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Construction and application of an *Escherichia coli* bioreporter for aniline and chloroaniline detection

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Abstract Aniline and chlorinated anilines (CAs) are classified as priority pollutants; therefore, an effective method for detection and monitoring is required. In this study, a green-fluorescence protein-based bioreporter for the detection of aniline and CAs was constructed in *Escherichia coli* DH5α, characterized and tested with soil and wastewater. The sensing capability relied on the regulatory control between a two-component regulatory protein, TodS/TodT, and the P_{todX} promoter of *Pseudomonas putida* T-57 (PpT57), since the gene expression of *todS*, *todT*, and *todC2* are positively induced with 4-chloroaniline. The bioreporter system (DH5α/pPXGFP–pTODST) is markedly unique with the two co-existing plasmids. The inducibility of the fluorescence response was culture-medium- and time-dependent. Cells grown in M9G medium exhibited a low background fluorescence level and were readily induced by 4CA after 3-h exposure, reaching the maximum induction level at 9 h. When tested with benzene, toluene, ethyl-benzene and xylene, aniline and CAs, the response data were best fit by a sigmoidal dose–response relationship, from which the $K_{1/2}$ value was determined for the positive effectors. 3CA and 4CA were relatively powerful inducers, while some poly-chlorinated anilines could also induce green fluorescence protein expression. The results indicated a broader recognition range of PpT57'sTodST than previously reported for *P. putida*. The test results with environmental samples were reliable, indicating the potential application of this bioreporter in the ecotoxicology assessment and bioremediation of areas contaminated with aniline- and/or CAs.

Keywords Bioreporter · Chloroaniline · BTEX · TodST · Organic-solvent-tolerant bacteria

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Introduction

Aniline and chlorinated anilines (CAs) are widespread in the environment because they have been intensively used in the industrial production of polyurethane, pesticides, and dyes, and are accumulated in agricultural areas from the natural degradation of pesticides [9]. Since they are known to be toxic to humans and other organisms, and are classified as priority pollutants in environmental risk assessments, their existence is of serious concern for public health and environmental safety [9]. Therefore, effective detection methods are required in order to ascertain the need for, and to monitor the progress of, remedial treatment.

Conventionally, environmental pollutants are determined using chemical analysis. However, these analytical methods

only determine the total amount of the pollutants presented in the samples and are not able to distinguish between bioavailable (potentially toxic) and non-bioavailable (nontoxic) fractions. Alternatively, bacterial-based sensing and reporting systems, i.e., bioreporters, have been increasingly applied as devices to monitor and assess the bioavailability of pollutants because they can be genetically engineered for the specific sensing of given target pollutants [13]. In addition, since the responses of bioreporters can also reflect the real toxicological potency of an environmental sample, bioreporters are potentially more appropriate tools to be applied for monitoring the fate and availability of pollutants regarding their toxicity and remediation. Bioreporters typically consist of a genetically engineered plasmid, which contains a transcriptional regulator with a specific sensing function to a particular chemical, a cognate promoter and a reporter protein, all within a bacterial host strain, such as *E. coli* [19] and *Pseudomonas putida* [8]. Currently, several bioreporters have been developed for the detection of aromatic pollutants, primarily focusing on benzene-toluene-ethylbenzene-xylene (BTEX), substituted benzenes, and substituted toluenes [1–4, 6, 15, 16, 18, 21]. Among the regulator genes and promoters from toluene catabolic gene clusters, the *todST* genes have previously been used as the basis of a sensing system in bioreporters for aromatic effectors. They encode a two-component regulatory system (TCS) that regulates the expression of the P_{todX} promoter, which then concomitantly governs the expression of the toluene dioxygenase (*tod*) gene cluster, in *P. putida* F1 (PpF1) [1, 2] and DOT-T1E (PpT1E) [6, 15].

The presence and toxicity of aniline and CAs in the environment raises safety awareness. Therefore, the availability of a bioreporter system for these pollutants is important, but has not yet been developed. In this study, a bioreporter for the detection of aniline and CA was developed based upon the *E. coli* DH5α host strain, transformed with two plasmid constructs, the first of which consists of an inducible reporter green-fluorescent protein (*gfp*) gene fused with the $P_{todX(T57)}$ and the second contains the constitutively expressed *todST* TCS genes from PpT57, a solvent-tolerant bacterium with toluene degradation ability [10]. This work not only expands the known range of the sensing capacity of the TodST regulatory proteins to aniline and CA pollutants, but also exhibits their potential application for monitoring and preliminary detection of these toxic pollutants.

Materials and methods

Chemicals and bacterial strains

Aniline and chloroanilines (mono-chloroanilines (MCAs): 2-chloroaniline (2CA), 3-chloroaniline (3CA), 4-chloroaniline (4CA); di-chloroanilines (DCAs): 2,3-dichloroaniline (23DCA), 2,4-dichloroaniline (24DCA), 3,4-dichloroaniline (34DCA), 3,5-dichloroaniline (35DCA); tri-chloroanilines (TCAs): 2,3,4-trichloroaniline (234TCA), 2,4,5-trichloroaniline (245TCA), 2,4,6-trichloroaniline (246TCA) were from Chem Service (USA). The bacterial strains, primers, and plasmids used in this study are listed in Table 1.

RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR)

Overnight-grown PpF1 and PpT57 in Luria–Bertani (LB) (28 °C, 200 rpm) were used as an inoculum (5 % v/v) for cell cultivation in the following conditions: a non-induction control of MP (minimal salt basal medium [10] supplemented with 20 mM pyruvate); two induction conditions of MP supplemented with 1 mM of either toluene or 4CA (prepared in ethanol), and the solvent control (MP with ethanol). Total RNA was then extracted with NucleoSpin® RNA II kit (Macherey–Nagel, PA, USA) according to the manufacturer's instructions. Then, cDNA was generated by using the One-Step SYBR PrimeScript RT-PCR kit (Takara Bio Inc., Japan) and used for quantitative reverse transcriptase (qRT)-PCR on a LightCycler 1.5 thermocycler (Roche Diagnostics, USA), which was performed at 42 °C (5 s) followed by 40 cycles of 95 °C (5 s), 57 °C (10 s), and 72 °C (6 s), with *todS*, *todT*, *todC2*, and *gyrB* primers (Table 1). The gene expression data were normalized to the expression of the endogenous reference gene, *gyrB* and were then reported as a relative value to that of the non-induction control.

Plasmid construction and transformation

To construct plasmids, two backbone plasmids, i.e., pQF50, a Gram-negative promoterless *lacZ* transcriptional fusion vector [11], and pSTV28, a low-copy number plasmid (Takara, Japan), were used. The 205-bp PCR product of the $P_{todX(T57)}$ was digested with *Sph*I and *Bam*HI and cloned into the *Sph*I and *Bam*HI sites of pQF50, creating pPXlacZ. Subsequently, *lacZ* was exchanged with *gfp* using the In-fusion® HD PCR cloning kit (Clontech, Japan), generating pPXGFP (Fig. 1). The 3.6-kb PCR product of *todST* was digested with *Sph*I and *Bam*HI and cloned into the corresponding sites of pSTV28, creating pTODST (Fig. 1). To create the bioreporter, pPXGFP was initially introduced into DH5α. Then, DH5α recombinants harboring pPXGFP were prepared as competent cells and used for a heat-shock transformation with pTODST. The double transformants were selected on LB with ampicillin (100 mg l^{-1}) and chloramphenicol (40 mg l^{-1}), and the positive clone, harboring pPXGFP and pTODST, of DH5α

Table 1 Bacterial strains, primers and source of sequence

Bacterial strains	Description	Source or reference
E. coli DH5α	F^- *endA*1 *glnV*44 *thi*-1 *recA*1 *relA*1 *gyrA*96 *deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR*17(r_K^- m_K^+), λ–	Gibco BRL Inc. (USA)
P. putida F1	Toluene-oxidizing bacterium	ATCC 700007
P. putida T-57	Organic-solvent tolerant, toluene-oxidizing bacterium	[10]

Primers	Region	Primer sequence (5′ → 3′)[a]	Source or reference
Primers for quantitative RT-PCR			
qPCR-F1-todS-F	*todS*	CAGCAACAACTTGTGTACGTTTCC	[Pput_2872][b]
qPCR-F1-todS-R		GCGGTTGGTTTATCTCGTGTG	3,262,301–3,262,220
qPCR-F1-todT-F	*todT*	GCAGCTTGGTGCGTTCAATC	[Pput_2871][b]
qPCR-F1-todT-R		CATCTCGGGCATACGGACATC	3,261,518–3,261,397
qPCR-F1-todC2-F	*todC2*	AGCGTCCCTTCATCATCGTG	[Pput_2880][b]
qPCR-F1-todC2-R		GACATTCACTACTTCATGCCCATTC	3,271,353–3,271,473
qPCR-F1 gyrB-F	*gyrB*	CAGACTTACGTTCACGGTGTTCC	[Pput_0004][b]
qPCR-F1 gyrB-R		GATGGCTTGAAGTGGATGTGG	4,602–4,690
Primers for PCR			
Ptx-F-SphI	P_{todX}	**ATGC**GCATGC(*Sph*I)TGGGTGCATATCCATCAGAA	PpT57 genomic DNA
Ptx-R-BamHI		**GCAT**GGATCC(*BamH*I)ACATCTTATTTTTTTTATAAAGTGAAG	
todST-F-BamHI	*todST*	**ATGC**GGATCC(*BamH*I)TTAAGTCAGAATCTTGTGAGTCAT	PpT57 genomic DNA
todST-R-SphI		**ATGC**GCATGC(*Sph*I)CTATTCCAGGCTATCCTTGA	
todST-F-BamHI	*todS* (partial)	**ATGC**GGATCC(*BamH*I)TTAAGTCAGAATCTTGTGAGTCAT	PpT57 genomic DNA
todS-EP-R-Blt		**ATGC**GATATC(*EcoR*V)ATTGCCTGAGAATGACAGGG	
Primers for an In-fusion cloning®			
Inf-GFP-F	*gfp*	TAGAGGGTATTAATAATGAGTAAAGGAGAAGAACTTTTC	$pGFP_{uv}$
Inf-GFP-R		CCTGCCCGGTTATTATTATTTGTAGAGCTCATCCATG	(Clontech, Japan)
Inf-pQF50-F	pQF50Δ*lacZ*	TAATAACCGGGCAGGCCATG	pQF50
Inf-pQF50-R		TATTAATACCCTCTAGCTAGAAGCTTCTA	(Laboratory stock)

[a] Additional nucleotides are shown in *boldface*; recognition sequences of restriction enzymes are *underlined* and shown in *parenthesis*

[b] Gene locus tag number and the amplified region of the gene from *P. putida* F1 genome database (GenBank: CP000712.1)

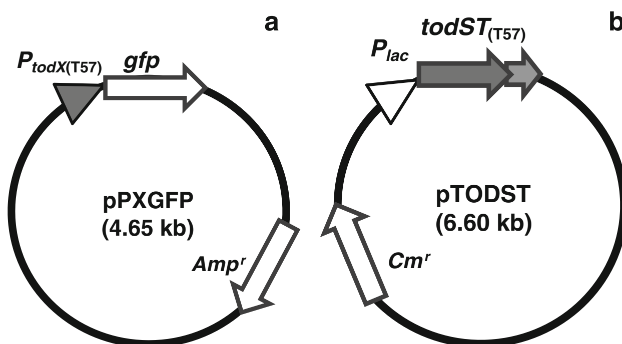

Fig. 1 Schematic organization of the two plasmids in the bioreporter DH5α/pPXGFP–pTODST: **a** pPXGFP was constructed on a pQF50 with $P_{todX(T57)}$, *gfp*, and Amp^r (ampicillin resistance). **b** pTODST was constructed with a pSTV28 with P_{lac} (lac promoter), *todST*, and Cm^r (chloramphenicol resistance)

bioreporter, is referred to as DH5α/pPXGFP–pTODST hereafter.

Bacterial growth, induction experiments, fluorescence measurement, and determination of effector range

The DH5α/pPXGFP–pTODST cells were grown in either LB, Terrific broth (TB), or M9-glucose medium (M9G), containing ampicillin (100 mg l^{-1}) and chloramphenicol (40 mg l^{-1}). Cells were cultivated for 12 h at 37 °C on an orbital shaker (150 rpm) to an optical density at 600 nm (OD_{600}) of 0.8–1.0 before being harvested, washed twice and re-suspended in the same medium and volume. The culture was aliquoted (2 ml) into sterile 30-ml glass vials containing different known concentrations of the respective test chemicals, incubated at 37 °C on an orbital shaker

(150 rpm), and the cell growth (OD_{600}) and fluorescence intensities, measured at 395 nm excitation and 509 nm emission wavelengths, were examined at intervals. The relative fluorescence unit (RFU) was calculated from the fluorescence intensity value obtained as the instrument's arbitrary unit (AU) relative to the culture biomass at OD_{600}. The data are reported as the induction ratio, defined as the RFU of a chemical-induced bioreporter divided by the RFU of the non-induced bioreporter (control). The tests were also conducted using DH5α/pPXGFP as a negative control. The experiments were conducted independently and at least in triplicate. To test the effector range, the test chemical, including alcohols, alkanes, BTEX, aniline, and CAs, was added to the bioreporter cell suspension to the final concentration of 1 mM. Measurement of the fluorescence response was conducted and reported.

Test of the bioreporter with 4CA-contaminated soil and wastewater

An agricultural soil sample from the central part of Thailand was identified as a loam soil and its physico-chemical properties were determined (Supplement 1). Industrial wastewater was obtained from a polymer production factory in Rayong Province, Thailand, and its properties were determined (Supplement 1). The original soil and wastewater samples were kept in the dark at 4 °C until used. In addition, for further tests, 4CA-contaminated soil and 4CA-contaminated wastewater were prepared by spiking 4CA to the soil (100 ppm;1 mM) and wastewater samples (25 ppm; 0.2 mM). The soil samples and the 4CA-contaminated soil were extracted with either deionized water or methanol (80 % v/v), using a soil-liquid phase ratio of 1:5 (w/v). The soil suspension supernatant was then harvested and used for HPLC analysis and the bioreporter assay.

For the bioassay, cells were prepared and tenfold concentrated. The composition of the assay was as follows: soil extract (with or without 4CA-spike) (600 μl) or wastewater (with or without 4CA-spike) (1200 μl), the 10× cell suspension (150 μl), 10 × M9G (150 μl), and deionized distilled water to a final volume of 1,500 μl. The samples for the bioassay included: (1) induced cells by environmental samples, (2) non-induced cells, (3) the assay component without the bioreporter cells (as a negative control to determine the influence of other bacteria in the environmental sample tested), and (4) the samples containing the known concentrations of 4CA as positive controls, to generate a standard curve, and to examine any possible inhibitory effect from other chemicals in the environmental sample. A standard curve was derived from the linear regression of the average induction ratio values obtained from the bioreporter when induced with 4CA over a linear concentration range from 0.1 to 1.0 mM. The concentration of 4CA in the environmental samples was then calculated as the 4CA-equivalent concentration from the 4CA standard curve (Supplement 2).

For HPLC analysis, the soil suspension (with and without 4CA-spike), either as a water-extracted or a methanol-extracted sample and the wastewater samples (with and without 4CA-spike) were analyzed at 30 °C using reverse phase HPLC [23]. A standard curve was derived from the linear regression of the average HPLC peak areas for each 4CA concentration over the 0.05–1.2 mM range (Supplement 2). The concentration of 4CA in each test sample was then calculated from the standard curve. Additionally, the soil suspension and wastewater samples were also analyzed by GC/MS (Agilent 7890A GC/7000B Triple Quadrupole MS). Data acquisition and analysis was conducted by comparison of the results with the mass spectra database (The NIST 11 mass spectral database, SIS Inc., USA).

Data analysis

GraphPad Prism 5.04 was used to analyze the data, to generate non-linear best-fit lines, and to estimate the $K_{1/2}$ value (i.e., the concentration at which the half-maximal effect is observed) [24] from the graph.

Results and discussion

Expression of *todST* and *todC2* gene transcripts in PpF1 and PpT57 in response to toluene and 4CA

PpT57 can utilize toluene as a sole carbon source, using the toluene dioxygenase pathway, and its *todC1C2BADE* sequence is homologous (>99 % similarity) to that of PpF1 [10]. The sequence immediately downstream of the *tod* operon comprises of two genes: *todS*, a sensory histidine kinase gene, and *todT*, a transcriptional response regulator gene. In this study, the *todST* sequence in PpT57 was preliminarily characterized. The 2,725-bp partial sequence of *todS* and the 3,579-bp *todST* were PCR-amplified from genomic DNA of PpT57 and PpF1 and digested with various restriction enzymes. The restriction-digest profiles, encompassing unique sites from PpT57 and PpF1, were similar (data not shown). The expression level of the PpT57 *todS*, *todT*, and *todC2* gene transcripts, analyzed by qRT-PCR, were markedly induced in the presence of toluene and 4CA, but not with ethanol as the solvent control (Fig. 2). Moreover, the expression profile of these three genes in PpT57 was broadly similar to that of PpF1, but occurred at different levels (Fig. 2). The fact that *todS* and *todT* genes were expressed in response to 4CA suggested

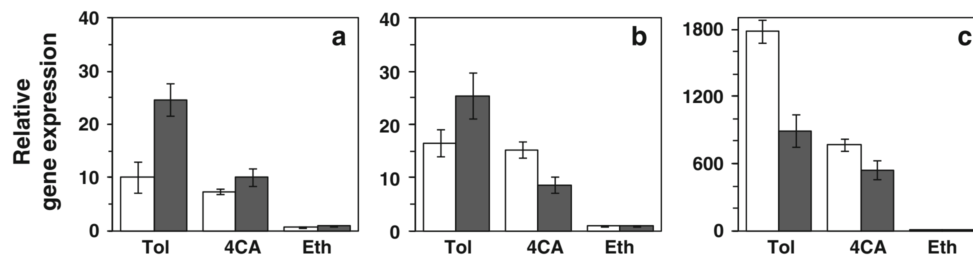

Fig. 2 Gene transcript expression levels from PpF1 (*open square*) and PpT57 (*filled square*): Cells were induced with 1 mM of: toluene (Tol), 4-chloroaniline (4CA) and ethanol (Eth) (the control). The expression level of the **a** *todS*, **b** *todT*, and **c** *todC2* transcripts were determined, normalized and reported as the relative value to that of the non-induced cells. Data are shown as the mean ± SD, derived from at least three independent repeats, and the *error bars* represent the standard errors of the mean

the potential of the TodST TCS as a CA-sensing moiety, and thus led us to construct the plasmids for a bioreporter to further investigate the recognition range and response of the PpT57's TodST system to aniline and a series of CAs.

Description of the bioreporter plasmids

The schematic organizations of the pPXGFP and pTODST plasmids are shown in Fig. 1. Both plasmids were transformed into DH5α, creating the DH5α/pPXGFP–pTODST bioreporter. In this study, the bioreporter was classified as an orthogonal-designed whole-cell bioreporter [22], in which the host cell is employed only for the production and maintenance of the sensor/reporter components, and has no indigenous function for biodegradation of the target aromatic compounds (data not shown). This design of the bioreporter system focuses on the sensing of the target chemicals and eliminates the sensing signal interference from structurally related intermediates generated from aromatic biodegradation pathways.

Generally, a plasmid-based bioreporter contains a single plasmid, in which TCS gene, suitable promoter, and reporter gene are all assembled together [22]. Nevertheless, there are a large variety of TCS in bacteria and the cross-regulation among TCS and promoters is not completely understood. Thus, the application of a bioreporter with a single-plasmid, harboring an entire genetic circuit, may generate a false-positive signal from the activation of the promoter by any other sensing protein that is capable of recognizing the target effector. This would then result in an over-estimated value of the bioreporting signal. Therefore, in this study, the adapted strategy was to serially introduce two plasmids into the exogenous host to create the bioreporter. The first plasmid contained the P_{todX} fused with the *gfp* reporter gene and was constructed using a pQF50-promoterless vector backbone, and was transformed into DH5α to create DH5α/pPXGFP. Subsequently, the second plasmid, pTODST that harbored *todST* gene under the control of P_{lac} in pSTV28, was introduced. By this approach, the positive signal from the target chemical would be directly relayed from the bioreporter, i.e., DH5α/pPXGFP–pTODST, whereas the interference signal generated from cross-talk between other TCS and the P_{todX}, would be detected using DH5α/pPXGFP and so would be subtracted from the actual bioassay.

The effect of the culture-medium composition and induction time on the bioreporter inducibility with toluene and 4CA

The effect of the culture-medium composition on the fluorescence induction by the DH5α/pPXGFP–pTODST in response to toluene and 4CA was examined as a time-course test. Toluene and 4CA induced *gfp* expression in a similar manner in all test media, but the expression levels varied among the different medium types (Supplement 3). When grown in LB or TB, cells exhibited a high background fluorescence level, even in the absence of the effectors. As a consequence, the induction ratio could not be distinguished. On the other hand, when the bioreporter obtained from cells were grown in M9G, a low background fluorescence intensity level was obtained, and so the induction ratio with either toluene or 4CA showed clear differences being markedly higher than that of the non-induced cell control. Therefore, the M9G medium was used to grow the bioreporter cells for subsequent tests.

In addition, the induction of GFP by toluene or 4CA was clearly both time-dependent. As the exposure time to the effector increased, so the GFP expression level increased. For M9G-grown bioreporter cells, although the fluorescence was maximal after a 9-h exposure, a 3-h exposure time resulted in clear induction differences between the treatment and the negative control and was sufficient for discrimination. Thus, a 3-h exposure time could be used for applications requiring a high throughput rate where a relatively low sensitivity is not a problem. However, compromising on increasing the assay time for an increased sensitivity, a 9-h induction period of M9G-grown cells was selected as the optimized induction time for further characterization in this study. Depending on several factors

including the characteristic of the bioreporter constructed plasmid, the host strain and the assay conditions, the previous reports of the optimal *gfp*-induction period was markedly varied. For example, the detection of toluene and related compounds by the *gfp*-containing bioreporter construct in *E. coli* was reported to require an induction of 2-h using P_{tbutA1}/*TbuT-gfp* [21], 8-h using P_r(*xylR*)/P_u-*gfp* [16], and 24-h using P_u(*xylU*)-*XylR* [17].

Effector range and sensitivity of the bioreporter to various concentrations of BTEX and CAs

The bioreporter systems developed in other studies have consisted of different genetic constructs and reporter genes, resulting in different in bioassay conditions, effector inducibility and response intensities [22]. As a consequence, a direct comparison of the characteristics of each bioreporter is generally impracticable and so the inducibility and response characteristics of any given bioreporter has to be individually evaluated prior to its application [12, 16, 20].

Although the biochemical and genetic information of the TodST TCS in *P. putida* has been comprehensively studied [5–7], its potential for development as a bioreporter system is poorly evaluated [1, 2]. Therefore, in this study, the bioreporter was examined for the inducibility with various effectors. Among the test alkanes and alcohols, a fluorescence induction was only observed with benzyl-alcohol (Fig. 3a). This result agrees with a previous report that TodS recognizes and binds to monoaromatic compounds and that benzyl-alcohol could be classified as an agonist that binds to TodS and induces the expression from P_{todX} [6].

In addition, to define the sensitivity of the $P_{todX(T57)}$-TodST system, the concentration-dependent inducibility of the bioreporter for BTEX was examined (Fig. 3b and c). It should be noted that no significant toxicity effect to the cells was observed within the range of BTEX concentrations used (Fig. 3). Moreover, the DH5α/pPXGFP cells did not exhibit any positive fluorescence signal, indicating that there is no cross-regulation by other TCS when BTEX was applied. The bioreporter positively responded to BTE (Fig. 3b), *m*-xylene (*m*-X), *p*-xylene (*p*-X), and the mixed xylenes, as the fluorescence intensity increased when the chemical concentration was increased, but not to *o*-xylene (*o*-X) (Fig. 3c). The response data were best fit by a sigmoidal dose–response relationship (data not shown) and nonlinear least-squares fit of the data yielded a $K_{1/2}$ value for BTE of 2.3, 0.15, and 378.2 μM, respectively, and for *m*-X, *p*-X, and the mixed xylenes of 78.8, 158.3, and 524.9 μM, respectively. The limit of detection (LOD) of the bioreporter for BTE was 0.05, < 0.01 and 50 μM, respectively, and for *m*-X, *p*-X, and the mixed xylenes was 5, 0.5 and 500 μM, respectively. The fact that *o*-X could not induce $P_{todX(T57)}$ activity is in agreement with a report of PpT1E. In fact, *o*-X is classified as an antagonist that can bind to TodS, but is unable to induce the promoter activity [6]. In addition, the presence of *o*-X in the mixed xylenes led to the partial inhibition of the bioreporter activity, as shown by its lower sensitivity (i.e., higher $K_{1/2}$ and LOD values) to the mixed xylenes. The inhibition phenomenon by *o*-X has been reported previously with respect to the toluene-mediated induction of $P_{todX(DOT\text{-}T1E)}$ activity [6].

The range of effectors detected by the bioreporter was further investigated with various concentrations of aniline and CAs. Aniline and MCAs were clearly able to induce the bioreporter activity (Fig. 4a). 3CA and 4CA were relatively powerful inducers with a 9.9 ± 1.0 and 9.6 ± 0.9 fold induction ratio at 1 mM, respectively. Concentrations of MCAs above 1 mM were toxic to the bioreporter cells and consequentially resulted in a reduced induction ratio. The $K_{1/2}$ value for aniline, 2CA, 3CA and 4CA was 27.8, 54.9, 47.2, and 2.7 μM, respectively. The LOD values for aniline, 2CA and 3CA were all approximately 5 μM, whereas it was <0.5 μM for 4CA. The fact that the $P_{todX(T57)}$ could be significantly induced by MCAs with a different substituent position in the aromatic ring at the C2, C3, and C4 position indicated the broader effector range of $P_{todX(T57)}$ than that of PpT1E since the $P_{todX(DOT\text{-}T1E)}$ was reported to be barely induced by *o*-substituted compounds (e.g., *o*-chlorotoluene and *o*-toluidine) [6].

With respect to polychlorinated anilines (Fig. 4b, c), 34DCA was the best inducer among the DCAs, whereas 35DCA was the worst inducer and the most toxic one for the bioreporter cells. The sensitivity expressed as the $K_{1/2}$ value, for 23DCA, 24DCA, 34DCA, and 35DCA was 56.9, 463.2, 66.5, and 1,267 μM, respectively, while the LOD values were approximately 50 μM for all DCAs. For the TCAs, the bioreporter responded to 234TCA and 245TCA with a $K_{1/2}$ value of 64.2 and 543.5 μM, respectively, and a LOD of approximately 50 μM. However, no significant GFP response was seen in response to 246TCA. Although the previous work on $P_{todX(DOT\text{-}T1E)}$ indicated that poly-substituted benzene derivatives could not induce $P_{todX(DOT\text{-}T1E)}$ expression [6], in contrast here several DCAs and TCAs were observed to stimulate the expression of $P_{todX(T57)}$, supporting the notion of the broader effector range of the DH5α/pPXGFP–pTODST bioreporter. In addition, the test conducted with DH5α/pPXGFP did not yield a positive signal, indicating that there is no cross-regulation by other TCS when aniline or the respective CAs was used as inducers.

Test of the bioreporter to soil and wastewater samples

The utility of the DH5α/pPXGFP–pTODST bioreporter was then evaluated on environmental samples. It is worthwhile to note here that because the TodST used for

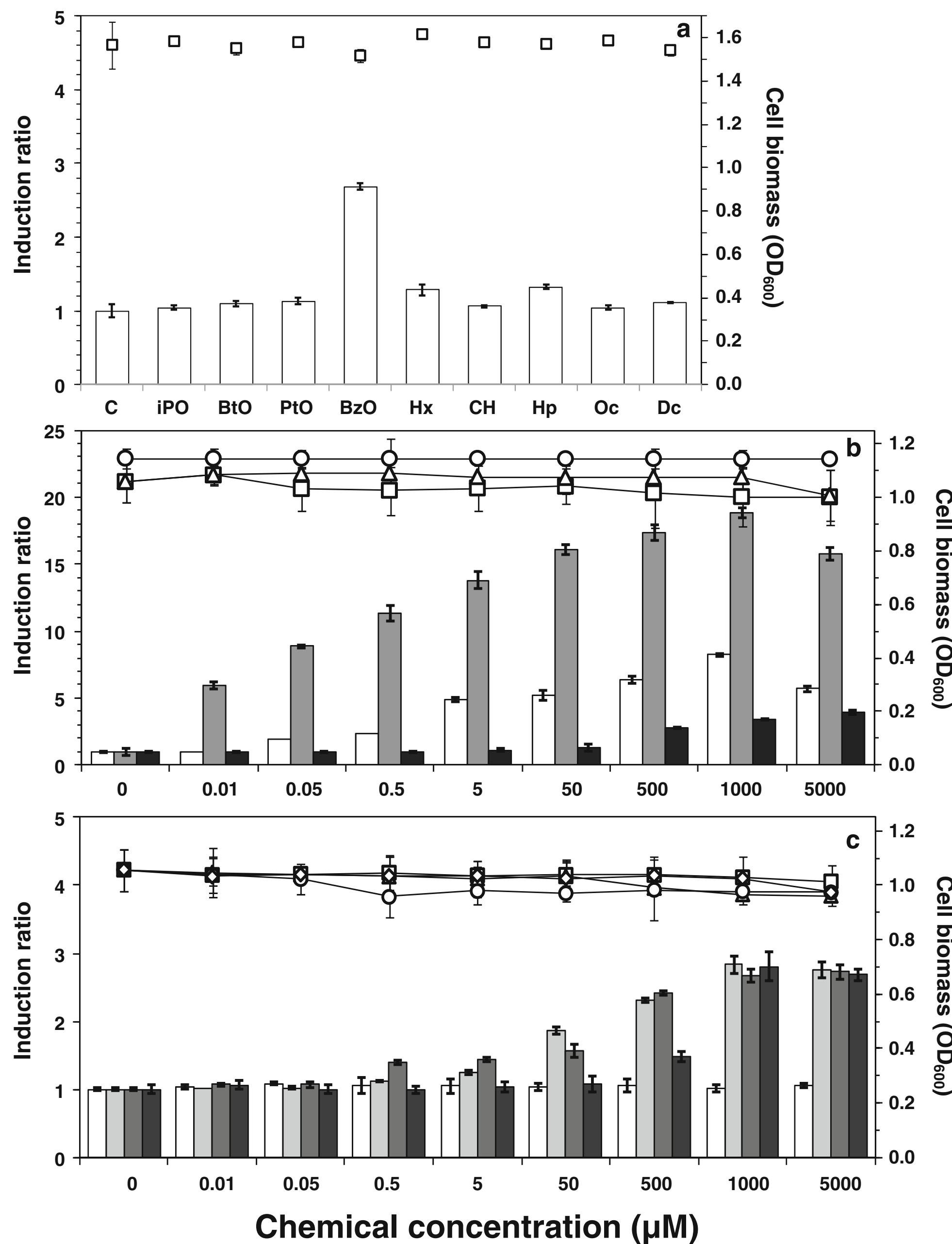

Fig. 3 Fluorescence induction ratio (*bar*) and cell biomass (*point and line*) of the DH5α/pPXGFP–pTODST bioreporter when exposed to: **a** 1 mM of iso-propanol (iPO), *n*-butanol (BtO), pentanol (PtO), benzyl alcohol (BzO), hexane (Hx), cyclohexane (CH), heptanes (Hp), octane (Oc), decane (Dc) or the non-exposed cell control (C); **b** various concentrations of (symbols are for the induction ratio and cell biomass, respectively): benzene (*open square*, *open square*), toluene (*dark gray square*, *open triangle*), and ethylbenzene (*filled square*, *open circle*); and **c** various concentrations of: *o*-xylene (*open square*, *open square*), *m*-xylene (*gray square*, *open triangle*), *p*-xylene (*filled square*, *open circle*), and the mixed xylenes (*filled square*, *open diamond*). Data are shown as the mean ± SD, derived from at least three independent repeats, and the *error bars* represent the standard errors of the mean

the reporting signal generation is generally capable of detecting a range of closely related chemicals, the readout result is accordingly expressed as an "equivalent target concentration". The use of "equivalent target concentration" may not be a direct interpretation of a single contaminant, but as the bioreporter assay is considered an initial, relatively rapid analysis, which additionally provides a toxicity sense of the contaminants, this expressing unit has been accepted for use [22]. Accordingly, the 4CA levels in the test samples estimated by the bioreporter

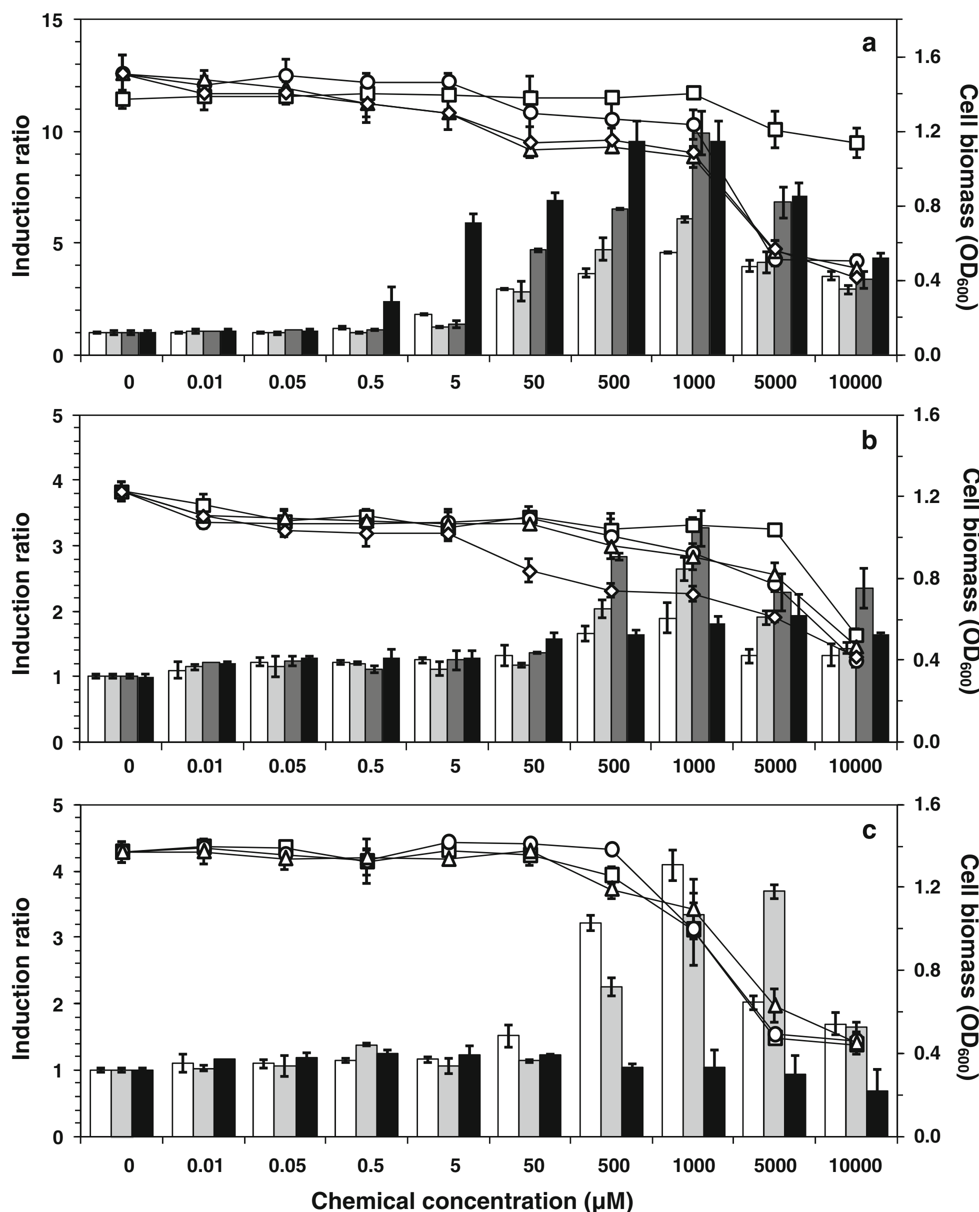

Fig. 4 Fluorescence induction ratio (*bar*) and cell biomass (*point*) of the DH5α/pPXGFP–pTODST bioreporter when exposed to various concentrations of (*symbols* are for the induction ratio and cell biomass, respectively): **a** aniline (*open square*, *open square*), 2CA (*gray square*, *open circle*), 3CA (*filled square*, *open triangle*), and 4CA (*filled square*, *open diamond*); **b** 23DCA (*open square*, *open square*), 24DCA (*gray square*, *open circle*), 34DCA (*dark gray square*, *open triangle*) and 35DCA (*filled square*, *open diamond*); **c** 234TCA(*open square*, *open square*), 245TCA (*gray square*, *open circle*) and 246TCA (*filled square*, *open triangle*). Data are shown as the mean ± SD, derived from at least three independent repeats, and the *error bars* represent the standard errors of the mean

analysis are reported as 4CA-equivalent concentrations and were then compared to those obtained by the HPLC analysis of the same samples (Table 2).

The analysis of soil by the bioreporter and HPLC analysis both indicated trace level of 4CA, but a 1.6-fold higher level when derived from the bioassay compared to the HPLC analysis. This may be due to the presence of other positive effectors in the soil sample that could slightly induce the bioreporter activity. In agreement with this notion is that the GC–MS analysis of the soil extract revealed the presence of trace aromatic derivatives (data not shown). In general, the potential drawback of the bioreporter application is that there are influences (either stimulation or inhibition) from several environmental factors on: (1) the bacterial survival, including extreme pH or toxic compounds, and (2) the bioreporter response level, including the presence of multiple compounds [14]. In this study, the chemicals presented in the soil extract were not severely toxic to the bioreporter cells (data

Table 2 Comparison of the detection of 4-chloroaniline (4CA) in soil and wastewater (WW) samples using the bioreporter and HPLC analyses

Bioreporter assay composition[a]				4CA-equivalent concentration (mM)[h]	Test purpose
Sample	M9G	Bioreporter	4CA		
Bioreporter assay[i]					
Soil ext[b]	+	+	–	0.014 ± 0.001	The test with original soil extract
Soil ext-S[c]	+	+	+	0.655 ± 0.014	The test with a water-extracted 4CA-spiked soil sample
Soil ext	+	–	–	–[e]	Influence of other soil bacteria
Soil ext	+	+	+[d]	0.877 ± 0.008	Possible influence from soil extract
Control	+	+	–	–	Control, non-induced cells
WW[f]	+	+	–	0.003 ± 0.001	The test with original wastewater
WW-S[g]	+	+	+[g]	0.264 ± 0.019	The test with 4CA-spiked WW, and possible influence from WW
WW	+	–	–	–[e]	Influence of other bacteria in WW
Control	+	+	–	–	Control, non-induced cells

Sample characteristics	4CA concentration (mM)[h]
HPLC analysis[i]	
Soil extract (original, non-spiked soil; water-extracted)	0.009 ± 0.001
Soil extract (4CA-spiked soil; water-extracted)[c]	0.534 ± 0.081
Soil extract (4CA-spiked soil; methanol-extracted)[c]	0.886 ± 0.069
WW (original, non-spiked WW)	ND
WW spiked with 4CA[g]	0.272 ± 0.014

[a] The bioreporter composition and quantity are as described in "Materials and Methods"

[b] Water-extract of the original soil sample

[c] Water-extract of the 1-mM 4CA-spiked soil sample (spiked prior to the extraction by water)

[d] Water-extract of original soil sample, plus 1-mM 4CA (added after extraction)

[e] The value of induction ratio was not significant and below that of the non-induced cell control

[f] Original wastewater

[g] Wastewater with the addition of 0.2-mM 4CA

[h] Calculated from the 4CA-derived standard curves (Fig. S1a and S1b)

[i] Data are shown as the mean ± SD, derived from at least three independent repeats, and the *error bars* represent the standard errors of the mean

ND not detected

not shown), but still adversely affected the bioreporter activity as shown by the 10 % decrease of the 4CA level (i.e., the 4CA equivalent concentration was 0.877 ± 0.008 mM as opposed to 1 mM).

In the soil sample that was spiked with 4CA at 1 mM prior to water extraction, the 4CA concentration was estimated to be 0.655 ± 0.014 mM using the bioassay. This value was approximately 10 % difference from the HPLC analysis, suggesting the acceptable ability of the DH5α/pPXGFP–pTODST bioreporter. This efficient ability of the bioreporter was also confirmed with the 4CA-spiked wastewater, where a similar result was obtained with both analyses (Table 2). The relative low extraction efficiency of 4CA from soil is likely to be due to the partial adsorption of 4CA onto soil particles and the partitioning into the extraction water of only the 4CA that was loosely attached to the soil or dissolved in soil moisture content. Although the extraction efficiency was improved by the use of methanol as the solvent instead of water (for HPLC analysis), in terms of ecotoxicology assessment, the use of water extraction could actually be an advantage of the bioreporter, in that it can distinguish the level of immediately leachable (potentially hazardous) pollutants in the environment to living organisms. In addition, for bioremediation application of the target pollutant, the chemical monitoring by the bioreporter can provide useful information of the remaining and bioavailable level of the target chemical, while the chemical analysis cannot.

In conclusion, the DH5α/pPXGFP–pTODST bioreporter was constructed based upon the functional relationship between the TodST TCS and the $P_{todX(T57)}$ of *P. putida* T-57. To our knowledge, the co-existing double plasmid bioreporting system is markedly unique and has the advantage in that when applied along with a negative

control (DH5α/pPXGFP), interference from other TCS cross-regulation can be ruled out, resulting in a potentially more accurate readout. This work not only expands the previously reported range of the sensing capacity of the TodST-based sensor from BTEX to include aniline and CAs, but the results also indicate a broader recognition range of PpT57's TodST towards *o*-substituted aromatic compounds, and several polychlorinated-substituted aniline derivatives. The successful test results with environmental samples indicated the potential application of this bioreporter in ecotoxicology assessment and bioremediation of soil or water contaminated with aniline and/or CAs.

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