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Identification of a novel 4-hydroxyphenylpyruvate dioxygenase from the soil metagenome

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ABSTRACT

4-Hydroxyphenylpyruvate dioxygenase (HPPD) is a Fe(II)-dependent, non-heme oxygenase that converts 4-hydroxyphenylpyruvate to homogentisate. Essential cofactors, such as plastoquinone and tocopherol, are produced by HPPD-dependent anabolic pathways in plants. To isolate a novel *hppd* using culture-independent method, a cosmid metagenomic library was constructed from soil in Korea. Screening of *Escherichia coli* metagenomic libraries led to the identification of a positive clone, YS103B, producing dark brown pigment in Luria–Bertani medium supplemented with L-tyrosine. *In vitro* transposon mutagenesis of YS103B showed that the 1.3 kb insert was sufficient to produce the hemolytic brown pigment. Sequence analysis of YS103B disclosed one open reading frame encoding a 41.4 kDa protein with the well-conserved prokaryotic oxygenase motif of the HPPD family of enzymes. The HPPD-specific β-triketone herbicide, sulcotrione, inhibited YS103B pigmentation. The recombinant protein expressed in *E. coli* generated homogentisic acid. Thus, we present the successful heterologous expression of a previously uncharacterized *hppd* gene from an uncultured soil bacterium.

4-Hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) catalyzes the second step in tyrosine catabolism, which is essential for all aerobic forms of life. Specifically, HPPD catalyzes the oxidative decarboxylation of 4-hydroxyphenylpyruvate (4-HPP) via 1, 2-(ortho)-rearrangement of the carboxymethyl group to yield 2,5-dihydrophenylacetic acid (homogentisic acid; HGA) [1]. In *Escherichia coli*, HGA interacts with indoles in host cells to produce red and orange colored triaryl cation pigments [2]. HPPD is the main molecular target for pharmaceutical drugs used to treat hereditary hypertyrosinemia. Active HPPD, along with fumarylacetoacetase deficiency in hypertyrosinemia patients, stimulates the accumulation of toxic compounds in the liver due to unregulated tyrosine degradation. Toxic by-products, such as succinylacetoacetate, succinylacetone and 5-aminolevulinic acid, cause severe liver and kidney damage. Inhibition of HPPD by 2-(2-nitro-4-trifluoromethylbenzoyl) cyclohexane-1,3-dione (NTBC) prevents the formation of harmful by-products, and is the major treatment mode for hypertyrosinemia [3,4]. In plants, however, HPPD is involved in a different bioconversion reaction. HGA produced by HPPD becomes a key precursor of tocochromanols and prenyl quinones [5]. Plant HPPD is additionally the target site for several β-triketone herbicides, including sulcotrione (2-[2-chloro-4-methanesulfonyl benzoyl]-cyclohexane-1,3-dione) and mesotrione (2-[4-methylsulfonyl-2-nitrobenzoyl]-cyclohexane-1,3-dione). Inhibition of plant HPPD disrupts carotenoid biosynthesis, followed by photosynthetic chlorophyll destruction at a later stage [6].

HPPDs have been isolated from several organisms, including human [7], plants [8], and prokaryotes [9,10]. Bacterial HPPDs are widely utilized in various agricultural and medical applications. For instance, bacterial HPPD isolated from *Pseudomonas fluorescens* confers strong herbicide resistance to plants [11]. Fungal HPPD from the human pathogen *Coccidioides immitis* elicits a proliferative response in a murine T-cell line, supporting the theory that the enzyme is an important drug target for human fungal diseases [12]. To date, bacterial HPPDs have been isolated from both culturable [9] and unculturable strains [2]. Since only 1% of the microbial community from soil is readily culturable [13], uncultured soil microorganisms are evidently the richest gene sources [14]. Here, we search for the novel *hppd* gene in Korean soil metagenomic libraries to establish whether the enzyme can be exploited from the unculturable bacterial genome.

Materials and methods

Construction of metagenomic libraries. Metagenomic libraries were constructed using three environmental soil samples from ecological conservation ecoparks in Korea (the "Upo" wetland, and the "Mujechi" and "Daebudo" sandbars). A soil depth of 10–25 cm was sampled, and stored at 4 °C until use. Crude soil DNA samples

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were extracted, as described previously [15]. Crude DNA was fractionated by pulsed-field gel electrophoresis (CHEF, BioRad) on 1% low melting point agarose at 14 °C for 12 h (4 V cm^{-1}). Gel blocks containing 100–190 kb chromosomal DNA were processed with agarase (1 U/100 mg slice, Takara, Japan). Purified chromosomal DNA was partially digested with *Sau3AI* (0.05 U μL^{-1} of DNA, 37 °C for 1 h), and separated by pulsed-field gel electrophoresis to isolate ~40 kb DNA fragments in 1% low melting point agarose. The purified ~40 kb DNA was ligated into BamHI-digested pSuperCosI (Stratagene), and packaged using MaxPLax Lambda Packaging Extracts (Epicentre, USA). Metagenomic cosmids were isolated using the Large-Construct Kit (Qiagen). The *E. coli* strain, DH5α, was employed for subcloning and sequencing.

Subcloning and sequence analysis. Pigment-producing cosmid clones were sequenced using the shotgun method. To form a shotgun library, metagenomic cosmids were randomly sheared with a sonicator (1-s intervals with a setting of 0.5 outputs, Misonix 3000, NY, USA). Sheared DNA fragments were end-repaired for ligation into pUC118/HincII/BAP at 16 °C. To screen for pigment-producing cells, individual colonies in 96-well plates were grown for 7 days at 28 °C in Luria–Bertani (LB) medium supplemented with ampicillin (100 $\mu g\ mL^{-1}$) and L-tyrosine (600 $\mu g\ mL^{-1}$) [9]. Color production was visually monitored in LB broth. Color screening was conducted on duplicate. *In vitro Mu* transposon mutagenesis was performed with the GeneJumper Primer Insertion kit (Invitrogen, CA, USA). Putative ORFs were identified using the BLASTX algorithm with the non-redundant protein database in NCBI. Amino acid sequences were aligned using MEGA software (ClustalW, ver. 4.0).

Herbicide inhibition and hemolysis analysis. The HPPD-specific bleaching herbicide, sulcotrione, was applied to monitor pigment inhibition among the metagenomic clones. Sulcotrione (2-[2-chloro-4-methanesulfonyl benzoyl]-cyclohexane-1,3-dione) was purchased from Sigma–Aldrich (Riedel-de Haen, Germany). The compound was dissolved in DMSO (maintained at a volume of less than 0.1% (v/v) in the real test setups) before adding to LB. Decolorization was measured at $OD_{440\ nm}$, as described previously [16]. Hemolysis activity was detected on LB agar containing 5% defibrinated sheep blood for 4 days at 28 °C.

Expression in E. coli. The ORF of YS103B was amplified with the primers, 5′-CACC ATGAATGCATCAGCGCAAGCG-3′ and 5′-CAGCACGCCGCGAGCCATCT-3′. The amplified product was cloned into pET101 Directional TOPO cloning vector (Invitrogen, CA, USA) to generate a plasmid, pCM103B, with a C-terminal 6× His-tag. The recombinant fusion protein from pCM103B was expressed in *E. coli* BL21 (DE3) at 37 °C in LB. Expression was induced with 1 mM IPTG during the exponential growth phase of bacterial culture (OD_{600} = 0.6). Cells were grown for another 6 h at 37 °C before harvest. The pellet was resuspended in cell lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole). Cells were sonicated using Misonix 3000 (NY, USA) fitted with a blunt tungsten tip (2 × 2 min at 10 W), followed by centrifugation at 12,000*g* for 30 min at 4 °C to obtain cell-free supernatant. Recombinant protein was purified using the Nickel–nitriacetic acid (Ni–NTA) agarose column, according to the manufacturer's recommendation (Qiagen, Germany). Protein concentrations were measured with the Bradford assay (BioRad, CA).

Enzyme assay and HPLC protocol. HPPD activity was assessed using 50 μg purified protein in 20 mM HEPES buffer (pH 7.0) [5]. The standard assay mixture included 500 μM β-mercaptoethanol (β-ME) and 10 μM Fe(II) under atmospheric oxygen. The enzyme reaction was initiated by adding 400 μM 4-hydroxyphenylpyruvate to obtain a total volume of 200 μL. The assay mixture was incubated for 4 h at 25 °C before terminating the reaction with perchloric acid (70 μL, 20% (v/v)). Residual protein was removed by centrifugation at 20,000*g* for 15 min, and the supernatant subjected to HPLC analysis. The injection volume of 40 μL was analyzed with a Shimadzu HPLC system (LC10AD) equipped with a photodiode array detector (SPD-M10A, Shimadzu, Japan). A Nova-Pak C18 reverse phase column (3.9 × 250 mm, Waters, Ireland) was isocratically run at 0.5 mL min^{-1} using 50% solvent A (0.1% (v/v) trifluoroacetic acid in distilled water) at ambient temperature. Solvent B was 80% acetonitrile containing 0.07% (v/v) trifluoroacetic acid. HGA was detected at UV absorbance of 288 nm [8].

Results and discussion

Genetic analysis of color-producing metagenomic clones

Soil for metagenomic libraries was acquired from the "Upo" wetland, and the "Mujechi" and "Daebudo" sandbars. These regions are ecologically well-conserved swamps in Korea. To preserve biodiversity, we did not enrich soil samples under stress conditions. The cosmid libraries contained 30,000 individual clones with an average insert size of 40 kb (data not shown). About 80% of the clones displayed different digestion patterns by EcoRI, indicating high diversity of metagenomic DNA libraries in soil. Screening of dark brown clones in LB-tyrosine broth for 7 days at 28 °C led to the initial isolation of five metagenome clones, which were designated 60B, 85C, 103B, 107D, and 180A (Supplementary Fig. S1). The insert sizes for the cosmid clones ranged from 38 to 40 kb, with unique enzyme digestion patterns. Among the clones examined, 103B developed brown pigment most efficiently, even after one day of incubation at 37 °C. To determine whether the pigment-producing isolate encodes a novel HPPD enzyme, we examined the genes in 103B.

Rather than sequencing whole inserts in 103B (~40 kb), we searched for genes that conferred brown pigmentation in *E. coli* by random subcloning and transposon mutagenesis (Supplementary Fig. S1). Mild sonication, followed by ligation into blunt-ended pUC118, led to the generation of a 103B random subclone library with an average insert size about 2 kb. Entire subclone libraries were re-screened to identify the pigment-producing subclones. The plasmid isolated was mutagenized with *Mu* transposon, and the resulting mutants screened for color production. Consequently, clear pigment disappearance in LB-tyrosine was observed for one subclone, YS103B. All non-color-producing random insertional mutants contained the transposon within the YS103B insert (data not shown).

Sequence analysis of the 1332 bp insert in YS103B disclosed one large predicted ORF homologous to several bacterial HPPDs (GenBank Accession Nos. DQ849081 and EU489065). The deduced polypeptide sequence, designated mHPPD, displayed the highest homology to *Stenotrophomonas maltophilia* HPPD (72% identity, 84% similarity), along with lower-level homology to other bacterial HPPDs (<58% identity). The *mHPPD* gene encoded 367 residues with a theoretical molecular weight of 41.4 kDa and isoelectric point of 5.4. Analogous to other HPPDs, we identified a well-conserved C-terminal dioxygenase signature domain for binding to Fe(II) [17] (Fig. 1). In multiple amino acid sequence alignments, the deduced mHPPD sequence did not match those of known HPPDs from human or plants, suggesting derivation from bacterial cells rather than eukaryotes (Supplementary Fig. S2).

Despite the lack of consensus patterns between species, central homogentisate pathway genes, including *hppd*, and subsequent catabolic tyrosine/phenylalanine pathway enzymes, are clustered often on the genomes of several bacteria [18]. Notably, clusters of genes encoding the homogentisate pathway provide advantages in preventing the accumulation of catabolic intermediates, such as maleylacetoacetate or fumarylacetoacetate, that confer toxicity to cells. To evaluate the mechanism of heterologous *mHPPD* expression in the *E. coli* background, we further sequenced several *Mu* transposon insertional mutants by primer walking. Both di- and tripeptide transporter and a putative MarR family transcriptional regulator sequences reside upstream of *mHPPD*. Analysis of the *mHPPD* native promoter region revealed the typical organization of σ^{70}-dependent promoters with a −10 signal box (TATCTT) located at a consensus distance (17 bp) from a −35 signal box (TTGCAA) (nucleotides matching those in the consensus sequences are underlined). The 3′ end of the *mHPPD* ORF contained an intergenic region (19 bp) between *mHPPD* and a putative homogentisate dioxygenase (*hmgA*) gene.

Hemolytic activity and inhibition by a HPPD-specific herbicide

In vivo bleaching of *P. fluorescens* HPPD by herbicides has been reported [11]. To determine whether metagenomic *mHPPD* encodes a novel HPPD enzyme, YS103B bleaching by the HPPD-specific herbicide, sulcotrione, was monitored [16,19]. Brown pigmentation was consistently observed following growth of cells containing intact *mHPPD* (pYS103B) for one day in LB-tyrosine, which was clearly inhibited by sulcotrione (Fig. 2A). Under our conditions, sulcotrione did not retard growth of *E. coli*, since wild-type cells containing a control vector with no inserts displayed normal growth. Significant reduction of brown pigment was evident in cells cultured in the presence of 10 μM sulcotrione.