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Potential Application of the Recombinant *Escherichia coli*-Synthesized Heme as a Bioavailable Iron Source

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To investigate the potential use of microbial heme as an iron source, recombinant *Escherichia coli* coexpressing ALA synthase (HemA) as well as the NADP-dependent malic enzyme (MaeB) and dicarboxylic acid transporter (DctA) were cultured. The typical red pigment extracted from the recombinant *E. coli* after 38 h showed highest absorbance at 407 nm, and the amount of iron in 38.4 mg of microbial heme extract derived from 6-l fermentation broth was 4.1 mg. To determine the commercial potential of the recombinant *E.coli*-synthesized iron-associated heme as an iron source, mice were fed the iron-free provender with the microbial heme extract. The average body weight reduction of mice fed non-iron provender was 2.3%, whereas no detectable weight loss was evident in mice fed microbial heme addition after 15 days. The heme content of the blood from microbial heme fed mice was 4.2 mg/ml whereas that of controls was 2.4 mg/ml, which implies that the microbial heme could be available for use as an animal iron source.

Keywords: Iron, microbial heme, application, bioavailability

Iron is an essential mineral nutrient as a catalytic center for a broad spectrum of metabolic functions in biological systems including humans [2]. Iron is required for the transport of oxygen and carbon dioxide in hemoglobin. Iron is also a component of various enzymes such as the cytochromes and dehydrogenases that are critical for energy production [9]. The important function of elementary iron comes from its redox reactivity, as it exists in two stable and interchangeable forms: ferrous (Fe^{2+}) and ferric (Fe^{3+}). The redox potential of cytochrome is different in the electron transport system, which allows electron transport from NADH to O_2 by sequential redox cycling of the iron atoms between the ferrous and ferric states.

The daily diet of iron is absorbed from the intestinal lumen, across the epithelial cells of the digestive tract, and into the circulation [10]. A 70 kg human male harbors 4–5 g of iron, which is composed of the functional iron of hemoglobin (60%), myoglobin (5%), and enzymes (5%), as well as storage iron in the form of ferritin (20%) and hemosiderin (10%). The body's iron is acquired only from the diet except for iron that is recycled from macrophages of the reticuloendothelial system that ingest old and damaged erythrocytes. No excretion mechanism is known and iron is lost from the body only by bleeding including menstruation. Iron's bioavailability (the proportion of a nutrient present in food that the body is able to absorb and utilize) differs according to the type of iron source, namely nonheme iron and heme iron. Since it is generally agreed upon that only soluble iron is absorbed, and dietary nonheme iron is not generally present in readily soluble forms, digestion must first release the dietary nonheme iron in a soluble form as a prerequisite for its bioavailability. The general bioavailability of nonheme iron is about 5% depending on the physiological iron stores and accompanying dietary factor (*i.e.*, ascorbic acid enhances nonheme iron absorption). The absorption of heme iron, an iron chelated porphyrin, occurs primarily by the uptake of heme iron directly into the mucosal cells as the intact iron–porphyrin complex that is further enzymatically cleaved to generate the iron in an inorganic form. Bioavailability of heme iron is, thus, higher (approximately 35%) than those of nonheme iron and is not affected by other dietary factors. Diets from animal sources contain 40% heme iron and 60% nonheme iron, whereas plant source diets contain only nonheme iron. No microbial heme has been reported as an iron source exceptfor the microbial ferritin complex [4].

The biochemical pathway for heme biosynthesis is well known and includes the assembly of eight 5-aminolevulinic

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acid (ALA) into cyclic tetrapyrrole, modification of the side chains, and incorporation of reduced iron into the molecule [15]. Except for the initial steps in the formation of ALA, the pathway is fairly well conserved throughout plants, animals, and bacteria. The ALA precursor of heme biosynthesis can be synthesized either by condensation of glycine and succinyl-CoA (C4 pathway) or from glutamate (C5 pathway) depending on species (Fig. 1) [5]. In either case, ALA is the first committed step in the formation of heme. *Escherichia coli* and related bacteria synthesize ALA *via* the C5 pathway in three steps; (1) ligation of tRNA (GTR) synthetase, (2) reduction of the resulting GTR to glutamate 1-semialdehyde (GSA) by GTR reductase, and (3) transamination of GSA to ALA by GSA aminotransferase. Some photosynthetic bacteria, yeast, as well as humans synthesize ALA *via* the C4 pathway, involving the condensation of glycine and succinyl-CoA into ALA by ALA synthase. In one study, recombinant *E. coli* coexpressing ALA synthase and NADP-dependent malic enzyme produced ALA, and the cells were able to bypass the rate-limiting step leading to the appearance of a typical red pigment considered to be heme iron [13].

Presently, we report on the use of a recombinant *E. coli* for ALA biosynthesis to produce microbial heme, and the use of this heme as a dietary iron source.

MATERIALS AND METHODS

Strains and Plasmids

The strains, plasmids, and oligonucleotides used in this study are listed in Table 1. Routine DNA manipulations were performed as described previously [12]. Plasmid pTrc (P_{lac}hemA$^+$-maeB) harboring the ALA synthase gene (*hemA* from *Rhodobacter sphaeroides*) and a NADP-dependent malic enzyme gene (*maeB* from *E. coli*) were obtained from our laboratory repository [13]. C4-dicarboxylic acid transport protein (*dctA*; GenBank AC_000091.1: 3956968..3958254) was amplified by polymerase chain reaction (PCR) using *E. coli* W3110 (Korean Collection of Type Culture, KCTC 2223) genomic DNA as the template with the oligonucleotides AAGCTTAGGAGG AACAGACATGAAAACCTCTCTGCTTTTA (HindIII site underlined and RNA Binding Site) and AAGCTTTTAAGAGGATAATTCGTG CGTTTT (HindIII site underlined). The 1.3-kb PCR fragment was ligated into the 8.5-kb HindIII-digested pTrc(P_{lac}hemA$^+$-maeB) after purification from a T-cloning vector to produce pTrc (P_{lac}hemA$^+$-maeB-dctA). The ligation candidates were verified by their DNA-fragmentation patterns (2.8 kb and 7.0 kb after PstI digestion) and full sequencing (Solgent, Daejeon, Korea). The pTrc(P_{lac}hemA$^+$-maeB-dctA) was electroporated using a 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.

Media and Culture

Luria–Bertani (LB) medium was used for all DNA manipulations. The medium for microbial heme synthesis (medium-S) contained 5 g of yeast extract, 10 g of tryptone, 5 g of KH_2PO_4, 10 g of succinate (disodium succinate hexahydrate), 2 g of glycine, and 40 mg of $FeCl_3$ per liter. Antibiotics (20 μg/ml ampicillin) and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were supplemented as needed. The pH of the medium was set to 6.5 before sterilization.

A single colony of *E. coli* W3110 harboring pTrc (P_{lac}hemA$^+$-maeB-dctA) was inoculated into a 15-ml test tube containing 4 ml of LB medium and precultivated in a rotary shaking incubator (37°C, 230 rpm) for 16 h. Aliquots (1 ml) of the cells were transferred into 250-ml Erlenmeyer flasks containing 50 ml of medium-S supplemented with ampicillin (20 μg/ml). After 4 h cultivation, the broth was used to inoculate a 5-l Biotron fermentor (Bucheon, Korea) containing 3 l of medium-S supplemented with 20 μg/ml ampicillin and 0.1 mM IPTG. The fermentor was operated at 37°C with 0.5 vvm aeration and 300 rpm agitation for 38 h.

Enzyme Activities

Actively growing cells were disrupted using a sonicator (UP200S Hielscher Ultrasonics GmbH, Teltow, Germany) set at 30 W for 1 min at 1-s intervals on ice. After removal of cell debris by centrifugation (10,000 rpm for 20 min), the supernatant was used for the enzyme activities. The activities of ALA synthase (HemA) and

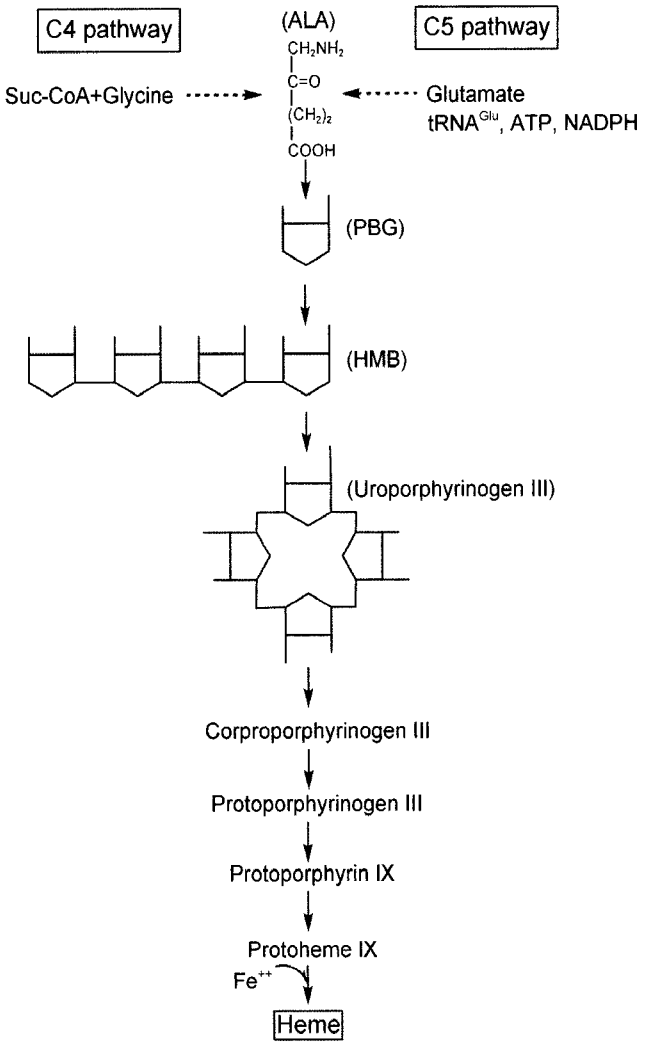

Fig. 1. Heme biosynthetic pathway from 5-aminolevulinic acid.

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

Strain, plasmid, or oligonucleotide	Description	Source or reference
E. coli strain:		
W3110	Wild-type *E. coli*	KCTC2223
DH5α	F-φ80 d*lacZ* ΔM15 Δ(*lacZYA-arg*F) U169 *endA1 recA1 hsdR17* (rK-mK+) *deoR thi-1 phoA supE44λ- gyrA96 relA1*	Invitrogen Co.
Plasmids:		
T vector	T&A cloning vector	RBC Co., Taiwan
pTrc99A	Expression vector, trc promoter, Ap^R	AP Biotech. Co.
pTrc(P_{lac}hemA$^+$)	pTrc99A with *lac* promoter, *hemA* gene, *hemA* flanking region from *Rhodobacter sphaeroides* at the XbaI–PstI site	[13]
pTrc(P_{lac}hemA$^+$-maeB)	PTrc(P_{lac}hemA$^+$) with RBS and *maeB* gene from *E. coli* at the PstI–HindIII site	[13]
pTrc(P_{lac}hemA$^+$-maeB-dctA)	pTrc(P_{lac}hemA$^+$-maeB) with RBS and *dctA* gene from *E. coli* at the HindIII site	This study
Oligonucleotides:		
For DctA (forward)	AAGCTT**AGGAGG**AACAGACATGAAAACCTCTCTGCTTTTTA (HindIII site underlined; RBS in bold)	This study
For DctA (Reverse)	AAGCTTTTAAGAGGATAATTCGTGCGTTTT (HindIII site underlined)	This study

NADP-dependent malic enzyme (MaeB) as well as total protein content were analyzed as described previously using a spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan) [13]. Enzyme activities of 1 unit were defined as the amount of enzyme converting 1 μmole of product per minute.

The C4-dicarboxylic acid transporter (DctA) activity was estimated by measuring the intracellular amount of succinate using a 500 MHz FT–NMR spectrometer (UI500; Varian Inc., Palo Alto, CA, U.S.A.) in a facility of KBSI (Metabolome Analysis Facility, Korea Basic Science Institute Seoul Center). Cells were harvested by centrifugation (12,000 rpm, 10 min) at 4°C and resuspended in a 50 mM phosphate buffer (pH 7.0) containing 10 g/l of disodium succinate hexahydrate. The cell suspension was stored at room temperature for 1 min to allow transporting succinate into cell. Cells were washed twice with distilled water to remove extracellular succinate and resuspended to an OD=1. The cell suspension (0.5 ml) in a 5-mm NMR tube was mixed with 100 μl of 0.75% trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) solution in D_2O for further quantification by ^{1}H-NMR.

Microbial Heme Extraction

Red-colored cells were harvested by centrifugation (3,000 ×*g* for 15 min at 4°C) and washed twice with distilled water. The cells were suspended in 15 ml of distilled water and then disrupted using a UP200S sonicator (Hielscher Ultrasonics GmbH, Teltow, Germany) set at 30 W for 20 min at 1-s intervals on ice. After removing the cellular debris by centrifugation (10,000 ×*g* for 10 min at 4°C), the supernatant was kept in a 65°C water bath for 30 min. Protein precipitate was removed by centrifugation (10,000 ×*g* for 10 min at 4°C), and the supernatant was used for the subsequent extraction procedure. The red pigment was extracted using cold acid–acetone extraction [3]. The cell extract supernatant was added drop–wise with stirring into 100 ml of acid–acetone (99.8 ml of acetone and 0.2 ml of 10 N HCl) at −20°C. The solution was centrifuged at 10,000 ×*g* for 30 min at −20°C. The operation was repeated once to complete the removal of the red color in the precipitate. The acid–acetone was then neutralized by adding 10 N NaOH and then evaporated using a rotary evaporator. The remaining solution was freeze-dried for further experimentation.

Mice and Diets

Twenty-one 12-week-old female mice (strain ICR) and iron-deficient provender (AIN-93G) [11] were purchased from Central Lab. Animal Inc. (Seoul, Korea). Body weights of these animals ranged from 32–34 g. Mice were randomly divided into three groups of seven mice. Each mouse was daily fed 6 g of iron-free provender pellet. The three solutions were orally injected using a syringe equipped with a round-head 39-mm-long needle. The solution containing microbial heme extract (1 mg extract in 0.5 ml) was administrated daily to mice in group 1, whereas 0.5 ml of the solution containing control bacterial extract (1 mg extract in 0.5 ml) and 0.5 ml of distilled water were dispensed to the mice in groups 2 and 3, respectively. Mice were studied for 15 days, during which time food intake and spillage were measured daily and body weight and food consumption were assessed weekly. At the conclusion of the study, all mice were killed under ether anesthesia and blood was collected *via* cardiac puncture. This study was approved by the Institutional Care and Use Committee of the Catholic University of Korea.

Analyses

Bacterial biomass was estimated by measuring optical density (OD) at 600 nm and transformed into dry cell weight (DCW) using the coefficient of 1 OD=0.31 g/l. Protein contents in the extracts were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. Iron content of microbial heme extract was determined by the *ortho*-phenanthroline colorimetric method using $[Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O]$ as the standard [14]. The spectrum of the purified microbial heme solution (0.1 mg extract/ml) was examined using a UV1240 spectrophotometer (Shimadzu, Kyoto, Japan) [1]. Red blood cell content, packed cell volume ratio of red blood cell, and heme content of the mice blood were measured using a MS9-5 hematocrit analyzer (Melet Schloesing, Osny, France). Heme amount was verified by a previously described colorimetric method

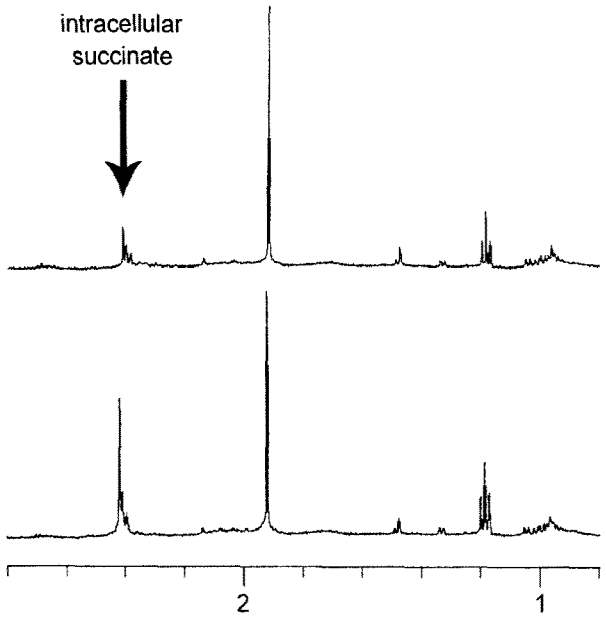

Fig. 2. ^{1}H-NMR spectrum for intracellular succinate concentrations of *E. coli* expressing dicarboxylic acid transporter (DctA).
The upper figure was obtained from control strain (W3110/pTrc99A) and lower figure from DctA-expressing strain [W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA)]. The arrow mark indicates the protons of succinate peaks comprising 2.399, 2.414, and 2.424 ppm. The quantities were estimated by area ratios to internal standard (TSP) compared with standard succinate.

at 557 nm using hemin (Sigma-Aldrich, St. Louis, MO, U.S.A.) as a standard [6]. The data were expressed as mean±standard error of the mean. Data were analyzed for normal distribution, and the statistical significance of the difference between groups was determined by the Student's t-test. Differences were considered significant at $P<0.05$.

RESULTS

Bacterial Cell Culture and Enzyme Activities

Wild-type *E. coli* W3110 harboring blank vector pTrc99A and W3110 harboring pTrc(P_{lac}hemA$^+$-maeB-dctA) were each cultured in a 5-l jar fermenter for microbial heme production. The recombinant strain expressed three genes upon induction by 0.1 mM IPTG: ALA synthase (HemA) from *Rhodobacter sphaeroides*, NADP-dependent malic enzyme (MaeB), and C4-dicarboxylic acid transport protein (DctA) from *E. coli*. Culture sample (30 ml) was taken to analyze the enzyme activities after 6-h inoculation. The ALA synthase and NADP-dependent malic enzyme activities of W3110/pTrc (P_{lac}hemA$^+$-maeB-dctA) were 1.22 and 0.75 units/mg-protein, whereas those of W3110/pTrc99A were less than 0.01 and 0.10 units/mg-protein, respectively. Intracellular succinate after DctA expression [W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA)] was 14.6 μmol/OD cell, whereas that before the DctA expression (W3110/pTrc99A) was 1.8 μmol/OD cell (Fig. 2). The above results indicated the three enzymes (HemA, MaeB, DctA) were functionally expressed in the *E. coli* at the given culture condition.

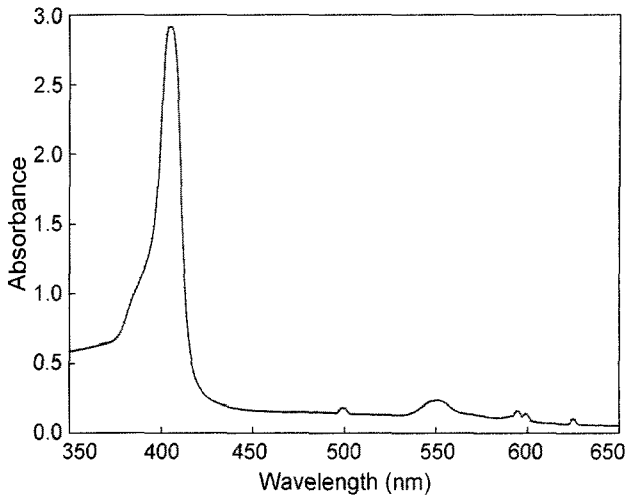

Fig. 3. Spectrum scan of microbial heme.

Heme Extract Preparation

Biomass increased to 0.44 g/l (W3110/pTrc99A) and 0.40 g/l [W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA)] in 16 h and was maintained until harvest at 38 h. The colors of both culture broths were yellowish at 16 h. The color of control cells did not change appreciably after 16 h, whereas that of W3110/ pTrc (P_{lac}hemA$^+$-maeB-dctA) cell gradually turned red with time. The red cells showed no variation in spectrum scan. The fermentations were repeated twice for each strain. The red-colored W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA) cells of 2.4 g of cells from 6-l culture as well as the yellowish control cells of 2.6 g of cells from 6 l culture were harvested (Table 2). The purification processes of the red pigment (heat precipitation, cold acid–acetone extraction, and freeze drying) yielded 0.6 g of extract from both W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA) cells and W3110/pTrc99A cells. The spectra of the extract from W3110/

Table 2. Fermentation production of microbial heme.

	Culture volume	Cell amount	Extract amount	[a]Heme amount	[b]Iron amount
W3110/ pTrc99A	6 l	2.6 g	0.6 g	<0.01 mg	<0.03 mg
W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA)	6 l	2.4 g	0.6 g	38.4 mg	4.1 mg

[a]Heme amount was determined by a colorimetric method using hemin as a standard.
[b]Iron amount was determined by a colorimetric method using $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ as a standard.

Table 3. Effect of microbial heme diet on mice blood.

	Iron source supplement	Mice body weight after 15 days	Blood		
			RBC (cells/μl)	Packed volume (%)	Heme content (mg/ml)
Group 1	Microbial heme extract	99.8±4.3 g	9.8±0.2	56.6±3.1	4.2±0.1
Group 2	Control bacterial extract	98.5±3.7 g	8.7±0.2	50.1±2.8	2.6±0.1
Group 3	Water	97.7±4.2 g	9.2±0.3	49.5±4.2	2.5±0.2

pTrc(P_{lac}hemA$^+$-maeB-dctA) cells (0.1 mg/ml) showed a major peak at 407 nm, as well as minor peaks at 500, 551, 594, 599, and 625 nm (Fig. 3). The heme amounts based on hemin standard were 64.0 mg/g extract and <0.01 mg/g extract from W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA) cells and control cells, respectively. The iron content of the extract from W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA) cells was 6.8 mg/g extract, whereas that from control cells was <0.05 mg/g extract. Based on these results, we considered that the red-colored pigment from W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA) cells represented microbial heme.

Effect of Microbial Heme Diet on Mice

The iron-deficient diet pellet (6 g/day) was provided to 12-week-old female mice (32–34 g in weight) for 15 days. The mice were randomly divided into three groups and each mouse received a daily oral injection of 0.5 ml of different iron sources. Group 1 received 1 mg of microbial heme extract. Group 2 received 1 mg of extract from control cells. Group 3 received distilled water. The body weights did not change appreciably during the diet experiment among mice from all three groups (Table 3). The body weight of group 3 mice decreased by 2.3%, whereas mice in groups 1 and 2 showed little variation; however, all points were in error range. Blood of the mice was retrieved after 15 days for the analyses of red blood cell content, packed cell volume ratio of red blood cell, and heme content. Although the differences were not big, blood from group 1 mice displayed the highest red blood cell content (9.8 cells/μl), and packed cell volume ratio of red blood cell (56.6 %) compared with blood from groups 2 and 3 mice. The most critical difference was found in the heme content of the blood. The heme content of group 1 was 4.2 mg/ml, whereas those of groups 2 and 3 were 2.6 mg/ml and 2.5 mg/ml, respectively.

Discussion

Heme, a higher bioavailable iron source found in meat and fish diet, was able to be produced from bacterial culture and was verified to be available to mice for iron source. The extract from W3110/ pTrc(P_{lac}hemA$^+$-maeB-dctA) cells showed the characteristic spectrum of heme and contained 6.8 mg of iron per gram of extract. In the mice diet experiment, the blood from microbial heme fed mice displayed higher red blood cell content, packed cell volume ratio of red blood cell, and heme content than those of the controls. The heme content especially showed a marked difference compared with the controls (4.2 vs. 2.5 mg/ml). Therefore, the microbial heme was considered suitable for use in an animal diet.

The recombinant strain W3110/pTrc(P_{lac}hemA$^+$-maeB-dctA) expressed three enzymes. The expression of ALA synthase (HemA), which mediates the condensation of succinyl-CoA and glycine into ALA, enabled the bacteria to bypass the feedback regulation of heme accumulation on the original C5 pathway as well as to provide a foreign C4 pathway. The expression of NADP-dependent malic enzyme (MaeB) mediated CO_2 fixation on pyruvate, leading to an enhanced C4 metabolite for succinyl-CoA [7, 8]. The C4-dicarboxylic acid transport protein (DctA) expression enhanced the succinic acid transport into cells. As in Fig. 2, the 8-times increase of succiate influx was found in the DctA expressing cell, which consequently increased heme synthesis through increase of precursor ALA. Altogether, the metabolism of the strain was directed to ALA, the committed precursor of heme synthesis. Further metabolic engineering of the strain, as well as the fermentation process optimization, might additionally enhance microbial heme synthesis.

Iron-supplemented products for humans have been manufactured from animal spleen and blood. The results of the present study enable us to conclude that microbial heme might be able to substitute the source of iron without the need to sacrifice source animals.

Acknowledgments

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