Assemblage of Ortho Cleavage Route for Simultaneous Degradation of Chloro- and Methylaromatics

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Genetic engineering is a powerful means of accelerating the evolution of new biological activities and has considerable potential for constructing microorganisms that can degrade environmental pollutants. Critical enzymes from five different catabolic pathways of three distinct soil bacteria have been combined in patchwork fashion into a functional ortho cleavage route for the degradation of methylphenols and methylbenzoates. The new bacterium thereby evolved was able to degrade and grow on mixtures of chloro- and methylaromatics that were toxic even for the bacteria that could degrade the individual components of the mixtures. Except for one enzymatic step, the pathway was fully regulated and its component enzymes were only synthesized in response to the presence of pathway substrates.

CHLORINATED AROMATIC COMpounds are major environmental pollutants (*1*). Although some compounds can only be degraded slowly by soil microbes, if at all, others are metabolized relatively rapidly. However, a readily metabolized compound may not be degraded when accompanied by other chemicals that are toxic or that cause misrouting of pathway intermediates from productive into nonproductive pathways. For example, methylaromatics, such as toluene, xylenes, or cresols, are often present in mixtures along with chloroaromatics, such as chlorobenzenes or chlorophenols. Aromatics in aerobic environments are generally transformed to dihydroxy derivatives, such as catechols, which serve as substrates for oxygenolytic cleavage of the aromatic ring (*2*). Chlorocatechols are metabolized by ortho cleavage pathways, whereas methylcatechols generally follow a meta cleavage route (Fig. 1). Ortho cleavage pathways can partially metabolize methylaromatics and meta pathways can cometabolize chloroaromatics, although dead-end products or reactive intermediates that constitute suicide substrates for the next enzyme in the pathway (such as 3-chlorocatechol for the meta cleavage enzyme) are generally formed (*3, 4*). Soil bacteria frequently possess both types of pathways, although normally only one type is active at any given time in a particular organism. When such bacteria are confronted with mixtures of chloro- and methylaromatics, however, both pathways are induced and unproductive misrouting of the catechol derivatives ensues (Fig. 1), thereby disrupting bacterial growth. Misrouting of substituted catechols could be circumvented by the experimental evolution of an organism able to degrade both chloro- and methylaromatics exclusively through a single type of aromatic ring cleavage pathway.

One general strategy for laboratory evolution of pathways for the degradation of poorly biodegraded compounds is the rational restructuring of existing pathways; this approach was recently used to construct a bacterium able to degrade 4-ethylbenzoate (*5*). An alternate, more versatile strategy involves the patchwork assembly of new pathways by the judicious combination of enzymes recruited from different pathways and different organisms. We describe the use of this approach to construct an ortho cleavage pathway for the degradation of methylaromatic compounds that consists of five discrete segments, namely the oxidation of methylbenzoates and methylphenols to methylcatechols, the conversion of methylcatechols to methyllactones, the transformation of 4-methyl-2-enelactone to 3-methyl-2-enelactone, and the conversion of 3-methyl-2-enelactone to Krebs cycle intermediates. The presence of this pathway in a *Pseudomonas* species, strain B13 (*Pseudomonas* sp. B13 or B13), enables the engineered bacterium to grow well on mixtures of 3-chlorobenzoate (3CB), 4-chlorobenzoate (4CB), and 4-methylbenzoate (4MB) and of 4-chlorophenol (4CP) and 4-methylphenol (4MP) without nonproductive misrouting of intermediates.

The B13 strain has a well-characterized ortho cleavage route for the degradation of 3CB and 4CP (Fig. 2A) and lacks detectable meta cleavage activity (*6*). The narrow spectrum of chloroaromatics degraded by B13 can be expanded by judicious recruitment of additional enzymes and gene regulators with relaxed substrate-effector specificities. For example, the recruitment of the TOL plasmid-encoded enzymes toluate dioxygenase and dihydroxycyclohexadiene carboxylate dehydrogenase, which transform methylbenzoates to methylcatechols (Fig. 2), permitted B13 to metabolize 4CB, and the recruitment of the NAH plasmid-encoded salicylate hydroxylase, which transforms chlorosalicylates to chlorocatechols, enabled B13 to catabolize all isomers of monochlorosalicylate (*7, 8*). The former hybrid bacterium could transform methylcatechols to 4-carboxymethyl-methylbut-2-ene-1,4-olides (methyl-2-enelactones) (*9*). However, the newly acquired catabolic phenotypes were unstable in all types of hybrid bacteria constructed (*10*). The strategy we adopted to construct a stable B13 derivative able to degrade chloro- and methylaromatics exclusively through ortho cleavage routes (Fig. 2) was as follows: (i) cloning and insertion into the B13 chromosome of genes of the above mentioned TOL enzymes to enable B13 to degrade 3CB and 4CB and to transform methylbenzoates first to methylcatechols and then to methyl-2-enelactones; (ii) recruitment of enzymes that transform methyl-2-enelactones to Krebs cycle intermediates, thus completing an ortho pathway for the degradation of methylbenzoates; and (iii) recruitment of a broad substrate specificity phenol hydroxylase, able to transform chloro- and methylphenols (cresols) to catechols, to permit degradation of substituted phenols via the constructed ortho route.

Toluate dioxygenase and dihydroxycyclohexadiene carboxylate dehydrogenase are encoded by the cistrons *xylXYZ* and *xylL*, respectively, of TOL plasmid pWW0 (*11*), which specifies a meta cleavage pathway for

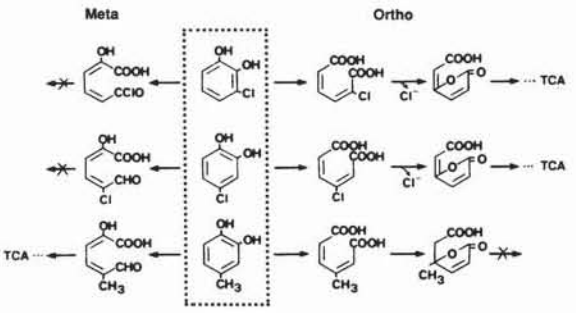

Fig. 1. Unproductive misrouting of substituted catechols by ortho and meta cleavage enzymes.

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the degradation of benzoate and alkylbenzoates (*12*). These four cistrons are the first in an operon whose promoter Pm is positively controlled by the XylS regulatory protein, which is itself activated by pathway substrates (*13–15*). A B13 derivative that stably expressed the TOL plasmid enzymes was constructed by inserting the genes for these enzymes and their promoter Pm and the *xylS* gene into transposon Tn*5*; the hybrid transposon was then transposed into the chromosome of B13 (*16–19*). One B13 derivative that carried the hybrid transposon was selected for further study and designated FR1 (Fig. 2B). This derivative not only completely degraded 3CB and 4CB, but also transformed 3- and 4-methylbenzoate (3MB and 4MB, respectively) to 4-carboxymethyl-2-methylbut-2-ene-1,4-olide (2-methyl-2-enelactone) and 4-carboxymethyl-4-methylbut-2-ene-1,4-olide (4-methyl-2-enelactone), respectively, and like B13 grew on 4-carboxymethyl-3-methylbut-2-ene-1,4-olide (3-methyl-2-enelactone) but not on the 2- and 4-methyl isomers as a sole source of carbon and energy. Moreover, it stably maintained the 4CB catabolic phenotype, since no loss of this activity was observed after 50 generations of growth on complete medium in the absence of selective pressure.

An ortho pathway for the degradation of 4-methylcatechol has been characterized in *Alcaligenes eutrophus* strain JMP134, in which 4-methyl-2-enelactone is formed and subsequently isomerized to 3-methyl-2-enelactone, which is in turn transformed to 4-methyl-3-oxoadipic acid and then to Krebs cycle intermediates (*20*). Since B13 can metabolize 3-methyl-2-enelactone, the degradation of 4MB by B13 derivative FR1 in principle requires recruitment only of the isomerase that converts 4-methyl-2-enelactone to 3-methyl-2-enelactone. Sau-3AI–cleaved total DNA of *A. eutrophus* JMP134 was cloned in the cosmid vector pLAFR3 to produce a gene bank in *Escherichia coli* HB101 (*21*). The bank of hybrid plasmids was mass transferred by conjugation into FR1, and transconjugant clones were selected that were resistant to tetracycline (the selection marker of the vector) and able to grow solely on 4MB. A number of such clones were isolated and the hybrid cosmid present in one of them, designated pFRC20P (Fig. 2B), was studied further. It contained a 26-kb DNA fragment from the *A. eutrophus* chromosome and, when retransferred into cosmid-free FR1 bacteria, again conferred the ability to grow on 4MB. Deletion and subcloning analysis of the inserted *A. eutrophus* DNA, with the vector pRK2501 (*19*), localized the region that encoded the 4-methyl-2-enelactone isomerase to a segment 3 kb in length. A pRK2501 hybrid with this segment was designated pFRC32P. Cell-free extracts of strains FR1(pFRC20P) and FR1(pFRC32P) exhibited enzymatic activities that were lacking from FR1 and that converted purified 4-methyl-2-enelactone to 3-methyl-2-enelactone (Table 1). High levels of activity were measured both in 4MB-grown cells and in acetate-grown cells of strain FR1-(pFRC20P), although the highest levels were obtained in 4MB-grown cells. This indicates that expression of the isomerase is specifically regulated in this strain, but that its basal level of synthesis is relatively high.

3-Methylbenzoate and 3-methylphenol (3MP) are not substrates for FR1 (pFRC20P), since they are mainly cometabolized to 2-methyl-2-enelactone (*22*), which is

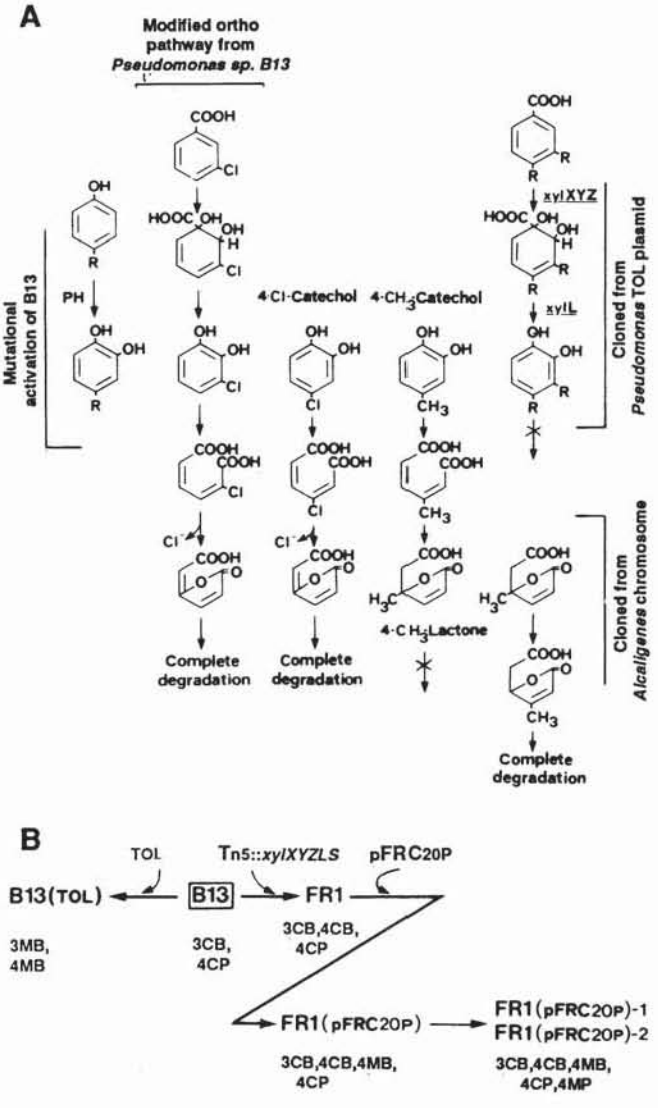

Fig. 2. Constructed hybrid pathway for the simultaneous degradation of chloro- and methylaromatics. (**A**) The pathway. The route is based on the modified ortho pathway for 3CB of *Pseudomonas* sp. B13. Introduction into B13 of the TOL plasmid genes that code for toluate 1,2-dioxygenase (*xylXYZ*) and dihydroxycyclohexadiene carboxylate dehydrogenase (*xylL*), together with that of the positive regulator of the *xylXYZL* operon (*xylS*), expands the degradation range to include 4CB and permits transformation of 4MB to 4-methyl-2-enelactone, which would accumulate as a dead-end metabolite. Recruitment of a 4-methyl-2-enelactone isomerase from *Alcaligenes eutrophus* allows transformation of 4-methyl-2-enelactone to 3-methyl-2-enelactone (bottom right), which is degraded by other enzymes of B13. Mutational activation of a phenol hydroxylase of B13 further extends its degradation capacities to chloro- and methylphenols. R = CH_3, Cl, or H. (**B**) The genetic steps for the formation of FR1(pFRC20P)-1 and -2.

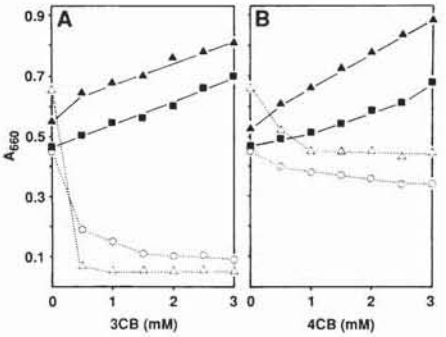

Fig. 3. Effect of chlorobenzoates on the growth of bacteria that degrade methylbenzoates. Bacteria were grown in minimal medium containing 4MB (5 m*M*) as sole source of carbon and energy. When the cultures reached an absorbance of A_{660} = 0.5, they were diluted tenfold with fresh medium containing both 4MB and either (**A**) 3CB or (**B**) 4CB at the indicated concentrations; the incubation was continued 20 hours. The final A_{660} was measured and plotted against the concentration of 3CB and 4CB initially present in the fresh medium. The strains used were: *Pseudomonas putida* KT2440(TOL) (○; possesses TOL plasmid meta pathway for degradation of methylbenzoates); *Pseudomonas* sp. strain B13(TOL) (△; possesses TOL plasmid meta pathway and an ortho pathway for 3CB); and B13 derivatives FR1(pFRC20P) and FR1(pFRC32P) (▲ and ■, respectively; lack meta pathways but possess ortho pathways for degradation of chloro- and methylbenzoates).

not further metabolized by B13 enzymes nor by the 4-methyl-2-enelactone isomerase cloned from *A. eutrophus*. However, derivatives of *A. eutrophus* have been isolated that grow on 2-methyl-2-enelactone (*10*). Recruitment into FR1(pFRC20P) of enzymes that transform 2-methyl-2-enelactone to Krebs cycle intermediates should enable metabolism of 3-methylsubstituted phenol and benzoate via the ortho cleavage pathway.

Pseudomonas sp. B13 can grow on phenol and, after adaptation, 4CP as sole sources of carbon and energy; catabolism is via an ortho cleavage route with catechol or 4-chlorocatechol as intermediates (*22*). In cell-free extracts, the B13 phenol hydroxylase also transformed 3MPs and 4MPs to the corresponding methylcatechols (*22*). Thus, derivative FR1(pFRC20P), which can mineralize 4-methylcatechol, should possess all of the enzymes necessary to grow on 4MP as sole source of carbon and energy. However, although phenol is a growth substrate for FR1(pFRC20P), 4MP is not. Spontaneous mutants of FR1(pFRC20P), such as FR1(pFRC20P)-1 and -2, could nevertheless be selected (frequency 10^{-7} to 10^{-8}) that grew on 4MP. Since synthesis of the phenol hydroxylase of B13 is inducible by phenol, the inability of FR1(pFRC20P) to grow on 4MP may be due to a lack of induction of this enzyme by 4MP; thus mutants that could grow with 4MP were presumed either to be constitutive for synthesis of this enzyme or to exhibit altered regulation of synthesis of the enzyme that resulted in production of the enzyme in response to 4MP. Measurement of phenol hydroxylase levels in FR1(pFRC20P) bacteria and mutant bacteria grown either in succinate, phenol, or 4MP revealed little or no enzyme in 4MP or succinate-grown cells and high activities in both parental and mutant bacteria grown on phenol and in mutant bacteria grown on 4MP. Enzyme synthesis is thus specifically regulated.

The addition of a chloroaromatic such as 3CB to bacteria such as *Pseudomonas putida* strain KT2440(TOL) or *Pseudomonas* sp. B13(TOL), which actively degrade a methylbenzoate through a meta ring-fission pathway, resulted in inhibition of cell growth as a consequence of misrouting of 3CB into the meta pathway and irreversible inactivation of the key enzyme of the pathway, catechol-2,3-dioxygenase (*3*), by the ring fission product of 3-chlorocatechol, a highly reactive acyl halide (Fig. 3). Addition of 4CB to such cells had a less severe impact on growth because the dead-end product that forms as a result of misrouting of 4CB into the meta pathway is less reactive (Figs. 1 and 3). Addition of 3CB or 4CB to the constructed B13 derivatives FR1(pFRC20P) and FR1(pFRC32P) growing on 4MB had no inhibitory effect, and in fact promoted further growth of the cultures (Fig. 3). The B13 derivatives simultaneously degraded the chloro- and methylbenzoates in the mixtures of 4MB and 4CB and of 4MB and 3CB (Table 2).

The newly evolved catabolic pathway was stably expressed and all of the pathway segments appeared to be specifically regulated, although a high basal activity of one step, that of methyllactone isomerization, was observed.

Although microbes have the capacity to evolve enzymes capable of attacking most chemical structures, the evolution of entire pathways may proceed very slowly, particularly if multiple genetic changes are required. A major advantage of experimental evolution of pathways is that laboratory selection conditions can be custom designed for each of the individual changes required. In this way the evolutionary process can be considerably accelerated.

Table 1. 4-Methyl-2-enelactone isomerase activity in cell-free extracts of FR1 bacteria harboring plasmids pFRC20P or pFRC32P. Exponentionally growing bacteria in 100-ml cultures were collected by centrifugation, resuspended in 2 ml of 100 m*M* tris (hydroxymethyl) aminomethane tris-HCl, *p*H 7.5, and disrupted in a French pressure cell. The particulate fraction was eliminated by ultracentrifugation, and 4-methyl-2-enelactone isomerase activity in the soluble fraction was assayed by monitoring the disappearance of 4-methyl-2-enelactone (4ML) and appearance of 3-methyl-2-enelactone in a reaction mixture with 0.5 ml of cell extract, 0.2 ml of 4ML (50 m*M*), and 4.3 ml of phosphate buffer, *p*H 6.5, that was incubated at 30°C. Samples were taken at different times, acidified to *p*H 2 with H_3PO_4, and analyzed by high-performance liquid chromatography. The activity of 4ML isomerase is shown for strain B13 derivative FR1 grown on 4MB (5 m*M*), 4CB (5 m*M*), 3CB (5 m*M*), or acetate (Ac, 10 m*M*) as carbon sources. The activity for *Alcaligenes eutrophus* strain JMP134 grown in either 4ML (fully induced activity) or in fructose (Fr, not induced) is shown for comparison. Values are averages for two to three independently prepared cultures. Standard errors were less than 10% of the reported mean.

Bacterial strain	Growth substrate (micromoles converted per minute per milligram of protein)					
	4MB	4CB	3CB	Ac	4ML	Fr
FR1(pFRC20P)	0.580	0.140	0.185	0.375		
FR1(pFRC32P)	0.095	0.075	0.090	0.160		
JMP134					0.600	0.010

Table 2. Simultaneous degradation of chloro- and methylbenzoate by FR1(pFRC20P). Strain FR1(pFRC20P) was grown to exponential phase in minimal medium that contained either 4MB (5 m*M*) or 4CB (5 m*M*) as the sole carbon source. The bacteria were harvested and resuspended in 100 m*M* tris-HCl, *p*H 7.0, to give a suspension with an absorbance A_{546} of 2. Either 4MB, 4CB, or 3CB (final concentration, 2 m*M*) or a mixture of 4MB and 3CB or of 4MB and 4CB (final concentration, 1 m*M* each) was then added to the cells, and the kinetics of degradation of the substrates were followed by HPLC analysis of samples of supernatant fluid of the cell suspensions. In the case of 4MB and 3CB, and of 4MB and 4CB, the first value of the pair refers to 4MB, whereas the second refers to 3CB or 4CB. Values are averages for two to three independently prepared cultures. Standard errors were less than 20% of the reported mean.

Growth substrate	Substrate added (micromoles converted per minute per milligram of protein)				
	4MB	4CB	3CB	4MB + 3CB	4MB + 4CB
4MB	0.140	0.155	0.140	0.065, 0.075	0.095, 0.050
4CB	0.012	0.185	0.195	0.095, 0.120	0.130, 0.100

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16. The strategy used was the following: a 1.8-kb Bam HI fragment that contained the *xylS* gene was excised from plasmid pJLR156 [a pEMBL9 derivative (*15*)] and ligated to Bam HI-cleaved pUC19 (*17*) to produce plasmid pFRC1P. A 5-kb Sac I fragment that contained the *xylXYZL* genes plus the Pm promoter (*13*) was then excised from plasmid pGSH2017, a pBR322 derivative that contained the whole TOL meta cleavage operon plus a Tn1000 insertion in the *xylE* gene (S. Harayama, unpub-

lished results), and ligated into Sac I–cleaved pFRC1P (that is, at a site adjacent to that of the inserted *xylS* gene), to obtain plasmid pFRC3P. Finally, a 7.3-kb fragment that contained the *xylXYZLS* genes was excised from pFRC3P by cleavage with Hind III and partial cleavage with Eco RI; the sticky ends were filled in with the Klenow fragment of DNA polymerase I, and the fragment was cloned into the Sma I site located in the Tn*5* transposon present on the narrow host range transposon donor plasmid pSUP2021 (*18*). The resulting hybrid plasmid, designated pFRC4P, was mobilized into B13 in a triparental mating that involved B13 as recipient, *E. coli* HB101 (pFRC4P) as donor, and *E. coli* HB101 (pRK2013) (*19*) as helper. Kanamycin-resistant derivatives of B13 were selected in which the Tn*5* hybrid transposon that carried the cloned TOL genes had transposed into the chromosome.

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