Biotechnology and Bioprocess Engineering 24: 915-923 (2019)
DOI 10.1007/s12257-019-0376-z

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Enhanced Production of 5-aminolevulinic Acid via Flux Redistribution of TCA Cycle toward L-Glutamate in *Corynebacterium glutamicum*

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Received: 4 October 2019 / Revised: 21 October 2019 / Accepted: 21 October 2019

Abstract 5-Aminolevulinic acid (ALA), a valuable nonproteinogenic amino acid, has received increasing attention in various fields including medicine, agriculture, and cosmetics. Here, we developed metabolically engineered *Corynebacterium glutamicum* to enhance ALA production. To achieve this object, we focused on the flux redistribution of the TCA cycle toward L-glutamate and introduction of the heterogenous ALA transporter in *C. glutamicum*. First, the oxoglutarate dehydrogenase inhibitor (OdhI) was mutated by site-directed mutagenesis to prevent the phosphorylation that abolishes the capability of OdhI protein to inhibit oxoglutarate dehydrogenase complex activity. The over-expression of the double-mutated OdhI, T14A/T15A, showed the highest L-glutamate and ALA production compared with that of the native and single-mutated OdhI. To increase ALA secretion from the engineered strain, the ALA exporter RhtA from *Escherichia coli* was introduced and allowed 2.46 ± 0.11 g/L of ALA production, representing a 1.28-fold increase in extracellular ALA production. In the final strain, the induction of triggers, including Tween 40 and ethambutol, was performed to amplify the effect of the flux redistribution toward ALA. A significant increase in ALA production was observed in the induction of triggers. In particular, ethambutol induction showed the best result, corresponding to 2.9 ± 0.15 g/L of ALA production. Therefore, this biotechnological model enables the efficient extracellular production of ALA from glucose in *C. glutamicum*.

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Keywords: 5-aminolevulinic acid, flux redistribution, TCA cycle, L-Glutamate, C5 pathway, *Corynebacterium glutamicum*

1. Introduction

5-Aminolevulinic acid (ALA) plays as an important role as a common precursor in biological processes for vitamin B12, heme, and cytochrome [1,2]. ALA has attracted much attention in various fields, including medicine and agriculture. In the medicine field, it has been used as a photodynamic agent for tumor and cancer therapy due to its safety and compatibility [3]. Furthermore, owing to its biodegradability, it has also been applied as a plant growth regulator, insecticide, and herbicide in agriculture [4,5]. Despite its many advantages and applications, ALA is commonly synthesized via chemical synthesis, leading to a low yield and making it a high-priced material [6,7].

Biological production of ALA has received much attention as an alternative approach until recently to increase the production yield and lower the production cost of ALA [8]. In living organisms, ALA is biosynthesized via two different routes, the C4 and C5 pathways. The C4 pathway, found in mammals, fungi and some photosynthetic bacteria, is catalyzed by ALA synthase (ALAS) and contributes to the condensation of glycine and succinyl-CoA to ALA. The C5 pathway, found in plants, algae, and some bacteria, proceeds via three enzymatic reactions by glutamyl-tRNA synthetase (GltX), NADPH-dependent glutamyl-tRNA reductase (HemA), and glutamate-1-semialdehyde amino-transferase (HemL) [6]. To increase ALA production, initially, many researchers had focused on engineering the

C4 pathway, due to the difficulty of understanding the regulatory mechanism of the C5 pathway [9,10]. Despite the shorter and more simple reaction, the C4 pathway has the critical issue that the main precursors glycine and succinyl-CoA should be exogenously added during fermentation for ALA production, with the exogenous supply limiting the productivity and yield of ALA [11,12]. To overcome the issue, recently, Ding *et al.* achieved 2.81 g/L of ALA production from glucose without the addition of glycine and succinyl-CoA by engineering the serine pathway in *Escherichia coli* [13].

Alternatively, the C5 pathway has attracted much attention for ALA production from glucose [6,8,14-17]. Notably, N-terminal engineering of HemA increased the stability and decreased the feedback regulation [14]. Indeed, the overexpression of Mutant HemA ($HemA^M$) and HemL with the serine/threonine transporter RhtA in *E. coli* contributed to the significant accumulation of ALA, corresponding to 4.13 g/L [15]. Since then, further engineering such as the flux redistribution of the glyoxylate cycle and optimization of modular pathways made it possible to obtain higher titer and productivity [16,17].

Corynebacterium glutamicum can also be utilized as a promising workhorse for ALA production through the C5 pathway, due to its innate ability to overproduce various organic acids and amino acids including L-glutamate [18-24]. For the first time, C5 pathway-engineered *C. glutamicum* showed enhanced ALA production, resulting in 2.2 g/L of ALA production [6]. At the same time, Yu *et al.* reported that $HemA^M$- and HemL-overexpressing *C. glutamicum* produced 1.79 g/L of ALA production after 144 h shake-flask cultivation [25]. Finally, it was reported that the blockade of secretion to the medium and conversion to arginine and proline of L-glutamate were effective in ALA production, resulting in 895 mg/L of ALA [26]. However, the production yield and productivity of ALA from glucose remain low in *C. glutamicum*, thus requiring additional engineering. In general, 2-oxoglutarate is an important branch point in the TCA cycle for L-glutamate biosynthesis and oxidative decarboxylation by the 2-oxoglutarate dehydrogenase complex (ODHC) competing with each other. The knockout of the *odhA* gene encoding the E1o subunit of ODHC induced L-glutamate overproduction [27]. Niebisch *et al.* revealed that the 2-oxoglutarate dehydrogenase inhibitor (OdhI) in the unphosphorylated status inhibited the ODHC activity [28]. Thereafter, Kim *et al.* demonstrated that overexpression of the OdhI protein allowed L-glutamate accumulation by inhibiting ODHC activity [19,29]. Biotin limitation and induction of various triggers such as Tween 40, penicillin G, and ethambutol, have also been used for L-glutamate overproduction [30-33]. However, few studies have increased ALA production via the flux redistribution of the TCA cycle toward L-glutamate by protein and metabolic engineering in *C. glutamicum*, although the potential exists.

In this study, we described the enhanced extracellular production of ALA via the flux redistribution of the TCA cycle toward L-glutamate and introduction of the heterogenous ALA transporter in *C. glutamicum*. The metabolic engineering strategy for the flux redistribution of the TCA cycle is the inactivation of ODHC by the OdhI protein (Fig. 1A). Next, protein engineering of the OdhI protein was conducted to increase the synthesis of unphosphorylated OdhI, which is the completely active form to inhibit ODHC activity (Fig. 1B). The effect on ALA accumulation by the overexpression of the engineered OdhI protein was investigated. To increase ALA secretion, the heterogenous ALA transporter RhtA from *E. coli* was overexpressed.

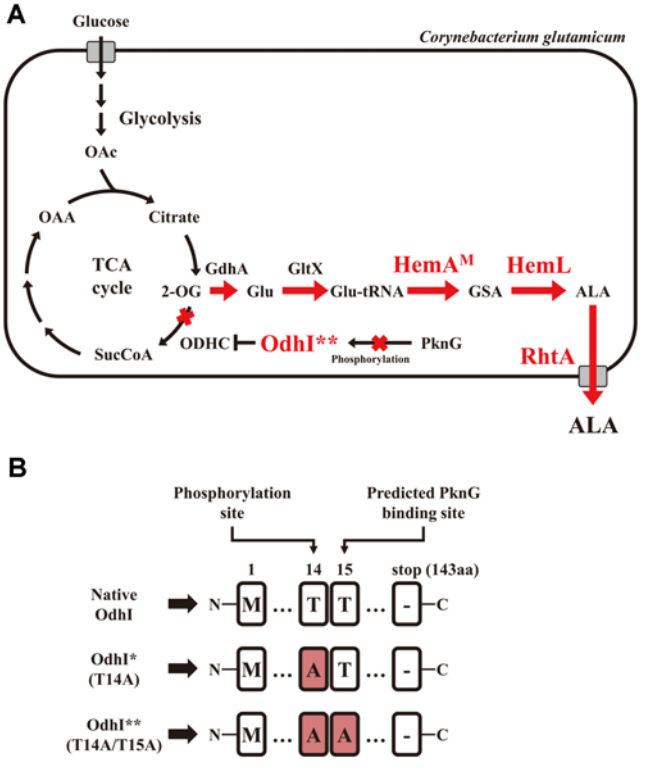

Fig. 1. (A) Schematic overview of the metabolic pathway for ALA production in *Corynebacterium glutamicum*. The red solid arrow indicates the predicted flux influenced by the protein and metabolic engineering implemented in this study. The blunt arrow indicates the ODHC inhibition by the double-mutated OdhI (T14A/T15A), named OdhI**. The large, bold, and red letter denotes overexpressed genes. The X shape indicates negative regulation related to the OdhI** protein. (B) Mutation strategy of the OdhI protein. The letters T and A indicate threonine and alanine, respectively. The colored box indicates target sites for mutation. Abbreviations: OAc, acetyl-CoA; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; SucCoA, succinyl-CoA; Glu, L-glutamate; Glu-tRNA, L-glutamyl-tRNA; GSA, L-glutamate 1-semialdehyde; ALA, 5-aminolevulinic acid.

Finally, the synergistic effect on ALA accumulation of the induction of triggers such as Tween 40 and ethambutol was tested.

2. Materials and Methods

2.1. Materials

Most of the materials for cell culture were purchased from BD Biosciences (USA) and Daejung Chemical Co. (Korea). Plasmid and genomic DNA in microbes were extracted using the GeneAll® Exprep Plasmid Kit (GeneAll, Korea) and Wizard DNA Purification Kit (Promega, USA), respectively. Oligonucleotide synthesis and gene sequencing were performed by Cosmogenetech (Korea) or Macrogen (Korea). KOD DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from Toyobo Co. (Japan), New England Biolabs (USA), and Promega, respectively. Chemicals such as acetonitrile and methanol for HPLC analysis were purchased from Duksan (Korea). 5-Aminolevulinic acid hydrochloride (Sigma-Aldrich, USA) and L-glutamate (Sigma-Aldrich, USA) were used as standard materials for quantitative analysis. The Glucose (GO) Assay Kit (Sigma-Aldrich, USA) was used to quantify residual glucose.

2.2. Strains and plasmids

All recombinant plasmids and bacteria used in this study are listed in Table 1. *E. coli* DH5α was chosen as a host strain for cloning, and *C. glutamicum* ATCC 13032 was used as an ALA producer. The *odhI* and *rhtA* genes were obtained from genomic DNA extracted in *C. glutamicum* and *E. coli*, respectively. All oligonucleotides in this study are listed in Table 2. pEKEx2 and pZ8C vectors, which are *E. coli* and *C. glutamicum* shuttle vectors, respectively, were used to overexpress target genes in *C. glutamicum* [34]. To construct the pZ8C vector, the *cmr* gene was amplified using CmR_AsiSI_F and CmR_DraIII_R primers

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construct	Reference or source
Strains		
Escherichia coli DH5α	F^-, *deoR*, *endA1*, *gyrA96*, *hsdR17*(rk^-mk^+), *recA1*, *relA1*, *supE44*, *thi-1*, Δ(*lacZYA- argF*)*U169*, (Phi80*lacZ*delM15)	Invitrogen[a]
Corynebacterium glutamicum		
WT	*C. glutamicum* ATCC 13032	ATCC[b]
KC	WT harboring pEKEx2 and pZ8C	This study
OI	WT harboring pEKEx2 and pZ8C::odhI	This study
AL	WT harboring pEKEx2::hemAL and pZ8C	This study
AL-OI	WT harboring pEKEx2::hemAL and pZ8C::odhI	This study
OI*	WT harboring pEKEx2 and pZ8C::odhI::T14A	This study
OI**	WT harboring pEKEx2 and pZ8C::odhI::T14A::15A	This study
AL-OI*	WT harboring pEKEx2::$hemA^M$L and pZ8C::odhI::T14A	This study
AL-OI**	WT harboring pEKEx2::$hemA^M$L and pZ8C::odhI::T14A::15A	This study
AL-Rh-OI**	WT harboring pEKEx2::$hemA^M$LrhtA and pZ8C::odhI::T14A::15A	This study
Plasmids		
pEKEx2	*C. glutamicum-E. coli* shuttle vector, P_{tac}, lacI, Kan^R, pBL1 ori	[34]
pJYS2cm_crtYf	*C. glutamicum-E. coli* shuttle vector, Pj23119-crRNA targeting *crtYf*, Cm^R, V ori, pMB1 ori	[35]
pZ8-Ptac	*C. glutamicum-E. coli* shuttle vector, $P_{tac,}$ lacI, Kan^R, pHM 1519 ori	[36]
pZ8C	*C. glutamicum-E. coli* shuttle vector, $P_{tac,}$ lacI, Cm^R, pHM 1519 ori	This study
pEKEx2::$hemA^M$L	pEKEx2 carrying $hemA^M$ from *Salmonella typhimurium* and *hemL* from *E. coli*	[37]
pEKEx2::$hemA^M$LrhtA	pEKEx2 $hemA^M$ from *Salmonella typhimurium* and *hemL* and *rhtA* from *E. coli*	This study
pZ8C::odhI	pZ8C carring *odhI* from *C. glutamicum*	This study
pZ8C::odhI::T14A	pZ8C carring *odhI* exchanging threonine codon 14 to alanine codon	This study
pZ8C::odhI::T14A::15A	pZ8C carring *odhI* exchanging threonine codon 14 and 15 to alanine codons	This study

[a]Invitrogen Corporation, Carlsbad, California, USA.
[b]American Type and Culture Collection, Manassas, USA.

Table 2. List of oligonucleotides used in this study

Primer name	Sequence (5'→3')
CmR_AsiSI_F	ACTGCGATCGCGAAGATCCTTTGATCTTTTCTACGGGG
CmR_DraIII_R	ATATCACGTTGTGTTACGCCCCGCCCTG
RhtA_BamHI_F	GGCGGGATCCAAGGAGATATAGATGCCTGGTTCATTACGT
RhtA_KpnI_R	GCGCGGTACCGGTTATGCATAACCATGCAGAAATG
OdhI_EcoRI_F	GCATGGAATTCATGAGCGACAACAACGGCA
OdhI_BamHI_R	GGCGGGATCCAGGAAGTGTTTTTACTCAGCAG
OdhI_SDM1_F	GAGCCACAGGTCGAGGCCACCTCAGTATTCC
OdhI_SDM1_R	GGAATACTGAGGTGGCCTCGACCTGTGGCTC
OdhI_SDM2_F	CACAGGTCGAGGCCGCCTCAGTATTCCGC
OdhI_SDM2_R	GCGGAATACTGAGGCGGCCTCGACCTGTG

and the pJYS2cm_crtYf vector as a template [35]. The resultant DNA fragment was ligated into the AsiS1- DraIII restriction sites of pZ8-Ptac by T4 DNA ligation, generating the pZ8C vector [36]. To construct the pZ8C::odhI vector, the *odhI* gene was amplified with OdhI_EcoRI_F and OdhI_BamHI_R primers using genomic DNA from *C. glutamicum* as a template. The resultant DNA fragment was ligated into the EcoRI-BamHI restriction sites of pZ8C by T4 DNA ligation, generating the pZ8C::odhI vector. For the first site-directed mutagenesis of the *odhI* gene, resulting in the pZ8C::odhI::T14A vector, PCR was performed using the pZ8C::odhI vector as a template with OdhI_SDM1_F and OdhI_SDM1_R primers. To construct pZ8C::odhI::T14A::T15A, the second site-directed mutagenesis was performed using pZ8C::odhI::T14A as a template with OdhI_SDM2_F and OdhI_SDM2_R primers. To construct pEKEx2::hemA^{M}LrhtA, the *rhtA* gene was amplified with RhtA_BamHI_F and RhtA_KpnI_R primers using genomic DNA from *E. coli* as a template. The amplified DNA fragment was ligated into the BamHI-KpnI restriction sites of the pEKEx2::hemA^{M}L [37] by T4 DNA ligation, generating the pEKEx2::hemA^{M}LrhtA vector. The ribosomal binding site "AAGGAG" was inserted upstream of the start codon in individual genes except for a first gene close to the promoter. All engineered vectors were transformed in *E. coli* and *C. glutamicum* through heat shock and electroporation methods as previously described [22,38].

2.3. Culture medium and conditions

The *E. coli* strains used in the construction of recombinant strains were grown overnight in a shaking incubator at 37°C and 200 rpm in Luria Bertani medium supplemented with 50 mg/L of ampicillin or 33 mg/L of chloramphenicol. The *C. glutamicum* strains for seed culture and preculture were grown overnight in a shaking incubator at 30°C and 200 rpm in brain heart infusion medium supplemented with 15 mg/L of kanamycin and/or 7.5 mg/L of chloramphenicol. For the main culture to produce ALA, the preculture was inoculated to an OD600 of 1 in 20 mL of CGAJ medium [39] containing 80 g/L of glucose, 15 mg/L of kanamycin, and 1 mM isopropyl thio-β-D-galactoside (IPTG) in a 100-mL baffled flask. L-Glutamate and ALA were produced for 24 h and 48 h cultivation in shaking incubator at 30°C and 200 rpm, respectively. Tween-40 (2 g/L) and ethambutol (25 mg/L) were added to the culture medium at 6 h.

2.4. Analytical methods

The cell growth of all the recombinant *C. glutamicum* strains was estimated at OD600 using a UV–vis spectrophotometer (Mecasys Co., Ltd., Korea). The dried cell weight (DCW) was calculated based on the correlation using the methods described by our previous study [37]. The glucose concentration was measured using the Glucose Assay Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. The concentration of L-glutamate was measured using a high-performance liquid chromatography (HPLC) system (Waters Corporation, USA) and a Waters AccQ-Tag Amino Acid Analysis System with a SUPELCOSIL LC-18-DB HPLC Column L × I.D. 250 × 4.6 mm and 5-μm particle size (Supelco Inc., USA). The HPLC system comprised a binary HPLC pump (Waters 1525), autosampler (Waters 717) and dual λ absorbance detector (Waters 2487). The ALA concentration was estimated using modified Ehrlich's reagent [40]. In detail, the supernatant or cell extract sample (1 volume) was reacted with acetylacetone (0.25 volume) in 1 M sodium acetate buffer pH 4.8 (0.5 volume) at 100°C for 15 min. The resultant mixture was cooled on ice. The modified Ehrlich's reagent, comprising 20 mg of 4-dimethylaminobenzaldehyde, 820 μL of acetic acid and 160 μL of 70% perchloric acid per 1 mL of deionized water, was added to the mixture and incubated at 25°C for 10 min. The absorbance at 553 nm was determined using an Epoch 2 microplate spectrophotometer (BioTek Instruments, USA).

3. Results and Discussion

3.1. Protein engineering of OdhI for the reinforcement of flux distribution toward L-glutamate

It was revealed that, when threonine codons 14 and 15 of the OdhI protein were exchanged to the alanine codon, phosphorylation by protein kinase G (PknG) was abolished [28]. To exist in the completely active form to inhibit ODHC activity, the OdhI protein must be unphosphorylated at threonines 14 and 15. Hence, we performed site-directed mutagenesis to exchange threonine codons 14 and 15 of the OdhI protein. To identify the effect of the native OdhI protein on L-glutamate production, pZ8C carrying the *odhI* gene was constructed and co-transformed with pEKEx2 in *C. glutamicum* (named OI). To construct the reference group, pZ8C was cotransformed with pEKEx2 in *C. glutamicum* (named KC). After 24 h batch cultivation, the L-glutamate production titer (213.67 ± 5.76 mg/L) and yield (8.81 ± 0.59 mg/g glucose) obtained using the OI strain was higher than that of the production titer (142.24 ± 6.65 mg/L) and yield (6.32 ± 0.3 mg/g glucose) obtained with the KC strain (Fig. 2A). To investigate the effect of single mutation of the OdhI protein, threonine codon 14 of the *odhI* gene in pZ8C::odhI was converted into alanine codon, resulting in pZ8C::odhI::T14A. This resultant vector was cotransformed with pEKEx2 in *C. glutamicum* (named OI*). Similarly, the double-mutated *odhI* gene exchanging threonines 14 and 15 to alanine codons was created, generating pZ8C::odhI::T14A::T15A. The resulting vector was also cotransformed with pEKEx2 in *C. glutamicum* (named OI**). During 24 h flask cultivation, the OI* strain showed the L-glutamate production titer (232.35 ± 8.28 mg/L) and yield (10.86 ± 0.71 mg/g glucose) (Fig. 2A). Flask cultivation of the OI** strain allowed production of 320.84 ± 13.92 mg/L of L-glutamate, 1.5-fold higher than that produced by the OI strain. The production yield (13.55 ± 0.78 mg/g glucose) in the OI** strain was also increased 1.5-fold compared with that of the OI strain. The OI** strain presented a similar DCW and specific growth rate compared with the OI and OI* strains as well as the KC strain (Fig. 2B).

In a previous study, the *odhA* gene deletion was used for L-glutamate overproduction [27]. This mutation showed a positive effect on L-glutamate overproduction but a substantial decrease in cell growth. Furthermore, the absence of the *odhA* gene provided genetic instability to the cell, with each clone showing other growth and production patterns, some of which produced less L-glutamate than that of the parental strain. The deletion of the *pknG* gene increased the unphosphorylated status of the OdhI protein, but some remained in the monophosphorylated status presumably due to additional protein kinases existing in *C. glutamicum*

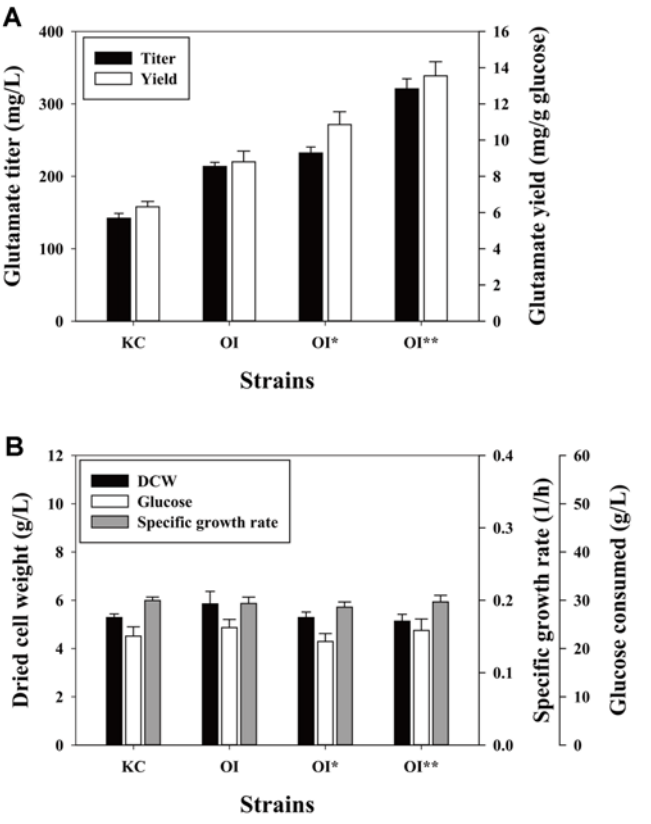

Fig. 2. Effect of OdhI mutants on L-glutamate production in *Corynebacterium glutamicum*. (A) Production characteristic of L-glutamate after 48 h cultivation of the KC, OI, OI*, and OI** strains. The black and white bars indicate the production titer and yield of L-glutamate, respectively. (B) The growth characteristic of KC, OI, OI*, and OI** strains. The black, white, and gray bars indicate the DCW, glucose consumed, and specific growth rate of each strain, respectively.

[28]. Furthermore, the effect of the *pknG* deletion mutant on L-glutamate production depends on the induction of additives [41]. On the other hand, overexpression of the OdhI protein showed a similar maximum cell growth compared to that of wild-type strain, although the lag phase was somewhat delayed [19,29]. Similar to previous studies, our results showed a stable growth pattern and L-glutamate overproduction without the addition of any triggers in OdhI protein-overexpressing strains (Fig. 2). The overexpression of native OdhI protein in *C. glutamicum* increased L-glutamate production, although the phosphorylated OdhI protein existed substantially [19]. Initially, the mutation of threonine codon 14 was more focused on the phosphorylation of the OdhI protein [28]. However, it was recently revealed that the mutation of threonine codon 15 was also important to inhibit ODHC activity to produce diamine putrescine from glutamate [42]. In this study, the double mutation of two threonine codons in the OdhI protein represented the highest L-glutamate production among the engineered

strains (Fig. 2A), and was tried for the first time, to our knowledge. These results indicate that the unphosphorylated OdhI protein by the double mutation inhibits the ODHC activity more efficiently than the native protein, resulting in increased metabolic flux toward L-glutamate. Therefore, the double mutation can be superior to native protein or single mutation for L-glutamate accumulation as well as ALA production.

3.2. Effect on ALA accumulation of OdhI mutants

Owing to the capability of OdhI mutants to overproduce L-glutamate, we hypothesized that the overexpression of OdhI mutants would induce increased ALA production via the flux redistribution of the TCA cycle toward L-glutamate in *C. glutamicum*. To identify the effect of the OdhI protein on ALA accumulation, pZ8C carrying the *odhI* gene was constructed and cotransformed with pEKEx2::hemAL in *C. glutamicum* (named AL-OI). Similarly, the AL-OI* (harboring pEKEx2::hemAL and pZ8C::odhI::T14A) and AL-OI** (harboring pEKEx2::hemAL and pZ8C::odhI::T14A::T15A) strains were also constructed. After 48 h batch cultivation, substantial differences in ALA production were observed among the recombinant strains. Similar to L-glutamate production, the ALA production titer and yield of the AL-OI, AL-OI*, and AL-OI** strains were greater than those of the AL strain harboring pEKEx2::hemAL and pZ8C (Fig. 3A). Among them, the AL-OI** strain showed the highest production titer (2.1 ± 0.07 g/L) and yield (63.38 ± 5.24 mg/g glucose) of ALA. Likewise, the AL-OI, AL-OI*, and AL-OI** strains showed similar DCW and specific growth rates to that of the AL strain (Fig. 3B).

The combinatorial overexpression of the OdhI, $HemA^M$, and HemL proteins in *C. glutamicum* had a synergistic effect on ALA accumulation compared with the $HemA^M$- and HemL-overexpressing strains (Fig. 3A). Furthermore, the double-mutated OdhI-overexpressing AL strain showed the highest ALA production similar to the results for L-glutamate production. These results indicate that the increase in ALA production results from the flux redistribution of the TCA cycle toward L-glutamate by the overexpression of native OdhI and its mutants. As mentioned above, the overexpression of single-mutated OdhI (T15A) showed increased production of diamine putrescine from L-glutamate [42]. Thus, reducing ODHC activity by overexpression of the double-mutated OdhI protein can be an efficient method for ALA accumulation.

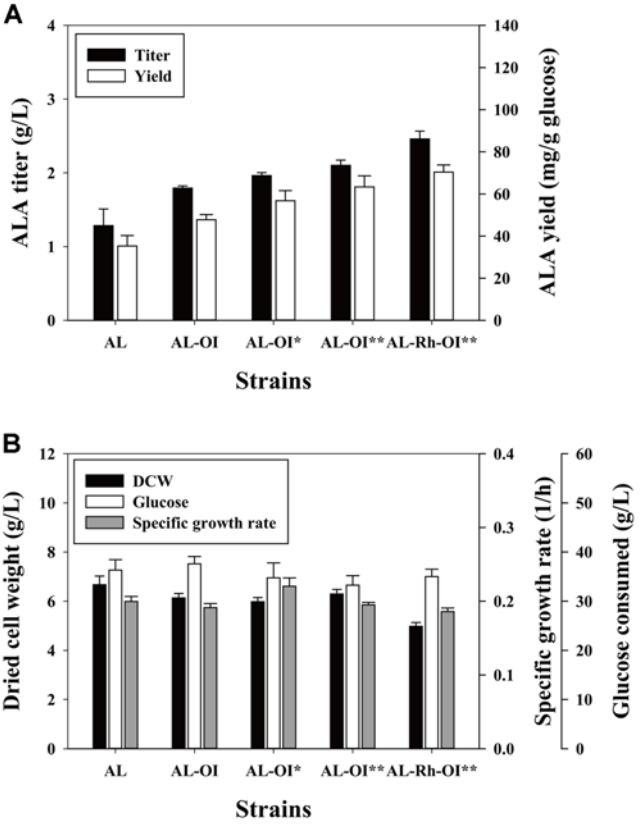

Fig. 3. Effect on ALA production of the overexpression of OdhI mutants in ALA-producing *Corynebacterium glutamicum*. (A) Production characteristic of ALA after 48 h cultivation of the AL, AL-OI, AL-OI*, AL-OI**, and AL-Rh-OI** strains. The black and white bars indicate the production titer and yield of ALA, respectively. (B) Growth characteristics of AL, AL-OI, AL-OI*, AL-OI**, and AL-Rh-OI** strains. The black, white, and gray bars indicate the DCW, glucose consumed, and specific growth rate of each strain, respectively.

3.3. Introduction of the heterogenous ALA exporter RhtA to increase ALA secretion

After engineering efforts toward better flux distribution, we aimed to increase ALA secretion because the more the strain was stepwise developed, the more ALA was accumulated

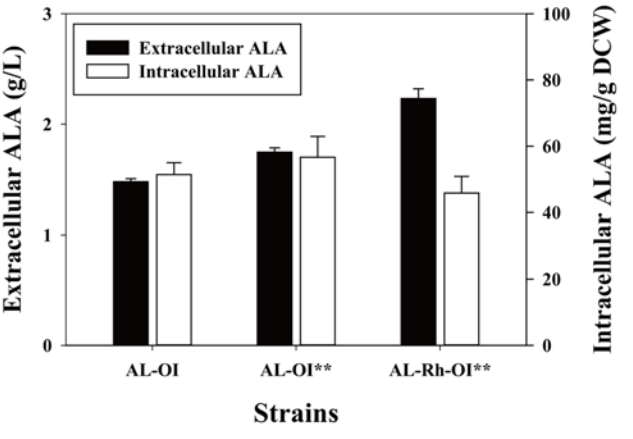

Fig. 4. Distribution of extracellular and intracellular ALA produced in the AL-OI, AL-OI**, and AL-Rh-OI** strains. The black and white bars indicate extracellular of ALA, respectively.

into the cells (Fig. 4). The serine/threonine transporter RhtA from *E. coli* was selected as the ALA exporter. The *rhtA* gene from *E. coli* was inserted into pEKEx2::hemAL, generating pEKEx2::hemALrhtA. The AL-Rh-OI** strain was constructed by coexpressing pEKEx2::hemALrhtA and pZ8C::odhI::T14A::T15A. Although flask cultivation of the AL-Rh-OI** strain showed slightly decreased DCW compared with the AL-OI** strain presumably due to heterologous overexpression of membrane protein, enhanced ALA production was observed with similar specific growth rate and glucose consumption (Fig. 3A, 3B). The AL-Rh-OI** strain showed an ALA production titer of 2.46 ± 0.11 g/L with a yield of 70.35 ± 3.43 mg/g glucose, the highest titer and yield, respectively, among other engineered strains (Fig. 3A). The extracellular ALA concentration of the AL-Rh-OI** strain was increased 1.28-fold compared with that of the AL-OI** strain (Fig. 4). These results indicate that heterologous overexpression of the *rhtA* gene from *E. coli* successfully induced ALA secretion in C5 pathway-engineered *C. glutamicum*, consistent with the result of the previous study that heterologous overexpression of RhtA successfully increased ALA export in the C4 pathway-engineered *C. glutamicum* [12].

3.4. Effect of various triggers on enhanced ALA production

In our previous study, it was reported that the induction of penicillin G showed enhanced ALA production in C5 pathway-engineered *C. glutamicum* via increased L-glutamate flux [41]. In addition to penicillin G, Tween 40 and ethambutol are good candidates for increasing ALA production, because they were verified as a trigger for enhanced L-glutamate production. To identify the effect on ALA production of other triggers, 2 g/L of Tween 40 and 25 mg/L of ethambutol were added for flask cultivation of the AL-Rh-OI** strain at 6 h. During 48 h cultivation, the induction of all triggers showed increased ALA production although with decreased growth patterns (Fig. 5). Among them, the induction of ethambutol showed the best ALA production (2.9 ± 0.15 g/L). Furthermore, the induction of Tween 40 and ethambutol presented 1.29-fold and 1.26-fold higher production yield than the normal culture condition, respectively.

Treatments with triggers generate structural and dynamic changes in the cell membrane or membrane-associated protein such as the Ncgl1221 protein, resulting in L-glutamate overproduction despite decreased growth [27,29]. The cultivation of *C. glutamicum* with the addition of ethambutol caused 56 mM of L-glutamate accumulation via the alteration of the lipid and arabinan composition of the cell membrane, whereas only traces of L-glutamate were detected in the cultivation without the addition of ethambutol [32].

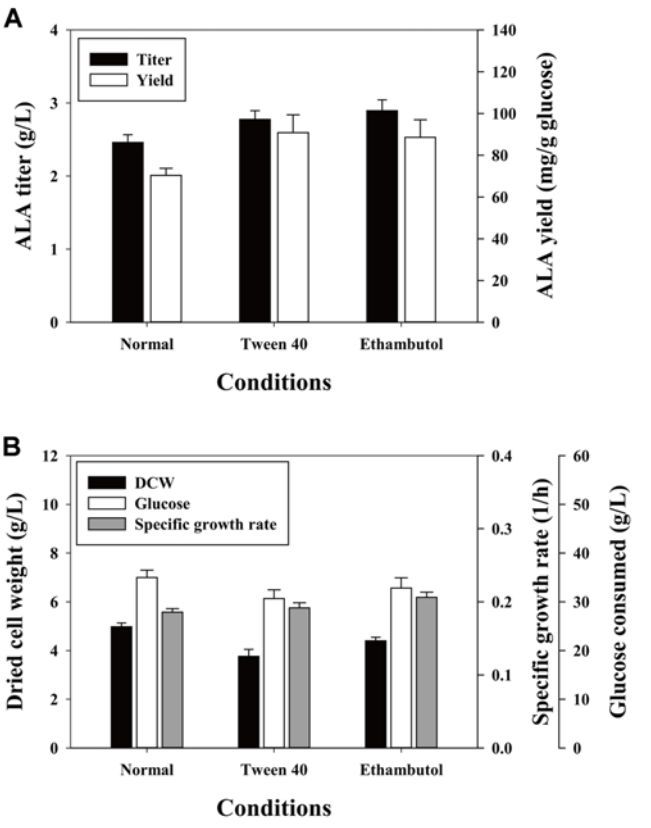

Fig. 5. Effect of the induction of triggers on ALA production in the AL-Rh-OI** strain. (A) Production characteristic of ALA after 48 h cultivation. The black and white bars indicate the production titer and yield of ALA, respectively. (B) Growth characteristic. The black, white, and gray bars indicate the DCW, glucose consumed, and specific growth rate of each strain, respectively.

The addition of Tween 40 increased the level of unphosphorylated OdhI and L-glutamate production [19]. Indeed, as mentioned above, increased L-glutamate production by the addition of penicillin G induced enhanced ALA production [41]. Thus, our results demonstrate that the induction of triggers has a significant effect on ALA overproduction via increased L-glutamate flux.

4. Conclusion

We demonstrated the possibility of the flux redistribution of the TCA cycle toward L-glutamate for enhanced ALA production in *C. glutamicum*. The overexpression of the double mutated OdhI protein contributed to the significant increase in the metabolic flux of L-glutamate and ALA. Furthermore, the introduction of the heterogenous ALA transporter increased ALA secretion successfully. Finally, the treatment of triggers induced ALA accumulation efficiently. Based on this study, the following can be

required for scalable ALA production in a further study: 1) genome recombineering such as gene deletion and attenuation for additional flux redistribution toward ALA and inhibition of byproduct biosynthesis, 2) strong expression of a target gene via a strong synthetic promoter, 3) introduction of target genes into the genome for expression stability.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2018R1A2B2003704) and supported by a Korea University Grant.

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