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Exploring Lysine Riboswitch for Metabolic Flux Control and Improvement of L-Lysine Synthesis in *Corynebacterium glutamicum*

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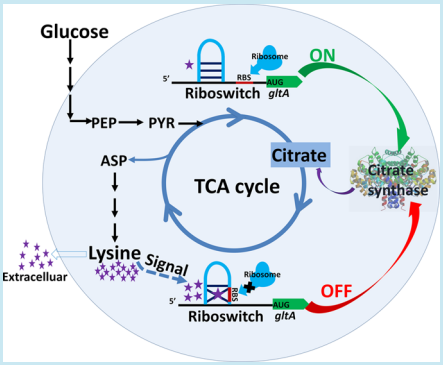
Supporting Information

ABSTRACT: Riboswitch, a regulatory part of an mRNA molecule that can specifically bind a metabolite and regulate gene expression, is attractive for engineering biological systems, especially for the control of metabolic fluxes in industrial microorganisms. Here, we demonstrate the use of lysine riboswitch and intracellular L-lysine as a signal to control the competing but essential metabolic by-pathways of lysine biosynthesis. To this end, we first examined the natural lysine riboswitches of *Eschericia coli* (ECRS) and *Bacillus subtilis* (BSRS) to control the expression of citrate synthase (*gltA*) and thus the metabolic flux in the tricarboxylic acid (TCA) cycle in *E. coli*. ECRS and BSRS were then successfully used to control the *gltA* gene and TCA cycle activity in a lysine producing strain *Corynebacterium glutamicum* LP917, respectively. Compared with the strain LP917, the growth of both lysine riboswitch-*gltA* mutants was slower, suggesting a reduced TCA cycle activity. The lysine production was 63% higher in the mutant ECRS-*gltA* and 38% higher in the mutant BSRS-*gltA*, indicating a higher metabolic flux into the lysine synthesis pathway. This is the first report on using an amino acid riboswitch for improvement of lysine biosynthesis. The lysine riboswitches can be easily adapted to dynamically control other essential but competing metabolic pathways or even be engineered as an "on-switch" to enhance the metabolic fluxes of desired metabolic pathways.



KEYWORDS: *dynamic metabolic control, lysine riboswtch, lysine biosynthesis, citrate synthase, C. glutamicum*

Metabolic flux control is a major issue in developing highly productive microorganisms for bioproduction. In particular, the control of metabolic pathways that are essential for cell growth but not desired for the production of a special compound (the so-called essential by-pathways) still represents a challenge. Conventional molecular biological approaches such as gene knockout are either biologically not possible or technologically not economic, because expensive precursors may need to be added into the growth medium after disrupting the pathway. Leakage of corresponding gene(s) by random mutation is the classic but less efficient approach, which may cause unexpected negative effects to the production strain. Ideally, the by-pathway should be controlled dynamically according to the need of the cells and the bioproduction process; that is, it should be active during the growth phase but down-regulated or even switched off in the production phase. Efforts have been made previously in this direction.[1−4] In a recent work, our group demonstrated the engineering of an artificial allosteric enzyme for a dynamic metabolic control by using the desired product as an intracellular signal.[5] Though it is feasible, the design of artificial allosteric enzymes and regulation with desired sensitivity and dynamic range is still difficult in most cases. In this regard, riboswitches that regulate gene expression in response to certain metabolites may provide an alternative and attractive way for the control of metabolic pathways. Here, we report on the use of riboswitch to improve lysine biosynthesis in *Corynebacterium glutamicum* by using lysine as an intracellular signal.

C. glutamicum is a Gram-positive bacterium widely used for producing amino acids and organic acids in industry.[6−8] With the development of genomic research, the whole genome sequence of *C. glutamicum* has been available since 2003.[9,10] Using metabolic engineering tools, many of the metabolic pathways in *C. glutamicum* have been reengineered or optimized, especially for the synthesis of L-aspartate-derived amino acids and vitamins.[10,11] Among others, inducible expression systems were widely used.[12,13] However, the inducibility is promoter dependent and requires often codon optimization. The stability may not be maintained for long time in different organisms. Moreover, the basal expression and exogenous molecules may interfere or lower the inducible expression. In addition, the costs of inducers needs to be considered for large-scale bioproduction.[13] To circumvent these drawbacks, riboswitches could be used as a promising tool for controlling gene expression. Riboswitch is the regulatory part of an mRNA, which is normally located in the upstream (5′UTR) of a specific gene. There are two components in a riboswitch: an aptamer and an expression platform. The aptamer domain can directly bind a metabolite, resulting in structural change of

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the expression platform and modulating thus gene expression by mechanisms such as transcription termination, translation initiation, and intron splicing.[14,15]

Till now, more than 20 riboswitches have been discovered.[14] Among them, only two amino acids riboswitches were found: a lysine riboswitch and a glycine riboswitch. A recent study predicted the existence of some riboswitches in *C. glutamicum*, which, however, do not include the lysine riboswitch.[16] Lysine riboswitch was first analyzed in *Bacillus subtilis* (designated here as BSRS),[17] which was found to be located in the 5′UTR of the *lysC* gene coding for the enzyme aspartkinase. Several previous studies examined the functionality and manipulation of the *B. subtilis* lysine riboswitch.[18−20] A lysine riboswitch was also found in *E. coli*, which revealed in fact the mechanism of lysine feedback repression of aspartkinase III in *E. coli*.[17] Interestingly, the lysine riboswitch of *E. coli* (ECRS) has been found to have dual functions and showed a different regulation mechanism from that of BSRS.[21] In the presence of lysine, the translation initiation of aspartkinase III in *E. coli* was repressed; at the same time, mRNA decay was accelerated.[21] However, only transcription inhibition was found for the lysine riboswitch from *B. subtilis*. Because of the different mechanisms and thus sensitivities to lysine, both of these lysine riboswitches are considered in this work for metabolic flux control. For this purpose, we first examined the possibility of metabolic pathway control by engineering the two lysine riboswitches in *E. coli*. To this end, the control of expression of the *gltA* gene coding for citrate synthase (CS) of the tricarboxylic acid cycle (TCA) was used as an example. We then checked the functionality of the two lysine riboswitches in lysine producing *C. glutamicum*. Finally, we integrated the riboswitches into the *C. glutamicum* genome to control *gltA* expression for an enhanced lysine production (Figure 1). To our knowledge, this is the first study

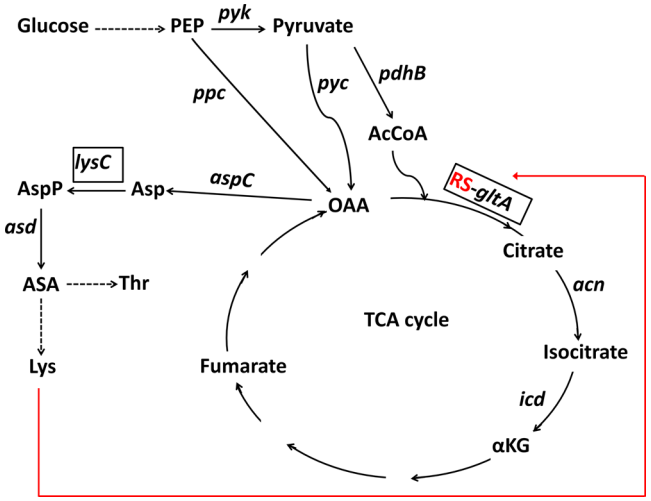

Figure 1. Simplified illustration of L-lysine biosynthetic pathway and the tricarboxylic acid (TCA) cycle as an essential but competing bypathway in *C. glutamicum*.[34] Red line indicates the control of citrate synthease (*gltA*) by riboswitch (RS) using lysine as a signal.

to demonstrate that an amino acid riboswitch can be used to improve the target molecule production in an industrial microorganism. It can be further explored for systematic metabolic engineering or synthetic biology of industrial microorganisms such as *C. glutamicum* for the production of various valuable bioproducts.

RESULTS AND DISCUSSION

Proof-of-Concept of Metabolic Flux Control by Riboswitches in *E. coli*. For the purpose of using lysine riboswitch for metabolic pathway control, we first studied the functionality of the two lysine riboswitches and introduced them into the chromosome of *E. coli*. Citrate synthase (CS), the first enzyme of the TCA cycle (Figure 1) and coded by the gene *gltA*, was selected as an example for the control of metabolic pathway. It has been proved that the reduction of CS activity can contribute to the increased lysine production in lysine producing microorganisms.[23] The regulatory region between the promoter and the start codon of *gltA* was replaced by the lysine riboswitch. After the riboswitch was intergraded into the chromosome of lysine producing strain *E. coli* DL2 by using the λ-red system, two mutant strains (DLECRS and DLBSRS) were obtained. At the same time, a mutant DLG31C was also generated by using the same strategy, in which the lysine riboswitch was mutated at codon 31 so that the expression of *gltA* will not be affected by lysine in this mutant. The strain *E. coli* DL2 and its derivatives were cultured in MSI medium for examining the performance. Notably, both the glucose consumption and the cell density in the mutants DLECRS and DLBSRS were much lower than those of the parent strain DL2, whereas the mutant DLG31C showed nearly the same results as the strain DL2 (Figure 2). In contrast, all

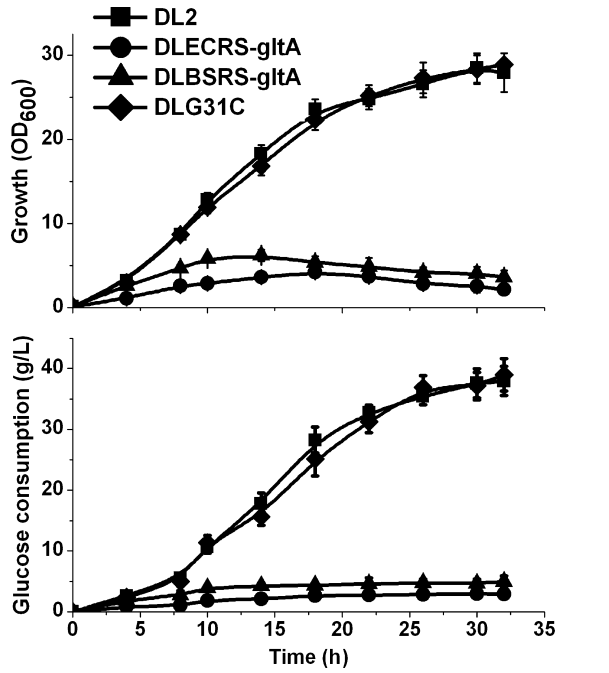

Figure 2. Cell growth and glucose consumption of *E. coli* DL2, DLECRS, DLBSRS, and DLG31C. The data represent mean values and standard deviations from three independent experiments. OD_{600} means the optical density at 600 nm.

the strains grew equally well in Luria−Bertani (LB) rich medium (data not shown). Obviously, both the lysine riboswitches from *E. coli* and *B. subtilis* function well in the mutants DLECRS and DLBSRS. Although the lysine riboswitch secondary structure was formed and stabilized in the mutant DLG31C, the lysine binding was inhibited due to the G31C mutation. The results demonstrated that the secondary structure of riboswitch is not responsible for the decrease in

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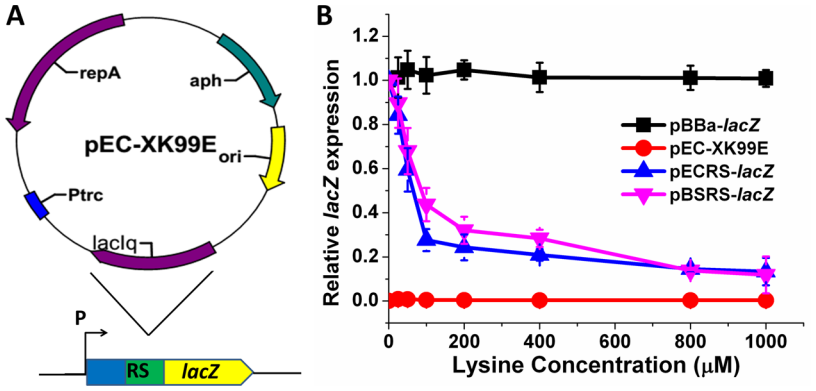

Figure 3. Characterization of the riboswitch-*lacZ* expression in *C. glutamicum*. (A) Construction for the *β*-galactosidase reporter system. P is the constitutive promoter BBa_J23100; RS means riboswitch, from either *E. coli* (ECRS) or *B. subtilis* (BSRS). (B) Relative *lacZ* expressions were measured in the absence and presence of different lysine concentrations. Plasmid pBBa-*lacZ*, which contains BBa_B0034-*lacZ* (strong RBS), was used as the positive control, plasmid pEC-XK99E as the negative control. The expressions were normalized to the value of pBBa-*lacZ* in the absence of lysine.

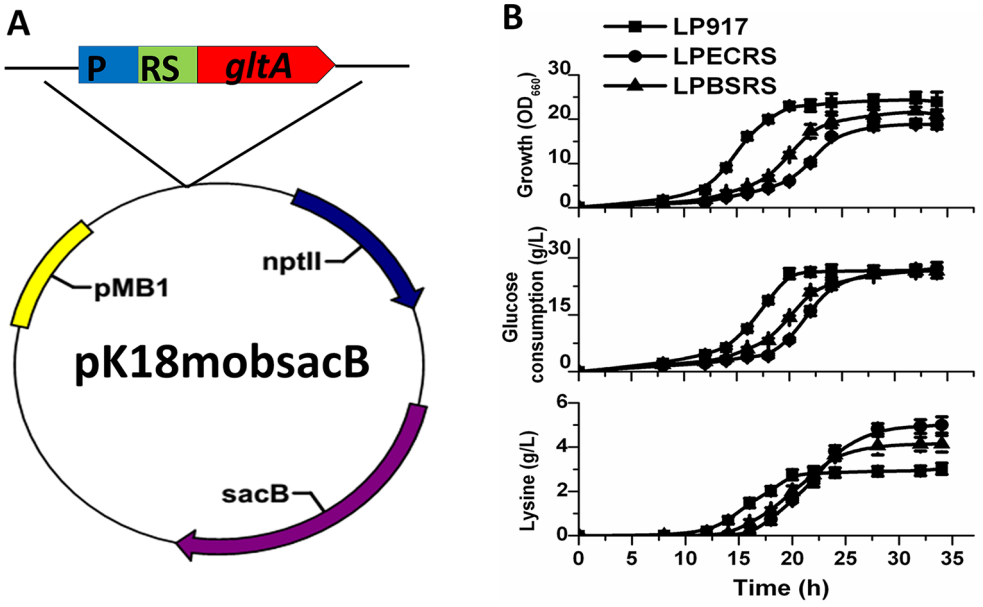

Figure 4. (A) Structure of the riboswitch-*gltA*. P: native promoter of *gltA*. RS: riboswitch. (B) Fermentation results with *C. glutamicum* LP917, LPECRS, and LPBSRS in shake flasks. The data represent mean values and standard deviations from three independent experiments. OD_{660} means the optical density at 660 nm.

cell growth. When the mutants DLECRS and DLBSRS were grown in MSI medium without exogenous lysine, the intracellular lysine concentration was obviously high enough to repress the *gltA* gene expression through the lysine riboswitch, resulting in strongly reduced TCA activity and cell growth. In cultures grown on LB medium, amino acids including lysine may be mainly taken up from the complex medium and the intracellular lysine concentration may not be high enough to repress the *gltA* expression in mutants. Alternatively, the reduced TCA cycle activity may be compensated by the rich nutrients in the LB medium.

Functionality Examination of Riboswitches in *C. glutamicum*. To use lysine riboswitch for metabolic control in *C. glutamicum*, we constructed plasmids to analyze the function of lysine riboswitch in this organism. First, we checked whether there is a native lysine riboswitch in the chromosome of *C. glutamicum* by using the software RiboSW.[22] No native lysine riboswitch could be found. Considering the different mechanisms of the lysine riboswitches from *E. coli* and *B. subtilis*, we amplified and introduced them into *C. glutamicum* ATCC 13032, respectively. To confirm the functioning of the introduced lysine riboswitch, the repression of *β*-galactosidase expression by lysine was analyzed using two constructs pECRS-*lacZ* and pBSRS-lacZ (Figure 3). The pBBa-*lacZ* fusion was used as a positive control and pEC-XK99E as a negative control. As shown in Figure 3, the *β*-galactosidase activity of riboswitch-*lacZ* was sharply decreased when the concentration of lysine was increased from 25 *μ*M to 100 *μ*M. Moreover, the lysine repression of pECRS-*lacZ* (translational "OFF" switch) is much stronger than that of pBSRS-*lacZ* (transcriptional "OFF" switch) in *C. glutamicum*, indicating a higher efficiency of ECRS. Results from previous studies have indicated that *E. coli lysC* riboswitch also controls initial mRNA decay,[17] which may result in the lower level of mRNA than in *B. subtilis*. Therefore, the results presented here showed that *β*-galactosidase activity was repressed in the presence of exogenously supplied lysine,

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indicating that the two lysine riboswitches are functional in *C. glutamicum*.

Exploring Riboswitches for Metabolic Pathway Control in *C. glutamicum*. After testing the function of lysine riboswitches in reporter strains, we decided to introduce them into the chromosome of *C. glutamicum* for metabolic pathway control. Both lysine riboswitches were tested because they have different efficiency of repression. Correspondingly, two plasmids pK18ECRS and pK18BSRS were constructed that carried the native *gltA* promoter, *lysC* riboswitch, and *gltA* gene (Figure 4A). The lysine producing *C. glutamicum* strain LP917 was selected to carry out the homologous replacement in the chromosome, in which the expression of *gltA* gene could be repressed by the endogenous lysine without adding any other exogenous inducers. Several colonies were identified that grew slowly on the LBHIS medium, indicating that the expression of *gltA* gene was repressed. After screening and sequencing, we succeeded in obtaining two strains LPECRS and LPBSRS that were further used for fermentation studies.

Fermentation with the Mutants for Enhanced Lysine Production. To examine lysine production in the mutants, batch fermentations were carried out in shake flasks. As depicted in Figure 4B, the cell growth of both LPECRS and LPBSRS strains was delayed in comparison with the strain LP917, indicating that the endogenous lysine levels are high enough to suppress the expression of the *gltA* gene at the beginning. Furthermore, the values of optical density of the LPECRS and LPBSRS cultures were also lower than that of LP917. We postulate that the citrate synthase and the TCA cycle were down-regulated by endogenous lysine. Meanwhile, the glucose consumption of LPECRS and LPBSRS were approximately identical in the end, which may result in more fluxes used for producing lysine but not for cell growth. Importantly, both the yield and the production rate of lysine in LPECRS and LPBSRS were significantly higher than those of LP917. The yield of lysine in the LPECRS strain was 0.227 ± 0.005 mol per mol of glucose consumed (Table 1), which is

Table 1. L-Lysine Production by Riboswitch Derivatives of *C. glutamicum*[a]

strain	lysine yield[b]	final growth (OD_{660})	specific activity of CS (μmol min^{-1} mg^{-1})
LP917	0.139 ± 0.003	23.96 ± 2.18	0.972 ± 0.058
LPECRS	0.227 ± 0.005	18.77 ± 1.03	0.287 ± 0.014
LPBSRS	0.193 ± 0.004	21.03 ± 1.15	0.416 ± 0.022

[a]The mean values were generated from three independent experiments. [b]Lysine yield: mol lysine per mol glucose consumed.

63% higher than that of the strain LP917. In the strain LPBSRS, the yield was 0.193 ± 0.004 mol/mol of glucose (Table 1), which is 38% higher than that of the strain LP917. Enzyme activity assays showed that the LPECRS and LPBSRS mutants had only a 30% and 43% CS activity compared to the parent strain, respectively. The increased yields in the mutants are obviously due to the repression of the citrate synthase and thus the reduced metabolic flux of the TCA cycle. The differences between the strains LPECRS and LPBSRS may be due to the different regulation mechanisms of the two riboswitches ECRS and BSRS as mentioned above and their different sensitivity toward lysine as mentioned in the introduction.

The successful application of lysine-responsive riboswitches in this work provides further opportunities for synthetic biology and metabolic engineering in *C. glutamicum*. Although highly efficient homologous recombination methods have been set up to study gene functions in the genome of *C. glutamicum*, especially for metabolites production, there are still lots of essential genes that could not be knocked out or mutated. Riboswitch can be used to realize a dynamic control of essential genes under the control of native promoters but not a weakened one.[23] The engineering and screening of weaker promoters is not only time-consuming, more importantly, it also cannot result in dynamic control of gene expression as with the riboswitch. Furthermore, the approach presented in this work is more convenient and straightforward. We do not need to change the promoter of the target gene. We simply replace the regulatory part between the promoter and the start codon of the target gene with a riboswitch. Though inducible expression systems could be used for improving production,[24] the use of endogenous lysine as a signal molecule to improve the production of a target metabolite as demonstrated in this work is more attractive. It does not rely on other exogenous inducer molecules. It should be mentioned that the dynamic range and sensitivity of natural lysine riboswitches may still not be optimal for dynamic control of metabolic fluxes. To this end, natural lysine riboswitches could be further re-engineered or tailor-made. Furthermore, the riboswitch can be also engineered as an "on-switch" to turn on certain metabolic pathways or enhance their fluxes using intracellular metabolites as signal according to the need of the bioproduction processes or the possible industrial applications. Thus, the riboswitch can be further explored for systematic metabolic engineering or synthetic biology of industrial microorganisms for improving the efficiency of bioproduction.

METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions. All the bacterial strains and plasmids used in this study are listed in Support Information (SI) Table S1. *E. coli* TOP10 (Invitrogen, Karlsruhe, Germany) was used for cloning and plasmids construction. The bacterial strains were inoculated and cultured on a rotary shaker (230 rpm) at 37 °C as described before.[27] *C. glutamicum* was used for genetic disruption and expression using plasmid pK18mobsacB derivative.[25] *C. glutamicum* was grown at 30 °C in LB or trypticase soy broth medium (DSMZ medium no. 535). For the generation of mutants of *C. glutamicum*, brain heart infusion broth with 0.5 M sorbitol (LBHIS) was used.[26] If necessary, antibiotics were used at the following concentrations: for *E. coli*, ampicillin, 100 μg/mL; kanamycin, 50 μg/mL; and for *C. glutamicum*, kanamycin, 15 or 25 μg/mL.

DNA Manipulation. Standard protocols were used for the genomic DNA extraction. DNA restriction enzyme digestion, ligation, and DNA polymerase chain reaction (PCR) were performed by following the manufacturer's instructions (Thermo Scientific, Germany). Oligonucleotides used for PCR amplification were purchased from Life Biotechnologies (Darmstadt, Germany). DNA fragments were separated by gel electrophoresis and purified using a gel extraction kit (MACHEREY-NAGEL, Dürren, Germany). Plasmids were extracted by using an isolation kit (Qiagen, Germany). The transformation of *C. glutamicum* was performed as described before.[26]

Construction of Riboswitch-*lacZ* Fusions. All primers are listed in SI Table S2. The lysine riboswitches of *E. coli* (ECRS) and *B. subtilis* (BSRS) were amplified from *E. coli*

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MG1655 and *B. subtilis* genomic DNA, respectively. As described before, the fragment of lysine riboswitch contains lysine-binding aptamer and expression platform (Support Information). To compare the activity of different lysine riboswitches in *C. glutamicum*, riboswitch-controlled *lacZ* gene constructs under the control of the constitutive promoter BBa_J23100 (BioBrick) were first amplified (SI Table S2). Then, the fragments were cloned into the pJET1.2 vector (Thermo Scientific, Germany). The *lacZ* gene was also amplified from *E. coli* MG1655 genomic DNA, and the backbone was amplified from pEC-XK99E. Then, the fragments were fused to the lysine riboswitch by using Gibson Assembly Master Mix (NEB) to generate the constructs pECRS-*lacZ* and pBSRS-*lacZ*.

The fusion constructs were transformed into *C. glutamicum* ATCC 13032 cells, respectively. The colonies that were transformed with riboswitch-*lacZ* fusion plasmids were designated as CgECRS*lacZ* and CgBSRS*lacZ*, which were cultured in a defined minimal medium as described before[29] in the absence and presence of various different lysine comcentration. The expression of riboswitch-*lacZ* fusions was measured with the β-galactosidase enzyme assay kit (Thermo Scientific, Germany) in 96-well plates. All the experiments were repeated three times.

Construction of Chromosomal Riboswitch-*gltA* Mutant in *E. coli* and *C. glutamicum*. In order to use the lysine riboswitch to improve lysine production in *E. coli*, we introduced the lysine riboswitch to control the *gltA* gene in *E. coli* DL2.[27] Considering the possibility of inhibition caused by the change of secondary structure in the regulatory region of *gltA*, a single-point mutation (G31C) was introduced into the lysine-binding domain of the riboswitch.[21] Replacement of the native regulatory part of *gltA* gene was performed by PCR-based λ-red recombination, which was carried out using plasmids pKD4 and pKD46.[28] Briefly, the standard protocol was followed to prepare the electrocompetent cells of *E. coli* DL2 carrying plasmid pKD46. Then, two primers were used to amplify the kanamycin resistance (Kan^R) gene from pKD4, with 50-nucleotides homologous to the genome (SI Table S2). After transformation, the deletion mutant, in which double homologous recombination took place, was verified by PCR. Then, the lysine riboswitch was fused with the *gltA* gene by overlapping PCR (SI Table S2). The fused fragment was transformed into deletion mutant to result in controlling the expression of *gltA* by the lysine riboswitch. After a second round homologous recombination in electrotransformation, the linear riboswitch-*gltA* fragment was expected to replace the kanamycin cassette in the chromosome of *gltA* deletion mutant of *E. coli* by using PCR-based λ-red recombination.[28] The M9 minimal medium was used for screening target mutants, as the *gltA* gene deletion mutant could not grow in this medium. The chromosomal replacement was verified by PCR and sequencing.

The suicide vector pK18mobsacB was used to integrate the riboswitch-*gltA* into lysine producing strain *C. glutamicum* LP917.[29] Lysine riboswitches of *E. coli* and *B. subtilis* were amplified from pJETECRS and pJETBSRS, respectively, and the fragments (upstream of *gltA* gene that includes the native promoter of *gltA*, the ORF of *gltA* gene, and the backbone of pK18mobsacB) were amplified by PCR primers, respectively. Then, the four fragments were fused to generate the plasmid pK18ECRS and pK18BSRS by using Gibson Assembly Master Mix (NEB), respectively. After sequencing, the transformation was carried out to insert the riboswitch into the chromosome by homologous recombination.[30] After positive and negative selections, the target strains were verified by sequence analysis.

Fermentation. *E. coli* was cultured in 300-mL conical flasks that contained 30-mL MSI medium.[31] The fermentation was performed as described before.[29,32] For the batch fermentation of *C. glutamicum*, an optimized minimal medium, which was described before,[29] was used: it was improved by adding 16 g/L K_2HPO_4 to control the pH to be 7.2 during the process. Shake flask fermentations were repeated three times for all the strains.

Analysis. The specific activity of citrate synthase was determined as described before.[23] The cell growth was determined by measuring the optical density at 600 nm (*E. coli*) or 660 nm (*C. glutamicum*) in 0.1 M HCl with a UV spectrophotometer. Glucose concentration was confirmed using the YSI glucose analyzer (YSI, Xylem Inc., U.S.A.). The concentrations of lysine were measured by using the ninhydrin method and HPLC as described before.[29,33]

■ ASSOCIATED CONTENT

Supporting Information

Sequence information on ECRS and BSRS described. List of strains and plasmids, Table S1. All the primers used in this work, Table S2. HPLC analysis of lysine concentration, Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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