UDC 575.224.46:579.842.14
ISSN 0352-9193

original scientific paper

The Phenomenon of the *Uvr*$^+$ Dependent Mutagenesis in *Salmonella typhimurium*

Pojava *uvr*$^+$ ovisne mutageneze u bakteriji *Salmonella typhimurium*

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Received: October 13, 1994
Accepted: February 28, 1995

Summary

The ***Salmonella typhimurium*** *phenotype is comparable to the* ***Escherichia coli ada*** *mutant in having a low but significant constitutively expressed cellular activity of enzyme O^6-methylguanine DNA-methyltransferase II (MT II) for repair of promutagen lesions O^6-methylguanine and O^4-methylthymine in DNA.*

The role of nucleotide excision repair in the mutagenicity of the monofunctional alkylating agents ***N****-methyl-****N****'-nitro-****N****-nitrosoguanidine (MNNG), methyl methanesulfonate (MMS),* ***N****-ethyl-****N****'-nitro-****N****-nitrosoguanidine (ENNG),* ***N****-ethyl-****N****-nitrosourea (ENU),* ***N****-propyl-****N****'-nitro-****N****-nitrosoguanidine (PNNG) and* ***N****-butyl-****N****'-nitro-****N****-nitrosoguanidine (BNNG) in* ***S. typhimurium*** *was examined. Comparing the* ***S. typhimurium uvrB*** *mutant with the nucleotide excision repair proficient strain it has been noticed that the latter is mutagenized more efficiently at low doses and less efficiently at high doses of methylating (MMS, MNNG) or ethylating (ENU, ENNG) agents. No* ***uvr***$^+$ *dependent mutagenesis has been noticed in* ***S. typhimurium*** *after treatment by propylating (PNNG) and butylating (BNNG) agents. Our results suggest that the* ***uvr***$^+$ *dependent mutagenesis is probably a consequence of the specific competition between enzyme activity involved in nucleotide excision repair enzymes and MT II which are responsible for the overall mutagenic effects of low doses of methylating and ethylating agent in* ***S. typhimurium.***

Sažetak

Po fenotipu bakterija ***Salmonella typhimurium*** *može se usporediti s bakterijom* ***Escherichia coli*** *mutantom* ***ada*** *jer imaju malu, ali značajnu količinu konstitutivno ekspresirane aktivnosti enzima O^6-metilgvanin DNA-metiltransferaze II (MT II), koja popravlja predmutagena oštećenja u DNA O^6-metilgvanin i O^4-metiltimin.*

Ispitana je uloga nukleotidnog ekscizijskog popravka u ***S. typhimurium*** *nakon mutagenog djelovanja monofunkcionalnih alkilirajućih agensa:* ***N****-metil-****N****'-nitro-****N****-nitrozogvanidina (MNNG), metil metansulfonata (MMS),* ***N****-etil-****N****'-nitro-****N****-nitrozogvanidina (ENNG),* ***N****-metil-****N****-nitrozouree (ENU),* ***N****-propil-****N****'-nitro-****N****-nitrozogvanidina (PNNG) i* ***N****-butil-****N****'-nitro-****N****-nitrozogvanidina (BNNG). Ustanovljeno je da male doze metilirajućih (MMS, MNNG) i etilirajućih agensa (ENU, ENNG) u* ***S. typhimurium*** *uzrokuju veću mutabilnost soja s ispravnim nukleotidnim ekscizijskim popravkom negoli u mutantu koji nosi deleciju u genu* ***uvrB. Uvr***$^+$ *ovisna mutageneza nije zapažena u* ***S. typhimurium*** *nakon djelovanja propilirajućih (PNNG) i butilirajućih (BNNG) agensa. Naši su rezultati pokazali da je* ***uvr***$^+$ *ovisna mutageneza vjerojatno posljedica natjecanja za oštećenje između dva stanična popravka, tj. enzima nukleotidnog ekscizijskog popravka i MT II.*

Introduction

The maintenance of the integrity of the information encoded in every genome is crucial to the survival of cells and organisms, and to the continuation of the species from generation to generation. The genome of every organism is continuously and relentlessly exposed to DNA damage, not just from agents in the environment but also from naturally occurring intermediary metabolites within each cell.

Presented at the 2nd Croatian Congress of food Technologists, Biotechnologists and Nutritionists, Zagreb, June 15–17, 1994

The repair of DNA damage protects cells from mutations, chromosome damages and cell death. Exposure of *E. coli* to sublethal levels of monofunctional methylating agents induces an adaptive response which protects the cells from both the toxic and mutagenic effect of alkylating agents *(1,2)*. In addition to the adaptive response, *E. coli* constitutively expresses three other repair functions that act on DNA alkylation damage. These are the Tag 3MeA DNA glycosylase *(3)*, the Ogt MTase *(4)*, and the nucleotide excision repair pathway for the repair of DNA alkylation damage *(5)*.

Thus a number of overlapping DNA repair functions protect *E. coli* against alkylation: proteins Tag and Ogt provide resistance at very low doses alkylation; adaptive response is induced to provide resistance at higher levels of chronic exposure; and nucleotide excision repair only plays a role at very high levels of DNA alkylation damage when the other pathways have been saturated *(6)*.

The UvrABC enzyme complex is responsible for the initial steps in nucleotide excision repair in *E. coli (7)*. Repair by the UvrABC proteins shows a broad specificity for many types of DNA damage induced by a variety of chemical agents and UV irradiation. These proteins are involved in the recognition of helical distortion in the DNA and incision of the damaged strand at both sides of the lesion. The incision reaction produces a 12–13 mer oligonucleotide containing the damaged residue(s) *(8)*.

S. typhimurium and *E. coli* are members of two related bacterial genera. The homology of their genomes is approximately 80 %. Their genetic maps are almost completely correspondent.

An adaptive response to alkylating agents has been found in many bacterial species but cannot be efficiently induced in *S. typhimurium* cells. The knowledge about the impact of DNA repair systems on mutation induction in *S. typhimurium* cells is limited, although many derivatives of this bacterium have widely been used in Ames test for the detection of environmental mutagens and carcinogens.

Alkylating agents are toxic, mutagenic and carcinogenic, and they represent one of the most potent classes of chemical DNA damaging agents present in our environment *(9–11)*. Alkylating agents such as the *N*-alkyl-*N'*-nitro-*N*-nitrosoguanidines are among the most widely used laboratory mutagens. They decompose within a cell to produce an alkyldiazonium ion which reacts with nucleophilic sites in DNA to produce a variety of N-alkylated and O-alkylated nucleotides *(12)*. The N-nitroso compounds produce GC to AT and TA to GC transition mutations because they alkylate O^6 of guanine and O^4 of thymine. These mutations occur because O^6-alkylguanine forms a stable mispair with thymine while O^4-alkylthymine forms a mispair with guanine.

In order to determine the influence of nucleotide excision repair on alkylating DNA, we are studying genetic effects of monofunctional alkylating agents in *S. typhimurium* nucleotide excision repair proficient and deficient cells. We have compared mutant induction by MMS, ENU, MNNG, ENNG, PNNG, BNNG over a wide dose range.

Materials and Methods

Bacterial strains

S. typhimurium LT2 strain G46 (*his*G46) and its isogenic nucleotide excision repair-deficient derivative TA1950 (*his*G46, *uvrB-bio-chlA) (13)* were obtained from Prof. B. N. Ames (Berkeley, CA). *His*G46 is a mis-sense mutation in the *S. typhimurium his*G46 gene, which replaces a CTC (leucine) with a CCC (proline) codon. Any base change at the first base, and GC-AT or GC-TA base changes at the second base of the codon give rise to a *his*$^+$ phenotype.

Chemicals tested for mutagenesis

PNNG and BNNG were purchased from Aldrich Chemical Co, ENNG and ENU from Pfaltz and Bauer, MMS from Eastman Kodak, and MNNG from Koch and Light. These alkylating agents were dissolved in phosphate-citrate buffer (PCB), pH = 6, immediately before the experiment.

Chemicals, media and buffers

The origin of chemicals used was as follows: bacto nutrient broth, bacto agar (Difco, American Scientific Products, MacGraw Park IL).

Media and buffers used were of the following composition: nutrient medium (nutrient broth 25 g/L); nutrient plates (for 1 L: nutrient broth 8 g, NaCl 5 g, 15 g agar); top agar (for 1 L: minimal essential salts, 0.5 g glucose, 1.2 mg biotin, 6 g agar); minimal essential salts (0.02 M $MgSO_47H_2O$, 0.24 M citric acid, 1.44 M K_2HPO_4, 0.42 M $NaNH_4HPO_44H_2O$); minimal medium *(14)* (for 1 L: minimal essential salts, 0.5 g glucose, 3 mg biotin); minimal plates (minimal media with 15 g/L agar); phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) phosphate-citrate buffer (PCB) (0.2 M Na_2HPO_4, 0.1 M citric acid).

Experimental protocol

The cell cultures in nutrient broth were grown with shaking to a concentration between 6 and 8×10^8 bacteria per mL. Then they were centrifuged, resuspended and concentrated 10 times in phosphate buffer (PBS) pH = 7, because of very low spontaneous frequency of revertants. The cell suspension of each strain was distributed in reaction tubes and the cold agent solution of various concentrations was added. Mutagen assay was carried out at 37 °C under aeration for one hour. The reaction was stopped by adding iced PBS pH = 6.5. The cells were washed and resuspended to the initial volume in PBS pH = 7. This is the modification of the Salmonella histidine reversion assay *(15)*. After 48 h at 37 °C the number of *his*$^+$ revertants was counted. The colonies on nutrient agar were counted after 1 day incubation at 37 °C. The mutation frequency was calculated as the number of *his*$^+$ revertants per 10^7 surviving cells.

Experiments were carried out at least five times.

Results and Discussion

The impact of the nucleotide excision repair on mutation induction in *S. typhimurium* LT2 after treatment with monofunctional alkylating agents MMS, MNNG, ENU, ENNG, PNNG and BNNG was investigated. The response to alkylating agents treatment was measured as the induction of *his*$^+$ revertants in the wild-type and nucleotide excision repair-deficient strains. The genetic system *his*G46 of *S.typhimurium* tester strains used in these experiments has a high specificity for the transition mutation GC to AT.

Mutant induction as a function of exposure concentrations of MMS and ENU is shown in Figs. 1 and 2. Mutant induction was not linearly related to exposure concentrations. Our results suggest that mutagenesis induced by methylating (MMS) and ethylating (ENU) agents in *S.typhimurium* strains is typically biphasic (Figs. 1 and 2). The mutagenicity of MMS is comparable to its ethyl analog EMS effects previously observed under the same experimental conditions *(16)*. The doses of MMS which were used were rather high, from 6 to 60 mM (Fig. 1), compared with other methylating agent (MNNG). The low level of mutagenic O-alkylation in DNA produced by MMS can account for the low mutagenic potential of this agent. We found previously that the mutagenic potential increased in the following order: MMS<ENU<ENNG<MNNG *(17)*. In addition, MMS mutagenesis is partially dependent on the UmuDC functions *(18)* and therefore the weak mutagenic response in *S. typhimurium* may reflect the weak UmuDC-like activity in this organism *(19)*. The influence of nucleotide excision repair was observed at low exposure concentrations. Although the uvrB strain was mutagenized to a greater extent than the *uvr*$^+$ strain at higher doses of MMS and ENU, it was more resistant to the mutagenic effect of MMS and ENU at concentrations below 24 mM and 0.8 mM, respectively (Figs. 1 and 2). The *uvr*$^+$ dependent mutagenesis previously described for low concentrations of EMS *(16)*, was observed for MMS and ENU (Figs. 1 and 2), as well as for MNNG and ENNG up to concentrations of 5 mM and 30 mM, respectively (Table 1).

Guttenplan *(20)* reported greater sensitivity of Salmonella strain TA1975 *(uvr*$^+$*)* compared to TA1535 *(uvrB)* to mutant induction by low doses of ethyl nitrosourea. Reports of the role of nucleotide excision repair in alkylation mutagenesis in *E. coli* are ambiguous. Several scientists have presented data on the involvement of nucleotide excision repair in the avoidance of mutant induction by EMS *(21,22)*. It was also found that the removal of O^6-ethylguanine from the DNA of *E. coli* cells treated with *N*-ethyl-*N*-nitrosourea was much slower in the *uvrA* mutant than in the wild-type strain *(23)*. On the other hand, there was no evidence that a *uvr*$^+$ dependent nucleotide excision repair can act on O^6-methylguanine *(24)*. Todd and Schendel *(25)* did not observe differences in sensitivity of *uvr*$^+$ and *uvrA* strains of *E. coli* to mutant

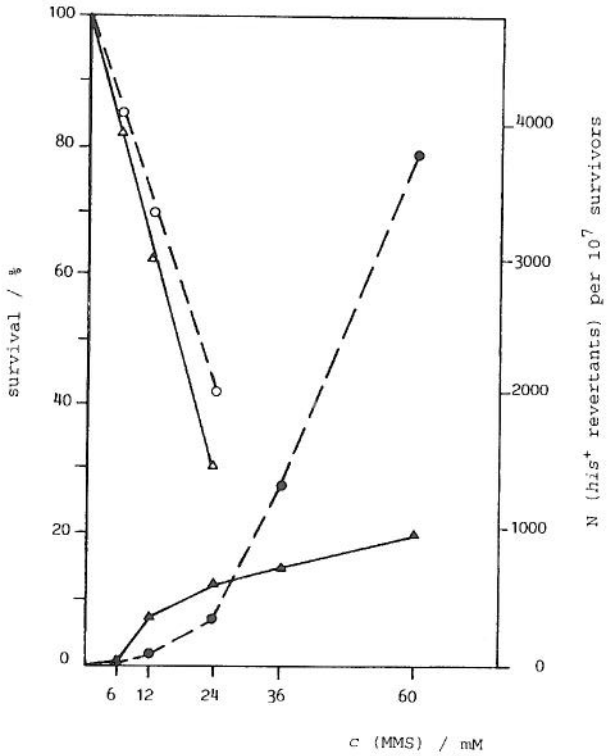

Fig. 1. Survival and mutation induction in *S. typhimurium* strain G46 (Δ—Δ, ▲—▲) compared with TA1950 (O– –O, ● – – ●) after 1 h exposure to MMS
Slika 1. Preživljavanje i indukcija mutacija u *S. typhimurium* soj G46 (Δ—Δ, ▲—▲) uspoređeno sa sojem TA1950 (O– –O, ● – – ●) nakon jednosatnog izlaganja MMS-u

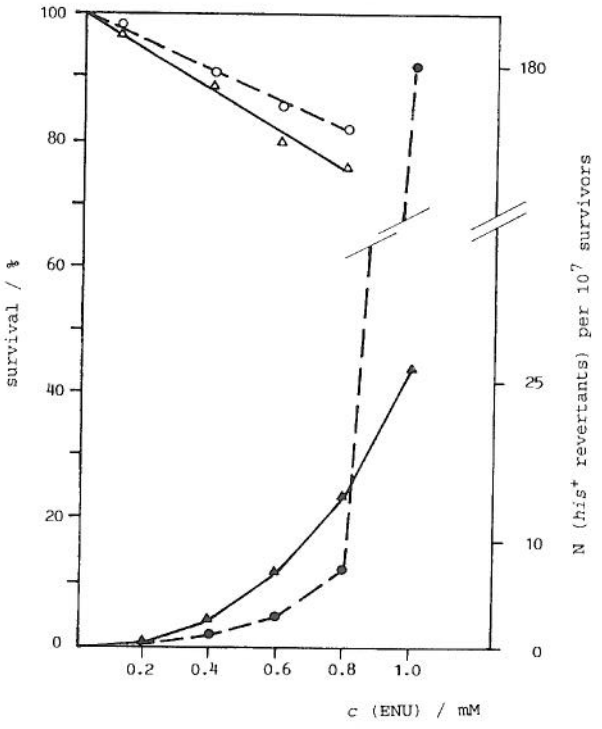

Fig. 2. Survival and mutation induction in *S. typhimurium* strain G46 (Δ—Δ, ▲—▲) compared with TA1950 (O– –O, ● – – ●) after 1 h exposure to ENU
Slika 2. Preživljavanje i indukcija mutacija u *S. typhimurium* soj G46 (Δ—Δ, ▲—▲) uspoređeno sa sojem TA1950 (O– –O, ● – – ●) nakon jednosatnog izlaganja ENU

induction by *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine. Garner et al. *(26)* obtained similar reversion rates in *uvr*$^+$ and *uvrA* strains of *E. coli* WP2 exposed to alkyl nitrosoureas. A study of alkylation mutagenesis using reversion of ochre mutations would give a somewhat distorted picture of the presence of alkylating agents for GC to AT transitions *(27)*.

In a previous study, Matijašević and Zeiger *(16)* determined the relationship between the EMS exposure concentrations and the actual dose to DNA (total ethylation of DNA). They reported that the differences in mutant induction between G46 *(uvr*$^+$*)* and TA1950 *(uvrB)* strains of *S. typhimurium* are not due to differential cell uptake or DNA binding of EMS at either low or high concentrations.

The adaptive response to alkylating damage has been demonstrated in several enterobacterial species *(28)*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Micrococcus luteus*, *Streptomyces frediae* *(29)*. Vaughan and Sedgwick *(28)* suggested that the weak induction of the adaptive response in *S. typhimurium* results from poor transcriptional activation by the self-methylated Ada protein. Thus phenotypicaly *S. typhimurium* is a naturally occurring *ada* mutant. The second MT, the Ogt protein, repairs O^6-methylguanine and O^4-methylthymine. The amino acid sequence of this 19-kDa protein has homology with the C-terminal domain of Ada protein. The expression of the ogt gene is constitutive *(30)*. Rebeck et al. *(31)* identified an active 19-kDa MT in unadapted *S. typhimurium* cells.

The low sensitivity range of *uvrB* cells corresponds to the limited capacity of Ogt-like protein in *S. typhimurium* to remove O^6-methylguanine and O^4-methylthymine, and the high sensitivity range corresponds to its accumulation in DNA after saturation of this repair process, when nucleotide excision repair pathway plays a major role in the repair of this highly mutagenic DNA lesion.

The nucleotide excision repair acts relatively slowly but can ultimately repair many lesions because, as a classical enzyme, it is not consumed in the repair process *(32)*. The UvrABC system repairs a wide variety of bulky (helix distorting) damage, which includes alkyl chain DNA products *(33)*. Kohda et al. *(34)* reported that an increase in alkyl chain length is accompanied by the increased involvement of UvrABC excinuclease.

PNNG and BNNG react with DNA like other monofunctional alkylating agents, and are capable of forming a variety of adducts at all oxygen and most nitrogen positions *(35)*.

Table 1 shows the induced mutant frequency in both *uvr*$^+$ and *uvrB* strains after treatment of MNNG, ENNG, PNNG and BNNG. The induced mutant frequency represents the difference between *his*$^+$ revertant frequency and spontaneous *his*$^+$ frequency. The induced mutagenesis

Table 1. The induction of *his*$^+$ revertants and survival in *S. typhimurium* strains G46 *(uvr*$^+$*)* and TA1950 *(uvrB)* after 1 h exposure to MNNG, ENNG, PNNG, and BNNG
Tablica 1. Indukcija revertanata *his*$^+$ i preživljavanje u *S. typhimurium* u sojevima G46 *(uvr*$^+$*)* i TA1950 *(uvrB)* nakon jednosatnog izlaganja MNNG, ENNG, PNNG i BNNG

Agent Agens	c/μM	Induced *his*$^+$ revertant frequency ($\times 10^{-7}$) mean ± S.D. Inducirana učestalost revertanata *his*$^+$ ($\times 10^{-7}$) srednja vrijednost ± S.D.			
		[a]G46 *(uvr*$^+$*)* [a]G46 *(uvr*$^+$*)*	Survival/% Preživ./%	[b]TA1950 *(uvrB)* [b]TA1950 *(uvrB)*	Survival/% Preživ./%
MNNG	0.5	0.06 ± 0.05	100	0.04 ± 0.02	100
	2.0	6.99 ± 0.7	93	0.47 ± 0.1	96
	5.0	690.71 ± 29.9	91	390.64 ± 8.5	91
	10.0	3384.81 ± 272.0	91	4723.90 ± 332.3	88
	15.0	4062.44 ± 502.3	89	13213.71 ± 643.1	81
ENNG	10	0.56 ± 0.04	100	0.05 ± 0.03	100
	15	3.92 ± 0.2	93	0.06 ± 0.02	98
	20	17.17 ± 3.5	91	0.57 ± 0.1	95
	30	30.71 ± 5.5	87	6.86 ± 1.1	93
	40	96.93 ± 14.6	86	203.59 ± 19.0	88
	60	170.46 ± 20.3	61	1965.78 ± 54.3	82
	80	326.09 ± 25.0	53	6440.31 ± 351.0	47
PNNG	10	0.04 ± 0.01	84	13.43 ± 2.3	94
	25	0.19 ± 0.1	82	39.61 ± 3.4	94
	50	1.77 ± 0.3	79	73.52 ± 11.1	91
	100	9.79 ± 0.5	72	184.77 ± 15.1	85
BNNG	10	1.74 ± 0.1	94	14.83 ± 0.4	91
	20	2.54 ± 0.6	94	30.99 ± 3.9	90
	50	9.11 ± 2.5	94	62.34 ± 7.0	82
	100	12.68 ± 1.5	90	158.36 ± 23.1	80

[a] spontaneous *his*$^+$ revertant frequency for G46, 0.07×10^{-7}
[a] spontana učestalost retromutanata *his*$^+$ za G46, 0.07×10^{-7}
[b] spontaneous *his*$^+$ revertant frequency for TA1950, 0.8×10^{-7}
[b] spontana učestalost retromutanata *his*$^+$ za TA1950, 0.8×10^{-7}

is significantly higher in *uvrB* strain than in wild type strain exposed to PNNG and BNNG within the range from 10 to 100 μM (Table 1). Thus damages caused by the influence of these agents are efficiently removed by the UvrABC system indicating that propyl and butyl adducts are not substrates for MT II.

In *S. typhimurium* LT2 the nucleotide excision repair influences the induced mutagenesis by methylating (MMS, MNNG) and ethylating (ENU, ENNG) agents in dose dependent manner. The presence of functional UvrABC system increases resistance to mutagenesis at high level of DNA alkylation. On the contrary, mutagenesis induced by low doses of MNNG, MMS, ENU, and ENNG is *uvr*$^+$ dependent (Figs. 1 and 2, Table 1). The UvrABC pathway may cooperate with or antagonize other repair pathways, especially following different levels of DNA damage (36). Thus, the higher mutability of *uvr* proficient strain by low doses of the mentioned mutagen could be the consequence of the competition for the lesions between nucleotide excision enzyme complex and constitutively present MT II. It is supported by the fact that above certain high doses (MMS, MNNG, ENU, ENNG) or the whole range (PNNG, BNNG) of examined agents the *uvr*$^+$ dependent mutagenesis was not noticed; presumably because the constitutive level of MT II was consumed. Pienkowska et al. (37) have suggested that UvrABC excision repair and constitutive MT II play a cooperative role in the removal of DNA lesions caused by EMS at lower doses.

Figs. 1 and 2 demonstrate as well the toxic effects of MMS and ENU for low concentration of examined agents. MMS was very toxic and was about equally so in both *uvr*$^+$ and *uvrB* cells. Survival was high, within the range of *uvr*$^+$ dependent mutagenesis, for both used strains after treatment by ENU (Fig. 2) as well as by MNNG and ENNG (Table 1).

It would be of interest to learn more about the nature of the low dose effect noted. This research is of great importance because of the impact of low doses on human health and environment as well as the general knowledge about cell metabolism.

Acknowledgment

This work has been supported by the Ministry of Science, Technology and Informatics, Republic of Croatia, No. 1-08- 101.

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