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SPECIFICATION

TITLE:

BIOCONVERSIONS CATALYZED BY THE TOLUENE
MONOOXYGENASE OF PSEUDOMONAS MENDOCINA KR-1

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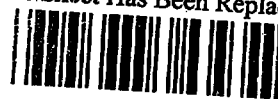
BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Serial No. 177,631 filed April 5, 1988, which is hereby incorporated by reference.

5 The present invention is directed to the use of recombinant DNA techniques to confer upon microorganism host cells the capacity for selected bioconversions. More specifically, the invention is directed to the cloning of toluene monooxygenase genes from a newly isolated and characterized Pseudomonas strain, Pseudomonas mendocina
10 KR-1. The present invention provides genetically engineered plasmids that allow production of toluene monooxygenase enzymes and proteins in a variety of Gram-negative bacteria in the absence of a toxic inducer, and provides more efficient means of conducting bioconversions dependent on this enzyme system.

15 A bacterial strain identified as Pseudomonas mendocina KR-1 (PmKR1) was isolated by Richardson and Gibson from an algal-mat taken from a fresh water lake. Whited, Ph.D. Dissertation, The University of Texas at Austin, Library Reference No. W586 (1986). PmKR1 utilizes toluene as a sole carbon and energy source. Other
20 bacterial strains have been isolated and described which metabolize or degrade toluene, including Pseudomonas putida mt-2 (Pp mt-2) (Williams and Murray, J. Bacteriol. 120: 416-423 (1974) and Pseudomonas putida PpF1 (PpF1) (Gibson, et al. Biochemistry 9:1626-1630 (1970)). In addition, a bacterial strain designated G4,
25 isolated from a waste treatment lagoon, can metabolize toluene (Shields et al., App. Environ. Microbiol. 55: 1624-1629 (1989)). However, the genes, the enzymes and the pathways for toluene

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metabolism in these various bacterial strains are distinct and non-overlapping.

The catabolic pathway for the degradation of toluene by Pp mt-2 has been designated TOL. The genes for the TOL pathway are encoded on isofunctional catabolic plasmids found in certain strains of Pseudomonas. The reference plasmid for the TOL degradative pathway is pWWO originally isolated from Pp mt-2. The genetics and biochemistry of the TOL pathway are well described. Kunz and Chapman, J. Bacteriol. 146:179-191 (1981); Williams and Murray, J. Bacteriol. 120:416-423 (1974); Williams and Worsey, J. Bacteriol. 125:818-828 (1976); Worsey and Williams, J. Bacteriol. 124:7-13 (1975); Murray, et al., Eur. J. Biochem. 28:301-310 (1972). In particular, detailed studies of the organization and regulation of the TOL pathway genes of plasmid pWWO have been performed. Franklin, et al., Proc. Natl. Acad. Sci. USA 78: 7458-62 (1981); Spooner et al., J. Gen. Microbiol. 132: 1347-58 (1986); Spooner, et al., J. Bacteriol. 169: 3581-86 (1987); Inouye et al., J. Bacteriol. 169: 3587-92 (1987); Inouye et al., Gene 66: 301-306 (1988). A brief summary of the TOL pathway is as follows: initial attack of toluene is at the methyl group which undergoes successive oxidations to form benzoic acid, which is further oxidized by formation of a cis-carboxylic acid diol, which is oxidized to form catechol, which is then degraded by enzymes of a meta cleavage pathway to acetaldehyde and pyruvate.

A second catabolic pathway for the degradation of toluene by PpFl has been established and designated TOD. In contrast to the TOL pathway, the genes for the TOD pathway are located on the



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bacterial chromosome and are not plasmid-encoded. Finette, et al., J. Bacteriol. 160:1003-1009 (1984); Finette, Ph.D. Dissertation, The University of Texas at Austin, Library Reference No. F494 (1984). The genetics and biochemistry of the TOD pathway has been studied by

5 Finette, et al. (supra); Finette (supra); Gibson, et al. Biochemistry 9:1626-1630 (1970); Kobal, et al., J. Am. Chem. Soc. 95:4420-4421 (1973); Ziffer, et al., J. Am. Chem. Soc. 95:4048-4049 (1973); Dagley, et al., Nature 202:775-778 (1964); Gibson, et al., Biochemistry 7:2653-2662 (1968). A brief summary of the TOD pathway

10 is as follows: the initial attack of toluene is by a dioxygenase enzyme system to form (+)-cis-1(S),2(R)-dihydroxy-3-methylcyclohexa-3,5-diene(cis-toluene dihydrodiol) which is oxidized to 3-methylcatechol which is further degraded by enzymes of a meta cleavage pathway. Zylstra and Gibson, J. Biol. Chem. 264: 14940-

15 46 (1989) and McCombie, Abstr. Annu. Meet. Am. Soc. Microbiol. K53: 155 (1984) have reported the cloning and sequencing of the *tod* genes which encode the first three enzymes in the TOD pathway.

A third catabolic pathway for the degradation of toluene has been recently identified in PmKR1. It has been found that PmKR1

20 catabolizes toluene by a novel pathway which is completely different than either of the two pathways described above. Richardson and Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. K54:156 (1984). The catabolic pathway for the degradation of toluene by PmKR1 has been designated TMO, because the first step in the pathway is catalyzed

25 by a unique enzyme complex, toluene monooxygenase. The biochemistry of the partially purified enzymes and proteins of this pathway has

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been recently studied by Whited, Ph.D. Dissertation, The University of Texas at Austin, Library Reference No. W586 (1986).

More recently, a toluene catabolic pathway, apparently distinct from the three above-described pathways, has been described in the trichloroethylene-degrading bacterium G4 by Shields et al., App. Environ. Microbiol. 55: 1624-1629 (1989). The bacterial strain designated G4 was isolated from a waste treatment lagoon. Strain G4 is uncharacterized with respect to genus and species. The toluene pathway of G4 appears to involve dihydroxylations of the aromatic ring by two monooxygenations, first ortho and then meta. The enzymes involved in these reactions have not been isolated and studied, and therefore remain completely uncharacterized.

The steps of the TMO pathway as outlined by Whited (supra) are diagrammed in Figure 1. In the initial step toluene is oxidized to p-cresol, followed by methyl group oxidation to form p-hydroxybenzoate, followed by hydroxylation to protocatechuate and subsequent ortho ring cleavage. In the first step of the TMO pathway, toluene is converted by toluene monooxygenase to p-cresol. PmkR1 elaborates a unique multicomponent enzyme system which catalyzes this first step monooxygenase reaction. The implications of the teachings of Whited, (supra), suggest that at least three protein components may be involved: component a (possibly NADH oxidoreductase, molecular weight unknown), component b (possibly an oxygenase, at least 2 subunits) and component c (red-brown, probably ferredoxin, 23,000d.).

Despite beginning biochemical studies of the enzymes and proteins of the TMO pathway (Whited, supra) and beginning genetic

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studies (Yen et al. Abstract, University of Geneva EMBO Workshop, August 31-September 4, 1986), the art has not been provided with information regarding the genes encoding the enzymes and proteins of the toluene monooxygenase system in PmKR1 or the usefulness of such genes and gene products in certain microbial bioconversions. The art has also not been provided with microorganism host cells harboring novel recombinant plasmids containing PmKR1 toluene monooxygenase genes, in which induction of the toluene monooxygenase genes does not involve use of toxic compounds or simultaneous induction of other undesirable genes and in which some of the microorganism host cells harboring such recombinant plasmids under certain conditions express toluene monooxygenase enzyme activity at levels that equal, or under certain assay conditions, exceed the activity of wildtype PmKR1 cells.

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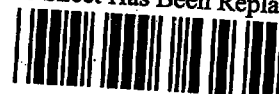
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SUMMARY OF THE INVENTION

The present invention provides novel gene segments, biologically functional plasmids and recombinant plasmids, and microorganism host cells, all of which contain the PmKR1 toluene monooxygenase genes. The present invention further provides a microorganism host cell harboring a novel recombinant plasmid containing PmKR1 toluene monooxygenase genes, in which synthesis of only toluene monooxygenase but not other undesirable enzymes can be induced specifically with an innocuous and inexpensive inducer and at levels that equal or, under certain conditions, exceed the activity of wildtype PmKR1 cells. In addition, the present invention provides a method for using transformed microorganism host cells containing the PmKR1 toluene monooxygenase genes in microbial bioconversions. Thus, the present invention provides microorganisms genetically engineered to produce toluene monooxygenase enzymes and proteins specifically and under innocuous conditions and therefore provides a more efficient means of conducting bioconversions with this enzyme system.

The present invention encompasses a biologically functional plasmid derived from PmKR1 containing toluene monooxygenase genes. This plasmid (designated pAUT1) can be transferred by conjugation to a microorganism host cell lacking the toluene monooxygenase gene system and thus unable to convert toluene to p-cresol. In a particularly preferred embodiment of the present invention, the microorganism host cell for the pAUT1 plasmid is Pseudomonas putida KT2440.

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The present invention also encompasses the toluene monooxygenase genes which have been isolated as various DNA gene segments from PmKR1 and cloned into a suitable, autonomously-replicating plasmid vector, resulting in a series of recombinant plasmids each of which contains a toluene monooxygenase gene segment. Each such recombinant plasmid is biologically functional and can be used to transform a microorganism host cell, conferring on the microorganism host cell the ability to convert toluene to p-cresol.

10 The present invention further encompasses a series of such transformed microorganism host cells. In a preferred embodiment of the present invention, the microorganism host cell is *E. coli* HB101, the recombinant plasmid is pMY402 and the inducer is isopropyl-thiogalactoside (IPTG). The pMY402 recombinant plasmid is the
15 pMMB66EH plasmid into which a 4.7 kb *Xho*I fragment encoding the PmKR1 toluene monooxygenase genes has been inserted. In another preferred embodiment of the present invention, the microorganism host cell is *E. coli* FM5, the recombinant plasmid is pKMY287 and the inducer is heat (42°C). The pKMY287 recombinant plasmid is the
20 pCFM1146 plasmid into which a 4.7 kb *Xho*I fragment encoding the PmKR1 toluene monooxygenase genes has been inserted. Under certain assay conditions, these resulting recombinant host cells express toluene monooxygenase enzyme activity at levels exceeding the activity of wildtype PmKR1 cells from which the toluene
25 monooxygenase genes were isolated.

Other preferred embodiments of the present invention include the recombinant plasmids pKMY336 and pKMY340 in *E. coli* FM5 cells

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and a particularly preferred embodiment is plasmid pKMY342 in PpY2500 cells (PpY2511). These cells synthesized the highest levels of TMO enzyme observed for recombinant microorganisms described herein.

5 Under alternative assay conditions, the levels of TMO activity detected in the recombinant host cells equal but do not exceed the activity detected in wildtype PmKR1 cells. However, it is advantageous to use the recombinant host cells for certain microbial bioconversions. Advantages of using recombinant host cells with
10 cloned TMO genes according to the present invention versus PmKR1 cells in these bioconversions include: (i) the ability to use innocuous inducers (e.g., IPTG, heat, salicylate) instead of toluene for TMO enzyme induction, and (ii) the ability to prevent further conversion of product by enzymes of subsequent steps in the TMO
15 pathway that are present in PmKR1 but not in the recombinant host cells. Thus, it is particularly advantageous to use the cloned TMO gene cluster to generate recombinant plasmids and recombinant host cells for certain bioconversions according to the present invention because the need for toluene, which is volatile and toxic, is
20 eliminated as inducer of TMO enzyme activity. For large-scale bioconversions using fermentors or otherwise, the disadvantages of toluene vapors in the bioconversion process are clearly evident. For safety reasons, some laboratories simply do not permit the use of toluene vapors in their fermentation processes. Additionally, it
25 is particularly advantageous to use the isolated and cloned TMO gene cluster in bioconversions because it can be manipulated and introduced into certain host cells that will not further convert the

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desired product obtained in the bioconversion using the TMO genes. In contrast, since wildtype PmKR1 cells contain the genes for the entire TMO pathway and not just the TMO genes for the first step of the pathway, further conversion of the product of the first step
5 monooxygenase reaction by the PmKR1 cells is likely.

The present invention is directed to the characterization and nucleotide sequence analysis of an isolated gene cluster of five TMO genes (*tmoA*, *B*, *C*, *D*, *E*), localized on the 4.7 kb XhoI fragment from PmKR1. The five-gene cluster, when expressed in *E. coli*, gave
10 significant TMO activity. Expression of this gene cluster carrying mutations in the individual genes demonstrated that each of the five genes is essential for TMO activity. The present invention is further directed to the characterization and nucleotide sequence analysis of a sixth TMO gene (*tmoF*), isolated on an ~1.3 kb HindIII-
15 XmaI fragment downstream of the five essential TMO genes. The product of the *tmoF* gene is useful to enhance the activity of the products of the *tmoABCDE* genes. The gene cluster of six TMO genes is localized on an ~6.7 kb HindIII-BamHI fragment or an ~4.7 kb HindIII-XmaI fragment isolated from PmKR1. Expression of the six-
20 gene cluster gave significantly higher TMO enzyme activity than expression of the five-gene cluster.

The present invention is directed to certain microbial bioconversions using PmKR1 or the transformed microorganism host cells containing the PmKR1 toluene monooxygenase genes, in
25 particular, the conversion of a selected phenyl compound to a selected phenolic compound. In a particularly preferred embodiment of the present invention, a method is provided for making p-

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hydroxyphenylacetic acid using the transformed microorganism host cells containing the PmKR1 toluene monooxygenase genes. The p-hydroxyphenylacetic acid is a valuable chemical intermediate in the preparation of certain antibiotics and certain β -adrenergic blocking agents. The present invention is also directed to a method for the microbial production of indigo from indole using PmKR1 cells or the transformed microorganism host cells containing the PmKR1 toluene monooxygenase genes.

The present invention is also directed to an improved method for the degradative bioconversion of trichloroethylene using transformed microorganism host cells containing the PmKR1 *tnoABCDEF* gene cluster. Further aspects and advantages of the present invention will become apparent to those skilled in the art upon consideration of the following detailed description.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the steps of the PmKR1 toluene monooxygenase (TMO) pathway.

Figure 2 shows a map of the pKMY235 plasmid vector.

5 Figure 3 illustrates a summary of recombinant plasmids, plasmid vectors and restriction maps of the PmKR1 DNA segments containing toluene monooxygenase genes.

Figure 4 illustrates a summary of recombinant plasmids and restriction maps of the PmKR1 DNA segments containing toluene
10 monooxygenase genes. Arrows indicate transcriptional direction and sizes of the TMO genes. Heavy lines underneath the map denote the different inserts in the plasmids indicated.

Figure 5 shows the nucleotide sequence of an -4.7 kb PmKR1 DNA region carrying the *tmoABCDE* genes. A region of dyad symmetry is
15 underlined.

Figure 6 shows the sequence homology of the TmoC protein with the ferredoxin (NahA₆) of naphthalene dioxygenase and the ferredoxin (BdoB) of benzene dioxygenase. Vertical lines indicate identical amino acid residues. Double dots indicate evolutionarily related
20 amino acid residues based on the Gap program in UWCCG software (Devereux et al., Nucleic Acids Res. 12: 387-395 (1984)) with the similarity threshold set at 0.5. The amino acid sequences of the TmoC protein and the naphthalene dioxygenase ferredoxin protein are deduced from nucleotide sequences of the *tmoC* gene and the *NahA₆* gene
25 of the NAH7 plasmid (see Yen and Serdar, supra), respectively. The amino acid sequence of the benzene dioxygenase ferredoxin protein was determined by Morrice et al., FEBS Lett. 231: 336-340 (1988)

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from the protein B of P. putida ML2 (NCIB 12190). A methionine residue is inserted at the N-terminus of this protein to reflect the nucleotide sequence.

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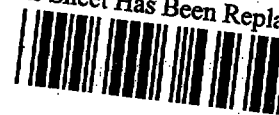
DETAILED DESCRIPTION

The methods and materials that provide an illustration of the practice of the invention and that comprise the presently preferred embodiments relate specifically to plasmid-borne DNA gene segments of PmKR1 origin encoding the genes for the toluene monooxygenase enzyme system. After cloning into a plasmid, these plasmid-borne DNA gene segments can be introduced and expressed in certain microorganism host cells, for example, by conjugation or transformation.

Microorganism host cells containing PmKR1 toluene monooxygenase genes are useful in certain bioconversions. For example, many phenyl compounds, including toluene, methylphenylacetic acid, ethylphenylacetic acid, acetanilide, 2-phenylethanol, fluorobenzene and ethylbenzene may serve as substrates and be converted to phenolic compounds by the TMO system as described herein. In addition, the broad substrate specificity of the TMO system makes it potentially useful in biodegradation of toxic compounds. Methods for the complete degradation of trichloroethylene (TCE) by the TMO system have been described in co-pending and co-assigned U.S. Patent Application Ser. No. 177,640, filed August 26, 1988, and hereby incorporated by reference in its entirety, and by Winter et al., Bio/Technology 7: 282-285 (1989). Improved methods for the complete degradation of TCE utilizing an isolated *tmoABCDEFG* gene complex are described herein.

The 4.7 kb XhoI fragment identified from PmKR1 that encodes TMO protein components has now been further characterized. This XhoI fragment originally designated as 4.6 kb by restriction enzyme

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analysis was determined by DNA sequence analysis to be 4.7 kb. Five TMO genes (*tmoABCDE*) have been identified within this region by DNA sequencing analysis and N-terminal amino acid determination. Expression of this five-gene cluster carrying mutations in the individual genes demonstrated that each of the five genes is essential for TMO activity. A sixth gene, *tmoF*, was isolated on an -1.3 kb HindIII-XmaI fragment downstream of the five essential TMO genes. The sixth gene, *tmoF*, is useful along with the *tmoABCDE* genes to yield enhanced TMO activity upon expression. The gene cluster of six TMO genes are localized on an ~6.7 kb HindIII-BamHI fragment or an ~4.7 kb HindIII-XmaI fragment from PmKRI. Expression of the six-gene cluster gave significantly higher TMO enzyme activity than expression of the five-gene cluster.

The potential exists, using recombinant DNA technology, to prepare variants, mutants or derivatives of one or more of the six TMO genes, which would encode a variant, mutant or derivative TMO protein. The complete gene sequence for each of the six TMO genes is disclosed in Figure 5. Various modifications might result in single or multiple amino acid deletions, substitutions, insertions or inversions, for example, by means of in vitro mutagenesis of the underlying DNA by methods well-known in the art. In addition, various fragments of one or more of the proteins encoded by the TMO genes, whether produced in vivo or in vitro, may possess the requisite useful TMO activity. Experiments have shown, for example, that changes in the N-terminal sequences of *TmoF* do not substantially change its functional activity. All such variations, modifications or fragments resulting in a variant, mutant or

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derivative of one or more of the six TMO genes are included within the scope of this invention so long as they encode the functional segment(s) of TMO protein(s) and the characteristic functional TMO activity remains substantially the same, e.g. unaffected in kind.

5 Functional TMO activity results from a functional five-gene or six-gene TMO gene complex, as measured by the assays described herein, for example, in Example 11. From the disclosure of the TMO DNA sequences herein and the amino acid sequence of each of the six TMO genes, a TMO variant, mutant or derivative may be prepared and
10 identified by those skilled in the art.

The invention is now illustrated by the following Examples, with reference to the accompanying drawings. Plasmids previously designated with the prefix "pKY" have been redesignated herein as "pKMY". The examples do not include detailed descriptions for
15 conventional methods employed in the isolation of DNA, the cleavage of DNA with restriction enzymes, the construction of vectors, the insertion of DNA gene segments encoding polypeptides of interest into such vectors (e.g. plasmids) or the introduction of the resulting recombinant plasmids into microorganism host cells. Such
20 methods are well-known to those skilled in the art of genetic engineering and are described in numerous publications including the following laboratory manuals: Maniatis et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982); Davis et al., Basic Methods in Molecular Biology, Elsevier Science
25 Publishing Co. (1986); Current Protocols in Molecular Biology, edited by Ausubel et al., Greene Publishing Associates and Wiley Interscience (1987); Sambrook et al., Molecular Cloning: A



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Laboratory Manual (Second Edition), Cold Spring Harbor Laboratory Press (1989). In addition to using published methods, methods for enzymatic cleavage, modification, and ligation of DNA may be achieved according to manufacturer's instructions distributed by various commercial suppliers of restriction enzymes, including New England Biolabs, Inc. (NEB), Beverly, MA 01915 and Boehringer Mannheim Biochemicals, Indianapolis, IN 46250.

EXAMPLE 1

Growth of PmKR1 Cells

10 Pseudomonas mendocina KR-1 was grown overnight at 30° in PAS medium or on a PAS agar plate (Chakrabarty, et al., Proc. Natl. Acad. Sci. U.S.A., 70:1137-1140 1973) with toluene (supplied as vapor) for growth and for induction of the toluene monooxygenase genes.

EXAMPLE 2

Construction of PmKR1 EgIII Library in E. coli HB101

A. Preparation of PmKR1 DNA

15 Total DNA was isolated from PmKR1 by conventional methods. Briefly, PmKR1 was inoculated into PAS medium containing toluene according to Example 1 and incubated with shaking at 30°C overnight (13-17 hours). After incubation, PmKR1 cells in the stationary growth phase were collected by centrifugation. The cells were lysed and total PmKR1 DNA was then extracted and purified as described by Dhaese et al., Nucleic Acid Res. 7: 1837-1849 (1979).



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B. Preparation of Plasmid DNA

Plasmid DNA may be isolated according to the method of Johnston and Gunsalus, Biochem. Biophys. Res. Comm. 75: 13-19 (1977). E. coli HB101 containing the pRK290 plasmid (Ditta, et al., Proc. Natl. Acad. Sci. U.S.A. 77: 7347-7351 (1980)) was inoculated into L broth and incubated with shaking at 37°C overnight. The bacterial cells were collected by centrifugation, lysed and the bulk of chromosomal DNA and cellular debris was removed by centrifugation. The pRK290 plasmid DNA was then purified by conventional techniques using cesium chloride/ethidium bromide density gradients.

C. Preparation of Recombinant Plasmid

Total PmkR1 DNA obtained in Part A above and pRK290 plasmid DNA obtained in Part B above were separately treated with the restriction endonuclease BglIII, under conditions of complete digestion. The BglIII digested PmkR1 DNA was mixed with BglIII digested pRK290 plasmid DNA and the mixture then incubated with DNA ligase.

D. Transformation with Recombinant Plasmid

Transformation of E. coli with plasmid DNA may be achieved by the calcium chloride procedure originally described by Mandel and Higa, J. Mol. Biol. 53: 159-162 (1970). The ligated DNA obtained in Part C above was used to transform E. coli HB101 and the transformed cells were plated on selection plates of L-agar containing 10 µg/ml tetracycline. Only those cells which are successfully transformed and which contain the pRK290 plasmid or a recombinant pRK290 plasmid with PmkR1 DNA can grow on the selection plates. Colonies which

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grew on the selection plates were tested for the presence of recombinant plasmids containing PmKR1 toluene monooxygenase genes by the conjugation and complementation screening assay of Example 3.

EXAMPLE 3

5 Conjugation and Complementation Screening Assay

A complementation assay involving plasmid transfer via bacterial conjugation was used to screen the PmKR1 BglIII library made according to Example 2 and the PmKR1 SacI library made according to Example 8 in order to detect recombinant plasmids
10 containing PmKR1 toluene monooxygenase genes. Accordingly, plasmids were transferred between bacterial strains by the conjugation ("mating") procedure (spot crosses) described by Yen and Gunsalus, Proc. Natl. Acad. Sci. U.S.A., 79:874-878 (1982). This procedure is summarized briefly as follows.

15 Colonies were removed from the selection plates of Example 2 or Example 8 by gentle scraping in L-broth with a slide. The resulting bacterial cell suspension was washed to remove any tetracycline and suspended in L-broth for the mating. Suspensions of donor cells, helper cells (if necessary) and recipient cells in
20 logarithmic phase were mixed in equal volumes. Small aliquots of the mixture were placed on L-agar plates thus allowing all cell types to grow. After overnight incubation at 30°C, the cells were replated on a PAS agar selection plate containing 50 µg/ml tetracycline. Toluene was provided as sole carbon source for
25 growth. Toluene vapor was supplied to the selection plate by taping a cotton-stoppered toluene containing tube to the lid of the plate.

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This selection plate permits only the desired trans-conjugates to grow. In all experiments performed, the donor cells were from an E. coli HB101 library (either the BglIII library of Example 2 or the SacI library of Example 8) carrying a recombinant plasmid (pRK290 in Example 2 or pKMY235 in Example 8 containing PmKR1 gene segments) to be transferred in the mating. The helper cells used were E. coli HB101 cells carrying the helper plasmid pRK2013 which provided the transferring functions for those transferring plasmids which do not carry the tra genes. Alternatively, the helper plasmid pRK2013 was introduced directly into the donor cells to provide its transferring function. The recipient strain was one of several mutant strains of Pseudomonas mendocina KR-1 (Pm Y4001, Pm Y4002, Pm Y4007) prepared as described in Example 4. Each of the mutant strains has a defective toluene monooxygenase gene and is unable to convert toluene to p-cresol. When a recombinant plasmid containing the specific PmKR1 toluene monooxygenase gene which is defective in the recipient strain has been successfully transferred during conjugation, the resulting transconjugate will be able to grow as a colony on the selection plates containing toluene as the sole carbon source for growth.

The colonies which grew on the selection plates were purified by restreaking each colony once or twice on a selection plate. These transconjugates are further manipulated according to Example 5.

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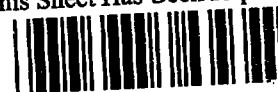
EXAMPLE 4Preparation of *Pseudomonas mendocina* KR-1 Mutant Strains

PmKR1 cells were mutagenized and the toluene monooxygenase defective mutants were isolated according to the following protocol.

- 5 Cells were grown in 5 ml of L broth to O.D.₆₈₀ of approximately 0.7 and resuspended into 2 ml of 50 mM citrate buffer pH 6.0 containing N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) at a concentration of 0.1 mg per ml. After incubation at room temperature for 20 minutes, the cells were washed twice with 2 ml of 1 M
- 10 phosphate buffer pH 7.0 and resuspended into 50 ml of L broth. After growth overnight, the cells were streaked on L agar plates for single colonies. The individual colonies were picked and streaked onto PAS plates containing toluene or p-cresol (2.5 mM) as sole carbon source. The toluene monooxygenase defective mutants,
- 15 PmY4001, PmY4002 and PmY4007 were isolated as strains which grew on p-cresol but not on toluene. The toluene monooxygenase assay as described in Example 11 further confirmed that these mutants have a defective toluene monooxygenase enzyme system.

- Similar mutagenesis techniques may be used to obtain mutants
- 20 defective in the enzyme p-cresol hydroxylase or p-hydroxybenzaldehyde dehydrogenase of the TMO pathway. After nitrosoguanidine treatment of PmKR1 cells as described above, p-cresol hydroxylase defective mutants can be isolated as strains which grow on p-hydroxybenzyl alcohol but not on p-cresol and p-
- 25 hydroxybenzaldehyde dehydrogenase defective mutants can be isolated as strains which grow on p-hydroxybenzoate but not p-hydroxybenzylalcohol or p-hydroxybenzaldehyde.

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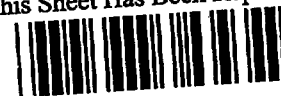
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EXAMPLE 5**Isolation of 9.4 kb BglIII Fragment**

A number (12) of the transconjugate colonies of PmY4001 containing PmKR1 toluene monooxygenase genes isolated according to Example 3 were further characterized as follows. Each colony was grown and plasmid DNA was isolated by conventional methods. The plasmid DNA from each isolate was used to transform *E. coli* HB101 cells. The plasmid in each transformant was transferred to PmY4001 by conjugation according to Example 3 except that the selection plates contained tetracycline and glucose (2 mg/ml). Each transconjugate was tested for growth on toluene by plating the cells on PAS agar supplemented with 50 µg/ml tetracycline and toluene vapor. After the toluene monooxygenase complementing activity of the plasmid was confirmed in the transconjugates each such HB101 transformant was grown and plasmid DNA was isolated by conventional methods.

The DNA was digested with BglIII and a 9.4 kb fragment was isolated from each transconjugate colony which complemented each PmKR1 mutant strain of Example 4 for toluene utilization. This result indicated that the 9.4 kb BglIII fragment from PmKR1 contained one or more toluene monooxygenase genes. Two SacI sites were mapped close to one end of the 9.4 kb BglIII fragment. No TMO activity could be detected from the pRK290 plasmid (Example 2) carrying this 9.4 kb BglIII insert in either *E. coli* HB101 or *P. putida* KT2440 (Bagdasarian et al., Gene 16: 237-247 (1981)). Subcloning of this insert into *E. coli* expression vectors pUC18 and pUC19 (Yanisch-

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Perron et al., Gene 33: 103-119 (1985)) did not lead to the detection of any TMO activity. Thus, the 9.4 kb BglII fragment did not appear to contain the entire TMO gene cluster required for activity.

5

EXAMPLE 6

Construction of pKMY235 Plasmid Vector

The starting material for the construction of the pKMY235 plasmid was the pKY217 plasmid described by Yen and Gunsalus, J. Bacteriol. 162: 1008-13 (1985). The pKMY235 plasmid was constructed according to the following series of steps. In the first step, two adjoining HindIII fragments (~1.1 and ~3.2 kb) from pKY217 containing the nahR and nahG genes was cloned into the HindIII site of the pKT240 plasmid described by Bagdasarian et al., Gene 26: 273-82 (1983). The resulting plasmid from this first step was designated pKMY219. In the second step, an -7 kb BamHI -SacI fragment from pKMY219 containing the nahR and nahG genes was cloned into the BamHI and SacI sites of the pKT231 plasmid described by Bagdasarian et al. Gene 16: 237-47 (1981). The resulting plasmid was designated pKMY223. In the next step, an -6 kb PstI fragment from pKMY223 containing the nahR gene, -200 base pairs of the nahG gene and the pKT231 gene conferring kanamycin resistance was cloned into the PstI site of the pUC19 plasmid described by Yanisch-Perron et al., Gene 33: 103-119 (1985). The resulting plasmid was designated pKMY256. The orientation of the -6 kb PstI fragment in pKMY256 placed the multi-cloning site of pUC19 from the SalI to the EcoRI site immediately downstream to the PstI site in the nahG gene.

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In the final step, an -5.4 kb BstEII - EcoRI fragment from pKMY256 containing the gene conferring kanamycin resistance, the nahR gene, -200 base pairs of the nahG gene and a multiple cloning site was end-filled with the large fragment of E. coli DNA polymerase I and inserted into the pRK290 plasmid described by Ditta et al., Proc. Natl. Acad. Sci. U.S.A. 77: 7347-7351 (1980) to replace the -1 kb SmaI fragment of pRK290. The resulting plasmid was designated pKMY235 and a map of pKMY235 is shown in Figure 2.

EXAMPLE 7

10

Construction of pCFM1146 Plasmid Vector

The plasmid pCFM1146 is an E. coli cloning vector similar to pCFM4722 (Burnette et al., Bio/Technology 6: 699-706-1988)). The starting material for the construction of the pCFM1146 plasmid was the pCFM836 plasmid. A detailed description of the construction of expression vectors, including pCFM836, is described in U.S. Patent No. 4,710,473, which is hereby incorporated by reference in its entirety. The pCFM836 plasmid contains a heat inducible promoter, a restriction site bank (cloning cluster), plasmid origin of replication, a transcription terminator, genes regulating plasmid copy number, and a gene conferring kanamycin resistance but no synthetic ribosome binding site immediately preceding the cloning cluster. The pCFM1146 plasmid (A.T.C.C. accession no. 67671, deposit date April 5, 1988) was derived from pCFM836 by substituting the small DNA sequence between the unique ClaI and XbaI restriction sites with the following oligonucleotide

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3' TAACTAAGATC 5'

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and by destroying the two endogenous NdeI restriction sites by cleavage with NdeI and then end-filling with T4 polymerase enzyme, followed by blunt end ligation.

EXAMPLE 8

5 Construction of PmKR1 SacI Library in E. coli HB101

The pKMY235 plasmid vector prepared according to Example 6 was used to construct a SacI library in E. coli HB101 according to conventional techniques for constructing genomic libraries. Total DNA from PmKR1 was isolated as described in Example 2, Part A. The
10 isolated PmKR1 DNA was treated with the restriction endonuclease SacI under conditions of partial digestion. In order to produce a population of DNA fragments enriched in those fragments containing some or all of the PmKR1 toluene monooxygenase genes for use in constructing this SacI library, the partially digested PmKR1 DNA
15 was fractionated by size using a 10%-40% sucrose density gradient according to conventional procedures. After centrifugation for 24 hours at 26,000 rpm in an SW-28 rotor, the DNA fractions were collected and tested by hybridization. The 9.4 kb BglII fragment isolated from the BglII library constructed according to Example 3,
20 is known to complement each PmKR1 mutant strain for toluene utilization according to Example 5 and thus is likely to contain at least one of the PmKR1 toluene monooxygenase genes. The plasmid pUC19 carrying the 9.4 kb fragment was radiolabeled and used as a probe to select hybridizing fractions from the sucrose gradient.
25 The hybridizing fractions were pooled to provide a population of DNA fragments enriched in PmKR1 toluene monooxygenase genes. This

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enriched population of DNA fragments was used to construct the SacI library in E. coli HB101. They were randomly cloned into the SacI site of plasmid pKMY235 as follows.

The enriched SacI digested PmKR1 DNA was mixed with SacI digested pKMY235 plasmid DNA and incubated with DNA ligase. The ligated DNA was used to transform E. coli HB101 and the transformed cells were plated onto selection plates of L-agar containing 10 µg/ml tetracycline. Only those cells which were successfully transformed and containing the pKMY235 plasmid or a recombinant pKMY235 plasmid with PmKR1 DNA can grow on the selection plates. Transformed colonies were tested for PmKR1 toluene monooxygenase genes by the conjugation and complementation assay of Example 3.

EXAMPLE 9

Isolation of 20.4 kb SacI Fragment

15. A number (10) of the transconjugates which utilized toluene as a sole carbon source were further characterized by isolating the plasmid DNA, transforming E. coli HB101, and conjugating into PmY4001 to test for growth on toluene according to Example 5. An E. coli HB101 transformant containing a recombinant pKMY235 plasmid (designated pKMY266, A.T.C.C. accession no. 67672, deposit date April 5, 1988) carrying toluene monooxygenase genes was grown and plasmid DNA was isolated by conventional methods. Restriction enzyme analysis of the insert in pKMY266 plasmid indicated that it carried two 10.2 kb SacI fragments (previously designated two SacI fragments of 10.2 kb and 10.3 kb, respectively). One of the 10.2 kb



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SacI fragments contains -8 kb of the -9.4 kb EglII fragment described in Example 5.

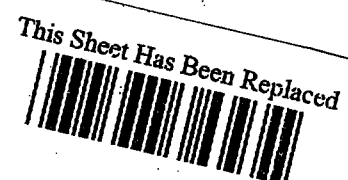
Plasmid pKMY266 containing the -20.4 kb (previously designated 20.5 kb) SacI insert described above, allowed the utilization of toluene by PmY4001. Despite the complementation pattern, no TMO enzyme activity (according to the alternative method of Example 11) could be detected in E. coli HB101 carrying pKMY266. Since the insert in pKMY266 consisted of two different SacI fragments of identical size, each was subcloned and expressed in the high-copy-number E. coli expression vector pUC19 described by Yanisch-Perron et al., Gene 33: 103-119 (1985). This experiment led to the successful mapping of the TMO genes as described in Example 10 below.

EXAMPLE 10

15 Construction of Recombinant Plasmids to Map the Toluene Monooxygenase Genes

This example presents results on the mapping of the TMO genes and determination of TMO activities in various recombinant strains based on earlier constructs and TMO assay procedure (Example 11, Part A). Further mapping of the TMO genes and determination of TMO activities in more recently constructed strains based on the alternative TMO assay procedure described in Example 11, Part B is presented in Example 16.

One of the SacI fragments described in Example 9, when cloned into pUC19 (pKMY277) and expressed in E. coli JM109, led to the synthesis of a blue pigment which was chloroform soluble and water

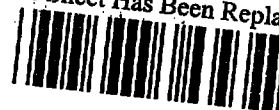


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insoluble. Production of the blue pigment was also observed from *E. putida* cells harboring pKMY266 and was dependent on the presence of indole. This blue pigment was identified as indigo. A low level of TMO enzyme activity was detected from the *E. coli* JM109 carrying pKMY277 (Table I). Further mapping of the toluene monooxygenase genes correlated the indigo-producing property with the presence of toluene monooxygenase activity. (See Table I in Example 11 and also Table III in Example 16).

The ~10.2 kb SacI fragment of pKMY277 was digested with a series of restriction enzymes and a partial restriction map was generated as shown in Figure 3. Based on this restriction map, a series of DNA fragments were deleted from one end of the 10.2 kb SacI fragment in pKMY277 to generate plasmids pKMY280, pKMY281, pKMY282 and pKMY283 shown in Figure 3. An ~4.7 kb XhoI fragment of pKMY282 was subcloned into the SalI site of pUC19 to generate the plasmid pMY401. An ~4.6 kb BamHI - SphI fragment of pMY401 was inserted into the *E. coli* expression vector pUC18 described by Yanisch-Perron et al., Gene 33: 103-119 (1985) to generate the plasmid pMY404. The pUC18 plasmid is identical to pUC19 except the polycloning site is in an opposite orientation with respect to the lac promoter. As a result, the ~4.7 XhoI fragment was inserted into the pUC18 plasmid in an opposite orientation to that in the pUC19 plasmid with respect to the lac promoter. The ~4.7 kb XhoI fragment of pKMY277 was also cloned into the broad host range plasmid vector pMMB66EH described by Furste et al., Gene 48: 119-131 (1986) to construct the plasmid pMY402. In addition, as shown in Figure 3, an ~2.2 kb SacI - BglII fragment was deleted from the left end of the

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-5.9 kb SacI - XmaI fragment of pKMY282 by digesting pKMY282 DNA with SacI and BglII, filling the ends with the large fragment of E. coli DNA polymerase I and ligating the ends. The resulting plasmid was designated pMY400.

5 As shown in Table I (according to the assay of Example 11), pMY402 containing cells responded to IPTG for induction of the toluene monooxygenase genes. This result located the toluene monooxygenase genes in the -4.7 kb XhoI fragment and revealed the direction of transcription of the toluene monooxygenase genes as
10 from left to right shown in Figure 3. The difference in the orientation of the -4.7 kb XhoI fragment in pMY401 and pMY404 as well as the difference in toluene monooxygenase activity in pMY401 and pMY404 containing cells (Table I) are also consistent with this transcriptional direction of the toluene monooxygenase genes. In
15 order to express the toluene monooxygenase genes at a high level, the -4.7 kb XhoI fragment of pKMY282 was also cloned into the XhoI site of the E. coli expression vector pCFM1146 (as described in Example 7) to construct pKMY287.

EXAMPLE 11

20 Toluene Monooxygenase Assays

A. Conditions for Assay

Cells were grown in PAS medium containing 0.4% glutamate or in L broth to saturation. They were resuspended into an appropriate volume of the same medium to an O.D.₅₅₀ of 3.0. An aliquot of the
25 cells was used for the determination of protein concentration by the method of Bradford, Anal. Biochem. 72: 248 (1976) using the Bio-Rad Protein Assay. An aliquot of 0.5 ml of cells was mixed with 4

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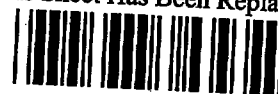
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5 μ moles of p-cresol in 10 μ l and 15 nmole of radioactive toluene (toluene-ring- 14 C, Sigma Chemical Co., 56.3 mCi/ μ mole) in 5 μ l and the mixture was incubated at room temperature with occasional vortexing for 20 minutes. After incubation, 20 μ l of the mixture were spotted on a small piece of a thin-layer chromatography plate and the plate was air-dried for twenty minutes. The nonvolatile radioactivity remained on the filter was determined in a liquid scintillation counter and was used to calculate the amount of toluene degradation product on the plate and the specific activity of toluene monooxygenase. The results are presented in Table I.

B. Conditions for Alternative Assay

15 Alternatively, toluene monooxygenase activity could be assayed by a procedure similar to the assay described for naphthalene dioxygenase by Ensley et al., in Microbial Metabolism and the Carbon Cycle (Hagedorn et al., eds.), Harvard Academic Publishers, New York (1988) at p. 437. Late log-phase cells were diluted into L-broth to a density of O.D.₅₅₀-0.5 for the assay. The reactions were initiated by adding 15 nmole of 14 C-toluene (Sigma, 40-60 mCi/ μ mol) to 0.5 ml of cells in L-broth. After incubation at room temperature for 5 minutes, aliquots of 20 μ l were spotted on small strips of thin-layer chromatography plate. The plates were air-dried for 20 minutes and counted in a scintillation counter to determine the remaining radioactivity. Specific activity of TMO was expressed as nmole of nonvolatile material produced from 14 C-toluene per minute per milligram of whole cell protein. The protein concentration was determined by the method of Bradford, Anal. Biochem. 72: 248, using the Bio-Rad protein assay kit obtained from Bio-Rad Laboratories,

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Richmond, CA 94804. For the protein determination, cells were resuspended in 0.1 N NaOH and incubated in a boiling water bath for 20 minutes, then assayed with the kit. The results of this alternative TMO assay are presented in Table III, Example 16.

5

TABLE I

Expression of the Toluene Monooxygenase (TMO) genes in E. coli and P. mendocina

10

15

20

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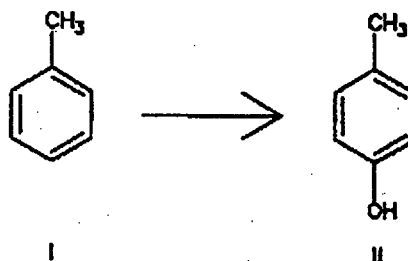
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Plasmid	Inducer	Host Cell	Specific Activity of TMO (nmole min ⁻¹ mg ⁻¹)	Indigo Formation
pAUT1	Toluene	<u>P. mendocina</u> KR1	0.130	+
pAUT1	None	<u>P. mendocina</u> KR1	0.010	+
pKMY266	None	<u>P. putida</u> KT2440	0.020	+
pKMY277	None	<u>E. coli</u> JM109	0.010	+
pMY405	None	<u>E. coli</u> HB101	0.005	-
pMY405	IPTG	<u>E. coli</u> HB101	0.015	+
pKMY280	None	<u>E. coli</u> JM109	0.010	+
pKMY281	None	<u>E. coli</u> JM109	0.010	+
pKMY282	None	<u>E. coli</u> JM109	0.010	+
pKMY283	None	<u>E. coli</u> JM109	0.005	-
pMY400	None	<u>E. coli</u> JM83	0.005	-
pMY401	None	<u>E. coli</u> JM83	0.035	+
pMY404	None	<u>E. coli</u> JM83	0.010	+
pMY402	None	<u>E. coli</u> HB101	0.005	-
pMY402	IPTG	<u>E. coli</u> HB101	0.200	+
pKMY287	Heat	<u>E. coli</u> FM5	0.500	+
pUC19	None	<u>E. coli</u> JM109	0.005	-
pMMB66EH	IPTG	<u>E. coli</u> HB101	0.005	-
pCFM1146	Heat	<u>E. coli</u> FM5	0.005	-

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EXAMPLE 12**Conversion of Certain Phenyl Compounds to
Certain Phenolic Compounds****A. Conversion by PmKR1 Cells**

- 5 Many phenyl compounds, including toluene, methylphenylacetic acid, ethylphenylacetic acid, 2-phenylethanol, acetanilide, fluorobenzene and ethylbenzene, may serve as substrates and thus be converted to phenolic compounds via para-hydroxylation by the toluene monooxygenase system of PmKR1. The following schemes
- 10 illustrate several possible conversions:

Scheme A

wherein:

I is toluene

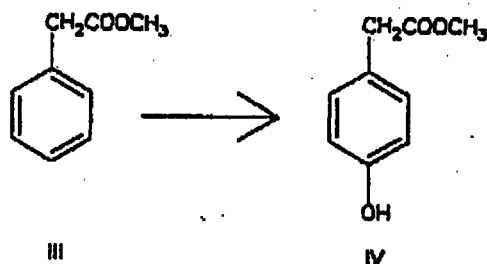
II is p-cresol

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Scheme B



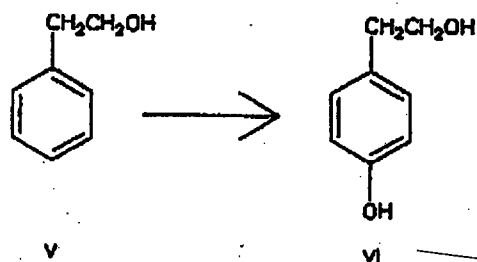
wherein:

III is methylphenylacetic acid

IV is p-hydroxymethylphenylacetic acid

5

Scheme C



wherein:

V is 2-phenylethanol

VI is p-hydroxy-2-phenylethanol

For each conversion, a phenyl compound substrate (for example,

10 Formulas I, III, or V) was mixed with PmKRI cells, incubated for a

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period sufficient to effect the bioconversion and then assayed for the presence of phenolic compounds as follows.

Pseudomonas mendocina KR1 cells were grown at 25°C-30°C in 50 ml PAS medium supplemented with 0.4% glutamate to stationary phase (12-16 hours) in the presence (induced) or absence (uninduced) of toluene vapor supplied from 2.5 ml toluene. An aliquot of 5-50 ml cells were resuspended into the same volume of the same medium or concentrated 2.5 fold in the same medium. A given amount of the substrate equivalent to form a 15-30 mM solution was mixed with the cells and the mixture was incubated at 25°C-30°C with vigorous shaking for 1-24 hours. Typically the mixture was incubated for 5-6 hours. Formation of phenolic compounds was determined according to the assay method of Gupta et al., Clin. Biochem. 16 (4): 220-221 (1983). The assay results for conversion of several phenyl substrates to phenolic compounds at various times and temperatures of incubation are shown in Table II.

TABLE II

Synthesis of Phenolic Compounds by Toluene
Monooxygenase of Pseudomonas mendocina KR1

20

Substrate (Time and Temperature of Incubation)	O.D. ₆₈₀ reading in Assay
acetanilide (6 hrs., 25°C)	1.07
25 fluorobenzene (24 hrs., 25°C)	0.73
methylphenylacetate (6 hrs., 30°C)	0.23
ethylphenylacetate (6 hrs., 30°C)	0.13
ethylbenzene (6 hrs., 30°C)	0.37
2-phenylethanol (5 hrs., 30°C)	0.16
30 substrate in uninduced culture	0.03

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B. Conversion by Microorganism Host Cells Containing Recombinant Plasmids encoding PmKR1 Toluene Monooxygenase Genes

The same conversions according to Part A may be accomplished by using microorganism host cells containing the recombinant plasmids of Examples 10, 16, and 19. Any of the recombinant plasmids (except pKMY283 or pMY400) which encode functional PmKR1 toluene monooxygenase genes as described in Example 10 may be used to transform an appropriate microorganism host cell. A preferred method is to use pMY402 as the recombinant plasmid, *E. coli* HB101 as the microorganism host cell and IPTG as the inducer, as described in Example 11. The resulting strain was designated EcY5072 (HB101/pMY402). Another preferred method is to use pKMY287 or pKMY336 as the recombinant plasmid, *E. coli* FM5 as the microorganism host cell and heat (42°C for 1.5 or 3 hrs.) as the inducer. The resulting strains were designated EcY5082 (FM5/pKMY287) and EcY5236 (FM5/pKMY336), respectively. A particularly preferred method is to use pKMY342 as the recombinant plasmid, PpY2500 as the microorganism host cell and sodium salicylate (0.35 mM in L-broth throughout cell growth) as the inducer. The resulting strain was designated PpY2511 (PpY2500/pKMY342).

For each conversion, a phenyl compound (for example, Formulas I, III or V) is mixed with EcY5072 (HB101/pMY402), EcY5082 (FM5/pKMY287), EcY5236 (FM5/pKMY336) or PpY2511 (PpY2500/pKMY342) cells. The mixture is incubated for a period sufficient to effect the bioconversion and then assayed as described in Part A for the presence of phenolic compounds. For each bioconversion with EcY5072 (HB101/pMY402) cells, the cells are grown and assayed in the

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presence of IPTG to induce PmkR1 toluene monooxygenase activity as follows. Cells are grown in PAS medium containing 0.4% glutamate and 1 mM IPTG or grown in L broth with 1 mM IPTG to saturation. The cells are resuspended in an appropriate volume of the same medium to an O.D.₆₆₀ of 3.0 and incubated with substrate and assayed as described in Part A. For each bioconversion with EcY5082 (FM5/pKMY287) or EcY5236 (FM5/pKMY336) cells, the cells are grown under the following temperature conditions to induce PmkR1 toluene monooxygenase activity. EcY5082 (FM5/pKMY287) or EcY5236 (FM5/pKMY336) cells are grown in L broth to an O.D.₆₆₀ of 0.4. The cultures are incubated with shaking at 42°C for 3 hours and then shifted to 30°C to incubate for another 2 hours. Cells are resuspended in fresh L broth to an O.D.₆₆₀ of 3.0 and incubated with substrate and assayed as described in Part A. For indigo production (Example 15) using these two strains, the cells are incubated at 30° for 24 hours after induction at 42°C. For each bioconversion with the PpY2511 strain, the cells are grown and induced under the following conditions. The cells are grown in L broth to saturation in the presence of 0.35 mM sodium salicylate to induce toluene monooxygenase production. Cells are resuspended in the same medium to an O.D.₆₆₀ of 3.0 and incubated with substrate and assayed as described in Part A.

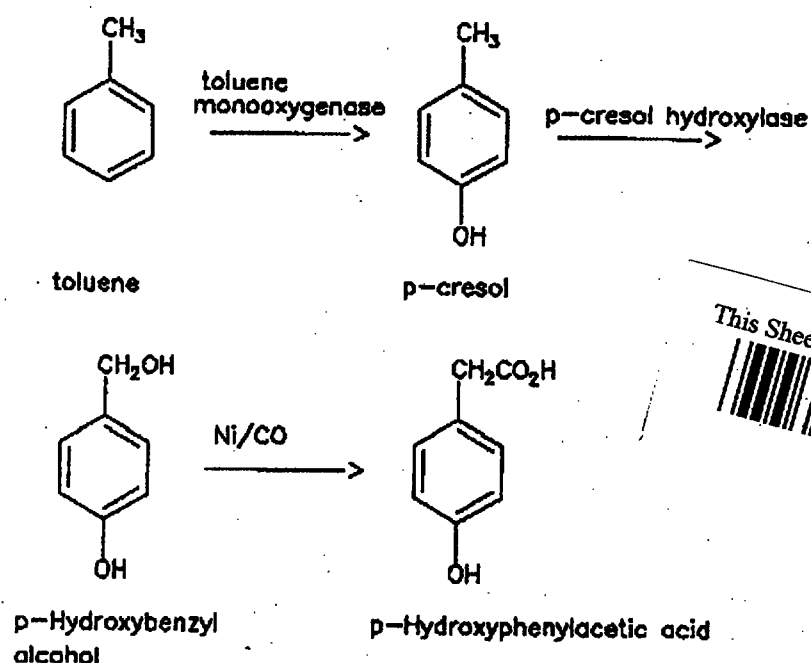
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EXAMPLE 13**Conversion of Toluene to p-Hydroxyphenylacetic Acid****A. Conversion by PmKR1 Cells**

For the conversion of toluene substrate to p-hydroxyphenylacetic acid, toluene is mixed with a PmKR1 mutant containing defective p-hydroxybenzaldehyde dehydrogenase as described in Example 4 and incubated for a period sufficient to effect the conversion of toluene to p-hydroxybenzyl alcohol. In the second step, the cell mixture containing the p-hydroxybenzyl alcohol intermediate is reacted with nickel (Ni) and carbon monoxide (CO) in such concentrations and at such temperatures sufficient to convert the p-hydroxybenzyl alcohol to p-hydroxyphenylacetic acid, according to the methods of U.S. Patents 4,482,497; 4,659,518; 4,631,348, which are hereby incorporated by reference. The conversion scheme is illustrated as follows:



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B. Conversion by Microorganism Host Cells Containing Recombinant Plasmids encoding PmKR1 Toluene Monooxygenase Genes

The same conversion according to Part A may be accomplished by using microorganism host cells harboring recombinant plasmid(s) carrying the p-cresol hydroxylase gene and the TMO genes. The p-cresol hydroxylase genes may be isolated by cloning of restriction fragments from PmKR1 or plasmid pND50 (Hewatson et al., Genet. Res. Camb. 32: 249-255, 1978) which allow p-cresol hydroxylase defective mutants of PmKR1 (Example 4) to use p-cresol as a carbon and energy source. Alternatively, it may be isolated by using the sequence of TMO genes as a probe to clone overlapping restriction fragments that contain the gene. The possibility exists that the ~10.2 kb SacI fragment containing the TMO gene cluster (Example 10) contains the p-cresol hydroxylase genes. For use in the bioconversion described in Part A, the p-cresol hydroxylase genes may be cloned and expressed in plasmid pMY402, pKMY287, pKMY336 or pKMY342 (Examples 10, 16, and 19) each of which contains a functional TMO gene cluster. Alternatively, the p-cresol hydroxylase genes may be cloned and expressed in another plasmid which can be introduced into strains which contain plasmid pMY402, pKMY287, pKMY336 or pKMY342.

For the conversion as illustrated in Part A, toluene is mixed with induced cells containing the p-cresol hydroxylase genes and the TMO genes. The mixture is incubated for a period sufficient to effect the conversion of toluene to p-hydroxybenzyl alcohol, and then is reacted with Ni and CO according to Part A to effect the conversion to p-hydroxyphenylacetic acid.

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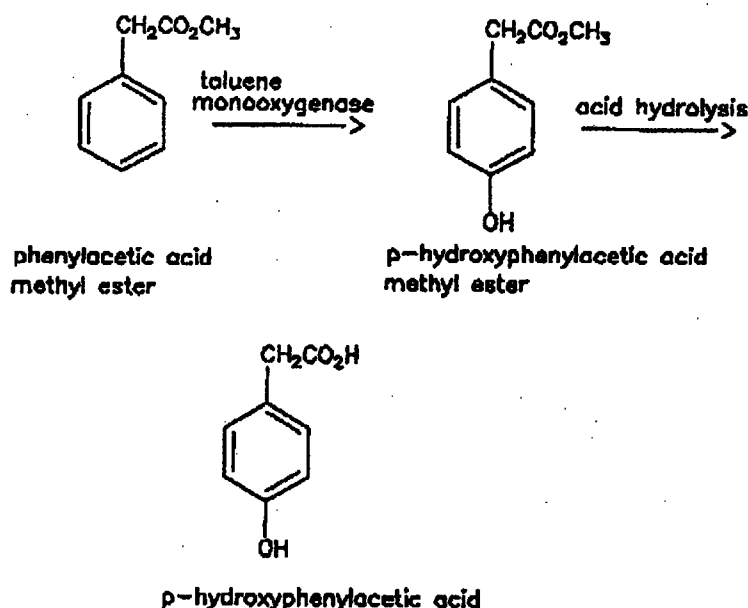


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EXAMPLE 14**Conversion of Methylphenylacetic Acid to
p-Hydroxyphenylacetic Acid****A. Conversion by PmKR1 Cells**

5 For the conversion of methylphenylacetic acid substrate to p-hydroxyphenylacetic acid, methylphenylacetic acid is mixed with PmKR1 grown as described in Example 12 and incubated for a period sufficient to effect the conversion of methylphenylacetic acid to p-hydroxymethylphenylacetic acid. In the second step, the cell

10 mixture containing the p-hydroxyphenylacetic acid intermediate is subjected to acid hydrolysis at acid concentrations and temperatures sufficient to convert the p-hydroxymethylphenylacetic acid to p-hydroxyphenylacetic acid. The conversion scheme is illustrated as follows:



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B. Conversion by Microorganism Host Cells Containing Recombinant Plasmids encoding PmKR1 Toluene Monooxygenase Genes

The same conversion according to Part A may be accomplished by using microorganism host cells containing the recombinant plasmids of Examples 10, 16, and 19 that carry a functional TMO gene cluster isolated from PmKR1. A preferred method is to use EcY5072 (HB101/pMY402) cells. Another preferred method is to use EcY5082 (FM5/pKMY287) or EcY5236 (FM5/pKMY336) cells. A particularly preferred method is to use PpY2511 (PpY2500/pKMY342) cells.

For the conversion as illustrated in Part A, methylphenylacetic acid is mixed with: EcY5072 (HB101/pMY402) cells grown and induced with IPTG, EcY5082 (FM5/pKMY287) or EcY5236 (FM5/pKMY336) cells grown and induced with heat, or, PpY2511 (PpY2500/pKMY342) cells grown and induced with sodium salicylate, as described in Example 12. The mixture is incubated for a period sufficient to effect the bioconversion of p-hydroxymethylacetic acid and then the mixture is subjected to acid hydrolysis at acid concentrations and temperatures sufficient to yield p-hydroxyphenylacetic acid.

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EXAMPLE 15

Conversion of Indole to Indigo

A. Conversion by PmKR1 Cells

For the conversion of indole substrate to indigo, 50 µg/ml indole was mixed with PmKR1 cells grown as described in Example 12 and incubated for a period sufficient to effect the conversion of indole to indigo, generally 48 hours. The indigo may be isolated



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from the cell mixture by the procedure described by Ensley in Example 5 of U.S. Patent No. 4,520,103.

5 B. Conversion by Microorganism Host Cells Containing
Recombinant Plasmids encoding PmKR1 Toluene
Monooxygenase Genes

The same conversion according to Part A may be accomplished by using microorganism host cells containing the recombinant plasmids of Examples 10 and 16 that carry a functional TMO gene cluster isolated from PmKR1. A preferred host strain is one that produces
10 indole endogenously in the presence of an inexpensive carbon source, such as glucose. An example of such a host is *E. coli* or a particular strain of *E. coli* with an enhanced rate of indole synthesis. A preferred method is to use EcY5082 (FM5/pKMY287) or EcY5236 (FM5/pKMY336) cells.

15 For the conversion as illustrated in Part A, EcY5082 (FM5/pKMY287) or EcY5236 (FM5/pKMY336) cells were grown in L-broth and induced with heat as described in Example 12. The mixture is incubated for a period sufficient to effect the bioconversion of indole to indigo. The indigo may be isolated from the cell mixture
20 according to the procedure of Part A.

EXAMPLE 16

Mapping and Nucleotide Sequence Analysis of ~~tmo~~ABCDEF Gene Cluster

A. Mapping

The region of the ~10.2 kb SacI fragment (see Example 10)
25 encoding TMO proteins was determined by deletion mapping. Deletion mapping was accompanied by DNA sequencing to reveal restriction sites. Various regions of the SacI fragment (Figures 3 and 4) were

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cloned individually into the *E. coli* expression vector pCFM1146 (Example 7) which can express foreign genes from a heat-inducible phage P_L promoter. Each of the recombinant plasmids was introduced into the *E. coli* strain FM5, which contains the integrated phage lambda repressor gene CI_{857} (Sussman and Jacob, Compt. Rend. Acad. Sci. 254: 1517-1519 (1962)), as described by Burnette et al., (supra). The resulting strains were assayed for TMO activity by the alternative assay described in Example 11 under induced and uninduced conditions.

10 The following plasmids were constructed for mapping: pMY421, pMY437, pKMY336, pMY448, pMY429, and pKMY340. Cloning of an -2.6 kb HindIII fragment of plasmid pMY401 (Example 10) containing the *tmoABCD* genes (Figure 3) into pUC19 (Yanisch-Perron et al., (supra)) produced plasmid pMY419. Substitution of the XbaI-Asp718 fragment of pKMY287 (previously designated pKY287 in Example 10) with a
15 corresponding XbaI-Asp718 fragment of pMY419 containing the *tmoAB* genes generated plasmid pMY421 (Figure 4).

Several intermediate plasmids were involved in the construction of pMY437 (Figure 4). Deletion of an -0.9 kb SspI
20 fragment downstream from the *tmoABCDE* genes in plasmid pMY401 (Example 10) produced plasmid pMY424. Insertion of a XhoI linker into the SspI site of pMY424 generated plasmid pMY436. Substitution of an Asp718-XhoI fragment of pMY421 containing the *tmoDE* genes with the corresponding Asp718-XhoI fragment of pMY436 generated plasmid
25 pMY437.

Construction of plasmid pMY448 (Figure 4) involved using the plasmids pMY476 and pKMY336. Insertion of the -0.8 kb HindIII

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fragment within the *tmoE* gene (Figure 4) into the SmaI site of pUC19 produced pMY476. Substitution of the Asp718-BamHI fragment of pMY421 containing the *tmoDE* genes with the longer Asp718-BamHI fragment of pKMY282 (previously designated pKY282 in Example 10; 5 Figures 3 and 4) containing the *tmoDEF* genes produced plasmid pKMY336. Substitution of the EspI-BamHI fragment of pKMY336 containing part of the *tmoE* gene with the ~0.76 kb EspI-BamHI fragment of pMY476 produced plasmid pMY448 (Figure 4). Deletion of an ~1.2 kb NcoI fragment from the 5' end of the *tmoA* gene in pKMY287 10 produced plasmid pMY429 (Figure 4).

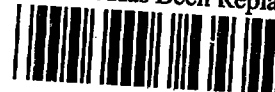
Construction of plasmid pKMY340 involved using the plasmids pKMY277 (previously designated pKY277 in Example 10) and pMY421 (described above). Deletion of an ~2.3 kb BamHI fragment from the region downstream from the *tmoABCDEF* gene cluster in pKMY277 15 generated a plasmid designated pKMY280 (previously designated pKY280 in Example 10). Replacement of the ~2 kb Asp718-BamHI fragment of pMY421 containing the *tmoDE* genes with the ~4.8 kb Asp718-BamHI fragment of pKMY280 containing the *tmoDEF* genes produced pKMY340.

Inducible TMO activity (as measured by the alternative assay 20 described in Example 11) was observed from a strain carrying any of the recombinant plasmids pKMY287 (Example 10), pMY437, pMY421, pKMY336 or pKMY340, but not from the strain carrying pMY429 or pMY448 (Figure 4, Table III). This result further demonstrated transcriptional direction of the TMO genes and defined more 25 precisely the minimal DNA region required for TMO activity. The TMO genes are transcribed from left to right based on the map shown in Figure 4. Plasmids pKMY287, pMY437 and pMY421 gave similar levels

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of TMO activity (Table III). Plasmids pKMY336 and pKMY340, each carrying the *tmoF* gene, along with the *tmoABCDE* genes gave enhanced levels of TMO activity (Table III). Among the recombinant plasmids yielding inducible TMO activity, pMY437 carries the smallest insert
5 (Figure 4). This insert is an ~3.6 kb fragment defined by the recognition sites of HindIII and SapI (Figure 4). The essential TMO genes are therefore located between these two sites on the ~10.2 kb SacI fragment.

There is a perfect correlation between the presence of TMO
10 activity and the indigo-producing capability among these strains tested. Indigo was produced only in strains having TMO activity but not in strains lacking TMO activity (Tables I and III). The indigo-plus strains all contain the intact *tmoABCDE* gene cluster and each of the indigo-minus strains misses an essential TMO component gene.
15 Indigo production can therefore serve as a good indicator for the presence of the TMO gene cluster when these genes are investigated.

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TABLE III

TMO Activities and Indigo-Forming Properties
of Recombinant *E. coli* Plasmids Carrying
Different PmKRI DNA Fragments.

Plasmid ^a	Specific Activity of TMO ^b (nmole min ⁻¹ mg ⁻¹)	Indigo Formation ^c
pCFM1146, Induced	0.1	-
10 pMY429, Uninduced	0.1	-
pMY429, Induced	0.1	-
pMY448, Uninduced	0.1	-
pMY448, Induced	0.1	-
15 pKMY287, Uninduced	2.0	+ --
PKMY287, Induced	7.0	+
pMY437, Uninduced	0.5	+
pMY437, Induced	7.3	+
pMY421, Uninduced	0.9	+
pMY421, Induced	10.0	+
20 pKMY336, Uninduced	0.6	+
PKMY336, Induced	19.0	+
pKMY340, Uninduced	0.7	+
PKMY340, Induced	20.0	+

25 ^aEach plasmid listed except pCFM1146 is the *E. coli* expression vector pCFM1146 carrying TMO genes. The different inserts in these plasmids are defined in Figure 4.

30 ^bTMO specific activities in toluene-induced and uninduced PmKRI cells are 30 and 0.5 nmole of non-volatile material formation from toluene per minute per mg of protein, respectively.

^c+, indigo formation; -, absence of indigo formation

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B. Sequencing

Initially, the XhoI-XhoI DNA region extending from coordinate 0.4 to 5.1 on the map shown in Figure 4 was sequenced in its entirety in both orientations. The nucleotide sequence of this XhoI fragment carrying the TMO genes was determined by the dideoxy method of Sanger et al., Proc. Natl. Acad. Sci. 74: 5463-67 (1977) on double-stranded DNA using the Sequenase™ DNA sequencing kit obtained from United States Biochemical Corporation, Cleveland, Ohio 44122. DNA samples were denatured in 0.2 M NaOH for 10 minutes and neutralized with 0.2 M ammonium acetate (pH 4.5) before use in the sequencing reactions. The -4.7 kb XhoI fragment and various deletion derivatives were cloned into pUC19 or pUC18 (Yanisch-Perron et al., (supra)) for DNA sequencing. Both commercially available and synthetic primers were used for sequencing reactions. The nucleotide sequence corresponding to the HindIII-SapI region required to give TMO activity is presented in Figure 5. Five open reading frames were identified in this region.

Each of the five open reading frames was confirmed by determining the N-terminal amino acid sequence of the corresponding gene product produced in E. coli from plasmid pMY421 (see Table VI in Example 18 below) and by cloning each of the regions containing an open reading frame and demonstrating corresponding activity (see Example 17 below). The genes defined by these five open reading frames were designated *tmoA*, *tmoB*, *tmoC*, *tmoD* and *tmoE* in the order of transcription (Figure 4). Part of a sixth open reading frame was also detected near the 3' end of the XhoI fragment. This led to further sequence analysis of the region downstream of the *tmoABCDE*

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gene cluster. The sequence of the entire sixth open reading frame is also shown in Figure 5. This sixth open reading frame was designated *tmoF*, continuing in the order of transcription. This open reading frame was confirmed by determining the N-terminal amino acid sequence of the corresponding gene product in *E. coli* from plasmid pMY440 (see Table VI in Example 18 below). Plasmid pMY440 encodes the sixth open reading frame and expresses a functional TmoF protein in the *E. coli* FM5 host cells. It was constructed by deleting the ~3.4 kb *HindIII* fragment from the 5' end of the *tmoABCDEF* gene cluster in pKMY336.

The TMO genes are organized as a very closely-spaced cluster. In addition to the Shine-Delgarno (S-D) sequence (*Nature* 254: 34-38 (1975)) preceding each gene, only very short non-coding regions separate the genes (Figure 5). Downstream from the *tmoABCDE* gene cluster, there is a GC-rich region of dyad symmetry followed by a series of thymidine residues (Figure 5), a structure characteristic of a rho-independent transcription terminator (Platt, *Ann. Rev. Biochem.* 55: 339-372 (1986)). However, further downstream, is a sixth open reading frame encoding TmoF. The fact that the plasmids pKMY336 and pKMY340 gave higher TMO activity than did the other plasmids as shown above in Table III (Part A of this Example) indicates transcriptional read-through at the termination region described above.

The base composition of the *tmoABCDEF* cluster is unusual for *P. mendocina* genes. The G+C content of the DNA fragment presented in Figure 5 is 48.8%. This low value is significantly different from the reported G+C content of 62.8-64.3% for the *P. mendocina*

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genome (Palleroni, et al., J. Gen. Microbiol. 60: 215-231 (1970)). Consistent with the low G+C content, there is no codon usage preference for guanine and cytosine at the third position of each codon in the TMO genes (Table IV). The PmKR1 strain, initially typed as described in Whited (supra), has been retyped and the identity of this strain has been confirmed. A simple explanation of the low G+C content of the TMO genes is that these genes originated from another bacterial species, that were later transferred into P. mendocina on a plasmid. Consistent with such a hypothesis is the observation that the toluene utilizing property can be transferred from PmKR1 to P. putida KT2440. This observation suggested the presence of the toluene plasmid pAUT1 (Table I of Example 11) in PmKR1.

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TABLE IV

Codon Usage of the *tmcABCDEF* Genes.

5	Amino Acid	Codon	A	B	C	D	E	F	Total
10	Gly	GGG	4	0	1	0	1	9	15
	Gly	GGA	5	0	3	3	1	5	17
	Gly	GGU	14	1	3	2	5	6	31
	Gly	GGC	10	1	4	1	1	3	20
	Glu	GAG	15	5	5	2	14	12	53
	Glu	GAA	24	3	7	10	12	17	73
	Asp	GAU	19	5	5	5	13	8	55
	Asp	GAC	15	1	3	2	6	4	31
15	Val	GUG	4	4	1	2	3	10	24
	Val	GUA	5	3	4	1	3	6	22
	Val	GUU	9	6	0	2	1	3	21
	Val	GUC	4	1	3	0	5	5	18
20	Ala	GCG	7	2	1	1	3	9	23
	Ala	GCA	18	2	1	3	5	7	36
	Ala	GCU	7	2	1	5	10	6	31
	Ala	GCC	12	1	2	1	5	5	26
25	Arg	AGG	1	0	0	1	2	1	5
	Arg	AGA	1	1	0	0	1	1	4
	Ser	AGU	4	0	2	0	10	3	19
	Ser	AGC	8	1	3	1	7	1	21
30	Lys	AAG	14	1	2	0	8	7	32
	Lys	AAA	11	2	3	3	7	11	37
	Asn	AAU	8	2	2	3	7	9	31
	Asn	AAC	9	1	2	3	6	7	28
	Met	AUG	21	4	3	3	12	8	51
	Ile	AUA	3	1	0	1	1	1	7
	Ile	AUU	7	0	4	3	7	11	32
	Ile	AUC	14	1	4	6	2	4	31
35	Thr	ACG	4	0	1	0	3	3	11
	Thr	ACA	4	0	3	3	3	2	15
	Thr	ACU	3	2	1	1	4	5	16
	Thr	ACC	11	1	2	2	5	3	24
40	Trp	UGG	22	0	2	0	13	2	39
	End	UGA	1	0	0	0	1	0	2
	Cys	UGU	1	1	3	0	1	2	8
	Cys	UGC	3	0	2	0	3	5	13

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TABLE IV Continued

	Amino Acid	Codon	A	B	C	D	E	F	Total
5	End	UAG	0	1	0	0	0	0	1
	End	UAA	0	0	1	1	0	1	3
	Tyr	UAU	14	0	1	1	6	6	28
	Tyr	UAC	8	1	3	1	8	4	25
10	Leu	UUG	10	1	0	4	10	4	29
	Leu	UUA	2	1	3	1	1	4	12
	Phe	UUU	12	3	1	2	4	11	33
	Phe	UUC	12	2	2	2	4	6	28
	Ser	UCG	4	1	0	0	4	6	15
15	Ser	UCA	3	1	0	0	4	6	14
	Ser	UCU	4	0	1	0	2	2	9
	Ser	UCC	4	1	2	1	3	5	16
	Arg	CGG	2	2	0	1	1	1	7
	Arg	CGA	0	1	0	1	3	2	7
20	Arg	CGU	15	3	0	1	6	7	32
	Arg	CGC	7	0	1	3	6	1	18
	Gln	CAG	14	1	2	5	11	5	38
	Gln	CAA	7	1	0	1	7	4	20
	His	CAU	9	2	3	1	3	4	22
25	His	CAC	6	1	2	0	10	1	20
	Leu	CUG	9	1	1	2	15	11	39
	Leu	CUA	3	1	1	1	3	6	15
	Leu	CUU	7	1	1	0	8	5	22
	Leu	CUC	4	0	1	1	1	6	13
30	Pro	CCG	9	0	1	3	5	7	25
	Pro	CCA	5	3	1	1	5	4	19
	Pro	CCU	5	1	1	0	2	5	14
	Pro	CCC	4	0	1	0	5	2	12

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EXAMPLE 17Identification of *tmoABCDE* as Essential TMO Genes

A. Plasmid pKMY341 and Single-gene Mutant Derivatives

To determine if each of the designated *tmoABCDE* genes as
5 described in Example 16 encodes a TMO protein component necessary
for TMO activity, a single mutation was introduced into each of the
genes and its effect on TMO activity was determined. A plasmid,
pKMY341, was initially constructed by cloning the *tmoABCDE* genes
into the *E. coli* plasmid T7-5, followed by introducing a DNA
10 sequence change into the individual genes. The pT7-5 plasmid is a
ColEI-based plasmid containing the β -lactamase gene and a multiple
cloning site downstream from a T7 RNA polymerase-specific promoter
obtained from S. Tabor (see, e.g., Tabor and Richardson, Proc. Natl.
Acad. Sci. 82: 1074-1078 (1985)).

15 The plasmid pKMY341 carrying the *tmoABCDE* genes was
constructed by cloning the -4.7 kb *XbaI*-*BamHI* fragment of pKMY336
(Example 16) into the *XbaI* and *BamHI* sites of pT7-5. The plasmids
pMY459, pMY458, pMY482, pMY484, and pMY472 carry mutations in the
tmoA, *tmoB*, *tmoC*, *tmoD*, *tmoE* genes, respectively. Cleavage of the
20 *NcoI* site in the *tmoA* gene (Figure 4) of pKMY341 followed by end-
filling and ligation generated plasmid pMY459. The same treatment
at the *NcoI* site in the *tmoB* gene, at the *Asp718* site in the *tmoC*
gene, and at the *ClaI* site in the *tmoD* gene, generated the mutations
in plasmids pMY458, pMY482, and pMY484, respectively (Figure 4).
25 Cleavage of the *BanII* site in the *tmoE* gene (Figure 4) followed by
removing the overhangs and ligation generated the mutation in
plasmid pMY472.

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Enzyme assays revealed that each of the mutations completely eliminated TMO activity in *E. coli* cells as shown in Table V.

TABLE V

Complementation Between Individually Cloned *tmo* Genes and the *tmoABCDE* Gene Cluster Carrying Corresponding Mutations.

	Bacterial Strain (EcY#) ^a	Plasmid ^b	<i>tmo</i> Genes	Specific Activity of TMO (nmole min ⁻¹ mg ⁻¹)
5				
10	5246	pKMY341	ABCDE	10.7
	5283	pMY459	A ⁻ BCDE	0.06
	5282	pMY458	AB ⁻ CDE	0.04
	5286	pMY482	ABC ⁻ DE	0.08
15	5287	pMY484	ABCD ⁻ E	0.05
	5285	pMY472	ABCDE ⁻	0.10
	5258	pMY438	A	0.09
	5265	pMY447	B	0.03
	5288	pMY474	C	0.04
20	5289	pMY479	D	0.02
	5224	pKMY327	E	0.04
	5291	pMY438, pMY459	A, A ⁻ BCDE	2.0
	5290	pMY447, pMY458	B, AB ⁻ CDE	3.9
25	5297	pMY474, pMY482	C, ABC ⁻ DE	7.4
	5298	pMY479, pMY484	D, ABCD ⁻ E	2.0
30	5292	pKMY327, pMY472	E, ABCDE ⁻	10.3

^aEach of the strains was constructed by introducing an appropriate plasmid or plasmids into the *E. coli* strain HB101. All cultures were grown in the presence of 0.35 mM sodium salicylate which induced the TMO genes cloned into pKMY319.

^bConstruction of all plasmids is described in Examples 16 and 17.

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B. Complementation Tests Between Mutant Derivatives of Plasmid pKMY341 and Plasmids Carrying a Single TMO Gene

To determine whether lack of TMO activity was due to lack of
5 expression of the TMO genes in the presence of a polar mutation
which itself is not located in a TMO gene, complementation tests
between plasmids pMY459, pMY458, pMY482, pMY484 and pMY472
(constructed as described in part A above) and plasmids carrying
only one of the *tmoABCDE* genes were conducted. Plasmids pMY438,
10 pMY447, pMY474, pMY479, and pKMY327 were constructed to contain the
tmoA, *B*, *C*, *D*, and *E* genes, respectively, in the broad host range
expression vector pKMY319. Plasmid pKMY319 is described and claimed
in co-pending and co-assigned U.S. Patent Application Serial No.
07/590,280, filed on September 28, 1990, hereby incorporated by
15 reference in its entirety. Plasmid pKMY319 is a plasmid vector in
which expression of foreign genes can be regulated by the NahR
protein and an inducer, such as sodium salicylate.

Plasmids pMY438, pMY447, pMY474, pMY479, and pMY327 are
derivatives of pKMY319 that carry *tmoA*, *tmoB*, *tmoC*, *tmoD*, and *tmoE*,
20 respectively, and were constructed as follows. For the construction
of plasmid pMY438, pMY430 was initially constructed by cloning an
-1.6 kb DraI fragment containing the *tmoA* gene (Figure 4) into the
SmaI site of pUC19 in an orientation that placed the XbaI site of
pUC19 at the 5' end of the *tmoA* gene. Plasmid pMY438 was
25 constructed by cloning the -1.6 kb XbaI-SacI fragment of pMY430
carrying the *tmoA* gene into the XbaI and SacI sites of pKMY319.

Plasmids pKMY332 and pMY446 were used in the construction of
pMY447. The plasmid pKMY332 was obtained by deleting an -2 kb HpaI-

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XmnI fragment of pKMY287 at the 5' end of the *tmo* gene cluster (Figure 4). Cloning of an ~0.7 kb XbaI-Asp718 fragment of pKMY332 containing the *tmoB* gene into the XbaI and Asp718 sites of the pKMY319 produced plasmid pMY446. Further deletion of an ~0.2 kb XbaI-NdeI fragment upstream from the *tmoB* gene in pMY446 (Figure 4) generated plasmid pMY447.

Construction of plasmid pMY474 involved constructing intermediate plasmids pMY414, pMY426, pMY452, and pMY466. Cloning of an ~0.4 kb Asp718-ClaI fragment of pKMY282 containing the 3' end portion of the *tmoC* gene (Figure 4) into the Asp718 and AccI sites of pUC19 produced intermediate plasmid pMY414. Deletion of an ~0.1 kb BspMI-SphI fragment downstream from the *tmoC* gene (Figure 4) generated intermediate plasmid pMY426. Ligation of a SacI linker to an end-filled HindIII site in pMY426 and insertion of the Asp718-SacI fragment of pMY426 into pKMY319 generated intermediate plasmid pMY452. Cloning of an ~1.9 kb ClaI-Asp718 fragment of pKMY341 containing the *tmoAB* genes and the 5' end of the *tmoC* gene (Figure 4) into the ClaI and Asp718 sites of pMY452 produced intermediate plasmid pMY466. Deletion of a 1.8 kb ClaI fragment upstream from the *tmoC* gene in pMY466 generated plasmid pMY474.

Intermediate plasmids pMY404, pMY470, and pMY478 were generated and used in the construction of plasmid pMY479. Cloning of the ~4.7 kb BamHI-SphI fragment of pMY401 carrying the *tmoABCDE* genes into the plasmid pUC18 (Yanisch-Perron et al., (supra)) produced intermediate plasmid pMY404 (see Example 10). Deletion of the ~3 kb BspMI fragment of pMY404 carrying the *tmoABC* genes (Figure 4) generated intermediate plasmid pMY470. An ~0.4 kb HindIII

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fragment of pMY470 carrying the *tmoD* gene (Figure 4) was end-filled and ligated along with another end-filled 0.8 kb HindIII fragment of pMY470 into the SmaI site of pUC19 (Yanisch-Perron et al., (supra)) to form intermediate plasmid pMY478. In pMY478, a NheI site was
5 generated upstream from the *tmoD* gene as a result of ligation of two end-filled HindIII sites. Cloning of the ~0.4 kb NheI-SacI fragment of pMY478 carrying the *tmoD* gene into the XbaI and SacI sites of pKMY319 generated plasmid pMY479.

Plasmid pKMY327 was constructed from the intermediate plasmid
10 pKMY324. Cloning of the XbaI fragment of pKMY282 carrying the *tmoABCDE* genes into pKMY319 produced intermediate plasmid pKMY324. Deletion of an ~3.1 kb Clal fragment of pKMY324 upstream from the *tmoE* gene (Figure 4) generated plasmid pKMY327.

Each of these plasmids was introduced by transformation into
15 a strain which contained a corresponding member of the plasmids pMY459, pMY458, pMY482, pMY484, and pMY472 and the TMO activity in each of the resulting strains was determined after induction as shown in Table V above. A plasmid carrying a mutation in any member of the *tmoABCDE* genes was complemented by the plasmid which carries
20 that particular gene in synthesizing the TMO enzyme (Table V). This result demonstrated that the mutation in each of the *tmo* genes, did not abolish the expression of the downstream *tmo* genes. Since each of the mutations prevented the synthesis of a functional TMO system (Table V), this indicated that each of the *tmoABCDE* genes plays an
25 essential role in directing the synthesis of the TMO enzyme system.

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EXAMPLE 18

Purification and Analysis of TMO Proteins

TMO proteins except TmoF were partially purified from PmKR1 or E. coli FM5 cells carrying the plasmid pMY421 by DEAE cellulose chromatography according to Whited, (supra), except that TEGD buffer (50 mM Tris, pH 7.45; 10% glycerol; 10% ethanol; 1 mM dithiothreitol) instead of PEG buffer was used in the column. The TmoF protein was purified from E. coli FM5 cells carrying the plasmid pMY440, by isolating the protein after polyacrylamide gel electrophoresis of the FM5/pMY440 lysate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli, Nature 227: 680-685 (1970). Protein samples were heated at 65°C for 15 minutes in a loading buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, and 62.5 mM Tris-Cl (pH 6.8) before they were loaded on the gel.

For determination of N-terminal amino acid sequences, partially purified TMO proteins were further purified by SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane according to Matsudaira, J. Biol. Chem. 262: 10035-38 (1987) with slight modifications. The protein bands immobilized on the polyvinylidene difluoride membrane were visualized by Coomassie blue staining and destained with 50% methanol and 7% acetic acid. The stained bands were excised with a razor blade and sequenced in an Applied Biosystems Model 477 automated protein sequencer as described by Lu et al., Int. J. Peptide Protein Res. 33: 237-49 (1989).

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Conditions to generate tryptic fragments were described by Klein et al., Arch. Biochem. Biophys. 276: 531-537 (1990). The tryptic peptides were isolated by reverse-phase HPLC in an acetonitrile-trifluoroacetic acid gradient elution system. The
5 isolated peptides were pooled and loaded onto a glass-fiber disc precycled with polybrene for automatic sequence analysis. In situ cyanogen bromide cleavage of protein samples and sequence analysis of the cleaved mixture were also described by Klein et al., (supra).

The N-terminal amino acid sequences of the *tmoA*, B, C, D, E
10 and F gene products are shown in Table VI. These N-terminal amino acid sequences for each of the TmoA, B, C, D and E proteins encoded by the HindIII-SspI region and by the HindIII-XmaI region for the TmoF protein (as described in Example 16 and Table IV) agrees completely with that predicted from the nucleotide sequence of the
15 corresponding open reading frame (Figure 5). The estimated molecular weights of the purified TmoA, B, C, D, E and F proteins are also shown in Table VI, along with their predicted molecular weights based on the nucleotide sequence of the *tmoA*, B, C, D, E and F genes shown in Figure 5. The observed and expected protein
20 molecular weights correlate closely for the *tmoA*, B, C, D, and E gene products, but not for the *tmoC* gene product. The nucleotide sequence of the *tmoC* gene has capacity to encode a protein with a molecular weight of approximately 12,000 as shown in Table VI. However, on SDS-PAGE the observed molecular weight of the TmoC
25 protein was approximately 25,000 (Table VI), almost exactly twice the size of the expected molecular weight. A 25 kilodalton (kDa) protein was also isolated from PmkR1 and identified to be the same

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25 kDa *tmoC* product isolated from the recombinant *E. coli* by comparing the N-terminal amino acid sequences. Further characterization of the 25 kDa protein demonstrated that it is encoded solely by the *tmoC* gene. This protein was isolated from *E. coli* and further purified from SDS polyacrylamide gels. Fragments of the protein were obtained in separate experiments after trypsin digestion or cyanogen bromide treatment. N-terminal amino acid sequence analysis demonstrated that all of the fragments were cleavage products of the TmoC protein. This result suggested that the 25 kDa protein was a dimer of the *tmoC* product not fully reduced under the conditions used for the SDS-PAGE.

TABLE VI

N-terminal Sequence and Molecular Weights
of the *tmoABCDEF* Gene Products Produced
from Recombinant *E. coli* FM5 Host Cells
Carrying Plasmid pMY421^a or pMY440^b

15

Gene	Region of Nucleotide Sequence	Product Molecular Weight		N-terminal Sequence Determined From Purified Protein ^c
		Predicted From Nucleotide Sequence	Estimated From Purified Protein	
25	<i>tmoA</i> ^a 37-1536	57,982	55,000	AMHPRKXDWYELTR
	<i>tmoB</i> ^a 1558-1809	9,588	9,500	SAFPVHAAFEKDFLVQLVV
	<i>tmoC</i> ^a 1818-2153	12,326	25,000	VDLNDSDMQVA
	<i>tmoD</i> ^a 2217-2525	11,618	11,500	SFEKIXSLDDIWVGEMETPETS
30	<i>tmoE</i> ^a 2539-3519	38,386	35,000	STLADQALHNNNVGPIIR
	<i>tmoF</i> ^b 3548-4575	35,983	38,000	IIRAGD
				SFESKXPMRTWSXL
				MFNIQSDDLHHE

^cThe letter X indicates undetermined amino acid.

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The definitive functions of each of the TMO genes is not known. However, there is evidence at least to suggest a role for the *tmoC* gene. Comparison of the amino acid sequences deduced from the TMO genes with those of known proteins reveal homology between the *tmoC* product and several other ferredoxin proteins functioning in dioxygenase systems. Among the 114 amino acid residues in the TmoC protein, 36 residues (31.6%) are identical to those of the benzene dioxygenase ferredoxin protein at corresponding positions and 14 residues (12.3%) are represented by evolutionarily related amino acids in the benzene dioxygenase ferredoxin at corresponding positions (Figure 6). Similar homology exists between the TmoC protein and the naphthalene dioxygenase ferredoxin protein (Figure 6). The ferredoxin component of the toluene dioxygenase system from PpFl differs from the benzene ferredoxin protein by only six amino acid residues (Zylstra and Gibson, J. Biol. Chem. 264: 14940-46 (1989)). It therefore shares similar homology with the TmoC protein. The region of maximum homology between the TmoC protein and the other ferredoxins is located between positions 53 and 77 (Figure 6). Among the 23 amino acid residues in this region, the TmoC protein shares 10 (43%) with the benzene dioxygenase ferredoxin and 9 (39%) with the naphthalene dioxygenase ferredoxin (Figure 6). In addition, the two dioxygenase ferredoxins share 13 (56.5%) amino acids in this region (Figure 6). It is interesting to note that this region contains two conserved cysteine residues (at positions 53 and 74, respectively) each of which is followed by a conserved histidine in the vicinity. Benzene dioxygenase ferredoxin (Geary et al., supra) and toluene dioxygenase ferredoxin (Subramanian et

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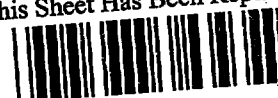
al., J. Biol. Chem. 260: 2355-63 (1985)), each contains a single [2Fe-2S] cluster with a redox potential significantly higher than [2Fe-2S] clusters coordinated to four cysteine residues. It has been suggested by Cline et al., J. Biol. Chem. 260: 3251-54 (1985),
5 that in addition to cysteines, histidine residues may provide nitrogen ligands to the [2Fe-2S] cluster which may contribute to the higher redox potential of the cluster. It is likely that this region of maximum homology between the two dioxygenase ferredoxins is involved in the binding of the [2Fe-2S] cluster. The fact that
10 the TmoC protein shares overall homology with these two ferredoxins and does so especially in this region suggests that it is a ferredoxin of the toluene monooxygenase system.

EXAMPLE 19

15 Degradative Bioconversion of TCE by Microorganism
Host Cells Containing Recombinant Plasmids Carrying
PmKRI Toluene Monooxygenase Genes

TCE degradation catalyzed by the toluene monooxygenase of PmKRI has been demonstrated by Winter et al., Bio/Technology 1: 282-285 (1989) and claimed in co-assigned U.S. Patent Application Serial
20 No. 177,640 incorporated by reference. Plasmid pKMY342 is plasmid pKMY319 carrying two copies of the *tmoABCDEF* gene cluster and is described in co-pending and co-assigned U.S. Patent Application
Serial No. 07/590,280, filed on September 28, 1990. It gives higher
TMO activity than the recombinant plasmids previously described for
25 TCE degradation and can replicate in all Gram-negative bacteria tested to date. It is therefore a particularly preferred and improved plasmid for use in TCE degradation. Other recombinant

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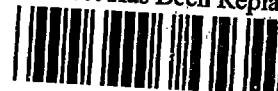


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plasmids such as pKMY336 and pKMY340 (Example 16) that give higher TMO activity than the plasmids described in U.S.S.N. 177,640 are also useful for TCE degradation.

In a particularly preferred embodiment, Pseudomonas putida
5 Y2511 cells harboring recombinant plasmid pKMY342 are grown to mid-log phase in L-broth with 0.35 mM sodium salicylate as inducer. The cells are washed in L-broth for the degradative bioconversion of TCE, as follows. Cells are resuspended to an OD₃₅₀ of ~0.5 in L-broth, and 4 ml of the cell suspension is added to serum vials. TCE
10 (Aldrich, (Milwaukee, Wisconsin), spectrophotometric grade) is diluted in N,N-dimethylformamide (DMF) (Aldrich, spectrophotometric grade) to 10 mM or 20 mM and 4 µl added to cell suspension to give a final TCE concentration of 10 µM (1.3 ppm) or 20 µM (2.6 ppm). Vials are stoppered, vortexed, and 10 µl of gas phase are withdrawn
15 using a gas-tight syringe at various times for analysis. Gas phase samples are analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a 25 meter 5% phenyl methyl silicone column (Hewlett Packard, Palo Alto, California) and a ⁶³Ni electron capture detector. The injector, oven, and detector temperatures are 120°, 100°, and
20 300°, respectively. The carrier gas is helium and the makeup gas is 95% argon-5% methane. Peak areas are calculated by a Hewlett-Packard 3392A Integrator. Data are reported as the percentage of TCE remaining at various times after addition to the cell suspension. The amount of TCE present at zero time is taken to be
25 100%.

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WHAT IS CLAIMED IS:

1. An isolated *tmoABCDE* gene complex.
2. An isolated *tmoABCDEF* gene complex.
3. A recombinant plasmid comprising an isolated *tmoABCDE* gene complex.
4. A recombinant plasmid comprising an isolated *tmoABCDEF* gene complex.
5. A recombinant plasmid comprising a DNA segment containing a toluene monooxygenase gene which encodes a Tmo protein.
6. A recombinant plasmid according to Claim 5, wherein the toluene monooxygenase gene encodes a Tmo A protein.
7. A recombinant plasmid according to Claim 5, wherein the toluene monooxygenase gene encodes a TmoB protein.
8. A recombinant plasmid according to Claim 5, wherein the toluene monooxygenase gene encodes a TmoC protein.
9. A recombinant plasmid according to Claim 5, wherein the toluene monooxygenase gene encodes a TmoD protein.
10. A recombinant plasmid according to Claim 5, wherein the toluene monooxygenase gene encodes a TmoE protein.
11. A recombinant plasmid according to Claim 5, wherein the toluene monooxygenase gene encodes a TmoF protein.
12. A microorganism host cell transformed with the recombinant plasmid according to Claim 3.
13. A microorganism host cell transformed with the recombinant plasmid according to Claim 4.
14. A microorganism host cell transformed with the recombinant plasmid according to Claim 5.

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15. A microorganism host cell transformed with the recombinant plasmid according to Claim 6.

16. A microorganism host cell transformed with the recombinant plasmid according to Claim 7.

17. A microorganism host cell transformed with the recombinant plasmid according to Claim 8.

18. A microorganism host cell transformed with the recombinant plasmid according to Claim 9.

19. A microorganism host cell transformed with the recombinant plasmid according to Claim 10.

20. A microorganism host cell transformed with the recombinant plasmid according to Claim 11.

21. A DNA sequence encoding substantially the same amino acid sequences for proteins having substantially the same activity as represented in Figure 5 for *tmoABCDEF* gene products.

22. A DNA sequence for *tmoABCDEF* genes as represented in Figure 5.

23. An isolated protein having an amino acid sequence of a TmoA protein as represented in Figure 5, or having substantially the same amino acid sequence and substantially the same activity of the TmoA protein as represented in Figure 5.

24. An isolated protein having an amino acid sequence of a TmoB protein as represented in Figure 5, or having substantially the same amino acid sequence and substantially the same activity of the TmoB protein as represented in Figure 5.

25. An isolated protein having an amino acid sequence of a TmoC protein as represented in Figure 5, or having substantially the

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same amino acid sequence and substantially the same activity of the TmoC protein as represented in Figure 5.

26. An isolated protein having an amino acid sequence of a TmoD protein as represented in Figure 5, or having substantially the same amino acid sequence and substantially the same activity of the TmoD protein as represented in Figure 5.

27. An isolated protein having an amino acid sequence of a TmoE protein as represented in Figure 5, or having substantially the same amino acid sequence and substantially the same activity of the TmoE protein as represented in Figure 5.

28. An isolated protein having an amino acid sequence of a TmoF protein as represented in Figure 5, or having substantially the same amino acid sequence and substantially the same activity of the TmoF protein as represented in Figure 5.

29. A microbial enzymatic method for making p-hydroxyphenylacetic acid from methylphenylacetic acid comprising (a) reacting methylphenylacetic acid with microorganism host cells according to Claim 12, the microorganism host cells having been
5 treated with an inducer of toluene monooxygenase genes, and (b) hydrolyzing with acid for a sufficient time until the methylphenylacetic acid is substantially converted to p-hydroxyphenylacetic acid.

30. A microbial enzymatic method for making indigo from indole comprising using microorganism host cells according to Claim 12, the microorganism host cells having been treated with an inducer of toluene monooxygenase genes.

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31. An improved method for the microbial degradation of TCE comprising reacting a TCE-containing substance with microorganism host cells according to Claim 13, the microorganism host cells having been treated with an inducer of toluene monooxygenase genes.



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