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Research Article

Mutagenic Potency of MMS-Induced 1meA/3meC Lesions in *E. coli*

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The mutagenic activity of MMS in *E. coli* depends on the susceptibility of DNA bases to methylation and their repair by cellular defense systems. Among the lesions in methylated DNA is 1meA/3meC, which is recently recognized as being mutagenic. In this report, special attention is focused on the mutagenic properties of 1meA/3meC which, by the activity of AlkB-dioxygenase, are quickly and efficiently converted to natural A/C bases in the DNA of *E. coli alkB*$^+$ strains, preventing 1meA/3meC-induced mutations. We have found that in the absence of AlkB-mediated repair, MMS treatment results in an increased frequency of four types of base substitutions: GC→CG, GC→TA, AT→CG, and AT→TA, whereas overproduction of PolV in CC101–106 *alkB*$^-$/pRW134 strains leads to a markedly elevated level of GC→TA, GC→CG, and AT→TA transversions. It has been observed that in the case of AB1157 *alkB*$^-$ strains, the MMS-induced and 1meA/3meC-dependent *argE3*→Arg$^+$ reversion occurs efficiently, whereas *lacZ*$^-$→ Lac$^+$ reversion in a set of CC101–106 *alkB*$^-$ strains occurs with much lower frequency. We considered several reasons for this discrepancy, namely, the possible variance in the level of the PolV activity, the effect of the PolIV contents that is higher in CC101–106 than in AB1157 strains and the different genetic cell backgrounds in CC101–106 *alkB*$^-$ and AB1157 *alkB*$^-$ strains, respectively. We postulate that the difference in the number of targets undergoing mutation and different reactivity of MMS with ssDNA and dsDNA are responsible for the high (*argE3*→Arg$^+$) and low (*lacZ*$^-$ → Lac$^+$) frequency of MMS—induced mutations. Environ. Mol. Mutagen. 50:791–799, 2009.

Key words: *E. coli alkB* mutants; MMS mutagenesis; PolIV; PolV; Arg$^+$/Lac$^+$ revertants

INTRODUCTION

Alkylating agents are widespread in the environment and also result from normal cellular metabolism. Their reactions with nucleic acid bases create products that are toxic, mutagenic, or neutral to cells [Singer and Kusmierek, 1982; Beranek, 1990; Taverna and Sedgwick, 1996]. Alkylating agents (i.e., methylating and ethylating) can be divided according to their chemistry into two types, SN_1 (e.g., *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)) and SN_2 (e.g., methylmethane sulfonate (MMS)). Both can methylate nitrogen and oxygen in DNA bases, however, the rate of methylation differs; SN_1 reagents produce much more *O*-methylated bases (e.g., O^6meG) than SN_2 reagents.

The most abundant methylated base in MMS-treated DNA is N^7meG, which accounts for 78–83% of the total methylated product and is neither harmful nor mutagenic [Singer and Grunberger, 1983]. The O^6meG adduct is not directly toxic but is highly mutagenic as a consequence of its ability to form base pairs with dTTP as well as dCTP during replication and induce GC→AT transitions. The most toxic and mutagenic lesions are the 3meA adducts (a substrate for Tag and AlkA *N*-glycosylases), AP sites transiently formed during base excision repair (BER), and

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1meA/3meC adducts, the substrates for AlkB dioxygenase [Sedgwick, 2004]. The presence of 3meA, AP sites, and 1meA/3meC arrests DNA replication, and to be bypassed and induce mutations, it requires *umuDC*-dependent, DNA polymerase V (PolV) [Nieminuszczy et al., 2006b].

Methylating agents can induce two global DNA repair systems in bacteria: the adaptive response (Ada response), involving four genes associated with the repair of alkylated DNA [Shevell et al., 1988; Fernandez De Henestrosa et al., 2000; Landini and Volkert, 2000; Sedgwick and Lindahl, 2002; Nieminuszczy and Grzesiuk, 2007] and the SOS response, involving more than 40 genes including those participating in replication, recombination, DNA repair, and synthesis of DNA polymerases [Fernandez De Henestrosa et al., 2000; Courcelle et al., 2001; Janion, 2008].

Following the induction of the Ada response in *E. coli*, four proteins – Ada, AlkA, AlkB, and AidB (encoded by the *ada, alkA, alkB,* and *aidB* genes, respectively) – are highly expressed [Lindahl et al., 1988; Shevell et al., 1988; Landini and Volkert, 2000; Sedgwick and Lindahl, 2002]. The Ada protein plays more than one role in DNA repair. It shows methyltransferase activity for O^6meG, O^4meT, and methylphosphotriesters in DNA, and when methylated at Cys38 (or Cys38 and Cys322), it serves as an inducer for all genes in the Ada regulon.

The recently identified AlkB protein shows Fe(II) and 2-oxoglutarate (2OG)-dependent oxidative demethylase activity; in the presence of O_2, 2OG is converted to succinate and CO_2, and concomitantly, the methyl groups on 1meA/3meC are oxidized to hydroxymethyl derivatives that rapidly dissociate to form formaldehyde and recover the A and C bases both in DNA and RNA [Falnes et al., 2002; Trewick et al., 2002; Aas et al., 2003; Drablos et al., 2004; Ougland et al., 2004; Falnes et al., 2007; Sedgwick et al., 2007]. In a similar way, AlkB oxidizes and dealkylates ethyl, ethano-1, and cyclic etheno-substituted bases in DNA [Delaney et al., 2005; Mishina et al., 2005; Frick et al., 2007].

In *E. coli*, three out of five DNA polymerases – PolII, PolIV, and PolV – are upregulated within the SOS system in response to DNA damage. PolII is able to restart arrested DNA replication in an error-free manner [Rangarajan et al., 1999], whereas PolIV and PolV bypass the non-coding lesions and induce mutations [Wagner et al., 2002; Fuchs et al., 2004]. The *dinB*-encoded PolIV is a low-fidelity polymerase that acts on damaged and undamaged DNA and produces untargeted mutations. Among the SOS polymerases, PolIV binds more frequently at a stalled replication fork and is involved in adaptive mutagenesis by inducing −1 frameshift mutations [Kim et al., 2001]. The *umuDC*-encoded PolV is the major error-prone polymerase participating in translesion synthesis (TLS). Under non-SOS conditions, there are 180 UmuD molecules per cell and a non-detectable number of UmuC molecules. Derepression of the SOS regulon by proteolytic cleavage of the LexA protein produces 2,500 UmuD and 200 UmuC molecules [Sommer et al., 1993]. PolV is formed by UmuC and two molecules of UmuD′ (UmuD′ is post-translationally modified and is 15 amino acids shorter than UmuD) [Wagner et al., 2002; Fuchs et al., 2004]. The content of PolV in the induced cell is restricted by the number of UmuC molecules.

In this report, our attention has been focused on the mutagenic activity of 1meA/3meC residues persisting in the DNA of MMS-treated *E. coli* strains defective in the *alkB* gene product. The involvement of PolV in the mutagenic processing of these lesions was of special interest. The markers for testing mutagenesis were as follows: (i) *argE3*→Arg^+ reversion in AB1157 situated on the chromosome and (ii) *lacZ*$^-$→Lac^+ reversion located on the episome in a set of CC101–106 strains used for determination of the specificity of mutations. We have found that in the absence of AlkB-mediated repair, the frequency of four types of transversions is elevated as a result of MMS treatment: GC→CG, GC→TA, AT→CG, and AT→TA. The overproduction of PolV in CC101–106 *alkB*$^-$/pRW134 strains resulted in a marked elevation of GC→TA, GC→CG, and AT→TA transversions.

Since the frequency of MMS-induced mutations in AB1157 *alkB*$^-$ strains measured in the *argE3*→Arg^+ reversion system was extremely high [Nieminuszczy et al., 2006b] compared with the level of Lac^+ reversions in CC *alkB*$^-$ strains [Dinglay et al., 2000; Nieminuszczy et al., 2006a], we considered several reasons for this discrepancy, namely, the influence of PolV synthesis and SOS induction, the effect of PolIV overproduction in CC strains, and the number and structure of targets leading to the Arg^+ or Lac^+ reversions. The results reported herein indicate that the latter explanation seems to be the most reasonable.

MATERIALS AND METHODS

Media and Plates

Luria-Bertani (LB) broth and LCA medium were the rich media [Miller, 1972]. The minimal medium E contained C-salts [Vogel and Bonner, 1956] supplemented with glucose (0.5%), casamino acids (0.2%), and thiamine (10 μg/ml). E-Arg and E-Pro plates contained E medium devoid of casamino acids but supplemented with His, Thr, Pro, and Leu (each at 25 μg/ml) (E-Arg) or Arg, His, Thr, and Leu (E-Pro). The LAC medium was E-Pro with 0.5% lactose instead of glucose. All plates were solidified with Difco agar at 1.5%. Bacteria harboring antibiotic resistance were selected on LB plates containing the appropriate antibiotics: carbenicillin (50 μg/ml), kanamycin (50 μg/ml), spectinomycin (50 μg/ml), and chloramphenicol (30 μg/ml).

Bacterial Strains and Plasmids

The *Escherichia coli* K12 strains and plasmids as well as their derivatives constructed in this study are listed in Table I. The constructed strains, except for Δ*lacZYA* derivatives, were obtained by P1-mediated transduction [Miller, 1972]. The *alkB* mutants were selected on appropriate plates

TABLE I. Bacterial Strains and Plasmids

Strain	Genotype	Source/method of strain construction
AB1157	*arg E3 hisG4 leuB6* Δ(*gpt-proA*)*62 thr-1 ara-14 galK2 lacY1 mtl-1 xylA5 thi-1 rpsL31 glnV44 tsx-33 rfbD1 mgl-51 kdgK51*	[Bachmann, 1987]
RW82	Δ*umuDC595*::*cat*donor	[Woodgate, 1992]
EC2413	as AB1157 but Δ*umuDC*	[Grzesiuk and Janion, 1994]
BS87	as AB1157 but a*lkB117*::Tn*3*	[Sedgwick, 1992]
GC4540	*pyrD sulA*::Tn*5*	[Fijalkowska et al., 1997]
JL2145	*lexA300*(Del)::spc	[Hill and Little, 1988]
AB1157 *sulA*	as AB1157 but *pyrD sulA*::Tn*5*	this work AB1157 × P1/GC4540
AB1157 *sulA lexA*	as AB1157 but *pyrD sulA*::Tn*5 lexA300*(Del)::spc	this work AB1157 *sulA* × P1/JL2145
AB1157 *sulA alkB117*	as AB1157 but *pyrD sulA*::Tn*5 alkB117*::Tn*3*	this work AB1157 *sulA* × P1/BS87
AB1157 *sulA lexA alkB117*	as AB1157 but *pyrD sulA*::Tn*5 lexA300*(Del)::spc*alkB117*::Tn*3*	this work AB1157 *sulA alkB117* × P1/JL2145
AB1157 Δ*lacZYA*	as AB1157 but Δ*lacZYA*(Kan^R)	this work according to [Datsenko and Wanner, 2000]
HK82 Δ*lacZYA*	as HK82 but Δ*lacZYA*(Kan^R)	this work according to [Datsenko and Wanner, 2000]
AB1157 *alkB117* Δ*lacZYA*	as AB1157 but *alkB117*::Tn*3* Δ*lacZYA*(Kan^R)	this work AB1157 Δ*lacZYA* × P1/BS87
CC101-CC106	*ara thi* Δ(*lac-proB*)$_{xiii}$, F′ *lacI*$^-$*Z*$^-$*proB*$^+$	[Cupples and Miller, 1989]
CC101-CC106 *alkB117*	as CC101-CC106, but *alkB117*::Tn*3*	[Nieminuszczy et al., 2006a]
CC101-CC106 Δ*umuDC*	as CC101-CC106, but Δ*umuDC*	this work
CC101-CC106 *alkB117* Δ*umuDC*	as CC101-CC106, but *alkB117*::Tn*3* and Δ*umuDC*	this work
CC102 (104, 105) *sulA lexA*	as CC102 (104, 105) but *sulA*::Tn*5 lexA300*(Del)::spc	this work CC102 (104, 105) *sulA* × P1/JL2145
CC102 (104, 105) *alkB117 sulA*	as CC102 (104, 105) but *alkB117*::Tn*3 sulA*::Tn*5*	this work CC102 (104, 105) *alkB117* × P1/AB1157 *sulA*
CC102 (104, 105) *alkB117 sulA lexA*	as CC102 (104, 105) but *alkB117*::Tn*3 sulA*::Tn*5 lexA300*(Del)::spc	this work CC102 (104, 105) *alkB117 sulA* × P1/JL2145
NR11152	*ara thi* Δ(*lac-proB*)$_{xiii}$, F′CC102	Schaaper's collection constructed for this work
NR11155	*ara thi* Δ(*lac-proB*)$_{xiii}$, F′CC105	
NR16625	*ara thi* Δ(*lac-proB*)$_{xiii}$, F′CC102/Δ*dinB*::kan	
NR16626	*ara thi* Δ(*lac-proB*)$_{xiii}$, F′CC105/Δ*dinB*::kan	
NR16627	*ara thi* Δ(*lac-proB*)$_{xiii}$ Δ*dinB*::kan, F′CC102/Δ*dinB*::kan	
NR16628	*ara thi* Δ(*lac-proB*)$_{xiii}$ Δ*dinB*::kan, F′CC105/Δ*dinB*::kan	
NR11152 (11155, 16625, 16626, 16627, 16628) *alkB117*	as NR11152 (11155, 16625, 16626, 16627, 16628) but *alkB117*::Tn*3*	this work NR11152 (11155, 16625, 16626, 16627, 16628) × P1/BS87
Plasmids		
pGB2	Spc^R, low-copy pSC101 derivative with mp8 polylinker	[Churchward et al., 1984]
pRW134	*umuD′C* genes inserted into pGB2	[Ho et al., 1993]
pKD46	*araC-ParaB* and γβ *exo* derivative of pINT	*E.coli* Genetic Stock Center, Yale University, USA
pKD4	Kan^R derivative of pANTSγ	

containing carbenicillin, and the presence of the *alkB* mutation was confirmed by the plate test measuring sensitivity to MMS as described by Nieminuszczy et al. [2006b]. The CC101–106 and AB1157 derivatives with *umuDC* deletion (the source of the deletion was RW82 strain) were obtained by P1-mediated transduction, selected on plates containing chloramphenicol, and confirmed by increased sensitivity to UV irradiation.

The *sulA::*Tn*5* and *lexA300* (Del) mutants were selected on appropriate plates containing kanamycin and spectinomycin, respectively. Disruption of *sulA* and *lexA* genes was verified by an analysis of the PCR products.

The set of CC101–106 strains constructed by Cupples bears a different point mutation at Glu461 in episomal *lacZ* gene encoding β-galactosidase. Each of the strains can be back-mutated to Lac$^+$ by all possible transitions or transversions that permit straight definition of the specificity of mutations [Cupples and Miller, 1989].

Transformation with pRW134 (harboring *umuD′C*) and pGB2 (empty control vector) was done according to Sambrook et al [1989]. Transformants were selected on appropriate plates containing spectinomycin. The presence of plasmid DNA was confirmed by agarose gel electrophoresis.

Construction of the AB1157 Strain Deleted in *lacZYA* and Transformed With F′ Episome

The one-step gene inactivation method described by Datsenko and Wanner [2000] was used to construct a *lacZYA* deletion. *E. coli* AB1157 strain was transformed with the pKD46 plasmid harboring phage λ Red recombinase. The PCR fragment was generated with pKD4 plasmid as a template and the following primers:

5′-GCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGGTGTAGGCTGGAGCTGCTTC-3′ and 5′-GTCATCGACAATGGTTAAATTGAAATTTGCATAAAACATATGAATATCCTCCTTAG-3′. The underlined sequences correspond to 20 bases that are specific for pKD4, whereas the remaining sequences correspond to the ends of *lacZYA*. The PCR products were used to transform strains carrying pKD46 by electroporation. Recombinant strains were selected on LB plates containing kanamycin. Deletion of *lacZYA* genes was verified by PCR.

Transfer of the F′ episome from the respective CC strains into AB1157 Δ*lacZYA alkB*$^+$ or *alkB*$^-$ derivatives was carried out by conjugation according to Miller [1972]. Logarithmic-phase cultures of donor and recipient strains were mixed (1:1) and incubated without shaking at

TABLE II. Effect of PolV Content on the Level of MMS–Induced (A) Lac$^+$ and (B) Arg$^+$ Reversions

Bacterial strains and specificity of mutations	*alkB*$^+$				*alkB117*			
	no plasmid	Δ*umuDC*	+pGB2	+ pRW134	no plasmid	Δ*umuDC*	+pGB2	+ pRW134
	A: Frequency of Lac$^+$ revertants (×10^{-8} cells) in strains							
CC101 AT → CG	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.5	3.1 ± 0.9	2.5 ± 2.4	0.0 ± 0.0	1.6 ± 2.2	7.6 ± 4.2
	[0.0 ± 0.0]*	[0.0 ± 0.0]	[0.07 ± 0.12]	[0.0 ± 0.0]	[0.1 ± 0.1]	[0.0 ± 0.0]	[0.1 ± 0.1]	[0.3 ± 0.2]
CC102 GC → AT	18.9 ± 3.3	7.2 ± 1.2	24.6 ± 3.2	15.7 ± 2.1	27.9 ± 8.4	3.7 ± 1.1	27.0 ± 10.7	18.0 ± 6.0
	[0.0 ± 0.0]	[3.8 ± 0.8]	[1.5 ± 0.8]	[1.4 ± 0.1]	[1.3 ± 1.1]	[0.3 ± 0.0]	[0.5 ± 0.5]	[1.3 ± 0.2]
CC103 GC → CG	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	1.1 ± 0.7	3.4 ± 2.5	0.0 ± 0.0	7.0 ± 3.2	29.3 ± 11.9
	[0.0 ± 0.0]	[0.0 ± 0.0]	[0.0 ± 0.0]	[0.0 ± 0.0]	[0.0 ± 0.0]	[0.2 ± 0.0]	[0.4 ± 0.7]	[0.0 ± 0.0]
CC104 GC → TA	0.9 ± 0.5	0.2 ± 0.0	2.1 ± 1.1	2.7 ± 1.6	6.6 ± 3.2	0.7 ± 0.1	20.3 ± 5.9	118.6 ± 16.3
	[0.2 ± 0.0]	[0.2 ± 0.0]	[1.0 ± 0.5]	[0.99 ± 1.4]	[0.6 ± 0.2]	[0.7 ± 0.1]	[0.2 ± 0.3]	[0.3 ± 0.5]
CC105 AT → TA	2.7 ± 1.2	1.6 ± 0.2	0.8 ± 0.7	7.5 ± 5.0	4.7 ± 1.1	0.0 ± 0.0	7.7 ± 3.7	30.3 ± 9.0
	[0.2 ± 0.0]	[0.7 ± 0.1]	[0.4 ± 0.5]	[0.3 ± 0.4]	[0.4 ± 0.2]	[0.2 ± 0.0]	[0.3 ± 0.2]	[0.1 ± 0.2]
CC106 AT → GC	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.7 ± 0.3	0.0 ± 0.0	0.5 ± 0.1	1.1 ± 0.8	1.7 ± 1.6
	[0.0 ± 0.0]	[0.0 ± 0.0]	[0.1 ± 0.1]	[0.0 ± 0.0]	[0.0 ± 0.0]	0.0 ± 0.0	[0.0 ± 0.0]	[0.0 ± 0.0]
	B: Frequency of Arg$^+$ revertants (×10^{-8} cells) in strains							
AB1157	140.9 ± 79.7	27.2 ± 3.4	176.2 ± 68.2	724.3 ± 151.2	1795.7 ± 492.1	35.0 ± 11.3	1436.6 ± 905.8	3353.6 ± 915.6
	[0.5 ± 0.0]	[0.0 ± 0.0]	[0.5 ± 0.1]	[0.8 ± 0.2]	[0.6 ± 0.03]	[0.0 ± 0.0]	[0.7 ± 0.1]	[0.9 ± 0.2]

*Values in brackets represent the frequency of spontaneous Lac$^+$/Arg$^+$ revertants.

37°C for 1 hr. Conjugants were selected on E-Pro plates containing kanamycin (recipient strains are Kan^R) and the presence of the F′ episome, which enabled growth on a medium devoid of proline.

MMS Mutagenicity Assay

Bacteria (overnight culture diluted 1:50), grown in fresh E medium to 2–4 × 10^8 cells/ml, were treated with 20 mM (0.17%) MMS for 15 min, spun down, washed twice, resuspended, diluted (1:10) in fresh E medium, and grown overnight to express the mutations. Subsequently, the bacteria were plated on LB or E-Pro (usually diluted 10^6) and on E-Arg or LAC plates (diluted when necessary) and incubated for 1 day for viable cells or for 2 days for Arg$^+$ and Lac$^+$ revertants. Following colony counting, the frequency of reversion (number of Arg$^+$ and Lac$^+$ revertants/10^8 viable cells) was determined. All experiments were repeated six to nine times in duplicate, and the standard deviations (± SD) were calculated.

RESULTS

Effect of PolV on the Frequency and Specificity of MMS-Induced Mutations

It has been shown previously that mutations in the *alkB* gene significantly increase the sensitivity of bacteria to the cytotoxic and mutagenic activities of MMS. It is also known that PolV is crucial for MMS-induced mutagenesis [Grzesiuk and Janion, 1994; Nieminuszczy et al., 2006b]. These latter observations prompted us to examine more closely the contribution of PolV to the mutagenic activity of MMS in the investigated *alkB*$^-$ strains. To increase the PolV level, we transformed CC101–106 and AB1157 *alkB*$^+$ as well as *alkB*$^-$ strains with a low-copy pRW134 plasmid carrying the *umuD′C* sequence (as a control an empty plasmid vector, pGB2 was used). To eliminate PolV from the cells, the *umuDC* operon was deleted from the above mentioned strains.

The results presented in Table II (Lac$^+$ revertants) indicate that MMS exerts different mutagenic effects in plasmid-free CC101–106 strains. There were few MMS-induced Lac$^+$ revertants monitored in these strains. The most numerous (about 19 Lac$^+$ revertants/10^8 cells) were the GC→AT transitions in CC102, and AT→TA transversions (about 3 Lac$^+$ revertants/10^8 cells) in CC105.

The *alkB117* mutation resulted in an elevated level of MMS-induced Lac$^+$ revertants that arose from unrepaired 1meA/3meC lesions. It is especially seen in CC103 *alkB*$^-$, which monitors GC→CG transitions, and in CC104 *alkB*$^-$, which monitors GC→TA transversions. In these strains, the level of Lac$^+$ revertants was 24- and 7-fold higher, respectively, than in the *alkB*$^+$ counterparts. The introduction of the pRW134 plasmid into the CC *alkB*$^+$ strains led to a 3-fold (CC101, CC104, CC105) and about 8-fold (CC103) increase in the level of MMS-induced Lac$^+$ revertants, whereas in the CC103, CC104, and CC105 *alkB*$^-$ strains harboring the pRW134 plasmid, the level of these revertants was 8-, 18-, and 6-fold higher, in comparison to their plasmid-free counterparts (Lac$^+$ revertants of Table II). Interestingly, in CC102 *alkB*$^+$ and CC102 *alkB*$^-$ bearing pRW134, the frequency of GC→AT transitions was slightly decreased.

In most cases, the presence of the pGB2 plasmid (the control for pRW134) did not influence the level of MMS-induced Lac$^+$ revertants. Two strains, CC104 *alkB*$^-$/pGB2 and CC103 *alkB*$^-$/pGB2, were exceptional with their 3- and 2-fold increase in the level of mutation. However, this was still 18- and 8-fold smaller, respectively, than the strains bearing the pRW134 plasmid encoding

TABLE III. Effect of Chronic Induction of the SOS Response on MMS–Induced (A) Lac^+ and (B) Arg^+ Revertants

	alkB$^+$		*alkB117*	
Bacterial strains	(−) MMS	(+) MMS	(−) MMS	(+) MMS
A: Frequency of Lac^+ revertants ($\times 10^{-8}$ cells) in strains				
CC102 but *sulA*::Tn*5*	3.9 ± 2.5	25.7 ± 3.3	3.1 ± 2.0	22.2 ± 3.7
CC102 but *sulA*::Tn*5 lexA300*(Del)::*spc*	0.7 ± 0.4	13.9 ± 6.0	2.0 ± 0.9	26.0 ± 1.3
CC104 but *sulA*::Tn*5*(*lexA*$^+$)	2.3 ± 0.4	1.0 ± 1.0	0.4 ± 0.6	52.4 ± 9.0
CC104 but *sulA*::Tn*5 lexA300*(Del)::*spc*	0.9 ± 1.3	0.4 ± 0.5	0.5 ± 0.8	75.3 ± 21.8
CC105 but *sulA*::Tn*5*(*lexA*$^+$)	0.3 ± 0.0	1.3 ± 1.2	0.3 ± 0.4	14.1 ± 5.7
CC105 but *sulA*::Tn*5 lexA300*(Del)::*spc*	1.8 ± 2.1	2.0 ± 0.6	0.4 ± 0.3	3.2 ± 3.1
B: Frequency of Arg^+ revertants ($\times 10^{-8}$ cells) in strains				
AB1157 but *sulA*::Tn*5*	2.2 ± 2.2	179.7 ± 153.4	1.7 ± 0.4	1304.0 ± 373.6
AB1157 but *sulA*::Tn*5 lexA300*(Del)::*spc*	2.1 ± 1.3	171.7 ± 141.9	3.5 ± 0.8	3268.0 ± 449.6

UmuD′C proteins, in terms of the frequency of Lac^+ revertants (Lac^+ revertants of Table II).

To confirm the involvement of PolV in MMS-induced mutagenesis in CC strains, a *umuDC* deletion was introduced into these strains. As expected, the level of MMS-induced Lac^+ revertants was dramatically decreased in all six CC strains. However, in CC102 Δ*umuDC*, which monitors GC→AT transitions, the frequency of MMS-induced mutations was still significant (7.2 Lac^+ revertants/10^8 cells). Interestingly, in the CC102 Δ*umuDC* *alkB*$^-$ strain, the level of MMS-induced Lac^+ revertants was 2-fold lower in comparison to CC102 Δ*umuDC* *alkB*$^+$ (Table II).

The data for AB1157 *alkB*$^+$ and *alkB117* strains, both with and without the pRW134 plasmid, are shown in Table II. The level of MMS-induced Arg^+ revertants was almost 13-fold higher in AB1157 *alkB*$^-$ than in the corresponding *alkB*$^+$ strain. However, in the *alkB*$^-$ background the elevated level of UmuD′C produced from pRW134 increased the frequency of Arg^+ revertants only by 1.8-fold, whereas in AB1157 *alkB*$^+$, the frequency increased by up to 5-fold. In contrast, both strains lacking PolV, AB1157 Δ*umuDC* *alkB*$^+$, and AB1157 Δ*umuDC* *alkB117*, showed an extremely low level of MMS-induced Arg^+ revertants.

The Effect of Constitutively Expressed SOS Response on the Level of MMS-Induced Lac^+/Arg^+ Revertants

In attempting to increase the level of PolV, we have constructed *alkB*$^+$ and *alkB*$^-$ derivatives of CC102, CC104, CC105, and AB1157, in which the SOS system is constitutively expressed. The product of the *lexA* gene, the LexA protein, is a repressor for all genes in the SOS regulon. The *lexA*300(Del) mutant is entirely devoid of the LexA protein since the *lexA* gene was replaced by a segment of DNA encoding spectinomycin resistance. The *sulA*-encoded SulA protein is exclusively expressed in the SOS-induced cells and plays a role in arresting cell division. As a result, the bacteria grow as long filaments. Mutations in *sulA* and *lexA* genes lead to normal cell division, and the bacteria grow as single cells under conditions of constitutive SOS induction. Bacteria mutated in *lexA* only are not able to grow [Hill and Little, 1988].

The results shown in Table III indicate that among the three pairs of *alkB*$^+$ and *alkB117* strains tested, CC102, CC104, and CC105, a high frequency of MMS-induced Lac^+ revertants was observed only in CC104 *alkB117* *sulA* and in CC104 *alkB117* *sulA* *lexA300* mutants. The level of MMS-induced Lac^+ revertants in these strains was 54- and 188-fold higher in comparison to the *alkB*$^+$ strains, respectively. However, under conditions of constitutive SOS induction, the level of MMS-induced Lac^+ revertants in the CC104 *lexA* *sulA* *alkB117* strain was almost as high as in the *lexA*$^+$ strain (75.5 vs. 52.4 Lac^+ revertants/10^8 cells, Table III). On the other hand, in CC105 *alkB117* *sulA* *lexA300*, the level of MMS-induced AT→TA transversions was the lowest among the three strains tested (3.2 Lac^+ revertants/10^8 cells) (Table III). In the same CC105 strain, with the *alkB117* mutation and harboring pRW134 plasmid, the frequency of MMS-induced mutations was 10-fold higher. Therefore, this class of mutation is exclusively PolV-dependent and not dependent on the SOS response as a whole.

In the AB1157 *sulA*$^-$*alkB*$^-$ strain, the MMS treatment led to a 7-fold increase in the frequency of Arg^+ revertants in comparison to that observed in the *alkB*$^+$ variant. In the AB1157 *sulA*$^-$*lexA*$^-$ mutant that constitutively expresses the SOS response, the level of Arg^+ revertants was significantly higher (19-fold) in comparison to the *alkB*$^+$ counterpart (Table III).

Does Elevated Level of PolIV in CC Strains May Decrease MMS-Induced Lac^+ Reversion?

The series of CC101–106 strains and the derivatives of AB1157 differ in the number of *dinB* genes encoding DNA polymerase IV (PolIV). The AB1157 strain bears only one *dinB* gene on its chromosome, expressing 250 PolIV molecules per one non-induced cell and 10-fold

TABLE IV. The Frequency of MMS-Induced Lac$^+$ Reversions in Strains Differing in *dinB* Copy Number

Specificity of mutation	Bacterial strain	*dinB* genotype		Frequency of Lac$^+$ revertants ($\times 10^{-8}$ cells) in strains:			
				alkB$^+$		*alkB117*	
		Chromosome	F′	control	+ MMS	control	+ MMS
GC → AT	NR11152	+	+	2.0 ± 2.1	18.7 ± 13.5	1.9 ± 2.2	17.8 ± 8.8
	NR16625	+	−	0.4 ± 0.4	24.0 ± 14.7	0.9 ± 0.8	23.7 ± 12.3
	NR16627	−	−	0.3 ± 0.1	16.9 ± 0.1	1.6 ± 2.3	19.9 ± 9.0
AT → TA	NR11155	+	+	0.2 ± 0.1	1.3 ± 1.2	0.3 ± 0.1	11.1 ± 6.0
	NR16626	+	−	0.2 ± 0.1	1.3 ± 1.2	0.2 ± 0.1	12.3 ± 7.3
	NR16628	−	−	0.5 ± 0.6	2.3 ± 1.5	0.8 ± 1.3	8.7 ± 4.5

All NR strains are derivatives of CC102 (GC → AT) and CC105 (AT → TA).

more per SOS-induced cell. In CC101–106 bacteria, apart from one copy of the *dinB* gene on the chromosome, there is one more copy on its F′ episome, which is expressed at a level of 750 and 7,500 PolIV molecules per non-induced, and SOS-induced cell, respectively. In other words, in AB1157 strains, there are 2,500 PolIV molecules per SOS-induced cell, in the CC101–106 strains, there is a 4-fold higher level of PolIV, i.e., 10,000 molecules per SOS-induced cell [Kim et al., 2001]. The latter suggests that a significantly higher amount of PolIV over PolV may be able to suppress PolV activity in the CC strains.

Recently, CC101–106 derivatives that differ in their number of *dinB* genes were constructed in Roel Shaaper's laboratory (Table I). We have chosen three derivatives of CC102 (NR11152, NR16625, NR16627), and three derivatives of CC105 (NR11155, NR16626, NR16628), bearing from none to two copies of the *dinB* gene, and introduced the *alkB117* mutation into them. The frequency of MMS-induced Lac$^+$ reversions was estimated in each of the aforementioned strains and their *alkB*$^-$ derivatives. The results are presented in Table IV and demonstrate that for all tested strains differing in the number of *dinB* genes, the frequency of MMS-induced mutations does not differ significantly in either the *alkB*$^+$ or *alkB*$^-$ derivatives. This indicates that the increased level of PolIV in CC101–106 does not influence the level of Lac$^+$ revertants; thus, either 1meA/3meC lesions are not bypassed by PolIV or bypass by PolIV does not induce mutations.

Measurement of MMS-Induced Lac$^+$ and Arg$^+$ Revertants in One Tester Strain

Our experimental approaches presented above did not resolve the problem of the poor MMS mutability in a set of the CC101–106 *alkB117* strains measured by *lacZ*$^-$ →Lac$^+$ reversion, and the high mutability in the AB1157 *alkB*$^-$ strains measured by *argE3*→Arg$^+$ reversion. To eliminate any metabolic reasons for this discrepancy, we constructed bacteria that permit the determination of reversions of both *argE3*→Arg$^+$ and *lacZ*$^-$ →Lac$^+$ markers in the same tester strain. Therefore, by genetic manipulation (see Materials and Methods for details), we deleted *lacZYA* from the chromosome of AB1157*alkB*$^+$ and AB1157*alkB117*, transformed these strains with episomal DNA isolated either from CC102 (F′102) or CC105 (F′105), and then tested the reversion frequency of each marker.

TABLE V. MMS-Induced Mutations in AB1157 Δ*lacZYA alkB*$^+$ or *alkB*$^-$ Derivatives Harboring the Indicated F′ Episome

Bacterial strain	F′-episome	Frequency of revertants ($\times 10^{-8}$ cells)	
		Lac$^+$	Arg$^+$
AB1157 *alkB*$^+$ Δ*lacZYA*	F′102	28.7 ± 4.5	118.0 ± 58.9
	F′105	0.3 ± 0.1	119.2 ± 20.0
AB1157 *alkB117* Δ*lacZYA*	F′102	45.6 ± 12.6	1409.1 ± 294.4
	F′105	2.7 ± 3.2	1316.8 ± 262.3

F′-102 and F′-105 denote F′ episomal DNA isolated from CC102 (GC → AT) or CC105 (AT → TA) strains.

The data shown in Table V indicate that in general the frequency of MMS-induced mutations in the constructed strains is comparable with that observed in the separate strains for each marker shown in Table II. Nevertheless, the level of Arg$^+$ revertants measured in separate *alkB*$^+$ and *alkB*$^-$ strains was slightly higher in comparison to the level observed in the strains lacking the *lacZYA* operon but containing introduced F′102 (141 vs. 118 Arg$^+$ revertants/10^8 cells in *alkB*$^+$ and 1,800 vs. 1,409 Arg$^+$ revertants/10^8 cells in *alkB*$^-$). Similar results were obtained in the strains with F′105 (141 vs. 119 Arg$^+$ revertants/10^8 cells in *alkB*$^+$ and 1,800 vs. 1,317 Arg$^+$ revertants/10^8 cells in *alkB*$^-$). In the case of Lac$^+$ revertants, the introduction of F′102 into *alkB*$^+$ and *alkB*$^-$ Δ*lacZYA* resulted in a slightly elevated level of Lac$^+$ revertants (29 vs. 19 Lac$^+$ revertants/10^8 cells in *alkB*$^+$ and 46 vs. 28 Lac$^+$ revertants/10^8 cells in *alkB*$^-$), whereas the introduction of F′105 into the *alkB*$^+$ Δ*lacZYA* strains led to a rather decreased level of Lac$^+$ revertants (0.3 vs. 2.7 Lac$^+$ revertants/10^8 cells). In the *alkB*$^-$

strain, the levels were identical (2.6- and 2.7 Lac^+ revertants/10^8 cells).

DISCUSSION

Modification of nucleic acid bases by the MMS treatment exerts a strong mutagenic and genotoxic effect on *E. coli*. However, there are many cellular defense systems and proteins that repair the modified DNA bases and protect cells against toxicity. One such protein is AlkB dioxygenase that repairs 1meA/3meC lesions that arise in MMS-treated *E. coli* cells and restore natural A and C bases in DNA through damage reversal.

It has been shown that mutations arising in *alkB*-defective strains are dependent on *umuDC*-encoded PolV [Nieminuszczy et al., 2006b; Fix et al., 2008]. Here, we have established more precisely the involvement of PolV in the processing of 1meA/3meC lesions arising after the MMS treatment in *E. coli alkB*$^-$ mutants, and also resolved the problem of the great frequency difference in MMS-induced Arg^+ and Lac^+ revertants measured in AB1157 and CC strains. With the use of the CC101–106 strains, we were able to determine the specificity of Lac^+ revertants according to the AlkB and UmuD(D′)C content. In testing CC *alkB*$^+$ strains, we have found that the great majority (83%) of MMS-induced Lac^+ revertants arose by GC→AT transitions monitored in CC102. This class of mutations was also the most numerous in the CC *alkB*$^-$ strains; however, it constituted only 62% of all types of Lac^+ revertants (Table II). In strains lacking the functional AlkB protein the level of other classes of Lac^+ revertants, namely the GC→TA (CC104), AT→TA (CC105), and GC→CG (CC103) transversions, was elevated by 15-, 11-, and 8-fold, in comparison to the CC *alkB*$^+$ strains, respectively. Transformation of the CC101–106 *alkB*$^+$ and *alkB117* strains with the pRW134 plasmid bearing *umuD′C* genes increased the level of MMS-induced Lac^+ reversions in all strains except CC102, which monitored GC→AT transitions. It is likely that the premutagenic lesions producing this class of mutations are the O^6meG residues leading to *umuDC*-independent mutations.

An extremely high increase in the frequency of Lac^+ revertants has been observed in the CC104 *alkB117*/pRW134 strain (118.6 $Lac^+/10^8$ cells), which monitors GC→TA transversions. The level of these mutations was 44-fold higher in comparison to the *alkB*$^+$ counterpart (Lac^+ revertants of Table II, last column). A significant increase in the Lac^+ revertants was noted also in CC103 (GC→CG transversions) and CC105 (AT→TA transversions) bearing the *alkB117* mutation and harboring the pRW134 plasmid. In these strains, the level of MMS-induced Lac^+ revertants of indicated specificity was 30- and 4-fold higher in comparison to the *alkB*$^+$ strain, respectively.

According to Delaney and Essigmann [2004], under conditions of SOS induction (here assured by MMS treatment) and in the absence of functional AlkB, 3meC produces 35% C→T transitions, 30% of C→A, and 5% of C→G transversions. Fix et al. [2008] have shown that the MMS-induced C→T mutations in *E. coli alkB*$^-$ strains are strongly sequence-dependent; therefore, specificity of mutations may vary depending on the test system used. The 1meG lesion, which is able to induce GC→TA transversions, can be also a substrate for the AlkB enzyme [Delaney and Essigmann, 2004]. However, as it has been shown here that this mutational specificity is detectable only in the CC104 *alkB117*/pRW134 which has an increased level of Pol V.

To find the reason for poor mutability of MMS in the *lacZ*$^-$→Lac^+ reversion system, we considered the following possibilities: (i) shortage of PolV in the CC strains; (ii) the effect of PolIV on mutation suppression, taking into account that PolIV is overproduced in the CC101–106 strains but not in AB1157; (iii) the role of SOS induction on MMS-induced mutations; and (iv) the structure and number of targets involved in the mutagenic pathway leading to Arg^+ or Lac^+ reversions.

As shown above, even under the condition of increased levels of PolV, in the CC strains harboring pRW134, the highest Lac^+ frequency was only 118 revertants/10^8 cells in CC104 *alkB117*/pRW134 (Table II). In addition, reduction of the PolIV content by deletion of one or both *dinB* genes (on the chromosome and/or episome) in the CC101–106 *alkB*$^+$ and *alkB*$^-$ strains did not change the frequency of MMS-induced mutations (Table IV).

The influence of constitutive expression of the SOS response on the level of MMS-induced Lac^+ reversions was studied in *alkB*$^+$ and *alkB*$^-$ CC102, CC104, and CC105 strains mutated in *sulA*, or in both *lexA* and *sulA* genes. Among the three strains and six variants tested (*lexA*$^{+/-}$ and *sulA*$^-$), elevated levels of MMS-induced Lac^+ revertants were observed only in CC104 *alkB117 sulA*::Tn*5 lexA300*(Del), and surprisingly, in its *lexA*$^+$ counterpart. Moreover, the levels of the Lac^+ revertants shown in Table III for CC104 *alkB117 sulA* and CC104 *alkB117 sulA lexA*(Del), are ~10-fold higher than the corresponding values for CC104 *alkB117 sulA*$^+$. This result indicates that disturbance in cell division and filamentous growth of MMS-treated *alkB*$^-$ *sulA*$^+$ strains results in lower frequency of Lac^+ revertants, in comparison to *sulA*$^-$ strains, and that inhibition in bacterial growth plays an important role in mutation avoidance. Additional mutation in *alkB* in the CC104 *sulA*$^-$ *lexA*$^+$ and CC104 *sulA*$^-$ *lexA*$^-$ strains led to an increase in MMS-induced Lac^+ revertants of over 50-fold and 188-fold, respectively (Table III). The high level of mutations in the CC104/pRW134 strains indicates that GC→TA transversions are

the main class of PolV-dependent mutations that occurs in *alkB*$^-$ strain.

A possible reason for the differences in MMS mutability of the *argE3* and *lacZ*$^-$ markers in the same cell background (Table V) is the different number of targeted sites and their various structures in the cells. Our previous studies showed that in the MMS-treated AB1157 strain about 68% of Arg$^+$ revertants arose by the *supL* suppressor formation by AT→TA transversions and 22% by the *supB* suppressor arising by GC→AT transitions. In the BS87 *alkB*$^-$ strain, these proportions are different; only 33% of Arg$^+$ revertants arise by the *supL* suppressor and as much as 56% by the *supB* and *supE* suppressor formation, both by GC→AT transitions. The remaining mutations arise by unknown *supX* suppressor formation. Back mutations occurring by base substitutions in the *argE3* site constitute about 4% of all types of Arg$^+$ revertants in the AB1157 strains as well as in BS87 strains [Nieminuszczy et al., 2006b]. In the CC101–106 strains, reversion of *lacZ*$^-$ → Lac$^+$ occurs only by back mutation at one point in the structural gene encoding the β-galactosidase protein that when not expressed would be primarily in a double-stranded DNA form. Reversion to Arg$^+$ occurs mostly by formation of a variety of *sup*tRNA (ochre) suppressors that are heavily transcribed and exist mostly as single stranded DNA. There are at least eight separate tRNA genes, which by point-mutations located at the anticodon part of tRNA, can form tRNA(ochre) suppressors able to suppress the ochre codon present at the *argE3* locus thus recovering the Arg$^+$ phenotype [Sledziewska-Gojska et al., 1992]. Moreover, DNA encoding tRNA genes most often exists in a single-stranded form that facilitates methylation of A/C to 1meA/3meC. In ssDNA, 1meA/3meC occurs at about 6- to 7-fold higher frequency than in dsDNA [Drablos et al., 2004; Nieminuszczy and Grzesiuk, 2007]. In conclusion, the great discrepancy in the frequencies of MMS-induced *lacZ*$^-$ → Lac$^+$ and *argE3*→Arg$^+$ revertants most probably depends on the number of targets undergoing mutations and results from differences in reactivity of MMS to form 1meA/3meC lesions in single vs. double stranded DNA.

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