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Biotransformation of anisole and phenetole by aerobic hydrocarbon-oxidizing bacteria

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Abstract

Wild type, mutant, and recombinant bacterial strains capable of oxidizing aromatic hydrocarbons were screened for their ability to oxidize anisole (methoxybenzene) and phenetole (ethoxybenzene). Toluene-induced cells of *Pseudomonas putida* F39/D transformed anisole to a compound tentatively identified as *cis*-1,2-dihydroxy-3-methoxycyclohexa-3,5-diene (anisole-2,3-dihydrodiol), 2-methoxyphenol, catechol, and trace amounts of phenol while phenetole was converted primarily to *cis*-1,2-dihydroxy-3-ethoxycyclohexa-3,5-diene (phenetole-2,3-dihydrodiol) and 2-ethoxyphenol. Induced cells of *Pseudomonas* sp. NCIB 9816/11 and *Beijerinckia* sp. B8/36 transformed anisole to phenol, and phenetole to phenol and ethenyloxybenzene. Toluene-induced cells of *P. putida* BG1 converted anisole to phenol but did not oxidize phenetole. In contrast, toluene-induced cells of *P. mendocina* KR1, which oxidize toluene via monooxygenation at the *para* position, transformed anisole to 4-methoxyphenol, and phenetole to 2-, 3- and 4-ethoxyphenol. The involvement of toluene and naphthalene dioxygenases in the reactions catalyzed by strains F39/D and NCIB 9816/11, respectively, was confirmed with recombinant *E. coli* strains expressing the cloned dioxygenase genes. The results show that the oxygenases from different *Pseudomonas* strains oxidize anisole and phenetole to different hydroxylated products.

Introduction

Anisole (methoxybenzene) and phenetole (ethoxybenzene) represent two simple examples of compounds known as alkyl aryl- or phenolic-ethers. These aromatic compounds, and many others with a variety of substituents, are widespread in nature and are most abundant as polymeric components of lignin (Ribbons & Harrison 1972). Due to their presence in the environment, it is not surprising that these compounds serve as substrates for both anaerobic (Young 1984) and aerobic bacteria (Ribbons & Harrison 1972; Bernhardt et al. 1988). The *O*-demethylation of phenolic ethers has been described for a number of aerobic bacteria with specificity requirements for the methoxy groups oriented *ortho*, *meta*, and *para* with respect to a carboxyl on the phenyl nucleus (Cartwright et al. 1971). For example, vanillate *O*-demethylase catalyzes the *O*-demethylation of vanillate and several other methoxybenzoates in strains of *P. fluorescens* (Cartwright & Smith 1967), *P. aeruginosa*, and *P. testosteroni* (Ribbons 1970; Ribbons 1971; Ribbons & Harrison 1972). *Pseudomonas putida* (DSM 1868) uses 4-methoxybenzoate monooxygenase to catalyze the *O*-demethylation of a variety of methoxybenzoic acids (Bernhardt et al. 1973; Bernhardt et al. 1988). *O*-Deethylation of 4-ethoxybenzoate by a *Nocardia*

sp. is also catalyzed by 4-methoxybenzoate *O*-demethylase (Cartwright et al. 1971). Recently, strains containing toluene dioxygenase have been shown to catalyze the transformation of anisole (Renganathan & Johnston 1989; Gibson et al. 1990), however, little information exists describing the aerobic transformation of phenolic ethers by other hydrocarbon-utilizing bacteria.

The purpose of this study was to identify the transformation products and types of reactions catalyzed by toluene-, naphthalene-, and biphenyl-oxidizing bacteria with respect to the substrates anisole and phenetole. The results obtained show that certain dioxygenases catalyze the *O*-demethylation of anisole, and *O*-deethylation and desaturation of phenetole, in addition to dioxygenation of their 'native' substrate.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *P. putida* F39/D (PpF39/D) is a dihydrodiol dehydrogenase mutant of *P. putida* F1 (PpF1) which oxidizes toluene quantitatively to (+)-*cis*-(*1S,2R*)-dihydroxy-3-methylcyclohexa-3,5-diene (Gibson et al. 1970; Ziffer et al. 1973). PpF39/D, PpF1, *P. putida* BG1, *P. mendocina* KR1, *P. pickettii* PKO1, and *P. cepacia* G4 (and G4-102) were grown at 30 °C in a mineral salts basal medium (MSB) (Stanier et al. 1966) at pH 7.2 containing 15 mM (0.16%) pyruvate in the presence of toluene vapor. *Pseudomonas* sp. NCIB 9816/11, a naphthalene dihydrodiol dehydrogenase mutant which oxidizes naphthalene to (+)-*cis*-(*1R,2S*)-dihydroxy-1,2-dihydronaphthalene (Jerina et al. 1971; Jeffrey et al. 1975), and *Beijerinckia* strain B8/36, a biphenyl dihydrodiol dehydrogenase mutant which oxidizes biphenyl to *cis*-(*1S,2R*)-dihydroxy-3-phenylcyclohexa-3,5-diene (Gibson et al. 1973; Ziffer et al. 1977), were grown as described above but were induced with 0.05% salicylate and *m*-xylene (vapor), respectively. Salicylate (Barnsley 1975) and *m*-xylene (Mahaffey et al. 1988) are known inducers of the naphthalene dioxygenase of NCIB 9816 and the biphenyl dioxygenase of *Beijerinckia* B1, respectively. The *E. coli* recombinant strains JM109(pDTG601A) and JM109(pDTG141) contain the genes encoding the toluene dioxyge-

Table 1. Bacterial strains used in this study.

Strain	Relavant phenotype[a]	Reference
Pseudomonas putida F1 (PpF1)	Oxidizes toluene through *cis*-toluene dihydrodiol (dihydrodiol pathway)	(Gibson et al. 1970)
P. putida F39/D (PpF39/D)	Mutant which oxidizes toluene to (+)-*cis*-(*1S,2R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol)	(Gibson et al. 1970)
P. mendocina KR1 (PmKR1)	Oxidizes toluene through *p*-cresol (toluene 4-monooxygenase pathway)	(Whited & Gibson 1991)
P. pickettii PKO1	Oxidizes toluene through *m*-cresol (toluene 3-monooxygenase pathway)	(Kukor & Olsen 1990)
P. cepacia G4	Oxidizes toluene through *o*-cresol (toluene 2-monooxygenase pathway)	(Shields et al. 1989)
P. cepacia G4-102	Mutant which oxidizes toluene to 3-methylcatechol	(Shields et al. 1991)
P. putida BG1 (PpBG1)	Oxidizes toluene through benzyl alcohol (TOL plasmid pathway)	(Whited et al. 1986)
Pseudomonas sp. NCIB 9816/11	Mutant which oxidizes naphthalene to (+)-*cis*-(*1R,2S*)-dihydroxy-1,2-dihydro-naphthalene (*cis*-naphthalene dihydrodiol)	(Jerina et al. 1971)
Beijerinckia sp. B8/36	Mutant which oxidizes biphenyl to (+)-*cis*-(*1S,2R*)-dihydroxy-3-phenyl-cyclohexa-3,5-diene (*cis*-biphenyl dihydrodiol)	(Gibson et al. 1973)
E. coli JM109(pDTG601A)	JM109 containing structural genes for toluene dioxygenase (*todC1C2AB*) in plasmid pKK223-3; IPTG-inducible; Amp^r	(Zylstra & Gibson 1991)
E. coli JM109(pDTG141)	JM109 containing structural genes for naphthalene dioxygenase (*nahAaAbAcAd*) in pKK223-3; IPTG-inducible; Amp^r	(Suen 1991)

[a] Amp^r, Resistant to ampicillin.

nase from *P. putida* F1 (Zylstra & Gibson 1991) and the naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816 (Suen 1991), respectively. These cultures and JM109(pKK223-3), the vector-only control, were grown at 37 °C in MSB containing 20 mM glucose, 1 mM thiamine, and 10 μg/ml ampicillin. When the turbidity of the culture reached 0.5 at 600 nm, cultures were incubated at 30 °C and isopropyl-β-D-thiogalactoside (IPTG) was added to give a concentration of 200 mM. After 1 hour (approximately 1 doubling) cells were harvested by centrifugation. Cultures contained 50 ml or 800 ml media, in 250 ml Erlenmeyer or 2.8 l Fernbach flasks, respectively, and were grown with rotary shaking (250 cycles/min).

Intact cell incubations

Induced cultures (800 ml) were harvested in the late exponential phase of growth by centrifugation (9000 g, 10 min, 4 °C), washed once in MSB, and resuspended in 200 ml MSB. Cell suspensions (50 ml, turbidity 2.0–2.5 at 600 nm) used for transformation studies contained 5 mM pyruvate and 0.05% (v/v) anisole or phenetole. Flasks (250 ml) were sealed with neoprene rubber stoppers (to prevent volatilization of substrates) and incubated for 4 h at 30 °C with shaking (250 cycles/min). Cells were then removed from 1.0 ml samples by centrifugation and the clear supernatant solutions analyzed for transformation products with a Beckman DU-70 spectrophotometer over the 200–400 nm range.

Extraction of transformation products

The contents of each flask were extracted three times with equal volumes of sodium hydroxide-washed ethyl acetate. The combined ethyl acetate solutions were dried over anhydrous sodium sulfate and concentrated to approximately 2 ml at 30 °C under reduced pressure prior to analyses.

Separation and identification of transformation products

Thin layer chromatography (TLC) of extracts was performed on 0.2 mm thickness silica gel sheets (Silica gel 60 F_{254}, Merck no. 5735). The solvent was chloroform/acetone (80:20). Compounds were visualized by observing quenching of fluorescence under shortwave UV light (254 nm) and by spraying with a 2% (w/v) methanolic solution of 2,6-dichloroquinone-4-chloroimide (Gibb's reagent). Dihydrodiols were derivatized by adding 0.1 ml of extract to 0.025 ml ethyl acetate containing 1 mg of phenylboronic acid (PBA). PBA has been shown to derivatize *cis*-1,2-diols of cyclohexane and cyclopentane in a 1:1 ratio, while *trans* isomers yielded dibenzeneboronates (Sugihara & Bowman 1958).

Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard model 5890 gas chromatograph equipped with a Hewlett-Packard Ultra-1 capillary column (25 m x 0.2 mm with 0.33 μm film thickness of crosslinked methyl silicone). The column temperature program used was from 70–240 °C at 10 °C/min, or from 50–80 °C at 3 °C/min then to 240 °C at 20 °C/min. Temperatures of the injection port and detector were 220 °C and 280 °C, respectively, and helium was the carrier gas (0.5 ml/min). Mass spectra of compounds eluting from the column were obtained with a Hewlett-Packard model 5970 mass selective detector. Samples (1.0 μl) were injected with a split ratio of 50:1. Authentic standards, when available, were used to confirm the identity of transformation products. Relative product distributions were calculated as percentages of total peak area derived from integration of the GC-MS total ion chromatograms.

Results

Transformations catalyzed by toluene dioxygenase

Toluene-induced cells of PpF39/D, incubated 4 hours with anisole and phenetole, formed products with UV-absorption maxima at 272 nm and 276 nm, respectively. TLC analysis indicated that the ani-

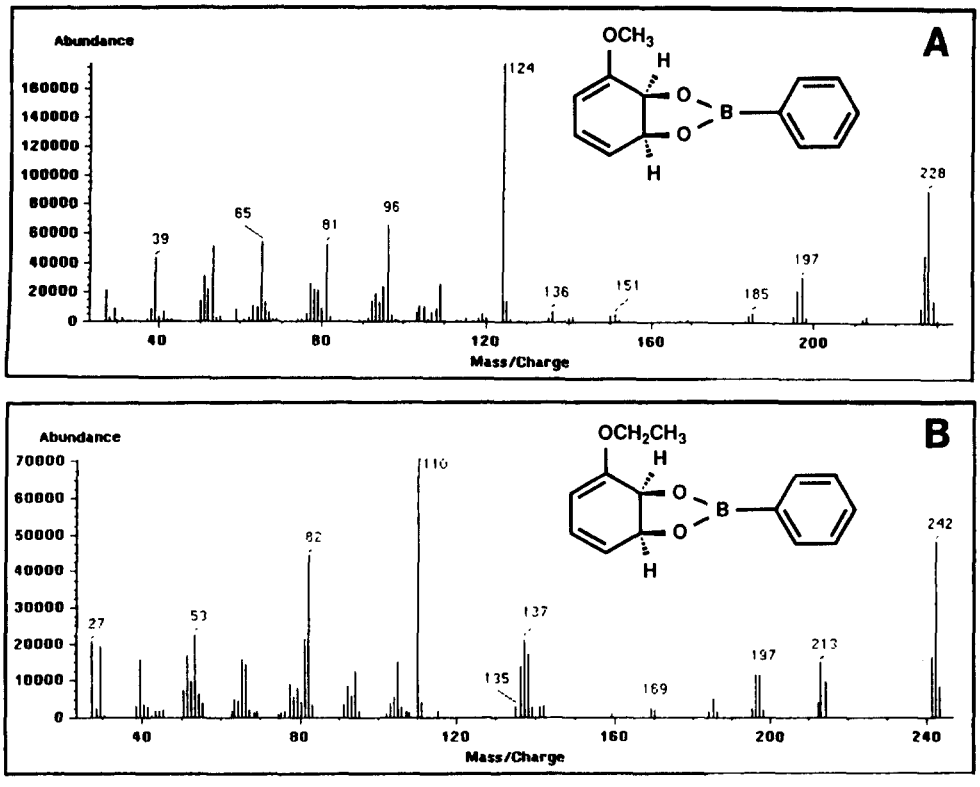

Fig. 1. Mass spectra of phenylboronic acid derivatives of (A) anisole-2,3-dihydrodiol and (B) phenetole-2,3-dihydrodiol formed by toluene dioxygenase.

sole transformation products consisted of a dihydrodiol ($R_f = 0.2$), a catechol ($R_f = 0.3$), and a phenol ($R_f = 0.5$). GC-MS analysis of the products formed from anisole identified 2-methoxyphenol (28%), catechol (7%), and a compound (16%) tentatively identified as 1,2-dihydroxy-3-methoxycyclohexa-3,5-diene (anisole dihydrodiol). Phenol was detected in small amounts (< 1%) and the remaining material was accounted for as unused anisole (36%). In a separate experiment, toluene-induced cells of PpF39/D oxidized phenol to catechol but did not oxidize 2-methoxyphenol. TLC analyses of the products formed from phenetole by PpF39/D showed the presence of a major ($R_f = 0.2$) and a minor component ($R_f = 0.5$). However, the only product detected by GC-MS was 2-ethoxyphenol (TLC, $R_f = 0.5$). These results indicated that the polar compound ($R_f = 0.2$) formed from phenetole was 1,2-dihydroxy-3-ethoxy-cyclohexa-3,5-diene (phenetole dihydrodiol). The putative dihydrodiols formed from anisole and phenetole were extremely unstable and rapidly aromatized to phenols during the work up of extracts for analysis. However, both dihydrodiols formed stable monobenzeneboronate derivatives with PBA indicating a *cis*-orientation of the hydroxyl groups (Fig. 1).

Anisole and phenetole were added to mid-log phase cultures of PpF39/D growing with pyruvate. After 24 hours the products formed from anisole were the same as those described above for toluene-induced cells. In contrast, phenetole inhibited the growth of PpF39/D on pyruvate and over a 24 hour period no phenetole metabolites were detected. These observations indicate that anisole, but not phenetole, is capable of inducing toluene dioxygenase in PpF39/D.

E. coli JM109(pDTG601A) contains the structural genes (*todC1C2BA*) for toluene dioxygenase cloned in the vector pKK223-3 where they are under the control of the *tac* promoter and inducible by

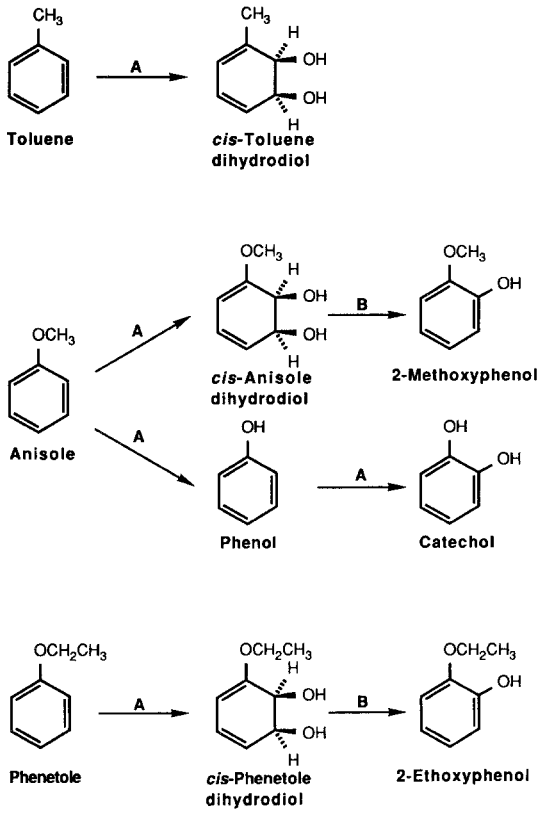

Fig. 2. Products formed from anisole and phenetole by PpF39/D and JM109(pDTG601A). A, reactions attributed to toluene dioxygenase. B, non enzymatic reactions due to rearomatization with loss of water.

IPTG (Zylstra & Gibson 1991). IPTG-induced cells of *E. coli* JM109(pDTG601A) oxidized anisole and phenetole to the same products as those produced by PpF39/D. In control experiments, IPTG-treated cells of *E. coli* JM109(pKK223-3) did not oxidize anisole or phenetole. A summary of the reactions catalyzed by PpF39/D and JM109(pDTG601A) is shown in Fig. 2.

Transformations catalyzed by naphthalene and biphenyl dioxygenases

Salicylate-induced cells of *Pseudomonas* sp. NCIB 9816/11, which oxidized naphthalene to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, converted anisole to phenol (64%). The same cells oxidized phenetole to phenol (18%) and ethenyloxybenzene (13%). Anisole and phenetole did not induce naphthalene dioxygenase activity in NCIB 9816/11 and phenetole added at concentrations of 0.05% inhibited growth of the organism on pyruvate (data not shown).

E. coli JM109(pDTG141) is a recombinant organism which contains the structural genes for naphthalene dioxygenase (*nahAaAbAcAd*) in the vector pKK223-3 where they are under the control of the *tac* promoter. IPTG-induced cells of this strain oxidized naphthalene to *cis*-naphthalene dihydrodiol as expected. The same cells oxidized anisole to phenol (29%), and phenetole to phenol (8%) and ethenyloxybenzene (6%). These results indicate that naphthalene dioxygenase is capable of *O*-dealkylation and desaturation reactions.

m-Xylene-induced cells of *Beijerinckia* B8/36 oxidized biphenyl to *cis*-2,3-dihydroxy-2,3-dihydrobiphenyl (*cis*-biphenyl dihydrodiol). The same cells oxidized anisole to phenol (33%), and phenetole to phenol (5%) and ethenyloxybenzene (5%). These results suggest that biphenyl 2,3-dioxygenase is responsible for the dealkylation and desaturation reactions observed. Anisole and phenetole did not induce biphenyl dioxygenase activity in *Beijerinckia* B8/36. The reactions catalyzed by naphthalene and biphenyl dioxygenases are shown in Fig. 3.

Transformations catalyzed by PpF1, G4, and PKO1

Toluene-induced cells of PpF1, G4 and PKO1 oxidized anisole and phenetole to their respective *meta* ring-fission products 2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoic acid (λ_{max}, 305 nm at pH 7.0) and 2-hydroxy-6-ethoxy-6-oxohexa-2,4-dienoic acid (λ_{max}, 306 nm at pH 7.0). The identity of the products was based on their absorption spectra and the analogous reactions reported for 3-substituted catechols (Dagley et al. 1964). The absorption spectrum of the yellow ring-cleavage product formed from anisole was identical to that given by the ring-fission product formed from 3-methoxycatechol by purified 3-methylcatechol 2,3-dioxygenase of PpF1 (Menn 1991). Trace amounts of 2-methoxyphenol and 4-methoxyphenol were formed from anisole by strain G4. In contrast, no neutral transformation products were detected from anisole by PpF1 or

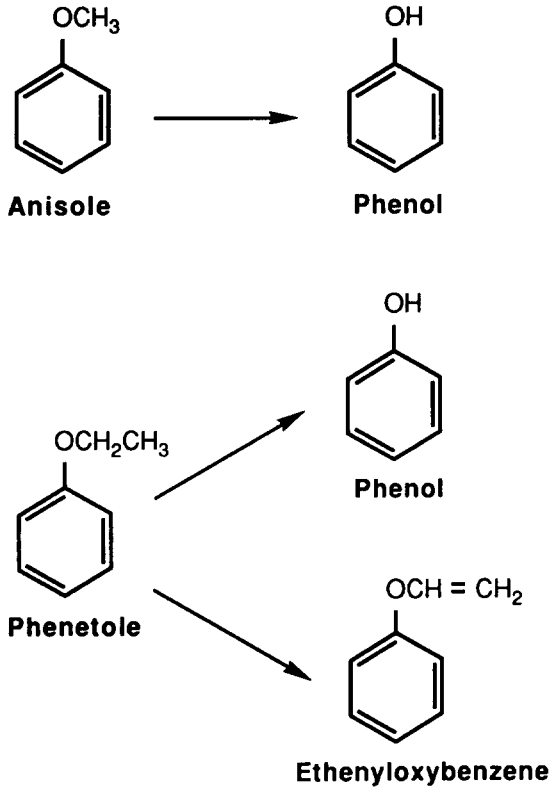

Fig. 3. Products formed from anisole and phenetole by naphthalene and biphenyl dioxygenases.

PKO1. Significant amounts of phenol were formed from phenetole by PpF1. The formation of phenol, by *O*-demethylation of phenetole, may have prevented the complete transformation of phenetole. The major metabolite formed from phenetole by G4 was the ring-fission product (2-hydroxy-6-oxo-6-ethoxyhexa-2,4-dienoic acid). In addition, 2-ethoxyphenol, 3-ethoxyphenol, and 4-ethoxyphenol were identified by GC-MS.

Transformations catalyzed by P. putida BG1, P. mendocina KR1, and P. cepacia G4-102

Toluene-induced cells of PpBG1, PmKR1, and G4-102 oxidized anisole to phenol (35%), 4-methoxyphenol (60%), and 3-methoxycatechol (58%), respectively, as the major products. Trace amounts of 2-methoxyphenol (< 1%) and phenol (< 1%) were also formed by PmKR1 and larger amounts of 2-methoxyphenol (9%) and 4-methoxyphenol (3%) were formed by G4-102. When phenetole was the substrate, 4-ethoxyphenol (41%), 3-ethoxyphenol (8%), and 2-ethoxyphenol (12.5%) were formed by PmKR1. In contrast, 3-ethoxycatechol (28%), 2-ethoxyphenol (35%), 3-ethoxyphenol (9%), and 4-ethoxyphenol (5%) were formed by G4-102. Phenetole was not oxidized by toluene-induced cells of PpBG1.

Discussion

A variety of hydrocarbon-oxidizing bacteria were examined for their ability to transform anisole and phenetole. These included mutant strains *P. putida* F39/D (Gibson et al. 1970; Gibson et al. 1990), *Beijerinckia* sp. B8/36 (Gibson et al. 1973) and *Pseudomonas* sp. NCIB 9816/11 which contain toluene, biphenyl, and naphthalene dioxygenases, respectively. In addition, we examined *P. cepacia* G4 (Shields et al. 1989), *P. pickettii* PKO1 (Kukor & Olsen 1990) and *P. mendocina* KR1 (Whited & Gibson 1991) which contain monooxygenases that initiate toluene oxidation at the *ortho*-, *meta*-, and *para*-positions, respectively.

Toluene dioxygenase in *P. putida* F39/D and *E. coli* JM109(pDTG601A) oxidized anisole and phenetole to unstable *cis*-dihydrodiols which dehydrated easily to form the *ortho*-phenols, 2-methoxyphenol and 2-ethoxyphenol, respectively (Fig. 2). The proposed structures of the dihydrodiols formed from anisole and phenetole are *cis*-(*1S,2S*)-dihydroxy-3-methoxycyclohexa-3,5-diene and *cis*-(*1S,2S*)-dihydroxy-3-ethoxycyclohexa-3,5-diene, respectively, based on ^{1}H NMR analyses [Resnick & Torok, unpublished data] and previous studies with toluene dioxygenase (Gibson et al. 1970; Ziffer et al. 1973; Gibson et al. 1990). The results obtained with PpF39/D indicate that toluene dioxygenase can catalyze the *O*-demethylation of anisole and the hydroxylation of phenol to catechol. This was confirmed by demonstrating that the cloned dioxygenase in *E. coli* (Zylstra & Gibson 1991) catalyzed the same reactions. The conversion of phenol to catechol may proceed through a 'triol intermediate' as previously suggested (Spain et al. 1989).

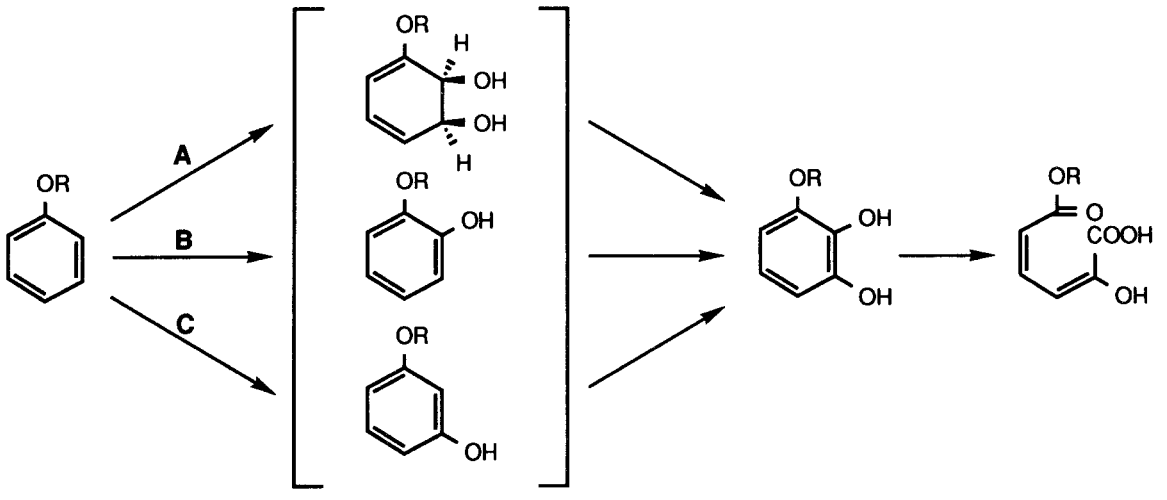

Fig. 4. Proposed reactions catalyzed by wild type strains of (A) *P. putida* F1, (B) *P. cepacia* G4, and (C) *P. pickettii* PKO1 for anisole (R = CH_3) and phenetole (R = CH_2CH_3). Minor products detected but not shown are described in the text.

Naphthalene and biphenyl dioxygenases of *Pseudomonas* sp. NCIB 9816/11 and *Bejerinckia* sp. B8/36, respectively, did not oxidize the aromatic nucleus of anisole or phenetole. The major reactions catalyzed by both enzymes were the *O*-demethylation of anisole, and the *O*-deethylation and desaturation of phenetole (Fig. 3). Both NCIB 9816/11 and B8/36 formed phenol from anisole and phenetole, presumably by attack at the electrophilic *O*-alkyl substituent resulting in a reaction similar to the *O*-dealkylation mechanism described for vanillate *O*-demethylase (Cartwright & Smith 1967; Ribbons & Harrison 1972) and 4-methoxybenzoate monooxygenase (Bernhardt et al. 1988). In these reactions, monooxygenases attack the alkyl moiety introducing a hydroxyl group and forming an unstable hemi-acetal intermediate which decays spontaneously to yield phenol and the corresponding aldehyde (Chapman 1972; Bernhardt et al. 1988).

The desaturation of phenetole to ethenyloxybenzene also results from attack on the alkyl sustituent. However, this reaction, catalyzed by strains containing naphthalene dioxygenase or biphenyl dioxygenase, represents an oxidation without the incorporation of oxygen into the substrate. Whole cell and enzymatic transformations have previously shown that the naphthalene dioxygenase catalyzes the desaturation of indan to indene, and 1-indanol to 1-indenol (Haigler 1986).

The wild type strains of *P. putida* F1, *P. cepacia* G4, and *P. pickettii* PKO1 oxidized anisole to a yellow ring-fission product which was identified as 2-hydroxy-6-oxo-6-methoxyhexa-2,4-dienoate, thus indicating that 3-methoxycatechol is a substrate for the catechol 2,3-dioxygenases of these organisms.

Table 2. Reactions catalyzed by bacteria in this study with respect to anisole and/or phenetole.

Reaction observed	Toluene dioxygenase PpF39/D & pDTG601A	Naphthalene dioxygenase 9816/11 & pDTG141	Biphenyl dioxygenase B8/36	PmKR1, PKO1, & G4
Dioxygenation	YES	NO	NO	NO
O-Dealkylation	YES	YES	YES	NO
Desaturation	NO	YES	YES	NO
Monooxygenation	YES[a]	NO	NO	YES

[a] A net monooxygenation was observed by the transformation of phenol to catechol.

Analogous reactions are proposed for phenetole (Fig. 4). The major product formed from anisole by *P. cepacia* G4-102, a mutant that oxidizes toluene to 3-methylcatechol, was 3-methoxycatechol. The organism also oxidized phenetole to 3-ethoxycatechol. Monohydroxylated products were formed in small amounts from both substrates. In contrast, *P. mendocina* KR1 oxidized anisole almost exclusively to 4-methoxyphenol. Similar results were obtained with phenetole, however, less specific oxygenation resulted in the formation of the 2- and 3-ethoxyphenols.

Conclusions

The reactions catalyzed by the bacteria in this study with respect to anisole and phenetole are summarized in Table 2. Results show that in addition to the benzylic monooxygenation of indan and indene described for toluene and naphthalene dioxygenases (Wackett et al. 1988), the toluene, naphthalene, and biphenyl dioxygenases are also capable of *O*-dealkylation with respect to the phenolic ethers, anisole and phenetole. The desaturation of phenetole by the naphthalene and biphenyl dioxygenases extends this activity, previously demonstrable for benzocyclic substrates (Haigler 1986), to the *O*-ethyl substituent of phenetole. The substrates influenced the types of reactions catalyzed by the oxygenases in this study. Conventional dioxygenase and monooxygenase reactions were not predictable for anisole and phenetole. Thus, in certain situations, the distinction between dioxygenase and monooxygenase activity can be dictated by the substrate rather than the enzyme.

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