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Effects of Intracellular Glutathione on Sensitivity of *Escherichia coli* to Mercury and Arsenite

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The effect of intracellular glutathione on sensitivity to mercuric cations and arsenite anions was studied in *Escherichia coli* mutants that lack glutathione (gshA) with or without an additional mutation affecting the osmotregulant trehalose. The absence of glutathione increased cellular sensitivity to both Hg^{2+} and AsO^{2-}. The double mutant was more sensitive to Hg^{2+} than the single mutant strain. The addition of plasmid resistance determinants of Hg^{2+} and AsO^{2-} showed additivity between chromosomal genes and plasmid genes. Mercury resistance was increased in the plasmid-containing cells but not up to the level of wild-type cells. Plasmid arsenite resistance was not expressed in the gshA mutant of *E. coli*. © 1998 Academic Press

Heavy metals exert very toxic effects on cells of all organisms (21,24). Human activities, such as mining operations and discharge of industrial, municipal, and sewage wastes have resulted in an excessive accumulation of meals on the environment (8). Generally, the microbial inhabitants of these polluted environments respond to this challenge by the evolution of resistant populations (6,7). The most studied mechanisms of heavy metals resistance in microorganisms are: a) reduction of intracellular accumulation of toxic metal ions mediated through efflux mechanisms (16,18), b) transformation of toxic metal ions to non toxic forms (22), and c) bioaccumulation of the heavy metals intracellularly or extracellularly in a form that is inaccessible to the target macromolecules (22).

Glutathione (GSH, the tripeptide gamma-glutamyl-cysteinyl-glycine) is the predominant low molecular weight thiol in all living organisms. GSH constitutes 95% of the acid-soluble sulfur in bacteria (1). It exists in both the reduced (GSH) and oxidized (GSSG) forms (13). GSH has been reported to be involved in many biochemical activities within cells (11). The maintenance of adequate levels of reduced glutathione in living organisms is thought to be necessary for the protection of the cellular membrane proteins and lipids against oxidation damage (2), free radicals (15), sulfhydryl-reactive agents (3) and thermosensitivity (20). In addition, GSH has been reported to play significant roles in the protection of both bacterial cells and higher organisms against heavy metals and xenobiotics toxicity (23, 25).

The aim of this study is to investigate the tolerance of different *E. coli* mutants (gshA20::Tn10 Km^r and/or ostA1::Tn10) to Hg^{2+} and/or AsO^{2-} and possible additivity of chromosomal genes and plasmid-determined resistances to these toxic ions.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strain FRAG5 F^- thi rha 1acZ kdpABC5 and its derivatives with additional mutations FRAG69 ostA1::Tn10, FRAG70 gshA20::Tn10 Km^r, and FRAG76 ostA1::Tn10 gshA20::Tn10Km^r (12) were obtained from W. Epstein (University of Chicago). Plasmids pGN120 Ap^r Hg-resistance (17) (with the intact mer operon cloned in plasmid pBR322 and pCL120) (Lien Chu, unpublished) Ap^r Hg-hypersensitivity (because of deletion of the mer operon beyond the EcoRI site in merA were obtained from Debrabarta Mukapadhyrah (University of Illinois, Chicago). Plasmids pGJ103 (10) and pBR322 were obtained from Guangyong Ji (University of Illinois, Chicago), see Table 1.

TRANSFORMATION AND GROWTH PATTERNS

Each strain of *E. coli* was transformed with the plasmid DNA after treatments that rendered them competent for uptake of exogenous DNA (19). Transformants were selected on LB agar supplemented with antibiotics and scored on either mercury or arsenite containing agar. Transformants from pBR322 were selected on L agar supplemented with either tetracycline or ampicillin and sometimes both antibiotics.

Luria broth (LB) (14) supplemented with mercuric chloride or sodium arsenite was inoculated 1:100 with overnight cultures and incubated at 37°C with constant aeration overnight. Growth was determined by turbidity measurements in a Klett-Summerson colorimeter. Growth susceptibility patterns of each strain were further examined by disk inhibition assays. Overnight cultures were concentrated to one-tenth of the original volume by centrifugation, 0.1 ml

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TABLE 1

Bacterial Strains and Plasmids

E. Coli K-12 strain or plasmid	Relevant characteristic or marker	Reference or source
	Strains	
FRAG5	F^- thi rha lacZ kdpABC5	Epstein
FRAG69	FRAG5 ostA1::Tn10	Epstein
FRAG70	FRAG5 gshA20::Tn10Km^r	Epstein
FRAG76	FRAG5 ostA1::Tn10 gshA20::Tn10Km^r	Epstein
	Plasmids	
pCL20	Ap^r Hg^r	D. Mukhopadhyay
pl A	Ap^r Hg^s	D. Mukhopadhyay
pGJ103	Tc^r As^r	G. Ji
pBR322	Tc^r Ap^r	G. Ji
pGN120	Ap^r Hg^r	Nucifora

of these cells were spread on LB agar plates and allowed 30 to 45 min. for the cells to be properly absorbed in agar. Paper disks (6 mm diameter) containing $HgCl_2$ were laid on the surface of LB agar and plates were incubated at 37°C overnight. The diameters of the growth inhibition zones for each strain were measured, and the data presented minus the 6 mm disk.

RESULTS AND DISCUSSION

Involvement of GSH in heavy metal tolerance. The effect of GSH content on mercury and arsenite sensitivity in *E. coli* strain FRAG5 and its derivatives were measured, both without and with the mercury-resistance plasmid present (Fig. 1a). The gshA mutation in strain FRAG70 slightly (but consistently, see also Figs. 2 and 3) increased bacterial sensitivity to Hg^{2+}. Sur-

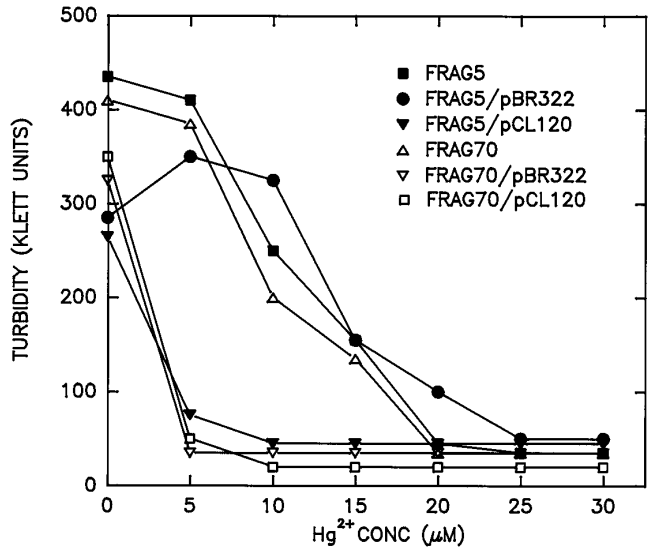

FIG. 2. The effect of the plasmid containing the merA deletion or plasmid pBR322 on the growth of FRAG strains in broth containing various concentrations of mercury. Overnight cultures were diluted 1 to 100 in L. broth containing various concentrations of $HgCl_2$ and incubated at 37°C with constant aeration for 16 h. Culture turbidity was measured in a Klett colorimeter containing green no. 54 filter. Symbols: FRAG5 (■), FRAG5/pBR322 (●), FRAG5/pCL120 (▼), FRAG70 (△), FRAG70/pBR322 (▽), FRAG70/pGN120 (□).

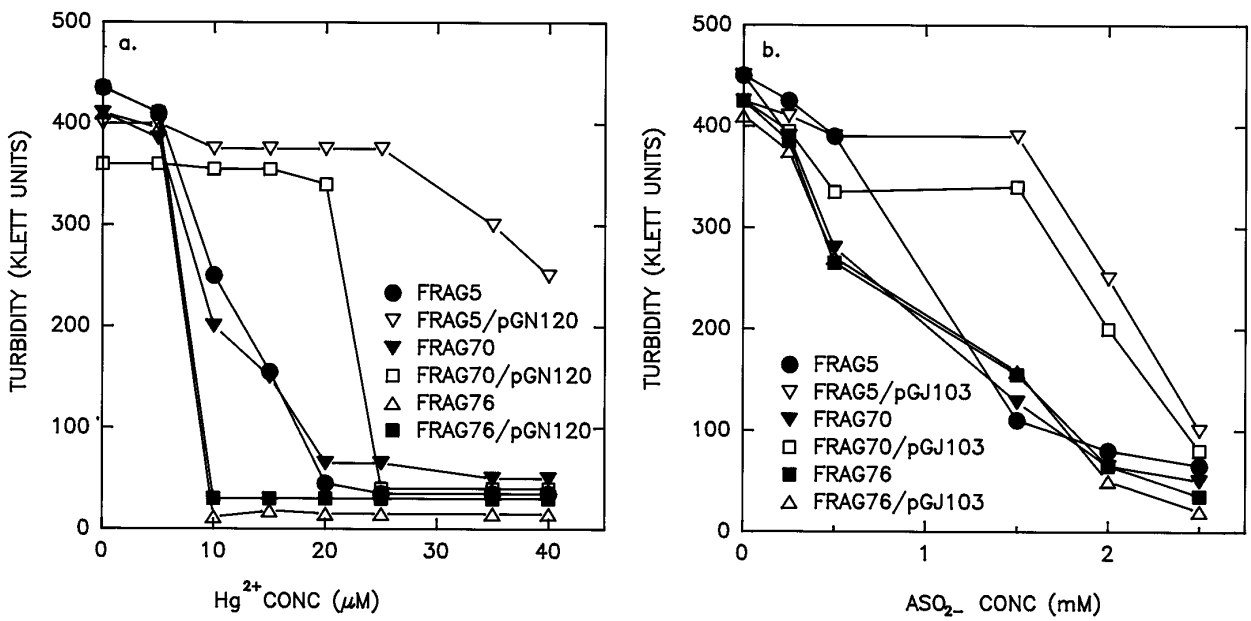

FIG. 1. Growth tolerance patterns of wild-type and mutants strains of *E. coli* (FRAG5, FRAG70, and FRAG76) to various concentrations of mercury and arsenite respectively were determined by turbidity measurement in a Klett colorimeter. (a) Overnight culture of either transformed or plasmid free strains were diluted 1 to 100 in L. broth with indicated concentrations of $HgCl_2$, and incubated at 37°C with continuous aeration for 16 h. (b) Inoculum and incubation conditions were as described above except arsenite was substituted for mercury. Symbols: (a) FRAG5 (●), FRAG5/pGN120 (▽), FRAG70 (▼), FRAG70/pGN120 (□), FRAG76 (△), FRAG76/pGN120 (■). (B) FRAG5 (●), FRAG5/pGJ103 (▽), FRAG70 (▼), FRAG70/pGJ103 (□), FRAG76 (■), FRAG76/pGJ103 (△).

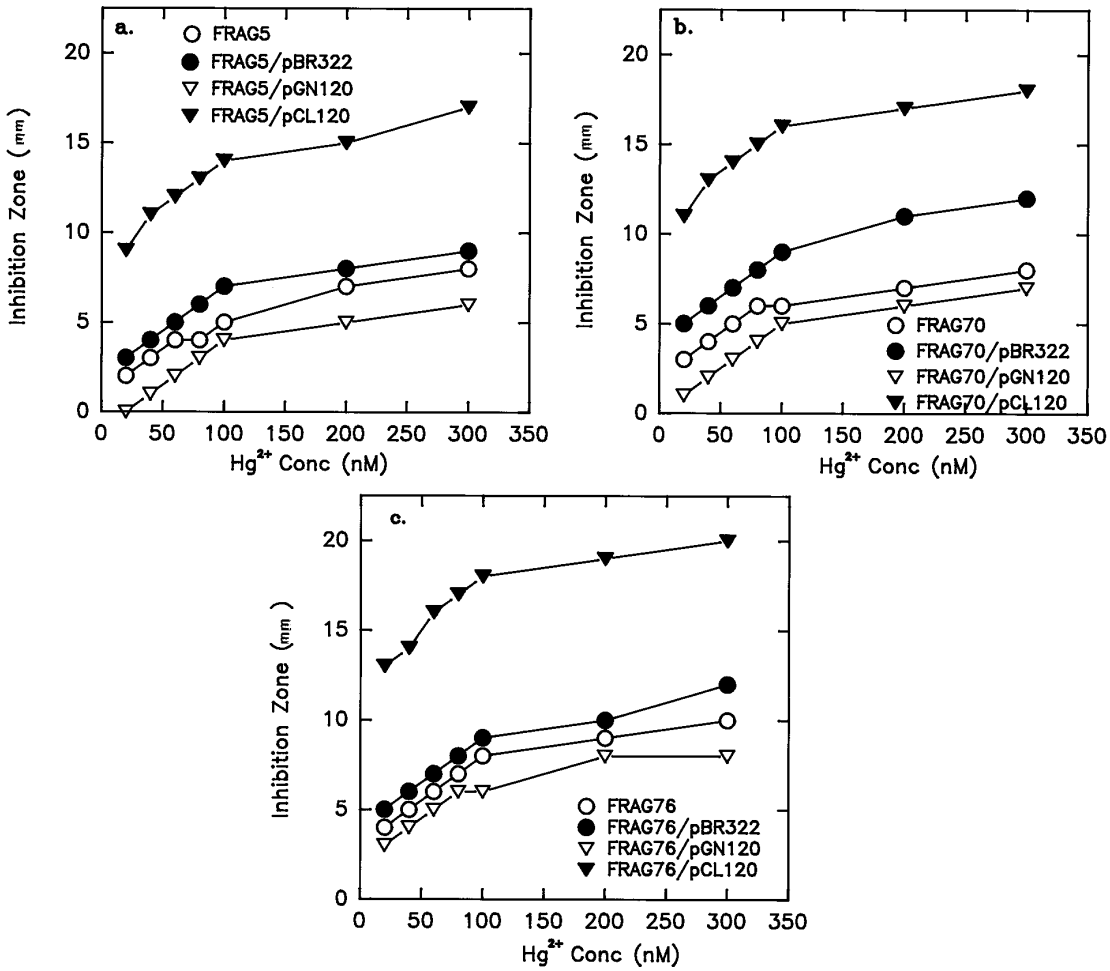

FIG. 3. Growth susceptibility to $HgCl_2$ in FRAG strains. Overnight cultures were concentrated by centrifugation and the pellet resuspended in one-tenth of the original volume. Cells suspensions were spread on LB agar and paper disks (6 mm diameter) containing $HgCl_2$ were laid on the surface of the agars. Inhibition zones were measured after 16 h at 37°C. Symbols: (a) FRAG5 (○), FRAG5/pBR322 (●), FRAG5/pGN120 (▽), FRAG5/pCL120 (▼); (b) FRAG70 (○), FRAG70/pBR322 (●), FRAG70/pGN120 (▽), FRAG70/pCL120 (▼); (c) FRAG76 (○), FRAG76/pBR322 (●), FRAG76/pGN120 (▽), and FRAG76/pCL120 (▼).

prisingly, the additional mutation in the ostA gene further increased bacterial sensitivity to Hg^{2+} (Fig. 1a) and to AsO^{2-} (Fig. 1b).

When the plasmid conferring mercury resistance was added to the *E. coli* strains, the resistance of both FRAG5 and FRAG70 to Hg^{2+} increased substantially, but there was no effect of the mer-determinant with strain FRAG76 (Fig. 1a). It was also the case that the ars-determinant for arsenite resistance did not function in FRAG76 (Fig. 1b).

Because the mercury resistance determinant was cloned into vector pBR322, which contains the tetracycline resistance determinant known function at the membrane transport level and to affect potassium as well as tetracycline uptake (5), the effect of vector pBR322 on Hg^{2+} resistance was determined (Fig. 2). Vector pBR322 increased sensitivity to Hg^{2+} (Fig. 2) both with strain FRAG5 and with strain FRAG70. Fig. 2 further shows that the hypersensitivity of plasmid pCL120 (due to the merT- and merP-gene function in the absence of mercuric reductase) is expressed in the FRAG70 gshA-minus strain, showing that glutathione does not have an essential role in Hg^{2+} transport into the cell.

Cation sensitivity and resistance can be carefully monitored on petri dishes as well, by measuring the zone of clearing (inhibition of growth) surrounding paper disks containing Hg^{2+} or other toxic heavy metals, much as is done in clinical settings for antibiotic susceptibility testing. The results of such measurements in Fig. 3 confirm and extend the liquid culture experiments. With all three strains, FRAG5, FRAG70 and FRAG76, the addition of the vector pBR322 slightly increased sensitivity to Hg^{2+}, while addition of the mer operon increased resistance. These two effects were quantitatively small but reproducible. Also for all three

strains, the addition of the mercury transport function on plasmid pCL120 caused a striking hypersensitivity to Hg^{2+} (Fig. 3), as had been previously reported in other chromosomal backgrounds. As a control Figure 2 shows that tetracycline resistance element of plasmid pBR322 could not confer resistance to the FRAGs at mercury concentrations of 15 μM and above. Data analysis of the zone of inhibition measurements from plasmid-transformed or non-transformed FRAGs grown on L agar previously overlaid with filters that contained mercury, showed mercury hypersensitivity in pBR322 and merA-deleted plasmid (pCL120) transformants (Fig. 3).

Many reports have implicated GSH and trehalose in tolerance mechanisms of higher organisms to hyperosmotic shock, heat, and other environmental toxicants (4,26). Neither GSH nor trehalose alone confer H_2O_2, heat, and gamma radiation adaptation to bacteria (9). We have found that the absence of glutathione (mutation in the gshA gene) or a mutation at ostA gene alone in *E. coli* K-12 (FRAG70 or FRAG69) is not sufficient to significantly increase the growth susceptibility of these bacterial cells to mercury or arsenite toxicity.

However, the double mutant (FRAG76) which could not synthesize glutathione (gshA::Tn10) and trehalose (ostA::Tn10) displayed remarkable growth sensitivity to both mercury and arsenite. The moderate tolerance to mercury and arsenite of the gshA mutant (FRAG70) tranformed with either arsenite or mercury resistance plasmids, suggest that the mercury reductase enzymes and arsenite efflux ATPase systems in *E. coli* do not require cellular GSH.

The plasmid-free (FRAG5 strain and its gshA mutant derivative FRAG70 compared to plasmid pBR322 or pCL120 transformed exhibited better growth response in various concentrations of mercury (Fig. 1a and 2). The inability of pBR322 and pCL120 transformed FRAG5 and FRAG70 to grow in low mercury concentration could be due to an increase in mercury uptake mediated by tetracycline resistance element of pBR322. The hypersensitivity of pCL120 transformed FRAG5 to mercury toxicity was also inferred to the consortium transport activities of transport system on mercury resistance operon of plasmid pCL120 and tetracycline resistance element.

Findings from this study clearly underline the independent and additive roles of glutathione and trehalose in *E. coli* response mechanisms to the toxic effects of heavy metals.

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