Journal of Microbiological Methods 175 (2020) 105997

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Detection of 2,4-dichlorophenoxyacetic acid herbicide using a FGE-sulfatase based whole-cell *Agrobacterium* biosensor

Benjarat Ritcharoon[a], Ratiboot Sallabhan[a], Neal Toewiwat[b], Skorn Mongkolsuk[a,b,c], Suvit Loprasert[a,b,c,*]

[a] Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210, Thailand
[b] Applied Biological Sciences Program, Chulabhorn Graduate Institute, Chulabhorn Royal Academy, Bangkok 10210, Thailand
[c] Center of Excellence on Environmental Health and Toxicology, Ministry of Education, Bangkok 10400, Thailand

ARTICLE INFO

Keywords:
Activator
Error-prone PCR
Genetically-engineered biosensor
Pesticide
Phenoxyacetic acid
Toxicant screening tool

ABSTRACT

2,4-Dichlorophenoxyacetic acid (2,4-D) has been widely used as a herbicide for agricultural purposes. Currently, the available methods for detecting 2,4-D require multi-step sample preparations and expensive instruments. The use of a whole cell biosensor is an interesting approach that is straightforward and simple to use. In this study, we constructed a genetic-based *Agrobacterium tumefaciens* biosensor based on a *cadA* promoter and *cadR* regulator from *Bradyrhizobium* sp. strain HW13 (2,4-D degrader) with a formylglycine generating enzyme (FGE)-sulfatase as the reporter gene. The performance of the biosensor was further improved through direct evolution of the *cadR* activator. The detection limit of *cadR* mutants for phenoxyacetic acid herbicides including 2,4-D and 4-Chlorophenoxyacetic acid (4-CPAA) were 1.56 μM (an eight-fold improvement compared to wild-type CadR). The biosensor could detect 2,4-D contamination in environmental samples without encountering interference from other complex compounds. The *Agrobacterium* biosensor was also stable after storing in a simple Luria-Bertani (LB) medium at 4 °C for 30 days where the activity remained at 82% when exposed to 100 μM of 2,4-D. This novel biosensor, with its high stability under simple storage conditions, exhibits promising potential to be used as an inexpensive and easy-to-use tool to screen for 2,4-D contamination in environmental sources.

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is an organic compound that is classified as a category D chemical (EPA, 2005) and is possibly carcinogenic to humans (group 2B) (Loomis et al., 2015). 2,4-D has been used as a herbicide since 1944 and has been commercially available since 1946 (Tayeb et al., 2011). It is the most widely used herbicide in agriculture for broadleaf weed control in crops and non-crops. In the USA, 2,4-D is one of the 10 most commonly used conventional active ingredients of pesticide in the agricultural sector. Excessive use of 2,4-D and improper remediation has led to environmental contamination.

2,4-D is known to negatively affect biological systems raising the possibility of causing adverse health effects in humans. These include negative effects on gene expression by triggering stress response leading to the disruption of cell cycle control as well as the disruption of immunological responses and DNA repair (Bharadwaj et al., 2005). 2,4-D exposure was found to decrease the levels of reduced glutathione in erythrocytes and significantly altered antioxidant enzyme activities (Bukowska, 2003). In aquatic ecosystems, 2,4-D has been reported to have effects on embryo development in zebrafish (*Danio rerio*) (Gaaied et al., 2020).

The half-life of 2,4-D ranges from a week or more under aerobic conditions, but can exceed 120 days in the absence of oxygen (WHO, 2003). 2,4-D can be degraded by microorganisms (Qurratu and Reehan, 2016). 2,4-D-degrading bacteria are categorized into three groups. The

Abbreviations: *A. tumefaciens*, *Agrobacterium tumefaciens*; BPA, Bisphenol A; 4-CPAA, 4-Chlorophenoxyacetic acid; CPF, Chlorpyrifos; 2,4-D, 2,4-Dichlorophenoxyacetic acid; 2,4-DCP, 2,4-Dichlorophenol; *E. coli*, *Escherichia coli*; ep-PCR, Error-prone PCR; FGE, Formylglycine generating enzyme; GC/MS, Gas chromatography mass spectrometry; HTH, α-Helix–turn–α-helix; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LB, Luria-Bertani; LC/MS, Liquid chromatography mass spectrometry; LC-MS/MS, Liquid chromatography with tandem mass spectrometry; 4-MPAA, 4-Methylphenoxyacetic acid; 4-MU, 4-Methylumbelliferone; 4-MUS, 4-Methylumbelliferyl sulfate; *P. putida*, *Pseudomonas putida*; PAA, Phenoxyacetic acid; PCR, Polymerase chain reaction; *S. meliloti*, *Sinorhizobium meliloti*; UHPLC-MS/MS, Ultra-high performance liquid chromatography coupled to tandem mass spectrometry

* Corresponding author at: Laboratory of Biotechnology, Chulabhorn Research Institute, Kamphaengphet 6 Rd., Laksi, Bangkok 10210, Thailand.
E-mail address: suvit@cri.or.th (S. Loprasert).

https://doi.org/10.1016/j.mimet.2020.105997
Received 13 May 2020; Received in revised form 29 June 2020; Accepted 1 July 2020
Available online 06 July 2020

Table 1
List of strains, plasmids and primers used in this study

Strains, plasmids and primers	Properties or products	Reference
Strains		
Bradyrhizobium sp. HW13	Wild-type, class III 2,4-D degrader	Kitagawa et al., 2002
Escherichia coli XL1- Blue	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac* [F' *proAB*lacI^q*lacZ*Δ *M15*Tn*10* (Tc^r)]	Stratagene Inc.
Sinorhizobium meliloti 1021	Host strain	Capela et al., 2001
Pseudomonas putida KT2440	Host strain	Nelson et al., 2002
Agrobacterium tumefaciens NTL4	Host strain	Luo et al., 2001
Plasmids		
pMPchpAp-atsBA	*chpA* promoter-*atsBA* transcriptional fusion vector; Tc^r	Whangsuk et al., 2016
pGEM-T Easy	Cloning vector; Ap^r	Promega
pBBR1MCS-2	Cloning vector; Tc^r	Kovach et al., 1994
pMPcadAp-atsBA	*cadA* promoter-*atsBA* transcriptional fusion	This study
pBBRcadR	*cadR* coding region in pBBR1MCS-2	This study
Primers		
BT6563 GTCGGTACCCCCAGAACAGC	Forward primer for *cadA* promoter	This study
BT7116 GCCTGCATGCGTTGAGGAAACAAC	Reverse primer for *cadA* promoter	This study
BT6715 GAAGGAGTTGAGAGATGCGCACAACCGTC	Full length *cadR* of *Bradyrhizobium* sp. strain HW13	This study
BT6716 CAGATCAGTGTCCCTGGTTGGCGC	Full length *cadR* of *Bradyrhizobium* sp. strain HW13	This study
BT7791 CGCGATGAGTTCAAAGCGACTACGAAGAAC	*cadR* E321K	This study
BT7792 GTTCTTCGTAGTCGCTTTGAACTCATCGCG	*cadR* E321K	This study
BT7793 CCCAACTCACCAAACTCCATATAGAAGGC	*cadR* E39K	This study
BT7794 GCCTTCTATATGGAGTTTGGTGAGTTGGG	*cadR* E39K	This study

first group comprises β- and γ-proteobacteria which have *tfdA*, a gene capable of converting 2,4-D to 2,4-Dichlorophenol (2,4-DCP) via α-ketoglutarate-dependent dioxygenase (Hayashi et al., 2016). The second group contains non-oligotrophic and fast-growing *Sphingomonas* sp. belonging to α-proteobacteria, which possess *cadA* and *cadB* genes that are responsible for 2,4-D monooxygenase large and small unit productions, respectively. This monooxygenase degrades 2,4-D to 2,4-DCP. The third group comprises oligotrophic and slow-growing α-proteobacteria belonging to *Bradyrhizobium* sp. isolated from non-contaminated soil. The members of this group express versions of *cadA* and *cadB* that share high amino acid identity with TftA and TftB from *Burkholderia cepacian* AC1100 (54 and 59%, respectively). They also share 43–46% identity with TfdAα towards TfdA from *Cupriavidus necator* JMP134 (Hayashi et al., 2016). Genes *cadRABKC* from *Bradyrhizobium* sp. HW 13 were characterized and transformed into *Sinorhizobium meliloti* Rm1021 by Kitagawa et al. (2002). The study suggested that *cadR* encodes for an AraC/XylS-type positive transcriptional regulator. The two adjacent genes, *cadA* and *cadB*, were predicted to encode for large and small subunits of 2,4-D oxygenase, respectively, whereas *cadC* was assumed to encode for the ferredoxin component of *cadAB*, while *cadK* was responsible for encoding the 2,4-D transporter. *Sinorhizobium* transformants required all three *cadABC* genes for 2,4-D degradation (Kitagawa et al., 2002).

The most common methods used to detect 2,4-D are gas chromatography mass spectrometry (GC/MS), liquid chromatography mass spectrometry (LC/MS), liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Solymosné Majzik et al., 2006), and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) (McManus et al., 2014). However, the drawbacks of these methods are complex sample extraction procedures, the need for skilled operators, and costly instrument requirements. As a result, these currently available detecting methods are not suitable for on-site monitoring, especially in developing countries.

Using a whole cell-based biosensor is an interesting alternative method for environmental pollutant detection since it is a simple and non-costly method with acceptable sensitivity that is appropriate for in situ detection (Gui et al., 2017). Whole cell-based biosensors have been successfully used to detect heavy metals and pesticides (2,4-D and chlorpyrifos) by using cyanobacteria (*Anabaena torulosa*). However, this biosensor, although sensitive to 2,4-D, was not specific to 2,4-D (Shing et al., 2013).

In this study, we developed the first genetic-based *Agrobacterium* cell biosensor for 2,4-D detection by using *cadR* and *cadA* genes from *Bradyrhizobium* sp. HW13. The *cadR* and *cadA* genes were inferred to encode an AraC/XylS type of transcriptional regulator and a large subunit of 2,4-D oxygenase, respectively.

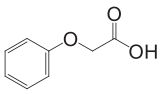
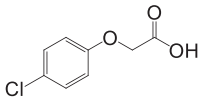
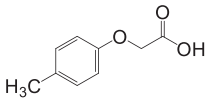
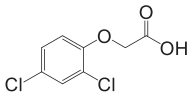
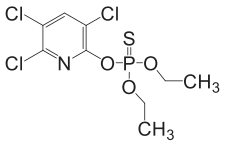
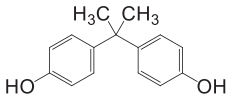
The aim of this work is to construct a genetically engineered whole cell biosensor based on the *cadR* transcriptional regulator and the *cadA* promoter, transcriptionally fused to a highly sensitive detection system, the AtsBA reporter. The reporter is composed of two genes: *atsB* and *atsA*, coding for the formylglycine-generating enzyme (FGE) and arylsulfatase, respectively. A sulfatase-based detection system was chosen for its high sensitivity and successful applications in our previous work for the detection of chlorpyrifos (Whangsuk et al., 2016), and arsenic (Sangkaew et al., 2019). Sulfatase (EC 3.1.6) catalyzes the hydrolysis of sulfate esters to yield phenol and inorganic sulfate. The activity of sulfatase can be readily measured using a small aromatic sulfate compound, 4-methylumbelliferyl sulfate (4-MUS). This fluorometric assay is very specific, extremely sensitive, inexpensive, and rapid (Whangsuk et al., 2016). We used *Agrobacterium tumefaciens* strain NTL4 as the host strain. This is a non-human pathogen and has never been reported as a host strain for a biosensor. We also improved the biosensor sensitivity through direct *cadR* evolution by using random mutagenesis. Biosensor performance was investigated against chlorophenoxyacetic acid herbicide and 4-methylphenoxyacetic acid (4-MPAA) to determine the detection limits, specificity and the importance of substrate functional groups.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bradyrhizobium sp. strain HW13 was grown at 30 °C in CPTYM (1.25 g BactoTryptone, 1.25 g Bacto Peptone, 1.25 g Casamino Acids, 2.50 g yeast extract, 1.50 g mannitol, 0.03 g $MgSO_4{\cdot}7H_2O$, and 0.0035 g $CaCl_2{\cdot}2H_2O$, each per liter), *Sinorhizobium meliloti* SM1021 and *Agrobacterium tumefaciens* strain NTL4 were grown in Luria-Bertani (LB) medium at 28 °C, *Pseudomonas putida* KT2440, and *Escherichia coli* XL1-Blue were grown in Luria-Bertani (LB) medium at 37 °C. A list of strains, plasmids, and primers are shown in Table 1. Antibiotics were used at final concentrations of 15 μg ml^{-1} for tetracycline and kanamycin.

Table 2
Compounds used in this study

Compounds	Abbreviation	Structure
Phenoxyacetic acid	PAA	
4-Chlorophenoxyacetic acid	4-CPAA	
4-Methylphenoxyacetic acid	4-MPAA	
2,4-Dichlorophenoxyacetic acid	2,4-D	
Chlorpyrifos	CPF	
Bisphenol A	BPA	

2.2. Chemicals

4-Methylumbelliferyl sulfate (4-MUS), Bisphenol A (BPA) and Chlorpyrifos (Sigma-Aldrich), 2,4-Dichlorophenoxyacetic acid (Fluka), 4-Chlorophenoxyacetic acid, Phenoxyacetic acid and 4-Methylphenoxyacetic acid (Tokyo chemical industry, TCI) were used (listed in Table 2). All phenoxyacetic acids were dissolved in 0.1 N NaOH.

2.3. Construction of recombinant plasmids and transformation

The DNA promoter region (*cadAp*) was amplified from the chromosome of *Bradyrhizobium* sp. strain HW13 by DreamTaqPCR master mix (Thermo Scientific, USA) using primers BT6563 and BT7116. The PCR cycles comprised a pre-run at 96 °C for 5 min, 30 cycles of denaturation at 96 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, followed by a post-run at 72 °C for 5 min. The PCR fragments were cloned into pGEM-T Easy vectors (Promega) and digested with *Acc*65I and *Sph*I restriction enzymes, before cloning into pMPchpAp-atsBA treated with the same enzymes to construct transcriptional fusion plasmid pMPcadAp-atsBA, which acted as the promoter-reporter fusion system. Next, the transcriptional regulator gene, *cadR*, was amplified from the chromosome of *Bradyrhizobium* sp. strain HW13 using primers BT6715 and BT6716. The PCR cycles comprised a pre-run at 96 °C for 5 min, 30 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a post-run at 72 °C for 5 min. The PCR fragments were cloned into pGEM-T Easy vectors (Promega), digested with *Apa*I and *Spe*I, and cloned into the *Apa*I and *Spe*I sites of pBBR1MCS-2 (Kovach et al., 1994) to generate pBBRcadR, which provided the activator. Both the promoter-reporter fusion, and the activator-carrying plasmid were co-transformed into *E. coli* XL1- Blue and the transformants were selected on LB agar medium supplemented with tetracycline and kanamycin (15 μg ml^{-1}), isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 μM final concentration), 4-MUS (100 μM final concentration), and 2,4-D (100 μM final concentration). Recombinant plasmids were verified by restriction analysis and PCR using the corresponding primers before transforming into *A. tumefaciens* by electroporation. Competent cells of *Agrobacterium* were prepared as previously described (McCormac et al., 1998). Competent cells were mixed with recombinant DNA plasmid and electroporated in electroporation cuvettes using Bio-Rad Gene Pulser (Bio-Rad MicroPulserTM) set to parameters 25 μF; 200 Ω; 2.5 kV. Prior to plating onto LB agar medium supplemented with appropriate antibiotics, cells were diluted with 1 ml LB medium and grown for an hour at 28 °C with shaking.

2.4. Sulfatase activity assays

Overnight cultures of recombinant bacteria were inoculated at 5% (v/v) into LB medium containing the antibiotics, tetracycline and kanamycin (15 μg ml^{-1}), and IPTG (100 μM) to induce the expression of *cadR*. Cultures were then incubated at 28 °C until the cells reached exponential phase (OD_{600} ~ 0.4–0.6). 4-MUS was added to the culture (100 μM final concentration) to serve as the substrate. Then, 200 μl of active culture was aliquoted into a 96-well plate before adding 2 μl of each concentration of 2,4-D, 4-CPAA, PAA, 4-MPAA, BPA, and CPF to a range of final concentrations. The 96 well-plate was incubated overnight with shaking at 225 rpm at 28 °C. Fluorescence intensity was measured with a SpectraMaxiD3 spectrofluorometer (Molecular Devices, USA) using the excitation and emission wavelengths of 365 and 460 nm, respectively (Obaya, 2006). The sulfatase activities were presented as the fold of control i.e. the fluorescence value in the presence of inducer was subtracted from the value in the absence of inducer, and then divided by the value in the absence of inducer. The experiment was performed in triplicate.

2.5. Random mutagenesis and mutant library construction

Random mutagenesis of *cadR* was performed by error-prone PCR (ep-PCR) using GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technology, USA). Briefly, primers BT6715 and BT6716 were used for amplifying megaprimers from pBBRcadR. The amplified megaprimers were used to generate mutant pBBRcadR. The PCR cycles comprised 1 cycle of 95 °C for 1 min, 25 cycles of 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 13 min. The PCR product was digested with *Dpn*I for 2 h at 37 °C to eliminate the wild-type parent template to generate the mutant library. The mutant library of *cadR* was transformed into *A. tumefaciens* NTL4 along with plasmid pMPcadAp-atsBA and transformants were selected on LB agar medium supplemented with tetracycline and kanamycin (15 μg ml^{-1}), 100 μM IPTG, 100 μM 4-MUS and 12.5 μM 2,4-D.

2.6. Site-directed mutagenesis

The *cadR* mutants were created using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, USA) to yield new mutants (BR55 and BR95) by substituting glutamate with lysine at positions 39 and 321 of BR37, respectively. The primers used for site-directed mutagenesis are listed in Table 1. PCR cycles comprised 1 cycle at 95 °C for 2 min, followed by 18 cycles at 95 °C for 20 s, 60 °C for 10 s, 68 °C for 4 min, and then a final incubation at 68 °C for 5 min. The mutagenic plasmids were digested with *Dpn*I at 37 °C for 5 min and transformed into *E. coli* XL1-Blue. The mutations were confirmed by nucleotide sequencing.

2.7. Statistical analysis

All analyses were performed in triplicate. The results are presented as the mean ± standard deviation (SD). Statistical analysis was performed using two-way ANOVA (Turkey's multiple comparison test) and two-tailed unpaired *t*-tests using Prism 7.00 software (GraphPad, San Diego, CA, USA). A p-value less than 0.05 was considered to be statistically significant.

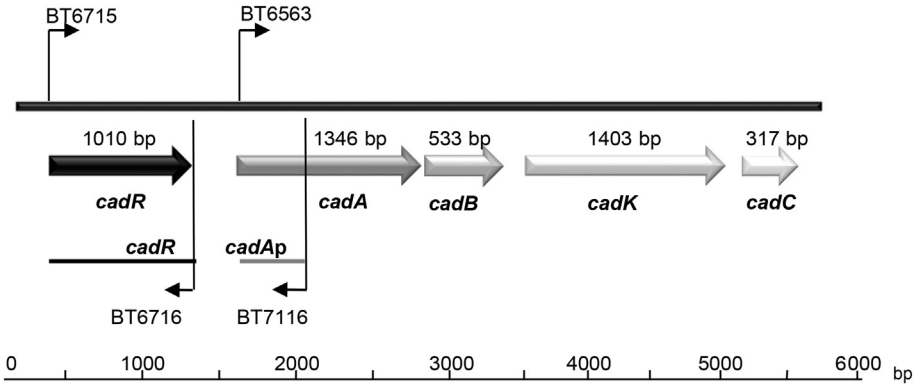


Fig. 1. The gene organization of *cad* operon in *Bradyrhizobium* sp. strain HW13.

3. Results and discussion

3.1. Construction and analysis of whole cell biosensors in response to phenoxyacetic acid

Two genes from the 2,4-D degrading operon (*cadR* and *cadA*) from *Bradyrhizobium* sp. HW13 were selected to develop a sensitive 2,4-D whole cell biosensor. In this operon, 2,4-D catabolic genes, *cadRABKC*, which belong to class III 2,4-D degrader, are responsible for the degradation of 2,4-D (Kitagawa et al., 2002) (Fig. 1).

To examine the two-plasmid whole cell biosensor system harboring pMPcadAp-atsBA and pBBRcadR, *E. coli* XL1-Blue, *P. putida* KT2440, and *S. meliloti* SM1021 were used as the three different candidate host strains to test the system. Each host strain of bacteria (*E. coli*, *P. putida*, and *S. meliloti*) harboring the biosensor system were grown in LB agar medium supplemented with 100 μM 2,4-D and the fluorescence of bacterial colonies was observed under UV light. The constructed biosensors in three tested bacterial strains did not respond to the presence of 2,4-D at 100 μM, which was in partial agreement with a study by Kitagawa et al. (2002) who were also unsuccessful in their attempts to use *E. coli* HB101 and *P. putida* PpY101 as host strains to study the functions of 2,4-D degrading genes from *Bradyrhizobium* sp. HW13. However, they were able to successfully utilize *S. meliloti* Rm1021 as the host strain to screen for 2,4-D degradation products and *cadA* induction by 2,4-D through Northern Blot analysis. The *cadABC* region encoding 2,4-D oxygenase is composed of three components: *cadA* and *cadB* are thought to encode for the terminal oxygenase, whereas *cadC* is likely to transcribe for ferredoxin components (Kitagawa et al., 2002). We suspected that the transcriptional response to the induction by 2,4-D might occur but it was not detectable by our system due to the lower sensitivity of the two-plasmid whole cell biosensor system, compared to Northern Blot analysis. Therefore, we were not able to detect the expression of sulfatase reporter in *S. meliloti*. A previous study in 2004 by Allenby et al. (2004) on the transcription of phosphate-specific ABC transporter (*pst* operon) using a transcriptional reporter system also showed that the results of their transcriptional reporter was less sensitive than Northern Blot analysis.

A non-pathogenic bacterium which is closely related to *Sinorhizobium* sp. and has never been reported as a biosensor, *Agrobacterium tumefaciens* NTL4, was chosen as the host strain for the whole cell biosensor system in this study. The colonies of *A. tumefaciens* transformants harboring plasmids pMPcadAp-atsBA and pBBRcadR fluoresced under UV light after exposure to 2,4-D. In the presence of 2,4-D, *cadR* was activated, leading to the transcription of pMPcadAp-atsBA fusion, which expressed a sulfatase enzyme that cleaved 4-MUS, resulting in the luminesced colonies under UV light. We succeeded in inducing the expression of *cadA*via the *cadR* activator of *Bradyrhizobium* sp. HW13 by using *A. tumefaciens* NTL4 as the host strain (Fig. 2).

The detection limits of the constructed biosensor for phenoxyacetic acid herbicide, including PAA, 2,4-D and 4-CPAA, were measured by sulfatase activity assays. The results showed that our biosensor detected 2,4-D and 4-CPAA at 12.5 μM, and exhibited a linear range from 0 to 100 μM. However, it did not respond to PAA in the range of concentrations from 0 to 750 μM.

To improve the sensitivity of our biosensor, *cadR* was mutated by error-prone PCR (ep-PCR) as described in the Materials and methods section. Eighteen mutants showing fluorescent in the presence of 12.5 μM 2,4-D on LB agar medium were isolated and verified by sulfatase activity assay (Fig. 3). The two mutants, which exhibited the highest folds of induction, were designated as BR37 and BR53, and used for further studies. Mutations of *cadR* were confirmed by sequence analysis. The *cadR* of BR37 was mutated at three amino acid residues: threonine was substituted for arginine at position 156 (T156R), leucine was substituted for phenylalanine at position 168 (L168F), and alanine was substituted for valine at position 296 (A296V). The *cadR* of BR53 was found to have two mutations; glutamate was substituted by lysine at positions 39 and 321 (E39K and E321K, respectively). To improve biosensor sensitivity and validate whether the point mutations of *cadR* found in BR53 contribute to an increase in biosensor capability in detecting 2,4-D, site-directed mutagenesis was used to introduce additional mutations. Two mutants, BR55 and BR95, were generated from BR37, where the amino acids were altered at positions E39K and E321K, respectively, resulting in four mutations in each clone (BR55- E39K, T156R, L168F, and A296V, and BR95- T156R, L168F, A296V, and E321K).

Comparison of the sulfatase activities of biosensors harboring wild-type and mutant *cadR* revealed that both BR37 and BR53 had an improved limits towards detection of 2,4-D concentrations from wild-type *cadR*, which was 12.5 μM, to 1.56 μM (an eight-fold improvement compared to wild type) (Fig. 4A). The mutant had significantly higher fold of induction than wild type ($p < .0001$). However, although the mutated biosensors had a lower detection limit towards 2,4-D, the linear detection range was shortened to 12.5 μM for BR37, whereas BR53 showed higher sulfatase sensitivity at 1.56 μM but did not give a good linearity range of detection (Fig. 4A). The same sensitivity of response was also found for 4-CPAA (Fig. 4B) and 4-MPAA (Fig. 4C). Both *cadR* mutants (BR37 and BR53) showed increased folds of induction of 2.02-fold ($p < .0001$) and 3.19-fold ($p < .0001$) at 1.56 μM 4-CPAA, respectively, compared to those of wild-type (Fig. 4B). The dose-dependent experiments of the *cadR* mutant biosensors on PAA showed that BR37 and BR53 were able to detect PAA at 100 μM, whereas no induction was observed in the wild-type biosensor (data not shown).

To our disappointment, BR55 and BR95, which were derived from BR37 (containing additional mutations at E39K and E321K, respectively), did not perform better than BR37 in terms of sensitivity to 2,4-D. BR55 and BR95 showed significantly lower fold-induction than BR53 when exposed to 12.5 μM 2,4-D ($p < .0001$) (Fig. 5).

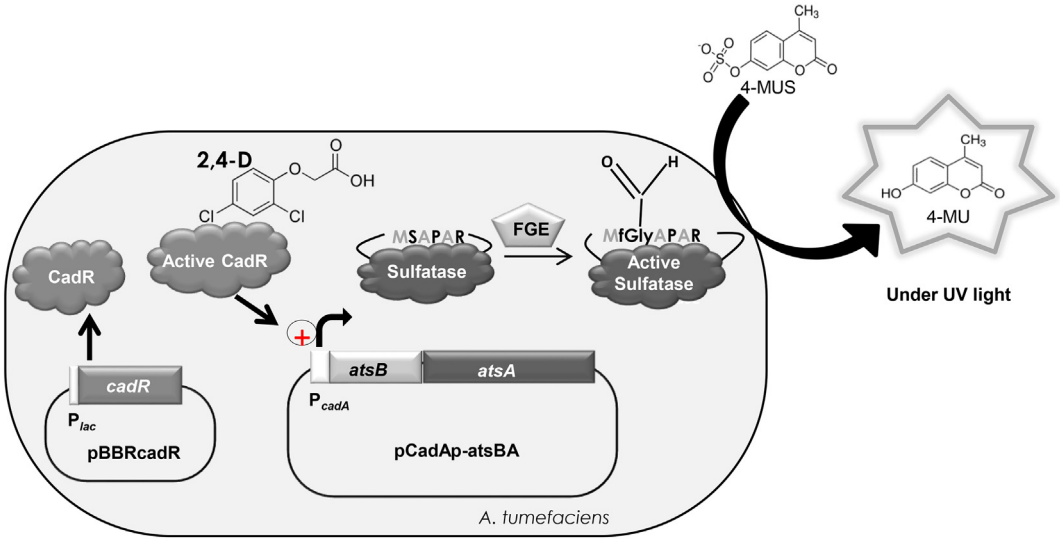


Fig. 2. Architecture of a whole cell genetic-based biosensor using sulfatase as a reporter system.

3.2. The para position of phenoxyacetic acid is important

We wanted to assess the importance of the inducer's structure because there was a report demonstrating that only 2,4-D and 4-CPAA were capable of inducing *cadA* expression via the *cadR* activator, whereas phenoxyacetic acid alone was incapable of induction (Kitagawa et al., 2002). In order to investigate the importance of the functional group of the phenoxyacetic acid inducer, biosensor harboring strains of wild-type and mutant *cadR* were exposed to 4-MPAA and appeared to be induced at the same concentrations as 2,4-D and 4-CPAA. BR37 and BR53 showed increased fold-induction of 3.43-fold ($p < .0001$) and 4.43-fold ($p < .0001$) at 1.56 μM, respectively (Fig. 4C). The results suggested that either chlorine or methyl substitution at the *para* position of phenoxyacetic acid is important, making it a good inducer for *cadA* expression.

To investigate the specificity of our systems, the biosensors were exposed to an endocrine disrupting agent, BPA, and an organophosphate pesticide CPF (chlorpyrifos), which are the common environmental contaminants. No induction was observed in all biosensors constructed in this study. The fluorescence intensities of the wild-type biosensor when exposed to 100 μM BPA, CPF and 2,4-D shown in Fig. 6 suggested that we have developed a novel genetic-based whole cell biosensor with specificity and sensitivity towards 2,4-D. However, we did not test the specificity of our systems to metals like cadmium. Therefore, further investigation on the specificity of our biosensors to metals is mandatory in future studies.

3.3. Structural analysis of CadR

Unfortunately, the crystal structure of *Bradyrhizobium* CadR is not available. Therefore, we attempted to predict the structure by using a homology template in the current protein structure database. The structure of *Bradyrhizobium* CadR was investigated using SWISS-MODEL (Biasini et al., 2014). The amino acid sequence analysis showed that *Bradyrhizobium* CadR was only 32.61% identical with a transcriptional regulator protein (AraC-type, DNA-binding, domain-containing protein) of *Chromobacterium violaceum* (SWISS-MODEL Repository Q7NTG7–1). The identity between these two proteins is limited to the C-terminal region (positions 241–319 of *Bradyrhizobium* CadR). The other protein that was most similar to our CadR is CuxR (SWISS-MODEL

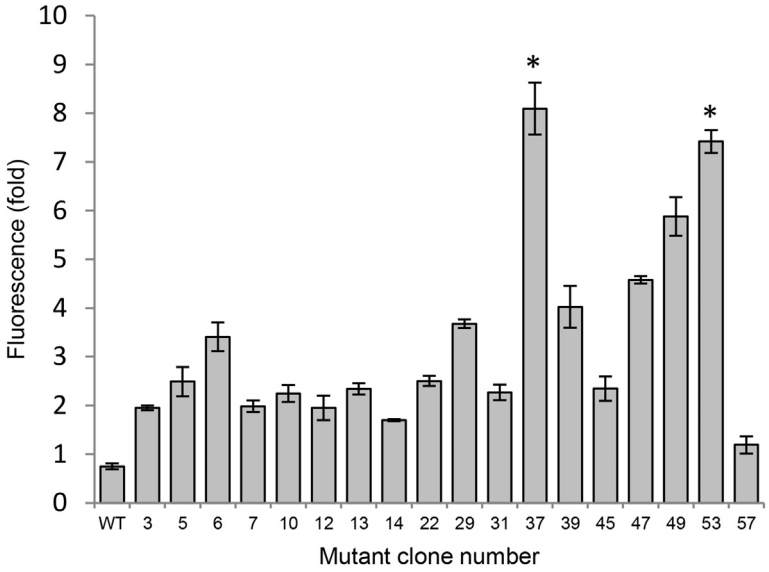


Fig. 3. Sulfatase activity of wild-type and various *cadR* mutants when exposed to 12.5 μM 2,4-D. Asterisks indicate clones (37 and 53) which were selected as candidates for further study.

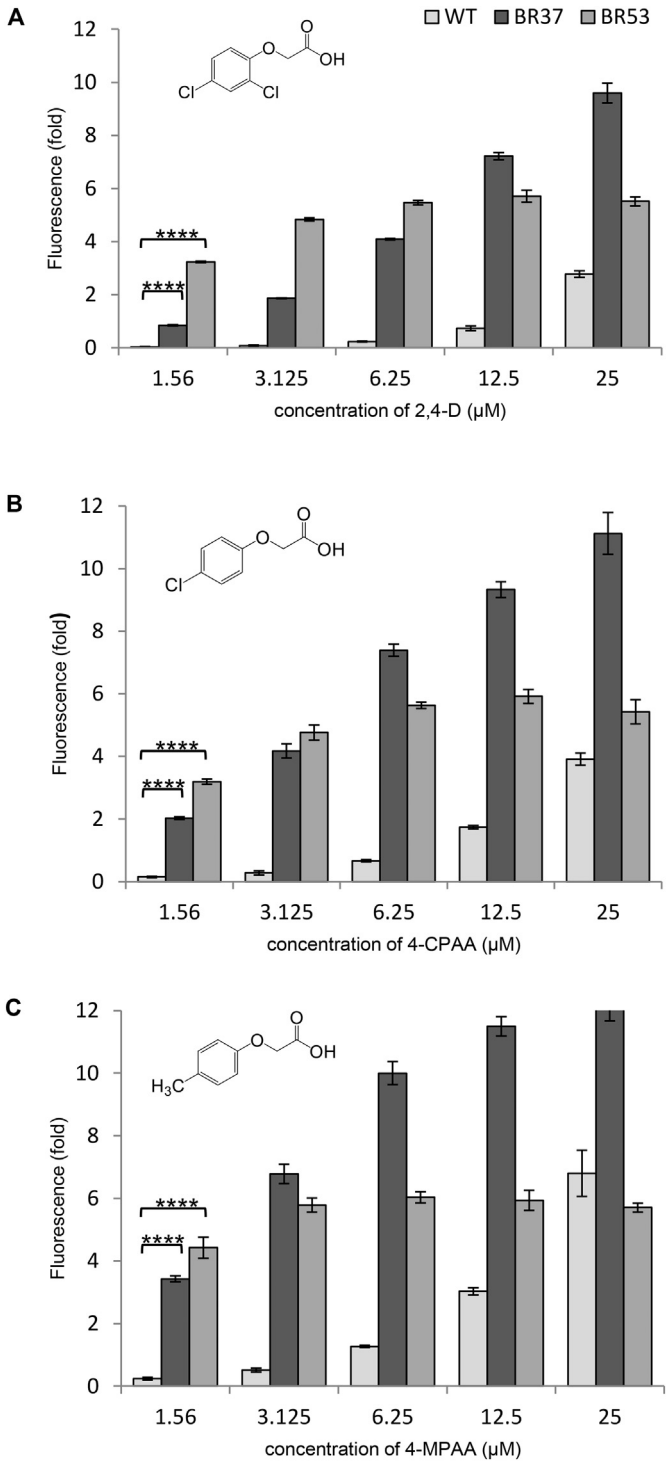


Fig. 4. The folds of relative fluorescence comparison between the wild-type *cadR* and two *cadR* mutant biosensors when exposed to 2,4-D (A), 4-CPAA(B), and 4-MPAA(C) at concentrations ranging from 0 to 25 μM. Mutant *cadR* systems were able to respond to 2,4-D, 4-CPAA and 4-MPAA at 1.56 μM. The data represents three independent experiments. Error bars represent standard deviation from the mean (n = 3).

Repository F7X866) from *S. meliloti* (spanning amino acid positions 11 to 321) but it has low identity (only 19%). Therefore, neither candidate could be used as the model to predict the structure of *Bradyrhizobium* CadR.

Gallegos et al. (1997) discovered that the secondary structure of AraC/XylS regulator members formed α-helix–turn–α-helix (HTH) DNA binding motifs. Most of the members in this group are composed of 250 to 300 amino acids (Gallegos et al., 1997). In the XylS regulator, the first and second HTH motifs are located at positions 228 to 251, and 281 to 305, respectively. The alignment of 99 amino acids within the C-terminal regions of members of the AraC/XylS family showed that 17 residues have a high degree of conservation and represent a consensus sequence for the family (A———S—L—F——G————————R—A—L————————(I/V)–(I/V)——G(F/Y)———F—F(R/K)—G–P) (Gallegos et al., 1997). The CadR of *Bradyrhizobium* sp. HW13 has 13 amino acid residues (A239———S—L—F—G————————R—A—L————————————G————F—F——G–P313) out of 17 residues at the C-terminal region that are identical to the aforementioned 17 residues at the consensus region of AraC/XylS (Fig. 7).

XylS consists of two parts: the C-terminal domain which carries DNA-binding and transcriptional activation abilities, and the N-terminal region which carries effector-binding and regulatory functions. The Kaldalu group discovered that a 112-residue C-terminal fragment of XylS binds specifically to the *Pm* operator (promoter of the *meta*-cleavage operon) *in vitro*, and 210-residue N-terminal portion is necessary for benzoate responsiveness of XylS (Kaldalu et al., 2000).

The mutations of CadR in BR37 (T156R, L168F, A296V) and BR53 (E39K, E321K) occurred throughout C-terminal and N-terminal, and three patterns were observed: the alteration of polar amino acid to positively charged amino acid (T156R), the substitution of a different amino acids from the same group (L168F and A296V), and the substitution of a negatively charged amino acid to a positively charged amino acid (E39K and E321K). Mutations at these positions might affect the conformation of CadR, making the regulator more sensitive to the 2,4-D inducer. Although the point mutations of *cadR* in BR37 and BR53 positively affected the sensitivity of the biosensors towards 2,4-D compared to the wildtype, *cadR*s with 4-point mutations (E39K, T156R, L168F, A296V and T156R, L168F, A296V, E321K) had decreased sensitivities instead. We suspected that the mutations might affect the folding of CadR and altered its structure. However, since the tertiary structure remains unknown, the secondary structure of the protein was predicted and observed. The secondary structure of CadR by the Jpred 4 secondary structure prediction server (Drozdetskiy et al., 2015) revealed that mutations of L168F in BR37 and E321K in BR53 occurred at the α-helix, which plays an important role in DNA binding. The alteration in the amino acids in that region might affect protein folding. Although the structures of the mutated CadR remained unknown, the mutations improved the detection limit of the biosensor towards 2,4-D to 1.56 μM.

3.4. Detection of 2,4-D in environmental samples

Wild-type and mutant CadR biosensors were used to detect 2,4-D in environmental samples from a canal. The sterile, filtered water samples were mixed with an equal volume of 2x LB medium containing tetracycline, kanamycin (15 μg ml^{-1}), and 100 μM IPTG, followed by the addition of overnight bacteria cultures to an A_{600} of 0.2. After incubation for 2.30 h, 100 μM 4-MUS was added together with varying concentrations of 2,4-D and incubated at 28 °C overnight. Results revealed that our biosensor could detect 2,4-D without encountering interference from other complex compounds in the environmental water when compared with the control that used deionized water (analysis by two-tailed unpaired *t*-test found no significant difference at $P < .05$) (Fig. 8). In addition, the biosensor performance was evaluated after storage in LB broth at 4 °C. The biosensor activity was tested every five days using the sulfatase assay. The activities remained at 82% when exposed to 100 μM 2,4-D after 30 days of storage, making it a promising alternative to be used in detecting 2,4-D contamination in the environment. The remaining biosensor activity dropped below 50% after 52 days of storage at 4 °C. Fluorescence intensity from the biosensor kept in Luria-Bertani (LB) medium containing 15% glycerol was not significantly different than that stored in Luria-Bertani (LB) without

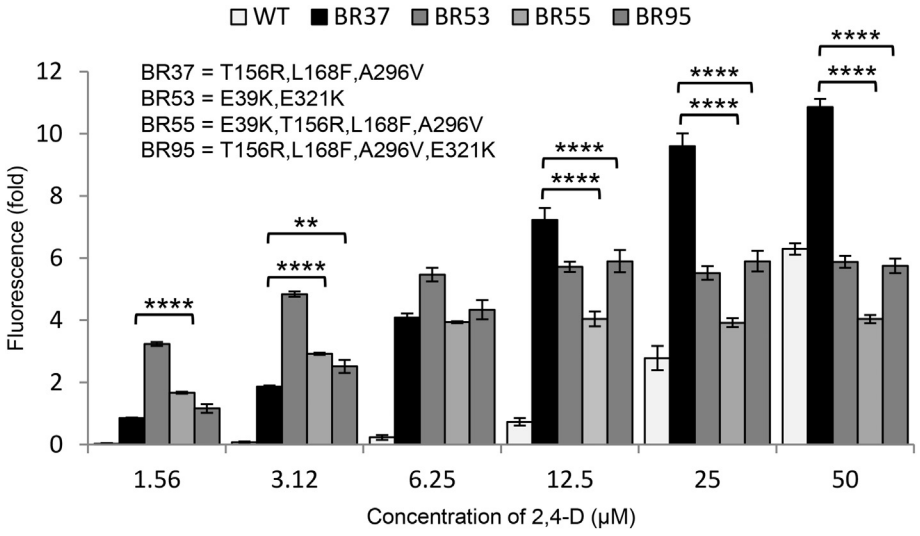


Fig. 5. Sulfatase activity of *Agrobacterium tumefaciens* NTL4 of wild-type and mutant *cadR* biosensors when exposed to 2,4-D concentrations ranging from 0 to 50 μM. The data represent three independent experiments. Error bars represent standard deviation from mean (n = 3).*indicates P value ≤.05; ** indicates P value ≤.01; *** indicates P value ≤.001. **** indicates P value ≤.0001.

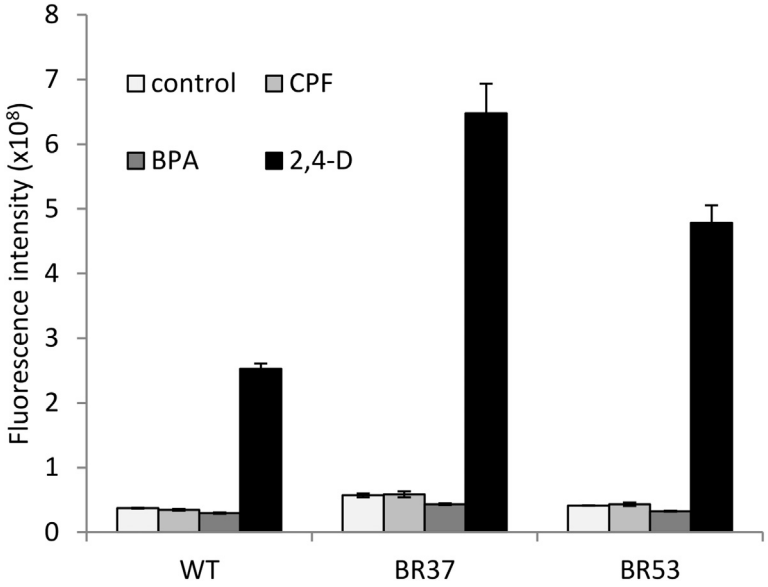


Fig. 6. Sulfatase activity of wild-type and *cadR* mutant biosensors when exposed to bisphenol A (BPA) and the organophosphate pesticide, chlorpyrifos (CPF), at 100 μM.

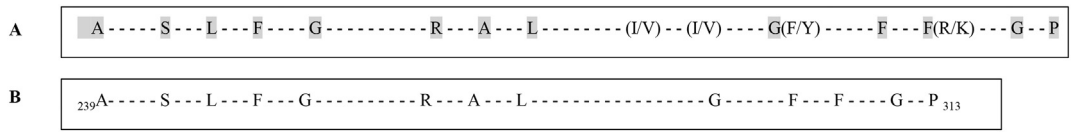


Fig. 7. Alignment of the C-terminal consensus amino acid sequences of AraC/XylS family regulators (A) and the C-terminal region of CadR of *Bradyrhizobium sp.* HW13 at (B). Residues highlighted in gray indicated amino acid identity.

15% glycerol (p = .8126) (Fig. 9). The biosensor offers a simple and low-cost storage method since no cryoprotectants such as glycerol are required. Our study is the first report to show that *Agrobacterium tumefaciens* can be used as the host for a whole cell-based biosensor that can detect 2,4-D contaminations with high sensitivity and specificity.

4. Conclusion

We constructed a genetic-based *Agrobacterium tumefaciens* biosensor for 2,4-D detection using the *cadR* and *cadA* genes of the 2,4-D degrading operon from *Bradyrhizobium* sp. HW13. The reporter gene was *atsBA* carrying FGE and sulfatase, which provided a fluorescent product after reacting with 4-methylumbelliferyl sulfates (4-MUS). The sensitivity in 2,4-D detection was improved via generations of random mutagenesis libraries of the *cad* gene transcription regulator (*cadR*). The developed biosensor was able to respond to the presence of 2,4-D and 4-CPAA at 1.56 μM. The biosensor could detect 2,4-D in environmental samples without encountering interference from other complex compounds. In addition, the *Agrobacterium* biosensor was stable after storage in a simple LB medium at 4 °C up to 52 days.

Declaration of Competing Interest

The authors declare that they have no known competing financial

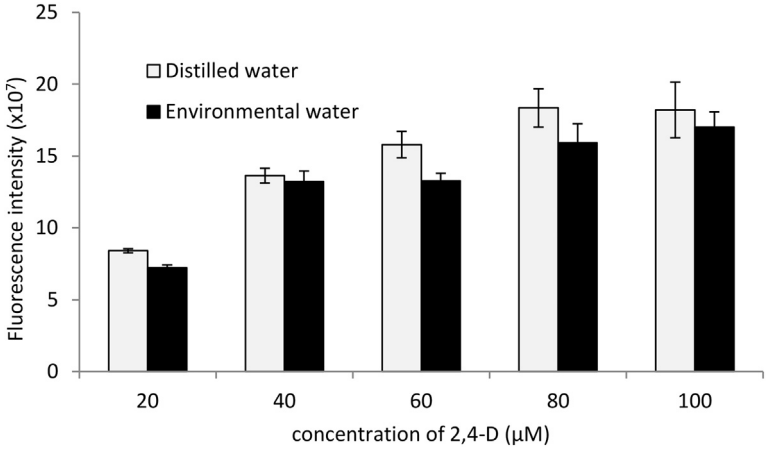


Fig. 8. Detection of 2,4 D in environmental samples using our whole-cell biosensor with deionized water as a control. The data represents three independent experiments. Error bars represent standard deviation from the mean (n = 3).

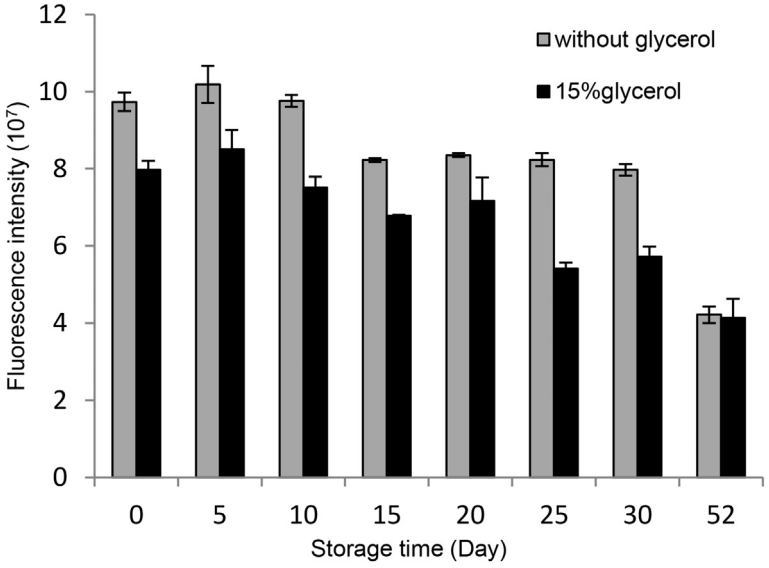


Fig. 9. A stability comparison of the biosensor after storage in a Luria-Bertani (LB) with and without 15% glycerol for 52 days at 4 °C.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research work was supported by a grant from the Center of Excellence on Environmental Health and Toxicology, Thailand. Particular thanks go to Wataru Kitagawa (Department of Bioengineering, Faculty of Technology, Nagaoka University, Japan) for the kind gift of the *Bradyrhizobium* sp. strain HW13 and James Dubbs for critical reading.

References

Allenby, N.E.E., O'Connor, N., Prágai, Z., Carter, N.M., Miethke, M., Engelmann, S., Hecker, M., Wipat, A., Ward, A.C., Harwood, C.R., 2004. Post-transcriptional regulation of the *Bacillus subtilis pst* operon encoding a phosphate-specific ABC transporter. Microbiology 150 (Pt 8), 2619–2628.

Bharadwaj, L., Dhami, K., Schneberger, D., Stevens, M., Renaud, C., Ali, A., 2005. Altered gene expression in human hepatoma HepG2 cells exposed to low-level 2,4-dichlorophenoxyacetic acid and potassium nitrate. Toxicol. In Vitro 19 (5), 603–619.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, G.T., Bertoni, M., Bordoli, L., Schwede, T., 2014. SWISS-MODEL: modeling protein tertiary and quaternary structure using evolutionary information. Nucl. Acids. Res. 42, W252–W258.

Bukowska, B., 2003. Effects of 2,4-D and its metabolite 2,4-dichlorophenol on antioxidant enzymes and level of glutathione in human erythrocytes. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 135 (4), 435–441.

Capela, D., et al., 2001. Analysis of the chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain 1021. Proc. Natl. Acad. Sci. U. S. A. 98 (17), 9877–9882.

Drozdetskiy, A., Cole, C., Procter, J., Barton, G.J., 2015. JPred4: a protein secondary structure prediction server. Nucl. Acids Res. 43 (W1), W389–W394.

EPA, 2005. Reregistration Eligibility Decision for 2,4-D. U.S. Environmental Protection Agency, Washington, D.C.

Gaaied, S., Oliveira, M., Domingues, I., Banni, M., 2020. 2,4-Dichlorophenoxyacetic acid herbicide effects on zebrafish larvae: development, neurotransmission and behavior as sensitive endpoints. Environ. Sci. Pollut. Res. Int. 27 (4), 3686–3696.

Gallegos, M.T., Schleif, R., Bairoch, A., Hofmann, K., Ramos, J.L., 1997. AraC/XylS family of transcriptional regulators. Microbiol. Mol. Biol. Rev. 61 (4), 393–410.

Gui, Q., Lawson, T., Shan, S., Yan, L., Liu, Y., 2017. The application of whole cell-based biosensors for use in environmental analysis and in medical diagnostics. Sensors 17 (7), 1623.

Hayashi, S., Sano, T., Suyama, K., Itoh, K., 2016. 2,4-Dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading gene cluster in the soybean root-nodulating bacterium *Bradyrhizobium elkanii* USDA94. Microbiol. Res. 188-189, 62–71.

Kaldalu, N., Toots, U., de Lorenzo, V., Ustav, M., 2000. Functional domains of the TOL plasmid transcription factor XylS. J. Bacteriol. 184 (4), 1118–1126.

Kitagawa, W., Takami, S., Miyauchi, K., Masai, E., Kamagata, Y., Tiedje, J.M., Fukuda, M., 2002. Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium* sp. HW13 isolated from a pristine environment. J. Bacteriol. 184 (2), 509–518.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., Peterson, K.M., 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques 16 (5), 800–802.

Loomis, D., Guyton, K., Grosse, Y., El Ghissasi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Mattock, H., Straif, K., 2015. Carcinogenicity of lindane, DDT, and 2,4-

dichlorophenoxyacetic acid. Lancet Oncol. 16 (8), 891–892.
Luo, Z.Q., Clemente, T.E., Farrand, S.K., 2001. Construction of a derivative of *Agrobacterium tumefaciens* C58 that does not mutate to tetracycline resistance. Mol. Plant-Microbe Interact. 14 (1), 98–103.
McCormac, A.C., Elliott, M.C., Chen, D.F., 1998. A simple method for the production of highly competent cells of *Agrobacterium* for transformation via electroporation. Mol. Biotechnol. 9 (2), 155–159.
McManus, S.L., Moloney, M., Richards, K.G., Coxon, C.E., Danaher, M., 2014. Determination and occurrence of phenoxyacetic acid herbicides and their transformation products in groundwater using ultra high performance liquid chromatography coupled to tandem mass spectrometry. Molecules 19 (12), 20627–20649.
Nelson, K.E., et al., 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. 4 (12), 799–808.
Obaya, A.J., 2006. Molecular cloning and initial characterization of three novel human sulfatases. Gene 372, 110–117.
Qurratu, A., Reehan, A., 2016. A review of 2,4-dichlorophenoxyacetic acid (2,4-D) derivative: 2,4-D dimethylamine salt and 2,4- butyl ester. Int. J. Appl. Eng. Res. 11 (19), 9946–9955.
Sangkaew, W., Sallabhan, R., Ritcharoon, B., Mongkolsuk, S., Loprasert, S., 2019. FGE-sulfatase-based bacterial biosensor with single copy evolved sensing cassette for arsenic detection. J. Chem. Technol. Biot. 95, 1173–1179.
Shing, W.L., Heng, L.Y., Surif, S., 2013. Performance of a cyanobacteria whole cell-based fluorescence biosensor for heavy metal and pesticide detection. Sensors 13 (5), 6394–6404.
Solymosné Majzik, E., Tóth, F., Benke, L., Kiss, Zs, 2006. SPE-LC-MS-MS determination of phenoxy acid herbicides in surface and ground water. Chromatographia 63, S105–S109.
Tayeb, W., Chaieb, I., Hammami, M., 2011. Environmental fate and effects of 2,4-dichlorophenoxyacetic herbicide. In: Piotrwosky, K.P. (Ed.), Herbicides: Properties, Crop Protection and Environmental Hazards. Nova Science Publisher, New York, pp. 161–187.
Whangsuk, W., Thiengmag, S., Dubbs, J., Mongkolsuk, S., Loprasert, S., 2016. Specific detection of the pesticide chlorpyrifos by a sensitive genetic-based whole cell biosensor. Anal. Biochem. 493, 11–13.
WHO, 2003. 2,4-D in Drinking-Water. Background Document for Preparation of WHO Guidelines for Drinking-Water Quality. World Health Organization (WHO/SDE/WSH/03.04/70), Geneva.