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Engineering of L-tyrosine oxidation in *Escherichia coli* and microbial production of hydroxytyrosol

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ABSTRACT

The hydroxylation of tyrosine is an important reaction in the biosynthesis of many natural products. The use of bacteria for this reaction has not been very successful due to either the over-oxidation to *ortho*-quinone when using tyrosinases from bacteria or plants, or the lack of the native cofactor, tetrahydrobiopterin (BH4), needed for the activity of tyrosine hydroxylases (TH). Here, we demonstrate that an *Escherichia coli* cofactor, tetrahydromonapterin (MH4), can be used as an alternative cofactor for TH in presence of the BH4 regeneration pathway, and tyrosine hydroxylation is performed without over-oxidation. We used this platform for biosynthesis of one of the most powerful antioxidants, hydroxytyrosol. An endogenous aromatic aldehyde oxidase was identified and knocked out to prevent formation of the side product, and this resulted in nearly exclusive production of hydroxytyrosol in engineered *E. coli*. Finally, hydroxytyrosol production from a simple sugar as a sole carbon source was demonstrated.

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1. Introduction

Hydroxylation of aromatic rings is an important reaction used for preparation of many valuable compounds including L-DOPA for treatment of Parkinson's disease, analgesic alkaloids such as morphine and codeine, and indole alkaloids such as serotonin and melatonin (Ali et al., 2007; Haq et al., 2003; Maskos et al., 1992; Nakagawa et al., 2011; Park et al., 2011). Compared with organic synthesis, which frequently uses metallic oxidants in organic solvents, enzymatic hydroxylation of aromatic rings is an interesting and promising method to synthesize the desired products in a single step with a high regioselectivity using mild conditions (Hollmann et al., 2011). Microbial aromatic hydroxylation is generally performed by oxygenases and tyrosinases, and occurs in the catabolism of aromatic compounds as carbon sources (Di Gennaro et al., 2011).

Tyrosinase is a type-3 copper protein found in fungi, plants, and animals (Claus and Decker, 2006; Olivares and Solano, 2009; Robb, 1984; Solomon et al., 1996). This enzyme catalyzes multiple oxidations of L-tyrosine using molecular oxygen as an oxidant; the first oxidation step is *o*-hydroxylation of L-tyrosine to L-DOPA and is known to be the slowest step, and the second oxidation step is the production of *ortho*-quinone from L-DOPA, which is fast and followed by non-enzymatic reaction to dopachrome, a colored intermediate in the melanin biosynthetic pathway. As conversion of tyrosine to L-DOPA is slow, over-oxidation to *ortho*-quinone is difficult to avoid when microbially-derived tyrosinase is used (Haq et al., 2003; Land et al., 2003).

In animals, however, the oxidation of L-tyrosine to L-DOPA is performed by tyrosine hydroxylase (TH) using tetrahydrobiopterin (BH4) as a cofactor (Daubner et al., 2011; Fitzpatrick, 1999; Kappock and Caradonna, 1996). The use of the pterin cofactor during the oxidation step is a unique feature of TH and related enzymes, such as phenylalanine hydroxylase (PAH) and tryptophan hydroxylase (TPH) (Fitzpatrick, 2003; Pribat et al., 2010). The requirement for this reduced cofactor (BH4) at the active site for the enzyme activity prevents the enzyme from performing the second oxidation by restricting the access of a fresh cofactor to the active site after the first oxidation, and this minimizes the over-oxidation of L-tyrosine to *ortho*-quinone (Maass et al., 2003). However, the use of TH for microbial biosynthesis of L-DOPA or related metabolites has not been reported due to the lack of the coenzyme BH4, which is only found in eukaryotes (Torres Pazmino et al., 2010).

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Here, we engineered an artificial pathway in *Escherichia coli* for the oxidation of L-tyrosine to L-DOPA using mouse tyrosine hydroxylase (Iwata et al., 1992) and an endogenous cofactor in *E. coli*. We also used this engineered pathway to produce hydroxytyrosol, a potent anti-oxidant from olive oil, in *E. coli* not only from supplemented tyrosine but also from endogenously-produced tyrosine (Fig. 1).

2. Material and methods

2.1. Bacterial strains and cultures

Strains used in this study are summarized in Table 1. *E. coli* DH10B (Invitrogen, Carlsbad, CA) was routinely used for plasmid construction. To confirm tetramonapterin (MH4) biosynthesis, *E. coli* BW25113 and its *folM*- and *folX*-knockout mutants in the Keio collection, JW1598 and JW2300, were employed (Baba et al., 2006). As for hydroxytyrosol production, *E. coli* JW1380, a *feaB*-knockout mutant derived from BW25113, was employed. These strains were used after eliminating the kanamycin-resistance gene on the chromosome, as described previously (Datsenko and Wanner, 2000).

The media used were LB broth medium (Lennox; Becton, Dickinson and Company, Franklin Lakes, NJ) and M9 minimal medium (M9 minimal salts (Becton, Dickinson and Company), 1% (*w*/*v*) glucose, 5 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.025% (*w*/*v*) of yeast extract (M9Y media). When needed, kanamycin and carbenicillin were added to the medium at 50 μg/mL and 100 μg/mL, respectively.

2.2. Plasmids construction

Plasmids used in this study are listed in Table 2. In order to enable rapid cloning and assembly of genes, we employed the BglBrick cloning strategy using BglBrick vectors (Anderson et al., 2010; Lee et al., 2011). The genes encoding tyrosine hydroxylase (TH) from mouse (accession number NP_033403), pterin-4a-carbinolamine dehydratase (PCD) from human (accession number NP_000272), dihydropteridine reductase (DHPR) from human (accession number P09417) and L-DOPA decarboxylase (DDC) from pig (accession number NP_999019), which were optimized for codon usage in *E. coli* by using Gene Designer 2.0 software (DNA2.0 Inc., Menlo Park, CA), were purchased from GenScript USA Inc. (Piscataway, NJ). The tyramine oxidase (TYO) gene was PCR amplified from the *Micrococcus luteus* genome (accession number AB010716). The TH gene was subcloned into the BglBrick compatible vector pBbE1k-RFP (ColE1 origin, *trc* promoter, *lacI*, Kan^r, red fluorescent protein (RFP) gene) (Lee et al., 2011) to construct pBbE1k-1 as described in Table 2. In order to construct an artificial operon of the PCD and DHPR genes based on BglBrick strategy, these genes were inserted in the order DHPR–PCD 3′ of the *trc* promoter in pBbE1k-RFP; this construct was designated pBbE1k-2. To obtain pBbE1k-3, which includes an operon of the TH-DHPR–PCD genes, the TH gene was inserted between the promoter and the DHPR gene in pBbE1k-2 using the BglBrick cloning strategy. The DDC gene was subcloned into the BglBrick compatible vector pBbE1k-RFP (ColE1 origin, *trc* promoter, *lacI*, Kan^r, red fluorescent protein (RFP) gene) (Lee et al., 2011) to construct pBbE1k-DDC, as described in Table 2. The TYO gene was subcloned into the BglBrick compatible vector pBbS1a-RFP (SC101 origin, *trc* promoter, *lacI*, Amp^r, red fluorescent protein gene) (Lee et al., 2011) to construct pBbS1a-1, as shown in Table 2. In order

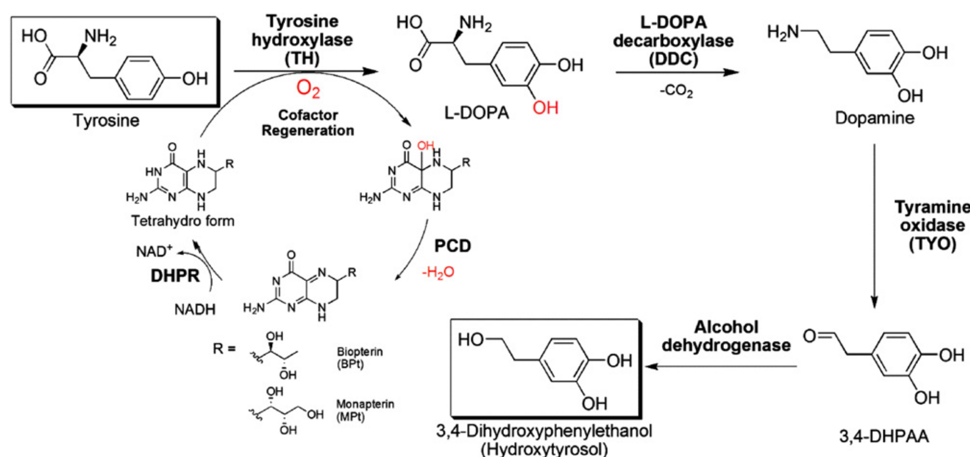

Fig. 1. Pathway for oxidation of tyrosine to hydroxytyrosol. L-tyrosine oxidation is catalyzed by tyrosine hydroxylase (TH) in the presence of the pterin cofactor. The tetrahydrobiopterin (BH4) cofactor regeneration system is composed of pterin-4 alpha-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR).

Table 1
Bacterial strains used in this study.

E. coli strain	Relevant charactristic or genotype	Source or reference
DH10B	F^- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ^- rpsL nupG/pMON14272/pMON7124	Invitrogen
BLR(DE3)	F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3) Δ(srl-recA)306::Tn10 (Tet^r)	Novagen
BW25113	rrnB ΔlacZ4787 hsdR514 Δ (araBAD)567 Δ (rhaBAD)568 rph-1	NBRP-*E. coli* at NIG[a]
JW1598	BW25113 derivative; $folM^-$	NBRP-*E. coli* at NIG
JW2300	BW25113 derivative; $folX^-$	NBRP-*E. coli* at NIG
JW1380	BW25113 derivative; $feaB^-$	NBRP-*E. coli* at NIG

[a] NBRP-*E. coli* at NIG: National Bioresource Project, National Institute of Genetics, Japan.

Table 2
Plasmids used in this study.

Plasmid name	Description	Reference
pBbE1k	BglBrick compatible vector; ColE1ori, *trc* promoter, Km^r	Lee et al. (2011)
pBbE1k-1	pBbE1k derivative with TH gene	This study
pBbE1k-2	pBbE1k derivative with DHPR and PCD genes	This study
pBbE1k-3	pBbE1k derivative with TH, DHPR, and PCD genes	This study
pBbE1k-DDC	pBbE1k derivative with DDC gene	This study
pBbS1a	BglBrick compatible vector; pSC101 ori, *trc* promoter, Amp^r	Lee et al. (2011)
pBbS1a-1	pBbS1a derivative with TYO gene	Satoh et al. (2012)
pBbS1a-3	pBbS1a derivative with DDC and TYO genes	This study

to construct an artificial operon of DDC and TYO based on the BglBrick cloning strategy, the DDC gene was inserted 5′ of TYO gene in pBbS1a-1; this construct was designated pBbS1a-3 (Table 2).

For construction of control plasmids pBbE1k and pBbS1a, the gene encoding red fluorescent protein was deleted in the pBbE1k-RFP and pBbS1a-RFP vectors, respectively.

2.3. L-DOPA production

E. coli harboring pBbE1k-derived plasmids were cultured in LB medium for 16 h at 37 °C. The aliquots (1 mL or 0.1 mL) were inoculated into 250 mL shake flasks containing 50 mL of medium. These were cultured at 30 °C for 3 h and then isopropyl β-D-thiogalactopyranoside (IPTG) was added at the concentration of 0.5 mM and the cells were cultured for addition 20 h at 30 °C. The supernatants of shake flask cultures were collected in test tubes to take a picture.

2.4. Hydroxytyrosol production

Shake flask experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of M9Y medium. The aliquots (50 μL) of overnight cultures were inoculated into 2 mL of fresh LB medium and cultured at 30 °C for 4 h. Then the cells were harvested and washed once with the same amount of M9Y medium. The all cells were inoculated into 50 mL of M9Y medium. They were cultured for 3 h at 30 °C and 160 rpm, and IPTG was added at final concentration of 0.5 mM. Optical density (OD) measurements at 600 nm were also taken using a Beckman spectrophotometer.

Samples (1 mL) collected at appropriate time points were analyzed by HPLC. The samples of culture supernatants (2 μL) were analyzed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc., Santa Clara, CA), equipped with a Discovery HS F5 column (15 cm × 2.1 mm, 3 μm; Sigma-Aldrich Co. LLC, St. Louis, MO). Buffer A (0.1% (v/v) formic acid solution) and buffer B (acetonitrile with 0.1% (v/v) formic acid) were used as a mobile phase, and compounds were eluted at 35 °C and a flow rate of 0.3 mL min^{-1}, with increasing concentrations of buffer B as follows: 5%, 0–2 min; 5–30%, 2–22 min. Eluted compounds were detected using a diode array spectrophotometer (Agilent Technologies) measuring absorbance at 280 nm or a time-of-flight mass spectrometer (Agilent Technologies). Tyrosine, L-DOPA, dopamine, 3,4-dihydroxyphenylacetate (DHPA), and hydroxytyrosol (Sigma-Aldrich) were used as standards.

2.5. In vitro activity assay of DDC

E. coli BLR(DE3) harboring pBbE1k or pBbE1k-DDC were cultured in LB medium for 16 h at 37 °C. The aliquots (1 mL) were inoculated into 250 mL shake flasks containing 50 mL of LB medium. These were cultured at 30 °C for 3 h and then IPTG was added at the final concentration of 0.5 mM. The cells were cultured for additional 20 h at 30 °C. The harvested cells were washed with 50 mL of chilled 20 mM Tris–HCl (pH7.2) and then resuspended in 5 mL of the same buffer. The supernatants after sonication and centrifugation at 20,000 × *g* at 4 °C for 30 min were used for in vitro enzyme assay.

The reaction mixture (1 mL) contained 1 mM dopamine or tyramine, 50 μM pyridoxal-5′-phosphate (PLP), 0.4 mM ascorbate, 400 μg proteins in 100 mM Tris HCl (pH 7.2). The reaction was started by addition of the enzyme solutions and carried out at room temperature. After 1 h or appropriate time point, the reaction mixtures were ultra-filtrated to prepare the samples for HPLC analysis.

The samples (2 μL) were analyzed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc.), equipped with a Discovery HS F5 column (15 cm × 2.1 mm, 3 μm; Sigma-Aldrich Co. LLC). As for analysis of in vitro DDC activity, buffer C (50 mM NH_4COOH, pH3) was used as a mobile phase, and compounds were eluted at 35 °C and a flow rate of 0.2 mL min^{-1}. Eluted compounds were detected by a diode array spectrophotometer (Agilent Technologies Inc.) measuring absorbance at 280 nm.

3. Results and discussion

3.1. Tyrosine hydroxylation

3.1.1. Expression of tyrosine hydroxylase and cofactor synthesis/regeneration

One of the issues for the application of tyrosine hydroxylase (TH) to bacteria would be availability of the coenzyme BH4, a unique cofactor for animals. To reconstitute L-tyrosine hydroxylation in *E. coli* using TH, we needed three components: an active enzyme (TH), a cofactor (BH4) biosynthetic pathway, and a cofactor (BH4) regeneration system. First, mouse TH was codon optimized for *E. coli*, synthesized, and cloned into pBbE1k using BglBrick standard cloning to obtain pBbE1k-1. TH expression was confirmed by SDS-PAGE (Supplementary Fig. S1A), and the activity of TH was judged by the color change of the culture since it has been reported that L-DOPA is easily oxidized in aerobic culture to melanin, which is black in color (Lee and Xun, 1998). However, cultures of *E. coli* harboring pBbE1k-1 did not show any obvious color change (Supplementary Fig. S1B), even though TH was expressed in a soluble form (Supplementary Fig. S1A). This was expected because the enzyme requires active cofactor BH4 to oxidize L-tyrosine, but neither the cofactor nor its regeneration pathway was present in *E. coli*. We then attempted to construct BH4 biosynthesis and regeneration pathways in *E. coli* (Figs. 1 and 2A). BH4 biosynthesis requires two heterologous enzymes in addition to the endogenous GTP cyclohydrolase (FolE), which produces 7,8-dihydroneopterin triphosphate (H2-NPt-P3) from GTP: the 6-pyruvoyl-tetrahydropterin synthase (PTPS) catalyzes the conversion of H2-NPt-P3 to 6-pyruvoyl-tetrahydropterin (P-H4-Pt), and sepiapterin reductase (SR) performs the two-step reduction of the diketo intermediate P-H4-Pt to BH4 (Fig. 2A) (Smith, 1987; Takikawa et al., 1986; Thony et al., 2000; Yamamoto et al., 2003). Even though the biosynthesis of BH4 in *E. coli* has been reported previously using a heterologous pathway (Yamamoto et al., 2003), we were not able to clone and express the SR gene in *E. coli* despite many attempts. This failure motivated us to

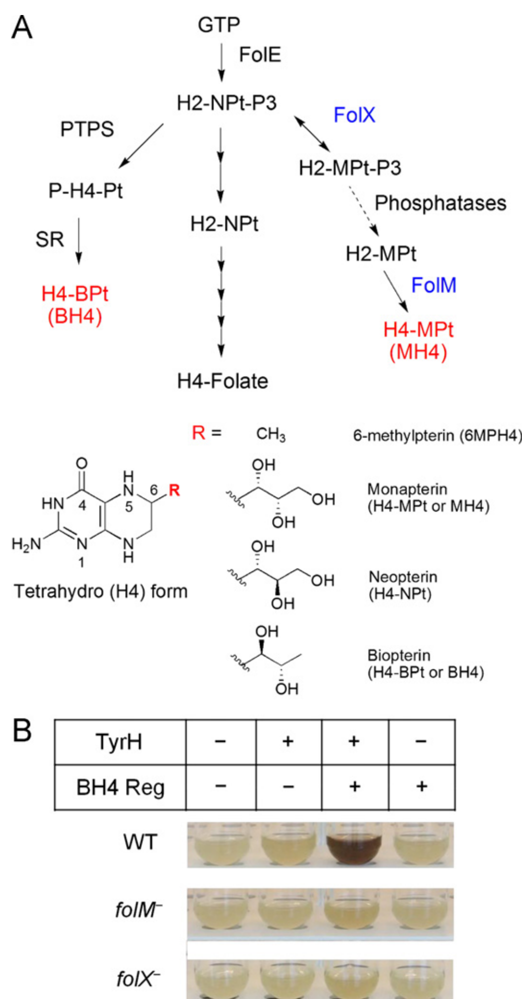

Fig. 2. Pterin cofactor biosynthesis and tyrosine oxidation in MH4 deficient mutants. (A) Biosynthesis of pterin cofactors from GTP. FolE is a GTP cyclohydrolase. PTPS (pyruvoyl tetraydropterin synthase) and SR (sepiapterin reductase) are essential for tetrahydrobiopterin (BH4 or H4-BPt) biosynthesis. FolX (dihydroneopterin triphosphate 2′-epimerase) and FolM (dihydrofolate reductase) are essential for tetrahydromonapterin (MH4 or H4-MPt) biosynthesis. (B) Tyrosine oxidation in wild-type *E. coli* BW25113 (WT) and mutants in MH4 biosynthesis (Δ*folX*, JW2300 and Δ*folM*, JW1598). The strain was transformed with a plasmid harboring the genes encoding either TH (tyrosine hydroxylase) or TH and the BH4 regeneration system and tested for L-DOPA production. The color change to black indicates the oxidation of L-tyrosine to L-DOPA and further to melanin. WT can produce L-DOPA when both TH and the BH4 regeneration system were expressed, but the *folX* and *folM* mutants were not able to produce L-DOPA under the same condition.

search for an alternative cofactor that can be used for TH expressed heterologously in *E. coli*.

3.1.2. Verification of an alternative cofactor

E. coli and other bacteria naturally produce tetrahydromonapterin (MH4 or H4-MPt); for example, it has been reported that MH4 is the cofactor for Pseudomonas phenylalanine hydroxylase (PAH) (Ikemoto et al., 2002; Pribat et al., 2010). It is also reported that 6-methyl-5, 6, 7, 8-tetrahydropterin (6-MPH4, Fig. 2(A)), a synthetic analog of tetrahydropterin species, was able to be used as a cofactor for the activity of mouse tyrosine hydroxylase in vitro (Ichikawa et al., 1991). Based on the functional and structural similarity of PAH and TH (Maass et al., 2003) and a potential broad cofactor specificity of TH, we hypothesized that mouse TH might be able to use MH4 as an alternative cofactor to BH4 in vivo. As cofactors are generally regenerated, we prepared a well-known BH4 regeneration pathway of animals and added it into the *E. coli* strain to regenerate either MH4 or any other putative cofactor needed for TH activity. We synthesized genes encoding pterin-4 alpha-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) from human (Thony et al., 2000), and then incorporated them into pBbE1k using BglBrick standard cloning to construct pBbE1k-2. For L-DOPA biosynthesis, the TH gene was subcloned into the operon encoding the cofactor regeneration pathway forming pBbE1k-3 (TH, DHPR, and PCD in this order), and this plasmid was transformed into *E. coli* BLR(DE3). Protein production was confirmed by SDS-PAGE analysis (Supplementary Fig. S1A).

To test the hypothesis that MH4 could replace BH4 in tyrosine hydroxylation by TH, L-DOPA production by *E. coli* harboring pBbE1k, pBbE1k-1, or pBbE1k-3 (Table 2) was analyzed in vivo. We assessed L-DOPA production by the color of the medium as previously described (Lee and Xun, 1998; Santos and Stephanopoulos, 2008). When *E. coli* BLR(DE3) harboring the pBbE1k-derived plasmids (pBbE1k, pBbE1k-1 or pBbE1k-3) were cultured without the addition of BH4 as a cofactor, the color change was observed only in cultures of the strain that expressed both TH and the BH4 regeneration pathway (Supplementary Fig. S1B). In contrast, cultures of the control strain, which does not express either TH or the BH4 regeneration pathway did not change color (Supplementary Fig. S1B). This result shows that the engineered strain expressing TH and the BH4 regeneration pathway genes successfully produced L-DOPA without the biosynthesis of the original cofactor BH4, and it supports the hypothesis that there is an endogenous cofactor in *E. coli* that can replace BH4 in tyrosine hydroxylation by TH. However, it is still not certain that MH4 is the responsible cofactor for TH activity in *E. coli*.

To confirm whether it was MH4 that replaced BH4 in tyrosine hydroxylation, we transformed pBbE1k-3 into *folM* (encoding dihydrofolate reductase) or *folX* (encoding dihydroneopterin triphosphate 2′-epimerase) deletion mutants of *E. coli* BW25113, which are incapable of producing MH4 (Fig. 2A) (Baba et al., 2006; Pribat et al., 2010). Cultures of these mutants harboring pBbE1k-3 did not turn black under the same condition that oxidized L-tyrosine to L-DOPA (Fig. 2B). In contrast, cultures of the wild-type strain changed color under the same cultivation conditions, indicating that MH4 biosynthesis is essential for TH activity. Therefore we could conclude that MH4 is an alternative to BH4 in tyrosine hydroxylation by TH in *E. coli*. Further in vitro studies using MH4 as a cofactor of TH activity would have strengthened and confirmed this hypothesis. However, the cofactor MH4 was not available from any commercial source, and we could not perform further in vitro verification of our hypothesis.

Even though we demonstrated the new pathway to oxidize L-tyrosine to L-DOPA by TH in *E. coli*, we did not quantify the actual titers of L-DOPA since L-DOPA is easily oxidized to *o*-quinone and further to melanin. Even though reducing agents such as ascorbic acid are known to reduce oxidation of L-DOPA (Ali et al., 2007), they usually make the purification of the product from fermentation broth more difficult and make the process less attractive for industrial application. Instead, we decided to make this process more industrially attractive by introducing downstream enzymes that can transform the unstable L-DOPA to more stable end products such as hydroxytyrosol (3,4-dihydroxyphenyl ethanol) (Fig. 1).

3.2. Hydroxytyrosol biosynthesis

Hydroxytyrosol is one of the most powerful antioxidants, having an oxygen radical absorbance capacity (ORAC) of 28,000 μmol TE/g, which is about 50% greater than resveratrol, a popular and

commercialized antioxidant found in red wine (Angelino et al., 2011). Additionally, hydroxytyrosol has potential as an anti-tumor, anti-atherogenic, anti-inflammatory and anti-platelet aggregation agent (Granados-Principal et al., 2010). Currently, hydroxytyrosol is obtained mostly from enriched olive extracts after chemical or enzymatic hydrolysis of an elenolic acid ester form of hydroxytyrosol (oleuropein). Recently microbial conversion of tyrosol to hydroxytyrosol has been reported (Liebgott et al., 2009). The biosynthetic pathway of hydroxytyrosol from tyrosine is shown in Fig. 1.

3.2.1. Stepwise conversion of L-DOPA into hydroxytyrosol

The first step in hydroxytyrosol biosynthesis after tyrosine oxidation is the decarboxylation of L-DOPA to dopamine. For this conversion, we needed an L-DOPA-specific decarboxylase that does not catalyze the decarboxylation of tyrosine, since the production of tyramine from tyrosine will eventually lead to the production of tyrosol, a side product with much weaker antioxidant potential (Angelino et al., 2011). After screening several aromatic amino acid decarboxylases including tyrosine decarboxylases (TDC) and L-DOPA decarboxylases (DDC), we employed DDC from pig (*Sus scrofa*) (Blechingberg et al., 2010), which converts L-DOPA to dopamine with high specificity while not decarboxylating tyrosine to tyramine in vitro (Supplementary Fig. S2). Interestingly, when expressed from a high-copy plasmid under the control of a strong promoter, the DDC activity was low when induced with IPTG but was higher in the absence of IPTG (Supplementary Fig. S3A). SDS PAGE analysis indicated that the DDC was largely insoluble in the induced cells but was soluble in the uninduced cells (Supplementary Fig. S3B), suggesting that expression from low-copy plasmids would improve production of the active DDC.

To achieve the downstream conversion of dopamine to hydroxytyrosol, we needed a monoamine oxidase (MAO) to convert dopamine to 3,4-dihydroxyphenyl acetaldehyde (3,4-DHPAA) and an alcohol dehydrogenase (ADH) to convert the aldehyde to hydroxytyrosol (Fig. 1). *E. coli* has endogenous MAO and several ADHs (Blattner et al., 1997; Roh et al., 1994). Unfortunately, MAO showed low activity and produced only a small amount of hydroxytyrosol even when the gene encoding the endogenous MAO has been overexpressed (data not shown). Instead, we chose tyramine oxidase (TYO) from *Micrococcus luteus*, which has been used previously for this conversion (Roh et al., 2000). *E. coli* BW25113 transformed with pBbS1a-1, which includes TYO from *M. luteus* (Table 1) (Satoh et al., 2012), produced hydroxytyrosol from dopamine, which confirms the activities of TYO and endogenous ADHs (Fig. 3A).

In addition to producing hydroxytyrosol, however, the culture also produced 3,4-dihydroxyphenyl acetic acid (3,4-DHPA) (Fig. 3A, Table 3), indicating that there is another endogenous enzyme in *E. coli* that oxidizes the aldehyde (3,4-DHPAA) to the acid (3,4-DHPA) and competes with ADHs for the same substrate. We observed a similar activity in the microbial production of tyrosol; phenylacetaldehyde dehydrogenase (FeaB) has been implicated in *E. coli* (Satoh et al., 2012). To improve the yield and purity of hydroxytyrosol, we used the *feaB* knockout strain *E. coli* JW1380 (Baba et al., 2006) for production. *E. coli* JW1380 transformed with pBbS1a-1, a TYO expressing plasmid (Table 2), successfully produced hydroxytyrosol as the main and almost exclusive product with about 70% yield when cultured in M9Y medium supplemented with dopamine (Fig. 3A, Table 3). Thus, knocking out *feaB* appears to be essential to achieve high yield and high purity of hydroxytyrosol.

Next, we tried to construct a pathway to convert L-DOPA to hydroxytyrosol. For this conversion, we added the L-DOPA-specific decarboxylase (DDC) into the strain that converts dopamine to hydroxytyrosol. As we observed that high-level expression of the gene encoding DDC was detrimental to its activity, we used a low

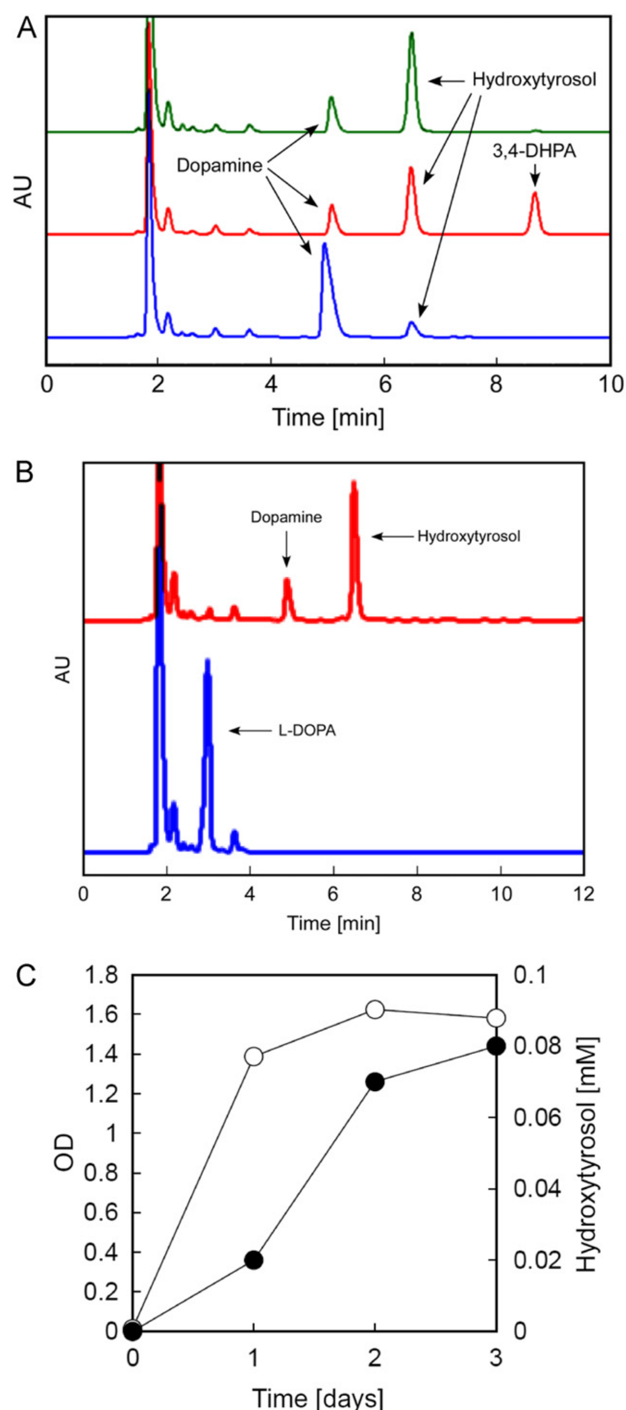

Fig. 3. Hydroxytyrosol production from various substrates. HPLC with diode array detector (at 280 nm) was used for analysis and the identity of peaks was confirmed with standards (A) Hydroxytyrosol production from dopamine. Top: *E. coli* JW1380 (Δ*feaB*) with TYO overexpression. Middle: *E. coli* BW25113 (wild type) with TYO overexpression. Bottom: *E. coli* BW25113 without TYO overexpression. (B) Hydroxytyrosol production from L-DOPA in *E. coli* JW1380. Top: *E. coli* JW1380 with DDC and TYO overexpression. Bottom: *E. coli* JW1380 without DDC and TYO. (C) Hydroxytyrosol production from glucose. *E. coli* JW1380 with TH, the BH4 regeneration system, DDC and TYO overexpression. Open circles represent OD_{600}, and closed circles represent hydroxytyrosol titers from cultures in which no exogenous tyrosine was added.

Table 3
Strains for hydroxytyrosol production.

E. coli host strain[a]	Genes added in plasmid (s)	Substrate added (1 mM)	Product (mM) (hydroxytyrosol)	Byproduct (mM) (3,4-DHPA)
BW25113[b]	TYO[d]	Dopamine	0.47 ± 0.014	0.31 ± 0.0059
BW25113	None	Dopamine	0.12 ± 0.0033	0
JW1380[c]	TYO	Dopamine	0.69 ± 0.0064	0
JW1380	None	Dopamine	0.09 ± 0.013	0
JW1380	DDC[e], TYO	L-DOPA	0.74 ± 0.088	0
JW1380	None	L-DOPA	0	0
JW1380	DDC, TYO	Tyrosine	0	0
JW1380	DDC, TYO	None	0	0
JW1380	TH[f], Regen[g], DDC, TYO	Tyrosine	0.19 ± 0.0056	0
JW1380	TH, Regen, DDC, TYO	None	0.08 ± 0.00060	0

[a] *E. coli* has endogenous MAO (monoamine oxidase) and ADH (alcohol dehydrogenase).
[b] BW25113 is an *E. coli* strain with a genotype of *rrnB ΔlacZ4787 hsdR514 Δ (araBAD)567 Δ (rhaBAD)568 rph-1*.
[c] JW1380 is a *feaB* knockout mutant of BW25113, from Keio collection of single-gene knockouts.
[d] Tyramine oxidase from *M. luteus*.
[e] Dopa decarboxylase from *Sus scrofa*.
[f] Tyrosine hydroxylase from mouse.
[g] BH4 regeneration pathway genes composed of pterin-4α-carbinolamine dehydratase (*PCD*) and dihydropteridine reductase (*DHPR*).

copy number vector to harbor this gene and constructed a plasmid co-expressing the genes encoding DDC and TYO (pBbS1a-3). When *E. coli* JW1380 harboring pBbS1a-3 was grown in M9Y medium supplemented with L-DOPA, 0.74 mM of hydroxytyrosol were detected (Fig. 3B, Table 3).

Under the experimental condition, L-DOPA was consumed and both dopamine and hydroxytyrosol were accumulated by HPLC analysis (Fig. 3B). This result suggests that the low expression of DDC has sufficient activity for the conversion of L-DOPA to dopamine, and the amine oxidation by TYO is probably a rate-limiting step. In the case of tyrosol biosynthesis as reported previously (Satoh et al., 2012), tyramine accumulation was not observed under the experimental conditions. The substrate specificity of TYO, which prefers tyramine to dopamine as a substrate (Roh et al., 2000), may account for these observations and hence it suggests that TYO expression and/or activity should be optimized for effective hydroxytyrosol production.

In addition, despite the fact that this process from L-DOPA to hydroxytyrosolrequires 3 steps (decarboxylation of L-DOPA, deamination of dopamine, and reduction of 3,4-DHPAA), the conversion (0.74 mM) was slightly higher than that (0.69 mM) from dopamine by *E. coli* JW1380 expressing TYO, which needed two steps (Table 2). This might be explained by the efficiency of substrate uptake; since the chemical structure of L-DOPA is quite similar to L-tyrosine, it could be transported into *E. coli* more efficiently than dopamine.

3.2.2. Hydroxytyrosol production from L-tyrosine and glucose using an engineered E. coli

Finally, we assessed whether the *feaB*-knockout mutant strain harboring both pBbE1k-3 (TH, PCD, and DHPR) and pBbS1a-3 (DDC and TYO), all of the enzymes necessary to convert tyrosine to hydroxytyrosol (Table 2), could produce hydroxytyrosol. When cultured in M9Y medium supplemented with L-tyrosine (1 mM), the culture of *E. coli* JW1380 harboring pBbE1k-3 and pBbS1a-3 did not turn black, while the culture of the control strain harboring pBbE1k-3 and pBbS1a became dark colored. This suggests that TH and the BH4 regeneration pathway (pBbE1k-3) worked well in the *feaB* knockout mutant and produced L-DOPA, which consecutively was converted to hydroxytyrosol by DDC, TYO (pBbS1a-3), and endogenous ADHs before undesired oxidation to melanin occurs. From 1 mM L-tyrosine supplemented, the culture of the engineered strain produced 0.19 mM hydroxytyrosol without any contamination by 3,4-DHPA or tyrosol (Table 3). However, a small amount of dopamine (0.03 mM) was detected under the experimental condition (Supplemental Table S1). This result also suggests that TYO activity is not fast enough in the engineered hydroxytyrosol biosynthesis cell as described above.

Glucose can be converted into L-tyrosine via an endogenous biosynthesis pathway in *E. coli* (Gibson and Pittard, 1968; Ikeda, 2006). There also have been engineering efforts to improve tyrosine production in *E. coli* (Juminaga et al., 2012; Lutke-Eversloh and Stephanopoulos, 2007; Patnaik et al., 2008). Since *E. coli* can produce small amount of tyrosine even without any further engineering of tyrosine biosynthesis pathway, we tested our hydroxytyrosol-producing strain to see whether it can produce hydroxytyrosol from glucose without tyrosine supplementation. As shown in Fig. 3C, the strain harboring both pBbE1k-3 and pBbS1a-3 successfully produced 0.08 mM hydroxytyrosol over 3 day without supplemented tyrosine. In *E. coli*, the tyrosine level is controlled by complex feedback regulation machinery (Ikeda, 2006), and it is hard to estimate the actual tyrosine level in the presence of the pathway that consumes tyrosine as a substrate. As a result, it is also not easy to estimate the conversion efficiency of tyrosine to hydroxytyrosol in this strain. Further metabolic engineering efforts using tyrosine overproducing *E. coli* strains engineered for hydroxytyrosol production may improve the hydroxytyrosol titer from glucose to the level that allows this microbial platform a good alternative to the olive-based hydroxytyrosol industry.

4. Concluding remarks

We succeeded in introducing an artificial pathway for L-tyrosine oxidation into *E. coli* using a mammalian tyrosine hydroxylase (TH) and an endogenous *E. coli* cofactor. Furthermore, in order to elucidate the availability of TH in *E. coli* for the biosynthesis of hydroxylated tyrosine derivatives, we have combined this pathway with an aryl alcohol biosynthetic pathway and successfully produced hydroxytyrosol, a powerful antioxidant. This engineered *E. coli* is capable of producing hydroxytyrosol not only from supplemented tyrosine but also from endogenously produced tyrosine.

The oxidation of aromatic amino acids such as L-tyrosine is a crucial reaction for the biosynthesis of important biochemicals in

many species, but tyrosine hydroxylase (TH) could not be used for this reaction in a bacterial host due to the lack of BH4, which was thought to be essential for the activity of this enzyme. Although it has been known that *E. coli* does not produce BH4, we found out that TH is functional and can oxidize tyrosine in *E. coli* using the endogenous cofactor MH4 and the BH4 regeneration system from animals. As mentioned previously, benzyl isoquinoline alkaloids, such as the analgesic compounds morphine and codeine, and indole alkaloids, such as serotonin and melatonin, are important natural products whose biosynthetic pathways include hydroxylation of aromatic amino acids (Nakagawa et al., 2011; Park et al., 2011). The aromatic amino acid oxidation platform we report here will allow *E. coli*, one of the most widely used workhorses for biochemical production, to be used to produce these compounds without unfavorable over-oxidation issues. Moreover, when combined in an aromatic amino acid overproducing strain, a simple sugar or even biomass could be used as a carbon source for the production, and this will make the microbial system more economically attractive.

Author contributions

Y.S., K.T., M.M, J.D.K. and T.S.L. designed the experiments. Y.S. and T.S.L. performed the experiments. Y.S., J.D.K, and T.S.L. wrote the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.08.002.

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