

Glutathione and Transition-Metal Homeostasis in *Escherichia coli*[∇]

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Glutathione (GSH) and its derivative phytochelatin are important binding factors in transition-metal homeostasis in many eukaryotes. Here, we demonstrate that GSH is also involved in chromate, Zn(II), Cd(II), and Cu(II) homeostasis and resistance in *Escherichia coli*. While the loss of the ability to synthesize GSH influenced metal tolerance in wild-type cells only slightly, GSH was important for residual metal resistance in cells without metal efflux systems. In mutant cells without the P-type ATPase ZntA, the additional deletion of the GSH biosynthesis system led to a strong decrease in resistance to Cd(II) and Zn(II). Likewise, in mutant cells without the P-type ATPase CopA, the removal of GSH led to a strong decrease of Cu(II) resistance. The precursor of GSH, γ -glutamylcysteine (γ EC), was not able to compensate for a lack of GSH. On the contrary, γ EC-containing cells were less copper and cadmium tolerant than cells that contained neither γ EC nor GSH. Thus, GSH may play an important role in trace-element metabolism not only in higher organisms but also in bacteria.

Under aerobic growth conditions, either glutathione (GSH; L- γ -glutamyl-L-cysteine-glycine) or the small 12-kDa protein thioredoxin (TrxB) is essential to maintain a reduced environment in the cytosol of *Escherichia coli* cells (5, 27, 60, 65). Since *E. coli* thioredoxin reductase can transfer electrons from NADH to glutaredoxin 4 (Grx4, GrxD, or YdhD) and Grx4 can reduce Grx1 (GrxA) and Grx3 (GrxC) (14), *E. coli* is able to catalyze the reduction of disulfides without GSH. Thus, GSH by itself is not essential for the survival of this bacterium (17).

The cellular GSH is kept almost completely reduced (2, 30): the reduced GSH-oxidized GSH (GSSG) couple has a standard redox potential at pH 7.0 of -240 mV (66). Using a potential of about -260 mV in vivo (29) and the Nernst equation results in the calculation of a GSH concentration of about 5 mM and a GSSG concentration of about 5 μ M. Therefore, any change in the GSH concentration is likely to influence the cellular metabolism by changing the redox potential of the cytoplasm and maybe also that of the periplasm (59). A decrease of the GSH concentration by half would increase the cytoplasmic redox potential by 18 mV.

GSH is also involved in the osmoadaptation of *E. coli* (39). As a response to highly osmotic conditions, a mutant strain unable to synthesize trehalose as an osmoprotectant accumulates GSH to a concentration about 10-fold that under normal conditions. The first and quickest response of *E. coli* to changing osmotic conditions is to change the cytoplasmic potassium concentration (39), and indeed, GSH is needed for the regulation of this pool (13), probably through interaction with the GSH-gated potassium efflux system KefC-YabF (43). Moreover, GSH is involved in the detoxification

of methylglyoxal (13), although there are GSH-independent pathways of methylglyoxal degradation (44), and resistance to chlorine compounds (7, 67). In bacteria other than *E. coli*, GSH is essential for thiamine synthesis (19) and is also involved in defense against oxidative (31) and acidic (64) stress. Bacteria unable to synthesize GSH are sometimes able to import GSH from the growth medium, as shown previously for *Haemophilus influenzae* and for lactic acid bacteria (34, 71, 77), or to use other thiol compounds like mycothiol or γ -glutamylcysteine (γ EC) instead (12, 45, 47). Some gram-positive bacteria use cysteine as the main cellular thiol against oxidative stress (26).

These data illustrate the importance of GSH and related compounds to the cellular biochemistry. In contrast, not much is known about the interplay of heavy metals and GSH in bacteria, except that GSH influences resistance to arsenite and mercury in *E. coli* (32) and cadmium tolerance in *Rhizobium leguminosarum* bv. *viciae* (15, 35). On the other hand, GSH is heavily involved in transition-metal homeostasis in eukaryotes. In *Saccharomyces cerevisiae*, GSH is essential for full cadmium resistance (18). In most plants and fungi, GSH is the major substrate for the synthesis of the heavy-metal binding polypeptide phytochelatin (8, 40). The sequestration of heavy metal cations by phytochelatin and the consecutive transport of the bis-glutathionato complexes into vacuoles seem to be the main route of heavy-metal detoxification in these organisms. Phytochelatins can be produced successfully in transgenic bacteria, leading to enhanced metal accumulation in the bacterial cytoplasm (3, 11, 28, 69, 72) without the compartmentation ability of a vacuole.

Why do bacteria detoxify heavy metals mainly by efflux (49, 50) even though they should be able to sequester metals to the phytochelatin educt GSH? In GSH, two cysteine residues and four carboxyl groups should be able to form octahedral bis-glutathionato complexes that are very stable (76). To address this question, we deleted the GSH synthesis pathway in *E. coli* and also genes for various efflux systems and investigated if

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GSH might be a crucial transition-metal binding factor of the bacterial cell.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* was grown in Luria-Bertani (LB) medium or in Tris-buffered mineral salts medium (41) containing 2 ml of glycerol and 3 g of Casamino Acids per liter (TMM). Solid medium contained 20 g of agar/liter. Antibiotics (chloramphenicol at 25 $\mu\text{g/ml}$, kanamycin at 25 $\mu\text{g/ml}$, and ampicillin at 125 $\mu\text{g/ml}$) and metals were added where appropriate.

Dose response growth experiments. Overnight cultures of *E. coli* strains were diluted 1:100 in fresh LB medium or 1:1,000 in fresh TMM. After 2 h, they were diluted 1:10,000 in fresh LB medium with increasing metal cation concentrations and cultivated for 16 h with shaking at 37°C. The turbidity at 600 nm was measured using a SmartSpec3000 photometer (Bio-Rad, Munich, Germany).

Growth experiments. Overnight cultures of *E. coli* strains were diluted 1:100 in fresh LB medium. After 2 h, they were diluted 1:100 in fresh LB medium with or without metal and cultivated with shaking at 37°C. The turbidity over 18 to 22 h was monitored by a Klett photometer.

Gene deletions and other genetic techniques. Genes were deleted by the insertion of resistance cassettes using the λ Red recombinase system (9). Initial deletions in *E. coli* strain BW25113, in which the target genes were exchanged for a chloramphenicol resistance cassette (*cat*), were transferred by general transduction with phage P1 into *E. coli* strain W3110 or its derivatives. The mutant strains harboring *copA::Km* or *zntA::Km* were constructed by insertion of kanamycin resistance genes through homologous recombination (62, 63). Multiple deletion mutants were constructed by the FLP recombination target-dependent elimination of the respective resistance cassette with the assistance of flippase from plasmid pCP20 (9) and subsequent general phage P1 transduction. Otherwise, standard molecular genetic techniques were used (68). PCR was performed with *Taq* or *Taq/Pwo* DNA polymerase (Roche, Mannheim, Germany). All primer sequences can be obtained upon request.

GSH content determination. Overnight cultures were diluted 1:100 in fresh LB medium or TMM with or without metals and cultivated with shaking at 37°C until the optical density (at 600 nm) reached 2.25 ± 0.25 (the late exponential phase of growth). Volumes of cells corresponding to 2.5 to 5 mg (dry weight) were harvested by centrifugation (15 min at $4,500 \times g$ and 4°C), and the cells were suspended in 1 ml of 0.1 N HCl and disrupted by sonication for 2 min on ice with a UniEquip UW60 at 60 W and a 70% time interval. Cell debris was removed by centrifugation (15 min at $15,300 \times g$ and 4°C). The supernatant was used to determine the protein concentration by the bicinchoninic acid assay with an assay kit from Sigma GmbH (Osterode) incorporating bovine serum albumin as the standard. The supernatant was also used to measure the GSH content by high-performance liquid chromatography (HPLC) analysis.

HPLC analyses of thiols were performed using monobromobimane derivatization as described previously (46). A volume of 0.12 ml of the cell-free supernatant was added to a mixture of 0.18 ml of 0.2 M CHES buffer [2-(cyclohexylamino)ethanesulfonate], pH 9.3] and 30 μl of 5 mM dithiothreitol. After incubation on ice for 1 h, 10 μl of monobromobimane (30 mM in methanol [MeOH]) was added for thiol derivatization and incubation was continued for 15 min in the dark at room temperature. The reaction was stopped with 5% acetic acid (vol/vol). HPLC analyses were carried out on a Lichrospher 60 RP Select B column (4 by 250 mm; particle size, 5 μm ; Merck, Darmstadt, Germany) using a Merck-Hitachi LaChrom system equipped with a D-7000 interface, an L-7100 pump, an L-7200 autosampler, and a D-7480 fluorescence detector (excitation wavelength, 420 nm; emission wavelength, 520 nm). Mobile phase A consisted of a solution of 2% MeOH (vol/vol) in H_2O with 2.5 ml of glacial acetic acid liter⁻¹, adjusted to pH 4.3 with 10 N NaOH. Mobile phase B was composed of a solution of 90% MeOH (vol/vol) in H_2O with 2.5 ml of glacial acetic acid liter⁻¹, adjusted pH 3.9 with 0.1 N NaOH. HPLC running conditions are available upon request.

RESULTS

Cellular GSH in *E. coli*. The *gshA* gene (encoding the γ -Glu-Cys synthetase), the *gshB* gene (encoding the GSH synthetase), or both genes were deleted from the chromosome of the *E. coli* wild-type strain W3110. The deletion of these genes resulted in a decrease of the cellular GSH concentrations in cells grown in TMM and in LB medium-grown cells (Fig. 1). The crude extracts from wild-type cells contained a mean \pm a standard

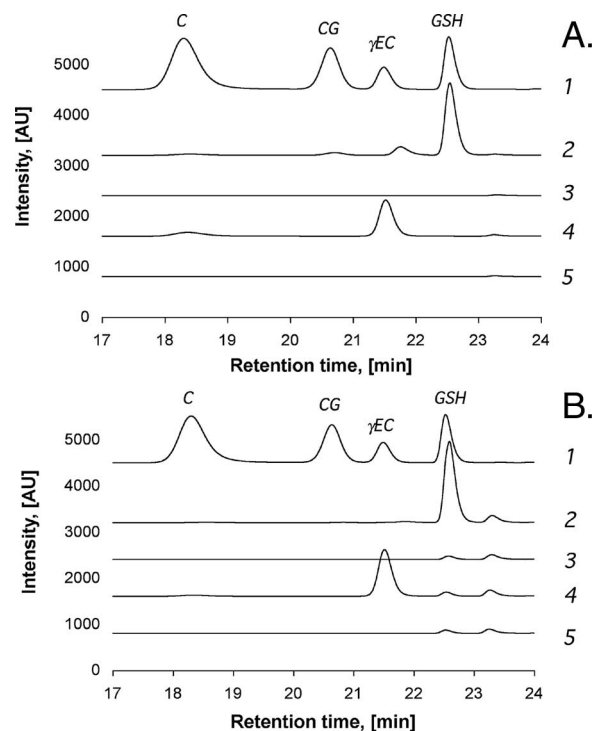


FIG. 1. Thiol compounds in the crude extracts from *E. coli* wild-type and Δgsh mutant strains. HPLC profiles of the crude extracts prepared from *E. coli* wild-type cells (lanes 2), the $\Delta gshA$ mutant strain (lanes 3), the $\Delta gshB$ mutant strain (lanes 4), and the $\Delta gshA \Delta gshB$ double mutant strain (lanes 5), each cultivated in TMM (A) or in LB medium (B), are shown. Lanes 1 represent a standard (8 $\mu\text{g/ml}$) containing peaks corresponding to cysteine (C), cysteine-glycine (CG), γEC , and GSH. All chromatograms were horizontally shifted for better visibility. The minor peak in panel A, lane 2, does not represent γEC but a nonreproducible artifact. AU, arbitrary units.

deviation of 13.7 ± 1.6 mg (TMM-grown cells) or 18.5 ± 2.4 mg (LB medium-grown cells) of GSH/g of total protein, and those from the $\Delta gshB$ mutant strain contained 27.4 ± 4.9 mg (TMM-grown cells) or 34.1 ± 6.4 mg (LB medium-grown cells) of γEC /g of total protein. Thus, the $\Delta gshB$ mutant strain contained a level of γ -Glu-Cys (γEC) higher than the level of GSH in the wild-type strain, which can be explained by the lack of GSH feedback inhibition of the γEC synthetase GshA in the $\Delta gshB$ mutant strain (1).

When cultivated in LB medium, all three types of mutant cells exhibited small GSH-derivative signals in HPLC analyses, indicating cytoplasmic GSH concentrations to be an order of magnitude smaller than that in wild-type cells (Fig. 1B). Since LB medium contains yeast extract and, therefore, GSH (mean value for various LB medium batches, 26 ± 6 μM) (data not shown), this residual GSH in the mutant cells may be the result of the uptake of external GSH by the GsiABCD uptake system (73). Even though LB medium-grown mutant cells were not entirely GSH free, *E. coli* strains with multiple deletions in *gsh* genes plus genes for metal efflux systems were cultivated in LB medium because that minor residual amount of the tripeptide (<5% of the wild-type level) (Fig. 1B) should not be able to affect toxic metal concentrations.

The deletion of *gshA* or of both *gsh* genes did not influence

the growth rate of *E. coli* in LB medium, but the deletion of *gshB* alone decreased the growth rate by half (data not shown). Growth rates, expressed as the increase in turbidity at 600 nm, were $1.52 \pm 0.34 \text{ h}^{-1}$ (wild type), $1.34 \pm 0.18 \text{ h}^{-1}$ ($\Delta gshA$ mutant), $0.77 \pm 0.17 \text{ h}^{-1}$ ($\Delta gshB$ mutant), and $1.43 \pm 0.36 \text{ h}^{-1}$ (double mutant). Thus, γEC , present in the $\Delta gshB$ mutant cells, was not able to compensate for the missing GSH since a decreased growth rate was observed, even without additional heavy-metal stress.

GSH and copper. In the presence of 3 mM Cu(II), the three Δgsh mutant strains and the wild type exhibited growth rates that were not significantly different (data not shown). Growth rates were $0.95 \pm 0.08 \text{ h}^{-1}$ (wild type), $1.23 \pm 0.26 \text{ h}^{-1}$ ($\Delta gshA$ mutant), $1.22 \pm 0.26 \text{ h}^{-1}$ ($\Delta gshB$ mutant), and $1.37 \pm 0.58 \text{ h}^{-1}$ (double mutant). However, the lag phase for the $\Delta gshB$ cells was markedly extended compared to those of the other strains (data not shown). This finding indicates that Cu(II) delayed the growth of the γEC -containing cells. As demonstrated by dose response curves for cells grown in LB medium, the deletion of *gshA* or of *gshA* and *gshB* did not influence copper resistance but $\Delta gshB$ mutant cells exhibited decreased copper resistance (Fig. 2A). Thus, GSH was not required for full copper resistance in this genetic background and copper toxicity was increased slightly in $\Delta gshB$ mutants. This increase was reversed in the double mutant, suggesting that the presence of γEC contributes to the increased copper toxicity. Similar effects were obtained in TMM but to a lesser extent (data not shown).

Cytoplasmic copper is detoxified mainly by the Cu(I)-exporting CPx-type ATPase CopA (62). As expected, the deletion of *copA* led to decreased copper resistance (Fig. 2B). In a $\Delta copA$ mutant background, the further deletion of *gshA* or of *gshB* led to a decrease in copper resistance (Fig. 2B), and again, the $\Delta copA \Delta gshB$ mutant strain was less copper resistant than the $\Delta copA \Delta gshA$ mutant strain. Thus, GSH was required for copper resistance in *E. coli* when the cells were unable to detoxify cytoplasmic copper by efflux, and increased γEC was not able to compensate for missing GSH. Interestingly, low copper concentrations slightly stimulated the growth of the $\Delta copA \Delta gshA$ strain but not that of the $\Delta copA \Delta gshB$ cells. This result may indicate a beneficial effect of GSH, but not of γEC , on cellular copper allocation (Fig. 2B).

As reported previously (24), the inactivation of the periplasmic detoxification systems CusCFBA and CueO decreased the copper resistance of *E. coli* such that the 50% lethal dose for the $\Delta copA$ mutant was 1.25 mM and that for the triple mutant strain was 0.83 mM (Fig. 2C). However, the additional deletion of *gshA* or of *gshB* in the triple mutant strain did not diminish copper resistance much further (Fig. 2C). In this mutant background, the deletion of *gshA* and that of *gshB* had similar effects on growth in the presence of copper.

GSH and zinc. In the wild-type background, the deletion of the *gsh* genes had no effect on zinc resistance, either in dose response experiments performed with LB medium (Fig. 3A) or TMM (data not shown) or in time-dependent growth experiments [performed with $850 \mu\text{M Zn(II)}$ in LB medium] (data not shown). Thus, in these types of cells, the presence of GSH did not correlate with full zinc resistance and the presence of γEC instead of GSH did not correlate with increased zinc sensitivity.

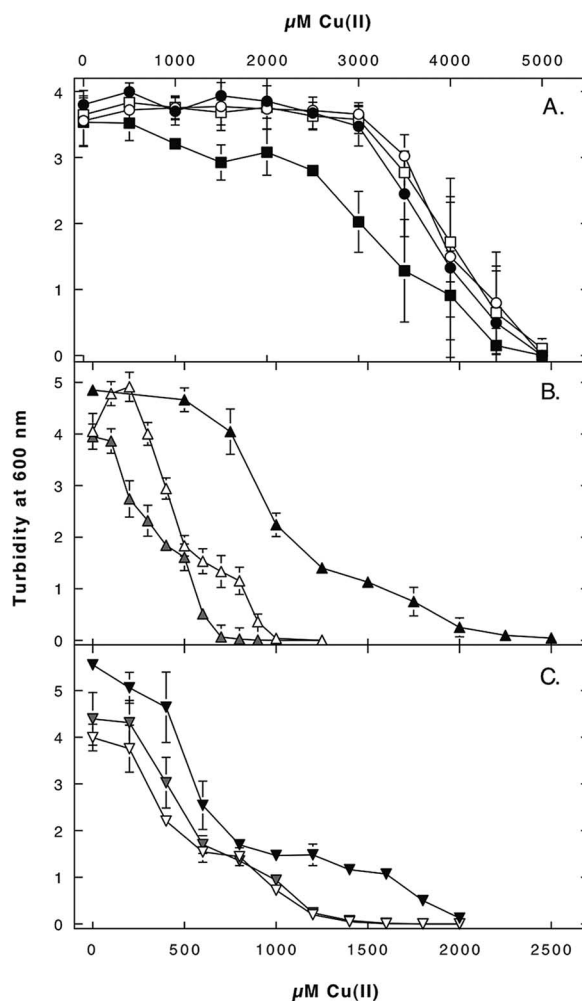


FIG. 2. Effects of deletions of *gsh* genes on copper resistance. Dose response curves (over 16 h at 37°C in LB medium) were recorded for the *E. coli* wild type (black circles) and the corresponding $\Delta gshB$ (black squares), $\Delta gshA$ (white circles), and $\Delta gshA \Delta gshB$ (white squares) mutants (A); the $\Delta copA$ (black triangles), $\Delta copA \Delta gshA$ (white triangles), and $\Delta copA \Delta gshB$ (gray triangles) mutant strains (B); and the $copA::Km \Delta cusA \Delta cueO$ (black inverted triangles), $\Delta copA \Delta cusCFBA \Delta cueO \Delta gshA$ (white inverted triangles), and $copA::Km \Delta cusA \Delta cueO \Delta gshB$ (gray inverted triangles) mutant strains (C). Note that the scales of the x axes in panels B and C are identical but different from that in panel A. Mean values with standard deviations (error bars) from at least three repeats are shown.

The deletion of the gene for the CPx-type ATPase ZntA led to strongly decreased zinc resistance, as expected (70). The deletion of *gshB*, *gshA*, or both (Fig. 3B) in the $\Delta zntA$ background led to a further decrease of zinc resistance in all three cases. The $\Delta zntA \Delta gshA$ mutant was slightly more zinc resistant than the other two strains. It is not clear why the absence of GSH in this mutant had an effect different from that of the absence of GSH in the $\Delta zntA \Delta gshA \Delta gshB$ mutant; the integrity of the constructs was verified continuously. Nevertheless, the differences were small, and as in the case of copper, GSH seemed to be important for zinc resistance in the absence of the cytoplasm-detoxifying CPx-type ATPase.

Another protein able to detoxify the cytoplasm of *E. coli* is

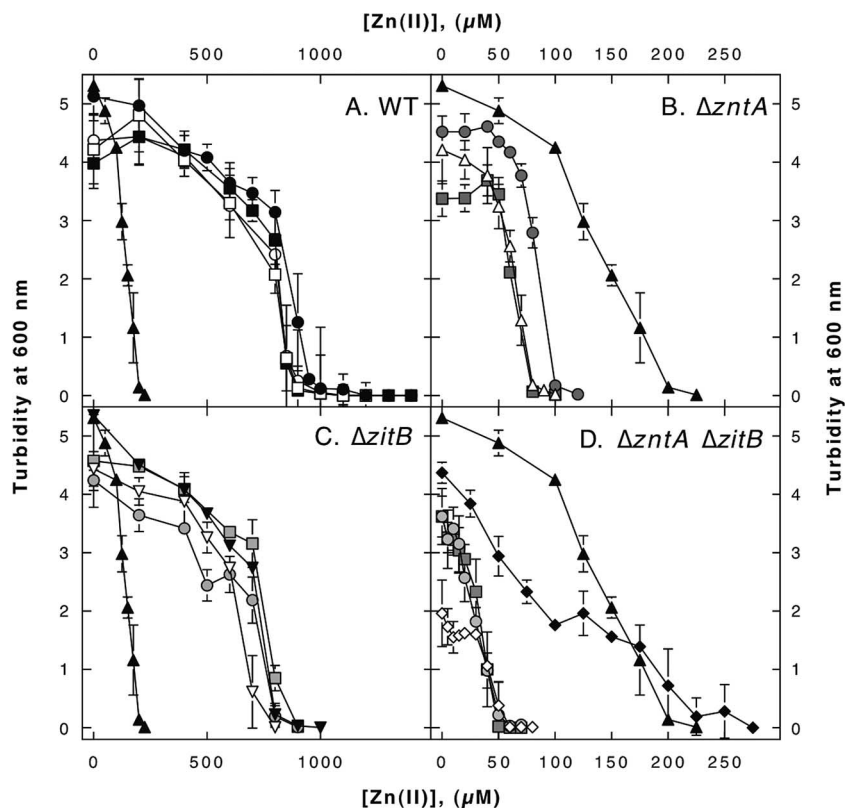


FIG. 3. Effects of deletions of *gsh* genes on zinc resistance. Dose response curves (over 16 h at 37°C in LB medium) were recorded for *E. coli* wild-type (WT) and mutant cells. For easier comparison, the dose response of the $\Delta zntA$ mutant cells (black triangles) is shown in all panels. (A) Results for wild-type (black circles), $\Delta gshB$ (black squares), $\Delta gshA$ (white circles), and $\Delta gshA \Delta gshB$ (white squares) cells. (B) Results for $\Delta zntA$ cells (black triangles) and $\Delta gshA$ (gray circles), $\Delta gshB$ (white triangles), and $\Delta gshA \Delta gshB$ (gray squares) mutants thereof. (C) Results for $\Delta zitB$ cells (black inverted triangles) and $\Delta gshA$ (gray circles), $\Delta gshB$ (white inverted triangles), and $\Delta gshA::Cm \Delta gshB$ (gray squares) mutants thereof. (D) Results for $\Delta zntA \Delta zitB$ cells (black diamonds) and $\Delta gshA$ (gray circles), $\Delta gshB$ (white diamonds), and $\Delta gshA::Km \Delta gshB::Km$ (gray squares) mutants thereof. The *x*-axis scales in panels A and C are identical, as are those in panels B and D. Mean values with standard deviations (error bars) from at least three repeats are shown.

the cation diffusion facilitator ZitB (20, 33). The deletion of *zitB* (in the presence of ZntA) led to no decrease in zinc resistance, and the further deletion of *gsh* genes in the $\Delta zitB$ mutant led to only small decreases in zinc resistance (Fig. 3C). Finally, the $\Delta zntA \Delta zitB$ double mutant displayed decreased zinc resistance compared to that of the $\Delta zntA$ mutant, and the deletion of *gsh* genes decreased zinc resistance even more (Fig. 3D), especially in the $\Delta gshB$ mutant containing only γEC . Thus, the absence of GSH correlated with decreased zinc resistance when the efflux pump ZntA was missing, and γEC seemed not to be able to compensate for missing GSH.

GSH and chromate resistance. GSH was also required for full chromate resistance (Fig. 4). The $\Delta gshA$ and $\Delta gshA \Delta gshB$ mutant strains showed some decrease in chromate resistance compared to that of the wild type, while the $\Delta gshB$ mutant was the most sensitive strain of those tested. There was no effect of the *gsh* mutations on resistance to Ni(II) or Co(II) (data not shown).

GSH and cadmium. In the presence of Cd(II), all mutant cells were less metal resistant than wild-type cells, in TMM and in LB medium (Fig. 5). While the $\Delta gshB$ mutant cells showed the smallest degree of cadmium resistance in either medium, the $\Delta gshA$ mutant cells and the $\Delta gshA \Delta gshB$ double mutant cells displayed intermediate levels of cadmium resistance (Fig. 5). Thus, in contrast to that in the cases of zinc and copper

resistance, the absence of GSH could not be fully complemented by efflux systems. The presence of γEC in the $\Delta gshB$ mutant correlated with decreased, not increased, cadmium resistance.

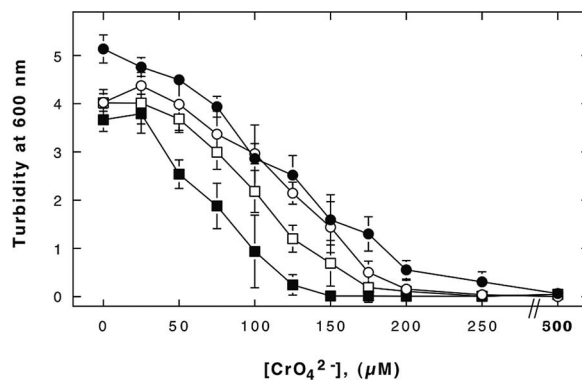


FIG. 4. Influence of GSH on chromate resistance. Dose response curves for the *E. coli* wild type (black circles) and the corresponding $\Delta gshA$ (white circles), $\Delta gshB$ (black squares), and $\Delta gshA \Delta gshB$ (white squares) mutant strains over 16 h at 37°C in LB medium containing increasing concentrations of potassium chromate were recorded. Mean values with standard deviations (error bars) from triple determinations are shown.

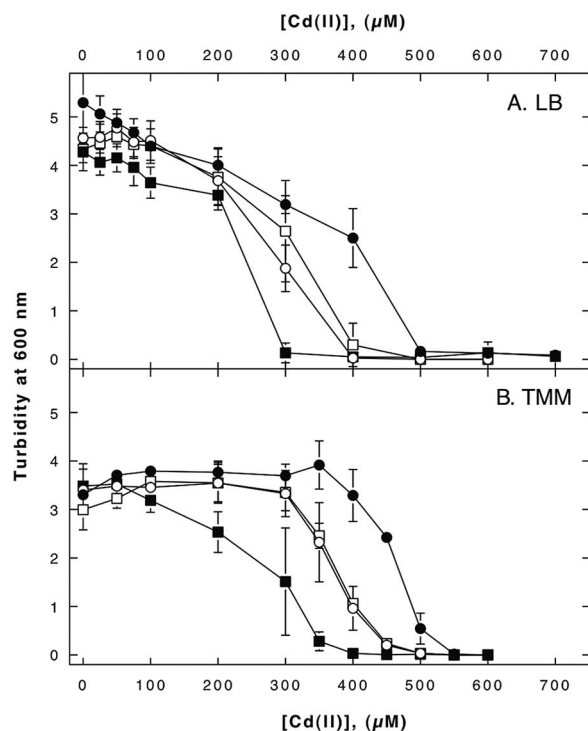


FIG. 5. Influence of GSH on cadmium resistance. Dose response curves for the *E. coli* wild type (black circles) and the corresponding $\Delta gshA$ (white circles), $\Delta gshB$ (black squares), and $\Delta gshA \Delta gshB$ (white squares) mutant strains over 16 h at 37°C in LB medium (A) or TMM (B) containing increasing concentrations of cadmium(II) chloride were recorded. Mean values with standard deviations (error bars) from at least three repeats are shown.

In the $\Delta zntA$ and $\Delta zntA \Delta zitB$ mutant strains, the deletion of *gsh* genes led to strongly decreased cadmium resistance (Fig. 6; the cadmium concentration is expressed on a logarithmic scale). The difference between $\Delta gshA$ mutant derivatives (containing GSH) and $\Delta gshB$ mutant derivatives (containing γ EC) with the wild-type background was greater than that between the corresponding mutant derivatives with $\Delta zntA$ and $\Delta zntA \Delta zitB$ mutant backgrounds (Fig. 5 and 6). Reminiscent of the findings for zinc and copper, this result confirmed GSH to be an important tool in metal homeostasis in the absence of the efflux system and γ EC to be unable to compensate for the missing GSH.

DISCUSSION

Transition-metal ion homeostasis in general. How can cellular heavy-metal homeostasis be described in terms of an interplay of transport and binding reactions? Transition-metal cations are taken up, bind to cytoplasmic and periplasmic components, and are exported by efflux systems. Upon first consideration, the cytoplasmic metal concentration seems to be in a flow equilibrium that is the result of uptake and efflux processes, but efflux systems may not be able to reach some of the metal cations in the cytoplasm because they are bound too tightly. Thus, superimposed on the kinetic flow equilibrium of uptake and efflux processes, there is a process of partitioning between cytoplasmic metal binding sites, including regulatory

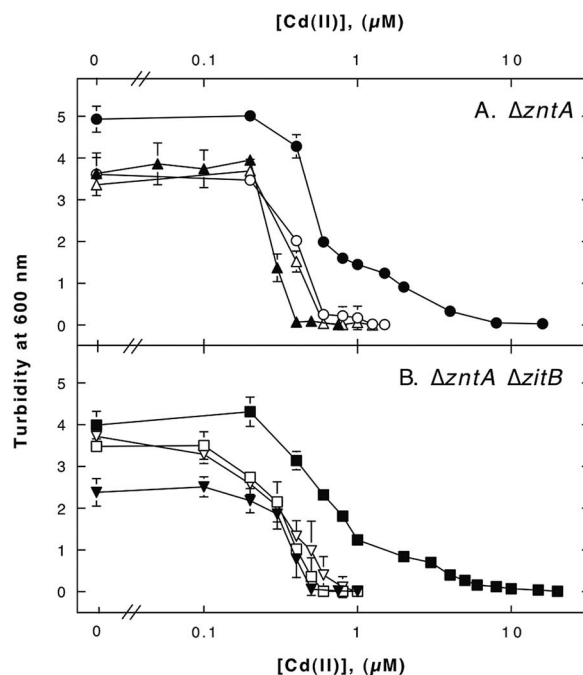


FIG. 6. Cadmium resistance of *E. coli* mutants with deletions in genes involved in cadmium detoxification. Dose response curves (over 16 h at 37°C in LB medium) were recorded for *E. coli* $\Delta zntA$ (black circles), $\Delta zntA \Delta gshA$ (white circles), $\Delta zntA \Delta gshB$ (black triangles), and $\Delta zntA \Delta gshA \Delta gshB$ (white triangles) cells (A) and for *E. coli* $\Delta zntA \Delta zitB$ (black squares), $\Delta zntA \Delta zitB \Delta gshA$ (white squares), $\Delta zntA \Delta zitB \Delta gshB$ (black inverted triangles), and $\Delta zntA \Delta zitB \Delta gshA \Delta gshB$ (white inverted triangles) cells (B). Mean values with standard deviations (error bars) from at least three repeats are shown. The x axis is on a logarithmic scale.

sites, and the substrate binding sites of the efflux proteins. Much is known about the uptake and efflux systems that compose the transport flow backbone of the cellular metal homeostasis, but binding and partitioning reactions have hardly been characterized.

Zinc. In the transport flow backbone of transition metals in *E. coli*, Zn^{2+} is imported into the *E. coli* cells by fast and unspecific uptake systems like ZupT (21). Additionally, conditions of zinc starvation induce the ZnuABC uptake system (58). Inside the cells, the essential trace element zinc binds to a multitude of proteins, especially to the RNA polymerase (56). Excess zinc in the cytoplasm induces the expression of the CPx-type efflux ATPase ZntA (70) and of the cation diffusion facilitator family protein ZitB (20, 33), which export transition metals. Additionally, *E. coli* contains another cation diffusion facilitator protein, FieF (YiiP), that binds Zn(II) and transports Zn(II) in vitro (37) but may be an Fe(II) efflux system in vivo (22). The expression of the ZnuABC uptake system is under the control of the Zur protein (58), a paralog of the main iron homeostasis regulator, Fur. The expression of the main zinc efflux pump, ZntA, is under the control of the MerR-type regulator ZntR (4, 57). The sigmoidal activity functions of Zur and ZntR overlap, keeping the free cytoplasmic Zn^{2+} concentration in a narrow window between 2×10^{-16} M (the half-maximum-induction point for Zur regulation) and 10^{-15} M (the half-maximum-induction point for ZntR regulation)

(57). In a previous study, the expression of the *zitB* gene was induced by zinc in a reporter gene assay (20), but the regulator has not yet been identified. So, partitioning events involved in cytoplasmic zinc homeostasis should occur between the unaccounted-for cytoplasmic binding sites of Zn^{2+} and the substrate binding sites of ZntA, ZitB, or even FieF.

Copper. Copper homeostasis in *E. coli* is a more complicated process than zinc homeostasis. A great deal of copper homeostasis occurs in the periplasm. The resistance-nodulation-cell division-driven (RND) efflux system CusCBA seems to transport periplasmic copper from the periplasm to the outside of the cell (16, 24). Additionally, copper is bound by the small periplasmic protein CusF (16, 36), and Cu(I) is oxidized by the multicopper oxidase CueO into the less toxic form Cu(II) (23). It is unclear how periplasmic copper enters the cytoplasm and when and where it is reduced to Cu(I). It is exported, however, by the CPx-type ATPase CopA (62), which together with CueO is under the regulatory control of CueR (6).

Cobalt. Any influence of GSH on cobalt resistance was not observed here (data not shown), although such influence in *Salmonella enterica* has been shown previously (74). However, the *gshA* mutant of *S. enterica* was a transposon mutant (74), indicating the possibility of polar effects and rearrangements, especially since the arrangement of genes on the *S. enterica* chromosome is slightly different from that on the *E. coli* chromosome (<http://biocyc.org>). In *E. coli*, cobalt toxicity is based mainly on the competition of Co(II) with Fe(II) during the synthesis of iron-sulfur clusters (61), which fits with the higher affinity of Co(II) than of Fe(II) for sulfur- and oxygen-containing ligands in complex compounds (48).

Chromate. Like in other bacteria (52, 53), chromate is probably imported into *E. coli* cells by sulfate uptake systems, but *E. coli* does not contain a CHR-type efflux system for chromate (51). In the cytoplasm, chromate should interact immediately with GSH (42), leading to the generation of GSSG and Cr(III). This process may produce free radicals but detoxify chromate, which otherwise may interfere with the sulfate metabolism. The absence of GSH increased chromate toxicity (Fig. 4). Thus, the beneficial effect of GSH-mediated chromate detoxification should compensate for the production of free radicals.

Cadmium. Similar to the findings for chromate, GSH-free cells suffered increased toxicity from cadmium. The deletion of the gene for the cadmium efflux pump ZntA had a much stronger effect on cadmium toxicity (Fig. 6) than the absence of GSH. Thus, GSH protects the cytoplasm by preventing the binding of Cd^{2+} to toxicity targets, but decreasing the absolute amount of cytoplasmic cadmium cations by efflux was more effective than decreasing the concentration of available cadmium by GSH-mediated sequestration.

GSH and transition metals. The cytoplasmic metal cation concentration is tightly controlled by the transport flow backbone of metal homeostasis. However, it is unclear what the cytoplasmic metal concentration is. With respect to the cytoplasmic concentration of GSH and the affinity of most transition metals for sulfur (48), every divalent transition metal cation entering an *E. coli* cell may form a bis-glutathionato complex immediately. The complex binding constants rely heavily on the pH and the buffer composition. Robust numbers are 10^{42} for the Hg^{2+} -bis-glutathionato complex (54) and 10^{39} for the Cu^{+} -monoglutathionato complex (55), which easily ex-

plains the sensitivity of the copper resistance regulator CueR to zeptomolar levels of copper (6).

The values for other transition metals should be below the copper and mercury values due to the lower affinity of these metals for sulfur. If a zinc quota of 200 μ M zinc, a GSH concentration of 5 mM, and a free zinc concentration of 4×10^{-16} M (between the half-maximum-induction points for Zur and ZntR regulation) (56) are assumed, the complex binding constant of the Zn^{2+} -bis-glutathionato complex should be 2×10^{16} , which fits with the data for copper and mercury, again considering the different affinities for sulfur. The rate constant for the formation of a Zn^{2+} -monoglutathionato complex is $3,900 \text{ M}^{-1} \text{ s}^{-1}$ (10). Thus, 200 μ M free zinc with 5 mM GSH should form the monoglutathionato complexes as a first step with an initial velocity of 3.9 mmol/s, leading to the rapid formation of zinc-bis-glutathionato complexes.

Thus, the absence of GSH should lead to increased metal sensitivity because target sites for metal toxicity are now readily available. However, this was not the case for the metal cations of the first transition period, like zinc and copper. The absence of GSH had no effect (Fig. 2 and 3) in wild-type cells. However, in the absence of the respective P-type ATPase efflux systems for Zn^{2+} and Cu^{+} (ZntA and CopA, respectively), the absence of GSH clearly decreased metal resistance. Thus, these transport systems complemented the absence of GSH and their detoxification activities were sufficient to prevent the binding of Zn^{2+} or Cu^{+} to cytoplasmic target sites.

So, cells needed GSH for full resistance to elements with high affinities for thiol groups, like Cd^{2+} and chromate, while metal cations like Zn^{2+} were sufficiently detoxified by efflux even in the absence of GSH. Cu^{+} was also sufficiently detoxified by efflux, although Cu^{+} has an even higher affinity for sulfur-containing ligands than Cd^{2+} (48). However, copper toxicity in *E. coli* seems to be a periplasmic rather than a cytoplasmic effect (38), and a copper uptake system in *E. coli* has not been identified yet.

If GSH detoxifies metal cations by sequestration and chromate by reduction, γ EC should be able to perform similar tasks. Cells of the Δ *gshB* mutant strain contained levels of γ EC higher than the levels of GSH in wild-type cells (Fig. 1). Nevertheless, Δ *gshB* mutant cells were less resistant to copper, cadmium, zinc, and chromate than Δ *gshA* mutant cells that did not contain considerable amounts of any cellular thiol compound. Even in the absence of efflux systems like ZntA and CopA, γ EC was not able to provide any degree of protection.

Zn^{2+} binds primarily to the thiol group of cysteine, followed by the imidazole ring of histidine and the carboxyl groups of aspartate and glutamate, but only if these groups are deprotonated (75). Cd^{2+} should bind in a similar manner with a stronger emphasis on thiols. GSH contains a thiol group and two carboxyl groups, which should be able to bind to both divalent cations, forming a six-ligand "cage" around them in the respective bis-glutathionato complex. In γ EC, the second carboxyl group of glycine is missing, leaving the cage open, allowing other molecules to attack and displace γ EC much more easily than GSH. Since the main reason behind cadmium toxicity in *E. coli* is the sequestration of sulfide (originating during assimilatory sulfate reduction or genesis or the disturbance of iron-sulfur clusters [25]), γ EC may not prevent this toxic action sufficiently in *E. coli*.

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