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## Redesigning Metabolic Routes: Manipulation of TOL Plasmid Pathway for Catabolism of Alkylbenzoates

JUAN L. RAMOS, ALAIN WASSERFALLEN, KEITH ROSE, KENNETH N. TIMMIS

Increasing quantities of man-made organic chemicals are released each year into the biosphere. Some of these compounds are both toxic and relatively resistant to physical, chemical, or biological degradation, and they thus constitute an environmental burden of considerable magnitude. Genetic manipulation of microbial catabolic pathways offers a powerful means by which to accelerate evolution of biodegradative routes through which such compounds might be eliminated from the environment. In the experiments described here, a catabolic pathway for alkylbenzoates specified by the TOL plasmid of *Pseudomonas* was restructured to produce a pathway capable of processing a new substrate, 4-ethylbenzoate. Analysis of critical steps in the TOL pathway that prevent metabolism of 4-ethylbenzoate revealed that this compound fails to induce synthesis of the catabolic enzymes and that one of its metabolic intermediates inactivates catechol 2,3-dioxygenase (C23O), the enzyme that cleaves the aromatic ring. Consequently, the pathway was sequentially modified by recruitment of genes from mutant bacteria selected for their production of either an altered pathway operon regulator that is activated by 4-ethylbenzoate or an altered C23O that is less sensitive to metabolite inactivation. The redesigned pathway was stably expressed and enabled host bacteria to degrade 4-ethylbenzoate in addition to the normal substrates of the TOL pathway.

MOST ORGANIC COMPOUNDS ARE ultimately degraded by soil microorganisms. Bacteria, for example, rapidly mineralize many substances and play important roles in the carbon cycle and in maintaining ecosystems in balance. In recent years, large quantities of synthetic organic chemicals have been released into the biosphere. Although many of these are rapidly biodegraded, others are catabolized only slowly or incompletely, if at all. Toxic members of this latter group constitute an important source of environmental pollution. Although soil and water microorganisms have considerable evolutionary potential and, under appropriate selective conditions, evolve pathways able to degrade many synthetic compounds, such conditions do not always arise in nature and the evolution of effective pathways for certain compounds may thus be relatively slow.

Genetic engineering offers considerable promise as an approach to accelerate the evolution of desired metabolic pathways (1). Two general strategies can be envisaged for the experimental evolution of new catabolic activities, namely, the restructuring of an existing pathway and the assembly of a new

route through the functional combination of appropriate sections of different pathways. Restructuring an existing pathway by substituting enzymes or other proteins through recruitment of new genes has been successfully used to evolve derivatives of *Pseudomonas* sp. B13 that can degrade an increased range of chlorinated aromatics (2–4). These earlier experiments involved recruitment of single enzymes exhibiting broad substrate specificities. We now describe the restructuring of a catabolic pathway for alkylbenzoates specified by the TOL plasmid pWWO of *Pseudomonas putida* (Fig. 1), through directed evolution of existing key pathway elements, to create a pathway able to process 4-ethylbenzoate (4EB). This process involved identifying the barriers that prevent degradation of 4EB through the TOL pathway, selecting mutants exhibiting broader substrate-effector specificities in the key barrier steps, and recruiting the mutant genes to produce derivatives of *P. putida* able to degrade 4EB and utilize it for growth.

Flasks containing M9 minimal medium (4) plus an alkylbenzoate as sole source of carbon and energy were inoculated with soil

samples from the Geneva area and incubated at 30°C. Although organisms were readily isolated that could grow on 3-methylbenzoate (3MB), 4-methylbenzoate (4MB), and 3,4-dimethylbenzoate, none was isolated that could grow on 4EB. The resistance of 4EB to degradation could in principle be due to (i) its toxicity, (ii) its failure to be taken up by the bacteria, (iii) its failure to act as an effector of regulators of transcription of catabolic operons, and (iv) its failure to be degraded by the pathway enzymes. Since 3-ethylbenzoate is catabolized by *P. putida* carrying TOL plasmid pWWO and related bacteria (5–7), the first two possibilities were unlikely. That they did not account for the resistance to degradation was confirmed by examining the metabolism of 4EB by *P. putida* carrying pNM72, a TOL plasmid derivative in which the benzoate *meta*-cleavage pathway (Fig. 1) is expressed constitutively at high levels (8). Although *P. putida* (pNM72) did not grow on 4EB as sole carbon source, when this bacterium was provided with glucose and 4EB it grew at the expense of the sugar and accumulated 4-ethylcatechol (4EC) (identified in culture supernatants by mass spectrometry after being purified by high-pressure liquid chromatography). This result suggests that 4EB is not particularly toxic, that it enters the bacteria, and that it is metabolized by the initial enzymes of the benzoate pathway.

The reason for the block in 4EB degradation at the level of 4EC is that 4EC (or its metabolite) inactivates the subsequent enzyme of the pathway, catechol 2,3-dioxygenase (C23O), the key enzyme for cleavage of the aromatic ring (Fig. 2). Inactivation was not reversed by addition of 100  $\mu$ M catechol (Fig. 2) or by dialysis of the inactivated enzyme.

Plasmid pNM185 (Table 1) is an expression vector with broad host range that contains the promoter ( $P_m$ ) of the TOL plasmid *meta*-cleavage pathway operon and *xylS*, the gene for the positive regulator of this promoter (9). Plasmid pNM189 (Table 1), a derivative of pNM185 containing *lacZ* downstream of  $P_m$  (9), has been used to quantify the ability of potential substrates of the TOL *meta*-cleavage pathway to activate the *xylS* protein and thereby synthesize the catabolic enzymes (4). Although 3MB and 4MB activated the *xylS* protein, 4EB did not (Table 2). Thus, there are at least two obstacles to the degradation of 4EB by the TOL plasmid *meta*-cleavage pathway. To redesign the TOL pathway so that 4EB could be processed through it, it was neces-

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

sary at least to broaden the effector specificity of the *xylS* protein and to generate a C23O mutant enzyme resistant to inactivation by 4EC.

To isolate regulatory mutants we constructed plasmid pJLR200, a derivative of pBR322 in which the promoter of the determinant for tetracycline resistance had been removed and replaced with the  $P_m$  promoter of the TOL *meta*-cleavage operon (4). *Escherichia coli* 5K cells concomitantly carrying pJLR200 and pNM185 (which carries *xylS*) are sensitive to tetracycline in the absence of a *xylS* protein effector, but resistant in the presence of an effector. The plating of such bacteria on medium containing a noneffector benzoate analog and the mutagen ethylmethane sulfonate (1 to 4 mg/ml) resulted in the selection of tetracycline-resistant mutants with altered *xylS*- $P_m$  properties (4). One such mutant was isolated on plates containing 4EB; the mutation was designated *xylS4E*, and the mutant pNM185-derivative carrying this mutant *xylS* allele was designated pERD4E. To analyze the inducibility of  $P_m$  by 4EB in *P. putida* containing the *xylS4E* allele, we introduced a 7.7-kb Eco RI fragment bearing promoterless *lacZ* and *lacY* genes isolated from pNM182 (9) into the unique Eco RI site of pERD4E located just downstream of the  $P_m$  promoter. This plasmid was designated pJLR111. Table 2 shows that 4EB induces synthesis of  $\beta$ -galactosidase in such bacteria. The *xylS4E* allele does not alter the ability of such bacteria to respond to 3MB and 4MB (10). Transfer of pERD4E into *P. putida*

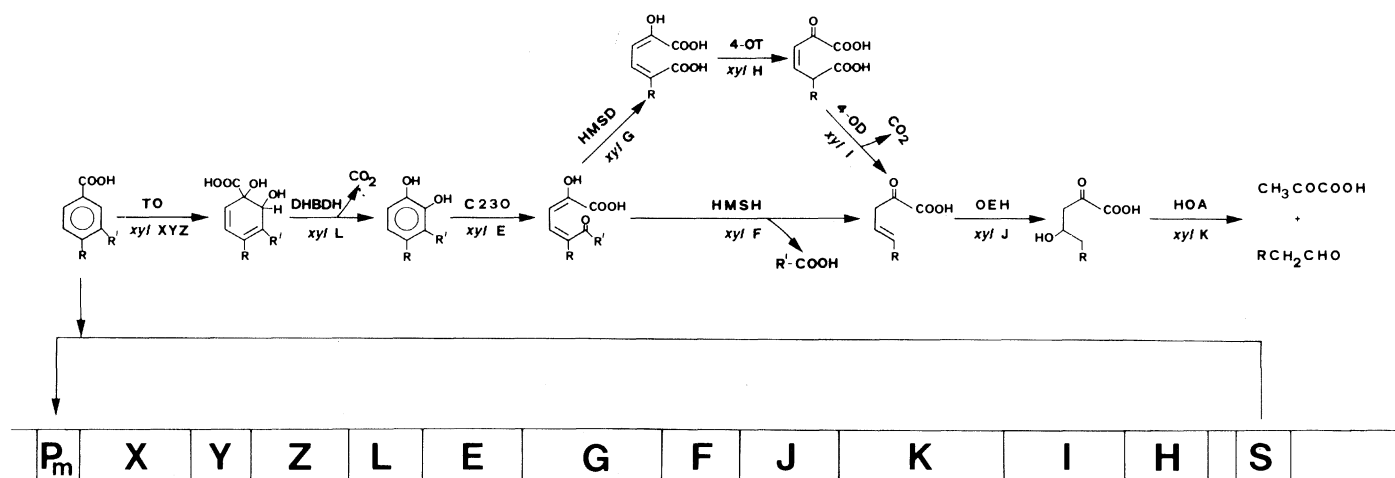
**Table 1.** Plasmids used in this study. Abbreviations: Km<sup>r</sup> and Ap<sup>r</sup>, resistance to kanamycin and ampicillin, respectively; 4EC, 4-ethylcatechol; C23O, catechol 2,3-dioxygenase;  $P_m$ , promoter of the *meta*-cleavage operon.

| Plasmid  | Pertinent characteristics   | Reference |
|----------|---|-----------|
| pWWO     | Archetypal TOL plasmid; specifies <i>meta</i> -cleavage pathway for benzoates                                 | (5)       |
| pWWO-EB1 | Mutant pWWO derivative; specifies a C23O resistant to inactivation by 4EC                                     | This work |
| pWWO-EB6 | Mutant pWWO derivative; specifies a C23O resistant to inactivation by 4EC                                     | This work |
| pNM72    | pKT231-pWWO hybrid plasmid carrying a constitutively expressed <i>meta</i> -cleavage pathway, Sm <sup>r</sup> | (8)       |
| pNM185   | <i>xylS</i> , $P_m$ , Km <sup>r</sup> , IncP4   | (9)       |
| pNM189   | pNM185 derivative, <i>xylS</i> , $P_m$ :: <i>lacZ</i> , Km <sup>r</sup>                                       | (9)       |
| pERD4E   | Mutant pNM185, <i>xylS4E</i>  | This work |
| pJLR111  | pERD4E derivative, <i>xylS4E</i> , $P_m$ :: <i>lacZ</i> , Km <sup>r</sup>                                     | This work |
| pJLR200  | $P_m$ :: <i>tet</i> , Ap <sup>r</sup> , pBR322 derivative   | (4)       |

(pWWO) enabled the bacteria to transform 4EB to 4EC, which accumulated in the medium.

If C23O inactivation represents the sole limiting step for degradation of 4EB by *P. putida* (pWWO; pERD4E), it might be possible to isolate mutants that produce an altered C23O resistant to inactivation and that are able to grow on 4EB. We therefore spread  $10^8$  to  $10^9$  cells of *P. putida* (pWWO; pERD4E) on plates containing M9 minimal medium with 5 mM 4EB as sole source of carbon, kanamycin (25  $\mu$ g/ml), and ethylmethane sulfonate (1 mg/ml). Six clones that grew on these plates were able, after purification, to grow in liquid minimal medium containing 4EB as the sole source of carbon. To confirm that the responsible mutations were located on the TOL plasmids (designated pWWO-EB) of these

clones, we transferred the TOL plasmids from *P. putida* KT2440, which is sensitive to antibiotic, to *P. putida* PaW340, which is resistant to nalidixic acid (Nal<sup>r</sup>) and streptomycin (Sm<sup>r</sup>), and first selected Nal<sup>r</sup>Sm<sup>r</sup> transconjugants able to grow on 4MB. Among 300 transconjugant clones tested (50 from each mating), none grew on 4EB. Since pWWO mobilizes pERD4E at low frequencies (about  $10^{-6}$ ), we repeated the transfer but selected for Nal<sup>r</sup>Sm<sup>r</sup>Km<sup>r</sup> transconjugants able to grow on 4MB, which should therefore have received both pWWO-EB and pERD4E. All 56 clones tested grew on 4EB. We therefore transformed pERD4E (Km<sup>r</sup>) into six transconjugants obtained in the first mating (one from each) and selected Km<sup>r</sup> clones on plates containing either 4MB or 4EB as the sole carbon source. The frequencies of transfor-



**Fig. 1.** TOL plasmid pWWO *meta*-cleavage pathway, its genetics and regulation. Gene order (XYZLEGFJKIHS) is as determined by Harayama *et al.* (13). Degradation of benzoate (R, R' = H), 3-methylbenzoate (3MB; R = H, R' = CH<sub>3</sub>), 3-ethylbenzoate (3EB; R = H, R' = CH<sub>2</sub>CH<sub>3</sub>), and 4-methylbenzoate (4MB; R = CH<sub>3</sub>, R' = H) proceeds to the corresponding catechols through the catalytic action of the multicomponent enzyme toluate dioxygenase (TO) and dihydroxycyclohexadiene carboxylate dehydrogenase (DHBDH). The rings of corresponding catechol derivatives are cleaved by catechol 2,3-dioxygenase (C23O). The semialdehydes formed from 3MB and 3EB are metabolized through the hydrolytic branch [hydroxymuconic semialdehyde hydrolase (HMSH)] (13, 14), whereas

those derived from benzoate and 4MB are metabolized through the oxalocrotonate branch [hydroxymuconic semialdehyde dehydrogenase (HMSD), 4-oxalocrotonate tautomerase (4-OT), and 4-oxalocrotonate decarboxylase (4-OD)]. The pathway branches reconverge at 2-oxopent-4-enoate or its derivatives, which are further metabolized by 2-oxopent-4-enoate hydratase (OEH) and 2-oxo-4-hydroxypent-2-enoate aldolase (HOA). Pathway enzyme genes (*xyl*) form an operon whose transcription originates at the  $P_m$  promoter and is regulated by the *xylS* gene product, a positive regulator of  $P_m$  that is activated by most initial pathway substrates, such as benzoate, 3MB, and 4MB.

**Table 2.**  $\beta$ -Galactosidase levels from Pm::lacZ gene fusions in *P. putida* bearing *xylS* and *xylS4E* genes. *Pseudomonas putida* KT2440 (pNM189) or *P. putida* KT2440 (pJLR111) were grown overnight at 30°C in LB medium with kanamycin (25  $\mu$ g/ml) and then diluted 100-fold in the same medium containing 1 mM of the indicated effector. After 5 hours of growth at 30°C,  $\beta$ -galactosidase activities were determined (4).  $\beta$ -Galactosidase units are as defined by Miller (16).

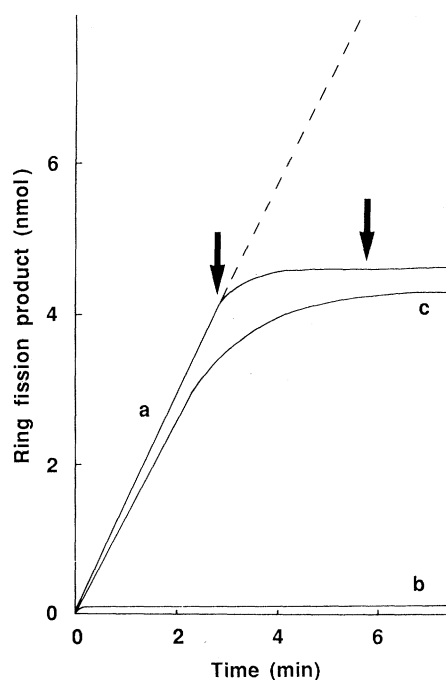
| Effector | pNM189 | pJLR111 |
|----------|--------|---------|
| None     | 192    | 178     |
| 3MB      | 1098   | 1142    |
| 4MB      | 820    | 781     |
| 4EB      | 175    | 740     |

mation were similar on the two types of plate. These results confirmed that the mutations located in both plasmids are required for the 4EB growth phenotype. The constructed *P. putida* (pWWO-EB; pERD4E) strains stably maintained the 4EB degradative phenotype: after cultivation for at least 40 generations under nonselective conditions (LB medium), greater than 95% of progeny clones could grow with 4EB as the sole source of carbon.

On the basis of bacterial growth characteristics with 3MB, 4MB, and 4EB as substrates, two groups of pWWO-EB plasmids could be distinguished. The parent strain *P. putida* (pWWO; pERD4E) had generation times of about 1.5 hours on 4MB, 1.2 hours on 3MB, and did not grow on 4EB. *Pseudomonas putida* (pWWO-EB1; pERD4E) had generation times of 5 hours on 4MB, 12 to 15 hours on 4EB, and did not grow on 3MB. The *P. putida* (pERD4E) containing the other mutant pWWO-EB plasmids (pWWO-EB2 to pWWO-EB6) had generation times of 3 to 3.5 hours on 4MB, 1.5 to 2 hours on 3MB, and 4.5 to 7 hours on 4EB. Analysis by high-pressure liquid chromatography, thin-layer chromatography, and mass spectrometry of pathway intermediates was carried out on cell-free culture supernatants. No significant amounts of 4-methylcatechol (4MC) (<0.05 mM) were found in culture supernatants of *P. putida* (pERD4E; pWWO, pWWO-EB1, or pWWO-EB6) growing on 4MB, nor of 3-methylcatechol (3MC) in supernatants of *P. putida* (pERD4E; pWWO or pWWO-EB6) growing on 3MB. Supernatants of *P. putida* (pERD4E; pWWO-EB1) growing on glucose plus 3MB contained significant concentrations (0.5 mM) of 3MC, and those of *P. putida* (pERD4E; pWWO) bacteria growing on glucose plus 4EB contained 4EC (about 0.4 mM). The *P. putida* (pERD4E; pWWO-EB1 or pWWO-EB6) growing on 4EB accumulated low amounts (<0.04 mM) of 4EC and an unidentified orange compound [ $R_F$  = 0.75 on cellulose

thin-layer chromatography plates; mobile phase *n*-butanol:acetone:acetic acid:water (3.5:3.5:1:2)], whose absorption spectrum showed a peak at 425 nm and shoulders at 375 and 270 nm. Thus, the bacterial phenotypes with regard to intermediate accumulation correlated with growth phenotypes.

These results suggested that plasmids pWWO-EB1 and pWWO-EB6 specify altered C23O enzymes. The Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) values for the C23O enzymes were therefore measured in cell-free extracts of *P. putida* carrying pERD4E plus pWWO, pWWO-EB1, or pWWO-EB6 grown in the presence of 4MB, 3MB, or 4EB. The kinetic parameters determined for the C23O enzymes of cells grown on 4MB are presented in Table 3. With catechol, 3MC, and 4MC as substrates, the  $K_m$  values for C23O enzymes specified by the three plasmids were similar (around 2  $\mu$ M). The C23O enzymes specified by pWWO-EB1 and pWWO-EB6 exhibited somewhat lower  $V_{max}$  values for these three substrates than the enzyme of pWWO. Although rapid inactivation of the enzyme made it impossible to determine  $K_m$  and  $V_{max}$  values for C23O from pWWO with 4EC as substrate (Fig. 2), such values for the enzymes specified by pWWO-EB1 and pWWO-EB6 were readily determined (Table 3) because of the resistance of these enzymes to inactivation by 4EC (see, for example, Fig. 2 for C23O from pWWO-EB6). The  $K_m$  values for these enzymes with 4EC as substrate were slightly higher (5  $\mu$ M) and the  $V_{max}$  values somewhat lower than the values obtained with catechol and 4MC as substrates.



When *P. putida* (pERD4E; pWWO or pWWO-EB6) was grown on 3MB, the kinetic parameters determined for C23O were similar to those determined in cells grown on 4MB. However, levels of C23O were negligible in cell-free extracts of *P. putida* (pERD4E; pWWO-EB1) grown on glucose plus 3MB. This does not reflect a lack of induction of the pathway enzymes, because the accumulation of 3MC from 3MB indicated that the two first enzymes of the pathway (Fig. 1) were synthesized and functional. Rather, it seems to reflect in vivo inactivation of C23O by 3MC. *Pseudomonas putida* (pERD4E; pWWO-EB1) grown on 4MB produces a C23O that in vitro linearly transformed catechol to its corresponding semialdehyde for at least 3 minutes. In contrast, linear transformation with 3MC as substrate was observed for only a few seconds, after which enzyme activity progressively decreased. Upon addition of 3MC (10  $\mu$ M) to enzyme linearly transforming catechol (30  $\mu$ M in the assay), progressive loss of enzyme activity occurred, which was not reversed after addition of 100  $\mu$ M catechol. The  $K_m$  values for the C23O enzymes specified by pWWO-EB1 and pWWO-EB6 isolated from cells grown on 4EB were similar to those determined with enzymes from cells grown on 4MB. Values of  $V_{max}$ , however, were variable (range, 0.2 to 4 U per milligram of protein), probably reflecting different degrees of inactivation of C23O in vivo by the 4EC formed.

The pioneering studies of Clarke and her colleagues demonstrated the evolutionary potential of one enzyme, an amidase of *Pseudomonas*, and the regulator of expression

**Fig. 2.** Time course of ring fission of catechol and 4EC by C23O and mutant C23O. *Pseudomonas putida* KT2440 (pERD4E; pWWO or pWWO-EB6) was grown in M9 minimal medium with 4MB (5 mM) as the sole source of carbon and kanamycin (25  $\mu$ g/ml) until the late exponential phase. Cell-free extracts were then prepared by sonication (9). A 1-ml portion of the assay mixture (15) contained phosphate buffer (pH 7.0, 100 mM), catechol or 4EC (30  $\mu$ M), and 0.22  $\mu$ g of protein of the pWWO extract or 0.78  $\mu$ g of protein of the pWWO-EB6 extract. Curve a shows catechol ring fission by C23O encoded by pWWO; the first arrow indicates the addition of 10  $\mu$ M 4EC, and the dotted line shows a control without this addition. The second arrow indicates the addition of 100  $\mu$ M catechol. Curves b and c are 4EC ring fission by C23O from pWWO and pWWO-EB6, respectively. The  $V_{max}$  value for C23O from pWWO with catechol was 6.7 U per milligram of protein and that for the enzyme from pWWO-EB6 with 4EC was 1.1 U per milligram of protein. Units are micromoles of product formed per minute. Extinction values for a 1M solution and 1-cm path length ( $E_{1cm}^{1M}$ ) of  $3.6 \times 10^4$  at 381 nm and  $3.6 \times 10^4$  at 375 nm were used for the ring cleavage products of 4EC and catechol, respectively (14).

**Table 3.** Kinetic parameters of catechol 2,3-dioxygenases specified by plasmids pWWO, pWWO-EB1, and pWWO-EB6. Growth of *P. putida* KT2440 (pERD4E; pWWO, pWWO-EB1, or pWWO-EB6) and preparation of cell-free extracts were as in the legend of Fig. 2. The assay procedure was also that described in Fig. 2 except that the concentrations of the substrates were in the range of 0.3 to 30  $\mu$ M and the initial reaction rates ( $V_0$ ) were determined in the first 15 seconds, with at least six different substrate concentrations ( $S$ ). The values of  $K_m$  ( $\mu$ M) and  $V_{max}$  (units per milligram of protein) were calculated from the representation of double reciprocal (Lineweaver-Burk) plots ( $1/V_0$  versus  $1/S$ ) that gave straight lines with correlation coefficients greater than 0.982. Values given are means of three independent determinations whose standard deviations were 10 to 30% of the given values.

| Substrate        | Plasmid |           |          |           |          |           |
|------------------|---------|-----------|----------|-----------|----------|-----------|
|                  | pWWO    |           | pWWO-EB1 |           | pWWO-EB6 |           |
|                  | $K_m$   | $V_{max}$ | $K_m$    | $V_{max}$ | $K_m$    | $V_{max}$ |
| Catechol         | 2.1     | 7.6       | 2.4      | 2.9       | 1.6      | 1.3       |
| 3-Methylcatechol | 1.8     | 5.4       | 3.4      | 1.4       | 1.3      | 2.4       |
| 4-Methylcatechol | 1.5     | 5.0       | 1.1      | 1.7       | 1.7      | 1.6       |
| 4-Ethylcatechol  |         |           | 5.1      | 0.5       | 5.0      | 1.2       |

of its gene (11). In this study, we have explored the evolutionary potential of an entire pathway, namely, the TOL plasmid *meta*-cleavage pathway for the catabolism of alkylbenzoates, and have identified key targets for genetic manipulation. The *xylS* gene product and C23O are critical elements in the TOL pathway, not only because the former acts as an inducer for the synthesis of all pathway enzymes and the latter effects the crucial aromatic ring cleavage, but also because their effector-substrate specificities constitute critical bottlenecks that determine the range of substituted benzoates that can be degraded. The narrow effector specificity of *xylS* gene product seems to reside in an inability of its effector binding site to accommodate benzoate derivatives with substitutions at position 4 larger than a methyl group (4). On the other hand, the narrow substrate specificity of C23O seems to result more from its inactivation by the substituted catechol or its ring cleavage product (or a transient intermediate of the reaction) than

from a strict inability to process the catechol derivative (12). Mutational manipulation of the *xylS* gene, to broaden the effector specificity of its product, and of the *xylE* gene, to reduce the susceptibility of C23O to inactivation by specific substrates or reaction products, are therefore important aspects of the experimental evolution of the TOL plasmid pathway. Not unexpectedly, the two C23O analogs generated in this study that are resistant to inactivation by 4EB are slightly less efficient (Table 3) in transforming the usual substrates of the original enzyme. In contrast, the broader effector specificity of the *xylS4E* protein is not accompanied by any loss in efficiency of activation of transcription of the catabolic genes.

One major objective of the genetic manipulation of soil microbes is to accelerate evolutionary events, such as the evolution of catabolic pathways for xenobiotics. Such acceleration can be achieved in different ways, but the use of highly selective conditions (for example, for antibiotic resistance)

unrelated to the phenotype ultimately to be evolved and the simultaneous introduction of multiple genetic changes may be critical for the rapid evolution of a particular property that can be readily attained in the laboratory, but that may occur infrequently in nature. The general strategy described here will be applicable to other pathways and to more complicated evolutionary objectives, particularly to the development of pathways for the catabolism of major environmental pollutants.

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