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# Heme Derived from *Corynebacterium glutamicum*: A Potential Iron Additive for Swine and an Electron Carrier Additive for Lactic Acid Bacterial Culture

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To investigate the potential applications of bacterial heme, aminolevulinic acid synthase (HemA) was expressed in a *Corynebacterium glutamicum* HA strain that had been adaptively evolved against oxidative stress. The red pigment from the constructed strain was extracted and it exhibited the typical heme absorbance at 408 nm from the spectrum. To investigate the potential of this strain as an iron additive for swine, a prototype feed additive was manufactured in pilot scale by culturing the strain in a 5 ton fermenter followed by spray-drying the biomass with flour as an excipient (biomass: flour = 1:10 (w/w)). The 10% prototype additive along with regular feed was supplied to a pig, resulting in a 1.1 kg greater increase in weight gain with no diarrhea in 3 weeks as compared with that in a control pig that was fed an additive containing only flour. To verify if *C. glutamicum*-synthesized heme is a potential electron carrier, lactic acid bacteria were cultured under aerobic conditions with the extracted heme. The biomasses of the aerobically grown *Lactococcus lactis*, *Lactobacillus rhamosus*, and *Lactobacillus casei* were 97%, 15%, and 4% greater, respectively, than those under fermentative growth conditions. As a potential preservative, cultures of the four strains of lactic acid bacteria were stored at 4°C with the extracted heme and living lactic acid bacterial cells were counted. There were more *L. lactis* and *L. plantarum* live cells when stored with heme, whereas *L. rhamosus* and *L. casei* showed no significant differences in live-cell numbers. The potential uses of the heme from *C. glutamicum* are further discussed.

**Keywords:** Bacterial heme, GRAS, *C. glutamicum*, iron additive, electron carrier, lactic acid bacteria

## Introduction

Heme is a biological molecule containing a porphyrin structure and a ferrous ion ($Fe^{2+}$) chelated by coordinate bonds [1]. Because heme has a hydrophobic ring structure on the outside and a high-density electron cloud in the center, heme is involved in many key biological processes, such as energy generation by electron transfer in prokaryotic cells and mitochondria, molecular oxygen circulation by red blood cells in animals, detoxification of reactive oxygen species, and several other redox transfer reactions in cells. Chlorophyll, a molecule critical for photosynthesis in plants and algae, has a structure similar to that of heme, except that magnesium ions ($Mg^{2+}$) are chelated. Hence, it is not surprising that all living creatures contain heme and its derivatives.

Although heme is necessary for many biological processes, excessive levels of heme are toxic to cells; excessive heme causes nonspecific interactions with molecular oxygen molecules, resulting in the modification of the cellular redox state and oxidative stress [2, 3]. Therefore, cells tightly regulate heme concentrations. Heme biosynthesis occurs via two distinct pathways in nature. The C4 pathway, found in phototrophic bacteria, yeast, and mammals, begins with the condensation of succinyl-CoA and glycine substrates into aminolevulinic acid (ALA). The C5 pathway

for ALA production, found in most chemotrophic bacteria, cyanobacteria, and algae, begins with a glutamate substrate along with ATP and NADPH co-substrates. The ALA synthesizing step is the critical step for regulating the concentration of heme [4]. Branch steps for syntheses of chlorophyll, siroheme, and vitamin $B_{12}$, along with the final ferrous ion chelation step, are also involved in the regulation of cellular heme concentrations [5].

Iron is an essential mineral for humans and it is supplied by diet. Dietary heme iron has been reported to have a higher bioavailability [6] and lower gastrointestinal side effects [7] than that of non-heme iron. Considering that 25% of the current human population is iron deficient [8], it is not surprising that a heme-iron-based diet efficiently improved the iron status of women in their reproductive ages [9]. Although heme-iron-rich blood food products and hemoglobin-based meat pigments have been used in the diet of many cultures around the world, large-scale purification processes present a risk of contamination through sources leading to animal-human infectious diseases (*e.g.*, viruses). Kwon *et al.* [10] have reported that heme synthesized from recombinant *E. coli* was a potential iron source for mice. Because there is no common infectious agent between prokaryotic bacteria and eukaryotic humans, microbe-synthesized heme iron could be a safer heme iron source. However, *E. coli* contains endotoxin lipopolysaccharides (LPS) in its outer membrane. *E. coli*-derived heme iron should be separated from LPS, a cost hurdle for commercial applications. Alternatively, *Corynebacterium glutamicum* is a gram-positive actinomycete from soil that contains no endotoxins. It is generally recognized as safe, and has been shown to play a variety of roles as an industrial host [11]. Since there is no endotoxin risk [12], *C. glutamicum*-synthesized heme iron could be supplied directly to animal feed without purification, which is a cost hurdle for *E. coli*-synthesized heme iron.

Pigs gain weight in a relatively short period: pigs weigh 1−1.5 kg at birth, reaching twice their weight by the first week, and 4 times their weight by the third week. The rapid growth of pigs can induce iron-deficient anemia by 3 weeks. Iron supplementation is necessary, otherwise pigs are susceptible to retarded growth, diarrhea, and even sudden death. To prevent iron deficiency, complementary measures are recommended, such as contact with soil or mud, iron injections for the mother and new-born pig, and feeding more than 15 g of iron salt per day [13]. Because of the massive production by the pig raising industry in recent years, these complementary measures to prevent iron deficiency have their limitations: there is a risk of soil or mud infections from bacterial and parasitic insects, iron injection requires veterinarian labor, and iron salts have low bioavailability.

Lactic acid bacteria (LAB) are gram-positive aero-tolerant or anaerobic bacteria that produce lactic acid from the fermentation of sugars. LAB have been widely used in fermented foods (such as dairy products and fermented vegetables) and probiotics. LAB genetically lack a heme biosynthetic pathway, although some LAB contain a cytochrome oxidase gene. Supplementation of bovine-originated heme was reported to enable a lactic acid bacterium to grow with respirational metabolism and increased biomass production and survivability [14, 15]. These reports encouraged us to hypothesize that *C. glutamicum*-synthesized heme also can be used as a supplemental electron carrier in the culture of LAB.

In this paper, we report the construction of heme-synthesizing *C. glutamicum*, using an adaptively evolved strain tolerant to oxidative stress. The constructed *C. glutamicum* strain may have industrial applications as an iron-deficient-swine feed additive and as a supplemental electron carrier for LAB cultures.

## Materials and Methods

### Strains and Plasmids

The adaptively evolved *C. glutamicum* HA strain resistant to oxidative stress (KCTC 12280BP) and the wild-type strain (ATCC 13032) were used [16]. The *hemA* gene encoding ALA synthase was PCR-amplified with oligonucleotides of CTGCAG**AGGAAA** CAGACCATGGACTACAATCTGG and CTGCAGAAACCTAGG GGATCCGCCAGCGGATCCTAG (*PstI* site underlined; ribosome binding site in bold) using the template DNA of pTrc($P_{lac}$*hemA*$^+$-*coaA*) as described in a previous study [5]. The amplified PCR product was subcloned into a TA-vector (T-blunt PCR Cloning Kit; SolGent, Korea) and transformed into *E. coli* DH10B. The subcloned vector was digested with *PstI* after sequence verification, and the DNA fragment (1.7 kb) was subcloned into PstI-digested pSK1cat-$P_{180}$, a *Corynebacterium* expression vector [17], resulting in the construct pSL360-*hemA*. The constructed vector was further transformed into the HA strain for heme production.

Four LAB strains (*Lactococcus lactis* subsp. *lactis* KCTC 2796, *Lactobacillus rhamosus* KCTC 5033, *Lactobacillus casei* KCTC 3109, and *Lactobacillus plantarum* KCTC 33131) were obtained from the Korean Collection of Type Cultures (KRIBB, Jeongeup, Korea).

### Culture Conditions and Sample Preparation

For DNA manipulations, *E. coli* DH10B was grown at 37°C in Luria-Bertani (LB) medium, and *C. glutamicum* HA was grown at 30°C in YS medium consisting of 40 g glucose, 10 g yeast extract, 10 g soytone, 1 g $MgSO_4$, 5 g $(NH_4)_2SO_4$, 1.5 g $K_2HPO_4$, 0.5 g

$NaH_2PO_4$, 0.4 g $CaCl_2$, and 0.02 g $FeSO_4$ per liter. Kanamycin was added at a concentration of 25 μg/ml.

For lab-scale heme production, the *C. glutamicum* HA strain harboring pSL360-*hemA* was grown in a Erlenmeyer flask containing MCGC minimal medium (consisting of 40 g glucose, 4 g $(NH_4)_2SO_4$, 3 g $KH_2PO_4$, 6 g $Na_2HPO_4$, 1 g NaCl, 1 g sodium citrate dehydrate, 0.2 mg biotin, 1 mg thiamine·HCl, 20 mg $FeSO_4·7H_2O$, 0.2 g $MgSO_4·7H_2O$, 2 mg $MnSO_4·H_2O$, 2 mg $FeCl_3$, 0.5 μg $ZnSO_4·7H_2O$, 0.2 μg $CuCl_2·2H_2O$, 0.1 μg $(NH_4)_6Mo_7O_{24}·4H_2O$, 0.2 μg $Na_2B_4O_7·10H_2O$, and 70 μg $CaCl_2$ per liter) supplemented with 25 mg kanamycin per liter. The flask was incubated for 72 h with shaking at 30°C, and the biomass was harvested for heme extraction. After harvesting the biomass by centrifugation (10,000 ×*g* for 10 min at 4°C), cells were dispersed in a 1 N NaOH solution (1 ml) and then subjected to vigorous shaking in a beadbeater (Minibeadbeater-16; BioSpec Products Inc., USA) for 1 min. Cellular debris was separated by centrifugation (10,000 ×*g* for 10 min at 4°C). The top layer of the lysate (200 μl) was mixed with 400 μl of acetonitrile:dimethylsulfoxide (4:1 (v/v)), vortexed, and centrifuged (10,000 ×*g* for 10 min at 4°C). The top layer was discarded, and the bottom layer was filtered using a polytetrafluoroethylene membrane filter [5]. The filtrate was further subjected to high-performance liquid chromatography (HPLC) quantification and was added to the LAB cultures.

Pilot-scale prototype additive was produced following a series of fermentations at a Bio Control Research Center facility (Korea). A single colony was inoculated into a 3 L baffled flask, containing 1 L YS medium (industrial grade), and was incubated for 24 h. The culture broth was inoculated into a 50 L fermenter (KBT Ltd., Korea) containing 30 L of YS medium. After 18 h of cultivation, the broth was aseptically transferred into a 5 ton fermenter (KBT Ltd., Korea) harboring 2,000 L of YS medium. Dissolved oxygen was maintained at 30−40% with aeration (1 vvm) and a varied agitation speed for 30 h at 30°C. After the stationary phase, aeration was stopped, and the culture was maintained for 3 days to allow for heme accumulation. The cells were harvested using a ceramic membrane filter system (Puretech EnG Co., Korea), and the harvested cells were mixed with 60 kg of flour. Spray drying (Cheilgigong, Korea) was performed by blowing hot air at 200°C. The dried product was used for the swine feeding.

**Swine Feed Preparation**

To test if heme in the *C. glutamicum* biomass could be used as an iron source for swine, the prototype additive (the spray-dried *C. glutamicum* 10% with flour 90%) was mixed with regular commercial provender (Don Don Step3; CJ Cheiljedang, Korea) in a 1:9 ratio (w/w). For control feed preparation, only flour was mixed with the regular provender in a 1:9 ratio (w/w). Two 3-week-old male pigs were fed the prepared feeds in a farm facility (Yang Sung Greenbio Inc., Korea) under daily inspection by a veterinarian for 3 weeks. The body weights of the pigs were monitored every week.

**LAB Culture Conditions**

Four strains of LAB were cultured in a deMan Rogosa Sharpe (MRS) medium (Difco, BD BioSciences, USA). LAB were aerobically grown in a 15 ml test tube plugged with a cotton stopper at 220 rpm for 48 h. Temperature was maintained at 37°C for the cultures of *L. casei* and *L. rhamosus*, and at 30 °C for culturing *L. lactis*. *C. glutamicum*-derived heme extract was added to the MRS medium at 2.5 or 5 μg-heme/ml; heme was not added in the control. To measure fermentative growth, LAB were grown in an anaerobic glass tube (Bellco Glass Inc., USA) sealed with a rubber stopper and an aluminum cap. Air in the headspace was flushed with nitrogen gas for 5 min. To avoid oxygen contamination, inoculation and sampling were performed using a 1 ml syringe. Temperature was maintained at the same level as that in the aerobic cultures. The biomasses of LAB were determined from at least three biological replicates.

To verify the effect of *C. glutamicum*-synthesized heme addition on the survival of LAB, cells were anaerobically cultured in tubes with rubber stoppers with MRS medium. The cultures were transferred to tubes with cotton stoppers with addition of the heme extract (5 μg-heme/l) and stored at 4°C without shaking. The control cultures without heme extract remained in tubes with rubber stoppers during the storage at 4°C. The culture was aseptically sampled (0.1 ml), diluted in a sterilized saline solution, and spread on a MRS agar plate. The plate was incubated for 3 days and colonies were counted.

**Analyses**

The biomasses of *C. glutamicum* and LAB were estimated by measuring optical density at 600 nm. The dried cell weight of *C. glutamicum* was converted using an extinction coefficient of 1 $OD_{600nm}$ = 0.25 mg/ml [18].

The spectrum of purified *C. glutamicum*-derived heme extract was monitored using a spectrophotometer (UV1240; Shimadzu, Japan) [19]. The iron content of *C. glutamicum*-derived heme extract was verified using the *ortho*-phenanthroline colorimetric method with $Fe(NH_4)_2(SO_4)_2·6H_2O$ as a standard [20].

ALA synthase enzyme activity was verified based on ALA formation from a 1 ml reaction mixture consisting of 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 in 50 mM Tris-HCl (pH 7.5) [21]. The enzyme reaction mixture was visualized by adding Ehrlich reaction buffer (0.02 g *para*-dimethylaminobenzaldehyde, 0.84 ml of glacial acetic acid, 0.16 ml of 70% (v/v) perchloric acid) and the absorbance was measured at 555 nm. The protein content of the extracts was determined using a Protein Assay Kit (Bio-Rad Inc., USA) with bovine serum albumin as a standard. One unit of ALA synthase activity was defined as the enzyme amount needed to convert 1 μmol of ALA per minute.

The heme concentration was determined by HPLC (Waters Co., USA) equipped with a C18 column (Xbridge, Waters Co., USA) and a UV detector at 400 nm [5]. The eluent (1 M ammonium

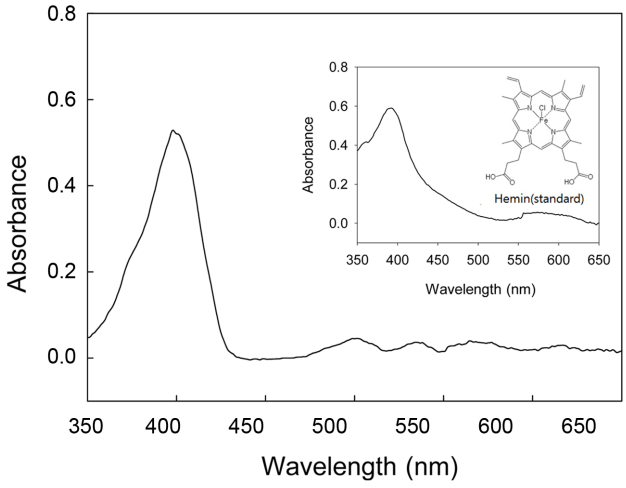


**Fig. 1.** Spectrum of heme extract from oxidative stress-tolerant *C. glutamicum* strain HA.
The inset graph represents the spectrum of hemin standard.

acetate mixed with 86% (v/v) methanol, pH 5.16) was allowed to flow at 1 ml/min. Bovine hemin chloride (Sigma-Aldrich, USA) was used as the standard.

## Results

### Construction of Heme-Producing *C. glutamicum* Strains

To bypass the most committed step of the heme biosynthetic pathway, the ALA synthase gene (*hemA*) was expressed in a vector under the control of the P180 promoter (a constitutive promoter) in wild-type *C. glutamicum* 13032. The extract of the actively growing transformed *C. glutamicum* showed ALA synthase activity (0.94 ± 0.09 μmol/min·mg-protein), whereas the wild type did not (<0.03 μmol/min·mg-protein), verifying the *hemA* gene was functionally expressed. Cultures of the plasmid harboring *C. glutamicum* in MCGC minimal medium were initially yellowish color, but turned red in 72 h. Because the red color was thought to come from heme, the pigment was purified and subjected to spectrum analysis (Fig. 1). The spectrum showed a typical heme peak at 407 nm along with minor peaks at 500 and 503 nm.

Because excessive heme production would have caused oxidative stress [22], heme accumulation was induced using an oxidative stress-tolerant *C. glutamicum* strain. The HA strain, a strain adaptively evolved against $H_2O_2$ stress [16], was used. Cultures of the HA strain expressing ALA synthase produced a biomass (OD = 47.7) similar to that of the wild-type host (OD = 47.6); however, the heme content was 0.74 ± 0.15 mg-heme/g-DCW for strain HA and 0.33 ± 0.08 mg-heme/g-DCW for the wild type (Fig. 2). Therefore, the HA host produced more than twice the amount of heme produced by the wild-type host.

### Effects of Spray-Dried *C. glutamicum* Feed on Pig Weight

To verify the potential use of *C. glutamicum* synthesized heme as an iron source, a prototype animal feed additive was manufactured at a pilot scale. The whole biomass was spray dried without a heme purification step to reduce the manufacturing cost. A prototype feed additive weighing 70 kg was prepared. The prototype feed additive contained 10% dried heme-producing *C. glutamicum* cells and 90% flour. The feed (prototype additive:regular provender = 1:9 (w/w)) was administered to a 3-week-old male pig for 3 weeks, and its body weight was monitored under veterinarian examinations (Fig. 3; Supplement 1). The amount of daily feed was set at 1 kg/day during the first week, 1.2 kg/day

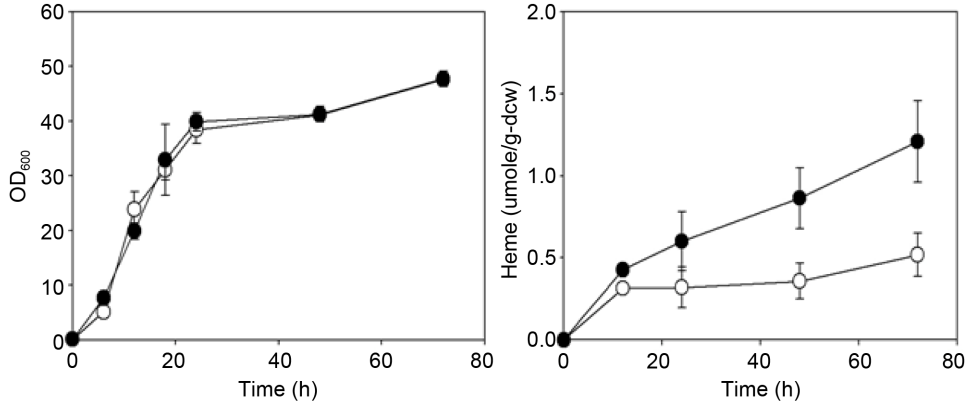


**Fig. 2.** Production of heme-iron in oxidative stress-tolerant *C. glutamicum* strain HA.
Closed circles represent the data from the HA host, and open circles from the wild-type host.

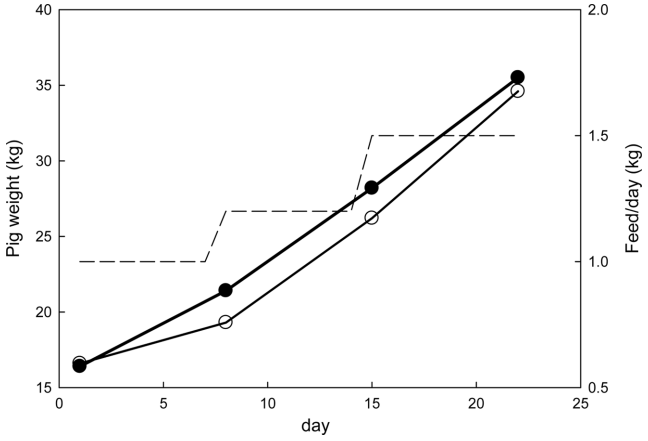


**Fig. 3.** Pig weight after the addition of heme-synthesizing *C. glutamicum* to the feed.

Closed circles represent the weight of the pig fed with the spray-dried *C. glutamicum* additive. Open circles represent the weight of the pig fed with control flour additive.

during the second week, and 1.5 kg/day during the third week. The control pig gained 18.0 kg, whereas the *C. glutamicum*-fed pig gained 19.1 kg in 3 weeks. Minor symptoms of diarrhea occurred once in the control pig, whereas the *C. glutamicum*-fed pig did not show any symptoms during the test period.

### Effects of *C. glutamicum*-Synthesized Heme Extract on LAB Culture

Some strains of strictly fermentative LAB have been reported to perform aerobic growth once respiratory components (*i.e.*, heme from bovine, menaquinone for other species) were exogenously added to the medium [14, 15, 23]. To verify whether *C. glutamicum*-synthesized heme extract addition might also enhance LAB biomass production, four strains of LAB were grown under aerobic or fermentative conditions, and their biomasses were compared (Table 1). *C. glutamicum*-synthesized heme addition (5 μg/ml) enabled

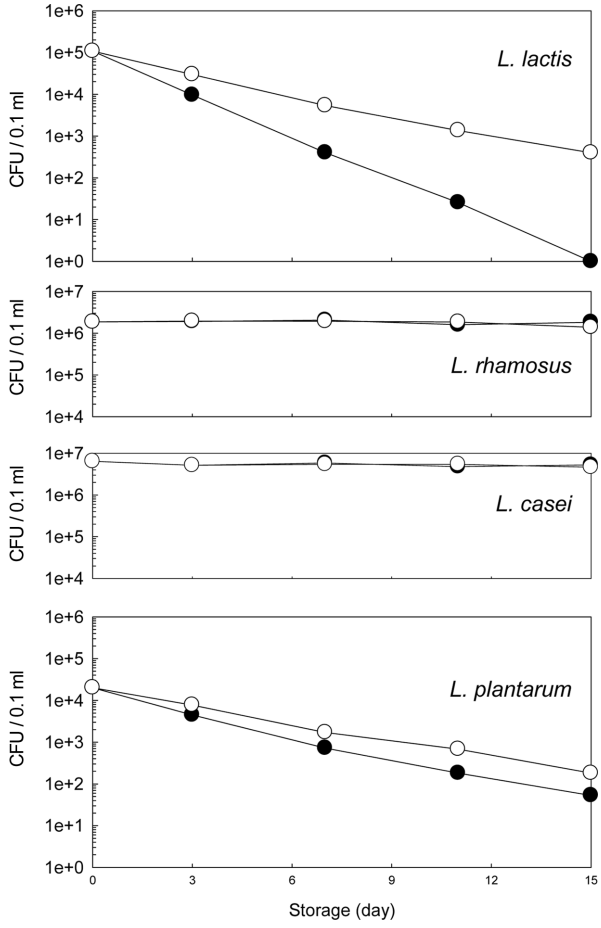


**Fig. 4.** Effect of heme extract from *C. glutamicum* on the survival of LAB.

LAB cultures were preserved at 4°C, and living cells were counted. Open circles represent the living cells in the cultures with *C. glutamicum*-synthesized heme extract, and closed circles represent living cells in the culture without heme extract. The tested bacterium is displayed in the graph.

*L. lactis*, *L. rhamosus*, and *L. casei* to produce 97%, 15%, and 4% more biomass, respectively, than that of the strains grown

**Table 1.** Effect of *Corynebacterium*-derived heme extract addition on the aerobic growth of lactic acid bacteria.

| Strains | Fermentative | Aerobic (no heme extract) | Aerobic (heme extract 2.5 μg/ml) | Aerobic (heme extract 5 μg/ml) |
|---|---|---|---|---|
| *Lactococcus lactis* subsp. *lactis* (KCTC 3769) | 1.67 ± 0.02 | 1.82 ± 0.08 | 2.94 ± 0.13 | 3.30 ± 0.57 |
| *Lactobacillus rhamosus* (KCTC 5033) | 12.27 ± 0.39 | 12.17 ± 0.05 | 13.64 ± 0.13 | 14.16 ± 0.35 |
| *Lactobacillus casei* (KCTC 3109) | 10.68 ± 0.50 | 6.25 ± 0.40 | 6.95 ± 0.31 | 11.07 ± 1.22 |
| *Lactobacillus plantarum* (KCTC 33131) | 10.25 ± 0.13 | 6.92 ± 0.27 | 8.35 ± 0.49 | 8.52 ± 0.01 |

Lactic acid bacteria were grown in MRS medium for 48 h at 30°C. Fermentative culture was performed in an anaerobic glass vial with a rubber stopper and an aluminum cap after air was removed and flushed with nitrogen gas. Aerobic culture was performed in an Erlenmeyer flask with a cotton stopper, with shaking at 220 rpm.

Data represent the mean ± SD of biomass measured by $OD_{600nm}$ from at least three biological replicates.

under fermentative conditions. The biomass of *L. plantarum* grown under aerobic culture conditions, however, was lower than that from fermentative culture. The enhanced biomass caused by heme addition was dose-dependent, implying that the biomass enhancement could be increased by supplementing more *C. glutamicum*-synthesized heme extract.

To confirm the potential use of *C. glutamicum*-synthesized heme for preservation, the heme extract was added to storage cultures at 4°C, and the living cells were counted (Fig. 4). Heme-added cultures of *L. lactis* and *L. plantarum* showed more live cells after 15 days of storage at 4°C. Cultures of *L. rhamosus* and *L. casei* in the presence or absence of heme were not significantly different after storage for 15 days.

## Discussion

ALA synthase-expressing *C. glutamicum* strain HA was able to accumulate more heme than ALA synthase-expressing wild-type *C. glutamicum* (Fig. 1). Functionally expressed ALA synthase enabled *C. glutamicum* to bypass feedback regulation in the heme biosynthetic pathway, resulting in heme accumulation. Because excess heme accumulation leads to stress and inhibits cellular activities [2, 22], more heme accumulation was induced in the oxidative stress-tolerant host (Fig. 2).

Pig, a naturally iron-deficient animal, was found to consume the dried cells of heme-producing *C. glutamicum*, resulting in increased weight gain and no diarrhea (Fig. 3 and Supplement 1). Using a feed additive composed of soil-derived bacterial biomass would be an alternative method to supply iron without the risk of soil-originated diseases, which may arise from direct contact with soil and mud [13], Furthermore, there was no need to purify heme from the microbial biomass, which is another benefit for the reduction of production costs and for commercial applications. For more statistically convincing results of the diet effect on the growth performance of swine, massive cell cultures followed by feeding to many numbers of animals should be tested before the industrial-scale application.

Three out of the four LAB tested showed increased biomass with the addition of heme-producing *C. glutamicum* extract (Table 1). The degree of biomass increase by heme addition varied amongst the tested LAB. Variation in the response may have been caused by differences in the minimum respiration machinery in the LAB. Once LAB possess a respiration machinery, exogenous addition of heme would enable the cells to grow using respiration, which would produce more energy than fermentation, thereby leading to increased biomass. Increased survival of LAB during storage was also observed with the addition of *C. glutamicum*-derived heme (Fig. 4). This might be a result of more energy derived from the proton motive force and used for the maintenance energy of some LAB species during cold storage, as is the case with bovine-derived heme addition [24] and the case with porphyrin addition, one of the intermediates in the heme biosynthetic pathway (Geppel *et al*. 2016. Porphyrin-containing latic acid bacterial cells and use thereof. US Patent 9,410,117 B2). Therefore, *C. glutamicum*-synthesized heme extract could be useful to increase biomass productivity and survivability, and to reduce the medium needed in LAB starter cultures for probiotics when the LAB possess other respiration machineries.

Excess heme-producing *C. glutamicum* was constructed in this study. The strain could be applied as a feed additive for swine to increase iron and as an electron carrier for LAB starter cultures. It is worth mentioning that the supernatants of the heme-synthesizing *C. glutamicum* culture broths used in this study also contained intermediates of the heme pathway, byproducts of microbial heme synthesis (*i.e.*, ALA, uroporpyrin, and coproporpyrin) [5]. The heme intermediates contained in the supernatant are the same intermediates involved in chlorophyll synthesis, which could therefore be used for plant and algae growth stimulation [25, 26]. Indeed, a preliminary experiment showed that watering with the culture broth of the heme-synthesizing *C. glutamicum* stimulated growth of a plant, garlic chive (Supplement 2). To develop a commercial application of the heme-synthesizing *C. glutamicum* culture, studies to validate the increase of swine weight and optimize microbial heme production, and strain development to overcome non-GMO/non-antibiotic marker issues, are further required.

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