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Identification of *syn*- and *anti*-Anethole-2,3-epoxides in the Metabolism of *trans*-Anethole by the Newly Isolated Bacterium *Pseudomonas putida* JYR-1

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Bacterial strain JYR-1, which utilizes high concentrations (up to 100 mM) of *trans*-anethole as the sole source of carbon and energy, was isolated from soil. It grew to $OD_{600nm} = 2.6$ with a doubling time of 8 h when grown on 20 mM *trans*-anethole. Strain JYR-1 was identified as *Pseudomonas putida* based on the partial gene sequence of its 16S rDNA. Elution profiles of culture extracts were examined by high-performance liquid chromatography and showed that four metabolites were produced from the bacterial culture containing *trans*-anethole that were not detected in control experiments. LC-MS analysis showed molecular weights of 138.2, 164.5, 164.3, and 152.3. The metabolites with molecular weights at 152.3 and 138.2 were confirmed to be *p*-anisic acid and *p*-hydroxybenzoic acid, respectively, when compared with HPLC retention times and molecular weights of authentic compounds. The metabolites with molecular weights at 164.5 and 164.3 were further analyzed by NMR and were proved to be stereoisomer *syn*- and *anti*-anethole epoxides. Therefore, strain JYR-1 most likely initiates the metabolism of *trans*-anethole through the formation of epoxides on the propene group of the compound.

KEYWORDS: ***trans*-Anethole; *Pseudomonas*; flavor; phenylpropanoid; biotransformation**

INTRODUCTION

The phenylpropanoid pathway is known to be one of the secondary pathways in plants that is involved in the production of lignin, flavonoids, anthocyanins, etc. (*1*). Indeed, compounds produced from this pathway are used by plants as part of their defense system against pathogens and herbivores (*2*). Demand for phenylpropanoid compounds in industry has increased due to their potential application in diverse areas such as in the production of antioxidants, pharmaceuticals, and food additives for flavor (*3*−*7*).

Biotransformations to produce value-added intermediates from natural resources have been given attention as an alternative tool to replace conventional chemical synthesis (*7*, *8*). For example, vanillin, one of the most extensively used aromatic flavor compounds, can be produced by microbial biotransformation of plant-produced phenylpropanoid compounds such as eugenol and ferulic acid (*8*−*10*). The biochemical process of vanillin production from eugenol and ferulic acid by microorganisms involves oxidation of the side chain prior to oxidation of the ring structure (*4*).

trans-Anethole (*p*-methoxypropenylbenzene), a type of phenylpropanoid compound formed in plants as a byproduct of terpene synthesis, is the major component present in the essential oils of anise, fennel, and star anise (*11*). *trans*-Anethole is commercially used as a flavor substance in baked goods, candy, ice cream, chewing gum, and alcoholic beverages (*9*). However, there have been few research studies regarding its metabolism (*6*, *7*, *12*). Recently, Shimoni et al. (*6*, *7*) elucidated a *trans*-anethole degradation pathway by *Arthrobacter* strain TA13 and its mutant strains. Its metabolic derivatives, anisic acid, anisic alcohol, and anisaldehyde, can be also used as a flavoring source for a variety of food additives (*6*, *7*).

This study documents the initial metabolites of *trans*-anethole by a newly isolated strain, JYR-1, which can grow on concentrations of *trans*-anethole of up to 100 mM.

MATERIALS AND METHODS

Chemicals. *trans*-Anethole was purchased from Aldrich (Milwaukee, WI). *p*-Anisic acid, *p*-hydroxybenzoic acid, and *p*-anisaldehyde were purchased from Sigma (St. Louis, MO). Organic solvents (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ).

Isolation of Bacterial Strain. Soil from Uiryung, South Korea, which was contaminated with industrial oils, was used as a basis for

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an enrichment culture to isolate bacterial strains. Soils (5 g) were suspended in 100 mL of Stanier's basal minimal salts buffer (MSB) (*13*) containing 0.5% (w/v) *trans*-anethole and were incubated with shaking at 200 rpm at 27 °C in the dark. After a series of transfers of the culture, the culture solution was spread on nutrient agar plates. One hundred colonies were isolated, purified, and re-inoculated into MSB medium containing 0.5% (w/v) *trans*-anethole. One colony capable of utilizing *trans*-anethole as a sole source of carbon and energy was isolated and called JYR-1.

Bacterial Identification by Sequence Analysis of 16S rDNA. Chromosomal DNA was extracted from strain JYR-1 using the procedure of Pitcher et al. (*14*) as modified by Chun and Goodfellow (*15*). The 16S rDNA was amplified by employing two universal primers, p27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and p1525R (5′-GYT ACC TTG TTA CGA CTT-3′). The PCR program used for amplification was as follows: 95 °C for 15 min, followed by 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a single final extension step consisting of 72 °C for 7 min. Amplified 16S rDNA fragments were purified by a PCR fragment purification kit (Qiagen, Valencia, CA). Partial nucleotide sequences were obtained by using an ABI 377 XL upgrade DNA Sequencer (Perkin-Elmer, Boston, MA) and software provided by the manufacturer. Sequence similarity searches were performed using the BLAST program. The sequence information was then imported into the PHYDIT program (*15*) for assembly and alignment. The sequence of 16S rDNA for strain JYR-1 was compared to sequences from type strains held in the GenBank database. Nucleotide substitution rates were calculated, and phylogenetic trees were constructed by the neighbor-joining method (*16*).

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was performed using a Varian ProStar HPLC equipped with a photodiode array (PDA) detector (Varian, Walnut Creek, CA) and a reverse phase C18 column (5 μm particle size, 4.6 mm × 25 cm, Milford, MA). The mobile phase, which was composed of acetonitrile containing 1% formic acid and water containing 1% formic acid, was programmed as follows: 10% acetonitrile at 0 min, 60% acetonitrile at 10 min, 90% acetonitrile at 20 min, and 90% acetonitrile at 30 min. The flow rate with an injection volume of 20 μL was 1 mL/min, and UV detection was performed at 270 nm. Preparative HPLC was performed to collect metabolites of *trans*-anethole. A Varian prep-HPLC equipped with a PDA detector (Varian) and a Rainin C18 ODS column (10 μL particle size, 21.4 mm × 25 cm, Varian) was used. The elution program with mobile phase, which was composed of ethanol containing 1% formic acid and water containing 1% formic acid, was as follows: 20% ethanol at 0 min, 50% ethanol at 10 min, 90% ethanol at 20 min, and 90% ethanol at 40 min. The flow rate with an injection volume of 1 mL was 15 mL/min, and UV detection was performed at 270 nm. Peaks corresponding to metabolites were collected and evaporated to dryness with an Automated Environmental Speed Vac AES1010 (ThermoProd, Riviera Beach, FL).

Liquid Chromatography−Mass Spectrometry (LC-MS). LC-MS was performed by coupling an HP 1100 system to a Quattro LC triple-quadruple tandem mass spectrometer (Micromass, Manchester, U.K.) in electrospray ionization (ESI^+) mode. The mobile phase, which was composed of water/acetonitrile containing 1% formic acid, was programmed linearly as follows: 20% methanol at 0 min, 40% methanol at 10 min, 70% methanol at 20 min, and 90% methanol at 50 min. The flow rate was 1 mL/min, and UV detection was performed at 270 nm. The injection volume was 20 μL, which was then split in the ratio of 1:3 for injection into the (ESI^+) mode. The source temperature, desolvation temperature, cone voltage, and capillary voltage were kept at 60 °C, 220 °C, 26 V, and 3.99 kV, respectively. An electron multiplier voltage of 640 V was used. The nebulizer gas and desolvation gas were ultrapure nitrogen set at 94 and 562 L/h, respectively.

Nuclear Magnetic Resonance (NMR) Spectrometry. All NMR measurements were performed on a Bruker Avance 400 spectrometer system (9.4 T) at 298 K. The ^1H and ^{13}C NMR, DEPT, COSY, HMQC, and HMBC spectra were collected in $CDCl_3$ (Aldrich, Chicago, IL) with TMS as an internal reference. The concentrations of metabolites **II** and **III** were 78 and 58 mM, respectively. For ^1H NMR analysis, 16 transients were acquired with a 1 s relaxation delay using 32K data points. The 90° pulse was 9.7 μs with a spectral width of 4000 Hz. The ^{13}C NMR and DEPT spectra were obtained for a spectral width of 20000 Hz, collecting 64K data points. Two-dimensional spectra were acquired with 2048 data points for t_2 and 256 for t_1 increments (*17*).

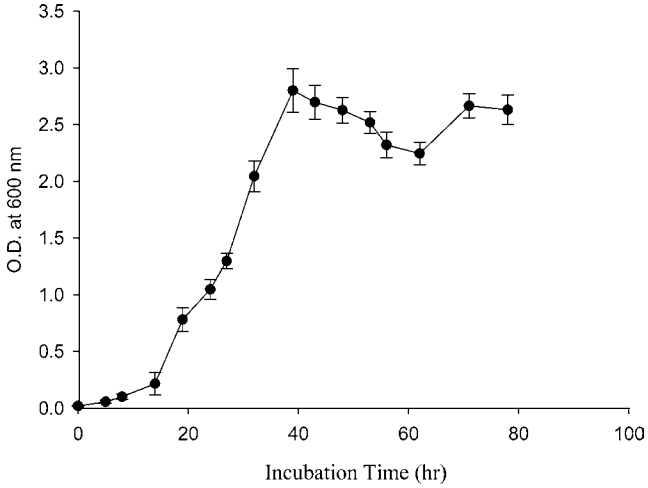


Figure 1. Growth of *P. putida* JYR-1 on *trans*-anethole at a concentration of 20 mM.

Biotransformation Kinetics of *trans*-Anethole by Resting Cells of Strain JYR-1. Biotransformation kinetics by whole cells of strain JYR-1 was investigated. The bacterial culture was harvested by centrifugation at 10000*g* at 4 °C for 15 min. The bacterial pellet was washed three times using MSB medium. The culture was resuspended in MSB medium, adjusting the optical density of the suspension at 600 nm to 1.0. The bacterial culture (25 mL) was resuspended into 125-mL septum vials containing *trans*-anethole at a concentration of 2.5 mM and incubated with shaking at 200 rpm and 25 °C. One milliliter of the bacterial culture was extracted with 5 mL of ethyl acetate once, and the solvent was evaporated to dryness with the Automated Environmental Speed Vac AES1010. Identification and quantification of the parent material and its metabolites were monitored using HPLC under conditions identical with those described above. Triplicate experimental analyses were performed. The same bacterial cultures grown on glucose as their carbon and energy source were used as the control experiments.

RESULTS

Bacterial Isolation and Identification. Growth of an enrichment culture containing 20 mM *trans*-anethole as a sole source of carbon and energy enabled us to isolate a Gram-negative soil bacterium strain, called JYR-1. Strain JYR-1 grew in minimal medium containing *trans*-anethole (20 mM) to 2.6 (OD_{600nm}) with a doubling time of 8 h (**Figure 1**). The concentration (20 mM) of *trans*-anethole was enough to kill *Pseudomonas putida* KTCT 1643 and *Escherichia coli* K12 when they were exposed to the same concentration level (data not shown). Strain JYR-1 was also able to grow at concentrations of up to 100 mM [∼1.5% (w/v)] *trans*-anethole with an extended lag time, which was the highest concentration tested in these experiments (data not shown). The partial sequence (568 bp) of its 16S rDNA, which was deposited in GenBank (Accession No. AY671902), showed that strain JYR-1 is *Pseudomonas putida* with similarity of 98.9% to the type strain. This bacterial strain has been deposited in the Biological Resource Center in Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea, under deposit no. KCTC 12301. The BBL CRYSTAL Identification System (Becton Co., Dickinson, MD) was used to investigate biochemical properties of *P. putida* JYR-1. *P. putida* JYR-1 was able to metabolize arabinose, mannose, and galactose but was not able to metabo-

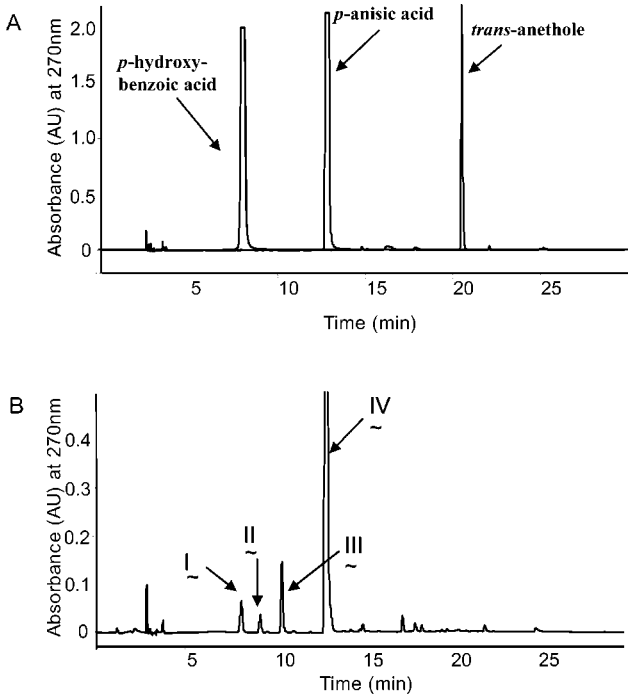
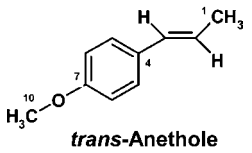


Figure 2. HPLC elution profile of the authentic compounds *trans*-anethole, *p*-anisic acid, and *p*-hydroxybenzoic acid (**A**) and the metabolites produced from *trans*-anethole by *P. putida* JYR-1 (**B**).

lize sucrose, melibiose, rhamnose, sorbitol, mannitol, adonitol, and inositol. It also metabolized glycine and arginine. It showed enzymatic hydrolysis reactions to the substrates proline nitroanilide and Γ-L-glutamyl *p*-nitroanilide. *P. putida* JYR-1 also showed urease activity and a positive result in the oxidative deamination reaction with *p*-nitro-D,L-phenylalanine.

Identification of Metabolites Produced from *trans*-Anethole by *P. putida* JYR-1. HPLC elution profiles showed four peaks corresponding to the metabolites produced from *trans*-anethole by *P. putida* JYR-1 (**Figure 2**). Metabolites **I**, **II**, **III**, and **IV** were eluted with retention times of 7.8, 8.8, 10, and 12.6 min, respectively. Metabolites **I** and **IV** showed the same retention times as did the authentic compounds, *p*-hydroxybenzoic acid and *p*-anisic acid, respectively. LC-MS analysis in the positive mode (**Figure 3**) showed molecular masses at 139.2, 165.5, 165.3, and 153.3 $[M + H]^+$ at *m*/*z* for metabolites **I**, **II**, **III**, and **IV**, respectively. The molecular weights of 138 and 152 for metabolites **I** and **IV** corresponded to the authentic compounds, *p*-hydroxybenzoic acid and *p*-anisic acid, respectively (**Figure 2**). Metabolites **II** and **III** shown on the HPLC elution profile, which have the same molecular weight of 164, were deduced to have an epoxide functional group on the propene side chain of *trans*-anethole. For further structural identification by NMR, metabolites **II** and **III** were prepared using the preparative HPLC chromatography. Metabolites were isolated, purified, and subjected to analysis by ^{1}H and ^{13}C NMR (**Table 1**). Eight peaks were observed in the ^{13}C NMR spectrum of metabolite **II**. Two peaks at 112.8 and 126.9 ppm showed double intensities, so the metabolite was composed of 10 carbons. Because the starting compound, *trans*-anethole, contained 10 carbons, too, the carbon moiety of the metabolite was considered to have not been changed. However, although the molecular weight of *trans*-anethole is 148, the molecular mass of a metabolite was observed at 165.5 $[M + 1]^+$ at *m*/*z*. Therefore, one oxygen was added to the metabolite. As listed in **Table 1**, two peaks at 112.8 and 126.9 ppm showing double intensities were assigned to C6/C8 and C5/C9 of the benzene ring, respectively, and two single carbons at 131.4 and 158.3 ppm were assigned to C4 and C7 of the benzene ring, respectively. Two quartet carbons at 16.5 and 54.3 ppm were assigned to C1 and C10, which were a methoxy group. Only two doublet carbons at 70.3 and 76.3 ppm should be decided. On the basis of their chemical shifts, the two doublet carbons were considered to be oxygenated. However, from the MS data one oxygen was added, meaning that an epoxidation reaction had occurred in this metabolic pathway. From the coupling constants of H1 (1.03 ppm, d, $J = 6.4$ Hz), H2 (3.92 ppm, dd, $J = 4.6$ Hz, 6.4 Hz), and H3 (4.50 ppm, d, $J = 4.6$ Hz), the position of the epoxidation was decided. The final structure of metabolite **II** was determined to be anethole-1,2-epoxide. The molecular weight of metabolite **III** showed only 0.1 difference with that of metabolite **II**. In addition, as listed in **Table 1**, the ^{13}C chemical shifts of metabolite **III** showed values similar to those of metabolite **II**. Their differences ranged within 1 ppm except for carbons of an epoxide. Therefore, the structure of metabolite **III** should have the same structure as metabolite **II** except for the spatial position of the epoxide group (**Figure 3**). The methyl group, C1, can be placed in the anti or syn position in the epoxide plane. Therefore, metabolites **II** and **III** must be *anti*- or *syn*-anethole-2,3-epoxide. Whereas the coupling constant of H2 and H3 in metabolite **II** is 4.6 Hz, the coupling constant in metabolite **III** is 7.5 Hz. Because $^3J_{cis}$ is greater than $^3J_{trans}$ in general, H2 and H3 in metabolite **III** have a cis conformation (*18*). As a result, we conclude that metabolite **III** is *syn*-anethole-2,3-epoxide.

Table 1. ^{1}H and ^{13}C NMR Data of Metabolites **II** and **III** Produced from *trans*-Anethole by Strain JYR-1



trans-Anethole

position of C	assignment for metabolite **II**			assignment for metabolite **III**		
	δ of ^{13}C	CHn	δ of ^{1}H	δ of ^{13}C	CHn	δ of ^{1}H
1	16.5	q	1.03 (d, 6.4)	17.7	q	0.97 (d,6.3)
2	70.3	d	3.92 (dd, 4.6, 6.4)	71.3	d	3.77 (dd, 7.5, 6.3)
3	76.3	d	4.50 (d, 4.6)	78.2	d	4.26 (d, 7.5)
4	131.4	s		132.2	s	
5, 9	126.9	d	7.22 (d, 8.7)	127.0	d	7.20 (d, 8.7)
6, 8	112.8	d	6.83 (d, 8.7)	112.9	d	6.82 (d, 8.7)
7	158.3	s		158.5	s	
10	54.3	q	3.74	54.3	q	3.74

Biotransformation Kinetics of *trans*-Anethole by Resting Cells of *P. putida* JYR-1. Figure 4 shows that *trans*-anethole (initial concentration of 2.5 mM) was completely biotransformed at 25 °C after 25 min of incubation by resting cells of *P. putida* JYR-1 grown previously in the presence of *trans*-anethole. This process followed a first-order reaction. However, *P. putida* JYR-1, which was previously grown on glucose, did not significantly biotransform *trans*-anethole in the same incubation time. As shown in **Figure 2B**, *p*-anisic acid was the compound that was most highly and transiently accumulated in the culture medium of *trans*-anethole. In addition, the production ratio of *anti*- and *syn*-anethole-2,3-epoxide produced from *trans*-anethole by the strain JYR-1 was about 1:4 with incubation time (**Figure 2B**).

DISCUSSION

P. putida JYR-1 initiated metabolism of *trans*-anethole most likely with formation of *syn*- and *anti*-anethole-2,3-epoxides as

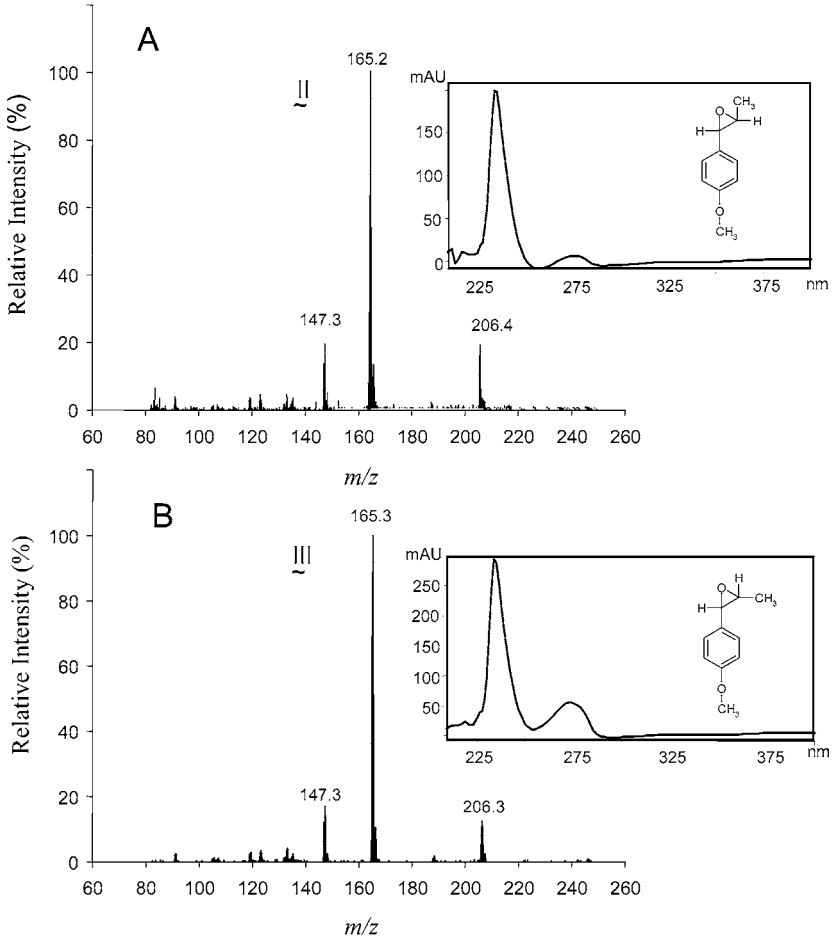


Figure 3. LC-MS spectra of metabolites **II** and **III** produced from *trans*-anethole by strain JYR-1. (Insets) UV spectra for the metabolites and parent compounds.

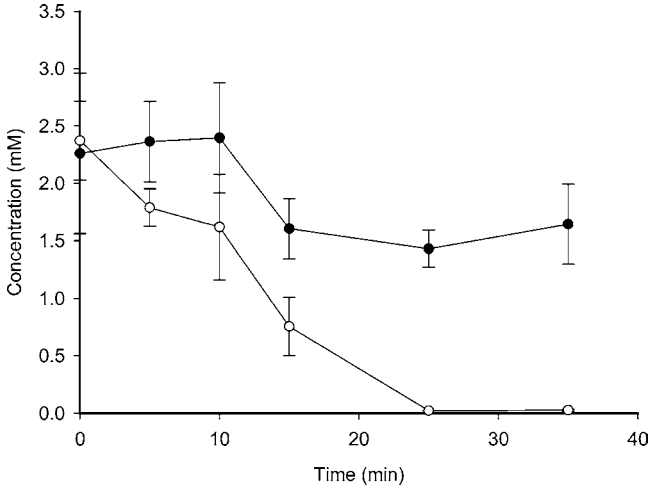


Figure 4. Biotransformation kinetics of 5 mM *trans*-anethole by *P. putida* JYR-1 previously grown with either *trans*-anethole (○) or glucose (●).

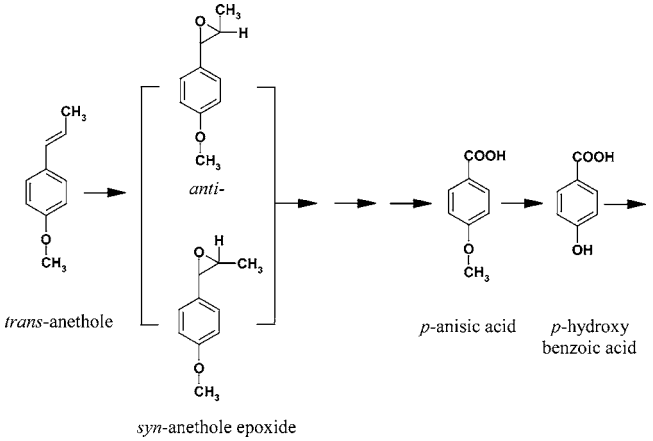


Figure 5. Proposed biotransformation pathway of *trans*-anethole by *P. putida* JYR-1.

initial products, on the propene side chain of *trans*-anethole instead of initiating the metabolism on the aromatic ring structure. Regardless of the stereochemistry of the anethole epoxides, *P. putida* JYR-1 could proceed with further metabolism, resulting in transient accumulation of *p*-anisic acid and *p*-hydroxybenzoic acid. The deduced metabolic pathway of *trans*-anethole by *P. putida* JYR-1 (**Figure 5**) is very similar to that by a Gram-positive *Arthrobacter* species, which was proposed by Shimoni et al. (*6*, *7*). That *Arthrobacter* species can also biotransform derivatives of *trans*-anethole, eugenol, isoeugenol, and isosafrole, to vanillic acid and ferulic acid, vanillic acid and vanillin, and piperonylic acid, respectively (*7*). However, they did not confirm the presence of anethole epoxides as the initial metabolites in the metabolism of *trans*-anethole by *Arthrobacter* species. They postulated production of anethole-2,3-epoxide indirectly by detection of *trans*-anethyldiol in the metabolism. Miller et al. (*19*) extensively studied the mechanism of monooxygenase activity of cytochrome *c* peroxidase, which can catalyze epoxidation of a π bond on the olefins, styrene, *trans*-β-methylstyrene, and *cis*-β-methylstyrene. CcP exclusively produced *trans*-epoxide from *trans*-β-methylstyrene. However, the enzyme oxidized styrene to the styrene epoxide and

phenylacetaldehyde and *cis*-*β*-methylstyrene to the *cis*- and *trans*-epoxide and 1-phenyl-2-propanone. Compared with the metabolites produced from the *trans*-olefins, the enzymatic mechanism of *P. putida* JYR-1, which would be involved in the epoxidation of *trans*-anethole, is most likely different from that of CcP. A similar epoxide formation has been well studied with styrene by enzymes of ammonia monooxygenase from *Nitrosomonas europaea* (*20*), soluble methane monooxygenases from *Methylococcus capsulatus* (Bath) (*21*), *Methylosinus trichosporium* OB3b (*22*), bacterial styrene monooxygenase from *Pseudomonas fluorescens* ST (*23*), chloroperoxidase (*24*), cytochrome *c* peroxidase (*19*), and hepatic and bacterial P-450 isozymes (*25*, *26*).

Considering the potential industrial applications of the strain, *P. putida* JYR-1 may be a valuable bacterium in terms of environmentally benign bioproduction of a versatile chemical synthone epoxide from naturally occurring phenylpropanoid compounds and production of value-added metabolites such as anisic acid and anisaldehyde. Indeed, chemical epoxidation of olefins catalyzed by metal complexes has become an important research area in both bulky production of fine and pharmaceutical grade chemicals and bio-inorganic modeling of oxygen-transfer metalloenzymes over the past decade (*27*, *28*). In addition, *P. putida* JYR-1 showed growth in high concentrations of *trans*-anethole, up to 100 mM, without the addition of Amberlite XAD-2, which was used to grow *Arthrobacter* species (*6*).

In conclusion, *P. putida* JYR-1, resistant to high concentrations of *trans*-anethole, facilitated an epoxidation reaction on the propene side chain of the compound, resulting in the formation of stereoisomer *anti*- and *syn*-anethole-2,3-epoxides.

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