Process Biochemistry 49 (2014) 751–757

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

A systems level engineered *E. coli* capable of efficiently producing L-phenylalanine

Shuang Ping Liu, Rui Xia Liu, Meng Rong Xiao, Liang Zhang, Zhong Yang Ding, Zheng Hua Gu, Gui Yang Shi*

The Key Laboratory of Industrial Biotechnology of Ministry of Education, National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, People's Republic of China

ARTICLE INFO

Article history:
Received 21 August 2013
Received in revised form 2 January 2014
Accepted 2 January 2014
Available online 8 January 2014

Keywords:
Metabolic engineering
Synthetic biology
Phenylalanine
Genetic switch
Systems level engineering

ABSTRACT

The biosynthesis of L-phenylalanine (Phe) is one of the most complicated amino acid synthesis pathways. In this study, the engineering of Phe producer was carried out to illustrate the effectiveness of systems level engineering: (1) inactivated glucose specific phosphoenolpyruvate-carbohydrate phosphotransferase (PTS) system by inactivation of *crr* to moderate the glucose uptake rate to reduce overflow metabolism; (2) genetic switch on or off the expression of phe^{fbr}, *aroG15*, *ydiB*, *aroK*, and *tyrB* to increase the supply of precursors; (3) employed a *tyrA* mutant strain to reduce carbon diversion and to result in non-growing cells; (4) enhanced the efflux of Phe by overexpressing *yddG* to shift equilibrium towards Phe synthesis and to release the feedback regulation in Phe synthesis. The mutants in PTS were firstly compared and a crr^- mutant was firstly screened. The mutant AroG15 was demonstrated to a thermostable mutant. The strains expressing *yddG* excreted Phe into the medium at higher rate and less intracellular Phe accumulated. By systems level engineering, an engineered Phe producer achieved 47.0 g/L Phe with a yield of 0.252 g/g which was the highest under the non-optimized fermentation condition.

1. Introduction

L-phenylalanine (Phe) is an essential amino-acid and it is also an important chiral substrate for the production of low calorie sweetener aspartame. With the advent of rational metabolic engineering, it becomes possible to construct a Phe producer by engineering specific genes in microorganisms [1]. However, the control architectures of a biosynthesis pathway for desired chemical or intermediate always form a control system [2], it is not easy to release all the restrictions generated by a high intracellular concentrations of desired chemical or intermediate. While, it might be much more successful if the engineering process was carried out at a systems level [3]. With the development of contributing tools (hosts, vectors, genetic controllers, and characterized enzymes) in synthetic biology [4], it is possible to develop a more effective and robust cell factory at the systems level with less and less costs and time.

The biosynthesis of Phe is one of the most complicated amino acid synthesis pathways; meanwhile its regulatory landscape has been delineated [2,5,6] which makes it possible to develop a Phe producer at the systems level. In *E. coli*, glucose is always assimilated by phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) in a high rate. The high rate of glucose uptake and glycolysis results in a rate of Acetyl coenzyme A (AcCOA) synthesis surpassing the capacity of the tricarboxylic cycle to completely consume this metabolite. Part of the excess AcCOA is diverted into the acetate kinase and phosphotransacetylase (Ack-Pta) pathway to generate acetic acid [7] which inhibits cell metabolism and also decreases the yield of Phe. Carbon flux partitioning at the phosphoenolpyruvate (PEP) node was the major determinant of yield for aromatic amino compounds synthesized from glucose. When *E. coli* grows in minimal medium containing glucose as the carbon source, the PTS consumes 50% of PEP, while the 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP) synthase consumes 3% [8].

The biosynthesis of Phe has ten reactions from PEP and D-Erythrose-4-phosphate (E4P). Among these reactions, six are feedback regulated, three of them (shikimate dehydrogenase, shikimate kinase and aminotransferases) can be deregulated by overexpression of the corresponding genes by a strong promoter, the other three (DAHP synthase, chorismate mutase and prephenate dehydratase) must be replaced by their uninhibited mutants. The enzyme characteristics of these uninhibited mutants need to be considered, as some mutants are thermally instable and produce less Phe than the original genes [9–11]. Furthermore, a higher intracellular concentration of desired chemicals or intermediates

* Corresponding author. Tel.: +86 510 85918229; fax: +86 510 85918229.
E-mail address: biomass_jnu@126.com (G.Y. Shi).

1359-5113/$ – see front matter © 2014 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.procbio.2014.01.001

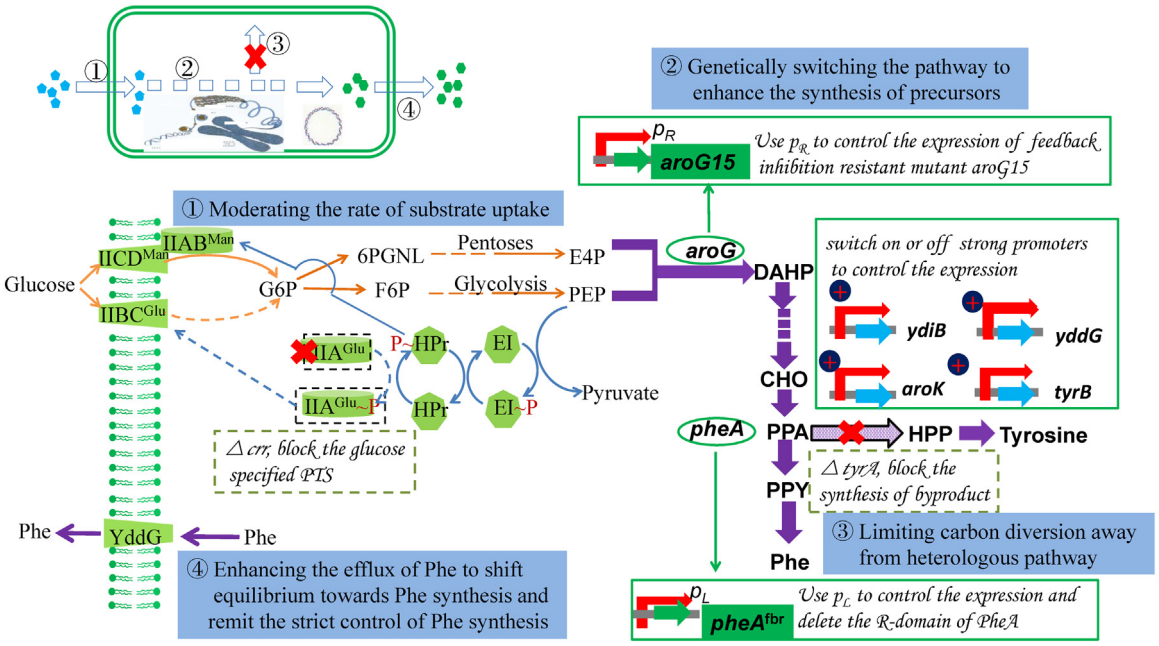

Fig. 1. A holistic view of the systems level engineered *E. coli* to effective Phe producer. Methods for manipulating the flux from glucose towards Phe were grouped into four categories. Metabolites abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PGNL, 6-phosphoglucono-δ-lactone; CHO, chorismate; PPA, prephenate; PPY, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate. Genes and enzymes: *aroG*, DAHP synthase; *pheA*, chorismate mutase and prephenate dehydratase; *ydiB*, shikimate dehydrogenase; *aroK*, shikimate kinase; *tyrB*, tyrosine aminotransferase; *yddG*, methyl viologen efflux pump; *ptsG*, fused glucose-specific PTS enzyme IIBC; *ptsH*, PTS system phosphohistidinoprotein-hexose phosphotransferase (HPr); *ptsI*, PEP-protein phosphotransferase of PTS system (EI); *crr*, PTS system glucose-specific transporter subunit IIA; *manX*, fused mannose-specific PTS enzyme IIAB; *manY*, PTS system mannose-specific transporter subunit IIC; *manZ*, PTS system mannose-specific transporter subunit IID.

may disturb cell metabolism, certain genetic approaches need to be developed to accurately regulate the synthesis of Phe and effectively elevate the rate of Phe excretion.

In view of the phenomena above, a systems level engineering was designed (Fig. 1) and carried out. The four steps in Fig. 1 were respectively studied and ideal methods were found to achieve a Phe producer by the systems level engineering. In this study, the systems level engineering was firstly used for producing Phe to illustrate the effectiveness of the systems level engineering.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains (Supplementary Table S1) with the plasmids containing temperature-controllable promoters p_L and p_R were cultured at 33 °C in Luria Bertani (LB) medium, others grew at 37 °C. Respective antibiotics (ampicillin 100 μg/L, kanamycin 30 μg/L, or chloramphenicol 25 μg/L) were added to maintain the plasmids.

2.2. Construction of deletion mutants

The genes *ptsG*, *ptsI*, and *crr* in *E. coli* W3110 or WSH-Z06 were inactivated by Red recombination [12]. The FRT-*kan*-FRT cassettes were amplified using pKD13 as template (primers in Supplementary Table S2). After the cassettes were transformed into *E. coli*, the deleted mutants were verified by antibiotic resistance and PCR products. Then the *kan* gene was removed [12].

2.3. Amplification and overexpression genes

The nucleotides coding the catalysis domains (chorismate mutase and prephenate dehydratase) of PheA were amplified from *E. coli* W3110 chromosome, thus the R-domain responsible for Phe binding was not contained in the resulted feedback inhibition resistant (fbr) PheA^{fbr} (1–300 amino acid residues of wild type PheA) [5,13]. The uninhibited allele *aroG15* (with an Asp-146-Asn substitution) of DAHP synthase was amplified from T-aroG15 (primers in Supplementary Table S3). The coding sequence of *tyrB* contained a strong TyrR box (TGTTTCAAAAAGTTGACG), this box was artificially changed by primer tyrB_*Eco*RV_SD_SB_FW. In this primer, only five bases were the same as the strong TyrR box, while in the weak TyrR box, there were at least seven same bases [2]. The genes *ydiB*, *aroK*, *and yddG* were respectively amplified from plasmid T-ydiBaroK or *E. coli* W3110 chromosome (primers in Supplementary Table S3). All the genes above were respectively connected with pSY130-14 [14] under the bacteriophage λ promoters p_R (for *aroG15*, *tyrB*) and p_L (for *pheA*^{fbr}, *ydiB*, *aroK*, *yddG*). This enabled a simple temperature change to efficient and rapid switch on or off the expression of target genes.

2.4. Purification of AroG and its mutants

The uninhibited alleles (*aroG15* and *aroG29*) and the original *aroG* were amplified from T-aroG15, T-aroG29 and *E. coli* W3110 chromosome (primers in Supplementary Table S3). They were respectively connected with pET28a and expressed in *E. coli* BL21(DE3). Single clone of *E. coli* BL21(DE3) harboring target plasmid was cultured in LB medium at 37 °C for 12 h, and then this culture was used to inoculate TB medium [15]. At OD_{600} = 1, the T_7 promoter was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, I5502, Sigma-Aldrich) and cultured at 25 °C for 16 h. The cells were harvested by centrifugation, washed with sodium phosphate buffer (pH 7.5) and broken by sonication. After centrifuging for 30 min at 16,000 × *g* to pellet cell debris, the clarified enzyme solutions were purified. The purification process was carried out with the AKTA purifier 100 and the HisTrap affinity columns (17-5247-01, GE Healthcare BioSciences, Sweden) in a linear gradient elution of imidazole. The peaks containing the target protein were determined by SDS-PAGE [15].

2.5. Denatured protein analysis

The peak containing the target protein was centrifugally concentrated by ultrafiltration tubes (Vivaspin 20 VS2041, Sartorius, Germany) with a molecular weight cut off of 100,000 Da, and the target protein was dissolved in 5 mM potassium phosphate buffer (pH7.5). The denatured protein analysis was performed on a circular dichroism (CD) spectrometer (MOS-450 AF, Bio-Logic, France) equipped with a temperature cell holder [16]. Conformational changes in the secondary structure of protein were monitored in the region between 190 and 250 nm at a protein concentration of 0.2 g/L with a path length of 1 mm. The Tm values were calculated at the optimum wavelength under the temperature period of 20–75 °C.

2.6. Fermentation conditions

The fermentation medium with 1 g/L tyrosine was prepared as previous report [17]. Shake flask fermentation was carried out in 500-mL conical flasks containing 70 mL fermentation medium inoculated with 5% (v/v) seed culture. It was incubated at 33 °C [18] for 4–5 h (log phase), then the temperature was elevated to 38 °C to induce the expression of the target genes. Fed-batch fermentation was performed in a 15-L jar fermentor with an initial broth volume of 6 L. Afer sterilization, the glucose