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6 AUTHOR(S)
David T. Gibson

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7 PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Univ of Iowa
202 Jessup Hall
Iowa City, IA 52242

8 PERFORMING ORGANIZATION REPORT NUMBER
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13. ABSTRACT (Maximum 200 words)

The absolute stereochemistry of the chiral dihydrodiols formed from ortho- and meta-dichlorobenzene were determined. Both diols were found to be enantiomerically pure with 1S,2S absolute configuration. Toluene-grown cells of Pseudomonas putida F1 and Pseudomonas sp. JS150 were found to oxidize 2- and 3-nitrotoluene to benzyl alcohols. These results represent the first demonstration of the oxidation of a methyl substituent by toluene dioxygenase. Both organisms oxidized 4-nitrotoluene to 2-methyl-5-nitrophenol and 3-methyl-6-nitrocatechol. The significance of these unexpected results was evaluated.

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# FINAL REPORT SUMMARY

FOR

GRANT NO. AFOSR-88-0225

# A BIOTECHNICAL APPROACH TO STUDIES ON THE BIODEGRADATION OF CHLOROBENZENES AND TRICHLOROETHYLENE

Submitted by

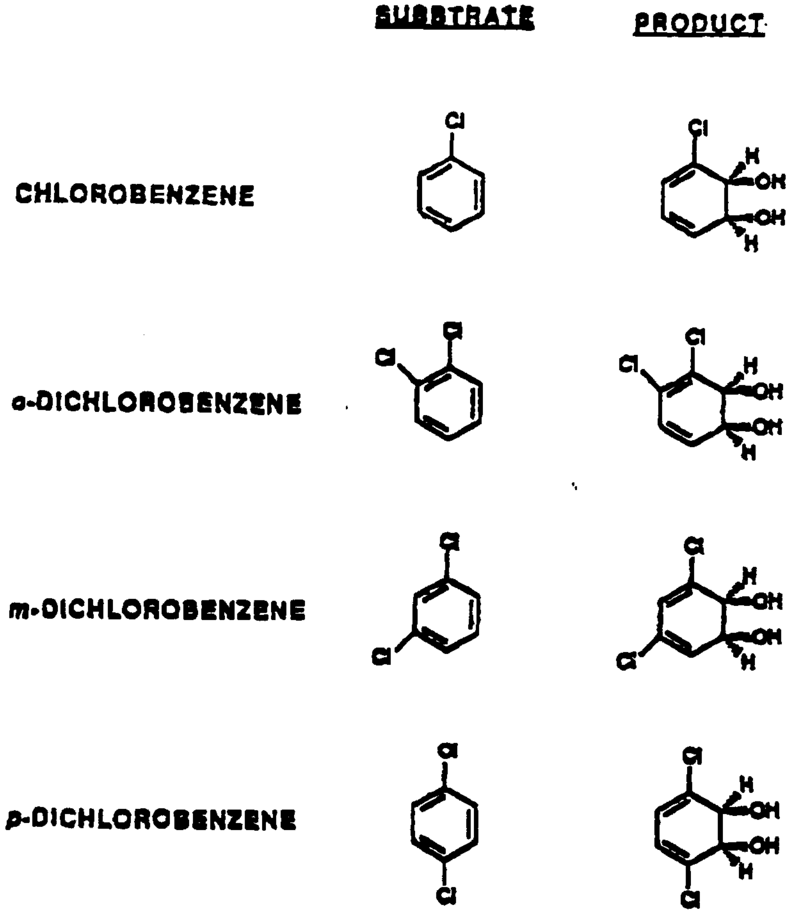
David T. Gibson, Principal Investigator
Department of Microbiology
Room 3-669 Bowen Science Building
The University of Iowa
Iowa City, Iowa 52242

Telephone: (319) 335-7980

FAX: (319) 335-9999

## 1. Degradation of Chlorobenzenes by *Pseudomonas putida*

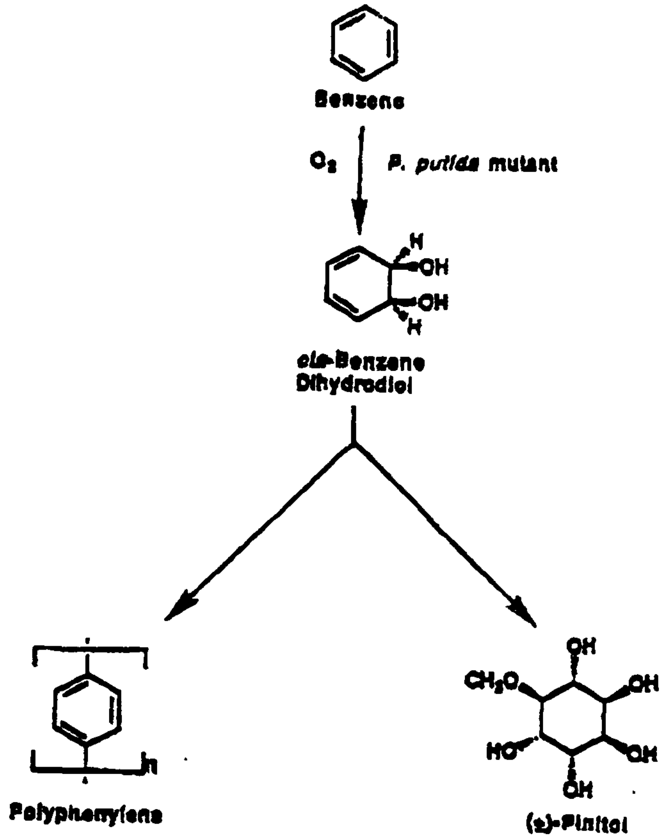
Our previous studies have shown that toluene-grown cells of *Pseudomonas putida* F39/D oxidize chlorobenzene, *ortho*-, *meta*-, and *para*-dichlorobenzene to dihydrodiols in which the hydroxyl groups have a *cis* relative stereochemistry. These reactions are shown below.

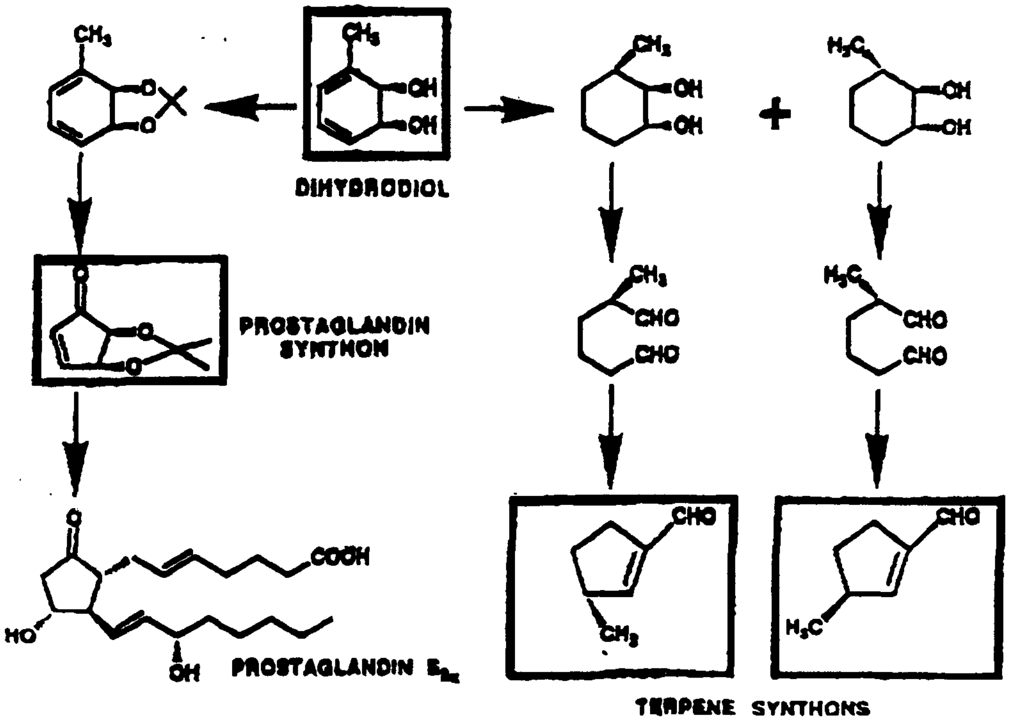


The absolute stereochemistry of chlorobenzene dihydrodiol has been determined previously. During the current grant period we have determined the absolute stereochemistry of the chiral dihydrodiols formed from *ortho*- and *meta*- dichlorobenzene. These studies, which were carried out in collaboration with Dr. Derek Boyd, Queen's University, Belfast, Northern Ireland, involved reacting the dihydrodiols formed by *P. putida* F39/D with 4-phenyl-1,2,4-

triazoline-3,5-dione. These Diels-Alder adducts were then converted to single crystalline diesters with chiral $\alpha$-methoxy-$\alpha$-(trifluoromethyl)-phenylacetic acid (MTPA). NMR analyses of the diMTPA diastereoisomers showed that both dihydrodiols are enantiomerically pure (>98% enantiomeric excess) and have a 1S,2S absolute configuration which is the same configuration found in the *cis*-dihydrodiol formed from chlorobenzene.

The significance of these studies relates not only to the base of knowledge required for the development of bioremediation technology, but also to the recent use of dihydrodiols formed by bacteria as chiral synthons in the production of specialty chemicals. For example, Hudlicky et. al., (Tetrahedron Letters, 30: 4053-4054, 1989) have shown that the enantiomerically pure *cis* dihydrodiol formed from chlorobenzene by *P. putida* 39/D can be used to synthesize D- and L- erythrose. The same group has used *cis*-toluene dihydrodiol to synthesize prostaglandin $E_{2\alpha}$ and terpenes with potential antitumor properties (Hudlicky et. al., J. Am. Chem. Soc., 110: 4735-4741, 1988). *cis*-Benzene dihydrodiol, formed by *P. putida*, has been used to synthesize the semiconductor polyphenylene (Ballard et. al., J. Chem. Soc. Chem., Commun. 954-955, 1983) and precursors of the physiologically-important inositol phosphates (Ley et. al., Tetrahedron Lett. 28:225-226, 1987). These reactions are summarized on the following page. In the past three years there have been many papers on the chemistry of enantiomerically-pure dihydrodiols formed by bacteria. Consequently, it is possible that many useful new products will be synthesized from aromatic hydrocarbons and their halogenated derivatives.


Benzene
O2
P. putida mutant
H
OH
OH
H
cis-Benzene Dihydrodiol
n
Polyphenylene
OH
CH3O
OH
HO
OH
OH
(±)-Pinitol

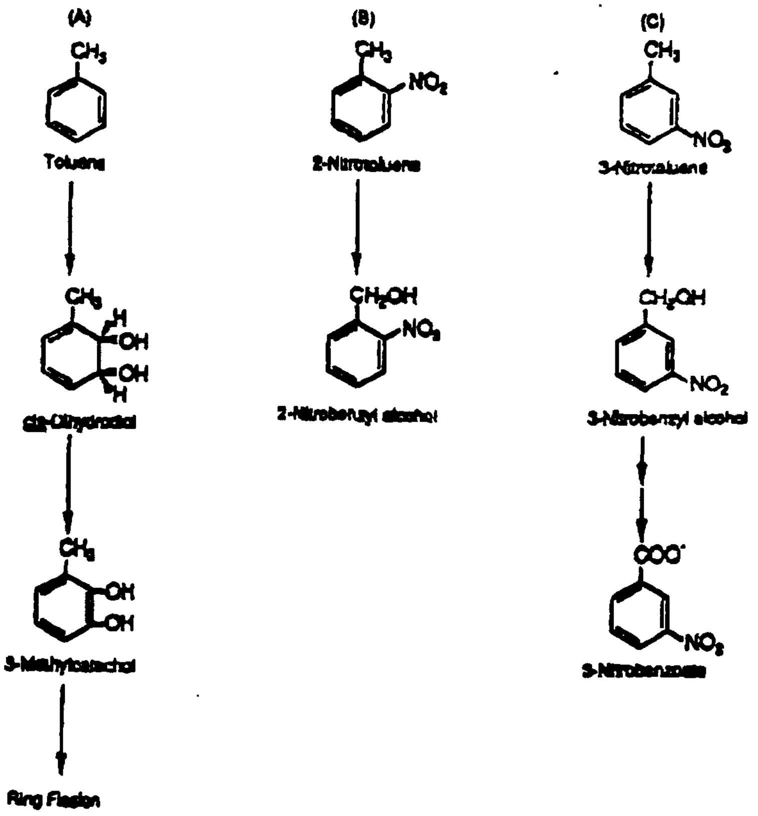

CH3
O
O
CH3
OH
OH
DIHYDRODIOL
CH3
OH
OH
+
H3C
OH
OH
O
O
O
PROSTAGLANDIN SYNTHON
CH3
CHO
CHO
H3C
CHO
CHO
O
COOH
HO
OH
PROSTAGLANDIN E2α
CHO
CH3
CHO
H3C
TERPENE SYNTHONS

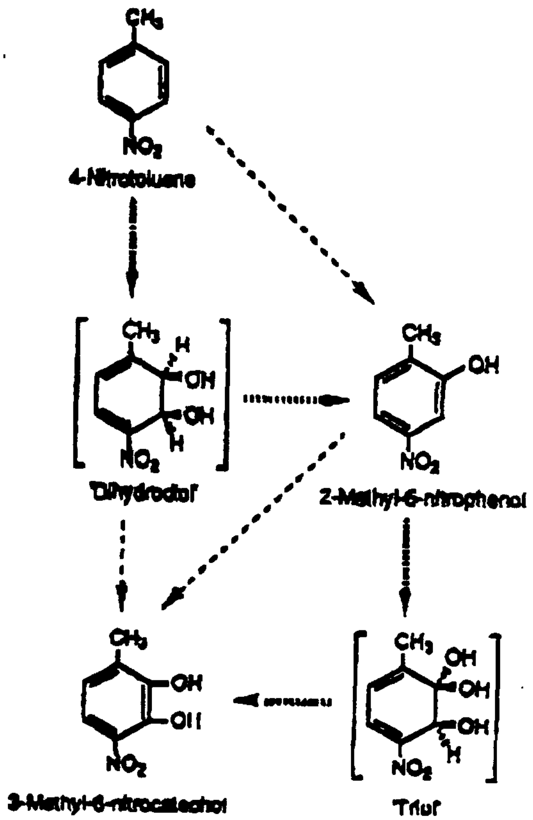
## 2. Oxidation of Nitrotoluenes by *Pseudomonas* strains and Genetically Engineered *Escherichia coli*.

The total U.S. production of the isomeric nitrotoluenes has been estimated to be about 44 million pounds as of 1980. The principal commercial use of these compounds are in the manufacture of azo and sulfur dyes and in the production of explosives. Nitrotoluenes have been detected in the waste water from paper mills and chemical plants and high levels of 2- and 4-nitrotoluene have been detected in waste water from TNT manufacturing facilities. Our previous studies supported by grant No. AFOSR-88-0225 have shown that growth of *Pseudomonas putida* F1 on toluene yields cells that can degrade chlorobenzenes, chlorophenols and trichloroethylene. We have now extended these studies to include the isomeric nitrotoluenes. The work described below was conducted in collaboration with Dr. Jim C. Spain, Tyndall Air Force Base, Florida.

Toluene-grown cells of *Pseudomonas putida* F1 and *Pseudomonas* sp. JS150 oxidize toluene to *cis*-toluene dihydrodiol as shown on the next page in sequence A. The same cell preparations were expected to catalyze analogous reactions for the oxidation of 2-, 3-, and 4-nitrotoluene. However both *Pseudomonas* strains oxidized 2- and 3-nitrotoluene to benzyl alcohols (sequences B and C). *Pseudomonas* sp. JS150 also oxidized 3-nitrobenzyl alcohol to 3-nitrobenzoate (sequence C). In contrast, both organisms oxidized 4-nitrotoluene to 2-methyl-5-nitrophenol and 3-methyl-6-nitrocatechol (see next page).

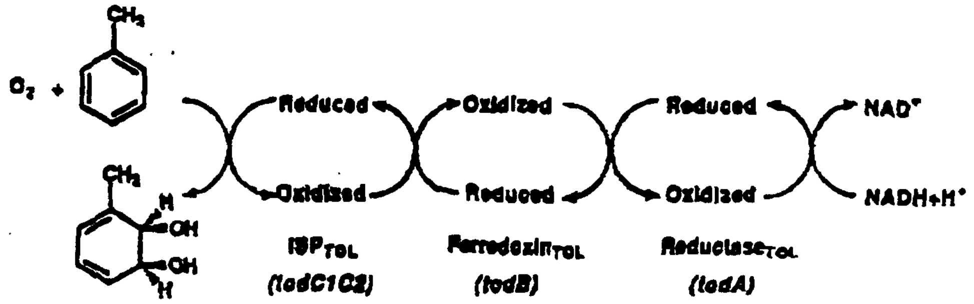
The oxidation of the methyl group in 2- and 3-nitrotoluene was unexpected and represents a novel and exciting reaction in the bacterial degradation of nitrotoluenes. The formation of 2-methyl-5-nitrophenol and 3-

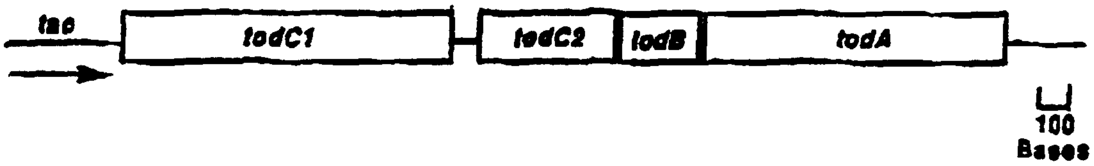

(A)
$CH_3$
Toluene
cis-Dihydrodiol
3-Methylcatechol
Ring Fission
(B)
$CH_3$
$NO_2$
2-Nitrotoluene
$CH_2OH$
2-Nitrobenzyl alcohol
(C)
$CH_3$
$NO_2$
3-Nitrotoluene
$CH_2OH$
3-Nitrobenzyl alcohol
$COO^-$
3-Nitrobenzoate
H
OH


$CH_3$
$NO_2$
4-Nitrotoluene
'Dihydrodiol'
2-Methyl-6-nitrophenol
3-Methyl-6-nitrocatechol
'Triol'
H
OH

methyl-6-nitrocatechol was also unexpected and is best explained at this time by the reactions shown by heavy dashed lines in the scheme on the previous page.

The results suggest that the enzyme catalyzing these reactions is toluene dioxygenase. This enzyme contains three proteins, a flavoprotein (reductase$_{TOL}$), a [2Fe.2S] iron-sulfur protein (ferredoxin$_{TOL}$), and a terminal iron-sulfur protein (ISP$_{TOL}$) which catalyzes the incorporation of both atoms of molecular oxygen into the aromatic nucleus of toluene to form *cis*-toluene dihydrodiol. ISP$_{TOL}$ consists of two non-identical subunits. Thus four genes are required for the synthesis of toluene dioxygenase. These genes *todABC1C2* have been cloned in *E. coli* JM109 where they are under the control of the *tac* promoter. The organization of the proteins in the toluene dioxygenase system and the organization of the genes in the recombinant organism, *E. coli* JM109(pDTG601), are shown below. When this organism was induced by growth in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) the recombinant organism oxidized the isomeric nitrotoluenes to the same metabolites as those





formed by *P. putida* F1 and *Pseudomonas* sp. JS150. These results demonstrate for the first time that toluene dioxygenase can oxidize a methyl substituent and extends the range of substrates known to be oxidized by this versatile enzyme.

## 3. Future Studies

1. Confirm the ability of recombinant strains of *E. coli* containing the cloned toluene and naphthalene dioxygenase to oxidize *O*-, *m*-, and *p*-dichlorobenzene to dihydrodiols.

2. Determine the stereochemistry of the dihydrodiols formed from the isomeric dichlorobenzenes by cloned toluene and naphthalene dioxygenases.

3. Initiate studies on the ability of naphthalene dioxygenase to oxidize the isomeric nitrotoluenes and 2,4-dinitrotoluene.