JOURNAL OF BACTERIOLOGY, Oct. 1994, p. 6074–6081
0021-9193/94/$04.00+0

Vol. 176, No. 19

Substrate Specificity of Catechol 2,3-Dioxygenase Encoded by TOL Plasmid pWW0 of *Pseudomonas putida* and Its Relationship to Cell Growth

PASCALE CERDAN, ALAIN WASSERFALLEN,† MONIQUE REKIK, KENNETH N. TIMMIS,‡ AND SHIGEAKI HARAYAMA*

Department of Medical Biochemistry, University Medical Centre, 1211 Geneva 4, Switzerland

Received 23 May 1994/Accepted 27 July 1994

Catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 of *Pseudomonas putida* consists of four identical subunits, each containing one ferrous ion. The enzyme catalyzes ring cleavage of catechol, 3-methylcatechol, and 4-methylcatechol but shows only weak activity toward 4-ethylcatechol. Two mutants of catechol 2,3-dioxygenases (4ECR1 and 4ECR6) able to oxidize 4-ethylcatechol, one mutant (3MCS) which exhibits only weak activity toward 3-methylcatechol but retained the ability to cleave catechol and 4-methylcatechol, and one phenotypic revertant of 3MCS (3MCR) which had regained the ability to oxidize 3-methylcatechol were characterized by determining their K_m and partition ratio (the ratio of productive catalysis to suicide catalysis). The amino acid substitutions in the four mutant enzymes were also identified by sequencing their structural genes. Wild-type catechol 2,3-dioxygenase was inactivated during the catalysis of 4-ethylcatechol and thus had a low partition ratio for this substrate, whereas the two mutant enzymes, 4ECR1 and 4ECR6, had higher partition ratios for it. Similarly, mutant enzyme 3MCS had a lower partition ratio for 3-methylcatechol than that of 3MCR. Molecular oxygen was required for the inactivation of the wild-type enzyme by 4-ethylcatechol and of 3MCS by 3-methylcatechol, and the inactivated enzymes could be reactivated by incubation with $FeSO_4$ plus ascorbic acid. The enzyme inactivation is thus most likely mechanism based and occurred principally by oxidation and/or removal of the ferrous ion in the catalytic center. In general, partition ratios for catechols lower than 18,000 did not support bacterial growth. A possible meaning of the critical value of the partition ratio is discussed.

It is becoming increasingly evident that all contemporary enzymes have developed from a limited number of ancestral species through successive changes in amino acid residues. Very often, catabolic enzymes of one superfamily have acquired different substrate specificities during evolution while their catalytic mechanisms have been conserved (5, 6). To understand the mechanisms of enzyme evolution, Clarke and colleagues, in their pioneering work, isolated a number of substrate specificity mutants of amidase of *Pseudomonas aeruginosa* by selecting clones grown on specific substrates (3). Similarly, Hall screened many substrate specificity mutants of so-called evolved β-galactosidase coded for by *ebg* in *Escherichia coli* (4).

Expansion of the substrate range of the catabolic enzymes and the effector range of the regulatory proteins for the toluene-degradative pathway encoded by TOL plasmid pWW0 of *Pseudomonas putida* has been studied by Ramos and coworkers again by virtue of growth selection (1, 21, 22). *P. putida* containing a TOL plasmid can grow on benzoate, *m*-toluate, and *p*-toluate because *meta*-cleavage pathway enzymes encoded by the TOL plasmid will metabolize all intermediates derived from these substrates. This strain, however, cannot grow on 4-ethylbenzoate, and this inability is due to (i) the positive regulatory protein, XylS, which induces the *meta*-cleavage enzymes in response to benzoate, *m*-toluate, and *p*-toluate but not to 4-ethylbenzoate, and (ii) catechol 2,3-dioxygenase, which oxidizes catechol, 3-methylcatechol, and 4-methylcatechol, intermediates derived from benzoate, *m*-toluate, and *p*-toluate, respectively, but does not oxidize 4-ethylcatechol, an intermediate derived from 4-ethylcatechol. From a strain of *P. putida* containing a TOL plasmid and synthesizing a mutant XylS recognizing 4-ethylcatechol, clones able to grow on 4-ethylbenzoate were isolated. These clones expressed the catechol 2,3-dioxygenase activity which oxidizes 4-ethylcatechol (22).

In this study, (i) a mutant catechol 2,3-dioxygenase which exhibits only weak activity toward 3-methylcatechol but retained the ability to cleave catechol and 4-methylcatechol and (ii) its phenotypic revertant which had regained the ability to oxidize 3-methylcatechol were isolated. These mutant catechol 2,3-dioxygenases were characterized in this study.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *P. putida* KT2440 (wild type) and PaW94 (benzoate-1,2-dioxygenase-negative mutant) have been described elsewhere (27). TOL plasmid pWW0 and its mutants pWW0-EB1 and pWW0-EB6, which synthesize catechol 2,3-dioxygenases capable of oxidizing 4-ethylcatechol, have also been described previously (22). Catechol 2,3-dioxygenases synthesized from pWW0-EB1 and pWW0-EB6 are called 4ECR1 (4-ethylcatechol resistant 1) and 4ECR6 (4-ethylcatechol resistant 6), respectively, in this study. pNM72 is a pKT231-based plasmid containing a set of

* Corresponding author. Present address: Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi-City, Iwate 026, Japan. Phone: 81-193-26-6544. Fax: 81-193-26-6592.

† Present address: Mikrobiologisches Institut, ETH Zürich, CH-8092 Zürich, Switzerland.

‡ Present address: Department of Microbiology, Gesellschaft für Biotechnologische Forschung mbH, D-3300 Braunschweig, Germany.

genes (*xylXYZLTEGFJQKIH* and *xylS*) required for the growth of KT2440 or of PaW94 on benzoate, *m*-toluate, and *p*-toluate (9). The methods for the isolation of pNM72 mutants pGSH2225 and pGSH2225R are described in Results. Plasmid pGSH2225 synthesizes a mutant catechol 2,3-dioxygenase called 3MCS (3-methylcatechol sensitive) which exhibits only weak activity toward 3-methylcatechol but retained the ability to cleave catechol and 4-methylcatechol, while plasmid pGSH2225R synthesizes a phenotypic revertant of 3MCS called 3MCR (3-methylcatechol resistant) which had regained the ability to oxidize 3-methylcatechol. Plasmid pBS(+) was purchased from Stratagene (La Jolla, Calif.). Complete and minimal media have been described previously (7).

Chemicals and enzymes. Catechol was purchased from Fluka (Buchs, Switzerland), and 3-methylcatechol and 4-ethylcatechol were purchased from Lancaster Synthesis (Morecambe, United Kingdom). All other chemicals were of the highest grade available. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.), and T7 DNA polymerase sequence kit was obtained from Pharmacia (Uppsala, Sweden).

DNA manipulations. The TOL plasmid DNA was isolated by the method of Hansen and Olsen (5), while other plasmids were isolated by the alkaline lysis procedure (11).

Purification of catechol 2,3-dioxygenases. Wild-type catechol 2,3-dioxygenase was purified from *P. putida* KT2440 containing TOL plasmid pWW0, mutant catechol 2,3-dioxygenases 4ECR1 and 4ECR6 were isolated from *P. putida* KT2440 containing plasmid pWW0-EB1 and pWW0-EB6, respectively, and the 3MCS and 3MCR enzymes were isolated from *P. putida* PaW94 containing pGSH2225 and pGSH2225R, respectively. Cells were grown overnight at 30°C in 3 liters of Luria broth containing 10 mM *m*-toluate. Cells were pelleted by centrifugation at 20,000 × *g* for 30 min at 4°C, resuspended in 80 ml of 10 mM ethylenediamine-hydrochloride buffer (pH 7.4) containing 10% (vol/vol) isopropanol, and disrupted by passage through a precooled French pressure cell (model FA-073; SLM Instruments Inc., Urbana, Ill.) at a pressure difference of 96.5 MPa. The cell extract was centrifuged at 40,000 × *g* for 30 min at 4°C, and the supernatant fluid was recentrifuged at 250,000 × *g* for 60 min at 4°C. The new supernatant fluid was filtered through a Nalgene filter (0.45-μm pore size; Nalgene Labware Div., Nalgene/Sybron Corp., Rochester, N.Y.) and loaded onto a Bio-Gel anion-exchange column (type TSK-DEAE-5PW; 150 by 21.5 mm; Tosoh, Tokyo, Japan) fitted to a Waters 600 liquid chromatography system (Millipore Corp., Bedford, Mass.). The protein was eluted by a linear gradient of 0 to 0.2 M Na_2SO_4 in 250 ml of 10 mM ethylenediamine-hydrochloride buffer (pH 7.4) at a flow rate of 5 ml/min. The eluate was collected in 10-ml fractions on ice. The wild-type and mutant catechol 2,3-dioxygenases were eluted at a salt concentration of 0.15 M. Fractions containing active catechol 2,3-dioxygenase were adjusted to 25% saturation of ammonium sulfate at 4°C, and the proteins which precipitated were removed by centrifugation at 40,000 × *g* for 10 min at 4°C. Most of the catechol 2,3-dioxygenase activity was recovered in the supernatant fluid. Further ammonium sulfate was added to 75% saturation at 4°C, and the suspension was centrifuged as described above. The pellet containing the catechol 2,3-dioxygenase activity was resuspended in 5 ml of 10 mM ethylenediamine-hydrochloride buffer (pH 7.4) containing 1 M ammonium sulfate, filtered through a Millex-GV filter, and loaded onto a hydrophobic interaction column (Bio-Gel Phenyl 5PW; Tosoh) preequilibrated with 10 mM ethylenediamine-hydrochloride buffer (pH 7.4) containing 1 M ammonium sulfate. Proteins were eluted from the column by a linear gradient from 1 to 0 M ammonium sulfate in 30 ml of 10 mM ethylenediamine-hydrochloride buffer (pH 7.4) at a flow rate of 1 ml/min. Purified enzymes were stored either at 4°C for a short-term storage (less than 3 days) or at −80°C in 20% (vol/vol) glycerol.

Assay of catechol 2,3-dioxygenase. Catechol 2,3-dioxygenase activity was assayed at 30°C by the method of Nozaki et al. (18) by following the absorbance changes at 375, 388, 382, and 381 nm to monitor the formation of the ring cleavage products of catechol, 3-methylcatechol, 4-methylcatechol, and 4-ethylcatechol, respectively. The molar absorption coefficients of the ring cleavage products of catechol, 3-methylcatechol, 4-methylcatechol, and 4-ethylcatechol were 33,000, 19,400, 28,100, and 35,000, respectively, at pH 7.4. One unit of enzyme was defined as the amount which oxidizes 1 μmol of catechol per min under the standard assay conditions. The protein concentration was determined by the method of Bradford (2).

Determination of partition ratio. The kinetic mechanism of catechol 2,3-dioxygenase has been characterized by Nozaki and colleagues (16, 19) to be an ordered bi-uni, with catechol (or its structural analogs) as the first substrate and molecular oxygen as the second substrate. A suicide inhibition of the enzyme is described by the following kinetic mechanism:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EAB \rightleftarrows EP)$$

$$\overset{k_3}{\searrow} E + P$$
$$\underset{k_4}{\nearrow} E^* + P$$

where E, A, B, P, and E* are catechol 2,3-dioxygenase, catechol (or its structural analogs), molecular oxygen, the ring cleavage product of catechol (2-hydroxymuconic semialdehyde or its analogs), and the inactive catechol 2,3-dioxygenase, respectively, while k_3 and k_4 are the rate constants of the productive reaction (ring cleavage) and the enzyme inactivation, respectively. The kinetics of the enzyme inactivation by suicide inhibition is described as

$$-\frac{d[E]}{dt} = k_4([EAB] + [EP]) \quad (1)$$

where [*E*] is the total concentration of active enzyme species. The steady-state kinetics (23) shows that [EAB + EP] is expressed as

$$[EAB + EP] = \frac{[E] \times k_1k_2[A][B]}{k_{-1}(k_{-2} + k_3) + k_2k_3[B] + k_1(k_{-2} + k_3)[A] + k_1k_2[A][B]} \quad (2)$$

If the concentrations of A and B are constant, equations 2 and 1 are rewritten as

$$[EAB + EP] = \gamma[E] \quad (3)$$

$$-\frac{dE}{dt} = k_4\gamma[E] \quad (4)$$

where γ is a constant. The differential equation (equation 4) is solved as

$$\log_e \frac{[E]}{[E_0]} = -k_4\gamma t \quad (5)$$

where E_0 is the initial concentration of the active enzyme. The speed of enzyme catalysis, $v(t)$, at time t is described as

$$v(t) = k_3[\mathrm{EAB} + \mathrm{EP}] = k_3\gamma[E] \quad (6)$$

At $t = 0$, the initial velocity, v_0, is expressed as

$$v_0 = k_3\gamma[E_0] \quad (7)$$

Equation 5 is rewritten as

$$k_4\gamma = \frac{\log_e \frac{v_0}{v(t)}}{t} \quad (8)$$

When the concentrations of A and B are significantly higher than their respective K_m values, the γ value becomes 1. The k_4 values were determined as follows. A wild-type or mutant catechol 2,3-dioxygenase of known activity was added to 1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing a catechol derivative at 50 μM ($\geqslant K_m$) and 10% (vol/vol) isopropanol (to minimize the spontaneous inactivation of the enzyme), and the formation of the ring cleavage product of the catechol derivative was monitored spectrophotometrically. The concentration of the reaction product increased until the enzyme was completely inactivated. The concentration of the enzyme was adjusted so that the complete inactivation of the enzyme occurred before 30% of the substrate was oxidized. The catalytic rate at each time point, $v(t)$, was obtained from the slope of the absorbance change:

$$v(t) = \frac{[A(t + \Delta t) - A(t)]V}{(\Delta t \times \varepsilon)} \quad (9)$$

where ε is the extinction coefficient of the ring cleavage product, V is the volume of the reaction mixture (1 ml), and Δt was usually 10 s. When the logarithm of the $v_0/v(t)$ values was plotted against t, a straight line was obtained. Equation 8 indicates that the slope of the straight line represent the k_4 value. Since γ is supposed to be close to 1 at the 50 μM substrate concentration, the slope was taken as k_4.

The suicide inhibition is best described by the partition ratio (24), which is the ratio of k_3 ($= k_{cat}$) to k_4. The k_{cat} values were obtained from a Lineweaver-Burk plot (23), while the k_4 values were obtained by the method described above, and the ratio k_{cat}/k_4, was calculated. The partition ratio also represents the number of product molecules formed per molecule of catechol 2,3-dioxygenase before the enzyme is inactivated. Thus, the ratio could also be calculated from the maximum amount of products formed before the complete inactivation of the enzyme, and the amount was divided by the initial concentration of catechol 2,3-dioxygenase. The two methods provided almost identical results.

Inactivation and reactivation of catechol 2,3-dioxygenase. A 3-ml sample containing about 150 U of the wild-type or 3MCS catechol 2,3-dioxygenase was incubated for 20 min at room temperature in 100 mM potassium phosphate (pH 7.4) containing 5 mM 4-ethylcatechol, 5 mM 3-methylcatechol, or 200 μM H_2O_2. When necessary, the sample was made anaerobic by evacuation with a vacuum pump followed by flushing nitrogen gas. The sample was then loaded on a gel filtration column (G200; Bio-Rad Laboratories, Richmond, Calif.) preequilibrated with 100 mM potassium phosphate (pH 7.4) and eluted by the same buffer. The elution of the enzyme was monitored by a UV detector. The protein-containing fraction (about 5 ml) was made anaerobic as described above. Freshly prepared $FeSO_4$ and/or ascorbic acid were added to the buffer to a final concentration of 1 mM. Incubation was continued for 60 min, and the level of catechol 2,3-dioxygenase activity in each sample was determined spectrophotometrically with 33 μM catechol as a substrate.

Determination of iron content of catechol 2,3-dioxygenase. The iron content of the catechol 2,3-dioxygenases was determined by using *o*-phenanthroline (12). This chelator is specific to ferrous ion, and ferric ion is detected only in the presence a reducing agent. The iron content was determined both in the presence and in the absence of 2.2 mM ascorbic acid.

RESULTS

Isolation of catechol 2,3-dioxygenase mutants. PaW94 is a *P. putida* mutant defective in benzoate 1,2-dioxygenase and is not therefore able to grow on benzoate (26). Plasmid pNM72 carries a complete set of the *meta*-cleavage operon of the TOL plasmid and confers upon *P. putida* the ability to grow on benzoate, *m*-toluate, and *p*-toluate (9). Strain PaW94(pNM72) was mutagenized with *N*-methyl-*N'*-methyl-*N*-nitrosoguanidine as described by Miller (13), and mutagenized bacteria were spread on M9 minimal plates containing 10 mM benzoate. Colonies that developed on the plates were replicated onto *m*-toluate minimal plates, and clones which were not able to grow on *m*-toluate were retained for further study. One such mutant, designated GSH2225, formed a dark brown product when plated on *m*-toluate, which suggested that it accumulated 3-methylcatechol. Thus, catechol 2,3-dioxygenase in GSH2225 may be defective in the oxidation of 3-methylcatechol. This strain, however, could grow on benzoate and *p*-toluate, indicating that catechol 2,3-dioxygenase of this mutant can oxidize catechol and 4-methylcatechol, which are metabolites of benzoate and *p*-toluate, respectively. To obtain revertants of GSH2225 able to grow on *m*-toluate, about 3×10^9 cells of GSH2225 were spread onto a *m*-toluate minimal plate and incubated at 30°C for 2 weeks. About 30 colonies developed on the *m*-toluate plate, and some of them exhibited a brown halo which PaW94(pNM72) did not produce. Such derivatives of GSH2225 were thus probably not true revertants but may carry suppressor mutations. One of the revertants was named GSH2225R, and the plasmids harbored in GSH2225 and GSH2225R were named pGSH2225 and pGSH2225R, respectively. As described later, the mutations responsible for these phenotypes were mapped in *xylE*, the structural gene for catechol 2,3-dioxygenase. Mutant catechol 2,3-dioxygenases synthesized from GSH2225 and GSH2225R are called 3MCS and 3MCR, respectively.

Purification of wild-type and mutant catechol 2,3-dioxygenases. Two mutants of TOL plasmid pWW0, pWW0-EB1 and pWW0-EB6, which synthesize catechol 2,3-dioxygenases capable of oxidizing 4-ethylcatechol have been isolated previously (22). Catechol 2,3-dioxygenases synthesized from pWW0-EB1 and pWW0-EB6 are called 4ECR1 and 4ECR6, respectively. The wild-type and mutant catechol 2,3-dioxygenases were purified as described in Materials and Methods (Table 1). The specific activity of the wild-type enzyme was 400 μmol of catechol oxidized per mg of protein, which is slightly higher than that reported previously (14). The specific activities of the mutant enzymes were very low compared with to the wild-type

TABLE 1. Purification of wild-type and mutant catechol 2,3-dioxygenases

Step	Wild type		3MCS		3MCR		4ECR1		4ECR6	
	SA[a]	TA[b]	SA	TA	SA	TA	SA	TA	SA	TA
Cell extract	4,300	9,300	1,000	1,800	1,200	1,900	820	3,000	1,100	2,300
Anion exchange	134,000	8,000	21,000	870	11,000	360	1,400	95	13,000	1,100
Ammonium sulfate	350,000	3,300	6,000	80	3,700	70	150	6	1,300	30
Hydrophobic interaction	400,000	1,900	5,300	30	3,500	17	150	0.4	1,400	13

[a] SA, specific activity (nanomoles of catechol oxidized per minute per milligram of protein).
[b] TA, total activity (micromoles of catechol oxidized per minute).

enzyme activity, probably because of the inactivation during the purification. The specific activity of pure catechol 2,3-dioxygenase from each mutant was estimated from the specific activity of the mutant enzyme in cell extracts, assuming that these mutant enzymes are expressed at the same level to the wild-type enzyme, using the equation (specific activity of the purified wild-type enzyme) × (specific activity of a mutant enzyme in cell extracts)/(specific activity of the wild-type enzyme in cell extracts). The values calculated from the activities of each enzyme in four independent cell extract preparations were 196, 115, 127, and 130 μmol of catechol oxidized per mg of protein for 3MCS, 3MCR, 4ECR1, and 4ECR6, respectively, which are the same orders of magnitude relative to the values for the wild-type enzyme. Anion-exchange chromatography of mutant enzymes except for 4ECR1 resulted in a 10-fold purification rather than the 30-fold purification achieved with the wild-type enzyme. For 4ECR1, the specific activity of the enzyme was not increased significantly by anion-exchange chromatography, suggesting significant inactivation in this purification step. Ammonium sulfate fractionation and hydrophobic interaction chromatography, which successfully purified the wild-type enzyme, gave very low yields of all four mutant enzymes. The wild-type and mutant enzymes were essentially pure after the hydrophobic interaction chromatography, as judged by sodium dodecyl sulfate-gel electrophoresis, but the purified mutant enzymes showed much lower specific activities than the calculated values, indicating that the majority of the purified enzyme molecules were inactive. About 8 h was required for the three purification steps, and the activity of each fraction was determined immediately after each fractionation. The purified wild-type enzyme was rather stable if the protein concentration was as high as 2 mg/ml: the enzyme lost about 20% of its activity after storage at 4°C for 3 days. In contrast, the mutant enzymes readily lost their activity upon storage at either 4 or −80°C. The purified 4ECR1 and 4ECR6 enzymes were extremely labile, and more than 95% of the activity of purified samples was lost upon storage at 4°C for 10 h. Storage at −80°C did not prevent the initial loss of activity, probably because these enzymes are sensitive to freezing and thawing.

Reactivation of purified enzyme. The low yield of the mutant enzymes may be due to spontaneous oxidation and/or loss of the iron cofactor during the purification process. If this is the case, inactivated enzymes may be reactivated by incubating them with $FeSO_4$ and ascorbic acid. The specific activities of the 4ECR1 and 4ECR6 enzymes were increased significantly by incubating them anaerobically with $FeSO_4$ and ascorbic acid. Incubation of purified 3MCS and 3MCR enzymes with $FeSO_4$ and ascorbic acid did not significantly increase their activities (Table 2).

In previous studies, it was demonstrated that inactive catechol 2,3-dioxygenase containing ferric ion could be reactivated by ascorbic acid alone, while inactive enzyme containing no iron cofactor could be reactivated by ascorbic acid plus $FeSO_4$ (19). In our experiments, catechol 2,3-dioxygenase inactivated by H_2O_2 (and hence containing ferric ion in the catalytic center, according to previous studies) could not be reactivated by ascorbic acid alone but could be reactivated by ascorbic acid plus $FeSO_4$ (data not shown). In our hands, therefore, reactivation experiments did not distinguish between inactive enzymes containing ferric cofactor and those containing no iron cofactor.

Determination of iron content. The monomer concentrations of the purified catechol 2,3-dioxygenases were estimated from their protein concentrations, while the concentration of iron was determined by *o*-phenanthroline. As shown in Table 3, the wild-type, 3MCS, and 3MCR enzymes contain one atom of iron per monomer of catechol 2,3-dioxygenase, while the iron contents of the 4ECR1 and 4ECR6 enzymes were lower than 1. The concentrations of iron determined in the presence and absence of ascorbic acid were almost identical, indicating that the majority of the iron in the purified enzymes is ferrous ion.

Determination of Michaelis-Menten constants. The K_m and k_{cat} values of the wild-type and mutant catechol 2,3-dioxygenases were determined by examining their initial velocities at substrate concentrations of between 0.67 and 300 μM (Table 4). The K_m and k_{cat} values for 3-methylcatechol of 3MCS, and for 4-ethylcatechol of the wild-type enzyme, of 3MCS, and of 3MCR could not be determined because of the rapid inactivation of these enzymes during the catalysis of these substrates. For these catalytic reactions, the initial velocities were obtained by extrapolation the $\log_e[1/v(t)]$ versus t plot to $t = 0$ using the relationship of equation 8. The extrapolated initial velocities of the wild-type enzyme, 3MCS, and 3MCR at 20

TABLE 2. Reactivation of catechol 2,3-dioxygenases by treatment with $FeSO_4$ and ascorbic acid

Enzyme	Activity (nmole of catechol oxidized/min/mg of protein)	
	Initial	After reactivation
XylE	123,000	127,000
4ECR1	<3	2,300
4ECR6	6	1,100
3MCS	940	980
3MCR	2,800	4,400

TABLE 3. Iron contents of purified catechol 2,3-dioxygenases[a]

Enzyme	Concn (μM)			Iron/subunit concn ratio
	Total iron[b]	Fe(II)[c]	Enzyme subunit	
XylE	60	60	54	1.1
4ECR1	8	8	30	0.25
4ECR6	40	40	68	0.58
3MCS	40	39	38	1
3MCR	41	39	39	1

[a] Nonreactivated enzymes were used.
[b] Determined in the presence of ascorbic acid.
[c] Determined in the absence of ascorbic acid.

μM 4-ethylcatechol were approximately equal to those at 50 μM 4-ethylcatechol. Similarly, the initial velocity of 3MCS for the oxidation of 3-methylcatechol at 20 μM was approximately equal to that at 50 μM. Therefore, the initial velocities of these reactions at 50 μM were considered to correspond to the V_{max} values, and the k_{cat} values of the wild-type enzyme, 3MCS, and 3MCR for 4-ethylcatechol and that of 3MCS for 3-methylcatechol were calculated. The k_{cat} values of the mutant enzymes thus determined, however, did not correspond to their real k_{cat} values, as the purified mutant enzymes contained significant fraction of inactive proteins. Therefore, the absolute k_{cat} values of the mutant enzymes were estimated as described in Table 4, footnote *a*. The k_{cat} values of the wild-type enzyme, of 3MCS, and of 3MCR for 4-ethylcatechol were less than 10% of those for catechol. The k_{cat} for 4-ethylcatechol of 4ECR6 was much higher than that of the wild-type enzyme. The 4ECR1 enzyme, which confers the same growth phenotype as 4ECR6, in contrast, has a k_{cat} value for 4-ethylcatechol similar to that of the wild-type enzyme. The k_{cat} value of 3MCS for 3-methylcatechol was much lower than that of the wild-type enzyme, but the 3MCR enzyme, which is a phenotypic revertant of 3MCS, has a k_{cat} for 3-methylcatechol similar to that of the 3MCS enzyme. Therefore, the substrate specificities of these enzymes deduced from growth phenotypes were not explained solely by a change in k_{cat}.

Determination of partition ratios. In the experiments described above, we noticed that some enzymes were rapidly inactivated during the catalysis of some of the substrates. We therefore determined rate constants of enzyme inactivation, k_4, of the wild-type and mutant enzymes for catechol, 3-methylcatechol, and 4-ethylcatechol as described in Materials and Methods. As shown in Table 5, 4-ethylcatechol was a potent inactivator of the wild-type enzyme, of 3MCS, and of 3MCR, as their k_4 values for 4-ethylcatechol were much higher than those for catechol. In contrast, the k_4 values of the 4ECR1 and 4ECR6 enzymes for 4-ethylcatechol were lower than that of the wild-type enzyme for 4-ethylcatechol, indicating that 4ECR1 and 4ECR6 are more resistant to the inactivating effect of 4-ethylcatechol. The k_4 values for 3-methylcatechol of 3MCS were two times higher than those of the wild-type enzyme. Those of 3MCR and of 4ECR1 were between the values of 3MCS and the wild-type enzyme.

The capacity of an enzyme to metabolize a given substrate is influenced by the speeds of catalysis and of enzyme inactivation and is best presented by a partition ratio, k_{cat}/k_4, which is the ratio of a productive catalysis characterized by k_{cat} (or k_3 in the equations in Materials and Methods) to a suicide catalysis characterized by k_4 (24). The partition ratios thus calculated are presented in Table 5. The partition ratio for 3-methylcatechol of 3MCS was low. Those of 3MCR and 4ECR1 were only slightly higher than that of 3MCS, indicating that the 3MCR and 4ECR1 enzymes are partially defective in the metabolism of 3-methylcatechol. The partition ratios for 4-ethylcatechol of the wild-type enzyme, of 3MCS, and of 3MCR were very low, but those of 4ECR1 and 4ECR6 at 50 μM 4-ethylcatechol were higher than the wild-type value.

In general, the partition ratios for those catechols which do not support bacterial growth (4-ethylcatechol for the wild-type enzyme, 3MCS, and 3MCR and 3-methylcatechol for 3MCS) were lower than 18,000. In contrast, those for catechols which support bacterial growth were much higher than 18,000, with a few exceptions. The coefficient of 4ECR1 for 4-ethycatechol was only 17,000, and *P. putida* containing 4ECR1 formed colonies on 4-ethylbenzoate only 4 days after inoculation, whereas *P. putida* containing 4ECR6 formed colonies on the same compound 24 h after inoculation. The coefficients of 3MCR and 4ECR1 for 3-methylcatechol were 27,000 and 22,000, respectively. *P. putida* containing these enzymes grew on *m*-toluate but accumulated 3-methylcatechol. Therefore, a partition ratio of around 18,000 may correspond to a lower limit that permits the growth of host cells.

Inactivation and reactivation of catechol 2,3-dioxygenases. To study whether inactivation of catechol 2,3-dioxygenase by catechol analogs requires molecular oxygen, inactivation of the wild-type enzyme by 4-ethylcatechol and that of 3MCS by 3-methylcatechol in the absence of molecular oxygen were investigated. The anaerobic incubation of the enzymes with

TABLE 4. Michaelis-Menten kinetic parameters of catechol 2,3-dioxygenases[a]

Enzyme	k_{cat} (s^{-1})			K_m (μM)		
	Catechol	3-Methylcatechol	4-Ethylcatechol	Catechol	3-Methylcatechol	4-Ethylcatechol
Wild type	933 (100)	476 (51)	30 (3.2)	1.4	1.7	ND[b]
3MCS	497 (100)	80 (16)	29 (5.9)	1.8	ND	ND
3MCR	215 (100)	90 (42)	18 (8.5)	1.1	0.8	ND
4ECR1	182 (100)	51 (28)	20 (11)	1.8	1.9	5.4
4ECR6	161 (100)	57 (35)	122 (76)	1.4	3.2	2.4

[a] Values were determined by using purified enzymes. The (apparent) k_{cat} values of the mutant enzymes thus obtained experimentally, however, did not correspond with their absolute k_{cat} values, since the purified fractions contained significant amounts of inactive enzymes. Therefore, the absolute k_{cat} values of the mutant enzymes for catechol were estimated by using the following equation: absolute k_{cat} value of a mutant enzyme for catechol = (k_{cat} value of the wild-type enzyme for catechol) × (specific activity for catechol of the mutant enzyme in cell extracts)/(specific activity for catechol of the wild-type enzyme in cell extracts). The absolute k_{cat} value of a mutant enzyme for 3-methylcatechol or for 4-ethylcatechol was calculated from the equation (absolute k_{cat} value of the mutant enzyme for catechol) × (apparent k_{cat} value for 3-methylcatechol or for 4-ethylcatechol obtained by using the purified mutant enzyme)/(apparent k_{cat} value for catechol obtained by using the purified mutant enzyme). Both the absolute k_{cat} values of mutant enzymes thus calculated and the relative activities of the mutant enzymes for 3-methylcatechol or for 4-ethylcatechol compared with those for catechol, (apparent k_{cat} value for 3-methylcatechol or 4-ethylcatechol)/(apparent k_{cat} value for catechol) × 100, are presented. Values in parentheses are percentages.
[b] ND, not determined.

TABLE 5. Rate constants for inactivation and partition ratio of catechol 2,3-dioxygenases

Enzyme	Rate constant for the inactivation of catechol 2,3-dioxygenase ($s^{-1} \times 10^{-6}$)			Partition ratio of catechol 2,3-dioxygenases		
	Catechol	3-Methylcatechol	4-Ethylcatechol	Catechol	3-Methylcatechol	4-Ethylcatechol
Wild type	690	2,300	4,600	1,400,000	210,000	6,500
3MCS	1,300	4,400	5,100	382,000	18,000	5,700
3MCR	440	3,400	9,000	490,000	27,000	2,000
4ECR1	710	2,300	1,200	260,000	22,000	17,000
4ECR6	520	460	1,700	310,000	120,000	34,000

each substrate for 20 min did not diminish the enzyme activity. In contrast, aerobic incubation of the wild-type enzyme with 4-ethylcatechol, or that of 3MCS with 3-methylcatechol, resulted in a great diminution of enzyme activity (Table 6). We therefore infer that inactivation of the wild-type enzyme by 4-ethylcatechol and that of 3MCS by 3-methylcatechol are mechanism based.

To investigate the mechanism responsible for 3-methylcatechol- and 4-ethylcatechol-driven inactivation, reactivation by $FeSO_4$ and ascorbic acid of the inactivated enzymes was examined. The wild-type enzyme inactivated by 4-ethylcatechol could be reactivated by ascorbic acid plus $FeSO_4$. Similarly, the 3MCS enzyme inactivated by 3-methylcatechol could be reactivated by ascorbic acid plus $FeSO_4$ (Table 7). These results suggest that the oxidation and/or removal of the iron cofactor is the major mechanism of the inactivation of catechol 2,3-dioxygenase by 4-ethylcatechol and by 3-methylcatechol.

Identification of the amino acid substitutions in the catechol 2,3-dioxygenase mutants. *Xho*I-I fragments of 2.2 kb containing the structural genes for 3MCS, 3MCR, 4ECR1, and 4ECR6 were isolated from pGSH2225, pGSH2225R, pWW0-EB1, and pWW0-EB6, respectively, and subcloned into the *Sal*I site of pBS(+). The *xylE* genes in these subclones were sequenced by the dideoxy-chain termination technique, using oligonucleotides complementary to the wild-type *xylE* sequence as primers. As shown in Table 8, each allele contained a single nucleotide substitution in the wild-type sequence (15) except that of the structural gene for 3MCR, which carried two nucleotide substitutions, one of which was identical to that found in the structural gene for 3MCS. Thus, phenotypic reversion in 3MCR is due to an intracistronic suppressor mutation.

DISCUSSION

Catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 consists of four identical subunits, each of which contains one ferrous ion associated with amino acid residues of the apoprotein (14, 16). This enzyme catalyzes the ring-cleavage of catechol, 3-methylcatechol and 4-methylcatechol by incorporating two atoms of molecular oxygen (16–19). In this study, substrate specificity mutants of catechol 2,3-dioxygenase were isolated and characterized.

All of the substrate-specificity mutant enzymes were less stable than the wild-type enzyme, and the greater part of the purified mutant enzyme preparations was inactive. The inactive 3MCS and 3MCR enzymes still contained the ferrous ion cofactor. Therefore, inactivation of these enzymes during purification was due neither to the oxidation or loss of ferrous ion cofactor nor to the complete denaturation of the proteins. Rather, local structural deformation may be responsible for the inactivation. The 4ECR1 and 4ECR6 enzymes, in contrast, contained reduced amount of ferrous ion, and part of the inactive enzymes could be reactivated by $FeSO_4$ and ascorbic acid. This observation indicated that the instability of these two enzymes was partly due to altered iron-cofactor binding.

A low efficiency in the oxidation of 4-ethylcatechol by the wild-type enzyme and that of 3-methylcatechol by the 3MCS enzyme was due to their low k_{cat} and high k_4 (rate constant for enzyme inactivation) values for respective substrates. These nonfavorable substrates inactivated the enzymes in the presence of molecular oxygen, the second substrate of catechol 2,3-dioxygenase, but did not inactivate them in the absence of molecular oxygen. The oxidation and/or removal of the ferrous ion in the catalytic center was responsible for the inactivation of the wild-type enzyme provoked by 4-ethylcatechol and of the 3MCS enzyme provoked by 3-methylcatechol because the inactivated enzymes could be reactivated by incubating them anaerobically with $FeSO_4$ and ascorbic acid.

In the catalytic reaction of catechol 2,3-dioxygenase, a catechol substrate binds first to the iron cofactor, followed by the binding of molecular oxygen to the iron cofactor (10). The iron-bound molecular oxygen may subsequently attack carbon 2 of the substrate. We assume that attack by the iron-bound molecular oxygen on carbon 2 of 4-ethylcatechol within the wild-type enzyme or the attack on carbon 2 of 3-methylcatechol within 3MCS is inefficient, since the k_{cat} values of these reactions are low. The consequence of this would be an increased probability of oxidation of the iron cofactor in the ternary enzyme-oxygen-substrate complex. Two different substitutions, Leu-226→Ser in 4ECR1 and Thr-253→Ile in 4ECR6, diminished the frequency of the suicide reaction and increased the catalytic reaction with 4-ethylcatechol. In a previous study, we showed that the amino acid substitution Ile-291→Val also diminished the frequency of the suicide reaction with 4-ethylcatechol (25). These substitutions may modify the structure of the substrate binding site so that the mutant enzymes better accept 4-ethylcatechol in their substrate-binding pockets. Interestingly, 4ECR1 and 4ECR6 exhibited reduced binding of the ferrous ion cofactor. Modification of the substrate binding site in 4ECR1 and 4ECR6 may also have modified the structure of the ferrous ion binding site.

TABLE 6. Inactivation of catechol 2,3-dioxygenases by 3-methylcatechol or 4-ethylcatechol

Enzyme substrate	Enzyme activity (nmole of catechol oxidized/min/mg of protein)			
	Initial	Aerobic		Anaerobic, +substrate
		+Substrate	No substrate	
Wild type 4-Ethylcatechol	68,000	0	44,000	41,000
3MCS 3-Methylcatechol	5,300	55	5,000	5,900

TABLE 7. Inactivation of catechol 2,3-dioxygenases by 3-methylcatechol or 4-ethylcatechol and subsequent reactivation with $FeSO_4$ and ascorbic acid

Enzyme substrate	Enzyme activity (μmol of catechol oxidized/min/mg of protein)				
	Initial	Incubation		Fe(II)+ ascorbic acid	
		Without substrate	With substrate	After incubation without substrate	After incubation with substrate
Wild type 4-Ethylcatechol	189	179	0	57	49
3MCS 3-Methylcatechol	110	51	7	42	37

[a] Purified only by anion-exchange chromatography.

Alternatively, modification of electrostatic environment in these mutant proteins might result in a decrease in the redox potential of the iron cofactor, and as a consequence, the cofactor may have an increased tendency to autooxidize and dissociate from the apoprotein. The change Ala-177→Val, made the enzyme sensitive to 3-methylcatechol, but the effect of this mutation was suppressed by a second mutation, Thr-196→Ile. The mutation in 4ECR1, Leu-226→Ser, also modified the catalytic activity toward 3-methylcatechol. Therefore, these residues seem to be involved in determination of the structure recognizing the methyl group at carbon 3 of catechol substrates.

Substrates with partition ratios lower than 17,000 did not support growth. Bacteria producing 4ECR1, which has a partition ratio of 17,000 for 4-ethycatechol, grew very slowly on 4-ethylbenzoate. Bacteria producing 3MCS, having a partition ratio of 18,000 for 3-methylcatechol, did not grow on *m*-toluate, while those producing 3MCR and 4ECR1, having the partition ratios for 3-methylcatechol of 27,000 and 22,000, respectively, accumulated 3-methylcatechol during growth on *m*-toluate. The partition ratio of 3MCS for 3-methylcatechol (18,000) was higher than that of 4ECR1 for 4-ethylcatechol (17,000), but *P. putida* containing 4ECR1 grew on 4-ethylbenzoate, and the bacteria containing 3MCS failed to grow on *m*-toluate. Although the calculated partition ratios may not be very accurate because of experimental errors in the determination of the k_{cat} values of the mutant enzymes (Table 4), the results indicated that a partition ratio of around 18,000 for catechol analogs may be a threshold allowing the growth of bacteria on these substrates, and a slight increase or decrease from the threshold value suffices to change the growth phenotype of hosts from negative to positive or vice versa.

In a previous study (20), we showed that catechol 2,3-dioxygenase inactivated in vivo is reactivated by a mechanism mediated by the product of the *xylT* gene. In a mutant defective in *xylT* hence defective in the reactivation of catechol 2,3-dioxygenase, the step catalyzed by catechol 2,3-dioxygenase becomes a limiting factor for the growth of cells on 4-methylbenzoate (20). From the data obtained for *P. putida* defective in *xylT*, the threshold value of the partition ratio required for cell growth was calculated to be about 200×10^3. In the present study, we determined the partition ratio value critical for cell growth to be 18×10^3 by examining the growth of *P. putida* containing the $xylT^+$ gene in which inactivated catechol 2,3-dioxygenase is reactivated. The fact that $xylT^+$ *P. putida* cells grew with the partition ratio much lower than 200×10^3 indicated that the *xylT*-mediated regeneration system of catechol 2,3-dioxygenase is very efficient. In the *xylT* mutant, the cell does not grow if the rate of the synthesis of catechol 2,3-dioxygenase is smaller than the rate of its inactivation, while in the $xylT^+$ strain, the cell does not grow only if the rate of the synthesis plus regeneration of catechol 2,3-dioxygenase is smaller than the rate of its inactivation.

TABLE 8. Mutation sites

Mutant	Nucleotide substitution	Amino acid substitution
3MCS	C-530[a]→T	Ala-177→Val
3MCR	C-530→T	Ala-177→Val
	C-587→T	Thr-196→Ile
4ECR1	T-674→C	Leu-226→Ser
4ECR6	C-758→T	Thr-253→Ile

[a] The first nucleotide of the ATG initiation codon of *xylE* corresponds nucleotide 1.

It is generally accepted that enzymes are biological catalysts which exhibit high specificity toward their substrates. If this is the case, the natural adaptation of a catabolic pathway to a new substrate may be a difficult process, since several simultaneous mutations are required for the total conversion of the substrate specificity of each catabolic enzyme. This view should be changed in light of the present observations. Many bacterial catabolic enzymes show relaxed substrate specificity catalyzing conversion of not only genuine substrates but also their structural analogs which are not growth substrates or intermediates. We showed that a slight improvement of the activity of a bottleneck enzyme can switch a nonpermissive compound to a growth substrate. Once an enzyme acquires a minimum activity required for the growth of host cells on a specific compound, environmental selective pressure works on the evolution of the enzyme, and the speed of fixation of additional genetic changes to improve the enzyme will be accelerated. Thus, the potential of the emergence of new catabolic properties is high if the substrate specificity of enzyme is broad and the threshold activity of the enzymes required for growth is low.

ACKNOWLEDGMENT

This work was supported by a grant from Swiss National Science Foundation.

REFERENCES

1. **Abril, M.-A., C. Michan, K. N. Timmis, and J. R. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171**:6782–6790.
2. **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.
3. **Clarke, P. H.** 1984. The evolution of degradative pathways, p. 11–27. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
4. **Hall, B. G.** 1990. Directed evolution of a bacterial operon. Bioessays **12**:551–558.
5. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
6. **Harayama, S., M. Kok, and E. L. Neidle.** 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. **46**:565–601.
7. **Harayama, S., R. A. Leppik, M. Rekik, N. Mermod, P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1986. Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzyl alcohol by the *xylA* product. J. Bacteriol. **167**:455–461.

8. **Harayama, S., and K. N. Timmis.** 1992. Aerobic biodegradation of aromatic hydrocarbons by bacteria, p. 99–155. *In* H. Shigel and A. Shigel (ed.), Metal ions in biological systems, vol. 28. Marcel Dekker, Inc., New York.
9. **Mermod, N., P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1984. Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of coordinately and positively regulated overlapping promoters. EMBO J. **3:**2461–2466.
10. **Mabrouk, P. A., A. M. Orville, J. D. Lipscomb, and E. L. Solomon.** 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.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Massey, V.** 1958. Studies on succinate dehydrogenase: valency state of the iron in beef heart succinic dehydrogenase. J. Biol. Chem. **229:**763–770.
13. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Nakai, C., K. Hori, H. Kagamiyama, T. Nakazawa, and M. Nozaki.** 1983. Purification, subunit structure, and partial amino acid sequence of metapyrocatechase. J. Biol. Chem. **258:**2916–2922.
15. **Nakai, C., H. Kagamiyama, M. Nozaki, T. Nakazawa, S. Inouye, Y. Ebina, and A. Nakazawa.** 1983. Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2. J. Biol. Chem. **258:**2923–2928.
16. **Nozaki, M.** 1979. Oxygenases and dioxygenases. Top. Curr. Chem. **78:**145–186.
17. **Nozaki, M., Y. Kojima, T. Nakazawa, H. Fujisawa, K. Ono, S. Kotani, O. Hayaishi, and T. Yamano.** 1966. Studies on the reaction mechanism of dioxygenases, p. 347–367. *In* K. Bloch and O. Hayaishi (ed.), Biological and chemical aspects of oxygenases. Maruzen Company, Tokyo.
18. **Nozaki, M., S. Kotani, K. Ono, and S. Seno.** 1970. Metapyrocatechase. 3. Substrate specificity and mode of ring fission. Biochim. Biophys. Acta **220:**213–223.
19. **Nozaki, M., K. Ono, T. Nakazawa, S. Kotani, and O. Hayaishi.** 1968. Metapyrocatechase. II. The role of iron and sulfydryl groups. J. Biol. Chem. **243:**2682–2690.
20. **Polissi, A., and S. Harayama.** 1993. *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. EMBO J. **12:**3339–3347.
21. **Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis.** 1986. Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA **83:**8467–8471.
22. **Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis.** 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235:**593–596.
23. **Segel, I. H.** 1993. Enzyme kinetics. John Wiley & Sons, Inc., New York.
24. **Tipton, K. F.** 1992. Principles of enzyme assay and kinetic studies, p. 1–24. *In* R. Eisenthal and M. J. Danson (ed.), Enzyme assays. A practical approach. IRL Press, New York.
25. **Wasserfallen, A., M. Rekik, and S. Harayama.** 1991. A *Pseudomonas putida* strain able to degrade *m*-toluate in the presence of 3-chlorocatechol. Bio/Technology **9:**296–298.
26. **Williams, P. A., S. J. Assinder, and L. E. Shaw.** 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.
27. **Worsey, M. J., F. C. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWW0) from *Pseudomonas putida* mt-2. J. Bacteriol. **134:**757–764.