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Production of Natural Fragrance Aromatic Acids by Coexpression of *trans*-Anethole Oxygenase and *p*-Anisaldehyde Dehydrogenase Genes of *Pseudomonas putida* JYR-1 in *Escherichia coli*

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ABSTRACT: A gene encoding *p*-anisaldehyde dehydrogenase (PAADH), which catalyzes the oxidation of *p*-anisaldehyde to *p*-anisic acid, was identified to be clustered with the *trans*-anethole oxygenase (*tao*) gene in *Pseudomonas putida* JYR-1. Heterologously expressed PAADH in *Escherichia coli* catalyzed the oxidation of vanillin, veratraldehyde, and piperonal to the corresponding aromatic acids vanillic acid, veratric acid, and piperonylic acid, respectively. Coexpression of *trans*-anethole oxygenase (TAO) and PAADH in *E. coli* also resulted in the successful transformation of *trans*-anethole, isoeugenol, *O*-methyl isoeugenol, and isosafrole to *p*-anisic acid, vanillic acid, veratric acid, and piperonylic acid, respectively, which are compounds found in plants as secondary metabolites. Because of the relaxed substrate specificity and high transformation rates by coexpressed TAO and PAADH in *E. coli*, the engineered strain has potential to be applied in the fragrance industry.

KEYWORDS: *p-Anisaldehyde, p-anisaldehyde dehydrogenase gene (paadh), trans-anethole, trans-anethole oxygenase gene (tao), p-anisic acid, vanillic acid, secondary metabolite*

INTRODUCTION

Biotechnological production of fragrance and flavor compounds has been given much attention recently because U.S. and European legislation has legally designated "natural" flavor substances as compounds prepared by either physical processes via extraction from natural sources or enzymatic or microbial processes from chemical precursors isolated from nature.[1] Phenylpropanoid compounds and their metabolic intermediates transformed by microorganisms have been traditionally used by the flavor and fragrance industry as starting materials for chemical syntheses.[2,3] Compounds with a backbone structure of 1-propenylbenzene, such as isoeugenol, anethole, isosafrole, etc., are types of aromatic compounds found widely in essential oils from plants and have often been attempted as the starting materials to produce aromatic fragrances and other value-added chemicals.

Several genes and enzymes have been reported to be involved in the transformation of 1-propenylbenzenes to oxidized products, such as benzaldehydes and benzoic acids.[4−9] Among them, metabolism for isoeugenol oxidation is relatively well-studied. The genes *iso* and *iem* encoding the enzymes that catalyze isoeugenol oxidation to vanillin have been isolated from *Pseudomonas putida* IE27[7] and *Pseudomonas nitroreducens* Jin1,[4] respectively. Another well-characterized metabolism for plant-originated 1-propenylbenzenes is in regard to *trans*-anethole, which has been found in the bacteria *Arthrobacter* sp. TA13[5] and *P. putida* JYR-1.[10,11] In a previous report, we isolated the *tao* gene from *P. putida* JYR-1 encoding *trans*-anethole oxygenase (TAO), which can catalyze *trans*-anethole to *p*-anisaldehyde. Interestingly, TAO was shown to have a relaxed substrate specificity toward isoeugenol, *O*-methyl isoeugenol, and isosafrole, all of which contain the propenyl functional group on the aromatic ring structure.[10] Aldehydes produced from plant-originated 1-propenylbenzene substrates may be further metabolized to the fragrance aromatic acid compounds[12] *p*-anisic acid, vanillic acid, veratric acid, piperonylic acid, etc., which have also been demonstrated in diverse industrial applications for producing flavors, spices, perfumes, cosmetics,[13] and pharmaceuticals with antibacterial, antifungal, anti-inflammatory, antispasmodic, and antioxidant activities.[14,15]

In this study, we tried to biologically produce the diverse aromatic acid compounds *p*-anisic acid, vanillic acid, veratric acid, and piperonylic acid from plant-originated 1-propenylbezene substrates. For this purpose, we cloned the *p*-anisaldehyde dehydrogenase (*paadh*) gene that is located downstream of the *trans*-anethole oxygenase gene (*tao*) in *P. putida* JYR-1 and coexpressed both *paadh* and *tao* genes in *Escherichia coli*.

MATERIALS AND METHODS

Chemicals. *trans*-Anethole, isoeugenol, *O*-methyl isoeugenol, isosafrole, *p*-anisaldehyde, vanillin, veratraldehyde, piperonal, *p*-anisic acid, veratric acid, and piperonylic acid were purchased from Sigma-Aldrich (Milwaukee, WI). Organic solvents [high-performance liquid chromatography (HPLC) grade] were purchased from Fisher Scientific (Fair Lawn, NJ).

Plasmids, Bacterial Strains, and Growth Conditions. All plasmids and bacterial strains used in this study are listed in Table 1. *P. putida* JYR-1 was grown in tryptic soy broth (TSB) or Stanier's minimal salt broth (MSB)[16] containing 10 mM *trans*-anethole and incubated with rotary shaking at 200 rpm and 25 °C. *E. coli* strains DH5α and BL21(DE3) were routinely grown in Luria-Bertani (LB) medium and incubated by rotary shaking at

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmid	description	source
	Strains	
P. putida JYR-1	*trans*-anethole transformation strain	ref 31
E. coli BL21(DE3)	host strain for expression vector, F^- *omp*T *hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
E. coli EC100	host strain for transposon Tn*5* insertion, F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *φ*80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G	Epicenter
E. coli EPI100	host strain for fosmid genomic library, F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *φ*80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G *trf*A *ton*A *dhf*r	Epicenter
E. coli DH5*α*	host strain for cloning vector, F^- *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G *φ* 80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169, *hsd*R17(r_K^- m_K^+), λ^-	ref 32
	Plasmids	
pET21-a	Ap^r; expression vector	Novagen
pGEM-Teasy	Ap^r; TA cloning vector	Promega
pETDuet-1	Ap^r; expression vector	Novagen
pTA163	Cm^r; 41 kb pEpiFos-5 containing *tao* gene	this study
pG-TAO	Ap^r; pGEM-Teasy cloning vector containing *tao* gene	this study
pG-PAADH	Ap^r; pGEM-Teasy cloning vector containing *tao* gene	this study
pET-PAADH	Ap^r; pET21-a expression vector containing *paadh* gene	this study
pETD-PAADH	Ap^r; pETDuet-1 expression vector containing *paadh* gene	this study
pETD-PAADH-TAO	Ap^r; pETDuet-1 expression vector containing *paadh* and *tao* genes	this study

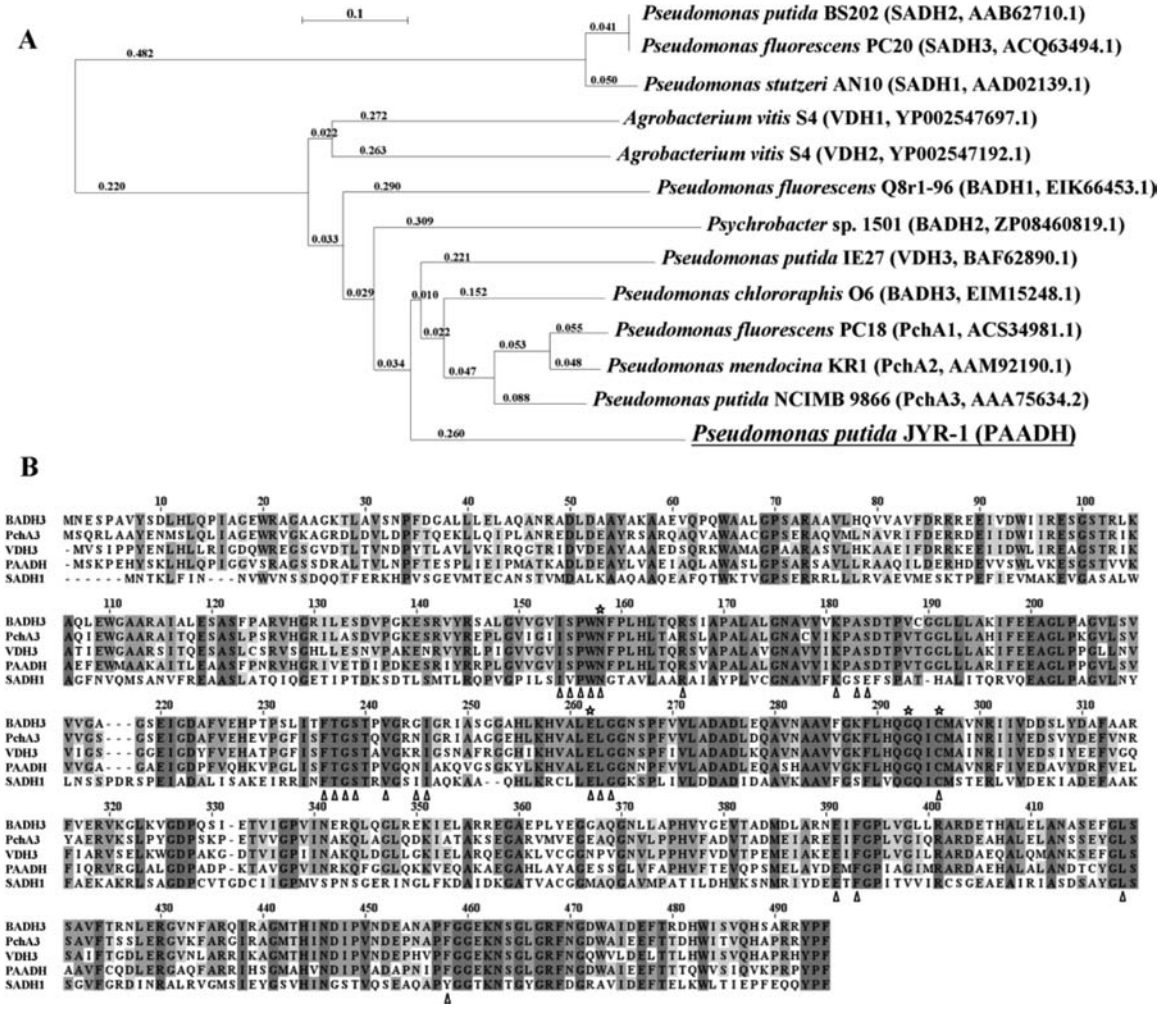

Figure 1. (A) Phylogenetic tree and (B) amino acid alignment among *p*-anisaldehyde dehydrogenases (PAADH), *p*-hydroxybenzaldehyde dehydrogenases (PchA), benzaldehyde dehydrogenases (BADH), and salicylaldehyde dehydrogenases (SADH). Catalytic residues and NAD(P) binding sites conserved in the ALDH superfamily are indicated by ☆ and △, respectively.

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 selecting the corresponding recombinant *E. coli* strains.

Subcloning of the Gene Encoding *p*-Anisaldehyde Dehydrogenase (*paadh*) and Expression of *paadh* in *E. coli*. To clone the open reading frame (ORF) 11 for gene *paadh* from Contig2 of pTA163,

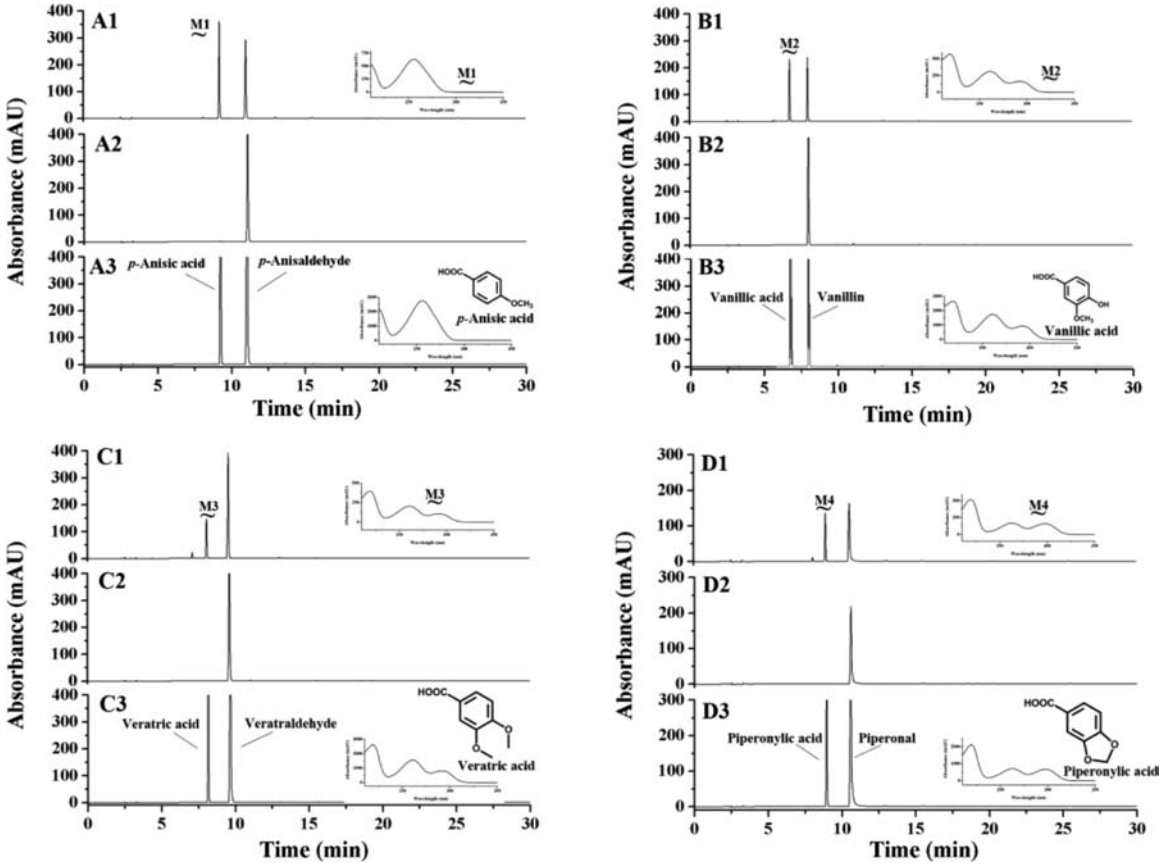

Figure 2. HPLC elution profile of the metabolites produced from (A) *p*-anisaldehyde, (B) vanillin, (C) veratraldehyde, and (D) piperonal by (A1, B1, C1, and D1) resting cells of *E. coli* BL21(DE3)(pET-PAADH) expressing PAADH after 30 min of incubation, (A2, B2, C2, and D2) resting cells of *E. coli* BL21(DE3)(pET21a) without PAADH, and (A3, B3, C3, and D3) authentic aldehyde and acid compounds.

a fosmid, carrying 35 kb of *P. putida* JYR-1 genomic DNA and containing the gene cluster encoding enzymes for metabolizing *trans*-anethole,[10] polymerase chain reaction (PCR) was performed by forward primer attachment to the *Nde*I recognition sequence, 5′-GGGAATTCCATATGAGCAAGCCAGAGCATTACA-3′, and reverse primer attachment to the *Bam*HI recognition site, 5′-CGCGGATCCTCAGAACGGGTAAGGGCGTG-3′. The PCR product digested by *Nde*I and *Bam*HI was ligated into an expression vector pET21-a (Novagen, Madison, WI) under the control of the T7 promoter. The resulting plasmid named pET-PAADH was transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI). A coexpression vector called plasmid pETD-PAADH-TAO for expressing *paadh* and *tao* under the contol of the T7 promoter was constructed by cloning the genes into plasmid pETDuet-1 (Novagen, Madison, WI). The gene *paadh* was amplified from pTA163 in *P. putida* JYR-1 by forward primer attachment to the *Nco*I recognition sequence, 5′-CATGCCATGGGCAAGCCAGAGCATTACA-3′, and reverse primer attachment to the *Bam*HI recognition site, 5′-CGCGGATCCTCAGAACGGGTAAGGGCGTG-3′, and the PCR product was ligated into the pETDuet-1 vector after digestion with *Nco*I and *Bam*HI, resulting in vector pETD-PAADH. The coding region of the *tao* gene and 134 nucleotides of its 5′-untranslated region were amplified from pTA163 in *P. putida* JYR-1 by forward primer attachment to the *Xho*I recognition sequence, 5′-CCGCTCGAGGAGACCGATGCGAAGGGCAA-3′, and reverse primer attachment to the *Xho*I recognition site, 5′-CCGCTCGAGTCAGTTAGTCCTCAAGTCGGAATT-3′. The PCR product was ligated into the pETD-PAADH vector after digestion with *Xho*I. Finally, the coexpression plasmid pETD-PAADH-TAO was confirmed by sequencing the inserted amplicons.

Kinetics of *trans*-Anethole and *p*-Anisaldehye Biotransformation. *E. coli* BL21(DE3)(pET-PAADH) and *E. coli* BL21(DE3)-(pETD-PAADH-TAO) were cultured in LB medium overnight at 37 °C, by rotary shaking at 200 rpm. Cells (1%, v/v) were transferred into fresh LB medium and cultured for 2.5 h at 37 °C by rotary shaking at 200 rpm. Expression of TAO was induced for 4 h after the addition of isopropyl-*β*-D-thiogalactoside (IPTG) to the medium at a final concentration of 1 mM. The cells were harvested by centrifugation at 10000*g* for 10 min and suspended in MSB medium. This process was repeated twice more for washing, and the cells were resuspended in MSB medium and adjusted to an optical density (OD) of 2 at 600 nm. The resuspended cells that were supplemented with 0.5 mM glucose as an energy source were reacted with 1 mM *p*-anisaldehyde, vanillin, veratraldehyde, and piperonal in the case of *E. coli* BL21(DE3)(pET-PAADH) and 1 mM *trans*-anethole, isoeugenol, *O*-methyl isoeugenol, and isosafrole in the case of *E. coli* BL21(DE3)(pETD-PAADH-TAO) (all from 100 mM stock solutions prepared in methanol). Reactions were performed by incubation by rotary shaking at 200 rpm and 30 °C for 10 h. Three volumes of ethyl acetate were used to extract the reaction solutions. The ethyl acetate extracts were evaporated in a speed vacuum centrifugal concentrator (Vision Scientific Co., Suwon, South Korea), and the residue was dissolved in an appropriate volume of methanol and filtered through polyvinylidene fluoride (PVDF) syringe filters (Whatman, Maidstone, U.K.). The amounts of remaining parent compounds and aldehyde and acid products in the reaction solutions were determined by HPLC. Each metabolite was identified by comparison of ultraviolet (UV) spectral data and liquid chromatography−mass spectrometry (LC−MS) data to those from the analyses of authentic compounds. All analyses were performed 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 μm particle size, 4.6 mm × 25 cm, Waters Corporation, 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%

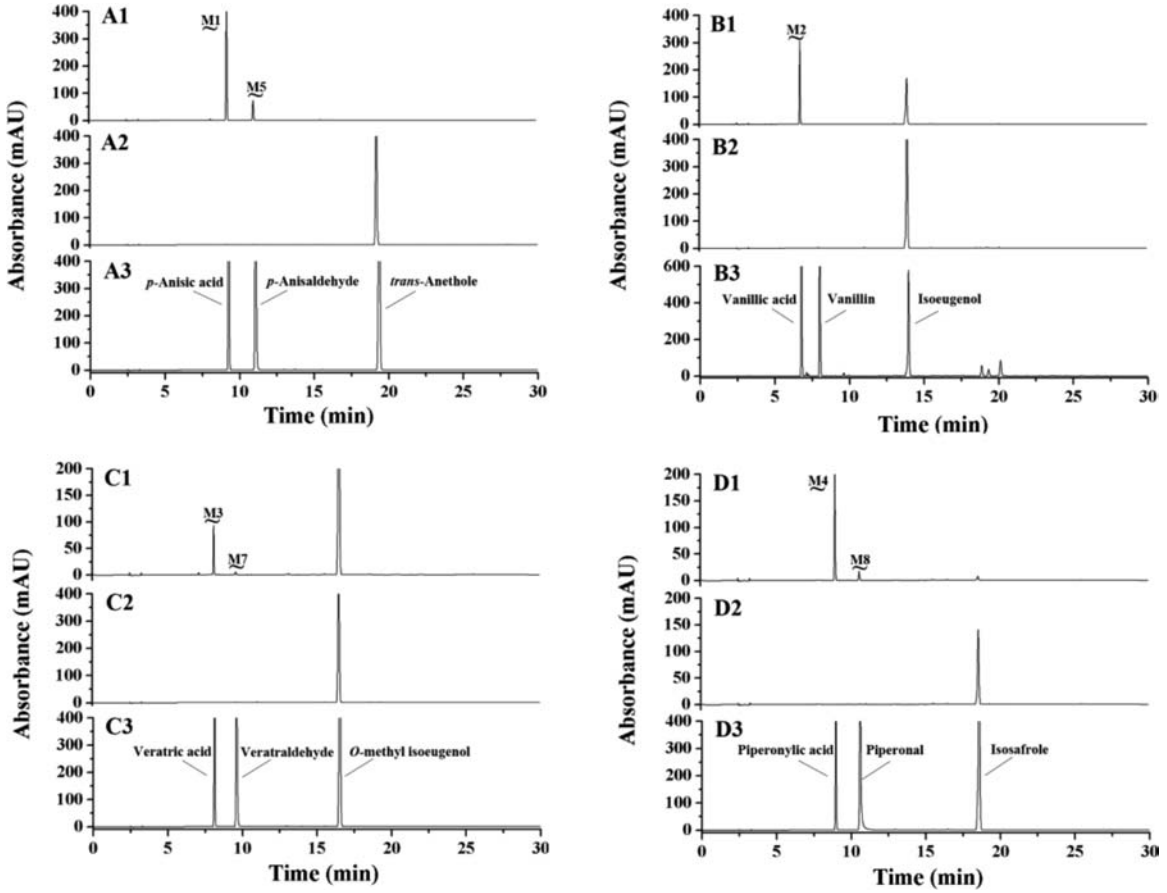

Figure 3. HPLC elution profile of the metabolites produced from (A) *trans*-anethole, (B) isoeugenol, (C) *O*-methyl isoeugenol, and (D) isosafrole by (A1, B1, C1, and D1) resting cells of *E. coli* BL21(DE3)(pET-PAADH-TAO) expressing PAADH and TAO together after 30 min of incubation, (A2, B2, C2, and D2) resting cells of *E. coli* BL21(DE3)(pET21Duet-1) without PAADH and TAO, and (A3, B3, C3, and D3) authentic compounds.

acetonitrile at 30 min. The flow rate with an injection volume of 10 μL 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 Corporation, Milford, MA) in positive electrospray ionization (ESI^+) mode. For LC analyses, a SunFire C18 column (3.5 m, 2.1 × 150 mm, Waters Corporation, Milford, MA) was used, and the mobile phase, elution program, and detection were identical to the analytical HPLC described above; the flow rate was 0.2 mL/min. For MS analyses, 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 and 500 h^{-1}, respectively. The protein concentration was determined by the Bradford assay method[17] with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA), and bovine serum albumin was used as the standard.

Nucleotide Sequence Accession Numbers. The DNA and deduced protein sequences described in this study have been deposited in the GenBank database under accession number JX104157.

RESULTS

Identification of the Gene *paadh* Encoding *p*-Anisaldehyde Dehydrogenase. ORF 11 of Contig2 of plasmid pTA163 in *P. putida* JYR-1, which is located downstream of the *trans*-anethole oxygenase gene, showed an amino acid identity of 66% with *p*-hydroxybenzaldehyde dehydrogenase (PchA) from *P. putida* NCIMB 9866,[18] 64% with vanillin dehydrogenase (VDH) from *P. putida* IE27,[7] 62% with benzaldehyde dehydrogenase (BADH) from *Pseudomonas chlororaphis* O6, and 35% with salicylaldehyde dehydrogenase (SADH) from *Pseudomonas stutzeri* AN10.[19] Therefore, it is assumed that ORF 11 is a putative aldehyde dehydrogenase gene and was named *p*-anisaldehyde dehydrogenase gene (*paadh*). A phylogenetic tree was constructed using the distance method with BioNJ algorithm,[20] and evolutionary distances were calculated using the LogDet method.[21] The phylogenetic tree showed that PAADH from *P. putida* JYR-1 was clustered with three *p*-hydroxybenzaldehyde dehydrogenases (ACS34981.1, AAM92190.1, and AAA75634.2), a benzaldehyde dehydrogenase (EIM15248.1), and a vanillin dehydrogenase (BAF62890.1) at a genetic distance of 0.260 (Figure 1A), suggesting that PAADH is evolutionally close to those five dehydrogenases, with the highest similarity to a vanillin dehydrogenase. This result, together with the biotransformation results, suggested that PAADH is a benzaldehyde dehydrogenase. PAADH contains not only NAD(P) binding sites but also active catalytic residues, which were also found in the ALDH family (Figure 1B).[22] PAADH activity was confirmed by cloning the gene *paadh* into a pET vector and expressing it in *E. coli* BL21(DE3)(pET-PAADH). Resting cells of *E. coli* BL21(DE3)(pET-PAADH) transformed 1 mM *p*-anisaldehyde in 4 h to *p*-anisic acid, which was identified by HPLC (Figure 2) and LC–MS (Figure 4).

Biotransformation of *p*-Anisaldehyde and Other Similar Aromatic Aldehyde Compounds by Resting Cells of *E. coli* BL21(DE3)(pET-PAADH) Expressing *paadh*. A total of 2 mL of resting cells (OD at 600 nm = 2) of *E. coli* BL21(DE3)(pET-PAADH) expressing *paadh* was equivalent to a total protein concentration of 0.32 mg/mL and was able to completely convert 1 mM *p*-anisaldehyde to *p*-anisic acid in 4 h of incubation

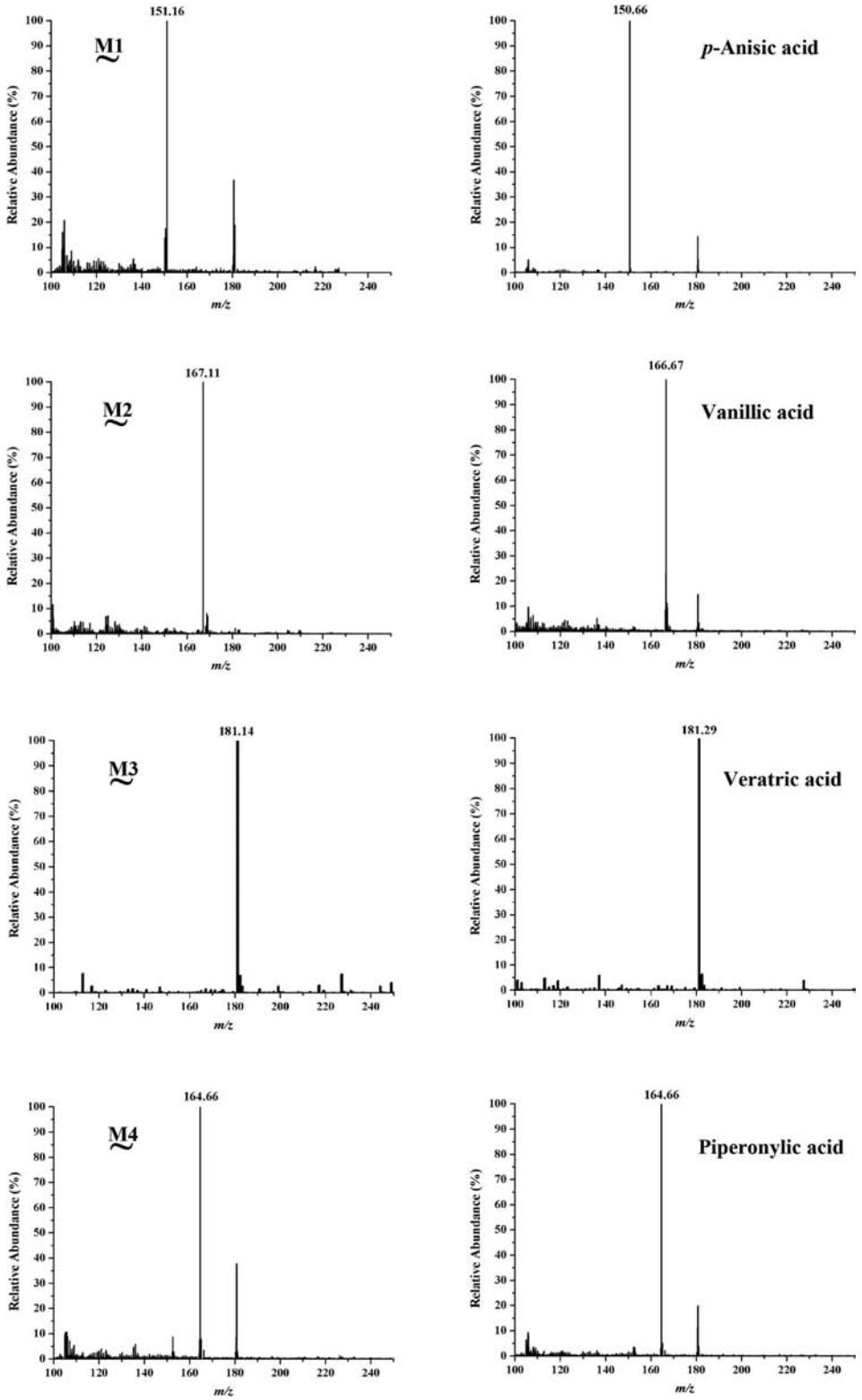

Figure 4. LC−MS spectra of aromatic acidic metabolites M1, M2, M3, and M4 produced from *trans*-anethole, isoeugenol, *O*-methyl isoeugenol, and isosafrole, respectively, by resting cells of *E. coli* BL21(DE3)(pETD-PAADH-TAO) expressing PAADH and TAO together and authentic chemicals *p*-anisic acid, vanillic acid, veratric acid, and piperonylic acid.

with a reaction rate of 6.51 nmol min^{-1} (mg of total protein)$^{-1}$ (Figure 5A). Metabolites of *p*-anisaldehyde produced by *E. coli* BL21(DE3)(pET-PAADH) were identified by retention time on HPLC chromatograms (Figure 2) and compared molecular mass detected by LC−MS spectroscopy (Figure 4) to those of the corresponding authentic compounds. HPLC elution profiles from the sample extracted at a reaction time of 30 min showed two peaks at 9.20 and 11.05 min, and these retention times were identical to the authentic compounds *p*-anisic acid and *p*-anisaldehyde, respectively (Figure 2A1). Electron impact−mass

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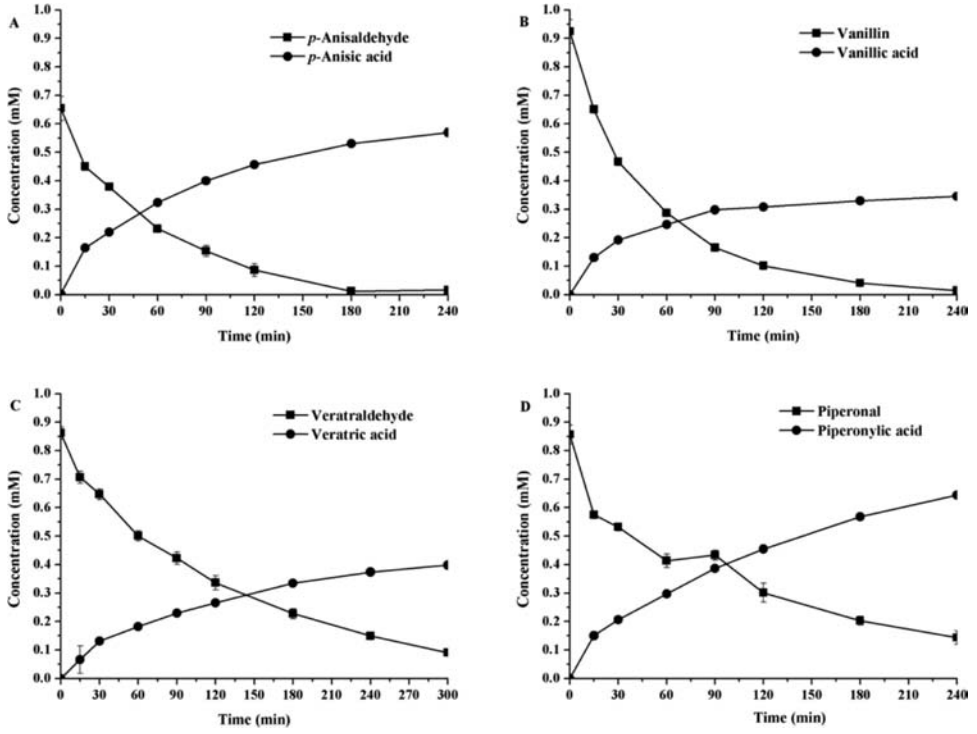

Figure 5. Biotransformation kinetics of (A) *p*-anisaldehyde, (B) vanillin, (C) veratraldehyde, and (D) piperonal by resting cells of *E. coli* BL21(DE3)(pET-PAADH) expressing PAADH. Substrates and products are represented by ■ and ●, respectively.

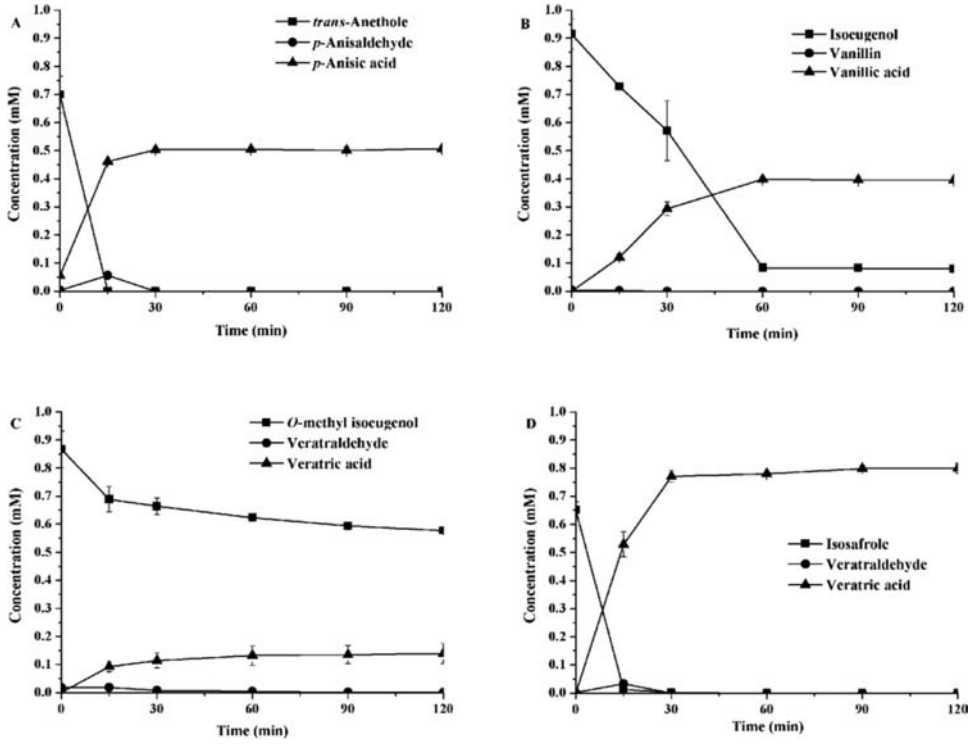

Figure 6. Biotransformation kinetics of (A) *trans*-anethole, (B) isoeugenol, (C) *O*-methyl isoeugenol, and (D) isosafrole by resting cells of *E. coli* BL21(DE3)(pETD-PAADH-TAO) expressing PAADH and TAO together. Substrates, intermediates, and products are represented by ■, ●, and ▲, respectively.

spectrometry (EI−MS) analyses in negative ionization mode (Figure 4) showed a molecular weight of 152 giving a $[M - H]^-$ molecular ion peak at 151, confirming *p*-anisic acid as a metabolite of *p*-anisaldehyde. In addition, resting cells of *E. coli* BL21(DE3)(pET-PAADH) were incubated for 4 h with 1 mM vanillin, veratraldehyde, and piperonal, which are structurally similar to *p*-anisaldehyde. HPLC and LC−MS analyses identified that acid products from vanillin, veratraldehyde, and piperonal were vanillic acid, veratric acid, and piperonylic acid, respectively (Figures 2 and 4). From the incubations, 0.55 mM *p*-anisic acid,

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0.32 mM vanillin, 0.4 mM veratric acid, and 0.63 mM piperonylic acid as final products were obtained from 1 mM of the each starting compound.

Production of Aromatic Acid Compounds from Plant-Originated Propenylbenzene by Resting Cells of *E. coli* BL21(DE3)(pETD-PAADH-TAO) Coexpressing *tao* and *paadh*. Engineered *E. coli* BL21(DE3) expressing both genes *tao* and *paadh* in similar amounts (data now shown) transformed *trans*-anethole to *p*-anisic acid (Figure 3A). In addition, the recombinant strain also produced vanillic acid, veratric acid, and piperonylic acid from isoeugenol, *O*-methyl isoeugenol, and isosafrole, respectively. These aromatic acidic products were also confirmed by HPLC and LC−MS analyses, as compared to the authentic compounds (Figures 2 and 4). Figure 6 shows the biotransformation kinetics of *trans*-anethole, isoeugenol, *O*-methyl isoeugenol, and isosafrole. The initial concentration of 0.7 mM *trans*-anethole rapidly decreased with the formation of *p*-anisic acid (Figure 6A). Within 2 h of incubation, *trans*-anethole was almost completely transformed. Similarly, the initial concentrations of 0.91 mM isoeugenol and 0.65 mM isosafrole decreased rapidly and were totally transformed to vanillic acid and piperonylic acid, respectively, after 2 h (panels B and D of Figure 6). However, the initial concentration of 0.86 mM *O*-methyl isoeugenol decreased to 0.6 mM with the production of veratric acid in 0.15 mM after 2 h (Figure 6C).

DISCUSSION

Aromatic aldehyde dehydrogenase was known to catalyze oxidation of benzaldehyde to benzoic acid, vanillin to vanillic acid, and veratraldehyde to veratric acid[23] and has been suggested to be applied in the aromatic fragrance industry.[24] The *paadh* gene encoding PAADH, which catalyzes the oxidation of *p*-anisaldehyde to *p*-anisic acid, is located downstream of the *tao* gene of *P. putida* JYR-1.[10] Similar activity of aromatic aldehyde dehydrogenase toward *p*-anisaldehyde has been characterized from maize (*Zea mays*).[25] However, *p*-anisaldehyde dehydrogenase activity has not been identified in microorganisms. Furthermore, there are also no reports on the biocatalytic oxidation of veratraldehyde and piperonal to veratric acid and piperonylic acid, respectively.

In the current research, by combining both *tao* and *paadh* genes under the control of a T7 promoter in *E. coli*, we successfully converted not only its physiological substrate *trans*-anethole to *p*-anisic acid but also isoeugenol, *O*-methyl isoeugenol, and isosafrole to the aromatic acid compounds vanillic acid, piperonylic acid, and veratric acid, respectively. In comparison to the other three substrates, the relatively low biotransformation rate of *O*-methyl isoeugenol to the veratric acid through veratraldehyde could be caused by the low catalytic activity of TAO to *O*-methyl isoeugenol[10] and not by PAADH to veratraldehyde. It was previously suggested that TAO has the least affinity to the aromatic compound with two methoxyl side chains.[10] As shown in Figure 5, PAADH catalyzed veratraldehyde significantly to veratric acid, as compared to other tested aldehyde compounds, suggesting that biotransformation of veratraldehyde to veratric acid by PAADH is not the rate-limiting step. In addition, the intermediate veratraldehyde and the end-product veratric acid produced from *O*-methyl isoeugenol could exert the antimicrobial activities onto *E. coli*.[15] During the isoeugenol biotransformation by the recombinant *E. coli*, stoichiometry was not observed, although isoeugenol fairly well decreased with the time of incubation. This is probably due to absorption to the cells and volatility of the compounds isoeugenol, vanillin, and vanillic acid.

Taken together, although various chemical methods have been applied for the synthesis of fragrance compounds,[26−28] there are usually limitations because of low yields, requirements for high temperatures and pressures, and issues with regard to low purity. Biological production of flavor aromatic acid compounds by genetically modified bacterial strains is considered an alternative way to the conventional chemical syntheses and physical extraction methods from their natural sources to effectively produce the legally designated "natural" flavor substances from the biomass containing the precursor compounds.[3,29,30] Indeed, the recombinant *E. coli* strain expressing TAO and PAADH, which show the relatively relaxed substrate specificity, has the potential to produce various fragrance aromatic acids with their high transformation rates toward plant-originated phenylpropanoid substrates *trans*-anethole, isoeugenol, *O*-methyl isoeugenol, and isosafrole.

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Notes

The authors declare no competing financial interest.

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