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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

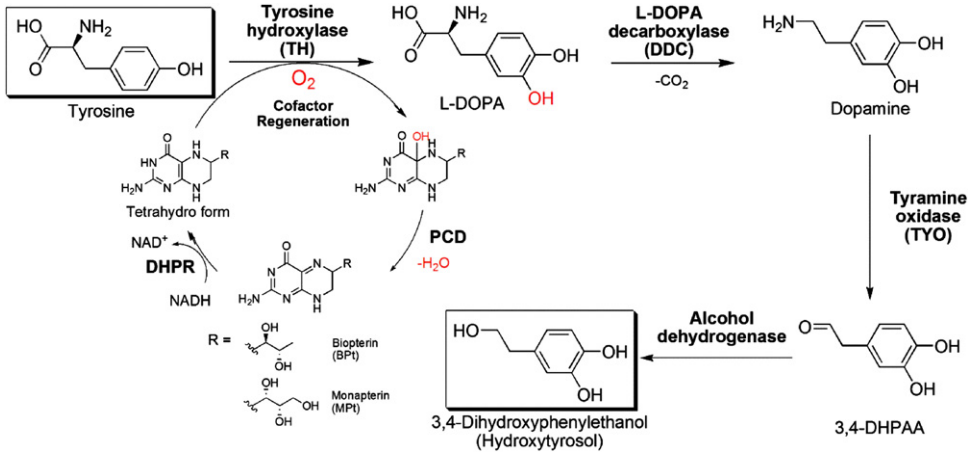

Fig. 1. Pathway for oxidation of tyrosine to hydroxytyrosol. L-tyrosine oxidation is catalyzed by tyrosine hydroxylase (TH) in the presence of the pterin cofactor. The tetrahydrobiopterin (BH4) cofactor regeneration system is composed of pterin-4 alpha-carbinolamine dehydratase (PCD) and dihydropteridine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