3 Biotech (2018) 8:247
https://doi.org/10.1007/s13205-018-1267-2

ORIGINAL ARTICLE

Pathway engineering in *Corynebacterium glutamicum* S9114 for 5-aminolevulinic acid production

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Received: 4 January 2018 / Accepted: 28 April 2018 / Published online: 8 May 2018
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Abstract

5-Aminolevulinic acid (ALA) is a non-protein amino acid with a significant potential for cancer treatment and plant stress resistance. Microbial fermentation has gradually replaced the traditional chemical-based method for ALA production, thus increasing the need for high-ALA-producing strains. In this study, we engineered the glutamate producing strain, *Corynebacterium glutamicum* S9114, for ALA production. To efficiently convert L-glutamate to ALA, *hemA* and *hemL* from *Salmonella typhimurium* and *Escherichia coli* were tandemly overexpressed. In addition, *ncgl1221* encoding a glutamate transporter was deleted to block glutamate secretion and thus improve ALA production. Furthermore, the intrinsic ribosome-binding site (RBS) of *hemB* was replaced by a relatively weak RBS to reduce the conversion of ALA to porphyrin. Transcriptional and fermentation data confirmed that inactivation of *lysE* and *putP* reduced the conversion of glutamate to arginine and proline, which also contribute to ALA production. The final SA14 strain produced 895 mg/L concentration of ALA after 72 h incubation in a shake flask. This amount was 58-fold higher than that obtained by the parent strain *C. glutamicum* S9114. The results demonstrate the potential of *C. glutamicum* S9114 for efficient ALA production and provide new targets for the development of ALA-producing strains.

Keywords *Corynebacterium glutamicum* · 5-Aminolevulinic acid · C5 pathway · Metabolic engineering

Abbreviations

RBS	Ribosome-binding sites
ALA	5-Aminolevulinic acid
ALAD	5-Aminolevulinic acid dehydratase

Introduction

Corynebacterium glutamicum is an aerobic gram-positive actinomycete that secretes a large amount of glutamate in the absence of biotin or the presence of penicillin (Becker and Wittmann 2016). It is used for the long-term production of various amino acids such as glutamate, arginine, lysine, ornithine, and alanine (Wieschalka et al. 2013), diamines such as putrescine (Nguyen et al. 2015) and cadaverine (Mimitsuka et al. 2007), dicarboxylic acids such as succinate (Chung et al. 2017; Jo et al. 2017), diols such as 1,3-propanediol (Huang et al. 2017) and 1,2-propanediol (Siebert and Wendisch 2015), and terpenes such as pinene and carotenoid (Heider et al. 2012; Kang et al. 2014). In China, *C. glutamicum* S9114, a subspecies of *C. glutamicum* derived from *Brevibacterium tianjinese* T_{6-13}, has been widely used for glutamate fermentation (Mei et al. 2016). To emphasize the importance of the microbe in glutamate production, its complete genome sequence was published in 2011 (Lv et al. 2011). Compared with other *C. glutamicum* strains, *C. glutamicum* S9114 secretes a large amount of glutamate, is resistant to high sugar content, and shows rapid growth, indicating that it is an ideal host for the production of glutamate-related compounds (Zhang et al. 2005).

5-Aminolevulinic acid (ALA), an intermediate metabolite produced during tetrapyrrole biosynthesis, has become noteworthy because of its efficient application in photodynamic therapy (Tetard et al. 2016) and as a photosensitizer in the photodynamic diagnosis for cancer patients (Ishikawa et al. 2015; Kennedy et al. 1990). Low ALA concentrations are used in agriculture to increase the tolerance of plants to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-018-1267-2) contains supplementary material, which is available to authorized users.

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low temperature and high salt concentrations (Akram and Ashraf 2013). However, the industrial scale production of ALA depends mainly on chemical synthesis methods that are unsustainable.

Recently, research attention has turned to microbial fermentation, which is an environmentally safe, economical, and sustainable method. Microbes such as *Rhodobacter sphaeroides* (Sasaki et al. 2002), *Escherichia coli* (Kang et al. 2011), and *C. glutamicum* (Yu et al. 2016) have been engineered to produce ALA. The biosynthesis of ALA occurs via the C4 and C5 pathways. In the C4 pathway, ALA is produced from succinyl-CoA and glycine, in a reaction that is catalyzed by the gene products of *hemA* from *R. sphaeroides*. Recently, a recombinant *C. glutamicum* strain was constructed by overexpressing codon-optimized *hemA* from *R. sphaeroides*, disrupting all known genes involved in acetate and lactate synthesis, overexpressing endogenous genes encoding phosphoenolpyruvate carboxylase and RhtA from *E. coli*, and by inactivating high-molecular weight penicillin-binding proteins (Feng et al. 2015). This recombinant *C. glutamicum* strain produced an ALA titer of 7.53 g/L in the presence of appropriate glycine and succinate concentrations in a 5-L bioreactor. Another study reported the engineering of a strain derived from *C. glutamicum* ATCC 13032 by overexpressing a codon-optimized *hemA* from *R. capsulatus* SB1003, inactivating *sucCD* genes, and overexpressing *rhtA* from *E. coli*. The engineered strain produced 14.7 g/L concentration of ALA in a fed-batch fermentation culture through the C4 pathway (Yang et al. 2016). However, the requirements for glycine and succinate as precursors in the fermentation medium in the C4 pathway have prompted researchers to shift attention to the C5 pathway, which is considered a potential pathway for the direct production of ALA from glucose. In the C5 pathway, ALA is produced from glutamate through two reactions catalyzed by enzymes that are the gene products of endogenous *hemA* and *hemL*. A mutant *C. glutamicum* strain was developed by constitutively coexpressing *hemA* and *hemL*, in which the addition of penicillin and baffled flask fermentation under optimized conditions resulted in an ALA titer of 2.2 g/L through the C5 pathway (Ramzi et al. 2015). At the same time, other researchers reported the construction of a recombinant *C. glutamicum* strain derived from the model strain *C. glutamicum* ATCC 13032 by the inducible coexpression of *hemA* and *hemL* (Yu et al. 2015). By optimizing dissolved oxygen and Fe^{2+} concentrations, the researchers attained an ALA yield of 1.79 g/L after 144 h of incubation in a shake flask.

In this study, we developed a new metabolically engineered *C. glutamicum* S9114 strain by overexpressing *hemA* with different synthetic ribosome-binding sites (RBSs) by replacing the original RBS of *hemB* to inhibit the conversion of ALA to porphyrin, and by disrupting arginine and proline transport systems to inhibit a competing metabolic pathway, which improved ALA production (Fig. 1). The present results indicate an efficient strategy for the development of ALA-producing industrial strains.

Materials and methods

Strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for routine cloning procedures. *C. glutamicum* S9114, origin to *C. glutamicum* CICC 20935 accessible in China Center of Industrial Culture Collection (CICC), was used as the parent strain to develop the engineered strains. The suicide vector pK18*mobsacB* containing the sucrose screening marker gene, *sacB*, from *Bacillus subtilis* was used for markerless gene deletion, insertion, and RBS replacement through double crossover recombination, as described previously (Xu et al. 2014). *E. coli*/*C. glutamicum* shuttle expression vector pXMJ19 was used to overexpress *hemA* and *hemL* (Jakoby et al. 1999). The *C. glutamicum* host strain was transformed with the plasmid using a method we previously described (ZHANG et al. 2015a). Luria Bertani medium containing an appropriate concentration of antibiotic (12.5 μg/mL chloromycetin or 30 μg/mL kanamycin) was used for bacterial growth and plasmid construction. *C. glutamicum* was cultured in brain

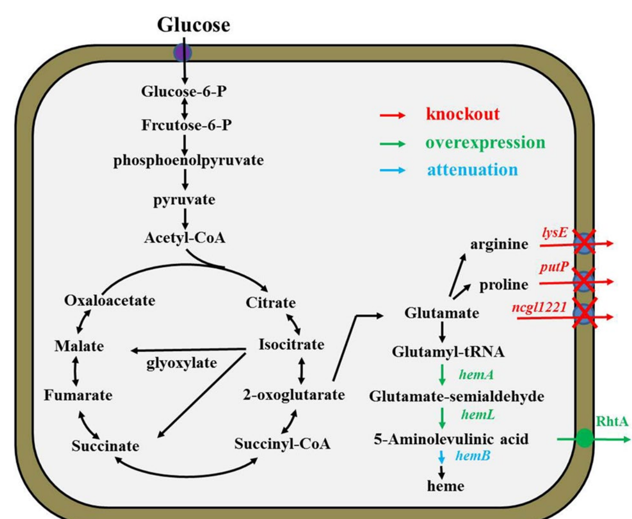

Fig. 1 Metabolic pathways associated with 5-ALA biosynthesis in *C. glutamicum* and the metabolic engineering strategies for 5-ALA overproduction. The genes that are involved are: *hemA*, encoding glutamyl-tRNA reductase; *hemL*, encoding glutamate-1-semialdehyde aminotransferase; *hemB*, encoding 5-aminolevulinic acid dehydratase; *rhtA*, encoding gene encoding inner membrane transporter for L-threonine; *ncgl1221*, encoding glutamate transporter; *lysE*, encoding lysine/arginine transporter; and *putP*, encoding L-proline transporter

Table 1 Strains and plasmids used in this study

Strain/Plasmid	Characteristics	Source
Strains		
E. coli DH5α	Clone host strain	TransGen
S. typhimurium LT-2	Provided template for *hemA* amplification	Lab store
C. glutamicum S9114	Industrial strain for glutamate production	Mei et al. (2016)
SA0	*C. glutamicum* S9114 transformed with pXMJ19	This study
SA1	*C. glutamicum* S9114 transformed with pXMJ19-SB1-*hemA*	This study
SA2	*C. glutamicum* S9114 transformed with pXMJ19-SB2-*hemA*	This study
SA3	*C. glutamicum* S9114 transformed with pXMJ19-SB3-*hemA*	This study
SA4	*C. glutamicum* S9114 transformed with pXMJ19-SB4-*hemA*	This study
SA5	*C. glutamicum* S9114 transformed with pXMJ19-SB1-*hemA-hemL*	This study
SA6	*C. glutamicum* S9114 transformed with pXMJ19-SB2-*hemA-hemL*	This study
SA7	*C. glutamicum* S9114 transformed with pXMJ19-SB3-*hemA-hemL*	This study
SA8	*C. glutamicum* S9114 transformed with pXMJ19-SB4-*hemA-hemL*	This study
SA9	SA7 strain with *ncgl1221* deletion	This study
SA10	SA9 strain with a change in *hemB* RBS100	This study
SA11	SA9 strain with a change in *hemB* RBS500	This study
SA12	SA9 strain with *lysE* deletion	This study
SA13	SA12 strain with *putP* deletion	This study
SA14	SA13 strain with a change in *hemB* RBS100	This study
SA15	SA14 strain with insertion of *E. coli-rhtA* in *ncgl1221*	This study
Plasmid		
pK18*mobsacB*	Mobilizable vector allows for selection of double crossover in *C. crenatum*, Km^R, and *sacB*	Schafer et al. (1994)
pXMJ19	A shuttle expression vector, Cm^R	Jakoby et al. (1999)
pXMJ19-SB1-*hemA*	A derivative of pXMJ19 harboring *S. typhimurium hemA* and synthetic RBS of 5341.55 au	This study
pXMJ19-SB2-*hemA*	A derivative of pXMJ19 harboring *S. typhimurium hemA* and synthetic RBS of 25210.78 au	This study
pXMJ19-SB3-*hemA*	A derivative of pXMJ19 harboring *S. typhimurium hemA* and synthetic RBS of 57705.62 au	This study
pXMJ19-SB4-*hemA*	A derivative of pXMJ19 harboring *S. typhimurium hemA* and synthetic RBS of 580091.25 au	This study
pXMJ19-SB1-*hemA-hemL*	A derivative of pXMJ19 harboring *S. typhimurium hemA*, synthetic RBS of 5341.55 au, and *E. coli hemL*	This study
pXMJ19-SB2-*hemA-hemL*	A derivative of pXMJ19 harboring *S. typhimurium hemA*, synthetic RBS of 25210.78 au, and *E. coli hemL*	This study
pXMJ19-SB3-*hemA-hemL*	A derivative of pXMJ19 harboring *S. typhimurium hemA*, synthetic RBS of 57705.62 au, and *E. coli hemL*	This study
pXMJ19-SB4-*hemA-hemL*	A derivative of pXMJ19 harboring *S. typhimurium hemA*, synthetic RBS of 580091.25 au, and *E. coli hemL*	This study
pXMJ19-*rhtA*	A derivative of pXMJ19 harboring *E. coli rhtA*	This study
pK18-Δ*ncgl1221*	A derivative of pK18*mobsacB* harboring Δ*ncgl1221* fragment	This study
pK18-*hemB100*	A derivative of pK18*mobsacB* harboring *hemB* RBS replacement fragment (92.52 au)	This study
pK18-*hemB500*	A derivative of pK18*mobsacB* harboring *hemB* RBS replacement fragment (553.57 au)	This study
pK18-Δ*lysE*	A derivative of pK18*mobsacB* harboring Δ*lysE* fragment	This study
pK18-Δ*putP*	A derivative of pK18*mobsacB* harboring Δ*putP* fragment	This study
pK18-Δ*ncgl1221-rhtA*	A derivative of pK18*mobsacB* harboring Δ*ncgl1221* fragment and *E. coli rhtA*	This study

^{R}Resistance to the following antibiotics: kanamycin (Km) and chloramphenicol (Cm)

heart infusion (BHI) broth and terrific broth (TB) as previously described (Ramzi et al. 2015), with some modification. For shake flask cultivation, each recombinant strain was inoculated in 10 mL BHI broth in a 100-mL unbaffled flask and was cultured for 14 h at 30 °C and 200 rpm. The pre-culture was inoculated in 25 mL TB (containing 12 g

tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, and 40 g glucose per liter of water) in a normal 250-mL flask to an optical density at 600 nm (OD_{600}) of 0.3. The pH of the culture medium was adjusted to 7.0. ALA production in shake flasks was performed at 30 °C and 200 rpm for 72 h.

Construction of plasmids and strains

To construct recombinant expression vectors of pXMJ19-SB-*hemA, hemA* encoding glutamyl-tRNA reductase from *S. typhimurium* LT2 was amplified by polymerase chain reaction (PCR). To maintain the stability of glutamyl-tRNA reductase, two lysine residues (KK) were inserted into three and four amino acid residues of *hemA* using specific primers (Ramzi et al. 2015). Synthetic RBSs with different translation initiation strengths were designed using the RBS Calculator (Tian and Salis 2015) https://www.denovodna.com/software/doLogin and were added upstream of *hemA* using specific primers. Sequences of the synthetic RBSs are listed in Additional file 1: Table S1. PCR products were purified, digested, and inserted into multiple cloning sites of pXMJ19. For the coexpression of *hemA* and *hemL* (the latter encodes glutamate-1-semialdehyde aminotransferase), *hemL* from *E. coli* DH5α was amplified, inserted at the end of *hemA* by overlap PCR, and cloned into pXMJ19 by enzyme digestion and ligation. All the enzymes used for constructing the recombinant plasmid are purchased from TakaRa Bio (Shiga, Japan).

For gene deletion, we used the recombinant plasmids pK18-Δ*ncgl1221*, pK18-Δ*lysE*, and pK18-Δ*putP*, which we previously constructed (Zhang et al. 2017a, b). For the chromosome RBS change of *hemB*, an RBS sequence was inserted into an overlapping region between the upstream and downstream fragments of *hemB* using rationally designed primers. Plasmids pK18-*hemB100* and pK18*hemB500* were constructed by standardized molecular cloning. To integrate *rhtA* into the *ncgl1221* region of *C. glutamicum, rhtA* was amplified from the genomic DNA of *E. coli* DH5α and was inserted into plasmid pK18-Δ*ncgl1221* using the Gibson assembly to generate plasmid pK18-Δ*ncgl1221-rhtA*. All the recombinant plasmids were transformed by electroporation into engineered *C. glutamicum* S9114-derived strains. The correct mutants were obtained after two rounds of homologous recombination. Randomly chosen strains from the second round of recombination were confirmed by colony PCR. All the primers are listed in Additional file 1: Table S2.

RT-PCR analysis

For RNA extraction, 500 µL fermentation samples were collected at 12 h. Total RNA was extracted using an RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech Co., Ltd., Beijing, China). RNA integrity was analyzed by 1% agarose gel electrophoresis, and the RNA concentration was determined using a microplate reader (BioTek Instruments, Winooski, VT, USA). Reverse transcription was performed using PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio). RT-PCR analysis was performed accordingly as we previously described (Yao and Ye 2015).

Enzyme assay of ALA dehydratase (ALAD)

For ALAD enzyme activity detection, cells were collected after 12 h of cultivation. Disposition of samples and the enzymatic reaction system have been previously described (Sassa 1982; Zhang et al. 2015c).

Analytical procedures

Cell growth was monitored by measuring OD_{600} with a microplate reader. Fermentation supernatant was filtered a 0.22-µm filter. Glucose, glutamate, and lactate concentrations were analyzed in the filtered sample using a model SBA-40C biosensor (Biology Institute of Shandong Academy of Sciences, Shandong, China). ALA concentration in the culture medium was measured as described previously using a modified Ehrlich reagent (Fu et al. 2007) and using a microplate reader at 554 nm.

Results and discussion

Expression levels of *hemA* and *hemL* affect ALA production

In previous study, expression of mutant glutamyl-tRNA reductase from *S. typhimurium* resulted in the highest ALA yield in *C. glutamicum* ATCC 13032 through the C5 pathway (Ramzi et al. 2015). In the present study, a medium copy number plasmid pXMJ19 with an isopropyl β-D-1-thiogalactopyranoside inducible *tac* promoter was used to express this enzyme in *C. glutamicum* S9114 and generate mutant strains having increased ALA production. RBS optimization is an efficient synthetic biological tool that has become widely used recently for pathway engineering, such as the optimization of metabolic flux for shikimic acid production (Zhang et al. 2015b), succinic acid production (Wang et al. 2015), and mevalonate-based farnesyl pyrophosphate biosynthesis (Nowroozi et al. 2014). To improve the ALA yield, four types of synthetic RBSs with gradient translation initiation strength were designed using the RBS calculator and inserted in the upstream of *hemA* to generate strains SA1, SA2, SA3, and SA4. SA3 produced the most ALA of all the strains (126 mg/L) during shake flask fermentation

(Fig. 2a). Mutant SA7 strain coexpressing *hemA* and *hemL* produced 509 mg/L of ALA (Fig. 2b), which was approximately 33-fold higher than that obtained by *C. glutamicum* S9114. The significant improvement of ALA production observed in the present study can be directly attributed to the heterologous expression of *S. typhimurium hemA* and *E. coli hemL*. The results are consistent with the previous observation that the overexpression of *hemA* and *hemL* lead to high ALA production (Ramzi et al. 2015). Interestingly, the SA7 strain with an RBS having a predicted translation initiation strength of 57705.62 au inserted in front of *hemA* showed the highest ALA production, which revealed that insertion of disparate RBS in front of *hemA* greatly affects the biosynthesis of ALA. Compared with the constitutive expression of *hemA* and *hemL* in *C. glutamicum* ATCC 13032 (457 mg/L), a higher concentration of ALA was produced by optimizing RBS of *hemA* in *C. glutamicum* S9114.

Improvement of precursor accumulation by deleting *ncgl1221* for ALA production

Glutamate is a direct precursor of ALA biosynthesis through the C5 pathway, indicating that accumulation of glutamate is a key factor for high ALA production. *Ncgl1221* encodes a membrane protein that transports glutamate, and inactivation of *ncgl1221* drastically decreases glutamate secretion by damaging the transportation system (Chen et al. 2015). Moreover, deletion of *ncgl1221* reportedly increases L-arginine production in *C. crenatum* and *C. glutamicum* (Chen et al. 2015; Park et al. 2014). To provide more glutamate for ALA synthesis, *ncgl1221* was deleted in the SA7 strain to produce a mutant strain, SA9. Shake flask fermentation was performed to evaluate the effect of *ncgl1221* disruption on ALA production. The deletion of *ncgl1221* did not affect the growth and glucose consumption by SA9 (Fig. 3a). SA9 produced approximately 616 mg/L of ALA in 72 h (Fig. 3b), which was 9.8% higher than that the ALA yield of 561 mg/L produced by the control strain, SA7. Glutamate concentration in the supernatant was decreased by approximately 43.7% by *ncgl1221* deletion, confirming that Ncgl1221 plays a key role in glutamate secretion (Fig. 3c). However, strains SA7 and SA9 produced lactate because of the presence of low oxygen concentration. Therefore, we conclude that inactivation of *ncgl1221* contributes slightly to ALA production. The results also demonstrate that in vivo glutamate accumulation affected ALA production. But, the improved titer of ALA was relatively lower than the reduction of glutamate, indicating that glutamate is not the only precursor of ALA in *C. glutamicum*. In addition to the previous study using penicillin and specific culture (Ramzi et al. 2015), this study provides a genetic strategy in which blocking of the glutamate transport system can improve glutamate accumulation, which in turn improves ALA production.

ALA production is enhanced by inhibiting its conversion

ALAD, which is encoded by *hemB*, is the first enzyme involved in the conversion of ALA to porphyrin. ALA production can be improved using ALAD inhibitors such as D-glucose, levulinic acid, phthalic acid, and maleic acid and using genetically engineered HemB, which is obtained by adding a degradation ASV tag at the C terminus of HemB, to reduce the ALAD reaction (Yu et al.

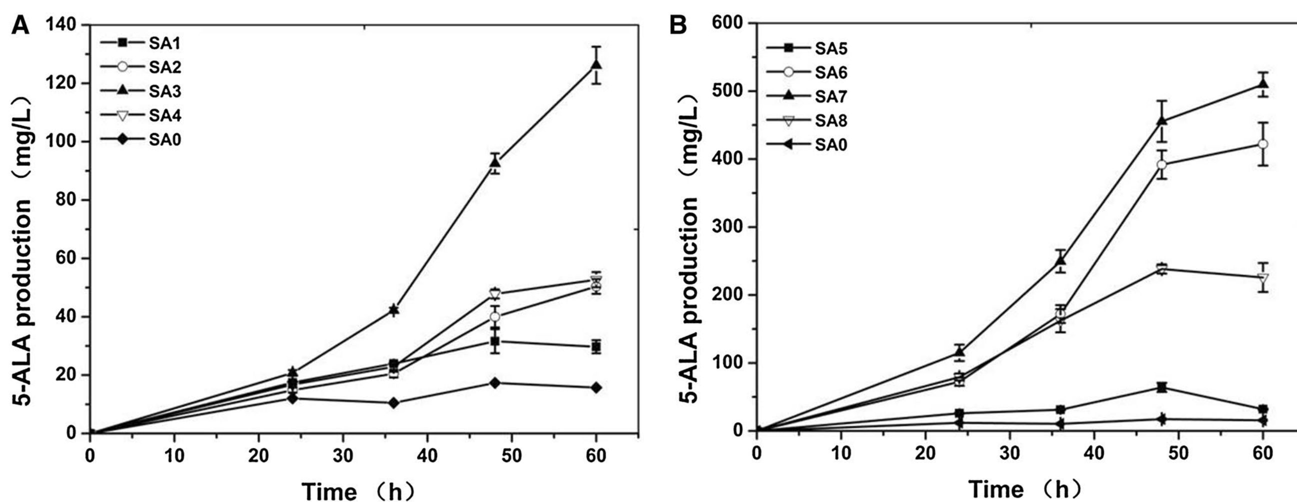

Fig. 2 Influence of different expression of *hemA* alone and in combination with *hemL* on ALA production during shake flask cultivations in *C. glutamicum* S9114. **a** ALA production by the sole expression of *hemA* with disparate RBS. SA1, predicted RBS of 5341.55 au; SA2, predicted RBS of 25210.78 au; SA3, predicted RBS of 57705.62 au; SA4, predicted RBS of 580091.25 au. **b** ALA production by coexpression of *hemA* and *hemL*. Results of standard deviations present in three individual experiments

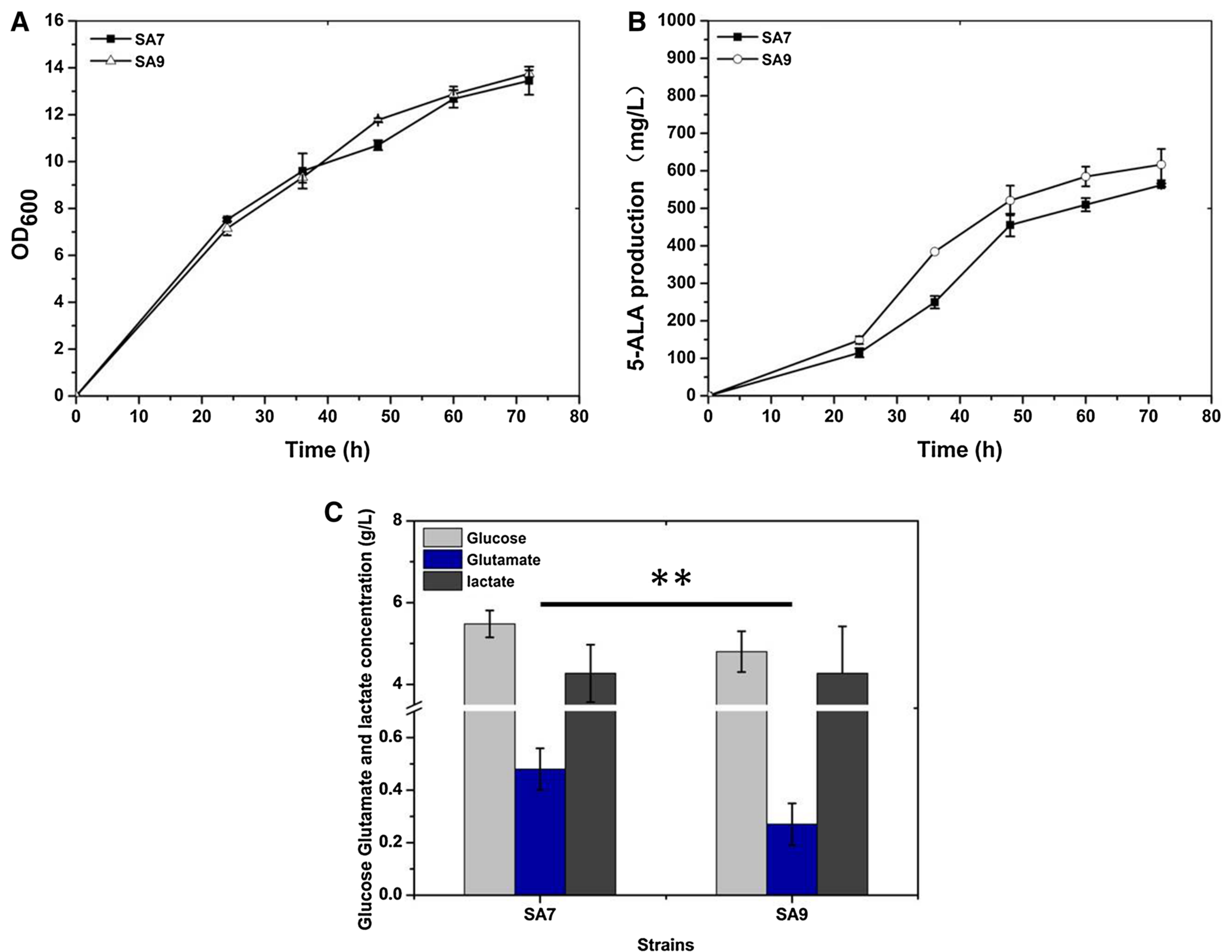

Fig. 3 Effect of *ncgl1221* deletion on ALA production and cell growth during shake flask cultivations. **a** The growth of SA7 and SA9 (SA7 with *ncgl1221* deletion); **b** L-ornithine curves with temporal change. **c** The residual glucose, glutamate, and lactate concentration at 72 h. Results of standard deviations present in three individual experiments. Compared with the control group, $^{**}p < 0.01$

2015). To inhibit ALA conversion to prophyrin, we were initially unsuccessful in our attempts to disrupt *hemB*, as the deletion of the gene resulted in poor cellular viability. We hypothesize that *hemB* might play a crucial role in cellular growth. Thus, we lowered the expression level of hemB by replacing its native RBS with a lower one. The translation initiation strength of the original *hemB* RBS was 5557.35 au, as predicted using the RBS Calculator (https://www.denovodna.com/software/doLogin). Therefore, two designed RBSs with translation initiation intensity of 92.52 and 553.57 au were designed using the RBS Calculator. They were used to replace the native RBS in the SA9 strain to produce strains SA10 and SA11. Shake flask fermentation was performed to evaluate the effect of RBS replacement on ALA production. Compared with the SA9 parent strain, the relative ALAD specific activity of strains SA10 and SA11 were reduced to 47 and 69% (see Table 2). After 72 h of incubation, the recombinant strains SA10 and SA11 produced approximately 827 and 730 mg/L ALA, respectively (Fig. 4b), which was 34.2 and 18.5% higher than that (616 mg/L) obtained using the control SA9 strain. The growth rate of the SA11 strain was similar to that of the parent SA9 strain (Fig. 4a). However, the growth of the SA10 strain was slightly affected, probably due to the low expression level of HemB. These results indicate that attenuation of *hemB* using a synthetic RBS promotes ALA production. These results also confirmed that ALAD regulation is a restrictive factor for enhancing ALA production, which is consistent with a previous study (Yu et al. 2015). Compared with the inhibition of ALAD achieved by addition of a degradation tag, the attenuated expression of ALAD via RBS replacement provides more strategic options based on the insertion of RBSs with differing strengths of translation initiation.

Table 2 HemB enzyme activity detected in engineered strains

Strains	Cell biomass (OD600)	ALA accumulation (mg/L)	Relative ALAD enzyme activity
SA9	13.75 ± 0.15	616.56 ± 41.71	1.00 ± 0.08
SA10	12.08 ± 0.28	827.82 ± 13.41	0.47 ± 0.03
SA11	12.43 ± 0.18	730.90 ± 10.43	0.69 ± 0.07

Fermentations were performed at 200 rpm for 72 h, ALA concentration was determinated at 72 h. ALAD enzyme activity was determinated at 12 h. Results are the means ± standard deviations in three individual experiments

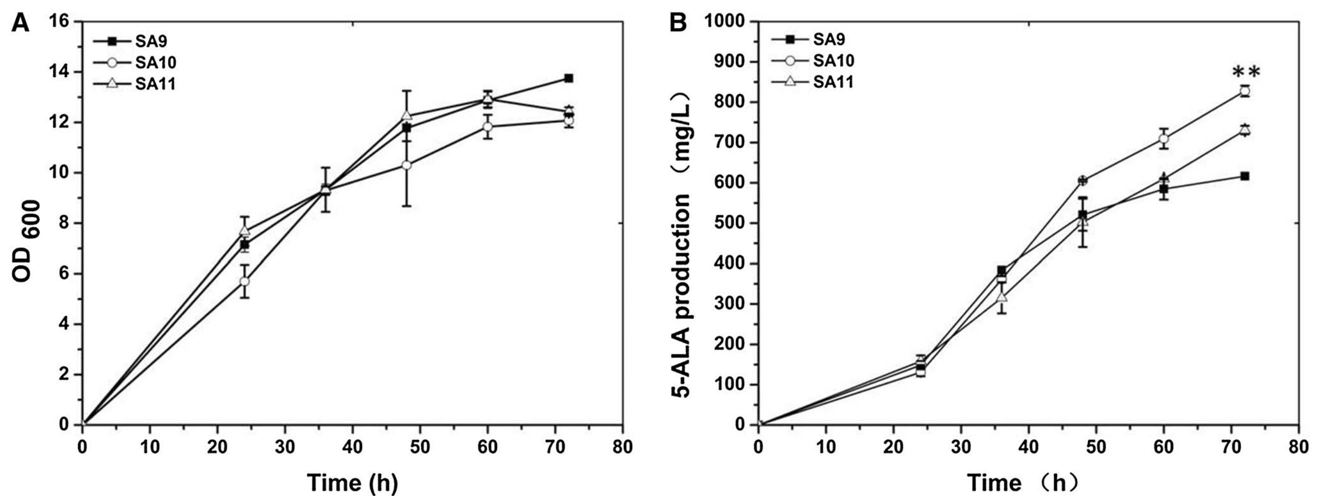

Fig. 4 ALA production and cell growth in engineered strains with attenuation of *hemB*. **a** The growth of SA9, SA10 (SA9 with a change in *hemB* RBS100), and SA11 (SA9 with a change in hemB RBS500). **b** ALA concentration in fermentation supernatant. Results of standard deviations present in three individual experiments. Compared with the control group, $*p < 0.05$; $**p < 0.01$

Effect of transport system modification on ALA production

Since glutamate is also converted to proline and arginine through multistep enzymatic reactions, proline and arginine are considered as competing metabolic by-products and are unfavorable for ALA biosynthesis. We inactivated LysE and PutP, two membrane proteins involved in the transportation of arginine and proline, respectively (Lubitz et al. 2016; Peter et al. 1997), to preserve the glutamate precursor, which promoted intracellular arginine and proline accumulation, and then inhibited its synthesis by feedback regulation to generate mutant strains SA12 and SA13. SA12 and SA13 produced approximately 718 and 734 mg/L of ALA, respectively (Fig. 5b), which was 16.5 and 19.1% higher than the 616 mg/L generated by the control strain, SA9. The growth was not affected by deletion of the two genes, consistent with the previous finding that *lysE* and *putP* were not necessary for cell growth (Dong et al. 2016). In addition, the expression levels of *argB* and *argJ* involved in arginine synthesis in strain SA12 were decreased to approximately half of the levels in strain SA9 (Fig. 5c). Moreover, the transcription level of *proC*, which encodes pyrroline-5-carboxylate reductase in the last step of proline synthesis, decreased by approximately 30% in strain SA13 (Fig. 5d). These results indicate that deletion of the transport proteins LysE and PutP promotes ALA production. In combination with the attenuation of *hemB*, recombinant strain SA14 was constructed. This strain produced 895 mg/L ALA, which was 45% higher than the 616 mg/L obtained by the control strain, SA9. These results confirmed that *hemB* attenuation and deletion of *lysE* synergistically enhanced ALA synthesis.

The transport protein RhtA from *E. coli* was demonstrated to be necessary for the transport of ALA, with no other protein having been identified (Feng et al. 2015). To further improve the yield of ALA, *rhtA* was introduced into the chromosome of strain SA14 to generate strain SA15. During shake flask fermentation, SA15 produced 872 mg/L ALA in the fermentation supernatant, which was lower than the production by SA14. Overexpression of *rhtA* did not exert a positive effect on the yield of ALA, possibly because the transport system is not a rate-limiting step. ALA production after the genetic modifications is still low compared with the highest level (2.2 g/L) that has been obtained (Ramzi et al. 2015). We anticipate that strategies such as the addition of penicillin or levulinic acid, or optimization of the

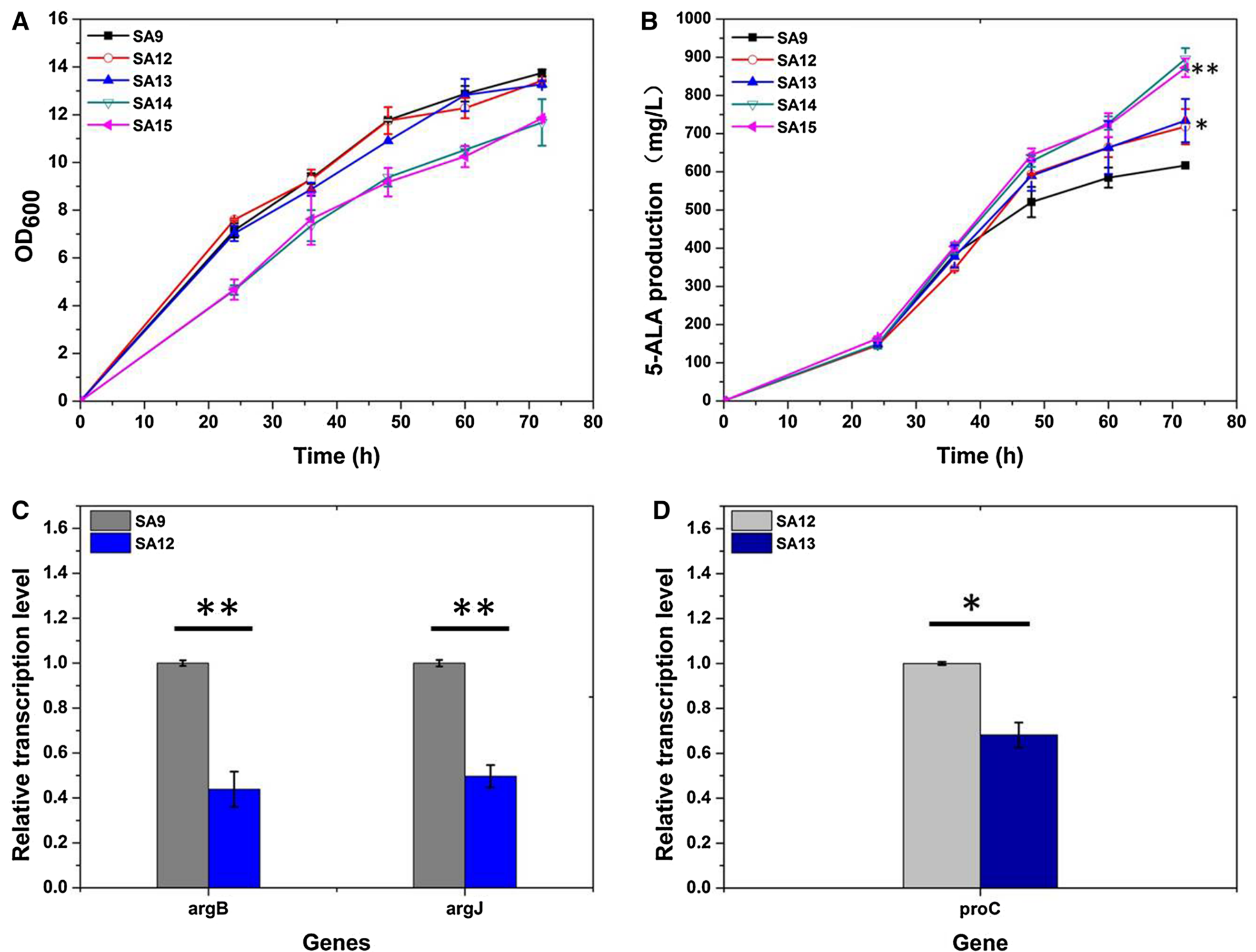

Fig. 5 Effect of modified transport system on cell growth and ALA production in engineered *C. glutamicum* S9114. **a** Growth of SA9, SA12 (SA9 with deletion of *lysE*), SA13 (SA12 with deletion of *putP*), SA14 (SA13 with the replacement of *hemB100*), and SA15 (SA14 with the insertion of *rhtA* in the site of *ncgl1221*) strains. **b** 5-ALA production by SA9, SA12, SA13, SA14, and SA15 strains. **c** Comparison of the transcription levels of L-arginine biosynthesis genes in SA9 and SA12 strains. **d** Comparison of *proC* transcription level in SA12 and SA13 strains. Samples were collected at 12 h for transcription level analysis. Results of standard deviations present in three individual experiments. Compared with the control group, $*p < 0.05$; $**p < 0.01$

fermentation conditions will further increase ALA production. This optimism is based on the demonstration of the success of such strategies in promoting ALA accumulation in other *C. glutamicum*, which is obviously appropriate for strain SA14 (Yu et al. 2015).

Conclusion

We successfully reconstructed the pathway involved in producing ALA from glucose to engineer an industrially-relevant, high-glutamate producing *C. glutamicum* S9114 strains for improved ALA production. This study provides some efficient strategies such as the optimized synthetic RBS of *hemA*, deletion of *ncgl1221, lysE*, and *putP*, and downregulated expression of *hemB* by RBS replacement, which could improve the fermentative production of ALA from renewable resources such as glucose. The present study is the first report of the combined application of those targets and their synergistic effect on ALA production, which will inform the development of ALA-producing strains. Additional genetic engineering and optimization of fermentation are needed to further increase ALA yield.

Acknowledgements We thank Dr. Andreas Burkovski and Dr. Xue-Lan Chen for providing pXMJ19 and pk18*mobsacB*. Moreover, we thank Dr. Zhong-Gui Mao and Dr. Li-Ming Liu for providing *C. glutamicum* S9114 strains. This study was supported by China NSF 21575089 and 21335003 and the National Key Technologies R&D Programs (2014AA021502).

Compliance with ethical standards

Conflict of interest All authors declared that they have no conflict of interest.

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