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SHORT COMMUNICATION

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Production of tyrosine through phenylalanine hydroxylation bypasses the intrinsic feedback inhibition in *Escherichia coli*

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Abstract Tyrosine is a proteinogenic aromatic amino acid that is often used as a supplement of food and animal feed, as well as a (bio-)synthetic precursor to various pharmaceutically or industrially important molecules. Extensive metabolic engineering efforts have been made towards the efficient and cost-effective microbial production of tyrosine. Conventional strategies usually focus on eliminating intrinsic feedback inhibition and redirecting carbon flux into the shikimate pathway. In this study, we found that continuous conversion of phenylalanine into tyrosine by the action of tetrahydromonapterin (MH4)-utilizing phenylalanine 4-hydroxylase (P4H) can bypass the feedback inhibition in *Escherichia coli*, leading to tyrosine accumulation in the cultures. First, expression of the P4H from *Xanthomonas campestris* in combination with an MH4 recycling system in wild-type *E. coli* allowed the strain to accumulate tyrosine at 262 mg/L. On this basis, enhanced expression of the key enzymes associated with the shikimate pathway and the MH4 biosynthetic pathway resulted in the elevation of tyrosine production up to 401 mg/L in shake flasks. This work demonstrated a novel approach to tyrosine production and verified the possibility to alleviate feedback inhibition by creating a phenylalanine sink.

Keywords Phenylalanine · Tyrosine · Phenylalanine 4-hydroxylase · Metabolic engineering

The authors J. Huang and Y. Lin contributed equally to this work.

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In recent years, the increasing demand for amino acids in the industries such as health food, animal feed, dietary supplements and cosmetics promoted the expansion of their market [3]. Tyrosine is an aromatic amino acid which serves not only as a protein building block, but also as the biosynthetic intermediate of the neurotransmitter dopamine [3]. Moreover, tyrosine is the precursor of *p*-hydroxystyrene and *p*-hydroxycinnamic acid, both of which can be used for the manufacture of novel materials, pharmaceuticals and nutraceuticals [11, 12]. Although tyrosine can be naturally biosynthesized through the shikimate pathway in *Escherichia coli*, the feedback inhibition mechanisms by 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (AroG) and chorismate mutase-prephenate dehydrogenase (TyrA) in combination with the TyrR-mediated transcriptional regulation usually prevent the accumulation of aromatic amino acids and strictly control them at very low concentrations in the cultures [12, 13]. To achieve the production of tyrosine by microorganisms, one of the commonly used rational strategies is to eliminate the intrinsic feedback inhibition by employing feedback inhibition-resistant (FBR) enzyme variants. For example, over-expression of the FBR variants of AroG and TyrA led to the deregulation of tyrosine biosynthesis and the high-level accumulation of tyrosine [6, 12]. Another study reported that the employment of an

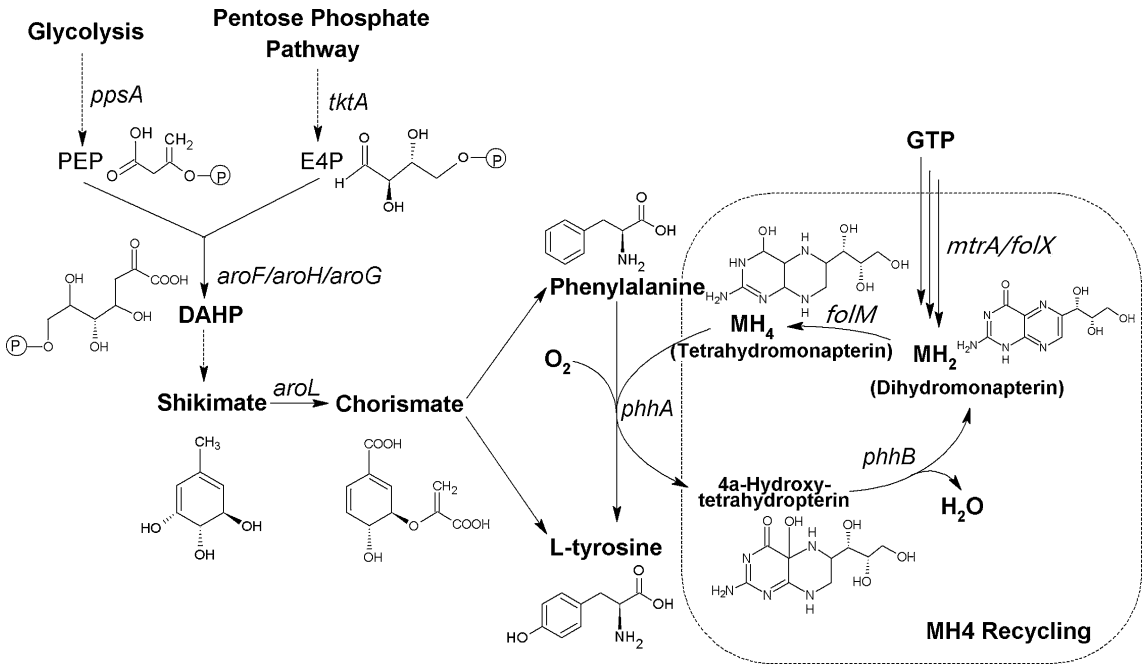

Fig. 1 A novel artificial pathway for the biosynthesis of tyrosine in *E. coli*. E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate. The genes encode: *ppsA,* phosphoenolpyruvate synthase; *tktA,* transketolase A; a*roG,* DAHP synthase; *aroL,* shikimate kinase; *phh*A, prokaryotic phenylalanine 4-hydroxylase; *phh*B, pterin 4a-carbinolamine dehydratase; *fol*M, dihydromonapterin reductase; *mtr*A, GTP cyclohydrolase I; *fol*X, dihydromonapterin reductase

FBR cyclohexadienyl dehydrogenase (TyrC) from *Zymomonas mobilis* and the chorismate mutase domain from native chorismate mutase-prephenate dehydratase (PheA-CM) improved the yield of tyrosine in *E. coli* [4]. Moreover, elevation of the availability of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP), the two precursors of the shikimate pathway, by over-expressing PpsA and TktA can further increase the tyrosine production [6, 12]. Recently, a study on modular engineering of tyrosine biosynthetic pathway involving a total of 11 genes allowed the yield to reach 80 % of the theoretical maximum yield [7].

In this work, we developed a new approach to produce tyrosine in *E. coli* by expressing a bacterial phenylalanine 4-hydroxylase (P4H) which can convert phenylalanine into tyrosine and bypass the intrinsic feedback inhibition. In fact, P4Hs belong to the class of pterin-dependent aromatic amino acid hydroxylases (AAAHs) which were widely identified and extensively studied in human and animals, because of their connections to phenylketonuria, Parkinson's disease, and neuropsychiatric disorders [15]. These AAAHs usually utilize tetrahydrobiopterin (BH4) as the coenzyme. Additionally, several P4Hs have also been identified in some bacteria, e.g., *Chromobacterium* [8]. Recent studies suggested that bacterial P4Hs may utilize tetrahydromonapterin (MH4) rather than BH4 as the native pterin coenzyme [14]. Interestingly, MH4 is a native metabolite in *E. coli*; however, its function has not been determined [14]. In our previous work, prokaryotic P4H activity was reconstituted in *E. coli* by introducing a MH4 recycling mechanism. Moreover, bioprospecting of P4Hs from different microorganisms allowed the identification of the most efficient homolog from *Xanthomonas campestris* [9]. On this basis, we hypothesized that if phenylalanine can be continuously depleted by the action of P4H, feedback inhibition effects may be alleviated, leading to the accumulation of tyrosine. To test this hypothesis, a wild-type *E. coli* strain BW25113 was transformed with pZE-XcABM to express the P4H from *X. campestris* in combination with the MH4 recycling enzymes (pterin 4a-carbinolamine dehydratase from *Pseudomonas aeruginosa* and dihydromonapterin reductase from *E. coli*) (Fig. 1). As we expected, the resulting strain (JH7) was able to accumulate 262 mg/L of tyrosine in the cultures after 48 h cultivation (all the plasmids and strains are listed in Table 1). The production followed the growth-dependent pattern, similar to that of other aromatic compounds (Fig. 2a). At 72 h, we observed the decrease in both the cell density and tyrosine titer, probably due to the tyrosine consumption during stationary and death phases. On the contrary, the control strain (BW25113 carrying the blank plasmid pZE12-luc) did not show detectable accumulation of tyrosine at any time, suggesting that continuous conversion of phenylalanine into tyrosine may have created a phenylalanine sink and therefore circumvented feedback inhibition.

Table 1 Plasmids and strains used in this study

	Description	Source
Plasmids		
pZE12-luc	$P_L$lacO1, *colE* ori, *luc*, Amp^r	[9]
pCS27	$P_L$lacO1, *P15A* ori, Kan^r	[9]
pZE-XcABM	pZE12-luc containing *phhA* from *X. campetris* ATCC 33913, *phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655	[9]
pCS-APTA	pCS27 containing *aroL*, *ppsA*, *tktA*, $aroG^{fbr}$ from *E. coli* MG1655	[10]
pCS-APTA-AX	pCS27 containing *aroL*, *ppsA*, *tktA*, $aroG^{fbr}$ and *folX* from *E. coli* MG1655; *mtrA* from *B. subtilis*	This study
pCS-AX	pCS27 containing *mtrA* from *B. subtilis* and *folX* from *E. coli* MG1655	This study
Strains		
JH7	*E. coli* BW25113 with plasmid pZE-XcABM	This study
JH8	*E. coli* BW25113 with plasmid pZE-XcABM and pCS-APTA-AX	This study
JH9	*E. coli* BW25113 with plasmid pZE-XcABM and pCS-AX	This study
JH10	*E. coli* BW25113 with plasmid pZE-XcABM and pCS-APTA	This study
JH18	*E. coli* BW25113 with plasmid pCS-AX	This study
JH19	*E. coli* BW25113 with plasmid pCS-APTA	This study

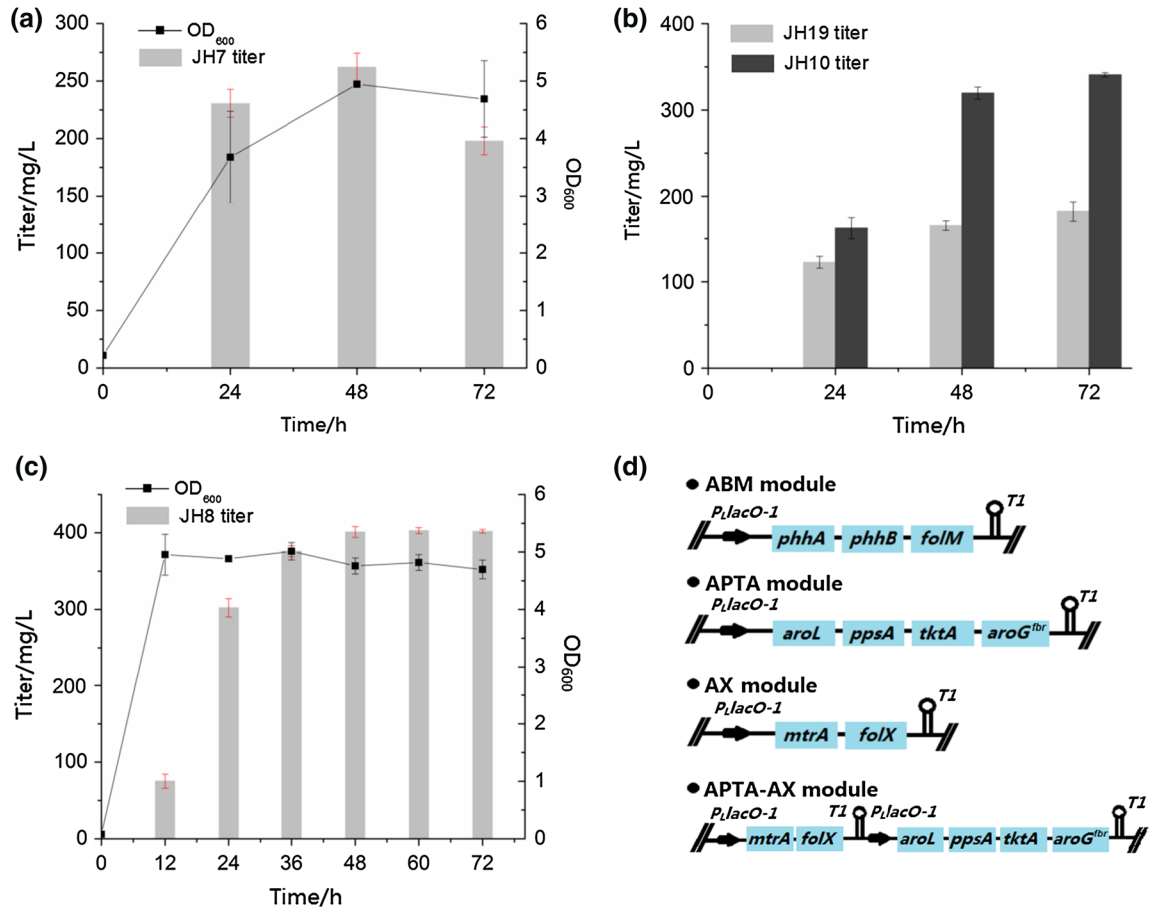

Fig. 2 Microbial production of tyrosine by the engineered *E. coli* strains and the modular structure of the constructed plasmids. **a** The production of tyrosine and cell concentrations by JH7; **b** the production of tyrosine and cell concentrations by JH10 and JH19; **c** the production of tyrosine and cell concentrations by JH8; **d** gene organization of the four modules: ABM, APTA, AX and APTA-AX, respectively

For strain JH7, we speculated that two possible bottlenecks might have constrained the tyrosine production. First, the low carbon flux in the native shikimate pathway may be a limiting factor for precursor supply. Second, the intracellular concentration of native MH4 coenzyme may not be sufficiently high to fully support the function of P4H. To examine first possible limitation, we employed a previously constructed chorismate-boosting plasmid pCS-APTA expressing the major rate-limiting enzymes associated with the shikimate pathway, including AroL, PpsA, TktA, and the feedback inhibition resistant mutant of AroG ($AroG^{fbr}$) [10]. When pCS-APTA and pZE-XcABM were co-transferred into *E. coli* BW25113, the resulting strain (JH10) was able to produce 320 mg/L tyrosine in 48 h (22 % increase compared with JH7), and finally to 340 mg/L in 72 h (Fig. 2b). However, to our surprise, the control strain JH19 (*E. coli* BW25113 carrying pCS-APTA only) can also accumulate tyrosine at 165 mg/L in 48 h and 181 mg/L in 72 h (Fig. 2b), indicating that this portion of tyrosine was produced through the native producing pathway due to the enhancement of carbon flux into the shikimate pathway rather than the hydroxylation of phenylalanine. We inferred that the boosted carbon flux into the shikimate pathway might have exceeded the feedback inhibition capacity of TyrA, resulting in tyrosine accumulation (Fig. 1).

In addition, we also evaluated the effect of MH4 availability on the tyrosine production. Although the function of MH4 is still unknown, its biosynthetic pathway has been proposed. GTP cyclohydrolase (encoded by *folE*) and dihydromonapterin reductase (encoded by *folX*) catalyze the two critical steps in the proposed pathway [2, 14]. Since it has been reported that the MtrA from *Bacillus subtilis* is more efficient than *E. coli* FolE [2], plasmid pCS-AX carrying *mtrA* from *B. subtilis* and *folX* from *E. coli* was constructed in order to enhance the supply of MH4. However, when pCS-AX and pZE-XcABM were simultaneously introduced into *E. coli* BW25113 as strain JH9, the tyrosine production was not improved compared with JH7. Strain JH9 can only produce around 80 mg/L in 48 h. We speculated that the effect of MH4 enrichment might not be significant unless the shikimate pathway is boosted at the same time. Therefore, a plasmid carrying both APTA and AX modules (pCS-APTA-AX) was constructed to boost the supply of MH4 and chorismate simultaneously. When pZE-XcABM and pCS-APTA-AX were co-transferred into *E. coli* (JH8), a further increase in tyrosine titer up to 401 mg/L was achieved (Fig. 2c), equivalent to a 53 % increase compared with JH7. Meanwhile, another control strain JH18 (*E. coli* carrying pCS-AX only) was tested. By the end of 72 h, no tyrosine accumulation was detected as expected. The gene organization of all the expression cassettes used in this study is shown in Fig. 2d.

During the past decades, the development of metabolic engineering allowed a number of valuable molecules to be manufactured [1, 5]. Although microbial production of tyrosine has been extensively studied, this work reported a novel strategy distinct from the conventional ones. We found that the action of P4H in combination with the MH4 recycling system can continuously convert phenylalanine into tyrosine. During this process, a phenylalanine sink was created and the carbon flux diverted to tyrosine production. As the result, 401 mg/L of tyrosine was obtained by the engineered *E. coli* strain in the shake flask experiments. This work demonstrated a novel approach for tyrosine production, verified the possibility to alleviate feedback inhibition by creating a sink of phenylalanine, and suggested a novel strategy to overcome the constraint of metabolic regulation, which is applicable for the microbial production of many other metabolites than tyrosine.

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