Isolation of a Gene Responsible for the Oxidation of *trans*-Anethole to *para*-Anisaldehyde by *Pseudomonas putida* JYR-1 and Its Expression in *Escherichia coli*

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A plasmid, pTA163, in *Escherichia coli* contained an approximately 34-kb gene fragment from *Pseudomonas putida* JYR-1 that included the genes responsible for the metabolism of *trans*-anethole to protocatechuic acid. Three Tn5-disrupted open reading frame 10 (ORF 10) mutants of plasmid pTA163 lost their abilities to catalyze *trans*-anethole. Heterologously expressed ORF 10 (1,047 nucleotides [nt]) under a T7 promoter in *E. coli* catalyzed oxidative cleavage of a propenyl group of *trans*-anethole to an aldehyde group, resulting in the production of *para*-anisaldehyde, and this gene was designated *tao* (*trans*-anethole oxygenase). The deduced amino acid sequence of TAO had the highest identity (34%) to a hypothetical protein of *Agrobacterium vitis* S4 and likely contained a flavin-binding site. Preferred incorporation of an oxygen molecule from water into *p*-anisaldehyde using ¹⁸O-labeling experiments indicated stereo preference of TAO for hydrolysis of the epoxide group. Interestingly, unlike the narrow substrate range of isoeugenol monooxygenase from *Pseudomonas putida* IE27 and *Pseudomonas nitroreducens* Jin1, TAO from *P. putida* JYR-1 catalyzed isoeugenol, *O*-methyl isoeugenol, and isosafrole, all of which contain the 2-propenyl functional group on the aromatic ring structure. Addition of NAD(P)H to the ultrafiltered cell extracts of *E. coli* (pTA163) increased the activity of TAO. Due to the relaxed substrate range of TAO, it may be utilized for the production of various fragrance compounds from plant phenylpropanoids in the future.

Bacterial metabolism as a means of producing value-added compounds from natural resources has been given much attention as an alternative method to replace conventional chemical syntheses (21, 27). If natural resources can be continuously supplied as the starting materials, biotransformation employing bacterial systems may become practical from the points of view of sustainability and applicability. One of the major groups of natural compounds targeted for the production of value-added compounds includes the group of chemicals that occur in plant phenylpropanoid pathways, which are involved in the production of lignin, flavonoids, anthocyanins, etc. (5, 16–19). For example, isoeugenol, eugenol, and ferulic acid produced by the phenylpropanoid pathway have often been attempted as the starting materials to produce vanillin, one of the most extensively used aromatic flavor compounds (25–27, 32).

trans-Anethole (*p*-methoxy propenylbenzene), the major component present in the essential oils of anise, fennel, and star anise, is also a type of phenylpropanoid compound formed by terpene synthesis in plants (10). *trans*-Anethole is used commercially as a flavor substance in baked goods, candy, ice cream, chewing gum, and alcoholic beverages (21). However, there have been few research reports regarding its metabolism (8, 12, 21, 22, 31). Indeed, to date, the only two bacterial strains isolated that are known to use *trans*-anethole as a sole carbon source are *Arthrobacter* sp. strain TA13 and *Pseudomonas putida* JYR-1 (14, 22). When strain TA13 was induced with *trans*-anethole, it was capable of growing on *p*-anisic alcohol, *p*-anisaldehyde, *p*-anisic acid, *p*-hydroxybenzoic acid, and protocatechuic acid as the sole carbon and energy sources. Based on the metabolism of *trans*-anethole by a series of mutant strains of TA13, metabolism of *trans*-anethole by strain TA13 was postulated to proceed to *p*-anisic alcohol via initial epoxide or diol formation. In the case of strain JYR-1, two

stereoisomeric epoxides, *syn*- and *anti*-anethole-2,3-epoxides, were in fact identified in the culture medium as metabolic intermediates during biotransformation of *trans*-anethole. *p*-Anisic acid and *p*-hydroxybenzoic acid were also detected in cell cultures of strain JYR-1 (14).

The two strains *Arthrobacter* sp. strain TA13 and *P. putida* JYR-1 can transform various compounds present in the phenylpropanoid pathway. In fact, strain TA13 can convert isoeugenol into vanillin and vanillic acid, eugenol into vanillic acid and ferulic acid, isosafrole into piperonylic acid, and safrole into hydroxychavicol (21). However, due to the absence of demethylase in *Arthrobacter* sp. strain TA13, the strain cannot cleave the aromatic ring structure and further utilization does not occur (21). In contrast, *P. putida* strain JYR-1 was able to utilize not only caffeic acid and *p*-coumaric acid as sole sources of carbon and energy, but also isoeugenol and ferulic acid, both of which possess a methoxy group at the *para* position of the aromatic ring. However, resting cells of strain JYR-1 previously grown on *trans*-anethole could not metabolize eugenol. These results indicate that there are likely slightly differ-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>Pseudomonas putida</i> JYR-1	<i>trans</i> -Anethole transformation strain	14
<i>Escherichia coli</i>		
BL21(DE3)	Host strain for expression vector; F [−] <i>ompT hsdS_B(r_B[−] m_B[−]) gal dcm</i> (DE3)	Novagen
EC100	Host strain for transposon Tn5 insertion; F [−] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU galK</i> λ [−] <i>rpsL nupG</i>	Epicentre
EPI100	Host strain for fosmid genomic library; F [−] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU galK</i> λ [−] <i>rpsL nupG trfA tonA dhfr</i>	Epicentre
DH5α	Host strain for cloning vector; F [−] <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> φ80 <i>dlacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>hsdR17</i> (r _K [−] m _K ⁺) λ [−]	2
Plasmids		
pEpiFos-5	Cm ^r ; 7.5-kb fosmid vector for construction of the genomic library	Epicentre
pET-TAO	Ap ^r ; pET21-a expression vector containing <i>tao</i> gene	This study
pET21-a	Ap ^r ; expression vector	Novagen
pGEM-T Easy	Ap ^r ; TA cloning vector	Promega
pG-TAO	Ap ^r ; pGEM-T Easy cloning vector containing <i>tao</i> gene	This study
pTA163	Cm ^r ; 41-kb pEpiFos-5 containing <i>tao</i> from JYR-1	This study
pTA163-3A, pTA163-1C, pTA163-7C	Cm ^r Km ^r ; transposon Tn5 insertion into <i>tao</i> of pTA163	This study

ent metabolic pathways for the biotransformation of phenyl-propanoid compounds by these two bacterial strains.

Considering the ability of strain JYR-1 to catalyze the propene functional group to an aldehyde, characterization of the corresponding genes from strain JYR-1 would be worthy of studying with the aim of producing value-added compounds from plant biomass materials. In the present study, we first report genes involved in the metabolism of *trans*-anethole, which are clustered in about a 12-kb nucleotide region of strain JYR-1. The *trans*-anethole oxygenase (TAO) gene (*tao*; 1,047 nucleotides [nt]), which initiated the first reaction in the metabolism of *trans*-anethole, was cloned and heterologously expressed in *Escherichia coli*. The NAD(P)H-dependent TAO was able to catalyze *trans*-anethole, isoeugenol, *o*-methyl isoeugenol, and isosafrole, all of which have the propene side chain, as does *trans*-anethole. The deduced amino acid sequence of TAO reveals that TAO is a novel oxygenase with no putative conserved domains reported until now.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. All plasmids and bacterial strains used in this study are listed in Table 1. *Pseudomonas putida* JYR-1 was grown in tryptic soy broth (TSB) or Stanier's minimal salt broth (MSB) (24) containing 10 mM *trans*-anethole and incubated by rotary shaking at 200 rpm and 25°C. *Escherichia coli* strains EPI100, EC100, DH5α (2), and BL21(DE3) were routinely grown in LB medium and incubated by rotary shaking at 200 rpm and 37°C. When required, ampicillin (Amp) at 50 μg/ml, kanamycin (Kan) at 50 μg/ml, and chloramphenicol (Chl) at 12.5 μg/ml were used for the corresponding recombinant *E. coli* strain selection.

Chemicals. *trans*-Anethole, *para*-anisaldehyde, isoeugenol, eugenol, propenyl guaethol, *O*-methyl isoeugenol, isosafrole, cinnamic acid, ferulic acid, and 4-coumaric acid were purchased from Sigma-Aldrich (Milwaukee, WI). Organic solvents (high-performance liquid chromatography [HPLC] grade) were purchased from Fisher Scientific (Fair Lawn, NJ).

Gene library construction. DNA of *P. putida* JYR-1 was extracted using a Qiagen DNA buffer set and Genomic-tip 100/G (Qiagen, Valencia,

CA) according to the manufacturer's instructions. Approximately 40-kb DNA fragments were prepared, and a fosmid library was constructed using the EpiFOS fosmid library production kit (Epicentre Biotechnologies, Madison, WI), also according to the manufacturer's instructions. Six hundred chloramphenicol-resistant (Chl^r) clones were picked, and 10 clones each were inoculated into 160-ml serum bottles that contained 20 ml of LB medium plus 500 μM *trans*-anethole. Cells were cultured overnight at 30°C by rotary shaking at 200 rpm, and equal volumes of ethyl acetate were added to all bottles to extract *trans*-anethole and its metabolites from the LB medium by vertical shaking at 120 rpm for 10 min. Extracts (4 ml) were dried *en vacuo* with a Speed vacuum (Vision Scientific Co., Suwon, South Korea), dissolved in 0.5 ml of methanol, and analyzed by high-performance liquid chromatography (HPLC) as described below. A single colony, *E. coli* EPI100(pTA163), from among 600 colonies was found to be able to metabolize *trans*-anethole. Fosmid DNA from pTA163 was extracted with a Qiagen large-construct kit (Qiagen, Valencia, CA) as described in the manufacturer's instructions and was sequenced by Macrogen, Inc. (Seoul, Republic of Korea).

Tn5 mutagenesis of plasmid pTA163. Plasmid pTA163 from *E. coli* EPI100(pTA163) was isolated and reacted with a transposon from the EZ-Tn5<KAN-2> insertion kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. *E. coli* TransdorMax EC100 (Epicentre Biotechnologies, Madison, WI) was transformed with the Tn5-inserted plasmid pTA163 by electroporation (1.8 kV, 5.9 ms) and spread onto LB agar plates. Transposon-inserted clones were selected from the LB agar plates containing both Kan (50 μg/ml) and Chl (12.5 μg/ml). Ninety-six colonies were selected and tested to screen for mutants that were defective in the biodegradation of *trans*-anethole. Each mutant strain was inoculated into 10 ml of LB medium containing the antibiotics overnight, and resting cells were prepared by centrifugation at 10,000 × g for 10 min and washed twice with 20 mM phosphate buffer (pH 7.0). Suspended cells (optical density at 600 nm [OD₆₀₀] of 2.0) in the same buffer were incubated with 2 mM *trans*-anethole for 4 h at 25°C by rotary shaking at 200 rpm. Ethyl acetate was added to the reaction mixture, the mixture was evaporated to dryness *en vacuo* as described above, and residue was dissolved in methanol and analyzed by HPLC. The mutants pTA163-1C, pTA163-3A, and pTA163-7C, which lost their ability to transform *trans*-anethole, were identified. For confirmation of the Tn5 transposon insertion sites of the three mutants, fosmid DNA from

FIG 1 Identification of genes related to *trans*-anethole degradation. (A) Contig 2 of fosmid clone pTA163, which contains a *P. putida* JYR-1 genomic DNA fragment, and (B) *tao* gene with arrows indicating the sites of Tn5 transposon insertions.

mutated colonies was extracted as described above and sequenced bidirectionally by Macrogen, Inc. (Seoul, Republic of Korea), using Ez-Tn5<KAN-2> transposon-specific primers (forward primer KAN-2 FP-1 and reverse primer KAN-2 RP-1, supplied with the Tn5 mutagenesis kit). Afterwards, the insertion sites were identified by mapping of the flanking sequences of the Tn5 transposon.

Subcloning of ORF 10 encoding TAO. In order to clone open reading frame 10 (ORF 10) (Fig. 1), PCR was performed by forward-primer-attaching NdeI recognition sequence (5'-GGGAATTCCATATGGAGGACATCATGCAAGGC-3') and reverse-primer-attaching BamHI recognition site (5'-CGCGGATCCTCAGTTAGTCTCAAGTCGGAATT-3'). The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI) to obtain plasmid pG-TAO, which was used for transformation of *E. coli* DH5 α . The ORF 10 region of plasmid pG-TAO was digested by NdeI and BamHI and ligated into expression vector pET21-a (Novagen, Madison, WI) under the T7 promoter. The resulting plasmid, pET-TAO, was transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI). As a control experiment, heat-killed cells of *E. coli* BL21(DE3)(pET-TAO) were used.

Biotransformation of *trans*-anethole and other related compounds by resting cells of *E. coli* BL21(DE3)(pET-TAO) expressing TAO. *E. coli* BL21(DE3)(pET-TAO) was cultured in LB medium overnight at 37°C, by rotary shaking at 200 rpm. Cells (1% [vol/vol]) were transferred into fresh LB medium and cultured for 2.5 h at 37°C by rotary shaking at 150 rpm. Expression of TAO was induced for 4 h after IPTG (isopropyl- β -D-thiogalactopyranoside) was added to the medium in a final concentration of 1 mM. The cells were harvested by centrifugation at 10,000 $\times$ g for 10 min and resuspended in MSB medium. After washing twice, the cells were finally resuspended in MSB medium and adjusted to an optical density of 2.0 at 600 nm. The resuspended cells supplemented with 0.5 mM glucose as an energy source were reacted with 1 mM *trans*-anethole, eugenol, isosafrole, *O*-methyl isoeugenol, propenyl guaethol, cinnamic acid, 4-coumaric acid, and ferulic acid (each from 100 mM stock solution prepared in methanol). The reaction was performed by rotary shaking at 250 rpm and 25°C for 6 h, and 3 volumes of ethyl acetate was used to extract the reaction solutions. The ethyl acetate extract was evaporated *en vacuo* as described previously, and the residue was dissolved in an appropriate volume of methanol and filtered with a polyvinylidene fluoride (PVDF) syringe filter. The amounts of the remaining substrates and metabolites produced from the reactions were determined by HPLC and by calculation from the standard curve of authentic chemicals, respectively. Each metabolite was identified by comparison of UV spectra and liquid chro-

matography-mass spectrometry (LC-MS) full-scan analyses with the results from analyses of authentic compound standards. All analyses were done in triplicate.

$^{18}\text{O}_2$ and H_2^{18}O incubations. $^{18}\text{O}_2$ incubation was performed in a closed system connected with two syringes. Air was removed from reaction buffer by flushing N_2 before closing the system. Fifty percent $^{18}\text{O}_2$ was added into the vial after removing the corresponding volume of N_2 . H_2^{18}O incubation experiments were carried out with resting cells of *E. coli* BL21(DE3)(pET-TAO) expressing TAO and 1 mM *trans*-anethole in 0.5 ml of MSB buffer prepared with 50% H_2^{18}O . The same methods described above were applied for reactions, extractions, and analyses.

Effect of cofactors, metal ions, chelator, and inhibitors on the biotransformation of *trans*-anethole by cell extracts of *E. coli* BL21(DE3)(pET-TAO) expressing TAO. *E. coli* BL21(DE3)(pET-TAO) was cultured, harvested, washed twice with 20 mM Tris buffer (pH 8.0), and stored at -70°C until use. The cell pellets of *E. coli* BL21(DE3)(pET-TAO) (10 g wet weight) were resuspended in 20 ml of 20 mM Tris (pH 8.0) containing 10% glycerol (TG buffer). The cell extracts were prepared using an ultrasonicator (Cole-Parmer, Chicago, IL) with 70% amplitude for 20 min (3.0 s on and 9.0 s off) and centrifuged at 18,000 $\times$ g for 30 min twice at 4°C using 20 mM Tris buffer (pH 8.0). The cell extracts were diluted with 5 volumes of TG buffer and concentrated using ultrafiltration through an Amicon YM-10 membrane (Millipore, Bedford, MA) two times at 4°C (13). Ultrafiltered cell extracts (1 mg) with 1 mM *trans*-anethole were transferred into 20 mM Tris-Cl (pH 8.0) in a total volume of 1 ml, which contained the cofactors NAD(P)H and metal ion Fe(II), Fe(III), Mg(II), Mn(II), Ni(II), Cu(II), or Zn(II). In addition, sodium 4,5-dihydroxybenzene-1,3-disulfonate (tiron), a metal chelator, and proadifen and *p*-hydroxymercuribenzoate, inhibitors of cytochrome P-450 monooxygenases and oxidoreductases, respectively, were also tested in biotransformation reactions of *trans*-anethole by cell extracts from *E. coli* BL21(DE3)(pET-TAO) expressing TAO. All of the cofactors, metal ions, and metal chelator tiron were added to the solutions at final concentrations of 1 mM. The reactions were initiated by addition of *trans*-anethole to the reaction solutions, carried out for 10 min at 30°C, and stopped by addition of 1 ml of 100% methanol. After centrifugation of the solutions at 13,000 $\times$ g for 20 min at 4°C, the supernatant solutions were analyzed by HPLC to determine the amounts of *p*-anisaldehyde that were produced. All analyses were done in triplicate.

Analytical methods. 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- μ m particle size, 4.6 mm by 25 cm; Milford, MA). The mobile phase, which was composed of acetonitrile containing 0.1% formic acid and water, 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 10 μ l was 1 ml/min, and UV detection was performed at 270 nm. LC-MS was performed by coupling an Alliance 2695 LC system (Waters Corporation, Milford, MA) to a Quattro LC triple-quadrupole tandem mass spectrometer (Waters, Milford, MA) in positive electrospray ionization (ESI⁺) mode. For LC analysis, a SunFire C₁₈ column (3.5 μ m, 2.1 by 150 mm; Waters) was used and the mobile phase, elution program, and detection were identical to those for analytical HPLC described above; the flow rate was 0.2 ml/min. For MS analysis, the source temperature, desolvation temperature, and capillary voltage were kept at 150°C, 350°C, and 3.2 kV, respectively. The cone voltage was 20 V. The cone gas and desolvation gas were ultrapure nitrogen set at 30 liters/h and 500 liters/h, respectively. Protein concentration was determined by the Bradford assay (3) with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as a standard.

Nucleotide sequence accession number. The DNA and deduced protein sequences described in this study have been deposited in the GenBank database under accession no. [HQ889281](#).

RESULTS

Genes involved in *trans*-anethole biotransformation. The positive fosmid clone pTA163 carrying 35 kb of *P. putida* JYR-1 genomic DNA was selected from 600 colonies, and *E. coli* EPI100(pTA163) was determined to possess the ability to transform *trans*-anethole to *para*-anisic acid. The inserted DNA of the pTA163 clone was sequenced and assembled into four contigs, no. 0, 1, 2, and 3, with 3.1-, 2.0-, 20.4-, and 8.6-kb nucleotides, respectively. Among them, contig 2 included 14 putative open reading frames (ORFs) (Fig. 1). Based on the deduced amino acid sequences of the 14 ORFs in contig 2, which were blasted against the NCBI protein database and annotated (see Table S1 in the supplemental material), ORFs 7, 8, 11, and 13 were expected to be involved in the metabolic pathway of *trans*-anethole. Deduced amino acid sequences of ORFs 7 and 8 possessed the highest identities to ferredoxin (62%; reductase component of *p*-anisic acid demethylase) and *p*-anisic acid demethylase (75%) from *P. putida* W619, which might be involved in the demethylation of *p*-anisic acid, and were therefore designated *aniA* and *aniB*, respectively. The deduced amino acid sequences of ORFs 11 and 13 exhibited 65 and 74% identity to *p*-hydroxybenzaldehyde dehydrogenase and *p*-hydroxybenzoate hydroxylase, respectively. Considering the structural similarities between *p*-hydroxybenzaldehyde and *p*-anisaldehyde, ORF 11 was expected to be responsible for the dehydrogenation of *p*-anisaldehyde and ORF 13 might be capable of hydroxylation of *p*-hydroxybenzoic acid. Therefore, ORFs 11 and 13 were designated *adh* and *pbh*, coding for *p*-anisaldehyde dehydrogenase and *p*-hydroxybenzoate hydroxylase, respectively (see Fig. 6). In addition, we identified the possible metabolism of formaldehyde that is derived from the demethylation of *p*-anisic acid. The genes involved in this, based on the deduced amino acid sequences of ORF 1 and ORF 4, which showed 97% and 98% identities to the formate dehydrogenase alpha subunit from *P. putida* F1 and glutathione-independent formaldehyde dehydrogenase (accession no. YP_001265877 and YP_001666600 in the GenBank database), respectively, were designated *fdh1* and *fdh2*, respectively.

Tn5 mutagenesis of plasmid pTA163. Ninety-six colonies were isolated and examined for their ability to biotransform

trans-anethole by HPLC. Insertion sites of the colonies which lost the ability for biotransformation were identified by bidirectional sequencing, and as shown in Fig. 1, three mutations occurred in ORF 10 of contig 2 from pTA163. These results indicated that ORF 10 is a related gene involved in the biotransformation of *trans*-anethole. We named ORF 10 (1,047 bp) the *tao* (*trans*-anethole oxygenase) gene (Fig. 1), which did not align with genes with more than 4% sequence similarity (data not shown) that were compared in the GenBank database. Also, its deduced amino acid sequence also aligned the most (34% identity) to the hypothetical protein Avi_3741 of *Agrobacterium vitis* S4 (accession no. YP_002550702.1) (see Table S2 in the supplemental material). In addition, the deduced amino acid sequence of TAO did not contain a conserved domain compared to other enzymes that have similar catalytic functions toward similar chemical structures, like the isoeugenol monooxygenases Iso from *P. putida* and Iem from *P. nitroreducens* (15, 34), apocarotenoid-15,15'-oxygenase (ACO) (7), and styrene monooxygenase (SMO) (28).

Biotransformation of *trans*-anethole and other related compounds by resting cells of *E. coli* BL21(DE3)(pET-TAO) expressing TAO. Two milliliters of resting cells of *E. coli* BL21(DE3)(pET-TAO) induced by 1 mM IPTG was equivalent to a total protein concentration of 0.38 mg/ml and was able to completely convert 1 mM *trans*-anethole to *p*-anisaldehyde in 4 h of incubation with the reaction rate of 5.48 nmol/min-mg total protein. Expression of TAO was proven by SDS-PAGE of cell extract from *E. coli* (pET-TAO) induced by 1 mM IPTG (see Fig. S1 in the supplemental material). Metabolites of *trans*-anethole that were produced by *E. coli* BL21(DE3)(pET-TAO) were identified by retention time on HPLC chromatograms (Fig. 2), UV-visible spectroscopy (data not shown), and molecular weights detected by LC-MS spectroscopy (Fig. 3) compared to those of the corresponding authentic compounds. HPLC elution profiles from the sample extracted at a reaction time of 40 min showed two peaks at 14.5 and 20.1 min, and these retention times were identical to the authentic compounds *para*-anisaldehyde and *trans*-anethole, respectively (Fig. 2A). HPLC elution profiles from the sample extracted at 4 h showed one peak at 14.5 min, which was identified as *para*-anisaldehyde (Fig. 2B). Electron impact MS (EI-MS) analyses in the positive ionization mode (Fig. 3) showed a molecular weight of 136, giving ions at 137 [M+H]⁺ and 108 [M-29+H]⁺, confirming *para*-anisaldehyde as a metabolite of *trans*-anethole. In this experiment, *syn*- and *anti*-anethole epoxides, which were detected in the culture medium of *P. putida* JYR-1, were not detected from resting cells of *E. coli* BL21(DE3)(pET-TAO). This is most likely due to the near instantaneous conversion of the epoxides to *p*-anisaldehyde by the recombinant *E. coli* cells. In addition, resting cells of *E. coli* BL21(DE3)(pET-TAO) were incubated for 4 h with 1 mM substrates eugenol, isoeugenol, *O*-methyl isoeugenol, isosafrole, propenyl guaethol, cinnamic acid, 4-coumaric acid, and ferulic acid, which are structurally similar to *trans*-anethole. As shown in Table 2, TAO accepted isoeugenol, *O*-methyl isoeugenol, and isosafrole as substrates and resulted in formation of aldehyde products, vanillin (0.63 mM), veratraldehyde (0.38 mM), and piperonal (0.38 mM), respectively. Identification of aldehyde products by HPLC and LC-MS is shown in Fig. S2 and S3, respectively, in the supplemental material. TAO also converted the compounds with 3,4-dimethoxy and 3,4-methylenedioxy functional groups, as shown from *O*-methyl isoeugenol and isosa-

FIG 2 HPLC elution profiles of the metabolite produced from *trans*-anethole by *E. coli* BL21(DE3)(pET-TAO) after 40 min of incubation (A) and 240 min of incubation (B) and that produced by *E. coli* BL21(DE3)(pET21a) (C), as well as authentic compounds (D) *para*-anisaldehyde and *trans*-anethole. AU, arbitrary units.

frole, respectively, at half the conversion rates of *trans*-anethole and isoeugenol. As shown with propenyl guaethol, TAO was unable to metabolize the compounds with an ethoxy functional group in the *meta* position (Table 2). Finally, eugenol, cinnamic acid, ferulic acid, and 4-coumaric acid were not transformed by TAO (Table 2). Considering these chemical structures, it was likely that TAO required a 1-propenylbenzene group, not 2-propenyl, with methoxyfunctional group(s) at either the *para* or *meta* position on the benzene ring.

¹⁸O-labeling experiment for incorporation of an oxygen atom into *para*-anisaldehyde by resting cells of *E. coli* BL21(DE3)(pET-TAO). In order to identify the origin of the ox-

ygen atom incorporated into *p*-anisaldehyde during oxidative cleavage of the propenyl group of *trans*-anethole by TAO, three different oxygen labeling combinations were tested as follows: (i) ¹⁶O₂ and H₂¹⁶O, (ii) ¹⁸O₂ and H₂¹⁶O, and (iii) ¹⁶O₂ and H₂¹⁸O. LC-MS was used to measure the increased mass of *p*-anisaldehyde produced by resting cells of *E. coli* BL21(DE3)(pET-TAO). The increased mass for *p*-anisaldehyde at 139 *m/z* was only detected in the cells incubated with the oxygen feeding combination—¹⁶O₂ and H₂¹⁸O (Fig. 3). Based on the reactions, the reaction mechanism of oxygen incorporation into *p*-anisaldehyde by TAO was proposed as shown in Fig. 4C. The pattern of oxygen incorporation by TAO is quite different from the incorporation of oxygen

FIG 3 LC-MS spectra of a metabolite produced from *trans*-anethole by *E. coli* BL21(DE3)(pET-TAO). (A) Incubation with O₂ and H₂O; (B) incubation with ¹⁸O₂ and H₂O; (C) incubation with O₂ and H₂¹⁸O; (D) authentic *para*-anisaldehyde.

TABLE 2 Biotransformation of *trans*-anethole and other related compounds by resting cells of *E. coli* BL21(DE3)(pET-TAO) expressing TAO

Substrate chemical name	Substrates	Availability from plants	Metabolites		
	Structure		Amt (mM) of metabolite produced	Chemical name	Structure
<i>trans</i> -Anethole		Yes	0.63 ± 0.00	<i>p</i> -Anisaldehyde	
Eugenol		Yes	ND ^a		
Isoeugenol		Yes	0.63 ± 0.02	Vanillin	
Isosafrole		Yes	0.38 ± 0.01	Piperonal	
O-Methyl isoeugenol		Yes	0.38 ± 0.02	Veratraldehyde	
Propenyl guaethol		No	ND		
Cinnamic acid		Yes	ND		
Ferulic acid		Yes	ND		
4-Coumaric acid		Yes	ND		

^a ND, not detected.

atoms from O₂ and water into vanillin by Iso of *P. putida* IE27 (34) and Iem of *P. nitroreducens* Jin1, in which an oxygen atom from either ¹⁸O₂ or H₂¹⁸O may be inserted into the aldehyde group of vanillin produced from isoeugenol. These results suggest that *E. coli* BL21(DE3)(pET-TAO) expressing TAO has a stereopreferential activity for incorporating oxygen atoms from water molecules into the carbon atom closely located next to the aromatic ring structure. However, we could not detect acetaldehyde, which is a possible metabolite from the reaction (34), possibly due to its fast metabolism by the resting cells.

Effect of metals ions on biotransformation of *trans*-anethole by cell extracts of *E. coli* BL21(DE3)(pET-TAO) expressing TAO. Ultrafiltered cell extracts of *E. coli* BL21(DE3)(pET-TAO) were prepared to test the effects of different metal ions on the biotransformation of *trans*-anethole. The activities of the ultrafiltered cell extracts of *E. coli* BL21(DE3)(pET-TAO) for 10 min of incubation were increased up to 13.0 and 9.7 times by addition of NADH and NADPH (Table 3), implying that TAO is likely to be

an NAD(P)H-dependent oxygenase. The activities of the ultrafiltered cell extracts of *E. coli* BL21(DE3)(pET-TAO) were also increased by addition of the inorganic cations Mg(II), Mn(II), Ni(II), and Zn(II), with relative activities of 238.8%, 171.9%, 149.5%, and 223.6%, respectively. However, addition of Fe(II), Fe(III), and Cu(II) appeared not likely to affect TAO activity (Table 3). Interestingly, addition of Fe(II) or Fe(III) to the reaction solution containing NAD(P)H was found to inhibit TAO activity. Addition of the metal chelator tiron did not inhibit TAO activity, indicating that TAO may not contain metal binding sites. Also proadifen, the inhibitor of cytochrome P-450 enzymes, had no effect on TAO activity. Finally, *p*-hydroxymercuribenzoate, the oxidoreductase inhibitor (11), inhibited TAO activity by 63.9% (Table 3).

DISCUSSION

The *tao* gene located in the constructed fosmid pTA163 from *P. putida* JYR-1 encoded *trans*-anethole oxygenase (TAO), which

FIG 4 Proposed mechanism of *trans*-anethole side chain cleavage by *E. coli* BL21(DE3)(pET-TAO) expressing TAO of *P. putida* JYR-1. Pathways A, B, and C were deduced from conditions A, B, and C in Fig. 3.

metabolized *trans*-anethole to *p*-anisaldehyde. Tn5 mutagenesis of plasmid pTA163 also confirmed that *tao* was responsible for encoding the enzyme for the initial metabolism of *trans*-anethole. In a parallel experiment, fosmid pTA163 was sequenced and contig 2 in the fosmid pTA163 was found to contain all of the proposed metabolic genes encoding *p*-anisaldehyde dehydrogenase (PAADH), two components of *p*-anisic acid demethylase (ANI), and *p*-hydroxy benzoate hydroxylase (PBH) (Fig. 1). Based on the genetic information, we postulated that *trans*-anethole is metabolized to protocatechuic acid through formation of *trans*-anethole

epoxide, *trans*-anethole diol, anisaldehyde, anisic acid, and *p*-hydroxybenzoic acid. A similar pathway for *trans*-anethole metabolism was previously suggested for *Arthrobacter* sp. strain TA13 (22). In addition, a mutant strain of *Arthrobacter* sp. strain TA13, which lost its 4-methoxybenzoate-*O*-demethylase activity, accumulated *trans*-anethole diol, *p*-anisic alcohol, *p*-anisaldehyde, and *p*-anisic acid in the culture medium (21).

TAO heterologously expressed in *E. coli* catalyzed the incorporation of an oxygen atom into the carbon-carbon double bond on the 1-propenyl group of the substrate *trans*-anethole, leading to the formation of *p*-anisaldehyde through epoxide and diol intermediates. Compared to the extreme narrow substrate range of isoeugenol monooxygenases, Iem from *Pseudomonas nitroreducens* Jin1 (15) and Iso from *Pseudomonas putida* IE27 (34), which metabolize isoeugenol alone, TAO exhibited a relatively broad substrate range that encompassed isoeugenol, *O*-methyl isoeugenol, and isosafrole (Table 2). However, when the methoxy group is replaced with an ethoxy group on the aromatic ring, TAO was unable to catalyze the compound, as shown in the case of propenyl guaethol. TAO was also unable to catalyze acrylic acid (2-propenoic acid) attached to the aromatic ring structure, such as in the cases of cinnamic acid, ferulic acid, and 4-coumaric acid. When the *tao* gene sequence and its deduced amino acid sequences were compared with sequences of Iem and Iso, they did not show any similarity, suggesting different enzymatic reaction mechanisms between TAO and Iem and Iso when exerted on isoeugenol.

Regarding the biochemical reaction mechanism mediated by TAO, Table 2 provides evidence that TAO is likely to be an NAD(P)H-dependent and metal ion-independent enzyme (Table 3). In addition, inhibition of TAO by *p*-hydroxymercuribenzoate suggested that an oxidoreductase component may be involved in

TABLE 3 Effect of cofactors, chelator, and inhibitors on biotransformation of *trans*-anethole to *p*-anisaldehyde by ultrafiltered cell extracts of *E. coli* BL21(DE3)(pET-TAO) expressing TAO

Cofactor(s) (1 mM)	% relative activity ^a
No cofactor	100 ± 0.0 (2.0 ± 0.0 μM) ^b
NADH	1297.8 ± 254.4
NADPH	967.8 ± 113.5
NADH + Fe(II)	443.8 ± 114.0
NADH + Fe(III)	731.9 ± 42.7
Fe(II)	112.5 ± 9.7
Fe(III)	103.3 ± 16.6
Cu(II)	102.1 ± 9.4
Mg(II)	238.8 ± 38.6
Mn(II)	171.9 ± 20.7
Ni(II)	149.5 ± 7.8
Zn(II)	223.6 ± 14.1
Tiron	104.7 ± 18.6
<i>p</i> -Hydroxymercuribenzoate	63.9 ± 4.1
Proadifen	106.3 ± 14.9

^a Values are means ± standard deviations obtained from triplicate experiments.

^b In parentheses is shown the amount of *p*-anisaldehyde produced after 10 min of incubation.

TAO		0
PAMO		0
CPMO		0
CHMO		0
HAPMO	MSAFNTTLPSLDYDDDTLREHLQGADIP TLLLTVAHLTGDLQILKPNWKP SIAMGVARS GMDLETAQVREFCLQRLIDFRDSGQPAPGRPTSDQLHLIG	100
TAO	 MEDIMQGN AA VSDNRG YK. WIAREIERLDPEKDPAEITRALSTTYVNDVFMNLVNTL	57
PAMO	 MAGQTTVDSRRQ. PPEEVDVLV GAGSGLYALYRLR. ELGRSVHVIETAGDVGGVFWNNYPPGARDIESIEYCY	74
CPMO	 MTTMTTMTTEQLGMNNSVNDKLDVLLIGAGFTGLYLYHLR. KLGYKVLVDAGADIGGITHWNYPPGARVDTHQIQY	79
CHMO	 MS. QKMDFDAIVIGSGGGLYAVKKLRDELELVQAFDKATDVAGITFWNNYPPGALDITETHLYCY	65
HAPMO	TWLMGPVIEPYLP LIAEEAVTAEDLRA PRWHKDHVASGRDFKVVIIGAGESG MIAALRFK. QAGVPFVIYEKNGDVGGITRENITPGCRVDINSFVTSF	199
TAO	GIP. APTQPPAGSVVMGVITEKAIK. POKRTDDILQHFHW. EYGPDDPRMQASLAHVNRGHAALAKRSFGTFPARDVI	135
PAMO	SFSEEVLEQNNWTERYASQPEILSYIN FVADKFDLRSGITFHTIVTAAAFIEATNTWVD. TNHGDRIARARYLIMASGQLSVQLENFGLKIFAGNL	171
CPMO	SIP. ELWQEFNNWKLFPNKAQ MREYFHFADKKLDLSDISENTAVQSAVFIEGTREWTVR. SIGHQPIQARFVIANLGFASPTSTPNVIGITFPGQW	175
CHMO	SDWKELLQSL EIKKKYVQGP DVRVYLQCAEKHDLKSYQENTAVQSAHYNEADALREVT. TEYGDKYTARFLITALGLLSAPNLPNINIGINQPKGEL	162
HAPMO	SFARGIWDDC. PAPAPQVFAYMQAVAREHGLVEHIBENTEVSDAHWIESTQRWLLYRDEGQTVDSNVVVFVAVGQLNBEMIPALGIEITFKGPM	294
TAO	YITAWIG. ANLHRLRLSVSLPGSTKNQDIASQRYTAICRQWSED. GLVTEYSPSEFAMQYIEDYEAQFWEQVESGRVLT	215
PAMO	YHTGNWPHEFVDFSGGVVIGTGSGGIQVSPQIAKQARELEVQRTPHFAVPARNAPLDPEFLADLKRYAEFREESRNTPGGTHRY. QGPKSALEVSD	270
CPMO	YHTALWPQGVNMGAGREVAIIGTGSGGVQVQAEALDAKQVTVYQRTPNLALPMHQKQLSAEDNLRMKPELPAAFERRGKCFAGFDFF. FIAKNAIETLSA	274
CHMO	HHTSRWP. DIVSFEGRVGVIGTGSGGVQVITAVAPAKHLTVFQBSAQVSPIGNDPLSEEDVKKIKDNYDKINDGVNNSALAFGLN. ESTVPAASVSA	260
HAPMO	PHSAQWD. HIVDWSGRVGVIGTGSAATQPIPLQATRELHVFARITNWLLPTP. DLHEKISDSCKWLLAHVPHYSLV. YRVAMAIPQSV	382
TAO	EAIKQFVDAYFP. GPLGWIGRQLYLSFQIPTINGIMQS. GKNPIMKXVMKRGWLFGLTLQERVFPDPKLSTPEKA	290
PAMO	EELVETLERYWQE. GGP. DILAAYRDILRRDANERVAEFIRNKIRNTVRIPVVAERLVP. KGYPFGTKRLILEID. YYEMFNRDVHLVDTLSAPKET	365
CPMO	AERTIELEELWNA. GGFYRWLANFQDYLFDKANDVYVEFWRDVRARIKIPKVAEPLAPMKKPHPYGAKRPSLEQW. YYEIPFNQNVHLVDVNETPFLR	372
CHMO	EERKAVPEKANQTGGGPRFMFETPGDIATNMEANIEAQNFIKKGIAEIVKIPATAQLMP. QDLYAKRPLCDSG. YYNTFNRDVVALEDVKANPVE	355
HAPMO	GFLEDVMVDVGYP. PTELAVSARNDRRLQDISAWMEPQFAD. SPDLREVLIP. DSPVGGKRIVRDNGTWISTLKRDNVEMIR. QPIEV	466
TAO	RRKP. VRPGQHIDPPTAEVKCPPE. GATSQSPISADSSGCPFHAGKANGEANNSDLRN.	348
PAMO	ITPRGVRTSERE. YELDSLVLATGFDALTGALFKIDIRGVGNVALKKWA. AGPRTYLGSLTAGFPNLFPIAGP. GSPSALSNNLVSIQHVWVUDHIA	462
CPMO	ITPKGIVTAEGE. AEPDLIVFATGFDVATGGLTSIDFRNNQGQSFKOVNS. DGIRTQLGVATAGFPNLLPGYGP. QSPAGFCNGPSSAEYQGDLLIQLMN	469
CHMO	ITPNGVLENGDFVELEMLICATGFDVADGNYVRMDIQGKNGLAMKQYWK. EGPSSYMGVTWNNYPMFMVLGP. NGP. FTLNPPSIEBQVWISDTIQ	451
HAPMO	ITPKGICCVDGTEHFPDLIVYGTGFHASK. FLMPINVTGRDGVALHDVWKGDDARAYLGMTVPQFPNMFPCMYGNTGLVVYSTVIQFSEATASYIVDAVR	565
TAO		348
PAMO	YMFKNGLTRSEAVLEKEDEWVEHVN. EIADETLYPMTASWYTGANVPGKPRVFMVLVGGFHRVQICDEVAARKGYEGFVLT.	542
CPMO	YLRDNNISRIEAAQSEAQEWKSLIA. DFWDSSLFPPRAKSNYQGSNIPGKKVESLNFPLGLPTIYSKFNESAERKGYAGFSLAS.	550
CHMO	YTVENNVSIEATKEAEQNTQTCA. NIAEMTLFPKAQSWIPGANIPGKNTVYFYLGGGLKEYSALANCKNHAYEGFDIQLQRSDIKQPAN	542
HAPMO	LLLEGGHQSMEVKTVPFESYNQRVDEGNALRAWGFSKVNSEYKNS. KGRVTQNFPFTAVEFNQRTHSVEPTDYQLG.	640

FIG 5 Amino acid sequence alignment among TAO and Baeyer-Villiger monooxygenases (BVMO), phenylacetone monooxygenase (PAMO), cyclopentanone monooxygenase (CPMO), cyclohexanone monooxygenase (CHMO), and 4-hydroxyacetophenone monooxygenase (HAPMO) from *Thermobifida fusca*, *Comamonas testosteroni*, *Acinetobacter calcoaceticus*, and *Pseudomonas fluorescens*, respectively.

the oxygenase reaction for conversion of *trans*-anethole to *p*-anisaldehyde (Table 3). We conclude that the oxygenase reaction of TAO may occur in accordance with the reaction catalyzed by Baeyer-Villiger monooxygenase (BVMO). When the deduced amino acid sequence of TAO was aligned with the amino acid sequence of the BVMO, phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* (9), TAO was found to contain Trp-38, Thr-43, and Tyr-55, which likely correspond to the conserved amino acids of Trp-55, Tyr-60, and Tyr-72 in PAMO (Fig. 5), all of which are known to make extensive van der Waals interactions with flavin on the *si* side of the enzyme (9). Furthermore, the basic side chain of the Arg-337 residue in PAMO may correspond to the Lys-261 residue in TAO (Fig. 5), which supposedly lay on the *re* side of the flavin ring (9). In addition, three other BVMOs also contain those conserved amino acids for flavin binding sites (Fig. 5). However, we could not find the putative conserved domain of FXGXXHXXXW(P/D) for binding NAD(P)H in TAO, as has been shown to occur in BVMOs (4). In related research that deals with the biotransformation of plant origin phenylpropanoid compounds, many investigators have focused on isoeugenol and eugenol, both of which can be metabolized into vanillin. For this purpose, diverse bacterial strains, including strains of *Bacillus fusiformis* (36), *B. pumilus* strain S-1 (32), *B. subtilis* HS8 (35), *B.*

subtilis B2 (23), *Brevibacillus agri* 13 (30), *Pseudomonas chlororaphis* CDAE5 (6), *P. nitroreducens* Jin1 (29), *P. putida* IE27 (33), *Psychrobacter* sp. strain CSW4 (1), and *Nocardia iowensis* DSM 45197 (20), have been isolated. However, little is known about the genetics and the biochemical mechanisms for the metabolism of isoeugenol and eugenol.

In summary, the present study first describes the gene that codes for *trans*-anethole oxygenase, which was able to catalyze the oxidative cleavage of the carbon-carbon double on the 1-propenyl side chain of *trans*-anethole to produce *p*-anisaldehyde, part of a proposed metabolic pathway from *trans*-anethole to protocatechuic acid by *P. putida* JYR-1, as shown in Fig. 6. Compared to the narrow substrate range of Iem and Iso, which only catalyze isoeugenol to vanillin, TAO can also convert isoeugenol, *O*-methyl isoeugenol, and isosafrole to vanillin, veratraldehyde, and piperonal, respectively. Considering the very low similarities of the *tao* gene sequence and its deduced amino acid sequence, it is likely to be a novel enzyme, which is worthy of further characterization with purification. In-depth biochemical knowledge of TAO may provide a greener biocatalytic tool for efficient bioproduction of flavoring materials from plant origin biomass.

FIG 6 Proposed metabolic pathway from *trans*-anethole to protocatechuic acid by *P. putida* JYR-1.

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