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1 ***Ergothioneine protects Streptomyces coelicolor A3(2) from oxidative stresses***

2

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12 Running title: Function of ergothioneine in a *Streptomyces* strain

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14 Key words: ergothioneine; mycothiol; *Streptomyces coelicolor*; biological function

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ABSTRACT

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1 Thiol compounds with low-molecular weight, such as glutathione, mycothiol (MSH),
2 bacillithiol, and ergothioneine (ERG), are known to protect microorganisms from oxidative
3 stresses. Mycobacteria and actinobacteria utilize both MSH and ERG. The biological functions of
4 MSH in mycobacteria have been extensively studied by genetic and biochemical studies, which
5 have suggested it has critical roles for detoxification in cells. In contrast, the biological functions
6 of ERG remain ambiguous because its biosynthetic genes were only recently identified in
7 *Mycobacterium avium*. In this study, we constructed mutants of *Streptomyces coelicolor* A3(2), in
8 which either the MSH or ERG biosynthetic gene was disrupted, and examined their phenotypes.
9 A *mshC* (*SCO1663*)-disruptant completely lost MSH productivity. In contrast, a disruptant of the
10 *egtA* gene (*SCO0910*) encoding γ -glutamyl-cysteine synthetase unexpectedly retained reduced
11 productivity of ERG, probably because of the use of L-cysteine instead of γ -glutamyl-cysteine.
12 Both disruptants showed delayed growth at the late logarithmic phase and were more susceptible
13 to hydrogen peroxide and cumene hydroperoxide than the parental strain. Interestingly, the
14 ERG-disruptant, which still kept reduced ERG productivity, was more susceptible. Furthermore,
15 the ERG-disruptant accumulated 5-fold more MSH than the parental strain. In contrast, the
16 amount of ERG was almost the same between the MSH-disruptant and the parental strain. Taken
17 together, our results suggest that ERG is more important than MSH in *S. coelicolor* A3(2).

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23 Microorganisms utilize several thiol compounds with low-molecular weight to maintain a
24 reducing environment (1). Glutathione is a representative compound found in gram-negative
25 microorganisms and glutathione reductases reduce oxidized glutathione for recycling.

1 Glutathione *S*-transferase and glutaredoxin also participate in the reduction of the oxidative state
2 using glutathione (1). In *E. coli*, glutathione and glutathione reductase are not essential for growth
3 but a glutathione-disruptant became more susceptible to chemical toxins and oxygen than the
4 parental strain. Moreover, disruption of all of the genes encoding glutathione reductase and
5 glutaredoxin was lethal (1).

6 In contrast to gram negative bacteria, gram-positive microorganisms do not use glutathione
7 to maintain reducing conditions but make use of mycothiol (MSH), bacillithiol, and
8 ergothioneine (ERG) compounds (1). MSH is widespread in actinobacteria. It was first identified
9 in a cell extract of a *Streptomyces* strain in 1993 (2) and then its structure was determined (3,4).
10 Recently, the biosynthetic pathway of MSH was established and the functions of four
11 biosynthetic genes were characterized (5,6). The biological function of MSH is essentially the
12 same as that of glutathione, and mycothione (oxidized form of MSH) reductase catalyzes the
13 reduction of the disulfide form into MSH (1). In *Mycobacterium tuberculosis*, MSH is essential
14 for in vitro growth (5). In contrast, MSH is not essential in *Mycobacterium smegmatis* but
15 MSH-disruptants showed increased sensitivity to oxidative stress, alkylating agents, and some
16 antibiotics (1). In *Firmicutes*, bacillithiol, the structure of which is similar to that of MSH, is
17 utilized (7). By taking advantage of the structural similarities, the biosynthetic genes of
18 bacillithiol were identified (8). A bacillithiol-disruptant of *Bacillus subtilis* showed the same
19 phenotype as glutathione- and MSH-disruptants (1).

20 In mycobacteria, actinobacteria, and certain fungi, ERG is also utilized together with MSH.
21 In the late 1960s, the building blocks of ERG were identified to be histidine, cysteine, and
22 methionine (9,10). Until recently, there were no reports on the biosynthetic genes and enzymes of
23 ERG. However, five genes, *egtA* to *E*, in *Mycobacterium avium* were recently confirmed to be
24 responsible for ERG biosynthesis (11). As for the biological function of ERG, a MSH-disrupted
25 *M. smegmatis* mutant was reported to accumulate 2 to 26-fold higher ERG levels (12,13),

1 suggesting that ERG has the same function as MSH. However, the detailed biological function of
2 ERG in bacterial cells is not well understood because it has only been a short period since the
3 biosynthetic genes were discovered. Recently, a MSH-disruptant, an ERG-disruptant, and a
4 disruptant of both were constructed in *M. smegmatis* and both single disruptants were shown to
5 be susceptible to peroxide (13). The double-disruptant was also significantly more susceptible to
6 peroxide than either of the single disruptants.

7 *Streptomyces* strains are industrially very important microorganisms because they produce a
8 variety of anti-bacterial compounds that can be candidates for antibiotic development. Recently,
9 different classes of antibiotics were shown to contribute to killing bacteria by generating
10 deleterious reactive oxygen species including hydrogen peroxide, in addition to their intrinsic
11 drug-target interactions (14). This result suggests that antibiotics induce redox alterations that
12 lead to cellular damage even in their producers. In this study, we used *Streptomyces coelicolor*
13 A3(2) as a model strain and constructed mutants, in which either the MSH or ERG biosynthetic
14 gene was disrupted, to investigate the biological functions of both of these thiol compounds. Both
15 the MSH-disrupted and ERG-disrupted *S. coelicolor* A3(2) were more susceptible to hydrogen
16 peroxide than the parental strain and showed different phenotypes from those of *M. smegmatis*
17 mutants. Furthermore, our results suggested ERG was more important than MSH in *S. coelicolor*
18 A3(2).

19

20

Materials and Methods

21

22 **Chemicals** ERG was obtained from Cosmo Bio Co. Ltd (Tokyo, Japan). Cumene hydroperoxide,
23 diamide, and monobromobimane (mBBR) were purchased from Sigma-Aldrich Japan (Tokyo,
24 Japan). All other chemicals were analytical grade.

25

1 **DNA isolation and manipulation** Plasmids from *E. coli* were prepared using a Qiagen plasmid
2 kit (QIAGEN Japan, Tokyo). All restriction enzymes, T4 DNA ligase, and calf intestinal alkaline
3 phosphatase were obtained from Toyobo (Osaka, Japan). Transformations of *E. coli* and *S.*
4 *coelicolor* A3(2) with plasmid DNA were performed by electroporation under standard conditions
5 using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research, Inc.,
6 San Diego, CA) and by the protoplast-polyethylene glycol assisted method (15). Other general
7 procedures were performed as described by Maniatis *et al.* (16).

8
9 **Preparation of SCO0910 recombinant enzyme** The SCO0910 gene was amplified by PCR
10 using gene-specific primers, P1 and P2 (Table S1). After subcloning and sequence confirmation,
11 1.3-kb fragments obtained by *Nde*I and *Hind*III digestion were ligated to the corresponding site of
12 pMAL-c5X (New England Biolabs Japan, Tokyo). The constructed plasmid was designated
13 pMAL-SCO0910. *E. coli* XL-1 blue harboring pMAL-c5X (control) and pMAL-SCO0910 were
14 separately grown in Luria-broth supplemented with 100 µg/mL ampicillin. The strain was grown
15 at 37°C until OD₆₀₀ reached 0.5 and then isopropyl β-D-thiogalactopyranoside (0.5 mM) was
16 added to the culture, followed by additional cultivation at 18°C for 18 h. After the cells were
17 harvested and washed once with chilled buffer A (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), they
18 were suspended in the same buffer and then disrupted by sonication. The debris was removed by
19 centrifugation, the supernatant was applied to amylose resin (New England Biolabs) and the
20 recombinant enzyme was purified according to the manufacturer's instructions. The eluent was
21 desalted and concentrated using an Amicon apparatus (Merck Japan, Tokyo, Japan). After the
22 purity of the recombinant enzyme was checked by SDS-PAGE, the enzyme was used for in vitro
23 assay.

24 The standard assay mixture for SCO0910 contained, in a final volume of 200 µL, 12.5 mM
25 of L-glutamate, 12.5 mM of L-cysteine, 12.5 mM of ATP, 12.5 mM of MgSO₄, 50 mM Tris-HCl

1 (pH 8.0), and a suitable amount of recombinant SCO0910. After the mixture was incubated at
2 37°C overnight, the products were analyzed by LC-ESI-MS (Waters ACQUITY UPLC equipped
3 with a SQ Detector2; Nihon Waters, Tokyo, Japan). The analytical conditions were as follows:
4 Merck SeQuant™ ZIC-HILIC PEEK HPLC column (2.1 × 150 mm, 5 μm); column temperature,
5 35°C; detection, positive mode; mobile phase, 20 mM ammonium acetate (pH 6.8):acetonitrile =
6 15:85 for 60 min; flow rate, 0.3 ml/min; cone voltage, 20 V.

7
8 **Disruption of the SCO0910 and SCO1663 genes** The *SCO0910* and *SCO1663* genes, which
9 encode EgtA (EC 6.3.2.2) and MshC (EC 6.3.1.13), respectively, were disrupted by
10 double-crossover homologous recombination. Two DNA fragments carrying the upstream and
11 downstream regions of each of the target genes (2 Kb) were amplified with a set of primers
12 (listed in Table S1 and Fig. S1) by PCR. After sequence confirmation, these two fragments were
13 inserted into the appropriate restriction sites of pUC18 in the same direction as in the genomic
14 region. DNA fragments carrying the thiostrepton resistance gene (Table S1 and Fig. S1) were
15 then inserted between the above-mentioned two fragments. The constructed plasmids were
16 digested with *ScaI* and used to transform *S. coelicolor* A3(2). Disruption of the genes in
17 thiostrepton resistant colonies was confirmed by PCR analysis (Fig. S1). Production of ERG and
18 MSH was confirmed by LC-ESI-MS analysis as described below.

19
20 **Growth of the disruptants** To examine the effects of ERG- or MSH-deficiency on growth, the
21 disruptants were cultivated with 3 ml of SK No. 2 medium (17) in a test tube for 3 days at 30°C.
22 An aliquot of the culture (0.5 mL) was inoculated into 50 ml of fresh SK No. 2 medium in a 200
23 ml Erlenmeyer flask and cultured for 50 h. Samples of the cell culture were collected over a time
24 course and growth was measured by dry cell weight (g/L).

25

1 **Analysis of productivities of thiol compounds** The disruptants and the parental strain were
2 cultivated in the same manner as described above. Mycelia were collected at late logarithmic
3 phase when the dry cell weight reached about 5 g/L. Since both of the disruptants grew slower
4 than the parental strain, the former and the latter were collected after 48 h and 36 h of cultivation,
5 respectively. After centrifugation, both the mycelia and the supernatant were used for
6 measurement of thiol compounds. For the analysis of intracellular compounds, the mycelia
7 collected from 20 mL of culture broth were washed with 10 ml of ultrapure water and then 200
8 mg of the collected cells were suspended in 500 μ L of buffer A containing 50% acetonitrile, 2
9 mM mBBr, and 20 mM Tris HCl (pH 8.0). The mycelia were disrupted with a sonicator (Branson
10 450D, Emerson CT, USA) and incubated at 60°C for 15 min to produce mBBr-derivatives (18).
11 After the reaction, 25 μ l of 5 M methanesulfonic acid solution was added and the supernatants
12 obtained by centrifugation were analyzed by LC-ESI-MS after appropriate dilution. For the
13 analysis of secreted compounds, the supernatant was filtrated and concentrated by free-drying.
14 The dried samples were dissolved with buffer A. The subsequent protocol was the same as that
15 for intracellular compounds. LC-ESI-MS analytical conditions were as follows: InertSustain C18
16 HP column (150 mm \times 2.1 mm ID, 3 μ m; GL Sciences Inc., Tokyo, Japan); column temperature,
17 40°C; detection, positive mode; mobile phase, 0.05% trifluoroacetic acid solution:methanol =
18 90:10 at 3 min, and a linear gradient to 15:85 for an additional 30 min; flow rate, 0.3 ml/min;
19 cone voltage, 40 V. The amounts of thiol compounds were normalized by cell growth (dry cell
20 weight). The productivities of the thiol compounds except for MSH were calculated with a
21 standard curve. MSH productivity was shown as relative values; the amount of MSH produced by
22 the parental strain was defined as 1 since MSH was not commercially available.

23
24 **Examination of sensitivity to peroxide** The sensitivity of the ERG- and MSH-disruptants
25 against peroxides was tested by a paper disk-agar diffusion assay, which is based on the

1 phenomenon that toxic compounds will diffuse from a paper disc into an agar medium containing
2 test microorganisms and form a growth-inhibitory zone. The two disruptants and the parental
3 strain (control) were cultivated in the same manner as described above. Molten soft agar
4 containing the test microorganisms was spread on ATCC5 plates (0.2% starch, 0.1% glucose,
5 0.1% Bacto yeast extract, 0.1% fish meat extract, 0.01% FeSO₄, 2% agar, pH 7.2). Paper disks
6 containing various concentrations of peroxides were put onto the lawn of cells. After incubation
7 for 2 days, the size of the halo was measured and the minimum inhibition concentration (MIC)
8 was determined.

9
10 **Examination of organic hydroperoxide resistance (Ohr) protein production** Induction of the
11 organic hydroperoxide resistance protein (SCO2396, SCO2986, and SCO7111) was examined by
12 SDS-PAGE analysis. Mycelia of the disruptants and the parental strain, which were cultivated by
13 the method described above, were harvested by centrifugation. After being suspended in 50 mM
14 of sodium-phosphate buffer (pH 8.0) containing 300 mM of sodium chloride, the cells were
15 disrupted by ultrasonic oscillation at 4°C for 5 min. Supernatants obtained by centrifugation of
16 the cell lysates were analyzed by SDS-PAGE on 15% gels. Protein concentration was determined
17 by a protein-dye standard assay (Bio-Rad) using bovine serum albumin as a standard.

18 19 20 **Results and Discussion**

21
22 **SCO0910 encodes γ -glutamyl-cysteine synthetase** Five genes, *egtA* to *egtE*, in *Mycobacterium*
23 *avium* were recently confirmed to be responsible for ERG biosynthesis (11) (Fig. 1). We searched
24 for orthologs of these genes in the genome database of *S. coelicolor* A3(2) and identified
25 *SCO0910* to *SCO0913* as genes corresponding to *egtA* to *egtD* in *M. avium*. *SCO1921*, which was

1 located outside of the gene cluster, was suggested to be an ortholog of *egtE*. To determine
2 whether these genes indeed encoded the ERG biosynthetic enzymes, the function of *SCO0910*,
3 which possibly encoded γ -glutamyl-cysteine synthetase (EgtA), was examined with a
4 recombinant enzyme. Maltose binding protein-fused recombinant SCO0910 (89.6 kDa) was
5 successfully expressed in a soluble form and the purified enzyme was subjected to SDS-PAGE
6 analysis to confirm its molecular size and purity (Fig. 2A).

7 The catalytic activity was then examined. The purified recombinant SCO0910 was incubated
8 with L-glutamate and L-cysteine in the presence of ATP, and the reaction product was analyzed by
9 LC-ESI-MS. As shown in Fig. 2B, the formation of γ -glutamyl-cysteine was specifically
10 detected.

11
12 **Construction of ERG- and MSH- disruptants** To investigate the biological functions of ERG
13 and MSH in *S. coelicolor* A3(2), we constructed two types of mutants, in which the respective
14 biosynthetic genes were disrupted by homologous recombination. In the ERG-disruptant, we
15 disrupted the *SCO0910* gene by homologous recombination since SCO0910 was confirmed to be
16 γ -glutamyl-cysteine synthetase as described above. A disruptant, in which the *SCO0910* gene was
17 replaced with the thiostrepton resistance gene, was constructed and the intended disruption was
18 confirmed by PCR (Fig. S1 and Table S1). After cultivation of the disruptant, we examined the
19 production of ERG. Since a significant portion of ERG was recently reported to be secreted into
20 the culture broth (13), both secreted and intracellular ERG were measured. Unexpectedly, the
21 disruptant still retained ERG productivity. Intracellular and secreted ERG levels were estimated
22 to be 20% and 85% of those of the parental strain (Table 1 and Fig. S2). In the biosynthesis of
23 ovoidthiol, which has similar structure and function to ERG (19), OvoA was shown to catalyze the
24 incorporation reaction of thiol with L-cysteine instead of γ -glutamyl-cysteine (20). In *S.*
25 *coelicolor* A3(2), EgtB corresponding to OvoA, might utilize L-cysteine in a similar manner to

1 OvoA.

2 In the case of the MSH-disruptant, *SCO4204*, *SCO1663*, and *SCO4151* encoding MshA, C,
3 and D, respectively, were previously confirmed to be essential for MSH biosynthesis (Fig. S3) by
4 a gene-disruption experiment (21). We therefore disrupted the *SCO1663* gene encoding *mshC* by
5 the same method as described above. We successfully obtained the intended disruptant, (Table S1
6 and Fig. S1), suggesting that MSH is not essential in *S. coelicolor* A3(2) similar to *M. smegmatis*
7 (6,12), and the absence of MSH production was confirmed by LC-ESI-MS as previously reported
8 (21) (Fig. S2).

9
10 **Effects of ERG or MSH-deficiency on growth** We first examined the effects of ERG- or
11 MSH-deficiency on growth. In the case of *M. smegmatis*, the $\Delta egtD$ and $\Delta mshA$ disruptants were
12 shown to grow normally (13) and slightly slower than the parental strain on agar plates (6),
13 respectively. The $\Delta SCO0910$ disruptant (ERG-disruptant), $\Delta SCO1663$ disruptant
14 (MSH-disruptant) and parental strain were cultivated in liquid medium for 50 h and cell growth
15 was measured by dry cell weight. No differences were observed until mid-logarithmic phase but
16 the growth of both disruptants was delayed at late-logarithmic phase (Fig. 3) in contrast to the *M.*
17 *smegmatis* mutant. These results suggested that both MSH and ERG are necessary for normal
18 growth at the late-logarithmic phase. In particular, the delayed growth of the ERG-disruptant,
19 which still produced a reduced amount of ERG, suggested that the ERG level for normal growth
20 at this stage was critical. ERG might play a critical role for differentiation at the late-logarithmic
21 phase such as secondary metabolite productions and spore formations.

22
23 **Productivity of thiol compounds in disruptants** Previously, $\Delta mshA$ disruptants of *M.*
24 *smegmatis* were shown to produce 2 to 26-fold more ERG than the parental strain, probably to
25 compensate for the loss of MSH (12,13). We therefore examined whether similar phenomena

1 were observed in the ERG- and MSH-disruptants of *S. coelicolor* A3(2). We measured the
2 amounts of ERG and MSH at the late-logarithmic phase since both compounds were suggested to
3 be required for normal growth at this stage as described above. The MSH-disruptant produced
4 almost the same amount of ERG as the parental strain, differing from the above-mentioned
5 $\Delta mshA$ disruptants of *M. smegmatis* (Table 1). On the other hand, the ERG-disruptant
6 accumulated 5-fold more MSH, despite the fact that the disruptant still retained ERG productivity.
7 This phenomenon was also quite different from that of a $\Delta ergD$ disruptant of *M. smegmatis*,
8 which accumulated the same amount of MSH as the parental strain (13).

9 We next measured the amounts of L-cysteine and γ -glutamyl-cysteine to examine whether
10 these compounds compensated for the loss of MSH and ERG. In the case of the MSH-disruptant,
11 both compounds increased approximately three times compared with the parental strain. On the
12 other hand, the ERG-disruptant, in which the γ -glutamyl-cysteine synthetase gene was disrupted
13 and no γ -glutamyl-cysteine was produced (Table 1), accumulated four times as much L-cysteine.
14 These results were different from those of the MSH-disruptant of *M. smegmatis* (*mshA*(G32D)
15 mutant), which accumulated a normal amount of L-cysteine (1).

16

17 **Production of organic hydroperoxide resistance protein (Ohr)** In *M. smegmatis*, a Δmsh
18 disruptant was shown to overproduce Ohr protein, which participates in resistance to cumene
19 hydroperoxide and isoniazid (12). Since *S. coelicolor* A3(2) was suggested to possess three
20 orthologs of Ohr (SCO2396, SCO2986, and SCO7111) by a Blast search, we examined whether
21 these proteins were also overproduced in the ERG- and MSH-disruptants. However, no obvious
22 proteins with the molecular size (15 kDa) of Ohr were overproduced in either disruptant, though
23 the patterns of some proteins were slightly different between them (Fig. S4).

24

1 **Sensitivity to various stresses** Both Δmsh and $\Delta egtD$ disruptants of *M. smegmatis* were reported
2 to have increased susceptibility to agents generating reactive oxygen species (1,12,13). To study
3 the biological functions of both compounds in *S. coelicolor*, A3(2), a paper disk-agar diffusion
4 assay was employed. We used hydrogen peroxide, cumene hydroperoxide, and diamide as agents
5 generating reactive oxygen species. As shown in Table 2, both the ERG- and MSH-disruptants
6 were more sensitive to hydrogen peroxide and cumene hydroperoxide than the parental strain.
7 The ERG-disruptant, which still produced a reduced level of ERG, was unexpectedly more
8 susceptible to both compounds, especially to hydrogen peroxide. The amounts of intracellular
9 ERG might be critical for the protection from hydrogen peroxide. Otherwise, the increased
10 amounts of L-cysteine in the ERG-disruptant (Table 1) might become toxic in the disruptants
11 since L-cysteine was known to show cytotoxicity at even low concentrations in *E. coli* (22).

12 In conclusion, we constructed disruptants of *Streptomyces coelicolor* A3(2), in which either
13 the MSH or ERG biosynthetic gene was disrupted, and examined their phenotypes to determine
14 the physiological roles of these thiol compounds. The *mshC* (*SCO1663*)-disruptant completely
15 lost MSH productivity. In contrast, the disruptant of the *egtA* gene (*SCO0910*) encoding
16 γ -glutamyl-cysteine synthetase unexpectedly kept reduced productivity of ERG. Both the ERG-
17 and MSH-disruptants showed delayed growth at late-logarithmic phase and were susceptible to
18 hydrogen peroxide and cumene hydroperoxide. These results suggested that both ERG and MSH
19 protect *S. coelicolor* A3(2) against oxidative stress. In particular, the ERG-disruptant, which still
20 retained reduced productivity of ERG, produced 5-fold more MSH and the MSH-disruptant
21 produced almost the same amount of ERG as the parental strain. Furthermore, the
22 ERG-disruptant was more sensitive to hydrogen peroxide than the MSH-disruptant. Together,
23 these results suggest that ERG and MSH play a critical role in *S. coelicolor* A3(2) and *M.*
24 *smegmatis*, respectively.

25 Supplementary data related to this article can be found at [http://](http://dx.doi.org/10.1016/j.jbiosc.2015.01.013)
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1

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5

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1 **TABLE 1.** Secreted and intracellular thiol compounds of the parental strain, ERG-disruptant,
 2 and MSH-disruptant.

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	WT		ERG- disruptant		MSH- disruptant	
	intracellular	secreted	intracellular	secreted	intracellular	secreted
6 ERG ^a	33 ± 24	180 ± 30	7 ± 2	140 ± 10	29 ± 1	120 ± 20
7 Cys ^a	140 ± 70	150 ± 10	590 ± 130	280 ± 30	450 ± 120	440 ± 30
8 γ-GC ^a	12 ± 2	ND	ND	ND	35 ± 10	ND
9 MSH ^b	1	ND	5.13	ND	ND	ND

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11 ^a; nM/DCW (g).

12 ^b; relative values; the amount of MSH produced by the parental strain was defined as 1.

13 ND, Not detected.

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1 **TABLE 2.** Minimum inhibition concentration (mM) of hydrogen peroxide, cumene
2 hydroperoxide (CuOOH), and diamide against the parental strain, ERG-disruptant, and
3 MSH-disruptant.

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	WT	ERG- disruptant	MSH- disruptant
6 H ₂ O ₂	13.8 ± 1.1	3.5 ± 0.5	10.1 ± 2.2
7 CuOOH	14.0 ± 1.4	6.2 ± 0.3	7.3 ± 0.6
8 Diamide	41.6 ± 3.0	31.6 ± 5.5	47.0 ± 4.4

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1 **FIGURE LEGENDS**

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3 **FIG. 1.** (A) Biosynthetic pathway of ERG and (B) structure of MSH.

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5 **FIG. 2.** In vitro assay of recombinant SCO0910 (EgtA). (A) Purified SCO0910 (EgtA) was
6 analyzed by SDS-PAGE. Lane 1, molecular mass markers; lane 2, purified maltose binding
7 protein-fused recombinant SCO0910 (89.6 kDa). (B) L-Glutamate and L-cysteine were
8 incubated in the presence of ATP without (upper) and with (lower) maltose binding
9 protein-fused recombinant SCO0910. The reaction product was analyzed by LC-ESI-MS. A
10 mass of 251, which correspond to $[M+H]^+$ of γ -glutamyl-cysteine, was selected.

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12 **FIG. 3.** Growth curves of the ERG-disruptant (circle), MSH-disruptant (square), and parental
13 strain (triangle). Growth was measured by dry cell weight.

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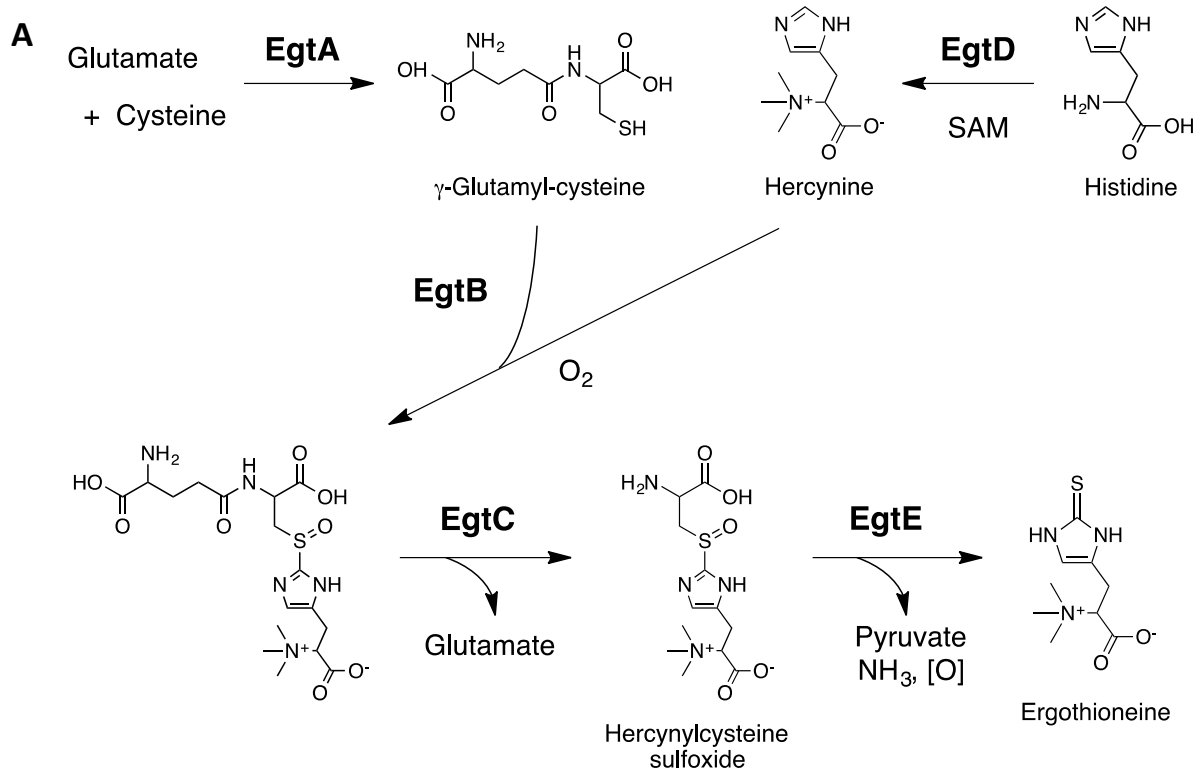
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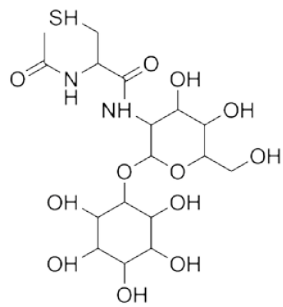
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1 **Figure 1**

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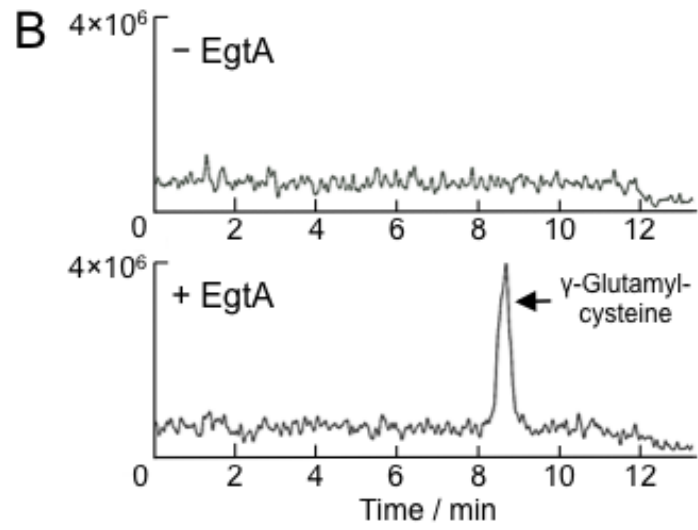
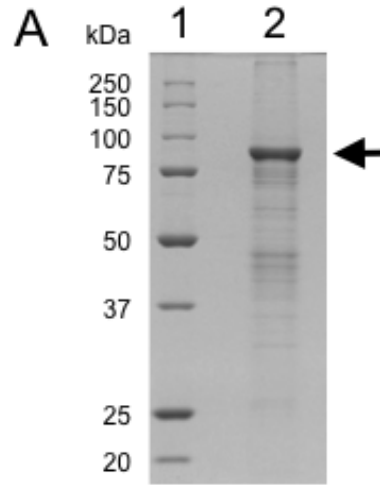
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1 **Figure 2**

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1 **Figure 3**

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