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Export of Glutathione by Some Widely Used *Salmonella typhimurium* and *Escherichia coli* Strains†

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Significant levels of extracellular glutathione (GSH) were detected in aeroobically grown cultures of some strains of *Salmonella typhimurium* LT-2 and in *Escherichia coli* K-12, B, and B/r but not in cultures of nine freshly isolated clinical isolates of *E. coli*. Cultures of *S. typhimurium* generally contained less total GSH (intracellular plus external) than did *E. coli* cultures. *S. typhimurium* TA1534 contained about 2 mM intracellular GSH and exported about 30% of its total GSH. The external GSH concentration increased logarithmically during exponential growth and peaked at about 24 μM in early-stationary-phase cultures. External accumulation of GSH was inhibited by 30 mM NaN_3. GSH was predominantly exported in the reduced form. Two-dimensional paper chromatography of supernatants from cultures labeled with $Na_2{}^{35}SO_4$ confirmed the presence of GSH and revealed five other sulfur-containing compounds in the media of *S. typhimurium* and *E. coli* cultures. The five unidentified compounds were not derivatives of GSH.

Glutathione, γ-glutamylcysteinylglycine, is the major nonprotein thiol in many gram-negative bacteria and in higher organisms, including humans. It is found inside cells at concentrations of up to 10 mM, almost all of which is in the reduced form (GSH) (2, 5, 7, 8, 10, 16, 20). Glutathione can also exist as the oxidized disulfide (GSSG), as mixed disulfides, and as conjugates with other compounds (20). Glutathione is a radioprotectant both in bacteria and in animal cells (8, 21, 23) and GSH performs accessory functions in *Escherichia coli*.

The only report of glutathione in the media of bacterial cultures was that of Roberts et al. (27), in which cultures of *E. coli* B were examined. They detected a spot of ^{35}S-labeled material that comigrated with GSSG in a two-dimensional paper chromatography system.

In this study, evidence is presented for the export of reduced glutathione by some widely used laboratory strains of *Salmonella typhimurium* and *E. coli*. In contrast, freshly isolated clinical *E. coli* strains did not export significant quantities of GSH.

MATERIALS AND METHODS

Strains. See Table 1 for a list of the strains used in this study. Wild-type prototrophic laboratory strains were obtained at the Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., in 1956 and maintained at The Johns Hopkins University since then, except for *E. coli* B (Black). This strain has been maintained separately at least since 1956 and was obtained from Lindsay W. Black. *E. coli* B and H originated as separate strains from the culture collection of J. Bronfenbrenner, Washington University School of Medicine, St. Louis, Mo., in the early 1940s (17; A. D. Hershey, personal communication).

Chemicals. $Na_2{}^{35}SO_4$ (33.4 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.) as a powder. Glutathione reductase [NAD(P)H:oxidized glutathione oxidoreductase; EC 1.6.4.2] was Type IV Sigma Chemical Co. (St. Louis, Mo.). GSH, GSSG, and 3-carboxy-4-nitrophenyl disulfide were obtained from Sigma. Di-γ-glutamylcystine was made by digestion of GSSG with carboxypeptidase A by the method of Strumeyer and Bloch (29). All other compounds used were reagent grade and were obtained from various companies.

Growth media. Vogel-Bonner E minimal (E) medium (31) was the base for the growth media used. For experiments in which only *S. typhimurium* was used, the medium was supplemented with 2.0 g of D-glucose per liter–0.1 mM L-histidine–0.5 μM D-biotin (his-bio-E). For experiments in which either strain AB1157 or 821 was used, E medium was supplemented with 2.0 g of D-glucose per liter–0.7 mM L-threonine–1.3 mM L-leucine–0.8 mM L-proline–10 μM thiamine–0.2 mM L-histidine–1.0 mM L-arginine–0.5 μM D-biotin (ABE). For plates, the media were solidified with 15 mg of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per ml.

Time course of glutathione accumulation. Two cultures of TA1534 were grown in parallel in his-bio-E medium. Cultures were grown at 37°C with shaking. At 1-h intervals 10- to 16-ml samples were withdrawn. A portion of each sample was diluted and plated for a viable count. The samples were centrifuged for 5 min at 12,000 × *g* and at 4°C. The pellets and supernatants were immediately frozen at −40°C. After all the samples were collected, the supernatants were thawed on ice and transferred to 75-ml lyophilization bottles (The VirTis Co., Inc., Gardiner, N.J.). The supernatants were frozen in a dry ice-ethanol bath and lyophilized to dryness. Each supernatant residue was then suspended in 1.5 ml of 0.3 M (5%) trichloroacetic acid (TCA) with 5 mM EDTA (TCA-EDTA). Each pellet was thawed and suspended in 2.0 ml of TCA-EDTA. The pellet samples were mixed in a Vortex mixer for 1 min to lyse the cells. After a

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TABLE 1. Bacterial strains used in this study

Species and strain	Genotype	Reference or source
S. typhimurium		
LT-2, LT-7, LT-8, LT-10	Wild-type prototrophs	15
LT-2 derivatives		
TA1534	*hisD3052 ΔuvrB chl bio*	1
hisG46	*hisG46*	1
TA1538	*hisD3052 ΔuvrB chl bio gal rfa*	1
hisD3052	*hisD3052*	1
TA1535	*hisG46 ΔuvrB chl bio gal rfa*	1
TA1950	*hisG46 ΔuvrB chl bio*	1
TA1975	*hisG46 rfa*	1
E. coli		
K-12, B, B/r, H	Wild-type prototrophs	This study
K-12 derivatives		
AB1157	*thr-1 leu-6 proA2 hisG4 argE3 thi-1 rpsL31 supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33* λ^s λ^-	2
821	*thr-1 leu-6 proA2 hisG4 argE3 thi-1 rpsL31 supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 gshA2* λ^s λ^-	2
Nine clinical isolates	Wild-type prototrophs	This study

20-min incubation period on ice, the pellet samples were centrifuged for 5 min at 12,000 × *g* at 4°C. The supernatant was taken for the assay. All samples in TCA-EDTA were kept on ice until the time of the assay.

Glutathione assay. GSSG and GSH were measured by the glutathione reductase recycling assay described by Tietze (30). The assay was calibrated with a set of five reduced glutathione standards in TCA-EDTA which spanned the entire linear range of 2 to 80 nmol/ml. Duplicate fractions from each sample were analyzed in a spectrophotometer (DU model 2400; Beckman Instruments, Inc., Fullerton, Calif.) with a chart recorder (model 6051; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Estimates obtained by this method were the sum of GSH plus GSSG, expressed as GSH equivalents (GSH + 1/2 GSSG) (30).

Export rate analysis with and without azide. A 400-ml culture of TA1534 was grown to late logarithmic phase in his-bio-E medium as described above. The culture was then split in half and centrifuged for 5 min at 12,000 × *g* at 4°C. The pellets were each washed once in 200 ml of unsupplemented E medium. One pellet was then suspended in 200 ml of unsupplemented E medium. The other pellet was suspended in E medium containing 30 mM sodium azide. The azide-containing culture was then split in half, and GSH was added to one half of the culture to a final concentration of 6.5 nmol/ml. The three cultures were then transferred to flasks and incubated at 37°C with shaking. After 63 min at 37°C, the culture without azide was split in half, and sodium azide was added to one half of the culture to a final concentration of 30 mM. Incubation with shaking at 37°C was then continued for all four cultures for another 87 min. At various times 10-ml samples of each culture were withdrawn for analysis. The samples were centrifuged, and the pellets and supernatants were processed and assayed for glutathione as described above for the time course experiments.

Strain survey. Cultures were grown in E or ABE medium at 37°C in a roller drum (model TC-7; New Brunswick Scientific Co., Inc., Edison, N.J.). Culture densities were measured with a colorimeter (Klett-Summerson) with a red filter (640 by 700 nm). When the cultures reached the late-logarithmic growth phase, the cultures were centrifuged and the pellets and supernatants were processed and assayed for glutathione as described above for the time course experiments.

Assay for reduced thiols. Total thiols were measured by the method of Ellman (6). A total of 0.2 ml of sample in TCA-EDTA was mixed with 3-carboxy-4-nitrophenyl disulfide in 0.1 M sodium phosphate buffer, containing 5 mM EDTA (pH 7.5) at room temperature. The optical density at 410 nm was measured exactly 2 min after mixing. The assay was calibrated with a set of GSH standards which spanned the linear range of 10 to 490 nmol/ml.

^{35}S-labeling of cells and supernatants. $Na_2{}^{35}SO_4$ (1.1 mCi, 33.4 mCi/mmol) was dissolved in 500 μl of supplemented E medium (his-bio-E or ABE). A total of 400 μl of the radiolabeled sulfate solution was added to 12 ml of ABE medium plus 0.2 ml of an overnight culture (grown in ABE medium) in a 125-ml flask. The culture was incubated at 37°C in a gyratory shaker until the early stationary phase (6 to 8 h). A total of 10 ml of culture was centrifuged for 5 min at 12,000 × *g* at 4°C. The supernatant was then mixed with an equal volume of 0.6 M TCA or 0.6 M TCA containing 0.167 mg of GSH per ml and 0.167 mg of GSSG per ml. The mixture was then incubated for 1 h on ice and centrifuged for 5 min at 12,000 × *g* at 4°C. The mixture was decanted (no pellet was observed) and frozen at −15°C until use. Samples of known compounds were made as 1-mg/ml solutions in supernatants of TA1534 grown in his-bio-E medium.

Paper chromatography. Whatman no. 1 filter paper was cut to 18 by 22 cm. For one-dimensional chromatography, samples were applied along a line 1 cm from the 22-cm side (20 μl/cm). Standard compounds (2 to 5 μl; 1 mg/ml in 50% [vol/vol] TA1534 supernatant in 50 mg of TCA per ml) were used. Ascending paper chromatography was then performed with 1-butanol–glacial acetic acid–water (2:1:1) (3) or 2-propanol–concentrated ammonia–water (25:1:7.33) as a solvent. For two-dimensional chromatography, 20 μl of the sample was spotted 1 cm from the bottom of an 18-cm solvent running length. The butanol-acetic acid-water solvent was used for the first dimension. The paper was then dried, rotated 90 degrees, and cut so that the origin was again 1 cm from the bottom of the 18-cm solvent running length. The 2-propanol–ammonia–water solvent was used for the second dimension. After development, the chromatograms were dried and stained by the cadmium-ninhydrin method (D. A. Powers [ed.]. 1985. Experimental molecular biology, p. 109. Department of Biology, The Johns Hopkins University).

Autoradiography was performed on chromatograms of radiolabeled samples for 10 to 31 days with Kodak XAR5 x-ray film. Chromatograms and autoradiograms were photographed with Kodak Ektachrome 160 tungsten film.

Cadmium-ninhydrin stain. Solution A was 56 mM ninhydrin in acetone. Solution B was 38 mM cadmium acetate in 50% (vol/vol) acetic acid. Both solutions were prepared within 1 h of use. Within 10 min of use, 6.67 parts of solution A was added to 1 part of solution B. The mixture was

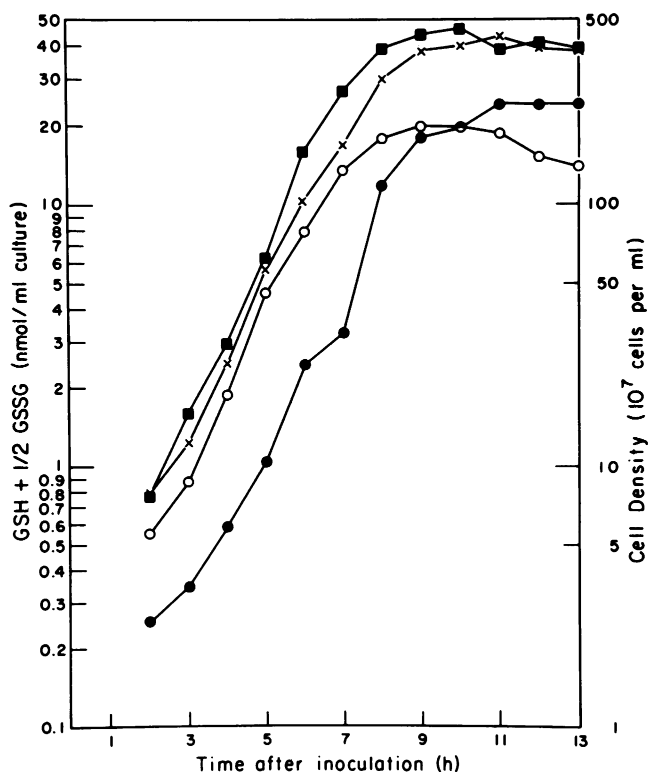

FIG. 1. Accumulation of glutathione (GSH and GSSG expressed as GSH equivalents) in the pellets (○) and supernatants (●) of TA1534 cultures. Values represent means of two different cultures grown in parallel. Cultures were grown at 37°C in his-bio-E medium. Other symbols: X, pellet and supernatant glutathione; ■, cell density.

sprayed onto the chromatogram as a fine mist (10 ml per 400 cm^2 of chromatogram). The sprayed chromatograms were heated at 65°C until color developed (about 30 min).

RESULTS

Time course of glutathione accumulation. In Fig. 1 it is shown that the accumulation of glutathione in the external media of cultures of *S. typhimurium* TA1534 is logarithmic and parallels the increase in cell density as well as the increase in intracellular glutathione. The internal glutathione level per cell was fairly constant at about 5 to 7 nmol per 10^9 cells during the logarithmic growth phase. This translates into an internal GSH concentration of about 2 mM, based on pellet volumes (data not shown).

The external GSH level continued to increase for about 1 h after the cells stopped dividing. During the log phase about one third of the glutathione in the culture was external. At the peak accumulation of glutathione in the culture, in the early stationary phase, the amount of GSH in the external medium (24.2 nmol/ml of culture) was slightly greater than the amount contained within the cells (18.6 nmol/ml of culture) (Fig. 1).

Almost identical amounts of extracellular glutathione to those shown in Fig. 1 were detected in filtrates through 0.45-μm-pore-size filters (Millipore Corp., Bedford, Mass.) made at 37°C (data not shown). No GSH or GSSG was detected in unused media or in media from cultures of the GSH^- *E. coli* mutant strain 821 (Table 2). The limit of detection was about 0.3 nmol/ml.

Suspension and azide treatment. Washed log-phase TA1534 cells suspended in fresh E medium (citrate as the sole carbon source and no histidine or biotin) accumulated glutathione linearly in the medium at a rate of approximately 0.3 nmol h^{-1} (10^9 cells)$^{-1}$. This external accumulation was inhibited by 30 mM NaN_3 (Fig. 2A). Azide treatment also

TABLE 2. Glutathione levels in several strains of *S. typhimurium* and *E. coli* in logarithmic growth phase

Species and strain	Estimated GSH + ½ GSSG (nmol/ml of cultures)/100 Klett units[a] ± SE			Average %	
	External	Internal	Total	N	External of total GSH
S. typhimurium					
LT-2 derivatives					
Wild type	0.3 ± 0.1	3.9 ± 0.6	4.2 ± 0.5	6	7
hisG46	<0.3	4.2 ± 0.4	4.2 ± 0.4	4	<7
TA1534	1.3 ± 0.1	3.0 ± 0.2	4.2 ± 0.2	12	30
TA1538	1.5	3.9	5.4	2	28
hisD3052	<0.3	3.9	3.9	2	<8
TA1535	0.6	4.4	5.0	2	12
TA1950	0.8	4.0	4.8	2	17
TA1975	1.3	2.9	4.2	2	31
LT-7 wild type	0.3 ± 0.1	3.7 ± 1.2	4.0 ± 1.2	3	8
LT-8 wild type	0.4 ± 0.1	3.8 ± 1.5	4.2 ± 1.4	3	10
LT-10 wild type	0.3 ± 0.2	3.5 ± 1.1	3.8 ± 1.1	3	8
E. coli					
K-12 derivatives					
Wild type	2.7 ± 0.7	8.7 ± 0.6	11.4 ± 0.7	7	24
AB1157	4.8 ± 1.6	9.6 ± 0.9	14.4 ± 2.1	3	33
821 (GSH^-)	<0.3	<0.4	<0.7	3	
B (Hopkins)	1.9	3.9	5.8	2	33
B (Black)	2.9	6.6	9.5	2	31
B/r	3.3 ± 1.2	3.7 ± 0.7	7.0 ± 0.9	4	47
H	0.4	4.6	5.0	2	8
Nine clinical isolates	<0.3	2.7 ± 6.8	2.7 ± 6.8	2	<4 ± 11

[a] The value of 100 Klett units equals approximately 6 × 10^8 cells per ml.

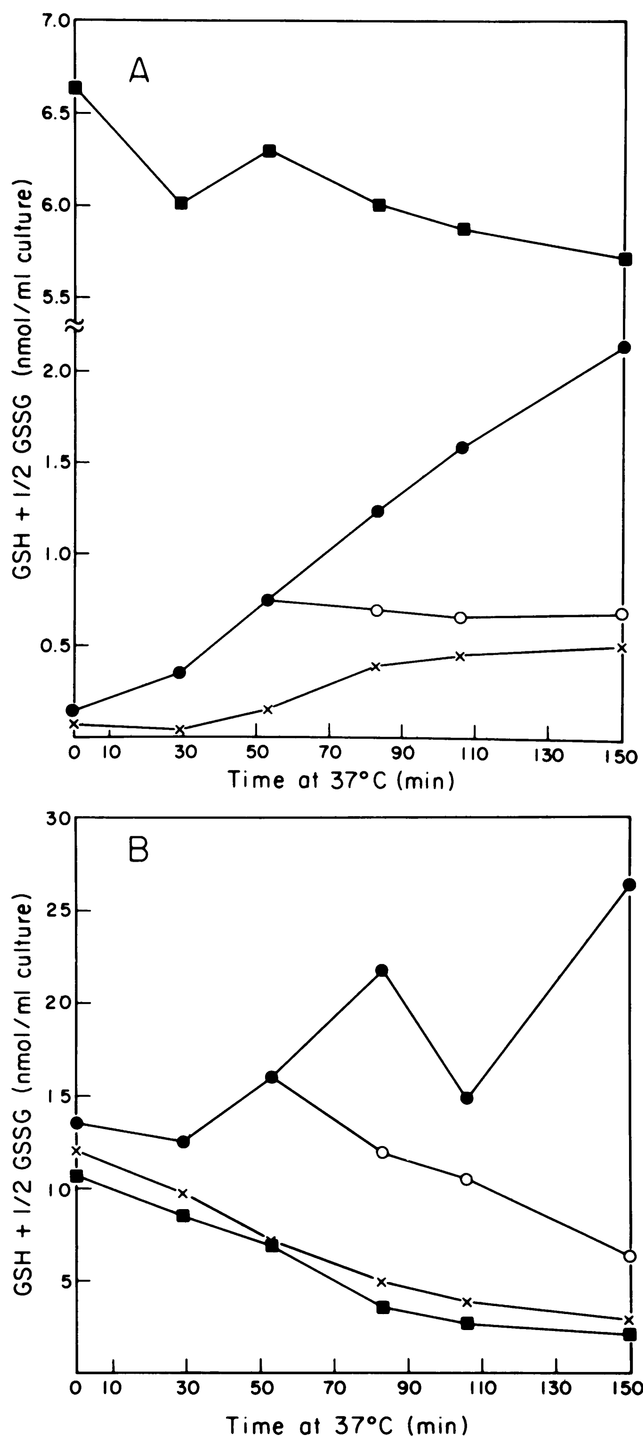

FIG. 2. Glutathione (as GSH equivalents) levels in supernatants (A) and pellets (B) of late-log-phase TA1534 cells washed and suspended in unsupplemented E medium. Symbols: ●, no azide; ○, azide added to 30 mM final concentration at time 63 min; X, azide added to 30 mM at time zero; ■, azide added to 30 mM and GSH added to 6.5 nmol/ml at time zero.

caused a decline in the intracellular glutathione levels (Fig. 2B). Azide did not interfere with the glutathione assay (Fig. 2A).

Internal and external glutathione: reduced versus oxidized. Measurements of TCA-soluble reduced thiols indicated that most, if not all, of the internal glutathione was in the reduced form (data not shown). GSH is known to be the predominant intracellular thiol in both *E. coli* and *S. typhimurium* (7, 8, 12). Cultures of strain 821 (GSH^-) contained very little TCA-soluble thiols (<10% of the level of its GSH^+ parent AB1157).

Measurements of thiols in the media of TA1534 cells indicated that 50% of the extracellular glutathione was in the reduced form (Table 3). Because results of reconstruction experiments showed that the oxidized glutathione in the media could be accounted for by oxidation during aerobic incubation as well as during sample processing (Table 3), it is concluded that the glutathione is predominantly exported in the reduced form.

TABLE 3. Levels of glutathione and reduced thiols in supernatants of early-stationary-phase cultures of TA1534[a]

Sample	Estimated GSH + $\frac{1}{2}$ GSSG[a] (nmol/ml)	Estimated total thiols (nmol/ml)	Estimated % GSH
Untreated supernatant	10	5	50
Supernatant + 23 nmol of GSH per ml[b]	31	17	55
70 nmol of GSH per ml[c]	68	37	54

[a] Expressed as reduced glutathione equivalents.
[b] GSH was added immediately before the bacteria were sedimented.
[c] GSH was added to the medium with no bacteria present.

Strain survey. *E. coli* strains generally contained higher GSH levels than did *S. typhimurium* strains (Table 2). In contrast to nine fresh clinical isolates of *E. coli* which did not export GSH, laboratory strains both of *E. coli* and *S. typhimurium* contained GSH and GSSG in the culture media. Exceptions were LT2 derivatives *hisG46* and *hisD3052*, which failed to export GSH. There was no correlation between the level of GSH export and any known genotype of the strains tested except GSH^- *E. coli* 821, which had neither internal nor external glutathione.

Paper chromatography of ^{35}S-labeled culture supernatants. Two-dimensional paper chromatography was performed on supernatants from cultures labeled with $Na_2{}^{35}SO_4$. Eight major spots of sulfur-containing material were detected in the media of *Salmonella* TA1534 and *E. coli* AB1157 (Fig. 3). Corresponding spots from the media of the two strains had identical R_f values.

Spot 1 comigrated with inorganic sulfate. Spot 2 comigrated with GSSG, but it may have contained another compound(s) because it was also seen in the media of *hisG46* cultures which did not export glutathione. Spot 4 comigrated with GSH. Spot 4 was not detected in the media of either strain *hisG46* or strain 821 (*E. coli* GSH^-) cultures (data not shown).

Spots 3, 5, 6, 7, and 8 were not identified. Media of cultures of AB1157, 821 (*E. coli* GSH^-), and *hisG46* contained compounds with identical R_fs (data not shown). Thus, the five unidentified compounds were not derivatives of GSH. No differences in the intensities of any of the ^{35}S-labeled spots could be detected when cultures of TA1534 were grown in the presence of unlabeled 134 μM L-methionine (data not shown). Therefore, they were probably not methionine derivatives or intermediates in methionine biosynthesis. Because glutathione could account for all the thiols in the media, the unknowns were probably not thiols. Spot 8 had a concentration in the media greater than that of glutathione, in terms of the ^{35}S label. All of the ^{35}S-labeled

unknowns were found in greater quantities in the media than in TCA extracts of the pellets (data not shown).

We attempted to identify these compounds by testing many known sulfur-containing, biologically relevant chemicals, separately and by cochromatography, in our paper chromatography systems. None of the compounds tested matched the R_fs of the ^{35}S-labeled unknowns. The compounds tested were *S*-adenosyl-L-methionine, *S*-carboxymethyl-L-cysteine, L-cystathionine, L-cysteine sulfinic acid, L-cysteine, L-cystine, *S*-ethyl-L-cysteine, L-cysteine ethyl ester, L-homocysteic acid, D,L-homocysteine, D,L-homocystine, L-methionine, *S*-methyl cysteine, *S*-methyl glutathione, L-cysteine methyl ester (all from Sigma); L-cysteic acid (Nutritional Biochemicals Corp., Cleveland, Ohio); *S*-sulfocysteine (Pierce); and di-γ-glutamylcystine and γ-glutamylcysteine (see above).

GSH uptake by strain 821. After strain 821 (GSH^-) cultures were grown for several generations in media containing micromolar levels of either GSH or GSSG, some of the glutathione was detected in the pellet fraction. With GSH, 10% of the glutathione was in the pellet. With GSSG, 3% of the glutathione was in the pellet. In either case, the pellet-associated glutathione was predominantly in the reduced form (Table 4). The internal level achieved with added GSH was about one sixth that present in pellets of the GSH^+ parent strain AB1157 (Table 4).

Cysteine-deficient mutants of *S. typhimurium* grew normally in the presence of 200 μM GSH, but grew only minimally (they underwent one doubling in 24 h) in the presence of 200 μM GSSG (unpublished data). In experiments with *cys* mutants it was not determined whether the GSH was taken up intact or hydrolyzed first.

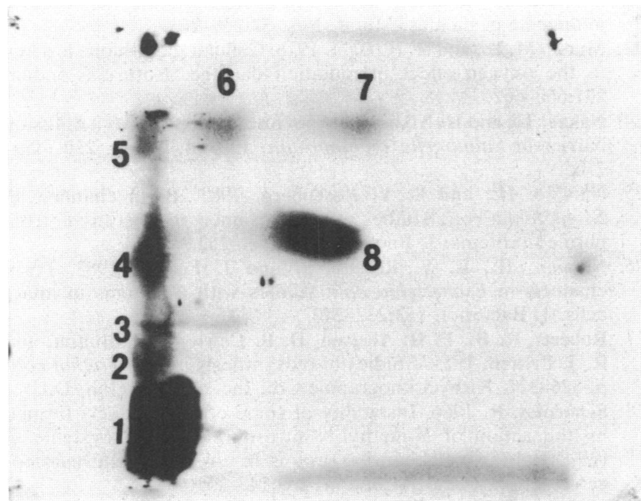

FIG. 3. Autoradiogram of a two-dimensional paper chromatogram of supernatant from a ^{35}S-labeled TA1534 culture. A culture of TA1534 was grown for 6 h at 37°C in 12 ml of his-bio-E medium containing 1.1 mCi of $Na_2{}^{35}SO_4$. At the early stationary phase, 10 ml of culture was centrifuged for 5 min at 12,000 × *g* and at 4°C. The supernatant was then mixed with an equal volume of 10% TCA. The mixture was then incubated for 1 h on ice and centrifuged for 5 min at 12,000 × *g* and at 4°C. A total of 20 μl of the decanted mixture was applied to Whatman no. 1 filter paper, and ascending paper chromatography was then performed with the butanol-acetic acid-water solvent for the first dimension (bottom to top) and the 2-propanol–ammonia–water solvent for the second dimension (left to right). Autoradiography was then performed for 21 days with Kodak XAR5 x-ray film. Spot numbers are identified in the text.

TABLE 4. Uptake of glutathione by strain 821 (*E. coli* GSH^-), after 6.5 h of growth at 37°C

Sample	Estimated internal GSH + $\frac{1}{2}$ GSSG (nmol/ml of culture)	Estimated internal total thiols (nmol/ml of culture)	Estimated % internal GSH
AB1157 (GSH^+)	23.3	20.9	90
821 (GSH^-)	<0.2	<1.0	
821 + 32.5 nmol of GSH per ml	3.6	2.8	78
821 + 16.3 nmol of GSSG per ml	0.9	1.0	100

DISCUSSION

The data presented here demonstrate the export of glutathione by several widely used laboratory strains of *S. typhimurium* and *E. coli* (Table 2 and Fig. 1 to 3). Some of the *E. coli* strains had higher external and internal GSH levels than did the *S. typhimurium* strains tested (Table 2). This apparent species difference may be reflective of a variability in glutathione levels between diverse strains, as found among both the *S. typhimurium* and the *E. coli* strains (Table 2), or it may reflect a true difference between the two species. Fahey et al. (7) observed considerable variability (<0.02 to 27 μmol/g) in the glutathione contents of different species of gram-negative bacteria. *E. coli* had the highest GSH level of the bacteria in that study, although no *Salmonella* strains were tested (7).

Micromolar levels of exogenous GSH can protect bacteria from otherwise toxic micromolar concentrations of heavy metals and other thiol-reactive agents (R. A. Owens and P. E. Hartman, Environ. Mutagen., in press). Extracellular GSH, cysteine, or both accelerate the chemical decomposition and inactivate the mutagenicity of nitrosocarbamates (3, 28), nitrosamides (3), *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (14, 18), and certain pesticides (22). Some standard laboratory media, to which laboratory strains have been repeatedly exposed over a period of several decades, contain direct-acting mutagens (19). It seems possible that laboratory strains, particularly during colonial growth or during growth in agar stab cultures, undergo beneficial adaptations that promote GSH export. This export could serve as an extracellular detoxification mechanism or it could be advantageous in some other way, for example, by promoting maintenance of cell surface thiols in a reduced state (13). On the other hand, freshly isolated *E. coli* strains fail to export GSH. It is very likely that these strains have experienced mobile and fluctuating environments, preventing localized extracellular GSH accumulation. Thus, important parameters of the physiology of traditional prototypic bacterial stock cultures may not precisely reflect the situation with regard to strains of the same species that exist under natural conditions.

The porin channels in the outer membranes of *E. coli* and *S. typhimurium* allow the passage of aqueous solutes as large as 600 daltons (4, 24–26). These porin channels, however, favor the passage of cations and uncharged compounds versus net anions such as GSH (307 daltons) or GSSG (612

daltons) (25, 26). External accumulation of glutathione in cultures of *S. typhimurium* TA1534 was inhibited by 30 mM NaN_3 (Fig. 2A). Azide treatment also caused a decline in the intracellular glutathione levels (Fig. 2B). Thus, either the maintenance of internal glutathione levels and glutathione export are both energy dependent or the two processes are coupled.

Five other sulfur-containing compounds detected in the culture media of laboratory strains (Fig. 3) remain unidentified. It is possible that these unknowns were fragments of surface macromolecules that were passively released into the media (cf. reference 9). This would explain why these five compounds were not detectable in the TCA extracts of bacterial pellets. On the other hand, the unidentified sulfur-containing compounds could be some other simple metabolites, the locations of which we did not determine on our chromatograms, for example thiosulfate, sulfite, or sulfide.

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