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ORIGINAL RESEARCH PAPER

# A genetically engineered *Escherichia coli*, expressing the fusion protein of green fluorescent protein and carboxylesterase B1, can be easily detected in the environment following degradation of pesticide residues

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**Abstract** Genetically engineered *Escherichia coli*, expressing the fusion protein of enhanced green fluorescent protein (EGFP) and carboxylesterase B1 (CarE B1), was successfully constructed by cloning the genes into the pET-28b vector and then transforming *E. coli* BL21 (DE3). Expression of the fusion protein was induced in *E. coli* BL21 (DE3) which could then degrade environmental pesticides and could be easily detected using fluorescence spectrophotometry or by the naked eye in daylight.

**Keywords** Carboxylesterase B1 · *Escherichia coli* · Fusion expression · Genetically engineered bacteria · Green fluorescent protein

## Introduction

Genetically engineered microorganisms (GEMs) constructed by recombinant DNA technology is an efficient way to solve the problem of pesticide residues in the environment. However, their use is still limited in the field because GEMs may cause new environmental contamination such as interference with the distribution and growth of the indigenous microbial populations. Therefore, a system that can readily detect and monitor the released organisms in the environment is needed. GFP is a natural protein expressed in bioluminescent jellyfish that can emit bright green fluorescence after UV light excitation. Enhanced green fluorescent protein (EGFP), which is a red-shifted variant of wild type GFP, fluoresces after visible light excitation or exposure to daylight and has much stronger fluorescence intensity than that of GFP (Cormack et al. 1996; Nolte et al. 2001). GFP requires no cofactors but $O_2$ for fluorescence (Lindow 1995) and can be fused with many other proteins and still fluoresce (March et al. 2003), making it ideal for monitoring GEM (Gory et al. 2001).

The carboxylesterase B1 (CarE B1) gene of *Culex pipiens quinquefasciatus* encodes an enzyme that confers pesticide resistance (Mouches et al. 1990). Reports indicate that CarE B1 also has a strong ability to degrade organic chloride pesticides, carbamates, and pyrethoid insecticides as well as organophosphorus pesticides (Vontas et al. 2000; Zhang et al. 2004b; Barata et al. 2004; Nishi et al. 2006). Although genetically engineered bacteria expressing CarE B1 have been generated (Zhang et al. 2004a, b; Lan et al. 2005), no study has investigated the use of a CarE B1 and EGFP fusion protein. Herein, we

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demonstrate the successful construction of a strain of GEM by a fusion strategy of EGFP and CarE B1. The GEM we produced can degrade pesticides and also emits green fluorescence, which allows monitoring of the GEM much easier.

## Materials and methods

### Construction of the expression vector pET-EGFP-B1

To construct the pET-EGFP-B1 expression vector, the pET-28b vector (Novagen, Madison, WI, USA) was used as a cloning vector. The *egfp* gene amplified by using two primers (5′-GA GCT AGC ATG GTG AGC A AG GGC GAG GAG C-3′ and 5′-TT GGA TCC CTT GTA CAG CTC GTC CAT GCC GAG-3′) from pEGFP-N3 (Clontech, Palo Alto, CA, USA) was cloned into the *Nhe*I and *Bam*HI sites of pET-28b to form plasmid pET-EGFP, which is located after the polyhistidine tag and the thrombin recognition site. The stop codon of the original *egfp* gene was deleted and the *Nhe*I and *Bam*HI sites were added to the ends of the primers. The B1 gene was amplified from plasmid pET-B1 (gifted from Dr. CL Qiao, Institute of Zoology, CAS, Beijing, China) by using a SacI-containing forward primer (5′-TG GAG CTC ATG AGT TTG GAA AGC TTA ACC GT-3′) and a *Xho*I-containing reverse primer (5′-TT GTT CTC GAG TCA AAA CAG CTC ATC ATT CAC GTA C-3′), and cloned into the *Sac*I and *Xho*I sites of pET-28b following the *egfp* gene to generate the resultant plasmid pET-EGFP-B1 (Fig. 1). *Pfu* DNA polymerase (Tiangen Biotech, Beijing, China) was used in all PCR amplification.

### Cell culture

*Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for the cloning and maintenance of plasmids, and *E. coli* strain BL21 (DE3) ($F^{-}$*ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3), Novagen) was used as host in expression studies. The bacteria for expression of the recombinant proteins were incubated overnight at 37°C in Luria-Bertani medium containing kanamycin (50 μg/ml) and then transferred into the same medium and continuously grown at 37°C in a shaker until cell dry weight reached approximately 0.28 g/l ($OD_{600}$ ~ 0.6). The culture was then divided into eleven flasks and induced by IPTG (0–1 mM). After incubation for 8 h at 28°C, the cultures were collected. The fluorescence intensity of each culture was measured spectroflurophotometrically (excitation wavelength, 488 nm; emission wavelength, 540 nm; the bandwidth, 5 nm).

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

*Escherichia coli* BL21 (DE3) harboring pET-EGFP-B1 was induced by 0.01 mM IPTG at 28°C and sampled hourly for 12 h. All samples were measured optical density ($OD_{600}$) and fluorescence intensity. Furthermore, to observe the fluorescence of the recombinant protein more intuitively, *E. coli* BL21 (DE3) harboring pET-EGFP-B1 were plated on LB agar plate, over which IPTG was spread. After

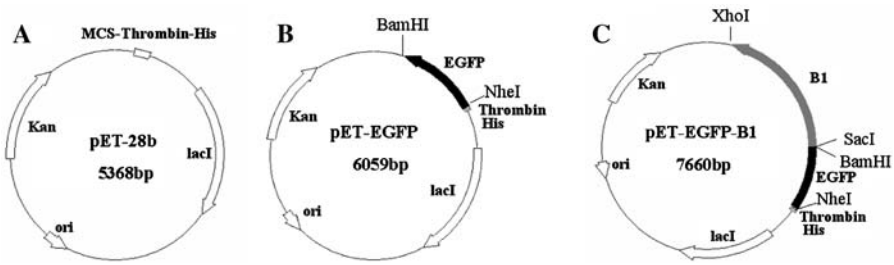


**Fig. 1** Construction maps of the recombinant plasmid. The *egfp* gene was amplified by PCR from pEGFP-N3 and then inserted into pET-28b vector (**A**) to construct pET-EGFP plasmid (**B**). The B1 gene was inserted into the downstream site of the *egfp* gene in the plasmid pET-EGFP to form the pET-EGFP-B1 plasmid, in which a stop codon of the original *egfp* gene was deleted (**C**). *MCS* is a compilation of multiple cloning sites. *His* is used to indicate the polyhistidine tag and *thrombin* is used to indicate the thrombin recognition site

incubation 24 h at 28°C, the colonies were detected by fluorescence microscopy (Leica, Germany) and observed by naked eye directly in daylight. Total cellular protein (TCP) of samples was prepared for analysis as follows: cells were harvested, washed with 200 mM phosphate/buffered saline (PBS) (pH 7.0) and then resuspended in one-fifth volume of the same buffer. The suspension was ultrasonicated on ice for 2 min and the TCP was determined by the Bradford method with bovine serum albumin as a standard.

### SDS-PAGE and Western blot analysis

Equal amounts of the TCP sample (4 μg) were electrophoresed on two identical 10% SDS-PAGE gels. After electrophoresis, one gel was stained by Coomassie Brilliant Blue, the other was transferred onto a Hybond ECL nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) to do Western blot analysis. The primary anti-His antibody (Tiangen Biotech, Beijing, China) raised in mouse was used at a dilution of 1:2000, and the secondary antibody was a goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma), which was used at a 1:5000 dilution. The Western blots were developed by enhanced chemiluminescence (Pierce, Rockford, IL, USA) and imaged using Bio-Rad ChemiDoc XRS gel imaging system

### Determination of activity and kinetics of carboxylesteras

Carboxylesterase (CarE) activity in TCP was assayed using $\beta$-naphthyl acetate ($\beta$-NA) as a substrate (Asperen 1962). About 25 μl of 10 mM $\beta$-NA was added into 1,975 μl of 200 mM PBS containing 5 μl TCP and then incubated at 37°C for 10 min. The reaction was terminated by adding of 500 μl stop solution (mixture of 1% Fast Blue B and 5% SDS, 2:5, v/v) and the absorbance was measured at 550 nm. Activities are expressed as nmol $\beta$-naphthol liberated per min per μg TCP.

## Results

### The optimal concentration of IPTG for induction of protein expression and detection of fluorescence

As shown in Table 1, all samples exhibited similar fluorescence intensity levels, except those samples grown in the absence of IPTG treatment, which remained at basal level (400–500). These data suggest that the lowest concentration (0.01 mM) of IPTG is sufficient for induction of expression of the recombinant protein. After induction, the fluorescence intensity of the cells containing pET-B1 approximated basal levels, while the fluorescence intensities of cells containing pET-EGFP-B1 and pET-EGFP increased proportionally with induction time. Of note, the intensity of pET-EGFP was much stronger than the pET-EGFP-B1. The results clearly indicate that the growth and fluorescence intensity for pET-EGFP-B1 plateaued at 8 h (Fig. 2). Furthermore, by fluorescence microscopy or by direct visualization in daylight, distinct green fluorescence could be observed from the colonies of *E. coli* BL21 (DE3) harboring pET-EGFP-B1 and pET-EGFP after incubation on LB agar plates containing IPTG (data not shown).

**Table 1** Cell dry weight and fluorescence intensity of *E. coli* BL21 (DE3) harboring pET-EGFP-B1 plasmid induced by IPTG at different concentrations

| Sample number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentration of IPTG (mM) | 0 | 0.01 | 0.02 | 0.04 | 0.06 | 0.08 | 0.1 | 0.16 | 0.2 | 0.6 | 1 |
| Cell dry weight (g/l) | 2.59 | 2.88 | 2.84 | 2.79 | 2.75 | 2.97 | 2.84 | 2.79 | 2.75 | 2.63 | 2.59 |
| Fluorescence intensity | 453 | 3501 | 3720 | 3729 | 3747 | 3870 | 3898 | 3724 | 3871 | 3778 | 3713 |

*Escherichia coli* BL21 (DE3) containing pET-EGFP-B1 plasmid was incubated at 37°C in a shaker until the cell dry weight reached 0.275 g/l. At this time, cultures were divided into eleven flasks and IPTG was added into each flask as indicated (0–1 mM). These cultures were allowed to incubate for 8 h at 28°C in a shaker with samples being collected. Then, cell concentrations and fluorescence intensity were measured respectively. The cell concentrations were determined turbidometrically at 600 nm and converted to the dry weight, which was determined gravimetrically

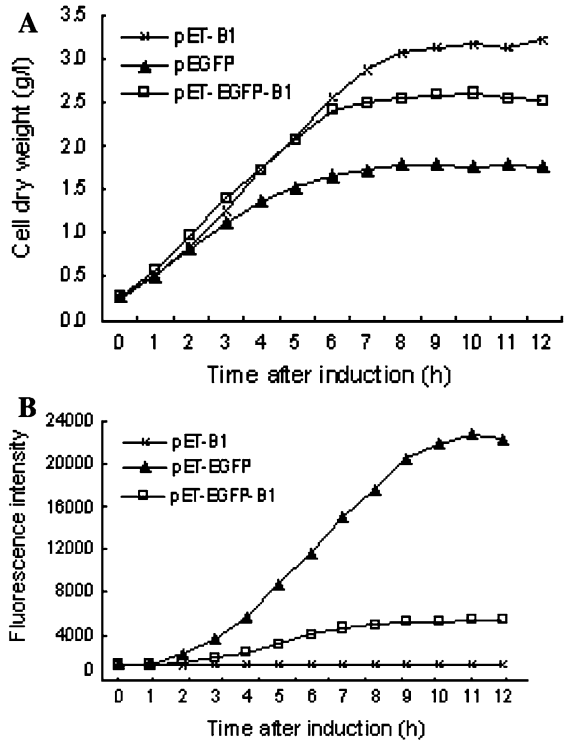


**Fig. 2** Growth curves and fluorescence intensity of *E. coli* BL21 (DE3) transformed with pET-EGFP-B1, pET-EGFP, or pET-B1 plasmid. The growth curve (**A**) and fluorescence intensity (**B**) of cultures containing *E. coli* BL21 (DE3) transformed with pET-EGFP-B1, pET-EGFP, or pET-B1 plasmid was monitored hourly by dry weight determinations and spectroflurophotometric method respectively, after induction with 0.01 mM IPTG at a dry weight of 0.275 g/l. 0 h indicates the start time of the induction

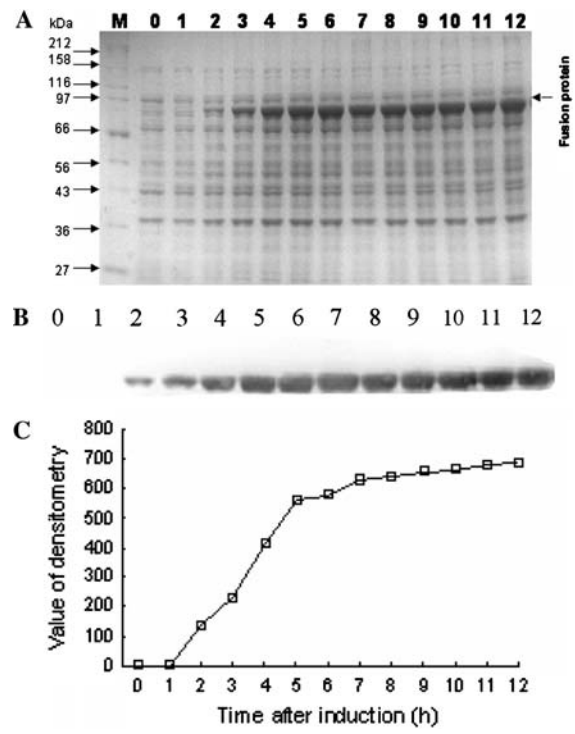


**Fig. 3** SDS-PAGE and Western blotting analysis of the EGFP-B 1 fusion protein in *E. coli* BL21 (DE3) carrying pET-EGFP-B1 plasmid after IPTG induction at different time. Equivalent amounts of total cellular proteins collected at different induction time were loaded per well, separated by SDS-PAGE (10% polyacrylamide). Lane M: protein marker (kDa); from lane 0 to lane 12 in (**A**) and (**B**): samples collected after 0–12 h induction, respectively. Location of fusion protein of EGFP and B1 is indicated. The gel in (**A**) was stained by Coomassie Brilliant Blue. Western blot analysis in (**B**) was performed using the antibody against His. (**C**) The result of Western blot quantified by computer-aided densitometry (Quantity One Version 4.6.2, Bio-Rad)

## Confirmation of expression of recombinant EGFP-B1 fusion protein by Western blot analysis

Expression of recombinant EGFP-B1 fusion protein was detected by SDS-PAGE and Western blot analysis from TCP of samples collected hourly after induction. The molecular weight of the fusion protein, EGFP-B1, is predicted to be approx. 90 kDa, including the $His_6$·Tag and the thrombin recognition sequence, since CarE B1 and EGFP are about 62 and 27 kDa respectively. As expected, a dark band of the recombinant protein was visualized at 90 kDa by Coomassie Brilliant Blue staining (Fig. 3A), and this was confirmed with antibodies against the His tag (Fig. 3B). The data indicate that recombinant EGFP-B1 was expressed in *E. coli* BL21 (DE3) harboring pET-EGFP-B1. Using SDS-PAGE or Western Blots, we also observed that the expression level of EGFP-B1 fusion protein gradually increased over time. In particular, the expression of EGFP-B1 plateaued at 5–6 h (Fig. 3C), which preceded detection by fluorescence spectrophotometry by 2–3 h (Fig. 2 vs. Fig. 3), perhaps due to the higher sensitivity of Western blot.

## Activity and kinetics of carboxylesterase in the genetically engineered bacteria

After 12 h induction by IPTG, CarE activities of TCP of *E. coli* BL21 (DE3) transformed with pET-B1 and pET-EGFP-B1 were 3.31 ± 0.05 and 0.91 ± 0.09 nmol

$\beta$-naphthol produced per min per μg protein, respectively. However, the basal activity of CarE in *E. coli* BL21 (DE3) transformed with pET-EGFP was only $(7.81 \pm 0.05) \times 10^{-3}$ nmol $\beta$-naphthol produced per min per μg protein. The Km value of EGFP-B1 and B1 were obtained by Lineweaver–Burk plots. The $K_m$ plots for pET-EGFP-B1 and pET-B1 were almost parallel and indicated non-competitive inhibition. The Km values of pET-EGFP-B1 and pET-B1 were 65 and 120 μM, respectively, and the Vmax of pET-EGFP-B1 and pET-B1 were 0.26 and 0.57 μmol/min.

## Discussion

The results of fluorescence spectrophotometry and enzyme activity assays indicate that the fusion protein, EGFP-B1, expressed in *E. coli* BL21 (DE3) possesses green fluorescence and esterase activity. However, the fluorescence intensity and CarE activity in the bacteria expressing the fusion protein were lower than those observed in bacteria expressing EGFP or B1 alone. To avoid the negative influence of the fusion expression strategy, one of the alternative possibilities for the use of GFP to monitor GEMs is to express GFP and B1 individually in the GEM. In this way, the two genes can be placed in a dual gene expression vector or, alternatively, one gene could be placed on the chromosome and the other in a plasmid. However, if the target gene is inserted into the chromosome, the expression level would probably decrease greatly since the copy number of the target gene once integrated into the chromosome is much lower than if the gene were introduced as a plasmid. However, the disadvantage of using a dual gene expression vector, is that the existence of green fluorescence cannot indicate the information of B1. Therefore, through the use of a fusion expression system, the recombinant protein can possess the characteristics of the two individual proteins, and thus fluorescence intensity can be correlated with enzyme activity (Wu et al. 2000). Indeed, fluorescence can be used to monitor the GEM and to indicate the enzyme activity. The individual colonies of the GEM on agar plates with IPTG could be identified in daylight by their green fluorescence, allowing the detection of the GEM to be performed much easier.

The Lineweaver–Burk plots of EGFP-B1 and B1 activities indicated non-competitive inhibition and thus the complex of EGFP-B1 and its substrate is more stable than the complex of B1 and its substrate. This resulted in a decrease of apparent $K_m$ and $V_{max}$. The powerful system of pET ensured the high output of target protein. From the results of SDS-PAGE (Fig. 3A), one can see that the product of recombinant protein increased quickly after IPTG induction and comprised more than 50% of the TCP a few hours later. The high output and the $His_6$·Tag were convenient for protein purification and both features make isolation of the target protein easy.

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## References

Asperen KV (1962) A study of house fly esterase by means of a sensitive colorimetric method. J Insect Physiol 8:401–416

Barata C, Solayan A, Porte C et al (2004) Role of B-esterases in assessing toxicity of organophosphorus (Chlorpyrifos, Malathion) and carbamate (Carbofuran) pesticides to *Daphnia magna*. Aquat Toxicol 66:125–139

Cormack BP, Valdivia R, Falkow S et al (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:37l–374

Gory L, Montel MC, Zagorec M (2001) Use of green fluorescent protein to monitor *Lactobacillus sakei* in fermented meat products. FEMS Microbiol Lett 194:127–133

Lan WS, Cong J, Jiang H et al (2005) Biodegradation of pesticide pollutants by two kinds of enzymes coexpressed in genetically engineered bacteria. Bull Environ Contam Toxicol 75:335–342

Lindow SE (1995) The use of reporter genes in the study of microbial ecology. Mol Ecol 4:555–566

March JC, Rao G, Bentley WE (2003) Biotechnological applications of green fluorescent protein. Appl Microbiol Biotechnol 62:303–315

Mouches C, Pauplin Y, Agarwal M et al (1990) Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. Proc Natl Acad Sci USA 87:2574–2578

Nishi K, Huang H, Kamita SG et al (2006) Characterization of pyrethroid hydrolysis by the human liver carboxylesterases hCE-1 and hCE-2. Arch Biochem Biophys 445:115–123

Nolte C, Matyash M, Pivneva T et al (2001) GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue. Glia 33:72–86

Vontas JG, Small GJ, Hemingway J (2000) Comparison of esterase gene amplification, gene expression and esterase activity in insecticide susceptible and resistant strains of the brown planthopper, *Nilaparvata lugens* (Stål). Insect Mol Biol 9:655–660

Wu CF, Cha HJ, Rao G et al (2000) A green fluorescent protein fusion strategy for monitoring the expression, cellular location, and separation of biologically active organophosphorus hydrolase. Appl Microbiol Biotechnol 54:78–83

Zhang J, Lan W, Qiao C et al (2004a) Bioremediation of organophosphorus pesticides by surface-expressed carboxylesterase from mosquito on *Escherichia coli*. Biotechnol Prog 20:1567–1571

Zhang JL, Qiao CL, Lan WS (2004b) Detoxification of organophosphorus compounds by recombinant carboxylesterase from an insecticide-resistant mosquito and oxime-induced amplification of enzyme activity. Environ Toxicol 19:154–159