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5-Aminolevulinic Acid Biosynthesis in *Escherichia coli* Coexpressing NADP-dependent Malic Enzyme and 5-Aminolevulinate Synthase

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Abstract 5-Aminolevulinate (ALA) synthase (E.C. 2.3.1.37), which mediates the pyridoxal phosphate-dependent condensation of glycine and succinyl-CoA, encoded by the *Rhodobacter sphaeroides hemA* gene, enables *Escherichia coli* strains to produce ALA at a low level. To study the effect of the enhanced C4 metabolism of *E. coli* on ALA biosynthesis, NADP-dependent malic enzyme (*maeB*, E.C. 1.1.1.40) was coexpressed with ALA synthase in *E. coli*. The concentration of ALA was two times greater in cells coexpressing *maeB* and *hemA* than in cells expressing *hemA* alone under anaerobic conditions with medium containing glucose and glycine. Enhanced ALA synthase activity *via* coupled expression of *hemA* and *maeB* may lead to metabolic engineering of *E. coli* capable of large-scale ALA production.

Keywords: ALA synthase, NADP-dependent malic enzyme, coexpression, 5-aminolevulinic acid

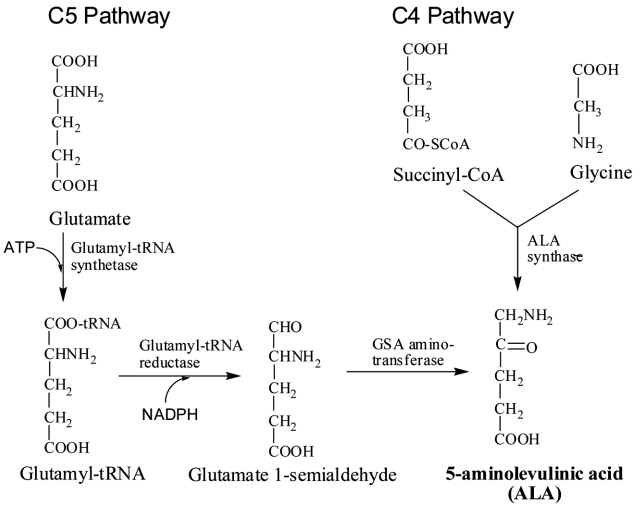

Fig. 1. Biosynthesis of ALA *via* the C4 and C5 pathways.

The C5 amino acid 5-aminolevulinic acid (ALA) is the committed biosynthetic precursor of such tetrapyrroles as heme, porphyrin, chlorophyll, and vitamin B_{12} analogs [1]. Biosynthesis of ALA occurs *via* two distinct pathways [8, 18, 23] (Fig. 1). The C4 pathway, which is found in photosynthetic bacteria as well as in yeast and mammalian cells, involves the pyridoxal-phosphate-dependent condensation of succinyl-CoA and glycine by ALA synthase (EC 2.3.1.37). The C5 pathway, which is found in photosynthetic algae and cyanobacteria, utilizes glutamate with ATP and NADPH as cosubstrates, and involves three steps catalyzed by glutamyl-tRNA synthetase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde aminotransferase. The formation of ALA is believed to be the rate-limiting step in tetrapyrrole biosynthesis because it is tightly regulated by feedback inhibition at the ALA level [29]. Cells treated with ALA are able to bypass this rate-limiting step, leading to an increased tetrapyrrole concentration; thus, ALA is of agricultural interest as a stimulator of plant growth as well as being a photodynamic herbicide and insecticide [22]. ALA has also been shown to increase the salt tolerance of cotton seedlings and the cold tolerance of rice plants [11]. Medical interest in ALA ranges from cosmetic uses, including the restoration of hair growth, to the diagnosis of heavy metal poisoning, porphyria, and brain tumors, as well as the photodynamic therapy of cancer and rheumatoid arthritis. In addition, several biotechnological applications have been reported for heme-containing enzyme production, plant and animal cell cultures, and porphyrin and vitamin B_{12} production [2, 21, 24]. Because of the many applications of ALA, significant effort has been made to optimize its production. For example, chemical synthesis has been proposed; however, such methods are cost-prohibitive, given the number of steps involved and their very low yields [16]. Photosynthetic bacteria such as *Rhodobacter sphaeroides* can also be used to produce ALA, but they require special light-illuminating bioreactors [21]. A

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Table 1. Strains, plasmids, and oligonucleotides used in this study.

Strain, plasmid, or oligonucleotide	Description	Reference or source
E. coli strain		
W3110	Wild-type *E. coli*	KCTC 2223
DH5α	F-φ80 d*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* ($r_K^-m_K^+$) *deoR thi-1 phoA supE44λ*$^-$ *gyrA96 relA1*	Invitrogen Co.
Plasmids		
T vector	T&A cloning vector	RBC Co., Taiwan
pALA7	pUC19 containing *lac* promoter, *hemA* gene from *Rhodobacter sphaeroides*, *hemA* flank region	[27]
pTrc99A	Expression vector, trc promoter, Ap^R	AP Biotech Co.
pTrc(P_{lac}hemA+)	pTrc99A with *lac* promoter, *hemA* gene, *hemA* flank region from pALA7 at the XbaI-PstI	This study
pTrc(P_{lac}hemA$^+$-maeB)	pTrc(P_{lac}hemA+) with RBS and *maeB* gene from *E. coli* at the PstI-HindIII	This study
Oligonucleotides		
For HemA (Forward)	TCTAGACCCCGCGCGTTGGCCGATTCATTAATG (XbaI site underlined)	This study
For HemA (Reverse)	CTGCAGGGATCCGCCAGCGGATCGCGCCCCTCGC (PstI site underlined)	This study
For MaeB (Forward)	CTGCAG**AGGAGG**AACAGACATGGATGACCAGTTAAAAC (PstI site underlined; RBS in bold)	This study
For MaeB (Reverse)	AAGCTTTTACAGCGGTTGGGTTTGCGCTTC (HindIII site underlined)	This study

biological method, based on genetically engineered *Escherichia coli* strains harboring ALA synthase from photosynthetic bacteria, has also been proposed, which produced a few grams of ALA per liter [9, 14, 27].

We recently discovered that the anaerobic C4 metabolism of *E. coli* can be enhanced by overexpression of NADP-dependent malic enzyme (*maeB*, E.C. 1.1.1.40) [12]. Our results suggested that *maeB* expression increases the level of succinyl-CoA in anaerobic C4 metabolism of *E. coli*, which is a substrate of ALA synthase, resulting in greater ALA production. Here, we describe the effect of coexpressing NADP-dependent malic enzyme and ALA synthase on ALA biosynthesis in *E. coli*. We also discuss the further ALA biosynthesis enhancement by metabolic engineering.

The strains, plasmids, and oligonucleotides used in this study are listed in Table 1. Routine DNA manipulations were performed as described by Sambrook and Russell [20]. The *hemA* gene (GenBank CP_000143) was amplified by PCR using pALA7 [27] as the template with the oligonucleotides TCTAGACCCCGCGCGTTGGCCGATTCATTAATG (XbaI site underlined) and CTGCAGGGATCCGCCAGCGGATCGCGCCCCTCGC (PstI site underlined). The PCR fragment (2.0 kb) was ligated into XbaI-PstI-digested pTrc99A after purification from a T-cloning vector (T&A Cloning Vector, RBC, Taiwan), to yield pTrc(P_{lac}hemA$^+$). The *maeB* gene (GenBank AC_000091) was amplified by PCR using *E. coli* W3110 (Korean Collection of Type Culture, KCTC 2223) genomic DNA as the template with the oligonucleotides CTGCAG**AGGAGG**AACAGACATGGATGACCAGTTAAAAC (PstI site underlined, ribosomal binding site in bold) and AAGCTTTTACAGCGGTTGGGTTTGCGCTTC (HindIII site underlined). The PCR fragment (2.3 kb) was ligated into PstI-HindIII-digested pTrc(P_{lac}hemA$^+$) after purification from a T-cloning vector, to produce pTrc(P_{lac}hemA$^+$-maeB). Each PCR fragment was fully sequenced for verification (Bionex Co., Seoul, Korea). The recombinant vectors were electroporated (Gene Pulser, Bio-Rad, Hercules, CA, U.S.A.) into *E. coli* DH5α for the DNA manipulations and into *E. coli* W3110 for the subsequent experiments.

Cells were disrupted using a UP200S sonicator (Hielscher Ultrasonics GmbH, Teltow, Germany) set at 30 W for 1 min at 1-s intervals on ice. After removing the cellular debris by centrifugation (10,000 rpm for 20 min), the supernatant was analyzed for protein content and enzyme activity. The protein content of the extracts was determined using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard. ALA synthase (HemA, E.C. 2.3.1.37) activity was estimated by measuring the rate of ALA formation. The reaction mixture (1 ml) consisted of 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 M disodium succinate, 0.1 M glycine, 0.1 mM pyridoxal phosphate, 15 mM ATP, 0.2 mM CoA, and 50 µl of cell extract. The reaction was allowed to run at 37°C for 30 min. The absorbance of the mixture was measured against standard ALA samples at 555 nm using a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan). One unit of ALA synthase activity was defined as the enzyme amount converting 1 µmole of ALA per min. NADP-dependent malic enzyme (MaeB, E.C. 1.1.1.40) activity was determined from the level of NADPH. The reaction mixture (1 ml) contained 0.1 M Tris-HCl (pH 8.1), 20 mM $MnCl_2$, 2 mM NH_4Cl, 1 mM DTT, 1 mM $NADP^+$, 10 mM malate, 20 mM Na-arsenate, and 25 µl of cell extract. After 15 min at 37°C,

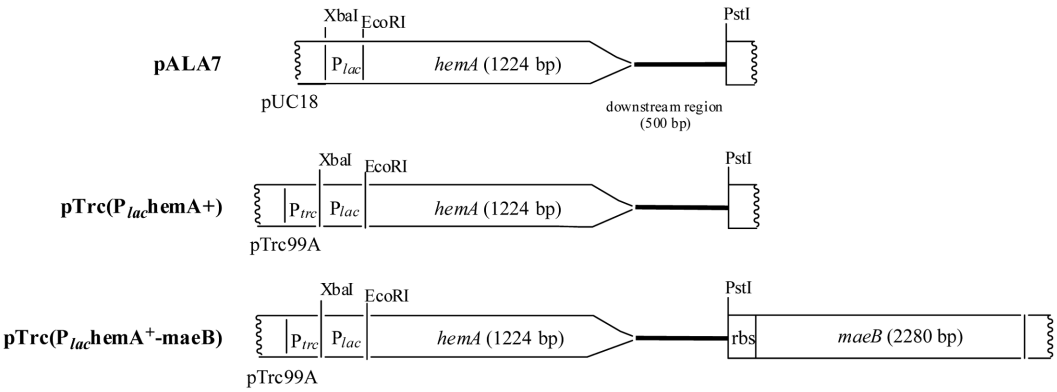

Fig. 2. Structures of the recombinant plasmids.

the absorbance of the mixtures was measured at 340 nm using a multiplate spectrophotometer (Benchmark, Bio-Rad, Hercules, CA, U.S.A.). The extinction coefficient for NADPH at 340 nm was 6.67 mM/cm. One unit of MaeB activity was defined as the enzyme amount converting 1 μmole of malate to pyruvate per min.

Luria-Bertani (LB) medium was used for all DNA manipulations. The glucose-containing medium (G-medium) contained (per liter) 5 g of yeast extract, 10 g of tryptone, 5 g of KH_2PO_4, 10 g of glucose, 2 g of glycine, and 20 g of $NaHCO_3$. The succinic acid-containing medium (S-medium) was the same as the G-medium except that 10 g of succinate (disodium succinate hexahydrate) was added instead of glucose and the bicarbonate was excluded. Antibiotics (20 μg/ml ampicillin) and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were supplemented as needed.

A single colony was transferred into a 15-ml test tube containing 4 ml of LB medium and cultivated in a rotary shaking incubator (37°C, 230 rpm) for 16 h. Aliquots (10 μl) of the cells were used to inoculate into 250-ml Erlenmeyer flasks containing 200 ml of G-medium (anaerobic glucose condition), 100 ml of S-medium (aerobic succinate condition), or 200 ml of S-medium (anaerobic succinate condition). A rubber stopper was used to maintain the anaerobic condition without agitation, whereas a cotton stopper was used for the aerobic condition with agitation at 230 rpm. The Erlenmeyer flasks were incubated in a chamber set at 37°C for 24 h, and 1-ml samples were collected every 4 h for analyses. All experiments were repeated at least three times.

The biomass was estimated by measuring the absorbance at 600 nm. The concentration of ALA in the medium was determined using a spectrophotometer. Briefly, the supernatant (10 μl) was mixed with 0.5 ml of 1 M sodium acetate buffer (pH 4.7), 0.5 ml of deionized water, and 50 μl of acetylacetone (2,4-pentanedione) in a 1.5-ml tube. The tubes were kept for 15 min in a boiling water bath. After cooling, 3 ml of freshly prepared modified Ehrlich's reagent (1 g of *p*-dimethylaminobenzaldehyde, 30 ml of glacial acetic acid, 8 ml of 70% [v/v] perchloric acid, and 12 ml of acetic acid) was added, and the mixture was incubated for 15 min at room temperature [15]. Absorbance of the mixture at 555 nm was measured against standard ALA samples.

Plasmids for *hemA* (ALA synthase) expression and *hemA* and *maeB* coexpression by IPTG induction were constructed as indicated in Fig. 2. PCR-amplified DNA from pALA7, which included the *hemA* structural gene from *R. sphaeroides* under control of a *lac* promoter plus the flanking region of *hemA*, was ligated into pTrc99A to produce pTrc(P_{lac}hemA$^+$). The *maeB* gene from *E. coli* with upstream ribosomal binding site (RBS) was also amplified by PCR, and the fragment was ligated downstream of the *hemA* flanking region in pTrc(P_{lac}hemA$^+$) to produce pTrc(P_{lac}hemA$^+$-maeB). The plasmids pTrc(P_{lac}hemA$^+$) and pTrc(P_{lac}hemA$^+$-maeB) were subsequently introduced into *E. coli* W3110, and the cells were grown in LB medium with 0.1 mM IPTG for 24 h. Distinguishing red-colored colonies were formed when pTrc(P_{lac}hemA$^+$) and pTrc(P_{lac}hemA$^+$-maeB) were induced by IPTG in *E. coli*, suggesting the accumulation of tetrapyrroles in the cells by ALA synthase expression. The enzyme activities of ALA synthase and NADP-dependent malic enzyme in extracts from cells harboring pTrc(P_{lac}hemA$^+$) and pTrc(P_{lac}hemA$^+$-maeB) are listed in Table 2. ALA synthase activity was not detected in the control strain, whereas it was present in the strains harboring pTrc(P_{lac}hemA$^+$) and pTrc(P_{lac}hemA$^+$-maeB). The level of NADP-dependent malic enzyme activity in W3110/pTrc(P_{lac}hemA$^+$-maeB) was more than 0.6 Unit/mg-protein, which is at least four times greater than that in the control strains [*i.e.*, the W3110/pTrc99A and W3110/pTrc(P_{lac}hemA$^+$), less than 0.15 Unit/mg-protein]. Both ALA synthase and NADP-dependent malic enzyme were expressed in W3110/pTrc(P_{lac}hemA$^+$-maeB) under the control of a *lac* promoter.

ALA biosynthesis in *E. coli* expressing *hemA* [W3110/pTrc(P_{lac}hemA$^+$)] or coexpressing *hemA* and *maeB* [W3110/

Table 2. ALA synthase and NADP-dependent malic enzyme activities in *E. coli* W3110.

	ALA synthase	NADP-dependent malic enzyme
W3110/pTrc99A	<0.01	0.12±0.08
W3110/pTrc(P_{lac}hemA$^+$)	1.20±0.18	0.08±0.04
W3110/pTrc(P_{lac}hemA$^+$-maeB)	1.21±0.18	0.65±0.11

The data are given as Units/mg-protein. Each experiment was repeated at least three times, and the numbers indicate the mean±SD of the replicates. Cells were grown for 24 h in LB medium containing 0.1 mM IPTG.

pTrc(P_{lac}hemA$^+$-maeB)] was compared in two different media: S-medium and G-medium. S-medium, an LB-based medium supplemented with succinate and glycine, was designed based on the medium used for ALA synthesis in the previous report [3]. G-medium is an LB-based medium supplemented with glucose, glycine, and bicarbonate, which was based on the medium used to increase C4 metabolism in *maeB*-expressing *E. coli* [12]. Cell growth and ALA biosynthesis in W3110/pTrc(P_{lac}hemA$^+$) and W3110/pTrc(P_{lac}hemA$^+$-maeB) grown under aerobic conditions in S-medium did not vary within the error range (Fig. 3). The maximum ALA concentrations were 0.92 g/l and 0.86 g/l for the *hemA*-expressing and *hemA-maeB*-coexpressing cultures, respectively. ALA biosynthesis followed a different trend, however, in those cultures grown in G-medium under anaerobic conditions (Fig. 4). Although the maximum ALA concentration was about one-twentieth that in the cultures grown in S-medium under aerobic conditions, *E. coli* coexpressing *hemA* and *maeB* produced 54.7 mg/l ALA in 24 h, whereas *E. coli* expressing *hemA* alone produced 35.3 mg/l ALA in 24 h. For those cells grown in G-medium, the highest levels of ALA concentration was observed in the *hemA-maeB* coexpressing cells, and it was 50% greater than the levels detected in the *hemA*-expressing cells, respectively.

Increased C4 metabolism *via* NADP-dependent malic enzyme expression results in increased ALA production by ALA synthase in *E. coli*. Since glycine was provided exogenously, succinyl-CoA availability was the limiting factor for ALA production in our system. NADP-dependent malic enzyme enhances C4 metabolism in cells grown under anaerobic conditions in G-medium, whereas ALA synthase uses the exogenous glycine to condense succinyl-CoA; thus, greater ALA biosynthesis is possible in cells coexpressing both enzymes than in those cells expressing ALA synthase alone (Fig. 4). Under anaerobic conditions, *E. coli* represses α-ketoglutarate dehydrogenase as well as succinate dehydrogenase, thereby transforming the TCA cycle into two branched pathways [17], and the major

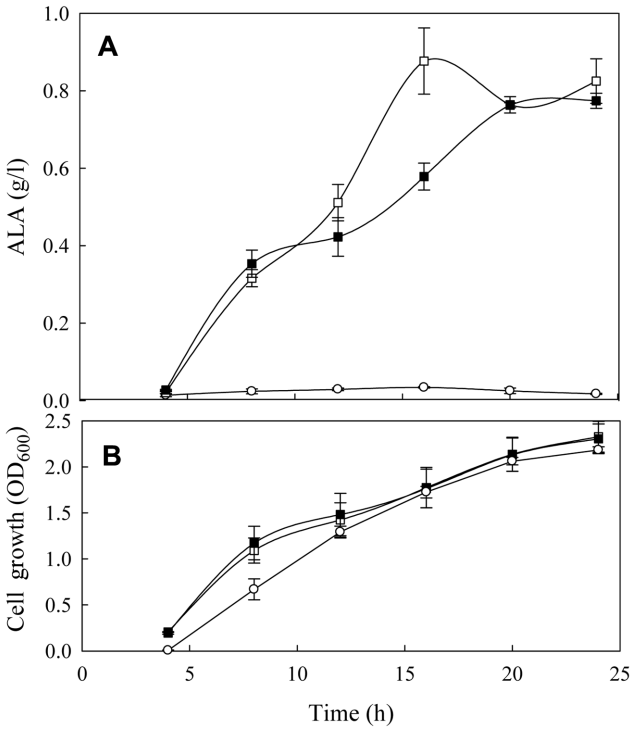

Fig. 3. ALA biosynthesis in recombinant *E. coli* in S-medium under aerobic conditions.
A. ALA biosynthesis. **B**. Cell growth. Symbols represent (○) for control W3110/pTrc99A; (□) for W3110/ pTrc(P_{lac}hemA$^+$); (■) for W3110/ pTrc(P_{lac}hemA$^+$-maeB).

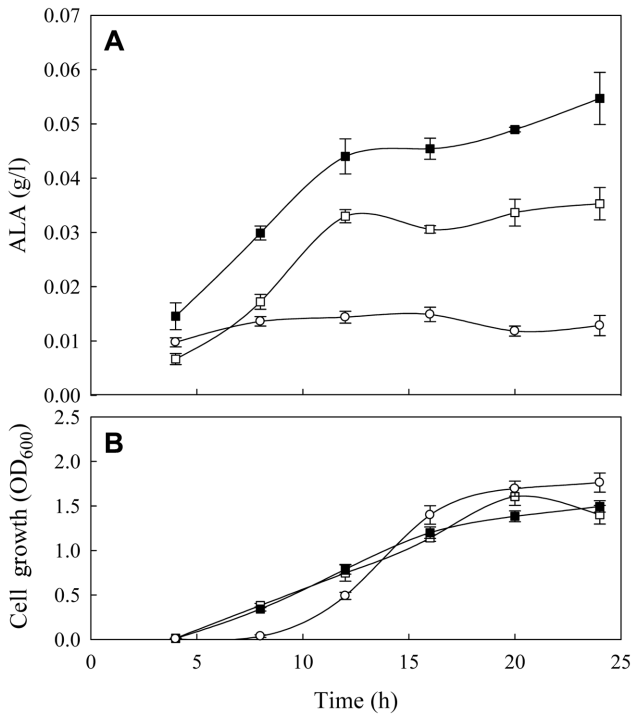

Fig. 4. ALA biosynthesis in recombinant *E. coli* in G-medium under anaerobic conditions.
A. ALA biosynthesis. **B**. Cell growth. Symbols represent (○) for control W3110/pTrc99A; (□) for W3110/pTrc(P_{lac}hemA$^+$); (■) for W3110/ pTrc(P_{lac}hemA$^+$-maeB).

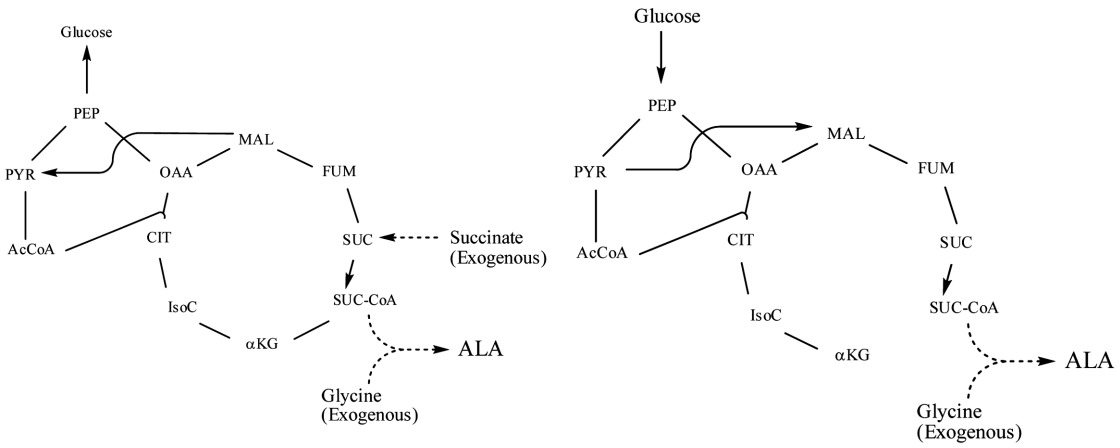

Fig. 5. Metabolic engineering strategy for ALA biosynthesis in *E. coli*.
PEP: phosphoenolpyruvate; PYR: pyruvate; AcCoA: acetyl-CoA; OAA: oxaloacetate; CIT: citrate; IsoC: isocitrate; αKG: α-ketoglutarate; MAL: malate; FUM: fumarate; SUC: succinate; SUC-CoA: succinyl-CoA; ALA: 5-aminolevulinic acid.

carbon flux of succinyl-CoA, which falls into the reductive branch, becomes a "dead end." MaeB expression with bicarbonate supplementation should accelerate the reductive TCA branch as long as there is enough reducing power in the cell [12, 13]. Therefore, the availability of succinyl-CoA for ALA synthase would be increased under these conditions (*i.e.*, glucose, anaerobic, MaeB coexpression). On the other hand, a succinate-based medium and aerobic conditions produced little effect on ALA biosynthesis in cells coexpressing *hemA* and *maeB* (Fig. 3). The succinate would be used for gluconeogenesis as well as for ALA biosynthesis, whereas NADP-dependent malic enzyme would promote the formation of pyruvate from malate, rather than the other way around, thereby decreasing the pool of C4 metabolites. This may be the reason why *hemA-maeB* coexpression had little positive effect on ALA biosynthesis (Fig. 5).

ALA production under anaerobic conditions in G-medium was one-twentieth that under aerobic conditions in S-medium, and cell growth in G-medium was about 60% of the value in S-medium. The high concentration of succinate in the S-medium may have promoted ALA biosynthesis. Metabolic efficiency (*i.e.*, fermentation under anaerobic conditions in G-medium *vs*. respiration under aerobic conditions in S-medium) may also have contributed, since fermentation would require more substrate, leaving less available carbon for ALA biosynthesis. This possibility is supported by the low level of cell growth observed.

The expression of *Rhodobacter hemA* gene in *E. coli* by pTrc99A vector required both an extra *lac* promoter and the flanking region. The pTrc(P_{lac}hemA$^+$) lacking either the *lac* promoter or the flanking region showed no ALA synthase activity, whereas the complete pTrc(P_{lac}hemA$^+$) vector produced consistent ALA synthase activity. The plausible reason might be that *E. coli* may have a PrrA-like protein, a DNA binding protein that is associated with the metabolic switch between aerobic growth and anoxygenic photosynthetic growth in *hemA* expression in *Rhodobacter sphaeroides* regulation, which requires the upstream and downstream regions of the *hemA* structural gene [19]. Another possibility is the involvement of two promoters for *hemA* gene expression [4].

To increase ALA biosynthesis in recombinant *E. coli*, several conditions must be met. As shown in this study, increased C4 metabolism coupled with *hemA* expression is a good method for increasing ALA biosynthesis. To increase the C4 metabolic rate of *E. coli*, strategies based on the expression of anaplerotic enzymes [10, 13], fermentation [25, 28], and increased reducing power [6, 7] have been proposed. In addition to *hemA* expression, succinyl-CoA synthase overexpression may be helpful for increasing ALA biosynthesis. Greater intracellular CoA concentration can be also considered for the higher succinyl-CoA leading ALA biosynthesis [26]. Our results suggest that co-feeding cells with glucose for growth and succinate for ALA biosynthesis might be ideal under anaerobic conditions. The co-feeding of glucose would downregulate the succinate transport system of *E. coli* [5], and artificial expression of a succinate transporter such as DctA (dicarboxylate DAACS transporter) would be required to accomplish the co-feeding. Based on these considerations, further increase of ALA biosynthesis in *E. coli via* metabolic engineering strategies are under way.

Acknowledgments

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