J. Microbiol. Biotechnol. (2013), **23**(5), 668–673
http://dx.doi.org/10.4014/jmb.1302.02022
First published online March 16, 2013
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of Gene Amplifications in Porphyrin Pathway on Heme Biosynthesis in a Recombinant *Escherichia coli*

Lee, Min Ju[1], Hye-Jung Kim[1†], Joo-Young Lee[1], An Sung Kwon[2], Soo Youn Jun[2], Sang Hyeon Kang[2], and Pil Kim[1*]

[1]*Department of Biotechnology, The Catholic University of Korea, Bucheon 420-742, Korea*
[2]*iNtRON Biotechnology Inc., Seongnam 462-120, Korea*

Received: February 13, 2013 / Revised: February 18, 2013 / Accepted: February 22, 2013

A recombinant *E. coli* co-expressing ALA synthase (*hemA*), NADP-dependent malic enzyme (*maeB*), and dicarboxylic acid transporter (*dctA*) was reported to synthesize porphyrin derivatives including iron-containing heme. To enhance the synthesis of bacterial heme, five genes of the porphyrin biosynthetic pathway [pantothenate kinase (*coaA*), ALA dehydratase (*hemB*), 1-hydroxymethylbilane synthase (*hemC*), uroporphyrinogen III synthase (*hemD*), and uroporphyrinogen III decarboxylase (*hemE*)] were amplified in the recombinant *E. coli* co-expressing *hemA-maeB-dctA*. Pantothenate kinase expression enabled the recombinant *E. coli* to accumulate intracellular CoA. Intracellular ALA was the most enhanced by uroporphyrinogen III synthase expression, porphobilinogen was the most enhanced by ALA dehydratase expression, uroporphyrin and coproporphyrin were the most enhanced by 1-hydroxymethylbilane synthase expression. The strain co-expressing *coaA*, *hemA*, *maeB*, and *dctA* produced heme of 0.49 μmol/g-DCW, which was twice as much from the strain without *coaA* expression. Further pathway gene amplifications for the porphyrin derivatives are discussed based on the results.

Key words: Porphyrin pathway, bacterial heme, pantothenate kinase, uroporphyrinogen III synthase, uroporphyrinogen III decarboxylase

Porphyrins are present in nature as ubiquitous pigment cofactors, based on the four pyrrole rings (tetrapyrrole) structure, for a variety of biological processes including the storage and transport of oxygen molecule (myoglobin and hemoglobin), electron transport for oxidative phosphorylation (cytochromes *b* and *c*), and oxidation of hydrocarbon (cytochrome P450 and cytochrome oxidase) [5]. One of the best-known porphyrins is the pigment of red blood cells, heme, that contains the coordination bound ferrous ion (Fe^{2+}) in a protoporphyrin structure. The fact that iron in the heme structure has a much higher bioavailability than non-heme iron [7] and the fact that heme iron absorption is not affected by other dietary constituents have enabled heme to be used for food or animal feed additives [11]. Even though heme can be chemically synthesized either by total synthesis or derivatization from heme or chlorophyll of other animals or plants, regio- and stereoselective functionality cannot be achieved easily from the above. Considering that chemical synthesis requires several steps that result in low yields, a biosynthetic approach might be an alternative way to obtain natural porphyrins in relatively high yields [4].

The most studied heme biosynthesis is the porphyrin pathway (Fig. 1). For the first synthesis of 5-aminolevulinic acid (ALA), two pathways has been reported. Phototrophic algae, cyanobacteria, and *Escherichia coli* use the C5 pathway comprising of 3 steps (glutamyl-tRNA synthase, glutamyl-tRNA reductase, glutamate-1-semialdehyde aminotransferase) from glutamic acid with cofactors of ATP, NADPH, and tRNA. Mammal cells, yeast, and phototrophic purple non-sulfur bacteria synthesize ALA *via* the C4 pathway comprising a single-step condensation of glycine and succinyl-CoA mediated by ALA synthase. Two molecules of ALA are condensed to porphobilinogen (PBG) and 4 molecules of PBG are further condensed to form 1-hydroxymethylbilane (HMB). HMB is cyclized to uroporphyrinogen III (spontaneously further oxidized to uroporphyrin by molecular oxygen), which is the general precursor of all tetrapyrroles in nature. Uroporphyrinogen III is decarboxylated to coproporphyrinogen III (spontaneously oxidized to coproporphyrin III), and further oxidation and another decarboxylation turn coproporphyrinogen III to protoporphyrin IX. Finally, ferrous iron is cooperatively bound to protoporphyrin IX to heme [1].

*Corresponding author
Phone: +82-2-2164-4922; Fax: +82-2-2164-4922;
E-mail: kimp@catholic.ac.kr

†Present address: R&D, Samyang Genex Co., Daejeon, Korea

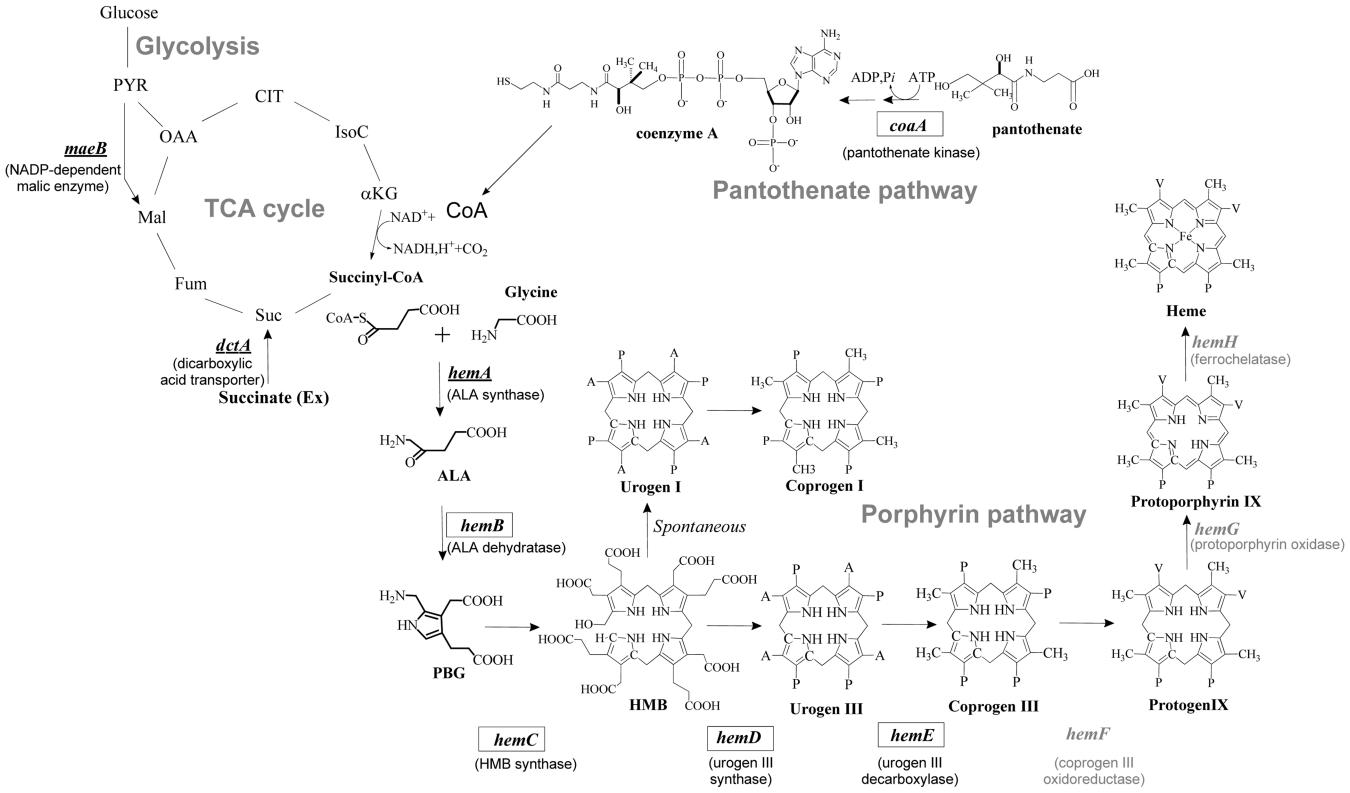

Fig. 1. Porphyrin pathway in the heme-synthesizing recombinant *E. coli*.
Genes underlined represent the ones in the control strain, and genes boxed the ones in this study. Ex: external; Urogen: uroporphyrinogen; Coprogen: coproporphyrinogen. Uroporphyrinogen and coproporphyrinogen were analyzed as their spontaneously oxidized forms, uroporphyrin and coproporphyrin.

We have reported that *E. coli*, which naturally possesses the C5 pathway for ALA synthesis, could produce ALA and porphyrins by introducing the ALA synthase (*hemA*) of *Rhodobacter sphaeroides*, a microorganism having the C4 pathway [10]. In addition to C4 pathway enzyme expression, co-expressions of NADP-dependent malic enzyme (*maeB*) and dicarboxylic acid transporter (*dctA*) enabled *E. coli* to produce heme as a bioavailable iron source [3]. We also reported an industry-feasible chemically defined medium for the heme synthesis using the recombinant *E. coli* [6]. Nevertheless, the three gene expressions enabled *E. coli* to synthesize heme, and an additional pathway engineering was needed considering the relatively many enzymatic steps were involved in the porphyrin pathway. This idea provoked the authors to further investigate the effect of additional gene expressions on the heme biosynthesis in the recombinant *E. coli*.

In this study, we expressed enzymes such as pantothenate kinase (*coaA*) to increase the CoA pool for a ALA precursor, succinyl-CoA, and enzymes in the porphyrin pathway including ALA dehydratase (*hemB*), HMB synthase (*hemC*), uroporphyrinogen III synthase (*hemD*), and uroporphyrinogen III decarboxylase (*hemE*) in the recombinant *E. coli*. The effect of the enzyme expressions are further discussed for the synthesis of heme and porphyrin derivatives.

Materials and Methods

Plasmids and Strains

The plasmids and strains used are listed in Table 1. The heme-synthesizing control strain was *Escherichia coli* W3110(DE3) with pTrc(p_{lac}*hemA*$^+$-*maeB-dctA*) containing ALA synthase (*hemA*), NADP-dependent malic enzyme (*maeB*), and dicarboxylic acid transporter (*dctA*) (short for HMD strain) as reported [3] and the DH5α strain for DNA manipulation. Oligonucleotides were synthesized at Bioneer Co. (Daejeon, Korea) and DNA sequencing was performed at Macrogen Co. (Seoul, Korea). The oligonucleotides for PCR amplifications were as follows: 5'-TTC TGC AG**A GGA GG**A ACA GAC ATG AGT ATA AAA GAG CAA ACG TTA ATG A-3' (*Pst*I site underlined, ribosomal binding site in bold) and 5'-TCT GCA GTT ATT TGC GTA GTC TGA CCT CTT CTA-3' (*Pst*I site underlined) for *coaA*; 5'-CATATG CCC CTC GAT TCC ACA AAC A-3' (*Nde*I site underlined), 5'-GGATCC TTA ACG CAG AAT CTT CTT CTC-3' (*Bam*HI site underlined) for *hemB*; 5'-GAGCTC **AGG AGG** CAA GCA TGT TAG ACA ATG TTT TAA G-3' (*Sac*I site underlined, ribosome binding site in bold) and 5'-GAG CTC ACT CAA TCA GCG GAA AAT GC-3' (*Sac*I site

Table 1. Plasmids and strains used in this study.

Plasmid and strain	Description	Source and reference
Plasmids		
T blunt vector	Cloning vector, Amp^R	Solgent, Korea
pTrc99a	Expression vector, trc promoter, Amp^R	AP Biotech Co. USA
pET24a	Expression vector, T7 promoter, km^R	Novagen, Germany
pTrc(P_{lac}*hemA*$^+$*-maeB-dctA*)	pTrc99A with lac promoter, *hemA* gene, *maeB* gene, and *dctA* gene from *E. coli*	Kwon *et al*. (2009)
pET(*coaA*)	pET24a with T7 promoter, *coaA* gene from *E. coli*	This study
pET(*hemB*)	pET24a with T7 promoter, *hemB* gene from *E. coli*	This study
pET(*hemC*)	pET24a with T7 promoter, *hemC* gene from *E. coli*	This study
pET(*hemD*)	pET24a with T7 promoter, *hemD* gene from *E. coli*	This study
pET(*hemE*)	pET24a with T7 promoter, *hemE* gene from *E. coli*	This study
Strains		
E. coli W3110(DE3)	*E. coli* W3110 λDE3(*imm*^{21}*Δnin5 Sam7*), a λ prophage carrying the T7 RNA *pol* gene	Kim *et al*. (2012)
Blank	W3110(DE3) harboring pTrc99a and pET24a	This study
HMD	W3110(DE3) harboring pTrc(P_{lac}*hemA*$^+$ *-maeB-dctA*)	This study
HMD-A	HMD strain harboring pET(*coaA*)	This study
HMD-B	HMD strain harboring pET(*hemB*)	This study
HMD-C	HMD strain harboring pET(*hemC*)	This study
HMD-D	HMD strain harboring pET(*hemD*)	This study
HMD-E	HMD strain harboring pET(*hemE*)	This study

underlined) for *hemC*; 5'-AAGCTT **AGG AGG** AGA TCC TCG CTG AAG TCT ATA A-3' (*Hin*dIII site underlined, ribosome binding site in bold) and 5'-AAGCTT ATG AGA GTT ATT GTA ATG CCC GTA A-3' (*Hin*dIII site underlined) for *hemD*; and 5'-CTCGAG **AGG AGG** CAC TAA GGA ACA GCC AAA ATG A-3' (*Xho*I site underlined, ribosome binding site in bold) and 5'-CTC GAG TAA TGA CGC GAG ATC CAT AAT CAC T-3' (*Xho*I site underlined) for *hemE*. Genomic DNA of *E. coli* W3110 (KCTC 2223) was used as the template. The PCR products (*coaA*: 980 bp; *hemB*: 1,008 bp; *hemC*: 1,043 bp; *hemD*: 803 bp; *hemE*: 1,116 bp) were subcloned into a T blunt vector (T Blunt Vector Cloning Kit; Solgent, Daejeon, Korea), and further purified using a gel purification kit (Cosmogene Tech Co., Seoul, Korea) after a corresponding restriction cut. The DNA fragments were ligated into a correspondingly restricted pET24a vector (Novagen, Darmstadt, Germany) yielding pET(*coaA*), pET(*hemB*), pET(*hemC*), pET(*hemD*), and pET(*hemE*), respectively. The constructed vectors were electroporated into the HMD strain (*E. coli* W3110[DE3]/pTrc[p_{lac}*hemA*$^+$*-maeB-dctA*]).

Media and Culture

Luria–Bertani (LB) medium was used for the DNA manipulating process. The medium for porphyrins and heme synthesis contained LB medium supplemented with glycine and succinate (GS medium) composed of 5 g yeast extract, 10 g tryptone, 5 g KH_2PO_4, 10 g $NaHCO_3$, 9 g glucose, 10 g succinate hexahydrate, and 2 g glycine per liter of medium. Antibiotics (20 μg/ml ampicillin and kanamycin) and isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) was added depending on the harboring vectors. The initial pH of the medium was set to 6.5 by addition of 6 N HCl.

A single colony was inoculated into a 15 ml tube containing LB medium (3 ml) and incubated for 16 h at 37°C and 250 rpm. The culture broth (0.5 ml) was transferred to a 250 ml Erlenmeyer flask containing GS medium (50 ml) for 48 h at 37°C and 250 rpm. Samples were taken at 24 h for precursors (CoA, ALA, and PBG) and at 48 h for porphyrins (uroporphyrin, coproporphyrin, and heme) for further analysis.

Analysis

The optical density at 600 nm of culture broth was measured and converted into biomass with the extinction coefficient (1 O.D. = 0.3 g/l). The porphyrins and intermediates were analyzed according to previous reports [3, 6]. The ALA and PBG concentrations of the culture broth were estimated by spectrophotometry at 550 nm after separation by an ion-exchange column (ALA/PBG Column Test Kit; Bio-Rad Laboratory, Hercules, CA, USA) and visualization with Ehrlich's reagent. The uroporphyrin and coproporphyrin concentrations were analyzed by gradient HPLC equipped with a UV detector (400 nm) after extraction from the culture broth and dissolving in a 1 N NaOH solution. The heme concentration was determined by isocratic HPLC equipped with a UV detector (400 nm) after cell harvesting, sonic cell disruption, and extraction with hemin standard (Sigma-Aldrich, USA).

The coenzyme A (CoA) concentration was decided by using an assay kit (Coenzyme A Assay Kit; Bio Vision, USA) according to the manufacturer's protocol. Cells were harvested from the culture broth by centrifugation (13,000 ×*g*, 4°C) and dispersed in 200 μl of distilled water. Cells were disrupted using a sonic vibrator (UP200S; Hielscher Ultrasonics GmbH, Germany) set at 30 W at 1 s intervals for 20 min on ice. After removal of the cell debris by centrifugation (13,000 ×*g*, 4°C), the supernatant was visualized. The CoA concentration was estimated by measuring the absorbance at 570 nm with standard material (Sigma-Aldrich, USA).

RESULTS

Effect of Gene Expressions on the Intracellular Coenzyme A, ALA, and PBG Synthesis

Coenzyme A (CoA) is an acyl group carrier molecule, and increase of the intracellular CoA pool was expected to contribute to porphyrin biosynthesis, considering succinyl-CoA is the one of the substrates of 5-aminolevulinic acid (ALA), the committed precursor of the porphyrin pathway (Fig. 1). The intracellular CoA concentration was determined in the bacterial heme-synthesizing HMD strain with co-expressions of *coaA*, *hemB*, *hemC*, *hemD*, and *hemE* (Fig. 2, left). The CoA concentration in the HMD strain co-expressing *coaA* (HMD-A strain) was 75.7 nmol/g-DCW, which was 60% greater than that in the HMD strain (47.8 nmol/g-DCW). The CoA concentrations in the HMD strain co-expressing *hemB*, *hemC*, and *hemE* were even lower than that in the HMD strain, and only *hemD* co-expression showed a slightly greater CoA concentration (58.4 nmol /g-DCW) than the control strain.

α-Ketoglutarate and CoA turn into succinyl-CoA after decarboxylation, and the succinyl-CoA combines with glycine to ALA mediated by ALA synthase (*hemA*) (Fig. 1). Two molecules of ALA are condensed into a PBG, mediated by ALA dehydratase (*hemB*). The variations of ALA and PBG concentrations were analyzed in the co-expressing strains (Fig. 2, right). The ALA concentrations in the co-expressions of *coaA*, *hemC*, *hemD*, and *hemE* were drastically increased to 2.6, 1.8, 3.4, and 2.3 mmol/g-DCW, respectively, compared with that in the control strain of 0.5 mmol/g-DCW.

The PBG concentration in HMD strain was 0.03 mmol/g-DCW, which corresponds to only 7% of ALA. When the *hemB* gene was co-expressed, its concentration was increased to 1.0 mmol/g-DCW. Co-expressions of *coaA*, *hemC*, *hemD*, and *hemE* showed no distinctive contribution to the PBG accumulation.

Effect of Gene Expressions on the Porphyrins Syntheses

Formation of uroporphyrin, the next step to the unstable tetrapyrrole structured 1-hydroxymethylbilane (HMB) in the porphyrin pathway, was increased the most by co-expression of *hemC* (10.5 μmol/g-DCW) followed by co-expressions of *hemB*, *coaA*, and *hemD* (2.8, 1.6, and 1.3 μmol/g-DCW, respectively), whereas the control HMD strain produced uroporphyrin of 0.6 μmol/g-DCW (Fig. 3, left). On the other hand, the uroporphyrin concentration in the *hemE* co-expression was 20% lower (0.5 μmol/g-DCW) than that in the control, representing that uroporphyrinogen was converted to coproporphyrinogen by the increased uroporphyrinogen III decarboxylase.

Coproporphyrins were the most enhanced by co-expressions of *hemC* (Fig. 3, right). The coproporphyrin I and III concentrations in the *hemC* co-expressing strain were 1.4 and 2.9 μmol/g-DCW, respectively, whereas those in the control strain were 0.21 and 0.15 μmol/g-DCW, respectively. Co-expressions of *coaA* (0.6 μmol/g-DCW), *hemB* (0.9 μmol/g-DCW), *hemD* (0.4 μmol/g-DCW), and *hemE* (0.8 μmol/g-DCW) also increased coproporphyrin III, even though the effect was not as great as the co-expression of *hemC*.

Effect of Gene Expressions on the Heme Synthesis

Heme production, the final product of the porphyrin pathway, was influenced by the co-expressions (Fig. 4). The heme

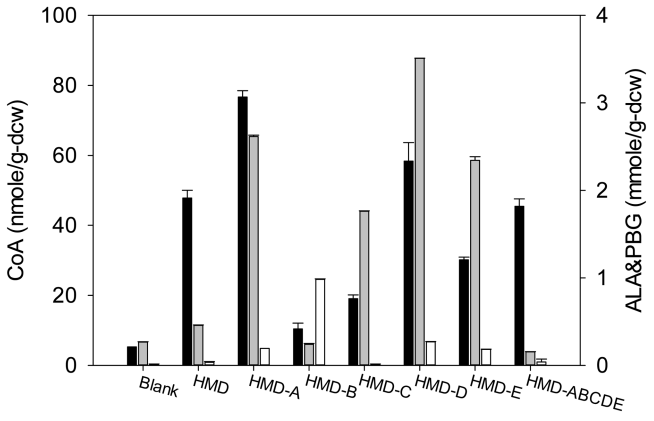

Fig. 2. Effect of gene expressions on the coenzyme A, ALA, and PBG concentrations in recombinant *E. coli*.
The black bar is for coenzyme A (left axis), grey for ALA (right axis), and white for PBG (right axis). Samples were taken at 24 h. Coenzyme A was determined using an enzyme-coupled assay kit. ALA and PGB were measured using spectrophotometry after separation on an ion-exchange column and visualization by Ehrlich's reagent.

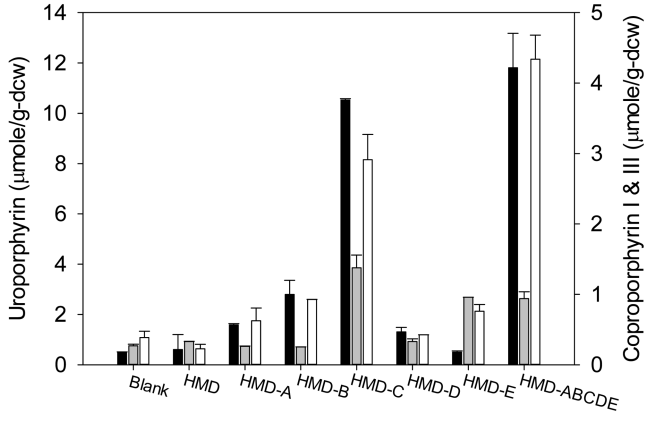

Fig. 3. Effects of gene expressions on the porphyrin derivatives concentrations in recombinant *E. coli*.
The black bar is for uroporphyrin (left axis), grey for coproporphyrin I (right axis), and white for coproporphyrin III (right axis). Each porphyrin concentration indicates its reduced form; that is, porphyrinogen. Samples were taken at 48 h. The porphyrin derivatives were measured using a gradient HPLC equipped with UV-detector (400 nm) after extraction from the sample.

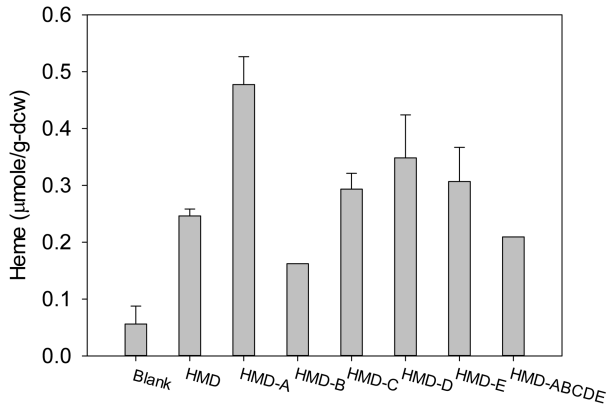

Fig. 4. Effect of gene expressions on heme production in recombinant *E. coli*.
Samples were taken at 48 h. The porphyrin derivatives were measured using an isocratic HPLC equipped with a UV-detector (400 nm) after extraction from the sample.

productions were increased by 20~100% by co-expressions of *coaA* (0.48 μmol/g-DCW), *hemC* (0.29 μmol/g-DCW), *hemD* (0.35 μmol/g-DCW), and *hemE* (0.31 μmol/g-DCW), compared with that in the HMD strain (0.25 μmol/g-DCW). The *hemB* co-expression, however, showed a reduction of heme production (0.16 μmol/g-DCW).

DISCUSSION

The *coaA* co-expression in addition to ALA synthase (*hemA*), NADP-dependent malic enzyme (*maeB*), and dicarboxylic acid transporter (*dctA*) enabled *E. coli* to produce twice as much heme (Fig. 4). The *coaA* co-expression also increased the CoA and ALA (Fig. 2), which would enhance the porphyrin pathway resulting in greater heme synthesis. Therefore, enrichment of the CoA pool by the pantothenate kinase co-expression was better pathway engineering to produce heme (Fig. 1).

The *E. coli* pantothenate kinase is a homodimer enzyme mediating pantothenate into 4-phosphopantothenate with the usage of ATP [8]. Increased coenzyme A, the final product of the pantothenate pathway (Fig. 1), feedback inhibits the pantothenate kinase by competitive binding on the ATP binding site (Lys101) [9]. The feedback inhibition of pantothenate kinase driven by increased CoA level would be reduced by increase of the ATP level [12]. Since artificial PEP carboxykinase (*pckA*) expression under glycolytic conditions enhances the intracellular ATP level [2], addition of this gene could be beneficial for emphasis of the effect of *coaA* expression by the reduction of feedback inhibition.

Even though the effect for heme synthesis was not clear as *coaA*, the co-expressions of the porphyrin pathway (*hemC*, *D*, and *E*) marginally contributed to the heme production (Fig. 4). The only one that did not contribute to heme production was the *hemB* co-expression. The possible reason might be the presence of an unknown regulation driven by the increase of intracellular PBG (Fig. 2, right), which might have inhibited the further pathway progress.

The molar ratios of heme/coproporphyrin III (based on Figs. 3 right and 4) of the *hemD*, *hemE*, and *coaA* expressions were relatively higher (81%, 41%, and 77%, respectively) than those of the *hemB* and *hemC* expressions (17% and 10%, respectively). Therefore, the *hemD* and *E* expressions as well as *coaA* expression would be a better engineering strategy for heme synthesis than the *hemB* and *C* expressions.

Accumulation of uroporphyrin by the HMB synthase (*hemC*) expression (Fig. 3, left) seemed reasonable because the increased HMB by the amplified enzyme must have rapidly turned into the next intermediate step, uroporphyrin. It is not clear, however, why coproporphyrin was also accumulated by the *hemC* expression (Fig. 3, right). The accumulation of uroporphyrin/coproporphyrin might have inhibited the enzyme reacting on coproporhyrin, which is *hemF* (coproporphyrinogen III oxidoreductase). Therefore, further understandings of the presence of allosteric inhibition by intermediates are required to enforce the porphyrin pathway.

In conclusion, we have previously reported that bacterial heme was synthesized by artificial expressions of ALA synthase, NADP-dependent malic enzyme, and dicarboxylic acid transporter [3], and the additional expression of pantothenate synthase enhanced the CoA pool, resulting in twice as much heme synthesis. Moreover, expressions of the porphyrin pathway genes (*hemBCDE*) have marginal effects on the pathway intermediates as well as heme; that is, ALA was enhanced by *hemCDE* expressions, PBG by *hemB* expression, uroporphyrin by *hemBC* expressions, and coproporphyrin by *hemBCE* expressions. Further studies on the regulations and enzymatic characteristics of the porphyrin pathway are required to achieve an industrial strain overproducing heme. The effects of the coproporphyrinogen III oxidoreductase (*hemF*), protoporphyrin oxidase (*hemG*), and ferrochelatase (*hemH*) expressions of the porphyrin pathway also remain to be studied.

Acknowledgments

This work was financially supported by the Intelligent Synthetic Biology Center of Global Frontier project (2012M3A6A8054887), to P. Kim.

REFERENCES

1. Battersby, A. R. 2000. Tetrapyrroles: The pigments of life. *Nat. Prod. Rep.* **17:** 507–526.

2. Kim, H. J., Y. D. Kwon, S. Y. Lee, and P. Kim. 2012. An engineered *Escherichia coli* having a high intracellular level of ATP and enhanced recombinant protein production. *Appl. Microbiol. Biotechnol.* **94:** 1079–1086.
3. Kwon, O. H., S. Kim, D. H. Hahm, S. Y. Lee, and P. Kim. 2009. Potential application of the recombinant *Escherichia coli*-synthesized heme as a bioavailable iron source. *J. Microbiol. Biotechnol.* **19:** 604–609.
4. Kwon, S. J., A. L. de Boer, R. Petri, and C. Schmidt-Dannert. 2003. High-level production of porphyrins in metabolically engineered *Escherichia coli*: Systematic extension of a pathway assembled from overexpressed genes involved in heme biosynthesis. *Appl. Environ. Microbiol.* **69:** 4875–4883.
5. Kwon, S. J., R. Petri, A. L. DeBoer, and C. Schmidt-Dannert. 2004. A high-throughput screen for porphyrin metal chelatases: Application to the directed evolution of ferrochelatases for metalloporphyrin biosynthesis. *Chembiochem* **5:** 1069–1074.
6. Lee, M. J., S. J. Chun, H. J. Kim, A. S. Kwon, S. Y. Jun, S. H. Kang, and P. Kim. 2012. Porphyrin derivatives from a recombinant *Escherichia coli* grown on chemically defined medium. *J. Microbiol. Biotechnol.* **22:** 1653–1658.
7. Monsen, E. R. and J. L. Balintfy. 1982. Calculating dietary iron bioavailability: Refinement and computerization. *J. Am. Diet. Assoc.* **80:** 307–311.
8. Rock, C. O., R. B. Calder, M. A. Karim, and S. Jackowski. 2000. Pantothenate kinase regulation of the intracellular concentration of coenzyme A. *J. Biol. Chem.* **275:** 1377–1383.
9. Rock, C. O., H. W. Park, and S. Jackowski. 2003. Role of feedback regulation of pantothenate kinase (CoaA) in control of coenzyme A levels in *Escherichia coli*. *J. Bacteriol.* **185:** 3410–3415.
10. Shin, J. A., Y. D. Kwon, O. H. Kwon, H. S. Lee, and P. Kim. 2007. 5-Aminolevulinic acid biosynthesis in *Escherichia coli* coexpressing NADP-dependent malic enzyme and 5-aminolevulinate synthase. *J. Microbiol. Biotechnol.* **17:** 1579–1584.
11. Turhan, S., T. B. Altunkaynak, and F. Yazici. 2004. A note on the total and heme iron contents of ready-to-eat doner kebabs. *Meat Sci.* **67:** 191–194.
12. Vallari, D. S. and S. Jackowski. 1988. Biosynthesis and degradation both contribute to the regulation of coenzyme A content in *Escherichia coli*. *J. Bacteriol.* **170:** 3961–3966.