Genome engineering *Escherichia coli* for L-DOPA overproduction from glucose

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Genome engineering has become a powerful tool for creating useful strains in research and industry. In this study, we applied singleplex and multiplex genome engineering approaches to construct an *E. coli* strain for the production of L-DOPA from glucose. We first used the singleplex genome engineering approach to create an L-DOPA-producing strain, *E. coli* DOPA-1, by deleting transcriptional regulators (tyrosine repressor *tyrR* and carbon storage regulator A *csrA*), altering glucose transport from the phosphotransferase system (PTS) to ATP-dependent uptake and the phosphorylation system overexpressing galactose permease gene (*galP*) and glucokinase gene (*glk*), knocking out glucose-6-phosphate dehydrogenase gene (*zwf*) and prephenate dehydratase and its leader peptide genes (*pheLA*) and integrating the fusion protein chimera of the downstream pathway of chorismate. Then, multiplex automated genome engineering (MAGE) based on 23 targets was used to further improve L-DOPA production. The resulting strain, *E. coli* DOPA-30N, produced 8.67 g/L of L-DOPA in 60 h in a 5 L fed-batch fermentation. This titer is the highest achieved in metabolically engineered *E. coli* having PHAH activity from glucose.

L-DOPA (3,4-dihydroxyphenyl-L-alanine) is an aromatic compound that is derived from L-tyrosine (Fig. 1). L-DOPA has been used to treat Parkinson's disease, which is caused by deficiency of the neurotransmitter dopamine. Since Monsanto developed a commercial process for L-DOPA synthesis by asymmetric hydrogenation, L-DOPA has been produced by asymmetric, enzymatic and microbial synthesis. However, the asymmetric synthesis has major disadvantages such as a poor conversion rate and low enantioselectivity. Thus, biotechnology approaches using microorganisms or enzymes have been explored as alternatives. Microorganisms with tyrosinase, tyrosine phenol-lase (*Tpl*) and *p*-hydroxyphenylacetate 3-hydroxylase (PHAH) activity have been used to produce L-DOPA. However, the microbial fermentations require tyrosine or catechol/pyruvate as substrates, leading to high production costs. Nakagawa et al. constructed an *E. coli* expressing *Streptomyces castaneoglobisporus* tyrosinase gene, which can produce 293 mg/L of L-DOPA from glucose. Muñoz et al. reported an engineered *E. coli* having PHAH activity, which can produce 1.5 g/L of L-DOPA from glucose. However, the titer of L-DOPA in the engineered *E. coli* is lower than that of the microbial fermentation from tyrosine or catechol/ pyruvate. Thus, further work must be carried out to increase L-DOPA production from glucose in *E. coli*.

Genome engineering is a powerful technique to manipulate entire genomes for obtaining desired phenotypes. The singleplex and multiplex genome engineering approaches have been successfully used for strain development. Thus, we first focus on increasing the supply of the precursor, tyrosine, by using a singleplex genome engineering approach. We then apply multiplex automated genome engineering (MAGE) to develop an *E. coli* strain overproducing L-DOPA.

Results and Discussion

*E. coli* W *hpaBC* has been successfully introduced into *E. coli* to produce L-DOPA from glucose. Figure 1 shows that tyrosine availability should first be increased to improve L-DOPA production from glucose. Successful strategies for engineering *E. coli* strains that can overproduce tyrosine include: (i) improving the carbon flow through the biosynthetic pathway of interest by removing transcriptional and allosteric regulation; (ii) increasing the availability of the direct precursors phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P); (iii) preventing loss of carbon to competing pathways; (iv) enhancing the first enzymatic reaction of the shikimate pathway.

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to yield 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP); (v) and identifying and relieving rate-limiting enzymatic reactions. Thus, we first used singleplex genome engineering to increase the supply of tyrosine.

Removal of transcriptional regulators. Tyrosine repressor (TyrR) is a transcriptional dual regulator that represses the transcription of several genes encoding enzymes involved in aromatic acid biosynthesis. Carbon storage regulator A (CsrA) is a regulator of carbohydrate metabolism. CsrA regulates the levels of three enzymes that participate directly in phosphoenolpyruvate (PEP) metabolism. It activates pyruvate kinase (PykF) and represses PEP carboxykinase (PckA) and PEP synthase (PpsA) in E. coli. It has been reported that the inactivation of tyrR and csrA improves aromatic compound production. Thus, we first deleted tyrR and csrA to obtain E. coli AROM-1 (Fig. 1), resulting in a slight increase in L-DOPA production from 138.7 ± 4.9 mg/L to 148.3 ± 11.7 (Table 1). Munoz et al. also reported that knocking out tyrR enhanced L-DOPA production in E. coli.

Increasing the availabilities of the precursor PEP by altering glucose transport. Increasing PEP availability is a common strategy for engineering E. coli strains for the overproduction of aromatic compounds. In E. coli, glucose is mainly transported and phosphorylated by the phosphotransferase system (PTS). Under standard growth conditions, 50% of the glycolytic intermediate PEP resulting from the catabolism of glucose is used as the phosphate donor for phosphorylation and translocation by the PTS. The properties of the PTS limit the production of compounds that have PEP as a precursor. Carmona et al. suggested that inactivation of the PTS is the primary strategy for engineering E. coli to overproduce aromatic metabolites. Thus, we deleted the PTS (ptsHIcrr) to further improve L-DOPA production. The inactivation of the PTS increased the L-DOPA titer to 176 ± 3.6 mg/L (Table 1). Non-PEP-mediated glucose transport and phosphorylation systems have successfully been used for the replacement of the PTS to increase PEP availability. Thus, we integrated the galP and glk genes into E. coli to increase PEP availability.

Figure 1. Schematic representation of metabolic pathways involved in L-DOPA biosynthesis and regulation in E. coli. The strategies for constructing a genetically defined strain for L-DOPA overproduction are also shown. The x’s indicate that the genes are deleted. Encircled + or − symbols indicate inhibition or activation, respectively. The genes targeted by MAGE are underlined. PTS: phosphotransferase system; TCA: tricarboxylic acid cycle; G6P: glucose 6-phosphate; 6PBL: 6-phospho-D-glucono-1,5-lactone; Ribu5P: D-ribulose 5-phosphate; X5P: D-xylulose 5-phosphate; R5P: D-ribose 5-phosphate; S7P: D-sedoheptulose 7-phosphate; F6P: fructose 6-phosphate; GAP: glyceraldehyde 3-phosphate; E4P: D-erythrose 4-phosphate; PEP: phosphoenolpyruvate; Pyr: pyruvate; Ac-CoA: acetyl-CoA; OAA: oxaloacetate; CIT: citrate; DAHP: 3-Deoxy-arabino-heptulosonate 7-phosphate; DHQ: Dihydroquinic acid; DHSH: Dihydroshikimate; SHK: shikimate; S3P: shikimate 3-phosphate; EPSP: 5-enolpyruvyl-shikimate 3-phosphate; CHA: Chorismate; Phe: phenylalanine; HPPH: 4-hydroxyphenylpyruvate; galP: galactose permease gene; glk: glucokinase gene; zwf: glucose-6-phosphate dehydrogenase gene; tktA: transketolase I gene; pckA: PEP carboxykinase gene; ppc: PEP carboxylase gene; ppsA: PEP synthase gene; pykFA: pyruvate kinase I/II gene; aroE, aroG and aroH: DAHP synthase gene; aroB: DHQ synthase gene; aroD: DHQ dehydratase; aroE/gyd: shikimate/quinate dehydrogenase gene; aroA: 3-phosphoshikimate-1-carboxyvinyltransferase gene; aroC: CHA synthase; tyrA: CHA mutase/prephenate dehydrogenase gene; tyrB: tyrosine aminotransferase gene; thrED: anthranilate synthetase gene; pheA: prephenate dehydrogenase gene; hpacBC: E. coli W p-hydroxyphenylacetate 3-hydroxylase gene. nadK: NAD kinase gene; rpoD: sigma 70 factor gene; rpoA: α subunit of RNA polymerase gene; csrA: carbon storage regulator A; tyrR: tyrosine repressor.
under the control of the P37 promoter into the E. coli knockout strain AROM-2 to obtain E. coli AROM-3. The titer of L-DOPA and growth of E. coli AROM-3 harboring pQE30-hpaBC showed no significant difference compared to E. coli AROM-2 (p < 0.05, Table 1).

**Knockout of Glucose-6-phosphate dehydrogenase gene.** Glucose-6-phosphate dehydrogenase (encoded by zwf) catalyzes the oxidation of glucose-6-phosphate to gluconate-6-phosphate. It has been reported that knocking out zwf drives more carbon flux into the Embden-Meyerhof-Parnas (EMP) pathway and tricarboxylic acid (TCA) cycle. They also found that the zwf mutant is able to synthesize pentose phosphate (PP) pathway-derived compounds independently from the oxidative part of the PP pathway by directing its carbon flow from the EMP pathway directly into the non-oxidative part of the PP pathway. Thus, we disrupted zwf in E. coli AROM-3 to obtain E. coli AROM-4. E. coli AROM-4 (pQE30-hpaBC) produced L-DOPA at 205.3 ± 2.5 mg/L, which was greater than E. coli AROM-3 (pQE30-hpaBC) (Table 1). The stoichiometric analysis demonstrated that the yield of the aromatic compound DAHP approaches the theoretical maximum when E4P is provided by the nonoxidative part of the PP pathway and pyruvate is recycled to PEP by PpsA. The improvement of L-DOPA titer after zwf deletion was experimentally demonstrated for the first time.

**Removal of competing pathway.** Prephenate can be converted into either tyrosine or phenylalanine. To eliminate the loss of prephenate to the competing reaction (phenylalaninebiosynthesis), we deleted prephenate dehydratase and its leader peptide genes (pheLA) in E. coli AROM-4 to obtain E. coli TYR-1. The pheLA deletion slightly increased the L-DOPA titer to 209.2 ± 0.9 mg/L (Table 1). Some other groups have previously reported that the pheLA deletion increases L-tyrosine production.

**Coordinating expression of the downstream pathway of chorismate.** The bifunctional enzyme Chorismate (CHA) mutase/prephenate dehydrogenase, TyrA, catalyzes the first and second step of L-tyrosine biosynthesis (Fig. 1). TyrA catalyzes both reactions in separate domains of the protein, and the CHA mutase/prephenate dehydrogenase is feedback-inhibited by L-tyrosine (up to 95% inhibition of the prephenate dehydrogenase and 45% of the CHA mutase activity). Feedback-resistant mutants of the TyrA E. coli enzyme have been used for L-tyrosine overproduction. Thus, TyrA [M53I/A354V] was used to deregulate the feedback inhibition by tyrosine. Substrate channeling is a powerful tool for balancing the expression of genes. It can increase the catalytic efficiency of the sequential reactions in a biosynthetic pathway. To increase the rate of CHA conversion to L-DOPA, we first fused the tyrA, tyrB and hpaBC genes with a (G4S)3 linker, then integrated the fusion protein chimera under the control of the T7P37 promoter into the chromosome of E. coli TYR-1 to obtain E. coli DOPA-1. E. coli DOPA-1 produced 307.4 ± 3.7 mg/L of L-DOPA.

**Multiplex automated genome engineering.** MAGE is an efficient and rapid tool for the genome engineering of bacterial strains. We selected aroF, aroG, aroB, aroD, ydiB, aroE, ppsA, iktA, nadK, aroL, aroK, aroA, tyrA, tyrB and tyrA [M53I/A354V] (M53I/A354V) as target sites to tune translation by ribosome binding site (RBS) replacement (Fig. 1). The RBS sequences were designed to be DRRRRRRRDDDD (D = A, G, T; R = A, G) with a total pool complexity of 3.5 × 10^4 (3^5 × 2^15). Six genes (aroF[p314]) and ppaD[p5218] and rpoA[p2578] were targeted for amino acid mutations in their open reading frames (ORF). The introduction mutations in aroF, aroG and tyrA were used to remove product feedback inhibition. The rpoD and rpoA mutants have been successfully used to increase tyrosine production. Two genes (trpD and trpE) were targeted for inactivation by introducing a revertible premature stop codon into each ORF. To increase the MAGE allelic replacement frequency, the methyl-directed mismatch repair protein gene (mutS) of E. coli DOPA-1 was first deleted to obtain E. coli DOPA-2. E. coli DOPA-2 (pSIM6) was used as the starting strain for MAGE. After 30 cycles of MAGE, 1.3 × 10^10 genetic variants (4.3 × 10^8 bp variations per cycle for 30 MAGE cycles) were generated. According to an allelic replacement efficiency calculation, 30 MAGE cycles generate 2.3% of genomes with at least 3 out of 23 targeted loci and 6.1 × 10^{-12} of genomes with all 23 targeted loci. One hundred clones from the 5th, 10th, 15th, 20th and 25th cycle and 1000 clones from the 30th cycle were screened in deep-well microplate culture. L-DOPA can be easily oxidized to dopachrome and then polymerized nonenzymatically to form the black pigment.

### Table 1. L-DOPA production in different E. coli strains.

*Experiments were conducted in triplicates, and measurements are presented with their means and s.d.

| Strain                | Genetic modification of the host strain | OD_{600} | Tyrosine (mg/L) | L-DOPA (mg/L) |
|-----------------------|----------------------------------------|----------|----------------|--------------|
| E. coli BW25113 (pQE-hpaBC) |                                      | 5.55 ± 0.08 | 292.5 ± 5.2 | 138.7 ± 4.9 |
| E. coli AROM-1 (pQE-hpaBC) |                                      | 6.13 ± 0.06 | 263.5 ± 60.8 | 148.3 ± 11.7 |
| E. coli AROM-2 (pQE-hpaBC) |                                      | 4.57 ± 0.04 | 366.2 ± 11.8 | 176.0 ± 3.6 |
| E. coli AROM-3 (pQE-hpaBC) |                                      | 4.56 ± 0.07 | 304.0 ± 25.5 | 173.9 ± 11.7 |
| E. coli AROM-4 (pQE-hpaBC) |                                      | 4.50 ± 0.03 | 256.9 ± 7.8  | 205.3 ± 2.5 |
| E. coli TYR-1 (pQE-hpaBC) |                                      | 4.59 ± 0.05 | 256.6 ± 4.8  | 209.2 ± 0.9 |
| E. coli DOPA-1         |                                      | 4.24 ± 0.09 | 241.3 ± 6.2  | 307.4 ± 3.7 |
As shown in Fig. 2, the strain produced 8.67 g/L of L-DOPA at 60 h. The OD600 of the culture reached 110. The strain produced more tyrosine and produced 614.3 mg/L of L-DOPA.

Table 2. L-DOPA production in MAGE strain harboring pSIM6*. *Experiments were conducted in triplicates, and measurements are presented with their means and s.d.

| Strain          | OD_{600} | Tyrosine (mg/L) | L-DOPA (mg/L) | Total L-DOPA plus tyrosine (mg/L) |
|-----------------|----------|-----------------|---------------|-----------------------------------|
| E. coli DOPA-2  | 4.87 ± 0.06 | 462.7 ± 7.0    | 287.7 ± 3.7  | 524.0                             |
| E. coli DOPA-30 (pQE30) | 7.10 ± 0.15 | 546.8 ± 10.4   | 490.3 ± 8.3  |                                    |
| E. coli DOPA-30 (pQE30-phaBCN) | 5.08 ± 0.02 | 650.3 ± 23.6   | 0.0 ± 0.0    |                                    |
| E. coli DOPA-30N | 4.87 ± 0.06 | 0.0 ± 0.0      | 614.3 ± 19.1 |                                    |

Table 3. Effect of overexpression of hpaBC on L-DOPA production*. *Experiments were conducted in triplicates, and measurements are presented with their means and s.d.

Fed-batch fermentation. Fed-batch fermentation of E. coli DOPA-30N was performed in a 5 L bioreactor. As shown in Fig. 2, the strain produced 8.67 g/L of L-DOPA at 60 h. The OD_{600} of the culture reached 110. The L-DOPA productivity was 144.5 mg/L/h. The L-DOPA yield from glucose was 62.7 mg/g. The titer and yield were 34% higher than that of the starting strain E. coli DOPA-30 (pQE30) 4.87 ± 0.06. Comparing the sequence of the hpaC in pQE30-2hpaBC with that reported by Munoz et al., the 5'-UTR sequence of the hpaC has been changed. The change may lead to the imbalanced expression between the hpaB and hpaC. Is this change resulted in the accumulation of L-tyrosine in the engineered strain? We re-amplified the hpaBC operon with the native 5'-UTR sequence of the hpaC to obtain pQE30-30N. As shown in Table 3, E. coli DOPA-30 harboring pQE30-30N cannot produce L-tyrosine. Thus, the hpaBC in E. coli DOPA-30 was replaced with the hpaBCN, which can produce L-DOPA more efficiently.

Comparison with other microorganisms. L-DOPA production by microorganisms is summarized in Table 4. The L-DOPA titer obtained in this study is higher by a factor of 3.7 than the highest level previously reported using metabolically engineered E. coli strain that have PHAH activity from glucose. The value is also higher than that obtained in microorganisms that have tyrosinase activity from tyrosine. However, the value in this study is lower than that obtained in some microorganisms with Tpl activity from catechol and pyruvate. It indicates that further works should be carried out for improving L-DOPA production.
Although the L-DOPA titer of our engineered \textit{E. coli} is considerably higher than that previously reported, all of the tyrosine was converted to L-DOPA only after 40 h (Fig. 2). It indicates that PHAH is the rate-limited step for L-DOPA biosynthesis in this strain. The catalytic efficiency of the PHAH encoded by \textit{hpaBC} should be improved. Directed evolution may be used to increase its catalytic efficiency. Because only three targets were found in the MAGE strain (Supplementary Table 1), we can apply other strategies to further enhance the availability of tyrosine, such as upregulating \textit{tktA}, increasing NADPH availability and upregulating \textit{hpaBC}.

In conclusion, we first constructed an L-DOPA-producing \textit{E. coli} strain, DOPA-1, using a singleplex genome engineering approach based on knockouts of genes and integration of the \textit{tyraFbr}, \textit{tyrB} and \textit{hpaBC} fusion protein chimera. MAGE based on 23 targets was then used to further improve L-DOPA production, which yielded the strain \textit{E. coli} DOPA-30N. \textit{E. coli} DOPA-30N produced 8.67 g/L of L-DOPA in 60 h in a 5L fed-batch fermentation. This titer is the highest reported in metabolically engineered \textit{E. coli} that has PHAH activity from glucose. This strain, \textit{E. coli} DOPA-30N, can serve as a base strain for developing more efficient strains capable of producing L-DOPA or other aromatic compounds. The rapid and efficient markerless deletion approach using the IPTG-inducible \textit{ccdB} as a counter-selectable marker will be generally useful for gene knockout of \textit{E. coli}.

### Methods

#### Strains, plasmids and primers.

The strains and plasmids used in this study are listed in Table 5. The primers are listed in Supplementary Table 2.

#### Genetic methods.

The genes \textit{hpaB} and \textit{hpaC} were amplified from \textit{E. coli} W using the primers \textit{hpaB-F/hpaB-R} and \textit{hpaC-F/hpaC-R}, respectively. The \textit{hpaB} fragment was cloned into the SacI/KpnI sites of pQE30 to obtain pQE30-hpaB. The \textit{hpaC} fragment was cloned into the KpnI/SalI sites of pQE30-hpaB to obtain pQE30-hpaBC. The \textit{hpaBC} genes were also amplified from pQE30-hpaBC using the primers hpabc-F/hpabc-R and then cloned into the SalI/HindIII sites of pQE30-hpaBC to obtain pQE30-2hpabc. The \textit{hpabc} operon was amplified from \textit{E. coli} W using the primers hpabc-F/hpabc-R and then cloned into the SacI/SalI to obtain pQE30-hpabcN.
**Strains/Plasmids**

| Strain | Reference |
|--------|-----------|
| E. coli BW25113 | lacPrrnR323, ΔlacZΔM15, hsdR514, ΔaraBAD, ΔrhaBAD, AαG129 | 42 |
| E. coli AROM-1 | E. coli BW25113, ΔtyrR, ΔcrA | This study |
| E. coli AROM-2 | E. coli AROM-1, ΔphrH1, Δacr | This study |
| E. coli AROM-3 | E. coli AROM-2, Psr-galP-Psr-glk | This study |
| E. coli AROM-4 | E. coli AROM-3, Δzwf | This study |
| E. coli TYR | E. coli AROM-4, AphaLA | This study |
| E. coli DOPA-1 | L-DOPA producer, E. coli TYR derivative integrated the tyrA′, tyrB and hpaBC fusion protein chimera under the control of 7P37 promoter | This study |
| E. coli DOPA-2 | ΔmutS | This study |
| E. coli DOPA-30 | MAGE strain with the artificial 5′-UTR sequence of the hpaC | This study |
| E. coli DOPA-30N | MAGE strain with the native 5′-UTR sequence of the hpaC | This study |

**Plasmid**

| pQE30 | Expression vector, T5 promoter, pBR322 ori, Amp′ | Invitrogen |
| pQE30-hpaBC | pQE30 containing E. coli W hpaBC | This study |
| pQE30-2hpaBC | pQE30 containing 2 copies of E. coli W hpaBC | This study |
| pOSIP-CF | Integration vector, HK022 integrase, attP, ccdB gene, cat′ | 46 |
| pXM19J | C. glutamicum E. coli shuttle expression vector, Puc, IPTG inducible, cat′; GenBank No. AJ133195 | 47 |
| pK-JL | pK18mob sacB derivative, sacB under the control of the tac-M promoter, Kan′ | 48 |
| pMD-ccdBKanS | ccdB-kan-IsceI cassette | This study |
| pBAD30 | Expression vector, P630, promoter, arabinose induction, pACYC184 ori, Amp′ | 49 |
| pBAD30-I-SceI | pBAD30 derivative with the I-SceI endonuclease gene | This study |
| pSIM6 | pSC101 replicon, P37-gam-bet-exo, B587, Amp′ | 50 |
| pSIMS | pSIM6 derivative with the arabinose-inducible I-SceI endonuclease gene | This study |
| pZSBP | Biobrick vector, P37 promoter, P630 ori, Kan′ | 38 |
| pZSBP-P37-glk | pZSBP derivative with the glk under the control of the P37 promoter, respectively | This study |
| pZSnP37 | pZSBP derivative with the nP37 promoter (n = 2, 3, 4, 5, 6, or 7) | This study |
| pZSBP-P37-galP | pZSBP derivative with the galP under the control of the P37 promoter | This study |
| pHKK5b | Integration expression plasmid, attPHK site, P630 promoter, Kan′ | 52 |
| pHKK5b-P37-glk | pHKK5b derivative with the glk under the control of the P37 promoter | This study |
| pHKK5b-P37-galP-P37-glk | pHKK5b derivative with the galP and glk under the control of the P37 promoter, respectively | This study |
| p21KT5b | Integration expression plasmid, attPP21 site, P630 promoter, Kan′ | 52 |
| p21KT5b-7P37-tyrA′-tyrB-hpaBC | p21KT5b derivative with the tyrA′-tyrB-hpaBC fusion chimera under the control of the 7P37 promoter | This study |
| pCas | repA101(Ts) ori, kan′, Pcas-car9, Para-B-Red, lacIq, Pric-sgRNA-pMB1 | 53 |
| pTargetF-hpaC | sgRNA plasmid, pMB1 ori, Spe′ | 53 |

**Table 5. Strains and plasmid used in this study.**

The knockouts of the csrA, tyrR and mutS genes were carried out according to the one-step inactivity method with the help of the pSIM6 plasmid expressing the lambda red recombination system. Gene knockouts were verified by colony PCR using appropriate primers (Supplementary Table 2).

The knockouts of other genes were carried out by a two-step recombination method using lambda red recombination and I-SceI cleavage as described as in Supplementary Fig. 1. The method was first reported by Yu et al. They used sacB as the counter-selectable marker. However, the efficiency of the first recombination is very low (24%) because sacB generally results in a certain number of false-positive colonies in the screening process due to mutation of sacB. Thus, we used the IPTG-inducible ccdB gene as the counter-selectable marker. The ccdB gene was amplified from pOSIP-CF using the primers ccdBF/ccdBR, then cloned into the HindIII/XbaI sites of pXM19J to obtain pXM1-ccdB. The plasmid pXM1-ccdB was digested by HindIII, blunted and self-ligated to obtain pEC-ccdB. The IPTG-inducible ccdB gene was amplified from pXM1-ccdB using the primers ccdB′F/ccdB′R, then cloned into pMD18 to obtain pMD-lacI-ccdB. A kan resistance gene (encoding amino-glycoside 3′-phosphotransferase) containing I-SceI recognition sites was amplified from pK-JL using the primers kanF/kanR and then cloned into the XhoI/SpeI sites of pMD-lacI-P630-ccdB to obtain pMD-ccdBKanS. The I-SceI endonuclease gene was synthesized by Suzhou GENEWIZ, Inc. (Suzhou, China) and ligated into pUC57 to obtain pUC57-I-SceI. The I-SceI was cut from pUC57-I-SceI by EcoRI/KpnI and cloned into pBAD30 to obtain pBAD30-I-SceI. The arabinose-inducible I-SceI was amplified from pBAD30-I-SceI using the primers...
overnight seed culture was then inoculated into 50 mL of fermentation medium with a starting OD₆₀₀ of 0.1. The Afbr by MluI/BamHI, then cloned into the integration expression vector pP21KT5b to yield pP21KT5b-7P37-galP-glk. The galP gene under the control of the P37 promoter was digested with BglII/Sall from pZSBP-galP, then ligated into BamHII/Sall-digested pHKKT5b to yield pHKKT5b-P37-galP-glk. The galP gene was amplified from pZSBP using the primers P37F/P37R and assembled into pZSBP by the BglBrick standard approach to produce pZSnP37 (n = 2, 3, 4, 5, 6 or 7), which has a tandem and stronger promoter. The tyrA and tyrB genes were amplified from E. coli using the corresponding primers and cloned into pMD-19T (simple) to obtain pMD-19T-tyrA and pMD-19T-tyrB, respectively. Site-directed mutagenesis was used to remove the BamHII/BglIII sites and feedback inhibition of the tyrA to obtain pMD-19T-tyrA′. The hpaBC gene was amplified from pQE30-hpaBC using the primers hpaBCF1/hpaBCCR2 and cloned into pMD-19T (simple) to obtain pMD-19T-hpaBC. The plasmid pMD-19T-tyrA′-tyrB-hpaBC containing the tyrA′-tyrB-hpaBC fusion protein chimera was assembled by the BglBrick standard approach. The fusion chimera fragment was cut from pMD-19T-tyrA′-tyrB-hpaBC by SphI/Apal, then ligated into SphI/Apal-digested pZS7P37 to yield pZS7P37-tyrA′-tyrB-hpaBC. The tyrA′-tyrB-hpaBC fragment under the control of the 7P37 promoter was cut from pZS7P37-tyrA′-tyrB-hpaBC by MulI/BamHII, then cloned into the integration expression vector p21KT5b to yield p21KT5b-7P37-tyrA′-tyrB-hpaBC.

For L-DOPA production, a single colony was inoculated into 5 mL of LB medium in a 20-mL conical tube which was cultured overnight at 37°C in a rotary shaker at 200 rpm. The overnight seed culture was then inoculated into 50 mL of fermentation medium with a starting OD₆₀₀ of 0.1. The fermentation medium (pH 7.0) contains (g/L): peptone 10, yeast extract 5, NaCl 10, glucose 14, ascorbic acid 0.45 and 10 mL of trace element solution. The trace element solution contains (g/L): FeSO₄·7H₂O 10, ZnSO₄·7H₂O 2.2, MnSO₄·4H₂O 0.58, CuSO₄·5H₂O 0.1, (NH₄)₆Mo₇O₂₄·4H₂O 0.1, Na₂B₄O₇·10H₂O 0.2 and HCl 10 mL. The main cultures were incubated at 37°C for 48 h in a rotary shaking incubator at 150 rpm. IPTG was added as an inducer to a final concentration of 0.1 mM after 6 h when needed.

Fed-batch culture for L-DOPA production. The seed culture produced in 5 mL of LB medium was sub-cultured in 6 × 50 mL LB medium for 10–12 h with shaking at 200 rpm at 37°C. The seed culture (~300 mL) was inoculated into a 5L fermenter (Biostat B5, B. Braun, Germany) containing 3L of fermentation medium with an initial OD₆₀₀ of approximately 0.4. The fermentation medium (pH 7.0) contains (g/L): peptone 10, yeast extract 5, NaCl 10, glucose 14, ascorbic acid 0.45 and 10 mL of trace element solution. The trace element solution contains (g/L): FeSO₄·7H₂O 10, ZnSO₄·7H₂O 2.2, MnSO₄·4H₂O 0.58, CuSO₄·5H₂O 0.1, (NH₄)₆Mo₇O₂₄·4H₂O 0.1, Na₂B₄O₇·10H₂O 0.2 and HCl 10 mL. The main cultures were incubated at 37°C for 48 h in a rotary shaking incubator at 150 rpm. IPTG was added as an inducer to a final concentration of 0.1 mM after 6 h when needed.
Samples were periodically withdrawn, and the following parameters were measured: OD$_{600}$ residual glucose concentration, tyrosine concentration and L-DOPA concentration. Fermentation experiments were carried out in triplicate.

**Analytical methods.** Growth was monitored by measuring the optical density at 600 nm. Tyrosine and L-DOPA in the supernatants were analyzed using a Shimadzu HPLC system (LC-20 A, Shimadzu, Japan) equipped with an Inertsil ODS-SP column (5μm, 4.6 × 150 mm, GL Sciences Inc., Tokyo, Japan). The mobile phase was 0.2% TFA in 40% methanol, with a flow rate of 0.5 mL/min, at 30°C. A photodiode array detector (SPD-M20A) operating at 280 nm was used, and a standard curve was constructed from serial dilutions of a standard stock solution. Glucose concentration was determined by using glucose oxidase and a glucose assay kit (Shanghai Rongsheng Biotech Corporation, Shanghai, China).

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Author Contributions
T.W. performed the experiments. B.-Y.C. developed the markerless deletion approach and performed gene deletions. J.-Z.L. directed the project and wrote the paper.

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