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Heterologous and High Production of Ergothioneine in *Escherichia coli*

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Supporting Information

ABSTRACT: Ergothioneine (ERG) is a histidine-derived thiol compound suggested to function as an antioxidant and cytoprotectant in humans. Therefore, experimental trials have been conducted applying ERG from mushrooms in dietary supplements and as a cosmetic additive. However, this method of producing ERG is expensive; therefore, alternative methods for ERG supply are required. Five *Mycobacterium smegmatis* genes, *egtABCDE*, have been confirmed to be responsible for ERG biosynthesis. This enabled us to develop practical fermentative ERG production by microorganisms. In this study, we carried out heterologous and high-level production of ERG in *Escherichia coli* using the *egt* genes from *M. smegmatis*. By high production of each of the Egt enzymes and elimination of bottlenecks in the substrate supply, we succeeded in constructing a production system that yielded 24 mg/L (104 μM) secreted ERG.

KEYWORDS: *ergothioneine, heterologous production, Escherichia coli*

INTRODUCTION

Ergothioneine (ERG), a histidine-derived thiol compound, was isolated from an ergot fungus, *Claviceps purpurea*, more than a century ago. ERG is also known to be synthesized in actinobacteria, cyanobacteria, and a fission yeast.[1−3] Recent studies show that ERG functions as an antioxidant, such as glutathione, mycothiol, and bacillithiol. There is no direct evidence for biosynthesis of ERG in humans. However, ERG has been reported to be accumulated in various cells and tissues at high concentrations, probably by intake from diets, such as mushrooms and red beans, that contain relatively large amounts of ERG through an ERG-specific organic cation transporter, OCTN1.[4,5]

The presence of the ERG-specific transporter and the extensive accumulation of ERG in tissues suggest that ERG should have significant biological functions in humans. Although the true physiological role of ERG in humans has yet to be fully understood, ERG has been shown by *in vitro* experiments to function as an antioxidant and a cytoprotectant. Therefore, applications of ERG in dietary supplements and as a cosmetic additive have been explored, and there is an increasing demand for ERG.[6] Mushrooms have traditionally been the source of ERG.[7] However, slow growth, low content, and time-consuming purification procedures lead to a high manufacturing cost. Therefore, alternative and sustainable sources of ERG are necessary.

One such reliable and practical method is a fermentative process using microorganisms, such as actinobacteria and cyanobacteria, that are known to produce ERG. However, their ERG productivities are very low (1.18 mg/g of dry weight after 4 weeks of cultivation of *Mycobacterium avium* and 0.8 mg/g of dry weight of *Oscillatoria* sp.),[2,8] and thus, genetic and metabolic engineering are indispensable for industrial production. Until recently, however, there were no reports on ERG biosynthesis genes and enzymes. In 2010, five genes in *Mycobacterium smegmatis*, *egtABCDE*, were confirmed to be responsible for ERG biosynthesis (Figure 1).[9] In the biosynthetic pathway, EgtD catalyzes the formation of hercynine (HER) by transfer of three methyl groups derived from *S*-adenosylmethionine (SAM) to L-histidine (L-His). Then, EgtB catalyzes O_2-dependent C−S bond formation between γ-glutamylcysteine (γGC) supplied by EgtA and HER to form hercynyl-γ-glutamylcysteine sulfoxide (γGC-HER). This is followed by removal of the L-glutamate (L-Glu) moiety by EgtC, an amidohydrolase, to produce hercynylcysteine sulfoxide (Cys-HER). Then, EgtE, a PLP-dependent C−S lyase, catalyzes the formation of ERG with concomitant formation of pyruvate and ammonia as side products.

In this study, we developed heterologous and high-level production of ERG in *Escherichia coli* using the *egt* genes from *M. smegmatis*. By high production of each of the Egt enzymes and elimination of bottlenecks in substrate supply, the production system yielded 24 mg/L (104 μM) secreted ERG.

MATERIALS AND METHODS

General Procedures. Lysogeny broth (LB, Lennox) medium was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan); L-methionine (L-Met) and L-His were obtained from Wako Pure Chemical Industry (Osaka, Japan); and HER was purchased from Shinsei Chemical Company, Ltd. (Osaka, Japan). Other chemicals

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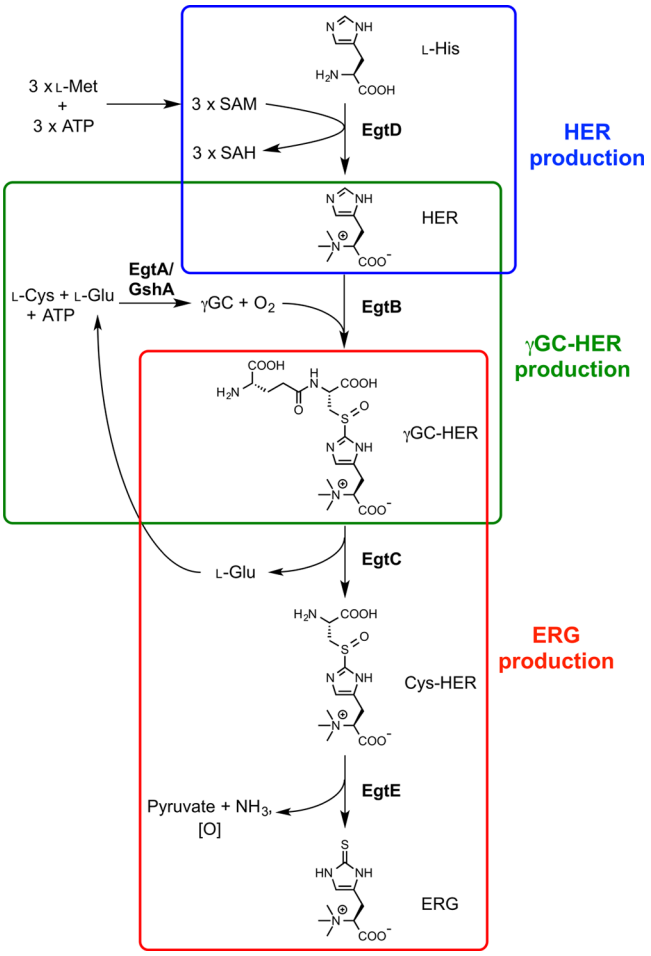

Figure 1. ERG biosynthetic pathway.

were of analytical grade and purchased from Wako Pure Chemical Industry or Sigma-Aldrich Japan. Primers were obtained from FASMAC Co., Ltd. (Kanagawa, Japan). Enzymes and kits for DNA manipulation were purchased from Takara Bio, Inc. (Shiga, Japan) or New England BioLabs Japan, Inc. (Tokyo, Japan). Polymerase chain reaction (PCR) was carried out using a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) with Tks Gflex DNA polymerase (Takara Bio). General genetic manipulations of *E. coli* were performed according to standard protocols. High-resolution electrospray ionization Fourier transform mass spectrometry (HR-ESI−FT−MS) analysis was performed using an Exactive system (Thermo Fisher Scientific, Inc.).

Bacterial Strains and Cultures. Microorganisms used in this study are summarized in Table 1. *E. coli* XL1-Blue (Nippon Gene Co., Ltd., Tokyo, Japan), BL21(DE3) (Merck KGaA, Darmstadt, Germany), and BW25113 (National Institute of Genetics, Shizuoka, Japan), which is a high producer of L-cysteine (L-Cys) used for a sulfur donor of ERG,[10] were used for plasmid construction, protein production, and ERG production, respectively. The media used were LB and M9Y minimal medium prepared by adding 1% (w/v) glucose, 5 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.1% (w/v) yeast extract (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) to M9 minimal salts (Becton, Dickinson and Company). Ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km), streptomycin (Sm), and tetracycline (Tc) were added to the media at concentrations of 100, 33, 25, 20, and 5 mg/L, if necessary. Optical density (OD) at 600 nm was measured with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Inc.).

Preparation of Egt Recombinant Enzymes. Detailed plasmid construction methods are described in Supplementary Methods 1 of the Supporting Information, and the plasmids are summarized in Table 2. Briefly, EgtB, EgtC, EgtD, and EgtE were amplified by PCR using *M. smegmatis* genomic DNA as the template and appropriate primers (Table S1 of the Supporting Information), in which restriction sites were introduced at the N and C termini. The PCR products were respectively cloned into the expression vectors. The plasmids obtained were introduced into *E. coli* BL21(DE3). A liquid culture of the transformant in LB supplied with appropriate antibiotics was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached about 0.6. The cultivation was continued for an additional 16 h at 20 °C. The production of each recombinant enzyme was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) with Coomassie Brilliant Blue staining. The purification of each recombinant protein was carried out using nickel−nitrilotriacetic acid (Ni−NTA) agarose

Table 1. Bacterial Strains Used in This Study

strain	description	source
M. smegmatis JCM6386	ERG producer	JCM[a]
E. coli		
XL1-Blue	*hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *supE*44, *relA*1, *lac*[F′, *proAB*, *lacI*^{q}ZΔM15, Tn10(Tc^R)]	Nippon Gene
BL21(DE3)	F^-, *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Merck
BW25113	*rrnB*3 Δ*lacZ*4787 *hsdR*514 Δ(*araBAD*)567 Δ(*rhaBAD*)568 *rph*-1	NIG[b]
ET1	BW25113 harboring pCF1s-MsD	this study
ET2	BW25113 harboring pCF1s-MsD, pQE1a-mMsB	this study
ET3	BW25113 harboring pCF1s-MsD, pQE1a-mMsB, pAC1c-hMsC/hMsE	this study
ET4	BW25113 harboring pCF1s-MsD, pQE1a-mMsB/EcA, pAC1c-hMsC/hMsE	this study

[a]Japan Collection of Microorganisms, Riken BioResource Center. [b]National Institute of Genetics.

Table 2. Plasmids Used in This Study

plasmid	description	source
pQE1a-Red	protein production plasmid, *tac* promoter, ColE1 ori, Ap^R	lab stock
pCF1s-Red	protein production plasmid, *tac* promoter, CDF ori, Sm^R	lab stock
pET-21a	protein production plasmid, *T7* promoter, pBR322 ori, Ap^R	Merck
pACYCDuet-1	protein production plasmid, *T7* promoter, p15A ori, Cm^R	Merck
pQE1a-MsB	pQE1a-Red derivative, production of EgtB	this study
pQE1a-mMsB	pQE1a-MsB derivative, production of MBP-fused EgtB	this study
pQE1a-mMsB/EcA	pQE1a-mMsB derivative, co-production of MBP-fused EgtB and GshA	this study
pQE1a-MsC	pQE1a-Red derivative, production of EgtC	this study
pQE1a-hMsC	pQE1a-MsC derivative, production of His-tagged EgtC	this study
pQE1a-MsE	pQE1a-Red derivative, production of EgtE	this study
pQE1a-hMsE	pQE1a-Red derivative, production of His-tagged EgtE	this study
pAC1c-hMsC/hMsE	pACYCDuet-1 derivative, co-production of His-tagged EgtC and EgtE	this study
pCF1s-MsD	pCF1s-Red derivative, production of EgtD	this study

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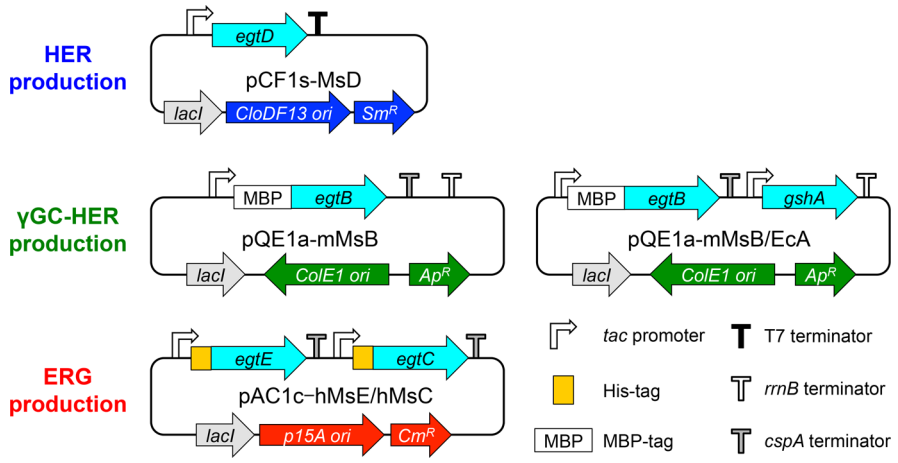

Figure 2. Plasmids for ERG production.

(QIAGEN K.K., Tokyo, Japan) or amylose resins (New England BioLabs) according to the protocols of the manufacturer. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) with bovine serum albumin as the standard.

ERG Production. *E. coli* harboring plasmids carrying ERG biosynthetic genes (Figure 2) was cultured in 3 mL of M9Y media at 30 °C for 16 h. The cultures (1 mL) were inoculated into 50 mL of M9Y media supplemented with 0.5 g/L L-His, 0.5 g/L L-Met, and 20 mg/L $FeSO_4 \cdot 7H_2O$ in 200 mL Erlenmeyer flasks and incubated at 30 °C with shaking (200 rpm) for up to 72 h. $Na_2S_2O_3$ was added to media at 20 mM, if needed. IPTG was added to a final concentration of 0.5 mM after 3 h of cultivation. Samples (1 mL) were collected at appropriate time points and analyzed by liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS).

LC–ESI–MS Analysis of Products. Mixtures of 0.05% (v/v) heptafluorobutyric acid (HFBA) solution (180 μL) and culture broth or enzymatic reaction solutions (20 μL) were analyzed. The LC–ESI–MS conditions were as follows: Waters ACQUITY UPLC system equipped with a photodiode array and a SQ Detector2 (Tokyo, Japan); XBridge BEH C18 XP column (150 mm length × 2.0 mm internal diameter, 2.5 μm, Waters); flow rate, 0.15 mL/min; temperature, 35 °C; mobile phase, water containing 0.05% HFBA and 7% methanol; injection volume, 2 μL; and detection, 210 nm for His and HER, 250 nm for Cys-HER and γGC-HER, and 258 nm for ERG.

RESULTS AND DISCUSSION

The most reliable strategy for high production of ERG is overproduction of each of the enzymes for ERG biosynthesis and then optimization of any inefficient biosynthetic step(s) (identified by intermediate accumulation) by molecular genetics and metabolic engineering. If no intermediates accumulate, supply of the initial substrates, L-His, L-Glu, L-Cys, and L-Met, should be enhanced.

ERG biosynthetic genes are present in some microorganisms, such as actinobacteria, cyanobacteria, and α-proteobacteria.[11] Among these bacteria, we selected *M. smegmatis* as the gene source, because the ERG biosynthetic pathway was first identified in this strain and the biosynthesis genes constitute an operon.[9] Because *egtB*, *egtC*, and *egtE* are translationally coupled, we first tried expressing the *egt* genes as the operon with *tac* and T7 promoters. We constructed several plasmids using pQE1a-Red and pET-21a, but ERG productivities of transformants harboring the plasmids were low (less than 0.2 mg/L). Therefore, we overexpressed *egt* genes stepwise under the control of individual promoters by checking production of recombinant enzymes and their activities by production of intermediate compounds (HER, γGC-HER, and Cys-HER) and ERG by *in vitro* experiment (Figure 1). Because *E. coli* has an *egtA* orthologue, *gshA*, which is responsible for glutathione biosynthesis,[12] we expressed the other genes, *egtB*, *egtC*, *egtD*, and *egtE*. We also prepared the intermediate compounds, which are not commercially available, with the recombinant enzymes to obtain standards for quantitative analysis (Supplementary Methods 2 of the Supporting Information).

Overproduction of Recombinant Enzymes. For overproduction of Egt enzymes, we used pQE1a-Red and pCF1s-Red, both of which are home-constructed and compatible vectors with the *tac* promoter for protein production (Figure S1 of the Supporting Information). As Figure S2 of the Supporting Information shows, transformants harboring pCF1s-MsD carrying *egtD* overproduced EgtD in soluble form. To confirm whether the recombinant protein had the expected activity, we carried out *in vitro* experiments with cell-free extracts containing the recombinant enzyme.[9,13] The cell-free extracts were incubated with L-His and excess SAM. After the reaction, the product was analyzed by LC–ESI–MS. A specific peak with the same retention time and mass spectrometry (MS) spectrum as the HER standard was clearly detected after 1 h of incubation, demonstrating that EgtD converted L-His into HER (Figure S3 of the Supporting Information).

We next overproduced recombinant EgtB using pQE1a-Red (Figure S1 of the Supporting Information) and changed the probable start codon TTG into ATG. However, recombinant EgtB formed inclusion bodies in multiple culture conditions. Therefore, a plasmid from which a recombinant enzyme is produced as a maltose binding protein (MBP)-fused enzyme was examined. We successfully produced a MBP-fused EgtB in a soluble form in transformants harboring pQE1a-mMsB. To confirm whether the recombinant enzyme had the expected activity, we carried out *in vitro* experiments with purified recombinant enzyme (Figure S4 of the Supporting Information).[9] The recombinant MBP-fused EgtB was incubated with HER and γGC for 2 h. As Figure S5 of the Supporting Information shows, HER completely disappeared and the formation of a new product with m/z 462.20 was detected. On the basis of HR-ESI–FT–MS analysis of the purified compound, the molecular formula of the product was determined to be $C_{17}H_{27}O_8N_5S$ (m/z: $[M + H]^+$ calculated for $C_{17}H_{28}O_8N_5S^+$, 462.165 31; observed, 462.165 07), which corresponded to that of γGC-HER, suggesting the formation of

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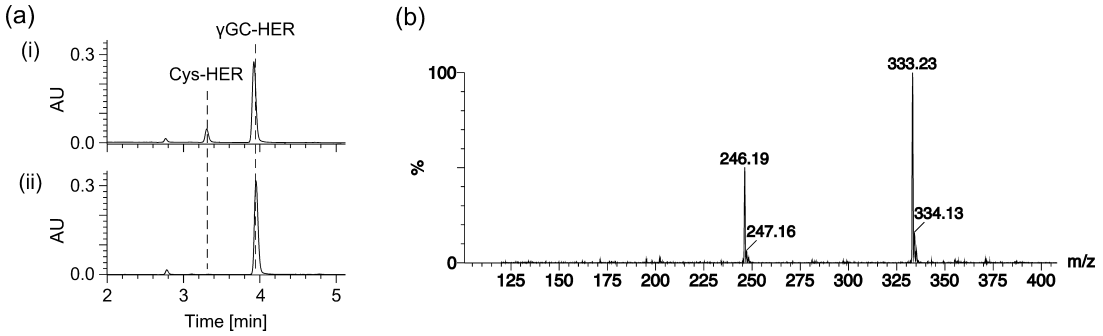

Figure 3. LC−ESI−MS analysis of EgtC reaction products. (a) Traces at 250 nm of reaction products. The reaction (40 μL) was carried out by adding (i) purified recombinant EgtC (3.9 μM) or (ii) boiled EgtC to the EgtB reaction solution at 25 °C for 17 h. (b) MS spectrum of the EgtC reaction product (ESI positive mode).

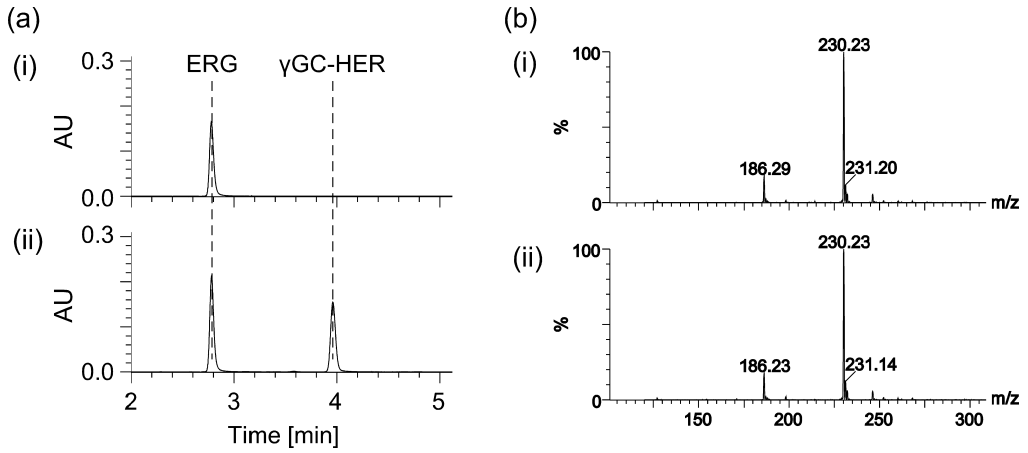

Figure 4. LC−ESI−MS analysis of EgtE reaction products. (a) Traces at 250 nm of (i) ERG standard and (ii) reaction products. The reaction (40 μL) was carried out by adding purified recombinant EgtE (2.5 μM) and EgtC (3.9 μM) (ii) to the boiled supernatant of the EgtB reaction mixture containing 2 mM dithiothreitol at 25 °C for 17 h. (b) MS spectra of (i) ERG standard and (ii) EgtE reaction product (ESI positive mode).

γGC-HER. The thus formed intermediate compound γGC-HER was purified by high-performance liquid chromatography (HPLC) (Supplementary Methods 2 of the Supporting Information) and used as a standard for quantitative analysis.

Finally, overproduction of recombinant EgtC and EgtE was examined. We first used the same vector as for EgtB production, but no production of either enzyme was observed. We then examined a plasmid from which a recombinant enzyme is produced as a His-tagged enzyme. In this case, both EgtC and EgtE were successfully overproduced. To confirm whether the recombinant enzymes had the expected activity, we carried out *in vitro* experiments with the purified recombinant enzymes (Figure S4 of the Supporting Information).[9] Recombinant EgtC was incubated with enzymatically prepared γGC-HER. After 17 h of reaction, the product was analyzed by LC−ESI−MS. As shown in Figure 3, a new peak with *m/z* 333.23 was detected and Cys-HER formation was suggested. On the basis of HR-ESI−FT−MS analysis of the purified compound, the molecular formula of the product was determined to be $C_{12}H_{20}O_5N_4S$ (*m/z*: $[M + H]^+$ calculated for $C_{12}H_{21}O_5N_4S^+$, 333.122 72; observed, 333.122 90), which corresponded to that of Cys-HER. The thus formed Cys-HER was purified (Supplementary Methods 2 of the Supporting Information) and used as a standard for quantitative analysis.

By adding recombinant EgtE together with EgtC into the EgtB reaction mixture, we confirmed the formation of ERG by LC−ESI−MS analysis (Figure 4), showing that all of the recombinant Egt enzymes possessed the expected activities.

Simultaneous Production of Egt Enzymes for ERG Production. To reconstruct the ERG-producing pathway in *E. coli*, we optimized the production conditions in a stepwise manner: HER, then γGC-HER, and finally ERG production (Figures 1 and 2).

The first step was HER production. *E. coli* BW25113 harboring pCF1s-MsD (named strain ET1) was cultured in M9Y medium, and 91 ± 2 mg/L HER (460 ± 10 μM) was produced in the culture broth after 48 h. We then carried out feeding experiments with L-His and L-Met, because amino acid biosynthesis in *E. coli* is strictly regulated by feedback inhibition and/or transcriptional repression. L-Met and L-His feeding increased the yield: 164 ± 3 mg/L HER (833 ± 15 μM) was produced after 48 h of cultivation (Figure 5). After this, L-His and L-Met feeding was employed in all *in vivo* production experiments.

We next carried out *in vivo* co-production of EgtD and EgtB for γGC-HER production. Transformants of *E. coli* BW25113 carrying both pCF1s-MsD and pQE1a-mMsB (strain ET2) were cultivated in the medium supplemented with L-His and L-Met. As Figure 6 shows, we confirmed 24 ± 1 mg/L γGC-HER (52 ± 2 μM) production after 24 h of cultivation, indicating that EgtB converted HER to γGC-HER using endogenous γGC in *E. coli*. However, accumulation of 3-fold higher amounts of HER (110 ± 5 mg/L) than Cys-HER (36 ± 2 mg/L) was

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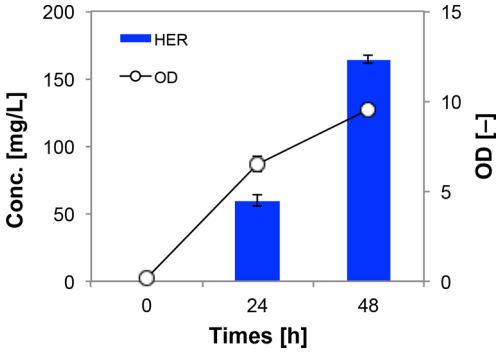

Figure 5. Culture profiles of strain ET1. *E. coli* BW25113 harboring pCF1s-MsD (strain ET1) was cultured in M9Y medium supplemented with L-His and L-Met. After 3 h of cultivation, 0.5 mM IPTG was added to the medium. Data are presented as mean values with standard errors from three independent experiments.

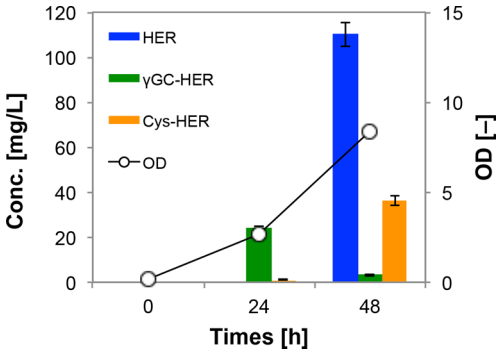

Figure 6. Culture profiles of strain ET2. *E. coli* BW25113 harboring pCF1s-MsD and pQE1a-mMsB (strain ET2) was cultured in M9Y medium supplemented with L-His and L-Met. After 3 h of cultivation, 0.5 mM IPTG was added to the medium. Data are presented as mean values with standard errors from three independent experiments.

detected, suggesting that γGC-HER production was rate-limiting.

For simultaneous expression of all *egt* genes, *egtC* and *egtE*, both of which were expressed from the *tac* promoter of pQE1a, were recloned into plasmid pACYCDuet-1, which is compatible with pQE1a-Red and pCF1s-Red, to construct pAC1c-hMsC/hMsE (Figure 2 and Supplementary Methods 2 of the Supporting Information). The plasmid was successfully constructed, and production of both enzymes in soluble forms in *E. coli* was confirmed by SDS−PAGE (Figure S6 of the Supporting Information). The plasmid was introduced into strain ET2 to construct strain ET3, and recombinant enzyme production was examined. As Figure S7 of the Supporting Information shows, all of the enzymes were produced in soluble forms. We then examined ERG production. Strain ET3 produced 19 ± 2 mg/L (83 ± 8 μM) ERG together with 73 ± 15 mg/L (370 ± 76 μM) HER in the culture broth after 72 h of cultivation (Figure 7 and Table 3). These results suggested that the EgtB-catalyzed reaction is a bottleneck.

ERG Production and Improvement of Rate-Limiting Steps. Considering that EgtB was overproduced in a soluble form in the producing strain ET3 and that it showed enough activity in *in vitro* experiments, we considered that insufficient supply of γGC, the substrate of EgtB, might cause the accumulation of HER. To test this hypothesis, overproduction of γGC synthetase was carried out. To produce γGC synthetase, the *gshA* gene from *E. coli*[12] was cloned and

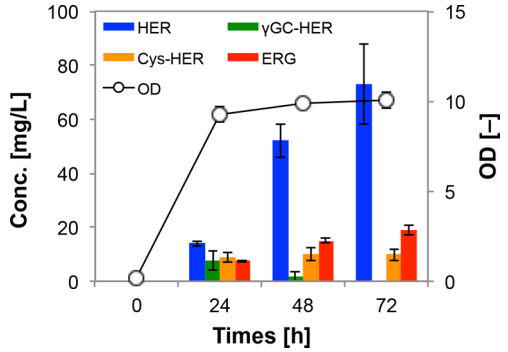

Figure 7. Culture profiles of strain ET3. *E. coli* BW25113 harboring pCF1s-MsD, pQE1a-mMsB, and pAC1c-hMsC/hMsE (strain ET3) was cultured in M9Y medium supplemented with L-His and L-Met. After 3 h of cultivation, 0.5 mM IPTG was added to the medium. Data are presented as mean values with standard errors from three independent experiments.

Table 3. Culture Profiles of ET3 and ET4 Strains[a]

strain	OD	HER (mg/L)	γGC-HER (mg/L)	Cys-HER (mg/L)	ERG (mg/L)
ET3[b]	10.1 ± 0.5	73 ± 15	ND[c]	10 ± 2	19 ± 2
ET4[b]	8.7 ± 0.2	121 ± 12	1 ± 1	9 ± 1	17 ± 1
ET3[d]	11.1 ± 0.5	48 ± 17	ND	9 ± 0	24 ± 4

[a]Data after 72 h of cultivation are presented as mean values with standard error from three independent experiments. [b]ET3 and ET4 were cultured in M9Y media supplemented with L-His and L-Met. [c]ND = not detected. [d]ET3 was cultured in M9Y media supplemented with L-His, L-Met, and thiosulfate.

inserted into pQE1a-mMsB to construct pQE1a-mMsB/EcA. Although MBP-fused EgtB and GshA were produced in soluble forms in *E. coli* BW25113 harboring the three plasmids (strain ET4) (Figure S8 of the Supporting Information), ERG productivity was decreased in comparison to that of strain ET3 to 17 ± 1 mg/L (72 ± 2 μM) after 72 h of cultivation (Table 3). In comparison of SDS−PAGE data for strains ET3 and ET4 (Figures S7 and S8 of the Supporting Information), soluble MBP-fused EgtB was found to be decreased in strain ET4. This could be the reason for the reduced productivity.

We then employed another strategy to enhance the γGC supply. In ERG biosynthesis, γGC is used as a sulfur donor. In particular, L-Cys is a net sulfur donor, because the L-Glu moiety of γGC is released by EgtC. Therefore, reinforcement of L-Cys flux may be effective in enhancing γGC flux and ERG production. Because L-Cys addition into media was reported to be toxic to *E. coli* cells,[14] we employed another strategy. We have been studying a L-Cys biosynthetic pathway in *E. coli* and demonstrated that thiosulfate ($S_2O_3^{2-}$) was a better sulfur source than sulfate (SO_4^{2-}) for high L-Cys production.[10] We therefore fed thiosulfate into the growth media. When strain ET3 was cultured in M9Y supplemented with L-Met, L-His, and thiosulfate, ERG productivity was increased to 24 ± 4 mg/L (104 ± 17 μM) (Table 3), indicating that reinforcement of L-Cys flux was very effective in enhancing the ERG production.

In conclusion, to establish a reliable and practical method for ERG supply, fermentative ERG production by microorganisms was investigated. We heterologously overexpressed genes *egtBCDE* of *M. smegmatis* in *E. coli* and succeeded in the production of ERG (19 mg/L). Reinforcement of the γGC supply by feeding of thiosulfate, a suitable sulfur donor for high L-Cys production, resulted in higher ERG production (24 mg/

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L). Considering that the reported ERG contents of mushrooms were from 0.15 to 7.27 mg/g of dry weight,[7] our system might become an alternative method for ERG supply. However, significant amounts of HER still accumulated, suggesting that more supply of γGC and L-Cys by metabolic engineering and use of another EgtB with higher activity are indispensable for high-level production of ERG.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04924.

Supplementary Methods 1 and 2, vectors pQE1a-Red and pCF1s-Red (Figure S1), SDS−PAGE analysis of EgtD production (Figure S2), LC−ESI−MS analysis of EgtD reaction products (Figure S3), SDS−PAGE analysis of purified recombinant EgtB, EgtC, and EgtE (Figure S4), LC−ESI−MS analysis of EgtB reaction products (Figure S5), SDS−PAGE analysis of recombinant EgtC and EgtE production (Figure S6), SDS−PAGE analysis of production of recombinant Egt enzymes in strain ET3 (Figure S7), SDS−PAGE analysis of production of recombinant Egt enzymes and GshA in strain ET4 (Figure S8), and primers used in this study (Table S1) (PDF)

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■ NOMENCLATURE

ERG = ergothioneine
HER = hercynine
γGC = γ-glutamylcysteine
γGC-HER = hercynyl-γ-glutamylcysteine sulfoxide
Cys-HER = hercynylcysteine sulfoxide
SAM = *S*-adenosylmethionine
SAH = *S*-adenosylhomocysteine
L-His = L-histidine
L-Glu = L-glutamate
L-Cys = L-cysteine
L-Met = L-methionine
HR-ESI−FT−MS = high-resolution electrospray ionization Fourier transform mass spectrometry
MBP = maltose-binding protein
SDS−PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

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