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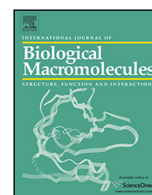

Construction and expression of mutagenesis strain of aroG gene from *Escherichia coli* K-12

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ABSTRACT

3-Deoxy-D-arabino-heptulonate-7-phosphate (DAHP) synthase is one of the key enzymes, which catalyzes the first step in the aromatic amino acid biosynthetic pathway and yields the three amino acids tyrosine (Tyr), tryptophan (Typ), and phenylalanine (Phe). In *Escherichia coli* (*E. coli*), three differently regulated DAHP synthases carry out the first regulated step in the aromatic amino acid biosynthetic pathway. The three DAHP synthases encoded by the genes aroG, aroF, and aroH are inhibited by phenylalanine, tyrosine and tryptophan, respectively. In this work, the aroG gene was cloned and mutated by site-directed mutagenesis using overlap extension PCR (SOE-PCR) technique. The feedback-resistant DAHP synthase encoded by aroG was achieved by replacing the residue Pro150 of aroG with Leu as to increase net carbon flow down the common pathway. SDS-PAGE and Western blots were used to assess the protein expression level of aroGM which showed the strain harboring the mutated aroGM150 gene achieving over-expression compared to the strain containing an empty plasmid pET-28b$^{(+)}$.

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1. Introduction

Trp is one of the protein amino acids that cannot be synthesized by humans and thus must be obtained from food or supplements [1]. As one of the essential amino acids for numerous mammalian species, including humans, L-Trp is widely used in food for humans and animals, pharmaceuticals [2] and food additives [3,4]. With the development of microbial fermentation techniques for the production of L-Trp, it is now becoming the predominant method for production. It is difficult to achieve good yields of L-Trp using a microbial fermentation approach at an industrial scale due to the complex biological synthetic pathway as well as the lack of highly active bacterial strains for fermentation. Recombinant DNA technology allows the introduction of specific genetic modifications within particular metabolic pathways [5] and is one of the many techniques used in metabolic engineering of pathways in host organisms using genetic engineering techniques [6]. DAHPS (3-deoxy-D-arabino-heptulosonate-7-phosphate synthase) is the pivotal enzyme in the synthesis of aromatic compounds in microorganisms. In microorganisms and plants, the first step in the biosynthetic pathway leading to most aromatic compounds, including Phe, Tyr, and Trp, is the stereospecific condensation of the glycolytic intermediate, phosphoenolpyruvate (PEP), and the pentose phosphate pathway intermediate, D-erythrose-4-phosphate (E4P), with the release of the phosphate of PEP and the formation of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) [7]. Metabolite flow in the aromatic biosynthetic pathway in *E. coli* is regulated primarily by feedback inhibition of its three DAHPS isozymes, one is inhibited by Phe [8], one by Tyr [9–11], and one by Trp [12]. In *E. coli*, DAHP synthase isoenzymes were encoded by aroG (80% of total activity), aroF (20% of total activity) and aroH (1% of total activity) genes, respectively. The feedback inhibition site of the aroG gene consists of Asp6, Asp7, Ile10, Ilel3, Prol50, Gin151, Leul75, Leul79, Ser180, Phe209, Ser211 and

Abbreviations: PEP, phosphoenolpyruvate; E4Pe, rythrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; PCR, polymerase chain reaction; SOE-PCR, splicing by overlap extension polymerase chain reaction; LB, Luria Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; AP, ammonium persulfate; *TEMED*, *N,N,N′,N′*-tetramethylethylenediamine; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate buffered saline.

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Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	Source
E. coli		
Escherichia coli k-12		Lab. save
E.coli BL21(DE3)	ompT, hsdS$_B$ (r$_B$$^-m_B$$^-$), gal dcm(DE3)	TIANGEN
E.coli Top10	mcrA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15ΔlacX74 recA1 ara Δ 139 Δ(ara-leu) 7697galU galK rpsL (Strr) endA1 nupG	TIANGEN
Plamid		
pGM-T	Amp^r	TIANGEN
pET-28b$^{(+)}$	Kan^r	Lab. save
pET-28b-aroGM150	Contains the aroGM150 gene	This work

Table 2
Oligonucleotides used in this study.

Primers	Sequence 5′–3′ numbers
AroG150-F1	*CCATGG*GCAATTATCAGAACGACGATTTACGCATC 35
AroG150-R2	GTCAGCGAGATATTGTAGGGTGATCATATCGAG 33
AroG150-F3	CTCGATATGATCACCCTACAATATCTCGCTGAC 33
AroG150-R4	*GGATCC*CGCCCGCGACGCGCTTTTACTGCATTC 33

Val221 and aroGM150 is the 150th feedback inhibition site of the aroG gene. In the previous work, the residue Pro150 of aroG was replaced with Leu and vectors pET-28b$^{(+)}$ contained aroGM150 gene achieved over-expressed in *E. coli* BL21 (DE3) [13].

In the present study, genetic manipulations in *E. coli* were attempted which would introduce an L-Trp-producing strain based on known regulatory and metabolic information. The feedback-resistant DAHP synthase is modified in order to achieve the maximum flow of carbon into the common pathway. Based upon this premise, the work performed in this study include: (1) The aroG gene of *E. coli* was cloned and mutated by site-directed mutagenesis using splicing overlap extension PCR (SOE-PCR) technique. The feedback-resistant DAHP synthase encoded by aroG was achieved by changing the residue Pro150 to Leu. The feedback-resistant aroG gene was named aroGM150. The DNA sequencing result of vector pGM-T-aroGM150 showed that the residue Pro150 of aroG was successfully replaced with Leu. (2) Mutated genes were successfully assembled on the vectors pET-28b$^{(+)}$ and expressed in *E. coli* BL21 (DE3). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were used in the study to test the expression level of protein. The results showed the mutation of residue Pro150 of aroG DNA sequences significantly achieved the over-expression of the aroG gene.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The genome of wild-type *E. coli* K-12 was used as template to obtain the aroG using polymerase chain reaction (PCR) technology. The pET-28b$^{(+)}$ vector which was modified as expression vector carry an N-terminal His·Tag/thrombin/T7·Tag configuration plus an optional C-terminal His·Tag sequence. Bacterial strains and plasmids used in this study are listed in Table 1. Strains and relevant details of their properties are also described. The *E. coli* strains and its derivatives were grown aerobically on a shaker at 37 °C for 12 h in Luria Bertani (LB) medium which contained 10 g/L trypeptone; 10 g/L NaCl; 5 g/L yeast extract or on LB plates which contained 10 g/L trypeptone; 10 g/L NaCl; 5 g/L yeast extract and 15 g/L agar powder. When required, 100 μg/mL ampicillin or kanamycin was added.

2.2. DNA extraction and manipulations

The Ex Taq DNA polymerase, T4 DNA ligase and restriction endonucleases were purchased from MBI Fermentas (Burlington, ON, Canada). The pGM-T vector was purchased from Tiangen (Beijing, China). All enzymatic reactions were performed according to the manufacturers' instructions. *E. coli* cells were transformed by heat shock using frozen competent cells prepared using the calcium chloride method described in *Molecular Cloning* [14]. Plasmid DNA was extracted from *E. coli* using the Biopin plasmid DNA Extraction Kit (BioFlux, China). Primer Premier 5.0 was used to design the primer.

2.3. Nucleotide sequence accession numbers and DNA sequences analysis

The complete genome sequences of *E. coli* str. K-12 substr. MG1655 studied in this paper are available from NCBI under accession number U00096, the sequences of the aroG gene are deposited in NCBI under version numbers GI: 48994873. Sequence comparisons were carried out using DNAMAN.

2.4. PCR protocol for amplification of the fragments aroGM150

The aroGM150^{12} (450 bp) and aroGM150^{34} (603 bp) fragments were synthesized from *E. coli* MG1655 genome by splicing overlap extension polymerase chain reaction (SOE-PCR) in Thermal Cycler (Thermo Electron Corporation, Milford, MA, USA) using primers aroGM150-F1/R2 and aroGM150-F3/R4, respectively. The PCR mixture (25 μL) contained 1 μL each forward and reverse primers, 12.5 μL 2 × Taq Plus Mix, 1 μL cooling *Escherichia coli* cell lysate (*E. coli* cell boiled at 95 °C for 10 min.), 9.5 μL distilled water. For amplification of aroGM150^{12} DNA fragment of the *E. coli* MG1655 the following PCR conditions were used: initial denaturation (94 °C for 5 min); 30 cycles of denaturation (95 °C for 1 min), annealing (56 °C for 30 s), polymerization (72 °C for 30 s), and an additional polymerization step (72 °C for 10 min) at the end. For amplification of the aroGM150^{34} fragments, the following PCR procedure was used: initial denaturation (94 °C; 5 min), 30 cycles of denaturation (94 °C; 30 s), annealing (55 °C; 30 s), and polymerization (72 °C; 45 s); and an additional polymerization step (72 °C; 10 min) at the end. The aroGM150 gene was amplified from *E. coli* MG1655 DNA using the same reaction conditions with the exception that the aroGM150^{12} and aroGM150^{34} fragments were substituted for the template and the same thermal program was used except for the extension time of 2 min at 72 °C. The primes for aroGM150 were aroGM150-F1 and aroGM150-R4. The primers used are listed in Table 2.

2.5. Transform and extraction of plasmid pGM-T-aroGM150

After the desired DNA fragments were isolated on a 1.0% agarose gel, the aroGM150 gene fragment was ligated into pGM-T vector at 16 °C for 12 h. Then, the mixture was transformed to competent cells of *E. coli* TOP 10 as described previously [13]. Plasmid DNA was extracted from *E. coli* using the Biopin plasmid DNA Extraction Kit (BioFlux, China). Then, the plasmids pGM-T-aroGM150 was cut at 37 °C with FastDigest NcoI and FastDigest BamHI (FastDigest enzymes are an advanced line of restriction enzymes for rapid DNA digestion) for 15 min. The reaction conditions were listed in Table 3. The double digested production of plasmid pGM-T-aroGM150 was identified using a 0.8% agarose gel electrophoresis and scan. The positive clones which contained target gene were verified by nucleotide sequencing.

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