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SPECIFIC FOR DNA DAMAGES *GFP* MICROBIAL BIOSENSOR AS A TOOL FOR GENOTOXIC ACTION ASSESSMENT OF ENVIRONMENTAL POLLUTION

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Abstract: In the presented paper, autofluorescent reporter of *Escherichia coli* K-12 *recA::gfpmut2* strain, which contained a plasmid-borne transcriptional fusion between DNA-damage inducible *recA* promoter involved in the SOS regulon response and fast folding GFP variant reporter gene-*gfpmut2*, have been used. GFP-based bacterial biosensors allowed the detection of bacterial cells response to selected tested genotoxic compounds such as mitomycin C (MMC), actinomycin D, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and formaldehyde (CH_2O). Experiment indicated that *E. coli* K-12 *recA::gfpmut2* biosensor strain is more specific and sensitive for especially two genotoxins: actinomycin D and MNNG and with very low response to other agents. So it was concluded that for formaldehyde and MMC *E. coli* K-12 *recA::gfpmut2* genetic system is disqualified for genotoxicity screening.

Key words: DNA damage, genotoxicity, *recA* promoter, SOS response.

1. Introduction

Contamination of environment with chemical compounds, originating from the industralisation and technological development, connected with widespread use of petroleum product and hazardous substances, mainly toxic compounds is highly toxic for natural ecosystems, in particular for public health. The hazards of mutagenic and carcinogenic effects connected with increasing levels of environmental pollution on living organisms, including human health requires specific, sensitive, rapid and effective tests for monitoring the presence of genotoxic agents in surface, subsurface water, soil, sediments, sewage, air and food products (Hansen and Sorensen, 2001; Stiner and Halverson, 2002; Belkin, 2003; Gu et al., 2004).

There are some conventional methods for toxicity assessment of environmental pollutants which rely mainly on extraction and chromathography, but these analytical techniques, although highly precise, suffer from the disadvantages of high cost, time-consuming or the need for trained personnel and all these methods are mostly laboratory bound. The assessment of mutagenic and carcinogenic ability of chemicals mainly are based on biological tests with using of living microorganisms and higher organisms (Bongaerts et. al., 2002; Casavant et al., 2003).

In addition to the classic Ames tests for measurement of mutagenicity and genotoxicity of chemicals a variety of tests have been developed with application of different promoters-reporter genes fusions which are mainly hosted by either *E. coli* (SOS chromotest) or *Salmonella* species (SOS *umu test*). Such promoters in fusion with a reporter gene- *lacZ* (*β*-galactosidase) for genotox biosensor construct, including promoters of the SOS response genes: *recA*, *umuC*, *sulA* from SOS regulon. There are some advantages in application of biosensors in comparison to the classical reverse mutation Ames tests. Firstly, the carcinogenic nature of a compound earlier was relied on the Ames test. Nowadays as a consequence of molecular genetics development it is possible to obtain biosensing cells which are more sensitive, faster and capable of classifying a compound on the basis of the manner in which DNA is damaged and there are not limited in the chemical make-up of the sample, as was the Ames test. Additionally, with the use of reporter genes it is possible to apply biosensors *in-situ*, that was impossible for the Ames test (Gu et al., 2004).

A microbial biosensors is an analytical device that couples microorganisms with a transducer to enable rapid, accurate and sensitive detection of target analytes in fields as diverse as medicine, environmental monitoring, defense, food processing and safety. Recently, genetically engineered microorganisms based on fusing of the *gfp*, *lux* or *lacZ* gene reporters to an inducible gene promoter have

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been used to developed biosensors for various environmental application, genotoxicity and bioavailability assessment of different compounds, for example: detecting toluene and related chemicals, SOS-inducing activity of genotoxic compounds, *N*-acyl homoserine lactones in soil, measuring water availability in microbial habitat, monitoring cell populations, (Kostrzyńska et. al., 2002; Lee et al., 2005; Lei et al., 2006; Rogers, 2006). Expression of reporter genes such as variants of *gfp* in transformed cells, can effectively used to reveal cellular and molecular changes associated with cancer, for example neoplasia in vivo (Contag, 2000). Recently, bioluminescent biosensors use *lux*, *luc* or *gfp* genes have been developed to detect a variety of chemicals, genotoxic agents and factors, which are responsible for DNA damage, oxidative damage or cell growth inhibition (Errampalli et al., 1999; Kim and Gu, 2003; Vollmer and Van Dyk, 2004).

These bacterial biosensors are based on analysis of the intensity of reporter gene expression, typically by creating transcriptional fusion between SOS promoter region and reporter gene in genetically engineered microorganisms (GEMs). The assessment of potential of genotoxicity rely on the response to DNA damage induced by genotoxins in bacteria cells.

In the presented experiment *E. coli* K-12 *recA*::*gfpmut2* microbial biosensor as reporters for detecting of activation of SOS promoter under genotoxic conditions has been used. The SOS regulon is one of the most thoroughly studied stress regulons for bacteria (Gu et al., 2004). The *recA* promoter transcription is induced upon DNA damage and induction of the SOS response is initiated by RecA protein activation to mediate the LexA repressor protein cleavage. With the cleavage of LexA, the promoters that it was bound to and repressing are then expressed that results in the induction of the SOS regulon, so each downstream gene product participates in the repair of the damaged DNA (Kostrzyńska et al., 2002; Gu et al., 2004). The popularity of application of *recA* promoter for creation of effective genotoxicity bacteria biosensors is connected with broad involvement of RecA protein in several DNA repair pathways, including the repair of daughter-strand gaps and double-strand breaks, es well as in an error prone damage tolerance mechanisms called SOS mutagenesis (Kostrzyńska et al., 2002). The mechanism of the induction of the SOS response regulon genes and its application in microbial biosensors was widely described by Gu et al., 2004. The examples of biosensors, limits of detection of analysed factors and environmental application of these devices are broadly reviewed in works Lei et al., 2006; Ron, 2007 and in earlier own papers (Rosochacki and Matejczyk 2002; Matejczyk, 2004; Matejczyk and Rosochacki, 2006 and 2007).

Living organisms-based biosensors, as like bacterial biosensors can perform functional sensing and provide measurement, such as bioavailability, genotoxicity or general toxicity. Above, due to their specificity, fast response time, low cost, portability, ease of use and giving a continuous real time signal they are famous for dynamic development and represent of the advantages compared with traditional methods (D'Souza, 2001; Stiner and Halverson 2002; Belkin 2003; Gu et al. 2004; Hazen and Stahl, 2006). In such living cell systems, bacteria are especially attractive due to their rapid growth rate, low cost, and easy handling (Kuang et al. 2004; Girotti et al., 2008).

The most popular reporter genes used in biosensors construction include *lacZ* gene from *Escherichia coli*, the *lux* genes from *Vibrio fischeri* or *gfp* from *Aequorea victoria*. These devices are being designed for the detection of chemical, physical or biological signals via the production of a suitable reporter protein, for example-GFP-green fluorescent protein. Generally, biosensors could be defined as a any system that detects the presence of a substrate by use of biological component which then provides a signal that can be quantified (Gu et al., 2004). Biosensors has been created to provide even cheaper, faster and potentially more cost effective alternatives and to accomodate high-throughput screening (Norman et al., 2006; Sørensen et al.,, 2006; Yagi, 2007).

Within bio-application the most popular and well-known fluorescent protein is green fluorescent protein (GFP). This protein has been isolated from coelenterates, for example the Pacific jellyfish *Aequorea victoria* (Gu et al., 2004). GFP is being used increasingly to construct whole-cell biosensors, because of its useful properties such as: high stability, minimal toxicity for life cells and the ability to generate the green fluorescence without addition of external cofactors. Additionally it is possible non-invasive detection of *gfp* expression with application of simple in use equipment, for instance UV lamp, fluorescence microscope or spectrofluorymeter. The chromophore is responsible for GFP light and is produced posttranslationally in the presence of oxygene from serine-tyrosine and glicyne. Wild type GFP absorbs blue light at 395 nm and emits green light at 509 nm. To increase a rate of chromophore maturation, stability and to obtain the emission of stronger light signal several mutants of GFP were developed. The most popular is GFP mut1 which has 35-fold-increased fluorescence intensity per unit protein excited at 488 nm when compared with the wild-type of GFP. Some variants with short live-time were created and they are very useful in measuring of activity and strength of promoters *in situ* and in real time monitoring (Willardson et al., 1998; Chirico et al., 2002; Kostrzyńska et al., 2002; Mitchell and Gu, 2003. The description of *gfp* and other reporter genes are broadly given elsewhere (Errampalli et al., 1999; Kain, 1999; Bae et. al., 2003; Jansson, 2003).

So in this work, the aim of research was the assessment of usefulness of GFP-protein based *Escherichia coli* K-12 MG1655 strain with plasmid-borne transcriptional fusion of SOS regulon-*recA* promoter and *gfp* mutated gene – *gfpmut2* variant (Fig. 1), as a biosensor for genotoxic activity monitoring of tested chemicals.

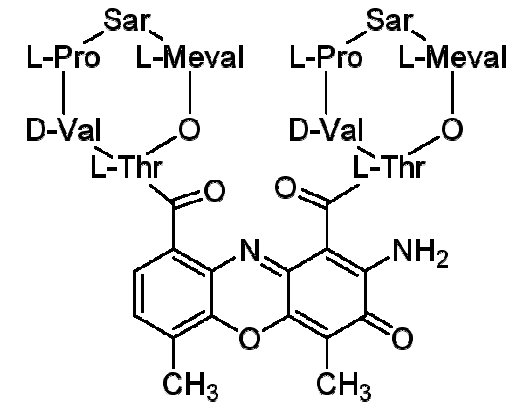
Fig. 1. Reporter plasmid pUA66 contains the gene *GFPmut2*. Vector include a *Bam*HI and *Xho*I cloning site for the promoter region, a low copy origin (SC101 origin) and a kanamycin resistance gene (Zaslaver, 2004).

The genetically modified strains of *E. coli* K-12 with *gfp* gene used in this work are the gift from Prof. Uri Alon, Department of Molecular Cell Biology & Department of Physics of Complex Systems, Weizmann Institute of Science, Rehovot, Israel.

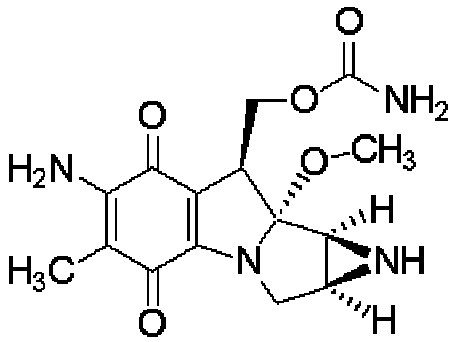
2. Experimental

The experiment was developed according to the method described by Cha et al., 1998 and Kostrzyńska et. al., 2002 with some modifications.

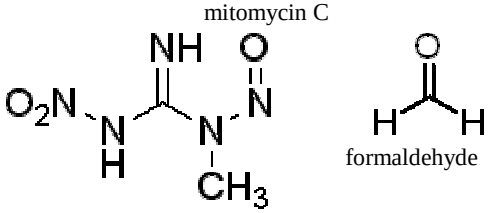
Escherichia coli K-12 MG1655 strain containing pUA66 plasmid with transcriptional fusion of *recA* promoter and *gfp* mutated gene – *GFPmut2* variant (Zaslaver et al., 2004) (Fig. 1) were cultured overnight in LB agar medium (Merck, Germany) at 30°C supplemented with 100 µg/ml of kanamycin (Sigma-Aldrich, Germany) in concentration of 100 µg/ml. During the whole experiment the 30°C as a temperature for strains incubation and room temperature for genotoxins treatment were selected to prevent overgrowth and reduce background fluorescence. Additionally, it is known that lower temperatures are optimal for correct GFP folding (Errampalli et al., 1999; Kostrzyńska et al., 2002). Colonies were carried to LB broth medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1000 ml of destilled water) with 100 µg/ml of kanamycin and incubated 20 hours at 30°C. After that, cells were washed with PBS buffer (1.44 g Na_2HPO_4, 0.24 g KH_2 PO_4, 0.2 g KCl, 8 g NaCl per 1000 ml of destilled water) and the Optical Density (OD) of bacterial cultures was standardized with spectrophotometer to 0.2 at wavelength of 600 nm. Cells were resuspended in 10 ml of PBS buffer and were tested for their ability to detect sublethal levels of known genotoxins: mitomycin C (Sigma-Aldrich, USA), actinomycin D (Sigma-Aldrich, USA), N-metyl-Nˇ-nitro-N-nitrosoguanidine (Sigma-Aldrich, USA) at concentration of 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 mg/ml and 10 mg/ml for each chemicals and formaldehyde (Sigma-Aldrich, Germany) at concentration of 50, 100, 300, 500, 700, 900, 1100, 1300 and 1800 mg/ml. The chemical structures of genotoxins used in experiment are presented in Fig. 2. As a negative control 4% ethanol and 4% acetone were used. Samples were incubated with chemicals for 90 minutes at room temperature with vortexing. The control samples of *Escherichia coli* K-12 *recA::gfpmut2* strain, not treated with chemical compounds were conducted in the same condition. Additionally, *Escherichia coli* K-12 strain containing pUA66 plasmid without the recA promoter was used as a negative control of fluorescence reactivity. After exposition of bacterial cultures to chemical pollutants, they were washed with PBS buffer. The intensity of fluorescence (IF) was measured with spectrofluorymeter (Hitachi Japan, F–2500). The measurements were done at excitation and emission wavelengths of 485 and 507 nm. The growth of bacteria strains was monitored with spectrophotometer at wavelength of 600 nm. Data showed beow include the specific fluorescence intensity (SFI) which is defined as the raw fluorescence intensity (IF) divided by the optical density (OD) measured at each time point. SFI values are averages of three independent experiments for the each tested chemicals.



actinomycin D



mitomycin C



N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)

formaldehyde

Fig. 2. The structure of compounds used in the experiment.

Specific fluorescence intensity was calculated according to the formula:

$$SFI = \frac{IF}{OD} \quad (1)$$

where:
SFI – Specific Fluorescence Intensity.
IF – The raw fluorescence of the culture treated with chemicals.
OD – Optical Density at 600 nm of treated with chemicals culture.

The percent of stimulation of gfp expression in comparison to the control was calculated according to the formula:

$$X\% = \frac{SFI_I \times 100\%}{SFI_0} \quad (2)$$

where:
X% – the percent of stimulation of gfp expression in comparison to the control.
SFI_0 – the specific fluorescence intensity of control sample.
SFI_I – the specific fluorescence intensity of the culture treated with chemicals.

3. Results

In experiment the positive fluorescence reactivity of *Escherichia coli* K-12 *recA::gfpmut2* was obtained for each tested chemicals. The highest stimulation of *gfp* expression, above 136%, 100% and 50% in comparison to the control was noticed with application of actinomycine D at concentration of 10 mg/ml, 1 mg/ml and 100 ng/ml, respectively. In the case of 10 ng/ml and 1 ng/ml concentration the higher about 14% and 17.47% levels of *gfp* expression in comparison to the control were detected. The increase of concentration of actinomycide D at 1 ng/ml to 10 mg/ml lifted the efficiency of *gfp* expression above 780%. Between the concentration of 1 mg/ml and 100 ng/ml in comparison to the 1 ng/ml we obtained above 575 and 280% of stimulation of *gfp* expression were obtained. At the concentration of 10 ng/ml the smallest stimulation of gfp expression, about 20% in comparison to the concentration of 1 ng/ml was noticed.

Different fluorescence reaction of *Escherichia coli* K-12 *recA::gfpmut2* was observed for N-metyl-Nˇ-nitro-N-nitrosoguanidine (MNNG). With using of this analyte the highest stimulation of *gfp* gene expression, 45.15% and 29.81% was noticed at concentration of 1 mg/ml and 10 ng/ml, respectively in comparison to the control. The changes in the fluorescence intensity of *gfp* in comparison to the control for 10 mg/ml, 100 ng/ml and 1 ng/ml were obtained, too. For 10 mg/ml it was 26.42% of stimulation, for 100 ng/ml 20.49% and for 1 ng/ml it was 5.33% of *gfp* gene expression activation in comparison to the control. Use of five different concentration of MNNG had developed stranger reaction of *recA-gfpmut2* genetic system. In concentration of 1 mg/ml, 10 ng/ml, 10 mg/ml and 100 ng/ml the 847%; 559.28%; 495.68% and 384.43% of *gfp* expression stimulation were registered in comparison to the concentration of 1 ng/ml.

The treatment of *Escherichia coli* K-12 *recA::gfpmut2* with mitomycin C differentiated *gfp* fluorescence response in comparison to the control. The highest stimulation of *gfp*: 16.08%, 10.36% and 8.36% were registered at concentration of 10 mg/ml, 100 ng/ml and 1 ng/ml, respectively. Less efficient flexibility in *gfp* expression system was observed after bacteria incubation with 1 mg/ml and 10 ng/ml of mitomycin C. It was 6.19% of *gfp* expression stimulation for 1 mg/ml and 1.89% for 10 ng/ml in comparison to the control. The application of mitomycin C from concentration of 1 ng/ml to 10 mg/ml had expanded fluorescence activity of *gfp* construct with *recA* promoter. The highest stimulation of *gfp* expression was noticed for concentration of 10 mg/ml and 100 ng/ml and it was 192.34% and 123.92% in comparison to the 1 ng/ml. At concentration of 1 mg/ml and 10 ng/ml the smallest stimulation of *gfp* expression, about 26% and 77.4% in comparison to the concentration of 1 ng/ml was noticed.

The incubation of *Escherichia coli* K-12 *recA::gfpmut2* with formaldehyde created highest *gfp* fluorescence response, about 17.43% in concentration of 900 mg/ml in comparison to the control. In the case of the different used concentration of formaldehyde the *gfp* expression were stimulated on a low levels. It was: 1.40% of stimulation at concentration of 50 mg/ml; 2.88% at 100 mg/ml; 0.95% at 300 mg/ml; 0.97% at 500 mg/ml; 5.97% at 700 mg/ml; 2.68% at 1100 mg/ml; 2.47% at 1300 mg/ml and 9.05% at 1800 mg/ml. The differentiation of *gfp* response with application of nine concentration of formaldehyde have made strange fluorescence activity in *E.coli* K-12 *recA::gfpmut2*. At concentration of 900 mg/ml and 1800 mg/ml the 1245% and 646.43% of *gfp* stimulation was obtained in comparison to the smaller concentration 50 mg/ml of formaldehyde. The efficiency of *gfp* expression was stimulated at the concentration of 100 mg/ml, 700 mg/ml, 1100 mg/ml and 1300 mg/ml in comparison to the 50 mg/ml of formaldehyde. The levels of stimulation were 205.71%; 426.43%; 191.43% and 176.43% , respectively for early pointed concentration. At the concentration of 300 mg/ml and 500 mg/ml the smallest stimulation of *gfp* expression, about 32.86% and 30.70% in comparison to the concentration of 50 mg/ml formaldehyde were assessed.

With application of 4% ethanol and 4% acetone the both chemicals have acted for recA promoter induction (data not shown), but no more than 6.43% for 4% ethanol and 5.22% for 4% acetone in comparison to the control. Our data indicated that *E. coli* K-12 *recA::gfpmut2* biosensor strain is more specific and sensitive for actinomycin D and MNNG and with very low response to other stressors.

In this work the fluorescence responses of *E. coli* K-12::*gfp* promoterless strain exposed to MMC,

actinomycin D, MNNG, CH_2O, ethanol and acetone were tested. None of these treatments increased fluorescence response (data not shown) more than 3.37% in comparison to the control. So, it was concluded that this strain is not sensitive enough for genotoxicity screening. As presented in Figs. 3-6, with use of *recA-gfpmut2* genetic fusion a more dramatic and sensitive fluorescence responses were obtained than with *gfpmut2 promoterless*.

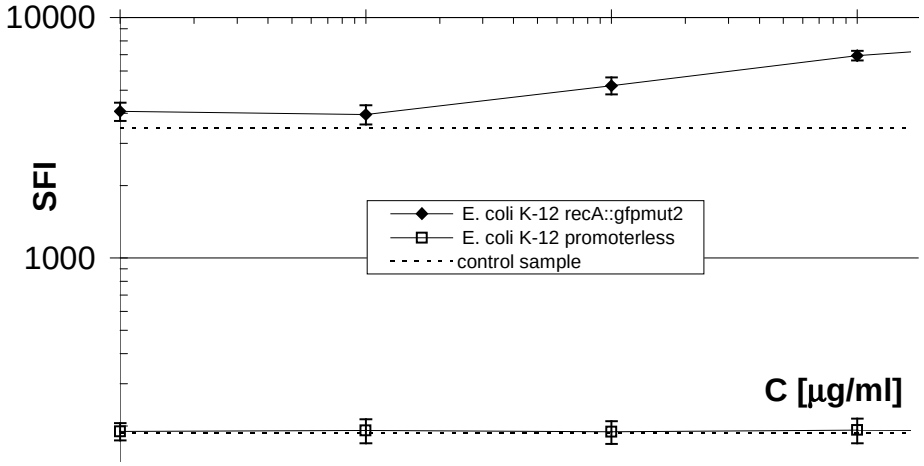


Fig. 3. Induction of *E. coli* K-12 *recA*::*gfpmut2* and *E. coli* K-12 *promoterless* by actinomycin D. Values are means ± u (x) (measurement uncertainty) for *n*=3. SFI – Specific Fluorescence Intensity; C – concentration.

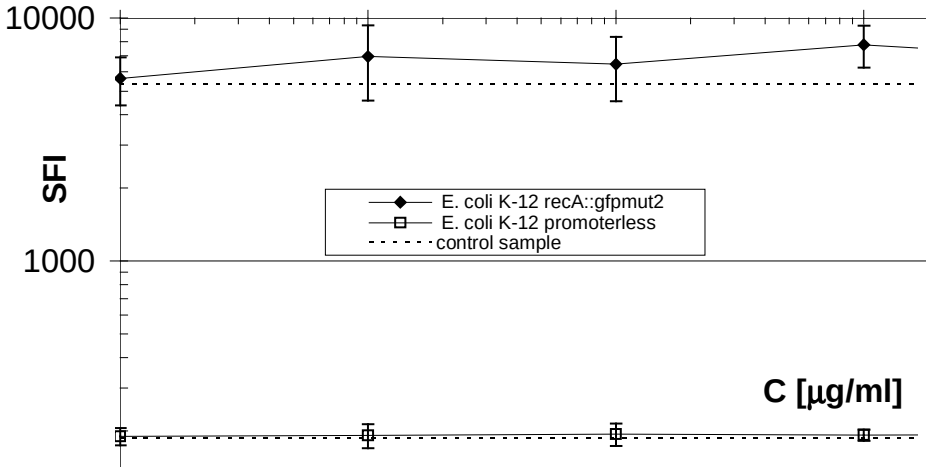


Fig. 4. Induction of *E. coli* K-12 *recA*::*gfpmut2* and *E. coli* K-12 *promoterless* by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Values are means ± u (x) (measurement uncertainty) for *n*=3. SFI – Specific Fluorescence Intensity; C – concentration.

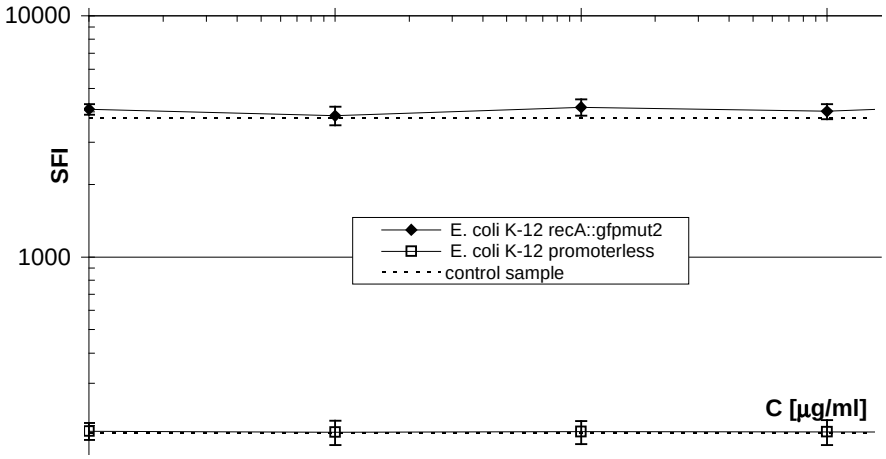


Fig. 5. Induction of *E. coli* K-12 *recA*::*gfpmut2* and *E. coli* K-12 *promoterless* by mitomycin C. Values are means ± u (x) (measurement uncertainty) for *n*=3. SFI – Specific Fluorescence Intensity; C – concentration.

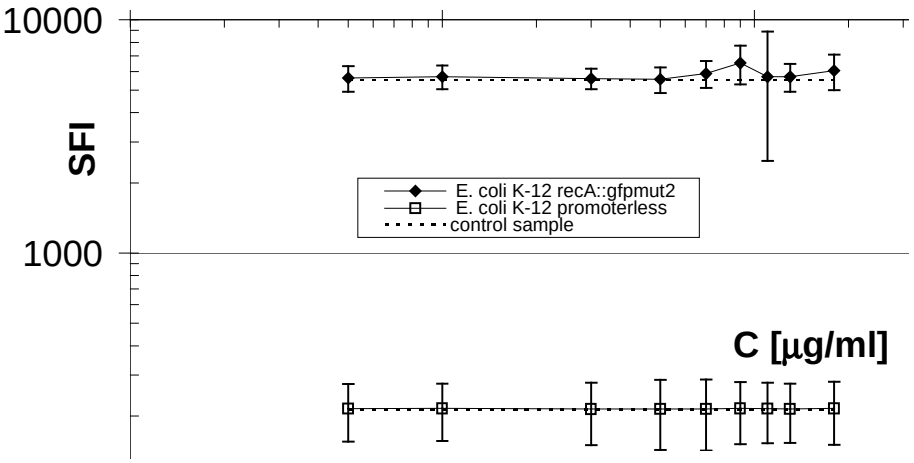


Fig. 6. Induction of *E. coli* K-12 *recA*::*gfpmut2* and *E. coli* K-12 *promoterless* by formaldehyde. Values are means ± u (x) (measurement uncertainty) for *n*=3. SFI – Specific Fluorescence Intensity; C – concentration.

4. Discussion

Results indicated that the chemical structure of tested genotoxins: mitomycin C (MMC), actinomycin D, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and formaldehyde (CH_2O) differentiated the strength of *recA* promoter induction in *E. coli* K-12 *recA:: gfpmut2* in comparison to *E. coli* K-12 carrying pUA66 – *gfpmut2* without *recA* promoter. The highest induction level of *gfp* expression was obtained after exposure of *Escherichia coli* K-12 *recA::gfpmut2* to actinomycine D (Fig. 3). For MNNG the fluorescence response of *recA-gfpmut2* fusion was smaller (Fig. 4). The fluorescence reactions to formaldehyde and MMC were included into the error of the measured broads (Figs. 5 and 6). So it was concluded that for formaldehyde and MMC *E. coli* K-12 *recA:: gfpmut2* genetic system is disqualified for practice application.

Results obtained in experiment are in agreement with studies of Kostrzyńska et al., 2002; Ahn et al., 2009; Ptitsyn et al., 1997 and the others who presented that reporter genes systems (with *gfp* and *lux* reporters) are sensitive and useful for measurement of genotoxic effect of the same compounds and various chemicals (Cha et al., 1999; Casavanth et al., 2003; Stiner and Halverson, 2002; Willardson et al., 1998; Baumstark-Khan et al., 2007).

In literature there are some discrepancies for results of sensitivity of *gfp* and *lux* genetic systems with specific for DNA damage promoters for the same tested compounds. Quite clear explanation we could find in the work of Ahn et al., 2009, where authors developed a novel approach to predict the mode of genotoxic action of chemicals using a group of seven different DNA damage sensing recombinant bioluminescent strains with genetic fusion of promoters involved in the SOS response (*nrdA*-, *dinI*-, *sbmC*-, *recA*-, *recN*-, *sulA*-, *alkA*-) and *lux* as a reporter in *E. coli*. Strains were tested against genotoxins such as: mitomycin C, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), nalidixic acid (Nal) and 4-nitroquinoline N-oxide (4-NQO). Each of these genotoxic compounds caused DNA damage by a different means. As a consequence of different responses these biosensors were grouped to a specific mode of action. It could be explanation for our results and other researchers. In the light of Ahn et al., 2009, experiment the basic mechanisms of genotoxins activity to DNA and efficiency of SOS promoters induction are strictly connected with chemical structure of tested genotoxins and scheme of their action to DNA. For example, the chemical mechanism of mitomycin C action include: oxygen radicals generation, DNA alkylation, and produces interstrand DNA cross-links, thereby inhibiting DNA synthesis. Mitomycin C also inhibits RNA and protein synthesis at high concentrations (Mao, 1999; Brander, 2001). The main mechanisms of action of actinomycin D rely on transcription inhibition. Also, Actinomycin D can bind DNA duplexes and interfere with DNA replication to inhibit DNA synthesis (Turan et al., 2006). *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) is a DNA damage alkylating agent known to covalently link alkyl groups at the position 6 of guanines in DNA (Ahn et al., 2009). The most relevant type of formaldehyde-induced DNA-damage are DNA-protein cross links (DPX) (Neuss and Speit, 2008). In own work each of tested genotoxins have had different chemical structure and mechanism of DNA damage. So, it was considered that it could be the main cause of differentiation of kinetic of *recA* promoter induction, after treatment of bacteria cells with the same concentration of MMC, MNNG, actinomycine D and used concentration of formaldehyde.

5. Conclusions

Current research indicated positive reaction of *E. coli* K-12 *recA::gfpmut2* genetic system for actinomycin D and MNNG.

The fluorescence reaction to formaldehyde and MMC were included into the error of the measured broads. So it was concluded that for formaldehyde and MMC

E. coli K-12 *recA:: gfpmut2* genetic system is disqualified for practice application.

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