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Effects of genetic disruption in thioredoxin and glutathione systems on selenium nanoparticle formation, selenite sensitivity, and selenoprotein biosynthesis in *Escherichia coli*

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Abstract

Escherichia coli uses selenite as a nutritional selenium source for the synthesis of selenoproteins, and excess selenite is converted to elemental selenium nanoparticles (SeNPs) through a detoxification process. The reduction of selenite is thought to be facilitated by two major redox systems: the thioredoxin (Trx) system and glutathione (GSH) system. However, the extent to which these redox systems are involved in selenoprotein synthesis and SeNP formation remains unclear. In this study, we investigated the effects of gene disruption in the Trx (*trxA*, *trxB*, and *trxC*) and GSH (*gshB* and *gor*) systems on SeNP formation, selenite sensitivity, and selenoprotein synthesis in *E. coli*. We found that the disruption of a single gene in either the Trx or GSH system did not drastically affect SeNP formation via selenite reduction in the presence of 1 mM selenite. However, *trxB*, *gshB*, and *gor* were observed to be important for the tolerance of the bacterium to > 5 mM selenite. The Δ*trxA* and Δ*trxB* strains exhibited lower activity of the selenoprotein formate dehydrogenase as compared to the wild-type strain, suggesting that *trxA* and *trxB* are important for selenoprotein biosynthesis. Selenite detoxification via SeNP formation involves both the Trx and GSH systems, but selenoprotein biosynthesis specifically depends on the Trx system.

Keywords: selenite reduction, selenium nanoparticles, selenoprotein biosynthesis, *Escherichia coli*, thioredoxin, glutathione

Statements about COI: The authors have no conflicts of interest associated with this manuscript to declare.

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Introduction

Selenium is an essential trace element found in many organisms [1-3]. It is primarily incorporated into selenoproteins as the 21st amino acid, selenocysteine (Sec) [4]. Selenoproteins include well-known examples such as formate dehydrogenase (FDH) and glycine reductase in bacteria as well as mammalian selenoprotein P and thioredoxin reductase [5, 6]. Although selenium plays a crucial role in biological processes, excess selenium can be toxic [1, 2, 7]. Several bacteria can reduce toxic selenium oxyanions, such as selenate (+VI) and selenite (+IV) [8]. This reduction is a part of their respiratory and detoxification mechanisms [9-11], leading to the formation of less toxic, insoluble elemental

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selenium nanoparticles (SeNPs) [9] or their conversion into volatile methylated selenium species [12, 13].

The biological conversion of selenite remains a central theme in selenium oxyanion metabolism because selenite is commonly employed as a nutritional supplement in bacterial and mammalian cell cultures. The reduction of selenite in bacteria is related to at least three processes: assimilative reduction for incorporating selenium into cellular components, such as Sec in selenoproteins or selenouridine in tRNAs; dissimilative reduction in respiration; and detoxification [8]. In various bacterial species, diverse redox molecules and enzymes, such as glutathione [14, 15], thioredoxin [16], fumarate reductase [17], nitrate reductase [18], nitrite reductase [19, 20], and sulfite reductase [21], have been identified as participants in the reduction of selenite.

In the extensively investigated bacterium *Escherichia coli* K-12, the reduction of selenite to selenide is postulated to occur within the cytoplasm. This selenide is then utilized in the assimilation pathway for the synthesis of the selenoprotein formate dehydrogenase (FDH) [22]. Conversely, excess selenide is converted into elemental selenium (Se^0) and accumulates as extracellular SeNPs in the detoxification pathway [23, 24]. The reduction of selenite in *E. coli* is hypothesized to be facilitated by two major redox systems: the thioredoxin (Trx) system and the glutathione (GSH) system [14]. The Trx system comprises thioredoxin 1 (Trx1) encoded by the *trxA* gene, thioredoxin reductase encoded by *trxB*, and NADPH [25]. Trx1 is a small, ubiquitous protein with two conserved cysteine residues that catalyze numerous redox reactions through the reversible oxidation of its active site dithiol to a disulfide. Oxidized thioredoxin is subsequently be reduced by the flavoenzyme thioredoxin reductase with NADPH. *E. coli* has an additional thioredoxin variant, thioredoxin 2 (Trx2), which is encoded by the *trxC* gene and characterized by an extended N-terminus and thermosensitivity [26]. The Trx system is present in all bacteria and functions in a wide variety of cellular processes [25, 27]. In contrast, the GSH system consists of GSH produced by GSH synthase, encoded by the *gshB* gene, GSH reductase, encoded by the *gor* gene, and NADPH [28]. GSH is the major low-molecular-weight thiol in *E. coli*, and it protects cells not only as an antioxidant, but also as a detoxifying molecule against reactive species and electrophiles. Oxidized GSH is reduced by GSH reductase with NADPH. Although GSH is abundant in many organisms and serves as a prominent thiol and antioxidant, it is scarce in most Gram-positive bacteria [29]. However, the extent to which these reducing systems are involved in the assimilation and detoxification-reduction pathways of selenite is not well understood. In this study, we investigated the effects of single-gene deletions in the Trx or GSH system of *E. coli* on SeNP formation, selenite sensitivity, and selenoprotein biosynthesis.

Materials and methods

Bacterial strains and culture conditions

E. coli K-12 BW25113 (a wild-type strain) and its single-gene mutant strains JW585-KC6 (Δ*trxA*), JW0871-KC (Δ*trxB*), JW2566-KC (Δ*trxC*), JW2914-KC (Δ*gshB*), and JW3467-KC (Δ*gor*) were obtained from the National BioResource Project (National Institute of Genetics, Japan). *E. coli* cells were pre-cultured overnight at 37°C in Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L sodium chloride) and then inoculated at an optical density at 600 nm (OD_{600}) = 0.01 into 5 mL of tryptic soy broth (TSB) medium (17 g/L pancreatic digest of casein, 3 g/L papaic digest of soybean, 2.5 g/L dextrose, 5 g/L sodium chloride, 2.5 g/L dipotassium phosphate) containing various concentrations of sodium selenite, followed by culturing at 37°C under aerobic conditions with shaking at 220 rpm. Kanamycin was added at a concentration of 50 mg/L to the pre-culture of the mutant strains.

Bacterial growth measurement

After 6, 24, and 48 h of culture under the conditions described above, the pellets containing bacterial cells and SeNPs were collected by centrifugation at 11,000 × *g* for 2 min. To remove SeNPs outside of bacterial cells, the pellets were treated with 100 mM DTT in 20 mM phosphate-buffered saline PBS (pH 7.5). The OD_{600} of bacterial cells was measured after the red color due to SeNPs disappeared.

Determination of selenite by hydride generation-atomic fluorescence spectrometry

Selenite in the bacterial culture supernatant was determined using a hydride-generation atomic fluorescence spectrometer (HG-AFS) (Millennium Excalibur, PSA, Orpington, UK) equipped with a selenium PS analytical lamp (P849SF Photoron Pty Ltd., Victoria, Australia), as previously described [30], after centrifugation of the culture supernatant sample at 15,000 × *g* for 15 min. The analytical conditions for HG-AFS were as follows: injection volume, 100 μL; acid carrier, 0.5% (w/v) KBr in 6 M

HCl; and reductant, 0.7% (w/v) $NaBH_4$ in 0.1 M NaOH.

Quantification and statistical analysis

All quantification experiments were performed in three replicates and the results are shown as mean ± standard deviation (SD). An unpaired *t*-test (Student's *t* test) is appropriate and was used for such comparisons. Dunnett test was applied to experiments with three or more groups. The statistical significance of differences between experimental groups was determined with R software. A *P* value of less than 0.01 was considered statistically significant.

Selenite sensitivity assay

E. coli cells were streaked onto TSB agar plates containing 0, 1, 5, 10, or 20 mM sodium selenite and incubated at 37°C for 48 h.

FDH assay on a solid agar medium

FDH was assayed using the benzyl viologen agar overlay method [31]. *E. coli* cells were anaerobically cultured overnight on solid LB medium containing 0.5% glucose at 37°C. The medium was then overlaid with 0.75% agar containing 1.0 mg mL^{-1} benzyl viologen, 3.4 mg mL^{-1} KH_2PO_4, and 17 mg mL^{-1} sodium formate.

Results and discussion

Effects of mutations in the Trx and GSH systems on SeNPs formation via selenite reduction

To assess the involvement of the Trx and GSH systems in selenite reduction, *E. coli* strains with deletions in thioredoxin-related genes (*trxA*, *trxB*, and *trxC*) or GSH-related genes (*gshB* and *gor*) were cultured in the presence or absence of 1 mM selenite. This selenite concentration was employed according to the previous studies [24, 32]. The reduction of selenite was assayed based on the development of a red color, attributed to the excitation of surface plasmon vibrations in the resulting SeNPs. As shown in **Figure 1**, all of the strains reduced selenite to produce a red color (i.e., SeNPs) after 24-h cultivation. On the other hand, the growth of each bacterial strain was significantly inhibited by the addition of 1 mM selenite, in contrast to growth in the absence of selenite (**Figure 2 (a)**). In all strains except Δ*gshB*, more than 96% of the selenite was removed from the medium at 24 h, while for the Δ*gshB* strain, the reduction was 66% at 24 h but reached 99% at 48 h (**Figure 2 (b)**). The selenite reduction efficiency, defined as the reduced selenite concentration normalized by the number of cells (OD_{600}), of Δ*gshB* at 24 h was slightly but significantly lower than that of the wild-type (WT) strain (**Figure 2 (c)**). Nevertheless, all strains were able to almost completely reduce selenite after 48 h (**Figure 2 (b)**). These results show that disruption of a single gene in either the Trx or GSH system did not drastically affect SeNP formation via selenite reduction by *E. coli* in the presence of 1 mM selenite.

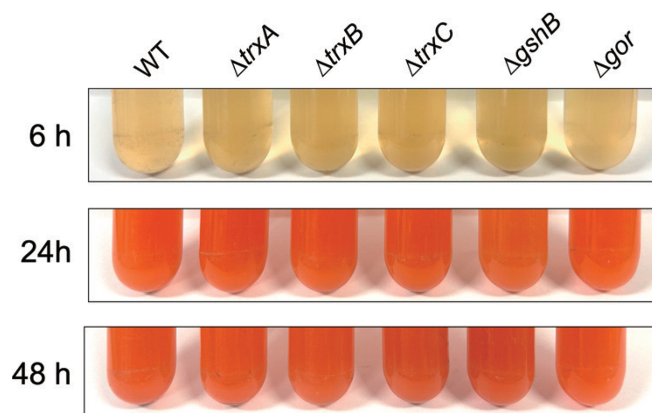

Figure 1. SeNP formation by the *E. coli* strains
Formation of red-colored SeNPs via selenite reduction by the WT and mutant strains of *E. coli*. The cells were cultured at 37°C in TSB medium containing 1 mM selenite.

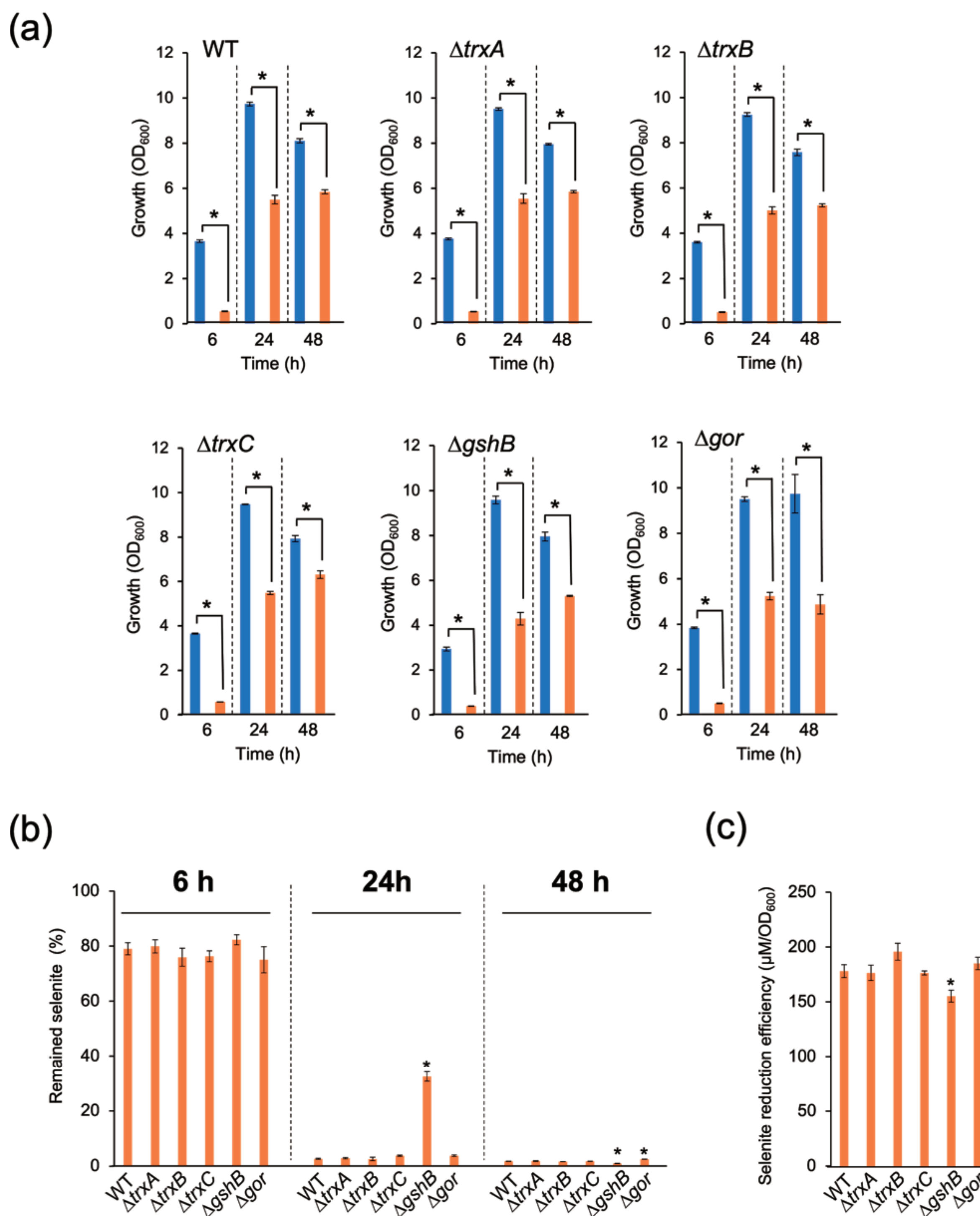

Figure 2. Growth and selenite reduction ability of the *E. coli* strains

(a) Growth (OD_{600}) of the WT and mutant strains cultured at 37°C in TSB medium with (orange) or without (blue) 1 mM selenite for 6 h, 24 h, and 48 h. The data are expressed as means ± SD (n = 3). Statistical analysis was performed by the t-test. *, $P < 0.01$ compared with WT.

(b) Amount of remained selenite in the culture supernatant of WT and mutant strains cultured at 37°C in TSB medium containing 1 mM sodium selenite for 6 h, 24 h, and 48 h. The data are expressed as means ± SD (n = 3). Statistical analysis was performed by the Dunnett test. *, $P < 0.01$ compared with WT.

(c) The selenite reduction efficiency, defined as the reduced selenite concentration normalized by the number of *E. coli* cells (OD_{600}) cultured at 37°C in TSB medium containing 1 mM sodium selenite for 24 h. The concentration of reduced selenite was calculated by subtracting the remained selenite concentration in the culture supernatant at 24 h from the initial selenite concentration at 0 h (1 mM). The data are expressed as means ± SD (n = 3). Statistical analysis was performed by the Dunnett test. *, $P < 0.01$ compared with WT.

Effect of mutations in the Trx and GSH systems on selenite sensitivity

To examine whether mutations in the Trx and GSH systems affect the sensitivity of *E. coli* to higher levels of selenite, WT and mutant strains were cultured on TSB agar medium containing 0, 1, 5, 10, or 20 mM selenite. All mutant strains grew similarly to the WT strain in the absence of selenite. However, marked growth inhibition was observed for the Δ*trxB*, Δ*gshB*, and Δ*gor* strains at 5–20 mM selenite compared with the WT (**Figure 3**), suggesting increased selenite sensitivity in these mutant strains. In contrast, deletion of *trxA* or *trxC* resulted in no drastic change in selenite sensitivity at 5–20 mM selenite compared with the WT. These results suggest that *trxB*, *gshB*, and *gor* are important for bacterial tolerance to > 5 mM selenite.

GSH is known to react with selenite to produce selenodiglutathione (GS-Se-SG) [14, 33]. The reduction of GS-Se-SG by GSH, thioredoxin, GSH reductase, or thioredoxin reductase is thought to form GSH selenopersulfide (GS-SeH), which is ultimately reduced to GSH and Se^0 [34-36]. Kessi and Hanselmann suggested that the same type of reaction is involved in the formation of SeNPs in *Rhodospirillum rubrum* and *E. coli* [15]. Our results are consistent with the previous observations and highlight the importance of thioredoxin reductase, GSH synthase, and GSH reductase in conferring selenite tolerance linked to SeNP synthesis via selenite reduction. The small effects of the deletion of either *trxA* or *trxC* on selenite tolerance and selenite reduction were probably due to the redundant functions of these thioredoxins.

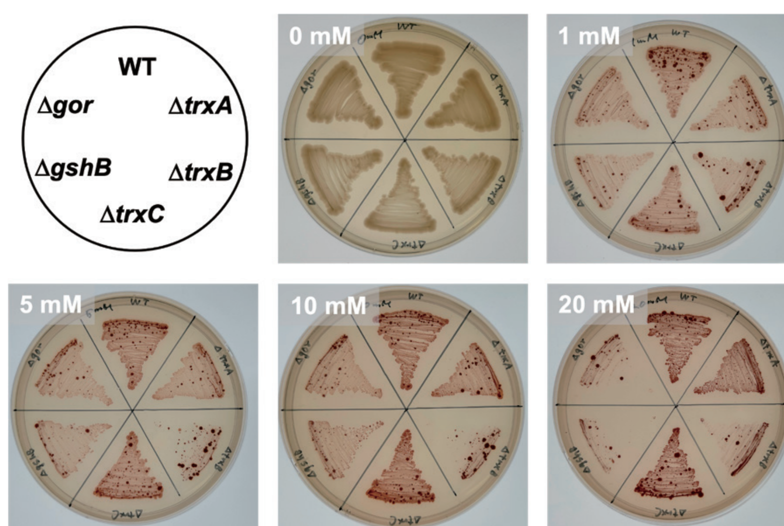

Figure 3. Selenite sensitivity of the *E. coli* strains
The WT and mutant strains of *E. coli* were streaked on TSB agar plates containing 0, 1, 5, 10, or 20 mM selenite. All plates were incubated at 37°C for 48 h.

Involvement of the Trx system in selenoprotein synthesis

To investigate whether the Trx and GSH systems are involved in selenoprotein biosynthesis, the activity of the selenoprotein FDH was assayed in whole *E. coli* cells anaerobically cultured on solid medium using benzyl viologen, as previously described [16]. **Figure 4** shows that WT cells were stained purple, indicating FDH activity. In contrast, Δ*trxA* cells were not stained in the assay, indicating a loss of FDH activity. Similarly, Δ*trxB* cells had slightly lower FDH activity than WT. In contrast, the loss of *trxC*, *gshB*, or *gor* had no effect on the FDH activity, which was comparable to that of the WT cells. These results suggest that the Trx system is important for selenite utilization during selenoprotein biosynthesis. Our results agree well with those of previous studies by Shimizu *et al.* [16] and Takahata *et al*. [22], which also suggest involvement of the Trx system in the selenium assimilation pathway. Interestingly, our results show that TrxC could not replace TrxA in selenoprotein biosynthesis, whereas these thioredoxins can be replaceable during SeNP synthesis. This suggests that there are at least two different pathways for

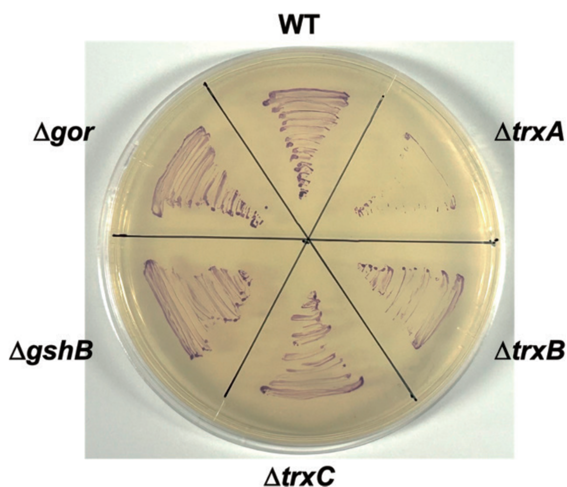

Figure 4. Whole-cell FDH assay of the *E. coli* strains
The WT and mutant strains of *E. coli* were anaerobically cultured at 37°C for 18 h on LB agar plate containing 0.5% glucose, followed by FDH assay using benzyl viologen.

selenite reduction in *E. coli*: a specific selenite assimilation (i.e., selenoprotein biosynthesis) pathway by the Trx system and a non-specific detoxification (i.e., SeNP synthesis) pathway involving both of the GSH and Trx systems.

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