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Notes & Tips

Specific detection of the pesticide chlorpyrifos by a sensitive genetic-based whole cell biosensor

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ABSTRACT

The *Sinorhizobium meliloti chpA* promoter is highly induced in the presence of the pesticide chlorpyrifos (CPF) through the action of the transcriptional activator, ChpR. A whole-cell biosensor for the detection of CPF was developed and is composed of an *Escherichia coli* strain carrying a *chpR* expression vector and a *chpA* promoter–*atsBA* transcriptional fusion plasmid encoding sulfatase (*atsA*) and formylglycine generating enzyme (*atsB*) from *Klebsiella* sp. The sulfatase is posttranslationally activated by formylglycine generating enzyme (FGE) and then converts 4-methylumbelliferyl sulfate (4-MUS) to the fluorescent product, 4-methyllumbelliferone (4-MU). This biosensor system exhibited a linear response range from 25 to 500 nM CPF.

Chlorpyrifos (CPF, *O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridylphosphorothioate) is a broad spectrum organophosphate insecticide widely used in agriculture to control insect pests. The use of CPF leads to atmospheric dispersal and its accumulation in soil and water and on foods such as vegetables and fruits [1]. CPF use raises environmental concerns because it is a neurotoxin and suspected endocrine disruptor that has been associated with asthma, reproductive and developmental toxicity, and acute toxicity [2]. The general chemical methods used to detect CPF are gas chromatography (GC) [3] and high-performance liquid chromatography (HPLC) [4]. Other biotechnology-based methods have been developed and include an immunochromatographic electrochemical biosensor to detect CPF metabolites as a biomarker of exposure [5] and a cholinesterase biosensor based on screen-printed electrodes that can detect amounts as low as 1 ppb CPF (2.86 nM) following an incubation time of 10 min [6]. However, these previously described methods involved high-cost equipment, were time-consuming, and required multi-step sample preparation. A cheaper, yet specific and sensitive, alternative for the detection of pesticides is the use of genetic-based whole cell biosensors. On exposure to a specific pesticide, a pesticide-sensing regulator stimulates the activity of a target promoter fused to a suitable reporter gene. This leads to high expression of the reporter gene as a detectable signal.

Previously, our research group reported the discovery of a CPF-sensing transcription factor together with a CPF-responsive promoter from *Sinorhizobium meliloti* [7]. We found that CPF highly induced *chpA* promoter activity in a manner that was dependent on *chpR*, a CadC family transcriptional regulator located upstream of, and divergently transcribed from, *chpAB* [7]. ChpR was found to mediate the CPF-inducible expression of the *S. meliloti chpA* promoter through direct interaction with the *chpA* promoter [7]. A genetic-based whole cell biosensor was developed based on the CPF-responsive transcription factor (ChpR) and the *chpA* promoter transcriptionally fused to *lacZ*. This biosensor consisted of the *chpA* promoter–*lacZ* transcriptional fusion plasmid, pMP–chpAp, which contains 800 bp of sequence upstream of *chpA* along with a *chpR* expression plasmid. When exposed to 100 μM CPF for 1 h, an *Escherichia coli* strain carrying both plasmids showed an induction in β-galactosidase activity that was at least 2.5-fold higher than values obtained with other organophosphate pesticides (dichlorvos, diazinon, ethion, and methylparathion) [7].

Abbreviations: CPF, chlorpyrifos; ONPG, *ortho*-nitrophenyl-β-galactoside; 4-MUS, 4-methylumbelliferyl sulfate; UV, ultraviolet; rRNA, ribosomal RNA; PCR, polymerase chain reaction; FGE, formylglycine generating enzyme; fGly, formylglycine; 4-MU, 4-methyllumbelliferone; IPTG, isopropyl-β-D-thiogalactopyranoside; 4-MUGA, 4-methylumbelliferyl β-D-galactopyranoside.

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The ultimate aim of this work was to improve the sensitivity of the reporter system to detect CPF at nanomolar (nM) levels. To reach this goal, a more sensitive detection assay system was employed to replace the spectroscopic detection of *ortho*-nitrophenyl-β-galactoside (ONPG). A sulfatase-based detection system was chosen for its high sensitivity and widespread use in biological research. Sulfatases (EC 3.1.6) catalyze the hydrolysis of sulfate esters to yield phenol and inorganic sulfate. The activity of sulfatase can be readily measured using the small aromatic sulfated compound, 4-methylumbelliferyl sulfate (4-MUS) [8]. The reaction, shown in Fig. 1B, involves enzymatic cleavage of the sulfate group of 4-MUS, resulting in the release of 4-methylumbelliferone, a highly fluorescent product with excitation and emission maxima at 365 and 460 nm, respectively. This fluorometric assay is very specific, extremely sensitive, inexpensive, and rapid [9].

The previously isolated strain, SLS5, was used as a source for sulfatase. This detergent-resistant, gram-negative, rod-shaped bacterium was previously isolated from detergent factory effluent (kindly provided by Witawat Jangiam, Burapha University, Thailand). This strain was found to fluoresce blue on ultraviolet (UV) illumination when grown on LB agar containing 4-MUS, indicating high sulfatase activity. To identify SLS5, part of the 16S ribosomal RNA (rRNA) gene was determined by sequencing a 1498-bp polymerase chain reaction (PCR) amplified fragment of the 16S rRNA gene amplified using universal forward 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse 1525R (5′-AAGGAGGTGATCCAGCC-3′) primers [10]. When compared with sequences reported in the GenBank database, the 16S rRNA fragment of SLS5 showed 99% identity to those from members of the genus *Klebsiella* (data not shown). Moreover, when strain SLS5 was grown on MacConkey agar at 37 °C for 24 h, it exhibited mucoid, round, and pink colonies with yellow halos that are characteristic of *Klebsiella* sp. Based on the morphological characteristics and 16S rRNA gene sequence analysis, SLS5 was designated as *Klebsiella* sp. strain SLS5.

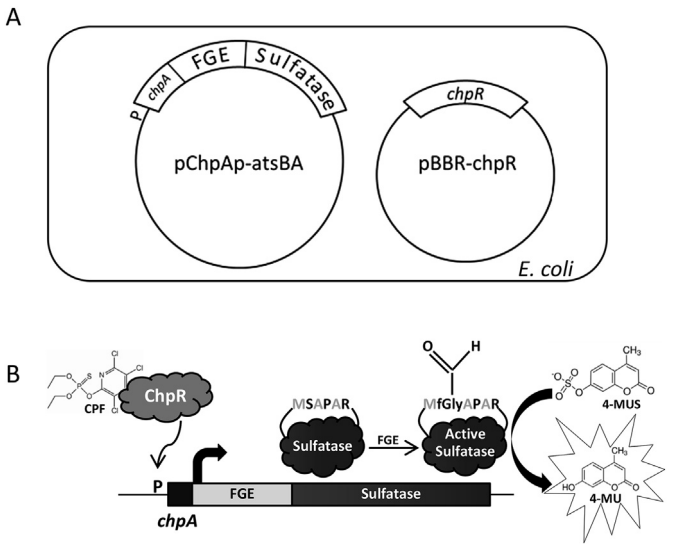

Fig.1. (A) Our *E. coli* biosensor system consists of two plasmids: pChpAp–atsBA and pBBR–chpR. The *atsBA* genes encode FGE and sulfatase, respectively, whereas ChpR is a chlorpyrifos-responsive transcription activator. (B) Architecture of whole cell genetic-based biosensor using sulfatase as a reporter system. On exposure of *E. coli* to CPF, active ChpR triggers the expression of FGE and sulfatase gene (via *chpA* promoter). FGE then modifies the serine residue at position 72 of sulfatase to fGly. The activated sulfatase cleaves the sulfate group from 4-MUS to produce the fluorescent product 4-MU. P stands for promoter.

The most common types of sulfatase are formylglycine-dependent sulfatases that are classified as group I sulfatases. They are often termed arylsulfatases and are widely distributed in both eukaryotes and prokaryotes [11]. In bacteria, the loci encoding aryl sulfatases generally contain two genes (*atsBA*). *atsA* encodes arylsulfatase, whereas *atsB* encodes a formylglycine generating enzyme (FGE), which is a posttranslational activator of arylsulfatase (see Fig. 1B). A 3-kbp PCR product spanning *atsBA* was amplified from the chromosome of *Klebsiella* sp. strain SLS5 by PCR using primers BT4662 (5′CAAGATCTGAGGATAAGCCGATGC3′) and BT4663 (5′GAAGATCTTCTTAATGGATCTGCT3′) (underlined sequences indicate *Bgl*II recognition sites), which are specific to conserved regions flanking *atsBA* that were identified from multiple sequence alignments of a number of *Klebsiella atsBA* loci (data not shown). The PCR fragment was then cloned into pGemTeasy to generate pGemT–atsBA. The complete DNA sequence of the entire 3-kbp PCR clone was determined and deposited at GenBank under accession number KR261597. The DNA sequence of *atsB* revealed an open reading frame (ORF) of 1188 bp that is predicted to encode a protein of 395 amino acids in length with a molecular mass of 43.9 kDa. The deduced amino acid sequence of SLS5 *atsB* is 97.97% identical to that of *Klebsiella pneumoniae atsB* (GenBank accession number AJ131525) [12]. SLS5 *atsA* is 1734 bp long and is predicted to encode a protein of 577 amino acids with a molecular mass of 64.2 kDa. The *atsA* of SLS5 shares 99.63% amino acid identity with the deduced amino acid sequence of *K. pneumoniae atsA* (GenBank accession number AJ131525) [12]. *atsA* from both organisms share the common pentapeptide SAPAR motif, which directs the posttranslational modification of the first serine residue at position 72 to formylglycine (fGly) (Fig. 1B).

To construct a *chpA* promoter–*atsBA* transcriptional fusion, the *Bgl*II fragment of pGemT–atsBA spanning SLS5 *atsBA* was inserted into the *Bam*H1 site of plasmid pMP–chpAp containing the *chpA* promoter [7] to generate the transcriptional fusion plasmid, pChpAp–atsBA. The ChpR expression plasmid, pBBR–chpR, contains a 2-kbp *Kpn*I–*Xho*I fragment from pDrive–ChpR spanning *S. meliloti chpR* inserted into pBBR1MCS5 [13]. pDrive-ChpR carries a 2-kbp PCR fragment, obtained by amplification of chpR from the *S. meliloti* chromosome using primers BT1709 (5′GATCACT CGGGCAATCCG3′) and BT1711 (5′CGGCAGCTGCAGCAGCCAGC3′), ligated into pDrive (Qiagen, USA). Both pChpAp–*atsBA* and pBBR–chpR were introduced into *E. coli* DH5α (Fig. 1A). Overnight cultures were subcultured at 1% (v/v) into LB medium containing tetracycline and gentamycin to an A_{600} of 0.4. 4-MUS (100 μM final concentration) was then added along with varying concentrations of CPF (25, 50, 100, 250, and 500 nM) and incubated at 37 °C for 4 h. Exposure to CPF induces ChpR-mediated expression of *atsAB* (via *chpA* promoter), which in turn modifies serine within the catalytic pentapeptide motif of AtsB to fGly, thereby activating the sulfatase. Active sulfatase then converts 4-MUS to 4-methyllumbelliferone (4-MU) (Fig. 1B). The fluorescence emission of 4-MU was measured spectrofluorometrically (SpectraMax M5, Molecular Devices, USA) using excitation and emission wavelengths of 365 and 460 nm, respectively, and sulfatase activity was calculated as described previously [14].

CPF detection using the conventional genetic-based whole cell biosensor based on the β-galactosidase reporter system was also performed for comparison. The *chpA* promoter–*lacZ* transcriptional fusion plasmid, pMP–chpAp, was cotransformed into *E. coli* DH5α along with plasmid, pBBR–chpR. For assays using ONPG as the chromogenic substrate, cells were grown in LB medium containing tetracycline and gentamycin to an A_{600} of 0.5 followed by the addition of varying concentrations of CPF and 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell cultures were then further incubated at 37 °C for 4 h. Lysis solution (50 μl; B-per, Thermo Scientific, USA) was added to 100 μl of induced cell culture followed by 20 μl of ONPG (4 mg/ml) and incubated for 10 min at room temperature, and

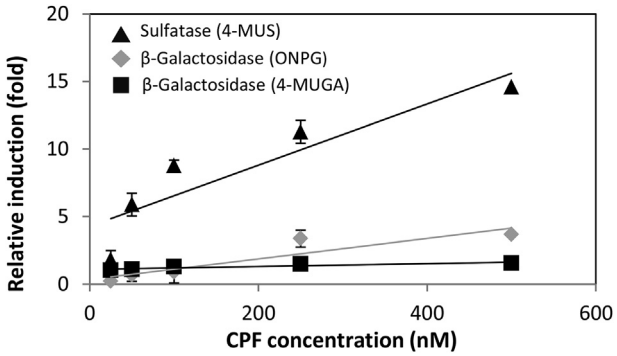

Fig.2. Chlorpyrifos detection by genetic-based whole cell biosensor comparing the two reporter systems: β-galactosidase and sulfatase. The results shown are the averages of three independent assays. Error bars show standard deviations.

then the reaction was terminated by the addition of 50 μl of 1 M Na_2CO_3 before the A_{420} value was determined [15]. In assays using the fluorescent substrate 4-methylumbelliferyl β-D-galactopyranoside (4-MUGA), overnight cultures were subcultured at 1% (v/v) into LB medium containing tetracycline and gentamycin to an A_{600} of 0.4. 4-MUGA (100 μM final concentration) was then added along with varying concentrations of CPF and incubated at 37 °C for 4 h. The fluorescence emission of 4-MU was measured using excitation and emission wavelengths of 365 and 460 nm, respectively.

A comparison of the results obtained between the two systems is shown in Fig. 2 and revealed that the sulfatase system gave a much more robust response than either of the β-galactosidase reporter systems. The sulfatase reporter system exhibited a linear response range from 25 to 500 nM CPF (Fig. 2). The fold induction observed with the sulfatase-based system was 8.7–9.7 times greater than that of the β-galactosidase reporter system (ONPG) (8.7-fold for 25 and 50 nM and 9.7-fold for 100 nM CPF) and was 1.8–6.7 times greater than that of the β-galactosidase reporter system using 4-MUGA (1.8-fold at 25 nM, 5.4-fold at 50 nM, and 6.7-fold at 100 nM CPF) at CPF concentrations of 100 nM and below. We noticed a strong background when using 4-MUGA as a substrate for β-galactosidase, which made it difficult to detect significant induction over the CPF concentration range tested. Analyses of the mean induction values for each CPF concentration using analysis of variance (ANOVA) showed statistically significant induction down to 250 nM CPF with the β-galactosidase system using either substrate. By contrast, fluorescence induction using the sulfatase system was statistically significant at 25 nM, making this system at least 10-fold more sensitive. The stronger signal of the sulfatase biosensor system (*E. coli* culture broth) allowed detection by eye under UV light at 25 nM (8.76 ppb) CPF, whereas the β-galactosidase system did not generate visible color under the same CPF concentration. Moreover, our sulfatase system was used to detect CPF in environmental water samples from a pond. The filter-steriled water samples were mixed with an equal volume of 2 × LB medium containing tetracycline, gentamycin, and 200 μM IPTG, followed by the addition of overnight bacterial cultures to an A_{600} of 0.1. After incubation for 3 h, 100 μM 4-MUS was added together with varying concentrations of CPF and incubated at 37 °C overnight. The result revealed statistically significant induction down to 250 nM CPF (data not shown).

One recent report dealing with organophosphate pesticide detection using a whole cell biocatalyst/biosensor yielded results that were similar to those reported here. The system consisted of an *E. coli* strain expression surface displayed organophosphorous hydrolase (OPH) and methyl parathion hydrolase (MPH) [16]. This system reported a linear relationship between the fluorescence signal and organophosphate pesticide concentration (parathion and methyl parathion) from 20 to 200 μM with a low detection limit of 2 μM (583 ppb)—three orders of magnitude less sensitive than the CPF detection system reported here. This study demonstrates the development of a sensitive genetic-based whole cell biosensor specific to CPF using sulfatase as a reporter system.

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