3 Biotech (2019) 9:85
https://doi.org/10.1007/s13205-019-1619-6

REVIEW ARTICLE

Metabolic engineering for the production of L-phenylalanine in *Escherichia coli*

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Received: 26 December 2018 / Accepted: 8 February 2019 / Published online: 15 February 2019

Abstract
As one of the three proteinogenic aromatic amino acids, L-phenylalanine is widely applied in the food, chemical and pharmaceutical industries, especially in production of the low-calorie sweetener aspartame. Microbial production of L-phenylalanine has become attractive as it possesses the advantages of environmental friendliness, low cost, and feedstock renewability. With the progress of metabolic engineering, systems biology and synthetic biology, production of L-phenylalanine from glucose in *Escherichia coli* with relatively high titer has been achieved by improving the intracellular levels of precursors, alleviating transcriptional repression and feedback inhibition of key enzymes, increasing the export of L-phenylalanine, engineering of global regulators, and overexpression of rate-limiting enzymes. In this review, successful metabolic engineering strategies for increasing L-phenylalanine accumulation from glucose in *E. coli* are described. In addition, perspectives for further improvement of production of L-phenylalanine are discussed.

Keywords *Escherichia coli* · Metabolic engineering · L-Phenylalanine · Glucose

Introduction

As one of the three proteinogenic aromatic amino acids, L-phenylalanine is widely used in food additives, animal feed, infusion fluids, or as a building block for drug synthesis. As it can be used in the biosynthesis of the low-calorie sweetener aspartame, demand for L-phenylalanine has increased worldwide (Sprenger 2007b). Because of its commercial importance and wide application, increasing attention has been paid to L-phenylalanine production. Chemical synthesis uses nonrenewable toxic materials and generates racemic mixtures of D- and L-phenylalanine, which is undesirable (Otrokhov et al. 2013). Accordingly, direct microbial fermentation from low cost raw materials has become more favorable than other strategies (Rodriguez et al. 2014). *Escherichia coli*, a model organism with the advantages of a clear genetic background, simple genetic manipulation and fast growth in cheap media, is widely used for the production of L-phenylalanine and other aromatic compounds (Gu et al. 2012, 2016; Liu et al. 2015). However, in wild strains of *E. coli*, titer of L-phenylalanine is limited by strong and complex regulation of the biosynthesis pathway.

Previous L-phenylalanine producers were mainly obtained by classical mutagenesis and screening procedures. To obtain L-phenylalanine producers, structural analogues fluorophenylalanine or chlorophenylalanine was firstly supplemented into the medium. And then, mutant strains with resistant against the antimetabolites will exhibit restored growth and can easily be selected (De Boer and Dijkhuizen 1990). Through DNA sequencing, it was found that the mutant strains usually possessed altered allosteric binding sites of key enzymes (Ikeda 2006). However, unexpected mutations will be inevitably generated in the process of classical mutagenesis, which may affect further improvement of the mutant strain (Dong et al. 2011). Accordingly, rational engineering technologies have been used to achieve deletion, overexpression, and genomic integration of target genes to improve L-phenylalanine production in *E. coli* strains (Table 1).

In *E. coli*, two precursors of the L-phenylalanine biosynthesis pathway are phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P). As the three aromatic amino acids share the same biosynthesis pathway from 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) to chorismate, the overall L-phenylalanine biosynthesis pathway is often

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Table 1 Comparison of L-phenylalanine production in different recombinant strains of *E. coli*

Strain	Relevant characteristics	Titer (g/L)	Conversion rate (mol/mol)	Productivity (g/L/h)	Culture methods	References
WSH-Z06 (pAP-B03)	*E. coli* K-12 with L-tyrosine auxotrophic	35.38	26%	N	3-L fed-batch fermentation	Haiyan et al. (2010)
HD-A2	Multiple random mutagenesis of *E. coli* W3110 and overexpressing $aroF^{WT}$, $pheA^{FBR}$ and *aroA*	62.47	25.74%	N	5-L fed-batch fermentation	Ding et al. (2016)
BR-42 (pAP-B03)	Multiple random mutagenesis of *E. coli* WSH-Z06 and overexpressing $aroF^{WT}$ and $pheA^{FBR}$	57.63	26.4%	1.153	3-L fed-batch fermentation	Zhou et al. (2011)
Xllp21	W3110-derived L-tyrosine auxotrophic with overexpressed $aroF^{WT}$, *aroD*, and $pheA^{FBR}$, inactivated *tyrR*, and genomic integrated *galP* and *glk*	72.9	28.3%	N	5-L fed-batch fermentation	Liu et al. (2018)
W3110 (pNpheABK15)	W3110 with overexpressed $pheA^{FBR}$, *ydiB, aroK* and *aroG15*	23.8	16.8%	0.073	15-L fed-batch fermentation	Liu et al. (2013)
pR15BABKG	WSH-Z06Δ*crr* with overexpressed *pheA, aroG15, ydiB, aroK, tyrB* and *yddG*	47	27.5%	N	5-L fed-batch fermentation	Liu et al. (2013)
W3110-4 (pF20)	W3110 (Δ*pheA*Δ*tyrA* Δ*aroF*) with overexpressed $aroF^{FBR}$ and $pheA^{FBR}$	32	N	N	300-L fed-batch fermentation	Gerigk et al. (2002b)
E. coli aroF-wt	w3110 Fnr^{+} (Δ*pheA*Δ*tyrA* Δ*aroF*)/pJF119EH-$aroF^{WT}$-$pheA^{FBR}$-$aroL^{WT}$	35	N	N	20-L fed-batch fermentation	Gerigk et al. (2002a)

N not reported

divided into the common pathway and the specific L-phenylalanine pathway that branches at the point of chorismate. As shown in Fig. 1, three DAHP synthase isoenzymes-AroF, AroG and AroH-are responsible for the first and rate limited step in the L-phenylalanine biosynthesis pathway (Umbarger 1978). AroG contributes about 80% of the overall DAHP activity, while the contributions of AroF and AroH to the total DAHP activity are only about 15% and 5%, respectively (Herrmann and Weaver 1999). Another rate-limiting enzyme in the L-phenylalanine pathway is chorismate mutase-prephenate dehydrogenase (CM-PDT), encoded by *pheA*, which is feedback inhibited by allosteric binding of L-phenylalanine. Notably, the last step in the formation of L-phenylalanine is a transamination reaction of phenylpyruvate with glutamate as the amino donor, and this step can be catalyzed by TyrB, AspC and IlvE.

Regulation of L-phenylalanine biosynthesis in *E. coli*

In *E. coli*, TyrR and TrpR are the main transcriptional regulators of genes in the L-phenylalanine biosynthesis pathway (Wallace and Pittard 1969). Transcription of *aroF, aroL*, and *tyrB* is repressed by TyrR (Pittard and Davidson 1991; Wilson et al. 1994). TrpR can repress the transcription of *aroH, aroL*, and also its own expression (Gunsalus and Yanofsky 1980). To increase the accumulation of L-phenylalanine, *tyrR* and *trpR* are often inactivated simultaneously or individually.

In addition, L-phenylalanine production is also limited by feedback inhibition of enzymes in *E. coli* such as DAHP synthases and CM-PDT. The three DAHP synthase isozymes are feedback inhibited by L-phenylalanine, L-tyrosine and L-tryptophan, respectively, but CM-PDT

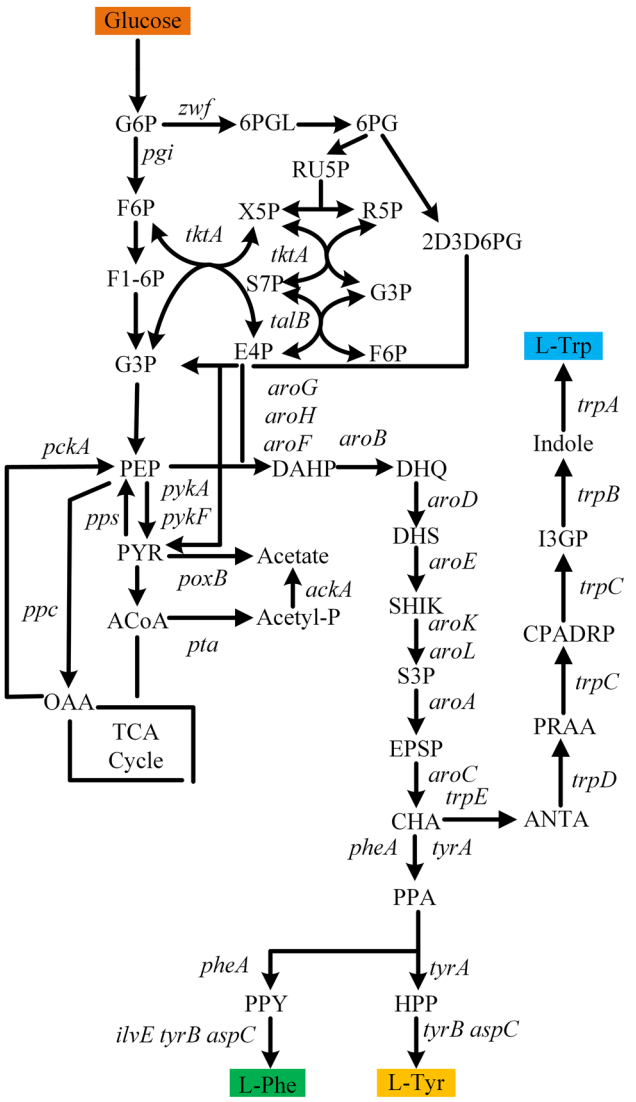

Fig. 1 The L-phenylalanine biosynthesis pathway in *Escherichia coli*

is only sensitive to the concentration of L-phenylalanine (Ikeda 2006). In addition, shikimate dehydrogenase, encoded by *aroE*, is feedback inhibited by shikimate. This is the only enzyme in the aromatic amino acid biosynthesis pathways of *E. coli* that is subject to feedback inhibition by an intermediate rather than an end-product (Sprenger 2007a).

In *E. coli*, the expression of *pheA* (encoding CM-PDT) is also regulated by an attenuation mechanism. When high concentrations of L-phenylalanine are present in the medium, the ribosomes can successfully translate the leader peptide sequence by enough charged $tRNA^{Phe}$, in turn preventing the formation of the second stem and loop and allowing formation of the third structure. As a result, transcription by RNA polymerase will be terminated. It was reported that 75% of the transcription of the *pheA* leader region was stalled when rich medium was applied for the cultivation of *E. coli* (Gavini and Davidson 1991).

Metabolic engineering for L-phenylalanine production in *E. coli*

Improving the intracellular levels of precursors of L-phenylalanine

To increase the intracellular concentration of E4P, transketolase (encoded by *tktA*) and transaldolase (encoded by *talB*) are often selected as engineering targets (Zhao and Winkler 1994). In addition, it was reported that inactivation of phosphoglucose isomerase was also advantageous for the supply of E4P by blocking glycolysis (Mascarenhas et al. 1991). By using glycerol instead of glucose as carbon source, the carbon flow directed into the pentose phosphate pathway can be obviously increased (Khamduang et al. 2009; Thongchuang et al. 2012).

When one glucose molecule is assimilated into *E. coli* cells by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), one molecule of PEP will be consumed (Gosset 2005; Postma and Lengeler 1985). It was reported that about 50% of the intracellular PEP is consumed by the PTS system, while only 1.5% of PEP is directed into aromatic amino acid biosynthesis (Flores et al. 2002; Sprenger 2007b). Accordingly, the PTS is often selected as the first engineering target to increase the PEP level. However, the growth of mutant strains with deficiency of PTS components is often impaired due to limitations on the transport of glucose. To solve this problem, adaptive evolution processes can be employed to improve the growth of PTS-defect strains on glucose, and versatile mutant strains with recovered growth on glucose were obtained, such as PB12 (Flores et al. 1996). It was found that this strain directs more PEP into the aromatic synthesis pathway than other strains (Flores et al. 2002), and it can be used to produce various aromatic compounds by further genetic modifications (Carmona et al. 2015; Escalante et al. 2010). Another optional strategy is replacement of the PTS by the glucose facilitator and glucokinase from *Zymomonas mobilis*, or the galactose permease and glucokinase from *E. coli* (Balderas-Hernandez et al. 2009; Yi et al. 2002).

Apart from engineering the PTS, decreasing the carbon flux from PEP into the tricarboxylic acid cycle was also necessary to further increase the PEP pool, including inactivation of pyruvate kinase PykF (Meza et al. 2012; Sabido et al. 2014) and phosphoenolpyruvate carboxylase Ppc, and improving the expression of PEP synthetase PpsA to recycle pyruvate to PEP (Patnaik and Liao 1994; Yi et al. 2002).

Engineering of global regulators

CsrA is a regulatory protein of carbohydrate metabolism and can also regulate the intracellular PEP titer. It was reported that the titer of PEP could be increased by deletion of *csrA* or increasing the expression of negative regulatory RNA of *csrA* encoded by *csrB* (Tatarko and Romeo 2001; Yakandawala et al. 2008). Engineering of *csrB* and/or *csrA* combined with *tktA* overexpression was more effective than traditional strategies in enhancing L-phenylalanine production (Yakandawala et al. 2008). In addition, Fis, a nucleoid-associated protein, was found to be abundant during the exponential phase and decreased obviously in the stationary phase. Perhaps Fis is needed for transcription of growth related genes, and overexpression of Fis can put the host into a more metabolically active state (Tyagi et al. 2017). To test this hypothesis, the *fis* gene was overexpressed in recombinant strain WF123456 and the L-phenylalanine titer was indeed increased 1.2-fold compared with the control (Tyagi et al. 2017). In addition, Ojima et al. found that excess expression of *yggG*, encoding a stress-responsive gene, could repress the secretion of acetate, which may be of benefit for production of L-phenylalanine in *E. coli* (Ojima et al. 2009). Accordingly, they introduced *yggG* into an L-phenylalanine producer (*E. coli* AJ12741) with feedback-resistant AroG; the recombinant strain could produce 6.4 g/L L-phenylalanine, 73% higher than the original strain in batch fermentation.

Overcoming transcriptional repression, feedback inhibition and attenuation of key enzymes

As DAHP synthase controls the amount of carbon flow into the L-phenylalanine biosynthesis pathway (Ogino et al. 1982), the removal of feedback inhibition of DAHP synthase isoenzymes may be helpful to increase L-phenylalanine yield. With the progress of structural analysis, the crucial amino acid residues of DAHP synthase isoenzymes have been identified. As a result, various feedback-resistant enzymes, such as AroG(Leu76Val), AroF(Pro148Leu), and AroF(Gln152Ile) have been obtained (Ger et al. 1994; Kikuchi et al. 1997), and some of them are summarized in Table 2.

Interestingly, it was found that wild-type *aroF* could achieve even higher final L-phenylalanine titers (34 g/L) than the $aroF^{FBR}$-containing strain (28 g/L) due to the higher activity of wild-type AroF (Gerigk et al. 2002a). In 2004, another group also constructed a recombinant *E. coli* strain containing $aroF^{WT}$ and $pheA^{FBR}$ inserted in an IPTG-inducible plasmid for L-phenylalanine production (Takors 2004). By overexpression of $pheA^{FBR}$ and $aroF^{WT}$ in L-tyrosine auxotrophic *E. coli* strain WSH-Z06, the L-phenylalanine titer reached 35.38 g/L in a 3-L fermentor, 2.81-fold higher than that of the parental strain (Haiyan et al. 2010). These examples imply that wild-type DAHP synthases can also be employed for L-phenylalanine production.

In *E. coli, ydiB*, encoding bifunctional enzyme shikimate dehydrogenase/quinate dehydrogenase, can transform 3-dehydroshikimate to shikimate like AroE. It was reported that *ydiB*-overexpressing strains could achieve a higher L-phenylalanine titer than *aroE* (Lutke-Eversloh and Stephanopoulos 2008), indicating this strategy could overcome the feedback regulation of *aroE*. In addition, *aroL* was reported as the preferred shikimate kinase for increasing L-phenylalanine yield because of its lower K_m. However, the activity of AroL is partly inhibited at high substrate concentration, while AroK works well in the same conditions (Oldiges et al. 2004). Accordingly, overexpression of *aroK* in *E. coli* will produce more L-phenylalanine than overexpression of *aroL*.

For CM-PDT, three mutants with substitution or an in-frame deletion located within codons 304–310 of the *pheA* gene were obtained. The mutated enzymes exhibited both high enzymatic activity and almost complete resistance to feedback inhibition when 200 mM L-phenylalanine was present (Nelms et al. 1992). In addition, PheA containing residues 1–285 and residues 1–300 retained full mutase and dehydratase activity and showed no feedback inhibition (Zhang et al. 1998). In 2004, through two

Table 2 Summary of deregulated key enzymes in L-phenylalanine biosynthesis pathway of *E. coli*

Enzyme	Mutant sites	References
AroG	D146 N, M147I	Ding et al. (2014)
AroG	S180F	Ger et al. (1994)
AroG	L76V	Kikuchi et al. (1997)
AroH	P18L, V147M, G149D, G149C, A177T	Ray et al. (1988)
AroF	Deletion of I11	Zhao et al. (2011)
AroF	P148L	Weaver and Herrmann (1990)
AroF	N8K	Jossek et al. (2001)
PheA	Deletion of T304 and Q306	Nelms et al. (1992)
PheA	Deletion of 301–386 amino acids residues	Zhang et al. (1998)
PheA	N5S, L8P, D54N, L55M, S235A	Baez-Viveros et al. (2004)

protein-evolutionary cycles of CM-PDT, two mutant genes, *pheA*^{ev1} and *pheA*^{ev2}, were obtained. Recombinant strain PB12 with overexpressed *tktA, aroG*^{FBR} and *pheA*^{ev2} could produce 0.33 g L-phenylalanine per gram of glucose, corresponding to 60% of the maximum theoretical yield (0.55 g/g) (Baez-Viveros et al. 2004).

Other engineering targets for L-phenylalanine production

The dissolved oxygen (DO) in culture is considered an important factor for bacterial growth and L-phenylalanine productivity. To increase the DO level, co-expression of a hemoglobin gene from *Vitreoscilla* with *aroF* and *pheA*^{FBR} was performed in *E. coli* CICC10245, which led to 21.9% more biomass and 16.6% more L-phenylalanine production, while only approximately 5% more glucose was consumed (Wu et al. 2018).

Another strategy to relieve the feedback inhibition of key enzymes is reduction of intracellular accumulation of L-phenylalanine. In *E. coli*, the protein YddG is responsible for the export of aromatic amino acids. It was reported that *E. coli* strains overexpressing *yddG* accumulated less L-phenylalanine within the cell and exported L-phenylalanine threefold faster than the control (Doroshenko et al. 2007).

In brief, to construct an L-phenylalanine producer from wild *E. coli* strains, the following engineering targets can be considered: repressor proteins TyrR and TrpR; feedback regulation of *aroG, aroE* and *pheA*; the intracellular levels of PEP and E4P; the L-phenylalanine transport system YddG; and the global regulators CsrA, CsrB, Fis and YggG.

Conclusions and perspectives

With the disadvantages of being labor-intensive, time-consuming, and producing undefined mutations, classical mutagenesis and screening have been replaced by rational metabolic engineering strategies in the construction of L-phenylalanine producing strains (Dong et al. 2011). However, only a few recombinant strains achieve a relatively satisfactory titer of L-phenylalanine. Further improvements can be expected by investigating novel engineering targets. An insight into intracellular flux distribution during L-phenylalanine production was obtained by flux variability analysis. According to the results, malic enzyme knockout mutants were constructed and exhibited well process performances in L-phenylalanine production (Michael et al. 2014).

As synthetic biology develops, more and more tools can be applied to the generation of large diversified libraries and high-throughput screening processes. In 2016, a *mtr* promoter-based biosensor was constructed and employed in FACS high-throughput screening of an *E. coli* MG1655 mutant library (Mahr et al. 2016). The best mutant could produce 4.3-fold L-phenylalanine levels compared with the wild-type strain. This suggests that a combination strategy can be implemented to obtain an improved L-phenylalanine producer. Firstly, random mutation and high-throughput screening can be carried out to obtain a base strain with deregulated feedback inhibition and transcriptional repression, and then whole genome sequencing implemented to verify which genes are affected. Next, defined metabolic engineering strategies focusing on traditional targets can be performed. Third, unwanted mutations generated in the random mutagenesis can be removed. By combining random mutation, rational engineering, and high-throughput screening methods, a recombinant strain with higher L-phenylalanine production may be generated.

Acknowledgements This work was financially supported by the National Natural Science Foundation of China (31600066, 31870105, 31741007), the Shandong Provincial Natural Science Foundation (ZR2016CB20, ZR2016CL02), and the State Key Laboratory of Microbial Technology Open Projects Fund (M2016-10).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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