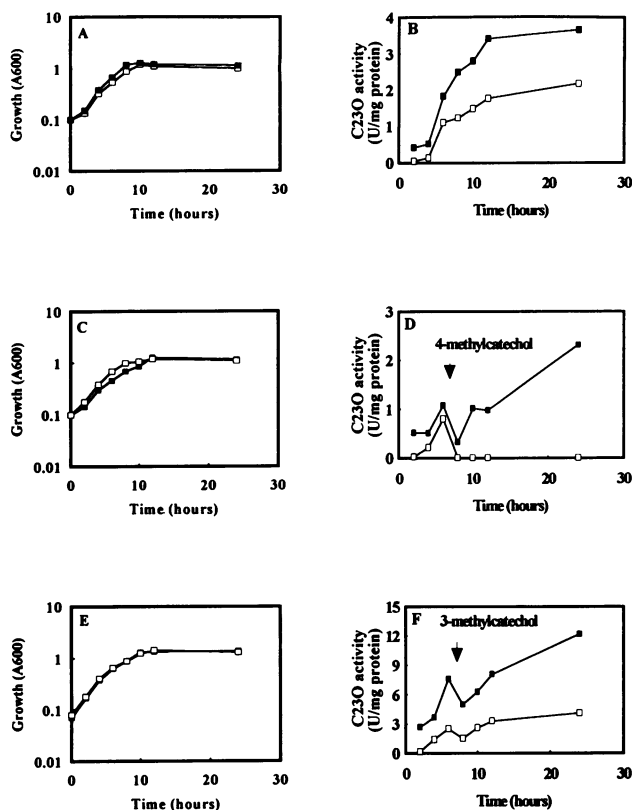
Fig. 3. Catechol 2,3-dioxygenase activities in the wild-type and *xylT* cells. Cultures of KT2440(pWW0-161) and of KT2440(pWW0-161 *xylT2*) were grown overnight in M9 minimal medium containing 5 mM *m*-toluate. Overnight cultures were diluted into the fresh medium of the same composition. Growth measured by absorbance (A600) (**A, C** and **E**) and catechol 2,3-dioxygenase activity (C23O) (**B, D** and **F**) of the wild-type (■) and *xylT2* (□) cells are presented. At *t* = 6 h, 2 mM 4-methylcatechol (**C** and **D**) or 2 mM 3-methylcatechol (**E** and **F**) was added to the cultures.

Fig. 4. Reactivation of catechol 2,3-dioxygenase inactivated by H_2O_2. KT2440(pWW0-161) and KT2440(pWW0-161 *xylT2*) cells were grown in M9 minimal medium containing 5 mM *m*-toluate, washed and resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 100 µg/ml chloramphenicol. After measuring the initial activity of catechol 2,3-dioxygenase, the cell suspensions of the wild-type and *xylT2* strains were added by 50 µM H_2O_2 (*t* = 0 min). 100 µl aliquots were harvested at different times, and the catechol 2,3-dioxygenase activities in these samples were measured. (**A**) Wild-type cells inactivated (■) or not inactivated (□) by H_2O_2. (**B**) *xylT1* cells inactivated (■) or not inactivated (□) by H_2O_2. The catechol 2,3-dioxygenase activity is expressed as nmol catechol oxidized per 10^7 cells per min. For experimental details see Materials and methods.

genase activity reincreased in a short period of time in the wild-type cells but not in the *xylT* cells. A plausible explanation of these observations was that wild-type cells, but not *xylT* cells, possess a mechanism to reactivate inactivated catechol 2,3-dioxygenase. To test this hypothesis, catechol 2,3-dioxygenase in chloramphenicol-treated cells was inactivated by treating them with H_2O_2, and subsequent regeneration of catechol 2,3-dioxygenase activity was observed in the wild-type and *xylT2* cells. H_2O_2 has been shown to inactivate catechol 2,3-dioxygenase by oxidizing the catalytic center ferrous ion to the ferric form (Nozaki *et al.*, 1968). The wild-type cells were capable of reactivating, within 10 min, the inactivated catechol 2,3-dioxygenase up to 50% of the initial activity (Figure 4A). In contrast, no reactivation of catechol 2,3-dioxygenase occurred in the *xylT2* mutant (Figure 4B). Our data thus demonstrated that the *xylT* product was necessary for the reactivation of catechol 2,3-dioxygenase inactivated either by 4-methylcatechol or by H_2O_2. The *xylT* product may reactivate catechol 2,3-dioxygenase by reducing the oxidized iron cofactor. The structure of the *xylT* product is consistent with this hypothesis: the *xylT* product has a structure similar to chloroplast-type ferredoxins (Harayama *et al.*, 1991a) which are involved in many different electron transport processes (Bruschi and Guerlesquin, 1988).

Determination of the rate constants for catechol 2,3-dioxygenase inactivation

All the results described above support the hypothesis that the inability of *xylT* mutants to grow on *p*-xylene, *p*-methylbenzyl alcohol and *p*-toluate is due to the inactivation of catechol 2,3-dioxygenase by 4-methylcatechol produced from these substrates. We then examined, in vitro, the inactivation of purified catechol 2,3-dioxygenase by 4-methylcatechol. Catechol 2,3-dioxygenase was purified from the wild-type strain and the *xylT2* mutant as described in Materials and methods. When these enzymes were incubated with 4-methylcatechol in the absence of oxygen (another substrate of catechol 2,3-dioxygenase), neither catalytic reaction nor enzyme inactivation occurred. In the presence of oxygen, the ring-cleavage reaction took place, and the enzyme inactivation occurred concomitantly. The degree of the enzyme inactivation was estimated by determining the rate constant for the enzyme inactivation, k_{inact}. As shown in Table II, the k_{inact} values of catechol 2,3-dioxygenase purified from the wild-type strain were identical to those of catechol 2,3-dioxygenase purified from the *xylT2* mutant, using as substrates catechol, 3-methylcatechol and 4-methylcatechol (each one at a concentration of 50 µM). Therefore, it is unlikely that post-translational modification by the XylT product stabilizes catechol 2,3-dioxygenase. As shown in Table II, catechol 2,3-dioxy-

Table II. Kinetic constants for the inactivation of catechol 2,3-dioxygenase purified from wild-type and *xylT2* strains

Substrates	C230 from wild-type k_{inact} (s^{-1})	C230 from *xylT2* k_{inact} (s^{-1})
Catechol	6×10^{-4}	n.d.[a]
3-methylcatechol	2.8×10^{-3}	2.5×10^{-3}
4-methylcatechol	5.8×10^{-3}	5.2×10^{-3}

[a]Not determined.
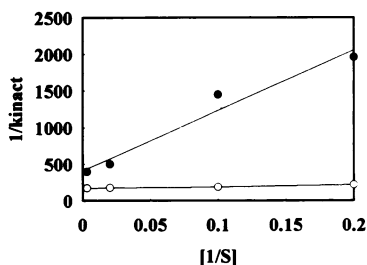

Fig. 5. Reciprocal plot of k_{inact} versus the concentration of 3-methylcatechol (●) and 4-methylcatechol (○) The k_{inact} values for 4-methylcatechol at concentrations of 5, 10, 50, and 300 μM were 4.5×10^{-3}, 5.3×10^{-3}, 5.6×10^{-3} and 5.7×10^{-3} s^{-1}, respectively. Those for 3-methylcatechol at these concentrations were 5.1×10^{-4}, 6.9×10^{-4}, 2.0×10^{-3} and 2.5×10^{-3} s^{-1}, respectively.

genase was inactivated very little during the oxidation of catechol. Higher inactivation of the enzyme was observed in the oxidation of methyl-substituted catechols: the k_{inact} for 4-methylcatechol was only 2-fold higher than that of 3-methylcatechol (Table II). We measured the k_{inact} for methyl-substituted catechols at different concentrations: k_{inact} values for 4-methylcatechol were almost constant at substrate concentrations between 5 and 300 μM while those for 3-methylcatechol decreased as the substrate concentration decreased from 300 to 5 μM. From the reciprocal plot presented in Figure 5, it was shown that the concentration of 4-methylcatechol required for the half maximum of k_{inact} was around 3 μM while the concentration of 3-methyl-catechol required for the half maximum of k_{inact} was >30 μM. Thus, although the k_{inact} value for 3-methyl-catechol at the concentration of 50 μM was only 2-fold lower than that for 4-methylcatechol at the concentration of 50 μM, the k_{inact} for 3-methylcatechol at the concentration of 5 μM was 10-fold lower than that for 4-methylcatechol at the concentration of 5 μM. These data showed that 4-methyl-catechol inactivated catechol 2,3-dioxygenase more strongly than 3-methylcatechol especially at low substrate concentrations, and explained the stronger effect of 4-methylcatechol than 3-methylcatechol in the catechol 2,3-dioxygenase inactivation observed *in vivo* (Figure 3).

Inhibition of the catechol 2,3-dioxygenase activity by ring-cleavage products

Previous studies (Hori *et al.*, 1973) demonstrated that the ring-cleavage product of catechol (2-hydroxymuconic semialdehyde) was a non-competitive inhibitor for the oxidation of catechol by catechol 2,3-dioxygenase. There-fore, the stronger inhibition of the catechol 2,3-dioxygenase activity exerted by the addition of 4-methylcatechol than by the addition of 3-methylcatechol (Figure 3) may be partly
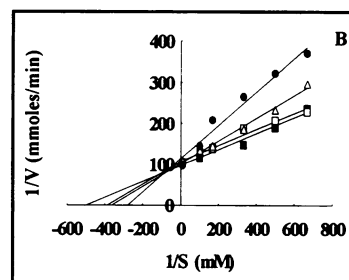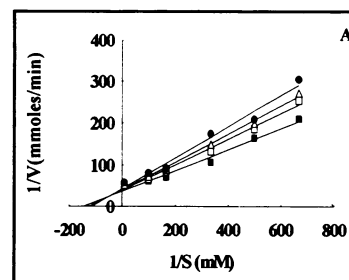
Fig. 6. Inhibition of the catechol 2,3-dioxygenase activity by the ring-cleavage products of 3-methylcatechol and 4-methylcatechol. Lineweaver—Burk plots of initial rates of catechol oxidation in the presence of various concentrations of the ring-cleavage products are presented. The reaction was carried out in 1 ml of 100 mM potassium phosphate buffer (pH 7.4) in the presence of 0.08 μg of enzyme. (A) The inhibition of the oxidation of 3-methylcatechol; the concentrations of 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate were: 82 μM (●), 53 μM (△), 34 μM (□), 0 μM (■). (B) the inhibition of the oxidation of 4-methylcatechol; the concentrations of 2-hydroxy-6-oxohepta-2,4-dienoate were: 117 μM (●), 82 μM (△), 30 μM (□), 0 μM (■).

due to the stronger inhibition of the enzyme activity by the ring-cleavage product of 4-methylcatechol than by the ring-cleavage product of 3-methylcatechol. We thus examined the inhibition of the catechol 2,3-dioxygenase-catalyzed oxidation of 4-methylcatechol and of 3-methylcatechol, respectively, by the ring-cleavage products of 4-methyl-catechol (2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate) and of 3-methylcatechol (2-hydroxy-6-oxohepta-2,4-dienoate). The kinetic analysis of the product inhibition shown in Figure 6A and B demonstrated that the ring-cleavage products are competitive inhibitors. Replots of slopes of Figure 6A and B versus the concentration of each inhibitor gave straight lines: the K_i values for 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate and for 2-hydroxy-6-oxohepta-2,4-dienoate, calculated as the abscissa intercepts of these replots were 1.6×10^{-4} and 10^{-4} M, respectively. Thus, the different sensitivity of catechol 2,3-dioxygenase to 3-methylcatechol and 4-methylcatechol was not due to the different degree of inhibition exerted by their ring-cleavage products.

Induction of catechol 2,3-dioxygenase

The capacity of *p*-xylene/*p*-toluate to induce the *meta* operon was lower than that of *m*-xylene/*m*-toluate. Figure 7 shows the induction pattern of catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxy-muconic semialdehyde hydrolase and 4-oxalocrotonate
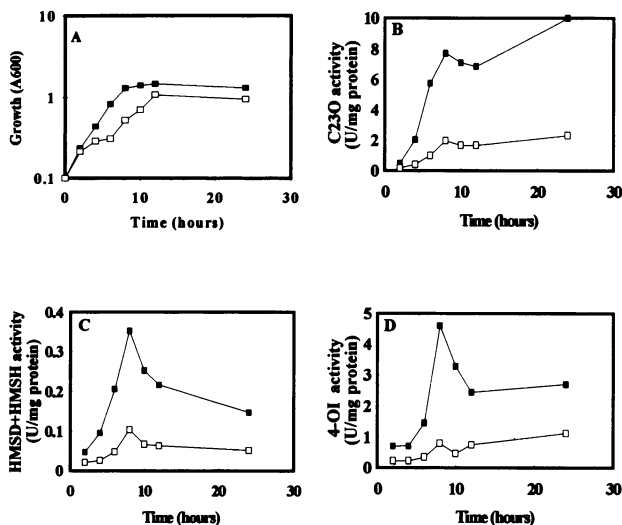
Fig. 7. Induction of *meta* pathway enzymes by *m*-toluate and *p*-toluate. A culture of KT2440(pWW0-161) was grown overnight in M9 minimal medium containing 10 mM glucose. The overnight culture was diluted into fresh medium containing 5 mM *m*-toluate (■) or 5 mM *p*-toluate (□) as the sole source of carbon. Growth (A) and the activities of catechol 2,3-dioxygenase (B), 2-hydroxymuconic semialdehyde dehydrogenase + 2-hydroxymuconic semialdehyde hydrolase (C) and 4-oxalocrotonate tautomerase (D). For enzyme abbreviations, see Figure 1.

tautomerase in wild-type cells grown in the presence of *m*-toluate or *p*-toluate. The activities of all the enzymes tested were significantly lower (~4-fold) in cells grown in the presence of *p*-toluate than those grown in the presence of *m*-toluate. This important difference in the induction mediated by *m*-toluate and *p*-toluate was only seen when these aromatic compounds were used as sole sources of carbon and energy. When *m*-toluate or *p*-toluate was added in minimal medium containing glucose or in L-broth, the *meta* operon was induced at the same level (Table I and our unpublished results).

Discussion

In order to clarify the physiological role of the *xylT* gene encoded by TOL plasmid pWW0, *xylT* mutants of pWW0 were constructed. *P.putida* KT2440 containing the *xylT* mutants of pWW0 did not grow on *p*-xylene, *p*-methylbenzyl alcohol or *p*-toluate. The inability of the *xylT* mutants to grow on *p*-methyl-substituted compounds was due to a deficiency in the regeneration of catechol 2,3-dioxygenase. Apparently, a significant amount of catechol 2,3-dioxygenase is inactivated during the metabolism of 4-methylcatechol, and without the regeneration of catechol 2,3-dioxygenase, cells are not able to metabolize 4-methylcatechol in an amount sufficient for growth. Although catechol 2,3-dioxygenase encoded *xylE* gene on the TOL plasmid pWW0 has been extensively studied since 1961 when this enzyme was discovered as one of the first examples of oxygenases (Kojima *et al.*, 1961), the *in vivo* regeneration of catechol 2,3-dioxygenase was not demonstrated before this study.

Catechol 2,3-dioxygenase consists of four identical subunits of 35 kDa and contains one catalytically essential Fe(II) ion per subunit (Nozaki *et al.*, 1968; Nozaki, 1979; Nakai *et al.*, 1983). From binding and kinetic studies, an ordered Bi Uni mechanism has been proposed in which the

enzyme combines first with catechol followed by the binding of oxygen (Hori *et al.*, 1973). Both the catechol and oxygen substrates bind to the catalytic iron which is coordinated to two or more amino acid ligands on the polypeptide (Mabrouk *et al.*, 1991). Catechol 2,3-dioxygenase oxidizes catechol, 3-methylcatechol and 4-methylcatechol (Nozaki *et al.*, 1970; Wallis and Chapman, 1990). However, we have demonstrated that 3-methylcatechol and 4-methylcatechol also act as suicide inhibitors.

It has been demonstrated that the enzyme was inactivated by oxidizing agents but reactivated in the presence of ferrous ions and reducing agents (Nozaki *et al.*, 1968; Bartels *et al.*, 1984). Catechol 2,3-dioxygenase was also inactivated during oxidation of 4-ethylcatechol, but the enzyme inactivated by 4-ethylcatechol could be reactivated in the presence of $FeSO_4$ and ascorbic acid (Wasserfallen, 1989; P.Cerdan, A.Wasserfallen, K.N.Timmis and S.Harayama, manuscript in preparation). These data indicate that the inactivation of catechol 2,3-dioxygenase, in these conditions, was mainly due to the oxidation and/or the removal of the catalytic iron. On the analogy of this interpretation, we infer that 3-methylcatechol and 4-methylcatechol inactivate the enzyme by oxidizing or removing the iron cofactor. Then the XylT product may be involved in the reduction of the oxidized iron cofactor of the enzyme inactivated by 3-methylcatechol, 4-methylcatechol or other agents. In fact, we found that catechol 2,3-dioxygenase inactivated by H_2O_2 was reactivated *in vivo* if the *xylT* product was expressed. The *xylT* product is most probably a chloroplast-type ferredoxin (Harayama *et al.*, 1991a). We therefore propose that the reduced form of XylT reactivates the inactivated catechol 2,3-dioxygenase by reducing the oxidized iron cofactor. In this reaction, the XylT product itself should be oxidized. The question then arose as to how the oxidized form of XylT is transformed to its reduced form. We have completed the DNA sequencing of the *meta* operon, and showed that there is no room for a gene which may have a role as electron donor specific to XylT (Harayama and Rekik, 1993). Therefore, a non-specific electron donor may reduce the oxidized XylT protein. For example, the *xylZ* gene product, which is an electron donor of toluate 1,2-dioxygenase (Harayama *et al.*, 1991b), may also donate electrons to XylT. Alternatively, electrons may be provided to XylT from an electron transfer system non-specific to the TOL catabolic pathway (e.g. respiratory chain).

After the addition of 3-methylcatechol, in the wild-type and *xylT* cultures, the catechol 2,3-dioxygenase activity decreased then reincreased (Figure 3). Both the *de novo* synthesis and reactivation of the enzyme may contribute to the recovery of the catechol 2,3-dioxygenase activity in the wild-type cells, while only *de novo* synthesis may be involved in the *xylT* cells. Such restoration of the enzyme activity was not observed in the *xylT* cells after the addition of 4-methylcatechol. This observation suggested that 4-methylcatechol was a more potent inactivator of the enzyme than 3-methylcatechol. This postulation was partly supported by the determination of the rate constants for enzyme inactivation: at a concentration of 5 μM 4-methylcatechol was an inactivator 10-fold stronger than 3-methylcatechol (Figure 5).

Another factor which may influence the growth of the *xylT* mutants on *m*-methyl- and *p*-methyl-substituted compounds is the difference in the induction of the *meta* operon by these compounds: we found that *p*-toluate was not as strong an inducer as *m*-toluate (Figure 7). Therefore, the amount of

active catechol 2,3-dioxygenase present in cells grown on *p*-toluate may be lower than that in cells grown on *m*-toluate, and may become a limiting factor in the absence of the XylT-dependent regeneration of the enzyme. The intracellular activity of catechol 2,3-dioxygenase in wild-type cells is determined by the rate of synthesis, inactivation and reactivation of the enzyme. In *xylT* mutants, the concentration of active enzyme may be determined solely by the rates of synthesis and inactivation of the enzyme. The rate of synthesis of catechol 2,3-dioxygenase may be proportional to the synthesis rate of total proteins, and expressed as: $D \times dP/dt$, where D and dP/dt represent the fraction of catechol, 2,3-dioxygenase synthetized among the total proteins, and the synthesis rate of total proteins, respectively. If we assume that the inactivation of catechol 2,3-dioxygenase in growing cells occurs principally by suicide catalysis, the rate of inactivation is proportional to the amount of the ES complex, [ES], which is expressed as $E(t) \times [S]/([S] + K_S)$ where $E(t)$, [S] and K_S are the total concentration of active enzyme, the concentration of the substrate and the dissociation constant of the substrate, respectively. Under these conditions the rate of enzyme inactivation is expressed as:

$$d[E^*]/dt = k_{inact} \times [ES] \tag{1}$$

where [E*] represents the concentration of the inactivated enzyme and k_{inact} the rate constant for the enzyme inactivation. If the concentration of catechol 2,3-dioxygenase in growing cells is the limiting factor for the total protein synthesis (which would be the case for *xylT* cells growing on *p*-toluate), the catalytic velocity of catechol 2,3-dioxygenase determines the rate of the total protein synthesis:

$$dP/dt = \alpha \times k_{cat} \times [ES] \tag{2}$$

where α is a yield of proteins per oxidation of substrate. The change in the intracellular concentration of catechol 2,3-dioxygenase, in this case, is described as:

$$d[E(t)]dt = (D\alpha k_{cat} - k_{inact}) \times [ES] \tag{3}$$

Equation (3) indicates that the catechol 2,3-dioxygenase concentration increases if $D\alpha \times k_{cat} > k_{inact}$. The catechol 2,3-dioxygenase activity in cell extracts of *m*-toluate-induced and *p*-toluate-induced cells were 7 and 1.5 U/mg protein, respectively (Figure 7). Since the specific activity of purified catechol 2,3-dioxygenase is 400 U/mg protein (Nakai *et al.*, 1983; Cerdan *et al.*, manuscript in preparation), the *D* values for *m*-toluate- and *p*-toluate-grown cells were $7/400 = 0.018$ and $1.5/400 = 0.0038$, respectively. It has been shown that *E.coli* growing on minimal media produces ~0.24 g of cell materials from 1 g of carbon substrates, and ~50% of the cell materials are proteins (Tempest and Neijssel, 1987). Assuming that 1 g of 3-methylcatechol or 4-methylcatechol produce 0.12 g of proteins, each 1 g (1/124 mole) of these catechols produce 2.1×10^{-3} ($=0.12 \times 0.018$) g and 0.45×10^{-3} ($=0.12 \times 0.0038$) g, respectively, of catechol 2,3-dioxygenase whose molecular weight per subunit is 35×10^3 (Nakai *et al.*, 1983). In other words, 1 mole of 3-methylcatechol and 4-methylcatechol produce $(124 \times 2.1 \times 10^{-3})/(35 \times 10^3)$ mole and $(124 \times 0.45 \times 10^{-3})/(35 \times 10^3)$ mole, respectively, of catechol 2,3-dioxygenase. The last two values correspond to $D\alpha$ of equation (3). Since k_{cat} for 3-methylcatechol and 4-methyl-

catechol are 480 s^{-1} and 860 s^{-1}, respectively (Cerdan *et al.*, manuscript in preparation), the $D\alpha k_{cat}$ values for 3-methylcatechol and 4-methylcatechol became 3.6×10^{-3} s^{-1} and 1.4×10^{-3} s^{-1}, respectively. The k_{inact} values for 3-methylcatechol and for 4-methylcatechol at a concentration of 5 μM, are 5×10^{-4} s^{-1} and 5×10^{-3} s^{-1}, respectively. This calculation thus explained why the activity of catechol 2,3-dioxygenase in the *xylT* mutants was zero when grown on *p*-toluate but increased when grown on *m*-toluate. Thus, the difference in the induction of the *meta* operon by *m*-toluate and *p*-toluate seems to be another critical factor in determining the growth of the *xylT* mutants on these substrates.

From the comparison of the primary structure of catabolic enzymes in different degradative pathways, it has been proposed that the recruitment of preexisting enzymes followed by the modification of substrate specificity represent the major mechanism to expand the substrate range of catabolic pathways (Harayama and Timmis, 1992; Harayama *et al.*, 1992). Here a novel mechanism to expand substrate specificity is reported: the XylT-dependent regeneration of inactivated catechol 2,3-dioxygenase allowed sufficient metabolism of 4-methylcatechol to support the growth of host cells on *p*-methyl-substituted compounds.

Materials and methods

Strain and plasmids
Strains and plasmids used in this study are listed in Table III.

Media and growth conditions
M9 minimal medium and L-broth have been described previously (Maniatis *et al.*, 1982; Harayama *et al.*, 1986). Carbon sources and inducers were added to the minimal medium at final concentrations of 10 mM for glucose, 5 mM for *m*-toluate, *p*-toluate and benzoate, or supplied as vapor in the case of toluene, *m*-xylene and *p*-xylene. When required, antibiotics were added at the following final concentrations: Ap, 20 μg/ml; Km, 20 μg/ml; Sp, 40 μg/ml; Tc, 25 μg/ml.

Chemicals
2-Hydroxymuconic semialdehyde, 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate and 2-hydroxy-6-oxohepta-2,4-dienoate were prepared enzymatically from catechol, 4-methylcatechol and 3-methylcatechol, respectively. 4-Oxalocrotonate was prepared as described previously (Harayama *et al.*, 1984) and stored at −20°C as an ethanolic solution. Aromatic compounds and other reagents were of the highest purity commercially available.

DNA manipulation and genetic methods
Methods for plasmid isolation, transformation, cleavage by restriction enzymes, ligation and agarose gel electrophoresis have previously been described (Maniatis *et al.*, 1982; Franklin *et al.*, 1983). Matings between *E.coli* and *P.putida* were performed as described by Lehrbach *et al.* (1984). Polymerase chain reaction amplification of the *xylT* DNA region was performed by using Vent$_R$ DNA polymerase (Biolabs). Reaction conditions were as described in the manufacturer's instructions. Amplification was carried out by using the primers 5'-ATGAACAGTGCCGGCTACGAG-3' (oligo 1) and 5'-CACGTGACCGCGGCTCATGCG-3' flanking the *xylT* region. Direct sequencing of PCR products was performed using the ds DNA Cycle Sequencing System kit (BRL) and oligo 1 as a primer.

Preparation of cell extracts and enzyme assays
Cultures were grown to late-exponential growth phase in M9 glucose medium containing an inducer (either *m*-toluate or *p*-toluate). Bacterial cells were harvested by centrifugation, washed and resuspended in 100 mM potassium phosphate (pH 7.4) containing 10% (v/v) acetone, and disrupted by sonication. Assays for 2-hydroxymuconic semialdehyde hydrolase, 2-hydroxymuconic semialdehyde dehydrogenase and 4-oxalocrotonate tautomerase were performed as described previously (Sala-Trepat and Evans, 1971; Harayama *et al.*, 1984). The catechol 2,3-dioxygenase activity was measured according to the method of Sala-Trepat and Evans (1971); the following wavelengths and molar absorption coefficients (M^{-1} cm^{-1}), indicated in brackets, were used: catechol, 375 nm (33 000); 4-methyl-

Table III. Strains and plasmids used for this study

Strain and plasmids	Relevant markers	Source or reference
Strains		
P.putida		
KT2440	PaW1 cured of pWW0	Franklin *et al.* (1981)
E.coli		
LE392	F⁻ *hsdR514 metB1 lacY1 supE44 supF58 galK2 galT22 trpR55*	Maniatis *et al.* (1982)
GSH3491	F⁻ *araD139* Δ*(ara-leu)7697* Δ*lacX74 galU galK hsdR rpsE*	Hugovieux-Cotte-Pattat *et al.* (1990)
Plasmids		
pACYC184	Cm^R Tc^R	Chang and Cohen (1978)
pGA1	pACYC184 derivative carrying 6 kb *Eco*RI–*Pst*I fragment of pWW0 carrying *xylXYZLTEG*	This study
pGA2	pGA1 derivative carrying a Km^R cassette in the *Nco*I site within the *xylT* gene	This study
pGA3	pGA1 derivative carrying a 21 bp linker in the *Nco*I site within the *xylT* gene	This study
pGA4	pGA1 derivative in which the *Nco*I site within *xylT* is modified by Klenow polymerase filling	This study
pPL392	pBR322 derivative carrying the *xylXYZLTEGFJQKIH* genes of pWW0	Harayama *et al.* (1984)
pRME1	pBR322 carrying 1.2 kb *Hae*II segment of Tn*903* conferring resistance to Km (this insertion is flanked by synthetic multiple restriction sites)	W.Messer provided by T.Chakrabarty
pWW0-161	pWW0 carrying Tn*401*, Ap^R, *m*-xylene⁺, *p*-xylene⁺	Franklin *et al.* (1981)
pWW0-161 *xylT1*::Km^R	Ap^R, Km^R, *m*-xylene⁺, *p*-xylene⁻ obtained by homologous recombination between pGA2 and pWW0-161	This study
pWW0-161 *xylT2*	Ap^R, Km^S, *m*-xylene⁺, *p*-xylene⁻ obtained by homologous recombination between pGA3 and pWW0-161 *xylT1*::Km^R	This study
pWW0-161 *xylT3*	Ap^R, Km^S, *m*-xylene⁺, *p*-xylene⁻ obtained by homologous recombination between pGA4 and pWW0-161 *xylT1*::Km^R	This study

catechol, 382 nm (28 100); 3-methylcatechol, 388 nm (13 800). One unit (U) for each enzyme was defined as the amount which transforms 1 mmol of substrate per min.

Purification of catechol 2,3-dioxygenase
Catechol 2,3-dioxygenase was purified from cultures of KT2440(pWW0-161) and of KT2440(pWW0-161 *xylT2*) grown overnight in L-broth containing 5 mM *m*-toluate. The purification method was similar to one described previously (Wasserfallen *et al.*, 1991) except that the final gel filtration step was replaced by a passage through a hydrophobic interaction column (TSK Phenyl 5-PW hydrophobic interaction column; 75 × 7.5 mm, Bio-Rad Laboratories). Active fractions from DEAE anion-exchange chromatography have been precipitated in ammonium sulfate at 70% saturation at 4°C, and precipitated proteins were resuspended in 10 mM ethylenediamine buffer (ED buffer) (pH 7.4) containing 1 M ammonium sulfate at a final concentration of 10 mg/ml. The protein suspension thus prepared was filtered through a Millex filter (pore size 0.2 μM; Millipore), and charged onto the hydrophobic interaction column pre-equilibrated with ED buffer containing 1 M ammonium sulfate. Proteins were eluted with a linear gradient formed by mixing ED buffer containing 1 M ammonium sulfate and ED buffer containing 10% (v/v) isopropanol.

Determination of kinetic constants for the inactivation of catechol 2,3-dioxygenase
The method for the determination of the kinetic constants for the inactivation of catechol 2,3-dioxygenase during catalysis is described elsewhere (Cerdan *et al.*, manuscript in preparation). Briefly, the purified enzyme was added to 1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing catechol, 3-methylcatechol or 4-methylcatechol at 50 μM, and the formation of the ring-cleavage product in this mixture was monitored using a UVICON spectrophotometer (Kontron). The concentration of the enzyme was adjusted so that the complete inactivation of the enzyme occurred before 20% of the substrate was oxidized. After the initiation of the enzyme reaction, the absorbance increased but the rate of the absorbance change decreased as

the enzyme became inactivated. This progress curve was followed for 30 min and later analyzed using the software Excell (Microsoft). In the analysis, the slope of the absorbance change, d$A(t)$/dt, was calculated. The d$A(t)$/dt value is described by the following equation:

$$dA(t)/dt = \epsilon k_{cat}[S]/([S] + K_S) \times E_0 \exp\{(-k_{inact} \times t \times [S])/([S] + K_S)\} \quad (4)$$

where ϵ, k_{cat}, $[S]$, K_S, E_0 and k_{inact} are the extinction coefficient of the product, the rate constant for the productive catalysis (ring-cleavage), the concentration of the substrate, the dissociation constant, the initial concentration of the enzyme and the rate constant for the enzyme inactivation, respectively. Since the substrate concentration used in the present experiments (50 μM) was significantly higher than the K_S value (<3 μM), $[S]/([S] + K_S)$ was equal to 1. The logarithm of the above equation is then given as:

$$\log \{dA(t)/dt\} = \log \epsilon k_{cat}E_0 - k_{inact} \times t \quad (5)$$

Thus, the k_{inact} value could be obtained from the slope of log $\{dA(t)/dt\}$ plotted against t.

In vivo reactivation of catechol 2,3-dioxygenase inactivated by H_2O_2
KT2440(pWW0-161) and KT2440(pWW0-161 *xylT1*) cells were grown at 30°C in M9 minimal medium containing glucose and *m*-toluate. The cells were harvested, washed and resuspended in an equal volume of 0.1 M potassium phosphate buffer (pH 7.4). To 4 ml of the cell suspension, 50 μM of H_2O_2 was added, and incubated at room temperature. At different times, 100 μl aliquots were taken, and their catechol 2,3-dioxygenase activities were determined in 0.1 M potassium buffer (pH 7.4) containing 600 μM catechol by measuring the change in absorbance at 375 nm. The catechol 2,3-dioxygenase activity determined using intact cells gave almost identical values to those determined using sonicated cell extracts.

Acknowledgements

References

Bartels,I., Knackmuss,H.J. and Reineke,W. (1984) *Appl. Environ. Microbiol.*, **47**, 500−505.

Bartilson,M., Nordlund,I. and Shingler,V. (1990) *Mol. Gen. Genet.*, **220**, 294−300.

Bruschi,M. and Guerlesquin,F. (1988) *FEMS Microbiol. Rev.*, **54**, 155−176.

Chang,A.C. and Cohen,S.N. (1978) *J. Bacteriol.*, **134**, 1141−1156.

Franklin,F.C., Bagdasarian,M., Bagdasarian,M.M. and Timmis,K.N. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7458−7462.

Franklin,F.C., Lehrbach,P.R., Lurz,R., Rueckert,B., Bagdasarian,M. and Timmis,K.N. (1983) *J. Bacteriol.*, **154**, 676−685.

Harayama,S. and Rekik,M. (1990) *Mol. Gen. Genet.*, **221**, 113−120.

Harayama,S. and Rekik,M. (1993) *Mol. Gen. Genet.*, in press.

Harayama,S. and Timmis,K.N. (1992) In Sigel,H. and Sigel,A. (ed.), *Metal Ions in Biological Systems*. Marcel Dekker Inc., Vol. XXVIII, pp. 100−151.

Harayama,S., Lehrbach,P.R. and Timmis,K.N. (1984) *J. Bacteriol.*, **160**, 251−255.

Harayama,S., Leppik,R.A., Rekik,M., Mermod,N., Lehrbach,P.R., Reineke,W. and Timmis,K.N. (1986) *J. Bacteriol.*, **167**, 455−461.

Harayama,S., Mermod,N., Rekik,M., Lehrbach,P.R. and Timmis,K.N. (1987) *J. Bacteriol.*, **169**, 558−564.

Harayama,S., Rekik,M., Wubbolts,M., Rose,K., Leppik,R.A. and Timmis,K.N. (1989) *J. Bacteriol.*, **171**, 5048−5055.

Harayama,S., Polissi, A. and Rekik,M. (1991a) *FEBS Lett.*, **285**, 85−88.

Harayama,S., Rekik,M., Bairoch,A., Neidle,E.L. and Ornston,L.N. (1991b) *J. Bacteriol.*, **173**, 7540−7548.

Harayama,S., Kok,M. and Neidel,E.L. (1992) *Annu. Rev. Microbiol.*, **46**, 565−601.

Hori,K., Hashimoto,T. and Nozaki,M. (1973) *J. Biochem.*, **71**, 375−381.

Hugovieux-Cotte-Pattat,N., Köhler,T., Rekik,M. and Harayama,S. (1990) *J. Bacteriol.*, **172**, 6651−6660.

Kojima,Y., Itada,N. and Hayaishi,O. (1961) *J. Biol. Chem.*, **236**, 2223−2230.

Lehrbach,P.R., Zeyer,J., Reineke,W., Knackmuss,H.J. and Timmis,K.N. (1984) *J. Bacteriol.*, **158**, 1025−1032.

Mabrouk,P.A., Orville,A.M., Lipscomb,J.D. and Solomon,E.I. (1991) *J. Am. Chem. Soc.*, **113**, 4053−4061.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Nakai,C., Kagamiyama,H., Nozaki,N., Nakazawa,T., Inouye,S., Ebina,Y. and Nakazawa,A. (1983) *J. Biol. Chem.*, **258**, 2923−2928.

Nakazawa,T., Inouye,S. and Nakazara,A. (1980) *J. Bacteriol.*, **144**, 222−231.

Nordlund,I., Powlowski,J. and Shingler,V. (1990) *J. Bacteriol.*, **172**, 6826−6833.

Nozaki,M. (1979) *Top. Curr. Chem.*, **78**, 145−186.

Nozaki,M., Ono,K., Nakazawa,T., Kotani,S. and Hayaishi,O. (1968) *J. Biol. Chem.*, **243**, 2682−2690.

Nozaki,M., Kotani,S., Ono,S. and Senoh,S. (1970) *Biochem. Biophys. Acta*, **220**, 213−223.

Sala-Trepat,J.M. and Evans,C.W. (1971) *Eur. J. Biochem.*, **20**, 400−413.

Shingler,V., Powlowsky,J. and Marklund,U. (1992) *J. Bacteriol.*, **174**, 711−724.

Tempest,D.W. and Neijssel,O.M. (1987) In Neidhardt,F.C. (ed.), *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*. ASM Press, Washington DC, Vol I, pp. 797−806.

Wallis,M.G. and Chapman,K. (1990) *Biochem. J.*, **266**, 605−609.

Wasserfallen,A. (1989) Ph.D thesis, University of Geneva.

Wasserfallen,A., Rekik,M. and Harayama,S. (1991) *Bio Technology*, **9**, 296−298.

Worsey,M.J. and Williams,P.A. (1975) *J. Bacteriol.*, **124**, 7−13.

Yen,K.M. and Gunsalus,I.C. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 874−878.

You,I.S., Ghosal,D. and Gunsalus,I.C. (1991) *Biochemistry*, **30**, 1635−1641.