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Isolation of Mutants of *Escherichia coli* with Increased Resistance to Alkylating Agents: Mutants Deficient in Thiols and Mutants Constitutive for the Adaptive Response

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Summary. A search has been made for mutants of *E. coli* that are constitutive for the adaptive response to alkylating agents. When selection was for resistance to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) the resulting strains all proved to be low in intracellular thiols. As MNNG has to be converted by thiols to the proximal mutagen methylnitrosamine, these strains were resistant simply because their low thiol levels made them less readily methylated by MNNG. The selection was therefore undertaken for strains resistant to N-methylnitrosourea (MNU) because this alkylating agent does not require activation by thiols. Four MNU resistant mutants proved to be constitutive for the adaptive response. Following exposure to (^{3}H) MNNG, they showed the same low ratio of O^6-methylguanine (O^6MeG) to 7-methylguanine (7MeG) as adapted wild type bacteria. The ratio of 3-methyladenine (3MeA) to 7MeG was the same in the wild type, the adapted wild type, and the constitutive strains. When exposed to very high MNNG doses, the constitutive mutants were even more resistant to mutation and killing than the adapted wild type. They have an even greater ability than the adapted wild type to remove O^6MeG from methylated DNA (Lindahl personal communication).

Introduction

When *E.coli* is exposed to various methylating agents the adaptive response is induced (Samson and Cairns 1977; Jeggo et al. 1977). This repair mechanism reduces the level in the DNA of the mutagenic lesion O^6MeG (Schendel and Robins 1978; Robins and Cairns 1979; Karran et al. 1979). Its induction results in an increased resistance of the cells to the mutagenic and killing effects of several different alkylating agents (Jeggo et al. 1977).

In order to increase our knowledge of the biochemistry and genetic control of the adaptive response, attempts have been made to generate mutations affecting the response. Jeggo (1979) has recently reported the isolation of *ada* mutants which cannot be adapted, but it is not known if these mutants are defective in the structural genes or in the regulation of expression of these genes. In order to determine which genes are involved in the regulation of adaptation, we have set out to isolate constitutive mutants. These mutants should be able to remove O^6MeG from their DNA without previous adaptation, and should therefore have an increased resistance to alkylating agents.

Two types of mutants were obtained. Those selected for increased resistance to MNNG proved to be low in cellular thiols. Mutants selected for their resistance to MNU were constitutive for the adaptive response.

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Materials and Methods

Chemical Reagents

N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was purchased from Sigma Chemical Co., and was stored as described previously (Jeggo et al. 1977). N-nitrosomethylurea (MNU) was a kind gift of P. Swann. (Methyl-^{3}H) MNNG (0.87 mCi/mg) was obtained from the Amersham Radiochemical Center.

Strains

AB1157 (F$^-$ *thr*1 *leu*6 *pro*A2 *his*4 *arg*E *thi*1 *lac*Y1 *gal*K *ara*1 *xyl*5 *mtl*1 *tsx*33 *str*A *sup*37) and W3110 (*thy*) are *E.coli* K12 strains. F26 (*thy his*) is an *E.coli* B/r strain. The mutants isolated in this paper are listed in Tables 1 and 2.

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Media

The liquid media and plates used here were M9 and LB media, described by Miller (1972), and antibiotic medium 3 (Difco). Where necessary these media were supplemented with amino acids at 40 μg/ml, vitamin B1 at 2 μg/ml, and thymine at 10 μg/ml.

Measurement of Survival and Mutation Frequency

Cultures of 2×10^8 cells per ml in supplemented M9 minimal medium were challenged with the mutagen for 10 or 20 min at 37°. To estimate survival samples were diluted in M9 salts and plated on L plates. To estimate the mutation frequency, the bacteria were centrifuged and resuspended in M9, diluted, and plated in soft agar (0.8%) on appropriate minimal plates for mutants and survivors.

Measurement of Methylation by MNNG

Exponential cultures were incubated with (^{3}H) MNNG (18.5 μCi/mg) for 10 min at 37°. TCA was added to a final concentration of 5%. The precipitates were collected on GF/C Whatman filters and counted in PPO toluene scintillation fluid in a Nuclear Chicago counter. The TCA precipitable counts per min were a measure of the overall methylation of macromolecules. Estimation of the methylation of purines in the cell DNA has been described in detail previously (Schendel and Robins 1978).

Measurement of Total Acid Soluble Thiols

The cellular level of acid soluble thiols was determined according to Lawley and Thatcher (1970) by a modification of Ellman's (1959) method. 7×10^9 cells from an exponential culture were centrifuged and resuspended in 0.5 ml 5% TCA. This suspension was centrifuged and 0.2 ml clear supernatant added to 1.4 ml dithiobis (2 nitrobenzoic acid) (200 μg/ml in 0.2 M sodium phosphate buffer pH 7.6). The absorption was measured at 410 nm. A standard curve was constructed using reduced glutathione.

Adaptation

The adaptive response was induced in strain F26 by growth in the presence of 1 μg MNNG per ml for 2 h.

Measurement of Spontaneous Mutation Frequency

The spontaneous mutation frequency was determined from the distribution of mutant clone size in replicate cultures according to the method described by Luria (1951). An overnight culture in LB broth or PAB was diluted to approximately 10^4 cells per ml. Forty-eight 3 ml samples of this diluted culture were shaken at 37° until they reached a cell density of 2×10^8 per ml (15 generations). The cultures were washed and plated as described above to determine the total number of cells and the number of *his*$^+$ revertants.

Results

E.coli induced for the adaptive response has an increased resistance to killing by alkylating agents (Jeggo et al. 1977). Thus, in the initial attempts to obtain strains constitutive for this DNA repair pathway, the selection was for mutants that are less readily killed by MNNG. Such strains have been previously reported, but their ability to remove O^{6}MeG from alkylated DNA has not been examined (Mandell et al. 1961; Zamenhof et al. 1966; Cerdá-Olmeda and Hanawalt 1968).

Isolation of MNNG-Resistant Mutants

Cultures of strains AB1157 and W3110 were mutagenized with 20 μg MNNG per ml for 10 min. This treatment resulted in approximately 25% survival and 10^{-3} *arg*$^+$ revertants per survivor of AB1157. The cells were centrifuged, resuspended, and diluted 5 fold before incubation overnight in minimal medium. To enrich for the presence of MNNG-resistant mutants, these mutagenised cultures were challenged with 50 μg MNNG per ml for 10 min, washed, diluted 5 fold and grown up overnight. This enrichment procedure was repeated several times. Survival of the wild types after such a challenge was 5 to 10%. After a succession of 4 such challenges, one out of four cultures of AB1157, and four out of four cultures of W3110, showed increased MNNG resistance. Single colony isolates were obtained from each culture (Table 1). To determine whether these isolates were constitutive mutants of the adaptive response, methylation of the cell DNA by MNNG at the O^6 and N^7 positions of guanine was measured. In all 5 strains the ratio of O^{6}MeG to 7MeG was approximately the same as in the wild types (Table 1). These isolates were therefore not constitutive for the adaptive response. However, total methylation of the cellular macromolecules was reduced by about 60% (Table 1), and this could account for the large increase in resistance of these mutants to killing by MNNG. The reduced methylation could result from a decreased permeability to MNNG, an increased ability to degrade MNNG to nonalkylating products, or a decreased ability to activate MNNG to its alkylating intermediate(s).

Cellular Thiol Content of MNNG-Resistant Strains

Thiols, such as glutathione and cysteine, are known to stimulate methylation by MNNG (Lawley and Thatcher 1970). Glutathione deficient strains have been isolated previously and shown to have a slightly increased sensitivity to a wide range of agents, but their sensitivity to MNNG has not been tested (Apontoweil and Berends 1975b). The acid soluble thiol levels of the MNNG resistant strains were estimated

Table 1. Characterisation of MNNG resistant mutants

Strain	% survival after 50 μg MNNG per ml	% survival after 2 mg MNU per ml	Methylation of DNA by (^{3}H) MNNG[a]			Total methylation of macromolecules (TCA insoluble cpm)[b]	Acid soluble thiols (μ moles per 10^{12} cells)[c]
			7MeG (cpm)	O^6MeG (cpm)	$\frac{O^6MeG}{7MeG} \times 100$		
AB1157 (wild type)	5	11	4,120	190	4.6	760	11.4
AB1157 BS53	94	7	500	20	4.0	300	0.5
W3110 (wild type)	3		10,100	920	9.1	620	9.9
W3110 BS55	87		410	30	7.1	230	0.2
W3110 BS56	57		1,070	70	6.6	300	2.6
W3110 BS57	60					320	0.4

[a] 100 ml cultures of 3×10^8 cells per ml were concentrated 100 fold and exposed to 10 μg MNNG per ml (0.7 mCi/mg) for 10 min. Estimation of methylation at the O^6 and N^7 positions of guanine was as described previously (7)

[b] 0.1 ml cultures (3×10^8 cells per ml) were exposed to 100 μg [^{3}H] MNNG per ml (19 μCi/mg) for 10 min. The TCA acid insoluble cpm were measured

[c] Acid soluble thiols were measured according to Lawley and Thatcher (5)

Table 2. Characterisation of MNU resistant mutants of F26

Strain	Survival after MNU (2 mg/ml) %	Methylation of cellular DNA by 50 μg [^{3}H] MNNG per ml for 10 min[a]				
		7MeG (cpm)	3MeA (cpm)	O^6MeG (cpm)	$\frac{3MeA}{7MeG} \times 100$	$\frac{O^6MeG}{7MeG} \times 100$
F26 (wild type)	10	15,800	450	2,280	2.9	14.4
F26 (wild type) adapted		14,680	486	110	3.3	0.8
BS11	92	11,170	270	10	2.5	0.09
BS21	62	21,230	740	110	3.5	0.5
BS31	74	7,460	240	30	3.2	0.4
BS41	88	11,650	270	120	2.3	1.0

[a] See footnote to Table 1

and found to be low (Table 1), and in the case of one strain the thiol pool was fractionated and shown to be deficient in glutathione (data not shown).

Methylation by the alkylating agent, methylnitrosourea, is not stimulated by thiols (Wheeler and Bowdon 1972). Indeed, the thiol deficient mutants were normal in their sensitivity to MNU (Table 1). In order to avoid the selection of strains low in thiols, MNU resistant mutants were therefore isolated in the continued search for a mutant constitutive for the adaptive response.

Isolation of Mutants Constitutive for the Adaptive Response

Strain F26 has a greater increase than strain AB1157 in its resistance to alkylating agents after induction of the adaptive response (data not shown). Thus, in order to increase our chances of isolating a mutant constitutively adapted to MNU, strain F26 was used. Cultures were mutagenised by MNNG, and then repeatedly challenged with 2 mg MNU per ml for 10 min. (The details of these procedures were as described above for the isolation of MNNG-resistant strains). After 6 challenges with MNU, isolates with increased resistance were obtained from 4 cultures (Table 2). On exposure to (^{3}H) MNNG, the ratio of O^6MeG to 7MeG produced in the DNA of these isolates was very low compared with the wild type, but was similar to the ratio in adapted F26. The ratio of 3MeA to 7MeG was not reduced in adapted F26 or in the new mutants (Table 2). The latter were thus considered to be constitutive mutants of the adaptive response, being able to remove O^6MeG from their DNA without previous induction of the response. This has been verified by Lindahl who has observed the removal of O^6MeG from alkylated DNA by extracts of the mutants prepared without prior adaptation (personal communication). All four mutants contained a similar level of this activity.

Killing and Mutation Induction by high MNNG Doses

Due to the high resistance of adapted F26 to the killing and mutagenic effects of MNNG, it was neces-

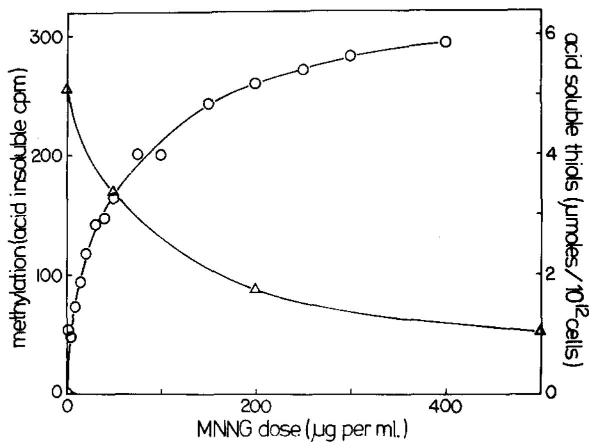

Fig. 1. Methylation of cellular macromolecules and the depletion of acid soluble thiols by MNNG. After incubation of F26 with (^{3}H) MNNG (18.5 μCi/mg) for 10 min at 37° C the acid insoluble counts per min (—○—) and acid soluble thiol levels (—△—) were determined

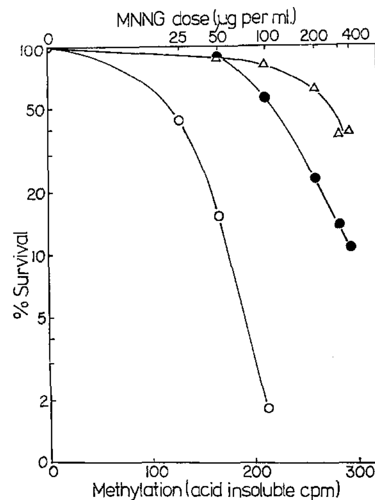

Fig. 3. Survival of F26 (—○—), F26 adapted (—●—), and BS21 (—△—) after exposure to various MNNG doses for 10 min at 37° C. Estimates of the amount of methylation by MNNG were obtained from Fig. 1

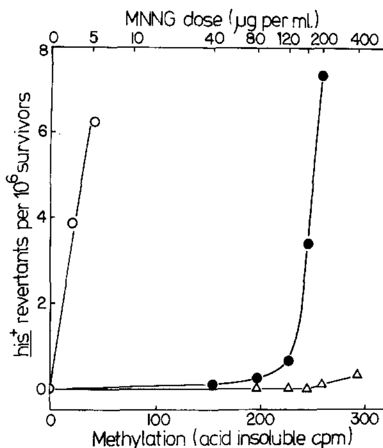

Fig. 2. Mutation induction in F26 and the strains constitutive for the adaptive response by varying doses of MNNG for 10 min. Strains BS11, BS31, and BS41 gave the same results as BS21 in that few *his*$^+$ revertants were induced by any of the MNNG doses used. Estimates of the amount of methylation of the cells by MNNG were obtained from Fig. 1. F26 —○—; F26 adapted —●—; BS21 —△—

sary to use very high doses to compare the behaviour of the constitutive mutants and the adapted wild type. At these high doses, methylation of the cell constituents was not proportional to the MNNG concentration, and may be limited because the thiol pool was being depleted by the high MNNG doses (Fig. 1). When challenging bacteria with high MNNG concentrations, the killing and mutagenic effects had, therefore, to be related to the amount of methylation rather than the MNNG concentrations. Figure 1 was used as a standard curve to obtain estimates of methylation of the cells at the MNNG doses used.

Strain F26 induced for the adaptive response was resistant to killing and mutation induction by MNNG up to a dose of approximately 50 μg per ml for 10 min (Figs. 2 and 3). At higher doses the adapted cells accumulated *his*$^+$ revertants at a similar rate to the unadapted wild type at low MNNG concentrations (Fig. 2); similar kinetics have been observed using AB1157, although adapted cells of this strain developed mutations at much lower MNNG doses (Schendel and Robins 1978). The constitutive mutants have a greater resistance to killing and mutation induction by MNNG than adapted F26 (Figs. 2 and 3); even when challenged with 400 μg MNNG per ml they developed very few *his*$^+$ revertants.

Spontaneous Mutation Frequency

If some spontaneous mutations were due to low levels of alkylating agents in bacterial growth media, the spontaneous mutation frequency of constitutive mutants should be less than that of F26. The parent strain, F26, and one of the mutants, BS21, proved to have the same spontaneous mutation frequency of 5×10^{-10} *his*$^+$ revertants per generation, whether grown in L broth or in PAB. In these conditions, therefore, alkylation of O^6 guanine does not appear to be contributing to the spontaneous mutation frequency.

Reversion of Constitutives to MNNG Sensitive Strains

When the constitutive mutants were subcultured from slopes that had been stored at room temperature for several months, they showed the paradoxical behaviour of being mutated by low doses of MNNG but not by high doses. Initially we had a rather complicated explanation for this effect (Cairns et al. 1980). However, we have now discovered that these strains show this unusual response because they have accumulated a large subpopulation of cells that cannot adapt at all; thus low doses of MNNG produce a large number of mutants in the subpopulation, whereas high doses kill the subpopulation but are not mutagenic for the constitutives that make up the rest of the population. We therefore believe that the constitutive mutants are at some survival disadvantage under certain conditions and are more easily reverted to non-constitutivity by the addition of a second *ada* mutation than by direct reversion of the original constitutive mutation.

Discussion

We have descibed the isolation and characterisation of two different types of *E.coli* mutants with increased resistance to certain alkylating agents. Five mutants with increased resistance to MNNG, but not to MNU, were low in cellular thiols. Thiols, such as cysteine and glutathione, stimulate the activation of MNNG, but not MNU, to its methylating intermediate, methylnitrosamine (Schulz and McCalla 1968; Lawley and Thatcher 1970; Wheeler and Bowdon 1972). Mutants low in thiols were therefore methylated less by MNNG, and this accounted for their increased resistance. Glutathione, which is the major component of the thiol pool of *E.coli* (Apontoweil and Berends 1975a), is generally considered to have a protective action against many agents (for a review see Chasseaud 1979). Indeed, mutants had already been described which were deficient in glutathione and, as a result are more sensitive to a wide range of chemicals (Apontoweil and Berends 1975b), but they were not tested at that time for resistance to MNNG.

Four mutants with increased resistance to MNU (and to MNNG) were characterised as constitutive mutants of the adaptive response. Production of O^6MeG, which is probably the main mutagenic lesion produced by these alkylating agents, was barely detectable in the mutants in vivo. An in vitro assay for the removal of O^6MeG from methylated DNA by extracts of adapted cells has been developed (Karran et al. 1979). This activity is present constitutively in extracts of the MNU resistant mutants (Lindahl, unpublished data).

We have found that the constitutive mutants are even more resistant to mutation induction by very high doses of MNNG than adapted wild type cells, and extracts of the constitutive mutants have been shown to contain a four fold greater activity for the removal of O^6MeG from methylated DNA than extracts of the adapted wild type (Lindahl, personal communication).

We have made two unsuccessful attempts to isolate similar constitutive mutants of *E.coli* K12 strains AB1157 and W3110. It may conceivably be harder to isolate such mutants in K12 strains because adaptation is rather less effective in K12 than in B/r, or because constitutive mutants may perhaps have an even lower survival advantage in K12 than in B/r.

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