APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1984, p. 653–657
0099-2240/84/040653-05$02.00/0

Vol. 47, No. 4

Leakage of Glutathione from Bacterial Cells Caused by Inhibition of γ-Glutamyltranspeptidase

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Received 4 November 1983/Accepted 30 January 1984

Glutathione leaked from cells of *Proteus mirabilis* grown in medium containing an inhibitor of γ-glutamyltranspeptidase. In medium containing 100 mM L-serine and borate, up to 300 μM glutathione accumulated. L-Serine in the medium was consumed during the logarithmic phase of growth, γ-glutamyltranspeptidase activity was restored, and glutathione decreased in the medium. In the presence of 2 mM 6-diazo-5-oxo-L-norleucine, cells increased normally, γ-glutamyltranspeptidase was inhibited completely, and the maximum concentration of glutathione which accumulated in the medium was 20 μM. The glutathione content of cells rose before leakage began. Glutathione leaked from intact cells of other bacteria, although to a lesser extent than was seen with *P. mirabilis*.

γ-Glutamyltranspeptidase (γ-GTP) catalyzes the hydrolysis of glutathione (GSH) to glutamate and cysteinylglycine and the transfer of the γ-glutamyl moiety of the substrate to certain amino acids and peptides (2, 25). The catalytic properties (28), reaction mechanism (15, 16), and localization (14, 29) of the enzyme from rat kidney have been investigated in detail. Several proposals have been made concerning the function of the enzyme (3, 7, 18, 26) in mercapturate biosynthesis (4, 5) and other biological processes. Inhibition of γ-GTP was reported to be accompanied by extensive glutathionuria in mice (12). Translocation of intracellular GSH to the medium was studied in lymphoid cells, and GSH exported from the cells was found to be recovered by the cell in the form of transpeptidation or hydrolysis products formed by the action of membrane-bound γ-GTP (13).

The breakdown of GSH and transpeptidation of its γ-glutamyl residue by whole cells and extracts of a bacterium (*Proteus vulgaris*) was reported for the first time by Talalay (27). γ-Glutamyl transfer activity was found to be widely distributed in bacterial species (20), and its specificity for γ-glutamyl acceptors was investigated with a cell-free preparation of *Proteus morganii*.

GSH plays many important physiological roles in mammals and is utilized as a treatment for hepatic diseases; it is also reported to repress liver tumors (23). In the course of investigating GSH production by bacteria, we found that *Proteus mirabilis* has high activities of γ-glutamylcysteine synthetase (EC 6.3.2.2) (17), glutathione synthetase (EC 6.3.2.3), and γ-GTP (EC 2.3.2.2). γ-GTP activity was detectable with intact cells of bacterium, and the addition of an inhibitor of the enzyme during cultivation resulted in leakage of GSH into the medium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this work were obtained from stock cultures in the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan. Each strain was grown on a medium containing citric acid · H_2O, 7.0 g; glucose, 10 g (separately autoclaved); peptone, 10 g; L-threonine, 25 mg; L-leucine, 50 mg; L-proline, 25 mg; L-arginine, 50 mg; L-histidine, 10 mg; thiamine-hydrochloride, 1 mg; K_2HPO_4, 10 g; $NaNH_4HPO_4 \cdot 4H_2O$, 10 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g, in 1 liter of tap water. The pH was adjusted to 8.0, and cultivation was carried out at 28°C in a reciprocal shaking flask containing 100 ml of the medium. Inhibitors of γ-GTP were added only to the main culture at the concentration specified, and the pH of the medium was adjusted to 8.0. 6-Diazo-5-oxo-norleucine (DON) solution was added to the autoclaved medium after filtration with a membrane filter.

γ-GTP assay. The enzyme activity of *P. mirabilis* cells was determined with L-γ-glutamyl-*p*-nitroanilide and glycylglycine (24). The assay solution contained 5.0 μmol of L-γ-glutamyl-*p*-nitroanilide, 120 μmol of glycylglycine, 100 μmol of Tris-hydrochloride (pH 8.0), and bacterial cells (approximately 10^5 cells) in a final volume of 2.0 ml. The cells were washed twice with 0.8% NaCl solution and suspended (10^6 cells per ml) in 50 mM Tris-hydrochloride (pH 8.0). Cell suspensions were prepared at 0 to 5°C. The reaction was started by the addition of 0.2 ml of cell suspension. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 1 ml of 3.5 N acetic acid, and cells were removed by centrifugation. The absorbance of the supernatant solution was measured at 410 nm. Activity was expressed as the number of micromoles of *p*-nitroaniline produced per minute. A blank test was carried out without glycylglycine, and the absorbance due to hydrolysis of the substrate was subtracted from the above value.

GSH and serine assay. GSH in the medium or cell extracts was assayed with glutathione reductase as described by Fahey et al. (9). It is a recycling enzymatic assay based on the oxidation of NADH linked to the enzymatic reduction of oxidized glutathione to GSH by glutathione reductase and the reoxidation of GSH by disodium tetrathionate, and it measures both GSH and oxidized glutathione. The assay solution contained 0.1 μmol of NADPH (Sigma Chemical Co.), 50 μmol of phosphate (pH 7.6), 2.5 μmol of EDTA, 0.5 U of yeast glutathione reductase (Sigma), and 50 μmol of disodium tetrathionate. The glutathione content of cells was determined by the same method after extraction by boiling cells in 80% ethanol. L-Serine in the medium was assayed with an automatic amino acid analyzer (K-101-AS; Kyowa Seimitsu Co. Ltd., Tokyo, Japan) attached to a column (4.6

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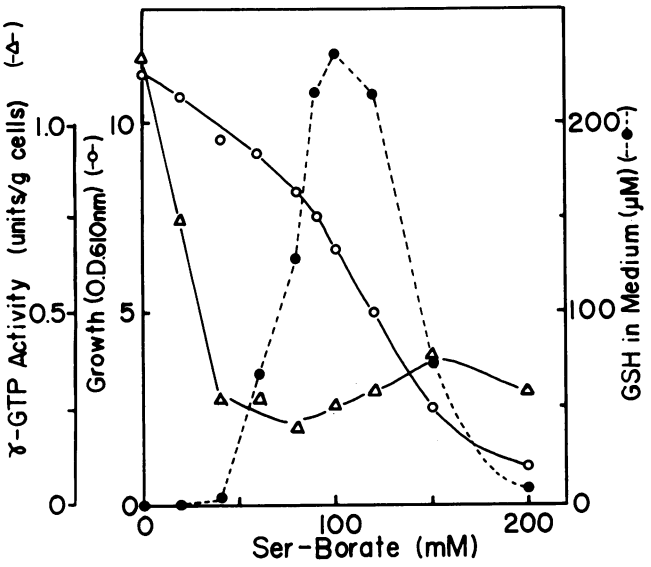

FIG. 1. Effect of concentration of serine-borate on leakage of GSH from *P. mirabilis*. Cultivations were carried out as described in the text for 24 h. Equal amounts of L-serine and borate were added to the medium. γ-GTP activity was expressed in units per gram of dry cells. O.D., Optical density.

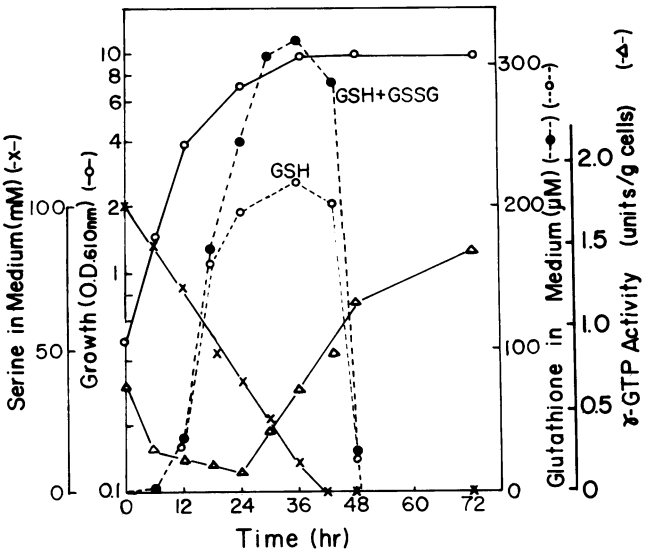

FIG. 2. GSH leakage during cultivation of *P. mirabilis* in the presence of serine-borate. Cultivation and assays were carried out as described in the text. --○--, Concentration of reduced thiol compounds in the medium. O.D., Optical density; GSSG, oxidized glutathione.

by 150 mm) of Kyowa gel 62210-S. Elution was carried out with 0.2 M citrate buffer (pH 3.25) containing 8% ethanol. Reduced-form thiol compounds were assayed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by the method of Ellman (8).

All cultivations and assays were carried out in duplicate.

RESULTS

Effect of serine-borate concentration on GSH leakage. Cell growth, γ-GTP activity, and the amount of GSH in the medium were measured after cultivation of *P. mirabilis* in medium containing various amount of L-serine and borate (Fig. 1). At concentrations of 100 mM L-serine-borate, cell growth was around half of that in the absence of the inhibitor, and at 200 mM L-serine-borate almost no growth was observed. The highest amount of leakage of GSH was observed at a concentration of 100 mM. γ-GTP activity decreased rapidly with inhibitor concentrations of up to 40 mM and remained almost constant at higher concentrations.

GSH leakage and γ-GTP activity during cultivation. *P. mirabilis* was cultivated in the presence of 100 mM L-serine-borate, and cell growth, GSH leakage, γ-GTP activity, and L-serine consumption were followed for 72 h (Fig. 2). In the absence of inhibitor, the bacteria usually reached the stationary phase after 24 h of cultivation; in the presence of L-serine-borate, growth was delayed. L-Serine in the medium decreased linearly and disappeared completely after 42 h. γ-GTP activity decreased up to 24 h and increased thereafter, suggesting a correlation with the decrease of L-serine in the medium. GSH accumulated in the medium until the early stationary phase (36 h) and decreased thereafter with the increase of γ-GTP activity of the cells. Finally, GSH decreased below the limit of detection by the assay method. The amount of thiol compounds in the medium as measured by DTNB was lower than that of GSH, as determined by glutathione reductase, suggesting the existence of oxidized GSH.

Effect of L-serine and D-serine on GSH leakage in the presence and absence of borate. The use of L-serine-borate for inhibition of the transpeptidase requires both compounds to be present in the system (19). As control experiments for the specific relation of γ-GTP inhibition and GSH leakage, L-serine, D-serine, or borate alone was added to the medium at a concentration 100 mM, and cell growth, serine consumption, and GSH leakage were followed in the same manner as described above. In the presence of borate alone, cells grew as slowly as in the presence of L-serine-borate, but accumulation of GSH was lower than in the presence of L-serine-borate. In the presence of L-serine or D-serine, *P. mirabilis* showed the same growth curve as that obtained in their absence, and GSH accumulation was lower than in the presence of L-serine-borate. D-Serine was found to be consumed by the bacterium, as was L-serine in the presence or absence of borate (Table 1).

GSH leakage in the presence of DON. The effect of DON on γ-GTP activity, cell growth, and GSH leakage was investigated by the addition of various concentrations of DON to the medium (Fig. 3). γ-GTP activity was inhibited completely at concentrations higher than 1 mM. Cell growth was partially decreased by mM and higher concentrations of the inhibitor. GSH leakage was maximal (44 μM) at 2 mM DON. At this concentration of the inhibitor, cell growth, γ-GTP activity, and the leakage of GSH were followed for 60 h of cultivation (Fig. 4). Cell growth showed a normal curve,

TABLE 1. Effect of L-serine or D-serine on GSH leakage in the presence and absence of borate

Addition	GSH leaked (nmol/mg of cells)	
	24 h	36 h
None	0	0
Borate	0.64	0.49
L-Serine	0.78	0.70
L-Serine-borate	5.03	5.37
D-Serine	0.68	0.38
D-Serine-borate	3.41	2.47

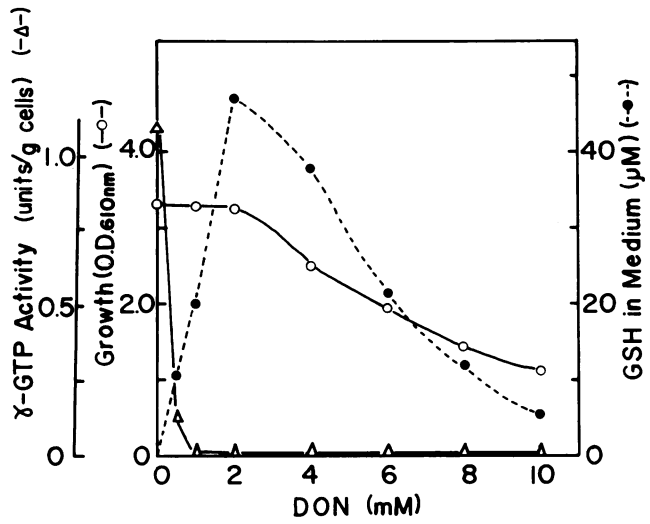

FIG. 3. Effect of concentration of DON on leakage of GSH from *P. mirabilis*. One-half milliliter of seed culture cultivated for 36 h without DON was inoculated into test tubes (1.6 by 16 cm) containing 5 ml of the medium and various concentrations of DON and cultivated at 30°C for 36 h with reciprocal shaking. O.D., Optical density.

whereas γ-GTP activity was lost after 6 h. GSH accumulated rapidly up to 12 h and then decreased gradually. The leakage of GSH was less than in the presence of L-serine-borate.

Intracellular concentration of GSH during cultivation of *P. mirabilis* in the presence of L-serine-borate. The GSH content of cells was followed during the cultivation of *P. mirabilis* in the presence of 100 mM L-serine-borate. The GSH content of cells rose just before leakage started and decreased thereafter (Table 2).

Leakage of GSH from cells of various bacterial strains.

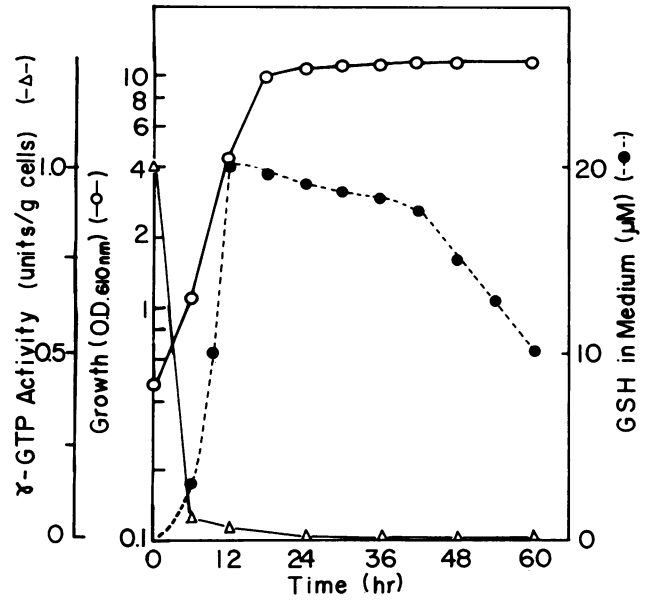

FIG. 4. GSH leakage during cultivation of *P. mirabilis* in the presence of DON. Cultivation was carried out as described in the legend to Fig. 3 in medium containing 2 mM DON. O.D., Optical density.

TABLE 2. Change in the GSH content of *P. mirabilis* during cultivation in the presence of L-serine-borate

Cultivation time[a] (h)	Intracellular GSH[b] (μmol/g of cells)
0	0.023
3	1.03
6	1.28
9	0.373
12	0.132
24	0.043
30	0.013
36	0.0056

[a] Cultivation conditions are described in the legend to Fig. 2.
[b] GSH content was measured after washing of cells and extraction of GSH.

Several bacterial strains were selected from the stock cultures and cultivated on the medium described above for 24 h without the inhibitor, since most bacterial strains employed could not grow well in the presence of 100 mM L-serine-borate. Harvested and washed cells were incubated in the same medium containing 100 mM L-serine-borate at 28°C. After 36 h of cultivation, the concentration of GSH in the supernatant of the incubation mixture was determined as described above. Leakage of GSH was observed in almost all bacterial strains (Table 3). A small amount of leakage was also observed in the absence of inhibition.

DISCUSSION

Although the presence of GSH in microorganisms has long been known (21), its distribution in bacteria has only recently been surveyed (10), and its basic function in microorganisms has not yet been clearly identified. Cheng et al. (6) found that GSH associated with vegetative cells disappeared during spore formation in *Bacillus cereus*. In *Neurospora crassa*, the status of GSH in conidia was reported to change during germination and aging (9). The isolation of GSH-deficient mutants of *Escherichia coli* (1, 11, 22) and investigation of the role of GSH with a mutant have elucidated its

TABLE 3. Leakage of GSH from bacterial cells by incubation with L-serine-borate

Strain	GSH leaked[a] (μmol/100 ml of culture)	
	−Serine-borate	+Serine-borate
Pseudomonas solanacearum	0.02	0.24
Agrobacterium radiobacter	0.20	0.99
Alcaligenes faecalis	0.00	1.03
Achromobacter superficialis	0.13	0.23
Escherichia coli	0.00	0.34
Serratia marcescens	0.22	0.27
Proteus mirabilis	0.79	4.60
Flavobacterium arborescens	0.33	1.15
Bacterium cadaveris	0.22	2.28
Micrococcus flavus	0.27	0.70
Sarcina aurantiaca	0.17	0.95
Corynebacterium pseudodiphteriticum	0.22	0.44
Arthrobacter simplex	0.27	0.54
Brevibacterium divaricatum	0.54	0.98

[a] Bacterial cells harvested from 100 ml of the medium described in the text were washed and suspended in the same medium with and without L-serine-borate at a cell concentration of 2 g of wet cells per 100 ml. After incubation at 28°C for 36 h, GSH in the medium was assayed as described in the text.

protective function against chemical challenges. Even though these recent investigations represent important advances toward understanding the function of GSH in microorganisms, its fundamental role in bacterial cells remains obscure.

γ-GTP activity is easily detectable with intact cells of *P. mirabilis* and is inhibited by treatment of the cells with antiserum to the purified enzyme. This suggests the localization of the enzyme near the cell surface, perhaps on the outside of the cytoplasmic membrane. We obtained a homogeneous preparation of γ-GTP from cells of *P. mirabilis* and found that it was inhibited by serine and borate or DON, which are known to be inhibitors of γ-GTP from mammalian sources (unpublished data).

The present data show that GSH leaked from cells of *P. mirabilis* upon the inhibition of γ-GTP by serine-borate. The concentration of L-serine, decreased activity of γ-GTP, and accumulation of GSH showed some correlation during the cultivation of *P. mirabilis*. This indicates that at first serine-borate inhibited γ-GTP, and GSH leaked from the cells and accumulated in the medium. Then, with the consumption of L-serine, γ-GTP regained its activity and degraded the accumulated GSH. When borate, L-serine, or D-serine alone was added to the medium, GSH did not appear in the medium to the extent seen with L-serine-borate. More than 65% of the total GSH (GSH plus oxidized glutathione) in the medium was found in the reduced form. The oxidation of GSH may take place non-enzymatically after leakage, with vigorous shaking during cultivation.

When DON was used as inhibitor, leakage of GSH did not increase after 12 h (Fig. 4), and the total accumulation of GSH was 15 times less than that when serine-borate was used, even though γ-GTP activity was inhibited throughout cultivation. These results indicate either that a GSH degradation system other than γ-GTP exists which operates after γ-GTP is inhibited or that synthesis of GSH is inhibited by the inhibitor. The large accumulation of GSH by serine-borate might also be explained by the bacterial utilization of L-serine as an energy source for growth or as a precursor of the amino acids constituting GSH. Since D-serine-borate is also an inhibitor for γ-GTP (19) and D-serine was not expected to be degraded by *P. mirabilis*, D-serine plus borate was expected to provide a control to eliminate the additional actions of L-serine unrelated to transpeptidase inhibition. However, D-serine as well as L-serine was consumed by *P. mirabilis* in the presence and absence of borate.

The GSH content of cells rose before it began to be leaked from cells (Table 2), suggesting that a GSH degradation system like γ-GTP also exists intracellularly and is inhibited by serine-borate.

The results in this paper suggest that GSH and γ-GTP have common roles and functions in bacterial cells, as has been suggested for mammalian cells on the basis of findings in lymphoid cells (13). Bacterial cells might thus be a convenient system with which to investigate the function of GSH in living cells. Also, the leakage of GSH from bacterial cells is an interesting phenomenon which might be used to search for an effective method to produce GSH industrially by using microorganisms.

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