



Title	High Production of Ergothioneine in Escherichia coli using the Sulfoxide Synthase from Methylobacterium strains
Author(s)	Kamide, Tomoyuki; Takusagawa, Shun; Tanaka, Naoyuki et al.
Citation	Journal of Agricultural and Food Chemistry, 68(23), 6390-6394 https://doi.org/10.1021/acs.jafc.0c01846
Issue Date	2020-06-10
Doc URL	https://hdl.handle.net/2115/81773
Rights	This document is the Accepted Manuscript version of a Published Work that appeared in final form in Journal of Agricultural and Food Chemistry, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see https://pubs.acs.org/doi/10.1021/acs.jafc.0c01846 , see http://pubs.acs.org/page/policy/articlesonrequest/index.html .
Type	journal article
File Information	aper.pdf



High production of ergothioneine in *Escherichia coli* using the sulfoxide synthase from *Methylobacterium* strains

Tomoyuki Kamide^{1†}, Shun Takusagawa^{1†}, Naoyuki Tanaka², Yasushi Ogasawara³, Yusuke Kawano², Iwao Ohtsu², Yasuharu Satoh^{3*}, and Tohru Dairi^{3*}

¹*Graduate School of Chemical Science and Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan.*

²*Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan.*

³*Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan.*

*Corresponding authors:

Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan.

Tel. +81-11-706-7815; Fax. +81-11-706-7118.

Email: dairi@eng.hokudai.ac.jp and syasu@eng.hokudai.ac.jp

[†] TK and ST contributed equally to this work.

ABSTRACT

We previously constructed a heterologous production system for ergothioneine (ERG) in *Escherichia coli* using five ERG biosynthesis genes (*egtABCDE*) from *Mycobacterium smegmatis*. However, significant amounts of hercynine (HER), an intermediate of ERG, as ERG was accumulated, suggesting that the reaction of EgtB catalyzing the attachment of γ -glutamylcysteine to HER to yield hercynyl- γ -glutamylcysteine sulfoxide was a bottleneck. In this study, we searched for other EgtBs and found many *egtB* orthologs in diverse microorganisms. Among these, *Methylobacterium* strains possessed EgtBs that catalyze direct conversion of HER into hercynylcysteine sulfoxide with L-Cys as a sulfur donor, in a manner similar to those of acidobacterial CthEgtB and fungal Egt1. *In vitro* study with recombinant EgtBs from *Methylobacterium brachiatum* and *M. pseudosasicola* clearly showed that both enzymes accepted L-Cys but not γ -glutamylcysteine. We reconstituted the ERG production system in *E. coli* with *egtB* from *M. pseudosasicola*; ERG productivity reached 657 mg L⁻¹.

Key words: ergothioneine; heterologous production; *Escherichia coli*; *Methylobacterium*

INTRODUCTION

Ergothioneine (ERG) (Figure 1) is synthesized in many mushrooms, fission yeast, actinobacteria and cyanobacteria, but not in animals¹⁻⁶. However, ERG is accumulated in various mammalian cells at high concentrations, probably by intake from the diet and transport via an ERG-specific transporter, ETT⁷. A knockout of this transporter from zebrafish resulted in reduced content of ERG and the study suggested that the role of ERG is to eliminate singlet oxygen⁸. Therefore, ERG would function as a specialized antioxidant and be useful for prevention of cardiovascular diseases. For this reason, demand for ERG as a dietary supplement and cosmetic additive is increasing. Mushrooms have traditionally been the source of ERG. However, edible mushrooms contained a low level of ERG (16 to 417 mg kg⁻¹ of wet weight)¹, so alternative and sustainable sources are necessary for commercial uses.

Microorganisms such as fungi, actinobacteria, and cyanobacteria produce ERG^{2,4,6}. However, their ERG productivities are very low. We therefore developed a heterologous production system for ERG in *Escherichia coli*⁹ using the *egtBCDE* biosynthesis cluster genes from *Mycobacterium smegmatis*¹⁰ (Figure 1). The transformant produced 24 mg L⁻¹ of ERG. However, 48 mg L⁻¹ of hercynine (HER), an intermediate compound in ERG biosynthesis and the substrate of EgtB, as ERG was accumulated⁹. By detailed analysis, it was found that this was perhaps caused by insufficient supply of L-Cys, which is used for biosynthesis of γ -glutamylcysteine (γ GC), the

counterpart substrate of EgtB. Therefore, L-Cys flux was enhanced by addition of thiosulfate ($\text{S}_2\text{O}_3^{2-}$), which is a better sulfur source than sulfate (SO_4^{2-}) for high L-Cys production, to culture broth. In these conditions, ERG production increased to 24 mg L^{-1} in Erlenmeyer flasks. We also produced 1.3 g L^{-1} ERG in fed-batch culture in a jar fermenter¹¹ using an L-Cys high producer strain with reinforced *S*-adenosylmethionine (SAM) supply. For L-Cys production, this strain carried feedback regulation-insensitive *serA* and *cysE* genes (encoding *E. coli* D-3-phosphoglycerate dehydrogenase and serine acetyltransferase respectively), and *ydeD* encoding the L-Cys exporter of *E. coli*. *metJ*, which encodes a transcriptional repressor controlling the SAM and methionine biosynthesis pathways, was disrupted to enhance the SAM supply.

However, even in this case, HER still accumulated, suggesting that the reaction catalyzed by EgtB that uses HER and γGC as substrates remained a bottleneck. Thus, in the present study, we searched for another EgtB. *egtB* orthologs (*Mb_egtB* and *Mp_egtB*) from *Methylobacterium* strains were revealed to encode EgtBs that catalyze the direct conversion of HER into hercynylcysteine sulfoxide (Cys-HER) with L-Cys as the sulfur donor. High ERG production (657 mg L^{-1}) was achieved by using *Mp_egtB* in an ERG production system in Erlenmeyer flasks.

MATERIALS AND METHODS

Detailed experimental procedures are described in Supporting Information.

74

75 **Bacterial Strains and Plasmids.** Microorganisms used in this study are summarized in Table
76 1. *E. coli* XL1-Blue (Nippon Gene Co. Ltd., Tokyo, Japan) and BL21(DE3) (Merck KGaA,
77 Darmstadt, Germany) were used for plasmid construction and protein production, respectively. *E.*
78 *coli* BW25113 (National Institute of Genetics, Shizuoka, Japan) and CH Δ *metJ*¹¹ were used for
79 ERG production in Erlenmeyer flasks. Their transformants were cultured in appropriate media
80 containing ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), kanamycin (Km), and
81 tetracycline (Tc), as necessary. *Methylobacterium brachiatum* NBRC103629, *M. pseudosasicola*
82 NBRC105203, *Aquabacterium olei* NBRC 110486, *Labilithrix luteola* NBRC 109946, and
83 *Tenacibaculum maritimum* NBRC 110778 were used as DNA sources of *egtB* genes.

84 Plasmids used in this study are summarized in Table 2. Detailed plasmid construction methods
85 are described in Supporting Information.

86

87 **RESULTS AND DISCUSSION**

88 Because the EgtB-catalyzed reaction was a bottleneck in the heterologous ERG production
89 system⁹, we searched for other EgtBs and found many *egtB* orthologs in diverse microorganisms
90 belonging to the α -, β -, and δ -proteobacteria, and Bacteroidetes¹². Recently, sulfoxide synthases
91 responsible for ERG biosynthesis were classified into five clades based on conserved amino acid

92 sequences and catalytic residues (Figure 2, Table S1)¹³. Mycobacterial EgtB, which was one of the
93 first identified sulfoxide synthases responsible for ERG biosynthesis¹⁰, belongs to the type I clade.
94 Egt1s from *Neurospora crassa*, *Aspergillus fumigatus*, and *Schizosaccharomyces pombe*, classified
95 into the type III clade, are bifunctional enzymes that catalyze the direct conversion of L-His to Cys-
96 HER with L-Cys as the sulfur donor (Figure 2). Recently, EgtB from *Chloracidobacterium*
97 *thermophilum* (CthEgtB), which possesses consecutive Tyr residues at the catalytic site and was
98 classified into the type II clade, was revealed to use HER and L-Cys as substrates for the direct
99 formation of Cys-HER¹³. Because the use of L-Cys, instead of γ GC, as the sulfur donor would
100 enable us to shortcut the reconstituted ERG biosynthetic pathway (Figure 1, boxed pathway), we
101 explored EgtBs in the type II clade.

102 EgtBs from *Methylobacterium* strains, ERG producers^{14,15}, possess the consecutive Tyr
103 residues and approximately 50% identity to CthEgtB (Figure 2, Table S1). Therefore, we examined
104 whether the EgtBs from *M. brachiatum* (Mb_EgtB) and *M. pseudosasicola* (Mp_EgtB) use L-Cys
105 as the sulfur donor. Recombinant Mb_EgtB and Mp_EgtB were prepared (Figure S1) and incubated
106 with HER and L-Cys. By liquid chromatography-electrospray ionization-mass spectrometry (LC-
107 ESI-MS) analysis, specific peaks with the same retention time and mass spectrum as those of Cys-
108 HER standard were clearly detected after 2 h of incubation at 30°C with either of the enzymes

(Figures 3 and S2), demonstrating that Mb_EgtB and Mp_EgtB accepted L-Cys. In contrast, no γ GC-HER were detected when γ GC was used as the sulfur donor (Figure 3).

We next examined whether the above mentioned EgtB orthologs found in β -proteobacteria, δ -proteobacteria, and Bacteroidetes use L-Cys or γ GC as the sulfur donor. We selected the EgtBs from *A. olei*, *L. luteola*, and *T. maritimum* for analysis because they possess the consecutive Tyr residues at the catalytic site and approximately 50% identity with each other (Figure 2, Table S1). His-tagged recombinant EgtBs were purified and used for *in vitro* assays (Figure S3). All the enzymes accepted only L-Cys, although their relative activities were lower than those of Mb_EgtB and Mp_EgtB (Figure S3).

We next investigated the enzymatic properties of Mp_EgtB. The product formation was optimal at 40°C and pH 8.0 (Figure S4). Kinetic parameters were determined. Because the enzyme activity was inhibited in the presence of >1.0 mM L-Cys, its concentration was varied from 0.2 to 1.0 mM. In these conditions, the enzyme followed Michaelis–Menten kinetics (Figure S5). The K_m values of Mp_EgtB were 0.53 ± 0.03 mM for HER, and 0.24 ± 0.03 mM for L-Cys. The k_{cat} value was approximately 2.1 s^{-1} . We also determined kinetic parameters for Mb_EgtB (Figure S6). K_m values were 0.48 ± 0.06 mM for HER, and 0.34 ± 0.04 mM for L-Cys. The k_{cat} value was about 2.3 s^{-1} . The k_{cat}/K_m values of Mb_EgtB ($7.4 \text{ s}^{-1} \text{ mM}^{-1}$) and Mp_EgtB ($9.0 \text{ s}^{-1} \text{ mM}^{-1}$) for L-Cys were slightly lower than that of EgtB from *M. smegmatis* ($13.5 \text{ s}^{-1} \text{ mM}^{-1}$ for γ GC)¹⁶. However, the

127 former and the latter utilize L-Cys and γ GC as a sulfur source, respectively, and L-Cys is also used
128 for γ GC biosynthesis. Considering that most of the intracellular L-Cys was reported to be exported
129 to the medium¹⁷ and that the slight difference of the enzymatic properties might be no effects on
130 product formation at low concentration, we expected that Mp_EgtB and Mb_EgtB that directly
131 utilize L-Cys have merit for heterologous ERG production in *E. coli*. *egtDE* from *M. smegmatis*
132 were introduced into *E. coli* BW25113 with *egtB* from either *Methylobacterium brachiatum* or *M.*
133 *pseudosasicola*. The transformant with Mb_EgtB produced $166 \pm 16 \text{ mg L}^{-1}$ of ERG, and that with
134 Mp_EgtB produced $225 \pm 5 \text{ mg L}^{-1}$ of ERG after 72 h of cultivation in conditions described
135 previously⁹ (Figure 4 and Table 3). The ERG productivity was 9.4 times that of *E. coli* expressing
136 *M. smegmatis egtBCDE* (24 mg L^{-1})⁹ and HER was reduced to $19 \pm 4 \text{ mg L}^{-1}$ (Mb_EgtB) and 34
137 $\pm 1 \text{ mg L}^{-1}$ (Mp_EgtB) (Table 3) possibly because of the direct use of L-Cys.

138 We next examined ERG productivity in the CH Δ *metJ* strain¹¹, which possesses the pCys^{HP}
139 plasmid carrying modified *E. coli cysE* and *serA* genes, which are insensitive to feedback regulation
140 by L-Cys or L-Ser, and the *E. coli* L-Cys exporter gene, *ydeD*. In addition, SAM supply is reinforced
141 by disruption of the gene encoding MetJ, a transcriptional repressor controlling the SAM and
142 methionine biosynthesis pathways. The transformants carrying *egtDE* from *M. smegmatis* and
143 *Mb_egtB* or *Mp_egtB* produced 475 ± 109 or $657 \pm 89 \text{ mg L}^{-1}$ of ERG, respectively, after 192 h of
144 cultivation in Erlenmeyer flasks. The ERG production was thus 2.4 times that of CH Δ *metJ* carrying

egtABCDE from *M. smegmatis* (275 mg L⁻¹)¹¹ and was comparable to that of mushrooms (16 to 417 mg kg⁻¹ of wet weight)¹.

We next tried a fed-batch culture in a jar fermenter using the transformant strains. However, the transformants showed slow growth; we are now trying to clarify the reason for the growth inhibition. Recently, ERG production in engineered *Saccharomyces cerevisiae* was reported¹⁸. By engineering of ERG transporters and nitrogen metabolism together with optimization of medium composition, the authors obtained 598 mg L⁻¹ ERG in fed-batch culture in a jar fermenter. Application of such approaches, especially ERG transporter engineering, might relieve the growth inhibition in our strains.

ABBREVIATIONS USED

ERG, ergothioneine; HER, hercynine; γ GC, γ -glutamylcysteine; γ GC-HER, hercynyl- γ -glutamylcysteine sulfoxide; Cys-HER, hercynylcysteine sulfoxide; L-His, L-histidine; L-Cys, L-cysteine; SAM, S-adenosylmethionine; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

We thank James Allen, DPhil, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at xxx:

Supplementary Methods; SDS-PAGE analysis of Mb_EgtB and Mp_EgtB production (Figure S1); mass spectra of *in vitro* reaction products of L-Cys and recombinant EgtBs from *Methylobacterium brachiatum* and *M. pseudosasicola* (Figure S2); comparison of various EgtBs activities (Figure S3); effect of pH and temperature on Mp_EgtB activity (Figure S4); Michaelis-Menten plots of the Mp_EgtB for HER and L-Cys (Figure S5); Michaelis-Menten plots of the Mb_EgtB for HER and L-Cys (Figure S6); ¹H NMR spectrum of HER (Figure S7); ¹³C NMR spectrum of HER (Figure S8); identities of amino acid sequences of type I–V EgtBs (Table S1); and primers used in this study (Table S2) (PDF).

FUNDING

This work was supported in part by Grants-in-Aid for Research on Innovative Areas from MEXT, Japan (16H06452 to T.D.), Research Grant from Asahi Group Foundation (to T.D.), and

the Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry from MAFF, Japan (26027AB to I.O.).

NOTES

The authors declare no competing financial interest.

REFERENCES

1. Kalaras, M. D.; Richie, J. P.; Calcagnotto, A.; Beelman, R. B. Mushrooms: a rich source of the antioxidants ergothioneine and glutathione. *Food Chem.* **2017**, *233*, 429–433.
2. Genghof, D. S. Biosynthesis of ergothioneine and hercynine by fungi and *Actinomycetales*. *J. Bacteriol.* **1970**, *103*, 475–478.
3. Sheridan, K. J.; Lechner, B. E.; Keefe, G. O.; Keller, M. A.; Werner, E. R.; Lindner, H.; Jones, G. W.; Haas, H.; Doyle, S. Ergothioneine biosynthesis and functionality in the opportunistic fungal pathogen, *Aspergillus fumigatus*. *Sci. Rep.* **2016**, *6*, 35306.
4. Takusagawa, S.; Satoh, Y.; Ohtsu, I.; Dairi, T. Ergothioneine production with *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **2019**, *83*, 181–184.

- 197 5. Pluskal, T.; Ueno, M.; Yanagida, M. Genetic and metabolomic dissection of the ergothioneine
198 and selenoneine biosynthetic pathway in the fission yeast, *S. pombe*, and construction of an
199 overproduction system. *PLoS One* **2014**, *9*, e97774.
- 200 6. Pfeiffer, C.; Bauer, T.; Surek, B.; Schömig, E.; Gründemann, D. Cyanobacteria produce high
201 levels of ergothioneine. *Food Chem.* **2011**, *129*, 1766–1769.
- 202 7. Gründemann, D.; Harlfinger, S.; Golz, S.; Geerts, A.; Lazar, A.; Berkels, R.; Jung, N.; Rubbert,
203 A.; Schömig, E. Discovery of the ergothioneine transporter. *Proc. Natl. Acad. Sci. USA* **2005**,
204 *102*, 5256–5261.
- 205 8. Pfeiffer, C.; Bach, M.; Bauer, T.; Campos da Ponte, J.; Schömig, E.; Gründemann, D.
206 Knockout of the ergothioneine transporter ETT in zebrafish results in increased 8-oxoguanine
207 levels. *Free Radic. Biol. Med.* **2015**, *83*, 178–185.
- 208 9. Osawa, R.; Kamide, T.; Satoh, Y.; Kawano, Y.; Ohtsu, I.; Dairi, T. Heterologous and high
209 production of ergothioneine in *Escherichia coli*. *J. Agric. Food Chem.* **2018**, *66*, 1191–1196.
- 210 10. Seebeck, F. P. In vitro reconstitution of Mycobacterial ergothioneine biosynthesis. *J. Am.*
211 *Chem. Soc.* **2010**, *132*, 6632–6633.
- 212 11. Tanaka, N.; Kawano, Y.; Satoh, Y.; Dairi, T.; Ohtsu, I. Gram-scale fermentative production of
213 ergothioneine driven by overproduction of cysteine in *Escherichia coli*. *Sci. Rep.* **2019**, *9*, 1895.

- 214 12. Jones, G. W.; Doyle, S.; Fitzpatrick, D. A. The evolutionary history of the genes involved in
215 the biosynthesis of the antioxidant ergothioneine. *Gene* **2014**, *549*, 161–170.
- 216 13. Stampfli, A. R.; Goncharenko, K. V.; Meury, M.; Dubey, B. N.; Schirmer, T.; Seebeck, F. P.
217 An alternative active site architecture for O₂ activation in the ergothioneine biosynthetic EgtB
218 from *Chloracidobacterium thermophilum*. *J. Am. Chem. Soc.* **2019**, *141*, 5275–5285.
- 219 14. Fujitani, Y.; Alamgir, K. M.; Tani, A. Ergothioneine production using *Methylobacterium*
220 species, yeast, and fungi. *J. Biosci. Bioeng.* **2018**, *126*, 715–722.
- 221 15. Alamgir, K. M.; Masuda, S.; Fujitani, Y.; Fukuda, F.; Tani, A. Production of ergothioneine by
222 *Methylobacterium* species. *Front. Microbiol.* **2015**, *6*, 1185.
- 223 16. Goncharenko, K. V.; Vit, A.; Blankenfeldt, W.; Seebeck, F. P. Structure of the sulfoxide
224 synthase EgtB from the ergothioneine biosynthetic pathway. *Angew. Chem. Int. Ed.* **2015**, *54*,
225 2821–2824.
- 226 17. Yamada, S.; Awano, N.; Inubushi, K.; Maeda, E.; Nakamori, S.; Nishino, K.; Yamaguchi, A.;
227 Takagi, H., Effect of drug transporter genes on cysteine export and overproduction in
228 *Escherichia coli*. *Appl. Environ. Microbiol.* **2006**, *72*, 4735–4742.
- 229 18. van der Hoek, S. A.; Darbani, B.; Zugaj, K. E.; Prabhala, B. K.; Biron, M. B.; Randelovic, M.;
230 Medina, J. B.; Kell, D. B.; Borodina, I. Engineering the yeast *Saccharomyces cerevisiae* for
231 the production of L-(+)-ergothioneine. *Front. Bioeng. Biotechnol.* **2019**, *7*, 262.

232

233 **FIGURE CAPTIONS**

234 **Figure 1. Ergothioneine (ERG) biosynthesis pathway.**

235 The pathway reconstituted in this study is boxed. SAM, *S*-adenosylmethionine; SAH, *S*-
236 adenosylhomocysteine.

237

238 **Figure 2. Multiple sequence alignment of type I–V EgtBs.**

239 A partial sequence alignment of EgtBs is shown. The amino acid sequences of EgtBs were analyzed
240 using the Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The consecutive
241 Tyr residues at the catalytic site in type II enzymes are shown in red. Origins of proteins analyzed:
242 SpoEgt1, *Schizosaccharomyces pombe*; NcrEgt1, *Neurospora crassa*; AfuEgt1, *Aspergillus*
243 *fumigatus*; MaeEgtB, *Microcystis aeruginosa*; TelEgtB, *Thermosynechococcus elongatus*;
244 MsmEgtB, *Mycobacterium smegmatis*; MthEgtB, *Mycobacterium thermoresistibile*; Tm_EgtB,
245 *Tenacibaculum maritimum*; Ao_EgtB, *Aquabacterium olei*; Ll_EgtB, *Labilithrix luteola*; CthEgtB,
246 *Chloracidobacterium thermophilum*; Mb_EgtB, *Methylobacterium brachiatum*; Mp_EgtB,
247 *Methylobacterium pseudosasicola*.

248

249 **Figure 3. *In vitro* assay of EgtBs from *Methylobacterium brachiatum* and *M. pseudosasicola*.**

250 High-performance liquid chromatography traces at 250 nm of the reaction products formed with L-
251 Cys (A) or γ GC (B) as sulfur donors. (i) Standards of Cys-HER and γ GC-HER; (ii to iv) lysates
252 prepared from *E. coli* expressing no EgtB, Mb_EgtB, or Mp_EgtB. In panel B, Cys-HER formation
253 was confirmed because of contamination of L-Cys in the γ GC used for the *in vitro* assay.

254
255 **Figure 4. Culture profiles of *Escherichia coli* BW25113 expressing *egtDE* and *egtBs* from**
256 ***Methylobacterium* strains.**

257 *E. coli* carrying *egtDE* from *M. smegmatis*, and Mb_egtB (i) or Mp_egtB (ii) were cultured for 72
258 h at 30°C. The compounds detected in the supernatants of culture broths are presented as mean
259 values with standard deviations from three independent experiments.

260

Table 1. Bacterial strains used in this study.

Strain	Description	Source
<i>E. coli</i>		
XL1-Blue	<i>hsdR17, recA1, endA1, gyrA96, thi-1, supE44, relA1, lac[F', proAB, lacI^qZΔM15, Tn10(Tc^R)]</i>	Nippon Gene
BL21(DE3)	F ⁻ , <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3)	Merck
BW25113	<i>rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</i>	NIG
CH	L-Cys overproducing strain, BW25113 harboring pCys ^{HP} plasmid	11
CHΔ <i>metJ</i>	CH derivative, <i>metJ</i> disruptant	11
<i>Methylobacterium</i> <i>brachiatum</i>	DNA source of <i>Mb_egtB</i>	NITE
NBRC103629		
<i>Methylobacterium</i> <i>pseudosasicola</i>	DNA source of <i>Mp_egtB</i>	NITE
NBRC105203		
<i>Aquabacterium olei</i>	DNA source of <i>Ao_egtB</i>	NITE
NBRC 110486		
<i>Labilithrix luteola</i>	DNA source of <i>Ll_egtB</i>	NITE
NBRC 109946		
<i>Tenacibaculum</i> <i>maritimum</i>	DNA source of <i>Tm_egtB</i>	NITE
NBRC 110778		

NITE, National Institute of Technology and Evaluation.

NIG, National Institute of Genetics.

Table 2. Plasmids used in this study.

Plasmid	Description	Source
pQE1a-Red	protein production plasmid, <i>tac</i> promoter, ColE1 ori, Ap ^R	9
pQE1a_MbB	pQE1a-Red derivative, production of Mb_EgtB	this study
pQE1a_MpB	pQE1a-Red derivative, production of Mp_EgtB	this study
pQE1a_hMbB	pQE1a-MbB derivative, production of His-tagged Mb_EgtB	this study
pQE1a_hMpB	pQE1a-MpB derivative, production of His-tagged Mp_EgtB	this study
pQE1a_hAoB	pQE1a-Red derivative, production of His-tagged Ao_EgtB	this study
pQE1a_hLIB	pQE1a-Red derivative, production of His-tagged LI_EgtB	this study
pQE1a_hTmB	pQE1a-Red derivative, production of His-tagged Tm_EgtB	this study
pCF1s_MsD	production of EgtD from <i>M. smegmatis</i> , <i>tac</i> promoter, CloDF13 ori, Sm ^R	9
pCF1s_MsD/hMsE	pCF1s_MsD derivative, production of EgtD from <i>M. smegmatis</i> and His-tagged EgtE from <i>M. smegmatis</i>	this study
pAC1c_hMsE	production of His-tagged EgtE from <i>M. smegmatis</i> , <i>tac</i> promoter, p15A ori, Cm ^R	9
pCys ^{HP}	L-Cys overproduction, p15A ori, Tc ^R , feedback-inhibition insensitive mutant genes of <i>E. coli serA</i> and <i>cysE</i> , inner membrane L-Cys exporter gene of <i>E. coli ydeD</i>	11

Table 3. Comparison of ERG production^a

host cell	genes expressed	cultivation time (h)	OD	ERG (mg L ⁻¹)	HER (mg L ⁻¹)	reference
BW25113	<i>egtBCDE</i>	72	11.1 ± 0.8	24 ± 6	48 ± 24	9
BW25113	<i>Mb_egtB, egtDE</i>	72	10.3 ± 0.5	166 ± 16	19 ± 4	This study
BW25113	<i>Mp_egtB, egtDE</i>	72	8.7 ± 0.1	225 ± 5	34 ± 1	This study
CHΔ <i>metJ</i>	<i>Mb_egtB, egtDE</i>	192	17.8 ± 1.2	475 ± 109	ND ^b	This study
CHΔ <i>metJ</i>	<i>Mp_egtB, egtDE</i>	192	14.0 ± 1.4	657 ± 89	ND ^b	This study

^aData are presented as mean values with standard deviations.

^bND, not determined.

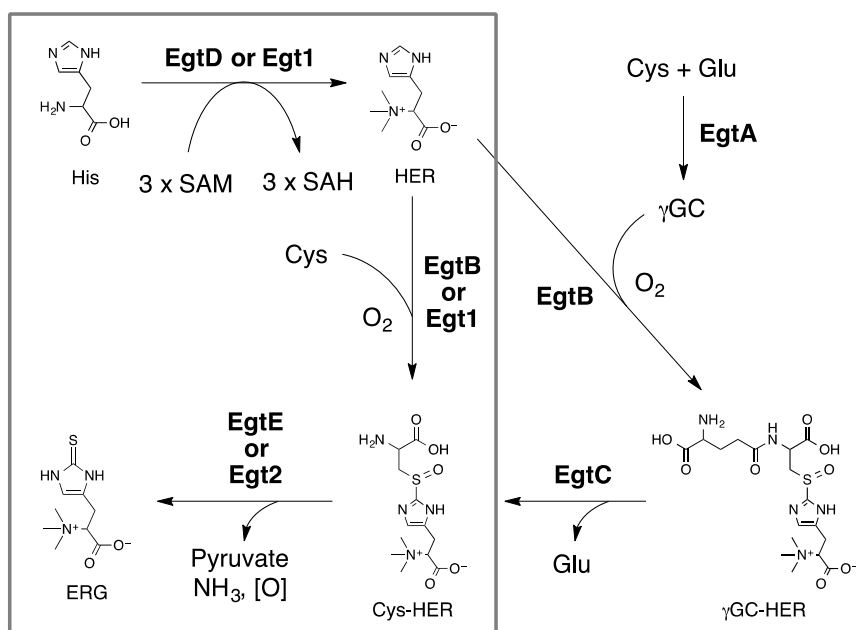


Figure 1. ERG biosynthetic pathway.

The pathway reconstituted in this study is boxed. SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

Type III	SpoEgt1	ANKKHFWEWFQRGIDPDIEDPSKCHWH-S	428
	NcrEgt1	SEPAHFCKIFERGIDPDVDNPELCHAH-S	458
	AfuEgt1	TEPRNYRQIFERGIDPDVDNPEHCHSH-S	437
Type IV	MaeEgtB	SPNPDYELLFGVGVDPAT----PEELNSA	103
Type V	TelEgtB	-FRERDRYW----YAADG-----RPKVE	95
Type I	MsmEgtB	MLAPEVDRLYDAFE-----HSRAS	84
	MthEgtB	LLEPAVEQLYDAFV-----HPRAS	89
Type II	Tm_EgtB	VYNTDFAYLFNSYYNNAG----KRVLRPN	90
	Ao_EgtB	PLRPGWHRLFNSYYEALG----PRHPRPE	116
	Ll_EgtB	PVDASYDYLFNSYYEAVG----KRHPRNK	88
	CthEgtB	PFHPRYDYIFNSYYEAVG----ARHPRPQ	105
	Mb_EgtB	IYDERLHYLFNSYYVAAG----PRQPRIQ	118
	Mp_EgtB	IYDERLHYLFNSYYVAAG----PRQPRIQ	118

Figure 2. Multiple alignment of type I – V EgtBs.

A part of multiple alignment of EgtBs is shown. The amino acid sequences of EgtBs were analyzed using Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The consecutive Tyr residues at the catalytic sites in type II enzymes are shown in red. SpoEgt1, *Schizosaccharomyces pombe*; NcrEgt1, *Neurospora crassa*; AfuEgt1, *Aspergillus fumigatus*; MaeEgtB, *Microcystis aeruginosa*; TelEgtB, *Thermosynechococcus elongatus*; MsmEgtB, *Mycobacterium smegmatis*; MthEgtB, *Mycobacterium thermoresistibile*; Tm_EgtB, *Tenacibaculum maritimum*; Ao_EgtB, *Aquabacterium olei*; Ll_EgtB, *Labilithrix luteola*; CthEgtB, *Chloracidobacterium thermophilum*; Mb_EgtB, *Methylobacterium brachiatum*; Mp_EgtB, *Methylobacterium pseudosasicola*.

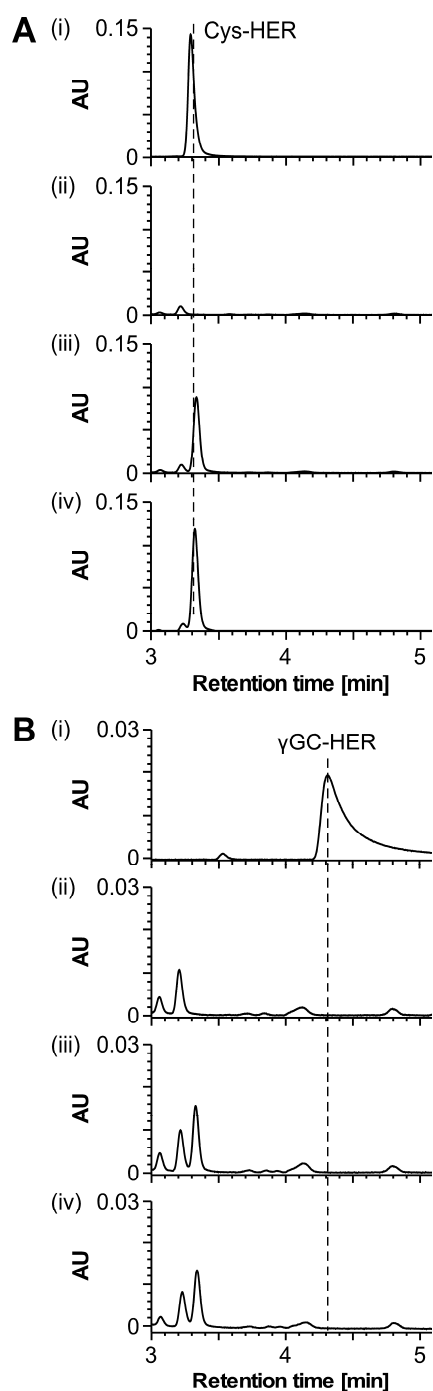


Figure 3. *In vitro* assay of EgtBs from *Methylobacterium brachiatum* and *M. pseudosasicola*.

High-performance liquid chromatography traces at 250 nm of the reaction products formed with L-Cys (A) or γ GC (B) as sulfur donors. (i) Standards of Cys-HER and γ GC-HER; (ii to iv) lysates prepared from *E. coli* expressing no EgtB, Mb_EgtB, or Mp_EgtB. In panel B, Cys-HER formation was confirmed because of contamination of L-Cys in the γ GC used for the *in vitro* assay.

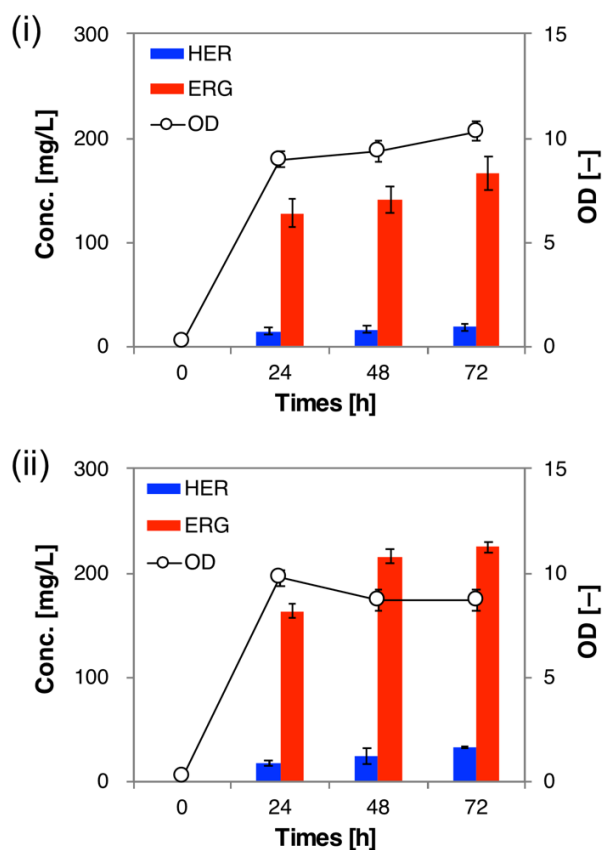


Figure 4. Culture profiles of *Escherichia coli* BW25113 expressing *egtDE* and *egtBs* from *Methylobacterium* strains.

E. coli carrying *egtDE* from *M. smegmatis*, and *Mb_egtB* (i) or *Mp_egtB* (ii) were cultured for 72 h at 30°C. The compounds detected in the supernatants of culture broths are presented as mean values with standard deviations from three independent experiments.