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Construction of genetically engineered bacteria that degrades organophosphorus pesticide residues and can be easily detected by the fluorescence

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Organophosphorus compounds (OPs) are widely used in agriculture and industry and there is increased concern about their toxicological effects in the environment. Bioremediation can offer an efficient and cost-effective option for the removal of OPs. Herein, we describe the construction of a genetically engineered microorganism (GEM) that can degrade OPs and be directly detected and monitored in the environment using an enhanced green fluorescent protein (EGFP) fusion strategy. The coding regions of EGFP, a reporter protein that can fluoresce by itself, and organophosphorus hydrolase (OPH), which has a broad substrate specificity and is able to hydrolyse a number of organophosphorus pesticides, were cloned into the expression vector pET-28b. The fusion protein of EGFP–OPH was expressed in *E. coli* BL21 (DE3) and the protein expression reached the highest level at 11h after isopropyl β-D-thiogalactopyranoside induction. The fluorescence of the GEM was detected by fluorescence spectrophotometry and microscopy, and its ability to degrade OPs was determined by OPH activity assay. Those GEM that express the fusion protein (EGFP and OPH) exhibited strong fluorescence intensity and also potent hydrolase activity, which could be used to degrade organophosphorus pesticide residues in the environment and can also be directly monitored by fluorescence.

Keywords: pesticide; degradation; fusion protein; genetically engineered microorganism; organophosphate

Introduction

Organophosphorus compounds (OPs) are a broad class of neurotoxic chemicals, including insecticides, herbicides, and plasticizers, which are widely used in agriculture and industry. However, the use of OPs results in severe environment pollution and their residues can be detected in water, soil, vegetables, fruits, milk, food products, and other living organisms.[1] OPs released into the environment can be detoxified by hydrolysis by exposure to sunlight, air, soil, and so on,[2] half-life of OPs varies considerably from days to months in different environment media for the same organophosphorus pesticide.[3–5]

Several methods, such as chemical oxidation with ozone, photo degradation, biological degradation, and membrane filtration and adsorption, have been developed to cleanup OP residues in the environment.[1] In particular, the use of microorganisms in detoxification and decontamination of OPs is considered a viable and environmental-friendly approach.[3] Various microorganisms capable of biodegrading OPs have been isolated from polluted environment.[2] In addition, the development and application of genetically engineered microorganism (GEM) that can degrade OPs would be an efficient way to reduce OPs residues.

Organophosphorus hydrolase (OPH) is an organophosphotriester hydrolysing enzyme discovered in the soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* spp.[6] OPH has broad substrate specificity and is able to hydrolyse a number of OPs.[7–9] Since the natural OPH cannot be easily obtained, the development of recombinant DNA technology provides a promising approach for generating GEM that overexpresses OPH. Many GEMs have been constructed to express recombinant protein that possess diversified functions by recombinant DNA technology.[10–12]

However, some problems exist which limit their practical use. In particular, when the novel GEMs are released into natural environments, they may reproduce, spread, and transfer their novel genetic materials to the indigenous microbial populations to cause ecological problems. Thus, detecting and monitoring released GEMs in the environment is necessary. In the past years, several monitoring methods such as using gene probes,[13–15] polymerase chain reaction (PCR),[16,17] monoclonal antibodies,[18] and bioluminescence [19–21] have been developed to identify GEM. Although these methods are often sensitive, these markers do not allow for direct detection of GEMs and thus their applications were restricted. The discovery and

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application of green fluorescent protein (GFP) enable easy and feasible detection. GFP is a natural protein expressed in bioluminescent jellyfish that can emits bright green fluorescence upon UV light excitation.[22,23] Enhanced GFP (EGFP) is a red-shifted variant of wild-type GFP, which fluoresces after visible light excitation and has much stronger fluorescence than that of wild-type GFP.[24] In this study, we demonstrate the successful construction of a GEM strain to express the fusion protein of EGFP and OPH, which can be used for monitoring the GEMs that degrade OPs in the polluted environment.

Materials and methods

Construction of the expression vector pET-EGFP-OPH

The *egfp* gene was amplified by PCR except the stop codon TAA from pEGFP-N3 (Clontech, Palo Alto, CA, USA) using the primers: 5′-GAG CTA GCA TGG TGA GCA AGG GCG AGG AGC-3′ and 5′-GTA AGC TTA TGG TGC GCT CCT CCA AGA ACG TC-3′. The PCR-amplified *egfp* gene and the vector, pET-28b (Novagen, Madison, WI, USA), were digested by *Nhe*I and *Bam*HI, and then ligated with the *egfp* gene positioned between the polyhistidine tag and the thrombin recognition site. The resulting plasmid was designated as pET-EGFP. The *opd* gene, which encodes OPH, was amplified from pGEMT-opd obtained from Prof. Qiao (Institute of Zoology, Chinese Academy of Sciences). The primers used for amplifying the *opd* gene were: 5′-TTT AAG CTT ATA TGC AAA CGA GAA GGG TTG TGC TCA AGT-3′ and 5′-TTA CTC GAG TCA TGA CGC CCG CAA GGT CGG TG-3′. Restriction sites of *Hind*III and *Xho*I were added to the ends of the two primers, respectively. The PCR-amplified *opd* gene was then inserted between the *Hind*III and *Xho*I sites of pET-EGFP, and the plasmid was designated as pET-EGFP-OPH (supplementary data Figure S1).

Cell culture

The plasmid pET-EGFP-OPH was transformed into *E. coli* strain BL21 (DE3) ($F^- ompT hsdS_B (r_B^- m_B^-)) gal dcm$ (DE3) (Novagen, Madison, WI, USA) using the calcium chloride procedure.[25] The seed cultures were incubated overnight at 37 °C and then transferred into Luria-Bertani medium (50 × volume of the seed culture) containing kanamycin (50 μg/ml). The culture was grown at 37 °C in a shaker until the optical density reached approximately 0.6 (OD_{600}). The cultures were then divided into 10 flasks and induced by isopropyl β-D-thiogalactopyranoside (IPTG) (0–1 mM). All cultures were incubated continuously for 10 h at 30 °C in a shaker, and samples were collected every 2 h. OD_{600} and fluorescence intensity were measured using Beckman DU800 spectrophotometer and Hitachi F-4500 fluorescence spectrophotometer (excitation wavelength, 488 nm; emission wavelength, 540 nm and the bandwidth, 5 nm), respectively.

Expression of the recombinant protein in E. coli *and preparation of total cellular protein*

The recombinant protein was expressed in *E. coli* strain BL21 (DE3) after induction at 30 °C by 0.01 mM IPTG. After addition of IPTG, the incubation was continued for 12 h and the samples were collected hourly after the induction. The OD_{600} value and fluorescence intensity of all samples were measured by using the spectrophotometer and fluorescence spectrophotometer, respectively. Furthermore, the fluorescence of the samples was also detected by using a Leitz DMIRB microscope (Leica, Germany). Samples were divided into two parts and one of them was smeared directly on microscope slides and then observed by fluorescence microscopy; the other one was washed with 50 mM Tris-HCl (pH 7.8) and then resuspended in the same buffer to a final OD_{600} of 0.1. The suspension was smeared on slides and then observed by fluorescence microscopy.

The bacteria were harvested by centrifugation at 10,000 × *g* for 1 min and the supernatant was discarded. Pellets were completely resuspended in one-fifth volume of 50 mM Tris-HCl buffer (pH 7.8) and then sonicated on ice for 2 min. The obtained total cell protein (TCP) was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the OPH activity assay. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard.[26]

SDS-PAGE and Western blotting analysis

Equal amounts of the TCP sample (4 μg) were mixed with 4× sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 300 mM dithiothreitol, 8% sodium dodecyl sulfate, 10% β-mercaptoethanol, and 0.02% bromophenol blue) and boiled at 100 °C for 3 min and then electrophoresed on two identical 10% SDS–polyacrylamide gels.[27,28]. After electrophoresis, one gel was stained by Coomassie bright blue, while the other was transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) at 10 V for 120 min with a Bio-Rad semi-dry blotter for Western blotting analysis. The primary antibody was anti-His antibody (1:2000, Tiangen Biotech, Beijing, China). The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG (1:5000, Sigma, St Louis, MO, USA). The Western blots were developed using standard ECL (Pierce, Rockford, IL, USA) and imaged using the Bio-Rad ChemiDoc XRS system (Bio-Rad, Munich, Germany).

OPH activity assay

OPH can hydrolyse parathion, an organophosphorus pesticide, into *p*-nitrophenol, which displays a strong absorption at 400 nm. The value of OD_{400} is proportional to moles of *p*-nitrophenol produced.[29,30] By making a standard

curve of *p*-nitrophenol, the amount of parathion hydrolysed can be calculated. Bacteria were washed with 50 mM Tris-HCl buffer (pH 7.8) and then resuspended in the same buffer and the OD_{600} of the suspension was adjusted to 0.1. The reaction was carried out at 30 °C for 20 min in 1 ml of 50 mM Tris-HCl buffer (pH 7.8), containing 3 μl of 50 mM parathion (99% purity, dissolved in ethanol) and 997 μl of the cell suspension or TCP (100 μg protein). After the parathion was added into the reaction system, the OD_{400} value was monitored continuously using a Beckman DU800 spectrophotometer. The degradation rate of parathion by OPH from the GEM was expressed as the remaining amount of parathion (%) and the value was expressed as mean ± SD.

Results and discussion

IPTG-induced expression of the recombinant protein in GEM

We found that the expression of the recombinant protein in *E. coli* strain BL21 (DE3) was induced by different concentrations of IPTG. All cells have a similar growth trend (Figure 1(a)) and each achieved final fluorescence intensity beyond 4000 except the sample without IPTG induction, which remained at a lower value (400–500) during the whole course (Figure 1(b)). This is consistent with that seen by fluorescence microscopy (Figure 2(a)). When the OD_{600} of GEM suspended in the buffer was adjusted to 0.1, bright

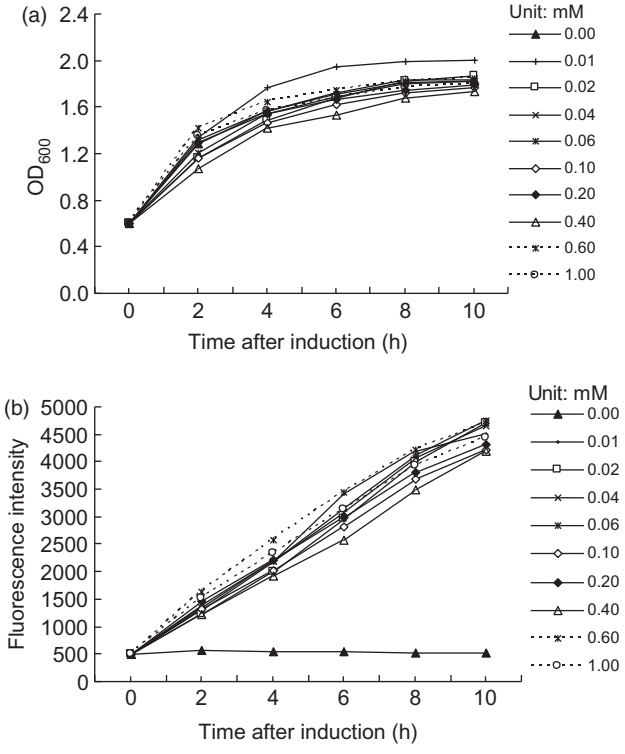

Figure 1. Growth curves and fluorescence intensity of the GEM induced by IPTG. The cultures of the GEM induced by different concentrations of IPTG (0–1 mM) were collected every 2 h and the OD_{600} (a) and fluorescence intensity (b) were measured.

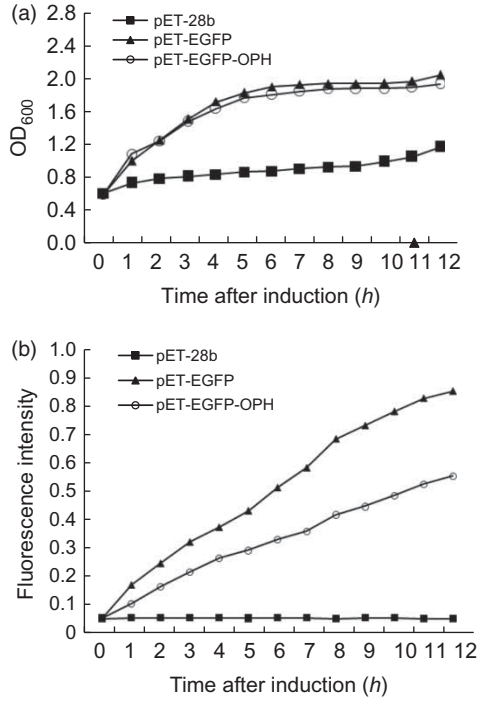

Figure 3. Growth curves and fluorescence intensity of the GEM induced by 0.01 mM IPTG. The OD (a) and fluorescence intensity (b) of cultures containing *E. coli* BL21 (DE3) transformed with pET-28b, pET-EGFP, and pET-EGFP-OPH were monitored hourly by a spectrophotometer and fluorescence spectrophotometer, respectively, after induction with 0.01 mM IPTG with an initial OD_{600} of 0.6. 0 h indicates the start time of the induction.

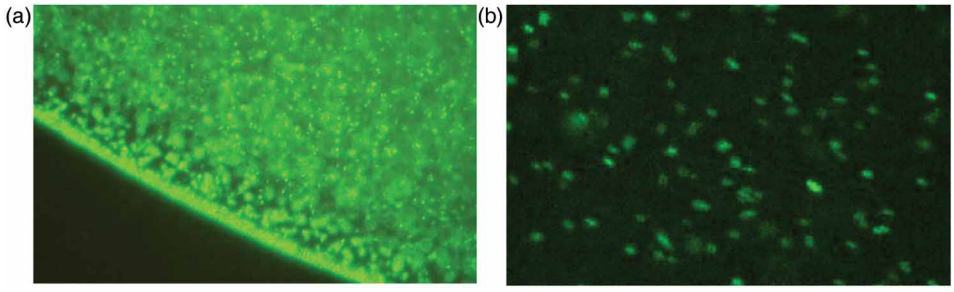

Figure 2. Fluorescence image of the GEM. Cells of the induced *E. coli* BL21 (DE3) transformed with pET-EGFP-OPH in Luria-Bertani medium (a) or in 50 mM Tris-HCl buffer (b) were smeared on microscope slides and then observed by fluorescence microscopy.

green fluorescence dots could be seen clearly under a fluorescence microscope (Figure 2(b)). However, no bright dot in the control sample could be seen in the microscope (data not shown). In addition, this result indicated that the induction with IPTG at 0.01 mM was sufficient to induce the expression of the recombinant protein. Thus, this concentration of IPTG, 0.01 mM, was chosen for all subsequent experiments.

This result showed that the concentration of IPTG we used is 100 times lower than the common dosage (1 mM), which makes this approach more feasible and practical since IPTG is a relatively expensive and toxic chemical and the reduce of IPTG dosage is desired.

Confirmation of the recombinant EGFP–OPH fusion protein expression

As shown in Figure 3(a), the OD_{600} of cells transformed with pET-EGFP and pET-EGFP-OPH was much higher than those of the bacteria cells with pET-28b, suggesting that the expressed short peptide by pET-28b is perhaps a little toxic to the host cells. The fluorescence intensity of both the bacteria containing pET-EGFP and pET-EGFP-OPH increased clearly with the increase in induction time, although the former was higher than the latter (Figure 3(b)). Those bacteria containing pET-28b displayed only a basal level of fluorescence during the whole process in spite of their ascending growth curve. The EGFP used in our study is a red-shifted variant of GFP and fluoresces 35 times brighter than wild-type GFP.[24] Because of this brighter fluorescence, detection of GEM is easier when observed by fluorescence microscopy, and thus this fluorescence could be used to monitor the dispersion and the cell density of GEM.[31,32] One thing to be noted here is that the fluorescence of the bacteria containing pET-EGFP-OPH is dimmer than that of the bacteria containing pET-EGFP, perhaps resulting from fusion with OPH in the bacteria.[33]

The expression level of recombinant EGFP–OPH fusion protein was determined by SDS-PAGE and Western blotting using TCP samples collected hourly after induction. The molecular weight of the fusion protein EGFP–OPH is predicted to be approx. 66 kDa including the His6·tag and the thrombin recognition sequence. As expected, a dark band of the recombinant protein was visualized at 66 kDa by Coomassie brilliant blue staining (Figure 4(a)), and this was verified by Western blotting with antibodies against

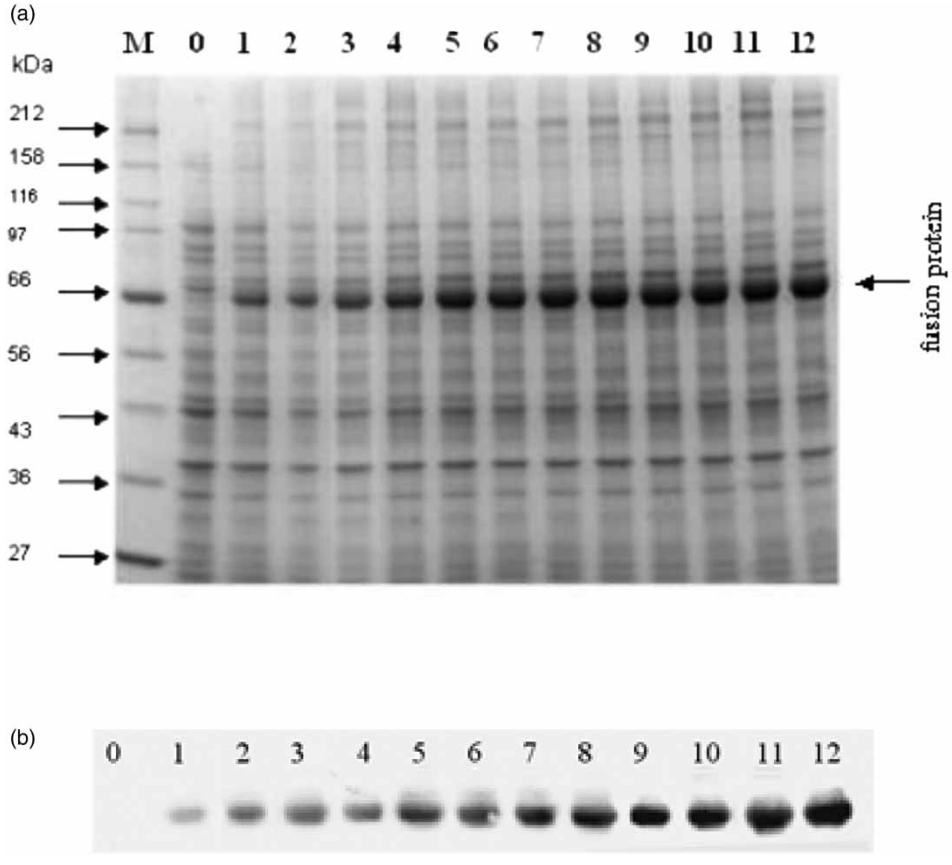

Figure 4. Detection of the fusion protein in the GEM. Equal amounts of TCPs collected from *E. coli* BL21 (DE3) carrying the pET-EGFP-OPH plasmid after different induction times were loaded per well, separated by SDS-PAGE (a) and detected by Western blotting analysis with anti-His tag antibody (b). Lane M: protein marker; lanes from 0 to 12: samples collected after 0–12 h induction, respectively. Position of fusion protein of EGFP–OPH is indicated.

the His tag (Figure 4(b)). The data indicated that recombinant EGFP–OPH was expressed in *E. coli* strain BL21 (DE3) harbouring pET-EGFP-OPH. In addition, Figure 4(a) showed that the level of EGFP–OPH fusion protein gradually increased over time in Western blotting analyses and reached the highest level at 11 h after IPTG induction (Figure 4(b)).

The pET system that was used in the study is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. After a few hours of induction, the target gene product comprised more than 50% of the TCPs. This high level of expression and the *N*-terminal His6·tag of the recombinant protein make it easy to isolate the protein by utilizing conventional chromatographic methods, thereby allowing convenient protein purification and a feasible means by which to detect the fusion protein.

Degradation of parathion by the genetically engineered bacteria

As shown in Figure 5, both TCP and whole-cell suspensions (WCS) of the *E. coli* BL21 (DE3) transformed with pET-EGFP-OPH plasmid have high catalytic activity to degrade OP. During the detection, the OD_{400} of the controls (*E. coli* BL21 (DE3) transformed with pET-28b, pET-EGFP plasmid) showed minimal change. For the TCP and WCS of GEM bacteria transformed with pET-EGFP-OPH, analyses of the first 5min of reaction showed that parathion was degraded by $33.84 \pm 2.28\%$ and $21.25 \pm 0.73\%$, respectively. The initial hydrolysis rate of the TCP was about $1.01 \pm 0.68 \times 10^{-4}$ μmol/min/μg protein. This finding of OPH activity in WCS indicates that the GEM can be used directly to degrade OPs.

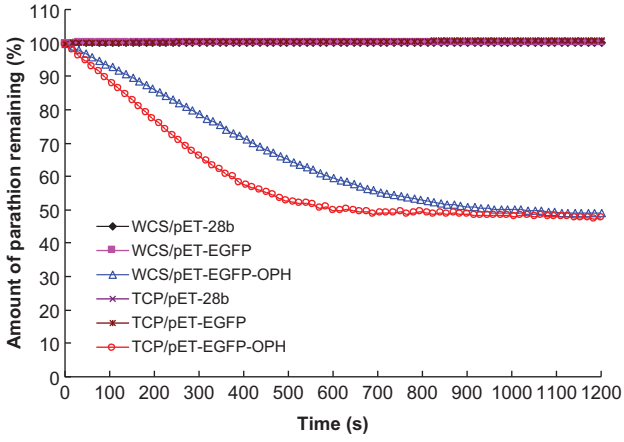

Figure 5. Degradation of parathion by the GEM. Samples were collected from *E. coli* BL21 (DE3) carrying pET-EGFP-OPH or pET-EGFP or pET-28b 11 h after induction with 0.01 mM IPTG at 30 °C. The TCP and WCS were prepared by ultrasonic processing and the OD_{400} value was monitored continuously by spectrophotometer. The degradation of parathion by OPH from the GEM was expressed as the remaining amount of parathion (%).

The high catalytic activity of the fusion protein EGFP–OPH to degrade OP as well as the high yield and the His6·tag make the GEM to be a better candidate strain for the preparation of degrading enzymes for organophosphorus pesticides.[34,35] Compared with the method of expressing hydrolase gene and marker gene separately, the prominent advantage of the EGFP fusion strategy is that the fluorescence intensity could directly reflect the OPH activity in GEM since the EGFP protein is fused to OPH.

Conclusions

In summary, the GEM we generated can exhibit strong green fluorescence and high OPH activity. The GEM could be directly used to degrade organophosphorus pollutants directly and also could be effectively monitored in practice by fluorescence detection. Moreover, this GEM could be a potential resource for preparation of degrading enzymes of organophosphorus pesticides since it is convenient to isolate and purify the target protein EGFP–OPH from the GEM due to its high expression level and the presence of His6·tag.

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Supplemental data

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