Some Properties of Glutathione Biosynthesis-deficient Mutants of *Escherichia coli* B

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Mutants of *Escherichia coli* B that contain essentially no detectable glutathione were isolated. These mutants had a very low activity of γ -glutamylcysteine synthetase or glutathione synthetase. No significant differences in growth in minimal medium were observed between the mutants and the parental strain. The mutants lacking γ -glutamylcysteine synthetase activity were more susceptible to toxic compounds than either the parental strain or a glutathione synthetase-deficient strain. The mutants lacking γ -glutamylcysteine synthetase activity were also susceptible to oxygen.

INTRODUCTION

We have been studying the metabolism of glutathione in order to elucidate its functions in *Escherichia coli* B. One way of doing this is to observe the effect of the intracellular oxidation of glutathione by chemical agents such as diamide (Kosower *et al.*, 1969), azoester (methylphenyldiazene carboxylate) or *t*-butyl hydroperoxide (Srivastava *et al.*, 1974). These agents have been shown to be effective in oxidizing intracellular glutathione, but in many cases they also cause a reduction in cell viability. Therefore, in studying the functions of glutathione in cells, it would be advantageous to use mutants deficient in glutathione biosynthesis. Recently, we developed an efficient method for isolating such mutants of *E. coli* B and have obtained mutants deficient in the activity of γ -glutamylcysteine synthetase or glutathione synthetase (Murata *et al.*, 1981). This paper reports the properties of the mutants and the physiological functions of glutathione in *E. coli* B.

METHODS

Chemicals. Reduced and oxidized glutathione and acetyl phosphate were purchased from Sigma. γ -Glutamylcysteine was prepared by the method of Strumeyer & Block (1962). All other chemicals were analytical grade reagents.

Bacterial strains, culture conditions and preparation of cell extracts. The strains used and their properties are listed in Table 1. All strains were grown in the minimal medium of Davis & Mingioli (1950), with or without nutritional supplements or other chemicals as indicated. Glucose was autoclaved separately. Other culture conditions and the preparation of cell extracts were as previously described (Murata *et al.*, 1980). Protein was determined by the Lowry method.

Isolation of mutants. The glutathione biosynthesis-deficient mutants were isolated by the method described previously (Murata *et al.*, 1981). Revertants of the γ -glutamylcysteine synthetase-deficient strain C912 were isolated by the following procedure. Cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) by the method of Adelberg *et al.* (1965). Incubation was carried out with 200 μ g NTG ml⁻¹ at 30 °C for 30 min. After the treatment, cells were washed with water and then suspended in L-broth [comprising 1.0% (w/v) polypeptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract and 0.1% (w/v) glucose (pH 7.2)]. The culture was grown at 37 °C for several hours to allow expression of the phenotype. After washing with saline solution, cells were spread on agar plates of minimal medium supplemented with 10 μ g 8-hydroxyquinoline ml⁻¹. Plates were incubated at 37 °C for 2 d and then the colonies were tested for their ability to synthesize γ -glutamylcysteine by the method of Jackson (1969). One of the revertants was designated RC912.

Table 1. *Escherichia coli* B strains used

Strain	Parent	Relevant properties*
355	—	Wild-type
M71	355	Cys ⁻
M910	M71	Mg ^R
C912	M910	Mg ^R , GshA ⁻
C915	M910	Mg ^R , GshA ⁻
RC912	C912	Mg ^R
C1001	M910	Mg ^R , GshB ⁻

* Cys⁻, requirement for cysteine; Mg^R, resistant to methylglyoxal; GshA⁻, deficient in γ -glutamylcysteine synthetase activity; GshB⁻, deficient in glutathione synthetase activity.

Enzyme assays. The activities of γ -glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) were determined by the methods of Jackson (1969) and Mooz & Meister (1967), respectively.

Determination of intracellular concentrations of glutathione and total thiol compounds. Cells growing exponentially in minimal medium were harvested and washed once with cold 0.85% (w/v) saline solution. Ethanol (3.0 ml; 80%, w/v) was added to 0.5 g wet wt cells and the thiol compounds were extracted by boiling for 5 min. The clear supernatant obtained after centrifugation was used for the determination of glutathione, as described by Murata *et al.* (1978), and total thiol compounds, by titration with Ellman's reagent (Ellman, 1959). All results were expressed as μmol glutathione or thiol compounds (g wet wt cells)⁻¹.

Determination of minimum inhibitory concentrations. The gradient plate technique of Szybalsky & Bryson (1952) was used. The method employed a press-bottom Petri plate (10 cm diam.) with two layers of agar. The lower layer, consisting of 10 ml minimal agar medium, was allowed to harden with the plate slanted to cover the entire bottom. The second layer, consisting of 10 ml minimal agar medium containing an appropriate concentration (C_0 , $\mu\text{g ml}^{-1}$) of the test compound, was added with the plate in the normal horizontal position. The plates were incubated at 37 °C for 24 h after streaking with cells. The length of the growing streak (L , cm) was measured and the minimum inhibitory concentration (C , $\mu\text{g ml}^{-1}$) was calculated using the equation: $C = LC_0/10$.

Survival curves. Cells growing exponentially in minimal medium were harvested and resuspended in 3.0 ml minimal medium containing the test compound at a density of 5×10^8 cells ml⁻¹. The suspension was incubated at 37 °C with shaking. At appropriate intervals, samples were taken, diluted, and spread on L-broth agar plates. The plates were incubated at 37 °C to determine the number of surviving cells.

RESULTS

Activities of enzymes involved in glutathione biosynthesis. The activities of γ -glutamylcysteine synthetase and glutathione synthetase were determined to locate the step in glutathione biosynthesis which was blocked in the mutant strains (Table 2). As reported previously (Murata *et al.*, 1981), strains C912 and C915 were deficient in γ -glutamylcysteine synthetase activity. Strain C1001 lacked glutathione synthetase activity. Strain RC912, a revertant of C912, had fully recovered the activity of γ -glutamylcysteine synthetase.

Intracellular concentrations of glutathione and total thiol compounds. The amounts of intracellular glutathione and total thiol compounds in the mutant and parental strains were determined (Table 3). In the parental strains (355 and M910), glutathione represents about 35–40% of the total thiol compounds. As suggested previously (Murata *et al.*, 1980), methylglyoxal resistance was associated with an increase in the contents of glutathione and total thiol compounds. In the mutant strains (C912 and C1001), no significant amount of glutathione was detected. Higher concentrations of glutathione and total thiol compounds were found in RC912, a revertant of mutant C912.

Growth in minimal medium. Figure 1 shows that the maximum levels of growth of the mutant strains in minimal medium were somewhat lower than that of the parental strain. The addition of glutathione to the medium did not improve the growth of strain C912.

Susceptibility to inhibitors. The susceptibility of the mutant strains to various chemicals was investigated, since glutathione is thought to be responsible for the detoxification of

Table 2. Activities of γ -glutamylcysteine synthetase and glutathione synthetase in mutant and parental strains

Strain	Parent	γ -Glutamylcysteine synthetase [nmol min ⁻¹ (mg protein) ⁻¹]	Glutathione synthetase [nmol min ⁻¹ (mg protein) ⁻¹]
C900	M910	22.1	26.8
C912	M910	0.00	24.9
C915	M910	0.02	26.9
C964	M910	21.9	23.9
C1001	M910	24.9	0.01
C1065	M910	23.4	24.8
C1198	M910	22.3	23.8
RC912	C912	24.8	23.7
355	Wild	14.2	16.2
M910	355	23.8	24.9

Table 3. Glutathione and total thiol contents of mutant and parental strains

Strain	Susceptibility to methylglyoxal	Total thiol [μ mol (g cells) ⁻¹]	Glutathione [μ mol (g cells) ⁻¹]
355	Sensitive	8.2	2.9
M910	Resistant	9.6	3.8
C912	Resistant	3.9	0.04
C1001	Resistant	4.9	0.02
RC912	Resistant	10.7	4.7

Fig. 1

Fig. 2

Fig. 1. Growth of mutant and parental strains in minimal medium. Overnight cultures in minimal medium were harvested and washed once with 0.85% saline solution. Samples of cells were resuspended in 3.0 ml minimal medium (supplemented with 0.1 mM-glutathione where indicated) and incubated at 37 °C with shaking. Growth was monitored by measurement of the absorbance at 660 nm as described previously (Murata *et al.*, 1980). Strains: ●, M910; ○, C1001; ◐, C912; ◑, C912 + 0.1 mM-glutathione.

Fig. 2. Decrease in cell viability during incubation in the presence of TMTD (a) and 8-HQ (b). Cells growing exponentially in minimal medium were harvested, washed once with 0.85% saline solution and resuspended in 3.0 ml minimal medium containing 50 μ g TMTD ml⁻¹ or 40 μ g 8-HQ ml⁻¹ (and supplemented with 0.1 mM-glutathione where indicated). The incubation was at 37 °C with shaking. The number of viable cells after each treatment was determined by counting the colonies that appeared on nutrient agar plates as described in the Methods. Strains: ●, M910; ○, C1001; ◐, C912; ◑, C912 + 0.1 mM-glutathione.

Table 4. *Minimum inhibitory concentrations of various compounds*

Compound	Strain ...	Minimum inhibitory concentration ($\mu\text{g ml}^{-1}$)			
		355	M910	C912	C1001
HgCl ₂		4.0	5.0	0.1	0.4
Iodoacetamide		75	92	20	40
Tetramethylthiuram disulphide (TMTD)		90	100	5.5	10
8-Hydroxyquinoline (8-HQ)		15	20	4.0	10
Propyl 4-hydroxybenzoate		95	120	40	75
Methylglyoxal		25	40	5.5	7.5
Chloramphenicol		3.5	4.0	8.0	8.5
Dibromophenol		120	140	75	85
<i>m</i> -Cresol		1000	1000	900	1000
Tetrabromo- <i>m</i> -cresol		500	550	50	120
Thymol		250	300	150	200
Tetrabromophenolphthalein		95	130	55	75
Chloromethylketone		250	250	80	110

Fig. 3. Effect of culture volume on the growth of mutant and parental strains. Cells were grown in various volumes of minimal medium containing $10 \mu\text{g TMTD ml}^{-1}$. Incubation was at 37°C , with shaking for 32 h, using 500 ml Sakaguchi flasks. Strains: ●, M910; ◐, C912; ○, C1001.

compounds unfavourable to growth. The chemicals tested were typical thiol-reactive agents (HgCl₂, iodoacetamide), a metal-chelating agent (8-hydroxyquinoline), an antibiotic (chloramphenicol) and others (mostly inhibitors of γ -glutamyltranspeptidase). As can be seen in Table 4, the γ -glutamylcysteine synthetase-deficient strain (C912) was highly susceptible to various inhibitors, with the exception of chloramphenicol, whereas the glutathione synthetase-deficient strain (C1001) was somewhat more resistant to these compounds. The specific interactions of these compounds with glutathione were not elucidated in this study.

Figure 2 shows the change in viability of mutant and parental strains exposed to tetramethylthiuram disulphide (TMTD) and 8-hydroxyquinoline (8-HQ). A marked decrease in viability was observed for the γ -glutamylcysteine synthetase-deficient strain (C912). The addition of glutathione to the mixture with TMTD or 8-HQ protected the cells of strain C912.

Susceptibility to oxygen. Figure 3 shows the growth of mutant and parental strains in different volumes of minimal medium containing $10 \mu\text{g TMTD ml}^{-1}$. Growth of the parental strain (M910) was unaffected by the volume of the medium, whereas inhibition of the growth of the γ -glutamylcysteine synthetase-deficient strain (C912) increased as the volume of the medium decreased. The effect of the culture volume on growth was less severe for the glutathione synthetase-deficient mutant (C1001).

DISCUSSION

Mutants of *E. coli* B deficient in glutathione biosynthesis were easily isolated by a previously described method (Murata *et al.*, 1981). An alternative method for the isolation of such mutants, a colony colour technique, has been reported by Apontowiel & Berends (1975). However, the application of their method is limited to bacterial strains with extremely low concentrations of intracellular thiol compounds other than glutathione. Cells containing larger quantities of thiol compounds will still produce a red colour even in the absence of glutathione. This is the case with *E. coli* B, and thus the method is unsuitable for isolating glutathione biosynthesis-deficient mutants in this organism. Our method, using a cysteine auxotroph in combination with a methylglyoxal-resistant mutant, is simple and easy, and may be applied to all bacterial strains regardless of the intracellular content of thiol compounds.

Two types of mutants were isolated. Enzymic analysis showed that one type lacked γ -glutamylcysteine synthetase activity (C912 and C915) and the other lacked glutathione synthetase activity (C1001). The defect in glutathione biosynthesis was further supported by the fact that the mutants contained little glutathione. All other mutants obtained showed both γ -glutamylcysteine synthetase and glutathione synthetase activity: these may be the revertants of M910, a methylglyoxal-resistant strain which excretes glutathione into the growth medium (Murata *et al.*, 1980).

The growth of the glutathione biosynthesis-deficient mutants in minimal medium was not significantly affected by glutathione deficiency. As originally suggested by Apontowiel & Berends (1975) and by Fuchs & Warner (1978), this indicates that glutathione is not specifically required for any process in the cells for their proliferation. This is of considerable interest, since glutathione represents approximately 40% of the total thiol compounds in *E. coli* B cells.

In agreement with the results of Apontowiel & Berends (1975), glutathione was found to be a significant factor for the protection of cells exposed to toxic compounds. The mutant deficient in γ -glutamylcysteine synthetase activity was more susceptible to various inhibitors than either the parental strain or the mutant deficient in glutathione synthetase activity. It seems likely that γ -glutamylcysteine, a product of the γ -glutamylcysteine synthetase reaction, can substitute for glutathione in some, but not all, instances. This result suggests that the mutation was brought about only in the region of *gshA*, not in *gshB*, though NTG treatment usually induces multiple mutations (Adelberg *et al.*, 1965). Kosower & Kosower (1969) showed that lysis occurs when a chemical challenge is made in the absence of glutathione and they termed this phenomenon the 'glutathione loss catastrophe'. The same phenomenon has been recognized by many other investigators, though the chemical interactions of glutathione with these substances remain obscure. The glutathione biosynthesis-deficient mutants were also susceptible to the amount of aeration, unlike the parental strains. In addition, a high susceptibility to lysozyme was found for the glutathione biosynthesis-deficient mutants (data not shown). Thus, glutathione may play an important role in the maintenance of the rigidity of cell walls.

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