Contribution of transcription-coupled DNA repair to MMS-induced mutagenesis in *E. coli* strains deficient in functional AlkB protein

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ABSTRACT

In *Escherichia coli* the alkylating agent methyl methanesulfonate (MMS) induces defense systems (adaptive and SOS responses), DNA repair pathways, and mutagenesis. We have previously found that AlkB protein induced as part of the adaptive (Ada) response protects cells from the genotoxic and mutagenic activity of MMS. AlkB is a non-heme iron (II), α-ketoglutarate-dependent dioxygenase that oxidatively demethylates 1meA and 3meC lesions in DNA, with recovery of A and C. Here, we studied the impact of transcription-coupled DNA repair (TCR) on MMS-induced mutagenesis in *E.coli* strain deficient in functional AlkB protein. Measuring the decline in the frequency of MMS-induced *argE3*→Arg^+ revertants under transient amino acid starvation (conditions for TCR induction), we have found a less effective TCR in the BS87 (*alkB*$^-$) strain in comparison with the AB1157 (*alkB*$^+$) counterpart. Mutation in the *mfd* gene encoding the transcription-repair coupling factor Mfd, resulted in weaker TCR in MMS-treated and starved AB1157 *mfd-1* cells in comparison to AB1157 *mfd*$^+$, and no repair in BS87 *mfd*$^-$ cells. Determination of specificity of Arg^+ revertants allowed to conclude that MMS-induced 1meA and 3meC lesions, unrepaired in bacteria deficient in AlkB, are the source of mutations. These include AT→TA transversions by *supL* suppressor formation (1meA) and GC→AT transitions by *supB* or *supE*(oc) formation (3meC). The repair of these lesions is partly Mfd-dependent in the AB1157 *mfd-1* and totally Mfd-dependent in the BS87 *mfd-1* strain. The nucleotide sequence of the *mfd-1* allele shows that the mutated Mfd-1 protein, deprived of the C-terminal translocase domain, is unable to initiate TCR. It strongly enhances the SOS response in the *alkB*$^-$ *mfd*$^-$ bacteria but not in the *alkB*$^+$ *mfd*$^-$ counterpart.

INTRODUCTION

The potentially mutagenic and genotoxic alkylating agents are widely spread in the environment and are also produced as a result of normal cellular metabolism [1-3]. They introduce into DNA lesions that block replication (e.g., 3meA), cause mutations (e.g., O^4meT and O^6meG), or, as in the case of 7meG, are neither cytotoxic nor mutagenic, but undergo spontaneous elimination or excision by glycosylases in the course of base excision repair (BER), leaving behind an apurinic (AP) site. Living organisms are well equipped with mechanisms protecting cells from the harmful effects of alkylating agents. In *E.coli* alkylating agents induce an adaptive response resulting in an increased expression of four genes: *ada, alkB, alkA* and *aidB* [4-8]. The key component of this response is the Ada protein, an activator of transcription of its own gene and of those encoding AlkA, AlkB, and AidB [5,9]. The function of AlkB protein has been established only recently [10-16]. It is a dioxygenase that oxidatively demethylates N^1meA (1meA) and N^3meC (3meC) in DNA in a reaction involving α-ketoglutarate, O_2, and Fe^{2+}, and resulting in the recovery of A and C bases.

Methyl methanesulfonate (MMS), an S_N2 alkylating agent, is not only an efficient inducer of adaptive response, but also induces the SOS system that increases the expression of over 40 genes involved in DNA recombination, repair, replication, and mutagenesis [17-19]. Two among these genes, *umuD* and *umuC*, encode the Y-family DNA polymerase V (PolV). In the mutagenic process of translesion synthesis (TLS) this low fidelity polymerase is able to bypass lesions, inserting a stretch of several nucleotides; subsequently, PolIII, the main replicative polymerase in *E.coli*, resumes DNA replication [20].

In *E.coli* AB1157 (*argE3*) strain, the induction of the SOS system and expression of PolV is a prerequisite for 70-80% of MMS-induced *argE3*→Arg^+ revertants. They arise by *de novo*

formation of *supL* suppressor created from tRNAlys2 by AT→TA transversions. In contrast to these *umuDC*-dependent mutations, the *umuDC*-independent ones arise by formation of *supB* (from tRNAgln1) or conversion of *supE44*(amber)→*supE*(ochre). Both *supB* and *supE*(ochre) suppressors arise by GC→AT transitions. The *umuDC*-independent mutations may also arise by back mutation at the *argE3* site [21].

The phenomenon of mutation frequency decline (MFD) was first described by Witkin [22,23] as a loss of UV-induced mutations in tRNA suppressor genes during transient inhibition of protein synthesis. Evelyn Witkin [24] also constructed the *E.coli* WU3610-45 *mfd-1* mutant, showing a smaller decline in the frequency of UV-induced mutations than the parental WU3610 strain. The *mfd-1* mutation has been used frequently, but is fully characterized only in the present study.

Further investigations of the MFD phenomenon have shown that this system, called transcription-coupled DNA repair (TCR), mediates selective removal of lesions from the transcribed DNA strand, coupling transcription and DNA repair. TCR requires *mfd*-encoded Mfd protein which removes transcription elongation complexes stalled at DNA non-coding lesions and recruits to these sites proteins involved in nucleotide excision repair (NER) [25].

E.coli Mfd is a multifunctional protein of 130 kDa consisting of eight domains. These domains can be grouped into several modules according to their function. Among others, there is a module homologous to the UvrB protein which in complex with Mfd can bind UvrA, thus bringing the NER machinery to the RNA polymerase (RNAP)-Mfd complex [26]. There is also a domain of interaction with RNAP, a translocase domain comprised of TD1 translocase module and TD2 with the TRG (Translocase in RecG) motif [27]. In this way, Mfd functions not as a helicase but as a double strand (ds) DNA translocase [28], cleansing DNA of RNAP stalled at a

lesion [29].

Here, we studied the effect of TCR on MMS-induced lesions under conditions of non-functional AlkB protein. Transient amino acid starvation was used to induce TCR, whereas mutation in the *mfd* gene served to switch it off. We found that during transient amino acid starvation, the decline in the level of MMS-induced Arg^+ revertants in the *alkB*$^-$ mutant was weaker than in the AB1157 *alkB*$^+$ strain. On the other hand, the effect of *mfd-1* mutation on the level of MMS-induced Arg^+ revertants in transiently starved bacteria was much stronger in the *alkB*$^-$ strain (total lack of TCR) than in the *alkB*$^+$ counterpart. We established that MMS-induced 1meA and 3meC lesions in *alkB*$^-$ bacteria are the source of AT→TA transversions by formation of *supL* suppressor (1meA) and of GC→AT transitions by formation of *supB* or *supE*(oc) (3meC). In AB1157 *mfd-1*, the repair of MMS-induced lesions occurred, in contrast to the BS87 *mfd-1* strain, where DNA repair was inhibited, therefore totally Mfd-dependent. Moreover, in an *alkB*$^-$ strain the additional mutation in the *mfd* gene resulted in elevated induction of the SOS response. The involvement of the *umuDC*-encoded PolV in the processing of 1meA/3meC lesions arising in MMS-treated *alkB*$^-$ bacteria is also shown and discussed.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* K12 strains and plasmids used in this study are listed in Table 1. Transductions and transformations were performed by routine methods [43]. Derivatives of AB1157 and BS87 harboring the *umuDC* deletion or *alkB117* and *mfd-1* mutations were constructed by P1 mediated transduction [43]. Selection of transductants was done on LB plates supplemented with either chloramphenicol (30 μg/ml), carbenicillin (50 μg/ml), or tetracycline

(12.5 μg/ml). Subsequently, the desired phenotype of the transductant, *umuDC* deletion, *alkB* or *mfd* mutations, was confirmed by testing sensitivity to UV, MMS or by sequencing, respectively.

2.2. Media

Luria–Bertani (LB) broth [43] was used as rich medium. Minimal medium (MM) contained C-salts supplemented with glucose (0.5%), casamino acids (0.2%), thiamine (10 μg/ml) and Arg, His, Thr, Pro and Leu at 25 μg/ml each. E-Arg plates were MM plates devoid of arginine and solidified with 1.5% Difco agar.

2.3. Survival and mutagenicity assay

To estimate the survival of tested strains, bacteria were treated with 20 mM MMS for 15 min, spun down, resuspended in complete MM or in MM devoid of arginine, histidine and casamino acids (starvation medium), and incubated with shaking for 60 min. Non-starved bacteria were plated on LB, whereas starved samples were supplemented with arginine, histidine and casamino acids and incubated for another 60 min before plating. After 18 h of incubation, the colonies were counted and the percent of survivors was calculated. MMS-treated and immediately plated samples were assigned as control.

For MMS mutagenesis, bacteria (overnight culture in LB diluted 1:50) were grown in MM with shaking. When the culture reached a density of 2-4 x 10^8 cells/ml, it was treated with 20 mM MMS for 15 min, centrifuged, washed and diluted 10-fold in MM devoid of arginine, histidine and casamino acids (starvation conditions for TCR expression). The missing amino acids were added either immediately (non-starved control) or after 30 or 60 min of starvation.

For UV mutagenesis, bacterial cultures were grown to a density of 2-4 x 10^8 cells/ml,

centrifuged and resuspended in C salts. Samples of 5 ml were irradiated for 35 s in Petri plates (φ 80 mm) with a Philips 15 W UV lamp, emitting mainly 254 nm light and placed 8 cm above the plate. The UV dose was 45 J/m^2, except for the AB1157 *mfd-1 uvrA6* strain, which was irradiated with 2.7 J/m^2, with the UV lamp placed 16 cm above the plate. The irradiated bacteria were diluted 10-fold in LB either immediately or after 20 min incubation in C salts supplemented with 0.5% glucose (starvation conditions). In both, MMS- and UV-treatment, bacteria were incubated overnight, appropriately diluted (usually 10^6-fold) and plated onto LB plates for total counts (CFU); for Arg^+ revertants non diluted or diluted 10-100-fold bacteria were plated onto E-Arg plates. Colonies growing on LB plates were counted after 24 h, and those growing on E-Arg plates after 48 h of incubation. Following colony counting, the frequency of reversion (number of Arg^+ revertants/10^8 viable cells) was determined. All incubations were at 37°C.

The CC101-CC106 strains [38] and their *mfd-1* and *alkB117* derivatives were grown, MMS treated, and plated as described above. For monitoring Lac^+ revertants the E-Arg plates were replaced with MM plates deprived of glucose, but enriched with 0.5% lactose. Lac^+ reversion frequencies were calculated as for Arg^+ revertants.

All experiments were repeated four to six times in duplicate, and standard deviation (±SD) was calculated.

2.4. Mutational specificity assay

Revertants to Arg^+ were classified according to their requirements for histidine and threonine, into four phenotypic classes: (I) Arg^+ His^- Thr^-, (II) Arg^+ His^+ Thr^-, (III) Arg^+ His^- Thr^+, and (IV) Arg^+ His^+ Thr^+. At least 200 Arg^+ colonies from each experiment (100 in

duplicate) were analysed for their phenotype, and about 20 members of each class were tested for sensitivity to amber (B17) and ochre (oc427, ps292, ps205) mutants of T4 phages as described previously [21]. This method allowed to identify the suppressor tRNA, and to deduce the following mutational pathways: GC→AT transition that arose by *supB* formation or by *supE*(am)→ *supE*(oc) conversion, and AT→TA transversion that arose by *supL* formation.

2.5. Expression of β-galactosidase

All the strains examined for β-galactosidase expression were transformed with plasmid pSK1002 bearing a *umuC*::*lacZ* fusion [42]. An overnight culture in LB was diluted 10-fold in MM, treated with 20 mM MMS for 15 min, centrifuged, washed, resuspended in the same volume of fresh MM, and further incubated to $OD_{600} \approx 0.350$. At zero and appropriate time points aliquots were removed and β-galactosidase activity was assayed according to Miller [43].

2.6. Microscopic observation of bacteria

To examine the morphology of growing bacterial cells, liquid cultures were treated with 20 mM MMS for 15 min, diluted in fresh MM deprived of arginine, histidine, and casamino acids, and incubated for 1 h. Starvation was stopped by the addition of the omitted amino acids, and the cultures were incubated for an additional hour. After that time, aliquots were taken, spread onto glass slides, fixed over a flame, stained with basic fuchsin and examined under a light microscope (Nikon Microphot S.A.) at a 1000× magnification. Bacteria not treated with MMS were used as controls.

2.7. Characterization of the *mfd-1* mutation

The *mfd-1* gene was amplified by PCR (Run Polymerase, A&ABiot) under standard conditions using primers mfd1 and mfddn. The PCR product was sequenced with primers listed in Table 2 (DNA Sequencing Laboratory, IBB). Partial sequences were assembled and the whole sequence was used as a query in the NCBI microbe genome database (BLAST).

3. Results

3.1. Effect of starvation on the level of MMS-induced Arg$^+$ reversion in *E.coli* AB1157 and BS87 strains

It has been shown previously that during transient amino acid starvation of *E.coli* AB1157 (with intact an *alkB* gene), the MMS-induced *argE3*→Arg$^+$ reversions are partially repaired by TCR, resulting in about 3-fold decline in mutation frequency (Fig. 1A). In UV irradiated and starved for 20 min AB1157 cells, we observed an 8-fold lower level of Arg$^+$ revertants in comparison to non-starved control. Introduction of *mfd-1* and *uvrA6* mutations into the AB1157 strain totally inhibited TCR in UV irradiated cells, whereas in MMS-treated and starved for 60 min AB1157 *mfd-1 uvrA6* bacteria, some decrease in the level of Arg$^+$ revertants *vs*. wild type AB1157 was still observed (Table 3).

In the BS87 (*alkB* $^-$) strain there was only a 2-fold decline in the frequency of MMS-induced Arg$^+$ revertants after 60 min of starvation (Fig. 1B). However, the absolute levels of Arg$^+$ revertants in the AB1157 *alkB*$^+$ and BS87 *alkB* $^-$ strains were fundamentally different. The frequency of MMS-induced Arg$^+$ revertants in non-starved AB1157 cells was about 160 Arg$^+$ revertants/10^8 cells and 54 Arg$^+$ revertants/10^8 cells after 60 min of starvation (Table 3). The corresponding values for BS87 were 1,800 *vs*. 846 Arg$^+$ revertants/10^8 cells. As established earlier [31], the *alkB* mutation substantially increased the frequency of MMS-induced mutations.

The majority of MMS-induced mutations in the AB1157 strain depend on the activity of PolV encoded by the *umuDC* genes [21,31]. In the absence of the *umuDC* operon, the level of MMS-induced Arg^+ revertants drops dramatically, 6-fold in the case of the AB1157Δ*umuDC* strain, and even 51-fold in the case of BS87Δ*umuDC* (Fig. 1 A and B). Under transient amino acid starvation, the pool of MMS-induced, *umuDC*-independent mutations was subjected to TCR. Our previous experiments indicate that these mutations are GC→AT transitions arising by *supE* suppressor formation, and that the source of these mutations is 3meC lesion present in MMS-treated cells devoid of functional AlkB protein [44].

Overproduction of PolV in AB1157 harboring pRW134 resulted in an over 4-fold increase in the frequency of MMS-induced Arg^+ revertants (160 *vs*. 680 Arg^+ revertants/10^8 cells in AB1157 and AB1157/pRW134, respectively) (Fig. 1A), whereas in BS87 overproducing PolV, the level of these mutations was only slightly increased (1,900 *vs*. 3,000 Arg^+ revertants/10^8 cells) (Fig. 1B).

3.2. Specificity of MMS-induced mutations

Arg^+ revertants obtained by MMS-treatment were analyzed for their requirements for histidine and threonine and for susceptibility to T4 phage mutants. The results of analysis of MMS-induced Arg^+ revertants are summarized in Table 4.

In the AB1157 strain, about 80% of MMS-induced Arg^+ revertants showed class II phenotype and within this class about 50% were due to AT→TA transversions by *supL* suppressor formation. The remaining Arg^+ revertants were of class I, arising by *supB* formation or by *supE*(am)→ *supE*(oc) conversion due to GC→AT transitions. Back mutations (any transition or transversion at AT base pairs inside the $argE3_{(ochre)}$ UAA locus) constituted about 10% of all Arg^+ revertants. Transient starvation of MMS-treated AB1157 strain resulted in a 10-

fold decrease in the level of GC→AT transitions , which arose by *supB* but not *supE*(oc) formation, and over 4-fold decrease in the level of AT→TA transversions. In the AB1157*mfd-1* mutant, there was no decline in the level of GC→AT transitions that arose by *supB*; however, there was a 2-fold decrease in the GC→AT transitions arose by *supE*(oc) suppressor formation as well as AT→TA transversions of *supL* origin (Table 4).

In the BS87 (*alkB* $^{-}$) strain, MMS-induced Arg^{+} revertants were split almost equally between phenotypic classes I and II. The level of AT→TA transversions was 2-fold higher in comparison to GC→AT transitions by *supB* and 3-fold higher in comparison to GC→AT transitions by *supE*(oc) (respective values were 931.5, 465.8, and 279.5 Arg^{+} revertants/10^{8} cells, Table 4). The level of GC→AT transitions by *supB* formation decreased over 5-fold during starvation, and that of AT→TA transversions by *supL* decreased 2-fold. The GC→AT transitions by *supE*(oc) remained at the same level, however, was twice as numerous as in the BS87 *mfd-1* strain. The other two classes, GC→AT transitions by *supB* and AT→TA transversions by *supL*, remained at similar level. In MMS-treated BS87 *mfd-1* bacteria, the frequency of all these types of Arg^{+} revertants were not affected by starvation.

3.3. Induction of the SOS response

The induced state of the SOS response was shown by measuring the level of β-galactosidase in MMS-treated AB1157(*alkB*$^{+}$), BS87(*alkB117*), and BS87*mfd-1* strains harboring the pSK1002 plasmid (Fig. 2). The plasmid bears an *umuC*::*lacZ* fusion [42] placing the *lacZ* gene encoding β-galactosidase under the control of the *umuC* promoter, and the fusion gene is expressed in response to SOS induction. The obtained results indicate that the level of β-galactosidase was 1.75- and over 2-fold higher in BS87 and BS87*mfd-1*, respectively, in

comparison to the AB1157 strain (Fig. 2).

The same conditions as for β-galactosidase induction were found to promote filamentous growth, a characteristic feature of induction of the SOS response in *E.coli* [45]. This type of growth results from the expression of the *sulA* gene induced as one of the latest within the SOS regulon. The SulA protein is an inhibitor of cell division. Delay in the division of cells allows for DNA repair after the action of DNA-damaging agents [46]. Figure 3 shows photomicrographs of bacteria treated with MMS for 15 min, starved for 60 min and incubated for a further 60 min in the growth medium. The filamentous growth of the *alkB* mutant indicates an induction of the SOS response. The additional mutation in the *mfd* gene resulted in an even stronger filamentation in $alkB^{-}\ mfd^{-}$, but not in $alkB^{+}\ mfd^{-}$ cells. This increased filamentation was not accompanied by MMS-mediated killing; on the contrary, MMS-treated and subsequently starved BS87 *mfd-1* strain survived better than its mfd^{+} counterpart (Fig. 4).

3.4. Characterization of the *mfd-1* mutation

The *mfd-1* allele was sequenced and found to have only one deletion of a thymine residue from among three Ts (2365-2367), resulting in a frameshift and premature stop codon. The mutated Mfd (Mfd-1) protein contains only 852 amino acids *vs* 1148 in wt Mfd. Mfd-1 protein is thereby deprived of the C-terminal translocase domain, TD2, and the D7 domain, which in free Mfd protein blocks the interaction with the UvrA protein [26].

The mutational spectra of strains with the *mfd-1* mutation were analyzed in a system of *E.coli* CC101-CC106 *lacZ* mutants. By measuring the frequency of Lac^{+} revertants, the system allows identification of all six types of base substitutions [38]. In these mfd^{+} strains, MMS induces Lac^{+} revertants that arise mainly by GC→AT transitions monitored in the CC102 strain

(about 19 Lac^+ revertants /10^8 cells), or by AT→TA transversions, observable in the CC105 strain (about 3 Lac^+ revertants /10^8 cells) [38].

In the *mfd-1* derivatives of the CC101-CC106 strains MMS induced almost exclusively (95%) GC→AT transitions (51 Lac^+ revertants/ 10^8 cells) (Table 5). The introduction of *alkB117* mutation (the source of this allele was BS87) into strains CC101-CC106 *mfd-1* resulted in the highest level of Lac^+ revertants in CC104, which shows GC→TA transversions (122 Lac^+ revertants/10^8 cells in comparison to 6.6 and 0.7 Lac^+ revertants/10^8 cells in single CC104 *alkB117* and CC104 *mfd*-1 mutants, respectively). The level of GC→AT transitions in CC102 *mfd-1 alkB117* was similar to that observed in CC102 *alkB117* (26.5 and 27.9 Lac^+ revertants/10^8 cells, respectively), and about 2-fold lower than in CC102 *mfd-1* (51 Lac^+ revertants/10^8 cells). Introduction of *mfd-1* to CC105 *alkB117* and CC103 *alkB117* led to an about 9-fold increase in the AT→TA, and a 150-fold increase in the GC→CG transversions, in comparison to the single CC105 *mfd-1* and CC103 *mfd-1* mutants.

3.5. Effect of *mfd-1* mutation on survival of bacteria and frequency of MMS-induced Arg^+ revertants

We compared the survival of MMS-treated and starved (or not) bacteria of four strains: AB1157 and BS87, and their mfd^- counterparts (Fig. 4). The *mfd-1* mutation did not influence the survival of all these strains unless they were starved after MMS treatment. Under starvation conditions, the *mfd* mutants (especially BS87 *mfd-1*) survived better than the mfd^+ counterparts. The MMS treatment by itself resulted in poorer survival of the mfd^- strains.

In the AB1157 mfd^+ strain there was a 3-fold decline in the frequency of MMS-induced Arg^+ revertants after 60 min of starvation: about 160 Arg^+ revertants/10^8 cells without starvation

297 to 54 Arg^+ revertants/10^8 cells with starvation. For the AB1157 *mfd*$^-$ mutant the MFD was small
298 but still observable (about 180 Arg^+ revertants/10^8 cells without and about 105 Arg^+
299 revertants/10^8 cells with starvation). In MMS-treated and starved for 60 min BS87 strain, a 2-fold
300 decrease in the level of Arg^+ revertants was observed, whereas in the BS87 *mfd-1* mutant, the
301 difference between non- starved and starved samples was barely visible (about 1370 *vs.* 1145
302 Arg^+ revertants/10^8 bacteria). Thus, in contrast to AB1157, in the BS87 strain all TCR seems to
303 be *mfd*-dependent.
304
305 **4. Discussion**
306 In the present study, we used the *argE3*→ Arg^+ reversion system of *E. coli* AB1157
307 (*argE3*) to monitor the involvement of transcription coupled DNA repair, TCR, in the repair of
308 MMS-induced lesions in *alkB*$^-$ strains deprived of a functional AlkB protein. The AlkB
309 dioxygenase demethylates 1meA/3meC lesions in DNA with recovery of A and C. To favor TCR
310 we applied transient (60 min) amino acid starvation (liquid MM was deprived of arginine and
311 histidine).
312 In the AB1157 *alkB*$^+$ strain, a 60-min starvation decreased the frequency of MMS-induced
313 Arg^+ revertants to about one-third of that found in non-starved control (Fig. 1A). This decline in
314 the level of MMS-induced mutations is much smaller compared with MFD described for UV
315 mutagenesis. In UV-irradiated and starved AB1157 strain, we observed an 8-fold lower level of
316 Arg^+ revertants than in non-starved control, whereas in the AB1157*mfd-1 uvrA*6 mutant, the
317 frequency of these revertants was independent on starvation (Table 3). In starved bacteria, UV
318 irradiation by creating TˆT dimers and 6-4 photoproducts in DNA, immediately induces SOS
319 response and UvrA protein that starts NER and TCR. We postulate that in contrast to UV-

induced lesions, UvrA endonuclease is not involved, or is involved to a lesser degree, in repair of MMS-induced damage.

In BS87(*alkB*$^-$), only a two-fold decrease in the frequency of Arg$^+$ revertants was caused by starvation (Fig. 1B). We have previously shown that 95-98% of MMS-induced Arg$^+$ revertants in *alkB*$^-$ strains are *umuDC*-dependent, which results in a strong reduction of mutation frequency in the BS87Δ*umuDC* strain [44]. In this strain, the level of MMS-induced Arg$^+$ revertants was 51-fold lower than in the *umuDC*$^+$ counterpart (AB1157Δ*umuDC* showed a 6-fold decrease in the level of the revertants comparison with the AB1157*umuDC*$^+$ strain) (Fig. 1). The remaining pool of MMS-induced, *umuDC*-independent mutations was subject to TCR and the decline in the level of MMS-induced Arg$^+$ revertants upon starvation was 2-fold larger in the Δ*umuDC alkB*$^-$ than in the Δ*umuDC alkB*$^+$ strain.

In AB1157, MMS-induced Arg$^+$ revertants arise in about 80% due to *supL* suppressor formation by AT→TA transversions, in about 15% due to *supB* and *supE*$_{(ochre)}$ suppressor formation by GC→AT transitions; the remaining revertants arise by back mutations, which can occur by transition or transversion at AT bases within the *argE3* site [21]. Here, we determined phenotypic classes (according to requirements for histidine and threonine) and suppressors of Arg$^+$ revertants in AB1157 and BS87 strains and their *mfd*$^-$ counterparts (Table 4). In the BS87 strain, during starvation, the decrease in the levels of Arg$^+$ revertants arising by *supB* suppressor formation due to GC→AT transitions and by *supL* formation due to AT→TA transversions, was 2-fold weaker in comparison with respective values for the AB1157 strain. We observed a very strong effect of the presence of a non-functional Mfd protein (*mfd-1* mutation) on the anti-mutational action of amino acid starvation, but only in the *alkB*$^-$ mutant. In the AB1157 *alkB*$^+$*mfd*$^-$ strain the short starvation still brought about an almost 2-fold decrease in the frequency of Arg$^+$

revertants (resulting from the decline in GC→AT transitions due to *supE*(oc) and in AT→TA transversions due to *supL* formation). On the other hand, in the *alkB*$^{-}$ counterpart (BS87 *mfd-1*), the starvation no longer affected the mutation frequency (Fig. 5 and Table 4).

The mutagenic targets of MMS-induced DNA damage were found to be: (i) 5'TTG3' and 3'AAC5'in the coding and transcribed DNA strands, respectively, of the *gln*-tRNA gene, causing *supB* suppressor formation, (ii) 5'CTA3' and 3'GAT5' in the coding and transcribed DNA strands, respectively, of *supE44*, encoding amber tRNA suppressor causing conversion to *supE*(oc), and (iii) 5'AAA3' in the transcribed strand of the *lys*-tRNA gene, causing *supL* formation (the targeted bases are underlined) [47]. Suppressors created by lesions in the transcribed DNA strand are repaired preferentially. Regarding repair of these lesions, other DNA repair systems, e.g., BER, should also be taken into consideration [48]. Among adducts created by MMS in DNA, there are 3meC, O^6meG, 3meA, 1meA and, the most numerous but not mutagenic until removed, 7meG. The above analysis indicates the following sources of suppressors: 3meC (unrepaired in AlkB-defective strain) for *supB*; O^6meG (7meG) for *supE*(oc); and 3meA and 1meA (the latter unrepaired in AlkB-defective strain) for *supL*.

The high level of MMS-induced lesions, poorly repaired during transient starvation, caused induction of the SOS response in the *alkB*$^{-}$ strain (Figs. 2 and 3). The additional *mfd-1* mutation resulted in an even stronger induction of this response in *alkB*$^{-}$ *mfd*$^{-}$, but not in *alkB*$^{+}$ *mfd*$^{-}$ cells. This result confirms the involvement of the Mfd protein in the repair of MMS-induced lesions in the BS87 strain.

We sequenced the *mfd-1* allele and found a deletion of one of the three thymine residues in positions 2365-2367. This results in a premature stop codon and a shorter, 852-amino acid, Mfd-1 protein (Mfd wt contains 1148 amino acids), deprived of the TD2 C-terminal translocase

366 domain and the D7 domain, the latter, blocking interaction with the UvrA protein [26].

367 The specific effects of the *mfd-1* mutation determined in strains CC101-106 showed
368 significant differences in the *alkB*$^+$ and *alkB*$^-$ backgrounds. Among the CC101-106 *mfd-1 alkB*$^+$
369 strains, 95% of the Lac$^+$ revertants arose by GC→AT transitions, whereas this class of mutations
370 constituted only 21.5% in the double mutant, *alkB117 mfd-1*. Conversely, the GC→TA
371 transversions monitored in this strain constituted 70% of all base substitutions (Table 5).

372 Observed differences in MMS mutability of the *argE3* and *lacZ*$^-$ markers are probably
373 caused by different number of targets and their various structure. The *lacZ*$^-$→ Lac$^+$ reversion
374 occurs only by back mutation at one point of the double-stranded gene encoding β-galactosidase.
375 The *argE3*→Arg$^+$ reversion occurs mostly by formation of at least 8 separate *sup*tRNA (ochre)
376 suppressors that are actively transcribed and exist as single-stranded (ss)DNA [49]. The
377 fragments of ssDNA are more accessible to MMS and cause strong induction of the SOS
378 response.

379 Summing up, MMS induces two global defense systems, Ada and SOS. UvrA protein
380 engaged in the error-free DNA repair systems, NER and TCR, is the first one induced within the
381 SOS response. A prolonged state of the SOS induction leads to the expression of *umuDC*-
382 encoded PolV, which allows error-prone translesion synthesis of MMS-modified bases. The
383 AlkB protein, a member of Ada response, oxidatively demethylates 1meA/3meC lesions with
384 recovery of the original A and C bases. In MMS-treated *alkB* mutant, unrepaired 1meA/3meC
385 lesions are processed by PolV during TLS, which results in an elevated level of Arg$^+$ revertants.
386 Here, we have found that MMS-induced TCR is less effective in the *alkB*$^-$ strain in comparison to
387 *alkB*$^+$ counterpart. The *mfd-1* mutation totally inhibits TCR and strongly enhances the SOS
388 response in *alkB*$^-$ *mfd* $^-$, but not in the *alkB*$^+$ *mfd* $^-$ strain. Studies on the specificity of MMS-

induced Arg^+ revertants showed that the decline in the level of all types of suppressor mutations during transient starvation is totally Mfd-dependent. We have established that not only 3meC, but also 1meA lesion may be a source of mutations, namely, AT→TA transversions arising by *supL* suppressor formation.

Aknowledgement

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Legends to figures

Fig. 1

Frequency of MMS-induced Arg^+ revertants under transient starvation conditions. *E.coli* strains AB1157 (A) and BS87 (B) harboring the indicated plasmids and/or *umuDC* deletion were treated with 20 mM MMS for 15 min and subsequently starved for indicated time (□ - 0 min; ▩ - 30 min; ■ - 60 min).

Fig. 2

Expression of β-galactosidase from a *umuC*::*lacZ* fusion in MMS-treated strains AB1157 (▲), BS87 (■), and BS87 *mfd*-1 (●) harboring the pSK1002 plasmid. The empty symbols represent the same strains not treated with MMS.

Fig. 3

Filamentous growth of *E.coli* AB1157 and BS87 cells and their mfd^- counterparts treated with 20 mM MMS for 15 min and starved for 60 min. Magnification 1000×. ctrl - control not treated with MMS.

Fig. 4

Survival of MMS-treated and starved *E.coli* strains AB1157 and BS87 and their *mfd-1* derivatives. Bacteria were treated with 20 mM MMS and immediately plated (□); incubated in full MM for 60 min (▨); starved for 60 min and incubated in full medium for 60 min before plating (▩) (see Materials and Methods for details). MMS-treated and immediately plated samples were assigned as control.

552 Fig. 5

553 Frequency of MMS-induced Arg^+ revertants under transient starvation in *mfd-1* mutants. *mfd-1*

554 derivatives of *E.coli* strains AB1157 and BS87 were treated with 20 mM MMS for 15 min and

555 subsequently starved for indicated time (□ - 0 min; ▒ - 30 min; ■ - 60 min).

Table 1

Bacterial strains and plasmids

Strains and plasmids	Genotype	Reference
AB1157	*argE3 hisG4 leuB6* Δ*(gpt-proA)62 thr-1 ara-14galK2 lacY1 mtl-1 xylA5 thi-1 rpsL31 glnV44 tsx-33rfbD1 mgl-51 kdgK51*	[30]
RW82	Δ*umuDC595::cat* donor, derivative of AB1157 but *thyA325*	[31]
NR10121	*ara thi zcf-117* :: Tn*10 mfd-1*; *mfd-1* donor	[32]
EC2423	as AB1157 but *mfd-1*	[33]
BS87	as AB1157 but *alkB117*::Tn3	[34]
MW21	as BS87 but *mfd-1*	this work
EC2413	as AB1157 but Δ*umuDC595::cat*	[35]
BS87Δ*umuDC*	as BS87 but Δ*umuDC595::cat*	this work
AB1886	as AB1157 but *uvrA6*	[36]
EC2424	as EC2423 but *uvrA6 malB*::Tn*9* cm^R	[37]
CC101-CC106	*ara thi* Δ(*lac-proB*)$_{xiii}$, F' *lacIZ*$^-$*proB*$^+$	[38]
CC101-CC106 *alkB117*	as CC101-CC106, but *alkB117*::Tn*3*	[39]
CC101-CC106 *mfd-1*	as CC101-CC106, but *mfd-1*	this work
CC101-CC106 *alkB117 mfd-1*	as CC101-CC106, but *alkB117*::Tn*3, mfd-1*	this work
pGB2	spc^R	[40]
pRW134	*umuD'C* genes inserted in pGB2	[41]
pSK1002	*umuC*::*lacZ* fusion	[42]

Table 2

Primers used in *mfd-1* sequencing

Name	Sequence
mfd1	AACAGCATTGCTTATCAG
mfd2	CCTTCGAAGTGAAGCGCG
mfd3	CGATACACTGATCCGTAA
mfd4	TCGATATTCTGATCGGTA
mfd5	CCTCGCTGGAAGATCTCG
mfddn	CAGTGTCGGATAGTGCAG

Table 3

Effect of UV irradiation and MMS treatment on the level of Arg^+ revertants in the indicated *E.coli* strains

Strain	Frequency of Arg^+ revertants ($x10^{-8}$ cells) in cultures:				
	Non-treated control	UV irradiated	UV irradiated and starved for 20 min	MMS-induced	MMS-induced and starved for 60 min
AB1157	3.0 ± 1.0	2242.3 ± 325.6	259.3 ± 72.7	157.0 ± 23.3	54.0 ±14.5
AB1157 *mfd-1*	4.1 ± 0.5	1283.3 ± 72.7	643.0 ± 162.2	180.0 ±31.4	105.0 ±20.3
AB1157 *mfd-1 uvrA*6	10.6 ± 2.5	340 ± 56.4	325.7 ± 75.7	210.4 ±35.8	129.2 ±19.8

Table 4

Specificity of MMS-induced Arg^+ revertants in *E.coli alkB* and *mfd* mutants

Strain	Duration of starvation (min)	Phenotypic classes of Arg^+ revertants (%)				Supressors and mutation pathway (Arg^+ revertants /10^8 cells)			
		I His^- Thr^-	II His^+ Thr^-	III His^- Thr^+	IV His^+ Thr^+	*supB* GC→AT	*supE*(oc) GC→AT	*supL* AT→TA	back AT→AT
AB1157	0	20	78	0	2	54.9	7.9	70.7	23.6
	60	24	76	0	0	5.1	8.5	15.3	3.4
AB1157 *mfd-1*	0	8	90	2	0	27.0	36.0	90.0	27.0
	60	7	90	2	1	36.8	15.8	42.0	10.5
BS87	0	51	44	37	2	465.8	279.5	931.5	186.3
	60	60	22	17	1	87.1	217.8	435.5	130.7
BS87 *mfd-1*	0	42	56	1	1	479.2	136.9	684.5	68.5
	60	33	67	0	0	399.7	171.3	513.9	57.1

The data are means of three independent experiments. SD=5-20%

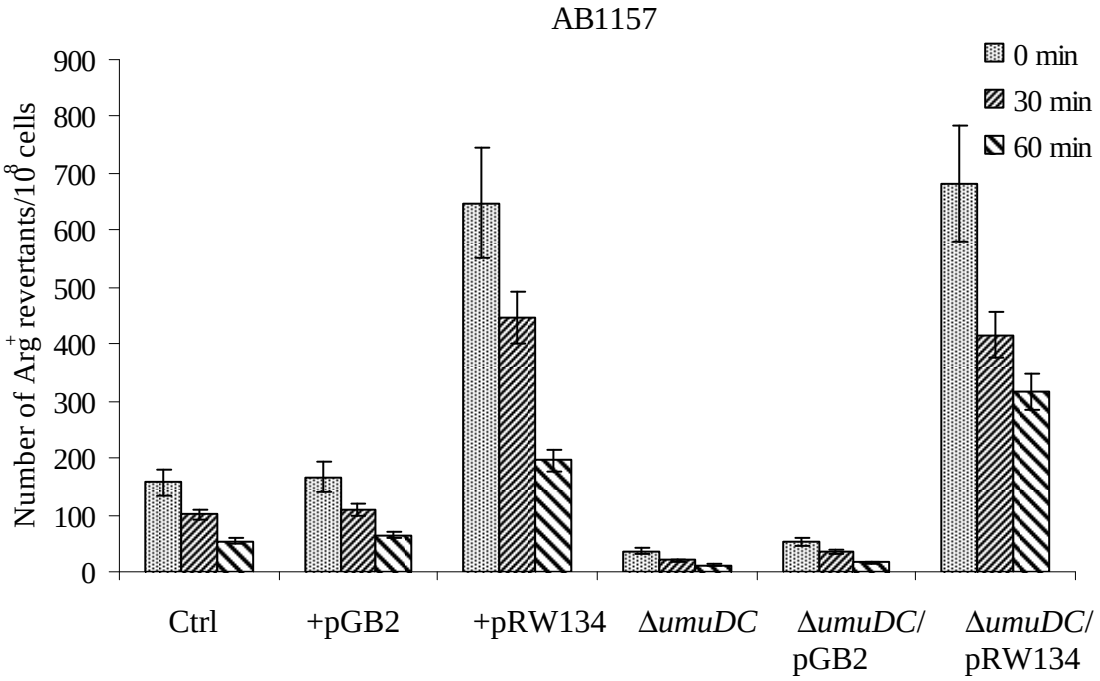
Table 5

Effect of *mfd-1* and *alkB117* mutations on the level of Lac^+ revertants of indicated specificity

Bacterial strain and specificity of mutation		Frequency of Lac^+ revertants /10^8 cells in strains:							
		mfd$^+$ *alkB*$^+$ *		*mfd*$^+$ *alkB117**		*mfd-1*		*mfd-1 alkB117*	
		Ctrl (no MMS)	+MMS	Ctrl (no MMS)	+MMS	Ctrl (no MMS)	+MMS	Ctrl (no MMS)	+MMS
101	AT→CG	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	2.5 ± 2.4	1.4 ± 1.5	0.3 ± 0.1	0.4 ± 0.6	1.6 ± 1.1
102	GC→AT	0.0 ± 0.0	18.9 ± 3.3	1.3 ± 1.1	27.9 ± 8.4	0.3 ± 0.2	51.1 ± 2.5	0.6 ± 0.4	26.5 ± 4.2
103	GC→CG	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	3.4 ± 2.5	0.2 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	15.2 ± 9.5
104	GC→TA	0.2 ± 0.0	0.9 ± 0.5	0.6 ± 0.2	6.6 ± 3.2	0.7 ± 0.2	0.7 ± 0.7	1.9 ± 1.5	122.2 ± 62.9
105	AT→TA	0.2 ± 0.0	2.7 ± 1.2	0.4 ± 0.16	4.7 ± 1.1	0.2 ± 0.3	1.5 ± 0.4	0.8 ± 0.3	12.3 ± 6.5
106	AT→GC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.5	0.2 ± 0.4

* Data for *mfd*$^+$ *alkB*$^+$ and *mfd*$^+$ *alkB117* have been published in Nieminuszczy et al. [39]

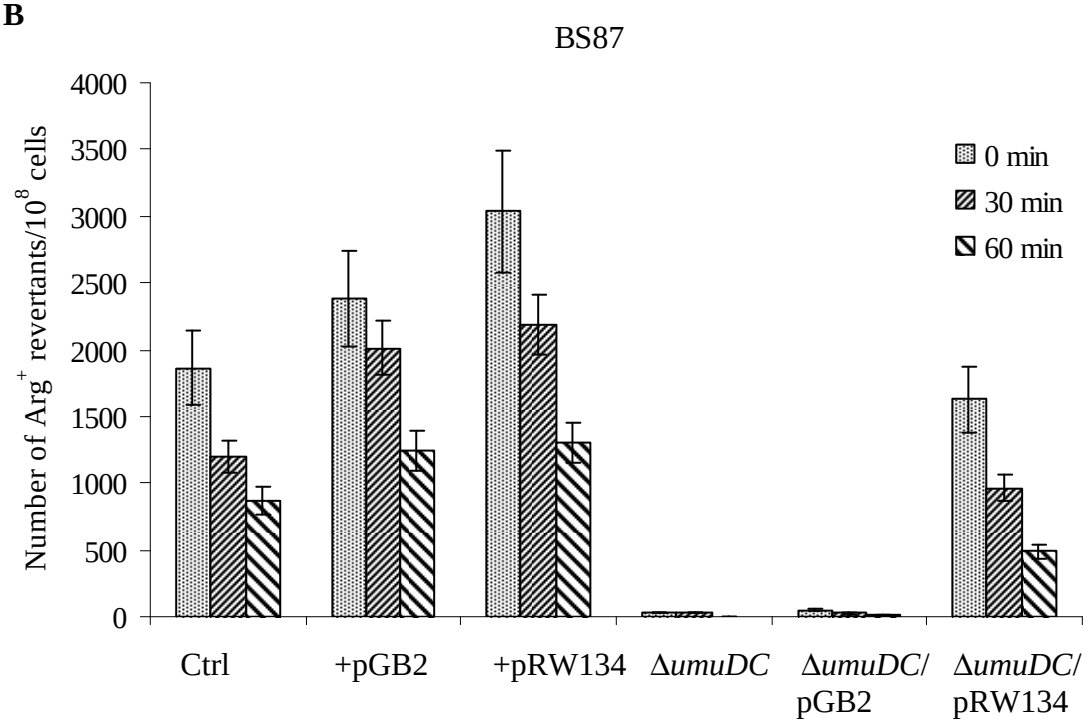
A



Duration of starvation (min)	Relative frequency of Arg^+ revertants * (%)					
	Ctr	+pGB2	+pRW134	Δ*umuD*	Δ*umuDC*/ pGB2	Δ*umuDC*/ pRW134
0	100	100	100	100	100	100
30	64	66	69	56	66	61
60	34	39	30	34	32	46

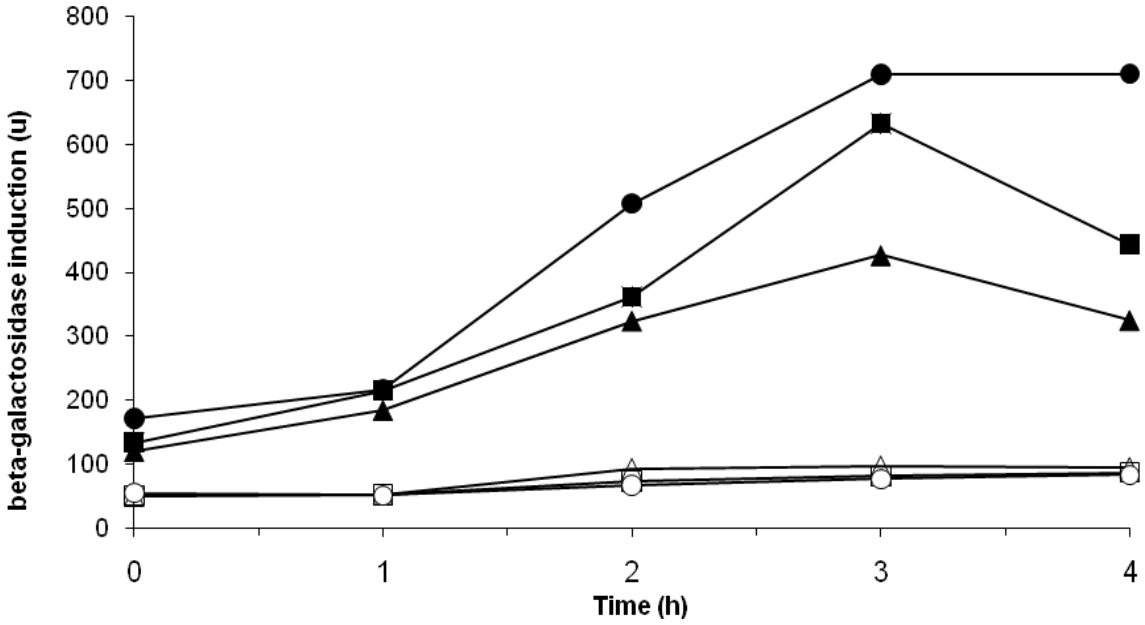
*Frequency of Arg^+revertants of respective strains not subjected to starvation was taken as 100%

B



Duration of starvation (min)	Relative frequency of Arg^+ revertants * (%)					
	Ctr	+pGB2	+pRW134	Δ*umuDC*	Δ*umuDC*/ pGB2	Δ*umuDC*/ pRW134
0	100	100	100	100	100	100
30	64	84	72	83	48	59
60	47	52	43	18	17	30

*Frequency of Arg^+ revertants of respective strains not subjected to starvation was taken as 100%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500
400
300
200
100
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beta-galactosidase induction (u)
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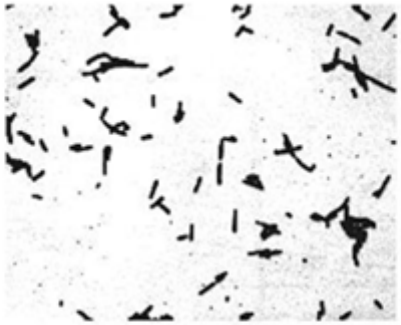
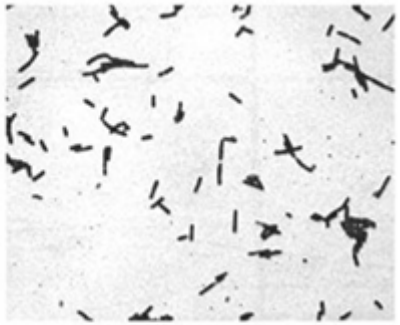
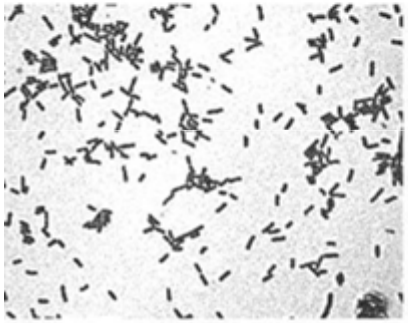
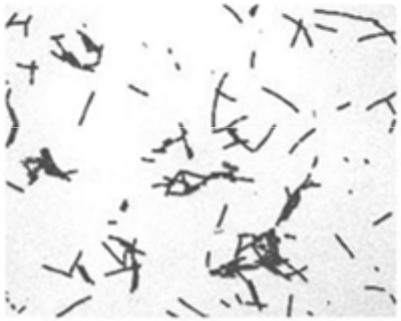
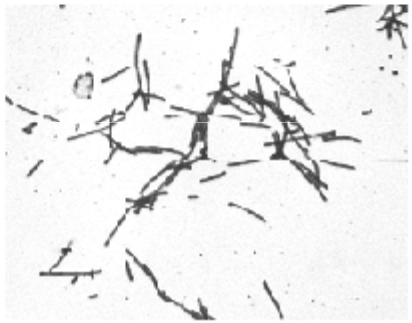
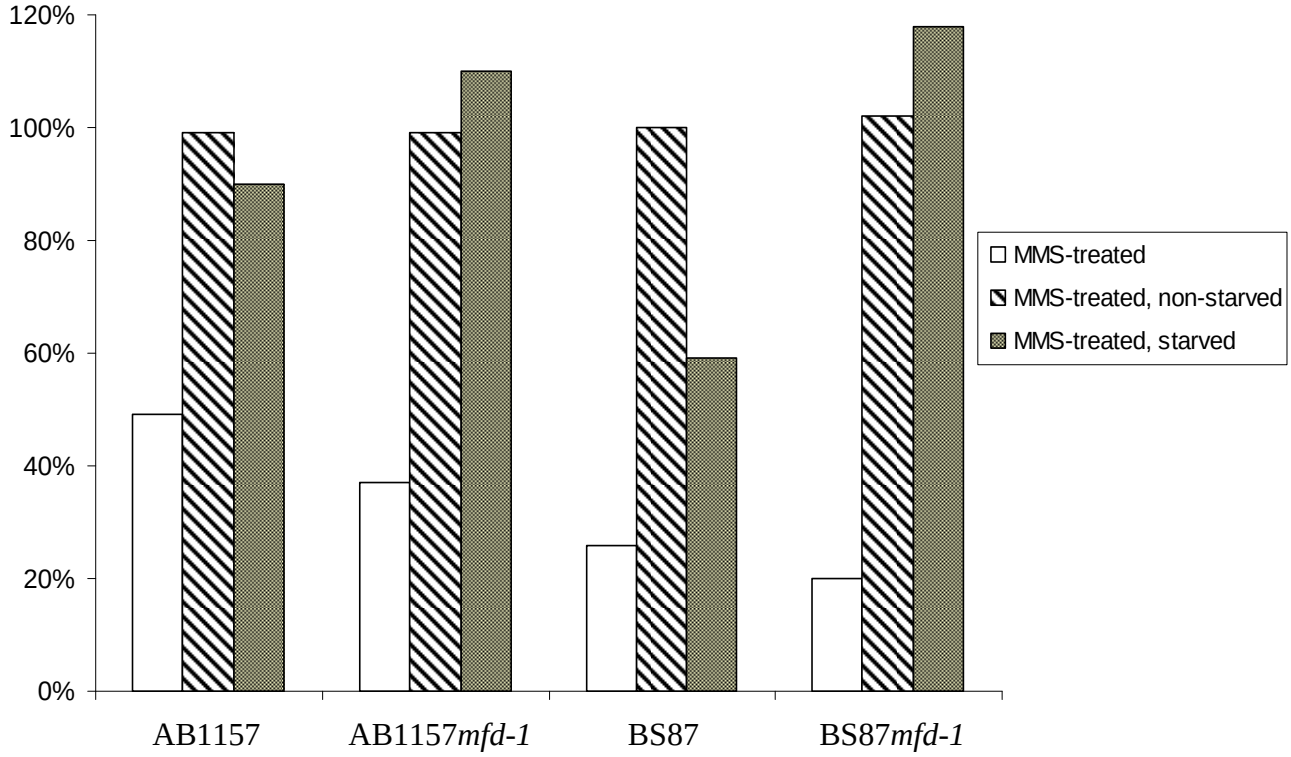
1
2
3
4
Time (h)

	ctr	*mfd* $^{+}$	*mfd - 1*
AB1157			
BS87			


120%
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80%
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AB1157
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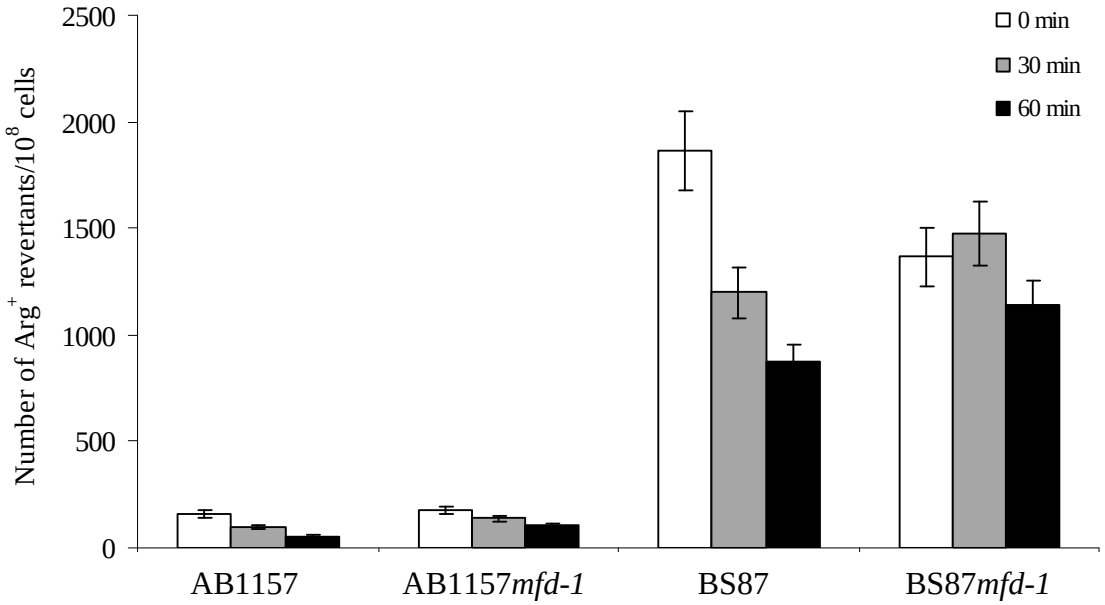
BS87
BS87mfd-1
MMS-treated
MMS-treated, non-starved
MMS-treated, starved



Duration of starvation (min)	Relative frequency of Arg^+ revertants * (%)			
	AB1157	AB1157 *mfd-1*	BS87	BS87*mfd-1*
0	100	100	100	100
30	64	77	64	108
60	34	58	47	83