Ribosome binding site libraries and pathway modules for shikimic acid synthesis with Corynebacterium glutamicum

Bo Zhang1,3, Nan Zhou1, Yi-Ming Liu1, Chang Liu1, Chun-Bo Lou2, Cheng-Ying Jiang1* and Shuang-Jiang Liu1*

Abstract

Background: The shikimic acid (SA) pathway is a fundamental route to synthesize aromatic building blocks for cell growth and metabolic processes, as well as for fermentative production of various aromatic compounds. Genes encoding enzymes of SA pathway are not continuous on genome and they are differently regulated.

Results: In this study, efforts were made to construct continuous genetic modules of SA pathway that are regulated by a same Ptac promoter. Firstly, aro genes [aroG (NCgl2098), aroB (NCgl1559), aroD (NCgl0408) and aroE (NCgl1567)] from Corynebacterium glutamicum and ribosome binding site (RBS) libraries that were tailored for the above genes were obtained, and the strength of each RBS in the 4 libraries was quantified. Secondly, 9 genetic modules were built up from the RBS libraries, a previously characterized ribozyme insulator (RiboJ) and transcriptional promoter (Ptac) and terminator, and aroG, aroB, aroD and aroE. The functionality and efficiency of the constructed genetic modules were evaluated in C. glutamicum by determination of SA synthesis. Results showed that C. glutamicum RES167ΔaroK carrying a genetic module produced 4.3 g/L of SA, which was 54 folds higher compared to that of strain RES167ΔaroK (80 mg/L, without the genetic module) during fermentation in 250-ml flasks. The same strain produced 7.4, and 11.3 g/L of SA during 5-L batch and fed-batch fermentations, respectively, which corresponding to SA molar yields of 0.39 and 0.24 per mole sucrose consumption.

Conclusion: These results demonstrated that the constructed SA pathway modules are effective in increasing SA synthesis in C. glutamicum, and they might be useful for fermentative production of aromatic compounds derived from SA pathway.

Keywords: Shikimic acid pathway, Corynebacterium glutamicum, Shikimate production, Synthetic biology, Genetic modules, Ribosome binding site (RBS)

Background

The shikimic acid (SA) pathway exists in prokaryotes and plants, and is the common route for the synthesis of aromatic amino acids (Trp, Phe, Tyr) [1–3] and vitamins such as phylloquinone [4]. Since its discovery, the SA pathway has attracted extensive interest from science and industries. Recent investigations have demonstrated that more chemicals can be produced by expanding the SA pathway [5]. Seven steps of reactions complete the SA pathway, leading to the conversion of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to chorismic acid [1]. In Corynebacterium glutamicum, the aro genes encoding DAHP synthase (aroG/ncgl2098), 3-dehydroquinate synthase (aroB/ncgl1559), 3-dehydroquinate dehydratase (aroD/ncgl0408) and shikimate dehydrogenase (aroE/ncgl1567) are involved in conversion of PEP and E4P to shikimic acid, and they are located at different transcriptional regulation units [6–9] (Fig. 1). Recent study showed that transcription of aroE was correspondent to the levels of shikimate in C. glutamicum [9]. Genes encoding the enzymes of SA pathway are not continuous on genome and are differently regulated; this would results in extra difficulties for genetic manipulation and metabolic engineering of SA pathway.

* Correspondence: jiangcy@im.ac.cn; liusj@im.ac.cn
1 State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beichen-Xilu No.1, 100101 Beijing, PR China
Full list of author information is available at the end of the article

© 2015 Zhang et al. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.
The development of synthetic biology brings new concepts to design and construct genetic modules or metabolic engineering for bioprocesses. Genetic elements that regulate transcription, translation or encode various enzymes are used as “parts” to build genetic modules [10, 11]. Ideally, the properties of the parts and modules can be accurately and quantitatively predicted when they are implanted into chassis cells [12, 13]. Recently, scientists have designed and constructed a series of parts libraries of promoters, ribosome binding sites (RBS) and terminators, which enabled the regulation of gene expression over wide dynamic ranges in Escherichia coli cells [14, 15]. For example, RBS of different strengths have been applied to optimize the metabolic flux of mevalonate-based farnesyl pyrophosphate biosynthetic pathway [16]. So far, synthetic parts and modules are very limited for C. glutamicum, an important industry workhorse that has been used for decades to produce amino acids, vitamins, nucleotides [17–20], and recently biofuels and chemicals [21–24].

In this study, efforts were made to construct continuous genetic modules for SA pathway with synthetic biology logistics. Four RBS libraries that were tailored for C. glutamicum and 9 genetic modules for SA synthesis were constructed. The functionality and efficiency of the constructed SA pathway modules were evaluated by determination of SA production with C. glutamicum. Results suggested that the newly constructed pathway modules were effective. During batch and fed-batch fermentation, SA production reached titers of 7.4 and 11.3 g/L, respectively. This represented the highest titer of fermentative production of SA with C. glutamicum.

**Results**

**Design, construction, and screening of RBS libraries for aroB, aroD, aroE and aroG**

RBS sequences such as AGAAAGGAGG and GAAAGGAGG had been previously identified in C. glutamicum. In addition, the sequence of AAAGGAGG had been used for expression of genes involving in biopolyester synthesis with C. glutamicum [28]. All these RBS sequences shared a common feature of AAAGGAGG, which is correspondent to the anti-Shine-Dalgarno sequence at the 3’-end of the 16S rRNA from cornyebacteria [26]. In addition, it was reported that the spaces between RBS and translational start codon were found to be dominantly 5–10 nucleotides in C. glutamicum [27]. Based on these observations, we generalized a seeding sequence of AAAGG(N)6–9. According to this design, a pool of RBS sequences was chemically synthesized.

For easy screening of RBS sequences of different strengths and for the purpose to prevent the influence of neighboring elements on gene translation, the enhanced green fluorescence protein (eGFP) [29] and the ribozyme-based insulator RiboJ [30] genes were applied to make constructions for screening tailored RBS libraries for individual aroG, aroB, aroD and aroE. Construction and screening of the tailored RBS libraries are diagramed in Fig. 2. As showed in Fig. 2, 146, 52, 59 and 54 clones were randomly selected for aroB, aroD, aroE and aroG, respectively. Plasmids harboring RBS sequences of different strengths were extracted from E. coli clones, and were further sequenced. These plasmids were then transferred into C. glutamicum. RBS of different strengths were screened
by quantification of fluorescence intensities in *C. glutamicum*, and finally 4 RBS libraries were obtained that had 33, 43, 49 and 42 members for *aroB*, *aroD*, *aroE* and *aroG*, respectively. The RBS sequences of these libraries and the strength of individual RBS are showed in Fig. 3. As seen from Fig. 3, the strengths of the RBS libraries spanned wide ranges. Specifically, the individual RBS strengths of *aroB*, *aroD*, *aroE* and *aroG* libraries had 70, 21, 19 and 10-folds differences, respectively.

**Construction and evaluation of genetic modules for SA pathway**

The above RBS libraries were exploited to build up genetic modules for SA pathway. Each genetic module had *aroB*, *aroD*, *aroE* and *aroG* genes that were independently regulated by RBS of different strengths. The organization of the genetic modules is generalized in Fig. 4a. To simplify the construction and evaluation of genetic modules, RBS with relative high (H), medium (M) or low (L) strength (Fig. 3) from each of the four libraries, were selected for *aroG*, *aroB*, *aroD* or *aroE*. Starting with these building blocks (3 RBS of different strengths and 4 genes with the order of *aroG*-*aroB*-*aroD*-*aroE*), there were theoretical 81 combinations (i.e. genetic modules that possible have different levels of gene expression). By using a mathematic model of combinatorial approach, such 81 combinations were scaled down to 9 combinations (Fig. 4c).

Genetic modules of the above 9 combinations were constructed and were inserted into pXMJ19. Thus, 9 pXMJ19 derivatives, namely plasmid-1 to plasmid-9, were obtained and were transferred into *C. glutamicum* RES167ΔaroK cells. To determine that if gene translations in the genetic modules were exactly correlated to their RBS strengths as they were previously determined, shikimate dehydrogenase (AroE) activities were determined. As shown in (Fig. 4b), those modules (*G*<sup>H</sup>*B*<sup>H</sup>*D*<sup>H</sup>*E*<sup>H</sup>, *G*<sup>M</sup>*B*<sup>M</sup>*D*<sup>M</sup>*E*<sup>M</sup>, and *G*<sup>L</sup>*B*<sup>L</sup>*D*<sup>L</sup>*E*<sup>L</sup>) harbored low strengths of RBS exhibited low AroE activities and those modules (*G*<sup>H</sup>*B*<sup>H</sup>*D*<sup>H</sup>*E*<sup>H</sup>, *G*<sup>M</sup>*B*<sup>M</sup>*D*<sup>M</sup>*E*<sup>M</sup>, and *G*<sup>L</sup>*B*<sup>L</sup>*D*<sup>L</sup>*E*<sup>L</sup>) harbored higher strengths of RBS exhibited higher AroE activities. These results suggested that levels of gene translations in the 9 genetic modules were highly correlated to RBS strengths determined previously via EGFP fluorescence intensities.

**Fig. 2** Procedures of construction and screening of RBS libraries tailored for *aroG*, *aroB*, *aroD* and *aroE*. Numbers of RBS sequences in each library are represented by the clone numbers of *E. coli* or *C. glutamicum*. 
Genetic modules increased SA synthesis with *C. glutamicum*

In order to obtain a mutant that accumulated SA, the *aroK* that encodes shikimate kinase was deleted from *C. glutamicum* RES167, generating the mutant RES167ΔaroK. Plasmids (Table 1) harboring the SA pathway modules (Fig. 4c) were transferred into *C. glutamicum* RES167ΔaroK cells and the effect of those genetic modules on SA production was observed. Results showed that the SA production varied significantly among different genetic modules (Fig. 5),

---

Fig. 3 Quantification of RBS strength in *C. glutamicum* by measuring fluorescence emitted from eGFP fusion proteins with AroG (a), AroB (b), AroD (c), or AroE (d). Columns appeared in dark were RBS selected for construction of genetic modules
although the growth of *C. glutamicum* was not affected by those genetic modules (Data not shown). The SA production with RES167ΔaroK plasmid-2 that carried genetic module of GHBMDE was 6.8 higher than that of RES167ΔaroK, suggesting that the module of GHBMDE was the most effective combination for SA synthesis in *C. glutamicum*.

Insertion of transcriptional terminators into genetic modules further increased SA production with *C. glutamicum*

The genetic module GHBMDE was designed that there is a tac promoter for each gene but only one terminator after the last gene (Fig. 4a). Since terminator regulates also gene transcription and subsequently translation, 3 new SA pathway modules with insertion of terminators were constructed (Fig. 6a). The SA productions with those new combinations by *C. glutamicum* are shown in Fig. 6b. It was found that insertion of a terminator between *aroB* and *aroD* (GHBMTE) resulted in improvement of SA production by about 56% (Fig. 6b).

**SA production in 250-mL flasks and 5-L fermenters with *C. glutamicum***

To evaluate SA productivity, *C. glutamicum* RES167ΔaroK/pXMIJ19-GBTDE was cultivated in 250-mL flasks and 5-L fermenters. Cell growth, SA production, consumption of sucrose and accumulation of 3-dehydroshikimate were monitored (Fig. 7a, 7b, 7c). SA productions were 4.3, 7.4, and 11.3 g/L during 250-mL flask, 5-L batch and fed-batch fermentations, respectively. SA yields from sucrose were 0.22, 0.39, 0.24 mol SA per mole sucrose consumption.
**Table 1 Bacterial strains and plasmids used in this study**

| Strains/plasmids | Relevant characteristics | Source/reference/notes |
|------------------|--------------------------|------------------------|
| **Strains**      |                          |                        |
| E. coli DH5α     | F- endA1thi-1 recA1 gyrA96deoA086xlacZΔlacZ158 (rK−,mK+) supE44 phoA | Invitrogen             |
| C. glutamicum RES167 | Restriction-deficient mutant of ATCC 13032, ΔcglIM-cglIR-cglIIIR | University of Bielefeld |
| Res167ΔaroK     | Res167 derivate, a fragment of DNA encoding for aroK was deleted | This study              |
| Res167ΔaroK/pZB-aroG | Res167ΔaroK derivate, containing plasmid pZB-aroG | This study              |
| Res167ΔaroK/pZB-aroB | Res167ΔaroK derivate, containing plasmid pZB-aroB | This study              |
| Res167ΔaroK/pZB-aroD | Res167ΔaroK derivate, containing plasmid pZB-aroD | This study              |
| Res167ΔaroK/pZB-aroE | Res167ΔaroK derivate, containing plasmid pZB-aroE | This study              |
| **Plasmids**     |                          |                        |
| pk18mob sacB     | Mobilizable vector, for gene disruption in C. glutamicum | University of Bielefeld |
| pk18mob sacB-aroK | Derived from pk18mob sacB, carrying aroK gene | This study              |
| pk18mob sacB-ΔaroK | Derived from pk18mob sacB-aroK, a 573 bp fragment of aroK was deleted | This study              |
| pUC19-RiboJ     | pUC19 carrying RiboJ | Sangon Biotech         |
| pACGFP           | Plasmid carrying enhanced green fluorescence protein (GFP) gene | Invitrogen             |
| pXM19            | Shuttle vector (Cam+, Ptac, lacZ, pBL1 onYC6, pK18 onYVE142) | University of Bielefeld |
| pXM19-RiboJ     | pXM19 carrying RiboJ gene | This study              |
| pZB              | Derived from pXM19, carrying both RiboJ and GFP genes | This study              |
| pZB-aroG        | Derived from pZB, carrying aroG gene with various RBS | This study              |
| pZB-aroD        | Derived from pZB, carrying aroD gene with various RBS | This study              |
| pZB-aroB        | Derived from pZB, carrying aroB gene with various RBS | This study              |
| pZB-aroE        | Derived from pZB, carrying aroE gene with various RBS | This study              |
| pXM19-aroGMU     | pXM19 carrying aroG of which recognition sites of HindIII and PstI were mutated | This study              |
| pXM19-aroKMU     | pXM19 carrying aroK of which recognition sites of BamHI and Sphl were mutated | This study              |
| pXM19-aroDMU     | pXM19 carrying aroD of which recognition site of PstI was mutated | This study              |
| pXM19-aroEMU     | pXM19 carrying aroE of which the recognition sites of EcoRI and Sall were mutated | This study              |
| pXM19-RiboJ-aroGMU-H | pXM19 carrying RiboJ and aroG gene with high strength RBS | This study              |
| pXM19-RiboJ-aroGMU-M | pXM19 carrying RiboJ and aroG gene with medium strength RBS | This study              |
| pXM19-RiboJ-aroGMU-L | pXM19 carrying RiboJ and aroG gene with low strength RBS | This study              |
| pXM19-RiboJ-aroDMU-H | pXM19 carrying RiboJ and aroD gene with high strength RBS | This study              |
| pXM19-RiboJ-aroDMU-M | pXM19 carrying RiboJ and aroD gene with medium strength RBS | This study              |
| pXM19-RiboJ-aroDMU-L | pXM19 carrying RiboJ and aroD gene with low strength RBS | This study              |
| pXM19-RiboJ-aroEMU-H | pXM19 carrying RiboJ and aroE gene with high strength RBS | This study              |
| pXM19-RiboJ-aroEMU-M | pXM19 carrying RiboJ and aroE gene with medium strength RBS | This study              |
| pXM19-RiboJ-aroEMU-L | pXM19 carrying RiboJ and aroE gene with low strength RBS | This study              |
| pXM19-GHBH       | Plasmid pXM19-RiboJ-aroGMU-H derivate, containing aroB-H module | This study              |
| pXM19-GHBH/ΔH    | pXM19 carrying aroB gene with high strength RBS | This study              |
| plasmid-1        | pXM19-GHBHΔH derivate, containing aroE-H module | This study              |
| plasmid-2        | Plasmid pXM19-RiboJ-aroGMU-H derivate, containing aroB-M module, aroD-M module, aroE-M module | This study              |
Discussion
Several methods, such as overexpression of aro genes [31, 32] and the use of enzymes with improved properties [33], have been reported to enhance the metabolic flux into SA pathway, thus finally increase the production of aromatic amino acids or shikimic acid. This current study revealed a new synthetic biology strategy: Four aro genes were organized as continuous genetic modules and their transcriptions were coordinated by the same tac promoter, Riboj and terminator. The translation levels of aro genes in the genetic modules were regulated by their RBS, which were quantitatively characterized in this study.

RBS is vital to initiate genetic translation, and are useful synthetic biology parts for construction modules [16]. In this study, four tailored-made RBS libraries were constructed and the strength of each RBS sequence was determined in the background of C. glutamicum cells. Although the RBS libraries were tailored for aroG, aroB, aroD and aroE, it is believed that these RBS would be applicable also for other purposes when C. glutamicum was used as host. Similarly, the constructed SA pathway modules were tested for SA production in this study, they should be also useful for productions such as aromatic amino acids that are derived from SA pathway.

Table 1 Bacterial strains and plasmids used in this study (Continued)

| Plasmid   | Description                                                                 | Source      |
|-----------|-----------------------------------------------------------------------------|-------------|
| plasmid-3 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-H derivate, containing aroB-L module, aroD-L module, aroE-L module | This study  |
| plasmid-4 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-M derivate, containing aroB-H module, aroD-M module, aroE-L module | This study  |
| plasmid-5 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-M derivate, containing aroB-M module, aroD-L module, aroE-H module | This study  |
| plasmid-6 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-M derivate, containing aroB-L module, aroD-H module, aroE-M module | This study  |
| plasmid-7 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-L derivate, containing aroB-H module, aroD-L module, aroE-M module | This study  |
| plasmid-8 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-L derivate, containing aroB-M module, aroD-H module, aroE-L module | This study  |
| plasmid-9 | Plasmid pXMJ19-RiboJ-aroG<sup>MU</sup>-L derivate, containing aroB-L module, aroD-M module, aroE-H module | This study  |
| pXMJ19-GBTDE | Plasmid 2 derivate, containing a terminator between aroB and aroD Module | This study  |
| pXMJ19-GBTDE | Plasmid pXMJ19-GBTDE derivate, containing a terminator between aroD and aroE module | This study  |
| pXMJ19-GBTDE | Plasmid pXMJ19-GBTDE derivate, containing a terminator between aroG and aroB module | This study  |
| pXMJ19-GTBTDTE | Plasmid pXMJ19-GTBTDTE derivate, containing a terminator between aroG and aroB module | This study  |
SA is a highly valued commercial compound. Efforts were made to improve SA production by de-repressing of feedback inhibition of enzymes involved in SA synthesis [33], increasing glucose availability [34], and optimizing metabolic fluxes [31], with *E. coli* or *B. subtilis*. So far as we know, *C. glutamicum* has not been exploited for SA production. By implementing the constructed genetic modules in the shikimate kinase deficient mutant, *C. glutamicum* was successfully engineered to produce SA at 11.3 g/L in 5-L fermenter. So far, this represents the highest titer of SA production with *C. glutamicum*. The SA production with *C. glutamicum* is comparable to the productivity with *B. subtilis* (19.7 g/L) [35]. Although this SA titer is lower when compared to SA production by *E. coli* (84 g/L) [33], *C. glutamicum* is still a promising SA producer due to its non-pathogenic nature, and its productivity can be further improved by optimization of fermentation process, or by replacement of the tryptophan- and prephenate-sensitive DAHP synthase [36, 37].

**Conclusion**

Synthetic biology tool boxes for manipulating *C. glutamicum* were expanded by including 4 RBS libraries, in addition to the previous reported promoters [38, 39] and CoryneBrick [40]. The RBS libraries represent the first set of RBS libraries that were quantitatively characterized in *C. glutamicum*. The selected RBS and *aro* genes could be organized as continuous genetic modules and their transcriptions could be coordinated. Genetic modules were successful constructed for SA pathway, and were demonstrated to be useful for increase of SA synthesis. In fed-batch fermentation, *C. glutamicum* harboring newly constructed SA pathway modules achieved 11.3 g/L SA, which represented the highest SA production with *C. glutamicum*.

**Materials and methods**

**Microorganisms, plasmids, medium, and cultivation**

The bacterial strains and plasmids used in this study are listed in Table 1. *C. glutamicum* was cultivated at 30 °C in Luria Bertani (LB) [41] broth or Brain Heart Infusion (BHI) medium [42]. *E. coli* was cultivated at 37 °C in 50 mL of LB broth in 250-ml flasks on a rotary shaker at 200 rpm. When needed, chloramphenicol at a final concentration of 10 or 20 μg/mL in medium was used for cultivation of *C. glutamicum* or *E. coli*. Expression of genes with *C. glutamicum* was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

Fermentative production of shikimic acid with *C. glutamicum* was carried out in 250 mL flasks and 5-L fermenter (Bioflo Model 3000 bioreactor, New Brunswick Scientific, N.J., U.S.A.). Seeding cultures were grown with Medium A (g/L): K$_2$HPO$_4$·3H$_2$O (0.5); KH$_2$PO$_4$ (0.5); (NH$_4$)$_2$SO$_4$ (10); glucose (40); MgSO$_4$·7H$_2$O (0.2); phenylalanine (0.15); tyrosine (0.15); tryptophan (0.15); CaCO$_3$ (30); FeSO$_4$·7H$_2$O (0.02); MnSO$_4$·4H$_2$O (0.02); biotin (50 μg); thiamine (200 μg), pH 7.4.

Fermentation was conducted with Medium B (g/L): K$_2$HPO$_4$·3H$_2$O (0.5); KH$_2$PO$_4$ (0.5); Urea (3); sucrose (38); MgSO$_4$·7H$_2$O (0.2); Yeast extract (10); peptone (4); FeSO$_4$·7H$_2$O (0.02); MnSO$_4$·4H$_2$O (0.02); biotin (50 μg); thiamine (200 μg), pH 7.4. The fermenter was stirred at 300 rpm, aerated at 3.0 vol/vol per minute, and pH was maintained at 7.0. Cell growth was monitored by measuring optical density at 600 nm (OD$_{600}$)
Fig. 7 The growth (solid squares), sucrose consumption (open squares), productions of shikimic acid (circles) and 3-dehydroshikimic acid (open circles) with recombinant C. glutamicum RES167ΔaroK harboring pXMJ19-GBTDE, during shake-flask (a), batch (b), and fed-batch cultivation (c). Data are averages of three parallel fermentations.
with a spectrophotometer (Biospec-1601 DNA/Protein Enzyme Analyzer, Shimadzu). Cellular dry weights were determined by centrifugation and lyophilization with 3 parallel samples.

*C. glutamicum* was cultivated in mineral salts (MS) medium when RBS strength were tested. The MS medium contained following components (g/L, pH 8.0): Na$_2$HPO$_4$·12H$_2$O (2); KH$_2$PO$_4$ (0.5); MgSO$_4$·7H$_2$O (0.03); NH$_4$Cl (0.53); trace element solution 2 mL. Trace element solution (g/L, pH 6.0): EDTA, (0.5); ZnSO$_4$·7H$_2$O, (0.22); CaCl$_2$, (0.055); MnCl$_2$·4H$_2$O, (0.051); FeSO$_4$·7H$_2$O, (0.0499); (NH$_4$)$_2$Mo$_7$O$_{24}$·4H$_2$O, (0.011); CuSO$_4$·5H$_2$O, (0.0157); CoCl$_2$·6H$_2$O, (0.0161); biotin (0.0125); thiamine (0.05).

### DNA extraction, amplification, plasmid construction and genetic transformation

Plasmid and chromosomal DNAs were isolated using the OMEGA Plasmid Mini Kit and the OMEGA Bacterial DNA Kit (Omega genetics, Beijing), respectively. DNA fragments from PCR amplification were purified with the OMEGA Cycle-Pure Kit (Omega genetics, Beijing). Restriction enzymes, ligases and other DNA-manipulating enzymes were used according to their manufacturer’s instructions. Genetic transformation of *C. glutamicum* and *E. coli* was carried out by electroporation, and recombinant strains were selected according to Tauch *et al.* [43].

#### Construction of pXMJ19-aroG$^{\text{MU}}$, pXMJ19-aroD$^{\text{MU}}$, pXMJ19-aroB$^{\text{MU}}$ and pZB

The *aro* genes, *i.e.*, *aroG* (GenBank accession number, NP_601382.1), *aroB* (NP_600835.1), *aroD* (NP_599670.1), and *aroE* (NP_600843.1) were PCR amplified from genomic DNA of *C. glutamicum* RES167 using primers listed in Table 2. Subsequently, these *aro* genes were cloned into pXMJ19, generating pXMJ19-aroG, pXMJ19-aroB, pXMJ19-aroD, and pXMJ19-aroE. For subsequent cloning, the following silent mutations were made with primers listed in Table 1: the HindIII and PstI of pXMJ19-aroB, XmaI and EcoRI of pXMJ19-aroD, and Sall and BamH1 of pXMJ19-aroE. The resulting plasmids were named pXMJ19-aroG$^{\text{MU}}$, pXMJ19-aroB$^{\text{MU}}$, pXMJ19-aroD$^{\text{MU}}$, and pXMJ19-aroE$^{\text{MU}}$.

pZB was derived from pXMJ19. Chemically synthesized gene of Ribol (27) was cloned into pXMJ19 at HindIII and PstI sites, resulting in pXMJ19-Ribol. This pXMJ19-Ribol was digested with EcoRI and KpnI, and a genetic fragment encoding the enhanced green fluorescence protein was cloned at the KpnI and EcoRI sites. The resulting plasmid was named pZB, and was used for later construction of RBS libraries.

### Design and construction of RBS libraries tailored for *aroG*, *aroB*, *aroD* and *aroE*, and evaluation of RBS strength according to fluorescence intensity

Based on the currently known RBS sequences from *C. glutamicum*, we designed a seeding sequence of AAAGGN$_{6-9}$, where "N" represents any nucleotide of A, T, G, or C. From this seeding sequence, oligonucleotides tagged as MU-RBSAG-F, MU-RBSAB-F, MU-RBSAD-F, and MU-RBSAE-F, were chemically synthesized. These oligonucleotides and their partner primers (Table 2) were used to amplify the *aro* genes from plasmid pXMJ19-aroG$^{\text{MU}}$, pXMJ19-aroB$^{\text{MU}}$, pXMJ19-aroD$^{\text{MU}}$, pXMJ19-aroE$^{\text{MU}}$. The amplified *aro* genes, each had a specific RBS sequence at its 5’-end, were digested with restriction endonuclease and were cloned into the sameyl digested pZB. Thus, four RBS libraries were constructed and were named as pZB-aroG, pZB-aroB, pZB-aroD, and pZB-aroE (Fig. 2).

The strength of each RBS for genetic translation was determined according to its fluorescence intensity. *C. glutamicum* cells harboring single plasmid (thus a single RBS) of libraries of pZB-aroG, pZB-aroB, pZB-aroD, and pZB-aroE were cultivated in the presence of 0.5 mM IPTG at 30 °C in MS medium. After incubation for 48 h at 30 °C and 200 rpm, 200 μl of cell suspension was transferred into a 96-well plate. The fluorescence from the eGFP in *C. glutamicum* cells and optical density were measured using a BioTek’ synergy H4 Hybrid Reader (Keruiente, Beijing, China).

#### Construction of genetic modules for SA pathway

To construct the nine plasmids with the combination of different strength RBS, *aroG* gene with high, middle and low strength RBS were amplified from pXMJ19-aroG$^{\text{MU}}$ and cloned between Sall and BamH1 cloning sites of plasmid pXMJ19-Ribol. These three plasmids were named as pXMJ19-Ribol-aroG$^{\text{MU}}$-H, pXMJ19-Ribol-aroG$^{\text{MU}}$-M, and pXMJ19-Ribol-aroG$^{\text{MU}}$-L, respectively. Taking the same way, we got plasmids pXMJ19-Ribol-aroB$^{\text{MU}}$-H, pXMJ19-Ribol-aroB$^{\text{MU}}$-M, pXMJ19-Ribol-aroB$^{\text{MU}}$-L, pXMJ19-Ribol-aroB$^{\text{MU}}$-H, pXMJ19-Ribol-aroB$^{\text{MU}}$-M, pXMJ19-Ribol-aroB$^{\text{MU}}$-L, pXMJ19-Ribol-aroD$^{\text{MU}}$-H, pXMJ19-Ribol-aroD$^{\text{MU}}$-M, and pXMJ19-Ribol-aroD$^{\text{MU}}$-L, which also have the high, middle and low strength RBS, accordingly. Then, Pta-Ribol-aroB$^{\text{MU}}$-H fragments with BamH1 and Xmal sites were amplified from plasmid pXMJ19-Ribol-aroB$^{\text{MU}}$-H and cloned into plasmid pXMJ19-Ribol-aroG$^{\text{MU}}$-H, resulting plasmid named pXMJ19-GHBBH. Then fragments Pta-Ribol-aroD$^{\text{MU}}$-H with Xmal and KpnI sites were cloned into plasmid pXMJ19-GHBBH, resulting plasmid named pXMJ19-GHBBHDH. From plasmid pXMJ19-Ribol-aroE$^{\text{MU}}$-H we got fragments Pta-Ribol-aroE$^{\text{MU}}$. H with KpnI and EcoRI sites and cloned the fragments...
| Primers | Sequences | Notes |
|---------|-----------|-------|
| **aroG-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroG from genome, SalI and KpnI underlined |
| **aroG-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroG from genome, SalI and KpnI underlined |
| **aroB-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB from genome, SalI and KpnI underlined |
| **aroB-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB from genome, SalI and KpnI underlined |
| **aroD-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroD from genome, SalI and KpnI underlined |
| **aroD-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroD from genome, SalI and KpnI underlined |
| **aroE-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroE from genome, SalI and KpnI underlined |
| **aroE-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroE from genome, SalI and KpnI underlined |
| **MU-aroG-1-F** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate HindIII in aroG |
| **MU-aroG-1-R** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate HindIII in aroG |
| **MU-aroG-2-F** | TCGCGCCACATGAAATGGGTTGATGTTG | Amplification of aroG from genome, SalI and KpnI underlined |
| **MU-aroG-2-R** | TCGCGCCACATGAAATGGGTTGATGTTG | Amplification of aroG from genome, SalI and KpnI underlined |
| **MU-aroG-3-F** | TCGCGCCACATGAAATGGGTTGATGTTG | Amplification of aroG from genome, SalI and KpnI underlined |
| **MU-aroG-3-R** | TCGCGCCACATGAAATGGGTTGATGTTG | Amplification of aroG from genome, SalI and KpnI underlined |
| **MU-aroB-1-F** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate BamHI in aroB |
| **MU-aroB-1-R** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate BamHI in aroB |
| **MU-aroD-1-F** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate BamHI in aroD |
| **MU-aroD-1-R** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate BamHI in aroD |
| **MU-aroE-2-F** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate EcoRI in aroE |
| **MU-aroE-2-R** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate EcoRI in aroE |
| **MU-aroE-3-F** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate EcoRI in aroE |
| **MU-aroE-3-R** | GGCCTTACCCGTTGCAACAATCCACGACCTTCCTGCTCAGCTCAAGTACCC | Mutate EcoRI in aroE |
| **Ribof-J-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of Ribof from pUC19, HindIII and PstI underlined |
| **Ribof-J-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of Ribof from pUC19, HindIII and PstI underlined |
| **EGFP-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of egfp from pACGFP, KpnI and EcoRI underlined |
| **EGFP-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of egfp from pACGFP, KpnI and EcoRI underlined |
| **MU-BSAG-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAG-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAB-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAB-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAD-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAD-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAE-F** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **MU-BSAE-R** | CGCGCGTACATGAAATGGGTTGATGTTG | Amplification of aroB with mutated RBS, SalI and KpnI underlined |
| **aroG-H-F** | CGCGCGTACATGAAATGGGTTGATGTTG | aroG with high strength RBS, SalI and BamHI underlined |
| **aroG-H-R** | CGCGCGTACATGAAATGGGTTGATGTTG | aroG with high strength RBS, SalI and BamHI underlined |
| **aroG-M-F** | CGCGCGTACATGAAATGGGTTGATGTTG | aroG with medium strength RBS, SalI and BamHI underlined |
| **aroG-M-R** | CGCGCGTACATGAAATGGGTTGATGTTG | aroG with medium strength RBS, SalI and BamHI underlined |
| **aroB-H-F** | CGCGCGTACATGAAATGGGTTGATGTTG | aroB with low strength RBS, SalI and BamHI underlined |
| **aroB-H-R** | CGCGCGTACATGAAATGGGTTGATGTTG | aroB with low strength RBS, SalI and BamHI underlined |
into plasmid pXMJ19-GHBHDH, resulting plasmid named plasmid-1. Plasmid-2 to plasmid-9 and derive plasmids were also got by the way describe above. Three terminator fragments with XmaI, BamHI and KpnI cloning sites were amplified from plasmid pXMJ19, respectively. After terminator with XmaI site was cloned in plasmid-2, we got plasmid pXMJ19-GBTDE. Then terminator with BamHI site was cloned in plasmid pXMJ19-GBTDE to get plasmid pXMJ19-GTBTDTE. Plasmid pXMJ19-GTBTDTE was constructed by cloning terminator with KpnI site.

### Measurement of SA dehydrogenase activity

The enzyme activities of the shikimate dehydrogenases were assayed by monitoring the absorbance of NADPH at 340 nm ($\varepsilon = 6230 \text{ M}^{-1} \text{ cm}^{-1}$) using a spectrophotometer (Specord 205 Analytk, Jena, Germany). The assays were conducted at 25 °C in a volume of 1 mL solution, containing 100 mM Tris–HCl buffer at pH 8.0, 1 mM NADP+, 2 mM SA, and 2 mM NADP+. Cellular lysates from C. glutamicum were added finally to trigger the reaction. One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of NADP+ per minute at 25 °C.

### Oligonucleotides used in this study (Continued)

| Oligonucleotide | Sequence |
|-----------------|----------|
| aroB-M-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroB-M-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| aroB-L-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroB-L-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| aroB-H-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroB-H-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| aroD-M-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroD-M-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| aroD-L-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroD-L-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| aroE-M-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroE-M-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| aroE-L-F        | CGCGGCCTGACAAAGGCATGTTCATGACGCGCAGTGCAGATTTTC |
| aroE-L-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| PB-F            | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| PB-R            | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| PD-F            | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| PD-R            | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| PE-F            | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| PE-R            | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| Terminator 1-F  | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| Terminator 1-R  | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| Terminator 2-F  | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| Terminator 2-R  | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| Terminator 3-F  | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| Terminator 3-R  | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| acok-F          | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| acok-R          | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| KTaroK-F        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| KTaroK-R        | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| V-KTaroK-F      | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |
| V-KTaroK-R      | CGCGGCATCTTCAGTGTGCTGTGCTAAGAAGCAGAAGCGG |

| aroB with medium strength RBS, SalI and BamHI underlined |
| aroB with low strength RBS, SalI and BamHI underlined |
| aroD with medium strength RBS, SalI and BamHI underlined |
| aroD with high strength RBS, SalI and BamHI underlined |
| aroE with medium strength RBS, SalI and BamHI underlined |
| aroE with high strength RBS, SalI and BamHI underlined |
| PB with medium strength RBS, SalI and BamHI underlined |
| PB with low strength RBS, SalI and BamHI underlined |
| PB with medium strength RBS, SalI and BamHI underlined |
| PB with low strength RBS, SalI and BamHI underlined |
| PB with medium strength RBS, SalI and BamHI underlined |
| PB with low strength RBS, SalI and BamHI underlined |
| PB with medium strength RBS, SalI and BamHI underlined |
| PB with low strength RBS, SalI and BamHI underlined |
| XmaI and KpnI underlined |
| XmaI and KpnI underlined |
| KpnI and EcoRI underlined |
| XmaI underlined |
| XmaI underlined |
| KpnI underlined |
| KpnI underlined |
| KpnI underlined |
| EcoRI and HindIII underlined |
| XmaI underlined |
| XmaI underlined |
| Primer used to verify ΔaroK |
| Primer used to verify ΔaroK |
For preparation of cellular lysates of *C. glutamicum*, cells were harvested by centrifugation (6000 g, 4 °C, 5 min) of culture samples. Supernatants were removed, the cell pellets were washed and re-suspended in 50 mM pH 8.0 Tris--HCl buffer. This cell suspension was subjected to sonication (Ningbo Sciento Biotechnology Co., LTD, China) and centrifugation (12,000 g, 4 °C, 10 min). The supernatants were collected and used for enzyme assays. Protein concentrations were determined using Bradford method [44].

**Construction of *C. glutamicum* RES167ΔaroK**

Disruption of the shikimate kinase gene, aroK, in *C. glutamicum* was performed using the suicide vector pK18mobsaC. The intact DNA fragment (2946 bp) of aroK was amplified from chromosomal DNA of *C. glutamicum*, using the primers aroK-F and aroK-R (Table 1). This intact aroK fragment was cloned into pK18mobsaC EcoRI/HindIII sites. The resulting plasmid was named pK18mobsaC-aroK, and was amplified with primers K TaroK-F and K TaroK-R, thus resulting DNA fragments with disrupted aroK gene. After digested with Xmal restriction endonuclease, DNA fragments were ligated and transformed into *E. coli*. The recombinant plasmid was named pK18mobsaC-ΔaroK and was electroporated into *C. glutamicum* RES167. Using the method described by Schäfer et al. [45], the aroK mutant RES167ΔaroK was screened out on BHI agar plates. The Disruption of aroK was verified by PCR amplification and sequence of the disrupted aroK gene from RES167ΔaroK.

**Determination of SA and 3-dehydroshikimic acid concentrations**

The concentrations of SA and 3-dehydroshikimic acid were determined with an HPLC system (Agilent 1200 series, Agilent Technologies, Inc., USA) equipped with a ZORBAX SB C18 column (4.6 mm x 250 mm x 5 μm) and detected at 215 nm wavelength. The HPLC was run with a mixture of solution A (phosphoric acid in water, pH 2.5) and solution B (methanol) as eluant and was operated at a flow rate of 0.35 mL/min. The following gradient was used: at 0–7.5 min, 95 % of solution A and 5 % of solution B; at 7.5–15 min, 100 % of solution B; at 15.0–22.5 min, 95 % of solution A and 5 % of solution B. Standard shikimic acid (Cat. No. S5375, Sigma-Aldrich, USA) and 3-dehydroshikimic acid (Cat. No. 05616, Sigma-Aldrich, USA) were eluted at 5.411 and 6.241 min, respectively, under these conditions.

**Determination of sucrose concentrations**

The sucrose concentrations in fermentation broth were determined with spectrometric method, as previously described [46].

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

BZ and NZ carried out the experimental work, BZ drafted the manuscript. YML and CL and CBL participated in experimental design. CTJ and SJL supervised the research and finalized the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by 973 Project from Ministry of Science and Technology (No. 2012CB7211-04).

**Author details**

1State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beichen-Xilu No.1, 100101 Beijing, PR China.

2CAS Key Laboratory of Microbial Physiology and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, 100101 Beijing, PR China.

3University of Chinese Academy of Sciences, 100101 Beijing, PR China.

**Received:** 3 March 2015 **Accepted:** 6 May 2015

**Published online:** 17 May 2015

**References**

1. Herrmann KM. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell. 1995;7:907–19.

2. Näränen M, Bongaerts J, Bovenberg R, Kremer S, Müller U, Of S, et al. Metabolic engineering for microbial production of shikimic acid. Metab Eng. 2003;5:277–83.

3. Tzin V, Galili G. New insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. Mol Plant. 2010;3:956–72.

4. Quiróz DCD, Carmona SB, Bolívar F, Escalante A. Current perspectives on applications of shikimic and aminoshaminic acids in pharmaceutical chemistry. Rep Prog Chem. 2014;68(3–46).

5. Weber C, Bruckner C, Weinreb S, Lehr C, Endl C, Boles E. Biosynthesis of cis, cis-Muconic acid and its aromatic precursors, catechol and protocatechue acid, from renewable feedstocks by Saccaromyces cerevisiae. Appl Environ Microbiol. 2012;78:421–30.

6. Liao HF, Lin LL, Chien HR, Hsu WH. Serine 187 is a crucial residue for allosteric of Corynebacterium glutamicum 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. FEMS Microbiol Lett. 2001;194:59–64.

7. Han MA, Lee HS, Cheon CI, Min KH MSL. Cloning and analysis of the aroB gene encoding dehydroquinate synthase from Corynebacterium glutamicum. Can J Microbiol. 1999;45:885–90.

8. Liu C, Liu YM, Sun QL, Jiang C, Liu SJ. Unwaveling the kinetic diversity of microbial 3-dehydroquinate dehydratases of shikimate pathway. AAB Express. 2015;5:1–7.

9. Kubota T, Tanaka Y, Hiraga K, Inui M, Yukawa H. Characterization of shikimate dehydrogenase homologues of Corynebacterium glutamicum. Appl Microbiol Biotechnol. 2013;97:839–49.

10. Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, et al. Insoprenoid pathway optimization for Taxol precursor overproduction in Eschenichia coli. Science. 2010;330:70–6.

11. Brophy JA, Voigt CA. Principles of genetic circuit design. Nat Methods. 2014;11:508–20.

12. Schindelior ZG, Dipping M, Gruberger A, Kohlheyer D, Yoshida A, Binder S, et al. Taking control over control: Use of product sensing in single cells to remove flux control at key enzymes in biosynthesis pathways. ACS Synth Biol. 2014;3:21–9.

13. Oyarzun DA, Stan GB. Synthetic gene circuits for metabolic control: design trade-offs and constraints. J R Soc Interface. 2012;10:13–26.

14. Mutalik VK, Guimaraes JC, Cambray G, Wang J, d'Inverno T, Mai QA, et al. Precise and reliable gene expression via standard transcription and translation initiation elements. Nat Methods. 2013;10:354–60.

15. Chen YJ, Liu P, Nielsen AA, Brophy JA, Clancy K, Peterson T, et al. Characterization of S82 natural and synthetic terminators and quantification of their design constraints. Nat Methods. 2013;10:659–64.

16. Nowroooz FF, Baidoo EE, Ernakov S, Redding-Johnson AM, Bath B, Pettold CI, et al. Metabolic pathway optimization using ribosome binding site variants and combinatorial gene assembly. Appl Microbiol Biotechnol. 2014;98:1567–81.
combined with optical nanosensors: a general strategy for fast producer strain generation. Nucleic Acids Res. 2013;41:6360–9.

18. Krause FS, Blomback B, Eikmanns BJ. Metabolic engineering of Corynebacterium glutamicum for 2-ketosovalerate production. Appl Environ Microbiol. 2010;76:8903–61.

19. Stabler N, Oikawa T, Bott M, Eggeling L. Corynebacterium glutamicum as a host for synthesis and export of D-Amino Acids. J. Bacteriol. 2011;193:1702–9.

20. Rittmann D, Lindner SN, Wendisch VF. Engineering of a glycerol utilization pathway for amino acid production by Corynebacterium glutamicum. Appl Environ Microbiol. 2008;74:216–22.

21. Rakavi P, Peiu S, Gramajo H, Merzella HG. Design and testing of a synthetic biology framework for genetic engineering of Corynebacterium glutamicum. Microb Cell Fact. 2012;11:147.

22. Litsanov B, Kabus A, Brocker M, Bott M. Efficient aerobic succinate production from glucose in minimal medium with Corynebacterium glutamicum. Microb Biotechnol. 2012;5:116–28.

23. Weschalka S, Blomback B, Bott M, Eikmanns BJ. Bio-based production of organic acids with Corynebacterium glutamicum. Microb Biotechnol. 2013;6:87–102.

24. Wieschalka S, Blomback B, Eikmanns BJ. Engineering Corynebacterium glutamicum for the production of pyruvate. Appl Microbiol Biotechnol. 2012;94:449–59.

25. Aronador E, Castro JM, Corêa A, Martin JF. Structure and organization of the rrnD operon of Brevibacterium lactofermentum: analysis of the 16 s rRNA gene. Microbiology. 1999;145:915–24.

26. Martin JF, Barreiro C, González-Lavado E, Banruoso M. Ribosomal RNA and ribosomal proteins in Corynebacter. J Biotecn. 2003;104:41–53.

27. Pfeifer-Sancar K, Mentz A, Rückert C, Kalinowski J. Comprehensive analysis of the Corynebacterium glutamicum transcriptome using an improved RNAseq technique. BMC Genomics. 2013:14.

28. Liu Q, Quyang SP, Kim J, Chen GQ. The impact of PHB accumulation on L-glutamate production by recombinant Corynebacterium glutamicum. J Biotecnol. 2007;132:237–9.

29. Cinelli RAG, Ferrari A, Beltram F, Pellegrini V, Tyagi M, Giacca M. The enhanced green fluorescent protein as a tool for the analysis of protein dynamics and localization: local fluorescence study at the single-molecule level. Photosynthet Photobiol. 2000;71:771–6.

30. Lou C, Stanton B, Chen YJ, Minsky B, Voigt CA. Ribozyme-based insulator parts buffer synthetic circuits from genetic context. Nat Biotechnol. 2012;30:1137–42.

31. Liu DF, Ai GM, Zheng QX, Liu C, Jiang CY, Liu LX, et al. Metabolic flux responses to genetic modification for shikimic acid production by Bacillus subtilis strains. Microb Cell Fact. 2014;13:40.

32. Cui YY, Ling C, Zhang YY, Huang J, Liu JZ. Production of shikimic acid from Corynebacterium glutamicum cells with an engineered glucose metabolism. J Biotechnol. 2007;132:273–82.

33. Escalante A, Calderon R, Valdivia A, de Anda R, Hernandez G, Ramirez OT, et al. Metabolic engineering for the production of shikimic acid in an evolved Escherichia coli strain lacking the phospho-enolpyruvate carboxykinase and phosphoglucomutase. Microb Cell Fact. 2010;9:21.

34. Iomantas YAV, Abalakina EG, Polanueva BM, Yampolskaya TA, Bachina TA, Koobov YI. Method for producing shikimic acid. U.S. 2002.

35. Liu YJ, Li PP, Zhao XW, Wang BJ, Liang CY, Drake HL, et al. Corynebacterium glutamicum strain constitutive expression of shikimate 3-dehydrogenase and chorismate mutase endows new regulation on DAHP synthase activity in Corynebacterium glutamicum. Appl Environ Microbiol. 2013;79:10373–80.

36. Pauling J, Rotger R, Tauch A, Azevedo V, Baumbach J. CoryneRegNet 6.0–Updated database content, new analysis methods and novel features focusing on community demands. Nucleic Acids Res. 2012;40:D610–4.

37. Patek M, Holatko J, Busche T, Kalinowski J, Nesvera J. Corynebacterium glutamicum promoters: a practical approach. Microb Biotechnol. 2013;6:103–17.