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DNA Alkylation Repair Limits Spontaneous Base Substitution Mutations in *Escherichia coli*

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The *Escherichia coli* Ada and Ogt DNA methyltransferases (MTases) are known to transfer simple alkyl groups from O^6-alkylguanine and O^4-alkylthymine, directly restoring these alkylated DNA lesions to guanine and thymine. In addition to being exquisitely sensitive to the mutagenic effects of methylating agents, *E. coli ada ogt* null mutants display a higher spontaneous mutation rate than the wild type. Here, we determined which base substitution mutations are elevated in the MTase-deficient cells by monitoring the reversion of six mutated *lacZ* alleles that revert via each of the six possible base substitution mutations. During exponential growth, the spontaneous rate of G:C to A:T transitions and G:C to C:G transversions was elevated about fourfold in *ada ogt* double mutant versus wild-type *E. coli*. Furthermore, compared with the wild type, stationary populations of the MTase-deficient *E. coli* (under lactose selection) displayed increased G:C to A:T and A:T to G:C transitions (10- and 3-fold, respectively) and increased G:C to C:G, A:T to C:G, and A:T to T:A transversions (10-, 2.5-, and 1.7-fold, respectively). *ada* and *ogt* single mutants did not suffer elevated spontaneous mutation rates for any base substitution event, and the cloned *ada* and *ogt* genes each restored wild-type spontaneous mutation rates to the *ada ogt* MTase-deficient strains. We infer that both the Ada MTase and the Ogt MTase can repair the endogenously produced DNA lesions responsible for each of the five base substitution events that are elevated in MTase-deficient cells. Simple methylating and ethylating agents induced G:C to A:T and A:T to G:C transitions in these strains but did not significantly induce G:C to C:G, A:T to C:G, and A:T to T:A transversions. We deduce that *S*-adenosylmethionine (known to be a weak methylating agent) is not the only metabolite responsible for endogenous DNA alkylation and that at least some of the endogenous metabolites that cause *O*-alkyl DNA damage in *E. coli* are not simple methylating or ethylating agents.

Some normal intermediary metabolites and some by-products of normal metabolism are cytotoxic compounds that can attack nucleic acids, proteins, and lipids (2, 12, 29, 36). The most extensively studied compounds of this type are active oxygen species (the by-products of oxidative metabolism), and these are now believed by some to play a major role in the onset of neurodegenerative, cardiovascular, and neoplastic diseases (2, 12). It is thus clearly important to identify endogenous toxic compounds and to identify the cellular defenses that normally ameliorate their effects.

If the damage inflicted by toxic intermediary metabolites on DNA and its precursors is allowed to remain, it can increase the rate of spontaneous mutation (3, 17, 23, 34, 45, 55, 61). Factors that influence spontaneous mutation rates are of considerable interest, since it was hypothesized that one of the early steps in carcinogenesis involves an elevation in the rate of spontaneous mutation (14, 18, 31, 54, 56). Indeed, it was recently shown that defects in a human gene whose product may be involved in the correction of DNA replication errors result in a predisposition to colon cancer (19, 28, 41). Extensive characterization of *Escherichia coli* has established that low spontaneous mutation rates are not only achieved by the prevention and correction of DNA replication errors (16, 38) but are also achieved by the prevention and removal of nucleotide damage (17, 23, 34, 45, 55). Thus, in *E. coli*, the repair of oxidized DNA and the hydrolysis of oxidized deoxynucleoside triphosphate DNA precursors have been shown to be significant contributors to the maintenance of low spontaneous mutation rates (17, 23, 34, 55).

We recently demonstrated that the repair of DNA alkylation damage also contributes to the maintenance of low spontaneous mutation rates in both prokaryotes (45) and eukaryotes (61). However, the identity of the endogenous cellular compounds responsible for producing the DNA alkylation damage remains unknown. Alkylating agents transfer alkyl groups to the nucleophilic nitrogens and oxygens in DNA, generating at least a dozen types of DNA adducts (30). Of these, O^6-alkylguanine (O^6alkylG) and O^4-alkylthymine (O^4alkylT) are thought to be responsible for the majority of alkylation-induced mutations because of their propensity to mispair during replication. It is well established that O^6methylG mispairs with thymine and O^4methylT mispairs with guanine and that, because of this, methylating agents cause G:C to A:T and A:T to G:C transition mutations (32, 44, 52); however, the O^6methylG-driven G:C to A:T transitions outnumber the O^4methylT-driven A:T to G:C transitions by about 100-fold (26), even though, after exposure to methylating agents, O^6methylG adducts only outnumber O^4methylT adducts by about 10-fold (6). The precise effects of larger alkyl groups (at O^6-G and O^4-T) on base pairing have not been examined in as much detail, but it is clear that agents transferring larger alkyl groups to DNA induce transversion in addition to transition mutations (26).

E. coli has two DNA repair methyltransferases (MTases), encoded by the *ada* and *ogt* genes, that mediate the irreversible transfer of methyl groups from either O^6MeG or O^4MeT to a specific cysteine residue in the MTase itself (47). These MTases also transfer ethyl, propyl, and butyl groups from DNA, but the efficiency of transfer decreases as the size of the

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TABLE 1. Bacterial strains and characteristics

Strain	Chromosomal genotype	Episomal genotype	Reference(s)
FC36	*ara*Δ(*lacproB*)$_{XIII}$ *thiA* Rif^r	F$^-$	7, 11
FC215	As FC36	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC102 G:C to A:T	13
FC219	As FC36	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC101 A:T to C:G	13
FC220	As FC36	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC103 G:C to C:G	13
FC221	As FC36	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC104 G:C to T:A	13
FC222	As FC36	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC105 A:T to T:A	13
FC223	As FC36	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC106 A:T to G:C	13
FC218	As FC215 but *ogt-1*::Kan^r Δ*ada-25*::Cam^r	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC102 G:C to A:T	13, 22, 45, 50
FC321	As FC219 but *ogt-1*::Kan^r Δ*ada-25*::Cam^r	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC101 A:T to C:G	13, 45, 50
FC322	As FC220 but *ogt-1*::Kan^r Δ*ada-25*::Cam^r	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC103 G:C to C:G	13, 45, 50
FC325	As FC221 but *ogt-1*::Kan^r Δ*ada-25*::Cam^r	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC104 G:C to T:A	13, 45, 50
FC323	As FC222 but *ogt-1*::Kan^r Δ*ada-25*::Cam^r	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC105 A:T to T:A	13, 45, 50
FC326	As FC223 but *ogt-1*::Kan^r Δ*ada-25*::Cam^r	*lacI*:*lacZ lacY*$^+$ *pro*$^+$; from CC106 A:T to G:C	13, 45, 50

alkyl group increases (35, 47). The Ogt MTase is expressed constitutively, while Ada is induced as part of the adaptive response to alkylating agents (30, 47). Ada has two active site cysteine residues, Cys-321 that transfers alkyl groups from O^{6}alkyG or O^{4}alkyT adducts and Cys-69 that transfers alkyl groups from alkylphosphotriesters. Once alkylated at Cys-69, Ada becomes a transcriptional activator of its own gene (*ada*) and three other genes involved in the adaptive response (the *alkA* 3-methyladenine DNA glycosylase gene plus *alkB* and *aidB* of unknown function). *E. coli ada ogt* null mutants are severely compromised in their ability to repair O^{6}alkylG and O^{4}alkylT DNA adducts (45, 48) and suffer an elevated spontaneous mutation rate which becomes most apparent in stationary cultures (45). In this study, we have investigated what types of mutational events are elevated in these DNA repair-deficient cells, in order to gain some insight into the identity of the endogenous alkylating agents responsible for mutation induction.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Strains FC219, FC215, FC220, FC221, FC222, and FC223 are isogenic derivatives of FC36, a spontaneous rifamycin-resistant isolate of P90C (7, 11, 22). All of the strains have a chromosomal deletion of the lactose operon, and all strains except FC36 carry one of six F'lac episomes, each with a different *lacZ* base substitution mutation affecting Glu-461 of β-galactosidase (13). The six episomes were designated CC101 to CC106 (13), and the *lacZ* mutant alleles on these episomes revert to the wild type via A:T to C:G (CC101), G:C to A:T (CC102), G:C to C:G (CC103), G:C to T:A (CC104), A:T to T:A (CC105), and A:T to G:C (CC106). P1 transduction (37) was used to transfer the Δ(*ada-25*)::Cam^r allele from GW7101 (50) and the *ogt-1*::Kan^r allele from GWR107 (45) into these strains to produce *ada ogt* single and double mutant derivatives of FC219, FC215 (22), FC220, FC221, FC222, and FC223. Patricia L. Foster (Boston University) used our *ogt-1*::Kan^r allele (45) and the Δ(*ada-25*)::Cam^r allele (50) to construct the MTase-deficient strains listed in Table 1, and we thank her for generously sharing the strains with us. E7014 is a strain carrying a deletion for the *lacZI* genes and can neither mutate nor recombine with F'lac to produce Lac$^+$ revertants (5a). Transformation of the *lac* mutant Miller strains with pUC19, pUCogt (45), and pSV2ada (47a) plasmids was performed as previously described (10). The *ada* and *ogt* genes were expressed from their own promoters in the pUCogt and pSV2ada plasmids.

Media. Bacteria were grown in either Luria-Bertani medium or M9 minimal medium supplemented with 0.025% glucose, 0.025% thiamine, and 40 μg of methionine per ml (37). Minimal plates contained M9 medium plus 0.025% glucose or lactose, 0.025% thiamine, 40 μg of methionine per ml, and 0.15% Bacto agar. Antibiotic concentrations (per milliliter) were 100 μg of ampicillin, 40 μg of kanamycin, 25 μg of tetracycline, and 25 μg of chloramphenicol.

Spontaneous Lac$^+$ mutation assays. A single colony was inoculated into glucose-minimal M9 medium and grown to stationary phase. From this culture, approximately 10^6 cells were transferred to each of 30 tubes containing 1.5 ml of glucose-minimal M9 medium plus the appropriate antibiotics, and the cultures were grown with aeration for about 16 h until they just reached stationary phase (approximately 1×10^9 to 2×10^9 cells per ml). From 10 cultures, a 10-μl aliquot was serially diluted, plated in duplicate onto glucose-minimal M9 medium plates, and scored the next day to determine the average number of viable cells per culture (N [see below]). The 30 cultures were each concentrated into a final volume of 100 μl, and the entire culture was spread onto minimal lactose plates. For the FC218 strain that exhibited a high mutation rate, approximately 10^8 cells were plated with 10^9 *lac* mutant E7014 scavenger cells to prevent growth of FC218 cells on the plate (1, 7, 25, 53). It is important to note that in separate experiments we checked that the spontaneous mutation rates were in the linear range; in other words, we checked that the number of Lac$^+$ revertant colonies remained (throughout the experiment) proportional to the number of cells plated. The importance of this was described by Cairns and Foster (7). From 48 h onwards, the plates were examined daily for Lac$^+$ revertants.

The spontaneous mutation rate in the growing phase of the cultures was calculated from the day 2 colony counts. The mean number of mutations per culture (m) was calculated either from the proportion of cultures that contained no mutants (P_0) (33) or from the median number (r) of mutants per distribution according to the equation $(r/m) - \ln(m) = 1.24$ (27). Mutation rates per generation were calculated as either $-\ln(2P_0)/N$ (33) or $m(\ln 2)/N$ (15, 27), where N is the average number of viable cells per culture. The frequency of mutations occurring after plating was calculated as the cumulative number of Lac$^+$ revertants/N; note that it has been demonstrated that N does not increase significantly on the lactose plates during the course of these experiments (7, 33a).

Alkylation-induced mutation. To measure induced mutation frequencies, *E. coli* cells were grown in minimal medium to 5×10^8 cells per ml and exposed to several doses of methyl

methanesulfonate (MMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG), all purchased from Sigma; note that only one dose for each agent is shown in Table 3, but several doses were tested to find the linear range. The treated cells were concentrated in M9 minimal medium and then diluted and plated on minimal plates containing either glucose (to estimate the number of viable cells) or lactose (to estimate the number of Lac^+ mutants), and the colonies were counted after 2 days at 37°C. Mutation frequencies are expressed as the number of induced Lac^+ revertants per 10^9 surviving cells.

RESULTS

We recently found that *E. coli ada ogt* null mutant strains display a higher rate of spontaneous mutation than the wild type and that the increased mutation rate appears to occur predominantly in nondividing cells (45). Spontaneous mutation was measured as the reversion (or suppression) of an uncharacterized *his* mutant allele, and so the precise mutational events that were elevated in these MTase-deficient bacteria were not determined. In order to gain some insight into the identity of the endogenous compounds responsible for alkylation-induced spontaneous mutation, we set out to determine which mutational events increase in *ada ogt* double mutant *E. coli* cells. Since base substitutions constitute well over 90% of the mutations induced by alkylating agents (26), we confined our analysis to the six possible base substitution events. A set of six *E. coli lacZ* mutant strains was recently developed by Cupples and Miller (13): each strain reverts to $lacZ^+$ via a different base substitution event, namely the G:C to A:T and A:T to G:C transition events and the G:C to C:G, A:T to C:G, G:C to T:A, and A:T to T:A transversion events. Spontaneous mutation was analyzed for the wild-type parental strains and for their *ada*, *ogt*, and *ada ogt* double mutant derivatives. Spontaneous mutation was measured in two ways. (i) The spontaneous mutation rate during log-phase growth (mutations per cell per generation) was measured as described by Luria and Delbruck (33) and Lea and Coulson (27). (ii) The accumulation of mutants in nondividing cultures was measured as described by Cairns et al. (8), Rebeck and Samson (45), and Cairns and Foster (7).

Spontaneous mutation during log-phase growth. Spontaneous mutation rates were determined (for wild-type and *ada ogt* double mutant strains) from the number of Lac^+ colonies present 2 days after plating 30 independent cultures that had undergone 10 to 12 cell doublings (see Materials and Methods). Table 2 shows that the *lacZ* mutant reversion rates in wild-type cells varied by over 100-fold for the six different *lacZ* mutant alleles, with the allele reverting via a G:C to C:G transversion having the lowest (4.28×10^{-11} per generation) and the allele reverting via a G:C to T:A transversion having the highest (5×10^{-9} per generation) rate of spontaneous reversion. These results are in general agreement with those reported by Hall for the same strains (25). The absence of the Ada and Ogt O^6alkylG and O^4alkyT DNA MTases increased the spontaneous mutation rate (by about fourfold) for just two of the six *lacZ* mutant alleles, namely those which revert via a G:C to A:T transition and via a G:C to C:G transversion (Table 2). The increase in the G:C to A:T spontaneous mutation rate for this MTase-deficient strain is presumably driven by endogenously produced O^6alkylG lesions mispairing with thymine during DNA replication, but the lesion responsible for the G:C to C:G transversions is unclear. However, since the G:C to C:G transversions are prevented by the Ada and Ogt MTases, they are also likely to be driven by O^6alkylG. (Note that the Ada and Ogt MTases are not thought to repair alkylated cytosines or any form of alkylated guanine other than O^6alkylG [30].)

TABLE 2. Number of spontaneous mutations per cell per generation

Base pair substitution	No. of mutations per cell per generation[a]		Wild-type/*ada ogt* double mutant ratio
	Wild-type rate	*ada ogt* double mutant rate	
G:C to A:T	2.2×10^{-9}	9.0×10^{-9}	4.1
	2.9×10^{-9}	1.0×10^{-8}	3.4
A:T to G:C	2.1×10^{-10}	1.3×10^{-10}	0.62
	2.2×10^{-10}	1.9×10^{-10}	0.86
G:C to C:G	4.28×10^{-11}	1.82×10^{-10}	4.25
	5.1×10^{-11}	2.3×10^{-10}	4.51
A:T to C:G	1.15×10^{-9}	1.04×10^{-9}	0.9
	1.3×10^{-9}	1.3×10^{-9}	1.0
G:C to T:A	4.2×10^{-9}	3.0×10^{-9}	0.71
	5.0×10^{-9}	3.2×10^{-9}	0.64
A:T to T:A	3.3×10^{-9}	2.8×10^{-9}	0.85
	4.1×10^{-9}	2.9×10^{-9}	0.71

[a] Mutant colonies were scored 48 h after plating.

The observed increase in spontaneous mutation rates in dividing cells was, at first, surprising because we had previously found that forward mutation to rifampin resistance was unaffected by the absence of the Ada and Ogt MTases (45). (Because sensitive cells die when plated on rifampin, only mutants that arise during cell growth prior to plating are measured.) The increased spontaneous mutation rate observed in the current experiments may be explained by the fact that only one kind of mutational event is monitored in each strain. Rifampin resistance can probably be achieved via all six base substitutions (plus other types of mutation), and so a four- to fivefold increase in G:C to A:T transitions and G:C to C:G transversions might not produce a significant change in the overall spontaneous mutation rate.

Since the Ada MTase acts as a transcriptional regulator for at least four genes, it was possible that a deficiency in one of the Ada-regulated gene products was responsible for the increased spontaneous mutation rate. However, the rate of spontaneous reversion of the G:C to A:T and G:C to C:G revertible *lacZ* mutant alleles was not elevated in growing cultures of the *ada* single mutants (i.e., *ada* ogt^+), compared with the wild type (data not shown). Similarly, the spontaneous mutation rate was not increased in ada^+ *ogt* single mutants. Thus, spontaneous mutation increases only in the complete absence of DNA repair MTase, and from this we infer that both the Ada and Ogt MTases can repair the DNA alkylation damage responsible for driving the G:C to A:T and G:C to C:G mutations. In support of this, expression of either the *ada* gene or the *ogt* gene in the *ada ogt* double mutant (from pSV2ada or pUCogt, respectively) abolished the increased G:C to A:T transition mutations seen in the MTase-deficient strain (data not shown). However, overexpression of the cloned *ada* or *ogt* genes in wild-type bacteria did not decrease G:C to A:T mutations (data not shown), indicating that wild-type MTase levels are more than adequate for the removal of the endogenously produced DNA alkylation damage that drives these spontaneous mutations.

Spontaneous mutation in nondividing cultures. A number of studies indicate that nondividing *E. coli* populations that are subjected to nonlethal selections continue to produce mutant colonies for many days (8, 20, 21). We previously showed that stationary populations of *ada ogt* double mutant *E. coli* cells

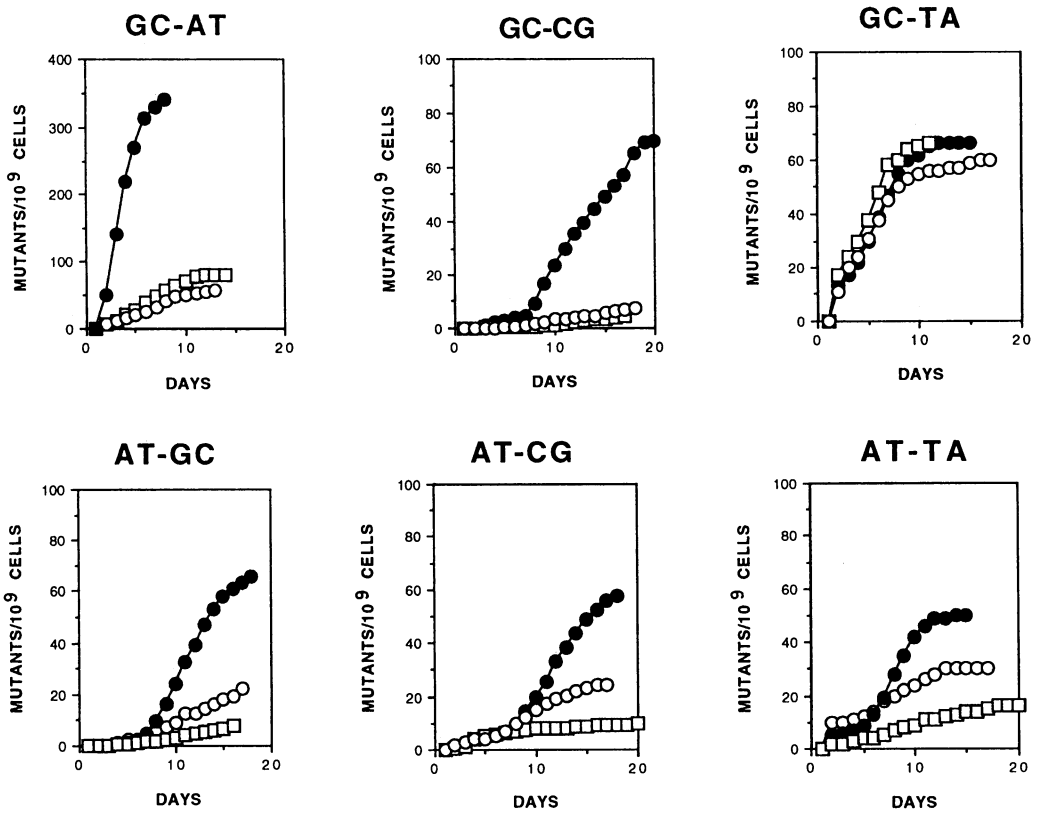

FIG. 1. The accumulation of spontaneous Lac$^+$ mutants in stationary cells. The postplating mutation rate was measured as described in Materials and Methods. Cells were plated on minimal lactose medium, and colonies were scored every day after plating; values reflect the average of 30 independent cultures. The base pair substitution that reverts the *lacZ* mutant allele in each strain is indicated above each panel. ○, *ada*$^+$ *ogt*$^+$ *E. coli*; ●, *ada ogt* double mutant *E. coli*; □, *ada ogt* double mutant *E. coli* containing the pUCogt plasmid expressing the Ogt MTase.

produced more His$^+$ revertant colonies than wild-type stationary populations (45). Here, we determined which particular mutational events are elevated in MTase-deficient *E. coli* by monitoring reversion of the six *lacZ* mutant alleles in stationary cultures of wild-type and *ada ogt* double mutant bacteria. Figure 1 shows the surprising result that five of the six base substitution events are significantly elevated in *ada ogt* double mutant *E. coli* cells compared with the wild type. Only the G:C to T:A transversion did not show a significant increase. This result raised the possibility that being MTase deficient somehow increased all spontaneous mutation rates in stationary bacteria. However, the reversion rate of a *lac* mutant allele via a frameshift mutation (7) was not elevated in *ada ogt* double mutant cells (data not shown). We infer that the levels of spontaneous G:C to A:T and A:T to G:C transition mutations (10-fold and 3-fold, respectively) and G:C to C:G, A:T to C:G, and A:T to T:A transversion mutations (10-fold, 2.5-fold, and 1.7-fold, respectively) are specifically elevated in cells that cannot repair O^{6}alkylG and O^{4}alkylT DNA lesions.

Reversion of the six *lacZ* mutant alleles in these stationary populations was not elevated in the *ada* or *ogt* single mutants compared with the wild type (data not shown), indicating that each MTase is capable of repairing the DNA damage responsible for driving the five base pair substitution events that were elevated in MTase-deficient cells. In support of this finding, the expression of either the *ada* gene or the *ogt* gene in the *ada ogt* double mutant (from pSV2ada or pUCogt, respectively) abolished the increased spontaneous mutation seen in nondividing MTase-deficient bacteria and reduced the accumulation of mutants to a level the same as, or slightly lower than, that in wild-type cells. The data for pUCogt are shown in Fig. 1 and are essentially the same as those for pSV2ada.

It is important to note the times at which extra mutants appear in MTase-deficient populations (Fig. 1). Increased numbers of G:C to A:T transition mutants steadily accumulate during the first week after plating at a rate of about 50 to 100/10^9 cells per day. Note that Foster and Cairns (22) saw a similar rate of accumulation of Lac$^+$ colonies in this same *ada ogt* double mutant strain, but a comparison with the wild-type *ada*$^+$ *ogt*$^+$ strain was not made. In contrast, accumulation of the four other mutant types only begins 7 to 9 days after plating (Fig. 1). It is therefore possible that the endogenous compounds that drive the G:C to A:T transitions are different from those that drive the other four base substitutions and that the latter compounds do not start to be produced until the cells have been stationary for about a week. However, it should be noted that if the DNA lesions which drive the A:T to G:C, G:C to C:G, A:T to C:G, and A:T to T:A mutations can also be repaired via an Ada- and Ogt-independent pathway, one could explain the delayed mutant accumulation in the following way: lesions may in fact be produced continuously but are only efficiently repaired by this hypothetical pathway during the first week of stationary phase; thus, the MTase deficiency may only become apparent when the hypothetical pathway does not operate.

The ability of different types of alkylating agents to revert the six *lacZ* mutant alleles. The nature of the endogenous compounds responsible for alkylation-induced spontaneous

TABLE 3. Induction of base substitution mutations in *ada ogt* double mutant *E. coli* cells by different alkylating agents

Base substitution	No. of induced mutants/10^9 surviving cells treated with:		
	MNNG (0.1 μg/ml)	ENNG (0.1 μg/ml)	MMS (0.1%)
G:C to A:T	150,953	412	57,646
A:T to G:C	51.7	70.6	17.5
A:T to C:G	ND[a]	0.8	ND
G:C to C:G	ND	ND	ND
A:T to T:A	ND	1.6	ND

[a] ND, No detectable increase above untreated bacteria.

mutation remains unclear. The ability of different alkylating agents to induce each of the six possible base pair substitutions has been inferred from mutational spectra in *E. coli* (26). The S_N1-methylating agents (e.g., MNNG) induce almost exclusively transition mutations with about 100-fold more G:C to A:T than A:T to G:C events and virtually no detectable transversion events (26). The S_N2-methylating agents (e.g., MMS) and alkylating agents with larger alkyl groups (e.g., ENNG) tend to produce a slightly lower proportion of G:C to A:T transitions with a concomitant increase in A:T to G:C transitions plus the four transversions (26). Mutational spectra such as these give the average value of all of the hot and cold spots for each base substitution event within a certain region of the genome, and it was not possible to use these data to accurately predict how susceptible the six *lacZ* mutant alleles are to alkylation-induced reversion (relative to each other). We therefore measured directly the ability of various alkylating agents to revert the six *lacZ* mutant alleles in order to gain possible insight into the nature of the endogenous alkylating agent or agents responsible for producing O^6alkylG and O^4alkylT in *E. coli*.

The initial burst of mutations seen after plating *ada ogt* double mutant cells is exclusively G:C to A:T transitions, and these reach a level of about 350 Lac^+ revertants per 10^9 cells after 7 days. During these 7 days, none of the other base substitution mutations are elevated above the wild-type level. The results in Table 3 show that the ratio of G:C to A:T versus A:T to G:C transitions induced in these strains by three different alkylating agents is approximately as follows: MNNG, 3,000; ENNG, 6; and MMS, 3,000. Thus, if the endogenous metabolite or metabolites responsible for the initial burst of G:C to A:T transitions were an S_N1-methylating agent (like MNNG) or an S_N2-methylating agent (like MMS), the concomitant increase in A:T to G:C transitions would have been undetectable in this assay. However, since ENNG can induce the A:T to G:C mutation at about one-sixth the level of the G:C to A:T mutation and since no extra A:T to G:C mutations appear during the first 7 days after plating, we infer that the endogenous metabolite or metabolites responsible for the initial burst of spontaneous G:C to A:T mutations are unlikely to be an S_N1-ethylating agent.

We also determined whether the S_N1-methylating (MNNG) and S_N1-ethylating agent (ENNG) or the S_N2-methylating agent (MMS) could induce transversion mutations in addition to transition mutations (Table 3). Note that the spontaneous A:T to G:C transitions and the four transversions appear at roughly equivalent rates in *ada ogt* double mutant *E. coli* cells, beginning after about a week in stationary phase (Fig. 1). We determined whether doses of alkylating agents that induce A:T to G:C transition mutations (at roughly the same frequency as they appear spontaneously) could also induce transversion mutations. The results in Table 3 clearly demonstrate that MNNG, ENNG, and MMS each failed to induce significant numbers of transversion mutations at doses that induced significant numbers of A:T to G:C transition mutations. We infer that the endogenous metabolites that alkylate DNA to drive transversion mutations in stationary cells are unlikely to be simple monofunctional methylating or ethylating agents.

DISCUSSION

Defects in a human gene whose product is involved in the maintenance of low spontaneous mutation rates were recently linked to Lynch syndrome, which manifests as a predisposition to colon and other cancers (19, 28, 41). The mutated human gene was identified, in part, because it is a homolog of the *E. coli mutS* gene, whose role in controlling spontaneous mutation rates via the correction of DNA replication errors has been thoroughly characterized (38). Since DNA repair mechanisms are highly conserved from bacteria to humans, it is likely that some of the other *E. coli* pathways that reduce spontaneous mutation may also have mammalian counterparts and that these may influence cancer predisposition. Here, we characterize one such *E. coli* pathway.

It is now quite clear that a deficiency in DNA alkylation repair MTases elevates the spontaneous mutation rate in both prokaryotic and eukaryotic cells (3, 45, 61). We have determined what kinds of mutations are elevated in MTase-deficient *E. coli* cells by using six different *lacZ* mutant strains, each of which detects just one of the six possible base substitution mutations (13). Unlike a classical determination of mutational spectra by the sequencing of randomly isolated mutants, these strains offer exquisite sensitivity for the detection of each individual base substitution event. Since alkylation-induced G:C to A:T transitions usually outnumber each of the five other base substitutions by up to 100-fold (and in some cases by many hundredfold) the spectrum is usually dominated by these high-frequency G:C to A:T mutational events (26). The use of such a sensitive assay system in this study may explain the unexpected finding that five types of base substitution mutations (the two transitions plus three of the four possible transversions) accumulate at a higher rate in MTase-deficient *E. coli* cells than in wild-type cells. However, it should be noted that simple methylating and ethylating agents were very poor inducers of transversion mutations in these strains; thus, at least some of the endogenously produced O^6-G and O^4-T lesions may bear adducts other than methyl or ethyl groups, and it may be these alkylated bases that induce the extra transversion mutations in MTase-deficient cells. Ultimately, sensitive analytical chemistry will be needed to detect and identify the extra *O*-alkyl lesions that remain unrepaired in the genome of DNA MTase-deficient cells.

The Ada and Ogt MTases transfer alkyl groups from the O^6 position of G and the O^4 position of T, and there is no reason to suspect that these proteins repair any other type of alkylated base (30, 35). We thus infer that endogenously produced O^6alkylG lesions are responsible for the extra mutation at G:C base pairs and that O^4alkylT lesions are responsible for the extra mutation at A:T base pairs in MTase-deficient cells. Since it is well established that O^6methylG can pair with T and O^4methylT can pair with G, we conclude that some of the unrepaired O^6alkylG and O^4alkylT lesions direct the G:C to A:T and A:T to G:C transition mutations, respectively, that are elevated in MTase-deficient cells. Furthermore, we suggest that some of the endogenously produced O^4alkylT lesions may pair with C or T to cause the A:T to C:G and A:T to T:A

transversions, respectively, and that some of the O^6alkylG lesions may pair with G to cause the G:C to C:G transversions. While most studies on the base pairing properties of O^4methylT and O^6methylG (5) have concentrated on their ability to pair with G and T, respectively, two nuclear magnetic resonance studies showed that O^6MeG could pair with G in DNA in vitro (42, 43), and our results suggest that O^6alkylG:G base pairs may indeed be formed in vivo. However, it should be noted that the presence of unrepaired alkylated bases in the genome may signal the induction of the SOS response and that SOS mutagenesis may account for the elevated rate of transversion mutations.

The facts that MTase deficiency results in a mutator phenotype and that alkylated bases can be detected in the DNA of cells that were not exposed to exogenous alkylating agents (40, 51, 57) indicate that the repertoire of endogenous cellular metabolites includes compounds that alkylate DNA. Thus, in parallel with the constant flux of oxidative DNA damage in aerobically metabolizing cells (2), there may also be a constant flux of DNA alkylation damage, the nature of which may depend upon the particular growth conditions. The identity of these endogenous alkylating agents remains unclear. *S*-Adenosylmethionine is an efficient methyl donor in vivo and is known to act as a weak S_N2-alkylating agent in vitro (4, 39, 46); this compound is widely believed to be the major cellular metabolite responsible for endogenous DNA alkylation (29). Reactive alkylating species may also arise from lipid peroxidation (59) and from amine nitrosation (9, 58, 60), and we expect that other endogenous alkylating agents remain to be identified. While *S*-adenosylmethionine may be responsible for the DNA lesions that drive the spontaneous G:C to A:T transitions seen in log-phase and relatively young stationary cultures, our results indicate that this metabolite is unlikely to account for DNA lesions that drive the spontaneous transversion mutations seen in old stationary cultures.

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