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Cloning and Expression of the *Rhodobacter capsulatus hemA* Gene in *E. coli* for the Production of 5-Aminolevulinic Acid

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Abstract The *hemA* gene encoding 5-aminolevulinic acid synthase (ALAS) was cloned from *Rhodobacter capsulatus*, and its nucleotide sequence was determined. DNA sequencing data revealed one open reading frame coding for a protein with 401 amino acids that displayed high similarity to the amino acid sequences of other known ALASs. The *hemA* gene was then cloned and expressed under the control of constitutive promotor in *E. coli*. The recombinant *E. coli* strain was able to accumulate 5-aminolevulinic acid to 21 mM in the liquid medium supplemented with 45 mM glycine and 120 mM succinate. In addition, a marked effect of the pH of the culture medium on ALA production was observed, and the optimum pH for culture medium was determined to be 5.8–6.3.

Key words: *Rhodobacter capsulatus*, *hemA*, ALA synthase, 5-aminolevulinic acid

5-Aminolevulinic acid (ALA) is the first compound in the tetrapyrrole biosynthesis pathway that leads to the formation of hemes, chlorophylls, corrins, and bile pigment [2, 11]. ALA is synthesized by either of two major pathways. In the C_4 pathway, ALA is formed by the condensation of glycine and succinyl-CoA [3, 16]. This reaction is catalyzed by ALA synthase (ALAS), which is found in animal cells, yeasts, fungi, and certain bacteria. In the C_5 pathway, ALA is formed from glutamate by a series of reactions, including the activation of glutamate by ligation to tRNA, reduction of the activated glutamate to yield glutamate-1-semialdehyde (GSA) by an NAD(P)H-dependent reductase, and transamination of GSA to form ALA by a GSA 2,1-aminotransferase [3, 4]. The C_5 pathway is found in plant chloroplasts, cyanobacteria, anaerobic archaebacteria, *Escherichia coli*, and *Salmonella typhimurium* [9, 13].

ALA has a variety of agricultural applications not only as an herbicide, insecticide, and growth promoting factor [25, 27], but also some based on its ability to confer salt and cold temperature tolerance to plants [14]. In animal fields, new applications to improve animal productivity are being tested. In addition, several medical applications, including tumor diagnosis and cancer treatment, have been reported [27]. Due to its physiological significance, genes encoding ALA synthase have been cloned from diverse organisms, including bacteria, fungi, plants, and animals. Much of the research on ALA production microbially have so far been focused on using whole-cell system [6, 7, 17, 20, 27, 30]. Photosynthetic bacteria, especially purple nonsulfur bacteria, accumulate ALA under light illumination in the presence of levulinic acid [8, 23]. However, the use of light illumination on an industrial scale is not economical, and the levels of ALA accumulated by these microorganisms were less than 2.0 mM [28]. Recently, ALA production from recombinant *E. coli* by overexpression of the *hemA* gene placed under the inducible promotor has been reported [6, 30].

We describe here the cloning of the *hemA* gene encoding ALA synthase from *Rhodobacter capsulatus*, and the production of ALA, without the addition of LA, higher than any concentration reported before, by recombinant *E. coli* containing *R. capsulatus hemA* gene.

Bacterial Strains, Plasmids, and Growth Conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium [26] at 37°C on a rotary shaker at 200 rpm. Where necessary, ampicillin was used at a final concentration of 50 μg/ml.

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Table 1. Bacterial strains and plasmids used.

Strains or plasmids	Relevant properties	Referenece or source
Rhodobacter capsulatus ATCC11166		KCCM
E. coli strains		
DH5α	supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Takara-Korea Biomedical
BLR(DE3)	F⁻ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcmΔ(srl-recA)306::Tn10(Tc*^{R}*)*(DE3)	Novagen
Plasmids		
pHCE-IIB	Expression vector; Ap^r	Takara-Korea Biomedical
pHEMA	pHCE IIB harboring a 1.2kb *Nde*I/*Hin*dIII fragment from *R. capsulatus* encoding hemA	This study
BH3	Ap^r; 1.2 kb *Nde*I/*Hin*dIII fragment inserted	This study

R. capsulatus was grown in PYE medium (0.3% Bacto peptone, 0.3% yeast extract) at 37°C on a rotary shaker at 200 rpm. For the extracellular ALA production, the transformant *E. coli* was cultivated in the ALA production medium, containing 1% NaCl, 0.7% yeast extract, 30 mM glycine, and 90 mM succinate [6].

Cloning of *hemA* Gene from *Rhodobacter capsulatus*

To clone the *hemA* gene from *R. capulatus*, genomic DNA of *R. capsulatus* ATCC11166 was extracted according to the method of Mak and Ho [21]. The consensus PCR primers, based upon a comparison of bacterial *hemA* gene sequences, were RC1 (5'-ggtgacgcatatggactacaatct-3') and RC2 (5'-cctcggcaagcttcacgcacagcg-3') for the amplification of the *hemA* gene from the genomic DNA. These primers were designed with *Nde*I and *Hin*dIII restriction sites, respectively, to allow in-frame insertion of the *hemA* gene into the *Nde*I/*Hin*dIII sites of the pHCE-IIB expression vector. PCR was performed in a 50 μl reaction mixture, containing primers (100 pmole), template DNA (100 ng), 5 μl of 10× Taq polymerase buffer, and 4 μl of 2.5 mM

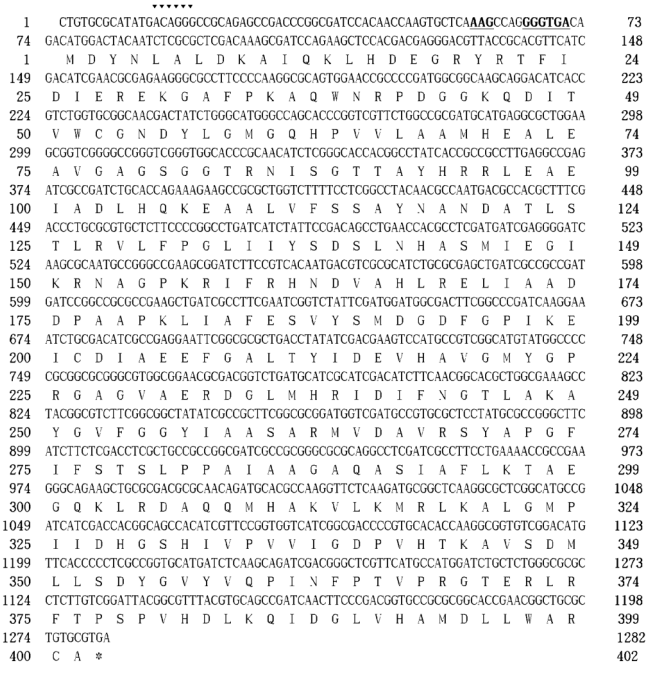

Fig. 1. Nucleotide and deduced amino acid sequences of a 1.2 kb DNA fragment from the *R. capsulatus hemA* region.
The putative Shine-Dalgarno sequence preceding the start codon is underlined. Arrowheads indicate a 6 bp motif which is similar to sequences at the transcriptional initiation sites of the *R. capsulatus puf* [1], *puc* [30] operons, and of the *Rhizobium meliloti* ALAS gene [16].

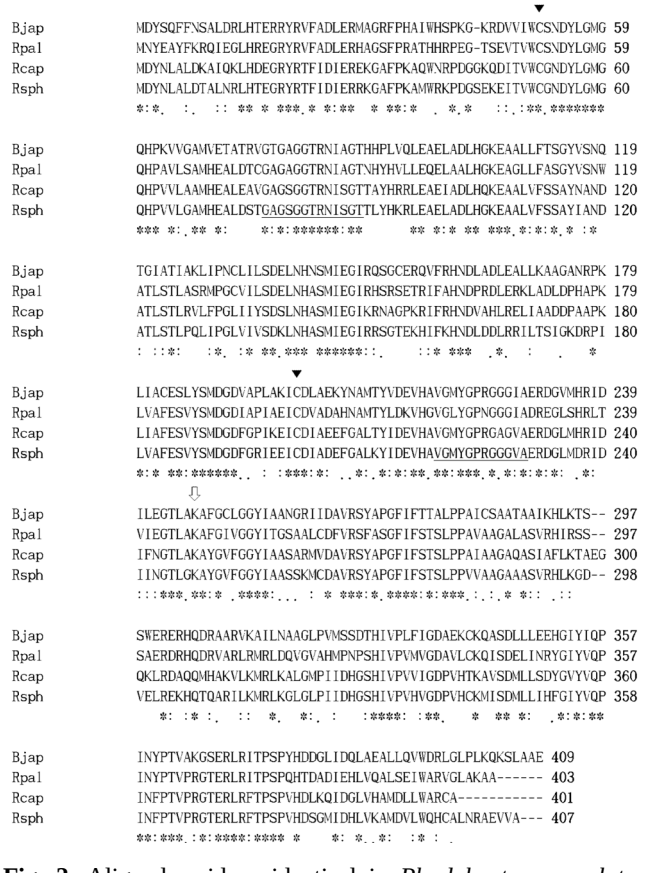

Fig. 2. Aligned residues identical in *Rhodobacter capsulatus* ATCC11166 HemA (Rcap), *Bradyrhizobium japonicum* (Bjap), *Rhodopseudomonas palustris* (Rpal), and *Rhodobacter sphaeroides* (Rsph) are indicated by an asterisk.
Residues likely to be involved in pyridoxal phosphate binding are indicated by open arrow. Two glycine-rich regions which could be involved in CoA or pyridoxal phosphate binding are underlined. Closed arrowheads show two cysteine residues conserved in all of the ALA synthase sequences.

dNTP. A 1.2-kb DNA fragment carrying *hemA* was isolated by PCR from chromosomal DNA of *R. capsulatus* ATCC11166 (data not shown), and cloned into the pSTBlue-1 cloning vector for sequencing.

Sequencing experiments showed that the *hemA* coding region was 1,206 bp long starting with an ATG. This sequence predicts a protein of 401 amino acids with a molecular weight of 43,675 Da. The entire *hemA* nucleotide sequence and translated amino acid sequence are shown in Fig. 1. A potential ribosome-binding sequence was found 6 bp upstream of the ATG initiation codon.

The deduced amino acid sequence of *R. capsulatus hemA* was aligned with those reported for other purple bacterial ALA synthases (Fig. 2). Overall, the deduced amino acid sequence of the *R. capsulatus* ATCC11166 *hemA* shares 75% identity with the *Rhodobacter sphaeroides hemA*, 55% identity with the *Bradyrhizobium japonicum hemA*, and 57% identity with the *Rhodopseudomonas palustris hemA*. Multiple amino acids sequence alignments showed a highly conserved domain. Specifically, the amino acid sequence around Lys248 of the cloned gene is well conserved. The ε-amino group of the active site lysine residue that is involved in the formation of an internal Schiff base between the formyl group of PLP and the residue was conserved among various ALA synthases [15]. Sequences implicated in catalytic activity and pyridoxal phosphate cofactor binding, characterized by arginine- and glycine-rich regions, are conserved [10]. Also, two cysteine residues (C52, C201) are conserved in all of the ALA synthase sequences.

Heterologous Expression of *hemA* Gene from *Rhodobacter capsulatus* in *E. coli*

For the expression of *hemA* gene encoding 5-aminolevulinic acid synthase, the constitutive expression vector pHCE-IIB was employed. Using the restriction enzyme *Nde*I and *Hin*dIII, the *hemA* gene from the recombinant pSTBlue-1 vector was digested and subsequently subcloned into the expression vector pHCE-IIB. This placed the *hemA* gene under the promotor of D-amino acid aminotransferase (D-AAT) gene from *Geobacillus toebii* [21], in-frame with an ATG start site located in the vector. The resulting plasmid, designated pHEMA, was transformed into *E. coli* BLR(DE3).

After cultivation on an ALA production medium containing 90 mM succinate and 30 mM glycine, cell extracts of *E. coli* containing pHEMA were analyzed by 10% SDS-PAGE [18]. As shown in Fig. 3, ALA synthase was detectable in the extracts of *E. coli* cells harboring the *R. capsulatus hemA* gene, while not detectable in the extracts of *E. coli* cells harboring vector only. ALAS encoded by *hemA* from *R. capsulatus* has a molecular mass of 44 kDa, which was estimated by its amino acid sequences. A distinct protein band with this molecular mass was observed only for *E. coli* BH3 containing pHEMA

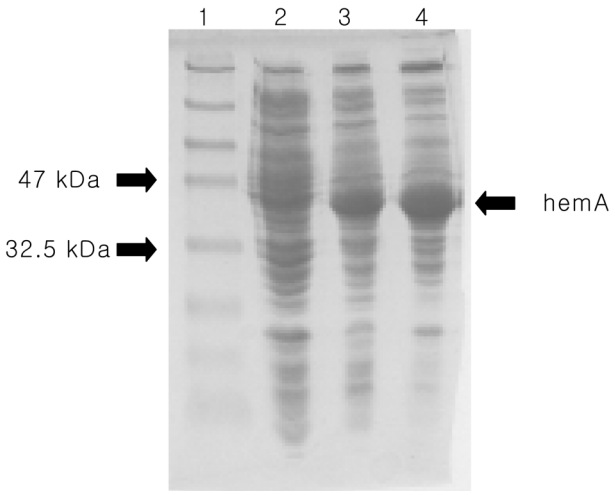

Fig. 3. SDS-PAGE of ALA synthase expressed by pHEMA.
The position of the *hemA* gene product is indicated at the right. Lane 1, marker; lane 2, control transformed with pHCE-IIB only; lanes 3 and 4, *E. coli* BLR(DE3) containing pHEMA after incubation for 24 h, 48 h.

(Fig. 3, lanes 3 and 4). In the extracts of cells containing plasmid pHCE-IIB only, the protein corresponding to the estimated molecular mass was not detected (Fig. 3, lane 2). There was no difference in the ALAS expression levels between 24 h and 48 h of cultivation, suggesting that ALAS is constitutively expressed.

Effect of pH of the Culture Medium on ALA Production

ALA in aquous solution is found to be unstable, which is dependent on pH and other factors [11]. Therefore, the effect of initial pH of the culture medium on the level of ALA production was investigated. ALA concentrations in the medium were measured colorimetrically [22] as follows. To 10 µl of supernatant, 1 ml of 0.5 M sodium acetate buffer (pH 4.7) and 50 µl of acetylacetone (2,4-pentanedione) were added. Then, tubes were boiled in a water bath for 10 min. ALA and acetylacetone were condensed to form a pyrrole compound, 2-methy-3-acetyl-4-(3-propionic acid) pyrrole, in the acetate buffer at 100°C. After cooling, 3 ml of freshly prepared modified Ehrlich's reagent (1 g of ρ-dimethylamino-benzaldehyde, 8 ml of 70% perchloric acid, and 42 ml of acetic acid solution) were added. The A_{555} of the mixture was measured after 5 min at room temperature. The pyrrole compound in each tube was visualized (purple-red) by allowing it to react with ρ-dimethylaminobenzaldehyde in Ehrlich's reagent.

As shown in Fig. 4, a marked effect of the initial pH of the medium on ALA production by recombinant *E. coli* was observed, and it was concluded that the optimum medium pH for ALA production was 5.8–6.3. A slightly lower level of ALA production was observed at below pH 5.8, because of inhibition of cell growth (data not shown). Additionally, a much lower level of ALA production was

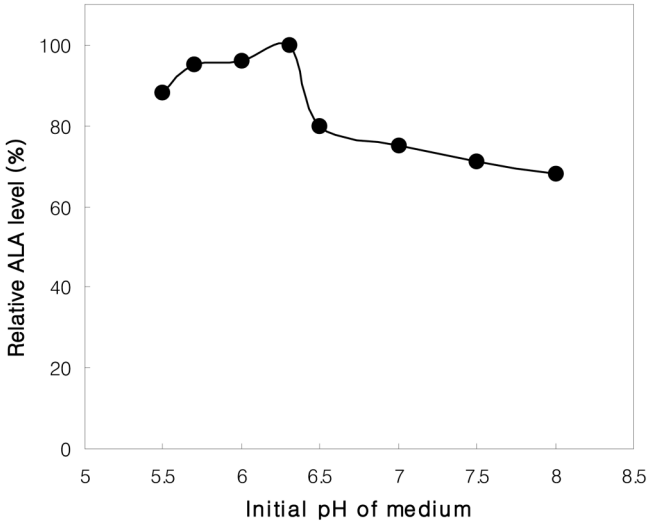

Fig. 4. Effect of pH of the culture medium on ALA production in recombinant *E. coli* containing the *hemA* gene.
Cells were grown at 30°C in ALA production medium.

observed in alkaline condition, indicating that ALA degrades into pyrazine compounds in alkaline solution [5]. This result suggests that ALA production should be carried out at a slightly acidic condition.

Effect of Succinate and Glycine on ALA Production

Because there are several reports that succinate and glycine enhance the ALA production as precusor or growth substrate [6, 27, 30], we first examined the effect of succinate level in the growth medium on ALA production. As shown in Fig. 5, cell growth rate was slightly inhibited as the succinate concentration increased. However, the addition of succinate up to 120 mM resulted in the increase of the extracellular ALA accumulation, and a slight increase in the level of ALA synthase was also observed (data not shown). When higher concentration (150 mM) of succinate was added to the medium, less ALA was produced due to the substrate inhibition [30].

Glycine is another important factor for ALA production, because it is a precursor for ALA production [6, 27, 30]. Therefore, we investigated the effect of glycine level in the growth medium on ALA production. As shown in Fig. 6, cell growth was inhibited as the glycine concentration increased. However, the addition of glycine up to 45 mM resulted in the increase of extracellular ALA accumulation, and a marked increase in the level of ALA synthase activity was also observed (data not shown). When glycine was added to the medium at higher concentration (60 mM), less ALA was produced. Strong inhibition of cell growth was probably due to both glycine and ammonia produced as a result of glycine metabolism [27]. Extracellular ALA accumulation continued for 50 h and then decreased slightly, indicating degradation of ALA into pyrazine compounds and tetrapyrroles [5, 11].

As shown in the above results and previous reports, physiological factors such as medium pH and substrates had an enormous effect on ALA production. When glycine and succinate were added together to the concentration of 45 mM and 120 mM, respectively, ALA accumulated in the medium up to 3.5 g/l. Much of the research on ALA production has so far been microbially focused. Recent investigations revealed the possibility of microbial ALA production by overexpression of *hemA* genes placed under the inducible promotor in *E. coli*. Werf and Zeikus [30] achieved up to 2.25 mM ALA by recombinant *E. coli* whole cell containing *hemA* gene, and Choi *et al.* [6] also reported extracellular accumulation of ALA up to 15 mM

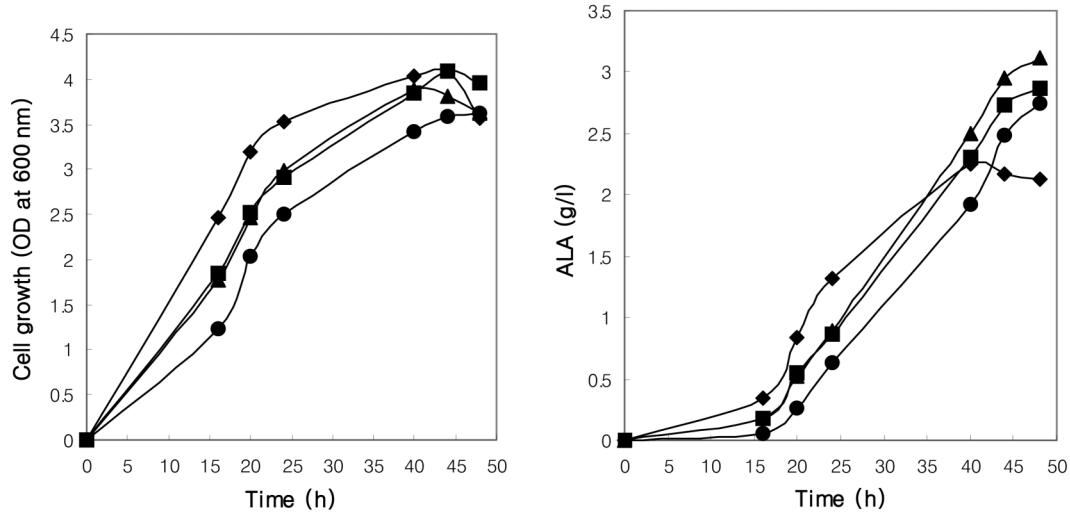

Fig. 5. Effect of succinate on the growth and ALA production of the recombinant *E. coli* containing the *hemA* gene.
Symbols used for 60 mM (◆); 90 mM (■); 120 mM (▲); 150 mM (●).

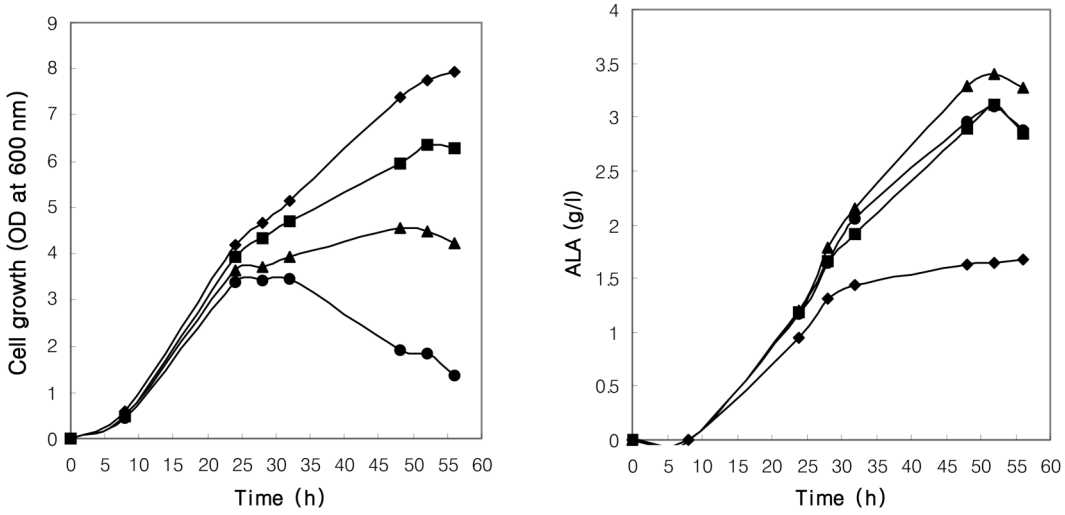

Fig. 6. Effect of glycine concentration on the growth and ALA production of recombinant *E. coli* containing the *hemA* gene.
Symbols used for 15 mM (◆); 30 mM (■); 45 mM (▲); 60 mM (●).

from recombinant *E. coli* containing *Bradyrhizobium japonicum* ALA synthase under the control of inducible promotor.

The recombinant *E. coli* containing *R. capsulatus hemA* gene under the control of constitutive promotor produced ALA at a maximum concentration of 21 mM in the absence of levulinic acid, an inhibitor of ALA dehydratase. Through a further metabolic pathway engineering and optimization of large-scale production conditions, we expect to be able to economically supply microbial-produced ALA for industrial applications.

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