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

Shunsuke Nakajima¹, Yasuharu Satoh¹, Kentaro Yanashima², Tomomi Matsui¹, and Tohru Dairi¹*

¹ Graduate School of Engineering, Hokkaido University, Hokkaido 060-8628, Japan
² Kyowa Hakko Bio Co. Ltd., Chiyoda-ku, Tokyo 100-8185, Japan

*Corresponding author, Graduate School of Engineering, Hokkaido University, Hokkaido 060-8628, Japan. Tel. +81-11-706-7815; Fax. +81-11-706-7118; E-mail: dairi@eng.hokudai.ac.jp

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

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

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

Microorganisms utilize several thiol compounds with low-molecular weight to maintain a reducing environment (1). Glutathione is a representative compound found in gram-negative microorganisms and glutathione reductases reduce oxidized glutathione for recycling.
Glutathione S-transferase and glutaredoxin also participate in the reduction of the oxidative state using glutathione (1). In *E. coli*, glutathione and glutathione reductase are not essential for growth but a glutathione-disruptant became more susceptible to chemical toxins and oxygen than the parental strain. Moreover, disruption of all of the genes encoding glutathione reductase and glutaredoxin was lethal (1).

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

In mycobacteria, actinobacteria, and certain fungi, ERG is also utilized together with MSH. In the late 1960s, the building blocks of ERG were identified to be histidine, cysteine, and methionine (9,10). Until recently, there were no reports on the biosynthetic genes and enzymes of ERG. However, five genes, *egtA* to *E*, in *Mycobacterium avium* were recently confirmed to be responsible for ERG biosynthesis (11). As for the biological function of ERG, a MSH-disrupted *M. smegmatis* mutant was reported to accumulate 2 to 26-fold higher ERG levels (12,13),
suggesting that ERG has the same function as MSH. However, the detailed biological function of ERG in bacterial cells is not well understood because it has only been a short period since the biosynthetic genes were discovered. Recently, a MSH-disruptant, an ERG-disruptant, and a disruptant of both were constructed in *M. smegmatis* and both single disruptants were shown to be susceptible to peroxide (13). The double-disruptant was also significantly more susceptible to peroxide than either of the single disruptants.

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

**Materials and Methods**

**Chemicals** ERG was obtained from Cosmo Bio Co. Ltd (Tokyo, Japan). Cumene hydroperoxide, diamide, and monobromobimane (mBBr) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). All other chemicals were analytical grade.
**DNA isolation and manipulation** Plasmids from *E. coli* were prepared using a Qiagen plasmid kit (QIAGEN Japan, Tokyo). All restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo (Osaka, Japan). Transformations of *E. coli* and *S. coelicolor* A3(2) with plasmid DNA were performed by electroporation under standard conditions using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research, Inc., San Diego, CA) and by the protoplast-polyethylene glycol assisted method (15). Other general procedures were performed as described by Maniatis *et al.* (16).

**Preparation of SCO0910 recombinant enzyme** The SCO0910 gene was amplified by PCR using gene-specific primers, P1 and P2 (Table S1). After subcloning and sequence confirmation, 1.3-kb fragments obtained by *Nde*I and *Hind*III digestion were ligated to the corresponding site of pMAL-c5X (New England Biolabs Japan, Tokyo). The constructed plasmid was designated pMAL-SCO0910. *E. coli* XL-1 blue harboring pMAL-c5X (control) and pMAL-SCO0910 were separately grown in Luria-broth supplemented with 100 µg/mL ampicillin. The strain was grown at 37°C until OD_{600} reached 0.5 and then isopropyl β-D-thiogalactopyranoside (0.5 mM) was added to the culture, followed by additional cultivation at 18°C for 18 h. After the cells were harvested and washed once with chilled buffer A (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), they were suspended in the same buffer and then disrupted by sonication. The debris was removed by centrifugation, the supernatant was applied to amylose resin (New England Biolabs) and the recombinant enzyme was purified according to the manufacturer’s instructions. The eluent was desalted and concentrated using an Amicon apparatus (Merck Japan, Tokyo, Japan). After the purity of the recombinant enzyme was checked by SDS-PAGE, the enzyme was used for in vitro assay.

The standard assay mixture for SCO0910 contained, in a final volume of 200 µL, 12.5 mM of L-glutamate, 12.5 mM of L-cysteine, 12.5 mM of ATP, 12.5 mM of MgSO_{4}, 50 mM Tris–HCl...
(pH 8.0), and a suitable amount of recombinant SCO0910. After the mixture was incubated at 37°C overnight, the products were analyzed by LC-ESI-MS (Waters ACQUITY UPLC equipped with a SQ Detector2; Nihon Waters, Tokyo, Japan). The analytical conditions were as follows: Merck SeQuant™ ZIC-HILIC PEEK HPLC column (2.1 × 150 mm, 5 µm); column temperature, 35°C; detection, positive mode; mobile phase, 20 mM ammonium acetate (pH 6.8):acetonitrile = 15:85 for 60 min; flow rate, 0.3 ml/min; cone voltage, 20 V.

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

Growth of the disruptants To examine the effects of ERG- or MSH-deficiency on growth, the disruptants were cultivated with 3 ml of SK No. 2 medium (17) in a test tube for 3 days at 30°C. An aliquot of the culture (0.5 mL) was inoculated into 50 ml of fresh SK No. 2 medium in a 200 ml Erlenmeyer flask and cultured for 50 h. Samples of the cell culture were collected over a time course and growth was measured by dry cell weight (g/L).
Analysis of productivities of thiol compounds The disruptants and the parental strain were cultivated in the same manner as described above. Mycelia were collected at late logarithmic phase when the dry cell weight reached about 5 g/L. Since both of the disruptants grew slower than the parental strain, the former and the latter were collected after 48 h and 36 h of cultivation, respectively. After centrifugation, both the mycelia and the supernatant were used for measurement of thiol compounds. For the analysis of intracellular compounds, the mycelia collected from 20 mL of culture broth were washed with 10 ml of ultrapure water and then 200 mg of the collected cells were suspended in 500 µL of buffer A containing 50% acetonitrile, 2 mM mBBr, and 20 mM Tris HCl (pH 8.0). The mycelia were disrupted with a sonicator (Branson 450D, Emerson CT, USA) and incubated at 60°C for 15 min to produce mBBr-derivatives (18). After the reaction, 25 µl of 5 M methanesulfonic acid solution was added and the supernatants obtained by centrifugation were analyzed by LC-ESI-MS after appropriate dilution. For the analysis of secreted compounds, the supernatant was filtrated and concentrated by free-drying. The dried samples were dissolved with buffer A. The subsequent protocol was the same as that for intracellular compounds. LC-ESI-MS analytical conditions were as follows: InertSustain C18 HP column (150 mm × 2.1 mm ID, 3 µm; GL Sciences Inc., Tokyo, Japan); column temperature, 40°C; detection, positive mode; mobile phase, 0.05% trifluoroacetic acid solution:methanol = 90:10 at 3 min, and a linear gradient to 15:85 for an additional 30 min; flow rate, 0.3 ml/min; cone voltage, 40 V. The amounts of thiol compounds were normalized by cell growth (dry cell weight). The productivities of the thiol compounds except for MSH were calculated with a standard curve. MSH productivity was shown as relative values; the amount of MSH produced by the parental strain was defined as 1 since MSH was not commercially available.

Examination of sensitivity to peroxide The sensitivity of the ERG- and MSH-disruptants against peroxides was tested by a paper disk-agar diffusion assay, which is based on the
phenomenon that toxic compounds will diffuse from a paper disc into an agar medium containing test microorganisms and form a growth-inhibitory zone. The two disruptants and the parental strain (control) were cultivated in the same manner as described above. Molten soft agar containing the test microorganisms was spread on ATCC5 plates (0.2% starch, 0.1% glucose, 0.1% Bacto yeast extract, 0.1% fish meat extract, 0.01% FeSO₄, 2% agar, pH 7.2). Paper disks containing various concentrations of peroxides were put onto the lawn of cells. After incubation for 2 days, the size of the halo was measured and the minimum inhibition concentration (MIC) was determined.

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

**Results and Discussion**

**SCO0910 encodes γ-glutamyl-cysteine synthetase** Five genes, egtA to egtE, in *Mycobacterium avium* were recently confirmed to be responsible for ERG biosynthesis (11) (Fig. 1). We searched for orthologs of these genes in the genome database of *S. coelicolor* A3(2) and identified SCO0910 to SCO0913 as genes corresponding to egtA to egtD in *M. avium*. SCO1921, which was
located outside of the gene cluster, was suggested to be an ortholog of \textit{egtE}. To determine whether these genes indeed encoded the ERG biosynthetic enzymes, the function of \textit{SCO0910}, which possibly encoded \(\gamma\)-glutamyl-cysteine synthetase (EgtA), was examined with a recombinant enzyme. Maltose binding protein-fused recombinant \textit{SCO0910} (89.6 kDa) was successfully expressed in a soluble form and the purified enzyme was subjected to SDS-PAGE analysis to confirm its molecular size and purity (Fig. 2A).

The catalytic activity was then examined. The purified recombinant \textit{SCO0910} was incubated with L-glutamate and L-cysteine in the presence of ATP, and the reaction product was analyzed by LC-ESI-MS. As shown in Fig. 2B, the formation of \(\gamma\)-glutamyl-cysteine was specifically detected.

\textbf{Construction of ERG- and MSH- disruptants} To investigate the biological functions of ERG and MSH in \textit{S. coelicolor} A3(2), we constructed two types of mutants, in which the respective biosynthetic genes were disrupted by homologous recombination. In the ERG-disruptant, we disrupted the \textit{SCO0910} gene by homologous recombination since \textit{SCO0910} was confirmed to be \(\gamma\)-glutamyl-cysteine synthetase as described above. A disruptant, in which the \textit{SCO0910} gene was replaced with the thiostrepton resistance gene, was constructed and the intended disruption was confirmed by PCR (Fig. S1 and Table S1). After cultivation of the disruptant, we examined the production of ERG. Since a significant portion of ERG was recently reported to be secreted into the culture broth (13), both secreted and intracellular ERG were measured. Unexpectedly, the disruptant still retained ERG productivity. Intracellular and secreted ERG levels were estimated to be 20\% and 85\% of those of the parental strain (Table 1 and Fig. S2). In the biosynthesis of ovothiol, which has similar structure and function to ERG (19), OvoA was shown to catalyze the incorporation reaction of thiol with L-cysteine instead of \(\gamma\)-glutamyl-cysteine (20). In \textit{S. coelicolor} A3(2), EgtB corresponding to OvoA, might utilize L-cysteine in a similar manner to
In the case of the MSH-disruptant, SCO4204, SCO1663, and SCO4151 encoding MshA, C, and D, respectively, were previously confirmed to be essential for MSH biosynthesis (Fig. S3) by a gene-disruption experiment (21). We therefore disrupted the SCO1663 gene encoding mshC by the same method as described above. We successfully obtained the intended disruptant, (Table S1 and Fig. S1), suggesting that MSH is not essential in S. coelicolor A3(2) similar to M. smegmatis (6,12), and the absence of MSH production was confirmed by LC-ESI-MS as previously reported (21) (Fig. S2).

Effects of ERG or MSH-deficiency on growth

We first examined the effects of ERG- or MSH-deficiency on growth. In the case of M. smegmatis, the ΔegtD and ΔmshA disruptants were shown to grow normally (13) and slightly slower than the parental strain on agar plates (6), respectively. The ΔSCO0910 disruptant (ERG-disruptant), ΔSCO1663 disruptant (MSH-disruptant) and parental strain were cultivated in liquid medium for 50 h and cell growth was measured by dry cell weight. No differences were observed until mid-logarithmic phase but the growth of both disruptants was delayed at late-logarithmic phase (Fig. 3) in contrast to the M. smegmatis mutant. These results suggested that both MSH and ERG are necessary for normal growth at the late-logarithmic phase. In particular, the delayed growth of the ERG-disruptant, which still produced a reduced amount of ERG, suggested that the ERG level for normal growth at this stage was critical. ERG might play a critical role for differentiation at the late-logarithmic phase such as secondary metabolite productions and spore formations.

Productivity of thiol compounds in disruptants

Previously, ΔmshA disruptants of M. smegmatis were shown to produce 2 to 26-fold more ERG than the parental strain, probably to compensate for the loss of MSH (12,13). We therefore examined whether similar phenomena
were observed in the ERG- and MSH-disruptants of *S. coelicolor* A3(2). We measured the amounts of ERG and MSH at the late-logarithmic phase since both compounds were suggested to be required for normal growth at this stage as described above. The MSH-disruptant produced almost the same amount of ERG as the parental strain, differing from the above-mentioned Δ*mshA* disruptants of *M. smegmatis* (Table 1). On the other hand, the ERG-disruptant accumulated 5-fold more MSH, despite the fact that the disruptant still retained ERG productivity. This phenomenon was also quite different from that of a Δ*ergD* disruptant of *M. smegmatis*, which accumulated the same amount of MSH as the parental strain (13).

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

Production of organic hydroperoxide resistance protein (Ohr) In *M. smegmatis*, a Δ*msh* disruptant was shown to overproduce Ohr protein, which participates in resistance to cumene hydroperoxide and isoniazid (12). Since *S. coelicolor* A3(2) was suggested to possess three orthologs of Ohr (SCO2396, SCO2986, and SCO7111) by a Blast search, we examined whether these proteins were also overproduced in the ERG- and MSH-disruptants. However, no obvious proteins with the molecular size (15 kDa) of Ohr were overproduced in either disruptant, though the patterns of some proteins were slightly different between them (Fig. S4).
Sensitivity to various stresses Both Δmsh and ΔegtD disruptants of *M. smegmatis* were reported to have increased susceptibility to agents generating reactive oxygen species (1,12,13). To study the biological functions of both compounds in *S. coelicolor* A3(2), a paper disk-agar diffusion assay was employed. We used hydrogen peroxide, cumene hydroperoxide, and diamide as agents generating reactive oxygen species. As shown in Table 2, both the ERG- and MSH-disruptants were more sensitive to hydrogen peroxide and cumene hydroperoxide than the parental strain. The ERG-disruptant, which still produced a reduced level of ERG, was unexpectedly more susceptible to both compounds, especially to hydrogen peroxide. The amounts of intracellular ERG might be critical for the protection from hydrogen peroxide. Otherwise, the increased amounts of L-cysteine in the ERG-disruptant (Table 1) might become toxic in the disruptants since L-cysteine was known to show cytotoxicity at even low concentrations in *E. coli* (22).

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

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2015.01.013.
Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (24651235) to T. Dairi from the Japan Society for the Promotion of Science (JSPS).
References


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

<table>
<thead>
<tr>
<th></th>
<th>WT intracellular</th>
<th>WT secreted</th>
<th>ERG-disruptant intracellular</th>
<th>ERG-disruptant secreted</th>
<th>MSH-disruptant intracellular</th>
<th>MSH-disruptant secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERGa</td>
<td>33 ± 24</td>
<td>180 ± 30</td>
<td>7 ± 2</td>
<td>140 ± 10</td>
<td>29 ± 1</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>Cysa</td>
<td>140 ± 70</td>
<td>150 ± 10</td>
<td>590 ± 130</td>
<td>280 ± 30</td>
<td>450 ± 120</td>
<td>440 ± 30</td>
</tr>
<tr>
<td>γ-GCa</td>
<td>12 ± 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>35 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td>MSHb</td>
<td>1</td>
<td>ND</td>
<td>5.13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a; nM/DCW (g).

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

ND, Not detected.
**TABLE 2.** Minimum inhibition concentration (mM) of hydrogen peroxide, cumene hydroperoxide (CuOOH), and diamide against the parental strain, ERG-disruptant, and MSH-disruptant.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ERG-disruptant</th>
<th>MSH-disruptant</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>13.8 ± 1.1</td>
<td>3.5 ± 0.5</td>
<td>10.1 ± 2.2</td>
</tr>
<tr>
<td>CuOOH</td>
<td>14.0 ± 1.4</td>
<td>6.2 ± 0.3</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>Diamide</td>
<td>41.6 ± 3.0</td>
<td>31.6 ± 5.5</td>
<td>47.0 ± 4.4</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

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

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

FIG. 3. Growth curves of the ERG-disruptant (circle), MSH-disruptant (square), and parental strain (triangle). Growth was measured by dry cell weight.
**Figure 1**

A) Glutamate + Cysteine $\rightarrow$ γ-Glutamyl-cysteine

- **EgtA**
  - Oxidizes Glutamate
  - Catalyzes the formation of γ-Glutamyl-cysteine

- **O2**
  - Required for the oxidation step

- **EgtB**
  - Converts γ-Glutamyl-cysteine to Hercynine

- **EgtC**
  - Forms Hercynylcysteine sulfoxide from Glutamate

- **EgtD**
  - Cycles Hercynine to Histidine

- **SAM**
  - S-Adenosylmethionine

B) Hercynylcysteine sulfoxide
Figure 2

A

kDa
250
150
100
75
50
37
25
20

B

$4 \times 10^6$

$4 \times 10^6$

- EgtA

+ EgtA

γ-Glutamyl-cysteine

Time / min
Figure 3