SHORT COMMUNICATION

Excretion of Glutathione by Methylglyoxal-resistant *Escherichia coli*

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(*Received* 19 *March* 1980; *revised* 19 *May* 1980)

A methylglyoxal-resistant mutant of *Escherichia coli* B excreted glutathione into the growth medium, especially during growth on medium containing methylglyoxal. In the presence of methylglyoxal, the total amount of glutathione excreted was increased about 50-fold over that of the wild-type strain. The resistant mutant had high activities of two enzyme systems: a glutathione-forming enzyme system (consisting of γ-glutamylcysteine synthetase and glutathione synthetase) and a glyoxalase system (consisting of glyoxalase I and glyoxalase II). Methylglyoxal resistance appeared to be due to the simultaneous increase in the activities of these two enzyme systems.

INTRODUCTION

Methylglyoxal (MG) is a toxic metabolite for *Escherichia coli* at millimolar concentrations (Ackerman *et al.*, 1974; Freedberg *et al.*, 1971; Krymkiewicz *et al.*, 1971). It is synthesized via a non-phosphorylated pathway from dihydroxyacetone phosphate (Cooper & Anderson, 1970). This pathway is considered to be finely regulated and regulation mechanisms have been studied in detail by Hopper & Cooper (1972), Freedberg *et al.* (1971) and many other investigators. Hopper & Cooper (1972) showed that the activity of MG synthase was regulated by inorganic phosphate. Freedberg *et al.* (1971) also showed that the increased capacity for MG metabolism in MG-resistant cells was due to elevated activity of the glyoxalase system, which requires the participation of glutathione for activity.

However, no report on the regulation of activity of the glyoxalase system has described the change in the concentration of glutathione in bacteria and the change in activity of the glutathione-forming enzyme system. To clarify the mechanism for MG resistance further, we have investigated the relationship between activities of enzymes involved in MG metabolism and glutathione synthesis in MG-resistant *E. coli.*

METHODS

Chemicals. Methylglyoxal (MG) and dihydroxyacetone phosphate (DHAP) were purchased from Sigma. All other chemicals were analytical grade reagents.

Micro-organisms. Micro-organisms used and their properties are listed in Table 1.

Isolation of an MG-resistant mutant (RM-9-10). Strain RM-7-23 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg *et al.* (1965) and spread on agar DM medium [comprising (per litre) 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0·1 g $MgSO_4.7H_2O$, 5 g glucose] containing 1·4 mM-MG. After 7 d at 30 °C, the largest colony formed was transferred to 2·0 ml of liquid DM medium containing

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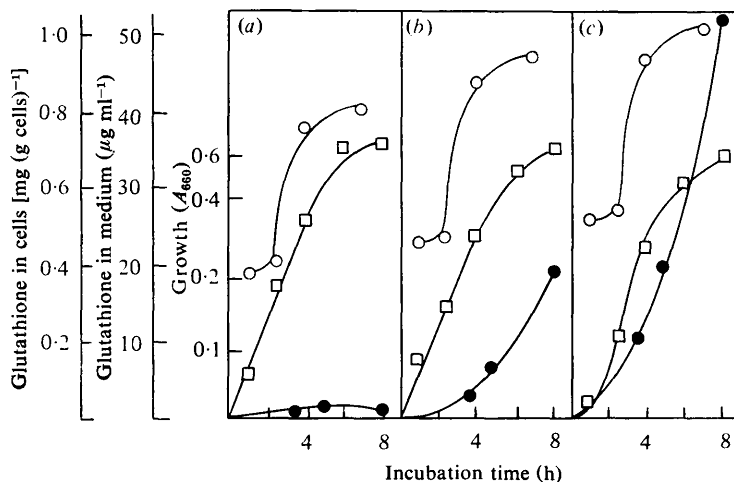

Fig. 1. Excretion of glutathione into the medium by wild-type *Escherichia coli* strain 355 and MG-resistant strain RM-9-10. The wild-type strain was grown in DM medium in the absence of MG (*a*). The MG-resistant strain was grown in DM medium in the absence (*b*) or presence (*c*) of 1·4 mM-MG. Growth was followed turbidimetrically at 660 nm. ●, Glutathione in medium; ○, glutathione in cells; □, growth.

Table 1. *Activities of enzymes involved in MG and glutathione metabolism*

Enzyme activities were determined using extracts prepared from cells growing exponentially on DM medium with the addition indicated. Assay conditions are described in Methods.

			Enzyme activities			
				Glyoxalase system†		
Strain	Properties	Addition to DM medium	MG synthase*	−GSH	+GSH	Glutathione-forming system‡
355	Wild-type	None	0·28	0·031	0·072	0·13
M-7	Cysteine auxotroph	L-Cysteine, 0·5 mM	0·27	0·030	0·068	0·12
RM-7-23	Revertant of M-7	None	0·25	0·030	0·061	0·11
RM-9-10	Resistant to 1·4 mM-MG	None	0·29	0·042	0·140	0·19
RM-9-10	Resistant to 1·4 mM-MG	MG, 1·4 mM	0·31	0·045	0·160	0·26

* Expressed as μmol MG formed min^{-1} (mg protein)$^{-1}$.

† Expressed as μmol MG degraded min^{-1} (mg protein)$^{-1}$; activities were assayed in the presence or absence of 1·0 mM-glutathione (GSH).

‡ Expressed as μmol glutathione formed h^{-1} (mg protein)$^{-1}$.

1·4 mM-MG. Cultivation was carried out with reciprocal shaking at 30 °C for 16 h and then samples of appropriately diluted culture were spread on agar DM medium containing 1·4 mM-MG. After 4 d at 30 °C, the largest colony formed was selected as an MG-resistant mutant and designated RM-9-10.

Assay of the glyoxalase system. Cells growing exponentially on 150 ml DM medium at 30 °C with shaking were harvested, washed once with 5·0 mM-KH_2PO_4/K_2HPO_4 buffer (pH 6·6) and suspended in the same buffer. This cell suspension was disrupted ultrasonically at 90 kHz for 5 min and then centrifuged at 25000 *g* for 30 min. The resultant supernatant was dialysed against the same buffer overnight. For determination of the activity of the glyoxalase system [glyoxalase I (EC 4.4.1.5) and glyoxalase II (EC 3.1.2.6)], a reaction mixture (1·0 ml) containing 0·5 mM-MG, 50 mM-KH_2PO_4/K_2HPO_4 buffer (pH 6·6) and dialysed extract (0·1 to 0·5 mg protein ml^{-1}) was incubated at 30 °C for 1 h and then the residual MG was determined colorimetrically by the method of Hopper & Cooper (1972).

Assay of the glutathione-forming enzyme system. Dialysed extracts were prepared as described above except that the buffer used contained 0·5 mM-L-cysteine. The activity of the glutathione-forming enzyme system [γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3)] was assayed by coupling with the ATP regeneration reaction catalysed by acetate kinase as described previously (Murata *et al.*, 1979). Glutathione formed was determined by the method of Tietze (1969).

Assay of MG synthase. Dialysed extracts were prepared as described above except that imidazole/HCl

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buffer (pH 7·0) was used instead of phosphate buffer. For the determination of MG synthase activity (EC 4.2.99.11), a reaction mixture (1·0 ml) containing 0·5 mM-DHAP, 50 mM-imidazole/HCl buffer (pH 7·0) and dialysed extract (0·1 to 0·5 mg protein ml^{-1}) was incubated at 30 °C for 1 h, and then the MG formed was determined colorimetrically by the method of Hopper & Cooper (1972). In all cases, protein was determined by the Lowry method.

Determination of glutathione. Cultures were cooled and centrifuged at 3000 rev. min^{-1} for 10 min. Extracellular glutathione was determined in this supernatant by the method of Tietze (1969). For the determination of intracellular glutathione, cells were washed once with chilled 0·85 % (w/v) NaCl and resuspended in water to give 10 mg wet wt cells ml^{-1}; 0·5 ml was then heated at 100 °C for 1 min, immediately cooled, centrifuged at 3000 rev. min^{-1} for 10 min and glutathione in supernatant was determined by the method of Tietze (1969).

RESULTS AND DISCUSSION

Under normal conditions most micro-organisms excrete little glutathione from the cells. However, in the course of a study on glutathione metabolism using a revertant (RM-7-23) of a cysteine auxotroph, we found that an MG-resistant strain (RM-9-10) derived from strain RM-7-23 excreted glutathione into the medium, though the parent strain did not (Fig. 1). In addition to the excretion of glutathione, the MG-resistant cells accumulated intracellular glutathione to considerably higher concentrations than did the parent cells. This excretion and accumulation of glutathione was further accelerated during growth on MG-containing medium. In the presence of MG, the concentration of glutathione excreted into the medium was about 50-fold higher than in the wild-type culture after 8 h (Fig. 1).

The reason for this glutathione overproduction was found to be that the activity of the glutathione-forming enzyme system (a combined enzyme system of γ-glutamylcysteine synthetase and glutathione synthetase) in the MG-resistant strain was much higher than in the parent strain. Furthermore, the activity of the glyoxalase system was also higher in MG-resistant cells (Table 1). Thus, it appears that MG resistance was caused by a simultaneous increase in glutathione synthetic activity and in MG disposal activity. As originally suggested by Freedberg *et al.* (1971), MG resistance was acquired through the increased capacity to remove MG rather than a simple tolerance of MG.

Thus, MG-resistant *E. coli* overproduced glutathione and excreted it, especially during growth on MG-containing medium. This excretion of glutathione was presumably due to a change in membrane structure or membrane components caused by contact with MG, since MG is thought to cause a drastic change in cell membranes (Szent-Györgyi, 1968).

We thank M. Hosoda for her helpful assistance.

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