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ENVIRONMENTAL APPLICATION OF REPORTER-GENES BASED BIOSENSORS FOR CHEMICAL CONTAMINATION SCREENING

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Abstract: The paper presents results of research concerning possibilities of applications of reporter-genes based microorganisms, including the selective presentation of defects and advantages of different new scientific achievements of methodical solutions in genetic system constructions of biosensing elements for environmental research. The most robust and popular genetic fusion and new trends in reporter genes technology – such as *Lac*Z (*β*-galactosidase), *xyl*E (catechol 2,3-dioxygenase), *gfp* (green fluorescent proteins) and its mutated forms, *lux* (prokaryotic luciferase), *luc* (eukaryotic luciferase), *pho*A (alkaline phosphatase), *gus*A and *gur*A (*β*-glucuronidase), antibiotics and heavy metals resistance are described. Reporter-genes based biosensors with use of genetically modified bacteria and yeast successfully work for genotoxicity, bioavailability and oxidative stress assessment for detection and monitoring of toxic compounds in drinking water and different environmental samples, surface water, soil, sediments.

INTRODUCTION

Nowadays, reporter genes technology is very useful and famous for widely application in different fields of sciences such as biology, ecology, medicine and environmental biotechnology [50]. Reporter genes are defined as a specific DNA sequences, used for genetic engineering creation of genetic constructs which are applied for transfer to the live cells – from bacteria to mammalian. The most popular reporter genes are: *Lac*Z (*β*-galactosidase), *xyl*E (catechol 2,3-dioxygenase), *gfp* (green fluorescent proteins) and its mutated forms, *lux* (prokaryotic luciferase), *luc* (eukaryotic luciferase), *pho*A (alkaline phosphatase), *gus*A and *gur*A (*β*-glucuronidase), antibiotics and heavy metals resistance genes. These genes give transformants some characteristic genotype or phenotype to enable later on monitoring of their activity, in bacteria, water, soil, biofilms and activated

sludge. Bacteria-based reporter genes genetic systems are suitable for on-line monitoring of the efficiency of pollutants biodegradation, promoter strength and activity, gene expression and cellular protein localization [23, 30]. They successfully work as a reporter cell systems for genotoxicity and oxidative stress assessment and as a biosensors and chemical pollution bioindicators for detection and monitoring of toxic compounds, such as EDCs (Endocrine Disrapting Compounds), dioxins and different chemicals in drinking water, environmental samples such as: surface water, soil, sediments [2, 18–20, 26, 29, 34, 38, 41, 45–46].

GENETICALLY ENGINEERED BACTERIA IN GENOTOXICOLOGY

Since Ames and coworkers created (early 1970s) *Salmonella* microsomal mutagenicity assay, there has been a significant development in the use of genetically engineered microorganisms for genotoxicity, bioavailability and oxidative stress assessment. Nowadays, bacterial-based genotoxicity test systems play an important role in pre-screening and detection of DNA damaging hazardous chemicals in environmental samples and surface or drinking water. Such bioreporter tests rely on transcriptional promoter-reporter gene fusions that produce a dose-dependent signal in the presence of DNA damaging compounds. These genetic systems are based on the fusion of different SOS-responsive promoters with reporter genes [6, 7, 17, 21, 24–25, 32–33, 37].

Biran et al., 2011 [6] constructed a bacterial genotoxicity reporter strain where *sul*A gene as a strong promoter of *Escherichia coli* SOS response system was fused to the alkaline phosphatase – *pho*A – reporter gene. The bioreporter successfully responded to known DNA-damaging agents – hydrogen peroxide, nalidixic acid (NA) and mitomycin C (MMC), in a dose-dependent manner and at a low concentration: 0,15 μM for MMC, 7,5 μM for nalidixic acid and 50 μM for hydrogen peroxide. Sensitivity was higher after single and double knockout mutations, so mutants displayed a five- and tenfold increase in sensitivity to MMC and NA [6].

Wasterink et al., 2009 [44] developed the Vitotox™ and RadarScreen assays as early screens for mutagenicity and clastogenicity. The Vitotox™ with application of *Salmonella typhimurium* was created by fusion of luciferase gene under the control of the *rec*N promoter from SOS-response. The specificity of the Vitotox™ assay was 94% for Ames test and that of the RadarScreen assay was 74% for clastogenicity. The Vitotox™ was more sensitive (86%) than RadarScreen (77%) assay in comparison with the tested compounds [44].

Microbial biosensors are good tool for monitoring of chemical quality in surface and drinking water, and that are broadly reviewed [15, 45]. The examples of environmental application of these devices are described in excellent work of Xu et al., 2013 [50].

To enhance the sensitivity of bacteria biosensors, various molecular manipulations have been developed. Yu et al., 2011 [48] engineered ribosome binding sequence (RBS), which in prokaryotes plays a very important role in mRNA translation and stability. They constructed *Escherichia coli* based biosensors responding for such compounds as: benzene, toluene, ethylbenzene and xylenes (BTEX). For three *E. coli* strains the same Pu promoter and XylR regulator from the *Pseudomonas putida* TOL plasmid were used,

but different in the engineered RBS in their reporter genes sequences. The luminescence activity induction of biosensors strains by 2-chlorotoluene showed 10–35 times stronger signal intensity for biosensors strain with engineered RBS (BTEX-SE and BTEX-SD) than the primary BTEX-W biosensor [48].

Yagur-Kroll et al., 2010 [47] created two bacterial sensors with *sulA* and *grpE* promoters fused to *lux*CDABE reporter gene (*sulA*:: *lux*CDABE and *grpE*:: *lux*CDABE). These genetic constructs were activated by nalidixic acid (NA) and ethanol. To improve sensitivity of this system (earlier response times and an increase in signal intensity) molecular manipulation of the promoter region was introduced. By introducing random or specific mutations in the promoter sequence or by duplicating the promoter sequence (*sulA* only), the length of the promoter-containing segments for both promoters was manipulated [47].

REPORTER-GENES BASED BACTERIA FOR ENVIRONMENTAL APPLICATIONS

The mechanism of gene regulation in bacteria is very important for their adaptation to changing conditions in the environment and in genetically engineering modification for biotechnological purposes. Reporter-genes based bacterial constructs appear as very useful tools for the analysis of promoter activity in a variety of Gram-negative population of bacteria and at single-cell levels [11, 14]. Silva-Rocha and de Lorenzo 2012 [38] described a bicistronic reporter system integrated to the bacterial chromosome, which utilizes an artificial operon containing *gfp* and *lac*Z genes. This genetic system was successfully applied for monitoring and characterization of two promoters – *Pb* and *Pc*, which are responsible for the expression of the benzoate and catechol degradation pathways of the soil bacterium *Pseudomonas putida* KT2440 [38].

Various biosensors for BTEX mainly rely on XylR – main transcriptional regulator of the TOL pathway in *Pseudomonas putida*. Behzadian et al., 2011 [5] described the construction of recombinant biosensors where in the pGLPX plasmid, the reporter *luc* gene is under the control of the *Pu* promoter. To enhance the sensitivity of bioreporting, the ability of two distinct nucleotide sequences functioning as SD elements and effect of introducing T_2rrnβ terminator on the specificity of the construct were evaluated. These manipulations showed that SD sequence (*taaggagg*) was important for biosensor sensitivity, but the presence of the T_2rrnβ terminator reduced biosensor sensitivity after exposition to inducers – varied concentrations of nitrotoluenes [5].

For improvement of the activity of the genetic circuit toward XylR inducers de Las Heras and de Lorenzo 2012 [13] utilized mini-transposon vectors and constructed a strain with transcriptional fusion between the *Pu* promoter and T7 RNA polymerase, along with a T7 promoter driving expression of the *lux*CDABE operon. This biosensor strain produced a dramatic increase of bioluminescence emission in comparison to the strain that carries direct fusion *Pu*::*lux*CDABE [13].

Nowadays, human activities very often result in environmental contamination with copper compounds that influence the ecosystem condition as well as human health. Ng et al., 2012 [28] in their experiment created a copper biosensor with genetic fusion of *cop* operon of *Achromobacter sp*. AO22. The construct had a high potential as biosensor for detecting copper bioavailability in a soil bacterial background [28].

Bacteria-based reporter genes systems are suitable for on-line monitoring of the efficiency of pollutants biodegradation [10, 37]. In the work of Shin et al., 2011 [36] a phenanthrene-degrading bacterium, *Sphingomonas paucimobilis* EPA505 was used to construct strain D with *gfp* as a reporter; this generated strain emits green fluorescence when it biodegrades phenanthrene. The reporter strain was activated during the changes of phenanthrene concentration and potential use of these strains in quantitatively determining biodegradable or toxic phenanthrene was discussed [36, 37].

Tecon et al., 2009 [42] created a double-tagged reporter strain *Burkholderia sartisoli* RP037-mChe, which is capable of mineralizing phenanthrene (PHE) and induces the expression of *gfp* as a function of the PAH flux to the cell. Simultaneously, this strain constitutively produces a second autofluorescent protein (mCherry). *Burkholderia sartisoli* RP037-mChe successfully expressed *gfp* proportionally to dosages of naphthalene or PHE in batch liquid cultures, so it could be useful as a bioreporter for the study of polycyclic aromatic hydrocarbon diffusion and bioavailability [42].

Abd-El-Haleem et al., 2007 [1] developed a *lux*CDABE-based *Klebsiella oxytoca* bioluminescent bioreporter for detection of nitrate/nitrite bioavailability in wastewater. 500-bp DNA fragment containing a nitrate/nitrite-activated nasR-like promoter was fused upstream of the *lux*CDABE gene cassette in a mini-Tn5 vector. Positive correlation between the concentrations of nitrate, nitrite and ammonium (from 1 to 11 ppm) and the intensity of bioluminescence has been noticed [1].

REPORTER-GENES BASED BACTERIA FOR ANTIBIOTIC BIOSENSORS CONSTRUCTIONS AND MONITORING OF BIOFILM FORMATION

Antimicrobial drugs are the most important medicines used in health care, more than any other class of drugs, and they have accounted for an increased life expectancy in humans. Inadequate application and widespread use of antibacterials in humans and animals and their occurrence in the environment and food accelerate bacterial resistance phenomenon. Cellular biosensors based on various microbial species, containing genetic fusion of reporter genes under the control of selected promoters that respond to antibiotic-induced stress are becoming increasingly popular for screening and characterization of inhibitors during the process of antibacterial drug discovery and to fight against the spread of bacterial resistance, and the development of new antibacterial compounds [22, 31, 43].

A particularly useful, as an antibiotic biosensor, strain has been developed by Urban et al., 2007 [43] using *Bacillus subtilis* 1S34 as the host. In the experiment authors generated and validated a set of five *Bacillus subtilis* promoters fused to the firefly luciferase reporter gene that signal the presence of inhibitors of the major biosynthetic pathways of bacteria: fatty acid synthesis (*fabHB* promoter), DNA synthesis (*yorB* promoter), cell wall synthesis (*ypuA* promoter), RNA synthesis (*yvgS* promoter) and protein synthesis (*yheI* promoter) [22, 43]. During large-scale validation of biosensor strains library 14,000 pure natural products, as a source of highly diverse chemical entities, were used. 6% of them had anti-*Bacillus subtilis* activity of ≤25 μg/ml. These biosensors represent promising candidates in antibiotic drug discovery and for detecting the mechanisms of action of a broad spectrum of antibiotics [43].

In work of Song et al., 2012 [39] three separate genetic strategies – with *lacZ*, *selA* and *nuoA* relied on gene induction from the Tn10 *tetA* promoter in *Escherichia coli* – were tested for specific detection of the antibiotic tetracycline (Tet). *LacZ* and *nuoA* biosensors successfully responded to tetracycline with detection limits of 0.11 µg/ml and 0.0026 µg/ml, respectively. The *selA*-based assay was not sensitive enough to detect Tet. It was the first report in which a novel and more sensitive respiratory gene – *nuoA* was used as a reporter gene in an amperometric biosensor [39].

Quorum sensing (QS) is a special kind of bacteria communication system with synthesis of signaling molecules and control of certain behaviors, including pathogenisity and biofilm formation. In the study of Struss et al., 2012 [40] a genetically engineered bioluminescent bacterial sensing systems as a tool to evaluate the ability of antibiotics commonly employed in the treatment of chronic inflammatory conditions to interfere with QS were created. The antibiotic effect of ciprofloxacin, metronidazole and tinidazole on quorum sensing was determined by monitoring the biosensor's bioluminescence response. Ciprofloxacin, metronidazole and tinidazole expressed a dose-dependent augmentation in bacterial sensing systems, showing an AHL-like effect. The results indicated that ciprofloxacin, metronidazole and tinidazole may interfere with bacterial communication systems and have beneficial effect in the treatment of intestinal inflammation [40].

The knowledge of main mechanisms of biofilm forming *Pseudomonas aeruginosa* and other bacteria in chronic infections, such as cystic fibrosis, bronchopneumonia and different infections is of major concern, especially in the development of novel anti-pathogenic drugs. The second messenger cyclic di-GMP is a positive regulator of biofilm formation. In the work of Rybtke et al., 2012 [35], authors described the creation of fluorescent biosensor by transcriptional fusion of the cyclic di-GMP responsive *cdrA* promoter and *gfp* reporter gene. That device can gauge the cellular level of cyclic di-GMP in *P. aeruginosa*. The reporter construct gave a fluorescent read-out of the intracellular level of cyclic di-GMP in *P. aeruginosa.* This reporter construct had a significant potential for use in the identification of novel anti-pathogenic compounds targeting cyclic di-GMP signaling, as well as for the better understanding of the biofilm biology of *P. aeruginosa* [35].

BACTERIAL AND YEAST BIOSENSORS FOR EVALUATING POTENTIAL IMPACT AND TOXICITY OF EDCS, DIOXINS AND DIFFERENT CHEMICALS

Contamination of the environment with some chemicals originating from the technological development, connected with widespread use of hazardous substances such as: polychlorinated biphenyls, dioxins, pesticides, fungicides, herbicides, plasticizers, drugs, antimicrobials, and flame retardants affects human health as a result of interference with endogenous hormones in the organisms. These chemicals called endocrine-disrupting compounds (EDCs) can modulate the activity of endocrine system and result in developmental, reproductive abnormalities and tumor formation in sensitive target tissues [9, 27]. Dioxins and dioxin-like polychlorinated biphenyls (DL-PCBs) are hazardous, toxic, ubiquitous and persistent chemicals with great ability to accumulate in food chain up to higher trophic levels [12]. So, there is a need of monitoring and limiting such dangerous

chemicals in water, air, soil and food. The biological activity of EDCs have been monitored by use of biomarkers such as vitellogenin, choliogenin and a DNA binding assay. Because of their diversity and simple nature, genetically engineered bioluminescent *E. coli* biosensors, which are sensitive to oxidative, DNA, membrane or protein damage, have been applied to EDCs screening and classification [8, 16]. Applications of reporter genes for screening of hormonal active compounds in the environment are broadly described and summarized by Svobodová and Cajthaml 2010 [41].

Much attention has been paid to chemicals that are able to interfere with the activity of the human estrogen receptor (hER) or androgen receptor (hAR) directly. These hormones are involved in the regulation of a wide variety of processes, including development, metabolisms, immune response, behaviour and stress resistance. Endocrine disrupting compounds (EDCs), which mimic the activity of estrogen or different hormones, also present in our environment, food and water can affect human health by disrupting the endocrine system. Many nonsteroid substances can bind to the human estrogen receptor α (ERSα) and mimic the natural estrogen 17β-estradiol and disrupt endocrine function in humans and animals [3–4, 9].

Bovee et al., 2011 [9] constructed a recombinant yeast cell that in response to glucocorticoids expresses the human glucocorticoid receptor alpha and GFP reporter protein. This yeast glucocorticoid bioassay was tested by exposures to cortisol and other related compounds and showed sensitivity to all chemicals, with the following order in relative potencies: budesonide > corticosterone > dexamethasone > cortisol = betamethasone > prednisolone > aldosterone. The obtained data were critically compared to a more sensitive GR-CALUX bioassay based on a human bone cell [9, 49].

Gierach et. al., 2011 [16] developed bacterial biosensors for evaluating potential impacts of estrogenic endocrine disrupting compounds in multiple species. *Escherichia coli* were incorporated with the ligand-binding domains (LBDs) of the *β*-subtype estrogen receptors (ERβ) from *Solea solea* (sole), and *Sus scrofa* (pig). The presence of ligands was indicated with use of strains for these receptors by changes in growth phenotype. It could differentiate agonist from antagonist and give a rough indication of binding affinity *via* dose-response curves. In tests the strains correctly identified estrogenic test compounds, so it can be useful in initial comparative analysis of EDCs impacts across multiple species [16].

Usually, traditional receptor-mediated reporter assays in yeast were performed twelve to twenty four hours after ligand addition and required high, nonphysiological concentrations of ligand, but Balsiger et al., 2010 [3] developed and described a yeast based screen that provides a rapid and sensitive method for EDCs, even in the four-hour and without sample extraction, concentration or sterilization.

In Table 1 selected genetically modified reporter-genes microorganisms and human cells biosensors are presented.

The main methodical problems in promoter-reporter gene systems constructions of biosensors is the specificity and sensitivity of promoter region, correct selection of the type of reporter gene/protein and genetic mechanisms of vectors integration (plasmids or chromosomal insertion). Table 2 presented defects and advantages of different methodical solutions in promoter – reporter gene systems constructions of biosensors with use of the most popular *gfp* or *luxAB/luc* reporter genes.

Table 1. Selected genetically modified reporter-genes microorganisms and human cells biosensors [3, 8–9, 49]

Biosensors	Analyte detection	Strain or promoter and the mechanisms of toxic action
Microbial biosensors:		
Microtox®	Water toxicity	*Vibrio fischeri*
ToxAlert®	Toxicity	*Vibrio fischeri*
Cellsense®	Toxicity	*Escherichia coli*
SPR-CELLIA	Toxicity	Whole cells
Micredox®	BOD toxicity	Microorganisms
BOD_5 Biosensor	BOD	*Pseudomonas putida*
Remedios	Toxicity	Luminescent bacteria
E. coli recA::lux	EDCs, dioxins	*RecA* – DNA damage
E. coli fabA::lux	EDCs, dioxins	*FabA* – membrane damage
E. coli katG::lux	EDCs, dioxins	*KatG* – oxidative damage
E. coli grpE::lux	EDCs, dioxins	*GrpE* – protein damage
E,coli lac::lux	EDCs, dioxins	*Lac* – general damage
Eucaryotic cells biosensors:		
Yeast biosensors		
Four-hour yeast-based bioassay*	Estradiol	Modified receptor-mediated *β*-galactosidase reporter assay
Yeast glucocorticoid bioassay**	Cortisol	Human glucocorticoid receptor alpha and *gfp* as a reporter
Recombinant cell lines biosensors – CALUX and CAFLUX***	Dioxins	Firefly luciferase or enhanced green fluorescent protein as a reporters

* biosensor developed and described by Balsiger et al., 2010 [3].
** biosensor developed and described by Bovee et al., 2011 [9].
*** biosensor developed and described by Zhao et al., 2010 [49].

SUMMARY

Human activities have an impact on the environment, resulting in the contamination of water, the atmosphere and soil with chemicals (heavy metals, EDCs, dioxins and the others) which have cytotoxic and genotoxic activity and very often can trigger living cell cancerogenesis. So, there is a need to develop rapid, cheap, effective and useful methods to detect and monitor such xenobiotics in the environment, especially in drinking water and food. The major limitations of analytical methods (physicochemical, mainly GC-MS or HPLC) include their cost, expensive equipment, well-trained stuff and the lack of hazard and toxicological information. Nowadays, for these reasons, the reporter genes and genetic engineering technology development of microbial biosensors which are very popular devices as a first-step in environmental, water and food hazardous substances pre-screening, as well as for medical and especially pharmaceutical research. On-site monitoring by microbial biosensors could be a useful alternative to chemical analysis.

Table 2. Defects and advantages of the most popular and useful *gfp* or *luxAB/luc* different methodical solutions in promoter-reporter gene systems constructions of biosensors for environmental application [8, 14, 17, 21, 32]

reporter genes	defects	advantages
gfp	Variability of GFP expression in different species unknown Plasmids may be unstable – use chromosomal insertion Influence of environmental conditions on GFP expression is unknown Interference by other fluorescent particles or bacteria Extended lifetime of fluorescence once cell had died or lysed GFP may not work under anaerobic conditions Strong stability of GFP is not very useful for the monitoring of the strength of different promoters	Principle of detection: Fluorescence Ease of detection by: (1) Epifluorescent microscopy (2) Fluorimetry (3) Flow cytometry (4) Plate counting No exogenous substrate needed No processing of cells required Single-cell detection with high resolution No fixing or staining of samples/cells necessary; but detection of fluorescence still possible in formaldehyde fixed cells Non-destructive; detection without disruption of microbial community Possible to monitor on-line or in real time Extremely stable – heat (658C); pH (6–12); resistant to denaturants and proteases. Different variants of *gfp* mutations are available for different experiments GFP expressed in cytoplasm; should have minimal effect on cell – surface dynamics Continually synthesized; minimizes fluorescence signal dilution during bacterial replication Allows analysis of living cells; repeated readings under various conditions for the same cell is possible No GFP background in indigenous bacterial populations Dual detection possible with different coloured markers
luxAB/luc	Additional substrate requirement for detection – n-decanal/luciferin Single-cell detection with low resolution Variability of *luxAB/luc* expression in different species unknown Plasmids may be unstable – use chromosomal insertion Influence of environmental conditions on *luxAB/luc* expression is unknown Interference by other luminescent particles or bacteria The work of *luxAB/luc* depends on metabolic status of bacteria cell	Principle of detection: Luminescence (1) Luminometry (2) CCD digitized camera (3) Flow cytometry (4) Plate counting

CONCLUSIONS

1. The current basic techniques in nanotechnology, genetic engineering, and microelectronics are tools for discovering and creation of novel, more robust reporter genes and their mutants, which provide stronger sensitivity, miniaturized, high-throughput, wireless/mobile and automated devices for ubiquitous multi-functional, *in situ* and *real-time* monitoring.
2. To improve sensitivity of this system (earlier response times and an increase in sensitivity and signal intensity) some new scientific achievements in molecular genetic manipulation have been developed, mainly:
   – introduction of random or specific mutations in the promoter sequence or by duplicating the promoter sequence,
   – manipulations of SD sequence (*taaggagg*) was important for improvement of biosensor sensitivity,
   – utilization of mini-transposon vectors could improve the strength of biosensor signal emission,
   – finding a new, more sensitive reporter genes (for example *nuoA* – respiratory gene) for enhancement of biosensors work,
   – using bacteria quorum sensing (QS) signaling system for improvement of biosensors sensitivity and evaluation of the ability of antibiotics action,

Microbial biosensors and their future genetic and technological improvements offer great promise for their application.

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ZASTOSOWANIE OPARTYCH NA GENACH REPORTEROWYCH BIOSENSORÓW W SCREENINGU CHEMICZNYCH ZANIECZYSZCZEŃ W ŚRODOWISKU

Prezentowana praca przeglądowa zawiera opis możliwości aplikacyjnych biosensorów opartych na genetycznie zmodyfikowanych mikroorganizmach wyposażonych w geny reporterowe. W pracy przedstawiono defekty i zalety nowych naukowych osiągnięć oraz metodologicznych rozwiązań dotyczących genetycznych systemów w biosensorach przeznaczonych do środowiskowego screeningu zanieczyszczeń. Opisano najbardziej użyteczne i popularne genetyczne fuzje sekwencji promotorowych z takimi genami reporterowymi jak: *lacZ* (β-galaktozydaza), *xylE* (katechol 2,3-dioxygenaza), *gfp* (gen białka zielonej fluorescencji) oraz jego zmutowane warianty, *lux* (prokariotyczna lucyferaza), *luc* (eukariotyczna lucyferaza), *phoA* (alkaliczna fosfataza), *gusA* i *gurA* (β-glukuronidaza), geny oporności na antybiotyki oraz metale ciężkie. Tego typu mikrobiologiczne biosensory znalazły szerokie zastosowanie w testach genotoksyczności, badaniach nad biodostępnością oraz stresem oksydacyjnym, a także w detekcji i monitoringu substancji toksycznych w wodzie pitnej, różnych próbach środowiskowych, wodach powierzchniowych, glebie i osadach.