Pathway optimization by redesign of untranslated regions for L-tyrosine production in *Escherichia coli*

Seong Cheol Kim1,*, Byung Eun Min2,*, Hyun Gyu Hwang†, Sang Woo Seo2,† & Gyoo Yeol Jung1,2

L-tyrosine is a commercially important compound in the food, pharmaceutical, chemical, and cosmetic industries. Although several attempts have been made to improve L-tyrosine production, translation-level expression control and carbon flux rebalancing around phosphoenolpyruvate (PEP) node still remain to be achieved for optimizing the pathway. Here, we demonstrate pathway optimization by altering gene expression levels for L-tyrosine production in *Escherichia coli*. To optimize the L-tyrosine biosynthetic pathway, a synthetic constitutive promoter and a synthetic 5′-untranslated region (5′-UTR) were introduced for each gene of interest to allow for control at both transcription and translation levels. Carbon flux rebalancing was achieved by controlling the expression level of PEP synthetase using UTR Designer. The L-tyrosine productivity of the engineered *E. coli* strain was increased through pathway optimization resulting in 3.0 g/L of L-tyrosine titer, 0.0354 g L-tyrosine/h/g DCW of productivity, and 0.102 g L-tyrosine/g glucose yield. Thus, this work demonstrates that pathway optimization by 5′-UTR redesign is an effective strategy for the development of efficient L-tyrosine-producing bacteria.

L-Tyrosine is a commercially important compound, as it is widely used as a precursor of pharmaceutical drugs and other valuable chemicals such as flavonoids and alkaloids1-5. Traditionally, L-tyrosine was produced by extraction methods; however, the increasing demand for L-tyrosine is not adequately met by such methods due to low-yield production6,7. Microbial production is currently being spotlighted as a mean to efficiently produce L-tyrosine due to the feasibility of which has been enhanced in recent years by greater understanding of genetic information, the existence of various engineering tools, and simple scale-up processes1,3,8.

For several decades, many attempts have been made to improve L-tyrosine production using rational or combinatorial approaches. Such rational approaches, which include overexpression of pathway enzymes, introduction of feedback-resistant enzymes, and deregulation of regulators (e.g. TyrR protein) have allowed for improved L-tyrosine production by rationally increasing the carbon flux toward L-tyrosine production and removing the bottlenecks in the L-tyrosine biosynthetic pathway9-13. For example, Juminaga et al. overexpressed and modulated genes of the L-tyrosine biosynthetic pathway yielding 2.169 g/L of titer, 0.055 g/h/g DCW of productivity, and 0.438 g L-tyrosine/g glucose yield in flask
culture. Metabolic flux imbalance by indiscriminate deletion or overexpression of genes has, however, meant that these rational modifications to L-tyrosine production methods have not satisfactorily maximized L-tyrosine production. For this reason, precise control of gene expression levels or optimization of modular engineering may be required for metabolic pathway optimization. Combinatorial approaches for optimizing L-tyrosine production include the introduction of small RNA regulators, global transcriptional machinery engineering (gTME), and melanin-based high-throughput screening. Recently, Na et al. achieved 2.0 g/L of titre, 0.034 g/h/g DCW of productivity, and 0.1 g L-tyrosine/g glucose yield in flask culture using small RNA regulators. Such approaches are advantageous to the production of large quantities of L-tyrosine because they overcome the limitations of rational approaches caused by a lack of understanding of complicated metabolic networks or pathways, and using such approaches, strain improvement is easily achieved via library screening. Despite these benefits, an efficient screening method and further optimization of metabolic pathways after screening are required in conjunction with combinatorial approaches in order to further enhance the efficiency of these methods.

The optimization of metabolic pathways for L-tyrosine production requires precise control of the expression levels of key enzymes at critical nodes, such as the phosphoenolpyruvate (PEP) node, which is both a precursor of the aromatic amino acid synthetic pathway and an intermediate of the glycolysis pathway. If carbon flux at the phosphoenolpyruvate (PEP) node was increased toward the L-tyrosine biosynthetic pathway through genetic engineering, carbon flux to the tricarboxylic acid (TCA) cycle would be decreased. This flux decrease results in cell growth inhibition due to non-optimal overexpression, and the low cell density may in turn lead to low L-tyrosine production. Thus, metabolic pathways should be optimized by precise control of the carbon flux between the L-tyrosine biosynthetic pathway and the TCA cycle at the PEP node for maximum L-tyrosine production.

Other researchers have previously reported on the regulation of expression levels by altering promoter strength or plasmid copy number; however, expression levels in these cases were not accurately regulated at a translational level. The design tools such as RBS Calculator (v1.0) which calculate translation initiation rate have been developed to predict and to design 5′-UTR sequences for desired expression level. It has been thoroughly tested and employed for optimizing various biological systems. We have also developed UTR Designer after the RBS Calculator with conceptually similar free energy model, but differed in terms of investigating specific mRNA folding regions critical for ribosome accessibility by statistical approach using 456 5′-UTR variants in order to better capture the effect of structure changes in translation rate. The UTR Designer was also proven to be useful designing short 5′-UTR sequences (25 base-pairs) to meet specific translation rate. A recent study offered a molecular explanation showing how mRNA structures at upstream regions control translation rate and developed a new version of RBS Calculator (v2.0) capable of predicting and designing longer structured 5′-UTR sequences. Combined engineering approaches which include promoter strength alteration and synthetic 5′-UTR sequence design may allow for the expression levels of specific genes to be precisely controlled and thus for the effective optimization of metabolic pathways.

In this study, an Escherichia coli strain was generated to produce large quantities of L-tyrosine by the replacement of nascent genetic elements of biosynthesis enzymes with synthetic promoters and a 5′-UTR for maximum expression. Using UTR Designer, the carbon flux at the PEP node was further optimized for maximum productivity by testing the effect of various expression levels of PEP synthetase gene (ppsA) on L-tyrosine yield. Using this approach, an E. coli strain that produces recombinant L-tyrosine at 3 g/L, which is higher than that produced by the wild-type strain, was generated. This work demonstrates that pathway optimization by UTR redesign can be an effective strategy for increasing the yield in recombinant L-tyrosine production.

Results and Discussion

Rational engineering of L-tyrosine overproduction pathway. In E. coli, the elements of the L-tyrosine synthetic pathway are encoded by eleven genes. The first step of this pathway consists of the condensation of erythrose-4-phosphate (E4P) and PEP to yield 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) by DAHP synthase (AroG). DAHP is then converted to chorismate (CHA) via six enzymatic reactions catalyzed by 3-dehydroquinate synthase (AroB), 3-dehydroquinate dehydratase (AroD), shikimate dehydrogenase (AroE), shikimate kinase II (AroL), 5-enolpyruvylshikimate-3-phosphate synthase (AroA), and CHA synthase (AroC). Tyrosine is then finally synthesized from CHA by chorismate mutase, prephenate dehydrogenase (TyrA), and transaminase (TyrB). In the tyrosine synthetic pathway, AroG, AroB, and TyrA are negatively regulated by L-tyrosine, which is higher than that produced by the wild-type strain, was generated. This work demonstrates that pathway optimization by UTR redesign can be an effective strategy for increasing the yield in recombinant L-tyrosine production.
Figure 1. The L-tyrosine biosynthetic pathway engineering strategy. (a) Each gene was under the control of synthetic expression design that substitutes native promoter and 5′-UTR with synthetic constitutive promoter and designed 5′-UTR specific to target gene. (b) Dashed lines indicate feedback regulation, ‘X’ denotes deletion of the tyrR gene, and thick red arrows represent overexpression of genes in the L-tyrosine synthetic pathway. Abbreviations: Glc, glucose; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl-CoA; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ, 3-dehydroquininate; DHS, 3-dehydroshikimate; SHIK, shikimate; S3P, shikimate-3-phosphate; EPSP, 5-enolpyruvylshikimate-3-phosphate; CHA, chorismate; PPA, prephenate; HPP, 3-hydroxyphenylpyruvate; L-tyr, L-tyrosine; KPP, keto-phenylpyruvate; L-Phe, L-phenylalanine. (c) Comparison of L-tyrosine productivity of wild-type (W3110) and rationally engineered (SCK1) E. coli strain. The specific productivity of L-tyrosine was increased to 0.0014 g/h/g DCW in the SCK1 strain while the wild type strain did not produce L-tyrosine. The y-axis represents specific productivity of L-tyrosine (g/h/g DCW) in each strain. Each point and error bar indicates means and standard deviations between measurements from biological triplicate cultures.

from the original UTR sequence of the gene on a chromosome using Red recombination. In case of aroB where we could not achieve high translation rate with native coding sequence, we further optimized the coding sequence with same codon preference to achieve high translation rate (Supplementary Table S1). Thus, each gene in the pathway was expressed as a monocistronic transcript under the control of the synthetic constitutive promoter and the 5′-UTR using inherent terminator (Fig. 1b). The tyrR gene encoding the TyrR protein was additionally knocked out to remove transcriptional regulation of the L-tyrosine pathway. Feedback-resistant variants of AroGfbr [D146N] and TyrAfbr [M53I; A354V] were then substituted for the wild-type enzymes to deregulate feedback inhibition by L-tyrosine8. In other studies, over-expression was performed using inducible promoter such as T7 promoter on a plasmid and only several target genes were engineered among all genes involved in tyrosine biosynthetic pathway8,44–46. However, in this study, each gene was under the control of constitutive strong promoters and synthetic 5′-UTRs on the chromosome to eliminate problems related with marker and plasmid origin incompatibilities as well as to eliminate the need for antibiotics and inducers15.

The rationally engineered E. coli strain SCK1 was cultivated in M9 minimal medium to allow for the L-tyrosine productivity of the strain to be determined. The SCK1 strain exhibited an L-tyrosine productivity of 0.0014 g/h/g dry cell weight (DCW), while the wild-type W3110 E. coli strain produced negligible levels of L-tyrosine (Fig. 1c). This indicates that the rationally maximized expression levels of specific genes at both the transcription and translation levels increases L-tyrosine production as a result of linear flux amplification in the L-tyrosine biosynthetic pathway.

Optimization of L-tyrosine biosynthetic pathway by fine-tuning expression level of ppsA. Although L-tyrosine productivity of E. coli was enhanced by amplification of the L-tyrosine biosynthetic pathway, we hypothesized that maximum L-tyrosine production would not be achieved by this method because of the metabolic imbalance between cell growth and L-tyrosine production around the PEP node. PEP is a starting material in the L-tyrosine pathway and is also used as a precursor of pyruvate that enters into the TCA cycle (Fig. 2a). Accordingly, an excessive increase of flux toward the TCA cycle (Fig. 2a). Accordingly, an excessive increase of flux toward the TCA cycle. Optimization of flux distribution at the PEP node must therefore be optimized in order for maximum L-tyrosine productivity to be achieved.

To optimize flux distribution at the PEP node, the ppsA gene (encoding PEP synthetase) was selected as the regulation target because it has been shown that the overexpression of ppsA increases tyrosine production by E. coli8,11,47. The growth of a ppsA-overexpressing strain could, however, be inhibited compared to that of a wild type strain because of decreased metabolic flux to the TCA cycle at PEP node, which causes decrease of L-tyrosine productivity despite of increase of flux toward L-tyrosine synthesis. Indeed, this was found to be the case when the growth rate of a ppsA-overexpressing strain was measured in this study (Supplementary Table S2). It means that overexpression or knock out of PpsA is not enough to optimize flux distribution at PEP node for maximum L-tyrosine production. For this reason,
optimization of carbon flux at the PEP node through precise control of the expression level of *ppsA* in both transcription and translation levels is essential for obtaining maximum L-tyrosine production. To precisely regulate the expression levels of genes, it is often not enough to control expression at a transcriptional level alone, as this does not control expression at a translational level especially when additional regulatory elements such as riboswitches are present. Furthermore, optimal catalytic activity, in many cases, cannot be easily achieved by modulating the promoter strength only. Accordingly, for maximum tyrosine production to be achieved, the expression level needs to be controlled at a translational level as well as at a transcriptional level.

Figure 2. Carbon flux redistribution at the PEP node by fine-tuning of *ppsA* expression. (a) Pathway optimization for fine-tuning the expression levels of the *ppsA* gene using UTR Designer. J23100 indicates a strong constitutive promoter from the Registry of Standard Biological Parts (BBa J23100; http://partsregistry.org). (b) Comparisons of predicted expression levels from UTR Designer and specific enzyme activities of *ppsA* variants. (c) The specific L-tyrosine productivity of each *ppsA* variant after 24 h cultivation in M9 minimal medium. Each point and error bar indicates means and standard deviations between measurements from biological triplicate cultures.

Optimization of culture condition for L-tyrosine overproduction. Although metabolic flux was optimized by controlling the expression level of *ppsA*, maximal L-tyrosine production in *E. coli* also requires optimization of the culture conditions. Cell growth and carbon uptake are inhibited in media with a low pH (resulting from the accumulation of L-tyrosine). For this reasons, the high L-tyrosine-producing *E. coli* strain engineered in this study (SCK5) produced only up to 0.3265 g/L L-tyrosine in batch cultures grown in M9 minimal medium. With pH control, L-tyrosine production was increased to 0.5155 g/L (Supplementary Table S2). We further tested the SCK5 *E. coli* strain as well as the other engineered *ppsA* variants in complex medium, after which L-tyrosine production was measured. As in the case of M9 minimal medium, L-tyrosine productivity among the *ppsA* variants grown in complex medium was highest in the SCK5 strain (Supplementary Fig. S2) and the highest titer was 1 g/L. After
culturing in complex medium with pH adjustment, the L-tyrosine titer and productivity increased to 3 g/L, 0.0354 g/h/g DCW and 0.102 g L-tyrosine/g glucose yield from the SCK5 strain (Fig. 3b) compared with negligible L-tyrosine production by the wild type W3110 E. coli strain (Fig. 3a). However, glucose uptake by the SCK5 strain was reduced after ~48 h in culture when L-tyrosine started to be precipitated. We are not sure why glucose uptake is inhibited after ~48 h. One possibility is that tyrosine precipitation may be accompanied by cell aggregation48 and reduce the performance of the batch culture. Collectively, our findings indicate that L-tyrosine was produced to capacity in the engineered SCK5 E. coli strain, and that this capacity is limited by low pH as a result of L-tyrosine accumulation.

In this study, we demonstrate that flux redistribution around the PEP node, the junction between flux to the TCA cycle and flux to the L-tyrosine synthetic pathway, is one of the most important factors to consider in engineered strain improvement for maximum L-tyrosine production. Pathway optimization using UTR Designer to achieve precise quantification of expression was also shown to contribute greatly to optimal L-tyrosine productivity of E. coli. This engineered E. coli strain for L-tyrosine production could be further applied to produce flavonoids by introducing several pathway enzymes in the flavonoid synthetic pathway49.

One of the major challenges associated with metabolic engineering is achieving precise control and optimized expression levels of genes to yield large amounts of metabolites of interest. Our findings show that UTR Designer is a useful tool by which fine control of protein expression can be gained for pathway optimization. Our previous work on the optimization of redox balance, genetic circuit construction, and control of heterologous expression32,36 also demonstrates that fine control of carbon flux redistribution can be achieved using UTR Designer.

Recently, we also developed UTR Library Designer with same biophysical model to generate UTR library covering large combinatorial space of expression level and demonstrated its usefulness for pathway engineering through lysine and hydrogen production37. We believe that both UTR Designer and UTR Library Designer can thus be used as efficient tools by which pathways can be optimized for the production of various chemicals, fuels, and recombinant proteins.

Figure 3. Fermentation profiles of E. coli strains cultivated in complex medium. Data for the (a) wild-type (W3110; open symbols) and (b) SCK5 (closed symbols) E. coli strains are shown. pH adjustments were made at 6-h intervals. The left y-offset and right y-axis represent glucose and L-tyrosine concentrations (g/L), respectively. The left y-axis represents OD_{600} and the x-axis represents culture time (h). Each point and error bar indicates means and standard deviations between measurements from biological triplicate cultures. Symbols: circle, OD_{600}; triangle, glucose; rectangle, L-tyrosine.
Methods

Bacterial strains, plasmids, and primers. The E. coli bacterial strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Table S4 and S5. Phusion polymerase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA) and the oligonucleotides used were synthesized by Genotech (Daejeon, South Korea) and Bioneer (Daejeon, South Korea). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

To construct the pTyra vectors, the tyrA gene was amplified by polymerase chain reaction (PCR) using V-tyra-F/R primers and was then inserted into a pMD20-T vector. The tyrA gene was constructed by site-directed mutagenesis using the M-tyra-F1/R1 and -F2/R2 primer pairs. A tyrA-FRT-Kan-FRT fragment was amplified by overlap PCR by using the P-tyra-F/R and P-FKF-F/R primers and the pGFKF2 and pTyra vectors, after which it was inserted into a pMD20-T vector. To construct the pAroG vectors, the aroG fragment amplified using the V-aroG-F/R and FRT-KanR-FRT primer set was inserted into a pMD20-T vector, after which feedback inhibition of AroG was stopped by site-directed mutagenesis using the M-aroG-F/R primer pair. The aroG vector was subsequently inserted into the pGFKF2 vector using the SacI and KpnI restriction sites.

All chromosomal manipulations were carried out using the Red recombination system with the pKD46 and pCP20 plasmids as described in previous studies. To construct the SCK1 E. coli strain, tyrR was deleted by insertion of the FRT-Kan-FRT fragment amplified with the D-tyrR-F/D-tyrR-R primer pair and by replacement of the native promoter and 5′-UTR of tktA, aroA, aroB, aroC, aroD, aroE, aroL, and tyrB with a BBA_J23100 promoter and a redesigned 5′-UTR, respectively, by using an FRT-Kan-FRT fragment amplified by each corresponding forward/reverse primer pair. The native aroG and tyrA genes were then replaced by aroG and tyrA with a BBA_J23100 promoter and a redesigned 5′-UTR by using an FRT-Kan-FRT fragment amplified by the O-aroG-F/O-aroG-R and O-tyrA-F/O-tyrA-R primer pairs, respectively. To verify the integrity of the genomic modifications, we performed sequencing after recombination.

To modulate ppsA expression, five different 5′-UTR sequences were designed using UTR Designer (http://sbi.postech.ac.kr/utr_designer). To construct the SCK2–SCK6 strains, the native promoter and 5′-UTR of ppsA was replaced with a BBA_J23100 promoter and a rationally redesigned 5′-UTR sequence (ppsA (v1–5)) using an FRT-Kan-FRT fragment amplified by the O-ppsA-F/O-ppsA-R primer pair.

Cell cultures and growth measurement. The wild type and engineered E. coli strains were cultivated in either M9 minimal medium (N salt solutions; Sigma-Aldrich, St. Louis, MO; 5 mM MgSO4, 0.1 mM CaCl2, 5 g/L glucose, and appropriate antibiotics) or complex medium (6.75 g KH2PO4, 2 g NH4Cl, 10 mL trace metal solution per liter (10 g FeSO4.7H2O, 2.2 g ZnSO4·7H2O, 0.58 g MnSO4·4H2O, 1 g CuSO4·5H2O, 0.1 g (NH4)6Mo7O24·4H2O, 0.2 g (NH4)2HPO4, 0.85 g citric acid, 3 g yeast extract, 40 g glucose, 10 mL trace metal solution per liter), 0.1 mM CaCl2, 5 or 40 g/L glucose, and appropriate antibiotics) or complex medium (6.75 g KH2PO4, 2 g NH4Cl, 10 mL trace metal solution per liter (10 g FeSO4.7H2O, 2.2 g ZnSO4·7H2O, 0.58 g MnSO4·4H2O, 1 g CuSO4·5H2O, 0.1 g (NH4)6Mo7O24·4H2O, 0.2 g (NH4)2HPO4, 0.85 g citric acid, 3 g yeast extract, 40 g glucose, 10 mL trace metal solution per liter) supplemented with 0.1 mM CaCl2, 5 or 40 g/L glucose, and appropriate antibiotics).

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Cells were cultured at 37 °C with shaking (200 rpm) and cell densities of E. coli cultures were measured at a wavelength of 600 nm (OD600) using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan).

To test PpsA activity, cells with different expression levels of ppsA were harvested in mid-log phase and then washed with cold phosphate-buffered saline (PBS). The resulting cell pellets were resuspended and lysed with Bug Buster Master Mix (EMD Bioscience, San Diego, CA, USA) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions. Total protein concentration in cell lysates was determined using the Bradford assay-based Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Total protein concentration in cell lysates was determined using the Bradford assay-based Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. PpsA activity was measured according to a previously described method. The assay mixture contained 4 μM pyruvate, 10 μM ATP, 10 μM MgCl2, and 100 μM Tris-HCl (pH 8.0). Assay reactions were initiated by the addition of 40 μL crude lysate into the assay mixture, after which reaction mixtures were incubated at 30 °C for 5 min. Reactions were terminated by the addition of 20 μL of the reaction mixture into a mixture containing 0.066 mL 0.1% aqueous solution of 2,4-dinitrophenylhydrazine and 0.18 mL H2O followed by incubation at 30 °C for 10 min. The resulting mixture was further incubated with 0.334 mL 10% (w/v) NaOH at 30 °C for 10 min. PpsA activity was determined by measuring the depletion of pyruvate at 445 nm using a VICTOR 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). PpsA activity was normalized to total protein content in cell lysates to obtain specific PpsA activity (U/mg).

L-Tyrosine production and detection of metabolites. The concentration of glucose consumed by the E. coli strains was determined by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) at a flow rate of 0.6 mL/min at 65 °C.
using 5 mM H₂SO₄ as the mobile phase. The glucose concentration was monitored using a Shodex RI-101 detector (Shodex, Klokkerfeldet, Denmark). L-tyrosine concentrations in culture broth were determined using a pre-column o-phthalaldehyde derivatization method coupled with reversed-phase liquid column chromatography (Acclaim 120 C18; Dionex, Sunnyvale, CA, USA) using an UltiMate 3000 analytical HPLC system (Dionex). Derivatized L-tyrosine was eluted at a flow rate of 1.5 mL/min with a gradient of an acetonitrile, methanol, and water solution (45:45:10 % [v/v]) and 50 mM sodium acetate buffer (pH 6.5). An ultraviolet-visible (UV-Vis) diode array detector was used to detect derivatized L-tyrosine at 338 nm.

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Author Contributions
S.C.K. and B.E.M. designed and performed the experiments, analyzed the data and wrote the manuscript. H.G.H. performed the experiments. S.W.S. and G.Y.J. designed experiment, analyzed the data and wrote the manuscript.

Additional Information
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