

Enhancement of 5-aminolevulinic acid production by metabolic engineering of the glycine biosynthesis pathway in *Corynebacterium glutamicum*

Yalan Zou · Tao Chen · Lili Feng · Shuanghong Zhang · Dongxu Xing · Zhiwen Wang

Received: 3 March 2017 / Accepted: 18 May 2017 / Published online: 23 May 2017
© Springer Science+Business Media Dordrecht 2017

Abstract

Objective To construct a strain of *Corynebacterium glutamicum* capable of efficiently producing 5-aminolevulinic acid (5-ALA) via the C4 pathway by modification of serine and glycine pathway using glucose as sole carbon source.

Results The recombinant *C. glutamicum* strain AP2 harboring a codon-optimized *hema* gene from *Rhodobacter sphaeroides* was used as host strain for 5-ALA production. A plasmid harboring the serine operon, which contained *serB*, *serC* and the site-specific mutant *serA*^{Δ197}, was constructed and introduced into *C. glutamicum* AP2, leading to an increase of 70% in 5-ALA production. Further overexpression of the *glyA* gene increased production of 5-ALA by 150% over the control. 5-ALA production was thus significantly enhanced by engineering the glycine

biosynthetic pathway. *C. glutamicum* AG3 produced 3.4 ± 0.2 g 5-ALA/l in shake-flask cultures in CGIIIM medium with the addition of 7.5 g glycine/l. **Conclusion** This is the first report of remodeling the serine and glycine biosynthetic pathway to improve the production of 5-ALA in *C. glutamicum*.

Keywords 5-Aminolevulinic acid · *Corynebacterium glutamicum* · Glycine synthesis · Serine pathway

Introduction

5-Aminolevulinic acid (5-ALA) is a common precursor in the biosynthetic pathways of porphyrin, heme and vitamin B₁₂, etc. (Kiatpapan and Murooka 2001). 5-ALA has attracted attention as it can be used in many fields, including as a photosensitizer in medicine, a herbicide or insecticide, as well as improving

Electronic supplementary material The online version of this article (doi:10.1007/s10529-017-2362-x) contains supplementary material, which is available to authorized users.

Y. Zou · T. Chen · L. Feng · S. Zhang · Z. Wang (✉)
Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, Peiyang Park, Tianjin 300354, People's Republic of China
e-mail: zww@tju.edu.cn

Y. Zou · T. Chen · L. Feng · S. Zhang · Z. Wang
Key Laboratory of Systems Bioengineering (Ministry of Education), Tianjin University, Tianjin, People's Republic of China

Y. Zou · T. Chen · L. Feng · S. Zhang · Z. Wang
SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), School of Chemical Engineering and Technology, Tianjin University, Tianjin, People's Republic of China

D. Xing
Sericulture and Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, People's Republic of China

tolerance to salts and cold in plants (Fu et al. 2008; Kiatpapan and Murooka 2001).

Biological synthesis has been developed into a viable alternative to produce 5-ALA (Fu et al. 2008). There are two major biosynthetic pathways of 5-ALA: the Shemin pathway (C_4 pathway) and the C_5 pathway. In the C_4 pathway, 5-ALA is synthesized via the condensation of succinyl-CoA and glycine, which is catalysed by 5-ALA synthase (Kiatpapan and Murooka 2001) (Fig. 1). As one of the precursors of 5-ALA synthesis, glycine plays an important role in 5-ALA accumulation. The effect of glycine addition on 5-ALA production has been investigated using recombinant *Escherichia coli* (Chung et al. 2005; Fu et al. 2010; Qin et al. 2006). 5-ALA reached its highest yield when glycine was exhausted (Fu et al. 2008). The same conclusion was obtained in *Propionibacterium freudenreichii* (Kiatpapan and Murooka 2001). Feng et al. (2016) found that 5-ALA production by *Corynebacterium glutamicum* increased considerably in the presence of 7.5 g glycine/l. Addition of glycine was necessary in almost all of the reported 5-ALA production strategies that utilise the C_4 pathway in different microorganisms.

In *C. glutamicum*, 3-phosphoglycerate is oxidized to phosphohydroxypyruvate by phosphoglycerate dehydrogenase (encoded by *serA*) (Peters-Wendisch et al. 2002). Subsequently, a transamination step

catalysed by phosphoserine aminotransferase (encoded by *serC*) yields phosphoserine, after which phosphoserine is dephosphorylated by phosphoserine phosphatase (encoded by *serB*) to yield L-serine (Peters-Wendisch et al. 2005). Glycine is then synthesised via the conversion of L-serine by serine hydroxymethyltransferase (encoded by *glyA*) (Schweitzer et al. 2009; Simic et al. 2002). By contrast, L-serine dehydratase (encoded by *sdaA*) is involved in L-serine degradation, catalyzing the conversion of L-serine to pyruvate (Hemanshu et al. 2016; Lai et al. 2012; Xu et al. 2015; Zhang et al. 2014) (Fig. 1).

In our previous work, we engineered *C. glutamicum* AP2 harboring a codon-optimized *hemA* gene from *Rhodobacter sphaeroides* for 5-ALA production, in which all known genes responsible for the formation of acetate and lactate were deleted, *ppc* gene was overexpressed, *pbp1b* was deleted (Feng et al. 2016). In the present work, we reconstructed a 5-ALA-producing *C. glutamicum* strain by metabolic engineering of the glycine pathway. The deregulated *serA*^{A197}, *serB* and *serC* genes were firstly co-overexpressed in a single operon to increase the flux from serine towards glycine. Secondly, the *sdaA* gene was deleted to explore its role in 5-ALA accumulation. Thirdly, the *glyA* gene was overexpressed to validate its effect on 5-ALA production. The final engineered

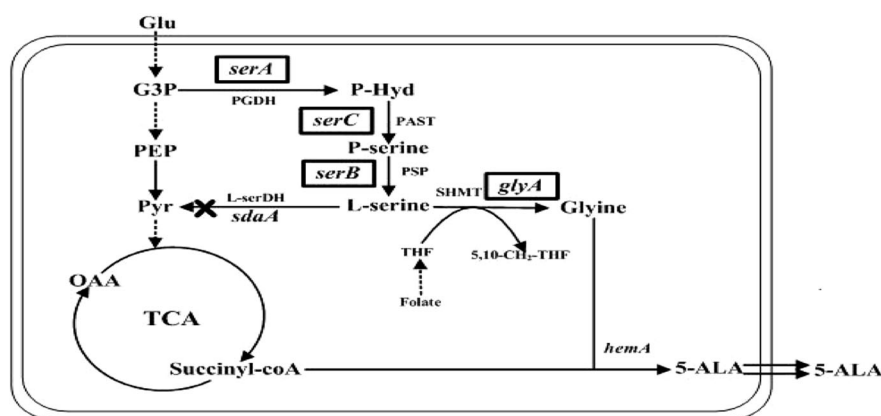


Fig. 1 Overview of the relevant pathways and engineering strategies for 5-ALA production via the C_4 pathway. Squares indicate genes that were overexpressed and the cross indicates a gene deletion. A codon-optimized *hemA* was derived from the corresponding *Rhodobacter sphaeroides* gene. Other non-standard abbreviations: *Glu* glucose, *G3P* glyceraldehyde 3-phosphate, *PEP* phosphoenolpyruvate, *Pyr* pyruvate, *OAA*

oxaloacetic acid, *PGDH* 3-phosphoglycerate dehydrogenase, *PAST* phosphoserine aminotransferase, *PSP* phosphoserine phosphatase, *SHMT* serine hydroxymethyltransferase, *L-SerDH* L-serine dehydratase, *P-Hyd* phosphohydroxypyruvate, *TCA* tricarboxylic acid cycle, *THF* tetrahydrofolate, *5,10-CH₂-THF* 5,10-methylenetetrahydrofolate

strain AG3 produced 3.4 ± 0.2 g 5-ALA/l in shake-flask cultures with the addition of 7.5 g glycine/l.

Materials and methods

Strains, media and culture conditions

The strains and plasmids used in this study are listed in Supplementary Table 1. *Corynebacterium glutamicum* ATCC 13,032 was the parent strain and *C. glutamicum* AP2 (Feng et al. 2016) was used as the chassis for 5-ALA production. *E. coli* DH 5 α was used to propagate vector DNA, and *E. coli* cells were cultured at 37 °C in lysogeny broth (LB) comprising 10 g tryptone/l, 5 g yeast extract/l and 10 g NaCl/l. Physiological characterizations were tested in defined CGXII medium (Feng et al. 2016; Unthan et al. 2014) supplemented with 10 g glucose/l. The CGXIIIM (CGXII with 7.5 g yeast extract/l) and CGIIM media, as described by Feng et al. (2016), were used for 5-ALA production in shake-flasks. Where appropriate, 0.5 mM IPTG, 10 μ g chloramphenicol/l and/or 25 μ g kanamycin sulfate/l was added to the media.

Construction of gene overexpression and deletion vectors

Fragments encoding *serA*^{A197}, *serB* and *serC* were amplified by PCR from *C. glutamicum* ATCC 13,032 genomic DNA using the primers *serA*^{A197}-F1 and *serA*^{A197}-R1, *serB*-F1 and *seB*-R1, *serC*-F1 and *seC*-R1, respectively. The amplified fragments were purified and subcloned into the constructed plasmid pXA, creating plasmid pXAA^{MBC}. Sequences upstream and downstream of *sdaA* were amplified from *C. glutamicum* genomic DNA using the primers *sdaA*-F1 and *sdaA*-R1, *sdaA*-F2 and *sdaA*-R2, respectively, and the two fragments were fused, purified and subcloned into pD-SacB, creating the plasmid pD-*sdaA*. The *sdaA* gene deletion was carried out as described previously (Zhu et al. 2013). The fragments encoding *glyA* were amplified from *C. glutamicum* genomic DNA using the primers *glyA*-F1 and *glyA*-R1, P*glyA*-F1 and P*glyA*-R1, T*glyA*-F1 and T*glyA*-R1, respectively. These amplified fragments were purified and subcloned into the plasmids pEC-XK99E, pEP2 and pEP2^{tuf}, generating the plasmids pEC-*glyA*, pEP-P*glyA* and pEP-T*glyA*. Primers used are listed in

Supplementary Table 2. The restriction endonuclease enzyme sites are underlined.

Cultivation conditions

The fermentation conditions were identical to the ones described by Feng et al. (2016). The fermentation experiments were carried out in 500 ml shake-flasks. The pre-seed culture was aseptically transferred into 50 ml of CGIIM medium with 10 g glucose/l in 500 ml shake-flasks and incubated for 10–12 h at 30 °C under constant orbital shaking at 220 rpm. An initial OD₆₀₀ value of 0.1 was used to inoculate the fermentation flasks.

Analytical methods

Supernatants were used directly for the determination of extracellular 5-ALA concentrations using modified Ehrlich's reagent (Feng et al. 2016; Kang et al. 2011). Cell concentrations were determined from the OD₆₀₀ values: 0.25 g cell dry weight (CDW)/l corresponded to an OD₆₀₀ value of 1. Glucose concentrations were measured using an SBA sensor machine (SBA-40E, Institute of Microbiology, Shandong, China) (Feng et al. 2016).

Results and discussion

Influence of the overexpression of a deregulated L-serine operon on 5-ALA accumulation

Peters-Wendisch et al. (2002) found that a truncation of the *serA* gene from *C. glutamicum* encompassing 197 amino acids at its C-terminus (encoded by the *serA*¹⁹⁷ allele) provided a 3-phosphoglycerate dehydrogenase devoid of feedback inhibition by L-serine. We thus deregulated the feedback inhibition of 3-phosphoglycerate dehydrogenase by L-serine, by truncating the 197 C-terminal amino acids of *serA* from *C. glutamicum*, and investigated whether the co-overexpression of *serA*^{A197}, *serB* and *serC* was able to increase 5-ALA accumulation in *C. glutamicum*. Accordingly, *serA*^{A197}, *serB* and *serC* were overexpressed to redirect additional carbon flux into the L-serine biosynthesis pathway, since it is the precursor of glycine, and also to decrease the secretion of by-products (Gu et al. 2014).

Corynebacterium glutamicum AG1, which harbors pXAA^{MBC}, was constructed to test the influence of the deregulated L-serine operon on 5-ALA accumulation. *C. glutamicum* AG1 produced 220 mg 5-ALA/l of with 10 g glucose/l without the addition of glycine, which was 69% higher than in the control strain AP2 (Fig. 2a), indicating that the co-overexpression of *serA*^{A197}, *serB* and *serC* was indeed beneficial for 5-ALA accumulation in *C. glutamicum*. The observed increase was most likely directly caused by an increase of the intracellular L-serine pool due to the co-overexpression of L-serine synthetic genes (Gu et al. 2014; Peters-Wendisch et al. 2005).

Effect of *sdaA* deletion on 5-ALA accumulation

Overexpression of the deregulated L-serine operon clearly increased the accumulation of 5-ALA. As shown in Fig. 1, L-serine was converted by serine hydroxymethyltransferase to generate glycine (Schweitzer et al.

2009). At the same time, L-serine dehydratase converted L-serine to pyruvate, and thus depleted the available L-serine pool (Netzer et al. 2004; Peters-Wendisch et al. 2005). In agreement with this, a deletion of the *sdaA* gene increased L-serine accumulation (Xu et al. 2015).

Thus, in order to increase the supply of glycine for 5-ALA production, the *sdaA* gene was knocked out in *C. glutamicum* AG1, yielding strain AG2. The resulting strain, however, did not exhibit any increase in the accumulation of 5-ALA. On the contrary, the production decreased significantly (Fig. 2b). Furthermore, the final biomass of AG2 decreased by 40% compared to the control strain AG1. This may be due to the fact that a knockout of *sdaA* can slightly increase L-serine production in *C. glutamicum* (Gu et al. 2014; Netzer et al. 2004). Nevertheless, the deletion of *sdaA* can markedly decrease the pyruvate concentration (Gu et al. 2014). Thus, a deletion of *sdaA* showed a negative effect on the growth of AG2 (Fig. 2b), which is in agreement with the literature (Zhu et al. 2013).

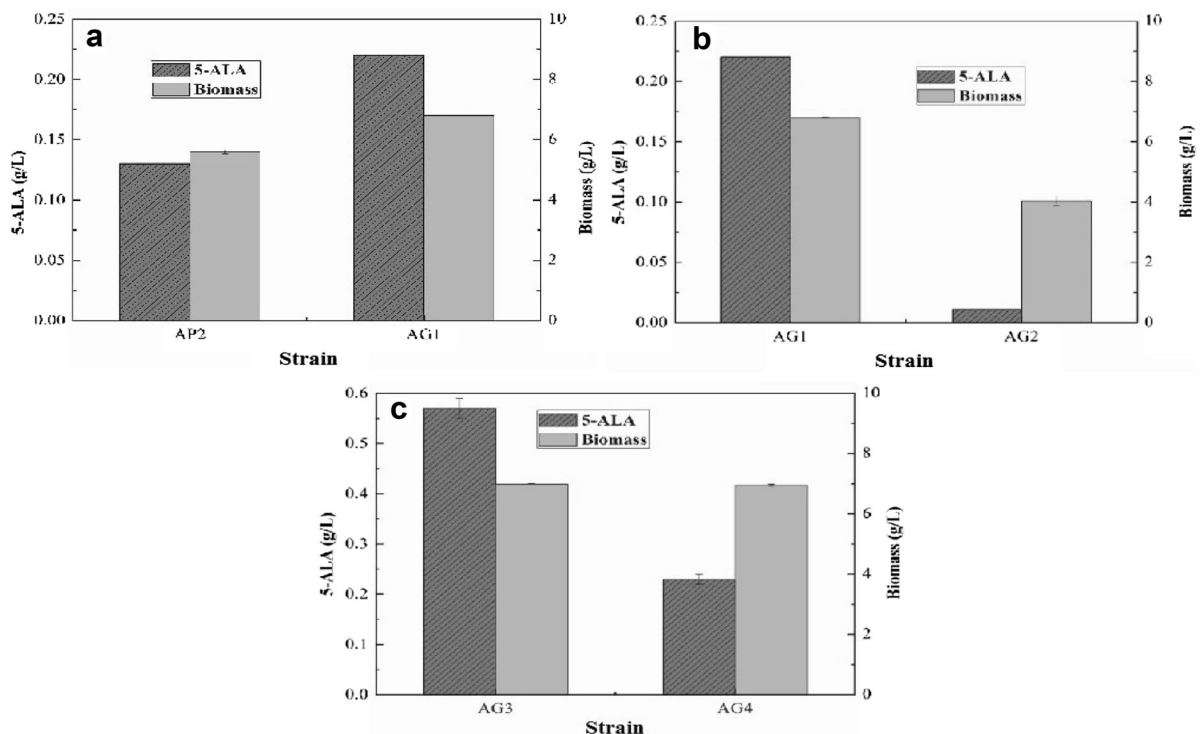


Fig. 2 Effects of gene regulation on biomass and 5-ALA production. **a** Overexpression of the serine operon. **b** Deletion of *sdaA*. **c** Overexpression of *glyA*. The cells were cultivated in CGXII medium supplemented with 10 g glucose/l. IPTG at

0.5 mM was added into the medium when OD₆₀₀ reached 1. Standard deviations were calculated from the results of three independent experiments

Therefore, the *sdaA* gene was not deleted in further *C. glutamicum* strains engineered for 5-ALA production.

Influence of *glyA* overexpression on 5-ALA accumulation

Since glycine is one of the precursors of 5-ALA, its supply of glycine is vital for the accumulation of 5-ALA. Schweitzer et al. (2009) concluded that the overexpression of *glyA* is essential to catalyse sufficient conversion of L-serine to glycine (Schweitzer et al. 2009). We thus overexpressed *glyA* to investigate whether it can improve 5-ALA production in *C. glutamicum*.

Consequently, the *C. glutamicum* strain AG3 and the corresponding control strain AG4, which harbor pEC-*glyA* and pEC-XK99E, respectively, were constructed based on AG1. To our delight, 5-ALA production of AG3 reached 590 mg/l, which was 2.5 times of the control. Importantly, the overexpression of *glyA* also had no effect on growth, and the biomass of the engineered strain was almost at the same level as the control (Fig. 2c). These results thus clearly demonstrate that the overexpression of *glyA* is beneficial for 5-ALA accumulation.

In order to investigate whether the synthesis of serine or the further *glyA* reaction is the limiting step for 5-ALA production in *C. glutamicum*, *glyA* was overexpressed with different promoters and different concentrations of serine were added to the medium for 5-ALA production. The results showed that 5-ALA production by strain AG5 was increased by nearly 60% over that of AG6 with overexpression of *glyA* under control of strong promoter *tuf*. The accumulation of

5-ALA was decreased when serine was added (data not shown). These results imply that the *glyA* reaction rather than synthesis of serine maybe the limiting step in glycine synthesizing pathway for 5-ALA production. However, the enzyme activity of SHMT should be controlled in an appropriate rang, not the more the better, and the excess serine addition can lead to cellular toxicity.

Glycine is the key factor of 5-ALA production

5-ALA production was significantly increased by engineering the glycine biosynthesis pathway. Nevertheless, the available glycine pool still seemed insufficient for high-level production of 5-ALA. In our previous work, the effect of glycine addition was investigated, and the 5-ALA production of AP2 reached 2.7 g/l in CGIIIM medium with 7.5 g glycine/l. However, the production of 5-ALA was not as high as expected in *C. glutamicum* AG3, which contains the engineered glycine biosynthetic pathway. We therefore studied how further, external supplementation of glycine would affect 5-ALA production in the engineered strain *C. glutamicum* AG3.

As shown in Fig. 3, supplementation of 7.5 g glycine/l was still optimal for the production of 5-ALA (Feng et al. 2016). The engineered strain *C. glutamicum* AG3 produced 3.4 ± 0.2 g 5-ALA/l with the addition of 7.5 g glycine/l in CGIIIM (Fig. 3a). This, to our knowledge, represents the highest yield obtained in *C. glutamicum* shake-flask cultures to date. Furthermore, *C. glutamicum* AG3 produced 2.6 ± 0.1 g 5-ALA/l with 5 g glycine/l, which was almost the same level as was achieved by AP2 with 7.5 g glycine/l (Feng et al. 2016). In contrast to

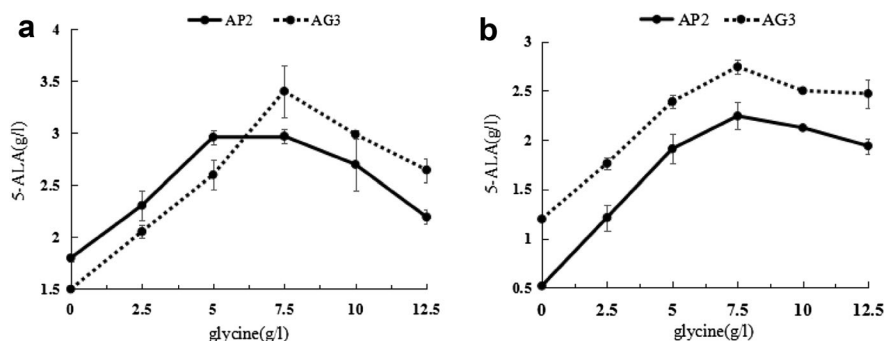


Fig. 3 Relationship between glycine addition and 5-ALA production in CGIIIM (a) and CGXIIIM (b) medium. The media were supplemented with 10 g glucose/l and the indicated amounts

of glycine. A final concentration of 0.5 mM IPTG was added to the medium when the OD_{600} reached 1. Standard deviations were calculated from the results of three independent experiment

the results in CGIIIM (Fig. 3a), *C. glutamicum* AG3 produced much more 5-ALA than the control strain when grown in CGXIIIM with different concentrations of glycine (Fig. 3b). These results thus imply that the supply of glycine is critical for 5-ALA production via the C4 pathway.

Conclusions

A 5-ALA producing *C. glutamicum* strain was constructed by metabolic engineering of the glycine biosynthesis pathway. The final strain produced 3.4 ± 0.2 g 5-ALA/l in 50 ml flask fermentations with the addition of 7.5 g glycine/l, which to our best knowledge represents the highest yield obtained in *C. glutamicum* shake-flasks to date. Furthermore, we confirmed that the supply of glycine is the most critical factor for 5-ALA production in *C. glutamicum* via the C4 pathway. Consequently, further metabolic strategies should be employed to enhance the intracellular glycine pool, in order to enhance 5-ALA production and reduce the necessary supplementation of this relatively expensive precursor.

Acknowledgements This work was supported by the Natural Science Foundation of Tianjin (No. 15JCQNJC06000).

Supporting information Supplementary Table 1—Strains and plasmids used.

Supplementary Table 2—Primers used.

References

- Chung S-Y, Seo K-H, Rhee JI (2005) Influence of culture conditions on the production of extra-cellular 5-aminolevulinic acid (ALA) by recombinant *E. coli*. *Proc Biochem* 40:385–394
- Feng L, Zhang Y, Fu J, Mao Y, Chen T, Zhao X, Wang Z (2016) Metabolic engineering of *Corynebacterium glutamicum* for efficient production of 5-aminolevulinic acid. *Biotechnol Bioeng* 113:1284–1293
- Fu W, Lin J, Cen P (2008) Enhancement of 5-aminolevulinic acid production with recombinant *Escherichia coli* using batch and fed-batch culture system. *Bioresour Technol* 99:4864–4870
- Fu W, Lin J, Cen P (2010) Expression of a *hemA* gene from *Agrobacterium radiobacter* in a rare codon optimizing *Escherichia coli* for improving 5-aminolevulinic acid production. *Appl Biochem Biotechnol* 160:456–466
- Gu P, Yang F, Su T, Li F, Li Y, Qi Q (2014) Construction of an L-serine producing *Escherichia coli* via metabolic engineering. *J Ind Microbiol Biotechnol* 41:1443–1450
- Hemanshu M, Konstantin S, Hanne BC, Alex TN (2016) Engineering of high yield production of L-serine in *Escherichia coli*. *Biotechnol Bioeng* 113:807–816
- Kang Z, Wang Y, Gu P, Wang Q, Qi Q (2011) Engineering *Escherichia coli* for efficient production of 5-aminolevulinic acid from glucose. *Metab Eng* 13:492–498
- Kiatpapan P, Murooka Y (2001) Construction of an expression vector for propionibacteria and its use in production of 5-aminolevulinic acid by *Propionibacterium freudenreichii*. *Appl Microbiol Biotechnol* 56:144–149
- Lai S, Zhang Y, Liu S, Liang Y, Shang X, Chai X, Wen T (2012) Metabolic engineering and flux analysis of *Corynebacterium glutamicum* for L-serine production. *Sci China Life Sci* 55:283–290
- Ma W, Wang X, Mao Y, Wang Z, Chen T, Zhao X (2015) Development of a markerless gene replacement system in *Corynebacterium glutamicum* using *upp* as a counter-selection marker. *Biotechnol Lett* 37:609–617
- Netzer R, Peters-Wendisch P, Eggeling L, Sahm H (2004) Cometabolism of a nongrowth substrate: L-serine utilization by *Corynebacterium glutamicum*. *Appl Environ Microbiol* 70:7148–7155
- Peters-Wendisch P, Netzer R, Eggeling L, Sahm H (2002) 3-Phosphoglycerate dehydrogenase from *Corynebacterium glutamicum*: the C-terminal domain is not essential for activity but is required for inhibition by L-serine. *Appl Microbiol Biotechnol* 60:437–441
- Peters-Wendisch PS, Etterich H, Kennerknecht N, Sahm H, Eggeling L (2005) Metabolic engineering of *Corynebacterium glutamicum* for L-serine production. *Appl Environ Microbiol* 71:7139–7144
- Pittard YZJPAHA (1994) Molecular analysis and characterization of a broad-host-range plasmid, pEP2. *J Bacteriol* 176:5718–5728
- Qin G, Lin J, Liu X, Cen P (2006) Effects of medium composition on production of 5-aminolevulinic acid by recombinant *Escherichia coli*. *J Biosci Bioeng* 102:316–322
- Schweitzer JE, Stolz M, Diesveld R, Etterich H, Eggeling L (2009) The serine hydroxymethyltransferase gene *glyA* in *Corynebacterium glutamicum* is controlled by GlyR. *J Biotechnol* 139:214–221
- Simic P, Willuhn J, Sahm H, Eggeling L (2002) Identification of *glyA* (encoding serine hydroxymethyltransferase) and its use together with the exporter ThrE to increase L-threonine accumulation by *Corynebacterium glutamicum*. *Appl Environ Microbiol* 68:3321–3327
- Unthan S et al (2014) Beyond growth rate 0.6: what drives *Corynebacterium glutamicum* to higher growth rates in defined medium. *Biotechnol Bioeng* 111:359–371
- Xu G et al (2015) Enhanced production of L-serine by deleting *sdA* combined with modifying and overexpressing *serA* in a mutant of *Corynebacterium glutamicum* SYPS-062 from sucrose. *Biochem Eng J* 103:60–67
- Zhang X, Xu G, Li H, Dou W, Xu H, Xu Z (2014) Effect of cofactor folate on the growth of *Corynebacterium glutamicum* SYPS-062 and L-serine accumulation. *Appl Biochem Biotechnol* 173:1607–1617
- Zhu N, Xia H, Wang Z, Zhao X, Chen T (2013) Engineering of acetate recycling and citrate synthase to improve aerobic succinate production in *Corynebacterium glutamicum*. *PLoS ONE* 8:e60659