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Lethal and mutagenic properties of MMS-generated DNA lesions in *Escherichia coli* cells deficient in BER and AlkB-directed DNA repair

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Methylmethane sulphonate (MMS), an S_N2-type alkylating agent, generates DNA methylated bases exhibiting cytotoxic and mutagenic properties. Such damaged bases can be removed by a system of base excision repair (BER) and by oxidative DNA demethylation catalysed by AlkB protein. Here, we have shown that the lack of the BER system and functional AlkB dioxygenase results in (i) increased sensitivity to MMS, (ii) elevated level of spontaneous and MMS-induced mutations (measured by *argE3* → Arg^+ reversion) and (iii) induction of the SOS response shown by visualization of filamentous growth of bacteria. In the *xth nth nfo* strain additionally mutated in *alkB* gene, all these effects were extreme and led to 'error catastrophe', resulting from the presence of unrepaired apurinic/apyrimidinic (AP) sites and 1-methyladenine (1meA)/3-methylcytosine (3meC) lesions caused by deficiency in, respectively, BER and AlkB dioxygenase. The decreased level of MMS-induced Arg^+ revertants in the strains deficient in polymerase V (PolV) (bearing the deletion of the *umuDC* operon), and the increased frequency of these revertants in bacteria overproducing PolV (harbouring the pRW134 plasmid) indicate the involvement of PolV in the error-prone repair of 1meA/3meC and AP sites. Comparison of the sensitivity to MMS and the induction of Arg^+ revertants in the double *nfo alkB* and *xth alkB*, and the quadruple *xth nth nfo alkB* mutants showed that the more AP sites there are in DNA, the stronger the effect of the lack of AlkB protein. Since the sum of MMS-induced Arg^+ revertants in *xth*, *nfo* and *nth xth nfo* and *alkB* mutants is smaller than the frequency of these revertants in the BER^- $alkB^-$ strain, we consider two possibilities: (i) the presence of AP sites in DNA results in relaxation of its structure that facilitates methylation and (ii) additional AP sites are formed in the BER^- $alkB^-$ mutants.

Introduction

Endogenous (by products of cellular metabolism) and exogenous (environmental chemicals) alkylating compounds introduce damage to DNA, blocking its replication and inducing mutations (1–4). Methylmethane sulphonate (MMS) is a classical S_N2-type alkylating agent that predominantly methylates nitrogen atoms in purines. Treatment with MMS creates the following adducts in DNA: 1-methyladenine (1meA), 3-methyladenine (3meA), 7-methyladenine (7meA), 3-methylguanine (3meG), 7-methylguanine (7meG), O^6-methylguanine (O^6meG), 3-methylcytosine (3meC) and methylphosphotriesters, which constitute ~3.5, 10.8, 1.8, 0.6, 83, 0.3, <1 and 0.8%, respectively, of the total adducts in double stranded DNA (dsDNA). In single stranded DNA (ssDNA) these adducts comprise, respectively, 18, 1.4, 3.8, 1.0, 68, 0, 10.0 and <1% of total adducts. In ssDNA, the participation of 1meA and 3meC in the pool of damaged bases increases significantly because, in contrast to dsDNA, the ring nitrogens at these positions are not protected by the complementary DNA strand. Most of the methylated bases are toxic to the cell, block DNA replication (3meA, 1meA, 3meC and 3meG) and can also be a source of base substitutions like GC → AT (O^6meG and 3meC), AT → GC (1-meA) and AT → TA (3meA and 1meA). The purines methylated at N3 or N7 position are easily hydrolysed and create apurinic/apyrimidinic (AP) sites in DNA (3–9).

The two types of direct reversal of alkylated bases and base excision repair (BER) are regarded as error-free repair systems playing an important role in cell response to alkylating agents. Direct repair in *Escherichia coli* involves two alkyltransferases, Ogt and Ada, and oxidative demethylase AlkB that remove alkyl groups with the recovery of natural bases in DNA. The BER system engages two 3meA DNA glycosylases, Tag and AlkA, and other universal proteins, AP endonucleases (Xth and Nfo), AP lyase, deoxyribophosphodiesterase (dRpase), DNA polymerase I and ligase. The *ada*, *alkA* and *alkB* genes are expressed after induction of adaptative (Ada) response (9). All these proteins participate in the repair of MMS-induced lesions depending mainly on the site of damage. The Ogt, DNA methyltransferase O^6meG, is expressed constitutively and in a suicide reaction transfers methyl groups from O^6meG to its own cysteine. The Ada protein possesses similar activity as the Ogt protein incorporating methyl groups into its Cys-321 residue. Moreover, it removes methyl groups from S_p-diastereoisomers of methylphosphotriesters transferring them onto its Cys-38 residue, thus becoming an inducer of the Ada response. Cys-38 residue can be also directly methylated by MMS. After induction of Ada response, the amount of Ada protein increases by 1500-fold (3,4,10).

AlkB protein belongs to a family of α-ketoglutarate- and non-haem Fe^{2+}-dependent dioxygenases. It acts both on DNA and RNA and reverts 1meA and 3meC residues directly to adenine and cytosine without excision of damaged bases, releasing the oxidized methyl group as formaldehyde. Other products of this reaction are CO_2 and succinate formed after conversion of α-ketoglutarate (4,11–13). It was shown in *in vitro* experiments that alkylated mRNA, tRNA, rRNA and viral RNA are all substrates for AlkB. However, in RNA, AlkB demethylates 1meA and 3meC residues 10-fold less efficiently than in DNA (14). Among other adducts induced by MMS, 1meG can also be repaired by AlkB protein, although with

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lower efficiently than the major substrates, 1meA and 3meC (8,15,16). MMS treatment of *E.coli alkB* mutants significantly decreases their survival, increasing the level of GC → AT, AT → TA and GC → TA base substitutions (17) and to lesser extent −1G and −2CG frameshifts (18). The level of MMS-induced mutagenesis depends on the test system used and is several orders of magnitude higher if the targets undergoing mutations are located in ssDNA [e.g. *argE3* → Arg^+ reversion arising mostly by forming tRNA (ochre) suppressors in *E.coli* AB1157 tester strain] than in dsDNA (e.g. *LacZ* → Lac^+ reversion in CC101–CC106 tester strains) (17–21).

Two proteins, the constitutively synthesized Tag protein (3meA DNA glycosylase I) and the inducible AlkA protein (3meA DNA glycosylase II), are monofunctional DNA *N*-glycosylases that remove 3meA and 3meG from DNA. Also 7meG and 7meA are substrates for AlkA protein (22). Recognition of the damaged base by the appropriate DNA glycosylase and cleavage of the N-glycosidic bond leads to appearance of AP sites in DNA. Formation of AP sites in bacterial DNA following MMS treatment is an initial step of BER. AP endonucleases, Xth exonuclease III and Nfo endonuclease IV create a ssDNA nick 5′ to the AP site generating 3′ OH group, a primer for DNA polymerase. The remaining 5′ terminal deoxyribose phosphate residue is removed by dRpases. In the next steps, DNA polymerase I fills in the nucleotide gap and DNA ligase seals the resulting nick after nucleotide incorporation. Moreover, AP endonucleases remove from DNA 3′-blocking lesions that result from the attack of reactive oxygen species and β-elimination reactions at AP sites. Nth (endo III) shows AP lyase activity via β-elimination. The Xth and Nfo proteins account for 85–90 and 5–10%, respectively, of the total AP endonuclease activity in *E.coli* (23–26).

When the direct reversal of methylated bases and removal of alkylated adducts by the BER system is insufficient, other repair pathways like mismatch repair, homologous recombination and lesion bypass become involved (9,27). The last two mechanisms are connected with induction of the SOS response, a bacterial defence system enabling the survival of cells whose DNA is damaged and replication arrested. The SOS system increases expression of >40 genes involved in DNA repair, replication and mutagenesis (28–30). The expression of genes of the SOS regulon is tightly regulated. The *umuD* and *umuC* genes encoding the Y-family DNA polymerase V (PolV) are expressed among the last ones. In the process of translesion synthesis (TLS), this low-fidelity polymerase, composed of UmuC and two particles of UmuD′ proteins, bypasses lesions inserting a patch of several nucleotides and allowing resumption of DNA replication by PolIII, the main replicative polymerase in *E.coli* (31).

In *E.coli* AB1157 $alkB^+$ and $alkB^-$ strains 60–70 and 95–98% of MMS-induced Arg^+ revertants, respectively, depend on Umu proteins. Moreover, the frequency of MMS-induced mutations has been dramatically reduced in $alkB^-$ strains bearing *umuDC* deletion, pointing to the involvement of PolV and the process of TLS in error-prone repair of 1meA/3meC lesions in DNA (17).

Here, we studied the effects of MMS treatment on survival and mutagenesis of *E.coli* cells mutated in *alkB* and the genes of the BER system. The extremely high sensitivity to MMS, significantly elevated frequency of Arg^+ revertants, and strong induction of SOS response leading to the 'error catastrophy' in the *nth xth nfo alkB* quadruple mutant indicate the role of 1meA/3meC lesions and AP sites in all these phenomena.

Materials and methods

Bacterial strains and media

The *E.coli* K12 AB1157 strain, its derivatives and the plasmids used in this work are listed in Table I. The *alkB* and Δ*umuDC* mutants were constructed by P1-mediated transduction. The *alkB* mutants were screened for MMS sensitivity; the lack of *umuDC* operon was confirmed by decreased level of mutagenesis induced by the *umuDC*-dependent mutagens (MMS or ultra violet).

The pMW1 plasmid was constructed by cloning the polymerase chain reaction (PCR) products encoding the *alkB* gene into pGB2 vector plasmid. The *alkB* gene was amplified with the following primers: reverse, TGAAACCGT-CAGTTATCAGCAACT; forward, CGCCAGACAAGTACAAGAAGTTCC. Following agarose gel electrophoresis, the PCR products were purified by Gel-out (A&A Biotechnology) according to the manufacturer's protocol and cloned with SmaI restriction enzyme.

BW535 and its *alkB* and Δ*umuDC* derivatives were transformed with pGB2, pRW134 and pMW1 plasmids according to Sambrook *et al.* (39).

The liquid incubation media were Luria–Bertani (LB) broth (40) and E medium consisting of C salts (41), glucose (0.5%), casamino acids (0.2%) and

Table I. Bacterial strains and plasmids.

Strains and plasmids	Genotype	Reference or source
	Strains	
AB1157	*argE3*, *hisG4*, *leuB6*, Δ(*gpt-proA*)*62*, *thr-1*, *ara-1*, *galK2*, *lacY1*, *mtl-1*, *xylA5*, *thi-1*, *rpsL31*, *glnV44*, *tsx-33*, *rfbD1*, *mgl-51*, *kdgK51*	(32)
BS87	As AB1157 but *alkB117*::Tn*3*	(33)
BH130	As AB1157 but *nfo-1*::kan	J. Laval collection
BH130 *alkB117*	As BH130 but *alkB117*::Tn*3*	This work (BH130 × P1/BS87)
BW9109	As AB1157 but Δ(*xth-pncA*)*90*	(26)
BW9109 *alkB117*	As BW9109 but *alkB117*::Tn*3*	This work (BW9109 × P1/BS87)
BW535	As AB1157 but *nth-1*::kan Δ(*xth-pncA*)*90 nfo-1*::kan	(26)
BW535 *alkB117*	As BW535 but *alkB117*::Tn*3*	This work (BW535 × P1/BS87)
AB1157 *nth*	As AB1157 but *nth*::*kan zbb-3055*::Tn*10*	(34)
AB1157 *nth alkB117*	As AB1157 *nth* but *alkB*::Tn*3*	This work (AB1157 *nth* × P1/BS87)
EC2413	as AB1157 but Δ*umuDC*::*cat*	(35)
BW535 *alkB117* Δ*umuDC*	As BW535 *alkB117*::Tn*3* but Δ*umuDC*::*cat*	This work (BW535 *alkB117*::Tn*3* × EC2413)
BW535 Δ*umuDC*	As BW535 but Δ*umuDC*::*cat*	(36)
	Plasmids	
pGB2	Spc^R, low-copy pSC101 derivative with mp8 polylinker	(37)
pRW134	*umuD′C* inserted in pGB2	(38)
pMW1	*alkB* inserted in pGB2	This work

thiamine (10 μg/ml). The solid media containing 1.5% Difco agar were LB and E-arg composed of C salts supplemented with thiamine, glucose and a mixture of amino acids (proline, leucine, threonine and histidine), each at 25 μg/ml. LCA medium (LB supplemented with calcium and magnesium: 1% trypton, 0.5% yeast extract, 1% NaCl, 0.25% $MgSO_4$ and 2.5 mM $CaCl_2$) was solidified with Difco agar at 0.6% (40). For bacteria bearing antibiotic resistance, appropriate antibiotics, carbenicillin (50 μg/ml), kanamycin (50 μg/ml), streptomycin (50 μg/ml) or chloramphenicol (30 μg/ml), were added to the media. Bacteria were grown at 37°C.

Survival and mutagenesis assays

Bacteria were grown in E medium to OD_{600} = 0.6–0.7, treated with 20 mM MMS for 5, 10 or 15 min, centrifuged, washed and suspended in the same volume of fresh medium. To test for mutagenicity, the MMS-treated bacteria were diluted 1:10 in E medium, grown overnight to express mutations and plated on the LB plates for viable cells (1 day of incubation) and on E-arg plates for Arg^+ revertants (2 days of incubation). Following the counts, the frequency of Arg^+ reversion (number of Arg^+ revertants per 10^8 of surviving cells) was calculated. The spontaneous level of mutations was assigned as a control.

To estimate the survival of tested strains, bacteria after MMS treatment were immediately diluted and plated on LB plates. After 1 day of incubation, the colonies of viable cells were counted and the percent of survivors was calculated. The sensitivity of bacteria to MMS was also checked in the plate test. In total, 0.1 ml of overnight culture was added to 5 ml of LCA medium and poured onto LB plate. Blotting paper discs soaked in 2 μl MMS were placed on the plate surface. After 1 day of incubation, the zone of inhibition of bacterial growth was measured.

Survival and mutagenesis experiments were repeated 4–10 times, each in duplicate, and standard deviation ± SD was calculated.

Microscopic observations

To examine the morphology of cell growth, bacterial liquid cultures were treated with 20 mM MMS for 5 min and diluted with fresh medium as described for the mutagenesis test. Probes of bacteria were taken after 1 and 2 h of growth after MMS treatment, spread onto glass slides, fixed over a flame, stained with basic fuchsin and examined under a light microscope (Nikon Microphot S.A.) with a ×100 objective lens. Bacteria not treated with MMS were used as controls. All photographs were taken at ×1000 magnification.

Results

Sensitivity of BER and alkB-mutated E.coli strains to MMS

It has been shown previously that a mutation in the *alkB* gene results in increased sensitivity of *E.coli* strains to MMS treatment (17,20). Here, a comparative study was performed on the survival of MMS-treated *E.coli alkB*$^-$ strains additionally mutated in genes involved in BER: *xth*, *nfo* and *nth*. Figure 1A and B and Table II include the results of two tests on bacterial sensitivity to MMS: measurement of bacterial survival after MMS treatment for the indicated time (5–30 min) in liquid medium and a spot plate test with bacteria being exposed to MMS for 18 h. The results of both tests were convergent. The examined *E.coli* mutants in the *alkB* gene or/and in three genes involved in BER, *xth*, *nfo* and *nth*, showed increased sensitivity to MMS in the following order: AB1157 < AB1157 *nth* ≤ BH130 (*nfo*) < BW9109 (*xth*) < BS87 (*alkB*) ≤ AB1157 *nth alkB* < BH130 *alkB* < BW535 (*nth xth nfo*) < BW9109 *alkB* ≤ BW535 *alkB*. Data shown in Figure 1A and Table II indicate that of the *nth* mutant's sensitivity to MMS resembles that of the *nfo* mutant rather than that of the wild type (wt). The *nth* gene encodes endonuclease III, a bifunctional glycosylase, which mainly excises oxidized pyrimidines and shows AP lyase activity incising the phosphodiester bond on the 3′ side of the deoxyribose in the β-elimination reaction (23,42,43). Here, we showed that *E.coli* cells mutated in the *alkB* gene (BS87 strain) were more sensitive to MMS than the *nth* (AB1157 *nth*), the *nfo* (BH130) or the *xth* (BW9109) single mutants and even the *nth xth nfo* (BW535) triple mutant, but only for short exposure to MMS (60 and 47% survival after 5 and 10 min of MMS treatment, respectively). For longer MMS treatment, BW535 showed stronger sensitivity to MMS in comparison to BS87 (Figure 1A and Table II).

Introduction of the *alkB117* mutation to the three single (*xth*, *nfo* and *nth*) and the triple (*nth xth nfo*) mutants dramatically increased their sensitivity to MMS (Figure 1B and Table II). Among all four *alkB*$^-$ strains tested, the *nth alkB* and *nfo alkB* double mutants were most resistant to MMS. The *xth alkB* double and the *nth xth nfo alkB* quadruple mutants showed nearly the same sensitivity to MMS when treated up to 30 min in liquid medium. However, in the spot test, the quadruple mutant was the most sensitive to MMS. Presented data indicate that the sensitivity to MMS observed in BW535 *alkB*$^+$ and *alkB*$^-$ strains results mostly from defects in the repair of AP sites.

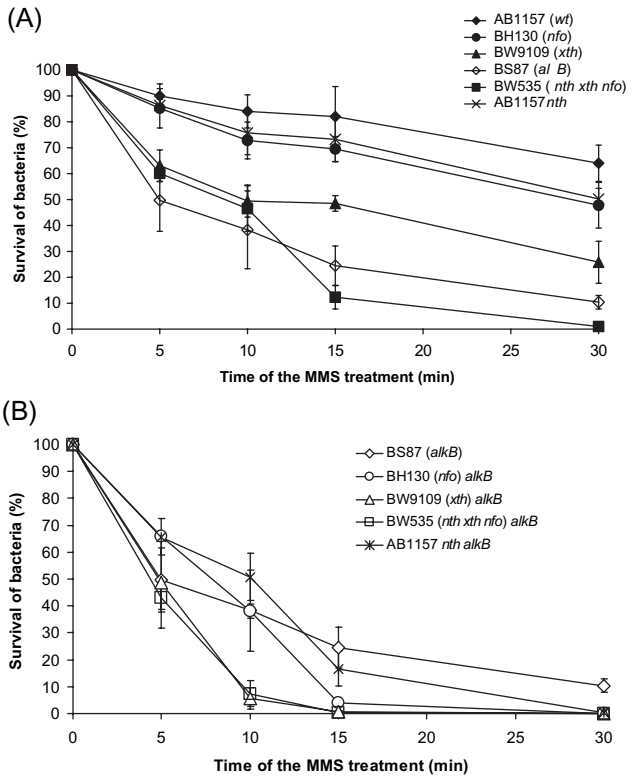

Fig. 1. Survival of bacterial strains treated with 20 mM MMS for the indicated times. (**A**) Strains mutated in BER. (**B**) Strains mutated in BER and *alkB*.

Table II. Sensitivity of *alkB* and BER (*xth*, *nfo* and *nth*) mutants measured by the zone of bacterial growth inhibition in a plate spot test

Strain	The zone of bacterial growth inhibition (mm)
AB1157 *wt*	11–12
BS87 (*alkB*)	29–30
AB1157 *nth*	13
AB1157 *nth alkB*	30–31
BH130 (*nfo*)	13
BH130 (*nfo*) *alkB*	30–32
BW9109 (*xth*)	19
BW9109 (*xth*) *alkB*	35
BW535 (*nth nfo xth*)	32–33
BW535 (*nth nfo xth*) *alkB*	40–45

Impact of the defect in BER on the level of spontaneous and MMS-induced Arg$^+$ revertants in E.coli alkB$^-$ strains

The *argE3* → Arg$^+$ reversion test system used here enables detection of spontaneous and MMS-induced Arg$^+$ revertants arising mostly by tRNA suppressor formation and indicates that the targets undergoing mutations are located in ssDNA (21). The frequency of spontaneous Arg$^+$ revertants was significantly elevated only in two strains, BW535 and AB1157 *nth*, and their *alkB*$^-$ counterparts (~57/71 and 30/33 Arg$^+$ revertants per 10^8 cells, respectively) (Figure 2). The *nth* is a known mutator gene that causes increased level of spontaneous mutations (44). Next, in BW535, a strain defective in *nth*, *xth* and *nfo*, a chronic induction of the SOS response occurs due to accumulation of AP sites in DNA (36).

The *alkB* mutation did not influence the level of spontaneous Arg$^+$ revertants in BH130 (*nfo*) and AB1157 *nth* strains, but increased the level of mutations in BW9109 (*xth*) by 2-fold (Figure 2).

For induced mutagenesis assay, all the strains bearing *alkB* and/or *xth*, *nfo* and *nth* mutations were treated with MMS for only 5 min because of the great sensitivity of *nth xth nfo alkB* mutant to MMS. Longer exposure (10–15 min) of this quadruple mutant led to extremely strong filamentation and even lysis of bacterial cells in overnight cultures (see Visualization of filamentous growth of bacteria after MMS treatment section). Introduction of the *alkB117* mutation to strains with a defective BER system, i.e. the *nfo* (BH130) and *xth* (BW9109) single and *nth xth nfo* triple (BW535) mutants, resulted in, respectively, 5-fold (345:1782), 15-fold (140:2162) and 7-fold (615:4327) increase in the frequency of MMS-induced Arg$^+$ revertants (Figure 2). Interestingly, the level of MMS-induced Arg$^+$ revertants in the *xth alkB* and *nfo alkB* double and *nth xth nfo alkB* quadruple mutants was higher than the sum of the reversion in the *alkB* and *xth*, and *nfo* single, and *nth xth nfo* triple mutants, respectively.

The frequencies of Arg$^+$ revertants were also estimated in the strains mutated in BER treated with MMS for 5, 10 and 15 min (Figure 3). Among these strains, BW535 showed the highest level of Arg$^+$ revertants. This result points to the accumulation of AP sites in the triple mutant, especially since the sum of Arg$^+$ revertants in AB1157 (wt), BW9109 (*xth*), BH130 (*nfo*) and AB1157 *nth* is lower than in BW535. In BW9109, MMS showed a more toxic effect than mutagenic effect, whereas in the *nfo* mutant, an opposite tendency was observed. It is worth mentioning that the *alkB xth* double mutant was significantly more mutable by and sensitive to MMS than the *alkB nfo* (Figure 2).

BS87(*alkB*) showed even higher level of Arg$^+$ revertants than BW535 when exposed to MMS for 5 min; however, longer 15-min exposure led to more numerous mutations in BW535 strain (Figure 3).

Visualization of filamentous growth of bacteria after MMS treatment

Filamentous growth of bacteria is a marker for the induction of the SOS response (36,45). This type of growth results from the expression of the *sulA* (*sfiA*) gene encoding an inhibitor of cell division and induced as one of the latest within the SOS regulon. Delay in cell division allows for DNA repair after the action of DNA-damaging agents (46). SulA interferes with the FtsZ protein and hinders the first step in the cell division process, i.e. FtsZ self-assembly into a ring structure at the future division site (47). Figure 4 shows filamentous growth of the indicated bacteria treated with MMS for 5 min and incubated for 1 or 2 h in growth medium allowing expression of the SOS response. Besides BW535 *alkB*$^+$ and BW535 *alkB*$^-$, none of the examined strains formed filaments unless treated with MMS. Filamentous growth of BW535 results from a chronic induction of the SOS response (36).

The inspected bacteria showed increasing filaments formation after MMS treatment in the following order: AB1157 = AB1157 *nth* (data not shown) = BH130 (*nfo*) < BW9109 (*xth*) < BS87 (*alkB*) = AB1157 *nth alkB* (data not shown) < BH130 *alkB* = BW9109 *alkB* < BW535 (*nth xth nfo*) < BW535 *alkB*. Analysis of data in Figure 4 reveals the following tendencies: (i) the filaments in MMS-treated *alkB*$^+$ strains are most evident after 1 h of incubation, and after 2 h the cells begin to return to the SOS uninduced state; (ii) in the *alkB*$^-$ cells, a stronger filamentation is observed after 2 h of incubation and (iii) for all the pairs of strains tested, the MMS-treated *alkB*$^-$ strains always show significantly higher filamentation than their *alkB*$^+$ counterparts.

There is a strong correlation between the extent of filamentation, MMS mutagenicity and MMS sensitivity in the

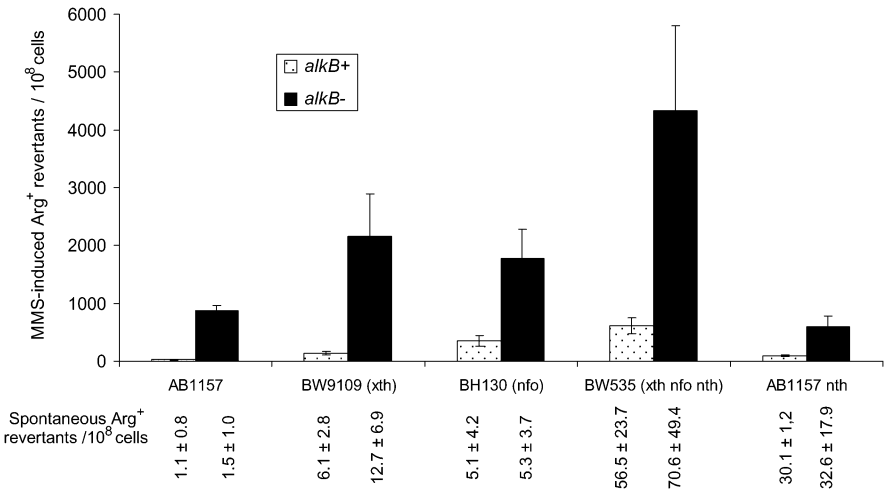

Fig. 2. Frequency of MMS-induced Arg$^+$ revertants in indicated *Escherichia coli* mutants after 5 min of MMS treatment. The table below presents the frequency of spontaneous Arg$^+$ revertants in these strains.

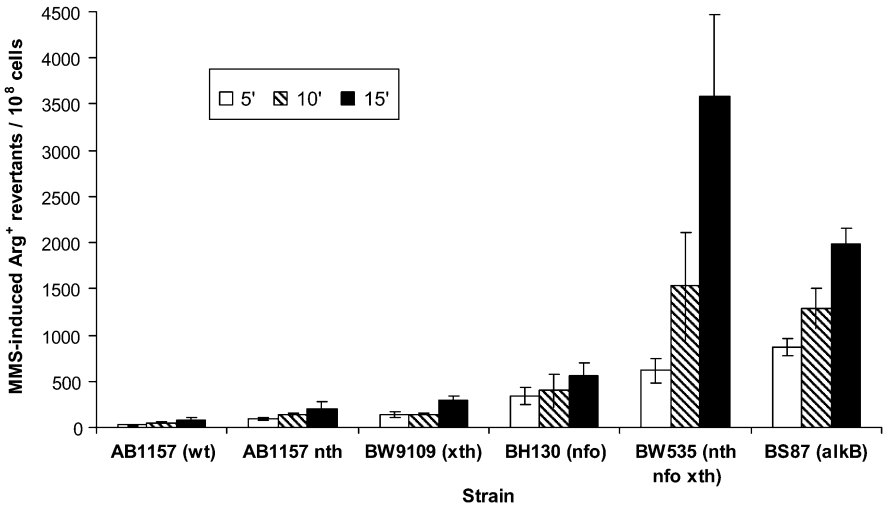

Fig. 3. Frequency of MMS-induced Arg$^+$ revertants in indicated *Escherichia coli* mutants treated with MMS for 5 min (open square), 10 min (striped square) or 15 min (filled square).

tested strains: the stronger filamentation of bacteria after MMS treatment, the higher the observed frequency of Arg$^+$ reversion and the stronger the sensitivity to MMS. This tendency was most striking in the BW535 *alkB* strain. The photograph of MMS-treated BW535 *alkB* shows (i) very strong filamentation of the cells, particularly after 2 h of incubation and (ii) lysis of the cells observed after 2 h of incubation. This is in accordance with the observation that the BW535 *alkB* strain treated with MMS for 10 and 15 min and subsequently incubated overnight in liquid medium showed lysis in the tube.

The involvement of the AlkB protein in the cell protection against lethal effect of MMS was additionally proved by observation of filamentous growth in BW535 *alkB* strain harbouring pMW1 plasmid. The presence of the plasmid bearing *alkB* gene resulted in shorter filaments and better shape of BW535 *alkB*/pMW1 cells. In contrast to the BW535 *alkB* (Figure 4) and BW535 *alkB*/pGB2 (pGB2 does not contain the *alkB* gene), in BW535 *alkB*/pMW1 no 'shadows' of dead bacteria have been observed (Figure 5). Nevertheless, the presence of pMW1 plasmid did not improve the survival of BW535 *alkB* strain, although it decreased the level of MMS-induced mutations by 3-fold in this strain. Note that the frequency of MMS-induced Arg$^+$ revertants in BW535 *alkB*/pMW1 was still 2.5-fold higher in comparison to BW535 *alkB*$^+$ (Table III).

Effect of PolV content on the frequency of mutations in BW535 alkB strains

We have previously shown that 95–98% of MMS-induced mutations in *alkB*$^-$ strains are *umuDC* dependent (17). In BW535 (*nth xth nfo*) strain, the deletion of the *umuDC* operon decreases the level of spontaneous Arg$^+$ revertants by 3.5-fold, although it still remains higher than in the AB1157 strain (36). This indicates that the *umuDC*-encoded PolV induced within the SOS response was responsible for only a part of the spontaneous mutations in BW535. In the same publication, we have reported that the yield of P1-mediated transduction of *umuDC* deletion was at least 100-fold lower in BW535 than in AB1157 and that BW535 Δ*umuDC* loses viability when kept on plates at room temperature. Here, we introduced *umuDC* deletion to BW535 *alkB* strain by P1-mediated transduction and the same problems appeared. Other authors reported that they could not obtain the *recA* and *recB* derivatives of the *nth xth nfo* mutants (48) and that *xthA nfo recB270* (Ts) cells were inviable at the non-permissive temperature (27).

In the BW535 *alkB* strain, the introduction of *umuDC* deletion further increased the sensitivity to MMS, from 43 to 11% after 5 min and from 7 to 0.06% after 10 min of MMS treatment (Table III). Prolonged MMS treatment resulted in total killing of BW535 *alkB* Δ*umuDC* cells. The most striking effect of the absence of UmuD′C proteins in the BW535 *alkB* strain was the dramatic decrease (∼193-fold) in the frequency of MMS-induced mutations. This effect was much weaker in the BW535 *alkB*$^+$ Δ*umuDC* strain where the level of MMS-induced Arg$^+$ revertants was 23-fold lower in comparison to the BW535 counterpart (Table III). This result may indicate preferential repair of 1meA/3meC lesions (substrates for AlkB dioxygenase) over AP sites (repaired by BER) by PolV during TLS. In BW535 *alkB* Δ*umuDC* and BW535 Δ*umuDC* strains, the level of MMS-induced Arg$^+$ revertants was only ∼1.5- and 2-fold higher, respectively, than of spontaneous ones, indicating again that most of the MMS-induced mutations are *umuDC* dependent.

The frequency of spontaneous Arg$^+$ revertants in BW535 and BW535 *alkB* strains was ∼4-fold higher in comparison to the strains with *umuDC* deletion, which suggests a role of PolV in spontaneous mutagenesis.

Introduction of pRW134 plasmid producing UmuD′C proteins (PolV) into strains deficient in BER, AlkB and PolV generally resulted in an increased level of MMS-induced Arg$^+$ revertants (Table III). In BW535/pRW134, the frequency of MMS-induced Arg$^+$ revertants was over 4-fold higher in comparison to the 'empty' strain, indicating involvement of error-prone TLS in the repair of MMS-induced lesions under condition of non-functional BER. In BW535 *alkB* strain, the presence of pRW134 plasmid did not change the level of Arg$^+$ revertants, although this level is ∼8-fold higher than in BW535 *alkB*$^+$. The lack of the two DNA repair systems and overproduction of PolV may explain the observed high dispersion in the results of frequency of Arg$^+$ revertants in BW535 *alkB*/pRW134 strain.

In the strains with deletion of the *umuDC* operon, where the level of MMS-induced mutations was extremely low, the presence of the pRW134 plasmid increased the frequency

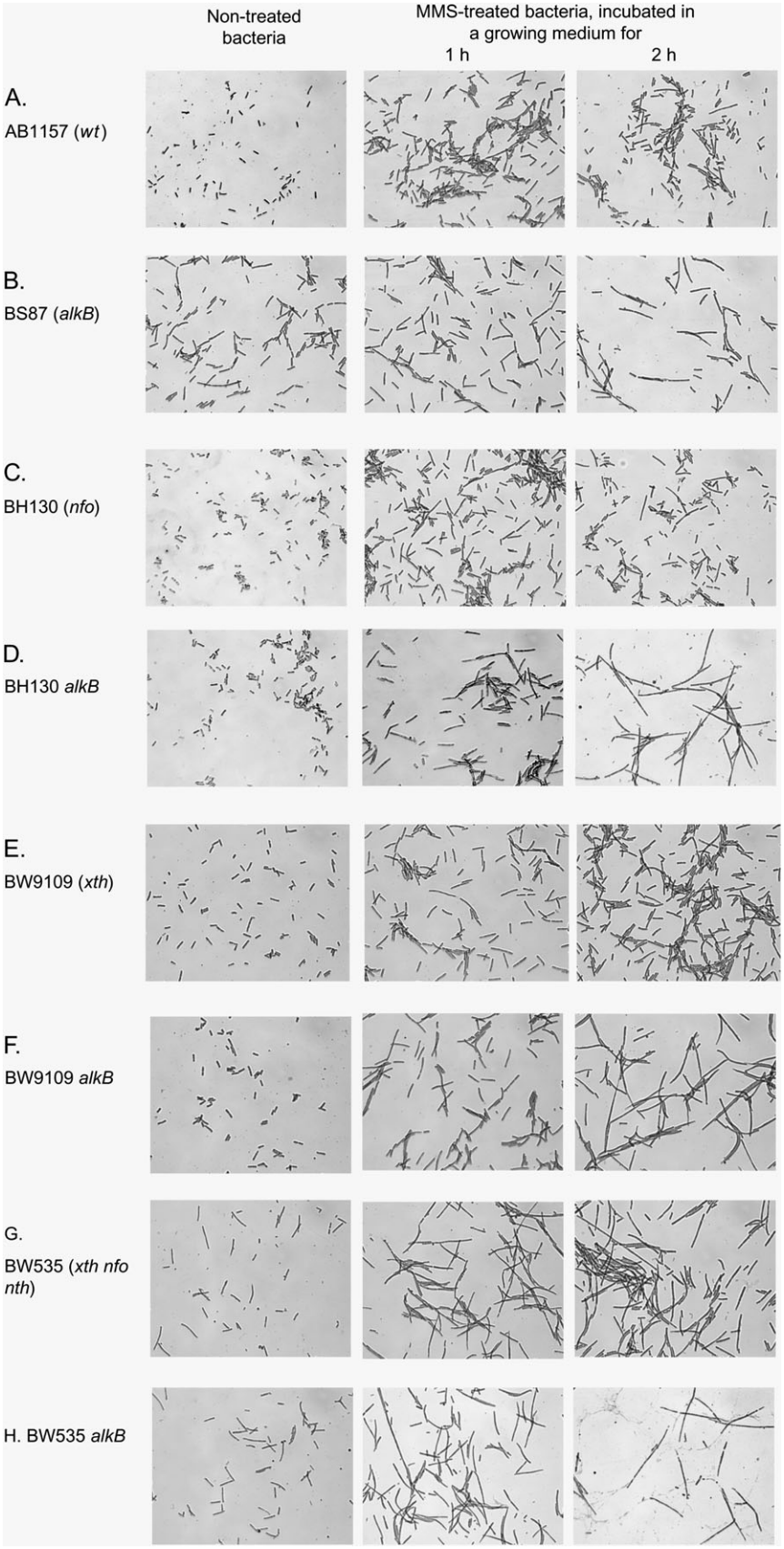

Fig. 4. Filamentous growth of the indicated bacteria resulting from the SOS induction after MMS treatment. (**A**) AB1157 (*wt*), (**B**) BS87, (**C**) BH130, (**D**) BH130 *alkB*, (**E**) BW9109, (**F**) BW9109 *alkB*, (**G**) BW535 and (**H**) BW535 *alkB*. Column I presents bacteria before MMS treatment. Columns II and III show bacteria treated with MMS for 5 min and incubated in the growth medium for 1 and 2 h, respectively.

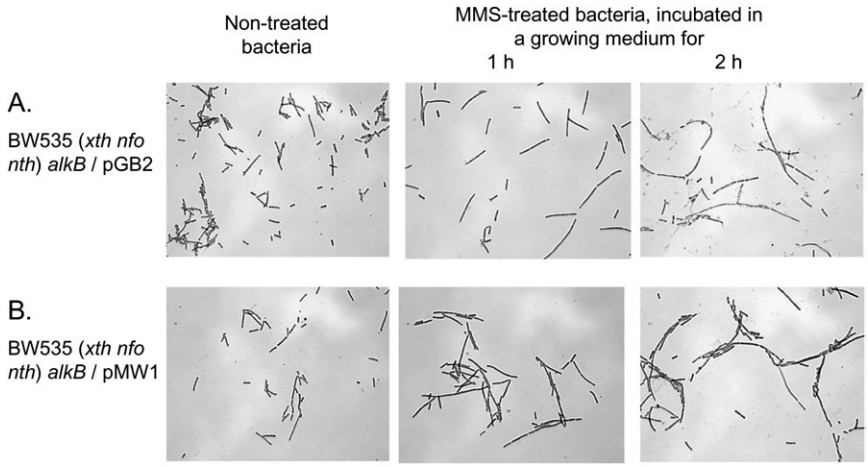

Fig. 5. Effect of overproduction of AlkB protein on filamentous growth of the *nth xth nfo alkB* mutants. (**A**) BW535 *alkB*/pGB2 and (**B**) BW535 *alkB*/pMW1. Column I presents bacteria before MMS treatment. Columns II and III show bacteria treated with MMS for 5 min and incubated in the growth medium for 1 and 2 h, respectively.

Table III. Survival and frequency of spontaneous and MMS-induced *argE3* → Arg$^+$ reversion in *alkB*$^-$ and defected in BER *Escherichia coli* strains bearing *umuDC* deletion or indicated plasmids

	Survival (%) after MMS treatment			Level of Arg$^+$ revertants per 10^8 cells	
Strain	5 min	10 min	15 min	Spontaneous	MMS induced for 5 min
BW535	60.1 ± 3.2	46.5 ± 8.8	12.3 ± 4.5	56.5 ± 23.7	615 ± 133
BW535/pGB2	–	–	–	47.9 ± 17	580 ± 44
BW535/pRW134	–	–	–	131.9 ± 81.9	2836 ± 1512
BW535/pMW1	76.3 ± 8.4	40.0 ± 5.2	13.8 ± 4.2	43.4 ± 10.0	523 ± 85.6
BW535 *alkB*	43.2 ± 4.5	7.4 ± 4.7	0.5 ± 0.4	70.6 ± 49.4	4327 ± 1478
BW535 *alkB*/pRW134	–	–	–	304.6 ± 158.2	4831 ± 3388
BW535 *alkB*/pMW1	36.8 ± 4.6	4.1 ± 1.9	0.6 ± 0.2	51.7 ± 8.9	1599 ± 59
BW535 Δ*umuDC*	48.1 ± 7.3	18.2 ± 8.3	1.2 ± 0.8	13.7 ± 0,5	26.8 ± 2.0
BW535 Δ*umuDC*/pGB2	–	–	–	28.1 ± 7.8	36.4 ± 5.9
BW535 Δ*umuDC*/pRW134	–	–	–	60.0 ± 15.8	1086 ± 37.9
BW535 *alkB* Δ*umuDC*	11.1 ± 4.9	0.06 ± 0.04	0.002 ± 0.002	18.2 ± 3.8	22.4 ± 2.7
BW535 *alkB* Δ*umuDC*/pGB2	–	–	–	19.5 ± 6.8	28.8 ± 4.5
BW535 *alkB* Δ*umuDC*/pRW134	–	–	–	68.3 ± 18.1	2009 ± 378

of Arg$^+$ revertants by 40- and 90-fold in BW535 Δ*umuDC* and BW535 *alkB* Δ*umuDC*, respectively. Interestingly, in BW535 Δ*umuDC*/pRW134, the frequency of MMS-induced Arg$^+$ revertants was almost 2-fold higher than in BW535 and, in contrast, over 2-fold lower in BW535 *alkB* Δ*umuDC*/pRW134 when compared to BW535 *alkB* (Table III). Introduction of the pGB2 plasmid (not producing PolV) into the same strains did not change the frequency of Arg$^+$ revertants, both spontaneous and MMS induced (data not shown). The presence of the pRW134 plasmid led also to an increased level of spontaneous Arg$^+$ revertants, by 2-fold in BW535 and 4-fold in BW535 *alkB*, BW535 Δ*umuDC* and BW535 *alkB* Δ*umuDC*.

Discussion

We have described the effects of absence of two independent error-free DNA repair systems, BER and AlkB-directed oxidative demethylation, on survival, mutagenesis and SOS induction in MMS-treated *E.coli* AB1157 mutants. Bacteria mutated in the genes of the BER system, *xth* and *nfo*, accumulate AP sites referred to as non-instructive lesions because they are incapable of forming Watson–Crick hydrogen bonds. Here, we have shown that the more AP sites are left unrepaired in DNA, the greater the sensitivity of bacteria to MMS (the order of mutants according to their sensitivity to MMS was *nfo* < *xth* < *nfo xth*) (Figure 1A and Table II). These results are in agreement with the data of Cunningham *et al*. (26), who observed an increased lethal effect of MMS on the *xth* and *nfo* mutants. Moreover, the *xth nfo* double and *nth xth nfo* triple mutants showed similar sensitivity to MMS, which was significantly higher than that of the *xth* and *nfo* single mutants.

Most studies concerning bacterial BER mutants focus on spontaneous mutagenesis and sensitivity to different DNA-damaging agents (25–27,42,48–50). Here, for the first time we have shown the involvement of the BER gene products in MMS-induced mutagenesis. As could be expected, among strains mutated in BER, the triple mutant, the *nth xth nfo* (BW535), showed the highest level of MMS-induced Arg$^+$ revertants (Figure 3). This result indicates the accumulation of AP sites in the BW535 strain. The sum of Arg$^+$ revertants in

the AB1157 (wt), BW9109, BH130 and AB1157 *nth* was lower than in the BW535. Surprisingly, a stronger mutagenic effect of MMS is observed in the *nfo* than *xth* single mutant, in spite of significantly more AP sites arising in the latter strain (Figure 3). This suggests that to some extent the AP sites show rather lethal than mutagenic effects. Following are the sources of AP sites in DNA of MMS-treated bacteria: (i) spontaneous depurination of methylated bases such as 7meA and 3-meA that constitutes 83 and 10.8%, respectively, of total adducts in dsDNA; (ii) intermediates arising during BER after excision of methylated bases, 3meA, 7meG, 3meG and 7meA, by DNA glycosylases. Up to 95% of these AP sites are left unrepaired in BER-deficient mutants (25,26). In these mutants, a chronic induction of the SOS system has been observed (36).

In bacteria deprived of functional AlkB protein, there is a pool of unrepaired 1meA and 3meC methylated bases resulting in increased sensitivity to MMS of *alkB* mutants in comparison to their *alkB*$^+$ counterparts. In the *xth*, *nfo* and *nth xth nfo* strains additionally mutated in the *alkB* gene, both types of lesions, AP sites and 1meA/3meC, are accumulated that results in high sensitivity to MMS, particularly in the quadrupled *nth xth nfo alkB* mutant (Figure 1 and Table II). The quadruple mutant was not able to survive >5 min of MMS treatment. Longer exposure to MMS led to filament formation and even cell lysis. Also, the level of MMS-induced Arg$^+$ revertants was the highest in BW535 *alkB* strain (4327 Arg$^+$ revertants per 10^8 cells in comparison to 615 and 867 Arg$^+$ revertants per 10^8 cells in BW535 and BS87, respectively) (Table III and Figure 3). Interestingly, the sum of the *argE3* → Arg$^+$ reversions in BW535 (*xth nfo nth*) and in BS87 (*alkB*) was ~3-fold lower than the level of these reversions in BW535 *alkB* after 5 min of MMS treatment. The mutation in *alkB* gene, however, did not increase the level of spontaneous Arg$^+$ revertants nor influenced the frequency of these revertants in the BW535 strain. Thus, in the BW535, a moderate mutator strain, the frequency of spontaneous Arg$^+$ revertants was only slightly lower in comparison to the BW535 *alkB* strain (~57 and 71 Arg$^+$ revertants per 10^8 cells, respectively) (Table III).

Here, we are dealing with two independent repair systems. However, the presented results indicate that in this specific case they influence each other. The most possible explanation of the observed phenomenon is that AP sites relax the dsDNA structure and more ssDNA appears in the bacterial chromosome. It is known that abasic sites change the conformation of DNA depending on the nature of the flanking and opposite lesions. In the case of apyrimidinic site, the purine usually remains stacked within the helix in an intrahelical conformation. In the case of apurinic site depending on the sequence context, the unrepaired pyrimidine may be stacked inside the helix, expelled outside or in equilibrium between the two states. This phenomenon is explained by base–base stacking interactions between the surrounding bases. AP sites do not change the global β-conformation of DNA but increase its flexibility (51). We suppose that apurinic sites formed in DNA spontaneously as a result of *xth* or *nfo* mutations cause a local relaxation of dsDNA structure that enables generation of 1meA and 3meC by MMS. The ssDNA is also a signal for induction of the SOS system (52). Comparing MMS sensitivity and induction of *argE3* → Arg$^+$ reversions in *nfo alkB*, *xth alkB* and *xth nfo nth alkB* mutants, it is clearly seen that the more AP sites in DNA, the stronger is the effect of the non-functional *alkB* gene. We suggest that in BW535 (*xth nfo nth*) and also, albeit to a lesser extent, in BW9109 (*xth*) and BH130 (*nfo*), the participation of 1meA and 3meC in chromosomal DNA of bacteria after MMS treatment is significantly higher than in the AB1157 (*xth*$^+$ *nfo*$^+$ *alkB*$^+$) strain. 1meA and 3meC as lesions blocking DNA synthesis (i) show a strong lethal effect and significantly increase the sensitivity of bacteria to MMS, (ii) are strong inducers of the SOS system and (iii) cause a dramatic increase in the rate of mutations when bypassed by PolV. Our system of mutation detection bases on *argE3* → Arg$^+$ reversion. The Arg$^+$ revertants arise mostly by formation of *sup*tRNA suppressors that exist as ssDNA, facilitating methylation of A/C to 1meA/3meC (21).

MMS-induced Arg$^+$ revertants are strongly *umuDC* dependent. In all the strains tested, deletion of the *umuDC* operon results in a dramatic decrease of MMS-induced mutations and, in contrast, the presence of the pRW134 plasmid overproducing UmuD'C (PolV) leads to an increased level of Arg$^+$ revertants. These results suggest that PolV is able to bypass AP sites, according to the A-rule, preferentially incorporating dA opposite these lesions (50,53). The frequency of MMS-induced Arg$^+$ revertants in the BW535*alkB* strain is extremely high and the same as in BW535 *alkB* harbouring pRW134, indicating some 'error catastrophe' in these strains. This error catastrophe was visualized by filamentous growth of bacteria and cell lysis. Overproduction of AlkB protein from the pMW1 plasmid in the BW535 *alkB* only partly reverses this effect, whereas the presence of this plasmid in the BS87 strain totally restores the *alkB*$^+$ phenotype (data not shown).

The presented results indicate that the higher level of DNA damage in a strain with defects in two different repair systems, BER and AlkB-directed demethylation, does not necessarily indicate that they both remove the same lesions. A defect in one repair system may indirectly promote the appearance of certain DNA lesions that are not its substrates. The excess of 1meA/3meC unrepaired in AlkB-deficient bacteria may result in the relaxation of DNA structure due to numerous AP sites and better accessibility of ssDNA to MMS. One could also consider the possibility that 1meA and 3meC may indirectly and by an unknown mechanism constitute a source of AP sites in DNA.

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