Improvement of L-Valine Production by Atmospheric and Room Temperature Plasma Mutagenesis and High-Throughput Screening in Corynebacterium glutamicum

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ABSTRACT: As one of the branched-chain amino acids, L-valine is an essential nutrient for most mammalian species. In this study, the L-valine producer Corynebacterium glutamicum ΔppcΔaceEΔalatΔp-qo was first constructed. Additionally, an improved biosensor based on the Lrp-type transcriptional regulator and temperature-sensitive replication was built. Then, the C. glutamicum strain was mutagenized by atmospheric and room temperature plasma. A sequential three-step procedure was carried out to screen L-valine-producing strains, including the fluorescence-activated cell sorting (FACS), 96-well plate screening, and flask fermentation. The final mutant HL2-7 obtained by screening produced 3.20 g/L of L-valine, which was 21.47% higher than the titer produced by the starting strain. This study demonstrates that the L-valine-producing mutants can be successfully isolated based on the Lrp sensor system in combination with FACS screening after random mutagenesis.

INTRODUCTION

L-Valine is one of the nine essential amino acids that must be supplied in the diet.1 It is largely produced through microbial fermentation, and the production capacity reached 2 thousand tons per year, with a market value of 45 million dollars.2 Microbial production of L-valine has been established using various industrial microorganisms, including Brevibacterium flavum,3 Escherichia coli,4 Corynebacterium glutamicum,5 and Bacillus subtilis.6 As a well-known workhorse for the production of amino acids such as L-glutamate,7 L-lysine,8 L-isoleucine9 and L-threonine,10 C. glutamicum is also viewed as an attractive microorganism for the production of L-valine.

Industrial strains for the production of L-valine were usually developed by traditional random mutagenesis or metabolic engineering. The traditional strategies, which utilize toxic analogues, have been used to produce the most efficient amino acid producing strains of C. glutamicum. Because of the inefficient genetic manipulation of C. glutamicum and heavy workload required to identify high-yield strains from very large mutant libraries,11−13 metabolic engineering has emerged as a more efficient solution for the production of specific amino acids and their precursors.14 Furthermore, the genome sequences of several C. glutamicum strains have been reported,15,16 and a high L-valine production phenotype was also obtained through rational genetic engineering manipulation.17−22 In C. glutamicum, the L-valine biosynthesis pathway competes with many by-product pathways, such as L-alanine and lactic acid. Consequently, the strategies to improve the yield of L-valine have largely focused on the enhancement of downstream synthesis pathways in combination with the inhibition of competing pathways.14

Rational design and subsequent metabolic engineering represents an important tool for developing industrial production strains.23 However, because of the high complexity of carbon and energy fluxes in living cells, rational approaches to strain development are often restricted by the current knowledge of bacterial physiology.24 However, the limits of rational strain engineering can be overcome by nonrational high-throughput approaches based on random mutagenesis and sequential efficient screening methods.25 The biosensors that detect intra- and extracellular metabolites or environmental signals are ubiquitous in nature.26 The use of biosensors has proven to be a highly valuable tool to specifically transform the intracellular product concentration into a screenable optical output, such as fluorescence.11,13,27 Transcriptional factor (TF)-based biosensors for the high-throughput screening of engineered bacteria11,28,29 and fast
selection of desirable mutations are widely used because of their ability to detect a wide variety of metabolites. In recent years, a large number of TF-based biosensors have been applied for the screening of high-value products such as amino acids, organic acids, and other microbial chemicals.

In this study, adaptive evolution driven by an l-valine biosensor in combination with fluorescence-activated cell sorting (FACS) approach following rational genetic manipulation and atmospheric and room temperature plasma (ARTP) mutagenesis was successfully applied to improve the L-valine production in C. glutamicum. This approach demonstrates the power of combining rational design with biosensor-driven laboratory evolution approaches to improve the production of specific amino acids.

**MATERIALS AND METHODS**

**Strains and Culture Conditions.** The strains and plasmids used in this study are listed in Table 1. E. coli DH5α was used for plasmid construction. E. coli cells were grown in an LB medium at 37 °C and 200 rpm. C. glutamicum was grown in an LBG medium at 37 °C and 200 rpm. Modified Epo medium (5 g/L of yeast extract, 10 g/L of tryptone, 10 g/L of NaCl, 30 g/L of glycerine and 0.1% Tween 80) was used for preparing competent cells of C. glutamicum. An LBHIS medium (2.5 g/L of yeast extract, 5 g/L of tryptone, 5 g/L of NaCl, 18.5 g/L of Brain Heart Infusion powder and 91 g/L of sorbitol) was used for C. glutamicum transformation. When necessary, 30 mg/L of kanamycin was added to the medium to promote plasmid maintenance.

**Construction of the Lrp-Based Biosensor.** For the construction of the biosensor, the rRNb terminator was amplified from the pTRCMob plasmid using the primer pair rrnB-F/R, digested with EcoRI and EcoRV, and ligated into the same sites of the pTRCMob backbone digested, resulting in pH2L01.

The fragment containing the C. glutamicum lrp ORF and the intergenic region of lrp-brnF was amplified using the primers lrp-F and lrp-brn-F. The egfp ORF was amplified by PCR using the primers gfp-F/R. The two resulting fragments lrp-brnF and gfp were ligated by overlap-extension PCR. The PCR product was digested with SacI/BamHI, and ligated into the same sites of pH2L01, resulting in the plasmid pH2L02.

The temperature-sensitive C. glutamicum replicon rep was amplified from pDTW109 using the primer pair rep-F/R. The PCR product was then digested with SpeI and EcoRV, and inserted into pH2L02, resulting in the plasmid pH2L03. The temperature sensitivity of pH2L03 was tested in C. glutamicum ATCC13032. The strains were grown in an LBG medium in the presence of kanamycin at 30 °C for 16 h, then introduced into a fresh LBG medium without kanamycin and grown at 30 or 37 °C. Samples were taken after 12 h, diluted, and spread onto LBG plates with or without kanamycin, and grown at 30 °C for 36 h. The colonies were counted on both plates and the proportion of kanamycin-resistant cells in the sample was calculated.

C. glutamicum was seeded into an LBG liquid medium and grown overnight, then transferred to a fresh LBG medium at an initial OD662 of 1. When the OD662 reached 2, L-valine was added to a final concentration of 0–40 mM. The fluorescence and OD662 were determined after continuous culture at 30 °C for 2 h. Fluorescence measurements were carried out using a SpectraMax M2 multidetection microplate reader (Molecular Devices, CA, USA). The eGFP fluorescence was measured at excitation and emission wavelengths of 488 and 530 nm, respectively.

**Construction of the Initial C. glutamicum Strain for ARTP.** Chromosomal inactivation of the ppc gene encoding phosphoenolpyruvate carboxylase (PEPC), aceE encoding the Elp subunit of the pyruvate dehydrogenase complex (PDHC), alat encoding aminotransferase (AT) and pqo encoding pyruvate/quinine oxidoreductase (PQO) in C. glutamicum ATCC1302 was performed using the suicide vector pK19mobSacB. The primers used in this study were designed according to the genome sequence of C. glutamicum and are listed in Table 2. The upstream fragment ppcU of the ppc gene was amplified from the genomic DNA of C. glutamicum ATCC1302 using the primer pair ppcU-F/R; the downstream fragment ppcD was amplified using the primer pair ppcD-F/R. The two fragments ppcU and ppcD were purified, mixed, ligated by overlap extension PCR, and the fusion product was ligated into the vector pK19mobSacB, resulting in the plasmid pH2L03.

The plasmid pK19mobSacB-Dppc was introduced into C. glutamicum ATCC1302 by electroporation and transformants were selected on LBHIS agar supplemented with 30 mg/L of kanamycin. The kanamycin-resistant transformants were further grown for 12 h in an LB medium, and subsequently selected again on the LBHIS plates supplemented with 10% sucrose. The sucrose-resistant and kanamycin-sensitive colonies were further grown in an LB medium. The deletion of ppc was further confirmed by PCR using the primers ppc-V and ppc-R. The cells of the mutant strain HL101 grew on the plate without antibiotics. Similarly, the aceE gene in the genome of HL101 was deleted, resulting in the strain HL102. Next, the alat gene in the genome of HL102 was deleted, resulting in the strain HL103. Finally, the pqo gene in the genome of HL103 was deleted, resulting in the strain HL104.

Table 1. Bacterial Strains and Plasmids Used in This Study

| Strain or Plasmid | Description | Source |
|------------------|-------------|--------|
| DH5α             | cloning strain of E. coli | Novagen |
| ATCC13032        | wild-type C. glutamicum | ATCC   |
| HL101            | ATCC13032Δppc | this work |
| HL102            | ATCC13032ΔppcΔaceE | this work |
| HL103            | ATCC13032ΔppcΔaceEΔalat | this work |
| HL104            | ATCC13032ΔppcΔaceEΔalatΔpqo | this work |
| ATCC13032/pHL203 | ATCC13032 harboring pHL203 | this work |
| HL104/pHL203     | HL104 harboring pHL203 | this work |

| Plasmids | Description | Source |
|----------|-------------|--------|
| pK19mobSacB | vector for gene deletion in C. glutamicum | 45 |
| pK19mobSacBΔppc | derived from pK19mobSacB by inserting the segment ppc (up + down) | this work |
| pK19mobSacBΔaceE | derived from pK19mobSacB by inserting the segment aceE (up + down) | this work |
| pK19mobSacBΔalat | derived from pK19mobSacB by inserting the segment alat (up + down) | this work |
| pK19mobSacBΔpqo | derived from pK19mobSacB by inserting the segment pqo (up + down) | this work |
| pHL200 | pTRCMob harboring lrp-brnE-egfp | this work |
| pHL201 | pTRCMob harboring rmmB | this work |
| pHL202 | pHL201 harboring lrp-brnE-egfp | this work |
| pHL203 | derived from pHL202 by inserting the temperature-sensitive replication origin from pDTW109 | this work |
Table 2. Primers Used for PCR in This Study

| name     | 5′−3′ sequence                        | restriction site |
|----------|---------------------------------------|------------------|
| ppcU-F   | GTAGGATCCATAAGTCTCGTAGTGGTTGAG        | BamHI            |
| ppcU-R   | GCAGAGCTTGGTCCGAGTACGATGAGCATGTCGAGG  |                  |
| ppcD-F   | CCATCCGCTTCTGCAATTCGAGCAGCATGTCGAGG  |                  |
| ppcD-R   | GTAGGATCCATGCTGATGATGAGCATGTCGAGG    | BamHI            |
| ppc-V    | AAGTGACACCGAGGAAACAGTG                |                  |
| aceEU-F  | GTAGGATCCAGATACGACATCTCACG           | BamHI            |
| aceEU-R  | GTCGCAATCCTCTGCAATTCGAGCAGCATGTCGAGG |                  |
| aceED-F  | CTCGGATGACTTCAAGCAGCATGGAACACCTGGAAC  |                  |
| aceED-R  | TACTCTGATTCCCTGAGTGGATGAGGC           | XbaI             |
| aceE-V   | AAGAACCCTTGGCAATTACCGAG               |                  |
| alatU-F  | ACGTCTTAGAGCATTACGATGAGGAAGG          | XbaI             |
| alatU-R  | GAGGAGCTACCTGAGATCAGCAGCTCTCTTCTCTCC |                  |
| alatD-F  | GATGAGGAGCTGCTGATGTCAGTCTCTCGTGG     |                  |
| alatD-R  | ACGTGATCAAGCTCTTTTCCTCTACTGAGCTGGC   | XbaI             |
| alat-V   | CCAGTAGGAAGCTGCTGAGCTTCG              |                  |
| pqoU-F   | GTAGGATCCATGCTCCGAGGTTCTG             | BamHI            |
| pqoU-R   | GTGGAGCCAGGTTTGCCGATATTGCGTGGATCCACC |                  |
| pqoD-F   | TGGGAGCCAGGCCATTTGCGGCAACACCTGCGCACC |                  |
| pqoD-R   | GTAGGATCCCTTCTTTGAGGCGGCTTACTGAGG    | BamHI            |
| pqo-V    | CTCCGACAAAGCAGATGTCG                  |                  |
| rnb-F    | CGATGGTAGTGGTGGGCTCTG                 | EcoRI            |
| rnb-R    | AGGAATTCGAAAAATTAAAACAAAAAGAG         | SacI             |
| Lrp-brnF-F | ATAGAGCTCATACACCTGGGCGGAGCTG          |                  |
| Lrp-brnR-R | TCTCTTCTTATTACTATATCTCTTCTTACTAAGTCC |                  |
| gfp-F    | AGAGGAGGATATATGAGTAAAGGGGAAAGACTTTCCTAC |              |
| gfp-R    | CCGGATACCCTATTGTATAGCTCATCCATGCA      | BamHI            |

*Restriction sites are underlined.

was deleted, resulting in the strain HL104. The replacement at the chromosomal loci was verified by PCR using the primer pairs aceE-V/aceED-R, alat-V/alatD-R, or pqo-V/pqoD-R, respectively.

**Mutagenesis by ARTP and Determination of Optimal Treatment Time.** An ARTP-IIIS instrument (Wuxi TMAX-TREE Biotechnology Co., Ltd, Wuxi, China) was used to carry out the ARTP mutagenesis.46 Pure helium was used as the plasma working gas to irradiate the samples on the stainless-steel plate. The operating parameters were as follows: (1) the radio frequency power input was 100 W; (2) the distance between the sample plate and the plasma torch nozzle exit was 2 mm; (3) the helium gas flow rate was 8 SLM (standard liter per minute); and (4) the temperature of the plasma jet was at 25−30 °C. The mutagenic effect was only dependent on the treatment time.

The *C. glutamicum* strain HL104/pHL203 was grown to an OD$_{562}$ of 10 in the LBHIS medium (mid-log phase). To determine the optimal treatment time, the cells were washed twice with 50 mL of phosphate-buffered saline (PBS), after which 10 μL of the cell suspension (1 × 10$^6$ cells per milliliter) was spread on the sterilized steel plate, and exposed to the ARTP nozzle exit for 0, 5, 10, 15, 20, 25, 30, and 40 s. After each treatment, the samples were washed down with 1 mL of PBS into fresh sterile tubes, properly diluted, and cultured on a solid medium with 30 mg/L of kanamycin at 30 °C for 2 days. An untreated culture was used as a control. The appropriate treatment time was selected based on the lethality rate, which was calculated as follows

Lethality rate (%) = \frac{\text{control colonies} − \text{survival colonies}}{\text{control colonies}} \times 100%

**Fluorescence Microscopy and Spectroscopy.** After ARTP treatment, HL104/pHL203 was cultivated on LBHIS plates at 30 °C for 12 h, transferred into shake flasks containing 25 mL of LBHIS with 30 mg/L of kanamycin, and grown for 2 days. The resulting cells were harvested by centrifugation at 8000g for 10 min at 4 °C, followed by washing in PBS three times. The washed cells were resuspended in PBS and vortexed for 5 min to obtain a uniform suspension. The cell suspension was diluted to an optical density OD$_{662}$ of 0.1 to avoid internal interference and collected to measure their GFP fluorescence by flow cytometry. The cells were screened using a MoFlo XDP Flow Cytometry Sorter (Beckman Coulter Inc., Fullerton, CA, USA). The fluorescence signal of the eGFP reporter was measured at an excitation wavelength of 488 nm, and the emission was detected using a 530 nm band-pass filter. The “purify model”44 was used to improve the ratio of positive cells in the library. All isolated cells were cultivated on LBHIS agar plates with 30 mg/L of kanamycin at 30 °C for 48 h for further analysis. The cells were transferred into 96-deep-well plates with 500 μL of a fresh fermentation medium per well, and cultivated at 30 °C for 48 h, at a shaking frequency of 1200 rpm and a shaking diameter of 3 mm in a Microtron shaker (Infors, Switzerland). The biomass and the eGFP fluorescence of the cultures was measured using a SpectraMax M2 multidetection microplate reader.44

**Fermentations.** The seed medium contained 25 g/L of glucose, 30 g/L of corn steep liquor (CS001, Angel Co., Ltd,
China), 5 g/L of urea, 1 g/L of KH₂PO₄·3H₂O, and 0.5 g/L of MgSO₄, and the pH was adjusted to 7.0 using 1 M KOH. The fermentation medium contained 120 g/L of glucose, 20 g/L of corn steep liquor, 20 g/L of (NH₄)₂SO₄, 1 g/L of KH₂PO₄, 0.5 g/L of MgSO₄, 0.01 g/L of FeSO₄·7H₂O and 0.01 g/L of MnSO₄·H₂O. The pH was adjusted to 7.0 and 20 g/L of CaCO₃ was added to balance the pH of the medium. When necessary, 30 mg/L of kanamycin was added into the medium.

For flask cultivation, seed cultures grown at 30 °C for 12 h were used to inoculate 25 mL of fermentation medium in 250 mL flasks to an initial OD₅₆₂ of 1.8. The cultures were incubated at 30 °C and 200 rpm in rotary shakers for 72 h. Samples were harvested for the determination of residual glucose, biomass, and amino acids as described below.

Analysis of Residual Glucose, Biomass and Amino Acids. The concentration of residual glucose was determined using the SBA-40C biosensor analyzer (Shandong Academy of Sciences, China). The cell density was determined by measuring the optical density at 562 nm (OD₅₆₂) using a UV-1800 spectrophotometer (Beijing Ruili Analytical Instrument Co., Ltd, China). The levels of amino acids were analyzed by HPLC on a 1200 series instrument (Agilent, USA) according to the method reported by Köös et al.²⁸

RESULTS AND DISCUSSION

Construction of the Lrp-Based Biosensor. To design a biosensor that responds to elevated intracellular concentrations of L-valine, a module based on the transcriptional regulator Lrp of C. glutamicum was constructed. The egfp coding sequence was cloned behind the plasmid region containing the open-reading frame of lrp and the intergenic region of lrp-brnF. Thus, the expression of egfp was placed under the control of the PbrnF promoter, which intrinsically responds to the intracellular concentration of L-valine. When the biosensor plasmid pHL200 was introduced into C. glutamicum ATCC13032, a discernible fluorescence signal above the autofluorescence of wild-type C. glutamicum was detected (Figure S1). To prevent this leaky expression, the rrnB terminator was cloned in front of Lrp to block the impact of other parts of the plasmid. Furthermore, the rep replicon was replaced by a temperature-sensitive replicon, resulting in the plasmid pHL203 (Figure 1a), which was again introduced into C. glutamicum ATCC13032. This construct effectively reduced the background fluorescence and improved the performance of the biosensor (Figure S1). The normalized fluorescence intensity (fluorescence/OD₅₆₂) was very low when no L-valine was added and did not increase significantly when added L-valine concentration was below 0.1 mM. Fluorescence intensity increased significantly with the increase of the added L-valine concentration when added L-valine concentration was higher than 1 mM (Figure 1b). The results were similar to that reported in a previous study. It may be attributed to the fact that the biosensor detects the intracellular rather than the extracellular L-valine concentration. Although this method is not particularly precise, it confirmed that the biosensor is functional. The results indicated that the biosensor could work to detect different levels of L-valine by generating corresponding levels of fluorescence intensity. Temperature-sensitive
plasmids can be maintained at a given permissive temperatures, which is favorable for further studies. The biosensor developed by Mustafi et al. could not be easily removed from *C. glutamicum*, which complicates the subsequent transformation for metabolic engineering. By contrast, the plasmid pH203 developed in this study can be easily removed by increasing the temperature. The temperature-sensitivity of pH203 was confirmed in ATCC13032/pH203 (data not shown) and was consistent with the results reported by Hu et al.

**Construction of *C. glutamicum* Knockout Mutants.**

The optimization of the intracellular pyruvate pool can increase the l-valine production in *C. glutamicum*. Pyruvate can be transformed into acetyl-CoA, oxaloacetate, L-alanine, acetate, and L-valine (Figure 2). PEPC encoding by ppc converts phosphoenolpyruvate to oxaloacetate, decreasing flow to L-valine. Deleting of gene ppc increased L-valine production (Figure 3b). The elp subunit of PDHC encoding by aceE converts pyruvate to acetyl-CoA. Therefore, gene aceE was deleted in HL102. As shown in Figure 3b, l-valine production increased and L-alanine became the major by-product of strain HL102. l-alanine was formed by catalyzing pyruvate with two enzymes encoding by alat and avat. However, L-alanine was still the major by-product of the strains HL103 and HL104, even though the alat gene was disrupted (Figure 3b). More L-valine was accumulated though the growth rate of HL104 was decreased because of the deletion of ppc. After 72 h of cultivation, HL104 produced 2.64 g/L of L-valine. Overall, the results demonstrated that the inactivation of the PEPC, PDHC, AT, and PQO improved the l-valine production. The inactivation of PEPC (encoded by ppc), which converts phosphoenolpyruvate to oxaloacetate, was reported to improve the L-valine production (36). A *C. glutamicum* strain with a mutant variant of aceE produced more L-valine than the control. The elimination of PQO, which converts pyruvate into acetate, was also reported to increase the L-valine production by increasing pyruvate levels. These above four candidate genes had been deleted individually, but not simultaneously for strain development in some previous studies.

Consequently, the genes ppc, aceE, alat, and ppo were deleted from *C. glutamicum* ATCC13032 before mutagenesis by ARTP. This engineering strategy, which was applied before the mutagenesis, provided a more favorable starting strain to reduce the labor-intensity of the whole project.

**High-Throughput Screening of the ARTP-Mutants Using the Biosensor.** To allow the high-throughput screening of strains with increased l-valine production, the plasmid pH203 was introduced into the l-valine producer strain HL104. Random mutations were induced by ARTP-irradiation, and high l-valine-producing mutants were screened. HL104 cells containing the biosensor plasmid were treated with helium-based ARTP for 5, 10, 15, 20, 25, 30, and 40 s, respectively. The lethality rate was over 90% when the exposure time exceeded 20 s (Figure 4). Therefore, the optimal exposure time was set at 20 s, with lethality ranging from 90 to 95%.

An efficient producer strain is one of the key factors that can make or break the industrialization of biotechnological processes. A rational design for the metabolic engineering of industrial strains has been proved to be a valuable approach, but the design and engineering of bacterial strains remains challenging because of the complexity of the bacterial physiology. Consequently, random mutagenesis and screening strategies are still widely used for strain development. The generation of genetic diversity is a key first step in high-throughput screening. The ARTP mutation breeding system is an effective method to generate genetic diversity and improve product yields. ARTP can produce a variety of high-energy, evenly distributed particles acting on the strands of DNA in the microbial cells under atmospheric pressure, causing mutations through an incomplete gene repair process. ARTP can generate hundreds of genomic modifications for FACS screening using a suitable biosensor. Moreover, beneficial mutation can be obtained by screening mutant libraries of strains that have first been modified using metabolic engineering. The affected sites can be identified by genome sequencing and bioinformatics analysis.

After mutagenesis, *C. glutamicum* cells were incubated in LBHIS medium at 30 °C for 12 h and were then subjected to shake-flask fermentation for 2 days to promote the l-valine
production, and were then analyzed and sorted by FACS. To ensure that the mutant library is sufficiently large, about $10^8$ cells were screened by FACS. The top 0.04% of the screened cells according to the fluorescence intensity were sorted and cultivated on LBHIS agar plates. The selected mutants were then seeded into individual wells of 96-deep-well plates containing a fermentation medium with 1% acetate. Acetate was added to support the growth of L-valine producing mutants with decreased pyruvate dehydrogenase activity. In this strategy, biomass is produced from acetate and the L-valine production from glucose is initiated during the stationary phase after the acetate is completely consumed.52

After 2 screening rounds, 900 isolates were preliminarily selected from the genetic library, and only 53 of the selected isolates were further confirmed that they exhibited obviously increased fluorescence intensities than the starting strain in 96-well plate fermentations. Eight improved mutants with the highest relative fluorescence intensity (Figure 5a) and three negative mutants showing no obvious fluorescence change (Figure 5a) were then chosen to inoculate in a 250 mL Erlenmeyer flask, and allowed to grow for 3 days at 30 °C and 200 rpm for the production of L-valine. In order to explore whether the changes of fluorescence intensity were caused by the sequence variations of the biosensor, the rrnB-lrp-brnE-gfp

**Figure 5.** Normalized fluorescence intensity (fluorescence/OD<sub>562</sub>) of mutants obtained by the ARTP treatment and fermentation analysis of C. glutamicum mutants. (a) Fluorescence intensity of mutants. The control is strain HL104/pHL203 with a fluorescence intensity of 49738, grown in a 96-deep-well plate at 30 °C for 72 h. (b) L-Valine production and cell growth of the strains. Strain HL104 harboring pHL203 was used as the control. (c) The titers of the by-products alanine and lysine. The data represent the means ± SEM (n = 3).
ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02747.

fluorescence intensity of pH203 and pH200; normalized fluorescence intensity and fermentation analysis of C. glutamicum mutants; and SV test, SNP test, and small InDel test results (PDF)

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Notes
The authors declare no competing financial interest.

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