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Construction of a Genetically Engineered Microorganism that Simultaneously Degrades Organochlorine and Organophosphate Pesticides

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Abstract Field contamination with pesticide mixtures of organophosphates (OPs) and organochlorines (OCs) is becoming global issues to be solved urgently. The strategy of utilizing engineered microorganisms that have an ability to simultaneously degrade OPs and OCs has increasingly received great interest. In this work, an OP degradation gene (*mpd*) and an OC degradation gene (*linA*) were simultaneously introduced into *Escherichia coli* by using two compatible plasmids, resulting in strains with both OP degradation and OC degradation capabilities. To overcome the potential substrate uptake limitation, MPH was displayed on the cell surface of *Escherichia coli* using the N- and C-terminal domains of ice nucleation protein (INPNC) as an anchoring motif. The surface localization of INPNC–MPH was verified by cell fractionation, Western blot, proteinase accessibility, and immunofluorescence microscopy. Furthermore, both LinA and green fluorescent protein (GFP) were functionally co-expressed in the MPH-displaying *Escherichia coli.* The engineered *Escherichia coli* degraded OPs as well as OCs rapidly, and it can be easily monitored by GFP fluorescence.

Keywords Organophosphate · Organochlorine · Co-contamination · Green fluorescent protein · Biodegradation

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Introduction

Organophosphate (OP) and organochlorine (OC) pesticides are widely used for pest control in developing countries. OPs are acute neurotoxins by virtue of their potent inhibition of acetylcholinesterase [1, 2]. Hexachlorocyclohexane (HCH) affects the nervous system, liver, and kidneys and tends to accumulate in biological tissues [3]. Bacterial enzymatic detoxification has attracted considerable interest as a strategy to economically and effectively degrade pesticides. The gene encoding methyl parathion degrading enzyme (*mpd*), isolated from a *Plesiomonas* sp. strain, is capable of hydrolyzing a wide range of oxon and thion organophosphates (OPs) [4, 5]. OCs degrading gene (*linA*) encoding γ-hexachlorocyclohexane dehydrochlorinase from the γ-hexachlorocyclohexane-degrading bacterium *Sphingomonas paucimobilis* UT26, which has the ability to catalyze the conversion of γ-hexachlorocyclohexane (γ-HCH) to 1,2,4-trichloro benzene (1,2,4-TCB) via γ-1,3,4,5,6-pentachlorocyclo hexene (γ-PCCH), was also characterized [3, 6].

The INP with the ability to protect cells in supercooled water is an outer membrane protein of *Pseudomonas syringae*. A total of two types of INPs identified have been used as anchoring motifs for display of foreign proteins on the cell surface [7, 8]. Compared to other anchors such as the Lpp-OmpA fusion system, it has special advantage of no cell lysis or growth inhibition, which makes it ideal for large-scale detoxification of pesticides. The green fluorescent protein (GFP) from the *jellyfish Aequorea victoria* can be expressed as a fluorescent protein in heterologous hosts without the need for specific co-factors or exogenous substrates [9] and can be detected noninvasively using fluorescence microscopy and flow cytometry [10]. In field studies, GFP has been used as a marker to assess the fate and activity of specific degrading microorganisms [11–13].

In previous study, we successfully constructed a recombinant *E. coli* strain over-expressing methyl parathion hydrolase (MPH) that was capable of efficiently degrading methyl parathion from off-gas during a 75-day bioreactor operation [14]. However, the disability of mineralize OCs limits its practical applications in the case where pesticide-contaminated sites are usually co-contaminated with OPs as well as organochlorine pesticides (OCs). Therefore, co-express enzyme of degrading methyl parathion and linden simultaneously will have great significance in usage. In this work, we aimed to construct the high effective engineered *E. coli* that can simultaneously degrade OPs and OCs by expressing MPH at the *E. coli* cell surface and over-expressing LinA as well as GFP by high copy plasmid pET system. The expression of GFP protein will easily monitor the engineering bacteria's fate in the environment by fluorescence, therefore manifesting a superior system in terms of both biodegradation capacity of two kinds of pesticides and detection feasibility.

Material and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The strains and plasmids used are listed in Table 1. *Escherichia coli* was grown aerobically at 37 °C in Luria Bertani (LB) growth medium. The final concentration of antibiotics was 50-μg kanamycin (Kana) and 100-μg ampicillin (Amp) per milliliter. For induction of gene expression, 0.1-mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the growth media.

Table 1 Strains and plasmids used in this work

Strain, plasmid, or primer	Description	Source or reference
Strains		
E. coli BL21 (DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
E. coli DH5α	*supE44* Δ*lacU169*(φ*80 lacZ*ΔM15) *recA1 endA1 hsdR17*(r_K^- m_K^+) *thi-1 gyrA relA1* F^- Δ(*lacZYA-argF*)	Tiangen
S. paucimobilis UT26	Source of *linA* genes	[21]
Plasmids		
pMDQ	Source of *mpd* gene	[15]
pEGFP-N3	Source of *egfp* gene	Clontech
pVLT33	*E. coli*/*Pseudomonas* shuttle vector, *oriT*, RSF1010, *oriV*, $lacI^q$, *tac* promoter, Km^r	[22]
pETDuet-1	Vector for the coexpression of two target genes, ColE1 replicon, two multiple cloning sites, two T7 promoters, Ap^r	Novagen
pINCM	pVLT33 derivative, control plasmid for expressing MPH on the surface	[15]
pCPM	pVLT33 derivative, control plasmid for expressing MPH in the cytoplasm	This study
pETLG	pETDuet-1 derivative, vector for the coexpression of *linA* and *gfp* genes	This study

Construction of Two Compatible Plasmids

Plasmid pINCM was used to express ice nucleation protein (INP)-MPH on the cell surface. The *mpd* fragment was PCR-amplified from pMDQ and subcloned into *Bam*HI/*Hin*dIII-digested pPNCO33, a medium copy-number vector, to give pINCM [15]. Plasmids pCPM was used for the intracellular expression. The amplified *mpd* gene was digested with *Eco*RI and *Hin*dIII and transferred into pPNCO33. PCR-amplification of the *mpd* gene was the same as the aforementioned PCR, except for forward primer 5′-ACGAATTCAGGAAA CAATGGCCGCACCGCAGGTGCGC-3′ (the *Eco*RI site is underlined).

pETDuet-1 was used as the vector for co-express genes of *linA* and *egfp*. The *linA* gene was amplified from *Sphingomonas paucimobilis UT26* with primers 5'-GACCATGG TGATGAGTGATCTAG-3' (*Nco*I) and 5'-GTAAGCTTTTATGC GCCGGACG-3' (*Hind*III) and was cloned into the first multiple cloning sites of pETDuet-1. Similarly, the *egfp* gene was amplified from pEGFP-N3 with primers 5'-GAGCTAGCATGGTGAGCAAG GGCGAGGAGC-3' (*Nhe*I) and 5'-TTGGATC CCTTGTACAGCTCGTCCATGCC GAG-3' (*BamH*I) was cloned into the second multiple cloning sites of pETDuet-1 to form plasmid pETLG. The recombinant plasmids were sequenced to verify the correct sequence of the inserted genes. Transformation of plasmid into *E. coli* BL21 (DE3) was carried out using the CaCl2 method. The resulting strain was named XL1-Blue/pETLG/pINCM.

Cell Fractionation

Cells were induced with 0.1-mM IPTG and cultured at 30 °C for 12 h, and then, 10-ml cells were collected by centrifugation at 3,500 g for 10 min. The harvested cells were washed and resuspended in potassium phosphate buffer (PBS) containing 1-mM EDTA and 1-mM PMSF, and then, we followed the method described by Yang et al. [15].

Western Blot Analysis

Each sample was mixed with SDS sample buffer, boiled for 7 min, and resolved by 10% (w/v) SDS-PAGE. For Western blot analysis, the gel was transferred onto nitrocellulose membranes (Millipore) with a tank transfer system (Bio-Rad). Blotted membranes were placed in a blocking solution of 3% BSA in TTBS buffer (100-mM Tirs-Cl, 0.9 % (w/v) NaCl, 0.1 % (v/v) Tween 20) for 1 h. For immunodetection, primary INPNC antiserum (1:1,000) was diluted in TTBS buffer. The second antibody was an alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1:1,000. Reaction of alkaline phosphatase was developed by a solution containing 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP)/ (Nitro blue tetrazolium chloride (NBT)).

Immunofluorescence Microscopy

E. coli cells were grown at 30 °C for 12 h upon induction with 0.1-mM IPTG, collected by centrifugation, washed three times with PBS buffer (pH 7.4), and fixed in 2% formaldehyde for 10 min. After three washes with PBS, blocking was performed by addition of 2% BSA at 4 °C for 2 h. Cells were centrifuged and resuspended in PBS buffer containing 2% BSA and anti-INPNC diluted 1:1,000 at 37 °C for 2 h. Cells were washed three times with PBS and then incubated with Rhodamine-labeled anti-rabbit IgG goat antibody (Molecular Probes, Eugene, OR) diluted 1:500 at 4 °C for 10 h. Finally, cells were washed three times with PBS and examined by a Nikon Optiophot fluorescence microscope.

Whole-Cell Activity Assay

Cells were grown in 50 ml of LB medium supplemented with 0.1-mM IPTG, 100-μg/ml Amp, and 20-μg/ml Km for 1 day, washed twice with 50 ml of 150-mM NaCl solution, and resuspended in 50-mM PBS (pH 8.0) to yield a final optical density at 600 nm of 1.0. Cells were incubated with 5-ppm γ-HCH at 30 °C. Aliquots were taken out from these reactions periodically and extracted with an equal volume of ethyl acetate. The mixture was vortexed for 2 min. After centrifugation (12,000 g, 5 min), the ethyl acetate layer was recovered. Of this extract, 1 ml was analyzed by GC-MS method [3]. One unit of activity was defined as the amount of OD600 cells required for the release of 1 nmol of lindane per min.

MPH assay was carried out as described by Yang et al. [15]. Similar whole-cell activity measurements were conducted with *E.coli* BL21 (DE3) harboring pCPM. In addition, for the reason that high transcription rate can block the translocation pathway and cause growth inhibition, we verified the IPTG from 0.005 to 1 mM to detect the optimal conditions.

Measurement of Whole-Cell Fluorescence

Cells harboring pETLG were suspended in a PBS buffer (pH 7.5) and diluted to an OD600 of 1.0, and the similarly diluted cells harboring pVLT33 were used as background references. The GFP fluorescence intensity was determined using a fluorescence spectrophotometer (F-4500, HITACHI, Japan) with a bandwidth of 5 nm, an excitation wavelength of 488 nm, and an emission wavelength of 510 nm.

Stability Study of Plasmids

Plasmid stability was estimated by cell counting in Petri dishes using the method described by Gupta et al. [16]. The ratio of the number of colony forming units on the Km/Amp-containing plate to that on the LB agar plate was used to determine the percentage of plasmid-carrying cells, which was used as an index of plasmid stability.

Results and Discussion

Construction and Characterization of the Vectors

Plasmids with the same origin of replication are usually considered incompatible [17, 18]. They cannot stably co-exist in a cell together because competition for replication factors leads to competition between plasmids. Ideally, a useful co-expression system should consist of a pair of vectors that have compatible origins of replication to prevent segregation and ultimate loss of one plasmid in culture. In addition, it is important that each vector contains a different antibiotic-resistance gene and a strong promoter [19]. To achieve this goal, we selected plasmid pVLT33 and pETDuet-1 to construct two vectors, pINCM with kanamycin resistance and pETLG with ampicillin resistance, to be used for co-expression. Plasmid pVLT33 is an RSF-1010-based, broad-host-range plasmid harboring the kanamycin resistance gene, which permits it to co-exist with the ColE1 replicon-carrying vector pETDuet-1 in the same *E. coli* host cell. The parent plasmid pVLT33 was stably maintained in several bacterial species at medium copy number [20] and was compatible with pET-based plasmids. In this work, the two different replicons are stably maintained in *E. coli* BL21 (DE3) simultaneously. About 60% of the cells were double antibiotic resistant (Km and Amp) after 50 generations in the absence of antibiotic.

Surface Localization of INPNC–MPH Fusion Protein

To verify the synthesis of INPNC–MPH fusion, whole-cell lysate was assayed by Western blot after induction with 0.1-mM IPTG. INPNC–MPH fusion was observed from cells carrying pINCM at a position of ca. 68 kDa, which matched well with the molecular mass of the fusion protein (Fig. 1). However, no band was found with the control cells carrying pVLT33 (Fig. 1, lane 4). Furthermore, total cell lysate, membrane, and soluble fractions were probed with anti-INPNC serum to assess the distribution of the fusion. As shown in

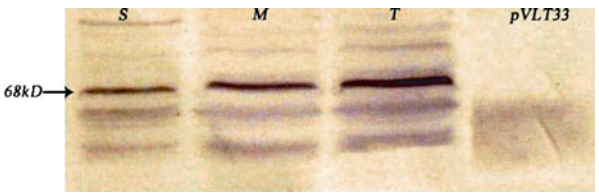

Fig. 1 Western blot analysis for subcellular location of expressed INPNC–MPH fusion in *E. coli* BL21 (DE3). The amount of INPNC–MPH in total cell lysate (*T*), soluble fraction (*S*), and membrane fraction (*M*) was detected with rabbit anti-INPNC serum at a 1:1,000 dilution. *Arrow* indicates the location of the fusion. Cells harboring pVLT33 were used as a negative control

Fig. 1, about 60% of the fusion was associated with the membrane fraction (Fig. 1, lane 2). Consistently, 60% of MPH activity was detected on the cell surface as assessed from the ratio of whole-cell activity to cell lysate activity. To investigate whether the fusion was displayed correctly on cell surface in a stable conformation, immunofluorescence microscopy was performed. Under the immunofluorescence microscopy, the orange fluorescence was detected on the cells harboring pINCM (Fig. 2b). In contrast, the control cells were not immunostained (Fig. 2a). These results indicated that the cell surface was covered with antibody-TRITC complex, which confirmed that INPNC–MPH fusion was successfully displayed on the surface of *E. coli* BL21 (DE3).

Assay for Conversion Activity of OPs and γ-HCH

The bottleneck, which was caused by the outer membrane to restrict the passage of moderate to large substrates into the cell, could be eliminated if enzyme is displayed onto the surface of cells. In this work, MPH was functionally expressed onto the cell surface using the truncated InaV (INPNC) from *Pseudomonas syringae* INA5 as the surface anchor. About 60% of MPH activity was present on the cell surface. Whole cells of BL21 (DE3)/pETLG/pINCM had seven-fold higher activity than BL21 (DE3)/pCPM (Fig. 3a), illustrating that surface-expressed MPH affords greater degradation than intracellularly expressed MPH. In addition, the impact of IPTG concentrations on MPH activity was researched. Result has shown that the best expression should be under the condition of about 0.1-mM IPTG added.

To overproduce HCH dehydrochlorinase in *E. coli*, the *linA* gene was inserted into high-copy expression vector pETDuet-1. After the recombinant plasmid pETLG was expressed in *E. coli* BL21 (DE3), two clear IPTG inducible bands corresponding to about 16.7 kDa (LinA) and 27 kDa (GFP) were observed in SDS-PAGE. We performed the conversion reaction by using whole cells bearing gene *linA*. The ability of *E. coli* BL21 (DE3)/pETLG/pINCM cells to rapidly degrade γ-HCH was investigated. Cells were induced under optimal conditions, and whole-cell LinA assay was carried out as described previously. The reaction mixture was stirred at 200 rpm and kept at 30 °C. The time course of γ-HCH degradation of the repeated-batch experiments is shown in Fig. 3b. γ-HCH (5 ppm) was completely hydrolyzed by induced cells of 1 OD600 within 16 h (Fig. 3b). And the same with degrading of OPs, the best induction condition is 0.1-mM IPTG.

GFP as a Biomarker

The *gfp* gene was one of the promising markers for monitoring because the protein fluoresces upon illumination with blue light and no other energy source or substrate addition is required. GFP has been optimized as a marker for bacteria in environmental samples [12]. In this work, GFP fluorescence remained at the original background level (56/OD600) at 0 h (postinduction). After 0.1-mM IPTG induction, the whole-cell bioactivities and fluorescence were rapidly and dramatically promoted in the following 24 h (max=3800/OD600) (Table 2). The engineered cells can be easily visualized in samples by microscopy (Fig. 2c).

Conclusion

In conclusion, we demonstrated the construction of a genetically engineered *E. coli* strain co-expressing MPH, LinA, and GFP. The end result is a cell line endowed with the abilities

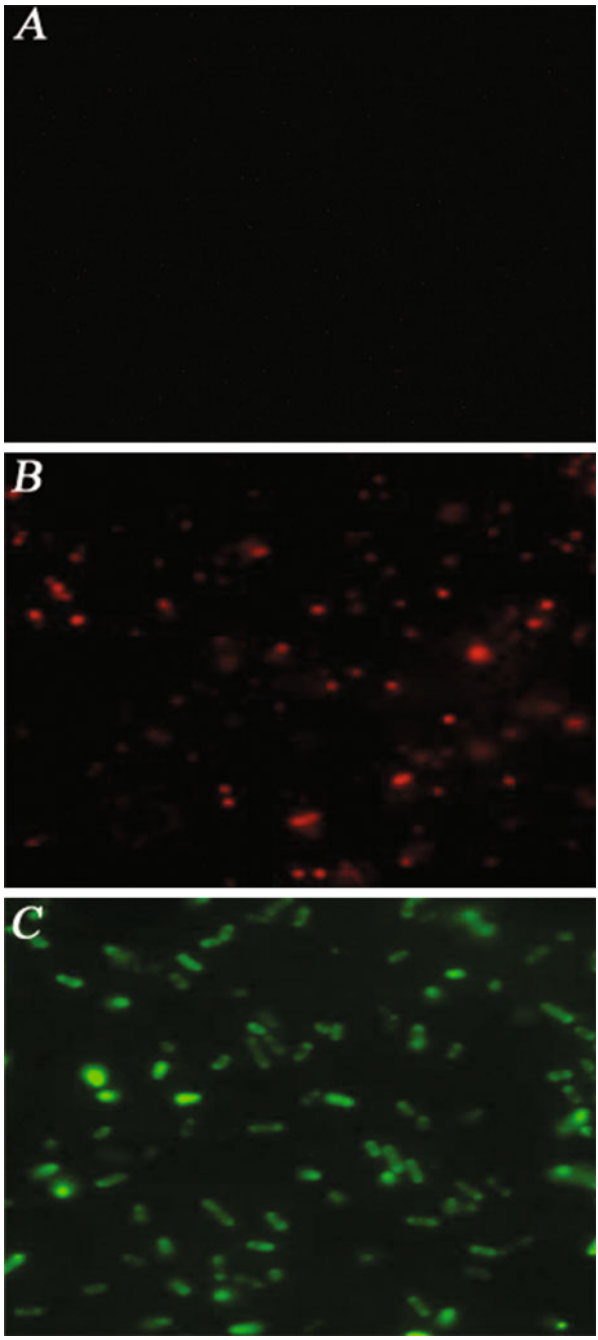

Fig. 2 **a** Immunofluorescence micrograph of *E. coli* BL21(DE3) harboring pVLT33. **b** Immunofluorescence micrograph of *E. coli* BL21(DE3) harboring pINCM. Cells were probed with rabbit anti-INPNC serum and fluorescently stained with goat anti-rabbit IgG-TRITC conjugate. **c** Green fluorescence micrograph after 24-h induction

to rapidly degrade organophosphorus compounds and organochlorine compounds and to fluoresce for detection. This is a superior system in terms of both biodegradation capacity of two kinds of pesticides and detection feasibility. The specific degradation rate for this surface expression system is more than seven-fold higher than that of the previously

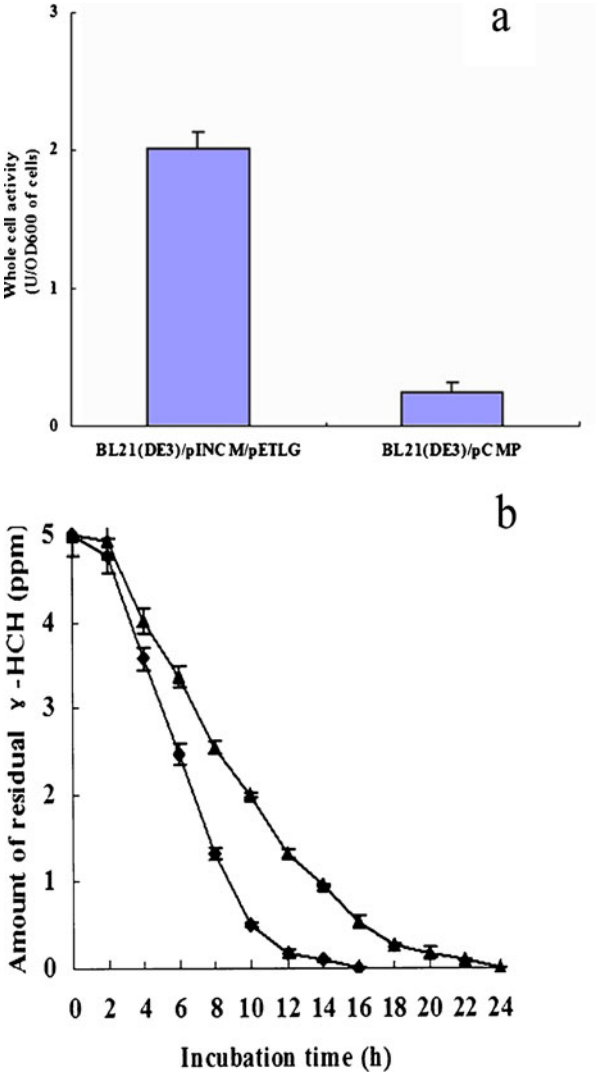

Fig. 3 **a** Whole-cell MPH activities of BL21(DE3)/pINCM/pETLG and BL21(DE3)/pCPM. **b** Degradation of 5-ppm γ-HCH by BL21(DE3)/pINCM/pETLG cells. Data are mean values ± standard deviations from three replicates

Table 2 Whole-cell bioactivity and GFP fluorescence assay

Postinduction (h)	MPH activity (U/OD_{600})	LinA activity (U/OD_{600})	Fluorescence intensity
0	ND	ND	56±7
6	0.39±0.043	1.12±0.061	1,979±26
12	1.10±0.091	2.17±0.10	3,648±31
24	1.22±0.010	2.39±0.095	3,800±19

Data are mean values ± standard deviations from three replicates

ND, not detected

described intracell system. More importantly, the GFP-based detection system is effective. For these bacteria's excellent advantage in simultaneously degrading OPs and OCs and its rapid degrading rate in both of them, it can be utilized as bioreactor of biosensor in on-site monitoring, and this work will lay the foundation of the investigation of the remediation of co-contamination of OPs and OCs by using it in a bioreactor.

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