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The expression of different gene constructs in *Escherichia coli* SM *lux* biosensor after exposure to drugs

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The research used bacterial biosensors containing bacterial luciferase genes to monitor changes in the environment in real-time. In this work to express four different gene constructs: *recA:luxCDABE*, *soxS:luxCDABE*, *micF:luxCDABE*, and *rpoB:luxCDABE* in *Escherichia coli* SM *lux* biosensor after exposure to three different antibiotics (nalidixic acid, ampicillin, kanamycin) and diclofenac was determined. It was found that incubation of the *E. coli* SM strain in various concentrations of analytes results in differentiation in gene expression at each of the tested concentrations (from 0.625 to 10 µg/mL) and during all three measurements, in "time 0", after 30 min. and after 1 h. The measurable signal is created as a result of the action of reporter genes (bacterial luciferase genes *luxCDABE)*, present in genetically modified bacterial cells. *E. coli* luminescent bioreporters in the stationary phase were used. In the analysis of the induction of the promoter (regulatory proteins) to the control (0 µg/ml), the highest biosensor response was shown in the case of kanamycin concentration equal to 0.625 µg/mL after 1-h incubation. The highest increase express gene construct was found for *micF:luxCDABE* in *E. coli* SM343 *lux* biosensor, where the *micF* promoter induction relative to the control at a concentration of 0.625 µg/mL is 73.9%.

Keywords Bacterial luciferase genes, *Escherichia coli* SM strain, Gene expression, Luminescence, Mechanism action, Microbial biosensors

According to the European Union Commission and World Health Organisation, environmental pollution by pharmaceutical residues is now one of the increasingly significant problems. This fact is forcing scientists to conduct more extensive ecotoxicological studies on the toxicity, genotoxicity and effects of drugs and their metabolites on living organisms. Microbial whole-cell biosensors have emerged as a promising tool for pre-screening the effects of such pollutants on living organisms.

Different antibiotics (ampicillin, kanamycin, nalidixic acid) and anti-inflammatory and analgesic drugs are commonly used to treat human and animal bacterial infections and inflammation[1]. Nalidixic acid, ampicillin and kanamycin are antibiotics detected in sewage and water worldwide. Nalidixic acid comes from the group of quinolones, which play an important part in bacterial infection treatment. The antibacterial mechanism of action of nalidixic acid is by interfering with bacterial DNA replication through inhibition of DNA synthesis. Ampicillin belongs to the β-lactam antibiotics of the aminopenicillin group, a broad-spectrum antibiotic that is sensitive to bacterial β-lactamases. The mechanism of action of ampicillin is by interfering with the bacterial cell wall biosynthesis. Kanamycin belongs to the group of aminoglycoside antibiotics and it exerts bactericidal activity by binding directly to the 16 S rRNA in the 30 S subunit of the ribosome and interfering with the translocation step of the translation process[2]. Due to the frequent use of analysed drugs in treatment, their metabolites end up in municipal sewage and become a water contamination problem. The presence of antibiotic residues in water brings the risk of resistance of pathogenic bacteria, as well as harmful effects on beneficial bacteria that inhabit the digestive system of mammals[3]. The solution to the problem of detecting harmful substances and their metabolites in water is bacterial biosensors[4,5]. Biosensors that use the entire microorganism (e.g. bacterial cells) as a biological component are called whole-cell biosensors[6]. Their action is based on physiological[7], and biochemical[8,9] changes occurring within the microorganism affected by the environmental change[10]. These are specific reactions between available molecules and receptors, as well the reactions involving nucleic acids and complementary sequences[11]. Bacterial biosensors are often based on the respiratory and metabolic processes of the bacterial cell, and the substance detected by the biosensor is usually a substrate or inhibitor of these processes[12]. As a result of the reactions taking place within the bacterial cell, a signal is created in the form of

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emission or absorption of light, uptake of gases, or their release[13,14]. At this time, the transducer transforms the biological signal into a measurable signal, which is amplified and processed and can be used for analyses[15].

The advantages of whole-cell biosensors are low price[16], versatility[17], and high sensitivity[18], as well as the ability to adapt to various environmental conditions[19], and the ability to modify the body depending on the detected substance[20]. The bacterial biosensors have the possibility of genetic modification of bacteria using mutations and DNA recombination[21]. Bacterial cells are also an exceptionally good source of obtaining intracellular enzymes[12]. Bacteria used as biosensors have plasmids with a regulatory promoter and a reporter protein that may occur naturally in the cell or be the result of genetic modification. As a result of contact of the biosensor with a given substance, the regulatory promoter is activated and then the regulatory protein produces a detectable, measurable signal[22]. This operating scheme is used by bioluminescent biosensors, due to the possibility of monitoring changes in real time[23]. They are a real solution for warning about the inflow of compounds that are highly toxic to microorganisms used in wastewater treatment[24,25]. Luminescent biosensors are characterized by fast response and high sensitivity[12]. In biosensors which use the luciferase gene, *lux* genes that encode bacterial luciferase, genes are located in a plasmid construct with a specifically induced promoter. Expression of the *lux* operon produces all the necessary components needed for the emission of light energy by bacteria[26]. These genes require active cellular metabolism. There are two patterns of analyte detection using luminescence-based biosensors: analyte-induced, which results in increased *lux* gene expression, or when the analyte suppresses cell life processes, resulting in decreased *lux* gene expression[27]. The detected substance can affect receptors on the cell wall surface or penetrate it and then bind to a specific receptor located inside the bacteria cell. In both cases, the signal is transmitted to the promoter, which is a consequence of the transcription of reporter genes (*luxCDABE*). With increasing luciferase synthesis, luminescence increases, which is a measurable signal. The signal coming from biosensors with the *lux* gene depends on the concentration of the tested substance (analyte). Low concentrations do not affect bacterial cells, and the luminescence is constant. At higher analyte concentrations, the promoter is activated, increasing luminescence. When the concentration of the analyte is very high and it is also toxic to the cell, vital functions are interrupted and the luminescence decreases[28].

Reporter genes that are used in luminescent biosensors are designed to transform the cell's response into a measurable signal. This is a process that is major for the operation of the biosensor. The most important genes used in bacterial biosensors are the *lacZ* gene encoding the enzyme β-galactosidase[28], the *crt* operon responsible for the synthesis of carotenoid[29], *gfp* (green fluorescent protein)[30,31], *lux* genes (encoding bacterial luciferase)[32], and *luc* genes (encoding eukaryotic luciferases)[28]. In the presented work we used *E. coli* luminescent bioreporter in the stationary phase.

Bacterial luciferase genes (*luxCDABE*) are obtained from strains of luminescent bacteria, including species such as *Vibrio fischeri*, *Vibrio harveyi*, *Photobacterium phosphoreum*, and *Photobacterium leiognathi*. In all species mentioned, the *luxA* and *lux B* genes encode luciferase subunits, while *luxCDE* encodes a fatty acid reductase complex[33]. Bacterial luciferases are heterodimers that oxidize long-chain aldehydes with atmospheric oxygen using reduced flavin mononucleotide[32]. The reaction results in the emission of blue-green light.

Results and discussion

This work studied the expression of four different gene constructs in *E. coli* SM *lux* biosensor strains—*E. coli* SM342 *recA:luxCDABE*, *E. coli* SM335 *soxS:luxCDABE, E. coli* SM343 *micF:luxCDABE* and *E. coli* SM346 *rpoB:luxCDABE* after exposure to three different antibiotics (nalidixic acid, ampicillin, kanamycin) respectively, and the anti-inflammatory drug—diclofenac. Together with a zero trial (without drugs)—five different series of results were obtained, depending on the gene construct used in the *E. coli* SM *lux* biosensor strains and the substance tested.

As a result of contact of the *E. coli* SM *lux* biosensor strains with a studied substance, the regulatory promoter is activated and then stimulation of the regulatory protein, which produces a detectable, measurable luminescence signal[22]. Signal induction was analysed for the concentration of the substance (analyte) incubated with every gene construct and the incubation time. It is a real solution for warning about the inflow of compounds that are highly toxic to microorganisms used in wastewater treatment.

The expression of *recA:luxCDABE* in *E. coli* SM342 *lux* biosensor after antibiotics—nalidixic acid

Incubation of the *E. coli* SM342 *recA:luxCDABE* strain in various concentrations of nalidixic acid results in the expression of the used gene construct. Gene expression occurs under the influence of each of the tested concentrations, increasing with the duration of incubation (Fig. 1). The highest increase in *recA* promoter induction in *E. coli* SM342 *recA:luxCDABE* culture was recorded at the concentrations of 10 µg/mL of nalidixic acid, after 30 min (3.5%) and 1 h (5.4%). The reaction was stronger with increasing concentration, where 10 µg/mL was the threshold of the positive reaction. The strongest inhibition of *recA* promoter after in *E. coli* SM342 *recA:luxCDABE* culture was recorded after 1 h at the concentrations from 0,625 to 5 µg/mL (respectively 67.5% and 56.03%) of nalidixic acid (Fig. 1). These are statistically significant differences (Supplementary Fig. S1a, Supplementary Fig. S2a). With bioreporters, a positive reaction shows that there is stress in the cell, whereas a negative reaction can be caused by, for example, the cell dying or inhibition of other cell processes. Nalidixic acid, or 1-ethyl-7-methyl-4-oxo-(1,8)naphthyridine-3-carboxylic acid, comes from the group of quinolones, which play an important part in bacterial infection treatment. This is confirmed by the research results where luminescent biosensor is characterized by fast response "time 0" and high sensitivity[12]. Nalidixic acid affects DNA gyrase in bacterial cells. Inhibitors bind to and stabilize a transient covalent DNA protein intermediate in the gyrase reaction cycle, termed the cleavage complex. Genotoxicity results from the conversion of cleavage complexes into DNA breaks by an as-yet-unknown mechanism. The use of quinolone antibiotics induces the SOS response in bacterial cells. Moreover, for a response to occur during the action of nalidixic acid, RecA protein and

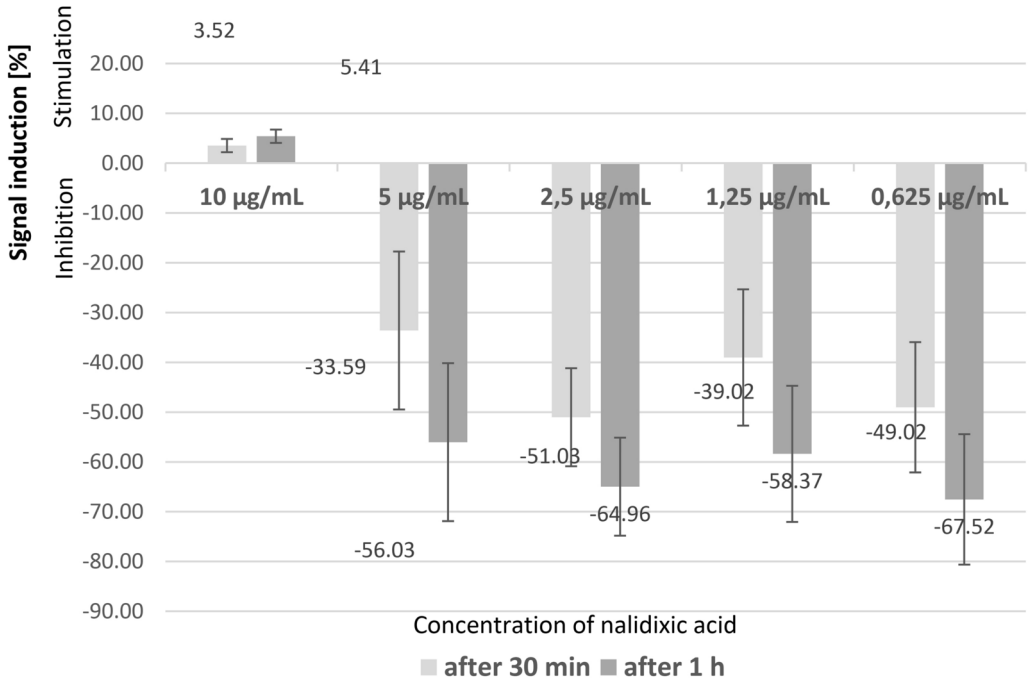


Fig. 1. Signal induction [%] pattern (standardised for control—100%) of the *recA* promoter after *E. coli* SM342 *recA:luxCDABE* incubation with nalidixic acid (NA). Standard error bars indicate the standard error from the mean of four independent replicates.

RecBC proteins must be present in the cell[34]. The RecA protein is a not genetically engineered bacterial protein that enhances the interaction and exchange of homologous DNA strands. It is sensitive to genotoxic stress[35]. It regulates repair mechanisms in the cell, catalyzes the formation of base pairs between a single-stranded DNA molecule and its complementary double-stranded DNA sequence, and induces the SOS response[36]. The RecA protein is an activator of SOS operons, which is an example of an adaptive response of bacteria to stress. SOS is activated when single-stranded DNA (ssDNA), oligonucleotides or free nucleotide triphosphates appear in the cell, which indicates inhibition of replication. When there is a significant damage in the cell, the signal sent by ssDNA and ATP causes a change in the conformation of the RecA protein, which stimulates the autoproteolysis of the LexA protein and the induction of the SOS system[15]. When the DNA damage signal disappears, the RecA protein returns to its inactive conformation, which no longer causes autoproteolysis of LexA. It has been shown that the concentration of RecA protein in cells reaches a maximum after induction of the SOS response, which occurs much later than the derepression of the *recA* promoter. The *recA* gene expression increase counteracts the RecA protein concentration weakening in cells, which is caused by cell fibrosis[37].

Descriptive statistics are presented in Supplementary Table S1. Variation in the induction value of the *recA* promoter in *E. coli* SM342 *recA:luxCDABE* after incubation with nalidixic acid depended on the concentration of the analyte and the incubation time of the strain (Fig. 1). Statistically significant differences occur between the incubation time of the *E. coli* strain versus to the control ("time 0"), respectively, to time after 30 min. ($p = 0.000207$) and after 1 h ($p = 0.000059$), and after 30 min. and after 1 h ($p = 0.000013$) (Supplementary Table S2). Taking into account the concentration of the analyte on the variability of promoter induction versus the control (0 µg/ml), statistically significant differences occur in concentrations 10 µg/mL ($p = 0.015825$) and 0.625 µg/mL ($p = 0.020013$) and between concentrations 2.5 µg/mL and 10 µg/mL ($p = 0.037487$) (Supplementary Table S2). The strongest gene expression over time was observed during incubation of the *E. coli* strain with the selected gene construct in the concentration of 10 µg/mL of the analyte (Supplementary Fig. S1b, Supplementary Fig S2b). It was found that after 30 min, and after 1 h of incubation at a concentration of 10 µg/ml, the stimulation of the promoter was the highest (Fig. 1). The RecA protein is the main regulator of SOS in the bacterial cell and works through three different mechanisms. These proteins catalyze the autoproteolysis of the transcription repressor LexA, inducing the expression of over 40 SOS genes. The inductive signal is DNA damage encountered at the replication fork. *RecA* also mediates recombinant DNA repair at double- and single-strand break sites.

The lowest gene expression and a negative reaction compared to the control (0 µg/mL) was obtained for nalidixic acid at a concentration of 0.625 µg/mL (67.5%) and after 1-h incubation of *E. coli* SM342 *recA:luxCDABE* biosensor strain (Fig. 1). The inhibition of *recA* promoter and luminescence signal in the analyte's lowest concentration (0.625 ug/mL) showed that it affects bacterial cycles and processes. Nalidixic acid is an antibiotic used in medicine, mainly in urinary tract bacterial infections[38], and has high antimicrobial activity. It is detected in hospital sewage in concentrations of up to several µg/l, also in industrial sewage, and even in treated sewage. The consequence is that the antibiotic and its residues enter the aquatic environment. As a result of the common use of this antibiotic, bacteria have developed drug resistance[39,40]. To improve the effect of nalidixic acid, changes were introduced in the basic quinolone nucleus to synthesize new derivatives of nalidixic acid, 1,2,4-triazole,

diacyl, and sulfonyl acyl hydrazine. The molecules produced by combining different pharmacophores show a better antimicrobial profile than the parent compound[41].

The expression *soxS:luxCDABE* in *E. coli* SM335 *lux* biosensor after ampicillin

Incubation of the *E. coli* SM335 strain with the *soxS:luxCDABE* gene construct in various concentrations of ampicillin causes gene expression at each of the tested concentrations (Supplementary Fig. S3). The contact of the biosensor with the ampicillin (after 30 min of incubation) produces strong *soxS* promoter inhibition in *E. coli* SM335 *soxS:luxCDABE* culture for all concentrations except 10 µg/mL (Fig. 2, Supplementary Fig. S4). At a concentration of ampicillin of 10 µg/mL, the stimulation of the *soxS* promoter in *E. coli* SM335 *soxS:luxCDABE* culture was recorded, at each time tested versus to control ("time 0"). Descriptive statistics are presented in Supplementary Table S1. Statistically significant differences occur between the incubation time of the *E. coli* strain versus to control occur, after 1 h ($p=0.000318$), and time after 30 min. and after 1 h ($p=0.003874$) (Supplementary Table S3). Taking into account the concentration of the analyte on the variability of promoter induction, statistically significant differences occur between concentration 0.625 µg/mL to concentrations 2,50 µg/mL ($p=0.033544$) and 5 µg/mL ($p=0.0195518$) and between concentration 5 µg/mL and 10 µg/mL ($p=0.041470$) (Supplementary Table S3).

The highest increase in *soxS* promoter induction in *E. coli* SM335 *soxS:luxCDABE* culture values were achieved at ampicillin concentrations lowest of 0.625 µg/mL and 1.25 µg/mL after 1 h versus to control (Fig. 2). This indicates the stimulation reaction of the *soxS* promoter in *E. coli* SM335 *soxS:luxCDABE* culture and increase luminescence under the influence of this analyte (Supplementary Fig. S3). The antibacterial effect of ampicillin is based on blocking one of the last stages of bacterial cell wall biosynthesis, the so-called transpeptidation, i.e. the process of cross-linking the original linear structure of the bacterial cell wall. Ampicillin binds to penicillin-binding proteins (PBPs) and inactivates them on the inner bacterial cell membrane. Inactivation of PBP disrupts the cross-linking of peptidoglycan chains necessary for the strength and stiffness of the bacterial cell wall. The ampicillin inhibits bacterial transpeptidase, which hinders the synthesis of the bacterial cell wall. This action activates autolytic enzymes in the bacterial cell wall, causing cell wall lysis and destruction of bacterial cells[42]. Ampicillin is a semi-synthetic penicillin antibiotic a β-lactam from the aminopenicillin group used to treat bacterial infections such as pneumonia and bronchitis, urinary tract inflammation, salmonellosis, and inflammation of the inner layer of the heart wall (endocardium). Moreover, ampicillin can bind tissue proteins in the body to form a complete antigen, which causes allergic reactions in people[43]. The popularity of this antibiotic has contributed to the emergence of bacterial strains that are fully resistant to its effects. The analysis of municipal wastewater showed that the number of drug-resistance genes reaches 9.50×10^5 gene copies/mL of municipal wastewater, which exceeds the number of gene copies in pharmaceutical wastewater[44,45]. Antibiotic degradation products are regularly detected in sewage, surface water, groundwater, fishponds, hospital effluent, and even seawater. Antibiotic residues are formed by biodegradation and chemical degradation, as well as during wastewater treatment processes[46].

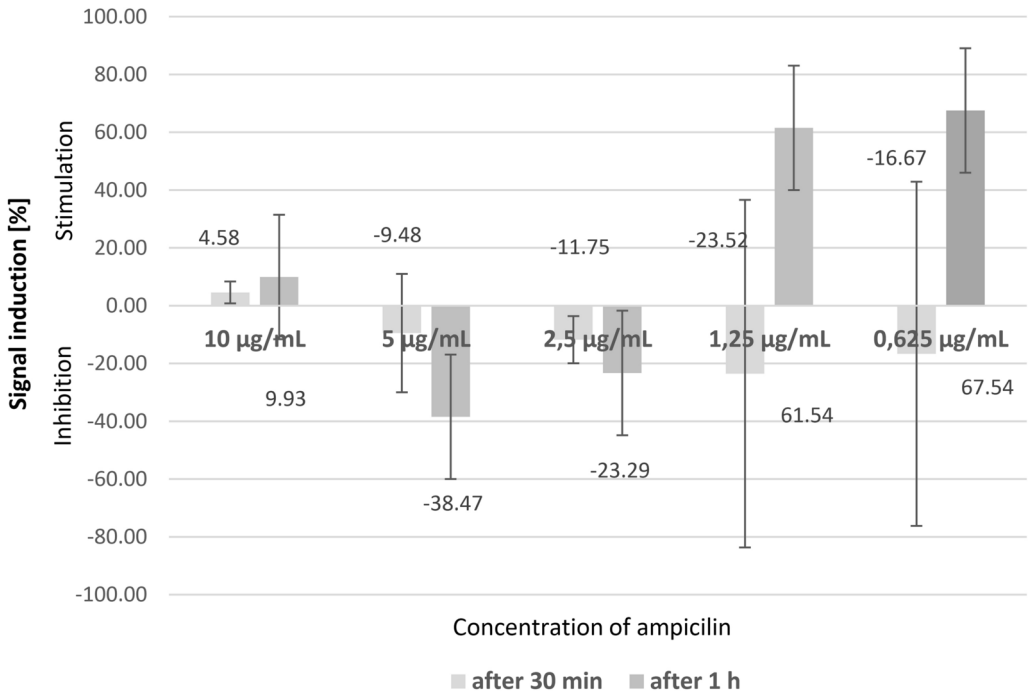


Fig. 2. Signal induction [%] pattern (standardised for control—100%) of the *soxS* promoter after *E. coli* SM335 *soxS:luxCDABE* incubation with ampicillin (AM). Standard error bars indicate the standard error from the mean of four independent replicates.

The lowest increase in *soxS* promoter induction in *E. coli* SM335 *soxS:luxCDABE* culture values were achieved at ampicillin concentrations of 2.5 µg/mL and 5 µg/mL, at each time tested versus to control (Fig. 2, Supplementary Fig. S4b). This inhibition of the *soxS* promoter leads to inhibition of *lux* gene expression and a decrease in luminescence signal. The soxS regulator is common in bacteria and it is an activator of the transcription of over 100 diverse genes involved in resistance to antibiotics. SoxS activates transcription through a "pre-recruitment" mechanism, where newly synthesized soxS molecules form binary complexes with RNA polymerase in solution, then scan soxS-dependent promoters and further activate transcription[47]. The soxS regulatory protein belongs to the group of AraC/XylS transcriptional regulators found in *Enterobacteriaceae*. Bacteria with the above-mentioned transcription regulators have three common regulatory functions: carbon metabolism, pathogenesis, and stress response[48]. These regulators play a particular role in antibiotic resistance, the pathogenicity of bacterial species, biofilm formation, and motility[49,50].

The response of the biosensor to incubation in various concentrations of ampicillin is characterized by an increased reaction in promoter *soxS* during the last measurement (after 1 h), relative to the control, except for the concentrations of 2.5 µg/mL and of 5 µg/mL (Fig. 2). It was found that ampicillin concentrations of 10 µg/ml, 1.25 µg/mL and 0.625 µg/mL caused the highest increase in induction *soxS* promoter after 1 h, by more than 9.9%, 61.5% and 67.5%, respectively, compared to the control (0 µg/ml). The highest increase in incubation *soxS* promoter after 1 h, may indicate that ampicillin shows the highest activity after a longer incubation time (Fig. 2). It has been shown that during the 3-month freezing of meat from pigs treated with ampicillin at − 20 °C and − 75 °C, the drug concentration in the meat does not decrease. Only after 8 months of storing meat at − 20 °C and − 75 °C did the concentration of ampicillin decrease[51]. Ampicillin in a changed form can remain in the animal's body[52], enter the muscles, or be partially excreted with milk by the mammary glands[53]. Because human and animal bodies are unable to completely metabolize ampicillin, using it results in residues of the substance in food of animal origin or the aquatic environment[52].

The expression of *micF:luxCDABE* in *E. coli* SM343 *lux* biosensor after antibiotics—kanamycin

Incubation of the *E. coli* SM343 *micF:luxCDABE* strain in various concentrations of kanamycin causes the biosensor to respond (Fig. 3). The *micF* gene from *E. coli* bacteria can control the expression of other genes and specifically inhibits the expression of the target *ompF* gene[54]. MicF RNA, a small regulatory RNA found in bacteria, posttranscriptionally regulates the expression of outer membrane protein F (OmpF) by interacting with the *ompF* mRNA 5'UTR[55]. Studies show that the *micF* gene is present in Gram-negative bacterial species such as *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. *Salmonella typhi*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* DNA sequences show over 96% homology in the *micF* gene compared to the *E. coli micF* sequence[56].

The expression of *micF:luxCDABE* in *E. coli* SM343 lux biosensor after kanamycin was during each of three measurements time "0", after 30 min. and 1 h. Statistically significant differences occur between the incubation time of the *E. coli* strain versus the control ("time 0"), respectively, to time after 30 min. ($p = 0.000423$) and after 1 h (0.005685), and time after 30 min. and after 1 h ($p = 0.000060$) (Supplementary Table S4). Kanamycin is an

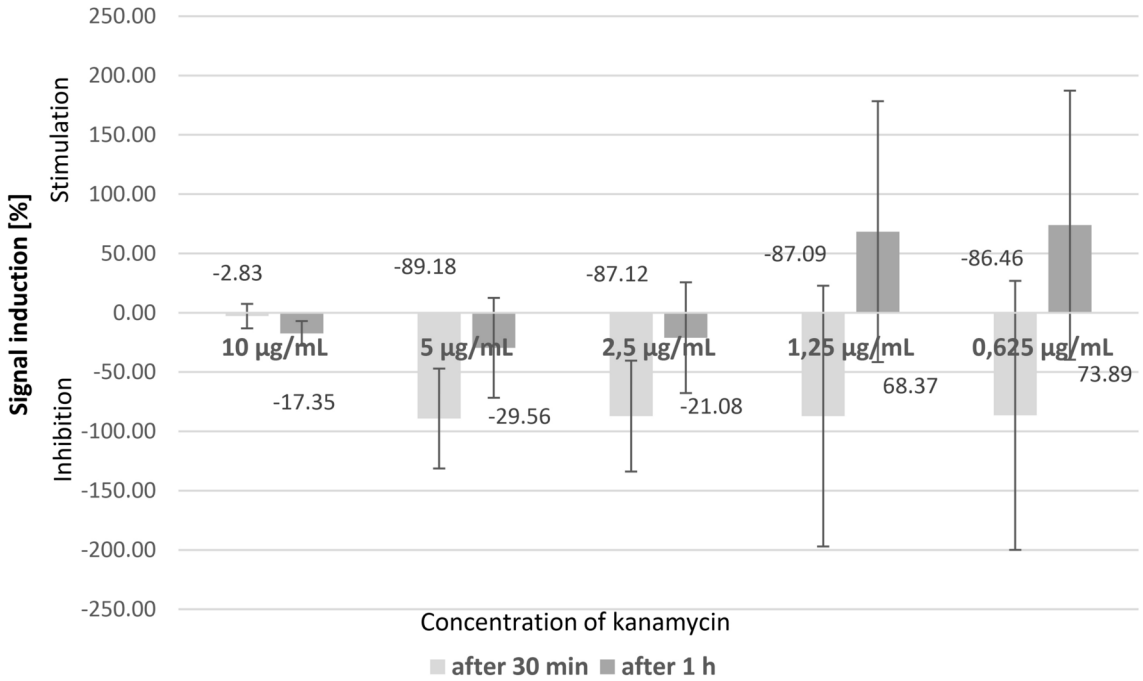


Fig. 3. Signal induction [%] pattern (standardised for control—100%) of the *micF* promoter after *E. coli* SM343 *micF:luxCDABE* incubation with kanamycin (KA). Standard error bars indicate the standard error from the mean of four independent replicates.

aminoglycoside antibiotic, whose main mechanism of action is related to the disruption of bacterial protein synthesis during the transfer of amino acids from aminoacyl-tRNA to ribosomes. Kanamycin promotes the retention of amino acids on the ribosome of aminoacyl-tRNAs that do not correspond to the codons formed in the A site of the ribosome. This coding synthesizes incorrect polypeptides with many errors, making kanamycin cytotoxic to bacterial cells.

In the first contact of the biosensor with the analyte in time "0", for each concentration, the induction of promoter *micF* is higher than the other two measurements (after 30 min and after 1 h) (Supplementary Fig. S5a, Supplementary Fig. S6a). After 30 min for each analyte concentration, an inhibition reaction of promoter *micF* and a decrease in luminescence was detected (Fig. 3). The strongest luminescence signal inhibition was demonstrated for kanamycin concentrations of 0.625–5 µg/mL after 30 min. 1-h *E. coli* SM343 *micF:luxCDABE* culture incubation with kanamycin at a concentration of 0.625 µg/mL and 1.25 µg/mL, triggered the *micF* promoter stimulation and *lux* gene expression growth and luminescence emission up to 73.9% and 68.4%, respectively.

Considering the concentration of kanamycin, differences are not statistically significant (Supplementary Fig. S5b, Supplementary Fig. S6b). Kanamycin is resistant to alkaline and acidic environments and is also thermally stable. To hydrolyze kanamycin needed a temperature not less than 100 °C, but according to studies on kanamycin removal, a removal efficiency of 96.4% was achieved at pH = 9 and a temperature of 160 °C. Therefore, it is very important to test industrial and drinking water for the presence of antibiotics and to monitor water sources in real time for kanamycin content[57,58].

In the analysis of the *micF* promoter induction in comparison to the control (0 µg/ml), the strongest luminescence signal inhibition was demonstrated for kanamycin concentrations of 0.625 µg/mL to 5 µg/mL after 30 min. For *micF:luxCDABE* in *E. coli* SM343 *lux* biosensor, the highest increase in gene expression and luminescence emission up to 73.9% was found after 1 h at a concentration of 0,625 µg/mL. Kanamycin is produced by microbial fermentation[59] and is commonly used in veterinary medicine to treat cattle. Kanamycin residues are detected in food of animal origin, honey, milk and meat. In addition to food, kanamycin residues also end up in the aquatic environment. Consuming food containing kanamycin residues leads to antibiotic resistance, kidney disease and hearing damage[60,61].

The expression *rpoB:luxCDABE* in *E. coli* SM346 *lux* biosensor after diclofenac

Incubation of the *E. coli* SM346 *rpoB:luxCDABE* strain in various concentrations of diclofenac results in gene expression at each of the tested concentrations (Fig. 4). These differences were found to be statistically significant (Supplementary Table S5). The *rpoB* gene encodes the β subunit of DNA-dependent RNA polymerase[62], and mutations within its sequence have been found to cause resistance to rifampicin, which belongs to the group of ansamycin antibiotics. Rifampicin is an antibiotic that binds to bacterial RNA polymerase, interfering with DNA transcription. Rifampicin resistance mutations in *rpoB* have been shown to be responsible for severe effects on transcription, cell fitness, bacterial stress response, and virulence[63]. Rifampicin resistance is common in several microorganisms and is usually caused by point mutations in the gene encoding the β subunit of RNA polymerase. Furthermore, *rpoB* DNA contains a conserved region in the bacterial cell that can be used for species classification. Using it, it is possible to identify intestinal bacteria, *Mycobacterium*, various species of spirochetes and *Legionella* species[64].

The response of the biosensor on diclofenac is characterized by the positive induction reaction of promoter *rpoB* after 30 min. for each concentration, except 10 µg/mL, compared to the other two measurements (in "time 0"

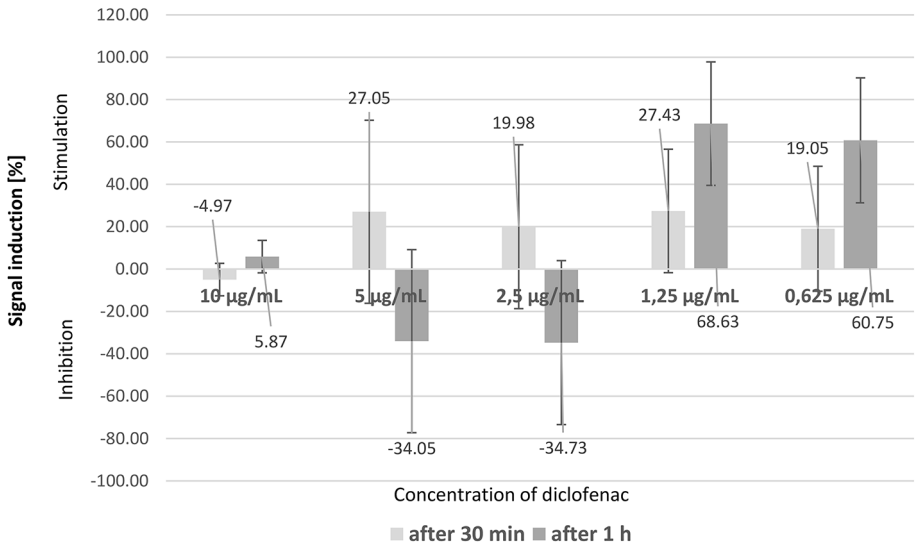


Fig. 4. Signal induction [%] pattern (standardised for control—100%) of the *rpoB* promoter after *E. coli* SM346 *rpoB:luxCDABE* incubation with diclofenac (DI). Standard error bars indicate the standard error from the mean of four independent replicates.

and after 1 h) (Supplementary Fig. S7a, Supplementary Fig. S8a). The induction of the *rpoB* promoter over time is statistically significant (Supplementary Table S5). The highest values of *rpoB* promoter induction were recorded for diclofenac concentration of 1.25 µg/mL after 30 min. and after 1 h, up to 27.4% and 68.6%, respectively (Fig. 4, Supplementary Fig. S7b, Supplementary Fig. S8b). After 30 min. high values of *rpoB* promoter induction were in the range from 19.1% at the concentration of 0.625 µg/mL to 27.4% at the concentration of 1.25 µg/mL (Fig. 4). After 1 h highest values of *rpoB* promoter induction were in the range from 60.8% at the concentration of 0.625 µg/mL to 68.6% at the concentration of 1.25 µg/mL (Fig. 4). Diclofenac, or 2-(2,6-dichlorineanilin) phenylacetic acid, is a non-steroidal anti-inflammatory and analgesic drug. The mechanism of its action is based on the inhibition of the enzyme cyclooxygenase or prostaglandin synthase, which catalyzes the conversion of arachidonic acid to prostaglandin by reducing the synthesis of prostanoids. Both enzymes (cyclooxygenases) have different activities in the inflammatory process and regulation of local homeostasis, depending on the effect of prostanoids on the kidneys, platelets and the vascular system[65].

The lowest values induction promoter *rpoB* were recorded for the concentration of 10 µg/mL for measurement after 30 min. and after 1 h (Fig. 4). It was found that the strongest gene expression occurs during incubation of the bacterial strain at a concentration of 1.25 µg/mL diclofenac. Diclofenac is excreted from the body with urine, in its original form or as metabolites, and then gets into sewage, where it is partially removed. Another reason for the presence of diclofenac in sewage is its improper disposal as solid waste. Unfortunately, the removal of diclofenac residues from wastewater is still not effective enough, so it is often carried out in water resources[66,67]. The maximum detection limit for diclofenac in municipal sewage is approximately 7 µg/l. In a long-term study of effluent from three sewage treatment plants lasting 10 months, diclofenac was detected at average concentrations of 1–10 µg/l, while in an EU-wide study monitoring effluent from sewage treatment plants, a maximum concentration of 174 ng/l was found, with mean values of 50 ng/l[23,68].

Analysis of the biosensor response relative to the control (0 µg/mL) shows that the highest values of *rpoB* promoter induction (up to 60–69%) occurs when the bacterial strain is incubated at a concentration of 1.25 µg/mL diclofenac after 30 min and after 1 h (Fig. 4). The strongest luminescence signal inhibition was demonstrated for diclofenac concentrations of 2.5–5 µg/mL after 1 h, up to 34.7% and 34.1%, respectively. These were the lowest induction values promoter *rpoB* and the decrease in luminescence compared to the control (Fig. 4).

Comparing the results obtained in our work with the results of antibiotic studies using microbial *E. coli* biosensors with plasmid gene constructs containing *recA*, *micF*, *soxS* and *rpoB* promoters in their structure[4,8], we found pronounced differences in promoter induction values and intensity of the luminescent cellular response (this is also explained in the Materials and Methods section of the paper). These discrepancies are due to the different growth phase of the *E. coli* cultures used in the study. In the experiments described in the present study, the *E. coli* cultures were in the stationary growth phase and in the work of Urban et al.[4] and Melamed et al.[8] in a logarithmic growth phase.

It is known that antibiotics target cellular biosynthetic processes. In the stationary growth phase, bacterial cells are no longer growing and dividing due to the lack of oxygen and/or nutrients, so those processes that are inhibited by antibiotics in the exponential growth phase no longer occur, and therefore, the stress level is low. Furthermore, luminescence requires energy, and it is doubtful that there is still glucose in the medium as an energy source.

The mechanism of antibiotics and diclofenac action in *E. coli*

Various species of bacteria can be used to construct a bacterial biosensor, but the most common are biosensors based on *E. coli* cells because they are the best-known gram-negative bacteria. *E. coli* bacteria with *lux* genes are used as semi-specific or induced sensors in response to damage to cell components. Toxic substances damage three types of biomolecules: DNA, proteins, and lipids. In *E. coli* cells, there are several regulons in which genes are simultaneously transcribed after the appearance of toxic factors. The SOS regulon (reacting to DNA damage), the "heat shock" regulon (reacting to protein damage), the regulon whose genes are expressed in the event of cell membrane damage, and two oxidative stress regulons: soxRS and oxyRS[28].

The studies showed that the appearance of an analyte (stressor) in various concentrations triggers a cascade of reactions leading to the expression of a reporter gene and the emission of a cellular signal. The possible mechanism of antibiotics and diclofenac action in *E. coli* was presented in Fig. 5. The organised heat shock proteins network in oxidative stress response and *recA*, *SoxS*, *micF* and *rpoB* genes activity (Drugs-Reactive Oxygene Species (ROS)- Heat Shock Proteins (HSPs), notation in brief Drugs-ROS-HSPs) was described in five important stages (Fig. 5).

Other genes with different activity and protein expression are also used in bacterial cells. The *lacZ* gene is also used in bacterial biosensors, but it is not popular due to low sensitivity, low efficiency, and length of the enzymatic reaction[28]. The *lacZ* gene encoding the enzyme β-galactosidase was isolated from the genome of *E. coli* bacteria. This enzyme hydrolyzes lactose into glucose and galactose. Enzyme activity is measured using photocolorimetry. When β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromine-4-chlorineindoxyl, which spontaneously dimerizes to form an insoluble blue pigment called 5,5′-dibromine-4,4′-dichloroine-indigo.

The *ctr* genes synthesise carotenoids in bacterial cells[29] and their activity is also measured using photocolorimetry. Bacteria that produce a visible red pigment (deinoxanthin) are used as biosensors, where the *ctr* gene has been removed from the bacterial chromosome and then inserted into a plasmid under the control of an inducible promoter. The disadvantage of biosensors with *ctr* genes is the reaction time and the complexity of the process because the dye is produced as a result of the reaction involving many proteins.

The next gene used in bacterial biosensors is a green fluorescent protein (*gfp*) that comes from the jellyfish species *Aequorea victoria*. GFP proteins are an invaluable tool for visualizing cellular structures and explaining the biochemical transport of single cells into entire organisms. The main *gfp* motif is the p-hydroxybenzylidene-

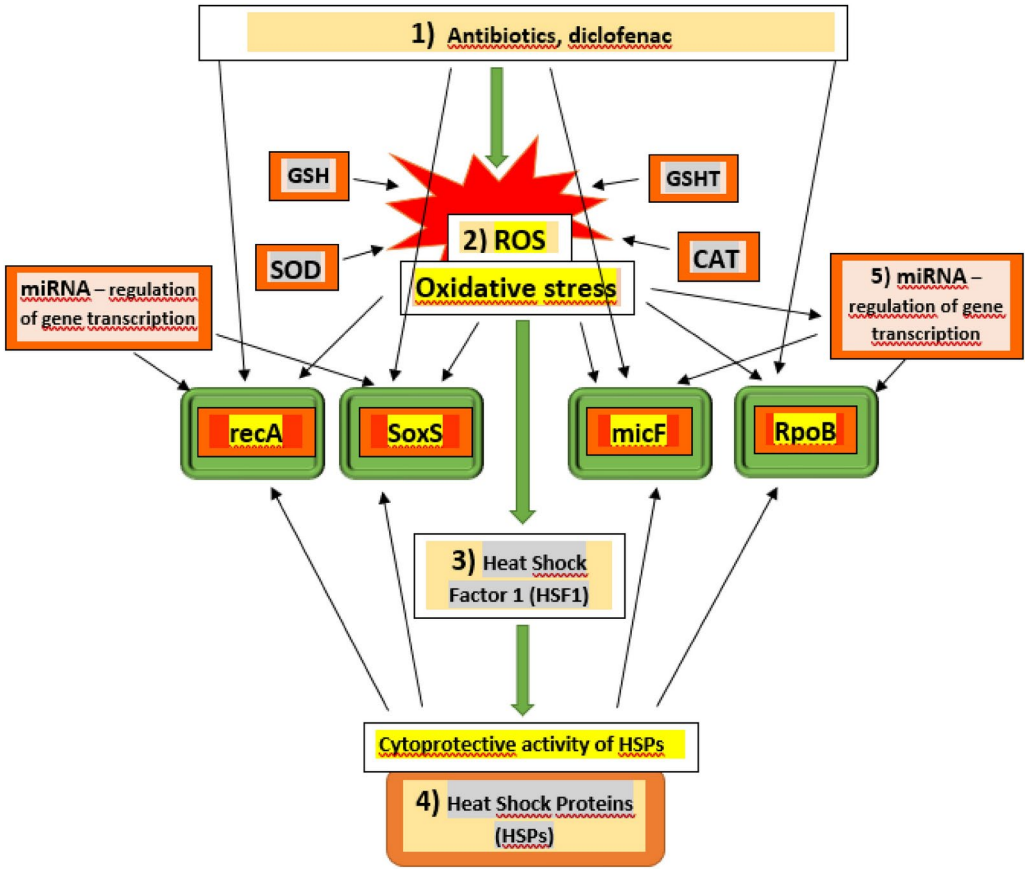


Fig. 5. The possible mechanism of antibiotics and diclofenac action in *E. coli*. The role of organised heat shock proteins network in oxidative stress response and *recA*, *SoxS*, *micF* and *rpoB* genes activity (Drugs-ROS-HSPs). (1) Drugs stress signal in *E. coli*; (2) Increase in ROS synthesis and oxidative stress activation, (3) Activation of oxidative stress-related cellular signal transduction pathways, including activation of the heat shock factor-1 (HSF-1), (4) Stimulation of expression of heat shock proteins genes and HSPs synthesis. HSPs have a cytoprotective function by reducing ROS levels and protecting nucleic acids and cellular proteins; HSPs may have a cytoprotective and supportive role in the activity of gene expression products *recA*, *SoxS*, *micF* and *rpoB* and the repair of damage after *E. coli* cell exposure to test drugs.; (5) Oxidative stress affects miRNA activity, which, can regulate the expression of many genes, including *recA*, *SoxS*, *micF* and *rpoB* and other genes whose expression products are involved in the antioxidant defence of the cell[69–71]. Abbreviations: CAT-catalase, GSH—Glutathione, GSHT—Glutathione transferase, miRNA—microRNA.

2,3-dimethylimidazolinone chromophore[30]. For the reason, no substrate is needed to activate fluorescence, *gfp* may have an advantage over other genes. However, for fluorescence to occur, the protein must develop sufficiently. Expression of fluorescent proteins involves transcription and translation. After translation, a fluorescent protein chromophore is formed. The time required to form the chromophore of a fluorescent protein is referred to as the maturation time. Genetic engineering allowed the optimization of *gfp* features, such as higher quantum yield and increased photostability[31].

Bacterial whole-cell biosensors are a valuable tool for detecting and assessing the toxicity of pollutants in the natural environment, and for monitoring the level of pharmaceuticals in surface waters, soil, air, sewage, and food[14,67]. They are an irreplaceable tool in research on gene expression, promoter activity, and regulation of bacterial cell response under stress conditions. Microbial biosensors with *gfp* and *lux* reporter genes are used in monitoring metals in contaminated soils, polychlorinated biphenyls (PCBs) in sewage, organic pollutants in treated sewage, chromium, arsenic, lead, pesticides and petroleum hydrocarbons in water[7,11]. *E. coli* strains with the *recA:luxCDABE* plasmid gene construct are used to determine the genotoxicity of many chemical compounds, including antibiotics[8], phenol, chromium (Cr^{6+}), lead (Pb^{2+}) and the potential of UV radiation to affect bacterial cells. In environmental monitoring, *E. coli recA:luxCDABE* strains are used to determine the genotoxicity of crude oil in surface water samples and the genotoxicity of municipal sewage[11,13]. In the work of Zappi et al.[19] two microbial *E. coli* biosensors containing the *gfp* and *lux* genes were used to determine the toxicity of nitrification inhibitors (allylthiourea, phenol and copper) in industrial and municipal sewage. Microbial biosensors with *lux* and *gfp* reporter genes are used to determine the cytotoxicity of drugs and other chemical compounds[7]. According to the International Agency for Research on Cancer[72] it is recommended that carcinogenic, mutagenic or teratogenic substances should be eliminated from treated wastewater. The organic

compounds with toxic effects and endocrinal active compounds are important for human health[73], and some drugs detected in wastewater, such as anticancer drugs[74] and hormones (17β-estradiol)[75] have these properties. The risks associated with the presence of pharmaceuticals in the environment cause their transport into the environment should be limited and constant monitoring of environmental matrices should be carried out[76]. Currently, bio-membrane-based methods and AOPs (Advanced Oxidation Processes) processes are becoming increasingly important in this area.

Conclusion

In the present study, the effect of selected antibiotics nalidixic acid, ampicillin, kanamycin, and antiinflammatory drug—diclofenac on *E. coli* SM biosensor strains, which contain *pBRlux-trp:recA::luxCDABE* plasmid constructs with *recA*, *SoxS*, *micF* and *rpoB* promoters and *luxCDABE* reporter gene was examined. The genotoxicity of the test nalidixic acid was measured based on the induction of genotoxin and DNA damage-inducible *recA* promoter in *E. coli* SM342 *recA:luxCDABE* biosensor strain. Using *E. coli* SM335 *soxS:luxCDABE* and *E. coli* SM343 *micF:luxCDABE*, the effects of ampicillin and kanamycin on the promoters of *soxS* and *micF* genes involved in the regulation of expression of many genes, including genes under SoxS regulation and genes involved in the removal of antibiotics, were investigated. Using the *E. coli* strain SM346 *rpoB:luxCDABE* the effect of the diclofenac tested on the RNA polymerase gene promoter (beta subunit) was determined. In these experiments, *E. coli* luminescent bioreporter in the stationary phase was used and the luminescence response was very low compared to the data in the cited scientific publications.

The results obtained in the present study are the basis for the following conclusions:

1. All of the drugs tested interacted with different intensities in *E. coli* SM strains on cellular pathways involving *recA*, *SoxS*, *micF* and *rpoB* gene promoters, indicating different sensitivity of the gene constructs tested to the drugs. Variation in the induction value of test promoters *recA*, *SoxS*, *micF* and *rpoB* in *E. coli* SM strains after incubation with drugs depended on the concentration of the analyte and the incubation time of the strain.
2. The highest increase in *recA* promoter induction in *E. coli* SM342 *recA:luxCDABE* culture was recorded at the concentrations of 10 µg/mL of nalidixic acid, after 30 min (3.5%) and 1 h (5.4%). The reaction was stronger with increasing concentration, where 10 µg/mL was the threshold of the positive reaction.
3. Among the drugs tested, one-hour incubation of *E. coli soxS:luxCDABE* with ampicillin at concentrations of 10 µg/ml, 1.25 µg/ml and 0.625 µg/ml induced the *soxS* promoter most intensively, by more than 9.9%, 61.5% and 67.5%, respectively, compared to the control (0 µg/ml).
4. In the analysis of the *micF* promoter induction in *E. coli micF:luxCDABE* in comparison with control (0 µg/ml), the highest biosensor response up to more than 68.4% and 73.9% was shown in the case of kanamycin concentrations of 1.25 and 0.625 µg/ml after 1 h.
5. Diclofenac tested at a concentration of 1.25 µg/ml after 1 h induced the *rpoB* promoter in *E. coli rpoB:luxCDABE* more intensively by more than 69% compared to the control (0 µg/ml).
6. The results obtained indicate that the *E. coli* biosensor strains with plasmid vectors containing *recA*, *SoxS*, *micF* and *rpoB* promoters and *luxCDABE* reporter gene used in this study can be used in gene expression analysis and as biosensors in the detection, genotoxicity testing and investigation of the toxicity effects of pharmacological contaminants present in the environment.

Materials and methods

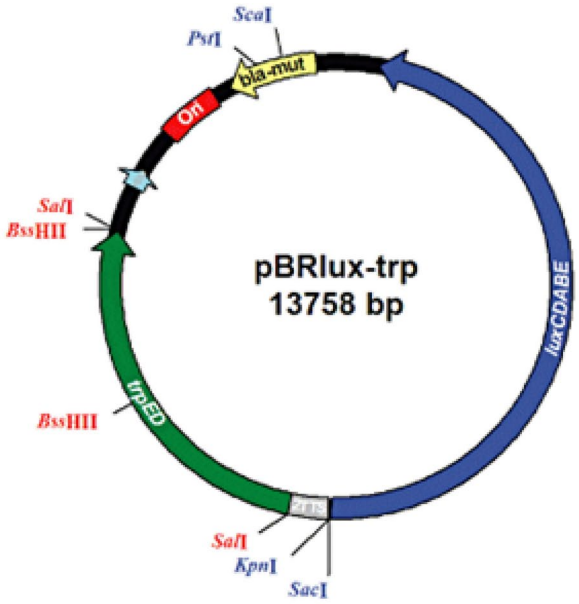
Material

Research about the expression level of plasmid constructs with *recA*, *soxS*, *micF* and *rpoB* promoters in transcriptional fusion with *luxCDABE* reporter gene in *E. coli* SM strains was conducted using luminometric indication. *E. coli* SM strains used in this study are presented in Table 1. A genetic map of the *pBRlux-trp* plasmid with the *luxCDABE* reporter gene used in this study is presented in Fig. 6. *E. coli* SM strains containing the *luxCDABE* plasmids with *recA*, *soxS*, *micF* and *rpoB* promoters are from the collection of Prof. Shimshon Belkin's laboratory, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel.

In selecting the promoters for the study, we were guided by their sensitivity to antibiotic stress-induced changes in the cell, as documented earlier in scientific studies[8,77,78]. These include DNA damage (*recA* promoter), the participation of dual transcriptional activator in the removal of antibiotics (*soxS* promoter), a small regulatory RNA that regulates of the translation of the OmpF porin (*micF* promoter), the regulation of RNA polymerase, beta subunit (*rpoB* promoter)[8,77]. It is known that some of the promoters e.g. *recA* are active at a certain level in the cell all the time. Furthermore, in *E. coli* cells in stationary growth, promoters can be induced by metabolic products of the bacteria that accumulate in the culture medium. Another factor capable of inducing the promoters under study and inducing significant luminescence in bacterial cells is reactive oxygen species (ROS), whose presence is always found at some level in the cells of aerobic organisms[79,80].

E. coli strain	Plasmid	Phenotype/genotype	Sensing element
SM342	*pBRlux-trp:recA::luxCDABE*	DtrpE/ptrpED	DNA recombination protein induces the SOS response to DNA damage
SM335	*pBRlux-trp:soxS::luxCDABE*	DtrpE/ptrpED	Dual transcriptional activator participates in the removal of antibiotics
SM343	*pBRlux-trp:micF::luxCDABE*	DtrpE/ptrpED	Antisense regulator of the translation of the OmpF porin, under SoxS regulation
SM346	*pBRlux-trp:rpoB::luxCDABE*	DtrpE/ptrpED	RNA polymerase, beta subunit

Table 1. Characterization of the *E. coli* strains used in the experiments described in this work[8].



Explanations: *luxCDABE* - reporter gene, *trpED* - tryptophan degradation gene, *ori* - sart replication site, *bla-mut* - mutated ampicillin resistance gene, T1, T2 - transcription terminators, *SacI*, *KpnI*, *SalI*, *BssHII*, *PstI*, *ScaI* - cut sites of the listed restriction enzymes

Fig. 6. Genetic map of the *pBRlux-trp* plasmid with the *luxCDABE* reporter gene and with the relevant restriction sites[8].

The analyses were performed using the GloMax*-Multi Detection System plate reader from Promega. The experiments were carried out in the culture medium and a specific dose of active substances (analytes). Among the antibiotics used were nalidixic acid, ampicillin, kanamycin, and the inflammatory drug diclofenac (Table 2). NA—a known genotoxin in this paper was used as a standard. The mechanism of action of NA is through inhibition of DNA replication in bacterial cells. Studies using *E. coli* strains or other bacteria often use nalidixic acid as a standard, especially in experiments with *E. coli* strains containing plasmids with a *recA* promoter and a specific reporter gene, e.g. *lux* or *gfp*[16]. β-lactam antibiotics (and ampicillin is one of these) are also among the most frequently detected contaminants of pharmaceutical origin in wastewater, water and dairy products[78].

All the tested drugs were dissolved in dimethyl sulfoxide (DMSO, Chempur). Each substance was prepared with a series of dilutions in DMSO with a dilution factor of q = 2. In all experiments, the drugs were tested at final concentrations of 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL and 0—only DMSO control sample. Four separate series of studies were performed using four different gene constructs in *E. coli* SM cells. For each series of studies, the gene construct contained a different promoter (*recA*, *soxS*, *micF* and *rpoB*), corresponding to the tested substance.

In the earlier works[76–78] the *E. coli* strains used in that work were already evaluated. However, the bacteria were in a different growth phase, a logarithmic one, whereas in our work we investigated how the sensitivity of luminescent *E. coli* biosensor strains changes in the stationary phase. This is because it is known that *E. coli* sensitivity to antibiotics is variable depending on the growth phase. The cultures tested in our work containing plasmids with the *luxCDABE* reporter gene were in the stationary growth phase. Furthermore, in our experiments, we used different concentrations of the test drugs and different incubation times to determine the intensity of the luminescence response. These methodological differences affected the results obtained[4,8,77].

Effects of test drugs on induction of *recA*, *soxS*, *micF* and *rpoB* promoters in the *pBRlux-trp-luxCDABE* plasmid in *E. coli* SM strains

The effect of test drugs was determined based on the induction parameters of the *recA*, *soxS*, *micF* and *rpoB* promoters in *E. coli* SM strains with the *luxCDABE* reporter gene in the plasmid vector *pBRlux-trp*. The determination of the effect is based on the quantitative determination of the intensity of luminescence of *E. coli* SM *recA:luxCDABE*, *micF:luxCDABE*, *soxS:luxCDABE* and *rpoB:luxCDABE* cultures incubated for a specified time with the tested active substances of the drugs compared to the control culture. The luminescent signal is proportional to the level of induction of a given promoter and the response potency of the active drug substances tested on bacterial cells. *E.coli* SM/pBRlux-trp strains with *recA*, *soxS*, *micF* and *rpoB* promoters were used for 24 h in LB broth at 37 °C. Luminescence measurement and data analysis were performed according to the method of Melamed et al.[8] and Kessler et al.[76]. Amount of 100 µl overnight broth culture of *E. coli* SM/pBRlux-trp strains with *recA*, *soxS*, *micF* and *rpoB* promoters was placed on a 96-well white plate (Grainer Bio-one,

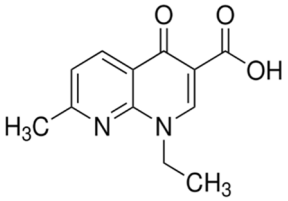
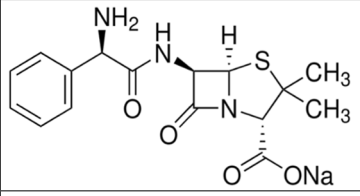
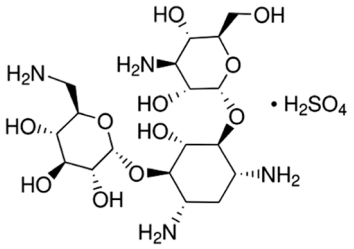
Summary formula	Structural formula	Chemical name
Antibiotics		
Nalidixic acid $C_{12}H_{12}N_2O_3$ https://www.sigmaaldrich.com/PL/pl/product/sial/n8878		Nalidixic acid CAS 389-08-2
Ampicillin $C_{16}H_{18}N_3NaO_4S$ https://www.sigmaaldrich.com/PL/pl/product/sial/a9518		Ampicillin sodium salt CAS 69-52-3
Kanamycin $C_{18}H_{36-37}N_{4-5}O_{10-11} \cdot H_2SO_4$ https://www.sigmaaldrich.com/PL/pl/product/sigma/60615		Kanamycin sulfate CAS 70560-51-9
Nonsteroidal anti-inflammatory drugs (NSAIDs)		
Diclofenac $C_{14}H_{10}Cl_2NNaO_2$ https://www.sigmaaldrich.com/PL/pl/product/sial/phr1144		Diclofenac sodium salt CAS 15307-79-6

Table 2. Chemical structure of the drugs used in the study.

	1	2	3	4	Applied substance dilution
A	100	100	100	100	10 µg/mL
B	100	100	100	100	5 µg/mL
C	100	100	100	100	2.5 µg/mL
D	100	100	100	100	1.25 µg/mL
E	100	100	100	100	0.625 µg/mL
F	100	100	100	100	0 µg/mL

Table 3. Scheme for adding broth culture and diluting the compound on the plate. 100—total sample volume, consisting of 50 µl of bacterial culture and 50 µl of the corresponding test drug solution; 10, 5, 2.5, 1.25, 0.625 µg/mL—final concentration of test drug in the total sample volume of 100 µl.

Germany) with a transparent bottom for simultaneous measurement of absorbance and luminescence (in wells A to F). The culture was repeated four times in horizontal orientation.

According to the scheme (Table 3), an equal volume of DMSO was added to 50 µl of culture of biosensor *E. coli* strains, so that the test volume was 100 µl. This volume of added DMSO had no toxic effect on the bacterial cells.

The prepared plate was placed in the measuring device. The measurement of luminescence and absorbance was performed at a wavelength of $\lambda = 600$ nm. The first measurement was performed after preparing the plate and was marked as "time 0", the next measurement was performed 30 min. after preparing the plate, and the next one after 1 h after preparing the plate. After obtaining the luminescence and absorbance values, the luminescence (L) was calculated using the formula:

$$L = \frac{l}{Abs}$$

L—luminescence, l—luminescence value from the luminometer, Abs—absorbance value for $\lambda = 600$ nm.

The effects of tested drugs on *E. coli* SM cells with plasmid gene constructs containing *recA*, *soxS*, *micF* and *rpoB* promoters with *luxCDABE* reporter gene are presented as luminescence values (L) of test and control

samples at time 0 and after 30 min and 1 h and as a percentage (%) increase/decrease in the luminescence of treated samples compared to the control (0 µg/mL).

Statistical analysis

Statistica ver. 13.3 (TIBCO Software Inc., 1984–2017) was used for the statistical evaluation of the expression of different gene constructs in *Escherichia coli* SM lux biosensor after drugs exposure. Statistically significant differences in the induction values of different promoters under the influence of active substances of tested drugs were assessed, taking into account different concentrations of analytes and time of promoter induction. A parametric T-test for dependent samples was performed for repeated measurements, with the condition of normal distribution using the Shapiro–Wilk W test and the lack of significant differences between variances. The T-test was used to assess differences between means in individual repetitions, as dependent samples "before" and "after" drug administration. Analysis of variance (ANOVA) compared descriptive statistics and correlations for dependent variables in each of the groups defined by one grouping variable, i.e. time or analyte concentration. Descriptive statistics, tests and statistical analyses are presented in the supplementary material. Values of $p < 0.05$ were regarded as significant and $p < 0.01$ as highly significant.

Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article and its supplementary information files.

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G.Ł. , M.M. conceptualization; G.Ł, M.M. methodology; G.Ł., U.D. investigation; G.Ł., M.M., U.D. contributed to writing-original draft preparation and editing, G.Ł., M.M. funding. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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