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ORIGINAL PAPER

Construction of 2,4,6-Trinitrotoluene Biosensors with Novel Sensing Elements from *Escherichia coli* K-12 MG1655

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Abstract Detection of 2,4,6-trinitrotoluene (TNT) has been extensively studied since it is a common explosive filling for landmines, posing significant threats to the environment and human safety. The rapid advances in synthetic biology give new hope to detect such toxic and hazardous compounds in a more sensitive and safe way. Biosensor construction anticipates finding sensing elements able to detect TNT. As TNT can induce some physiological responses in *E. coli*, it may be useful to define the sensing elements from *E. coli* to detect TNT. An *E. coli* MG1655 genomic promoter library containing nearly 5,400 elements was constructed. Five elements, *yadG, yqgC, aspC, recE*, and *topA*, displayed high sensing specificity to TNT and its indicator compounds 1,3-DNB and 2,4-DNT. Based on this, a whole cell biosensor was constructed using *E. coli*, in which green fluorescent protein was positioned downstream of the five sensing elements via genetic fusion. The threshold value, detection time, EC_{200} value, and other aspects of five sensing elements were determined and the minimum responding concentration to TNT was 4.75 mg/L. According to the synthetic biology, the five sensing elements enriched the reservoir of TNT-sensing elements, and provided a more applicable toolkit to be applied in genetic routes and live systems of biosensors in future.

Keywords Synthetic biology · Biosensor · TNT · GFP · Promoter

Introduction

The chemical, 2,4,6-trinitrotoluene (2,4,6-TNT), is the most common explosive material. With its wide use in military, civil, and other fields, TNT is also a toxic pollutant, contaminating the environment and causing injuries. TNT can be found in air, water, and soil, threatening human health. Because of mutagenicity and carcinogenicity of TNT and its degradation, it can greatly harm both aquatic and terrestrial organisms [1–3]. Long-term exposure to TNT increases the chances of developing anemia and cancer and may damage the liver, spleen, and immune system. In many military TNT testing and manufacturing sites, the soil and underground water are contaminated, even producing "pink water." Complete cleaning up of TNT-contaminated sites is very difficult and expensive [4].

As a cheap and efficient explosive, TNT has been used as the main filling for a variety of conventional explosive bombs and landmines used in war. Many landmines and bombs were forgotten after wars were over. Due to the long-term explosiveness of TNT, these landmines and other explosives continue to threaten people's lives up to today. According to UN statistics, at least 78 countries around the world are still affected by landmines and explosive remnants of war, which cause nearly 20,000 deaths and injuries each year [5]. China has been severely affected by landmines and explosive remnants. Many of

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China's border areas, such as Yunnan and Guangxi Provinces, are still affected by remaining minefields. Therefore, accurate and effective location and detection of the explosive remnants of war are of urgent need. Landmines are very difficult and dangerous to detect since they are hidden. Thus far, there is no fast, effective, and inexpensive method to detect large-scale minefields. The traditional detection methods include handheld metal detection, animal-based olfactory detection, and plant exploration. These methods are highly risky, time-consuming, and very costly. In addition, these methods have limitations. Handheld metal detection cannot detect plastic or ceramic mines, and for animal-based olfactory detection, the subjective uncertainty of animals is very high.

Currently, there are many laboratory techniques used for detecting TNT, such as high performance liquid chromatography (HPLC), ultraviolet, gas chromatography–mass spectrometry (GC–MS), laser surface-enhanced Raman spectroscopy (SERS), nuclear magnetic resonance, ion mobility spectrometry, etc., but they require expensive and complex equipment and/or complex sample preparation. The above methods are based on the physical properties of TNT. The recent development of synthetic biology suggests that the biological activity of TNT has the potential to be used to develop new detection methods.

Synthetic biology involves assembling specific biological modules in order to achieve a specific function, such as a genetic pathway [6]. The construction of bacterial biosensors using biological elements has been extensively studied and used for detection of specific compounds, chemical groups, and toxic compounds that are environmental pollutants [7–11]. Promoter elements, which sense target molecules and a reporter gene capable of providing a detectable signal, are generally the core elements used for building a biological sensor. A reporter element is positioned under the control of a promoter element. The target compound can activate the promoter in the sensor and induce the expression of a downstream reporter gene. A number of reporter genes have been developed. The most commonly used reporter genes are chemiluminescent (e.g., *luxCDABE* of photorhabdus luminescens [12] and fluorescent proteins (e.g., green fluorescent protein, GFP [13]).

Sensing elements are essential for developing effective biosensors. Recently, many biological sensing elements have been developed to detect toxic and hazardous compounds, including TNT [14]. In 1998, the first patent was granted for a recombinant bacterium engineered to detect TNT in soil to identify and label the location of mines in minefields [15, 16]. TNT can stimulate the sensing element of this bacterium in the environment to activate downstream GFP expression, which can then be visualized to locate mines in soil. Although a system for landmine detection was proposed based on this technology, it was never applied in real situation. Following this proposal, another biosensor system, in which the biosensor bacteria for TNT detection were wrapped in small beads, was developed. With technological improvement, key genes of bacterial stress response system as sensing elements for TNT [17], and the SOS/umu test was applied to detect the TNT content in soil. Using an error-prone polymerase chain reaction and DNA fragment shuffling technique, Garmendia et al. [18] constructed and obtained transcriptional regulatory protein XylR mutants that sense 2,4-DNT in the metabolic pathway of *Pseudomonas putida* mt-2 for toluene and its derivatives. The GFP gene was fused to the downstream of Pu promoter, regulated by XylR to construct biosensors to detect 2,4-DNT based on *P. putida*. Therefore, the essential step in construction of these biosensors is able to determine the biological sensing elements of target molecules.

TNT has been confirmed to activate some physiological pathways of *Escherichia coli* [17, 19], suggesting the existence of biological sensing elements in *E. coli* for the construction of biosensors to detect TNT. Previous studies have identified TNT-sensing elements in *E. coli* for constructing TNT biosensors [20, 21]. In the current study, different methods were used to build the genetic libraries to discover more genetic elements in the *E. coli* genome that were able to sense TNT. In this study, we first constructed an *E. coli* genomic promoter library. A rigorous vector, pTJ629, based on the pTJH521 vector, was built for *E. coli* promoter screening. An *E. coli* K-12 MG1655 promoter library was then built with pTJ629 as a backbone. A number of new promoter elements, which were responsive to TNT, were obtained from the nearly 5,000 promoters that were screened. The new promoter elements were then ligated to a pPROBE-TT vector [22]. Biosensors were constructed with GFP and their sensitivity and specificity in response to TNT was analyzed.

Materials and Methods

Compounds

Compound 2,4,6-TNT, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 1,3-dinitrobenzene (1,3-DNB), 1,4-dinitrobenzene (1,4-DNB), 1,4-dinitrobenzene (1,4-DNB), 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), 4-nitrotoluene (4-NT), benzene, toluene, and nitrobenzene were purchased from Sigma-Aldrich Corporation (USA). All hydrophilic compounds were dissolved in Tris buffer (200 mM, pH 7.0). Hydrophobic compounds were first dissolved in methanol and then diluted in Tris buffer. TNT was dissolved using an ultrasonic method. TNT powder was added to an excess of distilled water, followed by 800 W ultrasound for 15 min [20].

Strains and Growth Conditions

Information on the strains and plasmids used in this study are listed in Table 1.

Escherichia coli K-12 MG1655, a gift from Professor Wang Hengliang at the Beijing Institute of Biotechnology, was used as a clone template for this study and as a source for library construction. *E. coli* DH5α was the host strain for cloning and screening. Strains for GFP detection were cultured in TGA medium (10 g/L Bacto Tryptone, 5 g/L NaCl, 2 g/L D-(+) glucose, 11.9 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, C8H18N2O4S), pH 7.0). Since TGA medium exhibits a lower value of background fluorescence, all other culture conditions, unless described specifically, used Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0). Bacteria were cultured at 37 °C unless otherwise described. All bacteria were cultured under aerobic conditions.

96-well deep well plate was used for bacterial library construction and screening (Thermo Scientific). The 600 nm absorbance (OD_{600}) of the bacterial broth was measured in a Unic UV spectrophotometer.

Antibiotic streptomycin, tetracycline and kanamycin were used at a working concentration of 50 μg/mL.

Vectors used in this study were derived from pTJ629. Vector pTJH521 was constructed and stored in our laboratory. *E. coli* AS.1.1137, containing vector pSC101, was purchased from China General Microbiological Culture Collection Center. Cloning vector pMD18-T was purchased from China Dalian Takara Biotechnology Co., Ltd. pPROBE-TT was purchased from Addgene (USA).

Molecular Manipulation and DNA Sequencing

All molecular manipulations used in this study were performed according to standard procedures or kit instructions. The enzymes used in experiments were purchased from New England Biolabs, Inc. (USA). All plasmids were transformed using the chemical heat shock method. The polymerase chain reactions (PCR) were done using the NEB Q5 MIX high-fidelity PCR system. The PCR-amplified products (72 °C, 20 min) were elongated with dATP to form polyA and then cloned into pMD18-T (Takara, Japan) cloning vector by DNA ligation. T4 DNA ligase (New England Biolabs, USA) was used for all DNA ligation procedures, which were done overnight at 16 °C. A QIAGEN Plasmid Midi Kit (Qiagen, Germany) was used for plasmid extraction. The *E. coli* K-12 MG1655 genome was extracted

Table 1 Strains, plasmids, and primers used in this study

	Description	References
Strain		
MG1655	F-l-ilvG-rfb-50 rph-1	[39]
DH5α	F-endA1 hsdR17 (rk−, mk+) supE44 thi-1 λ-*recA*1 gyrA96 relA1deoR Δ(lacZYA-argF)-U169 ϕ80dlacZΔM15	[40]
Plasmid		
pSC101	Tet^r, with pSC101 ori	[23, 24]
pTJH521	Kan^r, promoter-less luxCDABE, with colE, oriV	Laboratory construction
PTJ629	Kan^r, promoter-less luxCDABE, with pSC101 ori	This work
pPROBE-TT	Tetr, promoter-less GFP	[22]
Primer		
*recE-Bam*HI	5′-CGGGATCCGCCACTCTGGACTA-3′	This work
*recE-Kpn*I	3′-GGGGTACCAAAATTCGTTTTGTTC-5′	This work
*topA-Bam*HI	5′-CGGGATCCTTCGTGAGCATGACGA-3′	This work
*topA-Kpn*I	3′-GGGGTACCAATTGCCCAGCAGCG-5′	This work
*yqgC-Bam*HI	5′-CGGGATCCAGGCTGCGATAGTCGTTAAC-3′	This work
*yqgC-Kpn*I	3′-CGGGATCCAAAGTGGAAATAGGGTGAA-5′	This work
*aspC-Bam*HI	5′-CGGGATCCATCGGCCAGGCCCAGAAT-3′	This work
*aspC-Kpn*I	3′-CGGGTACCACAGGACTTTGGTCCTGTT-5′	This work
*yadG-Bam*HI	5′-CGGGATCCATCCTCTTCCACCAGCATTTT-3′	This work
*yadG-Kpn*I	3′-CGGGTACCATTTACCAGAGAGCTGAT-5′	This work
*pSC101ori-Avr*II	5′-GGCCTAGGGCGGAACTGACTAAAGTAGT-3′	This work
*pSC101ori-Spe*I	3′-GCACTAGCTAAGTCTGTATGTAGAGTTAA-5′	This work

using a QIAamp DNA Mini Kit (Qiagen, Germany) and was used as a template for the construction of a promoter library. DNA gel electrophoresis and recovery of fragments that were about 5,000 bp was done using a 1 % agarose gel. Recovery of fragments that were more than 5,000 bp was done using a 0.6 % agarose gel. QIAquick Gel Extraction Kit (Qiagen, Germany) was used for recovery. DNA concentration was quantified in a Thermo Nano Drop 2000 (Thermo Scientific, USA).

pMD18-T was sequenced using the universal primer M13 (5′-TGTAAAACGACGGCCAGT-3′). PTJ629 vector was sequenced using primer 5′-GAGCTCGCATTTCCGA-3′. pPROBE-TT was sequenced using the sequencing primer 5′-AGCATTGAACACCATAAGTC-3′. All sequencings were performed by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China).

Vector Construction

The pTJH521 vector, a broad host promoter-screening platform, was constructed and preserved in the laboratory. It contained a promoter-deleted luxCDABE gene, a broad host replicon oriV and an *E. coli* ColE1 replicon, which is a high copy replicon. The new vector had to have a low copy number in *E. coli* in order to screen promoters, so the ColE1 replicon was replaced with the strict replicon, pSC101 [23, 24].

First, *E. coli* AS.1.1137 was cultured overnight in LB medium containing 50 ng/μL tetracycline and the pSC101 plasmid was extracted. The pSC101 vector replicon, ori, was then amplified by PCR to introduce *Spe*I and *Avr*II restriction sites (Fig. 1). After enzyme digestion, ori was ligated into the pTJH521 vector to replace the CoLE1, in order to obtain *E. coli* promoter-screening platform pTJ629 vector.

Random Promoter Library Construction

The *E. coli* K-12 MG1655 strain was recovered in LB medium containing 50 μg/mL streptomycin, cultured overnight for 12 h and diluted 100 times. The *E. coli* K-12 MG1655 strain was then inoculated into fresh LB medium containing 50 μg/mL streptomycin for another 12 h, after which the bacteria were collected for extraction and purification of the *E. coli* K-12 MG1655 chromosome genome. The restriction enzyme, *Sau3A*I, was used to partially digest the genomic DNA (37 °C, 2 h) and DNA fragments below 2 kb were purified and recovered by agarose gel electrophoresis. The pTJ629 vector was digested by a single restriction enzyme, *Bam*HI (*Bam*HI produces the same sticky end as *Sau*3AI). The digestion system was treated via heat inactivation in a 65 °C bath for 20 min and the digested vector was dephosphorylated using NEB CIP (0.5 U CIP enzyme per microgram of DNA) in a 37 °C bath for 1 h. Single-enzyme-digested fragments were dephosphorylated and finally ligated with MG1655 genomic fragments using NEB T4 DNA ligase. The ligation products were transformed into *E. coli* DH5α competent cells by a chemical transformation method. All detectable chemiluminescent clones were picked and cultured in 96 deep well plates (Thermo Scientific, USA) with medium containing 50 mg/L kanamycin, shaken at 37 °C for 18 h and preserved at 20 °C with glycerol.

Promoter Library Screening

The stored, cloned bacteria were inoculated to 96-well deep well plates, cultured overnight for 12 h and recovered in medium containing 50 mg/L kanamycin to screen for *E. coli* MG1655 genes regulated by TNT. The bacterial broth was then diluted 100 times for re-culture. When the 600 nm absorbance (OD_{600}) of the bacterial broth reached 0.6, TNT stock solution was added to a final concentration of 15 mg/L. The solution was then cultured for 8 h, 200 μL of the bacterial broth was added to each hole of black 96-well plates (Thermo Scientific, USA) and the chemiluminescence values were measured in a Perkin Elmer 2300 Multilabel Reader to screen for clones with high absorbance. The clones with promoters were screened a second time, with a concentration of 1 mg/L TNT in order to remove clones that were false positives. False positive and constitutively expressing clones were removed by comparing them with the chemiluminescence values obtained at 15 mg/L TNT. Clones were selected for a significant difference in chemiluminescence values between low and high concentrations of TNT.

Library Sequence Analysis

The 16 positive sensing elements of TNT were amplified by PCR and sequenced. The sequences were then compared with GenBank MG1655 genomic sequences, using the NCBI standard nucleic acid–nucleic acid BLAST program (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi), to align them in the genome for the corresponding gene names and functions.

Construction of Detecting Strains

After promoters regions were confirmed via NCBI BLAST comparison, *E. coli* K-12 MG1655 chromosomal DNA was used as a template and the promoters were amplified using the primers in Table 2 to introduce *Kpn*I and *Bam*HI restriction sites.

PCR-amplified products were separated and purified by gel electrophoresis, followed by double digestion with

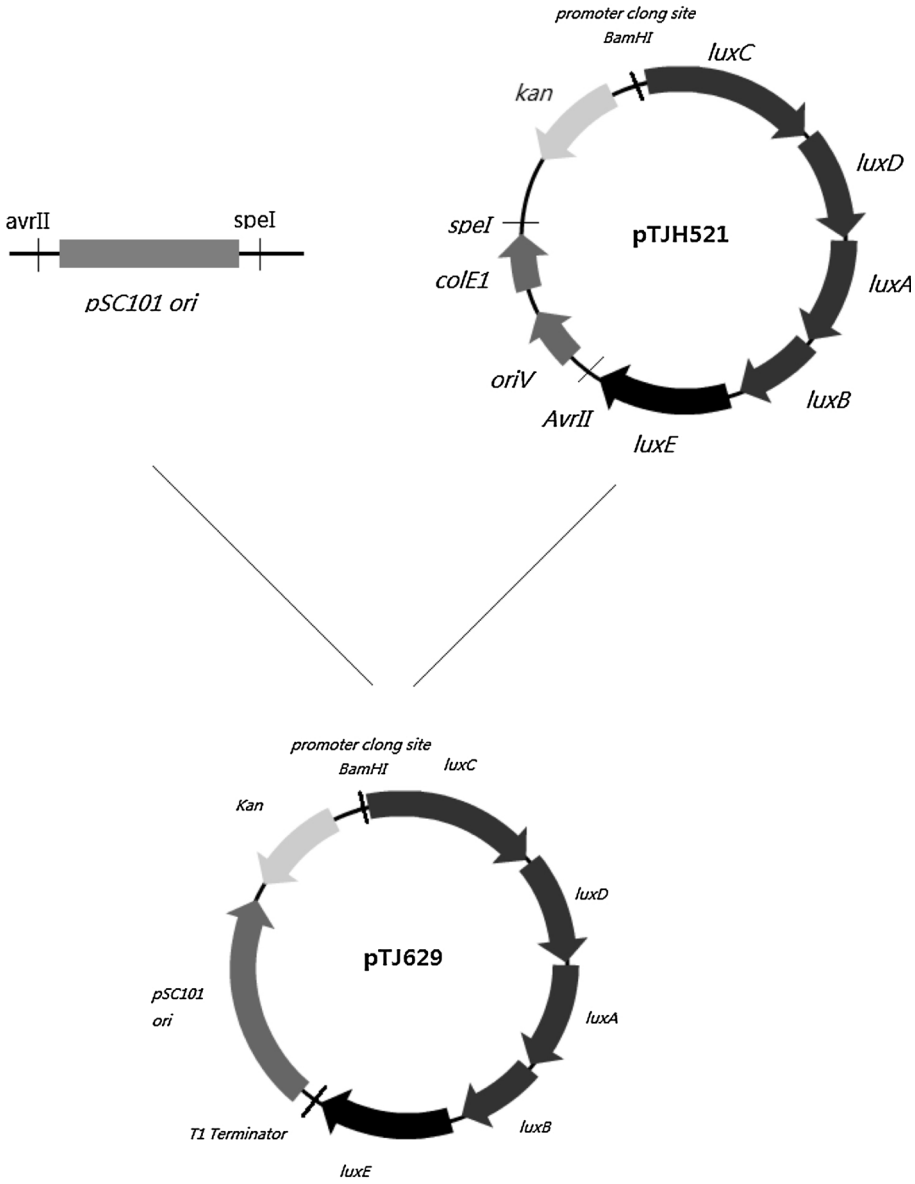

Fig. 1 pTJH521-based pTJ629 vector construction. Using the pSC101 vector as a template, the pSC101 ori was PCR amplified to introduce *avr*II and *Spe*I restriction sites. The pSC101 ori was then ligated into the pTJH521 vector to replace the pTJH521 colE1 replicon. The new pTJ629 vector has stringent replication in *E. coli*

NEB *Kpn*I and *Bam*HI restriction enzymes for 8 h at 37 °C. The corresponding target bands were recovered by gel electrophoresis and were ligated by T4 DNA ligase into the promoter-deleted, sensor detection platform pPROBE-TT vector, which had undergone the same restriction enzyme digestion. GFP was used as a reporter gene to construct biosensors containing promoter GFP. All new constructs were verified by sequencing.

luxCDABE and GFP Detection

All luminescent bacteria for test were removed from a −20 °C freezer. GFP-containing strains and luxCDABE strains were recovered in TGA medium and LB medium, respectively, at 37 °C for 12 h. They were then diluted 100 times and re-cultured in 96-well deep well culture plates. When cultured cells reached the early logarithmic growth state ($OD_{600} = 0.2$), mixed media was added. Different concentrations of enrichment media solutions were mixed with the corresponding compounds to reach the desired final concentration of compounds. When the bacteria grew to the respective detection state, 200 μL bacterial broth was added to 96-well polystyrene assay plates (Bio-Rad) and analyzed in a Perkin Elmer 2300 Multilabel Reader (GFP detection conditions: excitation/emission = 485/535 nm; luxCDABE detection uses general chemiluminescence detection program).

The fluorescence intensity of the instrument was used to indicate the sensing intensity of the promoters to target compounds. All of the fluorescence and chemiluminescence

Table 2 List of identified TNT-regulated genes

Gene name	CDS number	Fold change	Function or class
metG	AAC75175.1	23.14	A member of the family of aminoacyl tRNA synthetases
recE	AAC74432.1	24.23	SOS family
rssA	AAC74316.2	13.61	Putative patatin-like family phospholipase
rssB	AAC74317.1		Pcn-B degradosome ineraction factor; response regulator
lpxC	AAC73027.1	22.39	Catalyzes the second reaction and the first committed step in lipid A biosynthesis
murR	AAC75480.1	28.10	Repressor for murPQ, MurNAc 6-P inducible
yqgC	AAC75976.2	14.93	Uncharacterized protein
hrpB	AAC73259.2	12.82	Putative ATP-dependent helicase
yadG	AAC74355.1	42.39	Putative ABC superfamily
topA	AAC74356.1	19.93	S49 peptidase family protein
aspC	AAC74014.1	24.73	Aspartate aminotransferase
yaiO	AAC73461.1	10.03	Operon outer membrane protein
fnr	AAC75217.1	11.53	Oxygen-sensing anaerobic growth regulon transcriptional regulator autorepressor
ygjQ	AAC76121.1	10.98	DUF219 superfamily protein
acnB	AAC73229.1	40.32	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
pyrD	AAC74031.1	11.22	A membrane-bound flavoprotein

Fold change of gene expression under 15 mg/L TNT condition

values were normalized to that of the bacterial culture broth at same density and were represented by the relative fluorescence units (RFU) or relative luminescence units (RLU). For example, the induction intensity of GFP was represented by fluorescence difference between the presence and absence of the inducer ($\Delta RFU = RFU_{TNT} - RFU_{control}$).

In experiments using solid media plates, each bacterial broth was cultured overnight for 12 h and fresh bacterial broth (OD_{600} value is about 2.0) was diluted to 0.6 at OD_{600}. Then 1 μL broth was added to LB plates, containing different inducing agents. The TNT plates contained 15 mg/L TNT and the IPTG plates contained 1 mM IPTG. The plates were cultured for 8 h under conditions at 37 °C and constant humidity. Place images were taken in a ClinX fluorescence imaging system and the fluorescence intensities of colonies were compared.

EC_{200} Determination

As a common sensitivity indicator, EC_{200} is the concentration of an inducer that causes twofold of an indicator reaction. The smaller is the value of EC_{200}, the stronger of the reaction sensitivity [25, 26]. In the present study, the constructed sensors ($OD_{600} = 0.6$) were exposed to a certain range of concentrations of various compounds, at a increasing gradient. Untreated samples were used as negative controls. After 4 h of culture, the fluorescence change of the reaction was determined. The lowest concentration of a compound was recorded when it induced a twofold change in fluorescence compared to the control.

Statistical Analysis

All statistical analysis was done using SPSS 18.0 statistical software.

Results

Promoter Library Screening

First, the chromosome genome of *E. coli* K-12 MG1655 was extracted and Sau3AI endonuclease was used to digest the MG1655 genome. The fragments were then integrated into a pTJ629 promoter-screening platform. A promoter library with a storage capacity of 5,400 was constructed and downstream of each cloned promoter was coupled to the luxABCDE reporter gene. The response of each clone to target compounds was determined by the chemiluminescence value of luxABCDE. Theoretically, approximately every 2 kb DNA fragment contains one promoter. The total genomic length of *E. coli* K-12 MG1655 was 4.6 Mbp and the coverage rate of the promoter library is two times of total *E. coli* genomic promoters.

A total of 102 clones with high expression of the luxABCDE reporter gene were obtained with induction by 15 mg/L TNT. The 102 clones were also screened with 1 mg/L TNT. After two rounds of screening under high and low TNT concentrations, false positive clones and constitutively expressing clones were removed. The chemiluminescence values of these clones were compared and clones

with fluorescence ratios greater than 3 were selected (Fig. 2). A total of 16 clones were obtained that were regulated by TNT.

The corresponding positions in the *E. coli* K-12 MG1655 genome were identified after comparing the sequencing results of 16 promoter elements with the chromosome genomic sequence (Table 2). These genes belong to different families of proteins and physiological pathways of *E. coli*, such as *topA*/*recE* in the *E. coli* stress and injury pathway, *aspC*/*acnB*/*lpxC* in the amino acid or lipid metabolism pathway, *pyrD*/*yaiO* in the membrane-protein family, the transcription regulatory factors *rssA*/*rssB*/*murR*, as well as other genes with known and unknown functions.

Specificity Study

Using enzymatic digestion and ligation, 16 promoter elements were integrated into the pPROBE-TT platform to construct promoter::GFP biosensors. The sensitivity of the 16 promoters in the pROBE-TT vectors to toluene, 2,4-DNT and other TNT derivatives similar in structure to TNT, was examined. These promoters were further screened in order to identify promoter elements with a higher specificity to TNT or its derivative compounds.

The effective concentrations of different compounds to the five promoters were determined. Under these effective concentrations, the peak fluorescence of these promoters 8 h after different compound was measured. The comparison of these promoters is shown in Fig. 3. Five promoters with strong specificities were identified, namely *yqgC*, *recE, topA, yadG*, and *aspC*. *yqgC* and *recE*, which exhibited a higher response to 1,3-DNB and TNT. However, *yadG, topA*, and *aspC* were more sensitive to TNT.

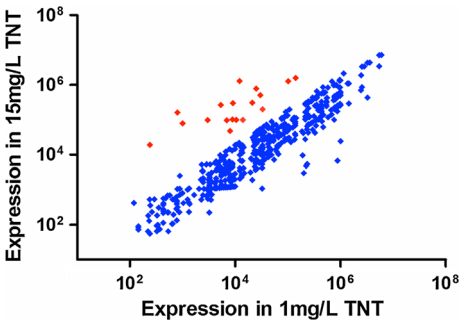

Fig. 2 Scatter plot of the chemiluminescence values of clones induced by high and low concentrations of TNT after 12 h treatment. *Red scatter spots* represent clones showing more than a threefold ratio of values between the high and low concentrations of TNT, indicating that the clone promoter may be induced by TNT. The *blue color* represents clones with a less than threefold ratio, indicating the library promoter fluorescence values are close to constitutively expressed levels or false positivity induced by TNT. *Red dots clones* were separated for further analysis (Color figure online)

The production of TNT unavoidably also produces its derivatives, including 1,3-dinitrophenyl (1,3-DNB), 2,4-DNT, and 2,6-DNT [27]. These compounds can also leak into the environment through production apparatus, mines and bombs as steams. Thus, TNT, 1,3-DNB, and DNT can also be used as target molecules to spot TNT-contaminated mines and other explosives [28]. Therefore, even without complete specificity and sensitivity to TNT, these five promoter elements can still be excellent TNT-sensing promoter components.

We focus on the above five promoter elements in this study, as the other 11 promoter elements did not show good specificity.

TNT Fluorescence Detection

The response of five promoters to liquid TNT was measured. As shown in Fig. 4, the linear responsiveness of the five promoters to TNT was dose-dependent with the concentration of 20–30 mg/L. However, the detection linearity reduced with increased TNT concentration, as the toxicity of increased TNT could disrupt the normal physiological response of the bacteria. It has been confirmed that many bacteria will not grow normally when the TNT concentration reaches 300 mg/L.

The activity of promoters in solid media was also measured. In the absence of inducers, the corresponding colonies of the respective promoter showed no significant fluorescent response, however, each promoter exhibited significant fluorescence after addition of TNT (Fig. 5). With IPTG, only T7-GFP colonies showed fluorescence, suggesting specificity of promoters to inducers. Unable to quantify the fluorescence, we observed that the induced activity of the promoter elements was comparable with the strong promoter T7.

Detection Sensitivity of EC_{200}

All five promoters displayed high detection sensitivities to TNT, among which *yqgC* showed highest EC_{200} value, which reached 4.75 mg/L. The EC_{200} values for 2,4-DNT, 2,6-DNT, and 1,3-DNB were as low as 27, 23, and 4 mg/L, respectively (Table 3).

Studies on Time Effectiveness

According to the results in Fig. 4, the best induction concentration was 20 mg/L for *aspC* and 30 mg/L for *yqgC, recE, topA*, and *yadG*. Thus, with these indicated concentrations, all five sensors started expressing the reporter gene in approximately 50 min. Fluorescence reached the peak in about 100 min and remain stable at 4 h for all five constructs (Fig. 6).

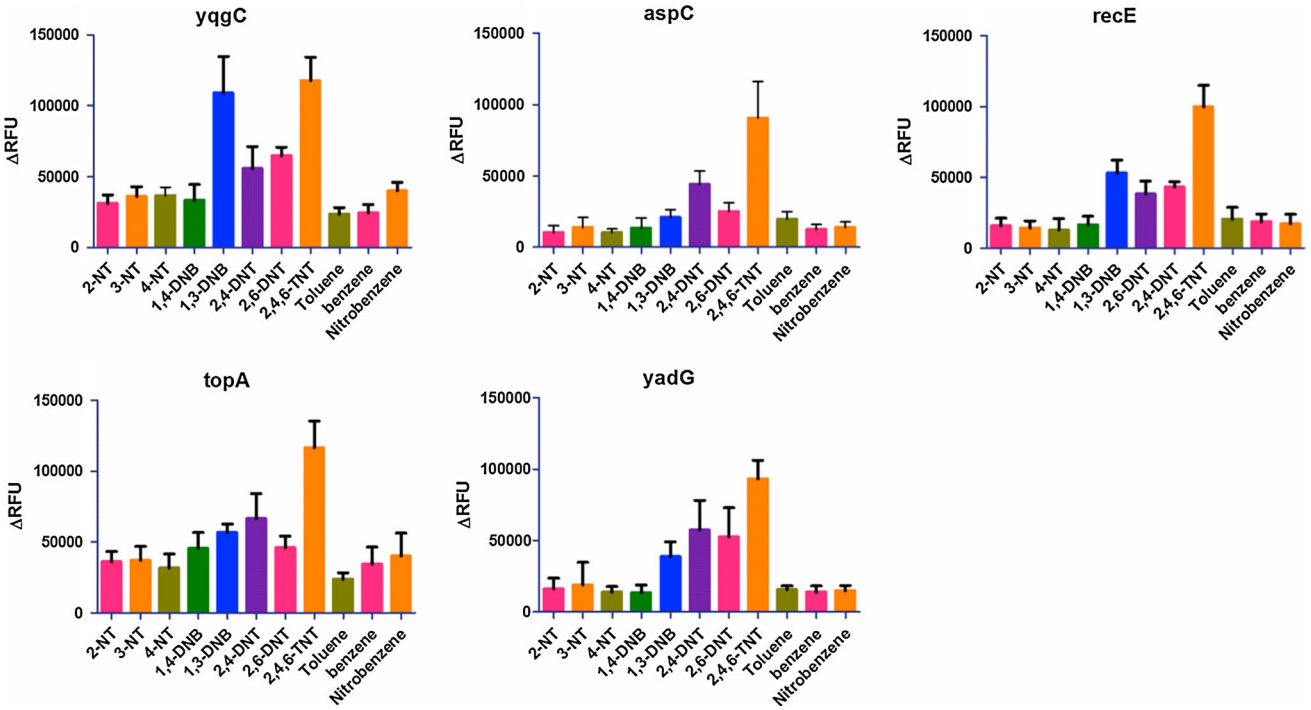

Fig. 3 Luminescence intensity between promoter elements after 8 h in the optimal induction conditions was compared. The 16 biosensors, constructed from the screened elements, reacted with the toluene derived compounds. After comparing the highest promoter activities at their respective optimum concentrations, the five promoter elements were screened out to specifically respond to TNT and its indicator compounds (1,3-DNB, 2,4-DNT, 2,6-DNT). As shown in the figure, *yqgC*, *aspC*, *recE*, *topA*, and *yadG* five demonstrated a significant difference in reaction to TNT, 1,3-DNB, 2,4-DNT, and 2,6-DNT compared with the other compounds ($P < 0.05$)

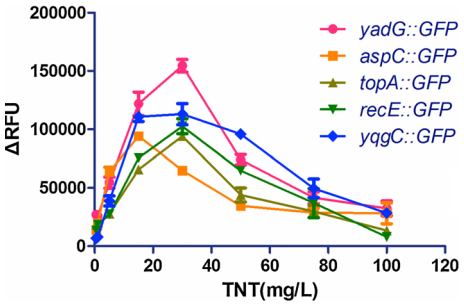

Fig. 4 Reaction strength of *YqgC*, *aspC*, *recE*, *topA*, and *yadG* five promoter elements to different concentrations of TNT. Bacterial broths of five biosensors were cultured to OD_{600} 0.6, induced by different concentrations of TNT and the reaction intensity was measured after 8 h. As shown in the figure, the optimal induction concentration for all five promoter elements was 20–30 mg/L. As the TNT concentration increased, the detection ability decreased, likely due to the high TNT concentration toxicity affecting the normal growth of bacteria

Discussion

As an efficient and high risk explosive, TNT is also a significant environmental pollutant. Biosensors have wide applications, including the identification of mines and other explosives during and after wars, and can also be used to detect trace amount of TNT in soil and water.

In this study, the promoter library of *E. coli* MG1655 constructed from the digested genome and five promoter elements, which could be induced TNT, were identified. Based on our results, biosensors were constructed with GFP as a reporter gene to be visualized for the detection of explosives and environmental TNT pollution.

Five promoter elements were tested, *yqgC, yadG, aspC, recE*, and *topA*. *YqgC* is a protein with a determined sequence, but unknown function. *recE* and *topA* are active factors in the bacterial SOS family [29, 30]. *recE* is one of recombination factors for SOS repair and *topA* is a DNA topoisomerase I. *TopA* is mainly involved in chromosome repair after DNA damage and resolve the chromosome supercoils [31]. It has been reported that TNT can cause genetic damage in bacteria and induce the bacterial SOS response [17]. Previously, our laboratory investigated stress and injury reactions caused by TNT and found that a certain concentration of TNT could induce the expression of some promoters of the SOS family, such as *recA*, *umuDC*, etc. In the current study, *recE* and *topA* were identified in the library, confirming our previous results. Therefore, the effect of *recE* and *topA* may be related to TNT-induced bacteria chromosomal damages. *YadG* is an ATP-binding component of a predicted ATP-binding cassette (ABC) superfamily efflux transporter and a putative

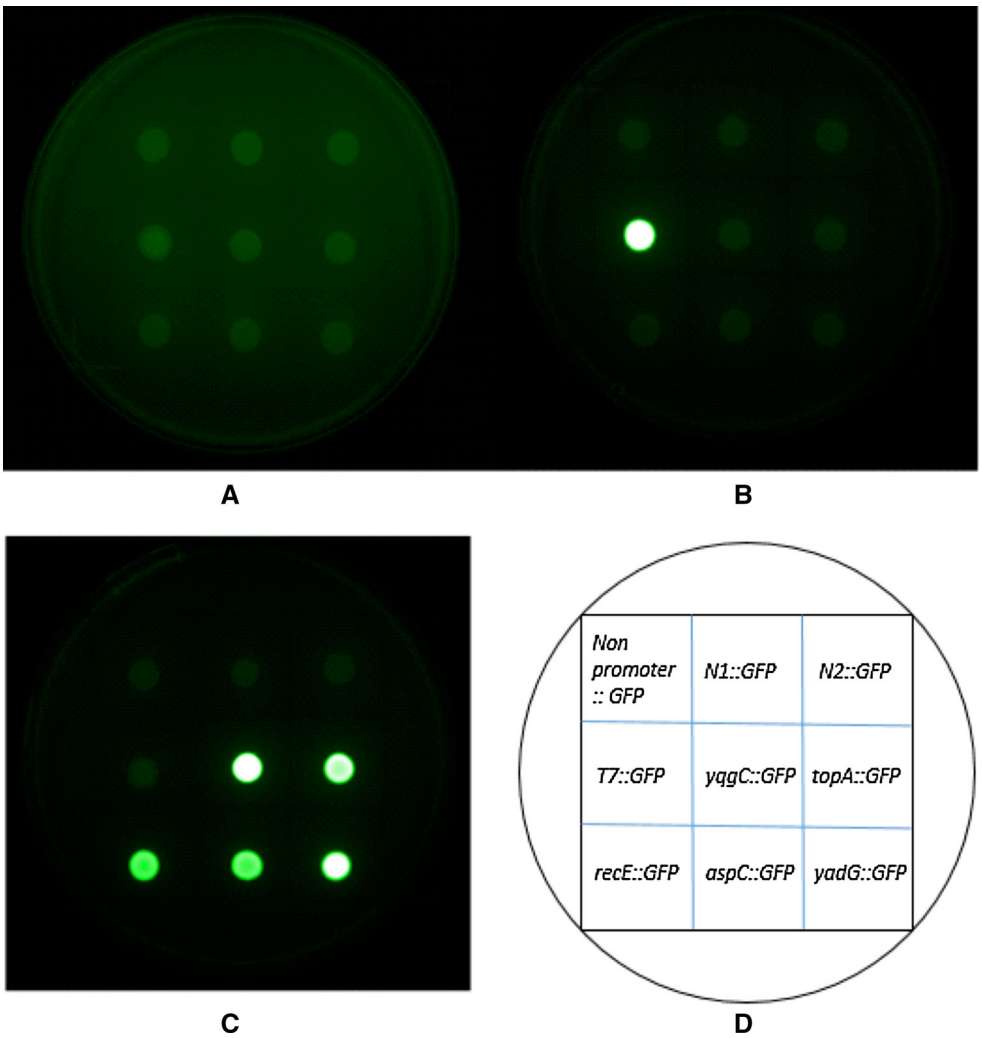

Fig. 5 Expression of each promoter in agarose solid medium under different conditions. Bacteria from each colony were cultured for 12 h and fresh broth ($OD_{600} = 2.0$) was diluted to OD_{600} 0.6. 1 μL broth was vertically dropped onto plates with different induction conditions and images were obtained with a fluorescent system, after 12 h. **a** No inducer, **b** 1 mM IPTG induction, **c** 15 mg/L TNT induction, and **d** promoter schema for each hole. No fluorescence was observed with under the condition of no inducer. The addition of TNT-induced apparent fluorescence in colonies containing *yqgC*, *topA*, *recE*, *aspC*, and *yadG*. The addition of IPTG only induced fluorescence in the T7-GFP colony, indicating that the five promoter elements were selective for the inducer. The induction activity was similar to the activity observed with the strong promoter, T7

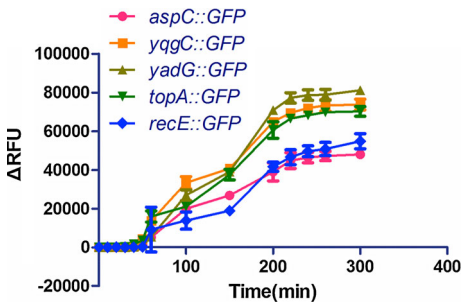

Fig. 6 Time curve of biosensor fluorescence intensity induced by one concentration of TNT. The optimal induction concentration was 20 mg/L for *aspC* and 30 mg/L for *yqgC*, *recE*, *topA*, and *yadG*. Therefore, the time response curve of the five promoter elements was examined under these induction conditions. As shown in Fig. 5, the five promoters had similar time response curves and displayed significant responses, of up to 2 or more times the change in fluorescence, in about 50 min

member of the ABC transporter superfamily. It has been reported that some factors of the plant ABC transporter family are sensitive to TNT [32]. For example, the ABC promoter At3g55130 has been shown to up-regulate promoter activity after exposure to TNT and can act as a sensing element for TNT [33]. Proteins in the same family may have some degree of structural and functional similarity, which may also be why *yadG* exhibited sensitivity to TNT. *AspC* is an aspartate synthase [34], which catalyzes the synthesis of phenylalanine, aspartic acid, and other compounds by ammonia transfer reaction. It is still unknown whether its functions and mechanisms are associated with TNT sensitivity.

The mechanisms by which these elements act on TNT and its derivatives have not yet been elucidated. These genes may perform physiological functions in response to TNT, such as the stress and injury response or source nutrition metabolism. There also may be a specific sequence consensus, which can be induced by TNT. In addition, other unknown mechanism may involve promoter-mediated expression of the downstream genes after TNT induction. Currently, the shortest responsive regions are being studied to determine their basic mechanisms of

Table 3 EC_{200} values of the identified promoter-based bioluminescent reporter strains

Chemical	EC_{200} (mg/L)				
	yadG	*recE*	*topA*	*yqgC*	*aspC*
2,4,6-Trinitrotoluene (2,4,6-TNT)	6.53 ± 1.05	5.32 ± 1.32	5.40 ± 1.65	4.75 ± 0.98	5.65 ± 1.91
2,4-Dinitrotoluene (2,4-DNT)	26.58 ± 2.55	43.43 ± 6.36	31.53 ± 4.07	27.55 ± 3.32	35.83 ± 6.65
2,6-Dinitrotoluene (2,6-DNT)	23.71 ± 1.56	39.54 ± 5.14	24.30 ± 3.46	23.38 ± 4.03	33.29 ± 5.75
1,3-Dinitrobenzene (1,3-DNB)	7.45 ± 2.22	6.31 ± 1.14	3.65 ± 1.31	4.45 ± 1.81	11.53 ± 7.05
1,4-Dinitrobenzene (1,4-DNB)	–	–	30.06 ± 2.37	–	–
2-Nitrotoluene (2-NT)	–	–	–	–	–
3-Nitrotoluene (3-NT)	–	–	–	–	–
4-Nitrotoluene (4-NT)	–	–	–	–	–
Benzene	–	–	–	–	–
Toluene	–	–	–	–	–
Nitrobenzene	–	–	–	–	–

action [21]. The five promoters identified in the current study have better sensitivity and quicker response times (50 min) compared to a previous report examining yqjF and ybiJ in *E. coli* K-12 MG1655 [21]. To our knowledge, it should be mentioned that the five promoters are the most sensitive elements to TNT in *E. coli.*

Biosensors to detect toxic and hazardous compounds have a long history. Back in 1999, Burlage et al. reported on a biosensor with GFP as a detection signal [16]. Based on this engineered bacterium, he proposed developing detection equipment for fermentation, spraying, and detection. In simulated minefields, the biosensor located the position of mines by detecting TNT in the soil. This observation received a lot of attention from the scientific community. However, no subsequent work has been reported since then. In 2011, Martin et al. developed a biosensor following this idea [35]. They jointly wrapped bacteria and a light-emitting device in a small bead, to construct the complete biosensor. However, these biosensors have not been used for various limitations, including a lack of strong specificity in signal detection, low intensity, and the bacterial growth response time. Current bacterial biosensors require at least overnight culture or even over 20 h [36]. Despite the progress in the construction of biosensors for the detection of toxic compounds, there is still a long way for their real life usage.

First proposed in the beginning of the twenty-first century. Synthetic biology is still a young cross-disciplinary science [37]. In 2005, Endy [38] published a review paper "Foundation for engineering biology," clearly introducing the commonly used approaches for engineering in synthetic biology: "standardization," "complex systems decoupling," and "abstraction" and divided the related biological system in synthetic biology into four levels: DNA, parts, devices, and systems. Two routes are included in synthetic biology: (1) the design and construction of new biological parts, components, and systems and (2) the re-design of existing, natural biological systems. Based on this, the BioBrick idea was proposed in synthetic biology. The BioBrick idea is a set of construction standards for developing new genetic routes and life systems in living cells by using and combining the existing biological elements. Finding a sensing element is the key step in building a biological sensor. Though lot of research is still needed, the sensing elements in the current study provide a reservoir of elements for the assembly of biological sensors for mine detection systems and life systems. In addition, we also offer some methods for finding natural biological sensing elements of other compounds. With further optimization, better elements with smaller fragments can be obtained and will be standardized in accordance with the BioBrick idea through bioinformatics and molecular biology methods to facilitate future assembly or usage. Current research on biological sensing elements is still at its infancy. Many natural compounds require more efficient detection methods and there are still many unknown elements. Therefore, more efficient and effective detection methods are needed, suggesting that, in the future, synthetic biology concept-based biosensors must have a broader application. The popularity of the synthetic biology concept in the field of bioengineering will no doubt produce more innovative ideas in the area of biological sensors and the development of biological elements and systems with new functions.

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References

1. Berthe-Corti, L., Jacobi, H., Kleihauer, S., & Witte, I. (1998). Cytotoxicity and mutagenicity of a 2,4,6-trinitrotoluene (TNT) and hexogen contaminated soil in *S. typhimurium* and mammalian cells. *Chemosphere, 37*, 209–218.
2. Reddy, G., Reddy, T. V., Choudhury, H., Daniel, F. B., & Leach, G. J. (1997). Assessment of environmental hazards of 1,3,5-trinitrobenzene. *Journal of Toxicology and Environment Health, 52*, 447–460.
3. U.S. Environmental Protection Agency. (1996). *Innovative treatment technologies: Annual status report* (8th ed.). Washington, DC: U.S. Environmental Protection Agency. Retrieved Novermber 8, 1996 from http://nepis.epa.gov/Exe/ZyNET.exe/10002Y79.TXT?ZyActionD=ZyDocument&Client=EPA&Index=1995+Thru+1999&Docs=&Query=&Time=&EndTime=&SearchMethod=1&TocRestrict=n&Toc=&TocEntry=&QField=&QFieldYear=&QFieldMonth=&QFieldDay=&IntQFieldOp=0&ExtQFieldOp=0&XmlQuery=&File=D%3A%5Czyfiles%5CIndex%20Data%5C95thru99%5CTxt%5C00000004%5C10002Y79.txt&User=ANONYMOUS&Password=anonymous&SortMethod=h%7C-&MaximumDocuments=1&FuzzyDegree=0&ImageQuality=r75g8/r75g8/x150y150g16/i425&Display=p%7Cf&DefSeekPage=x&SearchBack=ZyActionL&Back=ZyActionS&BackDesc=Results%20page&MaximumPages=1&ZyEntry=1&SeekPage=x&ZyPURL.
4. Frische, T. (2002). Screening for soil toxicity and mutagenicity using luminescent bacteria—A case study of the explosive 2,4,6-trinitrotoluene (TNT). *Ecotoxicology and Environmental Safety, 51*(2), 133–144.
5. *Resolution adopted by the General Assembly on 8 December 2005 on the report of the Special Political and Decolonization Committee (Fourth Committee) (A/60/473), sixtieth session Agenda item 27, Distr.: General 18 January 2006*.
6. Benner, S. A., & Sismour, A. M. (2005). Synthetic biology. *Nature Reviews Genetics, 6*(7), 533–543.
7. Leedjarv, A., Ivask, A., Virta, M., & Kahru, A. (2006). Analysis of bioavailable phenols from natural samples by recombinant luminescent bacterial sensors. *Chemosphere, 11*, 1910–1919.
8. Magrisso, S., Erel, Y., & Belkin, S. (2008). Microbial reporters of metal bioavailability. *Microbial Biotechnology, 1*, 320–330.
9. Robin, T., & van der Meer, J. R. (2008). Bacterial biosensors for measuring availability of environmental pollutants. *Sensors, 8*, 4062–4080.
10. Trang, P. T. K., Berg, M., Viet, P. H., Mui, N. V., & van der Meer, J. R. (2005). Bacterial bioassay for rapid and accurate analysis of arsenic in highly variable groundwater samples. *Environmental Science and Technology, 19*, 7625–7630.
11. Werlen, C., Jaspers, M. C. M., & van der Meer, J. R. (2004). Measurement of biologically available naphthalene in gas, and aqueous phases by use of a *Pseudomonas putida* biosensor. *Applied and Environment Microbiology, 70*, 43–51.
12. Meighen, E. A., & Szittner, R. B. (1992). Multiple repetitive elements andorganization of the lux operons of luminescent terrestrial bacteria. *Journal of Bacteriology, 174*, 5371–5381.
13. Kurvet, I., Ivask, A., Bondarenko, O., Sihtmäe, M., & Kahru, A. (2011). LuxCDABE-transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: Comparison with naturally luminous *Vibrio fischeri*. *Sensors, 11*(8), 7865–7878.
14. van der Meer, J. R., & Belkin, S. (2010). Where microbiology meets microengineering: Design and applications of reporter bacteria. *Nature Reviews Microbiology, 8*(7), 511–522.
15. Burlage, R., Youngblood, T., & Lamothe, D. (1998). *Bioreporter bacteria for landmine detection*. RNL report/CP-96972. Oak Ridge: Oak Ridge National Laboratory.
16. Burlage, R., Patek, D., & Everman, K. (1999). *Method for detection of buried explosives using a biosensor*. US Patent 5:972,638.
17. Kyoko, T., Sumio, G., Kiwao, K., Masana, H., Takao, I., & Manabu, S. (2001). Modification of umu Test using the bioluminescent bacteria and application to sediments and soils. *Journals in Environmental Chemistry, 11*(4), 841–848.
18. Garmendia, J., De Las Heras, A., Galvão, T., & De Lorenzo, V. (2008). Tracing explosives in soil with transcriptional regulators of *Pseudomonas putida* evolved for responding to nitrotoluenes. *Microbial Biotechnology, 1*(3), 236–246.
19. González-Pérez, M., Van Dillewijn, P., Wittich, R., & Ramos, J. (2007). *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. *Environmental Microbiology, 9*(6), 1535–1540.
20. Behzadian, F., Barjeste, H., Hosseinkhani, S., & Zarei, A. R. (2012). Zarei construction and characterization of *Escherichia coli* whole-cell biosensors for toluene and related compounds. *Current Microbiology, 62*, 690–696.
21. Sharon, Y., Chaim, L., Rachel, R., Neta, B., Yaara, M., & Shimshon, B. (2014). *Escherichia coli* bioreporters for the detection of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene. *Applied Microbiology and Biotechnology, 98*(2), 885–895.
22. Miller, W., Leveau, J., & Lindow, S. (2000). Improved Gfp and inaZ broad-host-range promoter-probe vectors. *Molecular Plant-Microbe Interactions, 13*, 1243–1250.
23. Lerner, C. G., & Inouye, M. (1990). Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. *Nucleic Acids Research, 18*, 4631.
24. Wang, R. F., & Kushner, S. R. (1991). Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene, 100*, 195–199.
25. Belkin, S., Smulski, D., Dadon, S., Vollmer, A., Van Dyk, T., & Larossa, R. (1997). A panel of stress-responsive luminous bacteria for the detection of selected classes of toxicants. *Water Research, 31*(12), 3009–3016.
26. Belkin, S. (1998). A panel of stress-responsive luminous bacteria for monitoring wastewater toxicity. *Methods in Molecular Biology, 102*, 247–258.
27. Nishino, S. F., Spain, J. C., Lenke, H., et al. (1999). Mineralizat ion of 2,4- and 2,6-DNT in soil slurries. *Environmental Science and Technology, 33*, 1060–1064.
28. Habib, M. (2007). Controlled biological and biomimetic systems for landmine detection. *Biosensors & Bioelectronics, 23*(1), 1–18.
29. Gillen, J. R., Willis, D. K., & Clark, A. J. (1981). Genetic analysis of the *RecE* pathway of genetic recombination in *Escherichia coli* K-12. *Journal of Bacteriology, 145*, 521–532.
30. Yang, Y., & Fix, D. (2006). Genetic analysis of the anti-mutagenic effect of genistein in *Escherichia coli*. *Mutation Research, 600*(1–2), 193–206.
31. Tse-Dinh, Y.-C., & Wang, J. C. (1986). Complete nucleotide sequence of the *topA* gene encoding *Escherichia coli* DNA topoisomerase I. *Journal of Molecular Biology, 191*(3), 321–331.
32. Rao, M. R., & Halfhill, M. D. (2009). Phytoremediation and phytosensing of chemical contaminants, RDX and TNT: identification of the required target genes. *Functional & Integrative Genomics, 9*(4), 537–547.
33. Pomeranz, M. C. (2005). ABC promoter up regulation using TNT and Kangenyacin. University of Tennessee Honors Thesis Projects. RDX and TNT: Identification of the required target genes. *Functional & Integrative Genomics*, *9*(4), 537–547.
34. Seiki, K., Sachiko, O., Tomoko, O., Hideyuki, O., & Hiroyuki, K. (1990). Aspartate aminotransferase of *Escherichia coli*: Nucleotide sequence of the *aspc* gene. *Journal of Biochemistry, 97*(4), 1259–1262.

35. Martin, W., Ulrich, T., Nicole, R., Christoph, K., & Stefan, S. (2012). Future security. *Communications in Computer and Information Science, 318*, 432–437.
36. De Las, Heras. A., Carreño, C., & De Lorenzo, V. (2008). Stable implantation of orthogonal sensor circuits in gram-negative bacteria for environmental release. *Environmental Microbiology, 10*(12), 3305–3316.
37. Gibbs, W. W. (2004). Synthetic life. *Scientific American, 290*(5), 48–55.
38. Endy, D. (2005). Foundations for engineering biology. *Nature, 438*, 449–453.
39. Blattner, F., Plunkett, G., Bloch, C., Perna, N., Burland, V., Riley, M., et al. (1997). Thecomplete genome sequence of *Escherichia coli* K-12. *Science, 277*(5331), 1453–1462.
40. Grant, S., Jessee, J., Bloom, F., & Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylationrestriction mutants. *Proceedings of the National Academy of Sciences of the United States of America, 87*(12), 4645–4649.