Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies

Mielecki D, Sikora A, Wrzesiński M, Nieminuszczy J, Detman A, Żuchniewicz K, Gromadka R, Grzesiuk E*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

In the memory of Prof. Celina Janion

Corresponding author: Elżbieta Grzesiuk,

E-mail: elag@ibb.waw.pl

Abstract

Within a decade the family of AlkB dioxygenases have been extensively studied as a one-protein DNA/RNA repair system in *E. coli* but also as a group of much wider function proteins in eukaryotes. Two strains, HK82 and BS87, are the most commonly used *E. coli* strains for *alkB* gene mutations. The aim of this study was to assess the usefulness of these *alkB* mutants in different aspects of research on AlkB dioxygenases functions not only in alkylated DNA repair but also in other metabolic processess in cells. Using of HK82 and BS87 strains we found the following differences among these $alkB^-$ strains: (i) HK82 has shown more than 10-fold higher MMS-induced mutagenesis in comparison to BS87; (ii) different specificity of Arg^+ revertants; (iii) increased induction of SOS and Ada responses in HK82; (iv) HK82 DNA, in comparison to AB1157 and BS87, contains additional mutations: *nalA*, *sbcC*, and *nuoC*. We hypothesize that in HK82 these mutations together with the non-functional AlkB protein may result in much higher contents of ssDNA, thus higher in comparison to BS87, MMS-induced mutagenesis.

In the light of our findings, we strongly recommend using BS87 strain in AlkB research as HK82, bearing several additional mutations in its genome, is not an exact derivative of the AB1157 strain, and shows additional features that may disturb proper interpretation of obtained results.

1. **Introduction**

The *alkB* locus was reported 30 years ago in Mutsuo Sekiguchi's laboratory, nonetheless, the biological function of AlkB protein has been discovered only 20 years later [1-3]. This enzyme has appeared to function as a one-protein DNA repair system whose action depends on one co-substrate, 2-oxoglutarate (2OG), and two cofactors, Fe(II) and O_2. It acts both, on DNA and RNA and reverts N1-methyladenine (1meA) and N3-methylcytosine (3meC) residues back to natural bases. Bioinformatic analysis placed AlkB in the superfamily of 2OG and Fe(II)-dependent dioxygenases found in almost all organisms [4]. Multiplicity of AlkB homologs, especially in eukaryotes, e.g. nine in humans (ALKBH1-8 and FTO), 13 in *Arabidopsis thaliana*, suggests a variety of functions exerted by these proteins.

In 1983 Kataoka and coworkers [5] isolated as *E. coli alkB⁻* mutant specified as HK82 that to this day serves as the most frequently used tool to determine a variety of AlkB features. The HK82 comes from the *E. coli* K12 AB1157 strain mutagenized with MNNG/EMS and the subsequent isolation of MMS sensitive and UV-light resistant colonies. Mutation localization was done by replica plating method, P1 transduction, and more precise mapping by crossing each mutant with Hfr strains. The mutation of one of the MMS-sensitive isolates, HK22 (*alkB22*), was mapped between *nalA* and *nrdB* markers. In the next step, a spontaneous nalidixic acid resistant (Nal^R) mutant of HK22 was isolated and *alkB22* was P1-transducted to AB1157. In this way, the HK82 (*alkB22 nalA*) and HK80 (*alkB⁺ nalA*) strains were constructed to determine the effect of *alkB22* mutation on bacterial growth, sensitivity to alkylating agents, and the type of MMS-induced mutations (Fig. 1A).

BS87 (AB1157 *alkB117*) strain has been obtained with the use of another construct by Kataoka and Sekiguchi (1985) *alkB* mutant, HK117. The genetic region encompassing gene *alkB*, on pHK12 plasmid (Cm^R) was interrupted with transposon Tn*3*, derived from temperature-sensitive pSC301::Tn*3* (Amp^R), in the background of HK82 strain, thus allowing the selection of pHK12 *alkB::*Tn*3* mutants sensitive to MMS. One of the plasmids with Tn*3* inserted into its *alkB* gene, pHK12-51, was recombined with the MM386 genome (*polA*^{ts}) producing the HK117 (*alkB*$^-$) strain [6]. Growing P1 phage on HK117 with subsequent transduction to AB1157, dr. Sedgwick obtained *E. coli* AB1157 *alkB*$^-$ marked as BS87 that harbors the *alkB117* with Tn*3* an insertional mutation [7] (Fig. 1B).

In our research published in 2006 [8], we have found at least 10-fold higher level of MMS-induced *argE3* □ Arg$^+$ reversion in HK82 strain in comparison to BS87 and the other *alkB*$^-$ mutant, MV1601 [9]. Here we showed that the high level of induced mutations in HK82 is due to a stronger induction of the SOS response. Using comparative analysis of the genome sequence we failed to indicate a single mutation responsible for this phenomenon, nevertheless, several divergences that we discovered in HK82 genome sequence may underline pecularly high activity of a mutagenic DNA repair network in this strain. At the same time, HK82 has been the most and preferably used strain in the AlkB studies, eg. [10-14]. By presenting results of our study, we wish to inform other research groups working on AlkB dioxygenase about the differences between HK82 and BS87 to conclude that the use of HK82 as a reference *alkB*$^-$ strain may cause false interpretation of experimental data.

2. **Materials and methods**

2.1. Bacterial strains, plasmids, and media

All bacterial strains and plasmids used in this study are presented in Table 1. The basis of this study are derivatives of *E. coli* K-12 AB1157, HK82 (*nalA alkB22*) constructed by Kataoka, Yamamoto and Sekiguchi (1983), and BS87

(*alkB117*) constructed by Sedgwick (1992). Strains representing various combinations of *alkB^wt^*, *alkB22*, *alkB117*, and *nalA* mutations in the backgrounds of AB1157, HK82, or BS87, as well as HK82 *sbcC^wt^* were prepared by P1 transduction [15]. The strains were selected on LB medium containing antibiotics indicated in Table 1. MMS-sensitivity of the strains was preliminarily checked by disc diffusion test with 2 μl of MMS applied onto each disc, and the *alkB22* and *alkB117* alleles distinguished by PCR reaction (data not shown).

In complementation experiments, pMW1 [16], pMW2, and pMW3 plasmids were used. Plasmid pMW2 was constructed by cloning the PCR product encoding the *ada-alkB* operon, produced with ada-pMW2-up 5'-AGGGCTGGCGGTTTATATGATCAC-3' and alkB-pMW1-dn 5'-CGCCAGACAAGTACAAGAAGTTCC-3' primers, into pGB2 vector plasmid cut with SmaI (NEB). The pMW3 was constructed by mutagenesis of the pMW2. Frameshift mutation and TAA stop codon were formed by MluI (NEB) digestion of pMW2, creation of blunt ends by PolI Klenow fragment (NEB) and relegation. Subsequently, only 98 amino acids of Ada protein could be formed from pMW3.

The liquid media were LB [15] and E medium consisted of C salts [17], glucose (0.5%), casamino acids (0.2%), and 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 was composed of trypton (1%), yeast extract (0.5%), NaCl (1%), $MgSO_4 \times 7H_2O$ (0.25%), $CaCl_2$ (2.5 mM), and Difco agar (0.6%) [15]. If necessary, carbenicillin (100 μg/ml), kanamycin (50 μg/ml), nalidixic acid (40 μg/ml), spectinomycin (50 μg/ml) or tetracycline (20 μg/ml) were added to the media. All bacterial variants were grown at 37°C.

Since both BS87 strain and pSK1002 plasmid bear the resistance to carbenicillin, BS87/pSK1002 transformants were selected on E-medium (C salts supplemented with thiamine (10 μg/ml), glucose (0.5%), and casamino

acids (0.4%)) containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 µg/ml) and MMS (2.5 mM). Blue colonies were selected and checked for the presence of pSK1002 plasmid DNA.

2.3. Mutagenesis assays

To test MMS-induced mutagenesis, bacteria were grown to OD_{600} 0.6–0.7 in E medium, treated with 20 mM MMS for 15 min, diluted 1:10 in E medium, grown overnight to express mutations, and spread on the LB plates for viable cells (one day of incubation at $37^{\circ}C$), LB was supplemented with rifampicin (100 µg/ml) for Rif^R mutants, and on E-arg for Arg^+ revertants (two days of incubation at $37^{\circ}C$). Following the counts, frequency of mutations was expressed as the number of mutants per 10^8 cells. The spontaneous level of mutations was assigned as a control.

Mutagenesis experiments were repeated 4-8 times, and standard deviation ± SD was calculated.

2.4. Expression of β-galactosidase

AB1157, AB1157 *alkB22 zfa-3145::Tn10kan*, HK82 *zfa-3145::Tn10kan alkB$^+$*, HK82, and BS87 were transformed with pSK1002 plasmid harboring a *umuC*::*lacZ* fusion [18, 19]. An overnight culture in LB was diluted 10-fold, treated with 20 mM MMS for 15 min, centrifuged, resuspended in the same volume of fresh medium, and incubated with shaking for 1-4 h. At appropriate time points, aliquots were taken and β-galactosidase activity was estimated according to [15].

2.5. Next generation genome sequencing

Genomic DNA (gDNA) was purified from the BS87 and HK82 cells by the CTAB/lysozyme method [20] and DNA concentrations were determined with the Qubit dsDNA BR Assay Kit (Invitrogen). The gDNAs were used to create the genomic libraries using the KAPA Library Preparation kit, Illumina series (Kapa Biosystems). The concentration of DNA libraries was checked by qPCR

6

using the Illumina Library Quantification kit LightCycler 480-qPCR mix. Sequencing was performed with v3, 2×300 bp chemistry on the MiSeq platform (Illumina).

The raw data was trimmed and cleaned for adaptors and assembled using Newbler v.3.0 (454 Life Sciences Corporation). Superscaffolds were annotated on the RAST server (Rapid Annotation Subsystem Technology) v.2.0. For SNP-calling, Geneious R6 v. 6.1.8 (Biomatters Ltd) was used with BS87 as a reference sequence.

3. **Results and discussion**
3.1. *Characterization of MMS-induced mutagenesis in BS87 and HK82 strains*

AlkB protein plays a protective role against the mutagenic action of MMS, thus mutations of its key amino acid residues sensitize bacterial cells to this alkylating agent. Our published results [8] have shown strongly elevated sensitivity of HK82 as well as BS87 mutants to MMS in comparison to the AB1157 counterpart. In spite of similar survival, these *alkB*⁻ mutants have presented different mutational responses to MMS treatment. In HK82 strain, the level of MMS-induced Arg$^+$ revertants has been at least 10-fold higher than that in BS87 (Fig. 2). Additionally, the specificity of MMS-induced Arg$^+$ revertants differs fundamentally between BS87 and HK82 strains. In BS87, Arg$^+$ revertants arise mainly by *supB* and *supEoc* suppressor formation due to the GC→AT transitions in contrast to HK82 strain where the AT→TA transversions arising by *supL* suppressor formation prevail [8].

In order to explain whether the differences between BS87 and HK82 result from the type of *alkB* mutation, i.e. the point mutation at *alkB22* locus *vs.* the transposon insertion in the *alkB* gene, *alkB117* (*alkB::Tn3*), a series of the strains with different *alkB* alleles and selected backgrounds were constructed (Table 1). Briefly, the *alkB22* was replaced for the *alkB117* in HK82 and *vice versa* in BS87. Additionally, the total *alkB* deletion allele, *alkB::kan*, was also tested in the HK82 genetic background. Moreover, the wild type *alkB*$^+$

phenotype was recovered in HK82 and the *alkB22* allele was introduced to the new AB1157 background. In all the constructs, the MMS-induced mutagenesis was tested. As it was shown in Fig. 2, the increased level of Arg$^+$ revertants was always observed in the HK82 background, irrespective of the type of mutated *alkB* allele. On the other hand, the *alkB22* showed the same effect as *alkB117* and *alkB::kan* when present in AB1157 or BS87 backgrounds.

Interestingly, the differences in the level of MMS-induced mutations between HK82 and BS87 concerned only the *argE3*☐Arg$^+$ reversions whereas the Rif^S☐Rif^R mutants remained at the same level (Fig. 2). As we proved before, the *argE3*☐Arg$^+$ reversion system enables detection of mutations arising from lesions in ssDNA and is especially useful for studies on induced mutagenesis in *E. coli alkB$^-$* strains [16, 21, 22]. Most of the MMS-induced Arg$^+$ revertants in the *alkB$^-$* mutants arise by tRNA suppressor formation [8]. The tRNA-encoding genes are heavily transcribed and exist mostly as ssDNA in cells. On the contrary, rifampicin resistance results from mutations in the *rpoB* gene encoding ☐ subunit of RNA polymerase, assumed to be in the form of dsDNA for most of the time. Moreover, the increased level of mutagenesis in HK82 in comparison to BS87 is possibly specific to the mutagenic agent (e.g. MMS) inducing both, the SOS and Ada regulons since no differences in the level of UV-induced mutations in both tested strains were observed (data not shown).

3.2. *Induction of SOS response in alkB mutants*

The difference between HK82 and BS87 was also noticed in the level of β-galactosidase induction measured in these strains harboring pSK1002 plasmid bearing *umuC::lacZ* fusion, where the *lacZ* gene remains under the control of *umuC* promoter. The fusion gene has been expressed in response to SOS induction. We have found 2-fold stronger β-galactosidase induction in MMS-treated HK82 in comparison to BS87 strain (Fig. 3). It is noteworthy that induction of β-galactosidase in the AB1157 *alkB22* strain is comparable to

that of BS87, and lower than that of HK82. Moreover, β-galactosidase induction in HK82 $alkB^+$ is stronger than in AB1157. Since these results indicate the induction of SOS response, we conclude that the HK82 strain shows elevated activation of essential DNA repair systems that remain under the control of SOS regulon. Taken together, the increased MMS-induced mutagenesis measured by the $argE3\square Arg^+$ reversion system, different spectrum of mutations, and induced SOS response point to the conclusion that there should be additional, AlkB-independent source of mutations in HK82 strain.

3.3. *The impact of Ada response on the frequency of MMS-induced mutations in alkB mutants*

Since the main repair system counteracting alkylating agents in *E. coli* is the Ada regulon, we performed a series of complementation assays in HK82 and BS87 expressing episomal copies of Ada regulon components. These components include bifunctional Ada protein, a transcription factor activating the Ada regulon and methyltransferase acting mainly on O^6meG and O^4meT, and AlkA glycosylase repairing broad range of substrates including replication blocking 3meA, and 7meA, 3meG, εA, and even excising normal bases from DNA [23, 24]. Interestingly, overproduction of AlkB protein (Fig. 4) led to the reduction of Arg^+ revertants to 16% in the BS87 strain, but only to about 48.4% in HK82. Further, simultaneous overexpression of three Ada proteins: Ada, AlkA, and AlkB decreased the level of Arg^+ revertants in MMS-treated BS87 and HK82 up to 6.4 and 3%, respectively, whereas in the presence of pMW3 plasmid mutated in *ada* gene respective values reached almost 10 and 34% (Fig. 4). These results suggest that AlkB protein alone is not sufficient to repair MMS-induced lesions in HK82 strain, but all three Ada response proteins (Ada, AlkA, AlkB) contribute to the repair of a spectrum of lesions in DNA produced by alkylating agent.

3.4. *Comparative analysis of genome sequence of HK82 and BS87 strains in the light of their different responses to MMS action*

To identify the factor(s) responsible for the malfunctioning of HK82 strain, we performed next generation genome sequencing of HK82 and BS87. NGS analysis identified several genes with non-synonymous mutations present in HK82, listed in Table 2. Among others, they include *nalA*, *sbcC*, and *nuoC* genes.

The *nalA* codes for DNA gyrase subunit A. As it was mentioned earlier, the spontaneous *nalA* mutant conferring the resistance against nalidixic acid and other quinolone-based antibiotics assisted the transduction of *alkB22* [5]. It is widely accepted that the NalA region encompassing 67 – 106 amino acids serves as the drug target and was dubbed the quinolone resistance-determining region [25, 26]. As we have established, HK82 strain carries the mutated *nalA* producing the NalA D87Y (Table 2). Although this mutation lies in the 67 – 106 region, Barnard and Maxwell [27] found that the GyrA D87A mutant was 2.5-fold less active in DNA supercoiling and 1.5-fold less active in DNA cleavage [27]. Taken together, we tested the mutagenesis levels in the *E. coli* cells with *nalA* alone and with combination with *alkB22* and *alkB117* alleles in different backgrounds. As it is shown in Fig. 2, the presence of *nalA* does not show any influence on the level of Rif^R mutants/Arg$^+$ revertants in AB1157 and its *alkB$^-$* mutants, BS87 and HK82.

The second selected candidate was the *sbcC* gene bearing the mutation producing protein with a H333Y substitution (Table 2). The SbcC protein is a subunit of SbcCD exonuclease complex dealing with hairpin and palindromic sequences in DNA. Recently, the involvement of SbcCD exonuclease in DNA repair, especially in the repair of double strand breaks, was established. Additionally, the *sbcC* and *sbcD* together act as a suppressor of *recB*/*recC* mutations [28-32]. *Bacillus subtilis* cells devoid of *sbcC* gene are much more sensitive to mitomycin C and γ-irradiation than wt strain [33]. Our bioinformatic analysis did not confirm the H333 position as laying in the

region important for the protein activity, and this region does not seem to be highly conserved (data not shown). Additionally, the HK $sbcC^{wt}$ $nalA$ strain shows similar level of mutations as HK82 counterpart (Fig. 2).

Recently, it was revealed that other genes not directly linked to DNA repair, function in the cell response to DNA double strand breaks [34]. The $nuoC$, producing one of the subunits of NADH-ubiquinone oxidoreductase takes part in this pathway. These genes are under the control of RpoE transcription factor responsible for the activation of stress responses to e.g. heat shock and oxidative stress [35]. To test whether HK82 is deficient in the response controlled by RpoE, the sensitivity of this strain to 0.5% SDS and 0.55 – 1.1 mM EDTA was checked by a serial dilution drop test (data not shown). The tested strain did not show an impaired membrane-protein stress response, thus proving to exert properly functioning RpoE pathway being in agreement with observed similar levels of Rif^R mutants in different variants of $alkB^-$ strains treated with MMS.

It can be speculated that while particular mutations in $nalA$, $sbcC$ and/or $nuoC$ alone do not show any effect on the DNA metabolism, when present in the $alkB^-$ background may be a source of ssDNA fragments leading to the induction of two protective responses, SOS and Ada, after MMS treatment. This induction is at a much higher level in the HK82 strain expressing mutated AlkB than in cells exclusively deprived of the functional enzyme.

4. **Conclusions**

As a tools for exploring the impact of AlkB dioxygenases on the repair of alkylation lesions in nucleic acids, we have used two $E. coli$ $alkB$ mutants, HK82 and BS87. However, we have found several undesirable features of the former strain and shared this knowledge at several international conferences. Unfortunately, we were not able to present precise data on the subject. Only now we could prove that HK82 is not an exact derivative of the original AB1157 strain because it bears several additional mutations in its genome that may influence MMS-induced SOS and Ada responses giving almost out of

control high levels of MMS-induced mutations. Thus, we recommend using BS87 as a reference *alkB⁻* strain for AlkB studies.

Funding

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

1.   S. Dinglay, S.C. Trewick, T. Lindahl, B. Sedgwick, Defective processing of methylated single-stranded DNA by E. coli AlkB mutants, Genes Dev 14 (2000) 2097-2105.
2.   S.C. Trewick, T.F. Henshaw, R.P. Hausinger, T. Lindahl, B. Sedgwick, Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage, Nature 419 (2002) 174-178.
3.   P.O. Falnes, R.F. Johansen, E. Seeberg, AlkB-mediated oxidative demethylation reverses DNA damage in Escherichia coli, Nature 419 (2002) 178-182.
4.   D. Mielecki, D.L. Zugaj, A. Muszewska, J. Piwowarski, A. Chojnacka, M. Mielecki, J. Nieminuszczy, M. Grynberg, E. Grzesiuk, Novel AlkB dioxygenases--alternative models for in silico and in vivo studies, PLoS One 7 (2012) e30588.
5.   H. Kataoka, Y. Yamamoto, M. Sekiguchi, A new gene (alkB) of Escherichia coli that controls sensitivity to methyl methane sulfonate, J Bacteriol 153 (1983) 1301-1307.
6.   H. Kataoka, M. Sekiguchi, Molecular cloning and characterization of the alkB gene of Escherichia coli, Mol Gen Genet 198 (1985) 263-269.
7.   B. Sedgwick, Oxidation of methylhydrazines to mutagenic methylating derivatives and inducers of the adaptive response of Escherichia coli to alkylation damage, Cancer Res 52 (1992) 3693-3697.
8.   J. Nieminuszczy, A. Sikora, M. Wrzesinski, C. Janion, E. Grzesiuk, AlkB dioxygenase in preventing MMS-induced mutagenesis in Escherichia coli: effect of Pol V and AlkA proteins, DNA Repair (Amst) 5 (2006) 181-188.
9.   M.R. Volkert, D.C. Nguyen, K.C. Beard, Escherichia coli gene induction by alkylation treatment, Genetics 112 (1986) 11-26.
10.   D. Zdzalik, A. Domanska, P. Prorok, K. Kosicki, E. van den Born, P.O. Falnes, C.J. Rizzo, F.P. Guengerich, B. Tudek, Differential repair of etheno-DNA adducts by bacterial and human AlkB proteins, DNA Repair (Amst) 30 (2015) 1-10.

11. J.C. Delaney, J.M. Essigmann, Mutagenesis, genotoxicity, and repair of 1-methyladenine, 3-alkylcytosines, 1-methylguanine, and 3-methylthymine in alkB Escherichia coli, Proc Natl Acad Sci U S A 101 (2004) 14051-14056.

12. S.C. Chang, B.I. Fedeles, J. Wu, J.C. Delaney, D. Li, L. Zhao, P.P. Christov, E. Yau, V. Singh, M. Jost, C.L. Drennan, L.J. Marnett, C.J. Rizzo, S.S. Levine, F.P. Guengerich, J.M. Essigmann, Next-generation sequencing reveals the biological significance of the N(2),3-ethenoguanine lesion in vivo, Nucleic Acids Res 43 (2015) 5489-5500.

13. G. Shivange, N. Kodipelli, M. Monisha, R. Anindya, A role for Saccharomyces cerevisiae Tpa1 protein in direct alkylation repair, J Biol Chem 289 (2014) 35939-35952.

14. G. Shivange, N. Kodipelli, R. Anindya, 2-Hydrazinobenzothiazole-based etheno-adduct repair protocol (HERP): a method for quantitative determination of direct repair of etheno-bases, DNA Repair (Amst) 28 (2015) 8-13.

15. J.H. Miller, Cold Spring Harbor Laboratory, 1972

16. A. Sikora, D. Mielecki, A. Chojnacka, J. Nieminuszczy, M. Wrzesinski, E. Grzesiuk, Lethal and mutagenic properties of MMS-generated DNA lesions in Escherichia coli cells deficient in BER and AlkB-directed DNA repair, Mutagenesis 25 (2010) 139-147.

17. H.J. Vogel, D.M. Bonner, Acetylornithinase of Escherichia coli: partial purification and some properties, J Biol Chem 218 (1956) 97-106.

18. H. Shinagawa, T. Kato, T. Ise, K. Makino, A. Nakata, Cloning and characterization of the umu operon responsible for inducible mutagenesis in Escherichia coli, Gene 23 (1983) 167-174.

19. C. Janion, A. Sikora, A. Nowosielska, E. Grzesiuk, E. coli BW535, a triple mutant for the DNA repair genes xth, nth, and nfo, chronically induces the SOS response, Environ Mol Mutagen 41 (2003) 237-242.

20. N.D. Connell, Mycobacterium: isolation, maintenance, transformation, and mutant selection, Methods Cell Biol 45 (1994) 107-125.

21. J. Nieminuszczy, D. Mielecki, A. Sikora, M. Wrzesinski, A. Chojnacka, J. Krwawicz, C. Janion, E. Grzesiuk, Mutagenic potency of MMS-induced 1meA/3meC lesions in E. coli, Environ Mol Mutagen 50 (2009) 791-799.

22. A. Sikora, C. Janion, E. Grzesiuk, Mutagenesis, InTech, Rijeka, Croatia, 2012

23. D. Mielecki, E. Grzesiuk, Ada response - a strategy for repair of alkylated DNA in bacteria, FEMS Microbiol Lett 355 (2014) 1-11.

24. D. Mielecki, M. Wrzesinski, E. Grzesiuk, Inducible repair of alkylated DNA in microorganisms, Mutat Res Rev Mutat Res 763 (2015) 294-305.

25. C.J. Willmott, A. Maxwell, A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex, Antimicrob Agents Chemother 37 (1993) 126-127.

26. H. Yoshida, M. Bogaki, M. Nakamura, S. Nakamura, Quinolone resistance-determining region in the DNA gyrase gyrA gene of Escherichia coli, Antimicrob Agents Chemother 34 (1990) 1271-1272.

27. F.M. Barnard, A. Maxwell, Interaction between DNA gyrase and quinolones: effects of alanine mutations at GyrA subunit residues Ser(83) and Asp(87), Antimicrob Agents Chemother 45 (2001) 1994-2000.

28. K.A. Storvik, P.L. Foster, The SMC-like protein complex SbcCD enhances DNA polymerase IV-dependent spontaneous mutation in Escherichia coli, J Bacteriol 193 (2011) 660-669.

29. S. Aedo, Y.C. Tse-Dinh, SbcCD-mediated processing of covalent gyrase-DNA complex in Escherichia coli, Antimicrob Agents Chemother 57 (2013) 5116-5119.
30. J. Ramirez-Santos, V. Garcia-Mata, S. Poggio, L. Camarena, M.C. Gomez-Eichelmann, Role of single-strand DNA 3'-5' exonuclease ExoI and nuclease SbcCD in stationary-phase mutation in Escherichia coli K-12, Arch Microbiol 191 (2009) 185-190.
31. G.A. Cromie, D.R. Leach, Recombinational repair of chromosomal DNA double-strand breaks generated by a restriction endonuclease, Mol Microbiol 41 (2001) 873-883.
32. J.C. Connelly, E.S. de Leau, D.R. Leach, Nucleolytic processing of a protein-bound DNA end by the E. coli SbcCD (MR) complex, DNA Repair (Amst) 2 (2003) 795-807.
33. J. Mascarenhas, H. Sanchez, S. Tadesse, D. Kidane, M. Krisnamurthy, J.C. Alonso, P.L. Graumann, Bacillus subtilis SbcC protein plays an important role in DNA inter-strand cross-link repair, BMC Mol Biol 7 (2006) 20.
34. A.A. Al Mamun, M.J. Lombardo, C. Shee, A.M. Lisewski, C. Gonzalez, D. Lin, R.B. Nehring, C. Saint-Ruf, J.L. Gibson, R.L. Frisch, O. Lichtarge, P.J. Hastings, S.M. Rosenberg, Identity and function of a large gene network underlying mutagenic repair of DNA breaks, Science 338 (2012) 1344-1348.
35. S.E. Ades, I.L. Grigorova, C.A. Gross, Regulation of the alternative sigma factor sigma(E) during initiation, adaptation, and shutoff of the extracytoplasmic heat shock response in Escherichia coli, J Bacteriol 185 (2003) 2512-2519.
36. B.J. Bachmann, Derivations and genotypes of some mutant derivatives of Escherichia coli K-12. , in: (Eds.), Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, American Society for Microbiology, Washington, DC, 1987, pp. 1192-1219.
37. M. Singer, T.A. Baker, G. Schnitzler, S.M. Deischel, M. Goel, W. Dove, K.J. Jaacks, A.D. Grossman, J.W. Erickson, C.A. Gross, A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of Escherichia coli, Microbiol Rev 53 (1989) 1-24.
38. G. Churchward, D. Belin, Y. Nagamine, A pSC101-derived plasmid which shows no sequence homology to other commonly used cloning vectors, Gene 31 (1984) 165-171.

Table 1. *E. coli* strains and plasmids used in this study.

Strain/Relevant genotype	P1 donor	P1 acceptor	Comments/Relevant fenotype	Reference
AB1157	-	-	*thr-1, ara-14, leuB6, Δ(gpt-proA)62, lacY1, tsx-33, supE44, galK2, rac⁻, hisG4(Oc), rfbD1, mgl-51, rpsL31, kdgK51, xyl-5, mtl-1, argE3*	[36]

			(Oc), thi-1, qsr	
CAG12080	-	-	λ-, mhpC281::Tn10, rph-1; Tet^R	[37]
CAG12148	-	-	λ-, tsx-247::Tn10, rph-1; Tet^R	[37]
CAG12183	-	-	λ-, gyrA-0(Nal^R), zfa-3145::Tn10kan, rph-1; donor of Tn10 insertion; Kan^R	[37]
HK82	-	-	As AB1157 but alkB22 nalA; Nal^R	[5]
BS87	-	-	As AB1157 but alkB117; Amp^R	[7]
DM12	-	-	As AB1157 but alkB::kan; Kan^R	[4]
HK82 zfa-3145::Tn10kan	CAG12183	HK82	As HK82 but with Tn10kan inserted in the vicinity of alkB22; Kan^R	this study
AB1157 nalA	HK82	AB1157	alkB$^+$ Nal^R	
AB1157 nalA	HK82 zfa-3145::Tn10 kan	AB1157	alkB$^+$ Nal^R Kan^R	
AB1157 alkB22 nalA	HK82 zfa-3145::Tn10 kan	AB1157	alkB22 Nal^R Kan^R	
HK82 alkB$^+$ nalA	CAG12183	HK82	alkB$^+$ Nal^R Kan^R	
HK82 alkB117	BS87	HK82	alkB117 Amp^R	
HK82 alkB117 nalA	BS87	HK82	alkB117 Amp^R Nal^R	
HK82 alkB::kan	DM12	HK82	alkB::kan Kan^R	
HK82 alkB::kan nalA		HK82	alkB::kan Kan^R Nal^R	
BS87 nalA	HK82	BS87	alkB117 Amp^R Nal^R	
BS87 nalA	HK82 zfa-3145::Tn10 kan	BS87	alkB117 Amp^R Nal^R Kan^R	
BS87 alkB22 nalA	HK82	BS87	alkB22 Nal^R	
BS87 alkB22 nalA	HK82 zfa-3145::Tn10 kan	BS87	alkB22 Nal^R Kan^R	
HK82 sbcC^{wt}	CAG12080	HK82	alkB22 Nal^R Tet^R	
HK82 sbcC^{wt}	CAG12148	HK82	alkB22 Nal^R Tet^R	

Plasmid	Genotype	
pSK1002	*umuC::lacZ* fusion	[18]
pGB2	Spc^R, low-copy pSC101 derivative with mp8 polylinker	[38]
pMW1	*alkB* inserted into pGB2	[16]
pMW2	*ada*, *alkB*, and *alkA* inserted into pGB2	this study
pMW3	As pMW2 but *ada*⁻	

Table 2. Non-synonymous mutations identified in HK82 genome.

BS87 base	HK82 base	Impact	SNP %	Gene ID (Locus name)	Feature Name	DNA Change	Protein Change
G	A	Non synonymous	100.0	946759 (b2286)	NADH-ubiquinone oxidoreductase NuoC	c.637G>A	E213K
G	T	Non synonymous	100.0	946614 (b2231)	DNA gyrase subunit A GyrA	c.259G>T	D87Y
G	A	Non synonymous	100.0	946708 (b2212)	Alkylated DNA repair protein AlkB	c.397G>A	D133N
A	G	Non synonymous	100.0	945011 (b0054)	Outer membrane protein Imp	c.1808A>G	M603T
A	C	Non synonymous	95.7	948243 (b3733)	ATP synthase gamma chain AtpG	c.194A>C	H65P
A	T	Non synonymous	96.2	949002 (b1211)	Peptide chain release factor 1 PrfA	c.401A>T	M134K
C	T	Non synonymous	100.0	949076 (b0397)	Exonuclease SbcC	c.997C>T	H333Y
T	-	Frameshift	90.0	948449 (b0022)	InsA-1	c.115T>-	Q38fs
T	C		52.2		5S rRNA		

Figure legend:

Fig. 1. Diagram representing the construction procedures for *E. coli* HK82 and BS87 strains. The basis for HK82 construction was chemical mutagenesis that allowed the isolation of the spontaneous *alkB22* mutant, the allele was
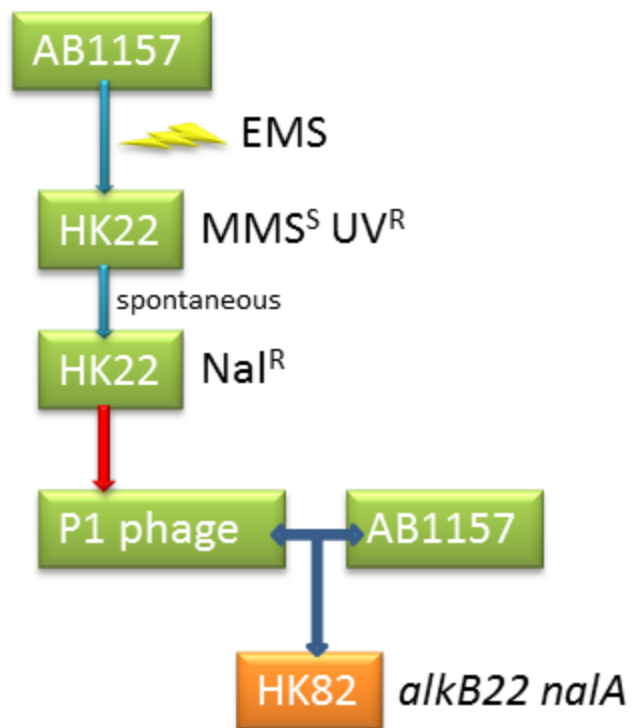
transferred to AB1157 strain by P1 trasnduction with *nalA* as a marker (A). Contrariwise, the BS87 was constructed owing to transposon insertional mutangenesis of *alkB* allele present on plasmid, subsequently recombined with *polA^{ts}* strain, and transduced to AB1157 (B).

Fig. 2. Frequency of MMS-induced mutagenesis measured by Arg$^+$ reversion and Rif^R mutation in *E. coli* AB1157, HK82, BS87, and their derivatives.
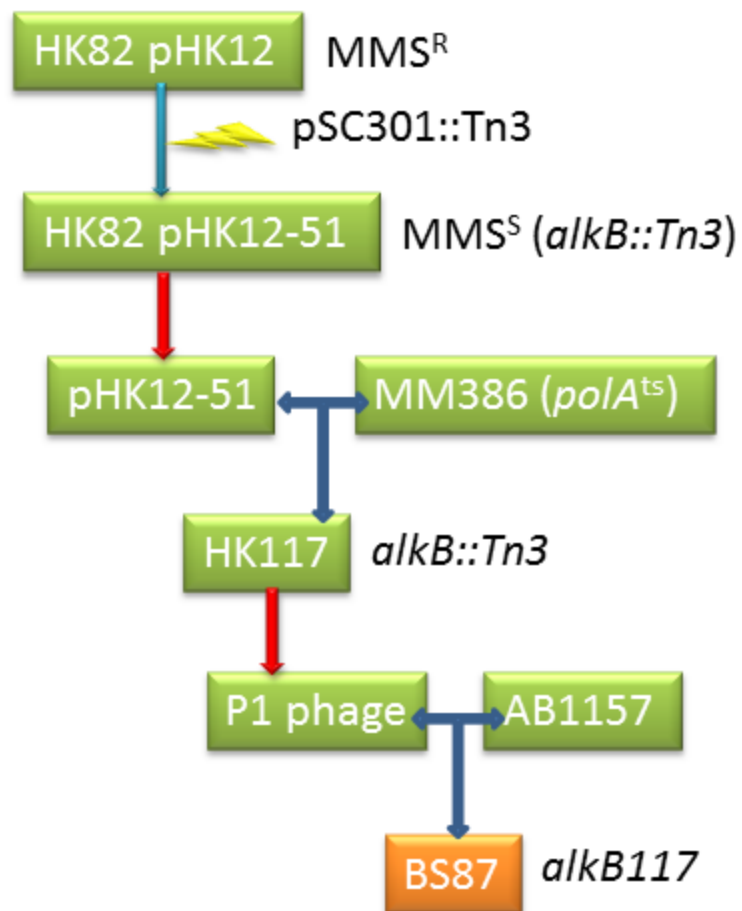
Fig. 3. Complementations studies of HK82 and BS87 strains with selected Ada response components bearing on plasmids: pMW1 – *alkB*, pMW2 – *alkB*, *alkA*, and *ada*, and pMW3 – *alkB*, *alkA*, and mutated *ada*.
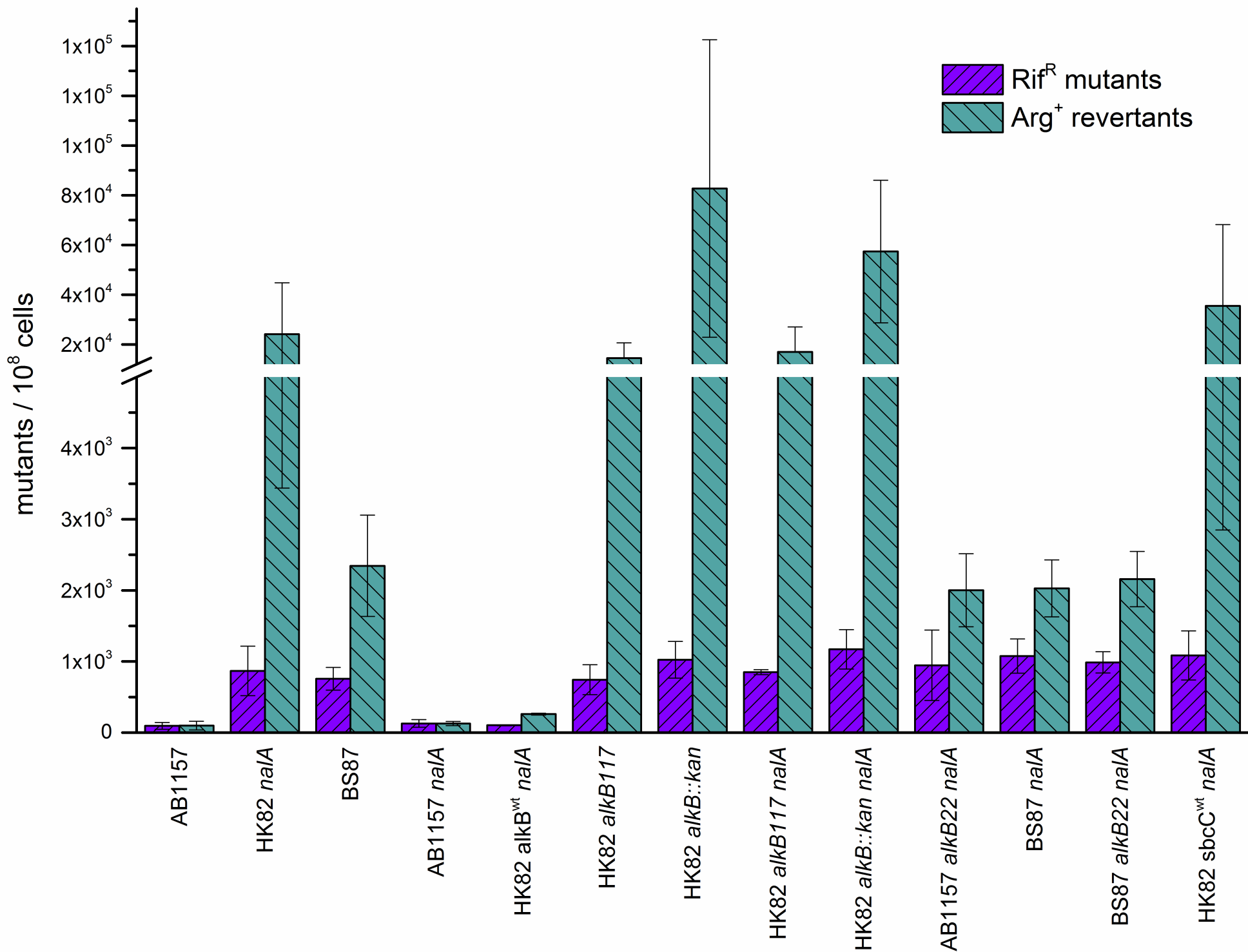
Fig. 4. The SOS induction in MMS-treated AB1157, HK82, and BS87 strains and their derivatives based on β-galactosidase expression from a *umuC*::*lacZ* fusion.

A: HK82
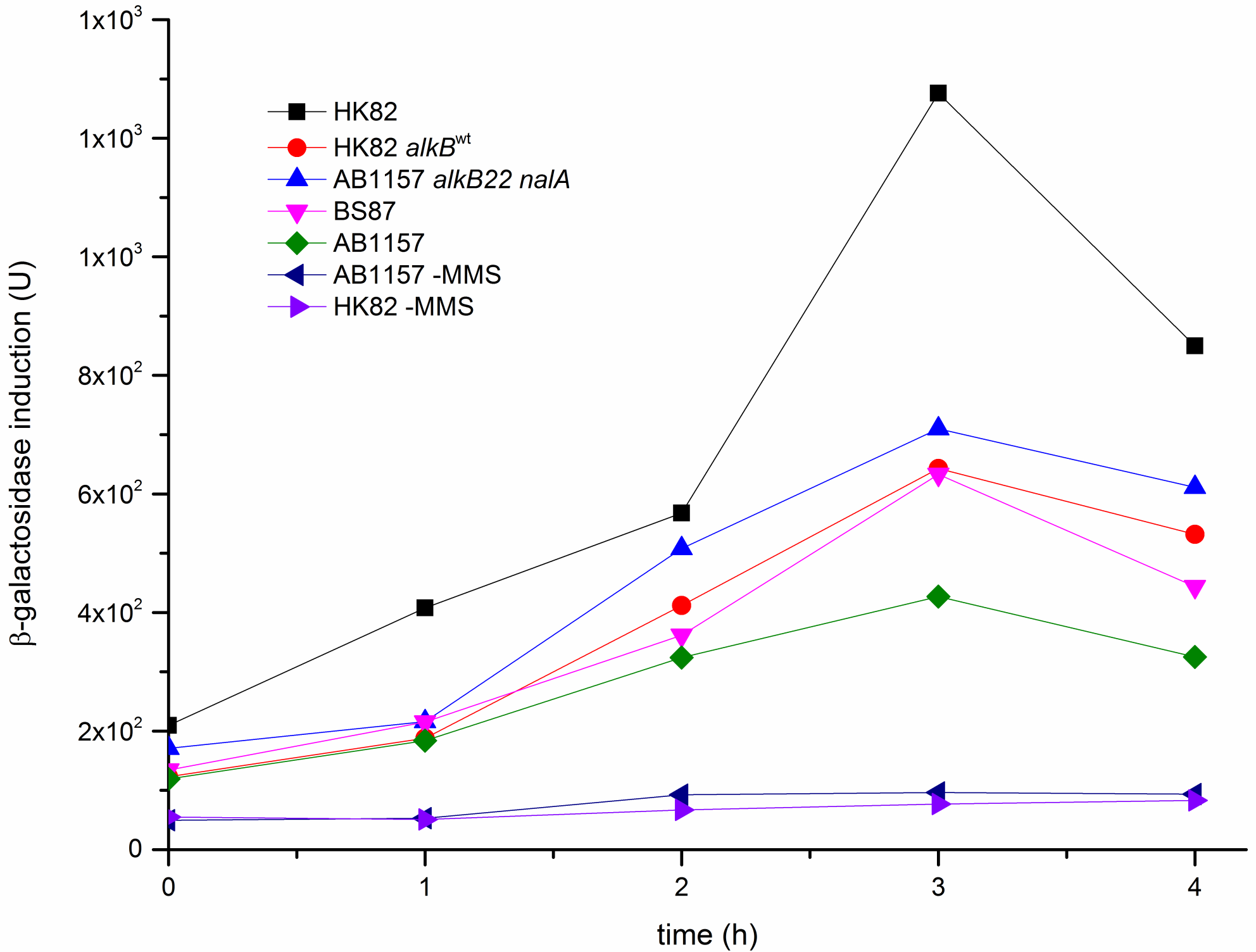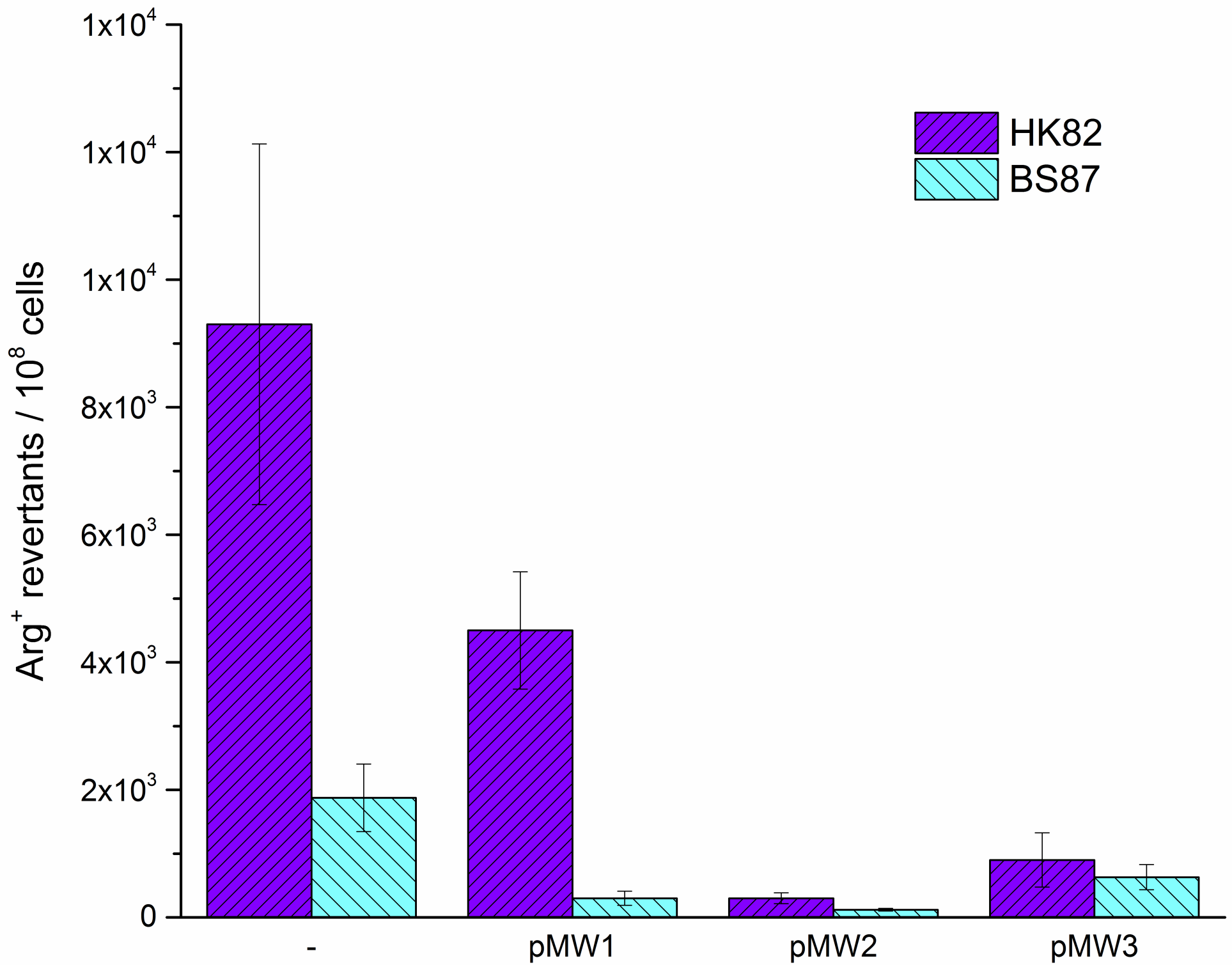
AB1157
↓ *EMS*
HK22 — MMS^S UV^R
↓ spontaneous
HK22 — Nal^R
↓
P1 phage ←→ AB1157
↓
HK82 — *alkB22 nalA*

B: BS87

HK82 pHK12 — MMS^R
↓ *pSC301::Tn3*
HK82 pHK12-51 — MMS^S (*alkB::Tn3*)
↓
pHK12-51 ←→ MM386 (*polA^{ts}*)
↓
HK117 — *alkB::Tn3*
↓
P1 phage ←→ AB1157
↓
BS87 — *alkB117*

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Elżbieta Grzesiuk

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

> The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Print name

GRZESIUK

ELSEVIER

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Damian Mielecki

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

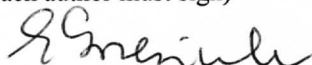> The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Damian Mielecki

Print name

DAMIAN MIELECKI

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.

Author name: Michał Wrzesiński

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Print name

M Wrzesiński   MICHAŁ WRZESIŃSKI

ELSEVIER

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Jadwiga Nieminuszczy

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

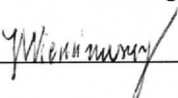The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

This work was funded by the National Science Centre, Poland, grant number UMO-2012/05/B/NZ1/00693.

Signature (a scanned signature is acceptable, but each author must sign)

_____

Print name

NIEMINUSZCZY

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Robert Gromadka

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Print name

GROMADKA

ELSEVIER

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Karolina Żuchniewicz

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Karolina Żuchniewicz

Print name

KAROLINA ŻUCHNIEWICZ

ELSEVIER

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Anna Sikora

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

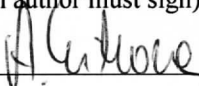The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Print name

ANNA SIKORA

DNA Repair
Conflict of Interest Policy

Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies.	Author name: Anna Detman

Declarations

DNA Repair requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

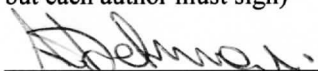The authors declare that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

Signature (a scanned signature is acceptable, but each author must sign)

Print name

ANNA DETMAN

Dear Editor,

Enclosed, please find the manuscript entitled „Evaluation of the *E. coli* HK82 and BS87 strains as tools for AlkB studies", authors: Mielecki D, Sikora A, Wrzesiński M, Nieminuszczy J, Detman A, Żuchniewicz K, Gromadka R, Grzesiuk E.

In the light of intensive studies on AlkB dioxygenases, it is of the great importance to have precisely defined mutant in *alkB* gene. Among two *E. coli* *alkB*⁻ strains, Kataoka's HK82 and Sedgwick's BS87, the former is the most frequently used even in papers published this year. Our team, led by prof. Celina Janion, have observed unlike features of HK82 e.g. extremely high and variable MMS-induced mutagenesis, but only now we get tools to prove that HK82 contains several additional mutations in its genome, thus is not exact AB1157 derivative and shows other features which disqualify it as a reference strain for AlkB studies. Consequently, BS87 is a proper AB1157 derivative and should be used instead.

I hope you find this work important for scientific community working on DNA repair, specifically on AlkB dioxygenases, and suitable for publication in DNA Repair.

Sincerely,

Prof. Elżbieta Grzesiuk.