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Site-specific integration and constitutive expression of key genes into *Escherichia coli* chromosome increases shikimic acid yields

Xianglei Liu, Jun Lin*, Haifeng Hu, Bin Zhou, Baoquan Zhu*

State Key Laboratory of New Drug and Pharmaceutical Process, Shanghai Institute of Pharmaceutical Industry, China State Institute of Pharmaceutical Industry, Shanghai 201203, China

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ABSTRACT

As the key starting material for the chemical synthesis of Oseltamivir, shikimic acid (SA) has captured worldwide attention. Many researchers have tried to improve SA production by metabolic engineering, yet expression plasmids were used generally. In recent years, site-specific integration of key genes into chromosome to increase the yield of metabolites showed considerable advantages. The genes could maintain stably and express constitutively without induction. Herein, crucial genes *aroG*, *aroB*, *tktA*, *aroE* (encoding 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase, dehydroquinate synthase, transketolase and shikimate dehydrogenase, respectively) of SA pathway and *glk*, *galP* (encoding glucokinase and galactose permease) were integrated into the locus of *ptsHIcrr* (phosphoenolpyruvate: carbohydrate phosphotransferase system operon) in a shikimate kinase genetic defect strain *Escherichia coli* BW25113 (Δ*aroL*/*aroK*, DE3). Furthermore, another key gene *ppsA* (encoding phosphoenolpyruvate synthase) was integrated into *tyrR* (encoding Tyr regulator protein). As a result, SA production of the recombinant (SA5/pGBAE) reached to 4.14 g/L in shake flask and 27.41 g/L in a 5-L bioreactor. These data suggested that integration of key genes increased SA yields effectively. This strategy is environmentally friendly for no antibiotic is added, simple to handle without induction, and suitable for industrial production.

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1. Introduction

Shikimic acid (3,4,5-tri-hydroxyl-cyclohexene-1-carboxylic acid, SA), a natural organic compound, is an industrially crucial chiral compound for the synthesis of many chemical substances like aromatic amino acids, indole, alkaloids, and many other aromatic metabolites [1–4]. In recent years, SA has attracted worldwide attention due to its characteristic pharmaceutical application as the key starting material of Oseltamivir phosphate (Tamiflu®), which is prescribed as the front-line treatment for serious cases of influenza [1,5–7]. At present, there are three main SA production methods—conventional plant extract, chemical synthesis and microbial fermentation [8,9]. Majority of commercial synthesis of SA is from the fruits of *Illicium vernum*, which requires extensive procedures to yield high purity SA, so it is cumbersome including number of drawbacks. The chemical synthesis method of SA is also known, but as per to environmental facets not commercially viable, waste streams containing environmental pollutants were generated. Fermentation procedure using microorganism proved to be a more efficient, simple and viable alternative source to achieve large scale production of SA. Several metabolic and genetic manipulations were conducted on different microorganisms like *Bacillus subtilis*, *Citrobacter freundii*, and *Esherichia coli* (*E. coli*) to obtain SA [1,10,11].

In *E. coli*, microbial production of SA involves the shikimate pathway (Fig. 1). In the past decades, an army of researchers have investigated and metabolically engineered *E. coli* strains to enhance the production of SA [12–16]. The strategy could be summarized as decrease expenditure and increase income. On the one hand, reduce expenditure. Knockout or interference of shikimate kinase isoenzymes (coded by *aroK* and *aroL*) could decrease the consumption of SA [12,17–19]. For the transportation of carbon source, phosphoenol pyruvate (PEP) is used for phosphorylation, while PEP is a crucial precursor for the synthesis of 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) with erythrose-4-phosphate (E4P). Replacement of phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS, coded by *ptsHIcrr* and *ptsG*) with other transport systems like galactose permease (coded by *galP*) and glucokinase (coded by *glk*) could significantly reduce the cost of PEP [12,16,20,21]. On the other hand, increase income. Augmentation of metabolic flux by overexpression of the key enzymes, like DAHP synthase isoenzymes (coded by *aroF*, *aroG*, and *aroH*), dehydroquinate synthase (coded by *aroB*), shikimate dehydrogenase (coded by *aroE*), transketolase

* Corresponding authors. Fax: +86 21 2057 2112.
E-mail addresses: junlin621@126.com (J. Lin), baoquanzhu@126.com (B. Zhu).

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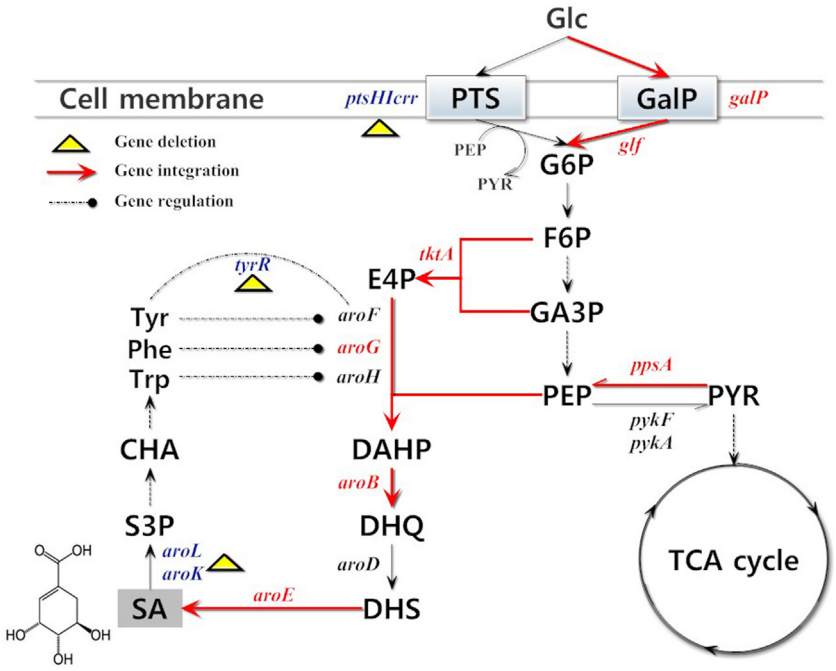

Fig. 1. Shikimate pathway in recombinant strain *E. coli* SA5. Abbreviations: phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS); tricarboxylic acid cycle (TCA); glucose-6-phosphate (G6P); fructose-6-phosphate (F6P); glyceraldehyde-3-phosphate (GA3P); phosphoenol pyruvate (PEP); pyruvate (PYR); erythrose-4-phosphate (E4P); 3-deoxy-d-arabinoheptulosonate-7-phosphate (DAHP); 3-dehydroquinic acid (DHQ); 3-dehydroshikimic acid (DHS); shikimic acid (SA); shikimate-3-phosphate (S3P); chorismic acid (CHA); galactose permease (*galP*); glucokinase (*glk*); transketolase I (*tktA*); DAHP synthase isoenzymes F, G and H (*aroF, aroG, aroH* respectively); phosphoenolpyruvate synthase (*ppsA*); pyruvate kinase I (*pykF*); pyruvate kinase II (*pykA*); DHQ synthase (*aroB*); DHQ dehydratase (*aroD*); shikimate dehydrogenase (*aroE*); shikimate kinase I (*aroK*); shikimate kinase II (*aroL*); Tyr regulator (*tyrR*).

(coded by *tktA* and *tktB*) and phosphoenolpyruvate synthase (coded by *ppsA*), could improve the utilization rate of substrate for the biosynthesis of SA [11,12,22].

However, the overexpression of genes relies on engineered plasmids mostly. Generally, antibiotic selection was indispensable for their stability, and isopropyl-β-D-thiogalactoside (IPTG) or other inducers were added for induction. While proving extraordinarily useful, the poor stability and operation complexity limit its application in industry. Consequently, it is advantageous to incorporate genes directly into the chromosome, which is stably maintained without the need for antibiotic selection and constitutively expressed without induction [23,24]. Recently, A SA-producing strain *E. coli* SA116 was constructed by triclosan-induced chromosomal evolution, with a production of 3.12 g/L [13]. It is the first report of an engineered SA-producing strain that lacks the expression plasmid and antibiotic selective marker in the chromosomal, however the sites of genes integration were limited and uncertain.

In our previous work, the recombinant strain *E. coli* BW25113 (Δ*aroL*/*aroK*, DE3)/pETDuet-GBAE was constructed, involved inactivating the *aroL* and *aroK* genes and overexpressing the *aroG*, *aroB*, *tktA*, and *aroE* genes by plasmid pETDuet-GBAE, antibiotic and IPTG were used inescapably [25]. In this study, the genes *aroG*, *aroB*, *tktA* and *aroE* were directly integrated into the locus of *ptsHIcrr*. Afterwards, the genes *glk* and *galP* were integrated to improve the efficiency of glucose transporter further. Due to the transcription expression of most genes of the TyrR regulon (such as *aroF* and *aroG*) is subject to repression control by the TyrR protein [26], the gene *tyrR* was deleted and integrated into the gene *ppsA* at last. On the whole, the key genes *aroG*, *aroB*, *tktA*, *aroE*, *ppsA*, *glk* and *galP* were site-specific integrated firstly into *E. coli* chromosome together with T7 promoter and T7 terminator, and genes *ptsHIcrr*, *tyrR* were deleted meanwhile. After the optimization of medium composition, SA production of the recombinant strain *E. coli* BW25113 (Δ*aroL*/*aroK*, Δ*ptsHIcrr::GBAEKP*, Δ*tyrR::ppsA*), named SA5, reached 2.83 g/L in shake flasks without antibiotics and IPTG induction. Another plasmid pETDuet-GBAE was added again, SA production increased to 4.14 g/L. In a 5-L bioreactor, SA accumulation of SA5/pGBAE increased to 27.41 g/L. The results implied that site-specific integration of key genes into *E. coli* chromosome could increase SA yields effectively.

2. Materials and methods

2.1. Microorganisms and reagents

All strains and plasmids used in this study are listed in Table 1, and primers used in this study are listed in Table 2. *E. coli* DH5α was used as host for plasmid construction. DNA polymerase (*LA Taq*®), restriction endonucleases and T4 DNA ligase were purchased from Takara (Dalian, China). Oligonucleotides, DNA polymerases *Taq*, *pfu*, *UltraTaq*, plasmid isolation and DNA purification kits were provided by Generay (Shanghai, China). DNA sequencing was completed by Sangon (Shanghai, China).

Strains were cultured in Luria-Bertani (LB) media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) during recombinant plasmid construction. EZ-Rich Defined Medium (RDM, Teknova, Inc.) supplemented with 0.5% glycerol were used for gene integration. Various concentrations of antibiotics (10 μg/mL tetracycline, 100 μg/mL spectinomycin, 34 μg/mL chloramphenicol, 100 μg/mL ampicillin, 30 μg/mL kanamycin) were added to culture media of plasmid-bearing *E. coli* strains.

2.2. Plasmid construction

Standard manipulation was carried out for polymerase chain reaction (PCR), DNA purification, enzyme digestion, and DNA ligation. The genes *glk*, *galP* and *ppsA* were cloned using the chromosome of *E. coli* BW25113 as the template. Primers used in the PCR

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