AlkA Glycosylase and AlkB Dioxygenase Constitute an Effective Protective System for Endogenously Arising Acrolein

E. coli AlkA Glycosylase Excises Acrolein Adduct to Adenine

We would like to honour the memory of our mentor and friend, Professor Jarosław T. Kuśmierek (1945-2024), the founder of the chemical approach to DNA damage research at IBB PAS, whose ideas and achievements continue to play a significant role in shaping current research directions.

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Abstract

Acrolein (ACR) is a ubiquitous environmental pollutant but also formed endogenously as a metabolite in oxidative stress conditions. Its adduct to adenine 1,N^6-α-hydroxypropanoadenine (HPA) is a mutagenic lesion effectively repaired by the AlkB dioxygenase. Here, we provide *in vivo*, *in vitro*, and *in silico* evidence that it is also the substrate for the AlkA glycosylase. We studied the role of AlkA and AlkB in *E. coli* cells under conditions of induced adaptive response. Both *alkA* and *alkB* defective strains were not more sensitive to exogenous ACR than the wild type was. To simulate endogenously arising adducts, we used acrolein-modified plasmids, allowing monitoring of all kinds of substitutions originating from the acrolein modification of adenine. Both the AlkA and AlkB proteins were engaged in alleviating HPA-induced mutagenesis. Moreover, HPA was effectively repaired by AlkA and AlkB *in vivo*, even without induction of adaptive response. These findings suggest that the main contribution to acrolein mutagenicity comes from its endogenous sources, whereas AlkA and AlkB can play an additional role in controlling the level of DNA adducts of endogenous origin. Acrolein does not induce the adaptive response. HPA contains an asymmetric carbon atom in the hydroxypropano ring and exists as two stereoisomers. AlkA excises both of them *in vitro*. Molecular modelling demonstrated how dsDNA carrying both HPA stereoisomers could be properly bound at the AlkA catalytic centre. So, in contrast to the reaction catalyzed by AlkB, the HPA repair by AlkA is not expected to be stereoselective.

Introduction

All living organisms are constantly exposed to harmful environmental factors and those arising endogenously as byproducts of cellular metabolism. Among the diverse effects these factors may cause is damage to their genetic material. Nevertheless, prokaryotic and eukaryotic cells are well-equipped with various DNA repair systems. One such repair system involves the adaptive response (Ada response). The Ada response phenomenon is defined as the ability of

microorganisms to develop resistance and adapt to higher concentrations of a mutagen when incubated in the presence of its lower amounts. In *E. coli,* the Ada operon consists of four genes, *ada*, *alkA*, *alkB*, and *aidB,* expressed after exposure to non-toxic doses of direct-acting methylating agents. Beyond its role in the repair of methylation damage to DNA, the Ada methyltransferase functions as an auto-activator of expression of the Ada operon.[1,2]

E. coli AlkA (3-methyladenine DNA glycosylase II; (EC 3.2.2.21) is a monofunctional DNA N-glycosylase that removes alkylated bases from DNA *via* the base excision repair (BER) pathway. AlkA is a broad-substrate-specificity enzyme catalyzing the excision of diverse nucleoside lesions caused by alkylating agents in DNA, such as N-3- and N-7-alkyl purines, O^2-alkyl pyrimidines including its primary substrate 3-methyladenine, deaminated bases such as hypoxanthine and xanthosine, as well as oxidative lesions oxanine, 5-formyluracil, and others.[2–4] Our *in vivo* studies have excluded the involvement of AlkA in the repair of 1,N^6-ethenoadenine (εA), 3, N^4-ethenocytosine (εC), and its precursor 3,N^4-α-hydroxyethanocytosine (HEC).[5,6] AlkA homologs are found in diverse prokaryotic and eukaryotic organisms, but in plants and vertebrates, it is replaced by a broadly specific non-homologous 3-methyladenine-DNA glycosylase AAG.[7]

E. coli AlkB (EC 1.14.11.33), a member of the superfamily of α-ketoglutarate- and iron-dependent dioxygenases, is conserved in almost all organisms and viruses.[8–11] The AlkB-like dioxygenases remove alkylation at the N1 position of purines and the N3 position of pyrimidines *via* an oxidative mechanism, eventually restoring the native bases.[12,13] We have found that AlkB removes the εC precursor HEC,[5] the acrolein adducts 3,N^4-α-hydroxypropanocytosine,[14] and 1, N^6-α-hydroxypropanoadenine (HPA),[15] and confirmed the *in vivo* involvement of AlkB in the repair of εC and εA[5,6], although not of their triphosphate forms in the cellular pool of DNA precursor.[16] Our *in vitro* and *in silico* studies employing a set of substrates that differed in their pK_a confirmed the hypothesis that AlkB preferentially recognizes and repairs substrates in the cationic, *i.e.*, protonated form. The pattern of the intermolecular interactions stabilizing the ligand in the AlkB active site indicated a role of the Asp135 residue in recognition of the N-alkylated base in its protonated form with Trp69 and His131 sandwiching the modified base and orienting it toward the Fe-redox centre.[14]

Recently, we have focused on a major family of DNA lesions, acrolein (propenal) adducts. ACR is a ubiquitous environmental pollutant and an endogenous metabolite. The α, β-unsaturated double bond makes it very active toward cellular nucleophiles. Humans are exposed to acrolein *via* oral (food and water), respiratory (cigarette smoke, automobile exhaust, and biocides), and dermal routes. ACR penetrates rapidly through the cell membrane and readily binds reactive species, disturbing the cellular redox balance, leading to oxidative stress, and, consequently, cytotoxicity. Endogenously, ACR is formed as a metabolic byproduct of lipid peroxidation enhanced during oxidative stress and as a cellular polyamine spermine decomposition product.[17–19] Endogenous exposure is suspected of outweighing exogenous ones.[20] At the cellular level, exposure to acrolein has diverse toxic effects, including DNA and protein adduction, oxidative stress, mitochondrial disruption, membrane damage, endoplasmic reticulum stress, and immune dysfunction. The facile reactivity of ACR toward DNA is particularly significant since the ACR-DNA lesions can initiate mutagenesis, thereby contributing to the etiology of disease processes, including carcinogenesis.[21] ACR has also been suggested to play a role in several diseases, including multiple sclerosis, Alzheimer's disease, cardiovascular disease, diabetes mellitus, as well as diverse liver, kidney, and nervous system disorders.[17–19]

Acrolein adduct to adenine – 1,N^6-α-hydroxypropanoadenine (HPA) belongs to exocyclic DNA base adducts of bifunctional electrophilic agents of both exogenous and endogenous origin. Although it was not studied extensively as a minor cyclic acrolein adduct, the formation of HPA has been detected in rat liver epithelial RL34 cells exposed to acrolein and in rats exposed to Fe^{3+}NTA. The latter is a carcinogenic iron chelate inducing oxidative stress in the rodent kidney.[22] HPA is responsible for over one-fifth of ACR-induced base substitutions in human fibroblasts.[23] A high level of acrolein-derived mutations (mainly originating from base substitutions) was observed in a *Salmonella typhimurium* TA104 tester strain using the Ames test in which the site undergoing mutagenesis was the nonsense codon TAA.[24] We have recently shown that the AlkB dioxygenase can quite efficiently repair HPA: the optimal pH and concentrations of Fe(II) and α-ketoglutarate for this reaction have been determined, and the protonated form of HPA has shown to be preferentially repaired by AlkB. The reaction also turned out to be stereoselective. HPA contains an asymmetric carbon atom in the hydroxypropano ring (Cα, Figure 1), so it can exist as two stereoisomers. The cationic form of the R stereoisomer of HPA is strongly favoured over the S one in the AlkB active centre. In parallel, in *in vivo* experiments, we have shown that HPA is mutagenic and, when generated in a plasmid DNA, causes the A → C and A → T transversions and, less frequently, the A → G transition.[15]

Here, we provide *in vivo*, *in vitro*, and *in silico* evidence that HPA can be effectively excised by another *E. coli* Ada response protein – the AlkA glycosylase. Since *alkA* and/or *alkB* defective strains were found to be no more sensitive to

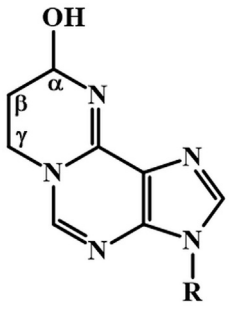

Figure 1. Structure of acrolein adduct to adenine – 1, N^6-α-hydroxypropanoadenine. Note that the Cα atom of the hydroxypropano ring is asymmetric.

exogenous ACR than the wild type, and HPA was repaired by AlkA and AlkB *in vivo* even without induction of the Ada response, we propose that the endogenous pool of ACR is responsible for its mutagenicity and discuss the role of these repair enzymes in controlling the level of DNA adducts of endogenous origin.

Materials and Methods

Bacterial strains

E. coli strains wt, and *alkA, alkB, and double alkAalkB* mutants have already been described and used.[5]

Growth inhibition test

E. coli strains were cultured under anaerobic conditions at 37 °C and 250 rpm. After the cultures reached OD_{600} = 0.4–0.6, MMS (Sigma) was added to the final concentration of 1 mM to induce the Ada response. The cultures were incubated for 15 min and then were used for the test. Control cells were left untreated with MMS. An aliquot of 0.1 mL of either culture was added to 5 mL of 0.6% top agar medium and poured onto a fresh LB plate. Blotting paper discs with different amounts of 15 M ACR were placed on the surface. After 18 h of incubation at 37 °C, growth inhibition zones were measured.

Plasmids modification

ACR modification of pIF101, pIF105, and pIF106 plasmids *in vitro* – was done as described elsewhere.[15]

Mutagenicity assay

Preparation of electrocompetent cells, induction of adaptive response, and electrotransformation were done according to 5. Briefly, ACR-modified or mock-treated plasmids were introduced by electroporation into cells prepared in two versions: with an induced (by pretreatment with 1 mM methyl methanesulphonate (MMS)) or uninduced Ada response. Appropriate dilutions of the transformation mixture were spread on LB plates containing chloramphenicol (30 μg/mL) to determine the total number of transformed cells and on lactose minimal plates to select Lac^+ revertants. Transformation efficiency was defined as the number of colony-forming units (cfu) produced by 1 μg of plasmid DNA in a transformation reaction. Mutation frequency (MF) was calculated as the number of revertants per 10^4 transformed cells.

Statistics

The statistical analysis was performed using the Statistica 10, statistical package software (StatSoft Inc., 2011, STATISTICA, version 10, https://www.statsoft.com). The distribution of variables was non-Gaussian, as stated by the Shapiro-Wilk test. The Kruskal-Wallis test with a post hoc Mann-Whitney *U* test was used to analyze the differences in mutagenesis (Figure 3). $P < 0.05$ with appropriate Bonferroni correction was considered statistically significant.

E. coli ada promoter assay

The experiment was conducted as described previously in 25,26. Promoterless pPROBE-NT and bearing kanamycin promoter pPROBE-KT: kanp were used as negative and positive controls, whereas pPROBE-NT-Ecadap bearing *E. coli ada* promoter was tested with 0.25, 0.5 mM ACR, and 1 mM MMS.

Purification of AlkA glycosylase

The *alkA* gene was cloned into the pET28a expression vector cut with the NdeI and XhoI restriction enzymes. AlkA glycosylase was overexpressed in *E. coli* BL21 pLysS strain and purified using His-Select Nickel Affinity Gel (Sigma-Aldrich) according to manufacturer protocol. In the sequence of the plasmid coding the AlkA glycosylase (pET28a::*alkA*), the spontaneous mutation resulting in substituting Pro9 with Ser has been identified (see Supplementary Figure S1). However, molecular modelling showed that despite Pro9 being identified with the *cis* peptide bond (as seen in pdb1diz)[27] its replacement with Ser only minutely affects the local conformation of the backbone and the packing with proximal sidechains (see Supplementary Figure S2) and would not interfere with DNA binding or the enzymatic activity of AlkA. The mass spectrometry profile shown in Supplementary Figure S3 confirmed AlkA sample purity. The purified protein was active against methylated DNA (data not shown) and was successfully used in the assay shown in Figure 5.

Preparation of HPA-containing oligodeoxynucleotide

The reaction mixture contained 30 nmole of 5′-d (TTT TTT ATT TTT ATT TTT ATT TTT T)-3′ (T22A3) oligodeoxynucleotide (Metabion, Martinsried, Germany), 0.65 M sodium acetate pH 4.5 and 1.5 M ACR (Fluka) in the final volume of 100 μL. The mixture was incubated for 15 min at 37 °C. Only adenine residues could be modified in those conditions, as thymine is unreactive in an acidic solution.[28] The oligodeoxynucleotide was purified from excess ACR by precipitation with 70% ethanol, lyophilized, and redissolved in TE buffer.

Annealing

Eight nmole of unmodified (T22A3, control) or modified [T22(HPA)3] oligodeoxynucleotide was associated with 16 nmole of the complementary one 5-d(AAA AAA TAA AAA TAA AAA TAA AAA)-3′ (A22T3) in 0.1 M NaCl in the final volume of 50 μL. The mixture was placed in a 90 °C water bath and cooled slowly to room temperature.

Excision of HPA by AlkA

The reaction mixture contained 20 nmole of double-stranded oligodeoxynucleotide, 100 pmole of AlkA glycosylase, 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM EDTA and 5 mM β-mercaptoethanol in the final volume of 120 μL. The mixture was incubated at 30 °C for 30 min and then purified using a GeneJET PCR purification kit (Thermo Fischer Scientific) to remove excised HPA.

Enzymatic hydrolysis of double-stranded oligodeoxynucleotides

The samples containing 5 nmole of either the HPA-containing- or unmodified (control) oligodeoxynucleotides were subjected to enzymatic digestion by snake venom phosphodiesterase (Sigma) and bacterial alkaline phosphatase (Thermo Fischer Scientific) (0.5 U of each enzyme) in 50 μL of 75 mM Tris/HCl buffer pH 8.8 and 5 mM $MgCl_2$. The mixture was incubated at 37 °C for 2.5 h and then analyzed by HPLC.

HPLC analysis

HPLC was performed using a Knauer dual pump system with a multi-channel UV spectrophotometer based on a diode array technology detector managed by Clarity Chrom controller V2.6.5.517. Separations were performed on a Waters Nova-Pak® C18 cartridge column (60 Å, 4 μm, 4.6 × 250 mm) at a flow rate of 1 mL/min. A linear gradient of 20 mM ammonium formate pH 6.5 × 20% aq. methanol over 30 min was applied, and detection was carried out at the respective maximum absorbance wavelengths for dA, dHPA, and T (257, 262, and 267 nm).

Molecular modelling of AlkA in complex with dsDNA containing T(HPA)T

Initial coordinates of both HPA stereoisomers, with a methyl group mimicking the deoxyribose moiety, were taken directly from the structure of 9-methyladenine deposited in the Cambridge Structural Database (BIFYOE).[29] Coordinates of the exocyclic hydroxypropane adduct were initially obtained by the MP3 method and further optimized using the B3LYP functional at 6-31G (d, -p) level. The resulting geometry and ESP-derived atomic charges were for both HPA stereoisomers incorporated into the Yasara2 forcefield used in molecular modelling. All *ab initio* calculations were performed using the Firefly program.[30] We have successfully applied such an approach to similar heterocyclic systems.[14,31,32] The atomic-resolution molecular model of dsDNA containing T(HPA)T bound to AlkA glycosylase was built based on the known crystal structure of AlkA in complex with dsDNA (PDB 1DIZ).[27] The location of the base of interest, lacking in the original PDB structure, was adopted from the known structure of AlkA with dsDNA carrying N^6-methyladenine (PDB 4NID).[33] The geometry of 1, N^6-α-hydroxypropanoadenine was prepared *ab initio* as described above. All molecular modelling of the protein-DNA complexes was done using the Simulated Annealing protocol implemented in the YASARA-Structure package (https://www.yasara.org) with the just-modified Yasara2 forcefield.[34]

Results and Discussion

Sensitivity of E. coli alkA and alkB defective strains to exogenous acrolein

AlkA and AlkB serve as DNA repair proteins, effectively countering the mutagenic and toxic effects of alkylated bases. They are both induced as a part of the *E. coli* Ada response system. Acrolein is a widely recognized environmental pollutant. Liu and co-workers reviewed its mutagenicity in different test systems. Several studies using the Ames test with *Salmonella typhimurium* and studies on *E. coli* or *Drosophila melanogaster* gave inconsistent results.[18] However, all those studies were performed with *alkA*$^+$ and *alkB*$^+$ backgrounds. Different extents of induction of those repair enzymes between studies were likely responsible for their conflicting outcomes. Therefore, to avoid this potential source of confusion, we attempted to estimate the impact of exogenous ACR on growing bacterial cells by comparing the wild type and strains devoid of AlkA and/or AlkB activity. Such an approach should allow us to demonstrate the mutagenic potential of the compound. Unfortunately, we could not obtain consis-

tent growth inhibition curves in liquid cultures of these strains dependent on ACR concentration. It turned out that commercially available ACR is unstable while diluted in water, so its concentration in liquid media changed over time. The acrolein dissipation effect has been reported for years.[35,36] Nevertheless, from those experiments, we found out that the window of acrolein concentration at which it would penetrate cellular DNA without killing the cell is very narrow, in the range between 1 to 3 mM (data not shown).

To avoid the problems encountered above, we performed a growth test on solid media, where the growth inhibition zone caused by increasing doses of undiluted ACR was determined for different *E. coli* strains (Figure 2). The inhibitory effect increased with the ACR dose, but somewhat unexpectedly, all four strains assayed showed a similar extent of growth inhibition at a given ACR dose. In agreement with this apparent irrelevance of AlkA and AlkB for the action of exogenous ACR on the bacteria, the induction of Ada response did not affect the extent of the growth inhibition zone. The only exception was the double *alkAalkB* mutant, which showed a significantly increased sensitivity to the lowest dose of ACR tested following Ada induction (Figure 2A). Strains lacking the AlkA and AlkB repair activities are extremely sensitive even at very low concentrations of the methylating agents,[5] such as MMS used to induce Ada response. Combined with ACR, this gave a more expansive growth inhibition zone. At the higher ACR doses (Figure 2B, C), ACR toxicity probably masked this effect. Altogether, this result indicates that the growth inhibition was likely due to acrolein cytotoxicity rather than mutagenicity.

In vivo repair of HPA by E. coli AlkA glycosylase and AlkB dioxygenase

To study the mutagenic potency of HPA, we used the test system elaborated and successfully applied in our previous studies on acrolein and chloroacetaldehyde (CAA) mutagenicity.[15,5,6] The system comprises a set of pIF plasmids[37] carrying lactose operon alleles of strains CC101-106 that allow monitoring of *Lac*$^+$ revertants arising from substitution mutations of individual bases in codon 461 of the β-galactosidase gene.[38] The three plasmids used, pIF101, pIF105, and pIF106, were indicatory for the AT → CG, AT → TA, and AT → GC substitutions. The plasmids were treated with different concentrations of ACR *in vitro* in a condition preventing thymine residue modification,[28] purified and then introduced by electrotransformation into *E. coli* wt, and *alkA, alkB* and double *alkAalkB* mutants. Although all plasmid bases may have been subject to modification, only one adenine residue was monitored. This approach allowed us to simulate endogenously arising HPA while avoiding the harmful effect of ACR on the cells. To examine the repair of HPA in bacteria, we expanded our previous study[15] by employing new strains, namely the *alkA* and double *alkAalkB* mutants, and using conditions of induced and uninduced Ada response.

The transformation efficiency of bacteria with mock-treated (control) plasmid varied in the range of $1\times$ to 2×10^6 cfu/μg DNA, depending on the batch of competent cells. This efficiency decreased with the increasing ACR concentration used for plasmid modification, dropping to 2×10^4 at 50 mM and 25 mM ACR for the single and double mutants, respectively. We observed a similar effect previously for chloroacetaldehyde

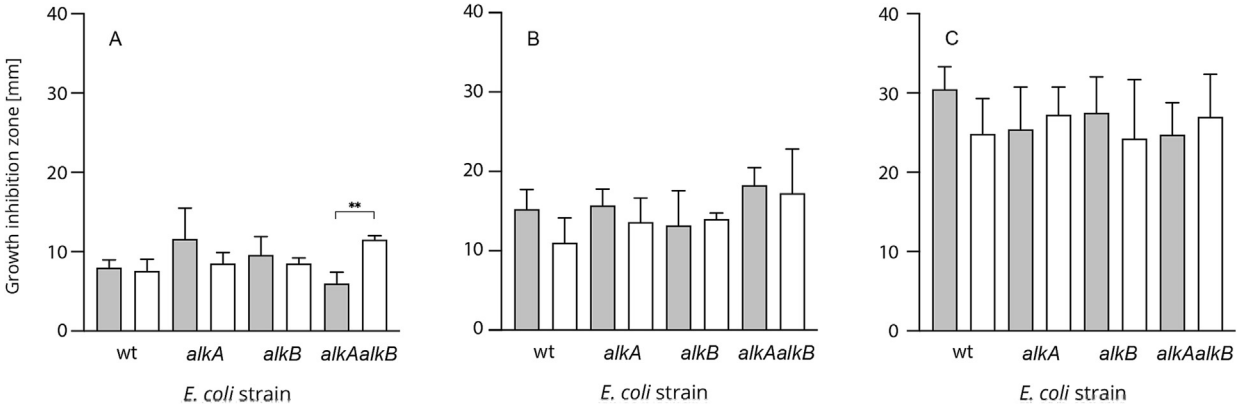

Figure 2. Inhibition of *E. coli* strains growth by acrolein. The effect of increasing doses of ACR (A) 9 μmole; (B) 18 μmole; (C) 27 μmole of ACR on the growth of indicated *E. coli* strains on solid media, was analyzed using a growth inhibition test. Grey bars represent bacteria with uninduced Ada response and empty bars – with the Ada response induced by pretreatment with MMS. Data are expressed as the mean ± standard deviation of three independent experiments. The statistical significance of the differences between these values is denoted by asterisks ($^{**}P < 0.01$).

mutagenesis[5]. We believe that in both cases, ACR and CAA, this phenomenon reflects the extent of modification-induced damage to the plasmid DNA and the inability of the transformed cells to repair the damaged plasmid, as discussed previously.[5]

The mutation frequencies for all three substitutions are shown in Figure 3. Numerical data and additional statistics concerning the significance of differences between various strains compared pairwise between variants with induced or uninduced Ada response (adapted or non-adapted, respectively) are presented in Supplementary Materials Tables S1–S3.

Spontaneous mutation frequency. The effect of *the alkA, alkB, and alkAalkB* mutations on the spontaneous mutation frequency (SMF) was different for the different plasmids analyzed. For pIF101 and pIF105, monitoring transversions, the SMF in non-adapted cells was up to 4-fold higher in single mutants and up to 8 times higher in the double *alkAalkB* mutant compared to wt strains. In contrast, for the pIF106 monitoring the A → G transitions, the effect of the single or double *alkA/alkB* mutations was much less pronounced, with around a 2–4 fold increase in SMF relative to wt. The spontaneous mutation frequency background also varied depending on the plasmid used. It was about an order of magnitude lower for transversions, whereas the difference between wt and mutant strains was much less evident for transitions.

The induction of the adaptive response affects SMF to varying degrees. Notably, in the case of A → C transversions, the induction of the adaptive response has no effect on SMF in wt and *alkA* mutant strains. However, in the *alkB* strain, there is a significant decrease in SMF levels. This suggests that AlkB dioxygenase plays a role in the repair of spontaneous mutations. No similar effect was observed in cases of A → T and A → G reversions; the reasons for this are unclear.

HPA-induced mutations. The frequency of mutations (MF) caused by HPA presence in the plasmid increased with increasing doses of ACR used for *in vitro* modification across all three plasmids and all the strains tested. In non-adapted cells, the frequency of A → T and A → C transversions was up to 5–6 and 6–8-fold higher for the single *alkA* and *alkB* mutants, respectively and up to over 20 times for the double mutant compared to the wt. In the case of A → G transversions, MF frequency was around 2 and 4 times higher for the two single and the double mutant, respectively. Notably, the amount of modifications caused by 50 mM ACR appeared too high for the double *alkAalkB* mutant, resulting in very low transformation efficiency. Virtually no mutants grew on minimal lactose plates, making it impossible to determine MF.

These results confirmed our earlier observation that HPA predominantly causes the A → T and A → C transversions and, much less frequently, the A → G transitions.[15] The data indicate that HPA is a non-instructive lesion.

The effect of the induction of Ada response on MF was visible for all the plasmids and strains tested. However, in the case of pIF101 and pIF105 replicated in wt, statistical significance was obtained only at higher ACR concentrations used for the modification. There is a visible, more significant difference between bacteria with uninduced and induced Ada responses in A → C MF caused by acrolein modification of the pIF101 plasmid in alkB strain compared to wt and alkA strains. The result appears to reflect a cumulative effect of (apparently independent) spontaneous mutations and those induced by the HPA. It is important to note that Ada induction substantially reduced the mutation frequency not only when both AlkA and AlkB were able to increase their activities but also in both single mutants, *alkA* and *alkB*. The latter convincingly demonstrated that both AlkB and AlkA proteins are involved in removing the HPA lesion. On the other hand, Ada response induction did not decrease MF in the double mutant. Conversely, an opposite effect was observed, *i.e.*, MMS pretreatment made the strain very susceptible, resulting in an up to 70-fold higher MF than in the adapted wt strain case. A similar effect was previously noted for CAA mutagenicity, which we attributed to the activation of the SOS and UVM responses (see 5 for details). We posit that the same explanation is valid for HPA mutagenicity.

The results obtained for all three plasmids demonstrated that the products of both studied genes, *alkA* and *alkB*, are engaged in repairing the ACR adduct to adenine. Our previous work showed that AlkB repairs HPA *in vivo,* even without prior Ada induction.[15] A similar effect is observed for AlkA in the present results. AlkB also repairs some ethenoadducts even at its low constitutive level.[39,5,6] Although AlkB is believed to be present in the cells at a much lower constitutive level than AlkA,[3,40] the quantities of both these proteins are sufficient to handle the small amounts of endogenously generated lesions. Therefore, we hypothesize that, in addition to preparing the cell to cope with the consequences of high concentrations of exogenous alkylating agents, the Ada-response enzymes (at least AlkA and AlkB) could also constitutively control the level of DNA adducts of endogenous origin.

Acrolein is the strongest electrophile among the α,β-unsaturated aldehyde series, readily interacting with diverse cellular molecules. In the case of exogenous exposure to ACR, cell membrane components are among the first 'to meet the enemy'. Consequently, membrane disruption may contribute significantly to ACR cytotoxicity. Nevertheless, ACR can more easily access DNA when produced endogenously,

particularly in dividing cells. The balance between the cytotoxicity and genotoxicity of acrolein has been under debate for years.[41] Here, by employing two fundamentally different approaches, but both using the same set of strains and the same induction of Ada response conditions, we determined the effect of ACR on bacteria and how it is affected by the Ada response. In the first approach, a direct impact of ACR on the cells was studied. In contrast, in the second, acrolein-modified plasmids were used to determine the effects of DNA lesions caused by the presence of acrolein adduct to adenine in DNA. HPA has been confirmed to be formed in DNA *in vivo* (along with an adduct to lysine) under induced oxidative stress conditions.[22] Thus, our method based on plasmids modified *in vitro* seems a good approximation of *in vivo* events at cellular conditions, enabling a direct comparison of the mutation frequencies in the wt and *alkA* and *alkB* strains. While the mutagenic effect of exogenous acrolein seems to be masked by its general cytotoxicity (Figure 2), we confirmed its mutagenic potential by introducing the acrolein adduct to adenine rather than acrolein itself to bacterial cells (Figure 3). If an acrolein molecule reaches DNA, which is more likely for an endogenously produced one, it can cause mutagenesis. Moreover, AlkB dioxygenase (as shown in 15) and AlkA glycosylase (demonstrated here for the first time) can alleviate its consequences. What is important is that both proteins remove HPA even at their low constitutive level.

Acrolein, one of the most abundant reactive species formed endogenously in oxidative stress, is known for its impact on the development of various pathological processes through its adducts to DNA bases.[42–44] There is little but compelling evidence that Ada response proteins (or its homolog) take part in mitigating the consequences of oxidative stress. AlkA glycosylase efficiently repairs an oxidized base, 5-formyluracil,[40,45] while the human AlkB dioxygenase homolog, ALKBH3, was shown to be involved in repairing endogenous 3-methylcytosine in genomic DNA.[46] An additional function of DNA repairing Ada response enzymes could be protecting cells against harmful metabolic byproducts appearing in oxidative stress. Further research is needed to explore this hypothesis and deepen our understanding of these protective mechanisms. This perspective provides a promising foundation for future investigation into the role of DNA repair enzymes in cellular defence.

Acrolein does not induce Ada response

To investigate further exogenous ACR impact, we examined whether sub-lethal ACR doses could

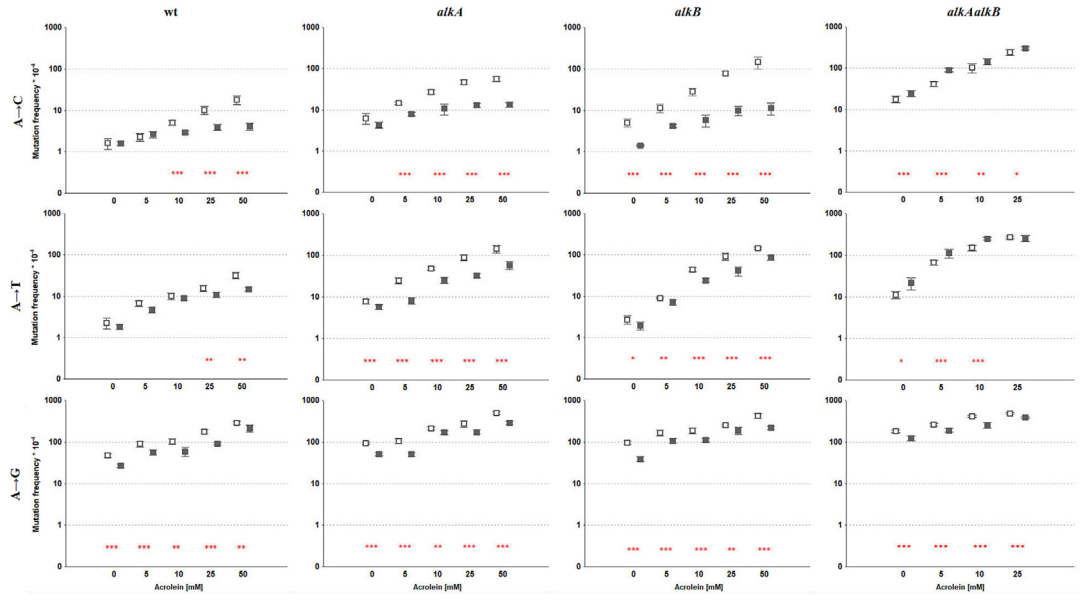

Figure 3. Frequencies of HPA-induced *Lac*$^+$ reversions in plasmids *lacZ* gene. Plasmids pIF101 indicatory for A → C transversions, pIF105 indicatory for A → T transversions, and pIF106 indicatory for A → G transitions were treated with increasing doses of ACR *in vitro* and replicated in wild type, *alkA, alkB* and *alkAalkB E. coli* strains. On the x-axis, the concentration (mM) of ACR used for modification of plasmids is given; on the logarithmic y-axis, mutation frequency (MF), expressed as the number of revertants per 10^4 transformed cells, is provided. The vertical panels show the MF results for different substitutions within a given strain, whereas the horizontal panels allow the comparison of MF results for a specific substitution across the studied strains. Data are expressed as mean ± standard deviation of at least three independent experiments. White and grey bars represent MF values obtained in bacteria with uninduced and induced Ada responses, respectively. The statistical significance of the differences between these values is denoted by asterisks ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

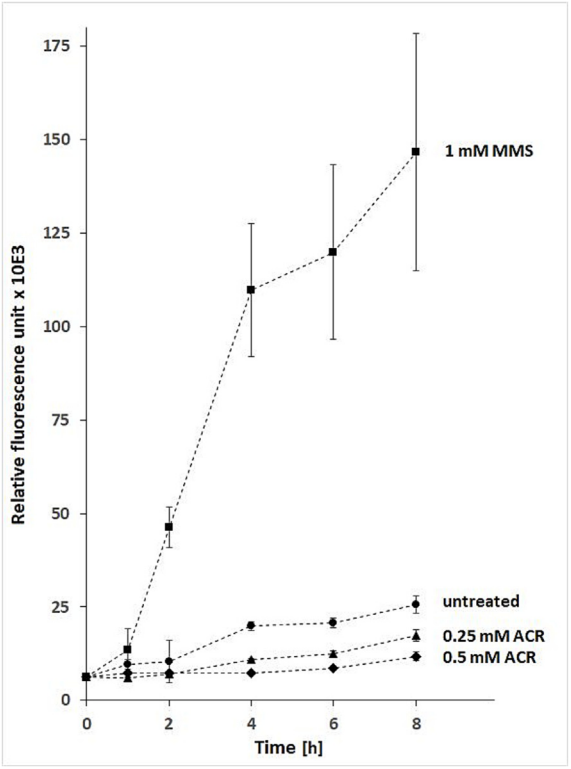

Figure 4. Effect of acrolein on Ada response promoter activity. Cultures of *E. coli* carrying pPROBE-NT:Ecadap vector was left untreated or treated with ACR or MMS, and GFP fluorescence was determined at time points indicated. Data are shown as mean ± standard deviation of three independent experiments.

induce the *E. coli* Ada response transcription activator. The activity of the *ada* gene promoter was monitored with a green fluorescence protein gene (GFP) reporter expressed from the pPROBE-NT:Ecadap vector carrying the *E. coli* ada gene promoter sequence upstream of the gene coding for the GFP constructed by us previously.[26] The results are presented in Figure 4. The GFP fluorescence in control cells carrying the promoterless vector was minimal (data not shown). Similarly, the fluorescence remained low in cells with the *ada* promoter driving GFP expression, both under untreated conditions and following ACR treatment, where it even slightly decreased. Interestingly, although not high, the difference in relative fluorescence level between untreated vector and treated by 0.25 and 0.5 mM ACR might suggest a direct toxic acrolein effect. As expected, MMS induced the Ada response and substantially enhanced the GFP reporter level. These results indicate that the Ada promoter stays uninduced upon ACR treatment. The finding aligns with the established knowledge that the Ada response is activated only by methylating agents. Similarly to ACR, the alkylating agent CAA also does not induce the Ada response[47] despite its DNA adducts being substrates for AlkB.[39,5,6] Thus, elements of the Ada response, whose primary function is to protect the bacterial cell against methylation-induced damage, may also be active against a broader spectrum of mutagens. AlkB has previously been shown to repair both CAA and ACR adducts, and now AlkA has also turned out to process one of the latter.

HPA is excised by AlkA glycosylase in vitro

We confirmed the ability of AlkA glycosylase to remove ACR adenine adducts from DNA using an *in vitro* assay. The adduct was generated under controlled conditions, allowing selective modification of adenine residues only[28] in a single-stranded oligodeoxynucleotide containing three adenine (A) and 22 thymine (T) residues. The extent of adenine modification was estimated by comparing HPLC profiles of nucleotides derived from the unmodified oligodeoxynucleotide and following treatment with ACR. Since the molar absorption coefficient of HPA is unknown, the calculations could only be approximate. We considered that the absorption maxima of A and HPA are different – 257 and 262 nm, respectively. As expected, the unmodified oligodeoxynucleotide contained only the T and dA nucleosides (Figure 5B), and following ACR treatment, a third peak corresponding to dHPA appeared (Figure 5C). We concluded from the integrals of appropriate peaks that approximately 45% of adenines were converted to HPA. Such partial modification was advantageous since HPA may destabilize DNA duplexes. The partially modified T22(HPA)3 oligodeoxynucleotide was annealed with a complementary unmodified one to form a double-stranded substrate for AlkA. In the control reaction without the enzyme, dHPA remained unchanged (Figure 5D), while following AlkA treatment, the dHPA peak disappeared, demonstrating the enzyme's ability to excise the adduct (Figure 5E). Only T and dA peaks were observed in parallel reactions performed with unmodified substrate (data not shown). These results confirm that the HPA adduct can be excised by *E. coli* AlkA glycosylase *in vitro*.

Molecular modelling of HPA poses in the substrate-binding pocket of AlkA glycosylase

Modifying adenine with ACR results in two adduct stereoisomers, R and S, each of which can adopt either the *anti* or *syn* conformation around the glycosidic bond. We have recently shown that AlkB dioxygenase specifically recognizes the isomers and repairs them with different efficiency.[15] The complete removal of HPA by AlkA implies that the enzyme processes both forms of HPA. We employed molecular modelling to propose the molecular mechanism of their repair, and to assess whether they could be repaired with different efficacy.

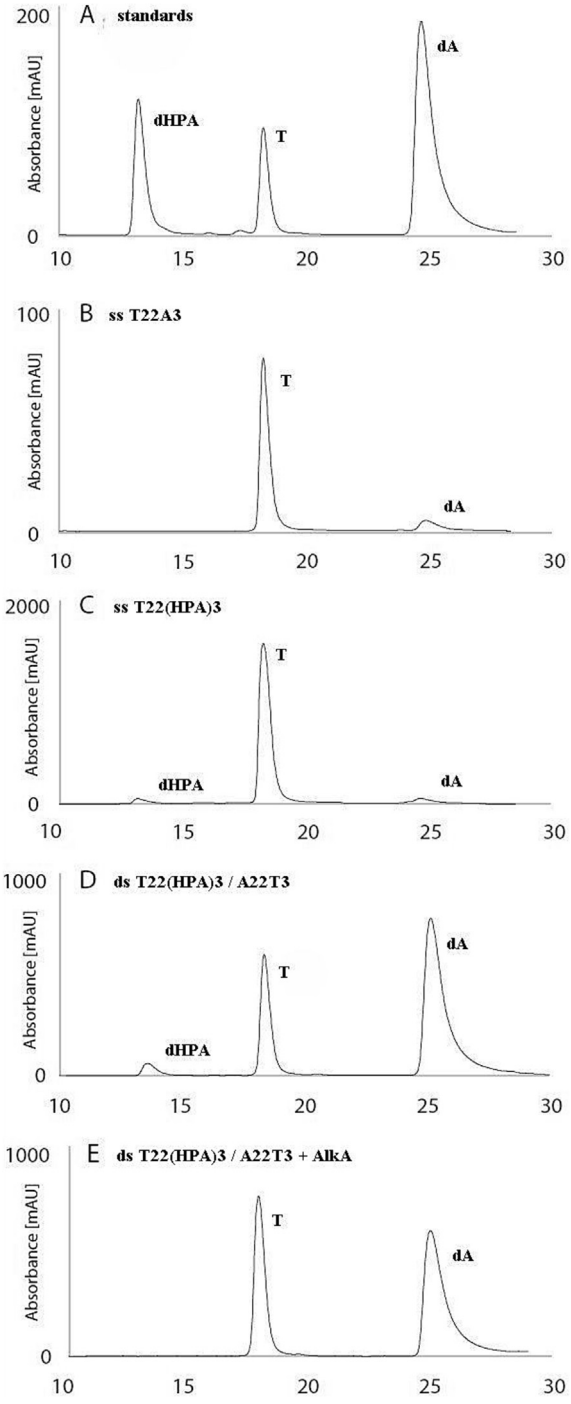

Figure 5. Excision of HPA by AlkA glycosylase *in vitro*. (A) Standards for the expected deoxynucleosides (dHPA, retention time 13.2 min; T, 18.6 min; dA, 25.1 min). (B–E) Oligodeoxynucleotides after enzymatic digestion; (B) Unmodified single-stranded T22A3; (C) Single-stranded T22(HPA)3, *i.e.* T22A3 treated with ACR; (D) double-stranded T22(HPA)3, *i.e.* annealed to complementary A22T3, – control without AlkA; (E) as (D), but after reaction with AlkA glycosylase.

Based on the available crystallographic structures,[27,33] we modelled the location of the two HPA stereoisomers, each in either the *syn* or *anti*-conformation of the glycosidic bond at the catalytic centre of the AlkA – dsDNA processive complex. Both isomers in the *anti*-conformation fit perfectly in the binding pocket. Numerous AlkA residues are involved in the ligand recognition (F18, L19, A21, R22, G123, V126, S127, V128, W218, Y222, D238, Y239, L240, and W272), among which F18, Y222, and W272 make π–π interactions with the HPA base, while R22, R119, V128, R226, and Y273, together with F18, interact with the hydroxypropano exocyclic group. For both HPA stereoisomeric forms, the glycosidic bond is proximal to the two catalytic residues (W218 and D238), adopting an orientation facilitating the acid-catalyzed cleavage of the glycosidic bond. With such orientation, the enzyme should not distinguish between the two HPA stereoisomers. In both cases, the location of the modified base to be excised in the enzyme's active site agrees with the S_N1-type mechanism of the glycosylase reaction, in which the essential catalytic D238 assists the base removal directly.[27] A slight binding preference for the S stereoisomer is due to better stacking of the positively charged alkylated base in this configuration against the aromatic side chain of W272, which should enhance the cleavability of the glycosidic bond, expected to be destabilized in modified nucleosides.[27] For both stereoisomers, the binding of the *syn* conformer is strongly disfavored due to numerous steric hindrances, indicating that HPA is likely to be recognized in the *anti*-conformation. Since purine nucleosides/nucleotides undergo a dynamic *syn-anti* equilibrium,[48] the preferences towards the *anti* forms do not strongly affect enzymatic processivity.

In summary, molecular modelling demonstrated how DNA carrying either the R or the S HPA stereoisomer can bind at the AlkA catalytic site and undergo hydrolysis of the glycosidic bond. In agreement with our experimental data, this finding indicates that both HPA isomers can be excised from DNA by AlkA glycosylase with comparable efficiency (Figure 6).

Conclusions and Perspectives

We have shown that AlkA glycosylase, like AlkB dioxygenase, removes HPA from DNA through experimental and computational approaches. AlkA and AlkB are broad-substrate-specificity DNA repair enzymes induced in *E. coli* as part of the Ada response to alkylating agents. AlkA initiates base excision repair, whereas AlkB constitutes a direct, single-protein repair system protecting the cellular DNA and RNA from various lesions and working on ss- rather than dsDNA. HPA has been shown to arise in single- and double-stranded DNA.[49] Those authors did not study it, but the adduct probably occurs more frequently in ssDNA since in dsDNA, the N1 and N^6 positions of adenine are protected by the A-T Watson-Crick pairing. Therefore, AlkB preferentially removing modifications from

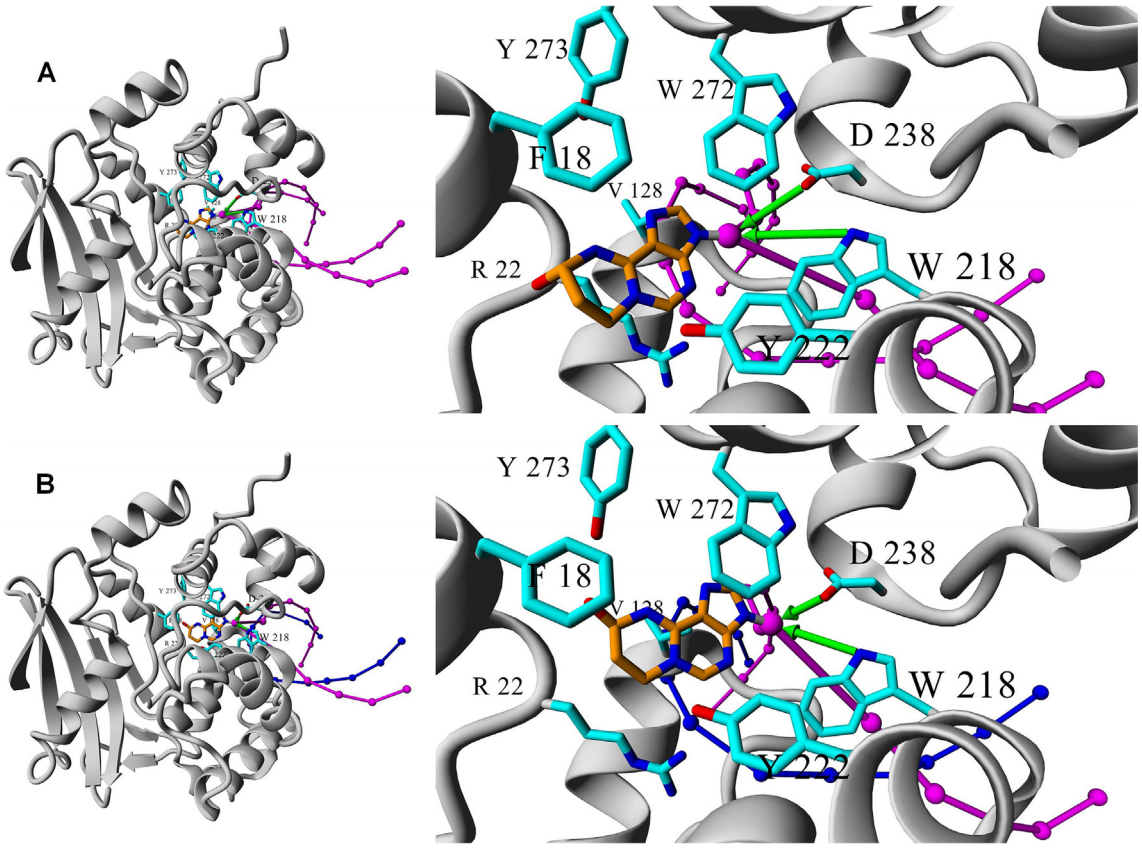

Figure 6. Structure of AlkA with ds DNA containing different stereoisomers of dHPA. 1DIZ and 4NID PDB structures[27,33] were used to fit the R (A) and S (B) stereoisomers of HPA into the active site of AlkA. HPA and some crucial amino acid residues, including D238 and W272, involved in the catalysis, are shown in stick representation.

single-stranded substrates[50] could be 'the first on the front line'. AlkB removes the R stereoisomer more readily and, as we have shown recently, can only perform a limited number of catalytic cycles, likely being inactivated by the reaction byproduct malondialdehyde.[15] Acting on dsDNA, AlkA complements the lesion removal process. Importantly, AlkA removes both isomers of HPA, excising the whole modified base without forming harmful byproducts, while BER machinery can efficiently repair the resulting abasic site. Two independent repair systems are involved in removing the HPA lesion, direct repair by AlkB and AlkA-assisted base removal followed by BER, underscoring the dangerous effect of exocyclic adducts on genome integrity. The two enzymes repair the acrolein adduct also at their constitutive levels, not only when induced. Acrolein is the most active α,β-unsaturated aldehyde formed endogenously during oxidative stress and is known for its extreme cytotoxicity. Most likely due to this feature, *alkA* and/or *alkB* defective *E. coli* strains were not more sensitive to exogenous ACR than the wild type was. Notably, ACR treatment did not induce the Ada response. We conclude that the mutagenicity of acrolein stems from its endogenous rather than exogenous sources. So, AlkA glycosylase and AlkB dioxygenase not only play crucial roles in responding to alkylating agents but also control the level of DNA adducts that arise during oxidative stress. Acting together, they constitute an effective protective system against endogenously arising threats. Interestingly, under moderate basic conditions, 1,N^6-α-hydroxypropanoadenine can undergo Dimroth rearrangement, spontaneously converting to its γ-isomer.[49] In light of our present findings, it would be worthwhile to investigate whether 1,N^6-γ-hydroxypropanoadenine has an independent role in mutagenesis. Moreover, AlkB dioxygenase repairs *in vitro* acrolein adduct to cytosine – 3,N^4-α-hydroxypropanocytosine.[15] Studies on its mutagenicity and excision by AlkA glycosylase are in progress.

The microbial adaptive response to alkylating agents is not conserved evolutionary, and AlkA glycosylase does not exist in higher organisms. However, non-homologues, but acting similarly to AlkA human 3-alkyladenine DNA glycosylase (AAG), and human AlkB homologs involved in DNA repair are promiscuous enzymes that could also be engaged in the removal of endogenously arising lesions – products of cellular oxidative stress, including HPA. Since such lesions are

widely known to play an essential role in the development of severe diseases, including inflammation, chronic infections, and cancer,[51] studies on the role of the human DNA repair enzymes in the etiology and prevention of those pathologies, their substrate specificities and mechanisms of action need to be continued. The insight gained from simple model organisms, such as *E. coli*, can provide helpful guidance for such investigations.

CRediT authorship contribution statement

Małgorzata Dylewska: Writing – original draft, Visualization, Investigation, Formal analysis. **Izabela Dąbrowska:** Investigation. **Karolina Ćwiek:** Investigation. **Katarzyna Padoł:** Investigation. **Damian Mielecki:** Investigation. **Beata Sokołowska:** Formal analysis. **Jarosław Poznański:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Agnieszka M. Maciejewska:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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Abbreviations:
Ada, adaptive response; ACR, acrolein; CAA, chloroacetaldehyde; ε, etheno; GFP, green fluorescence protein; HPA, 1,N^6-α-hydroxypropanoadenine; MMS, methyl methanesulfonate; m, methyl; MF, mutation frequency; SMF, spontaneous mutation frequency; wt, wild type

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